INSIGHTS OF FERMENTED FOODS AND BEVERAGES: MICROBIOLOGY AND HEALTH-PROMOTING BENEFITS

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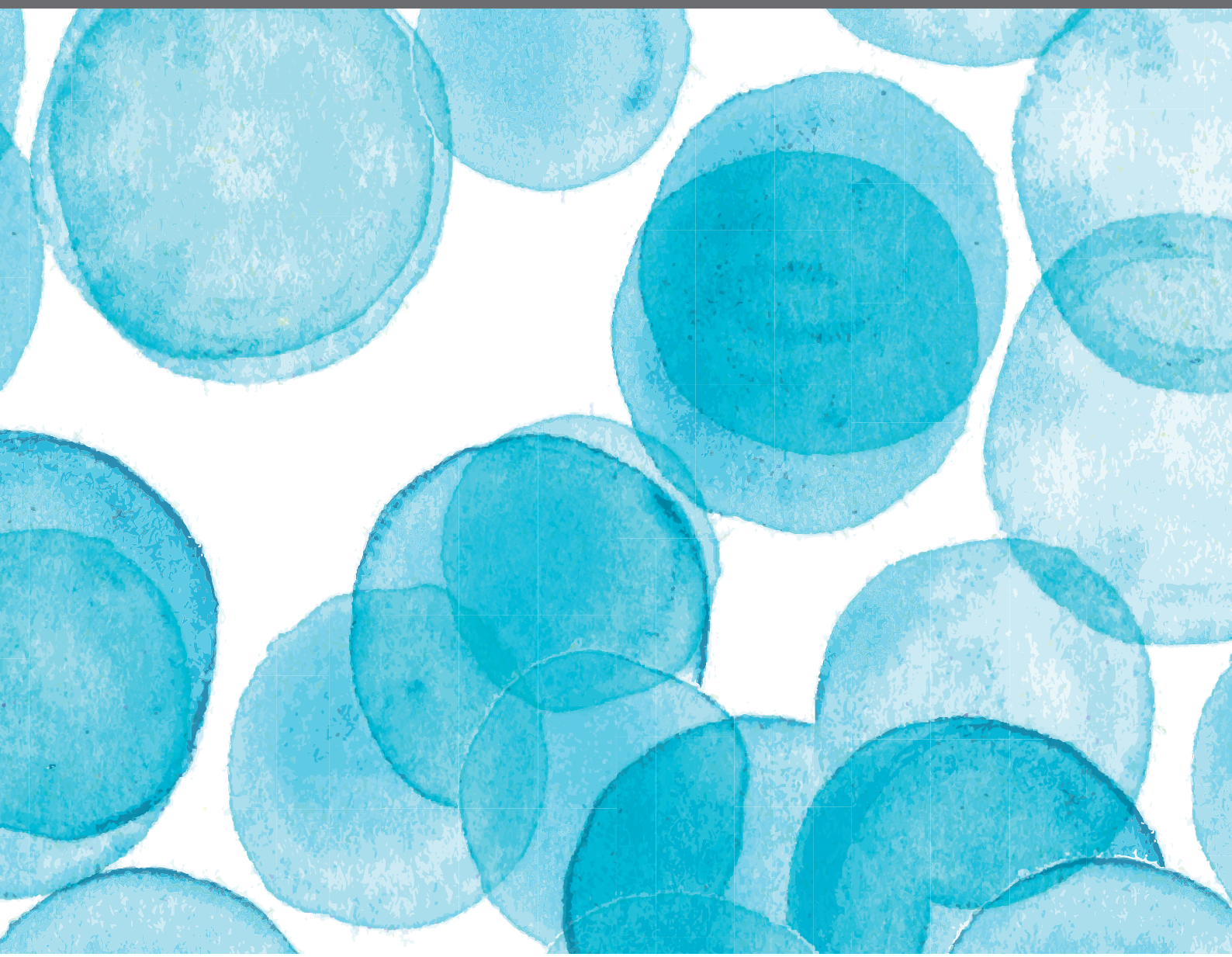

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INSIGHTS OF FERMENTED FOODS AND BEVERAGES: MICROBIOLOGY AND HEALTH-PROMOTING BENEFITS

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REVIEW
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Fermented Foods as a Dietary Source of Live Organisms

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The popularity of fermented foods and beverages is due to their enhanced shelf-life, safety, functionality, sensory, and nutritional properties. The latter includes the presence of bioactive molecules, vitamins, and other constituents with increased availability due to the process of fermentation. Many fermented foods also contain live microorganisms that may improve gastrointestinal health and provide other health benefits, including lowering the risk of type two diabetes and cardiovascular diseases. The number of organisms in fermented foods can vary significantly, depending on how products were manufactured and processed, as well as conditions and duration of storage. In this review, we surveyed published studies in which lactic acid and other relevant bacteria were enumerated from the most commonly consumed fermented foods, including cultured dairy products, cheese, fermented sausage, fermented vegetables, soy-fermented foods, and fermented cereal products. Most of the reported data were based on retail food samples, rather than experimentally produced products made on a laboratory scale. Results indicated that many of these fermented foods contained 10^{5-7} lactic acid bacteria per mL or gram, although there was considerable variation based on geographical region and sampling time. In general, cultured dairy products consistently contained higher levels, up to 10^9/mL or g. Although few specific recommendations and claim legislations for what constitutes a relevant dose exist, the findings from this survey revealed that many fermented foods are a good source of live lactic acid bacteria, including species that reportedly provide human health benefits.

Keywords: fermented foods, live microbes, lactic acid bacteria, health benefits, probiotics

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INTRODUCTION

Fermentation has long been used to preserve and enhance the shelf-life, flavor, texture, and functional properties of food (Hutkins, 2018). More recently, the consumption of fermented foods containing live microorganisms has emerged as an important dietary strategy for improving human health (Marco et al., 2017). In general, lactic acid bacteria (LAB) from several genera, including *Lactobacillus*, *Streptococcus*, and *Leuconostoc* are predominant in fermented foods, but other bacteria as well as yeast and fungi also contribute to food fermentations. Commercially-produced fermented foods also frequently serve as carriers for probiotic bacteria. Despite this interest and the potential public health benefits of these foods, there is still considerable confusion about which fermented foods actually contain live microorganisms, as well as understanding the role of these microbes on the gut microbiome (Slashinski et al., 2012).

Nonetheless, yogurt and other cultured dairy products are generally perceived by consumers as good sources of live and health-promoting organisms (Panahi et al., 2016). Moreover, in a survey of

335 adults, yogurt was the main food associated with probiotic bacteria (Stanczak and Heuberger, 2009). However, the actual concept of fermentation is evidently not so familiar—a survey of 233 college students attending Brescia University College in London, Ontario revealed that nearly two-thirds were unfamiliar with the term "fermented dairy products," and about the same percent were unsure that several cultured dairy products were fermented (Hekmat and Koba, 2006).

That a particular food or beverage is produced by fermentation does not necessarily indicate that it contains live microorganisms. Bread, beer, wine, and distilled alcoholic beverages require yeasts for fermentation, but the production organisms are either inactivated by heat (in the case of bread and some beers) or are physically removed by filtration or other means (in the case of wine and beer). Moreover, many fermented foods are heat-treated after fermentation to enhance food safety or to extend shelf-life. Thus, fermented sausages are often cooked after fermentation, and soy sauce and sauerkraut and other fermented vegetables are made shelf-stable by thermal processing. Some products, such as many of the commercial pickles and olives, are not fermented at all, but rather are placed into brines containing salt and organic acids. Even non-thermally processed fermented foods may yet contain low levels of live or viable organisms simply due to inhospitable environmental conditions that reduce microbial populations over time. It is important to note, however, that the absence of live microbes in the final product does not preclude a positive functional role. For example, food fermentation microbes may produce vitamins or other bioactive molecules *in situ* or inactivate anti-nutritional factors and yet be absent at the time of consumption.

LABELING LIVE MICROBES IN FERMENTED FOODS AND BEVERAGES

Yogurt, kefir, and other cultured dairy product manufacturers have long promoted the presence of live cultures. Indeed, the "live and active" seal was created by the National Yogurt Association (NYA), for yogurt products in the United States containing at least 100 million cells or cfu per gram at the time of manufacture (Frye and Kilara, 2016). According to the NYA, the "live and active" seal refers only to yogurt cultures, and specifically to the two species that comprise such cultures, *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*. However, frozen yogurt, kefir and other cultured dairy products also claim the presence of live and active cultures, even though the microorganisms may be different than those found in yogurt. In the U.S., there is no regulatory requirement to state microbial levels, thus these label declarations are strictly voluntary.

In contrast, in other regions, the number of live microbes present in yogurt and other cultured dairy products must satisfy regulatory requirements. For example, according to the CODEX standards for fermented milk products, the minimum number of starter culture bacteria in yogurt is 10^7 cfu per g (CODEX STAN 243-2003). If other organisms are indicated on the label, they must be present at 10^6 cfu per g. Nonetheless, in Europe, to make a claim for yogurt containing live cultures for improving lactose digestion, the European Food Safety Agency requires a minimum of 10^8 cfu per g of live bacteria (EFSA Panel on Dietetic Products, Nutrition and Allergies, 2010). In contrast, in Australia and New Zealand, a minimum of only 10^6 cfu per g is required (Commonwealth of Australia Gazette, 2015).

For many years, cultured dairy products were the only fermented foods that included label declarations regarding the presence of live microorganisms. Label declarations on sauerkraut or kimchi or miso, had, until recently, been rare. The popularity of artisan-style fermented foods (Johnson, 2016) and interest in their health properties (Marco et al., 2017) has led more manufacturers to inform consumers, via food labels, that their products contain live microorganisms. In some cases, the species in these types of foods have been identified and then compared to label claims (Yeung et al., 2002; Scourboutakos et al., 2017). However, to our knowledge, data on the actual levels of live microorganisms in most fermented retail products has not readily been reported or summarized in an organized form. Therefore, consumers, despite their interest in probiotics and functional fermented foods (Linares et al., 2017), have had little access to this useful information.

SURVEY DESIGN

The purpose of this study, therefore, was to survey the scientific literature and identify published papers in which the number of live microorganisms in a range of fermented foods was reported. Included were so-called western-fermented foods such as yogurt, cheese, and sausage, as well as soy-based and cereal-based fermented foods that are widely consumed in other regions (Tamang et al., 2016). We then organized and summarized the quantitative data from those reports. Our interest was focused on those reports in which foods were obtained from retail locations or were made under manufacturing conditions. Thus, reports describing results from experimentally-produced fermented foods on a laboratory or pilot scale were excluded, in part because they do not reflect commercial processing, distribution, and storage conditions as do retail products. A large number of the reports in the literature in which levels of microbes in fermented foods were described were of this sort. In addition, many reports have analyzed the importance of microbial food safety and hygienic conditions of fermented food products and have reported the presence of spoilage microorganisms or food pathogens. However, the organisms responsible for fermentation and that are commonly present in the finished products were the focus of this current study.

Search Criteria

Scientific articles were chosen that satisfied specific parameters relevant to our stated goals. Specifically, our database search (Google Scholar, WorldCat, Scopus, and PubMed) focused on those studies that enumerated microorganisms exclusively in fermented food products. Keywords for these searches included, but were not limited to, the type of fermented food analyzed and, "commercially produced," "commercial product," "enumerated," "lactic acid bacteria," "microbial characterization," "probiotic,"

and "culture." Food products that served only as vehicles for delivery of probiotic microorganisms were not included. Thus, studies that reported counts for frozen yogurt were included, but studies on ice cream containing probiotic microorganisms were not. In general, results were only included for commercial products, bought at retail locations, or those experimentally-produced under industrial manufacturing conditions. Thus, strictly experimental products (e.g., made in a laboratory or under small experimental-scale conditions) were not considered. The only exceptions were for products for which little or no data from retail or industrially manufactured sources was available. In those cases, lab- or pilot-scale-produced products were included, provided they were made using traditional manufacturing methods. No restrictions for date, location, or language were applied.

Data Reporting

For most products, quantitative data relied on cultural methods using well-established types of differential, selective, and general purpose media, as well as appropriate incubation conditions. LAB were the main group described, although other bacterial groups were occasionally reported. Some studies reported single microbial counts, whereas other reported ranges. Although papers reported counts either as log or as actual values, all of the data described in this review are shown as logs. For some products, values were estimated from graphs or figures. When products were held for shelf-life or aging studies, the counts from multiple times points are shown. Otherwise, single time-point data was reported. The region or origin of product manufacture was also noted.

GENERAL SURVEY RESULTS

Approximately 400 published studies were reviewed in which fermented foods were characterized for the presence of live microorganisms. However, about three-fourths were excluded and not used in our results. Several excluded studies focused on development of selective methods for distinguishing between different species of LAB, determining ratios (e.g., cocci-to-rods in yogurt), or for enumerating only probiotics organisms. Although most studies reported data based on traditional plating methods, many of the more recent studies reported abundance data (i.e., 16S rRNA-based community sequencing). Because the latter 16S-based methods also detect non-viable cells, these studies were excluded unless total counts were also reported. Ultimately, more than 140 studies were included in our survey. Although the literature from which the results were assembled covers a 50 year period and a range of different regions and methodologies, the results are remarkably consistent. As summarized below, nine groups of fermented foods were reviewed in this survey. These included yogurt and other cultured dairy products, cheese, fermented meats, fermented vegetables, traditional fermented Asian products, fermented cereals, beer, and fermented tea (Kombucha).

Yogurt and Other Cultured Dairy Products

Studies were conducted for retail or commercially manufactured yogurts and other cultured dairy products obtained in the U.S., Australia, Spain, France, Norway, Greece, Argentina, and South Africa (**Table 1**). All of the yogurts examined contained the yogurt culture organisms, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, at levels ranging from $<10^4$ to 10^9 cfu/g or ml. In general, counts for *S. thermophilus* were somewhat higher than for *L. delbrueckii* subsp. *bulgaricus*. In several studies, other microorganisms, including *Bifidobacterium* spp. and *Lactobacillus* spp., were also enumerated. Levels of the latter ranged from undetectable (<10 cfu/g) to 10^8 cfu/g. The addition of these probiotic bacteria did not appear to have any effect on levels of the yogurt culture organisms. Although most studies reported counts at only a single time point, other studies reported initial counts as well as at a second time point, usually considered end-of-shelf-life. In such cases, counts were generally similar at both time points ($>10^6$ cfu/g), provided samples were stored at refrigeration temperatures (Hamann and Marth, 1984).

In addition to fresh yogurt, frozen yogurt was also examined for bacteria. Results from several studies indicates that when these products were assessed for the relevant yogurt LAB, levels were generally similar to fresh yogurt, with counts ranging from 10^4 to 10^9 cfu/g. The stability of lactic cultures in frozen yogurt during long-term storage at freezer temperature (-23 C) has also been studied (Lopez et al., 1998). In general, LAB (*S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*) survived beyond the designated shelf-life period (1 year), with less than a 0.5 log reduction for most samples.

The number and type of live microorganisms in other cultured dairy products have also been reported (**Table 2**). These include kefir, cultured buttermilk and simply "fermented milk." As for other cultured dairy products, populations of LAB were in the 10^5–10^9 cfu/g range.

Cheese

Although considerable microbiological data for cheese exists, most of these reports are concerned with microorganisms having public health or cheese quality implications. Still, levels of lactic acid and related bacteria were reported for more than 30 types of cheese from 18 countries including the United States, Italy, France, Germany, Mexico, Ireland, and South Africa (**Table 3**). Many papers reported the microorganisms as mesophilic streptococci, lactococci, and lactobacilli or as thermophilic streptococci and lactobacilli. Others reported total microorganisms and total LAB. For most products, only one time period was recorded (usually the most aged sample). Microbial counts ranged from undetectable ($<10^3$ cfu/g) to 10^9 cfu/g, with the highest levels found in Tilsit cheese (typically aged 2–4 months). In contrast, Grana Padano aged 1 year, Parmesan aged greater than 1 year, and Swiss Gruyere aged greater than 1 year all showed no detectable microorganisms ($<10^3$ cfu/g). As noted for other products, the methods used by the investigators may have influenced the reported data. Thus, enumeration of selected organisms (e.g., *S. thermophilus*) was only possible if the appropriate medium and growth conditions were used.

TABLE 1 | Organisms in commercial yogurt products by region.

Region	Type	Source	Analyzed microorganisms	Initial Count (log cfu/mL or g)	Final Count (log cfu/mL or g)	Age	CP*	References
Argentina	Full and reduced fat yogurt	Retail	*S. thermophilus*	8.87–9.46	–	Within shelf life	6	Vinderola and Reinheimer, 2000
			L. bulgaricus[a]	5.58–7.95	–			
			Bifidobacteria[a]	2.60–8.71	–			
			L. acidophilus[a]	4.62–8.39	–			
			L. casei[b]	8.02–8.33	–			
	Set, skimmed set, drinking, and set with "dulce de leche" yogurt[c]	Industrially manufactured	Total LAB	7.54–8.62	–	Within shelf life	25	Birollo et al., 2000
			S. thermophilus	7.72–8.58	–			
			L. bulgaricus	7.29–7.38	–			
Australia	Full and reduced fat yogurt[d]	Commercially Manufactured	Streptococci	9.15–9.6	8.79–9.15	After manufacture and by expiration	4	Micanel et al., 1997
			L. bulgaricus	9.08	8.36			
			L. acidophilus	6.66–8.08	6.38–8.04			
			Bifidobacteria	5.81	7.54			
	Skim milk and regular yogurt[e]	Did not specify	*L. casei*	–	3.41–7.49	Did not specify	2	Ravula and Shah, 1998
	Variety of flavored, natural, and skinny yogurt[f]	Retail	*S. thermophilus*	8.62–9.17	–	After purchase	5	Tharmaraj and Shah, 2003
			L. bulgaricus	4.92–7.68	–			
			L. rhamnosus	7.36–7.72	–			
			L. casei	4.01–5.53	–			
			B. lactis	6.36–7.4	–			
			L. acidophilus	5.23–7.83	–			
	Variety of flavored yogurts[g]	Retail	*L. acidophilus*	< 2–8.34	<2–8	After purchase (around 20–30 days before expiration) and at expiration	26 CP from 14 companies	Shah et al., 2000
			Bifidobacteria	<2–6.86	<2–6.18			
			L. casei	5.65–8.18	<2–8.08			
	Yogurt[h]	Did not specify	*L. acidophilus*	–	6.56	–	18	Talwalkar and Kailasapathy, 2004
			Bifidobacteria	–	6.54			
			L. casei	–	6.38			
	Yogurt[i,j]	Obtained from manufacturer	*L. acidophilus*	4–8.5	NVO–7.7	After manufacture and 30 days	5	Shah et al., 1995

(Continued)

TABLE 1 | Continued

Region	Type	Source	Analyzed microorganisms	Initial Count (log cfu/mL or g)	Final Count (log cfu/mL or g)	Age	CP*	References
			B. bifidum	3.3–7	NVO–2.5			
China	Yogurt	Retail	*S. thermophilus* + *Lactobacillus*	–	4.0–8.18	End of shelf life	31	Dong et al., 2014
England	Yogurt[k]	Retail	Bifidobacteria	–	4.9–7.62	Does not specify	8	Iwana et al., 1993
Greece[i]	Greek type yogurt	Obtained from manufacturer	*S. thermophilus*	9.1	8.5	50 days (product shelf life)	1	Alexopoulos et al., 2017
			L. delbrueckii subsp. bulgaricus	8.8	7.9			
Italy	Plain stirred style yogurt	Retail	*S. thermophilus*	7.71–8.9	–	10 days after manufacture	11	De Noni et al., 2004
			L. bulgaricus	5.48–8.41	–			
	Sweetened stirred style yogurt	Retail	*S. thermophilus*	8.3–9.59	–	10 days after manufacture	11	De Noni et al., 2004
			L. bulgaricus	<4–8.18	–			
South Africa	Low fat, fruit flavored[j]	Obtained from manufacturer	*S. thermophilus*	8.7–9.5	7.9–9.5	Directly after production, and at expiration date	3	Lourens-Hattingh and Viljoen, 2002
			L. bulgaricus	7–8.6	5.5–7			
			L. acidophilus	7–8.7	4.9–7			
			B. bifidum	2–5.2	2.2–4.9			
United States	Custard style yogurt—plain and flavored[j]	Retail	Total LAB	9.1	–	15 days after manufacture	2 CP from 1 manufacturer	Hamann and Marth, 1984
			S. thermophilus	9.1	–			
			L. bulgaricus	8.1	–			
	Dannon, Breyers, Yoplait, YoBaby, Wal-Mart, and Kroger varieties	Retail	*Lactobacillus*	–	7.68–8.98	before expiration	10	Dunlap et al., 2009
	Flavored yogurt	Retail	*L. bulgaricus*	5.2–8.87	6.15–8.69	0 and 4 weeks after purchase	58 CP/7 brands	Ibrahim and Carr, 2006
			S. thermophilus	7.51–8.94	7.9–8.99			
			Bifidobacteria	<1–4.7	NVO**			
	Plain nonfat yogurt	Retail	*S. thermophilus*	8.14–9.83	–	After manufacture	3	Laye et al., 1993
			L. bulgaricus	9.04–9.33	–			
	Stirred style yogurt–flavored[j]	Retail	Total LAB	8.6	–	6 days after manufacture	1	Hamann and Marth, 1984

(Continued)

TABLE 1 | Continued

Region	Type	Source	Analyzed microorganisms	Initial Count (log cfu/mL or g)	Final Count (log cfu/mL or g)	Age	CP*	References
			S. thermophilus	8.6	–			
			L. bulgaricus	7.3	–			
	Yogurt	Retail	Total LAB	-	7.2–8.1	At expiration date	2	Shin et al., 2000
			Bifidobacteria	–	6.5–7.1			

*CP, commercial products.

**NVO, No viable organisms.

[a]Only viable in 4 of 6 CP.

[b]Only viable in 3 of 6 CP.

[c]Reported as average on duplicate agar plates.

[d]*L. delbrueckii* spp. *bulgaricus*—reported in only one product. *L. acidophilus* – 1 of 4 CP had NVO. *Bifidobacteria*—1 of 4 CP had NVO and 1 product had no detectable counts at initial enumeration (week 0).

[e]Lower end of range are microbial counts for skim milk yogurt and higher end are for regular yogurt. Both products claimed to contain *L. casei*.

[f]*S. thermophilus* —Seen in 5 of 5 CP, "yogurt culture" claimed in all 5 CP. *L. bulgaricus*—Seen in 2 of 5 CP, "yogurt culture" claimed in all 5 CP. *L. rhamnous*—Claimed in 2 of 5 CP. *L. casei*—Claimed in 2 of 5 CP. *B. lactis*—Claimed in 4 of 5 CP. *L. acidophilus*—Claimed in 4 of 5 CP.

[g]*L. acidophilus*—Claimed in 24 CP. *Bifidobacteria*—Claimed in 18 CP. *L. casei*—Claimed in 8 CP.

[h]*L. acidophilus*—9 of 18 CP. *Bifidobacteria*—8 of 18 CP. *L. casei*—6 of 18 CP.

[i]Interpreted from graph.

[j]*L. acidophilus*—2 of 5 CP had NVO. *B. bifidum*—4 of 5 CP had NVO.

[k]Observed in 5 of 8 CP, claimed in all products.

Fermented Meats

Microbial counts for fermented sausages are shown in **Table 4**. In general, samples were either obtained from retail, directly from manufacturers, or were produced via industrial conditions. Most samples were from the United States, Spain, Portugal, and Italy and were composed of pork and/or beef. The levels of microorganisms (LAB and total) ranged from undetectable ($<10^2$ cfu/g) to 10^{10} cfu/g. Data were reported as either within the product shelf life or after ripening or maturation of the sausage. Counts of viable microorganisms in sausages from the United States were generally lower ($<10^7$ cfu/g) compared to sausages from other countries. In particular, LAB levels were all $<10^6$ cfu/g. In contrast, several of the European sausages contained high levels of LAB ($>10^8$ cfu/g.). European sausages were more often artisan sausages from smaller manufacturers, although similar microorganisms are used in comparison to sausages from the United States.

Fermented Vegetables

Microbial counts for fermented vegetables, including sauerkraut, olives, mustard pickles, pickles, and kimchi are summarized in **Table 5**. Fermented cucumbers products were also considered (listed as pickles). Laboratory-manufactured products, using industrial or traditional practices, were included due to the lack of literature on fermented vegetables from retail sources.

Microbial counts for sauerkraut were generally reported as LAB with counts ranging from 10^3 to 10^8 cfu/g. Reported samples were for sauerkraut originating from the United States, Finland, and Croatia. Levels of LAB and *Lactobacillus* were reported for olives produced in Italy, Greece, Portugal, Spain, and the United States. These products contained 10^4 to 10^8 cfu/g and were between 30 and 200 days.

Other products for which quantitative data were reported included mustard pickles and kimchi from Taiwan and pickled cucumbers from China, India, and the United States. Microbial counts ranged from undetectable ($<10^1$) to 10^8 cfu/g. For several of these products, levels of species (e.g., *Lactobacillus plantarum*, *Lactobacillus brevis*, and *Pediococcus cerevisiae*) were reported. Species of *Leuconostoc*, *Weissella* and *Lactobacillus* were also reported for Korean kimchi, where they were generally present between 10^7 and 10^8 cfu/g.

Traditional Asian Fermented Products

Another group of fermented foods that contain lactic acid bacteria and other bacteria are those products traditionally manufactured in Asia and that rely on grain or legume substrates. One important difference in the fermentation of these food products compared to other fermented foods is the reliance on fungal enzymes to convert complex carbohydrates to simple sugars. Aerobic conditions are another unique characteristic used in various parts of the fermentation process. Data were collected for several products, including miso, tempeh, fish sauce, and fermented fish (**Table 6**). Similar to the fermented vegetables, there were few reports on products from retail sources. Therefore, laboratory manufactured products made using industrial or traditional practices were included. In general, aerobic bacteria counts of miso ranged from 10^2 to 10^7 cfu/g. Similar bacterial

TABLE 2 | Organisms in commercial cultured dairy products separated by product.

Dairy product	Region	Source	Analyzed microorganisms	Initial Count (log cfu/mL or g)	Final Count (log cfu/mL or g)	Age	CP*	References
Amasi	South Africa	Retail	LAB	5.1–6.29	–	Did not specify	5	Moyane and Jideani, 2013
			Total bacteria count	3.62–4.96	–			
Cultured Buttermilk	Ethiopia[a]	Dairy farms and processing units	Lactococci	6.07–9.25	–	Does not specify	16	Gebreselassie et al., 2016
			Lactobacilli	6.07–8.61	–			
	India	Restaurant	Total viable count	6	–	Does not specify	1	Jayashree et al., 2013
	United States	Retail	Total bacteria count	7.3–8.64	6.08–7.24	After purchase and 7 days after	8	Vasavada and White, 1979
Fermented Milk	Argentina	Retail	*S. thermophilus*	9.11–9.49	–	Within shelf life	2	Vinderola and Reinheimer, 2000
			L. acidophilus	4.62–6.60	–			
	Spain	Commercially Manufactured	*S. thermophilus*	8.42	8.37	After manufacture and at shelf life (24 days)	50	Medina and Jordano, 1994
			L. bulgaricus	7.71	6.87			
			Bifidobacteria	6.87	6.62			
	Spain[b]	Retail	*S. thermophilus*	9	7	30 days	10	Gueimonde et al., 2004
			Lactobacillus	7–7.3	5.1–6.8			
			Bifidobacteria	5.6–7.5	4.1–7.6			
	Spain	Retail	*S. thermophilus*	9.27	–	Within shelf life (28 days)	1	García-Cayuela et al., 2009
			L. bulgaricus	7.64	–			
			L. acidophilus	6.65	–			
			L. casei	6.79	–			
			B. lactis	8.2	–			
Frozen Yogurt	France	Obtained from manufacturer[c]	*S. thermophilus*	8.19	–	5 weeks after manufacture	1	Lopez et al., 1998
	Spain	Obtained from manufacturer	*S. thermophilus*	7.57–7.58	–	1 week after manufacture	2	Lopez et al., 1998
			L. bulgaricus	4.29–6.79	–			
	United States	Variety of flavors soft/hard from retail and the manufacturer[d]	Total bacteria	<5.52–8.81	–	Does not specify	34	Kosikowski, 1981
		Vanilla flavors from retail[e]	LAB	6.11–9.32	–	Does not specify	10	Schmidt et al., 1997
		Variety of flavors from retail	Total viable bacteria	2.30–8.53	–	Within shelf life	19	Tieszen and Baer, 1989
Kefir	Greece[f]	Retail	Yeast	5	–	15 days before expiration	9	Kalamaki and Angelidis, 2017

(Continued)

TABLE 2 | Continued

Dairy product	Region	Source	Analyzed microorganisms	Initial Count (log cfu/mL or g)	Final Count (log cfu/mL or g)	Age	CP*	References
	Korea	Manufactured with commercial grain	LAB	9.62	–	After fermentation	–[g]	Kim et al., 2015
			Acetic acid bacteria	9.52	–			
			Yeast	7.67	–			
	Norway[b,h]	Obtained from TINE Meieret dairy company	*Leuconostoc*	7.1	6.3	After production and at expiration	5	Grønnevik et al., 2011
			Lactobacillus	8.1	6.4			
			Lactococcus	8.1	5.8			
			Yeast	3.3	3.9			
	Turkey	Retail	*Lactobacillus*	6.51–8.01	–	Does not specify	4	Kesmen and Kacmaz, 2011
			Lactococcus	7.53–8.30	–			
	United States[i]	Manufactured with commercial starter culture	*Lactobacillus*	9.15	–	After fermentation	–[g]	OBrien et al., 2016
			Lactococcus	9	–			
			Yeast	7.2	–			

CP, Commercial Products.

[a]*Analyzed sour cream buttermilk and sour milk buttermilk.*

[b]*Interpreted from graph.*

[c]*No significant decrease in S. thermophilus over time. L. bulgaricus was absent in this CP.*

[d]*Only 23 CP of 34 CP had viable organisms.*

[e]*NVO in 6 CPs (<1 log).*

[f]*Only viable counts seen in 8 of the 9 CPs.*

[g]*Lab-scale fermentation with commercial kefir grain/starter*

[h]*Presumptive (95:5 ratio) for lactobacillus and lactococcus.*

[i]*Reported as average from triplicate agar plates.*

TABLE 3 | Organisms in commercial cheese separated by product.

Cheese	Region (Type)	Source	Analyzed microorganisms	Count (log CFU/g)	Age	CP*	References
Afuega'l Pitu	Spain	Traditionally manufactured	Total viable bacteria count	8.06	60 days	2	Cuesta et al., 1996
			Lactococci	6.77			
			Leuconostocs	6.76			
			Lactobacilli	8.01			
Armada[a]	Spain	Traditionally manufactured	Aerobic Mesophiles	4.39–8.14	16 weeks	2	Tornadijo et al., 1995
			Lactococci	4.17–6.38			
			Lactobacilli	4.19–8.09			
			Leuconostocs	3.38–7.58			
Asiago	Italy (Asiago Allevo)	Commercial sample	Meso. streptococci	5.7	3–10 months	1	Gatti et al., 1999
			Therm. streptococci	8.9			
			Meso. lactobacilli	4.5			
			Therm. lactobacilli	7.2			
Blue Cheese[b]	United States	Retail	Total plate count	7.32	Within shelf life	1	Genigeorgis et al., 1991
Brie	Italy	Commercial samples	Meso. streptococci	5.3	1–2 months	1	Gatti et al., 1999
			Therm. streptococci	<3			
			Meso. lactobacilli	n.d.**			
			Therm. lactobacilli	<3			
	South Africa[a]	Commercially manufactured	LAB	7–8.8	8 weeks	8	Viljoen et al., 2003
Burgos	Spain	Retail	LAB	4.6–8.8	Time of purchase	36	Garcia et al., 1987
Cabrales	Spain	Obtained from manufacturers	Aerobic mesophiles	7.45–8.36	90 days	2	Flórez et al., 2006
			Lactococci	7.44–8.12			
			Lactobacilli	5.85–7.15			
			Leuconostoc spp.	5.40–6.14			
		Obtained from manufacturers[c,d]	Total viable count	6.8–7.9	120 days	2	Nuñez, 1978
			Streptococci	3.5–5.9			
			Leuconostocs	3–3.8			
			Lactobacilli	3.2–6.5			
			Yeast+Molds	4.1–7.2			
Camembert[a]	South Africa	Commercially manufactured	LAB	7.6–8.5	8 weeks	8	Viljoen et al., 2003

(Continued)

TABLE 3 | Continued

Cheese	Region (Type)	Source	Analyzed microorganisms	Count (log CFU/g)	Age	CP*	References
Cheddar	Ireland	Commercially manufactured	*L. paracasei*	8	39 weeks	3	Fitzsimons et al., 2001
	Ireland[c]	Obtained from manufacturer	NSLAB***	1.70–6.90	8 weeks	8	Jordan and Cogan, 1993
			NSLAB	6.15	52 weeks	2	
	U.S.[e]	Traditionally manufactured with commercial starter culture	*Lactobacillus*	5.1	180 days	–	Madkor et al., 2000
Colby[b]	United States	Retail	Total plate count	7.6	Within shelf life	1	Genigeorgis et al., 1991
Comte	France[f]	Obtained from manufacturer	*Lb. paracasei*	6.28–7.59	168–280 days	4	Depouilly et al., 2004
			Lb. rhamnosus	5.37–6.9			
	Switzerland[c,g]	Commercially manufactured	Thermophilic streptococci	6.75	24 weeks	3	Bouton et al., 1998
			Thermophilic lactobacilli	7			
			Facultative heterofermentative lactobacilli	7.5			
			Propionibacteria	7.75			
Danbo	Denmark	Industrially manufactured	*Lactococcus*	5.76	6 weeks	1	Gori et al., 2013
			Lactobacillus	5.82–5.87			
Edam	Egypt (Edam-like cheese)[h]	Manufactured with commercial starter culture	Total viable bacteria count	7.76	15 weeks	1	Ayana and El-Deeb, 2016
	Italy	Commercial samples	Meso. streptococci	2.9	1–2 months	1	Gatti et al., 1999
			Therm. streptococci	4.3			
			Meso. lactobacilli	5.8			
			Therm. lactobacilli	5.3			
Feta	Greece	Obtained from manufacturer[b]	LAB	6.1	60 days	1	Alexopoulos et al., 2017
		Retail[i]	*Lactobacillus*	5.95–7.19	>60 days	4	Rantsiou et al., 2008
			Lactococcus	4.18–< 5			
	Iran (Probiotic feta)	Commercially manufactured	*Lactobacillus acidophilus*	6.7	Did not specify	1	Mohammadmoradi et al., 2015
			Bifidobacterium lactis	6.7			
Fontina	Italy	Commercial sample	Meso. streptococci	8.3	3–10 months	1	Gatti et al., 1999
			Therm. streptococci	8.3			

(Continued)

TABLE 3 | Continued

Cheese	Region (Type)	Source	Analyzed microorganisms	Count (log CFU/g)	Age	CP*	References
			Meso. lactobacilli	4.6			
			Therm. lactobacilli	8.6			
	Italy (Fontal)	Commercial samples	Meso. streptococci	<3	1–2 months	1	Gatti et al., 1999
			Therm. streptococci	5.2			
			Meso. lactobacilli	<3			
			Therm. lactobacilli	4.4			
Galotyri[j]	Greece	Retail	Total viable count	8.03	Time of purchase	1	Samelis and Kakouri, 2007
			Lactobacilli	7.55			
			Lactococci	8.11			
Gorgonzola	Italy	Commercial sample	Meso. streptococci	3.5	3–10 months	1	Gatti et al., 1999
			Therm. streptococci	7.4			
			Meso. lactobacilli	3.1			
			Therm. lactobacilli	6.4			
		Obtained from manufacturer[d]	Total mesophilic bacteria	7.36–7.56	86 days	1	Gobbetti et al., 1997
			S. thermophilus	7.85–7.92			
			Lb. delbrueckii subsp. *bulgaricus*	3.67–5.77			
			Mesophilic lactobacilli	5.57–5.69			
			Lactococci	7.73–7.87			
			Mold	6.81–7.44			
Gouda	Belgium[k]	Commercially manufactured	Total microflora count	5.8	42 days	1	Messens et al., 1999
			LAB	7.1			
			Lactococcus lactis	6.1			
	Belgium (Bellie)[c]	Commercial starter culture	*Enterococcus*	6.45–6.90	12 weeks	1	Van Hoorde et al., 2008
			Lactobacillus	6.3–7.3			
			Lactococcus	7.2–7.7			
			Leuconostoc	7.4–7.6			
	Belgium (Dulses)[c]	Commercial starter culture	*Enterococcus*	6.40–6.55	12 weeks	1	Van Hoorde et al., 2008
			Lactobacillus	6.90–7.20			
			Lactococcus	7.50–7.70			
			Leuconostoc	7.60–7.90			
	South Africa	Commercially manufactured	*Lactobacillus*	8.96	32 days	1	Welthagen and Viljoen, 1998

(Continued)

TABLE 3 | Continued

Cheese	Region (Type)	Source	Analyzed microorganisms	Count (log CFU/g)	Age	CP*	References
			Lactococcus	9.1			
			Total plate count	9.04			
Gubbeen[l]	Germany[m]	Traditionally manufactured with commercial starter culture	Total bacterial count	7.3	16 days	1	Mounier et al., 2006
Grana Padano	Italy[n]	Commercially manufactured	*Lactobacillus*	4.94–6.22	9 months	1	Monfredini et al., 2012
			Lactococcus	3.15–6.05			
	Italy	Commercial samples	Meso. streptococci	<3	>1 year	3	Gatti et al., 1999
			Therm. streptococci	<3			
			Meso. lactobacilli	<3			
			Therm. lactobacilli	<3			
	Italy	Commercial samples	Meso. streptococci	<3	3 days ripened	1	Gatti et al., 1999
			Therm. streptococci	<3			
			Meso. lactobacilli	4.4			
			Therm. lactobacilli	7			
	Italy[f]	Obtained from manufacturer	*Lactobacillus*	4.53	13 months	6	Santarelli et al., 2013
			Total viable count	7.11			
Havarti	Denmark (Pasteurized milk havarti)	Traditionally manufactured	*Lactococcus*	5.69	12 weeks	1	Gori et al., 2013
			Lactobacillus	3.65–5.54			
	Denmark (Raw milk Havarti)	Traditionally manufactured	*Lactococcus*	7.56	12 weeks	1	Gori et al., 2013
			Lactobacillus	6.45–7.75			
Livarot	France	Retail	Total bacteria count	8.58	Does not specify	1	Mounier et al., 2009
			Yeast	6.38			
Limburger[b]	United States	Retail	Total plate count	7.98	Within shelf life	1	Genigeorgis et al., 1991
Manchego	Spain	Retail	LAB	4.6–10.03	Time of purchase	36	Garcia et al., 1987
		Manufactured with commercial starter culture[c]	*Lactococcus*	5.9	150 days	1	Poveda et al., 2003
			Lactobacillus	5.5			

(Continued)

TABLE 3 | Continued

Cheese	Region (Type)	Source	Analyzed microorganisms	Count (log CFU/g)	Age	CP*	References
Monterey Jack[b]	United States	Retail	Total plate count	>6.0–8.62	Within shelf life	4	Genigeorgis et al., 1991
Mozzarella	Italy	Commercial Samples	Meso. streptococci	6.3	<20 days	1	Gatti et al., 1999
			Therm. streptococci	7.6			
			Meso. lactobacilli	4.3			
			Therm. lactobacilli	<3			
	Italy (Buffalo milk)	Retail	LAB	4.82	Within expiration date	18	Pisano et al., 2016
	Italy (Mozzarella Bufala)	Commercial samples	Meso. streptococci	5.6	<20 days	1	Gatti et al., 1999
			Therm. streptococci	5.6			
			Meso. lactobacilli	4.8			
	Italy (Mozzarella Bufala Campana)	Local markets	LAB	4.0–7.8	Within shelf life	3	Devirgiliis et al., 2008
	Italy (Cow milk)	Commercially manufactured with commercial starter	Therm. lactobacilli	4.6	15 days	1	De Angelis et al., 2008
			Meso. lactobacilli	4.81			
			Streptococcus	7.85			
			Enterococcus	3.87			
	Italy (Cow milk)	Retail	LAB	7.08	Within expiration date	14	Pisano et al., 2016
Muenster[b]	United States	Retail	Total plate count	4.53	Within shelf life	1	Genigeorgis et al., 1991
Parmesan	Italy (Parmigiano Reggiano)	Obtained from manufacturer	LAB	7.52	150 days	15	Coppola R. et al., 2000
	Italy (Parmigiano Reggiano)	Commercially manufactured	LAB	6.18	2 months	1	Gatti et al., 2008
			LAB	2.3	24 months		
	Italy (Parmigiano Reggiano)	Commercial samples	Meso. streptococci	<3	>1 year	1	Gatti et al., 1999
			Therm. streptococci	<3			
			Meso. lactobacilli	<3			
			Therm. lactobacilli	<3			
Puzzone di Moena[o]	Italy	Traditionally manufactured	*Lactobacillus*	7.1–7.7	3 months	2	Franciosi et al., 2008
			Lactococcus	7.5–7.7			

(Continued)

TABLE 3 | Continued

Cheese	Region (Type)	Source	Analyzed microorganisms	Count (log CFU/g)	Age	CP*	References
Pecorino Romano	Italy	Commercial sample	Meso. streptococci	3.5	3–10 months	1	Gatti et al., 1999
			Therm. streptococci	5.5			
			Meso. lactobacilli	3.7			
			Therm. lactobacilli	3			
Provolone	Italy (Piquant provolone)	Commercial sample	Meso. streptococci	2.5–3.4	3–10 months	2	Gatti et al., 1999
			Therm. streptococci	5.4–8.3			
			Meso. lactobacilli	2.8–<3			
			Therm. lactobacilli	5.5–7.2			
	Italy (Sweet provolone)	Commercial sample	Meso. streptococci	<3–4.3	3–10 months	2	Gatti et al., 1999
			Therm. streptococci	4.5–7.1			
			Meso. lactobacilli	<3			
			Therm. lactobacilli	<3–7.1			
Queso Fresco[p]	Mexico	Obtained from manufacturer	Mesophilic streptococci	6.85–9.07	Within 5 days of manufacturer	6	Renye et al., 2008
			Thermophilic streptococci	5.04–9.02			
			Mesophilic lactobacilli	7.13–8.99			
			Thermophilic lactobacilli	5.01–9.01			
			Leuconostoc	5.86–9.23			
			Enterococcus	5.05–7.91			
Serrano[l]	Brazil	Retail	*Lactococcus*	8.60–9.10	Within shelf life	10	Delamare et al., 2012
			Lactobacillus	7.95–9.10			
Stilton	United Kingdom[q]	Retail	Mesophilic LAB	8.87	Within shelf life	16	Ercolini et al., 2003
			Lactobacillus	7.76			
			Mesophilic streptococci	8.97			
			Mesophilic, anaerobic LAB	8.85			
	United Kingdom (blue-veined raw milk cheese)[d]	Obtained from manufacturer	LAB	6.90–7.41	After aging (12 weeks)	1	Yunita and Dodd, 2018
			Lactobacillus	4.85–6.18			

(Continued)

TABLE 3 | Continued

Cheese	Region (Type)	Source	Analyzed microorganisms	Count (log CFU/g)	Age	CP*	References
			Lactococcus	7.83–8.65			
Swiss[c,r]	France	Traditionally manufactured	Propionibacteria	7.5–7.6	24 weeks	2	Demarigny et al., 1996
			Facultatively heterofermentative *Lactobacillus*	7.4–7.9			
			Thermophilic streptococci	3.0–5.6			
			Thermophilic lactobacilli	2.6–5.9			
Swiss Gruyere	Italy	Commercial sample	Mesophilic streptococci	<3	>1 year	1	Gatti et al., 1999
			Thermophilic streptococci	<3			
			Mesophilic lactobacilli	<3			
			Thermophilic lactobacilli	<3			
Tilsit	Austria	Obtained from manufacturer	Total bacterial count	8.4–9.7	21 days	13	Eliskases-Lechner and Ginzinger, 1995

*CP, Commercial Products.
**n.d., not determined.
***NSLAB, non-starter LAB count.
[a]Winter and summer cheese analyzed on surface and in center.
[b]Did not support L. monocytogenes surface growth when enumerated.
[c]Interpreted from graph.
[d]Surface and interior of cheese was analyzed.
[e]Lactobacillus count of control cheese (not adjunct culture added).
[f]Lb. rhamnosus and Lb. paracasei were the only microorganisms enumerated in all 4 CP.
[g]Average of CP.
[h]The control from an Edam-like cheese experiment of goat's diet.
[i]3 of 4 CP reported "not applicable" (<5 log cfu/g).
[j]Industrial Cheese with commercial starter cultures.
[k]Pressure treatment of 0.1 MPa.
[l]Only licensed cheeses analyzed.
[m]Measurement of bacterial growth on cheese surface.
[n]Grana Trentino cheese; Measurements from middle section and core.
[o]Winter and summer cheese at 30°C.
[p]Raw and pasteurized milk cheese.
[q]Reported as average of triplicate agar plates.
[r]Raw and microfiltered milk reported.

TABLE 4 | Organisms in commercial sausage products by region.

Country	Type	Source	Analyzed microorganisms	Count (log CFU/g)	Age	CP	References
France	Dry fermented sausage	Obtained from manufacturer	LAB	6.50–7.74	End of drying (9 weeks)	1	Chevallier et al., 2006
Greece	Dry fermented sausage	Obtained from manufacturer	LAB	7.63–8.20	28 days after formulation	1	Samelis et al., 1994
		Commercially produced[a]	LAB	8.1–8.2	End of curing period	2	Papamanoli et al., 2003
Italy	Ciauscolo salami	Commercially produced[a]	LAB	7.5	End of ripening (45 days)	1	Aquilanti et al., 2007
			Yeast	5.5			
		Obtained from manufacturer	Total bacteria	2.7–5.95	End of ripening	22	Silvestri et al., 2007
			LAB	6.77–8.65			
	Fermented Sausage, Friuli Venezia Giulia region	Commercially produced[a]	Total bacteria	6.1	End of ripening (45 days)	1	Cocolin et al., 2001
			LAB	8.3			
		Commercially produced[b]	Total aerobic count	6.62–9.11	End of ripening (21 days)	3	Comi et al., 2005
			LAB	8.39–8.47			
		Obtained from manufacturer	Total bacteria	4.19–9.11	End of maturation	3	Rantsiou et al., 2005
			LAB	8.34–8.78			
	Salami bergamasco	Obtained from manufacturer	Total bacteria	6–7.17	After maturation of 60 days	2	Cocolin et al., 2009
			LAB	9–9.14			
	Salami Brianza	Local markets	Mesophilic lactobacilli	8.6	After purchase	1	Di Cagno et al., 2008
	Salami cremonese	Obtained from manufacturer	Total bacteria	5.17–6.69	After maturation of 60 days	5	Capita et al., 2006
			LAB	7.54–9.38			
	Salami Mantovano	Obtained from manufacturer	Total bacteria	4.23–9.87	After maturation of 60 days	4	Capita et al., 2006
			LAB	7.6–9.38			
		Commercially produced[c]	Lactobacilli	8.01–8.73	End of ripening (60 days)	2	Pisacane et al., 2015
	Salami Milano	Obtained from manufacturer	LAB	8.0	End of ripening (60 days)	1	Rebecchi et al., 1998
	Salami Napoli	Obtained from manufacturer[a]	Mesophilic lactobacilli	6.7	End of ripening (30 days)	1	Coppola et al., 1995
		Commercially produced[d]	Mesophilic LAB	5.5	End of ripening (41 days)	1	Coppola S. et al., 2000
	Salami Piacentino	Local markets	Mesophilic lactobacilli	8.3	After purchase	1	Di Cagno et al., 2008
		Obtained from manufacturer[e]	LAB	8.02–8.84	End of ripening (63 days)	6	Połka et al., 2015
	Salami Piedmontese	Commercially produced	LAB	7.84	End of ripening (45 days)	1	Greppi et al., 2015
	Salami Varzi	Local markets	Mesophilic lactobacilli	8.6	After purchase	1	Di Cagno et al., 2008
	Salsiccia Basilicata[a]	Commercially produced	LAB	4–7.23	End of ripening (40 days)	10	Parente et al., 2001
			Yeast	6–6.6			
	Soppressata Basilicata[a]	Commercially produced	LAB	8–8.4	End of ripening (40 days)	9	Parente et al., 2001
			Yeast	5.2–7			
	Soppressata Molisana[a]	Commercially produced	LAB	8.4	End of ripening (28 days)	2	Coppola et al., 1998

(Continued)

TABLE 4 | Continued

Country	Type	Source	Analyzed microorganisms	Count (log CFU/g)	Age	CP	References
Spain and Portugal	Alheiras	Retail	LAB	5.9–10.5	Within shelf life	12	Capita et al., 2006; Ferreira et al., 2006
	Androlla	Obtained from manufacturer	Total aerobic mesophilic bacteria	7.81–9.52	After 20–30 days of ripening	20	García Fontán et al., 2007b
			LAB	8.78–9.36			
	Botillo	Obtained from manufacturer	Total aerobic mesophilic bacteria	7.63–9.37	After 15–20 days of ripening	15	García Fontán et al., 2007a
			LAB	8.34–9.56			
	Chorizo Ostrich	Retail	Total bacteria	7.3	Within shelf life	8	Capita et al., 2006
			LAB	6.23			
	Chorizo Deer	Retail	Total bacteria	5.46	Within shelf life	6	
			LAB	5.15			
	Chorizo Pork	Retail	Total bacteria	8.25	Within shelf life	18	
			LAB	8.46			
	Salchicón Ostrich	Retail	Total bacteria	6.09	Within shelf life	22	
			LAB	5.61			
	Salchicón Deer	Retail	Total bacteria	6.28	Within shelf life	8	
			LAB	6.26			
	Salchicón Pork	Retail	Total bacteria	8.09	Within shelf life	19	
			LAB	7.5			
United States	Dry salami	Retail	Total bacteria	3–6	Does not specify	11	Acton and Dick, 1976
			LAB	3–5			
	Genoa salami	Retail	Total bacteria	3–7	Does not specify	8	
			LAB	2–6			
	Lebanon bologna	Retail	Total bacteria	7–8	Does not specify	5	
			LAB	<3			
	Pepperoni	Retail	Total bacteria	4–7	Does not specify	14	
			LAB	2–6			
	San Francisco dry salami	Retail	Total bacteria	6–7	Does not specify	4	
			LAB	3–6			
	Semidry salami	Retail	Total bacteria	3–4	Does not specify	8	
			LAB	<2			
	Summer sausage	Retail	Total bacteria	3–4	Does not specify	19	
			LAB	4			
	Thuringer	Retail	Total bacteria	3–7	Does not specify	13	
			LAB	5–6			

[a] *Interpreted from graph.*
[b] *Three seasons were analyzed.*
[c] *Crespone casings and Gentile casings were used.*
[d] *Core and edge data reported.*
[e] *With and without commercial starter cultures.*

TABLE 5 | Organisms in fermented vegetables separated by product.

Product	Region (Type)	Source/Fermentation style	Analyzed microorganisms	Count (log cfu/mL or g)	Age	References
Kimchi	Taiwan[a]	Supermarkets	Aerobic bacteria	1–7.2	Within shelf life	Tsai et al., 2005
	South Korea	Industrially produced with a spontaneous fermentation[b,c]	*Leuconostoc citreum*	7.4	90 days	Cho et al., 2006
			Leuconostoc gasicomitatum	8		
			Weissella koreensis	8		
			Lactobacillus sakei	7.4		
		Retail (online and markets) with starter cultures and spontaneous fermentations	LAB	7.14–9.23	5 days after purchase	Kim et al., 2016
		Obtained from commercial distributors[b,d]	Total viable bacteria	7.9–8.3	4 weeks of fermentation	Lee et al., 2018
			LAB	7.8–8.3		
		Obtained from commercial distributors[b,e]	Total viable bacteria	7.9	4 weeks of fermentation	Lee M. et al., 2017
			LAB	7.8		
Mustard Pickles	Taiwan[f]	Supermarkets	Aerobic bacteria	<1.0–4.2	Within shelf life	Kung et al., 2006a
Olives	Greece (Conservolea naturally black olives)	Laboratory manufactured with a spontaneous fermentation	LAB count	7.9	30 days	Panagou et al., 2008
		Laboratory manufactured with a commercial starter culture	LAB count	8	30 days	Panagou et al., 2008
	Italy (Bella Di Cerignola -Debittered green table olives)[b,g]	Commercially manufactured with a spontaneous fermentation	LAB count	5.5	90 days	De Bellis et al., 2010
	Italy (Nocellara del Belice–Spanish-style green olives)[h]	Industrially manufactured with a spontaneous fermentation	Viable cell count	6.58–7.40	131 days	Aponte et al., 2012
			Lactobacillus	7.21–7.35		
			Lactic streptococci	6.49–6.95		
	Italy (Nocellara del Belice–green table olives)	Obtained from commercial manufacturer with spontaneous fermentation	LAB	4.53	7–10 months	Romeo et al., 2012
	Portugal (Galega and Cordovil)[b]	Laboratory manufactured with a spontaneous fermentation	Viable LAB count	4.9	150 days	Silva et al., 2011
	Southern Spain (Spanish-style green olives)[b]	Industrially manufactured with a spontaneous fermentation	*Lactobacillus*	5.5	120 days	Ruiz-Barba and Jiménez-Díaz, 2012
			Lactic cocci	NVO*	120 days	
		Industrially manufactured with commercial starter culture[b]	*Lactobacillus*	5.9	120 days	Ruiz-Barba and Jiménez-Díaz, 2012
			Lactic cocci	4	120 days	

(Continued)

TABLE 5 | Continued

Product	Region (Type)	Source/Fermentation style	Analyzed microorganisms	Count (log cfu/mL or g)	Age	References
	United States (Sicilian-style green olive–colossal Sevillano olives)[b]	Commercially manufactured with a spontaneous fermentation	LAB count	7.4	200 days	Golomb et al., 2013
Pickles	India[b,i]	Laboratory manufactured with a spontaneous fermentation	LAB	7.1	3 days	Singh and Ramesh, 2008
	United States[b,j]	Laboratory manufactured with a pure culture fermentation	*P. cerevisiae*	8.26–8.77	Did not specify	Etchells et al., 1964
			L. plantarum	8.72–8.96		
			L. brevis	7.79–8.45		
Sauerkraut	United States[b]	Commercially manufactured with starter culture	LAB	8.3	10 days	Johanningsmeier et al., 2004
			Heterofermentative LAB	2.7		
	United States[b]	Commercially manufactured with a spontaneous fermentation	Total microbial count	7	60 days	Lu et al., 2003
			LAB	7		
	Croatia[k]	Laboratory manufactured with a spontaneous fermentation	Total microbial count	6.04	42 days	Beganović et al., 2011
			LAB	3.79	42 days	
	Finland[l]	Large-scale manufacturing with a spontaneous fermentation	LAB	7.3	15 days	Viander et al., 2003

[*] *NVO, No viable organisms.*
[a] *20 commercial products.*
[b] *Interpreted from graph.*
[c] *Incubation of microorganisms were at 15°C.*
[d] *Three seasons were analyzed.*
[e] *19 out of 44 Chinese cabbage samples (88 total samples using other vegetables) were provided by commercial suppliers.*
[f] *14 CP (Commercial Products).*
[g] *Data from control set (no inoculation) with 8% NaCl.*
[h] *Olive from both irrigated and not irrigated fields.*
[i] *30 cucumber samples were used.*
[j] *Individual fermentations of each microorganism.*
[k] *Fermentations with 4% NaCl.*
[l] *Fermentations with 1.2% NaCl.*

counts were reported for fish sauce. LAB counts for tempeh and fermented fish were between 10^3 to 10^7 cfu/g with fermented fish being at the lower end of the range.

Fermented Cereals

Fermented porridges and gruels are widely consumed in many African countries. Here, studies were reported from Burkina Faso, Uganda, Ghana, Benin, Tanzania, and Mexico (**Table 7**). These cereals were made using pearl millet, millet, sorghum, and maize as starting grains. In general, the cereals contained LAB and mesophilic aerobic bacteria with a range of 10^5 to 10^9 cfu/g.

Beer

Several sour beer products from Belgium, such as lambic and gueuze, were included in the survey (**Table 8**). LAB counts were reported for these products, ranging from 10^2 to 10^5 cfu/g. The age of the products reported in the table refers to the longest time the beer was left to age. This maximum aging time was found to range from 40 days to 5 years across the different products.

Fermented Tea (Kombucha)

Kombucha is a fermented beverage made from sweetened tea to which a specialized culture is added. The latter is comprised of a *s*ymbiotic *c*ulture *o*f *b*acteria and *y*east or SCOBY, normally within a cellulose-type membrane. Bacteria commonly found in kombucha include the acetic acid bacteria belonging to the genera, *Acetobacter*, *Gluconacetobacter*, and *Gluconobacter*, as well as LAB. Most of the yeasts associated with kombucha are species of *Saccharomyces*, although other yeast genera may also be present (Teoh et al., 2004; Coton et al., 2017). While this product is now widely consumed, and manufacturers promote the presence of live microorganisms on product labels, there are few published data on the levels of microbes present in retail products. One recent study reported both bacterial and yeast counts for two kombucha products that were produced under industrial manufacturing conditions (Coton et al., 2017). In general, acetic acid bacteria levels ranged from 10^6 to 10^7 cfu/mL at the end of the fermentation, and similar counts were reported for LAB and total aerobic bacteria. Total yeast counts of about 10^7 cfu/mL were also reported.

DISCUSSION

Food-Associated Microbes Travel and Interact in the Gut

The human gastrointestinal tract is home to more than 10^{12} microbes. This diverse ecosystem provides protection against pathogens, extracts nutrients from dietary components, and modulates the immune system (Lozupone et al., 2013). The gut microbiota is also very stable, although several factors, including exposure to antibiotics, stress, and disease can disrupt this community, leading to dysbiosis (Sommer et al., 2017). The ability of diet and dietary components to modulate the gastrointestinal microbiota, redress dysbiosis, and enhance human health is now well- established (David et al., 2014; Graf et al., 2015; Sonnenburg and Bäckhed, 2016).

TABLE 6 | Organisms present in traditional Asian fermented products separated by product.

Product	Region (Type)	Source	Analyzed microorganism	Count (log cfu/g)	Age	References
Fermented Fish	Japan (Funazushi—fermented sushi)	Obtained from commercial manufacturer	LAB	3.48–5.43	Does not specify	Tsuda et al., 2012
Fish Sauce	Malaysia (anchovy)[a]	Obtained from commercial manufacturer	Aerobic bacteria	4.92–5.53	6–12 months	Zaman et al., 2010
Miso	Taiwan[b]	Supermarkets	Aerobic bacteria	2.1–7.1	Within shelf life	Kung et al., 2006b
	Japan	Laboratory manufactured with a spontaneous fermentation	Aerobic bacteria	4.3	15 weeks	Onda et al., 2003
Tempeh	Netherlands	Laboratory manufactured with industrial processes and a spontaneous fermentation[c]	LAB	7.01	Does not specify	Nout et al., 1987
		Shops, production places, and restaurants[d,e]	LAB	3–9	24 h after purchase	Samson et al., 1987

[a] *5 CP.*
[b] *27 CP (Commercial Products).*
[c] *Measure of tempeh and not the soak.*
[d] *81% of samples > 10^7 CFU/g.*
[e] *110 samples were analyzed.*

TABLE 7 | Organisms in commercial fermented cereals from Africa and Mexico.

Product (Region)	Source	Analyzed microorganisms	Count (log CFU/g)	Grain	CP	References
Ben-saalga (Burkina Faso)	Obtained from manufacturer	Total aerobic mesophiles	7.1	Pearl millet	12	Tou et al., 2006
		LAB	7			
		Yeast	5.5			
Bushera (Uganda)	Markets	LAB	8.1–8.4	Millet	5	Muyanja et al., 2003
		LAB	8.4	Sorghum	5	
		LAB	8.9–9	Millet and Sorghum	5	
Fura (Ghana)	Obtained from manufacturer	LAB	6.6–8	Does not specify	8	Owusu-Kwarteng et al., 2012
Koko Sour Water (Ghana)[a]	Obtained from manufacturer	LAB	8	Does not specify	3	Lei and Jakobsen, 2004
Mawè (Benin)	Market and manufacturer	Total aerobic mesophiles	8.8	Does not specify	30	Hounhouigan et al., 1993
		LAB	8.9			
		Yeast	6.4–6.9			
Pozol (Mexico)[b]	Market	Total bacteria	9.5	Does not specify	1	Omar and Ampe, 2000
		LAB	9			
Togwa (Tanzania)[c]	Obtained from manufacturer	LAB	9	Sorghum, maize, millet, and maize	36	Mugula et al., 2003
		Yeast	7			

[a]*Koko is porridge that have been heat treated. Koko sour water is the edible untreated water byproduct.*
[b]*Interpreted from graph. Measured outside and inside of sample in triplicate.*
[c]*Samples were obtained from manufacturer before fermentation.*

Among the food components known to influence the composition of the microbiota are fermentable fibers and prebiotics that enrich for particular members of the gut microbiota. Another route by which the gastrointestinal microbiota may be modulated is via consumption of probiotics—live microbes consumed at a dose sufficient to provide beneficial effects (Hill et al., 2014). Probiotics, however, are temporary members of the microbiome and rarely persist more than a few days (Tannock, 2003; Derrien and van Hylckama Vlieg, 2015; Zhang et al., 2016).

Perhaps the easiest and most common way to introduce potentially beneficial microbes to the gastrointestinal tract is via consumption of microbe-containing foods, and fermented foods and beverages, in particular. Like many probiotics, many microbes associated with fermented foods may also have the capacity to survive digestion, reach the gastrointestinal tract, and ultimately provide similar health benefits (Marco et al., 2017). When consumed regularly, these fermentation-associated microbes form what some researchers have called the "transient microbiome" (Derrien and van Hylckama Vlieg, 2015).

In general, the microorganisms present in fermented foods and beverages originate via one of two ways. For so-called natural or spontaneous fermented foods, the microorganisms are autochthonous and are naturally present in the raw material or manufacturing environment. To survive fermentation and processing, the LAB, yeasts, and any other microorganisms present in the finished product must manage a range of selective and competitive pressures, including salt, organic acids, ethanol, anaerobiosis, and low pH. Many of the fermented foods reviewed in this survey, including fermented cereals, sauerkraut, kimchi, and other fermented vegetables, and fermented soy-based products are made by natural fermentation. In addition, many wines and even some fermented sausages and beers are made in this manner.

Other fermented foods rely on the addition of a starter cultures. Cultured dairy products, cheese, and fermented sausages are commonly made using starter cultures. When cultures are used, their selection is based on the performance characteristics specific to the product. In addition, the incubation temperature during fermentation and the nutrient content are usually well-suited to the needs of the microorganisms. In many cases, the culture is added at such high inoculum levels, there would be little competition from other organisms. Collectively, most food fermentation microorganisms are well-adapted to the food environment.

In contrast, once the organisms present in fermented foods are consumed, they become foreign or allochthonous to the gastrointestinal tract. In most cases, they lack the physiological and biochemical resources to compete in this ecological niche. If they survive transit, they do not become stable members of this community (Zhang et al., 2016). Nonetheless, the presence of food fermentation-associated microorganisms in the GI tract, even if they are just "passing through," is now well-documented (Lee et al., 1996; Walter et al., 2001; Dal Bello et al., 2003; David et al., 2014; Derrien and van Hylckama Vlieg, 2015; Zhang et al., 2016; Lisko et al., 2017).

TABLE 8 | Organisms in commercial sour beer products.

Product	Region	Source	Analyzed microorganisms	Count (log CFU/g)	Age	References
Gueuze	Belgium	Obtained from a traditional brewery	LAB	5.25–5.31	2 years	Spitaels et al., 2015a
			LAB	3.87–3.88	4 years	
			LAB	3.49–3.96	5 years	
Lambic	Belgium	Obtained from a traditional brewery[a]	LAB	3.08–4.26	24 months	Spitaels et al., 2014
		Obtained from industrial brewery	LAB	4.33–4.38	12 months	Spitaels et al., 2015b
		Obtained from two breweries[b]	LAB	2.3–2.75	40 days	Martens et al., 1991

[a] *Incubated at 28°C aerobically or 20°C anaerobically on MRS agar.*
[b] *Interpreted from graph.*

Evidence of Health Benefits Associated With Fermented Foods

The evidence for the potential health benefits of fermented foods is based on numerous epidemiological as well as clinical reports (reviewed in Marco and Golomb, 2016; Kok and Hutkins, in press). In general, epidemiological studies have shown that consumption of fermented foods is associated with improvements of health status or reductions in disease risk. For example, yogurt-rich diets were associated with a reduced risk of metabolic syndrome in older Mediterranean adults (Babio et al., 2015). A similar finding was reported in another large cohort study that showed cultured milk consumption reduced the risk of bladder cancer (Larsson et al., 2008). Yogurt consumption has also been associated with reduced weight gain (Mozaffarian et al., 2011). Epidemiological data also suggests that consumption of other fermented foods may be correlated to beneficial health outcomes. Consumption of kimchi and other fermented vegetables, for example, correlated with reduced incidence of asthma and atopic dermatitis in Korean adults (Park and Bae, 2016; Kim et al., 2017). Reduced risks of type 2 diabetes and high blood pressure among Japanese adults was associated with consumption of fermented soybean foods rich in phytoestrogens and bioactive peptides (Kwon et al., 2010; Nozue et al., 2017). In contrast, the large European Prospective Investigation into Cancer and Nutrition cohort study from the Netherlands reported no association between fermented foods consumption and overall mortality (Praagman et al., 2015).

Although many human clinical studies have assessed the effects of probiotic-containing fermented foods on health biomarkers, fewer randomized controlled trials (RCT) have considered fermented foods alone. Nonetheless, several reports provide evidence that fermented foods, such as kimchi, fermented soy products, and yogurt, can improve relevant biomarkers. For example, kimchi consumption improved fasting blood glucose and other metabolic syndrome symptoms in overweight and obese adults (Kim et al., 2011), and similar improvements were observed in healthy adults (Choi et al., 2013). Consumption of a fermented soybean paste also improved plasma triglyceride levels in obese adults (Lee Y. et al., 2017). Perhaps the strongest evidence is for yogurt and improved lactose tolerance, due to *in vivo* expression and release of β-galactosidase by the yogurt culture microbes, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* (Kolars et al., 1984; Martini et al., 1987; Pelletier et al., 2001; Savaiano, 2014). This is the only approved health claim approved by the European Food Safety Authority (EFSA Panel on Dietetic Products, Nutrition and Allergies, 2010).

As noted previously, some fermented foods could impart health benefits even in the absence of live microorganisms in the finished products. For example, in sour dough bread manufacture, LAB may express phytase enzymes that degrade phytates and therefore enhance mineral absorption (Nuobariene et al., 2015). In the manufacture of red wine, ethanol produced early in the fermentation enhances extraction of polyphenolic compounds from the grape skins. Fermented foods may also contain vitamins and other bioactive molecules produced *in situ* from microbial metabolism that are not present in the original food. Recently, Saubade et al. (2017) noted that folic acid deficiency is a global health problem and suggested that fermented foods could be a food-based alternative for delivering folic acid to at-risk populations. Although some LAB are able to produce modest levels of folate (Leblanc et al., 2011), amounts produced in foods may be too low to be reach required levels (Saubade et al., 2017). Thus, selection of over-producing strains, as well as combining strains with non-LAB may be necessary to enhance production of this vitamin in foods.

If present, fermentation-derived microorganisms, despite their transient nature, may yet have the potential to influence gut microbiota diversity, structure, and function (Zhang et al., 2016). Notably, they may also affect health due to their ability to out-compete pathogens for resources, produce short chain fatty acids from available carbohydrates, secrete anti-microbial agents, contribute to immune homeostasis, and produce vitamins, *in situ* (Derrien and van Hylckama Vlieg, 2015).

The Number of Fermentation-Associated Microbes Depends on Region and Product Age

In this survey, we reviewed the literature for studies that included quantitative data on microorganisms present in commercial

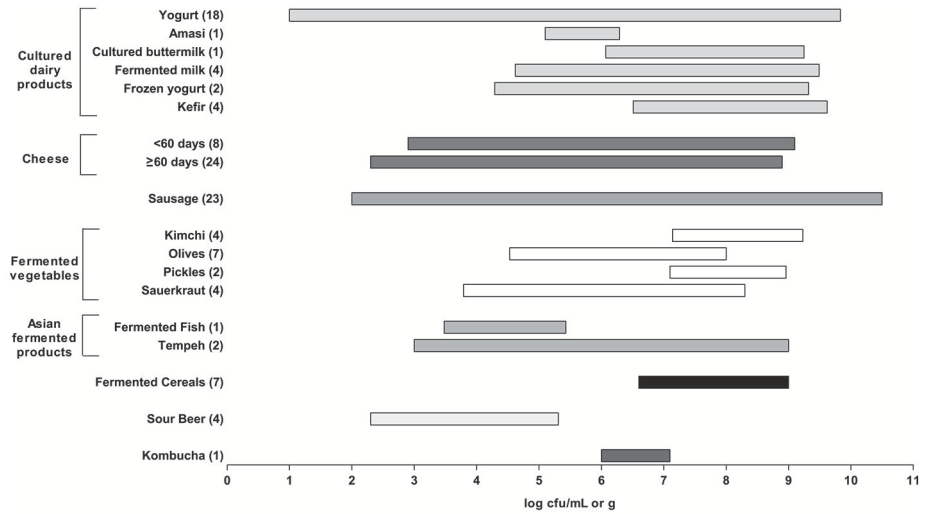

FIGURE 1 | Summary of lactic acid bacteria (LAB) counts in all fermented foods as reported in **Tables 1–8**. The bar plots represents a range (minimum to maximum) of counts found across the studies surveyed. The number of studies used here for each fermented food is shown in brackets. Products were excluded if they had no viable counts or when LAB counts were not reported. For yogurt, initial counts were used for products that had counts for more than one timepoint. For cheese, the products were divided by aging time (60 days) and were excluded if aging time was not reported.

fermented food products. To our knowledge, this is the first time that there has been a compilation of the hundreds of previous studies that enumerated microbes in fermented foods from retail samples or commercial products. In general, most of the products for which data were available contained at least 10^6 cells/mL or g. However, there was considerable variation depending on product age and region, and several relevant bacterial species or groups were present at less than that amount.

Although regular consumption of yogurt is often included in dietary guidelines (Smug et al., 2014), recommendations for other fermented foods rarely exist (Chilton et al., 2015). Likewise, to our knowledge, there are few guidelines for what constitutes a minimum dose of live microorganisms. The one exception is the yogurt health claim for "improved lactose tolerance" that was approved in 2010 by the European Food Safety Authority (EFSA Panel on Dietetic Products, Nutrition and Allergies, 2010). The claim states that yogurt should contain at least 10^8 cfu live starter microorganisms per gram- the same count the NYA requires for the "live and active" seal, as noted above.

Even in the absence of a seal or stamp, many commercial yogurt products, as well as kefir, fermented vegetables, and miso, also provide numerical information on their labels. Recently, Derrien and van Hylckama Vlieg (2015) suggested that consumption of 10^{10} cells would be necessary to induce an effect on the microbiota and host health. This could be achieved by consuming 100 g of fermented food containing 10^8 cells/g.

According to the results reported in this survey, many commercial fermented food products would be close to meeting this requirement (**Figure 1**). However, several caveats are relevant. First, there was a wide range of reported microbial counts (over several logs) within the various product groups. Some products also reported total LAB, whereas other reported specific genera or species or as thermophilic or mesophilic. Second, for most products, enumeration relied on standard cultural methods for LAB (including medium and incubation conditions), which may have under-estimated more fastidious species. This can be attributed to the high stress conditions of fermented products that can occasionally lead to injured microorganisms that are viable but not culturable.

Finally, the age or time at which the products were analyzed also varied considerably. In general, "fresher" products had higher numbers. These would include yogurt and cultured dairy products, as well as kimchi, sauerkraut, and other fermented vegetables. The counts from the cheeses also varied widely, with the longer aged cheeses (e.g., Parmesan, Grana) consistently having the lowest counts.

Recommendation of Fermented Foods as Part of Dietary Guidelines

In many cultures, fermented foods containing live microorganisms are consumed on a regular or even daily basis (Hutkins, 2018). Based on the data reported in this survey, consumption of fermented foods would not only provide important macronutrients, they could also deliver large numbers of potentially beneficial microorganisms to the gastrointestinal tract. For example, if Korean kimchi contains 10^8 lactic acid bacteria per g (**Table 5**), and given per capita consumption of kimchi is estimated at 100 g per person per day, then the daily consumption of live microbes from kimchi alone would be 10^{10}. Likewise, in the Netherlands, where yogurt consumption is also around 100 g per day, similar levels of microbes (i.e., 10^{10} cfu per day) would be ingested. These are the doses noted above that can influence the gut microbiota and provide a potential health benefit (Derrien and van Hylckama Vlieg, 2015).

Recently, the concept of "shared core benefits" was introduced to explain how and why phylogenetically related organisms

could deliver similar health benefits (Sanders et al., 2018). Thus, although the microbes in fermented foods cannot, by definition, be considered probiotic, many of them are evolutionarily highly related to probiotic organisms, and they often share the same molecular mechanisms responsible for health-promoting properties in probiotic organisms. The application of various omic approaches to understand functional properties of fermentation-derived microbes will also likely reveal new attributes relevant to the health benefits these microbes may provide (Macori and Cotter, 2018).

In part, this is why several prominent groups have recommended that health care professionals should promote fermented foods containing live microbes as part of public health policy (Ebner et al., 2014; Sanders et al., 2014; Chilton et al., 2015; Bell et al., 2017; Hill et al., 2017). In particular, including fermented foods in dietary guidelines for specific populations has also been recommended. For example, Bell et al. (2018) recently suggested fermented foods should be introduced to children early in life and incorporated into their everyday meal plans. In addition, regular consumption of fermented foods could be especially important for low income, resource-challenged communities that are disproportionally susceptible to gastrointestinal infections (Kort et al., 2015).

AUTHOR CONTRIBUTIONS

SR, CK, and RH each contributed 30% to data collection. MH contributed 10% to data collection. SR, CK, and RH wrote the manuscript.

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Novel Pathway for Corrinoid Compounds Production in *Lactobacillus*

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Vitamin B_{12} or cobalamin is an essential metabolite for humans, which makes it an interesting compound for many research groups that focus in different producer-strains synthesis pathways. In this work, we report the influence of key intermediaries for cobalamin synthesis added to the culture medium in two *Lactobacillus* (*L.*) strains, *L. reuteri* CRL 1098 and *L. coryniformis* CRL 1001. Here, we report that addition of Co^{2+} and 5,6-dimethylbenzimidazole increased the corrinoid compounds production in both strains while addition of L-threonine increased only the corrinoid compounds production by CRL 1001 strain. Then, we purified and characterized by LC-MS the corrinoid compounds obtained. Physiological studies besides *in silico* analysis revealed that *L. reuteri* CRL 1098 and *L. coryniformis* CRL 1001 follow different pathways for the last steps of the corrinoid compounds synthesis.

Keywords: lactic acid bacteria, *Lactobacillus*, corrinoid synthesis, cobalamin gene cluster, biosynthetic intermediaries

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INTRODUCTION

Vitamin B_{12} belongs to group B vitamins and is the most complex water-soluble vitamin. Many analogous of vitamin B_{12} have been described; all of them are porphyrin compounds with a common structure: a corrinoid ring contracted with a chelated cobalt ion at the center of the macrocycle. In this vitamin, the cobalt ion is covalently bound to an upper or β-ligand, and is coordinated with a lower or α-ligand (Rucker et al., 2001). In nature, the vitamin B_{12} analogs present either an adenosyl group or a methyl group as β-ligand. The best-studied vitamin B_{12} is cobalamin, a cobamide where 5,6-dimethylbenzimidazole (DMB) is the α-ligand aglycon (Johnson and Escalante-Semerena, 1992). In the vitamin B_{12} synthetic preparations, the cyano group is present as β-ligand in contrast to naturally occurring analogs. Furthermore, changes in the α-ligand result in different vitamin B_{12} analogs. Several vitamin B_{12} analogs containing benzimidazoles, purines, and phenolic compounds as bases of the α-ligand have been described (Chan et al., 2015).

De novo biosynthesis of all these corrinoid compounds can be divided into three main steps: (i) the Uroporphyrinogen III synthesis; (ii) the corrinoid ring synthesis, and (iii) the adenosylation, the amino-propanol arm attachment and the nucleotide loop bridging assembly of the lower ligand to the cobalt of the corrinoid ring core (Martens et al., 2002). Corrinoid ring synthesis follows two different pathways according to oxygen requirements. In the aerobic pathway, the cobalt chelation occurs as one of the final steps of the corrinoid ring formation while in the anaerobic pathway this reaction takes place in the first step of the synthesis (Warren et al., 2002).

Animals cannot synthesize vitamin B_{12}, which is involved in many important enzymatic reactions, thus making it an essential metabolite. These reactions are the conversion of homocysteine to methionine and the interconversion of (2R)-methylmalonyl-CoA to succinyl-CoA. Certain bacteria strains and archaea are able to synthetize vitamin B_{12} by *de novo* biosynthetic pathway (Roth et al., 1996). For this reason, many research studies are focused on the production of vitamins by bacteria in food and on improving the synthesis in large-scale production as well.

Currently, there are several *Lactobacillus* (*L.*) strains described as vitamin B_{12} producers, such as *L. reuteri* CRL 1098, *L. rossiae* DSM 15814, *L. coryniformis* CRL 1001 and *L. plantarum* BCF 20, BHM 10 and LZ 95 (Taranto et al., 2003; De Angelis et al., 2014; Torres et al., 2016; Bhushan et al., 2017) whose genomes have been partially sequenced.

Previous genome analysis of *L. reuteri* CRL 1098 and *L. coryniformis* CRL 1001 showed that both strains contain all necessary genes for *de novo* corrinoid compound biosynthesis. In this work, we put in evidence key differences in the last steps of the biosynthetic pathway of these strains. Besides, an increased corrinoid compounds synthesis was observed when adding certain intermediaries to the culture medium. These results were confirmed by HPLC quantification, and compounds were characterized by mass spectrometry. The expression profile of key genes of the biosynthetic pathway was also studied. Finally, we demonstrated the existence of two different biosynthetic pathways of cobalamin-type corrinoid compounds in *Lactobacillus* strains.

MATERIALS AND METHODS

Strains, Media, and Culture Conditions

Lactobacillus reuteri CRL 1098 and *L. coryniformis* CRL 1001 were previously described as cobalamin producer strains (Taranto et al., 2003; Torres et al., 2016). These strains belong to CERELA-CONICET culture collection. The strains CRL 1098 and CRL 1001 were grown overnight without shaking in Man-Rogosa-Sharpe (MRS) broth and in Vitamin B_{12} Assay Medium (Merck, Germany), at 37°C.

Salmonella (S.) Typhimurium AR 2680 was used as indicator strain in the bioassays for cobalamin determination in minimal A medium (NaCl, 0.5 g/l, Na_2HPO_4, 6 g/l; KH_2PO_4, 3 g/l, NH_4Cl 1 g/l; glucose, 4 g/l; $MgSO_4$, 2 mM, $CaCl_2$ 0.1 mM). This strain has two mutations in *metE* and *cbiB* genes. The AR 2680 strain was grown with aeration in Luria-Bertani (LB) broth at 37°C. As negative control, *L. plantarum* ATCC 8014 strain was used.

Cultures and Cell-Extracts

The biosynthetic intermediaries added to the Vitamin B_{12} Assay Medium were: 5-aminolevulinic acid [ALA; final concentration (FC): 25 ng/ml]; Cobalt Chloride ($CoCl_2$, FC: 250 ng/ml), 5,6-dimethylbenzimidazole (DMB; FC: 200 ng/ml); Porphobilinogen (PBG, FC: 250 ng/ml); L-threonine (L-Thr, FC: 50 μg/ml) and Uroporphyrinogen III (UIII, FC: 250 ng/ml). The corrinoids produced in the presence of different intermediaries were extracted as described by Torres et al. (2016).

Cobalamin Detection

The production of corrinoid compounds in the cell extract (CE) obtained from each strain grown for 16 h was analyzed with the bioassay using *S.* Typhimurium AR 2680 as indicator strain. *L. plantarum* ATCC 8014 and a commercial cyanocobalamin solution (0.5 μg/ml) were used as negative and positive control, respectively. In addition, the concentration of vitamin B_{12} was determined by commercial enzyme immunoassay (RIDASCREEN-FAST Vitamin B_{12}. R-Biopharm, Rhone Ltd., Glasgow, Scotland). The competitive immunoassay for the vitamin B_{12} determination was performed following the protocol described by the manufacturer.

Purification, Characterization, and Quantification of the Corrinoid Produced by *Lactobacillus* Strains

Based on previous results of our research group, key intermediaries (alone or in combination) were added to the growth culture media in order to increase the corrinoid concentration produced. The following conditions were used for *L. coryniformis* CRL 1001 and *L. reuteri* CRL 1098: (i) $CoCl_2$, (ii) $CoCl_2$ plus DMB, and (iii) $CoCl_2$ plus L-Thr only for CRL 1001 strain.

Then, the cells were broken and the corrinoid in the intracellular fraction (cell extract -CE-) was evinced by bioassay. The corrinoid produced was purified and quantified by RP-HPLC. The corrinoid concentration was calculated from the peaks areas and a commercial CN-Cbl standard curve made with 0.5, 1, and 2 μg/ml. The peaks of the chromatogram were collected for the mass spectrometry analysis. RP-HPLC and mass spectrometry were carried out as described by Torres et al. (2016). Experiments were performed three times in duplicate and results were expressed as means ± SD. Statistical analysis was conducted using MINITAB software (version 15 for Windows). Tukey's *post hoc* test was used to test for differences between the mean values. Significance was set at $P < 0.05$.

Relative Expression of Key Genes of Cobalamin Synthesis in *Lactobacillus* Strains

The primers for relative gene expression analysis (RT-qPCR) were designed on the basis of the corresponding gene sequences of *L. coryniformis* CRL 1001 (NZ_LNUL00000000.1) and *L. reuteri* CRL 1098 (NZ_LYWI00000000.1) using Primer Design tool (Bio-Rad) (**Table 1**). As normalizing reporter, *16S rRNA* gene was used.

Total RNA from *L. coryniformis* CRL 1001 and *L. reuteri* CRL 1098 grown in presence of the intermediaries were extracted at different growth phases (lag, exponential, and late exponential). Briefly, 10 mL of the cell pellet was suspended in 500 μL TE buffer (Tris–HCl 10 mM pH 8 – EDTA 1 nM pH 8) and added to 170 μL macaloid 2%, 500 μL TE buffer saturated with phenol-chloroform-isoamylic solution (chloroform-isoamylic 24:1, phenol-chloroform-isoamylic 1:1), 50 μL SDS 10% and 0,6 g glass beads (Sigma-Aldrich, Buenos

TABLE 1 | Primer sequences of key genes of *de novo* biosynthesis of cobalamin-type corrinoid compounds.

Strain	Gene	Forward primer (5′–3′)	Reverse primer (5′–3′)	Amplicon length (bp)
CRL 1098	*16S rRNA*	ACGTGCTACAATGGACGGTA	ACTAGCGATTCCGACTTCGT	120
	cbiF	TGCGTTCCTGGTGTTAGTTC	GTCCTGCCATACGCGTAATAA	106
	hemB	CGCGAAGTAGCTAGTGATGAA	CAACTAACGGCAGCAAAGTATG	115
	cobS	AAGCACATGGCGAATTATTG	TTCGTGACCTTTCGTCGAT	132
	cobT	AAACAGCAGCAGAAGTTGTTGGGC	CATTGCTCCAGCCATTGCACCTAA	182
CRL 1001	*16S rRNA*	GACGAAAGTCTGATGGAGCA	TTCTGGTTGGATACCGTCAA	122
	cbiF	AGTGTTTCTGGGACTTTGGC	CGGTATTCATGGGTGAAATG	146
	hemB	GATGGTATCGTGCAACAAGC	ACGACACCACAATGACCAGT	113
	cobS	TCTTCACGAACACCCGATAA	TTGCAATGGTGTTACCTGCT	141
	pduX	GTACGGCAGATCTAGTGGCA	GCATCGATCACAGTCAATCC	148
	cblS	TCAGTCGGTGTAACCGTGAT	GTCGCACTTCTTGGTAAGCA	148
	cblT	CAATTATGCATCAAGCCGTT	TGGTAAGGCCACGTAGACAA	98

Aires, Argentina). This suspension was subjected to Mini-BeadBeater-8 cell disrupter (BioSpec Products Inc.) at maximum speed in 10 cycles of 1 min each cycle, with intervals of 1 min on ice, to disrupt the cells. Subsequently, samples were centrifuged (5,000 × *g*, 15 min, 4°C). The upper phase was separated and added phenol-chloroform-isoamylic solution (chloroform-isoamylic 24:1, phenol-chloroform-isoamylic 1:1). Again, the samples were centrifuged (5,000 × *g*, 15 min, 4°C). After separating the upper phase, RNA precipitation was done with absolute ethanol and 3 M sodium acetate at −70°C overnight. Next day, samples were centrifuged (10,000 × *g*, 15 min, 4°C), washed with ethanol 70°, and dried at room temperature. RNA samples were suspended in 20 μL miliQ water. DNase treatment was performed adding 10 μL of DNase (Turbo Dnase, Ambion, Thermo Fisher Scientific, Buenos Aires, Argentina) followed by incubation at 37°C for 180 min. DNase was inactivated by adding 1 μl of RQ1 DNase Stop Solution (Ambion) to the reaction mixture and heating at 65°C for 10 min. After verifying the absence of DNA by conventional PCR using the purified RNA as template, the samples were quantified with a Qubit®2.0 fluorometer (Invitrogen™, Life Technologies Co., Carlsbad, CA, United States) using Qubit®HS RNA Assay Kit (Molecular Probes™, Life Technologies Co.). All RNA samples were stored at −70°C until use.

The cDNA was synthetized using 1 μg of total RNA and qScript™ cDNA SuperMix kit (Quanta Biosciences™) according to the manufacturer's instructions in a T100™ Thermal Cycler (Bio-Rad). A conventional PCR was performed to confirm cDNA synthesis. The cDNA was stored at −70°C until use.

Quantitative PCR assays was performed using an iQ™5 Multicolor Real-Time PCR Detection System iCycler (Bio-Rad Laboratories Inc.). Amplicons were detected with PerfeCta™ SYBER® Green SuperMix for iQ™ (Quanta Biosciences™). Each reaction contained 1X SYBER® Green SuperMix, 300 nM of each primer and 50 ng of total cDNA, genomic DNA as positive control and no template as negative control. All reactions were done in duplicate. The amplification program consisted of 1 cycle of 94°C for 5 min and 40 cycles of amplification (94°C for 1 min, 55°C for 1 min, and 72°C for 30 s) followed by a melting curve (81 cycles of 10 s at 60°C). The relative expression of the *cbiF*, *hemB*, *cobS*, *pduX*, *cblS*, and *cblT* genes for *L. coryniformis* CRL 1001 and *cbiF*, *hemB*, *cobS*, and *cobT* for *L. reuteri* CRL 1098 with the addition of different synthesis intermediaries at 3, 6, and 9 h was estimated according to the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001). The condition with the addition of $CoCl_2$ at 3, 6, and 9 h was used as control for each point of the growth curve for each condition. The reported values are the changes in the gene expression of the strain grown with the intermediaries and without (control, given value = 1) and normalized against *16S rRNA* gene expression.

RESULTS

In silico Comparative Genomic Analysis of Cobalamin Biosynthetic Gene Cluster

Lactobacillus reuteri CRL 1098 and *L. coryniformis* CRL 1001 are well-known cobalamin producer strains and they have gained attention for novel functional foods development. Previous *in silico* analyses of genome sequences of both strains revealed the presence of all the necessary genes for the *de novo* synthesis of cobalamin-type compounds although some differences in the cobalamin biosynthetic gene cluster were evidenced (**Figure 1A**). The genes *hemALBCD* encoding proteins involved in the first steps, the synthesis of uroporphyrinogen III from L-glutamyl-tRNA(glu), are present in both genomes. In addition, both strains produce the corrinoid ring by the anaerobic pathway and they bear all the *cbi* genes necessary for the complete ring formation. However, the last biosynthetic steps showed genes involved in different pathways for the aminopropanol arm formation and the assembly of the lower ligand.

The genome of *L. reuteri* CRL 1098 possesses *cobT* gene encoding for a nicotinate mononucleotide (NaMN): base phosphoribosyltransferase, which activates the lower ligand base. In contrast, *L. coryniformis* CRL 1001 does not own *cobT* gene but possesses the *cblTS* genes encoding for an α-ribasol transporter and a kinase protein, respectively, as described for other *Firmicutes* strains. The α-ribazole salvaging and α-ribazole-P synthesis were reported previously and presented as an alternative pathway of lower ligand activation

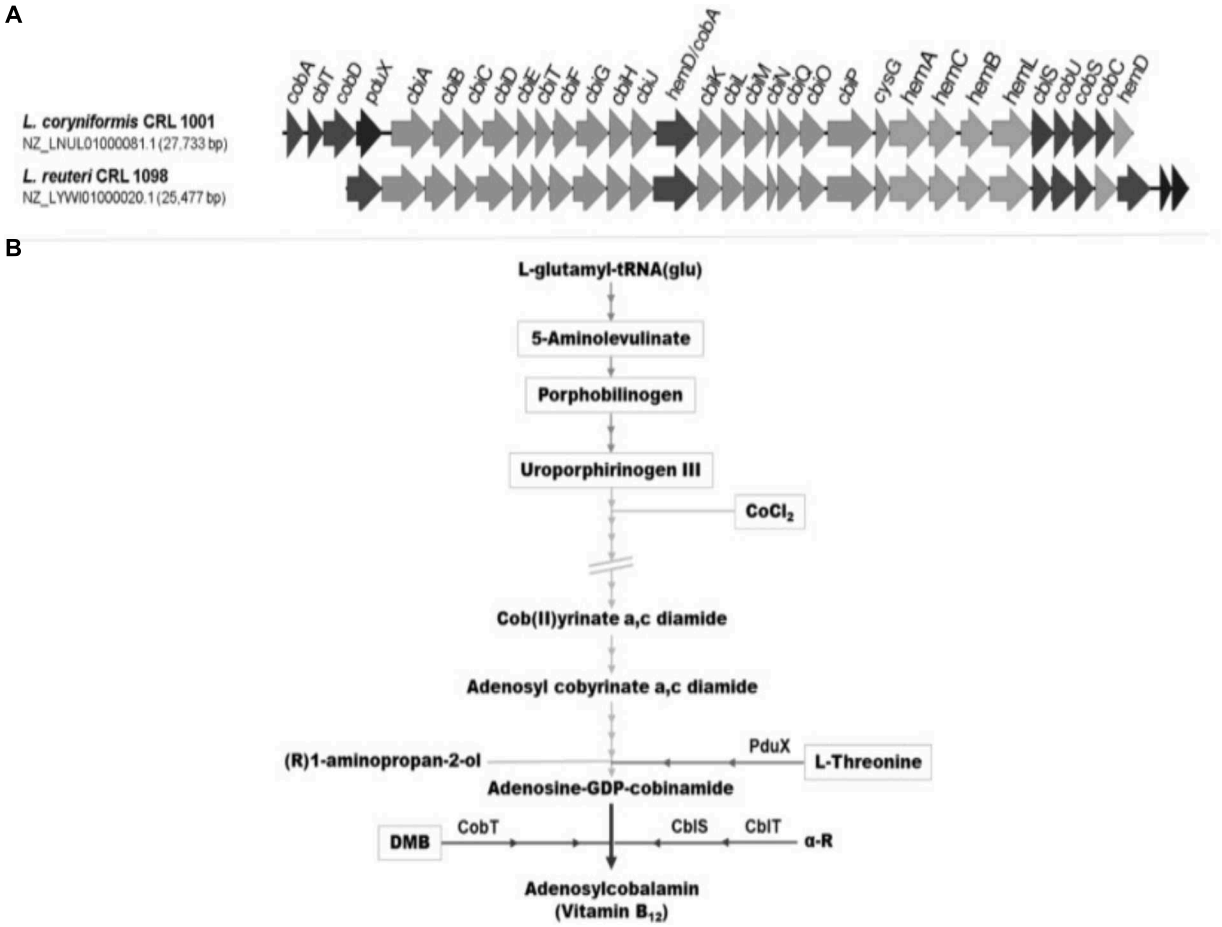

FIGURE 1 | (A) *cbi-cob-hem* gene clusters of *L. coryniformis* CRL 1001 and *L. reuteri* CRL 1098. **(B)** Simplified cobalamin anaerobic synthesis pathway. Addition of different pathway intermediaries are highlighted with a box.

(Gray and Escalante-Semerena, 2010). It is important to note that only *L. coryniformis* CRL 1001 harbors the *pduX* gene that encodes a kinase able to phosphorylate L-Thr. This compound is a precursor of the aminopropanol arm that binds the lower ligand to the corrinoid (**Figure 2**). The *in silico* studies point out differences in the set of genes involved in the cobalamin-type compounds synthesis. On this basis, the production of these compounds by the strains under study in the presence of different intermediaries was analyzed.

Effect of Biosynthetic Pathway Intermediaries on Corrinoid Compounds Production

Lactobacillus coryniformis CRL 1001 and *L. reuteri* CRL 1098 were grown in vitamin B_{12}-free culture medium in the presence of the following biosynthetic pathway intermediaries: ALA, PBG, UIII, $CoCl_2$, DMB plus $CoCl_2$, L-Thr plus $CoCl_2$. The sequential addition and roles of intermediaries have been shown in **Figure 1B**. The effect of DMB and L-Thr was evaluated in the presence of supplementary $CoCl_2$ considering that the addition of this ion is an essential condition for the synthesis of corrinoids in stages subsequent to the insertion of this element. The **Figure 3** show the relative quantification using a commercial enzyme immunoassay of total corrinoids in the presence of different intermediaries respect to the condition without intermediaries. The corrinoid production by *L. coryniformis* CRL 1001 was greater with the $CoCl_2$, DMB + $CoCl_2$ and L-Thr + $CoCl_2$ compared with the condition without intermediaries; ALA, PBG, and UIII did not improve the corrinoid synthesis in this strain. In *L. reuteri* CRL 1098, only DMB + $CoCl_2$ and $CoCl_2$ increased vitamin B_{12} production while the other intermediaries evaluated (L-Thr, ALA, PBG, and UIII) had no positive effect.

Purification, Quantification, and Characterization of the Corrinoid Compounds Produced by *Lactobacillus* Strains

Bioassay and immunoassay demonstrated that both $CoCl_2$ and DMB + $CoCl_2$ addition increased the production of corrinoid compound by *L. coryniformis* CRL 1001 and *L. reuteri* CRL 1098; while the presence of L-Thr + $CoCl_2$ induced the corrinoid

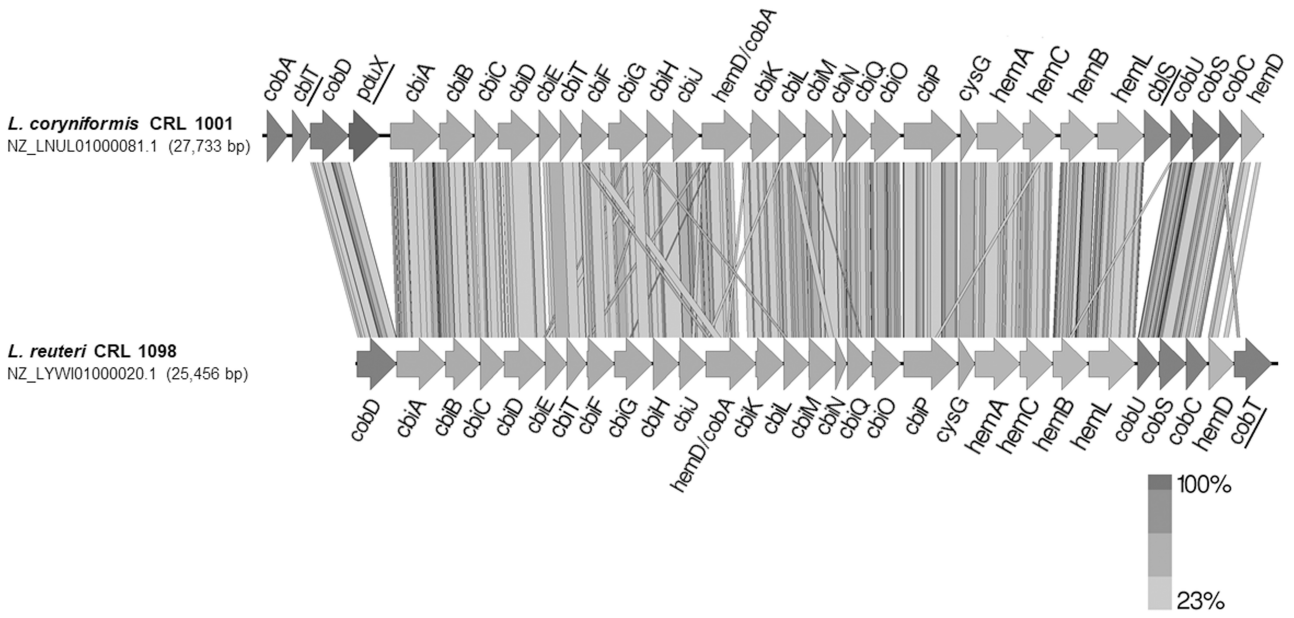

FIGURE 2 | *cbi-cob-hem* gene clusters comparison between *L. coryniformis* CRL 1001 and *L. reuteri* CRL 1098. Arrows indicate the transcription direction depict genes. Orthologous conserved genes are depicted as gray and black bars.

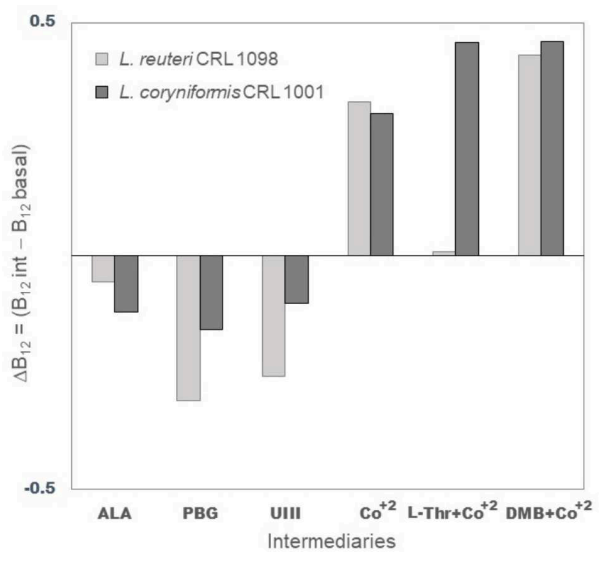

FIGURE 3 | Relative quantification of corrinoid compounds produced by *L. reuteri* CRL 1098 and *L. coryniformis* CRL 1001 with addition of different intermediaries. Results are presented as ΔB_{12} (net difference of the corrinoid quantification with [B12 int.] and without intermediary [B_{12}basal]). ALA (5-aminolevulinic acid), PBG (Porphobilinogen), UIII (Uroporphyrinogen III), $CoCl_2$ (Cobalt, Co^{+}), L-Thr (L-threonine) + $CoCl_2$, DMB (5,6-dimethylbenzimidazole) + $CoCl_2$.

production only in CRL 1001 strain. L-Thr was not evaluated in further assays for CRL 1098 strain as it had no effect on the corrinoid formation. To evaluate the optimum cobalamin production of CRL 1001 strain, a set of different growth conditions were evaluated (i) $CoCl_2$, (ii) $CoCl_2$ + DMB, and (iii) $CoCl_2$ + L-Thr. In subsequent experiments, the corrinoid production by *L. reuteri* CRL 1098 was evaluated only in the presence of $CoCl_2$ and $CoCl_2$ + DMB. In order to purify the corrinoid compounds, the peaks with retention time (RT) close to cyanocobalamin (CN-Cbl) RT (24.98 min) were collected (**Figure 4**) and analyzed for cobalamin activity by bioassay using *S.* Typhimurium AR 2680 as indicator strain. All the collected peaks showed the same B_{12} complementation ability than CN-Cbl standard (data not shown). Furthermore, higher corrinoid levels with DMB + $CoCl_2$ addition compared with the basal condition ($CoCl_2$ alone) were obtained. The corrinoid production in the presence of DMB was twofold and 2.2-fold greater for CRL 1098 and CRL 1001 strains, respectively, compared to the basal condition. The L-Thr + $CoCl_2$ addition to the medium increased by 2.6-fold the corrinoid synthesis in *L. coryniformis* CRL 1001 respect to the basal condition (**Table 2**).

To characterize the corrinoid compounds, liquid chromatography–electrospray ionization/tandem mass spectrometry (LC/ESI–MS/MS) to the collected peaks was performed. Transitions were sought in the MS and MS/MS spectra of the peaks with cobalamin activity. The transitions 678.3 (m/z) [M + 2H+]++ to 358.7 (m/z) and 678.3 (m/z) [M + 2H+]++ to 146.9 (m/z) were examined for corrinoid compounds where DMB is the aglycon attached to ribofuranose 3-phosphate. For corrinoid compounds where adenine is the aglycon attached to ribofuranose 3-phosphate, transitions 672.5 (m/z) [M + 2H+]++ to 347.8 (m/z) and 672.5 (m/z) [M + 2H+]++ to 135.9 (m/z) were sought. For all active peaks analyzed, the MS spectra indicated that a double charged ion with an approximately m/z of 673 [M + 2H+]++ was

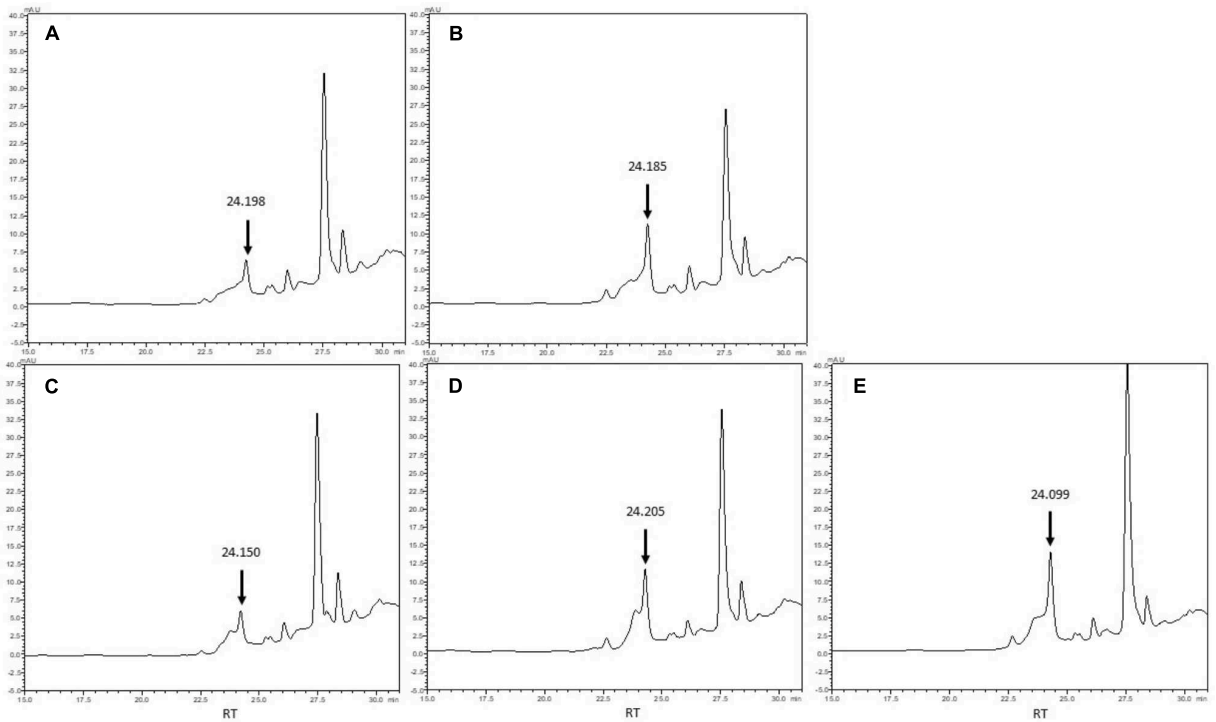

FIGURE 4 | RP-HPLC chromatogram of the cell extract (CE) from *L. reuteri* CRL 1098 (**A** $CoCl_2$ **B** DMB + $CoCl_2$) and *L. coryniformis* CRL 1001 (**C**- $CoCl_2$ **D**- DMB + $CoCl_2$ **E**- L-Thr + $CoCl_2$). An arrow indicates peaks corresponding to the corrinoid compound.

prominent. The MS/MS spectrum showed that the dominant ions with a value approximate m/z 347.8 [M + 2H+]++ were attributable to Coα-[α-(7-adenyl)]-Coβ-cyanocobamide (Pseudo B12). In this compound type, adenine is the aglycon attached to ribofuranose-3-phosphate in the lower ligand (**Figure 5**).

Expression of Key Genes of the Corrinoid Biosynthesis Pathway

Since the addition of biosynthetic intermediaries improved cobalamin production in both strains, the relative expression of key genes of the biosynthetic pathway in cells grown during 3 h (lag phase), 6 h (exponential phase), and 9 h (late-exponential phase) in B_{12} free medium in the presence of different intermediaries was tested by qPCR. The time of incubation (3, 6, and 9 h) in the B_{12} free medium with $CoCl_2$ was the condition used as reference, to which the arbitrary value of 1 was assigned for both strains. Basal level of expression of key genes in the vitamin B_{12} free medium with different intermediaries was observed for both strains at all growth phases. The relative expression levels of the key genes with DMB and L-Thr addition remained unchanged in the strains under study after 3, 6, and 9 h of incubation with respect to the basal condition (data not shown). These data demonstrate that the key genes studied for *L. reuteri* CRL 1098 and *L. coryniformis* CRL 1001 may be regulated at a post-transcriptional or translational level.

TABLE 2 | Corrinoid compounds quantification by HPLC.

L. reuteri CRL 1098	Intermediaries	Corrinoid (μg/mL)[#]
	$CoCl_2$	1.28 ± 0.26*
	DMB + $CoCl_2$	2.52 ± 0.20
L. coryniformis CRL 1001	Intermediaries	Corrinoid (μg/mL)
	$CoCl_2$	1.34 ± 0.18
	DMB + $CoCl_2$	2.98 ± 0.43
	L-Thr + $CoCl_2$	3.45 ± 0.48

*The corrinoid concentration in CEs was calculated with a commercial CN-Cbl standard curve and the active peaks areas. [#]Corrinoid concentration in CEs was calculated with a commercial CN-Cbl standard curve and the active peaks areas. *Standard deviation.*

DISCUSSION

We have previously reported that *L. reuteri* CRL 1098 supplementation efficiently correct the nutritional vitamin B12 deficiency in an *in vivo* model (Molina et al., 2008, 2009). In this work, cobalamin biosynthesis intermediaries were added to the vitamin B_{12} free medium to analyse their effect on the corrinoid production by two different *Lactobacillus* strains.

The common precursor of all tetrapyrrole molecules (cobalamin, heme, and chlorophyll) is ALA. The result of two condensed ALA molecules is PBG. Finally, four PBG molecules are polymerized and cyclized to form UIII, the last common intermediary (Kang et al., 2012). Mohammed et al. (2014) reported an increase in vitamin B_{12} production by *Bacillus*

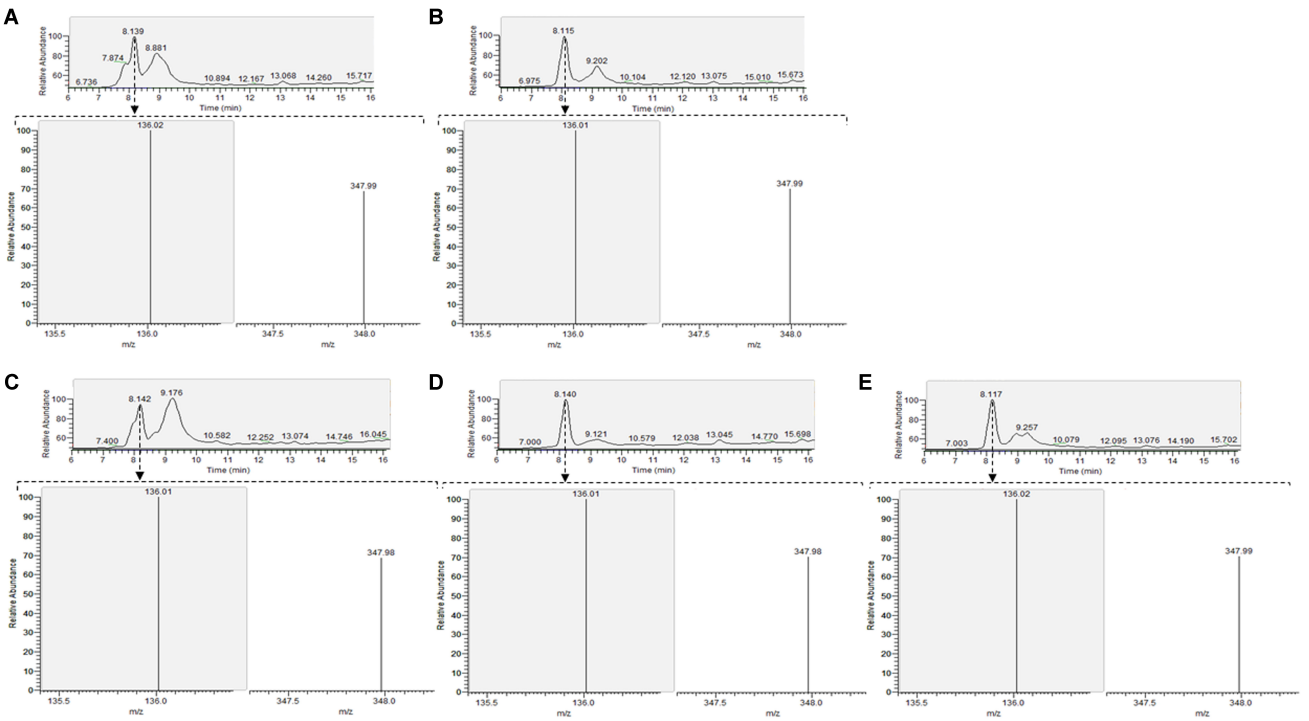

FIGURE 5 | Liquid chromatography–electrospray ionization/tandem-mass spectrometry (LC/ESI–MS/MS) chromatograms of peaks with cobalamin activity. The transitions are shown together (SRMs, 672.5 m/z – > 136.0 m/z calculated for adenine y 672.5 m/z – > 348.0 m/z correspond to the lower ligand in which adenine is the aglycon attached to ribofuranose 3-phosphate). Total ion chromatogram (TIC) of the active peaks of *L. reuteri* CRL 1098 (**A**- $CoCl_2$ **B**- DMB + $CoCl_2$) and *L. coryniformis* CRL 1001 (**C**- $CoCl_2$ **D**- DMB + $CoCl_2$ **E**- L-Thr + $CoCl_2$).

(*B.*) *megaterium* with addition of ALA to the culture medium (Mohammed et al., 2014). Different results were obtained for both *L. reuteri* CRL 1098 and *L. coryniformis* CRL 1001 strains, in which the decreased corrinoid compound production with ALA, PBG, and UIII addition may be due to a negative regulation by a classical feedback control (Robin et al., 1991).

Regarding $CoCl_2$ and DMB addition, an increased cobalamin production is described for *Propionibacterium* and *Bacillus* strains (Mohammed et al., 2014; Wang et al., 2015). Similar results are reported for both *Lactobacillus* strains, CRL1001 and CRL1098, in the present study.

In the anaerobic pathway of the cobalamin biosynthesis, precorrin-2 is chelated with cobalt a reaction that is catalyzed by CbiK enzyme. Cobalt requirements for cobalamin-type corrinoids synthesis is proposed as screening criteria for detecting producer strains of *Propionibacterium* and *Lactobacillus* genera (Seidametova et al., 2004; Bhushan et al., 2016). In this work, we report that cobalt addition as chloride salt allowed the growth of both CRL 1098 and CRL 1001 *Lactobacillus* strain. Moreover, the cobalt addition also increased the corrinoid production in both strains compared with our previous data without cobalt addition (Taranto et al., 2003; Torres et al., 2016).

In another step of the biosynthesis, DMB is the nucleotide attached to the aminopropanol arm as lower ligand in the cobalamin molecule (Hazra et al., 2015). The cobalamin-type corrinoid compounds produced by CRL 1001 and CRL 1098 strains increased with DMB addition. Nevertheless, LC-MS results were different as expected since the molecules synthesized corresponded to pseudo-B_{12} despite the positive regulation on the synthesis. This compound, with adenine instead of DMB as lower ligand base is synthesized in most bacteria able to produce cobalamin-type corrinoid compounds via the anaerobic pathway (Taga and Walker, 2008). Hazra et al. (2013) proposed that CobT enzyme of *L. reuteri* CRL 1098 expressed in *E. coli* activates DMB rather than other molecules as base of the lower ligand (Hazra et al., 2013). According to our results, CRL 1098 strain is not able to use DMB instead of adenine for vitamin B_{12} synthesis. As previously published, *L. coryniformis* CRL 1001 does not have the *cobT* gene but the genes encoding CblS (a kinase enzyme) and CblT (a transporter protein) (Torres et al., 2016). Mattes and Escalante-Semerena (2017) observed that *cblST* genes of *Geobacillus* (*G.*) *kaustophilus* expressed in *S. enterica* Δ*cobT* that CblT transports DMB into the cell and CblS activate preferably ribazole over adenine (Mattes and Escalante-Semerena, 2017). Our results are not in agreement with this statement since pseudo-B_{12} is obtained, thus suggesting that CblS is unable to phosphorylate DMB despite entering the cell. Further studies are oncoming in both strains for a better understanding of the changes taking place in the lower ligand nucleotide of the molecule.

In a different way to the previously described, the L-Thr addition to the vitamin B_{12} free medium showed different results in *L. reuteri* CRL 1098 and *L. coryniformis* CRL 1001 strains. The increased production of cobalamin-type corrinoid compounds in CRL 1001 strain could be ascribed to the presence of *pduX* gene in the genome (Torres et al., 2016). The *pduX* encodes a protein kinase able to phosphorylate free L-Thr prior to the formation of lower ligand aminopropanol arm (Fan and Bobik, 2008). For this reason, we propose that L-Thr may exert some positive regulation in the synthesis of this type of compounds.

The study of the expression profile of key genes encoding proteins involved in cobalamin-type corrinoid compound synthesis showed no expression change despite the increased cobalamin production in both strains. Data obtained by different work groups showed that cobalamin synthesis is regulated by a riboswitch mechanism (Nahvi et al., 2004). Briefly, binding of cobalamin to the *cob* mRNA inhibits the initiation of translation and stabilizes the complex formed between them (Polaski et al., 2016). For these reasons, we suggest that the regulation is at the post-transcriptional or translational level. Our results are in agreement with previous reports since no changes in regulation of key synthesis genes of the mRNA transcription phase were evidenced (Nahvi et al., 2004).

In this study, we demonstrate that addition of key intermediaries to the vitamin B_{12} free medium increases the corrinoid production by both CRL1001 and CRL 1098 lactobacilli strains. In addition, the existence of two different cobalamin-type corrinoid compound biosynthetic pathways in two close phylogenetic strains was confirmed. The results obtained in this study would improve the production of these cobalamin-type corrinoid compounds by the food grade *L. coryniformis* CRL 1001 and *L. reuteri* CRL 1098 strains.

AUTHOR CONTRIBUTIONS

AT carried out biochemical and molecular genetic studies and participated in the drafting the manuscript. VV participated in physiological studies. GF participated in the discussion of the study. LS participated in the design, discussion, and coordination of this study and drafting the manuscript. MT carried out the coordination of this study and participated in the design, discussion, and drafting the manuscript. All authors read and approved the final manuscript.

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Diversity of Yeasts and Molds by Culture-Dependent and Culture-Independent Methods for Mycobiome Surveillance of Traditionally Prepared Dried Starters for the Production of Indian Alcoholic Beverages

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Marcha, *thiat*, *dawdim*, *hamei*, *humao*, *khekhrii*, *chowan*, and *phut* are traditionally prepared dried starters used for production of various ethnic alcoholic beverages in North East states of India. The surveillance of mycobiome associated with these starters have been revealed by culture-dependent methods using phenotypic and molecular tools. We identified *Wickerhamomyces anomalus*, *Pichia anomala*, *Saccharomycopsis fibuligera*, *Pichia terricola*, *Pichia kudriavzevii*, and *Candida glabrata* by ITS-PCR. The diversity of yeasts and molds in all 40 samples was also investigated by culture-independent method using PCR-DGGE analysis. The average distributions of yeasts showed *Saccharomyces cerevisiae* (16.5%), *Saccharomycopsis fibuligera* (15.3%), *Wickerhamomyces anomalus* (11.3%), *S. malanga* (11.7%), *Kluyveromyces marxianus* (5.3%), *Meyerozyma* sp. (2.7%), *Candida glabrata* (2.7%), and many strains below 2%. About 12 strains of molds were also identified based on PCR-DGGE analysis which included *Aspergillus penicillioides* (5.0%), *Rhizopus oryzae* (3.3%), and sub-phylum: *Mucoromycotina* (2.1%). Different techniques used in this paper revealed the diversity and differences of mycobiome species in starter cultures of India which may be referred as baseline data for further research.

Keywords: mycobiome, dried starters, PCR-DGGE analysis, yeasts, filamentous molds

INTRODUCTION

Essence of alcoholic fermentation depends on different types of starters that copulate the uniqueness to organoleptic segmentations for ethnic values (Hesseltine, 1983; Steinkraus, 1996; Tamang et al., 2016b). Yeasts have several economic significances and have been used for centuries in the production of fermented foods and alcoholic beverages (Fleet, 2003; Tamang and Fleet, 2009;

Jolly et al., 2017). In Asia, preparation of amylolytic (related to conversion of starch to sugar) and alcoholic (production of alcohol) starter is an innovative back sloping technique of cultivation of native microbiota in the form of dry, flattened, or round balls made up of rice/wheat for production of different traditional alcoholic beverages (Tamang, 2010), locally known as *marcha* in India, Nepal and Bhutan, *benh men* in Vietnam, *bubod* in the Philippines, *chiu/chu/daque* in China and Taiwan, *loogpang* in Thailand, *ragi* in Indonesia, and *nuruk* in Korea (Tamang, 2016). Traditional methods of preparation of Asian amylolytic dry starters are similar with slight variation in terms of wrapping materials, incubation period, size, and shapes of particular starters. Ethnic people practicing the age-old traditional preservation or sub-culturing amylolytic and alcohol-producing as well as flavor-enhancing fungi and bacteria have attracted many researchers to study the microbial diversity in such starters. In recent years, few researchers have reported the fungal and bacterial species using both culture-dependent and-independent techniques in some common starter cultures of Asia such as *marcha* of India (Tsuyoshi et al., 2005; Sha et al., 2017), *daqua* of China (Wang et al., 2008; Zheng et al., 2012; Lv et al., 2013; Chen et al., 2014; Xu et al., 2017), *benh men* of Vietnam (Dung et al., 2007; Thanh et al., 2008); *nuruk* of Korea (Jung et al., 2012), and *dombea* of Cambodia (Ly et al., 2018).

North East regions of India[1] have several varieties of traditionally prepared and sun-dried starters prepared by different linguistic ethnic groups of people that include *marcha* of Sikkim, *humao* of Assam, *hamei* of Manipur, *chowan* of Tripura, *thiat* of Meghalaya, *khekhrii* of Nagaland, *dowdim* of Mizoram, and *phut* of Arunachal Pradesh (**Figure 1**). These starter cultures except *khekhrii* of Nagaland are traditionally prepared from soaked rice with some wild herbs, and then mixed with previously prepared starter powder (1–2%) as an inoculum (back-sloping). The mixtures are ground in a wooden mortal with addition of water to make a thick dough which are kneaded into round to flattened balls/cakes of different size and shape. Dough cakes are covered with fern fronds/paddy straws/jute sags, fermented at room temperature for 1–3 days; and fresh balls/cakes are sun dried for few days (Anupma et al., 2018). *Khekhrii* of Nagaland is prepared by naturally fermenting sprouted-rice grains and then sun-dried to use as dry starter culture to prepare *zutho*, local alcoholic beverage. Some species of yeasts *Saccharomycopsis fibuligera*, *S. capsularis*, *Pichia anomala*, *P. burtonii*, *P. guilliermondii*, *P. fabianii*, *Trichosporon* sp., *Candida tropicalis*, *C. parapsilosis*, *C. montana*, *C. glabrata*, *Torulaspora delbrueckii*, *Saccharomyces cerevisiae*, *S. bayanus*, and *Wickerhamomyces anomalus* were previously reported from some samples of *marcha* and *hamei* of India (Hesseltine and Kurtzman, 1990; Tamang and Sarkar, 1995; Tsuyoshi et al., 2005; Jeyaram et al., 2008, 2011, Sha et al., 2016, 2017).

One of the common methods for culture-dependent identification is by the analysis of the Internal Transcribed Spacer (ITS)1-5.8S-ITS2 region, which is widely applied in explorations of diversity of fungi associated with many traditional fermented foods (Caggia et al., 2001; Las Heras-Vazquez et al., 2003). ITS analysis may provide the fast and easy means for accurate identification at species level (Esteve-Zarzoso et al., 1999), due to greater sequence variation, the ITSl/ITS2 domains are more suited for species and strain identification than the 18s region (small subunit), the 5.8s region, and the 28s region (large subunit) (Iwen et al., 2002; Korabecna, 2007; Susan Slechta et al., 2012). However, the culture-dependent methods may not detect the whole microbial community in foods (Ercolini, 2004). The culture-independent methods such as PCR denaturing gradient gel electrophoresis (DGGE) analysis, are highly useful to detect the whole microbial communities in food samples (Chen et al., 2014; Puerari et al., 2015; Tamang et al., 2016a). PCR-DGGE analysis method has been designed to profile microbial communities directly from substrates including fermented foods, and is based on sequence-specific distinctions of 16SrRNA and 26SrRNAmplicons (Cocolin et al., 2000; Ercolini, 2004; Ercolini et al., 2004; Alegría et al., 2011).

No studies have been conducted on traditionally prepared starters of India except *marcha* (Tamang and Sarkar, 1995; Tsuyoshi et al., 2005; Sha et al., 2016, 2017), and *hamei* (Tamang et al., 2007; Jeyaram et al., 2008, 2011). Based on our preliminary analysis of microbial load in traditionally prepared starters of North East India, fungi mostly yeasts and filamentous molds ($>10^6$ cfu/g) predominate over bacteria. Hence, we aimed to study the mycobiome diversity in dried starters of India by culture-dependent and -independent methods to underline the continuous interest in the characterization of microbial consortia associate to poorly studied food fermentations to isolate new potential pro-technological and functional strains, to improve the conservation of microbial diversity, to characterize and limit spoilage microbes, microbial producers of toxic compounds, and pathogens (Capozzi and Spano, 2011; Russo et al., 2016; Tamang et al., 2016a,b; Gonelimali et al., 2018).

MATERIALS AND METHODS

Sample Collection

Forty different samples of traditionally prepared starter (five samples of each starter) *marcha* of Sikkim, *thiat* of Meghalaya, *hamei* of Manipur, *phut* of Arunachal Pradesh, *chowan* of Tripura, *dawdim* of Mizoram, *humao* of Assam, and *khekhrii* of Nagaland were collected immediately after the preparation (fermentation and sun-dried drying) from local people of eight states of North East India, and were transferred to gamma irradiated sterile bottles, sealed, and stored in desiccator at room temperature for the further analysis.

Isolation of Microorganisms

Ten grams of sample was homogenized with 90 ml of 0.85% (w/v) sterile physiological saline in a stomacher lab-blender 400 (Seward, United Kingdom) for 1 min and serially diluted in the same diluents. Yeasts were isolated on

[1]http://www.northeasttourism.gov.in

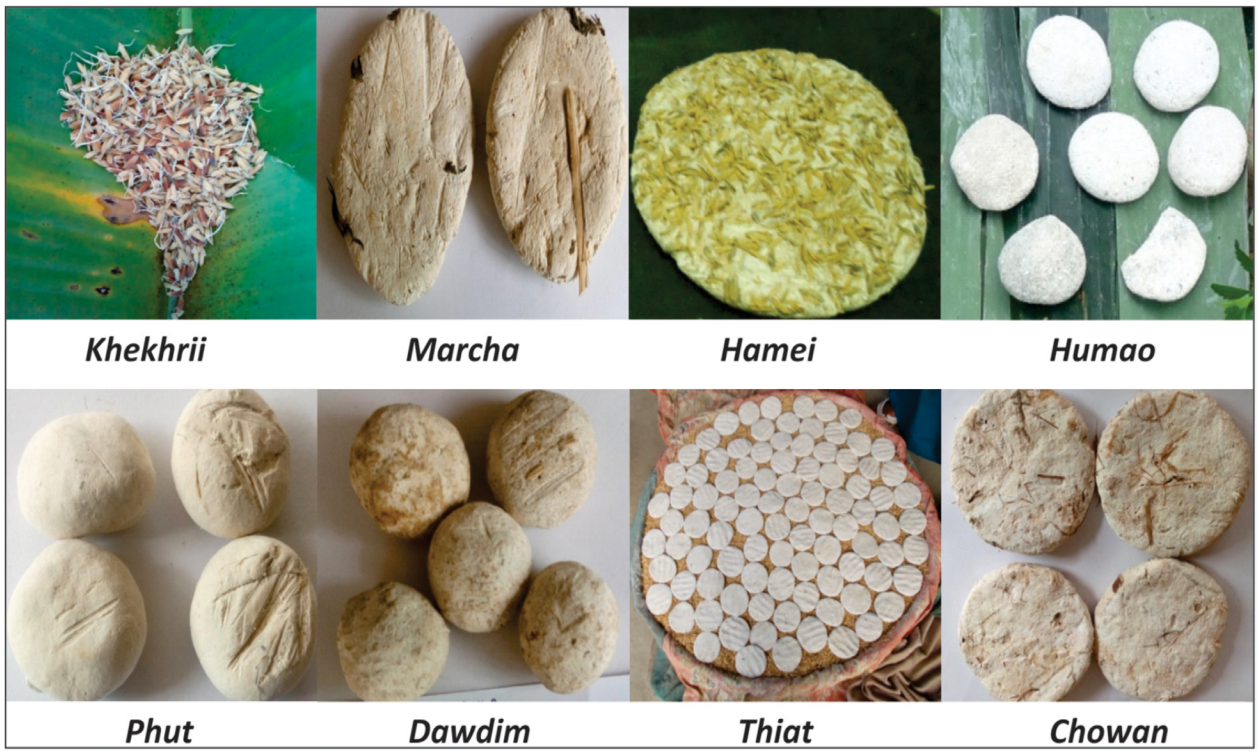

FIGURE 1 | Traditionally prepared dried starters collected from different parts of North East India.

yeast-malt extract agar (M424, HiMedia, India) and molds were isolated on potato dextrose agar (M096, HiMedia, India) supplemented with 10 IU ml^{-1} benzyl penicillin and 12 mg ml^{-1} streptomycin sulfate, and were incubated aerobically at 28°C for 3 days. Purity of the isolates was checked by streaking again on fresh agar plates of the same isolation medium, followed by microscopic examination. Isolation of yeast strains were typically based on morphotypes and criterion included size, color, shape, and appearance of fully grown culture on growth media. Colonies were counted as colony forming units (cfu)/g sample. Identified strains of yeasts were preserved in 20% glycerol at −20°C (Thapa and Tamang, 2004).

Culture-Dependent Approach for Diversity Analysis

Phenotypic and Biochemical Characterization

A total of 386 yeasts strains were isolated from 40 samples of eight different starters of North East India. Characterizations of yeasts were phenotypically tested on the basis of colony and cell morphology, sugar fermentation, and sugar assimilation tests. Cell morphology of actively growing yeast isolates was determined using a phase-contrast microscope (CH3-BH-PC; Olympus, Tokyo, Japan). Yeast cultures have been characterized on the basis of mycelium type, ascospore type, nitrate reduction, growth at 37 and 45°C, sugar fermentation, and sugar assimilation following the methods of Kurtzman et al. (2011).

Biolog System

Commercial Biolog Identification System (MicroLog TM System Release 4.2 User Guide 2001, Biolog, Inc.) based on the utilization of 95 substrates in 96-welled plate, were used for biochemical characterization of yeast isolates. Aliquots of the cultures were transferred to biolog plate wells and incubated at 37°C for 24–48 h, where positive results were recorded according to color changes. The results obtained were automatically read and analyzed using BiologMicrolog Reader and compared with the database of the Biolog Microlog database software (Biolog Inc.), which provided the most probable genera and species of the tested cultures.

Molecular Identification of Yeast Isolates

Identification of yeast isolates were done by ITS region sequencing wherein DNA extraction, PCR for ITS region, sequencing, and phylogenic affiliations were performed subsequently. Briefly, yeast DNA was extracted using ProMega DNA kit (ProMega). One gram of yeast cell pellet was suspended in lysis solution and incubated at 65°C for 15 min. Subsequently, the RNA was eliminated from the cellular lysate by administering the RNase solution following incubation at 35°C for 15 min. The residual proteins were removed by adding protein precipitation solution and centrifugation at maximum speed. Finally, the DNA was precipitated by adding isopropanol, which was purified with two washes of 70% ethanol. The quality of DNA was checked on 0.8% agarose gel and concentration was measured using Nano-Drop ND-1000 spectrophotometer (Nano Drop

Technologies, Wilmington, DE, United States) as described by Banskar et al. (2016). The DNA was stored at −20°C until further processing. For amplification of the ITS region, the forward primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and reverse primer ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (White et al., 1990) were used and PCR mixture and the thermal cycling protocol conditions were applied as described by (Esteve-Zarzoso et al., 1999). Products were analyzed on 1.5% agarose gel containing 0.7 mg/ml of ethidium bromide and visualized under UV light (UV source Gel-Doc 1000, Bio-Rad). Approximate size of amplicons was determined using standard molecular weight markers (Himedia-100-bp DNA Ladder) (Lv et al., 2013). All PCR-amplified products were purified and sequenced using ABI-DNA-Sequencer (ABI Genetic Analyser 3500, HITACHI, Japan). The sequences were compared with the GenBank database using the BLAST program (Altschul et al., 1990; Zhao and Chu, 2014). Sequences were visualized and edited using Chromas Version 1.45[2] (Pryce et al., 2003).

Culture-Independent Approach for Diversity Analysis

DNA Extraction, PCR Amplification From Starter Cultures

About 10 g of starters was homogenized in 90 ml of 0.85% w/v sterile physiological saline, and subsequently filtered through four layers of sterile cheese-cloth. The resulting filtered solutions were centrifuged at 14,000 *g* for 10 min at 4°C (Lv et al., 2013). Then, the pellets were subjected to DNA extraction using the ProMega DNA extraction kit (ProMega, United States) according to the manufacturer's instructions. Quality of resultant DNA was checked on 0.8% agarose gel and concentration was measured using Nano-Drop ND-1000 spectrophotometer (Nano Drop Technologies, Wilmington, DE, United States) as previously described (Banskar et al., 2016). The 250 nucleotides of the 5′-end D1/D2 region of the 26SrRNA gene was amplified by PCR using the primer NL1 (5′-CGC CCG CGC GCG GGC GGG GCG GGG GCC ATA TCA ATA AGC GGA AAA G-3′) (the GC clamp sequence used is underlined) and a reverse primer LS2 (5′-ATT CCC AAA CAA CTC GAC TC-3′) (Cocolin et al., 2000; El Sheikha et al., 2009). PCR was performed in a final volume of 50 μl containing 10 mM Tris–HCl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dATP, dCTP, dGTP, and dTTP, 0.2 mM of the primers, and 1.25 IU *Taq*-DNA polymerase (Promega, United States) and 2 μl of the extracted DNA (approximately 50 ng) using Thermal Cyclers (Applied Biosystems, United States). The reactions were run for 30 cycles at 95°C for 60 s for denaturation, at 52°C for 45 s for annealing, and at 72°C for 60 s for extension and finally for 7 min at 72°C (Cocolin et al., 2002). The PCR products were analyzed on 2.0% agarose gel containing 0.5 μg/ml ethidium bromide and were visualized in UV source GelDoc (Bio-Rad) (Cocolin et al., 2000). The concentration was again measured using Nano-DropND-1000 spectrophotometer.

PCR-DGGE Fingerprinting and Sequencing of DGGE Eluted Bands

The PCR products were analyzed by DGGE using DCode™ Universal Mutation Detection System (DGGEK-1001, CBS Scientific, San Diego, CA, United States) following the procedure of El Sheikha et al. (2009). Samples containing approximately equal amounts of PCR products were loaded into 8% w/v polyacrylamide gels (acrylamide:*N*,*N*′-methylene bisacrylamide, 37.5:1; Promega) in 1 × TAE buffer (40 mM Tris–HCl, pH 7.4, 20 mM sodium acetate, and 1.0 mM Na_2-EDTA). All electrophoresis experiments were performed at 60°C using a denaturing gradient in the range of 30–50% (100% corresponded to 7 M urea and 40% v/v formamide; Promega) (Cocolin et al., 2002). The gels were electrophoresed at 20 V for 10 min and then at 80 V for 12 h (El Sheikha et al., 2009). The gels were stained with SYBR Gold for 30 min (reconstituted according to the manufacturer's directions; Molecular Probes, Invitrogen, United States) and photographed in UV source GelDoc (Bio-Rad, United States) as described by Grizard et al. (2014). The DGGE bands were excised using sterile micro pipette tips. DNA of each band was eluted in 50 μl sterile water overnight at 4°C and 2 μl of the eluted DNA was reamplified as following the method of Cocolin et al. (2000). The PCR products which yielded only one band in DGGE electrophoresis were amplified with the primers without GC-clamp, purified and finally sequenced with the help of ABI-DNA-Sequencer (ABI Genetic Analyser 3500, HITACHI, Japan). The sequences were compared with the GenBank database using the BLAST program (Altschul et al., 1990; Zhao and Chu, 2014). The DNA sequences obtained from sequencing of total 202 bands was submitted to GeneBank.

Bioinformatics and Statistical Analysis

Quality of raw ITS region from yeast isolates and PCR-DGGE band sequencing data was checked with the help of Sequence Scanner software (Applied Bio systems, United States) and the data alignment and analysis were done with the help of SEQMANN software (DNASTAR, United States). After the

[2]http://www.technelysium.com.au/chromas.html

TABLE 1 | Average populations of yeasts in starters of North East India.

