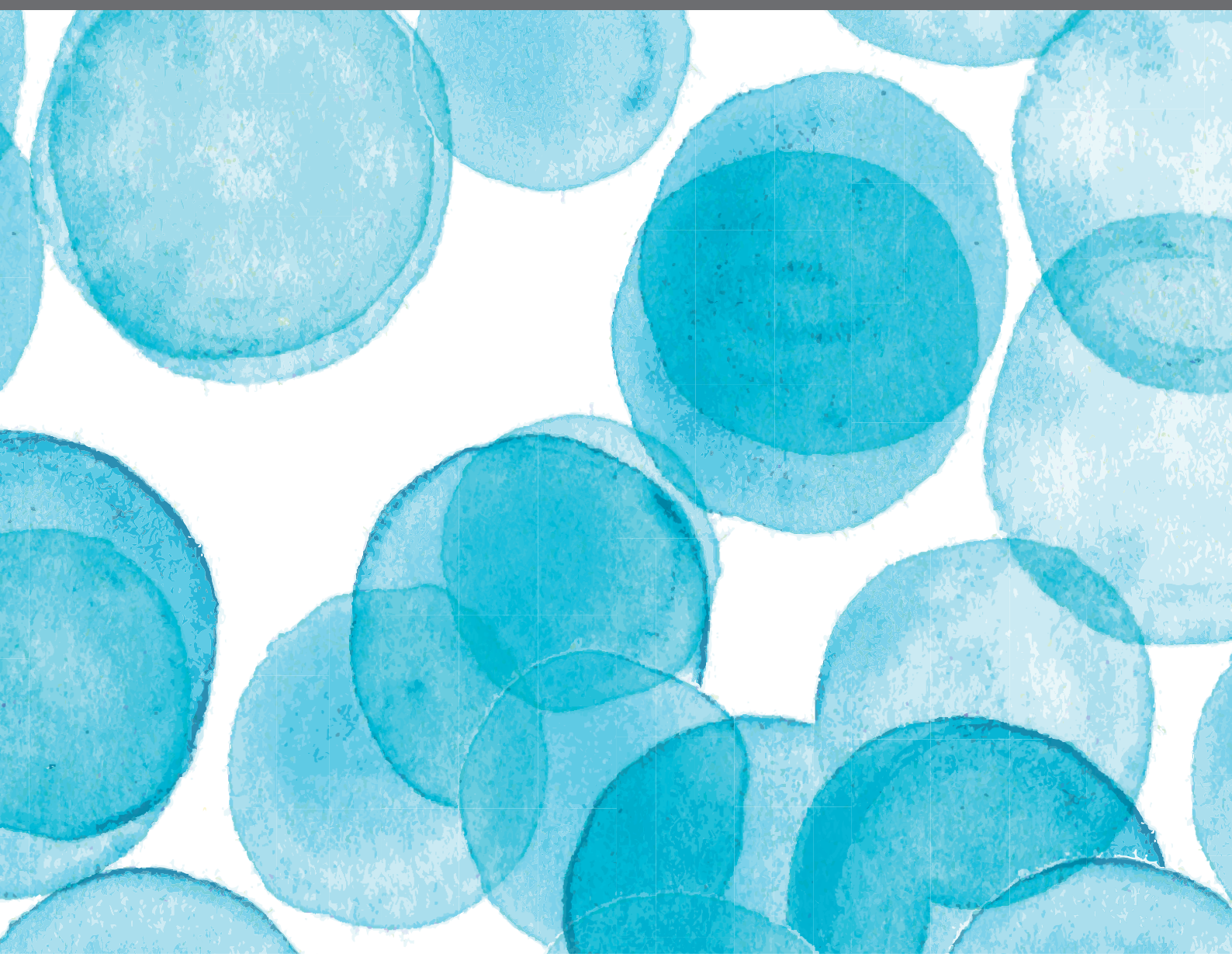
NOVEL RESEARCH ON METABOLITES SECRETED BY GRAM-POSITIVE BACTERIA

EDITED BY: Dennis Ken Bideshi, Hyun-Woo Park, José E. Barboza-Corona and Rubén Salcedo-Hernández

PUBLISHED IN: Frontiers in Microbiology



frontiers Research Topics

frontiers

ISSN 1664-8714
ISBN 978-2-83250-798-8
DOI 10.3389/978-2-83250-798-8

About Frontiers

Frontiers is more than just an open-access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers Journal Series

The Frontiers Journal Series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the Frontiers Journal Series operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to Quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews.
Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the Frontiers Journals Series: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area! Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers Editorial Office: frontiersin.org/about/contact

NOVEL RESEARCH ON METABOLITES SECRETED BY GRAM-POSITIVE BACTERIA

Topic Editors:
Dennis Ken Bideshi, California Baptist University, United States
Hyun-Woo Park, California Baptist University, United States
José E. Barboza-Corona, University of Guanajuato, Mexico
Rubén Salcedo-Hernández, University of Guanajuato, Mexico

Citation: Bideshi, D. K., Park, H.-W., Barboza-Corona, J. E., Salcedo-Hernández, R., eds. (2023). Novel Research on Metabolites Secreted by Gram-Positive Bacteria. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-83250-798-8

Table of Contents

frontiers | Frontiers in Microbiology

TYPE Editorial
PUBLISHED 27 June 2023
DOI 10.3389/fmicb.2023.1237790

OPEN ACCESS

EDITED AND REVIEWED BY
Eric Altermann,
Massey University, New Zealand

*CORRESPONDENCE
José E. Barboza-Corona
✉ josebar@ugto.mx

RECEIVED 10 June 2023
ACCEPTED 14 June 2023
PUBLISHED 27 June 2023

CITATION
Barboza-Corona JE, Bideshi DK, Park H-W and Salcedo-Hernández R (2023) Editorial: Novel research on metabolites secreted by gram-positive bacteria.
Front. Microbiol. 14:1237790.
doi: 10.3389/fmicb.2023.1237790

Editorial: Novel research on metabolites secreted by gram-positive bacteria

José E. Barboza-Corona[1]*, Dennis K. Bideshi[2], Hyun-Woo Park[2] and Rubén Salcedo-Hernández[1]

[1]Departamento de Alimentos, Posgrado en Biociencias, Universidad de Guanajuato, Campus Irapuato-Salamanca, Irapuato, Guanajuato, Mexico, [2]Department of Biological Sciences, Program in Biomedical Sciences, California Baptist University, Riverside, CA, United States

KEYWORDS

bacteria, secretion proteins, antimicrobial peptides, hydrolytic enzymes, secondary metabolites

Editorial on the Research Topic
Novel research on metabolites secreted by gram-positive bacteria

Bacteria play essential roles in nature and have a ubiquitous distribution. Their mutualistic, commensal, and parasitic associations with humans, animals, fungi, and plants have been extensively documented. Many of these synergistic interactions are mediated by biomolecules they secrete, which are of industrial and pharmaceutical interests. Bacteria are classified as Gram-positive or Gram-negative based primarily on their cell wall structure. Gram-positive bacteria secrete metabolites directly into culture media, facilitating rapid recovery for downstream applications. The papers presented in "*Novel research on metabolites secreted by gram-positive bacteria*" include original research, reviews, and *in silico* analyses of compounds with antimicrobial and bioactive properties produced by Gram-positive bacteria from different ecological niches.

In the first paper, *In silico Prediction and Exploration of Potential Bacteriocin Gene Clusters Within the Bacterial Genus Geobacillus*, Egan et al. utilized the BAGEL3 software to scan publicly available genomes that harbor putative bacteriocin gene clusters. They identified the endospore-forming genus *Geobacillus* as a potential source of novel bacteriocins that could have commercial and therapeutic value in treating antibiotic-resistant bacteria of clinical significance.

Recognized as probiotic bacteria, the ability of *Lactococcus* and *Lactobacillus* species to support immune balance and gut epithelial integrity is of crucial interest. Sokovic Bajic et al. showed that *Lactobacillus brevis* BGZLS10-17, a strain isolated from Zlatar cheese, can produce high levels of gamma amino butyric acid (GABA) and reduced adhesion of *Salmonella enterica* and *Escherichia coli* to colonocyte-like Caco-2 cells. Additionally, the monolayer of differentiated Caco-2 cells treated with GABA-containing supernatants alleviated an inflammatory response and increased the production of tight junction proteins. Their findings demonstrated the possibility of developing novel functional dairy products and beverages that can be useful in modulating and suppressing immunological responses that give rise to clinically significant inflammatory conditions in the digestive system.

One of the technical problems associated with acquiring sufficient bacteriocins for basic and applied studies is that they are synthesized in low amounts by the native producer strains. Within the *Lactococcus* group, Telke et al. reported that *Lactococcus garvieae* KS1546

produces a bacteriocin called Garvicin, that is active against clinically significant bacteria. By combining different strategies, including genetic engineering, and modifying culture media and conditions, they produced a KS1546 strain that exhibited a 2000-fold increase in Garvicin synthesis.

Optimizing the growth of beneficial microbes in natural niches using "helper" microbes is also of significant interest. Toward this end, Xu et al. showed that adding *Lactobacillus plantarum* and *L. buchneri* to corn silage favored the proliferation of native beneficial *Lactobacillus* species while suppressing the growth of other species. Interestingly, the study also showed that corn silage treated with *L. plantarum* and *L. buchneri* increased metabolite yields of antimicrobial and antioxidant activities and bioactive compounds that can reduce cholesterol and help control depression.

Another intriguing microbial group is represented by *Bacillus* spp., which produces metabolites of interest to the food, pharmaceutical, and veterinary industries. Taking this into account, Caulier et al. provide a timely review of antimicrobial metabolites produced by the *Bacillus subtilis* group and propose a classification system based on the biosynthetic pathways, i.e., ribosomal peptides (RPs), non-ribosomal peptides (NRPs), volatiles, polyketides (PKs), compounds, and hybrids between PKs and NRPs, and the chemical nature of these microbes.

In addition to antimicrobials, *Bacillus* and actinobacteria species synthesize compounds beneficial to plants, and their symbiotic interactions with plants result in the generation of biomolecules with broad applications. In their paper titled, *Bioactive products from plant-endophytic gram-positive bacteria*, Ek-Ramos et al. provide a comprehensive review of metabolites produced by *Bacillus* and actinobacteria endophytes. These metabolites include aromatic compounds, lipopeptides, plant hormones involved in promoting plant growth, polysaccharides, enzymes, and other compounds that potentially have applied use in agriculture and human and veterinary medicine.

Compared to hydrated biomes, relatively fewer studies have been conducted on microbial competition in arid soils. The study by Nasfi et al. found that the dominant species in arid rhizosphere soil were *Bacillus* sp. Interestingly, 93% of the isolates were active against Gram-positive and Gram-negative bacteria. Among these isolates was *Bacillus* sp. M2 that showed promising antimicrobial activities against Gram-negative bacteria. The inhibitory activity was attributed to the organic compound 1-acetyl-β-carboline, which also has antitumor, antiviral, and antiparasitic properties. *Bacillus* sp. M2 also produced fungicides such as bacillomycin, fengycin and surfactins, which could be helpful in controlling other organisms. Regarding the latter, Rodriguez et al. showed that *Bacillus atrophaeus* produces lipopeptides, including surfactins, fengycins, and bacillomycins that damage the cuticle membrane and decrease the foraging capability of *Rhopalosiphum padi*, an aphid that elicit significant economic losses to cereal crops.

Gram-positive bacteria can also be used as microbial cell factories to produce metabolic precursors of biomolecules that can treat human diseases such as cancer. Abdallah et al. successfully produced taxadiene, a precursor of taxol, through metabolic engineering of *B. subtilis* 168 by transforming this strain to produce taxadiene synthetase (TXS). TXS converts geranylgeranyl pyrophosphate (GGPP) to taxa-4,11-diene. Moreover, by further engineering the TXS-producing *B. subtilis* to express the *ispA* and *crtE* genes and a synthetic operon that increases the supply and flux of the GGPP precursor, they increased the production of taxadiene 81-fold. Finally, small molecules, such as pipecolic acid or L-PA, have gained importance in the pharmaceutical and chemical industries, as these compounds are important in synthesizing specific amino acids, neurotransmitters, and in plant immune defenses against bacterial pathogens. These metabolites can be synthesized in *Corynebacterium glutamicum*. Understanding L-PA's physiological function in microbial factories is essential to the commercial development of these microbial factories. Pérez-Garcia et al. showed that the external addition of L-AP to a culture medium or its *de novo* synthesis is advantageous for bacterial growth under hyper-osmotic stress conditions and that proline permease ProP and the mechanosensitive channel YggB are involved in the export and import of L-PA.

Conclusion

In conclusion, Gram-positive bacteria continue to be a resource for secreted biomolecules, including antimicrobial metabolites, lipopeptides, metabolites (GABA, 1-acetyl-β-carboline), and organic acids, which not only naturally influence the establishment and maintenance of diverse ecological niches, but also for multiple applications in human and animal health, plant crop protection, and the food industry. Current developments in genetic manipulations focused on engineering Gram-positive bacteria to be used as microbial factories to express proteins or metabolites with biotechnological applications are intriguing. Indeed, synthetic biology will prove to be indispensable in translational endeavors. Although this Research Topic highlighted, arguably, of the most significant resource species, i.e., *Lactococcus*, *Lactobacillus*, and *Bacillus*, it is clear that current and future exploration of less studied microbes, including *Geobacillus,* will undoubtedly identify novel metabolites secreted by a broad range of Gram-positive bacteria that can be exploited for applied purposes.

Author contributions

JB-C and DB wrote the editorial. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

frontiers in Microbiology

ORIGINAL RESEARCH
published: 20 September 2018
doi: 10.3389/fmicb.2018.02116

In silico Prediction and Exploration of Potential Bacteriocin Gene Clusters Within the Bacterial Genus *Geobacillus*

Kevin Egan[1], *Des Field*[1,2], *R. Paul Ross*[1,2], *Paul D. Cotter*[2,3] *and Colin Hill*[1,2]*

[1] School of Microbiology, University College, Cork, Ireland, [2] APC Microbiome Institute, Cork, Cork, Ireland, [3] Teagasc Food Research Centre, Moorepark, Fermoy, Co., Cork, Ireland

OPEN ACCESS

Edited by:
José E. Barboza-Corona, Universidad de Guanajuato, Mexico

Reviewed by:
Gustavo Hernandez Guzman, Universidad de Guanajuato, Mexico
Jin Zhong, State Key Laboratory of Microbial Resources, Institute of Microbiology (CAS), China
Agustino Martínez Antonio, Unidad Irapuato (CINVESTAV), Mexico

***Correspondence:**
Colin Hill
c.hill@ucc.ie

Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: *20 June 2018*
Accepted: *20 August 2018*
Published: *20 September 2018*

Citation:
Egan K, Field D, Ross RP, Cotter PD and Hill C (2018) In silico Prediction and Exploration of Potential Bacteriocin Gene Clusters Within the Bacterial Genus Geobacillus. Front. Microbiol. 9:2116. doi: 10.3389/fmicb.2018.02116

The thermophilic, endospore-forming genus of *Geobacillus* has historically been associated with spoilage of canned food. However, in recent years it has become the subject of much attention due its biotechnological potential in areas such as enzyme and biofuel applications. One aspect of this genus that has not been fully explored or realized is its use as a source of novel forms of the ribosomally synthesized antimicrobial peptides known as bacteriocins. To date only two bacteriocins have been fully characterized within this genus, i.e., Geobacillin I and II, with only a small number of others partially characterized. Here we bioinformatically investigate the potential of this genus as a source of novel bacteriocins through the use of the *in silico* screening software BAGEL3, which scans publically available genomes for potential bacteriocin gene clusters. In this study we examined the association of bacteriocin gene presence with niche and phylogenetic position within the genus. We also identified a number of candidates from multiple bacteriocin classes which may be promising antimicrobial candidates when investigated *in vitro* in future studies.

Keywords: bacteriocin, antimicrobial, bioinformatics, *in silico* screen, *Geobacillus*

INTRODUCTION

The genus *Geobacillus* is composed of thermophillic, rod shaped, spore-forming, aerobic or facultative anaerobic bacteria. Their defining feature is their ability to grow at elevated temperatures of up to 80°C, with most isolates having growth temperature optima between 45° and 70°C (Nazina et al., 2001; Zeigler, 2014). Their sporulating nature makes their presence particularly challenging in food as they may survive intensive thermal processing methods and germinate when optimum conditions exist at a later period (Egan et al., 2016). In recent years this genus has attracted ever greater attention due an increased appreciation of its biotechnological potential, e.g., as sources of thermostable enzymes, as well as the biofuel and bioremediation industries (Cripps et al., 2009; Hussein et al., 2015; Kananavičiūtė and Citavičius, 2015; Studholme, 2015). One application of *Geobacillus* which has not yet been fully explored relates to their usefulness as a source of novel and highly potent antimicrobial peptides called bacteriocins.

Bacteriocins are ribosomally synthesized, narrow or broad-spectrum, antimicrobial peptides produced by bacteria. They can be broadly classified into three classes: class I post translationally modified, class II unmodified and class III <10 kDa in size (Arnison et al., 2013; Cotter et al., 2013). In past decades bacteriocins have been isolated primarily from lactic acid bacteria (LAB) due to their generally recognized as safe (GRAS) status which allows them to be used in food (Cotter et al., 2005). With the widespread use of *in silico* screening (Marsh et al., 2010; Azevedo et al., 2015; Walsh et al., 2015; Collins et al., 2017) and large culture based screening projects (Rea et al., 2010), bacteriocin candidates have been identified from alternative bacterial genera isolated from environmental, food or clinical samples. However, relatively few *Geobacillus*-associated bacteriocins have been identified to date (Pokusaeva et al., 2009; Garg et al., 2012; Özdemir and Biyik, 2012; Alkhalili et al., 2016), with very little genetic or structural information available with respect to these peptides. Geobacillin I and II represent the only two well characterized lantibiotic (class I) bacteriocins from this genus, with a large amount of information available with regard to antimicrobial spectrum, physiochemical characteristics and genetic determinants (Garg et al., 2012).

In silico screening of bacterial genomes for novel bacteriocins has become a staple element of bacteriocin discovery and characterisation over the past decade. Its widespread use and popularity has been driven by its ability to reduce time and cost relative to culture based bacteriocin screening studies. First generation *in silico* screening of bacterial genomes required the use of "driver genes" to predict potential new bacteriocin genes within genomes (Begley et al., 2009; Marsh et al., 2010). However, in recent years the bacteriocin prediction software BAGEL3 (van Heel et al., 2013) has become the tool of choice for *in silico* bacteriocin discovery. BAGEL3 searches bacterial genomes in DNA FASTA format using two different approaches to discover new bacteriocins, i.e., (1) detection of bacteriocin structural genes and (2) detecting other genes commonly associated with bacteriocin production. Those bacteriocins which are identified using both approaches are compared and filtered to remove duplicate candidates. Furthermore this software can be supplemented with traditional "driver gene" *in silico* screening or even with other programs such as antismash 3.0, which can detect other classes of antimicrobial peptides such as Non-Ribosomal PolyKetide (NRPK) antimicrobials (Weber et al., 2015).

This study set out to use BAGEL3 (van Heel et al., 2013) to perform an *in silico* screen of publically available *Geobacillus* genomes in an attempt to identify bacteriocin candidates for future *in vitro* experiments. The specific objectives were to (1) identify potential structural peptides within *Geobacillus* genomes; (2) investigate the possibility of a relationship between genome phylogenetic position and gene presence; and (3) examine any homology between structural peptide-encoding and surrounding genes with previously characterized bacteriocin gene clusters.

Abbreviations: LAPs, Linear Azole-containing Peptides; PTM, Post Translational Modification; PBGCs, Potential Bacteriocin Gene Clusters; WGS, Whole Genome Sequencing.

MATERIALS AND METHODS

Bacteriocin Identification and Visualization

Using the *in silico* bacteriocin prediction tool BAGEL3 (van Heel et al., 2013), genome sequences belonging to the genus *Geobacillus* (**Table 1**) were aquired and analyzed. Amino acid sequences of all 16 class III bacteriocins were aquired from Bactibase (Hammami et al., 2007) and aligned against the genomes as driver sequences using blastP (Altschul et al., 1990). Where necessary NisP (NCBI protein ID: AAA25200.1) and NisT (NCBI protein ID: AAA25191.1) driver sequences were used to seek and identify LanT and LanP-determinants in genome sequences. Those bacteriocin genes predicted were further visualized using Artemis genome visualization tool (Rutherford et al., 2000). Blastn and blastP (Altschul et al., 1990) were used to determine the % identities between putative peptides/genes and those accurately curated. Structural peptides were aligned using the Multiple Sequence Alignment (MSA) tool MUSCLE (Edgar, 2004) and then visualized using Jalview (Waterhouse et al., 2009). The previously generated MUSCLE peptide alignments were then input into the MEGA 7 software package (Kumar et al., 2016) for phylogenetic analysis. Using a neighbor-joining method, an unrooted phylogenetic tree was generated using a Jukes–Cantor method (Dukes and Cantor, 1969) and bootstrap replication values of 1,000 similarly to that by Zhang et al. (2015). In alignments where specific sequences contained no common sites, these were deleted. The resulting nexus tree files were exported to the interactive tree of life (itol) (Letunic and Bork, 2016) for graphical adjustment.

Phylogenetic Analysis of *Geobacillus* Species

Where available, 16S sequences were acquired from genbank, however if no 16S sequence was available the *in silico* prediction tool RNAmmer (Lagesen et al., 2007) was used. The *B. cereus* ATCC14579 16S sequence was selected as a root for the final version of the tree. All 16S sequences were then collated and aligned as before using the MSA tool MUSCLE (Edgar, 2004). The resulting alignment output was then input into MEGA 7 (Kumar et al., 2016). Similar to Cihan et al. (2011), a neighbor-joining tree was generated using bootstrap values based on 1,000 replications and the resulting nexus tree file was then input into the itol software (Letunic and Bork, 2016) for final graphic adjustments. Where no common sites were found for specific peptides in the generation of the phylogenetic tree they were not included in the phylogenetic arrangement. The strains which had nether pre-determined or non-predictable 16S rRNA sequences were excluded from the overall study. The bacteriocin predictions by BAGEL3 were subsequently overlaid onto the phylogenetic tree using microsoft Powerpoint.

RESULTS

Bacteriocin Cluster Distribution Across the Genus of *Geobacillus*

This study sets out to use an *in silico* approach to determine both the prevalence and diversity of bacteriocin gene clusters within

TABLE 1 | List of *Geobacillus* genomes examined in this *in silico* screen.

Number	Species	Strain ID	Accession no.	Source	Country	Sample type	Bacteriocin encoded?
1	*G. galactosidius*	DSM18751	GCA_002217735.1	Compost	Italy	Environmental	Lantibiotic; LAPs
2	*G. icigianus*	G1w1	GCA_000750005.1	Hydrothermal samples	Russia	Environmental	No
3	*G. kaustophilus*	Et2/3	GCA_000948165.1	Geyser	Chile	Environmental	Circular; Sactipeptide
4	*G. kaustophilus*	Et7/4	GCA_000948285.1	Geyser	Chile	Environmental	Circular
5	*G. kaustophilus*	HTA426	GCA_000009785.1	Deep sea sediment	Marina trench	Environmental	Lantibiotic; circular
6	*G. litanicus*	N-3	GCA_002243605.1	High temp oilfield	Litunia	Environmental	Lantibiotic; Circular
7	*G.* sp.	Y4.1MC1	GCA_000166075.1	Hot Spring	USA	Environmental	LAPs; Class II
8	*G.* sp.	FJ8	GCA_000445995.2	Compost	Japan	Environmental	No
9	*G.* sp.	44B	GCA_002077755.1	Deep subsurface	USA	Environmental	Sactibiotic; LAPs
10	*G.* sp.	44C	GCA_002077865.1	Deep subsurface	USA	Environmental	Lantibiotic; Circular; LAPs
11	*G.* sp.	WCH70	GCA_000023385.1	Compost	USA	Environmental	Class II; LAPs
12	*G.* sp.	46C-IIa	GCA_002077765.1	Deep subsurface	USA	Environmental	No
13	*G.* sp.	47C-IIb	GCA_002077775.1	Deep subsurface	USA	Environmental	Sactibitoic
14	*G.* sp.	PA-3	GCA_001412125.1	Soil	Litunia	Environmental	Lantibitoic; Sactibitoic
15	*G.* sp.	12AMOR1	GCA_001028085.1	Deep sea hydrothermal vent	Unknown	Environmental	Sactibiotic
16	*G.* sp.	LEMMY01	GCA_002042905.1	Soil	Brazil	Environmental	Lantibitoic; Sactibiotic; Circular
17	*G.* sp.	1017	GCA_001908025.1	Oil water	China	Environmental	Lantibiotic
18	*G.* sp.	GHH01	GCA_000336445.1	Soil sample	Germany	Environmental	No
19	*G.* sp.	Y4.12MC61	GCA_000024705.1	Hot spring	USA	Environmental	Circular
20	*G.* sp.	Y4.12MC52	GCA_000174795.2	Hot spring	USA	Environmental	Circular
21	*G.* sp.	Sah69	GCA_001414205.1	Soil	Algeria	Environmental	Sactibitoic
22	*G.* sp.	JS12	GCA_001592395.1	Compost	South Korea	Environmental	Lantibiotic; Sactibiotic
23	*G.* sp.	T6	GCA_001025095.1	Hot water spring	Argentina	Environmental	Circular
24	*G.* sp.	BC02	GCA_001294475.1	Bore well isolate	Australia	Environmental	Circular; Sactibiotic
25	*G.* sp.	WSUCF1	GCA_000422025.1	Soil	USA	Environmental	No
26	*G.* sp.	FJAT-46040	GCA_002335725.1	Hot spring	China	Environmental	No
27	*G.* sp.	ZGt-1	GCA_001026865.1	Hot spring	Jordan	Environmental	Lantibiotic
28	*G.* sp.	A8	GCA_000447395.1	Deep mine	South africa	Environmental	No
29	*G.* sp.	CAMR5420	GCA_000691465.1	Unknown	Unknown	Environmental	No
30	*G. stearothermophilus*	10	GCA_001274575.1	Hot spring	USA	Environmental	Sactibiotic; Circular
31	*G. stearothermophilus*	22	GCA_000743495.1	Hot spring	Russia	Environmental	No
32	*G. stearothermophilus*	53	GCA_000749985.1	Hot Spring	Russia	Environmental	No
33	*G. stearothermophilus*	C1BS50MT1	GCA_001620045.1	Water sediment	Australia	Environmental	Circular
34	*G. subterraneus*	KCTC3922	GCA_001618685.1	Subsurface Oil field	China	Environmental	No
35	*G. subterraneus*	K	GCA_001632595.1	Oilfield	Russia	Environmental	No
36	*G. thermocatenulatus*	KCTC3921	GCA_002243665.1	Gas well isolate	USSR	Environmental	Lantibiotic; Circular
37	*G. thermocatenulatus*	BGSC93A1	GCA_002217655.1	Oilfield	Russia	Environmental	Lantibiotic; Circular
38	*G. thermocatenulatus*	SURF-48B	GCA_002077815.1	Deep subsurface	USA	Environmental	No
39	*G. thermodenitrificans*	NG80-2	GCA_000015745.1	Deep subsurface	China	Environmental	Geobacillin I; Geobacillin II
40	*G. thermodenitrificans*	T12	GCA_002119625.1	Compost	Neatherlands	Environmental	No
41	*G. thermoleovorans*	CCB US3 UF5	GCA_000236605.1	Hot spring	Malaysia	Environmental	Lantibiotic; Circular
42	*G. thermoleovorans*	FJAT-2391	GCA_001719205.1	Soil	China	Environmental	No
43	*G. thermoleovorans*	KCTC3570	GCA_001610955.1	Soil	USA	Environmental	No
44	*G. thermoleovorans*	N7	GCA_001707765.1	Hot spring	India	Environmental	Circular
45	*G. thermoleovorans*	B23	GCA_000474195.1	Deep oil reserve	Japan	Environmental	Lantibiotic
46	*G. uzenesis*	BGSC92A1	GCA_002217665.1	Oilfield	Russia	Environmental	No
47	*G.* sp.	B4113	GCA_001587475.1	Mushroom soup	Neatherlands	Food	LAPs; Circular
48	*G. kaustophilus*	NBRC102445	GCA_000739955.1	Pasteurized milk	Unknown	Food	Lantibiotic
49	*G. stearothermophilus*	A1	GCA_001183895.1	Milk powder facility	New Zealand	Food	Sactibiotic; Circular

(Continued)

TABLE 1 | Continued

Number	Species	Strain ID	Accession no.	Source	Country	Sample type	Bacteriocin encoded?
50	*G. stearothermophilus*	B4114	GCA_001587395.1	Buttermilk power	Neatherlands	Food	Sactibiotic; Circular
51	*G. stearothermophilus*	D1	GCA_001183885.1	Milk powder facility	New Zealand	Food	Sactibiotic; Circular
52	*G. stearothermophilus*	P3	GCA_001183915.1	Milk powder facility	New Zealand	Food	Sactibiotic; Circular
53	*G. stearothermophilus*	DSM 458	GCA_002300135.1	Sugar beet juice	Austria	Food	Circular
54	*G. stearothermophilus*	GS27	GCA_001651555.1	Casein pipeline	Neatherlands	Food	Sactibiotic; Circular
55	*G. stearothermophilus*	ATCC 12980	GCA_001277805.1	Spoilled canned food	USA	Food	Sactibiotic; Circular
56	*G thermodenitrificans*	DSM 465	GCA_000496575.1	Sugar beet juice	Austria	Food	Lantibiotic
57	*G. thermodenitrificans*	KCTC3902	GCA_002072065.1	Sugar Beet juice	Austria	Food	Lantibiotic
58	*G. jurassicus*	NBRC107829	GCA_001544315.	Unknown	Unknown	Unknown	Sactibiotic
59	*G. kaustophilus*	GBlys	GCA_000415905.1	Unknown	Unknown	Unknown	Circular
60	*G.* sp.	G11MC16	GCA_000173035.1	Unknown	unknown	unknown	Lantibiotic
61	*G.* sp.	LC300	GCA_001191625.1	Bioreactor	USA	Unknown	Circular
62	*G.* sp.	C56-T3	GCA_000092445.1	Unknown	Unknown	Unknown	Circular
63	*G.* sp.	CAMR12739	GCA_000691445.1	Unknown	Iceland	unknown	Sactibitoic; Circular
64	*G.* sp	FW23	GCA_000617945.1	Oil well	India	unknown	Lantibiotic
65	*G. stearothermophilus*	ATCC7953	GCA_000705495.1	Unknown	Unknown	unknown	Circular
66	*G. subterraneus*	PSS2	GCA_000744755.1	Unknown	Unknown	unknown	Lantibiotic; Circular
67	*G. vulcani*	PSS1	GCA_000733845.1	Human Microbiome isolate	Japan	Human	Circular

Also included is their accession numbers, location and type of bacteriocin predicted by BAGEL3.

the genus *Geobacillus*. Utilizing the genome sequences available in the public databases, 67 genomes (**Table 1**) representing 12 *Geobacillus* species, including *galactosidius*, *iciganius*, *jerrasicus*, *kaustophilus*, *liticanus*, *stearothermophilus*, *subterraneus*, *thermogalactosidius*, *thermoleovorans*, *thermocatenulatus*, *uziensis* and *vulcani* were analyzed. This screen resulted in the prediction of 88 bacteriocin gene clusters, of which 2 matched the previously characterized Geobacillin I and II (Garg et al., 2012) discovered in *Geobacillus thermodenitrificans* NG80-2. The other 86 clusters represented potentially novel bacteriocin candidates belonging to class I (modified) and class II (unmodified) bacteriocin families. When characterized class III bacteriocins were used as "driver" sequences and blasted against the entire *Geobacillus* genome database, no homologies were found. Furthermore no class III bacteriocins were predicted by BAGEL3.

In order to reveal associations between bacteriocin cluster gene presence within genomes and their phylogenetic position within the overall *Geobacillus* genus, we superimposed the BAGEL3 bacteriocin predictions onto a *Geobacillus* neighbor-joining phylogentic tree constructed from 16S rRNA sequences. Where possible, 16S rRNA sequences previously determined before whole genome sequencing (WGS) were used to construct the tree. However where no sequence was available, the 16S rRNA genes were predicted using *in silico* prediction software RNAmmer (Lagesen et al., 2007). Here we can see that bacteriocin clusters are both diverse and common across those genomes examined in this study (**Figure 1**). While lantibiotics and circular bacteriocin clusters are spread across the whole genus, Linear Azole-containing Peptides (LAPs), are associated with those strains for which a species has been designated but cluster closely with the species *G. galactosidius* and *G. thermodenitrificans*. A higher frequency of sactibiotics can also been seen within the species *G. stearothermophilus* but these are also present in other species. Furthermore, there are a number of strains included whose genomes have not been fully sequenced and therefore it is not possible to state definitively that alternative bacteriocin clusters are absent from these genomes other than those predicted in this screen.

Similarly to Walsh et al. (2015), the homology of predicted Potential Bacteriocin Gene Clusters (PBGCs) to existing genes and the arrangement of those genes was examined. Below we group PBGCs by bacteriocin class. These arrangements will display only those genes whose function is predicted to be involved in bacteriocin bioactivity and not those genes of unknown function that exist within these clusters.

Class I Bacteriocins

Lantibiotics

Twenty-nine putative lantibiotic gene clusters within 18 genomes were identified by BAGEL3 as part of this genome led bacteriocin screen (**Figure 2**). Lantibiotics belong to class I bacteriocins, which undergo significant post-translational modifications. These peptides are small and usually contain thioether internal bridges due to the interaction of dehydroalanine or dehydrobutyrine with intrapeptide cysteines, resulting in the formation of lanthionine or β-methyllanthionine residues. The structural gene (LanA) typically encodes a leader at the N-terminal of the prepeptide, which is transported across the cell membrane by LanT, then cleaved by LanP. The Post Translational Modification (PTM) enzyme LanB catalyzes the dehydration of amino acids, while LanC catalyzes thioether formation. The

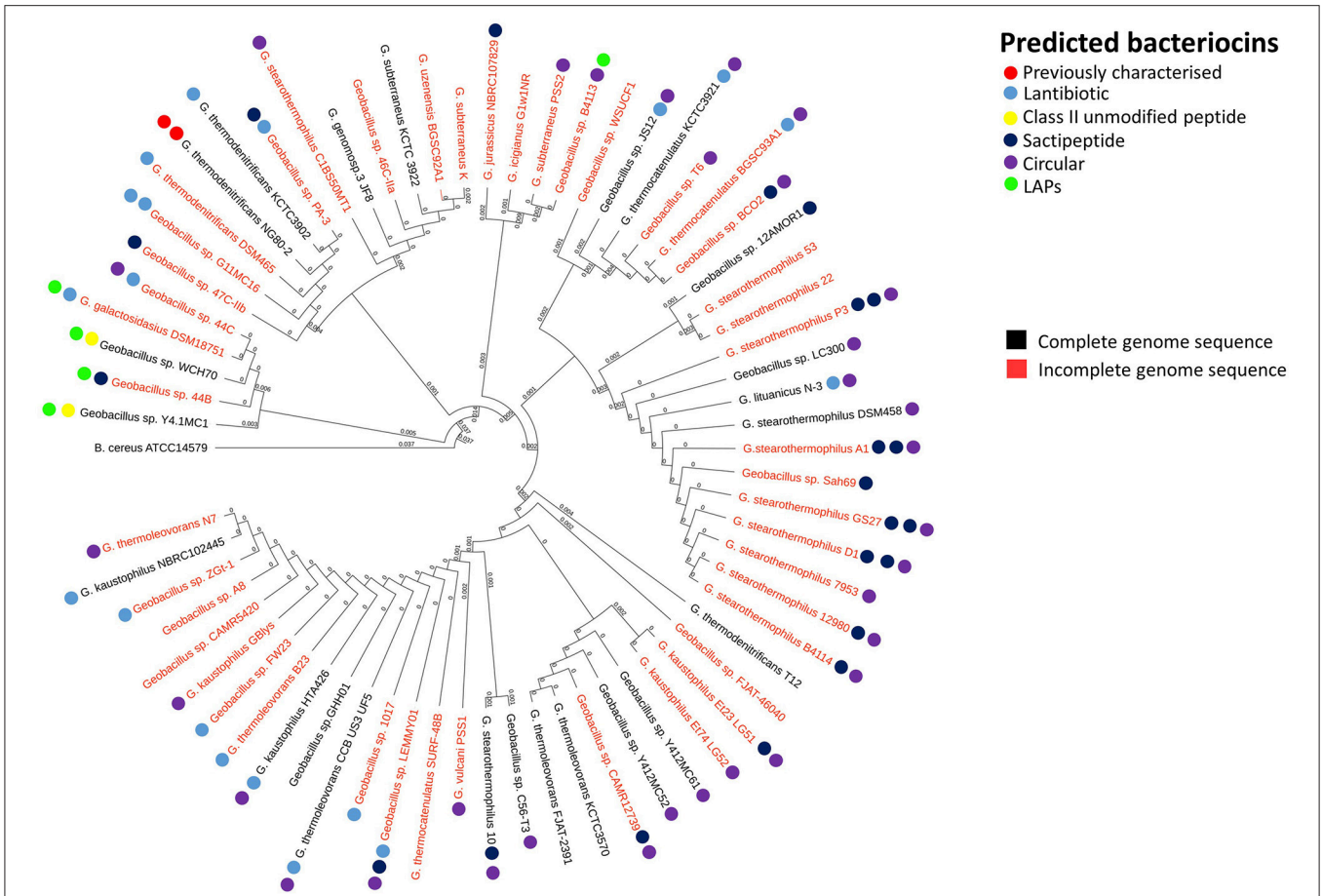


FIGURE 1 | Phylogenetic arrangement of *Geobacillus* genomes investigated in this study. The BAGEL3 peptide predictions are overlaid in order to examine associations between bacteriocin gene presence and position within the *Geobacillus* phylogenetic arrangement.

two component regulatory system genes, *lanR* and *lanK*, encode a response regulator and histidine kinase, respectively (Marsh et al., 2010; Draper et al., 2015; Field et al., 2015). While there are other PTM enzymes associated with lantibiotics they were not observed in this study so will not be described further, but they are discussed in greater detail elsewhere (McAuliffe et al., 2001).

The lantibiotics predicted in this study (**Figure 2**) were grouped according to their amino acid similarity. Grouping the predicted peptides in this way facilitates a comparison with characterized bacteriocins in Bactibase (Hammami et al., 2007). When aligned with the Bactibase bacteriocin peptide database the following highest homology hits was seen for each peptide group; Group 1: no hits; Group 2: 98% similarity to Geobacillin I; Group 3: no hits; Group 4: 12% similarity to LsbB; Group 5: 19% identity to nisin U; Group 6: 16% identity to nisin U; Group 7: 25% identity to nisin U; Group 8: 100% identity to Geobacillin I; Group 9: 5% identical to cinnamycin; Group 10: no hits. Furthermore, a phylogenetic analysis of those predicted peptides was carried out (**Figure S1**) resulting in the arrangement of 5 phylogroups. Phylogroups 1, 3, 4, and 5 were relatively homogenous showing little evolutionary distance between the group nodes. Phylogroup 2 however displayed a larger level of heterogenity with large evolutionary distances existing between the various nodes of the group.

The putative lantibiotics discovered consisted of 7 PBGCs (**Figure 3**) with some containing multiple peptide candidates per PBGC (**Figure 2**). These PBGCs were then typed according to their cluster structure so they could be easily compared with one another. The first cluster (lantibiotic cluster type 1) was contained within 9 genomes (*Geobacillus* sp. 1017, *G. thermocatenulatus* KTCT3921, *G. thermodenitrificans* KCTC3902, *Geobacillus* sp. PA-3, *Geobacillus* sp. Lemmy01, *G. kaustophilus* NBRC102445, *G. thermoleovorans* B23, *G. thermodenitrificans* DSM465, *G. thermocatenulatus* BGSC93A1). It consisted of genes predicted to encode a LanB, LanT, LanC, LanR and LanK consecutively and is similar to the Geobacillin I cluster with regard to its gene makeup. However, within this cluster structure, the predicted lantibiotic peptides were not completely homologous, showing differences in their amino acid composition (**Figure 2**). Additionally two adjacent lantibiotic peptides were predicted within this cluster type for the genomes: *G. thermocatenulatus* KTCT3921, *G. thermocatenulatus* BGSC 93A1 *G. thermoleovorans* B23 and *G. kaustophilus* NBRC102445. There were a number of exceptions to this general cluster structure: *G. thermoleovorans* CCB

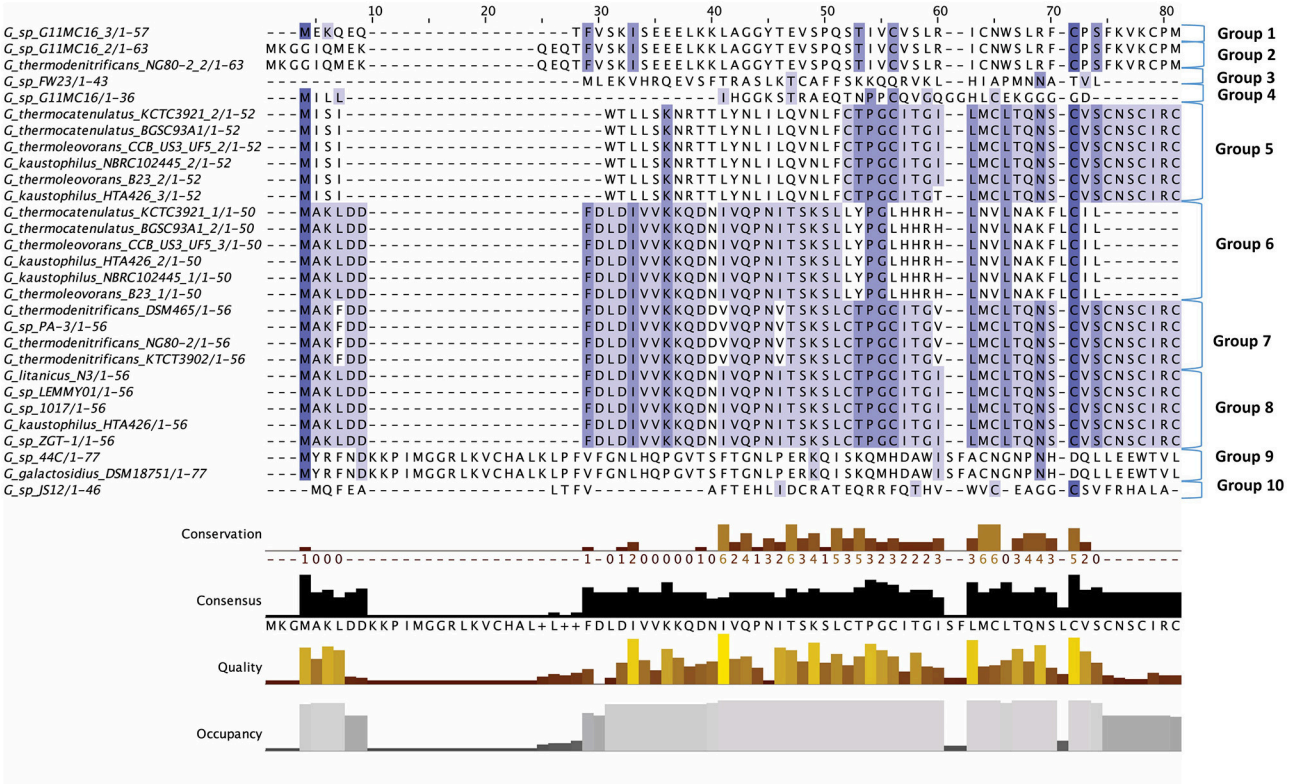


FIGURE 2 | Multiple Sequence Alignment (MSA) of those lantibiotic peptides predicted. In some genomes multiple peptides were predicted within a single bacteriocin cluster and were therefore included as part of this alignment.

US3 UF5 and *G. litanicus* N3 lacked a LanK-determinant (lantibiotic cluster type 2), while *Geobacillus* sp. JS12 contained an extra LanC-encoding gene (lantibiotic cluster type 3). *G. thermoleovorans* CCB US3 UF5 encodes two peptides within this cluster type and they are located adjacent to each other. *Geobacillus* sp. 44C (Lantibiotic cluster type 4) encodes an identical peptide to *G. galactosidius* DSM18751 (lantibiotic type 5), but the PBGC of *G. galactosidius* DSM 18751 contains an additional ABC transporter after the LanC homolog. The genome for *Geobacillus* sp. G11MC16 is predicted to encode three LanA peptides. The first and second peptides are encoded within a distinct cluster from the third. These two peptides are within a cluster that also contains genes predicted to encode a PD2_2 homolog, sigma70, structural peptide, a LanM and LanT homolog (lantibiotic cluster type 6). The third putative peptide-encoding gene is not within an obvious PBGC, but is encoded 10kbs downstream of a region predicted to encode PTM enzymes SpaB-C, ABC transporter, LanC, LanR, and LanK. The peptide predicted to be encoded by *Geobacillus* sp. FW23 is within a cluster consisting of genes predicted to encode a LanB, LanT, LanC, structural peptide and response regulator (lantibiotic cluster type 7).

There were two putative LanAs encoded within the genome of *G. kaustophilus* HTA426. The gene corresponding to the first peptide was located upstream of three ABC transporter-determinants, while the gene corresponding to the second peptide was downstream of these three genes. There was a putative LanC and a further ABC transporter encoded approximately 10 kbs downstream from these predicted structural peptides which appear to exist within a neighboring gene cluster. However, no corresponding LanA-encoding gene was detected. The genome for *Geobacillus* sp. ZGt1 was predicted to encode one LanA that is situated upstream of two ABC transporter-encoding genes. However, the nearest putative LanB, ABC transporter and LanC determinants are located 10 kbs upstream of these genes. Finally NisP driver sequences were aligned against all genomes containing lantibiotics, however there were no definitive results which indicted the presence of these determinants.

Sactipeptides

Sactibiotics, like lantibiotics, are post-translationally modified and thus are a subclass of class I bacteriocins. These post-translational modifications take place in the form of intramolecular bridges of cysteine sulfur to α-carbon linkages (Mathur et al., 2015). 20 sactibiotics peptides were predicted within 17 *Geobacillus* genomes as part of this *in silico* screen (**Figure 4**). No conservation of amino acid residues was observed when these peptides were aligned with known sactibiotic structural peptides. Furthermore when these predicted peptides

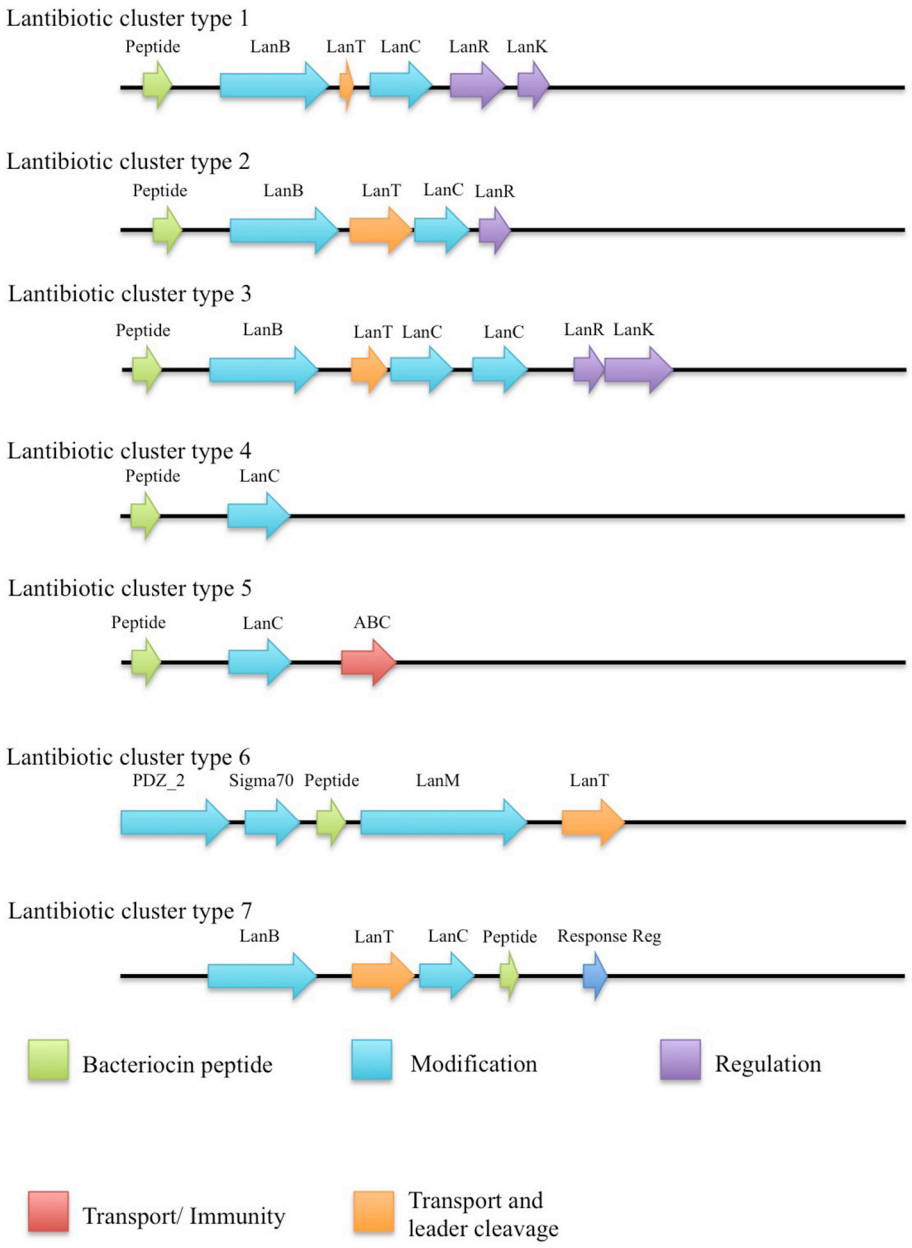


FIGURE 3 | Lantibiotic cluster types predicted by BAGEL3.

were aligned against the Bactibase bacteriocin peptide database (Hammami et al., 2007), no strong homologies with existing sactibiotics were found. Furthermore when a phylogenetic analysis of the predicted peptides (**Figure S2**) was carried out 3 phylogroups were observed. Phylogroup 1 contained the Trnα peptide while phylogroup 3 contained all other previously characterized sactibiotic peptides. Phylogroup 2 however did not contain any of the previously characterized sactibiotics.

When the sactibiotic biosynthetic gene clusters were further investigated, it was seen that 8 different types of predicted sactibiotic gene clusters were encoded within the *Geobacillus* genomes (**Figure 5**). The putative *G. stearothermophilus* A1,

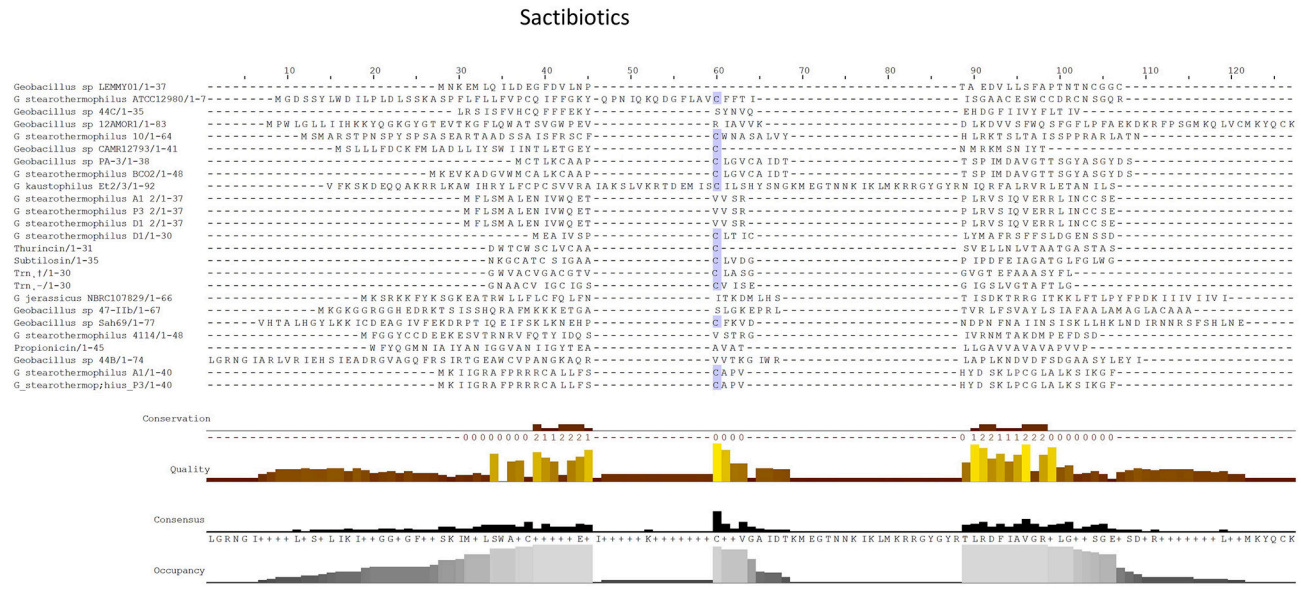


FIGURE 4 | MSA alignment of Sactibiotic peptides predicted by BAGEL3 with characterized sactibiotic peptides.

Geobacillus sp. GS27, *Geobacillus* sp. 47-IIb, *Geobacillus* sp. Sah69, *Geobacillus* sp. 44C, G. *stearothermophilus* ATCC 12980, *G. stearothermophilus* P3 and *Geobacillus* sp. BC02 SacA-determinants were all located upstream of a putative PTM enzyme SacCD-encoding gene (sactibiotic cluster type 1). *Geobacillus* sp. Lemmy 01 contained putative SacCD, LanK, LanR and LanD-encoding genes (sactibiotic cluster type 2). *G. jerrasicus* 107829 contained putative SacCD and LanD-determinants (cluster type 3). *Geobacillus* sp. CAMR12793 and *G. stearothermophilus* B4114 genomes encoded putative SacCD and an ABC transporter-determinants (Sactibiotic cluster type 4). *Geobacillus* sp. PA-3 contains putative SacCD, two ABC transporters and a Radical SAM enzyme-determinants (sactibiotic cluster type 5). The genomes for *Geobacillus* sp. 12AMOR1 and *G. kaustophilus* et2/3 contain putative SacCD and a radical sam enzyme-determinants (sactibiotic cluster type 6). *G. stearothermophilus* D1 and *G. stearotherophilus* A1 are predicted to encode peptides located downstream of a SacCD enzyme-determinant (cluster type 7).

The *G. stearothermophilus* 10 genome encoded a predicted structural peptide, radical SAM and two ABC transporters. While the structural peptide was encoded on the positive strand of the genome the two secondary enzymes were encoded on the negative strand and therefore are not part of the same operon but could however be part of this PBGC. A second putative sactibiotic gene cluster, predicted to be encoded within the *G. stearothermophilus* D1 genome, contains a structural peptide and SacCD enzyme-determinant, which are separated by 13 genes. The genome of *Geobacillus* sp. GS27 was predicted to encode a second sactibiotic peptide other than that predicted previously, however the SacCD-determinant driving this prediction was located on the opposite strand so is not encoded within the same operon but could still be part of the PBGC.

Linear Azole Containing Peptides (LAPS)

Linear Azole containing Peptides (LAPs) are another subclass of class I bacteriocins that are distinguished by virtue of containing a variety of heterocyclic rings of thiazole and (methyl)oxazole. These are formed through an ATP-dependant cyclodehydration and further flavin mononucleotide-dependant dehydrogenation of the amino acid residues cysteine, serine and threonine. The most notable of the LAPs is streptolysin S, which is modified by the cyclodehydratase SagCD (Melby et al., 2011; Cox et al., 2015; Alvarez-Sieiro et al., 2016). Six putative LAPs were identified in six *Geobacillus* genomes (**Figure 6**), five of which were identified in those strains for which a species was not assigned. These peptides did not return any strong homologies to known LAPs or other bacteriocins when aligned against the bactibase bacteriocin database (Hammami et al., 2007). When a phylogenetic analysis of the predicted LAP peptides was performed (**Figure S3**), 3 phylogroups were observed, each consisting of two nodes.

Five out of six peptides (**Figure 7**) are contained within a gene cluster containing a structural peptide followed by a SagD-like and SagB-like determinants (LAP cluster type 1). For *Geobacillus* sp. B4113, the only gene which is predicted to be involved in the PTM of the associated peptide is a cyclodehydration enzyme-determinant upstream of the structural peptide (LAP cluster type 2). There is a LapBotD enzyme-derminant on the opposite strand which is close to the structural peptide, so while it is not part of the same operon it may still be part of this PBGC.

Class II Bacteriocins

Circular (a)

Circular bacteriocins belong to class IIc bacteriocins and are characterized primarily by the C to N terminal covalent linkage.

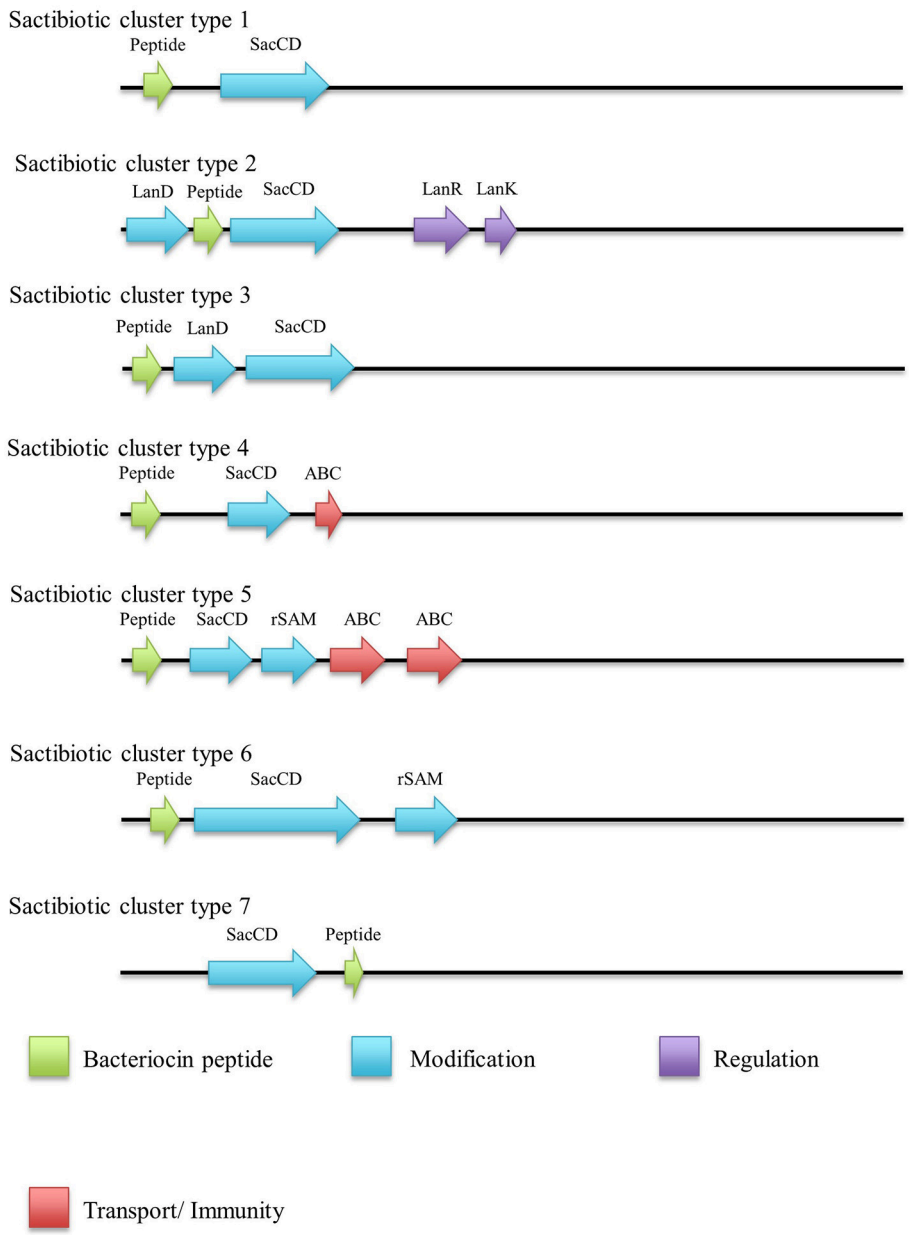


FIGURE 5 | Sactibiotic cluster types predicted by BAGEL3.

They are known for their proteolytic, heat and pH resistance along with their size of 5.6–7.2 kDa, however to date only a handful have been characterized (Gabrielsen et al., 2014). Recently *in silico* software has been used to predict a new circular bacteriocin pumilarin (van Heel et al., 2017) and assisted in the characterisation of plantaricyclin from WGS data (Borrero et al., 2017).

Thirty-one circular peptides were predicted within 29 genomes in this screen (**Figure 8**). These peptides displayed a weak homology (∼30–40%) to known circular peptides when aligned against the bacteriocin database bactibase (Hammami et al., 2007). Five phylogroups were observed when a phylogenetic analysis of the peptides was performed (**Figure S4**). Three peptides from the strains *G. kaustophilus* Et7/4, *G.*

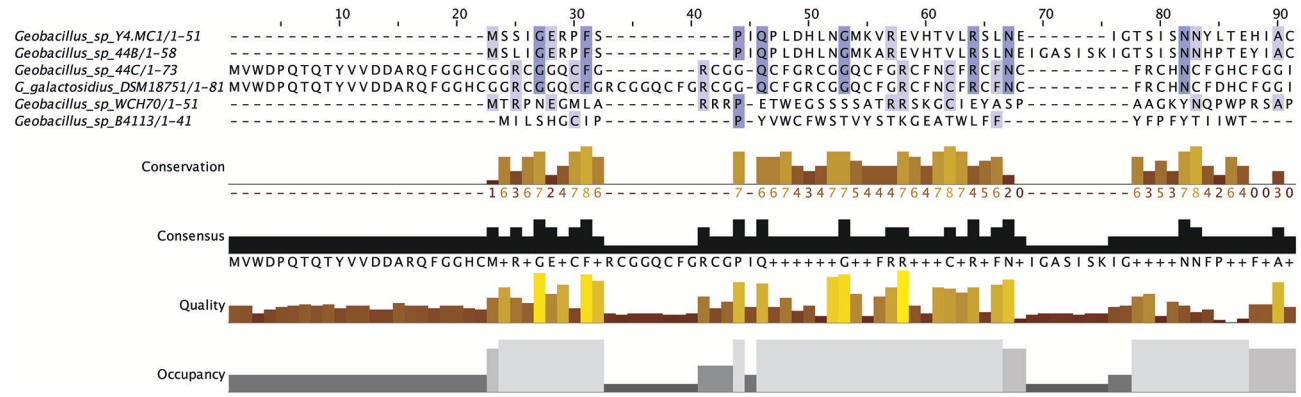


FIGURE 6 | Linear Azole containing peptides predicted by BAGEL3.

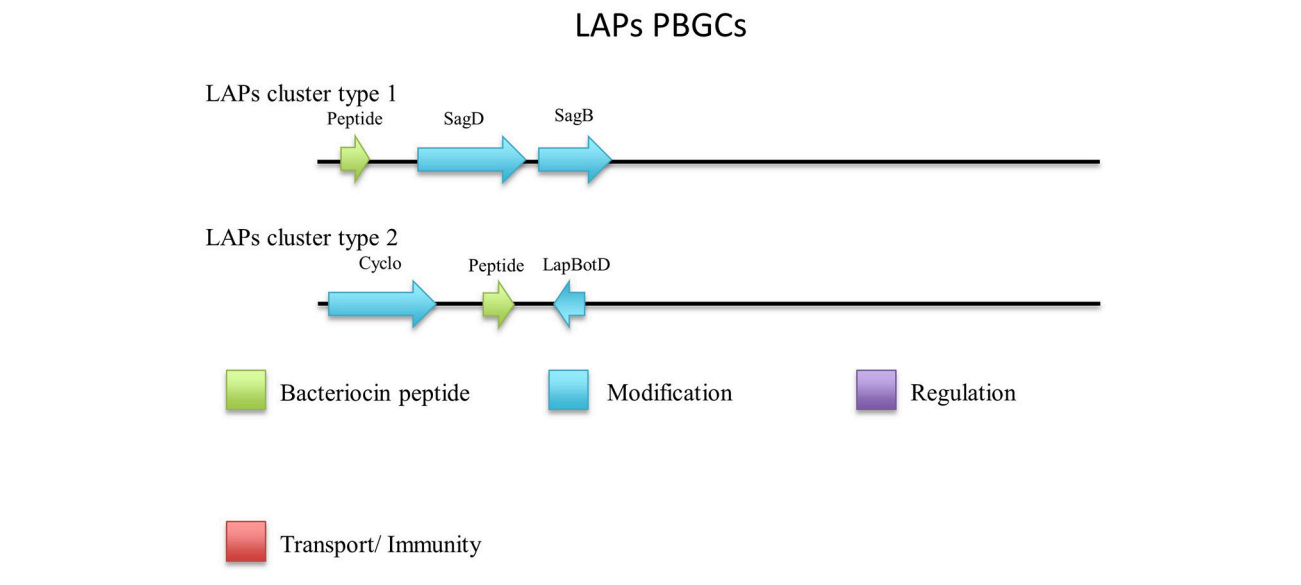


FIGURE 7 | Linear Azole containing peptides (LAPs) cluster types predicted by BAGEL3.

kaustophilus Et2/3 and *G. stearothermophilus* 10 were not included in the phylogenetic tree due to the absence of common sites. While circular peptides have been predicted recently within the genomes of *Geobacillus* (van Heel et al., 2017), they have not been examined in terms of those genes which surround their structural peptide. For those circular structural peptides predicted within the genus, there are 6 general gene cluster structures (**Figure 9**). The genomes of *G. stearothermophilus* B4114, *G. stearothermophilus* GS27, *G. stearothermophilus* B4109, *G. stearothermophilus* 10, *G. stearothermophilus* ATCC12980, *G. stearothermophilus* A1, *G. stearothermophilus* ATCC7953, *G. stearothermophilus* P3, *Geobacillus* sp. 4113, *Geobacillus* sp. T6, *Geobacillus* sp. Y4.MC52, *G. thermocatenulatus* KTCT3921, *G. thermocatenulatus* BGSC93A1, *G. stearothermophilus* DSM458, *G. subterraneus* PSS2 and *Geobacillus* sp. Y412MC61 contain a cluster predicted to encode a structural peptide, a modification gene and two ABC transporter-determinants (Circular cluster type 1). The genomes of *Geobacillus* sp. JS12, *Geobacillus* sp. C56-T3, *Geobacillus* sp. LC300, *G. kaustophilus* Gbly and *G. thermoleovorans* CCB US3 UF5 contain a structural peptide gene, a modification gene, two ABC transporter genes and an additional 3 genes further downstream, putative LanK and Sigma5 determinants (circular cluster type 2). While it is unclear what role these gene products could play in the activity of the structural peptide, we do know that these genes are homologs of lantibiotic regulation machinery. The *Geobacillus* sp. BCO2 genome is predicted to encode a structural peptide and three ABC transporter-determinants (circular cluster type 3). *Geobacillus* sp. CAMR12739 is predicted to encode a structural peptide, a modification protein and three ABC transporter-determinants (circular cluster type 4). *G. kaustophilus* Et7/4 encodes a structural peptide and an ABC

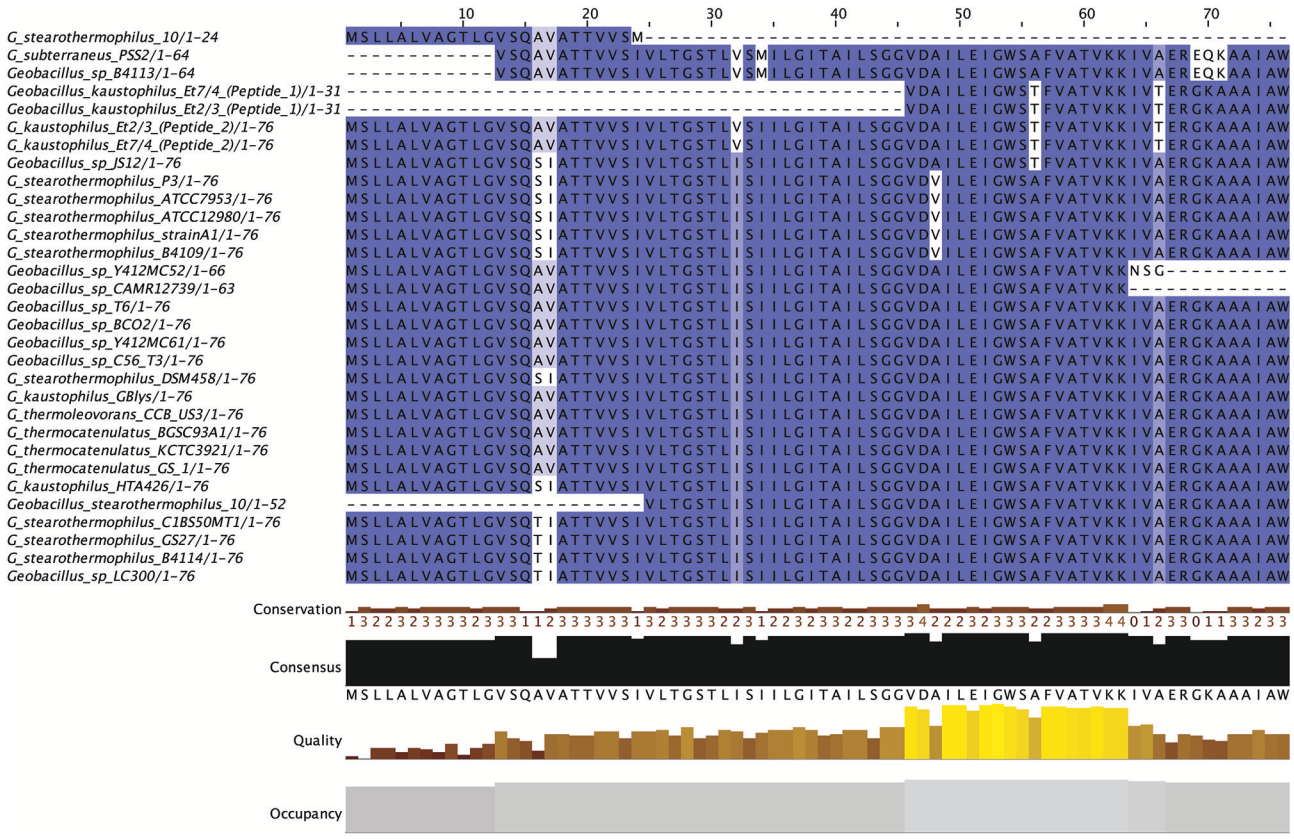


FIGURE 8 | MSA of circular peptides predicted by BAGEL3.

transporter-determinants (circular cluster type 5). The genome of *G. kaustophilus* Et7/4 encodes a modification and an ABC transporter-determinant following the structural peptide (circular cluster type 6).