Samples	*Marcha*	*Humao*	*Hamei*	*Thiat*	*Phut*	*Khekhrii*	*Chowan*	*Dawdim*
States	Sikkim (*n* = 10)	Assam (*n* = 5)	Manipur (*n* = 5)	Meghalaya (*n* = 5)	Arunachal Pradesh (*n* = 5)	Nagaland (*n* = 5)	Tripura (*n* = 5)	Mizoram (*n* = 5)
Log cfu/g	6.865 (±0.06)	6.834 (±0.14)	6.852 (±0.03)	6.839 (±0.08)	6.836 (±0.05)	6.851 (±0.04)	6.852 (±0.03)	6.851 (±0.04)

n, number of samples analyzed; cfu, colony forming unit; standard deviation are given in parenthesis.

TABLE 2 | Grouping of total isolates of yeasts from starters of North East India on the basis of fermentation, and assimilation of sugars and other phenotypic tests.

Parameters	Tentative identity							
	Saccharomyces	***Pichia***	***Candida***	***Issatchenkia***	***Kluyveromyces***	***Schizosaccharomyces***	***Saccharomycopsis***	***Torulopsis***
Total isolates	43	60	56	51	41	52	23	60
Sugar fermented								
Lactose	+ (3), − (40)	−	+ (6), − (50)	−	−	−	−	−
Raffinose	+ (37), − (6)	+ (56), − (4)	+ (57), − (3)	+	+	+	+ (18), − (5)	+ (56), − (4)
Xylose	+ (39), − (4)	+ (55), − (5)	+ (54), − (6)	+ (45), − (5)	+	+ (50), − (2)	+ (20), − (3)	+ (55), − (5)
Sugar assimilated								
Trehalose	+ (40), − (3)	+ (50), − (10)	+ (55), − (5)	+ (46), − (5)	+	+ (50), − (2)	+ (20), − (3)	+ (50), − (10)
Lactose	+ (3), − (40)	+	+ (4), − (52)	−	+	+ (4), − (48)	+ (3), − (20)	−
Raffinose	+ (39), − (4)	+ (51), − (5)	+ (50), − (6)	+ (47), − (4)	+ (38), − (3)	+ (47), − (5)	+ (20), − (3)	+ (56), − (4)
Melibiose	+ (40), − (3)	+ (55), − (5)	+ (54), − (6)	+	+	+	+	+
True/pseudo-mycelia	Pseudo-mycelia	Pseudo-mycelia	True mycelia	Pseudo-mycelia	Pseudo-mycelia	Pseudo-mycelia	Pseudo-mycelia	Pseudo-mycelia
Ascospore	Hat-shaped	Hat-shaped	Oval	Spheroidal	Ellipsoidal	Globose	Hat-shaped	Spheroidal
Representative strains	GM:Y12, AS:Y12, HM:Y15, ST:Y46, AP:Y45, M:Y1, CH:Y22	GM:Y34, AS:Y3, HM:Y3, ST:Y3, AP:Y4, KY:Y3, M:Y49, CHY:34	GM:Y37, AS:Y7, HM:Y7 ST:Y41, AP:Y22, KY:45, MY:47, CHY:37	GM:Y4, AS:Y4, HM:Y50, ST:Y24, AP:Y3,KY:Y4, M:Y3, CHY:36,	GM:Y29, AS:Y6, HM:Y26, ST:Y36, AP:Y6, KY:33, M:Y6, CM:Y10,	AS:Y45, HM:Y9, ST:Y49, AP:Y15, KY:Y5, M:Y9, CH:Y15	GM:Y22, AS:Y2, HMY12, ST:Y12, AP:Y2, KY:Y42, M:Y2, CH:Y22	GM:Y1, AS:Y1, HM:Y28, ST:Y30, AP:Y38, KY:Y10, M:Y38, CM:Y18

All isolates fermented glucose, maltose, trehalose, sucrose, cellobiose, starch, and galactose. All isolates assimilated arabinose, rhamnose, sucrose, xylose, cellobiose, starch, and maltose.

data alignment, BLAST program was used for comparing DNA databases for sequence similarities available on the server[3] (Altschul et al., 1990; Zhao and Chu, 2014). Construction of a phylogenetic tree by the neighbor-joining method (Saitou and Nei, 1987) was performed using the CLUSTAL W program (Thompson et al., 1994). Shannon index of general diversity

[3]http://blast.ncbi.nlm.nih.gov/Blast.cgi

TABLE 3 | Biolog identification of yeasts isolated from starters.

Yeast species	Probability (%)	Similarity	Distance	Status
Pichia anomala	0.943	0.683	4.185	Identified
Pichia terricola	0.974	0.768	3.182	Identified
Pichia sydowiorum	0.834	0.652	3.285	Identified
Pichia onychis	0.834	0.737	3.234	Identified
Pichia guilliermondii	0.834	0.652	3.223	Identified
Pichia subpeliculum	0.834	0.734	3.764	Identified
Pichia trelalophila	0.834	0.794	3.234	Identified
Candia glabrata	0.834	0.786	3.864	Identified
Saccharomycopsis fibuligera	0.934	0.739	3.123	Identified
Zygosaccharomyces bailii	0.834	0.783	3.652	Identified
Phaffia rhodozyma	0.734	0.768	3.223	Identified
Debaryomyces	0.934	0.752	3.682	Identified
Debaryomyces castelli	0.834	0.754	3.285	Identified
Debaryomyces polymorphus	0.834	0.783	2.876	Identified
Issatchenkia orientalis	0.834	0.656	3.987	Identified
Saccharomyces cerevisiae	0.834	0.765	3.243	Identified
Rhodotorula bacarum	0.834	0.784	2.239	Identified
Rhodotorula aurantaea	0.834	0.618	2.285	Identified
Rhodotorula acheniorium	0.916	0.742	3.947	Identified

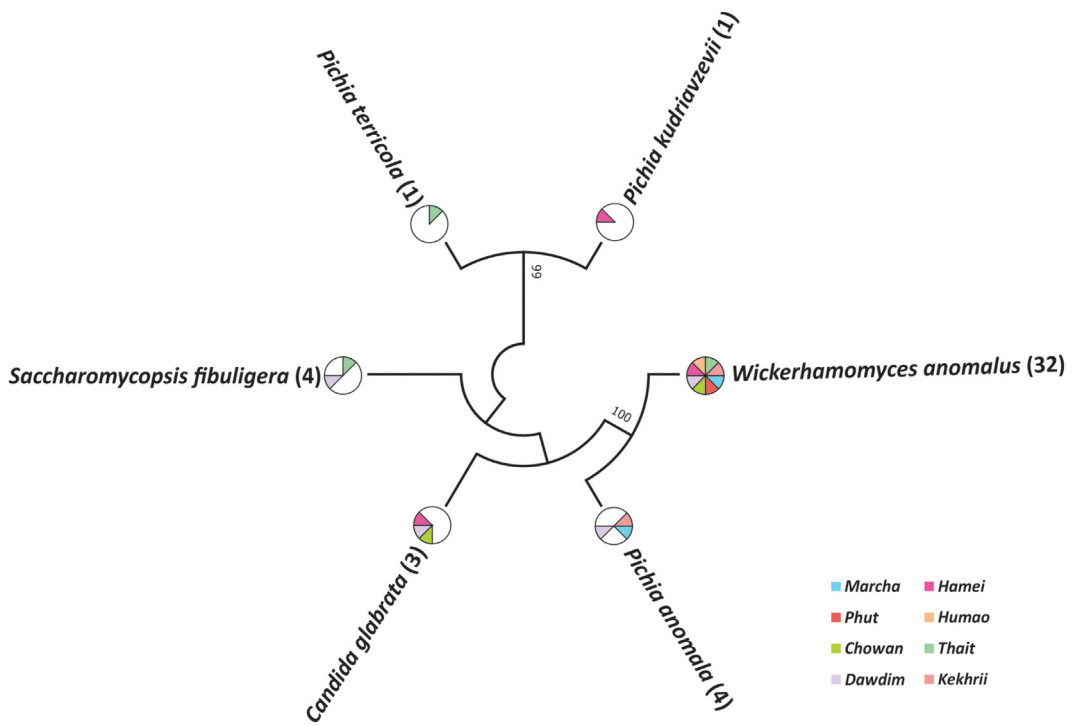

FIGURE 2 | Molecular phylogenetic analysis of 46 yeast isolates recovered from a starters based on ITS region sequencing. The bootstrap consensus tree derived with 1000 replicates to neighbor-joining method and Kimura two-parameter model. Numbers on branches depict the percent occurrence of a given branch during 1000 replicates. The origin distribution patterns of these isolates were depicted in subsequent pi-charts.

(H) and the richness of the microbial community as microbial diversity indices were determined by following the method of Oguntoyinbo et al. (2011). Other graphical emphasis was done on *igraph* package in R Software (Csardi and Nepusz, 2006).

Nucleotide Accessions

The sequences obtained from ITS region sequencing of isolated 46 yeast strains have been deposited in the GenBank under accessions: KY587119–KY626335 and 26S rRNA gene of 202

TABLE 4 | Molecular characterization and identification results of 46 yeast strains from starters of North East India by PCR ITS1-5.8S ITS2.

Product	Isolate code	[a]AP	[b]H	[c]R	GenBank accession number	Species
Marcha	GM:29	554	0.642	2	KY605141	*Wickerhamomyces anomalus*
	GM:Y1	582	0.613		KY605153	*Wickerhamomyces anomalus*
	GM:Y5	548	0.623		KY605154	*Wickerhamomyces anomalus*
	GM:Y12	529	0.626		KY587129	*Pichia anomala*
	GM:Y29	483	0.625		KY587130	*Wickerhamomyces anomalus*
	GM:Y46	604	0.623		KY587131	*Wickerhamomyces anomalus*
	M:Y5	658	0.622		KY605150	*Wickerhamomyces anomalus*
Thiat	ST:Y21	793	6.000		KY605140	*Saccharomycopsis fibuligera*
	ST:Y6	705	0.911	3	KY605145	*Wickerhamomyces anomalus*
	ST:Y24	840	0.941		KY605146	*Pichia terricola*
	ST:Y15	624	0.921		KY605147	*Saccharomycopsis fibuligera*
	ST:Y12	702	0.901		KY605148	*Wickerhamomyces anomalus*
	ST:Y3	596	6.911		KY605149	*Wickerhamomyces anomalus*
	ST:Y49	661	0.921		KY626330	*Wickerhamomyces anomalus*
Hamei	M:Y8	661	0.911	3	KY587121	*Wickerhamomyces anomalus*
	HS:Y7	1031	0.921		KY626335	*Pichia kudriavzevii*
	AH:45	458	0.921		KY605155	*Candida glabrata*
	H:Y7	710	0.941		KY605152	*Pichia kudriavzevii*
Huamo	AS:Y3	515	0.441	1	KY587126	*Wickerhamomyces anomalus*
	AS:Y5	601	0.441		KY587127	*Wickerhamomyces anomalus*
	AS:Y7	594	0.401		KY587128	*Wickerhamomyces anomalus*
	AS:Y4	565	0.431		KY605162	*Wickerhamomyces anomalus*
Chowan	CH:Y28	801	0.621	2	KY605143	*Candida glabrata*
	CH:Y39	574	0.601		KY605144	*Wickerhamomyces anomalus*
	CX:44	258	0.621		KY605159	*Wickerhamomyces anomalus*
	CH:X26	594	0.611		KY605160	*Wickerhamomyces anomalus*
	CH:X39	918	0.631		KY626331	*Wickerhamomyces anomalus*
	CH:Y22	845	0.601		KY626334	*Wickerhamomyces anomalus*
Phut	ST:Y53	927	0.410	1	KY626332	*Wickerhamomyces anomalus*
	ST:Y20	919	0.400		KY626333	*Wickerhamomyces anomalus*
Dawdim	M:Y9	592	1.100	4	KY587136	*Wickerhamomyces anomalus*
	M:Y20	484	1.030		KY587137	*Wickerhamomyces anomalus*
	M:Y30	529	1.002		KY587138	*Candida glabrata*
	M:Y47	588	1.001		KY587139	*Wickerhamomyces anomalus*
	M:Y57	585	1.1 11		KY587140	*Wickerhamomyces anomalus*
	M:Y3	629	1.121		KY587119	*Wickerhamomyces anomalus*
	M:Y6	627	1.120		KY587120	*Pichia anomala*
	ST:Y15	692	1.120		KY605157	*Saccharomycopsis fibuligera*
	XT:Y20	610	1.131		KY605156	*Pichia anomala*
	XT:Y15	654	1.113		KY605147	*Saccharomycopsis fibuligera*
Khekhrii	K:Y8	558	0.630	2	KY605151	*Wickerhamomyces anomalus*
	K:Y20	589	0.600		KY605152	*Wickerhamomyces anomalus*
	K:Y18	529	0.601		KY587132	*Wickerhamomyces anomalus*
	K:Y27	599	0.611		KY587133	*Pichia anomala*
	K:Y38	604	0.620		KY587134	*Wickerhamomyces anomalus*
	K:Y45	599	0.612		KY587135	*Wickerhamomyces anomalus*

[a]AP, arbitrary primers = sizes in base pairs; [b]H, Shannon's index; [c]R, species richness. Only gene bank percent of strains with more than 90% were shown in the table.

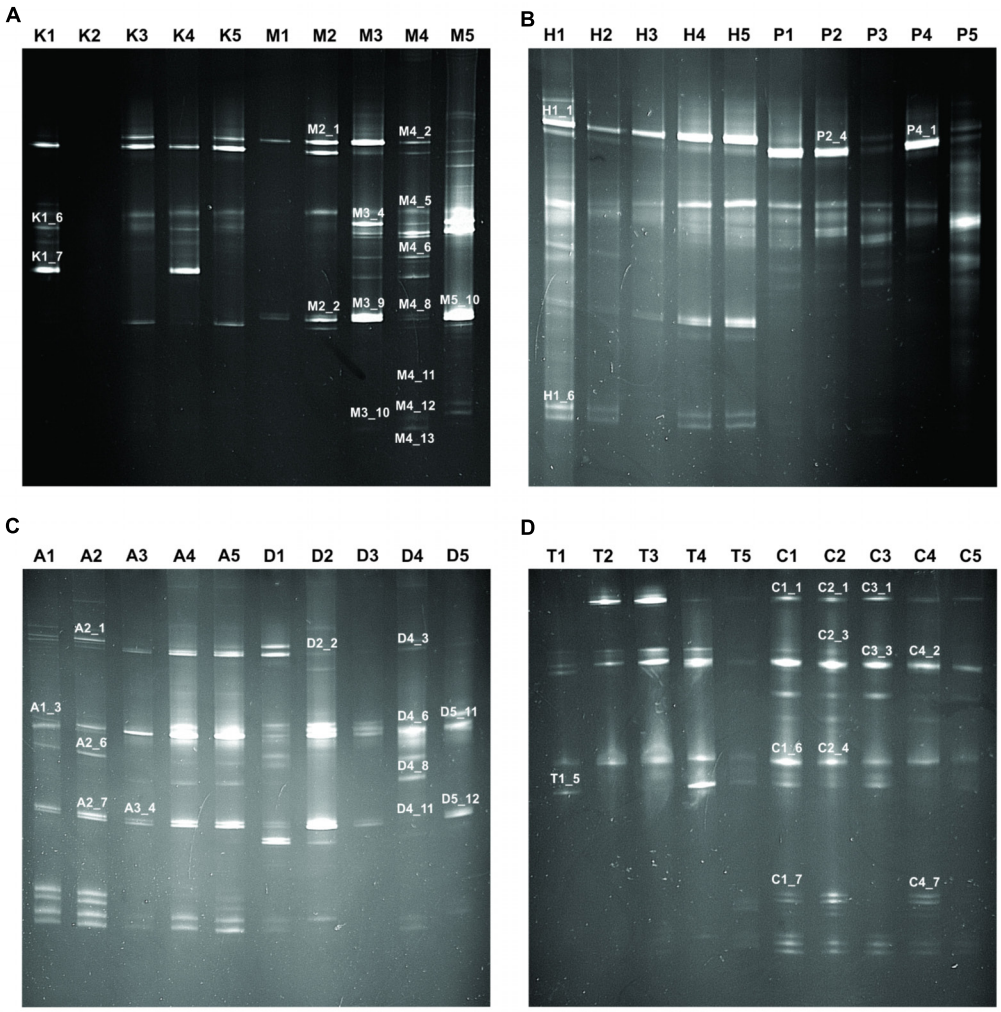

FIGURE 3 | Fingerprint of PCR-DGGE analysis of different samples. Total 40 samples were taken for PCR-DGGE wherein five samples from each amylolytic starter used for fingerprinting. Samples and respective band patterns are demonstrated like: **(A)** *Khekrii* (K) and *Marcha* (M); **(B)** *Hamai* (H) and *Humao* (P); **(C)** *Phut* (A) and *Dawdim* (D); **(D)** *Thiat* (T) and *Chowan* (C). Representation of band numbers of respective bands on fingerprint were those which showed ≥98% sequence identity to GeneBank nucleotide database.

bands excised from PCR-DGGE under accessions: KY594045–KY594246.

RESULTS

Culture-Dependent Approach

The average populations of yeast in all eight starters was 7.2×10^6 cfu/g (**Table 1**). Ascertaining the cultured diversity, a total of 386 yeasts strains were isolated from 40 samples and characterized by phenotypic assessment on the basis of colony morphology, cell morphology, sugar fermentation, and sugar assimilation tests (**Table 2**). Tentatively the following yeast genera were phenotypically identified using the taxonomical keys of Kurtzman et al. (2011) as *Saccharomyces*, *Pichia*, *Candida*, *Issatchenkia*, *Kluyveromyces*, *Schizosaccharomyces*, *Saccharomycopsis*, and *Torulopsis* (**Table 2**). Their metabolic capacities were also assessed by using the Biolog system. By comparing with the yeast database (MicroLog TM System Release 4.2 User Guide 2001, Biolog), the result revealed that maximum identified yeast species were associated with starter having ≥0.75% probability and ≥0.7 similarities index value (**Table 3**). The yeasts strain *Pichia terricola* showed highest ≥0.974% probability with ≥0.77 similarities index value. It was observed that the results from Biolog were revealing more diversity of yeasts than phenotypic characterization and it presented in **Supplementary Data Sheet S1**.

Out of 386 isolates, 46 representative isolates were grouped based on colony appearance, cell shape, type of mycelia and ascospores, pellicle formation, nitrate reduction, and growth at 37 and 45°C. Precisely, species level identification was done with molecular methods by ITS-region gene sequence analysis. We found that all cultures were identified in six species only as: *Wickerhamomyces anomalus*, *Pichia anomala*, *Saccharomycopsis*

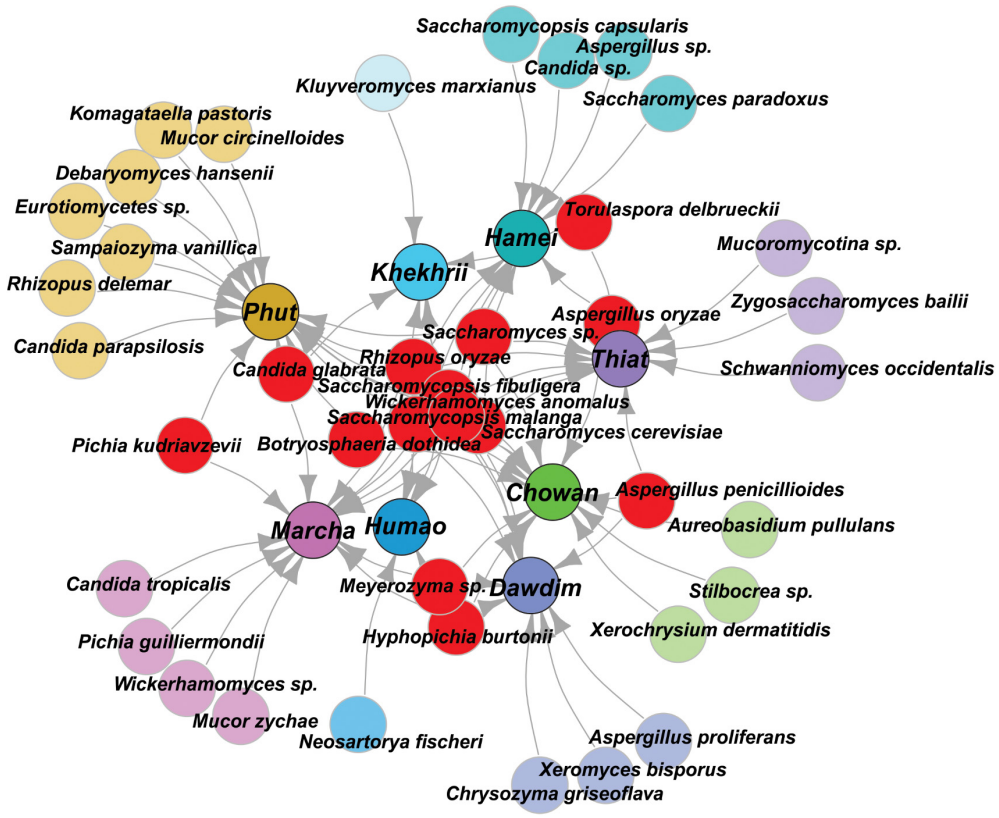

FIGURE 4 | Graphical representation of all species identified in PCR-DGGE of 26SrRNA gene after sequencing. Shared species were represented in red color, and sample specific unique species were represented in respective colors to the starter samples and arrow indicated the origin distribution patterns of these isolates.

fibuligera, *Pichia terricola*, *Pichia kudriavzevii*, and *Candida glabrata* which was reported in **Supplementary Data Sheet S2**. The average distributions in all starters and molecular phylogenetic relationship with neighbor-joining method were shown in **Figure 2**. From the sequencing results of ITS region gene; it was observed that species richness (R) was higher in *dawdim*, *hamei*, *thiat* than *marcha khekhrii*, *chowan*, and *phut* (**Table 4**). *Wickerhamomyces anomalus* was dominant in all starters. The Shannon index (H) of yeasts isolates was higher in *dawdim* than other starters (**Table 4**).

Culture-Independent Approach

In this study, we targeted D1 and D2 domain of 26S rRNA gene (large ribosomal subunit) of fungi from 40 samples of starters using PCR-DGGE fingerprint analysis. We used NL-1 forward primer and a new LS2 reverse primer to amplify the portion of 26S rRNA gene. These primers amplified a product of approximately 250 bp covering most of the D1 expansion loop. In PCR-DGGE fingerprint, diversity map distributions in the form of band patterns of yeasts and molds had been observed in different starters (**Figure 3**). Total 202 DGGE bands were selected on the basis of visualizing the prominent and differential band patterns inside the gels, after analysis of raw sequenced data with the help of BLAST comparison in GenBank as presented in **Supplementary Data Sheet S2**. More than 98% similar identity with the closest species of yeasts and molds has different phylum and genus level distribution pattern in different starters (**Figure 4**). Interestingly, we observed the distinct species were more than the shared species and *phut* was found to have highest diversity (**Figure 5**).

All these different techniques revealed the diversity and their differences of mycobiome species in different starters (**Figure 6**). Notably, the average distributions of yeasts in all samples were summarized as *Saccharomyces cerevisiae* (16.5%), *Saccharomycopsis fibuligera* (15.3%), *Wickerhamomyces anomalus* (11.3%), *S. malanga* (11.7%), *Kluyveromyces marxianus* (5.3%), *Meyerozyma* sp. (2.7%), *Candida glabrata* (2.7%), *Saccharomyces* sp. (1.3%), *Hyphopichia burtonii* (1.2%), *Schwanniomyces occidentalis* (1.1%), *Pichia kudriavzevi* (1.0%), *Torulaspora delbrueckii* (1.0%), *Zygosaccharomyces bailii* (1.0%), *Pichia guilliermondii* (1.0%), *Candida parapsilosis* (0.4%), *Komagataella pastoris* (0.3%), *S. capsularis* (0.6%), *S. Paradoxus* (0.6%), and *C. tropicalis* (0.1%). Similarly, the average distributions of molds in amylolytic starters were *Aspergillus penicillioides* (5.0%), *Rhizopus oryzae* (3.3%), sub-phylum: *Mucoromycotina* (2.1%), *Cryptococcus amylolentus* (1.7%), *Xerochrysium dermatitidis* (1.6%), *Aspergillus oryzae* (1.3%), *Neosartorya fischeri* (0.8%), *A. proliferans* (0.6%), *Chrysozyma griseoflava* (0.6%), *Stilbocrea*sp. (0.6%), *Mucor*

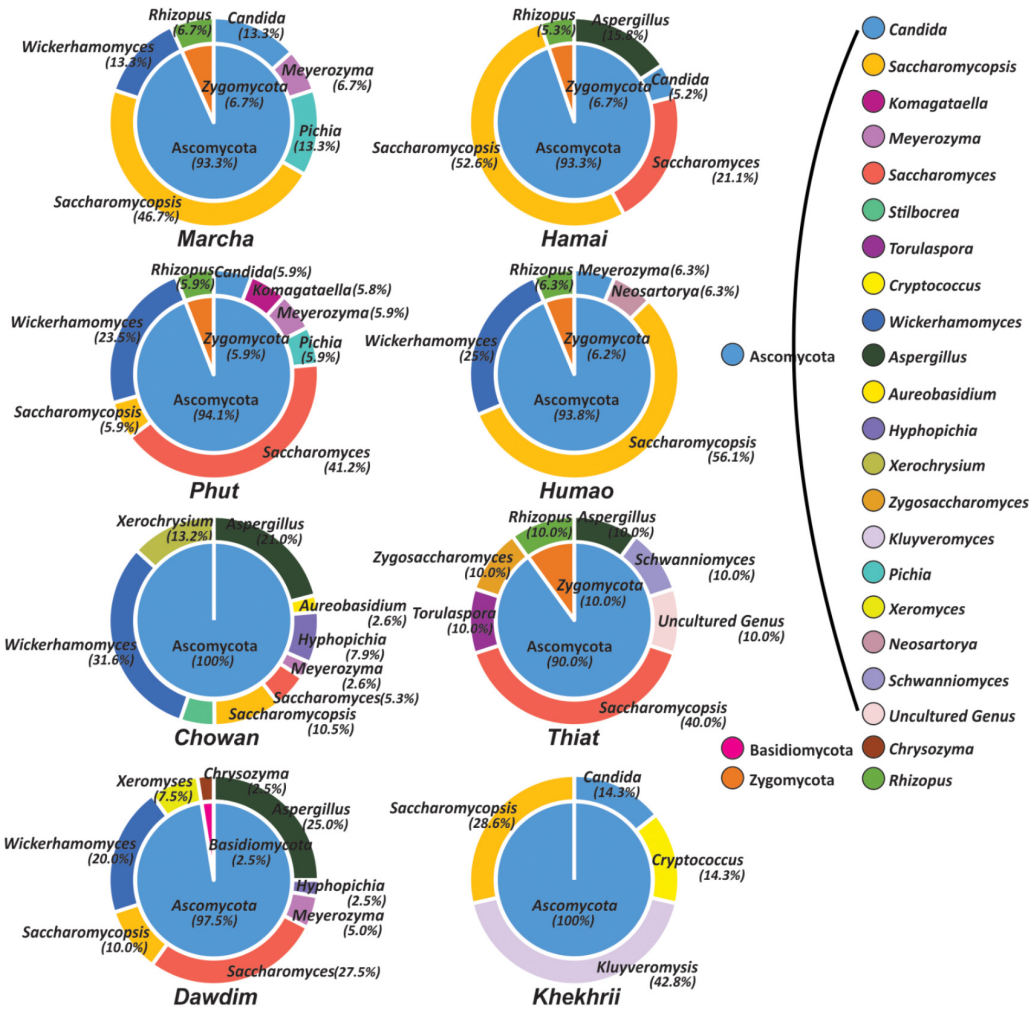

FIGURE 5 | Genus and phylum level distribution of yeast and molds diversity in starters. Genus and respective phylum presented here was based on 98% identity cutoff value to the GeneBank database.

circinelloides (0.5%), *Aureobasidium pullulans* (0.4%), and *Xeromyces bisporus* (0.3%).

DISCUSSION

Due to geographical locations, starters may have different and distinct mycobiome species diversity (Jeyaram et al., 2011). Going forward with this hypothesis, we examined and produced extensive surveillance report in different starters used in Indian alcoholic beverage production as an ethnic constituent. Results from Biolog system, where the profile of growth responses provides a metabolic fingerprint for each isolate (Praphailong et al., 1997), showed more diversity of yeasts in starters of North East India than phenotypic characterization based on probability and similarities index value. Even with high reliability rates, both phenotypic and Biolog tests did not coincide with the molecular reference tests for the majority of the isolates: when the identification results by Biolog were compared to 18S rRNA gene sequencing and species-specific PCR reactions (Nisiotou and Nychas, 2007). It has been previously reported that the ITS region gene analysis is a reliable routine technique for the differentiation of yeasts at species level (Clemente-Jimenez et al., 2004; Combina et al., 2005; Zott et al., 2008). Considering that species-specific PCR protocols target specific genes of genera and species, the reliability of ITS region gene sequences was considered to be 100% (Moraes et al., 2013). Another advantage of molecular culture-dependent method, which includes ITS, is that it allows a collection of pure cultures that may be used for further selection of suitable yeast strains to improve quality of alcoholic beverages (Lv et al., 2013).

In this study, *Wickerhamomyces anomalus*, *Pichia anomala*, *Saccharomycopsis fibuligera*, and *Candida glabrata* were identified in starters using ITS analysis. The previous studies also reported *Candida glabrata*, *Pichia anomala*, and *Saccharomycopsis fibuligera* from *marcha* based on 18S rDNA sequences (Tsuyoshi et al., 2005). It has been reported that *Candida glabrata*, which

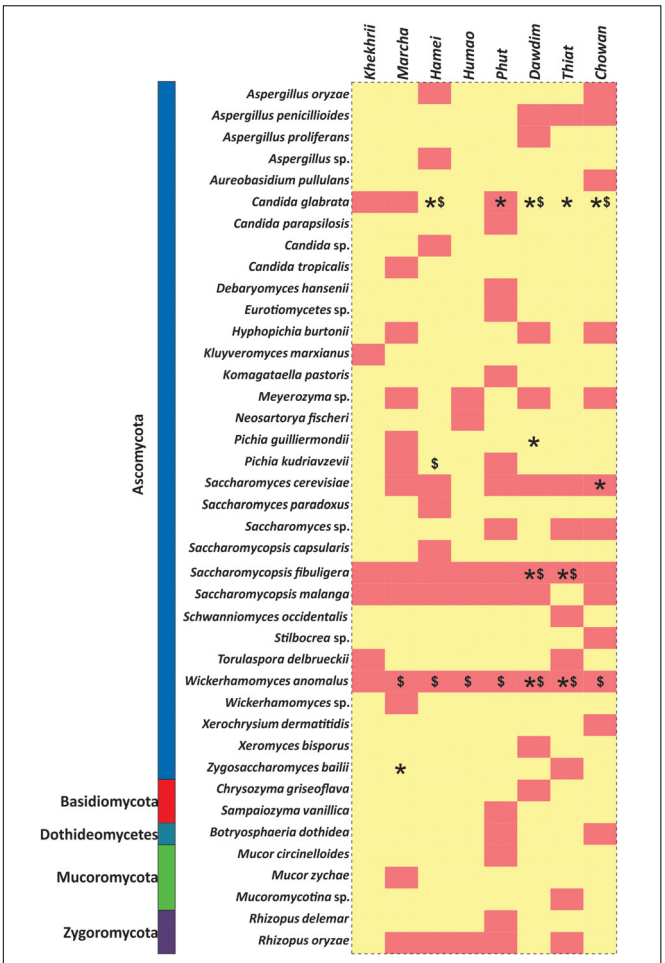

FIGURE 6 | Heatmap showing the consensus species diversity observed during PCR-DGGE, Biolog identification hits and ITS region gene sequencing of yeast isolates. We used presence–absence value of PCR-DGGE species data to generate heatmap whereas red color indicates the presence and in other hand yellow color represents absence value. Other datasets were mapped over the heatmap like: Biolog identification (*) and ITS-region gene sequencing of yeast isolates ($).

is a moderate alcohol producer, has also been recovered in *kodo ko jaanr*, ethnic fermented finger millet beverage prepared by using *marcha* (Thapa and Tamang, 2004) and some traditional Vietnamese starters (Dung et al., 2007), indicating that it is involved in alcohol production. Non-*Saccharomyces* yeasts may contribute to flavor or aroma formation in the alcoholic beverage (Rojas et al., 2001; Fleet, 2003; Moreira et al., 2005; Dung et al., 2006; Jolly et al., 2017). *Saccharomycopsis fibuligera*, *Saccharomyces cerevisiae*, *Wickerhamomyces anomala*, *Pichia* sp., and *Candida* sp. are the most common yeasts present in rice-based starters of Asia (Lee and Fujio, 1999; Xie et al., 2007; Jeyaram et al., 2008). Interestingly, *Wickerhamomyces anomalus*, probably the most abundant yeast, was reported for the first time from all the eight amylolytic starters of North East India using ITS-PCR method. The multiple sequence alignment of the ITS region gene sequences of *Wickerhamomyces anomalus* may be used for many purposes including inferring the presence of ancestral relationships between the sequences (Rampersad, 2014). It may be noted that protein sequences that are structurally very similar can be evolutionarily distant which is referred to as distant homology (Li and Durbin, 2010).

Genomic DNA extracted directly from samples of dried starters of India using the PCR-DGGE tools showed diversity of yeasts *Wickerhamomyces anomalus*, *Saccharomyces cerevisiae*, *S. malanga*, *S. paradoxus*, *Saccharomycopsis fibuligera*, *Sm. Capsularis*, *Candida glabrata*, *C. tropicalis*, *Meyerozyma* sp., *Pichia guilliermondii*, and *P. kudriavzevi*. Some researchers have reported the microbial community in some traditionally prepared dried starters for production of alcoholic beverages using PCR-DGGE analysis such as principal amylase-producer yeast *Sm. fibuligera* and ethanol-producers *S. cerevisiae* in *banh men* of Vietnam (Thanh et al., 2008), *nuruk* of Korea (Jung et al., 2012), and *yaa qu* and *hong qu* of China (Lv et al., 2012, 2013; Chen et al., 2014), respectively. *Sm. fibuligera* secretes considerable amount of α-amylase, glucoamylase, acid proteases, and β-glucosidase, which are applied in the fermentation industry (Chi et al., 2009).

The dominance of *S. cerevisiae* in *marcha*, *thiat*, *dawdim*, and *phut* might be due to its competitive growth in the presence of fermentable sugars and its ethanol tolerance may be due to fast growth during various alcoholic fermentations (Dung et al., 2006, 2007; Jeyaram et al., 2008). *S. cerevisiae* has also found to be one of the dominant yeasts in all starters of North East India, because of its competitive growth under strict anaerobic conditions and its tolerance to ethanol (Romano et al., 2006). *Wickerhamomyces anomalus*, a regular component in several types of Asia-Pacific alcohol fermentation starters (Limtong et al., 2002; Thanh et al., 2008), was detected in all analyzed samples. *P. guilliermondii* which was observed in *marcha* was also reported from wheat-based *qu* for Chinese Shaoxing rice wine (Xie et al., 2007) and *hamei* of Manipur in India (Jeyaram et al., 2008), which can produce volatile phenols and esters in the initial stages of alcoholic fermentation (Moreira et al., 2005). *Pichia kudriavzevii*, *Wickerhamomyces anomalus*, *S. malanga*, *Kluyveromyces marxianus*, *Torulaspora delbrueckii*, *Hyphopichia burtonii*, *S. capsularis*, and *Debaryomyces hansenii* were also reported from other Asian starters for the production of flavor and ethanol (Dung et al., 2006; Xie et al., 2007; Thanh et al., 2008; Zhang et al., 2008; Jung et al., 2012; Lv et al., 2013; Chen et al., 2014). *Zygosaccharomyces bailii* is widely present in various food fermentations, such as wine, tea, and vinegar fermentations (Garavaglia et al., 2015), and also produced various flavor compounds including alcohol in Chinese *Maotai* liquor (Xu et al., 2017).

In *chowan*, few pathogenic fungi were also detected such as *Xerochrysium dermatitidis*, which is a pathogenic fungus causing skin diseases (Pitt et al., 2013); and *Aureobasidium pullulans*, a ubiquitous black, yeast-like human fungal pathogen found in soil, water, air, and limestone (Chan et al., 2011). These pathogenic fungi may be contaminated through various raw substrates including wild herbs, water, etc. during crude preparation of *chowan* by village people in Tripura. The presence of sub-phylum: *Mucoromycotina*, which is the earliest mutualistic symbiosis fungus with *Haplomitriopsida* liverworts (Field et al., 2015),

probably passed through the plants used during preparation of *thiat*.

Besides yeast community, some molds *Rhizopus* spp. and *Aspergillus* spp. were also detected by PCR-DGGE analysis in starters except in *khekhrii* samples of Nagaland (prepared by naturally fermenting germinated sprouted rice grains). Species of *Rhizopus* spp. and *Aspergillus* were reported from many Asian amylolytic starters (Tamang et al., 1988; Oda et al., 2006; Yang et al., 2013; Zhu and Tramper, 2013). The distributions of yeasts communities in amylolytic starters of North East India were higher in comparison to molds, this may be due to low temperatures of that particular environment in North East India and also the substrates used for fermentation (Chi et al., 2009). These traditional starters are the result of long-term selection for preserving and cultivation the amylolytic and alcohol-producing native yeasts and fungi by ethnic people which has been practicing the traditional process for centuries (Tamang, 2010; Londoño-Hernández et al., 2017). The DGGE analysis has some disadvantages due to its inability to determine the relative abundance of dominant species, differentiate between viable and nonviable cells, and difficulties in interpretation of multi-bands (Nam et al., 2012; Dolci et al., 2015). Besides, DNA extraction efficiencies vary between microorganisms since DGGE band intensity is not always correlated with population density (Ercolini, 2004; Prakitchaiwattana et al., 2004; Lv et al., 2013). Sub-culturing or back sloping of desirable inocula from previous batch during the traditional preparation of starters under uncontrolled fermentation may pose health risks (Rossetti et al., 2009). However, combination of culture-dependent and -independent analysis may be used to assess the safety of the microbiota associated with spontaneous/natural fermentation that may help to predict the possible risks for human health (Capozzi et al., 2007; Van Hijum et al., 2013).

CONCLUSION

Starter making technology reflects the traditional method of "sub-culturing" of desirable inocula from previous batch to new culture using rice as base substrates by back-sloping, in North East India. Selection of ethnic starters from different geographical regions with diverse mycobiome is gaining the importance of species diversity as indigenous property. We performed one of the successful trials to find out the mycobiome associated with eight different dried starters of North East India analyzed by ITS-PCR and PCR-DGGE techniques. These results may enrich our knowledge of cultivable indigenous mycobiota present in the starters (amylolytic and alcoholic) of Asia that may be used to promote the production technology of unique ethnic alcoholic beverages high quality and typical attributes; moreover, data of starters of India can be used as reference data base for the further research.

AUTHOR CONTRIBUTIONS

SS contributed to this present work as a part of his research work. MS and KJ helped and assisted in some molecular work Bioinformatics analysis. AS, YS, and JT framed and prepared this paper critically with final approval of JT.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02237/full#supplementary-material

DATA SHEET S1 | GenBank accessions number of identified species of yeasts.

DATA SHEET S2 | Biolog identification of yeast strains isolated from different amylolytic starters of North East India.

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Characterization of Diversity and Probiotic Efficiency of the Autochthonous Lactic Acid Bacteria in the Fermentation of Selected Raw Fruit and Vegetable Juices

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The diversity of indigenous lactic acid bacteria (LAB) in fermented broccoli, cherry, ginger, white radish, and white-fleshed pitaya juices was analyzed using culture-independent and -dependent approaches. The major properties of selected probiotic strains, including dynamic variations in pH, viable cell counts, antibiotic resistance, bacterial adhesion to hydrophobic compounds, and survivability during simulated gastrointestinal transit, were investigated using broccoli as the fermentation substrate. In broccoli and ginger juices, the genus *Lactobacillus* occupied the dominant position (abundances of 79.0 and 30.3%, respectively); in cherry and radish juices, *Weissella* occupied the dominant position (abundances of 78.3 and 83.2%, respectively); and in pitaya juice, *Streptococcus* and *Lactococcus* occupied the dominant positions (52.2 and 37.0%, respectively). *Leuconostoc mesenteroides*, *Weissella cibaria/soli/confusa*, *Enterococcus gallinarum/durans/hirae*, *Pediococcus pentosaceus*, *Bacillus coagulans*, and *Lactococcus garvieae/lactis* subspecies were identified by partial 16S rRNA gene sequencing. In general, the selected autochthonous LAB isolates displayed no significant differences in comparison with commercial strains with regard to growth rates or acidification in fermented broccoli juice. Among all the isolates, *L. mesenteroides* B4-25 exhibited the highest antibiotic resistance profile (equal to that of *L. plantarum* CICC20265), and suitable adhesion properties (adhesion of 13.4 ± 5.2% ~ 36.4 ± 3.2% and 21.6 ± 1.4% ~ 69.6 ± 2.3% to ethyl acetate and xylene, respectively). Furthermore, *P. pentosaceus* Ca-4 and *L. mesenteroides* B-25 featured the highest survival rates (22.4 ± 2.6 and 21.2 ± 1.4%, respectively), after simulated gastrointestinal transit. These results indicated a high level of diversity among the autochthonous bacterial community in fermented fruit and vegetable juices, and demonstrated the potential of these candidate probiotics for applications in fermentation.

Keywords: autochthonous lactic acid bacteria, microbial diversity, fermentation, fruit and vegetable juice, probiotic viability

INTRODUCTION

Fermented fruit and vegetable juices (FVJs) containing lactic acid bacteria (LAB) are important research targets with regard to providing additional value and choices for vegetarians and individuals with lactose intolerance (Di Cagno et al., 2013). Abundant sources of autochthonous LAB exist in the spontaneous fermentation of fruits and vegetables, which is carried out in exclusive uncontrollable environmental conditions (Sanni, 1993; Steinkraus, 1997). Researchers have explored the use of indigenous LAB strains isolated from food materials and their addition to more complex food systems, with the aim of improving the quality characteristics and functional properties of the end products (Galvez et al., 2007; Di Cagno et al., 2009a; Ong et al., 2012). For instance, autochthonous LAB obtained by the fermentation of mango juice can be employed to compensate for the loss of antioxidant substances, increase the contents of nutrients such as organic acids and mannitol, and provide better sensory characteristics such as acidity and sweetness (Liao et al., 2016). However, different probiotics have different survival characteristics and functional performance in various juices. A previous screening study was conducted to determine which, among apple, grape, and orange juices, was the best substrate for the growth of *Lactobacillus* strains with respect to bacterial viability, superoxide dismutase activity, folate production, and hedonic characteristics (Espirito-Santo et al., 2015). Researchers have revealed that indigenous probiotics isolated from raw materials have an inherent stability, which may contribute to improving the survival rate and persistence observed in food matrices (Ong et al., 2012; Reina et al., 2015). Therefore, the characterization and identification of indigenous probiotics from various FVJs could provide diverse microbiological resources with enhanced fermentative capabilities for the manufacturing of products with greater stability and production efficiency (Gibbons and Rinker, 2015; Bokulich et al., 2016).

The combined utilization of culture-independent and -dependent analyses is useful for profiling complex microbial taxonomic communities and assessing the viability of cultivable microbial populations (Kesmen et al., 2012; Davis, 2014). High-throughput sequencing has emerged as an innovative culture-independent technique to quantitatively investigate the biodiversity of microbial communities in foods, and has been proven to be reliable in the study of dominant, as well as minor, microbial populations (Medina et al., 2016). The method that is based on the cultivation of microorganisms in selective media has a specific advantage in that it can yield single colonies of the bacteria that are present in the fermentation ecosystem, enabling their selective isolation, cultivation, and identification (Ellis et al., 2003). However, the majority of previous studies that used these two methods have focused on fermented seafoods, soybean paste, kimchi, wine, sourdough, soil etc. (Nam et al., 2012; Park et al., 2012; Adewumi et al., 2013; Ercolini et al., 2013; Jung et al., 2013; Pinto et al., 2015), whereas few studies have assessed fermented FVJs. The studies that assessed fermented FVJs employed a single method and/or used commercial LAB cultures (Aneja et al., 2014; Nicomrat and Chamutpong, 2016). Moreover, the environments inside different fermented FVJs with surface microorganisms are markedly dissimilar with regard to multiple factors, including the variety of carbohydrates/carbon sources and nutritional compositions, and discrepancies in the initial pH. In addition, the presence of amino acids, vitamins, dietary fibers, phenolic compounds, mixed oligosaccharides, and other bioactive substances gives rise to food substrates that have probiotic properties, such as antioxidant activity, antiproliferative effects on cancer cells, and the capacity to stimulate the growth of *Lactobacillus* and *Bifidobacterium* species (Granato et al., 2010; Nematollahi et al., 2016). Therefore, by selecting a wide diversity of raw materials, we were able to investigate dissimilarities in their microbial profiles and identify promising LAB strains in these FVJs. In this study, cherry (Jacob et al., 2003; Chaovanalikit and Wrolstad, 2004; Kim et al., 2005), white-fleshed pitaya (Wichienchot et al., 2010; Garcia-Cruz et al., 2017), white radish (Hashimoto et al., 2006; Lee et al., 2012; Kaymak et al., 2015), broccoli (Keck et al., 2003; Moreno et al., 2006; Berenbaum, 2014; Armah et al., 2015), and ginger (Kruth et al., 2004; Palatty et al., 2013; Daily et al., 2015), which have been shown to exhibit a great variety of bioactive characteristics, were chosen as substrates for the isolation of LAB strains and follow-up testing.

To the best of our knowledge, detailed investigations of variations in the indigenous bacterial community in fermented FVJs are limited. The objective of this study was to characterize and identify the microbial diversity of fermented non-pasteurized fresh FVJs using culture-independent and -dependent methods and to determine whether isolated indigenous microbes habituated on the surface of fruits and vegetables could be successfully cultivated and used for inoculating commercial products. In addition, we aimed to identify ideal substrates that could be selected for delivering such isolated LAB.

MATERIALS AND METHODS

Sampling

Broccoli (*Brassica oleracea*), cherry (*Prunus avium*), ginger (*Zingiber officinale*), white radish (*Raphanus sativus*), and white-fleshed pitaya (*Hylocereus undatus*) were collected from a local market (Beijing, China) and stored at 4°C prior to use. The fermented samples were prepared according to a previously reported method (Di Cagno et al., 2016). Fifty grams of each sample were suspended in 50 mL MRS broth separately and fermented for 48 h at 37°C in an anaerobic incubator (LAI-3-T, Shanghai Longyue Instruments Equipment Co., Ltd., Shanghai, China). Unfermented samples were prepared according to a previously described method (Di Cagno et al., 2009a). Ten grams of each sample were suspended in 90 mL of sterile sodium chloride (0.9% w/v) solution and homogenized (FB-110Q, Shanghai Litu Mechanical Equipment Engineering Co., Ltd., Shanghai, China) for 2 min at room temperature.

Microbial Diversity Analysis

The fermented juices were successively filtered through 0.45- and 0.22-μm membranes. Microbial DNA was extracted using the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek,

Norcross, GA, United States) according to the manufacturer's protocols. The final concentration and purification of DNA were determined using a NanoDrop 2000 UV-vis spectrophotometer (Thermo Scientific, Wilmington, DE, United States), and DNA quality was checked via 1% agarose gel electrophoresis. The V3 and V4 hypervariable regions of the bacteria 16S rRNA genes were amplified with primers 338F (5′- ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTA AT-3′) using a thermocycler polymerase chain reaction (PCR) system (GeneAmp 9700; ABI, Carlsbad, CA, United States). PCR was conducted using the following program: denaturation for 3 min at 95°C; 27 cycles of 30 s at 95°C, annealing for 30 s at 55°C, and elongation for 45 s at 72°C; and a final extension at 72°C for 10 min. The PCR was performed in triplicate, with 20-μL reactions containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM 2′-deoxynucleoside 5′-triphosphate (dNTPs), 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA. The PCR products were extracted from a 2% agarose gel and further purified using an AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, United States) and quantified using a QuantiFluor™-ST fluorometer (Promega, Madison, WI, United States) according to the manufacturer's protocol. Purified amplicons were pooled in equimolar ratios and paired-end sequenced (2 × 300) on an Illumina MiSeq platform (Illumina, San Diego, CA, United States) according to the standard protocols of Majorbio Bio-Pharm Technology Co., Ltd. (Shanghai, China).

The raw fastq files were demultiplexed, quality-filtered using the Trimmomatic tool, and merged using FLASH software with the following criteria: (i) the reads were truncated at any site that received an average quality score of less than 20 over a sliding window of 50 bp. (ii) Primers were exactly matched allowing two-nucleotide mismatching, and reads containing ambiguous bases were removed. (iii) Sequences with overlap longer than 10 bp were merged according to their overlap sequence. Operational taxonomic units (OTUs) were clustered with 97% similarity cutoff using UPARSE software (version 7.1[1]), and chimeric sequences were identified and removed using UCHIME. The taxonomy of each 16S rRNA gene sequence was analyzed using RDP classifier algorithm[2] by reference to the Silva (SSU123) 16S rRNA database with a confidence threshold of 70%.

Isolation and Identification of LAB Strains

The isolation of strains was carried out according to a previously described method (Di Cagno et al., 2009a). Each sample was serially diluted $10^{-1} \sim 10^{-7}$-fold with sterilized saline. Thereafter, 100-μL dilutions were spread onto MRS agar plates. After incubation at 37°C for 48 h under anaerobic conditions, colonies with different morphotypes from the highest dilutions were collected in MRS broth supplemented with 20% glycerol, and stored at −80°C for further analyses (Park et al., 2016). Gram-positive, catalase-negative, non-motile rods and cocci were cultivated in MRS broth at 37°C for 24 h, and then re-streaked onto MRS agar. Identification of the screened LAB strains was performed by sequencing the 16S rDNA gene. Genomic DNA of selected LAB strains was extracted from cultures grown at 37°C for 24 h in MRS broth using TRIzol reagent (Tiangen Biotechnology Co., Ltd., Beijing, China) and amplified by PCR using two universal primers, namely, 27F (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 1492R (5′-GGY TAC CTT GTT ACG ACT T-3′) (Ding et al., 2017). Fifty microliters of each PCR mixture contained: 4 μL 2.5 mM of dNTPs, 1 μL of both forward and reverse primer, 2 μL template, and 0.5 μL 5 U of Taq DNA polymerase [Takara Biomedical Technology (Beijing) Co., Ltd., Beijing, China], in 5 μL supplied buffer. The expected amplicons of about 1465 bp after amplification with the primer pair were eluted from the gel and purified. PCR products were sequenced by Majorbio Biotechnology Co., Ltd. (Shanghai, China), and the sequences were compared with the sequence database in the National Center of Biotechnology Information (NCBI) using the basic local alignment search tool (BLAST[3]) to identify the strains at the species level (Altschul et al., 1990). The sequences of highly homologous type strains were downloaded from GenBank database, and a phylogenetic study was carried out with MEGA version 5 (Tamura et al., 2011). The obtained sequences were lined up by ClustalX software (Kohli and Bachhawat, 2003), and the neighbor-joining algorithm was used to construct a phylogenetic tree based on distance estimates calculated by the Kimura-2 parameter, which includes a bootstrap test with 1000 replicates (Saitou and Nei, 1987).

Determination of pH and Viable Cell Count in Fermented Broccoli Juice

Broccoli was blended with purified water (1:3, g/mL) and then pasteurized for 5 min at 80°C. From the MRS broth cultures of the selected LAB strains (10^9 CFU/mL), 0.4 mL was centrifuged for 10 min at 10000 × *g*, and the resulting precipitate of bacteria was added to 40 mL broccoli juice to obtain an initial count of mesophilic LAB of 10^8 CFU/mL in the final juice sample. Fermentation experiments were conducted in 50-mL sterile centrifuge tubes, each containing 40 mL juice. The juice was then incubated for 48 h at 37°C in an anaerobic incubator (LAI-3-T, Shanghai Longyue Instruments Equipment Co., Ltd., Shanghai, China). Meanwhile, the progress of fermentation was monitored every 12 h by quantifying the colony forming units (CFUs) on MRS agar plates using the standard method of decimal dilution and measuring the pH using a pH meter (Medidor pH basic 20, Crison Instruments, Spain) (Di Cagno et al., 2008).

Antibiotic Resistance

The standard disk diffusion assay was used to determine the sensitivity or resistance of LAB to conventional antibiotics. Paper disks containing ampicillin (10 μg), penicillin (10 μg), amoxycillin (10 μg), norfloxacin (10 μg), levofloxacin (5 μg), gentamicin (120 μg), streptomycin (10 μg), amikacin (30 μg), and erythromycin (15 μg), which were purchased from Solarbio Technology Co., Ltd. (Beijing, China), were employed for the

[1]http://drive5.com/uparse/

[2]http://rdp.cme.msu.edu/

[3]http://blast.ncbi.nlm.nih.gov/Blast.cgi

antibiotic resistance tests (Lee et al., 2014). From the MRS broth culture of each one of the test strains, 100 μL was mixed with 8 mL of liquid MRS agar, over-layered on a pre-solidified agar plate and allowed to solidify, and then disks were aseptically placed onto the center of plates using sterile forceps. The plates were incubated for 48 h at 30°C in an anaerobic chamber. The results were recorded according to the interpretive category defined by the Clinical and Laboratory Standards Institute (CLSI) (Sharma et al., 2017). The tests were carried out in triplicate.

Determination of Hydrophobicity

The hydrophobicity of LAB isolates was assessed using a modified version of a previously reported method (Bautista-Gallego et al., 2013). Ethyl acetate and xylene were used as the hydrophobic substances for the hydrophobicity assays according to the recommendations of previous reports (Vanhaecke and Pijck, 1988; Guo et al., 2010). One milliliter of fermented broccoli juice (10^9 CFU/mL) was centrifuged for 15 min at 8000 × *g* and washed twice with phosphate-buffered saline (PBS). The pellet was resuspended in PBS, and the optical density was assessed at 600 nm (A_0). Equal proportions of ethyl acetate and xylene were blended with the bacterial cells and vortexed for about 5 min, incubated for 1 h at room temperature, and the optical density was assessed at 600 nm (A_1). The capability of the bacteria to adhere to the hydrophobic compound (BATH) was calculated as follows:

$$BATH\% = [(A_0 - A_1)/A_0] \times 100$$

Assessment of the Survivability of LAB in Simulated Gastrointestinal Transit

The survivability of the LAB isolates in the presence of artificial gastrointestinal juices was measured by the method described by Baruah et al. (2017): (i) First, 1 mL fermented broccoli juice (10^9 CFU/mL) was centrifuged for 10 min at 8000 × *g* and the pellet was washed twice with sterile PBS before being resuspending in 10 mL of simulated gastric juice (GJ), and incubated for 90 min at 37°C. The GJ consisted of pepsin (1000 U/mL) in PBS, with the pH adjusted to 2.5 with 10% hydrochloric acid. (ii) The solution was then centrifuged for 10 min at 8000 × *g*, the supernatant was removed, and the precipitate was re-suspended in 10 mL simulated duodenal juice (DJ) and incubated for 10 min at 37°C. The DJ was composed of 1% (w/v) bile salts, and the pH was adjusted to 8.0 with 1M NaOH. (iii) The solution was then centrifuged for 10 min at 8000 × *g*, the supernatant was removed, and the precipitate was re-suspended in 10 mL simulated intestinal juice (IJ) and incubated for 120 min at 37°C. The intestinal fluid was composed of 0.3% (w/v) bile salts and 1000 U/mL of trypsin solution, and its pH was adjusted to 8.0. The viable bacterial cell counts were determined by serial dilution in physiological saline solution at the beginning of each step and at the end of the last step.

Data Analysis

All experiments were performed in triplicate. Data fitting was performed using the software Statistica for Windows ver. 10. Data were also analyzed via one-way ANOVA and Tukey's test ($P < 0.05$). The results are expressed as the mean ± standard deviation. Each of the bars represents the standard deviation from the mean.

RESULTS AND DISCUSSION

The Structure of the Uncultured Microbial Community in Fermented Samples

Throughout the five different samples, a total of 246 OTUs at a distance of 3% was obtained, with an average of 98 OTUs in each sample, including repetitive OTUs. Rarefaction analysis demonstrated the abundances in the different samples, and rarefaction curves for a similarity of 97% indicated that the sufficient coverage of sequencing could account for the majority of the bacterial diversity within each sample. The coverage indices, which were greater than 99%, also indicated that the microbial community was reflected accurately (Wang et al., 2017). A total of 16 phyla were detected via taxonomic analyses. The five most abundant phyla were *Bacteroidetes* (0.2%), *Cyanobacteria* (0.4%), *Actinobacteria* (6.2%), *Proteobacteria* (8.6%), and *Firmicutes* (84.6%). A total of 154 bacterial genera were identified. The most abundant genera were characterized to elucidate which might be the most important bacteria present in the fermented FVJs ecosystem. The relative abundances (%, abundances >5%) and distributions of the dominant microorganisms in the different juices, as determined via the genus analysis, can be clearly ascertained in **Figure 1**. The microbiota was found to be almost exclusively dominated by members of the phylum *Firmicutes*; in particular, of the five principal OTUs in all five materials, three belonged to LAB, namely *Weissella* (46.0%), *Lactobacillus* (24.2%), and *Streptococcus* (12.1%), and the other two corresponded to *Rhodococcus* (6.7%) and *Enterobacteriaceae* (4.6%). However, the dominant genera were different in each juice. In broccoli juice, *Lactobacillus* occupied the dominant position with an abundance of 79.0%, and the number of 125 OTUs was the highest recorded among the five raw materials, which may be correlated to the specific structure of the broccoli flowering head. *Lactobacillus* and *Weissella* were the most abundant genera in ginger juice, with abundances of 30.3 and 25.8%, respectively. In cherry and radish juices, *Weissella* occupied the dominant position (78.3 and 83.2%, respectively), whereas *Streptococcus* and *Lactococcus* occupied the dominant position (52.2 and 37.0%, respectively) in pitaya juice. Naturally occurring microbial populations in food ecosystems are responsible for spontaneous fermentation that leads to a variety of traditionally fermented products, which represent a valuable reservoir of novel strains of environmental origin (Tamang et al., 2016). In this study, high-throughput sequencing enabled the analysis of the microbial community as a whole, whereas culture techniques provide isolates for further applications (Perez-Cataluna et al., 2018). We observed that the dominant genera in the different fermented juices varied, which was partially congruent with the results of dominant genera previously identified within the microbiota of banana, kimchi,

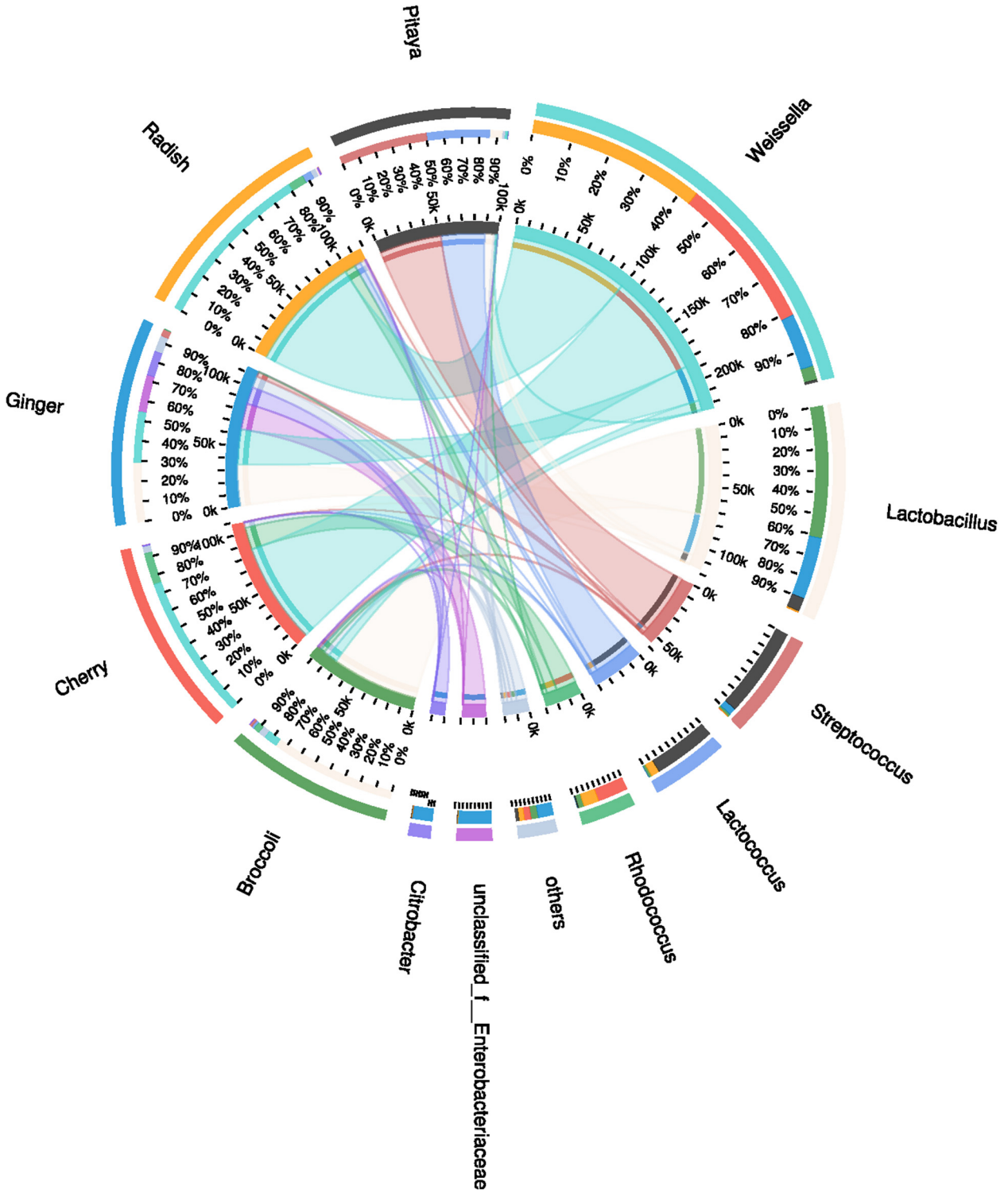

FIGURE 1 | Distribution of the microbial community for each sample at the genus level. The data were visualized by Circos. The small semi-circle **(left)** shows the composition and abundance of species in each sample. The large semi-circle **(right)** shows the distribution ratio of samples within the dominant species, and the width of the bars for each genus indicates the relative abundance of that genus in the sample. From the outer circle to the inner circle, the outer circle shows the different samples and species using specific colors, and the length is related to the distribution proportion. The second circle shows the percentage. In the innermost circle, the two ends of each chromatic stripe connect the sample with the dominant genus; the width of the stripe at the endpoint represents the abundance and distribution proportion, and the numerical value outside the circle represents the abundance values of the corresponding samples and species.

cucumber, tomato, chard, and other fruits and vegetables (Choi et al., 2003; Lee et al., 2004; Di Cagno et al., 2009a; Nicomrat and Chamutpong, 2016). Such differences in diversity were probably associated with the geographical location, harvesting season, storage position, processing techniques used, and other complex and various factors (Yoon et al., 2017). For example, the distribution of genera of *Enterobacteriaceae* differed significantly between the samples, and their relative abundance in ginger juice reached 19.0% whereas it was less than 0.5% in the other four samples. Some species in the *Enterobacteriaceae* family are known to be pathogenic or opportunistic. The ubiquity of *Enterobacteriaceae* genera in the studied ginger samples may be ascribed to unhygienic handling, inappropriate processing or storage conditions in the market (Stoops et al., 2016). Evidence suggests that the structural diversity of bacterial communities is closely associated with the organoleptic attributes, nutrients and, the quality of the fermented products (Liu and Tong, 2017). For instance, some species in the genus *Weissella* have potential as probiotics, owing to their ability to produce exopolysaccharides (e.g., *Weissella cibaria* and *Weissella confusa*) (Fusco et al., 2015). Moreover, a number of studies have reported the dominant effective microbes present in traditionally fermented pickles, fermented dough, yogurt, and fermented wine, as well as the production of foods fermented with LAB strains from their natural microbiota (Ben Omar and Ampe, 2000; Pinto et al., 2015; Fan et al., 2017; Motato et al., 2017). These analyses, highlighting the diversity and richness of microbial communities among the fermented FVJs, provided the foundation for the separation of LAB isolates (Pinto et al., 2015).

Variance Analysis of Samples and Dominant Species

The calculated values of the Shannon index of microbial diversity for the fermented juices (α-diversity) showed no significant differences, as demonstrated by an independent *t*-test. Moreover, the abundance matrix that was obtained from the fermented juices was subjected to principal component analysis (PCA) and hierarchical clustering analysis (β-diversity) (**Figures 2A,B**). The differences in the distributions among the fermented samples did not indicate significant dissimilarities, and the individual variations that were observed may be related to the preparation processes. Clustering of the various samples, which was based on the unweighted pair-group method with arithmetic mean (UPGMA), also did not show a statistically significant difference in the microbial diversity between pitaya, broccoli, ginger, cherry, and radish. Previous knowledge regarding such microbial biodiversity was mainly based on studies that assessed the processing of products such as olives, fermented sausage, and fermented cabbage (Giello et al., 2018; Medina et al., 2018; Wang and Shao, 2018). Comparatively, there is little research on the microbial composition of fermented FVJs using high throughput sequencing. In our study the microbial community structure in different samples showed no significant differences in α- and β- diversity; however, the dominant genera were variable. This contradicts with the results of the microbial profiles reported in other fermented vegetables, which significantly differed based on their region of origin and raw materials used (Peng et al., 2018). The results of difference analysis concerning the abundance of predominant genera in the five samples are depicted in **Figure 3**. The significance testing used strict statistical methods to detect obvious differences between genera on the basis of the data on abundances in the communities. The abundance of *Weissella* and *Lactobacillus* presented highly significant differences among the five samples ($P < 0.01$). In addition, *Streptococcus*, *Citrobacter*, *Enterococcus*, and *Enterobacter* also displayed significant differences between the samples ($P < 0.05$). *Weissella* has occasionally been found in fermented foods in comparison with *Lactobacillus* (Karovicova and Kohajdova,

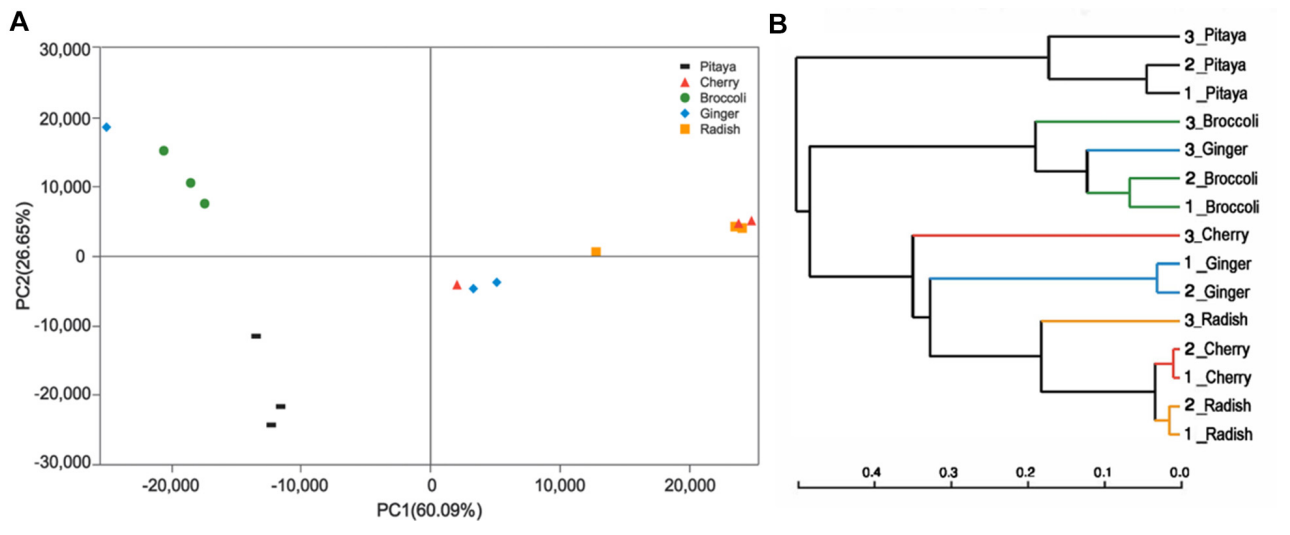

FIGURE 2 | Variance analysis of the bacterial communities among the fermented juices. **(A)** PCA plots indicate the abundance of diverse bacteria in the fermented juices. The first principal component (PC1) and second principal component (PC2) shows 26.65 and 60.09%, respectively, of the variance in the unweighted Unifrac metrics. Each point represents the microbiota from a single sample. **(B)** Hierarchical clustering of the group means based on UPGMA.

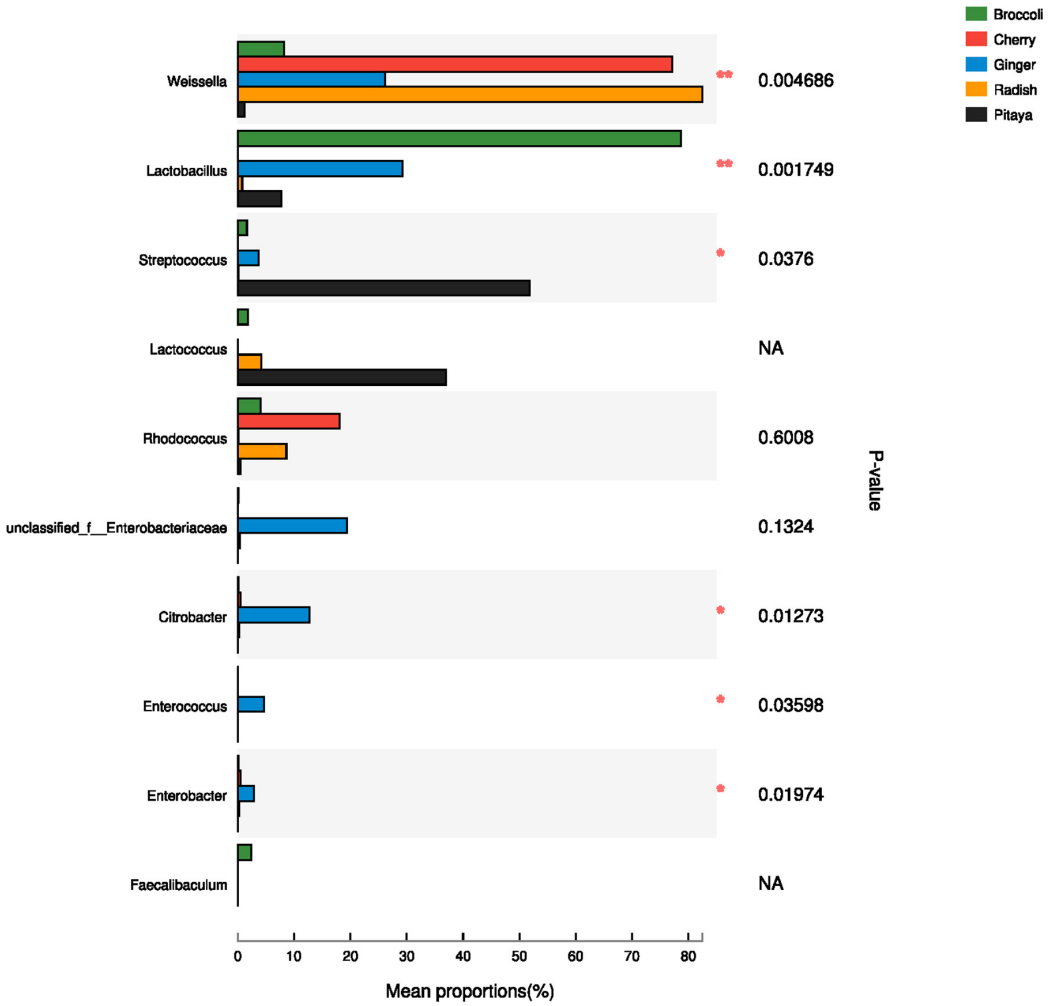

FIGURE 3 | Comparison of dominant genera in five samples using one-way ANOVA. In the vertical axis, the different identified bacteria genera are depicted; the length of the corresponding column indicates the average of the relative abundance of the genus in the different samples, $^{**}P < 0.01$, $^{*}P < 0.05$; NA, not available.

2005); these typical genera used in fermentation may promote various quality properties and are valuable sources of functional ingredients. For instance, *W. cibaria* RBA12 from pomelo can generate dextran and the survival of *Staphylococcus aureus* can be suppressed by *Lactobacillus rhamnosus* LOCK900 from carrot juice (Trząskowska and Gasentzer, 2016; Baruah et al., 2017). The verification of microbial diversity of food-inherent ecosystems is essential for revealing the natural processes and reconstructing such ecosystems under optimized and controlled conditions.

Identification and Typing of Isolated LAB Strains

Lactic acid bacteria strains were isolated from fermented FVJs and freshly squeezed juice without sterilization. The number of strains in each sample was determined according to macroscopic (colony morphology) and microscopic (cell morphology) characteristics. After confirmation of negative catalase reaction, Gram staining, and 16S rRNA sequencing analysis, the presumptive mesophilic LAB present in the highest dilution of the different fermented juices were identified. Phylogenetic relationships of the isolates together with representative 16S bacterial sequences were analyzed using the neighbor-joining method (Saitou and Nei, 1987). The resulting tree showed that the 32 isolates could be classified into six clusters on the basis of similarities in 16S rRNA sequences (**Figure 4**), namely *Leuconostoc* (3 isolates), *Weissella* (5 isolates), *Lactococcus* (5 isolates), *Pediococcus* (3 isolates), *Enterococcus* (15 isolates), and *Bacillus* (1 isolates). All isolates of different genera were separated into unique clusters. Notably, the similarity could be visualized among phylogenetically related isolates in **Figure 4**. At the similarity level of 70%, the lowest percentage of the isolates was grouped in genus *Enterococcus*, three isolates of *Leuconostoc mesenteroides* were closely related to *L. mesenteroides* ATCC 8293 with 95% identity, and other isolates were put in separate branches of the tree and showed 100% of identity with related type strain 16S rRNA sequences. The following species were identified for

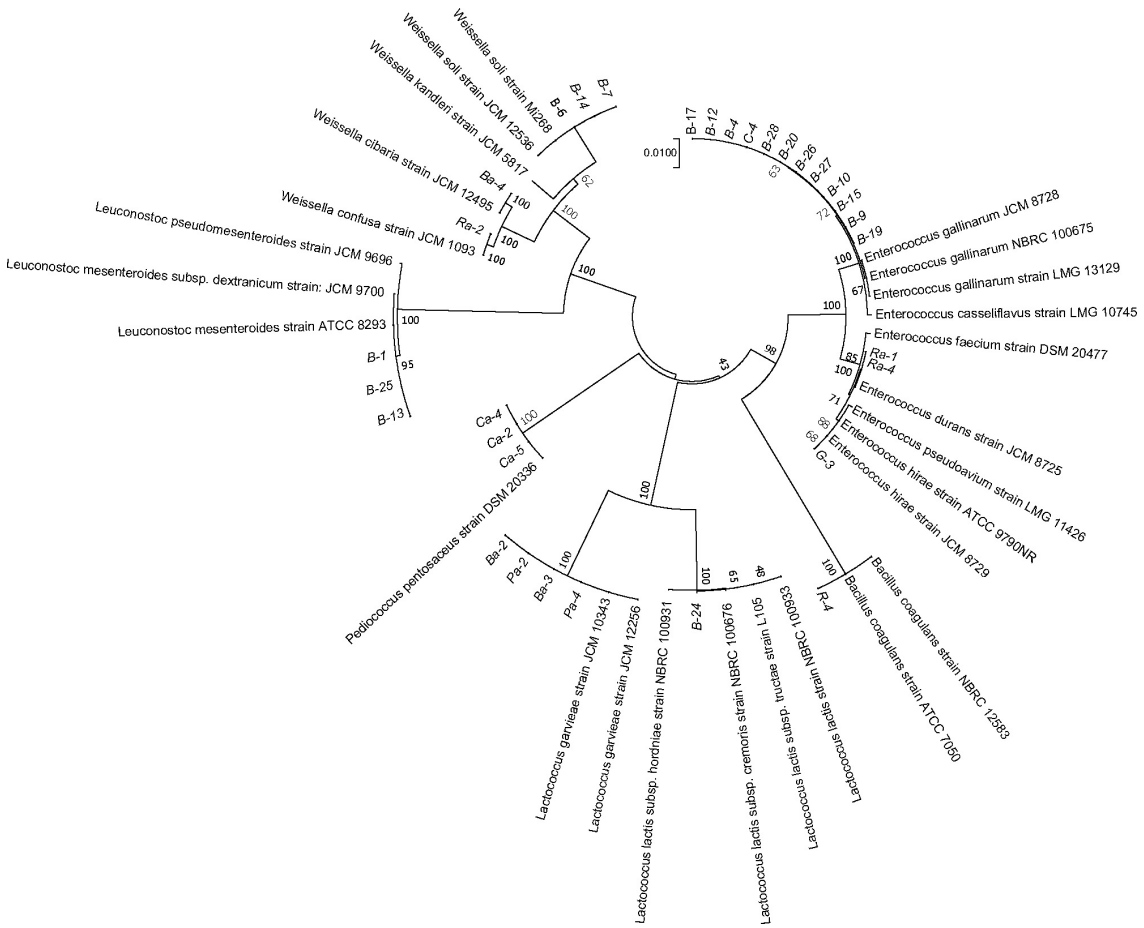

FIGURE 4 | Phylogenetic tree constructed with sequences of the partial 16S rRNA gene of selected LAB strains. The 16S sequences of the isolated bacteria are aligned with reference strains. The data of type strains of related species were from GenBank database. Branch lengths are proportional to distance. Bootstrap values are indicated near the internodes. B, R, C, G, and P represent juices of broccoli, radish, cherry, ginger, and pitaya, respectively; Ba, Ra, Ca, Ga, and Pa represent juices of broccoli, radish, cherry, ginger, and pitaya after fermentation, respectively.

each sample: broccoli, *L. mesenteroides* (3 isolates), *Weissella cibaria/soli* (4 isolates), *Enterococcus gallinarum* (11 isolates), *Lactococcus garvieae/lactis subspecies* (3 isolates); cherry, *Pediococcus pentosaceus* (3 isolates), *E. gallinarum* (1 isolates); radish, *W. confusa* (1 isolate), *Enterococcus durans* (2 isolates), *Bacillus coagulans* (1 isolate); pitaya, *Lactococcus garvieae* (2 isolates); ginger, *Enterococcus hirae* (1 isolate). *Lactococcus lactis* subspecies isolate B-24 from broccoli juice needed a further identification based on the 16S rRNA and *recA*, *groEL* genes (Le Bourgeois et al., 2015). The results of the culture-dependent analysis demonstrated that species in fermented radish and pitaya juices with the highest concentration were in accordance with the most highly abundant species detected by culture-independent analysis, namely, *Weissella* and *Lactococcus* species, respectively. However, the results of *P. pentosaceus* in fermented cherry juice (approximately 10^6 CFU/mL) as well as *Lactococcus* and *Weissella* (approximately 10^7 CFU/mL) in fermented broccoli juice did not match the high throughput sequencing results. The dominant microbiota in fermented broccoli and ginger juices were *Lactobacillus* species, but no isolates from this genus were detected in the highest dilutions of fermented ginger juice. Probably the necessary conditions for successful isolation of the different *Lactobacillus* species might not be fully efficient with MRS as the selecting medium, since some species of this genus require enrichment conditions for their successful isolation from environmental samples. This inconsistency has also been observed during the detection of potential foodborne pathogens during the kimchi elaboration process (Lee et al., 2017). This phenomenon may be ascribed to the facts that not all the isolates in the different dilutions were identified and culture-independent analyses did not discriminate between live and dead microbial cells (Fusco and Quero, 2014; Liu and Tong, 2017). In addition, changes in the fermentation conditions also played a role in the distribution of colonies. For example, it has been shown that the *Pediococcus* species can be detected in table olives through culture-independent analysis, but cannot be isolated which could be due to their low survival rate in acidic conditions (Sanchez et al., 1995). Species in the genus *Enterococcus* can play a positive role in various fermented products and have attracted more attention

in recent years, than the normally relatively common species in LAB groups (M'hir et al., 2012). Although species mainly from humans and domestic animals have been studied in some detail, limited information is available on plant-associated species. **Figure 4** shows that *E. gallinarum, E. durans,* and *E. hirae* were identified in the fermented juices. Even though *Enterococcus* species are considered indicators of fecal contamination (e.g., in water), or even as potentially pathogenic microorganisms, they possess many desirable properties, such as improvements in sensory characteristics, natural preservation, and health-related benefits, that could increase the value of vegetable-based fermented foods (Ben Omar et al., 2004). The heterogeneous nature of fermented products, with variations in microbial diversity, quality, and properties, requires the exploitation of appropriate starter cultures to initiate fermentation and obtain consistent products with acceptable quality. Undoubtedly, the identification of relevant strains can provide the foundation of a mixed fermentation starter for the elaboration of compound juices.

Dynamic Variance in Viable Cell Counts and pH in Fermented Broccoli Juice

Weissella cibaria Ba-4, *L. mesenteroides* B-25, *L. lactis* subspecies B-24, *P. pentosaceus* Ca-4, *E. hirae* G-3, *L. garvieae* Pa-2, and *W. confusa* Ra-2 were selected for further investigation. Each of these strains belonged to different clusters among the various LAB strains that were identified. To assess their adaptation to broccoli juice, dynamic variations in pH and viable cell counts were determined and compared with those of the commercial starters, *L. plantarum* CICC20265 and *S. thermophilus* CICC6220. The cell densities of all the autochthonous strains increased from 7.0 Log CFU/mL to values that ranged from 10.2 ± 0.39 to 11.0 ± 0.58 Log CFU/mL. Overall, the stationary growth phase was reached after 18 h fermentation at 37°C, when both the commercial and the autochthonous strains reached a cell density of 10.52 ± 0.37 Log CFU/mL (**Figure 5A**). Based on previous research, we know that allochthonous strains tend to demonstrate poor growth characteristics in comparison with autochthonous isolates (Di Cagno et al., 2009a), as has been reported for fermented carrots (8.57/7.62 Log CFU/mL), French beans (8.95/8.08 Log CFU/mL), marrows (8.48/7.40 Log CFU/mL), mangoes (10.33/7.71 Log CFU/mL), and tomatoes (9.8/8.52 Log CFU/mL) (Di Cagno et al., 2008, 2009a,b; Liao et al., 2016). Although almost a similar behavior was observed for the commercial species, the indigenous isolates may certainly have influenced the fermentation and the characteristics of the final product.

Furthermore, dynamic changes in pH were directly associated with the cell density of LAB. As shown in **Figure 5B**, the pH of *L. lactis* B-24 and *W. confusa* Ra-2 cultures decreased significantly after 18 h of fermentation in comparison with broccoli juice fermented without a starter (pH 6.63 ± 0.02), and the average pH of *L. plantarum* and *S. thermophilus* fermented juices was as low as 3.65 ± 0.12. *P. pentosaceus* Ca-4 (pH 3.83 ± 0.24) and *L. garvieae* Pa-2 (pH 3.98 ± 0.06) reflected the best acidification characteristics in fermentation. As previously reported, indigenous strains of *L. mesenteroides* can reduce the pH of fermented prickly pear from 6.01 to 4.07, *W. confusa* reduced the pH of fermented peppers from 5.0 to 3.7, and *L. plantarum* reduced the pH of fermented tomato juices from 4.3 to 3.78 (Di Cagno et al., 2009a,b, 2016). However, the strains isolated from broccoli juice did not show any obvious superiority in growth rate and capability to decrease pH, and this probably might be attributed to inherent characteristics of the raw material used for growing the LAB strains (Santo et al., 2011). Previous reports have indicated that *Lactobacillus* and *Bifidobacterium* strains sustain higher viability in orange and pineapple juices in comparison with cranberry juice (Sheehan et al., 2007), and similar results have also been observed for

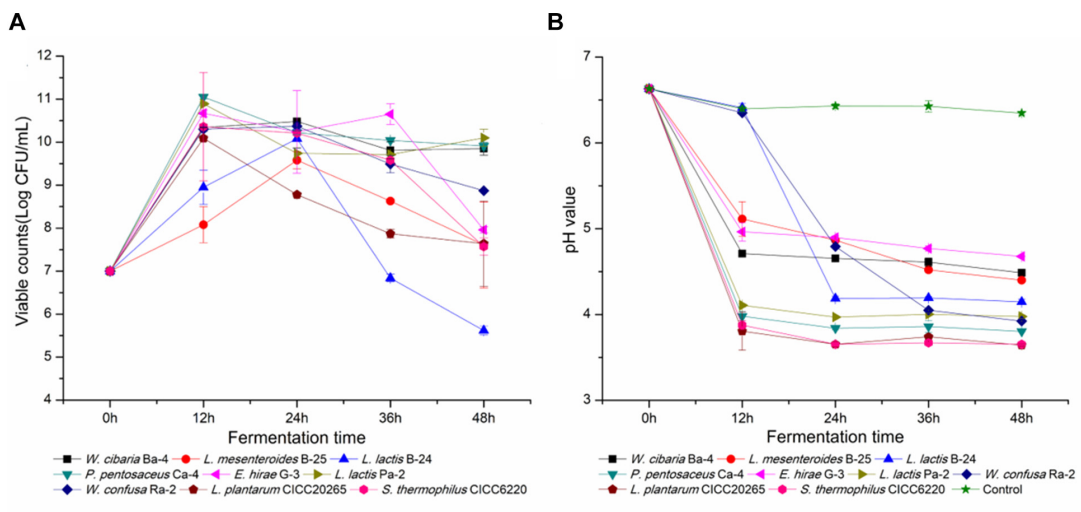

FIGURE 5 | Viable cell counts and pH values throughout the fermentation of broccoli juices incubated with the nine selected LAB strains. **(A,B)** Represent the change in viable cell counts and pH, respectively.

pomegranate juice when different starters were used (Mousavi et al., 2011). Our observations indicated that broccoli juice was appropriate for LAB fermentation, as it enabled a rapid bacterial growth and a sufficient population of viable cells, consistent with the results of tomato, carrot, cabbage, artichokes, and reed beet juices in regards to suitability as a fermentation substrate (Valerio et al., 2006; Rivera-Espinoza and Gallardo-Navarro, 2010; Di Cagno et al., 2013). It would be beneficial to optimize a combination of species isolated from fermented raw fruits and vegetables, with the aim of comprehensive utilization in a wide range of fermented foods.

Antibiotic Resistance

Lactic acid bacteria strains have been widely used in commercial applications and have been specifically selected to discourage the spread of antibiotic resistance and prevent the exchange of transferable resistance genes (Ouwehand et al., 2016). According to the breakpoints recommended by the European Food Safety Authority (European Food Safety Authority [EFSA], 2012) and the interpretive category defined by CLSI, the antibiotic resistance was shown in **Table 1**, our results demonstrated that the nine selected isolated strains were all susceptible to amoxicillin (10 μg) and resistant to amikacin (30 μg). Charteris et al. (1998) tested 46 *Lactobacillus* strains from human and dairy sources for susceptibility to 44 antibiotics, and all strains were resistant to 14 antibiotics, including amikacin (30 μg), gentamicin (10 μg), streptomycin (10 μg), and norfloxacin (10 μg) (Charteris et al., 1998), antibiotics that were also assessed in our study. In contrast to this previous report, the nine strains tested in the present study exhibited sensitivity or intermediate susceptibility to gentamicin (10 μg). There have been reports that corroborate our findings regarding the susceptibility of LAB to gentamicin, for example, Jiang et al. (2016) reported the intermediate susceptibility to gentamicin of *Lactobacillus* strains isolated from human milk. Furthermore, isolates belonging to the same species may show several sensitivities to the same antibiotic; for instance, most *Leuconostoc* species tested were resistant to gentamicin (10 μg), but *L. mesenteroides* B-25 was susceptible (Ammor et al., 2007). It has also been demonstrated that the source of indigenous isolates influences the antibiotic resistance; 31 indigenous *Lactobacillus* isolates from curd and human milk showed strong resistance to streptomycin (10 μg) (Sharma et al., 2017). In the three isolates of our study, only an intermediate susceptibility was observed. All the isolates tested in the present study exhibited strong resistance to erythromycin (15 μg) except for *L. lactis* subspecies B-24. However, earlier studies showed that low resistance frequencies (0.7% in each case) among LAB isolates of *Lactobacillus*, *Pediococcus,* and *Lactococcus* species have potential for probiotic or nutritional use (Klare et al., 2007). In general, previous results have indicated that variations in source, species, inoculum size, incubation temperature and time, and even the test medium can influence the activity of probiotics including the pattern of antibiotic sensitivity (Herrero et al., 1996). The high resistance and sensitivity of LAB strains to a range of antibiotics used in the medical practice is considered highly significant, since there is the probability of transferring antibiotic resistance from LAB strains to other undesirable

TABLE 1 | Antibiotic susceptibility profile of selected LAB isolates.

Strains	Ampicillin (10 μg)	Penicillin (10 μg)	Amoxycillin (10 μg)	Norfloxacin (10 μg)	Levofloxacin (5 μg)	Gentamicin (120 μg)	Streptomycin (10 μg)	Amikacin (30 μg)	Erythromycin (15 μg)
W. cibaria Ba-4	S	R	S	R	IS	S	R	R	R
L. mesenteroides B-25	R	R	S	R	S	S	R	R	R
L. lactis B-24	S	R	S	S	S	S	R	R	S
P. pentosaceus Ca-4	IS	R	S	R	IS	S	R	R	R
E. hirae G-3	IS	S	S	IS	IS	S	IS	R	R
L. garvieae Pa-2	S	R	S	IS	IS	IS	IS	R	R
W. confusa Ra-2	S	R	S	R	S	S	IS	R	R
L. plantarum CICC20265	S	R	S	R	R	S	R	R	R
S. thermophilus CICC6220	S	R	S	R	IS	S	R	R	R

Strains showing resistant (R), susceptibility (S) and intermediate susceptibility (IS).

and detrimental organisms. The ability to transfer antibiotic resistant factors must be considered as an important parameter in the selection of probiotic strains. Most studies on antibiotic resistance that have been conducted so far in LAB have involved members of the genus *Enterococcus*, which occupies a peculiar position among food microorganisms. The *Enterococcus* species play a pivotal role in traditionally fermented foods, but their role as opportunistic pathogen has also been acknowledged. Our results demonstrated that *E. hirae* G-3 showed susceptibility and intermediate susceptibility to most of the tested antibiotics with the exception of amikacin (30 μg) and erythromycin (15 μg). It is important to investigate the location of the antibiotic resistance genes and to determine their potential transfer prior to the commercial use of these isolates. This study facilitates an understanding of the differences in antibiotic resistance profiles among various LAB strains and establishes a basis for optimally selecting probiotics to manufacture high-quality fermented products.

Surface Hydrophobicity

Adhesion to the intestinal epithelial mucosa is related to many beneficial functions that are attributed to probiotics (Dunne et al., 2001). This is a complicated process that involves contact of bacteria with the intestinal mucosa surface and is influenced by multiple factors. In previous studies, the cell adhesion capability of *Streptococcus*, *Lactobacillus*, and *Bifidobacterium* species has been assessed by testing their adhesion to hydrocarbons. A positive correlation between adhesion ability and hydrophobicity has been observed (Wadstrom et al., 1987; Colloca et al., 2000; Nikolic et al., 2010). Some researchers have proposed that surface hydrophobicity could be used to identify *Bifidobacterium* species with adhesion potential to enterocytes (Del Re et al., 2000). Therefore, the reliability of the use of bacterial adhesion to hydrophobic compounds to measure the adhesion ability of LAB is clear (Vinderola et al., 2004). In our study, the phase separation of bacterial cells between the aqueous phase and ethyl acetate and xylene is shown in **Figure 6A**. The data demonstrated adhesion percentages of 13.4 ± 5.2% ∼ 36.4 ± 3.2% and 21.6 ± 1.4% ∼ 69.6 ± 2.3% to ethyl acetate and xylene, respectively, supporting the hypothesis that the cells possessed good adhesion properties. Previous reports have shown that the percentage of adhesion to ethyl acetate and xylene of *Propionibacterium* species ranged from 7.0 ± 2.8 to 71.0 ± 2.1% and from 2.0 ± 1.0 to 79.0 ± 1.6%, respectively (Darilmaz et al., 2012). Similar reports have shown that the binding percentage of *Bifidobacterium* and *Lactobacillus* to xylene was in the range of 17.4 ± 8.5% ∼ 75.2 ± 9.0% and 13.5 ± 5.0% ∼ 67.1 ± 10.7%, respectively (Collado et al., 2008). In this study, the most hydrophobic strains were *L. mesenteroides* B-25 (36.4 ± 3.2% to ethyl acetate) and *L. garvieae* Pa-2 (69.6 ± 2.3% to xylene). Moreover, *L. garvieae* Pa-2 presented binding proportions of 23.3 ± 4.0 and 69.6 ± 2.3% to ethyl acetate and xylene, respectively, which represented a significant difference. Our results revealed a great heterogeneity in adhesion to hydrophobic compounds. High or low affinity for a solvent did not exclude simultaneous affinity for the other solvent, suggesting that the cell surface was very complex. This may be due to the presence of proteins or polysaccharides on the cell surface leading to differences in hydrophobicity (Walker, 2008; Giri et al., 2018). The cell surface hydrophobicity test results can be used for preliminary screening in order to identify probiotic bacteria that are suitable for human or animal use.

Response to Simulated Gastrointestinal Tract Conditions

Tolerance to low pH and bile salts during transit through the gastrointestinal tract is essential for LAB to survive, grow, and

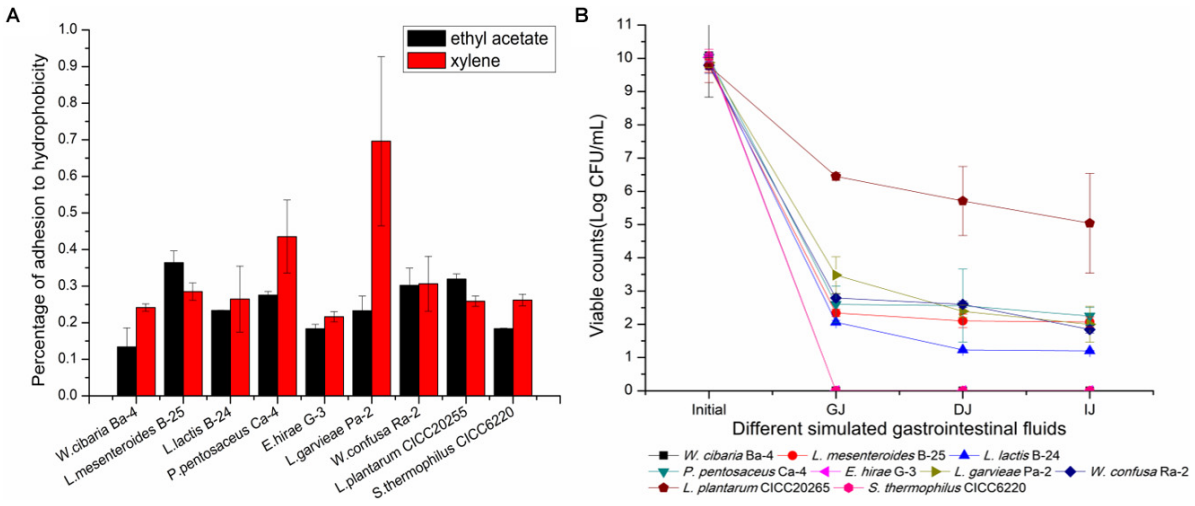

FIGURE 6 | Evaluation of hydrophobicity to ethyl acetate and xylene and survival of simulated gastrointestinal digestion of the nine selected LAB strains. **(A)** Percentage of hydrophobicity. **(B)** Survival capacity of LAB strains during simulated gastrointestinal digestion. GJ, gastric juice; DJ, duodenal juice; IJ, intestinal juice.

exert their beneficial functions (Jena et al., 2013). The loss of viability after exposure to simulated gastrointestinal tract conditions has been reported in several previous studies (Santos et al., 2016; Freire et al., 2017). The survival rate or loss of viability was calculated by a comparison of bacterial counts during the gastrointestinal transit *in vitro*. As shown in **Figure 6B**, *P. pentosaceus* Ca-4 and *L. mesenteroides* B-25 exhibited the highest survival rates after the gastrointestinal transit of 22.4 ± 2.6 and 21.2 ± 1.4%, respectively. However, the viable population only maintained 3.0 Log CFU/mL, which was significantly lower than that of *L. plantarum* CICC20265. *W. cibaria* Ba-4, *E. hirae* G-3, and *S. thermophilus* CICC6220 lost their viability during the transit. The LAB present in fermented FVJs must sustain their viability during gastrointestinal transit and achieve eventual engraftment in the host gut mucosa (Ranadheera et al., 2012). According to the literature, several strains exhibit different cell survival rates under harsh environmental conditions. For instance, cell counts of 6.40 Log CFU/g of *Lactobacillus bulgaricus*, 8.70 Log CFU/mL of *L. casei* DN-114 001, and 5.86 Log CFU/g of *P. pentosaceus* Q3 remained after gastrointestinal transit. Survival rates of 0.1–40% for *L. lactis* and 36.6% for *L. mesenteroides* IM082 were reported, which suggests that microencapsulation and other protective technologies may be beneficial for extending the application of probiotics (Oozeer et al., 2004; Mainville et al., 2005; Dobson et al., 2011; Jensen et al., 2012; Chen et al., 2017). After transiting through simulated GJ for 3 h, the *Bifidobacterium* species exhibited a viable bacterial cell count of 7.32 Log CFU/mL, with a survival rate of 72.1%, and these results may be associated with the anaerobic fermentation characteristics of the *Bifidobacterium* species (Watson et al., 2008). Notably, the colonization level and the capacity to remain in the gastrointestinal tract were somewhat inconsistent among different strains. Hence, it is important to highlight that the isolates identified from broccoli juice did not show specific superiority when compared with other strains, although some studies have reported that strain variation as well as an appropriate carrier food matrix can potentially improve the survival of probiotics in the presence of simulated gastric and small intestinal juices (Saxelin et al., 2010; Ranadheera et al., 2012). Fruits and vegetables are valuable nutrient sources, making them ideal substrates for growing probiotics (Shori, 2016). Researchers revealed that the composition of the carrier food matrix such as fat content may provide additional protection for probiotic species (Pigeon et al., 2002; Vinderola and Reinheimer, 2003). Even though the isolates in this study were all indigenous phytogenic strains, there were observable differences in tolerance to acidic conditions and bile salts in terms of different survival rates during passage through the gastrointestinal tract. The results showed that the application of probiotic cultures in different food matrices could represent a great challenge for the viability of probiotics. It is essential for the isolated strains to have a protection system to withstand the low pH in the stomach and digestive enzymes and bile of the small intestine (Jensen et al., 2012). The findings of this study suggest that adequate measurement of probiotic potential LAB starters should be carried out in the intended carrier foods. In summary, based on our study results, potential LAB starters used to obtain reliable and controlled fermentation processes can be selected from the isolates of autochthonous microbiota of raw FVJs, for example, *L. garvieae* Pa-2 (GenBank accession number: MH198321), *P. pentosaceus* Ca-4 (GenBank accession number: MH198320), and *L. mesenteroides* B-25 (GenBank accession number: MH198322).

CONCLUSION

Both the traditional culture-dependent method and molecular technique were used to determine the composition of LAB populations in fermented FVJs. A wide diversity of autochthonous bacterial communities was identified among the five fermented FVJs, namely, broccoli, ginger, pitaya, cherry, and radish juices. The fermentation characteristics of strains in broccoli juice, as well as their antibiotic resistance, hydrophobic properties, and survivability in the simulated gastrointestinal tract environment, which are all important factors that influence the efficacy of probiotics, were also investigated. The results indicated similarities and differences in bacterial abundance between the various fermented products, with isolated indigenous microbes present on the fruit and vegetable surface, as well as inoculated commercial species, having potential use in the processing of fermented FVJs.

The use of indigenous microbes and appropriate fermentation conditions are crucial for the elaboration of high-quality fermented FVJs. The species obtained in this study demonstrated their potential to be used as starter cultures to overcome unstable and/or unmanageable fermentation conditions encountered in the production of FVJs. Further investigations will aim to better understand the mechanisms underlying the observed diversity among different materials. In addition, further studies are still required to clarify how the endogenous microbiome can affect the properties of fermented juices and to identify the bacteria responsible for the quality of fermentation foods. Such research will aid in the development of functional autochthonous starters and help to diversify the availability of processed high-quality fruit and vegetable products.

AUTHOR CONTRIBUTIONS

JW and XL conceived and designed the experiments. XX wrote the paper. XX, DL, and YB revised the manuscript and performed the experiments.

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Characterization of Bacterial Communities in Mexican Artisanal Raw Milk "Bola de Ocosingo" Cheese by High-Throughput Sequencing

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The dynamics of bacteria community of "Bola de Ocosingo" cheese, a Mexican artisanal raw milk cheese was investigated by high-throughput sequencing (454 pyrosequencing). Dairy samples (raw milk, curd, cheese at 50 and 110 days of ripening) were collected at dry (March-June) and rainy season (August-November) from three producers located in Chiapas, Mexico. In general, raw milk contained high bacterial diversity which was reduced throughout cheese manufacture. However, in two productions an important increase during cheese ripening was observed probably due to cross-contamination. Species such as *Streptococcus thermophilus*, *Lactococcus lactis*, *Lactobacillus helveticus*, *L. delbrueckii* and *L. plantarum* from which potential probiotic strains may be obtained, predominated during processing, varying its prevalence from one producer to another. Furthermore, low proportions of *Escherichia coli*/*Shigella flexnerii* were detected in almost all processes, however, could not be recovered by traditional methodology, indicating presence of non-cultivable cells. This work provides insights into bacteria communities of Bola de Ocosingo cheese for starter culture development, many of which are reported to provide health related benefits, and the usefulness of high-throughput sequencing to evidence cross-contamination during processing.

Keywords: artisanal cheese, lactic acid bacteria, microbial communities, pyrosequencing, raw milk

INTRODUCTION

"Bola de Ocosingo" cheese is a Mexican artisanal short-ripened cheese elaborated with raw milk produced in Chiapas, Mexico, and is characterized for uneven quality as a result of differences in processing conditions, the use of traditional acidification by autochthonous lactic acid bacteria (LAB) present in the raw materials, and the absence of controls in storage. These factors limit the distribution to regional level and furthermore, represent a health risk for consumers as the raw milk may contain foodborne pathogens (Cervantes et al., 2006).

For cheese-making producers, Mexican normativity indicates the use of pasteurized milk (Nom-243-SSA1-2010, 2010). However, pasteurization eliminates some of the indigenous microflora in the raw milk, affecting adversely sensorial characteristics such as flavor, odor and texture quality (Albenzio et al., 2001). Adding microorganisms (such as LAB) selected from artisanal production process, as starter culture to produce cheese using pasteurized milk would allow the development of desirable sensorial characteristics, constant quality and safety in the final product (Torres-Llanez et al., 2006).

Studies focused on bacterial biodiversity during cheese processing may serve as the first step in the development of starter cultures containing technologically relevant microorganisms from the cheese under study (Riquelme et al., 2015). Furthermore, these microorganisms could provide probiotic effects in the host, if properly isolated and characterized (Monnet and Bogovic, 2012). To address the diversity of microbial communities throughout cheese manufacture and ripening, culture-independent molecular techniques such as pyrosequencing has been applied enabling rapid insight into composition, structure and dynamics of microbial communities (Alegria et al., 2012).

In this study, high-throughput sequencing was used to describe dynamics of bacterial communities during processing of Bola de Ocosingo cheese.

MATERIALS AND METHOS

Cheese

Bola de Ocosingo cheese was made with raw milk from Braunvieh, Brown Swiss and Zebu cows. A portion of the raw milk was skimmed, obtaining cream that was added to the batch process, in a portion of 4 Kg of cream per 100 L of milk. The mixture was curdled using 2.5 mL of commercial calf rennet 1:10,000 (Cuamex, Mexico) for 5–12 h without addition of $CaCl_2$. After 4–8 h, the curd was cut, rested for 12 h, transferred to a muslin cloth sack, hanged to drain whey for up to 5 days, and then finally salted (3–4%). Dry curd was transferred to a new muslin sack, hanged for up to 5 days, and this step repeated for 50 days to obtain a ripened curd. Curd was then crumbled and mixed with butter only if needed, in a portion of 0.3–0.5 Kg per 10 Kg of ripened curd, obtaining a texture similar to double-cream cheese, shaped in balls (200–300 g) and covered with *pasta filata* cheese obtained from recently skimmed milk which was acidified with vinegar, curdled, whey was drained, then boiling water was added to melt the curd. It can be sold fresh (50 days of ripening), or aged an additional 2 months (110 days of ripening). All the processing, distribution and storage in the market is at room temperature, the final product is sold without cover other than the hardened *pasta filata*, which is not consumed.

Sample Collection

Twenty-four samples were obtained from three producers (A, B, and C) in the state of Chiapas, Mexico, collected at dry (March-June) and rainy seasons (August-November). One sample of raw milk, curd, fresh cheese (curd at 50 days of ripening mixed with butter and covered with *pasta filata*) and ripened cheese (110 days of ripening) from the same batch was obtained from each manufacture and season. Samples were stored at −20°C and transported to the Universidad Autónoma de Querétaro for analysis.

DNA Extraction

Total genomic DNA was extracted as previously reported (Aldrete-Tapia et al., 2014) consisting in a pre-treatment to remove food lipids, proteins and salts, obtaining the cell pellet. Cells were subjected to lysis by heat and powdered glass and DNA was purified with phenol-chloroform, and ethanol precipitation.

Pyrosequencing (454 Sequencing)

DNA sequencing was performed at MR DNA[1] (Shallowater, TX, United States). Briefly, the 16S rRNA gene was amplified using the primers 27Fmod (AGRGTTTGATCMTGGCTCAG) and 530R (CCGCNGCNGCTGGCAC); 454-adaptors were included in the forward primer as well as a barcode for each sample. The sequencing was performed utilizing a Roche 454 FLX titanium (Roche diagnostics Ltd., West Sussex, United Kingdom) instrument and reagents, following the manufacturer's instructions. The sequence data were processed using Mothur version 1.31.2 software (Schloss et al., 2009) with a modified pipeline. Briefly, sequences were subjected to quality controls and the 454-adaptors trimmed; unique sequences were aligned to the SILVA reference database. Chimeras were removed from aligned sequences with the uchime algorithm and classified to obtain the taxonomic assignment using the Silva 16S rRNA gene database. The final sequences were normalized to the lower number of sequences reads (data was subsampled to 1834 sequences). Good's coverage, Chao1 richness and Inverse Simpson diversity indices were calculated and rarefaction curves produced. Quality sequences were aligned to the 16S rRNA gene sequences using the BLAST tool from NCBI. Permutational multivariate analysis of variance (PERMANOVA) calculating Bray-Curtis dissimilarity was used to determine if proportions of main species detected were different across seasons, producers and sample type with the function *Adonis* in the library *vegan* (Oksanen et al., 2016) for R software (R Core Team, 2016).

RESULTS

Bioinformatic Analysis of Sequences

A total of 160,617 quality reads with average length of 280 bp (range = 264 to 327) were obtained from 24 dairy samples after Mothur pipeline. Number of sequence reads and OTUs, as well as diversity, richness and coverage estimators (calculated with an identity at 3% sequence similarity level) are shown in **Table 1**. Rarefaction curves approached a plateau, meaning most microbial diversity was captured within the number of

[1]http://www.mrdnalab.com

TABLE 1 | OTUs identified at 97% similarity, species richness estimate (Chao1), diversity index (inverse Simpson) and coverage for 16S rRNA sequencing of dairy samples from Bola de Ocosingo cheese production.

Manufacturer	Season	Sample	Reads	OTUs	Chao1		Inverse Simpson		Good's coverage
A	Dry	Milk	7984	217	295.96	(259.19–364.80)	12.97	(12.43–13.56)	99.16%
		Curd	2186	39	73	(49.10–153.42)	3.89	(3.77–4.01)	99.22%
		Cheese 50	2006	30	35	(31.16–51.57)	2.80	(2.69–2.92)	99.5%
		Cheese 110	4014	24	36	(26.66–78.20)	1.12	(1.1–1.13)	99.78%
	Rainy	Milk	12267	311	505.24	(430.29–627.28)	8.22	(7.98–8.49)	99.01%
		Curd	30084	82	122.07	(98.95–176.73)	1.36	(1.35–1.37)	99.89%
		Cheese 50	21104	77	111.36	(90.39–165.16)	2.48	(2.46–2.50)	99.87%
		Cheese 110	19618	162	279.30	(223.46–385.86)	4.22	(4.17–4.27)	99.65%
B	Dry	Milk	3034	19	26	(20.34–55.54)	1.21	(1.19–1.24)	99.77%
		Curd	2054	35	53.20	(40.1–99.89)	3.58	(3.48–3.69)	99.32%
		Cheese 50	2725	56	96.63	(71.02–165.89)	1.52	(1.47–1.58)	99.05%
		Cheese 110	3563	24	27	(24.5–41.95)	1.41	(1.38–1.45)	99.83%
	Rainy	Milk	6654	26	27.50	(26.15–41.08)	5.21	(5.06–5.36)	99.95%
		Curd	1834	46	63	(51.34–100.16)	2.14	(2.03–2.27)	99.07%
		Cheese 50	4828	81	100.71	(88.32–134.09)	5.12	(4.91–5.34)	99.50%
		Cheese 110	8410	145	291.30	(223.32–418.28)	5	(4.87–5.12)	99.08%
C	Dry	Milk	2388	76	106.67	(87.15–160.35)	4.74	(4.52–4.99)	99.00%
		Curd	7223	77	93.87	(83.15–123.26)	2.44	(2.38–2.50)	99.68%
		Cheese 50	3335	46	69.75	(53.99–116.61)	1.84	(1.78–1.91)	99.4%
		Cheese 110	2180	50	100.14	(68.47–186.09)	1.46	(1.41–1.52)	98.76%
	Rainy	Milk	3589	92	119.60	(102.11–167.35)	9.64	(9.21–10.11)	99.33%
		Curd	2341	41	92	(55.99–214.50)	2.12	(2.03–2.23)	99.23%
		Cheese 50	3386	41	53.75	(45.14–80.28)	1.53	(1.49–1.58)	99.47%
		Cheese 110	3810	26	31.63	(27.32–50.06)	1.18	(1.16–1.2)	99.74%

samples (**Supplementary Figure S1**). Good's coverage indicated a satisfactory overall sampling with levels above 99%.

Higher richness and diversity were observed in samples obtained in the rainy season, particularly from manufacturer A. In general, Chao1 and Inverse Simpson estimators decreased with steps in the cheese process, and following ripening. Despite this, an increase in both estimators was observed during manufacture from manufacturer A and B in the rainy season.

Diversity of Bacterial Species During Processing

Pyrosequencing revealed the bacterial community at species level present in dairy samples collected during Bola de Ocosingo cheese production in two seasons (dry and rainy) among three producers (**Figure 1**).

Bacterial composition differed across samples type ($P < 0.05$) as main effect (**Table 2**), in which a reduction in the number of species was observed, some of them becoming predominant at final stages of processing. Also, the interaction between producer and season was statistically significant ($P < 0.05$), in which the predominance of certain species among samples analyzed between producers at the two seasons had an effect.

In raw milk, the dominant species included bacteria that naturally occur in milk, such as *Streptococcus thermophilus*, *Lactobacillus delbrueckii* and *Lactococcus lactis* (**Table 3**). *Macrococcus caseolyticus* was found in low abundance, with exception of the sample obtained from manufacturer C in the rainy season, accounting for 22% of reads. Also, environmental microorganisms were observed, including *Acinetobacter junii*, *Bacillus licheniformis*, *Acidovorax* sp. or *Diaphorobacter* sp., and *Elizabethkingia meningoseptica*. For some raw milk samples, there was sporadic occurrence of species known to cause mastitis (i.e., *Enterobacter aerogenes* or *Routella planticola*, *Stenotrophomonas maltophilia*, *Citrobacter freundii*, *Staphylococcus epidermidis* and *L. garviae*).

In curd, *L. lactis* was in all samples (1.54 to 66.96 %), in combination with different LAB, such as *S. thermophilus* (0 to 85.24 %), *L. delbrueckii* (0 to 24.2 %), and/or *L. helveticus* (0 to 32.52 %). Curd sample from manufacturer A in dry season contained a high proportion of *E. aerogenes* or *R. planticola* (30.24 %) and *C. freundii* (30.6 %).

During ripening of cheese, species abundances (e.g., *S. thermophilus, L. helveticus, L. delbrueckii, L. lactis* and *L. plantarum*) were different across manufacturers and seasons. For instance, *S. thermophilus* dominated in cheese at 50 days (44.02 to 80.48%) and 110 days (32.37 to 94.67 %) of ripening among all manufacturers in both seasons. However, in cheese at 50 days of ripening made by manufacturer B in the rainy season and in cheese at 110 days made by manufacturer A in the rainy season, there was low abundance of this bacteria (0.023 and 5.43, respectively). For the former, there were other bacteria such as *L. lactis* (37.05 %), *L. plantarum* (19.64 %) *and L. brevis* (9.61 %), and for the latter, *L. plantarum* (26.04 %), *L. helveticus* (30.88 %) and *E. aerogenes* or *R. planticola* (26.30 %).

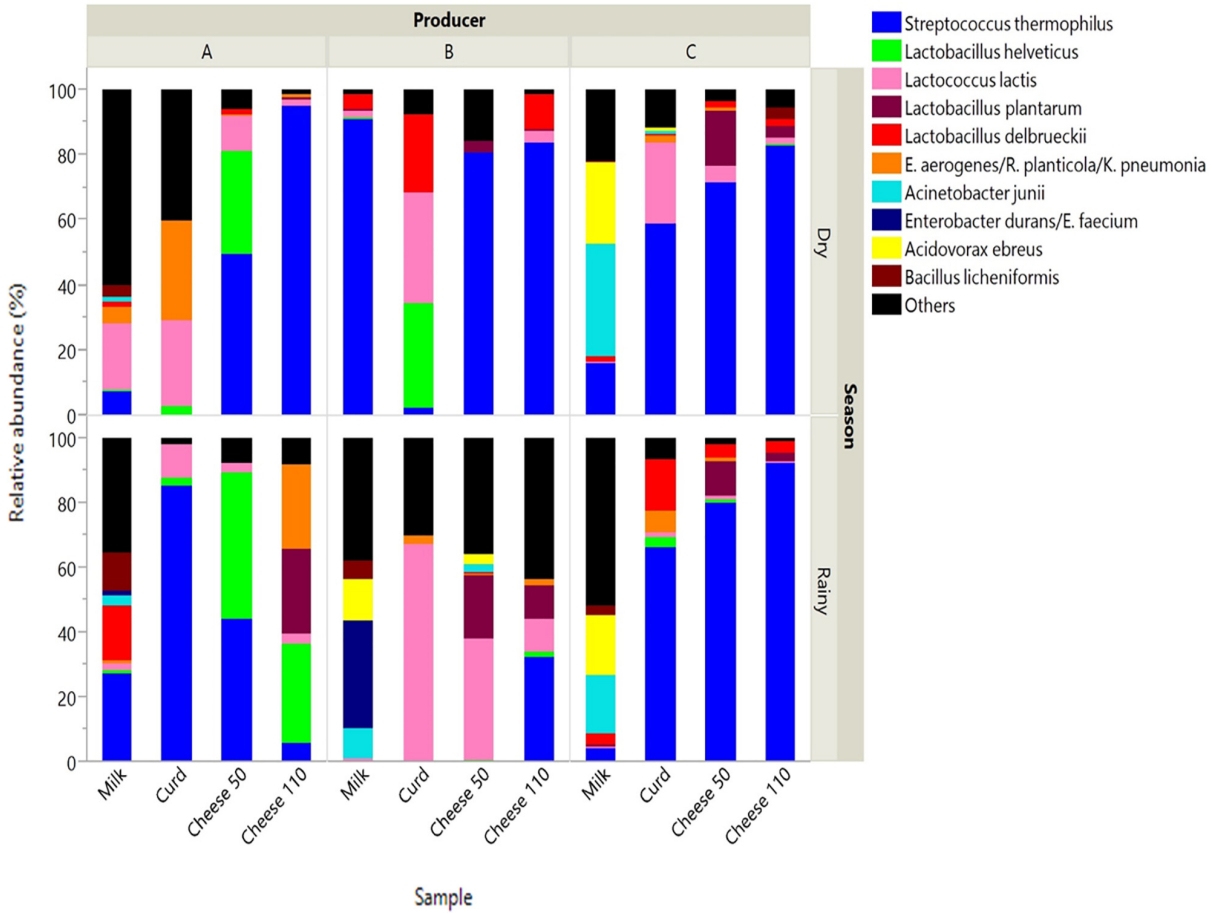

FIGURE 1 | Relative abundance of species in dairy samples collected from Bola de Ocosingo cheese production. Cheese 50: cheese ripened during 50 days. Cheese 110: ripened during 110 days.

TABLE 2 | PERMANOVA results of Bray-Curtis dissimilarities for main bacteria species found in Bola Cheese production process during two season from three producers.

Terms	Degrees of freedom	Sum of squares	*F*- value by permutation	R^2	*P*
Producer	2	0.5355	2.1919	0.10197	0.077
Season	1	0.2253	1.8443	0.0429	0.118
Sample	3	1.4692	4.0093	0.27978	0.005*
Producer x Season	2	0.7253	2.9687	0.13811	0.04*
Producer x Sample	6	1.0878	1.4842	0.20714	0.187
Season x Sample	3	0.4754	1.2974	0.09053	0.287
Residuals	6	0.7329		0.13957	
Total	23	5.2514		1	

*P-values are based on 999 permutations. *Statistical significance ($P < 0.05$).*

DISCUSSION

This study provides insight into the microbiota present during artisanal production of Bola de Ocosingo cheese from three manufacturers, in dry and rainy seasons. Differences depending of the producer between seasons were observed, in which mainly rainy season samples contained higher diversity. This suggests milk composition and environmental conditions, such as temperature and humidity, affect the types of bacteria introduced during Bola de Ocosingo cheese manufacture. Similar results have been observed during production of other types of cheeses in different seasons (Bonetta et al., 2008a,b; Hinz et al., 2012; Aldrete-Tapia et al., 2014).

TABLE 3 | Predominant species identified using BLAST from pooled sequences.

Species	% of total reads	Accession number of closest relative	Identity (%)	*E* value
Streptococcus thermophilus	44.88	NR 074827.1	98	9E-136
Lactobacillus helveticus	11.33	NR 075047.1	99	3E-151
Lactococcus lactis subsp. lactis	8.69	NR 103918.1	100	9E-146
Lactobacillus plantarum	5.13	NR 075041.1	100	9E-156
Enterobacter aerogenes/Raoultella planticola	4.33	NR 102493.1/NR 113701.1	100	2E-141
Lactobacillus delbrueckii	2.65	NR 043183.1	100	2E-153
Acinetobacter junii	1.74	NR 117623.1	100	4E-139
Enterococcus durans/E. faecium	1.56	NR113257.1/NR 114742.1	100	2E-152
Acidovorax ebreus	1.45	NR 074591.1	100	5E-138
Bacillus licheniformis	1.45	NR 118996.1	100	2E-146
Lactobacillus futsaii	1.43	NR 117973.1	99	2E-153
Enterococcus italicus	1.13	NR 025625.1	100	3E-150
Elizabethkingia meningoseptica	1.06	NR 115236.1	100	4E-139
Macrococcus caseolyticus	0.95	NR 074941.1	100	2E-147
Citrobacter freundii	0.83	NR 113596.1	100	2E-142
E.coli/ Shigella flexneri	0.76	NR 114042.1/NR 026331.1	99	8E-141
Lactococcus garvieae	0.69	NR 102968.1	100	7E-147

Environmental species were observed in raw milk, as well as other bacteria associated with mastitis in cows. A sample from manufacturer C contained high proportions of *M. caseolyticus*, a species in raw milk causing casein breakdown and contributing to formation of aroma precursors (Fuka et al., 2013). All of the species detected have been found in manufacture of other raw milk cheeses, such as Poro (Aldrete-Tapia et al., 2014), Danish (Masoud et al., 2011), Mozzarella (Ercolini et al., 2012), Fontina (Dolci et al., 2014) and Pico cheese (Riquelme et al., 2015), and usually disappear during subsequent processing. This reduction in bacterial diversity is attributed to selection of microorganisms during fermentation and manufacturing processes, attributed to changes in environmental conditions such as pH, moisture, oxygen, water activity, nutrients, and microbial growth inhibitors produced by bacteria or added -externally (Monnet et al., 2014). Yet, as Bola de Ocosingo cheese production includes several processing steps where other raw materials are added by hand, such as adding butter during curd ripening with hand-mixing, and raw milk-derived *pasta filata* used to cover the soft curd and making a round shape cheese. These manufacturing practices represent contamination opportunities, and could explain the presence of other bacteria (e.g., *B. licheniformis*, *E. aerogenes*, *R. planticola*, *Acidovorax* sp.) as observed in manufacturers A and B during the rainy season.

Dominant species in Bola de Ocosingo cheese were LAB, including *S. thermophilus*, *L. delbrueckii*, *L. helveticus* and *L. lactis.* These bacteria are common natural starter cultures, or are inoculated during production of yogurt and Emmental, Gruyere, Parmigiano, Grana, Mozzarella and Cheddar cheese (Bouton et al., 2002; Randazzo et al., 2002; Hols et al., 2005; Delorme, 2008; Aldrete-Tapia et al., 2014). Their principal role is the reduction of pH during manufacture by production of lactic acid (Beresford et al., 2001). Additionally, LAB contribute to aroma and flavor of fermented products (Morales et al., 2003; Leroy and De Vuyst, 2004).

Low proportions of *S. thermophilus* were detected in most raw milk samples. However, during Bola de Ocosingo cheese processing, *S. thermophillus* increased in abundance, while another LAB such as *L. delbrueckii* or *L. helveticus* also increased. This could be due to low molecular weight nitrogen compounds in milk (principally peptides and aminoacids), which are necessary for growth of *S. thermophilus* (Giraffa et al., 2001). Some *S. thermophilus* strains required commensal associations to fulfill nitrogen requirements, possibly provided by the metabolic activity of *L. delbrueckii* and *L. helveticus* (Courtin et al., 2002; Dandoy et al., 2011).

In general, *L. lactis* was present in low levels, but reached higher numbers when *S. thermophilus* was not predominant, for example in curd sample of manufacturer B in rainy season. In this same production, after further processing the proportions of *L. lactis* reduced, overwhelmed by the increase of others bacteria species. This may suggest that *L. lactis* could not dominate during processing, providing an open entrance to other bacteria which could affect quality, even though *L. lactis* is known to rapidly acidify cheese during curd production, preventing proliferation of pathogenic and spoilage species (Wouters et al., 2002; Dandoy et al., 2011).

Lactobacillus plantarum was detected in high proportion in some batches, principally during the aging process. This species is a member of the so-called Non-starter LAB (NSLAB) group; NSLAB introduce variability in the ripening process, improving sensory characteristics, but also producing defects (Beresford et al., 2001; Settanni and Moschetti, 2010).

Probiotic effect has been reported for some strains of species detected in bola cheese such as *L. helveticus* (Giraffa, 2014) and potential probiotic properties of *L. delbrueckii*, *S. themophilus* (Mater et al., 2006; Guglielmotti et al., 2007), *L. lactis* (Beck et al., 2015) and *L. plantarum* (Blana et al., 2014).

Interestingly, *Escherichia coli* an indicator of fecal contamination (or the pathogen *Shigella flexneri*) was detected in all samples, with the exception of those from manufacturers A and C during the rainy season. The abundance of this microorganism was very low in raw milk, and remained so until the end of ripening. However, detection of *E. coli* by traditional culture dependent methods was negative in all samples evaluated, which could indicate that cells were dead already, due to changes in physical and chemical parameters during cheese making caused by LAB and the potential antimicrobial compounds generated by their metabolism (Macori and Cotter, 2018). In fact, many LAB isolated from the production of Bola de Ocosingo cheese inhibited the growth of *Salmonella* Typhimurium and *Listeria monocytogenes* in plate wells assays (data not shown), which could possibly validate the production of antimicrobial metabolites, however, more studies should be carried out.

This study provides an insight in microbial community dynamics during Bola de Ocosingo cheese production. *S. thermophilus*, *L. lactis*, *L. helveticus*, *L. delbrueckii* and *L. plantarum* dominated during the cheese processing, all reported with potential probiotic effect. Prevalence of these bacteria differed across manufacturers and seasons which could account to differences in final product quality. Pyrosequencing revealed the presence of *E. coli*/*S. flexnerii* in very low proportions even in the ripened cheese. However, detection by traditional methodology was negative. Therefore, to achieve homogeneous cheese quality would be desirable to develop a starter culture by selecting strains with technological characteristics and health benefits, which could be added to pasteurized milk to generate the traditional characteristics of Bola de Ocosingo cheese.

AUTHOR CONTRIBUTIONS

AA-T acquired, analyzed, and discussed the data and wrote the manuscript. CE-R did the experimental design, sampled, funded, and revised the draft. MT did the experimental design, funded, analyzed the data, revised the draft, and did the English language editing. MH-I did the experimental design, received funding, revised draft, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02598/full#supplementary-material

FIGURE S1 | Rarefaction curves of the pyrosequencing reads from dairy samples collected from three producers **(A–C)** of Ocosingo Bola cheese in dry season (continuous line) and rainy season (discontinuous line).

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The Cheese Matrix Modulates the Immunomodulatory Properties of *Propionibacterium freudenreichii* CIRM-BIA 129 in Healthy Piglets

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Propionibacterium freudenreichii is a beneficial bacterium, used as a cheese starter, which presents versatile probiotic properties. These properties are strain-dependent. We hypothesized they may also be delivery vehicle-dependent. In this study, we thus explored in healthy piglets how the cheese matrix affects the immunomodulatory properties of *P. freudenreichii*. During 2 weeks, three groups of weaned piglets consumed, respectively, *P. freudenreichii* as a liquid culture (PF-culture), *P. freudenreichii* under the form of a cheese (PF-cheese), or a control sterile cheese matrix (Cheese-matrix). The *in vivo* metabolic activity of *P. freudenreichii* was assessed by determining short chain fatty acids (SCFA) concentration and bifidobacteria population in feces. Whatever the delivery vehicle, *P. freudenreichii* was metabolically active in piglets' colon and enhanced both bifidobacteria and SCFA in feces. *P. freudenreichii* consumption decreased the secretion of TNFα and of IL-10 by peripheral blood mononuclear cells (PBMC). It did not alter IL-10, IFNγ, IL-17, and TNFα secretion in mesenteric lymph node immune cells (MLNC). PF-cheese enhanced significantly Treg phenotype, while PF-culture decreased significantly Th17 phenotype in PBMC and MLNC. Remarkably, only PF-cheese induced an increase of Th2 phenotype in PBMC and MLNC. *Ex vivo* stimulation of PBMC and MLNC by Lipopolysaccharides and Concanavalin A emphasized the difference in the immunomodulatory responses between PF-culture and PF-cheese group, as well as between PBMC and MLNC. This study shows the importance to consider the delivery vehicle for probiotic administration. It confirms the anti-inflammatory potential of *P. freudenreichii*. It opens new perspectives for the use propionibacteria-fermented products as preventive agents for inflammatory bowel diseases and intestinal infectious diseases.

Keywords: probiotics, *P. freudenreichii*, delivery vehicle, cheese matrix, immunomodulation, T lymphocytes phenotype, PBMC, MLNCv

INTRODUCTION

Propionibacterium freudenreichii is a beneficial bacterium, belonging to the Actinomycetales order. It has been recognized as safe (GRAS status) in the United States of America, and qualified presumption of safety (QPS status) in Europe. *P. freudenreichii* is a cheese starter used in Swiss-type cheeses manufacture such as Emmental. It moreover revealed versatile, strain-dependent, probiotic functionalities (Thierry et al., 2011; Rabah et al., 2017). These properties are strain-dependent and result from the production of several beneficial metabolites by propionibacteria (Cousin et al., 2011; Rabah et al., 2017). Short chain fatty acids (SCFA), especially propionic and acetic acids derived from lactate fermentation by propionibacteria, have anti-inflammatory and anti-cancerous effects on colonic intestinal cells (Jan et al., 2002; Lan et al., 2008; Cousin et al., 2012b, 2016). *P. freudenreichii* produces also bifidogenic factors, including 1,4-dihydroxy-2-naphtoic acid (DHNA),which enhances bifidobacteria growth and reduces inflammation in intestinal epithelial cells (Isawa et al., 2002; Okada, 2006; Suzuki et al., 2006; Rabah et al., 2017). Finally, the surface proteome of *P. freudenreichii* is involved in host-bacteria interaction, with a prominent role of non-covalently surface-bound proteins such as S-layer proteins (Slps) (Le Maréchal et al., 2015; Colliou et al., 2017; Deutsch et al., 2017; Do Carmo et al., 2018). The development of dairy fermented products as functional foods by screening specific starter bacteria which possess both probiotic and food fermentation abilities is a promising perspective. It will provide an alternative tool to prevent several inflammatory diseases, as inflammatory bowel diseases (IBD). A functional food is defined as an "ingredient that affects beneficially one or more target functions in the body, beyond adequate nutritional effects, in a way that is relevant to either an improved state of health and well-being and/or reduction of risk disease" (Saris et al., 1998; Diplock et al., 1999). Understanding how *P. freudenreichii* impacts health, specifically intestinal health, is crucial to develop functional dairy foods. Indeed, effects of dairy products, fermented solely by propionibacteria, or in combination with lactic acid bacteria, already revealed beneficial effects in a mice model of colitis (Plé et al., 2015, 2016; Foligné et al., 2016). The ingestion of these fermented dairy foods reduced the severity of chemically induced colitis. Recently, *P. freudenreichii* was shown to be part of the human milk microbiota and to participate in the prevention of necrotizing enterocolitis (NEC) in preterm infants (Colliou et al., 2017). These detailed studies highlighted the potential of *P. freudenreichii* consumption to prevent intestinal inflammatory diseases. However, investigations on the impact of the delivery vehicle on the probiotic functionalities of *P. freudenreichii* in healthy subjects are lacking. Here, we investigated the influence of the cheese matrix on *in vivo* probiotic functionalities of *P. freudenreichii*. Indeed, dairy matrices were shown to enhance propionibacteria tolerance toward digestive stresses, via overexpression of a panel of proteins involved in acid and bile salts stress responses (Leverrier et al., 2005; Saxelin et al., 2010; Gagnaire et al., 2015). In addition, the high concentration of dairy proteins in cheese plays a role as buffering agent toward gastric acids, in addition to the presence of lipids, which limits the toxic effect of bile salts on bacterial membranes (Rabah et al., 2017, 2018). Such tolerance may favor propionibacteria survival, enhance their metabolic activity, and consequently their immunomodulatory effects, within the gut. Furthermore, adhesion and immunomodulation, both mediated by S-layer proteins, may be promoted, since the cheese matrix protects these proteins from digestive proteolysis *in vitro* (Do Carmo et al., 2017; Rabah et al., 2018). In this study, we compared the effect of two delivery vehicles, a single-strain cheese (PF-cheese) and fresh culture in milk ultrafiltrate (PF-culture), in healthy piglets. Both delivery vehicles were fermented by the strain *P. freudenreichii* CIRM-BIA 129, which has been selected previously as the most anti-inflammatory one (Foligné et al., 2010, Foligné et al., 2013). The impact of the delivery vehicle on *in vivo* metabolic activity and on immunomodulation by *P. freudenreichii* was assessed in healthy piglets. Then, to seek a functional role of *P. freudenreichii* consumption by healthy animals against inflammation, we assessed piglets' immune cell responses to exogenous proinflammatory stimulations.

MATERIALS AND METHODS

Bacterial Strain and Dairy Matrices Preparation

The strain *P. freudenreichii* CIRM-BIA 129 (equivalent to ITGP20 strain) was provided by the French Dairy Interbranch Organization (Centre National Interprofessionnel de l'Economie Laitière, CNIEL) and maintained by the International Centre for Microbial Resources (Centre International de Ressources Microbiennes-Bactéries d'Intérêt Alimentaire, CIRM-BIA). Dairy propionibacteria were routinely cultivated at 30°C in yeast-extract-lactate medium (YEL). For the PF- culture, *P. freudenreichii* CIRM-BIA 129 was grown in milk ultrafiltrate supplemented with 100 mM sodium DL-lactate (50% in H_2O, Sigma) and 5 g/L casein hydrolysate (Organotechnie, La Courneuve, France) (Cousin et al., 2012c) at 30°C, without agitation, in microaerophilic conditions until stationary phase (60 h of incubation). PF-cheese is a single-strain cheese fermented by *P. freudenreichii* CIRM-BIA 129 as described previously (Plé et al., 2015). The biochemical composition of the cheese was: dry matter 58 g/100 g, lipids 28 g/100 g, proteins 29 g/100 g, carbohydrates 0 g/100 g, and calcium 840 mg/100 g (Plé et al., 2015). The cheese matrix is a sterile dairy matrix prepared in the same way as the single strain cheese. Glucono delta-lactone was used to acidify the sterile supplemented milk before cheese matrix manufacturing procedure, as described previously (Plé et al., 2015). The propionibacteria amounts reached 5.10^9 CFU/ml in PF-culture, and 1.10^{10} CFU/g in PF-cheese.

Ethics Statement

The experimental protocol was performed in accordance with recommendations of the French law (2001-464 29/05/01)

and EEC (86/609/CEE) for the care and use of laboratory animals. The protocol was approved by the ethical committee on animal experimentation of Rennes (France), under the certificate of authorization to experiment 2017010922379066-V2. Pigs were sacrificed by electronarcosis followed by exsanguination, and every effort was made to minimize animal suffering.