Circular (b)

There were an additional 5 identical circular peptides predicted (**Figure 10**) that had distinctly different amino acid sequences to the group a circular peptides described above and therefore were designated as a separate group. When aligned against the bacteriocin database bactibase these circular peptides displayed low homology of 17% to lacticin 481. Four of these predicted peptides were encoded within the genomes of *G. kaustophilus* HTA426, *G. thermoleovorans* N7, *G. kaustophilus* Gblys, *Geobacillus* sp. CAMR12739, *Geobacillus* sp. LC300. These circular peptides (**Figure 11**) were predicted to be encoded within the previously described type 2 circular PBGC (**Figure 9**). These structural genes were the last genes encoded within the cluster after the putative modification, 2 ABC transporters, LanK and Sigma 5 determinants (circular (b) cluster stype 1). It is unclear if these genes have any functional role within this cluster due to a lack of any strong amino acid similarity to known bacteriocins such as to circularin (Kawai et al., 2004) and enterocin AS-48 (Grande Burgos et al., 2014), however they could represent a family of potentially novel bacteriocins which may merit further *in vitro* testing.

The structural peptide predicted to be encoded by *Geobacillus* sp. CAMR 12793 is located approximately 10 kbs downstream of the previously described type 4 circular (a) cluster (b). It was accompanied by upstream putative histidine kinase and response regulator determinants (circular (b) cluster type 2) (**Figure 11**). As before it is unclear whether this peptide would be biologically active in this gene cluster so it may merit further *in vitro* experimentation.

Non Circular Class II Bacteriocins

There were a number of non-circular class II bacteriocins predicted by BAGEL3 (**Figure 12**), which are heterogenous with regard to both their predicted amino acid composition and those genes predicted to be involved in their bioactivity (**Figure 13**). When a phylogenetic analysis was performed, 3 phylogroups were observed for these predicted peptides (**Figure S5**). The predicted peptide encoded within the genome of *Geobacillus* sp. Y4MC1 structural peptide is 54% identical to Lacticin Z, however it is located on the opposite strand to its predicted ABC transporters so it is unclear whether they may have a role in its production (class II cluster type 3). The *G. stearothermophilus* D1 predicted cluster contained a circularisation enzyme and two ABC transporters, meanwhile the predicted structural peptide could be potentially novel as it displayed no homology to any

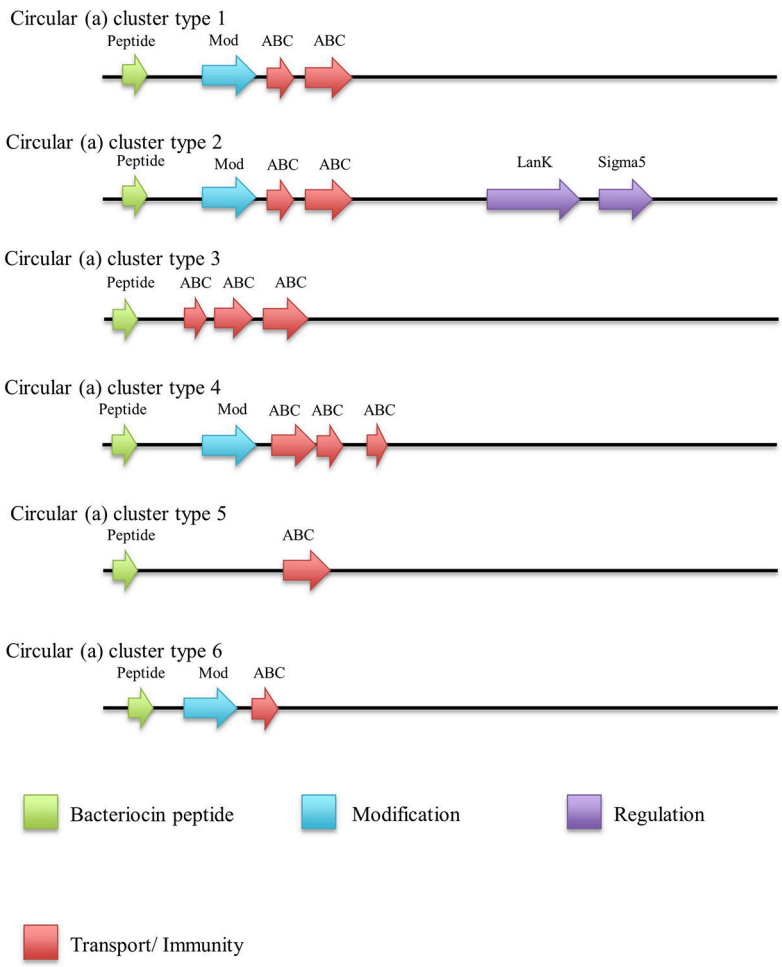


FIGURE 9 | Circular (a) cluster types predicted by BAGEL3.

known bacteriocins (class II cluster type 2). The *Geobacillus* sp. Lemmy 01 putative peptide did not display any homology to known bacteriocins and its prediction as a class II peptide was most likely based on the presence of a circularisation gene-determinant located 16 genes downstream of the structural peptide (class II cluster type 4). *G. stearothermophilus* 10 is predicted to produce a class II unmodified peptide (class II cluster type 1), which is encoded before the previously described circular (a) cluster type 1 (**Figure 9**). It is unclear if either or both peptides are bioactive. *G. litanicus* N3 is predicted to encode a bacteriocin which is two genes upstream of a circularisation gene-determinant, however it has no further transport or modification genes associated with it (class II cluster type 5). *G. vulcani* PSS1 encodes a class II peptide with no homology to existing bacteriocins and is situated on the opposite strand to four ABC transporter and modification gene-determinants (class II cluster type 6).

DISCUSSION

Bacteriocin prospecting has typically been a long and expensive process, based on trial and error in order to isolate bacteriocin producing bacteria and then optimize their growth conditions for bacteriocin production and protein purification. Further characterisation of these bacteriocins then typically required the use of trained personnel to carry out High Performance Liquid Chromatography (HPLC), mass spectrometry and other steps. Since the advent of *in silico* screening this process of bacteriocin discovery has been significantly reduced in terms of time and cost. Indeed this technology allows the bioinformatician to characterize to a high level putative bacteriocin candidates in terms of their amino acid content, physiochemical characteristics and surrounding genes which may be related to its function. Interestingly, it is these elements which had previously been

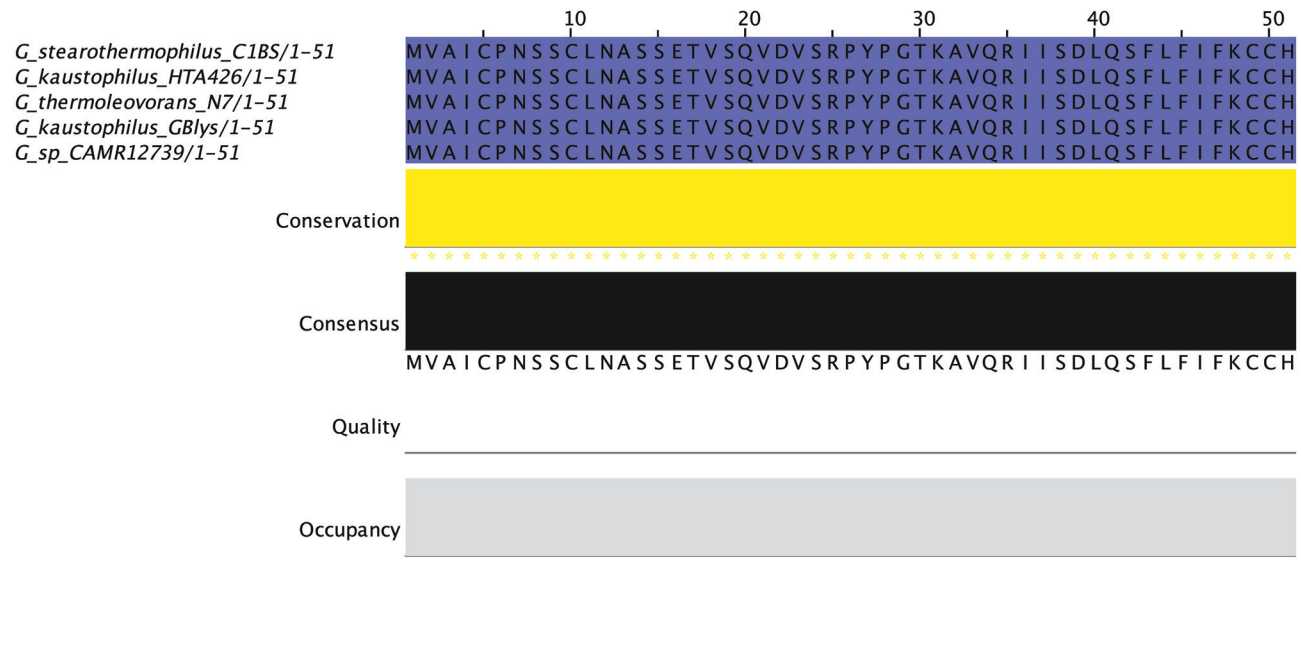


FIGURE 10 | Circular peptides (b) predicted by BAGEL.

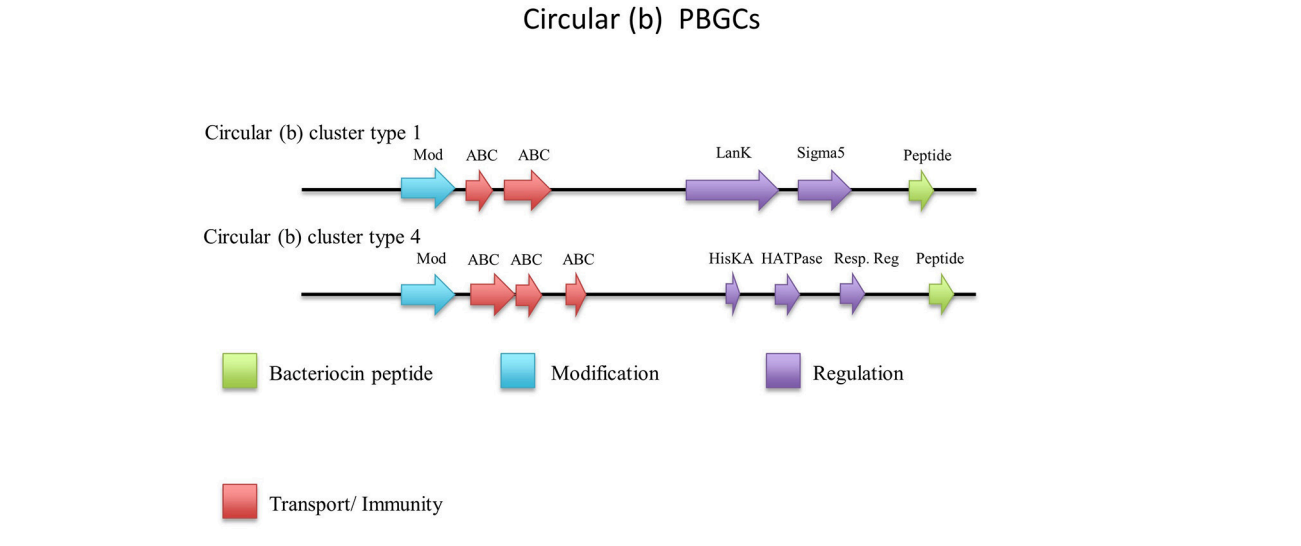


FIGURE 11 | Circular (b) cluster types predicted by BAGEL.

the most laborious and expensive elements of bacteriocin discovery. This ability to identify candidates *in silico* ultimately removes a large portion of this trial and error process as so much is known about the bacteriocin once it is produced *in vitro*.

This *in silico* screen resulted in the identification of 7 lantibiotic, 7 sactibiotic, 2 LAPs, and 8 circular and 6 class II PBGCs which are potentially novel. The putative bacteriocins identified through this *in silico* screening approach will require further investigation through *in vitro* experimentation. However, it was possible to study the genes surrounding the structural peptide to more accurately predict that the bacteriocin cluster was indeed likely to be functional. Notably, in some cases those genes predicted by BAGEL3 were situated within annotated genes and could be determined to be pseudogenes. This study serves to therefore validate and critically assess BAGEL3 as a tool for bacteriocin discovery which could be advantageous for future improvements. When we consider the report that 30-99% of bacteria produce at least one bacteriocin Riley and Wertz (2002), it does seem likely that this may also be the case for *Geobacillus*,

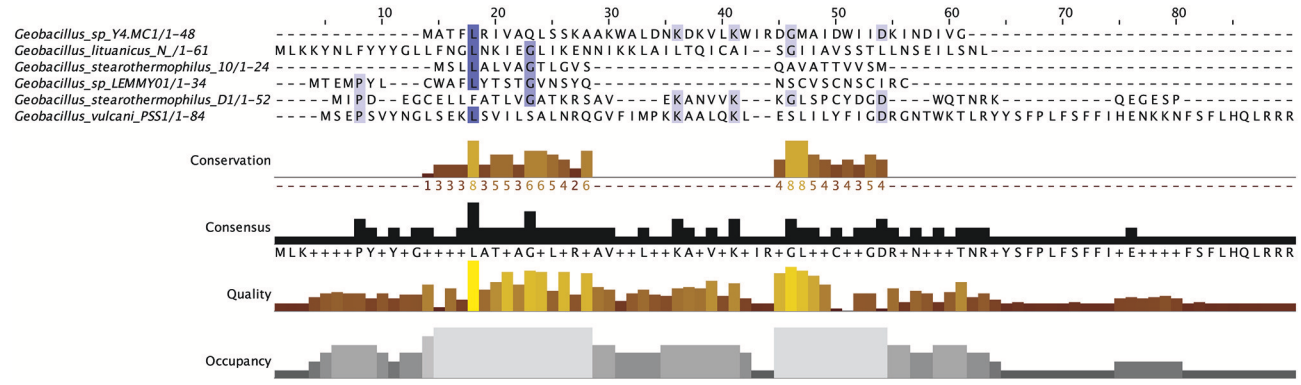


FIGURE 12 | MSA of Class II bacteriocins predicted by BAGEL3.

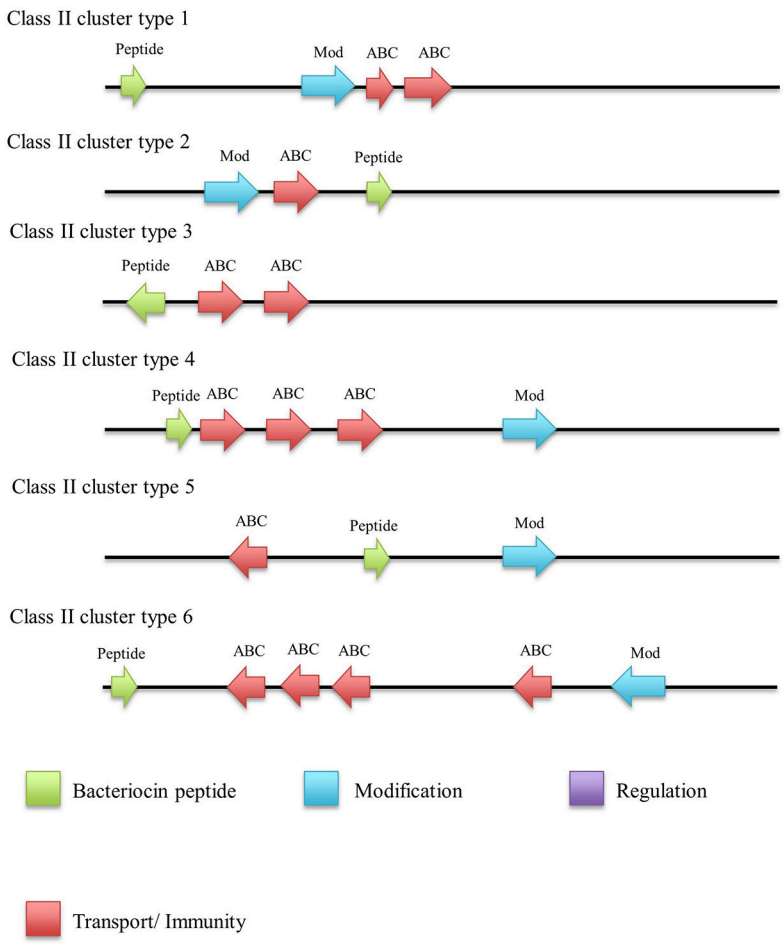


FIGURE 13 | Class II cluster types predicted by BAGEL3.

though a more complete picture will not become apparent until *in vitro* experiments are carried out to validate the findings of this study. Within the genomes examined here, only 23 of 67 were completely sequenced genomes. Where a genome only contains a partially sequenced bacteriocin cluster BAGEL3 will likely return a bacteriocin hit due to its dual detection method, distinguishing both structural peptides and associated bacteriocin genes. In order to fully explore the potential of *Geobacillus* as a reservoir of bacteriocin discovery, the generation of complete assembled genome sequences would be advantageous. A more conclusive picture of its potential will be revealed when the magnitude of genome sequences reaches that of *Lactobacillus*, which was examined recently *in silico* for its bacteriocinogenic potential (Collins et al., 2017). It could be expected that over the next number of years the amount of completely sequenced *Geobacillus* genomes will increase due to the wealth of data generated by way of the widespread use of metagenomic sequencing technologies and the ease/lower cost of WGS which is enabled by third generation sequencing technologies, such as PacBio (Rhoads and Au, 2015) and or Oxford Nanopore (Lu et al., 2016) instruments, that allow for *de novo* genome assembly. With this expected increase in genome sequence data, associations between niche and bacteriocin presence could be investigated in the future.

In the case of lantibiotic peptide predictions, LanT-determinants were not however identified always by BAGEL3 and in most cases LanT-determinant identification was made possible through the alignment of putative ABC transporter-determinants and NisT driver sequences, highlighting the importance of using a hybrid approach of BAGEL and driver sequence homology searching to peptide prediction. Furthermore, a LanK-determinant was absent from a number of lantibiotic gene clusters yet was found in circular PBGCs predicted in the same genomes. It is unclear what role (if any) these LanK-determinants play in these lantibiotic PBGCs. Another interesting observation which merits further investigation was the absence of LanP-determinants from the *Geobacillus* genomes as was seen in the study of the geobacillin I and II biosynthetic genes (Garg et al., 2012). This could be due to effects of incomplete genome sequencing or perhaps the absence of LanP-homologs for peptide leader cleavage as seen in geobacillin I and II. Issues surrounding absent bacteriocin gene-determinants have however been overcome in various studies through the use of heterologous expression systems and such technology will be important for future validation of the various *in-silico* screening studies that have taken place to date (Piper et al., 2011; van Heel et al., 2016; Mesa-Pereira et al., 2017).

A common method of bacteriocin molecular mass size determination involves the use of Native Sodium Dodecyl Polyacrylamide Gel Electrophoresis (SDS-PAGE), where the protein preparation is loaded onto an SDS gel and subjected to electrophoresis. It is then washed and overlaid with agar containing a sensitive indicator bacteria. A zone of inhibition surrounding a protein band provides an estimation of its molecular mass when compared to a molecular-weight size marker or ladder. While we have seen this method used to estimate the molecular mass of a bacteriocin produced by *Geobacillus* sp. ZGt-1 of 15–20 kDa, no such class III bacteriocin was predicted within this genome in our *in silico* screen (Alkhalili et al., 2016). This may indicate the presence of a potentially highly novel class III bacteriocin within *Geobacillus* sp. ZGt-1 given the lack of homology to any known class III peptide, the presence of an uncommon gene cluster not identified in this study or the presence of another type of peptide antimicrobial other than a bacteriocin. Toebicin 218 is produced by *G. stearothermophilus* DSM22 with a molecular mass of 5.5 kDa (Özdemir and Biyik, 2012) and it is interesting to note that no bacteriocin was detected within this genome in the current study. Pokusaeva et al. (2009) used this method to estimate the size of bacteriocins produced by various *G. stearothermophilus* at 6.8, 5.6, 7.1, and 7.2 kDa. However, the genomes of these strains have not been sequenced and therefore the identity of potential bacteriocin candidates cannot be determined through bioinformatics. This is also the case for *G. toebii* HBB-247, that has been shown to produce a bacteriocin with an estimated mass of 38 kDa (Başbülbül Özdemir and Biyik, 2012; Özdemir and Biyik, 2012). There are a number of other bacteriocins of undetermined mass which have been characterized within *Geobacillus* prior to modern sequencing or mass spectrometry methods (Shafia, 1966; Yule and Barridge, 1976; Sharp et al., 1979; Fikes et al., 1983). Indeed, it is notable that there is a significant lack of mass spectrometry data for all *Geobacillus*-associated bacteriocins other than the lantibiotics Geobacillin I and II discovered within *G. thermodenitrificans* NG80-2.

While *Geobacillus* appears to represent a potential reservoir for novel bacteriocin discovery, its route to commercial application in food or medicine remains unclear. The nature of *Geobacillus* when in the form of a thermally resistant spore makes it difficult to remove once introduced into an processing environment (Egan et al., 2016). Furthermore, the associated high temperature growth requirements would translate to high processing and energy costs. Typically its direct addition to food, albeit a GRAS bacterial genus, is not applicable due to its history as a bacterial spoilage agent. Despite this, *Geobacillus* do already have applications in the biotechnology industry in a number of ways (such as biofuel and chemical production), so perhaps it is within this niche where bacteriocins produced by *Geobacillus* could be of commercial relevance. Additionally, these bacteria could serve as a platform for research into protein thermostability and as a source of not only heat stable bacteriocins but also post translational modification enzymes. Finally, with the oncoming antimicrobial resistance (AMR) crisis, humankind is looking outside of the traditional antimicrobial candidate reservoirs and increased investment in other classes of antimicrobials such as defensins (Oppedijk et al., 2015) are visibly apparent. Given the abundance of potentially novel bacteriocins identified by this study, *Geobacillus* spp. could yet develop their full potential as a source of new peptide structures with enhanced functionality.

AUTHOR CONTRIBUTIONS

KE drafted the manuscript. KE and CH conceived the manuscript. DF, RR, PC, and CH revised and approved the final manuscript.

FUNDING

KE, DF, RR, PC, and CH are supported by the Irish Government under the National Development Plan, through the Food Institutional Research Measure, administered by the Department of Agriculture, Fisheries and Food, Ireland (DAFM 13/F/462). RR and CH are supported by the SFI Investigator awards (10/IN.1/B3027). DF, PC, CH, and RR are also supported by the APC Microbiome institute under Grant Number SFI/12/RC/2273.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02116/full#supplementary-material

Figure S1 | Phylogenetic arrangement of predicted lantibiotics.

Figure S2 | Phylogenetic arrangement of predicted sactibiotics.

Figure S3 | Phylogenetic arrangement of predicted LAPs.

Figure S4 | Phylogenetic arrangment of predicted circular bacteriocins.

Figure S5 | Phylogenetic arrangement of predicted class II bacteriocins.

REFERENCES

Alkhalili, R. N., Bernfur, K., Dishisha, T., Mamo, G., Schelin, J., Canbäck, B., et al. (2016). Antimicrobial protein candidates from the thermophilic geobacillus sp. strain ZGt-1: production, proteomics, and bioinformatics analysis. *Int. J. Mol. Sci.* 17, 1363. doi: 10.3390/ijms17081363

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2

Alvarez-Sieiro, P., Montalbán-López, M., Mu, D., and Kuipers, O. P. (2016). Bacteriocins of lactic acid bacteria: extending the family. *Appl. Microbiol. Biotechnol.* 100, 2939–2951. doi: 10.1007/s00253-016-7343-9

Arnison, P. G., Bibb, M. J., Bierbaum, G., Bowers, A. A., Bugni, T. S., Bulaj, G., et al. (2013). Ribosomally synthesized and post-translationally modified peptide natural products: overview and recommendations for a universal nomenclature. *Nat. Prod. Rep.* 30, 108–160. doi: 10.1039/C2NP20085F

Azevedo, A. C., Bento, C. B., Ruiz, J. C., Queiroz, M. V., and Mantovani, H. C. (2015). Distribution and genetic diversity of bacteriocin gene clusters in rumen microbial genomes. *Appl. Environ. Microbiol.* 81, 7290–7304. doi: 10.1128/AEM.01223-15

Başbülbül Özdemir, G., and Biyik, H. H. (2012). Isolation and characterization of a bacteriocin-like substance produced by *Geobacillus toebii* strain HBB-247. *Indian J. Microbiol.* 52, 104–108. doi: 10.1007/s12088-011-0227-x

Begley, M., Cotter, P. D., Hill, C., and Ross, R. P. (2009). Identification of a novel two-peptide lantibiotic, lichenicidin, following rational genome mining for LanM Proteins. *Appl. Environ. Microbiol.* 75, 5451–5460. doi: 10.1128/AEM.00730-09

Borrero, J., Kelly, E., O'Connor, P. M., Kelleher, P., Scully, C., Cotter, P. D., et al. (2017). Purification, characterization and heterologous production of plantaricyclin A, a novel circular bacteriocin produced by Lactobacillus plantarum NI326. *Appl. Environ. Microbiol.* 84:e01801-17. doi: 10.1128/AEM.01801-17

Cihan, A. C., Ozcan, B., Tekin, N., and Cokmus, C. (2011) Phylogenetic diversity of isolates belonging to genera Geobacillus and Aeribacillus isolated from different geothermal regions of Turkey. *World. J. Microbiol. Biotechnol.* 27, 2683–2696 doi: 10.1007/s11274-011-0742-2

Collins, F. W. J., O'Connor, P. M., O'Sullivan, O., Gómez-Sala, B., Rea, M. C., Hill, C., et al. (2017). Bacteriocin Gene-Trait matching across the complete Lactobacillus Pan-genome. *Sci. Rep.* 7:3481. doi: 10.1038/s41598-017-03339-y

Cotter, P. D., Hill, C., and Ross, R. P. (2005). Bacteriocins: developing innate immunity for food. *Nat. Rev. Microbiol.* 3, 777–788. doi: 10.1038/nrmicro1273

Cotter, P. D., Ross, R. P., and Hill, C. (2013). Bacteriocins - a viable alternative to antibiotics? *Nat. Rev. Microbiol.* 11, 95–105. doi: 10.1038/nrmicro2937

Cox, C. L., Doroghazi, J. R., and Mitchell, D. A. (2015). The genomic landscape of ribosomal peptides containing thiazole and oxazole heterocycles. *BMC Genomics* 16:778. doi: 10.1186/s12864-015-2008-0

Cripps, R. E., Eley, K., Leak, D. J., Rudd, B., Taylor, M., Todd, M., et al. (2009). Metabolic engineering of geobacillus thermoglucosidasius for high yield ethanol production. *Metab. Eng.* 11, 398–408. doi: 10.1016/j.ymben.2009.08.005

Draper, L. A., Cotter, P. D., Hill, C., and Ross, R. P. (2015). Lantibiotic resistance. *Microbiol. Mol. Biol. Rev.* 79, 171–191. doi: 10.1128/MMBR.00051-14

Dukes, T. H., and Cantor, C. R. (1969). Evolution of protein molecules. *Mammal. Protein Metabol.* 3:132.

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res.* 32, 1792–1797. doi: 10.1093/nar/gkh340

Egan, K., Field, D., Rea, M. C., Ross, R. P., Hill, C., and Cotter, P. D. (2016). Bacteriocins: novel solutions to age old spore-related problems? *Front. Microbiol.* 7:461. doi: 10.3389/fmicb.2016.00461

Field, D., Cotter, P. D., Ross, R. P., and Hill, C. (2015). Bioengineering of the model lantibiotic nisin. *Bioengineered* 6, 187–192. doi: 10.1080/21655979.2015.1049781

Fikes, J. D., Crabtree, B. L., and Barridge, B. D. (1983). Studies on the mode of action of a bacteriocin produced by *Bacillus stearothermophilus*. *Can. J. Microbiol.* 29, 1576–1582. doi: 10.1139/m83-240

Gabrielsen, C., Brede, D. A., Nes, I. F., and Diep, D. B. (2014). Circular bacteriocins: biosynthesis and mode of action. *Appl. Environ. Microbiol.* 80, 6854–6862. doi: 10.1128/AEM.02284-14

Garg, N., Tang, W., Goto, Y., Nair, S. K., and van der Donk, W. A. (2012). Lantibiotics from *Geobacillus thermodenitrificans*. *Proc. Natl. Acad. Sci. U.S.A.* 109, 5241–5246. doi: 10.1073/pnas.1116815109

Grande Burgos, M. J., Pulido, R. P., Del Carmen López Aguayo, M., Gálvez, A., and Lucas, R. (2014). The cyclic antibacterial peptide enterocin AS-48: isolation, mode of action, and possible food applications. *Int. J. Mol. Sci.* 15, 22706–22727. doi: 10.3390/ijms151222706

Hammami, R., Zouhir, A., Ben Hamida, J., and Fliss, I. (2007). BACTIBASE: a new web-accessible database for bacteriocin characterization. *BMC Microbiol.* 7:89. doi: 10.1186/1471-2180-7-89

Hussein, A. H., Lisowska, B. K., and Leak, D. J. (2015). "Chapter One - The Genus Geobacillus and Their Biotechnological Potential," in *Advances in Applied Microbiology,* eds Sariaslani S, and Gadd G. M (Academic Press), 1–48.

Kananavičiūtė, R., and Čitavičius, D. (2015). Genetic engineering of Geobacillus spp. *J. Microbiol. Methods* 111, 31–39. doi: 10.1016/j.mimet.2015.02.002

Kawai, Y., Kemperman, R., Kok, J., and Saito, T. (2004). The circular bacteriocins gassericin A and circularin A. *Curr. Protein Pept. Sci.* 5, 393–398. doi: 10.2174/1389203043379549

Kumar, S., stecher, G., and Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874. doi: 10.1093/molbev/msw054

Lagesen, K., Hallin, P., Rødland, E. A., Stærfeldt, H.-H., Rognes, T., and Ussery, D. W. (2007). RNAmmer: consistent and rapid annotation of ribosomal RNA genes. *Nucleic Acids Res.* 35, 3100–3108. doi: 10.1093/nar/gkm160

Letunic, I., and Bork, P. (2016). Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Res.* 44, W242–245. doi: 10.1093/nar/gkw290

Lu, H., Giordano, F., and Ning, Z. (2016). Oxford nanopore MinION sequencing and genome assembly. *Genomics Proteomics Bioinformatics* 14, 265–279. doi: 10.1016/j.gpb.2016.05.004

Marsh, A. J., O'Sullivan, O., Ross, R. P., Cotter, P. D., and Hill, C. (2010). In silico analysis highlights the frequency and diversity of type 1 lantibiotic gene clusters in genome sequenced bacteria. *BMC Genomics* 11, 679–679. doi: 10.1186/1471-2164-11-679

Mathur, H., Rea, M. C., Cotter, P. D., Hill, C., and Ross, R. P. (2015). The sactibiotic subclass of bacteriocins: an update. *Curr. Protein Pept. Sci.* 16, 549–558. doi: 10.2174/1389203716666150515124831

McAuliffe, O., Ross, R. P., and Hill, C. (2001). Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbiol. Rev.* 25, 285–308. doi: 10.1111/j.1574-6976.2001.tb00579.x

Melby, J. O., Nard, N. J., and Mitchell, D. A. (2011). Thiazole/oxazole-modified microcins: complex natural products from ribosomal templates. *Curr. Opin. Chem. Biol.* 15, 369–378. doi: 10.1016/j.cbpa.2011.02.027

Mesa-Pereira, B., O'Connor, P. M., Rea, M. C., Cotter, P. D., Hill, C., and Ross, R. P. (2017). Controlled functional expression of the bacteriocins pediocin PA-1 and bactofencin A in Escherichia coli. *Sci. Rep.* 7:3069. doi: 10.1038/s41598-017-02868-w

Nazina, T. N., Tourova, T. P., Poltaraus, A. B., Novikova, E. V., Grigoryan, A. A., Ivanova, A. E., et al. (2001). Taxonomic study of aerobic thermophilic bacilli: descriptions of *Geobacillus subterraneus* gen. nov., sp. nov. and Geobacillus uzenensis sp. nov. from petroleum reservoirs and transfer of Bacillus stearothermophilus, Bacillus thermocatenulatus, Bacillus thermoleovorans, Bacillus kaustophilus, Bacillus thermodenitrificans to Geobacillus as the new combinations G. stearothermophilus, G. thermocatenulatus, G. thermoleovorans, G. kaustophilus, G. thermoglucosidasius and G. thermodenitrificans. *Int. J. Syst. Evol. Microbiol.* 51, 433–446. doi: 10.1099/00207713-51-2-433

Oppedijk, S. F., Martin, N. I., and Breukink, E. (2015). Hit 'em where it hurts: The growing and structurally diverse family of peptides that target lipid-II. *Biochim. Biophys. Acta* 1858, 947–957. doi: 10.1016/j.bbamem.2015.10.024

Özdemir, G. B., and Biyik, H.H. (2012). Isolation and characterization of toebicin 218, a bacteriocin, produced by *Geobacillus toebii* HBB-218. *Afr. J. Biotechnol.* 11:7711. doi: 10.5897/AJB11.1331

Piper, C., Hill, C., Cotter, P. D., and Ross, R. P. (2011). Bioengineering of a Nisin A-producing *Lactococcus lactis* to create isogenic strains producing the natural variants Nisin F, Q and Z. *Microb. Biotechnol.* 4, 375–382. doi: 10.1111/j.1751-7915.2010.00207.x

Pokusaeva, K., Kuisiene, N., Jasinskyte, D., Rutiene, K., Saleikiene, J., and Chitavichius, D. (2009). Novel bacteriocins produced by *Geobacillus stearothermophilus*. *Cent. Eur. J. Biol.* 4, 196–203. doi: 10.2478/s11535-009-0009-1

Rea, M. C., Sit, C. S., Clayton, E., O'Connor, P. M., Whittal, R. M., Zheng, J., et al. (2010). Thuricin CD, a posttranslationally modified bacteriocin with a narrow spectrum of activity against Clostridium difficile. *Proc. Natl. Acad. Sci. U.S.A.* 107, 9352–9357. doi: 10.1073/pnas.0913554107

Rhoads, A., and Au, K. F. (2015). PacBio sequencing and its applications. *Genomics Proteomics Bioinformatics* 13, 278–289. doi: 10.1016/j.gpb.2015.08.002

Riley, M. A., and Wertz, J. E. (2002). Bacteriocins: evolution, ecology, and application. *Annu. Rev. Microbiol.* 56, 117–137. doi: 10.1146/annurev.micro.56.012302.161024

Rutherford, K., Parkhill, J., Crook, J., Horsnell, T., Rice, P., Rajandream, M., et al. (2000). Artemis: sequence visualization and annotation. *Bioinformatics* 16, 944–945. doi: 10.1093/bioinformatics/16.10.944

Shafia, F. (1966). Thermocins of *Bacillus stearothermophilus*. *J. Bacteriol.* 92, 524–525.

Sharp, R. J., Bingham, A. H. A., Comer, M. J., and Atkinson, A. (1979). Partial characterization of a bacteriocin (*Thermocin*) from *Bacillus stearothermophilus* RS93. *Microbiology* 111, 449–451. doi: 10.1099/00221287-111-2-449

Studholme, D. J. (2015). Some (bacilli) like it hot: genomics of *Geobacillus* species. *Microb. Biotechnol.* 8, 40–48. doi: 10.1111/1751-7915.12161

van Heel, A. J., de Jong, A., Montalbán-López, M., Kok, J., and Kuipers, O. P. (2013). BAGEL3: automated identification of genes encoding bacteriocins and (non-)bactericidal posttranslationally modified peptides. *Nucleic Acids Res.* 41, W448–453. doi: 10.1093/nar/gkt391

van Heel, A. J., Kloosterman, T. G., Montalban-Lopez, M., Deng, J., Plat, A., Baudu, B., et al. (2016). Discovery, production and modification of Five novel lantibiotics using the promiscuous nisin modification machinery. *ACS Synth. Biol.* 5, 1146–1154. doi: 10.1021/acssynbio.6b00033

van Heel, A. J., Montalban-Lopez, M., Oliveau, Q., and Kuipers, O. P. (2017). Genome-guided identification of novel head-to-tail cyclized antimicrobial peptides, exemplified by the discovery of pumilarin. *Microbial Genomics* 3:e000134. doi: 10.1099/mgen.0.000134

Walsh, C. J., Guinane, C. M., Hill, C., Ross, R. P., O'Toole, P. W., and Cotter, P. D. (2015). *In silico* identification of bacteriocin gene clusters in the gastrointestinal tract, based on the Human Microbiome Project's reference genome database. *BMC Microbiol.* 15:183. doi: 10.1186/s12866-015-0515-4

Waterhouse, A. M., Procter, J. B., Martin, D. M., Clamp, M., and Barton, G. J. (2009). Jalview Version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25, 1189–1191. doi: 10.1093/bioinformatics/btp033

Weber, T., Blin, K., Duddela, S., Krug, D., Kim, H. U., Bruccoleri, R., et al. (2015). antiSMASH 3.0-a comprehensive resource for the genome mining of biosynthetic gene clusters. *Nucleic Acids Res.* 43, W237–243. doi: 10.1093/nar/gkv437

Yule, R., and Barridge, B. (1976). Isolation and characterization of a bacteriocin produced by *Bacillus stearothermophilus* strain NU-10. *Can. J. Microbiol.* 22, 1743–1750. doi: 10.1139/m76-257

Zeigler, D. R. (2014). The Geobacillus paradox: why is a thermophilic bacterial genus so prevalent on a mesophilic planet? *Microbiology* 160, 1–11. doi: 10.1099/mic.0.071696-0

Zhang, X., Luo, H., Xu, Z., Zhu, Y., Ji, A., Song, J., et al. (2015). Genome-wide characterisation and analysis of bHLH transcription factors related to tanshinone biosynthesis in Salvia miltiorrhiza. *Sci. Rep.* 5:11244. doi: 10.1038/srep11244

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer GG and handling Editor declared their shared affiliation

frontiers in Microbiology

ORIGINAL RESEARCH
published: 13 November 2018
doi: 10.3389/fmicb.2018.02742

Soil Bacteria Isolated From Tunisian Arid Areas Show Promising Antimicrobial Activities Against Gram-Negatives

Zina Nasfi[1,2,3], Henrik Busch[4], Stefan Kehraus[4], Luis Linares-Otoya[2], Gabriele M. König[4], Till F. Schäberle[2,5] and Rafik Bachoual[1]*

[1] Laboratory of Plant Improvement and Valorization of Agroresources, National School of Engineering of Sfax, Sfax, Tunisia, [2] Institute for Insect Biotechnology, Justus-Liebig-University Giessen, Giessen, Germany, [3] Faculty of Sciences of Gabès, University of Gabès, Gabès, Tunisia, [4] Institute for Pharmaceutical Biology, University of Bonn, Bonn, Germany, [5] Department of Bioresources of the Fraunhofer Institute for Molecular Biology and Applied Ecology, Giessen, Germany

Arid regions show relatively fewer species in comparison to better-watered biomes, but the competition for the few nutrients is very distinct. Here, in total 373 bacterial strains were isolated from rhizospheric soils obtained from three different sampling sites in Tunisia. Their potential for the production of antimicrobial compounds was evaluated. Bacterial strains, showing antibacterial activity against pathogenic bacteria, were isolated from all three sites, one strain from the Bou-Hedma national park, 15 strains from Chott-Djerid, and 13 strains from Matmata, respectively. The dominant genus was *Bacillus*, with 27 out of 29 strains. Most interestingly, 93% of the isolates showed activity against Gram-positive and Gram-negative test bacteria. Strain *Bacillus* sp. M21, harboring high inhibitory potential, even against clinical isolates of Gram-negative bacteria, was analyzed in detail to enable purification and identification of the bioactive compound responsible for its bioactivity. Subsequent HPLC-MS and NMR analyses resulted in the identification of 1-acetyl-β-carboline as active component. Furthermore, fungicides of the bacillomycin and fengycin group, which in addition show antibiotic effects, were identified. This work highlights the high potential of the arid-adapted strains for the biosynthesis of specialized metabolites and suggest further investigation of extreme environments, since they constitute a promising bioresource of biologically active compounds.

Keywords: *Bacilli*, natural products, antibiotics, carboline, fungicides

OPEN ACCESS

Edited by:
José E. Barboza-Corona, Universidad de Guanajuato, Mexico

Reviewed by:
Norma Margarita de la Fuente Salcido, Universidad Autónoma de Coahuila, Mexico
Mariusz Cycoń, Medical University of Silesia, Poland

***Correspondence:**
Till F. Schäberle
till.f.schaeberle@agrar.uni-giessen.de

Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: *19 September 2018*
Accepted: *26 October 2018*
Published: *13 November 2018*

Citation:
Nasfi Z, Busch H, Kehraus S, Linares-Otoya L, König GM, Schäberle TF and Bachoual R (2018) Soil Bacteria Isolated From Tunisian Arid Areas Show Promising Antimicrobial Activities Against Gram-Negatives. Front. Microbiol. 9:2742. doi: 10.3389/fmicb.2018.02742

INTRODUCTION

The discovery of antibiotics to treat infectious diseases has revolutionized the field of medicine in the mid-twentieth century (Wohlleben et al., 2016). However, due to the misuse of antibiotics or extensive use for clinical and veterinary purposes, many of the relevant pathogens became resistant to antibiotic therapy (Ventola, 2015). These pathogens have accumulated a large number of resistance elements by mutational adaptations, acquisition of genetic material, or alteration of gene expression. This resulted in (i) modification of the antimicrobial target, (ii) a decrease in drug-uptake, (iii) activation/increase of efflux mechanisms to extrude the harmful molecule, or (iv) global changes in important metabolic pathways via modulation of regulatory networks (Munita and Arias, 2016).

The genes encoding for the respective resistance mechanism can be found both, within the genome and on plasmids; thereby, greatly limiting the therapeutic options (Wright, 2012). The number of resistant bacterial isolates increases at an alarming rate (Frieri et al., 2017), threatening treatment options of modern medicine. Hence, there is an immense need for the discovery and development of novel antibiotics with resistance-breaking properties to effectively target the multi drug resistant (MDR) pathogen bacteria that cause life-threatening infections (Morens and Fauci, 2013).

Medicinal drugs based on natural products obtained from microorganisms are playing the most important role in the treatment of bacterial infections and appear to be still the most promising source of future antibiotics (Kumar and Kumar, 2016). This became clear, since the standard research approach performed in the recent decades, from gene via target using high-throughput screening to generate a lead, delivered no innovative antibiotics. Chemical compound libraries, optimized for oral bioavailability, have non-suitable physicochemical properties, especially for passing through Gram-negative cell membranes. On the other hand, natural products, optimized for bacterial activity, but not for human application can be associated with toxicity and low *in vivo* activity. A drawback in natural product research is that it seems like the low-hanging fruits are already harvested. Often, known compounds had been re-isolated. Combined with the fact that the economic value of a new antibiotic can be close to zero, thereby facing development costs of around 1.000 Mio€, since for innovative novel resistance breaking antibiotics, only small margins can be expected. An innovative novel antibiotic will receive the status of a reserve antibiotic, which will result in relatively low sales figures. Therefore, companies and research groups left the field: Today only 50 groups worldwide are active in antibiotic research with a total of ∼500 people (The Boston Consulting Group, 2017).

To increase the chance of success for bioprospecting projects, which aim to identify novel lead structures for antibiotic development, these must be based on a good rational. In nature, there will be still many different potential sources to discover such leads. Rhizospheric soil in general, with its enormous biological diversity, remains a most important target for screening projects; since there is a universal dissemination of antibiotics among rhizospheric microorganisms. The latter seem to shape the microbiome of the specific biological niche and using specialized metabolites of interest for communication and antagonism (Raaijmakers and Mazzola, 2012; Ghanmi et al., 2016). Many pharmaceutically important antibiotics have been identified in the past from this bioresource, e.g., vancomycin produced by *Streptomyces orientalis* isolated from a soil sample from Borneo (Griffith, 1981), kanamycin produced by a soil bacterium *Streptomyces kanamyceticus* (Umezawa et al., 1957), and erythromycin first isolated in 1952 from the soil bacterium *Saccharopolyspora erythraea* (Staunton and Wilkinson, 1997). Bacterial genera reported so far as a bioresource with a high chance to detect compounds of interest are *Actinomycetes* (Hotam et al., 2013; Tiwari and Gupta, 2013), *Bacilli* (Sumi et al., 2015), and *Pseudomonads* (Mukherjee et al., 2014; de Oliveira et al., 2016). In the present project so far unexplored arid sampling sites of Southern Tunisia were investigated, since the arid environment results in high competition between organisms. Several strains with antimicrobial properties were isolated and characterized. From one isolated *Bacillus* strain, an antimicrobial compound was isolated and further bioactive natural products were identified by LC/MS.

MATERIALS AND METHODS

Sampling Sites

Samples were collected from different arid areas located in South Tunisia (**Figure 1**). Three rhizospheric soil samples, of herbaceous vegetation, were collected aseptically from Matmata (33°54′86″ N, 9°96′13″ E), the national park of Bou-Hedma (34°47′45″ N, 9°48′21″ E) and an arid shallow aquifer in Chott-Djerid (33°94′16″ N, 8°44′52″ E). Matmata has an arid climate with hot, dry summers and a short, highly variable humid period in winter with mean annual precipitation < 150 mm (Dearing et al., 1996). Bou-Hedma national park has a low arid climate with an approximate mean annual rainfall of 180 mm, a mean annual temperature of 17.2°C, and minimum and maximum monthly mean temperatures of 3.8°C in December and 36.2°C in July, respectively (Le Houérou, 2001). The Chott-Djerid is a flat area, with a mean altitude of 15 m. The mean annual rainfall for the area is around 100 mm (Richards and Vita-Finzi, 1982). The texture of the three soil samples was sandy to sandy-loamy.

Soil Sampling and Isolation of Bacterial Strains

Each soil collection was made from 10 to 15 cm depth below ground surface. Samples were placed in sterile flasks and transported immediately to the laboratory, where they were air-dried for 2 h at 45°C and sieved prior use. For isolation purpose 1 g of the dried samples was dissolved in 10 mL of sterile distilled water. Soil samples were shaken vigorously for 2 min and the suspensions were treated by heat for 10 min at 80°C, except the soil suspension of Chott-Djerid. A serial dilution in sterile physiological salt solution (0.9% NaCl) up to 10^{-4} was prepared and an aliquot of 0.1 mL was spread over Luria-Bertani (LB) medium agar plates. Plates were incubated at 37°C for 24 h. After incubation, colonies were isolated, recorded and subjected to antimicrobial activity screening. Purified strains that inhibited the growth of at least one of the test microorganisms, were selected, cryo-conserved in 20% v/v glycerol stocks, and stored at −20°C. The code used for the isolates is (B) for Bou-Hedma national park, (C) for Chott-Djerid, and (M) for Matmata.

Growth Conditions

The isolated bacterial strains were cultivated in LB broth, which contains 10 g peptone, 5 g yeast extract and 10 g NaCl per L. The pH was adjusted to 7 with 0.01M HCl and 0.01M NaOH. Incubation was performed at 37°C and agitation of 200 rpm. The cultures were inoculated into broth medium with 1% (v/v) inoculum.

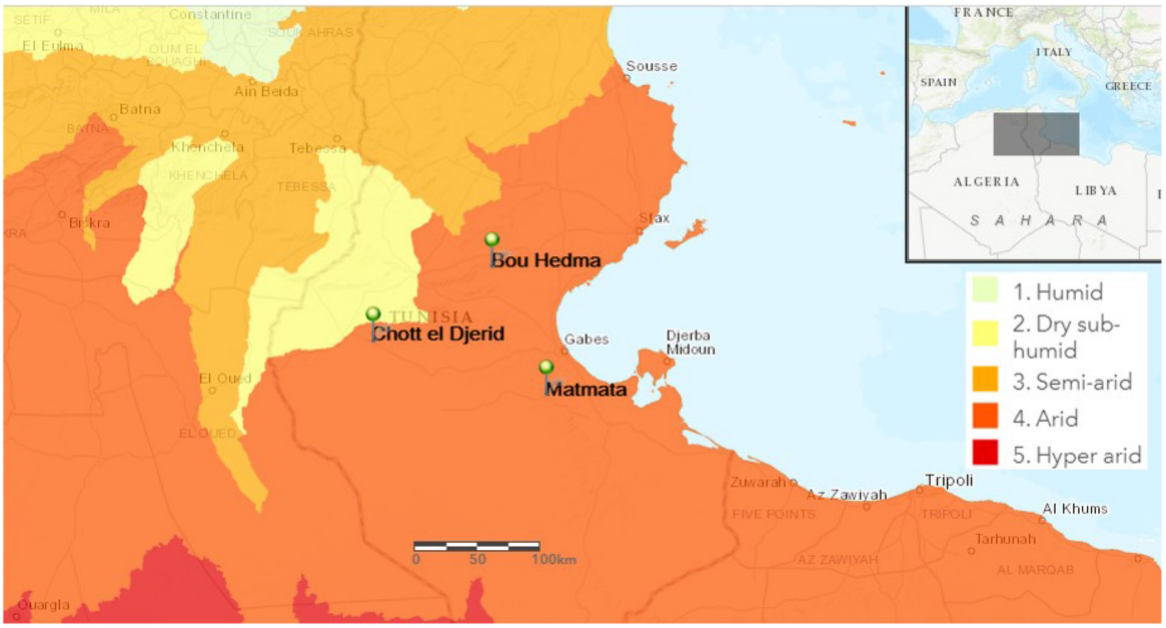


FIGURE 1 | Map of Tunisia showing the location of the sampling sites. Bou-Hedma national park (Sidibouzid), Chott-Djerid (Tozeur), and Matmata (Gabès).

Bacterial Strains

Gram-positive test bacteria used: The reference strain *Staphylococcus aureus* ATCC 29213, and three clinical isolates, i.e., *S. aureus*, *S. epidermidis*, and *S. saprophyticus*. The last three bacterial strains were kindly provided by the Tunisian Sahloul hospital at Sousse. Gram-negative test bacteria: Two clinical isolates of *Salmonella typhimurium* and six *Escherichia coli* strains (one clinical isolate *E. coli* MA, three reference strains *E. coli* ATCC 25922, ATCC 35218 and KL 16; two *in vitro* mutants *E. coli* KL 16.2a and *E. coli* KL 16.2b, derived from the reference strain *E. coli* KL 16 by selection at twofold MIC of ciprofloxacin. These strains carry point mutations in the *gyrA* and *parC* genes (Ser83Leu in GyrAand Ser80Ile in ParC, respectively). *E. coli* MA, carry three mutations, two in *gyrA* (Ser83Leu and Asp87Asn) and one in *parC* (Ser80Ile). The mutant strains were recovered from patients with urinary tract infection (Bachoual et al., 1998); and a clinical *E. coli* isolate from the strain collection of the Tunisian Sahloul hospital at Sousse.

Antimicrobial Activity

Primary Screening

The antibacterial activity of pure isolates was determined by spot assay on Mueller Hinton (MH) agar. In brief, the test bacteria, i.e., *E. coli* ATCC 25922 and *S. aureus* ATCC 29213 were plated on agar plates before isolated soil bacteria were spotted on top. Plates were incubated for 24 h at 37°C. After incubation, bacterial spots showing an inhibition zone were selected for a secondary screening, which was performed in triplicate by disk agar diffusion on MH agar plates against several test organisms. Cell free supernatant (50 μL) was used to saturate a sterilized Whatman filter paper disc (6 mm), allowed to dry at room temperature and placed onto agar plates inoculated with 10^7 CFU/mL of the test bacteria. Plates were incubated at 37°C for 24 h. Antimicrobial activity was evaluated by measuring the diameter of the inhibition zone around each paper disc. All tests were performed in triplicate. The average results are presented in **Supplementary Table 1**.

Screening of Large-Scale Extract

Strain *Bacillus* M21a was fermented as described at point 2.9. The organic phase of this fermentation was obtained by liquid/liquid extraction between fermentation broth and ethyl acetate. The resulting organic phase was subjected to FLASH chromatography (BUCHI Reveleris X2, column silica 40 g). The collected fractions were tested for their antibacterial activity on agar plates and on 96 well plates. For the 96 well plate assay, wells were loaded with respective amounts of 200, 100, and 50 μg of extract. The antibiotic ampicillin (final concentration of 20, 10, and 5 μg per well) and DMSO (10, 5, and 2.5 μL per well) were used as positive and negative control against the test bacteria, respectively. Volume per well was 200 μL. After loading the samples, the 96 well plate was measured at 600 nm to determine the absorbance (time T0). At T0, the OD_{600} was 0.1. After incubation at 30°C for 24 h, the absorbance was measured again (time T1). The growth inhibition of the test bacteria (in %) was calculated by using the formula: % inhibition = $(OD_{T1} - OD_{T0})/[AV\ (-\ control)] * 100$. Wells with values ≤ 30% were considered to have an antibacterial effect.

Identification of the Producer Strains

The isolates were preliminary characterized based on colony morphology and cell characteristics. Subsequently, identification by 16S rDNA sequencing was performed. Therefore, the 16S

rDNA of the isolates was amplified by PCR using universal primers pA (5′AGAGTTTGATCCTGGCTCAG 3′) and pH (5′AAGGAGGTGATCCAGCCCCA 3'). Amplification was done according to the following profile: an initial denaturation step at 94°C for 10 min, followed by 30 amplification cycles of 94°C for 45 s, 56°C for 45 s, and 72°C for 2 min, and a final extension step of 72°C for 4 min. The PCR product was subjected to agarose gel electrophoresis at 80V for 30 min. The DNA fragment in the agar was visualized by ultraviolet fluorescence after ethidium bromide staining, then excised and purified using a kit (Wizard® SV Gel and PCR Clean Up System, Promega).

The resulting fragments were cloned into pGEM-T vector (Promega, Madison, WI, United States), introduced into chemical competent *E. coli* XL-1 Blue cells by transformation. The plasmids were isolated using the PureYieldMiniprep kit (Promega, Madison, WI, United States) and the inserts were sequenced from both sides using T7and SP6 primers (GATC BioTech AG Company, Konstanz, Germany). Vector fragments in the sequencing result were removed using VecScreen-Blast and the full 16S rDNA were assembled. The 16S rDNA sequences were blasted with the available 16S rRNA gene sequences contained in the GenBank database[1].

Gram Staining and Mobility Test of Bioactive Bacterial Isolates

A smear of bacterial isolate was applied onto a clean glass slide and heated gently over a flame. The fixed bacteria were covered with a thin film of crystal violet for 1 min and washed gently under slow running water. Gram's iodine solution was flooded over the glass slide for 1 min and washed with water. Ethanol (80%) was used to decolorize the sample. Counter staining was performed with fuchsine for 2 min. Glass slides were washed, air dried, and analyzed.

For the bacterial mobility test, a small drop of bacterial culture was placed in the center of a clean microscope slide, covered with a cover glass, and then examined microscopically for the motility of the bacterial cells.

Phylogenetic Analysis

Sequences of each isolate were refined using BioEdit sequence Alignment Editor (Hall, 1999), in which the sequences obtained from both primers were assembled to obtain consensus sequences. To analyze the relationships of the isolates to known bacterial species, the 29 sequences from this study and sequences of type strains, which had the closest relationship, were initially aligned using the MUSCLE Multiple alignment (Edgar, 2004). Based on the homology of 16S rDNA sequences, the evolutionary distances were computed through MEGA (version 7.0) program (Kumar et al., 2016) with p-distance using neighbor-joining method (Saitou and Nei, 1987). Gaps were treated as missing data. Further, the bootstrap values were calculated from 1000 replications to represent the evolutionary history of the bacterial isolates (Felsenstein, 1985).

[1]http://www.ncbi.nlm.nih.gov/

Isolation of 1-acetyl-β-carboline

Two *Bacillus* strains, i.e., M21 (Nasfi et al., 2018) and M21a, were identified positive for the production of 1-acetyl-β-carboline. For isolation, both strains were inoculated into LB broth and incubated at 30°C under shaking condition. However, strain M21 lost its viability and therefore all downstream steps were performed using strain M21a. In order to isolate the bioactive compound(s), a single colony of *Bacillus* sp. M21a was subcultured in LB broth and incubated at 30°C for 24 h. The resulting culture was used to inoculate forty 5 L flasks containing 1 L LB broth each. Flasks were incubated at 30°C for 48 h with agitation at 140 rpm in presence of 20 g/L autoclaved amberlite XAD-16, a polymeric resin often used as a first step in downstream processing to separate bioactive compounds from bacterial growth media. The resin was collected by filtration and washed sequentially with 5 L distilled water to remove impurities. Thereafter, the resin was resuspended in a mixture of MeOH-acetone and maintained at 26°C for 18 h with agitation followed by filtration. The resulting filtrate was concentrated by a rotary evaporator at 40°C under vacuum to yield the crude extract. This extract was partitioned using liquid-liquid separation between MeOH 60% in water and DCM. The organic phase was bioassayed for activity by disc diffusion method against *E. coli* and *B. megaterium* and was subsequently subjected to successive chromatographic purification. Medium pressure ("Flash") chromatography was performed on a silica gel 40 column, using an elution gradient with A: Petroleum ether and B: acetone with A:B from 100–0 to 0–100% (Reveleris Silica 40 μm, 12 g; Flow rate 40 mL/min; 400 mg – 8 g sample). Purity of the active fraction(s) was further examined using HPLC with an RP column (Xterra RP 18–5 μm). At a concentration of 5 mg/mL, a 20 μL aliquot was applied onto the RP column. The mobile phase consisted of an isocratic gradient of 60% MeOH (solvent B) in Milli-Q water (solvent A). The flow rate of the mobile phase was set at 0.8 mL/min for 45 min. Elution was monitored at a wavelength of 210 nm and fractions were collected manually. Resulting fractions were dried using rotary evaporators. Resulting precipitates were dissolved in MeOH for antibacterial activity testing. Fractions from a given retention time that showed antibacterial activity were pooled from different HPLC runs and concentrated.

Structure Elucidation of the Purified Compound

High-resolution mass analysis of the bioactive compound was performed using a MicrOTOF-QII mass spectrometer instrument (Bruker Daltonics GmbH, Germany).

^{1}H and ^{13}C-NMR spectra were recorded on a Bruker 600 Ascend spectrometer. Purified and lyophilized bioactive compound was dissolved in MeOH-d_4. NMR spectra were referenced to residual solvent signals of methanol at δ_H3.35 and δ_C49.0. Coupling constants (J) are given in Hz. ^{1}H and ^{13}C-NMR spectroscopic assignment analyses were performed using correlation spectroscopy (COSY) and heteronuclear multi-bond correlation (HMBC) spectroscopy.

LC-MS/MS Data Processing – Molecular Networking

Mass data were converted to mzXML format with Bruker's Data Analysis software and uploaded to the Global Natural Product Social (GNPS) molecular networking tool[2]. In GNPS Wang et al. (2016), the data were subjected to molecular networking using the online workflow at GNPS. The data were clustered with MS-Cluster with a parent mass tolerance of 0.5 Da and a MS/MS fragment ion tolerance of 0.2 Da to create consensus spectra. A network was then created where edges parameters were cosine score above 0.2 and more than three matched peaks. The network was then searched against GNPS's spectral libraries. The network analysis was exported from GNPS and analyzed in Cytoscape (Shannon et al., 2003).

RESULTS

Soil Bacteria Isolation and Antibacterial Activities

In total 373 bacterial strains were isolated from rhizosphere samples of pseudo-savannah vegetation from three different arid regions in Tunisia. Thereunder, 25 isolates from Bou-Hedma, 140 from Chott-Djerid and 208 from Matmata. All strains were fast growing organisms and produced single colonies after 24h incubation at 37°C. Of these strains 29 (7.8%) showed moderate to strong antibacterial activity in the first screenings against *E. coli* ATCC 25922 and *S. aureus* ATCC 29213. These active strains were analyzed for their morphology and all bacteria showed a rod shape appearance. Gram-staining revealed that except for isolate C6, all isolates were Gram-positive bacteria. Ten of the 29 appeared non-motile, while the majority showed motility by microscopic investigation. The morphological characteristics (shape, margin, color, cell shape, motility, and Gram-staining) and the motility of the 29 soil bacteria are summarized in **Supplementary Table 2**.

To evaluate if the initial screening results could be verified, all strains were re-tested against a panel of Gram-positive and Gram-negative test strains; thereby all strains retained their activity (**Supplementary Table 1**). The most active strains were derived from all three sampling sites. Most interestingly, 93% were active against Gram-negative bacteria. Against Gram-positive bacteria very prominent activities were observed. Only strain C2 showed no activity against Gram-negative strains. Indeed, strain C2 showed the narrowest spectrum of activity, inhibiting solely the Gram-positives *S. aureus* ATCC 29213 and *S. epidermidis* (**Supplementary Table 1**). Four strains, i.e., C5, C6, C7, and C11, all from one sampling site, showed the largest activity spectrum with inhibition zones against all tested bacteria, except the clinical *E. coli* isolate.

16S rDNA Sequence Analysis

To obtain an insight into the diversity and phylogeny of the isolated strains showing antibiotic activity, these isolates were identified by 16S rRNA gene sequence analysis. The sequences obtained for the strains were compared to sequences deposited in publicly accessible databases. This revealed that the strains belong to three different bacterial phyla, i.e., *Firmicutes*, *Actinobacteria,* and *Proteobacteria*. However, *Bacillaceae* represented by far the most prominent family (86%), followed by *Brevibacteriaceae* (2%) and *Pseudomonadaceae* (2%). In fact, out of the 28 soil isolates, 26 were members of the genus *Bacillus*, one was *Brevibacterium halotolerans* and one was *Pseudomonas stutzeri* (**Figure 2** and **Table 1**). Hence, in this project, strains of the genus *Bacillus* were found to dominate the strain collection. It seemed that they represent the most isolated bacterium from rhizospheric soil samples, which is in accordance with published data, since it was reported that the genus *Bacillus* is very common in soil by Amin et al. (2015). However, it must be considered that their ability to form highly resistant endospores is on the one hand the key for their successful colonization of a wide variety of environmental habitats, especially dry arid biological niches. On the other hand, the isolation strategy applied clearly favors spore formers.

Isolation of 1-acetyl-β-carboline

Determination of the Fermentation Conditions

To get first insights into the metabolomic basis of the antibacterial activities observed, it was projected to isolate the underlying compound. First, a favorable culture medium was selected, to enable isolation of the antibacterial compound by high enough production yields. Therefore, strain M21a was cultured in 14 different media for 48 h at 30°C. Then the fermentation broth was tested by agar diffusion test against the test strains *E. coli* and *B. megaterium*. The composition of the culture media tested is given in **Supplementary Table 3**. All media selected promoted the growth of *Bacillus* sp. M21a, yielding visible biomass production. However, concerning the antibacterial activity, LB medium gave the best results against both, Gram-positive and Gram-negative test bacteria. In contrast, fermentation in M8 medium resulted in the lowest antibacterial activity observed. For all other 12 culture media, antibacterial activity was detected only against the Gram-positive test strain *B. megaterium* and not against the Gram-negative *E. coli*. Since we aimed to find the reason for the anti-Gram-negative activity, LB medium was chosen for the up-scaling.

Extraction, Purification and Structure Elucidation of 1-acetyl-β-carboline

For the isolation of the target compound, the producer strain *Bacillus* sp. M21a was fermented in 40 L LB medium. The organic phase of this fermentation broth was obtained by liquid-liquid extraction. The isolation of the compound was performed in a bioactivity-based manner. From the fermentation, 8 g dry weight were obtained and revealed a strong activity against *E. coli* (18 mm) and *B. megaterium* (30 mm) at a concentration of 5 mg/mL and 1 mg/mL, respectively. The bioactive crude extract was fractionated using Flash chromatography, which resulted in seven fractions. Subsequent bioactivity assay revealed the loss of activity against *E. coli*. However, one fraction, i.e., fraction

[2]https://gnps.ucsd.edu/ProteoSAFe/static/gnps-splash.jsp

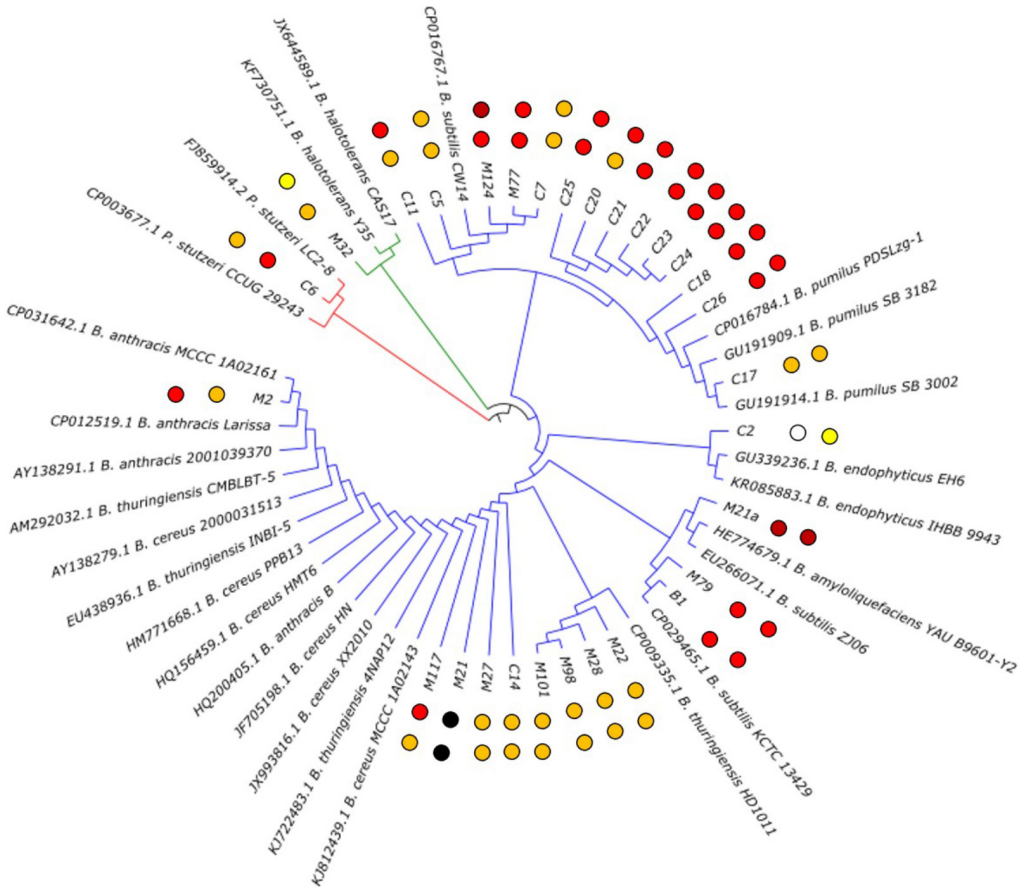


FIGURE 2 | Phylogenetic tree of the isolated bioactive bacteria. The two closest homologoues based on 16 rDNA analysis for each isolate are given. The isolates from this study are carrying their identifier (compare **Table 1**). The observed antibacterial activity against *E. coli* ATCC25922 (inner dots) and *S. aureus* ATCC 29213 (outer dots) is indicated by dark red (strong activity), red (good activity), orange (moderate activity) and yellow (weak activity) dots. Strain M21 lost its viability during the project; therefore, the values could not be determined.

1, maintained activity against *B. megaterium*. For further fractionation, fraction 1 was separated by an analytical HPLC run. Three fractions had been collected and a 96 well plate assay was performed against the Gram-positive *Arthrobacter crystallopoietes*. We found that 200 μg of Fr1.1 showed a good antibacterial activity, whereby the other fractions showed no inhibition.

To finally purify the active compound, fraction Fr1.1 was purified by an additional HPLC separation. A peak with an adsorption maximum of 210 nm was observed at 12 min. This peak was manually collected and we obtained 1.8 mg of the bioactive compound.

By high-resolution LC-MS analysis, the mass was determined to be *m/z* 211.0907 $[M + H]^+$ (**Supplementary Figure 1**). This mass fitted to a compound with the molecular formula $C_{13}H_{10}N_2O$ (calculated *m/z* for $[M + H]^+$: 211.0871). To identify the bioactive compound unambiguously, NMR analyses were performed. The ^{1}H-NMR spectrum revealed signals for six aromatic protons (δ_H7.32; 7.61; 7.71; 8.23; 8.47; and 8.32), two ortho-coupled doublets at δ_H7.71 and 8.23 (J = 7.9 Hz) and four multiplet protons of which two were triplets at δ_H7.32 and 7.61 (J = 7.9 Hz) (**Supplementary Table 4** and **Supplementary Figure 2**). These data indicated the presence of a 1,2-disubstituted aromatic ring. In the aliphatic region, the ^{1}H-NMR spectrum showed the presence of an aromatic bounded methyl group singlet at δ_H2.83. The ^{13}C-NMR spectrum showed signals for 13 carbon atoms (**Supplementary Figure 3**). Among these, six aromatic methine *C*-atoms (δ_C129.8; 130.2; 113.4; 122.5; 138.54; and 120.1) and five sp^2 quaternary carbons (δ_C143.3; 121.5; 132.3; 130.7; and 137.1) (Tab. S4). The remaining two carbons were considered as an acetyl group attached to C-9, indicated by the HMBC correlation between H-13 and C-9. The COSY (**Supplementary Figure 4**) and HMBC data (**Supplementary Figure 5**) led to reveal the structure of 1-acetyl-β-carboline (**Figure 3**) that was confirmed by the comparison with literature data (Lee et al., 2013).

Identification of Bioactive Lipopeptides

In addition to the here isolated carboline, derivatives of the known fungicides bacillomycin and fengycin were identified by mass-based analyses (**Figure 4**). These bioactive lipopeptides

TABLE 1 | Closest relative of the 29 bioactive soil isolates based on 16S rDNA sequence.

Strain	Species[a]	Accession	Identity	Origin
B1	*Bacillus subtilis* KCTC 13429	CP029465.1	99	Bou-Hedma national park
C2	*Bacillus endophyticus* IHBB 9943	CP011974.1	98	Chott-Djerid
C5	*Bacillus subtilis* strain CW14	CP016767.1	99	Chott-Djerid
C6	*Pseudomonas stutzeri* strain LC2-8	FJ859914.2	99	Chott-Djerid
C7	*Bacillus subtilis* strain CW14	CP016767.1	99	Chott-Djerid
C11	*Bacillus subtilis* KCTC 13429	CP029465.1	99	Chott-Djerid
C14	*Bacillus cereus* strain XX2010	JX993816.1	99	Chott-Djerid
C17	*Bacillus pumilus* strain SB 3002	GU191914.1	99	Chott-Djerid
C18	*Bacillus pumilus* strain PDSLzg-1	CP016784.1	99	Chott-Djerid
C20	*Bacillus pumilus* strain PDSLzg-1	CP016784.1	99	Chott-Djerid
C21	*Bacillus pumilus* strain PDSLzg-1	CP016784.1	99	Chott-Djerid
C22	*Bacillus pumilus* strain PDSLzg-1	CP016784.1	99	Chott-Djerid
C23	*Bacillus pumilus* strain PDSLzg-1	CP016784.1	99	Chott-Djerid
C24	*Bacillus pumilus* strain PDSLzg-1	CP016784.1	99	Chott-Djerid
C25	*Bacillus pumilus* strain PDSLzg-1	CP016784.1	99	Chott-Djerid
C26	*Bacillus pumilus* strain PDSLzg-1	CP016784.1	99	Chott-Djerid
M2	*Bacillus anthracis* strain MCCC 1A02161	CP031642.1	99	Matmata
M21	*Bacillus thuringiensis* strain CMBLBT-5	AM292032.1	100	Matmata
M21a	*Bacillus subtilis* strain ZJ06	EU266071.1	99	Matmata
M22	*Bacillus thuringiensis* strain HD1011	CP009335.1	99	Matmata
M27	*Bacillus anthracis* strain B	HQ200405.1	99	Matmata
M28	*Bacillus anthracis* strain B	HQ200405.1	99	Matmata
M32	*Brevibacterium halotolerans* strain CAS17	JX644589.1	99	Matmata
M77	*Bacillus subtilis* strain CW14	CP016767.1	99	Matmata
M79	*Bacillus subtilis* KCTC 13429	CP029465.1	99	Matmata
M98	*Bacillus anthracis* strain MCCC 1A02161	CP031642.1	99	Matmata
M101	*Bacillus thuringiensis* strain HD1011	CP009335.1	99	Matmata
M117	*Bacillus thuringiensis* strain CMBLBT-5	AM292032.1	99	Matmata
M124	*Bacillus subtilis* strain CW14	CP016767.1	99	Matmata

[a]*The best hit obtained by BlastN analysis is given.*

from the methanolic extract of a solid cultivation of *Bacillus* sp. M21a were detected using LC/MS in positive ion mode. In the MS spectra, three clusters of molecular mass ions belonging to the iturin, surfactin and fengycin lipopeptide families were found. These included four bacillomycins, members of the iturin family, having fatty acyl chain lengths of C13–C16, three fengycins A (from C14 to C16 and three fengycins B (C15–C17), and six surfactins (C12–C17).

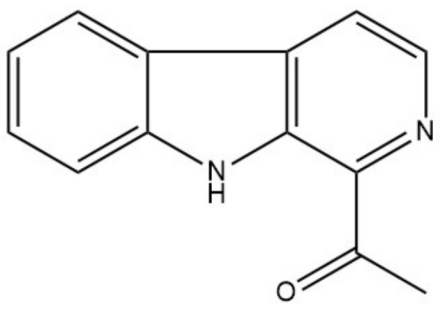


FIGURE 3 | Structure of 1-acetyl-beta-carboline.

Bacillomycin Identification

The protonated molecular mass ions $[M + H]^+$ at m/z 1017.51, 1031.52, 1045.53, and 1059.55 (**Supplementary Figure 3A**) were assigned as putative homologs of bacillomycin D, a variant of the iturin molecule group, with C13, C14, C15, and C16 β-amino fatty acids, respectively (Cao et al., 2012). On the basis of NORINE's database, which contains the most common non-ribosomal peptides, these compounds were identified as C13-bamD ($C_{47}H_{72}N_{10}O_{15}$), C14-bamD ($C_{48}H_{74}N_{10}O_{15}$), C15-bamD ($C_{49}H_{76}N_{10}O_{15}$), and C16-bamD ($C_{50}H_{78}N_{10}O_{15}$). The sequence of the compound C14-Bam D with an *m/z* of 1,031.5 was determined from series of *N*-terminal and proline-directed fragments (**Supplementary Figure 4A**). The peptide ring of this compound was cleaved both at the peptide bond between its amino fatty acid residue (β-NH_2-FA) and threonine at position 7 (Thr_7) as well as at the N-terminal of proline 4 (Pro_4). In the first case, fragment ions at *m/z* 340.2, 504.2, and 617.3 were detected and corresponded to $[M + H\text{-Tyr-Asn-Pro-Glu-Ser-Thr}]^+$, $[M + H\text{-Asn-Pro-Glu-Ser-Thr}]^+$, and

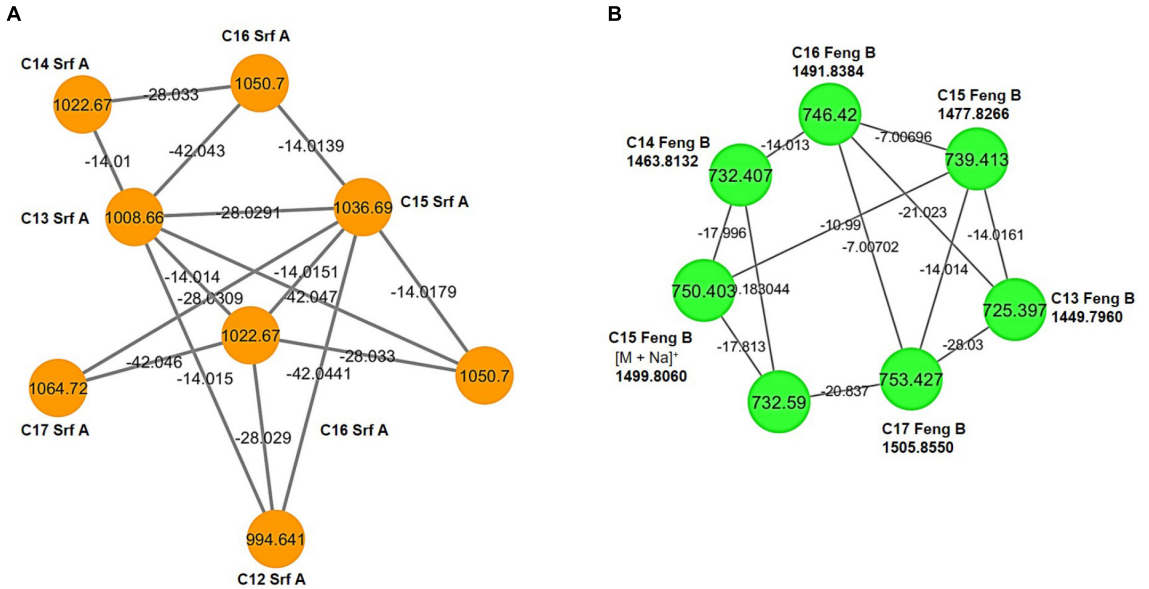


FIGURE 4 | Molecular networks of *Bacillus* sp. M21a extracts. **(A)** Surfactin A homologs ($[M + H]^+$; orange), the *m/z* differences of 14.01, 28.03, and 42.04 indicate molecules with different lengths of fatty acid chains within this cluster. **(B)** Fengycin B cluster from double charged precursor ions *m/z* ($[M + 2H]^{2+}$; green). Nodes represent the precursor ion and bridges indicate the mass difference between individuals nodes. Labels at the nodes name the respective dereplicated molecule. Srf, surfactin; Feng, fengycin, the number indicates the length of the lipid tail.

$[M + H\text{-Pro-Glu-Ser-Thr}]^+$, respectively. In the second case, fragment ions at *m/z* 227.1, 314.1, 441.3, 617.3, 754.4, and 917.4 were observed and corresponded to $[M + H\text{-Ser-Thr-FA-Asn-Tyr-Asn}]^+$, $[M + H\text{-Thr-FA-Asn-Tyr-Asn}]^+$, $[M + H\text{-FA-Asn-Tyr-Asn}]^+$, $[M + H\text{-Asn-Tyr-Asn}]^+$, $[M + H\text{-Asn-Tyr}]^+$, and $[M + H\text{-Asn}]^+$, respectively. The MS/MS spectrum of $[M + H]^+$ ion at *m/z* 1045.5 presented fragments with *m/z* at 517.3, 618.3, 654.4, 732.4, 768.4, 829.4, 931.5, and 958.5. The corresponding amino acid sequence is given in **Supplementary Figure 4B**. In lower intensity *Bacillus* sp. M21a produced C16-Bam D and its fragmentation pattern is given in **Supplementary Figure 4C**.

Fengycin Identification

The LC-MS data and the molecular networking analysis showed that the ions corresponding to fengycins were $[M + 2H]^+$ (*m/z* 718.392) for C14-fenA ($C_{70}H_{106}N_{12}O_{20}$), $[M + 2H]^+$ (*m/z* 725.397) for C15-fenA ($C_{71}H_{108}N_{12}O_{20}$), $[M + 2H]^+$ (*m/z* 732.407) for C16-fenA ($C_{72}H_{110}N_{12}O_{19}$), $[M + 2H]^+$ (*m/z* 739.413) for C15-fenB ($C_{72}H_{110}N_{12}O_{20}$), $[M + 2H]^+$ (*m/z* 746.42) for C16-fenB ($C_{74}H_{114}N_{12}O_{20}$), and $[M + 2H]^+$ (*m/z* 753.427) for C17-fenB ($C_{75}H_{116}N_{12}O_{20}$) (**Supplementary Figure 3B**). The fragmentation patterns of C15-FenA, C16-FenA, C15-FenB and C17-FenB are given in **Supplementary Figure 5**.

Surfactin Identification

The isolate M21a was also able to produce surfactins. Ion peaks at *m/z* 994.64, 1008.65, 1022.67, 1036.68, 1050.69, and 1064.71 were obtained and correspond to C12 to C17-srf, respectively (**Supplementary Figure 3C**). The MS/MS spectra of surfactin isoforms showed common fragments $[M + H]^+$ at *m/z* 685.45, 671.43, and 699.46 (**Supplementary Figure 6**). These ions are from characteristic amino acid sequences, previously reported as Val/Leu/Asp/Val/Leu/Leu, Leu/Leu/Asp/Val/ Leu/Leu, Leu/Leu/Asp-OMe/Val/Leu/Leu and Leu/Leu/Asp/Leu/Leu/Leu, respectively (Bonmatin et al., 1995; Tang et al., 2010; Biniarz and Lukaszewicz, 2017). The MS/MS spectrum of $[M + H]^+$ ion at *m/z* 1036.6869 presented fragments corresponding to losses of amino acids Leu/Leu/Asp/Val/Leu/Leu, with *m/z* 923.6029, 810.5212, 695.4953, 596.4275, 483.3442, and 370.2595, respectively (**Supplementary Figure 4**). The *m/z* 370.2595 corresponded to glutamic acid residue with aliphatic fatty acid chains containing 15 carbons, indicating similarities between the proposed surfactin A (C15) produced by *Bacillus* sp. M21a and the known surfactin (Liao et al., 2016). Additional fragment ions $[M + H]^+$ confirmed the presence of amino acids sequence as *m/z* 227.1766 $[Leu + Leu + H]^+$, *m/z* 328.1873 $[Leu + Asp + Val + H]^+$, *m/z* 441.2721 $[Leu + Asp + Val + Leu + H]^+$, and *m/z* 554.3560 $[Leu + Asp + Val + Leu + Leu + H]^+$ (**Supplementary Figure 5**). The molecular networking analysis showed compounds with *m/z* differences of 14.01, 28.03, and 42.04, suggesting molecules with different lengths of fatty acid chains within the same isoform family (**Figure 4**). Surfactin isoforms $[M + H]^+$ are displayed in **Table 2**.

DISCUSSION

The development of effective medicinal drugs has been revolutionizing the treatment of many human diseases. Lead structures for these drugs were detected by natural product

TABLE 2 | Lipopeptide identified in *Bacillus* sp. M21a.

Structure	Molecular formula	$[M + H]^+$	Observed $[M + H]^+$
BACILLOMYCIN D			
C13	$C_{47}H_{72}N_{10}O_{15}$	1017.5000	1017.5102
C14	$C_{48}H_{74}N_{10}O_{15}$	1031.5395	1031.5245
C15	$C_{49}H_{76}N_{10}O_{15}$	1045.5551	1045.5382
C16	$C_{50}H_{78}N_{10}O_{15}$	1059.5707	1059.5520
C17	$C_{51}H_{81}N_{10}O_{15}$	1073.5863	1073.5680
FENGYCIN			
C14 – Fen A	$C_{70}H_{106}N_{12}O_{20}$	1435.7725	1435.7854
C15 – Fen A	$C_{71}H_{108}N_{12}O_{20}$	1449.7881	1449.7960
C16 – Fen A	$C_{72}H_{110}N_{12}O_{20}$	1463.8038	1463.8132
C15 – Fen B	$C_{72}H_{110}N_{12}O_{20}$	1477.8194	1477.8266
C16 – Fen B	$C_{74}H_{114}N_{12}O_{20}$	1491.8351	1491.8384
C17 – Fen B	$C_{75}H_{116}N_{12}O_{20}$	1505.8510	1505.8550
SURFACTIN A			
C12	$C_{50}H_{87}N_7O_{13}$	994.6440	994.6401
C13	$C_{51}H_{89}N_7O_{13}$	1008.6597	1008.6550
C14	$C_{52}H_{91}N_7O_{13}$	1022.6753	1022.6701
C15	$C_{53}H_{93}N_7O_{13}$	1036.6912	1036.6843
C16	$C_{54}H_{95}N_7O_{13}$	1050.7059	1050.6999
C17	$C_{55}H_{97}N_7O_{13}$	1078.7379	1064.7100

research, since it seems that nature represents a most important resource for biologically active compounds. However, concerning antibiotics, which can be regarded as option of choice to combat bacterial infections, most of the drugs in application were already discovered several decades ago (Pidot et al., 2014). The increase of highly resistant pathogenic bacteria could put us back into a situation comparable to the pre-antibiotic era. Thus, there is an urgent need for a new age of antibiotic discovery (Khan and Khan, 2016). Ninety percent of all antibiotics, which are in clinical use today, are derived from microorganisms (Katz and Baltz, 2016) and the majority of the modern classes of antibiotics was discovered by antimicrobial activity-based screening approaches of microorganisms isolated and cultured predominantly from soil (Rolain et al., 2016). In other words, bacteria have so far been and can be also expected to remain the most promising resource for antibiotics in the future, since the majority of drugs has been developed from lead structures on the basis of bacterial natural products (Elbandary et al., 2018). Proven proliferative antibiotic-producing bacterial species include Actinomycetes, predominantly the genus *Streptomyces* (Hug et al., 2018), myxobacteria (Mulwa et al., 2018), cyanobacteria (Senhorinho et al., 2015), *Bacilli* (Sumi et al., 2015), and Pseudomonads (Gross and Loper, 2009). Even though many compounds have been already isolated from the before mentioned bacteria, it must be considered that uncultured bacteria sum up to over 99% of all species in external environments (Ling et al., 2015). Hence, there could still be a multitude of metabolites awaiting discovery, and even for well-studied producer organisms it was shown that they harbor many more biosynthetic gene clusters (BGC) for the production of specialized metabolites than natural products described from them (Pidot et al., 2014).

Only a few reported surveys exist on the bacterial diversity in arid areas, where bacterial species should be able to deal with relatively high temperatures, salt concentrations, and radiation. Hence, arid and desert habitats represent special ecosystems, and it can be expected that the chance to isolate uncommon bacteria with new metabolic capabilities is high (Trabelsi et al., 2016). In the present study, bacteria were isolated from Tunisian underexplored arid areas. Thereunder, 28 rhizospheric bacteria showed the ability to produce natural products effective against both Gram-positive and Gram-negative bacteria. The majority, 26 isolates, belonged to the Firmicutes phylum (genus *Bacillus*); one isolate belonged to the Actinobacteria phylum (genus *Brevibacterium*) and one to the Proteobacteria phylum (genus *Pseudomonas*). This is in accordance to the fact that *Bacillus* species are dominant soil bacteria. Their abilities of endospore-formation and antibiotics production have to be regarded as an advantage for the colonization of environments (Moshafi et al., 2011; Amin et al., 2015). Antimicrobial peptides of *Bacilli* were regarded as a promising starting point in the search for new antibacterial drugs (Sumi et al., 2015), and several known antibacterial compounds had been isolated, e.g., subtilin (Kim et al., 2012), surfactin (Jacques, 2011), and macrolactin A (Lu et al., 2008). Even though in this screening only one actinobacterial strain was isolated, they have been shown to be a resource with an unprecedented diversity of biosynthetic pathways, awaiting to be exploited for the identification of novel scaffolds. The latter could then contribute to the filling of the antibiotics development pipeline. *Brevibacterium halotolerans* SA87, a close homolog to the here isolated strain, has been shown to produce a variety of specialized metabolites with distinct bioactivities. It was shown that *B. halotolerans* SA87inhibits the growth of both Gram-positive and Gram-negative pathogens (Ahmed et al., 2015). Concerning *Pseudomonas* species, many specialized metabolites are reported and reviewed (Gross and Loper, 2009). This group of bacteria adapts to different stress environments and produces a wide range of bioactive metabolites with antimicrobial activity and various *Pseudomonas* species are able to biosynthesize compounds with a broad biological activity (Robles-Huízar et al., 2017). *Pseudomonas stutzeri* CMG1030, the closest homolog to strain C6, was reported to biosynthesize zafrin, an antibacterial compound, which showed strong antibacterial activity against Gram-positive as well as against Gram-negative bacteria (Uzair et al., 2008).

The strain *Bacillus* sp. M21a showed strong antimicrobial activity against Gram-positive and Gram-negative test strains and was chosen for further investigation and isolation of the bioactive compound. However, after fractionation the activity against *E. coli* was lost. This might be due degradation of the active compound(s), or to synergistic effects between different compounds, which were separated by the fractionation. The Gram-positive active compound was identified as 1-acetyl-β-carboline. The structure of this compound was unambiguously proven by NMR experiments and the data obtained were in good agreement with reported literature values (Huang et al., 2011; Lee et al., 2013). This compound was previously isolated from several types of organisms. Thereunder plants like *Ailanthus malabarica* and *Hypodematiumsquamuloso-pilosum*

(Joshi et al., 1977; Zhou et al., 1998), a fungus *Neosartorya pseudofischeri* (Lan et al., 2016), a sponge *Tedaniaignis* (Dillman and Cardellina, 1991), as well as several bacteria, e.g., *Steptomyces* and *Pseudomonas* spp. (Proksa et al., 1990; Shin et al., 2010; Huang et al., 2011; Chen et al., 2013; Lee et al., 2013; Broberg et al., 2017). The β-carboline alkaloid displays various biological activities, e.g., antitumor, antimicrobial, antiviral, and antiparasitic (Cao et al., 2007; Lee et al., 2013). 1-acetyl-β-carboline showed antibacterial activity against both, MSSA and MRSA strains. The reported activities have to be considered as moderate, however, the MRSA strain tested was inhibited more efficiently by 1-acetyl-β-carboline (MICs in the range of 32–128 μg/mL) than by the β-lactams tested (ampicillin, penicillin and oxacillin, MICs in the range of 64–512 μg/mL). Further, 1-acetyl-β-carboline was described to possess synergistic effects with ampicillin and penicillin. Shin et al. (2010) found that the fractional inhibitory concentration (FIC) indices of 1-acetyl-β-carboline in combination with ampicillin and of 1-acetyl-β-carboline in combination with penicillin were in the range of 0.156–0.313 and of 0.188–0.375 in combination with 32 and 64 μg/mL of 1-acetyl-β-carboline against the MRSA strains tested. Thereby, a synergistic effect was observed. However, it did not exhibit a synergistic antibacterial effect against most of the MRSA strains tested, if combined with oxacillin. The same difference in synergistic effects has been also observed in combinations of dieckol and β-lactams (Lee et al., 2008), as well as epigallocatechin gallatein combination with β-lactams (Zhao et al., 2001). The mechanism of the observed synergism between 1-acetyl-β-carboline and β-lactams is unknown. It was speculated that a possible reason for the synergistic effect can be attributed to the fact that both molecules attack the same target of the cell wall, but on different sites due to their different structures (Shin et al., 2010). Another study also reported that 1-acetyl-β-carboline in contrast to β-lactams was active against both MRSA and MSSA (Lee et al., 2013). In light of these findings, it seems reasonable that the antibacterial activity of 1-acetyl-β-carboline is not related to PBP2, the main target site of β-lactams in the MSSA cell wall, since it was also active against MRSA cells, which have PBP2a in the cell wall. The latter has a low affinity toward β-lactams. The biosynthesis of 1-acetyl-β-carboline was described for *Marinactinospora thermotolerans*. In this bacterium, a BGC, consisting of the three genes *mcbA, mcbB* and *mcbC*, was identified to be responsible for the generation of the β-carboline scaffold. This scaffold is generated by a Pictet-Spengler (PS) reaction, using tryptophan (or tryptamine) and an aldehyde as substrates. The PS reaction is an important reaction for the synthesis of natural products especially in those bearing indole and isoquinoline alkaloids as scaffolds in their complex structures (Heravi et al., 2018). The novel enzyme McbB encoded in this BGC was characterized to catalyze the PS cyclization/decarboxylation/oxidation process. By heterologous expression experiments in *Streptomyces lividans* TK64 and *Escherichia coli* BL21, it was found that *mcbB* was sufficient in *E. coli* for the production of 1-acetyl-3-carboxy-β-carboline, which represents the major product. Furthermore, two minor products, i.e., 1-acetyl-β-carboline and 1-acetyl-3-hydroxy-β-carboline, are generated (Chen et al., 2013). By *in vivo* gene inactivation and bioinformatic tools, Chen et al. (2013) assigned the other proteins encoded in the BGC as a CoA ligase (McbA) and a decarboxylase (McbC).

It was reported before that 1-acetyl-β-carboline produced by *Streptomyces* sp. 04DH52 exhibits only a moderate antibacterial activity against Gram-negative bacteria with MICs ranging from 64 to 256 μg/mL (Shin et al., 2010). Also Lee et al. (2013) reported that 1-acetyl-β-carboline, isolated from *Pseudomonas* sp. UJ-6, showed antibacterial activity against Gram-negative bacteria with MICs ranging from 32 to 128 μg/mL. However, all the reported activities are in the moderate to low range. In our hands, *Bacillus* sp. M21a extracts lost *E. coli* activity after fractionation by Flash chromatography. This can be explained by a number of possible reasons. When we tested the organic phase of *Bacillus* sp. M21, many compounds were present in this mixture and might be interacting with one another to result in the inhibition observed. When the compounds were separated by using Flash chromatography, these could not interact anymore, which resulted in the loss of *E. coli* inhibition. It can be speculated that the compound 1-acetyl-beta-carboline was likely working with an unkown compound to yield the inhibitory effect against *E. coli*. Separation of the compounds abolished the synergistic effects they had in combination. Another reason why this occurred might be the instability of the active component during the Flash fractionation and dryness process, due to light or high temperature sensitivity leading to changes in the conformation of the active molecule, before testing the activity again. Another factor could have accounted for such a loss of activity; it might be a question of concentration that means that after fractionation we lost an amount of the active compound and its concentration, in one of the collected fractions, wasn't sufficient to inhibit the test *E. coli* strain.

In addition to the antibacterial compound, further fungicides were identified. These natural products are involved in the plant growth promoting effects described for various *Bacilli*. Fengycins, composed of 10 amino acids linked to a fatty acid chain with 14–19 carbon atoms, exhibit a strong antifungal activity due to their interaction with the cell membrane, which results in an increased cell permeability, finally leading to an ultrastructural destruction of pathogenic fungi (Yang et al., 2015). Fengycins were described to possess an antibacterial activity against the Gram-positive *S. epidermidis* and the Gram negative *E. coli* (Huang et al., 2006; Roy et al., 2013). Huang et al. (2006) reported also that fengycins showed an antiviral activity against Newcastle disease virus and bursal disease virus. An antitumoral activity has also been attributed to this decalipopeptide (Sivapathasekaran et al., 2010; Yin et al., 2013; Ditmer, 2014). The inhibition of plant fungal pathogens by bacillomycin D and fengycins, was shown to be based on the induction of ROS production (Tang et al., 2014; Han et al., 2015; Gu et al., 2017). The bacillomycin D analogs have been mostly assayed for their antifungal activities (Tabbene et al., 2016), especially against phytopathogenic fungi (Moyne et al., 2001; Tanaka et al., 2014; Kim et al., 2016). Recently, bacillomycin D has showed antitumoral activities (Hajare et al., 2013; Gong et al., 2014; Qian et al., 2015; Sun et al., 2018). Surfactin possesses a number of biological activities. It is known for its antiviral,

antibacterial and antitumoral properties (Meena and Kanwar, 2015; Moro et al., 2018).

CONCLUSION

It was shown that the strain collection of rhizospheric bacteria from Tunisian arid areas has a potential for the discovery of compounds with antibacterial and antifungal activities. Bacillomycin D, fengycin A and B, and surfactin were identified based on LC-MS data and molecular networking analysis. In addition, 1-acetyl-β-carboline was isolated as pure compound, which is known to have synergistic effects with other antibiotics. Overall, the strain collection is dominated by the Gram-positive genus *Bacillus*, which seems to play an important role in shaping the microbiome by the production of bioactive compounds.

AUTHOR CONTRIBUTIONS

GK, TS, and RB designed and planned the research. ZN isolated and identified the bacterial strains. ZN and HB isolated natural products. SK guided laboratory work and analyzed the NMR data. All authors analyzed and interpreted the results and commented on the manuscript prepared by ZN and TS.

FUNDING

The main part of this research was supported by a bilateral grant (TUNGER-7) of the Tunisian Ministry of Higher Education and Scientific Research and the German Federal Ministry of Education and Research (BMBF, 01DH16010). ZN obtained a fellowship from the Tunisian ministry of higher education, scientific research and information and communication technologies.

ACKNOWLEDGMENTS

We thank Alexander Bogdanov and Antonio Dávila-Céspedes for their support in NMR and Flash experiments. ZN gratefully acknowledges her fellowship from the Tunisian ministry of higher education, scientific research and information and communication technologies.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.02742/full#supplementary-material

REFERENCES

Ahmed, H. A., Ebrahim, W., Mikhailovna, P. A., Henrich, B., and Proksch, P. (2015). Extraction and Identification of some metabolites produced by antagonistic apple plant bacteria *Brevibacterium halotolerans*. *Int. J. Adv. Res.* 3, 1208–1217.

Amin, M., Rakhisi, R., and Ahmady, A. Z. (2015). Isolation and Identification of *Bacillus* species from soil and evaluation of their antibacterial properties. *Avicenna J. Clin. Microb. Infec.* 2:e23233. doi: 10.1007/s10534-012-9606-y

Bachoual, R., Tankovic, J., and Soussy, C. J. (1998). Analysis of the mutations involved influoroquinolone resistance of in vivo and in vitro mutants of *Escherichia coli*. *Microb. Drug. Resist.* 4, 271–276. doi: 10.1089/mdr.1998.4.271

Biniarz, P., and Lukaszewicz, M. (2017). Direct quantification of lipopeptide biosurfactants in biological samples via HPLC and UPLC-MS requires sample modification with an organic solvent. *Appl. Microbiol. Biotechnol.* 101, 4747–4759. doi: 10.1007/s00253-017-8272-y

Bonmatin, J. M., Labbe, H., Grangemard, I., Peypoux, F., Maget-Dana, R., Ptak, M., et al. (1995). Production, isolation and characterization of [Leu4]- and [Ile4]surfactins from *Bacillus subtilis*. *Lett. Pept. Sci.* 2, 41–47. doi: 10.1007/BF00122922

Broberg, A., Bjerketorp, J., Andersson, P., Sahlberg, C., and Levenfors, J. J. (2017). Labradorins with antibacterial activity produced by *Pseudomonas* sp. *Molecules* 22:1072. doi: 10.3390/molecules22071072

Cao, R., Peng, W., Wang, Z., and Xu, A. (2007). β-Carboline alkaloids: biochemical and pharmacological functions. *Curr. Med. Chem.* 14, 479–500. doi: 10.2174/092986707779940998

Cao, Y., Xu, Z., Ling, N., Yuan, Y., Yang, X., Chen, L., et al. (2012). Isolation and identification of lipopeptides produced by *B. subtilis* SQR 9 for suppressing Fusarium wilt of cucumber. *Sci. Hortic.* 135, 32–39. doi: 10.1016/j.scienta.2011.12.002

Chen, Q., Ji, C., Song, Y., Huang, H., Ma, J., Tian, X., et al. (2013). Discovery of McbB, an enzyme catalyzing the β-carboline skeleton construction in the Marinacarboline biosynthetic pathway. *Angew. Chem. Int. Ed.* 52, 9980–9984. doi: 10.1002/anie.201303449

de Oliveira, A. G., Spago, F. R., Simionato, A. S., Navarro, M. O. P., da Silva, C. S., Barazetti, A. R., et al. (2016). Bioactive organocopper compound from *Pseudomonas aeruginosa* inhibits the growth of *Xanthomonas citri* subsp. *citri*. *Front. Microbiol.* 7:113. doi: 10.3389/fmicb.2016.00113

Dearing, J. A., Livingstone, I. P., and Zhou, L. P. (1996). A late quaternary magnetic record of tunisian loess and its climatic significance. *Geophys. Res. Lett.* 23, 189–192. doi: 10.1029/95GL03132

Dillman, R. L., and Cardellina, J. H. II (1991). Aromatic secondary metabolites from the sponge tedaniaignis. *J. Nat. Prod.* 54, 1056–1061. doi: 10.1021/np50076a021

Ditmer, E. M. (2014). *Evaluation of the Anti-Proliverative Effect of the Lipopeptides Iturin A and Fengycin of Bacillus spp. and the Viral Fusionprotein PTD4-VP3 on Human Cancer and Normal Cells*. Ph.D. thesis, Universidade Federal do Rio Grande do Sul, Brazil.

Edgar, R. C. (2004). "MUSCLE: multiple sequence alignment with improved accuracy and speed," in *Proceedings of the IEEE Computational Systems Bioinformatics Conference (CSB)*, (Stanford, CA: IEEE). doi: 10.1109/CSB.2004.1332560

Elbandary, A. A., Hessain, A. M., El-Hariri, M. D., Seida, A. A., Moussa, I. M., Mubarak, A. S., et al. (2018). Isolation of antimicrobial producing actinobacteria from soil samples. *Saud. J. Biol. Sci.* 25, 44–46. doi: 10.1016/j.sjbs.2017.05.003

Felsenstein, J. (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39, 783–791. doi: 10.1111/j.1558-5646.1985.tb00420.x

Frieri, M., Kumar, K., and Boutin, A. (2017). Antibiotic resistance. *J. Infect. Public Health* 10, 369–378. doi: 10.1016/j.jiph.2016.08.007

Ghanmi, F., Carré-Mlouka, A., Vandervennet, M., Boujelben, I., Frikha, D., Ayadi, H., et al. (2016). Antagonistic interactions and production of halocin antimicrobial peptides among extremely halophilic prokaryotes isolated from the solar saltern of Sfax. Tunisia. *Extremophiles* 20, 363–374. doi: 10.1007/s00792-016-0827-9

Gong, Q., Zhang, C., Lu, F., Zhao, H., Bie, X., and Lu, Z. (2014). Identification of bacillomycin D from *Bacillus subtilis* fmbJ and its inhibition effects against Aspergillus flavus. *Food Control* 36, 8–14. doi: 10.1016/j.foodcont.2013.07.034

Griffith, R. S. (1981). Introduction to vancomycin. *Rev. Infect. Dis.* 3, 200–204. doi: 10.1093/clinids/3.Supplement_2.S200

Gross, H., and Loper, J. E. (2009). Genomics of secondary metabolite production by *Pseudomonas* spp. *Nat. Prod. Rep.* 26, 1408–1446. doi: 10.1039/b817075b

Gu, Q., Yang, Y., Yuan, Q., Shi, G., Wu, L., Lou, Z., et al. (2017). Bacillomycin D produced by *Bacillus amyloliquefaciens* is involved in the antagonistic interaction with the plant -pathogenic fungus *Fusarium graminearum*. *Appl. Environ. Microbiol.* 83:e01075-17. doi: 10.1128/AEM.01075-17

Hajare, S. N., Subramanian, M., Gautam, S., and Sharma, A. (2013). Induction of apoptosis in human cancer cells by a *Bacillus lipopeptide* bacillomycin D. *Biochimie* 95, 1722–1731. doi: 10.1016/j.biochi.2013.05.015

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser.* 41, 95–98.

Han, Q., Wu, F., Wang, X., Qi, H., Shi, L., Ren, A., et al. (2015). The bacterial lipopeptide iturins induce *Verticillium dahliae* cell death by affecting fungal signalling pathways and mediate plant defence responses involved in pathogen -associated molecular pattern -triggered immunity. *Environ. Microbiol.* 17, 1166–1188. doi: 10.1111/1462-2920.12538

Heravi, M., Zadsirjan, V., and Malmir, M. (2018). Application of the asymmetric pictet–spengler reaction in the total synthesis of natural products and relevant biologically active compounds. *Molecules* 23:943. doi: 10.3390/molecules23040943

Hotam, S. C., Bhavana, S., Anju, R. S., and Saurabh, S. (2013). Diversity and versatility of actinomycetes and its role in antibiotic production. *J. Appl. Pharm. Sci.* 3, S83–S94.

Huang, H., Yao, Y., He, Z., Yang, T., Ma, J., Tian, X., et al. (2011). Antimalarial β-carboline and indolactam alkaloids from *Marinactinospora thermotolerans*, a deep sea isolate. *J. Nat. Prod.* 74, 2122–2127. doi: 10.1021/np200399t

Huang, X., Lu, Z., Zhao, H., Bie, X., Lü, F., and Yang, S. (2006). Antiviral activity of antimicrobial lipopeptide from *Bacillus subtilis* fmbj against pseudorabies virus, porcine parvovirus, newcastle disease virus and infectious bursal disease virus in vitro. *Int. J. Peptide Res. Ther.* 12, 373–377. doi: 10.1007/s10989-006-9041-4

Hug, J. J., Bader, C. D., Remškar, M., Cirnski, K., and Müller, R. (2018). Concepts and methods to access novel antibiotics from actinomycetes. *Antibiotics* 7:44. doi: 10.3390/antibiotics7020044

Jacques, P. (2011). "Surfactin and other lipopeptides from *Bacillus* spp," in *Biosurfactants. Microbiology Monographs*, Vol. 20, ed. G. Soberón-Chávez (Berlin: Springer).

Joshi, B. S., Kamat, V. N., and Gawad, D. H. (1977). Some β-carboline alkaloids of ailanthus malabarica DC. *Heterocycles* 7, 193–200. doi: 10.3987/S-1977-01-0193

Katz, L., and Baltz, R. H. (2016). Natural product discovery: past, present, and future. *J. Ind. Microbiol. Biotechnol.* 43, 155–176. doi: 10.1007/s10295-015-1723-5

Khan, S. N., and Khan, A. U. (2016). Breaking the spell: combating multidrug resistant 'superbugs. *Front. Microbiol.* 7:174. doi: 10.3389/fmicb.2016.00174

Kim, J. D., Jeon, B. J., Han, J. W., Park, M. Y., Kang, S. A., and Kim, B. S. (2016). Evaluation of the endophytic nature of *Bacillus amyloliquefaciens* strain GYL4 and its efficacy in the control of anthracnose. *Pest. Manag. Sci.* 72, 1529–1536. doi: 10.1002/ps.4181

Kim, S. Y., Lee, N. K., Han, E. J., and Paik, H. D. (2012). Characterization of subtilin KU43 Produced by *Bacillus subtilis* KU43 isolated from traditional Korean fermented foods. *Food Sci. Biotechnol.* 21, 1433–1438. doi: 10.1007/s10068-012-0188-9

Kumar, D., and Kumar, S. (2016). Antimicrobial metabolites and antibiotics obtained from different environmental sources. *Int. J. Pharm. Res. Allied Sci.* 5, 85–90.

Kumar, S., Stecher, G., and Tamura, K. (2016). MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets. *Mol. Biol. Evol.* 33, 1870–1874. doi: 10.1093/molbev/msw054

Lan, W. J., Fu, S. J., Xu, M. Y., Liang, W. L., Lam, C. K., Zhong, G. H., et al. (2016). Five new cytotoxic metabolites from the marine fungus *Neosartorya pseudofischeri*. *Mar. Drugs* 14:18. doi: 10.3390/md14010018

Le Houérou, H. N. (2001). Biogeography of the arid steppeland north of the Sahara. *J. Arid. Environ.* 48, 103–128. doi: 10.1006/jare.2000.0679

Lee, D. S., Eom, S. H., Jeong, S. Y., Shin, H. J., Je, J. Y., Lee, E. W., et al. (2013). Anti-methicillin-resistant *Staphylococcus aureus* (MRSA) substance from the marine bacterium *Pseudomonas* sp. UJ-6. *Environ. Toxicol. Pharm.* 35, 171–177. doi: 10.1016/j.etap.2012.11.011

Lee, D. S., Kang, M. S., Hwang, H. J., Eom, S. H., Yang, J. Y., Lee, M. S., et al. (2008). Synergistic effect between dieckol from *Ecklonia stolonifera* and β-lactams against methicillin-resistant *Staphylococcus aureus*. *Biotechnol. Bioprocess Eng.* 13:765. doi: 10.1007/s12257-008-0162-9

Liao, J.-H., Chen, P.-Y., Yang, Y.-L., Kan, S.-C., Hsieh, F.-C., and Liu, Y.-C. (2016). Clarification of the antagonistic effect of the lipopeptides produced by *Bacillus amyloliquefaciens* BPD1 against *Pyricularia oryzae* via in situ MALDI-TOF IMS analysis. *Molecules* 21:1670. doi: 10.3390/molecules21121670

Ling, L. L., Schneider, T., Peoples, A. J., Spoering, A. L., Engels, I., Conlon, B. P., et al. (2015). A new antibiotic kills pathogens without detectable resistance. *Nature* 517:455. doi: 10.1038/nature14098

Lu, X. L., Xu, Q. Z., Liu, X. Y., Cao, X., Ni, K. Y., and Jiao, B. H. (2008). Marine drugs–macrolactins. *Chem. Biodivers.* 5, 1669–1674. doi: 10.1002/cbdv.200890155

Meena, K. R., and Kanwar, S. S. (2015). Lipopeptides as the antifungal and antibacterial agents: applications in food safety and therapeutics. *BioMed Res. Int.* 2015:473050. doi: 10.1155/2015/473050

Morens, D. M., and Fauci, A. S. (2013). Emerging infectious diseases: threats to human health and global stability. *PLoS Pathog.* 9:e1003467. doi: 10.1371/journal.ppat.1003467

Moro, G. V., Almeida, R. T. R., Napp, A. P., Porto, C., Pilau, E. J., Lüdtke, D. S., et al. (2018). Identification and ultra-high-performance liquid chromatography coupled with high-resolution mass spectrometry characterization of biosurfactants, including a new surfactin, isolated from oil-contaminated environments. *Microb. Biotechnol.* 11, 759–769. doi: 10.1111/1751-7915.13276

Moshafi, M. H., Forootanfar, H., Ameri, A., Shakibaie, M., Dehghan-Noudeh, G., and Razavi, M. (2011). Antimicrobial activity of *Bacillus* sp. strain FAS 1 isolated from soil. *Pakistan J. Pharm. Sci.* 24, 269–275.

Moyne, A. L., Shelby, R., Cleveland, T. E., and Tuzun, S. (2001). Bacillomycin D: an iturin with antifungal activity against *Aspergillus flavus*. *J. Appl. Microbiol.* 90, 622–629. doi: 10.1046/j.1365-2672.2001.01290.x

Mukherjee, K., Mandal, S., Mukhopadhyay, B., Mandal, N. C., and Sil, A. K. (2014). Bioactive compound from *Pseudomonas synxantha* inhibits the growth of Mycobacteria. *Microbiol. Res.* 169, 794–802. doi: 10.1016/j.micres.2013.12.005

Mulwa, L. S., Jansen, R., Praditya, D. F., Mohr, K. I., Wink, J., Steinmann, E., et al. (2018). Six heterocyclic metabolites from the Myxobacterium *Labilithrix luteola*. *Molecules* 23:542. doi: 10.3390/molecules23030542

Munita, J. M., and Arias, C. A. (2016). Mechanisms of antibiotic resistance. *Microbiol. Spectrum* 4:10. doi: 10.1128/microbiolspec.VMBF-0016-2015

Nasfi, Z., Poehlein, A., Harms, H., Goralski, E., Fisch, K. M., Daniel, R., et al. (2018). Draft genome sequence of *Bacillus* sp. Strain M21, isolated from the arid area of Matmata, Tunisia. *Genome Announc.* 6, e323–e318. doi: 10.1128/genomeA.00323-18

Pidot, S. J., Coyne, S., Kloss, F., and Hertweck, C. (2014). Antibiotics from neglected bacterial sources. *Int. J. Med. Microbiol.* 304, 14–22. doi: 10.1016/j.ijmm.2013.08.011

Proksa, B., Uhrín, D., Šurdíková, M., and Fuska, J. (1990). 1-Acetyl-β-carboline, a new metabolite of *Streptomyces kasugaensis*. *Acta Biotechnol.* 10, 337–340. doi: 10.1002/abio.370100405

Qian, S., Lu, H., Meng, P., Zhang, C., Lv, F., Bie, X., et al. (2015). Effect of inulin on efficient production and regulatory biosynthesis of bacillomycin D in *Bacillus subtilis* fmbJ. *Bioresour. Technol.* 179, 260–267. doi: 10.1016/j.biortech.2014.11.086

Raaijmakers, J. M., and Mazzola, M. (2012). Diversity and natural functions of antibiotics produced by beneficial and plant pathogenic bacteria. *Annu. Rev. Phytopathol.* 50, 1–22. doi: 10.1146/annurev-phyto-081211-172908

Richards, G. W., and Vita-Finzi, C. (1982). Marine deposits 35,000–25,000 years old in the Chott el Djerid, southern Tunisia. *Nature* 295, 54–55. doi: 10.1038/295054a0

Robles-Huízar, M., De La Torre-Zavala1, S., De La Garza-Ramos, M. A., and Galan-Wong, L. J. (2017). Antimicrobial activity of secondary metabolites obtained with different carbon sources at different stages of the *Pseudomonas* growth curve isolated from Fresnillo, Zacatecas, México mineral soils. *Afr. J. Bacteriol. Res.* 9, 30–36.

Rolain, J. M., Abat, C., Jimeno, M. T., Fournier, P. E., and Raoult, D. (2016). Do we need new antibiotics? *Clin. Microbiol. Infect.* 22, 408–415. doi: 10.1016/j.cmi.2016.03.012

Roy, A., Mahata, D., Paul, D., Korpole, S., Franco, O. L., and Mandal, S. M. (2013). Purification, biochemical characterization and self-assembled structure of a fengycin-like antifungal peptide from *Bacillus thuringiensis* strain SM1. *Front. Microbiol.* 4:332. doi: 10.3389/fmicb.2013.00332

Saitou, N., and Nei, M. (1987). The neighbor-joining method: a new method for reconstruction of phylogenetic trees. *Mol. Biol. Evol.* 4, 406–425.

Senhorinho, G. N., Ross, G. M., and Scott, J. A. (2015). Cyanobacteria and eukaryotic microalgae as potential sources of antibiotics. *Phycologia* 54, 271–282. doi: 10.2216/14-092.1

Shannon, P., Markiel, A., Ozier, O., Baliga, N. S., Wang, J. T., Ramage, D., et al. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res.* 13, 2498–2504. doi: 10.1101/gr.1239303

Shin, H. J., Lee, H. S., and Lee, D. S. (2010). The synergistic antibacterial activity of 1-acetyl-beta-carboline and beta-lactams against methicillin-resistant *Staphylococcus aureus* (MRSA). *J. Microbiol. Biotechnol.* 20, 501–505.

Sivapathasekaran, C., Das, P., Mukherjee, S., Saravanakumar, J., Mandal, M., and Sen, R. (2010). Marine bacterium derived lipopeptides: characterization and cytotoxic activity against cancer cell lines. *Int. J. Peptide Res. Ther.* 16, 215–222. doi: 10.1007/s10989-010-9212-1

Staunton, J., and Wilkinson, B. (1997). Biosynthesis of erythromycin and rapamycin. *Chem. Rev.* 97, 2611–2630. doi: 10.1021/cr9600316

Sumi, C. D., Yang, B. W., Yeo, I.-C., and Hahm, Y. T. (2015). Antimicrobial peptides of the genus *Bacillus*: a new era for antibiotics. *Can. J. Microbiol.* 61, 93–103. doi: 10.1139/cjm-2014-0613

Sun, J., Li, W., Liu, Y., Lin, F., Huang, Z., Lu, F., et al. (2018). Growth inhibition of *Fusarium graminearum* and reduction of deoxynivalenol production in wheat grain by bacillomycin D. *J. Stored Prod. Res.* 75, 21–28. doi: 10.1016/j.jspr.2017.11.002

Tabbene, O., Azaiez, S., Di Grazia, A., Karkouch, I., Ben Slimene, I., Elkahoui, S., et al. (2016). Bacillomycin D and its combination with amphotericin B: promising antifungal compounds with powerful antibiofilm activity and wound-healing potency. *J. Appl. Microbiol.* 120, 289–300. doi: 10.1111/jam.13030

Tanaka, K., Ishihara, A., and Nakajima, H. (2014). Isolation of anteiso-C17, iso-C17, iso-C16, and iso-C15 bacillomycin D from *Bacillus amyloliquefaciens* SD-32 and their antifungal activities against plant pathogens. *J. Agric. Food Chem.* 62, 1469–1476. doi: 10.1021/jf404531t

Tang, J.-S., Zhao, F., Gao, H., Dai, Y., Yao, Z.-H., Hong, K., et al. (2010). Characterization and online detection of surfactin isomers based on HPLC-MSn analyses and their inhibitory effects on the overproduction of nitric oxide and the release of TNF-a and IL-6 in LPS-induced macrophages. *Mar. Drugs* 8, 2605–2618. doi: 10.3390/md8102605

Tang, Q. Y., Bie, X. M., Lu, Z. X., Lv, F. X., Tao, Y., and Qu, X. X. (2014). Effects of fengycin from *Bacillus subtilis* fmbJ on apoptosis and necrosis in *Rhizopus stolonifer*. *J. Microbiol.* 52, 675–680. doi: 10.1007/s12275-014-3605-3

The Boston Consulting Group (2017). *Breaking Through the Wall: a Call for Concerted Action on Antibiotics Research and Development*. Berlin: Follow-up report for the German Guard Initiative.

Tiwari, K., and Gupta, R. K. (2013). Diversity and isolation of rare actinomycetes: an overview. *Crit. Rev. Microbiol.* 39, 256–294. doi: 10.3109/1040841X.2012.709819

Trabelsi, I., Oves, D., Manteca, A., Genilloud, O., Altalhi, A., and Nour, M. (2016). Antimicrobial activities of some actinomycetes isolated from different rhizospheric soils in Tunisia. *Curr. Microbiol.* 73, 220–227. doi: 10.1007/s00284-016-1053-5

Umezawa, H., Ueda, M., Maeda, K., Yagishita, K., Kondo, S., Okami, Y., et al. (1957). Production and isolation of a new antibiotic: kanamycin. *J. Antibiot.* 10:181.

Uzair, B., Ahmed, N., Ahmad, V. U., Mohammad, F. V., and Edwards, D. H. (2008). The isolation, purification and biological activity of a novel antibacterial compound produced by *Pseudomonas stutzeri*. *FEMS Microbiol. Lett.* 279, 243–250. doi: 10.1111/j.1574-6968.2007.01036.x

Ventola, C. L. (2015). The antibiotic resistance crisis. *P T* 40, 277–283.

Wang, M., Carver, J. J., Phelan, V. V., Sanchez, L. M., Garg, N., Peng, Y., et al. (2016). Sharing and community curation of mass spectrometry data with GNPS. *Nat. Biotechnol.* 34:828. doi: 10.1038/nbt.3597

Wohlleben, W., Mast, Y., Stegmann, E., and Ziemert, N. (2016). Antibiotic drug discovery. *Microb. Biotechnol.* 9, 541–548. doi: 10.1111/1751-7915.12388

Wright, G. D. (2012). Antibiotics: a new hope. *Chem. Biol.* 19, 3–10. doi: 10.1016/j.chembiol.2011.10.019

Yang, H., Li, X., Li, X., Yu, H., and Shen, Z. (2015). Identification of lipopeptide isoforms by MALDI -TOF -MS/MS based on the simultaneous purification of iturin, fengycin, and surfactin by RP -HPLC. *Anal. Bioanal. Chem.* 407, 2529–2542. doi: 10.1007/s00216-015-8486-8

Yin, H., Guo, C., Wang, Y., Liu, D., Lv, Y., Lv, F., et al. (2013). Fengycin inhibits the growth of the human lung cancer cell line 95D through reactive oxygen species production and mitochondria-dependent apoptosis. *Anticancer Drugs* 24, 587–598. doi: 10.1097/CAD.0b013e3283611395

Zhao, W. H., Hu, Z. Q., Okubo, S., Hara, Y., and Shimamura, T. (2001). Mechanism of synergy between epigallocatechin gallate and β-lactams against methicillin-resistant *Staphylococcus aureus*. *Antimicrob. Agents Chemother.* 45, 1737–1742. doi: 10.1128/AAC.45.6.1737-1742.2001

Zhou, T. S., Ye, W. C., Wang, Z. T., Che, C. T., Zhou, R. H., Xu, G. J., et al. (1998). β-Carboline alkaloids from Hypodematiumsquamuloso-pilosum. *Phytochemistry* 49, 1807–1809. doi: 10.1016/S0031-9422(98)00232-5

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 18 December 2018
doi: 10.3389/fmicb.2018.03114

Aphicidal Activity of Surfactants Produced by *Bacillus atrophaeus* L193

*Miguel Rodríguez[1,2], Ana Marín[1,2], Marta Torres[1,2,3], Victoria Béjar[1,2], Mercedes Campos[4] and Inmaculada Sampedro[1,2]**

[1] Department of Microbiology, Pharmacy Faculty, Microbial Exopolysaccharide Research Group, Granada, Spain, [2] Biomedical Research Center (CIBM), Biotechnology Institute, Granada, Spain, [3] Institute for Integrative Biology of the Cell (I2BC), CEA/CNRS/University of Paris-Sud, University Paris-Saclay, Gif-sur-Yvette, France, [4] Department of Plant Protection, Estación Experimental del Zaidín, Spanish National Research Council, Granada, Spain

The biosurfactants produced by *Bacillus atrophaeus* L193 was examined by their use in the control of the aphid *Rhopalosiphum padi* in order to suggest a friendly alternative to chemical pesticides. A screening of different culture media demonstrated the highest biosurfactant production by L193 in TSB supplemented with colloidal chitin. Surfactants, which are produced in large quantities (2.04 g/L), reduce surface tension to 33 mN/m. Electrospray Q-TOFS MS analysis demonstrated that lipopeptides, such as surfactins, fengycins, bacillomycins and iturins, are the predominant metabolites present in biosurfactants produced by strain L193. Treatment with L193 surfactants led to an aphid mortality rate of 59.8% within 24 h. Microscopy analysis showed that these compounds caused insect death by affecting cuticle membranes. An evaluation of aphid feeding activity also demonstrated that aphid feeding capacity is affected by treatment with surfactants. Moreover, microbial cultures of strain L193 and their supernatants also showed high levels of activity against *R. padi,* which is probably due to the presence of surfactants and hydrolytic enzymes such as proteases and glucanases. This study demonstrates that *B. atrophaeus* L193 is an effective treatment for plants affected by aphids.

Keywords: *Bacillus atrophaeus* L193, biosurfactants, lipopeptides, aphids, insecticidal activity

OPEN ACCESS

Edited by:
José E. Barboza-Corona, Universidad de Guanajuato, Mexico

Reviewed by:
Fauzia Yususf Hafeez, COMSATS Institute of Information Technology, Pakistan
Varinia Lopez, Instituto Tecnológico Superior de Irapuato, Mexico

****Correspondence:***
Inmaculada Sampedro
isampedro@ugr.es

Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: *31 July 2018*
Accepted: *03 December 2018*
Published: *18 December 2018*

Citation:
Rodríguez M, Marín A, Torres M, Béjar V, Campos M and Sampedro I (2018) Aphicidal Activity of Surfactants Produced by Bacillus atrophaeus L193. Front. Microbiol. 9:3114. doi: 10.3389/fmicb.2018.03114

INTRODUCTION

Aphid species *Rhopalosiphum padi* affects several cereal crops, resulting in severe economic losses in agriculture. These hemiptera insects are considered to be some of the most abundant and economically important aphids affecting both winter and spring wheat crops. *R. padi* leads to plant decline and transmits different phytopathogenic viruses including the barley yellow dwarf virus (BYDV) which causes chlorosis, stunting and yield loss (Biurrun et al., 2010; Liu et al., 2014). It also promotes the growth of fungi, such as *Cladosporium* sp. and *Alternaria* sp. on leaf surfaces through the excretion of sugars, and decreases the rate of plant photosynthesis (Dhami et al., 2013).

Currently, this pest is managed through the application of chemical insecticides. However, this can lead to resistance and pollute subterranean water due to soil lixiviation. In recent years, many

predatory insects, though highly inefficient in open fields, have been used to control this pest in greenhouse crops. The use of microorganisms as biocontrol agents, which are typically members of the genus *Bacillus,* is a safe and environmentally friendly alternative to chemical pesticides (Copping, 2004).

Various studies have analysed the efficiency of *Bacillus thuringiensis* in the control of chewing insects such as *Lepidoptera*, *Diptera,* and *Coleoptera* (Bravo et al., 2007; Sanahuja et al., 2011). Nevertheless, few published studies have examined the insecticidal activity of the genus *Bacillus* against sucking insects such as aphids. Thus, studying the potential of these microorganisms as biocontrol agents against aphids could be worthwhile and contribute to the development of environmentally friendly approaches in agriculture.

The objective of this study is to analyse the biopesticidal activity of *Bacillus atrophaeus* L193 against the aphid *R. padi*. Thus, the production and physicochemical characteristics of L193 biosurfactants, as well as their anti-aphid insecticidal activity under controlled conditions were studied. The effect of *B. atrophaeus* L193 surfactant treatment on the aphid membrane was evaluated using microscopy analysis and by studying aphid feeding. These tests were also carried out on *B. atrophaeus* cultures and cell-free supernatants. To the best of our knowledge, this is the first study to evaluate the anti-aphid insecticidal activity of *B. atrophaeus*.

MATERIALS AND METHODS

Microorganism Isolation and Identification

The strain used in this study was isolated from the Malahá salt works (Granada, Spain, 37°06′11.9"N 3°43′14.6"W) after 200 bacteria isolated from several hypersaline environments were screened in order to find the best producers of biosurfactants with insecticidal potential. The methodology used in this screening was the drop collapsing test described by Youssef et al. (2004). The 16S rRNA gene of strain L193 was amplified by PCR and sequenced using universal bacterial primers (Lane, 1991) 16F_B27 (5′AGAGTTTGATCMTGGCTCAG-3′) and 16R_B1488 (5′-CGGTTACCTTGTTAGGACTTCACC-3′). 16S rRNA fragments obtained by PCR were cloned into the pGEM-T vector (Promega®) and later transformed into *Escherichia coli* DH5α. Vector plasmids were purified with the aid of an Illustra GFX DNA kit (GE Healthcare®) according to the manufacturer's instructions. Sequencing was carried out in an Illumina NextSeq™ 500 system. The resulting sequences were analysed with the DNAstar Lasergene Seqman programme (Madison, WI, United States). The sequences were identified using the GenBank and EMBL databases (Altschul et al., 1990) and the EzTaxon-e programme (Yoon et al., 2017).

Biosurfactant Production

Biosurfactant production of L193 strain was tested in the following media: Luria Bertani (LB), LB supplemented with 1% (w/v) colloidal chitin, tryptic soy broth (TSB), TSB supplemented with 1% (w/v) colloidal chitin, Cooper supplemented with 1% (w/v) colloidal chitin, Cooper supplemented with 4% (w/v) glucose and Cooper supplemented with 1% (w/v) colloidal chitin and 4% (w/v) glucose (Cooper et al., 1981). Colloidal chitin was obtained as described by Wu et al. (2009).

To determine biosurfactant production (Song et al., 2013), strain L193 was cultured in 100 mL of each medium as described above at 28°C for 7 days and 130 rpm rotary shaking. The cultures were centrifuged at 13,000 rpm for 20 min, and the cell pellets were freeze-dried. The supernatants were subjected to acid extraction in order to obtain the lipopeptides within it. The supernatant lipopeptides were then freeze-dried, and the difference in yield between cell biomass and biosurfactant was calculated.

Biosurfactant Detection in L193 Culture on TSB Chitin Medium

In order to detect biosurfactant production on strain L193, the evolution of surface tension in the strain culture was evaluated during 24 h. Additionally, the surfactant activity of the strain L193 culture was studied using the drop-collapsing test, the oil spreading test and the emulsification index. All tests were carried out in triplicate using 1% (v/v) Triton X-100 and sterile water as positive and negative controls, respectively.

Surface tension was measured at 25°C during 24 h using the Wilhelmy plate method (Biswas et al., 2001) with the aid of a Kruss K11 tensiometer. Samples were taken every hour during 24 h from a culture of strain L193 in TSB supplemented with 1% (w/v) colloidal chitin. They were then centrifuged to obtain cell-free supernatants. Three replicates were measured per each sample.

The drop-collapsing test (Youssef et al., 2004) was performed in 96-well micro-titter plates by adding 2 μL mineral oil to each well. After 1 h at room temperature to allow the stabilisation of the oil drops, 5 μL of L193 supernatant were deposited on the mineral oil drop, and the shape of the drop was analysed by visual inspection after 1 min. The reduction in surface tension caused by the presence of biosurfactant in the supernatant produced a flattened oil drop, while a round oil drop was produced in the absence of the biosurfactant.

For the oil-spreading test (Morikawa et al., 2000), 15 μL crude oil were deposited on the surface of a 150-mm diameter Petri dish containing 40 mL destilled water. 10 μL of the biosurfactant were then carefully deposited in the centre of the dish, and the radius of the clearly formed circle was measured in order to calculate the area of oil displacement.

The emulsification index (Cooper and Goldenberg, 1987) of the culture supernatants and the purified biosurfactant were determined in haemolysis tubes by mixing 4 mL of diferent hydrocarbons (such as almond oil, isopropyl mirystate and mineral oil) with an equal volume of cell-free supernatant or biosurfactant. After vortexing at maximum speed for 5 min, the mixtures were left to stabilise at room temperature for 24 h. The emulsification index (E_{24}) (%) was expressed as the height of the emulsified fraction in relation to the total height of the mixture.

Determination of Hydrolytic Enzyme Production

In order to quantify hydrolytic activity, strain L193 was cultured in 100 mL TSB medium supplemented with 1% (w/v) colloidal chitin at 28°C for 5 days in a shaking incubator at 130 rpm. To obtain cell-free supernatants, the cultures were centrifuged at 13,000 rpm for 20 min. Protease, chitinase and amylase activities of the supernatants were tested in 6-mm-diameter wells done in TSB media in Petri dishes containing 50% (v/v) skimmed milk, 1% (w/v) colloidal chitin and 1% (w/v) anhydrous starch, respectively (Barrow and Feltham, 1993). Each activity was tested using 100 μL of the pure supernatant (100% concentration) and the supernatant diluted in water at concentrations of 50% and 25%. For the protease and chitinase determination, plates were incubated at 28°C for 10 days. The radius of the transparent halos around the wells was measured after 5 and 10 days of incubation. Amylase activity was measured after 5 days using a Lugol's solution (Barrow and Feltham, 1993). A transparent halo arround the wells indicated a positive result for amylase activity, while if the medium remained dark blue/black meant that starch was not hydrolysed, therefore reacting with iodine of the Lugol's solution and dyeing the medium.

Genetic Identification of Biosurfactant

Non-ribosomal peptide synthetase (NRPS) genes coding for biosurfactant production were detected by PCR using specific degenerated primers described in **Table 1**. PCR amplifications were achieved in 50 μL mixtures containing PCR buffer, 2 mM $MgCl_2$, 4 mM of each primer, 5U Taq polymerase, 0.2 mM of each dNTP and 80–100 ng of genomic DNA. Amplification conditions were as follows: 95°C for 5 min, 40 cycles at 94°C for 1 min, annealing temperature for 1 min, extension at 72°C for 1 min and a final extension at 72°C for 10 min. Annealing temperatures were 45°C, 43°C, 50°C, and 53°C for Af2/Tf1, As1//Ts2, BmyBF/BmyBR and ItuDF/ItuDR, respectively (Chung et al., 2008; Tapi et al., 2010; Mora et al., 2011). The amplification products were analysed by electrophoresis in a 2% (w/v) agarose gel after staining with RedSafe™ (Intron).

Physicochemical Characterisation of Biosurfactant

The L193 purified biosurfactant was characterised as explained above using the oil-spreading test (Morikawa et al., 2000), the emulsification index (Cooper and Goldenberg, 1987) and by measuring surface tension reduction and critical micelle concentration (CMC) (Phale et al., 1995). Finally, the chemical composition of the surfactant was determined by ultra performance liquid chromatography time-of-flight mass spectrometry (UPLC-TOF MS).

To determine the CMC, the reduction in surface tension in a 0.2% (w/v) surfactant solution and its serial dilutions were measured at 25°C using a Kruss K11 tensiometer. The CMC of the L193 biosurfactant corresponded to the highest dilution of the surfactant that produced the maximum reduction in surface tension.

The chemical composition of the biosurfactant was analysed by UPLC-TOF MS using an Acquity UPLC® BEH300 C4 column (1.7 μm, 2.1 × 50 mm). The mobile phase used was a mixture of acetonitrile and water buffered with a solution containing 0.1% (v/v) formic acid in water. Analysis conditions were as follows: 10 μL of sample at 40°C and an analysis time of 12 min.

Mass spectrometry analysis was carried out by positive electrospray ionisation (ESI+) using a MS Waters Synapt-G2 device. The analysis was done under the following conditions: capillary 3 Kv, source temperature 100°C, desolvation temperature 500°C, cone gas flow 40 L/h, desolvation gas flow 800 L/h, mass interval of detection from 100 to 2,400 Da and survey scan time 0.1 s. The data obtained were processed with the aid of MassLynx™ software (Waters).

Insecticidal Activity Bioassay

In order to determine the insecticidal potential of strain L193, the bacterial culture, supernatant and purified biosurfactant were tested *in vivo* against *R. padi* aphids (Agrobio S.L.).

To obtain the different treatments, strain L193 was cultured in TSB medium supplemented with 1% (w/v) colloidal chitin at 28°C in a shaking incubator at 130 rpm for 5 days. The supernatant was obtained by centrifugation at 13,000 rpm for 20 min and was then filtred through a 0.22-μm pore filtre. The biosurfactant was extracted from the 250 mL culture supernatant as described above and was dissolved in 250 mL of sterile water.

Imidacloprid (50 μg/mL) was used as positive control. As negative controls, sterile cell-free TSB medium with 1% (w/v) colloidal chitin was used for comparison with the whole culture and the supernatant treatments, while sterile water was used for comparison with the biosurfactant treatment.

The experimental model consisted of sixty individually grown barley (*Hordeum vulgare*) seedlings per treatment and control. Plants were grown in 0.2 pots filled with artificial soil at 23°C. Three-day barley seedlings (plant height: 6–7 cm) were initially infested with three 3rd-instar *R. padi* aphids and grown for 7 days in a growth chamber at 25°C, 60% humidity and for a 16 h light/8 h dark photoperiod. To avoid aphid dispersion, each plant was covered with transparent plastic film.

The insecticidal experiment lasted 5 days after the 7 days of plant growth. The treatments and controls were sprayed (0.5 mL per plant) on day 1 and 3 of the assay while ensuring that the whole aphid population and plant surface were covered with the spray solution. The dead and living aphids of each plant were counted daily.

Aphid mortality was calculated using Abbot's formula (Abbott, 1925), which compares living aphids in each treatment (At) with living aphids in each control (Ac):

$$Mortality\,(\%) \;=\; (1 - \frac{At}{Ac}) \times 100.$$

Determination of Aphid Feeding

To test whether the treatments affected aphid feeding capacity, ten barley seedlings infested each with three 3rd-instar aphids were used for each treatment and control. The experiment

TABLE 1 | PCR primers of lipopeptide biosynthesis genes in *Bacillus atrophaeus* L193.

Lipopeptide	Gene	Primers	Primer sequences (5′→3′)	PCR product size (bp)	Reference
Fengycin	*fen*C	Af2 (F)	GAATAYMTCGGMCGTMTKGA	443–455	Tapi et al., 2010
		Tf1 (R)	GCTTTWADKGAATSBCCGCC		
Surfactin	*srf*A-A	As1 (F)	CGCGGMTACCGVATYGAGC	419–431	Tapi et al., 2010
		Ts2 (R)	ATBCCTTTBTWDGAATGTCCGCC		
Bacillomycin	*bmy*B	BmyB (F)	GAATCCCGTTGTTCTCCAAA	370	Mora et al., 2011
		BmyB (R)	GCGGGTATTGAATGCTTGTT		
Iturin	*itu*D	ItuD (F)	TTGAAYGTCAGYGCSCCTTT	482	Chung et al., 2008
		ItuD (R)	TGCGMAAATAATGGSGTCGT		

conditions were the same as described above, but in this case 90-mm diameter filtre paper discs were placed at the base of each plant stem to collect honeydew drops from the aphids (Kim and Jander, 2007).

In order to avoid contamination of the filtre paper disc by the soil, a Petri dish (with an opening for the stem) was placed between the filtre and the soil. Likewise, when the treatments and controls were sprayed on the seedlings, the filtre and dish were removed to avoid their contamination. Discs containing honeydew from the aphids were collected after 96 h and were immersed in a 0.1% (w/v) ninhydrin solution in acetone. Discs were oven-dried at 65°C for 30 min and cut into strips. The purple/pink spots were extracted with 4 mL of 90% (v/v) methanol by vortexing for 1 h at maximum speed. The tubes were then centrifuged at 5,000 rpm for 10 min, and supernatant absorbance was measured at 500 nm using 90% (v/v) methanol as blank (Nisbet et al., 1994).

Microscopy Analysis

After the *in vivo* assay described above, dead aphids from plants treated with the bacterial culture or the biosurfactant, as well as water-control aphids, were collected in order to analyse the effect of treatments using optical, scanning electron and transmission electron microscopy. The samples were prepared as described by Kim et al. (2011). Briefly, for scanning electron microscopy analysis, aphid samples were fixed with 2% (v/v) glutaraldehyde and 2% (v/v) paraformaldehyde in 50 mM cacodylate buffer at pH 7.4 for 48 h at room temperature. After fixing, the samples were washed three times in the cacodylate buffer and dehydrated through 50, 70, 90, and 100% ethanol for 5 min in each stage. The samples were dried and examined with a scanning electron microscope. For transmission electron microscopy analyses, aphid samples were immersed in 2.5% (v/v) glutaraldehyde in 0.1 M PBS buffer at pH 7.4 for 48 h at 4°C. Following washing with the PBS buffer, the samples were immersed in a mixture of 2% osmium tetroxide and 3%(v/v) ferrocyanide (1:1, v/v) in the same buffer for 1 h at 4°C. After a washing with the PBS buffer, the samples were dehydrated in a series of ethanol solutions and embedded in Epon 812. Ultrathin sections were mounted on Formvar-coated nickel grids (200 mesh) and stained with aqueous uranyl acetate and alkaline lead citrate for 5 min. The samples were then examined with a transmission electron microscope.

Statistical Analyses

Data normality was checked using the Shapiro-Wilk test. Since the data did not follow a normal distribution pattern, even after natural logarithmic and Box-Cox transformation, Kruskal-Wallis and Mood's median tests were used to determine whether the five treatments differed significantly ($p \leq 0.05$) in terms of aphid mortality. The Mann-Whitney U test with the Bonferroni correction was then used for pairwise comparisons of the treatments.

RESULTS

Microorganism Isolation and Identification

The strain used in this study was isolated from a soil sample taken from the Malahá salt works (Granada) in the south-east of Spain. The 16S rRNA gene sequence (1,355 bp) indicated that the strain belongs to the genus *Bacillus*. It showed 16S rRNA gene-sequence similarity of 99.5% to its closest relative *B. atrophaeus*. The isolate was named *B. atrophaeus* L193.

Biosurfactant Production

Different culture media were screened in order to determine in which of these media the biosurfactant production was higher (**Table 2**). TSB supplemented with colloidal chitin, in which strain L193 produced 2.04 g/L of biosurfactants, was selected and used as the culture medium to perform the subsequent experiments.

Biosurfactant Detection in L193 Culture on TSB Chitin Medium

The cell-free supernatant of strain L193 grown in TSB medium supplemented with 1% (w/v) colloidal chitin produced a clear

TABLE 2 | Surfactant production by *B. atrophaeus* L193 in different culture media.

	LB	LB chitin	TSB	TSB chitin	Cooper chitin	Cooper glucose	Cooper glucose chitin
Surfactant (mg/L)	502.4	281.2	1620.8	2040.0	203.6	271.6	284.0
Biomass (mg/L)	1640	1424	1908	1840	1516	416	1450
Yield	0.31	0.20	0.85	1.11	0.13	0.65	0.20

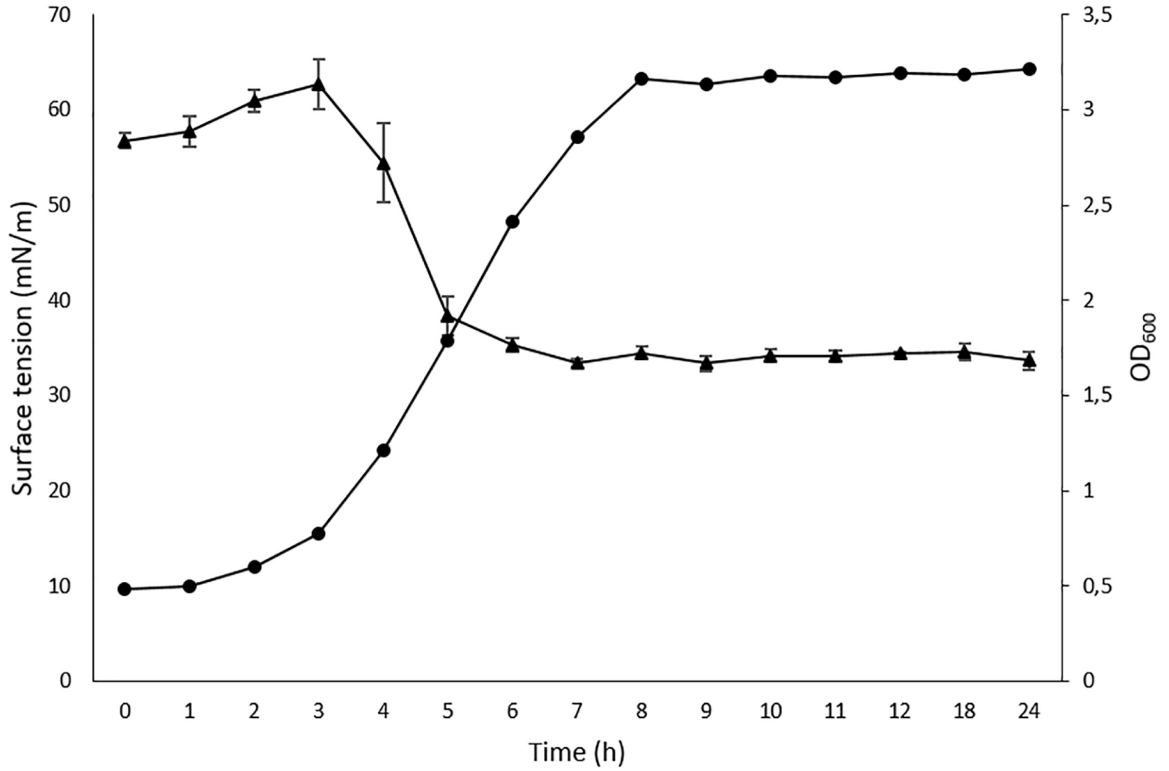


FIGURE 1 | Time-course cell density (●) and surface tension (▲) values for *Bacillus atrophaeus* L193. Data represent mean ± standard deviation of the triplicates.

halo zone of 24.95 cm^2 in the oil displacement test, while the drop-collapsing test was also positive (data not shown). The emulsification index (E_{24}) for cell-free supernatant activity against mineral oil, almond oil and isopropyl myristate was 8.08% (±1.24), 42.63% (±2.63), and 3.73% (±1.31), respectively. The reduction in surface tension reached a maximum (33.0 mN/m) after 4.5 h of incubation (**Figure 1**).