Animal Procedures and Immune Cell Isolation

Twenty one [(Pietrain × Landrace) × (Large White)] 8-week old piglets (13.3 ± 0.4 kg) from the experimental herd of INRA St-Gilles (UEPR, France) were used. Three groups of seven piglets were constituted: (1) Cheese matrix (10 g), (2) PF-culture (1.10^{11} CFU of *P. freudenreichii*) and (3) PF-cheese (1.10^{11} CFU of *P. freudenreichii*). PF-cheese and cheese matrix were mixed by a turrax (Ultra-turrax T8 IKA, Fischer Scientific, 20,000 tr/min-2 min) in four volumes of sterile physiological water. Piglets were gavaged using syringes every morning (between 9.00 and 10.00 am) during 14 days. They were fed with a standard pig diet that was given at 10.00 am. Food was removed from the cage at 4:00 pm to monitor daily food intake. Animals were fasted from 4:00 pm to 9:00 am but had free access to water. Piglets were weighed five times (d0, d1, d4, d7, d10, and d14) and fecal samples were collected at day 0, day 7, and day 14. At the end of the 14-day treatment period, pigs were sacrificed 30 min after their last gavage by electronarcosis then exsanguination. Blood was collected in sterile BD vacutainer®CPT™ tubes (containing sodium heparin as well as Ficoll™Hypaque™ density fluid and a polyester gel barrier) at room temperature. Following centrifugation at 1500 *g* for 20 min without brake, we isolated the peripheral blood mononuclear cells (PBMC) by carefully pipetting the interface above the gel barrier. Additional washing in Hank's balanced saline solution (HBSS), supplemented with 200 UI/ml penicillin and 200 μg/ml streptomycin, and centrifugation steps resulted in a suspension of concentrated mononuclear cells. After laparotomy, 4 g of intestinal mesenteric lymph nodes (MLN) were placed in ice-cold HBSS for mononuclear cell isolation, as already described (Ferret-Bernard et al., 2010).

Quantification of Fecal Propionibacteria, Lactobacilli and Bifidobacteria Populations

Propionibacteria, Lactobacilli, and Bifidobacteria were quantified in feces collected at day 0 (before treatment) and day 14 (end of the treatment). Samples were analyzed in duplicate. For propionibacteria quantification, QIAamp DNA Stool Kit was used to extract DNA, as described previously (Hervé et al., 2007; Cousin et al., 2012a). For lactobacilli and bifidobacteria quantification, DNeasy Blood & Tissue Kits (Qiagen) was used to extract DNA from pure cultures of *Bifidobacterium longum* CIRM-BIA 1336 or *Lactobacillus pentosus* CIRM-BIA 660. Propionibacteria concentrations were measured by qPCR of 5S subunit gene of transcarboxylase as described previously (**Supplementary Table S1**) (Hervé et al., 2007; Cousin et al., 2012a). Briefly, ten-fold dilutions of the *P. freudenreichii* CIRM-BIA 129 were prepared in saline solution and enumerated using YELA medium. One hundred microliters of each dilution was then added to 200 mg of feces from naive pig (exempt of propionibacteria) and thoroughly mixed. DNA was then extracted in the same way as unknown samples. A standard curve was generated and results are expressed as log [bacteria] per gram of sample. For lactobacilli and bifidobacteria quantification, 10-fold serial dilutions of target genomic DNA extracted from pure cultures of *B. longum* CIRM-BIA 1336 or *L. pentosus* CIRM-BIA 660 were performed (**Supplementary Table S1**). The linear equation for the standard curve was then used to interpolate the numbers of copies present in the unknown samples.

Short Chain Fatty Acids Analysis

Short chain fatty acid concentration was determined in fecal samples at days 7 and 14. Immediately after collection, fecal samples were diluted in ortho-phosphoric acid (50% V/V) to stop fermentation and samples were stored at −20°C until analysis. SCFA were separated on a BP20 (SGE) column and quantified by a flame ionization detector as previously described (Jouany et al., 1981). Isocaproic acid was used as an internal standard. Samples were analyzed in duplicate, and the results are expressed as micromolar per g of feces.

PBMC and MLNC Stimulation

Immune cells were suspended in complete RPMI 1640 medium (Sigma) supplemented with 10% fetal calf serum (FCS), 100 IU/ml penicillin and 100 mg/ml streptomycin to achieve cell concentration of 5×10^6 cells/ml for PBMC and 10×10^6 cells/ml for MLNC in 96-well flat-bottomed plates. Cells were stimulated for 72 h at 37°C, under an atmosphere containing 5% CO_2, in unstimulated condition (complete RPMI alone) or in presence of 200 μg/ml of *P. freudenreichii*' S-layer proteins (Slps). Slps were extracted (Le Maréchal et al., 2015), partially purified by size exclusion chromatography as previously described (De sa Peixoto et al., 2015) and proteins were concentrated by filtration using VivaSpin-10 kDa. PBMC and MLNC were also cultivated in presence of 10 μg/ml of Lipopolysaccharides (LPS) from *Escherichia coli* 0111:B4, or in presence of 0.5 μg/ml of Concanavalin A (ConA, sub-optimal concentration), or a combination of Slps + LPS and Slps + ConA. Culture supernatants of PBMC and MLNC were harvested and stored at −20°C until assayed for cytokine detection. Remaining cells were re-suspended in FCS 10% DMSO (Hybri-max, Sigma) and stored at −150°C until mRNA extraction.

Cytokine Patterns of PBMC and MLNC

Concentrations of IL-10, IFNγ, and TNFα were measured in culture supernatants of PBMC and MLNC, using capture sandwich ELISA porcine ELISA kit (R&D Systems, Lille, France) according to the manufacturer's instructions. IL-17 concentration was measured also using capture sandwich swine ELISA kit (VetSet™, Kingfisher Biotech, United States). Cytokine concentrations after stimulation were given in pictograms per ml of supernatant.

RT-qPCR

Quantitative PCR was performed to determine *Tbet*, *GATA3*, *FOXP3,* and *RORγt* mRNA levels in PBMC and MLNC. Primers used for mRNA quantification are listed in **Supplementary Table S1**. Total RNA from cells was isolated by Trizol reagent (Invitrogen Ambion), and cDNA was synthesized using a qScript cDNA synthesis kit (Quanta Biosciences). Amplification was performed as previously described (Rabah et al., 2018). The transcripts level of the target genes was normalized to the transcript level of *hprt* gene (Ledger et al., 2004) (housekeeping gene, see **Supplementary Table S1**). These primers were described previously (Muráni et al., 2007; Delroisse et al., 2008; Hernández et al., 2009; Young et al., 2012; Hermann-Bank et al., 2013; Zhu et al., 2014). *Hprt* expression was not affected by the tested matrices. The results are expressed as expression level ($2^{-\Delta CT}$), in duplicate analysis for each piglets (n = 7) for the three groups.

Statistical Analysis

We analyzed all data with non-parametric tests after checking the non-Gaussian distribution of data. The effect the consumption of *P. freudenreichii* on the fecal propionibacteria amounts was analyzed with the Mann–Whitney test by comparing only PF-culture and PF-cheese groups. The difference in lactobacilli and bifidobacteria was analyzed separately by comparing the concentration at day 0 to the concentrations determined at day 14 using non-parametric ANOVA with Dunn's multiple comparison test as a *post hoc* test. The same test was also performed to compare the three groups in term of SCFA concentration, basal cytokine concentrations and gene expression. To analyze cytokine secretion after ConA and LPS stimulation, 2-way non-parametric ANOVA was performed with the sidak' s multiple comparison test as a *post hoc* test. Statistical significance was set at $p < 0.05$. Calculations were performed using GraphPad Prism Software (Prism 7 for Windows). All data were expressed as mean values and standard error of the mean (SEM) ($n = 7$).

RESULTS

Piglet Growth and Food Intake

Food intake was similar among the three experimental groups (**Supplementary Figure S1A**). Piglets had a similar growth without significant difference between experimental groups (**Supplementary Figure S1B**). Feed efficiency was also similar among experimental groups (**Supplementary Figure S1C**). No health problems was encountered during the 14-day experimental period.

Quantification of Fecal Population of Propionibacteria, Bifidobacteria, and Lactobacilli

Fecal propionibacteria population, as determined by qPCR, was undetectable before treatment (day 0, data not shown). Propionibacteria remained also undetectable in the cheese matrix piglets group's feces (**Figure 1A**). After 2 weeks of gavage, propionibacteria reached 7.0 ± 0.1 log/g of feces in the PF-culture piglets and 6.9 ± 0.1 log/g of feces in the PF-cheese piglets, with no significant difference between the two groups ($p > 0.05$). The impact of *P. freudenreichii* consumption on piglet's microbiota was investigated, focusing on two genera, using qPCR. Lactobacilli and bifidobacteria populations between the different treated groups at day 14 were compared to the population level at day 0. *P. freudenreichii* ingestion significantly enhanced bifidobacteria in feces (**Figure 1B**). By contrast, none of the treatments induced significant change in the population of lactobacilli, regardless of the delivery vehicle (**Figure 1B**).

SCFA Quantification in Piglet Feces

To assess the *in vivo* metabolic activity of *P. freudenreichii,* SCFA concentration were determined in feces at days 7 and 14. At day 7, SCFA concentrations in feces were equivalent among the different groups (**Supplementary Figure S2**). However, at day 14, *P. freudenreichii* consumption enhanced significantly total SCFA concentration in feces, regardless

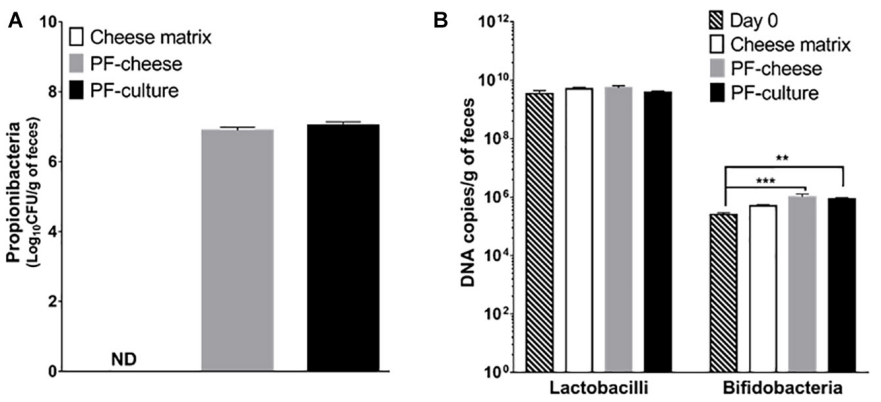


FIGURE 1 | Propionibacteria total population **(A)**, lactobacilli and bifidobacteria **(B)** total population in feces were assessed at day 14 in the different piglets groups: cheese matrix, PF-culture or PF-cheese fermented both by *P. freudenreichii* CIRM-BIA 129 (PF). Propionibacteria, lactobacilli, and bifidobacteria concentrations were determined by qPCR in feces. Results are represented as means ± SEM (n = 7). $^{**}P < 0.005$ and $^{***}P < 0.0005$.

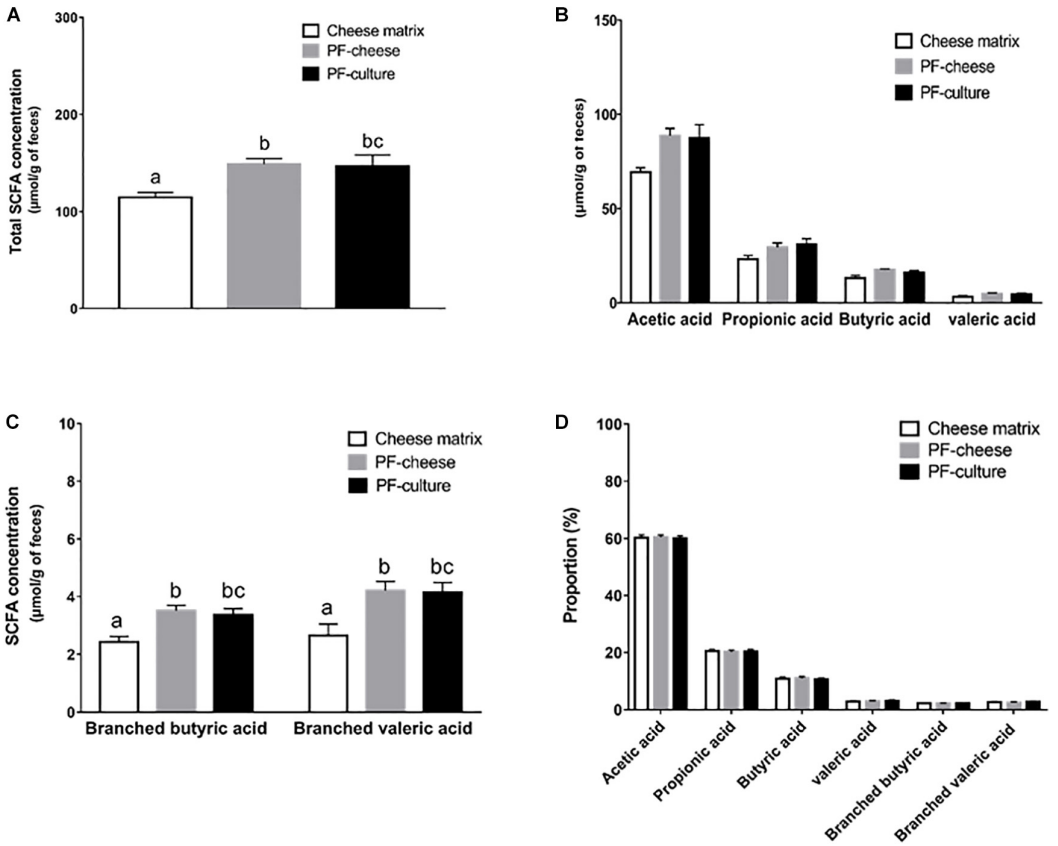


FIGURE 2 | Analysis of short chain fatty acids (SCFA) concentration in feces contents at day 14 of the three piglets groups fed with: cheese matrix, PF-culture or PF-cheese fermented by *P. freudenreichii* CIRM-BIA 129 (PF). The total SCFA concentration **(A)**, the concentration of each SCFA type (C2, C3, C4, and C5) **(B)**, and the concentration of branched short chain fatty acids **(C)** were measured by gas-phase chromatography, thus the proportion of each type of SCFA **(D)** was determined. Results are represented as means ± SEM (n = 7). Letters denotes significant difference, $P < 0.05$.

of the delivery matrix (PF-culture or PF-cheese, p = 0.98) (**Figure 2A**). *P. freudenreichii* consumption tended to increase acetic acid concentration, compared to the cheese matrix group (p = 0.062 for PF-culture group and p = 0.069 for PF-cheese group) (**Figure 2B**). By contrast, propionic, butyric or valeric acid concentrations were not significantly enhanced (**Figure 2B**). Regarding the concentrations of branched chain fatty acids (BCFA), PF-culture piglets and PF-cheese piglets displayed increased fecal branched valeric and butyric acids concentration, compared to cheese matrix group (**Figure 2C**). In addition, compared to cheese matrix group, no significant difference in BCFA concentrations between the PF-culture and the PF-cheese groups were observed (**Figure 2C**). The proportions of the different SCFA were not modified by the experimental treatments (**Figure 2D**).

Anti-inflammatory Properties of *P. freudenreichii* Slps Proteins on Naive Swine PBMC and MLNC

To verify the effect of *P. freudenreichii* Slps on naive swine immune cells as already demonstrated in other species (Le Maréchal et al., 2015), PBMC and MLNC from cheese matrix piglets were stimulated by Slps in the presence of LPS or ConA. Slps significantly increased IL-10 secretion by both PBMC and MLNC (**Figures 3A,B**). In the presence of ConA, Slps did not influence secretion of IL-10 by both cells types (**Figures 3A,B**). In the presence of LPS, Slps tended to increase IL-10 secretion by PBMC (p = 0.0763) (**Figure 3A**). Slps significantly reduced LPS or ConA-induced TNFα secretion by PBMC and MLNC (**Figures 3C,D**). Only ConA triggered a high secretion of IFNγ by PBMC, which was significantly reduced by the co-stimulation with Slps (**Figures 3E,F**).

Basal Cytokines Secretion by PBMC and MLNC

Propionibacterium freudenreichii consumption decreased basal secretion of IL-10 by PBMC, compared to cheese matrix group, whatever the delivery vehicle (**Figure 4A**). Basal TNFα secretion by PBMCs was significantly lower in PF-culture and PF-cheese groups, compared to cheese matrix group (**Figure 4C**). Finally, basal secretion of IFNγ and IL-17 by PBMC was similar in all piglet groups (**Figures 4B,D**). By contrast, in MLNC, IL-10, TNFα, IL-17, and IFNγ secretion were similar among the different experimental groups (**Supplementary Figures S3A–D**).

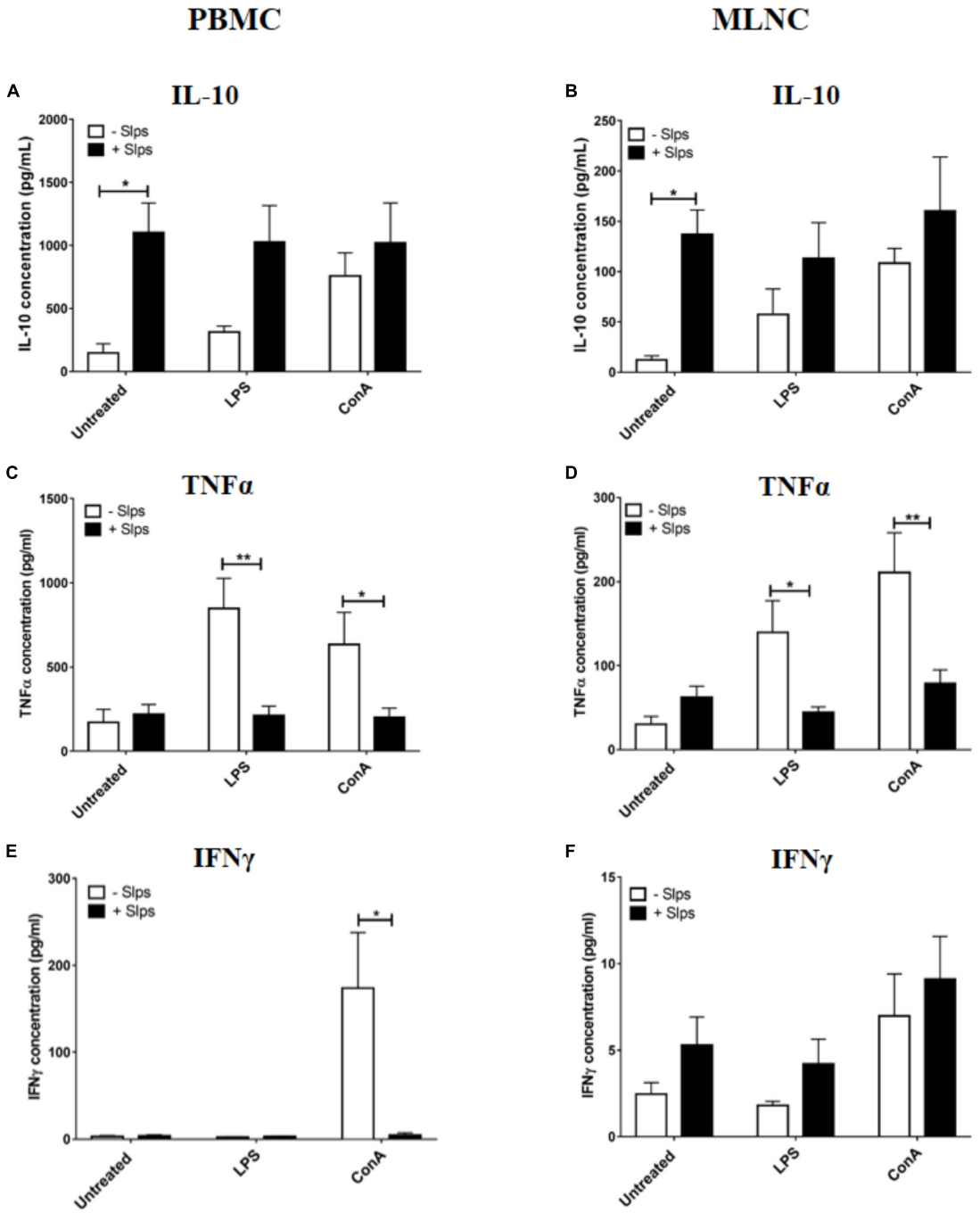


FIGURE 3 | Secretion of **(A,B)** IL-10, **(C,D)** TNFα, and **(E,F)** IFNγ by peripheral blood mononuclear cells (PBMC) and mesenteric lymph nodes cells (MLNC) from naive piglets (cheese matrix group). Cells were stimulated *ex vivo* with Lipopolysaccharides (LPS), Concanavalin A (ConA) in combination with *P. freudenreichii*' S-layer proteins (Slps). Cytokine concentrations were measured by ELISA and represented as means ± SEM ($n = 7$). *$P < 0.05$ and **$P < 0.01$.

T Lymphocytes Phenotype in PBMC and MLNC

Phenotype of T lymphocytes population in PBMC and MLNC was assessed by analyzing the expression of transcriptions factors: Tbet, GATA3, Foxp3, and RORγt, respectively, specific of Th1, Th2, Treg, and Th17 lymphocytes populations. Tbet expression in PBMC was similar among the different piglets groups (**Figure 5A**). PF-cheese, but not PF-culture consumption, increased significantly GATA3 expression in PBMC compared to the cheese matrix group (**Figure 5B**). The Th1/Th2 ratio was significantly lower in PF-cheese piglets, compared to cheese matrix ones, without significant difference with PF-culture piglets

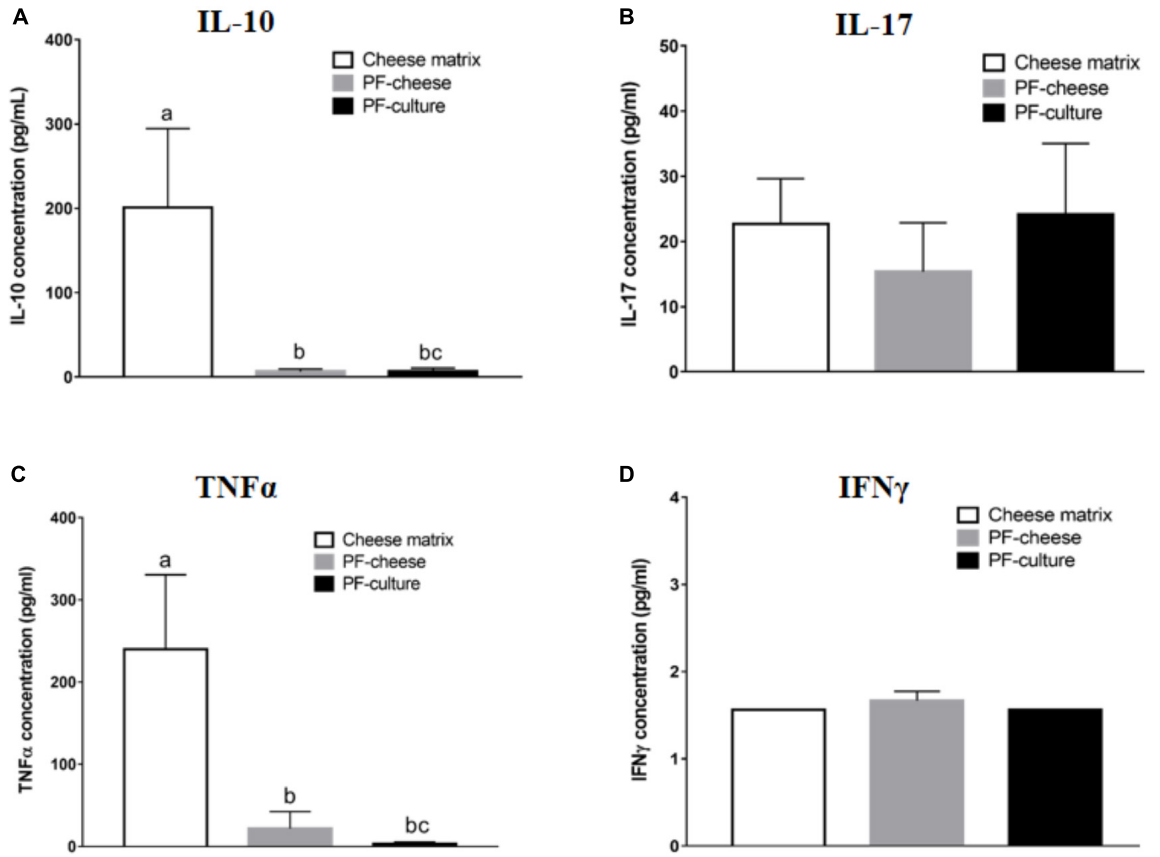


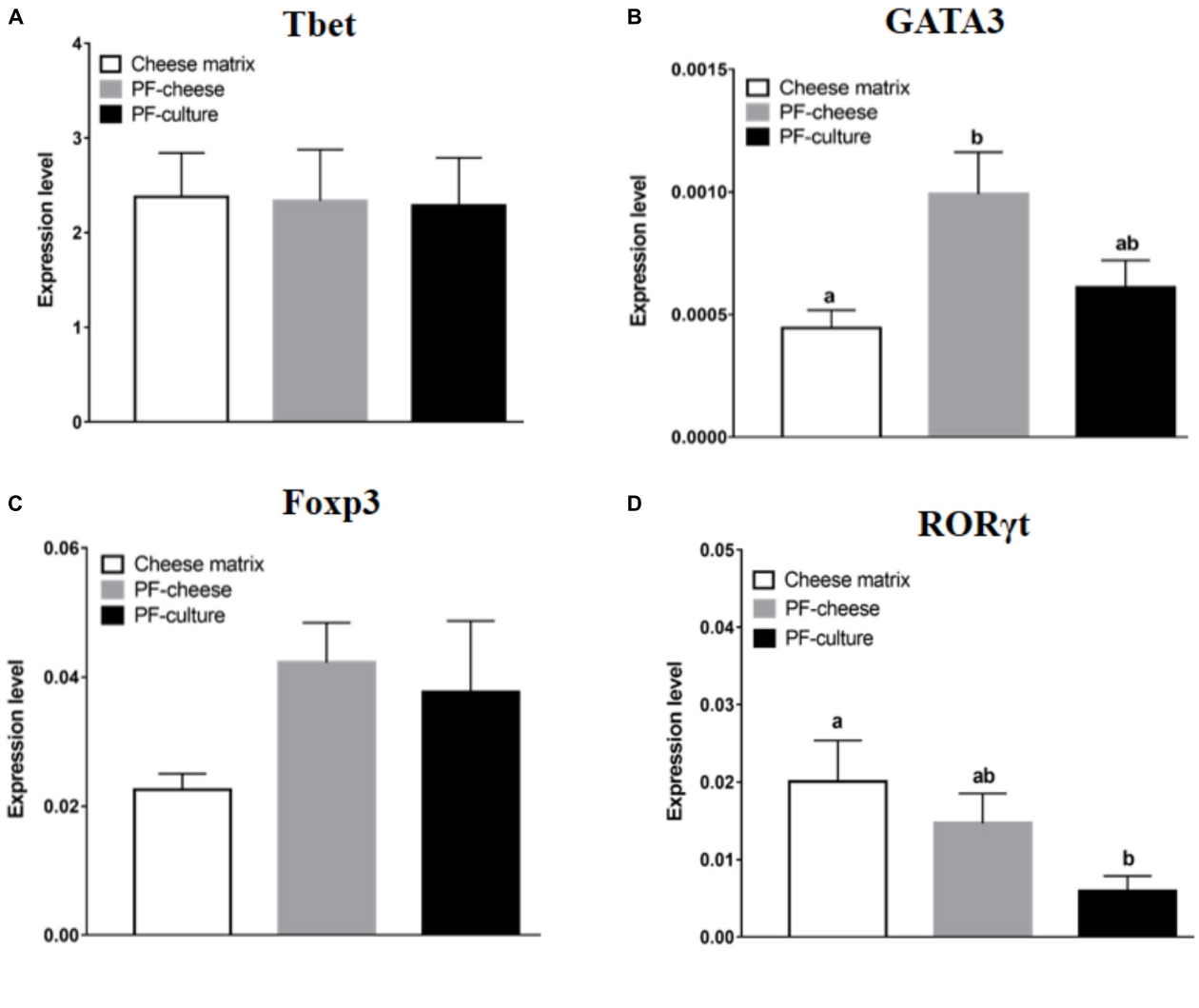
FIGURE 4 | Basal secretion of **(A)** IL-10, **(B)** IL-17, **(C)** TNFα, and **(D)** IFNγ by PBMC of different piglets groups fed with: cheese matrix, PF-culture or PF-cheese both fermented by *P. freudenreichii* CIRM-BIA 129 (PF). Cytokine concentrations were determined by ELISA and represented as means ± SEM (n = 7). Letters denotes significant difference, $P < 0.05$.

(**Figure 5E**). PBMC from PF-cheese piglets tended to express more Foxp3, compared to cheese matrix ones (p = 0.0636) (**Figure 5C**). PBMC from PF-culture piglets, but not from PF-cheese ones, had a significantly lower expression of RORγt compared to cheese-matrix piglets (**Figure 5D**). By determining Treg/Th17 ratio, we observed that only PF-culture piglets displayed a significant increase of Treg/Th17 ratio, compared to the cheese matrix piglets (**Figure 5E**). The Treg/Th17 ratio tended also to be higher in PF-cheese piglets, compared to cheese matrix ones (p = 0.0666) (**Figure 5E**).

Tbet expression was similar in MLNC from the different piglet groups (**Figure 6A**). Consumption of PF-cheese significantly increased GATA3 expression in MLNC, compared to the cheese matrix group, but without significant difference compared with PF-culture group (**Figure 6B**). MLNC from PF-cheese piglets displayed greater Foxp3 expression, compared to MLNC from both cheese matrix and PF-culture groups (**Figure 6C**). Consumption of PF-cheese significantly decreased Th1/Th2 ratio, compared to PF-culture group, but without significant difference with cheese matrix group. RORγt expression was lower in MLNC from PF-culture piglets compared to PF-cheese and cheese matrix groups (**Figure 6D**). Consumption of *P. freudenreichii*, regardless of the delivery vehicle, significantly increased the Treg/Th17 ratio (**Figure 6E**).

Cytokine Secretion by PBMC and MLNC in Response to *ex vivo* Stimuli

The LPS stimulation of PBMC induced a significant increase of IL-10 secretion, compared to untreated cells, only by PBMC from PF-culture and PF-cheese groups (**Figure 7A**). LPS stimulation induced also a significant increase of TNFα secretion by PBMC, compared to untreated cells, in all groups (**Figure 7B**). However, IFNγ secretion by PBMC was not triggered by LPS in any group (**Figure 7C**). ConA stimulation of PBMC induced a significant increase of IL-10 secretion compared to untreated cells, with no difference between groups (**Figure 7D**). ConA stimulation triggered a significant increase of TNFα secretion compared to untreated cells, only in PBMC from PF-cheese group. PBMC from PF-culture group tended also (p = 0.0676) to secrete more TNFα in response to ConA stimulation (**Figure 7E**). Stimulation by ConA induced a significant increase of IFNγ secretion, compared to untreated cells, only in PBMC from cheese matrix group (**Figure 7F**).



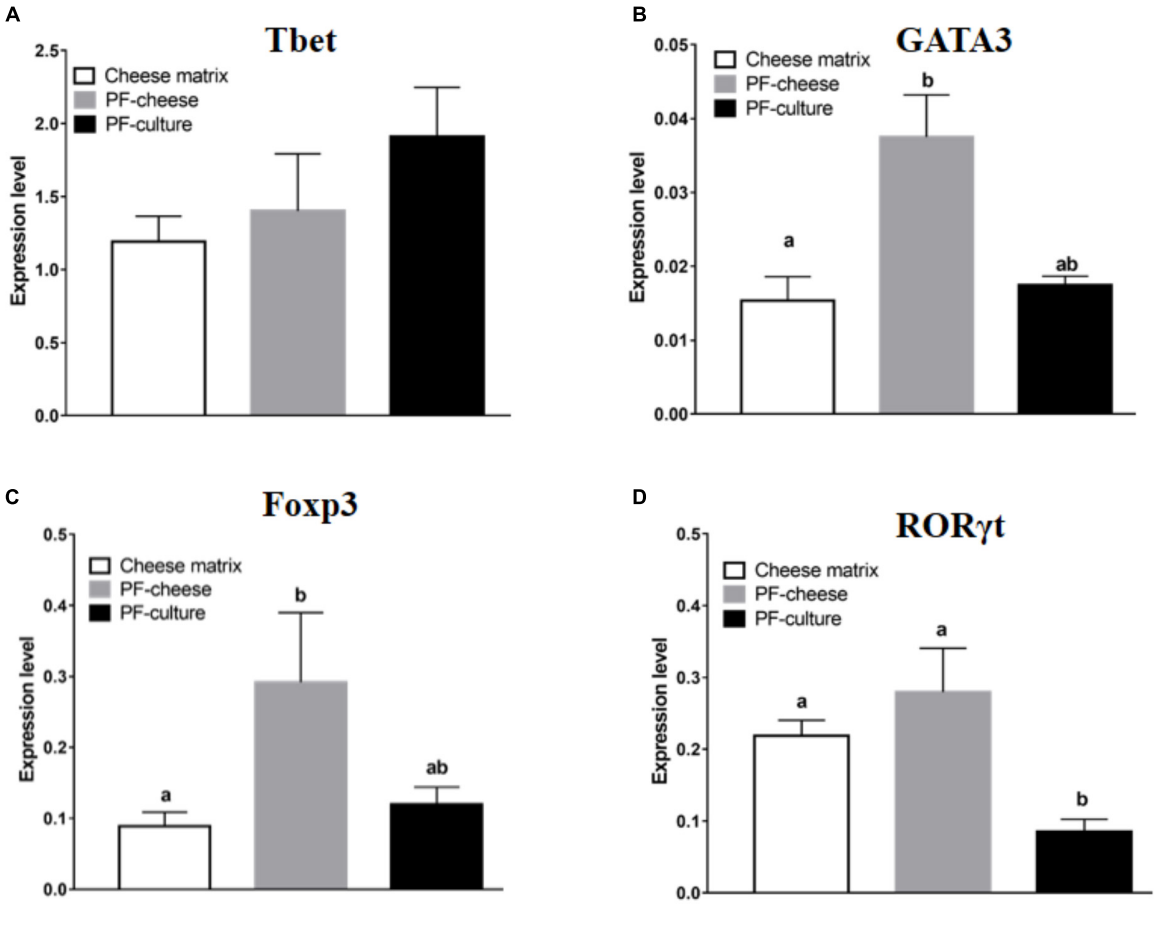
E

Groups	Th1/Th2 ratio	Treg/Th17 ratio
Cheese-matrix	7180 ± 1475 ^{a}	1.20 ± 0.32 ^{a}
PF-cheese	2600 ± 801.3 ^{b}	5,45 ± 2,08 ^{ab}
PF-culture	4008 ± 721.5 ^{ab}	9.65 ± 3.45 ^{b}

FIGURE 5 | Basal expression of **(A)** Tbet, **(B)** GATA3, **(C)** FOXP3, and **(D)** in PBMC from different piglets groups fed with cheese matrix, PF-culture or PF-cheese both fermented by *P. freudenreichii* CIRM-BIA 129 (PF). The level of expression was determined by RT-PCR. Ratios of Th1/Th2 and Treg/Th17 **(E)** were determined by calculating, respectively, Tbet/GATA3 and Foxp3/RORγt ratios. Results are represented as means ± SEM ($n = 7$). Letters denotes significant difference, $P < 0.05$.

The LPS Stimulation of MLNC increased significantly IL-10 secretion compared to untreated cells, only in MLNC from PF-culture group (**Figure 8A**). It induced also a significant increase of TNFα secretion by MLNC from cheese matrix and PF-culture piglets but not PF-cheese ones (**Figure 8B**). In addition, LPS stimulation induced a slight but significant secretion of IFNγ compared to untreated cells, only in MLNC from PF-cheese group (**Figure 8C**).

ConA triggered a significant rise of IL-10 secretion only in MLNC from PF-cheese group (**Figure 8D**). ConA stimulation of MLNC from all groups induced a significant secretion of TNFα compared to untreated cells (**Figure 8E**). Similarly, ConA



E

Groups	Th1/Th2 ratio	Treg/Th17 ratio
Cheese matrix	71.19 ± 12,99 ^{ab}	0.27 ± 0.11 ^{a}
PF-cheese	41.23 ± 14,2 ^{a}	1.35 ± 0.33 ^{b}
PF-culture	159.2 ± 51,27 ^{b}	1.22 ± 0.32 ^{b}

FIGURE 6 | Basal expression of **(A)** Tbet, **(B)** GATA3, **(C)** FOXP3, and **(D)** RORγt were analyzed in MLNC from different piglets groups fed with: cheese matrix, PF-culture or PF-cheese both fermented by *P. freudenreichii* CIRM-BIA 129 (PF). The level expression was determined by RT-PCR. Ratios of Th1/Th2 and Treg/Th17 **(E)** were determined by calculating, respectively, Tbet/GATA3 and Foxp3/RORγt ratios. Results are represented as means ± SEM (n = 7). Letters denotes significant difference, $P < 0.05$.

stimulation significantly increased IFNγ secretion compared to untreated cells, only in MLNC from PF-cheese piglets (**Figure 8F**).

DISCUSSION

The aim of this study was to investigate the impact of the delivery vehicle on the probiotic functionalities of *P. freudenreichii* in healthy piglets. For this purpose, three groups of piglets were fed during 2 weeks, with sterile cheese matrix, or a fresh culture of *P. freudenreichii* (PF-culture, 10^{11} CFU/day) or a single-strain cheese (PF-cheese, 10^{11} CFU/day) fermented solely by *P. freudenreichii* CIRM-BIA 129.

We investigated firstly if *P. freudenreichii* was metabolically active in piglet colon. *P. freudenreichii* metabolically active enhanced SCFA concentration in rats (Lan et al., 2007) and increased bifidobacteria in humans' fecal contents

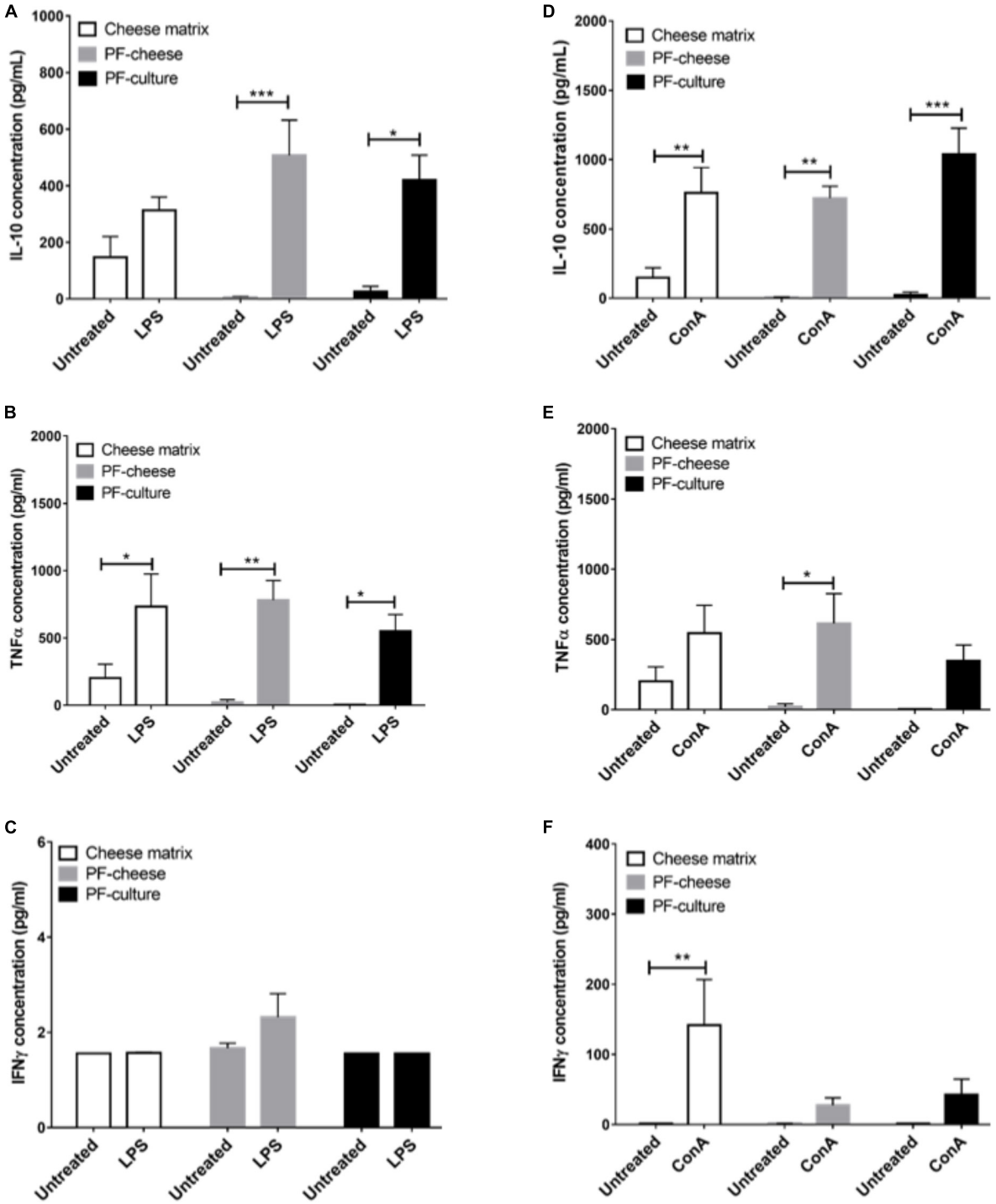


FIGURE 7 | Secretion of IL-10, TNFα, and IFNγ of stimulated PBMC from different piglets groups fed with: cheese matrix, PF-culture or PF-cheese fermented by *P. freudenreichii* CIRM-BIA 129 (PF). Cells were stimulated ex vivo with **(A–C)** LPS or **(D–F)** Concanavalin A (ConA). Cytokine concentrations were measured by ELISA and represented as means ± SEM ($n = 7$). $^{*}P < 0.05$, $^{**}P < 0.005$, and $^{***}P < 0.001$.

(Bouglé et al., 1999). The ability of a probiotic to be metabolically active in the colon depends on its adaptability toward colonic environment. *In vitro* studies suggested that propionibacteria digestive stresses tolerance is strain-dependent (Leverrier et al., 2005 ; Cousin et al., 2012c), but also matrix-dependent (Leverrier et al., 2005; Cousin et al., 2012c; Huang et al., 2016; Rabah et al., 2018). By contrast, our study demonstrated that both vehicles delivered metabolically

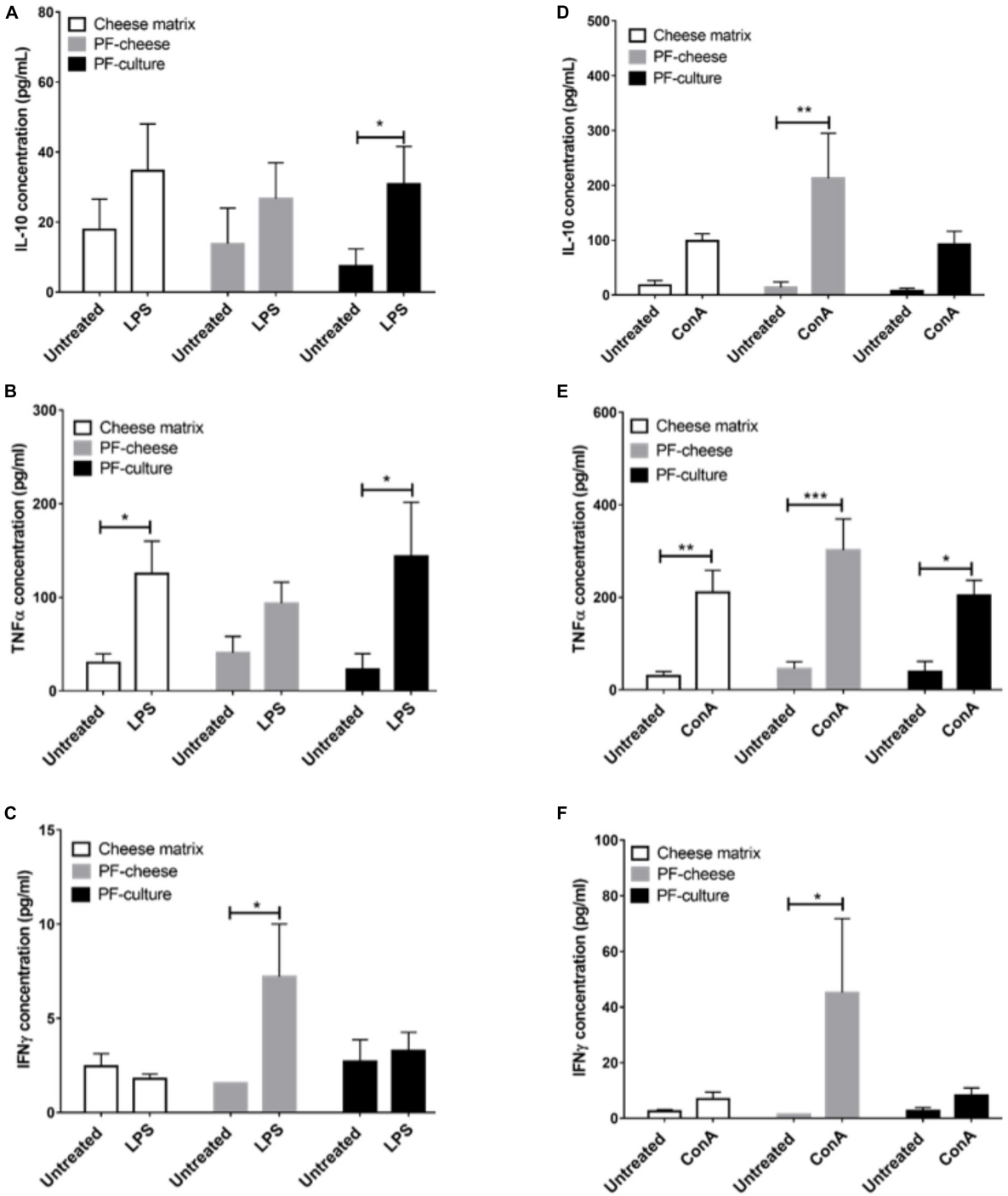


FIGURE 8 | Secretion of IL-10, TNFα, and IFNγ of stimulated MLNC from different piglets groups fed with: cheese matrix, PF-culture or PF-cheese fermented by *P. freudenreichii* CIRM-BIA 129 (PF). Cells were stimulated ex vivo with **(A–C)** Lipopolysaccharides (LPS) or **(D–F)** Concanavalin A (ConA). Cytokine concentrations were measured by ELISA and represented as means ± SEM ($n = 7$). $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$.

active *P. freudenreichii* to the piglets' colon. To attest of the *in vivo* metabolic activity of *P. freudenreichii,* we investigated its bifidogenic effect. Indeed, numerous studies demonstrated that *P. freudenreichii* has a bifidogenic effect (Bouglé et al., 1999; Hojo et al., 2002; Colliou et al., 2017) due to the *in situ* production of several metabolites (Yamazaki et al., 1999; Isawa et al., 2002; Kouya et al., 2007). *P. freudenreichii* consumption enhanced bifidobacteria population, without affecting lactobacilli population, in fecal contents, compared to cheese matrix group. *P. freudenreichii* metabolic activity was also assessed by analyzing

fecal SCFA concentration. In our study, *P. freudenreichii* CIRM-BIA 129 significantly increased total SCFA concentration, in particularly BCFA concentrations. The ability of *P. freudenreichii* to produce BCFA has already been demonstrated (Dherbécourt et al., 2008). These results are consistent with a previous transcriptomic study demonstrating that *P. freudenreichii*, within the colon, represses the wood-werkman cycle pathway (suggesting decreased propionic and acetic acid production), while inducing branched-chain amino acid degradation (suggesting an increase of BCFA production) (Saraoui et al., 2013). The modulation of SCFA content could also result indirectly from the increase of bifidobacteria population (Ríos-Covián et al., 2016; Hemalatha et al., 2017; LeBlanc et al., 2017). In a previous study, *P. freudenreichii* CIRM-BIA 129, with a dose of 1.10^{10} CFU/day, did not change colonic SCFA concentration in healthy piglets (Cousin et al., 2012a). However, in our study, piglets receiving 10^{11} CFU of *P. freudenreichii* per day showed an increase of fecal SCFA concentration. These results suggest the major importance of the dose of live propionibacteria when seeking *in vivo* beneficial metabolic effects.

We examined *ex vivo* the immunomodulatory effect of extracted *P. freudenreichii* Slps on PBMC and MLNC from naive piglets (cheese matrix group). As already demonstrated with human PBMC (Le Maréchal et al., 2015; Deutsch et al., 2017), Slps induced high IL-10 secretion in swine PBMC. We further extended this observation to swine MLNC. In addition, Slps inhibited the secretion of TNFα induced by LPS and ConA stimulation, in both cell types. Slps also inhibited ConA-induced IFNγ secretion in PBMC. Indeed, these results are consistent with a previous study, showing that *P. freudenreichii* CIRM-BIA 129 Slps decreased IFNγ induced by the pro-inflammatory *Lactococcus lactis* MG1363 strain in human PBMC (Le Maréchal et al., 2015). The molecular mechanisms by which propionibacteria Slps suppress induction of pro-inflammatory cytokines triggered by ConA and LPS remain unexplained. Few studies showed the ability of Slps from *P. freudenreichii* (Colliou et al., 2017) or from other probiotics (Konstantinov et al., 2008; Martínez et al., 2012; Prado Acosta et al., 2016) to bind to C-type lectins receptors of monocytes. More investigations are, however, needed to elucidate the detailed molecular mechanism.

To explore the immunomodulatory effects of *P. freudenreichii* consumption at the systemic level, immune cells from blood (PBMC) were isolated. At the basal state, cytokines secretion and T lymphocytes phenotypes were analyzed. *P. freudenreichii* consumption, whatever the delivery vehicle, modulated Treg/Th17 ratio, compared to cheese matrix group. Nevertheless, PF-culture decreased significantly Th17 phenotype, compared to cheese matrix group. Contrastingly, PF-cheese tended to enhance Treg phenotype, compared to the cheese matrix group. In addition, only PF-cheese significantly modulated PBMC Th1/Th2 ratio toward a Th2 phenotype. However, basal cytokine secretions were not consistent with T lymphocytes PBMC phenotypes investigated by qPCR. *P. freudenreichii* consumption, in both delivery vehicles, decreased basal PBMC secretion of IL-10 (a cytokine produced by Treg and Th2 cells) and of TNFα (a cytokine produced by Th1 cells), compared to cheese matrix group. Basal cytokine secretion is a global response of T, B cells and innate immune cells (macrophages and dendritic cells), which may explain these discrepancies. Moreover, we did not evaluate IL-4 basal secretion, a marker of Th2 T cells. Finally *in vitro* studies showed *P. freudenreichii* CIRM-BIA 129 as an inducer of IL-10 and TNFα in human PBMC (Kekkonen et al., 2008; Le Maréchal et al., 2015; Deutsch et al., 2017), which is in contradiction to the *in vivo* results obtained in this study. This result suggests that chronic ingestion of *P. freudenreichii* affects differently immune cells than *ex vivo* acute stimulation. Noteworthy, *P. freudenreichii* consumption inhibited IFNγ secretion by PBMC in response to ConA stimulation, regardless of the delivery vehicle. Further research is needed to understand this delivery vehicle-induced switch in TNFα/IFNγ secretion.

The intestinal immune response to *P. freudenreichii* consumption was also investigated. *P. freudenreichii*, regardless of the delivery vehicle, did not affect basal secretion of IL-10, IL-17, TNFα, or IFNγ. Once again, cytokine secretion patterns were not consistent with the phenotype of T lymphocytes in the different piglet groups. The effects of *P. freudenreichii* on T lymphocytes phenotypes of MLNC were similar to that of PBMC. PF-cheese enhanced Treg phenotype and PF-culture decreased Th17 phenotype, compared to cheese matrix group. Moreover, PF-cheese enhanced Th2 phenotype, compared to cheese matrix group, but without significant difference with the PF-culture group. MLNC responses to LPS and ConA stimulation showed different responses between PF-cheese and PF-culture groups from that of PBMC. Indeed, in response to LPS stimulation, PF-culture consumption enhanced IL-10 and TNFα secretion by MLNC, compared to untreated cells, while PF-cheese consumption induced a slight, yet significant increase in IFNγ secretion. In addition, only MLNC from PF-cheese group showed a high secretion of IL-10 and IFNγ, compared to untreated cells. ConA in naive MLNC did not induce secretion of IFNγ. Moreover, there was no significant difference between the three groups in Th1 phenotypes, an IFNγ-secreting phenotype (Takaoka and Yanai, 2006; Kak et al., 2018). IFNγ may be also secreted by others innate immune cells (Takaoka and Yanai, 2006; Kak et al., 2018). This result suggests that PF-cheese modulates innate immune cells differently from PF-culture. IFNγ plays a primordial role in immune cells to fight intestinal infections: it activates macrophages and Th1 expansion, which induces an effective immune response against pathogens (Kak et al., 2018). In addition, only PF-cheese enhanced Th2 phenotype. Thus, the secretion of IFNγ may be a way to control Th2 cells expansion. These results suggest the potential of using *P. freudenreichii* CIRM-BIA 129 in functional foods to prevent intestinal infections, as already shown with *Listeria monocytogenes* infection in mice model (Kato-Mori et al., 2010; Colliou et al., 2018). This should be confirmed in the future by *in vivo* pathogens challenge experiments in piglets.

Altogether, *P. freudenreichii* showed an anti-inflammatory effect on the systemic and intestinal immune system by enhancing Treg and Th2 phenotypes or decreasing Th17 phenotype, depending on the delivery vehicle. Th2 and Treg responses triggered by *B. breve* was shown to protect mice from chemically induced colitis (Zheng et al., 2014). This may partially explain the protective role of *P. freudenreichii* in cheese against

colitis (Plé et al., 2015, 2016; Foligné et al., 2016). Previously, *P. freudenreichii* P.UF1 was shown to increase Th17 population, to sustain Treg population and to decrease Th1 population, via a S-layer protein, in mice (Colliou et al., 2017, 2018).

In this study, since a similar metabolic activity of *P. freudenreichii* between the two delivery vehicles was observed, we assumed also that the matrix-dependent immunomodulatory effect may be related to surface proteins that would be protected by the cheese matrix (Rabah et al., 2018). More investigations are needed to confirm this hypothesis and to elucidate the molecular mechanism triggered by *P. freudenreichii* CIRM-BIA 129 to interact with host immune system.

CONCLUSION

The present study demonstrated that *P. freudenreichii* exerts an anti-inflammatory effect, regardless of the delivery vehicle. The difference between vehicles in term of immunomodulatory modulation was obvious after *ex vivo* stimulation of immunes cells by LPS and ConA, in particularly at the intestinal level. Our study shows that the delivery vehicle should be carefully considered. It opens the perspective to use *P. freudenreichii* in cheeses to prevent IBD or intestinal infectious diseases.

AUTHOR CONTRIBUTIONS

GB, GJ, and SF-B designed the study. HR and SH prepared all matrices. HR, SH, FG, RJ, SF-B, LLN, GB, and GJ participated in animal experiment and laboratory analyses. HR and GB analyzed data and prepared figures. HR wrote the manuscript with the help of GB, FC, SF-B, and GJ. RJ, GB, and GJ supervised the project. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02584/full#supplementary-material

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Influence of *Lactobacillus plantarum* P-8 on Fermented Milk Flavor and Storage Stability

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Previously, we demonstrated that the flavor of milk fermented with *Lactobacillus delbrueckii* subsp. *bulgaricus* (IMAU20401) and *Streptococcus thermophilus* (IMAU40133) at a 1:1000 ratio was superior to that of other ratios of the two strains. In this study, *Lactobacillus plantarum* P-8 was used as the probiotic bacterium. Six ratios (1:1, 1:5, 1:10, 1:50, 1:100, and 1:1000) of *L. plantarum* P-8 to yogurt starter were evaluated. A total of 66 volatile compounds including aldehydes, ketones, acids, alcohols, esters, alcohols, and aromatic compounds were identified in milk fermented with the six different *L. plantarum* P-8 to yogurt starter ratios at 0 d of storage. In particular, key flavor compounds, such as 3-methylbutanal, hexanal, (E)-2-octenal, nonanal, 2-heptanone, 2-nonanone, and acetoin, were identified in the 1:100 ratio treatment. Furthermore, the viable cell count, pH, titratable acidity, viscosity, and syneresis of the milk samples were analyzed during fermentation over 14 d of storage at 4°C. The results indicated that milk can be fermented with *L. plantarum* P-8 in combination with *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*, and the physicochemical characteristics of the milk were not affected by the probiotic bacteria.

Keywords: fermented milk, *L. plantarum* P-8, SPME–GC–MS, volatile flavor compounds, storage stability

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INTRODUCTION

Probiotics are live microorganisms that confer health benefits to a host when they are consumed in adequate amounts (Food and Agriculture Organization of the United Nations/World Health Organization [FAO/WHO], 2006). Yogurt, which is considered to be a source of probiotics, is made from milk by adding starter cultures and is valued for its unique flavor, desirable texture, and nutritional value (Manilópez et al., 2014). However, there has been some debate regarding the survival of yogurt starter bacteria, including *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, which have the ability to survive gastric passage to colonize the gut (Mater et al., 2010). Probiotic bacteria are mostly consumed as a component of food and must overcome physical and chemical barriers in the gastrointestinal tract, particularly acid and bile stresses (Tamang et al., 2016a). Today, it is common to find yogurt and fermented milk products that contain probiotic bacteria in the market, such as Jelley Brown (United States) and Zott (Germany), which have added *Lactobacillus acidophilus*, or Yili Changqing (China), which has added *Bifidobacterium* and *Lactobacillus rhamnosus*.

Research over the past decade has demonstrated the health benefits of probiotic bacteria such as *Lactobacillus* and *Bifidobacterium* (Bao et al., 2010; Ashraf and Shah, 2011), including antioxidant properties (Zhang et al., 2017) and effects on lowering blood pressure (He et al., 2017), reducing serum cholesterol levels (Guan et al., 2017), and stimulating the immune system

(Ashraf and Shah, 2014). *L. plantarum* is distributed worldwide and is present in meat, fish, dairy products, and plant-based fermented foods (Siezen et al., 2010; Tamang et al., 2016b; Shangpliang et al., 2018). *Lactobacillus plantarum* P-8 was isolated from traditional fermented milk. It possesses excellent fermentation properties and is considered to be a probiotic bacterium (Bao et al., 2012a,b; Zhang et al., 2015). The complete genome of *L. plantarum* P-8 consists of a circular 3.03 Mb chromosome and seven plasmids (Bao et al., 2012a). *L. plantarum* P-8 can significantly reduce lipid levels, enhance immune function, and improve the intestinal microbiome (Bao et al., 2012b; Zhang et al., 2015). In addition, *L. plantarum* P-8 can be used synergistically with *S. thermophilus* as a starter to improve the flavor and texture of fermented dairy products (He et al., 2012). However, the relationship between fermented milk quality and probiotic effects is poorly understood.

Solid-phase microextraction coupled with gas chromatography–mass spectrometry (SPME–GC–MS) has been used extensively to analyze flavor compounds, including those in fermented milk (Pan et al., 2014), goat milk cheese (Chiofalo et al., 2004), and fermented soymilk (Yin et al., 2013). The combined fermentation of probiotics and yogurt starters can improve the health benefits and flavor profile of fermented milk. Due to its probiotic properties, *L. plantarum* P-8 has been used extensively in the production of dairy products such as fermented soymilk (Wang et al., 2013) and fermented milk (Guo et al., 2013). As living standards improve, consumers place greater value on the flavor and probiotic content of fermented milk when choosing such drinks. The objective of this study was to evaluate the flavor and shelf life, as well as the pH, titratable acidity (TA), viable cell counts, viscosity, and syneresis, of milk fermented using a 1:100 ratio of *L. plantarum* P-8 to *S. thermophilus* and a 1:1000 fixed ratio of *L. delbrueckii* subsp. *bulgaricus* to *S. thermophilus* during 14 d of storage at 4°C.

MATERIALS AND METHODS

Strain Culture and Reagents

Streptococcus thermophilus (IMAU40133), *L. delbrueckii* subsp. *bulgaricus* (IMAU20401), and *L. plantarum* P-8 were obtained and cryopreserved from the Lactic Acid Bacteria Collection Center of Inner Mongolia Agricultural University. These isolates were activated in M17 (HB0391, QuingDoa HopeBiol Co., Quingdau, China) and De Man, Rogosa, and Sharpe (MRS) (027312, Huankai Microbial, Guangdong, China) liquid media at 37°C for 24 h, respectively. After subculturing in 50 ml M17 and 500 ml MRS media for two consecutive passages at 37°C for 24 h, the cells were collected and resuspended in PBS buffer (0.8% NaCl, 0.02% KH_2PO_4, 0.115% Na_2HPO_4, 1% tryptone, and 0.1% sodium glutamate inactivated at 121°C for 15 min). 1,2-Dichloro-benzene, which was used as an internal standard (ISTD), was purchased from Sigma-Aldrich (Steinheim, Germany). MRS broth and whole milk powder were purchased from OXOID (Hampshire, United Kingdom) and NZMP (Wellington, New Zealand), respectively.

Fermented Milk Manufacture

Whole milk powder (11.5%) was stirred and dissolved in distilled water at 50°C. The water temperature was increased to 60°C, and 6.5% sucrose was added and mixed well and then hydrated for 30 min. Homogenization was performed twice in succession (65°C at 15 and 35 MPa, respectively) by high-pressure homogenization (Shanghai, China), and the resulting homogenized milk was pasteurized at 95°C for 5 min and quickly cooled in ice water to 4°C until use. The yogurt starters were compounded from *L. delbrueckii* subsp. *bulgaricus* (IMAU20401) isolated from traditional fermented dairy products and *S. thermophilus* 40133 at a 1:1000 ratio (Dan et al., 2017b). *L. plantarum* P-8 cultures were compounded with the yogurt starters at ratios of 1:1, 1:5, 1:10, 1:50, 1:100, and 1:1000. Using the amount of *S. thermophilus* (40133) added to reach 5×10^7 CFU/ml as the benchmark, *L. delbrueckii* subsp. *bulgaricus* (IMAU20401) and *L. plantarum* P-8 were added to the homogenized whole milk, which was added to a 15 ml gas-phase flask and fermented in an incubator at 42°C. When the pH of the sample reached 4.5 and the TA reached 70–90°C, the milk was transferred to 4°C for storage (0 d) to determine the volatile flavor compounds.

Physicochemical Characteristics of Fermented Milk

Determination of pH

The pH of the fermented milk was measured at 20°C using a pHSJ-3F pH meter (Leici, Shanghai, China) in parallel.

Determination of TA

A 5 g sample of the fermented milk was weighed accurately using an electronic balance and placed in a 100 ml conical flask. To the conical flask, 20 ml CO_2-free distilled water and three drops of phenolphthalein indicator agent were added, and the flask was shaken well. A 0.1 mol/l NaOH standard solution was added for titration until a reddish color developed. If the color of the solution did not disappear within 30 s, the volume of the NaOH standard solution added was recorded. Triplicates of each fermented milk sample were performed in parallel, and the following formula was used:

$$X = \frac{c \times V \times 100}{m \times 0.1},$$

where "X" represents the acidity of the fermented milk sample in degrees (°T), "c" represents the molar concentration (mol/l) of the NaOH standard solution, "V" represents the volume (ml) of the NaOH standard solution consumed at time of titration, "m" represents the mass (g) of the sample, and 0.1 is the molar concentration (mol/l) of NaOH, as defined by the acidity theory.

Determination of Viable Cell Counts

The fermented milk sample (0.5 ml) was placed in 4.5 ml of sterilized physiological saline and the mixture was shaken to mix well. A serial dilution was performed. Viable bacterial counts of *S. thermophilus* 40133, *L. delbrueckii* subsp. *bulgaricus* IMAU20401, and *L. plantarum* P-8 in the fermented milk were

determined by culturing the diluted samples at 37°C in an incubator for 48 h using the MRS solid medium decanter method and counting the resulting colonies.

Determination of Viscosity

The fermented milk (40 ml) was centrifuged in triplicate using a viscometer at 20–22°C at 100 rpm for 30 s.

Determination of Syneresis

A 20 g sample of fermented milk was weighed and placed in a funnel with a piece of filter paper (New Star Medium-Speed Qualitative Filter Paper, Hangzhou Special Paper Industry, Hangzhou, China) and allowed to stand at 4°C for 2 h. The filtrate was collected and weighed. The following formula was used to calculate syneresis:

$$\text{Syneresis}(\%) = \text{Filtrateweight}(g)/\text{Sampleweight}(g) \times 100\%.$$

Determination of Volatile Flavor Compounds

Isolation of volatile flavor compounds

The SPME fibers were inserted into the injection port of the Agilent 7890B gas chromatograph (Agilent Technologies Inc., Palo Alto, CA, United States) at 250°C for 5 min for preconditioning. They were then inserted above the gas-phase bottle for extraction for 60 min. Desorption was conducted at 250°C for 3 min.

A temperature-programmed route was used for chromatography. The temperature was maintained at 35°C for 3 min and then increased by 4°C/min to 140°C. The temperature was maintained at 140°C for 1 min and increased to 250°C for 3 min. The transfer line temperature was set to 250°C. The carrier gas was helium, the flow rate was 1.0 ml/min, and no split sampling was performed.

For MS, electron ionization was performed at 70 eV. The ion source temperature was 230°C, the mass scan range was m/z 33–450 AMU, and the emission current was 100 μA.

Qualitative Analysis

We used the National Institute of Standards Technology Mass Spectral Database 11 to reference the published literature and identify compounds. We calculated the relative peak area ratio of all components based on normalization of the peak area (the percentage of each component's peak area relative to the total peak areas for all substances in the ion chromatograms). We calculated the retention index of each component using a temperature-programmed method to identify the compounds. The retention index (RI) was determined by the following equation:

$$\text{RI} = 100 \times \left[z + \frac{\text{RT}_{(X)} - \text{RT}_{(Z)}}{\text{RT}_{(Z+1)} - \text{RT}_{(Z)}} \right],$$

where "RT" represents the retention time (min) and the retention times according to the carbon number of *n*-alkanes follow the order RT (z) < RT (X) < RT ($Z + 1$). *n*-Alkane standards (C3–C25) were obtained from AccuStandard (New Haven, CT, United States).

1,2-Dichlorobenzene solution (Sigma-Aldrich, St. Louis, MO, United States) was added to the fermentation sample as the ISTD. The concentrations of all flavor components in the samples were used in the following formula to calculate the concentration of each compound:

$$c_i = \frac{A_i}{A_s} \times c_s,$$

where "c_i" represents the concentration (μg/l) of the compound in the test sample, "c_s" represents the concentration (μg/l) of 1,2-dichlorobenzene, "A_i" represents the chromatographic peak area of the test substances in the sample, and "A_s" represents the chromatographic peak area of the ISTD.

Evaluation of Odor Activity

To quantify the volatile flavor compounds in the fermented milk, we used the flavor threshold value for each flavor compound in water and calculated the physical parameters of the compounds, namely the odor activity value (OAV), which indicates the flavor contribution from each flavor compound. The following formula was used:

$$\text{OAV}_i = \frac{C_i}{\text{OT}_i},$$

where OAV_i represents the flavor of compound i, C_i represents the concentration of compound i in fermented milk (μg/l), and OT_i represents the flavor threshold value of the compound in water.

Sensory Evaluation

A total of 10 trained panelists conducted a sensory assessment of the flavor of the milk samples fermented with different *L. plantarum* P-8 to yogurt starter ratios at 0 d of storage, based on the requirements specified by RHB 103-2004 of China's dairy industry for assessing the sensory quality of cultured milk.

Statistical Analysis

Data were analyzed using Microsoft Excel, SPSS v19.0, SIMCA-P v11.5, and SAS v9.0. Normalized data were assessed by principal component analysis, significance tests, and correlation analysis. Principal component analysis was performed to determine the most important volatile compounds in milk fermented with the six different ratios of *L. plantarum* P-8 to yogurt starter. We used Origin v8.6 and Heml v1.0 to create principal component loading plots and score plots. Similarities were analyzed in the chromatograms obtained from the fermented milk samples using the Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (version A) and GC fingerprints were obtained.

RESULTS

Volatile Flavor Compounds in Fermented Milk

Lactobacillus plantarum P-8 was compounded and fermented using *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* (1:1000) yogurt starter at six different inoculation ratios (1:1,

1:5, 1:10, 1:50, 1:100, and 1:1000). At 0 d of storage, 66 volatile flavor compounds were identified in milk fermented with the six different ratios of probiotic bacteria using the HS–SPME–GC–MS technique (**Table 1**). These compounds included various types of aldehydes, ketones, carboxylic acids, alcohols, esters, and aromatic hydrocarbons.

Principal Component Analysis of Volatile Compounds

Principal component analysis was performed to examine the differences among the volatile compounds from milk fermented with different ratios of *L. plantarum* P-8 to starter culture at 0 d of storage. The distribution of the scores in the first two scatter plots (**Figure 1A**) revealed two separate clusters that corresponded to the six different ratios of the probiotic bacteria. The volatile flavor compounds in milk fermented with the 1:100 and 1:1000 ratios of probiotic strains were clustered together on the positive axis, whereas the components in milk fermented with the 1:1, 1:5, 1:10, and 1:50 ratios were clustered together on the negative axis.

The volatile flavor compounds in the fermented milk were classified into six major types: aldehydes, ketones, acids, alcohols, esters, and aromatic hydrocarbons (**Figure 1B**). On the positive axis, aldehydes, ketones, alcohols, and acidic compounds were associated with the flavor of milk fermented with the 1:100 and 1:1000 ratios of probiotic bacteria. On the negative axis, esters and aromatic hydrocarbon compounds were associated with the flavor of milk fermented with the 1:1, 1:5, 1:10, and 1:50 ratios of probiotic bacteria. Aldehydes, ketones, alcohols, esters, and aromatic hydrocarbon compounds were located on the positive axis of the plane, whereas acidic compounds were located on the negative axis of the plane.

Overall, aldehydes, ketones, and alcohols were present in the samples fermented with the 1:100 and 1:1000 ratios of probiotic bacteria, indicating that a better flavor, compared with the samples fermented with the other ratios of probiotic bacteria.

GC Fingerprint Analysis and Similarity Evaluation

The GC fingerprints of six samples of milk fermented with different ratios of *L. plantarum* P-8 to starter culture were examined using the Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicines (ver. 2004A, SFDA, China) (**Figure 2** and **Table 2**). The similarity values of all samples, prepared in triplicate, ranged from 0.923 to 0.992, indicating that all experiments had good repeatability. The similarity values between the 1:100 ratio and 1:1, 1:5, 1:10, 1:50, and 1:1000 ratio treatments were 0.59, 0.42, 0.46, 0.57, and 0.95, respectively. These values indicated higher similarity between the 1:100 and 1:1000 ratio treatments but lower similarity between the 1:100 ratio treatment and the other four ratio treatments.

Key Volatile Compounds in Fermented Milk

Generally, compounds with an OAV 0.1–1 are flavor compounds and confer an important modifying effect on the flavor of fermented milk, whereas compounds with an OAV $\geq$ 1 are key contributors to the flavor of fermented milk. The OAVs of volatile compounds in milk fermented with the 1:100 and 1:1000 ratios of probiotic bacteria are shown in **Table 3**. The odor threshold concentrations of these compounds that have been reported in the literature are presented in **Table 3**. The volatile compounds in the milk fermented with the 1:1, 1:5, 1:10, and 1:50 ratios consisted of 7, 7, 7, and 6 important flavor compounds, respectively. In particular, the OAV for hexanal was 5.1 in the 1:50 ratio samples, which indicated that this compound could be a significant contributor to the aroma of the fermented milk. Similar results were found in the 1:100 and 1:1000 ratio samples consisting of 10 important flavor compounds, 4 and 6 of which had OAVs of 0.1–1 and >1, respectively. Six characteristic compounds, 3-methylbutanal, hexanal, (E)-2-octenal, nonanal, 2-heptanone, and 2-nonanone, were detected in milk fermented with the 1:100 and 1:1000 ratios of probiotic bacteria. In the 1:100 and 1:1000 ratios, hexanal had an OAV of 10.99 and 10.69, respectively, which suggests that the compound could be a significant contributor to the aroma of Parmigiano-Reggiano cheese.

pH and TA

Table 4 shows the changes in pH and TA during fermentation and storage, caused by the residual activity of microorganisms. After 2 h of fermentation, the pH of the milk began to decrease rapidly, reaching ∼4.5 in less than 6 h. In particular, the pH of the fermented milk supplemented with *L. plantarum* P-8 reached 4.01 at the end of the 14-d storage period. The TA value of the fermented milk supplemented with *L. plantarum* P-8 increased steadily during fermentation and storage, reaching 93.28°T at the end of the 14-d storage period.

Viable Cell Counts

The viable cell counts during fermentation and storage were not significantly affected by the addition of probiotics at the 1:100 ratio (**Table 4**). The viable cell counts in the 1:100 ratio treatment increased rapidly during fermentation (0–4 h) and storage (0–3 d), reaching 9.72 $\log_{10}$ CFU/ml after 3 d of storage, and then decreased significantly thereafter. Similar results were found in the yogurt prepared with a fixed ratio (1:1000) of *L. delbrueckii* subsp. *bulgaricus* to *S. thermophilus*, in which the viable cell counts peaked at 2 d during storage (9.45 log10 CFU/ml).

Viscosity and Syneresis

Table 4 presents the viscosity and syneresis values of milk inoculated with the 1:100 ratio during fermentation and storage. During fermentation and storage, the viscosity of the fermented milk increased significantly over time and peaked at 1280 mPa s at 1 d of storage. Similarly, the viscosity increased steadily in the fermented milk supplemented with *L. plantarum* P-8, reaching 1166 mPa s after 3 d of storage. However, the change in viscosity during storage (at 7 and 14 d) was not significant. The fermented milk supplemented with *L. plantarum* P-8 demonstrated more syneresis than did the yogurt during refrigeration storage. Syneresis (31–36%) was observed in the fermented milk during storage.

TABLE 1 | Volatile compounds produced by milk fermented with different ratios of *L. plantarum* P-8 to starter culture at 0 d of storage.

No.	Volatile compound	Chemical formula	RT[1]	RI[2]	RI[3]	Method[4]	μg/l					
							1:1	1:5	1:10	1:50	1:100	1:1000
Aldehyde compounds												
1	3-Methyl-butanal	$C_5H_{10}O$	3.63	700.59	697	MS, RI	–	2.28 ± 0.002	2.02 ± 0.001	5.18 ± 0.034	8.51 ± 0.006	11.32 ± 0.053
2	Hexanal	$C_6H_{12}O$	6.86	809.1	809	MS, RI	–	–	5.5 ± 0.09	15.3 ± 0.517	32.96 ± 0.067	32.08 ± 0.085
3	(E)-2-Hexenal	$C_6H_{10}O$	8.97	864.56	861	MS, RI	1.17 ± 0.103	0.65 ± 0.174	0.67 ± 0.062	1.47 ± 0.258	2.9 ± 0.713	2.73 ± 0.507
4	(Z)-4-Heptenal	$C_7H_{12}O$	10.39	901.97	902	MS, RI	–	–	–	0.44 ± 3.462	–	0.75 ± 0.287
5	Heptanal	$C_7H_{14}O$	10.85	914.27	910	MS, RI	1.51 ± 0.103	2.79 ± 0.000	2.89 ± 0.004	3.76 ± 0.705	8.72 ± 0.902	8.42 ± 0.318
6	(Z)-2-Heptenal	$C_7H_{12}O$	12.87	968.82	–	MS	5.61 ± 0.051	–	–	1.06 ± 0.603	–	7.82 ± 0.804
7	(E)-2-Heptenal	$C_7H_{12}O$	12.87	968.86	967	MS, RI	–	–	3.44 ± 0.001	–	10.3 ± 0.519	9.24 ± 0.702
8	(E,E)-2,4-Heptadienal	$C_7H_{10}O$	14.81	1023.72	1023	MS, RI	–	2.16 ± 1.068	0.83 ± 0.309	2.67 ± 0.405	3.76 ± 2.001	4.86 ± 0.004
9	Benzaldehyde	C_7H_6O	12.94	970.7	970	MS, RI	0.46 ± 0.079	0.74 ± 0.007	0.81 ± 0.043	1.08 ± 0.025	0.65 ± 0.002	–
10	(E)-2-Octenal	$C_8H_{14}O$	16.4	1071.64	1065	MS, RI	5.51 ± 0.06	3.81 ± 0.051	3.8 ± 0.051	4.96 ± 0.3615	10.03 ± 0.405	8.03 ± 1.280
11	Nonanal	$C_9H_{18}O$	17.92	1118.74	1119	MS, RI	1.03 ± 0.071	1.05 ± 0.069	0.63 ± 0.194	0.56 ± 0.003	2.38 ± 0.051	1.99 ± 0.147
12	(E)-2-Nonenal	$C_9H_{16}O$	19.65	1175.39	1174	MS, RI	–	3.67 ± 0.372	–	–	–	–
13	(E)-2-Decenal	$C_{10}H_{18}O$	22.51	1274.41	1279	MS, RI	–	7.69 ± 2.826	–	–	–	–
14	(Z)-2-Decenal	$C_{10}H_{18}O$	22.61	1277.94	1280	MS, RI	12.53 ± 0.921	9.89 ± 0.724	9.58 ± 0.003	9.38 ± 0.035	19.7 ± 0.084	15.6 ± 6.932
15	2-Undecenal	$C_{11}H_{20}O$	24.72	1355.87	1359	MS, RI	–	0.69 ± 0.047	1.04 ± 0.078	–	–	–
16	(E)-2-Undecenal	$C_{11}H_{20}O$	25.29	1377.31	1374	MS, RI	1.66 ± 0.229	1.00 ± 0.173	–	1.50 ± 0.145	–	1.46 ± 0.042
17	(E)-2-Dodecenal	$C_7H_{14}O$	27.43	1452.66	1452	MS, RI	–	–	–	14.6 ± 0.029	2.57 ± 0.073	1.76 ± 0.132
Ketone compounds												
18	3-Methyl-2-butanone	$C_5H_{10}O$	3.19	667.69	666.1	MS, RI	–	–	–	0.46 ± 0.025	0.77 ± 0.115	0.89 ± 0.023
19	Acetoin	$C_4H_8O_2$	4.08	716.09	712	MS, RI	–	–	–	–	15.5 ± 0.097	–
20	2-Heptanone	$C_7H_{14}O$	10.42	902.86	902	MS, RI	13.55 ± 0.270	10.54 ± 0.034	8.16 ± 0.027	3.03 ± 0.158	27.84 ± 0.395	25.18 ± 0.906
21	5-Methyl-3-heptanone	$C_8H_{16}O$	12.71	964.55	962	MS, RI	0.5.0 ± 0.851	–	0.46 ± 0.004	11.11 ± 0.016	0.82 ± 0.072	–
22	2-Propyl-1-heptanone	$C_{10}H_{22}O$	12.94	970.85	–	MS, RI	2.07 ± 0.048	1.38 ± 0.009	1.33 ± 0.026	0.92 ± 0.007	3.78 ± 0.003	4.52 ± 0.165
23	2-Nonanone	$C_9H_{18}O$	17.53	1106.15	1104	MS, RI	13.27 ± 0.004	9.94 ± 0.058	8.44 ± 0.029	1.77 ± 0.076	21.7 ± 0.148	18.17 ± 0.009
24	2-Undecanone	$C_{11}H_{22}O$	23.52	1310.53	1305	MS, RI	2.31 ± 0.047	1.65 ± 0.064	1.79 ± 0.005	1.99 ± 0.092	3.45 ± 0.036	2.76 ± 0.005
Carboxylic acids												
25	3-Heptenoic acid	$C_7H_{12}O_2$	11.82	940.54	947	MS, RI	–	–	–	0.47 ± 0.085	0.78 ± 0.172	–
26	Hexanoic acid	$C_6H_{12}O_2$	14.37	1010.42	1013	MS, RI	6.88 ± 0.427	1.45 ± 0.005	4.24 ± 0.044	4.29 ± 0.018	–	–
27	Heptanoic acid	$C_7H_{14}O_2$	16.24	1063.81	1065	MS, RI	–	–	–	2.86 ± 0.138	5.64 ± 0.004	5.16 ± 0.032

(Continued)

TABLE 1 | Continued

No.	Volatile compound	Chemical formula	RT[1]	RI[2]	RI[3]	Method[4]	μg/l					
							1:1	1:5	1:10	1:50	1:100	1:1000
28	7-Oxo-octanoic acid	$C_8H_{14}O_3$	17.4	1101.69	–	MS, RI	1.15 ± 0.067	1.19 ± 0.205	–	–	2.7 ± 0.105	3.95 ± 1.312
29	Cyclohexanecarboxylic acid	$C_7H_{12}O_2$	19.17	1159.69	1157	MS, RI	–	–	0.27 ± 0.065	–	–	–
30	2-Undecenoic acid	$C_{11}H_{20}O_2$	22.59	1277.25	–	MS	1.58 ± 0.015	–	1.11 ± 0.007	0.79 ± 0.320	1.46 ± 0.405	0.84 ± 0.018
31	Z-8-Methyl-9-tetradecenoic acid	$C_{15}H_{28}O_2$	33.17	1727.78	–	MS	1.68 ± 0.402	–	9.06 ± 5.004	2.79 ± 0.108	–	–
Alcohol compounds												
32	3-Methyl-1-butanol	$C_5H_{12}O$	5.2	752	749	MS, RI	2.67 ± 0.436	1.87 ± 0.270	0.86 ± 0.089	–	4.25 ± 0.371	4.43 ± 0.001
33	Dicyclopropyl carbinol	$C_7H_{12}O$	6.53	800.26	–	MS	0.43 ± 0.054	–	–	–	–	1.01 ± 0.054
34	4-Hepten-1-ol	$C_7H_{14}O$	9.26	872.19	870	MS, RI	0.81 ± 0.006	–	–	34.73 ± 0.104	1.53 ± 0.241	1.61 ± 0.003
35	Hexanol	$C_6H_{14}O$	9.65	882.36	880	MS, RI	15.6 ± 0.190	11.71 ± 0.165	8.53 ± 0.054	5.4 ± 0.208	23.84 ± 0.002	22.33 ± 0.418
36	2-Ethenyl-bicyclo [2.1.1]hexan-2-ol	$C_6H_{14}O$	9.65	882.5	880	MS, RI	–	–	–	13.01 ± 0.154	1.08 ± 0.208	0.63 ± 0.002
37	(Z)-3-Hepten-1-ol	$C_7H_{14}O$	12.36	955.11	959	MS, RI	–	0.32 ± 0.418	–	–	–	–
38	*cis*-Hept-4-enol	$C_7H_{14}O$	13.26	979.26	–	MS	0.99 ± 0.454	0.62 ± 2.343	–	0.87 ± 0.007	–	1.39 ± 0.903
39	Heptanol	$C_7H_{16}O$	13.43	983.99	975	MS, RI	33.63 ± 0.438	27.2 ± 0.117	22.75 ± 0.004	1.43 ± 0.205	64.93 ± 0.437	59.65 ± 0.005
40	1-Octen-3-ol	$C_8H_{16}O$	13.75	992.48	991	MS, RI	1.34 ± 0.101	1.14 ± 0.392	0.77 ± 0.052	0.54 ± 0.006	2.28 ± 0.181	2.63 ± 0.060
41	3-Methyl-hepta-1,6-dien-3-ol	$C_8H_{14}O$	14.01	999.53	–	MS	1.3 ± 0.001	–	0.82 ± 0.114	–	–	1.89 ± 0.120
42	3-Decyn-2-ol	$C_{10}H_{18}O$	14.26	1007.1	1101	MS, RI	–	0.51 ± 0.004	–	–	–	–
43	3,5-Octadien-2-ol	$C_8H_{14}O$	15.41	1041.72	1039	MS, RI	0.56 ± 0.187	0.46 ± 0.405	–	0.78 ± 0.009	1.07 ± 0.203	1.06 ± 0.158
44	(Z)-2-Octen-1-ol	$C_8H_{16}O$	16.07	1061.59	1067	MS, RI	0.55 ± 0.903	–	–	–	0.71 ± 0.002	0.68 ± 0.194
45	9-Oxabicyclo[6.1.0] nonan-4-ol	$C_8H_{14}O_2$	17.69	1111.4	–	MS	1.03 ± 0.146	–	0.38 ± 0.055	–	1.71 ± 0.103	0.84 ± 0.166
46	3,4-Dimethylcyclo hexanol	$C_8H_{16}O$	18.02	1121.96	–	MS, RI	0.7 ± 0.052	0.3 ± 0.008	0.39 ± 0.049	4.83 ± 0.481	0.96 ± 0.007	0.9 ± 0.173
47	2-Nonen-1-ol	$C_9H_{18}O$	18.49	1137.37	–	MS, RI	0.55 ± 0.061	–	1.39 ± 1.294	0.48 ± 0.141	–	–
48	(E)-2-Nonen-1-ol	$C_9H_{18}O$	19.52	1171.14	1171	MS, RI	–	1.33 ± 0.264	–	–	–	–
49	Nonanol	$C_9H_{20}O$	19.99	1186.62	1186	MS, RI	6.22 ± 0.076	4.74 ± 0.367	3.84 ± 0.043	0.72 ± 0.324	9.74 ± 0.286	8.34 ± 0.006
50	2-Butyl-1-octanol	$C_{12}H_{26}O$	25.05	1368.32	–	MS, RI	–	0.97 ± 0.307	3.01 ± 0.256	0.65 ± 0.156	1.7 ± 1.009	–
51	2-Methyl-1-hexadecanol	$C_{17}H_{36}O$	34.45	1823.35	–	MS	2.13 ± 0.043	–	3.77 ± 0.627	18.86 ± 0.516	–	–

(Continued)

TABLE 1 | Continued

No.	Volatile compound	Chemical formula	RT[1]	RI[2]	RI[3]	Method[4]	μg/l					
							1:1	1:5	1:10	1:50	1:100	1:1000
Ester compounds												
52	Butanoic acid, 2-ethyl-1,2,3-propanetriyl ester	$C_{21}H_{38}O_6$	21.46	1237.22	–	MS, RI	1.07 ± 0.003	–	0.74 ± 0.109	0.93 ± 0.007	–	1.18 ± 0.038
53	Allyl 2-ethyl butyrate	$C_9H_{16}O_2$	21.86	1251.46	1254	MS, RI	1.03 ± 0.614	–	0.49 ± 0.325	1.35 ± 1.086	1.59 ± 0.156	–
54	Acetic acid, 3,7,11,15-tetramethyl-hexadecyl ester	$C_{22}H_{44}O_2$	34.31	1811.51	–	MS	0.4 ± 0.130	0.45 ± 0.031	0.27 ± 0.298	–	–	–
Aromatic hydrocarbons												
55	*n*-Hexane	C_6H_{14}	2.27	–	–	MS	1.89 ± 0.927	0.77 ± 0.316	0.27 ± 0.141	1.68 ± 0.041	–	2.79 ± 0.782
56	Heptane	C_7H_{16}	3.65	701.31	–	MS	1.34 ± 1.483	–	–	–	–	–
57	2,4-Dimethyl-hexane	C_8H_{18}	4.85	742.61	738.9	MS, RI	–	5.22 ± 0.052	–	–	–	–
58	Octane	C_8H_{18}	5.45	763.28	760	MS, RI	3.66 ± 0.003	–	–	–	–	–
59	Octene	C_8H_{16}	6.52	800.18	799	MS, RI	0.62 ± 0.014	–	–	0.42 ± 0.005	0.87 ± 0.018	–
60	1-Nonene	C_9H_{18}	10.15	895.61	893	MS, RI	–	–	–	27.65 ± 0.074	–	–
61	1,2-Dimethyl-cyclooctene	$C_{10}H_{18}$	18.53	1138.74	–	MS	–	–	0.2 ± 0.092	–	–	0.97 ± 0.327
62	7-Methyl-3-octyne	C_9H_{16}	18.53	1138.78	–	MS	0.61 ± 0.271	0.55 ± 0.764	–	0.31 ± 0.373	1.08 ± 0.158	–
63	Tetradecane	$C_{14}H_{30}$	26.33	1415.01	–	MS, RI	–	0.57 ± 0.089	0.4 ± 0.274	0.65 ± 0.148	0.98 ± 0.231	0.81 ± 0.520
64	Pentadecane	$C_{15}H_{32}$	29.27	1520.11	–	MS	–	–	–	1.29 ± 0.589	–	–
65	2,6,10-Trimethyl-tetradecane	$C_{17}H_{36}$	31.43	1620.08	–	MS, RI	–	–	–	4.41 ± 0.625	–	–
66	Hexadecane	$C_{16}H_{34}$	31.44	1620.2	–	MS	–	0.56 ± 0.259	–	1.65 ± 0.610	–	–

[1]Retention time. [2]Retention indices (RI) of unknown compounds on an HP-5MS column calculated against the GC–MS retention time of n-alkanes (C3–C25). [3]RI from database (http://webbook.nist.gov/chemistry). [4]RI, agreed with retention index from the literature; MS, compared with Nist 11 Mass Spectral Database; STD, agreed with the mass spectrum of standard chemical. "–", not detected.

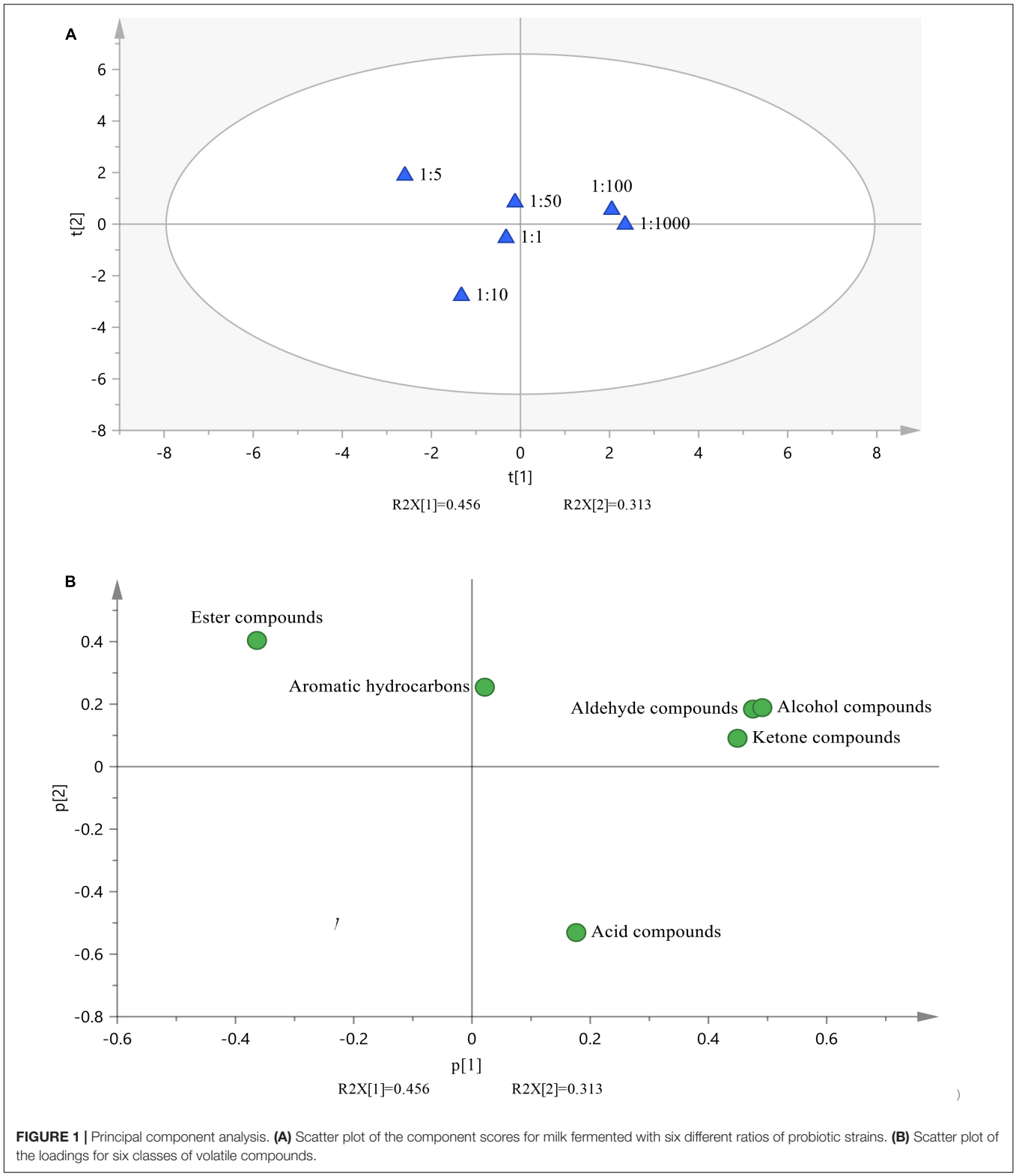

FIGURE 1 | Principal component analysis. **(A)** Scatter plot of the component scores for milk fermented with six different ratios of probiotic strains. **(B)** Scatter plot of the loadings for six classes of volatile compounds.

Sensory Assessment

The sensory evaluations of the flavor of the milk samples fermented with different *L. plantarum* P-8 to yogurt starter ratios were made by panelists at 0 d of storage. Samples fermented with *L. plantarum* P-8 to yogurt starter ratios of 1:100 were considered to have better yogurt characteristics than those of the other combinations, which were also considered to have good flavor.

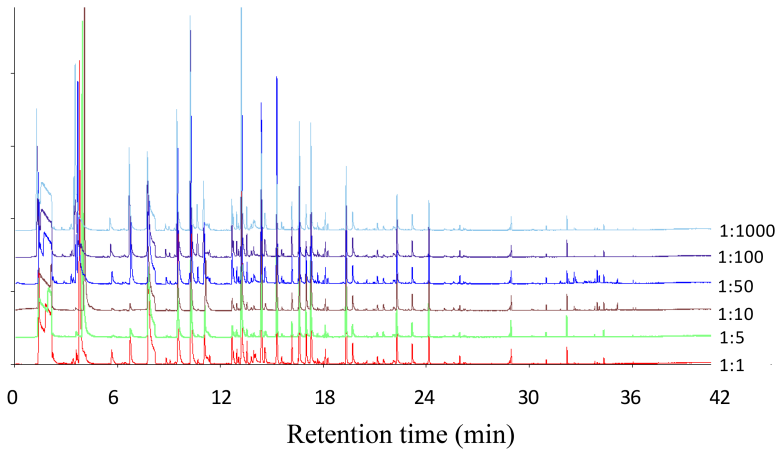

FIGURE 2 | Chromatographic fingerprints of all samples of milk fermented with different ratios of *L. plantarum* P-8 to starter culture at 0 d of storage.

TABLE 2 | Similarities in the gas chromatographic fingerprints among samples treated with six different ratios (1:1, 1:5, 1:10, 1:50, 1:100, and 1:1000) of *L. plantarum* P-8 to starter culture at 0 d of storage.

	1:1	1:5	1:10	1:50	1:100	1:1000	Reference
1:1	1.00	0.61	0.76	0.68	0.59	0.55	0.85
1:5	0.61	1.00	0.61	0.62	0.42	0.42	0.76
1:10	0.76	0.61	1.00	0.79	0.46	0.43	0.84
1:50	0.68	0.62	0.79	1.00	0.57	0.55	0.86
1:100	0.59	0.42	0.46	0.57	1.00	0.95	0.80
1:1000	0.55	0.42	0.43	0.55	0.95	1.00	0.78
Reference	0.85	0.76	0.84	0.86	0.80	0.78	1.00

DISCUSSION

The effect of *L. plantarum* strains as probiotic bacteria on the production of volatile aromatic compound metabolites in fermented milk has been described previously (Cheng, 2010; de Bok et al., 2011). *L. plantarum* plays an important role as a safe starter culture in food fermentation. In this study, a total of 66 volatile compounds, including aldehydes, ketones, acids, alcohols, esters, alcohols, and aromatic compounds, were identified in milk fermented with six different inoculation ratios (1:1, 1:5, 1:10, 1:50, 1:100, and 1:1000) of *L. plantarum* P-8 to *S. thermophilus* and a fixed ratio (1:1000) of *L. delbrueckii* subsp. *bulgaricus* to *S. thermophilus*.

Aldehydes have a greater impact on the flavor of fermented milk because of their lower threshold (Bránayik et al., 2012). Amino acid degradation forms 3-methylbutanal, which is a potent odorant in fermented milk (Madruga et al., 2009), and 3-methylbutanal was detected in the 1:5, 1:10, 1:50, 1:100, and 1:1000 ratio treatments. High levels of 3-methylbutanal were found in milk fermented with the 1:1000 *L. plantarum* P-8 to starter culture (11.32 μg/l) ratio and 1:1000 *L. delbrueckii* subsp. *bulgaricus* to *S. thermophilus* (7.8 μg/l) treatments, indicating

TABLE 3 | Odor activity values (OAVs) of the compounds produced in milk fermented with 1:100 and 1:1000 ratios of *L. plantarum* P-8 to *S. thermophilus* (compared with a 1:1000 ratio of *L. delbrueckii* subsp. *bulgaricus* to *S. thermophilus*).

Volatile compound	Odor threshold (μg/L)	OAV						Reference
		1:1	1:5	1:10	1:50	1:100	1:1000	
3-Methyl-butanal	5.4	–	0.42	0.37	0.96	1.58	2.10	Qian and Reineccius, 2003
Hexanal	3	–	–	1.83	5.1	10.99	10.69	Gemert, 2003
Heptanal	750	0.002	0.0037	0.0038	0.005	0.01	0.01	Qian and Reineccius, 2003
(E)-2-Heptenal	13	–	–	0.26	–	0.79	0.71	Leffingwell and Leffingwell, 1991
(Z)-2-Heptenal	13	0.43	–	–	0.08	–	0.60	John, 2001
(E)-2-Octenal	3	1.84	1.27	1.27	1.65	3.34	2.68	John, 2001
Nonanal	1	1.03	1.05	0.63	0.56	2.38	1.99	Gemert, 2003
2-Heptanone	5	2.71	2.11	1.63	0.61	5.57	5.04	Attaie, 2009
2-Nonanone	5	2.65	1.99	1.69	0.35	4.34	3.63	Attaie, 2009
3-Methyl-1-butanol	4750	–	–	–		–	–	Qian and Reineccius, 2003
1-Octen-3-ol	10	0.13	0.11	0.08	0.05	0.23	0.26	Molimard and Spinnler, 1996
Hexanol	120	0.13	0.10	0.07	0.05	0.20	0.19	Qian and Reineccius, 2003
Acetoin	55	–	–	–	–	0.28	–	Qian and Reineccius, 2003

TABLE 4 | The physiochemical characteristics of milk fermented with a 1:100 ratio of *L. plantarum* P-8 to *S. thermophilus* (compared with a 1:1000 ratio of *L. delbrueckii* subsp. *bulgaricus* to *S. thermophilus*) during fermentation (0, 2, and 4 h) and storage (0 h, 12 h, 1 d, 2 d, 3 d, 7 d, and 14 d).

	pH		TA		Viable count (log cfu/ml)		Viscosity(mPa s)		Syneresis (%)	
Time	**Lb-St-P8**	**Lb-St**	**Lb-St-P8**	**Lb-St**	**Lb-St-P8**	**Lb-St**	**Lb-St-P8**	**Lb-St**	**Lb-St-P8**	**Lb-St**
0 h (F)	6.79 ± 0.02	6.60 ± 0.03	12.3 ± 0.15	10.83 ± 0.05	7.65 ± 0.04	7.68 ± 0.00	110 ± 1.00	112 ± 2.1	42 ± 3.1	50 ± 4.1
2 h (F)	6.24 ± 0.01	6.12 ± 0.00	18.96 ± 0.04	18.34 ± 0.13	8.26 ± 0.03	7.89 ± 0.04	110 ± 5.00	206 ± 1.8	43 ± 3.0	47 ± 3.2
4 h (F)	5.25 ± 0.00	5.6 ± 0.04	40.18 ± 0.05	40.62 ± 0.32	8.86 ± 0.00	8.34 ± 0.01	256 ± 3.00	354 ± 2.7	39 ± 0.0	41 ± 0.9
0 d (S)	4.36 ± 0.02	4.46 ± 0.01	69.7 ± 0.04	70.99 ± 0.12	9.08 ± 0.04	9.16 ± 0.03	362 ± 3.00	558 ± 2.5	35 ± 2.1	28 ± 1.2
12 h (S)	4.26 ± 0.00	4.21 ± 0.02	76.36 ± 0.08	73.79 ± 0.25	9.17 ± 0.01	9.26 ± 0.00	688 ± 4.00	986 ± 1.9	36 ± 2.1	30 ± 2.1
1 d (S)	4.23 ± 0.01	4.13 ± 0.01	79.44 ± 0.04	81.77 ± 0.31	9.57 ± 0.02	9.4 ± 0.01	720 ± 1.00	1280 ± 10.56	32 ± 3.7	29 ± 2.6
2 d (S)	4.21 ± 0.01	4.05 ± 0.00	77.9 ± 1.18	91.19 ± 0.07	9.7 ± 0.03	9.45 ± 0.03	986 ± 26.63	1146 ± 7.2	31 ± 1.1	29 ± 1.5
3 d (S)	4.24 ± 0.03	3.94 ± 0.01	79.54 ± 0.04	94.87 ± 0.16	9.72 ± 0.01	9.3 ± 0.02	1166 ± 6.00	1027 ± 9.12	31 ± 3.2	30 ± 1.0
7 d (S)	4.09 ± 0.01	3.79 ± 0.02	87.23 ± 0.23	100.34 ± 0.31	9 ± 0.04	9.19 ± 0.00	870.67 ± 4.00	834 ± 8.21	36 ± 1.3	33 ± 2.1
14 d (S)	4.01 ± 0.02	3.72 ± 0.01	93.28 ± 0.18	103.44 ± 0.17	8.25 ± 0.01	8.98 ± 0.01	870 ± 3.00	830 ± 1.05	33 ± 3.0	28 ± 1.6

F, fermentation; S, storage.

that 3-methylbutanal formation in fermented milk is closely related to fermentation by *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*. Aldehydes, such as hexanal, are transitory compounds in fermented milk because they are easily reduced to acidic compounds or alcohols due to their relatively active chemical properties (Franciscojosé et al., 2010). Straight-chain aldehydes, including hexanal, heptanal, and nonanal, are quite common in fermented milk and originate from auto-oxidation of unsaturated fatty acids in milk fat. These compounds give grassy and herbaceous aromas to fermented milk. High levels of hexanal were detected in milk fermented with the 1:100 and 1:1000 ratios of bacteria (32.08 and 32.96 μg/l, respectively). Heptanal imparts a fatty aroma to fermented milk (Ferreira et al., 2000), and its maximum value (8.72 μg/l) was observed in the 1:100 ratio treatment. Heptanal levels increased with decreasing inoculation amounts of *L. plantarum* P-8, suggesting that *L. plantarum* P-8 inhibits the formation of heptanal. Nonanal has a low threshold value and provides citrus and fatty aromas to fermented milk (Piombino et al., 2008). Hexanal, heptanal, and nonanal were the most commonly observed odorants in this study and were detected in all six ratio treatments. (E)-2-Heptenal was found in milk fermented with the 1:10, 1:100, and 1:1000 ratios of probiotic bacteria, with the peak value (10.23 μg/l) at 1:100. Benzaldehyde is an important aromatic aldehyde formed from phenylacetaldehyde via α-oxidation or from cinnamic acid via β-oxidation (Dan et al., 2018). At lower levels, benzaldehyde provides an almond flavor to fermented milk, and at higher levels a fruity aroma (Chu and Yaylayan, 2008). Low levels of benzaldehyde (0.46–1.08 μg/l) were found in almost all treatment combinations, except the 1:1000 ratio. Benzaldehyde is an important compound frequently detected in dairy products such as fresh goat cheese (Condursoa et al., 2008). (E)-2-Octenal and (Z)-2-decenal were detected in milk fermented with all six ratios of bacteria, with the highest levels seen at 1:100 and 1:1000.

Ketones are produced mainly by thermal degradation of amino acids, oxidation of unsaturated fatty acids, and the Maillard reaction. As common constituents, ketones are known primarily for their effect on the aroma of most dairy products because of their low perception thresholds. A total of eight volatile ketones were detected in our milk samples. Diacetyl was detected at the beginning of fermentation (data not shown). As a byproduct of lactic acid bacteria metabolism, acetoin is produced by the chemical oxidation of diacetyl (Ott et al., 1999), which was found in milk fermented with the 1:100 ratio of probiotic bacteria. Acetoin gives fermented milk a weak creamy flavor and is an important taste compound that ameliorates the strong cream odor caused by diacetyl (Cheng, 2010). Methyl ketones including 2-heptanone, 2-nonanone, and 2-undecenone, which are known primarily for their contribution to the aroma of surface mold-ripened and blue-veined cheeses (Curioni and Bosset, 2002), were detected in our samples. As the predominant ketone compounds, 2-heptanone and 2-nonanone were detected in all six ratio treatments, with the highest levels reached at 1:100 (Pionnier and Hugelshofer, 2006; Dan et al., 2017a). 2-Undecenone was also detected in all six treatment ratios at levels ranging from 1.65 to 3.45 μg/l.

Carboxylic acids in fermented milk usually originate from lipolysis, proteolysis, or lactose fermentation (Franciscojosé et al., 2010). Studies have reported that acid compounds improve the taste of fermented milk and are the main source of sourness (Cheng, 2010). Hexanoic and heptanoic acids may be released via lipolytic activity. These short-chain fatty acids have a strong flavor; for instance, hexanoic acid gives a rancid, sweet cheese-like flavor to the fermented milk (Patton, 1964). Similar results have been reported by Chammas et al. (2006), who detected hexanoic acid in fermented milk (Chammas et al., 2006). In this study, hexanoic acid was found in the 1:1, 1:5, 1:10, and 1:50 ratio treatments, indicating that *L. plantarum* P-8 may promote the generation of hexanoic acid. Carboxylic acids are not major compounds in fermented milk due to their higher threshold values. Even though major acidic compounds were detected in all six ratio treatments, these compounds had OAV values <1 and did not significantly contribute to the overall flavor of fermented milk.

Considering the adverse effects on post-acidification and the variations in these volatile aromatics, especially acetic acid and 2-butanone as well as non-volatile metabolites, these

characteristics may considerably influence the organoleptic quality of the product.

Alcohols in fermented milk may be associated with lactose metabolism, methyl ketone reduction, and amino acid metabolism (Molimard and Spinnler, 1996). High levels of 3-methylbutanol, hexanol, heptanol, and nonanol were detected in milk fermented with the different ratios of probiotic bacteria. 3-Methylbutanol can confer a pleasant aroma of fresh cheese (Galvaþo et al., 2011), and its concentration was highest (4.43 μg/l) in milk fermented with 1:1000 ratio. Hexanol, heptanol, and nonanol are major flavor compounds in fermented milk (Cheng, 2010). These compounds were found in all six ratio treatments, with the highest levels (23.84 μg/l hexanol, 64.93 μg/l heptanol, and 9.74 μg/l nonanol) seen at the 1:100 ratio. Similar results were found in milk fermented with 1:1000 *L. delbrueckii* subsp. *bulgaricus* to *S. thermophilus* (Dan et al., 2017b). As the most common alcohol, 1-octen-3-ol has been identified as an important flavor compound in most dairy products investigated (Cheng, 2010; Ning et al., 2011); however, low levels were detected in our milk fermentation treatments. This compound has green and mushroom-like notes and contributes significantly to the aroma profiles of foods due to a low perception threshold (Curioni and Bosset, 2002).

Esters are produced primarily via the esterification of fatty acids and alcohols. Among the esters, ethyl esters have an important role in the formation of the fruity characteristics of dairy products (Curioni and Bosset, 2002). Allyl 2-ethyl butyrate as a common flavor compound was found in milk fermented with 1:1, 1:10, 1:50, and 1:100 ratios of the probiotic bacteria. Most esters provide fermented milk with fruity and floral flavors and weaken the pungent and astringent odors of fatty acids and amines (Cheng, 2010).

Aromatic hydrocarbon compounds have high flavor threshold values and do not have significant effects on the flavor of fermented milk, but at certain concentrations, they give fermented milk a fuller taste. Fifteen aromatic hydrocarbon compounds were found in all six ratio treatments and potentially play roles as supplementary flavor compounds in fermented milk.

The results of the principal component analysis and similarity evaluation revealed that the flavor of milk fermented with the 1:100 and 1:1000 ratios of probiotics was superior to the flavor of the milk prepared with the other ratios of probiotic bacteria. In this work, six key flavor compounds were found in the milk fermented with the 1:100 and 1:1000 ratios of probiotic bacteria, which were 3-methylbutanal, hexanal, (E)-2-octenal, nonanal, 2-heptanone, and 2-nonanone. All of these except for 3-methylbutanal were present in higher amounts in the treatment with a 1:100 ratio than in the treatment with a 1:1000 ratio of probiotic bacteria. In addition, acetoin was found in the milk fermented with a 1:100 ratio of probiotic bacteria. Acetoin is an important volatile compound that can influence the flavor of fermented milk. Therefore, the optimal ratio of *L. plantarum* P-8 to yogurt starter was determined to be 1:100. These results were consistent with the sensory assessment results.

The changes in the viable cell count, pH, TA, viscosity, and syneresis values in the milk fermented with the 1:100 ratio of *L. plantarum* P-8 to starter culture are shown in **Table 4** during fermentation (0, 2, and 4 h) and storage (0 h, 12 h, 1 d, 2 d, 3 d, 7 d, and 14 d). The pH and TA values in fermented milk supplemented with *L. plantarum* P-8 were similar to those observed in yogurt during fermentation and storage. In this study, the pH and TA values of fermented milk supplemented with *L. plantarum* P-8 decreased or increased steadily during fermentation and storage. Similar results were obtained when milk was fermented with *S. thermophilus*, *L. acidophilus*, *Bifidobacterium* species, or *L. casei* after 35 d of refrigeration (Gilliland et al., 2010). Gueimonde et al. (2004) also reported that the pH of commercially fermented milk is between 3.9 and 4.2 (Gueimonde et al., 2004). The TA is a key indicator of the acidity of fermented milk that reflects the summed total acidic groups that include peptides and free amino-acid residues; generally, the higher the acidity, the higher the TA (Li et al., 2017). Donkor et al. (2006) reported that the taste of fermented milk improves when the TA is maintained at 70–110°T (Donkor et al., 2006). However, another study reported that consumers prefer fermented milk with a TA around 120°T (Olson and Aryana, 2008). In this study, the fermented milk pH was consistently above 4 and the acidity below 100°T during fermentation and storage, indicating that the acidity of milk fermented with our ratios of probiotic bacteria is acceptable to consumers. In general, the post-acidification of fermented milk was closely related to the lactic acid bacteria used for milk fermentation. **Table 4** indicates that the milk supplemented with *L. plantarum* P-8 can delay post-acidification. These results indicated that incorporation of *L. plantarum* P-8 reduced the post-acidification of yogurt during storage.

The viable probiotic cell count is a key property of fermented milk. It is important for the milk industry to improve the number of viable bacteria in its final products. In this study, the viable cell counts in the 1:100 ratio treatment remained stable (>8.25 log CFU/g) toward the end of storage. These results are in accordance with the regulations of the International Dairy Federation, which states that the viable cell counts should exceed 10^7 CFU/ml during the shelf life of the product. At the beginning of fermentation, the counts of *S. thermophilus* remained higher than the counts of *L. delbrueckii* subsp. *bulgaricus* (1:1000). Kneifel et al. (1993) also reported that most commercial yogurts had higher counts of *S. thermophilus* than *L. delbrueckii* subsp. *bulgaricus*. As a lactic acid-producing bacterium, *L. delbrueckii* subsp. *bulgaricus* can lead a loss in viability of *S. thermophilus* and *L. plantarum* during refrigerated storage; however, it is an essential component of the starter culture that plays critical roles in the production of lactic acid and the development of the flavor of the yogurt. Fermented milk is the most common means for the delivery of probiotic cells to the intestinal tract. The number of probiotic microorganisms in the final products is generally the most important characteristic, as probiotic products must contain an adequate amount of viable probiotic cells, which should exceed 10^6 CFU/ml at the time of consumption (Sohrabvandi et al., 2010). In a preliminary experiment, the count of viable *L. plantarum* P-8 in the 1:100 ratio

treatment was not less than 10^7 during fermentation and storage (data not shown). He et al. (2012) also reported a similar result whereby a higher count of *L. plantarum* P-8 was detected in milk fermented with *L. plantarum* P-8 and *S. thermophilus* at various ratios.

Syneresis is the ability of fermented milk gels to bind to various components of milk, especially the water phase. Syneresis is a reversible indicator of the quality of fermented milk. Syneresis (31–36%) was observed in the fermented milk supplemented with *L. plantarum* P-8 during storage because probiotic bacteria grow slowly in basic cultures of fermented products due to the lack of proteolytic enzymes. Similar results were reported by González-Martiìnez et al. (2002), in that syneresis of yogurt supplemented with whey protein ranges from 23 to 36%. The viscosity markedly increased with fermentation time, reaching 1166 mPa s after 3 d of storage. The change in viscosity was consistent with the viable cell count in fermented milk. During fermentation and storage, the viable cell count and viscosity of the sample increased rapidly, peaking after 3 d of storage (9.72 log CFU/ml and 1166 mPa s, respectively). *L. plantarum* P-8 was reported to increase the viscosity of fermented milk, consistent with our results (Bao et al., 2012a).

CONCLUSION

In this study, the quality of the fermented dairy products was determined using a starter culture and probiotics; 66 volatile flavor compounds were identified in milk fermented with six different inoculation ratios of *L. plantarum* P-8 to *S. thermophilus* and a fixed ratio (1:1000) of *L. delbrueckii* subsp. *bulgaricus* to *S. thermophilus*, including aldehydes, ketones, acids, alcohols, esters, alcohols, and aromatic compounds. There were significant changes in the volatile profiles depending on the ratio of *L. plantarum* P-8 to starter culture. Some important volatile flavor compounds, such as 3-methylbutanal, hexanal, (E)-2-octenal, nonanal, 2-heptanone, 2-nonanone, and acetoin, were identified in the 1:100 ratio treatment. In addition, the stability of milk fermented with the 1:100 ratio of *L. plantarum* P-8 to *S. thermophilus* during fermentation and storage was supported. Our results indicated that the ratio of *L. plantarum* P-8 to starter culture used is important for determination of the volatile profiles and overall flavor of the final milk products.

AUTHOR CONTRIBUTIONS

TD and TS designed the experiments. HC, TL, JT, and WR performed the experiments. TD, TS, and HZ drafted the manuscript. All authors read and approved the final manuscript.

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High-Throughput Sequence Analyses of Bacterial Communities and Multi-Mycotoxin Profiling During Processing of Different Formulations of *Kunu*, a Traditional Fermented Beverage

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Kunu is a traditional fermented single or mixed cereals-based beverage popularly consumed in many parts of West Africa. Presently, the bacterial community and mycotoxin contamination profiles during processing of various *kunu* formulations have never been comprehensively studied. This study, therefore, investigated the bacterial community and multi-mycotoxin dynamics during the processing of three *kunu* formulations using high-throughput sequence analysis of partial 16S rRNA gene (hypervariable V3-V4 region) and liquid chromatography tandem mass spectrometry (LC-MS/MS), respectively. A total of 2,303 operational taxonomic units (OTUs) were obtained across six processing stages in all three *kunu* formulations. Principal coordinate analysis biplots of the Bray-Curtis dissimilarity between bacterial communities revealed the combined influences of formulations and processing steps. Taxonomically, OTUs spanned 13 phyla and 486 genera. Firmicutes (phylum) dominated (relative abundance) most of the processing stages, while Proteobacteria dominated the rest of the stages. *Lactobacillus* (genus taxa level) dominated most processing stages and the final product (*kunu*) of two formulations, whereas *Clostridium sensu stricto* (cluster 1) dominated *kunu* of one formulation, constituting a novel observation. We further identified *Acetobacter*, *Propionibacterium*, *Gluconacetobacter,* and *Gluconobacter* previously not associated with *kunu* processing. Shared phylotypes between all communities were dominated by lactic acid bacteria including

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species of *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Weissella*.Other shared phylotypes included notable acetic acid bacteria and potential human enteric pathogens. Ten mycotoxins [3-Nitropropionic acid, aflatoxicol, aflatoxin B_1 (AFB_1), AFB_2, AFM_1, alternariol (AOH), alternariolmethylether (AME), beauvericin (BEAU), citrinin, and moniliformin] were quantified at varying concentrations in ingredients for *kunu* processing. Except for AOH, AME, and BEAU that were retained at minimal levels of < 2 μg/kg in the final product, most mycotoxins in the ingredients were not detectable after processing. In particular, mycotoxin levels were substantially reduced by fermentation, although simple dilution and sieving also contributed to mycotoxin reduction. This study reinforces the perception of *kunu* as a rich source of bacteria with beneficial attributes to consumer health, and provides in-depth understanding of the microbiology of *kunu* processing, as well as information on mycotoxin contamination and reduction during this process. These findings may aid the development of starter culture technology for safe and quality *kunu* production.

Keywords: bacterial diversity, fermented beverage, food safety, high-throughput sequencing, lactic acid bacteria, *kunu*, mycotoxins

INTRODUCTION

Fermented beverages constitute a major part of the diets of traditional African homes (Tafere, 2015). In Nigeria, traditional beverages are widely consumed and mostly preferred to commercial soft drinks by individuals from low income settings due to their relatively low cost of production and high nutritional benefits (Ezekiel et al., 2015).

Kunu is a traditional beverage produced principally from single or mixed cereals such as maize, millet, rice, or sorghum. In some cases, peanut is added to the cereals to make-up the raw material input. The grain (cereals and nuts) input could also be supplemented with additives such as cloves, pepper, ginger, sweet potato and tiger nut, which are added as homogenized mixtures just before the fermentation of the beverage. The sets of cereal and nut applied to the production of *kunu* determine its variety. For example, *kunu-zaki* comprises of millet, sorghum or maize; *kunu-tsamiya*, millet, sorghum or rice; *kunu-gyada*, rice, peanut, millet or sorghum; and *kunu-gayamba*, solely millet (Gaffa et al., 2002). *Kunu-zaki* is the commonest of the *kunu* varieties due to its nutritional and health benefits (Adelekan et al., 2013). Generally, *kunu* is consumed whilst in an active state of fermentation by both adults and children (Efiuvwevwere and Akona, 1995). The nutritional content of *kunu* includes 9.84–12% carbohydrate, 1.56–3% protein, 0.1–0.3% fat, and 0.61–075% dietary minerals (Adeyemi and Umar, 1994; Badifu et al., 1999), and its health benefits range from purging the bowels and relief of flatulent conditions (Omakwu, 1980), to the enhancement of lactation in nursing mothers (Efiuvwevwere and Akona, 1995).

The production process of *kunu* comprises six critical steps: steeping of the cereals in water to allow for softening and fermentation by autochthonous bacteria, wet milling, gelatinization of a large portion of milled grains by addition of boiling water, addition of a mix of milled additives and the remainder portion of the milled grains to the gelatinized gruel, fermentation of the mixture, and sieving of the fermented slurry to obtain *kunu* (Gaffa and Ayo, 2002). The pH of *kunu* is usually acidic (pH 3–5.46) (Efiuvwevwere and Akona, 1995; Gaffa et al., 2002; Adelekan et al., 2013). The steeping duration varies and is largely dependent on the type of cereal used (Gaffa et al., 2002). Similarly, the duration of the fermentation step of the gelatinized gruel mix varies from 8 to 24 h depending on the complexity of the food matrices used for *kunu* processing and the proportion of mixture of milled grains and additives to the gelatinized gruel (Gaffa and Ayo, 2002; Osuntogun and Aboaba, 2004; Oluwajoba et al., 2013; Olosunde et al., 2015).

The fermentation stages of *kunu-zaki* are driven by consortia of bacteria (mostly lactic acid bacteria) (Efiuvwevwere and Akona, 1995; Gaffa and Gaffa, 2004; Osuntogun and Aboaba, 2004; Oguntoyinbo et al., 2011; Ikpoh et al., 2013; Aboh and Oladosu, 2014) and a few yeasts (notably *Saccharomyces cerevisiae*) (Efiuvwevwere and Akona, 1995; Gaffa and Gaffa, 2004) that contribute to the breakdown of complex macromolecules into simpler compounds. Although, there is a recent culture-independent (sanger-based sequencing technology) microbiological study of a *kunu* variety (Oguntoyinbo et al., 2011), most of the previous microbiological studies on *kunu* are based on conventional isolation methods and classical identification techniques which are prone to biases, low taxonomic resolution, misidentification of species and underestimation of species richness and diversity (Cocolin and Ercolini, 2015; Ezeokoli et al., 2016). Currently, there are no high-throughput sequencing (HTS)-based studies on *kunu* microbial ecology. The application of HTS technologies (also referred to as next generation sequencing) may help unravel hitherto unidentified bacterial species associated with *kunu* processing and *kunu* products (Franzosa et al., 2015; Ezeokoli et al., 2018). In addition, there is a paucity of information on the microbial diversity of different formulations of *kunu* at different stages of processing. Consequently, an in-depth understanding of the microbiology of *kunu* will provide insight into the community structure and functional roles of microbes

in the production of varieties of this beverage. Furthermore, the knowledge of *kunu* microbial community will facilitate the selection of starter cultures for improvement of the safety and quality of this widely consumed beverage.

Chemical food contaminants (e.g., mycotoxins) may, however, distort the safety and quality of *kunu* due to the use of diverse cereals and nuts that have been reported to be prone to several mycotoxins in the beverage formulation (Adetunji et al., 2014; Afolabi et al., 2015; Ezekiel et al., 2015, 2018; Oyedele et al., 2017). Thus, there is a need to evaluate the extent to which mycotoxins can be carried over into various *kunu* formulations, considering the diverse grain inputs into the production of this beverage. Previous studies have shown that a few mycotoxins can be present in *kunu-zaki*, albeit at reduced levels (Ezekiel et al., 2015; Olosunde et al., 2015). However, the influence of specific processing steps on the levels of mycotoxins during the processing of one or many formulations of *kunu* is yet to be elucidated. Such data coupled with information on the microbial community structure is highly relevant to food safety and protection of consumer health in view of the adverse health effects that may arise from dietary mycotoxin exposures (International Agency for Research on Cancer [IARC], 2015). Furthermore, it has been postulated that traditional beverages can contribute to increased mycotoxin exposures in high cereal -dependent regions such as sub-Saharan Africa (SSA) (Ezekiel et al., 2018). Thus, there is a need to determine the processing steps and grain combinations that are critical to the reduction of mycotoxin exposure through beverage consumption. This information may be useful for recommending safe *kunu* formulation(s) for consumer benefits.

Therefore, this study aimed to determine the bacterial community diversity and dynamics during the processing of three *kunu* formulations by using HTS-based technology, and to evaluate the effect of processing on the mycotoxin profiles at various stages of processing by using liquid chromatography tandem mass spectrometry (LC-MS/MS).

MATERIALS AND METHODS

Source of Ingredients

Samples of millet, sorghum (red and white varieties), ginger, peanut, sweet potato, tiger nut and cloves were purchased in January 2017 from the local market in Ilishan Remo (6.8932°N, 3.7105°E), Ogun state, Nigeria. Only ingredients without visible insect infestation, discolorations and rot were used for this study.

Kunu Formulations and Processing

Three different formulations of *kunu* designated as A, B, and C were prepared using the ingredients. Formulation A comprised of millet, white sorghum, peanut, cloves, ginger and tiger nut while formulation B was made from millet, white sorghum, cloves, ginger and sweet potato. Formulation C consisted of millet, red sorghum, cloves, ginger and tiger nut. In the formulations, cloves, ginger, sweet potato and tiger nut served as additives to millet, sorghum (white or red variety) and peanut, which were used as grain bases for the beverage. Precisely 1 kg of each grain and 100 g of each additive were used in the formulations. Maize was excluded from the formulations in order to eliminate extremely high levels of diverse mycotoxins from the beverage.

For *kunu* processing, rudimentary utensils and tap water were used in order to replicate as much as possible the traditional processing method. The exact process undertaken for the preparation of the *kunu* formulations in this study is outlined in **Figure 1**. For clarity, all processing steps, including the fermentation step, were carried out under prevailing ambient temperature (33 ± 2°C). Precisely 3 L and 300 mL of tap water were used for the steeping of grains (1 kg) and additives (100 g), respectively; this step and the fermentation step were performed in prewashed 5 L wide mouth plastic containers with lids and without agitation or additional aeration. Prewashing of the containers was performed with detergent and thorough rinsing with tap water. Ginger and sweet potato were not steeped, but peeled and washed with tap water prior to wet milling. Milling (wet milling) was performed for approximately 10 min using a commercial milling machine to make fine slurry. The commercial milling machine was rinsed twice with tap water to clear off debris from previous use. Gelatinized portions of the gruels were left to cool down to a temperature of 37 ± 2°C (typically for duration of at least 3 h) before proceeding to the homogenization step. Sieving of the fermented substrate was performed using a clean muslin cloth.

Sampling

Samples were collected from various core processing steps based on relevance of each step to either the bacterial community profile study or mycotoxin reduction analysis. For both (bacterial and mycotoxin) analyses, samples of steep liquor, milled grains and *kunu* were collected, while samples of raw grains, milled additives, cooled gruel (after gelatinization) and pomace (after sieving) were taken for mycotoxin analysis, and samples of the fermenting substrate were collected at 0, 6, and 12 h of fermentation for microbial analysis. Approximately 20 mL and 20 g subsamples of liquid and semi-/solid samples, respectively, were randomly collected for analysis. All samples were immediately frozen at −20°C prior to shipment on dry ice and analyses at the Agricultural Research Council–Biotechnology Platform, South Africa for HTS analysis, and the Center for Analytical Chemistry, IFA–Tulln, Austria for multi-mycotoxin analysis.

Total Community DNA Extraction

Genomic DNA was extracted from samples using the Quick-DNA Fecal/Soil microbe kit (Zymo Research, Irvine, CA, United States) according to the manufacturer protocol. For steep liquor, 20 ml of samples was vacuum filtered through a sterile 0.2 μm pore size membrane filter (Whatman Plc, Maidstone, United Kingdom) and DNA extracted directly from the membrane filter whereas for milled grains and fermenting gruels, 0.25 g (wet weight) of sample homogenate were used for DNA extraction. For *kunu*, samples were centrifuged at 12,000 rpm for 5 min and DNA was extracted from 0.25 g (wet weight) of the sediment. DNA integrity and concentration

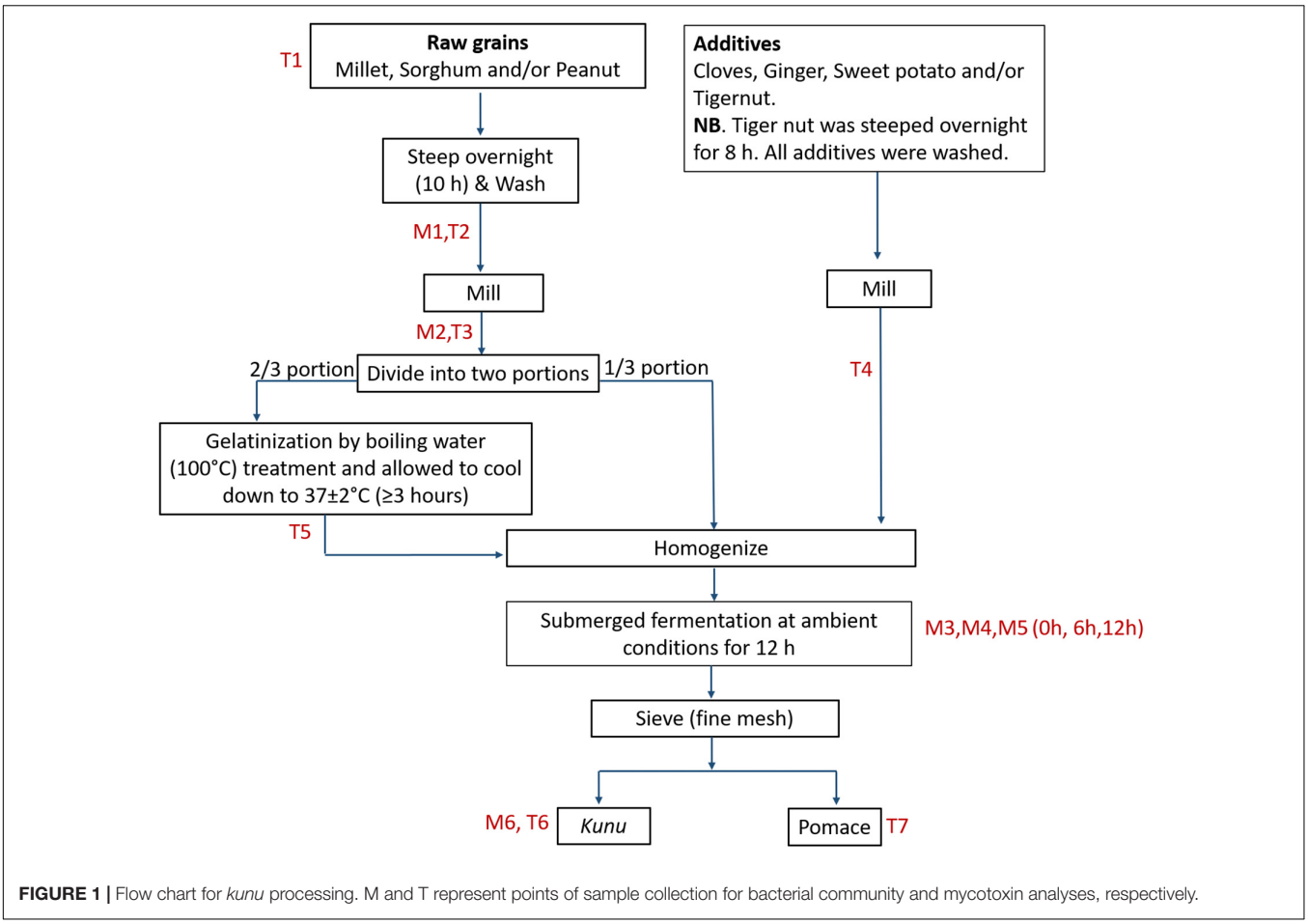

FIGURE 1 | Flow chart for *kunu* processing. M and T represent points of sample collection for bacterial community and mycotoxin analyses, respectively.

were verified by agarose (1% w/v) gel electrophoresis and fluorometric quantification (Qubit 2.0, Invitrogen, Carlsbad, CA, United States), respectively.

High-Throughput Sequencing of Bacterial Communities

Partial 16S rRNA gene (hypervariable V3-V4) libraries were amplified using universal bacterial primers 341F (forward) and 805R (reverse) (Klindworth et al., 2013). Each forward and reverse primer contained Illumina overhang adapters (Illumina Inc., United States). Library preparation steps were performed as described previously (Mashiane et al., 2017; van Wyk et al., 2017). Briefly, PCR amplicons were purified using Agent Court AMPure XP beads (Beckman Coulter, Brea, CA, United States), and each sample amplicon uniquely indexed with dual indexes. Uniquely indexed amplicons were purified again with AMPure beads, quantified using a Qubit fluorometer (Invitrogen, Carlsbad, CA, United States), normalized to equal concentration in a resuspension buffer (Illumina Inc., San Diego, CA, United States) library, pooled in equimolar proportions, denatured in 0.2 M NaOH and loaded along with denatured PhiX (control) library onto a MiSeq V3 cartridge for a 2 X 300 bp paired-end sequencing run on the Illumina MiSeq sequencer (Illumina Inc, San Diego, CA, United States).

Bioinformatics

Demultiplexed sequence reads were checked for quality using FastQC software (v. 0.11.7, Babraham Institute, United Kingdom) and subsequently trimmed of low-quality regions (10 bp) at both 5′- and 3′-ends using Trimommatic (Bolger et al., 2014). PANDAseq (Masella et al., 2012) was used to assemble (merge) forward and reverse reads with a minimum overlap of 50 bp, as well as eliminate merged reads with ambiguous nucleotide bases (N) and spurious lengths (>465 bp) at a threshold (t) of 0.7 and by using the simple Bayesian algorithm. Merged reads were then binned (closed reference OTU picking) into operational taxonomic units (OTUs) (97% 16S rRNA gene similarity) against the SILVA rRNA reference (Release 128) (Quast et al., 2013) by using Usearch61 (Edgar, 2010; Edgar et al., 2011) in Quantitative Insights Into Microbial Ecology (QIIME) software (v. 1.9.1) (Caporaso et al., 2010). Singletons were removed from the OTU count table before normalization (rarefaction) to even depths across samples in QIIME. Alpha diversity and principal coordinate analyses were performed in QIIME and/or R software version 3.4.0 (R Core Team, 2013). Multivariate analysis was performed based on the relative abundance of OTU counts. Statistical tests for differences in multivariate space were not performed because treatments were not replicated.

Data Availability

Raw sequence reads generated in this study are available in the Sequence Read Archives[1] of the National Centre for Biotechnology Information under the bioproject accession number PRJNA482055.

Multi-Microbial Metabolite Analysis of *Kunu* Formulations

Samples of grains, by-products (steep liquor and pomace) and *kunu* were analyzed for the presence of over 295 microbial metabolites including the major mycotoxins (e.g., aflatoxins, fumonisins, dexoynivalenol, ochratoxins, and their metabolites) by a liquid chromatography tandem mass spectrometric (LC-MS/MS) method described by Malachova et al. (2014). Please see full list of 295 metabolites in Malachova et al. (2014). For the grains, 5 g of each ground sample was extracted with 20 mL of acetonitrile/water/acetic acid (79:20:1, v/v/v) in a 50 mL polypropylene tube (Sarstedt, Nümbrecht, Germany) for 90 min using a GFL 3017 rotary shaker (GFL 3017, Burgwedel, Germany). In the case of liquid samples (steep liquor and *kunu*), mycotoxins were extracted from 2.5 mL of the samples in 15 mL polypropylene tubes containing 7.5 mL of extraction solvent and centrifuged at 10,000 rpm for 3 min at ambient temperature. All extracts were diluted with acetonitrile/water/acetic acid (20:79:1, v/v/v) solvent and injected into the LC system as described in detail by Sulyok et al. (2006). LC-MS/MS screening of target fungal metabolites was performed using a QTrap 5500 LC-MS/MS System (Applied Biosystems, Foster City, CA, United States) equipped with TurboionSpray electrospray ionization source and a 1290 Series HPLC System (Agilent, Waldbronn, Germany). Chromatographic separation was performed at 25°C on a Gemini®C18-column, 150 × 4.6 mm i.d., 5 mm particle size, equipped with a C18 4 × 3 mm i.d. security guard cartridge (Phenomenex, Torrance, CA, United States). Confirmation of positive analyte identification was obtained by the acquisition of two scheduled multiple reaction monitoring (MRMs) which yielded 4.0 identification points according to the European Commission decision 2002/657. In addition, the LC retention time and the intensity ratio of the two MRM transitions agreed with the related values of an authentic standard within 0.1 min and 30% rel., respectively. Apparent recoveries of the metabolites were determined by spiking 0.25 mL of five different *kunu* samples. The spiked samples were stored overnight at ambient temperature to establish equilibrium between the metabolites and samples. The extraction (in 1 mL of solvent), dilution and analysis were as described earlier. The accuracy of the method was crosschecked by participation in inter-laboratory comparison studies organized by BIPEA (Gennevilliers, France). Only mycotoxins that were positive in the samples are reported in the results section.