Determination of Hydrolytic Enzyme Production

In this study, we analysed the production of glucosidase enzymes (amylase and chitinase) and proteases (caseinase) by strain L193 in order to determine its potential insecticidal capacity. The undiluted L193 culture supernatants on 1% (w/v) TSB chitin-containing media displayed enhanced amylase, chitinase and caseinase production (**Table 3**).

TABLE 3 | Amylase, chitinase and caseinase activities of *B. atrophaeus* L193 supernatants on TSB medium supplemented with 1% (w/v) colloidal chitin.

	Amylase*	Chitinase	Caseinase
Supernatant (100%)	15	3	10
Supernatant (50%)	14	2	7
Supernatant (25%)	11	1	4

**Enzimatic activity was determined by measuring the hydrolysis halo (mm) produced in the different culture media.*

Genetic Characterisation of Biosurfactant

Genomic analysis indicates that *B. atrophaeus* L193 contains non-ribosomal lipopeptide synthetase gene clusters, which include genes involved in producing fengycin, surfactin, bacillomycin and iturin, such as *fenC*, *srf* A-A, *bmy*B and *itu*D (**Supplementary Figure S1**).

Physicochemical Characterisation of Biosurfactant

In order to analyse the efficiency of biosurfactants extracted from strain L193, critical micelle concentration (CMC) as well as emulsification and oil spreading (displacement) activity were measured. The value obtained for CMC was 9.38 mg/L. The oil spreading test showed a displacement halo area of 33.87 cm^2 for L193 relative to 55.42 cm^2 for the positive control Triton X-100 (**Supplementary Figure S2**). The emulsification index (E_{24}) for L193 biosurfactant activity against mineral oil, almond oil and isopropyl myristate was 51.53% (±1.39), 46.87% (±1.46), and 38.05% (±11.21), respectively (**Supplementary Table S1**). These emulsification indices for the surfactant produced by L193 were slightly lower than those for the positive control Triton X-100. Except with regard to almond oil, all the values for the biosurfactant were higher than those for the supernatant of L193 cultures. The emulsification index of water (negative control) for these compounds was 0% (**Supplementary Figure S3**).

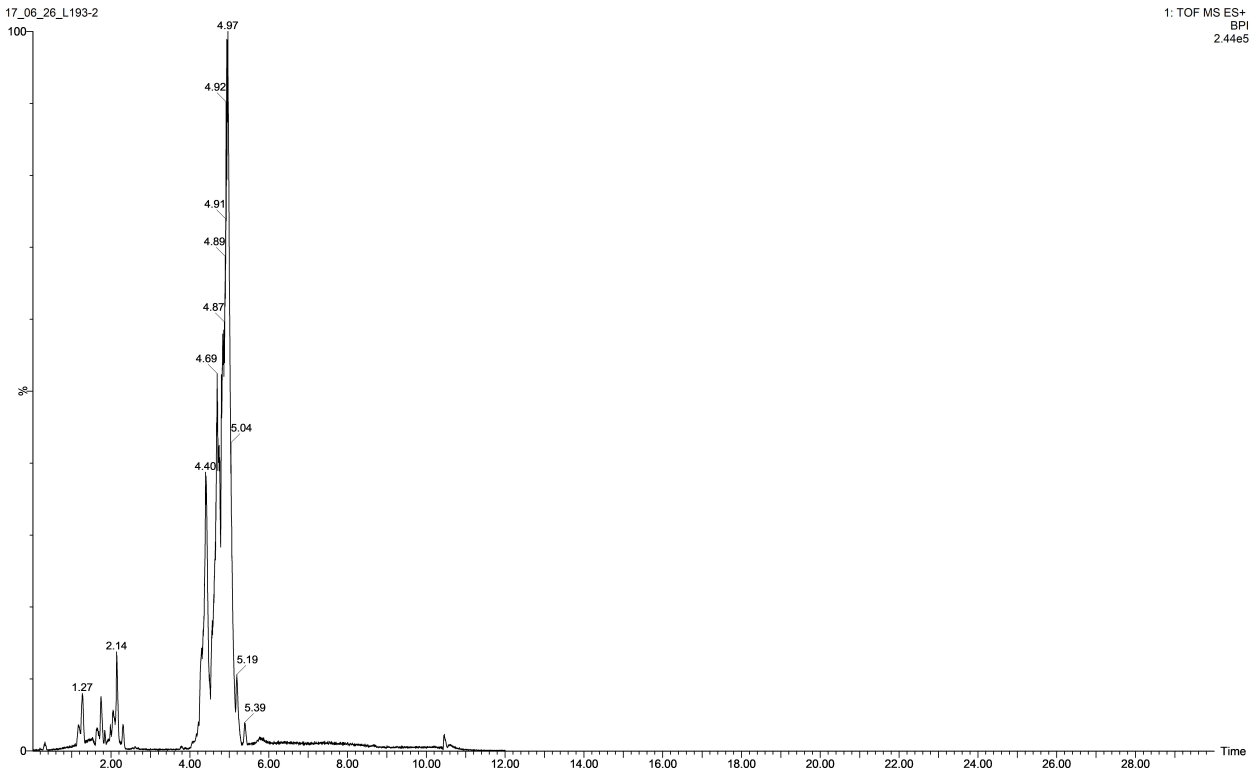


FIGURE 2 | TIC spectrum obtained from the surfactant of *B. atrophaeus* L193 cultured in TSB medium supplemented with 1% (w/v) colloidal chitin.

Ultra-performance liquid chromatography time-of-flight mass spectrometry (UPLC-TOFS MS) was used to identify metabolites produced by the isolate tested. **Figure 2** illustrates the total ion chromatogram (TIC) spectrum of the *B. atrophaeus* L193 culture extract.

The analyses show that strain L193 can produce various types of lipopeptides. Four known surfactins, with an acyl chain ranging from C12 to C15, as well as two known types of bacillomycin F (C15 and C16) were detected. Iturin A and fengycin B peaks were also observed (**Table 4**). UPLC-TOF MS detected two predominant compounds: an [M+H] peak at m/z 1,057.5736 for iturin A with molecular formula $C_{49}H_{76}N_{12}O_{14}$ and an [M+H] peak at m/z 1,036.6947 for surfactin with molecular formula $C_{53}H_{93}N_7O_{13}$ (**Figures 3A,D**, respectively). Also an [M+H] peak at m/z 1,085.6064, for bacillomycin F with a molecular formula $C_{51}H_{80}N_{12}O_{14}$ and an [M+H] peak at m/z 1,477.8317 for fengycin B, with a molecular formula $C_{73}H_{112}N_{12}O_{20}$ (**Figures 3B,C**, respectively) were detected. No mass signals were assigned to other lipopeptides. This agrees with the results obtained by the PCR analysis (**Supplementary Figure S1**).

TABLE 4 | Lipopeptide production by *B. atrophaeus* L193 as detected by Q-TOF MS.

Lipopeptide	Fatty chain length	[M+H]	Retention time (min)	Area (%)	Peak intensity
Iturin A	C15	1057.5736	1.27	2.00	1.95e4
Bacillomycin F	C15	1071.5880	1.53	0.37	4.77e3
	C16	1085.6064	1.74	1.18	1.86e4
Fengycin B	C15	1477.8317	2.15	2.54	5.99e3
Surfactin	C12	994.6420	4.21	0.05	9.92e3
	C13	1008.6595	4.40	15.47	9.55e4
	C14	1022.6754	4.69	2.31	1.29e5
	C15	1036.6947	4.97	72.50	2.46e5

Insecticidal Activity Bioassay

In order to evaluate the aphicidal activity of *B. atrophaeus* L193, *in vivo* studies were conducted in barley plants infested with three 3rd-instar *R. padi* aphids, and treated or untreated with strain L193 culture, cell-free supernatant or purified extract. The results show high levels of activity against *R. padi* for all of the treatments. The differences between the treatments and control were, in all cases, highly significant ($p \leq 0.05$). The highest mortality rate of aphids was obtained 24 h after the first treatment (**Figure 4**). Imidacloprid, a neonicotinoid commonly employed for aphid control, with 100% mortality after 24 h, was used as positive control (data not shown in **Figure 4**).

Given the corrected mortality based on Abbot's formula, an enhanced insecticidal activity was observed in the biosurfactant (59.8%) as compared to the culture (50.6%) and the cell-free supernatant (47.7%) of *B. atrophaeus* L193 (**Supplementary Table S2**).

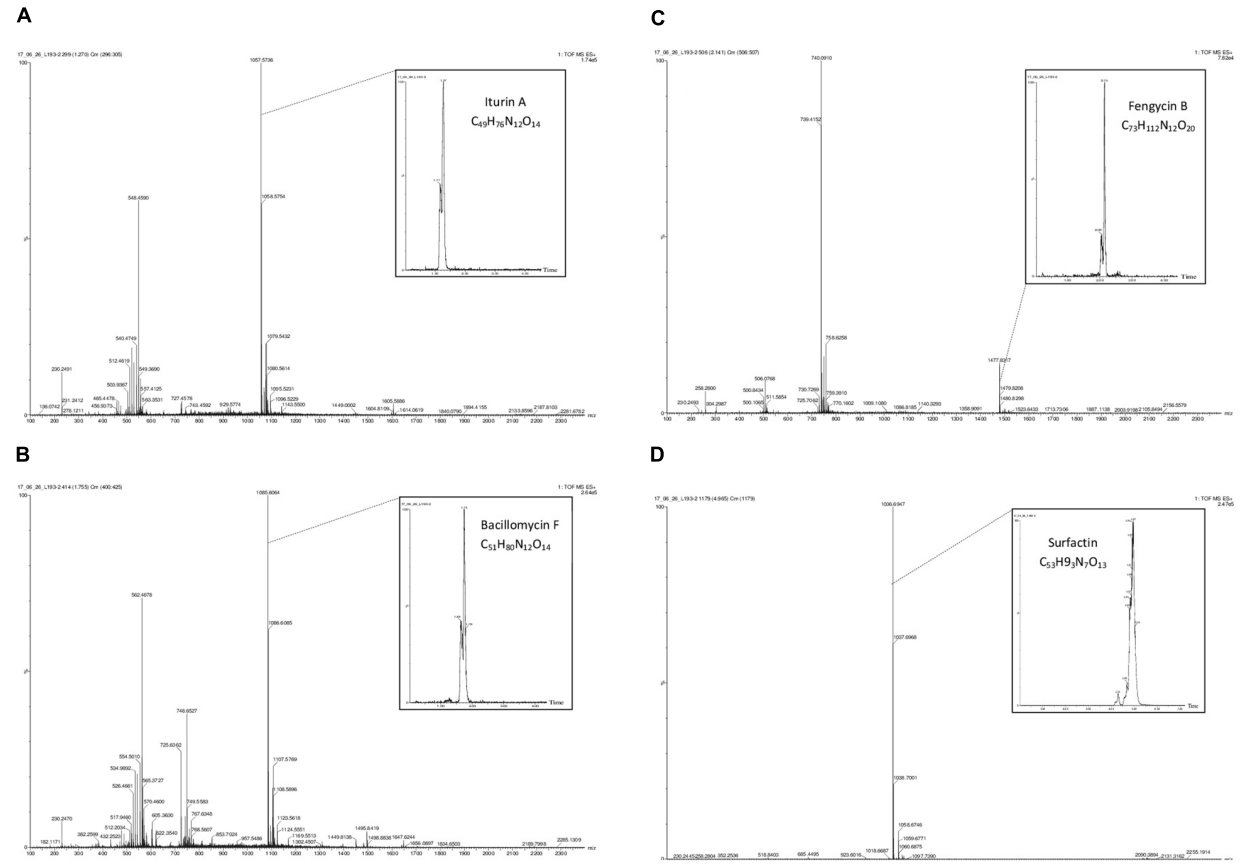


FIGURE 3 | Q-TOF MS spectra obtained from the surfactant produced by *B. atrophaeus* L193: protonated linear derivatives of the [M+H] of iturin A **(A)**, bacillomycin F **(B)**, fengycin B **(C)**, and surfactin **(D)**.

Determination of Aphid Feeding

Aphid feeding capacity was evaluated by measuring the amount of honeydew excreted by the whole aphid population after 96 h. The samples corresponding to the L193 culture and cell-free supernatant treatments showed a reduction of 38.5 and 42.9% in the honeydew excreted, respectively, as compared to controls. L193 surfactant treatment samples showed a reduction of 28.7% as compared to controls.

Microscopy Analysis

The signs of *R. padi* infection caused by topic treatment with *B. atrophaeus* L193 were observed by optical microscopy and are shown in **Figure 5**. Aphids exposed to L193 ceased to move, their bodies were dehydrated and their green colour darkened.

Scanning electron microscopy (SEM) and Transmission electron microscopy (TEM) data on aphids treated with TSB medium and the L193 culture or the biosurfactant produced by L193 are shown in **Figure 6**. SEM analysis of aphids treated with TSB medium alone showed an intact cuticle (**Figure 6A**), while clear evidence of damaged cuticle was detected in aphids treated with L193 culture and the biosurfactant (**Figures 6B,C**, respectively). TEM and optical microscopy confirmed that treatment with strain L193 affects the cuticular membrane of the aphids, with a 36.23% reduction in thickness in the case of the bacterial culture treatment (**Figure 6E**) and a 58.35% reduction with the L193 surfactant treatment (**Figure 6F**). With regard to treatment with the biosurfactant, a clear change in cuticle structure was also observed.

With regard to treatment with the L193 culture, TEM micrographs also highlighted the presence of a bacterial cell monolayer firmly attached to the cuticle surface of aphids (**Figure 6E**), with bacterial cells sometimes appearing to cause the development of a pseudopod-like structure in the aphid cuticle (**Supplementary Figure S4A**). SEM micrographs showed cuticle concavity in the aphids (**Supplementary Figure S4B**).

DISCUSSION

The strain used in this study showed 16S rRNA gene-sequence similarity of 99.5% to its closest relative *B. atrophaeus*. The isolate was named *B. atrophaeus* L193.

Several studies have previously highlighted the potential of different *Bacillus* strains to act as biocontrol agents against insect pests. The effect of these bacteria has been tested in relation to a wide range of agronomically important pests including aphids (Dutton et al., 2003; Palma et al., 2014; Rashid et al., 2017). As

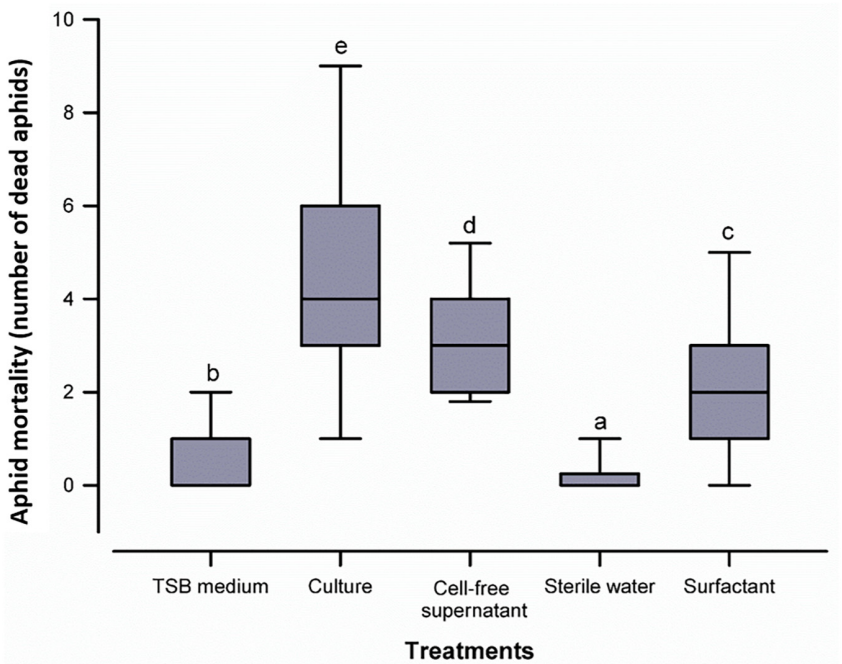


FIGURE 4 | Box plot representing aphid mortality with the five treatments tested. The centre line of the box depicts the median, the edges of the box reflect the 25th and 75th percentiles (interquartile range), and the whiskers depict the 10th and 90th percentiles. Different letters above the whiskers indicate significant differences ($p \leq 0.05$) between treatments according to the Mann-Whitney U test.

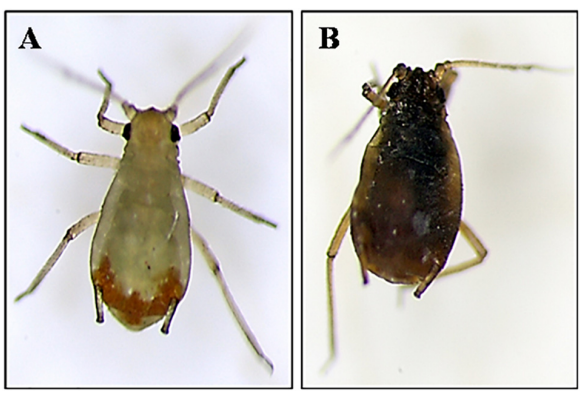


FIGURE 5 | Signs of infection in *Rhopalosiphum padi* caused by *B. atrophaeus* L193 observed by stereo microscopy. **(A)** Treatment with water (control). **(B)** Treatment with L193 surfactant.

explained elsewhere, these insects are considered to be the most destructive agents affecting several crops and the agricultural economy (Leroy et al., 2011).

Most studies have analysed the biopesticidal activity of the genus *Bacillus* against the green peach aphid *Myzus persicae*, one of the most destructive pests, which causes huge crop loss (Torres-Quintero et al., 2015). The most common *Bacillus* species tested against *M. persicae* is the entomopathogenic bacterium *Bacillus thuringiensis* (Torres-Quintero et al., 2015). This bacterium produces crystalline inclusions composed of insecticidal crystal proteins (ICP) and endotoxins. Although these ICPs and toxins are highly active against several orders of insects, various studies have shown low to moderate toxicity to aphids (Porcar et al., 2009). It would therefore be interesting to study the potential of other *Bacillus* species to act as biocontrol agents against these insects. To the best of our knowledge, this is the first study to analyse the anti-aphid insecticidal activity of *B. atrophaeus,* which we tested against *Rhopalosiphum padi* (L.) (*Homoptera*: *Aphididae*), one of the most abundant and economically important aphids in both winter and spring wheat crops.

Biosurfactant production by L193 was tested in different culture media. The data showed that the culture medium used in the growth of the microorganism can decisively influence biosurfactant production. TSB supplemented with colloidal chitin was selected for the experiments.

The use of biosurfactants from microorganisms is a potentially effective pest management strategy. In order to detect biosurfactant production by L193, the oil displacement test, drop-collapsing test and the emulsification index of the cell-free supernatant of strain L193 grown in TSB medium supplemented with colloidal chitin were used. The pattern observed was also found with respect to other biosurfactants produced by microorganisms (Lee et al., 2008).

The efficiency of biosurfactants extracted from strain L193 was evaluated by measuring the critical micelle concentration (CMC) as well as emulsification and oil spreading (displacement) activity. CMC values for the different biosurfactants tested range from 9 mg/L (Sivapathasekaran et al., 2009) to 140 mg/L (Mukherjee and Das, 2005). The low CMC value (9.38 mg/L) for L193

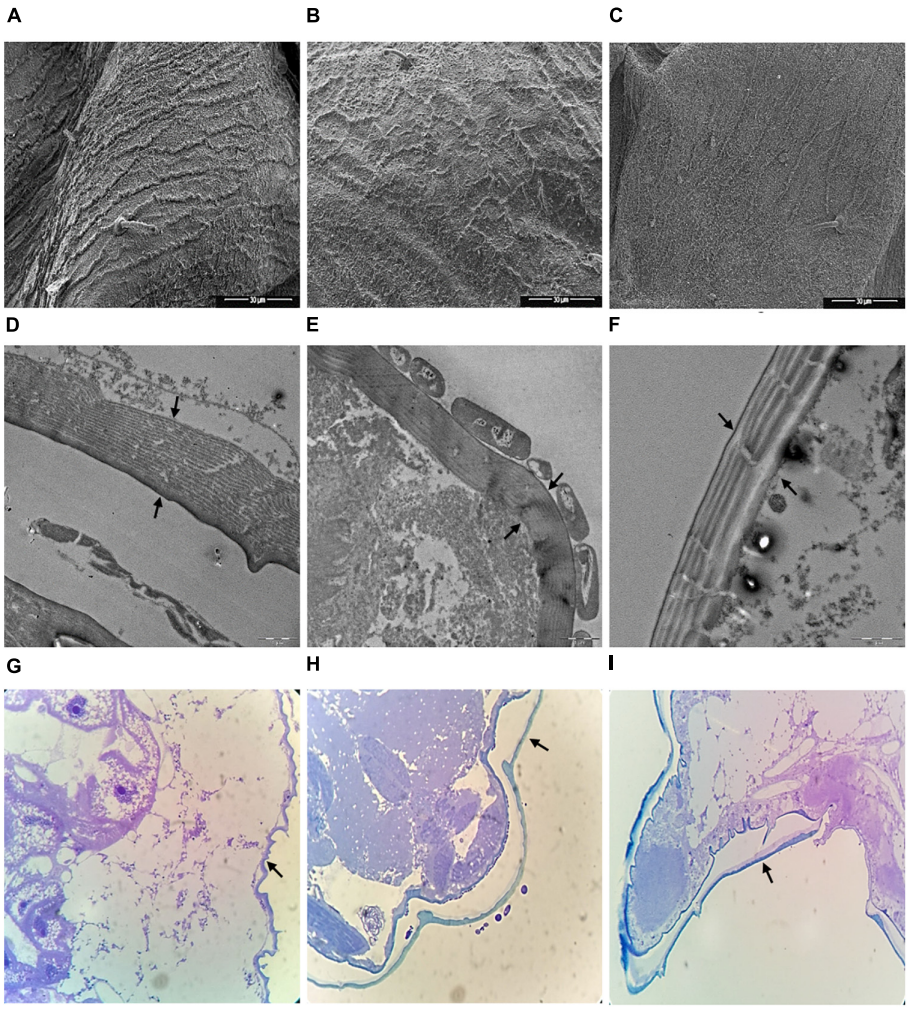


FIGURE 6 | Micrographs of aphid thoraxes produced by scanning electron microscopy **(A–C)**, transmission electron microscopy **(D–F)** and optical microscopy **(G–I)** of aphids treated only with TSB medium **(A**, **D,** and **G)**, the L193 culture **(B**, **E,** and **H)** and the biosurfactant produced by L193 **(C**, **F,** and **I)**. Arrows indicate aphid membranes.

indicates that this biosurfactant is more efficient than other biological surfactants (Sriram et al., 2011).

The oil spreading test and the emulsification indices used for the surfactant produced by L193 showed results very similar to those for positive controls such as Triton X-100. These properties of L193 lipopeptides, together with their CMC, highlight their potential biotechnological, biomedical and environmental applications due to their ability to reduce interfacial tension between aqueous and oleous mixtures.

In this study, we also analysed the production of glucosidase enzymes (amylase and chitinase) and proteases (caseinase) by strain L193 in order to determine its potential insecticidal capacity, as the insect cuticle is composed mainly of chitin nanofibres embedded in a matrix of proteins, polyphenols and water with small amounts of lipid. Chitin is also a common constituent of insect exoskeletons which support the epidermal cuticles and the peritrophic matrices lining the gut epithelium (Merzendorfer and Zimoch, 2003).

The role of such lytic enzymes in the insecticidal activity of this strain has previously been observed in the genus *Bacillus* (Driss et al., 2011). The undiluted L193 culture supernatants on 1% (w/v) chitin-containing media displayed enhanced chitinase, amylase and caseinase production. Driss et al. (2011) have highlighted the important role played by the chitinase enzyme in *B. thuringiensis* whose integration into ICPs enhanced their insecticidal activity. The synergistic effect of proteases and chitinases on the cuticle degradation mechanism has also been demonstrated (Leger et al., 1986). Moreover, our study demonstrates that L193 produces glucosidases and proteases, thus confirming the insecticidal potential of these enzymes.

Different *Bacillus* sp., which produce lipopeptides with surfactant properties, have been described (Raaijmakers et al., 2010). These metabolites are regarded as the most common class of compounds produced by *Bacillus* sp. (Stachelhaus and Marahiel, 2002). The antifungal activity of lipopeptides isolated from *B. atrophaeus* has also been described

(Huang et al., 2015). Genomic analysis carried out in our study indicates that *B. atrophaeus* L193 contains non-ribosomal lipopeptide synthetase gene clusters, which include genes involved in producing fengycin, surfactin, bacillomycin and iturin genes.

Aleti et al. (2016) have reported that the lipopeptides fengycins, iturins and surfactins produced by *B. atrophaeus* 176s display antifungal activity and can protect different crops against *Rhizoctonia solani* infection. However, very few of these compounds produced by *Bacillus* spp. have been reported to be involved in aphid control, and their insecticidal activity against this insect has not been studied to any significant extent.

The metabolites produced by L193 were identified using electrospray quadrupole time-of-flight mass spectrometry (Q-TOFS MS), which show that strain L193 can produce four types of lipopeptides: surfactins, bacillomycin F, iturin A, and fengycin B. All these lipopeptides have been previously observed by PCR. Although some studies have described the control of plant pathogens by surfactins (Chen et al., 2013), little research has been carried out on the aphicidal activity of these molecules produced by the genus *Bacillus*. To our knowledge, only two studies have analysed the role of surfactins produced by different *Bacillus* sp. acting as aphicidal metabolites (Yun et al., 2013; Yang et al., 2017), while the aphicidal role played by other lipopeptides, such as iturin, bacillomycin and fengycin, remains unclear.

In vivo studies of barley plants infested with three 3rd-instar *R. padi* aphids and treated with *B. atrophaeus* L193. The results show high levels of activity against *R. padi* for all of the treatments tested, L193 culture, cell-free supernatant and biosurfactant.

The highest mortality rate was observed after 24 h of incubation. As indicated above, the predominant metabolites present in the biosurfactant produced by strain L193 were lipopeptides, whose insecticidal activity against *R. padi* was also demonstrated. The mechanisms involved in the anti-aphid activity of lipopeptides produced by the genus *Bacillus* sp. remain unclear. Yun et al. (2013) identified a surfactin acting as an aphicidal metabolite produced by *B. amyloliquefaciens* G1. More recently, Yang et al. (2017) showed that *B. subtilis* Y9 produces biosurfactants which act as insecticidal metabolites against this aphid species. Both studies investigated aphid mortality rates using a topic assay method similar to that used in our study. However, most studies report that the anti-aphid insecticidal activity of the genus *B. thuringiensis* involves ingestion (Bravo et al., 2007).

Aphid feeding was evaluated by measuring the amount of honeydew excreted by the whole aphid population. The samples of L193 culture, cell-free supernatant and surfactant treatments showed a reduction in the honeydew excreted as compared to controls. These results indicate that L193 topic treatments not only affected cuticle integrity but also aphid feeding capacity. Similar results were found in a study of *Bacillus velezensis* YC7010 as an inductor of systemic anti-aphid resistance by plants (Rashid et al., 2017), although, in this case, the treatment was by ingestion.

Microscopy data on aphids treated with the L193 culture or the biosurfactant produced by L193 are given in this study. Scanning electron microscopy (SEM) analysis shows clear evidence of damage to the cuticle in aphids treated with L193 culture and the biosurfactant. Transmission electron microscopy (TEM) and optical microscopy confirmed that treatment with L193 affects the cuticular membrane. With regard to treatment with the biosurfactant, a clear change in cuticle structure was also observed. Lipopeptides appear to induce aphid cuticle dehydration due to interaction with cuticle molecules such as phospholipids and fatty acids (Puterka et al., 2003; Jang et al., 2013). We hypothesise that the biosurfactant produced by L193 affects cuticle lipids and leads to membrane perturbation. This mechanism is corroborated by microscopy observations which showed that the treatment tested in this study causes clear changes in the endocuticle layer of aphids. Similar results have been reported for other biosurfactants, such as rhamnolipids, produced by different species of *Pseudomonas* sp. (Kim et al., 2011; Jang et al., 2013).

Bacillus atrophaeus L193 produces a biosurfactant with enhanced physicochemical properties as compared to other biological surfactants described to date. In addition, genomic analysis indicated that L193 contains gene clusters for the biosynthesis of non-ribosomal lipopeptide synthetases. This was confirmed by Q-TOF MS, which detected the presence of different types of lipopeptides in the biosurfactant. The *in vivo* studies carried out suggest that the biosurfactants produced by *B. atrophaeus* L193 may be useful for controlling aphids. To the best of our knowledge, this is the first study to confirm the insecticidal activity of *B. atrophaeus* against *R. padi*, one of the most abundant and economically important aphids affecting both winter and spring wheat crops.

AUTHOR CONTRIBUTIONS

MR assisted with experimental techniques and statistical analysis. AM and MT assisted with experimental techniques related to previous screening and taxonomic identification. VB assisted with analysing the results and critical revision of the manuscript. MC designed the insecticidal assay, assisted with analysing the results, and critical revision of the manuscript. IS designed the experimental techniques, analysed the results, and drafted the manuscript.

ACKNOWLEDGMENTS

IS wishes to thank MINECO for her Ramón y Cajal contract. The authors also wish to thank Michael O'Shea for proofreading the English version of this manuscript, José Antonio Siles Martos for the statistical analysis, Encarnación Jurado Alameda for surface tension determination, and Maria Luisa Fernández for the technical support with regard to *in vivo* analyses.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.03114/full#supplementary-material

REFERENCES

Abbott, W. S. (1925). A method of computing the effectiveness of a insecticide. *J. Econ. Entomol.* 18, 265–267. doi: 10.1093/jee/18.2.265a

Aleti, G., Lehner, S., Bacher, M., Compant, S., Nikolic, B., Plesko, M., et al. (2016). Surfactin variants mediate species-specific biofilm formation and root colonization in *Bacillus*. *Environ. Microbiol.* 18, 2634–2645. doi: 10.1111/1462-2920.13405

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J. (1990). Basic local alignment search tool. *J. Mol. Biol.* 215, 403–410. doi: 10.1016/S0022-2836(05)80360-2

Barrow, G., and Feltham, R. (1993). *Cowan and Steel's Manual for the Identification of Medical Bacteria*, 3rd Edn. Cambridge: Cambridge University Press, doi: 10.1111/j.2042-7158.1965.tb07589.x

Biswas, S. C., Dubreil, L., and Marion, D. (2001). Interfacial behavior of wheat puroindolines: study of adsorption at the air-water interface from surface tension measurement using wilhelmy plate method. *J. Colloid Interface Sci.* 244, 245–253. doi: 10.10006/jcis.2001.7940

Biurrun, R., Lezáun, J. A., Zúniga, J., Garnica, I., and Llorens, M. (2010). Virus del enanismo amarillo de la cebada (BYDV). *Navarra Agrar.* 183, 24–28.

Bravo, A., Gill, S. S., and Soberon, M. (2007). Mode of action of *Bacillus thuringiensis* cry and cyt toxins and their potential for insect control. *Toxicon* 49, 423–435. doi: 10.1016/j.toxicon.2006.11.022

Chen, Y., Yan, F., Chai, Y., Liu, H., Kolter, R., Losick, R., et al. (2013). Biocontrol of tomato wilt disease by *Bacillus subtilis* isolates from natural environments depends on conserved genes mediating biofilm formation. *Environ. Microbiol.* 15, 848–864. doi: 10.1111/j.1462-2920.2012.02860.x

Chung, S., Kong, H., Buyer, J. S., Lakshman, D. K., Lydon, J., Kim, S. D., et al. (2008). Isolation and partial characterization of *Bacillus subtilis* ME488 for suppression of soilborne pathogens of cucumber and pepper. *Appl. Microbiol. Biotechnol.* 80, 115–123. doi: 10.1007/s00253-008-1520-4

Cooper, D. G., and Goldenberg, B. G. (1987). Surface-active agents from two *Bacillus* species. *Appl. Environ. Microbiol.* 53, 224–229.

Cooper, D. G., Macdonald, C. R., Duff, S. J., and Kosaric, N. (1981). Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions. *Appl. Environ. Microbiol.* 42, 408–412.

Copping, L. G. (2004). *The Manual of Biocontrol Agents*. Alton: British Crop Protection Council.

Dhami, M. K., Weir, B. S., Taylor, M. W., and Beggs, J. R. (2013). Diverse honeydew-consuming fungal communities associated with scale insects. *PLoS One* 8:e70316. doi: 10.1371/journal.pone.0070316

Driss, F., Rouis, S., Azzouz, H., Tounsi, S., Zouari, N., and Jaoua, S. (2011). Integration of a recombinant chitinase into *Bacillus thuringiensis* parasporal insecticidal crystal. *Curr. Microbiol.* 62, 281–288. doi: 10.1007/s00284-010-9704-4

Dutton, A., Romeis, J., and Bigler, F. (2003). Assessing the risks of insect resistant transgenic plants on entomophagous arthropods Bt-maize expressing Cry1Ab as a case study. *BioControl* 48, 611–636. doi: 10.1023/A:1026313719424

Huang, X. F., Zhou, D., Guo, J., Manter, D. K., Reardon, K. F., and Vivanco, J. M. (2015). Bacillus spp. from rainforest soil promote plant growth under limited nitrogen conditions. *J. Appl. Microbiol.* 118, 672–684. doi: 10.1111/jam.12720

Jang, J. Y., Yang, S. Y., Kim, Y. C., Lee, C. W., Park, M. S., Kim, J. C., et al. (2013). Identification of orfamide A as an insecticidal metabolite produced by *Pseudomonas* protegens F6. *J. Agric. Food Chem.* 61, 6786–6791. doi: 10.1021/jf401218w

Kim, J. H., and Jander, G. (2007). *Myzus persicae* (green peach aphid) feeding on Arabidopsis induces the formation of a deterrent indole glucosinolate. *Plant J.* 49, 1008–1019. doi: 10.1111/j.1365-313X.2006.03019.x

Kim, S. K., Kim, Y. C., Lee, S., Kim, J. C., Yun, M. Y., and Kim, I. S. (2011). Insecticidal activity of rhamnolipid isolated from *Pseudomonas* sp. EP-3 against green peach aphid (*Myzus persicae*). *J. Agric. Food Chem.* 59, 934–938. doi: 10.1021/jf104027x

Lane, D. J. (1991). "16S/23S rRNA sequencing," in *Nucleic Acid Techniques in Bacterial Systematics*, eds E. Stackebrandt and M. Goodfellow (Chichester: John Wiley and Sons), 115–175.

Lee, S. C., Lee, S. J., Kim, S. H., Park, I. H., Lee, Y. S., Chung, S. Y., et al. (2008). Characterization of new biosurfactant produced by *Klebsiella* sp. Y6-1 isolated from waste soybean oil. *Bioresour. Technol.* 99, 2288–2292. doi: 10.1016/j.biortech.2007.05.020

Leger, R. J. S., Charnley, A. K., and Cooper, R. M. (1986). Cuticle-degrading enzymes of entomopathogenic fungi: synthesis in culture on cuticle. *J. Invertebr. Pathol.* 48, 85–95. doi: 10.1016/0022-2011(86)90146-1

Leroy, P. D., Sabri, A., Heuskin, S., Thonart, P., Lognay, G., Verheggen, F. J., et al. (2011). Microorganisms from aphid honeydew attract and enhance the efficacy of natural enemies. *Nat. Commun.* 2:348. doi: 10.1038/ncomms1347

Liu, X. F., Hu, X. S., Keller, M. A., Zhao, H. Y., Wu, Y. F., and Liu, T. X. (2014). Tripartite interactions of barley yellow dwarf virus, *Sitobion avenae* and wheat varieties. *PLoS One* 9:e106639. doi: 10.1371/journal.pone.0106639

Merzendorfer, H., and Zimoch, L. (2003). Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. *J. Exp. Biol.* 206, 4393–4412. doi: 10.1242/jeb.00709

Mora, I., Cabrefiga, J., and Montesinos, E. (2011). Antimicrobial peptide genes in *Bacillus* strains from plant environments. *Int. Microbiol.* 14, 213–223. doi: 10.2436/20.1501.01.151

Morikawa, M., Hirata, Y., and Imanaka, T. (2000). A study on the structure-function relationship of lipopeptide biosurfactants. *Biochim. Biophys. Acta* 1488, 211–218. doi: 10.1016/S1388-1981(00)00124-4

Mukherjee, A. K., and Das, K. (2005). Correlation between diverse cyclic lipopeptides production and regulation of growth and substrate utilization by *Bacillus subtilis* strains in a particular habitat. *FEMS Microbiol. Ecol.* 54, 479–489. doi: 10.1016/j.femsec.2005.06.003

Nisbet, A. J., Woodford, J. A. T., and Strang, R. H. C. (1994). Quantifying aphid feeding on non-radioactive food sources. *Entomol. Exp. Appl.* 72, 85–89. doi: 10.1111/j.1570-7458.1994.tb01805.x

Palma, L., Munoz, D., Berry, C., Murillo, J., de Escudero, I. R., and Caballero, P. (2014). Molecular and insecticidal characterization of a novel cry-related protein from *Bacillus thuringiensis* toxic against *Myzus persicae*. *Toxins* 6, 3144–3156. doi: 10.3390/toxins6113144

Phale, P. S., Savithri, H. S., Rao, N. A., and Vaidyanathan, C. S. (1995). Production of biosurfactant "Biosur-Pm" by *Pseudomonas maltophila* CSv 89. Characterization and role in hydrocarbon uptake. *Arch. Microbiol.* 163, 424–431. doi: 10.1007/BF00272131

Porcar, M., Grenier, A. M., Federici, B., and Rahbe, Y. (2009). Effects of *Bacillus thuringiensis* delta-endotoxins on the pea aphid (*Acyrthosiphon pisum*). *Appl. Environ. Microbiol.* 75, 4897–4900. doi: 10.1128/AEM.00686-09

Puterka, G. J., Farone, W., Palmer, T., and Barrington, A. (2003). Structure-function relationships affecting the insecticidal and miticidal activity of sugar esters. *J. Econ. Entomol.* 96, 636–644. doi: 10.1603/0022-0493-96.3.636

Raaijmakers, J. M., De Bruijn, I., Nybroe, O., and Ongena, M. (2010). Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. *FEMS Microbiol. Rev.* 34, 1037–1062. doi: 10.1111/j.1574-6976.2010.00221.x

Rashid, M. H., Khan, A., Hossain, M. T., and Chung, Y. R. (2017). Induction of systemic resistance against aphids by endophytic *Bacillus velezensis* YC7010 via expressing phytoalexin deficient4 in arabidopsis. *Front. Plant Sci.* 8:211. doi: 10.3389/fpls.2017.00211

Sanahuja, G., Banakar, R., Twyman, R. M., Capell, T., and Christou, P. (2011). *Bacillus thuringiensis*: a century of research, development and commercial applications. *Plant biotechnol. J.* 9, 283–300. doi: 10.1111/j.1467-7652.2011.00595.x

Sivapathasekaran, C., Mukherjee, S., Samanta, R., and Sen, R. (2009). High-performance liquid chromatography purification of biosurfactant isoforms produced by a marine bacterium. *Anal. Bioanal. Chem.* 395, 845–854. doi: 10.1007/s00216-009-3023-2

Song, B., Rong, Y. J., Zhao, M. X., and Chi, Z. M. (2013). Antifungal activity of the lipopeptides produced by *Bacillus amyloliquefaciens* anti-CA against *Candida albicans* isolated from clinic. *Appl. Microbiol. Biotechnol.* 97, 7141–7150. doi: 10.1007/s00253-013-5000-0

Sriram, M. I., Gayathiri, S., Gnanaselvi, U., Jenifer, P. S., Raj, S. M., and Gurunathan, S. (2011). Novel lipopeptide biosurfactant produced by hydrocarbon degrading and heavy metal tolerant bacterium *Escherichia fergusonii* KLU01 as a potential tool for bioremediation. *Bioresour. Technol.* 102, 9291–9295. doi: 10.1016/j.biortech.2011.06.094

Stachelhaus, T. M. H., and Marahiel, M. A. (2002). "Nonribosomal assembly of peptide antibiotics on modular protein templates," in *Bacillus Subtilis and its Closest Relatives—From Genes to Cells*, eds A. L. Sonenshein, J. A. Hoch, and R. M. Losick (Washington, DC: ASM Press), 415–435. doi: 10.1128/9781555817992.ch30

Tapi, A., Chollet-Imbert, M., Scherens, B., and Jacques, P. (2010). New approach for the detection of non-ribosomal peptide synthetase genes in *Bacillus* strains by polymerase chain reaction. *Appl. Microbiol. Biotechnol.* 85, 1521–1531. doi: 10.1007/s00253-009-2176-4

Torres-Quintero, M. C., Peña-Chora, G., Hernández-Velázquez, V. M., and Arenas-Sosa, I. (2015). Signs of *Bacillus thuringiensis* (Bacillales: Bacillaceae) infection in *Myzus persicae* (Hemiptera: Aphididae): koch's postulates. *Fla. Entomol.* 98, 799–802. doi: 10.1653/024.098.0264

Wu, Y. J., Cheng, C. Y., and Li, Y. K. (2009). Cloning and expression of chitinase a from *Serratia marcescens* for large-scale preparation of N,N -Diacetyl chitobiose. *J. Chin. Chem. Soc.* 56, 688–695. doi: 10.1002/jccs.200900103

Yang, S. Y., Lim, D. J., Noh, M. Y., Kim, J. C., Kim, Y. C., and Kim, I. S. (2017). Characterization of biosurfactants as insecticidal metabolites produced by *Bacillus subtilis* Y9. *Entomol. Res.* 47, 55–59. doi: 10.1111/1748-5967.12200

Yoon, S. H., Ha, S. M., Kwon, S., Lim, J., Kim, Y., Seo, H., et al. (2017). Introducing EzBioCloud: a taxonomically united database of 16S rRNA and whole genome assemblies. *Int. J. Syst. Evol. Microbiol.* 67, 1613–1617. doi: 10.1099/ijsem.0.001755

Youssef, N. H., Duncan, K. E., Nagle, D. P., Savage, K. N., Knapp, R. M., and McInerney, M. J. (2004). Comparison of methods to detect biosurfactant production by diverse microorganisms. *J. Microbiol. Methods* 56, 339–347. doi: 10.1016/j.mimet.2003.11.001

Yun, D. C., Yang, S. Y., Kim, Y. C., Kim, I. S., and Kim, Y. H. (2013). Identification of surfactin as an aphicidal metabolite produced by *Bacillus amyloliquefaciens* G1. *J. Korean Soc. Appl. Biol. Chem.* 56, 751–753. doi: 10.1007/s13765-013-3238-y

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 23 January 2019
doi: 10.3389/fmicb.2018.03299

Modulation of Metabolome and Bacterial Community in Whole Crop Corn Silage by Inoculating Homofermentative *Lactobacillus plantarum* and Heterofermentative *Lactobacillus buchneri*

*Dongmei Xu[1,2], Wurong Ding[1,2], Wencan Ke[1,2], Fuhou Li[2,3], Ping Zhang[1,2] and Xusheng Guo[1,2]**

[1] State Key Laboratory of Grassland and Agro-ecosystems, School of Life Sciences, Lanzhou University, Lanzhou, China, [2] Probiotics and Biological Feed Research Center, Lanzhou University, Lanzhou, China, [3] Stay Key Laboratory of Grassland Agro-ecosystems, College of Pastoral Agriculture Science and Technology, Lanzhou University, Lanzhou, China

OPEN ACCESS

Edited by:
José E. Barboza-Corona,
Universidad de Guanajuato, Mexico

Reviewed by:
Sonia Arriaga,
Instituto Potosino de Investigación Científica y Tecnológica (IPICYT), Mexico
Nagamani Balagurusamy,
Universidad Autónoma de Coahuila, Mexico

***Correspondence:**
Xusheng Guo
guoxsh07@lzu.edu.cn

Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: 07 September 2018
Accepted: 18 December 2018
Published: 23 January 2019

Citation:
Xu D, Ding W, Ke W, Li F, Zhang P and Guo X (2019) Modulation of Metabolome and Bacterial Community in Whole Crop Corn Silage by Inoculating Homofermentative Lactobacillus plantarum and Heterofermentative Lactobacillus buchneri. Front. Microbiol. 9:3299. doi: 10.3389/fmicb.2018.03299

The present study investigated the species level based microbial community and metabolome in corn silage inoculated with or without homofermentative *Lactobacillus plantarum* and heterofermentative *Lactobacillus buchneri* using the PacBio SMRT Sequencing and time-of-flight mass spectrometry (GC-TOF/MS). Chopped whole crop corn was treated with (1) deionized water (control), (2) *Lactobacillus plantarum*, or (3) *Lactobacillus buchneri*. The chopped whole crop corn was ensiled in vacuum-sealed polyethylene bags containing 300 g of fresh forge for 90 days, with three replicates for each treatment. The results showed that a total of 979 substances were detected, and 316 different metabolites were identified. Some metabolites with antimicrobial activity were detected in whole crop corn silage, such as catechol, 3-phenyllactic acid, 4-hydroxybenzoic acid, azelaic acid, 3,4-dihydroxybenzoic acid and 4-hydroxycinnamic acid. Catechol, pyrogallol and ferulic acid with antioxidant property, 4-hydroxybutyrate with nervine activity, and linoleic acid with cholesterol lowering effects, were detected in present study. In addition, a flavoring agent of myristic acid and a depression mitigation substance of phenylethylamine were also found in this study. Samples treated with inoculants presented more biofunctional metabolites of organic acids, amino acids and phenolic acids than untreated samples. The *Lactobacillus* species covered over 98% after ensiling, and were mainly comprised by the *L. acetotolerans*, *L. silagei*, *L. parafarraginis*, *L. buchneri* and *L. odoratitofui*. As compared to the control silage, inoculation of *L. plantarum* increased the relative abundances of *L. acetotolerans*, *L. buchneri* and *L. parafarraginis*, and a considerable decline in the proportion of *L. silagei* was observed; whereas an obvious decrease in *L. acetotolerans* and increases in *L. odoratitofui* and *L. farciminis* were observed in the *L. buchneri* inoculated

silage. Therefore, inoculation of *L. plantarum* and *L. buchneri* regulated the microbial composition and metabolome of the corn silage with different behaviors. The present results indicated that profiling of silage microbiome and metabolome might improve our current understanding of the biological process underlying silage formation.

Keywords: corn silage, metabolomics, bacterial community, GC-TOF/MS, SMRT, *Lactobacillus plantarum*, *Lactobacillus buchneri*

INTRODUCTION

Ensiling is a conventional and global practice method for preserving green forage crops under anaerobic conditions. The goal of making silage is to obtain a high-quality feed for livestock. The high-quality silage should be avoided of undesirable compounds that could negatively affect animal performance, the environment, or net farm income (Kung et al., 2018). The epiphytic microbial communities of fresh forages play a critical role in the start and whole process of forage fermentation. Microbial fermentation in the silo produces an array of metabolites and can change many nutritive aspects of forage (Kung et al., 2018). However, the process of fermentation is exceedingly complex and involves many types of microorganisms, resulting in a variety of metabolites. Therefore, improved understanding of the metabolome and bacterial community in ensiled forages may provide an important scientific basis for making high-quality silages.

In the past decade, molecular tools such as denaturing gradient gel electrophoresis (DGGE), real-time PCR, terminal restriction fragment length polymorphism (TRFLP), ribosomal intergenic spacer analysis (RISA), and next generation sequencing (NGS) have been used to uncover the epiphytic microbiota in fresh forages and microbiota in ensiled forages (Stevenson et al., 2006; Brusetti et al., 2011; Li and Nishino, 2011a; Pang et al., 2011; Ni et al., 2017). However, these techniques only reflected a few of the most abundant operational taxonomic units (OTUs) present and did not reveal detailed information regarding the composition of the complete microbial community (Guo et al., 2018). Even though NGS technologies have high-throughputs and can facilitate the discovery of higher microbiota diversity as well as the detection of organisms that are presented in low numbers, partial sequence of the 16S rRNA gene analysis only provides microbiota profiles that are restricted to genus precision (Bokulich and Mills, 2012; Mayo et al., 2014). A vital metagenomic approach, the PacBio single molecule in conjunction with real-time sequencing technology (SMRT), can almost cover the full read length of the DNA fragment multiple times, resulting in a reduced error rate and increased ability to depict the bacterial profile to species level precision (Schloss et al., 2016). Hence, the SMRT sequencing platform should be considered suitable for precisely assessing the microbial community at the species level in ensiled forages with a low microbial biodiversity (Bao et al., 2016; Guo et al., 2018).

The fermentation of forage is very complex and involves many types of microorganisms mainly comprised by lactic acid bacteria (LAB), resulting in a variety of different metabolites during ensiling. Common fermentation end products of silages, such as lactic acid, acetic acid, propionic acid, butyric acid, 1,2-propanediol and ethanol, are conventionally detected to evaluate the fermentation quality of ensiled forages. Among them, acetic acid, 1,2-propanediol and propionic acid are good for improving silage aerobic stability after aerobic exposure. Some other metabolites with antifungal ability, such as 3-hydroxydecanoic acid, 3-(R)-hydroxytetradecanoic acid, 4-hydroxybenzoic acid, vanillic acid, 2,3-butanedione, acetaldehyde, and bacteriocins were also reported to be produced by LAB strains or liquid cultures of grass silage (Cleveland et al., 2001; Sjögren et al., 2003; Broberg et al., 2007; Arasu et al., 2013). In addition, LAB can produce a large number of metabolites during fermentation, such as oligosaccharides, amino acids, fatty acids, vitamins, and aromatic compounds (Sun et al., 2012). These results indicate that many other metabolites in ensiled forage might have not been identified.

To produce high quality silage, the LAB inoculants are usually used to promote fermentation process. Based on the fermentation pattern, inoculants are divided into homofermentative and heterofermentative cultures. These two types of inoculants use different approaches for directing fermentation during ensiling. Homofermentative LAB is often used to dominate the fermentation by rapid production of lactic acid and the consequent decrease in pH, which prevents growth of mold, yeast and other undesirable microbes, and helps to preserve the forage mass (Hu et al., 2009). The most common homofermentative inoculant is *Lactobacillus plantarum* (Koc et al., 2017). In order to improve aerobic stability, heterofermentative LAB such as *Lactobacillus buchneri* was developed as a silage inoculant. *Lactobacillus buchneri* improves aerobic stability by fermenting lactic acid to acetic acid and 1,2-propanediol (Elferink et al., 2001). However, how the homofermentative or heterofermentative LAB affect the bacterial community and metabolites in whole crop corn silage is unclear. Therefore, modulation of bacterial community and metabolome in corn silage, by inoculating *Lactobacillus plantarum* and *Lactobacillus buchneri,* were profiled in the present study.

MATERIALS AND METHODS

Silage Making

The whole crop corn (*Zea mays* L.) was mowed with a precision chop harvester at a 234.6 g DM kg^{-1} of fresh forage. The pH of raw corn was 5.08. The epiphytic LAB, mold and yeast in the fresh corn were 7.08, 5.66, and 6.56 log_{10} cfu g^{-1}, respectively. Vacuumed silos of whole crop corn (vacuum-sealing polyethylene plastic bags packed with approximately 300 g of fresh forage)

were individually prepared for each of the following treatments: (a) untreated (control), (b) *L. plantarum*, and (c) *L. buchneri*. The application rate of each inoculant into the fresh forage was 1×10^6 cfu g^{-1} FM, and an equal volume of distilled water was sprayed onto the fresh corn for the control group. The silos were then stored at ambient temperature (22–25°C) in dark conditions and sampled at 90 days of fermentation.

Characteristics of Fresh Forage and Silage

A 20 g fresh sample was put in a juice exactor and squeezed with 180 mL distilled water for 30 s at a high speed, then filtered via medical gauze with four layers. The filtrate pH was measured with a glass electrode pH meter immediately. A portion of the filtrate of each sample was acidulated with H_2SO_4 (7.14 mol L^{-1}) and filtered with a 0.45-μm dialyzer. Lactic acid, acetic acid, propionic acid, and butyric acid were analyzed by High Performance Liquid Chromatography (HPLC), (KC-811 column, Shodex; Shimadzu: Japan; oven temperature 50°C; flow rate 1 mL min^{-1}; SPD 210 nm). Enumeration of LAB, yeast and mold in fresh corn were detected according to the methods described by Reich and Kung (2010). Briefly, samples (10 g) were homogenized in 100 mL of sterile Ringer's solution (Oxide BR52) for 1 min and serially diluted (10-fold). The number of LAB was detected on spread plates using Rogosa agar (Oxoid CM627, Oxoid, Basingstoke, United Kingdom) and incubated at 37°C for 48–72 h. Yeast and mold were determined by pour plating serial 10-fold dilutions of water extracts on malt extract agar (Oxoid CM0059) that had been acidified with lactic acid (concentration of 850 g kg^{-1} added at 50 g kg^{-1}, vol/vol). Plates were incubated at 32°C for 48–72 h. Colonies were counted from plates where appropriate dilutions yielded 30–300 colonies.

SMRT Analysis of Bacteria Composition

Fresh and ensiled corn silages of each treatment were sampled for total bacteria DNA extraction. Total DNA was extracted using a DNA isolation kit (Tiangen, DP302-02, Tiangen, China) according to the manufacturer's specification. The quality of extracted DNA samples was evaluated by 1% agarose gel electrophoresis and spectrophotometry. All extracted DNA samples were stored at −20°C for further analysis. The PCR amplification of the full-length 16S rRNA gene for SMRT sequencing was carried out with the forward primer 27F (5′-GAGAGTTTGATCCTGGCTCAG-3′) and reverse primer 1492R (5′-TACCTTGTTACGACTT-3′). The two primers contained a set of 16-nucleotide barcodes. The PCR program was as follows: 95°C for 3 min, 25 cycles of 98°C for 20 s, 57°C for 30 s and 72°C for 90 s, with a final extension of 72°C for 2 min.

The 16S rRNA library was built with a Pacific Biosciences Template Prep Kit. Sequencing of the amplicons was performed on a PacBio Sequel instrument (Pacific Biosciences, Menlo Park, CA, United States). The quality control for PCR amplifications and sequences pre-processing were performed as described by Mosher et al. (2013). Raw data was extracted and filtered with the Circular Consensus Sequencing (CCS) software of the SMRT Link (minfullpass = 3, polish minPredictedAccuacy = 0.8, minLength = 500) to obtain the Raw CCS Reads. The barcode reads of every sample were recognited with Lima[1] to acquire Raw CCS. Whereafter the CCS (accuracy above 99%) of each sample was tested and chimeric reads were removed with UCHIME[2] to acquire the optimized sequence. Subsequently, representative sequence was compared using the Mothur[3] software with the Silva (Release 128[4]) database (classified at a bootstrap threshold of 0.9) to gain classified information (Quast et al., 2013). The Shannon-Wiener, Simpson's diversity, Chao1 and rarefaction estimators were calculated to evaluate the alpha diversity.

Metabolite Profiling Analysis

Samples of fresh and ensiled forages (5 g ± 1 mg) were extracted with 20 mL extraction liquid (VMethanol:VE.A = 1:1) in EP tubes. Then 666 μL of adonitol (0.5 mg mL^{-1} stock in dH_2O) as internal standard was added and vortex mixing for 30 s. The mixture was oscillated for 1 h, and then filtered with membrane (0.22 μm). The supernatant (0.6 mL) was dried with a vacuum concentrator without heating in a GC/MS glass vial, and then 80 μL of methoxyamine hydrochloride (20 mg mL^{-1} in pyridine) was added into each dried metabolite and incubated for 30 min at 80°C. The quality control (QC) samples consisted of partial extract (75 μL) from each sample. For derivatization, 100 μL of the BSTFA regent (containing 1% TMCS, vol/vol) was added into each sample, incubated for 1.5 h at 70°C. Finally, 10 μL of FAMEs (standard mixture of fatty acid methyl esters, C8-C16:1 mg mL^{-1}; C18-C24:0.5 mg mL^{-1} in chloroform) was added into the QC sample after cooling to the room temperature. All samples were analyzed by a Agilent 7890 gas chromatograph system coupled with a Pegasus 4D time-of-flight mass spectrometer (GC-TOF-MS).

The GC-TOF-MS system used a DB-5MS capillary column coated with 5% diphenyl and cross-linked with 95% dimethylpolysiloxane (30 m × 250 μm inner diameter, 0.25 μm film thickness; J&W Scientific, Folsom, CA, United States). Samples (1 μL) were injected in split mode (split ratio 20:1), with helium used as the carrier gas at a flow rate of 1.0 mL min^{-1}. The oven temperature ramp was as follows: initial temperature was 80°C for 1 min, then raised to 290°C at a rate of 10°C min^{-1}, and finally kept at 290°C for 13 min. The injection, transfer line, and ion source temperatures were 280, 295, and 220°C, respectively. The energy was −70 eV in electron impact mode. The mass spectrometry data was acquired in full-scan mode with an m z^{-1} range of 50–600 at a rate of 10 spectra per second, after a solvent delay of 7.9 min.

Chroma TOF 4.3X software of LECO Corporation and the LECO-Fiehn Rtx5 database were used for raw peak exaction, data baseline filtration and calibration of the baseline, as well

[1]https://github.com/PacificBiosciences/barcoding

[2]http://drive5.com/uchime

[3]https://mothur.org/wiki/Classify.seqs

[4]http://www.arb-silva.de

as peak alignment, deconvolution analysis, peak identification and integration of the peak area (Kind et al., 2009). Both the mass spectrum match and retention index match were considered in metabolites identification. Peaks with poor repeatability (<50% of QC samples or RSD >30%) in QC samples were removed (Dunn et al., 2011). The NIST[5] and KEGG[6] commercial databases were used to search for metabolites. The followed method was used to calculate the relative concentration of each detected metabolite. Briefly, with the fixed volume of sample injected into equipment, a peak area of each metabolite as its relative concentration in the sample was obtained. The relative concentrations of metabolites were calculated as the peak area rate of each metabolite and internal standard substance (adonitol).

Statistical Analysis

All metabolite data was normalized using SIMCA software (version 14, Umetrics AB, Umea, Sweden) before hierarchical cluster analysis and principal component analysis (PCA). PCA and projections to latent structure-discriminant analysis (PLS-DA) models were tested for all samples. The OPLS-DA model was employed with first principal-component of VIP (variable importance in the projection) values (VIP > 1) combined with Student's *T*-test ($P < 0.05$) to find differentially expressed metabolites.

RESULTS

Fermentation Quality of Whole Crop Corn Silage

The fermentation characteristics of whole crop corn silage ensiled for 90 days are shown in **Table 1**. Corn silage inoculated with *L. buchneri* had a higher pH, and greater acetic acid but less lactic acid compared with *L. plantarum*-treated and control groups ($P < 0.05$). Silage treated with *L. buchneri* had higher aerobic stability than samples treated with *L. plantarum,* but there was no significant difference with control group.

TABLE 1 | Fermentation characteristics of whole crop corn silage for 90 days.

	Treatment[1]		
Item[2]	Control	LP	LB
pH	3.66b	3.68b	3.74a
LA g/kg	223.1a	218.7a	175.4b
AA g/kg	45.9b	41.7b	54.7a
PA g/kg	15.8a	13.5b	13.6b
LAB $\log_{10}$ cfu/g FM	8.03b	7.81b	8.32a
Yeast $\log_{10}$ cfu/g FM	3.3a	0b	2.85a
Mold $\log_{10}$ cfu/g FM	0b	3a	0b
Aerobic stability h	136.5a	114b	121.5ab

[1]LP, samples treated with L. plantarum; LB, samples treated with L. buchneri. [2]LA, lactic acid; AA, acetic acid, PA, propionic acid; LAB, lactic acid bacteria. Means within the same line with different letters are significantly different ($P < 0.05$).

Metabolomic Profiles of Whole Crop Corn Silage

Based on the GC-TOF-MS of 12 samples, a total of 979 substances were detected, and 316 different metabolites were identified with their relative concentrations (**Supplementary Table S1**). According to PCA (**Figure 1**), the metabolites in *L. plantarum*, *L. buchneri* and control group samples were clearly separated by PC1, which represented 79.4% of variations among samples with different treatments. The altered metabolites in samples with or without inoculants were found from the first component of the PCA model, and the metabolites in *L. buchneri*-treated samples were separated by the second component of the PCA model. The contribution of metabolites to PC1 was dominated by lyxose, serine, oxoproline, proline, palmitic acid, succinic acid, and iminodiacetic acid (**Supplementary Table S2**). However, the application of multivariate analysis PLS-DA could be more useful for distinguishing control samples from *L. buchneri* and *L. plantarum*-treated samples (**Figure 2**).

The relative concentration and fold-changes of differentially expressed metabolites in whole crop corn silage with or without inoculants treatments are shown in **Table 2**. After 90 days of ensiling, samples treated with inoculants presented more amino acids, such as phenylalanine, lysine, tyrosine and glycine, than untreated samples. Compared to the control silage, up-accumulations of the phenolic acids of 4-hydroxycinnamic acid and 3,4-dihydroxycinnamic acid, the flavoring agent of gluconic lactone, and the organic acids of lauric acid, 3-hydroxypropionic acid, pentadecanoic acid, oxamic acid and isocitric acid were also observed in the inoculants treated silages. Inoculation of *L. buchneri* dramatically increased the relative concentrations of 2-hydroxybutanoic acid, saccharic acid, mannose, saccharic acid, 2-keto-L-gulonic acid, conduritol-β-epoxide, cholesterol and alpha-D-glucosamine-1-phosphate (a substance beneficial to health), in whole crop corn silage as compared to the *L. plantarum*-treated and control groups. Additionally, inoculation of *L. plantarum* remarkably increased the relative concentrations of cytosine, pyrogallol and tetrahydrocorticosterone in silages as compared to the control and samples inoculated with *L. buchneri*.

Among the identified metabolites, some substances with biological functions in the whole crop corn silage were also detected (**Table 3**). Inoculants significantly decreased the relative concentration of phenylethylamine (PEA) as compared to the control silage. Some other metabolites with bacteriostatic activity, such as catechol, azelaic acid, ferulic acid, 3-phenyllactic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid and glycolic acid, were found in the present whole crop corn silage although there were no significant differences in these metabolites among different treatments. Among those metabolites, catechol and ferulic acid have antioxidant properties, and 4-hydroxybutyrate has nervine activity. Linoleic acid with cholesterol lowering effects was detected at a higher relative concentration in the control silage compared to silages given inoculants. Myristic acid, a kind of flavoring agent, was also detected but no differences

[5]http://www.nist.gov/index.html

[6]http://www.genome.jp/kegg/

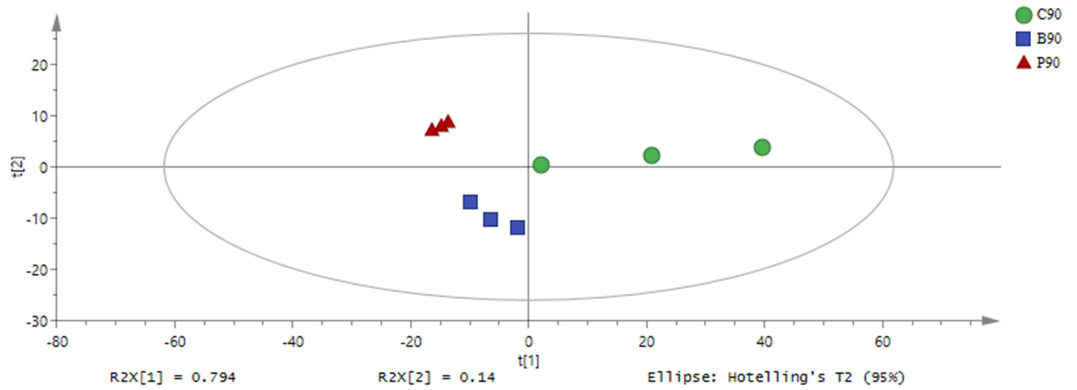


FIGURE 1 | Principal component analysis (PCA) of metabolic profiles in whole crop corn silage inoculated without (green circle) or with *L. plantarum* (red triangle) or *L. buchneri* (blue square) (*n* = 3). Input data were the total mass of the signal integration area of each sample, and the signal integration area was normalized with method of internal standard normalization for each sample.

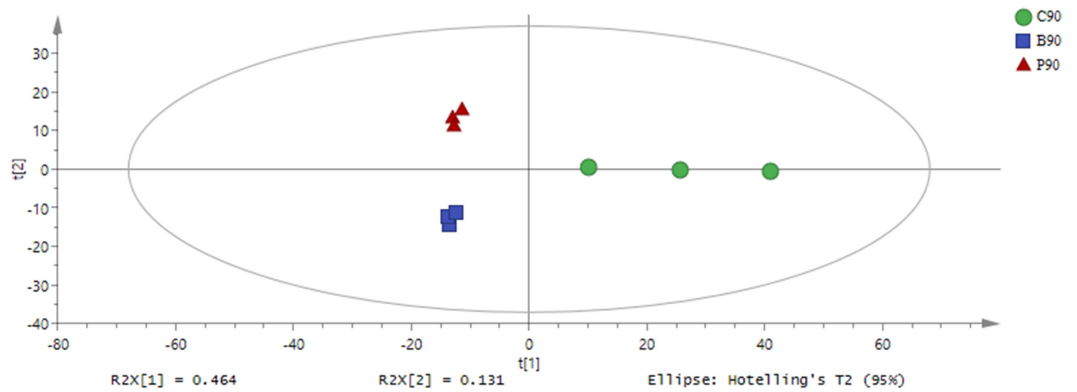


FIGURE 2 | Partial least squares-discriminate analysis (PLS-DA) of metabolic profiles in whole crop corn silage inoculated without (green circle) or with *L. plantarum* (red triangle) or *L. buchneri* (blue square) (*n* = 3). Input data were the total mass of the signal integration area of each sample, and the signal integration area was normalized with method of internal standard normalization for each sample.

were found among treatments. The *L. plantarum*-treated samples contained less 4-aminobutyric acid than the control and samples treated with *L. buchneri*.

Bacterial Microbiota of Whole Crop Corn Silage

Based on SMRT sequencing of the full-length 16S rRNA gene in silage bacteria, an average of 22,240 CCS sequences were obtained from each sample. The Good's coverage of samples showed that the sequence depth was adequate in the present study (**Table 4**). The α-diversity (Shannon index, Simpson index, Chao1 index), and alpha diversity index curves (**Supplementary Figure S1**) indicated high bacterial biodiversity in the present fermentation systems of whole crop corn silage.

At the genus level, the epiphytic microflora of fresh corn was mainly comprised by *Agrobacterium*, *Microbacterium*, *Sphingobacterium*, *Chryseobacterium*, *Candidatus Phytoplasma*, unclassified *Enterobacterales*, unclassified *Gammaproteobacteria*, *Leuconostoc* (2.69%), *Klebsiella*, *Stenotrophomonas*, *Lactobacillus* (2.44%), *Frigoribacterium*, unclassified *Bacteroidetes* and others (**Figure 3**). After 90 days of ensiling, the dominated microflora was the genus *Lactobacillus* (>98%), regardless of treatments. At the species level (**Figure 4**), fresh corn exhibited the greatest species richness and was harbored by many undesirable bacteria such as unclassified *Chryseobacterium* (4.6%), *Sphingobacterium siyangense* (3.5%), unclassified *Enterobacterales* (3.5%), *Microbacterium testaceum* (3.5%), unclassified *Sphingomonas* (2.6%), and so on. Meanwhile, the epiphytic LAB on the fresh corn mainly consisted of *Lactococcus lactis* (1.58%), *Leuconostoc pseudomesenteroides* (1.13%), *Lactobacillus paralimentarius* (1.06%), *Lactobacillus plantarum* (0.37%) and *Lactobacillus farciminis* (0.03%). After fermentation for 90 days, *Lactobacillus* species such as *L. acetotolerans*, *L. silagei*, *L. parafarraginis*, *L. buchneri*, and *L. odoratitofui* dominated the bacterial community. Compared to control group, samples inoculated with *L. plantarum* increased the relative abundances of *L. acetotolerans*, *L. parafarraginis*, *L. buchneri*, and decreased the relative abundance of *L. silagei*. Meanwhile, inoculation of *L. buchneri* increased the relative abundances of *L. silagei*, *L. odoratitofui*, *L. parafarraginis*,

TABLE 2 | Relative concentration and fold-changes in differential metabolites in whole crop corn silage with inoculation of *L. buchneri* or *L. plantarum* after 90 days of ensiling.