[1]https://www.ncbi.nlm.nih.gov/sra

RESULTS

Diversity and Community Structure of Operational Taxonomic Units

A total of 2,647,697 high-quality sequences were obtained from all the samples of the three *kunu* formulations after quality filtering and binning into OTUs. Normalization (rarefaction) of data to a depth of 13,920 sequences per sample was sufficient to estimate community diversity in all samples (**Figure 2**).

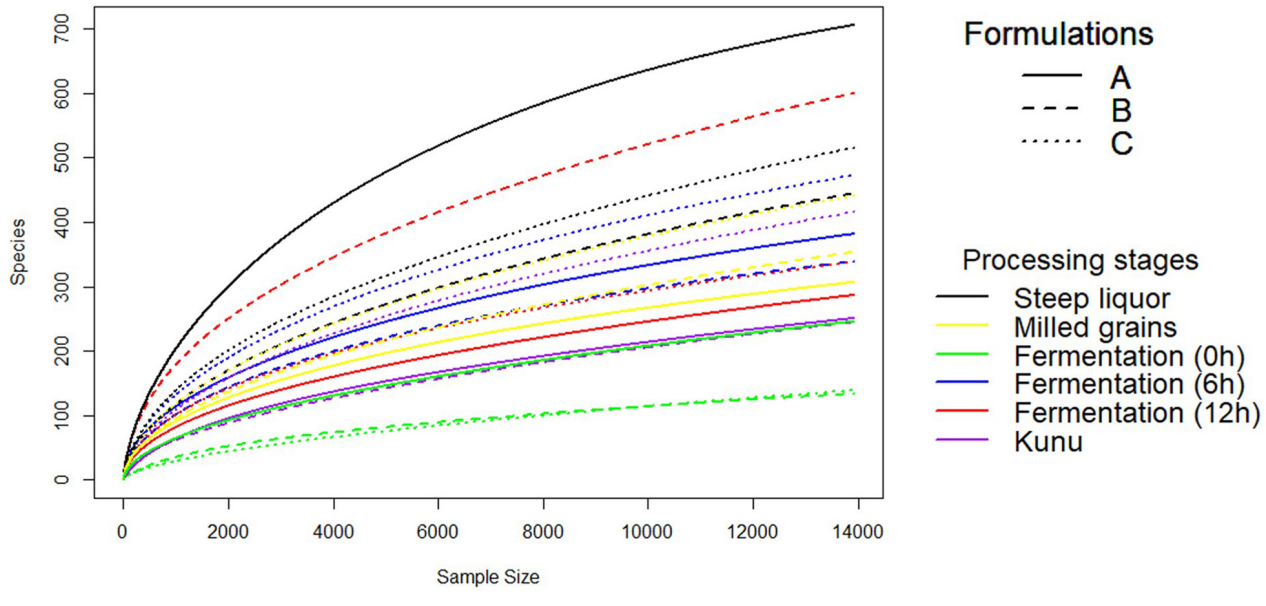

FIGURE 2 | Rarefaction Curve of OTUs to even subsampling depth. Rarefaction curve was constructed using the vegan package of R software after single rarefaction to a depth of 13920 sequences per sample in QIIME software.

A total of 2,303 OTUs were obtained from all samples (**Figure 3A**), with 526 OTUs shared between all formulations (**Figure 3A**). Steep liquor had the highest number of OTUs in both formulations A (706 OTUs) and C (516 OTUs), while fermentation at 12 h had the highest number of OTUs (600 OTUs) in formulation B (**Table 1**). As expected, trends in Chao1—, a species richness estimation that accounts for possible rare species in the community that might have been missed due to under sampling—, phylogenetic diversity—an indices based on evolutionary distances between species in a given sample—, and Shannon-Weiner index of diversity observed among stages of the different formulations were similar to trends observed in the number of OTUs (**Table 1**). In comparison to stages within a formulation and between formulations, higher values of these indices indicate higher species richness (Observed OTUs, Chao1) and diversity (Shannon–Weiner index, Phylogenetic diversity). In all formulations, the least OTU diversity was observed at the 0 h fermentation stage. Similarly, the least number of shared OTUs among all the formulations was at the 0 h fermentation, while the most number of shared OTUs was mid-way through the fermentation (6 h) (**Figures 3B–G**). Overall, pair-wise comparisons of shared OTUs between formulations revealed that the most number of OTUs (206 OTUs) were shared between formulation A and C (**Figure 3A**), while the least number of OTUs (119 OTUs) were shared between formulations A and B (**Figure 3A**).

The unweighted (on the basis of presence/absence of OTUs) Bray-Curtis dissimilarity principal coordinate analysis (PCoA) biplot for OTUs distribution revealed that the bacterial community structure of the processing stages of formulation A were the most diverse compared to other formulations (**Figure 4A**). However, close similarities in community structure were observed between stages of the same formulations and between stages of different formulations (**Figures 4A,B**). For example, the bacterial community of fermentation at 0, 6, and 12 h were similar for formulation B (**Figure 4A**), while the bacterial community of the fermenting substrate at 0 h was similar for both formulations B and C (**Figure 4A**). Similarly, the bacterial community of steep liquor and *kunu* for formulation B were similar, while the bacterial community of the fermenting substrate at 12 h and *kunu* of formulation B were closely similar. In the weighted (on the basis of presence/absence and relative abundance of OTUs) Bray-Curtis dissimilarity PCoA biplot for the absence/presence and relative abundance of OTUs (**Figure 4B**), the similarities between communities were more pronounced, particularly for communities between *kunu* of

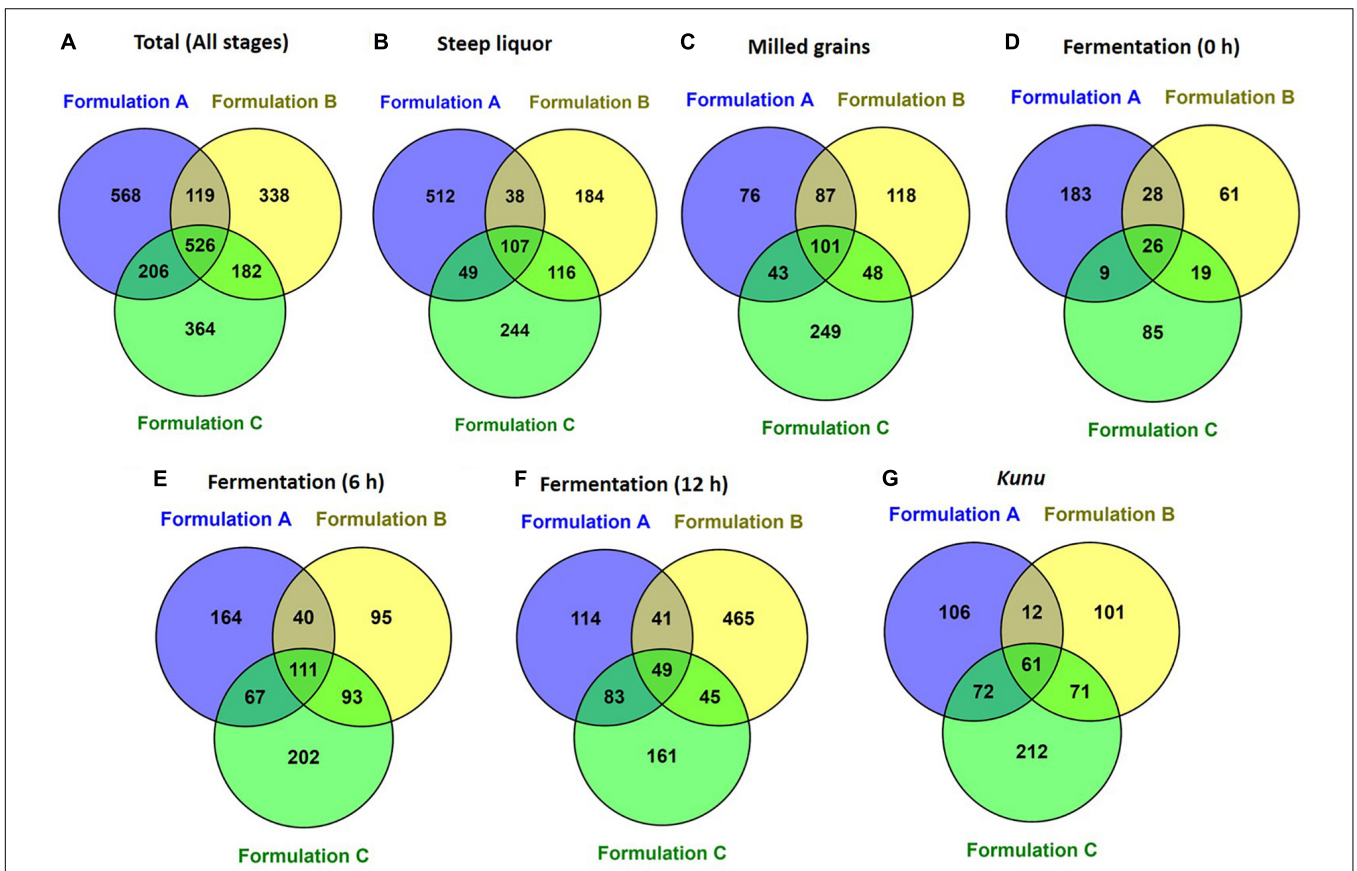

FIGURE 3 | Shared operational taxonomic units between formulations **(A)** Total (all stages) **(B)** Steep liquor. **(C)** Milled grains. **(D)** Fermentation at 0 h. **(E)** Fermentation 6 h. **(F)** Fermentation 12 h. **(G)** *Kunu*. Venn diagram was constructed by using the online Venny tool v.2.1. (Oliveros, 2007; http://bioinfogp.cnb.csic.es/tools/venny/).

TABLE 1 | Operational taxonomic units (OTUs) diversity metrics in different formulations of *kunu* after single rarefaction to even depth of 13,920 sequences per sample.

	Processing stage	Observed OTUs	Chao1	†Phylogenetic diversity	Shannon-Weiner
Formulation A	Steep liquor	706	811.99	49.40	6.21
	Milled grains	307	515.41	17.81	4.42
	Fermentation (0 h)	246	448.78	18.32	2.99
	Fermentation (6 h)	382	575.80	21.12	4.85
	Fermentation (12 h)	287	521.50	19.03	3.97
	Kunu (final product)	251	493.00	19.96	1.77
Formulation B	Steep liquor	445	720.34	27.14	5.15
	Milled grains	354	665.73	21.42	3.95
	Fermentation (0 h)	134	249.56	10.72	1.50
	Fermentation (6 h)	339	505.96	20.33	4.45
	Fermentation (12 h)	600	918.31	33.64	6.23
	Kunu (final product)	245	477.23	17.09	2.86
Formulation C	Steep liquor	516	874.50	29.79	4.65
	Milled grains	441	738.01	27.55	4.56
	Fermentation (0 h)	139	293.29	15.13	1.70
	Fermentation (6 h)	473	738.42	25.03	5.19
	Fermentation (12 h)	338	582.17	20.18	4.53
	Kunu (final product)	416	818.21	26.69	3.96

†*Based on PD_whole_tree; Observed OTUs or OTU Richness is the number of observed unique operational taxonomic units (defined by 97% 16S rRNA gene sequence similarity). Chao1 is a richness estimation or prediction based on the adjustment of species richness for rare OTUs that may have been missed due to under sampling. Chao 1 is often referred to as the true species richness of a given community; PD (phylogenetic distance) whole tree is a diversity estimate calculated from the phylogenetic distances of OTUs present within a sample. Shannon-Weiner index of diversity accounts for the abundance and evenness (equitability) of species in a community. The higher the Shannon-Weiner index, the more diverse are the species in that community.*

formulation A and milled grains of formulation B (**Figure 4B**). Within formulations, steep liquor, fermentation at 0 h and *kunu* had closely similar bacterial community structures (**Figure 4B**). Other closely similar bacterial communities included those of steep liquor in formulation C and fermentation at 6 h in formulation A.

Taxonomic Diversity and Dynamics

All 2,303 OTUs obtained from the processing stages of the different *kunu* formulations taxonomically spanned at least 13 phyla and 486 genera, with several of the OTUs being unclassified at these taxonomic ranks. At the phylum taxonomic level, Firmicutes dominated most of the processing stages of all *kunu* formulations (**Figure 5**), except for the steeping stages of formulation A, and at 6 and 12 h fermentation stages of formulations A and B, respectively, which were dominated by Proteobacteria (**Figure 5**). Other phyla that constituted at least 1% relative abundance in all stages included Actinobacteria, Bacteriodetes, and Cyanobacteria (**Figure 5**).

The phylotypes (at the genus taxonomic rank) with at least 1% relative abundance in any of the processing stages of each formulation are presented in **Figure 6**. Overall, *Lactobacillus* dominated most of the processing stages, especially in the *kunu* of formulations A and B (**Figures 6A,B**). In contrast, *Lactobacillus* dominated all stages but *kunu* of formulation C, where *Clostridium sensu stricto* (cluster 1) was found to dominate *kunu* of formulations C (**Figure 6C**). In formulation A, *Acetobacter*, *Brevundimonas*, *Clostridium*, *Delftia*, *Devosia*, *Elizabethkingia*, *Enterobacter*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Propionibacterium*, *Pseudomonas*, *Staphylococcus*, *Streptococcus*, *and Weissella* each constituted at least 5% relative abundance in at least one processing stage (**Figure 6A**). In formulation B, *Lactobacillus*, *Lactococcus*, *Acetobacter*, *Burkholderia*/*Paraburkholderia*, *Weissella*, *Enterobacter*, *Clostridium sensu stricto* (cluster 1), *Sphingobacterium*, *Acinetobacter*, and *Pseudomonas* each constituted at least 5% relative abundance in at least one processing stage (**Figure 6B**). In formulation C, *Burkholderia/Paraburkholderia*, *Clostridium sensu stricto* (cluster 1), *Enterobacter*, *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Streptococcus*, *and Weissella* each constituted at least 5% relative abundance in at least one processing stage (**Figure 6C**). Across formulations, some genera which constituted at least 1% (but less than 5%) relative abundance (**Figures 6A–C**) and whose species have potential roles in food fermentations include *Gluconobacter* and *Gluconacetobacter*.

Bacterial succession pattern was observed at the fermentation stages of all the *kunu* formulations. For formulation A, *Pediococcus* (67.5%) dominated the initial fermentation (0 h) (**Figure 6A**), while *Clostridium* (35.6%) and *Enterobacter* (33.8%) dominated at 6 h and *Lactobacillus* (68.9%) at 12 h fermentation. In contrast, *Weissella* dominated the initial fermentation (97.3%) and mid-stream (6 h) fermentation (32.9%) in formulation B while *Acinetobacter* (12.8%), *Sphingobacterium* (14.6%), *Pseudomonas* (11.5%) and *Enterobacter* (8.7%) were the relatively more abundant phylotypes in the final fermentation (12 h) stage of the same

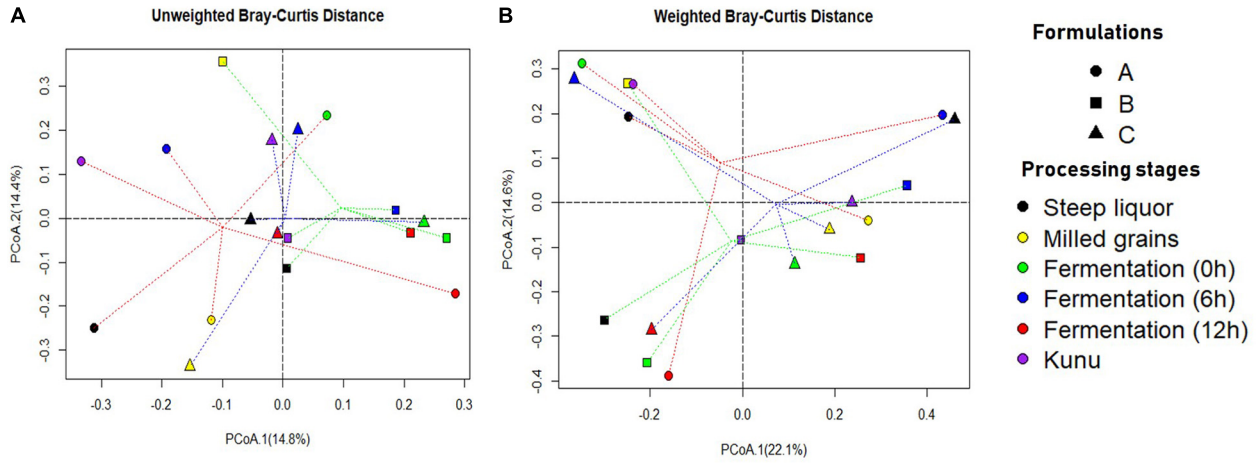

FIGURE 4 | Principal coordinate analysis (PCoA) Biplot of Bray-Curtis Dissimilarity between operational taxonomic units of different *kunu* formulations. **(A)** Unweighted (presence/absence of OTUs). **(B)** Weighted (presence/absence and relative abundance of OTUs). Dotted lines red, green and blue show distance of every sample to formulation A, formulation B, and formulation C group centroid, respectively. PCoA was constructed using the 'ape' package of R software. Bray-Curtis dissimilarity is a statistic used to estimate differences in the composition of species between two or more sites/samples based on counts.

formulation (**Figure 6B**). On the other hand, in formulation C, *Lactobacillus* spp. dominated all the three stages of fermentation (**Figure 6C**).

A total of 35 OTUs, comprising 16 phylotypes (at the genus taxonomic rank) were common to all processing stages and formulations (**Figure 7**). These "shared phylotypes" were dominated by *Lactobacillus* spp. (17%). Other shared phylotypes included species of *Enterobacter, Gluconacetobacter Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus,* and *Weissella*.

Multiple Mycotoxins in *Kunu* Formulations

Occurrence of Mycotoxins in the Ingredients (Grains and Additives)

A total of 10 mycotoxins were found in the ingredients used for processing the three *kunu* formulations (**Table 2**). Aflatoxins were found in almost all grains (except the white sorghum variety) and additive. Only the B-aflatoxins were detected in the grains. The mean levels of aflatoxin B_1 (AFB_1) were higher in red sorghum (113 μg/kg), peanut (51.5 μg/kg), and tiger nut (26.1 μg/kg) compared to millet (0.67 μg/kg), while almost similar mean concentrations of AFB_2, 6.56 and 9.27 μg/kg, were quantified in peanut and red sorghum, respectively. AFM_1 was detected in peanut and red sorghum at mean concentrations of 1.7 and 4.22 μg/kg, respectively, while citrinin (CIT) was only quantified in millet at mean concentration of 5.64 μg/kg. The concentrations of aflatoxicol, alternariol (AOH), alternariolmethylether (AME), and beauvericin (BEAU) in grains did not exceed 5 μg/kg. In contrast, the concentrations of 3-Nitropropionic acid (3-NPA) were very high in peanut (1121 μg/kg) and red sorghum (936 μg/kg). Moniliformin (MON) levels were higher in millet (68.7 μg/kg) and white sorghum (44.2 μg/kg) than in red sorghum (2.93 μg/kg) and peanut (1.42 μg/kg).

Changes in Mycotoxin Levels During Processing of the Three *Kunu* Formulations

The distribution of mycotoxin concentration data were normalized by logarithmic (Log_{10}) transformation. The value "1" was first added to the mycotoxin concentration in a sample, and then the resulting value was transformed to give the data reported in **Figure 8**. Estimates of percentage reduction of mycotoxins due to processing were based on percentage differences between mycotoxin levels in the raw grains and processing steps and *kunu* (Okeke et al., 2015). The changes in mycotoxin concentrations during processing of *kunu* formulation A is shown in **Figure 8A**. Steeping and milling of the raw grains caused a reduction below the detectable limits of 3-NPA, AFB_2, AFM_1, aflatoxicol, AOH, AME, BEAU and CIT levels in the milled grains, as well as 99.5% and 95.2% reduction of AFB_1 and MON contents of the raw grains from 52.2 and 114 μg/kg to 0.25 and 5.53 μg/kg, respectively, in the milled grains (**Figure 8A**). The residual AFB_1 and MON levels in the milled grains were further reduced to undetectable levels in the cooled gruel. The milled additives re-introduced all the 10 mycotoxins into the beverage process system at varying levels (**Figure 8A**). However, the fermentation stage caused complete reduction of 3-NPA, aflatoxicol, AFB_2, AFM_1, BEAU and MON to undetectable levels in the *kunu* product. Although AFB_1, CIT and AOH were also not found in *kunu* of formulation A, about 2, 26, and 32% of the respective 26.5, 6.79, and 2.26 μg/kg in the milled additives were residual in the pomace (a bye-product of *kunu* processing; please see **Figure 1**). Only 9% of 3.5 μg/kg AME in the milled additives was carried over into the *kunu* (**Figure 8A**).

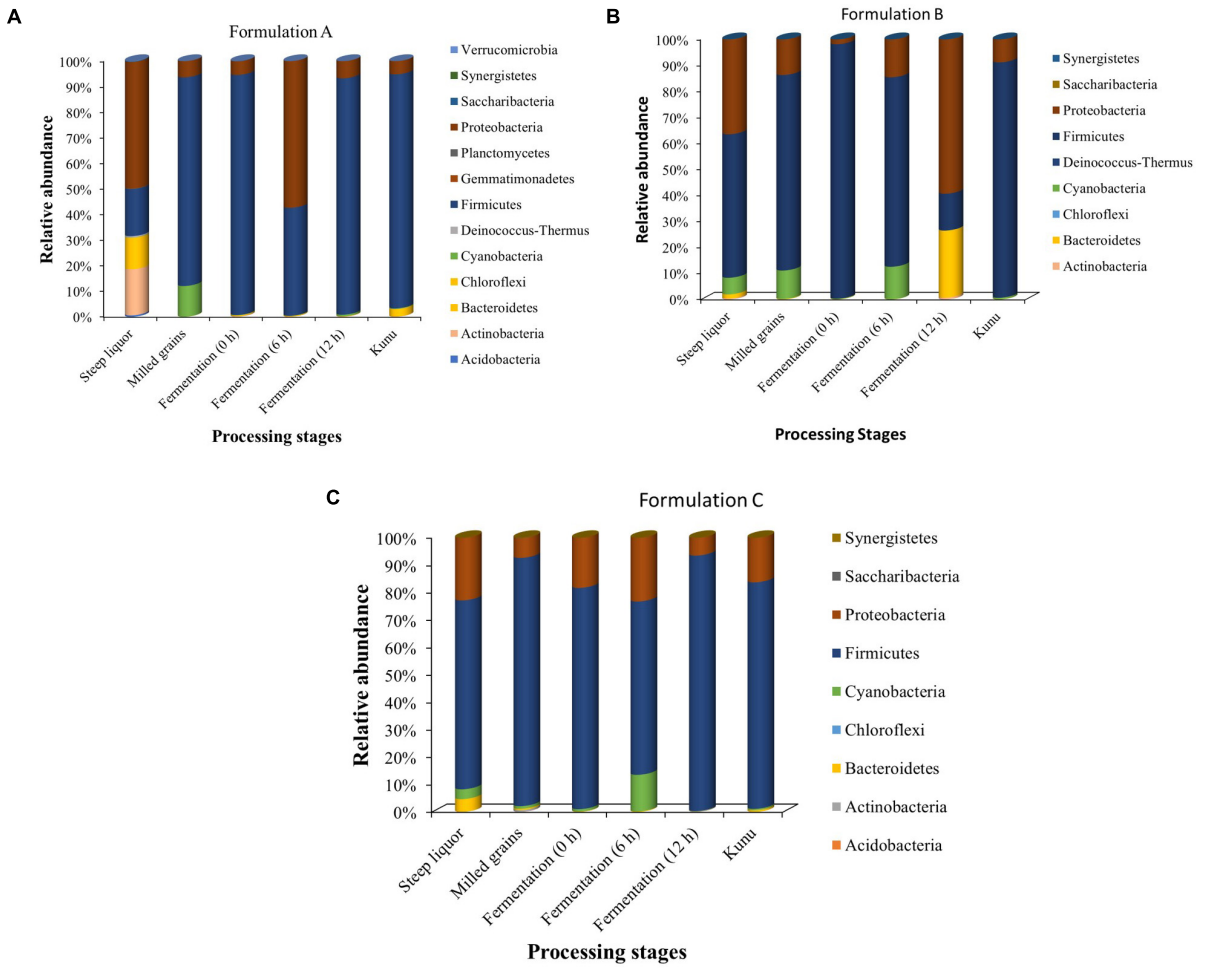

FIGURE 5 | Relative abundance of classifiable OTUs at the phylum taxonomic rank. **(A)** Formulation A **(B)** Formulation B **(C)** Formulation C. OTUs not assigned to a phylum taxonomic rank are excluded from the bar plot.

Only five of the 10 mycotoxins present in the ingredients were detected during the processing of formulation B *kunu* (**Figure 8B**). 3-NPA, AFB_1 and MON were not detected in the *kunu* despite their occurrence at different processing steps. For 3-NPA and MON, at least 75% of the levels in the raw grains (10.8 and 106 μg/kg, respectively) were quantified in the steep liquor. About 9% (0.43 μg/kg) of the BEAU in the ingredients (4.97 μg/kg) and 40% of CIT in the milled grains (4.32 μg/kg) were carried over into *kunu* (**Figure 8B**). During the processing of *kunu* formulation C (**Figure 8C**), steeping and milling of the raw grains as well as the fermentation stage resulted in reduction of the concentrations of 3-NPA, AFB_1, AFB_2, AFM_1, aflatoxicol, AOH and AME to undetectable levels. In addition, 16% (0.45 μg/kg) of the BEAU in the ingredient (milled grains and additives; 2.91 μg/kg) was carried over to *kunu*, with the sieving step removing the remainder portion of the toxin (**Figure 8C**). No detectable levels of the 8.7 μg/kg of CIT and 52.3 μg/kg of MON inputted into the processing system by the grains and additives were found in the *kunu*. However, significant reduction of MON and CIT were accomplished by the steeping and fermentation stages, respectively. Sieving also contributed to further reduction of CIT levels (**Figure 8C**).

DISCUSSION

This study reports, for the first time, on the application of HTS in the delineation of bacterial community structure during processing of *kunu vis-a-vis* changes in mycotoxin concentrations. Overall, this study clearly indicates the core bacterial communities and succession during the various stages of processing of three formulations of *kunu*, and the possible role of the microbial communities in reducing the mycotoxin levels (from ingredients to the final *kunu* product) irrespective of formulations.

The high number of OTUs (species richness) obtained during the steeping stage of the various *kunu* formulations suggests a rich genetic diversity of bacteria in the grains. Although potable tap water used for the steeping was not evaluated independently for microbial diversity, it is also likely that potable

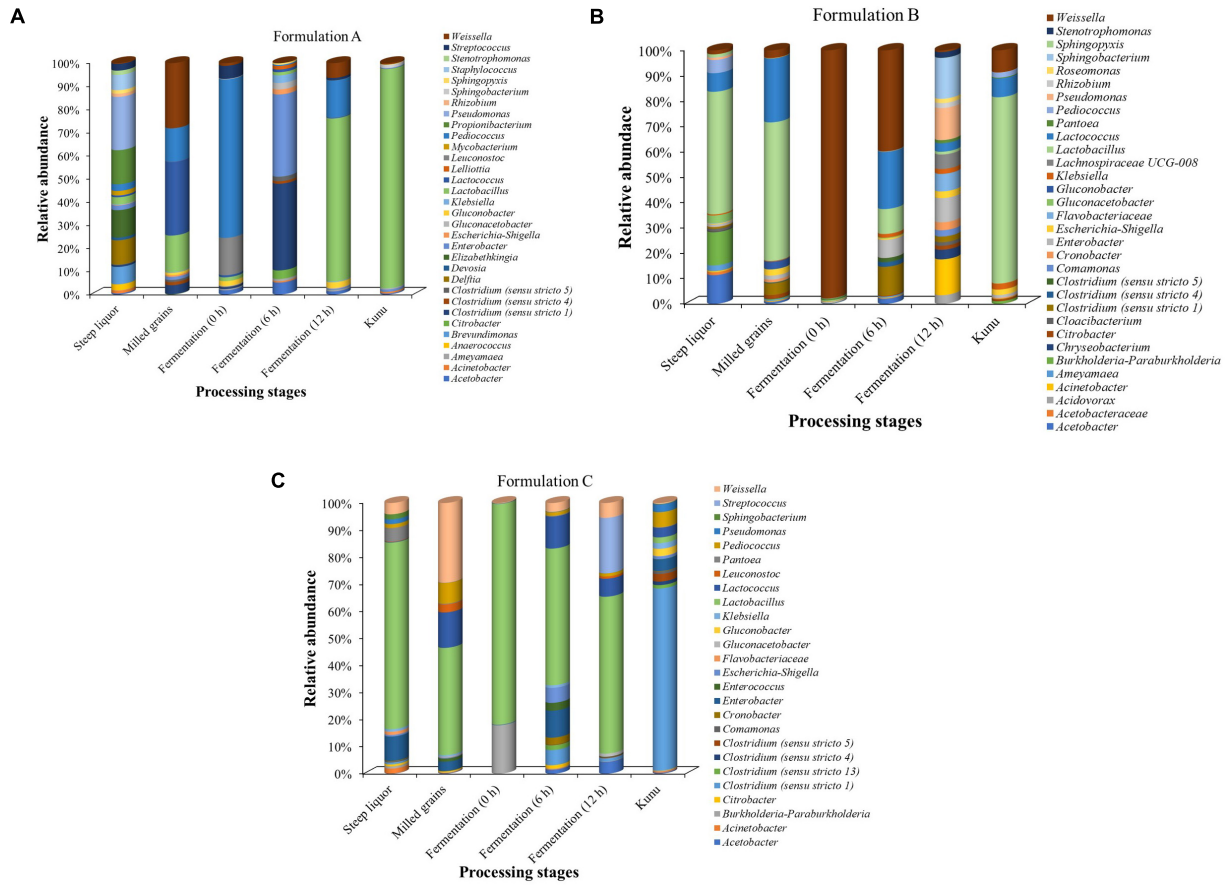

FIGURE 6 | Relative abundance of classifiable OTUs with at least 1% relative abundance at the genus taxa level. Family names are shown where OTUs are unclassifiable at the genus level. **(A)** Formulation A. **(B)** Formulation B. **(C)** Formulation C. OTUs not classifiable to genus taxonomic rank were excluded from the bar plot.

water contributed to the species diversity at the steeping stage. Furthermore, this observation of high species richness may be due to the stepping environment and duration serving as a broth and permitting the proliferation, respectively, of the autochthonous microbial species. Similar observations of diverse bacteria occurring during the steeping of raw grains for *kunu-zaki* production were suggested in previous studies (Efiuvwevwere and Akona, 1995; Gaffa and Gaffa, 2004). The high number of OTUs in formulations A and C, and shared OTUs between these two *kunu* formulations in contrast to the relatively lower number of OTUs obtained for the formulation B could be attributed to the composition of ingredients used for the various formulations: only formulations A and C contained tiger nut. This observation suggests that tiger nut may serve as the source of the additional OTUs in formulations A and C. Tiger nut grows underground and has been reported to harbor a plethora of microbiota consequent on its direct interaction (not enclosed in pods) with the soil environment (Ayeh-Kumi et al., 2014; Gambo and Da'u, 2014; Ike et al., 2017).

Herein, we put forward some hypotheses to explain the observed changes in OTU richness between successive stages of *kunu* processing. The lower OTU richness observed in the milled grains in comparison to OTU richness in steep liquor could be attributed to the fact that during the overnight steeping, bacterial species associated with the grains and steep water proliferate, giving rise to a fermented broth—the steep liquor (usually with bubbles or foams due to CO_2 release observed). Since this fermented broth (or steep liquor) are decanted and discarded before subsequent washing of the steeped grains, a large number of species present in the steep liquor are not carried over to the milling stage. Furthermore, since the steep liquor is not used in subsequent processing step, the OTUs detected in the milled grains are likely those that are within the grains, OTUs not completely eliminated during washing of the steeped grains, and/or any OTUs introduced by chance contamination during milling of the grains. Hence, the milled grain is expected to contain significantly lower species than the steep liquor as observed in all formulations. Between the milled grains and onset of fermentation, a reduction in OTUs was observed. This relatively low OTU diversity observed at the 0 h fermentation time compared to the pre-gelatinization stage in all the *kunu* formulations is attributable to the hot water (100°C) treatment during the preceding gelatinization of the milled grains. Certainly, such heat treatment will have at least a

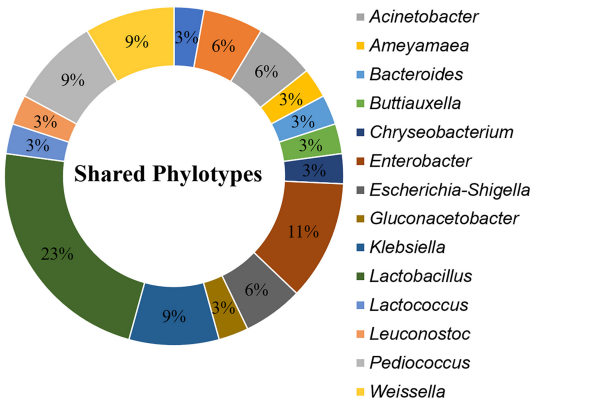

FIGURE 7 | Shared phylotypes (genus taxa level) between all processing stages of *kunu* formulations. OTUs unclassified at genus taxa level are excluded from the plot. Pie slices indicate relative abundance of individual phylotypes in the dataset. Shared phylotypes were computed using the "shared_phylotypes.py script in QIIME software.

bacteriostatic (if not significantly bactericidal) effect on most of the indigenous microbiota, consequently contributing to reduced species diversity (Russell, 2003). In a study by Akharaiyi and Omoya (2008), following heat treatment of fermented maize (ogi) at 100°C, the total viable count of microorganisms drastically reduced to minimal levels. Despite expected increase in species after the addition of the un-heated 1/3 portion, the restoration of species richness to the pre-gelatinization state may have not been detected due to dilution effect thereby resulting in a lower chance of species (or their DNA) recovery during DNA extraction. Bias in the efficiency of recovery and detection of rare species is a well-known limitation of metagenomics approaches (Wooley and Ye, 2010; Breitwieser et al., 2017).

During the fermentation stage, an increase in OTUs was observed between the onset and first 6 h of fermentation. *Kunu* fermentation is a lactic acid fermentation type, with initial slightly acidic pH of about 6.0 (Gaffa and Gaffa, 2004), which gradually reduces to an acidic pH of about 4.76 (Gaffa and Gaffa, 2004) at the end of 12 h and then to 3.0 in the *kunu* product (Efiuvwevwere and Akona, 1995; Gaffa et al., 2002; Adelekan et al., 2013). Increase in acidity is a selective pressure on the microbial community during fermentation (Wolfe and Dutton, 2015; Zabat et al., 2018). We hypothesize that the observed increase in OTUs between the first 6 h was due to the moderate pH and other favorable conditions (e.g., temperature, oxygen availability, absence of growth inhibition metabolites) during this period, permitting the growth of species which were suppressed by the heat treatment in the gelatinization stage (Yang et al., 2016). However, as the pH further decreases (selection pressure) toward the end of fermentation (at 12 h), only adapted species (mostly lactic acid bacteria) proliferate (Gaffa and Gaffa, 2004). Hence, a reduction (compared to at 6 h) in OTU diversity at the end of fermentation was observed in formulations A and C. In contrast, an increase in OTU richness was observed between the 6 and 12 h of fermentation in formulation B. The reason for this observation may be due to the presence of sweet potato and the absence of tiger nut in formulation B compared to formulations A and C. Difference in substrate types influences the genetic and functional diversity and dynamics of bacteria during fermentation, and also play a role in the equilibrium of biochemical reactions and conditions (e.g., pH, oxygen availability, redox reactions, enzymatic activities, and temperature) during fermentation (Giraffa, 2004; Ijabadeniyi, 2007; Van der Meulen et al., 2007). Furthermore, the observed general reduction in OTUs after the sieving step may be attributed to the removal of a large number of species in the pomace; the pomace contains fibrous material which provides a larger surface area for the adhesion of microorganisms compared to the liquid *kunu* fraction.

In view of the afore discussions on OTU diversity, the trend in numbers of OTUs obtained from the mid-stream (6 h) fermentation stage up to the final product stage for the formulations suggests the proliferation of adapted (after heat treatment) functional species (key drivers of biochemical conversions of macromolecules in the fermenting substrate)

TABLE 2 | Mycotoxin levels in grains and nuts for *kunu* formulation.

Mycotoxins	Limit of detection (μg/kg)	Mycotoxin concentrations (μg/kg) ± standard deviation[1]				
		Peanut	Millet	White sorghum	Red sorghum	Tiger nut
3-Nitropropionic acid	0.8	nd	4.15 ± 0.61	0.28 ± 0.01	nd	0.25 ± 0.02
Aflatoxin B_1	0.24	1121 ± 231	10.8 ± 0.01	nd	936 ± 514	9.97 ± 0.50
Aflatoxin B_2	0.4	3.69 ± 1.52	nd	nd	4.12 ± 1.81	1.68 ± 0.02
Aflatoxin M_1	0.4	51.5 ± 20.1	0.67 ± 0.01	nd	113 ± 34.2	26.1 ± 0.40
Aflatoxicol	1	6.56 ± 0.93	nd	nd	9.27 ± 1.20	2.50 ± 0.01
Alternariol	0.4	nd	1.89 ± 0.61	nd	nd	5.64 ± 0.40
Alternariolmethylether	0.032	1.42 ± 1.19	44.2 ± 20.4	68.7 ± 13.7	2.93 ± 0.28	5.06 ± 0.20
Beauvericin	0.008	3.35 ± 0.07	nd	0.61 ± 0.04	nd	1.73 ± 0.20
Citrinin	0.16	1.71 ± 1.41	nd	nd	4.22 ± 2.11	0.82 ± 0.00
Moniliformin	1.6	3.60 ± 0.11	nd	nd	nd	1.51 ± 0.10

[1] *Standard deviation from the mean (number of samples = 3). nd, not detected.*

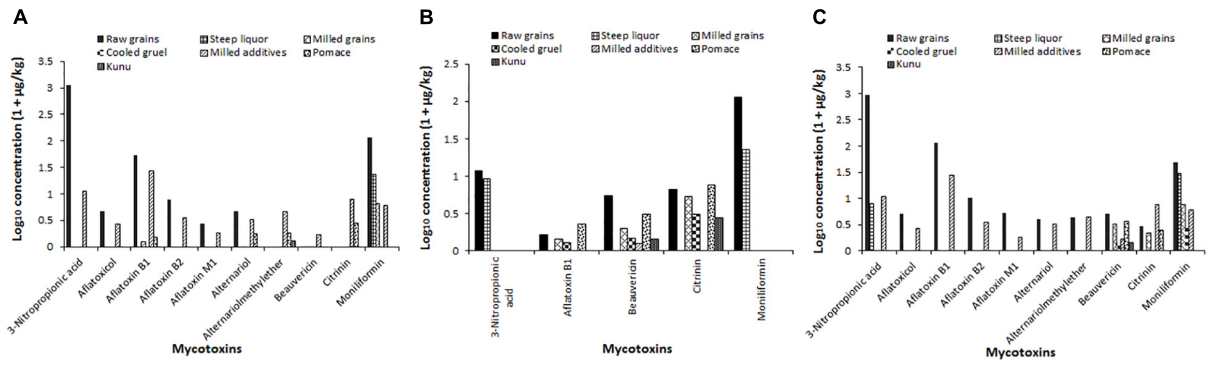

FIGURE 8 | Changes in mycotoxin levels during the processing of three *kunu* formulations (**A**: *kunu* made from millet, white sorghum, peanut, cloves, ginger, and tiger nut; **B**: *kunu* from millet, white sorghum, cloves, ginger, and sweet potato; **C**: *kunu* made from millet, red sorghum, cloves, ginger, and tiger nut).

and development of a climax/streamlined bacterial community observed in the *kunu* product. The associations observed between the bacterial community structure (as indicated by the ordination plots) of processing stages and/or different formulations suggest that, indeed, ingredients and processing steps influence microbial community composition and dynamics.

Taxonomically, the Firmicutes was the dominant phylum during most of the *kunu* processing stages and in the final product. Also, similar to observations of several culture-dependent microbiological studies on *kunu* (Efiuvwevwere and Akona, 1995; Gaffa and Gaffa, 2004; Osuntogun and Aboaba, 2004) and a culture-independent study of retailed *kunu* (Oguntoyinbo et al., 2011), lactic acid bacteria (LAB) were generally dominant during various processing stages of the three *kunu* formulations. With this finding, the dominant role of LAB during *kunu* production becomes more obvious and explanations for the acidic pH of *kunu* reported in earlier studies become clearer (Efiuvwevwere and Akona, 1995; Gaffa et al., 2002; Adelekan et al., 2013). In particular, *Lactobacillus* spp. was the most dominant of the LAB species in most of the fermentation stages of all three formulations and in *kunu* of most formulations. Similar observation for the dominance of *Lactobacillus* spp. in *kunu-zaki* was reported by Inyang and Dabot (1997) and Oguntoyinbo and Narbad (2012). The high occurrence of *Lactobacillus* in these formulations suggests that *Lactobacillus* spp. play a vital role during *kunu* processing and further adds to the growing scientific evidence on the predominance of this bacteria during processing of cereal-based fermented products such as *ogi* (Oguntoyinbo et al., 2011; Okeke et al., 2015), *boza* (Gotcheva et al., 2000), and *bushera* (Muyanja et al., 2003). In addition, *Lactobacillus* spp. are multifunctionally diverse, including many species with probiotic properties (Sanni et al., 2013; Oh et al., 2018) and physiological capabilities for the breakdown of complex polysaccharides in human and animal diets (Barrangou et al., 2006); thus, they could be a reason for some of the nutritional and health benefits generally associated with *kunu* consumption (Omakwu, 1980; Efiuvwevwere and Akona, 1995).

In contrast to the general assertions that the microbial community of *kunu* is dominated by *Lactobacillus*, *Clostridium sensu stricto* (cluster 1) dominated bacterial community of *kunu* product in formulation C. Although species of *Clostridium* have been previously reported in some *kunu* variety (e.g., *kunu-zaki*) (Oguntoyinbo et al., 2011), this is the first report associating the *Clostridium sensu stricto* (cluster 1) group with *kunu* as well as the dominance of the cluster. Until recently, the phylogenetic differentiation (and hence taxonomic classification) of some species within the *Clostridium sensu stricto* from other *Clostridium* species has been unclear, making their functional role in human diet and health, as well as in food fermentations unclear (Collins et al., 1994; Wiegel et al., 2006; Gupta and Gao, 2009; Kaur et al., 2014). A few species in the *Clostridium sensu stricto* cluster are being reclassified and functionally annotated (Wiegel et al., 2006; Gupta and Gao, 2009; Lopetuso et al., 2013; Yutin and Galperin, 2013). In fact, there is growing scientific evidence for their association with the human gut (Magne et al., 2006; Jacquot et al., 2011) and potential applications as hetero-fermenters in food fermentations and industry (Wiegel et al., 2006). Based on the findings of the study, *kunu* is a suitable source for the targeted isolation of species of the *Clostridium sensu stricto* (cluster 1) group. Subsequently, studies may explore the functional and metabolic traits of these species in *kunu* as well as their suitability as starter cultures for *kunu* fermentation or other cereal-based fermented beverages. Similarly, *Acetobacter*, *Gluconobacter*, *Gluconacetobacter* and *Propionibacterium* genera were present during *kunu* processing, especially in the steeped grains. To the best of our knowledge these species have not been previously reported in the microbiology of *kunu* processing. *Acetobacter*, *Gluconobacter* and *Gluconacetobacter* are acetic acid fermenters that have relevant industrial usefulness due to their ability to convert several sugars and alcohols into industrially important organic acids (e.g., vinegar) (Gupta et al., 2001; Sengun and Karabiyikli, 2011; Mamlouk and Gullo, 2013). *Acetobacter* have been identified in other cereal-based fermented foods such as *burukutu*, a fermented traditional sorghum-based *beer* (Oguntoyinbo, 2014), while *Gluconobacter* and *Gluconacetobacter* are associated with acidic beers (Bokulich and Bamforth, 2013; Mamlouk and Gullo, 2013; Spitaels et al., 2014a). Conversely, *Propionibacterium* is mainly associated with dairy and fermented dairy products (Moslemi et al., 2016), with

some species being able to improve the probiotic properties of lactic acid bacteria when incorporated into the vegetables during the production (fermentation) of sauerkraut and other vegetable salads (Babuchowski et al., 1999).

The succession in specific taxa during the processing of *kunu* formulations elucidate functional roles of the dominant species at specific time points as well as their adaptation to the prevailing fermentation conditions. The observation of a streamlined and relatively dominant community of lactic acid bacteria, including species of *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Weissella* and possibly species within the *Clostridium sensu stricto* clusters, toward the end of fermentation and in the final *kunu* product is related to their adaptation to the acidity pH of *kunu* fermentation which is expected as they are the drivers of the biochemical reactions and generators of lactic acid during the process (Gaffa and Gaffa, 2004; Amadou et al., 2011; Oguntoyinbo et al., 2011; Oguntoyinbo, 2014). As discussed earlier, these species are the drivers of the fermentation and are involved in the metabolic interconversions during fermentation as well as instigators of the selective pressure predisposing succession patterns in species/OTU diversity observed during the fermentation stages of *kunu* processing.

In the present study, several phylotypes common to all processing stages and final product of all formulations were identified. These common (or shared) phylotypes may constitute the core bacterial diversity of *kunu* production. The occurrence of these phylotypes throughout the fermentation stages and among all formulations suggest their key roles in *kunu* fermentation and earmark them as potential starter cultures for *kunu* processing. Some of these shared phylotypes, including species of *Enterobacter*, *Gluconacetobacter*, *Lactobacillus*, *Leuconostoc Pediococcus* and *Weissella*, have been previously reported in *kunu* (Amusa and Odunbaku, 2009; Nwachukwu et al., 2010; Oguntoyinbo et al., 2011), lactic acid-fermented west African cereal-based beverages such as *ogi*, *koko,* and *akasa*(Adebayo-tayo and Onilude, 2008; Oguntoyinbo, 2014; Okeke et al., 2015), cocoa fermentation (Nielsen et al., 2007), Spanish farm cheese (Abriouel et al., 2008) and *suan-tsai* (fermented mustard) from Taiwan (Chao et al., 2009). The role of these core microbiota during the fermentation of *kunu* may include the breakdown complex polysaccharides into simpler useful monomers (e.g., mannitol, a low-calorie sugar) (Hemme and Foucaud-Scheunemann, 2004; Ozogul and Hamed, 2018) and generation of aroma compounds that impact characteristic flavor to fermented food products. Some of these species, for example *Leuconostoc* spp., may also synthesize antibacterial compounds such as bacteriocins (Sawa et al., 2010) that help to eliminate pathogenic bacteria during fermentation or in the fermented food product (Ozogul and Hamed, 2018). The possible production of these useful chemical compounds by these species and other known beneficial/probiotic species found in the *kunu* samples, further underlines the health benefits associated with *kunu* consumption. Similarly, *Enterobacter hormaechei* have been previously reported during processing of fermented products such as lambic beer (Spitaels et al., 2014b) and *inyu*, a fermented black bean sauce (Wei et al., 2013). Also, strains of *E. hormaechei* are known to produce food additives and stabilizers such as trehalose (Richards et al., 2002). However, some *E. hormaechei* strains can cause infections in humans (Davin-Regli et al., 1997). Certainly, isolation and whole genome sequencing of some *E. hormaechei* strains associated with *kunu* processing may help elucidate its direct role (beneficial or pathogenicity) in this traditional beverage. As earlier mentioned, *Gluconacetobacter* is an acetic acid bacterium; however, its role in food fermentation is not yet completely understood. It has been associated with traditional balsamic vinegar (Gullo et al., 2006), however, *Gluconacetobacter is* also regarded as a spoilage organism in acidic beer (Bokulich and Bamforth, 2013; Mamlouk and Gullo, 2013). Nonetheless, the role of species of *Gluconacetobacter* during *kunu* processing could be a possible involvement in the oxidation of ethanol to acetic acid (Gomez-Manzo et al., 2010).

It is obvious from this study that the grains used for producing the various formulations of *kunu* were contaminated with different concentrations of several mycotoxins, albeit at levels that were lower than typical levels in maize batches applied to production of traditional fermented beverages in SSA (Okeke et al., 2015; Ogara et al., 2017; Ezekiel et al., 2018). Mycotoxin contamination of millet, sorghum, peanut and tiger nut have been previously reported in Nigeria (Adebajo, 1993; Makun et al., 2007, 2009; Rubert et al., 2013; Oyedele et al., 2017) and the associated public health concerns with such contaminations are well documented. A worrisome finding is the detection of all 10 mycotoxins in tiger nut; this suggests that tiger nut contributes significantly to the levels of mycotoxins during *kunu* processing. The AFB_1 level in tiger nut was about 13 times higher than 2 μg/kg; this is of concern because tiger nut is not only applied as an ingredient during *kunu* production but also popularly consumed as snacks (Belewu and Abodunrin, 2006; Gambo and Da'u, 2014). Thus, extracting the juice from this nut before adding it as ingredients during *kunu* processing and discarding the bran (Olaoye et al., 2015) could be explored to lower the mycotoxin contribution from this ingredient. A safer option is to exclude tiger nut from the list of ingredients intended for *kunu* processing and replace it with coconut or bambara nut. This is in view of the frequent feeding of *kunu* to young children as a complementary beverage in some parts of Nigeria (Olaoye et al., 2015).

Aside quantifying the mycotoxin levels in the ingredients, one main objective of the mycotoxin aspect of this study was to determine whether toxins present in the ingredients used for the various *kunu* formulations will be reduced and to what extent in the final product. This point was clearly established in this study as concentrations of all the mycotoxins reported in this study were reduced to non-detectable levels in the *kunu* irrespective of the formulations, except for AME, BEAU and CIT which retained very minimal levels (<2 μg/kg) in the final product of the various formulations. The findings of this study agree with previous reports that suggested that traditional processing significantly reduced the levels of mycotoxins in fermented foods and the extent of reduction depends on the mycotoxin content of grain inputs (Ezekiel et al., 2015; Okeke et al., 2015, 2018). The data on changes in mycotoxin levels during processing of the *kunu* indicated that steeping of the grains

contributed the most to reducing several of the mycotoxins. This observation may be substantiated by the diverse phylotypes and high number of OTUs observed at this processing stage. During steeping, diverse bacterial genera were observed including those previously reported to be associated with various mechanisms of mycotoxin reduction (Shetty and Jespersen, 2006; Adebo et al., 2015); although in this study we did not search for the degradation products of the mycotoxins that were reduced. A further reduction of the contents of many of the mycotoxins was observed during the fermentation stage, and microbial data suggest community succession and competitive exclusion of species leading toward the development of a climax community. Lactic acid fermentation has been reported to play a significant role in mycotoxin reduction because the microbiota in the fermenting substrate could either bind mycotoxins to their cell wall (Byun and Yoon, 2003; Oluwafemi and Da-Silva, 2009; Huang et al., 2017) or degrade/biotransform them (Shetty and Jespersen, 2006; Adebo et al., 2015). Aside fermentation, simple dilution that occurs during the gelatinization step and sieving were also useful to reduce the levels of some of the mycotoxins as has been previously reported (Okeke et al., 2018).

In summary, our study indicates that the processing of *kunu* is mediated by several core bacterial phylotypes dominated by members of the LAB, although acetic acid bacteria and other bacteria with unknown functional roles constitute the core bacterial community. We have also further established that *kunu* could be a safe beverage for consumption in terms of mycotoxin contents depending on the grain inputs. Thus, we propose the combination of millet, white sorghum, cloves, ginger and sweet potato, which are usually minimally contaminated by mycotoxins, as possible combination of grains and additives for *kunu* production. This proposal will help to minimize mycotoxin exposure in consumers. In addition, the aforementioned ingredients could be supplemented with some underutilized crops (e.g., Bambara nut, coconut, finger millet (*acha*) and sesame seed) that may be less prone to mycotoxins. The extract from tiger nut may be more useful compared to the whole tiger nut; this will further reduce mycotoxin exposure while enhancing nutritional content and imparting additional flavor. Furthermore, it is imperative to source high quality grains and apply simple first-line grain processing interventions (e.g., sorting and floatation washing) to keep mycotoxin levels in the starting materials at the barest minimum in order to encourage a further mycotoxin reduction to non-detectable/safe limits in the final *kunu* product. There is a need to explore the core phylotypes reported in this study by isolating and ascertaining their probiotic properties and suitability as starters for up-scaling of some of the cereal-based traditional fermented foods. This study has further laid the foundation for the potential discovery of novel mycotoxin detoxifiers from *kunu*. Overall, the data provided herein are highly relevant to experts in the food microbiology and safety, food processing and technology, microbial ecology and molecular biology horizons.

AUTHOR CONTRIBUTIONS

CNE conceived the study. CNE, MS, OO, RA, CN, and RK designed the study. IC-O, KA, OO, OA, OE, CNE, and MS performed the experiments in Nigeria, South Africa, and Austria. MS, OE, DvW, CNE, and KA analyzed the data. CNE, CN, RA, JH, CTE, and RK supervised the overall study. KA, OE, DvW, and CNE drafted the manuscript. All authors reviewed and approved the manuscript.

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Production of Naturally γ-Aminobutyric Acid-Enriched Cheese Using the Dairy Strains *Streptococcus thermophilus* 84C and *Lactobacillus brevis* DSM 32386

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The cheese-derived strains *Streptococcus thermophilus* 84C isolated from Nostrano cheese, and *Lactobacillus brevis* DSM 32386 isolated from Traditional Mountain Malga cheese have been previously reported as γ-aminobutyric acid (GABA)-producers *in vitro*. In the present study, the ability of these strains to produce GABA was studied in experimental raw milk cheeses, with the aim to investigate the effect of the culture and the ripening time on the GABA concentration. The cultures used consisted on *S. thermophilus* 84C alone (84C) or in combination with *L. brevis* DSM 32386 (84C-DSM). The control culture was a commercial *S. thermophilus* strain, which was tested alone (CTRL) or in combination with the *L. brevis* DSM 32386 (CTRL-DSM). The pH evolution, microbiological counts, MiSeq Illumina and UHPLC-HQOMS analysis on milk and cheese samples were performed after 2, 9, and 20 days ripening. During the whole ripening, the pH was always under 5.5 in all batches. The concentration of GABA increased during ripening, with the highest content in 84C after 9 days ripening (84 ± 37 mg/kg), in 84C-DSM and CTRL-DSM after 20 days ripening (91 ± 28 and 88 ± 24 mg/kg, respectively). The data obtained support the hypothesis that *S. thermophilus* 84C and *L. brevis* DSM 32386 could be exploited as functional cultures, improving the *in situ* bio-synthesis of GABA during cheese ripening.

Keywords: lactic acid bacteria, model cheese, GABA-enriched cheese, health-promoting bacteria, MiSeq Illumina, Ultra High Performance Liquid Cromatography - Orbitrap Q-Exactive Mass Spectrometry

INTRODUCTION

Milk and dairy products are a good food source of high-quality proteins, minerals and vitamins. Because of the presence of saturated fatty acids, some people believe that dairy foods may be detrimental to health, and limit or exclude dairy foods from their diet, especially if they are overweight or predisposed to cardiovascular disease (Rozenberg et al., 2016). However, observational evidences do not support the hypothesis that dairy fat contributes to obesity,

and its relation with the increase of low-density lipoprotein cholesterol and development of cardio-vascular disease is still unclear (Muehlhoff et al., 2013).

GABA is a non-protein amino acid acting as inhibitory neurotransmitter in the mammalian central nervous system. GABA has proven effects on brain function, preventing or alleviating anxiety, depression, sleeplessness, memory loss; it stimulates the immune system, prevents inflammation processes, hypertension and diabetes, and regulates the energy metabolism (Dhakal et al., 2012). GABA is naturally present in small quantities in many vegetal foods, and at high concentration in fermented products, especially fermented dairy products and soy sauces (Diana et al., 2014). The concentration of GABA detected in 22 different Italian cheese varieties ranged between 0.260 and 391 mg/kg (Siragusa et al., 2007), and between 320 and 6773.5 mg/kg in Cheddar cheese (Wang et al., 2010; Pouliot-Mathieu et al., 2013). Several authors reported the ability of selected lactic acid bacteria (LAB) and bifidobacteria to produce this GABA *in vitro* from the precursor L-glutamic acid (Siragusa et al., 2007; Li and Cao, 2010; Carafa et al., 2015; Franciosi et al., 2015), and investigated the GABA-producing ability of LAB belonging to *S. thermophilus*, *L. plantarum*, *L. paracasei*, *L. delbrueckii subsp. bulgaricus*, and *Lactococcus lactis* in fermented cows' milk and yogurt. The GABA concentration detected in the latter studies ranged between 15 and 5000 mg/kg, even though L-glutamate was added to milk before starting the fermentation process (Siragusa et al., 2007; Lacroix et al., 2013; Nejati et al., 2013; Linares et al., 2016). i.e., we reported the ability of *Lactobacillus brevis* BT66 (hereafter DSM 32386) and *Streptococcus thermophilus* 84C isolated from traditional alpine cheeses, to produce high concentration of GABA (Carafa et al., 2015; Franciosi et al., 2015). In the present study, the hypothesis that both strains (*S. thermophilus* 84C as starter and *L. brevis* DSM 32386 as non-starter) are able to produce GABA in cheese and to increase the concentration of GABA over ripening (2, 9, and 20 days) was tested. The use of raw milk was chosen for enhancing the natural production of free amino acids (including L-glutamate) by the proteolytic activity of milk resident bacteria on the peptides released by the hydrolytic action of the calf rennet on caseins (McSweeney, 2004).

To our knowledge, this is the first research addressed to the production of naturally GABA-enriched raw milk cheese, where the effect of two GABA-producing strains and the ripening time are considered as GABA producing factors.

MATERIALS AND METHODS

Microorganisms and Inoculum Preparation

The strains *S. thermophilus* 84C and *L. brevis* DSM 32386 belong to the culture collection of the Department of Food Quality and Nutrition - Fondazione Edmund Mach (FEM, San Michele all'Adige, TN, Italy). Both strains were isolated from traditional alpine cheeses (Nostrano and Traditional Mountain Malga cheese, respectively) and were phenotypically, genotypically, and technologically characterized in previous studies (Carafa et al., 2015; Franciosi et al., 2015). Both strains showed the ability to produce GABA *in vitro*, which was the reason for interest in the present study.

The commercial *S. thermophilus* was isolated and purified from the commercial lyophilized starter mix culture LYOFAST MOT 086 EE (Sacco, Cadorago, CO, Italy), which did not show any GABA-producing activity *in vitro*, and was selected as control strain. The *S. thermophilus* 84C and the commercial *S. thermophilus* were grown in M17 broth at 45°C, and *L. brevis* DSM 32386 was grown at 30°C in de Man, Rogosa and Sharpe (MRS) broth, acidified to pH 5.5 with lactic acid 5 mol/L. After 48 h incubation, the strains were individually sub-cultured in M17 or MRS (1%, v/v) and grown for 24 h at their optimal temperature. Cultures were harvested by centrifugation at 3,220 g for 10 min at 4°C, the pellets were suspended in Skim Milk (SM, Oxoid; 10:1, v/v; e.g., 400 mL pure culture in 40 mL SM), divided in 1 mL aliquots and frozen in liquid nitrogen. The cellular concentration of each culture was calculated in triplicate by plate counting onto M17 agar for *S. thermophilus* strains and MRS for *L. brevis*. All media were purchased from Oxoid (Milan, Italy).

Experimental Cheese Manufacture

We produced four mini-cheese batches, as follows: the control (CTRL) mini-cheese was manufactured inoculating raw cow's milk with the commercial *S. thermophilus* starter strain; the 84C mini-cheese (84C) was produced inoculating *S. thermophilus* 84C as starter strain; the CTRL-DSM mini-cheese was produced adding the commercial *S. thermophilus* as starter strain and *L. brevis* DSM 32386 as adjunct culture, and the 84C-DSM mini-cheese was produced inoculating *S. thermophilus* 84C as starter and *L. brevis* DSM 32386 as adjunct culture. Both commercial and 84C *S. thermophilus* strains were inoculated into bulk milk at concentration 10^6 CFU/mL, and *L. brevis* DSM 32386 at concentration 10^1 CFU/mL. Experimental cheeses were produced according to the method described by Cipolat-Gotet et al. (2013), with some modifications (**Figure 1**). Raw cow's milk (1.5 L) was heated at 37°C, inoculated with the bacterial mixture and coagulated with the addition of calf rennet (Naturen Plus 215 Hansen, Pacovis Amrein AG, Bern, Switzerland, 215 IMCU/mL). After coagulation, curd was cut into nut-size grains, cooked at 48°C for 10 min, and finally rested at this temperature for 20 more min. After separation, draining and molding, curd was pressed for 30 min at room temperature and salted for 30 min in a saturated brine solution (17°Be, 9°SH/50, 28°C). After two days ripening at 15°C and 85% relative humidity, all batches were stored under-vacuum and ripened for 18 more days. For each batch were produced three wheels to be opened and analysed after 2, 9 and 20 days ripening, and three replicates for each time point were produced in three consecutive weeks for a total of 36 mini-cheese batches. The chemical composition analysis of milk was performed by MilkoScan™ FT6000 (Foss Electric A/S, Hillerød, Danimarca), while the chemical composition of whey and cheese was determined by the FoodScan™ Lab (Foss Electric A/S).

[1]https://docs.tibco.com

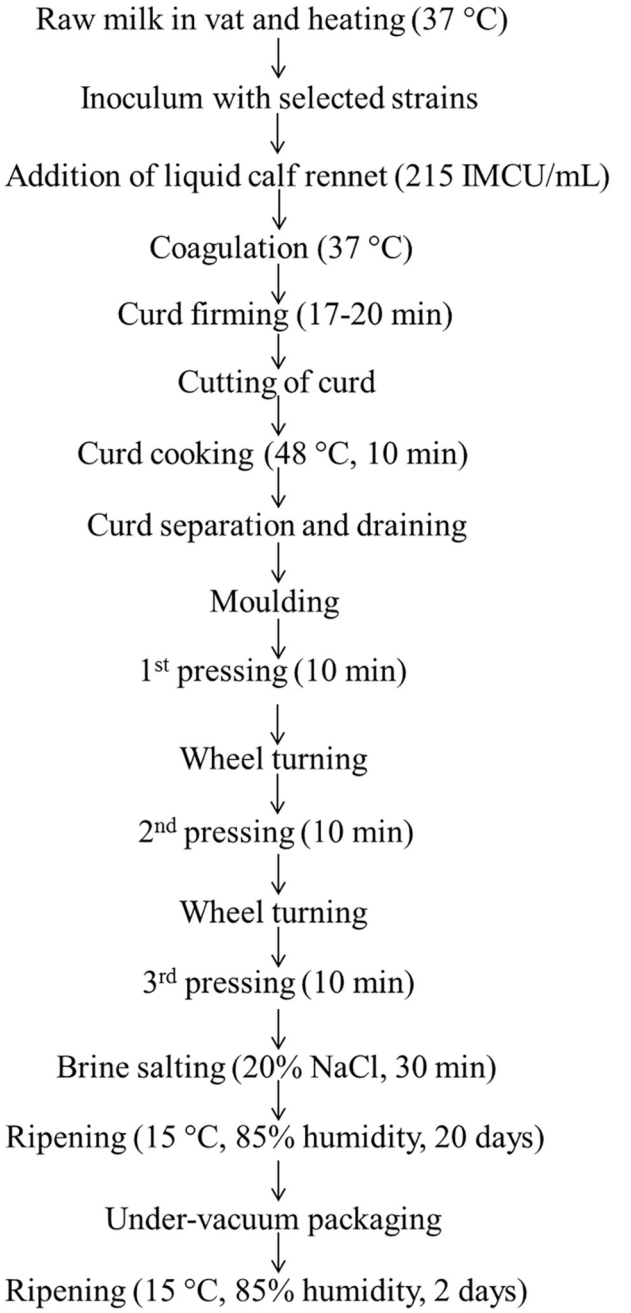

FIGURE 1 | Description of the model cheese-making process.

Microbiological Analysis

Milk, curd, and cheese samples were submitted to microbiological analysis. Four grams of cheese were homogenized with 36 g of sterile Na-citrate 2% (w/w) solution by ULTRA-TURRAX® (IKA® Werke GmbH & Co., KG, Staufen, Germany) for 5 min at 15,000 rpm, inside the microbiological cabinet. Then, milk and cheese samples were decimally diluted and plated onto selective agar media and incubated as follows: MRS agar acidified to pH 5.5 with 5 mol/L lactic acid, anaerobiosis, 48 h at 30°C and 45°C for mesophilic and thermophilic LAB rod-shaped, respectively; M17 agar for 48 h, aerobiosis at 30°C and anaerobiosis at 45°C for mesophilic and thermophilic LAB cocci-shaped, respectively; violet red bile agar (VRBA) for 24 h, aerobiosis at 37°C for coliforms; plate count agar (PCA) with SM (10 g/L, w/v) for 24 h, aerobiosis at 30°C for total bacterial count (TBC). All culture media were purchased from Oxoid.

DNA Extraction and MiSeq Library Preparation

Illumina analysis was performed on all milk and cheese samples. Ten mL of milk and homogenized cheese samples were centrifuged at 3,220 g for 15 min at +4°C. The genomic DNA was extracted from the pellet using the Power Food™ Microbial DNA Isolation Kit (Mo Bio Laboratories Inc., Carlsbad, CA, United States) according to the manufacturer's instructions. All DNA samples were purified by PowerClean DNA Clean-up Kit (Mo Bio Laboratories Inc.) and quantified by Nanodrop8800 Fluorospectrometer (Thermo Scientific, United States).

A 464-nucleotide sequence of the bacterial V3-V4 region (Baker et al., 2003; Claesson et al., 2010) of the 16S rRNA gene (*Escherichia coli* positions 341 to 805) was amplified. Unique barcodes were attached before the forward primers to facilitate the pooling and subsequent differentiation of samples. To prevent preferential sequencing of smallest amplicons, the amplicons were cleaned using the Agencourt AMPure kit (Beckman coulter) according to manufacturer's instructions. The DNA concentration of amplicons was determined using the Quant-iT PicoGreen dsDNA kit (Invitrogen) following the manufacturer's instructions. In order to ensure the absence of primer dimers and to assay the purity, the generated amplicon libraries quality was evaluated by a Bioanalyzer 2100 (Agilent, Palo Alto, CA, United States) using the High Sensitivity DNA Kit (Agilent). Following quantitation, the cleaned amplicons were mixed and combined in equimolar ratios. Pair-end sequencing was carried out at CIBIO (Center of Integrative Biology) – University of Trento (Trento, Italy) using the Illumina MiSeq system (Illumina, United States).

Illumina Data Analysis and Sequences Identification by QIIME2

Raw paired-end FASTQ files were demultiplexed using idemp[2] and imported into Quantitative Insights Into Microbial Ecology (Qiime2, version 2018.2). Sequences were quality filtered, trimmed, de-noised, and merged using DADA2 (Callahan et al., 2016). Chimeric sequences were identified and removed via the consensus method in DADA2. Representative sequences were aligned with MAFFT and used for phylogenetic reconstruction in FastTree using plugins alignment and phylogeny (Price et al., 2009; Katoh and Standley, 2013). Alpha and beta diversity metrics were calculated using the core-diversity plugin within QIIME2 and emperor (Vazquez-Baeza et al., 2013). Taxonomic and compositional analyses were conducted by using plugins feature-classifier[3]. A pre-trained Naive Bayes classifier based on

[2]https://github.com/yhwu/idemp/blob/master/idemp.cpp

[3]https://github.com/qiime2/q2-feature-classifier

the Greengenes 13_8 99% Operational Taxonomic Units (OTUs) database[4], which had been previously trimmed to the V4 region of 16S rDNA, bound by the 341F/805R primer pair, was applied to paired-end sequence reads to generate taxonomy tables.

The data generated by Illumina sequencing were deposited in the NCBI Sequence Read Archive (SRA) and are available under Ac. PRJNA497423.

Quantification of GABA and Glutamate

The amino acid composition of milk and all cheese samples was quantified by Ultra High Performance Liquid Cromatography - Orbitrap Q-Exactive Mass Spectrometry (UHPLC-HQOMS; UHPLC Ultimate 3000RS, ThermoScientific, Rodano, Italy), at the Technology Transfer Centre, Fondazione Edmund Mach (FEM, San Michele all'Adige, Italy).

Two grams of cheese were mixed to 0.4 g of sulfosalicylic acid, suspended in 29.7 mL of perchloric acid (0.01 M) and 0.3 mL of β-glutamic acid (500 mg/L) and homogenized with a ULTRA-TURRAX® for 10 min at 15,000 rpm. The suspensions were submitted to sonication for 30 min and centrifuged at 3,220 g for 20 min. The supernatant was filtered through a 0.22 μm pore size filter (Minisart, Sartorius Stedim Biotech, Goettingen, Germany) and diluted 1:50 with a water/methanol solution (50:50, *v/v*). For milk, 20 grams of sample were mixed to 0.4 g of sulfosalicylic acid, cooled in ice for 10 min and centrifuged at 3,220 g for 10 min. Two grams of supernatant were suspended in 29.7 mL of perchloric acid (0.01 M) and 0.3 mL of β-glutamic acid (500 mg/L) and homogenized. Samples were filtered through a 0.22 μm filter (Minisart, Goettingen) and diluted 1:50 with a water/methanol solution (50:50, *v/v*).

The separation was carried out with formic acid 0.1% (v/v; eluent A; Sigma) and methanol with formic acid 0.1% (v/v; eluent B; Sigma), injecting 5 μL of sample through an Acclaim Trinity P1 column (3 μm particle size, 100 mm × 2.1 mm I.D.; Merk, Germany) at 35°C. The flow rate was set at 0.4 mL/min. The analytical gradient for eluent B was: 1 min at 2%, 4 min at 30%, up to 50% in 0.5 min, to 100% in 0.5 min, held at 100% for 3 min for cleaning, and to 2% for reconditioning in 0.5 min.

Mass spectra were acquired in positive mode through a full MS analysis at mass resolving power 70,000. For ionization, HESI II parameters were set as follow: heated capillary temperature to 330°C; sheath gas flow rate at 40 arbitrary units; auxiliary gas flow rate at 20 arbitrary units; spray voltage at 3.0 kV; auxiliary gas heater temperature at 300°C.

GABA, glutamic acid and the internal standard β-glutamic acid were detected in the extracted ion chromatograms (EICs) corresponding to the protonated molecules $[M+H]^+$ (mass tolerance < 5 ppm) used for quantification, whereas dd-MS/MS spectra compared with those collected from available standards were used for confirmation.

Calibration curves were obtained by plotting the peak area ratio of the quantifier ions ($A_{standard}/A_{internalstandard}$), multiplied by the internal standard concentration, versus the corresponding concentration level. Precision, expressed as Relative Standard Deviation (RSD%) of repeatability was tested injecting the same sample four times within the sequence and must be ≤20% (SANTE guidelines 11945, 2015).

Statistical Analysis

One-way analysis of variance (one-way ANOVA) was performed on all data using STATISTICA data analysis software system, version 13 (TIBCO Software Inc., 2017). Multiple comparison of means was performed using Tukey's test at a p value of <0.05.

RESULTS

Physico-Chemical Characteristics and Microbial Counts of the Experimental Cheeses During the Ripening Time

The physico-chemical characteristics of milk, whey and cheese after 20 days of ripening are shown in **Table 1**. The different batches did not show any significant difference in terms of chemical composition ($p > 0.05$). The pH evolution was recorded in milk and during the cheese making process, as shown in **Figure 2**. The pH value of milk ranged between 6.47 and 6.51 and slightly decreased in curd after extraction with no significant differences among the four batches (6.41–6.47, $p > 0.05$). After 2 days ripening a further reduction of pH was observed in all batches, and cheese samples manufactured with the commercial *S. thermophilus* culture showed a significantly lower pH (5.05) than the cheeses produced with the cultures including

TABLE 1 | Chemical composition of milk, whey and cheese used in the experimental cheese production.

	MV	*SD*
Milk		
Fat, %	3.58	0.08
Protein, %	3.24	0.02
Lactose, %	5.10	0.01
Caseins, %	2.64	0.02
Total solids, %	12.49	0.09
pH	6.50	0.02
SCS[1], units	3.33	0.31
Whey		
Fat, %	0.66	0.07
Protein, %	0.87	0.02
Lactose, %	5.08	0.02
pH	6.46	0.03
Cheese		
Weight, g	119.18	16.29
Fat, %	32.70	4.49
Protein, %	25.71	2.58
Salt, %	2.04	0.07
Total solids, %	69.73	9.94
pH	5.30	0.32
Moisture, %	29.92	5.95
FDM[2], %	47.21	2.30

[1]SCS: somatic cell count = 3+log$_2$(SCC/100). [2]FDM: fat dry matter.

[4]http://greengenes.secondgenome.com/

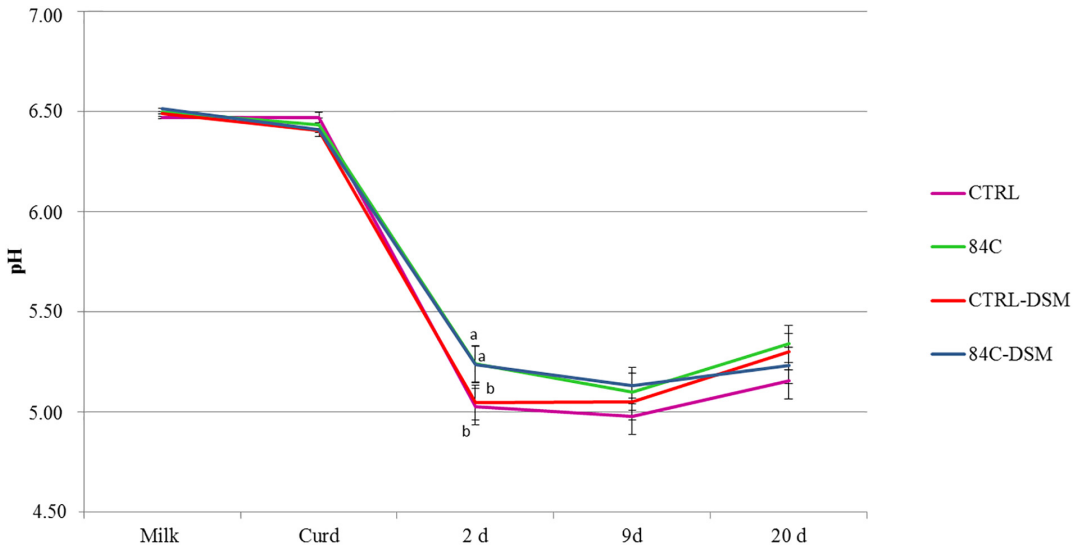

FIGURE 2 | pH dynamic of the experimental cheeses during ripening. Values are expressed as mean value ± standard deviation. Different letters (a–c) indicate a significant difference ($p < 0.05$) among the batches at the same sampling point (Tuckey's test) indicate significant differences.

S. thermophilus 84C (5.24). The lowest pH value was reached after 9 days ripening, with no significant differences ($p > 0.05$) among the four batches (the pH ranged between 4.98 and 5.13). At the end of the ripening period, the pH slightly increased to 5.16–5.34 with no significant differences ($p > 0.05$) among the cheese batches studied.

The total bacterial counts in milk, curd, and experimental cheeses after 2, 9, and 20 days ripening are shown in **Table 2**.

The count of all microbial groups detected in milk increased significantly ($p \leq 0.001$) in the curd with the exception of coliforms. The highest total bacterial counts were observed after 2 days ripening (8.6–9.0 log CFU/g), with no significant differences among the different batches. After 9 and 20 days ripening, total bacterial counts showed a little decreasing trend with no significant differences ($p < 0.05$) among batches.

LAB (M17 and MRS counts) showed a similar trend of total aerobic bacteria throughout the ripening. LAB counts increased for 2 days, and then decreased approximately one logarithmic cycle ($p > 0.05$) until the end of ripening.

The load on M17 30°C showed a trend similar to TBC, without any significant difference among batches. The bacterial concentration on M17 45°C was very low in bulk milk (<1 log CFU/mL) and increased in curd after inoculating the tested strains, ranging between 7.2 ± 0.2 and 7.4 ± 0.3 log CFU/g. After 2 days ripening, the count on M17 45°C increased by about one logarithmic unit ($p > 0.05$) in cheese samples inoculated with the commercial *S. thermophilus*, and was stable in samples containing the autochthonous *S. thermophilus* 84C. Milk contained 4.1 ± 0.27 log CFU/mL bacteria on MRS, which increased by about 3 and 4–5 logarithmic units in curd and cheese, respectively.

Very low counts on VRBA were detected in milk (1.5 ± 0.6 log CFU/mL), which reached the maximum count in cheese after 9 days ripening (range between 4.7 ± 0.3 and 5.3 ± 1.1 log CFU/g).

MiSeq Sequencing Data

The sequences obtained by MiSeq Illumina analysis were first submitted to merging and quality trimming, and 1,741,006 reads were subsequently analyzed. After alignment, the remaining Operational Taxonomic Units (OTUs) were clustered at 3% distance. In order to analyze the bacterial community richness in milk and cheese, the number of OTUs and the diversity Shannon index were determined using QIIME 2 at 97% similarity levels. Data are shown in **Table 3**.

The differences between samples were also evaluated by Bray Curtis phylogenetic metric (**Figure 3**). Cheeses inoculated with the 84C strain (84C and 84C-DSM) clustered together (orange and violet icons) on the third axis and were separated from cheeses inoculated with the commercial *S. thermophilus* starter strain. The PERMANOVA analysis, showed a higher dissimilarity among samples inoculated with different *S. thermophilus* strains ($p < 0.01$) than samples inoculated with or without *L. brevis* DSM 32386.

Microbial Communities Identified in Milk and Cheese Samples

The milk community was mainly constituted by *Moraxellaceae* (35%), *Flavobacteriales* (18%) and other *Gammaproteobacteria* (*Chryseobacterium* and *Pseudomonas*, 16%) (**Figure 4**). *Streptococcaceae* and *Lactobacillaceae* were detected at 8 and 2% relative abundance, respectively.

The cheese microbiota after 2 days ripening (**Figure 4**) showed that *Streptococcaceae* was the most abundant family in all batches (49% relative abundance in CTRL, 51% in CTRL-DSM, 54% in 84C and 56% in 84C-DSM). Furthermore, we observed other dominant microbial families as *Enterococcaceae* (29% in CTRL,

TABLE 2 | Changes in microbial counts of milk and cheese batches throughout ripening (curd, 2, 9, and 20 days).

	TBC		M17 30°C		M17 45°C		MRS 30°C		VRBA	
	MV	***SD***	**MV**	***SD***	**MV**	***SD***	**MV**	***SD***	**MV**	***SD***
Milk	5.4^{a}	0.8	5.0^{a}	0.5	0.4^{a}	1.0	4.1^{a}	0.3	1.5^{a}	0.6
CTRL										
curd	7.6^{b}	0.9	7.1^{b}	0.3	7.2^{b}	0.2	7.2^{b}	0.3	1.6^{a}	0.9
2 days	8.9^{c}	0.1	8.7^{c}	0.2	$8.3^{b,c}$	0.2	9.0^{c}	0.2	$4.9^{b,c}$	1.5
9 days	$8.1^{b,c}$	0.3	9.0^{c}	1.1	$8.0^{b,c}$	0.1	$8.2^{b,c}$	0.4	4.7^{b}	0.6
20 days	8.9^{c}	0.8	$7.7^{b,c}$	1.2	7.1^{b}	0.7	$8.3^{b,c}$	1.6	4.4^{b}	0.9
84C										
curd	7.6^{b}	0.6	7.2^{b}	0.3	7.4^{b}	0.2	7.3^{b}	0.3	2.0^{a}	1.1
2 days	8.6^{c}	0.4	8.6^{c}	0.4	$7.9^{b,c}$	0.1	8.9^{c}	0.3	4.4^{b}	0.4
9 days	7.9^{b}	0.3	$7.7^{b,c}$	0.1	7.1^{b}	0.4	$8.0^{b,c}$	0.2	5.3^{c}	1.1
20 days	$8.3^{b,c}$	0.9	$7.8^{b,c}$	0.3	7.4^{b}	0.3	8.5^{c}	0.9	$5.1^{b,c}$	0.4
CTRL-DSM										
curd	7.3^{b}	0.4	7.3^{b}	0.5	7.2^{b}	0.1	7.1^{b}	0.3	1.9^{a}	1.0
2 days	9.0^{c}	0.4	8.8^{c}	0.4	8.7^{c}	0.3	9.1^{c}	0.4	4.5^{b}	1.6
9 days	$8.4^{b,c}$	0.2	$8.4^{b,c}$	0.3	$7.7^{b,c}$	0.4	$8.2^{b,c}$	0.4	$5.0^{b,c}$	0.6
20 days	$8.0^{b,c}$	0.7	$7.8^{b,c}$	0.7	$7.6^{b,c}$	0.2	$8.3^{b,c}$	1.6	$4.9^{b,c}$	0.5
84C-DSM										
curd	$7.8^{b,c}$	1.1	7.4^{b}	0.9	7.4^{b}	0.3	7.2^{b}	0.3	2.2^{a}	0.8
2 days	8.7^{c}	0.9	8.6^{c}	0.8	$7.6^{b,c}$	0.4	$8.4^{b,c}$	0.8	$4.9^{b,c}$	1.1
9 days	$8.5^{b,c}$	0.2	8.5^{c}	0.2	$7.9^{b,c}$	0.4	$8.4^{b,c}$	0.5	5.1^{c}	0.5
20 days	8.6^{c}	0.5	$7.6^{b,c}$	0.4	7.3^{b}	0.3	8.5^{c}	1.4	$4.9^{b,c}$	0.7

The microbial counts in milk were performed before Streptococcus thermophilus (both commercial and 84C) and Lactobacillus brevis DSM 32386. Control (CTRL) was produced adding the commercial S. thermophilus, 84C cheese (84C) was produced inoculating S. thermophilus 84C; DSM cheese (CTRL-DSM) was produced adding the commercial S. thermophilus and L. brevis DSM 32386, and 84C-DSM cheese (84C-DSM) was produced inoculating S. thermophilus 84C and L. brevis DSM 32386. a, b, c, and d: Different letters in the same column indicate significant statistical differences (Tukey's Test $p < 0.05$).

TABLE 3 | Number of sequences analyzed (N reads), diversity richness (Chao 1), Observed OTUs (OTUs), and diversity index (Shannon) for experimental cheeses and milk samples.

Sample	N reads	Observed OTUs	Chao1	Shannon
Milk	17.167	82	84.05	4.76
CTRL				
2 days	50.419	235	272.97	6.43
9 days	47.698	193	209.70	5.99
20 days	37.841	155	190.83	5.41
84C				
2 days	52.458	288	331.32	6.58
9 days	46.364	311	362.34	6.25
20 days	47.323	261	313.69	6.24
CTRL-DSM				
2 days	51.551	261	296.26	6.49
9 days	46.493	229	260.42	6.45
20 days	48.336	239	279.26	6.10
84C-DSM				
2 days	47.072	219	248.95	5.75
9 days	44.886	237	275.37	5.94
20 days	42.727	196	226.01	5.47

27% in CTRL-DSM, 15% in 84C and 11% in 84C-DSM) and *Enterobacteriaceae* (18% in CTRL, 27% in CTRL-DSM, 23% in 84C and 25% in 84C-DSM).

After 9 days ripening the microbial composition did not show significant change, with the exception of cheese samples inoculated with *L. brevis* DSM 32386 strain where *Lactobacillaceae* were detected at 1.9 (CTRL-DSM) and 0.9% (84C-DSM), while in the other cheeses this microbial family was under 0.01% relative abundance.

At the end of ripening there was a change of the microbiota, in particular in cheese samples inoculated with the commercial *S. thermophilus* starter. We observed a significant decrease in *Streptococcaceae* from 45 to 30% and an increase of *Enterococcaceae* from 27 to 47% that became the dominant microbial family in this cheese. *Enterobacteriaceae,* ranging between 11 and 14%, decreased in all batches (**Figure 4**).

GABA Detection in Milk and Cheese Samples

The GABA concentrations are shown in **Figure 5A**. The UHPLC-HQOMS analysis on milk and cheese samples showed an increase of GABA during ripening, with the highest content in 84C after 9 days ripening (84 ± 37 mg/kg), in 84C-DSM and CTRL-DSM after 20 days ripening (91 ± 28 and 88 ± 24 mg/kg, respectively). Milk contained 1.9 ± 0.9 mg/kg GABA, which slightly increased ($p > 0.05$) in all cheese batches after 2 days ripening. The first significant change was observed in CTRL-DSM, 84C-DSM and 84C samples after 9 days ripening, which contained significantly more GABA (63 ± 19, 47 ± 22, and 84 ± 37 mg/kg, respectively)

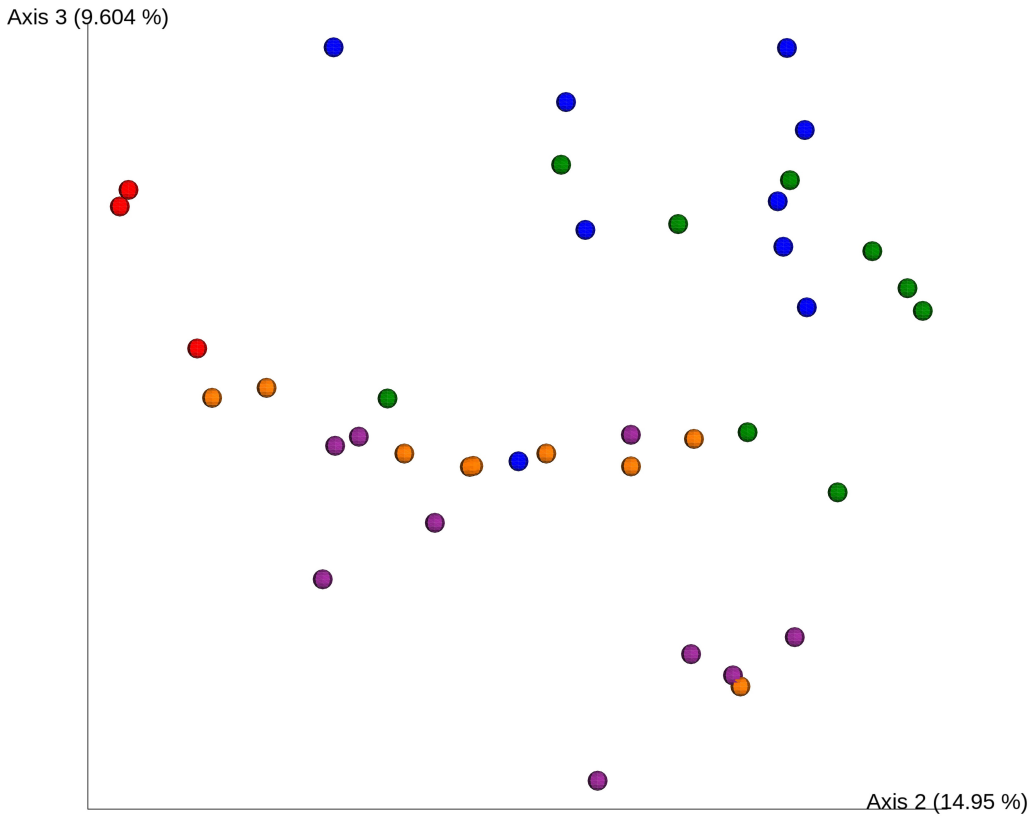

FIGURE 3 | Bray Curtis analysis of milk and cheese samples after 2, 9, and 20 days ripening. Red dots show milk samples (n = 3), the blue dots show the CTRL cheese (n = 9), green dots show CTRL-DSM cheese (n = 9), purple dots show 84C cheese (n = 9), and orange dots show 84C-DSM cheese samples (n = 9).

than CTRL (9.1 ± 7.8 mg/kg, $p < 0.05$). At the end of ripening (20 days), 84C-DSM cheese had the highest level of GABA (91 ± 22 mg/Kg), which was not significantly different from CTRL-DSM (88 ± 20 mg/Kg) and 84C (73 ± 21 mg/Kg), but significantly higher than CTRL cheese (11 ± 10 mg/Kg).

Glutamic acid was also detected in milk (36 ± 7 mg/kg) and cheese during ripening (**Figure 5B**). After 2 days ripening the content of glutamate ranged between 36 ± 10 and 62 ± 39 mg/kg, and increased after 9 days ripening between 47 ± 17 and 117 ± 8 mg/kg. We observed a significantly higher content of glutamate in 84C-DSM after 9 days ripening (117 ± 8 mg/kg), compared to CTRL (64 ± 37 mg/kg) and 84C batches (47 ± 17 mg/kg). At the end of ripening, the concentration of glutamate fluctuated between 48 ± 38 and 114 ± 40 mg/kg glutamate, with no significant differences between batches.

DISCUSSION

In the present study, the use of raw milk was chosen in order to exploit the ability of the resident microbiota to release naturally free amino acids (including L-glutamate) from the peptides originated from the hydrolytic action of the calf rennet on caseins (McSweeney, 2004).

The experimental mini-cheese batches were produced in the laboratory, developing a cheese making protocol which could simulate as better the technology of alpine raw milk cheeses in very small vats. The processing of small volumes of milk (1.5 L) has the advantage that up to 20 cheese batches/day can be produced using the same milk, but the disadvantage that the wheels are very small, and the high surface/volume ratio makes the cheese subjected to a fast drying process. For this reason the ripening process was conducted under-vacuum in order to mimic the anaerobic conditions of the cheese core and avoiding the formation of thick rinds.

All cheese batches had similar chemical composition at the end of ripening, suggesting that the tested strains operated similarly.

The pH evolution was monitored during the cheese making process because the role of pH is not only related to the outcome of the fermentation, but also to the production of GABA. The commercial *S. thermophilus* reduced the pH of cheese in a shorter time than the strain *S. thermophilus* 84C within 2 days ripening. Conversely, any of the tested strain (both alone and in combination with *L. brevis* DSM 32386) had not statistically significant effect ($p > 0.05$) on pH after 9 and 20 days ripening.

With regards to the microbiological concentration, we observed an increase of the plate counts in curd samples for all microbial batches, which is probably due to the physical retention of bacteria in the curd and to the multiplication of the inoculated

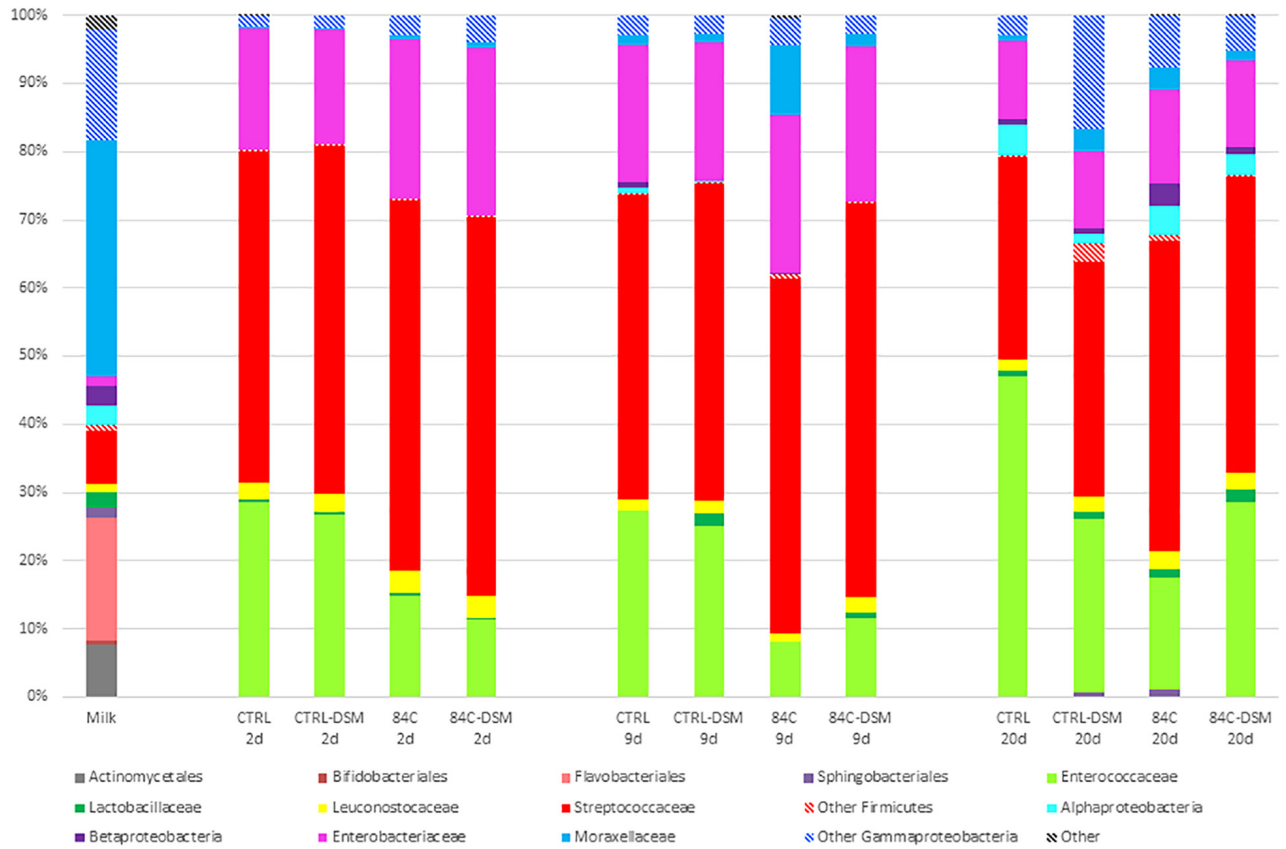

FIGURE 4 | Relative abundance of the microbial taxonomic groups detected in milk and cheese samples after 2, 9, and 20 days ripening. Data are expressed in percentage.

strains during the coagulation phase. The addition of *L. brevis* DSM 32386 was not detectable on MRS 30°C, and it might be related to the initial concentration of lactobacilli in raw milk (4.1 log CFU/mL), which was similar to the concentration of the inoculated *L. brevis*.

Analyzing the sequences obtained by MiSeq Illumina, we obtained much information looking at the quality of the reads and alpha and beta-diversity. The rarefaction analysis based on OTUs at 97% similarity showed approximation to asymptote, and we concluded that the recovered sequences represented properly the diversity of the bacterial communities in the 39 samples.

Based on the OTUs number and the Shannon and Chao indexes, there was no difference related to the inoculated strain. There was a decreasing richness and diversity of microbial population with fermentation time. In fact, the number of OTUs and Chao and Shannon indexes were higher in samples collected after 2 than after 20 days but these differences were not significant. The presence or absence of *L. brevis* DSM 32386 did not significant influence the Bray Curtis index, as also confirmed by PERMANOVA analysis.

Both alpha- and beta-diversity analysis suggested that *L. brevis* had no effect on the microbiota of experimental cheeses at the concentration used in this work. Furthermore, the differences in cheese microbiota are likely due by the starter used even if belonging to the same species and added at the same concentration.

The presence in milk of *Chryseobacterium* and *Pseudomonas* which are gram-negative spoilage bacteria was not unexpected because they are usually present in raw milk and are able to grow at low temperature during refrigerated storage (Quigley et al., 2013; von Neubeck et al., 2015). Conversely, *Streptococcaceae* and *Lactobacillaceae*, which are involved in dairy fermentation and in the determination of organoleptic, flavor and texture properties of the final product (Quigley et al., 2013) are totally desired. The dominance of *Streptococcaceae* after 2 days ripening suggested that bacteria belonging to this family (including the added *S. thermophilus* strains) started the fermentation process. After 9 days ripening *Lactobacillaceae* were significantly higher in CTRL-DSM and 84C-DSM cheeses, suggesting that the sequences identified as *Lactobacillaceae* corresponded to the inoculated *L. brevis* DSM 32386. Taxonomy data at the end of ripening suggested that only the commercial *S. thermophilus* starter strain influenced significantly the cheese microbiota and that all the tested strains had a decreasing effect on the relative abundance of *Enterobacteriaceae*.

During cheese ripening, L-glutamate which is naturally present in caseins (Zoon and Allersma, 1996) might be

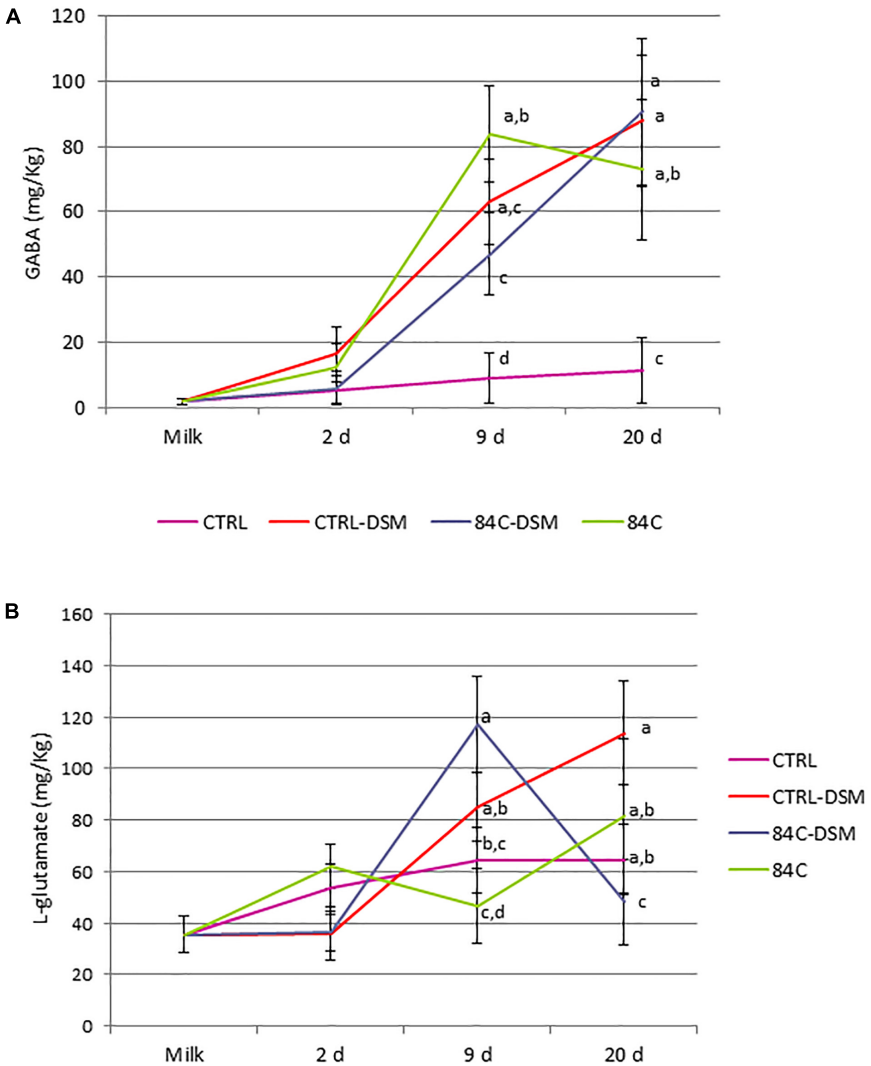

FIGURE 5 | GABA **(A)** and L-glutamic acid **(B)** concentration detected in milk and cheese samples after 2, 9, and 20 days ripening. The mean value ± standard deviation of both amino acids is expressed in mg/kg. Different letters (a, b, c) indicate a significant difference ($p < 0.05$) among the batches at the same sampling point (Tuckey's test) indicate significant differences.

released from caseins proteolysis. In cheese, the ripening period can facilitate this process, and L-glutamate can be converted to GABA by GABA-producing bacteria. For this reason we chose cheese as GABA-carrier food, even though many factors play a key role on the final content of GABA, like as the cheese-making process, the presence of starter or adjunct cultures and the ripening conditions (Siragusa et al., 2007). In order to determine when GABA is produced and if it persist during ripening, we decided to monitor the content of GABA at different ripening stages. We detected very interesting variations of GABA and glutamate between the four batches. High production of GABA by fermentation is correlated to the activity of glutamic acid decarboxylase (GAD) but also on the inhibition of GABA-decomposing enzymes. GABA transaminase (GABA-T) promotes the reversible conversion of GABA to succinic semi-aldehyde using either pyruvate-dependent GABA transaminase (GABA-TP) or α-ketoglutarate-dependent GABA transaminase (GABA-TK), and succinic semi-aldehyde dehydrogenase catalyzes the reversible conversion of succinic semi-aldehyde to succinate (Takayama and Ezura, 2015).

Overlapping GABA and glutamate concentration we hypothesized that the presence of *L. brevis* DSM 32386 enhanced the release of glutamate from caseins between day 2 and day 20, and the consequent conversion of L-glutamate to GABA. In fact, the increase of glutamate in CTRL-DSM samples after 9 and 20 days ripening corresponded to the increase of GABA. These results suggested that glutamate was gradually consumed over its release and converted to GABA.

By contrast, *S. thermophilus* 84C showed a different trend; the GABA production significantly ($p < 0.05$) increased in 84C cheese samples between 2 and 9 days ripening, but it was related

to a slight reduction of L-glutamate. Afterwards, between day 9 and day 20, we observed a slight decrease of GABA and an increase of L-glutamate 84C samples. These data suggest that the strain *S. thermophilus* 84C is likely not involved in the release of caseins, and that GABA is metabolized to succinate and reconverted to L-glutamate after reentering the Krebs cycle. This hypothesis is supported by 84C-DSM cheese samples, where we observed a constant increase of GABA over all the ripening period and a strong and significant decrease ($p < 0.05$) of L-glutamate between 9 and 20 days ripening. Since we did not perform any metatranscriptome or gene expression analysis, we are not able to confirm this hypothesis, and more investigation needs to be done.

Several studies demonstrated that GABA can reduce high blood pressure in animals and humans, as reviewed by Diana et al. (2014). In human intervention trials, a 12 weeks treatment with 100 ml of fermented milk containing between 10 and 12 mg of GABA, or 50 g of GABA-enriched cheese containing 16 mg of GABA, decreased blood pressure in hypertensive patients (Inoue et al., 2003; Pouliot-Mathieu et al., 2013). The experimental cheese produced in the present study contained about 117 mg/kg, thus a portion of 100 g would provide about 11.7 mg of GABA, covering the total intake needed in order to detect positive effects on human health.

The strains tested in the present study would be useful for the production of bioactive traditional alpine cheeses, which are mostly produced from raw milk. Cheese-makers from Trentino Alps are strongly faithful to tradition, and they are not willing to make any change that could compromise or modify the biodiversity and sensory characteristics of their cheeses. Whereas, they usually agree when they are suggested to use autochthonous strains that are able to improve the product. In this contest, a naturally GABA enriched cheese produced from raw alpine milk would satisfy the needs of both producers and consumers, who increasingly ask for healthy foods, and the marketing of local dairy products would rise. On the other hand, the utilization of raw milk might affect the production of GABA in the experimental cheeses, through a synergistic effect between the indigenous microbiota and the tested GABA-producing strains. From an industrial point of view, the use of pasteurized milk would facilitate the optimization of the GABA production in cheese, the standardization and reproducibility of results. For this reason, a new project aiming to develop a naturally GABA-enriched cheese from pasteurized milk is in progress.

CONCLUSION

The aim of this study was to demonstrate that GABA is produced in raw milk cheese and accumulates during ripening. The data obtained showed that after 20 days ripening, 84C-DSM, CTRL-DSM and 84C cheeses had a GABA content significantly higher than CTRL cheese, confirming the hypothesis that *S. thermophilus* 84C and *L. brevis* DSM 32386 could be exploited as bio-functional cultures, facilitating the *in situ* bio-synthesis of GABA during cheese ripening, and providing an option to replace chemical GABA with natural GABA.

We need to take into account that the production of experimental mini-cheeses had some limitations, which are the size of the wheels and the ripening conditions. The ripening took place in aerobic condition only during the first two days ripening and from day 3 to day 20 under-vacuum. The small size allowed producing many cheese batches within the same cheese-making process, while the storage and ripening under-vacuum had the double purpose of miming the anaerobic conditions of the cheese core and avoiding the total drying of cheese. However, this is the first study addressed to the manufacture of GABA-enriched raw milk cheese by LAB and more trials will be carried out in order to optimize the production and the accumulation of GABA in cheese and reduce as much as possible its degradation.

DATA AVAILABILITY

Most of the relevant data are included within the manuscript. Any additional raw data supporting the conclusions will be made available by the authors on request, without undue reservation, to any qualified researcher.

AUTHOR CONTRIBUTIONS

EF and GB devised the study. IC and EF drafted the manuscript. EF, IC, GS, and TN performed the experiments. EF, GB, RL, and KT provided resources and intellectual input that supported the study.

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ORIGINAL RESEARCH
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A Preliminary Report on the Use of the Design of Experiments for the Production of a Synbiotic Yogurt Supplemented With Gluten Friendly™ Flour and *Bifidobacterium infantis*

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The main goal of this paper was to design a synbiotic yogurt containing *Bifidobacterium infantis* and Gluten Friendly Flour™; the proposed approach relies upon milk fermentation through the classical starter of yogurt (*Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*) to avoid a strong production of acetic acid by bifidobacterial and inoculum of *B. infantis* after the fermentation. The research was divided in 3 steps. The aim of the first step was the optimization of fermentation kinetic by *L. delbureckii* and *S. thermophilus*, by combining the amount of flour (either Gluten Friendly Flour-GF- or Control Flour-CF) in milk, temperature and inoculum level; the factors were combined through a mixture design. As a result of this step, the best combination was pointed out: flour at 2.5 g/l; *L. delbrueckii* subsp. *bulgaricus* at 6 log cfu/ml; temperature at 37–40°C. The goal of the second step was to study the effect of flour (2.5 g/l) on the viability of *B. infantis*. GF prolonged the viability of the probiotic for 14 days. In the last step, a synbiotic yogurt, supplemented with GF and fermented with *L. delbureckii* and *S. thermophilus*, and then inoculated with *B. infantis*, was produced. The product was stored at 8 and 15°C. A positive effect of GF was found at 15°C, with *B. infantis* at 7.0 log cfu/g in GF sample and 5.5.5.7 log cfu/g in CF sample.

Keywords: synbiotic, gluten friendly, *Bifidobacterium* spp., centroid, desirability, multifactorial analysis of variance

INTRODUCTION

Nowadays consumers believe that foods can significantly contribute to their health and wellness (Mollet and Rowland, 2005; Hassani et al., 2016) and functional products meet this increasing demand of healthy diet, as they satisfy hunger and provide essential nutrients, but they can also prevent diseases and promote physical and mental wellness (Menrad, 2003).

Dairy products are functional foods since they are sources of calcium and they are vehicles of probiotic microorganisms (Plessas et al., 2012). Fermented milks are the classical carriers for probiotic microorganisms, due to the high consumers' preference for these beverages and for their proven health benefits (Batista et al., 2015). The survival of probiotics in fermented milks is a challenge, due to the influence of several intrinsic and extrinsic factors (Granato et al., 2010). There are several regulatory requirements for probiotic yogurts; the most important one is that the microorganisms are alive at the time of use, and the viable count must be at the level proven to confer a health benefit, i.e., at least 7 logcfu/g (Rosburg et al., 2010).

Bifidobacterium is a member of intestinal microbiota of mammals and is among the first microbial colonizers of the intestines of newborns; it plays key roles in the development of their physiology, including maturation of the immune system and use of dietary components. Some *Bifidobacterium* strains are probiotic microorganisms because of their beneficial effects (Hidalgo-Cantabrana et al., 2017).

Probiotic microorganisms can be added to fermented milks using different methods. They might be added as non-fermenting microorganisms after fermentation and cooling with the sole aim of being delivered through gastrointestinal tract (nonstarter probiotics), or be added as starter cultures to ferment milk base (starter probiotics) (Mohammadi et al., 2012).

Bifidobacteria were used to produce fermented milks, but they showed lower performances than lactobacilli, thus hindering their possible applications (Prasanna et al., 2012b). Moreover, their growth and viability are challenges, as they require longer fermentation times, strict anaerobic conditions and a low redox potential (Prasanna et al., 2012a). In addition, acetic acid and the lack of acetaldehyde could lead to off-flavors and off-odoursc(Mohammadi et al., 2012). The *Bifidus* fermentation pathway produces acetic acid and lactic acid at a ratio 3:2, and the production of acetic acid at high levels could be responsible of a "vinegary taint" (Mortazavian et al., 2011). Therefore, it is not advisable to use bifidobacteria as the only starter for fermented milk, while they should be combined with other lactic acid bacteria.

The most popular way is to add the probiotic microorganisms together with adjunct lactic starter cultures (mainly, traditional yogurt bacteria: *Lactobacillus delbrueckii* ssp. *bulgaricus* and *Streptococcus thermophilus*), since the adequate fermentation in milk rarely occurs by probiotics alone.

Lamacchia and coworkers (Lamacchia et al., 2013, 2015) designed a new and innovative method (Gluten Friendly^{TM})(PCT/IB2013/000797) for grain seeds to reduce the immunogenicity of gluten *in vitro*; this approach is based on the use of microwave on hydrated wheat kernels. Microwaves induce structural modifications to endosperm content, and as a result, the immunogenicity is significantly reduced (Lamacchia et al., 2016). An additional effect of this treatment is the functional effect of Gluten Friendly^{TM} on microorganisms, like the antimicrobial activity toward *Salmonella* sp., and the prolonged the viability of *Lactobacillus acidophilus* La5 in a model system (Bevilacqua et al., 2016) and in a fermented milk during a refrigerated storage (Speranza et al., 2018). Moreover, Gluten Friendly^{TM} flour or bread promoted a partial restoration of gut microbiota of coeliac subjects both in batch systems and in a gut model system (Bevilacqua et al., 2016; Costabile et al., 2017), as well as the increase of mucin production (Lamacchia et al., 2018).

The aim of this research was to design a yogurt, fermented by *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*, and supplemented with Gluten Friendly Flour and *Bifidobacterium infantis*. The research was divided into three different steps: (i) evaluation of the effects of Gluten Friendly flour on the acidification kinetics of the starter strains; (ii) study of the effects of flour on the viability of *B. infantis*; (iii) validation at laboratory level and production of a yogurt, supplemented with both flour and *B. infantis*.

MATERIALS AND METHODS

Microorganisms and Raw Materials

Bifidobacterium infantis Bb02 was purchased from Chr. Hansen (Hørsholm, Denmark); the strain was grown in MRS broth (Oxoid, Basingstoke, UK) supplemented with 0.5% cysteine (Sigma-Aldrich, Milan, Italy) at 37°C for 24 h under anaerobic conditions. *Lactobacillus delbrueckii* subsp. *bulgaricus* DSM 20081 was purchased from the German Collection of Microorganisms (DSMZ, Braunschweig, Germany) while *Streptococcus thermophilus* was purchased from Clerici-Sacco Group (Como, Italy). *L. bulgaricus* and *S. thermophilus* were grown in MRS broth (37°C for 24 h; anaerobiosis). Before each experiment the strains were centrifuged at 4,000 g for 10 min and suspended in sterile distilled water (9 log cfu/ml).

Gluten Friendly Flour (GF) (Lamacchia et al., 2015) was prepared as described by Speranza et al. (2018). Gluten Friendly^{TM} method is an innovative approach to treat wheat grains. Wheat grains was dampened until reaching 15–18% humidity, and then heated through microwaves (approximately 1 min between 1,000 and 750 watts); microwave heating was followed by a slow evaporation of the water. This combination (heating/evaporation) was repeated until reaching a final temperature of 80–90°C, and a moisture degree of 13–13.5%. After the treatment, the kernels were cooled and dried at 24°C for 12–24 h. A flour not treated with microwaves was used as control (CF).

Fresh whole pasteurized homogenized cow's milk (3.35 g/l protein; 5.0 g/l carbohydrates; 3.75 g/l fats) was used. Before each experiment, lactobacilli, lactococci and spoiling microorganisms (enterobacteria, spore formers, and pseudomonads) were checked if they were below the detection limit (standard plate count).

Evaluation of Acidification Kinetics of *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*

L. bulgaricus and *S. thermophilus* were inoculated to 4, 6, and 8 log cfu/ml, depending on the experimental plan, in 15 ml pasteurized milk supplemented with either GF or CF (0.0–2.5–5.0 g/l); the samples were incubated at 30–45°C for 72 h and the

pH was measured after 4, 6, 15, 18, 21, 24, 28, 30, 39, 48, and 72 h. Flour, inoculum and temperature were combined through a simplex centroid. The combinations of the design are in **Table 1**.

Four different designs were run: (a) *L. bulgaricus*+GF; (b) *L. bulgaricus*+CF; (c) *S. thermophilus*+GF; (d) *S. thermophilus*+CF.

The experiments were performed on two different samples; for each sample the measurements were repeated twice. The results were modeled as acidification (ΔpH) through the lag-exponential model (Van Gerwen and Zwietering, 1998; Baty and Delignette-Muller, 2004; Delignette-Muller et al., 2006), modified by Speranza et al. (2018):

$$\Delta pH = \begin{cases} 0 & t \le \alpha \\ \Delta pH_{max} - \log\{1 + (10^{\Delta pH}{}_{max} - 1) \\ \quad * \exp[-d_{max}(t - \alpha)]\} & t > \alpha \end{cases}$$

Or

$$\Delta pH = \Delta pH_{max} - \log\{1 + (10^{\Delta pHmax} - 1) * \exp(-d_{max}\, t)\}$$

where:

α is the time before the beginning of the acidification kinetic (h); d_{max} is the maximal acidification rate (1/h); ΔpH_{max} is the maximum level of acidification.

TABLE 1 | Combinations of the centroid.

	Coded values			Effective values		
Samples	**Inoculum**	**Flour**	**Temperature**	**Inoculum (log cfu/ml)**	**Flour (g)**	**Temperature (°C)**
A	1	0	0	8	0	30
B	0	1	0	4	5	30
C	0	0	1	4	0	45
D	0.5	0.5	0	6	2.5	30
E	0.5	0	0.5	6	0	37.5
F	0	0.5	0.5	4	2.5	37.5

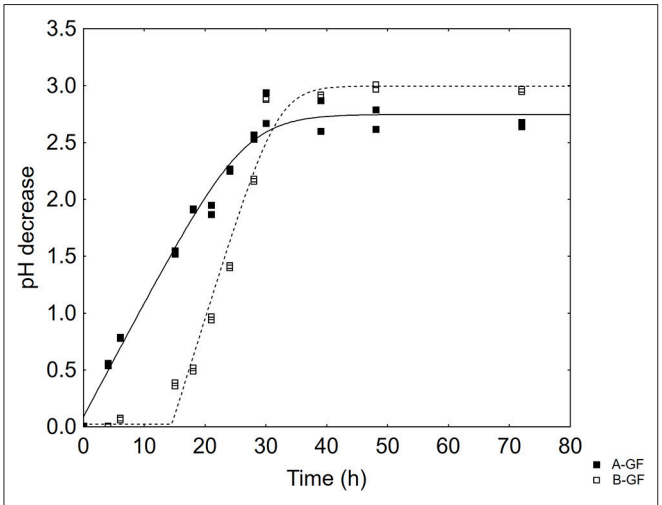

FIGURE 1 | Acidification of *L. delbrueckii* subsp. *bulgaricus* in two selected combinations of the design. The lines represent the best fit through the lag-exponential equation. GF, Gluten Friendly Flour.

Then, ΔpH_{max} and α were analyzed through the theory of the Design of the Experiments, to assess the significant effect of temperature, inoculum and flour as well as of their interactions. The analysis was done through the software Statistica for Windows (Statsoft, Ulsa, Okhla). The significance of the whole approach, as well as of each variable, was evaluated through the adjusted regression coefficients, the mean square residual, and the Fisher test ($P < 0.05$). The effect of each factor of the design (inoculum, temperature, flour) was also evaluated through the individual desirability functions, estimated as follows:

$$d = \begin{cases} 0, & y \le y_{min} \\ (y - y_{min})/(y_{max} - y_{min}) & y_{min} < y < y_{max} \\ 1, & y \ge y_{max} \end{cases}$$

Where y_{min} and y_{max} are the minimum and maximum values of the dependent variable, respectively.

The desirability is a dimensionless parameter ranging from 0 to 1; it i the answer to the question: how much desired is a result? Generally, desirability is set to 0 for the lowest or the worst value of the dependent variable and 1 for the maximum or the most desired value (in this paper it was set to 0 for the lowest value of ΔpH_{max} and α and 1 for their maximal values). It is a mathematical function estimated by focusing on the effect of each factor *per* time, while the other variables/factors of the design were set to a constant value. For this research, the desirability profiles were built by setting the variables to the coded level 0.33 (inoculum to 5.3 log cfu/ml, temperature to 35°C, flour to 1.65 g/l).

The desirability profile has two main applications: (a) to study the effect of each variable, without the influence of possible interactive or synergistic effects; (b) to compare variables or parameters with different units.

Effect of GF on the Viability of *Bifidobacterium infantis*

Saline solution was supplemented with either GF or CF (2.5 g/l) and inoculated with *B. infantis* to 7.70 log cfu/ml. The samples were stored at 4 and 37°C and analyzed after 1, 2, 4, 9, 14, and 21 days by pour plating (MRS Agar+cysteine, 37°C for 48–72 h under anaerobic conditions).

Saline solution without flour but inoculated with *B. infantis* was used as control. The experiments were performed twice on two independent samples.

Yogurt

Three different productions of yogurt were prepared as follows: milk supplemented with Gluten Friendly flour (2.5 g/l) (GF), control flour (2.5 g/l) (CF) or without flour (CNT, control). Then, the three batches were inoculated with *L. delbrueckii* subsp. *bulgaricus* (6 log cfu/ml) and *S. thermophilus* (6 log cfu/ml) and incubated at 40°C and let to ferment until a pH 4.0 was attained.

After the fermentation, the batches were inoculated with *B. infantis* (6.5–6.8 log cfu/ml), divided in samples of 25–30 g in sterile 50 ml-tubes and stored at 8 and 15°C. Immediately after the inoculation of bifidobacteria and after 3, 7, 10, 14,

and 17 days microbiological and chemico-physical analyses were done.

For microbiological analyses, the following media were used: MRS Agar and M17 Agar+lactose, incubated at 30°C or 44°C for 48–72 h for mesophilic and thermophilic lactobacilli and lactococci; MRSAgar+cysteine+NNLP antibiotic solution (**N**eomycin-**N**alidixic acid-**L**ithium chloride-**P**aromomycine: 2 g/l neomycin sulphate, 4 g/lparomomycine sulphate, 0.3 g/l nalidixic acid, and 60 g/l lithium chloride; all reagents were purchased from Sigma-Aldrich), incubated at 37°C for 48–72 h for bifidobacteria; Slanetz/Bartley Agar incubated at 37°C for 48 h, for enterococci; Plate Count Agar (PCA) incubated at 5 C for a week or 32°C for 48 h for psychrotrophic bacteria and mesophilic bacteria, respectively; Baird-Parker agar base, with egg yolk tellurite emulsion, incubated at 37°C for 48 h for staphylococci and *Micrococcaceae*; Pseudomonas Agar Base (PAB) with CFC Selective Supplement incubated at 25°C for 48 h for *Pseudomonas* spp.; Violet Red Bile Glucose Agar (VRBGA), incubated at 37°C for 24 h for *Enterobacteriaceae;* Violet Red Bile Agar (VRBA) incubated at 37°C or 42°C for 18–24 h for total and fecal coliforms, respectively; Sabouraud dextrose agar, supplemented with chloramphenicol (0.1 g/l) (C. Erba, Milan, Italy), incubated at 25°C for 48 h or 5 days, for yeasts and molds, respectively. All the media and the supplements were from Oxoid.

The viable count of *Bifidobacterium* was confirmed by a random isolation of some colonies, and microscopic examination.

pH was measured by a pH-meter and a_w through an AQUALAB CX-2 (Decagon Device, Pullman, WA, USA). Color was evaluated by a colorimeter Chroma Meter (Minolta, Japan) by measuring CIE L* (lightness), a* (redness), and b* (yellowness) values.

The experiments were repeated twice on two independent samples. The results of *Bifidobacterium* were analyzed by a

TABLE 2 | Standardized effects of inoculum, flour and temperature on acidification (ΔpH_{max}) of *L. delbrueckii* subsp. *bulgaricus* (Lb) and *S. thermophilus* (St) and on the time before the beginning of acidification (α) of *L. bulgaricus*. R^2_{ad}, adjusted regression coefficient.

	ΔpH_{max} Lb	α Lb	ΔpH_{max} St
GLUTEN FRIENDLY FLOUR			
Inoculum	68.97	–*	12.43
Flour	75.24	20.88	12.39
Temperature	53.95	4.93	–
Inoculum by flour	−5.51	4.67	−3.44
Inoculum by temp.	–	–	–
Temp. by flour	6.14	−2.64	2.33
R^2_{ad}	0.947	0.950	0.835
CONTROL FLOUR			
Inoculum	70.09	–	6.81
Flour	78.88	19.98	7.45
Temperature	59.69	4.88	–
Inoculum by flour	5.83	6.82	–
Inoculum by temp.	–	–	–
Temp. by flour	10.39	−4.44	3.24
R^2_{ad}	0.961	0.956	0.645

**Not significant.*

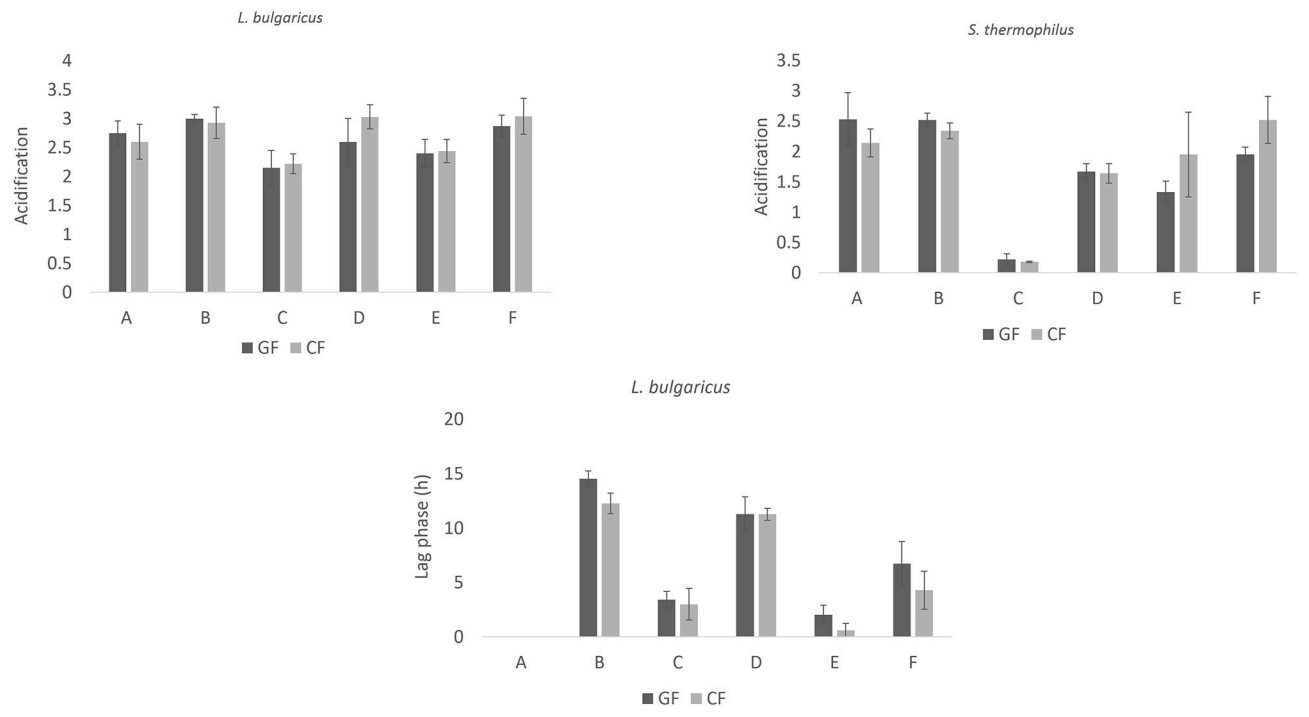

FIGURE 2 | Parameters (mean values ± standard error) of the lag-exponential model for *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* in the combinations of the design. Acidification: parameter ΔpH_{max}; lag phase: parameter α. GF, Gluten Friendly Flour; CF, Control Flour.

multifactorial analysis of variance and Tukey's test as the *post-hoc* comparison test. Storage time, temperature and kind of samples (control, GF or CF) were used as categorical predictors; the critical P-level was set to 0.05.

RESULTS

Acidification Kinetics

The aim of the first step was to study the effect of either GF or CF on acidification kinetics *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*. The microorganisms experienced a sigmoid-like acidification (see as an example **Figure 1**), with an exponential-like step (fast decrease of pH) and a steady state (a maximum value of acidification); in some combinations, there was a preliminary step when pH was constant (a lag-like period).

Figure 2 shows the most important parameters of these kinetics: ΔpH_{max} (acidification), and α (time before the beginning of acidification kinetic) for *L. bulgaricus* and *S. thermophilus*. ΔpH_{max} was 2.5–3.0 for *L. bulgaricus* and 1.8–2.7 for *S. thermophilus*. This microorganism did not experience an acidification kinetic in the combination C. The parameter α was never found for *S. thermophilus*, while it was from 0 (combination A) to 14 h (combination B) for *L. bulgaricus*.

ΔpH_{max} and α were used as inputs to run a DoE analysis and to study the effect of flour, inoculum and temperature. The significance of these effects is in **Table 2**. The approach was highly significant for *L. bulgaricus*, as shown by the high regression coefficients for both GF and CF (from 0.947 to 0.961).

The statistical weight of the variables was the same for both GF and CF; acidification (ΔpH_{max}) was affected by all factors as individual terms and by the interactions "inoculum/flour" and "temperature/flour," but the most significant term was

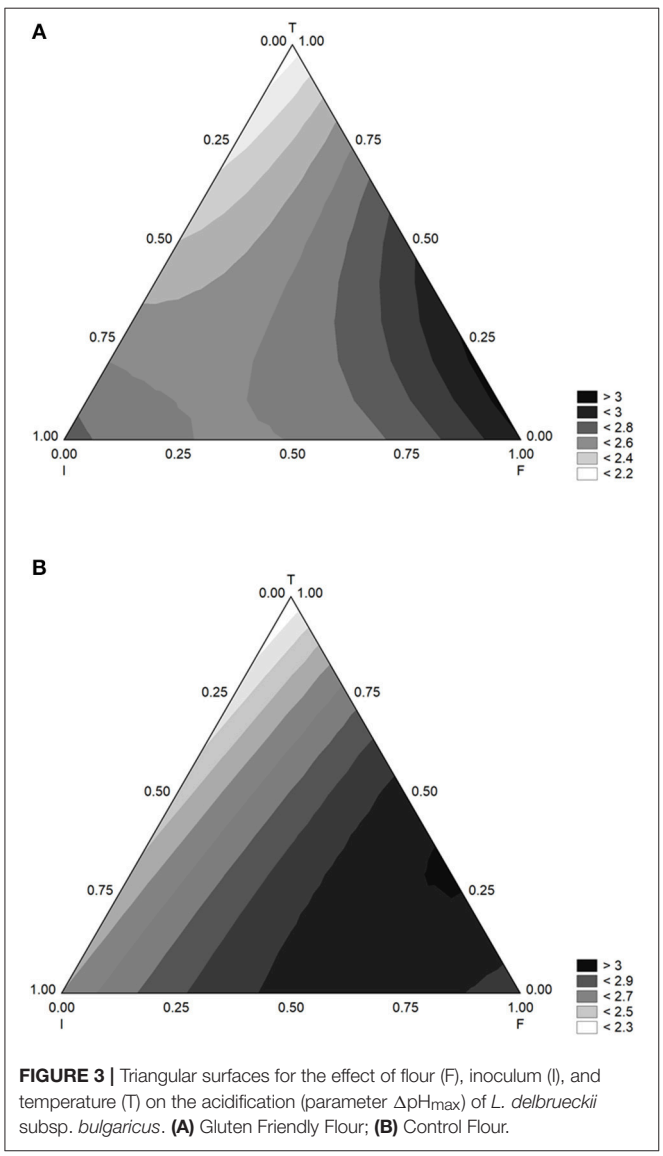

FIGURE 3 | Triangular surfaces for the effect of flour (F), inoculum (I), and temperature (T) on the acidification (parameter ΔpH_{max}) of *L. delbrueckii* subsp. *bulgaricus*. **(A)** Gluten Friendly Flour; **(B)** Control Flour.

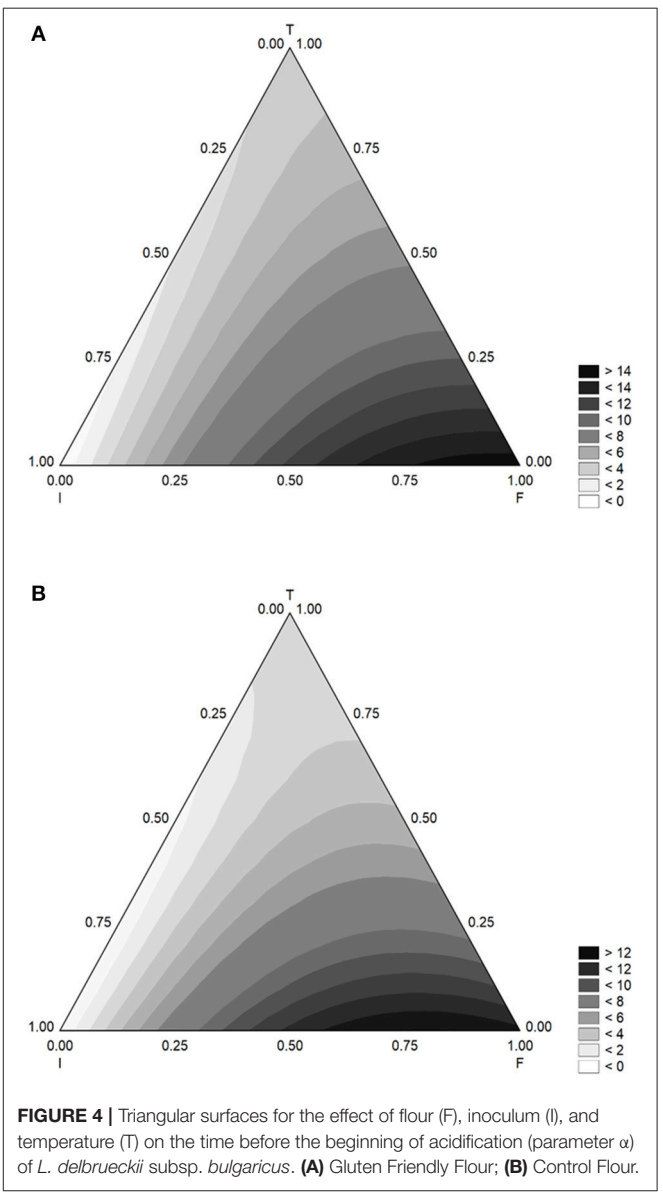

FIGURE 4 | Triangular surfaces for the effect of flour (F), inoculum (I), and temperature (T) on the time before the beginning of acidification (parameter α) of *L. delbrueckii* subsp. *bulgaricus*. **(A)** Gluten Friendly Flour; **(B)** Control Flour.

the individual effect of flour. The parameter α was affected by the individual terms of temperature and flour and by the interactive factors "inoculum/flour" and "temperature/flour." On the other hand, the significance of the approach was lower for *S. thermophilus*.

A table of standardized effect offers a qualitative output; some details on the quantitative correlation of the factors with ΔpH_{max} and α can be achieved by mean of ternary plots. A ternary plot is a triangular-like graphs and shows the effects of three variables (inoculum, flour, and temperature) in a 2D space. **Figure 3** shows the triangular plots for ΔpH_{max} with GF (A) and CF (B). The effects were similar, and the model predicted the maximum level of acidification (ΔpH 3) with flour at coded levels 0.5–1.0 (effective value from 2.5 to 5.0 g/l) and inoculum at 0.25–0.50

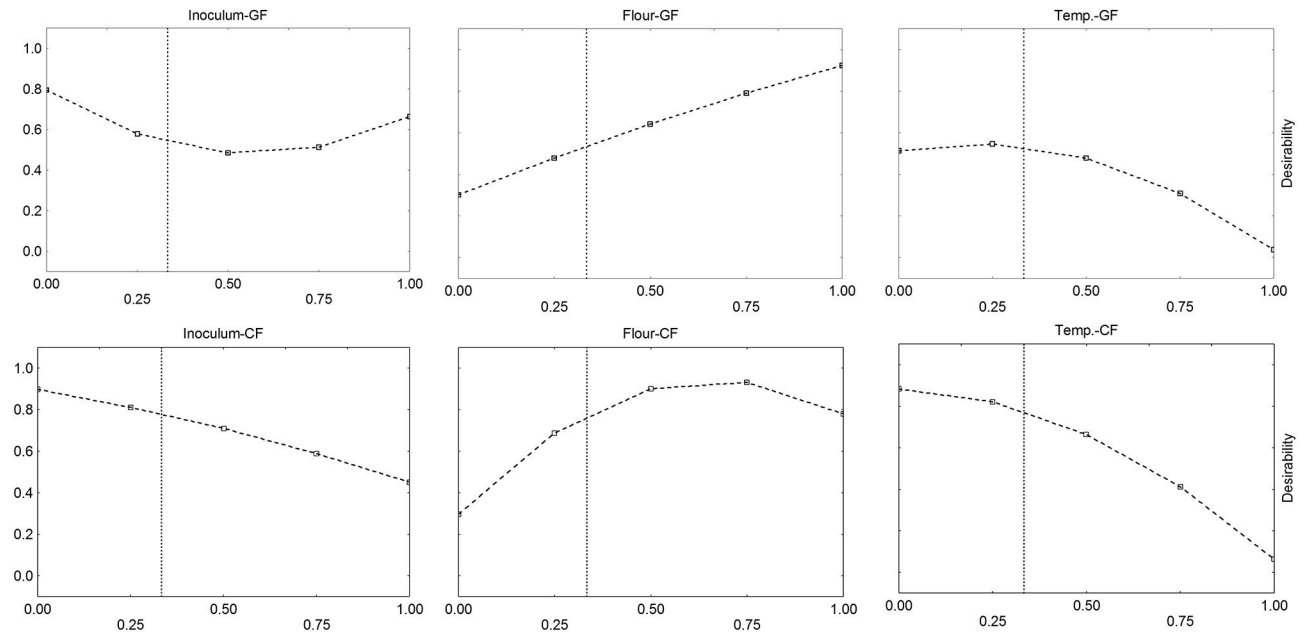

FIGURE 5 | Desirability profiles for the effect of flour (F), inoculum (I), and temperature (T) on the acidification (parameter ΔpH_{max}) of *L. delbrueckii* subsp. *bulgaricus*. GF, Gluten Friendly Flour; CF, Control Flour.

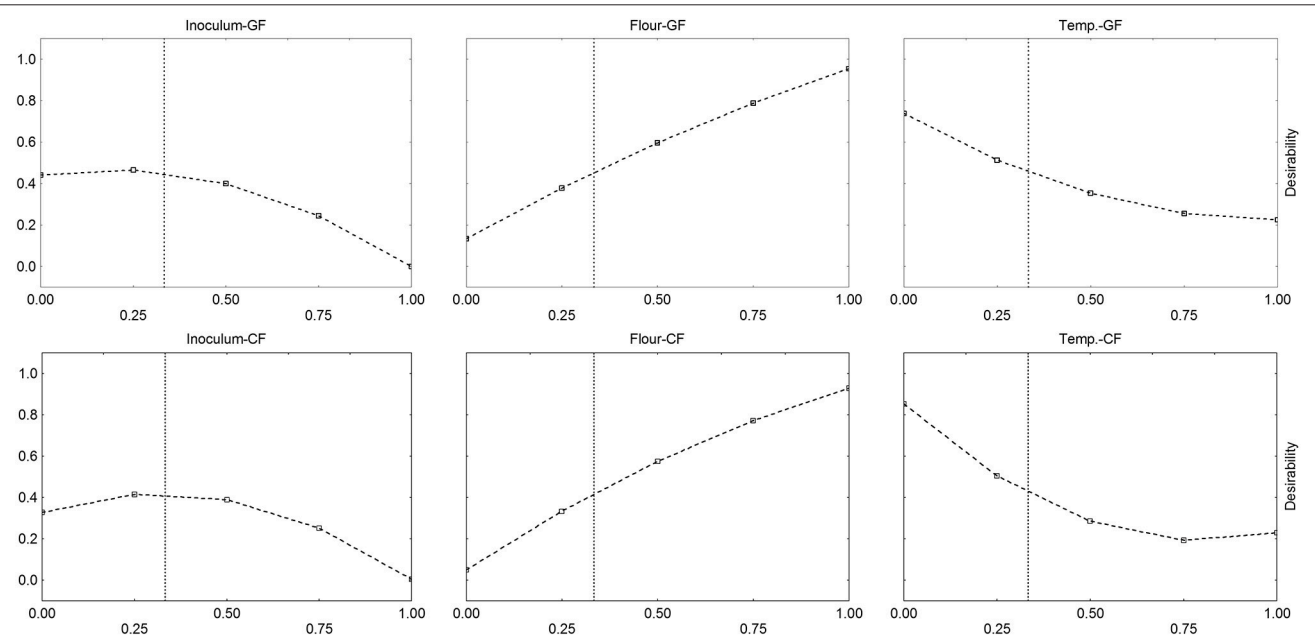

FIGURE 6 | Desirability profiles for the effect of flour (F), inoculum (I), and temperature (T) on the time before the beginning of acidification (parameter α) of *L. delbrueckii* subsp. *bulgaricus*. GF, Gluten Friendly Flour; CF, Control Flour.

(effective values, 5–6 log cfu/ml); the effect of temperature was negative, as an increase caused a decrease of ΔpH_{max}.

The triangular plots for α are in **Figure 4**. The lag phase increased when flour increased and was maximum (12–14 h) for the coded level 1 (flour at 5.0 g/l); inoculum and temperature negatively acted, and the lag phase was at its relative minimum values (1 or 3 days) when inoculum or temperature were at the coded level 1.

Ternary plots suffer a main drawback: they offer quantitative trends for interactions, but it is not possible to evaluate the effect of each factor alone. This goal can be achieved through the desirability profiles. The desirability profiles of the individual effects of inoculum, flour, and temperature on ΔpH_{max} are in **Figure 5**. The correlation inoculum/acidification was negative, as desirability decreased by increasing inoculum, while the correlation was positive for flour with slight differences between GF and CF. For GF the correlation flour/acidification was strictly linear, while for CF the correlation flour/acidification was quadratic, with a maximum for a coded level of flour at 0.75 (effective values 3.75 g/l). Finally, the effect of temperature was negative.

The same approach was used for the lag phase (**Figure 6**). Desirability profiles show that the desirability, i.e., the lag phase, increased by increasing both flour and temperature: the effect of flour was strong, as desirability increased from 0.1 to 1.0 by increasing flour to the coded level 0 (0 g/l) to 1 (5 g/l). On the other hand, the profiles for the temperature confirmed what found on the triangular plots: the correlation lag phase/temperature was negative, and the lag phase decreased by increasing temperature.

Desirability profiles and triangular plots were built for *S. thermophilus*, too (see as an example **Figure 7**); however, as reported above, the model was less significant, and the desirability profiles could not be used for an optimization process.

The last step of the statistical modeling was the optimization, i.e., the choice of the best combinations of the variables. For this research, the best combination would ideally result in the highest level of ΔpH_{max} and the lowest value of α (lag phase). However, the results of the DoE approach highlighted an uncoupling between the level of acidification and the lag phase: the combinations resulting in the highest level of acidification caused a significant prolongation of the lag phase. Therefore, a different goal was chosen: acidification of 2.0–2.5 with a lag phase of 6–7 h. The combination of all desirability profiles (saddle point approach) suggest that this goal could be achieved by setting the variables as follows:

a) flour at 2.5 g/l
b) *L. delbrueckii* subsp. *bulgaricus* at 6 log cfu/ml
c) Temperature at 37–40°C

Effect of GF on *B. infantis*

Before producing the synbiotic yogurt, the effect of flour was studied on *B. infantis*. The results are in **Table 3**. Both in the control and in the sample supplemented with CF, the microorganism was below the detection limit after 4 days, while a

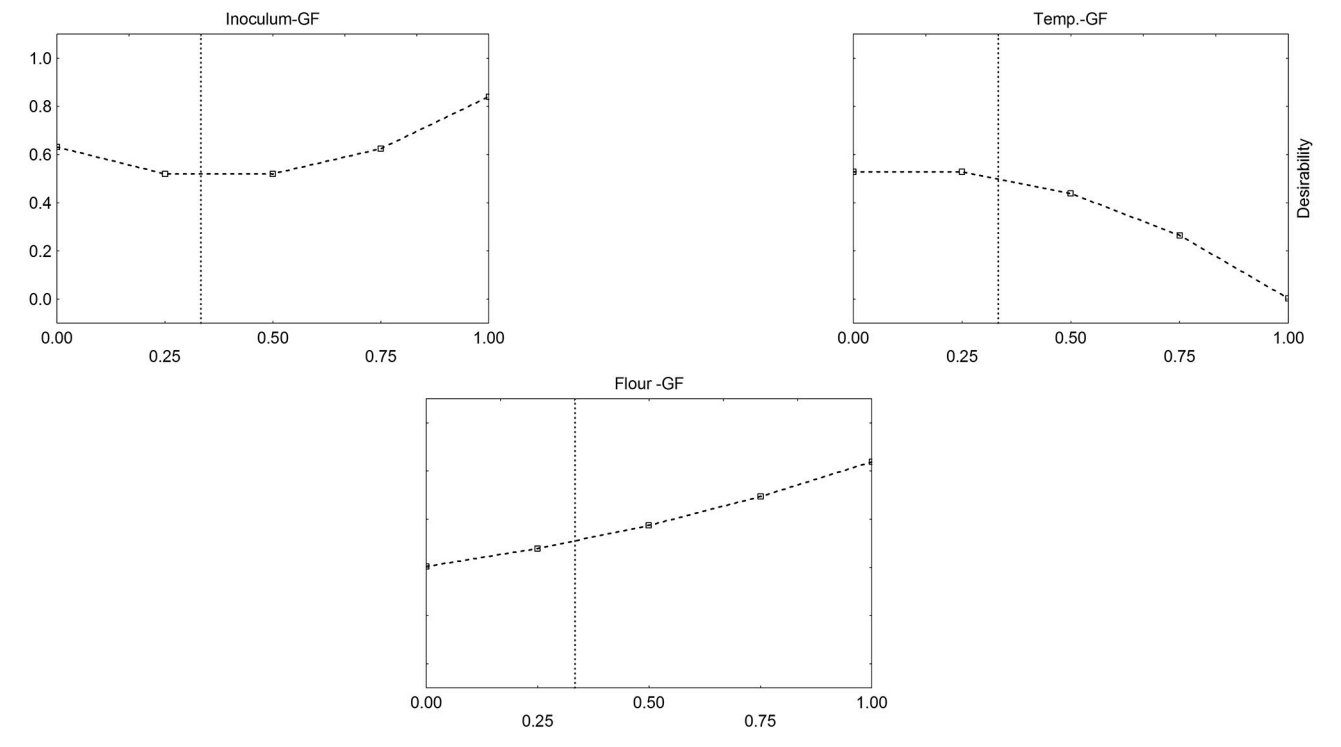

FIGURE 7 | Desirability profiles for the effect of Gluten Friendly Flour (F), inoculum (I), and temperature (T) on the acidification (parameter ΔpH_{max}) of *S. thermophilus*.

residual population was found in the sample with GF after 4 (5.08 log cfu/ml), 9 (4.08 log cfu/ml), and 14 days (3.60 log cfu/ml).

Yogurt

The synbiotic yogurt was produced through the classical starter strains and then supplemented with *B. infantis*. The level of mesophilic and termophilic lactobacilli and lactococci was always higher than 6 logcfu/g, while enterobacteria, pseudomonads, yeasts and staphylococci were below the detection limit (data not shown).

The viable count of *B. infantis* was analyzed through a multifactorial ANOVA; the results are in **Table 4** and in **Figure 8**. *B. infantis* was affected by time, temperature, and flour (**Table 4**).

A second output of multifactorial ANOVA is the decomposition of the statistical hypothesis, which shows the correlation of each factor or interactive term with the dependent variable. The decomposition of the statistical hypothesis for the viable count of *Bifidobacterium* has 3 outputs: correlation storage time vs. viable count (8A); qualitative correlation kind of flour vs. viable count (8B); actual data (8C). The decomposition of the statistical hypothesis, does not show effective or actual trends but it is a mathematical extrapolation of their statistical effect.

Figure 8A shows the effect of the storage time; as expected, time negatively acted on the viable count of *B. infantis*, as the cell number decreased within storage. **Figure 8A** shows the effect of the supplementation of flour; it does not report the viable count over time but it shows "mean" values for the entire running time. Flour supplementation exerted a positive effect, as in the sample with GF the analysis predicted the highest value and the lowest in the control; the sample supplemented with CF showed an intermediate trend (**Figure 8B**).

The decomposition of the statistical hypothesis for the interaction time/temperature/treatment shows when GF acted (**Figure 8C**). The positive effect of GF was found at 15°C (thermal abuse conditions), when it determined an increase of Bb02 in the last days of storage: the viable count of *B. infantis* was 7.0 log cfu/g in GF sample and 5.5–5.7 log cfu/g in the control and in CF sample.pH (3.8–4.0), Aw (0.98) and color did not undergo significant changes (data not shown).

TABLE 3 | Viable count of *B. infantis* (log cfu/ml) in saline solution (9 g/l NaCl) supplemented with either Gluten Friendly (GF) or Control Flour (CF) (2.5 g/l).

Time (days)	CNT	CF	GF
0	7.72 ± 0.23	7.72 ± 0.23	7.72 ± 0.23
1	5.41 ± 0.08	6.72 ± 0.13	6.15 ± 0.13
2	4.30 ± 0.17	5.54 ± 0.21	6.08 ± 0.21
4	–*	–	5.08 ± 0.19
9	–	–	4.08 ± 0.33
14	–	–	3.60 ± 0.21
21	–	–	–

CNT, control (saline solution). Mean values ± standard deviation. The samples were incubated at 37°C.
**Below the detection limit (1 log cfu/ml).*

TABLE 4 | Standardized effects of treatment (addition of GF or CF), duration of storage (time) and storage temperature on the viable count of *B. infantis*.

	SS	Degree of freedom	MS	Fishet-test	*P*-level
Intercept	2483.37	1	2483.37	54220.85	< 0.0001
Time	18.78	5	3.76	82.01	< 0.0001
Temperature	–*	–	–	–	–
Treatment	2.35	2	1.17	25.62	< 0.0001
Time*Temp.	9.41	5	1.88	41.07	< 0.0001
Time*Treatment	2.30	10	0.23	5.02	0.0001
Temp.*Treatment	–	–	–	–	–
Time*Temp.*Treat	1.59	10	0.16	3.48	0.003

*SS, sum of squares; MS, mean square residual. *Not significant.*

DISCUSSION

The optimization of a product is a complex process, as many variables could play a significant role and affect the final output; in this paper, the optimization of synbiotic yogurt was done through a step-by-step approach as the proposed product (traditional yogurt supplemented with both *B. infantis* and GF flour) involves different microorganisms.

First, the effect of GF on the acidification kinetics was studied, as the main requirement of an ingredient is that it must not delay or significantly affect the performances of a starter culture (Speranza et al., 2018). The optimization was based on two parameters: ΔpH_{max}, as a tool to measure the performances (acidification), and α, to highlight possible delays in the kinetic or a reversible inhibition.

The results show that the acidification kinetics of *L. bulgaricus* was significantly affected by the factors of the design (flour, inoculum, temperature). Namely, flour positively acted on acidification and this result was different from what found on *L. acidophilus*, which experienced a positive trend up to 2.5 g/l (Speranza et al., 2018). However, the most important result was the uncoupling between the performances of the microorganism in terms of pH decrease (ΔpH_{max}) and the parameter α. The combinations resulting in the highest reduction of pH also caused a delay of the kinetic. To the best of our knowledge, this trend was never found on *L. bulgaricus* and *S. thermophilus* and represents a challenge, as it was not possible to find out a combination with the highest acidification and the lowest value of α. The lack of adequate acidification could lead to long fermentation times with unsuitable economic and hygienic consequences (Mohammadi et al., 2012).

Therefore, the optimization was done by using a risk-benefit approach. As a cut-off point, two criteria were set: acidification of 2–2.5, in order to attain a final pH of 4.0, and α of 6–7 h; as a result, the combination able to fulfill this requirement was as follows: flour 2.5 g/l; inoculum 6 log cfu/ml; temperature 37°C.

Inoculum size of probiotic bacteria is an important key factor to ensure sufficient viable cells in the final food product (Lourens-Hattingh and Viljoen, 2001). The controversial effect of the inoculum (increase of performances up to a break-point and then

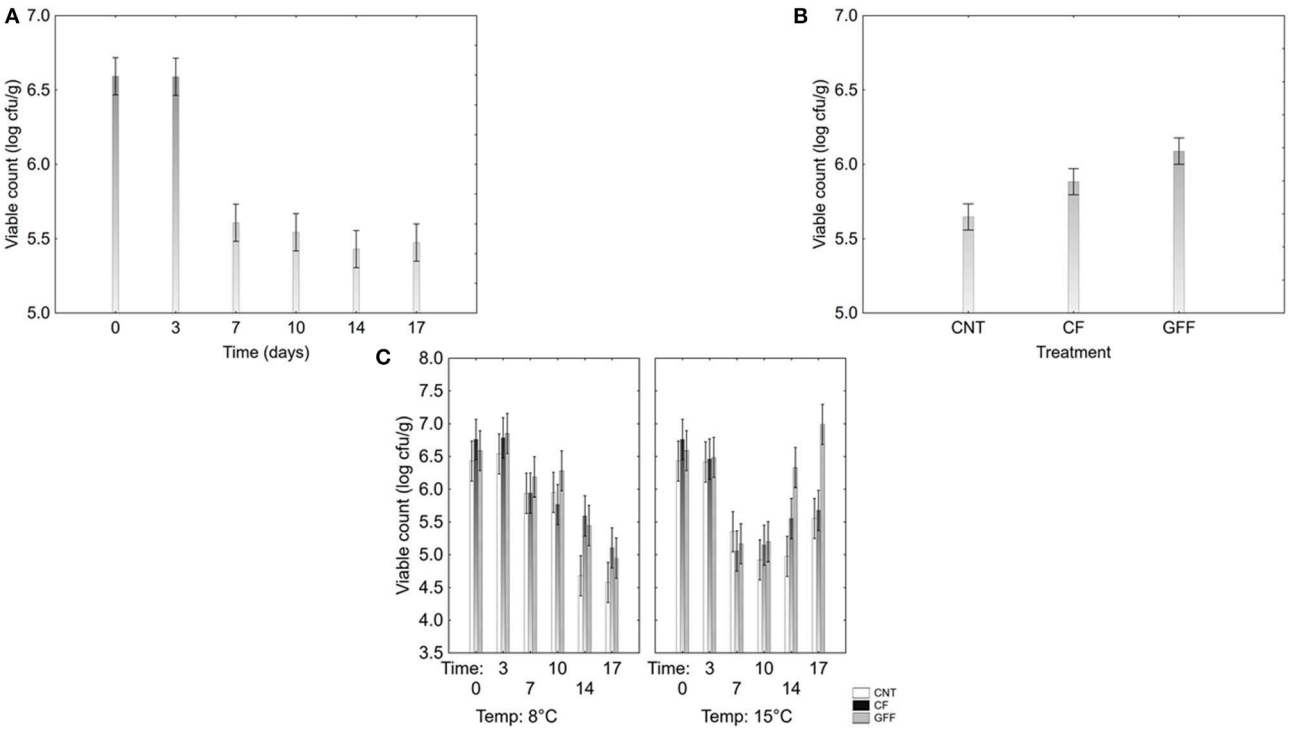

FIGURE 8 | Decomposition of the statistical hypothesis of multifactorial ANOVA on the viable count of *B. infantis*. In the yogurt. **(A)**, effect of the storage time; **(B)**, effect of flour addition; **(C)**, interaction time*temperature*flour addition. CNT, without flour (control); CF, Control Flour; GF, Gluten Friendly Flour. The bars represent the 95%-confidence intervals.

decrease of acidification) confirmed the preliminary reports on *L. acidophilus* La5 (Speranza et al., 2018) and was also reported by other authors; Khosravi-Darani et al. (2015) studied the effect of inoculum size in mono- and co-colture of *L. bulgaricus*, *L. acidophilus,* and *S. thermophilus* and found that *S. thermophilus* multiplication in yogurt was higher once a lower concentration of inoculum had been used. This effect was attributed to a possible competition for nutrients.

After the optimization, the effect of GF on the viability of the probiotic *Bifidobacterium* was assessed, as some preliminary reports (Bevilacqua et al., 2016; Costabile et al., 2017) highlighted the importance of GF (flours or bread) and pointed out a positive effect and a partial restoration of bifidobacteria in coeliac subjects, along with a protective effect and a lowering of death kinetic of *L. acidophilus* La-5 (Bevilacqua et al., 2016; Speranza et al., 2018).

The results of the second step confirmed the protective effect and the lowering of the death kinetic. However, the data of the last part, which surprisingly denoted a partial increase of bifidobacteria in the last days of the storage, highlighted that the effect of GF was not a mere protection, but also a possible stimulation of bifidobacterial population (Costabile et al., 2017).

GF technology is based on the use of microwaves for few seconds on hydrated wheat kernels; this process, coupled with rest times and water evaporation, probably induces a modification in secondary and tertiary structures of proteins. As a result of this change, the different spatial configuration of amino acids is able to drastically reduce the immunogenicity (Landriscina et al., 2017) and exerts a positive effect on some Gram-positive bacteria (namely lactobacilli and bifidobacteria).

Previously, it was suggested that the gluten modified by GF technology could be used as an alternative source under stressful conditions (Speranza et al., 2018), like those encountered by bifidobacteria after a prolonged storage at 15°C.

A key-factor for the optimization/design of functional foods is the viable count of probiotic. The traditional threshold is 10^6 cfu/g or cfu/ml by the Italian legislation (Fortina, 2007) and recently increased to 10^7 cfu/g or 10^9 *per* day (Rosburg et al., 2010; Italian Ministry of Health, 2013). The level of *B. infantis* for both the second and the third assay is lower than this break-point; namely in the second step the inoculum was 7.7 log cfu/ml, and this assured a probiotic shelf-life for 1 day in the control and in the sample supplemented with CF and 2 days for the sample supplemented for GF. The low inoculum affected the duration of shelf life; however, the main goal of this step was not the evaluation of shelf life but a focus on the effect of GF on the viability.

In the validation in synbiotic yogurt, the inoculum was lower (6.5–6.8 log cfu/g) as preliminary reports suggested that in complex systems GF could promote or enhance growth; thus, a compromise between the viable count required for a viability test (7–8 log cfu/g) and a growth assay (4–5 log cfu/g) was chosen. Therefore, this step, as previously reported, was not aimed at defining the shelf life but at studying the effect of GF in a complex

system. Further investigations are required to define shelf life by adding to the yogurt higher concentration of *B. infantis* (8–9 log cfu/g).

In addition, the results of 2nd and 3rd step both suggest that the supplementation of GFF at 2.5 g/l in a yogurt is a good compromise between technological performances of starter cultures and prolongation/enhancement of the viability of the probiotic microorganism *B. infantis*. However, the design of this synbiotic yogurt should be completed with the optimization of the inoculum of *B. infantis* after the fermentation to fulfill the basic requirements of law (probiotic at least 7 log cfu/g for the entire storage time).

This paper proposes a DoE approach to produce a traditional yogurt supplemented with GF and a probiotic *Bifidobacterium*. The most important findings can be summarized as follows:

a) The flour is responsible on an uncoupling on the fermentation kinetics of the starter microorganisms of the yogurt (increase of acidification and induction of a delay in the kinetic). Thus, it is not advisable to use flour amounts > 2.5 g/l.
b) The supplementation of GF exerted a positive effect in a simple system, as it prolonged the viability of *B. infantis*.
c) It is possible produce a synbiotic yogurt, fermented with *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* and supplemented with *B. infantis* and a prebiotic-like compound (GF). GF was able to cause an increase of bifidobacteria in the last days of storage.

In conclusion, this paper suggests a way to produce a synbiotic yogurt containing bifidobacterial and GF; the combination of these two factors could prolong and enhance the viability of the probiotic. The protocol hereby proposed also shows a way to counteract the drawback of bifidus pathway (production of acetic acid), by using the traditional starter cultures of yogurt. The combination of all these data suggests that the approach proposed in this paper is a promising way; however, further efforts are required to translate these preliminary data in an effective protocol to produce a new functional food. Some requirements have to be fulfilled: the definition of a unique death kinetic of the probiotic, along with a focus on the mode of action of GF, in order to evaluate and define a commercial shelf life as requested by the Regulatory Agencies.

AUTHOR CONTRIBUTIONS

AB, MS, CL, and MC conceived the study. AB, BS, DC, and MC designed the experiments. CL prepared the GF. BS, and DC performed the experiments. AB performed the statistic and wrote the paper. CL funded the research. All authors reviewed the paper.

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Mycobiome Diversity in Traditionally Prepared Starters for Alcoholic Beverages in India by High-Throughput Sequencing Method

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Chowan, dawdim, humao, hamei, khekhrii, and *phut* are sun-dried starters used for preparation of alcoholic beverages in North East regions of India. We attempted to profile the mycobiome community in these starters by high-throughput sequencing (HTS) method. All fungal populations were found to be restricted to Ascomycota (67–99%), Zygomycota (0.7–29%), Basidiomycota (0.03–7%), and Chytridiomycota (0.0003%). We found 45 core operational taxonomic units (OTUs) which were universally present and were further weighed to 41 genera level and 22 species level taxonomy. A total number of 594 fungal species were detected by HTS including common species (224), unique species (133) and rare-species (237) in samples of starters. Unique species were recorded in *phut* (40 species), *khekhrii* (28), *hamei* (23), *dawdim* (21), *chowan* (13), and *humao* (8), respectively. Most of the fungal families were found to correlate to a type of nutritional mode and growth morphologies of the community, where saprotrophic mode of mold species were more dominant, whereas morphotypes were more dominant in yeast species.

Keywords: dry starter, mycobiome, yeasts, molds, high-throughput sequencing

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INTRODUCTION

Traditionally prepared sun-dried cereal-based amylolytic/alcoholic starters, in the form of round/oval/flattened balls of varied sizes for production of mild-alcoholic beverages, are common in South East Asia (Hesseltine, 1983; Steinkraus, 1996; Nout and Aidoo, 2002; Tamang, 2010a). Usually three types of mixed cultures are traditionally used as starters to convert cereal starch to sugar and then to alcohol and organic acids (Hesseltine et al., 1988; Tamang and Fleet, 2009; Tamang, 2010a,b). These are (1): dried starter consisting of consortia of amylase/alcohol producing-yeasts, filamentous molds and bacteria, which are locally called *marcha* in India, Nepal and Bhutan (Tsuyoshi et al., 2005), *chiu/chu/daque* in China (Chen et al., 2014; Xu et al., 2017), *nuruk* in Korea (Jung et al., 2012), *ragi* in Indonesia (Surono, 2016), *loog-pang* in Thailand (Limtong et al., 2002), *benh men* in Vietnam (Dung et al., 2007) and *dombea* in Cambodia (Ly et al., 2018); (2): mixed culture of molds *Aspergillus oryzae* and *A. sojae* in the form of a starter called *koji* in Japan for making *saké,* distilled liquor, and several fermented

soybean products such as *miso* and *shoyu* (Kitamura et al., 2016), and (3): large compact cakes made up of whole-wheat flour with yeasts and filamentous molds to ferment starchy substrates for production of alcohol, mostly in China (Tamang, 2010a). Microbiota associated with traditionally prepared Asian dried starters are starch-degrading genera of molds *Actinomucor, Amylomyces, Aspergillus, Mucor, Neurospora, Penicillium, Rhizopus* (Hesseltine et al., 1988; Tamang et al., 1988; Nikkuni et al., 1996; Nout and Aidoo, 2002; Chen et al., 2014; Tamang et al., 2016a); amylolytic and alcohol-producing yeasts genera mostly *Candida, Debaryomyces, Dekkera*, *Galactomyces, Geotrichum, Hansenula, Hanseniaspora, Issatchenkia*, *Kazachstania*, *Kluyveromyces, Pichia, Saccharomyces, Saccharomycodes, Saccharomycopsis, Schizosaccharomyces, Torulaspora*, *Torulopsis, Wickerhamomyces*, and *Zygosaccharomyces* (Hesseltine and Kurtzman, 1990; Tamang and Sarkar, 1995; Tsuyoshi et al., 2005; Jeyaram et al., 2008; Lv et al., 2012, 2013; Chakrabarty et al., 2014; Sha et al., 2016, 2017, 2018) and few genera of bacteria, mostly *Pediococcus, Lactobacillus* (Hesseltine and Ray, 1988; Tamang and Sarkar, 1995; Sujaya et al., 2001; Tamang et al., 2007; Chakrabarty et al., 2014).

Since the culture-dependent method can only isolate the culturable microorganisms from samples using media, the culture-independent method may profile all microbial communities, including both those that are culturable and unculturable in food samples, by extracting the whole genomic DNA directly from small amount of samples (Roh et al., 2010; Jung et al., 2012; Puerari et al., 2015; Sha et al., 2018). Culture-independent methods, including pyrosequencing and high-throughput amplicon sequencing, are commonly applied for profiling microbiome of natural food fermentation within a short time and with more accuracy (Alegría et al., 2011; Cocolin et al., 2013; Chen et al., 2014; Mayo et al., 2014; Puerari et al., 2015; Tamang et al., 2016b; Shangpliang et al., 2018). Application of the amplicon-based high-throughput sequencing has been demonstrated for the monitoring of microbial populations between different strains within a species (Ercolini et al., 2012), and inter- and intra-species diversity within a particular genus or among genera (Yan et al., 2013).

Drinking of traditional alcoholic beverages and drinks is the distinct dietary culture and practices of ethnic people of North East India[1] with strong ritualistic and ethnical importance (Tamang, 2010a; Tamang et al., 2016a). Traditionally prepared sun-dried starters such as *dawdim, hamei, humao, khekhrii, chowan*, *phut,* etc., in North East states of India (Anupma et al., 2018) are commonly used by diverse groups of ethnic people to prepare mild-alcoholic (4–5%) beverages with sweet taste, providing a high source of calories and minerals (Thapa and Tamang, 2004, 2006; Tamang and Thapa, 2006; Tamang et al., 2012). In this study we selected six different starters, such as *chowan* of Tripura*, dawdim* of Mizoram*, hamei* of Manipur*, humao* of Assam, *khekhrii* of Nagaland and *phut* of Arunachal Pradesh, from North East states of India (**Figure 1**). All these amylolytic/alcoholic starters are dry, hard, with different shapes of round to flattened solid ball like structure, sizes ranging from 1.2 to 11.2 cm in diameter, and all creamy to dusty white in color. Except for *khekhrii,* all other starters are traditionally prepared from soaked rice/wheat, mixed with some locally available wild plants, added with previously prepared powdered starters (1–2%), and kneaded into round to flattened cakes by adding water. The mixtures are covered with fern fronds/paddy straws/jute sags, fermented for 1–3 days at room temperature; and finally sun dried (2–3 days) to get dry starters, which can be kept for a year or more (Tamang et al., 2016a; Anupma et al., 2018). *Khekhrii* is the only amylolytic/alcoholic starter in North East India, which is prepared by fermenting germinated sprouted-rice grains and then sun-dried to use as dry starters to prepare the local alcoholic drink (Anupma et al., 2018). Sha et al. (2018) studied the fungal diversity in *chowan, dawdim, hamei, humao, khekhrii,* and *phut,* based on the culture-dependent method using ITS-PCR and a culture-independent approach by PCR-DGGE analysis. In this paper, we attempted to understand the "ethno-microbiology" of mycobiome diversity in *chowan, dawdim, hamei, humao, khekhrii,* and *phut* by using the high-throughput sequencing method supported by bioinformatics interpretation.

MATERIALS AND METHODS

Sample Collection

Six samples of starter: *chowan* of Tripura*, dawdim* of Mizoram*, hamei* of Manipur*, humao* of Assam, *khekhrii* of Nagaland and *phut* of Arunachal Pradesh (**Figure 1**) were collected immediately after the preparation (fermentation and sun-drying) from different places of North East India. The average pH of these starter samples was 4.9 ± 0.2. Samples were kept in sterile containers, leveled, transported to the laboratory and stored at room temperature in a desiccator; dried starter can retain its potency *in situ* for more than a year in the moist-free condition (Tamang et al., 1988).

Community DNA Extraction

Firstly, dried starter samples were powdered with the help of sterile mortar and pestle and 1g of powdered sample were taken and homogenized in 9 ml of 0.85% physiological saline and subsequently filtered through 4 layers of sterile cheese-cloth. The resulting filtered solutions were centrifuged at 14000 *g* for 10 min at 4°C (Lv et al., 2013; Sha et al., 2018) and then the pellets were subjected to total community DNA extraction using the ProMega DNA extraction kit (ProMega, United States) according to the manufacturer's instructions. Subsequently, the RNA was eliminated from the cellular lysate by administering the RNase solution after incubation at 35°C for 15 min. The residual proteins were removed by adding protein precipitation solution and then centrifuging at maximum speed. Finally, the DNA was precipitated by adding isopropanol, and purified with two washes of 70% ethanol. Quality of DNA was checked on 0.8% agarose gel by measuring the concentration using Nano-Drop spectrophotometer (AG-6135, Eppendorf, Germany). The DNA was kept at −20°C until further processing.

[1] www.northeasttourism.gov.in

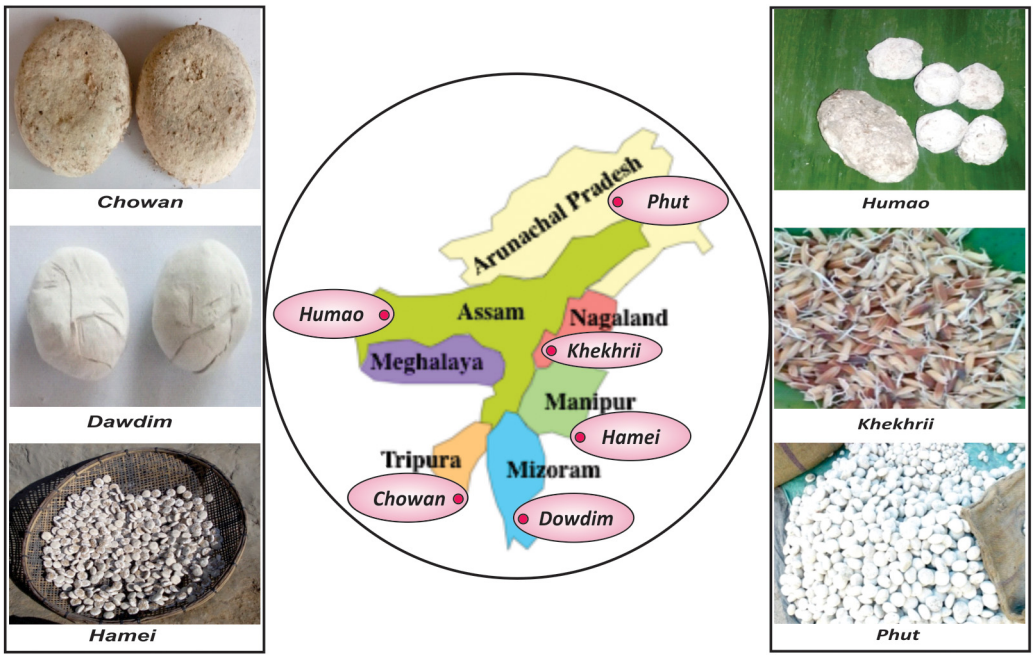

FIGURE 1 | Origin and types of sun-dried alcoholic starter cultures in eight North East states of India.

Sequencing of Fungal ITS2 Gene Region and Pre-processing

Internal Transcribed Spacer (ITS) 2 region of fungi was targeted for taxonomic profiling by amplification using ITS2F (GCATCGATGAAGAACGCAGC) and ITS2R (TCCTCCGCTTATTGATATGC) primers (Blaalid et al., 2013) due to its high interspecific variability which also conserved primer sites with multiple copies (Schoch et al., 2012). A composite sample for sequencing was made by combining equimolar ratios of amplicons from the samples, followed by gel purification with a QiagenMinElute gel extraction kit to remove potential contaminants and PCR artifacts. Amplicon libraries were purified by 1X AMpureXP beads, which were checked on an Agilent DNA 1000 chip on Bioanalyzer 2100, and finally quantified by Qubit Fluorometer 2.0 using Qubit dsDNA HS Assay kit (Life Technologies). MiSeqIllumina platform using 2 × 250 bp chemistry sequencing was performed. Pre-processing of downstream analysis for raw read was completed as described by Comeau et al. (2017), as follows: firstly, raw read quality from sequencer was checked for the average and range of the Phred quality scores along the reads (1 to 300 bp), for both forward and reverse reads independently, to pass it to the next steps using FastQC[2]; secondly, removal of adapter sequences through cut adapt tool (Martin, 2011); thirdly, adapter cleaned paired-end reads files merged using the PEAR (v0.9.10) program (Zhang et al., 2014) with default settings; fourthly, FASTQ stitched files were converted to FASTA and removed any sequences that had an "N" in them with run_fastq_to_fasta.pl command lin; and lastly, chimeric sequences were removed with VSEARCH tool (Rognes et al., 2016) using UNITE_uchime_ITS2only_01.01.2016.fasta reference dataset.

Downstream Analysis of ITS Gene Region Reads

The downstream analysis of chimera free FASTA files was done for detecting the taxonomic classification and their functional guided activity. For taxonomic classification of each sequence, we performed the diversity analysis in the QIIME 1.9 environment (Caporaso et al., 2010). Sequence reads were combined in a single FASTA file with guided metadata files and further steps were done accordingly as described by Comeau et al. (2017). Fungal operational taxonomic units (OTUs) were analyzed by an open reference-based OTU picking approach using UNITE reference database as UNITE_sh_refs_qiime_ver7_dynamic_20.11.2016.fasta. The OTU picking was carried out using the sortmerna_sumaclust method with a similarity threshold of 97%. Taxonomic assignments were performed using mother classifier (Schloss et al., 2009).

We performed the analysis with PIPITS (Gweon et al., 2015) and FUNGuild environments (Nguyen et al., 2016) for functional guided activity determination. The ITS2 region was extracted with ITSx, clustered into OTUs with VSEARCH[3] at 97% sequence similarity, and chimera removal was performed using the UNITE UCHIME reference data set. Representative sequences were assigned taxonomic classification with the RDP classifier against the UNITE fungal ITS reference data set at a

[2]http://www.bioinformatics.babraham.ac.uk/projects/fastqc/

[3]https://github.com/torognes/vsearch

TABLE 1 | Sequence statistics, alpha diversity matrix and species level taxonomy number observed for starter culture samples.

Samples	*Chowan*	*Dawdim*	*Hamei*	*Humao*	*Khekhrii*	*Phut*
Sequences used for analysis	292996	292996	292996	292996	292996	292996
Alpha Diversity Indices						
Observed OTUs	836	750	3037	702	1122	692
Goods coverage	0.997812	0.998276	0.990399	0.998198	0.996997	0.998601
Shannon	2.33745	3.489771	2.566343	2.645813	2.473556	2.075269
Simpson	0.689639	0.837917	0.759854	0.771975	0.684789	0.498496
Chao1	5200.255	3457.66	47476.08	3726.522	6894.537	2066.508
Taxonomy based analysis:						
Total number of observed species	82	108	67	82	113	142
Number of rare-species	31	39	30	24	54	59

confidence threshold of 0.85. We generated otu_table_funguild file by using pipits_funguild.py command line. This OTU table was used to run the online Guilds application to assign functional information to OTUs in high-throughput sequencing datasets[4].

Other Data Analysis

Alpha diversity analyses of the mycobiome were tested using QIIME platform and with the alpha_diversity.py script. For the continuous variables, non-parametric t-test was used, and for categorical variables between groups, either the Pearson chi-square or Fisher's exact test was used depending on assumption validity. Data analyses were performed by statistical environment R[5]. Phylum level abundance plots, bubble plots and heatmap were derived by ggplot2 package (Wickham, 2016), core microbiome heatmap were derived by microbiome package (Lahti and Shetty, 2017) and correlation plot by corplot package (Wei and Simko, 2017). The filtered OTUs based (less than 1% abundance value) rare-phylotypes heatmap were derived by ggplot2 package (Wickham, 2016). UPGMA based dendrogram was created using the Pearson similarity coefficient. Alpha diversity indices like Chao, Shannon, and Simpson were calculated after rarefying all samples to the same sequencing depth (Cox et al., 2017). Non-metric multidimensional scaling plots (NMDS) based on Bray-Curtis distance matrix was constructed to carry out the beta-diversity analysis.

Data Availability

The sequences obtained from high-throughput sequencing effort were submitted to NCBI, which are available under SRA accession: SRP150043 and BioProject ID:PRJNA474271.

RESULTS AND DISCUSSION

The present study reveals the mycobiome diversity in the same samples of *chowan, dawdim*, *hamei, humao*, *khekhrii,* and *phut* by culture-independent method using high-throughput sequencing approach, which permits the analysis of hundreds of nucleotide sequences (Roh et al., 2010). We generated 5213436 paired end sequences and were clustered into operational taxonomic units (OTUs) by single linkage clustering with 97% sequence similarity. About 2488812 high quality sequences (sequence lengths: 374 ± 31 nucleotides) and normalization were done on 292996 per sample for the study, which were assembled into 6097 global and species-level OTUs. All OTUs with <2 reads in total and those not representing fungi were omitted. OTU-table was generated for further taxonomy-based analysis. Samples diversity surveillance for the fungal population was analyzed by intra-sample variations through the alpha diversity measures (**Table 1**). The diversity indices provide an idea about the expected diversity values, like *goods coverage* index within 0.990 to 0.998. Observed OTUs values were found to be wide and within the range of 702 to 3037. Among the six starters, *hamei* had the highest OTUs. Phylum level abundance varied in each sample and was mostly limited to taxa Ascomycota, Basidiomycota, Chytridiomycota and Zygomycota (**Figure 2A**). The samples were discrete on the NMDS plots using OTUs level variations (**Figure 2B**). All fungal populations were found to be restricted to only Ascomycota (67–99% of abundance), Zygomycota (0.7–29%) and Basidiomycota (0.03–7%), however, Chytridiomycota (0.0003%) was also enlisted in *khekhrii* sample. High prevalence of Ascomycota phylum was also reported in similar types of dry starters of Asia such as *nuruk* of Korea (Jung et al., 2012; Bal et al., 2016), and *daqua* of China (Li et al., 2011; Chen et al., 2014; Xu et al., 2017). In the present study quantitative differences were observed for the presence of fungal taxa in all six starters, which could be the consequence of differences in the traditional methods of production of starters, use of wrapping materials and varied fermentation time (Jeyaram et al., 2011; Bora et al., 2016; Anupma et al., 2018). The Alpha diversity estimation of all starters using species richness and non-parametric Shannon index showed dominance of phylum Ascomycota over the Zygomycota. A similar observation was also reported in similar types of dry starters of India: *thiat* of Meghalaya state (Sha et al., 2017), and in *marcha* of Sikkim state (Tamang et al., 1988).

We found 45 core OTUs which were universally present in all starter samples tested and were further weighed to 41 genera level (**Figure 3A**) and 22 species level taxonomy

[4]http://www.stbates.org/guilds/app.php

[5]https://www.r-project.org/

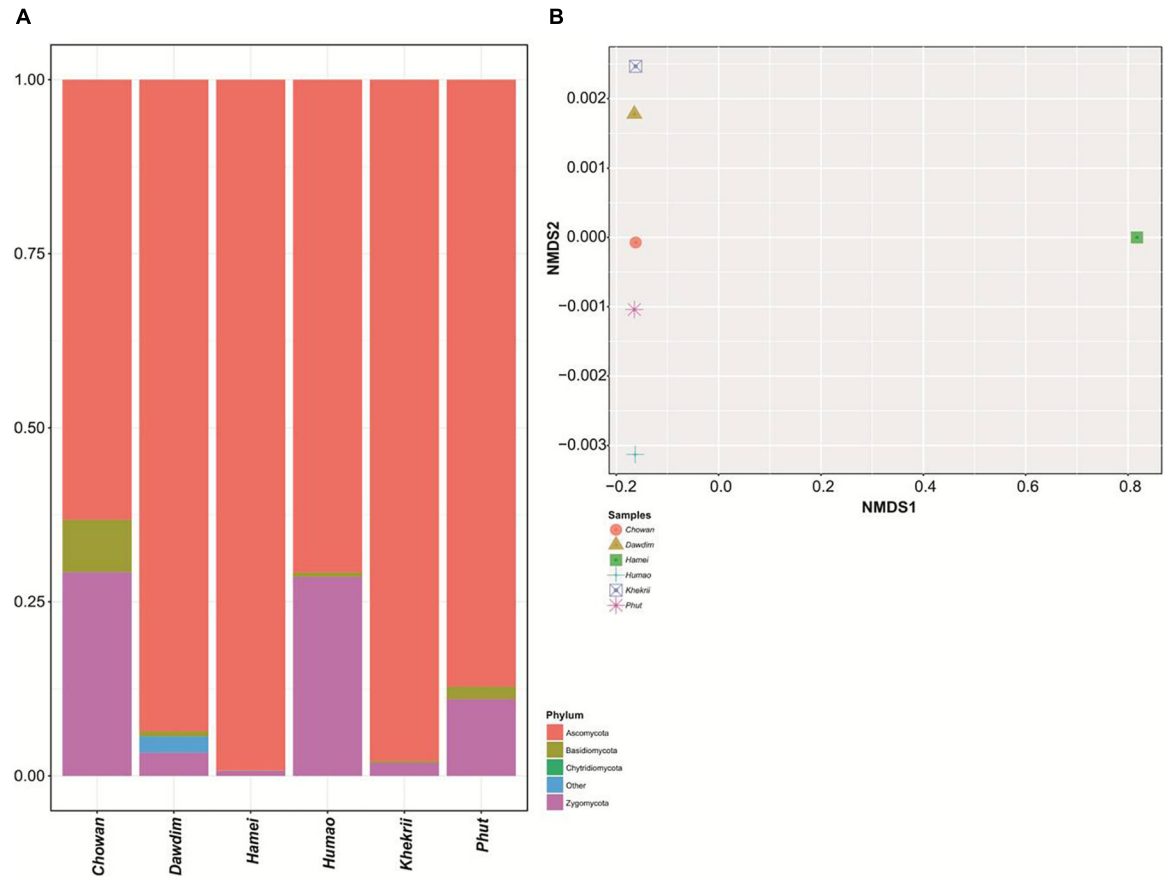

FIGURE 2 | (A) Barplot showing the phylum level diversity and their abundance distribution found in starters. **(B)** NMDS plot showing the beta diversity clustering pattern in tested subjects.

(**Figure 3B**). A wide diversity of fungal species, as well as various unique species in samples has been observed in this study. A total number of 594 fungal species were detected by HTS including noble or unique species (133), common species (224) and rare-species (237), in samples of *chowan, dawdim, hamei*, *humao, khekhrii,* and *phut* (**Supplementary Table 1**). A total of 133 fungal species were found to be noble or unique species with reference to diversity compared to the common species, out of which 40 species were sample-specific in *phu,t* followed by *khekhrii* (28), *hamei* (23), *dawdim* (21), *chowan* (13), and *humao* (8), respectively (**Supplementary Table 1**). Dominant unique species based on abundance were *Tetracladium setigerum* in *khekhrii*, *Saccharomyces eubayanus* in *chowan*, *Solicoccozyma terrea* in *hamei*, *Penicillium sumatraense* in *phut*, *Acremonium implicatum* in *humao*, and *Thermomyces lanuginosus* in *hamei*. Species with less than 1% abundances are known as rare-phylotypes (Li et al., 2018). We found 237 species within the rare-phylotypes category [those with less than 1% abundances (**Supplementary Table 1**)] including 19 different class level taxa (**Supplementary Figure 1**). Interestingly, samples of *phut* had the highest number of 59 rare-species, followed by *khekhrii* (54), *dawdim* (39), *chowan* (31), *hamai* (30), and *humao* (24), respectively (**Table 1**). A phylotype, often referred to as OTUs, is an environmental DNA fragment or group of sequences sharing greater than 97–98% similarity of a particular gene marker (Bhadury et al., 2011; Rivett and Bell, 2018). Importantly, in such lesser-known traditionally prepared dry starters, the presence of sizable number of rare-phylotypes may have some functional or biochemical properties, and sometimes these rare-species may have human health perspectives (Bhute et al., 2017).

The unique mold species recorded in dry starters of North East India are *Aspergillus penicillioides, Rhizopus arrhizus, Rhizopus microsporus* and the unique yeast species are *Kluyveromyce smarxianus, Trichomona scusciferrii, Candida humilis, Candida metapsilosis, Saccharomyces paradoxus, Saccharomycopsis malanga,* and *Wickerhamomyces sydowiorum*. Earlier reports demonstrated the presence of common yeasts in most of the Asian dried starters, which were similar to starters of North East India, including *Candida glabrata, Cryptococcus heveanensis, Cry. albidus, Pichia fabianii, P. guilliermondii, Rhodosporidium toruloides, Rhodotorula mucilaginosa, Saccharomyces cerevisiae, Saccharomycopsis fibuligera, Saccharomycopsis malanga, Sporobolomyces nylandii,* and *Wickerhamomyces anomalus* (Tsuyoshi et al., 2005; Xie

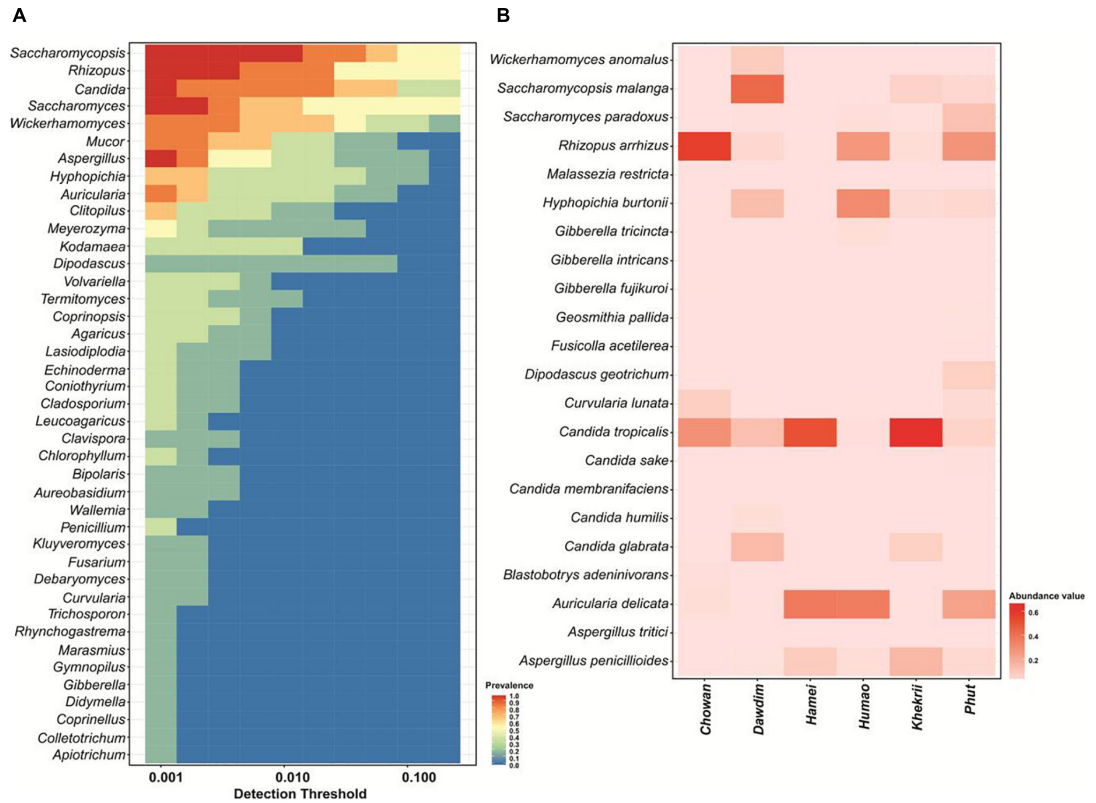

FIGURE 3 | Heatmap of OTUs showing **(A)** Core genus distribution pattern and **(B)** Core species and their relative abundance present in all samples.

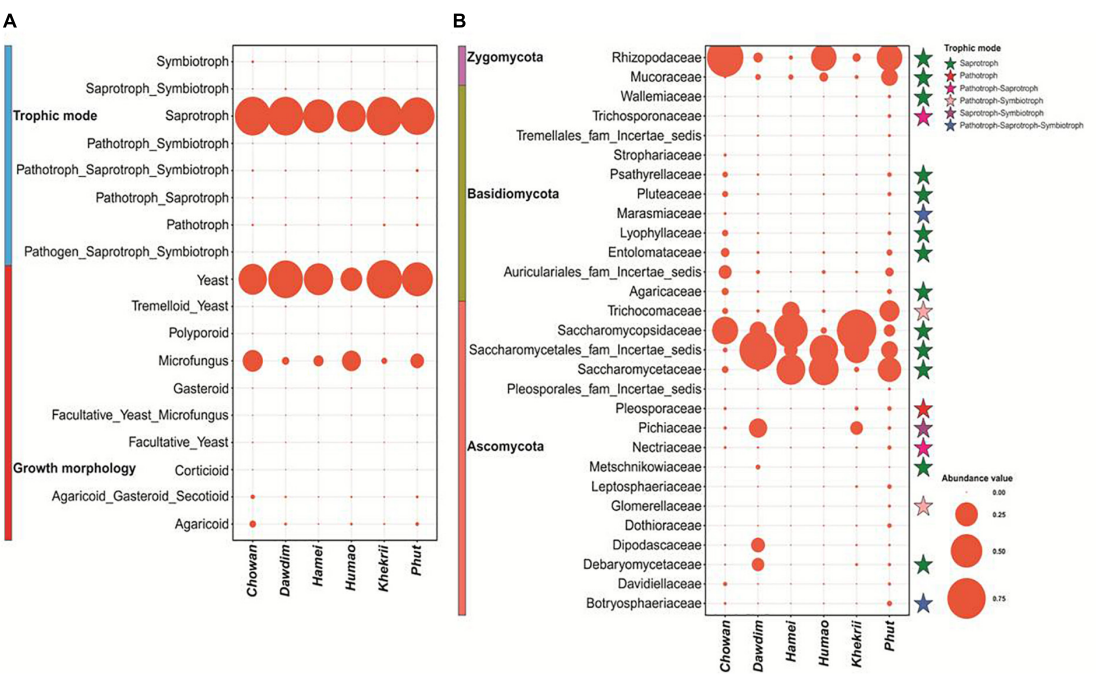

FIGURE 4 | (A) Bubble plot describing the features of mycobiome with relative abundance distribution present in starters. **(B)** Bubble plot describing the relative abundance distribution of family level diversity and their trophic mode of nutrition found in dried starters. Family level diversity having >1.0% abundance in each sample was taken for this plot.

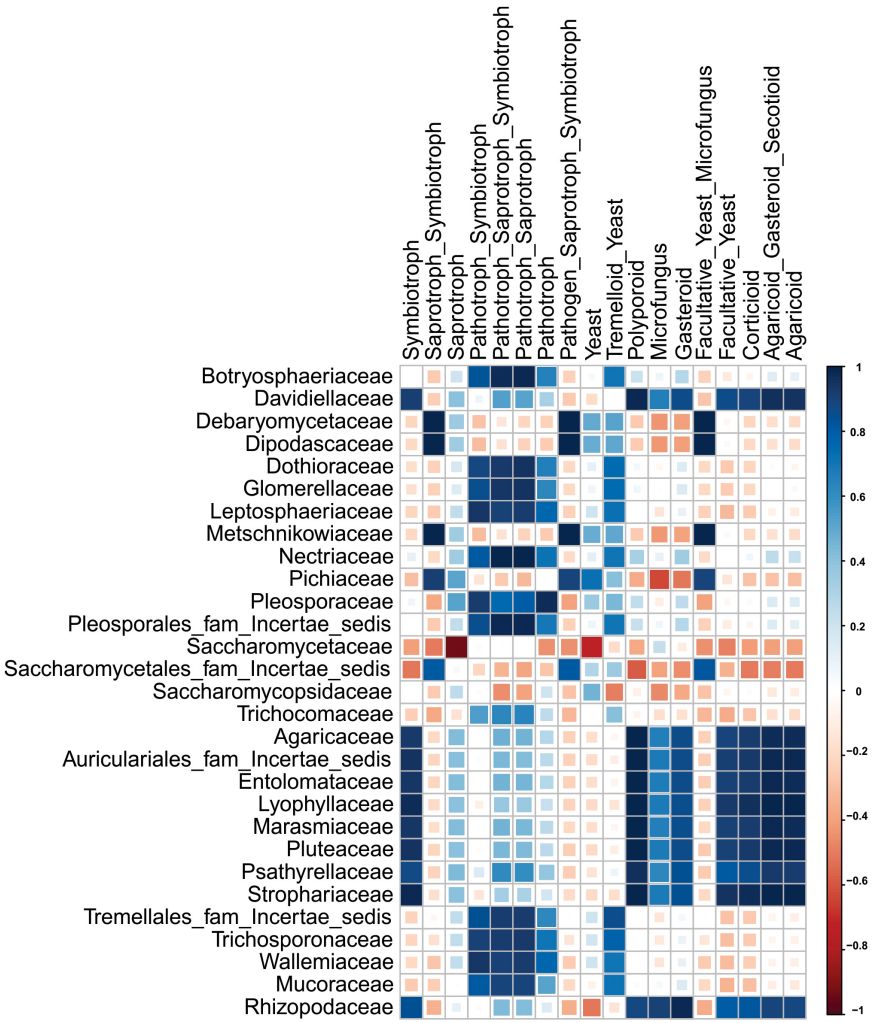

FIGURE 5 | Plot showing the spearman correlation values for family level diversity having > 1.0% abundance in each sample and different features of mycobiome.

et al., 2007; Jeyaram et al., 2008; Thanh et al., 2008; Lv et al., 2013; Bora et al., 2016; Sha et al., 2016, 2017, 2018). We assume that the higher yeast diversity in our study could have resulted from a larger sampling population. The yeast *Saccharomycopsis fibuligera,* possessing amylase and ethanol producing capacity, is one of the most common yeasts present in dried starters of Asia (Hesseltine and Kurtzman, 1990; Limtong et al., 2002; Tsuyoshi et al., 2005; Thanh et al., 2008; Sha et al., 2018).

We correlated mycobiome diversity which was earlier detected in six samples of dry starters of India viz. *(chowan, dawdim, hamei*, *humao, khekhrii,* and *phut*) by the culture-dependent method (ITS-PCR) (6 species) and culture-independent method using PCR-DGGE analysis (24 species) (Sha et al., 2018), with that of 594 fungal species detected by high-throughput sequencing method (**Supplementary Figure 2**). Based on OTUs, the HTS method could detect 594 fungal species showing a diverse profile of mycobiome communities in the six different types of starters in this study, which were not earlier detected by ITS-PCR and PCR-DGGE methods (Sha et al., 2018). The read length required by HTS platforms for DNA metabarcoding is preferably 200–400 bp (Banchi et al., 2018), which is used for ITS2 gene amplification that can generate the amplicons up to 400bp in size necessary for library preparation on Illumina platform (Blaalid et al., 2013). The shorter sequences for HTS platform using ITS2 primers favor the identification of a wide range of fungi, which is a major advantage of the ITS2 primer (Bellemain et al., 2013). Whereas in ITS-PCR, the read length of ITS gene sequence amplified by primers, ITS1 and ITS4, is ranging from 600 to 750 bp (White et al., 1990), which may not be used for the library preparation in Illumina sequencing platform for HTS (Banchi et al., 2018). Amplicon-based high-throughput sequencing reveals comprehensive microbial communities with superior sequence coverage and inter- and intra-species diversity within a particular genus or among genera (Bokulich and Mills, 2013; Yan et al., 2013; Połka et al., 2015), comparable to other molecular tools. This is because HTS can generate far more reads

than traditional culture-independent methods such as PCR-DGGE and facilitates the discovery of more microbiota diversity (Ercolini, 2013). However, a combined (culture-dependent and culture-independent) approach can be an appropriate strategy to investigate entire microbial communities of any food sample.

We assume that the geographical environment (including altitudes and climate) play important roles, over the production methods of dried starter cultures, when influencing the composition of microbiota (Jeyaram et al., 2011; Nam et al., 2012; Lv et al., 2012, 2013). Besides these, other factors that may affect the composition of mycobiome communities in dried starters include the level of hygienic conditions, quality of the glutinous rice or other cereal substrates, quality and source of water, as well as the back-slopping techniques used by the ethnic people (Capozzi and Spano, 2011; Gonelimali et al., 2018; Sha et al., 2018). There may also be the possibility of air-borne resources of fungal diversity in these tested samples (Cuadros-Orellana et al., 2013; Aguayo et al., 2018), probably during traditional preparation of starter.

The percentage distribution of total yeast and mold species found in different starters with their respective morphology and mode nutrition is shown in **Supplementary Table 2**. Saprotrophic mode of mold species was encountered in starters with a dominance range of 64 to 99% over other modes. In other hands, yeast morphotypes were more dominants in all samples (**Figure 4A**). Several families were enlisted for the diversity players inside the starters; most of them were saprophytes irrespective of the taxonomy. The Saccharomycopsidaceae family showing the saprotrophic mode of nutrition were found to be abundant, and the pathogenic Pleosporaceae family (Ariyawansa et al., 2015) had a lower abundance in Ascomycota phylum (**Figure 4B**). Most of the families were associated with the functional attributes to the KEGG Orthologous for the eubacterial diversity. Some important correlations have been observed between families and functional guilds (**Figure 5**). Interestingly, Pichaceae was negatively correlated to the micro-fungus morphotypes, and such correlations have been suggesting the extrusion of the diversity simulation (Schoch et al., 2012).

Functional attributes of the fungal diversity were formulated with bioinformatics tools, based on methods described by Gweon et al. (2015) and Nguyen et al. (2016). Since the ITS region has been recognized as the universal barcode for identification of fungi (Schoch et al., 2012), we used this region for fungal bar code with reference to database UNITE for OTU assignment. We applied the PIPITS pipeline since it extracts the ITS sub-region from raw reads and assigns taxonomy with a trained RDP Classifier. Total 662461 sequences were identified out of 689459 sequences, as containing an ITS2 sub-region. After quality filtering and removal of contaminants, we obtained results in 2402833 quality sequences. We set 59612 sequences per sample for further analysis to form 354 OTUs, which yielded 190 phylotypes. Several functions of the mycobiome were observed after the funguild function analysis (**Supplementary Table 3**). However, comparing with culture-independent method, the culturable diversity is more relevant for development of a potent starter in beverage industries (Sha et al., 2018).

CONCLUSION

Our study has shown a wide diversity of yeast and mold species (594 fungal species) in dry starters of North East India, based on nucleotides sequences clustered into OTUs, following the amplicon sequencing using a high-through sequence platform as well as bioinformatics tools. Taxonomical identifications of some sample-specific species of mycobiome in these starters are a remarkable observation in lesser-known, traditionally prepared dry starters for alcohol production in India. The present study demonstrated the baseline data for mycobiome diversity in traditionally prepared dry starters of India.

AUTHOR CONTRIBUTIONS

SS conducted the major experiments. MS has assisted with the bioinformatics. JT supervised the experiments and finalized the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00348/full#supplementary-material

FIGURE S1 | Distribution of rare-phylotypes category with 19 different class level taxa in six starters of North East India.

FIGURE S2 | Correlation among mycobiome diversity of six starters (*chowan, dawdim, hamei*, *humao, khekhrii,* and *phut*) **A**: 6 species of yeasts by culture-dependent method (ITS-PCR) (Sha et al., 2018), **B**: 24 species (yeasts = 6 and molds = 24) by PCR-DGGE analysis (Sha et al., 2018) and **C**: Total 594 species (yeasts = 83 and molds = 511) by high-throughput sequencing technique. Number and identity of each species are presented in **Supplementary Table 1**.

TABLE S1 | Table showing total number of 594 fungal species detected by HTS including common species (224), unique species (133) and rare-species (237), and their taxonomic affiliations in samples of *chowan, dawdim, hamei*, *humao, khekhrii,* and *phut*. Table also shows number of yeast species (6) detected by ITS-PCR, and yeast (18) and mold species (6) by PCR-DGGE in the same samples as reported earlier by Sha et al. (2018).

TABLE S2 | Table showing percent distribution of total yeast and mold species in different starters with their respective morphology and mode nutrition.

TABLE S3 | Different operational taxonomic units (OTUs) with taxonomy and their respective functional attributes observed in different dried starters. Generation of OTUs and functional attributes were derived through PIPITS (Gweon et al., 2015) and FUNGuild (Nguyen et al., 2016) environments.

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The Effects of Unfermented and Fermented Cow and Sheep Milk on the Gut Microbiota

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A variety of fermented foods have been linked to improved human health, but their impacts on the gut microbiome have not been well characterized. Dairy products are one of the most popular fermented foods and are commonly consumed worldwide. One area we currently lack data on is how the process of fermentation changes the gut microbiota upon digestion. What is even less well characterized are the possible differences between cow and other mammals' milks. Our aim was to compare the impact of unfermented skim milk and fermented skim milk products (milk/yogurt) originating from two species (cow/sheep) on the gut microbiome using a rat model. Male Sprague-Dawley rats were fed a dairy-free diet supplemented with one of four treatment dairy drinks (cow milk, cow yogurt, sheep milk, sheep yogurt) for 2 weeks. The viable starter culture bacteria in the yogurts were depleted in this study to reduce their potential influence on gut bacterial communities. At the end of the study, cecal samples were collected and the bacterial community profiles determined via 16S rRNA high-throughput sequencing. Fermentation status drove the composition of the bacterial communities to a greater extent than their animal origin. While overall community alpha diversity did not change among treatment groups, the abundance of a number of taxa differed. The cow milk supplemented treatment group was distinct, with a higher intragroup variability and a distinctive taxonomic composition. *Collinsella aerofaciens* was of particularly high abundance (9%) for this group. Taxa such as Firmicutes and *Lactobacillus* were found in higher abundance in communities of rats fed with milk, while Proteobacteria, Bacteroidetes, and *Parabacteroides* were higher in yogurt fed rats. *Collinsella* was also found to be of higher abundance in both milk (vs. yogurt) and cows (vs. sheep). This research provides new insight into the effects of unfermented vs. fermented milk (yogurt) and animal origin on gut microbial composition in a healthy host. A number of differences in taxonomic abundance between treatment groups were observed. Most were associated with the effects of fermentation, but others the origin species, or in the case of cow milk, unique to the treatment group. Future studies focusing on understanding microbial metabolism and interactions, should help unravel what drives these differences.

Keywords: gut microbiome, fermentation, cow, sheep, cecal microbiota

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INTRODUCTION

The old adage "You are what you eat," now appears to accurately reflect the evidence behind the composition and function of the gut microbiome. While other factors such as genetics (Turnbaugh et al., 2009) appear to play a part, environmental factors such as diet have amassed a large amount of evidence as to their influence in building and modifying gut microbial communities (Spor et al., 2011; David et al., 2014; Bokulich et al., 2016). The microbiome has a vital and complex symbiotic relationship with their host, and a fine balance between the two appears necessary to maintain optimal health. Although there are limited studies that concretely prove a cause and effect (i.e., is it microbiota dysbiosis that causes disease or disease that shifts the microbiota), a number of health conditions, including obesity, diabetes, and inflammatory bowel disease (IBD), have been linked to shifts in the gut microbiome (Ley et al., 2005; Frank et al., 2007; Cho and Blaser, 2012). Trying to elucidate what drives and constitutes a balanced gut microbiome and how we can maintain or directionally alter it favorably, has driven research into understanding the effects of food on the gut microbiome.

Fermented foods have a long history of use in humans and have been associated with a number of health benefits (Hata et al., 1996; Kawase et al., 2000; Guyonnet et al., 2007). Consumption of fermented products has been linked to improvements in cholesterol and blood pressure levels, a reduced risk of type 2 diabetes, and reduction in gut irregularity (Chen et al., 2014; Fekete et al., 2015; Cardoso Umbelino Cavallini et al., 2016; Díaz-López et al., 2016; Laatikainen et al., 2016; Nagata et al., 2016). To produce fermented food products, live microorganisms are added to a food, and metabolic activities modify the physical and nutritional characteristics by breaking down complex components and forming by-products.

Although many studies credit the observed health benefits to the live microorganisms in fermented foods, some studies have shown beneficial effects of fermented products containing no viable microorganisms (Ouwehand and Salminen, 1998). These effects have been attributed to the interaction of non-viable bacteria directly with the immune system or the products of microbial fermentation, such as bioactive peptides (Ouwehand and Salminen, 1998). Purified versions of these peptides have been shown to have an effect on bacterial growth (Hartmann and Meisel, 2007; Erdmann et al., 2008) which could potentially modify the composition and function(s) of the gut microbiota.

Yogurt is one of the most commonly consumed fermented foods. Although cow milk is most commonly utilized, a variety of other mammalian milks are available for the commercial production of yogurt. The use of non-bovine milk products has become more popular in Western markets, as people with allergies or sensitivities to cow milk may be able to tolerate non-bovine products (El-Agamy, 2007). The milks across mammalian species also have different nutritional and flavor profiles, which may encourage their consumption.

Currently, we lack an understanding of how consuming fermented foods differs in impact on the gut microbiome from their unfermented forms. Fermentation is known to alter nutritional availability and bioactive compounds, including bioactive peptides, which might be expected to impact the microbiota (Azuma et al., 1984; Liepke et al., 2002). There is also some debate on the extent that fermented foods are capable of affecting gut microbial communities (Veiga et al., 2010, 2014; McNulty et al., 2011). As composition of milk constituents differs among mammals, differences in their effect on the microbiota and pre- and post-fermentation may also occur.

Our aim was to characterize the effects of unfermented and fermented milk (yogurt) originating from cow and sheep on the composition of the gut microbiota. We hypothesized that we would observe differences in bacterial communities due to fermentation, but animal origin would more robustly influence those differences. Rats were fed a solid dairy-free diet supplemented with one of four dairy treatments for 2 weeks. Their cecal bacterial communities were then characterized by looking at diversity, community composition, and correlations with metadata.

MATERIALS AND METHODS

The rats used in this study were part of another experiment (parallel) that examined the effect of these dairy treatments on transit time and characterized their peptide profiles and potential bioactivity (Dalziel et al., 2018). Here we separately assessed the cecal contents following the completion of that study. The methods regarding the animal study and dairy drinks are described in Dalziel et al. (2018) but reiterated here for clarity.

Dairy Drinks Treatments

Cow skim milk powder [SMP 001 (111115)] (38% protein, <0.1% fat, 45% lactose) was donated by NZ Food Innovation Ltd. (Hamilton, New Zealand) while sheep skim milk powder (031215 Cipher number KY03) (52% protein, 1% fat, 37% lactose) was contributed by Blue River Dairy (Invercargill, New Zealand). Powdered milk rather than fresh was used due to the variable availability of large quantities of fresh skimmed sheep milk and for nutrient batch consistency. The pH of the reconstituted cow and sheep milk was measured and found to be 6.5 ± 0.1 for both species. To produce the yogurt, the cow (140 g/L) and sheep (105 g/L) skim milk powders were rehydrated in water using a stick blender for 2 h. Each milk was then slowly heated to 85°C over 2 h and held at that temperature for 30 min, while under constant stirring. The milks were then cooled to 43°C and a starter culture mix (0.26 U/L) of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* ssp. *bulgaricus* (CHR Hansen YF-L811 – YoFlex®) was added. This starter culture was chosen for its reliability in producing a consistent product as a drinkable yogurt. Inoculated milks were incubated at 43°C until the pH dropped to 4.5 (4 to 5 h). The yogurts were then frozen at −20°C in shallow trays. To reduce bacterial viability and improve freeze-drying, the yogurts were annealed by thawing to −5°C and refreezing at −20°C before freeze-drying.

The dairy drinks were prepared for usage by reconstituting the milk or yogurt powder to a 3% protein concentration in water and blending for 30 s. Drinks were made up daily and provided as two feeds with half kept at 4°C before use in the animal experiment.

A 3% protein concentration was chosen so the yogurt could be delivered as a free-flowing drinkable substance.

To determine the bacterial viability of *S. thermophilus* and *L. delbrueckii* ssp. *bulgaricus* in dairy drinks, milks and yogurts were resuspended in water at 3% protein by blending (30 s). Serial dilutions were performed in phosphate buffered saline (PBS) and 100 μL spread on selective agar plates in triplicate. *S. thermophilus* was grown at 37°C for 24–48 h on Mitis Salivarius agar under 5% CO_2 conditions. *L. delbrueckii* ssp. *bulgaricus* was grown at 45°C for 72 h on MRS agar (pH 5.2) (Fort Richard Laboratories Ltd, Auckland, New Zealand) under anaerobic conditions. *L. delbrueckii* ssp. *bulgaricus* was not detected in any milk or yogurt samples. *S. thermophilus* was recovered for the cow yogurt (4.5×10^6 CFU/mL) and sheep yogurt (1.5×10^4 CFU/mL), but not recovered from the milks.

Animal Care and Study Design

The animal study was approved by the AgResearch Grasslands Animal Ethics Committee (Palmerston North, New Zealand) (AE13501) in compliance with the Animal Welfare Act (1999, New Zealand). Forty male Sprague-Dawley rats (initial weight 404 ± 27 g), were obtained from the AgResearch Small Animal Breeding Unit (Hamilton, New Zealand). The rats were individually housed at a constant 21°C with a 12/12 h light/dark cycle. Starting from 10 weeks of age, the rats were fed a dairy-free solid AIN-93M OpenSource Rodent Diet (Research Diets Inc., New Brunswick, NJ, United States) with an egg white protein source. The composition of the rat solid diet is provided in **Supplementary Table S1**. At 12 weeks of age, one of four dairy drinks [CM, cow milk; CY, cow yogurt; SM, sheep milk; SY, sheep yogurt] were provided *ad libitum* for 14 days (10 rats per group). General health score, weight, and dietary intake were recorded three times a week. Following the completion of the study, the rats were euthanized via CO_2 overdose inhalation and cervical dislocation. Cecal samples were aseptically collected from rats and immediately stored at −80°C.

Microbiota Sequencing and Analysis

DNA was extracted from cecal samples that were thawed on ice using the Macherey Nagel Nucleospin Soil kit following the manufacturer's instructions with the addition of a 5 min bead-beating step (0.6–0.8 mm ceramic beads, FastPrep 120). A NanoDrop™ (Thermo Fisher Scientific) was used to quantify the DNA. DNA samples were then submitted to Omega Bioservices (Norcross, GA, United States) for library preparation and high-throughput sequencing. The libraries were prepared using the Illumina 16S Metagenomic Sequencing kit (Illumina, Inc., San Diego, CA, United States) according to the manufacturer's protocol. The V3-V4 region of the bacterial 16S rRNA gene was amplified using the 16S Amplicon PCR Forward Primer (5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG) and 16S Amplicon PCR Reverse Primer (5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC). Amplicon PCR was performed to amplify template out of input DNA samples. Briefly, each 25 μL of polymerase chain reaction (PCR) reaction contained 12.5 ng of sample DNA as input, 12.5 μL 2× KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Wilmington, MA, United States) and 5 μL of 1 μM of each primer. PCR reactions were carried out using the following protocol: an initial denaturation step performed at 95°C for 3 min followed by 25 cycles of denaturation (95°C, 30 s), annealing (55°C, 30 s), extension (72°C, 30 s), and a final elongation of 5 min at 72°C. PCR products were purified using Mag-Bind RxnPure Plus magnetic beads (Omega Bio-tek, Inc., Norcross, GA, United States). A second index PCR amplification, used to incorporate barcodes and sequencing adapters into the final PCR product, was performed in 25 μL reactions, using the same master mix conditions as described above. Cycling conditions were as follows: 95°C for 3 min, followed by eight cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. A final 5 min elongation step was performed at 72°C. The libraries were normalized with the Mag-Bind®EquiPure Library Normalization Kit (Omega Bio-tek, Inc., Norcross, GA, United States) then pooled. The pooled library ∼600 bases in size was checked using an Agilent 2200 TapeStation and sequenced (2 × 300 bp paired-end read setting) on a MiSeq (Illumina, San Diego, CA, United States).

The sequence data was analyzed using mothur following the methods of Schloss et al. (2009, 2011). Briefly, paired-end reads (3,519,095 pre-quality control paired reads) were assembled and underwent quality control that removed reads containing uncalled bases and homopolymers of greater than eight. The average quality scores across all bases was greater than 30 and the average sequence length was 416 bp. Sequences were then aligned against the SILVA database (release 132) (Quast et al., 2013). Following sequencing alignment and filtering, the sequencing reads underwent a pre-clustering step (4 bp) designed to denoise and reduce the effect of sequencing errors. Chimeric sequences were detected and removed using VSEARCH (Rognes et al., 2016). Following taxonomic classification, non-bacterial sequences were removed from the analysis and sequences were clustered into OTUs at a 97% cutoff. A total of 1,434,251 reads remained following all quality control and filtering steps with an average of 29,880 reads per sample. The number of sequences per sample was subsampled to 17,323 to equalize the number of reads across samples for all downstream analyses. BIOM tables were exported from mothur for use in other analysis programs. Raw output from mothur taxonomic classifications is available in **Supplementary Table S2**.

Alpha-diversity was calculated using the Shannon index. Taxonomic graphs were generated using classifications from mothur. LEfSe was used to identify taxonomic features significantly different ($p < 0.05$) and most likely to explain the differences in bacterial communities (LDA ≥ 2) (Segata et al., 2011). An OTU most likely species identity was determined by a BLAST nr search (Altschul et al., 1990), utilizing the top hit. Bar chart graphics for taxonomic graphs and LEfSe results were generated using Microsoft Excel®. Multiple linear regression analyses were performed using Calypso (Zakrzewski et al., 2017) to determine bacterial OTUs that correlated with previously collected intestinal transit scores (Dalziel et al., 2018). Rats with transit scores of less than 10 (6 rats) were removed from the analysis (2 CY, 2 SM, 2 SY), as it has been previously shown

that ∼10% of animals can have significantly delayed stomach emptying resulting in unusable transit data (Dalziel et al., 2016).

Statistical Analyses

Comparisons between rat dietary intake values and weights were analyzed using a one-way ANOVA. Standard deviations (SD) were also calculated and reported for average intake and body weight increases. PERMANOVA and PERMDISP analyses were perform with Calypso. P values less than 0.05 were considered significant.

RESULTS

Rat Dietary Intake and Weights

Rats in this study were fed a solid dairy-free diet to remove the potentially confounding effects of other dairy components in the diet. Powdered skimmed milks were used and the dairy drinks were diluted to 3% protein to level the fat and protein intake.

Rats across all treatment groups had an average daily intake of 27 ± 3.5 g (SD) of solid food and body weight increased by 19 ± 3.9% (SD) over 14 days (**Supplementary Table S3**) (Dalziel et al., 2018). There was no significant difference between daily solid food intake or treatment group weights on Day 0 or 14 (**Supplementary Table S3**). For the dairy drinks, the CM group (87.48 mL) had the lowest daily mean intake and the CY group the highest (103.73 mL), but the intake difference between all groups was not statistically significant (ANOVA, $p = 0.07$) (**Supplementary Table S3**).

Cecal Microbiota Differs Among Dairy Drink Treatments

To determine if there were differences among the four dairy drink treatment groups, the high-throughput sequencing data was analyzed for diversity, community composition, taxonomy, and correlations with metadata. To look at alpha diversity, the Shannon indices were determined. We found no significant differences between the treatment groups (**Supplementary Figure S1**).

Next, we determined differences in overall bacterial community composition. A Bray-Curtis principal component analysis (PCA) plot did not show a distinct separation between treatment groups, but rather more of a gradient-like distribution with the cow milk treatment showing the largest spread between individual rats (**Figure 1**). To determine if there might be a significant difference between groups, PERMANOVA was performed, which suggested a difference between the treatment groups ($p < 0.001$). To look at the possible effects of intragroup variation, PERMDISP was performed which indicated a significant difference ($f = 4.6861$, $p = 0.0063$). The combined results from the PERMANOVA and PERMDISP analyses indicate that while there was a difference between treatment groups, at least some of those differences were due to variance in intragroup variability.

The taxonomic composition of the treatment groups on phylum and family levels are visualized in **Figure 2**. At phylum level (**Figure 2A**), the cow milk (CM) group was particularly distinct with increased Actinobacteria and decreased Bacteroidetes. The milks (CM, SM) had similar amounts of Firmicutes while the yogurts (CY, SY) contained comparable ratios of both Firmicutes and Bacteroidetes. At a family-level taxonomic classification (**Figure 2B**), taxonomic abundance differences could also be seen in specific treatments (e.g., higher *Coriobacteriaceae* in CM) and between unfermented and fermented products (e.g., higher *Lactobacillaceae* in milks). The difference in community intragroup variation noted by PERMDISP analysis, suggested higher taxonomic variability among individual rats in some treatment groups (e.g., CM) (less uniform taxonomic composition). Taxonomic graphs of individual rats highlight these samples (**Supplementary Figure S2**).

In order to identify robust and meaningful differences in taxonomic abundances between treatment groups, we chose to use LEfSe to identify taxonomic groups with significant differences ranging from phylum to OTU. LEfSe identified 16 taxa with significant changes (**Figure 3**). While a number of taxa were identified, similar to the taxonomic composition graphs, the CM dairy drink treatment clearly had a more unique taxonomic representation. Its particularly high abundance in *Collinsella aerofaciens* (OTU6) (*Collinsella*, *Coriobacteriaceae*, *Coriobacteriales*, *Coriobacteriia*, Actinobacteria) and lower abundance in *Acidaminococcaceae* (*Selenomonadales*, *Negativicutes*) were absent from the other dairy drink groups.

Fermentation Status Influences Cecal Microbial Communities

The treatment-specific analysis identified that fermentation status (milk vs. yogurt) appeared to influence the composition of the microbial communities. With this in mind, we decided to use LEfSe to compare the combined milk (CM + SM) to the combined yogurt (CY + SY) bacterial communities (**Figure 4**). Fifteen of the sixteen taxa identified in the comparison of the four treatments, also were significant in the milk vs. yogurt comparison. An additional 12 taxa were also identified as significantly different. Despite attempts to deplete the starter culture bacteria from the yogurts, some viable *Streptococcus thermophilus* (CY: 4.5×10^6 CFU/mL, SY: 1.5×10^4 CFU/mL) were introduced into the rats. These counts are multiple logs lower than the commonly observed abundances in commercial yogurts (10^8–10^9 CFU/mL) (Ibrahim and Carr, 2006), however, the viable number of *S. thermophilus* introduced into the rats may have been greater than the initial plate counts as the yogurts sat at room temperature for several hours during the feeding. Thus unsurprisingly, *S. thermophilus* (OTU27) was found in higher abundances in the yogurt, while it was nearly undetectable in the milk samples. Interestingly, *Lactobacillus*, including *Lactobacillus murinus*, was found to be higher in the milks possibly due a greater substrate availability which may have been depleted during fermentation. While *Collinsella* was still found in a higher overall abundance in milk, *C. aerofaciens* (OTU6) was not significantly different in the fermentation status comparison. The genera *Phascolarctobacterium*, *Desulfovibrio*, and *Parabacteroides*

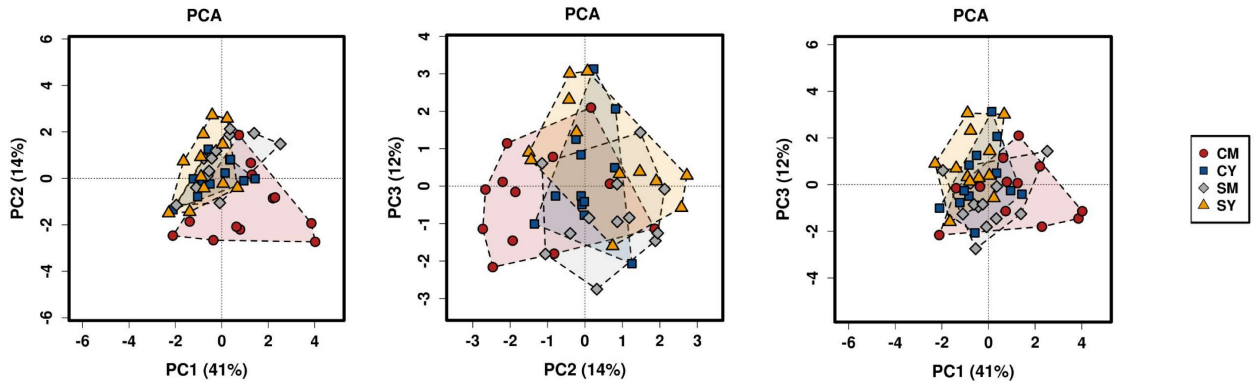

FIGURE 1 | A PCA plot of the differences in bacterial community composition between the four dairy drink treatment groups [CM, cow milk; CY, cow yogurt; SM, sheep milk; SY, sheep yogurt] as calculated using the Bray-Curtis index. The analysis was based on OTU-level taxonomic data.

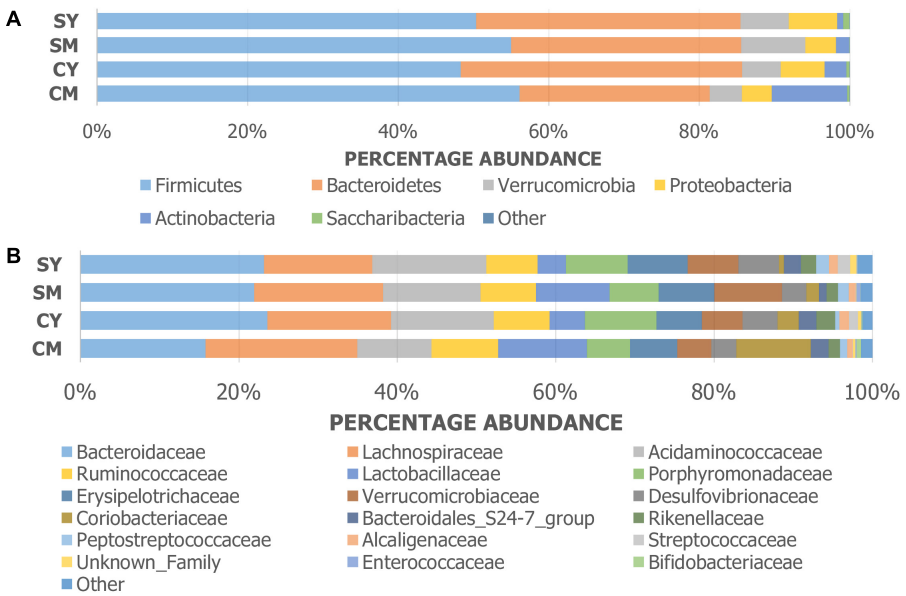

FIGURE 2 | Taxonomic graphs representing the **(A)** phylum and **(B)** family level bacterial communities (group average) found in each dairy drink treatment group [CM, cow milk; CY, cow yogurt; SM, sheep milk; SY, sheep yogurt].

were more abundant in yogurt. At a higher phylum taxonomic level, we noted a decrease in the Firmicutes:Bacteroidetes ratio in the yogurt along with an increase in Proteobacteria and decrease in Actinobacteria.

Product Species Origin Has Little Effect on Cecal Microbial Communities

Since fermentation status appeared to influence the cecal bacterial community composition, we also decided to test if species origin (cow vs. sheep) might affect the bacterial communities using LEfSe (**Figure 5**). While six taxa were identified as significantly different, the majority of the shift could be linked to a single OTU (*C. aerofaciens*, OTU6) suggesting a lesser influence of species origin on the overall bacterial community. This taxon was also identified in the treatment analyses and the genus *Collinsella* in the fermentation status analysis, suggesting multiple factors affect its abundance.

Transit Times Do Not Strongly Correlate With Bacterial Taxa Abundance

A parallel study run on the same rats, measured gut transit to determine if the different dairy products affected transit times. It was reported that transit was altered due to treatment method. Those effects appeared to be more related to the origin of the product (cow vs. sheep), than whether they were fermented or not (Dalziel et al., 2018). To determine whether the observed changes in transit scores could be linked to the bacterial communities, we performed regression analysis on the top 100 OTUs, utilizing the combined samples for greater statistical power. Only a single weak significant negative correlation was

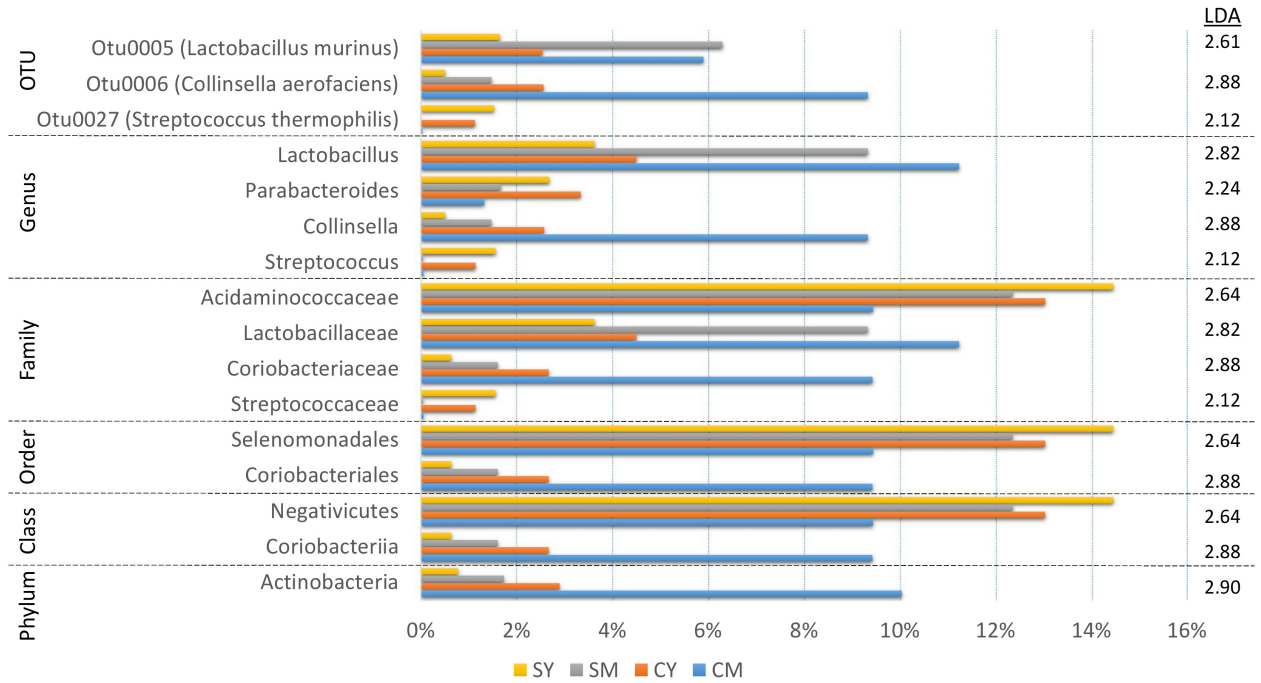

FIGURE 3 | A bar graph of the taxonomic groups indicated by LEfSe analysis to be the most likely to explain the differences between the four dairy drink treatment groups [CM, cow milk; CY, cow yogurt; SM, sheep milk; SY, sheep yogurt] ($p < 0.05$, LDA score ≥ 2). The bars represent the abundance of the taxa found in each treatment group. The LDA scores are listed on the right.

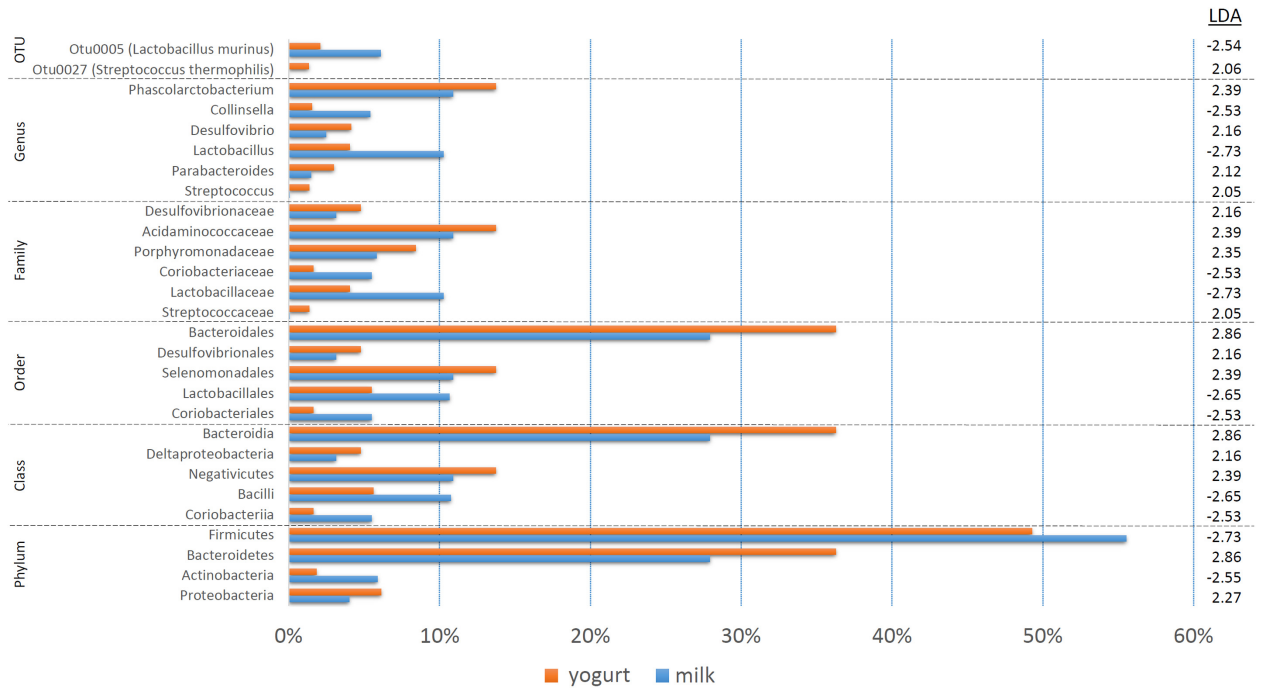

FIGURE 4 | A bar graph of the taxonomic groups indicated by LEfSe analysis to be the most likely to explain the differences between the product types (milk vs. yogurt) ($p < 0.05$, LDA score ≥ 2). The bars represent the abundance of the taxa found in each group. The LDA scores are listed on the right.

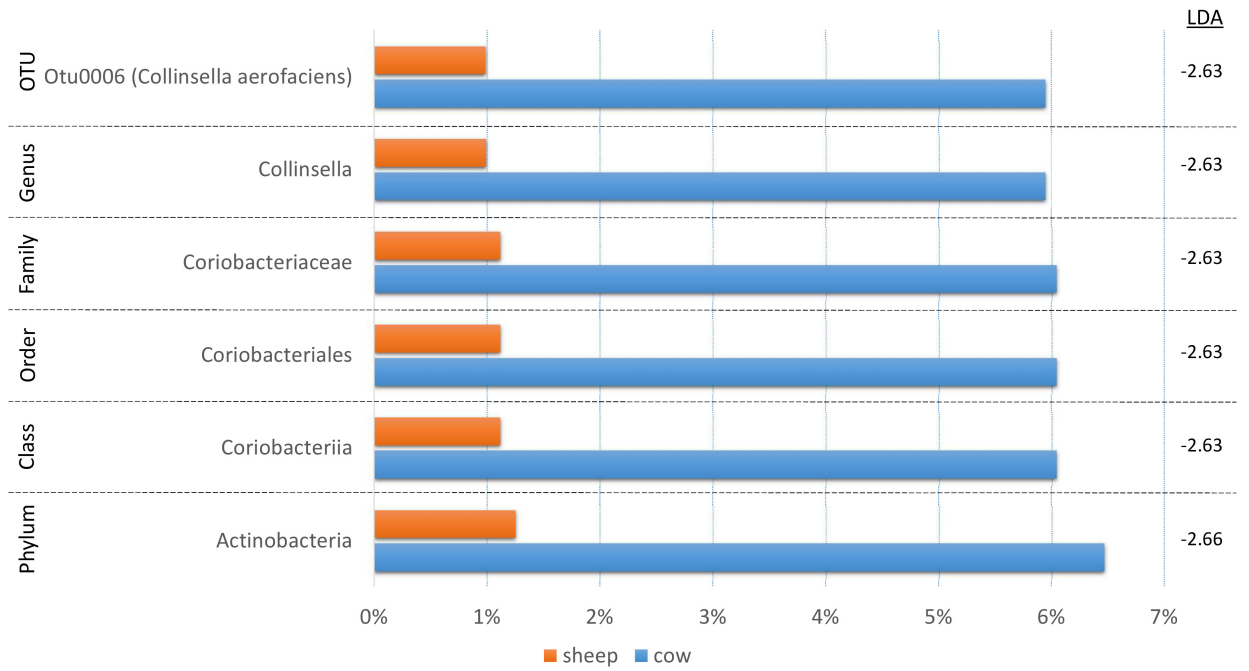

FIGURE 5 | A bar graph of the taxonomic groups indicated by LEfSe analysis to be the most likely to explain the differences in product origins (cow vs. sheep) ($p < 0.05$, LDA score ≥ 2). The bars represent the abundance of the taxa found in each group. The LDA scores are listed on the right.

discovered ($r = -0.3722$, $p = 0.015$) between *Ruminococcaceae* UCG-014 (OTU17) (mean abundance = 1.5%) and total transit score, suggesting abundance of specific taxa was not strongly associated with gut transit (**Supplementary Figure S3**).

DISCUSSION

Here we report novel findings of the impact of unfermented and fermented milk (yogurt) from cows and sheep on the rat cecal bacterial composition. The use of healthy rodent models in our study allowed us to control both dietary and environmental factors that would be difficult to implement in human studies and removes confounding factors that make data interpretation more difficult. We showed that the effects of fermentation appeared to have a moderate influence on the composition on the gut microbiota, while animal species origin of the drinks had a small impact. This is in contrast to the parallel gut transit study in which dairy species origin was the dominant influence (Dalziel et al., 2018).

While consumption of fermented milk products containing active bacterial cultures has frequently shown increased recovery of those bacteria within the gut microbiota (Mater et al., 2005; Elli et al., 2006; Oozeer et al., 2006), any effects on the rest of the gut microbiota are less well characterized. Few studies have looked at the effects of fermented milks on the gut microbiota in healthy individuals, and particularly absent are comparisons between unfermented and fermented products. We observed differences in bacterial community composition between consumption of milk and yogurt that could be linked to both the fermentation starter culture (*S. thermophilis*) and other gut community members. Although the viable bacteria in the yogurt starter culture were heavily depleted or eliminated, non-viable/dead cells still may have influenced the composition of the gut microbiota. In contrast, a study in healthy human twins consuming fermented cow milk showed no detectable effect on bacterial community composition (McNulty et al., 2011), although the number of reads per sample was small (1,640 reads), suggesting a lack of depth to detect differences. Other investigations in healthy humans have reported similar results for yogurt (Filteau et al., 2013).

Studies that have compared the effects of both fermented and unfermented milks have been done in both humans and rodents, but they have used patients or rodent models that have or mimic gut dysfunction such as irritable bowel syndrome (IBS) or IBD (Veiga et al., 2010, 2014; Rooks et al., 2014). Rodent studies comparing fermented and unfermented cow milk treatments identified increases in *Desulfovibrio* and Proteobacteria in fermented milk-treated mice and increases in *Lactobacillus* in the unfermented milk-treated mice that match with results observed in our study (Veiga et al., 2010; Rooks et al., 2014). While increases in Proteobacteria and *Desulfovibrio* have been observed in several disease states vs. healthy subjects, we cannot yet discern if and what taxonomic load might represent or lead to a disease status (Gibson et al., 1991; Rowan et al., 2010; Shin et al., 2015). Most *Lactobacillus* are generally thought to be commensal or indicative of a healthy gut microbiota (Fijan, 2014). We did not observe an increase in *Anaerostipes* and *Eubacterium* or decrease in *Enterobacteriaceae* in the fermented milk as compared to unfermented milk. A human study identified

an increase in *Roseburia inulinivorans* in a fermented milk treatment compared to the unfermented milk treatment gut bacterial communities (Veiga et al., 2014). Butyrate producers, such as *R. inulinivorans*, may play an important role in preventing GI disorders such as Crohn's disease (Takahashi et al., 2016).

In our study, we also observed an increase in *Phascolarctobacterium* and *Parabacteroides* in the yogurts. At a higher phylum taxonomic level, we saw a decrease in the Firmicutes:Bacteroidetes ratio in the yogurts along with a decrease in Actinobacteria. *Phascolarctobacterium* has been correlated with positive mood so may play a role in the gut-brain axis (Li et al., 2016). *Phascolarctobacterium* is also a known producer of short-chain fatty acids, including butyrate (Lecomte et al., 2015), so it may mirror some of metabolic effects induced by the increase in *R. inulinivorans* observed in the previously mentioned study (Veiga et al., 2014). A decrease in the Firmicutes:Bacteriodetes ratio has previously been associated with a decrease in body mass index (BMI) (Ley et al., 2006; Turnbaugh et al., 2009). It is unsurprising to note differences in the results between our study and others, as differences in methodology (e.g., diseased vs. healthy host, mice vs. rats vs. human host, fecal vs. cecal samples, 16S vs. shotgun sequencing) substantially influence the outcome. Another factor that may have influenced the results, is that powdered milks were utilized in this study while raw or pasteurized fresh milk have been studied previously.

The fecal microbiota has previously been shown to differ between rats fed cow and other mammals' milks (e.g., yaks, camels) (Wen et al., 2017). Our analysis also indicated differences in taxonomic abundances between cow and sheep milk, but overall the effects of milk origin were less pronounced than fermentation. The cow milk treatment group was distinct, as it exhibited treatment-specific effects not seen in the other groups. It also showed the greatest intragroup variability which indicates that cow milk may cause more variable gut microbial communities. This suggests particular substrates present in cow milk may trigger a non-uniform response on bacterial communities. Since these effects were not noted in the cow yogurt treatment, fermentation seemingly removes this result. It also appears to be animal species specific, as it was not seen in either sheep treatment group. To help further illustrate the variability in the cow milk treatment group, a pair-wise Morisita-Horn dissimilarity index matrix was generated in mothur (**Supplementary Figure S4**). Variability (e.g., SM44), was also observed in other treatment groups but was limited as compared to the cow milk treatment samples (**Supplementary Figure S4**). The high intragroup variability among cow milk samples, also led us to reconfirm the LEfSe results by removing the extreme outliers and performing a one-way ANOVA ($p < 0.05$) on the bacterial abundances (e.g., *Collinsella*, *Acidaminococcaceae*). The differences remained significant with or without the outliers.

Bacteria of particular interest from this research included *Collinsella* and *Lactobacillus*. The abundance of *Collinsella* appeared to be influenced by both fermentation (milk vs. yogurt) and species origin (cow vs. sheep) and was found in higher abundance in milk (as compared to yogurt from same animal species), particularly cow milk. *Collinsella aerofaciens*, generally considered a gut commensal, is commonly found in the human intestine (Rajilić-Stojanović and de Vos, 2014). Increased *Collinsella* abundance has been associated with both positive and negative health conditions (Malinen et al., 2010; Joossens et al., 2011; Lambeth et al., 2015; Chen et al., 2016), but there is really no consensus of its health effects. Further study is needed as it is not well characterized and has been reported in very low abundance, including those linked to health outcomes. *C. aerofaciens* is known to be able to ferment a range of different carbohydrates, including starches (Kageyama et al., 1999), and there is evidence that it plays a major role in gut lactose fermentation (Kovatcheva-Datchary, 2010). Studies have linked the use of fructooligosaccharides (FOS) and galactooligosaccharides (GOS) to increased activity (Tannock et al., 2004) or abundance (Dewulf et al., 2013; Azcarate-Peril et al., 2017). In a 15 member human gut model in gnotobiotic mice, *C. aerofaciens* was reduced after addition of fermented milk bacterial strains, possibly due to competition with lactic acid bacteria (McNulty et al., 2011).

Lactobacilli are capable of metabolizing a wide range of oligosaccharides and some are considered probiotic (Gänzle and Follador, 2012). *Lactobacillus* sp. have been linked to a number of beneficial effects including improvement in depression symptoms and gut function (Verna and Lucak, 2010; Wallace and Milev, 2017). Although lactic acid bacteria such as *Lactobacillus* are often associated with yogurt (Adolfsson et al., 2004), this may be due to their delivery as live fermentation starter cultures rather than actual growth stimulation of lactobacilli *in vivo*. We found increased abundances of *Lactobacillus* in both cow and sheep milk treatments as compared to their yogurt counterparts which may have been influenced by differences in substrate availability. Lactose can decrease more than 50% post-fermentation (yogurt vs. milk) (Alm, 1982) and the milks used in this study also differed in lactose concentrations (cow milk 3.6%, sheep milk 2.2%). The by-products of lactose fermentation (e.g., lactic acid, ethanol) in yogurt by beta-galactosidases, may be linked to our observed increase in *Desulfovibrio* which can use lactate as an electron acceptor (Price et al., 2014). In this study, there appears to be additional factors at play since changes in abundance of *Collinsella*, another lactose utilizer, do not parallel *Lactobacillus*. Lactic acid bacteria in starter cultures also utilize proteolysis to help successfully drive the fermentation process (Savijoki et al., 2006). This has downstream effects on the availability of types of substrates in fermented products, such as peptides, which could influence microbial abundances (Raveschot et al., 2018).

Bacterial strains exhibit individual preferences for nutrients that affect their ability to compete for specific resources (Fischbach and Sonnenburg, 2011). Both milk species origin and fermentation status influence the availability of nutrients and bioactive compounds in milk products (Pessione and Cirrincione, 2016; Balthazar et al., 2017). Raw sheep milk has a greater amount of protein, fat, and total energy compared to cow (Balthazar et al., 2017). The concentration of vitamins, minerals, and lactose concentrations can vary between animal species (Balthazar et al., 2017). In this study, the use of skim milks reduced the influence of absolute fat. While the milks used

in this study were also balanced for protein concentration, the types of proteins in cow and sheep milk still differ and may have influenced the composition of the cecal microbiota.

While lactose is the only major carbohydrate found in cow and sheep milk, the concentrations of the different types of protein fractions varies greatly. There is roughly an 80:20 ratio of total casein and whey proteins in both species, but sheep milk contains mostly β-casein (∼62%) while cow milk has 50% α-casein (40% being αS1-casein) (Balthazar et al., 2017). Cow milk only contains half the β-casein of sheep but 6× more αS1-casein. Specific types of casein have been previously shown to stimulate growth or particular functions, such as biofilm formation, in certain lactic acid bacteria (Azuma et al., 1984; Smid et al., 1991; Varhimo et al., 2011). Amino acid composition also varies among protein fractions types (e.g., α/β-casein, whey) and by animal origin (Gordon et al., 1949; Rafiq et al., 2016). The most abundant amino acid fermenters in the small intestine include genera from *Clostridiales*, Proteobacteria, and the *Bacillus-Lactobacillus-Streptococcus* group (Dai et al., 2011). While we didn't observe any substantial changes in these groups associated with animal origin, we did observe changes in Proteobacteria, *Lactobacillus*, and *Streptococcus* in the yogurts that may have been influenced by the availability of amino acids. The changes in lactic acid bacterial abundance are more likely due the difference in availability of casein between milk and yogurt. The lactic acid bacteria used in yogurt starter cultures can have casein-specific proteases which may break down casein during the fermentation process (Liu et al., 2010; Atanasova et al., 2014); this would leave less available to be delivered to the gut microbial community.

Bioactive compounds, such as peptides derived from proteolysis during fermentation, represent another mechanism by which bacterial communities can be influenced. Bioactive peptide composition and activity has been shown to differ between ruminants (e.g., cow, sheep, etc.) following *in vitro* digestion of milk (Tagliazucchi et al., 2018). Many bacterial starter and adjunct cultures used in yogurt manufacture are known to induce the release of bioactive peptides and bacterial strain influences which bioactive peptides are produced (Nguyen et al., 2015). In the parallel study, the number of different bioactive peptides present in milk increased with fermentation (Dalziel et al., 2018). Bioactive peptides from both whey and casein protein fractions of cow milk have been previously shown to stimulate the growth of *Bifidobacterium* and *Lactobacillus* (Azuma et al., 1984; Idota et al., 1994). Other milk peptides, such as lactoferrin, are known to have antimicrobial activity while their derivatives are capable of stimulating bifidobacteria growth (Liepke et al., 2002). The increased diversity and availability of these peptides following fermentation in our study, may have influenced the composition of the microbial community as some of the observed taxonomic changes are known to be influenced by bioactive peptides. In the parallel study, species origin affected total gut transit time (sheep faster than cow) while fermentation did not (milk and yogurt from same origin species were not different) (Dalziel et al., 2018). We did not note any correlations between bacterial abundance and transit time that appeared to be influenced specifically by origin species or fermentation status. A peptide analysis run in the parallel study, identified a β-casomorphin-7 peptide in both the cow milk and yogurt which may have contributed to slower transit (Dalziel et al., 2018).

Complex bacterial communities are not driven by simple direct substrate to strain-specific utilization dynamics, but rather complex interactions such as competition and cross-feeding that function more like a metabolic network (Fischbach and Sonnenburg, 2011). These types of microbial interactions may have driven observed changes in bacterial community composition in this study due to both origin species (composition of the milks) effects and fermentation. We currently lack an understanding of the outcomes of competition and cooperation for specific nutrients among gut microbial communities in their native environment, so it is difficult to predict the size and direction of their impact.

CONCLUSION

In summary, this study offers insights into the effects that fermented milk (yogurt) has on the gut microbiota as compared with unfermented milk in a healthy host. It also examined how the differences in animal origin of the milk (cow vs. sheep) might influence gut bacterial communities. We hypothesized, based partially on data from the parallel study, that animal origin would drive gut microbial composition to a greater extent than fermentation. However, while animal origin only impacted the abundance of a single genus (*Collinsella*), fermentation appeared to have a greater effect. The cow milk treatment was shown to produce changes in taxa and wider intra-treatment variation not observed in the other treatment groups. Of particular note, the abundance of *Collinsella aerofaciens* was much higher in the CM treatment than other groups. This may reflect a uniqueness in the availability of substrates and microbial interactions, including cross-feeding, that allowed it to flourish. Future studies looking at the effects of fermentation are needed to gain a greater understanding behind the microbial metabolic processes taking place by measuring bacterial metabolites and surveying microbial gene capability through shotgun metagenomics or RNA-Seq.

DATA AVAILABILITY

The sequencing data has been deposited into the NCBI SRA archive under Bioproject # PRJNA473571.

AUTHOR CONTRIBUTIONS

JD designed the study. ER analyzed and interpreted the data and wrote the manuscript. ER, EA, NR, and JD were involved in manuscript discussion and revision and approved the final version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00458/full#supplementary-material

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Unraveling the Contribution of High Temperature Stage to Jiang-Flavor Daqu, a Liquor Starter for Production of Chinese Jiang-Flavor Baijiu, With Special Reference to Metatranscriptomics

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Jiang-flavor (JF) daqu is a liquor starter used for production of JF baijiu, a well-known distilled liquor in China. Although a high temperature stage (70°C) is necessary for qualifying JF daqu, little is known regarding its active microbial community and functional enzymes, along with its role in generating flavor precursors for JF baijiu aroma. In this investigation, based on metatranscriptomics, fungi, such as *Aspergillus* and *Penicillium*, were identified as the most active microbial members and 230 carbohydrate-active enzymes were identified as potential saccharifying enzymes at 70°C of JF daqu. Notably, most of enzymes in identified carbohydrate and energy pathways showed lower expression levels at 70°C of JF daqu than those at the high temperature stage (62°C) of Nong-flavor (NF) daqu, indicating lowering capacities of saccharification and fermentation by high temperature stage. Moreover, many enzymes, especially those related to the degradation of aromatic compounds, were only detected with low expression levels at 70°C of JF daqu albeit not at 62°C of NF daqu, indicating enhancing capacities of generating special trace aroma compounds in JF daqu by high temperature stage. Additionally, most of enzymes related to those capacities were highly expressed at 70°C by fungal genus of *Aspergillus*, *Coccidioides*, *Paracoccidioides*, *Penicillium,* and *Rasamsonia*. Therefore, this study not only sheds light on the crucial functions of high temperature stage but also paves the way to improve the quality of JF baijiu and provide active community and functional enzymes for other fermentation industries.

Keywords: flavor generation, Chinese baijiu, metatranscriptomics, Jiang-flavor daqu, saccharification, high temperature stage, degradation of aromatic compounds

INTRODUCTION

Baijiu (Chinese liquor), one of the oldest known distilled liquors with an approximate 2000-year history, is the largest consumed spirit globally (over 13 billion liters in 2016) (Liu and Sun, 2018). Compared with whisky and brandy, baijiu is well known for its taste with more flavor compounds (>1870 volatile compounds) in liquor, including alcohols, aldehydes, organic acids, esters, phenols, lactones, heterocycles, terpenes, aromatic compounds, amino acids, and peptides, which leads to the final special aroma and health of baijiu (Jin et al., 2017; Liu and Sun, 2018). Thus, based on its distinctive flavor characteristics, baijiu can be divided into three major categories [i.e., Jiang-flavor (JF, also called sauce-flavor) baijiu, Nong-flavor (NF) baijiu and Qing-flavor (QF) baijiu] and nine minor categories, among which JF baijiu is with a full-bodied long-lasting aroma (Zheng and Han, 2016; Liu and Sun, 2018). The representative JF baijiu is moutai, the most famous baijiu, having the distinction as "the national liquor" and largely dominating the market in China (Zheng and Han, 2016; Jin et al., 2017). JF baijiu is fermented and distilled under solid-state conditions with a production process that mainly includes four distinct stages; i.e., daqu preparation (approximately 4 months), stacking fermentation (2–4 days), alcoholic fermentation and distillation processes (**Figure 1**) (Fan et al., 2012; Zheng and Han, 2016).

Typically, daqu, a liquor starter used to initiate the alcoholic fermentation process, constitutes the most essential component for alcoholic fermentation, not only providing the microbial community and enzymes (as a saccharifying and fermenting agent) for alcoholic fermentation but also significantly contributing to the final liquor flavor (Zheng and Han, 2016; Liu and Sun, 2018). Similar liquor starters can be found in many Asian countries, e.g., xiaoqu/fuqu in China (Zheng and Han, 2016; Jin et al., 2017), meju in Korea (Kim et al., 2011), ragi in Indonesia (Fibri and Frøst, 2019), marcha/thiat/dawdim/hamei/chowan in India (Sha et al., 2018), bubod in Philippines (Tamang et al., 2016), and banh men in Vietnam (Thanh et al., 2008). All those liquor starters are prepared in an open system with starchy materials (wheat, rice, etc), shaped into different sizes and shapes, and cultured under different conditions (temperature and time) (Liu and Sun, 2018; Sha et al., 2018; Waché et al., 2018). Among them, JF daqu is made from wheat, shaped into brick, and produced with two steps of spontaneous fermentation for approximately 1 month in a Qu-room and drying for another 3 months without ventilation in a storage room (Huang et al., 2017b; Jin et al., 2017). During the spontaneous fermentation process, the cultivation of JF daqu is controlled by manually turning over the bricks and opening/closing the windows to change the ventilation and temperature, with the special microbial community being enriched from raw materials and the working environments by environmental variables (temperature and moisture), among which temperature serves as a key driving force (Huang et al., 2017a; Xiao et al., 2017; Liu and Sun, 2018). According to the maximum temperature in the daqu preparation process, JF daqu is grouped into high-temperature (60–70°C) daqu and requires cultivation at the high temperature stage for approximately 7–8 days (Huang et al., 2017b; Liu and Sun, 2018). Owing to this high temperature stage, the thermophilic microbial community may be enriched in JF daqu and various thermostable enzymes (i.e., proteinase, glucoamylase, cellulase, alpha-amylase, and esterase) may also be produced to degrade materials and generate special flavor compounds.

Recently, the daqu microbial community has been studied throughout fermentation by culture-dependent and -independent methods, and their diversity and dynamics are well understood (Yan et al., 2013; Wang and Xu, 2015; Huang et al., 2017b; Xiao et al., 2017). However, little is known regarding the active microbial community and their metabolic functions. In addition, although numerous crude enzymes have been identified in daqu (Li et al., 2015; Liu et al., 2018), active enzymes and their relationships with the microbial community are yet unknown. Metatranscriptomics constitutes an ideal tool for studying daqu microbial ecology, as it directly analyzes mRNA from environments and provides information not only on the microbial community composition but also on active members and their specifically expressed enzymes (Bokulich et al., 2016). This technology has been successfully applied in microbial ecological systems; e.g., compost (Mello et al., 2017), mouse gut (Just et al., 2018), cattle rumen (Pandit et al., 2018), sludge (Xia et al., 2018), ocean (Yoshida et al., 2018), and human feces (Abu-Ali et al., 2018). Nevertheless, owing to the complicated conditions in baijiu brewing systems, such as the high content of starch and fermentation products along with strongly colored materials, it remains challenging to extract high-quality RNA from baijiu fermentation samples, especially from the high temperature stage (70°C) of JF daqu, in which greater amounts of fermentation products were generated with strong colors than in all the other daqus' making stages. Thus, to our knowledge, only samples from the JF alcoholic fermentation process (42.8°C) (Song et al., 2017) and Nong-flavor (NF) daqu (a medium-temperature daqu) preparation process (62°C) (Huang et al., 2017a) have previously been studied using metatranscriptomics.

The cultivation temperatures in the production process for JF daqu, a typical high-temperature daqu, are largely higher than those in other daqus including NF daqu (Huang et al., 2017b). The high temperature condition constitutes the most striking difference among the daqu production processes of JF daqu and other daqus, as well as their subsequent alcoholic fermentation processes, which results in unique microbial community, enzymes, and aroma compounds being generated in the JF daqu and fermented feedstock (Wu et al., 2009; Wang et al., 2014; Xiao et al., 2016; Liu and Sun, 2018). Compared with NF daqu, JF daqu has a lower capacity for saccharification, liquefaction, and fermentation (Zheng and Han, 2016; Liu and Sun, 2018), thus requiring the use of a large amount of JF daqu (nearly 1:0.9 ratio of daqu versus feedstock) in the alcoholic fermentation process, which is higher than that of NF daqu (approximately 1:2 ratio). Thus, the flavor precursors, enzymes, and microbial community enriched in JF daqu would likely be more strongly associated with the final liquor flavor than those in NF daqu. Recently, we have published breakthrough research wherein significant differences were predictively shown in energy, carbohydrate metabolism,

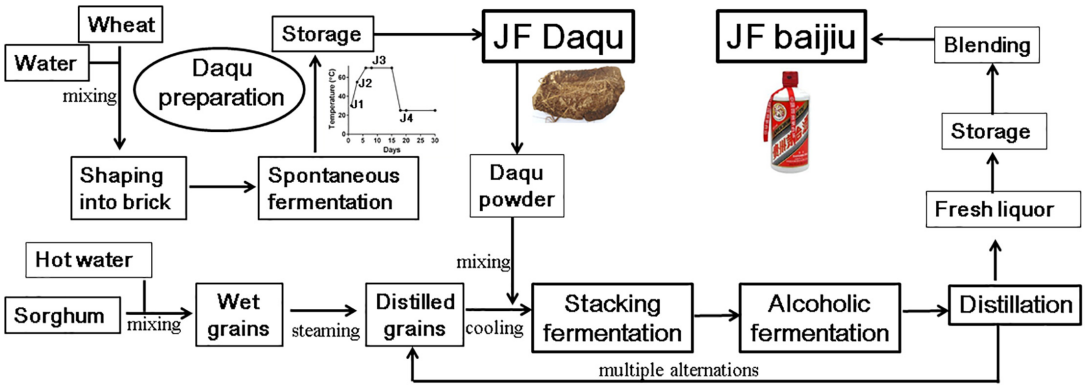

FIGURE 1 | Process diagram of traditional production of Jiang-flavor (JF) baijiu. JF daqu was produced with two steps of spontaneous fermentation in a Qu-room and drying without ventilation in a storage room, and its maximum cultural temperature was usually between 60 and 70°C. Besides stage of daqu preparation, JF baijiu was produced with other three distinct stages of stacking fermentation, multiple alternations of alcoholic fermentation and distillation processes.

and degradation of aromatic compounds between the JF daqu and NF daqu bacterial community (Huang et al., 2017b), and the active microbial community was found to highly express pivotal enzymes at the high temperature stage of NF daqu making process (Huang et al., 2017a). However, the active microbial community and important enzymes, as well as their functional correlations in JF daqu remain to be identified. More specific understanding regarding differences of the high temperature stage between JF and NF daqu have not been clarified. Therefore, in this study, we first employed metatranscriptomics to explain the structure and function of the actual microbial community and its pivotal enzymes at the high temperature stage of JF daqu making process. Moreover, a comprehensive and global comparison was performed between JF and NF daqu to shed light on functions of the high temperature stage with regard to saccharification and fermentation along with flavor compound generation. This study provides fundamental information related to the active microbial community and functional enzymes and may facilitate a comparative understanding of the pivotal role of the high temperature stage in the JF daqu making process and JF baijiu brewing.

MATERIALS AND METHODS

Sample Collection

JF daqu samples were collected at different time points from a fermentation workshop of Kweichow Hanwang Group Co., Ltd. in Renhuai, Guizhou, China, as described previously (Huang et al., 2017b). Briefly, Sample J1 was harvested at the beginning of daqu production (30°C); J2 was harvested after 3 days of daqu preparation (55°C); J3 was harvested after 8 days of daqu preparation (70°C); and J4 was harvested from the mature daqu after fermentation for 20 days (25°C) (**Figure 1**). In addition, all samples were selected and mixed from three locations in the same Qu-room at each time point. For RNA extraction, the daqu samples were frozen in liquid nitrogen immediately after collection, transferred to the Chengdu Biology Institute, Chinese Academy of Sciences on that day and stored in a −80°C freezer. For enzyme analysis, all the samples were suspended in 0.1% (v/v) Tween 80 solution and transferred to the institute at room temperature (Huang et al., 2017a).

Carbohydrate-Degrading Enzyme Activities

A total of 18 polymer analogs of insoluble chromogenic AZurine Cross-Linked (AZCL) polysaccharides (Megazyme, Ireland) were selected for detecting enzyme activities on cellulose, hemicellulose, starch, chitin, and glucan degradation (**Table 1**). As in our prior study (Huang et al., 2017a), all daqu samples in 0.1% (v/v) Tween 80 solution were incubated at 25°C and 100 rpm overnight, then their supernatants were added directly onto the wells of solid plates with AZCL polysaccharides according to the manufacturer's protocol. After incubation at 35, 45, or 55°C for 22 h, carbohydrate-degrading enzyme activities were determined by measuring the diameter of the blue haloes, which were recorded in millimeters.

RNA Extraction and Sequencing

Similar to the RNA extraction from NF daqu (Huang et al., 2017a), total RNA was extracted from JF daqu samples using borate buffer, cleaned with the RNeasy Midi Kit (Qiagen #75142, Venlo, Netherlands) and treated with DNase I (Fermentas, Waltham, MA, United States) according to the manufacturer's protocols. The RNA integrity was evaluated by gel electrophoresis and RNA integrity number (RIN) was checked using an Agilent2100 Bioanalyzer (Santa Clara, CA, United States). RNA samples with RIN value greater than 7.0 and OD260/OD280 ratio greater than 1.8 were selected for deep sequencing.

Total RNA (approximately 20 μg) from J3 was used for the RNA sequencing. Prior to metatranscriptomic library construction, using a previously reported method (Huang et al., 2017a), mRNA was isolated using magnetic beads with Oligo (dT) for eukaryotes, and for prokaryotes, mRNA was obtained after removing ribosomal RNA. The isolated mRNA was first fragmented and then used as template for subsequent

TABLE 1 | Relative abundances of highly active fungal and bacterial taxa according to their designated gene numbers by the NR database.

Classification			Relative abundance (%)
Fungi			97.7
	Aspergillus		53.2
		Neosartorya fischeri	9.9
		Aspergillus oryzae	9.9
		Aspergillus fumigatus	7.7
		Aspergillus niger	6.3
		Aspergillus clavatus	5.8
		Aspergillus terreus	5.4
		Aspergillus kawachii	3.9
		Aspergillus nidulans	2.3
		Aspergillus flavus	2.1
	Penicillium		29.2
		Penicillium stipitatus	14.7
		Penicillium marneffei	11.1
		Penicillium chrysogenum	2.2
		Penicillium digitatum	1.2
	Ajellomyces		3.6
		Ajellomyces dermatitidis	2.0
		Ajellomyces capsulatus	1.6
	Coccidioides		2.2
		Coccidioides posadasii	1.1
		Coccidioides immitis	1.1
	Paracoccidioides		1.4
		Paracoccidioides brasiliensis	1.4
	Uncinocarpus		0.9
		Uncinocarpus reesii	0.9
	Arthroderma		0.7
	Exophiala		0.6
		Exophiala dermatitidis	0.6
	Trichophyton		0.6
	Macrophomina		0.5
		Macrophomina phaseolina	0.5
Yeast			0.2
Bacteria			2.1
	Saccharopolyspora		0.6
	Acinetobacter		0.3
	Kurthia		0.2

first- and second-strand cDNA synthesis with random primers. Short cDNA fragments were purified and resolved with EB buffer for end reparation and poly(A) addition. Thereafter, the short cDNA fragments were ligated to sequencing adapters and suitable sized cDNA fragments were purified as templates for polymerase chain reaction amplification. RNA sequencing of the library was performed using platform (Illumina, San Diego, CA, United States) at the Beijing Genomics Institute (BGI)- the HiSeqTM 2000 Shenzhen, China.

Metatranscriptomics Assembly and Annotation

As for our previous metatranscriptomics assembly of NF daqu samples (N1–4), raw sequenced reads of J3 were first filtered by removing adaptors, low quality reads, and the rRNA sequences (Li et al., 2009). The clean reads of J3 were then *de novo* assembled using Trinity[1] (Grabherr et al., 2011), by which unigene sequences were generated. To annotate the metatranscriptome, the unigene sequences were aligned using Blastx (version 2.5.0) with protein and nucleotide databases including Non-redundant protein (NR), Non-redundant nucleotide (NT), Swiss-Prot, Kyoto Encyclopedia of Genes and Genomes (KEGG), Clusters of Orthologous Groups (COG), and Gene Ontology (GO) (e-value $< 10^{-5}$), and identified according to the highest similarity to known sequence. In cases of the non-alignment of unigenes against one of the listed databases, ESTScan was used to determine their coding directions. Thereafter, according to the standard codon usage, coding DNA sequences (CDSs) were translated into protein sequences. KEGG pathways were extracted from the KEGG web server[2] (Kanehisa et al., 2017). WEGO software[3] was used for GO classification (Ye et al., 2006). Carbohydrate-active enzymes (CAZymes) were retrieved from the Carbohydrate-Active Enzymes database (CAZy)[4] (Lombard et al., 2014).

Identification of Differentially Expressed Genes (DEGs) and Pathway Analysis

To compare the gene expression levels among J3 and NF daqu samples (N1–4), the predicted ORFs were combined after removing redundancy using cd-hit (Version 4.6.1)[5] (Li and Godzik, 2006). Gene expression levels were calculated using the Reads Per Kilobase per Million mapped reads (RPKM) method (Mortazavi et al., 2008). DEGs among J3 and NF daqu samples were identified using a method based on the Poisson distribution (Audic and Claverie, 1997). DEGs between two samples were identified using p-value ≤ 0.05, $\mathrm{Log_2}$(RPKM ratio) ≥ 1, and false discovery rate (FDR) value ≤ 0.001 (Benjamini and Yekutieli, 2001). To analyze GO enrichment, all DEGs were mapped to terms of the GO database.

Accession Number

The raw and assembled metatranscriptomics data of J3 have been deposited to the GenBank database under accession numbers SRR7785758 and GGWC00000000, respectively.

RESULTS

RNA Sequencing and Metatranscriptomics Assembly

After RNA sequencing of the J3 sample, 5.882 Gbp of raw data was generated, from which 5.663 Gbp of clean data was then obtained by filtering (**Supplementary Table S1**). These clean data were *de novo* assembled, from which 38,899 unigenes were identified with a total length of 46,187,298 nucleotides (nt) and

[1]http://trinityrnaseq.sourceforge.net/

[2]https://www.kegg.jp/kegg/

[3]http://wego.genomics.org.cn/cgi-bin/wego/index.pl

[4]http://www.cazy.org/

[5]http://weizhong-lab.ucsd.edu/cdhit_suite/cgi-bin/index.cgi

N50 length of 2232 bp (**Supplementary Table S2**). As shown in **Supplementary Figure S1**, there were 3585 unigenes with sequence size > 3000 nt.

Functional Annotation and Classification of Unigenes

To annotate the unigenes of J3, blastx alignment against the protein and nucleotide databases of NR, NT, Swiss-Prot, KEGG COG and GO was performed; the results are shown in **Supplementary Table S3**. The CDSs that mapped to the protein database and were predicted by ESTscan numbered 30,615 and 1041, respectively. A total of 31,279 known unigenes were identified by blastx, among which 14,912 genes were annotated by COG classification. There were 25 classes in the COG classification with the largest number of unigenes being found solely in the class of "general function prediction" (15.1%; **Supplementary Figure S2**). In addition, 19,468 unigenes were also annotated by the GO database, which accounted for 50.1% of all the unigenes, with the annotations grouped into three categories (biological process; cellular component; and molecular function) (**Supplementary Figure S3**). "Metabolic processes," "cell" and "catalytic activities" were dominant in the categories of biological processes, cellular components, and molecular functions, respectively.

Overall, 30,793 genes (e-value $< 10^{-5}$) were annotated using the NR database (**Supplementary Table S3**), which is far higher than those by other databases; the composition of active bacterial and fungal taxa in J3 is presented in **Table 1**. Based on their gene numbers, the active fungal community was more prevalent than the bacterial community and accounted for 97.7% in J3. In the fungal component, *Aspergillus* and *Penicillium* were the pivotal genera with high relative abundances of 53.2 and 29.2%, respectively. In addition, the active yeast showed low relative abundances of 0.2% at this high temperature stage.

As shown in **Figure 2**, starch and sucrose metabolism had the highest number of unigenes in J3, and except for the citrate cycle (TCA cycle) and oxidative phosphorylation, all of the 30 most abundant KEGG pathways showed higher numbers of unigenes in J3 than those in the high temperature stage (N3) of NF daqu. Moreover, large differences were found between J3 and N3 in basic metabolisms (i.e., purine metabolism, RNA degradation, RNA transport, meiosis-yeast, MAPK signal pathway-yeast, cell cycle-yeast, spliceosome, and mRNA surveillance pathway), degradation of aromatic compounds (aminobenzoate degradation, naphthalene degradation, benzoate degradation and bisphenol degradation), starch and sucrose metabolism, and amino sugar and nucleotide sugar metabolism. Conversely, comparable numbers of unigenes were found between J3 and N3 in oxidative phosphorylation,

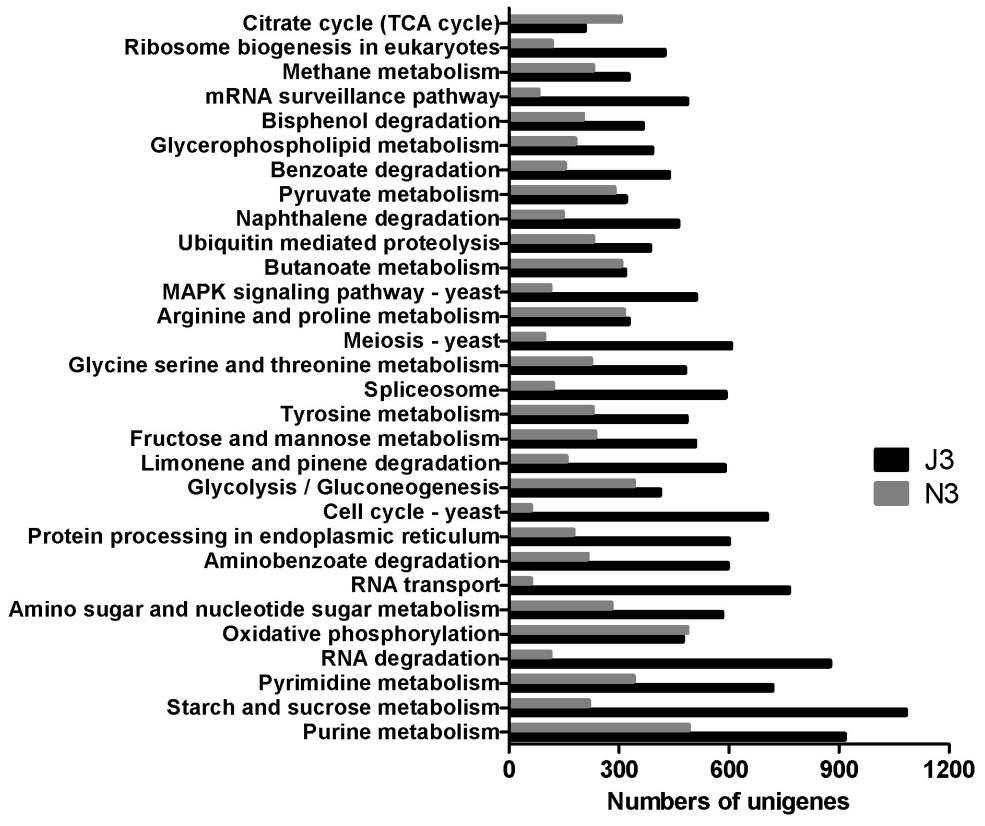

FIGURE 2 | The 30 most abundant KEGG pathways in high temperature stage samples of J3 and N3. J3 was harvested after 8 days of JF daqu preparation and N3 was harvested after 9 days of NF daqu preparation. The temperatures of J3 and N3 were 70 and 62°C, respectively.

glycolysis/gluconeogenesis, butanoate metabolism, and pyruvate metabolism. Additionally, metabolism of amino acids; i.e., tyrosine, glycine, serine, threonine, arginine, and proline, were also ranked in the top 30 of both J3 and N3.

Identification of CAZymes

Screening for genes encoding putative CAZymes from the metatranscriptomic library of J3 identified glycoside hydrolases (GH) (119) and glycosyl transferases (GT) (91) as having higher numbers of unigenes than carbohydrate esterases (CE) (13) and carbohydrate-binding modules (CBM) (7) (**Figure 3A**). Additionally, the total number (230) of these CAZymes in J3 was lower than that (932) in the N3 sample of NF daqu (Huang et al., 2017a). The expression level for all CAZymes in J3 showed a total RPKM value of 460.8, which was markedly lower than that of 20789.9 in N3 (**Supplementary Table S4**). The CAZyme classes with relatively high expression levels in J3 comprised GH15 (28%), GH1 (22%), GH18 (15%), GT2 (10%), GH28 (6%), GT20 (6%), and GH79 (4%), which totally differed from those in N3 (**Figure 3B** and **Supplementary Table S4**). These major GH families may have activities of glucoamylase (GH15), beta-glucosidase (GH1), chitinase (GH18), cellulose synthase and chitin synthase (GT2), polygalacturonase (GH28), alpha,alpha-trehalose phosphate synthase (GT20), and β-glucuronidase (GH79).

Moreover, insoluble chromogenic AZCL polysaccharide assays at different reaction temperatures (35, 45, and 55°C), clearly detected only endo-β-1,3-1,4-glucanase, endo-1,4- β -D-galactanase, and rhamnogalacturonanase as exhibiting activity at the high temperature stage of J3 (**Table 2**), which to some extent was complementary to the metatranscriptic results. In comparison, a broad spectrum of CAZymes was detected in the initial sage (J1) and mature stage (J4), and four CAZymes were also found in the high temperature stage of J2. Notably, only one CAZyme of α-amylase with thermophilic activity was obviously present, showing higher activity at higher temperatures in J1, whereas more CAZymes were clearly found with thermophilic activities in J2, J3, and J4, such as α-amylase, endo-β-1,3-1,4-glucanase, endo-proteases, and endo-1,4- β -D-xylanase.

DEGs Among J3 and NF Daqu Samples

The DEGs between the J3 and NF daqu samples were identified and a heatmap of hierarchical clustering of DEGs was constructed using $\log_2$(RPKM ratio) to visualize the respective patterns of DEGs. As shown in **Figure 4**, numerous DEGs (union) in J3, N2, N3, and N4 were clearly up-regulated with high $\log_2$(rations) values when compared with N1; thus, J3 together with N2–4 exhibit the largest differences in DEGs compared with N1. Alternatively, J3 presented the smallest differences in DEGs with N3. Similar results among J3 and NF daqu samples were also observed by analysis of hierarchical clustering of inter DEGs (**Supplementary Figure S4**). Furthermore, a comprehensive comparison performed between J3 and N3 identified a total of 14,149 unigenes as significant DEGs including 506 up- and 13,642 down-regulated genes (**Supplementary Figure S5**). In addition, for the GO functional classification (J3/N3), numerous DEGs were grouped into four dominant categories: "cellular processes," "metabolic processes," "binding," and "catalytic activities" (**Supplementary Figure S6**).