	Relative concentration[1]			Fold-changes[2]		
Metabolite name	**Control**	***L. buchneri***	***L. plantarum***	**Log_2 (B/C)**	**Log_2 (P/C)**	**Log_2(P/B)**
Phenylalanine	0.000	0.014	0.054	−13.162	−15.104**	−1.942*
Lysine	0.000	0.325	0.741	−17.705	−18.894**	−1.189
Tyrosine	0.000	0.087	0.217	−15.809**	−17.125*	−1.315*
Glycine	0.000	0.006	0.006	−11.917**	−12.057**	−0.141
Oxamic acid	0.000	0.055	0.059	−14.701**	−14.794**	−0.093
2-Hydroxybutanoic acid	0.109	0.508	0.091	−2.218*	0.264	2.482*
3-Hydroxypropionic acid	0.000	0.010	0.010	−15.023**	−15.012**	0.011
2-Methylglutaric acid	0.018	0.001	0.003	4.883	2.858	−2.025*
Lauric acid	0.000	0.021	0.026	−13.724*	−14.069*	−0.345
Isocitric acid	0.000	0.029	0.110	−14.231	−16.145*	−1.914
4-Hydroxycinnamic acid	0.000	0.299	0.323	−17.586**	−17.696**	−0.110
Pentadecanoic acid	0.000	0.005	0.005	−11.594**	−11.611**	−0.016
3,4-Dihydroxycinnamic acid	0.000	0.028	0.029	−14.160*	−14.197*	−0.037
Quinic acid	0.011	0.007	0.000	0.647*	15.988**	15.341**
Mannose	0.000	0.080	0.000	−15.677*	−5.911	9.766*
Melibiose	0.058	0.003	0.014	4.506**	2.047**	−2.459*
Cellobiose	0.000	0.031	0.039	−14.304**	−14.637**	−0.333*
Saccharic acid	0.025	0.217	0.035	−3.128*	−0.513	2.615*
2-Keto-L-gulonic acid	0.000	0.054	0.000	−15.127**	3.169	18.297**
Cytosine	0.000	0.000	0.158	3.211	−16.669**	−19.880**
Pyrogallol	0.000	0.000	0.008	3.211	−12.415*	−15.625*
Gluconic lactone	0.000	0.005	0.005	−11.753**	−11.543**	0.210*
Alpha-D-glucosamine-1-phosphate	0.000	0.361	0.000	−17.859*	3.169	21.028*
4-Methyl-5-thiazoleethanol	0.000	0.017	0.004	−13.414*	−11.367*	2.047*
Conduritol-β-epoxide	0.000	0.016	0.010	−13.323*	−12.753**	0.570
N-Acetyl-D-galactosamine	0.010	0.012	0.090	6.134	3.187	−2.947*
Purine riboside	0.018	0.000	0.012	16.703*	0.549	−16.154**
Phytosphingosine	0.005	0.001	0.003	2.503*	0.807	−1.696
Tetrahydrocorticosterone	0.000	0.001	0.002	−9.059	−10.500**	−1.440
Cholesterol	0.000	0.001	0.000	−9.500**	3.169	12.669**

*[1]The relative concentration of each metabolite is an average of data from three biological replicates using GC-TOF-MS. [2]The fold-changes were calculated using the formula log_2(X/Y). X and Y refer different treatments: C, control; P, Lactobacillus plantarum treatment; B, Lactobacillus buchneri treatment; *0.001 < P < 0.05; **P < 0.001. The major metabolites were selected based on at least one of fold-changes [log_2 (B/C), log_2 (P/C), log_2 (P/B)] contrast was statistically significant.*

L. farciminis, and diminished the relative abundance of *L. acetotolerans.*

Correlations Between the Relative Abundance of Bacteria and Metabolites of Ensiled Whole Crop Corn

Spearman correlations between the dominated bacteria and the metabolites that were differentially presented in ensiled samples within three treatments were calculated. This indicated perfect negative to perfect positive correlations (ranges from −1 to 1; *P*-values are shown as $^*0.01 < P \leq 0.05$, $^{**}P \leq 0.01$) for the 90-day fermented corn silages (**Figure 5**). Many of metabolites were positively correlated with LAB species, and were negatively correlated with undesirable bacteria presented during ensiling. Mannose was positively correlated with *L. farciminis*, *L. parafarraginis, L. odoratitofui, L. panis*, *L. futasaii* and unclassified *Lactobacillus,* but it was negatively correlated with unclassified *Sphingomonas* and unclassified *Chryseobacterium*. Generally, some end products of fermentation were positively correlated with some species of LAB but were negatively correlated with the other LAB species. For instance, 2-keto-L-gulonic acid and the health beneficial substance (alpha-D-glucosamine-1-phosphate) were positively correlated with *L. farciminis* and *L. panis*, whereas they were negatively correlated with *L. buchneri*, *L. kefiri* and undesirable bacteria.

The correlations between the dominant bacterial species and other metabolites with biological functions, which were not screened out as differential metabolites among treatments by using OPLS-DA analysis, were also analyzed by Spearman correlation analysis (**Figure 6**). The results exhibited that the *L. acetotolerans* positively correlated with 4-hydroxybutyrate, 3-phenyllactic acid and azelaic acid. Lauric acid positively correlated with *L. brevis*. In addition, most of metabolites with

TABLE 3 | Relative concentration and fold-changes in metabolites with biological functions in whole crop corn silage with inoculation of *L. buchneri* or *L. plantarum* after 90 days of ensiling.

	Relative concentration[1]			Fold-changes[2]		
Metabolite name	**Control**	***L. buchneri***	***L. plantarum***	**Log_2 (B/C)**	**Log_2 (P/C)**	**Log_2 (P/B)**
Phenylethylamine	0.179	0.153	0.101	0.227*	−0.831**	−0.604**
Catechol	0.037	0.020	0.024	5.336	−5.086	0.250
Linoleic acid	0.216	0.077	0.098	3.850*	−3.510*	0.340
Ferulic acid	0.465	0.196	0.242	2.369	−2.065	0.304
Myristic acid	0.042	0.024	0.028	0.801	−0.575	0.226
Azelaic acid	0.019	0.020	0.023	3.875	−3.709	0.167
Arachidonic acid	0.009	0.009	0.008	3.428	−3.628	−0.201*
3-Phenyllactic acid	0.412	0.706	0.875	−0.779*	1.089*	0.309*
3,4-Dihydroxybenzoic acid	0.409	0.247	0.085	0.728	−2.268*	−1.539**
4-Hydroxybenzoic acid	0.132	0.135	0.145	3.046	−2.943	0.103
4-Hydroxybutyrate	0.182	0.139	0.173	0.390	−0.072	0.318*
4-Aminobutyric acid	1.446	1.194	0.576	0.276	−1.327**	−1.051*
Glycolic acid	0.002	0.001	0.001	0.646	−2.417	−1.770

*[1]The relative concentration of each metabolite is an average of data from three biological replicates using GC-TOF-MS. [2]The fold-changes were calculated using the formula log_2 (X/Y). X and Y refer different treatments: C, control; P, Lactobacillus plantarum treatment; B, Lactobacillus buchneri treatment; *0.001 < P < 0.05; **P < 0.001. The major metabolites were selected based on at least one of fold-changes [log_2 (B/C), log_2 (P/C), log_2 (P/B)] contrast was statistically significant.*

TABLE 4 | Sequence and bacterial diversity estimation of fresh forage and experimental treatment groups.

Sample ID	Average length (bp)	Observed species	Chao1	Shannon	Simpson	Goods coverage
FM.1	1464	1007	2741.3	8.06	0.99	0.88
FM.2	1469	1314	4485.7	8.13	0.99	0.88
FM.3	1460	892	1935.4	7.59	0.99	0.92
C90.1	1499	267	437.0	1.09	0.21	1.00
C90.2	1501	200	538.7	1.89	0.59	1.00
C90.3	1486	350	717.1	1.49	0.40	0.99
P90.1	1498	355	701.2	3.10	0.75	0.99
P90.2	1500	386	891.6	1.79	0.39	0.99
P90.3	1500	493	895.2	2.45	0.57	0.99
B90.1	1504	349	640.2	2.30	0.56	0.99
B90.2	1504	448	1103.1	2.49	0.63	0.99
B90.3	1490	235	398.5	2.19	0.57	1.00

FM, fresh material; C, control; B, samples treated with L. buchneri; P, samples treated with L. plantarum; samples were fermented for 90 days. Goods coverage is calculated as C = 1 − (s/n), where s is the number of unique CCSs and n is the number of individuals in the sample. This index gives a relative measure of how well the sample represents the larger environment.

biological function were negatively correlated with LAB species. It is interesting that the correlation between metabolites and *L. acetotolerans* was diametrically opposite with the correlation between corresponding metabolites and *L. silagei*.

DISCUSSION

Ensiling is a complex process dominated by the epiphytic microbial community of LAB. Metabolomic and bacterial community analysis of silage fermentation system is proposed to have contributed important information on screening targeted LAB for modulating silage fermentation in order to make high-quality silage, and even for broadening silage function from nutritive value with perspective to being beneficial to animal health and welfare. This study, combining metabolomic and bacterial community analysis, for the first time revealed the characteristics of metabolome and bacterial community in whole crop corn silage inoculated by homofermentative *L. plantarum* and heterofermentative *L. buchneri*. Moreover, the present study also revealed that homofermentative and heterofermentative inoculants directed the fermentation process of whole crop corn silage with different manners.

Effects of Inoculants on Fermentation Characteristics in Whole Crop Corn Silage

Lactic acid bacteria are often inoculated into forage crops at ensiling for improving silage fermentation. Theoretical

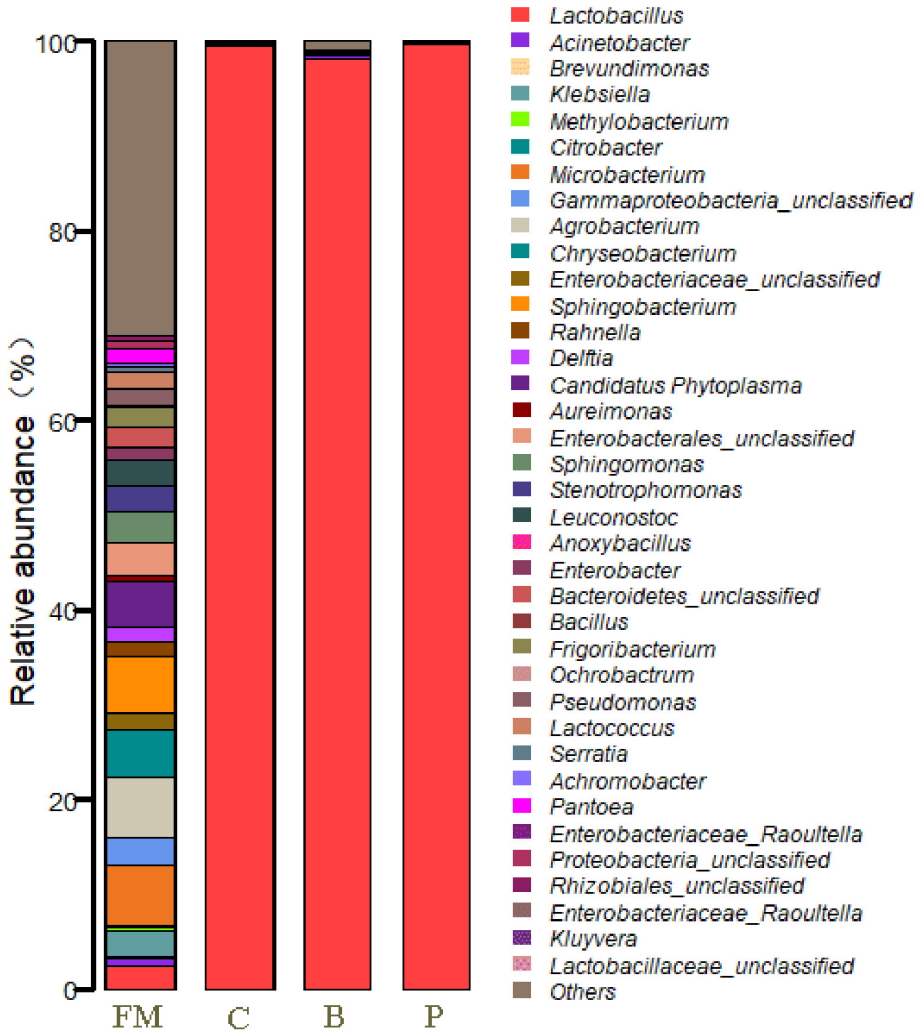


FIGURE 3 | Relative abundances of the corn silage bacterial community before and after fermentation with different treatments for different ensiled times at genus level. FM, fresh material; C, control group; B, samples treated with *L. buchneri*; P, samples treated with L. plantarum.

homofermentative LAB can rapidly reduce the pH of silage and help to preserve more forage mass, while heterofermentative LAB can produce much more acetic acid to improve aerobic stability. Fermentation parameters in the present study verified the theory. Compared with *L. plantarum*-inoculated samples, *L. buchneri*-treated samples had a higher pH and concentration of acetic acid, better aerobic stability and a lower concentration of lactic acid. There was no significance between the *L. plantarum*-inoculated and untreated group on parameters of fermentation characteristics. This study showed a similar effect of inoculation of *L. plantarum* and *L. buchneri* on forage fermentation quality to previous research (Ranjit and Kung, 2000; Weinberg et al., 2002).

Effects of Inoculants on Metabolomic Profiles in Whole Crop Corn Silage

Metabolome profiles of the present whole crop corn silage indicated that inoculation of the homofermentative *L. plantarum* or heterofermentative *L. buchneri* differently modulated the metabolite composition pattern during the ensiling. Over the past two decades, researches on metabolites in ensiled forages were mainly focused on the organic acids and 1, 2-propanediol in order to evaluate fermentation quality and aerobic stability of silages. Our results showed that samples treated with inoculants resulted in an up-accumulation of some free amino acids, which agrees with the results of previous studies on alfalfa silage (Guo et al., 2018). However, there was a big difference in metabolite types and compositions between the present whole crop corn silage and alfalfa silage previously studied (Guo et al., 2018). It might be due to there being a different microbial community present or due to the different fermentation that occurred in the various ensiled forage species. Some metabolites with antimicrobial activity were detected in the present study, such as 4-hydroxycinnamic acid, 3,4-dihydroxycinnamic acid, catechol, azelaic acid, 3-phenyllactic acid, 3,4-dihydroxybenzoic acid, 4-hydroxybenzoic acid, ferulic acid and glycolic acid. Among these metabolites, catechol, azelaic acid, 3-phenyllactic acid, 4-hydroxybenzoic acid and ferulic acid

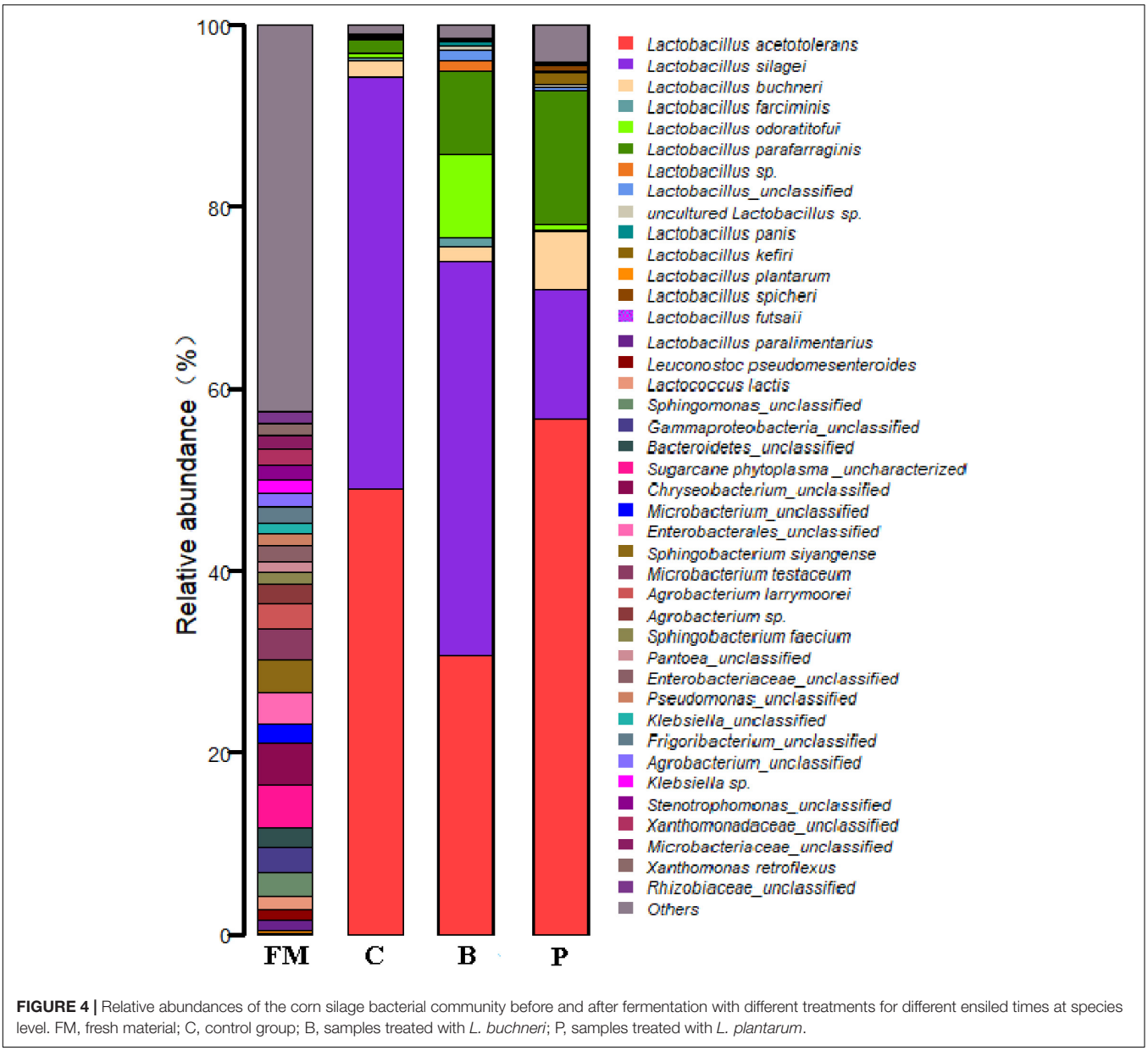


FIGURE 4 | Relative abundances of the corn silage bacterial community before and after fermentation with different treatments for different ensiled times at species level. FM, fresh material; C, control group; B, samples treated with *L. buchneri*; P, samples treated with *L. plantarum*.

had also been detected in grass silage, and higher catechol, azelaic acid, 3-phenyllactic acid and ferulic acid levels were observed in grass silages inoculated with LAB strains compared to the control silage (Broberg et al., 2007). In the present study, except for 4-hydroxycinnamic acid, 3,4-dihydroxycinnamic acid and 3-phenyllactic acid, no difference was observed in the other antimicrobial compounds between the control silage and inoculants treated silages. It might be because these metabolites with antimicrobial activity are produced by different bacteria, or because the interaction or competition with different microbes affected the end accumulation of these metabolites.

Some substances with other biological functions were detected in the ensiled whole crop corn silages after 90 days of fermentation. Catechol, pyrogallol and ferulic acid with antioxidant property, 4-hydroxybutyrate with nervine activity, and linoleic acid with cholesterol lowering effects, were discovered in the present study. In addition, a flavoring agent of myristic acid and a depression mitigation substance of PEA were also found in the whole crop corn silage. Previously, these metabolites had not been paid close attention to, nor their biofunctional activity in silages for animal health and welfare. As a non-protein amino acid in animals, 4-aminobutyric acid is a major inhibitory neurotransmitter and can decrease blood pressure (Pouliot-Mathieu et al., 2013). Our previous study showed that *L. buchneri* up-accumulated 4-aminobutyric acid in ensiled alfalfa (Guo et al., 2018), but the results from the present study indicated that *L. plantarum* resulted in a down-accumulation of 4-aminobutyric acid, and there

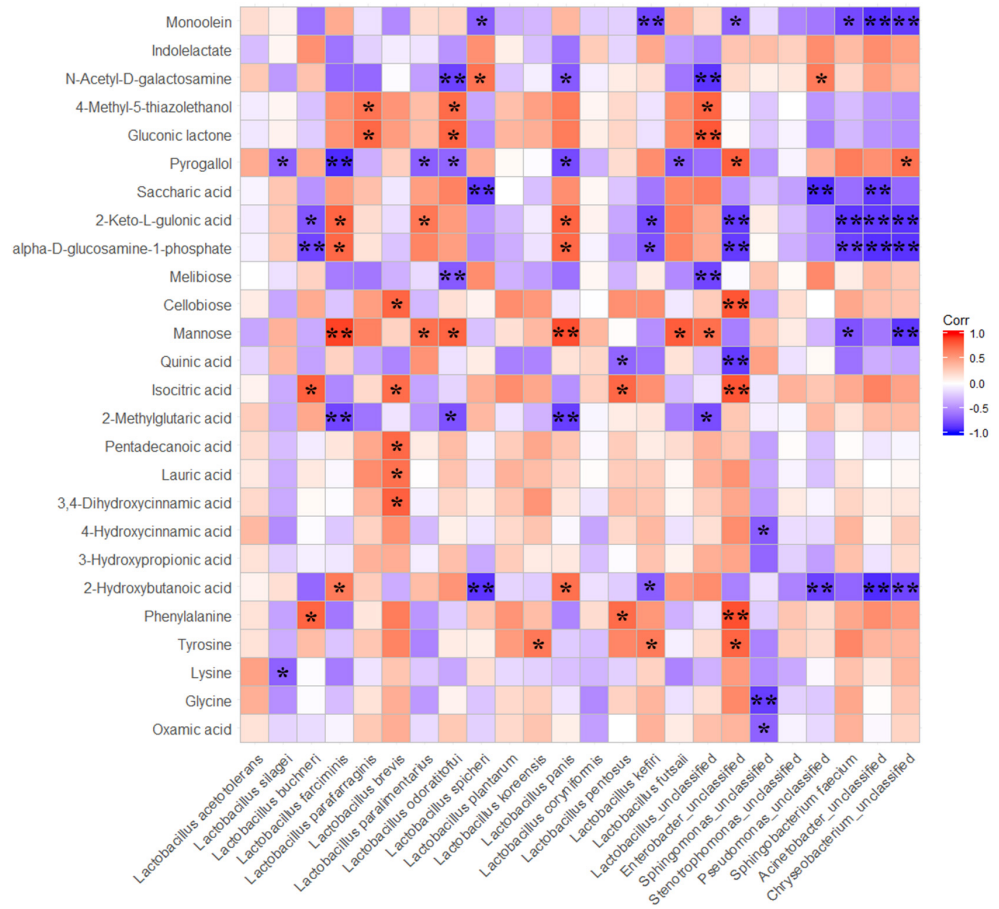


FIGURE 5 | Spearman correlations between main bacteria species and differentially presented metabolites. The differentially presented metabolites during fermentation were screened by OPLS-DA; P-values are shown as $^{*}0.01 < P \leq 0.05$, $^{**}P \leq 0.01$.

was no difference in 4-aminobutyric acid between the control and *L. buchneri*-treated silages. These results suggested that inoculants had different modulation manners in different forages with various epiphytic microorganisms at ensiling. However, numerous functional ingredients and flavoring agents were detected in the whole crop corn silages in the present study, which suggested that metabolomic analysis is an effective way to comprehensively evaluate the fermentative, nutritive, and functional profiles of ensiled forages. As for the detection method, however, we did not optimize the oven temperature ramp for metabolite analysis through the GC-TOF-MS system. Thus, it is necessary to explore an optimal temperature program suitable for analyzing metabolites in the fermentative system of silage.

Effects of Inoculants on Bacterial Microbiota in Whole Crop Corn Silage

It is well established that natural fermentation of forges depends on epiphytic microbial community especially LAB in anaerobic environment (Jones, 1991). In addition, various microbial community and succession were found in different pro- and after-ensiled forages (Parvin et al., 2010). Therefore, microbial community composition plays a crucial role in fermentation of ensiled forages, and it is necessary to know the community composition to understand the complex process of ensiling. In the present study, *Agrobacterium*, *Microbacterium*, *Sphingobacterium*, *Chryseobacterium*, *Candidatus Phytoplasma*, unclassified *Enterobacterales*, unclassified *Gammaproteobacteria* and a small proportion of LAB (2.69% *Leuconostoc* and 2.44% *Lactobacillus*) dominated the microbial composition in fresh whole crop corn before ensiling. While, the results of a previous report indicated that *Leuconostocaceae*, *Acetobacteraceae*, *Enterobacteriaceae*, *Moraxellaceae,* and *Lactobacillaceae* were the dominant bacteria in fresh whole crop corn before ensiling (Gharechahi et al., 2017). The differences between studies suggest that colonization of plant surfaces by bacteria depends on many factors including plant species, climate, period of duration, geographical location, solar radiation intensity and the type of fertilizer used (Cai et al., 1999a; Pang et al., 2011; Mcgarvey et al., 2013). After fermentation, most of the undesirable microorganisms were inhibited at anaerobic condition, which was confirmed well in the present study because *Lactobacillus* occupied over 98% in silages stored for 90 days. Furthermore, organic acids and metabolites with antimicrobial activity

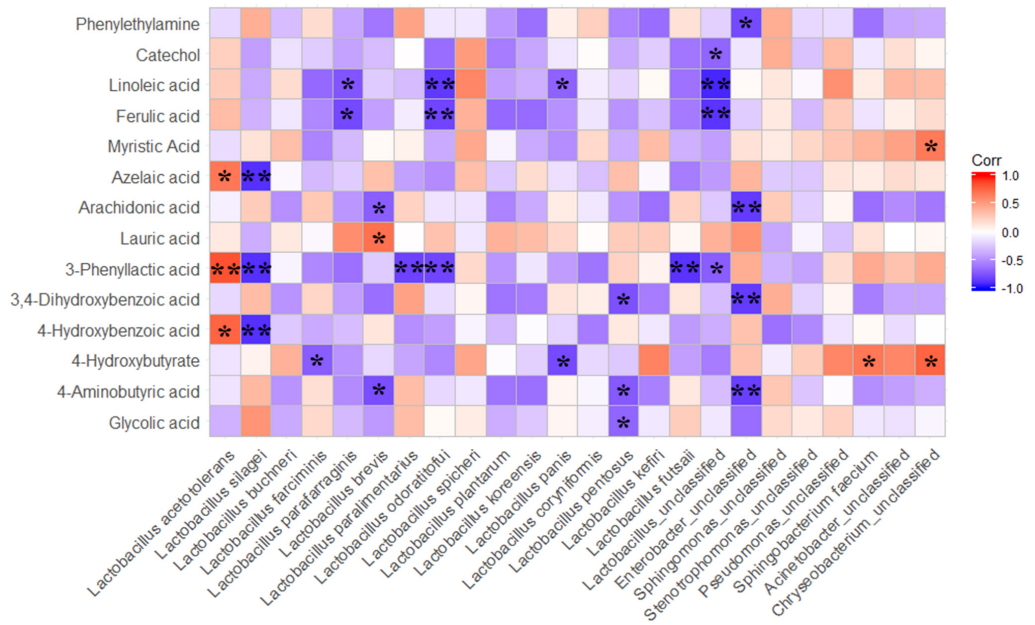


FIGURE 6 | Spearman correlations between metabolites with biofunctional activity and main bacteria species. The differentially presented metabolites during fermentation were screened by OPLS-DA; P-values are shown as *$0.01 < P \leq 0.05$, **$P \leq 0.01$.

produced by LAB inhibited the undesirable microbes during ensiling. The present study indicated that *L. acetotolerans*, *L. silagei*, *L. buchneri*, *L. odoratitofui*, *L. farciminis* and *L. parafarraginis* were the dominant microbial flora in the whole crop corn silage after 90 days of fermentation. Based on the previous studies, *L. acetotolerans* was detected in traditional pot fermentation of rice vinegar (Entani et al., 1986; Haruta et al., 2006); *L. farciminis* was isolated from fermenting mushroom, which exhibited potential for a bacteriocin and antibiotic assay (Halami et al., 2000), or was isolated from ensiled corn stover with feruloyl esterase (FAE) activities (Xu et al., 2017a); *L. Paralimentarius* was isolated from sourdough (Cai et al., 1999b); *L. odoratitofui* and *L. silagei* were isolated from orchardgrass silage or stinky tofu brine (Chao et al., 2010; Tohno et al., 2013); and *L. parafarraginis* was isolated from sudan-grass silage or corn stover silage (Liu et al., 2014; Xu et al., 2017b). However, these dominated LAB species had not been previously reported in the whole crop corn silage. It has been verified that *L. acetotolerans is* a homofermentative species resistant to high concentrations of acetic acid, while some physiological properties differ from other homofermentative species of the genus *Lactobacillus* (Entani et al., 1986). In the present study, the *L. buchneri*-treated group had a much greater abundance of *L. parafarraginis*. *Lactobacillus parafarraginis* has been reported to improve the aerobic stability of silages (Liu et al., 2014), which provided further evidence that inoculation of *L. buchneri* can improve aerobic stability of silage.

As for modulation of the bacterial community in the whole crop corn silage by inoculants, different microbial communities were observed in the control silage and silages treated with the two inoculants after 90 days of fermentation. In the control silage, *L. acetotolerans* and *L. silagei* were the most dominant LAB species, as they accounted for 93.2% of the total bacterial community: the relative abundances of *L. acetotolerans* and *L. silagei* were 48.9% and 45.3%, respectively. The microbial community in the *L. plantarum* treated silage was mainly comprised of *L. acetotolerans* (56.6%), *L. parafarraginis* (14.7%), *L. silagei* (14.2%), *L. buchneri* (6.4%), *L. kefiri* (1.3%), and *L. odoratitofui* (0.7%). In the *L. buchneri* inoculated silage, the microbial community mainly consisted of *L. silagei* (43.4%), *L. acetotolerans* (30.7%), *L. odoratitofui* (9.3%), *L. parafarraginis* (9.1%), *L. buchneri* (1.4%), and *L. farciminis* (0.97%). These results indicated that inoculation of *L. plantarum* markedly increased the relative abundances of *L. acetotolerans*, *L. buchneri*, and *L. parafarraginis*, but resulted in a considerable decline in the proportion of *L. silagei*; whereas an obvious decrease in *L. acetotolerans* and increases in *L. odoratitofui* and *L. farciminis* were observed in the *L. buchneri* inoculated silage. According to a previous study, *L. buchneri* inoculation did not alter the indigenous bacterial community in whole crop corn silage, and the *L. buchneri* was detected only as additions with DGGE (Li and Nishino, 2011b). Parvin et al. (2010) also studied the bacterial communities in whole crop maize silage inoculated with *L. buchneri* and *L. plantarum* by DGGE The results indicated that the bands of *L. lactic*, *Weissella paramesenteroides* and *Pediococcus pentosaceus* became faint after treatment with *L. plantarum*, and the band of *L. lactic* was extensive in *L. buchneri*-treated silage. Inoculation of *L. plantarum* promoted the growth of *L. buchneri* in the present whole crop corn silage, however, lower *L. buchneri* was observed in *L. buchneri* inoculated silage, which supports our previous results on alfalfa silage (Guo et al., 2018). Therefore, heterofermentative

L. buchneri and homofermentative *L. plantarum* both modulated silage fermentation in different ways as shown by the various microbial communities and metabolite composition in ensiled forage.

Correlations Between Main Bacterial Species and Metabolites of Whole Crop Corn Silage

To explore the correlations between main bacteria and the main metabolites, Spearman correlation analysis was performed. The results showed that metabolites were positively correlated with LAB species and were negatively correlated with undesirable bacteria presented during ensiling. These results suggested that LAB were more competitive than the undesirable bacteria in the airtight environment of silos. However, some end products of fermentation were positively correlated with some of LAB species but were negatively correlated with the other LAB species. This indicated that competition and synergy were concurrent between different species of bacteria during ensiling. Additionally, crop characteristics of dry matter content, sugar content, and sugar composition in combination with lactic acid bacterial properties, such as acid and osmotolerance, and substrate utilization, will decisively influence the competitiveness of the lactic acid bacterial flora during silage fermentation (Jones, 1991). After fermentation for 90 days, abundances of *L. acetotolerans* and *L. silagei* in total reached 70%. However, correlations between the two dominant species and those differentially presented metabolites were not significant, with the exception of the negative correlation between the two strains and the metabolites of pyrogallol and lysine. It suggested that the differences of metabolites between treatments probably did not result from the dominant species. However, there was a considerable difference in the abundance of *L. buchneri* and *L. odoratitofui* among the treatments, and a positive correlation between these two LAB species and some metabolites was also observed. Based on the positive correlation between *L. buchneri* and phenylalanine and the up-accumulation of phenylalanine in *L. plantarum*-treated samples, it can be inferred that *L. buchneri* could improve phenylalanine in ensiled forage. In addition, the up-accumulations of phenylalanine, lysine and tyrosine were also detected in the present whole crop corn silage inoculated with *L. buchneri*. All these results indicated that *L. buchneri* could produce some amino acids during fermentation of ensiled forages as it was suggested by our previous research on alfalfa silage (Guo et al., 2018). Higher abundance of *L. odoratitofui* in *L. buchneri*-treated samples was detected, which was positively correlated with mannose, gluconic lactone and 4-methyl-5-thiazoleethanol and was negatively correlated with 2-methylglutaric acid, melibiose, pyrogallol and *N*-acetyl-D-galactosamine. The results indicated that mannose, gluconic lactone and 4-methyl-5-thiazoleethanol were contributed by *L. odoratitofui*. Although a small proportion of *L. farciminis* was observed in *L. buchneri*-treated samples, the positive correlation between this species and metabolites showed that 2-hydroxybutanoic acid, mannose, 2-keto-L-gulonic acid and the health beneficial substance of alpha-D-glucosamine-1-phosphate were contributed by *L. farciminis*. Moreover, positive correlations between some observed metabolites and the minimal proportions of *L. brevis*, *L. paralimentarius*, *L. koreensis*, *L. panis*, *L. pentosus*, *L. kefiri* and *L. futsaii* indicated that these LAB species also played considerable roles in accumulation of those metabolites, in spite of their minute amounts. No significant correlation was observed between *L. plantarum* and all the detected metabolites because only a small proportion of *L. plantarum* was observed in *L. buchneri*-treated samples after 90 days of fermentation.

As for the correlations between bacterial species and metabolites with biofunctions, the result indicated that the correlation between the metabolites (4-hydroxybenzoic acid, 3-phenyllactic acid, azelaic acid) and *L. acetotolerans* was diametrically opposite with the correlation between the corresponding metabolites and *L. silagei*. The *L. plantarum*-inoculated samples presented a higher abundance of *L. acetotolerans* and a lower abundance of *L. silagei*, however, contrary results were observed in *L. buchneri*-treated samples. Correspondingly, *L. plantarum*-treated samples showed higher relative concentrations of 3-phenyllactic acid, 4-hydroxybutyrate and azelaic acid compared to the *L. buchneri*-treated samples. These results suggested that the antimicrobial compounds of 4-hydroxybenzoic acid, 3-phenyllactic acid and azelaic acid were probably produced by *L. acetotolerans*. Metabolites with biofunctions negatively correlated with LAB species, which might be a result of the concentrations in extracts of samples being too low to be detected accurately, yet still high enough to affect other microorganisms and animal health and welfare. However, correlation is not causation (Geer, 2011), it is based on statistics and correlates parameters to obtain a result that represents nothing more than speculation. It should also be noted that lack of a significant correlation between some bacterial taxa and metabolites in the correlation analysis does not necessarily mean those bacterial taxa or metabolites are not related. Furthermore, the functional features of a small number of species can also have a large impact on community structure and ecosystem functioning (Kruger Ben Shabat et al., 2016). Even so, the correlations between LAB and the metabolites with biological functions in the ensiled whole crop corn silage provided important information on screening targeted LAB for modulating silage fermentation in order to make high quality silage, and even for extending silage function from nutritive value with perspective to being beneficial to animal health and welfare. Therefore, the screening and application of inoculants for silage with secretion of biological substances, such as amino acids, small peptides, flavor agents, bacteriocin and other antibacterial agents (vitamins, polysaccharides, etc.), will be another prosperous research field on silage for future study.

CONCLUSION

Inoculation of *L. plantarum* and *L. buchneri* altered the microbial composition and fermentative metabolites in ensiled whole crop

corn silage in very different ways. Heterofermentative *L. buchneri* resulted in an up-accumulation of 2-hydroxybutanoic acid, saccharic acid, mannose, saccharic acid, 2-keto-L-gulonic acid and alpha-D-glucosamine-1-phosphate (a substance beneficial to health), and increased the abundances of the *L. silagei*, *L. odoratitofui*, *L. parafarraginis*, *L. farciminis* but diminished the abundance of *L. acetotolerans.* Homofermentative *L. plantarum* markedly increased the relative concentrations of pyrogallol and tetrahydrocorticosterone and increased the abundances of *L. acetotolerans*, *L. parafarraginis*, *L. buchneri*, but decreased the abundance of *L. silagei*. The correlations of metabolites and bacterial species revealed that dominant species *L. acetotolerans* and *L. silagei* did not result in the differences of metabolites. The LAB species which were markedly modulated by the two types of inoculants were closely correlated to some of metabolites. Thus, correlations between metabolites and bacterial species can provide important scientific information on screening targeted LAB for modulation of silage fermentation. Some new metabolites such as substances with bacteriostatic activity, antioxidant properties, nervine activities, cholesterol lowering effects or other flavoring agents were detected in the present whole crop corn silage. Some LAB species such as *L. acetotolerans*, *L. farciminis*, *L. Paralimentarius*, *L. odoratitofui* and *L. silagei* were discovered in the present whole crop corn silage. Therefore, profiling of silage microbiome and metabolome can improve our current understanding of the biological process underlying silage formation, and will be helpful to re-evaluate silage, with respect to nutritive value and fermentation quality but also to animal health and welfare.

AUTHOR CONTRIBUTIONS

DX and XG designed the study and wrote the manuscript. DX, WK, WD, FL, and PZ performed the experiments. DX and XG analyzed the data. All authors reviewed the manuscript.

FUNDING

This research was supported by the National Natural Science Foundation of China (31672487), the National Key R&D Program of China (Project Numbers: 2017YFD0502102-1, 2017YFE0104300) and the Fundamental Research Funds for the Central Universities of China (lzujbky-2017-ot08).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.03299/full#supplementary-material

FIGURE S1 | Alpha diversity index curves showing the diversity of taxa present in each sample [rarefaction curve (left) and rank abundance curve (right)]. D0.1–D0.3, whole crop corn before ensiling.

TABLE S1 | The relative concentration of 316 indentified metabolites in the control, *Lactobacillus plantarum*- and *Lactobacillus buchneri*-inoculated silages with triplicate for each treatment.

TABLE S2 | The contribution of 316 indentified metabolites to the first principal component (PC1) and standard error.

REFERENCES

Arasu, M. V., Jung, M., Ilavenil, S., Jane, M., Kim, D., Lee, K., et al. (2013). Isolation and characterization of antifungal compound from *Lactobacillus plantarum* KCC-10 from forage silage with potential beneficial properties. *J. Appl. Microbiol.* 115, 1172–1185. doi: 10.1111/jam.12319

Bao, W., Mi, Z., Xu, H., Zheng, Y., Kwok, L. Y., Zhang, H., et al. (2016). Assessing quality of *Medicago sativa* silage by monitoring bacterial composition with single molecule, real-time sequencing technology and various physiological parameters. *Sci. Rep.* 6:28358. doi: 10.1038/srep28358

Bokulich, N. A., and Mills, D. A. (2012). Next-generation approaches to the microbial ecology of food fermentations. *BMB Repo.* 45, 377–389. doi: 10.5483/BMBRep.2012.45.7.148

Broberg, A., Jacobsson, K., Ström, K., and Schnürer, J. (2007). Metabolite profiles of lactic acid bacteria in grass silage. *Appl. Enviro. Microbiol.* 73, 5547–5552. doi: 10.1128/AEM.02939-6

Brusetti, L., Crotti, E., Tamburini, A., Cittaro, D., Garavaglia, V., Rolli, E., et al. (2011). Influence of transgenic Bt176 and non-transgenic corn silage on the structure of rumen bacterial communities. *Annal. Microbiol.* 61, 925–930. doi: 10.1007/s13213-011-0215-8

Cai, Y., Kumai, S., Ogawa, M., and Benno, Y. (1999a). Characterization and identification of *Pediococcus* Species Isolated from forage crops and their application for silage preparation characterization and identification of *Pediococcus* Species isolated from forage crops and their application for silage prepa. *Appl. Environ. Microbiol.* 65, 2901–2906.

Cai, Y., Okada, H., Mori, H., Bennol, Y., and Nakase, T. (1999b). Lactobacillus paralimentarius sp. nov., isolated from sourdough. *Int. J. Syst. Bacteriol.* 49, 1451–1455. doi: 10.1099/00207713-49-4-1451

Chao, S. H., Sasamoto, M., Kudo, Y., Fujimoto, J., Tsai, Y. C., and Watanabe, K. (2010). Lactobacillus odoratitofui sp. nov., isolated from stinky tofu brine. *Int. J. Syst. Bacteriol.* 60, 2903–2907. doi: 10.1099/ijs.0.019307-0

Cleveland, J., Montville, T. J., Nes, I. F., and Chikindas, M. L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *Int. J. Food Microbiol.* 71, 1–20. doi: 10.1016/S0168-1605(01)00560-8

Dunn, W. B., Broadhurst, D., Begley, P., Zelena, E., Francis-mcintyre, S., Anderson, N., et al. (2011). Procedures for large-scale metabolic profiling of serum and plasma using gas chromatography and liquid chromatography coupled to mass spectrometry. *Nat. Protoc.* 6, 1060–1083. doi: 10.1038/nprot.2011.335

Elferink, S. J. W. H. O., Krooneman, J., Jan, C., Spoelstra, S. F., and Faber, F. (2001). Anaerobic conversion of lactic acid to acetic acid and 1, 2-Propanediol by *Lactobacillus buchneri* anaerobic conversion of lactic acid to acetic acid and. *Appl. Environ. Microbiol.* 67, 125–132. doi: 10.1128/AEM.67.1.125

Entani, E., Masai, H., and Suzuki, K. I. (1986). *Lactobacillus acetotolerans,* a new species from fermented vinegar broth. *Int. J. Syst. Evol. Microbiol.* 36, 544–549. doi: 10.1099/00207713-36-4-544

Geer, D. E. (2011). Correlation is not causation. *IEEE Secur. Priv.* 9, 93–94. doi: 10.1109/MSP.2011.26

Gharechahi, J., Kharazian, Z. A., Sarikhan, S., Jouzani, G. S., Aghdasi, M., and Hosseini Salekdeh, G. (2017). The dynamics of the bacterial communities developed in maize silage. *Microb. Biotechnol.* 10, 1663–1676. doi: 10.1111/1751-7915.12751

Guo, X. S., Ke, W. C., Ding, W. R., Ding, L. M., Xu, D. M., Wang, W. W., et al. (2018). Profiling of metabolome and bacterial community dynamics in ensiled *Medicago sativa* inoculated without or with *Lactobacillus plantarum* or *Lactobacillus buchneri*. *Scie. Repo.* 8, 357. doi: 10.1038/s41598-017-18348-0

Halami, P. M., Chandrashekar, A., and Nand, K. (2000). *Lactobacillus farciminis* MD, a newer strain with potential for bacteriocin and antibiotic assay. *Lett. Appl. Microbiol.* 30, 197–202. doi: 10.1046/j.1472-765x.2000.00691.x

Haruta, S., Ueno, S., Egawa, I., Hashiguchi, K., Fujii, A., Nagano, M., et al. (2006). Succession of bacterial and fungal communities during a traditional pot fermentation of rice vinegar assessed by PCR-mediated denaturing gradient gel electrophoresis. *Int. J. Food Microbiol.* 109, 79–87. doi: 10.1016/j.ijfoodmicro.2006.01.015

Hu, W., Schmidt, R. J., McDonell, E. E., Klingerman, C. M., and Kung, L. (2009). The effect of *Lactobacillus buchneri* 40788 or *Lactobacillus plantarum* MTD-1 on the fermentation and aerobic stability of corn silages ensiled at two dry matter contents. *J. Dairy. Sci.* 92, 3907–3914. doi: 10.3168/jds.2008-1788

Jones, D. J. C. (1991). The biochemistry of silage. *J. Agric. Sci.* 117, 386. doi: 10.1017/S0021859600067162

Kind, T., Wohlgemuth, G., Lee, D. Y., Lu, Y., Palazoglu, M., Shahbaz, S., et al. (2009). FiehnLib: mass spectral and retention index libraries for metabolomics based on quadrupole and time-of-flight gas chromatography/mass spectrometry. *Anal. Chem.* 81, 10038–10048. doi: 10.1021/ac9019522

Koc, F., Aksoy, S. O., Okur, A. A., Celikyurt, G., Korucu, D., and Ozduven, M. L. (2017). Effect of pre-fermented juice, *Lactobacillus plantarum* and *Lactobacillus buchneri* on the fermentation characteristics and aerobic stability of high dry matter alfalfa bale silage. *J. Anim. Plant Sci.* 27, 1766–1773.

Kruger Ben Shabat, S., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Berg Miller, M. E., et al. (2016). Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. *ISME J.* 10, 2958–2972. doi: 10.1038/ismej.2016.62

Kung, L., Shaver, R. D., Grant, R. J., and Schmidt, R. J. (2018). Silage review: interpretation of chemical, microbial, and organoleptic components of silages 1. *J. Dairy Sci.* 101, 4020–4033. doi: 10.3168/jds.2017-13909

Li, Y., and Nishino, N. (2011a). Bacterial and fungal communities of wilted Italian ryegrass silage inoculated with and without *Lactobacillus rhamnosus* or *Lactobacillus buchneri*. *Lett. Appl. Microbiol.* 52, 314–321. doi: 10.1111/j.1472-765X.2010.03000.x

Li, Y., and Nishino, N. (2011b). Effects of inoculation of *Lactobacillus rhamnosus* and *Lactobacillus buchneri* on fermentation, aerobic stability and microbial communities in whole crop corn silage. *Grass. Sci.* 57, 184–191.

Liu, Q. H., Yang, F. Y., Zhang, J. G., and Shao, T. (2014). Characteristics of *Lactobacillus parafarraginis* ZH1 and its role in improving the aerobic stability of silages. *J. Appl. Microbiol.* 117, 405–416. doi: 10.1111/jam.12530

Mayo, B., Rachid, C., Alegria, A., Leite, A., Peixoto, R., and Delgado, S. (2014). Impact of next generation sequencing techniques in food microbiology. *Curr. Genom.* 15, 293–309. doi: 10.2174/1389202915666140616233211

Mcgarvey, J. A., Franco, R. B., Palumbo, J. D., Hnasko, R., Stanker, L., and Mitloehner, F. M. (2013). Bacterial population dynamics during the ensiling of medicago sativa (alfalfa) and subsequent exposure to air. *J. Appl. Microbiol.* 114, 1661–1670. doi: 10.1111/jam.12179

Mosher, J. J., Bernberg, E. L., Shevchenko, O., Kan, J., and Kaplan, L. A. (2013). Efficacy of a 3rd generation high-throughput sequencing platform for analyses of 16S rRNA genes from environmental samples. *J. Microbiol. Methods* 95, 175–181. doi: 10.1016/j.mimet.2013.08.009

Ni, K., Minh, T. T., Tu, T. T. M., Tsuruta, T., Pang, H., and Nishino, N. (2017). Comparative microbiota assessment of wilted Italian ryegrass, whole crop corn, and wilted alfalfa silage using denaturing gradient gel electrophoresis and next-generation sequencing. *Appl. Microbiol. Biotechnol.* 101, 1385–1394. doi: 10.1007/s00253-016-7900-2

Pang, H., Qin, G., Tan, Z., Li, Z., Wang, Y., and Cai, Y. (2011). Natural populations of lactic acid bacteria associated with silage fermentation as determined by phenotype, 16S ribosomal RNA and recA gene analysis. *Syst. Appl. Microbiol.* 34, 235–241. doi: 10.1016/j.syapm.2010.10.003

Parvin, S., Wang, C., Li, Y., and Nishino, N. (2010). Effects of inoculation with lactic acid bacteria on the bacterial communities of Italian ryegrass, whole crop maize, guinea grass and rhodes grass silages. *Anim. Feed Sci. Technol.* 160, 160–166. doi: 10.1016/j.anifeedsci.2010.07.010

Pouliot-Mathieu, K., Gardner-Fortier, C., Lemieux, S., St-Gelais, D., Champagne, C. P., and Vuillemard, J.-C. (2013). Effect of cheese containing gamma-aminobutyric acid-producing lactic acid bacteria on blood pressure in men. *PharmaNutrition* 1, 141–148. doi: 10.1016/j.phanu.2013.06.003

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al. (2013). The Silva ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, 590–596. doi: 10.1093/nar/gks1219

Ranjit, N. K., and Kung, L. (2000). The effect of *Lactobacillus buchneri*, *Lactobacillus plantarum*, or a chemical preservative on the fermentation and aerobic stability of corn silage. *J. Dairy Sci.* 83, 526–535. doi: 10.3168/jds.S0022-0302(00)74912-5

Reich, L. J., and Kung, L. (2010). Effects of combining *Lactobacillus buchneri* 40788 with various lactic acid bacteria on the fermentation and aerobic stability of corn silage. *Anim. Feed Sci. Technol.* 159, 105–109. doi: 10.1016/j.anifeedsci.2010.06.002

Schloss, P. D., Jenior, M. L., Koumpouras, C. C., Westcott, S. L., and Highlander, S. K. (2016). Sequencing 16S rRNA gene fragments using the PacBio SMRT DNA sequencing system. *PeerJ* 4:e1869. doi: 10.7717/peerj.1869

Sjögren, J., Magnusson, J., Broberg, A., Schnürer, J., and Kenne, L. (2003). Antifungal 3-hydroxy fatty acids from *Lactobacillus plantarum* MiLAB 14 antifungal 3-hydroxy fatty acids from *Lactobacillus plantarum* MiLAB 14. *Appl. Enviro. Microbiol.* 69, 7554–7557. doi: 10.1128/AEM.69.12.7554

Stevenson, D. M., Muck, R. E., Shinners, K. J., and Weimer, P. J. (2006). Use of real time PCR to determine population profiles of individual species of lactic acid bacteria in alfalfa silage and stored corn stover. *Appl. Microbiol. Biotechnol.* 71, 329–338. doi: 10.1007/s00253-005-0170-z

Sun, M.-C., Li, A.-L., Huo, G.-C., and Meng, X.-C. (2012). Progress on the metabolomics of lactic acid bacteria. *Microbiol. Tongbao* 39, 1499–1505. doi: 10.1371/journal.pone.0190040

Tohno, M., Kitahara, M., Irisawa, T., Masuda, T., Uegaki, R., Ohkuma, M., et al. (2013). Lactobacillus silagei sp. nov., isolated from orchardgrass silage. *Int. J. Syst. Evol. Microbiol.* 63, 4613–4618. doi: 10.1099/ijs.0.053124-0

Weinberg, Z. G., Ashbell, G., Hen, Y., Azrieli, A., Szakacs, G., and Filya, I. (2002). Ensiling whole-crop wheat and corn in large containers with *Lactobacillus plantarum* and *Lactobacillus buchneri*. *J. Ind. Microbiol. Biotechnol.* 28, 7–11. doi: 10.1038/sj/jim/7000207

Xu, Z., He, H., Zhang, S., Guo, T., and Kong, J. (2017a). Characterization of feruloyl esterases produced by the four lactobacillus species: *L. amylovorus, L. acidophilus, L. farciminis and L. fermentum,* isolated from ensiled corn stover. *Front. Microbiol.* 8:941. doi: 10.3389/fmicb.2017.00941

Xu, Z., He, H., Zhang, S., and Kong, J. (2017b). Effects of inoculants *Lactobacillus brevis* and *Lactobacillus parafarraginis* on the fermentation characteristics and microbial communities of corn stover silage. *Sci. Rep.* 7:13614. doi: 10.1038/s41598-017-14052-1

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 20 February 2019
doi: 10.3389/fmicb.2019.00218

Metabolic Engineering of *Bacillus subtilis* Toward Taxadiene Biosynthesis as the First Committed Step for Taxol Production

Ingy I. Abdallah[1†], Hegar Pramastya[1,2†], Ronald van Merkerk[1], Sukrasno[2] and Wim J. Quax[1]*

[1]*Department of Chemical and Pharmaceutical Biology, Groningen Research Institute of Pharmacy, University of Groningen, Groningen, The Netherlands,* [2]*Pharmaceutical Biology Research Group, School of Pharmacy, Institut Teknologi Bandung, Bandung, Indonesia*

OPEN ACCESS

Edited by:
José E. Barboza-Corona, Universidad de Guanajuato, Mexico

Reviewed by:
Shawn Chen, Global Health Drug Discovery Institute, Tsinghua University, China
Hwan You Chang, National Tsing Hua University, Taiwan

***Correspondence:**
Wim J. Quax
w.j.quax@rug.nl

[†]These authors have contributed equally to this work

Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: *02 November 2018*
Accepted: *28 January 2019*
Published: *20 February 2019*

Citation:
Abdallah II, Pramastya H, van Merkerk R, Sukrasno and Quax WJ (2019) Metabolic Engineering of Bacillus subtilis Toward Taxadiene Biosynthesis as the First Committed Step for Taxol Production. Front. Microbiol. 10:218. doi: 10.3389/fmicb.2019.00218

Terpenoids are natural products known for their medicinal and commercial applications. Metabolic engineering of microbial hosts for the production of valuable compounds, such as artemisinin and Taxol, has gained vast interest in the last few decades. The Generally Regarded As Safe (GRAS) *Bacillus subtilis* 168 with its broad metabolic potential is considered one of these interesting microbial hosts. In the effort toward engineering *B. subtilis* as a cell factory for the production of the chemotherapeutic Taxol, we expressed the plant-derived taxadiene synthase (TXS) enzyme. TXS is responsible for the conversion of the precursor geranylgeranyl pyrophosphate (GGPP) to taxa-4,11-diene, which is the first committed intermediate in Taxol biosynthesis. Furthermore, overexpression of eight enzymes in the biosynthesis pathway was performed to increase the flux of the GGPP precursor. This was achieved by creating a synthetic operon harboring the *B. subtilis* genes encoding the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway (*dxs*, *ispD*, *ispF*, *ispH*, *ispC*, *ispE*, *ispG*) together with *ispA* (encoding geranyl and farnesyl pyrophosphate synthases) responsible for providing farnesyl pyrophosphate (FPP). In addition, a vector harboring the *crtE* gene (encoding geranylgeranyl pyrophosphate synthase, GGPPS, of *Pantoea ananatis*) to increase the supply of GGPP was introduced. The overexpression of the MEP pathway enzymes along with IspA and GGPPS caused an 83-fold increase in the amount of taxadiene produced compared to the strain only expressing TXS and relying on the innate pathway of *B. subtilis*. The total amount of taxadiene produced by that strain was 17.8 mg/l. This is the first account of the successful expression of taxadiene synthase in *B. subtilis*. We determined that the expression of GGPPS through the *crtE* gene is essential for the formation of sufficient precursor, GGPP, in *B. subtilis* as its innate metabolism is not efficient in producing it. Finally, the extracellular localization of taxadiene production by overexpressing the complete MEP pathway along with IspA and GGPPS presents the prospect for further engineering aiming for semisynthesis of Taxol.

Keywords: *Bacillus subtilis*, metabolite, MEP, GGPPS, taxadiene, Taxol

INTRODUCTION

Terpenoids represent the largest, structurally and functionally most varied group of natural products. This diversity is based on a structural complexity that cannot be simply reproduced using chemical synthetic processes. Nowadays, there are over 50,000 known terpenoids, a lot of which are biosynthesized by plants. Numerous terpenoids have attracted commercial interest for their medicinal value or use as flavors and fragrances. Among the most famous medicinally important terpenoids are the antimalarial artemisinin from the plant *Artemisia annua* and the anticancer paclitaxel (Taxol®) from the yew trees (*Taxus brevifolia* or *Taxus baccata*). The majority of terpenoids are naturally produced in low amounts and their extraction is usually labor intensive, and it entails considerable consumption of natural resources. For instance, the production of enough Taxol® to

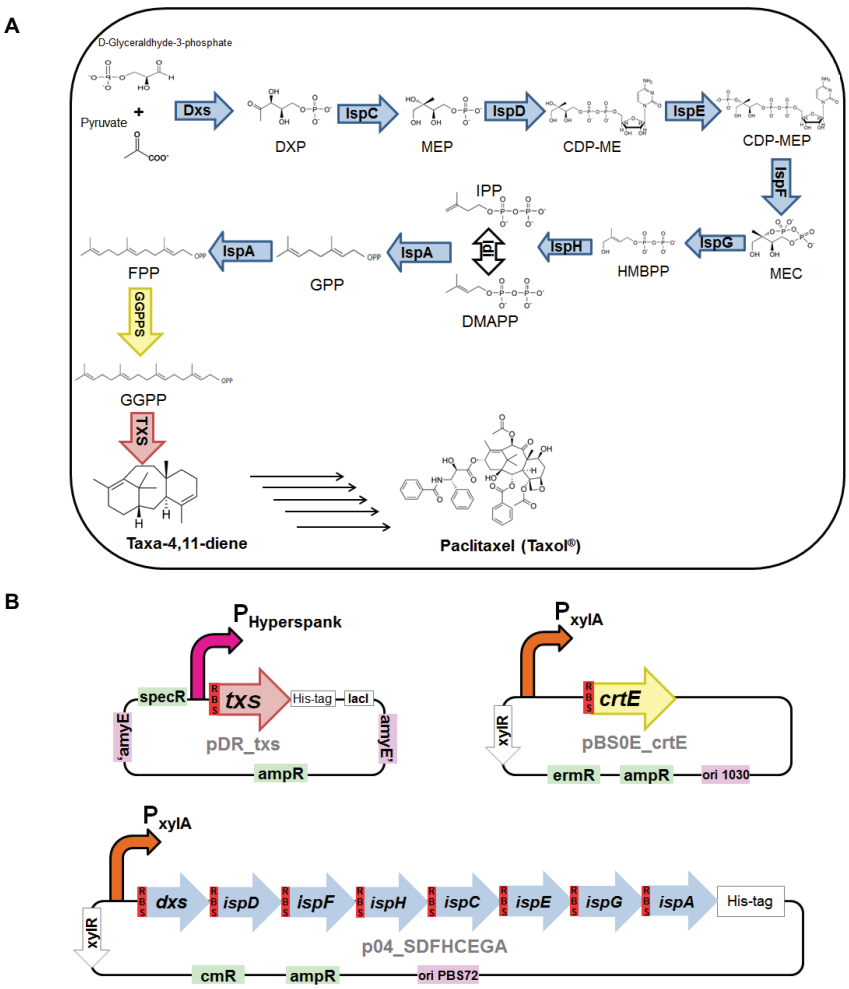


FIGURE 1 | (A) Biosynthesis of taxa-4,11-diene *via* the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway in *Bacillus subtilis*. **Intermediates in the metabolic pathway:** 1-deoxy-D-xylulose 5-phosphate (DXP), 2-C-methyl-D-erythritol 4-phosphate (MEP), 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol (CDP-ME), 2-phospho-4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol (CDP-MEP), 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MEC), (E)-4-hydroxy-3-methylbut-2-en-1-yl diphosphate (HMBPP), isopentenyl diphosphate (IPP), dimethylallyl diphosphate (DMAPP), geranyl pyrophosphate (GPP), farnesyl pyrophosphate (FPP), and geranylgeranyl pyrophosphate (GGPP). **Enzymes in the biosynthesis pathway:** 1-deoxy-D-xylulose-5-phosphate synthase (Dxs), 1-deoxy-D-xylulose-5-phosphate reductoisomerase, or 2-C-methyl-D-erythritol 4-phosphate synthase (Dxr, IspC), 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase (IspD), 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol kinase (IspE), 2-C-methyl-D-erythritol 2,4-cyclodiphosphate synthase (IspF), (E)-4-hydroxy-3-methylbut-2-enyl-diphosphate synthase (IspG), 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (IspH), isopentenyldiphosphate delta-isomerase (Idi), IspA which act as geranyl pyrophosphate synthase (GPPS) and farnesyl pyrophosphate synthase (FPPS), geranylgeranyl pyrophosphate synthase (GGPPS), and taxadiene synthase (TXS). **(B)** Plasmid constructs used for engineering *B. subtilis*. pDR_txs contains *txs* gene (red), preceded with *B. subtilis mntA* ribosomal binding site (dark red), to be inserted into the genome of *B. subtilis* between the *amyE* front flanking region and *amyE* back flanking region (purple), IPTG inducible hyperspank promoter (pink), and ampicillin and spectinomycin resistance cassettes (green). pBS0E_crtE contains *crtE* gene (yellow) encoding for GGPPS and preceded with *B. subtilis mntA* ribosomal binding site (dark red), xylose inducible promoter (orange), and ampicillin and erythromycin resistance cassettes (green). p04_SDFHCEGA contains seven genes of the MEP pathway, *dxs*, *ispD*, *ispF*, *ispH*, *ispC*, *ispE*, and *ispG*, along with the gene *ispA* (blue), each preceded with *B. subtilis mntA* ribosomal binding site (dark red), in a synthetic operon controlled by xylose inducible promoter (orange) and ampicillin and chloramphenicol resistance cassettes (green).

treat one cancer patient would approximately require six 100-year-old Pacific yew trees, and similarly, there are reports of enormous shortfalls in artemisinin production due to seed shortage. In addition, chemical synthesis and modification of most terpenoids is tremendously difficult and problematic because of the complexity and chirality of their chemical structures. Hence, researchers in the last few decades focused on metabolic engineering of the terpenoid biosynthetic pathways in host microorganisms as an alternate method of production (Connolly and Hill, 1991; Wagner et al., 2000; Koehn and Carter, 2005; Julsing et al., 2006; Klein-Marcuschamer et al., 2007).

The backbone of all terpenoids originates from 2 five-carbon precursors, isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP), which can be produced *via* the mevalonate (MVA) pathway or the 2-C-methyl-D-erythritol-4-phosphate (MEP) pathway. The consecutive condensation of IPP and DMAPP catalyzed by a group of prenyl pyrophosphate synthase enzymes produces the starting precursors of the different classes of terpenoids. These are (1) geranyl pyrophosphate (GPP; C_{10}) produced by geranyl pyrophosphate synthase (GPPS) for the synthesis of monoterpenoids, (2) farnesyl pyrophosphate (FPP; C_{15}) produced by farnesyl pyrophosphate synthase (FPPS) for the construction of sesquiterpenoids and triterpenoids, and (3) geranylgeranyl pyrophosphate (GGPP; C_{20}) synthesized by geranylgeranyl pyrophosphate synthase (GGPPS) for the production of diterpenoids and tetraterpenoids. Finally, these starting precursors are cyclized and/or rearranged by terpene synthase enzymes to yield the different terpenoids (Withers and Keasling, 2007; Muntendam et al., 2009; Abdallah and Quax, 2017).

Paclitaxel (Taxol®) is a diterpenoid known for its chemotherapeutic effect and is found in the bark and needles of different *Taxus* trees. Similar to all terpenoids, the extraction from the natural source is problematic, thus various *Taxus* species are now endangered due to high demand. Total synthesis of paclitaxel has been established, but the complexity of its chemical structure made the process commercially inapplicable (Nicolaou et al., 1994). Hence, nowadays paclitaxel is synthesized semisynthetically from 10-deacetylbaccatin III that is more easily extracted from *Taxus* needles. Also, docetaxel, which has been gaining more attention recently due to its higher water solubility leading to improved pharmacokinetic properties and better potency, can be synthesized from this precursor. However, this means that production still relies on the yew trees (Wuts, 1998; Baloglu and Kingston, 1999; Dewick, 2001). The first step in the production of paclitaxel is the production of the compound taxa-4,11-diene (**Figure 1A**). Taxadiene is produced from the cyclization of the diterpenoid precursor GGPP *via* the enzyme taxadiene synthase. The GGPP precursor can be synthesized *via* the MVA and/or the MEP pathway as previously explained. Taxadiene is converted to the final product, paclitaxel, through approximately 19 enzymatic steps involving hydroxylation and other oxygenation reactions of the taxadiene skeleton (Hezari and Croteau, 1997; Julsing et al., 2006; Abdallah and Quax, 2017).

The first committed intermediate in biosynthesis of paclitaxel, taxadiene, has been produced *via* metabolic engineering in *Escherichia coli* (Huang et al., 2001; Ajikumar et al., 2010), *Saccharomyces cerevisiae* (DeJong et al., 2006; Engels et al., 2008), and the transgenic plant *Arabidopsis thaliana* (Besumbes et al., 2004). Based on the success of taxadiene production in these hosts, *Bacillus subtilis* represents an interesting microbial host for the production of taxadiene where it has higher growth rate compared to *S. cerevisiae* and is mostly considered as GRAS (Generally Regarded As Safe) by the Food and Drug Administration unlike *E. coli* (Zhou et al., 2013). Also, *B. subtilis* possesses an innate MEP pathway that can be manipulated to increase the flux of precursors. An optimally regulated synthetic operon encompassing MEP pathway genes has been reported to lead to a high production of C_{30} carotenoids in *B. subtilis* (Xue et al., 2015). Also, the sesquiterpenoid amorphadiene, which is the first precursor for the production of artemisinin, has been successfully produced in *B. subtilis* (Zhou et al., 2013). In the current study, we aim at the metabolic engineering of *B. subtilis* for the biosynthesis of taxadiene as a first step in the semisynthetic production of paclitaxel. For the first time, we describe the successful expression of the enzyme taxadiene synthase essential for the synthesis of taxadiene in *B. subtilis*. Moreover, the production levels of taxadiene were increased by overexpression of the MEP pathway, IspA, and GGPPS enzymes. The reported *B. subtilis* strain with the highest level of production of taxadiene can strive to exceed yeast and *E. coli*, besides having the additional advantages provided from the use of *B. subtilis*. This study can serve as a stepping stone for further fine tuning of the biosynthetic pathway of paclitaxel in *B. subtilis* targeted at a sustainable and efficient production process.

MATERIALS AND METHODS

Bacterial Strains, Vectors, and Growth Conditions

Bacterial strains and expression vectors used in this research are listed in **Table 1**. *E. coli* DH5α strains were cultured in Luria-Bertani broth (LB), while *B. subtilis* 168 strains were grown in 2xYT medium. When necessary, growth media were supplemented with antibiotics in the following concentrations: 100 μg/ml ampicillin or 100 μg/ml erythromycin for *E. coli* DH5α and 5 μg/ml chloramphenicol, 10 μg/ml erythromycin, or 10 μg/ml spectinomycin for *B. subtilis* 168.

Construction of Different Strains of *B. subtilis* 168

Three different constructs were utilized to produce different *B. subtilis* strains expressing taxadiene synthase (TXS). The first construct consists of *txs* gene from the plant *Taxus baccata* in pDR111 plasmid. The *txs* gene was truncated by deleting the first 60 amino acids to remove the signal peptide targeting the plastid in order to improve expression and solubility of the protein (Williams et al., 2000). Circular polymerase extension cloning (CPEC) (Quan and Tian, 2011) was used to create the pDR_txs construct

TABLE 1 | Bacterial strains and vectors used in this research.

Bacterial strain	Genotype	Reference
B. subtilis 168	*trp*C2	(Kunst et al., 1997; Barbe et al., 2009)
B. subtilis 168_txs	168 *amyE*::$P_{hyperspank}$-*txs*; Sp^R	This study
E. coli DH5α	F^-*end*A1 *hsd*R17 (r_k^-,m_k^+) *sup*E44 *thi*$^-$1 λ$^-$*rec*A1 *gyr*A96 *rel*A1 φ80d*lac*ZΔM15	Bethesda Research Lab 1986
Vector	**Pertinent properties**	**Reference**
pDR111	*B. subtilis* integration vector; ori-pBR322; $P_{hyperspank}$ IPTG-inducible promoter; Sp^R; Amp^R	(Overkamp et al., 2013)
pBS0E	*B. subtilis* and *E. coli* shuttle vector; ori-1030 (theta replication); copy number ~15–25; P_{xylA} xylose-inducible promoter; Erm^R; Amp^R	(Popp et al., 2017)
pHCMC04G	*B. subtilis* and *E. coli* shuttle vector; ori-pBS72 (theta replication); copy number ~6; P_{xylA} xylose-inducible promoter; Cm^R; Amp^R	(Xue et al., 2015)

(**Figure 1B**) where *B. subtilis mntA* ribosomal binding site (RBS) plus spacer (AAGAGGAGGAGAAAT) were introduced before the *txs* gene along with a N-terminal 6× His-tag. Ampicillin and spectinomycin resistance cassettes are available for selection in *E. coli* and *B. subtilis*, respectively. The expression of TXS is controlled by IPTG inducible hyperspank promoter. The second construct consists of the *crtE* gene, encoding the GGPPS of *Pantoea ananatis*, together with the *mntA* RBS preceding the coding region. The gene was cloned into pBS0E plasmid containing xylose inducible promoter using CPEC method resulting in the construct pBS0E_crtE (**Figure 1B**). Finally, a construct expressing MEP pathway genes in pHCMC04G plasmid was used. The construct p04_SDFHCEGA (**Figure 1B**) expresses all the seven genes of the MEP pathway, *dxs*, *ispD*, *ispF*, *ispH*, *ispC*, *ispE*, and *ispG*, along with the gene *ispA*, each with its own engineered RBS, in one synthetic operon controlled by a xylose inducible promoter. p04_SDFHCEGA (16.4 Kb) was cloned using previously available constructs, p04_SDFH and p04_CEGA, by applying circular polymerase extension cloning (CPEC) (Quan and Tian, 2011; Xue et al., 2015). All these cloning steps were performed in *E. coli* DH5α, and the sequences of all the generated recombinant plasmids were confirmed by sequencing (Macrogen, Europe). The constructed plasmids were used to transform competent *B. subtilis* 168 cells following previously published protocol (Kunst and Rapoport, 1995). Three different strains of *B. subtilis* 168 were produced by transforming different combinations of the constructs.

Expression of Taxadiene Synthase in *B. subtilis* 168

Overnight culture of the *B. subtilis* strain containing pDR_txs construct was grown in 2xYT medium containing spectinomycin antibiotic. The next day, the overnight culture was diluted to an OD_{600} of 0.07–0.1 in 10 ml 2xYT medium with spectinomycin in 100-ml Erlenmeyer flask. The culture was incubated for 3 h at 37°C and 220 rpm till OD_{600} of 0.7–0.9. Then, IPTG was added to a final concentration of 1 mM to start induction. The culture was divided into 1 ml cultures in 15 ml round bottom tubes and then grown overnight at 37, 30, or 20°C and 220 rpm. The following day, certain volumes of the cultures normalized to the OD_{600} = 1 were pelleted by centrifugation for 10 min at 11,000 rpm, 4°C and then resuspended in Birnboim lysis buffer (25 mM Tris-HCl, pH 8.0, 50 mM glucose, cOmplete™ Protease Inhibitor Cocktail tablet from Sigma, and 25 mg/ml lysozyme) using an amount of 5 ml buffer per 1 g pellet and incubated at 37°C for 30 min. The soluble protein fractions were obtained by centrifugation for 15 min at 11,000 rpm. The total protein concentration in each soluble protein fraction was estimated using NanoDrop® spectrophotometer. The samples from the expression experiment at different temperatures were loaded with the same total protein concentration on NuPAGE® gels (Invitrogen) for separation by SDS-PAGE and then analyzed by Western blotting where mouse peroxidase-conjugated anti-His antibody (catalog no. A7058; Sigma) was used followed by visualization using Amersham ECL Prime Western blotting detection reagent (catalog no. RPN2232; GE Healthcare).