Pathway Comparisons of Starch and Sucrose Metabolism, Glycolysis, Pyruvate Metabolism, and the Citrate Cycle Between J3 and N3

For further functional comparison of DEGs between J3 and N3, metabolic pathways were analyzed based on the KEGG database. Moreover, several key carbohydrate and energy metabolisms that were associated with relatively high numbers of unigenes were selected for comparative analysis including starch and sucrose metabolism, glycolysis, pyruvate metabolism, and citrate cycle pathways. As shown in **Figure 5**, enzymes related to these selected pathways were mainly present, and a complete metabolic process of converting polymers into end-products was apparent in both J3 and N3. In addition, the majority of enzymes in these four key pathways exhibited lower expression levels in J3 than in N3, with the exception of e.g., aldehyde reductase (1.1.1.21), polygalacturonase (3.2.1.15), beta-fructofuranosidase (3.2.1.26), 4-alpha-glucanotransferase (2.4.1.25), 1,4-beta-cellobiosidase (3.2.1.91), and 2,3-bisphosphoglycerate-independent phosphoglycerate mutase (5.4.2.12) (**Figure 5** and **Supplementary Tables S5–S8**).

According to KEGG annotation, in starch and sucrose metabolism, glucoamylase (3.2.1.3), glucan 1,3-beta-glucosidase (3.2.1.58), endoglucanase (3.2.1.4), and beta-glucosidase (3.2.1.21) showed relatively high expression levels with RPKM values of 172.9, 70.1, 19.0, and 13.7, respectively, in J3; these are responsible for degrading polymers of starch, dextrin, 1,3-beta-glucan, and cellulose into glucose (**Figure 5** and **Supplementary Table S5**). Moreover, phosphoglucomutase (5.4.2.2), which efficiently collaborates with starch phosphorylase (2.4.1.1) in converting starch into glucose-6P, also exhibited relatively high expression abundance with an RPKM value of 83.6 in J3. Additionally, most types of these enzymes were highly expressed by fungal species; e.g., *Rasamsonia emersonii*, *Aspergillus fumigatus*, *Coccidioides immitis*, and *Aspergillus oryzae* (**Table 3**). In comparison, some enzymes showed very low expression levels in J3, such as xylan 1,4-beta-xylosidase, alpha-amylase, and alpha-glucosidase, which to some extent was consistent with their low activities in AZCL polysaccharides assays of J3 (**Table 2** and **Supplementary Table S5**).

In glycolysis and pyruvate metabolisms, J3 contained an integral serial of enzymes for converting glucose into the important product, pyruvate, which would then be reversibly converted to acetyl-coA under aerobic conditions (**Figure 5**). Hexokinase (2.7.1.1), 6-phosphofructokinase (2.7.1.11), and pyruvate kinase represent three key enzymes in glycolysis, although only pyruvate kinase (2.7.1.40) showed relatively high expression abundance (RPKM value of 75.5) in J3, which could irreversibly produce pyruvate from phosphenol-pyruvate (**Figure 5** and **Supplementary Tables S6, S7**). Alternatively, pyruvate dehydrogenase E1 (1.2.4.1), pyruvate dehydrogenase E2 (2.3.1.12), and acetyl-CoA synthase (6.2.1.1) are responsible for producing acetyl-CoA from pyruvate and acetate, respectively;

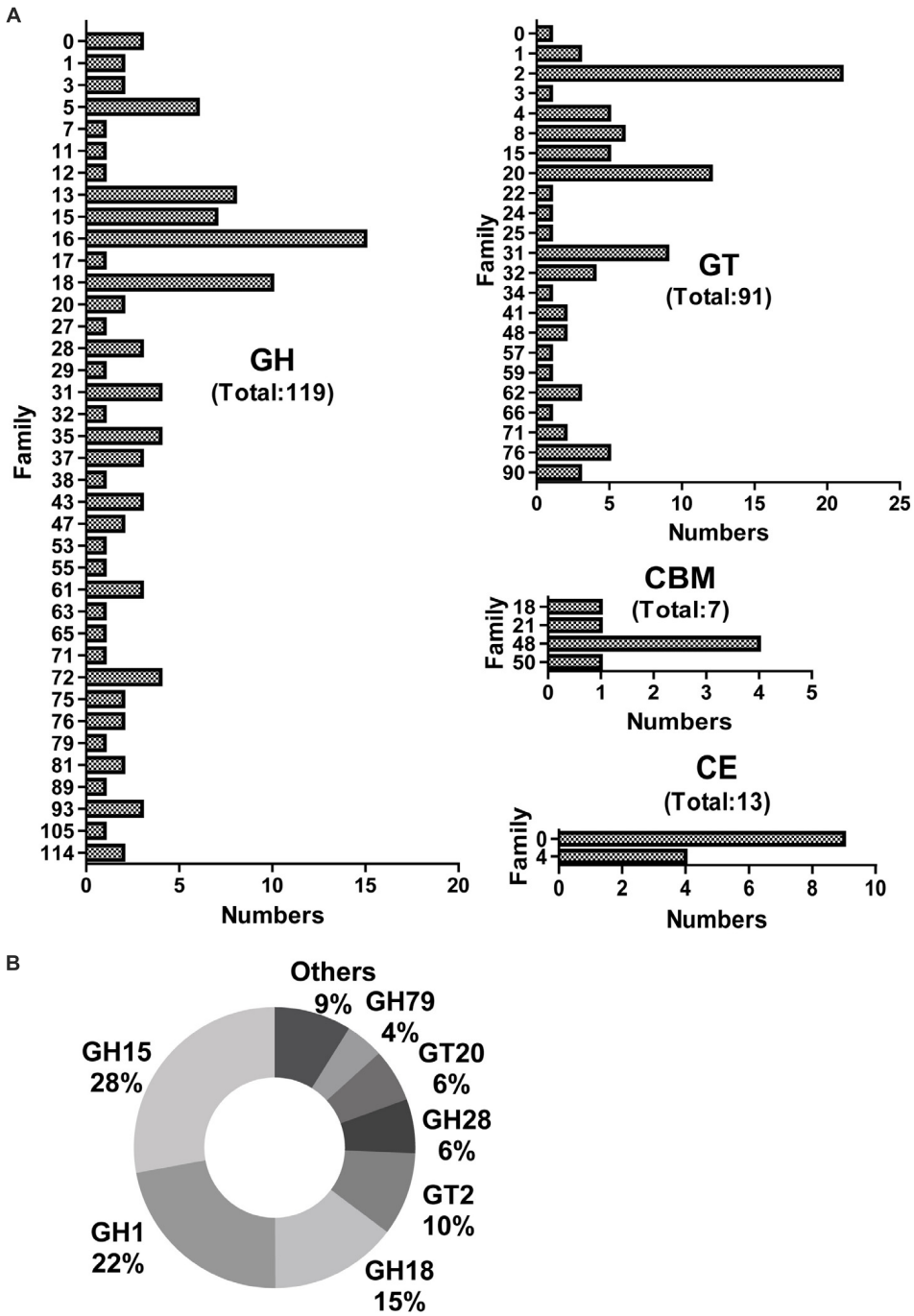

FIGURE 3 | Numbers and expression levels of carbohydrate-active enzymes in J3. Matched unigenes of carbohydrate-active enzymes are shown with numbers **(A)** and expression levels **(B)**. GH, glycoside hydrolase; GT, glycosyl transferase; CBM, carbohydrate-binding module; and CE, carbohydrate esterase.

all of these showed relatively low RPKM values in J3. Moreover, aldehyde dehydrogenase (NAD+) (1.2.1.3), which reversibly produced acetate from acetaldehyde, had the highest relative expression level with an RPKM value of 515.0, and acetyl-CoA hydrolase (3.1.2.1), which irreversibly produced acetate from acetyl-CoA, was also highly expressed in glycolysis and pyruvate metabolisms of J3 (**Figure 5** and **Supplementary Tables S6, S7**). Thus, acetaldehyde could be then converted to ethanol under anaerobic conditions by alcohol dehydrogenases (1.1.1.1 and 1.1.1.2) (**Figure 5** and **Supplementary Table S6**), which were expressed at low levels by fungal species of *A. fumigatus*, *C. immitis*, *Aspergillus terreus*, *Coccidioides posadasii*, *Penicillium marneffei*, and *Neosartorya fischeri* in J3 (data not shown). Therefore, high concentration of acetate

TABLE 2 | Carbohydrate-active enzyme analysis of Jiang-flavor daqu.

Substrate	Enzyme	Diameter (mm)			
		J1 35/45/55°C	J2 35/45/55°C	J3 35/45/55°C	J4 35/45/55°C
AZCL-curdlan	Endo-1,3- β -D-glucanase	15/13.5/7	7/3/6		
AZCL-beta-glucan	Endo-β-1,3-1,4-glucanase	6/9/7	0.5/0/2	7/8/13	1/5.5/11
AZCL-he-cellulose	Endo-β-1,4-glucanase				
AZCL-dextran	Endo-1,6-α-D-glucanase				
AZCL-xyloglucan	Endo-β-1,4-xyloglucanase				
AZCL-amylose	α-amylase	16.5/18/20			13/14/16
AZCL-casein	Endo-proteases		0.5/2/3		6/6/8
AZCL-collagen	Endo-proteases				
AZCL-debranched arabinan	Endo-1,5-α-L-arabinanase	6/6/6			6/6/5
AZCL-galactomannan	Endo-1,4- β -D-mannanase	4/3/3			
AZCL-galactan	Endo-1,4- β -D-galactanase	5/5.5/2		0/2/1	6/6/7
AZCL-rhmnogalacturonan I	Rhamnogalacturonanase	3/5/5	3/4/5	2/4/5	9/10/8
AZCL-chitosan	Chitosanase				
AZCL-pullan	Microbial pullulanase				
AZCL-xylan	Endo-1,4- β -D-xylanase				12/15.5/17
AZCL-arabinoxylan	Endo-1,4- β -D-xylanase				15/16/18

and low concentration of ethanol could be accumulated by collaborations of aldehyde dehydrogenase (NAD+), acetyl-CoA hydrolase, and alcohol dehydrogenases. In addition, aldehyde reductase (1.1.1.21), phosphoglycerate kinase (2.7.2.3), and lactoylglutathione lyase (4.4.1.5), which could produce lactaldehyde, phosphoglycerate, glucose, and lactoylglutathione, respectively, also showed relatively high expression levels and made large contributions in glycolysis and pyruvate metabolisms of J3. Highly expressed types of these enzymes also originated from fungal species, such as *Paracoccidioides sp.*, *Penicillium stipitatus*, *A. fumigatus*, *Aspergillus clavatus*, *Aspergillus oryzae*, and *N. fischeri* (**Table 3**). Considering that pyruvate and acetyl-coA serve as important intermediates for Val, Leu, and Ile biosynthesis, fatty acid biosynthesis, butanoate metabolism, leucine biosynthesis, and the synthesis and degradation of ketone bodies (**Figure 5**), the J3 samples thus showed capacities for converting glucose to pivotal intermediates of pyruvate and acetyl-coA for fatty acids, amino acids, and carbohydrates, which would further make large contributions for generating specific flavor in JF daqu.

In conditions of insufficient oxygen, pyruvate can be reversibly converted to lactate by L-lactate dehydrogenase (1.1.1.27), D-lactate dehydrogenase (1.1.1.28), D-lactate dehydrogenase (cytochrome) (1.1.2.4), or L-lactate dehydrogenase (cytochrome) (1.1.2.3); in particular, D-lactate dehydrogenase (cytochrome) showed relatively high expression abundance with an RPKM value of 162.1 in J3 (**Figure 5** and **Supplementary Table S7**). Members of D-lactate dehydrogenase (cytochrome) were specifically highly expressed by *P. marneffei* and *Trichophyton rubrum* (**Table 3**).

Almost all of the enzymes of the citrate cycle were present in the J3 sample (**Figure 5**). Among these, isocitrate dehydrogenase (NAD+) (1.1.1.41), fumarate hydratase (4.2.1.2), succinate dehydrogenase (1.3.5.1), and malate dehydrogenase (1.1.1.37) showed relatively high expression abundances with RPKM values of 94.1, 81.3, 63.0, and 37.8, respectively (**Supplementary Table S8**). Highly expressed members of these enzymes were mostly derived from fungal species, such as *A. oryzae*, *T. stipitatus*, *A. clavatus*, and *A. terreus* (**Table 3**). In addition, 2-oxoglutarate dehydrogenase E2 (2.3.1.61), citrate synthase (2.3.3.1), and aconitate hydratase 1 (4.2.1.3) were also clearly detected. Moreover, ATP citrate (pro-S)-lyase (2.3.3.8), phosphoenolpyruvate carboxykinase (ATP) (4.1.1.49), and pyruvate carboxylase (6.4.1.1), which are key enzymes that connect the citrate cycle and pyruvate metabolism, were still detected in J3. As shown in **Figure 5**, oxaloacetate, fumarate, and 2-oxoglutarate are pivotal intermediates for alanine, aspartate, and glutamate metabolism, arginine and proline metabolism, tyrosine metabolism, and D-Gln and D-Glu metabolism, which would then also contribute to specific flavor generation in JF daqu.

Abundant Comparisons of Enzymes for the Degradation of Aromatic Compounds Between J3 and N3

As large differences of unigene numbers were found between J3 and N3 with regard to the degradation of aromatic compounds (**Figure 1**), this study also focused on DEGs related to the degradation of aromatic compounds; i.e., aminobenzoate degradation (ABD), benzoate degradation (BD), bisphenol degradation (BPD), chlorobenzene degradation (CBD), ethylbenzene degradation (EBD), fluorobenzoate degradation (FBD), naphthalene degradation (ND), polycyclic aromatic hydrocarbon degradation (PD), styrene degradation (SD), toluene degradation (TD), and xylene degradation (XD)

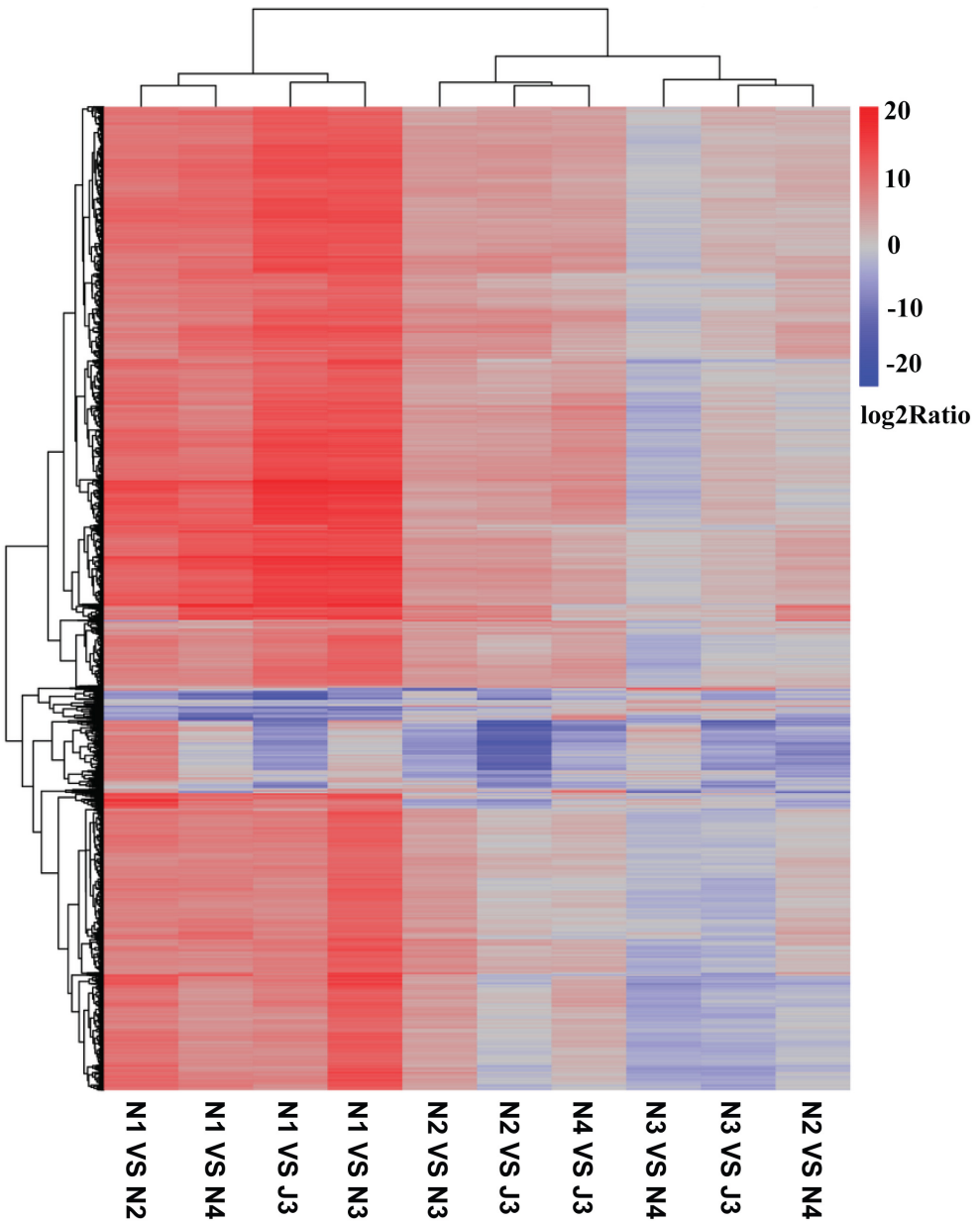

FIGURE 4 | Hierarchical clustering of differentially expressed genes (DEGs) (Union) among J3 and NF samples (N1, N2, N3, and N4). DEGs between two samples were identified using p-value ≤ 0.05, Log_2(RPKM ratio) ≥ 1, and false discovery rate (FDR) value ≤ 0.001.

(**Figure 6** and **Supplementary Table S9**). Notably, pathways in degradation of aromatic compounds are unnecessary for microbes, and many enzymes have not previously been identified in these pathways. As shown in **Figure 6**, among these pathways, benzoate degradation (BD) serves as the key pathway to connect most of other pathways via its pivotal products, benzoate, benzoyl-coA, catechol, and maleylacetate. A portion of enzymes related to these pathways was detected in J3 and N3, many of which showed lower expression levels in J3 than in N3. In particular, large numbers of enzymes were detected in J3 that were not identified in N3. Moreover, some of these, i.e., 2-deoxy-D-gluconate 3-dehydrogenase (1.1.1.125), 2-hydroxychromene-2-carboxylate isomerase (5.99.1.4), benzaldehyde dehydrogenase (NAD) (1.2.1.28), D-3-phosphoglycerate dehydrogenase (1.1.1.95), NADPH2:quinone reductase (1.6.5.5), L-iditol 2-dehydrogenase (1.1.1.14), 5-carboxymethyl-2-hydroxymuconate isomerase (5.3.3.10), and aldehyde reductase (1.1.1.21) clearly showed relatively high transcript abundances, with RPKM values ranging from 9.8 to 102.0, in pathways of BD, ND, BPD, ABD, EBD, XD, and TD,

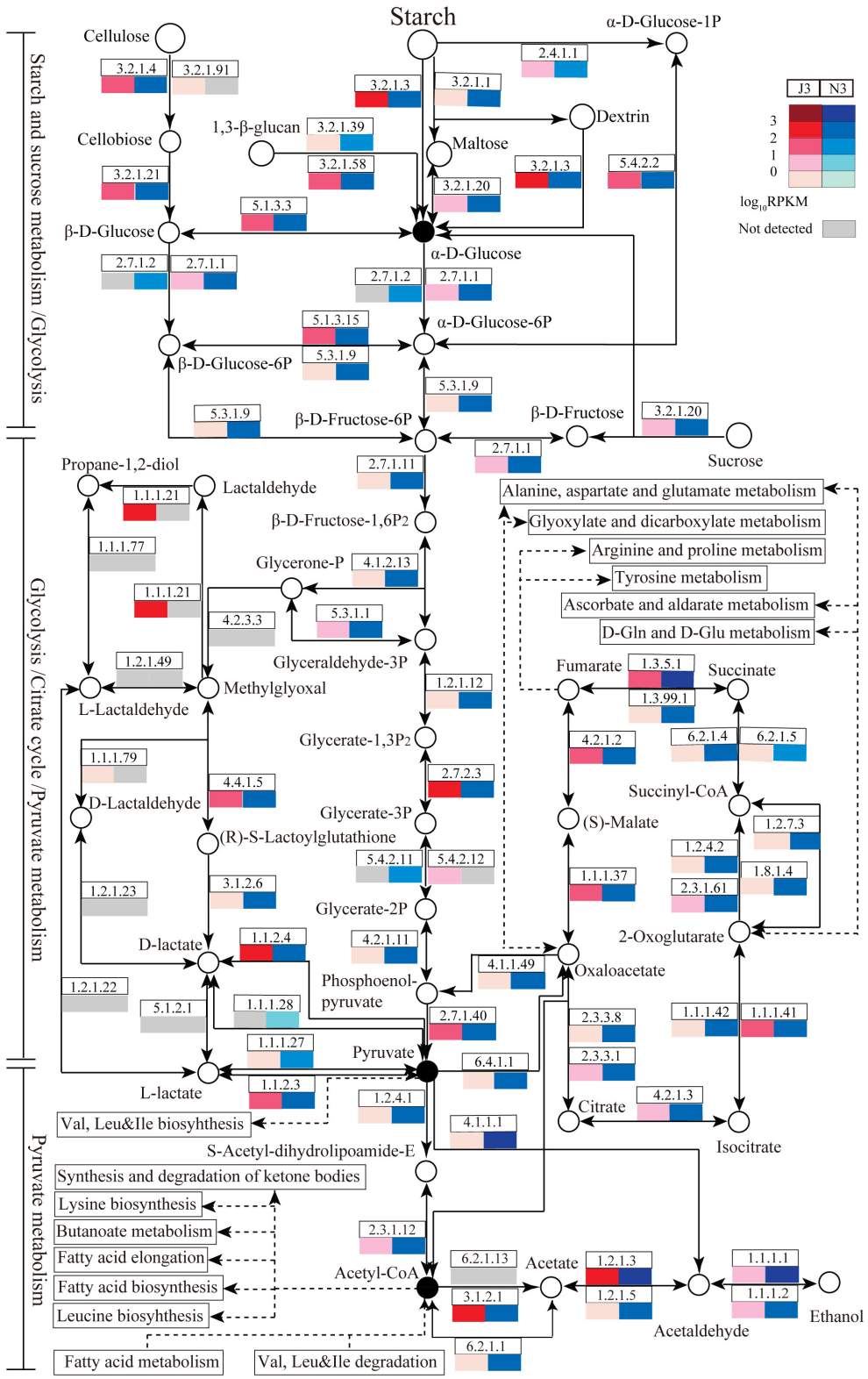

FIGURE 5 | Relative abundances of enzymes related to carbohydrate and energy metabolisms in J3 and N3. Four abundant carbohydrate and energy metabolisms were analyzed including starch and sucrose metabolism, glycolysis, pyruvate metabolism, and the citrate cycle. In these pathways, enzymes with relatively high expression levels are partly presented by EC number and total RPKM. Relative expression [log_{10}RPKM)] is shown in red for J3 and blue for N3. The key products are highlighted with black closed circles.

TABLE 3 | The top 20 expressed enzymes in starch and sucrose metabolism, glycolysis, pyruvate metabolism, and the citrate cycle in J3.

Gene ID	K0 ID	EC ID	Definition	RPKM	Species	Pathways
J3_2021	K01178	3.2.1.3	Glucoamylase	117.0	*Rasamsonia emersonii*	Starch and sucrose metabolism
J3_2988	K01835	5.4.2.2	Phosphoglucomutase	83.2	*Aspergillus fumigatus*	Starch and sucrose metabolism, Glycolysis
J3_3775	K01210	3.2.1.58	Glucan 1,3-beta-glucosidase	60.9	*Coccidioides immitis*	Starch and sucrose metabolism
J3_3326	K01178	3.2.1.3	Glucoamylase	29.4	*Aspergillus oryzae*	Starch and sucrose metabolism
J3_382	K01184	3.2.1.15	Polygalacturonase	28.0	*Aspergillus fumigatus*	Starch and sucrose metabolism
J3_2314	K00697	2.4.1.15	Alpha,alpha-trehalose phosphate synthase	26.6	*Aspergillus oryzae*	Starch and sucrose metabolism
J3_1666	K00128	1.2.1.3	Aldehyde dehydrogenase (NAD+)	514.9	*Paracoccidioides sp.*	Glycolysis, Pyruvate metabolism
J3_668	K00927	2.7.2.3	Phosphoglycerate kinase	134.7	*Penicillium stipitatus*	Glycolysis
J3_223	K00873	2.7.1.40	Pyruvate kinase	68.9	*Aspergillus fumigatus*	Glycolysis, Pyruvate metabolism
J3_870	K01785	5.1.3.3	Aldose 1-epimerase	18.3	*Aspergillus clavatus*	Glycolysis
J3_2941	K00102	1.1.2.4	D-lactate dehydrogenase (cytochrome)	141.2	*Penicillium marneffei*	Pyruvate metabolism
J3_2495	K01067	3.1.2.1	Acetyl-CoA hydrolase	134.6	*Aspergillus clavatus*	Pyruvate metabolism
J3_701	K00011	1.1.1.21	Aldehyde reductase	101.4	*Aspergillus oryzae*	Pyruvate metabolism
J3_3373	K01759	4.4.1.5	Lactoylglutathione lyase	63.2	*Neosartorya fischeri*	Pyruvate metabolism
J3_10280	K00026	1.1.1.37	Malate dehydrogenase	27.3	*Aspergillus oryzae*	Citrate cycle, Pyruvate metabolism
J3_1923	K00102	1.1.2.4	D-lactate dehydrogenase (cytochrome)	16.3	*Trichophyton rubrum*	Pyruvate metabolism
J3_909	K01679	4.2.1.2	Fumarate hydratase, class II	81.3	*Aspergillus oryzae*	Citrate cycle
J3_1885	K00235	1.3.5.1	Succinate dehydrogenase (ubiquinone) iron-sulfur subunit	62.4	*Penicillium stipitatus*	Citrate cycle
J3_1073	K00030	1.1.1.41	Isocitrate dehydrogenase (NAD+)	54.1	*Aspergillus clavatus*	Citrate cycle
J3_655	K00030	1.1.1.41	Isocitrate dehydrogenase (NAD+)	39.0	*Aspergillus terreus*	Citrate cycle

respectively (**Supplementary Table S9** and **Figure 6**). Highly expressed members of these enzymes in J3 mostly originated from fungal species, e.g., *N. fischeri*, *A. clavatus*, *Ajellomyces dermatitidis*, *Marssonina brunnea*, *Trichophyton equinum*, *Botryotinia fuckeliana*, and *A. oryzae* (**Table 4**). Therefore, it was considered reasonable to postulate that trace aromatic derivatives would be differently produced between J3 and N3, some of which might be only produced in J3. In addition, both J3 and N3 could degrade aromatic compounds into important end-products, such as acetyl-coA, fumarate, acetoacetate, succinate, and glycolate, which serve as intermediates for the citrate cycle, propanoate metabolism, and glycoxylate and dicarboxylate metabolism (**Figure 6**).

DISCUSSION

The daqus of Chinese JF and NF liquor, the most consumed liquors in China, undergo markedly different production processes that make large contributions to their special flavors. To ascertain the underlying factors, in comparison with our previous work of NF daqu, the present study comprehensively revealed the active microbial community and enzymes at the high temperature stage (J3) of JF daqu, and comparatively analyzed the active enzyme profiles at high temperature stages of JF and NF daqus. The active fungal community produced more diverse enzymes than those of the bacterial community, with *Aspergillus* and *Penicillium* representing the dominant genera at J3. This finding was complementary to the previous microbial diversity revealed for JF daqu by 16S rRNA and ITS sequencing, which indicated that the bacterial community was more diverse than the fungal community at J3 (Huang et al., 2017b). Meanwhile, low abundances of active yeast might be due to high temperature condition at J3, which may be well consistent with previous finding that yeast decreased quickly from J2 (55°C) to J3 (70°C) (Huang et al., 2017b). Additionally, the prevailing role of the active fungal community was also revealed in NF daqu samples by metatranscriptomics analysis (Huang et al., 2017a). Therefore, the present study further confirmed the suitability of metatranscriptomics for obtaining the active microbial community profiles in daqus.

JF daqu exhibited lower numbers and expression levels of CAZymes at the high temperature stage of J3 than those at the high temperature stage of N3 (Huang et al., 2017a). In addition, except for the initial stage of J1, most CAZymes were detected with lower activities and less diversities in the production process of JF daqu samples than those of NF daqu samples (**Table 2**) (Huang et al., 2017a). Lower activities and diversities of amylases (one kind of CAZymes) were similarly found in JF than in NF daqu via activities assay and protein electrophoresis (Liu et al., 2018). Thus, these findings might to some extent be consistent with the lower capacities of saccharification and liquefaction in JF daqu than those in NF daqu (Liu and Sun, 2018; Liu et al., 2018). Additionally, several thermostable CAZymes were detected in special stages of JF daqu samples, such as α-amylase in J1 and J4, endo-β-1,3-1,4-glucanase in J3 and J4, endo-proteases in J2 and J4, and endo-1,4- β -D-xylanase in J4, which suggests the feasibility of mining thermostable enzymes from special stages in the future.

Based on the functional annotation, starch and sucrose metabolism was the most abundant pathway in J3, which might imply that the microbial community has full capacity

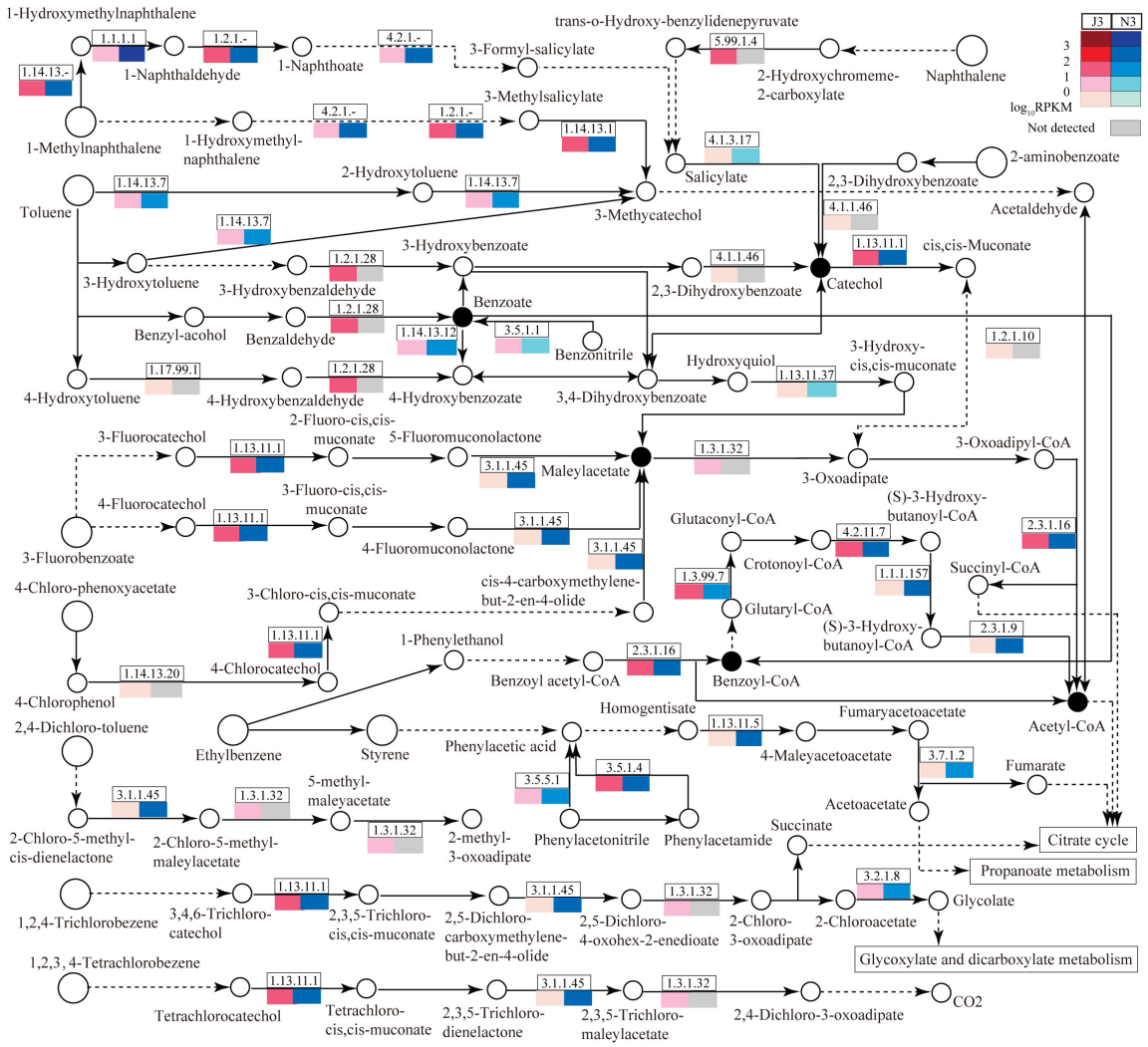

FIGURE 6 | Relative abundances of enzymes related to the degradation of aromatic compounds in J3 and N3. A total of 11 abundant pathways associated with the degradation of aromatic compounds were analyzed: aminobenzoate degradation, benzoate degradation, fluorobenzoate degradation, chlorobenzene degradation, ethylbenzene degradation, naphthalene degradation, bisphenol degradation, styrene degradation, xylene degradation, polycyclic aromatic hydrocarbon degradation, and toluene degradation. In these pathways, only the portion of enzymes with relatively high expression levels is presented by EC number and total RPKM. Relative expression [$\log_{10}$RPKM)] is shown in red for J3 and blue for N3. The key products are highlighted with black closed circles.

for degrading different polymers into glucose in J3. Upon comprehensive comparison between J3 and N3, most pathways showed higher diversities with more unigenes in J3 than in N3, which indicated more complicated metabolism for the microbial community in J3. Moreover, large differences of diversities were observed in basic metabolisms, degradation of aromatic compounds, starch and sucrose metabolism, and amino sugar and nucleotide sugar metabolism between J3 and N3, which further suggested that the microbial community of J3 might produce higher diversities of metabolites, some of which, such as phenol, benzaldehyde, and phenylethanol, might serve as precursors for aroma compounds (Fan et al., 2012; Wang et al., 2014; Xiao et al., 2016). In contrast, similar diversities were found in oxidative phosphorylation and glycolysis between J3 and N3, which indicated that the microbial communities released considerable bio-heat to maintain the high temperatures of 62 and 70°C for several days in NF and JF daqu, respectively (Huang et al., 2017b). In addition, butanoate metabolism and pyruvate metabolism were also similarly active between J3 and N3, suggesting that their intermediates, such as butanoate and acetate, represented important substrates for flavor compounds of e.g., butanol, acetic acid, butanoic acid, ethyl hexanoate, hexyl acetate, and isopentyl butanoate in JF and NF liquor (Fan et al., 2012; Wang et al., 2014; Xiao et al., 2016). Furthermore, six amino acid metabolisms were dominant in both J3 and N3, the products of which, i.e., amino acids, a-keto acids, and aldehydes, may serve as pre-substrates for important flavor precursors such as pyrazine, alcohol, and acids (Badrinarayanan and Sperry, 2012; Nashalian and Yaylayan, 2014; Scalone et al., 2015; Xu et al., 2017). Therefore, the microbial community was both active at the

TABLE 4 | The top 20 expressed enzymes for the degradation of aromatic compounds in J3.

Gene ID	K0 ID	EC ID	Definitions	RPKM	Species	Pathways
J3_701	K00011	1.1.1.21	Aldehyde reductase	101.4	*Aspergillus oryzae*	BD, ND, BPD
J3_3491	K00100	1.1.1.-	Dehydrogenase	55.0	*Paracoccidioides brasiliensis*	BD, ND, BPD
J3_1294	K00155	1.2.1.-	Dehydrogenase (NAD)	31.9	*Aspergillus oryzae*	ABD, ND, PD
J3_2545	K01826	5.3.3.10	5-carboxymethyl-2-hydroxymuconate isomerase	29.6	*Botryotinia fuckeliana*	BD
J3_3772	K00008	1.1.1.14	L-iditol 2-dehydrogenase	26.4	*Trichophyton equinum*	BD, ND, BPD
J3_787	K01113	3.1.3.1	Alkaline phosphatase D	26.1	*Aspergillus fumigatus*	ABD
J3_635	K00100	1.1.1.-	Dehydrogenase	21.1	*Penicillium marneffei*	BD, ND, BPD
J3_1295	K00100	1.1.1.-	Dehydrogenase	18.4	*Aspergillus terreus*	BD, ND, BPD
J3_20772	K00344	1.6.5.5	NADPH2:quinone reductase	16.6	*Marssonina brunnea*	ND
J3_3179	K00141	1.2.1.28	Benzaldehyde dehydrogenase (NAD)	16.1	*Aspergillus clavatus*	ABD, XD, TD
J3_124	K00058	1.1.1.95	D-3-phosphoglycerate dehydrogenase	16.0	*Ajellomyces dermatitidis*	BD
J3_2472	K00252	1.3.99.7	Glutaryl-CoA dehydrogenase	14.5	*Neosartorya fischeri*	BD
J3_988	K00517	1.14.-.-	Oxygenase	12.6	*Aspergillus niger*	ABD, BPD, PD
J3_300	K00632	2.3.1.16	Acetyl-CoA acyltransferase	12.5	*Aspergillus terreus*	BD, EBD
J3_1243	K00493	1.14.14.1	Unspecific monooxygenase	11.5	*Aspergillus oryzae*	ABD
J3_634	K14584	5.99.1.4	2-hydroxychromene-2-carboxylate isomerase	10.9	*Neosartorya fischeri*	ND
J3_1352	K03381	1.13.11.1	Catechol 1,2-dioxygenase	10.0	*Neosartorya fischeri*	BD, FBD, CBD, TD
J3_1961	K00065	1.1.1.125	2-deoxy-D-gluconate 3-dehydrogenase	9.3	*Neosartorya fischeri*	BD, ND, BPD
J3_4262	K01426	3.5.1.4	Amidase	9.2	*Penicillium chrysogenum*	ABD, SD
J3_246	K01692	4.2.1.17	Enoyl-CoA hydratase	8.5	*Aspergillus niger*	ABD, BD

ABD, Aminobenzoate degradation; BD, benzoate degradation; BPD, bisphenol degradation; CBD, chlorobenzene degradation; EBD, ethylbenzene degradation; FBD, fluorobenzoate degradation; ND, naphthalene degradation; PD, polycyclic aromatic hydrocarbon degradation; SD, styrene degradation; TD, toluene degradation; XD, xylene degradation.

high temperature stages of JF and NF daqu for generating bio-heat (Huang et al., 2017a,b; Xiao et al., 2017) and releasing flavor precursors (Wu et al., 2009; Zheng et al., 2011), and JF daqu could provide larger diversities of flavor precursors than NF daqu from most of the active pathways, in particular from the degradation of aromatic compounds.

Similar DEG profiles were observed between the high temperature stages of J3 and N3; thus, detailed functional comparisons of DEGs were performed between these stages with regard to four key carbohydrate and energy metabolisms: starch and sucrose metabolism, glycolysis, pyruvate metabolism, and the citrate cycle, as their intermediates are essential for ethanol and flavor generation. The results showed that both J3 and N3 contained an intact process for converting polymers into glucose, pyruvate, acetyl-coA, and ethanol, indicating a complete system for saccharification, liquefaction, and fermentation. In general, the majority of enzymes related to these four key pathways showed lower expression levels in J3 than in N3, indicating lower activities for enzymes in J3 than in N3 to a degree that is consistent with the lower capacities in saccharification, liquefaction, and fermentation exhibited by high-temperature JF daqu than those by medium-temperature NF daqu (Liu and Sun, 2018; Liu et al., 2018). Low expression levels of enzymes might result from the inhibition caused by the high temperature (70°C) in J3. However, some enzymes were only detected in J3, albeit with relative low expression levels, indicating that a large number of minor intermediates would likely be specifically generated in J3. Notably, among enzymes related to saccharification and liquefaction in J3, glucoamylases were clearly active with high expression levels, indicating their collaborative roles along with high temperature in degrading starches, which would be spontaneously decomposed under high temperature, as well as suggesting a feasible way to mine thermostable glucoamylases from J3. The majority of enzymes related to saccharification and liquefaction in J3 were highly expressed by fungal species of *R. emersonii*, *A. oryzae*, *A. fumigatus*, and *C. immitis*, some of which have been found to secrete numerous carbohydrate-active enzymes and show high capacities toward degrading polymers, such as *Aspergillus* (Culleton et al., 2013; de Vries et al., 2017; Cologna et al., 2018) and *R. emersonii* (Hua et al., 2014; Martínez et al., 2016). In addition, J3 showed considerable potential for converting glucose to pivotal intermediates, such as acetate, ethanol, pyruvate, and acetyl-coA, which might then serve as direct or indirect substrates for JF flavor compounds including ethyl acetate, ethyl butanoate, ethyl propanoate, ethyl 2-hydroxypropanoate, ethyl 2-hydroxyhexanoate, acetic acid, 2-acetylpyridine, hexyl acetate, benzyl acetate ethyl, ethyl 3-methylbutanoate, ethyl benzeneacetate, and 3-methylbutyl acetate (Fan et al., 2012; Wang et al., 2014; Xiao et al., 2016; Gao et al., 2017). The highly expressed enzymes related to glycolysis and pyruvate metabolism were mostly derived from fungal species, some of which have been applied to the production of fermented foods and drugs, such as *A. fumigatus* (Qin et al., 2012; Wakefield et al., 2017), *A. clavatus* (Mo et al., 2008; Zutz et al., 2013; Li et al., 2017), and *A. oryzae* (Park et al., 2018; Son et al., 2018; Zhong et al., 2018). Furthermore, low concentration of ethanol might be generated by several fungi in J3, which to some extent agreed with the earlier finding

that a small amount of ethanol could be directly produced by co-culture of fungi (Takano and Hoshino, 2012). Additionally, relatively high expression levels of D-lactate dehydrogenase (cytochrome) might indicate high concentration of lactate in J3, which may be consistent with the high level of lactate in the subsequent mature JF daqu (Wu et al., 2009). Moreover, intermediates of the citrate cycle also serve as pre-substrates for flavor compounds, and the highly expressed enzymes related to this pathway also originated from the fungal community, some of which have been applied to the saccharification and fermentation process of foods and drugs, including *A. oryzae*, *A. clavatus*, and *A. terreus*.

Numerous aromatic compounds, such as tannin, ferulic acid, and lignin have been identified in the materials of cereals, the degradation of which is strongly related to liquor flavor generation (Liu and Sun, 2018). Several laccases, feruloyl esterase and ferulic acid decarboxylase were detected with low expression levels from *A. clavatus*, *C. posadasii*, *P. marneffei*, *A. terreus* or *Pseudomonas aeruginosa* in J3 (data not shown), which might clearly confirm the degradations of ferulic acid and lignin during high temperature stage of JF daqu. Similarly, many aromatic compounds and phenols were identified in both JF and NF liquors (Wang et al., 2014; Xiao et al., 2016); consistent with this, in the present study some enzymes related to the degradation of aromatic compounds were also found to be expressed in JF and NF liquor starters, with most showing lower expression in the former. However, the remainder constituted those enzymes that were only detected (at low levels) in J3, indicating that trace aroma compounds were likely particularly associated with JF liquor flavor, such as ethyl benzeneacetate and benzaldehyde (Xiao et al., 2016). Highly expressed members of these enzymes were mostly derived from fungal species in J3, which appears consistent with the contributions of some fungi toward the degradation of aromatic compounds (Godoy et al., 2016; Sun et al., 2016; Vieira et al., 2018). Alternatively, enzymes expressed at low levels from bacteria may also substantively contribute to degradation of aromatic compounds (Pérez-Pantoja et al., 2015; Van der Waals et al., 2017). Therefore, both the fungal and bacterial communities appear to have an active role in degrading aromatic compounds in JF daqu (Bhattacharya et al., 2017; Wang et al., 2017; Kamyabi et al., 2018; Ma et al., 2018), especially in the high temperature and mature stage (Huang et al., 2017b).

In addition to the microbial community, temperature also makes large contributions to generate flavor compounds in JF daqu, such as pyrazines and their derivatives, which comprise pivotal impact aroma compounds of JF liquor (Zhu et al., 2007; Fan et al., 2012). In particular, their generation may be thermally induced from microbial metabolites by non-enzymatic browning via the Maillard reaction at 70°C in J3 (Richards et al., 2011; Nashalian and Yaylayan, 2014). Overall, JF liquor flavor thus appears to be determined by a highly complicated process and further analysis of the active microbial community, enzymes, and metabolites from the daqu preparation in addition to stacking fermentation and alcoholic fermentation processes are required to unravel the mystery of JF liquor flavor generation.

CONCLUSION

In the present study, fungi including *Aspergillus* and *Penicillium,* were identified as the most active microbial community members at the high temperature stage (J3: 70°C) of JF daqu by metatranscriptomics. Furthermore, the high temperature stage was found to not only lower the capacities of JF daqu toward saccharification and fermentation, but also enhance its ability in generating diverse minor flavor compounds, e.g., derivatives of aromatic compounds. Additionally, most of enzymes related to those capacities were highly expressed at 70°C by fungal genus of *Aspergillus*, *Coccidioides*, *Paracoccidioides*, *Penicillium,* and *Rasamsonia*. These exploratory findings shed light on our understanding of the JF baijiu fermentation system, in which the high temperature stage plays key roles in improving JF daqu by providing unique active microbiota and enzymes, and strongly contributing to the final distinctive aroma and taste of JF baijiu.

AUTHOR CONTRIBUTIONS

HZ, ZY, YF, YJ, LT, and KH designed the experiment. ZY performed the experiments and analyzed the data. ZY, YX, DL, and HL collected samples and communicated with the liquor factory. ZY and LC wrote the main manuscript. ZY, AD, YF, and HZ revised the manuscript. All authors revised and approved the final version of the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00472/full#supplementary-material

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REVIEW
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Mechanistic Insights Into Probiotic Properties of Lactic Acid Bacteria Associated With Ethnic Fermented Dairy Products

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Gut microbes and their metabolites maintain the health and homeostasis of the host by communicating with the host *via* various biochemical and physical factors. Changing lifestyle, chronic intake of foods rich in refined carbohydrates and fats have caused intestinal dysbiosis and other lifestyle-based diseases. Thus, supplementation with probiotics has gained popularity as biotherapies for improving gut health and treating disorders. Research shows that probiotic organisms enhance gastrointestinal health, immunomodulation, generation of essential micronutrients, and prevention of cancer. Ethnically fermented milk and dairy products are hotspots for novel probiotic organisms and bioactive compounds. These ethnic fermented foods have been traditionally prepared by indigenous populations, and have preserved unique microflora for ages. To apply these unique microflora for amelioration of human health, it is important that probiotic properties of the bacterial species are well studied. Majority of the published research and reviews focus on the probiotic organisms and their properties, fermented food products, isolation techniques, and animal studies with their health pathologies. As a consequence, there is a dearth of information about the underlying molecular mechanism behind probiotics associated with ethnically prepared dairy foods. This review is targeted at stimulating research on understanding these mechanisms of bacterial species and beneficial attributes of ethnically fermented dairy products.

Keywords: probiotics, ethnic fermented food, *Lactobacillus*, dairy, cancer, fermentation

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INTRODUCTION

A century ago, Élie Metchnikoff observed the beneficial role of fermented milk on the health of Bulgarian peasants and postulated the positive effect of selected microbiota on gastrointestinal health of the villagers due to the daily intake of soured or fermented milk (Kaufmann, 2008). A century later, this observation has led to a revolutionary concept of treating gastrointestinal diseases associated with dysbiosis with gut microbiota. Although, modern researchers are currently exploiting the idea by introducing functional foods (nutraceuticals) and fecal transplantations, yet indigenous communities unaware of the underlying principle have been naturally preparing and consuming fermented milk and milk products since the domestication of livestock species. Through fermented products, ethnic communities have been conserving

unique microbial diversity historically. Lactic acid bacteria have been employed to ferment milk over 6,000 years ago, mostly when Babylonian, Egyptian, and Indus valley civilization flourished (Fox, 1993; Robinson et al., 2006). Traditional fermented foods are those which are being consumed for centuries, predating historical evidences, and proved essential for the welfare of the community (Hesseltine, 1965). The process of milk fermentation was a natural phenomenon, as lactic acid-producing microorganism in milk could normally ferment and acidify milk. The early tribal people noticed that with incubation of the organisms, they could preserve food for a longer time with increased flavor, texture, and aroma (Mayo et al., 2010). Thus, a long tradition of handling and storing food with certain microbes was handed down from generation to generation in communities and tribes (Caplice and Fitzgerald, 1999).

Early people in tropical countries were the heavy consumers of fermented milk products, whereas, people from North America and Europe were hefty consumers of milk (Mayo et al., 2010). Currently, about 20–30% of the foods consumed are fermented products. Milk being a rich source of nutrition produced by almost all domesticated mammals in every part of the world identifies itself as a popular culture for all kinds of societies. Buffalo and cow milk are ubiquitous, whereas, yak and camel milk and its products were consumed by ethnic and tribal people of Himalaya and mid-west Asia and Northern Africa respectively (Mayo et al., 2010; Tamang et al., 2012, 2016a,b,c). Milk in the different regions comprises of different levels of micronutrients, proteins, sugars, and fats, depending upon conditions of the region and producer animals, which subsequently alter the characteristics of the microorganisms and their functions. Due to changing lifestyle and globalization, there are alterations in preparation for some of the fermented dairy products as compared to the ethnic preparations; however, the microorganisms are nearly conserved. These fermented milk products preserve the nutritional value through the development of lactic acid, acetic acid, flavor, enhancement of essential amino acids, metabolites, essential fatty acids, and detoxification of undesirable metabolites. In 1965, Lilley and Stillwell termed these microorganisms as "probiotics" and demonstrated that the substances secreted by these organisms could stimulate the growth of other microorganisms and hosts (Fuller, 1992).

Probiotics isolated from traditional dairy products comprise species of lactic acid bacteria with a history of safe use. The employment of live bacteria incorporated in food is included in the European Qualified Presumption of Safety list (Leyva Salas et al., 2018). Many probiotic lactic acid bacterial strains isolated from different dairy products have also undergone review and testing and have fulfilled the FDA "GRAS" status for use in fermented dairy product and other food items. Even though, probiotic properties have been studied and used safely for decades, yet the absence of molecular details about the mechanism of their healthy attributes (cause-and-effect relationship) concerns the regulatory authorities for their safe usage. Thus, an understanding of the mechanisms will enable therapeutic usage of scientifically supported probiotic supplements with well-defined health claims in desired fermented products. To get an insight into the mechanisms, a number of probiotic lactic acid bacteria have been isolated either from the ethnic fermented dairy products or native microbiota of healthy individuals. It has been observed that all the probiotic bacteria do not share all the attributes. Specific strain depicts precise function which is not observed in other strains of same species. However, it has also been observed that not all the probiotics are unique and many of them share some common functions (Sanders et al., 2018). The role of probiotics in maintaining the health of the human gut has been mostly documented (Butel, 2014; Vandenplas et al., 2015). Using probiotics and metabolites as gut modulators, the function and composition of the bacterial community can be selectively altered. To improve the role of probiotics isolated from an ethnic fermented dairy product in stimulating human health, it is crucial to study their mode of action. This review summarizes some of the underlying molecular mechanisms behind different attributes such as, gastrointestinal health, immune modulation, cholesterol reduction, cancer mitigation, and production of bioactive metabolites by microbiota associated with indigenous fermented milk products.

TRADITIONAL DAIRY PRODUCTS AND MICROBIOTA ASSOCIATED WITH THE PRODUCTS

Fermented milk products also known as cultured dairy products are dairy foods which have been fermented by a consortium of lactic acid bacteria (LAB) responsible for the curdling or souring of milk (FAO/WHO, 2003). LAB are best-suited organisms for milk fermentation while preserving the taste and nutritional properties. These bacteria are non-sporulated, mostly anaerobic in nature, and are only capable of growing in rich nutritional conditions that provide growth factors like vitamins, amino acids, and nucleotides. Bacterial members associated with fermented dairy products belong to the genera of *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Bacillus*, *Propionibacterium*, and *Bifidobacterium*. These bacteria live in same ecological niches and act mutualistically. There are approximately 400 traditional and fermented milk products comprising a diverse group of microorganisms giving rise to different sensory properties. There are two different classes of milk products based on fermentation:

Class I: Bacterial lactic acid fermentation: (1) Fermentation by mesophilic bacteria (acidified milk, buttermilk, filmjolk, and langfil), (2) fermentation by thermophilic and mesophilic bacteria (yoghurt, dahi, Bulgarian buttermilk, zabadi).

Class II: Fungal and bacterial lactic acid fermentation: Fermentation by bacteria as well as fungi. E.g. alcoholic milk (Acidophilus yeast milk, Koumiss, and kefir) and Moldy milk (Villi).

The varieties of milk products depend upon different types of milk and starter cultures, sugars, aromatic compounds, and grains. These varieties are developed using primary starter cultures (which participate in primary acidification) and secondary starter cultures (which participate in generating aroma, flavor, and texture). Genera used in primary culture are *Lactobacillus* sp.,

Leuconostoc sp., *Streptococcus* sp. (Parente and Cogan, 2004), whereas, the genera associated with secondary starter cultures are *Propionibacterium* sp., *Brevibacterium* sp., *Debaryomyces* sp., *Geotrichum* sp., *Penicillium* sp., and *Enterococcus* sp.

Characteristics of naturally fermented milk depend upon the availability of the milk in respective regions. However, fermented milk like Zeer, Kad, Zabady, Laban, Rayeb, and Shubat from Northern Africa, Morocco, and mid-west Asian countries; Ergo from Ethiopia; Amasi from Zimbabwe; Roub from Sudan; Chhurpi, Mohi, Philu, Somar, and Shoyu from Himalayan region; and flmjölk and långfl from Sweden have same characteristics of fermentation. These products are majorly dominated by mesophilic lactic acid bacteria, which lower pH, improve sensory properties, inhibit other bacterial spoilage, and improve health (**Table 1**). In primary fermentation, *Lactococcus lactis* and *Lactococcus lactis* subsp. *cremoris* are found to be most dominating ones. Other important bacteria commonly found in these products are *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus paracasei*, *Leuconostoc* sp., *Enterococcus* sp., and *Pediococcus* sp. In tropical countries, *Lactobacillus* sp. like *Lactobacillus helveticus*, *Lactobacillus fermentum*, *Lactobacillus acidophilus*, and *Lactobacillus brevis* are also prevalent (Gonfa et al., 2001; Mathara et al., 2004; Patrignani et al., 2006). In raw milk, a very high number of yeast species are also found (Gadaga et al., 2000; Gonfa et al., 2001; Benkerroum and Tamime, 2004). They enhance the flavor and texture of milk products. Major yeast species found are *Candida lusitaniae*, *Saccharomyces cerevisiae*, and *Kluyveromyces marxianus* (Gadaga et al., 2000; Benkerroum and Tamime, 2004). Some of the milk products are partly dried like Leben, Zeer, and Than; while some are maintained in oil like Shanklish. Few are mixed with spices like Mish and some are mixed with wheat and cereals like Kishk and Kadhi. Salted cheeses like Feta, Lighvan, and Domiati are heavily consumed in Middle Eastern Asia and Balkans, which also represent air-dried and sundried cheeses from Northern Africa (Kosikowski and Mistry, 1997). Many of the North African and middle western Asian fermented products are made up of camel milk like Chal, Unda, Shubat, and Susa. A rich diversity of traditional fermented milk products is present in the Himalayan region, mainly fermented from yak, buffalo, and cow milk. Chilika curd is one of the ethnic fermented foods with an exceptionally extended shelf life that is prepared by ethnic community of Chilika in Odisha state of South-Eastern India. Chilika is made up of special cup made up of bamboo basket using milk of Chilika Buffalo (Nanda et al., 2013). The lactic acid bacteria present in Chilika curd have been observed to exhibit higher antifungal activity due to the presence of compounds such as 3-hydroxy fatty acid, caproic acid, and fungicins. Nunu, a fermented milk consumed in Ghana and western part of Africa, is known to harbor strains of *Lactobacillus*, *Leuconostoc*, *Enterococcus*, *Weissella*, and *Pediococcus spp.* with health beneficial properties (Akabanda et al., 2013). Fermented milk products also represent an important part in the staple diet for countries like Afghanistan, Pakistan, India, Nepal, Bhutan, China, and Myanmar. Some of the indigenous fermented products are Dahi, Chhurpi, Churkam, Chhu, Somar, Mohi, Philu, Maa, and Shoyow. Some of the products are ubiquitous to the Indian subcontinent like Dahi (yoghurt), Mohi (buttermilk), whereas products like Chhu, Churpi, and Somar are restricted to inhabitants of Himalayan foothills where yaks are reared (Dewan and Tamang, 2006; Tamang and Samuel, 2010; Rai et al., 2016). Some naturally fermented milk products found in Himalayan regions were prepared from the old technique known as back-slopping and it is still used to preserve the microflora present in these fermented products. Such products include ethnic fermented products of Bhutan such as dahi, datshi, hard-chhurpi (churkam/chugo) mohi, gheu, and hitpa (Shangpliang et al., 2017). The traditional back-slopping in dairy fermentation is different from mono-culture fermentation in enhancing the probiotic characteristics as these contain wild-type strains with enriched biosynthetic capacity, higher genetic diversity, and enhanced ability to produce antimicrobials such as bacteriocins. Presence of a higher number of bacilli in contrast to cocci in the Himalayan fermented milk products implies that the milk in different regions supports a set of consortium for their particular characteristic fermentation and qualities (Dewan and Tamang, 2007). The dominant species in the Himalayan fermented milk products are *L. plantarum*, *Lactobacillus bifermentans*, *L. lactis* subsp. *cremoris*, *L. paracasei*, *L. alimentarius*, *L. kefir*, *L. bulgaricus*, and *Enterococcus faecium* (Dewan and Tamang, 2007). Indigenous fermented foods have been prepared and consumed for thousands of years and maintain the natural microflora present in them. The variety of microorganisms present in these fermented food are able to create flavors that are difficult to imitate in commercial products where pure starter cultures are used for preparing them (Thapa and Tamang, 2015). Since isolation and identification of these bacteria are based on culture-dependent methods, it is difficult to comprehend the true landscapes of their diversities and benefits. Molecular techniques such as denaturing gradient gel electrophoresis have revealed the presence of *Leuconostoc mesenteroides, Lactobacillus helveticus, L. kefiranofaciens, L. lactis, L. kefir, and L. casei* as the dominant microorganisms present in Tibetan kefir (Zhou et al., 2009). In another study carried out on the diversity of Mongolian traditional fermented dairy products using pyro-sequencing, it was found that there was a correlation between animal species and the genus *Lactobacillus* which was found to be the core foundation in Mongolian fermented milks. *L. kefiranofaciens, L. helveticus,* and *L. delbrueckii* were the predominant species sequenced using NGS for ethnic Khoormog, Airag, and Tarag fermented samples, respectively (Oki et al., 2014).

Recently, metagenomic investigations of the naturally ethnic fermented milk products such as churkam, churpi, mar, and dahi have shown that Proteobacteria (*Acetobacteraceae*) and Firmicutes (*Streptococcaceae, Lactobacillaceae*) were the two most predominant members of the microbial communities in these traditional fermented products. *L. helveticus* and *L. lactis* were the predominant lactic acid bacteria while *Gluconobacter and Acetobacter* spp. were the predominant acetic acid bacteria present in these fermented products (Shangpliang et al., 2018). Therefore, these metagenomic and culturonomic approaches will provide a wider spectrum of microbes associated with

TABLE 1 | Therapeutic and beneficial properties of ethnically fermented dairy products and associated microorganisms.

Traditional fermented dairy foods	Microbial flora	Associated actions	References
Koumiss	*L. casei* Zhang (LCZ)	Increased host immunity in gut by systemic immune response by secretion of IL-12, IFN-γ, sIgA, IL-10, and reduced level of pro-inflammatory cytokines (IL-1). Suppressed effect on pathogens such as *Acinetobacter* and *Pseudomonas*. Increased level of short-chain fatty acids (SCFA)	Ya et al., 2008; Dong et al., 2015; Wang et al., 2014; Hor et al., 2018; Guo et al., 2015
	Lactobacillus sp.	Helped in cholesterol assimilation. Enhanced synthesis of ACE inhibitors and GABA.	Sun et al., 2009
Kefir	*Lactobacillus kefir, Lactobacillus kefiranofaciens, and Lactobacillus kefirgranum*	Showed antibacterial activity by production of bacteriocin	Luo et al., 2011
		Reduced inflammation in epithelial cells of intestine	Seo et al., 2018
		Reduced cholesterol level in serum. Produced an EPS known as kefiran.	Wang et al., 2008 Bonczar et al., 2016
	L. plantarum MA2	Reduced cholesterol, LDL, and triglyceride in male Sprague–Dawley (SD) rats	Huang et al., 2013
	L. kefiri D17, *L. plantarum* B23 and *L. acidophilus* LA15	Induced apoptosis of Caco-2 and HT-29 cancer cells and decreased transforming growth factor (TNF-α and TNF-β) in HT-29	Zheng et al., 2013
		Displayed anti-proliferative effect in different cancer cell lines	Khoury et al., 2014 Furukawa et al., 2000 Chen et al., 2007
Katak	*L. brevis*	Showed antifungal activity against *Aspergillus* and *Penicillium* sp.	Tropcheva et al., 2014
Dahi	*L. rhamnosus* S1K3	Produced antimicrobial compounds to resist foodborne pathogens.	Kemgang et al., 2016
		Enhanced integrity of tight junction protein by up-regulating claudin 1 gene. Increased expression of human β-Defensin-2 and β-Defensin-3.	
		Induced the expression level of IL-4, Toll-like receptor (TLR) at Peyer's patches and IgA level in serum	
	L. acidophilus	Produced EPS	Vijayendra et al., 2008
		Enhanced riboflavin production	Jayashree et al., 2010
Camel milk fermented products	*L. plantarum*, *L. acidophillus* and *L. reuteri* and *L. lactis*	Produced EPS	Abushelaibi et al., 2017
		Displayed anti-proliferation of MCF-7, Caco-2 and HeLa cells. Production of ACE inhibitors	Ayyash et al., 2018
Tibetan Kefir	*L. plantarum* YW11 *Butyricoccus* sp. and *Blautia* sp.	Produced of EPS and elevated level of superoxide dismutase, catalase, glutathione peroxidase to protect from oxidative damage. Decreased level of malondialdehyde	Zhang et al., 2017
	L. plantarum Lp27	Decrease serum total cholesterol, LDL-cholesterol, and triglycerides in hypercholesterolemic SD rats	Huang et al., 2013
Iranian dairy product	*L. brevis*	Assimilated cholesterol	Iranmanesh et al., 2014
Tulum Cheese	*L. fermentum*	Assimilated cholesterol	Tulumoğlu et al., 2014
Khadi	*P. pentosaceus* GS4	Showed anti-proliferation activity in HCT-16 mammalian cells, increased expression of pro-apoptotic molecules NF-κB and p-Akt and produced conjugated linoleic acid	Dubey et al., 2016
Tarkineh, Shiraz	*L. plantarum* and *L. lactis subsp*. lactis	Induced of apoptois and produced anticancer peptides	Haghshenas et al., 2015
	Kluyveromyces marxianus AS41	Showed anti-proliferative activity on cancer cells. Down-regulated Bcl-2 expression and up-regulated BAD expression	Saber et al., 2017
Swiss Cheese	*Lactobacillus helveticus* R389	Enhanced immune system by increasing IgA and CD4 positive cells. Decreased IL-6 and increased IL-10 expression.	De LeBlanc et al., 2005
Kajmak	*L. mesenteroides*, *L. lactis* and *L. paracasei*	Enhanced flavor by production of diacetyl, acetate, and ethanol	Jokovic et al., 2008
Rabadi	*L. plantarum* RYPR1	Exhibited hypocholesterolemic effect due to bile salt hydrolase activity	Yadav et al., 2016
Brazilian Kefir	*L. lactis* subsp. cremoris MRS47	Modulated lipid profile by generation of SCFA	Vieira et al., 2017
Italian Cheese	*L. helveticus* PR4	Produced ACE inhibitors, antibacterial peptides and GABA	Minervini et al., 2003; Siragusa et al., 2007
Chhurpi (Yak cheese)	*L. fermentum*	Produced phytase, exhibited dephytanation in finger millets and Durum wheat under *in vitro* gastrointestinal conditions	Sharma et al., 2018

these indigenous products, along with a better perspective of their attributes contributing toward the welfare of human being.

MOLECULAR PERSPECTIVE OF ETHNIC DAIRY PRODUCTS IN GASTROINTESTINAL HEALTH

The Human gut microbiome consists of all the living microorganisms existing in association with the gut of human. These living microorganisms include bacteria, archaea, eukaryotes, and virus. These gastrointestinal microbes present in a continuum with ingested bacteria maintain metabolic homeostasis. Moreover, the single layer of specialized epithelial cells present in the intestine form a highly complex structured network which is the major intestinal defense system present in gut against pathogens and designated as intestinal barrier function. The epithelium of gut uses different defense mechanisms against the microbiota such as immune response (innate or acquired), mucus layer secretion, as well as integrity and turnover of the epithelial cell (Bron et al., 2012). In order to maintain the intestinal barrier function, adjacent epithelial cells of the gut form tight junctions with each other. These junctions act as a barrier that is impermeable to particulate things and liquid materials. Other cells of epithelium i.e. Goblet and Paneth cells also support barrier function, thereby contributing as a part of innate immune system. Collectively, all these barriers decrease the load of pathogens at the interface between epithelium and lumen. However, various intestinal linked inflammatory diseases such as inflammatory bowel disease (IBD) have been manifested by a leaky intestinal barrier. Transient passage of probiotic bacteria in upper gastrointestinal tract (GIT) at a concentration higher than 10^7 bacteria overwhelms the normal population of microbiota. These probiotic bacteria, therefore, possess a higher access to the microvilli, mucosa, and other cells of the immune system. A number of researchers have found that probiotics can activate both the innate and adaptive immune systems, and thus provide better protection against pathogens (McFarland, 2006; Sánchez et al., 2017). In different studies, consumption of ethnic probiotic bacteria in dairy product and their interaction with intestinal cells initiate an immunological response. The surface marker present on probiotic bacteria such as exopolysaccharides, lipoteichoic acid, fibronectin-binding proteins, and mucus-binding proteins are key factors responsible for crosstalk with the host intestinal epithelium cells (Sanders et al., 2018). These interactions are important because they influence the production of chemokines and cytokines that are secreted by intestinal enterocytic cells (**Figure 1**; Gill and Prasad, 2008). For example, in a study conducted on Mongolian Koumiss, a potent *L. casei* Zhang (LCZ) strain was isolated after screening 240 isolates of *Lactobacillus* sp. This strain has proven to have high binding affinity toward intestinal Caco-2 cell lines. Further role of LCZ probiotic in maintaining host immunity homeostasis was observed using *in vivo* experiments where it was observed that the LCZ induced gut immune response by secretory IgA (sIgA) secretion in intestine and also undergoes systemic immune response by secretion of IL-12 and IFN-γ in mice model (Ya et al., 2008; Dong et al., 2015). In a clinical study on this strain, it has been found that LCZ carried an ability to modulate the composition of fecal microbiota in both elderly and adult subjects. The strain exhibited growth-suppressive effect on pathogens such as *Acinetobacter* and *Pseudomonas*. Furthermore, it was also observed that there was an increase in the level of short-chain fatty acids (SCFA) for a prolonged period in the intestine (Wang et al., 2014). A very recent 12-month randomized clinical trial using this same strain was carried out on Malaysian populations where the study showed that LCZ strain alleviated gastrointestinal disorders and upper respiratory tract infections in the full-fledged population. It was observed the strain activated both B and T cells, and increased the level of anti-inflammatory cytokines such as IL-10 and reduced the level of pro-inflammatory cytokines (IL-1) (Hor et al., 2018). Regulatory T cells (T_{reg}) constitute a key source of anti-inflammatory cytokine IL-10, and are further involved in maintenance of immune tolerance and regulation of appropriate immune response mediated by T cells. Recent, randomized, double-blind clinical trial using *L. plantarum* showed that there was significant lowering of sepsis and lower respiratory tract infections among infants in rural India. The findings suggest that the probiotics LAB can effectively prevent a large proportion of neonatal sepsis in developing countries (Panigrahi et al., 2017).

Other modes of action of probiotics include competitive exclusion of the pathogens. The antimicrobial properties associated with probiotic kefir were reported on various pathogens. The production of bacteriocin and lactic acid by the probiotic *Lactobacillus* present in the kefir has been found to inhibit *Bacillus subtilis, Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa* (Luo et al., 2011). However, kefir-derived yeast probiotics have also been reported to produce organic acid, hyaluronic acid, acetic acid, and ethanol depending on the prevalent probiotic strain present during fermentation. The pathogens and probiotics behave in different manner to these metabolites. Dairy yeast strains generally respond to ethanol stress by reprogramming their metabolism and survive during various stresses of fermentation condition in a better form as compared with the pathogens (Lara-Hidalgo et al., 2017; Saini et al., 2017a, 2018). Another ethnic, curd-like product of Bulgaria called Katak has also been used traditionally for centuries using eve's milk. Katak has proven as a promising candidate for isolation of bacteria showing antibacterial and antifungal activity. *L. brevis* have been isolated from Katak which have the ability to suppress the growth of *Aspergillus* and *Penicillium* sp. (Tropcheva et al., 2014). Only a few studies conducted on probiotic bacteria derived from ethnic fermented dairy products have addressed human response to *Lactobacillus* at the molecular level. *L. rhamnosus* HN001 isolated from dairy products has been found to improve the immune system against multiple pathogens. Recently, in phase II clinical trial, *L. rhamnosus* was able to decolonize *S. aureus* in geriatric patients.

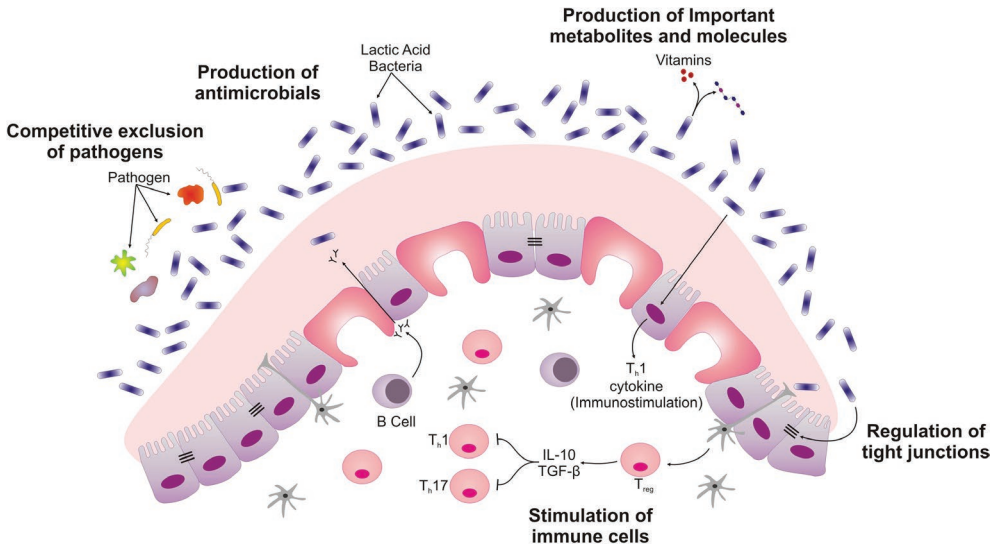

FIGURE 1 | Schematic displaying different ways by which probiotics exert their beneficial roles in intestine. LAB isolated from ethnic dairy product are responsible for competitive exclusion of pathogens, secretion of important metabolites and molecules such as bacteriocins. These Probiotics create mucus barrier by stimulating the Goblet cells. The interaction of *Lactobacillus* with intestinal epithelial cells also differentiates immune cell and regulates the barrier function of intestinal epithelial cell.

It was concluded that, probiotic strain *L. rhamnosus* outcompetes pathogens for important resources, thereby preventing colonization of pathogenic strains. *L. rhamnosus* HN001 was able to stimulate and help immune system in preventing colonization of *S. aureus* (Eggers et al., 2018). In a randomized clinical trial, consumption of probiotic cheese containing *L. rhamnosus* HN001 and *L. acidophilus* by elderly volunteers changed intestinal microbiota, lowered the count of *Clostridium difficile*, and increased IgA concentration compared to the cheese consumed without probiotics.

In human intestinal epithelium, the interaction between adjacent cells and cell-basement membranes form a crucial barrier that prevents the translocation of the microbes to the sub-epithelial layer. This adherence is governed by tight junction, gap junction, adherence junction, and desmosomes. The mechanistic role of probiotics reported in various studies associated with the strengthening of mucosal barrier function is mainly directed toward examining the ability of probiotic bacteria to prevent alterations in bridging proteins and tight junction present in the epithelial cell by various *in vitro* and *in vivo* models. A study conducted on *L. rhamnosus* S1K3 isolated from local Indian fermented milk product, dahi, showed that consumption of the strain for a period of 30 days could reduce *Salmonella enterica* due to the production of antimicrobial compounds. In this study, it was also observed that the pathogen was responsible for disruption of the tight junction proteins by down-regulating the expression of claudin 1 gene. Interestingly, when the probiotic strain S1K3 was administrated, it was again able to strengthen the integrity of the tight junction protein by up-regulating claudin 1 gene. There are two major antimicrobial peptides produced by intestinal epithelial cells in intestinal fluid, termed as defensins and cathelicidins. In the same study, probiotic bacteria induced an increase in the expression of human β-Defensin-2 and β-Defensin-3. The strain was able to induce the expression level of IL-4, Toll-like receptor (TLR) at Peyer's patches, and IgA level in serum and intestinal fluid (Kemgang et al., 2016). These factors present in the probiotic-fed group are collectively responsible for lowering down the level of pathogenic *Salmonella enterica* in feces. Therefore, probiotic strains depict enhanced intestinal barrier function by the production of the antimicrobial peptide, increased production of tight junction protein, stimulated intestinal mucosa, increased IgA responses, and prevented epithelial cell apoptosis.

Few studies have shown that probiotic *Lactobacilli* isolated from ethnic fermented product possess the ability to modulate gut residential population as a way to treat diseases like irritable bowel syndrome (IBS) and IBD. IBD includes both the ulcerative colitis (UC) and Crohn's diseases. These diseases occur in the area where the microbes (e.g. *Helicobacter hepaticus*) are prevalent in higher numbers. Microbiota present in a normal healthy individual differs from that of the person who is suffering from IBD. Firmicutes and *Bacteroides* are the predominant residential communities which are present in healthy individuals, whereas during dysbiosis of the gut in IBD patients, altered levels of predominant community and expansion in the level of Enterobacteriaceae family have been observed. Higher diversity of microbes present in normal individual provides colonization resistance against pathogens. The overall response of the normal residential microbes of the intestine is immunological tolerance and homeostasis. In contrast, during dysbiosis, the altered microbes activate deregulated T_h17 and T_h1 effector cells and in turn mediate inflammation of the intestine. The inflammation further leads to the formation of UC and Crohn's diseases in genetically susceptible host. The modulation of intestinal gut bacteria through the fermented dairy food containing probiotic bacteria offers a promising strategy to alleviate IBD diseases. A number of microbial strains, especially from the genera of *Lactobacillus, Bifidobacterium,* and *Faecalibacterium*, protect the host from mucosal inflammation

by a number of different mechanisms which include stimulation of anti-inflammatory cytokine IL-10 and down-regulation of inflammatory cytokines (Llopis et al., 2008). IL-10 has been found to stimulate T_h2 cells and is considered as a typical marker for anti-inflammatory effects. TNF and IL-12 are pro-inflammatory markers associated with stimulation of T_h1 cell and induction of IFN-γ by T-cell. An *in vivo* study conducted on colitis mice model fed with probiotic *L. plantarum* showed an increased anti-inflammatory effect on micro-integral membrane protein (MIMP), gut barrier, and inflammatory cytokines. These MIMPs may further act as target of clinical therapy for IBD patients (Yin et al., 2018). An *in vivo* study on extracellular vesicle (EV) of three *Lactobacillus* sp. (*Lactobacillus kefir, Lactobacillus kefiranofaciens*, and *Lactobacillus kefirgranum*) exhibited significant reduction of inflammation in epithelial cells of intestine. Administration of EV into IBD-induced mice models alle*via*ted rectal bleeding and weight loss, and increased stool consistency. Earlier, IL-8 has been reported as a crucial factor in stimulating the inflammation-based pathogenesis in IBD. It tends to be produced from the epithelial mucus lining in patients suffering from IBD. This study also reported that EV of *Lactobacillus* inhibits the expression of IL-8 in the intestine (Seo et al., 2018).

Furthermore, probiotic microorganisms and other commensals present in the gut can build a tolerant state which is mediated by the action of Toll-like receptors (TLR). Sensing of probiotics by dendritic cells (DCs), epithelial cells, and macrophages is governed by the TLR receptors which work as pattern recognition receptors (PRRs). Activation of these receptors induces pathways that trigger adaptive immune cells and pro-inflammatory T_h17 and T_h1 helper cells. It has been observed that TLR9 signaling is critical to facilitate anti-inflammatory effect of probiotics. However, there are studies where TLRs, such as TLR3 and TLR7, have also been implicated in the tolerance induced by probiotic cells. After activation by probiotic bacteria, DCs initiate differentiation of T_h0 to T_{reg}, which has been observed to exhibit an inhibitory effect on T_h1 and T_h17 inflammatory responses. The probiotics have been found to suppress the intestinal inflammation *via* down-regulation of TLR expression, decreased secretion of TNF, and inhibition of NF-κB signaling pathway. It has been observed that different *Lactobacillus* strains have ability to elicit a variable response in terms of cytokine production in different immune cells. For example, Van-Hemert et al. (2010), while working on different strains of *L. plantarum*, isolated from 48 different sources, observed induction of different concentrations of IL-10 and IL-12 during immune modulation. Future clinical trials guided by these parameters will provide further insights into the exact role of individual probiotic strain in immune modulation. Looking forward, there might be a need of focused selection and smarter manipulation of gut microbiota with the best strain of probiotics (Van-Hemert et al., 2010).

The presence of EPS in LAB allows the surface molecules to interact with host and protects probiotics from the harsh gut environment as demonstrated from various studies on LAB isolated from dairy products. EPS produced by dairy LAB has been observed to provide various physiological benefits such as induction of cytokines (IFNγ and IL-1), antitumor activity, macrophage activation, cholesterol reduction ability, and also enhanced colonization of the probiotics in gastrointestinal tract (Kitazawa et al., 1998, 2000; Pigeon et al., 2002; Chen and Chen, 2013). *L. kefiranofaciens* isolated from ethnic kefir grains produces an exopolysaccharide known as kefiran. Other isolates like *Lactobacillus* sp. KPB-167B, *L. kefiranofaciens, and Lactobacillus kefir* had been also described to produce EPS (Wang et al., 2008). In other studies, exopolysaccharide-producing probiotics, *L. lactis*, *and L. plantarum* were also isolated from camel milk, dahi, and other ethnic dairy products (Vijayendra et al., 2008; Abushelaibi et al., 2017). In a study by Zhang et al. (2017), the effect of EPS produced by *L. plantarum* YW11 isolated from ethnic Tibetan kefir on the gut microbiota and oxidative stress in an aging mouse model was investigated. A dose of 50 mg/kg per day was able to relieve oxidative stress in mice by increasing the level of superoxide dismutase, catalase, and glutathione peroxidase, and decreasing the level of malondialdehyde. EPS was also able to modulate the gut microbiota selectively by increasing the abundance of *Butyricicoccus* sp. and *Blautia* sp. and led to enhanced secretion of SCFA (Zhang et al., 2017).

Currently, researchers are making effort to exploit the benefits of gut microbiota by replicating the natural milieu of intestine. Fecal microbiota transplantation (FMT) is one such promising therapy which is gaining acceptance for treating autoimmune and infectious diseases. Ulcerative colitis and *Clostridium difficile* infection have been successfully treated using FMT; however, issues regarding the presence of unknown components still cast uncertainty about the safety of this approach (Gupta et al., 2016). Mostly, microbial strains present in FMT responsible for therapeutic effect are still unknown. Some bacterial taxa associated with FMT are *Bacteroides*, *Bifidobacteria*, *Clostridial* clusters, and *Lactobacilli* (Gupta et al., 2016). Incorporation of these known probiotic strains (which confer protection to IBD and colitis) in FMT therapy will eliminate risks associated with transfer of foreign unknown materials of the microbiota from fecal matter of donors. The ability for creating such probiotic incorporated therapies requires a detailed understanding of the *in vivo* mechanism of action of probiotic strains and disease pathogenesis. Strengthening the intestinal barrier using FMT can protect host from toxins released during CDI and other colonic infections. Microorganisms derived from ethnic dairy products can be a source of novel beneficial microorganisms which can limit unusual inflammatory responses and metabolic disorders (Bakken, 2014; Spinler et al., 2016).

Cardiovascular diseases are cause of serious threat to human life, as more than 17 million people died from these diseases in 2015 (Hajar, 2016). Different epidemiological studies carried out in last two decades have confirmed the correlation between cardiovascular diseases and total cholesterol (TC). In a study conducted on fermented dairy products like kefir, yoghurt, and cultured milk prepared from different milk sources, reduced cholesterol level was found compared to only milk (Bonczar et al., 2016). Cholesterol-lowering properties of only a few ethnic dairy products have been validated in animal models. A number of mechanisms have been put forward underlying the ability of probiotic bacteria to remove the cholesterol. These include hydrolysis of conjugated bile acid, assimilation of cholesterol, and precipitation of cholesterol along with bile salts.

A study conducted on traditional Iranian dairy products (made of ewe milk) showed that both dead and live *L. brevis* could assimilate cholesterol (Iranmanesh et al., 2014). Another study conducted on *L. plantarum* showed similar anti-cholesteremic effect as shown by *L. brevis* in Iranian dairy product. Scanning electron microscopic (SEM) images in the study depicted that cholesterol gets adhered to the cell surface by both enzymatic assimilation and cell surface binding. (Choi and Chang, 2015).

A number of studies have indicated the role of kefir grains in cholesterol reduction. *L. plantarum* MA2 isolated from kefir has also shown hypocholesterolemic activity in male Sprague–Dawley (SD) rats fed with high cholesterol diet. Huang et al. (2013) observed that probiotic *L. plantarum* Lp27 isolated from Tibetan kefir grains was able to decrease serum total cholesterol, LDL-cholesterol, and triglycerides in hypercholesterolemic SD rats that consumed a diet supplemented with *Lactobacillus*. They further found that the Lp27 strain was able to reduce cholesterol absorption in Caco-2 cells by down-regulating the expression of Niemann-PickC1-like 1 (NPC1L1) in Caco-2 cells (Huang et al., 2013).

In another study carried out in SD rats fed with high cholesterol-containing diet, Zheng et al. (2013) observed that three strains, *L. kefiri* D17, *L. plantarum* B23, and *L. acidophilus* LA15, were able to lower the serum total cholesterol, LDL, and triglyceride levels as well (Zheng et al., 2013). In a similar *in vivo* study, milk fermented with *Lactococcus lactis* subsp. *lactis* IS-10285 was found responsible for reducing LDL cholesterol, total serum cholesterol, and total bile acids (Pato et al., 2004). In a recent clinical study on hypertensive overweight women, the impacts of *Lactobacillus casei* 01 (concentration-10^8 cfu/g) when incorporated in Minas Frescal cheese was studied at pilot scale. The clinical study revealed that *L. casei* incorporation improved the low- and high-density lipoprotein cholesterol, total cholesterol, triacylglycerides, hematocrit, and hemoglobin count (Sperry et al., 2018).

In a recent study, 115 cultures isolated from ethnic fermented Tibetan yak milk were used to screen the cholesterol reduction ability. *L. plantarum* Lp3 was found to reduce cholesterol by 73.3% when administrated in rats fed with cholesterol-rich diet. A significant decline in liver and serum cholesterol was also detected (Ding et al., 2017). A study on yak milk fermented product concluded that the *Lactobacillus* isolated from ethnic yak milk has higher cholesterol reduction ability in comparison to other dairy products (Pan et al., 2011). The study showed that the mechanism behind the lowering effect of Lp3 was by assimilation and removal of cholesterol in feces due to deconjugation of bile acids.

A study using commercial and traditional kefir showed that there was a lower plasma cholesterol level when mouse models were fed with traditional kefir; however, when fed with commercial kefir, there was no such significant lowering of cholesterol. The study showed the beneficial role of traditional fermented milk product in lowering cholesterol level (Bourrie et al., 2018). A similar study conducted in Turkey on *L. fermentum* strains, isolated from Tulum cheese, showed that *L. fermentum* strains differ in their ability to assimilate cholesterol from media. It was observed that cholesterol assimilation in these strains ranged from 20.7 to 71.1% in media with bile. The authors also found that adhesion rates of some of these strains onto the Caco-2 cells were higher than that of control probiotic strain *L. rhamnosus* GG (Tulumoğlu et al., 2014). Furthermore, to find out whether traditional fermented food or traditional milk products serve as a better source of probiotic *Lactobacillus*, a comparative study was conducted on traditional homemade fermented cabbage called Suan-tsai and Koumiss. It was observed that there is a difference in cholesterol reduction ability of isolated probiotics from these sources. *Lactobacillus* isolated from Koumiss exhibited a higher cholesterol removal and bile tolerance ability than that of Suan-tsai samples. Thus, the traditional fermented dairy product can be a better source of potential probiotics when dealing with cholesterol assimilation ability (Guo et al., 2015). It can, therefore, be concluded that cholesterol reduction ability varies among different strains of lactic acid bacteria and ethnic foods can be potent source of these LAB.

The gut microbiota is comprised of resident commensal bacteria and transient probiotic bacteria which are consumed by fermented milk products (FMPs) and healthy foods. A correlation between gut microbiota and native inhabitants of FMPs has been observed in many studies. Veiga and coworkers, in a metagenomic study to observe the effect of FMPs on gut health, revealed that FMPs could substantially increase native beneficial bacteria like *Bifidobacteria* spp, along with the strains present in FMPs like *Streptococcus thermophilus* CNCM I-1630, *Lactobacillus delbrueckii* subsp. *bulgaricus* CNCM I-1632, *Lactobacillus delbrueckii* subsp. *bulgaricus* CNCMI-1519, and *Lactococcus lactis* CNCM I-1631. Three non-commensal species *Parabacteroides distasonis*, *Bilophila wadsworthia*, and *Clostridium* sp. HGF-2 were observed to be mitigated. FMPs also increased the native butyrate producers like *Roseburia intestinalis*, *Roseburia inulinivorans*, *Butyriovibrio crossotus*, *Clostridium* L2–50, *Faecalibacterium prausnitzii*, *Eubacterium hallii*, *Lachnospiraceae bacterium* 5-1-63FAA, *Coprococcus* ART55/1 and *Acidaminococcus*, *Bifidobactericae*, and *Firmicutes* (Veiga et al., 2014). Another study by Volokh and coworkers found significant augmentation of *Bifidobacterium* and *Firmicutes*, *Streptococcus isothermophilus*, and *Lactobacillus delbrueckii* after 30 days of FMP feeding. *Slackia isoflavoniconvertens* and *Adlercreutzia equolifaciens* were found to be increased with specific ability to metabolize isoflavone to equol, suggesting potential for multi-faceted positive impact of FMP (Volokh et al., 2017). Indian population belonging to the tribal parts exhibits higher consumption of FMPs than those consuming westernized foods, which may have increased beneficial gut microbiota. In a study, Mojibur R. Khan and coscientists have analyzed the gut bacterial profile of Mongoloid and Proto-Australoid tribes of India, where prevalence of *Prevotellaceae, Ruminococcaceae, Eubacteriaceae, Lachnospiraceae, Clostridiaceae, Veillonellaceae, Bacteroidaceae, Bifidobacteriaceae, Erysipelotrichaceae, Lactobacillaceae*, and *Coriobacteriaceae* have been observed. Among these strains, *Bifidobacteriaceae, Lactobacillaceae, Veillonellaceae, Clostridiaceae*, and *Eubacteriaceae* showed significant differences in their abundance across the population (Dehingia et al., 2015). Another study conducted

using 1,000 subjects across India in a project called "Landscape Of Gut Microbiome - Pan-India (LogMPIE)" showed dominant bacteria belonging to the phyla of *Firmicutes, Bacteroidetes and Proteobacteria*. Rarer factions belonging to the phyla of *Verrucomicrobia* and *Spirochaetes* have been observed. This study also reports prevalence of *Prevotella* dominance in Indian cohorts (Shetty, 2018).

ANTICANCER ATTRIBUTES OF FERMENTED MILK PRODUCTS AND ITS ASSOCIATED MICROBIOTA

Alteration or disturbances in gut milieu has an apparent effect on cancer development. Native gut inhabitants possess negative effect on cancer metabolism and prognosis (Zitvogel et al., 2017). Diverse mechanisms attribute to the anticancer properties of probiotics, prebiotics, and synbiotics (**Figure 2**). These include inhibition of mutagen- and carcinogen-producing microbes, protection from oxidative stress, metabolism of carcinogen and xenobiotics, immunomodulation, and altering expression of different genes in cancer metabolism like metastasis, cell cycle control, apoptosis and cell death, cancer stem cell inhibition, modulation of intestinal microflora, and inhibition of tyrosine kinase pathway (Raman et al., 2013; Liévin-Le Moal and Servin, 2014). It has been seen that probiotic bacteria have modulatory and anti-proliferative effect on different cells and cell types. Probiotics have been shown to affect different stages of metastasis like cellular adhesion, invasion and intravasation, maintenance of tumor microenvironment, and cancer stem cell homeostasis. Cell-to-cell adhesion plays a critical role in protecting cellular integrity of the tissues and any flaw in the system augments the metastasis process. Probiotics affect different stages of metastasis like cellular adhesion, invasion and intravasation, and maintenance of tumor microenvironment and cancer stem cell homeostasis (Motevaseli et al., 2017). The mechanisms span a wide range of lactic acid bacteria isolated from individual and indigenous ethnic milk fermented products. Although, the anticancer potential of extant human microbiota is being thoroughly investigated, yet, exploring mechanistic insights of anticancer activity of indigenous fermented milk products and their inhabiting microbiota is of paramount importance.

Several studies have been conducted on anti-proliferative effect of kefir. Kefir is a popular, indigenous fermented milk product of West Asia, comprised of a diverse group of lactic acid bacteria and yeast. Both kefir and cell-free extract of kefir could regulate expression of various genes involved in apoptosis and survivability in Caco-2 and HT-29 cancer cells. Kefir could arrest cell cycle in G2 phase and induce cell death. Using real-time and western blot studies, the authors showed that kefir could successfully decrease transforming growth factors (TNF-α and TNF-β) in HT-29 cell line which are essential for cell proliferation. Kefir also increases Bax-Bcl-2 ratio, which substantiates pro-apoptic effect of the extract. Interestingly, they observed no alteration in expression of matrix metalloproteinase and intravasation/mitigation of breast and colon cancer cells (Khoury et al., 2014). However, another study showed that cell-free extract of kefir displayed anti-metastasis effect on lung carcinoma and B16 melanoma cells (Furukawa et al., 2000). Metastasis does not only depend upon metabolites, but also on microenvironment, intravasation of vessels, and colonization of the secondary sites (Fidler, 2003). Chen et al. (2007) have demonstrated the effect of kefir in mammary cancer cells (MCF-7) and compared kefir with yoghurt extract. They found that kefir displayed anti-proliferative effect on cancer cell population upto 56%, whereas yoghurt

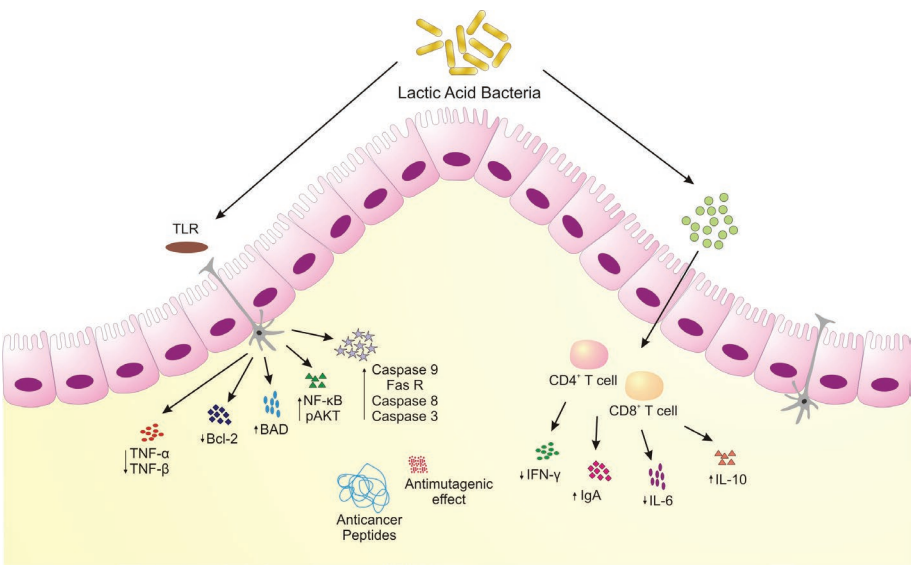

FIGURE 2 | Schematic representing anticancer attributes of microorganisms associated with traditional fermented milk foods. LAB induce apoptosis, increased anti-inflammatory cytokines, produce bioactive peptides to restrain colonic and colon-associated cancer.

could reduce only 14%. Capillary electrophoresis result showed that the content of peptide in kefir was much more than that of yogurt, which could be a probable reason for its enhanced anti-proliferative effect (Chen et al., 2007). Numerous studies have demonstrated anticancer effect of peptides (Deslouches and Di, 2017). *L. rhamnosus* GG, a bacterium isolated from human infant feces, expresses proteins p50 and p75, which are established to have anti-proliferative effect on cancer cells (Liévin-Le Moal and Servin, 2014). Kefir also delayed tumor development when fed to a breast cancer model of mouse for 2–7 days in a dose-dependent manner. Within 2 days, both the kefir and kefir-free extract increased IL-10 and reduced IL-6 expression in serum, which perturbs estrogen homeostasis in mammary gland. It was furthermore noticed that kefir could reduce TNF-α and INF-γ concentrations in breast cancer cells, factors required for cell proliferation. Kefir also augmented cellular apoptosis by reducing Bcl-2 protein in mammary glands. Involvement of peptides from the probiotic organisms and contents of the kefir was held as an important factor for tumor mitigation (Furukawa et al., 1990; Vinderola et al., 2005). Shiomi et al. (1982) observed that the molecules exerted by the fermentation of the bacteria displayed cytotoxicity to the cancer cells rather than the bacteria itself (Shiomi et al., 1982). Moreover, a number of probiotics that were isolated from fermented dairy products have also been found to have anti-*H. pylori* effects (Nair et al., 2016).

Dubey et al. (2016) showed promising effect of *P. pentosaceus* GS4 on colon cancer mitigation. *P. pentosaceus* GS4 strain was isolated from khadi, an Indian fermented dairy-based food. It showed anti-proliferation activity of HCT-16 mammalian cells, and increased expression of pro-apoptotic molecules NF-κB and p-Akt. In animal model, it decreased the severity of the cancer, augmented oxidative stress and necrosis (Dubey et al., 2016). The authors demonstrated the effect of conjugated linoleic acids on cancer mitigation. The gut mirobiota probably helps biohydrogenation to produce conjugated linoleic acid (CLA) which in turn triggers apoptosis, caspase activity, and cleavage of poly-ADP ribose polymerase (PARP). It was previously demonstrated that *P. pentosaceus* could increase Bax/Bcl-2 ratio which subsequently increases mitochondria-mediated membrane permeability and inhibits cancer cells. It has been seen that local delivery of CLA could modulate gut microenvironment in mitigation of colon cancer (Bassaganya-Riera et al., 2012; Dubey et al., 2015). Linoleic acid found in gut could be bio-transformed to CLA by intestinal microbiota which explains the pro-apoptotic and anti-proliferative effect (Edionwe and Kies, 2001).

Traditional Iranian dairy products like tarkineh, shiraz, yogurt, and cheese contain various species like *L. plantarum* and *L. lactis subsp*. lactis which showed anticancer activity against HT-29, AGS, MCF-7, and HeLa cells. The pro-apoptotic activity of the bacterial secreted products can be compared to the cytotoxic potential of taxol, an anticancer phytochemical. Haghshenas et al. (2015) demonstrated that the anti-proliferative effect of the fermented products reduced after pronase (a protease cocktail) treatment, which depicted proteinaceous nature of the bioactive metabolites (Haghshenas et al., 2015). *L. plantarum* strain is also a dominating lactic acid bacterial species found in ethnic dairy products like Armada cheese, Batzos cheese, yoghurt, Laban zeer, Kulenaoto, M'Bannick, Kwerionik, Koumiss, and Zincica (Psoni et al., 2003; Bernardeau et al., 2008). In another study, Haghshenas et al. (2015), while working with Iranian traditional yogurt, found two *Acetobacter* species, *Acetobacter indonesiensis* and *Acetobacter syzygii*, responsible for exhibiting cytotoxicity toward HeLa, MCF-7, AGS, and HT-29 cancer cells (Haghshenas et al., 2015). The secreted products of both the strains showed significant inhibition of cell proliferation without hindering the physiology of normal cells. Similar to the previous studies by the group, pronase treatment significantly reduced the effect of secreted products, due to the involvement active peptides or enzymes responsible for transformation of carcinogenic and xenobiotic compounds (De LeBlanc and Perdigón, 2010). The secreted metabolites also displayed pro-apoptotic behavior when treated with the above-mentioned cell lines, with observable physiological disorders like membrane blebbing, nucleus fragmentation, cell shrinkage, and apoptotic body formation (Haghshenas et al., 2015). Microbial samples isolated from yoghurt and cheese from rural areas of Kurdistan province of Iran contain highly beneficial yeast *K. marxianus* AS41. Saber et al. (2017) observed that *K. marxianus*-secreted metabolites significantly down-regulated Bcl-2 expression and up-regulated BAD and Caspase 9 gene expression in epithelial cancer cells, which subsequently increased apoptosis and reduced cell proliferation. In addition, Fas R, caspase 8, and caspase 3 were also observed to be up-regulated, the factors involved in intrinsic apoptotic pathway. The secreted metabolites demonstrated the apoptotic effect on cellular physiology by DNA fragmentation, chromatic condensation, and membrane blebbing in HT-29, Caco-2, and Hep-2 and Hep-G2 cells (Saber et al., 2017).

De LeBlanc et al. (2005) showed that *Lactobacillus helveticus* R389 (a strain isolated from Swiss Cheese) when fed to a mouse with breast cancer, increased IgA and CD4 positive cells 4 days post injection. The strain also showed decreased IL-6 and increased IL-10 expression. The fermented milk of *L. helveticus* R389 increased the IgA positive cells, eventually stimulated immune system, and inhibited growth of immune-dependent fibrosarcosoma in mouse model. *L. helveticus* also delayed the growth of breast cancer in balb/c mouse. It was inferred that the metabolites possibly modulated endocrine system, as decreased IL-6 expression repressed estrogen-dependent tumor development (De LeBlanc et al., 2005). *L. helveticus* also exerted anti-mutagenic effect in Ames test, where milk fermented by the proteolytic strain could inhibit mutagenesis significantly (Matar et al., 1997).

Camel milk fermented products like Chal and Shubat are very important part of daily cuisine in North Africa and mid-western Asia. Still, limited literature is available on mechanistic insight of anticancer properties of fermented camel milk. In a recent study, anticancer property of water-soluble extracts (<3 KDa) of fermented bovine and camel milk was evaluated against MCF-7, Caco-2, and HeLa carcinoma cells. It was observed that the proliferation of MCF-7, Caco-2, and HeLa cells was significantly inhibited by water-soluble extracts of camel milk rather than bovine milk fermented

by strains like *L. plantarum*, *L. acidophilus*, *L. reuteri*, and *L. lactis*. The authors concluded that the high anti-proliferation activity of fermented camel milk prepared using these strains may have contributed to the difference in peptides derived from fermented camel milk rather than those from fermented bovine milk. They correlated proliferation inhibition with angiotensin-converting-enzyme (ACE)-inhibitors, which suggested that peptides derived from fermented camel milk have multifunctional bioactivity (Ayyash et al., 2018).

Anti-mutagenic effect of probiotic metabolites is also of paramount importance for prevention of cancer. Ahmadi et al. studied 25 bacterial strains isolated from the Tarhana, an indigenous Turkish food, based on grain and yoghurt or fermented milk, a similar dish like Kishk. Species of *L. brevis*, *L. plantarum*, and *L. casei* isolated from Tarhana showed high anti-mutagenic and anticancerous effect (Abbas Ahmadi et al., 2014). In a study conducted on strains isolated from Dadih, an Indonesian ethnic fermented milk of West Sumatra, *Enterococcus faecium* IS-27526 was found to lower down the fecal mutagenicity in rats as compared to the milk cultured with *L. plantarum IS-20506* (Surono et al., 2009).

Recently, Navani et al. (2018) filed a patent on asparaginase produced by *L. brevis* isolated from Himalayan yak cheese Chhurpi. Asparaginase is an anticancer enzyme which depletes free L-asparagine in blood, leading to death of leukemic cells resulting from starvation (Navani et al., Patent no. 201811019299/ New Delhi, India; unpublished data). Proteins like asparaginase may hold a key to explore more metabolites associated with anticancer attributes. Previously, Haghshenas and coworkers have discussed the role of anticancerous peptides and proteins in traditional fermented milks, and proteins like asparaginase may have a role in their study (Haghshenas et al., 2015). Probably enzyme like asparaginase in combination with peptides and small metabolites exert synergistic effect toward anticancer activities of fermented dairy products.

The beneficial effect of probiotic dairy products and their extant microbes in cancer are being studied thoroughly throughout the world and found to be implicated in several pathways of cancer metabolism, angiogenesis, and metastasis. The bacteria or their bioactive molecules alter the expression of different genes and restrain varied pathways in cancer progression. Studies on the strain-specific bioactive compounds, immunoregulation, time-dependent transcriptomics, and metabolic studies will guide a better picture toward the application of the fermented products and their inhabitant microbes. Furthermore, randomized clinical studies should be conducted to translate the observations for medical use.

BENEFICIAL PERSPECTIVE OF BIOACTIVE METABOLITES PRESENT IN ETHNIC FERMENTED DAIRY PRODUCTS

During fermentation, microorganisms metabolize complex food matrix and synthesize bioavailable and bioactive compounds leading to healthy consequences for humans (**Figure 3**). In dairy industry, LAB are widely used as starter culture as they play crucial role in fermentation. At industrial scale, LAB are used for the synthesis of numerous primary or secondary metabolites like enzymes, organic acids, and vitamins (Patel et al., 2013). Microflora (*L. mesenteroides*, *L. lactis,* and *L. paracasei*) present in Kajmak (a fermented dairy product of soft and creamy texture found in mid-western Asia and Eastern Europe) contribute in developing aroma and flavor of the product by producing diacetyl, acetate, and ethanol (Jokovic et al., 2008). Acetate acidifies its surrounding environment, resulting in inhibition of pathogenic bacteria. Acetate and propionate control sugar metabolism *in vivo* by alleviating glycaemia and improving insulin sensitivity (Turnbaugh et al., 2006). *L. acidophilus, Streptococcus cremoris*, and *Streptococcus lactis* majorly found in camel's milk and Chal are used as starters in dairy products. Wide varieties of lactic acid bacteria present in camel's fermented milk assimilate carbohydrates like galactose, mannose, lactose, and xylose. Dairy yeast *K. marxianus* and *K. lactis* also carry ability to assimilate sugars like lactose and galactose (Yam et al., 2015; Saini et al., 2017b). *L. plantarum* RYPR1, an indigenous probiotic strain isolated from Indian fermented beverage Raabadi, showed hypocholesterolemic property due to bile salt hydrolase activity. Antibacterial activity of isolated strain was also observed against tested pathogens like *E. coli*, *S. aureus*, *P. aeruginosa,* and *S. albony* (Yadav et al., 2016).

Enzymatic activity of microflora present in fermented food contributes to improved digestibility and nutritional value of food. In other words, beneficial microorganisms present in fermented food may be used for the synthesis of enzyme extracts which are stable under native environment of fermentation and transform complex food matters into simpler ones (Tamang et al., 2016a,b,c). For example, thermophilic strain *L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus* used for the production of yogurt contain substantial amount of β-D-galactosidase enzyme which has been found to improve lactose malabsorption in people suffering from lactose intolerance (Tamang et al., 2016a,b,c). Many strains of LAB isolated from different fermented products produce also antimicrobial compounds such as bacteriocin by *L. lactis* and pediocin by *P. pentosaceus* (Tamang et al., 2016a,b,c). Likewise, cheese, a fermented dairy food is also consumed by lactose-intolerant people as some amount of lactose present in the milk is fermented and the rest is processed into the whey at the time of cheese manufacturing (Kokkiligadda et al., 2016; Marco et al., 2017). Enzymatic activities of LAB have been extensively studied and were found to produce diverse enzymes which affect food quality, texture, and organoleptic attributes. Proteolytic and lipolytic activity of LAB improves sensory quality of cheese. *Lactococcus lactis* subsp. *cremoris* produces peptidases which improve flavor and aroma of cheese (Guldfeldt et al., 2001, González et al., 2010). Enzymatic activity of LAB strains—*Lactococcus* sp. and *Enterococcus* sp.—associated with Spanish cheese Genestoso showed high activity of dipeptidase, leading to their enterolytic activity (González et al., 2010). A study showed that strains belonging to the genera *Lactobacillus* produce hydrolytic enzymes in gastrointestinal tract which positively influenced digestive process and reduced probability

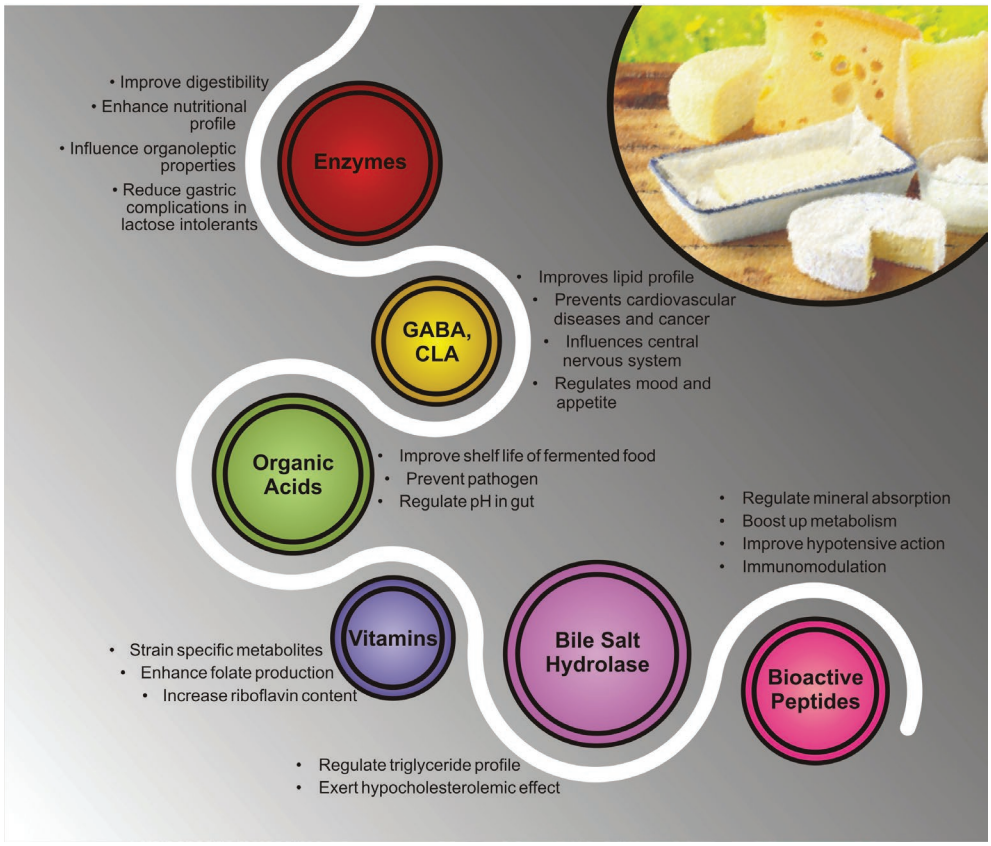

FIGURE 3 | Different health benefits of bioactive metabolites present in ethnic fermented dairy products.

of malabsorption (Naidu et al., 1999). Fermented foods also harbor *Lactobacillus*, *Lactococcus*, *Bifidobacterium*, and *Pediococcus* which synthesized enzymes responsible for carbohydrate degradation such as xylanases, glucosidases, and amylases (Novik et al., 2007; Patel et al., 2012).

Conjugated linoleic acid (CLA) has numerous beneficial effects on many health-related complications such as inflammatory diseases, cancer, metabolic issues, and cardiovascular diseases. LAB inherent in fermented dairy products enriches the product with increasing content of CLA. One study corroborating the fact showed that *L. lactis* subsp. *cremoris* MRS47 from Brazilian kefir grains has ability to modulate lipid profile (increased SCFA) of milk by fermentation. During fermentation, elevated amount of polyunsaturated fatty acid and reduced saturated fatty acid content were also observed (Vieira et al., 2017).

LAB produce amino acid derivatives and oligopeptides because of their proteolytic activity in fermented food. These bioactive molecules play important role in regulating many vital activities such as mineral absorption, metabolism, cardiovascular activities, immune modulations, and mood alteration (Pessione and Cirrincione, 2016). Proteolytic system of LAB produces many bioactive peptides from milk protein, especially from casein and whey protein. It was found that casein hydrolysis-generated peptides influenced gut-brain axis activities, cardiovascular functions, antimicrobial activity, nutrition, and immunomodulatory attributes (Thakur et al., 2012). *L. helveticus* CRL 1062, a common constituent of lactic starter culture used in cheese industry, hydrolyzed α- and β-caseins through cell envelop proteinase (Hébert et al., 1999). *L. helveticus* CRL 581, isolated from cheese, hydrolyzed β-casein more quickly in comparison to α-casein due to proteinase enzyme associated with the cell membrane (Hébert et al., 1999). Bioactive peptides released from hydrolysis of milk whey proteins (alpha-lacto-albumin, beta-lacto-globulin, lactoferrin, and immunoglobulins) produced bioactive peptides, had hypocholesterolemic property which can minimize absorption, and increased excretion of cholesterol in feces (Nagaoka et al., 2001).

ACE plays an imperative role in increasing blood pressure by generation of vasoconstrictor angiotensin II (a component of renin-angiotensin system) and down-regulating the expression of vasodilator bradykinin. Drugs to restrain ACE in blood are commonly used in hypertension, myocardial infarction (heart attack), and diabetes (Ma et al., 2010). Renin-angiotensin-aldosterone is the primary blood pressure controlling system. ACE inhibitory peptides casokinins and lactokinins are produced by proteolytic digestion of casein (FitzGerald and Meisel, 2000; FitzGerald et al., 2004). Blocking the generation of Angiotensin II, the peptides lower arterial

resistance, increase intravenous capacity, decrease cardiac output and index, and lowered resistance in blood vessels in kidneys and increased excretion of sodium and urine. Dairy isolates *Lactobacillus delbrueckii* subsp. *bulgaricus* SS1 and *L. lactis* subsp. *cremoris* FT4 are responsible for the synthesis of ACE inhibitor peptides in fermented dairy products. In another study, homemade Argentinian hard cheese-isolated strain *Lactobacillus delbrueckii* subsp. *lactis* CRL 581 was investigated for its cell envelope-associated proteinase (CEP) activity. It was found that *Lactobacillus delbrueckii* subsp. *lactis* CEP was able to hydrolyze both α- and β-casein except k-casein and produced antihypertensive peptides (Hebert et al., 2008). Generally, LAB peptidases enhance ACE inhibitor activity by minimizing the chain composed of poly/oligopeptide. Oligopeptides having antihypertensive property, derived from casein, are 2–6 amino acids long (Pessione and Cirrincione, 2016). *L. helveticus* PR4, obtained from Italian cheese, produced ACE inhibitory and antibacterial peptides by hydrolyzing casein milk protein. Antibacterial peptides showed wide range of inhibition against tested pathogens *Enterococcus faecium*, *Bacillus megaterium*, *Escherichia coli*, *Salmonella* spp., *Yersinia enterocolitica*, and *Staphylococcus aureus* (Minervini et al., 2003).

Some LAB species can decarboxylate amino acids such as gamma-amino-butyrate (GABA) which is a decarboxylated product of glutamate. Likewise histamine, β-phenylethylamine and tyramine are decrboxylated products of histidine, phenylalanine, and tyrosine respectively (Konings, 2006). These amines such as histamine, tyramine, tryptamine, beta-phenylethyl amine, and GABA have beneficial impact on vascular or central nervous system of humans (Moreno-Arribas et al., 2003). GABA and beta-phenylethyl amine are responsible for relaxing gut smooth muscles, controlling appetite as well as the mood. *L. brevis* PM17, *L. plantarum* C48, *L. paracasei* PF6, *L. delbrueckii* subsp. *bulgaricus* PR1, and *L. lactis* PU1 were reported as the maximum producer of GABA in comparison to other isolated species from Italian cheese (Siragusa et al., 2007). In another study, 81 strains of *Lactobacillus* were isolated from the Koumiss procured from Xinjiang, China, and screened for ACE inhibitory activity and GABA production. Koumiss is traditional fermented milk made of mare's or camel's milk. It was found that 16 strains showed ACE inhibitory activity, out of which two strains showed significant GABA-producing ability. *Lactobacillus helveticus* ND01 showed good ACE inhibitory and high GABA synthesis capability as well (Sun et al., 2009).

Several metabolites produced during fermentation are strain specific. LAB produce vitamins (e.g. folate) in variety of fermented dairy products such as curd, yoghurt, cultured butter milk, cheeses etc. Amount of folate in yoghurt depends on starter cultures (Wouters et al., 2002). Folate is a water-soluble vitamin B and very important for human health. Deficiency of folate may lead to a variety of health complications like osteoporosis, Alzheimer's disease, coronary heart disease, and high risk of breast and colorectal cancer (Rossi et al., 2011). Folate biosynthesis is strain-dependent property, as many *Lactobacillus* spp. and *Lactococci* spp. Like *L. plantarum, L. bulgaricus, L. lactis*, *S. thermophilus*, and *Enterococcus* spp. are able to produce folate while some lactobacilli (*L. gasseri, L. salivarius, L. acidophilus*, and *L. johnsonii*) cannot due to absence of genes responsible for folate biosynthesis (LeBlanc et al., 2007). In a study, cow's milk-isolated folate-producing lactic acid bacteria were screened for probiotic properties. It was found that *L. lactis* subsp. *cremoris* and *L. lactis* subsp. *lactis* showed efficient probiotic properties with significant folate production (Gangadharan et al., 2010). These folate-producing strains can be used for developing fermented dairy food with good nutrition profile. Riboflavin, a precursor of the coenzymes flavin mononucleotide (FMN) and flavin adenine dinucleotide (FAD) have been shown to be produced by *L. acidophilus* isolated from yoghurt samples in Vellore, India (Jayashree et al., 2010). Whey was recommended as a better fermentation medium compared with skim milk for riboflavin production (Guru and Viswanathan, 2013). The gut bacteria can also transform anti-nutritive factors present in cereals or plant products into nutritional metabolites improving nutritional value of the food product. Phytase-producing strains have long been used in degradation of phytate in wheat dough to increase calcium, phosphorus, and magnesium in food (Lopez et al., 1983, 2000; Reale et al., 2004; Palacios et al., 2008). Sharma et al. (2018) have recently reported a novel tyrosine phosphate like phytase from a probiotic bacterium *L. fermentum* NKN51 isolated from Himalayan yak cheese Chhurpi. The enzyme showed high specificity to its substrate phytate, a compound which chelates micronutrients and cationic proteins and limits their availability in food. Phytase from *L. fermentum* NKN51 also showed significant dephytanation of finger millet and Durum wheat under *in vitro* gastrointestinal conditions and displayed potential as food and feed additive (Sharma et al., 2018). Novel phytase have also been reported in other probiotic strains like *Bifidobacterium pseudocatenulatum* (Tamayo-Ramos, 2012), and *Lactobacillus sanfranciscensis* (De Angelis et al., 2003).

Probiotic strains improve the nutritional profile of fermented food by introducing their metabolites in food matrix. Bioactive metabolites constitute functional peptides, small molecules, short-chain fatty acids, vasodilators, vitamins, immunoregulators, and numerous other factors facilitate healthy and proper vital activities in human body. Thus, incorporating fermented food in daily eating habit can be a smart approach to attain healthy living.

RECENT ADVANCEMENTS IN GUT MICROBIOME RESEARCH

Recent advances in Next Generation Sequencing (NGS) approaches have completely altered gut microbiome research. "Omics" studies have facilitated researchers to explore *in situ* microbiome both temporally and spatially, complex microbial communities targeted phenotype studies to explore potential role in human health. Nicola Segata and coworkers have provided the largest ever catalog of human-associated microbes across

the world population. They analyzed bacterial genomes from oral cavity, skin, vagina, and stool samples of 32 countries taking westernized and non-westernized lifestyle and also included cohorts from Madagascar. The metagenomic samples of nearly 9,500 individuals reconstituted 154,723 new microbial genomes from 5,000 species. Around 77% is found to be unexplored till date. Many of the beneficial bacteria like *Succinatimonas* spp., *Bifidobacteria* spp., and *Firmicutes* spp. were found to be prevalent in non-westernized communities suggesting host lifestyle relating to healthier food habits. Whereas, bacteroids were majorly found in westernized factions. The study has reflected on the diversity of bacteria related to antibiotics, complex industrialization, and unhealthy lifestyle (Pasolli et al., 2019). A study on the presence of lactic acid bacteria in ethnic fermented milk of North-Eastern India by Tamang and coworkers depicts presence of Firmicutes (*Streptococcaceae, Lactobacillaceae*) and Proteobacteria (*Acetobacteraceae*) as the predominant species, *L. lactis* and *L. helveticus* being major lactic acid bacteria and *Acetobacter* spp. and *Gluconobacter* spp. as the chief acetic acid bacteria present in these products (Shangpliang et al., 2018). Moreover, recent NGS studies on cheese from pasteurized and unpasteurized milk showed difference in bacterial compositions relating to ripening, aging, coloration, and beneficial role of probiotic organisms (Salazar et al., 2018). The discovery of bacterial populations in culture-independent method has rejuvenated the significance of microbial ecology in fermented food and important functionality of the products.

Studies with large cohorts have shown pivotal role of early life microbiota in obesity and type I diabetes (T1D). Researchers identified biomarkers for assessing the role of these organisms later in adulthood. These findings depict that genes related to various functions like fermentation and biosynthesis of SCFA have more relevance than particular taxa in prognosticating development in metabolic and autoimmune diseases. Moreover, the study also conveyed that attention has to be paid in developing the patterns of beneficial microbial communities in childhood for infant health (Stanislawski et al., 2018, Stewart et al., 2018, Vatanen et al., 2018). World Gastroenterology Organization has recently launched new guidelines for physicians with science-based formulations to improve gut health *via* diet. Scientists have started exploring the effect of macronutrients like omega-3 polyunsaturated fatty acids, micronutrients, and food additives in broadening the dietary patterns. Food groups are recommending diet with different fibers, based on fermentability rather than solubility. Overall, scientists provided guidelines around various fibers and macronutrients affecting the gut health instead of isolated nutrients for thriving individual taxa of microbe (Halmos et al., 2015; Staudacher et al., 2017; Makharia et al., 2018). Researchers have elucidated the role of non-antibiotic drugs like proton-pump inhibitors, antipsychotics, and metformin in altering gut microbiota, which may have role in gastrointestinal side effects, therapeutic actions, and antibiotic resistance. In a study by Maier and coworkers, it was perceived that out of 835 human-targeted drugs, only a few strains were affected by these drugs; however, 40 drugs affected more than 10 strains. Species affected were *Eubacterium rectale, Roseburia intestinalis, Coprococcus, Bacteroides vulgatus*, *Prevotella copri*, *Blautia obeum*, whereas gamma-proteobacteria were almost resistant to those drugs (Maier et al., 2018). New evidence in host–microbe interaction studies revealed new mechanistic insights into role of *Lactobacillus rhamnosus* CNCM I-3690 in maturation of intestinal barrier's structure and functionality in mice (Natividad et al., 2013; Tlaskalova-Hogenova et al., 2015; Hayes et al., 2018). One of the major findings in recent years showed that significant source of antibiotic resistance genes in infant gut microbiota was from mother's gut and breast milk. Scientists have developed new methods for determining genes conferring resistance in gut microbiota and role of these genes in bacterial pathogenesis (Pärnänen et al., 2018). These show concern in propagation of antibiotic resistance and multiple antibiotic-resistant bacteria in infants. New studies have also shown that probiotics can regulate the need of antibiotics from childhood, which subsequently can mitigate the rise of antibiotic resistance. The studies showed that probiotics supplementation is more effective than placebo for reducing certain illnesses, which ultimately reduces the use of antibiotics (King et al., 2018). The past decade has seen a huge development in understanding microbiota gut-brain axis and its importance in ailment of neurodegenerative disorders. Recently, with the discovery of "neuropod cells," scientists have displayed how the gut lumen communicates rapidly with the brain after meal and role of microbiota in regulating homeostasis and proper signaling (Kaelberer et al., 2018).

CONCLUSION AND FUTURE PERSPECTIVES

The near explosion in the knowledge about gut microbiome and their role in metabolism has advanced our understanding about how intimately human health is related to microbes. Metabolism has direct relation with homeostasis of body functions as microbiota related to food and beverages play a pivotal role in modulation of host defense, host–microbe interaction, and epigenetic changes. As fermented indigenous foods have been serving communities since the dawn of civilization, microbiota associated with these foods is interconnected with healthy and safe attributes. The beneficial effect of these indigenous foods and associated microbiota includes up-regulation of immune system, strengthening gut-brain barrier, regulation of immune modulators, mitigation of carcinogens, induction of apoptotic pathways, and production of numerous metabolites. Diseases like IBD, colitis, IBS, lactose intolerance, peptic ulcers, vaginosis, and hypercholesterolemia can be treated successfully with probiotics. New evidences suggest that these microorganisms also help in improving brain functions, alleviate age-related diseases, and reduce hazardous metabolites from the body.

For optimized and safe utilization of these microbes for human welfare, deeper understandings of mechanistic details of their functional attributes with controlled clinical trials are required. It will also be important to know microbial

interactions and understand the molecular mechanism of such interactions so that such properties can lead to designer fermented products with predefined health attributes. Furthermore, comprehensive knowledge of metabolites and regulatory networks of these microorganisms could provide a platform for mimicking natural modulations and implementing them in treating disorders. Genetic manipulations and strain improvement will certainly enhance the valuable attributes. It is imperative for the food and dairy industries to study these indigenous dairy products and improve on prolonging shelf life, better adhesion, and survival in intestine for desired benefits and production for the global consumers. In addition, archiving microbiota from indigenous foods is also important as it will provide information about the attributes of starter cultures for improvement of aroma, texture, and flavor in dairy foods for posterity.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript.

AUTHOR CONTRIBUTIONS

TG, AB, and AS participated in all steps of preparation of this manuscript. AB and NN participated in the editing of the manuscript and revised it critically.

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Some Functional Properties of *khambir*, an Ethnic Fermented Cereal-Based Food of Western Himalayas

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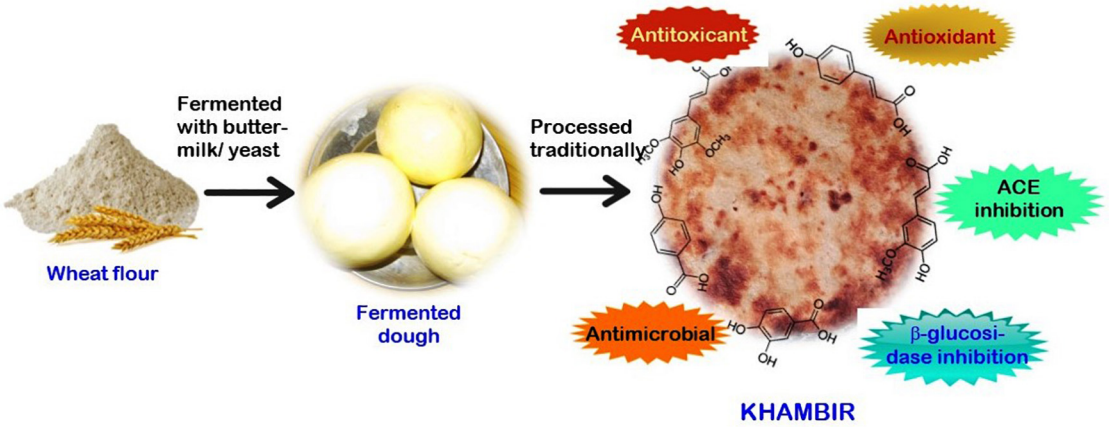
Traditional leavened wheat-based flat bread *khambir* is a staple food for the high-altitude people of the Western Himalayan region. The health promoting abilities of two types of *khambir*, yeast added *khambir* (YAK) and buttermilk added *khambir* (BAK), were evaluated. A group of microbes like yeast, mold, lactic acid bacteria (LAB), and *Bifidobacterium* sp. were abundant in both *khambir* but in varied proportions. Both are enriched with phenolics and flavonoids. The aqueous extracts of both breads strongly inhibited the growth of enteropathogens. Molecular docking experiments showed that phenolic acid, particularly *p*-coumaric acid, blocked the active sites of β-glucosidase and acetylcholine esterase (AChE), thereby inhibiting their activities. YAK and BAK showed antiradical and antioxidant activity ranging from 46 to 67% evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS), and ferric reducing/antioxidant power (FRAP) assays. The aqueous extract of both *khambir* samples protected the arsenic toxicity when examined under an *in situ* rat intestinal loop model study. The arsenic induced elevated levels of superoxide dismutase (SOD), catalase (CAT), reduced glutathione, lipid peroxidation (LPO) and DNA fragmentation, and transmembrane mitochondrial potential was alleviated by *khambir* extract. These results scientifically supported its age-old health benefit claims by the consumer at high altitude and there are enough potentialities to explore *khambir* as a medicinal food for human welfare.

Keywords: fermented *khambir*, antimicrobial, docking, antioxidant, antitoxicant activity



GRAPHICAL ABSTRACT | Health supportive properties of *khambir*.

INTRODUCTION

Traditional fermented foods have greater preference in certain communities due to typical characteristics such as flavor, color, and texture (Mondal et al., 2016). Most of the fermented foods contain an increased amount of health beneficial nutraceuticals, bioactive components, and good microbes compared to their unfermented substrate (Tamang et al., 2016). Due to age-old safety and beneficial experiences, scientists have been focused on exploring their nutrient profile, wild microbial resources, and therapeutic components, standardizing process parameters for the welfare of mankind.

Wheat-based handmade flat bread is a traditional and popular staple food, particularly in the Middle East, North Africa, and Central Asia (Parimala and Sudha, 2015). Several types of wheat-based flat bread in Middle Eastern households were documented by Al-Dmoor (2012) and in India by Mir et al. (2014) and Parimala and Sudha (2015). India is the second largest producer of wheat and native people prepare a variety of flat breads with different tastes and textures. Different ingredients like rice/a rice–wheat mix (e.g., *selroti*), finger millet (e.g., a*mbali*), or wheat (e.g., *nan*, *bhaturu* or *bhatooru*, *chilra, seera*, etc.) are commonly used for the preparation of delicious fermented flat bread in India. There are exceptionally few places where yeast, curd, or butter milk is added to wheat flour, fermented overnight, and then baked using traditional methods. *Tagi Khambir* or commonly called *khambir* is a "browned sourdough bread" – a very popular staple food at the high altitudes of the Himalayas like in Leh – Ladakh region of Jammu and Kashmir state, India (Angchok et al., 2009) and in a few places in Tibet and China (Tamang, 2010). According to the belief of the native people, this leavened bread can protect them against harsh environmental stresses (extreme cold at around −25°C during winter, strong wind, and low humidity) and provide adequate energy and mouth feeling (Angchok et al., 2009). The culinary practice is also unique and seems to be an inherited food culture of the Indus valley civilization. Rural women have the required knowledge of the proper art of baking this bread. Cleaned wheat flour is mixed with an adequate amount of salt, water, and buttermilk or yeast powder (marketed Baker's yeast). Then the dough is wrapped with a clean cloth and kept in a traditional kitchen overnight. Layers of cloth are wrapped over the container to maintain the temperature. The next day, the fermented dough is divided and hand-shaped into small ball-like structures (each having the weight around 200 g). It is then baked initially on hot stones and finally, directly in a fire made of wood or cow dung. It is finished off on the embers inside the fire, using edible oil (**Figure 1**). It can be stored at room temperature for more than a week. These traditional flat breads are gaining popularity among outsiders due to the rapid growth of "village tourism," "home stay," or "ethnic food tourism" in these regions (Tamang, 2010).

Although the native people believe in the health benefits of *khambir*, surprisingly no such study has been conducted to validate this. Considering this, we examined the health benefits (antimicrobial, trypsin, acetylcholine, and β-glucosidase inhibitory activities, antioxidant, and detoxicant activity) of homemade *Tagi Khambir*. Moreover, the ameliorative role of its extract was tested against arsenic (a globally recognized environmental pollutant and Gr. A carcinogen) induced toxicity, in an *in situ* loop model study of rat intestine, to prove its detoxification activity.

MATERIALS AND METHODS

Chemicals

All the chemicals used in this study are of analytical grade and was procured from standard companies.

Sample Collection and Preparation

Khambir samples were collected from households in two villages (*Sabu* and *Pheyang* which are about 10 km away from the town of Leh) in the Leh district, Jammu and Kashmir state, India. Three types of preparation, *viz.*, standard white wheat bread (unfermented, used as control), yeast (marketed Baking yeast) added *khambir* (YAK), and buttermilk added *khambir* (BAK)

FIGURE 1 | | Traditional process of *khambir* preparation. After overnight incubation of wheat flour and starter (yeast or buttermilk), the fermented dough is divided, and ball shaped by hand **(A)**. The handmade round-shaped dough is baked over a hot stone and then under direct fire **(B)**. The final cleaned and polished brown bread is ready for consumption **(C)**.

were collected. Then the samples were transferred into a sterile container and transported to the laboratory through an ice box. Bread samples were dried in a food dryer at 55°C for 10 h, and then dissolved into sterile distilled water (0.1%, w/v) by homogenization and centrifuged at 2000 *g* for 10 min. The supernatant was used as a food extract for further studies.

Microbiological Analysis

The quantity of the prevalent group of microbes in the food samples (direct sample) was enumerated on the basis of colony-forming units (cfu). The counts of different bacterial group were performed based on their colony morphology and color in various selective and differential agar media. Briefly, 10 g of the raw sample was mixed with a 100 ml of phosphate buffer saline (pH 7.2) and used as stock for the microbial count. The group of lactic acid bacteria (LAB) and *Bifidobacterium* sp. were cultivated in Rogosa SL agar (supplemented with 0.132% acetic acid) and Bifidobacterium agar supplemented with Bifidobacterium Selective Supplement (HiMedia, FD285), respectively, and plates were incubated in a CO_2 incubator (5% CO_2), at 37°C (Adak et al., 2013). All of the luxuriant growing colonies were enumerated for the above-mentioned bacteria. Total aerobic bacteria were quantified using Plate Count Agar (PCA) media and incubated at 37°C (Adak et al., 2013). Yeast and mold were enumerated by using yeast and mold agar, and Potato Dextrose Agar (PDA) media, respectively, and incubated at 30°C. The mycelial and round convex colonies were recorded for the mold and yeast counts, respectively. MacConkey agar and Salmonella differential agar were used for the determination of *Escherichia coli* and *Salmonella* sp., respectively. The plates were incubated at 35°C for 24 to 48 h. The pink red with bile precipitated colonies grown on the MacConkey agar were enumerated for *E. coli*. Moreover, the colorless and pink red colonies were counted for *Salmonella* sp. For *Vibrio* sp. enumeration, the yellow and bluish green color colonies grown on Thiosulfate Citrate Bile salt Sucrose (TCBS) agar base were selected.

Estimation of Total Phenolic Content

Total phenolic content was determined by the Folin–Ciocalteu method as described elsewhere. One milliliter of bread extract (100 mg/ml), 5 ml of diluted Folin–Ciocalteu phenol reagent (1:10 distilled water), and 4 ml of sodium carbonate solution (7%, w/v) were added sequentially. Soon after mixing the reactants, the test tubes were placed in the dark for 1 h and the absorbance was recorded at 725 nm against a reagent blank. The total content of phenolic compounds in extracts was expressed as a gallic acid equivalent (GAE) and mg/g of the dry sample.

Extraction of Phenolics and Chromatographic Analysis

The samples (300 mg) were extracted with 3 ml of methanol/water (80/20, v/v), for 10 min by sonication at room temperature. After centrifugation at 8000 rpm for 5 min, the supernatant was removed, and the extraction was repeated two times in a similar way. The combined supernatants were evaporated to dryness by centrifugal evaporation. The residues were dissolved in 400 μl of methanol/water (80/20, v/v) and filtered through a 0.2 μm PTFE membrane filter. A 20 μl of the final solution was injected into the HPLC system.

Phenolic compounds were separated on a LUNA-PFP (2) (3 μm, 150 mm × 4.6 mm) column thermostated at 35°C. Mobile phase A consisted of methanol/water (10/90, v/v) containing 0.1% acetic acid, while methanol containing 0.1% acetic acid served as mobile phase B. The gradient elution was performed as follows: 0.0 min, 5% B; 6.5 min, 25% B; 30.5 min, 37% B; 35.0 min, 55% B; 37.0 min, 95% B; 44.0 min, 95% B; 45.0 min, 5% B and 50.0 min; 5% B for re-equilibration of the column. The flow rate was adjusted to 0.7 ml/min. The injection volume was 20 μl. Phenolic compounds were monitored at 280 and 320 nm. For quantification, standards (Sigma–Aldrich, United States) of two subgroups of phenolic acid, viz., hydroxybenzoic acid (protocatechuic acid and *p*-hydroxybenzoic acid) and hydroxycinnamic acid (*p*-coumaric acid, ferulic acid, and sinapic acid) were employed.