Production and Extraction of Taxadiene in *B. subtilis* 168

All strains of *B. subtilis* 168 were grown with the suitable antibiotics using the abovementioned protocol for expression. In addition to IPTG, xylose was added at OD_{600} of 0.7–0.9 to a final concentration of 1% to start induction of pBS0E and pHCMC04G constructs in txs + crtE and txs + crtE + SDFHCEGA *B. subtilis* strains. After which the cultures were divided into 1 ml cultures in 15 ml round bottom tubes and overlaid with 100 µl dodecane containing 10 µl of 700 µM *β*-caryophyllene as internal standard and then grown overnight (24 h) at 37, 30, or 20°C with 220 rpm shaking. The dodecane layer was added to trap the taxadiene released by the cells. The 24-h time point was chosen to ensure maximum production of taxadiene (Zhou et al., 2013). The following day OD_{600} of all 1 ml cultures was measured. A comparison between taxadiene production at the different incubation temperatures along with the difference between direct extraction of taxadiene from the culture and extraction of taxadiene from cell lysate was performed. First, extraction without lysis was performed by adding 200 µl hexane to the 1 ml cultures overlaid with dodecane (1:3 dilution of dodecane), and then the cultures were centrifuged for 10 min at 11,000 rpm to separate the aqueous and organic phases. The dodecane-hexane layer was extracted for GC-MS analysis. Secondly, lysis followed by

extraction was performed by addition of 100 µl lysis buffer (50 mM Tris-HCl, pH 8.0, 70 mM NaCl, 10 mM $MgCl_2$, 25 mg/ml lysozyme, and 0.1 mg/ml Dnase) to the 1 ml cultures overlaid with dodecane and incubation at 37°C for 30 min. Then, 200 µl 12% SDS (sodium dodecyl sulfate) was added. Finally, 200 µl hexane was mixed (1:3 dilution of dodecane), and the cultures were centrifuged for 10 min at 11,000 rpm. The dodecane-hexane layer was extracted for GC-MS analysis and comparison to the extract without lysis. All cultures and extractions were performed in triplicates.

Quantification of the Sampled Taxadiene

The dodecane-hexane samples were analyzed on an HP-5MS (5% Phenyl)-methylpolysiloxane column (Agilent J&W 0.25 mm inner diameter, 0.25 µm thickness, 30 m length) in a Shimadzu GCMS-QP5000 system equipped with a 17A gas chromatograph (GC) and AOC-20i autoinjector. The samples (2 µl) were injected splitless onto the GC column, and helium was used as the carrier gas. The injector temperature was 250°C, and the oven initial temperature was 100°C with an increase of 15°C per minute up to 130°C and then 5°C per minute till 210°C. After 210°C was reached, the temperature was raised to 280°C with an increase of 35°C per minute and held for 2 min. The solvent cutoff was 8 min. The MS instrument was set to selected ion mode (SIM) for acquisition, monitoring *m*/*z* ion 122 for taxadiene and *β*-caryophyllene. The chromatographic peak areas for taxadiene and *β*-caryophyllene were determined using the integration tools in GCMSsolution 1.20 software (Shimadzu, Den Bosch, The Netherlands). A calibration curve of standard *β*-caryophyllene with concentration range of 0.5–28 mg/L was created based on chromatographic peak areas in SIM mode (*m*/*z* ion 122). For quantification of taxadiene, the peak area for each sample was corrected by using the peak corresponding to the internal standard *β*-caryophyllene (i.e., by multiplication of the taxadiene peak area for the sample by the peak area of reference *β*-caryophyllene, then division by the *β*-caryophyllene peak area of the sample). This correction is to avoid errors due to injection or loss during extraction. The taxadiene concentration in the diluted dodecane sample was calculated by applying the linear regression equation resulting from the calibration curve to each adjusted taxadiene peak area. Finally, the dilution factor was applied to calculate the concentration of taxadiene in the neat dodecane phase, and then this value was divided by 10 to determine the amount of taxadiene produced per liter of culture as the dodecane layer constitutes a second phase (10%) in the culture. The taxadiene concentration obtained for each sample was divided by the OD_{600} of the corresponding culture to calculate the specific taxadiene production value (mg/L/OD_{600}) *β*-caryophyllene equivalent (Rodriguez et al., 2014).

Analysis of Segregational Stability of the Constructs in txs + crtE + SDFHCEGA *B. subtilis* Strain

Segregational stability was measured by evaluating the growth of *B. subtilis* 168 strain harboring the pDR_txs, pBS0E_crtE, and p04_SDFHCEGA constructs in 2xYT medium without antibiotics for 30 generations involving several subcultures by adapting a previously reported protocol (Shao et al., 2015). The cells of *B. subtilis* 168 were first grown in 2 ml 2xYT broth containing 10 µg/ml spectinomycin, 10 µg/ml erythromycin, and 5 µg/ml chloramphenicol to select the different constructs, respectively. The overnight cultures were inoculated into 2 ml fresh 2xYT broth without antibiotics and incubated at 37°C for 24 h, attaining full growth. The cultures were diluted 1:1,000 by fresh 2xYT broth without antibiotics and further incubated for 24 h where they were grown for the first 3 h at 37°C and then the temperature was reduced to 20°C to resemble the best conditions used for expression and production of taxadiene (growth of 1:1,000 dilution accounts for about 10 generations of cultivation, 2^{10} = 1,024). These cultures were diluted 10^6 fold and plated onto 2xYT agar plates without antibiotics. Next day, 100 colonies were picked and transferred onto four different 2xYT agar plates supplemented with 10 µg/ml spectinomycin, 10 µg/ml erythromycin, 5 µg/ml chloramphenicol, or a combination of the three antibiotics. This treatment was successively repeated three times to obtain 30 generations of cultivation. The presence of the constructs was confirmed by the growth of the colonies on the antibiotic plates corresponding to the antibiotic resistance gene in each plasmid, thus indicating that the plasmid hosted by the colonies is segregationally stable. The whole experiment from beginning to end was performed in duplicate. The segregational stability of each construct was represented as the average of the % of colonies retaining the plasmid construct, which is equal to [colonies on 2xYT plate with antibiotic/colonies on 2xYT plate without antibiotic * 100%].

Nucleotide Sequence Accession Number

The nucleotide sequence of the taxadiene synthase gene from the plant *T. baccata* was previously reported with the accession number: AY424738. The nucleotide sequence of the complete genome of *P. ananatis* was previously reported with the accession number: FUXY01000004. The *crtE* gene encoding the GGPPS enzyme was amplified from genomic DNA of *P. ananatis*, and the protein was assigned the accession number: SKA77365. The nucleotide sequence of the complete genome of *B. subtilis* 168 was previously reported with the following accession numbers: AL009126 and NC000964. The MEP pathway genes used in this study were amplified from the genomic DNA of *B. subtilis* 168.

RESULTS

Construction of Different Strains of *B. subtilis* 168 Expressing Taxadiene Synthase

The first construct to be cloned was pDR_txs where the taxadiene synthase gene was cloned into pDR111 plasmid and then transformed into *B. subtilis*. This construct is designed to integrate the plant *txs* gene from *T. baccata* into the genome of *B. subtilis* where it is inserted into the *amyE* locus between the *amyE* front flanking region and *amyE* back flanking region (Overkamp et al., 2013). The *txs* gene expresses the enzyme taxadiene synthase,

TABLE 2 | *Bacillus subtilis* 168 strains generated in this study.

Strain	Constructs	Vectors	Genes in the operon
txs	pDR_txs	pDR111	*txs*
txs + crtE	pDR_txs	pDR111	*txs*
	pBS0E_crtE	pBS0E	*crtE*
txs + crtE + SDFHCEGA	pDR_txs	pDR111	*txs*
	pBS0E_crtE	pBS0E	*crtE*
	p04_SDFHCEGA	pHCMC04G	*dxs + ispD + ispH + ispF + ispC + ispE + ispG + ispA*

which is responsible for converting GGPP into taxa-4,11-diene. The expression of TXS from this construct is controlled by an engineered *B. subtilis mntA* ribosomal binding site and the strong IPTG inducible hyperspank promoter. The second construct produced was pBS0E_crtE, which is resulting from cloning the *crtE* gene from *P. ananatis* that encodes the GGPPS enzyme into pBS0E replicative plasmid. The GGPPS enzyme is responsible for the production of GGPP, which is the precursor of taxadiene. The pBS0E_crtE construct was transformed into *B. subtilis* strain containing pDR111_txs generating the txs + crtE strain. Finally, the construct overexpressing all genes of the MEP pathway along with *ispA* in one operon controlled by a xylose inducible promoter (p04_SDFHCEGA) was transformed into the txs + crtE strain generating the txs + crtE + SDFHCEGA *B. subtilis* strain. The aim was to increase the production of the precursors leading to the formation of taxadiene. All produced strains are listed in **Table 2**. The successful transformation of the constructs into the different *B. subtilis* strains was confirmed by colony PCR.

Expression of Taxadiene Synthase in *B. subtilis* 168

The taxadiene gene used is the natural gene from the plant *T. baccata* and was combined with the *B. subtilis mntA* RBS and a N-terminal 6× His-tag. pDR111 plasmid was chosen to integrate the *txs* gene into the genome of *B. subtilis*. The cell culture was lysed, and in the soluble protein fraction, the expression of TXS (approximately 89 kDa) has been clearly detected on Western blot by using specific antibodies against the His-tag as shown in **Figure 2**. Different growth temperatures (37, 30 and 20°C) were tried to determine the best temperature for the expression of TXS using the pDR_txs construct following induction by IPTG. Since the samples from expression at different temperatures were loaded with the same total protein concentration on SDS-PAGE gel, a comparison of the TXS band can determine the temperature resulting in the best expression. A clear band representing TXS was seen on Western blot (**Figure 2**) following expression at 20°C, while a very faint band was the result of expression at 30°C and no TXS band was visible after expression at 37°C. Hence, the temperature that showed best TXS expression upon induction was 20°C. This will later be corroborated by the level of taxadiene production after incubation at the different temperatures.

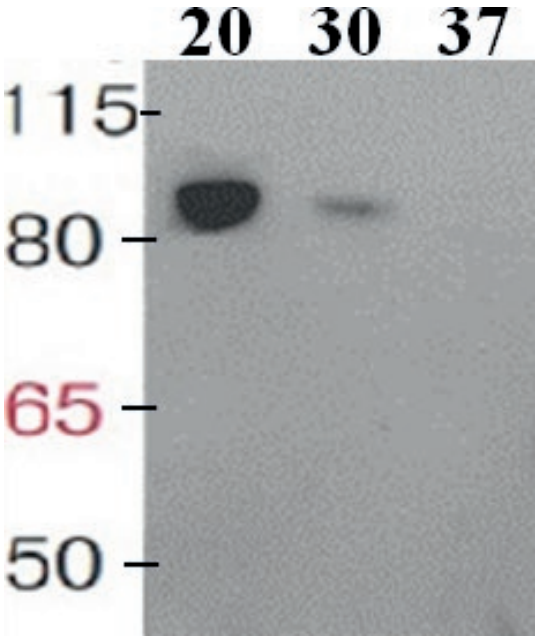


FIGURE 2 | Western blot of the soluble protein fraction showing taxadiene synthase expressed in *Bacillus subtilis* 168 after integration into the chromosome using pDR111 plasmid. The lanes show TXS bands (~89 kDa) resulting from expression at different temperatures as follows: 20, 30, and 37°C. The enzyme was visualized by using specific antibodies against the His-tag.

Level of Production of Taxadiene in Different Strains of *B. subtilis* 168

The OD_{600} of the *B. subtilis* strains ranged from 7 to 9. The produced taxadiene was detected using GC-MS. The GC-MS chromatograms of the different *B. subtilis* strains showed the internal standard β-caryophyllene peak at retention time 9.25 min. and the taxadiene peak at retention time 20.05 min (**Figure 3A**). The SIM mode was used to monitor *m*/*z* ion 122 in the mass spectrum of both β-caryophyllene (**Figure 3B**) and taxadiene (**Figure 3C**). The peak areas were calculated and used to determine the concentration of produced taxadiene. The total amount of taxa-4,11-diene (mg/L/OD_{600}) produced in the *B. subtilis* strains at the different incubation temperatures was compared (**Figure 4**). The highest level of taxadiene production was observed after incubation at 20°C, which coincides with the best expression of TXS. After that, the effect of extraction of taxadiene with and without cell lysis was also compared in the different strains when incubated at 20°C (**Figure 5**). The txs strain, which only contains the pDR_txs construct, relies on the innate MEP pathway, IspA (acting as GPPS and FPPS), and GGPPS for the production of taxadiene in *B. subtilis*. It showed the lowest production of taxadiene (0.024 mg/L/OD_{600}) and was used as a control to compare the effect of overexpression of other genes on the production level. Introduction of *crtE* gene, encoding GGPPS enzyme, in the txs + crtE strain significantly increased the amount of taxadiene produced (0.471 mg/L/OD_{600}), which is around 20 times higher than the control strain. This is probably due to the increased production

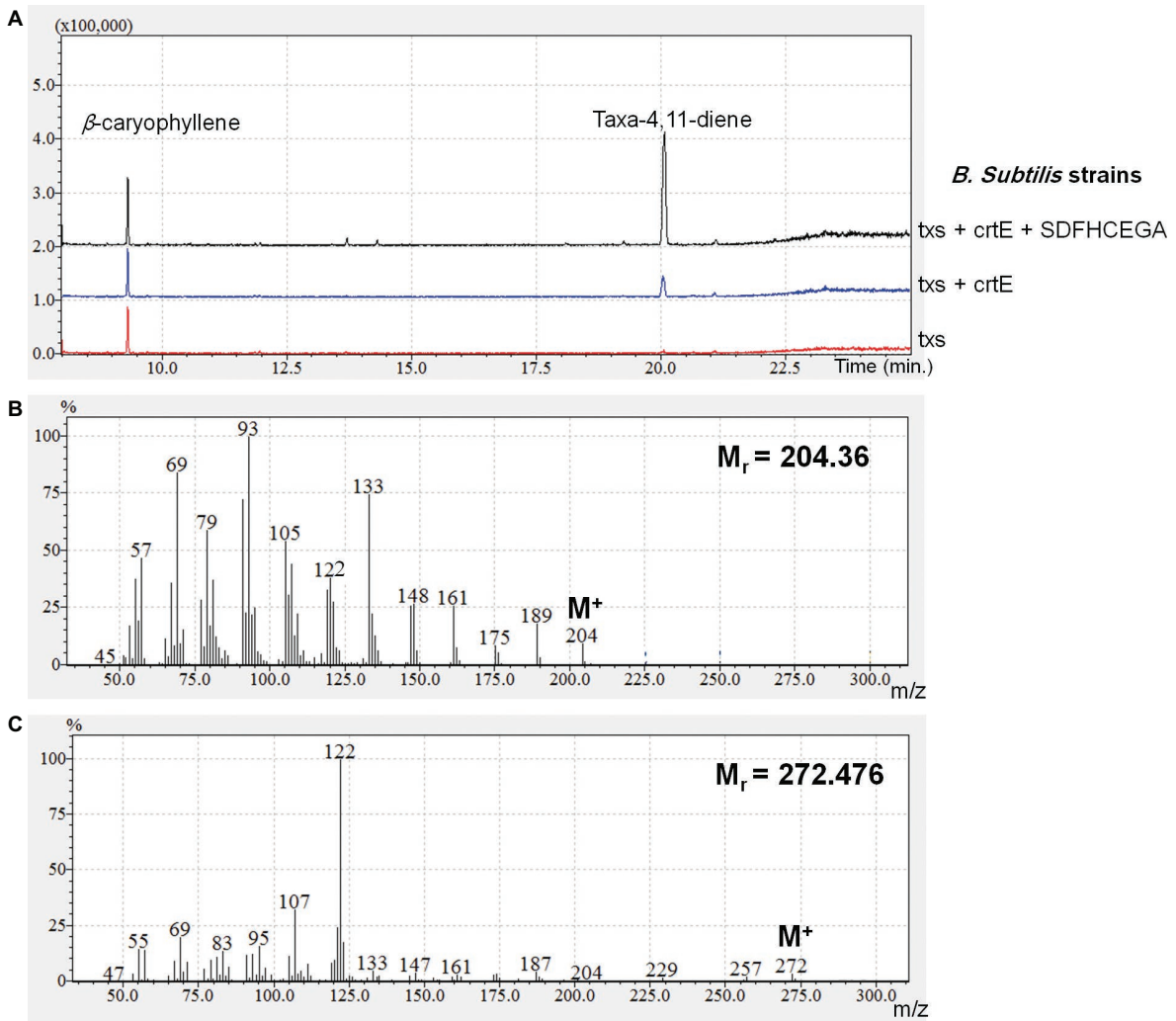


FIGURE 3 | (A) GC chromatograms of the different strains of *Bacillus subtilis* in selected ion mode (SIM) for acquisition, monitoring m/z ion 122 to show the internal standard β-caryophyllene and the produced taxadiene peaks. **(B)** Mass spectrum of β-caryophyllene. **(C)** Mass spectrum of taxa-4,11-diene.

of the precursor GGPP. Finally, combining the txs + crtE strain with overexpression of the MEP pathway genes and the *ispA* gene showed much higher amounts of taxadiene with the strain expressing all the genes showing the highest production levels (1.981 mg/L/OD_{600}), which is approximately 83 times higher than the control strain. In addition, cell lysis before extraction did not show significant increase in the production levels compared to extraction without lysis (**Figure 5**). This indicates that taxadiene is released from the cells into the dodecane layer during growth. In fact, the omission of a cell lysis treatment resulted in a reduced contamination of the taxadiene due to the lysis buffer and SDS used. Actually, the non-lysed fermentation broth showed a thick cell pellet that could be removed easily by centrifugation, whereas the lysed broth showed a completely clear solution. Also, the chromatogram of the lysed culture showed extra peaks especially due to SDS. The addition of SDS was necessary for complete lysis, which could not be achieved with lysozyme alone. It is important to note that through following OD_{600} of the engineered strains compared to wild-type *B. subtilis*, it was clear that the engineered strains have slower growth rate especially at the best expression temperature which is 20°C. However, after the 24 h growth, both the engineered strains and wild type reached the same OD_{600}.

Segregational Stability of the Constructs in txs + crtE + SDFHCEGA *B. subtilis* Strain

A bacterial strain is considered segregationally stable when all daughter cells get at least one plasmid during cell division. The development of plasmid-free cells can lead to a significant loss in productivity. The segregational stability of the pDR_txs, pBS0E_crtE, and p04_SDFHECEGA constructs in the txs + crtE + SDFHCEGA *B. subtilis* strain was evaluated. The strain showed 100% ability to retain the pDR_txs and pBS0E_crtE constructs until the 30th generation along with 90% ability to retain the p04_SDFHECEGA construct (**Figure 6**) in the absence of antibiotics. Thus, this stability should be sufficient for large-scale fermentations in the absence of antibiotics.

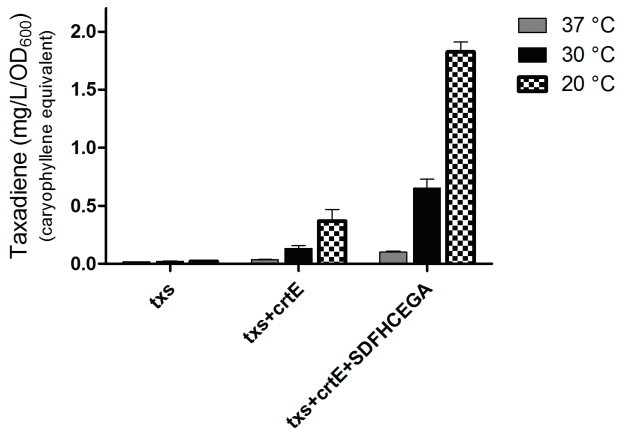


FIGURE 4 | Amount of taxa-4,11-diene (mg/L/OD_{600}) β-caryophyllene equivalent produced by the strains of *Bacillus subtilis* after incubation at 37, 30, or 20°C. The extraction of taxadiene was carried out without lysis of the cultures. The experiment was performed in triplicates.

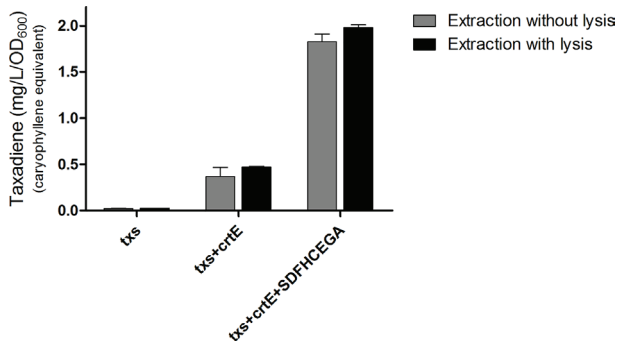


FIGURE 5 | Amount of taxa-4,11-diene (mg/L/OD_{600}) β-caryophyllene equivalent produced by the different strains of *Bacillus subtilis* after incubation at 20°C. A comparison of the amounts produced by extraction of taxadiene with and without lysis of the cultures is presented. The experiment was performed in triplicates.

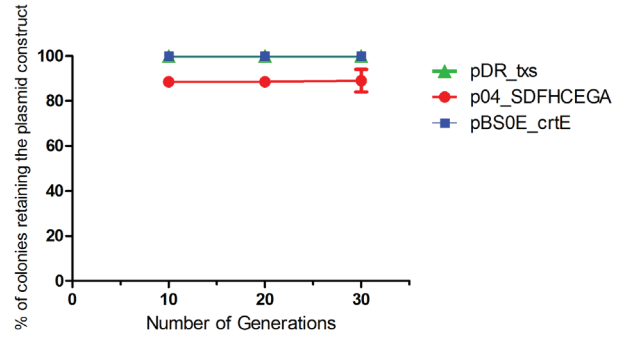


FIGURE 6 | Segregational stability of pDR_txs, pBS0E_crtE, and p04_SDFHCEGA constructs in txs + crtE + SDFHCEGA *Bacillus subtilis* 168 strain. The stability is represented as the % of colonies retaining the plasmid constructs formed on the spectinomycin, erythromycin, or chloramphenicol-containing analytical plates, respectively, after successive subculturing in the absence of antibiotics (30 generations). The experiment was performed in duplicate.

DISCUSSION

In the past few decades, researchers focused on the use of metabolic engineering and synthetic biology to manipulate a variety of hosts for biosynthesis of numerous terpenoids. Biosynthetic pathways of terpenoids have been studied, and the majority of the genes involved have been identified (Barkovich and Liao, 2001; Kirby and Keasling, 2008; Niu et al., 2017; Wang et al., 2017). Among these terpenoids is the chemotherapeutic paclitaxel. This study focused on the engineering of *B. subtilis* for the production of the first committed intermediate, taxa-4,11-diene, in the biosynthesis of paclitaxel. The biosynthesis of taxadiene in *B. subtilis* (**Figure 1A**) proceeds *via* the MEP pathway to produce IPP and DMAPP that would eventually be converted to the precursor GGPP. The enzyme taxadiene synthase cyclizes GGPP to produce taxa-4,11-diene (Croteau et al., 2006). Hence, the expression of the enzyme taxadiene synthase in *B. subtilis* is a prerequisite for the production of taxadiene. Moreover, tuning of the MEP pathway in *B. subtilis* has been previously explored (Guan et al., 2015; Xue et al., 2015) and can serve as a basis for improved production of taxadiene.

As a first step for the production of taxadiene in *B. subtilis*, the enzyme taxadiene synthase needs to be expressed. The gene encoding taxadiene synthase was amplified from the genome of the plant *T. baccata* that produces a protein of 862 amino acids. It has been reported that a pseudomature form of TXS where 60 amino acids were removed from the preprotein was superior in terms of level of expression, solubility, stability, and catalytic activity with kinetics analogous to that of the native enzyme (Williams et al., 2000). Hence, a truncated TXS protein eliminating the first 60 amino acids (plastid targeting sequence) was expressed in *B. subtilis*. For successful expression in *B. subtilis*, the *txs* gene along with the *B. subtilis mntA* RBS and N-terminal 6× His-tag was integrated into the chromosome using the pDR111 plasmid. The areas of the *amyE* gene flanking the *txs* gene in pDR111 allowed integration at the *amyE* locus in the *B. subtilis* chromosome. Following induction by IPTG at 20°C, a soluble TXS protein (~89 kDa) was expressed in *B. subtilis* and detected by Western blotting (**Figure 2**). The incubation at 20°C provided the best condition for expression of TXS as opposed to incubation at 30 or 37°C. The amount of taxadiene produced in this txs strain was very low, around 0.024 mg/L/OD_{600}, indicating that further tuning of the pathway leading to the formation of the taxadiene precursor GGPP is essential to boost the taxadiene production level.

In pursuance of increasing the production of taxadiene, higher levels of the precursor GGPP is required. In the biosynthetic pathway (**Figure 1A**), IPP and DMAPP are converted to GPP with the enzyme GPPS, and then GPP is elongated to FPP using the enzyme FPPS, both encoded by the gene *ispA*. Finally, GGPPS enzyme produces GGPP from FPP. Studies on prenyl synthases of *B. subtilis* from the gene or protein perspective are still limited. *B. subtilis* is known for producing FPP, the precursor for sesquiterpenes,

and triterpenes, through its *ispA* encoded GPPS and FPPS (Kunst et al., 1997; Kobayashi et al., 2003; Julsing et al., 2007; Barbe et al., 2009). The bacterium is also known to produce heptaprenyl (C_{35} terpenes) metabolites utilizing *hepT* and *hepS* encoding heterodimeric heptaprenyl diphosphate synthase enzymes that are responsible for producing heptaprenyl metabolite precursor from FPP (Zhang et al., 1998; Kontnik et al., 2008). Up until now, albeit it was reported that *B. subtilis* possesses an enzyme capable of producing GGPP from experiments with a semipurified protein of *B. subtilis* long before the genome sequence of *B. subtilis* was revealed (Takahashi and Ogura, 1981, 1982), the definite annotation of the gene encoding GGPPS is still uncertain. The gene that encodes GGPPS can be found in microorganisms that produce C_{40} carotenoids, such as lycopene, where GGPP precursor is needed in their biosynthesis. Examples of these microorganisms are the *Pantoea* genus with its *crtE* gene and *Corynebacterium glutamicum* with its *crtE* and *idsA* genes (Hara et al., 2012; Heider et al., 2014; Walterson and Stavrinides, 2015). *CrtE* of *Pantoea* has been widely used in metabolic engineering to produce high level of carotenoids and also C_{20} terpenoids in *E. coli* (Yoon et al., 2007). Hence, the *crtE* gene from *P. ananatis* was cloned in the pBS0E vector and transformed into the *B. subtilis* txs strain. The amount of taxadiene produced (**Figure 5**) by the txs + crtE strain (0.471 mg/L/OD_{600}) is approximately 20 times higher than the control txs strain (0.024 mg/L/OD_{600}). This indicates that overexpression of the GGPPS enzyme is essential to increase the formation of GGPP precursor in *B. subtilis* and in turn improve the production level of taxadiene.

Finally, overexpression of the MEP pathway leads to increased production of IPP and DMAPP. The *idi* gene (the final gene in the MEP pathway encoding for an IPP isomerase) was not overexpressed by us due to previous indications that it is non-essential in *B. subtilis* (Takagi et al., 2004). Past research also revealed that a knockout of the *idi* gene in *B. subtilis* is viable and produces isoprene, which implies that the synthesis of the isomer DMAPP does not rely on the isomerase (Eisenreich et al., 2004; Julsing et al., 2007). Note that, toxicity associated with the accumulation of prenyl diphosphates in *B. subtilis* with improved flux of MEP pathway has been reported (Sivy et al., 2011). Hence, it was important to control the p04_SDFHCEGA construct with an inducible promoter. The *B. subtilis* strain expressing TXS, GGPPS, all the MEP pathway, and IspA (acting as GPPS and FPPS) enzymes proved that high supply of the GGPP precursor is essential. The txs + crtE + SDFHCEGA strain showed the highest level of production of taxadiene (1.981 mg/L/OD_{600}), which is approximately 83 times higher than the txs control strain. The OD_{600} of this strain was approximately 9; hence, the total production of taxadiene is around 17.8 mg/L. This production level is higher than the amount reported in yeast 8.7 mg/L (Engels et al., 2008) and in *E. coli* 11.3 mg/L (Bian et al., 2017) on shake flask fermentation level. Also, the taxadiene production in *B. subtilis* is comparable to amorphadiene production that was previously reported in *B. subtilis* (Zhou et al., 2013). Moreover, our strain showed segregational stability of the different constructs up to the 30th generation of cultivation, which allow large-scale fermentations in the absence of antibiotics.

It is worth mentioning that the amount of taxadiene extracted from the cell culture with and without lysis is nearly the same (**Figure 5**). This indicates that taxadiene is released from the cells during growth and captured by the dodecane layer. It is also reported that the C_{15} amorphadiene produced in *B. subtilis* is trapped in the dodecane layer (Zhou et al., 2013) as we observed with our C_{20} taxadiene. Probably, due to their lipophilicity, they are able to permeate through the bacterial membrane and dissolve in the non-polar dodecane. However, C_{30} carotenoids are not exported from *B. subtilis* and have to be isolated from the cell pellet after lysis (Yoshida et al., 2009; Xue et al., 2015). Also, C_{35} heptaprenyl terpenes are produced in the spores of *B. subtilis* and not secreted (Bosak et al., 2008). The fact that taxadiene is not trapped in the cells and that lysis is not necessary, makes large-scale production much simpler where the *B. subtilis* cell culture in fermenters can be overlaid with dodecane to collect the released taxadiene. In addition, contaminants from the lysis solution can be avoided. As for *E. coli*, lysis is more likely to be needed due to its double membrane compared to *B. subtilis* which has a single membrane.

In conclusion, the successful expression of the enzyme taxadiene synthase in *B. subtilis* is reported for the first time. The expression of GGPPS in *B. subtilis* is crucial for the sufficient production of the essential precursor GGPP. Hence, the production level of taxadiene is boosted by overexpression of GGPPS enzyme along with MEP pathway and IspA (acting as GPPS and FPPS) enzymes. This is a proof of concept that taxadiene can be efficiently produced in *B. subtilis* and can serve as the basis for engineering *B. subtilis* as a cell factory for paclitaxel production. The txs + crtE + SDFHCEGA strain can be further engineered with additional enzymes (acyltransferases, cytochrome P450) necessary to produce 10-deacetylbaccatin III, which can be extracted and chemically converted to docetaxel or even paclitaxel (Walker and Croteau, 2000). In the future, efforts to improve titer such as optimization of the growth medium and/or deletion of competing pathways in *B. subtilis* that divert the flux of FPP or GGPP can be explored with caution to ensure avoiding pathways essential for the survival of the bacteria (Julsing et al., 2007; Guan et al., 2015; Zhao et al., 2016). Furthermore, process engineering to optimize pH, stirring, and aeration control will be required to develop the large-scale fed-batch fermentation system. Using the *B. subtilis* cell factory will eliminate the need to rely on the natural resources of the yew trees and thus avoid the endangering of the species and prevent shortfalls due to crop conditions.

AUTHOR CONTRIBUTIONS

IA and HP executed the experiments and interpreted the data. IA wrote the manuscript. HP revised the manuscript. RvM assisted with the experiments and revised the manuscript. S revised the data and manuscript. WQ conceived the project,

confirmed the data, and revised the manuscript. All authors read and approved the final manuscript.

FUNDING

Funding for this work was obtained through EuroCoRes SYNBIO (SYNMET), NWO-ALW 855.01.161, EU FP-7 grant 289540 (PROMYSE). IA is a recipient of Erasmus Mundus Action 2, Strand 1, Fatima Al Fihri project ALFI1200161 scholarship and is on study leave from Faculty of Pharmacy, Alexandria University. HP is a recipient of Bernoulli sandwich scholarship from The University of Groningen.

ACKNOWLEDGMENTS

We thank Jan-Willem Veening and David Rudner for kindly providing the pDR111 plasmid. We also thank Torsten Mascher and Jara Radeck for the generous gift of the pBS0E plasmid.

REFERENCES

Abdallah, I. I., and Quax, W. J. (2017). A glimpse into the biosynthesis of terpenoids. *KnE Life Sci.* 81–98. doi: 10.18502/kls.v3i5.981

Ajikumar, P. K., Xiao, W. H., Tyo, K. E., Wang, Y., Simeon, F., Leonard, E., et al. (2010). Isoprenoid pathway optimization for Taxol precursor overproduction in *Escherichia coli*. *Science* 330, 70–74. doi: 10.1126/science.1191652

Baloglu, E., and Kingston, D. G. I. (1999). A new semisynthesis of paclitaxel from baccatin III. *J. Nat. Prod.* 62, 1068–1071. doi: 10.1021/np990040k

Barbe, V., Cruveiller, S., Kunst, F., Lenoble, P., Meurice, G., Sekowska, A., et al. (2009). From a consortium sequence to a unified sequence: the *Bacillus subtilis* 168 reference genome a decade later. *Microbiology* 155, 1758–1775. doi: 10.1099/mic.0.027839-0

Barkovich, R., and Liao, J. C. (2001). REVIEW: metabolic engineering of isoprenoids. *Metab. Eng.* 3, 27–39. doi: 10.1006/mben.2000.0168

Besumbes, O., Sauret-Gueto, S., Phillips, M. A., Imperial, S., Rodriguez-Concepcion, M., and Boronat, A. (2004). Metabolic engineering of isoprenoid biosynthesis in *Arabidopsis* for the production of taxadiene, the first committed precursor of Taxol. *Biotechnol. Bioeng.* 88, 168–175. doi: 10.1002/bit.20237

Bian, G., Yuan, Y., Tao, H., Shi, X., Zhong, X., Han, Y., et al. (2017). Production of taxadiene by engineering of mevalonate pathway in *Escherichia coli* and endophytic fungus *Alternaria alternata* TPF6. *Biotechnol. J.* 12:1600697. doi: 10.1002/biot.201600697

Bosak, T., Losick, R. M., and Pearson, A. (2008). A polycyclic terpenoid that alleviates oxidative stress. *Proc. Natl. Acad. Sci. USA* 105, 6725–6729. doi: 10.1073/pnas.0800199105

Connolly, J. D., and Hill, R. A. (1991). *Dictionary of terpenoids*. (London, New York: Chapman & Hall).

Croteau, R., Ketchum, R. E. B., Long, R. M., Kaspera, R., and Wildung, M. R. (2006). Taxol biosynthesis and molecular genetics. *Phytochem. Rev.* 5, 75–97. doi: 10.1007/s11101-005-3748-2

DeJong, J. M., Liu, Y., Bollon, A. P., Long, R. M., Jennewein, S., Williams, D., et al. (2006). Genetic engineering of taxol biosynthetic genes in *Saccharomyces cerevisiae*. *Biotechnol. Bioeng.* 93, 212–224. doi: 10.1002/bit.20694

Dewick, P. M. (2001). *The mevalonate and deoxyxylulose phosphate pathways: terpenoids and steroids*. Chichester: John Wiley & Sons, Ltd.

Eisenreich, W., Bacher, A., Arigoni, D., and Rohdich, F. (2004). Biosynthesis of isoprenoids via the non-mevalonate pathway. *Cell. Mol. Life Sci.* 61, 1401–1426. doi: 10.1007/s00018-004-3381-z

Engels, B., Dahm, P., and Jennewein, S. (2008). Metabolic engineering of taxadiene biosynthesis in yeast as a first step towards Taxol (Paclitaxel) production. *Metab. Eng.* 10, 201–206. doi: 10.1016/j.ymben.2008.03.001

Guan, Z., Xue, D., Abdallah, I. I., Dijkshoorn, L., Setroikromo, R., Lv, G., et al. (2015). Metabolic engineering of *Bacillus subtilis* for terpenoid production. *Appl. Microbiol. Biotechnol.* 99, 9395–9406. doi: 10.1007/s00253-015-6950-1

Hara, Y., Kadotani, N., Izui, H., Katashkina, J. I., Kuvaeva, T. M., Andreeva, I. G., et al. (2012). The complete genome sequence of *Pantoea ananatis* AJ13355, an organism with great biotechnological potential. *Appl. Microbiol. Biotechnol.* 93, 331–341. doi: 10.1007/s00253-011-3713-5

Heider, S. A. E., Peters-Wendisch, P., Beekwilder, J., and Wendisch, V. F. (2014). IdsA is the major geranylgeranyl pyrophosphate synthase involved in carotenogenesis in *Corynebacterium glutamicum*. *FEBS J.* 281, 4906–4920. doi: 10.1111/febs.13033

Hezari, M., and Croteau, R. (1997). Taxol biosynthesis: an update. *Planta Med.* 63, 291–295. doi: 10.1055/s-2006-957684

Huang, Q., Roessner, C. A., Croteau, R., and Scott, A. I. (2001). Engineering *Escherichia coli* for the synthesis of taxadiene, a key intermediate in the biosynthesis of taxol. *Bioorg. Med. Chem.* 9, 2237–2242. doi: 10.1016/S0968-0896(01)00072-4

Julsing, M. K., Koulman, A., Woerdenbag, H. J., Quax, W. J., and Kayser, O. (2006). Combinatorial biosynthesis of medicinal plant secondary metabolites. *Biomol. Eng.* 23, 265–279. doi: 10.1016/j.bioeng.2006.08.001

Julsing, M. K., Rijpkema, M., Woerdenbag, H. J., Quax, W. J., and Kayser, O. (2007). Functional analysis of genes involved in the biosynthesis of isoprene in *Bacillus subtilis*. *Appl. Microbiol. Biotechnol.* 75, 1377–1384. doi: 10.1007/s00253-007-0953-5

Kirby, J., and Keasling, J. D. (2008). Metabolic engineering of microorganisms for isoprenoid production. *Nat. Prod. Rep.* 25, 656–661. doi: 10.1039/b802939c

Klein-Marcuschamer, D., Ajikumar, P. K., and Stephanopoulos, G. (2007). Engineering microbial cell factories for biosynthesis of isoprenoid molecules: beyond lycopene. *Trends Biotechnol.* 25, 417–424. doi: 10.1016/j.tibtech.2007.07.006

Kobayashi, K., Ehrlich, S. D., Albertini, A., Amati, G., Andersen, K. K., Arnaud, M., et al. (2003). Essential *Bacillus subtilis* genes. *Proc. Natl. Acad. Sci.* 100, 4678–4683. doi: 10.1073/pnas.0730515100

Koehn, F. E., and Carter, G. T. (2005). The evolving role of natural products in drug discovery. *Nat. Rev. Drug Discov.* 4, 206–220. doi: 10.1038/nrd1657

Kontnik, R., Bosak, T., Butcher, R. A., Brocks, J. J., Losick, R., Clardy, J., et al. (2008). Sporulenes, heptaprenyl metabolites from *Bacillus subtilis* spores. *Org. Lett.* 10, 3551–3554. doi: 10.1021/ol801314k

Kunst, F., Ogasawara, N., Moszer, I., Albertini, A. M., Alloni, G., Azevedo, V., et al. (1997). The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. *Nature* 390, 249–256. doi: 10.1038/36786

Kunst, F., and Rapoport, G. (1995). Salt stress is an environmental signal affecting degradative enzyme synthesis in *Bacillus subtilis*. *J. Bacteriol.* 177, 2403–2407.

Muntendam, R., Melillo, E., Ryden, A., and Kayser, O. (2009). Perspectives and limits of engineering the isoprenoid metabolism in heterologous hosts. *Appl. Microbiol. Biotechnol.* 84, 1003–1019. doi: 10.1007/s00253-009-2150-1

Nicolaou, K. C., Yang, Z., Liu, J. J., Ueno, H., Nantermet, P. G., Guy, R. K., et al. (1994). Total synthesis of taxol. *Nature* 367:630–634. doi: 10.1038/367630a0

Niu, F.-X., Lu, Q., Bu, Y.-F., and Liu, J.-Z. (2017). Metabolic engineering for the microbial production of isoprenoids: carotenoids and isoprenoid-based biofuels. *Synth. Syst. Biotechnol.* 2, 167–175. doi: 10.1016/j.synbio.2017.08.001

Overkamp, W., Beilharz, K., Detert Oude Weme, R., Solopova, A., Karsens, H., Kovacs, A., et al. (2013). Benchmarking various green fluorescent protein variants in *Bacillus subtilis*, *Streptococcus pneumoniae*, and *Lactococcus lactis* for live cell imaging. *Appl. Environ. Microbiol.* 79, 6481–6490. doi: 10.1128/AEM.02033-13

Popp, P. F., Dotzler, M., Radeck, J., Bartels, J., and Mascher, T. (2017). The *Bacillus* BioBrick Box 2.0: expanding the genetic toolbox for the standardized work with *Bacillus subtilis*. *Sci. Rep.* 7:15058. doi: 10.1038/s41598-017-15107-z

Quan, J., and Tian, J. (2011). Circular polymerase extension cloning for high-throughput cloning of complex and combinatorial DNA libraries. *Nat. Protoc.* 6, 242–251. doi: 10.1038/nprot.2010.181

Rodriguez, S., Kirby, J., Denby, C. M., and Keasling, J. D. (2014). Production and quantification of sesquiterpenes in *Saccharomyces cerevisiae*, including

extraction, detection and quantification of terpene products and key related metabolites. *Nat. Protoc.* 9, 1980–1996. doi: 10.1038/nprot.2014.132

Shao, H., Cao, Q., Zhao, H., Tan, X., and Feng, H. (2015). Construction of novel shuttle expression vectors for gene expression in *Bacillus subtilis* and *Bacillus pumilus*. *J. Gen. Appl. Microbiol.* 61, 124–131. doi: 10.2323/jgam.61.124

Sivy, T. L., Fall, R., and Rosenstiel, T. N. (2011). Evidence of isoprenoid precursor toxicity in *Bacillus subtilis*. *Biosci. Biotechnol. Biochem.* 75, 2376–2383. doi: 10.1271/bbb.110572

Takagi, M., Kaneda, K., Shimizu, T., Hayakawa, Y., Seto, H., and Kuzuyama, T. (2004). *Bacillus subtilis ypgA* gene is *fni*, a nonessential gene encoding type 2 isopentenyl diphosphate isomerase. *Biosci. Biotech. Bioch.* 68, 132–137. doi: 10.1271/bbb.68.132

Takahashi, I., and Ogura, K. (1981). Farnesyl pyrophosphate synthetase from *Bacillus subtilis*. *J. Biochem.* 89, 1581–1587. doi: 10.1093/oxfordjournals.jbchem.a133352

Takahashi, I., and Ogura, K. (1982). Prenyltransferases of *Bacillus subtilis*: undecaprenyl pyrophosphate synthetase and geranylgeranyl pyrophosphate synthetase. *J. Biochem.* 92, 1527–1537. doi: 10.1093/oxfordjournals.jbchem.a134077

Wagner, W. P., Helmig, D., and Fall, R. (2000). Isoprene biosynthesis in *Bacillus subtilis* via the methylerythritol phosphate pathway. *J. Nat. Prod.* 63, 37–40. doi: 10.1021/np990286p

Walker, K., and Croteau, R. (2000). Molecular cloning of a 10-deacetylbaccatin III-10-O-acetyl transferase cDNA from *Taxus* and functional expression in *Escherichia coli*. *PNAS* 97, 583–587. doi: 10.1073/pnas.97.2.583

Walterson, A. M., and Stavrinides, J. (2015). *Pantoea*: insights into a highly versatile and diverse genus within the Enterobacteriaceae. *FEMS Microbiol. Rev.* 39, 968–984. doi: 10.1093/femsre/fuv027

Wang, C., Zada, B., Wei, G., and Kim, S.-W. (2017). Metabolic engineering and synthetic biology approaches driving isoprenoid production in *Escherichia coli*. *Bioresour. Technol.* 241, 430–438. doi: 10.1016/j.biortech.2017.05.168

Williams, D. C., Wildung, M. R., Jin, A. Q., Dalal, D., Oliver, J. S., Coates, R. M., et al. (2000). Heterologous expression and characterization of a "Pseudomature" form of taxadiene synthase involved in paclitaxel (Taxol) biosynthesis and evaluation of a potential intermediate and inhibitors of the multistep diterpene cyclization reaction. *Arch. Biochem. Biophys.* 379, 137–146. doi: 10.1006/abbi.2000.1865

Withers, S. T., and Keasling, J. D. (2007). Biosynthesis and engineering of isoprenoid small molecules. *Appl. Microbiol. Biotechnol.* 73, 980–990. doi: 10.1007/s00253-006-0593-1

Wuts, P. G. (1998). Semisynthesis of Taxol. *Curr. Opin. Drug Discov. Devel.* 1, 329–337.

Xue, D., Abdallah, I. I., de Haan, I. E. M., Sibbald, M. J., and Quax, W. J. (2015). Enhanced C_{30} carotenoid production in *Bacillus subtilis* by systematic overexpression of MEP pathway genes. *Appl. Microbiol. Biotechnol.* 99, 5907–5915. doi: 10.1007/s00253-015-6531-3

Yoon, S.-H., Kim, J.-E., Lee, S.-H., Park, H.-M., Choi, M.-S., Kim, J.-Y., et al. (2007). Engineering the lycopene synthetic pathway in *E. coli* by comparison of the carotenoid genes of *Pantoea agglomerans* and *Pantoea ananatis*. *Appl. Microbiol. Biotechnol.* 74, 131–139. doi: 10.1007/s00253-006-0623-z

Yoshida, K., Ueda, S., and Maeda, I. (2009). Carotenoid production in *Bacillus subtilis* achieved by metabolic engineering. *Biotechnol. Lett.* 31, 1789–1793. doi: 10.1007/s10529-009-0082-6

Zhang, Y.-W., Koyama, T., Marecak, D. M., Prestwich, G. D., Maki, Y., and Ogura, K. (1998). Two subunits of heptaprenyl diphosphate synthase of *Bacillus subtilis* form a catalytically active complex. *Biochemistry* 37, 13411–13420. doi: 10.1021/bi972926y

Zhao, H., Sun, Y., Peters, J. M., Gross, C. A., Garner, E. C., and Helmann, J. D. (2016). Depletion of undecaprenyl pyrophosphate phosphatases disrupts cell envelope biogenesis in *Bacillus subtilis*. *J. Bacteriol.* 198, 2925–2935. doi: 10.1128/jb.00507-16

Zhou, K., Zou, R., Zhang, C., Stephanopoulos, G., and Too, H. P. (2013). Optimization of amorphadiene synthesis in *bacillus subtilis* via transcriptional, translational, and media modulation. *Biotechnol. Bioeng.* 110, 2556–2561. doi: 10.1002/bit.24900

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 25 February 2019
doi: 10.3389/fmicb.2019.00340

Function of L-Pipecolic Acid as Compatible Solute in *Corynebacterium glutamicum* as Basis for Its Production Under Hyperosmolar Conditions

*Fernando Pérez-García, Luciana F. Brito and Volker F. Wendisch**

Genetics of Prokaryotes, Faculty of Biology – CeBiTec, Bielefeld University, Bielefeld, Germany

Pipecolic acid or L-PA is a cyclic amino acid derived from L-lysine which has gained interest in the recent years within the pharmaceutical and chemical industries. L-PA can be produced efficiently using recombinant *Corynebacterium glutamicum* strains by expanding the natural L-lysine biosynthetic pathway. L-PA is a six-membered ring homolog of the five-membered ring amino acid L-proline, which serves as compatible solute in *C. glutamicum*.

Here, we show that *de novo* synthesized or externally added L-PA partially is beneficial for growth under hyper-osmotic stress conditions. *C. glutamicum* cells accumulated L-PA under elevated osmotic pressure and released it after an osmotic down shock. In the absence of the mechanosensitive channel YggB intracellular L-PA concentrations increased and its release after osmotic down shock was slower. The proline permease ProP was identified as a candidate L-PA uptake system since RNAseq analysis revealed increased *proP* RNA levels upon L-PA production. Under hyper-osmotic conditions, a Δ*proP* strain showed similar growth behavior than the parent strain when L-proline was added externally. By contrast, the growth impairment of the Δ*proP* strain under hyper-osmotic conditions could not be alleviated by addition of L-PA unless *proP* was expressed from a plasmid. This is commensurate with the view that L-proline can be imported into the *C. glutamicum* cell by ProP and other transporters such as EctP and PutP, while ProP appears of major importance for L-PA uptake under hyper-osmotic stress conditions.

Keywords: pipecolic acid, osmo regulation, compatible solute, proline, *Corynebacterium glutamicum*, RNAseq analysis, solute export, solute uptake

OPEN ACCESS

Edited by:
Peter Neubauer, Technische Universität Berlin, Germany

Reviewed by:
Xiaoyuan Wang, Jiangnan University, China
Marco Oldiges, Forschungszentrum Jülich, Helmholtz Association of German Research Centers (HZ), Germany

****Correspondence:***
Volker F. Wendisch
volker.wendisch@uni-bielefeld.de

Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: *13 November 2018*
Accepted: *08 February 2019*
Published: *25 February 2019*

Citation:
Pérez-García F, Brito LF and Wendisch VF (2019) Function of L-Pipecolic Acid as Compatible Solute in Corynebacterium glutamicum as Basis for Its Production Under Hyperosmolar Conditions. Front. Microbiol. 10:340. doi: 10.3389/fmicb.2019.00340

INTRODUCTION

In nature, all living organisms must respond to environmental fluctuations to survive. For example, bacteria have developed defense mechanisms for hyper- and hypo-osmotic external conditions to maintain cell viability including the *de novo* synthesis or uptake of osmo compatible solutes such as betaines. Since plants also contain these osmo compatible solutes, they are commonly present in production media and, thus, relevant for biotechnological fermentations (Farwick et al., 1995). The industrial workhorse *Corynebacterium glutamicum* disposes of mechanosensitive channels (MSCs) which perform as emergency release valves (Ruffert et al., 1999). After an osmotic downshift

and to avoid cell lysis, MSCs are immediately activated by membrane turgor pressure to release solutes and to decrease in the internal osmolality (Ruffert et al., 1997; Morbach and Krämer, 2003). *C. glutamicum* can synthesize proline, glutamine, and trehalose and use them as compatible solutes, whereas it cannot synthesize ectoine and betaine, which therefore only function as compatible solutes when present in the environment (Frings et al., 1993; Farwick et al., 1995; Guillouet and Engasser, 1995; Wolf et al., 2003). Proline is the major *de novo* synthetized compatible solute in *C. glutamicum* (Skjerdal et al., 1996; Wolf et al., 2003). *De novo* biosynthesis of proline is induced under osmostress-conditions (Rönsch et al., 2003) unless nitrogen is scarce, a condition when trehalose is synthesized instead of proline (Wolf et al., 2003). Externally added proline can be taken up into the *C. glutamicum* cell by the import systems EctP, ProP, and PutP (Peter et al., 1998). The import of proline by the carriers EctP and ProP is osmoregulated (Peter et al., 1998), while the import of proline by PutP is not (Peter et al., 1997).

The cyclic amino acid L-pipecolic (L-PA), also known as homoproline, is a non-proteogenic amino acid and an intermediate of the catabolism of D,L-lysine (Neshich et al., 2013). L-PA is similar in chemical structure to L-proline since they only differ in ring size by one carbon (Pérez-García et al., 2016). L-PA plays many roles in microorganisms, plants, and animals; including the interactions between organisms and as precursor of natural bioactive molecules (Vranova et al., 2013). Notably, L-PA was reported as compatible solute for the microorganisms *Silicibacter pomeroyi*, *Sinorhizobium meliloti,* and *Escherichia coli* (Gouesbet et al., 1994; Gouffi et al., 2000; Neshich et al., 2013). *E. coli* does not degrade lysine to L-PA, but to cadaverine by the lysine decarboxylases LdcC or CadA (Mimitsuka et al., 2007; Kind et al., 2010). However, externally added L-PA protected *E. coli* cells under high osmolarity conditions (Gouesbet et al., 1994). *C. glutamicum* lacks lysine catabolic pathways, although the production of L-lysine-derived compounds has been established in *C. glutamicum* by metabolic engineering (Kind et al., 2010; Pérez-García et al., 2016; Jorge et al., 2017). A lysine producing *C. glutamicum* strain was engineered to overproduce L-PA by heterologous expression of the lysine dehydrogenase gene (*lysDH*) from *S. pomeroyi* and overexpression of the native pyrroline-5-carboxylate reductase gene (*proC*) from *C. glutamicum* (Pérez-García et al., 2016). However, the physiological role of L-PA for *C. glutamicum* has not yet been described. Here, we characterized the effect of L-PA on *C. glutamicum* (either added to the culture medium or synthesized *de novo*) under different conditions of osmotic pressure by physiological and RNAseq experiments. We provide evidence that YggB may be involved in export of L-PA and ProP in its import into the *C. glutamicum* cell.

MATERIALS AND METHODS

Strain, Plasmids, and Culture Conditions

The strains and plasmids used in this work are listed in **Table 1**. *E. coli* strains were routinely cultivated in LB medium (10 g tryptone, 5 g yeast extract and 10 g NaCl per liter) or on LB agar plates at 37°C. *C. glutamicum* strains were routinely precultivated in brain heart infusion (BHI, ROTH®) plates or liquid medium overnight at 30°C. For *C. glutamicum* main cultures in flask, CGXII medium (Eggeling and Bott, 2005) was inoculated to an initial OD_{600} of 1 using 4% (w/v) glucose as sole-carbon source. For *C. glutamicum* main cultures in BioLector (m2p-labs, Baesweiler, Germany), growth experiments were performed in Flowerplates at 1,000 rpm, 95% humidity, 30°C and backscatter gain 15, inoculated to an initial OD_{600} of 1 and using 4% (w/v) glucose as sole-carbon source. When necessary, the growth medium was supplemented with kanamycin (25 $\mu g\ mL^{-1}$), spectinomycin (100 $\mu g\ mL^{-1}$) and isopropyl β-D-1-thiogalactopyranoside (IPTG) (1 mM). For growth in hyperosmolar conditions 100, 200, or 400 mM of NaCl were added to the medium.

Molecular Biology Methods

As host for gene cloning *E. coli* DH5α was used (Hanahan, 1983). *E. coli* was transformed by heat shock following the method described elsewhere (Hanahan, 1983), while *C. glutamicum* was transformed by electroporation following the method described elsewhere (Eggeling and Bott, 2005). The pair of primers YgFw/YgRv (**Table 2**) were used to amplified *yggB* from genomic DNA of *C. glutamicum* ATCC 13032. The pair of primers PrFw/PrRv (**Table 2**) were used to amplified *proP* from genomic DNA of *C. glutamicum* ATCC 13032. The amplified genes were cloned by Gibson assembly (Gibson, 2011) into the vector pEKEx3 (Stansen et al., 2005) digested with BamHI, yielding the vectors pEKEx3-*yggB* and pEKEx3-*proP*. Positive clones were verified by colony PCR using the pair of primers X1Fw/X1Rv (**Table 2**). The up- and downstream regions of *proP* gene were amplified by PCR from genomic DNA of *C. glutamicum* ATCC 13032 using the pair of primers PrDA/PrDB and PrDC/PrDD. The up and down amplified fragments were fused by cross-over PCR with primer pair PrDA/PrDD and cloned by ligation (Eggeling and Bott, 2005) into the vector pK19*mobsacB* (Schäfer et al., 1994) restricted with BamHI. Positive clones were verified by colony PCR using the pair of primers 196F/197R (**Table 2**). The resulting vector pK19mobsacB-*gdh* was transferred to *E. coli* S17-1. In-frame deletion of the *yggB* and *proP* genes from *C. glutamicum* was performed via a two-step homologous recombination method (Eggeling and Bott, 2005). The pK19*mobsacB* vectors were transferred to the GSL strain via conjugation using *E. coli* S17-1 (Simon et al., 1983). The deletions of *yggB* and *proP* were verified by colony PCR using the pair of primers DE31/DE32 and PrDE/PrDF, respectively.

Internal Amino Acids Extraction

For the quantification of intracellular L-PA 2 mL of liquid medium were collected. One milliliter was centrifuged at 14,000 rpm and 4°C for 10–15 min. The resulting pellets were resuspended and treated with 5% $HClO_4$ in an ice bath for 30 min. Then, the supernatant was neutralized with K_2CO_3 solution and centrifuged again at 14,000 rpm and 4°C for 10–15 min. Afterward, the supernatants were directly used for L-PA quantification or stored at −20°C

TABLE 1 | Strains and plasmids used in this work.

Strains and plasmids	Description	Source
Strains		
GSL	*C. glutamicum* ATCC13032 with the following modifications: Δ*pck,* Δ*sugR,* Δ*ldhA, pyc*^{P458S}, *hom*^{V59A}, two copies of *lysC*^{T311I}, two copies of *asd*, two copies of *dapA*, two copies of *dapB*, two copies of *ddh*, two copies of *lysA*, two copies of *lysE,* in-frame deletion of prophages CGP1 (cg1507-cg1524), CGP2 (cg1746-cg1752), and CGP3 (cg1890-cg2071)	Pérez-García et al., 2016
GSLΔ*yggB*	In-frame deletion of *yggB* (cg1434) in GSL	This work
GSLΔ*proP*	In-frame deletion of *proP* (cg3395) in GSL	This work
JJ001	*C. glutamicum* ATCC13032 with the following modifications: Δ*argF,* Δ*argR* (auxotrophic for L-arginine); carrying the vector pVWEx1	Jensen and Wendisch, 2013
JJ004	JJ001 strain carrying the vector pVWEx1-ocdPp(TAA)	Jensen and Wendisch, 2013
E. coli DH5α	F$^-$ *thi*-1 *endA*1 *hsdr*17 (r$^-$, m$^-$) *supE*44 Δ*lacU*169 (Φ80*lacZ*ΔM15) *recA*1 *gyrA*96 *relA*1	Hanahan, 1983
E. coli S17-1	*recA*, *thi*, *pro*, *hsd* R-M+ (RP4: 2-Tc:Mu-:Km, integrated into the chromosome)	Simon et al., 1983
Plasmids		
pVWEx1	Km^R, *C. glutamicum*/E. coli shuttle vector (Ptac, *lacI*, pHM1519 *oriV*$_{Cg}$)	Peters-Wendisch et al., 2001
pEKEx3	Spec^R, *C. glutamicum*/*E. coli* shuttle vector (Ptac, *lacI*, pBL1 *oriV*$_{Cg}$)	Stansen et al., 2005
pVWEx1-*lysDH-proC*	Km^R, pVWEx1 overexpressing *lysDH* from *S. pomeroyi* DSS-3 and *proC* from *C. glutamicum* ATCC 13032	Pérez-García et al., 2016
pEKEx3-*yggB*	Spe^R, pEKEx3 overexpressing *yggB* from *C. glutamicum* ATCC 13032	This work
pEKEx3-*proP*	Spe^R, pEKEx3 overexpressing *proP* from *C. glutamicum* ATCC 13032	This work
pK19*mobsacB*	Km^R; *E. coli*/*C. glutamicum* shuttle vector for construction of insertion and deletion mutants in *C. glutamicum* (pK18 *oriVEc sacB lacZ*α)	Schaffer et al., 2001
pK19*mobsacB*-Δ*yggB*	pK19*mobsacB* with a *yggB* (cg1434) deletion construct	Lubitz and Wendisch, 2016
pK19*mobsacB*-Δ*proP*	pK19*mobsacB* with a *proP* (cg3395) deletion construct	This work

TABLE 2 | Oligonucleotide sequences used in this work for molecular cloning and in-frame deletion.

Primer	Sequence (5→3)
PrFw	GCATGCCTGCAGGTCGACTCTAGAGGAAAGGAGGCCCT TCAGGTGAGCCCGATTCGCTC
PrRv	AATTCGAGCTCGGTACCCGGGGATCTTATGCGTTTTGCTTT TCAG
YgFw	GCATGCCTGCAGGTCGACTCTAGAGGAAAGGAGGCCCTTCA GATGATTTTAGGCGTACCC
YgRv	AATTCGAGCTCGGTACCCGGGGATCCTAAGGGGTGGACG TCGG
PrDA	GCATGCCTGCAGGTCGACTCTAGAGTTCGGTGCCCTCCACG GCAC
PrDB	GGGTAGGTGATTTGAATTTGTGAGTAAAACCTCTCGTCATATC
PrDC	ACAAATTCAAATCACCTACCCCCGTAAAGCCCGCTGCAAGG
PrDD	AATTCGAGCTCGGTACCCGGGGATCGTAACGATGCAGACCG CCGG
PrDE	CGGTGCCCTCCACGGCACC
PrDF	AACGATGCAGACCGCCGGCG
DE31	CTTTTGGCGCTCCAAGTACT
DE32	TCCTCGAGCGATCGAACAAT
X1Fw	CATCATAACGGTTCTGGC
X1Rv	ATCTTCTCTCATCCGCCA
196F	CGCCAGGGTTTTCCCAGTCACGAC
197R	AGCGGATAACAATTTCACACAGGA

(Sun et al., 2016). It has to be noted that the water space of the pellet in such a centrifugation step will contain compounds presents in the extracellular volume and this will affect the determination of the intracellular concentration. On the other hand, intracellular compounds may leak out of the cell during washing steps and this will also affect the determination of the intracellular concentration. Since all samples were processed in the same way, the possible fluctuations/errors in the measurements should affect all samples similarly. Exact quantitation would require methods such as described by Klingenberg and Pfaff (1967) which combine centrifugation through silicone oil for fast separation of supernatant and pellet by centrifugation and inactivation of the pellet by perchloric acid. The second collected mL was used to determine the biomass according to the correlation CDW [g L^{-1}] = 0.35 OD (Bolten et al., 2007).

Determination of L-PA by High Pressure Liquid Chromatography

The concentration of L-PA was quantified by using high-pressure liquid chromatography. The samples from the cell cultures were collected by centrifugation (14,000 rpm, 15 min and at room temperature), and further used for analysis. The samples were derivatized with fluorenylmethyl chloroformate (FMOC) as described (Schneider and Wendisch, 2010). Amino acid separation was performed on a system consisting of a pre-column (LiChrospher 100 RP18 EC-5μ (40 × 4 mm), CS-Chromatographie Service GmbH, Langerwehe, Germany) and a main column (LiChrospher 100 RP18 EC-5μ (125 × 4 mm), CS Chromatographie Service GmbH). The detection was carried out with a fluorescence detector with the excitation and emission wavelength of 230 nm and 310 nm, respectively (FLD G1321A, 1200 series, Agilent Technologies).

RNAseq Analysis

For extraction of *C. glutamicum* bacterial cell pellets grown under the experimental conditions were harvested at mid-exponential phase. Harvesting procedure was done according to Irla et al. (2015) and cell pellets were kept at −80°C for further RNA isolation. Then, the pellets were thawed in ice and RNA was isolated individually for each sample using NucleoSpin RNA isolation kit (Macherey-Nagel, Düren, Germany). RNA samples with genomic DNA contamination were treated with the RNase-free DNase set (Qiagen, Hilden, Germany) (Brito et al., 2017). The concentration of isolated RNA was determined by DropSense^{TM} 16 (Trinean, Ghent, Belgium; software version 2.1.0.18). To verify the quality of RNA samples, we performed capillary gel electrophoresis (Agilent Bioanalyzer 2100 system using the Agilent RNA 6000 Pico kit; Agilent Technologies, Böblingen, Germany). The extracted RNA samples were pooled in equal parts and the pool of total RNA was subsequently used for the preparation of the cDNA libraries. The preparation and sequencing of the libraries were performed as described elsewhere (Mentz et al., 2013; Irla et al., 2015). Then, the reads were trimmed to a minimal length of 20 base pairs and in paired end mode with the Trimmotatic ver. 0.33 (Bolger et al., 2014). Trimmed reads were mapped to the reference genome of *C. glutamicum* ATCC13032 (Kalinowski et al., 2003) using the software Bowtie (Langmead et al., 2009). In order to perform differential gene expression analysis (DEseq) (Anders and Huber, 2010), we used the software for visualization of mapped sequences ReadXplorer (Hilker et al., 2014).

Real-Time Quantitative Reverse Transcription-PCR

The real-time quantitative reverse transcription-PCR (qRT-PCR) was performed in order to validate the data obtained by DEseq analysis by using the CFX Connect^{TM} Real-Time PCR Detection System (Bio-Rad Laboratories, Irvine, CA, United States). Same RNA samples utilized in the RNAseq analysis were utilized as templates for qRT-PCR. All samples RNA concentration was adjusted to 50 ng μL^{-1}. Afterward, 1 μL for each sample was pipetted into a reaction mix of the SensiFAST^{TM} SYBR® No-ROX Kit (Bioline, Luckenwalde, Germany), following manufacturer's instructions. Differentially expressed genes in DEseq analysis were selected as targets for qRT-PCR amplifications (primers listed in **Table 3**). The melting-curve data-based quantification cycle (Cq) values, from the LightCycler® output files, were used for further calculation as it is described elsewhere (Crooks et al., 2004).

RESULTS

C. glutamicum Can Use L-PA for Osmoprotection

Structurally, L-PA is related to L-proline. To check whether L-PA functions as osmoprotectant in *C. glutamicum* the L-PA producer GSL(pVWEx1-*lysDH-proC*) (Pérez-García et al., 2016) was grown in glucose-minimal medium supplemented with 0, 100, 200, and 400 mM of NaCl using a BioLector system. Production of L -PA was induced by adding IPTG. When not induced for L-PA production, strains GSL(pVWEx1) and GSL(pVWEx1-*lysDH-proC*) showed decreasing growth rates (**Figure 1A**) and maximal biomass formation (**Figure 1B**) with increasing NaCl concentration. However, when L-PA production was induced, *C. glutamicum* GSL(pVWEx1-*lysDH-proC*) grew faster (**Figure 1A**) and to higher biomass concentrations (**Figure 1B**) in the presence of NaCl than the parent strain GSL(pVWEx1) (**Figure 1**). This indicated that biosynthesis of L-PA helps *C. glutamicum* to withstand hyperosmolar conditions.

To test if also exogenously added L-PA is beneficial for *C. glutamicum* when grown under hyperosmolar conditions, 40 mM of either L-PA or L-proline were added to the glucose-minimal medium and growth of *C. glutamicum* GSL(pVWEx1) was monitored (**Figures 1C,D**). The exogenous addition of L-PA or L-proline improved growth of strain GSL(pVWEx1) in the presence of all NaCl concentrations tested (**Figures 1C,D**). Thus, under the chosen conditions L-PA functions as well as the known osmoprotectant of *C. glutamicum*, L-proline, in promoting growth under hyperosmolar conditions.

TABLE 3 | Oligonucleotide sequences (5→3) used for amplification of gene fragments in qRT-PCR.

Gene identity	Forward	Reverse	Gene product length (bp)
betP	GCGGGCTTGCTTGAGAATCC	TGAAGGCCCAGCCGAGATTG	232
cg0569	AGCTTTGGCTGCTTCAGTAG	AGATTCCATGCCGGAACTTG	241
cg1665	GCTGCCAACTCTGCAACCTC	CCATTCGGGCCTTCTTCCAC	245
cg2677	GGCTCTGCCTCCATTCTTTG	GGTTGTGCCTTGACCTCTTC	210
cg2851	CAACGTGAACACGGTGTATC	CACATCGTCGAATCCGTTTG	210
cg3254	ATGCTTGCCCTAGGTTGG	CCGAGTGAAGAACTGCACG	255
cg3282	ATGACCTGCGGACACTGC	TCAGGACAAGACGGTGTAG	180
gntV	TCCGTCGGTAAAGCCCTAGC	CGGTTCCTGGGCATTTGGTG	238
proC	CGCGGCCAACATGAATCCAC	GGCCATGCTGACCACAACAC	232
proP	TCGACTGGTGGTGAATATGC	GAATACGCCAACCGAAATCC	202
pstC	AATGCGAACTCCTCTCAGAC	AATCCGCCAATACCTTCAGC	206
pstS	TCCGCAATGGACTACTTTGG	AACTGGGCCGATAACGAATG	222

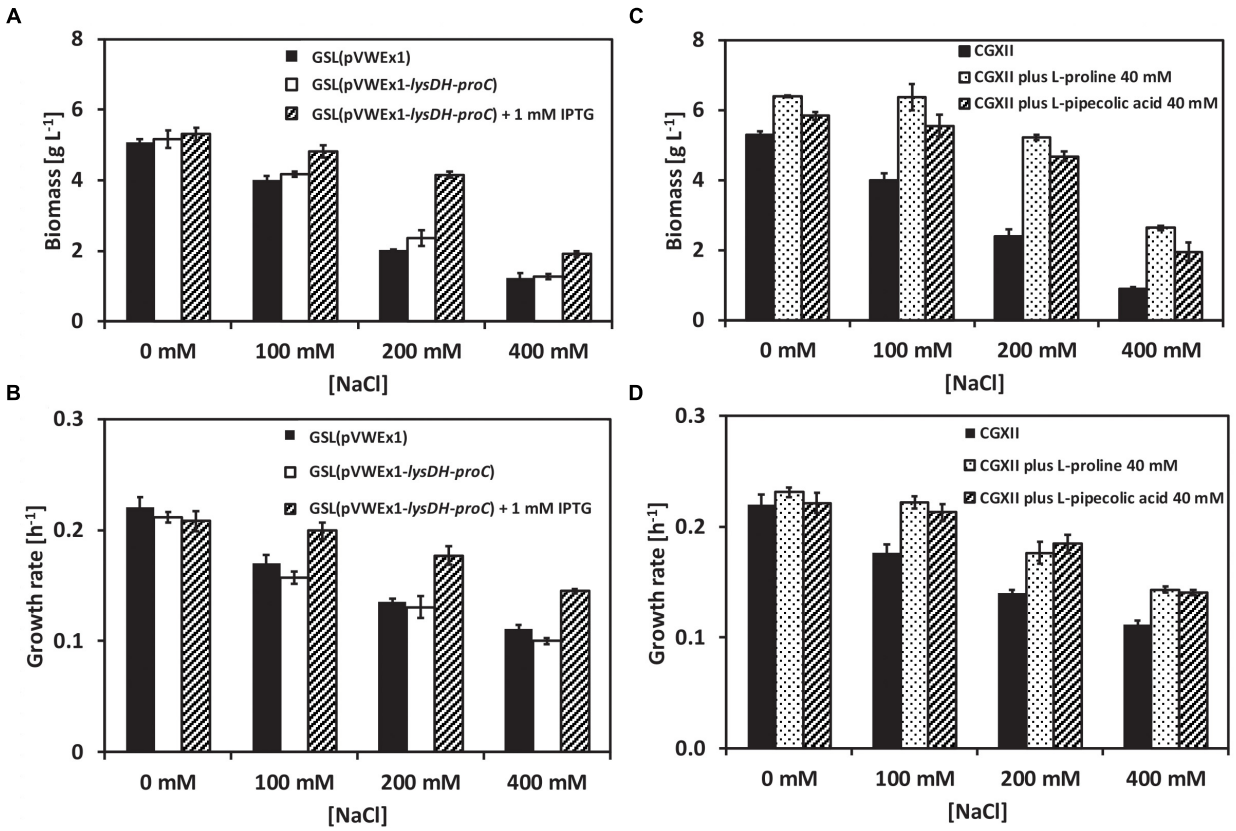


FIGURE 1 | Growth of the *C. glutamicum* strains GSL(pVWEx1) and GSL(pVWEx1-*lysDH-proC*) under different osmotic conditions. **(A)** Biomass formation in g L^{-1} of GSL(pVWEx1) and GSL(pVWEx1-*lysDH-proC*) strains in glucose minimal medium supplemented with 0, 100, 200, or 400 mM of NaCl. **(B)** Growth rates values in h^{-1} of GSL(pVWEx1) and GSL(pVWEx1-*lysDH-proC*) strains in glucose minimal medium supplemented with 0, 100, 200, or 400 mM of NaCl. **(C)** Biomass formation in g L^{-1} of GSL(pVWEx1) strain in glucose minimal medium supplemented with 0, 100, 200, or 400 mM of NaCl with 40 mM of L-proline or 40 mM of L-PA or nothing. **(D)** Growth rates values in h^{-1} of GSL(pVWEx1) strain in glucose minimal medium supplemented with 0, 100, 200, or 400 mM of NaCl with 40 mM of L-proline or 40 mM of L-PA or nothing. Values represent means and standard deviations.