Estimation of Total Flavonoids Content

For the estimation of the flavonoid, the bread extract of 0.5 ml was mixed with 0.1 ml of 5% $C_4H_4O_6KNa{\cdot}4H_2O$ [potassium sodium L-(+) tartrate]. After 5 min, 0.1 ml of 10% aluminum chloride was added to the mixture and made up to 3 ml using distilled water. After incubation at room temperature for 1 h, the absorbance of the reaction mixture was measured at 430 nm against a blank that

contained 0.1 ml of distilled water in place of aluminum chloride. The total flavonoid content was expressed (mg/g) as a quercetin equivalent.

Bioactivities of *Khambir* Sample

Antimicrobial Activity

Antimicrobial activity of *khambir* extract was tested (disc diffusion method, 6 mm) against different strains of human pathogens (some were locally isolated and characterized, and some were type cultures) like *Aeromonas hydrophila* SBK1, *Salmonella typhi* B3274, *S. typhi* E1590, *S. typhi* MTCC 734, *Shigella dysenteriae* 4717, *Shigella sonnei* RS 1, *Staphylococcus aureus* MB 13, *Streptococcus faecalis* MB 15, *Micrococcus luteus* ATCC 9341, and *Vibrio harveyi* MTCC 7954. The aqueous extract of the *khambir* samples (0.1%, w/v) was filter sterilized and 50 μl of the sample was tested against the above-mentioned pathogenic bacteria, which were spread onto the Mueller–Hinton agar (HiMedia, India) media and grown at 37°C for 24 h. Tetracycline (30 μg) was used as positive control. Thereafter inhibition zones that formed around the disc were measured and compared with an antibiotic.

Effect of *Khambir* Extract on the Activity of Some Health Indicator Enzymes

The bread sample was mixed with 10% diaion HP 20 resin (Sigma) under shaking for 30 min on a magnetic stirrer. Then the flask contents were eluted with 20 ml methanol. The collected methanol fractions were evaporated in a rotary evaporator (EYELA, Japan) and the residue was dissolved in DMSO and stored at −20°C for further analysis.

β-Glucosidase Inhibition Assay

The assay was performed according to the plate assay method as described by Pandey et al. (2013). Briefly, a 10 ml agar solution was prepared by mixing 0.07 g of agar powder in 0.1 M acetate buffer (pH 5.0) and dissolved at 80–100°C; followed by the addition of 1.2 ml of $FeCl_3$ (0.5%, w/v) solution and 40 μl (0.01 U/ml) of β-glucosidase (Sigma–Aldrich, source-almonds). This mixture was poured onto petri dishes and allowed to settle and firm up. The bread extract of 5 μl was spotted on the surface of the agar plate. Similarly, conduritol β-epoxide (Sigma), an irreversible inhibitor (0.75 μg), was used as a positive control and DMSO without extract was used as a negative control. The plates were incubated at room temperature for 15 min for an interaction between the enzyme and inhibitor. Later on, 7.0 ml (0.2%, w/v) of esculin (Himedia, India), the specific substrate for β-glucosidase, was floated on the surface of an agar plate and again incubated at room temperature for 30 min. Clear zones (CZs) were measured and compared to express the percentage (%) of enzyme activity or inhibition.

Determination of Trypsin Inhibition

The trypsin activity was assayed by the casein digestion method (Tripathi et al., 2011). Briefly, 1 ml of enzyme (SRL, India; Bovine pancreas, 1000 U/mg, 0.1 mg/ml) was incubated alone or with the bread extract for 20 min followed by the addition of 3.0 ml of 1% casein (in 100 mM Tris-HCl buffer; pH 8.0) at 37°C for 20 min. The reaction was stopped by the addition of 3.0 ml of 10% (w/v) trichloroacetic acid (TCA). The mixture was then centrifuged at 10,000 *g* and absorbance of the supernatant was measured at 280 nm to estimate the released tyrosine. One unit of trypsin activity was defined as the amount of enzyme that liberates 1.0 μg of tyrosine $min^{-1}ml^{-1}$ under standard assay conditions.

Acetylcholine Esterase (AChE) Inhibition Assay

The acetylcholine esterase (AChE) inhibitory activity of the bread extract was evaluated following the method of Elumalai et al. (2015). Briefly, 0.1 M phosphate buffer (pH 8.0, 150 μl), food extract solution (10 μl), and enzyme solution (earthworm head extract, 20 μl) were mixed and incubated for 15 min at 25°C; 10 μl of DTNB (10 mM) was then added. The reaction was then initiated by the addition of substrate (10 μl of acetyl thocholine, 14 mM solution). The formation of the colored product was measured at 410 nm after 10 min of incubation. One unit of AChE activity was defined as the amount of enzyme that liberates 1.0 μg of choline $min^{-1}ml^{-1}$ under standard assay conditions.

In silico Molecular Docking Experiment

For the molecular docking study, an X-ray crystallography structure of AChE (PDB ID: 1FSS) with a resolution of 3.0 Å and β glucosidase (BG) (PDB ID: IOGS) with a resolution of 2.0 Å were retrieved from the Protein Data Bank (PDB). Active sites or cavities of the selected target proteins were identified using the CAStp server[1]. The target proteins were developed for docking by deleting water and adding polar hydrogen. The structure of the positively correlated phenolic compound (*p*-coumaric acid) was downloaded from the NCBI PubChem database[2] and converted into pdb (.pdb) format using Open Babel (O'Boyle et al., 2011). Then docking was performed by using Autodock Tool (version 1.5.6) while PyMol (version 2.0) was used for visualization of the docked structure (Sanner, 1999; Morris et al., 2009).

Assay of *in vitro* Antioxidant Activity

DPPH (2,2-Diphenyl-1-Picrylhydrazyl) Free Radicals Scavenging Test

The water extraction of *khambir* (150 μl) with a concentration of 100 mg/ml was mixed with 37.5 μl methanolic 2,2-diphenyl-1-picrylhydrazyl (DPPH) (0.75 mM) solution. DPPH without *khambir* extract served as a control. After 20 min of incubation, absorbance was measured at 517 nm (Ghosh et al., 2015).

$$\text{DPPH scavenging activity}(\%) = (A_{control} - A_{sample} / A_{control}) \times 100$$

where A_{Sample} is the absorbance of the sample and $A_{control}$ is the absorbance of the control.

[1]http://sts.bioe.uic.edu/castp/

[2]https://pubchem.ncbi.nlm.nih.gov/

ABTS (2,2-Azino-Bis-3-Ethylbenzothiazoline-6-Sulfonic Acid) Assay

2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonic acid radical-scavenging activity was assayed, following the method of Halder et al. (2014), with necessary modifications. The mixture (1:1 ratio) of ABTS (7.0 mM) and potassium persulfate (2.45 mM) was incubated at 25°C overnight before use. The working solution was prepared by diluting the stock solution with methanol to reach an absorbance of 0.7 ± 0.02 at 734 nm ($A_{control}$). For measurements, 0.9 ml of the ABTS/ persulfate mixture and 0.1 ml of aqueous extract of *khambir* were mixed and absorbance was taken immediately after 15 min at 734 nm. The radical-scavenging activities (%) in both cases were calculated as follows:

$$\text{Antioxidant activity (\%)} = (A_{control} - A_{sample} / A_{control}) \times 100$$

where A_{Sample} is the absorbance of the sample and $A_{control}$ is the absorbance of the control.

Measurement of Ferric Reducing/Antioxidant Power (FRAP)

The reducing power of the *khambir* extract was measured (Yen and Chen, 1995) by mixing it with an equal volume of phosphate buffer (0.2 M, pH 6.6) and then incubating it at 50°C for 20 min with potassium ferricyanide (1%, w/v). The reaction was stopped by addition of TCA (10%, w/v) followed by centrifugation at 3000 rpm for 10 min. The supernatant was mixed with distilled water and ferric chloride (0.1%, w/v) solution, and the absorbance was measured at 700 nm. The reducing power (%) was calculated using the same equation used for DPPH or ABTS.

In situ Intestinal Loop Model Study to Assess Antitoxic Effect of *Khambir*

Inbreed male albino rats (150 ± 10 g) were used and fed rat specific standard food for 2 weeks prior to the experiment with Vidyasagar University Animal Ethical clearance (ICE/7-8/6-8/16 dt. 26.08.2016).

An *in situ* intestinal loop experiment was conducted as per the method described by Acharyya et al. (2015). Under anesthetize condition (by intramuscular injection of Ketamine-HCl, 22–24 mg/kg body wt.), the small intestinal portion was exposed sparingly from the abdominal cavity through a small cut on the cutaneous and abdominal muscle layers. In the small intestine, four loops (each having 2.0–2.5 inch in length) were created by creating five knots with a sterile cotton thread. These loops were filled (1 ml) sequentially by a syringe with a saline (control), aqueous extract of *khambir* (100 mg/ml), sodium arsenite ($NaAsO_2$, 250 mM), and an aqueous mixture of *khambir* and sodium arsenite, respectively. The intestine was carefully placed back in its original location and the cut site was stitched up. After 24 h, animals were again anesthetized and euthanized by cervical dislocation. The intestinal portion was removed quickly, cleaned, and immediately perfused with ice-cold saline (0.85% sodium chloride). Epithelial cells in the inner layer were scraped out from different demarcated locations of the intestine using a Teflon scrapper and homogenized in ice-cold buffer (phosphate buffer, 0.1 M, pH 7.4). The homogenate was initially centrifuged at 3000 rpm for 10 min at 4°C in a Remi Cooling Centrifuge (C-24 DL) to separate the nuclear debris. The aliquot obtained was again centrifuged at 10,000 rpm for 20 min at 4°C to obtain the post-mitochondrial supernatant, which was used as a source of various enzymes. The protein content of the homogenate was estimated by the Lowry et al. (1951) method, using bovine serum albumin as standard.

Estimation of Superoxide Dismutase (SOD) Activity

The activity of superoxide dismutase (SOD) was measured following the method of Marklund and Marklund (1974). A reaction mixture was prepared comprising of 2.875 ml Tris–HCl buffer (50 mM, pH 8.5), 100 µl tissue homogenate, and pyrogallol (24 mM in 10 mM HCl), and the total volume was made 3.0 ml. The activity of the enzyme was measured at 420 nm and the unit (U) was expressed in units/mg protein. One unit of enzyme activity was defined as inhibition of the 50% auto-oxidation of pyrogallol, and calculated as:

$$\text{SOD (units/mg protein)} = (\Delta\text{OD sample} \times \text{OD blank} \times 100) / (\Delta\text{OD sample} \times 50 \times \text{Vol. of sample}).$$

Estimation of Catalase (CAT) Activity

Catalase (CAT) activity was measured following the method of Aebi (1984). For assay, a reaction mixture was prepared with 2.0 ml phosphate buffer (0.1 M, pH 7.4), 0.05 ml of tissue homogenate, and 0.95 ml hydrogen peroxide (0.019 M) and the total volume was 3.0 ml. The activity of the enzyme was measured by taking absorbance at 240 nm. The CAT activity (U) was calculated in terms of nmol H_2O_2 consumed/minute/mg protein, with the help of the following formula:

$$\text{Catalase (unit/mg of protein)} = (\Delta\text{OD / min} \times \text{Vol. of assay}) / (0.081 \text{ of vol. of enzyme solution} \times \text{protein content}).$$

Estimation of Reduced Glutathione (GSH)

The method of Jollow et al. (1974) was adopted to measure the GSH level in the tissue. Briefly, 1.0 ml of sulfosalicylic acid (4%) was mixed with 1.0 ml of tissue homogenate. The sample was incubated for at least 1 h at 4°C and then centrifuged at 1500 rpm for 15 min at 4°C and used as the tissue mixture. The assay mixture contained 2.2 ml phosphate buffer (0.1 M, pH 7.4), 0.4 ml tissue mixture, and 0.4 ml of 5, 5'-dithiobis-2-nitrobenzoic acid, (DTNB, 10 mM) and absorbency was measured at 412 nm. The GSH content was calculated as

μmol DTNB conjugate formed/g tissue using a molar extinction coefficient of $13.6 \times 10^3\ M^{-1}\ cm^{-1}$ with the help of the following formula:

$$GSH = (\Delta OD / min \times Vol.\ of\ assay \times 100) / (1.36\ of\ mole\ GSH\ conjugate/g\ tissue)$$

Measurement of Lipid Peroxidation (LPO)

The level of membrane lipid peroxidation (LPO) was assayed following the method of Wright et al. (1981) with some modifications. The reaction mixture comprised of 1.0 ml cell homogenate, 1.0 ml of TCA (10%), and 1.0 ml TBA (0.67%). Then all the tubes were kept in a boiling water bath for 45 min. The tubes were then cooled at room temperature and centrifuged at 5000 rpm for 10 min. The optical density of the supernatant was measured at 532 nm. The level of LPO was measured with respect to malondialdehyde (MDA) formation and results were expressed as the mmol MDA formed/g tissue using a molar extinction coefficient of $1.56 \times 10^5\ M^{-1}\ cm^{-1}$, with the help of the following formula:

$$LPO = (Vol.\ of\ assay \times OD \times 10^9)/(1.56 \times 10^5 \times 10^3\ g\ tissue).$$

Analysis of the Mitochondrial Membrane Potential

The alteration of mitochondrial membrane potential of intestinal epithelial cells of different treatment was measured spectrofluorometrically using Rhodamine 123 (Dash et al., 2014). Cells were seeded in six-well plates at a density of around 2×10^4/well and incubated with 10 μl of 1.5 μM Rhodamine 123 at 37°C in the dark for 30 min. Then, fluorescence emitting from the Rh123 was measured for 2 min in a fluorescence spectrophotometer (Hitachi F-7000). The mitochondrial membrane potential was expressed as an emitting fluorescence level at an excitation wavelength of 493 nm and an emission wavelength of 522 nm.

DNA Fragmentation Study

The alkaline comet assay was done according to the method of Acharyya et al. (2015). A total of 75 ml of low melting point agarose (0.5%) in PBS at 37°C was added to a 25 ml cell suspension (~10^5 cells/ml). The mixture was then dropped onto a glass slide precoated with 1% agarose. The solidified slides were immersed in ice-cold lysis buffer (2.5 mM NaCl, 85 mM EDTA, 10 mM Trizma base, 1% Triton X-100, 10% DMSO, and 1% sodium lauryl sarcosinate, adjusted to pH 10.0) for 1 h. After lysis, the slides were repeatedly washed with PBS and placed in a submarine gel electrophoresis chamber filled with alkaline electrophoresis buffer (0.3 M NaOH and 1 mM EDTA). Then electrophoresis was done at 25 V and the current was adjusted to 300 mA. Slides were then neutralized with PBS and stained with a solution of 2 mg/ml ethidium bromide for 2 min. Slides were examined by fluorescence microscope.

Statistical Analysis

Collected data were presented as the arithmetic mean (mean ± SD). The variations in different analysis results were examined by one-way ANOVA [least significant difference (LSD) testing]. A significant variation was accepted at the level of 5 and 1% (i.e., $p < 0.05$ and $p < 0.01$) was measured using Sigmastat 11.0 (United States) statistical software. Multiple correlations between the beneficial properties of the control, YAK, and BAK were performed by IBM-SPSS (version 19).

RESULTS AND DISCUSSION

India is a country which has not lost all of its culture, food habits, and traditions. Most ethnic people still prefer traditional food as a staple diet and these foods are commonly served to celebrate functions, marriages, and rituals. *Khambir* is a traditional flat bread prepared and consumed in the Ladhak region. The native people believe that it plays a health protective role in this extreme environment. However, this claim has not been scientifically validated thus far. Considering this, we evaluated its functional properties to justify its age-old health benefit claims at high altitudes.

Total Count of Microbes

The microbial populations of *khambir* samples were examined and are represented in **Table 1**. There were no significant differences in the microbial content of mold, Bifidobacteria, *Vibrio* sp., *E. coli* between both types of the *khambir* samples ($P \leq 0.05$). However, significant differences of LAB and yeast counts were observed between the YAK and BAK ($P \leq 0.05$). The addition of yeast or butter milk as a starter in wheat flour leads to a profound microbial growth during the overnight incubation at room temperature and facilitated sourdough fermentation. Sourdough fermentation with LAB and yeasts leads to leavening and production of acid and CO_2 in bread. The created anaerobiosis may have facilitated the growth of *Bifidobacterium* sp. in the dough. Surprisingly, the amount of mold was significantly higher which might be due to the fermented dough being wrapped with

TABLE 1 | | Enumeration of different group of microbes in both YAK and BAK.

	Microbial *composition* (log_{10} cfu/g)							
	Total aerobic	**Yeast**	**Mold**	**LAB**	***Bifidobacterium* sp.**	***E. coli***	***Salmonella* sp.**	***Vibrio* sp.**
YAK	8.90 ± 0.33	7.96 ± 0.82	6.30 ± 1.22	1.32 ± 0.14	2.86 ± 0.49	5.30 ± 1.44	0	3.30 ± 0.28
BAK	11.64 ± 0.77	3.30 ± 0.46	6.60 ± 0.76	2.4 ± 0.13	3.23 ± 0.86	5.77 ± 1.74	0	3.77 ± 0.24

TABLE 2 | | Phenolics and flavonoid content in *khambir* samples.

	Total phenolics (mg/g)	Protocatechuic acid (mg/kg)	p-Hydroxy-benzoic acid (mg/kg)	p-Coumaric acid (mg/kg)	Ferulic acid (mg/kg)	Sinapic acid (mg/kg)	Total flavonoids (mg/g)
YAK	2.37 ± 0.21	18.31 ± 0.46	13.87 ± 0.57	2.26 ± 0.23	16.42 ± 0.82	ND	2.23 ± 0.4
BAK	1.29 ± 0.2	16.52 ± 0.43	7.53 ± 0.60	1.34 ± 0.33	18.34 ± 1.68	ND	1.60 ± 0.2

Data are presented as means ± standard deviation of three replicates.

a wet cloth and the storage of the baked product in room temperature for several days. Fermented flat breads in the Indian cuisine have its own unique taste, way of preparation, and use of ingredients, and that leads to different microbial associations. *Selroti* from the Himalayan region (Yonzan and Tamang, 2010) and *ambali* from South India (Ramakrishnan, 1980) contain mostly LAB. Whereas, wheat-based bread like *Bhatooru* from Himachal (Savitri and Bhalla, 2013), *seera* from the Middle and North of India (Savitri et al., 2012), and *nan* (Batra, 1986) is dominated by yeasts and very meager populations of LAB. The presence of *Vibrio* sp. in *khambir* is of great concern for its hygienic status which may be related to the quality of water (as the native people in the Himalayan region use glacier water directly for household purpose). However, the pathogenic property of an organism depends on the strain and host specificity as reflected by the regular consumption of such breads by the native people of the Himalayan region. Tamang et al. (2015) mentioned that about 80% of traditional fermented foods that are prepared through natural fermentation may contain functional, non-functional, and pathogenic microorganisms. The prevalence of pathogenic bacteria such as *S. aureus*, *Bacillus cereus*, *E. coli*, *Campylobacter*, *Vibrio cholerae, Aeromonas*, *Klebsiella*, *Shigella* sp., and *Salmonella* among others in traditional fermented foods was also documented by Abriouel et al. (2017). Additionally, microbial interplay (enzymes and metabolites) during the course of sourdough fermentation can delay starch digestibility leading to lower glycemic responses, reduced gluten content, and other antinutrients, modulates accessibility of bioactive components, and improves mineral bioavailability, thus enhancing gut health (Poutanen et al., 2009).

TABLE 3 | | Antimicrobial activity of aqueous extract of two types of *khambir* products.

Target microbes	YAK (zone of inhibition, mm)	AI	BAK (zone of inhibition, mm)	AI
Aeromonas hydrophila SBK1	ND	–	ND	–
Salmonella typhi B3274	6.5 ± 0.6,	0.56	ND	–
Salmonella typhi E1590	6.0 ± 0.6	0.36	3.5 ± 0.3	0.21
Salmonella typhi MTCC 734	5.5 ± 0.4	0.47	4.5 ± 0.4	0.39
Shigella dysenteriae 4717	5.2 ± 1.04	0.46	5.5 ± 0.5	0.49
Shigella sonnei RS 1	ND	–	ND	–
Staphylococcus aureus MB 13	7.5 ± 0.8	0.63	ND	–
Streptococcus faecalis MB 15	6.2 ± 0.6	0.65	ND	–
Micrococcus luteus ATCC 9341	7.0 ± 0.6	0.56	7.2 ± 0.7	0.58
Vibrio harveyi MTCC 7954	8.5 ± 0.8	0.94	6.5 ± 0.7	0.76

Data are presented as means ± standard deviation of three replicates. Activity index (AI) = inhibitory zone of test sample (excluding disc diameter, 5 mm)/inhibitory zone of a standard drug (tetracycline, 10 mg/disc).

Phenolic and Flavonoid Content of *Khambir*

It was estimated that the phenolic content of YAK and BAK was 2.37 and 1.29 mg/g, respectively (**Table 2**), which is a much higher value than the unfermented or unprocessed wheat flour of Indian varieties (Punia and Sandhu, 2016) as well as Chinese varieties (Li et al., 2015). Additionally, a significant or comparatively higher amount of phenolic acids like protocatechuic acid, *p*-hydroxy-benzoic acid, *p*-coumaric acid, and ferulic acid were also present in the YAK and BAK (**Table 2**). Similarly, flavonoid content in the fermented leavened bread was also increased many fold (1.63–2.23 mg/g in the *khambir* and 80–100 mg/g in unfermented wheat flour as reported by Punia and Sandhu, 2016]. During fermentation, microbes originating from hydrolytic enzymes (cellulases, esterases, glycosidase, polyphenol hydrolase, etc.) may lead to the branching and defabrication of the cellulosic backbone as well as polyphenolic structures; therefore, phenolics and flavonoid compounds are detached from the anchoring molecule and become free. Dietary flavonoids and phenolic acids have attracted much interest recently because they have a variety of beneficial biological properties and play an important role in the protection and prevention of many human diseases (Jalal et al., 2015).

Antimicrobial Activity of Water Extract of *Khambir*

The antimicrobial activity of the aqueous extracts of *khambir* samples was tested against different strains of enteropathogens

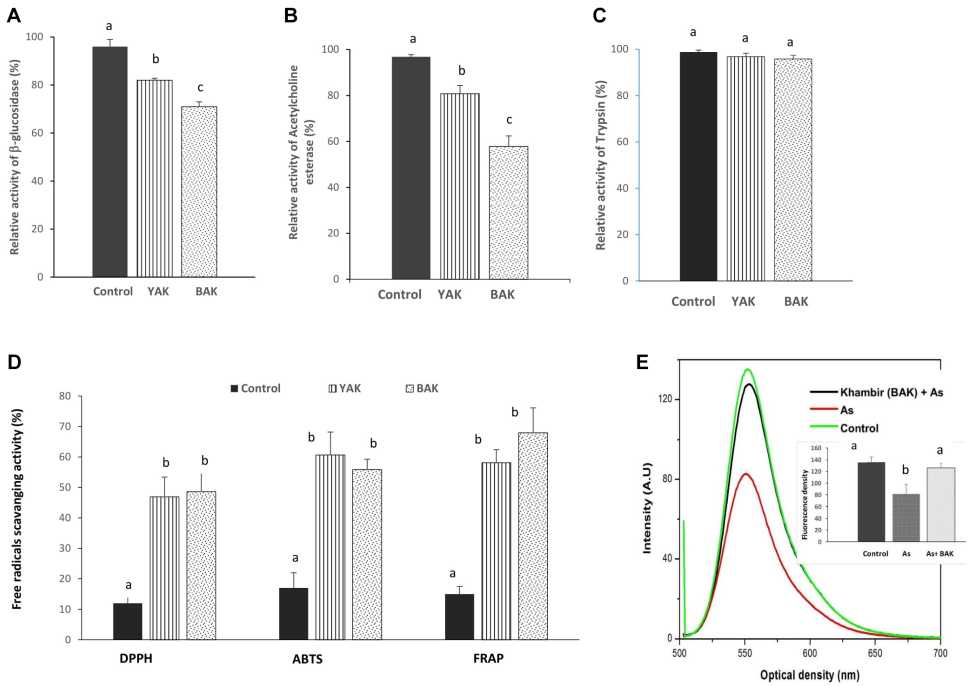


FIGURE 2 | | Evaluation of bioactivities of aqueous extract of yeast added *khambir* (YAK) and buttermilk added *khambir* (BAK). Changes of β-glucosidase **(A)**, acetylcholine esterase **(B)**, trypsin **(C)** inhibitory activities, and *in vitro* antioxidant **(D)** activities (DPPH, ABTS, FRAP) of fermented *khambir*, i.e., YAK and BAK in respect to control (unfermented) were determined. The activity of enzymes (without any additive) was considered as 100%. **(E)** The alteration of mitochondrial transmembrane potential in respect to emitted fluorescence level of Rhodamine 123 during exposure of arsenic and arsenic + *khambir* extract (BAK) in *in situ* intestinal loop model study. The control is indicated the fluorescence intensity of control tissue. Data presented as the mean ± standard deviation of five replicates. Different superscripts (alphabet) on the bar indicated the level of significance difference ($p < 0.05$) among respective groups.

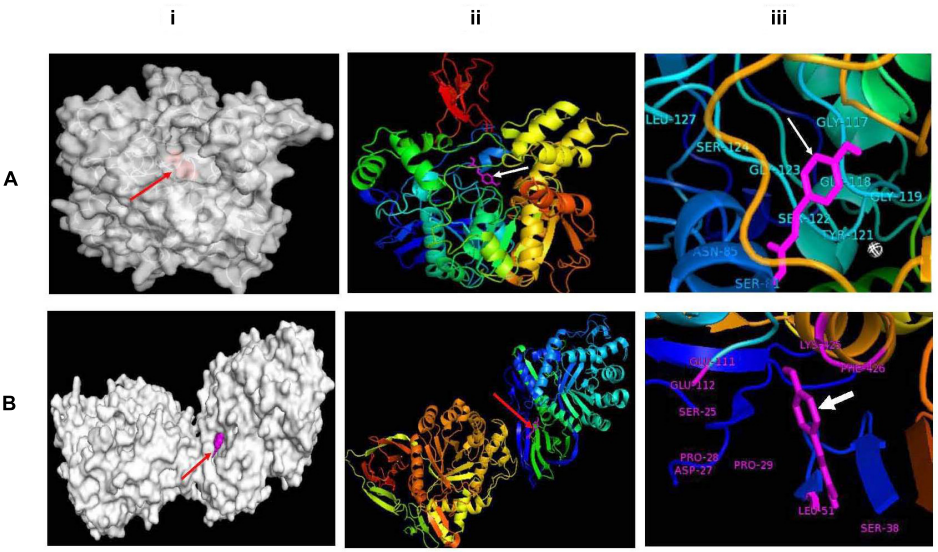


FIGURE 3 | | Molecular docking between p-coumaric acid with acetylcholine esterase **(A)** and β-glucosidase **(B)**. Docked proteins have been shown as gray surface models (i); proteins have been visualized as ribbons docked with p-coumaric acid (stick, magenta) (ii); and docked p-coumaric acid (stick, magenta) at the active sites of proteins (iii).

and other organisms (**Table 3**). It was found that YAK exerted more strain specific antibiosis, particularly against enteropathogens like *S. typhi*, *S. dysenteriae*, *S. faecalis*, and *V. harveyi* than BAK. The YAK also showed significant inhibitory effects against *S. aureus*, whereas the killing effect of BAK was more prominent against *M. luteus*. The results

TABLE 4 | | Activity of antioxidant defense related biomarkers in intestinal epithelia during exposure of arsenic.

Group	SOD (U)	Catalase (U)	MDA (nM/g)	GSH (mg/g)
Untreated control	1.72 ± 0.03	1.87 ± 0.07	119.42 ± 6.65	31.43 ± 1.27
Khambir	1.75 ± 0.12	1.92 ± 0.06	92.46 ± 4.34*	31.87 ± 2.83
Arsenic	0.64 ± 0.04**	1.19 ± 0.06**	165.24 ± 6.2**	49.82 ± 5.86**
YAK + arsenic	1.17 ± 0.04*	1.55 ± 0.05	104.42 ± 5.72	38.43 ± 5.66*
BAK + arsenic	1.62 ± 0.03	1.85 ± 0.05	96.04 ± 3.45*	35.19 ± 4.11

*Data are presented as means ± standard deviation (n = 5). *$p < 0.05$ and **$p < 0.01$ are the level of significance difference compared with the control group.*

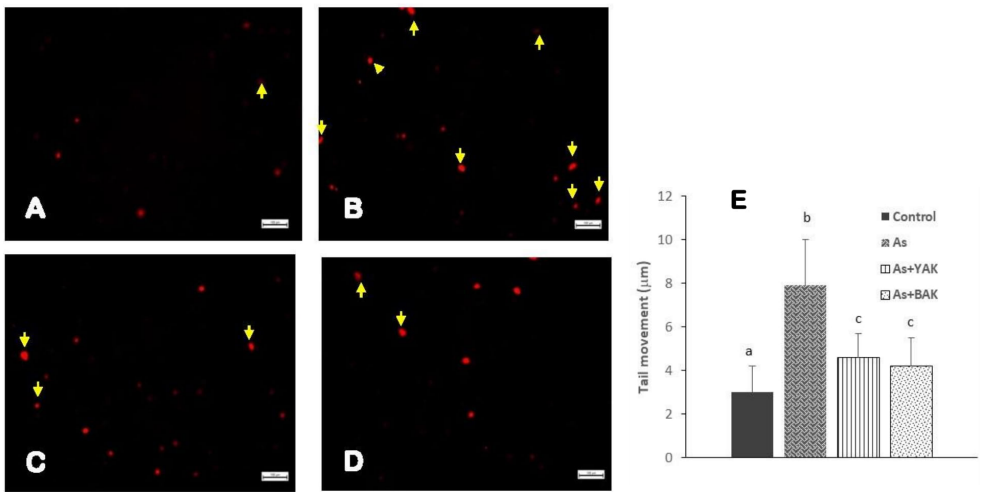


FIGURE 4 | | Determination of the genotoxic protective effects of *khambir* extracts against arsenic toxicity by Comet assay. Fluorescent microscopic image of **(A)** control tissue, **(B)** arsenic treated, **(C)** co-supplementation of arsenic and YAK, and **(D)** co-supplementation of arsenic and BAK; **(E)** graphical representation of the comet tail length of different experiments. The comet tail length was calculated as the distance between the end of nuclei heads and end of each tail. Values are expressed as the mean ± SD (n = 100); different superscripts (alphabet) on the bar indicate the different levels of significance ($p < 0.05$) among respective groups.

indicated that the reactive metabolites that evolved during fermentation act as a natural preservative in this food. The strain specific and variable antimicrobial effect of natural medicines is a common phenomenon and many factors like pH, extracting solvent and techniques, dilution, culturing media, and source of microorganism are very important and can alter the interaction of the active ingredients with medicinal flora (Rios and Recio, 2005). The antibiosis of the tested food samples was very significant and comparable with commercial antibiotics like tetracycline, which is commonly used as a food preservative (**Table 3**).

β-Glucosidase (BG), Acetylcholine Esterase (AChE), and Trypsin Inhibitory Potentialities of *Khambir* Extracts

Glucosidase inhibitors have significant therapeutic potential in the treatment of metabolic diseases and disorders like diabetes, obesity, human immune deficiency virus infection, metastatic cancer, lysosomal storage disease, etc. (Pandey et al., 2013). Over 100 glycosidase inhibitors have been isolated from plants and microorganisms (Pandey et al., 2013). A group of microbes originating from β-glucosidase inhibitors like acarbose, voglibose, valienamine, adiposin-1, and trestatin-B are commercially exploited as anti-diabetic drugs which can reduce sugar digestion (undigested resistant starch) and assimilation into the body (Kulkarni-Almeida et al., 2011). Both *khambir* preparations, YAK and BAK exhibited 18 and 29% inhibition of β-glucosidase activity (**Figure 2A**), establishing them as a useful diabetic diet. Our experimental results provide clues for the blood sugar lowering abilities of sourdough bread as it has previously been reported that sourdough bread consumption can lower post-prandial blood glucose and improve insulin and GLP-1 responses in human subjects (Maioli et al., 2008). In addition, the aqueous extract of YAK and BAK also exerted anti-AChE activity by inhibiting 19.2 and 42.2% of the original activity (**Figure 2B**). The occurrence of AChE inhibitors in natural resources has been well documented and characterized, but the quest for new inhibitors remains crucially important owing to their therapeutic potential in the treatment of neurological disorders such as Alzheimer's disease, senile dementia, ataxia, myasthenia Gravis, and Parkinson's disease (Elumalai et al., 2015). The potentialities of BAK have been proven to be more profound, with respect to the inhibition of β-glucosidase and AChE activity, than YAK, and this may relate to the abundance of a consortium of reactive metabolites particularly phenolic constituents in the *khambir* product.

TABLE 5 | Multiple correlation test among the phenolic, *in vitro* and *in vivo* antioxidant profiles, and other health beneficial effects.

	Total phenolics	Total flavonoids	Protocatechuic acid	p-hydroxy-benzoic acid	p-coumaric acid	Ferulic acid	ACE	BG	DPPH	ABTS	FRAP	SOD	Catalase	MDA	GSH
Total phenolics	1	0.956	0.895	0.992	0.872	0.675	−0.282	−0.442	0.765	0.849	0.673	0.423	0.427	−0.718	−0.644
Total flavonoids		1	0.987	0.911	0.689	0.862	−0.552	−0.687	0.921	0.967	0.861	0.671	0.675	−0.892	−0.841
Protocatechuic acid			1	0.832	0.562	0.933	−0.680	−0.796	0.972	0.995	0.932	0.782	0.786	−0.953	−0.918
p-Hydroxy-benzoic acid				1	0.927	0.576	−0.159	−0.326	0.678	0.775	0.574	0.305	0.310	−0.625	−0.543
p-Coumaric acid					1	0.227	0.224	0.053	0.352	0.481	0.224	−0.075	−0.070	−0.286	−0.188
Ferulic acid						1	−0.898	−0.960	0.992	0.963	1.000	0.954	0.956	−0.998*	−0.999*
ACE							1	0.985	−0.834	−0.747	−0.900	−0.989	−0.988	0.870	0.915
BG								1	−0.916	−0.850	−0.961	−1.000	−1.000	0.942	0.971
DPPH									1	0.990	0.991	0.907	0.909	−0.998*	−0.985
ABTS										1	0.962	0.838	0.841	−0.978	−0.951
FRAP											1	0.955	0.957	−0.998*	−0.999*
SOD												1	1.000	−0.934	−0.965
Catalase													1	−0.936	−0.967
MDA														1	0.995
GSH															1

**Correlation is significant at $p < 0.01$ and *correlation is significant at $p < 0.05$.

Together with the experimental results and statistical relationship, molecular docking experiments were performed for a better understanding of how the phenolic compound especially *p*-coumaric acid interacts with both the AChE and BG enzyme. In the presence of certain functional groups, such as hydroxyl, carboxyl, and acrylic acid groups, *p*-coumaric acid can act as a hydrogen bond acceptor or donor, which seems to increase the potency of inhibiting the activity of AChE and BG. Molecular docking analysis showed that *p*-coumaric acid has an optimum binding affinity (ΔG of −6.8 kcal/mol) with a molecular target in the second cavity of the predicted active sites of AChE and the amino acids in this site of the enzyme like SER-81, ASN-85, GLY-117, GLY 118, GLY-119, TYR-121, SER-122, GLY-123, SER-124, and LEU-127 formed H-bonds with the phenolic compound (**Figure 3A**). On the other hand, *p*-coumaric acid has blocked the activity of BG by possibly binding at the third cavity of the predicted active sites with an optimum binding energy or binding affinity of −7.6 kcal/mol. This interaction occurs via amino acids like ASP-27, PRO-28, PRO-29, SER-38, LEU-51, GLU-111, GLU-112, LYS-425, and PHE-426 at the active site (**Figure 3B**). The calculated absolute binding free energies in between −6.8 to −7.6 kcal/mol indicated that a number of relatively weak chemical interactions (non-covalent bonds) stabilize the conformations and the interactions between the molecules. This result clearly documents that a functional component of *khambir* is phenolics, which can specifically bind and inhibit the activities of both BG and AChE. A similar pattern of interaction for the inhibition of angiotensin-converting enzyme with the phenolic acids was also observed by Shukor et al. (2013).

Both extracts of *khambir* were non-responsive to trypsin activity (**Figure 2C**). A reduction in trypsin inhibitory activity during natural lactic acid fermentation of cereals was reported by Osman (2011). This indicates that the *khambir* has no such adverse effect on protein digestion.

Assay of *in vitro* Antioxidant Activity

Recently, there has been increased interest on antioxidant nutrients, which have the ability to scavenge free radicals in the system and neutralize them before they do any damage to body cells. A number of methods are also available to determine *in vitro* antioxidant activity each with their own specific reaction principles; therefore, researchers employ many methods simultaneously to obtain a clear picture of the antioxidant activity. In this study, DPPH, ABTS, and ferric reducing/antioxidant power (FRAP) methods were employed to evaluate the antioxidant potentialities of the aqueous extract of *khambir*.

The activity of DPPH, ABTS, and FRAP of control (unfermented), YAK, and BAK are shown in **Figure 2D**. The DPPH, ABTS, and FRAP activity of the control sample was 12, 17, and 15%; for YAK was 46.90, 60.67, and 58.11%; and for BAK was 48.62, 55.85, and 67.92%, respectively (**Figure 2D**). This result indicated that antioxidative potentialities of wheat flour were significantly ($p < 0.05$) improved due to fermentation by a consortium of microbes.

The "antioxidant power" of the *khambir* is greatly related to its high phenolic content, as these molecules have the innate ability to donate a hydrogen or electron. They have the ability to delocalize the unpaired electron of free radicals within the aromatic structure (Jalal et al., 2015; Li et al., 2015; Punia and Sandhu, 2016), thereby exhibiting various physiological activities including anti-inflammatory, antiallergic, anticarcinogenic, antihypertensive, anti-arthritic, and antimicrobial activities.

Antioxidant and Antitoxic Action of *Khambir*: *In situ* Experiment in Rat Intestine

The activities of SOD and CAT in the control and experimental groups are shown in **Table 4**. Arsenic-induced depletion ($p < 0.01$) of SOD and CAT activity was found in the respective group compared to the controls. Co-supplementation of the *khambir*, along with arsenic, significantly ($p < 0.05$) elevated the levels of SOD and CAT compared to the arsenic alone-exposed groups.

The administration of *khambir* alone diminished the level of MDA formation compared to normal levels in intestinal tissues. Arsenic treatment resulted in a significant ($p < 0.01$) elevation of the MDA level compared to the control group. Administration of *khambir* (YAK and BAK), along with arsenic, antagonized the toxic effects of arsenic that were reflected by the significant ($p < 0.05$) decrease of MDA levels compared to the arsenic alone-exposed groups (**Table 4**).

The content of GSH increased about 36% ($p < 0.05$) in the intestinal epithelia when exposed to arsenic compared to the control. Simultaneous *khambir* (both YAK and BAK) treatment with arsenic significantly ($p < 0.05$) decreased the GSH level in the tissue compared to the arsenic alone-treated groups (**Table 4**).

Arsenic toxicity leads to the disruption of the mitochondrial membrane architecture, which is reflected by the significant reduction ($p < 0.05$) of the mitochondrial transmembrane potential compared to the control (**Figure 2E**). Mitochondrial membrane permeability disruption is associated with a lack of rhodamine 123 retention and a decrease in fluorescence. *Khambir* extract protects the mitochondrial membrane from arsenic toxicity which was noted by the level of fluorescence intensity near the control level (**Figure 2E**).

The comet assay was carried out to measure the single-strands DNA breaks in the intestinal epithelial cells of the control, arsenic treatment, and arsenic along with *khambir* treatment (**Figure 4**). Results showed an enhanced number of tail migration (DNA strand break) in the arsenic-treated group, which was significantly restrained in the *khambir* supplemented arsenic-exposed group. The extrusion of the damaged DNA from the majority of cells in the arsenic treated group was clearly visualized (morphometric analysis) by noting the comet tail length (**Figure 3**). Supplementation of *khambir* extracts antagonized the toxic effects of arsenic, resulting in a lower amount of DNA damage as well as comet tail length as compared to arsenic alone.

Khambir, particularly BAK extract, exhibited strong protection against arsenic induced modification of the enzymatic antioxidant defense system, by restoring the activities of SOD and CAT, preventing LPO, restoring the GSH pool and mitochondrial transmembrane potential, and above all preventing DNA fragmentation from the harsh toxic effects of arsenic. *Khambir* extract, particularly its phenolic constituents, may alleviate arsenic toxicity by means of its antioxidant components via a number of mechanisms, including the protection of target molecules (lipids, proteins, and nucleic acids) from oxidative damage (by neutralizing the generated free radicals), suppressing the inflammatory response, modulating vascular homeostasis, and improving the cellular defense system by altering the expression at the gene level (Jalal et al., 2015; Li et al., 2015). The dietary fiber (1.2 g%) of *khambir* may also play a pivotal role in detoxification by entrapping arsenic before exerting any toxicity (Abdel-Salam et al., 2010).

Multiple Correlation

Table 5 shows the correlation coefficient (r) between total phenolic, flavonoid, phenolic acids, inhibition of AChE and β-glucosidase, *in vitro* antioxidant activities, and *in vivo* antioxidant parameters altered in arsenic treated rats during supplementation of BAK. The phenolics and flavonoids have a strong relationship with the parameters like *in vitro* and *in vivo* antioxidant properties, and inhibition of AChE and β-glucosidase activities as an r-value lies in between +1 to −1. A strong positive correlation was observed between total phenolics, total flavonoids, protocatechuic acid, *p*-hydroxy-benzoic acid, ferulic acid, *p*-coumaric acid, DPPH, ABTS, FRAP, SOD, and CAT. Among the phenolics, *p*-coumaric acid exhibited a strong position correlation with the inhibition of AChE and β-glucosidase. In contrast, a negative correlation was obtained between the phenolics and the content of GSH and MDA.

CONCLUSION

This study clearly demonstrated that both types of *khambir*, YAK and BAK, carry health benefits and are rich in phenolics, as they exhibit significant antimicrobial, antioxidant, antiradical, and anti-toxic effects. Additionally, a group of food graded microbes in the product can provide some added advantages to consumers. Thus, the experimental evidence supports its age-old claim as a healthy and protective food source against environmental stresses. Further, scientific intervention is urgently needed to improve its hygienic status which will expand its market demand as well as pave the way for economic and livelihood development of the ethnic people of the Leh-Ladakh regions.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of Vidyasagar University Animal Ethical clearance (ICE/7-8/6-8/16 dt. 26.08.2016) with written informed consent from all subjects. The study and protocol were reviewed and approved by the Institutional Ethics Committee (IEC) of Vidyasagar University.

AUTHOR CONTRIBUTIONS

The trial was conceived by PH, MR, SM, PD, and KM. KM designed the study. PH, MR, and SM conducted the research. SP, MT, KG, and PH analyzed the data. DB, SS, SD, and KM prepared the manuscript. KM had the primary responsibility of the final content. All authors read and approved the final manuscript.

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Transcriptional Comparison Investigating the Influence of the Addition of Unsaturated Fatty Acids on Aroma Compounds During Alcoholic Fermentation

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The levels of unsaturated fatty acids (UFAs) in grape must significantly influence yeast metabolism and the production of aroma compounds. In this work, cDNA microarray technology was applied to analyze the transcriptional discrepancies of wine yeast (commercial wine yeast Lalvin EC1118) fermenting in synthetic grape must supplemented with different concentrations of a mixture of UFAs (including linoleic acid, oleic acid, and α-linolenic acid). The results showed that the initial addition of a high level of UFAs can significantly enrich the intracellular UFAs when compared to a low addition of UFAs and further increase the cell population and most volatiles, including higher alcohols and esters, except for several fatty acids. Microarray analyses identified that 63 genes were upregulated, and 91 genes were downregulated during the different fermentation stages. The up-regulated genes were involved in yeast growth and proliferation, stress responses and amino acid transportation, while the repressed genes were associated with lipid and sterol biosynthesis, amino acid metabolism, TCA cycle regulation, mitochondrial respiration, and stress responses. Unexpectedly, the genes directly related to the biosynthesis of volatile compounds did not vary substantially between the fermentations with the high and low UFA additions. The beneficial aromatic function of the UFAs was ascribed to the increased biomass and amino acid transportation, considering that the incorporation of the additional UFAs in yeast cells maintains high membrane fluidity and increases the ability of the cells to resist deleterious conditions. Our results highlighted the importance of UFAs in the regulation of aroma biosynthesis during wine fermentation and suggested that the improvement of the resistance of yeast to extreme stresses during alcoholic fermentation is essential to effectively modulate and improve the production of aroma compounds. A potential way to achieve this goal could be the rational increase of the UFA contents in grape must.

Keywords: unsaturated fatty acids, microarray analyses, *Saccharomyces cerevisiae*, volatile aroma compounds, wine

INTRODUCTION

The production of aroma compounds during wine fermentation is largely influenced by the nutrition status of grape must. Even a small change in the must composition and nutrition concentration, such as sugar concentration, nitrogen source (amino acids), vitamins and fatty acids, could result in a significant impact on the profile of aroma compounds (Bell and Henschke, 2005; Luan et al., 2018). In this context, the effect of assimilable nitrogen sources (YAN) on the formation of volatile compounds has been investigated exhaustively because changes in the YAN content (ammonium salts or amino acids) have a direct and specific effect on the aroma quality of wine (Marks et al., 2003; Mendes-Ferreira et al., 2007). In recent years, the importance of unsaturated fatty acids (UFAs) for yeast fermentation performance and volatile formations has been recognized by winemakers. UFAs are required by *Saccharomyces cerevisiae* to grow under anaerobic conditions. The incorporation of more UFAs into yeast cells can maintain membrane integrity and increase their ability to resist fermentation stresses, such as high sugar and ethanol toxicity (Holcberg and Margalith, 1981; You et al., 2003). In addition, the degree of unsaturation of the cell membrane can influence the activity of membrane-associated enzymes and transporters (such as ATPase and general amino acid permease) and modulate the production of aroma compounds (Calderbank et al., 1984; Rosa and Sa-Correia, 1992). The absence of oxygen during wine fermentation suppresses the fatty acid desaturation of yeast. An alternative to biosynthesis is the uptake of UFAs from grape juice to avoid stuck fermentation (Varela et al., 2012). Several works demonstrated that UFAs influence the production of volatile compounds via their regulation of the formation of precursor acyl-CoA and the expression of related genes (Yoshioka and Hashimoto, 1983; Trotter, 2001; Swiegers et al., 2005; Duan et al., 2015; Rollero et al., 2016). The aromatic functions of UFAs are largely dependent on the type and concentration of UFAs, which might explain why the results reported by different researchers are inconsistent. For example, the addition of oleic acid and ergosterol can increase the production of higher alcohols and acetate esters but inhibit 1-butanol and 1-pentanol (Mauricio et al., 1997). Casu et al. (2016) found that increasing the concentration of linoleic acid was unfavorable for acetate ester formation but improved the production of higher alcohols. Fujii et al. (1997) confirmed that supplementation with linoleic acid can inhibit AATase (alcohol acetyltransferase) activity and reduce acetate ester synthesis. In a study of this synergistic effect, Tween 80, containing 70% oleic acid and 30% palmic acid and stearic acid, was added to improve the content of esters, higher alcohols and volatile fatty acids of wine (Varela et al., 2012).

Grape berries contain 0.15–0.24% (wet weight basis) lipids (Gallander and Peng, 1980), with UFAs being the major components of the total lipids. Linoleic acid (C18:2n6) is the most abundant lipid, followed by oleic (C18:1n9) and α-linolenic acids (C18:3n3). UFA concentrations in grape must change with grape cultivars (Ancín et al., 1998), fermentation technologies such as grape must clarification (Varela et al., 1999) and grape-skin maceration (Valero et al., 1998). Therefore, from the wine production perspective, it is essential to investigate the synergistic effect of UFAs on aroma compound synthesis during wine fermentation. Duan et al. (2015) indicated that rationally increasing the concentrations of UFA mixtures (linoleic, oleic and α-linolenic acids) can improve yeasts growth and most volatile compounds in wine, including higher alcohols, acetate esters (isoamyl acetate and 2-phenylethyl acetate) and ethyl esters. Numerous efforts have been made to characterize the entire gene expression profiles under different nitrogen conditions during vinification (Marks et al., 2003; Mendes-Ferreira et al., 2007; Liu et al., 2018). However, to our knowledge, no related information is available on the response of *S. cerevisiae* to UFA variation during wine fermentation. In this work, cDNA microarray technology was therefore applied to analyze the transcriptional discrepancies of the wine yeast *S. cerevisiae* EC1118 fermenting in two different culture media with different levels of UFAS (including linoleic, oleic, and α-linolenic acids), in which various aromatic compound profiles were detected. To facilitate this investigation, a simplified, chemically defined medium (MS300) that resembles the nutrient composition of grape juice was used, which is often employed in the transcriptional research of wine yeasts (Rossignol et al., 2003; Rossouw et al., 2010).

MATERIALS AND METHODS

Yeast Strain and Culture Media

The commercial *S. cerevisiae* var. *bayanus* strain EC1118 (Lallemand Inc., Blagnac, France) was used in this study. It is used for both red and white winemaking worldwide and is considered a fast and robust fermenting strain (Brice et al., 2014). The nitrogen synthetic grape must MS300 was used in this work (Varela et al., 2004). The pH of the medium was adjusted to 3.3. According to previous data by Duan et al. (2015) two UFA mixture concentrations (including linoleic, oleic and α-linolenic acids purchased from Sigma-Aldrich Company, St. Louis, MO, United States) were added to the MS300 medium, and a high UFA concentration medium (with 390 mg/L linoleic, 130 mg/L oleic, and 104 mg/L α-linolenic acids) and a low UFA concentration medium (with 30 mg/L linoleic, 10 mg/L oleic, and 8 mg/L α-linolenic acids) required to ensure normal cell growth and fermentation, considered the control, were obtained.

Fermentation Conditions and Samples

Saccharomyces cerevisiae EC1118 strain was inoculated into 200 mL YEPD medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose) and cultivated for approximately 15 h with shaking (120 rpm) at 28°C. After harvesting and washing twice with sterile water, 5 mL of yeast cells suspension was added into the flask, and the initial viable population was approximately 10^6 CFU/mL. Before the inoculation, nitrogen was sparged to eliminate oxygen from the medium. The 500 mL flasks with 350 mL MS300 medium were sealed with a fermentation lock, which guarantees carbon dioxide exhaustion and prevents oxygen from entering the flasks to achieve anaerobic conditions. Fermentations were carried out without shaking at 25°C and in triplicate. The fermentation lasted 170 h. The progress

of fermentation was monitored daily by measuring the cell density (OD_{600}) and sugar consumption. A total of 25 mL samples were taken from the fermentation flasks with a puncture needle and were immediately centrifuged to collect the cell-free supernatants for the analysis of the main fermentation products and aroma compounds.

Analytical Methods

General parameters (glucose, ethanol, glycerol, acetic acid, malic acid, lactic acid, and succinic acid) were determined by high-performance liquid chromatography (HPLC, 1200 series, Agilent Technologies, Inc., Palo Alto, CA, United States) as described by Duan et al. (2015). The system was equipped with an HPX-87H Aminex ion-exchange column (300 mm × 7.8 mm, Bio-Rad Laboratories, Hercules, CA, United States) with 5 mM sulfuric acid as the mobile phase. The volatile compounds of the wines were determined by headspace solid-phase microextraction coupled with gas chromatography-mass spectrometry (HS-SPME-GC-MS) as previously described (Zhang et al., 2011; Xu et al., 2015). An Agilent 6890A equipped with a 5975C MS system and an HP-INNOWAX column (60 m × 0.25 mm × 0.25 μm) was used in this system. The aroma compounds were identified by a comparison of the retention indices (RI) of reference standards and mass spectra that matched in the NIST 08 MS database. The quantification was applied with the calibration curves of aroma standards as described by Xu et al. (2015). Analyses were performed in triplicate. Significant differences of metabolites among the treatments were identified using one-way analysis of variance (ANOVA) followed by Duncan's test ($p < 0.05$) (SPSS 17.0, SPSS Inc., Chicago, IL, United States).

Microarray Procedures

For the DNA microarray analyses, the RNA of yeasts in mid-exponential (30 h), early-stationary (87 h) and late-stationary growth phases (123 h) were extracted, corresponding to the time points that 19.4, 67.2, and 88.1% sugars were consumed by yeast in high UFA culture, respectively, and 16.7, 57.6, and 89.9% sugars were consumed in low UFA culture, respectively. Three independent cultures were prepared for the biological repeats. Total RNA was isolated using the hot phenol method (Deed et al., 2011) and assessed by agarose gel electrophoresis and a NanoDrop spectrophotometer ND-1000 (NanoDrop products, DE, United States). The RNA samples were subjected to whole-genomic gene expression profiles (CapitalBio Corporation, Beijing, China). After purification, cDNA and biotin-labeled cRNA syntheses, the cRNA samples were hybridized with Yeast Genome 2.0 (Affymetrix GeneChip) and processed as described by the manufacturer (Affymetrix, CA, United States[1]). Pretreatments were applied to eliminate the sample variation, including background rectification and normalization. The pretreated data were analyzed with the RMA algorithm. A significance analysis of microarray (SAM) was applied to identify the genes that were differentially expressed between the high UFA and low UFA cultures. The threshold for significance was set to allow a median of one false positive per analysis for a false discovery rate (FDR) of <0.05%. Genes of the yeast in high UFA culture whose expression levels were greater than twofold or less than 0.5-fold, relative to the yeast in low UFA culture, were considered to be induced or repressed, respectively. These genes were further categorized by biological process using the *Saccharomyces* Genome Database Gene Ontology Slim Mapper tools (*SGD* GO Slim Mapper[2]).

[1]www.affymetrix.com

RESULTS

Cell Growth, Sugar Consumption, and Major Aroma Compounds in Low and High UFA Cultures

Yeast cell growth (OD_{600}) and sugar consumption in the treatments were monitored during fermentation (**Figure 1**). To facilitate the comparison, some key kinetic parameters were calculated, such as the duration of fermentation, maximum biomass, time to reach the maximum biomass, maximum specific growth, and rate of fermentation, which are shown in **Table 1**. In general, a relatively higher level of UFAs improved the yeast population and fermentation activity. The highest cell population (OD_{600}) obtained at late-stationary phase (123 h) in the high UFA fermentation (HUF) was 15.0% higher than that in the low UFA fermentation (LUF). Similarly, the fermentation rate (rate of sugar consumption) in HUF was slightly enhanced in comparison to the LUF (2.06 vs. 1.99 g/L·h of maximum fermentation rate). The production profiles of ethanol and glycerol were also determined (**Supplementary Figure S1**). The results showed that the ethanol concentration did not differ between treatments, while glycerol was higher in the HUF (6.34 ± 0.12 vs. 5.93 ± 0.07 g/L in final samples). No significant differences were

[2]https://yeastmine.yeastgenome.org/yeastmine/tools.do

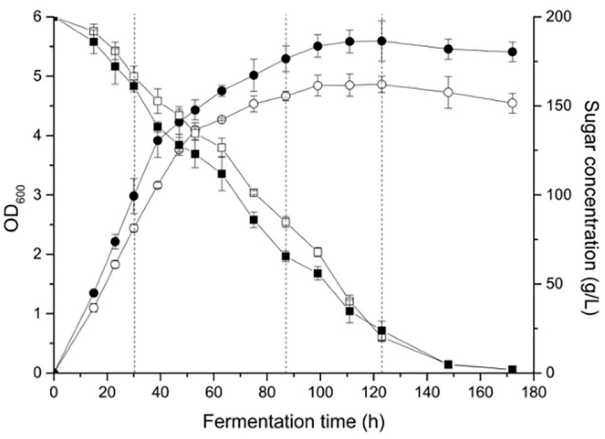

FIGURE 1 | Cell growth and sugar concentration profiles during the fermentation of MS 300 media with high (●, ■) or low (○, □) UFA concentrations. Data points represent the mean value from triplicate fermentations, and the vertical bars show ±SD. Dashed lines represent the three growth phases in which the microarray analyses were conducted.

TABLE 1 | Important parameters of yeast fermentation in MS300 media supplemented with high (HUFA) or low (LUFA) concentrations of UFAs.

Time to reach the end of fermentation (h)		Maximum OD	Time to reach maximum biomass (h)	Maximum specific growth rate (1/h)	Maximum fermentation rate (g/L·h)
LUFAs	172	4.86 ± 0.14a	123	0.015 ± 0.01a	1.99
HUFAs	172	5.59 ± 0.34b	123	0.016 ± 0.01a	2.06

found in the number of other metabolites, such as acetic, citric and malic acids, in the final samples of both fermentations (data not shown). We further determined the content of extracellular and intracellular UFAs during fermentation (**Figure 2**). The data indicated that the UFAs added were rapidly taken up by the cells. No UFAs were detected after fermentation in LUF, while few UFAs remained in the HUF sample. As expected, in both treatments, the concentration of intracellular UFAs increased before 30 h fermentation, after which the values slowly decreased. However, the concentration of particular cellular UFAs was always higher in the HUF than in the LUF samples.

To demonstrate the effects of UFAs on the generation of aromatic compounds, the main volatile compounds (higher alcohols, acetate esters, ethyl esters, and fatty acids) produced by yeasts at different fermentation times were determined, as shown in **Figures 3**, **4**. In general, the supply of a relatively higher UFA mixture improved the generation of most aroma compounds, including higher alcohols (2-methyl-1-propanol, 3-methyl-1-butanol, and phenylethyl alcohol) and esters (3-methyl-1-butanol acetate, ethyl acetate, 2-phenylethyl acetate, ethyl hexanoate, ethyl heptanoate, ethyl octanoate, ethyl nonanoate, ethyl decanoate, ethyl dodecanoate, ethyl myristate, and ethyl palmitate). As a result, the total content of higher alcohols, acetate esters and ethyl esters in the final samples of HUF was 45.5, 40.0, and 49.5% higher than those in LUF, respectively. The response of fatty acid formation to the addition of UFA depended to a large extent on the types of fatty acids added. Butanoic acid and octanoic acid showed higher trends, while hexanoic acid, decanoic acid, and dodecanoic acid showed decreased profiles. As a result, no significant difference in total fatty acid content was observed between the two treatments (10.35 ± 0.28 vs. 12.31 ± 0.79 mg/L).

Analysis of Yeast Global Gene Expression by DNA Microarray

Consistent with the results of Duan et al. (2015), the above data indicated that increasing the UFA content in synthetic grape juice improved yeast growth and the production of most volatiles. To gain insight into the mechanism at the molecular level,

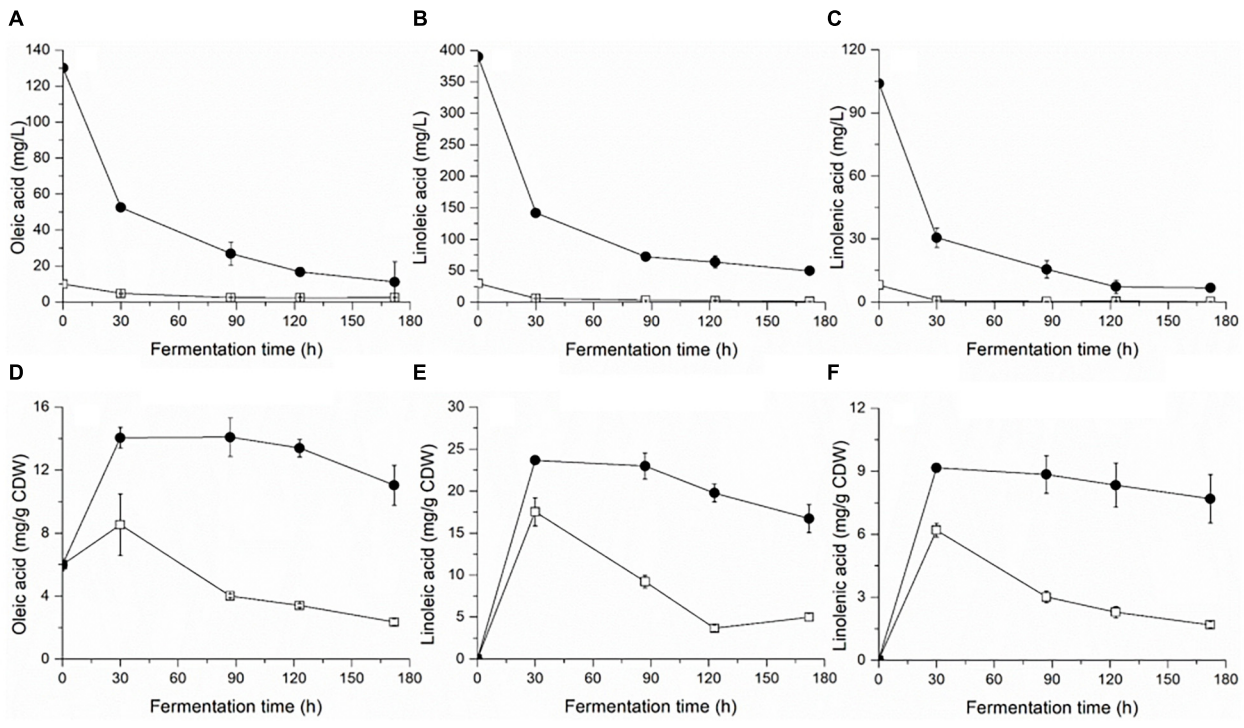

FIGURE 2 | Profiles of extracellular **(A–C)** and intracellular **(D–F)** fatty acids during the fermentation of MS300 media with high (●) and low (□) concentrations of UFAs. Data points represent the mean value from triplicate fermentations, and the vertical bars show ±SD.

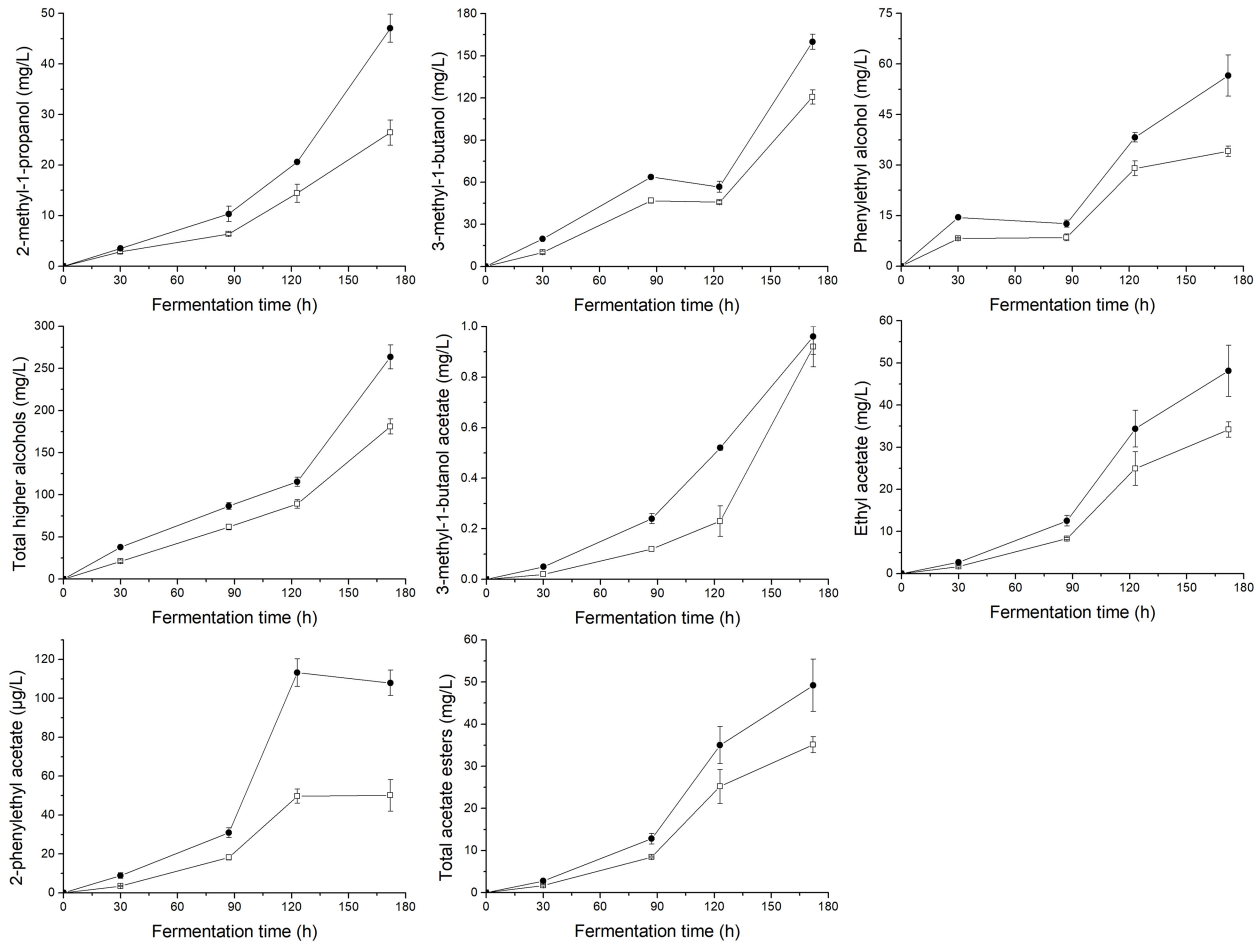

FIGURE 3 | Profiles of the major higher alcohols and total content of higher alcohols, acetate esters and total content of acetate esters during the fermentation of MS300 medium with high (●) or low (□) UFA concentrations. Data points represent the mean value from triplicate fermentations, and the vertical bars show ±SD.

a comparative transcriptome analysis using DNA microarray technology was applied. The RNA of yeast in the mid-exponential (30 h), early-stationary (87 h), and late-stationary growth phases (123 h) was used for pair wise comparisons of HUF compared to LUF. In total, 63 genes were upregulated (greater than twofold expression), and 91 were down-regulated (less than 0.5-fold expression), as shown in **Tables 2**, **3**. Most of the affected genes were found in the late-stationary growth phase. To confirm the results of the DNA microarray analyses, six genes were randomly selected, including *FAA4*, *BAP3*, *CLB1*, *SPS100*, *ALD3*, and *MGA2*, and qPCR experiments were performed. Correlation analysis showed that the correlation coefficient between the microarray chip and qPCR determinations exceeded 0.887, indicating that the data obtained by DNA microarray are reliable (**Supplementary Figure S2**).

The affected genes were further categorized according to their biological and functional processes as assigned by the *Saccharomyces* Genome Database (SGD). Eight genes were upregulated, and four genes were downregulated in the mid-exponential growth phase. In addition to two unknown function genes (*YML083C* and *YCR102C*), the upregulated genes included *PAU3*, *PAU14*, *PAU8*, and *AAD4*, which are involved in the processes of stress response. In addition, *AAD4* encodes a putative aryl-alcohol dehydrogenase and is involved in the oxidative stress response. *NRG2* mediates glucose repression, and *HES1* is associated with ergosterol biosynthesis. The downregulated genes included *TIP1* (involved in wall protein synthesis), *HXT5* (encodes hexose transporter), *IZH4* (involved in zinc ion homeostasis), and *HBT1* (encodes shmoo tip protein, the substrate of Hub1p ubiquitin-like protein).

Fewer genes were induced in the early-stationary phase compared to the mid-exponential phase, including *HXT9* and *IMA5*, and two unknown function genes (*YMR317W* and *YKL068W-A*). *HXT9* and *IMA5* are involved in sugar transportation and utilization. The expression of genes involved in lipid and fatty acid biosynthesis was downregulated in HUF. Exogenous fatty acids can strongly repress the *de novo* synthesis of lipids and fatty acids in yeasts, as has been reported by Chirala (1992). Although we did not observe the repression of *OLE1* transcription, which encodes delta (9) fatty acid desaturase

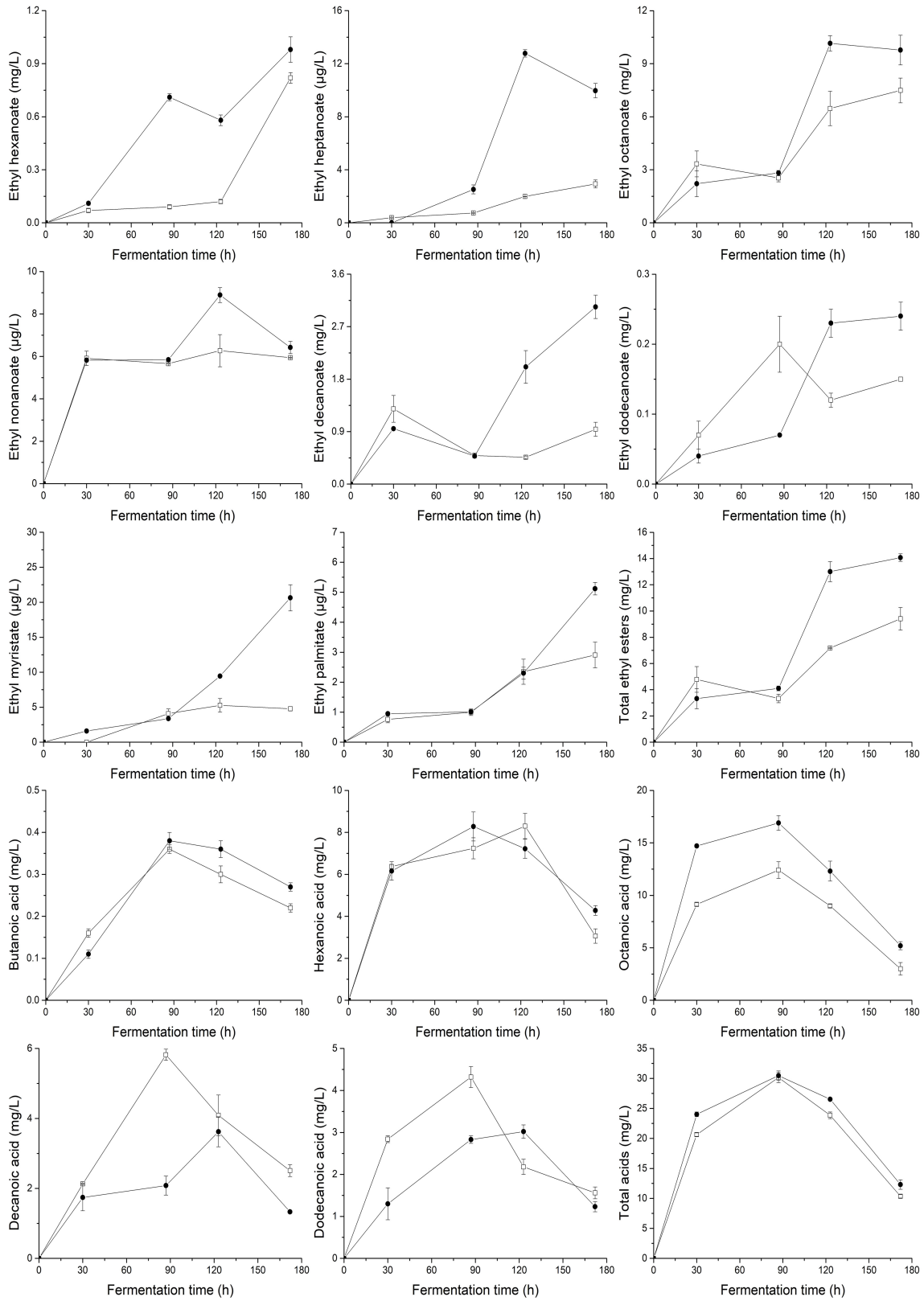

FIGURE 4 | Profiles of major ethyl esters and the total content of ethyl esters, fatty acids and the total content of fatty acids during the fermentation of MS300 media with high (●) and low (□) UFA concentrations. Data points represent the mean value from triplicate fermentations, and the vertical bars show ±SD.

TABLE 2 | Genes upregulated in MS300 media with high vs. low concentrations of UFAs, in different stages of yeast growth categorized by biological process (double or more).

Open reading frame (ORF)	Gene name	Description	Fold change
Mid-exponential phase			
Stress response			
YCR104W	*PAU3*	Member of the seripauperin multigene family	3.92
YIL176C	*PAU14*	Member of the seripauperin multigene family	2.58
YAL068C	*PAU8*	Member of the seripauperin multigene family	2.90
YDL243C	*AAD4*	Aryl-Alcohol Dehydrogenase	2.62
Glucose repression			
YBR066C	*NRG2*	Negative Regulator of Glucose-controlled genes	2.56
Ergosterol biosynthesis			
YOR237W	*HES1*	Protein implicated in the regulation of ergosterol biosynthesis	2.78
Unknown function			
YML083C	–	Protein of unknown function	2.42
YCR102C	–	Protein of unknown function	2.06
Early-stationary phase			
Sugar utilization			
YJL219W	*HXT9*	Putative hexose transporter that is nearly identical to Hxt11p	3.47
YJL216C	*IMA5*	Alpha-glucosidase	2.78
Unknown function			
YMR317W	–	Putative protein of unknown function	2.58
YKL068W-A	–	Putative protein of unknown function	2.19
Late-stationary phase			
Cell wall biosynthesis			
YHR143W	*DSE2*	Daughter cell-specific secreted protein with similarity to glucanases	4.59
YER124C	*DSE1*	Daughter cell-specific protein	2.08
YNR067C	*DSE4*	Daughter cell-specific secreted protein	3.55
YNL327W	*EGT2*	Glycosylphosphatidylinositol (GPI)-anchored cell wall endoglucanase	3.33
YGL028C	*SCW11*	Cell wall protein with similarity to glucanases	3.05
YNL066W	*SUN4*	Cell wall protein related to glucanases	3.77
YHR126C	*ANS1*	Putative GPI protein	2.18
YHR139C	*SPS100*	Protein required for spore wall maturation	2.12
Cell cycle, RNA, and ribosome			
YLR286C	*CTS1*	Endochitinase	2.97
YIL158W	*AIM20*	Altered inheritance rate of mitochondria	3.02
YGR108W	*CLB1*	B-type cyclin involved in cell cycle progression	2.29
YDR146C	*SWI5*	Transcription factor that recruits the mediator and Swi/Snf complexes	2.22
YPR119W	*CLB2*	B-type cyclin involved in cell cycle progression	2.02
YGL029W	*CGR1*	Protein involved in nucleolar integrity and processing of pre-rRNA	2.39
YIL016W	*SNL1*	Ribosome-associated protein	2.24
Metal and ions homeostasis			
YOR079C	*ATX2*	Golgi membrane protein involved in manganese homeostasis	2.23
YLR034C	*SMF3*	Putative divalent metal ion transporter involved in iron homeostasis	2.28
YNL259C	*ATX1*	Cytosolic copper metallochaperone	2.17
Stress response			
YLR461W	*PAU4*	Member of the seripauperin multigene family	3.86
YCR104W	*PAU3*	Member of the seripauperin multigene family	3.81
YIL176C	*PAU14*	Member of the seripauperin multigene family	2.48
YLL064C	*PAU18*	Member of the seripauperin multigene family	2.46
YAL068C	*PAU8*	Member of the seripauperin multigene family	3.73
Amino acid transportation			
YHL036W	*MUP3*	Low affinity methionine permease, similar to Mup1p	2.31
YDR046C	*BAP3*	Branched-chain amino acid permease	2.29
YNL217W	*PPN2*	Putative serine/threonine-protein phosphatase	2.11

(Continued)

TABLE 2 | Continued

Open reading frame (ORF)	Gene name	Description	Fold change
YOR115C	*TRS33*	Core component of transport protein particle (TRAPP) complexes I–III	2.02
YMR169C	*ALD3*	Cytoplasmic aldehyde dehydrogenase	2.04
Other process			
YOL155C	*HPF1*	Haze-protective mannoprotein	2.18
YLR084C	*RAX2*	N-glycosylated protein	2.18
YDR033W	*MRH1*	Membrane protein Related to Hsp30p	2.06
YJL219W	*HXT9*	Putative hexose transporter that is nearly identical to Hxt11p	2.74
YBR092C	*PHO3*	Constitutively expressed acid phosphatase similar to Pho5p	2.08
YGR131W	*FHN1*	Functional Homolog of Nce102	2.01
YBR161W	*CSH1*	Mannosylinositol phosphorylceramide (MIPC) synthase catalytic subunit	2.05
YDL059C	*RAD59*	Protein involved DNA double-strand break repair	2.09
YDR139C	*RUB1*	Ubiquitin-like protein with similarity to mammalian NEDD8	2.12
Unknown function			
YLR346C	*CIS1*	Putative protein of unknown function found in mitochondria	2.55
YNL046W	–	Putative protein of unknown function	2.04
YNL058C	–	Putative protein of unknown function	2.03
YMR317W	–	Putative protein of unknown function	2.38
YNL277W-A	–	Putative protein of unknown function	2.32
YNR034W-A	–	Putative protein of unknown function	2.31
YDL085C-A	–	Putative protein of unknown function	2.14
YDR524W-A	–	Putative protein of unknown function	2.11
YML007C-A	–	Putative protein of unknown function	2.10
YOL014W	–	Putative protein of unknown function	2.08
YLR285C-A	–	Putative protein of unknown function	2.53
YMR030W-A	–	Putative protein of unknown function	2.50
YML018C	–	Protein of unknown function	2.01
YIL169C	–	Putative protein of unknown function	2.27

and is involved in the formation of UFAs, two other genes, *MGA2* (encoding an ER membrane protein that regulates *OLE1* transcription) and *FAA4* (encoding the long chain fatty acyl-CoA synthetase responsible for the importation of long-chain fatty acids), were found to be downregulated. *IZH2* and *IZH4* were continuously repressed in HUF (still depressed in the late-stationary phase); both genes play an important role in zinc metabolism and homeostasis and exhibit elevated expression in zinc-deficient cells. It has been confirmed that their expression is lipid- and oxygen-dependent and linked with sterol metabolism (Lyons et al., 2004). The down-regulation of these genes might be due to the incorporation of more UFAs into the cell membrane, which disturbs *de novo* lipid syntheses. The decreased expression of *TIP1* was observed in this and the last yeast growth stage of HUF.

Most genes were affected after cells entered the late-stationary phase (51 and 78 genes were upregulated and downregulated, respectively). This could be because the nutritional status at this stage became sterile compared to other stages (Backhus et al., 2001; Marks et al., 2003). The induced genes in this stage were mainly associated with cell wall formation, cell cycle, RNA and ribosome biosynthesis, metal and ion metabolism, stress response, amino acid metabolism, process, and unknown function. The repressed genes were related to lipid, sterol, amino acid, carbohydrate metabolism, stress response, zinc ion homeostasis, process, and unknown function. Cell wall formation, cell cycle, RNA and ribosome biosynthesis are associated with cell growth and proliferation. The high expression of the genes *DSE2*, *DSE1*, *DSE4*, *EGT2*, *SCW1*, *SUN4*, *ANS1*, *SPS100*, *CTS1*, *AIM20*, *CLB1*, *SWI5*, *CLB2*, *CGR1*, and *SNL1* was correlated with an increase in the cell population in HUF. Members of *PAU* (*PAU3*, *PAU14*, *PAU14*, *PAU8*, and *PAU18*) and the genes *MUP3*, *BAP3*, and *TRS33*, all involved in amino acid transportation, were induced at this stage in HUF. In addition, *MUP3* encodes a low affinity methionine permease, and *BAP3* encodes a specific branched-chain amino acid permease. Another upregulated gene was *ALD3*, which encodes cytoplasmic aldehyde dehydrogenase and plays a critical role in the conversion of acetaldehyde to acetyl-CoA during growth on non-fermentable carbon sources, which can be induced in response to ethanol stress (Navarro-Aviño et al., 1999). The upregulation of *ALD3* might enable cells to generate more acetyl-CoA for producing esters in HUF. *PHO3* (encodes acid phosphatase to hydrolyze thiamine phosphates) was also upregulated by high UFA addition.

Compared to upregulated genes, more genes were repressed in the late-stationary phase. In addition to *MGA2* and *FAA4* (which were downregulated in the early-stationary phase), the genes *FAT3*, *LPX1*, *FMP45*, *SUT1*, and *MGA1* involved in lipid and sterol metabolism were repressed. *FAT3* encodes the

TABLE 3 | Genes downregulated in MS300 media with high vs. low concentrations of UFAs, in different stages of yeast growth categorized by biological process (double or more).

Open reading frame (ORF)	Gene name	Description	Fold change
Mid-exponential phase			
Glucose transportation		Hexose transporter with moderate affinity for glucose	
YHR096C	*HXT5*		0.45
Zinc ion homeostasis			
YOL101C	*IZH4*	Membrane protein involved in zinc ion homeostasis	0.28
Other process			
YBR067C	*TIP1*	Major cell wall mannoprotein with possible lipase activity	0.47
YDL223C	*HBT1*	Shmoo tip protein, substrate of Hub1p ubiquitin-like protein	0.47
Early-stationary phase			
Lipid and sterol biosynthesis			
YIR033W	*MGA2*	ER membrane protein involved in regulation of *OLE1* transcription	0.47
YMR246W	*FAA4*	Long chain fatty acyl-CoA synthetase	0.41
Zinc ion homeostasis			
YOL101C	*IZH4*	Membrane protein involved in zinc ion homeostasis	0.10
YOL002C	*IZH2*	Plasma membrane proteins thought to affect zinc homeostasis	0.43
Other process			
YMR175W	*SIP18*	Phospholipid-binding hydrophilin	0.39
YHR033W	–	Putative glutamate 5-kinase	0.48
YPR192W	*AQY1*	Spore-specific water channel	0.45
YBR067C	*TIP1*	Major cell wall mannoprotein with possible lipase activity	0.44
YLR413W	*INA1*	Putative protein of unknown function	0.24
Late-stationary phase			
Lipid and sterol biosynthesis			
YIR033W	*MGA2*	ER membrane protein involved in regulation of *OLE1* transcription	0.47
YKL187C	*FAT3*	Protein required for fatty acid uptake	0.40
YMR246W	*FAA4*	Long chain fatty acyl-CoA synthetase	0.39
YOR084W	*LPX1*	Peroxisomal matrix-localized lipase	0.49
YGR249W	*MGA1*	Protein similar to heat shock transcription factor	0.47
YDL222C	*FMP45*	Integral membrane protein localized to mitochondria	0.29
YGL162W	*SUT1*	Transcription factor of the Zn(II)2Cys6 family	0.50
YCR091W	*KIN82*	Putative serine/threonine protein kinase	0.48
Amino acid metabolism			
YBR132C	*AGP2*	Plasma membrane regulator of polyamine and carnitine transport	0.50
YMR136W	*GAT2*	Protein containing GATA family zinc finger motifs	0.48
YOR348C	*PUT4*	Proline permease	0.43
YMR042W	*ARG80*	Transcription factor involved in regulating arginine-responsive genes	0.41
YPL111W	*CAR1*	Arginase	0.41
TCA cycle, mitochondrial respiratory			
YLL041C	*SDH2*	Succinate dehydrogenase	0.49
YDR216W	*ADR1*	Alcohol dehydrogenase regulator	0.46
YML120C	*NDI1*	NADH: ubiquinone oxidoreductase	0.39
YDL085W	*NDE2*	Mitochondrial external NADH dehydrogenase	0.41
YMR303C	*ADH2*	Glucose-repressible alcohol dehydrogenase II	0.38
YLR393W	*ATP10*	Assembly factor for the F0 sector of mitochondrial F1F0 ATP synthase	0.45
YLL018C-A	*COX19*	Protein required for cytochrome c oxidase assembly	0.42
Zinc ion homeostasis			
YDR492W	*IZH1*	Membrane protein involved in zinc ion homeostasis	0.46
YOL101C	*IZH4*	Membrane protein involved in zinc ion homeostasis	0.28
Stress response			
YMR276W	*DSK2*	Nuclear-enriched ubiquitin-like polyubiquitin-binding protein	0.49
YMR280C	*CAT8*	Zinc cluster transcriptional activator	0.47
YOR028C	*CIN5*	Basic leucine zipper (bZIP) transcription factor of the yAP-1 family	0.44

(Continued)

TABLE 3 | Continued

Open reading frame (ORF)	Gene name	Description	Fold change
YMR070W	*MOT3*	Transcriptional repressor and activator with two C2-H2 zinc fingers	0.46
YER143W	*DDI1*	DNA-damage inducible 1 homolog 1 (*S. cerevisiae*)	0.48
YPL190C	*NAB3*	RNA-binding protein, subunit of Nrd1 complex (Nrd1p-Nab3p-Sen1p)	0.48
YOR178C	*GAC1*	Regulatory subunit for Glc7p type-1 protein phosphatase (PP1)	0.48
YLR116W	*MSL5*	Component of commitment complex	0.47
YAR073W	*IMD2*	Inosine monophosphate dehydrogenase	0.47
YMR164C	*MSS11*	Transcription factor	0.46
YPR065W	*ROX1*	Heme-dependent repressor of hypoxic genes	0.41
YGR088W	*CTT1*	Cytosolic catalase T	0.40
YPL230W	*USV1*	Putative transcription factor containing a C2H2 zinc finger	0.38
YBL066C	*SEF1*	Putative transcription factor; has homolog in *Kluyveromyces lactis*	0.37
YIL101C	*XBP1*	Transcriptional repressor	0.36
YHR205W	*SCH9*	AGC family protein kinase	0.36
YOR140W	*SFL1*	Transcriptional repressor and activator	0.30
YER064C	*VHR2*	Null mutation has global effects on transcription	0.45
YDR169C	*STB3*	Ribosomal RNA processing element (RRPE)-binding protein	0.43
YLL010C	*PSR1*	Plasma membrane associated protein phosphatase	0.44
Other process			
YLR315W	*NKP2*	Central kinetochore protein and subunit of the Ctf19 complex	0.50
YEL070W	*DSF1*	Putative mannitol dehydrogenase	0.48
YNL307C	*MCK1*	Meiotic and centromere regulatory ser, tyr-Kinase	0.50
YMR104C	*YPK2*	Protein kinase similar to serine/threonine protein kinase Ypk1p	0.47
YBR067C	*TIP1*	Major cell wall mannoprotein with possible lipase activity	0.46
YLR094C	*GIS3*	Glg1-2 suppressor	0.50
YLR446W	–	Putative hexokinase	0.43
YLL013C	*PUF3*	Protein of the mitochondrial outer surface	0.50
YGL169W	*SUA5*	Protein involved in threonylcarbamoyl adenosine biosynthesis	0.49
YPL119C	*DBP1*	Putative ATP-dependent RNA helicase of DEAD-box protein family	0.49
YAL039C	*CYC3*	Cytochrome c heme lyase	0.48
YHR199C-A	*NBL1*	Subunit of the conserved chromosomal passenger complex (CPC)	0.47
YDL223C	*HBT1*	Shmoo tip protein, substrate of Hub1p ubiquitin-like protein	0.39
YBR212W	*NGR1*	RNA binding protein that negatively regulates growth rate	0.36
YHR033W	–	Putative glutamate 5-kinase	0.33
YJR094C	*IME1*	Master regulator of meiosis that is active only during meiotic events	0.44
YGR068C	*ART5*	ADP-ribosyltransferase 5	0.50
Unknown function			
YLR267W	*BOP2*	Protein of unknown function	0.48
YPL054W	*LEE1*	Zinc-finger protein of unknown function	0.39
YBL081W	–	Non-essential protein of unknown function	0.48
YDR505C	*PSP1*	Asn and gln rich protein of unknown function	0.44
YPR153W	–	Putative protein of unknown function	0.47
YHR131C	–	Putative protein of unknown function	0.46
YMR291W	*TDA1*	Protein kinase of unknown cellular role	0.45
YNL269W	*BSC4*	Protein of unknown function	0.45
YMR147W	–	Putative protein of unknown function	0.45
YHR105W	*YPT35*	Endosomal protein of unknown function	0.44
YOL084W	*PHM7*	Protein of unknown function	0.44
YGL056C	*SDS23*	Protein involved in cell separation during budding	0.45
YJR115W	–	Putative protein of unknown function	0.43
YDL129W	–	Protein of unknown function	0.40
YLR413W	*INA1*	Putative protein of unknown function	0.40
YNR014W	–	Putative protein of unknown function	0.40
YDL037C	*BSC1*	Protein of unconfirmed function	0.39
YMR206W	–	Putative protein of unknown function	0.27
YGR067C	–	Putative protein of unknown function	0.37

transporter protein Fat3p and is responsible for fatty acid uptake; *SUT1* encodes the Zn(II)2Cys6 family transcription factor and positively regulates sterol uptake genes under anaerobic conditions; *FMP45* encodes an integral membrane protein located in the mitochondria and is required for sporulation and the maintenance of sphingolipid content. These data suggest that *de novo* synthesis and the metabolism of fatty acids and sterol in cells in the HUF were strongly negatively influenced in the late stationary phase. Additionally, several genes involved in amino acid and nitrogen metabolism were downregulated. It should be noted that these amino acids were not different from those present in the group of upregulated genes. They mainly included *AGP2*, *GAT2*, *PUT4*, *ARG80*, and *CAR1*. *AGP2* encodes the plasma membrane regulator of polyamine and carnitine transport and can act as a sensor that transduces environmental signals. *GAT2* encodes the protein containing the GATA family zinc finger motifs and is repressed by leucine. *PUT4* encodes a proline permease with high affinity proline transport. *ARG80* encodes a transcription factor and is involved in the regulation of arginine-responsive genes. *CAR1* encodes arginase that catabolizes arginine to ornithine and urea and controls the formations of ethyl carbamate (EC). Interestingly, some genes associated with the TCA cycle and mitochondrial respiratory chains were repressed, including *SDH2*, *ADR1*, *NDI1*, *NDE2*, and *ADH2*.

It should be noted that a majority of the stress-response genes were upregulated in LUF compared to HUF at this stage, including *DSK2*, *CAT8* (respond to DNA replication stress), *CIN5* (mediates pleiotropic drug resistance and salt tolerance), *MOT3* (transcriptional repressor, activator, cellular adjustment to osmotic stress), *MSS11* (a transcription factor controlling the activation of *FLO11* and *STA2* in response to nutritional signals), *ROX1* (involved in the hyperosmotic stress resistance), *CTT1* (protects from oxidative damage), *USV1* (responds to salt stress and cell wall biosynthesis), *XBP1* (transcriptional repressor, induced by stress or starvation during mitosis), and *PSR1* (plasma membrane associated protein phosphatase and involved in general stress response). These data suggest that yeast cells in LUF might be subjected to more stresses at this stage than those in HUF.

DISCUSSION

It is well known that wine yeast is challenged by simultaneous and sequential stresses during alcoholic fermentation, especially ethanol toxicity. The cellular membrane is the cell structure most affected by ethanol, which causes an increase in its permeability and leads to unfavorable effects, such as the inhibition of sugar, ammonium and amino acid uptake (Diniz et al., 2017). To maintain membrane stability, *S. cerevisiae* increases the synthesis of UFAs and enriches the plasma membrane UFA content. Alternatively, in the presence of exogenous UFAs, yeast cells can absorb UFAs into the cell membrane directly and improve their resistance to inhospitable environments, which can lead to increased biomass and fermentative activity and consequently modify the production of aroma compounds (Martin et al., 2007; Duan et al., 2015). To better understand the positive aromatic function of UFAs during wine fermentation, in this work, the transcriptional profiles of wine yeast in response to low and high UFA mixture additions were investigated by DNA microarray analyses in synthetic grape medium. The data described indicate that the initial supplementation of a high UFA mixture can promote cell growth and the production of most aromatic compounds, including higher alcohols, acetate esters and ethyl esters, with the exception of several fatty acids (hexanoic acid, decanoic acid, and dodecanoic acid). Microarray analyses identified that sixty-three and ninety-one genes were upregulated (greater than twofold expression) or downregulated (less than 0.5-fold expression), respectively. Because the aim of the study is to reveal the molecular effect of UFAs on aroma compounds, we focused on the groups of genes associated with volatile production in the discussion below, especially the upregulated genes.

The improvement of cell wall formation can increase the resistance of yeast to environmental stresses (Brennan et al., 2013). The expression of genes involved in cell wall formation, cell cycle, RNA, and ribosome biosynthesis was elevated in HUF, which corresponded to an increase in the biomass in HUF. Increasing the cell population in wine fermentation directly promotes the formation of aroma compounds (Varela et al., 2004; Duan et al., 2015). Thus, the beneficial effect of UFAs on aroma compounds observed in this work could be partially ascribed to an increased cell population able to resist deleterious conditions during wine fermentation. *PAU* genes comprise the largest multiple gene family in *S. cerevisiae*, with 24 members, which are induced by different stresses, such as low temperature, low oxygen and wine fermentation conditions (Luo and van Vuuren, 2009). In this study, *PAU3*, *PAU14*, *PAU8*, and *PAU18* were induced at different stages in HUF, which is consistent with the data of Rossignol et al. (2003), who found that 15 PAU/TIR genes were strongly upregulated during wine alcoholic fermentation. Wilcox et al. (2002) suggested that the function of PAU protein involves sterol transport, which might explain the substantial induction of *PAU* expression in HUF. Increased biomass can consume more nutrients (such as sterol, ions, copper, and thiamine) and cause nutrition deficiency, which can induce the expression of related functional genes to replenish these compounds (Liu et al., 2018). This could account for the increased expression of several genes (*PHO3*, *ATX1*, and *ATX2*) by high UFA additions. *PHO3*, which encodes an acid phosphatase, can hydrolyze thiamine phosphates in the periplasmic space and increase cellular thiamine uptake. Thiamine pyrophosphate is a cofactor essential for the activity of pyruvate decarboxylase, and its depletion has a negative effect on yeast carbon metabolism (Hohmann and Meacock, 1998). Increasing the yeast biomass can consume a large amount of thiamine pyrophosphate, resulting in its deficiency in the must (Liu et al., 2018). The upregulation of *ATX1* and *ATX2*, which encode the Mn^{2+} transporter, implies that the metal could also be limited in the cells (Lin and Culotta, 1996).

Amino acid metabolism is of particular interest from a winemaking perspective, as amino acids serve as the precursors of important volatile aroma compounds (Rossouw et al., 2010). In this study, the expression levels of genes directly related to the formation of aroma compounds, such as *BAT1*, *PDC1*, *ATF1*, *EEB1*, *EHT1*, and *IAH1*, were not significantly different between the two treatments, while the genes encoding amino acid permeases were greatly induced in HUF, for example, *BAP3* that encodes a one branched-chain amino acid permease. Branched-chain amino acids (including L-valine, L-leucine, and L-isoleucine) are important flavor precursors in grape must. The elevated expression of *BAP2* and *BAP3* can enable yeast to transport more extracellular amino acids into the cells to produce higher alcohols and corresponding esters (Hazelwood et al., 2008; Trinh et al., 2010). Thus, the upregulation of *BAP3* might be another reason that an increased number of high alcohol and esters were produced in HUF. It should be mentioned that *BAP3* is subject to nitrogen catabolite repression (NCR) and is strongly repressed by yeast-preferred nitrogen (such as ammonium and glutamine) but is depressed when the cells are starved for nitrogen (McCusker and Haber, 1990; Magasanik and Kaiser, 2002). The induction of *BAP3* in the late-stationary growth phase of HUF could be due to the increased biomass, which causes the nitrogen available for yeast to be deficient in comparison with LUF. The incorporation of abundant UFAs into the cell membrane can help the cells maintain normal membrane fluidity and protect the activity of membrane-associated enzymes and transporters, which might, at least partially, lead to the increased production of aroma compounds. Interestingly, we found that *CAR1* and *ARG80*, which are positively involved in ethyl carbamate (EC) formation, were downregulated in HUF. In grape musts, the catabolism of arginine by wine yeasts can produce ornithine and urea. The secreted urea spontaneously reacts with ethanol to generate EC, which causes different cancers in a variety of test animals (Beland et al., 2005). The disruption of *CAR1* can decrease the production of the carcinogen EC during wine fermentation (Schehl et al., 2007). As a result, it is believed that increasing the UFA content in grape must or enriching UFAs in the cell membrane might be a potential way to reduce the formation of EC in wines. This hypothesis merits further study.

The *de novo* synthesis of lipids and fatty acids is repressed in the presence of exogenous fatty acids (Chirala, 1992), and the absorption of UFAs from the environment can inhibit the biosynthesis of UFAs and FA in yeasts (McDonough et al., 1992). The reduction of medium-chain fatty acids (MCFAs) by exogenous UFAs has been reported by Redon et al. (2011). Similarly, we found that MCFA formation and several genes involved in fatty acid transportation and synthesis were repressed in HUF. It was also found that some genes associated with the TCA cycle and mitochondrial respiration (*SDH2*, *ADR1*, *NDI1*, *NDE2*, and *ADH2*) were induced in LUF in comparison with HUF. Currently, we cannot explain these data well. However, the data of Backhus et al. (2001) showed that the wine yeast response to low nitrogen in the late time point of wine fermentation is to switch from a fermentative mode of metabolism to respiration characteristic with a general relief of TCA and respiration genes from glucose repression, which was accompanied by the increased expression of *ADH2*.

It is important to highlight that some results obtained in this study are not consistent with previous data. For example, the supplementation of single UFAs, such as linoleic acid, oleic acid or α-linolenic acid, can inhibit AATase (alcohol acetyltransferase) activity and reduce acetate ester synthesis (Yoshioka and Hashimoto, 1983; Fujii et al., 1997). The inconsistency might be due to differences in the culture medium or (and) the added UFA compositions (single or combined addition) and concentrations. Additionally, many genes with unknown functions were upregulated (14) or downregulated (19) in HUF vs. LUF. For example, *YLR346C* is regulated by transcription factors involved in pleiotropic drug resistance, Pdr1p and Yrr1p (Le Crom et al., 2002). The expression of *YNR034W-A* is regulated by *Msn2*/*Msn4* (Lai et al., 2005). *Msn2* and *Msn4*, which encode stress-responsive transcriptional activators, are activated in response to various stress conditions. These observations highlight the limitation of our understanding of the molecular mechanisms involved in wine yeast survival and metabolism during wine fermentation. Revealing the functions of these genes could help us to rationally control the process of wine fermentation and effectively modulate the formation of aroma compounds.

CONCLUSION

The results of the present work indicate that adding high contents of an UFA mixture into synthetic grape medium increased cell growth and the production of most yeast-derived volatile compounds compared to the low UFA-added culture, including higher alcohols and the corresponding esters, with the exception of several fatty acids. Sixty three and ninety one genes were identified by microarray analyses to be upregulated or downregulated, respectively, during alcoholic fermentation. Most of the upregulated genes were involved in yeast growth and proliferation, stress response, and nitrogen compound transportation. There were no genes directly involved in the formation of higher alcohols, and esters were found to be significantly upregulated in HUF vs. LUF. The improvement of aroma compounds in HUF is ascribed to the increased resistance of yeast to various stresses due to the incorporation of more UFAs into cells and the increased biomass and amino acid transportation. Our results highlighted the importance of UFAs in the regulation of aroma biosynthesis during wine fermentation and suggested that improving the resistance of yeast to extreme stresses is essential to effectively manipulate and improve the production of aroma compounds.

AUTHOR CONTRIBUTIONS

C-QD and G-LY designed the experiments. L-LD and P-TL conducted the experiments. L-LD, P-TL, and G-LY analyzed the experimental data and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

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frontiers in Microbiology

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Fermented Dairy Foods: Impact on Intestinal Microbiota and Health-Linked Biomarkers

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The intake of fermented foods is gaining increasing interest due to their health-promoting benefits. Among them, fermented dairy foods have been associated with obesity prevention, and reduction of the risk of metabolic disorders and immune-related pathologies. Fermented foods could lead to these health benefits by providing the consumer with both easily metabolizable nutrients and beneficial microorganisms. Our aim was to evaluate the relationship between the consumption of fermented dairy products and the intestinal microbiota, serum lipid profile, and the pro-oxidant/inflammatory status. 130 healthy adults were evaluated. Dietary fermented food intake was assessed by an annual food frequency questionnaire (FFQ), including 26 fermented dairy products. Levels of the major phylogenetic types of the intestinal microbiota were determined by qPCR, and concentration of fecal short chain fatty acids were assessed by gas chromatography. Serum glucose and lipid profile, as well as serum malondialdehyde (MDA), C-reactive protein (CRP), and leptin levels were determined by standardized protocols. Among fermented dairy foods, natural yogurt, sweetened yogurt and matured/semi-matured cheese were the most consumed. While natural yogurt consumers showed increased fecal levels of *Akkermansia* with respect to non-consumers, sweetened yogurt intake was associated to lower levels of *Bacteroides*. Serum levels of CRP were also significantly reduced in yogurt consumers. Our results underline the interest in exploring the potential effects of the different yogurt types and the role the microbiota may play in such effects.

Keywords: fermented foods, yogurt, inflammation, microbiota, oxidative stress

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INTRODUCTION

Fermented foods have played an important role in human diet since the development of civilization and represent a special feature of some dietary patterns, such as the Mediterranean one. The initial goal of the fermentation process was to prolong the useful-life of some foods and beverages, and improving their safety, digestibility and organoleptic properties, however, nowadays fermented products have become more popular than ever before due to their health-promoting benefits (Şanlier et al., 2017). Fermented dairy foods have received special attention because of their

association in epidemiological studies with obesity prevention, and with the reduction on the risk of different diseases, including metabolic disorders, cardiovascular and immune-related diseases or cognitive decline, among others (Guo et al., 2017; Salas-Salvadó et al., 2017; Kok and Hutkins, 2018; Sivamaruthi et al., 2018). Apart from their content of fatty acids, vitamins, and minerals, these products contain bioactive peptides and living microorganisms that could modulate the immune responses and impact on the intestinal microbiota (IM) composition and functionality (Chakrabarti et al., 2014; Severyn and Bhatt, 2018). The human IM is a complex and dynamic community, represented by trillions of microorganisms, that plays an important role in the maintenance of health. Indeed, recent studies have consistently identified disease-specific microbiota signatures in different health disorders (Duvallet et al., 2017). The microbiota of healthy adults is represented mainly by anaerobic bacteria from the Firmicutes and Bacteroidetes phyla (Eckburg et al., 2005). While the genera *Clostridium, Enterococcus, Lactobacillus* and *Faecalibacterium* are predominant within the Firmicutes phylum, others such as *Bacteroides* and *Prevotella* are the most representative of the Bacteroidetes phylum (Eckburg et al., 2005). All of them are present in different proportions depending on the specific microbial composition of each individual. The disruption and alteration of the microbiota may be related to different pathologies and, for this reason, the search for strategies capable of reversing the IM dysbiosis in order to improve the health status of the host has become a key area of interest for the scientific community. In this regard, long-term dietary habits, as well as specific food constituents, such as fiber or phenolics, have been identified as critical drivers of gut microbiota composition (Wu et al., 2011; Fernández-Navarro et al., 2018). Fermented products may also modulate the IM (Kato-Kataoka et al., 2016), however, the association between fermented foods as part of the regular diet and the IM composition has not been sufficiently studied yet (Alvaro et al., 2007; Uyeno et al., 2008). In this regard, a recent work examining the impact of consuming a fermented milk containing microorganisms from the genera *Lactobacillus* and *Bifidobacterium* on the IM has reported a gender-specific increase in the levels of these two bacteria in the feces of volunteers (Lisko et al., 2017). The administration of a probiotic fermented milk, containing *Streptococcus thermophilus*, *Lactobacillus bulgaricus, Lactobacillus acidophilus LA5* and *Bifidobacterium animalis* subsp. *lactis* BB12, during the third trimester of pregnancy has been related with a reduced risk of maternal insulin resistance (Asemi et al., 2013). Yogurt consumption has been associated with immune effects, including a reduced concentration of inflammatory markers in pregnant woman (Asemi et al., 2011). It has also been reported that yogurt modulates both humoral (Meyer et al., 2007) and cellular (Chaves et al., 2011) immunity. Unfortunately, very often observational nutritional studies do not inform us as to whether the positive effect of fermented dairy foods is mediated by the microorganisms present, by some specific components of the product, or by the potential role of some of these products, i.e., yogurt, as a marker of a good overall diet (Kok and Hutkins, 2018). Nevertheless, it is worth underlining that some studies draw attention to the impact yogurt could have, independent of diet (Panahi et al., 2018).

Based on this evidence, it seems reasonable to hypothesize that some of the described beneficial effects of fermented dairy product on several pathologies, such as those affecting the cardiovascular and metabolic systems, might be partly explained by the potential changes induced in the gut microbiota (Marco et al., 2017; Kok and Hutkins, 2018). Thus, in this study we aimed at evaluating the relationship between the consumption of fermented dairy products within the regular diet and the intestinal microbiota. In addition, selected blood markers related with the metabolic profile of the subjects were also analyzed.

MATERIALS AND METHODS

This cross-sectional study sample comprised of 130 subjects from the Principality of Asturias Region (Northern Spain). Inclusion criteria were: not being diagnosed with diseases related to intestinal function, not being currently treated with corticoids, nor having consumed pro- and prebiotic supplements or antibiotics during the previous month. Participants were mentally and physically able to participate in the study and gave written informed consent. Ethical approval was obtained from the Bioethics Committee of CSIC and from the Regional Ethics Committee for Clinical Research of the Principality of Asturias in compliance with the Declaration of Helsinki of 1964. All experiments were carried out in accordance with approved guidelines and regulations.

Blood Biochemical Analysis

Blood samples were kept on ice and centrifuged (1000 × *g*, 15 min) within 2–4 h after collection. Plasma and serum aliquots were kept at −20°C until analyses were performed. Plasma glucose, cholesterol, and triglycerides were determined by standard methods. Serum levels of C-reactive protein (CRP) were assessed using a CRP Human Instant ELISA kit (eBioscience, San Diego, CA, United States), and those of malondialdehyde (MDA) with a colorimetric assay of lipid peroxidation (Bioxytech LPO-586, Oxis International SA, Paris, France); the within-run coefficient of variation ranged from 1.2 to 3.4%, depending on the concentration of MDA (Gerard-Monnier et al., 1998). Serum leptin was measured by a sensitive ELISA test (Human Leptin ELISA Development Kit, PeproTech Inc., Rocky Hill, CT, United States); the detectable concentration range was 63–4000 pg/mL and the intra-assay and inter-assay coefficients of variation were 5.21 and 5.20%, respectively.

Microbial Analysis

Fecal samples were immediately frozen at −20°C and transported to the laboratory. For analyses fecal samples were melted, weighed, diluted 1/10 in sterile PBS, and homogenized at full-speed in a LabBlender 400 Stomacher (Seward Medical, London, United Kingdom) for 4 min. The samples were then centrifuged and the supernatant was taken for SCFA analyses whereas the fecal pellet was used for DNA extraction using the QIAamp DNA

stool mini kit (Qiagen, Hilden, Germany) as previously described (Arboleya et al., 2012).

Quantification of different bacterial populations was performed with a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA, United States) using SYBR Green PCR Master Mix (Applied Biosystems), and covered the major bacterial groups present in the gut microbial ecosystem. One microliter of template fecal DNA (∼5 ng) and 0.2 μM of each primer were added to the 25 μL reaction mixture. PCR cycling consisted of an initial cycle of 95°C 10 min, followed by 40 cycles of 95°C 15 s, and 1 min at the appropriate primer-pair temperature. The number of cells was determined by comparing the Ct values obtained from a standard curve. Fecal DNA extracts were analyzed and the mean quantity per gram of fecal wet weight was calculated as indicated elsewhere (Arboleya et al., 2012).

The analysis of SCFA was performed by gas chromatography in system composed of a 6890N GC injection module (Agilent Technologies Inc., Palo Alto, CA, United States) connected to a flame injection detector (FID) and a mass spectrometry (MS) 5973N detector (Agilent), as described previously (Arboleya et al., 2016).

Nutritional Assessment

Dietary intake was assessed in a personal interview by means of an annual semi-quantitative food frequency questionnaire (FFQ) method validated in previous studies (Cuervo et al., 2014). The FFQ was organized by food groups and open-ended, allowing foods consumed by the subject and not present in the questionnaire to be recorded. Among the dairy products group, 26 items were listed, including the three major fermented food groups: yogurt, cheese, and fermented milk. Food intake was analyzed for energy, macronutrients, and total dietary fiber content by using the nutrient Food Composition Tables developed by CESNID (Centro de Enseñanza Superior de Nutrición Humana y Dietética [CESNID], 2008). Additionally, the following fiber components were ascertained using (Marlett and Cheung, 1997) food composition tables: soluble fiber, insoluble fiber based on the enzymatic-chemical method developed by Theander and Westerlund (1986).

Height and weight were recorded after an overnight fast, using the standardized procedures described previously (Fernández-Navarro et al., 2017) for BMI [weight (Kg)/height (m^2)]. Body fat percentage was measured by bioelectrical impedance (BIA) with ± 1% variation (Tanita Corporation of America, Inc., Arlington Heights, IL, United States).

Statistical Analysis

Statistical analysis was performed using the IBM SPSS program version 22.0 (IBM SPSS, Inc., Chicago, IL, United States). Goodness of fit to the normal distribution was analyzed by means of the Kolmogorov-Smirnov test. Categorical variables were summarized with percentages while continuous variables were summarized using mean and standard deviations. The chi-squared test and independent samples *t*-test were used for group comparisons where appropriate. Pearson bivariate correlation was used to investigate linear association between the intake of total fermented dairy products and each subgroup, with the major food groups consumed in the diet and to describe the relationship between the consumption of fermented dairy foods with fecal microbial levels and serum health-related biomarkers. Heatmap was generated under R version 3.5.1 package heatmap.2. The conventional probability value for significance (0.05) was used in the interpretation of results.

RESULTS

The general characteristics of the study sample defined a group of 130 healthy adults with a mean age of 58.2 ± 17.1 years, and a moderate overweight (**Table 1**). 12.3% of the sample were smokers, and 55% lived sedentary lifestyles. To avoid potential confounding factors, like age or gender, these variables were included as covariables in any further analysis.

The total consumption of milk and dairy products (388.23 g/day) (**Table 1**) corresponded, in 33% of the sample, to the intake of fermented dairy foods, mainly yogurt and cheese (75 and 19%, respectively), as shown in **Figure 1A**. Among fermented dairy foods, natural yogurt (77.82 ± 102.38 g/day), sweetened yogurt (18.64 ± 51.40 g/day) and matured/semi-matured cheese (13.83 ± 22.29 g/day) were the most consumed (**Table 1**). Among them, natural yogurt was the main contributor (**Figure 1B**). The relationship between fermented dairy products and major food groups from the diet is shown in **Figure 2**. The consumption of fermented dairy foods presented a significant positive association with the intake of total dairy products, oils and fats, and dried fruits. In more detail, yogurt was negatively related to the intake of non-alcoholic beverages, and the consumption of cheese presented a direct relation with cereals, and fruits from the regular diet. Focusing on yogurt types, natural yogurt was directly related to the intake of dairy products and fruits, and negatively associated with sugars, sauces and non-alcoholic beverages; on the contrary, the intake of sweetened yogurt was positively related to these latter food groups (**Figure 2**). In the case of cheese, matured/semi-matured cheese consumption presented a positive relationship with the intake of cereals, while fresh cheese did it with fruits. Fermented milk has not been significantly associated with the intake of none of the other assessed food groups (**Figure 2**).

Regarding fecal microbial composition, natural yogurt consumers showed significantly higher fecal levels of *Akkermansia,* and sweetened yogurt consumers displayed significantly lower fecal levels of *Bacteroides* than non-consumers. Moreover, cheese consumers (considering all types jointly) presented significantly higher levels of the major fecal SCFA, acetate, propionate and butyrate, whereas the consumers of fresh cheese specifically presented higher levels of propionate and butyrate than non-consumers (**Table 2**).

Delving into the impact of fermented dairy foods on health status, the association between them and serum health biomarkers was analyzed. While the intake of yogurt, especially natural yogurt, showed a direct association with LDL/HDL ratio values, serum CRP was significantly lower in yogurt consumers (5.5 ± 10.5 vs. 2.1 ± 4.6 mg/L). Moreover, natural yogurt was associated with the oxidant status, the consumers of this

TABLE 1 | General description of the study sample.

	Total	Gender	
	***n* = 130**	**Male *n* = 38**	**Female *n* = 92**
Age (y)	58.18 ± 17.10	57.95 ± 17.20	58.28 ± 17.20
BMI (kg/m^2)	27.04 ± 4.40	27.73 ± 3.19	26.75 ± 4.80
Sedentary (%)	55.3	42.1	**61.0***
Current smoker (%)	12.3	15.8	10.9
Energy intake (Kcal)	1919.34 ± 552.4	2079.39 ± 652.48	**1853.23 ± 494.4***
Total lipids (g/day)[a]	80.04 ± 28.14	76.35 ± 30.47	81.56 ± 27.18
PUFA	14.03 ± 7.67	13.99 ± 8.20	14.05 ± 7.67
MUFA	32.73 ± 15.66	31.00 ± 19.18	33.45 ± 14.03
SFA	26.84 ± 10.12	25.16 ± 7.27	27.54 ± 11.11
Total protein (g/day)[a]	80.01 ± 26.71	84.43 ± 32.10	**90.90 ± 24.31***
Animal protein	59.52 ± 21.91	53. 36 ± 25.72	**62.06 ± 20.22***
Vegetal protein	27.18 ± 10.01	29.30 ± 12.38	**26.31 ± 8.35***
Total carbohydrates (g/day)[a]	200.22 ± 66.37	210.41 ± 76.70	**196.01 ± 58.79***
Total fiber (g/day)[a]	19.94 ± 7.56	19.89 ± 7.75	19.96 ± 7.45
Soluble fiber	2.57 ± 1.15	2.53 ± 1.27	2.58 ± 1.10
Insoluble fiber	12.85 ± 5.56	12.33 ± 5.81	13.06 ± 12.65
Total dairy products (g/day)[a]	388.23 ± 219.24	331.29 ± 208.87	411.75 ± 222.15
Milk and non-fermentable dairies (g/day)	255.37 ± 183.54	223.78 ± 176.60	268.42 ± 186. 20
Fermented dairy products (g/day)	129.46 ± 111.29	101.59 ± 110.70	140.98 ± 111.0
Yogurt (g/day)	96.46 ± 102.19	85.81 ± 103.35	100.86 ± 102.06
Natural yogurt	77.82 ± 102.38	69.74 ± 102.44	81.16 ± 102.78
Sweetened yogurt	18.64 ± 51.40	16.08 ± 43.02	19.70 ± 54.69
Cheese (g/day)	24.92 ± 35.56	13.92 ± 21.09	**29.47 ± 39.79***
Matured/semi-matured cheese	13.83 ± 22.29	11.83 ± 19.14	14.65 ± 26.57
Fresh cheese	11.16 ± 26.48	2.04 ± 12.23	**14.93 ± 30.03***
Fermented milk (ml/day)	8.08 ± 33.70	1.86 ± 16.22	10.65 ± 38.54

*Results are presented as estimated marginal mean ± SD and percentage (%). [a]Univariate analysis adjusted by total energy intake. PUFA, polyunsaturated fatty acids; MUFA, monounsaturated fatty acids, SFA, saturated fatty acids. Variables included in natural yogurt: whole natural yogurt, skimmed natural yogurt and lactose-free natural yogurt. Variables included in sweetened yogurt: whole flavored yogurt, whole sweetened yogurt, whole yogurt with fruits, skimmed flavored yogurt, skimmed sweetened yogurt, skimmed yogurt with fruits and Greek yogurt. Variables included in matured/semi-matured cheese: blue cheese, matured/semi-matured cow cheese, matured/semi-matured goat cheese and processed cheese. Variables included in fresh cheese: fresh goat cheese and fresh cow cheese. Variables included in fermented milks: natural milk with Bifidobacterium, milk with Bifidobacterium and fruit, natural milk with Lactobacillus and milk with Bifidobacterium and sterols*p value ≤ 0.05. Bold characters indicate statistically significant differences (p < 0.05).*

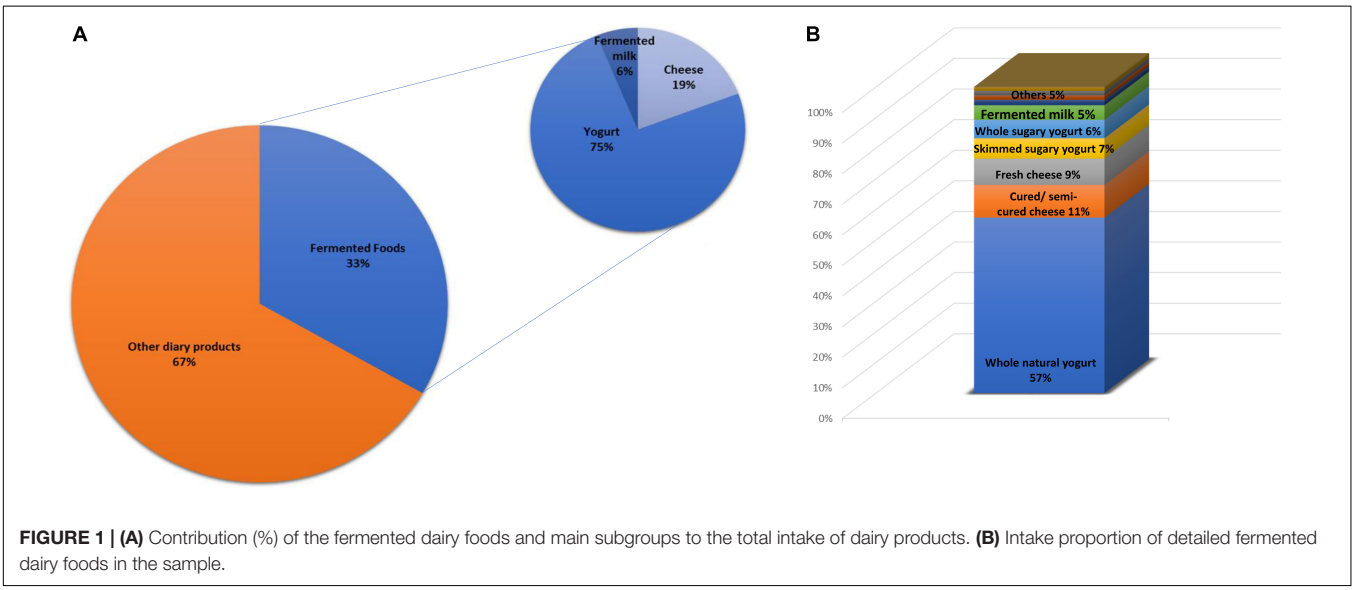

FIGURE 1 | (A) Contribution (%) of the fermented dairy foods and main subgroups to the total intake of dairy products. **(B)** Intake proportion of detailed fermented dairy foods in the sample.

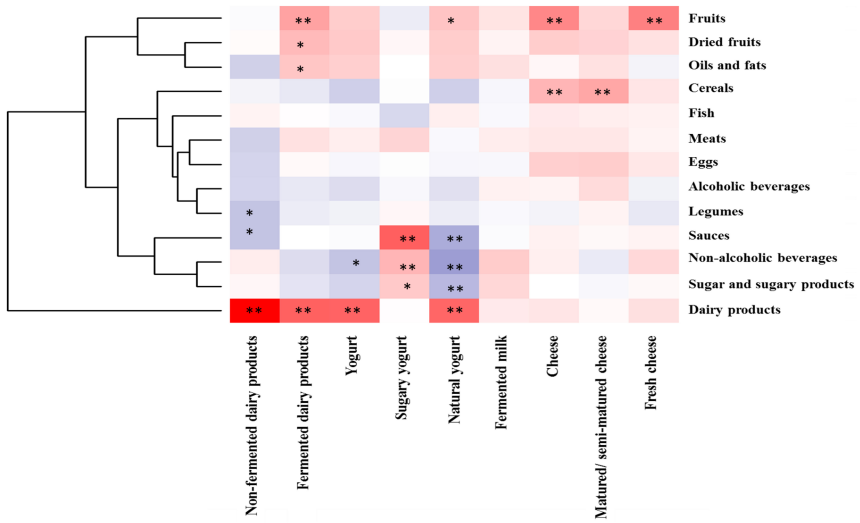

FIGURE 2 | Pearson correlation between the intake of major food groups (g/day) with fermented dairy foods (g/day) in the sample. Columns correspond to main fermented dairy products whereas rows correspond to food groups. Blue and red colors denote negative and positive association, respectively. The intensity of the color represents the degree of association between the fermented dairies consumed in the sample and major food groups in the diet. Asterisks indicate significant associations: *$p < 0.05$; **$p \leq 0.01$.

product showing also lower levels of serum MDA (2.80 ± 1.33 vs. 2.28 ± 0.59 μM) than non-consumers (**Table 3**). The intake of cheese and its different types or fermented milk did not show any association with any health-related biomarker (**Table 3**).

DISCUSSION

The present study is a pioneer report analyzing the relationship between the intake of fermented dairy foods within the regular diet, the gut microbial profile and health related biomarkers, considering the subject's global diet. Previous studies identified diets rich in fruits, vegetables or whole grains as critical modulators of the gut microorganisms, based on their content in fibers, phenolic compounds and prebiotics (Cuervo et al., 2014; Fernández-Navarro et al., 2018). However, the association between the different live microorganisms provided by the diet within the intestinal ecosystem offers a novel way to look into gut microbiota composition and its metabolic activity (Kok and Hutkins, 2018). In this regard, our results showed that, among the fermented dairy products assessed, yogurt was the product which showed higher ability to modulate the fecal microbiota. Interestingly, while the consumption of natural yogurt was directly associated with *Akkermansia* levels, the sweetened yogurt was inversely related with *Bacteroides* counts. The consumption of yogurt has been correlated with a good quality diet and some studies pointed out differences among yogurt types (Gómez-Gallego et al., 2018). A Danish cohort study suggested that consumption of whole-fat yogurt instead of low-fat products may be associated with a lower risk of type-2 diabetes (Ibsen et al., 2017). In the present sample, unfortunately, the low consumption of skimmed yogurt (consumed by only 6 out of the 80 volunteers consuming natural yogurt) precluded a skimmed vs. whole-fat comparison, however, it is worth mentioning that we have observed differences among the yogurt types assessed (natural vs. sweetened) with regards to the microbiota profile. These results underline the need for a full subcategorization of yogurt types in intervention and epidemiological studies, since different types may differ in their effects on health.

Given the descriptive nature of our study, we are not able to elucidate the mechanism of action explaining the observed associations. In spite of the lack of information about the modulation of intestinal *Akkermansia* in humans, recent research in mice treated with antibiotics has reported an increase in this bacterial group after the administration of a probiotic mix of *Lactobacillus* (Shi et al., 2018). Therefore, it may be plausible that the intake of such microorganisms, present in yogurt, might play a role in this association (Hill et al., 2014; Rezac et al., 2018). At this point, it should be mentioned that since labels of products do not provide information about the viable microorganisms present, we cannot know the exact amount and specific strains consumed by the study sample. According to the CODEX regulation (CODEX STAN 243-2003), yogurt must include a minimum bacterial counts of 10^7 cfu per gram from the symbiotic cultures of *Streptococcus thermophilus* and *Lactobacillus bulgaricus*. This, according to the intake data obtained, would correspond with intakes between 5×10^8 and 10^9 bacterial cells/day of each of these microorganisms. Nevertheless, although as shown in this study these levels can be easily reached within the context of a normal diet, it is also true that in interventional studies higher levels have been often used (Meyer et al., 2007; Asemi et al., 2011).

Results from intervention studies, both in animals and humans, have shown that the increase in *Akkermansia muciniphila* is associated with lower adiposity and a better

TABLE 2 | Differences in the concentration of major microbial groups and short chain fatty acids according to the intake of the different types of fermented dairy foods consumed by the sample.

	Yogurt (g/day)		Natural Yogurt (g/day)		Sweetened Yogurt (g/day)		Cheese (g/day)		Matured/semi-matured cheese (g/day)		Fresh cheese (g/day)		Fermented milk (mg/day)	
	Non-consumers (*n* = 27)	Consumers (*n* = 103)	Non-consumers (*n* = 50)	Consumers (*n* = 80)	Non-consumers (*n* = 106)	Consumers (*n* = 24)	Non-consumers (*n* = 27)	Consumers (*n* = 103)	Non-consumers (*n* = 47)	Consumers (*n* = 83)	Non-consumers (*n* = 86)	Consumers (*n* = 44)	Non-consumers (*n* = 122)	Consumers (*n* = 8)
Microbial target (log no. cells per gram of feces)														
Akkermansia	4.9 ± 2.4	5.6 ± 2.3	4.9 ± 2.3	**5.8 ± 2.2***	5.6 ± 2.3	5.0 ± 2.2	5.4 ± 2.5	5.5 ± 2.3	5.2 ± 2.3	5.6 ± 2.4	5.6 ± 2.4	5.2 ± 2.3	5.6 ± 2.4	4.6 ± 2.5
Bacteroides group	8.3 ± 2.0	8.3 ± 1.8	8.0 ± 2.0	8.5 ± 1.7	8.5 ± 1.7	**7.6 ± 1.9***	8.9 ± 1.0	8.3 ± 1.8	8.2 ± 2.0	8.4 ± 1.7	8.4 ± 1.6	8.3 ± 2.2	8.4 ± 1.9	8.1 ± 1.1
Bifidobacterium sp	7.4 ± 1.6	7.4 ± 1.7	7.3 ± 1.7	7.7 ± 1.7	7.6 ± 1.7	7.3 ± 1.8	7.6 ± 1.7	7.5 ± 1.7	7.6 ± 1.8	7.5 ± 1.7	7.5 ± 1.7	7.6 ± 1.8	7.6 ± 1.8	7.5 ± 0.5
Clostridium cluster XIVa	7.0 ± 2.4	7.0 ± 2.3	6.9 ± 2.7	7.0 ± 2.1	7.0 ± 2.2	6.9 ± 3.0	6.9 ± 2.3	7.0 ± 2.3	7.1 ± 2.2	6.9 ± 2.4	6.9 ± 2.3	7.2 ± 2.3	7.1 ± 2.3	6.0 ± 2.8
Lactobacillus group	5.5 ± 1.6	5.8 ± 1.9	5.7 ± 1.8	5.8 ± 1.8	5.7 ± 1.8	5.9 ± 2.0	6.2 ± 2.2	5.6 ± 1.7	6.0 ± 2.1	5.6 ± 1.7	5.9 ± 1.8	5.6 ± 1.9	5.8 ± 1.9	5.6 ± 1.1
Faecalibacterium prausnitzii	6.8 ± 1.5	6.8 ± 1.8	6.8 ± 1.6	6.8 ± 1.7	6.8 ± 1.7	6.9 ± 1.7	6.3 ± 2.4	7.0 ± 1.5	6.5 ± 2.1	7.0 ± 1.4	6.8 ± 1.8	6.8 ± 1.6	6.9 ± 1.8	6.7 ± 0.9
SCFA concentration (mM)														
Acetate	33.2 ± 15.9	36.5 ± 19.6	37.5 ± 18.2	34.7 ± 17.9	34.2 ± 17.5	**42.6 ± 17.1***	28.9 ± 14.5	**37.6 ± 18.5***	34.6 ± 20.7	36.5 ± 17.4	34.4 ± 17.4	38.4 ± 20.4	36.4 ± 18.8	27.4 ± 19.9
Propionate	12.5 ± 6.5	13.1 ± 7.6	13.1 ± 7.2	13.0 ± 7.3	12.8 ± 7.1	13.8 ± 7.6	9.8 ± 5.9	**13.9 ± 7.2***	12.6 ± 7.7	13.3 ± 7.1	12.1 ± 7.3	**14.7 ± 7.1***	13.2 ± 7.4	10.6 ± 7.6
Butyrate	11.7 ± 9.0	10.0 ± 7.1	11.2 ± 7.2	9.8 ± 7.6	10.4 ± 8.1	10.1 ± 4.4	7.7 ± 5.7	**11.1 ± 7.7***	9.8 ± 7.0	10.7 ± 7.8	9.4 ± 6.9	**12.1 ± 8.4***	10.5 ± 7.7	8.7 ± 5.6

*Results from univariate analyses, adjusted by age and gender, were presented as mean ± standard deviation. Bacteroides group, Bacteroides-Prevotella-Porphyromonas; SCFA, Short chain fatty acids. Variables included in natural yogurt: whole natural yogurt, skimmed natural yogurt and lactose-free natural yogurt. Variables included in sweetened yogurt: whole flavored yogurt, whole sweetened yogurt, whole yogurt with fruits, skimmed flavored yogurt, skimmed sweetened yogurt, skimmed yogurt with fruits and Greek yogurt. Variables included in matured/semi-matured cheese: blue cheese, matured/semi-matured cow cheese, matured/semi-matured goat cheese and processed cheese. Variables included in fresh cheese: fresh goat cheese and fresh cow cheese. Variables included in fermented milks: natural milk with Bifidobacterium, milk with Bifidobacterium and fruit, natural milk with Lactobacillus and milk with Bifidobacterium and sterols. *p value ≤ 0.05. Bold characters indicate statistically significant differences ($p < 0.05$).*

TABLE 3 | Differences in anthropometric parameters and mean concentrations of serum health related biomarkers according to the intake of the different types of fermented dairy foods consumed by the sample.

	Yogurt (g/day)		Natural Yogurt (g/day)		Sweetened Yogurt (g/day)		Cheese (g/day)		Matured/semi-matured cheese (g/day)		Fresh cheese (g/day)		Fermented milk (mg/day)	
	Non-consumers (*n* = 27)	Consumers (*n* = 103)	Non-consumers (*n* = 50)	Consumers (*n* = 80)	Non-consumers (*n* = 106)	Consumers (*n* = 24)	Non-consumers (*n* = 27)	Consumers (*n* = 103)	Non-consumers (*n* = 47)	Consumers (*n* = 83)	Non-consumers (*n* = 86)	Consumers (*n* = 44)	Non-consumers (*n* = 122)	Consumers (*n* = 8)
BMI (kg/m^2)	27.0 ± 4.5	27.0 ± 4.4	27.5 ± 4.9	26.7 ± 4.1	26.8 ± 4.2	27.9 ± 5.2	26.7 ± 5.0	27.1 ± 27.1	27.2 ± 4.7	26.93 ± 4.2	27.0 ± 4.1	27.1 ± 4.9	27.1 ± 4.4	25.9 ± 3.7
Body fat (%) n63	37.3 ± 10.6	35.1 ± 12.0	38.1 ± 12.4	33.9 ± 11.3	34.8 ± 11.1	38.7 ± 13.9	34.7 ± 12.8	35.6 ± 11.6	35.7 ± 13.4	35.44 ± 11.0	35.1 ± 11.1	36.1 ± 12.1	–	–
Blood parameters														
Glucose (mg/dL)	96.0 ± 9.6	100.3 ± 20.3	96.7 ± 9.9	101.0 ± 22.1	99.5 ± 19.9	98.7 ± 10.5	97.2 ± 19.7	100.0 ± 18.6	97.0 ± 16.2	101.0 ± 20.2	100.5 ± 21.4	97.1 ± 11.1	99.6 ± 19.1	96.2 ± 11.5
Triglycerides (mg/dL)	115.4 ± 70.1	116.6 ± 60.0	117.0 ± 79.0	116.1 ± 50.2	115.9 ± 55.3	119.2 ± 90.9	126.9 ± 60.7	113.5 ± 62.2	117.7 ± 65.1	115.6 ± 60.3	118.4 ± 63.8	112.34 ± 57.7	117.5 ± 62.4	94.7 ± 47.8
Total cholesterol (mg/dL)	212.0 ± 40.8	212.4 ± 40.3	207.6 ± 37.6	2015.1 ± 41.6	214.2 ± 41.3	202.6 ± 33.6	201.4 ± 47.6	215.3 ± 38.0	206.1 ± 42.8	216.2 ± 38.5	209.7 ± 41.6	217.7 ± 37.3	211.6 ± 40.1	226.4 ± 43.8
LDL/HDL ratio	2.1 ± 0.8	**2.6 ± 0.9***	2.2 ± 0.8	**2.7 ± 0.9***	2.5 ± 0.9	2.4 ± 0.9	2.4 ± 0.9	2.5 ± 0.9	2.4 ± 0.9	2.6 ± 0.9	2.6 ± 0.9	2.4 ± 0.8	2.5 ± 0.9	2.0 ± 1.0
Leptin (ng/mL)	11.1 ± 7.9	9.9 ± 6.5	11.2 ± 6.9	9.6 ± 6.5	9.9 ± 6.8	10.9 ± 6.0	9.4 ± 7.4	10.3 ± 6.5	9.7 ± 7.1	10.2 ± 6.4	9.9 ± 6.8	10.6 ± 6.3	–	–
CRP (mg/L)	5.5 ± 10.5	**2.1 ± 4.6***	4.2 ± 9.1	2.0 ± 3.9	2.8 ± 5.9	1.6 ± 7.3	3.4 ± 4.2	2.4 ± 6.5	3.9 ± 7.0	1.9 ± 5.4	2.5 ± 5.6	3.1 ± 7.1	–	–
MDA (μM)	2.6 ± 1.7	2.4 ± 0.6	2.8 ± 1.3	**2.3 ± 0.6***	2.4 ± 0.87	2.9 ± 0.8	2.7 ± 0.5	2.3 ± 1.0	2.6 ± 1.1	2.3 ± 0.69	2.5 ± 0.6	2.3 ± 1.3	–	–

*Results from univariate analyses, adjusted by age and gender, were presented as mean ± standard deviation. CRP, C-reactive protein; MDA, malondialdehyde. Variables included in natural yogurt: whole natural yogurt, skimmed natural yogurt and lactose-free natural yogurt. Variables included in sweetened yogurt: whole flavored yogurt, whole sweetened yogurt, whole yogurt with fruits, skimmed flavored yogurt, skimmed sweetened yogurt, skimmed yogurt with fruits and Greek yogurt. Variables included in matured/semi-matured cheese: blue cheese, matured/semi-matured cow cheese, matured/semi-matured goat cheese and processed cheese. Variables included in fresh cheese: fresh goat cheese and fresh cow cheese. Variables included in fermented milks: natural milk with Bifidobacterium, milk with Bifidobacterium and fruit, natural milk with Lactobacillus and milk with Bifidobacterium and sterols. (–) Data not available. *p value ≤ 0.05. Bold characters indicate statistically significant differences ($p < 0.05$).*

metabolic status, suggesting this microorganism could be a potential candidate for obesity control (Everard et al., 2013; Dao et al., 2016; Rodríguez-Carrio et al., 2017). In the current study, we found that natural yogurt consumers presented not only higher intestinal *Akkermansia* levels with respect to non-consumers, but also a "healthier metabolic profile" based on lower inflammation and serum lipid peroxidation, measured through serum CRP and MDA. These immune variables have been reported to be moderately reduced in intervention studies with probiotic yogurt by other authors (Mohamadshahi et al., 2014; Burton et al., 2017). These findings are coherent with recent data from the Kuopio Ischaemic Heart Disease Risk Factor Study showing a cardiovascular protective effect in men consuming fermented dairy products (Koskinen et al., 2018), and with several epidemiological studies supporting a protective role of fermented dairy products against the chronic "low-grade" inflammation associated with the metabolic syndrome and related diseases (Baothman et al., 2016; Kim et al., 2017; Salas-Salvadó et al., 2017). Despite the values of LDL/HDL ratio in our sample were higher for yogurt consumers than for non-consumers, these are far from the established levels of atherogenic risk (>4.5). It is also important to underline that, in contrast to some of the previous studies (Asemi et al., 2013), age and gender have been introduced as covariates in the analyses performed in our study, and global diet has been determined.