While several transport proteins for the compatible solute L-proline are known, transport of L-PA has not yet been studied.

The Mechanosensitive Channel YggB May Play a Role as Escape Valve for L-PA

Osmo compatible solutes accumulate intracellularly under hyperosmolar conditions and are released from the cell upon osmotic downshift. Since YggB has been shown to be a key player in osmoregulation in *C. glutamicum* (Börngen et al., 2010), *yggB* deletion mutants were also analyzed. Here, the accumulation and release of L-PA by *C. glutamicum* cells were analyzed (**Figure 2**). *C. glutamicum* cells were cultivated in 50 mL glucose-minimal medium without (blue columns) or with (red columns) 200 mM NaCl. When glucose was depleted 1 mL supernatant and 2 mL pellet were collected to measure the extracellular (dashed columns) and intracellular (filled columns) concentrations of L-PA (**Figure 2**, left panels). The rest of the pellet of cells that grew in CGXII (blue lines) or CGXII + 200 mM NaCl (red lines) was transferred to 35 mL milliQ-water 0.9% NaCl to force an osmotic downshift while keeping the cells intact. The extracellular (dashed lines) and intracellular (solid lines) concentrations of L-PA were monitored over time (**Figure 2**, right panels). The experiment was performed with the L-PA producing strain GSL(pVWEx1-*lysDH-proC*) (**Figure 2A**); a *yggB* deletion mutant of this strain lacking the MSC YggB, GSLΔ*yggB*(pVWEx1-*lysDH-proC*) (**Figure 2B**); and a derived strain expressing *yggB* from a plasmid for complementation of the *yggB* deletion, GSLΔ*yggB*(pVWEx1-*lysDH-proC*)(pEKEx3-*yggB*) (**Figure 2C**). After growth in CGXII minimal medium with 200 mM NaCl, the intracellular L-PA concentrations were higher than after growth in CGXII minimal medium without added NaCl (**Figure 2A**), left). Upon osmotic downshift, L-PA was released from cells grown without NaCl and accumulated in the medium with a rate of 0.97 ± 0.04 mM h^{-1}. However, when cells grown with 200 mM NaCl were subjected to osmotic downshift, L-PA was released with a 75% higher rate and to an about 1.6 higher concentration (**Figure 2A**, right and **Table 4**).

When growing the strain GSLΔ*yggB*(pVWEx1-*lysDH-proC*) on CGXII with or without 200 mM NaCl the internal accumulation of L-PA increased 80 and 40%, respectively, as compared with the strain GSL(pVWEx1-*lysDH-proC*)

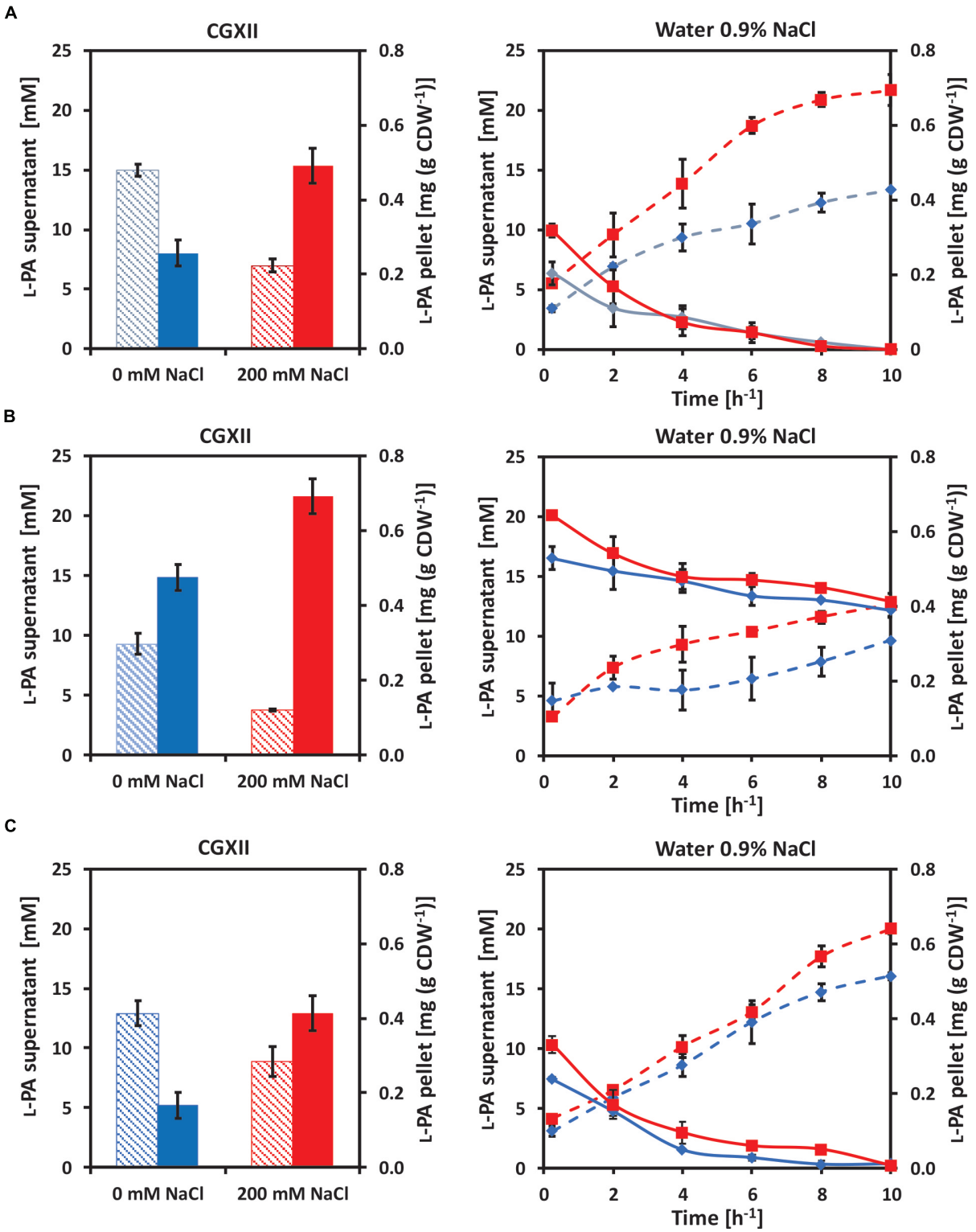


FIGURE 2 | Intracellular (filled columns, straight lines) and extracellular (dashed columns, dashed lines) L-PA concentration profiles of the strains **(A)** GSL(pVWEx1-*lysDH-proC*), **(B)** GSLΔ*yggB*(pVWEx1-*lysDH-proC*) and **(C)** GSLΔ*yggB*(pVWEx1-*lysDH-proC*)(pEKEx3-*yggB*) after growth in glucose-minimal medium (left panels) without added NaCl (blue) or with 200 mM NaCl (red) and after osmotic downshift to 0.9% NaCl (right panels). Values represent means and standard deviations.

TABLE 4 | Rates of decrease of intracellular L-PA concentrations and of L-PA accumulation in the cultivation medium upon osmotic downshock of different *C. glutamicum* strains after growth in glucose minimal medium with or without 200 mM NaCl.

Strain	Rates of decrease of intracellular L-PA concentrations (μg g CDW^{-1} h^{-1})		Rates of L-PA accumulation in the supernatant (mM h^{-1})	
	After growth with 0 mM NaCl	After growth with 200 mM NaCl	After growth with 0 mM NaCl	After growth with 200 mM NaCl
GSL(pVWEx1-*lysDH-proC*)	19.3 ± 0.2	30.2 ± 0.4	0.97 ± 0.04	1.73 ± 0.02
GSL**Δ*yggB***(pVWEx1-lysDH-proC)	14.0 ± 0.2	20.8 ± 0.3	0.47 ± 0.13	0.88 ± 0.17
GSL**Δ*yggB***(pVWEx1-*lysDH-proC*)(pEKEx3-***yggB***)	22.9 ± 0.3	28.9 ± 0.4	1.37 ± 0.16	1.68 ± 0.14

Values represent means and standard deviations. Important strain features are depicted in bold.

(**Figure 2B**, left). On 0.9% NaCl, the L-PA external accumulation rates decreased to the half (**Figure 2B**, right and **Table 4**). Therefore, the deletion of *yggB* slowed down L-PA export but was not enough to fully avoid it. The strain GSLΔ*yggB*(pVWEx1-*lysDH-proC*)(pEKEx3-*yggB*) showed similar L-PA internal/external profiles in both CGXII and water as compared with the strain GSL(pVWEx1-*lysDH-proC*) (**Figure 2C**, right and **Table 4**).

Comparative RNAseq Analysis of a L-PA Producing *C. glutamicum* Strain, a L-Proline Producing Strain and the Respective Control Strains

Under the assumption that genes relevant for production of either L-PA or L-proline are differentially expressed when comparing L-PA producing *C. glutamicum* strain GSL(pVWEx1-*lysDH-proC*) with its parent strain GSL(pVWEx1) and of L-proline producing *C. glutamicum* strain JJ004 with its parent strain JJ001, comparative RNAseq analysis was performed. *C. glutamicum* strains GSL(pVWEx1-*lysDH-proC*), GSL(pVWEx1), JJ004 and JJ001 were grown in glucose minimal medium with 1 mM IPTG after inoculation with an initial OD600 of 1. Samples for RNA preparation were harvested after 8 h of inoculation at an OD600 of 7.6 ± 0.4 and 7.7 ± 0.4, respectively, for strains GSL(pVWEx1-*lysDH-proC*) and GSL(pVWEx1). Sample for strains JJ004 and JJ001 were harvested 6 h after inoculation at an OD600 of 7.6 ± 0.1 and 8.0 ± 0.2, respectively. RNA and library preparation, sequencing, read mapping and differential gene expression analysis using the statistical method DEseq (Naville et al., 2011) was performed as described in Section "Materials and Methods." Full data is available as Gene Expression Omnibus GSE122249 data set at http://www.ncbi.nlm.nih.gov/geo/. As compared to the respective control strains, 5 genes showed increased RNA levels and 17 genes decreased RNA levels in the L-PA producing strain, while 26 genes showed increased RNA levels and 33 genes decreased RNA levels in the L-proline producing strain (**Table 5**).

The results obtained in the RNAseq analysis were validated by the analysis of gene expression patterns by qRT-PCR. For each analysis eight genes were selected, four upregulated and four downregulated genes. As shown in **Figure 3**, the relative gene expression levels obtained in qRT-PCR confirmed the pattern of their differential gene expression (fold change value) obtained in the RNAseq analysis.

In the L-proline producing strain JJ004, *pstSCAB* encoding phosphate ABC uptake system, genes for divalent metal transporter proteins (*ctpA*, cg0569, cg3281, cg3282, and cg3402), for transcriptional regulators (*glyR*, *whiB3,* and *whcE*) and for enzymes of central carbon metabolism (*pck*, *ldh* and *mez*) showed higher RNA levels than in the control strain JJ001 (**Table 5**). As compared to JJ001, RNA levels were lower in JJ004 for genes encoding iron-siderophore ABC uptake systems (cg0770, *irp1*, cg0924 and cg0926), the lysine/arginine permease gene *lysE* and genes for uptake of L-proline and other compatible solutes (*betP*, *putP* and *proP*) (**Table 5**). Thus, it appears that upon overproduction of L-proline, genes for its uptake from the culture medium are downregulated.

As expected, RNAseq analysis of the L-PA producing strain revealed increased expression of pyrroline-5-carboxylate reductase gene *proC* since it is expressed from plasmid pVWEx1-*lysDH-proC* (**Table 5**). Other genes showing increased RNA levels upon L-PA production were the divalent metal transporter protein gene cg0569, the gluconokinase gene *gntV* and the catechol 1,2-dioxygenase gene *catA* (**Table 5**). Genes showing decreased RNA levels upon L-PA production were the putative site-specific recombinase gene *xerC* and the compatible solute transport gene *proP* (**Table 5**). Notably, the genes *betP* and *putP* coding for uptake systems of L-proline and other compatible solutes did not show increased RNA levels. Thus, ProP was chosen as potential candidate for import of L-PA into the *C. glutamicum* cell.

Role of the Carrier ProP During Growth With L-PA as Osmo Compatible Solute

Deduced from the RNAseq data, we speculated that the carrier ProP may play a role with regard to L-PA as osmo compatible solute of *C. glutamicum*. ProP is used by *C. glutamicum* as the main osmoregulated uptake system for L-proline (Peter et al., 1998). To test if the absence of ProP affects the use of *C. glutamicum* of L-PA under hyperosmolar conditions, strain GSLΔ*proP* was constructed. The strains GSL(pEKEx3) (**Figure 4**, black columns), GSLΔ*proP*(pEKEx3) (**Figure 4**, red columns) and GSLΔ*proP*(pEKEx3-*proP*) (**Figure 4**, green columns) were grown in glucose minimal medium supplemented with 0, 100, 200, or 400 mM of NaCl using a BioLector system. CGXII

TABLE 5 | Comparative RNAseq analysis of L-proline producing strain JJ004, its isogenic non-producing control strain JJ001, L-PA producing *C. glutamicum* strain GSL(pVWEx1-*lysDH-proC*) and its isogenic non-producing control strain GSL(pVWEx1).

		Log_2 fold change of RNA level (*P*-value < 0.01)	
Locus or gene	**Product**	**JJ004/ control JJ001**	**GSL(pVWEx1-*lysDH-proC*)/ control GSL(pVWEx1)**
cg0018	Conserved hypothetical membrane protein	0.8	−1.0
cg0107	Putative secreted protein	0.3	−1.0
cg0175	Putative secreted protein	−0.8	−1.2
cg0282	CsbD family protein involved in stress response	−0.8	−0.6
ctpA	Putative Cu^{2+} transporting P-type ATPase	1.1	0.2
proC	Pyrroline-5-carboxylate reductase	0.0	4.7
glyR	Transcriptional activator of *glyA*, ArsR-family	1.0	−0.5
cg0569	Putative Cd^{2+} transporting P-type ATPase	1.0	1.7
whiB3	Transcriptional regulator protein, WhiB-family	2.6	−0.5
prpC2	2-Methylcitrate synthase	0.9	0.7
cg0770	ABC-type putative iron-siderophore transporter, permease subunit	−1.9	0.3
irp1	ABC-type putative iron-siderophore transporter, substrate-binding lipoprotein	−2.3	−0.3
whcE	Transcriptional regulator, WhiB-family	0.8	−0.4
pdxS	Pyridoxal 5'-phosphate (PLP) synthase subunit S	−0.8	0.0
pdxT	Pyridoxal 5'-phosphate (PLP) synthase subunit T	−1.0	0.5
cg0924	ABC-type putative iron-siderophore transporter, substrate-binding lipoprotein	−1.7	−0.5
cg0926	ABC-type putative iron-siderophore transporter, permease subunit	−1.3	−0.2
cg0935	Conserved hypothetical protein	−0.4	−1.3
rpf1	RPF-protein precursor	0.9	−0.4
cg0952	Putative integral membrane protein	−0.9	−0.3
betP	Na^+/glutamate symporter	−1.2	−0.7
cg1091	Hypothetical protein	−1.6	−1.0
cg1091	Hypothetical protein	−1.6	−1.0
cg1109	Hypothetical protein	−0.3	−1.1
cg1279	Putative secreted protein	0.8	0.6
cg1291	Putative membrane protein	1.2	−0.1
cg1293	Putative secreted protein	−0.8	−0.4
putP	Na^+/proline symporter	−0.8	−0.1
cg1419	Putative secondary Na^+/bile acid symporter, bile acid:Na^+ symporter (BASS) family	−1.7	−0.2
lysE	L-Lysine efflux permease	−5.4	−0.5
leuC	3-Isopropylmalate dehydratase, large subunit	−0.8	0.0
ptsG	Phosphotransferase system (PTS), glucose-specific enzyme IIBCA component	−0.9	0.1
cg1604	Secreted protein, putative channel protein	−0.9	0.1
cg1665	Putative secreted protein	−1.7	−0.9
cg1746	Putative membrane protein	0.8	*n.d.*
cg1897	Putative secreted protein	−3.1	*n.d.*
cg1930	Putative secreted hydrolase	−1.1	*n.d.*
cg2068	Hypothetical protein	−1.0	*n.d.*
psp1	Putative secreted protein	−1.6	*n.d.*
int2'	Putative phage Integrase (N-terminal fragment)	−1.6	*n.d.*
cg2181	ABC-type putative dipeptide/oligopeptide transporter, substrate-binding lipoprotein	−0.8	0.1
xerC	Putative site-specific recombinase	−0.2	−1.2
cg2402	Secreted protein NLP/P60 family	0.8	−0.1
cg2425	Putative permease	0.7	0.3
cg2477	Conserved hypothetical protein	0.0	−0.8
cg2564	Conserved hypothetical protein	−1.7	−0.5
catA	Catechol 1,2-dioxygenase	0.3	0.7
cg2651	Conserved hypothetical protein, pseudogene	−0.6	−1.4

(Continued)

TABLE 5 | Continued

Locus or gene	Product	Log_2 fold change of RNA level (*P*-value < 0.01)	
		JJ004/ control JJ001	GSL(pVWEx1-*lysDH-proC*)/ control GSL(pVWEx1)
cg2677	ABC-type putative dipeptide/oligopeptide transporter, permease subunit	0.0	0.9
gntV	Gluconokinase	−0.5	1.8
rpmJ	50S ribosomal protein L36	−0.3	−1.0
pstB	ABC-type phosphate transporter, ATPase subunit	1.3	0.1
pstA	ABC-type phosphate transporter, permease subunit	1.2	0.5
pstC	ABC-type phosphate transporter, permease subunit	1.5	0.3
pstS	ABC-type phosphate transporter, substrate-binding lipoprotein	1.3	0.1
cg2851	Branched-chain amino acid aminotransferase, AT class III/4-amino-4-deoxychorismate lyase	0.0	−1.6
cg2875	Hypothetical protein	0.8	−0.3
cg2908	Putative membrane protein	−1.0	0.1
pck	Phosphoenolpyruvate carboxykinase (GTP)	0.8	0.0
gntP	Gluconate:H^+ symporter	−1.0	0.1
cg3218	Pyruvate kinase-like protein	1.2	0.4
ldh	L-Lactate dehydrogenase, NAD-dependent	0.9	0.0
cg3254	Putative membrane protein	−0.8	−0.9
cg3271	SAM-dependent methyltransferase	0.3	−1.1
cg3281	Putative Cu^{2+} transporting P-type ATPase	1.2	−0.4
cg3282	Putative Cu^{2+} transporting P-type ATPase	1.4	0.0
cg3326	Hypothetical protein	1.1	0.8
mez	Malic enzyme	0.9	0.3
proP	Proline/betaine permease	−1.2	−1.2
cg3402	Putative Hg^{2+} permease, MerTP-family	1.2	0.0
cg3404	ABC-type putative iron(III) dicitrate transporter, substrate-binding lipoprotein	−2.1	−0.4
cg4014	Conserved hypothetical protein, possibly involved in stress response	0.3	−1.0
cg4019		0.8	0.1
cg4021		−0.6	−1.1

Names of genes used for subsequent qRT-PCR analysis are given in bold. n.d., not detected.

contains 200 mM MOPS buffer, thus, has a relatively high osmolarity: about 1,1 osmol/kg without added NaCl as compared to about 1,3 osmol/kg CGXII medium with 400 mM NaCl (Börngen et al., 2010). In addition, the effect of externally added L-proline or L-PA was tested. Data for final biomass formation and growth rate was collected for all conditions (**Figure 4**). It was observed that the strains carrying the deletion of *proP* suffered more from the hyperosmotic conditions as compared to the control *C. glutamicum* strain GSL(pEKEx3) or the complementation strain GSLΔ*proP*(pEKEx3-*proP*) (**Figure 4**). When 40 mM of L-proline was supplemented as osmo compatible solute to the minimal medium the growth rates and final biomass concentrations were reduced to a lesser extent than when 40 mM L-PA was added (**Figure 4**). Thus, ProP plays an important role when L-proline and L-PA are used as osmo compatible solutes in *C. glutamicum*. These findings are commensurate with the view that ProP does not only import L-proline into the *C. glutamicum* cell, but also L-PA. However, future in depth biochemical analysis of L-PA uptake are needed to determine the respective kinetic parameters of L-PA uptake.

DISCUSSION

In this study, L-PA was shown to be an osmo compatible solute for *C. glutamicum*. L-PA cannot be synthesized by *C. glutamicum* wild type, but can be imported from the environment. The synthesis and/or accumulation of compatible solutes is a widespread microbial strategy against osmolarity fluctuations (da Costa et al., 1998; Kempf and Bremer, 1998; Wood et al., 2001; Czech et al., 2018). High cytoplasmic concentrations of compatible solutes also stabilize protein folding and ribosomes and protect the DNA, increasing the resistance to other types of stress such as high and low temperatures and radiation (Li and Gänzle, 2016; Sajjad et al., 2018; Tribelli and López, 2018). The osmoprotection mechanisms for the microbial cell factories *E. coli*, *Bacillus subtilis* and *C. glutamicum* are described (Kempf and Bremer, 1998; Wood et al., 2001; Morbach and Krämer, 2003; Hoffmann and Bremer, 2017). These and other non-halophilic bacteria accumulate K ions, Na ions or glutamate after an osmotic upshock as first response before these ions are exchanged against compatible solutes either by synthesis or uptake (Wood, 1999). *C. glutamicum* either synthesizes glutamine, proline or

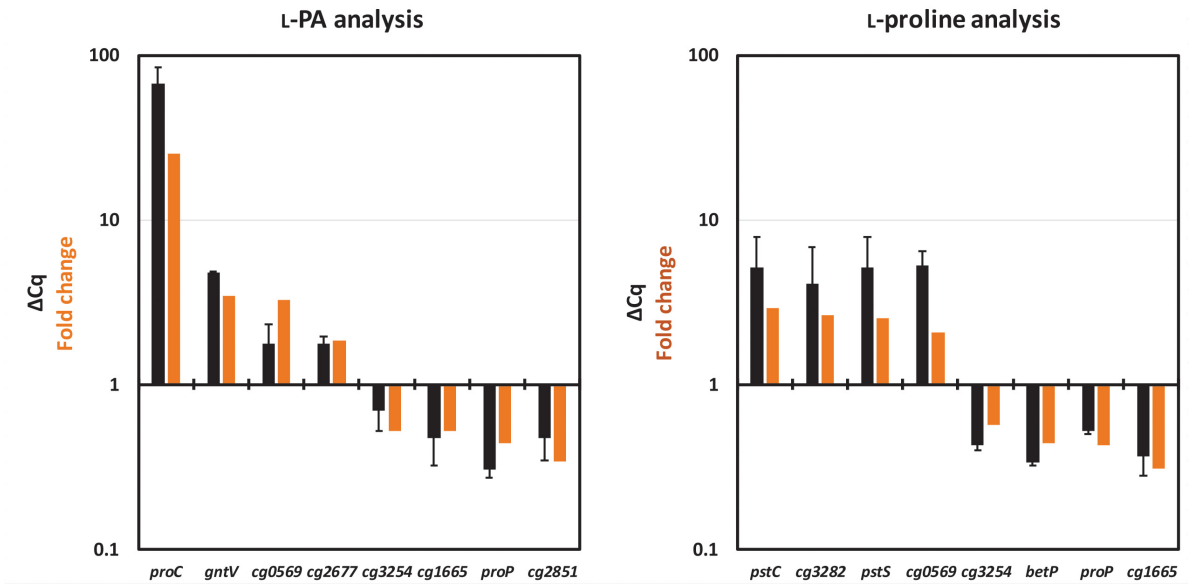


FIGURE 3 | Comparison of relative gene expression values obtained by qRT-PCR analysis (black bars) with those obtained by RNAseq analysis (orange bars). RNAseq data from **Table 5** and qRT-PCR data (ΔCq) collected for the L-PA analysis (**left**) and for the L-proline analysis (**right**) are listed. The values from the qRT-PCR are given as means and standard deviations.

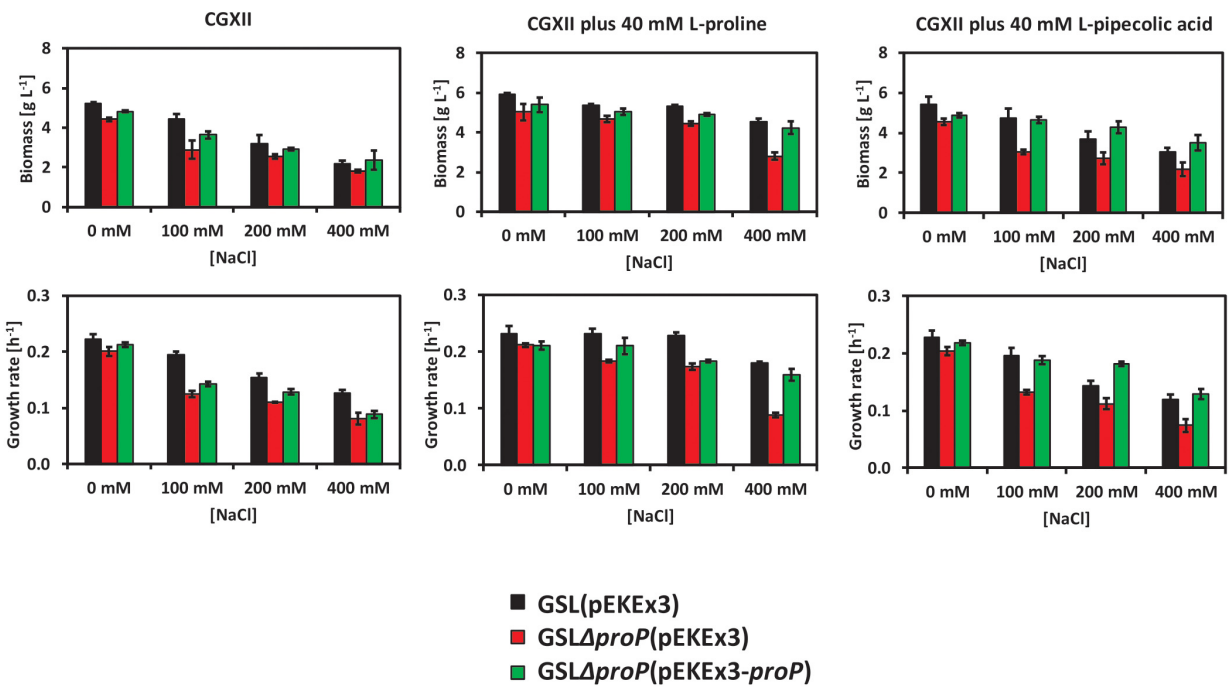


FIGURE 4 | Growth behavior of the *C. glutamicum* strains GSL(pEKEx3) (black bars), GSLΔ*proP*(pEKEx3) (red bars) and GSLΔ*proP*(pEKEx3-*proP*) (green bars) under different osmotic conditions. Biomass formation in g L^{-1} (top-left) and growth rate in h^{-1} (down-left) when growing the strains in glucose minimal medium supplemented with 0, 100, 200, or 400 mM of NaCl. Biomass formation in g L^{-1} (top-middle) and growth rate in h^{-1} (down-middle) when growing the strains in glucose minimal medium supplemented with 0, 100, 200, or 400 mM of NaCl an in presence of 40 mM of L-proline. Biomass formation in g L^{-1} (top-right) and growth rate in h^{-1} (down-right) when growing the strains in glucose minimal medium supplemented with 0, 100, 200, or 400 mM of NaCl an in presence of 40 mM of L-PA. Values represent means and standard deviations.

trehalose after an osmotic upshift or imports glycine betaine, proline or ectoine (Frings et al., 1993; Guillouet and Engasser, 1995; Skjerdal et al., 1996). In *C. glutamicum*, biosynthesis of proline involves one set of genes (*proA* for g-glutamyl phosphate reductase, *proB* for g-glutamyl kinase and *proC* for pyrroline 5-carboxylate reductase), while *B. subtilis* possesses one set of proline biosynthesis genes for anabolic purposes and a second osmostress-induced set (Bremer, 2000). Similarly, biosynthesis and internal content of L-PA is regulated by external osmolality in *Brevibacterium ammoniagenes* (Gouesbet et al., 1992). It has been described that *C. glutamicum* prefers uptake of compatible solutes to their synthesis because uptake of compatible solutes is faster and energetically more favorable than their synthesis (Morbach and Krämer, 2003). In *C. glutamicum*, glycine betaine is the most effective osmoprotectant among those that can be imported from the environment, followed by ectoine and proline (Farwick et al., 1995). Here, we have shown that 40 mM L-PA are almost as effective as 40 mM proline for osmoprotection of a lysine producing *C. glutamicum* strain (**Figures 1**, **4**). The improved growth in the presence of increasing salt concentrations is not due to catabolism of L-PA since *C. glutamicum* can use L-PA neither as carbon source nor as nitrogen source (Pérez-García et al., 2016). Rather, the external addition of L-PA is advantageous since resources for biosynthesis of L-proline for osmoprotection are not required. An osmoprotective role of L-PA has also been shown for *E. coli* since the addition of 1 mM of DL-PA decreased the inhibitory growth effect of 200–700 mM NaCl in 0.2% glucose minimal medium (Gouesbet et al., 1994). When growing *S. pomeroyi* in a mineral salts medium containing 400 mM NaCl, the supplementation of 5–20 mM of L-PA improved the growth of the microorganism (Neshich et al., 2013). In *Sinorhizobium meliloti*, both isomers, L-PA and D-PA have to be added for osmoprotective activity (Gouffi et al., 2000). Previously, we have developed strains for sustainable production of L-PA (Pérez-García et al., 2016, 2017a). Here, we have shown that recombinant *C. glutamicum* engineered for L-PA overproduction showed improved growth characteristics under hyperosmolar conditions (**Figure 4**). Thus, L-PA functions as osmo compatible solute not only when imported from the environment, but also when synthesized *de novo*. In these recombinant *C. glutamicum* strain synthesis is not osmostress-induced as described for certain plants. For example, in rapeseed leaf tissues, L-PA synthesis from L-lysine via the lysine-ketoglutarate reductase/saccharopine dehydrogenase pathway is osmo-dependent (Moulin et al., 2006).

Although uptake of osmo compatible solutes is important and energetically favored over their *de novo* synthesis, a *C. glutamicum* mutant devoid of the five known uptake systems for compatible solutes survived under hyperosmolar conditions (Steger et al., 2004) which has been attributed to *de novo* synthesis of proline, glutamine, and trehalose (Rönsch et al., 2003). The secondary carriers PutP, BetP, EctP, LcoP, and ProP differ by their substrate spectrum and substrate affinities, however, they show a degree of substrate redundancy that is typical for soil bacteria (Peter et al., 1997, 1998; Wood et al., 2001; Weinand et al., 2007). BetP is a high affinity carrier specific for betaine (Peter et al., 1997). EctP is a low affinity carrier for betaine, ectoine and proline and LcoP a low affinity carrier for betaine and ectoine, whereas the carrier ProP shows high affinity for its substrates proline and ectoine (Peter et al., 1998; Steger et al., 2004). PutP imports proline with high affinity for anabolic purposes (Peter et al., 1997). Based on differential gene expression analysis ProP was identified as possible L-PA import system in this work (**Table 5**). In *E. coli* various structural analogs of L-proline such as azetidine-2-carboxylate, L-pipecolic acid or 5-hydroxy-L-pipecolic acid enter the cell through ProP or ProU transport systems (Gouesbet et al., 1994). The *E. coli* proline/glycine betaine transporter ProP shares 36.8% identical amino acids with ProP from *C. glutamicum*. Here, growth analysis of *C. glutamicum* mutants lacking *proP* revealed perturbed growth under hyperosmolar conditions in the absence of *proP* (**Figure 4**). Since mutants lacking *proP* still possess functional EctP and PutP these carriers apparently do not contribute to uptake of L-PA under the chosen conditions, and, thus, ProP may act as major L-PA import system in *C. glutamicum*. Detailed biochemical transport assays will have to be performed in the future to characterize L-PA uptake by ProP.

It was also shown in this work that the MSC YggB performs as a major escape valve for L-PA in *C. glutamicum* (**Figure 2**). After an osmotic downshift compatible solutes are released to the medium involving MSCs (Morbach and Krämer, 2003). In particular, the MSC YggB was described as the main export system of L-glutamate in *C. glutamicum* (Nakamura et al., 2007). In *C. glutamicum* it is known that the use of biotin limitation, penicillin treatments or surfactants alter membrane tension by inhibiting lipid or peptidoglycan synthesis which triggers conformational changes in YggB allowing L-glutamate export (Duperray et al., 1992; Gutmann et al., 1992). In addition, betaine efflux induced by osmotic downshock was reduced upon deletion of *yggB* (Nottebrock et al., 2003). Thus, the decreased rate of L-PA accumulation in the supernatant as consequence of *yggB* deletion suggests that L-PA may be exported from the *C. glutamicum* cell by YggB. In depth biochemical analysis is required to characterize export of L-PA by YggB.

Due to their diverse applications in drug development, food industry, skin care products and cosmetics (Graf et al., 2008; Jorge C.D. et al., 2016; Li and Gänzle, 2016) the biotechnological production of compatible solutes has gained increasing momentum recently (Sauer and Galinski, 1998; Jensen and Wendisch, 2013; Tan et al., 2016; Chen et al., 2017). This included the establishment of strains that produce and secrete compatible solutes such as ectoine, L-PA or α-D-glucosylglycerol that are not synthesized by the wild-type strains (Ning et al., 2016; Pérez-García et al., 2017a,b; Ying et al., 2017; Roenneke et al., 2018). Production of L-PA by recombinant *E. coli* expressing the gene for lysine cyclodeaminase from *Streptomyces hygroscopicus* was established with a titer of 5.33 g L^{-1} L-PA and a yield of 0.13 g L^{-1} of glucose obtained in fed-batch cultivation and a titer of 0.64 g L^{-1} L-PA in shake flasks (Ying et al., 2017). Our previous work on establishing L-PA production in *C. glutamicum* led to superior values: 14.4 g L^{-1} L-PA and a yield of 0.20 g g^{-1} in fed-batch cultivation and a titer of 3.9 g L^{-1} L-PA in shake flasks (Pérez-García et al., 2017a). Although *de novo* synthesized L-PA protected *C. glutamicum* against high salt

conditions, we have observed that in the presence of 200 mM NaCl the L-PA titer in the supernatant was reduced from about 15 mM (about 1.9 g L^{-1}) to about half (**Figure 2A**, left panel). Thus, hyperosmolar conditions are not favorable for L-PA production by the *C. glutamicum* recombinant strains described here. However, while less L-PA was secreted under hyperosmolar conditions, more L-PA accumulated intracellularly (**Figure 2A**, left panel). After osmotic downshift, L-PA was released to the culture medium accumulating to about 22 mM (about 2.8 g L^{-1}) (**Figure 2A**, right panel). Therefore, in principle, the described *C. glutamicum* strains could be used in a process called "bacterial milking" (Sauer and Galinski, 1998). The Gram-negative bacterium *Halomonas elongata* was grown to high-cell-density (48 g cell dry weight per liter) before being exposed to alternating hyper- and hypo-osmolar conditions. Ectoine released to the hypo-osmolar medium was harvested by crossflow filtration and by this procedure 0.16 g of ectoine per cycle per gram cell dry weight could be produced (Sauer and Galinski, 1998). As use of high-salinity media in fermentation processes is costly and poses challenges with regard to the design and durability of bioreactors, it is generally assumed that direct fermentative production is preferred over the "bacterial milking" process. To determine if this notion holds true for the L-PA producing *C. glutamicum* strains described here, a head-to-head comparison of these strains operated in a fed-batch fermentation process vs. a "bacterial milking" process will have to be performed after each process has been thoroughly optimized by process intensification. In addition, strain optimization by transport engineering, as described for the production of amino acids (Nakamura et al., 2007; Blombach et al., 2009), non-proteinogenic amino acids (Jorge J.M.P. et al., 2016; Pérez-García et al., 2017a), diamines (Kind et al., 2014; Nguyen et al., 2015) or organic acids (Huhn et al., 2011), may be required.

DATA AVAILABILITY

The datasets generated for this study can be found in http://www.ncbi.nlm.nih.gov/geo/, GSE122249.

AUTHOR CONTRIBUTIONS

FP-G, LB, and VW designed the study. FP-G and LB performed the experiments. FP-G, LB, and VW analyzed the data. FP-G and LB drafted the manuscript. VW finalized the manuscript. All authors read and approved the final version of the manuscript.

FUNDING

FP-G was funded by the University of Bielefeld (Bielefeld Young Researchers Fund, Germany). LB was funded by the Science without Borders program (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior, Brazil). The funding agencies were not involved in the design of the study, collection, analysis, and interpretation of data and in writing the manuscript.

ACKNOWLEDGMENTS

We thank Dr. Tobias Busche and Prof. Dr. Jörn Kalinowski from the technology platform Genomics of CeBiTec for the kind assistance with the preparation and sequencing of the cDNA libraries.

REFERENCES

Anders, S., and Huber, W. (2010). Differential expression analysis for sequence count data. *Genome Biol.* 11:R106. doi: 10.1186/gb-2010-11-10-r106

Blombach, B., Hans, S., Bathe, B., and Eikmanns, B. J. (2009). Acetohydroxyacid synthase, a novel target for improvement of L-lysine production by *Corynebacterium glutamicum*. *Appl. Environ. Microbiol.* 75, 419–427. doi: 10.1128/AEM.01844-08

Bolger, A. M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinforma. Oxf. Engl.* 30, 2114–2120. doi: 10.1093/bioinformatics/btu170

Bolten, C. J., Kiefer, P., Letisse, F., Portais, J.-C., and Wittmann, C. (2007). Sampling for metabolome analysis of microorganisms. *Anal. Chem.* 79, 3843–3849. doi: 10.1021/ac0623888

Börngen, K., Battle, A. R., Möker, N., Morbach, S., Marin, K., Martinac, B., et al. (2010). The properties and contribution of the *Corynebacterium glutamic*um MscS variant to fine-tuning of osmotic adaptation. *Biochim. Biophys. Acta* 1798, 2141–2149. doi: 10.1016/j.bbamem.2010.06.022

Bremer, E. (2000). Coping with osmotic challenges: osmoregulation through accumulation and release of compatible solutes in B. subtilis. *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* 126:17.

Brito, L. F., Irla, M., Kalinowski, J., and Wendisch, V. F. (2017). Detailed transcriptome analysis of the plant growth promoting *Paenibacillus riograndensis* SBR5 by using RNA-seq technology. *BMC Genomics* 18:846. doi: 10.1186/s12864-017-4235-z

Chen, R., Zhu, L., Lv, L., Yao, S., Li, B., and Qian, J. (2017). Optimization of the extraction and purification of the compatible solute ectoine from *Halomonas elongata* in the laboratory experiment of a commercial production project. *World J. Microbiol. Biotechnol.* 33:116. doi: 10.1007/s11274-017-2281-y

Crooks, G. E., Hon, G., Chandonia, J.-M., and Brenner, S. E. (2004). WebLogo: a sequence logo generator. *Genome Res.* 14, 1188–1190. doi: 10.1101/gr.849004

Czech, L., Hermann, L., Stöveken, N., Richter, A. A., Höppner, A., Smits, S. H. J., et al. (2018). Role of the extremolytes ectoine and hydroxyectoine as stress protectants and nutrients: genetics, phylogenomics, biochemistry, and structural analysis. *Genes* 9:E177. doi: 10.3390/genes9040177

da Costa, M. S., Santos, H., and Galinski, E. A. (1998). An overview of the role and diversity of compatible solutes in bacteria and archaea. *Adv. Biochem. Eng. Biotechnol.* 61, 117–153.

Duperray, F., Jezequel, D., Ghazi, A., Letellier, L., and Shechter, E. (1992). Excretion of glutamate from *Corynebacterium glutamicum* triggered by amine surfactants. *Biochim. Biophys. Acta Biomembr.* 1103, 250–258. doi: 10.1016/0005-2736(92)90094-3

Eggeling, L., and Bott, M. (2005). *Handbook of Corynebacterium glutamicum*. Boca Raton, FL: CRC Press.

Farwick, M., Siewe, R. M., and Krämer, R. (1995). Glycine betaine uptake after hyperosmotic shift in *Corynebacterium glutamicum*. *J. Bacteriol.* 177, 4690–4695.

Frings, E., Kunte, H. J., and Galinski, E. A. (1993). Compatible solutes in representatives of the genera *Brevibacterium* and *Corynebacterium*: occurrence of tetrahydropyrimidines and glutamine. *FEMS Microbiol. Lett.* 109, 25–32. doi: 10.1111/j.1574-6968.1993.tb06138.x

Gibson, D. G. (2011). Enzymatic assembly of overlapping DNA fragments. *Methods Enzymol.* 498, 349–361. doi: 10.1016/B978-0-12-385120-8.00015-2

Gouesbet, G., Blanco, C., Hamelin, J., and Bernard, T. (1992). Osmotic adjustment in *Brevibacterium ammoniagenes*: pipecolic acid accumulation at elevated osmolalities. *Microbiology* 138, 959–965. doi: 10.1099/00221287-138-5-959

Gouesbet, G., Jebbar, M., Talibart, R., Bernard, T., and Blanco, C. (1994). Pipecolic acid is an osmoprotectant for *Escherichia coli* taken up by the general osmoporters ProU and ProP. *Microbiol. Read. Engl.* 140(Pt 9), 2415–2422. doi: 10.1099/13500872-140-9-2415

Gouffi, K., Bernard, T., and Blanco, C. (2000). Osmoprotection by pipecolic acid in *Sinorhizobium meliloti*: specific effects of d and l isomers. *Appl. Environ. Microbiol.* 66, 2358–2364.

Graf, R., Anzali, S., Buenger, J., Pfluecker, F., and Driller, H. (2008). The multifunctional role of ectoine as a natural cell protectant. *Clin. Dermatol.* 26, 326–333. doi: 10.1016/j.clindermatol.2008.01.002

Guillouet, S., and Engasser, J. M. (1995). Sodium and proline accumulation in *Corynebacterium glutamicum* as a response to an osmotic saline upshock. *Appl. Microbiol. Biotechnol.* 43, 315–320. doi: 10.1007/BF00172831

Gutmann, M., Hoischen, C., and Krämer, R. (1992). Carrier-mediated glutamate secretion by *Corynebacterium glutamicum* under biotin limitation. *Biochim. Biophys. Acta* 1112, 115–123.

Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J. Mol. Biol.* 166, 557–580.

Hilker, R., Stadermann, K. B., Doppmeier, D., Kalinowski, J., Stoye, J., Straube, J., et al. (2014). ReadXplorer–visualization and analysis of mapped sequences. *Bioinformatics* 30, 2247–2254. doi: 10.1093/bioinformatics/btu205

Hoffmann, T., and Bremer, E. (2017). Guardians in a stressful world: the Opu family of compatible solute transporters from *Bacillus subtilis*. *Biol. Chem.* 398, 193–214. doi: 10.1515/hsz-2016-0265

Huhn, S., Jolkver, E., Krämer, R., and Marin, K. (2011). Identification of the membrane protein SucE and its role in succinate transport in *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.* 89, 327–335. doi: 10.1007/s00253-010-2855-1

Irla, M., Neshat, A., Brautaset, T., Rückert, C., Kalinowski, J., and Wendisch, V. F. (2015). Transcriptome analysis of thermophilic methylotrophic *Bacillus methanolicus* MGA3 using RNA-sequencing provides detailed insights into its previously uncharted transcriptional landscape. *BMC Genomics* 16:73. doi: 10.1186/s12864-015-1239-4

Jensen, J. V. K., and Wendisch, V. F. (2013). Ornithine cyclodeaminase-based proline production by *Corynebacterium glutamicum*. *Microb. Cell Fact.* 12:63. doi: 10.1186/1475-2859-12-63

Jorge, C. D., Borges, N., Bagyan, I., Bilstein, A., and Santos, H. (2016). Potential applications of stress solutes from extremophiles in protein folding diseases and healthcare. *Extremophiles* 20, 251–259. doi: 10.1007/s00792-016-0828-8

Jorge, J. M. P., Nguyen, A. Q. D., Pérez-García, F., Kind, S., and Wendisch, V. F. (2016). Improved fermentative production of gamma-aminobutyric acid via the putrescine route: systems metabolic engineering for production from glucose, amino sugars and xylose. *Biotechnol. Bioeng.* 114, 862–873. doi: 10.1002/bit.26211

Jorge, J. M. P., Pérez-García, F., and Wendisch, V. F. (2017). A new metabolic route for the fermentative production of 5-aminovalerate from glucose and alternative carbon sources. *Bioresour. Technol.* 245, 1701–1709. doi: 10.1016/j.biortech.2017.04.108

Kalinowski, J., Bathe, B., Bartels, D., Bischoff, N., Bott, M., Burkovski, A., et al. (2003). The complete *Corynebacterium glutamicum* ATCC 13032 genome sequence and its impact on the production of L-aspartate-derived amino acids and vitamins. *J. Biotechnol.* 104, 5–25.

Kempf, B., and Bremer, E. (1998). Uptake and synthesis of compatible solutes as microbial stress responses to high-osmolality environments. *Arch. Microbiol.* 170, 319–330.

Kind, S., Jeong, W. K., Schröder, H., and Wittmann, C. (2010). Systems-wide metabolic pathway engineering in *Corynebacterium glutamicum* for bio-based production of diaminopentane. *Metab. Eng.* 12, 341–351. doi: 10.1016/j.ymben.2010.03.005

Kind, S., Neubauer, S., Becker, J., Yamamoto, M., Völkert, M., Abendroth, G., et al. (2014). From zero to hero - production of bio-based nylon from renewable resources using engineered *Corynebacterium glutamicum*. *Metab. Eng.* 25, 113–123. doi: 10.1016/j.ymben.2014.05.007

Klingenberg, M., and Pfaff, E. (1967). "Means or terminating reactions," in *Methods of Enzymology*, Vol. X, eds R. W. Estabrook and M. R. Pullamnn (New York, NY: Academic press), 680–684.

Langmead, B., Trapnell, C., Pop, M., and Salzberg, S. L. (2009). Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 10:R25. doi: 10.1186/gb-2009-10-3-r25

Li, H., and Gänzle, M. (2016). Some like it hot: heat resistance of *Escherichia coli* in food. *Front. Microbiol.* 7:1763. doi: 10.3389/fmicb.2016.01763

Lubitz, D., and Wendisch, V. F. (2016). Ciprofloxacin triggered glutamate production by *Corynebacterium glutamicum*. *BMC Microbiol.* 16:235. doi: 10.1186/s12866-016-0857-6

Mentz, A., Neshat, A., Pfeifer-Sancar, K., Pühler, A., Rückert, C., and Kalinowski, J. (2013). Comprehensive discovery and characterization of small RNAs in *Corynebacterium glutamicum* ATCC 13032. *BMC Genomics* 14:714. doi: 10.1186/1471-2164-14-714

Mimitsuka, T., Sawai, H., Hatsu, M., and Yamada, K. (2007). Metabolic engineering of *Corynebacterium glutamicum* for cadaverine fermentation. *Biosci. Biotechnol. Biochem.* 71, 2130–2135. doi: 10.1271/bbb.60699

Morbach, S., and Krämer, R. (2003). Impact of transport processes in the osmotic response of *Corynebacterium glutamicum*. *J. Biotechnol.* 104, 69–75.

Moulin, M., Deleu, C., Larher, F., and Bouchereau, A. (2006). The lysine-ketoglutarate reductase–saccharopine dehydrogenase is involved in the osmo-induced synthesis of pipecolic acid in rapeseed leaf tissues. *Plant Physiol. Biochem.* 44, 474–482. doi: 10.1016/j.plaphy.2006.08.005

Nakamura, J., Hirano, S., Ito, H., and Wachi, M. (2007). Mutations of the *Corynebacterium glutamicum* NCgl1221 gene, encoding a mechanosensitive channel homolog, induce L-glutamic acid production. *Appl. Environ. Microbiol.* 73, 4491–4498. doi: 10.1128/AEM.02446-06

Naville, M., Ghuillot-Gaudeffroy, A., Marchais, A., and Gautheret, D. (2011). ARNold: a web tool for the prediction of Rho-independent transcription terminators. *RNA Biol.* 8, 11–13.

Neshich, I. A. P., Kiyota, E., and Arruda, P. (2013). Genome-wide analysis of lysine catabolism in bacteria reveals new connections with osmotic stress resistance. *ISME J.* 7, 2400–2410. doi: 10.1038/ismej.2013.123

Nguyen, A. Q. D., Schneider, J., Reddy, G. K., and Wendisch, V. F. (2015). Fermentative production of the diamine putrescine: system metabolic engineering of *Corynebacterium glutamicum*. *Metabolites* 5, 211–231. doi: 10.3390/metabo5020211

Ning, Y., Wu, X., Zhang, C., Xu, Q., Chen, N., and Xie, X. (2016). Pathway construction and metabolic engineering for fermentative production of ectoine in *Escherichia coli*. *Metab. Eng.* 36, 10–18. doi: 10.1016/j.ymben.2016.02.013

Nottebrock, D., Meyer, U., Krämer, R., and Morbach, S. (2003). Molecular and biochemical characterization of mechanosensitive channels in *Corynebacterium glutamicum*. *FEMS Microbiol. Lett.* 218, 305–309.

Pérez-García, F., Max Risse, J., Friehs, K., and Wendisch, V. F. (2017a). Fermentative production of L-pipecolic acid from glucose and alternative carbon sources. *Biotechnol. J.* 12:1600646. doi: 10.1002/biot.201600646

Pérez-García, F., Ziert, C., Risse, J. M., and Wendisch, V. F. (2017b). Improved fermentative production of the compatible solute ectoine by *Corynebacterium glutamicum* from glucose and alternative carbon sources. *J. Biotechnol.* 258, 59–68. doi: 10.1016/j.jbiotec.2017.04.039

Pérez-García, F., Peters-Wendisch, P., and Wendisch, V. F. (2016). Engineering *Corynebacterium glutamicum* for fast production of L-lysine and L-pipecolic acid. *Appl. Microbiol. Biotechnol.* 100, 8075–8090. doi: 10.1007/s00253-016-7682-6

Peter, H., Bader, A., Burkovski, A., Lambert, C., and Krämer, R. (1997). Isolation of the *putP* gene of *Corynebacterium glutamicum* and characterization of a low-affinity uptake system for compatible solutes. *Arch. Microbiol.* 168, 143–151.

Peter, H., Weil, B., Burkovski, A., Krämer, R., and Morbach, S. (1998). *Corynebacterium glutamicum* is equipped with four secondary carriers for compatible solutes: identification, sequencing, and characterization of the proline/ectoine uptake system, ProP, and the ectoine/proline/glycine betaine carrier, EctP. *J. Bacteriol.* 180, 6005–6012.

Peters-Wendisch, P. G., Schiel, B., Wendisch, V. F., Katsoulidis, E., Möckel, B., Sahm, H., et al. (2001). Pyruvate carboxylase is a major bottleneck for glutamate and lysine production by *Corynebacterium glutamicum*. *J. Mol. Microbiol. Biotechnol.* 3, 295–300.

Roenneke, B., Rosenfeldt, N., Derya, S. M., Novak, J. F., Marin, K., Krämer, R., et al. (2018). Production of the compatible solute α-D-glucosylglycerol by metabolically engineered *Corynebacterium glutamicum*. *Microb. Cell Fact.* 17:94. doi: 10.1186/s12934-018-0939-2

Rönsch, H., Krämer, R., and Morbach, S. (2003). Impact of osmotic stress on volume regulation, cytoplasmic solute composition and lysine production in *Corynebacterium glutamicum* MH20-22B. *J. Biotechnol.* 104, 87–97.

Ruffert, S., Berrier, C., Krämer, R., and Ghazi, A. (1999). Identification of mechanosensitive ion channels in the cytoplasmic membrane of *Corynebacterium glutamicum*. *J. Bacteriol.* 181, 1673–1676.

Ruffert, S., Lambert, C., Peter, H., Wendisch, V. F., and Krämer, R. (1997). Efflux of compatible solutes in *Corynebacterium glutamicum* mediated by osmoregulated channel activity. *Eur. J. Biochem. FEBS* 247, 572–580.

Sajjad, W., Qadir, S., Ahmad, M., Rafiq, M., Hasan, F., Tehan, R., et al. (2018). Ectoine: a compatible solute in radio-halophilic *Stenotrophomonas sp. WMA-LM19* strain to prevent ultraviolet-induced protein damage. *J. Appl. Microbiol.* 125, 457–467. doi: 10.1111/jam.13903

Sauer, T., and Galinski, E. A. (1998). Bacterial milking: a novel bioprocess for production of compatible solutes. *Biotechnol. Bioeng.* 57, 306–313.

Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G., and Pühler, A. (1994). Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene* 145, 69–73.

Schaffer, S., Weil, B., Nguyen, V. D., Dongmann, G., Günther, K., Nickolaus, M., et al. (2001). A high-resolution reference map for cytoplasmic and membrane-associated proteins of Corynebacterium glutamicum. *Electrophoresis* 22, 4404–4422.

Schneider, J., and Wendisch, V. F. (2010). Putrescine production by engineered *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.* 88, 859–868. doi: 10.1007/s00253-010-2778-x

Simon, R., Priefer, U., and Pühler, A. (1983). A broad host range mobilization system for in vivo genetic engineering: transposon mutagenesis in gram negative bacteria. *Nat. Biotechnol.* 1, 784–791. doi: 10.1038/nbt1183-784

Skjerdal, O. T., Sletta, H., Flenstad, S. G., Josefsen, K. D., Levine, D. W., and Ellingsen, T. E. (1996). Changes in intracellular composition in response to hyperosmotic stress of NaCl, sucrose or glutamic acid in *Brevibacterium lactofermentum* and *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.* 44, 635–642. doi: 10.1007/BF00172497

Stansen, C., Uy, D., Delaunay, S., Eggeling, L., Goergen, J. L., and Wendisch, V. F. (2005). Characterization of a *Corynebacterium glutamicum* lactate utilization operon induced during temperature-triggered glutamate production. *Appl. Environ. Microbiol.* 71, 5920–5928. doi: 10.1128/AEM.71.10.5920-5928.2005

Steger, R., Weinand, M., Krämer, R., and Morbach, S. (2004). LcoP, an osmoregulated betaine/ectoine uptake system from *Corynebacterium glutamicum*. *FEBS Lett.* 573, 155–160. doi: 10.1016/j.febslet.2004.07.067

Sun, Y., Guo, W., Wang, F., Peng, F., Yang, Y., Dai, X., et al. (2016). Transcriptome and multivariable data analysis of *Corynebacterium glutamicum* under different dissolved oxygen conditions in bioreactors. *PLoS One* 11:e0167156. doi: 10.1371/journal.pone.0167156

Tan, X., Luo, Q., and Lu, X. (2016). Biosynthesis, biotechnological production, and applications of glucosylglycerols. *Appl. Microbiol. Biotechnol.* 100, 6131–6139. doi: 10.1007/s00253-016-7608-3

Tribelli, P. M., and López, N. I. (2018). Reporting key features in cold-adapted bacteria. *Life* 8:E8. doi: 10.3390/life8010008

Vranova, V., Lojkova, L., Rejsek, K., and Formanek, P. (2013). Significance of the natural occurrence of L- versus D-pipecolic acid: a review. *Chirality* 25, 823–831. doi: 10.1002/chir.22237

Weinand, M., Krämer, R., and Morbach, S. (2007). Characterization of compatible solute transporter multiplicity in *Corynebacterium glutamicum*. *Appl. Microbiol. Biotechnol.* 76, 701–708. doi: 10.1007/s00253-007-0938-4

Wolf, A., Krämer, R., and Morbach, S. (2003). Three pathways for trehalose metabolism in *Corynebacterium glutamicum* ATCC13032 and their significance in response to osmotic stress. *Mol. Microbiol.* 49, 1119–1134.

Wood, J. M. (1999). Osmosensing by bacteria: signals and membrane-based sensors. *Microbiol. Mol. Biol. Rev.* 63, 230–262.

Wood, J. M., Bremer, E., Csonka, L. N., Kraemer, R., Poolman, B., van der Heide, T., et al. (2001). Osmosensing and osmoregulatory compatible solute accumulation by bacteria. *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* 130, 437–460.

Ying, H., Tao, S., Wang, J., Ma, W., Chen, K., Wang, X., et al. (2017). Expanding metabolic pathway for de novo biosynthesis of the chiral pharmaceutical intermediate L-pipecolic acid in *Escherichia coli*. *Microb. Cell Fact.* 16:52. doi: 10.1186/s12934-017-0666-0

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

REVIEW
published: 26 February 2019
doi: 10.3389/fmicb.2019.00302

Overview of the Antimicrobial Compounds Produced by Members of the *Bacillus subtilis* Group

Simon Caulier[1,2†], Catherine Nannan[1†], Annika Gillis[1], Florent Licciardi[1], Claude Bragard[2] and Jacques Mahillon[1*]*

[1] *Laboratory of Food and Environmental Microbiology, Earth and Life Institute, Université catholique de Louvain, Louvain-la-Neuve, Belgium,* [2] *Laboratory of Phytopathology-Applied Microbiology, Earth and Life Institute, Université catholique de Louvain, Louvain-la-Neuve, Belgium*

Over the last seven decades, applications using members of the *Bacillus subtilis* group have emerged in both food processes and crop protection industries. Their ability to form survival endospores and the plethora of antimicrobial compounds they produce has generated an increased industrial interest as food preservatives, therapeutic agents and biopesticides. In the growing context of food biopreservation and biological crop protection, this review suggests a comprehensive way to visualize the antimicrobial spectrum described within the *B. subtilis* group, including volatile compounds. This classification distinguishes the bioactive metabolites based on their biosynthetic pathways and chemical nature: *i.e.*, ribosomal peptides (RPs), volatile compounds, polyketides (PKs), non-ribosomal peptides (NRPs), and hybrids between PKs and NRPs. For each clade, the chemical structure, biosynthesis and antimicrobial activity are described and exemplified. This review aims at constituting a convenient and updated classification of antimicrobial metabolites from the *B. subtilis* group, whose complex phylogeny is prone to further development.

Keywords: *Bacillus subtilis* group, bacteriocins, biocontrol, biosynthetic pathways, lipopeptides, polyketides, siderophores, volatile

OPEN ACCESS

Edited by:
José E. Barboza-Corona, Universidad de Guanajuato, Mexico

Reviewed by:
Stefan Junne, Technische Universität Berlin, Germany
Jeongdae Im, Kansas State University, United States

***Correspondence:**
Claude Bragard
claude.bragard@uclouvain.be
Jacques Mahillon
jacques.mahillon@uclouvain.be

† These authors have contributed equally to this work

Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: *05 November 2018*
Accepted: *05 February 2019*
Published: *26 February 2019*

Citation:
Caulier S, Nannan C, Gillis A, Licciardi F, Bragard C and Mahillon J (2019) Overview of the Antimicrobial Compounds Produced by Members of the Bacillus subtilis Group. Front. Microbiol. 10:302. doi: 10.3389/fmicb.2019.00302

INTRODUCTION

The genus *Bacillus* comprises 377 species[1] (last update in January 2019) of Gram-positive, rod-shaped bacteria (Gordon et al., 1973). Their ability to form endospores, their diversity in physiological properties, as well as their capacity to produce numerous antimicrobial compounds (AMCs) favor their ubiquitous distribution in soil, aquatic environments, food and gut microbiota of arthropods and mammals (Nicholson, 2002).

Bacteria from the *Bacillus subtilis* group consist of small vegetative cells (<1 μm-wide) for which the strain *B. subtilis* subsp. *subtilis* 168 is considered as model organism (Barbe et al., 2009). They are usually mesophilic and neutrophilic, although some can tolerate high pH. The four original species of the group (*B. subtilis, Bacillus licheniformis*, *Bacillus pumilus*, and *Bacillus amyloliquefaciens*) were discovered more than 40 years ago (Gordon et al., 1973; Priest et al., 1987). Since then, the evolution of their molecular, chemotaxonomic and physiological characterizations led to regular re-evaluations and (re-)description of numerous novel species and subspecies (see current taxonomy of the group in **Figure 1**) (Fan et al., 2017).

[1] http://www.bacterio.net/bacillus.html

The potential of *B. subtilis* group strains to produce a wide diversity of secondary metabolites mediating antibiosis was recognized for decades. For any given strain of the *B. subtilis* group, it is now estimated that at least 4–5% of its genome is devoted to antimicrobial compounds (AMCs) production (Stein, 2005). These molecules are mainly antimicrobial peptides (AMPs). Their structures are usually cyclic, hydrophobic and contain peculiar moieties such as D-amino acids (AA) or intramolecular thioether bonds. In addition to AMPs, volatile metabolites also constitute a large family of antimicrobials exhibiting numerous metabolic and functional roles.

Due to the wide diversity of these molecules, their classification is rather complex and can be based on several criteria such as their biosynthetic machinery, sources, biological functions, properties, three-dimensional structure, covalent bonding pattern or molecular targets (Tagg et al., 1976; Wang et al., 2015). Here a classification of the *B. subtilis* group antimicrobial molecules is proposed, based on their biosynthetic pathways and their chemical nature as shown in **Figure 2**. This review will emphasize the biosynthesis pathway and the bioactivity of the main clades of AMCs within the *B. subtilis* group: *i.e.*, the ribosomal peptides (RPs) (bacteriocins and enzymes), the polyketides (PKs), the non-ribosomal peptides (NRPs) and the volatiles. A full overview of this chart is provided as **Supplementary Material** (**Supplementary Figure S1**).

RIBOSOMAL PEPTIDES

Ribosomally synthesized peptides (RPs) are usually derived from short precursors (*ca.* 100 AA) and are processed to mature compounds through post-translational modifications (Oman and van der Donk, 2009). Various enzymes mediate these modifications and therefore generate a wide diversity of chemical structures. Most of these peptides were originally referred to as "bacteriocins," characterized as low molecular weight molecules that exhibit inhibiting growth activities against bacteria closely related to the producing strain (Klaenhammer, 1988; Chopra et al., 2015). In addition to bacteriocins, other types of enzymes exhibiting antagonistic activities are also ribosomally synthesized. However, those compounds display diverse metabolic activities such as quorum sensing (QS) mediation, cell lysis or induction of genetic competence (Schmidt, 2010; Shafi et al., 2017). It should also be noted that molecules referred to as BLIS (bacteriocins-like inhibitory substances) include AMPs for which the ribosomal synthesis has not been confirmed yet (Abriouel et al., 2011).

B. subtilis Group Bacteriocins

It is estimated that 99% of the bacteria and archaea are able to produce at least one bacteriocin. Historically, lactic acid bacteria (LAB) were studied as main bacteriocin producers, mostly because of their long history of safe use in food fermentation (O'Sullivan et al., 2002). Nisin (**Figure 3C**), produced by *Lactobacillus lactis* subsp. *lactis*, was approved as a food additive in the 1960s and has since then been used in over 50 countries for its antimicrobial activity against Gram-positive pathogens such as *Clostridium* spp. and *Bacillus* spp. (Klaenhammer, 1988; Delves-Broughton, 1990). However, the search for new bioactive molecules has rapidly expanded to other bacteriocin-producing genera, with a particular attention, in the late 1990s, to the GRAS (generally recognized as safe) *Bacillus* species whose bacteriocin antimicrobial spectra were broader than those of LAB (Pedersen et al., 2002; Riley and Wertz, 2002; Sumi et al., 2015).

The generic biosynthetic pathway of *Bacillus* species bacteriocins includes several post-translational modifications, including the proteolytic cleavage of the leader peptide at the N-terminal end (McIntosh et al., 2009). The modifications of active peptides, its secretion and the immunity to the bacteriocin (as described below) vary depending on the bacteriocin class.

While many classifications have been suggested over the years, one reasonable way to cope with the diversity of the *Bacillus* bacteriocins is to sort them on the basis of their biosynthetic pathway as previously reported for *Streptococcus* spp. and *Enterococcus* spp. bacteriocins (Nes et al., 2007) and reviewed in Abriouel et al. (2011). Accordingly, three main classes subdivided into several subclasses can be distinguished for the *B. subtilis* group. As detailed in **Table 1**, Class I includes the post-translationally modified peptides such as the lantibiotics whereas the non-modified peptides are grouped in Class II; Class III involved bacteriocins larger than 10 kDa (Abriouel et al., 2011). **Supplementary Table S1** summarizes the different RPs produced by the strains belonging to the *B. subtilis* group, as well as their reported antimicrobial activities.

Class I includes small AMPs (19–38 AA) with extensive post-translational modifications. Subclasses I.1, I.2, and I.3 have in common their lantibiotic structure, which refers to inter-residual thioester bonds made of modified AA residues. As illustrated in **Figure 3**, lantibiotics involve 2,3-didehydroalanine (Dha) and (Z)-2,3-didehydrobutyrine (Dhb), resulting from the dehydration of serine and threonine residues, respectively. The intra-molecular addition of Dha or Dhb on a cysteine residue leads to the respective formation of lanthionine and methyllanthionine bridges (Willey and Donk, 2007). Subtilin (**Figure 3B**), from subclass I.1, is one of the most studied bacteriocins from the *B. subtilis* group. Its structure shares several similarities with nisin A lantibiotics, shown in **Figure 3C** (Guder et al., 2000; Abriouel et al., 2011). Peptides from subclass I.4 undergo other types of modifications. For instance, subtilosin A is a head-to-tail cyclic peptide with unusual inter-residue linkages (*i.e.*, Cys-Phe bond) (Marx et al., 2001; Kawulka et al., 2004).

Class II bacteriocins include small (<10 kDa), linear and non-modified peptides, resistant to heat and acido-basic treatments. They are divided in three subclasses based on a conserved AA motif near their N-terminus. The YGNGVXC (X is any AA) motif is associated to pediocin-like peptides from subclass II.1 whereas DWTXWSXL is specific to thuricin-like peptides from subclass II.2. Subclass II.3 comprises the small non-modified AMPs without any typical motif in their AA sequence (Abriouel et al., 2011). Finally, class III bacteriocins consist into large and heat labile molecules, generally characterized by a phospholipase activity (Cleveland et al., 2001).

Because of their wide diversity, bacteriocins display different modes of action such as protoplasm vesicularization, pore formation or cell disintegration (Sumi et al., 2015). They are

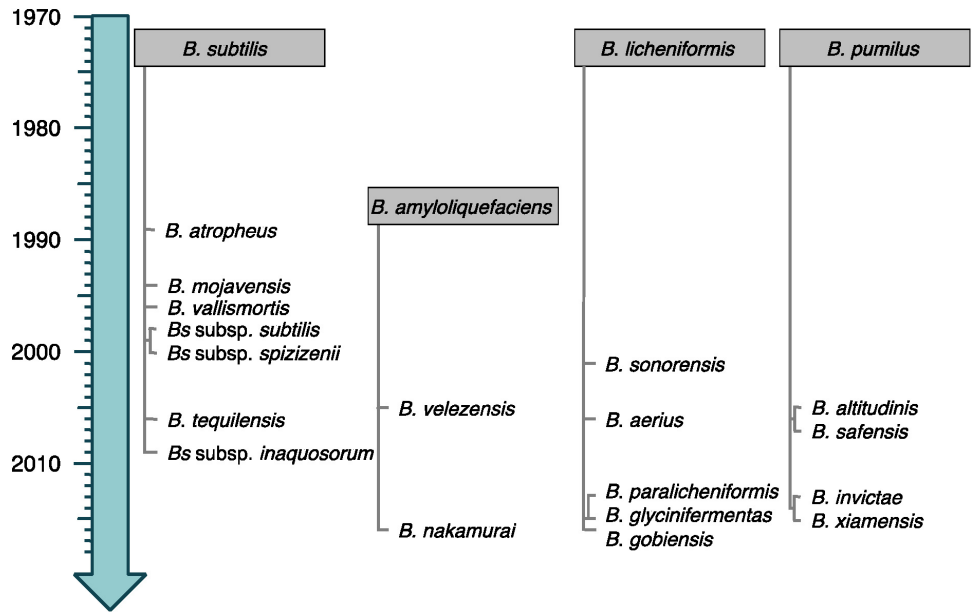


FIGURE 1 | Timeline emergence of the species from the *B. subtilis* group. The species are classified following their relatedness to the closest original member of the group (gray boxes). Heterotypic synonyms are not shown.