Fermented dairy foods may present nutritional properties independent of the presence of microorganisms, as seems to occur with the sweetened yogurts. Although the lower levels of *Bacteroides* observed in the consumers of sweetened yogurt in our sample could be *a priori* surprisingly; this result is in consonance with previous reports indicating a reduction in the intestinal level of *Bacteroides* associated with the consumption of certain sweeteners such as sucralose (Uebanso et al., 2017). Therefore, it could be interesting to examine if the addition of additives (flavors, sweeteners, etc.) to traditionally considered healthy products, such as yogurt, could influence on the gut microbiota and, therefore, on the health status of the host.

No statistical differences were found in the levels of intestinal microbial groups as related to cheese consumption. However, cheese consumers showed higher fecal concentrations of the major SCFA. These compounds have been widely related with different metabolic effects, directly modulating host health through a range of tissue-specific mechanisms (den Besten et al., 2013; Rios-Covian et al., 2016; Uebanso et al., 2017). From a nutritional point of view, differences in the relationship with health may be expected depending on the types of cheese considered. Notwithstanding, we have not observed differences in our sample in health-related parameters according to cheese intake.

It is also important to be aware that this study contains some limitations. As mentioned before, although the FFQ has been carried out with a high grade of detail, it has not been possible to collect information on the specific microbial strains contained in the products. On the other hand, even though the multivariate models were adjusted by age and gender, we cannot rule out possible residual confounders often present in this sort of study. In spite of this, the present work has the strength of being conducted within the context of the habitual and global dietary pattern of the volunteers, and points out natural yogurt as a healthy product that, as previously suggested (Gómez-Gallego et al., 2018), should have a more visible role in dietary recommendations and guidelines. Our data suggests that fermented dairy products in general, and yogurt in particular, could be a key element affecting the relationship between diet and health by means of the modulation of gut microbial composition and functionality.

DATA AVAILABILITY

The datasets generated for this study are available on request to the corresponding author.

ETHICS STATEMENT

This study was carried out in accordance with the recommendations of the Bioethics Committee from CSIC and the Regional Bioethics Committee from the Principality of Asturias (Spain) with written informed consent from all subjects. All subjects gave written informed consent in accordance with the Declaration of Helsinki. The protocol was approved by the Bioethics Committee from CSIC and the Regional Bioethics Committee from the Principality of Asturias (Spain).

AUTHOR CONTRIBUTIONS

MG and SG had the primary responsibility in the study design and protocol development, and confirm that they had full access to the data in the study and final responsibility for the decision to submit for publication and drafted the manuscript. SG and TF-N were involved in data collection and contributed to the dietary and nutritional data analysis and interpretation. CR-G, SA, and NS conducted the microbial analysis and data processing and supervised the execution of the study and data analysis. All authors critically reviewed the manuscript and approved the final version submitted for publication.

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Phenotypic and Genotypic Identification of Bacteria Isolated From Traditionally Prepared Dry Starters of the Eastern Himalayas

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Preparation of dry starters for alcohol production is an age-old traditional technology in the Eastern Himalayan regions of east Nepal, the Darjeeling hills, Sikkim, and Arunachal Pradesh in India, and Bhutan. We studied the bacterial diversity in 35 samples of traditionally prepared dry starters, represented by *marcha* of Nepal, Sikkim, the Darjeeling hills, and Bhutan, *phab* of Bhutan, and *paa, pee*, and *phut* of Arunachal Pradesh, respectively. Populations of bacteria in these starters were 10^5 to 10^8 cfu/g. A total of 201 bacterial strains were isolated from starter samples, phenotypically characterized, and their identities confirmed by the 16S rRNA sanger sequencing method. The dominant phylum was *Firmicutes* (85%), followed by *Proteobacteria* (9%), and *Actinobacteria* (6%). Lactic acid bacteria (LAB) (59%) formed the most abundant group, followed by non-LAB (32%) and Gram-negative bacteria (9%). Based on the 16S rRNA gene sequencing result, we identified LAB: *Enterococcus durans, E. faecium, E. fecalis, E. hirae, E. lactis, Pediococcus acidilactici, P. pentosaceus, Lactobacillus plantarum* subsp. *plantarum, Lb. pentosus, Leuconostoc mesenteroides,* and *Weissella cibaria*; non-LAB: *Bacillus subtilis* subsp. *inaquosorum, B. circulans, B. albus, B. cereus, B. nakamurai, B. nitratireducens, B. pseudomycoides, B. zhangzhouensis, Kocuria rosea, Staphylococcus hominis* subsp. *hominis, S. warneri, S. gallinarum, S. sciuri, Lysinibacillus boronitolerans, Brevibacterium frigoritolerans,* and *Micrococcus yunnanensis*; Gram-negative bacteria: *Pseudomonas putida, Klebsiella pneumoniae, Enterobacter hormaechei* subsp. *xiangfangensis, E. hormaechei* subsp. *steigerwaltii*, and *Stenotrophomonas maltophilia*. We characterized diversity indexes of the bacterial community present in traditionally prepared dry starters. This is the first report on the bacterial diversity of traditionally dry starters of the Eastern Himalayas by sanger sequencing.

Keywords: Eastern Himalayas, starters, 16S rRNA sequencing, bacterial diversity, lactic acid bacteria

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INTRODUCTION

The Himalayas, well known for high mountains with natural beauty and rich biological resources, extend from peak Nanga Parbat in Pakistan to peak Namcha Barwa across India, Nepal, and Bhutan (Le Fort, 1975). Based on geo-morphology and demography, the Himalayas are divided into three regions, the Western, Central, and Eastern Himalayas (Nandy et al., 2006). The geographical

location of the Eastern Himalayas extends from eastern Nepal, North East India (Darjeeling hills, Sikkim, and Arunachal Pradesh), Bhutan, and Tibet Autonomous Regions in China (Saha, 2013). Agrarian and pastoral types of mountain farming dominate the agriculture and animal husbandry systems in the Eastern Himalayas, and these are practiced by diverse ethnic communities (Sharma et al., 2007; Bhasin, 2013). Many major and rare types of ethnic fermented foods and beverages are traditionally produced from locally available plant and animal resources and are made into a wide variety of flavorsome cuisine that is consumed as staple diets, side-dishes, curries, soups, condiments, and alcoholic drinks by ethnic people of the Eastern Himalayas (Tamang, 2010; Tamang et al., 2012). The majority of ethnic Himalayan people drink home-made traditional alcoholic beverages and distilled liquor prepared from cereals (rice, finger millets, and maize) as per socio-compulsion but also for enjoyment. Vinification, malting, and brewing processes for alcohol production are completely unknown in the food culture of the Himalayan people; instead, rice or finger millets are fermented into mildly alcoholic (∼4%) beverages (Thapa and Tamang, 2004) by using dry starters, which are unique to these regions.

The Himalayan people have been practicing the art of starter-making using indigenous technology for centuries by using overnight-soaked and pounded rice flours mixed with wild herbs, spices, and 1–2% of previously prepared dry starters in powder form to make doughs. Doughs mixtures with desirable shapes and sizes are placed in fresh fern leaves and allowed to ferment for 2–3 days at room temperature, and the freshly fermented doughs are then sun dried for 2–3 days to get dry starters (Thakur et al., 2015; Anupma et al., 2018). Every ethnic community in the Western, Central, and Eastern Himalayas prepare amylase and alcohol-producing starters with slight variation in the use of substrates, such as rice or wheat, and wrapping materials, such as fern fronds, paddy straw, or plant leaves. In local languages, these are termed *marcha* in Nepal, the Darjeeling hills, and Sikkim in India (Shrivastava et al., 2012; Thakur et al., 2015; Anupma et al., 2018), *mana* and *manapu* in Nepal (Nikkuni et al., 1996), *phab* in Bhutan (Tamang, 2010), *chowan* in Tripura, *dawdim* in Mizoram, *humao, modor pitha* in Assam, *hamei* in Manipur, *khekhrii* in Nagaland, and *phut* in Arunachal Pradesh (Anupma et al., 2018) in India. Similar types of alcohol-producing starters are also prepared in South East Asia by ethnic Asian communities, such as the Vietnamese *benh* (Dung et al., 2007), Korean *nuruk* (Jung et al., 2012), Indonesian *ragi* (Surono, 2016), Philippine *bubod* (Kozaki and Uchimura, 1990), Chinese *daque* or *chiu* or *chu* (Chen et al., 2014), Thai *loogpang* (Limtong et al., 2002), and Cambodian *dombea* (Ly et al., 2018). The most remarkable advent in the traditional preparation of starter cultures is the practice of the "back-slopping method" (terminology in modern food microbiology) used by ethnic Asians irrespective of their geographical locations for sub-culturing the desirable and essential microbiota.

Traditionally prepared dry starters show coexistence of mixed microbiota represented by different genera and species of filamentous molds (Hesseltine et al., 1988; Tamang et al., 1988; Sha et al., 2019), yeasts (Hesseltine and Kurtzman, 1990; Jeyaram et al., 2008, 2011; Sha et al., 2017, 2018, 2019), and bacteria (Hesseltine and Ray, 1988; Tamang et al., 2007; Sha et al., 2017) for saccharification (Lee and Lee, 2002; Thapa and Tamang, 2004), liquefaction (Pervez et al., 2014), and ethanol production (Tsuyoshi et al., 2005; Zheng et al., 2011) to produce traditional alcoholic beverages and distilled liquor in many South East Asian countries, including Nepal, India, and Bhutan in the Himalayas. Filamentous molds (species of *Rhizopus, Mucor, Aspergillus*), and yeasts (species of *Saccharomyces, Pichia, Sacharomycopsis, Candida*) are involved in saccharification and liquefaction; they produce amylolytic enzymes for degrading starch into sugars, and the main alcohol-producing yeasts are *Saccharomyces* for alcohol production (Nout and Aidoo, 2002; Thapa and Tamang, 2004; Li et al., 2012; Nile, 2015). Besides the saccharifying and alcohol-producing ability of mycelia molds and yeasts, some bacterial species present in starters also contribute by imparting flavor, antagonism, and acidification onto the fermenting substrates (Tamang et al., 2007; Huang et al., 2017). Extensive profiling of the diversity of yeasts and mycelial molds in various traditionally prepared dry starters collected from different places of North East India have been reported earlier (Tamang et al., 1988; Tamang and Sarkar, 1995; Tsuyoshi et al., 2005; Jeyaram et al., 2008, 2011; Bora et al., 2016; Sha et al., 2017, 2018, 2019). Samples of *marcha* collected from the Darjeeling hills and Sikkim were analyzed earlier and reported few species of bacteria: *Pediococcus pentosaceus* (Tamang and Sarkar, 1995), *Pediococcus pentosaceus* and *Lb. brevis* (Tamang et al., 2007), *Acetobacter*, *Fructobacillus, Lactococcus*, *Lactobacillus, Leuconostoc*, *Burkholderia*, and *Gluconacetobacter* (Sha et al., 2017). However, no published reports on bacterial diversity associated with *marcha* in Nepal and Bhutan, *phab* in Bhutan, and *paa, pee*, and *phut* in Arunachal Pradesh are available to date. *Marcha* (**Figures 1A–D**) is a dry rice-based starter, prepared by the Gorkha/Nepali community in the Darjeeling hills and Sikkim in India, east Nepal, and south Bhutan, to ferment boiled finger-millets into a sweet-sour, mildly alcoholic beverage called *kodo ko jaanr* or *chyang* (Tamang et al., 1996). *Marcha* is prepared

FIGURE 1 | Different types of dry starters from the Eastern Himalayas: **(A)** *Marcha* from Nepal, **(B)** *Marcha* from Darjeeling, **(C)** *Marcha* from Sikkim, **(D)** *Marcha* from Bhutan, **(E)** *Phab* from Bhutan, **(F)** *Paa* from Arunachal Pradesh, **(G)** *Pee* from Arunachal Pradesh, and **(H)** *Phut* from Arunachal Pradesh.

from soaked and pounded rice flours mixed with some wild herbs, few spices, 1–2% of previously prepared powdered *marcha* by the back-slopping method to make doughs that are placed in fresh ferns leaves, are allowed to ferment for 2–3 days, and are then sun dried for 2–3 days to get dry starters. *Phab* or *pho* (**Figure 1E**) is a dark brown, flattened, cake-like starter prepared from powdered maize by the Drukpa community in Bhutan to produce a home-made distilled alcoholic drink called *ara* from barley and finger millets (Anupma et al., 2018). *Paa* (**Figure 1F**), *pee* (**Figure 1G**), and *phut* (**Figure 1H**) are dry starters prepared from rice by the Nyshing, Apatani, and Mongpa communities of Arunachal Pradesh, respectively (Anupma et al., 2018). *Pee* is used to ferment rice into a mildly alcoholic beverage called *opo* by the Nyshing tribes, a mildly alcoholic drink called *apong* by the Apatani, and *phut* is used to prepare a sweet-sour, mildly alcoholic beverage called *themsing* by the Mongpa tribes of Arunachal Pradesh (Shrivastava et al., 2012). Preparation of *marcha, phab, paa, pee*, and *phut* is more or less similar except for some variation in the use of substrates, such as rice in the case of *marcha, phut, paa*, and *pee* and maize-rice husk in *phab*, and wrapping materials, of which fern leaves are used for fermenting rice flour during *marcha* preparation, dry paddy straws are used for *phab* preparation, and locally available plant leaves are used for the preparation of *paa, pee*, and *phut*. We collected dry samples of *marcha, pee, paa, phut*, and *phab* from different places in the Eastern Himalayan regions of Nepal, India, and Bhutan to profile the bacterial diversity as information on yeasts and the mycelial molds community is already available (Sha et al., 2017, 2018, 2019). The present study aimed to profile bacterial diversity isolated from *marcha, pee, paa, phut*, and *phab* based on phenotypic and biochemical tests that use the 16S rRNA gene sequencing method.

MATERIALS AND METHODS

Samples

A total of 35 samples of traditionally prepared dry starters were collected in pre-sterile poly bags from different places located in the Eastern Himalayas viz *marcha* (8 samples) from Nepal, *marcha* (5) from the Darjeeling hills, *marcha* (8) from Sikkim, *marcha* (5) from Bhutan, *paa* (2), *pee* (3), and *phut* (2) from Arunachal Pradesh, and *phab* (2) from Bhutan (**Table 1**). Collected samples were transported and kept in a desiccator at room temperature since traditionally sun-dried starters are stored in a dry place for more than a year (Tamang et al., 1996).

Analysis of Moisture and pH

The moisture content of the samples was estimated by a moisture analyzer (OHAUS/MB-45, United States). The pH of the samples was determined by homogenizing 1 g of sample in 10 mL of distilled water, and the readings were taken using a digital pH-meter (Orion 910003, Thermo Fisher Scientific, United States).

Microbiological Analysis

Dry starter samples were taken from a desiccator, coarsely crushed by a sterile spatula, and 10 g of the powered sample was then homogenized with 90 mL of 0.85% physiological saline in a stomacher lab blender 40 (Seward, United Kingdom) for 2 min. The homogenized samples were serially diluted in the same diluents, and 1 mL of appropriate diluents was then plated using specific media by the pour plate method. Nutrient agar (MM102, HiMedia, Mumbai, India) for aerobic mesophilic bacterial count, MRS (Man-Rogosa-Sharpe) agar (M641, HiMedia, Mumbai, India) and M17 Agar Base (M929, HiMedia, Mumbai, India)

TABLE 1 | Bacterial load of dry starters from the Eastern Himalayas.

Sample	Region	Sample Collection Site	Altitude (Meter)	Latitude	Longitude	Moisture content (%)	pH	cfu/g ($\times 10^7$)
Marcha	Nepal	Dharan	371	26°48′ N	87°17′ E	12.5	5.6	2.1
		Dhankuta	1154	26°53′ N	87°8 ′ E	(9.6–17.0)	(5.5–5.9)	(1.1–2.9)
		Hiley	857	27°02′ N	87°24′ E			
		Hathikharka	1394	27°01′ N	87°32′ E			
Marcha	Darjeeling hills	Darjeeling	2059	27°04′ N	88°26′ E	13.1	5.4	15.3
		Kalimpong	1176	27°07′ N	88°47′ E	(12.9–13.3)	(5.2–5.6)	(11.0–19.6)
Marcha	Sikkim	Pakyong	1341	27°24′ N	88°59′ E	11.8	5.7	18.5
		Gangtok	1637	27°32′ N	88°61′E	(10.0–13.4)	(5.6–5.9)	(10.2–26.5)
		Recab	1072	27°21′ N	88°50′E			
		Basilakha	906	27°22′ N	88°60′ E			
Marcha	Bhutan	Gedumari	1045	26°90′ N	89°39′E	13.76	5.7	0.01
		Thimphu	2401	27°47′ N	89°62′ E	(11.8–15.72)	(5.5–5.9)	(0.01–0.02)
Paa	Arunachal Pradesh	Lower Subansiri	661	27°8′ N	93°6′E	11.7	5.1	2.3
						(11–12)	(5–5.2)	(2.0–2.6)
Pee		Ziro valley	1576	27°53′ N	93°81′E	12.1	5.5	17.6
						(11–13)	(5.2–5.8)	(16.8–18.4)
Phut		Upper Subansiri	1816	28°3′ N	94°E	11.6	5.2	11.5
						(11.4–11.8)	(5.1–5.3)	(9.8–13.2)
Phab	Bhutan	Dhonakha	2311	27°66′ N	89°70′ E	6.17	5.2	0.03
						(6.13–6.2)	(5.0–5.4)	(0.02–0.04)

for lactic acid bacteria (LAB), and VRBGA (violet red bile glucose agar) (M581, HiMedia, Mumbai, India) for Gram-negative bacteria were used for the enumeration of bacteria in respective plates. Nutrient agar plates and VRBGA plates were incubated at 37°C for 24 h, and MRS plates and M17 plates were incubated at 30°C for 24–48 h aerobically. The number of colonies was counted as colony forming unit cfu/g. The purity of colonies was maintained by re-streaking them into fresh medium, and this was further confirmed by microscopic examination. The pure colonies were then preserved in 50% glycerol at −20°C for further identification and analysis.

Phenotypic and Biochemical Characterization

Bacterial isolates were phenotypically characterized for their presumptive identification, and groupings were done on the basis of cell morphology, Gram's reaction, colony morphology, catalase test, sporulation tests, gas production from glucose, and ammonia production from arginine (Holt et al., 1994). The physiological tests including growth at different pHs, temperatures, and salt tolerance were performed (Tamang et al., 2007). Biochemical characterization of isolates such as sugar fermentation tests, IMViC (Indole, Methyl red; Voges-Proskauer and Citrate) tests specifically for Gram-negative isolates, nitrate reduction tests, and urease tests were also performed using the method of Hammes and Hertel (2003).

Genotypic Characterization

Genomic DNA Extraction

The genomic DNA of each bacterial isolate was extracted by the standard phenol/chloroform method of Cheng and Jiang (2006) with slight modifications. A total of 1 ml of culture grown overnight in MRS broth (M369, HiMedia, Mumbai, India) at 30°C was centrifuged at 8,000 rpm for 10 min. The pellets were centrifuged at 3,000 rpm, suspended in 40 μl 1× TE buffer, and freshly prepared 15 μl lysozyme and 15 μl RNAse enzyme were added to the pellets and incubated at 37°C for 3 h. After incubation, 15 μl of 20% SDS (sodium dodecyl sulfate) and 15 μl of proteinase-K were added and further incubated at 55°C for 3 h. An equal volume of phenol-chloroform solution (49:48) was added to the above mixture, centrifuged at 10,000 rpm for 15 min, and the aqueous upper layer formed was transferred to a fresh vial containing chloroform-isoamyl solution (48:1). It was centrifuged again at 10,000 rpm for 15 min, and the upper aqueous layer formed was transferred to a fresh vial containing 15 μl of 3M sodium acetate and 400 μl of cold absolute alcohol and kept at −20°C for 1 h. The mixture was again centrifuged at 10,000 rpm for 30 min, and the pellets were washed with 70% ethanol and further centrifuged at 10,000 rpm for 30 min. The pellets were then collected, air dried, and suspended in 30 μl 1× TE buffer and stored at −20°C for further analysis. The quality of the genomic DNA was checked by electrophoresis in 0.8% agarose gel and quantified using a NanoDrop spectrometer (ND-1000 spectrometer, NanoDrop technologies, Willington, CT, United States) (Kumbhare et al., 2015).

PCR Amplification

The PCR of the 16S rRNA gene from the isolated genomic DNA was amplified using a universal oligonucleotide primer pair 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′) (Lane, 1991) in a Thermal cycler (Applied Biosystems-2720, United States). The reaction mixture, conditions, and protocol for the polymerase chain reaction amplification were performed following the method of Chagnaud et al. (2001). PCR amplification was performed in a mixture containing a final volume of 50 μl of Go green Taq master mix (1×) (NEB), 10 μM of F primer, 10 μM of R primer, and nuclease-free water (NEB). The PCR reaction program was set under the following PCR conditions: 94°C for 10 min; 94°C for 1 min, 65°C for 1 min, 72°C for 30 s for 35 cycles, and 72°C for 7 min. PCR products were detected by electrophoresis using 1% agarose, and the bands were stained with 7 μl/100 mL of ethidium bromide (RM813, HiMedia, Mumbai, India) and visualized in UV source Gel-Doc 1000 (Bio-Rad, 97-0186-02, United States). A standard 100 base pair DNA ladder (HiMedia, Mumbai, India) was used for the verification of amplicon size.

Purification of the PCR Amplicons

The amplified PCR products were then purified using PEG (polyethylene glycol)-NaCl (sodium chloride) precipitation (20% w/v of PEG, 2.5 M NaCl) precipitation method with little modifications of method described by Schmitz and Riesner (2006). About 0.6 volume of 20% PEG-NaCl was added to final volume of PCR products and incubated at 37°C for 30 min. After centrifugation at 12,000 rpm for 30 min, the aqueous solution was discarded, the pellet was washed twice with freshly prepared ethanol (70%) by centrifugation at 12,000 rpm for 30 min. The collected pellet was then air-dried overnight and 20 μl of nuclease-free water was added, and the final purified product was loaded in 1% agarose gel.

16S rRNA Gene Sequencing

PCR products were set up in 5 μl volume for single primer amplification with the same universal primers 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′) (Lane, 1991) for separate reactions for each primer. PCR reaction was set as follows: denaturation for (96°C, 10 s), annealing (50°C, 5 s), and elongation (60°C, 2 min) with a stop reaction at 4°C. The amplicons were then precipitated with 1 μl sodium acetate (3M, pH 5.2) and 24 μl of absolute alcohol, mixed briefly in vortex and incubated at room temperature for 15 min, spun at 12,000 rpm for 20 min, further washed with 70% ethanol, air-dried, and suspended in 10 μl formamide. Sequencing of the amplicons was performed by the Sanger Sequencing method or the Chain-termination DNA (Sanger et al., 1977), the automation of a modified Sanger method that is commonly used to check the sequence of the templates (Heather and Chain, 2016), was carried out in an automated DNA Analyzer (ABI 3730XL Capillary Sequencers, Applied Biosystems, Foster City, CA, United States).

Bioinformatics

The sequence quality was checked by Sequence Scanner v.1.0 (Applied Biosystems, Foster City, CA, United States). After checking the sequence quality, the sequences were assembled using a ChromasPro 1.5 (McCarty, 1998). The orientation of the assembled sequences was checked using an orientation checker v.1.0. The identity of bacterial isolates was assigned by comparing their DNA sequences with those available in the GenBank NCBI (National Center for Biotechnology Information) database using a BLAST (basic local alignment search tool) 2.0 program (Altschul et al., 1990). The sequences were then aligned by pairwise alignment using clustalW, and the phylogenetic tree was constructed using MEGA7.0 software by the neighbor joining method (Gascuel and Steel, 2006; Kumar et al., 2016). Diversity indices were calculated using a PAST (PAleontological STatistics) v.3.25, which is a comprehensive statistics package used in many fields of life sciences, economics, earth science, engineering, and paleontology (Hammer et al., 2001). The Chao 1 value for species richness was calculated following the method of Chao and Chiu (2016).

Data Availability

The sequences retrieved from the 16S rRNA sequencing were deposited at GenBank-NCBI under the nucleotide accession number: MK748250-MK748278, MK202997-MK203032, and MK752675-MK752677.

RESULTS

Microbial Population

Populations of bacteria in 35 samples of traditionally prepared dry starters collected from different regions of the Eastern Himalayas were 1.0×10^5 to 2.7×10^8 cfu/g (**Table 1**). The moisture contents of all samples analyzed were 10%–17% except for *phab* of Bhutan in which the moisture content was comparatively low (<6%). Average pH of all samples was 5.5 (**Table 1**).

Phenotypic Characterization

We isolated 201 total bacterial isolates from 35 different samples of traditionally prepared starters collected from the Eastern Himalayas, which were represented by 139 isolates from *marcha* (Sikkim 49; Darjeeling 38; Nepal 34, Bhutan 18), 12 isolates from *paa* (Arunachal Pradesh), 17 isolates from *pee* (Arunachal Pradesh), 11 isolates from *phut* (Arunachal Pradesh), and 22 isolates from *phab* (Bhutan). All 201 bacterial isolates were phenotypically characterized based on various biochemical and physiological parameters (**Table 2**). A total of nine different bacterial genera including unidentified group were presumptively identified based on phenotypic results following Bergey's manual of bacteriological classification (Holt et al., 1994), which were mostly represented by Gram-positive bacteria (*Pediococcus, Lactobacillus, Enterococcus, Leuconostoc, Bacillus*, and *Staphylococcus*) and two Gram-negative bacteria (*Enterobacter* and *Citrobacter*). We randomly grouped 201 isolates into 68 representative bacterial strains based on phenotypic characterization results (data not shown).

Molecular Identification of Bacterial Isolates

The genomic DNA of each isolate of all 68 representative bacteria strains was extracted and PCR products were prepared for identification by 16S rRNA gene sequence using the Sanger method. DNA sequences of bacterial isolates were assigned by comparing them with those available in the GenBank NCBI database using a BLAST 2.0 program (Altschul et al., 1990) for identification. The phylogenetic trees of the nucleotide sequences of 68 bacteria isolates from samples of *marcha, paa, pee, phut*, and *phab* were constructed using the Neighbor-joining method with 1,000 bootstrap value replicates (**Figure 2**). The 16S rRNA sequencing results showed three bacterial phyla represented by *Firmicutes* (85%), *Proteobacteria* (9%), and *Actinobacteria* (6%). The phylum distribution of the *marcha* samples from Nepal showed *Firmicutes* (80%) followed by *Actinobacteria* (20%); Darjeeling showed *Firmicutes* (100%); Sikkim showed *Firmicutes* (92%), and *Actinobacteria* (8%); Bhutan showed *Firmicutes* (100%). In starters from Arunachal Pradesh the variable distribution pattern in phyla level was observed. Samples of *paa* showed *Firmicutes* (80%), and *Proteobacteria* (20%), *pee* showed *Firmicutes* (67%), *Proteobacteria* (16%), and *Actinobacteria* (17%), and *phut* showed *Firmicutes* (75%), and *Proteobacteria* (25%). Similarly, phylum distribution in *phab* from Bhutan showed *Firmicutes* (57%) and *Proteobacteria* (43%). Based on results of the 16S rRNA gene sequencing, 15 different genera viz. *Leuconostoc, Enterococcus, Bacillus, Staphylococcus, Lactobacillus, Enterobacter, Klebsiella, Pseudomonas, Pediococcus, Stenotrophomonas, Kocuria, Brevibacterium, Lysinibacillus, Weissella*, and *Micrococcus* with 32 species from starters of the Eastern Himalayas were identified (**Tables 3**, **4**). A wide diversity of bacteria (mainly LAB) was reported for the first time in traditionally prepared dry starters of the Eastern Himalayas (**Table 5**). The dominance of species of LAB was observed with 59% of total isolates in samples over non-LAB isolates (31%) (**Figure 3**). *Enterococcus durans, E. faecium, E. fecalis, E. hirae, E. lactis, Pediococcus acidilactici, P. pentosaceus, Lactobacillus plantarum* subsp. *plantarum, Lb. pentosus, Leuconostoc mesenteroides,* and *Weissella cibaria* were lactic acid bacterial species found in starter samples. *Enterococcus durans* (54.5%) was the most dominant species present in *marcha* samples from India (Darjeeling), whereas *Pediococcus pentocaseus* (5.8%) showed the lowest prevalence in *marcha* samples from Bhutan (**Figure 4**). LAB were found in all samples with highest occurrence in *marcha* samples of Darjeeling (91%). Non-LAB species were also recovered in many samples of starters, which were represented by *Bacillus subtilis* subsp. *inaquosorum, B. circulans, B. albus, B. cereus, B. nakamurai, B. nitratireducens, B. pseudomycoides, B. zhangzhouensis, Kocuria rosea, Staphylococcus hominis* subsp. *hominis, S. warneri, S. gallinarum, S. sciuri, Lysinibacillus boronitolerans, Brevibacterium frigoritolerans,* and *Micrococcus yunnanensis.* Interestingly, we detected few Gram-negative bacteria in

TABLE 2 | Phenotypic characterization of bacterial isolates from dry starters from the Eastern Himalayas.

Presumptive Identification (Total number of isolates)	Sugar fermentation													Tolerance							IMViC test					
														NaCl		pH			Temperature(°C)			Indole	MR	VP	Citrate	Urease	Nitrate
	Cellobiose	Raffinose	Sorbitol	Arabinose	Melibiose	Xylose	Lactose	Ribose	Melizitose	Glucose	Sucrose	Mannitol	Rhamnose	5%	10%	3.6	9.6	10.6	15	10	45						
Leuconostoc (15)	+(6) −(9)	+(7) −(8)	+(5) −(10)	+(4) −(11)	+(9) −(6)	−(10) +(5)	+(9) −(5) v(1)	−(6) +(8) v(1)	+(10) −(5)	+	+(8) −(6) v(1)	+(12) −(3)	+(4) −(10) v(1)	+	+(1) −(14)		+		+(5) −(10)	+(9) −(6)	+(12) −(3)	IMViC test was not determined for Gram-positive bacteria.					
Enterococcus (41)	+(18) −(23)	+(29) −(12)	+(23) −(18)	+(14) −(27)	+(34) −(7)	+(18) −(24)	+(34) −(6) v(1)	+(34) −(7)	+(33) −(3) v(5)	+	−(7) +(34)	+(7) −(34)	+(23) −(17) v(1)	+(36) −(5)	+(6) −(30) v(5)	+(6) −(35)	+(35) −(6)		+(13) −(28)	+(32) −(4) v(5)	+(35) −(6)						
Pediococcus (57)	+(19) −(38)	+(27) −(30)	+(19) −(38)	+(23) −(26) v(8)	+(28) −(29)	+(23) −(29) v(5)	+(36) −(12) v(9)	+(49) −(5) v(3)	+(32) −(24) v(1)	+	+(33) −(24)	+(13) −(44)	+(38) −(18) v(1)	+(48) v(9)	+(4) −(51) v(2)	+(3) −(51) v(3)	+(40) −(16) v(1)	+(5) −(52)	+(7) −(44) v(6)	+(38) −(13) v(6)	+(46) −(9) v(2)						
Lactobacillus (15)	+(12) −(3)	+(5) −(10)	+(10) −(5)	+(5) −(10)	+(10) −(5)	+(5) −(10)	+	+(12) −(3)	+(4) −(11)	+	+(12) −(3)	+(10) −(2) v(3)	+(4) −(11)	+	+(7) −(8)	+(10) −(5)	+(3) −(6) v(6)		+	+(9) −(6)	+(5) −(5) v(5)						
Bacillus (21)	+(17) −(4)	+(3) −(18)	+(3) −(18)	+(3) −(18)	+(3) −(18)	+(6) −(15)	+(15) −(6)	+(17) −(4)	+(9) −(12)	+(18) −(3)	−(11) +(10)	+(15) −(6)		+	+(7) −(14)	+(6) −(15)	+(17) v(4)		+	+(19) v(2)	+						
Staphylococcus (13)	+(8 −(5)	+(6) −(5) v(2)	+(5) −(8)	+(4) −(9)	+(6) −(7)	+(5) −(6) v(2)	+(9) −(2) v(2)	+(5) −(8)	+(3) −(10)	+	+(8) −(4) v(1)	+(8) −(5)	+(8) −(3) v(2)	+(6) −(2) v(5)	+(2) −(9) v(2)	+(2) −(9) v(2)	+(5) −(7) v(1)		+(2) −(10) v(1)	+(5) −(7) v(1)	+(9) −(3) v(1)						
Unidentified (21)	+(12) −(9)	+(7) −(14)	+(4) −(17)	+(11) −(8) v(2)	+(8) −(13)	+(9) −(10) v(2)	+(12) −(6) v(3)	+(18) −(3)	+(12) −(9)	+	+(11) −(10)	+(3) −(18)	+(12) −(9)	+	+(2) −(19)	+(1) −(19) v(1)	+(14) −(6) v(1)	+(3) −(17) v(1)	+(11) −(8) v(2)	+(15) −(3) v(3)	+(15) −(4) v(2)						
Enterobacter (8)	+	−	+(2) −(6)	+	−	+	−	+(6) −(2)	−	+	+	+	+	Physiological tests were not done for Gram–negative bacteria								−	−	+	+	+	+
Citrobacter (10)	+(5) −(5)	-	−	+(5) −(5)			+(8) −(2)	+(7) −(3)		+(8) −(2)	−(7) +(3)	−(5) v(5)	−(7) +(3)									−	−	−	+(6) −(4)	−(6) +(4)	+(6) −(4)

+,Positive; −,Negative; v, Variable; Numbers in parenthesis indicates number of isolates; All strains of Gram-negative bacteria were tested for IMViC either +/−. +,Positive; −,Negative; v, Variable; Numbers in parenthesis indicates number of isolates; All strains of Gram-negative bacteria were tested for IMViC either ±.

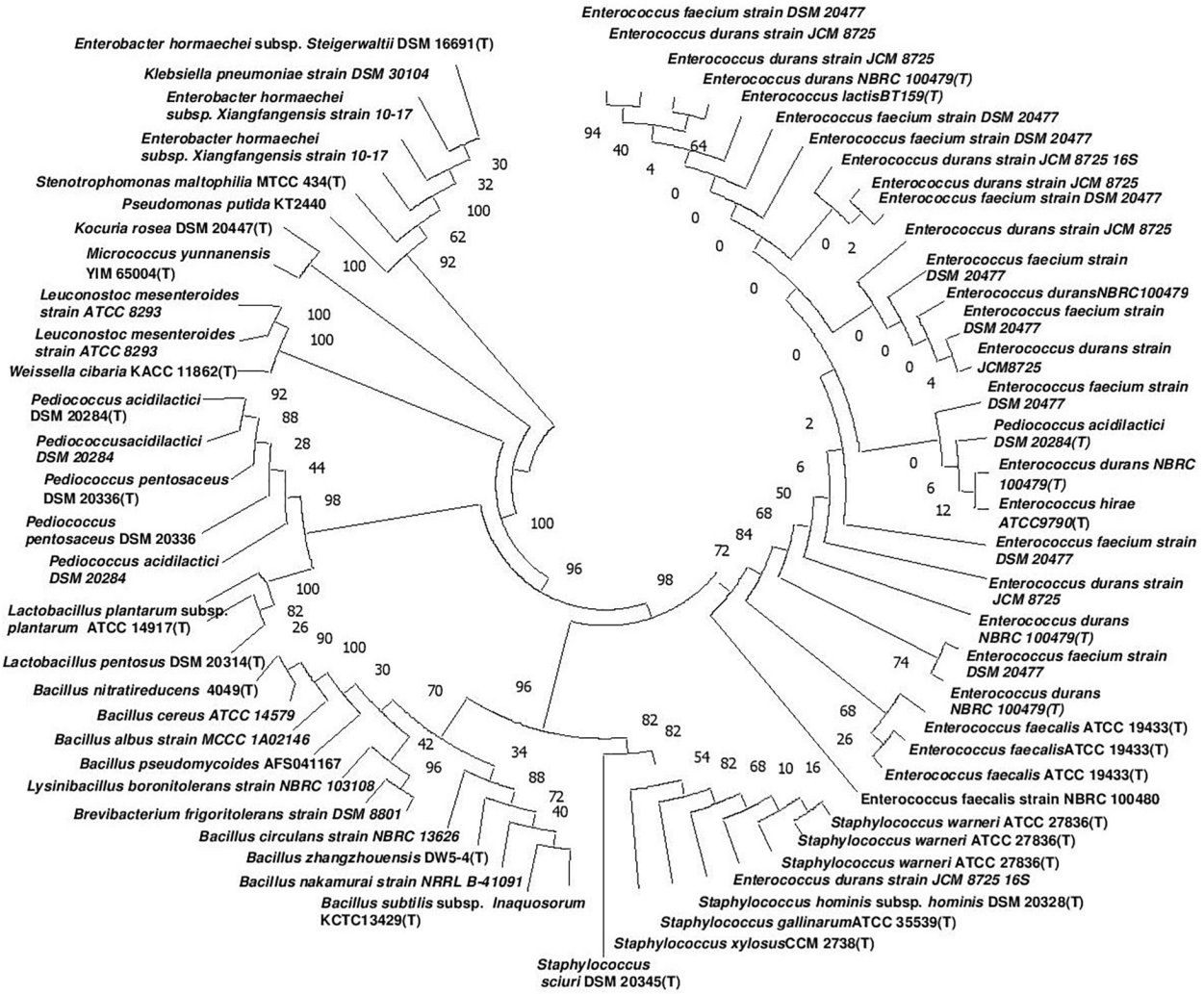

FIGURE 2 | Phylogenetic tree of the nucleotide sequences of 68 bacteria isolates from 35 different samples of dry starter from the Eastern Himalayas based on 16S rRNA sequencing. The tree was constructed by using the Neighbor-joining method (Gascuel and Steel, 2006) with bootstrap values for 1,000 replicates shown at the nodes of the tree using MEGA-7 (Kumar et al., 2016). The optimal tree with the sum of branch length = 0.98855936 is shown. The evolutionary distances were computed by the Maximum Composite Likelihood method (Varin et al., 2011) and are expressed in the units of the number of nucleotide substitutions per site. All positions containing gaps and missing data were eliminated. There were 308 total positions in the final dataset.

some of the starter cultures from Arunachal Pradesh such as *Stenotrophomonas maltophilia* in *paa, Klebsiella pneumoniae* in *pee, Pseudomonas putida* in *phut,* and *Enterobacter hormaechei* subsp. *xiangfangensis,* and *E. hormaechei* subsp. *steigerwaltii* in some samples of *phab* from Bhutan (**Table 5**).

Diversity indexes of bacterial communities of different starter cultures were characterized by the Shannon diversity index *H*, the Simpson's index, and the Dominance and Chao1 index (**Table 6**). The Shannon diversity index *H* for evaluating bacterial diversity recorded highest in *marcha* from Sikkim (*H*:2.305) and lowest in *marcha* from Darjeeling (*H*:1.121). Simpson's diversity index (1-*D*) values were 0.8878, 0.8711, 0.86, and 0.8374 for starters from Sikkim, Arunachal Pradesh, Nepal, and Bhutan, respectively. An estimation of species richness based on abundance was shown by the Chao 1 index. The dominance *D*-values were recorded as being highest for *marcha* samples from Darjeeling and lowest for *marcha* samples from Sikkim.

DISCUSSION

In this study five types of traditionally prepared dry starters (*marcha, pha, paa, pee*, and *phut*) were collected from different regions of the Eastern Himalayas, and they were analyzed for microbial load, pH, and moisture. The average bacterial population of all samples was 10^8 cfu/g, which was not reported earlier except for *marcha* from the Darjeeling hills and Sikkim (Tamang and Sarkar, 1995; Tsuyoshi et al., 2005; Tamang et al., 2007). The bacterial load of *marcha* from Sikkim was 10^6 to 10^8 cfu/g (**Table 1**), which was almost the same as that

TABLE 3 | Identification of LAB isolates from dry starters from the Eastern Himalayas based on 16S rRNA gene sequencing.

Isolate code	Sample (Place)	Identity	Type species (% similarity)	GenBank Accession No.	Size (base pair)
AKB6	*Marcha* (Darjeeling)	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* ATCC 8293 (99.54%)	MK748250	1315
BPB18	*Marcha*(Bhutan)	*Enterococcus durans*	*Enterococcus durans* JCM 8725 16S (99.52%)	MK748251	1254
DMB4	*Marcha* (Darjeeling)	*Enterococcus durans*	*Enterococcus durans* JCM 8725 (99.55%)	MK748252	1325
SMB13	*Marcha* (Sikkim)	*Leuconostoc mesenteroides*	*Leuconostoc mesenteroides* ATCC 8293 (99.55%)	MK748253	1339
AKB3	*Marcha* (Darjeeling)	*Pediococcus acidilactici*	*Pediococcus acidilactici* DSM 20284 (99.62%)	MK748254	833
AOB14	*Pee* (Arunachal Pradesh)	*Enterococcus durans*	*Enterococcus durans* JCM 8725 (99.62%)	MK748255	1333
AOB15	*Pee* (Arunachal Pradesh)	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (98.11%)	MK748256	1430
AOB25	*Phut* (Arunachal Pradesh)	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (99.71%)	MK748258	1406
AOB4	*Paa* (Arunachal Pradesh)	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (99.38%)	MK748259	1460
BPB11	*Marcha*(Bhutan)	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (98.91%)	MK748260	1476
BPB31	*Phab*(Bhutan)	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (99.28%)	MK748264	1390
BPB33	*Phab*(Bhutan)	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (99.51%)	MK748265	1432
DMB11	*Marcha* (Darjeeling)	*Enterococcus durans*	*Enterococcus durans* JCM 8725 (99.78%)	MK748267	1342
DMB12	*Marcha* (Darjeeling)	*Pediococcus acidilactici*	*Pediococcus acidilactici* DSM 20284 (99.59%)	MK748268	1462
DMB6	*Marcha* (Darjeeling)	*Enterococcus durans*	*Enterococcus durans* JCM 8725 (99.38%)	MK748269	1443
MBV14	*Pee* (Arunachal Pradesh)	*Enterococcus durans*	*Enterococcus durans* JCM 8725 (99.86%)	MK748270	1436
SMB15	*Marcha* (Sikkim)	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (99.86%)	MK748274	1447
SMB21	*Marcha* (Sikkim)	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (99.64%)	MK748276	1400
SMB5	*Marcha* (Sikkim)	*Enterococcus faecium*	*Enterococcus faecium* DSM 20477 (99.78%)	MK748277	1391
SMB7	*Marcha* (Sikkim)	*Enterococcus durans*	*Enterococcus durans* JCM 8725 (98.71%)	MK748278	1158
AOB5	*Paa* (Arunachal Pradesh)	*Enterococcus faecalis*	*Enterococcus faecalis* ATCC 19433(T) (99.86%)	MK202997	1421
BPB13	*Marcha*(Bhutan)	*Pediococcus pentosaceus*	*Pediococcus pentosaceus* DSM 20336(T) (99.73%)	MK203008	1456
BPB21	*Phab*(Bhutan)	*Enterococcus durans*	*Enterococcus durans* NBRC 100479(T) (99.79%)	MK203010	1430
BPB4	*Marcha*(Bhutan)	*Enterococcus durans*	*Enterococcus durans* NBRC 100479(T) (99.65%)	MK203013	1437
DMB3	*Marcha* (Darjeeling)	*Enterococcus durans*	*Enterococcus durans* NBRC 100479(T) (99.72%)	MK203015	1441
AOB24	*Phut* (Arunachal Pradesh)	*Enterococcus hirae*	*Enterococcus hirae* ATCC 9790(T) (99.86%.)	MK202998	1411
DMB13	*Marcha* (Darjeeling)	*Enterococcus durans*	*Enterococcus durans* NBRC 100479(T) (99.58%)	MK203017	1433
DMB14	*Marcha* (Darjeeling)	*Pediococcus acidilactici*	*Pediococcus acidilactici* DSM 20284(T) (99.52%)	MK203018	1461
DMB11	*Marcha* (Darjeeling)	*Pediococcus acidilactici*	*Pediococcus acidilactici* DSM 20284(T) (99.66%)	MK203019	1456
DMB15	*Marcha* (Darjeeling)	*Enterococcus durans*	*Enterococcus durans* NBRC 100479(T) (99.72%)	MK203020	1437
NMB3	*Marcha* (Nepal)	*Lactobacillus pentosus*	*Lactobacillus pentosus* DSM 20314(T) (97.44%)	MK203022	1276
NMB8	*Marcha* (Nepal)	*Lactobacillus plantarum* subsp. *plantarum*	*Lactobacillus plantarum* subsp. *plantarum* ATCC 14917(T) (99.65%)	MK203024	1441
AOB26	*Phut* (Arunachal Pradesh)	*Enterococcus lactis*	*Enterococcus lactis* BT159 (T) (98.0%)	MK202999	1398
NMB7	*Marcha* (Nepal)	*Lactobacillus plantarum* subsp. *plantarum*	*Lactobacillus plantarum* subsp. *plantarum* ATCC 14917(T) (100%)	MK203027	1435
SMB9	*Marcha* (Sikkim)	*Weissella cibaria*	*Weissella cibaria* KACC 11862(T) (99.66%)	MK203028	1455
SMB13	*Marcha* (Sikkim)	*Pediococcus pentosaceus*	*Pediococcus pentosaceus* DSM 20336(T) (99.79%)	MK203029	1433
AOB2	*Paa* (Arunachal Pradesh)	*Enterococcus faecalis*	*Enterococcus faecalis* ATCC 19433(T) (99.79%).	MK203002	1420
AOB11	*Paa* (Arunachal Pradesh)	*Enterococcus faecalis*	*Enterococcus faecalis* ATCC 19433(T) (99.58%).	MK203003	1421
SMB11	*Marcha* (Sikkim)	*Enterococcus durans*	*Enterococcus durans* JCM 8725 (96.44%)	MK752677	1432
SMB3	*Marcha* (Sikkim)	*Enterococcus faecalis*	*Enterococcus faecalis* NBRC 100480 (97.42%)	MK752675	1123

ATCC, American Type Cell Culture; JCM, Japan Collection of Microorganisms; DSM, Deutsche Sammlung von Mikroorganismen; MCCC, Microbial Culture Collection; NBRC, Biological Resource Centre, NITE; CCM, Czech collection of microorganisms; KACC, Korean Agricultural Culture Collection; YIM, Yunnan Institute of Microbiology; KCTC, Korean Collection for Type Cultures; NRRL, Agricultural Research Service Culture Collection.

of populations of yeasts and filamentous molds in *marcha* from Sikkim (Tsuyoshi et al., 2005). This shows that bacterial populations in traditionally prepared starters of the Eastern Himalayas may have co-existed equally with filamentous molds and yeasts (Hesseltine et al., 1988; Zheng et al., 2015). The moisture content of all starters was low due to the sun-drying process that followed immediately after fermentation, the step necessary to maintain the potency of traditionally prepared starters to be able to be stored in a dry place at room temperature for future use. The pH of all samples was mildly acidic, which may be due to the dominance of LAB ($\sim 10^8$ cfu/g) in dry starters (Tamang and Sarkar, 1995).

TABLE 4 | Identification of non-LAB and Gram-negative bacteria from dry starters from the Eastern Himalayas based on 16S rRNA gene sequencing.

Isolate code	Sample (Place)	Identity	Type species (% similarity)	GenBank Accession No.	Size (base pair)
AOB48	*Phut* (Arunachal Pradesh)	*Pseudomonas putida*	*Pseudomonas putida* KT2440 (99.85%)	MK203004	1379
AOB18	*Pee* (Arunachal Pradesh)	*Klebsiella pneumoniae*	*Klebsiella pneumoniae* DSM 30104 (99.3%)	MK748257	1439
BPB23	*Phab* (Bhutan)	*Enterobacter hormaechei* subsp. *xiangfangensis*	*Enterobacter hormaechei subsp. Xiangfangensis* 10−17 (99.58%)	MK748261	1431
BPB27	*Phab* (Bhutan)	*Enterobacter hormaechei* subsp. *Xiangfangensis*	*Enterobacter hormaechei* subsp. *Xiangfangensis* 10−17 (98.88%)	MK748263	1446
BPB26	*Phab* (Bhutan)	*Enterobacter hormaechei* subsp. *steigerwaltii*	*Enterobacter hormaechei* subsp. *Steigerwaltii* DSM 16691(T) (99.23%)	MK203011	1422
AOB9	*Paa* (Arunachal Pradesh	*Stenotrophomonas maltophilia*	*Stenotrophomonas maltophilia* MTCC 434(T) (99.79%)	MK203000	1416
NMB10	*Marcha* (Nepal)	*Bacillus zhangzhouensis*	*Bacillus zhangzhouensis* DW5−4(T) (99.58%)	MK203023	1432
NMB23	*Marcha* (Nepal)	*Staphylococcus xylosus*	*Staphylococcus xylosus* CCM 2738(T) (99.86%)	MK203021	1426
BPB24	*Phab* (Bhutan)	*Bacillus albus*	*Bacillus albus* MCCC 1A02146 (99.02%)	MK748262	1437
BPB8	*Marcha* (Bhutan)	*Bacillus circulans*	*Bacillus circulans* NBRC 13626 (98.64%)	MK748266	1412
NMB11	*Marcha* (Nepal)	*Bacillus cereus*	*Bacillus cereus* ATCC 14579 (100%)	MK748271	1460
NMB12	*Marcha* (Nepal)	*Brevibacterium frigoritolerans*	*Brevibacterium frigoritolerans* DSM 8801 (99.72%)	MK748272	1426
NMB13	*Marcha* (Nepal)	*Brevibacterium frigoritolerans*	*Brevibacterium frigoritolerans* DSM 8801 (100%)	MK748273	1388
SMB19	*Marcha* (Sikkim)	*Lysinibacillus boronitolerans*	*Lysinibacillus boronitolerans* NBRC 103108 (99.59%)	MK748275	1220
BPB1	*Marcha* (Bhutan)	*Staphylococcus warneri*	*Staphylococcus warneri* ATCC 27836(T) (99.72%)	MK203006	1437
BPB10	*Marcha* (Bhutan)	*Staphylococcus warneri*	*Staphylococcus warneri* ATCC 27836(T) (99.79%)	MK203007	1432
BPB17	*Marcha* (Bhutan)	*Staphylococcus warneri*	*Staphylococcus warneri* ATCC 27836(T) (99.72%)	MK203009	1429
BPB3	*Marcha* (Bhutan)	*Staphylococcus warneri*	*Staphylococcus warneri* ATCC 27836(T) (98.92%)	MK203012	1490
BPB7	*Marcha* (Bhutan)	*Bacillus nitratireducens*	*Bacillus nitratireducens* 4049(T) (99.36%)	MK203014	1404
DMB5	*Marcha* (Darjeeling)	*Staphylococcus hominis* subsp. *hominis*	*Staphylococcus hominis* subsp. *hominis* DSM 20328(T) (99.93%)	MK203016	1425
NMB20	*Marcha* (Nepal)	*Staphylococcus gallinarum*	*Staphylococcus gallinarum* ATCC 35539(T) (99.86%)	MK203025	1437
NMB22	*Marcha* (Nepal)	*Staphylococcus sciuri*	*Staphylococcus sciuri* DSM 20345(T) (99.65%)	MK203026	1439
SMB22	*Marcha* (Sikkim)	*Micrococcus yunnanensis*	*Micrococcus yunnanensis* YIM 65004(T) (99.64%)	MK203030	1379
SMB1	*Marcha* (Sikkim)	*Bacillus subtilis* subsp. *inaquosorum*	*Bacillus subtilis* subsp. *Inaquosorum* KCTC 13429(T) (99.65%)	MK203031	1425
SMB8	*Marcha* (Sikkim)	*Bacillus pseudomycoides*	*Bacillus pseudomycoides* AFS041167 (99.93%)	MK203032	1407
AOB19	*Pee* (Arunachal Pradesh)	*Kocuria rosea*	*Kocuria rosea* DSM 20447(T) (99.79%.)	MK203001	1399
AOB20	*Pee* (Arunachal Pradesh)	*Bacillus subtilis* subsp. *inaquosorum*	*Bacillus subtilis* subsp. *Inaquosorum* KCTC 13429(T) (99.79%)	MK203005	1431
SMB14	*Marcha* (Sikkim)	*Bacillus nakamurai*	*Bacillus nakamurai* NRRL B-41091 (96.65%)	MK752676	1103

First, we phenotypically characterized all 201 bacterial strains isolated from samples of *marcha, paa, pee, phut*, and *phab* and presumptively identified four genera of LAB- *Enterococcus, Pediococcus, Leuconostoc*, and *Lactobacillus,* two genera of non-LAB-*Bacillus* and *Staphylococcus*, and two Gram-negative bacterial genera, *Enterobacter* and *Citrobacter.* We grouped 201 isolates into 68 representative bacterial strains on the basis of phenotypic and biochemical tests for confirmation of their identity and assigned the taxonomical nomenclature by using 16S rRNA gene sequencing. In our study, we found a dominance of phylum *Firmicutes* (85%) over *Proteobacteria* (9%) and *Actinobacteria* (6%) in starters from the Eastern Himalayas. *Firmicutes* was also reported as the major abundant phylum in *daqu,* a starter for Chinese strongly flavored

TABLE 5 | Bacterial diversity in dry starters from the Eastern Himalayas.

Country/place	Starter	Bacterial species	
Nepal	*Marcha*	LAB:	*Lactobacillus pentosus, Lb. plantarum* subsp. *plantarum*
		Non-LAB:	*Bacillus cereus, B. zhangzhouensis, Brevibacterium frigoritolerans, Staphylococcus xylosus, S. gallinarum, S. sciuri*
		Gram-ve bacteria:	NR
India (Darjeeling hills)	*Marcha*	LAB:	*Enterococcus durans, Pediococcus acidilactici, Leuconostoc mesenteroides*
		Non-LAB:	*Staphylococcus hominis* subsp. *hominis*
		Gram-ve bacteria:	NR
India (Sikkim)	*Marcha*	LAB:	*Pediococcus pentosaceus, Leuconostoc mesenteroides, Enterococcus faecium, E. faecalis, E. durans, Weissella cibaria*
		Non-LAB:	*Lysinibacillus boronitolerans, Micrococcus yunnanensis, Bacillus subtilis* subsp. *inaquosorum, B. pseudomycoides, B. nakamurai*
		Gram-ve bacteria:	NR
India (Arunachal Pradesh)	*Paa*	LAB:	*Enterococcus faecalis, E. faecium*
		Non-LAB:	NR
		Gram-ve bacteria:	*Stenotrophomonas maltophilia*
	Pee	LAB:	*Enterococcus faecalis, E. durans, E. faecium*
		Non-LAB:	*Kocuria rosea, Bacillus subtilis* subsp. *inaquosorum*
		Gram-ve bacteria:	*Klebsiella pneumoniae*
	Phut	LAB:	*Enterococcus hirae, E. lactis, E. faecium*
		Non-LAB:	NR
		Gram-ve bacteria:	*Pseudomonas putida*
Bhutan	*Marcha*	LAB:	*Pediococcus pentosaceus, Enterococcus durans, E. faecium*
		Non-LAB:	*Staphylococcus warneri, Bacillus nitratireducens, B. circulans*
		Gram-ve bacteria:	NR
	Phab	LAB:	*Enterococcus durans, E. faecium*
		Non-LAB:	*Bacillus albus*
		Gram-ve bacteria:	*Enterobacter hormaechei* subsp. *xiangfangensis, Enterobacter hormaechei* subsp. *steigerwaltii*

LAB, lactic acid bacteria; NR, not recovered.

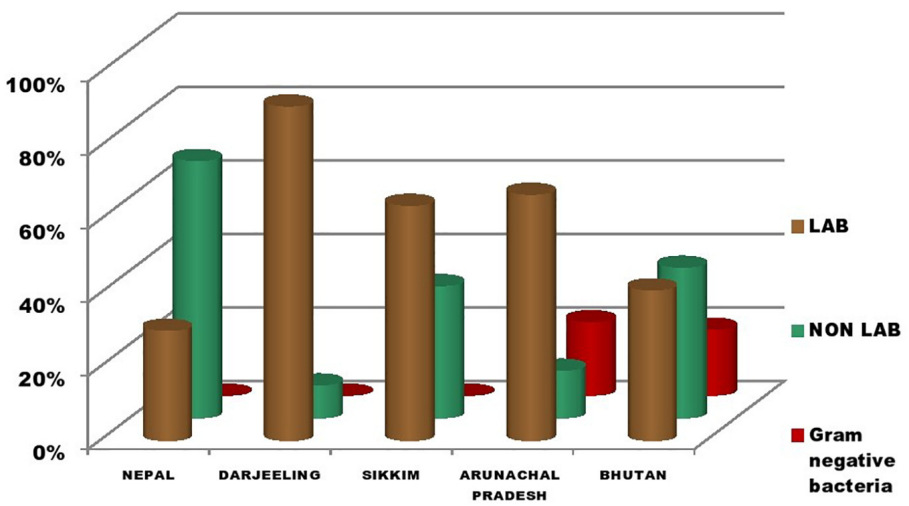

FIGURE 3 | Distribution of LAB, non-LAB, and Gram-negative bacteria in dry starters from the Eastern Himalayas.

liquor (Zou et al., 2018; He et al., 2019), and in *nuruk*, a starter from Korean used to produce *makgeolli,* a Korean alcoholic beverage (Jung et al., 2012). The sequence data based on a constructed phylogenetic tree revealed a dominance of LAB (59%) with five different genera and 11 species represented by *Enterococcus durans, E. faecium, E. fecalis, E. hirae, E. lactis, Pediococcus acidilactici, P. pentosaceus, Lactobacillus plantarum* subsp. *plantarum, Lb. pentosus, Leuconostoc mesenteroides,* and *Weissella cibaria*. Only two genera of LAB represented by *Pediococcus pentosaceus* and *Lactobacillus brevis* were reported

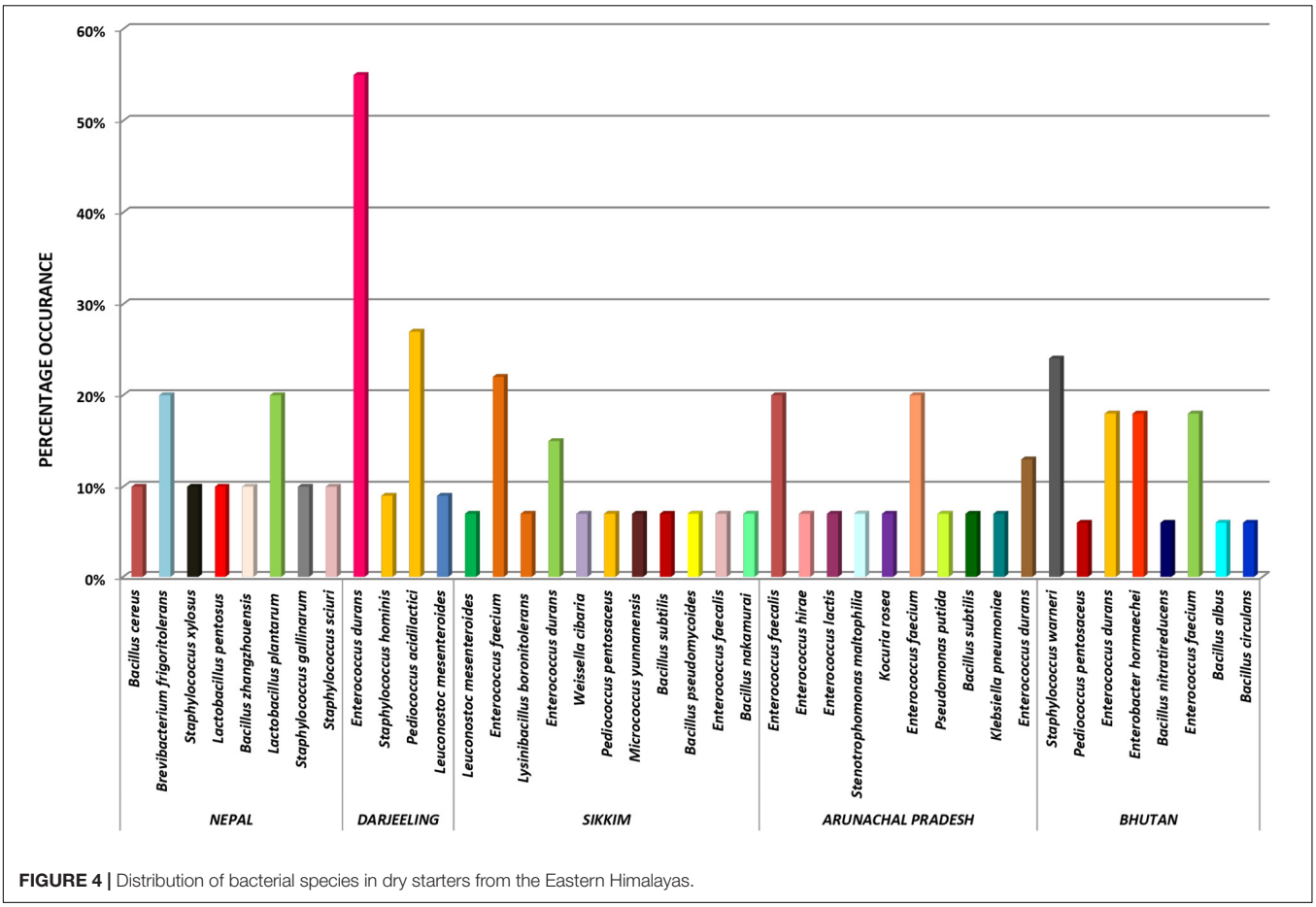

FIGURE 4 | Distribution of bacterial species in dry starters from the Eastern Himalayas.

earlier from *marcha* samples from Sikkim and the Darjeeling hills (Tamang and Sarkar, 1995; Tamang et al., 2007). However, in this study we found a wide diversity of LAB in samples of *marcha* collected from the Darjeeling hills and Sikkim in India, which included *Pediococcus pentosaceus, P. acidilactici, Enterococcus faecium, E. durans, E. faecalis, Leuconostoc mesenteroides*, and *Weissella cibaria*, whereas, *Lactobacillus pentosus* and *Lb. plantarum* subsp. *plantarum* were found only in *marcha* samples from Nepal. Variations in altitude and other geographical factors may affect the composition of microbiota in dry starters (Jeyaram et al., 2011; Lv et al., 2012). Traditional methods of preparation of *marcha, phab, paa, pee*, and *phut* are more or less similar except for some variations that were observed in the use of substrates, such as rice for *marcha, phut, paa*, and *pee*, and maize-rice husk for *phab* from Bhutan, and also wrapping materials for fermenting substrates such as fern leaves (*Glaphylopteriolopsis erubeseens*) for *marcha* preparation, dry paddy straws for *phab*, and locally available plant leaves for the preparation of *paa, pee*, and *phut*. Bacterial diversity in dry starters from the Eastern Himalayas may be influenced by hygienic conditions, quality of cereal substrates, wrapping materials, and sources of natural or tap water during traditional methods of preparation (Peter-Ikechukwu et al., 2016; Gonelimali et al., 2018; Sha et al., 2019).

The bacterial profile of *marcha* from Nepal and Bhutan, *paa, pee*, and *phut* of Arunachal Pradesh, and *phab* from Bhutan has been reported for the first time in our study. A similar type of dry starter for Assam in North East India called *xaj-pitha* also contained several species of LAB such as *Lactobacillus plantarum, Lb. brevis, Weissella cibaria, W. paramesenteroides, W. confusa, Lactococcus lactis, Lactobacillus casei group, Leuconostoc lactis, Leuconostoc pseudomesenteroides, Pediococcus pentosaceus, Lactococcus garvieae,* and *Enterococcus* sp. (Bora et al., 2016). Thanh et al. (2008) reported many species of LAB in Vietnamese *banh men*, which included *Pediococcus pentosaceus, Lactobacillus plantarum, Lb. brevis, Lb. fermentum, Lb. agilis, W. confusa, W. paramesenteroides,* and *Lactococcus lactis. Enterococcus faecium, Lactobacillus*

TABLE 6 | Diversity indices of different dry starters from the Eastern Himalayas.

Country/Region	Diversity indices			
	Simpson's index (1-*D*)	Shannon's index (*H*)	Dominance (*D*)	Chao-1
Nepal	0.86	2.025	0.14	13
India (Darjeeling hills)	0.6116	1.121	0.3884	5
India (Sikkim)	0.8878	2.305	0.1122	29
India (Arunachal Pradesh)	0.8711	2.176	0.1289	20.5
Bhutan	0.8374	1.925	0.1626	14

plantarum, Leuconostoc mesenteroides, Pediococcus acidilactici, P. pentosaceus, Weissella paramesenteroides, and *W. cibaria*, were reported in *nuruk* from Korea (Hoon et al., 2013). Several species of LAB in Cambodian *dombea* were also reported: *Weissella cibaria, Lactobacillus plantarum, Lactococcus lactis, Pediococcus pentosaceus*, and *Enterococcus durans* (Ly et al., 2018). This indicates that species of LAB predominate the microbial composition of traditionally prepared dry starters in Asia, including the Eastern Himalayas. LAB have been considered as favorable bacteria in cereal-based beverages due their ability to improve protein digestibility, enhance organoleptic quality, and increase nutritional bioavailability (Luana et al., 2014). Species of *Weissella, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus*, and *Enterococcus* are known for flavor development, the production of organic acids, and antimicrobial activities in Chinese *daqu* used for liquor production (Gou et al., 2015). *Enterococcus* sp. has been reported to produce enterocins, which play a major role in preventing the growth of foodborne and spoilage-causing pathogens (Javed et al., 2011).

Non-LAB species formed the next abundant group (32%) in starters from the Eastern Himalayas with the dominance of *Bacillus* spp. The abundance of *Bacillus* sp. may be due to its ability to survive in environments with low moisture and high temperature (Nuding et al., 2017). Also, the *Bacillus* species are important sources of amylase and protease enzymes, which are involved in saccharification and flavor production (Beaumont, 2002). A dominance of *Bacillus* sp. was also reported in *daqu* from China (Wang et al., 2008; Zheng et al., 2012) and *banh men* from Vietnam (Thanh et al., 2008). The next most abundant bacterium was *Staphylococcus* spp., found in the Himalayan starters, which secretes amylase (Li et al., 2014) and protease in Chinese *daqu* (Yang et al., 2017) and also produces lipases for the production of esters for flavor (Talon et al., 1996); thus, this group of bacteria probably plays a major role in the flavor enhancement of the final product. The prevalence of phylum *Actinobacteria* in some starters of the Eastern Himalayas was only 6%, represented by *Kocuria rosea*, *Micrococcus yunnanensis*, and *Brevibacterium frigoritolerans*. The presence of *Actinobacteria* has been reported in Chinese *daqu* (Zou et al., 2018) and Indian *marcha* and *thiat* (Sha et al., 2017).

Few species of opportunist pathogens and environmental contaminants such as *Micrococcus*, *Stenotrophomonas*, *Enterobacter*, *Klebsiella*, and *Pseudomonas* were detected, and they were found only in samples of *paa, pee,* and *phut* from Arunachal Pradesh, and *phab* from Bhutan. However, both the prevalence and populations of these contaminants were low and it is presumed that these organisms might have contaminated the samples during the traditional method of preparation from substrates, herbs, water, utensils, wrapping materials, etc., Gram-negative bacteria were not detected in any samples of *marcha* collected from Nepal, India, or Bhutan. In our previous study on *marcha*, no Gram-negative bacteria were found at the genus level, and this was discovered through an analysis using a high-throughput sequencing method (Sha et al., 2019). Although most of these bacteria are opportunists and probable foodborne pathogens, some of them, such as *Enterobacter* sp., are involved in the production of amylases and lipases and also the formation of flavor in *daqu* (Li et al., 2015). The presence of LAB inhibits the growth of pathogenic and spoilage microorganisms in foods (Cizeikiene et al., 2013; Castellano et al., 2017) and produces flavor compounds (Mukisa et al., 2017).

A diversity index, or phylogenetic metric, is a quantitative measure to show phylogenetic relations within different species in a community (Birtel et al., 2015). We characterized diversity indexes of the bacterial community present in starters from the Eastern Himalayas by using the Shannon diversity index *H*, Simpson's index, and Dominance and Chao1 index (**Table 6**). The Shannon diversity index *H* for evaluating bacterial diversity was recorded as being highest in *marcha* from Sikkim (*H*:2.305) and lowest in *marcha* from Darjeeling (*H*:1.121), indicating a higher bacterial diversity in *marcha* from Sikkim as compared to other starters. The Simpson's diversity index (1-*D*) index, which considers both the number of species as well as the relative abundance of each species for evaluating diversity, showed the highest values for *marcha* from Sikkim. The dominance *D*-values were recorded as being highest for *marcha* samples from Darjeeling and lowest for *marcha* samples from Sikkim, which supports the above inference regarding bacterial diversity. The dominance *D*-value ranged between 0–1, where the value 0 indicated that all taxa were equally present and value 1 indicated the dominance of one taxon over the whole community (Wagner et al., 2018). Thus, the values near zero indicate a highly diverse ecosystem and values near 1 indicate a less diverse or homogenous ecosystem (Lv et al., 2012). Hence, the phylogenetic matric of the bacterial community present in dry starters from the Eastern Himalayas showed high diversity within the community. The Eastern Himalayas are known for their rich floral and faunal diversity within a wide ecosystem (Chettri et al., 2010). Our findings thus highlight the richness of microbial diversity in the food ecosystem of the Eastern Himalayas.

The microbial communities and their interactions in starters are extremely important for proper fermentation, which may determine the productivity and flavor quality of the final alcoholic beverage (Cai et al., 2018). There has been an increasing amount of concern regarding the safety of fermented beverages due to the presence of ethyl carbamate, which is considered to be carcinogenic (Ryu et al., 2015), biogenic amines (Liu et al., 2016), mycotoxin (Sivamaruthi et al., 2018), and contamination by opportunistic microbial pathogens (Hong et al., 2016). All these considerations mandate a deep understanding of the microbial community in starters. Also, the profile of native microbiota in these starters opens a possibility of finding novel strain(s) with functional properties for industrial purposes. This study also records the bacterial diversity of *phab* from Bhutan, which is found to be produced rarely by a few ethnic people of Bhutan. This is probably due to their preference for commercial *marcha,* similar to *phab*, which is sold in local markets. Bacteria present in traditionally prepared dry starters have no amylolytic activities (Thapa and Tamang, 2004); however, they may contribute to the acidification of fermenting substrates and impart flavor with a mildly acidic and sour taste to traditional alcoholic beverages (*kodo ko jaanr, opo, apong*, and *themsing*) preferred by the Himalayan people (Thapa and Tamang, 2006; Tamang et al., 2007).

CONCLUSION

Information on the microbial composition of traditionally prepared dry starters of the Eastern Himalayan regions of India, Nepal, and Bhutan viz. *phab, paa, pee*, and *phut,* was unknown except for *marcha* from Sikkim in India. These traditional starters are used by the Himalayan people to ferment cereals into various alcoholic beverages for home consumption. The main objective of this study was to profile and assign the taxonomical identity of bacteria isolated from these traditional starters of the Eastern Himalayas based on 16S rRNA sequencing. *Firmicutes* was the most dominant phylum in all starters and was represented by several genera and species of LAB and also by some non-LAB. Interestingly our study showed high diversity within the bacterial community in traditionally prepared starters of the Eastern Himalayas, which may supplement the richness of microbial conservation in the food ecosystem of the regions. Besides diversity, some bacteria isolated from these traditional starters may have commercial and industrial importance. This is the first report on the bacterial diversity of dry starters of the Eastern Himalayas by Sanger sequencing.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the sequences retrieved from the 16S rRNA sequencing were deposited at GenBank-NCBI under the nucleotide accession number: MK748250-MK748278, MK202997-MK203032, and MK752675-MK752677.

AUTHOR CONTRIBUTIONS

PP performed the majority of the experiments. JT supervised the experiments and finalized the manuscript.

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Diversity of Filamentous Fungi Isolated From Some Amylase and Alcohol-Producing Starters of India

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Filamentous fungi are important organisms in traditionally prepared amylase and alcohol-producing dry starters in India. We collected 40 diverse types of amylase and alcohol-producing starters from eight states in North East India viz. *marcha*, *thiat*, *humao*, *hamei*, *chowan*, *phut*, *dawdim*, and *khekhrii*. The average fungal population was 4.9 × 10^5 cfu/g with an average of pH 5.3 and 10.7%, respectively. In the present study, 131 fungal isolates were isolated and characterized based on macroscopic and microscopic characteristics and were grouped into 44 representative fungal strains. Based on results of morphological characteristics and ITS gene sequencing, 44 fungal strains were grouped into three phyla represented by Ascomycota (48%), Mucoromycota (38%), and Basidiomycota (14%). Taxonomical keys to species level was illustrated on the basis of morphological characteristics and ITS gene sequencing, aligned to the fungal database of NCBI GenBank, which showed seven genera with 16 species represented by *Mucor circinelloides* (20%), *Aspergillus sydowii* (11%), *Penicillium chrysogenum* (11%), *Bjerkandera adusta* (11%), *Penicillium citrinum* (7%), *Rhizopus oryzae* (7%), *Aspergillus niger* (5%), *Aspergillus flavus* (5%), *Mucor indicus* (5%) *Rhizopus microsporus* (5%), *Rhizopus delemar* (2%), *Aspergillus versicolor* (2%), *Penicillium oxalicum* (2%), *Penicillium polonicum* (2%), *Trametes hirsuta* (2%), and *Cladosporium parahalotolerans* (2%). The highest Shannon diversity index *H* was recorded in *marcha* of Sikkim (*H*: 1.74) and the lowest in *hamei* of Manipur (*H*: 0.69). Fungal species present in these amylolytic starters are morphologically, ecologically and phylogenetically diverse and showed high diversity within the community.

Keywords: filamentous molds, amylolytic starter, India, *Mucor*, *Rhizopus*, *Aspergillus*, *Penicillium*

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INTRODUCTION

Drinking alcoholic beverages has a cultural connotation in India from the Indus Valley Civilization dating back to 8,000 years (Sarkar et al., 2016), mostly through fermentation (Singh et al., 2010) and distillation (Achaya, 1991). Traditionally malting, brewing (such as beer), and vinification (fermentation of grapes into wine) processes are unknown in Indian food culture. Instead, traditional alcoholic beverages are prepared either by natural fermentation of plants or cereals, or by using traditionally prepared dry starters in India (Tamang, 2010). Some ethnic people in India traditionally prepare amylase and alcohol-producing starters to ferment alcoholic beverages for

home consumption, which are known by different names in different languages spoken locally in regions such as *marcha* in Sikkim and Darjeeling hills, *thiat* in Meghalaya, *humao* in Assam, *hamei* in Manipur, *chowan* in Tripura, *phut* in Arunachal Pradesh, *dawdim* in Mizoramand *khekhrii* in Nagaland (Anupma et al., 2018), *dhehli, balam, maler, treh,* and *bakhar* of Himachal Pradesh and Uttarakhand (Thakur et al., 2015), and *ranu dabai/goti* of West Bengal, Odisha and Jharkhand (Ghosh et al., 2015). Traditional methods of the preparation of Indian starters are almost the same with some differences in use of starch-rich substrates such as rice or wheat or barley, and wrapping materials either in fern fronds or dry paddy-straw, or in fresh leaves of locally available wild plants (Shrivastava et al., 2012; Tamang et al., 2016). Soaked, dewatered, and ground cereal (rice/wheat/barley) flours are mixed with some wild plants, with a few spices such as sun-dried chilies or garlics and supplemented with 1–2% of previously prepared dry starters in powder forms ("back-slopping method" for sub-culturing the microbiota) to make thick doughs with addition of water. Thoroughly mixed dough mixtures are made into round or flat cakes of varying shapes and sizes, placed on fresh ferns or other plant leaves/dry paddy straws and allowed to ferment under semi-anaerobic conditions for 2–3 days at room temperature inside the room. After desirable fermentation, fermented doughs are then sun dried for 2–3 days to obtain dry starters which are exclusively used to ferment cereals into mild/strong alcoholic beverages (Tamang, 2010; Anupma et al., 2018). However, *khekhrii,* a dry starter from Nagaland in India is prepared by naturally fermenting sprouted-rice grains which are then dried in the sun to obtain dry starter granules to prepare an alcoholic beverage locally called *zutho*. Indian amylase and alcohol-producing starters are similar to starters from Asian countries such as *daqu* or *chiu* from China (Zheng et al., 2012), *benh* from Vietnam (Dung et al., 2007), *nuruk* from Korea (Jung et al., 2012), *ragi* from Indonesia (Roslan et al., 2018), *bubod* from the Philippines (Fronteras and Bullo, 2017), *loogpang* from Thailand (Daroonpunt et al., 2016) and *dombea* or *medombae* from Cambodia (Ly et al., 2018).

Several species of filamentous molds (Hesseltine et al., 1988; Yang et al., 2011; Lv et al., 2012a; Chen et al., 2014; Das et al., 2017); yeasts (Hesseltine and Kurtzman, 1990; Tamang and Sarkar, 1995; Tsuyoshi et al., 2005; Jeyaram et al., 2008, 2011; Thanh et al., 2008; Fronteras and Bullo, 2017; Sha et al., 2017, 2018, 2019), and bacteria (Hesseltine and Ray, 1988; Tamang et al., 2007; Sha et al., 2017; Roslan et al., 2018) are found to coexist in traditionally prepared dry starters as "micro-resources" which have been sub-cultured to preserve essential microbiota for alcohol production by Asian people for centuries (Tamang et al., 2020). Filamentous fungi present in traditional starters from Asia have several functionalities such as saccharification (Lee and Lee, 2002; Thapa and Tamang, 2004), liquefaction (Suesse et al., 2016), and ethanol production (Dung et al., 2007; Chen et al., 2014) to produce different types of low-alcoholic beverages and high-alcoholic distilled liquor. Filamentous molds are also responsible for the quality of alcoholic beverages including nutritional values and organoleptic properties such as flavor, taste, and color (Zhang et al., 2015; Tamang et al., 2016). Taxonomical identification of filamentous molds isolated from traditionally prepared dry starters from India have not been reported yet except from *marcha* (Tamang et al., 1988; Sha et al., 2017, 2019), *thiat* (Sha et al., 2017, 2019), *amou*, and *perok-kushi* (Das et al., 2017). *Mucor circinelloides*, *Rhizopus chinensis*, and *Rhizopus stolonifer* were reported earlier from *marcha* samples collected from Nepal, Darjeeling, and Sikkim (Tamang et al., 1988; Tamang and Sarkar, 1995; Thapa and Tamang, 2006; Sha et al., 2017, 2018), *Amylomyces rouxii* and *Rhizopus oryzae* from samples of *amou*, and *perok-kushi*, traditional starters of Assam (Das et al., 2017). Sha et al. (2017) reported fungal Phylum Ascomycota (98.6%) followed by Mucoromycota (1.4%), while in *marcha* samples only Phylum Ascomycota by high-through sequencing was reported. The present study aimed to identify the filamentous molds isolated from eight different types of traditionally prepared starters from North East India, viz. *marcha, thiat, humao, hamei, chowan, phut, dawdim*, and *khekhriii*, to species level by morphological and molecular identifications, and to profile their diversity within the fungal community.

MATERIALS AND METHODS

Sample Collection

A total of 40 samples of traditionally prepared dry starters viz *marcha* from Sikkim, *thiat* from Meghalaya, *humao* from Assam, *hamei* from Manipur, *chowan* from Tripura, *phut* from Arunachal Pradesh, *dawdim* from Mizoram, and *khekhrii* from Nagaland (**Table 1**) were collected directly from local markets and the homes of local producers in North East India (**Figure 1**) in pre-sterile containers. Dry starter samples were transported to the laboratory and stored in desiccators at room temperature as traditionally prepared dry starters have a shelf life of more than 1 year (Sha et al., 2018).

Analysis of pH and Moisture Content

The pH of homogenized samples was recorded by digital pH-meter (Orion 910003, Thermo Fisher Scientific, United States). The moisture content of the samples was estimated by a moisture analyzer (OHAUS/MB-45, United States).

Microbiological Analysis

Each dry sample starter was taken from the desiccator, then crushed coarsely by sterile spatula and 10 g of the crushed powered sample was homogenized with 90 mL of 0.85% physiological saline in a stomacher lab blender 40 (Seward, United Kingdom) for 2 min to obtain serial dilutions. One milliliter of each diluted sample (10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7}) was poured onto malt extract agar (M137, HiMedia, Mumbai, India) and potato dextrose agar (M096, HiMedia, Mumbai, India) with an addition of antibiotics (1% streptomycin) to suppress the growth of bacteria, and plates were then incubated under 28°C and observed for the appearance of colonies for up to 1 week. The colonies that appeared on plates were counted as a colony forming unit (cfu/g) on the dry weight of starters. Colonies were selected on the basis of macroscopic and microscopic characteristics. Selected filamentous molds were sub-cultured

TABLE 1 | Geographical locations, pH, moisture content, and fungal populations of dry starters from North East India.

Sample (n[a])	Region	Collection Site	Altitude (Meter)	Moisture content (%)	pH	cfu/g (×10^5)
Marcha (*n* = 8)	Sikkim	Gangtok	1637	11.6 (10.1 – 12.1)	5.2 (4.9 – 5.7)	5.0 (4.8 – 5.1)
		Basilakha	906			
		Pakyong	1341			
		Recabe	1072			
Thiat (*n* = 4)	Meghalaya	Shillong	1550	9.4 (8.7 – 10.0)	4.7 (4.5 – 5.0)	4.8 (4.5 – 5.1)
		Non-grem	1547			
Humao (*n* = 7)	Assam	Kokrajhar	49	9.7 (8.8 – 10.6)	4.9 (4.6 – 5.2)	4.6 (4.3 – 5.3)
		Jorhat	95			
		Sivsagar	93			
		Moran	100			
Hamei (*n* = 3)	Manipur	Kangchup	773	8.5 (8.0 – 9.6)	4.6 (4.1 – 5.4)	2.6 (2.5 – 3.2)
		Kakching	769			
		Phayeng	813			
Chowan (*n* = 4)	Tripura	Bangsul	116	9.1 (9.0 – 9.3)	5.6 (5.4 – 5.9)	3.1 (3.0 – 3.4)
		Dharmanagar	98			
Phut (*n* = 6)	Arunachal Pradesh	Doimukh	152	11.2 (11.4 – 11.8)	5.4 (5.5 – 5.7)	5.6 (4.9 – 5.9)
		Pasighat	155			
		Itanagar	361			
		Banderdewa	462			
		Nirjuli	151			
Dawdim (*n* = 3)	Mizoram	Saitual	438	13.7 (13.1 – 13.9)	6.2 (6.1 – 6.3)	7.4 (7.1 – 7.9)
Khekhrii (*n* = 5)	Nagaland	Kohima	1092	12.8 (12.3 – 13.1)	5.6 (5.5 – 5.9)	6.0 (5.7 – 6.8)

[a]*n = number of samples.*

on new plates and purified and stored on slants at 4°C for further studies.

Morphological and Physiological Identification

For each isolate, one- or three-point inoculations on petri plates containing ˜25 mL of media were applied. Fungal morphology was studied macroscopically by observing the colony features (surface color, reverse side color, shape, and diameter), and microscopically by observation of fruiting bodies using a stereomicroscope, and the vegetative and asexual stages were observed by a DE/Axio Imager A1 microscope (Carl Zeiss, Oberkochen, Germany) after staining freshly grown mycelia stained with cotton blue in MEA plates (Gaddeyya et al., 2012). Filamentous molds were identified on the basis of morphological features using the taxonomical keys described by Samson et al. (2004) and Pitt and Hocking (2009).

Genomic DNA Extraction

The genomic DNA was extracted from mold cultures following the methods of Umesha et al. (2016). Mycelial mass from the culture plate was scraped out by a sterile surgical blade and ground in a sterile mortar and pestle using 500 μL extraction buffer [100 mM Tris–HCl (pH 8.0), 20 mM EDTA (pH 8.0), 1.4 M NaCl, 2% CTAB, and 0.2% 2 mercaptoethanol]. The mixture was transferred to a fresh 1.5 mL tube with addition of 4-μL RNase, vortexed and incubated for 60 min at 37°C, and kept in a water bath for 60 min at 55°C. 500 μL phenol: chloroform: isoamyl alcohol (25:24:1) was added to the solution, mixed thoroughly for 5 min, and then centrifuged at 14,000 rpm for 10 min. The aqueous clear phase was recovered and mixed with chloroform: isoamyl alcohol (24:1), centrifuged at 12,000 rpm for 5 min, and the aqueous phase was recovered, adding 0.8 volume of cold 7.5 M ammonium acetate and 0.54 volume of ice-cold isopropanol, and finally mixed well and stored overnight for precipitation of DNA in a deep freezer. The solution was centrifuged at 14,000 rpm for 3 min and precipitated with absolute ethanol to recover DNA. The DNA was then rinsed twice with 1 mL of 70% ethanol and resuspended in 100 μL of 1X TE [200 mM Tris–HCl (pH 8.0), 20 mM EDTA (pH 8.0)] buffer for further use and stored at −20°C. The quality of DNA was checked on agarose gel and the concentration was measured using a nanodrop spectrometer (ND-1000 spectrometer, NanoDrop Technologies, Willington, United States) (Kumbhare et al., 2015).

PCR Amplification

Polymerase chain reactions (PCR) of the internal transcribed spacer (ITS) region of filamentous molds was amplified using the primer ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) (Adekoya et al., 2017). PCR reactions were performed in 25 μL of PCR pre-master mix solution (Promega, United States). The amplification steps were followed: initial denaturation at 94°C for 5 min followed by 35 cycles consisting of 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min, respectively; and a final extension at 72°C for 10 min in a Thermal Cycler (Applied biosystems-2720, United States). The PCR products were verified by electrophoresis on 1.0% agarose gel containing 0.7 mg/mL of

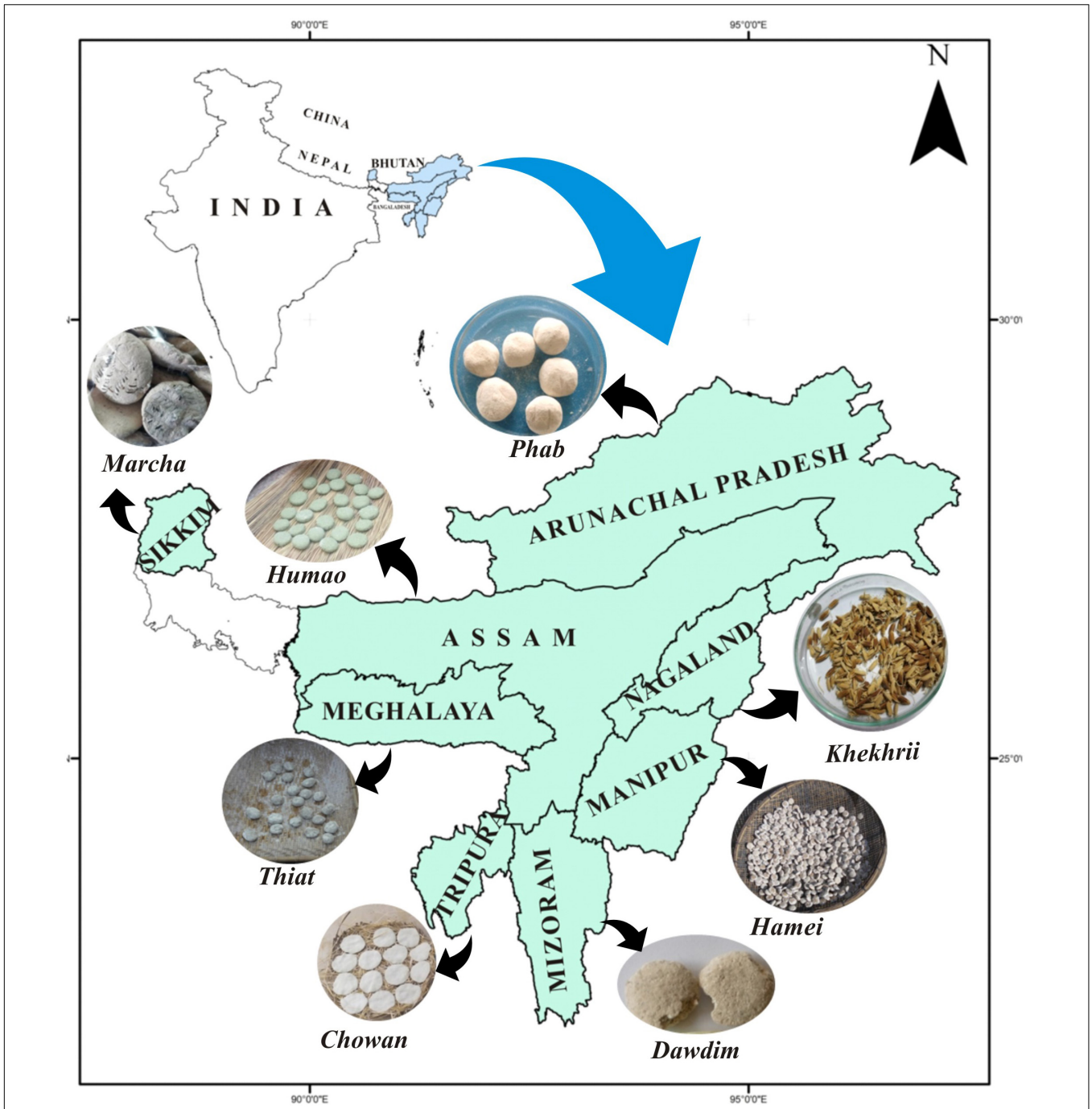

FIGURE 1 | Location map of North East India showing collection sites of traditionally prepared dry starters.

ethidium bromide and visualized under UV light (Gel doc 1000, Bio-Rad, 97-0186-02, United States). Approximate size of amplicons was determined using standard molecular markers (Himedia-100 bp DNA ladder, Mumbai, India).

Purification of the PCR Amplicons

The amplified PCR products were purified using PEG (polyethylene glycol)-NaCl (sodium chloride) and precipitation solution (20% w/v of PEG, 2.5 M NaCl) with the addition of 0.6 volumes of 20% PEG-NaCl to the final volume of the PCR products (Schmitz and Riesner, 2006). The mixture was centrifuged at 12,000 rpm for 30 min, incubated at 37°C for 30 min, the aqueous solution was discarded, and the pellet was washed twice with 1 mL ice cold 70% freshly prepared ethanol (70%). The collected pellet was then air dried prior to elution in 20 μL of nuclease-free

water, and finally, the purified product was loaded in 1% agarose gel.

ITS Sequencing

PCR-amplified products had been sequenced in a forward and reverse direction using ITS1 primer (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 primer (5′-TCCTCCGCTTATTGATATGC-3′), respectively, as per the method described by Martin and Rygiewicz (2005). The PCR reaction was carried out in 50 μL reaction volume containing 2.0 mM MgCl2, 0.2 μM each primer, 0.2 mM dNTP, 0.5 mg $[mL]^{-1}$ bovine serum albumin (BSA) and 0.04 U $[\mu L]^{-1}$ tTaq DNA polymerase on a thermal cycler equipped with a heated lid. The thermal program included initial denaturation, enzyme activation at 95°C (6–10 min) followed by 35 cycles to complete the step [95°C (1 min), 40°C (2 min) and 72°C (1 min)] and one cycle at 72°C (10 min). The amplified products were sequenced by an automated DNA Analyzer (ABI 3730XL Capillary Sequencers, Applied Biosystems, Foster City, CA, United States). These high-quality, double-stranded sequence data were analyzed with the help of the BLASTn program and multiple sequence alignment.

Bioinformatics

The qualities of the raw sequences were checked by Sequence Scanner version 1.0 (Applied Biosystems, Foster City, CA, United States) and were edited using software ChromasPro version 1.34. Sequences were compared with sequence entries in the GenBank of NCBI (National Center for Biotechnology Information)[1] using the Basic Local Alignment Search Tool for nucleotides (BLASTn) on the NCBI website (Pinto et al., 2012). For phylogenetic analysis, the available sequence of similar related organisms was retrieved in FASTA format and aligned using the clustal-W. Sequence alignment and a phylogenetic tree were constructed using MEGA7.0 software by Neighbor-Joining methods using 1000-bootstrap replicates (Lutzoni et al., 2004).

Statistical Analysis

Percentages of frequency and relative density of fungal species in samples were determined as per the method described by Doi et al. (2018). Frequency (%) was calculated by the equation:

$$\text{Frequency (\%)} = \frac{\text{Number of quadrats in which the species occurred}}{\text{Total number of quadrats studied}} \times 100$$

Relative Density (%) was calculated by the equation:

$$\text{Density} = \frac{\text{Total number of individuals of a species in all quadrats}}{\text{Total number of quadrats studied}} \times 100$$

Diversity indexes of filamentous molds in samples were calculated by species richness (R), Shannon's diversity index (H), and species evenness (E) (Panda et al., 2010) using PAST (Paleontological STatistics) software version 3.26 (Hammer et al., 2001).

[1]http://www.ncbi.nlm.nih.gov/Blast.cgi

Nucleotide Sequence Accession Numbers

The sequences obtained in this study were deposited at the GenBank-NCBI database under accession numbers: MK396469–MK396484, MK396486–MK396500, MK778442–MK778449, and MK796041–MK796045.

RESULTS

Microbial Load, pH, and Moisture

The microbial load of filamentous molds in 40 samples of traditionally prepared dry starters collected from different regions of North East India were 2.5 to 7.9 × 10^5 cfu/g (**Table 1**). The pH and moisture contents of all samples analyzed were pH 4.1–6.3 and 8.0–13.9%, respectively (**Table 1**).

Morphological Characterization

We isolated 131 total fungal isolates from 40 different samples of traditionally prepared dry starters (*marcha*, *thiat*, *humao*, *hamei*, *chowan*, *phut*, *dawdim*, and *khekhrii*) collected from eight states of North East India (**Table 1**). Based on the morphological characteristics (such as color, texture, size, and appearance of colony), microscopic characteristics (sporangia, sporangiospores, chlamydospores, conidia, conidiophore, and rhizoid structure), 44 representative fungal isolates were grouped (seven isolates from *marcha*, five from *thiat*, six from *humao*, two from *hamei*, five from *chowan*, six from *phut,* six from *dawdim*, and seven from *khekhrii*). *Mucor*, *Rhizopus*, *Aspergillus, Penicillium,* and *Cladosporium* and a few unidentified basidiomycetes fungi were tentatively identified on the basis of detailed morphological characters using the taxonomical keys described by Samson et al. (2004) and Pitt and Hocking (2009) (**Supplementary Table S1**).

Molecular Identification of Fungal Isolates

Genomic DNA of each isolate of 44 representative fungal strains was extracted and PCR products were prepared for identification by ITS gene sequencing. DNA sequences of fungal isolates were assigned by comparison with those available in the GenBank of NCBI database using the ITS gene sequence (ITS1 and ITS4) based on the Basic Local Alignment Search Tool (BLAST) 2.0 program (Raja et al., 2017). The phylogenetic trees of nucleotide sequences of the 44 fungal isolates from the samples were constructed using the Neighbor-joining method with 1000 replicates bootstrap values (**Figure 2**). ITS gene sequencing results showed three fungal phyla represented by Ascomycota (48%), Mucoromycota (38%), and Basidiomycota (14%) (**Figure 3**). Distribution percentage of the phyla in the starter showed the highest percentage of Ascomycota (86%) in

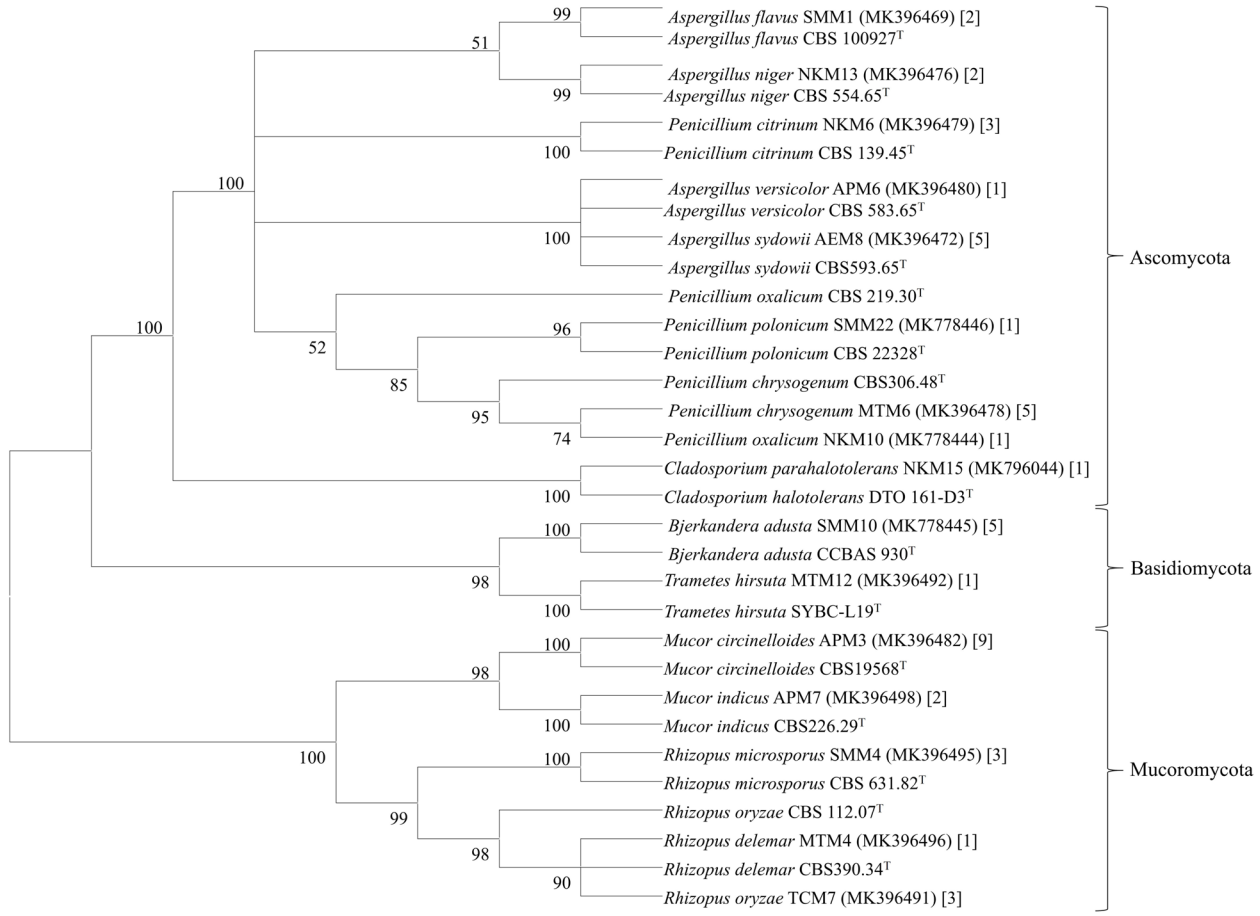

FIGURE 2 | Molecular phylogenetic analysis of 44 filamentous fungal isolates from starters from North East India using the Neighbor-Joining method in MEGA7 software. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branch. The tree is drawn to scale with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method and are in the units of the number of base substitutions per site. The phylogenetic tree branches are collapsed at 50%.

khekhrii, Mucoromycota (60%) in *dawdim*, and Basidiomycota (20%) in *chowan, dawdim*, and *thiat*, respectively. Phyla Ascomycota and Mucoromycota were present in all starters, whereas Basidiomycota was present only in *marcha*, *thiat, chowan*, and *dawdim.*

Based on results of morphological characteristics and ITS gene sequencing, 44 representative strains of filamentous molds isolated from traditionally prepared dry starters from India were grouped into seven genera with 16 species, which were represented by *Mucor circinelloides* (20%), *Aspergillus sydowii* (11%), *Penicillium chrysogenum* (11%), *Bjerkandera adusta* (11%), *Penicillium citrinum* (7%), *Rhizopus oryzae* (7%), *Aspergillus niger* (5%), *Aspergillus flavus* (5%), *Mucor indicus* (5%) *Rhizopus microsporus* (5%), *Rhizopus delemera* (2%), *Aspergillus versicolor* (2%), *Penicillium oxalicum* (2%), *Penicillium polonicum* (2%), *Trametes hirsuta* (2%), and *Cladosporium parahalotolerans* (2%) (**Table 2** and **Figure 4**). Interestingly we detected few basidiomycetes fungi represented by *Bjerkandera adusta* and *Trametes hirsuta* in *marcha, thiat, chowan* and *dawdim* samples. Colony morphology and microscopic images of 16 species of seven genera of filamentous molds isolated from dry starters from India were illustrated for fungal taxonomy (**Figure 5**).

Frequency and density of fungal species in samples showed that *Aspergillus niger* was colonized with *khekhrii;* a species from the *Mucor circinelloides* complex was observed with a high dominance in samples, whereas *Trametes hirsuta* was less diversified and observed only in *thiat* samples (**Table 3**).

Diversity indexes of filamentous molds of dry starters were characterized by species richness (R), Shannon's diversity index (H), and species evenness (E) (**Table 3**). The Shannon diversity index *H* was recorded as the highest in *marcha* from Sikkim (*H*: 1.74) and the lowest in *hamei* from Manipur (*H*: 0.69). Species Evenness (E) values were 0.97 in *marcha* followed by *humao* from Assam and *phut* from Arunachal Pradesh. The Species Richness (R), values were recorded highest in *marcha* and *khekhrii* samples (**Table 3**).

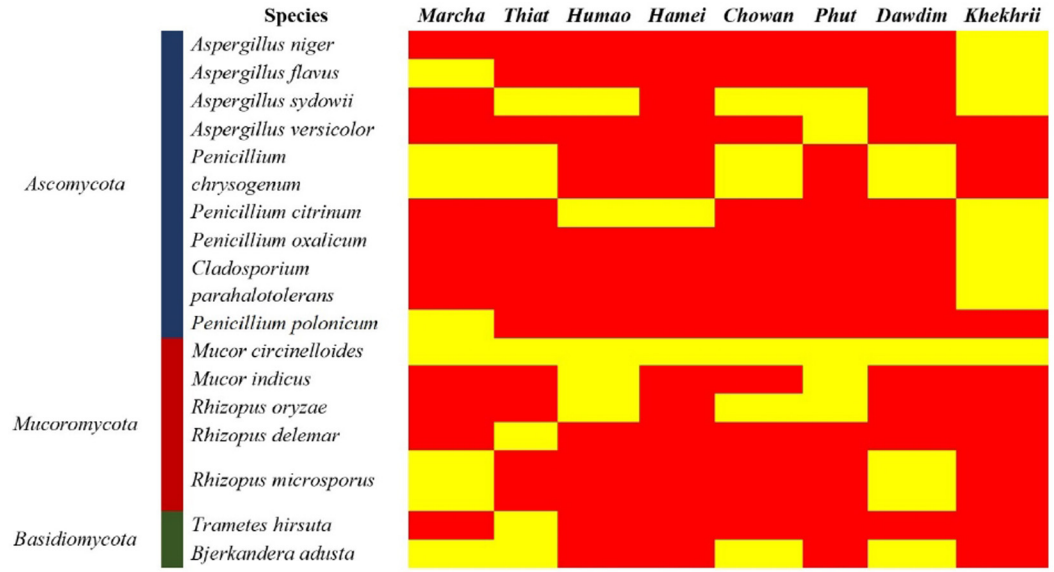

FIGURE 3 | Heatmap showing the consensus species diversity resulted by ITS-region gene sequencing of filamentous fungal isolates. We used presence/absence value for fungal species to generate the heatmap, where the yellow color indicates the presence and red indicates absence.

DISCUSSION

Drinking of cereal-based mild to strong alcoholic beverages produced by traditionally prepared amylase and alcohol-producing starters has been a traditional food culture of the ethnic people from the North East states of India for centuries. Traditionally prepared dry starters have consortia of co-existed microbiota containing filamentous molds, yeasts, and bacteria and are crudely sub-cultured through a "back-slopping" process by traditional starter-makers (Hesseltine et al., 1988; Tamang and Sarkar, 1995; Tamang et al., 2007; Sha et al., 2018, 2019), for alcohol production by the Indian people. The pH of traditionally prepared dry starters from India were slightly acidic in nature, perhaps due to accumulation of metabolic organic acids (Ma et al., 2019). Moreover, low pH is favorable for the growth of mycelial fungi (Abubakar et al., 2013). Low content of moisture in starter cultures is due to the sun-drying process during the traditional method of preparation practiced by the ethnic people of India, which may increase the shelf life of the starter for a year or more at room temperature (Tsuyoshi et al., 2005; Tamang, 2010).

Some traditionally prepared starters from North East India have been microbiologically analyzed in earlier works and several species of yeasts (Tsuyoshi et al., 2005; Jeyaram et al., 2008, 2011; Sha et al., 2017, 2018, 2019) and bacteria (Tamang et al., 2007; Pradhan and Tamang, 2019) were reported. However, detailed taxonomical studies of filamentous molds isolated from traditionally prepared dry starters from North East India have not been reported yet, except for *marcha* (Tamang et al., 1988; Tamang and Sarkar, 1995; Sha et al., 2017, 2019), *thiat* (Sha et al., 2017, 2019), *amou, perok-kushi* (Das et al., 2017). Hence, we studied the taxonomy and diversity of filamentous fungi associated with traditionally prepared dry starter cultures from North East India viz., *marcha* from Sikkim, *thiat* from Meghalaya, *humao* from Assam, *hamei* from Manipur, *chowan* from Tripura, *phut* from Arunachal Pradesh, *dawdim* from Mizoram, and *khekhrii* from Nagaland based on morphological characters and molecular identifications. The average fungal population in traditionally prepared dry starters from North East India was 10^5 cfu/g, which was in accordance with earlier reports on fungal populations in *marcha* of Sikkim, and the Darjeeling hills in India (Tamang et al., 1988; Tamang and Sarkar, 1995). No such data on fungal population in other starters of India are available except for *marcha*. In the present study, we first isolated and characterized 131 fungal isolates from 40 different starters from North East India based on macroscopic and microscopic characteristics and grouped them into 44 representative fungal strains. Morphological examination and identification of fungi are useful for identification up to the family or genus level (Alsohaili and Bani-Hasan, 2018). However, morphological-based identification is not adequate to identify the fungi up to species level (Lutzoni et al., 2004). The sequence-based identification tool is widely applied to confirm the exact identify of the fungal species (Romanelli et al., 2010; Xu, 2016).

We applied polymerase chain reactions (PCR) of the internal transcribed spacer (ITS) region of 44 strains of filamentous fungi isolated from starters from North East India using the primers ITS1 and ITS4 and grouped into three phyla represented by Ascomycota (48%), Mucoromycota (38%), and Basidiomycota (14%). A similar type of phylum distribution was also reported earlier in a *nuruk* dry starter from Korea (Carroll et al., 2017) and *daqu* from China (Shoubao et al., 2019). Seven genera with 16 species of filamentous fungi, isolated from Indian amylase

TABLE 2 | Molecular identification of filamentous molds isolated from starters from North East India by ITS gene sequence (ITS1 and ITS4) based on BLAST.

Product	Isolate code	Identity	GenBank accession number	Size in base pair (arbitrary primers)
Marcha	SMM-1	*Aspergillus flavus*	MK396469	519
	SMM-3	*Mucor circinelloides*	MK396489	642
	SMM-4	*Rhizopus microsporus*	MK396495	703
	SMM-10	*Bjerkandera adusta*	MK778445	675
	SMM-16	*Penicillium chrysogenum*	MK396477	577
	SMM-22	*Penicillium polonicum*	MK778446	582
	SMM-35	*Penicillium chrysogenum*	MK778447	552
Thiat	MTM-1	*Mucor circinelloides*	MK396487	636
	MTM-4	*Rhizopus delemar*	MK396496	768
	MTM-6	*Penicillium chrysogenum*	MK396478	583
	MTM-12	*Trametes hirsuta*	MK396492	637
	MTM-16	*Bjerkandera adusta*	MK396500	651
Humao	AEM-1	*Penicillium citrinum*	MK396481	437
	AEM-3	*Rhizopus oryzae*	MK396483	613
	AEM-4	*Mucor circinelloides*	MK396484	648
	AEM-8	*Aspergillus sydowii*	MK396472	467
	AXM-1	*Aspergillus sydowii*	MK396475	546
	AMM-3	*Mucor indicus*	MK778442	565
Hamei	MHM-1	*Mucor circinelloides*	MK796043	601
	MHM-15	*Penicillium citrinum*	MK796042	469
Chowan	TCM-1	*Bjerkandera adusta*	MK396494	520
	TCM-4	*Mucor circinelloides*	MK778449	636
	TCM-7	*Rhizopus oryzae*	MK396491	637
	TCM-9	*Aspergillus sydowii*	MK796041	541
	TCM-12	*Penicillium chrysogenum*	MK778448	541
Phut	APM-1	*Aspergillus sydowii*	MK396473	577
	APM-3	*Mucor circinelloides*	MK396482	645
	APM-6	*Aspergillus versicolor*	MK396480	417
	APM-7	*Mucor indicus*	MK396498	627
	APM-12	*Rhizopus oryzae*	MK396490	621
	APM-15	*Aspergillus sydowii*	MK396474	574
Dawdim	MDM-1	*Mucor circinelloides*	MK396497	645
	MDM-10	*Bjerkandera adusta*	MK396493	569
	MDM-11	*Rhizopus microsporus*	MK396488	696
	MDM-14	*Mucor circinelloides*	MK396486	641
	MDM-16	*Bjerkandera adusta*	MK396499	680
	MDM-18	*Penicillium chrysogenum*	MK778443	554
Khekhrii	NKM-1	*Mucor circinelloides*	MK796045	490
	NKM-6	*Penicillium citrinum*	MK396479	519
	NKM-7	*Aspergillus flavus*	MK396470	519
	NKM-8	*Aspergillus niger*	MK396471	551
	NKM-10	*Penicillium oxalicum*	MK778444	581
	NKM-13	*Aspergillus niger*	MK396476	602
	NKM-15	*Cladosporium parahalotolerans*	MK796044	546

and alcohol-producing starters, were identified based on the morphological and microscopic characteristics, and molecular identification which were represented by *Aspergillus flavus, A. niger*, *A. sydowii*, *A. versicolor*, *Bjerkandera adusta*, *Cladosporium parahalotolerans*, *Mucor circinelloides*, *M. indicus*, *Penicillium chrysogenum*, *P. citrinum*, *P. oxalicum*, *P. polonicum, Rhizopus delemar, R. microsporus, R. oryzae*, and *Trametes hirsuta*. Illustration of taxonomical keys based on morphological and molecular identification is more accurate and reliable in fungal taxonomy (Xing et al., 2018). Our earlier findings of *Rhizopus oryzae* and species from the *Mucor circinelloides* complex in traditionally prepared starters of North East India by PCR-DGGE method (Sha et al., 2018) supported the present study. Hesseltine and Kurtzman (1990) reported species from the *M. circinelloides* complex in *bubod* from the Philippines. Species from the *M. circinelloides* complex, *M. indicus, Rhizopus oryzae,* and *R. microsporus* were reported i*n benh men* from Vietnam (Dung et al., 2007; Thanh et al., 2008). In *marcha* and *khekhrii*

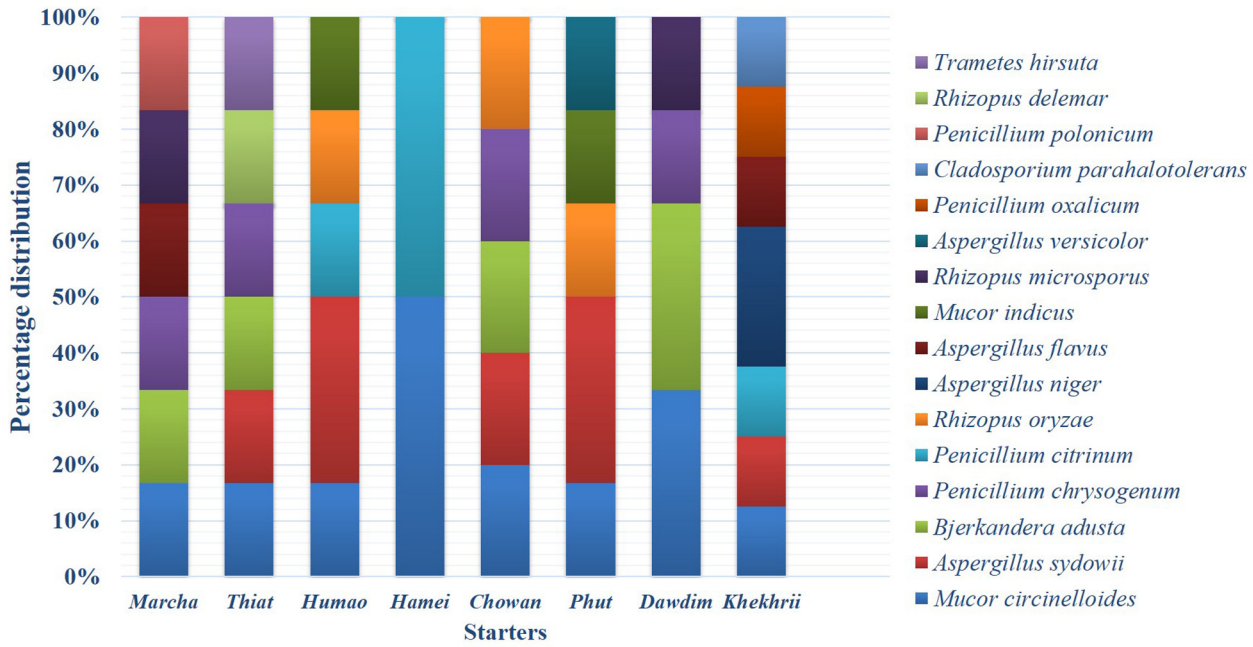

FIGURE 4 | Abundance distribution of the filamentous fungi isolated from dry starters from North East India.

we detected *Aspergillus flavus*, which was also reported in *mana*, an amylolytic starter from Nepal (Nikkuni et al., 1996).

Aspergillus belonging to order Eurotials is a phenotypically polythetic genus and is widely distributed in the environment (Tsang et al., 2018). Samson et al. (2014) proposed phylogenic identification of *Aspergillus* with ITS sequence data, and calmodulin as a secondary identification marker, according to the decision of the International Commission of *Penicillium* and *Aspergillus*[2]. Application of ITS with β-tubulin sequences for identification of *Aspergillus* species has also been reported by Zulkifli and Zakaria (2017). However, in this study we have applied both ITS sequence and morphological characteristics, such as the conidiophore with straight ending in a large vesicle from where primary and secondary sterigmata arise bearing conidia in chains, for identification of species of *Aspergillus*. *Aspergillus niger* and *A. flavus* cannot be distinguished only by their ITS sequences, the morphological characters are also essential in species identification (Zulkifli and Zakaria, 2017). We identified genus *Aspergillus* with four species in dry starter samples from India which included *A. niger*, *A. flavus*, *A. sydowii,* and *A. versicolor*. Among *Aspergillus A. flavus, A. niger* and *A. sydowii* were most prevalent in food samples due to their sporulating ability in the environment (Adekoya et al., 2017). *Aspergillus* is a dominant fungal genus in *daqu* from China (Ji et al., 2018), and may contribute to the saccharification process (Wang et al., 2019). We detected two strains of *Aspergillus flavus* in a *marcha* sample from Sikkim (*Aspergillus flavus* SMM-1) and in a *khekhrii* sample from Nagaland (*A. flavus* NKM-7). Though the distribution percentage was only 5%, the presence of *A. flavus* in samples of *marcha* and *khekhrii* is alarming. *A. flavus* is a saprotrophic with cosmopolitan distribution (Ramírez-Camejo et al., 2012), which produces aflatoxin (Saori and Keller, 2011; Priyanka et al., 2012; Mudili et al., 2014). Probable sources of *A. flavus* in starters may be from contaminated rice grains (Lai et al., 2015) since rice is the main base substrates for the preparation of starters for the production of alcohol. Moreover starter-makers commonly use low-quality, old-stocked and discarded rice grains for preparation of starters. However due to co-existence of other species of filamentous molds, yeasts and lactic acid bacteria in traditionally prepared starters may antagonize against *A. flavus* in *marcha* and *khekhrii,* which may reduce aflatoxin production in the sample (Karlovsky et al., 2016; Adebo et al., 2019). Lactic acid bacteria isolated from *marcha* showed an antagonistic property (Tamang et al., 2007), similarly, some bacteria have antifungal activity against aflatoxin-producing *A. flavus* (Shakeel et al., 2018). *Rhizopus* spp. from *tempeh,* a fermented soybean food from Indonesia, were reported for detoxification of alfatoxins (Nakazato et al., 1990). *A. sydowii* present in samples *humao*, *phut* and *chowan*, is an industrially important filamentous mold, which produces monosaccharides and indole alkaloids (Zhou et al., 2018). None of the amylolytic starters of North East India showed the presence of *A. versicolor* except in *phut* samples from Arunachal Pradesh. *A. versicolor* is a slow-growing filamentous fungus commonly found in/on damp indoor environments (Samson et al., 2004), foods, and feeds (Jurjevic et al., 2012), and produces toxic metabolites (Piontek et al., 2016). Contamination of *A. versicolor* in *phut* samples might be from the damp room where preparation of *phut* is often practiced by starter-producers in Arunachal Pradesh.

[2]www.aspergilluspenicillium.org

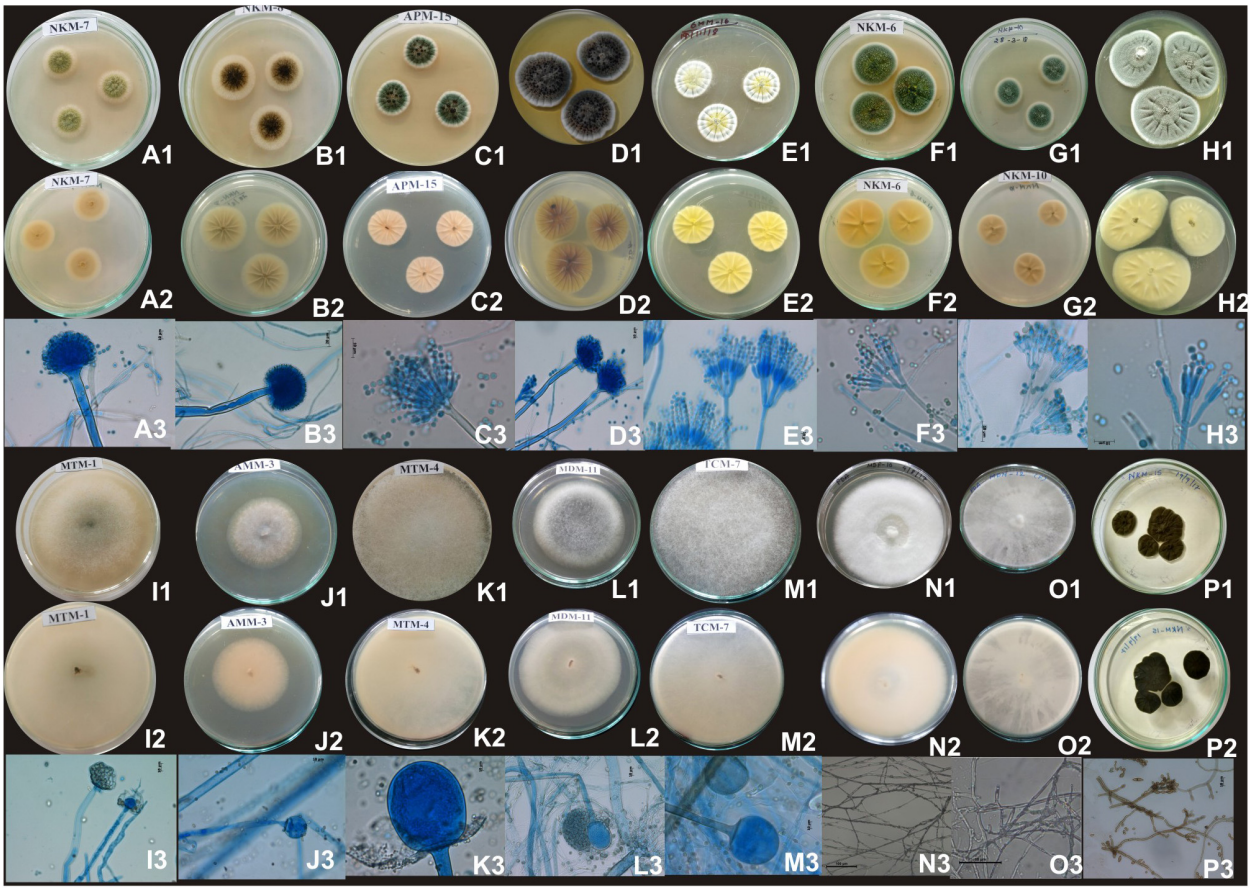

FIGURE 5 | Images of colony morphology and microscopic features of filamentous molds that grew on MEA media: *Aspergillus flavus* colonies top **(A1)**, reverse **(A2)**, Conidiophores **(A3)**; *Aspergillus niger* colonies top **(B1)**, reverse **(B2)**, mature conidia globose conidial head contain conidia **(B3)**; *Aspergillus sydowii* colonies top **(C1)**, reverse **(C2)**, mature conidiophore with vesicle bearing conidiogenous metulae and phailides (biserate) **(C3)**; *Aspergillus versicolor* colonies top **(D1)**, reverse **(D2)**, conidial heads supported vesicles with which are biseriate with metulae about the same size of philiades **(D3)**; *Penicillium chrysogenum* colonies top **(E1)**, reverse **(E2)**, smooth-walled conidiophores stipes (150–280 μm) and biverticillate **(E3)**; *Penicillium citrinum* colonies top **(F1)**, reverse **(F2)**, conidiophores stipes (150–280 μm) and biverticillate, phialides ampuliform (flask-shaped) **(F3)**; *Penicillium oxalicum* colonies top **(G1)**, reverse **(G2)**, mature conidiophores monoverticillate, or biverticillate and asymmetrical, phialides were cylindrical; *Penicillium polonicum* colonies top **(H1)**, reverse **(H2)**, conidiophore were terverticillate, phialides **(H)**; *Mucor circinelloides* colonies top **(I1)**, reverse **(I2)**, mature sporangiosphores contain sporangiospores **(I3)**; *Mucor indicus* colonies top **(J1)**, reverse **(J2)**, mature sporangiosphores contain sporangiospores **(J3)**; *Rhizopus delemar* colonies top **(K1)**, reverse **(K2)**, globose sporangium **(K3)**; *Rhizopus oryzae* colonies top **(L1)**, reverse **(L2)**, sporangiophores were usually straight, mostly 10∼20 μm **(L3)**; *Rhizopus microsporus* colonies top **(M1)**, reverse **(M2)**, sporangia globose, smooth and released spore **(M3)**; *Trametes hirsuta* colonies top **(N1)**, reverse **(N2)**, hyphal structure **(N3)**; *Bjerkandera adusta* colonies top **(O1)**, reverse **(O2)**, dichotomously branched hyphae **(O3)**; *Cladosporium parahalotolerans* colonies top **(P1)**, reverse **(P2)**, conidiophores and conidial chain **(P3)**.

Mucor circinelloides was found to be the most dominant fungus in dry starter cultures from North East India. *M. circinelloides* has a sub-globose sporangiospore with a sympodial branching pattern. Using the ITS sequencing tool, it is difficult to distinguish among the different species of the *Mucor circinelloides* complex (MCC) which include *M. circinelloides, M. griseocyanus, M. janssenii, M. lusitanicus, M. ramosissimus, M. variicolumellatus*, and *M. velutinosus* (Wagner et al., 2019). We therefore used species from the *Mucor circinelloides* complex. *Mucor circinelloides* contributes in saccharification and liquefaction of cereal during fermentation of *kodo ko jaanr*, an alcoholic product of Sikkim fermented by starter *marcha* (Thapa and Tamang, 2004; Tamang and Thapa, 2006). *M. circinelloides* is an oleaginous fungus (Qiao et al., 2018) which produces lipids (Wei et al., 2013), cellulose degrading enzymes (Huang et al., 2014), and has several functional properties including antioxidants (Hameed et al., 2017). Phylum Mucoromycota does not produce mycotoxins, however, some species that belong to this *M. circincelloides* forma *circinelloides* group has been described to be putatively responsible for human illnesses after consumption of mold-contaminated yogurt (Lee et al., 2014) although its involvement was not clearly proven. *M. circinelloides* was also reported earlier in *marcha* samples (Tamang et al., 1988; Tamang and Sarkar, 1995). *M. indicus*, isolated from *humao* from Assam and *phut* from Arunachal Pradesh, is a dimorphic and ethanolic fungus which is able to produce ethanol from glucose, mannose, fructose and galactose (Karimi and Zamani, 2013) and oil, protein, and glucosamine (Sharifyazd and Karimi, 2017).

TABLE 3 | Frequency, density, and diversity indices of filamentous molds observed in dry starters from North East India.

Filamentous molds	*Marcha*		*Thiat*		*Humao*		*Hamei*		*Chowan*		*Phut*		*Dawadim*		*Khekhari*	
	%															
	Fr	**RD**	**Fr**	**RD**	**Fr**	**RD**	**Fr**	**RD**	**Fr**	**RD**	**Fr**	**RD**	**Fr**	**RD**	**Fr**	**RD**
Aspergillus niger	0	0	0	0	0	0	0	0	0	0	0	0	0	0	25	0.25
Aspergillus flavus	16.6	0.16	0	0	0	0	0	0	0	0	0	0	0	0	12.5	0.12
Aspergillus sydowii	0	0	16.6	0.16	33.3	0.33	0	0	20	0.2	33.3	0.33	0	0	12.5	0.12
Aspergillus versicolor	0	0		0	0	0	0	0		0	16.6	0.16	0	0	0	0
Penicillium chrysogenum	16.6	0.16	16.6	0.16	0	0	0	0	20	0.2	0	0	16.6	0.16	0	0
Penicillium citrinum	0	0	0	0	16.6	0.16	50	0.5	0	0	0	0	0	0	12.5	0.12
Penicillium oxalicum	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12.5	0.12
Cladosporium parahalotolerans	0	0	0	0	0	0	0	0	0	0	0	0	0	0	12.5	0.12
Penicillium polonicum	16.6	0.16	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Mucor circinelloides	16.6	0.16	16.6	0.16	16.6	0.16	50	0.5	20	0.2	16.6	0.16	33.3	0.33	12.5	0.12
Mucor indicus	0	0		0	16.6	0.16	0	0	0	0	16.6	0.16	0	0	0	0
Rhizopus oryzae	0	0		0	16.6	0.16	0	0	20	0.2	16.6	0.16	0	0	0	0
Rhizopus delemar	0	0	16.6	0.16	0	0	0	0	0	0	0	0	0	0	0	0
Rhizopus microsporus	16.6	0.16667		0	0	0	0	0	0	0	0	0	16.6	0.16	0	0
Trametes hirsuta	0	0	16.6	0.16	0	0	0	0	0	0	0	0	0	0	0	0
Bjerkandera adusta	16.6	0.16667	16.6	0.16	0	0	0	0	20	0.2	0	0	33.3	0.33	0	0
DIVERSITY INDICES																
Species richness (R)	6		5		5		2		5		5		4		6	
Shannon's diversity index (H)	1.74		1.6		1.56		0.69		1.6		1.56		1.32		1.46	
Species evenness (E)	0.97		1		0.96		1		1		0.96		0.95		0.82	

Fr, Frequency of fungal species; RD, Relative density of fungal species in samples.

Phylogenetic and phylogenomic approaches show that genus *Rhizopus* has three major clades viz. *R. microsporus with* its sister taxon *R. stolonifer*, *R. arrhizus*, and *R. delemar* (Gryganskyi et al., 2018). *Rhizopus oryzae,* commonly inhabits soils, animal excrement, and rotting vegetables (Ghosh and Ray, 2011), and is very similar to *Rhizopus stolonifer*, except for its smaller sporangia with air-dispersed sporangiospores (Pitt and Hocking, 2009). *R. oryzae* and *R. microsporus* are detected in *yao qu* from China and *banh men* from Vietnam, which are strong amylase producers (Dung et al., 2007; Thanh et al., 2008; Lv et al., 2012b). *R. oryzae* is considered as a GRAS filamentous fungus (Londoño-Hernández et al., 2017), which is commonly used for production of some Asian fermented foods (Tamang et al., 2016). *Rhizopus microsporus* is the major fungus in *tempe*, a fermented soybean food from Indonesia (Hartanti et al., 2015). *R. delemar* was found in the *thiat* sample only, which naturally accumulates fumaric acid with a fruity taste (Odoni et al., 2017), and it probably imparts taste and flavor in *kiad,* an alcoholic product fermented by the starter *thiat*. *R. delemar* has also been reported in *xajpitha,* starter from Assam in India (Bora et al., 2016). Presence of *Rhizopus* spp. in starters from North East India may contribute functionalities in end products during acholic fermentation.

Penicillium chrysogenum was found in only four types of starters viz. *marcha* (Sikkim), *thiat* (Meghalaya), *chowan* (Tripura), and *dowdim* (Mizoram). The probable entry of *P. chrysogenum* during traditional preparation may be from damp and moist rooms where preparation for such starters is usually done, since *P. chrysogenum* is also found in damp buildings (Andersen et al., 2011). Due to the ability of *P. chrysogenum* to produce antibiotics, mostly penicillin (Bajaj et al., 2014), its presence in starters may have an antagonist property in the end product. *P. citrinum* was recovered in samples of *humao, hamei* and *khekhrii*, probably from indoor environments (Samson et al., 2004). *P. oxalicum* was found in samples of *khekhrii* (Nagaland) and *P. polonicum* in *marcha* samples. *P. oxalicum* produces various enzymes and natural products (Li et al., 2016). *P. polonicum* has also been reported in fermented black table olives (Bavaro et al., 2017).

It is interesting to note that we detected Basidiomycetous fungi represented by *Bjerkandera adusta* in samples of *marcha, thiat, dawdim,* and *chowan*, and also *Trametes hirsuta* in *thiat* samples. *Bjerkandera adusta* and *Trametes hirsuta* are wood decaying white-rot fungi (Rosales et al., 2005; Horisawa et al., 2019). *B. adusta* grows on a natural cellulosic substrate, imparts a refreshing aroma (Zhang et al., 2015), contributes to saccharification (Quiroz-Castañeda et al., 2009), and produces ethanol (Horisawa et al., 2019). *Trametes hirsuta* is lignin-degrading fungus due its ability to synthesize laccase (Cilerdzic et al., 2011). Traditional methods of preparation of these amylolytic starter cultures require locally grown wild herbs and spices used as ingredients by local starter-makers (Anupma et al., 2018). We assume that during collection of wild herbs from forest grounds, people might have collected whole wild plants *in situ*, where wood-rooting fungi have been reported in forests of North East India (Chuzho et al., 2017). There is no practice of filtering and cleaning of collected wild plants during starter preparation, hence chances for contamination of these basidiomycetous fungi may be possible during preparation. *B. adusta* and *T. hirsuta* were not reported earlier in any starter culture or in any fermented food.

Cladosporium parahalotolerance was found only in samples of *khekhrii*. *C. parahalotolerance* mostly occurred in plant debris, foods, and indoors (Bensch et al., 2012). Source of *Cladosporium* in *khekhrii* might be from wild herbs used as ingredients during traditional preparation of *khekhrii* in Nagaland. Species of *Bjerkandera, Trametes,* and *Cladosporium* have not been reported in any fermented foods elsewhere.

Diversity indexes determine the phylogenetic relations within different fungal species in a community (Fernandes et al., 2015). We calculated diversity indexes of fungal community present in starters of North East India by Shannon's diversity index (H), species evenness (E), and species richness (R). Shannon diversity index *H* for evaluating fungal diversity was recorded highest in *marcha* samples collected from Sikkim (H: 1.74) and lowest in *hamei* samples of Manipur (H: 0.69) indicating higher fungal diversity in *marcha* samples of Sikkim as compared to starters of other states. The diversity index, which considers both the number of species as well as relative abundance of each species for evaluating diversity (Lucas et al., 2017), showed the highest value for *marcha* of Sikkim. Species richness is the number of different species represented in an ecological community, where it reflects the abundances of species or their distributions (Unterseher et al., 2008). Species Richness (R) values in samples of *marcha* and *khekhrii* were recorded as the highest showing more diversity in species level of filamentous molds. Species evenness refers to how equal the community is numerically, ranging from 0 to 1 (Savary et al., 2018) signifying that the value 1.0 in *thiat, hamei,* and *chowan* have a complete evenness in comparison to other starters. Hence diversity index of filamentous fungal community present in dry starters of North East India showed high diversity within the community. It was observed that there was variation in fungal species distribution in each type of amylolytic starters in North East India which determines the quality of the acholic product, preferred by the local consumers. This might be due to varied geographical regions, environmental conditions, and different plant species used in the preparation methods of amylolytic starters. It therefore shows that fungal diversity, present in amylase and alcohol-producing starters, traditionally prepared by ethnic Indian people using their indigenous knowledge of "back-slopping," are morphologically, ecologically, and phylogenetically diverse. Our findings on fungal diversity in amylolytic starters from North East India may supplement the microbial diversity in ecosystems of North East India, which is one of the biodiversity hot spots of the world.

CONCLUSION

Traditionally prepared amylolytic starters are consortia of filamentous fungi, yeasts, and bacteria which are traditionally sub-cultured and preserved using traditional methods of

"back-slopping" by the ethnic people of North East India for production of alcoholic beverages. Yeasts and bacteria present in these starters have already been reported in earlier studies. However, no information on fungal communities and their diversity in Indian amylolytic starters is available. We therefore identified the filamentous molds isolated from *marcha, thiat, humao*, *hamei*, *chowan*, *phut*, *dawdim,* and *khekhrii* based on morphological and sequence-based identifications. We identified seven genera with 16 species represented by *Aspergillus flavus, Aspergillus niger*, *Aspergillus sydowii*, *Aspergillus versicolor*, *Bjerkandera adusta*, *Cladosporium parahalotolerans*, *Mucor circinelloides*, *Mucor indicus*, *Penicillium chrysogenum*, *Penicillium citrinum*, *Penicillium oxalicum*, *Penicillium polonicum, Rhizopus delemar, Rhizopus microsporus, Rhizopus oryzae,* and *Trametes hirsuta*. Fungal species present in these traditionally prepared dry starters are morphologically, ecologically, and phylogenetically diverse and showed high diversity within the community.

DATA AVAILABILITY STATEMENT

The sequences of the internal transcribed spacers (ITS) region obtained in this study were deposited at the GenBank-NCBI database 6S rRNA sequencing were deposited at GenBank-NCBI numbers: MK396469-MK396484, MK396486-MK396500, MK778442-MK778449, MK796041-MK796045.

AUTHOR CONTRIBUTIONS

AA performed the experiments. JT supervised the experiments and finalized the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2020.00905/full#supplementary-material

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