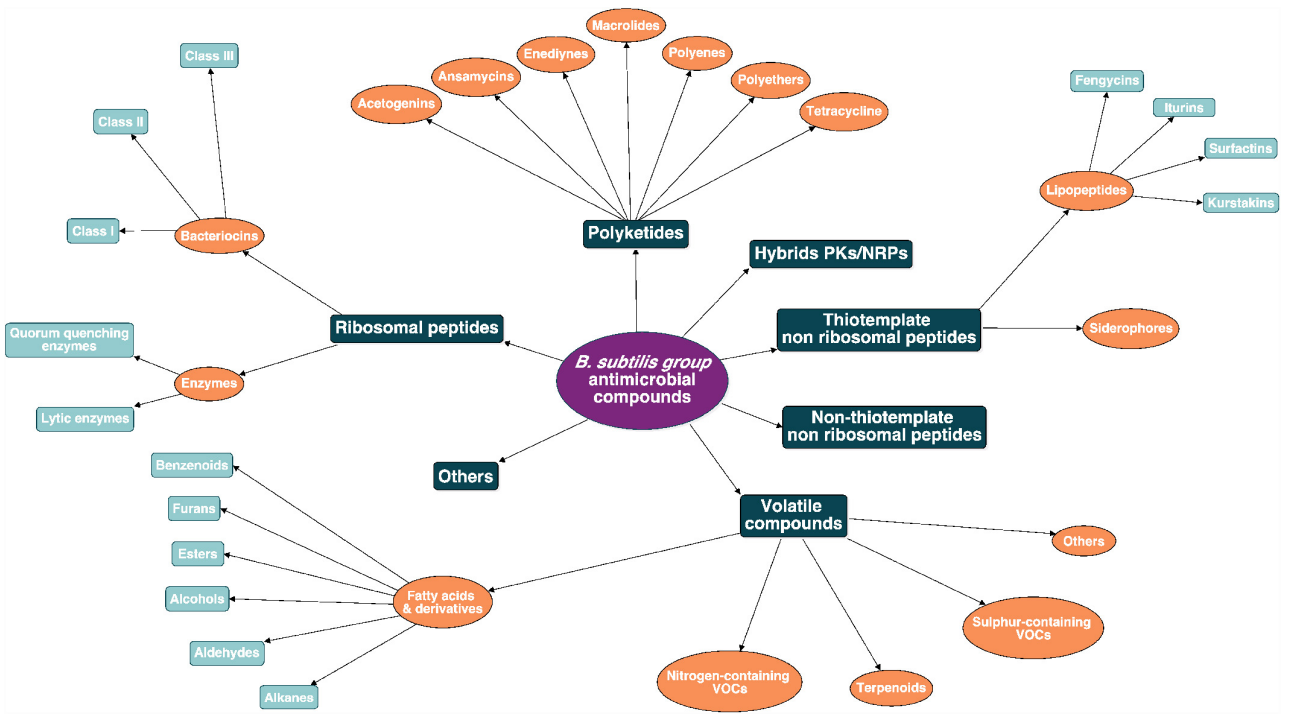


FIGURE 2 | Antimicrobial molecules classes from the *B. subtilis* group. The subdivision between the classes is based on the biosynthetic pathway (*i.e.*, ribosomal peptides, polyketides, hybrids, non-ribosomal peptides, and volatile compounds).

generally bactericidal with some exceptions that exhibit bacteriostatic activities (Gautam and Sharma, 2009). For most class I and II bacteriocins, the target of their activity is the bacterial envelope due to their amphiphilic or hydrophobic properties. For instance, lantibiotics from subclass I.1 have a dual mode of action. On the one hand, they can inhibit the cell wall synthesis of the targeted bacteria through binding to lipid II, the major transporter of peptidoglycan subunits

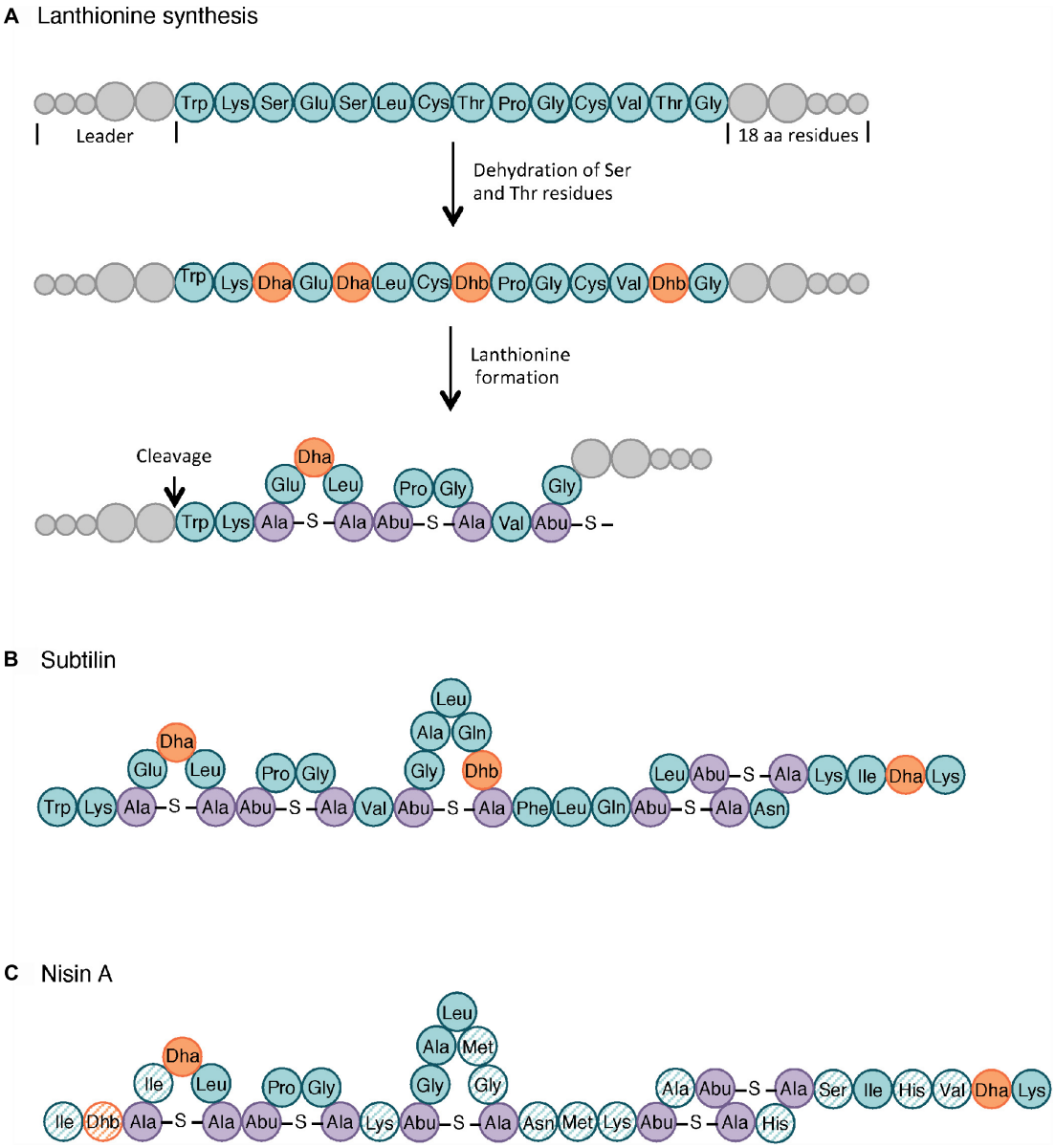


FIGURE 3 | Lanthionine biosynthesis. General pathway of the lanthionine synthesis **(A)**, structure of subtilin **(B)** and nisin A **(C)**. Non-modified AA are indicated in teal whereas dehydrated serine (Dha, dehydroalanine) and threonine (Dhb, dehydrobutyrine) are colored in orange. The lanthionine (Ala-*S*-Ala, alanine-*S*-alanine) and *R*-methyllanthionine (Abu-*S*-Ala, aminobutyrate-*S*-alanine) bridges are shown in purple. The AA of nisin that differ from those in subtilin are highlighted as hatched circles. Adapted from Cotter et al. (2005) and Spieß et al. (2015).

across the inner cell membrane. On the other hand, lipid II can be used as a docking molecule to insert the lantibiotic in the membrane leading to pore formation and ultimately to cell death as well described in Chatterjee et al. (2005) and Cotter et al. (2005). This duality has been reported for subtilin, a class I bacteriocin which is active against a broad range of Gram-positive bacteria such as *Staphylococcus simulans*, *B. subtilis,* and *Bacillus stearothermophilus* (Linnett and Strominger, 1973; Parisot et al., 2008).

Many regulation systems mediate bacteriocin production, secretion and immunity. Bacteriocin production is usually linked to particular cellular events such as stress responses. For instance, subtilin production depends on cell density and is increased under starvation conditions (Abriouel et al., 2011). Lantibiotic production is also mediated by QS. For subtilin, it has been demonstrated that the peptide itself acts as an auto-inducer of its own production (Kleerebezem, 2004). The export of bacteriocins is generally ensured by a dedicated membrane-associated ATP-Binding Cassette (ABC) transporter. For some lantibiotics, the cleavage of the leader peptide often occurs in a proteolytic domain present in the ABC transporter as described in McAuliffe et al. (2001) and Cotter et al. (2005). The immunity

TABLE 1 | Classification of the *B. subtilis* group bacteriocins.

Class	Class description	Subclass	Subclass description
I	Post-translationally modified peptides	I.1	Single-peptide, elongated lantibiotics
		I.2	Other single-peptide lantibiotics
		I.3	Two-peptide lantibiotics
		I.4	Other modified peptides
II	Non-modified peptides	II.1	Pediocin-like peptides
		II.2	Thuricin-like peptides
		II.3	Other linear peptides
III	Large peptides (>10 kDa)		

Adapted from Abriouel et al. (2011).

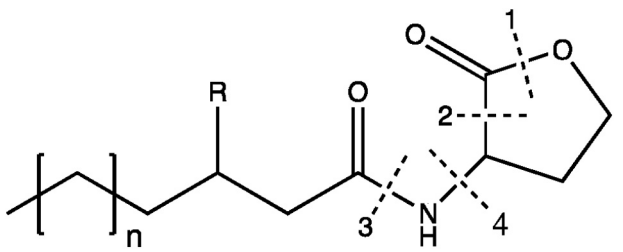


FIGURE 4 | AHLs structure and its corresponding enzymatic degradations by QQ. The broken lines show the cleavages sites of four enzymes: (1) lactonase; (2) decarboxylase; (3) acylase; (4) deaminase. Adapted from Czajkowski and Jafra (2009).

of the producing strains to its own active bacteriocin(s) can be achieved by several mechanisms like the secretion of immunity proteins sequestering the peptide, the bacteriocin re-export through an ABC transporter system or the alteration of the targeted peptidoglycans bonds (*e.g.*, modification of the cell wall or cytoplasmic membrane charge) (Cotter et al., 2005; Dubois et al., 2009).

B. subtilis Group AMP Enzymes

Among the *B. subtilis* group, two major types of enzymes exhibit antagonistic activities (**Supplementary Table S1**): the lytic enzymes and those involved in quorum quenching (QQ). Several strains from the *B. subtilis* group have indeed been identified as capable to produce lytic enzymes with biocontrol potential (Herrera-Estrella and Chet, 1999; Kumar et al., 2012; Shafi et al., 2017). They include cellulases, glucanases, proteases and chitinases and are generally referred to as cell wall degrading enzymes (CWDE) (Ariffin et al., 2006; Alamri, 2015; Caulier et al., 2018). They are particularly active against fungi since chitin and glucan are the major constituents of their cell wall where various glycoproteins are embedded (Bowman and Free, 2006; Geraldine et al., 2013; Gomaa, 2012).

Quorum quenching is able to silence or block QS which is generally defined as the cell-to-cell communication mechanism through the production of signal molecules (Czajkowski and Jafra, 2009). *N*-acyl-homoserine lactones (AHLs), composed of a fatty acid side chain and a homoserine lactone (**Figure 4**) are the most characterized signal autoinducers in Gram-negative bacteria. When a bacterial population proliferates, concentration of AHLs increases so that all the cells coordinate their metabolic activities (*e.g.*, biofilm formation, sporulation, virulence factors or antibiotic production) (Dong et al., 2004). As the QS system brings ecological advantages to a coordinate population, QQ is able to counteract QS. Four types of enzymes (*i.e.*, lactonase, decarboxylase, acylase, and deaminase) are able to inactivate AHLs, as illustrated in **Figure 4** (Czajkowski and Jafra, 2009). *B. subtilis* AHL-lactonases have for instance attracted interest for biocontrol since they affect the growth of deleterious microbial pest such as *Pectobacterium carotovorum* subsp. *carotovorum* causing potato soft rot (González and Keshavan, 2006).

POLYKETIDES

Among the bioactive compounds produced by microorganisms, PKs are well known from the human health sector for their broad spectrum of activity encompassing antibacterial, immunosuppressive, antitumor and many more antagonistic abilities. Typical PKSs structures from the *B. subtilis* group are presented in **Figure 5**. They are synthetized from acyl CoA precursors such as malonate and methyl malonate. Their biosynthesis depends on multifunctional polyketide synthases (PKSs). Their structure was first extrapolated from fatty acid synthases (FASs) that share similarities in terms of chain extension mechanisms, precursors and overall architecture design (Smith and Tsai, 2007). As shown in **Figure 6A**, PKS are composed of a succession of elongation modules, flanked by initiation and termination modules. The reactive mechanism of these three PKS domains is illustrated in **Figure 7A** and is well summarized in Hertweck (2009). The initiation module is composed of two domains: an acyltransferase (AT) domain that recruits and catalyzes the binding of a monomer substrate to an acyl carrier protein (ACP) domain. The ACP then acts as an arm with a second catalytic domain located on the next elongation module. This domain, a β-ketoacyl synthase (KS), catalyzes the chain-elongation reaction that occurs through a decarboxylative Claisen thioester condensation (Cane and Walsh, 1999; Hertweck, 2009). In addition to the three core domains, auxiliary domains can also be present on elongation modules (gray domains in **Figure 6A**). These auxiliary domains mediate ketoreduction (KR), dehydration (DH), or enoylacyl reduction (ER) occurring before the chain-elongation reaction. These modifications considerably enrich the structural complexity and diversity of mature PKs (Hertweck, 2009). Finally, a termination module harboring an additional thiosterase (TE) domain catalyzes the macrolactonization and the release of the mature PK (Cane and Walsh, 1999).

Polyketide synthases have been classified in three canonical types based on the structural organization of their functional domains. Type I PKSs involve large multifunctional enzymes housing several domains linearly arranged and covalently bonded. Type II PKSs are multienzyme complexes composed of separate monofunctional enzymes combined during the PK

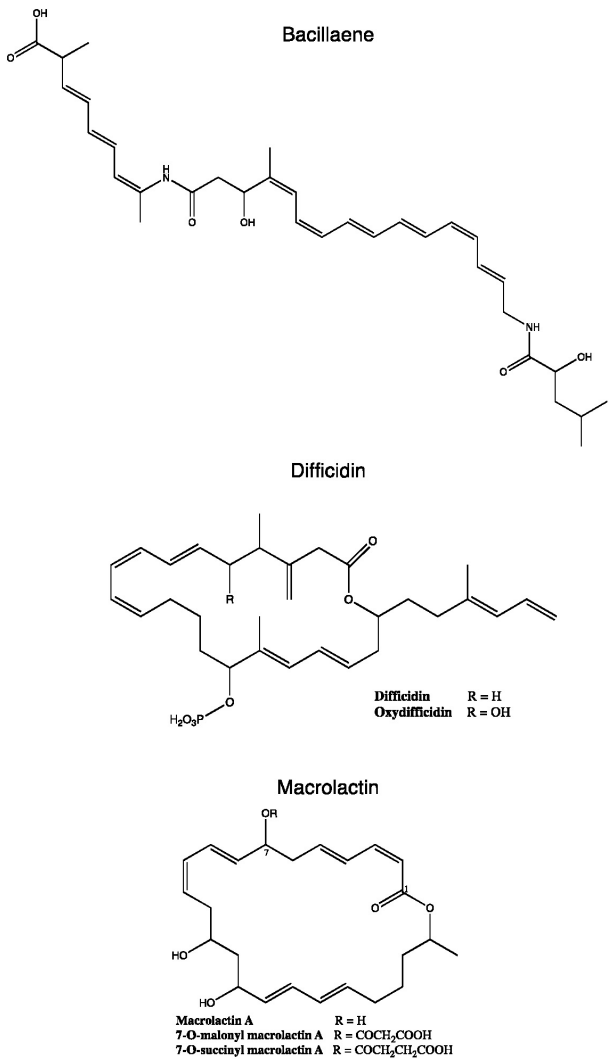


FIGURE 5 | Chemical structures of some *B. subtilis* group polyketides. Variants from macrolactin and difficidin are presented.

synthesis. Type III PKSs are chalcone synthase-like PKSs that operate the acid CoA thioesters directly without any ACP domain (Chen and Du, 2016). Beside these structural differences, PKSs are classified as iterative or non-iterative depending on how many KS domains are used in the biosynthetic process. Within prokaryotes, the non-iterative type I PKSs is the most represented. They produce PK compounds that harbor a one-to-one correspondence with the PKS modular architecture. This conservation of collinearity is used for PKS discovery via genome mining (Challis, 2008).

Due to the diversity of PKSs, many exceptions and transition states between the three main types are observed. In some cases, mixed PKs pathways combine different types of PKSs or can even be associated with FASs or NRP synthetases (NRPSs) to form PK-peptide hybrid metabolites such as bacillaene, compactin, fusarin C or salinosporamide A (Moldenhauer et al., 2007; Hertweck, 2009; Fisch, 2013).

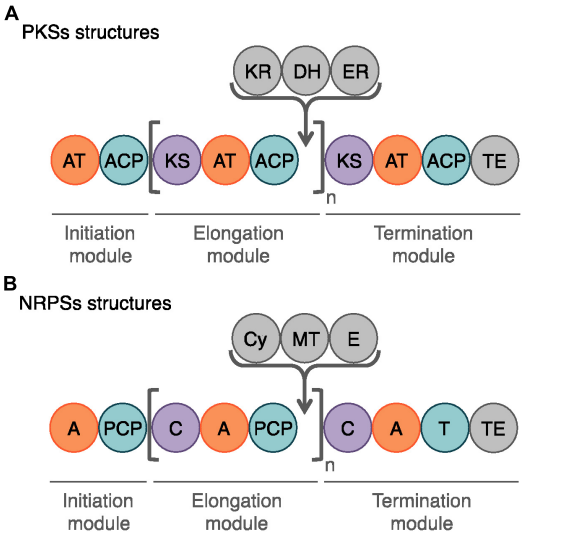


FIGURE 6 | Schematic representation of the modules and domains mediating PKS and NRP biosynthesis. **(A)** The domains involved in the PK synthesis are the acyltransferase (AT), the acyl carrier protein (ACP), the ketosynthase (KS) and the chain-terminating thiosterase (TE) domains. In gray, the auxiliary domains can mediate ketoreduction (KR), dehydration (DH), and enoylacyl reduction (ER) at each elongation step (n). **(B)** The core domains for NRP biosynthesis are the adenylation (A), the peptidyl carrier domain (PCP), the condensation (C), and the final thioesterase (TE) domains. The auxiliary domains consist in cyclization (Cy), *N*-methylation (MT), and epimerization (E) domains.

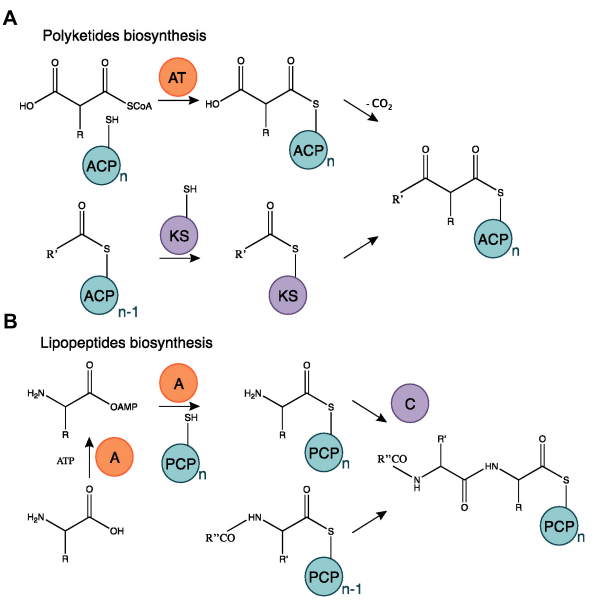
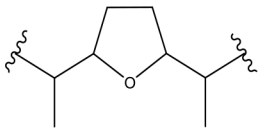
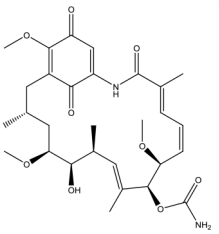
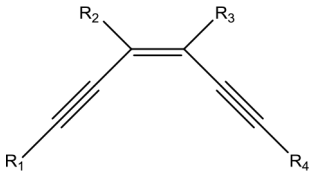
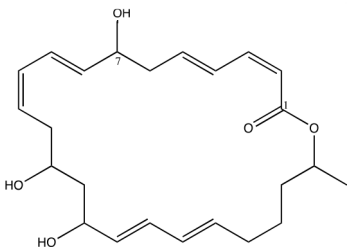
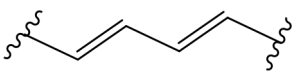
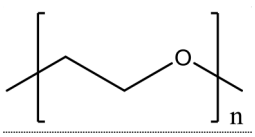
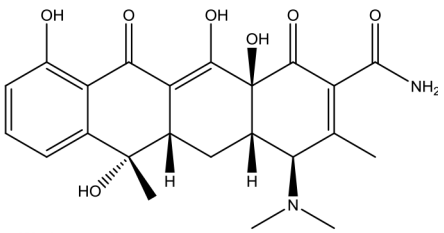


FIGURE 7 | Polyketides and lipopeptides biosynthesis mechanism. **(A)** The AT domain catalyzes the binding of the monomer substrate and the ACP domain. The KS domain is acetylated on the acyl residue of a polyketide starter or in elongation and catalyzes the transfer of the substrate subunit carried by the ACP. **(B)** The A domain activates an AA chain extension subunit and its transfer to the PCP carrier domain. The C domain catalyzes the bond mediating the chain elongation. Adapted from Cane and Walsh (1999) and Challis and Naismith (2004).

TABLE 2 | Major classes of polyketides.

Polyketide class	Structure description	Typical core unit or example structure	Reference
Acetogenins	Linear 32- or 34-carbon chains with oxygenated functional groups bearing a terminal γ-lactone ring		Li et al., 2008
Ansamycins	Bridge between an aromatic moiety and an aliphatic chain		Williams, 1975
Enediynes	Compounds characterized by a core structure formed by a double C–C bond conjugated to two acetylenic groups		Horsman et al., 2009
Macrolides	Large macrocyclic lactone ring with one or more deoxy sugars		Yuan et al., 2012a
Polyenes	Poly-unsaturated organic compounds containing at least three alterning single and double C-C bonds		Hertweck, 2009
Polyethers	Polymers containing more than one ether group		Hertweck, 2009
Tetracyclines	Compounds family characterized by a typical four-ring system		Rohr, 1992

To date, seven PKs families have been recognized based on their carbon skeletons and typical structures, as summarized in **Table 2** (Eustáquio et al., 2009). However, to our knowledge, only three antimicrobial PKs and their variants are produced within the *B. subtilis* group: bacillaene, difficidin, and macrolactin. These compounds exhibit antibacterial activities through selective inhibition of protein synthesis (**Table 3**). Bacillaene is a polyene PK resulting from a hybrid synthesis by a type I PKS and a NRPS *bae* operon (*baeJ, baeL, baeM, baeN* and *baeR*) (Chen et al., 2006; Moldenhauer et al., 2007). Its exhibits antimicrobial activity against various bacteria (*e.g.*, *Myxococcus xanthus* or *Staphylococcus aureus*) and fungi (*e.g.*, *Trichoderma* spp. or *Fusarium* spp.) (Patel et al., 1995; Um et al., 2013; Müller et al., 2014). Difficidin, and its oxidized form oxydifficidin,

TABLE 3 | PKS and hybrids NRPS/PKS produced by strains of the *B. subtilis* group.

PKS or hybrids class*	Compound	Antimicrobial activity**		References
		Antibacterial activity	Antifungal activity	
Macrolides	7-*O*-malonyl-macrolactin A	*B. cepacia*[c], *Enterococci faecalis*[c], *R. solanacearum*[c], *S. aureus*[c]	*F. oxysporum* f. sp. *cubense*[c]	Romero-Tabarez et al., 2006; Yuan et al., 2012a
Macrolides	7-*O*-succinyl-macrolactin F	*B. subtilis*[c], *S. aureus*[c]	–	Jaruchoktaweechai et al., 2000; Nagao et al., 2001
Macrolides	7-*O*-succinyl-macrolactin A	*B. subtilis*[c], *R. solanacearum*[c], *S. aureus*[c]	*F. oxysporum* f. sp. *cubense*[c]	Jaruchoktaweechai et al., 2000; Yuan et al., 2012a
Macrolides	Macrolactin A	*R. solanacearum*[c]	*F. oxysporum* f. sp. *cubense*[c]	Yuan et al., 2012a
Macrolides	Macrolactin D	*S. aureus*[c]	*A. solani*[c], *Pyricularia oryzae*[c]	Xue et al., 2008
Macrolides	Macrolactin F, G, H, I, J, K, L, M	*B. subtilis*[c], *S. aureus*[c]	–	Jaruchoktaweechai et al., 2000; Nagao et al., 2001
Macrolides	Macrolactin N	*E. coli*[c], *S. aureus*[c]	–	Yoo et al., 2006
Macrolides	Macrolactin Q	*B. subtilis*[c], *E. coli*[c], *P. aeruginosa*[c], *S. aureus*[c]	–	Mojid Mondol et al., 2011
Macrolides	Macrolactin S	*B. subtilis*[c], *E. coli*[c], *S. aureus*[c]	*P. oryzae*[c]	Lu et al., 2008
Macrolides	Macrolactin T	*S. aureus*[c]	*A. solani*[c], *P. oryzae*[c]	Xue et al., 2008
Macrolides	Macrolactin W	*B. subtilis*[c], *E. coli*[c], *P. aeruginosa*[c], *S. aureus*[c]	–	Mojid Mondol et al., 2011
Polyenes	Bacillaene A	*B. thuringiensis*[c], *E. coli*[c], *Klebsiella pneumoniae*[c], *M. xanthus*[c], *P. vulgaris*[c], *Serratia marcescens*[c], *S. aureus*[c]	*Coriolopsis* spp.[c], *Fusarium* sp.[c], *Pseudoxylaria* sp.[c], *Trichoderma* sp.[c], *Umbelopsis* sp.[c]	Patel et al., 1995; Um et al., 2013; Müller et al., 2014
Polyenes	Difficidin	*Actinomyces naeslundii*[c], *Bacteroides distasonis*[c], *C. perfringens*[c], *E. amylovora*[c], *E. coli*[c], *Eubacterium limosum*[c], *K. pneumoniae*[c], *P. vulgaris*[c], *P. aeruginosa*[c], *S. marcescens*[c], *S. aureus*[c], *Streptococcus faecalis*[c], *X. oryzae*[c]	–	Zimmerman et al., 1987; Chen et al., 2009; Wu et al., 2015b
Polyenes	Oxydifficidin	*A. naeslundii*[c], *B. distasonis*[c], *C. perfringens*[c], *E. coli*[c], *E. limosum*[c], *K. pneumoniae*[c], *P. vulgaris*[c], *P. aeruginosa*[c], *S. marcescens*[c], *S. aureus*[c], *S. faecalis*[c]	–	Zimmerman et al., 1987
Hybrids PKs/NRPs	Kanosamine	–	*C. albicans*[p], *Saccharomyces cerevisiae*[p]	Janiak and Milewski, 2001; van Straaten et al., 2013

*[c] Activity of isolated compound confirmed by compound purification or mutant deletion, [p] putative activity of the compound contained in a broth mixture. * Two PKs classes are reported in this review (macrolides and polyenes) as well as the hybrids between PKs and NRPs. ** –, no activity known.*

are polyenes synthesized by a type I PKS encoded in the *dif* operon. They both inhibit bacterial pathogens such as *Clostridium perfringens, Erwinia amylovora, Escherichia coli* or *Xanthomonas oryzae* (Zimmerman et al., 1987; Chen et al., 2009; Aleti et al., 2015; Wu et al., 2015b). Finally, macrolactins and their 7-*O*-succinyl- or 7-*O*-malonyl-derivatives are synthetized via a type I PKS. They show antibacterial and antifungal activities against *Burkholderia cepacia*, *Ralstonia solanacearum*, *S. aureus* or *Fusarium oxysporum* (Romero-Tabarez et al., 2006; Yoo et al., 2006; Yuan et al., 2012a). Some macrolactins, such as the macrolactin A, apparently also displays antiviral properties (*e.g.*, against Herpes simplex viruses) (Gustafson et al., 1989).

NON-RIBOSOMAL PEPTIDES

Non-ribosomal peptides form a versatile family of secondary metabolites with growing interest in many industrial fields as antibiotics, siderophores, surfactants, pigments, immunosuppressors or antitumor molecules (Wang et al., 2014). NRPs show a broad structural diversity, from linear to cyclic or branched structures (Kopp and Marahiel, 2007). As illustration, the Norine database counts almost 1.200 NRP molecules, including their structure, synthesis and evolution[2] (last update in January 2019) (Caboche et al., 2008).

Two categories of NRPs can be distinguished whether they are synthetized through a multi-enzyme thio-template mechanism or not (Sumi et al., 2015). The first ones usually result in structures with two to *ca.* 50 residues and other moieties such as fatty acid chains [*i.e.*, lipopeptides (LPs) and siderophores] whereas the second ones are generally smaller. **Figure 8** shows the chemical structures of typical NRPS from the *B. subtilis* group.

[2]http://bioinfo.lifl.fr/norine

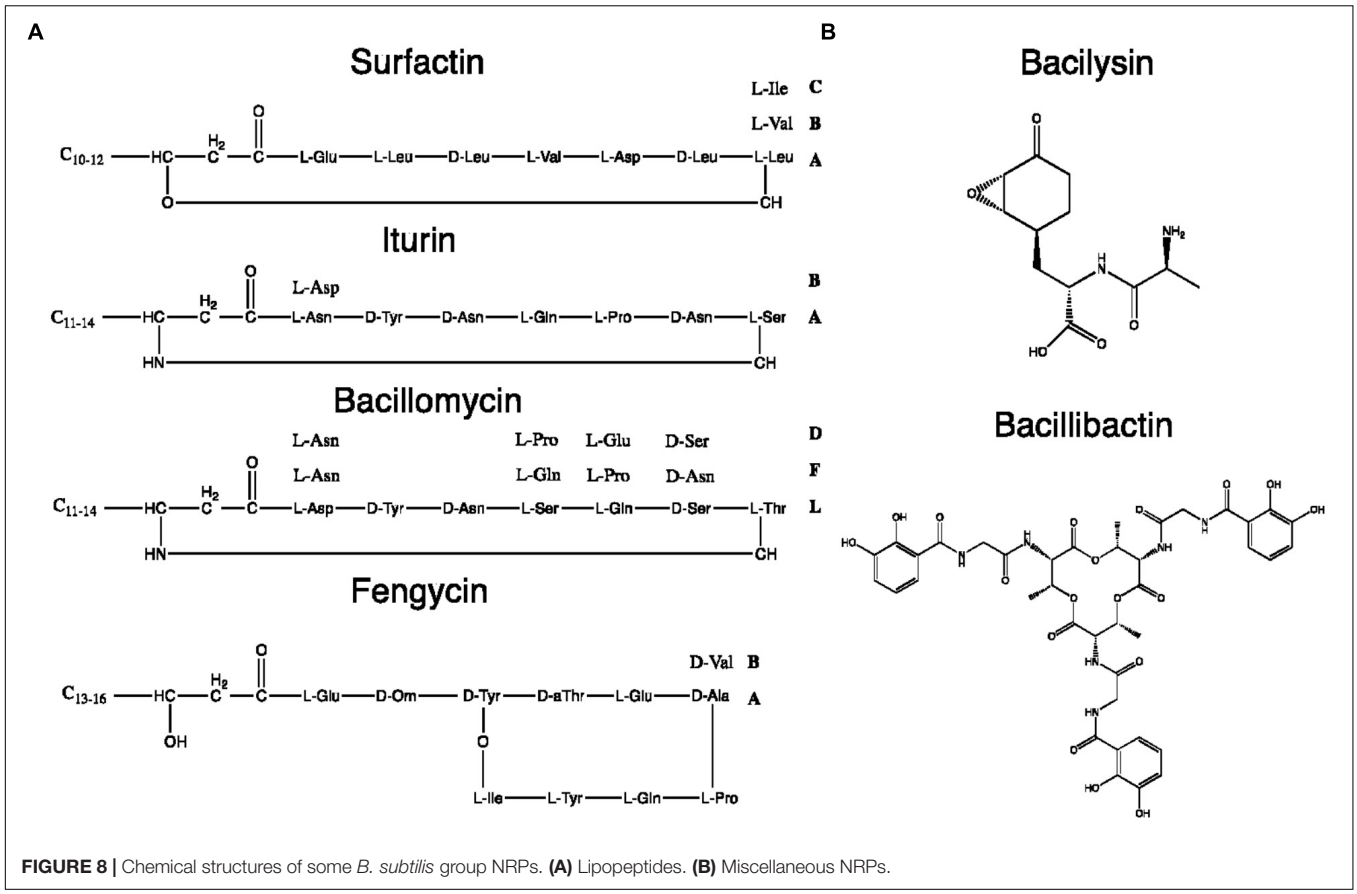


FIGURE 8 | Chemical structures of some *B. subtilis* group NRPs. **(A)** Lipopeptides. **(B)** Miscellaneous NRPs.

Thiotemplate NRPs – Lipopeptides

Lipopeptides are usually synthetized through a NRPS sequential addition of AA residues, either in an iterative or non-iterative way. Similarly to PKSs, NRPSs have a modular organization implementing the initiation, elongation, and termination modules (**Figure 6B**). Each module is subdivided in core domains whose catalytic and carrier domains slightly differ from PKSs, as shown in **Figure 7B**. The biosynthesis which was previously summarized in Ongena and Jacques (2008) and Raaijmakers et al. (2010) starts with an adenylation domain (A domain) that recruits and phosphorylates an AA monomer into an aminoacyl adenylate intermediate. The intermediate is then linked to the corresponding peptidyl carrier protein or thiolation domain (PCP or T domain) through a thioester bond. The PCP acts as a bridge and ensures the link with the condensation domain (C domain) that forms the C–N bond between the recruited aminoacyl and the peptide acyl chain in formation. The termination module contains a thioesterase domain (TE) that catalyzes the release of the final peptide acyl chain (Ongena and Jacques, 2008; Raaijmakers et al., 2010). The elongation modules can be supplemented with accessory domains such as cyclization domain (Cy), epimerization domain (E) and methylation domain (M). Those domains are able to modify the growing peptide chain which leads to diverse mature compounds structure (Cane and Walsh, 1999; Challis and Naismith, 2004).

Since the LP biosynthetic pathways are highly flexible, the range of produced LPs is extremely heterogeneous. Among LPs produced by *Bacillus* spp., four main families have been distinguished: kurstakins, surfactins, iturins, and fengycins (Jacques, 2011). Each family shares the same structural features based on the nature and organization of the peptide moiety or fatty acid tail, as summarized in **Table 4**. Strains from the *B. subtilis* group produce surfactins, iturins and fengycins whereas kurstakins are produced by *B. thuringiensis* strains (Béchet et al., 2012). Among the three LP families produced by *B. subtilis*, at least eight fengycins, 13 surfactins and 14 iturins variants have been described so far, as detailed in **Supplementary Table S2**.

For each LP family, the compounds production is mainly regulated by environmental factors such as carbon sources, oxygen availability, pH and temperatures (Yakimov et al., 1995;

TABLE 4 | Classification of the *Bacillus* spp. lipopeptides.

Family*	Surfactin	Iturin	Fengycin	Kurstakins
Peptide length	Heptapeptide	Heptapeptide	Decapeptide	Heptapeptide
Chiral sequence	LLDLLDL	LDDLLDL	LDDDLDLLLL	Not described
FA type	β-hydroxy FA	β-amino FA	β-hydroxy FA	β-hydroxy FA or not
FA length	13–15 carbons	14–17 carbons	16–19 carbons	11–14 carbons
Structure	Cyclic lactone	Cyclic peptide	Cyclic lactone	Cyclic lactone

**FA refers to fatty acid.*

Kim et al., 1997; Cosby et al., 1998). Warm temperature (≥37°C) and anaerobic conditions increase the production of surfactins while lower temperatures (25–37°C) and aerated bioreactors favor fengycins and iturins family metabolites (Jacques, 2011). The production of surfactins by *B. subtilis* is also QS-dependent and involves ComX and PhrC. These pheromones trigger complex cascades regulating cell density-dependent processes such as sporulation and competence (Hamoen et al., 2003; Ongena et al., 2005).

Iturins and fengycins are mainly known for their strong antifungal activity against several plant and human pathogenic fungi (**Supplementary Table S2**). In addition, iturin-like mycosubtilin, bacillomycin R, subtulene A and eumycin show antibacterial properties (Besson et al., 1976; Leclere et al., 2005; Thasana et al., 2010). Contrary to iturins and fengycins, surfactins mainly display antiviral and antibacterial activities (Ongena and Jacques, 2008). Their antiviral activity essentially targets enveloped viruses (*e.g.*, herpes simplex or porcine epidemic diarrhea viruses). They also inhibit pathogenic bacteria such as *Legionella pneumophila*, *Listeria monocytogenes*, *R. solanacearum* or *X. oryzae* (Naruse et al., 1990; Yakimov et al., 1995; Sabaté and Audisio, 2013; Loiseau et al., 2015; Luo et al., 2015). However, some surfactins are able to control important fungal plant and human pathogens such as *Botrytis cinerea, Candida albicans, F. oxysporum* or *Rhizoctonia solani* (Jenny et al., 1991; Lee et al., 2007; Qi et al., 2010; Dimkić et al., 2013; Romano et al., 2013).

The mere composition of LPs, where a peptide moiety is bound to a lipid tail, gives them an amphiphilic property. This nature makes them excellent surfactants and plays a significant role in their biological functions and antimicrobial properties. Indeed, LPs are able to destabilize the plasma membrane via a pore forming activity leading to the cell death of the target microbes. Their antiviral activity is the result of a similar disintegration of the bi-lipid envelope of virions explaining the weak LPs activity against plant viruses among which very few are enveloped (Ongena and Jacques, 2008).

Bacillus spp. LPs have many other biological and ecological functions as fully documented by Raaijmakers et al. (2010). They are also known to impact other metabolic mechanisms such as biofilm formation, motility, virulence, plant root colonization, and plant defenses. Moreover, it has been suggested that their participation to the degradation of hydrophobic substrates could be used for polluted soils bioremediation (Mulligan et al., 2001). Although some lipopetides have already been exploited as food biopreservatives or crop protection products, the industrial interest for LPs in specific applications is unsurprisingly continuously growing.

Thiotemplate NRPs – Siderophore

Itoic acid is a mono-peptide composed of a 2,3-dihydroxy-benzoate (DHB) molecule bound to a glycine. It is used as a precursor by trimodular NRPS machinery to produce bacillibactin which is obtained after a condensation of three units of DHB-glycine-threonine (May et al., 2001). The synthesis of the final hexapeptide is catalyzed by a terminal thioesterase domain leading to the production of a methylated trilactone ring link to three catecholates moieties. It is this cyclic structure that enables the sequestration of the metal atom (Dertz et al., 2006). Itoic acid and bacillibactin are both catecholic siderophores that chelates iron reducing its bioavailability. This is limited access to iron that allows *B. subtilis* to antagonize the growth of other surrounding microbes such as, for instance, *F. oxysporum* f. sp. *capsici* (Yu et al., 2011).

Non-thiotemplate NRPs

Bacteria from the *B. subtilis* group are also able to synthesize other antimicrobial NRPs through non-thiotemplate mechanism. Rhizocticins are di- and tri-phosphono-peptides. They are constituted of a L-2-amino-5-phosphono-3-*cis*-pentenoic acid (APPA) linked to an arginine (rhizocticin A). They can be supplemented with an additional valine (rhizocticin B), isoleucine (rhizocticine C) or leucine (rhizocticine D). After their integration into the target microbes, their cleavage by host cell peptidases releases the fungitoxic L-APPA moiety that interferes with threonine metabolism in fungal cells. Interestingly, rhizocticin A has also an antagonistic activity against nematodes such as *Caenorhabditis elegans* (Kugler et al., 1990).

In addition to rhizocticin compounds, two other dipeptide NRPs are produced by *B. subtilis*: bacilysin (also known as tetaine) and its chlorinated derivative, chlorotetain. They contain L-alanine (or chlorine-L-alanine) bound to the non-proteinogenic L-anticapsin (Kenig and Abraham, 1976; Rapp et al., 1988). Despite their simple composition, these bioactive compounds display strong antibacterial activity mediated by the anticapsin moiety that inhibits the glucosamine-6-phosphate synthase. Its inhibition suppresses the biosynthesis of peptidoglycans that are the main constituents of bacterial cell wall (Steinborn et al., 2005; Mahlstedt and Walsh, 2010). For the fungi, it has been proposed that because anticapsin is able to inhibit the production of chitin and fungal membrane mannoproteins, bacilysin and chlorotetain exhibit antifungal activity against *Aspergillus fumigatus* or *C. albicans* (Milewski et al., 1986; Rapp et al., 1988).

Finally, bacitracin and mycobacillin are two cyclic polypeptides produced by *B. subtilis*. Bacitracins are dodeca-peptides containing a cyclic heptapeptide linked to a thiazoline ring (Johnson et al., 1945). They are mostly active against Gram-positive bacteria where they inhibit the bacterial cell-wall biosynthesis by preventing the lipid carrier from re-entering in the reaction cycle of peptidoglycan synthesis (Siewert and Strominger, 1967). Besides this primary mode of action, bacitracin might also act through other mechanisms affecting membrane functions, hydrolytic enzymes and/or the biosynthesis of ubiquinone precursors (Konz et al., 1997). Mycobacillin is an antifungal cyclic tridecapeptide altering the membrane of fungi like *Aspergillus niger* (Majumdar and Bose, 1958). Interestingly, its biosynthesis is rather peculiar. Although it is catalyzed by a large NRPS complex, it is divided in three fractions (A, B, and C) and does not use a thio-template mechanism (Zuber et al., 1993). Each fraction of the enzymatic complex contains a single enzyme polypeptide that catalyzes the polymerization of a first pentapeptide (A), a second nonapeptide (B) and the final tridecapeptide.

VOLATILES

Besides RPs, NRPs and PKs, strains from the *B. subtilis* group are able to produce a wide diversity of volatile compounds encompassing important roles especially in soil, one of the major habitats of this group (**Supplementary Figure S1**). Volatiles are notably involved in the bioconversion of the food chain, in the biogeochemical cycles of essential elements, in many physiological and metabolic reactions (*e.g.*, nitrification, nitrogen mineralization, electron acceptor or donor reactions) as well as in communication signals triggering QS/QQ or defense mechanisms well reviewed in Effmert et al. (2012). Volatile compounds are generally classified into inorganic (VICs) and organic (VOCs) categories.

Volatile Inorganic Compounds (VICs)

Volatile inorganic compounds synthesized by microorganisms are mainly by-products of primary metabolism. They are carbonated, hydrogenated, sulfur or nitrogen-containing compounds such as CO_2, CO, H_2, HCN, H_2S, N_2, NH_3 and NO. Nitrogen-containing compounds are mostly released in aerated upper sediments layers by denitrifying bacteria. In this process, nitric oxide is enzymatically produced by the nitric-oxide reductase or the nitric-oxide synthase (Adak et al., 2002). The range of antimicrobial activities exhibited by VIC nitrogen-containing compounds from the *B. subtilis* group is wide. For instance, NO is able to induce systemic acquired resistance (SAR) in plants against bacterial pathogens such as *R. solanacearum* (Wang et al., 2005). *A contrario*, ammonia, a secondary metabolite from the catabolism of the amino acids L-aspartate, is known to be active against soil-borne Oomycetes such as *Pythium* spp. (Howell et al., 1988). Hydrogen cyanide, derived from the glycine catabolism, shows a direct antagonistic activity against aerobic microorganisms by inhibiting metal-containing enzymes such as the cytochrome c oxidase active in the respiration chain (Cherif-Silini et al., 2016).

Deeper in the soil, under low oxygen concentration, bacteria tend to produce different VICs such as H_2 or H_2S. Those compounds can serve as electron acceptors, AA precursors or antimicrobial metabolites. Hydrogen sulfide could be produced by *B. subtilis* from sulfate reduction or as a by-product of L-methionine and L-cysteine catabolism via a direct cleavage of L-methionine or a transamination followed by reductive demethiolations (Even et al., 2006; Schulz and Dickschat, 2007). It is known to exhibit antifungal activity against several plant pathogens such as *A. niger* or *Penicillium italicum* but also against some food-borne bacteria or human pathogens (Fu et al., 2014). Curiously, it is also known to act as a bacterial defense mechanism against antibiotics (Shatalin et al., 2011). Interestingly, ammonia increases the resistance of several Gram-negative and Gram-positive bacteria to antibiotics too (Bernier et al., 2011).

Volatile Organic Compounds (VOCs)

Volatile organic compounds are small compounds with fewer than 20 carbon atoms and are characterized by low molecular mass (100–500 Da), high vapor pressure, low boiling point and a lipophilic moiety. These features ensure an easy evaporation and a long distance distribution which is convenient in a complex matrix like soil (Schmidt et al., 2015). Their diffusion and production by soil-borne microbes are strongly dependent on various factors such as nutrient and oxygen availability, temperature, pH, physiological state of microorganisms, soil moisture, texture and architecture (McNeal and Herbert, 2009; Insam and Seewald, 2010; Effmert et al., 2012). The majority of VOCs derives from glucose oxidation involving glycolysis and the subsequent cycles such as the tricarboxylic acid cycle (TCA) as it has been well summarized in Korpi et al. (2009) and Schmidt et al. (2015). However, their production can also result from various other pathways such as aerobic heterotrophic carbon metabolism, fermentations, AA degradation, terpenes synthesis or sulfur reduction (Peñuelas et al., 2014). Based on previous reviews presented in Schulz and Dickschat (2007); Peñuelas et al. (2014) and Audrain et al. (2015), five categories of VOCs can be distinguished: (1) fatty acids and derivatives, (2) terpenoids, (3) nitrogen-containing VOCs, (4) sulfur-containing VOCs, and (5) metalloid- or halogenated-containing VOCs. To date, about 2,000 compounds produced by almost 1,000 species of microorganisms have been listed in the mVOC 2.0 database (Lemfack et al., 2018). According to this database, almost 70% of recorded *Bacillus* VOCs are fatty acids derivatives (alcohols, ketones, alkanes, aldehydes, alkenes, and acids) followed by sulfur- and nitrogen-containing compounds. **Supplementary Table S3** displays the VOCs produced within the *B. subtilis* group and their antimicrobial activity.

Since many volatile fatty acids and their derivatives result from the glucose metabolism, their precursors mostly derive from the Embden-Meyerhof (glycolysis), Entner-Doudoroff, heterolactic and homolactic fermentation pathways (Peñuelas et al., 2014). *B. subtilis* bacteria, for instance, ferment pyruvate to produce ketone compounds such as acetoin (3-hydroxy-2-butanone) or 2,3-butanedione under anaerobic conditions (Ryu et al., 2003). Other intermediates coming from fatty acid biosyntheses or their β-oxydations are also used as precursors by microbes and transformed into VOCs through a decarboxylation reaction or a reduction of their carboxyl group (Schulz and Dickschat, 2007). They provide essential hydrocarbons but also other fatty acid derivatives. An oxidative deamination of several amino acids can lead to the production of aldehyde, ketone or alcohol volatile too. For instance, the degradation of L-phenylalanine or L-tyrosine can be the first step of the aromatic volatile compounds synthesis such as benzene or its carbohydrate derivatives. Finally, benzenoid volatiles can also be synthesized by microbes through the shikimate pathway that leads to the formation of chorismate, a natural precursor of aromatic amino acids (Bentley and Haslam, 1990). Degradation of intermediates from the shikimate pathway or aromatic amino acids can also lead to the production of benzenoid volatiles (Dickschat et al., 2005).

This wide variety of volatile fatty acids and their derivatives make them the most important group of VOCs produce by microbes and represent up to 87% of known antimicrobial VOCs produced by *B. subtilis* bacteria (**Supplementary Table S3**). They can be divided in two main categories: hydrocarbons (alkanes, alkenes, alkynes) or carbohydrates (acids, alcohols, aldehydes, esters, furans, ketones, lactones, benzenoids). Among them,

benzenoids is the most represented sub-category followed by alkanes, aldehydes, ketones, acids, and alcohols. Even though benzenoids could be considered as an individual category, they can also be seen as fatty acids derivatives because a large majority of antimicrobial benzenoid volatile produced by *B. subtilis* harbor a benzene core linked to a fatty acid derivatives.

There is an important diversity of benzenoids, sometimes linked with carbohydrate chains containing nitrogen, sulfur or both. Most of these antimicrobial volatile exert fungicidal activities but some have been characterized for their antibacterial or nematicidal abilities, too. Their mode of action is rarely fully characterized. For instance, morphological abnormalities on fungal and bacterial cells have been documented after an exposition to *B. subtilis* VOCs (Tahir et al., 2017). Volatile such as 1,3-butadiene or 2,3-butanediol are also known to induce modifications in the expression of genes linked to the pathogenicity of *R. solanacearum* and *Pectobacterium carotovorum* (Marquez-Villavicencio et al., 2011; Tahir et al., 2017). In addition to direct antimicrobial activities, fatty acids volatile have also several other biological functions. For instance, acetoin and 2-butanone have the ability to stimulate plant defenses or to induce plant stress tolerance which then promote plant growth (Ryu et al., 2003; Ryu et al., 2004; Ryu, 2015). They are essentially produced by strains of *B. amyloliquefaciens*, *B. velezensis* or *B. subtilis* (Audrain et al., 2015).

Terpenes and their derivatives (also known as terpenoids or isoprenoids) are among the most abundant secondary metabolites found in living systems (Fisher et al., 2001; Gershenzon and Dudareva, 2007). They originate from two main precursors: isopentenyl pyrophosphate (IPP) and its allylic isomer the dimethylallyl pyrophosphate (DMAPP) (Schulz and Dickschat, 2007). IPP and DMAPP are also the end-products of the deoxy-xylulose phosphate pathway (DOXP) starting with pyruvate and glyceraldehyde-3-phosphate originating from the glucose metabolism (Fisher et al., 2001). Terpenoids can be synthesized from isoprene molecules too. Julsing et al. (2007) showed that, in *B. subtilis*, isoprene is not formed by the MVA or DOXP pathways but, as in plant systems, might be a product of the methylerythritol phosphate (MEP) pathway (Guan et al., 2015).

Isoprenoid compounds are produced by all living organisms for essential physiological functions such as electron transport, membrane fluidity, light harvesting, photoprotection, anchoring of molecules to specific membranes and signaling (Fisher et al., 2001). The signaling ability is particularly important and is associated with several antagonistic, mutualistic or multi-trophic interactions (Shrivastava et al., 2015). More than 25,000 terpenic compounds have been listed and, for the vast majority, their biological functions and roles remain unknown (Buckingham, 1997). Volatile terpenes are generally recognized for their ability to inhibit bacteria (Scortichini and Rossi, 1991), fungi (Hammer et al., 2003; Dambolena et al., 2008), nematodes (Gu et al., 2007) or insects (Lee et al., 2003; Justicia et al., 2005). They can be classified in three categories: isoprene, monoterpenes (C_{10}) and sesquiterpenes (C_{15}) (Schmidt et al., 2015).

The mode of action of these compounds might be linked to their lipophilic nature allowing them to destabilize the cell membrane integrity (Cox et al., 2000; Inoue et al., 2004). To our knowledge, only two terpenes produced by *B. subtilis* show antimicrobial abilities: isoprene and monoterpene α-terpineol exhibit antagonistic activities against cyanobacteria and nematodes (Wright and Thompson, 1985; Gu et al., 2007).

Little is known about the biosynthetic pathways of nitrogen-containing VOCs. Nevertheless, it is accepted that two main routes can be used: a non-enzymatic amination of acyloins, that can lead to the formation of pyrazines (Schulz and Dickschat, 2007) or derived from α-aminoketone intermediates resulting from AA catabolism (Owens et al., 1997; Zhu et al., 2010).

Nitrogen-containing VOCs can be distinguished based on their cyclization rate. Within non-cyclic compounds, three groups are identified (amides, amines and imines) while there are five categories of cyclic compounds (azoles, pyrazines, pyridines, pyridazines, and pyrimidines). Pyrazines are strongly represented among microbial volatile and are separated in two classes: lower-alkylated and higher-alkylated pyrazines (Schulz and Dickschat, 2007). These compounds are characterized by a strong odor and several *B. subtilis* coming from the rhizosphere or from food fermentations have already been recognized as pyrazines producers (Sugawara et al., 1985; Kosuge and Kamiya, 1962; Larroche et al., 1999; Leejeerajumnean et al., 2001). Pyrazines from *B. subtilis* strains are known to exhibit antifungal and nematicidal activities (Gu et al., 2007; Chen et al., 2008; Chaves-López et al., 2015; Haidar et al., 2016). For instance, tetramethylpyrazine inhibits the growth of *Moniliophthora perniciosa* and *F. oxysporum* f. sp. *lactucae*. Additionally, it acts on sporulation and elongation of the germ-tube of *B. cinerea* (Chen et al., 2008; Chaves-López et al., 2015). It is interesting to note that *B. subtilis* pyrazines can also exhibit antibacterial activities such as pulcherriminic acid which inhibits the growth of *S. aureus*, *E. coli* and *Proteus vulgaris* (Coutts et al., 1965). Beside pyrazines, strains from the *B. subtilis* group are able to produce other nitrogen VOCs such as 1H-imidazole,1-ethyl showing antifungal activities against numerous soil-borne phytopathogens (Lupetti et al., 2002; Liu et al., 2008; Snelders et al., 2009; Schmidt et al., 2015).

Microbial VOCs containing sulfur (VSCs) derive from two main pathways originated from inorganic or organic sources (Schulz and Dickschat, 2007): inorganic sulfate reduction in methylated inorganic sulfides compounds or, for some microbial VSCs, originate from catabolism of AA such as L-methionine or more rarely, L-cysteine (Schulz and Dickschat, 2007). Some VSCs are produced as secondary volatiles via the production of hydrogen sulfide or methanethiol. Indeed, these two compounds are important precursors for subsequent VSCs synthesis (Schulz and Dickschat, 2007; Sourabié et al., 2012). Within the *B. subtilis* group, multiple VSCs such as dimethyl disulfide (DMDS), dimethyl trisulfide (DMTS), *S*-methyl thioacetate or *S*-methyl butanethioate have been characterized for their antifungal and nematicidal activities (Coosemans, 2005; Gerik, 2005; Gu et al., 2007; Kai et al., 2009; Wang et al., 2009; de Vrieze et al., 2015; Schmidt et al., 2015; Velivelli et al., 2015; Gotor-Vila et al., 2017). A putative antibacterial effect of DMDS is not to exclude. Indeed, DMDS is known to affect the bacterial cell-to-cell communications through a decrease in the

amount of *N*-acyl homoserine lactone (AHL) mediating QS (Chernin et al., 2011).

Other volatile organic compounds such as halogenated, metalloids, tellurium or selenium compounds have also been described. However, at the time of writing, no *B. subtilis* strains have been proved to produce these type of VOCs (Schulz and Dickschat, 2007), although related bacteria, like *Bacillus arsenicoselenatis*, have been shown to generate them (Switzer Blum et al., 1998).

CONCLUSION AND PERSPECTIVES

The *B. subtilis* group offers a plethora of antagonistic compounds displaying a broad range of biological functions. This huge versatility increases the industrial and environmental interest of *B. subtilis* strains, especially when considering their range of action against foodborne or phytopathogenic flora as well as their history of safe use in food. The present review on known AMCs from the *B. subtilis* group proposes a consistent classification frame based on their biosynthetic pathways (*i.e.*, RPs, PKs, NRPs, volatiles) and chemical nature.

The present classification suggests to establish systematic approaches for novel molecules discoveries and characterizations (biosynthesis, chemical nature and activity). Indeed, most current publications report antimicrobial activity of partially purified fractions which can involve mixtures of bioactive compounds. To assess the activity of an unique compound, implementations of genetic confirmation such as knockout strategy are needed. Besides, very few studies have focused on the putative synergistic effects within these bio-active mixtures. Also, the concentration of purified or semi-purified compound(s) often remains uncharacterized or biologically irrelevant. Finally, there is no doubt that novel AMCs originating from *B. subtilis* bacteria remain to be identified, characterized and properly classified.

AUTHOR CONTRIBUTIONS

SC, CN, and FL conducted the bibliographic search. SC and CN wrote the manuscript. AG, CB, and JM edited and reviewed the manuscript. All authors have read and approved the final version.

FUNDING

This work was supported by the National Fund for Scientific Research (FNRS), the Université catholique de Louvain (UCLouvain), and the Brussels Institute for Research and Innovation (Innoviris, Doctiris programme to CN). SC was supported by the Foundation for Training in Industrial and Agricultural Research (FRIA, FNRS), AG holds a Chargé de Recherche fellowship from the FNRS (Grant 1.B.208.16F).

ACKNOWLEDGMENTS

We gratefully acknowledge members of SC's Ph.D. committee Prof A. Legrève, UCLouvain, Prof. M. Ongena, ULiège and Dr. J.-P. Goffart, CRAw for their valuable comments on this manuscript. We also acknowledge the Walloon Region for the long-term financial support through the WACOBI and ANTAGONIST projects (Conventions N° DGO3-D31-1330 and N° DGO3-D31-1383/S1, respectively).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00302/full#supplementary-material

REFERENCES

Abriouel, H., Franz, C. M., Omar, N. B., and Gálvez, A. (2011). Diversity and applications of *Bacillus* bacteriocins. *FEMS Microbiol. Rev.* 35, 201–232. doi: 10.1111/j.1574-6976.2010.00244.x

Adak, S., Aulak, K. S., and Stuehr, D. J. (2002). Direct evidence for nitric oxide production by a nitric-oxide synthase-like protein from *Bacillus subtilis*. *J. Biol. Chem.* 277, 16167–16171. doi: 10.1074/jbc.M201136200

Agrios, G. N. (1988). "1 - INTRODUCTION TO PLANT PATHOLOGY," in *Plant Pathology*, 3rdEdition Edn, ed. G. N. Agrios (New York, NY: Academic Press), 3–39. doi: 10.1016/B978-0-12-044563-9.50005-0

Alamri, S. A. (2015). Enhancing the efficiency of the bioagent *Bacillus subtilis* JF419701 against soil-borne phytopathogens by increasing the productivity of fungal cell wall degrading enzymes. *Arch. Phytopathol. Plant Prot.* 48, 159–170. doi: 10.1080/03235408.2014.884671

Aleti, G., Sessitsch, A., and Brader, G. (2015). Genome mining: prediction of lipopeptides and polyketides from *Bacillus* and related Firmicutes. *Comput. Struct. Biotechnol. J.* 13, 192–203. doi: 10.1016/j.csbj.2015.03.003

An, J., Zhu, W., Liu, Y., Zhang, X., Sun, L., Hong, P., et al. (2015). Purification and characterization of a novel bacteriocin CAMT2 produced by *Bacillus amyloliquefaciens* isolated from marine fish *Epinephelus areolatus*. *Food Control* 51, 278–282. doi: 10.1016/j.foodcont.2014.11.038

Arguelles Arias, A., Ongena, M., Devreese, B., Terrak, M., Joris, B., and Fickers, P. (2013). Characterization of amylolysin, a novel lantibiotic from *Bacillus amyloliquefaciens* GA1. *PLoS One* 8:e83037. doi: 10.1371/journal.pone.0083037

Ariffin, H., Abdullah, N., Md Shah, U. K., Shirai, Y., and Hassan, M. A. (2006). Production and characterization of cellulases by *Bacillus pumilus* EB3. *Int. J. Eng. Technol.* 3, 47–53.

Arrebola, E., Sivakumar, D., and Korsten, L. (2010). Effect of volatile compounds produced by *Bacillus* strains on postharvest decay in citrus. *Biol. Control* 53, 122–128. doi: 10.1016/j.biocontrol.2009.11.010

Audrain, B., Farag, M. A., Ryu, C.-M., and Ghigo, J.-M. (2015). Role of bacterial volatile compounds in bacterial biology. *FEMS Microbiol. Rev.* 39, 222–233. doi: 10.1093/femsre/fuu013

Bais, H. P., Fall, R., and Vivanco, J. M. (2004). Biocontrol of *Bacillus subtilis* against Infection of *Arabidopsis* Roots by *Pseudomonas syringae* is facilitated by biofilm formation and surfactin Production. *Plant Physiol.* 134, 307–319. doi: 10.1104/pp.103.028712

Barbe, V., Cruveiller, S., Kunst, F., Lenoble, P., Meurice, G., Sekowska, A., et al. (2009). From a consortium sequence to a unified sequence: the *Bacillus subtilis* 168 reference genome a decade later. *Microbiology* 155, 1758–1775. doi: 10.1099/mic.0.027839-0

Béchet, M., Caradec, T., Hussein, W., Abderrahmani, A., Chollet, M., Leclère, V., et al. (2012). Structure, biosynthesis, and properties of kurstakins,

nonribosomal lipopeptides from *Bacillus* spp. *Appl. Microbiol. Biotechnol.* 95, 593–600. doi: 10.1007/s00253-012-4181-2

Begley, M., Cotter, P. D., Hill, C., and Ross, R. P. (2009). Identification of a novel two-peptide lantibiotic, lichenicidin, following rational genome mining for LanM proteins. *Appl. Environ. Microbiol.* 75, 5451–5460. doi: 10.1128/aem.00730-09

Bentley, R., and Haslam, E. (1990). The shikimate pathway — a metabolic tree with many branche. *Crit. Rev. Biochem. Mol. Biol.* 25, 307–384. doi: 10.3109/10409239009090615

Bernier, S. P., Létoffé, S., Delepierre, M., and Ghigo, J.-M. (2011). Biogenic ammonia modifies antibiotic resistance at a distance in physically separated bacteria. *Mol. Microbiol.* 81, 705–716. doi: 10.1111/j.1365-2958.2011.07724.x

Besson, F., Peypoux, F., Michel, G., and Delcambe, L. (1976). Characterization of iturin A in antibiotics from various strains of *Bacillus subtilis*. *J. Antibiot.* 29, 1043–1049. doi: 10.7164/antibiotics.29.1043

Besson, F., Peypoux, F., Michel, G., and Delcambe, L. (1979). Antifungal activity upon *Saccharomyces cerevisiae* of iturin A, mycosubtilin, bacillomycin L and of their derivatives; inhibition of this antifungal activity by lipid antagonists. *J. Antibiot.* 32, 828–833. doi: 10.7164/antibiotics.32.828

Bowman, S. M., and Free, S. J. (2006). The structure and synthesis of the fungal cell wall. *Bioessays* 28, 799–808. doi: 10.1002/bies.20441

Brötz, H., Bierbaum, G., Markus, A., Molitor, E., and Sahl, H. G. (1995). Mode of action of the lantibiotic mersacidin: inhibition of peptidoglycan biosynthesis via a novel mechanism? *Antimicrob. Agents Chemother.* 39, 714–719. doi: 10.1128/aac.39.3.714

Buckingham, J. (1997). *Dictionary of Natural Products*. London: CRC press. doi: 10.1007/978-1-4899-6850-0

Caboche, S., Pupin, M., Leclère, V., Fontaine, A., Jacques, P., and Kucherov, G. (2008). NORINE: a database of nonribosomal peptides. *Nucleic Acids Res.* 36, D326–D331. doi: 10.1093/nar/gkm792

Cane, D. E., and Walsh, C. T. (1999). The parallel and convergent universes of polyketide synthases and nonribosomal peptide synthetases. *Chem. Biol.* 6, R319–R325. doi: 10.1016/S1074-5521(00)80001-0

Caulier, S., Gillis, A., Colau, G., Licciardi, F., Liépin, M., Desoignies, N., et al. (2018). Versatile antagonistic activities of soil-borne *Bacillus* spp. and *Pseudomonas* spp. against *Phytophthora infestans* and other potato pathogens. *Front. Microbiol.* 9:143. doi: 10.3389/fmicb.2018.00143

Cawoy, H., Debois, D., Franzil, L., De Pauw, E., Thonart, P., and Ongena, M. (2015). Lipopeptides as main ingredients for inhibition of fungal phytopathogens by *Bacillus subtilis/amyloliquefaciens*. *Microb. Biotechnol.* 8, 281–295. doi: 10.1111/1751-7915.12238

Challis, G. L. (2008). Genome mining for novel natural product discovery. *J. Med. Chem.* 51, 2618–2628. doi: 10.1021/jm700948z

Challis, G. L., and Naismith, J. H. (2004). Structural aspects of non-ribosomal peptide biosynthesis. *Curr. Opin. Struct. Biol.* 14, 748–756. doi: 10.1016/j.sbi.2004.10.005

Chatterjee, C., Paul, M., Xie, L., and van der Donk, W. A. (2005). Biosynthesis and mode of action of lantibiotics. *Chem. Rev.* 105, 633–684. doi: 10.1021/cr030105v

Chaves-López, C., Serio, A., Gianotti, A., Sacchetti, G., Ndagijimana, M., Ciccarone, C., et al. (2015). Diversity of food-borne *Bacillus* volatile compounds and influence on fungal growth. *J. Appl. Microbiol.* 119, 487–499. doi: 10.1111/jam.12847

Chen, H., and Du, L. (2016). Iterative polyketide biosynthesis by modular polyketide synthases in bacteria. *Appl. Microbiol. Biotechnol.* 100, 541–557. doi: 10.1007/s00253-015-7093-0

Chen, H., Xiao, X., Wang, J., Wu, L., Zheng, Z., and Yu, Z. (2008). Antagonistic effects of volatiles generated by *Bacillus subtilis* on spore germination and hyphal growth of the plant pathogen, *Botrytis cinerea*. *Biotechnol. Lett.* 30, 919–923. doi: 10.1007/s10529-007-9626-9

Chen, X., Scholz, R., Borriss, M., Junge, H., Mogel, G., Kunz, S., et al. (2009). Difficidin and bacilysin produced by plant-associated *Bacillus amyloliquefaciens* Dare efficient in controlling fire blight disease. *J. Biotechnol.* 140, 38–44. doi: 10.1016/j.jbiotec.2008.10.015

Chen, X., Vater, J., Piel, J., Franke, P., Scholz, R., Schneider, K., et al. (2006). Structural and functional characterization of three polyketide synthase gene clusters in *Bacillus amyloliquefaciens* FZB42. *J. Bacteriol.* 188, 4024–4036. doi: 10.1128/jb.00052-06

Cherif-Silini, H., Silini, A., Yahiaoui, B., Ouzari, I., and Boudabous, A. (2016). Phylogenetic and plant-growth-promoting characteristics of *Bacillus* isolated from the wheat rhizosphere. *Ann. Microbiol.* 66, 1087–1097. doi: 10.1007/s13213-016-1194-6

Chernin, L., Toklikishvili, N., Ovadis, M., Kim, S., Ben-Ari, J., Khmel, I., et al. (2011). Quorum-sensing quenching by rhizobacterial volatiles. *Environ. Microbiol. Rep.* 3, 698–704. doi: 10.1111/j.1758-2229.2011.00284.x

Chopra, L., Singh, G., Choudhary, V., and Sahoo, D. K. (2014). Sonorensin: an antimicrobial peptide, belonging to the heterocycloanthracin subfamily of bacteriocins, from a new marine isolate, *Bacillus sonorensis* MT93. *Appl. Environ. Microbiol.* 80, 2981–2990. doi: 10.1128/aem.04259-13

Chopra, L., Singh, G., Jena, K. K., Verma, H., and Sahoo, D. K. (2015). Bioprocess development for the production of sonorensin by *Bacillus sonorensis* MT93 and its application as a food preservative. *Bioresour. Technol.* 175, 358–366. doi: 10.1016/j.biortech.2014.10.105

Cleveland, J., Montville, T. J., Nes, I. F., and Chikindas, M. L. (2001). Bacteriocins: safe, natural antimicrobials for food preservation. *Int. J. Food Microbiol.* 71, 1–20. doi: 10.1016/S0168-1605(01)00560-8

Coosemans, J. (2005). Dimethyl disulphide (DMDS): a potential novel nematicide and soil disinfectant. *Acta Hortic.* 698, 57–64. doi: 10.17660/ActaHortic.2005.698.6

Cosby, W. M., Vollenbroich, D., Lee, O. H., and Zuber, P. (1998). Altered *srf* expression in *Bacillus subtilis* resulting from changes in culture pH is dependent on the Spo0K oligopeptide permease and the ComQX system of extracellular control. *J. Bacteriol.* 180, 1438–1445.

Cotter, P. D., Hill, C., and Ross, R. P. (2005). Bacteriocins: developing innate immunity for food. *Nat. Rev. Microbiol.* 3, 777–788. doi: 10.1038/nrmicro1273

Coutts, R. T., Pitkethly, W. N., and Wibberley, D. G. (1965). Antibacterial activity of some quinolines containing a cyclic hydroxamic acid group. *J. Pharm. Sci.* 54, 792–795. doi: 10.1002/jps.2600540530

Cox, S. D., Mann, C. M., Markham, J. L., Bell, H. C., Gustafson, J. E., Warmington, J. R., et al. (2000). The mode of antimicrobial action of the essential oil of *Melaleuca alternifolia* (tea tree oil). *J. Appl. Microbiol.* 88, 170–175. doi: 10.1046/j.1365-2672.2000.00943.x

Crane, J. M., Gibson, D. M., Vaughan, R. H., and Bergstrom, G. C. (2012). Iturin levels on wheat spikes linked to biological control of fusarium head blight by *Bacillus amyloliquefaciens*. *Phytopathology* 103, 146–155. doi: 10.1094/PHYTO-07-12-0154-R

Czajkowski, R., and Jafra, S. (2009). Quenching of acyl-homoserine lactone-dependent quorum sensing by enzymatic disruption of signal molecules. *Acta Biochim. Pol.* 56, 1–16.

Dambolena, J. S., López, A. G., Cánepa, M. C., Theumer, M. G., Zygadlo, J. A., and Rubinstein, H. R. (2008). Inhibitory effect of cyclic terpenes (limonene, menthol, menthone and thymol) on *Fusarium verticillioides* MRC 826 growth and fumonisin B1 biosynthesis. *Toxicon* 51, 37–44. doi: 10.1016/j.toxicon.2007.07.005

de Vrieze, M., Pandey, P., Bucheli, T. D., Varadarajan, A. R., Ahrens, C. H., Weisskopf, L., et al. (2015). Volatile organic compounds from native potato-associated *Pseudomonas* as potential anti-oomycete agents. *Front. Microbiol.* 6:1295. doi: 10.3389/fmicb.2015.01295

Delves-Broughton, J. (1990). Nisin and its application as a food preservative. *Int. J. Dairy Technol.* 43, 73–76. doi: 10.1111/j.1471-0307.1990.tb02449.x

Dertz, E. A., Xu, J., Stintzi, A., and Raymond, K. N. (2006). Bacillibactin-mediated iron transport in *Bacillus subtilis*. *J. Am. Chem. Soc.* 128, 22–23. doi: 10.1021/ja055898c

Dickschat, J. S., Bode, H. B., Wenzel, S. C., Müller, R., and Schulz, S. (2005). Biosynthesis and identification of volatiles released by the myxobacterium *Stigmatella aurantiaca*. *Chembiochem* 6, 2023–2033. doi: 10.1002/cbic.200500174

Dimkić, I., Živković, S., Berić, T., Ivanović, Ž, Gavrilović, V., Stanković, S., et al. (2013). Characterization and evaluation of two *Bacillus* strains, SS-12.6 and SS-13.1, as potential agents for the control of phytopathogenic bacteria and fungi. *Biol. Control* 65, 312–321. doi: 10.1016/j.biocontrol.2013.03.012

Dong, Y.-H., Zhang, X.-F., Xu, J.-L., and Zhang, L.-H. (2004). Insecticidal *Bacillus thuringiensis* silences *Erwinia carotovora* virulence by a new form of microbial antagonism, signal interference. *Appl. Environ. Microbiol.* 70, 954–960. doi: 10.1128/aem.70.2.954-960.2004

Dubois, J. Y. F., Kouwen, T. R., Schurich, A. K. C., Reis, C. R., Ensing, H. T., Trip, E. N., et al. (2009). Immunity to the bacteriocin sublancin 168 is determined by the SunI (YolF) protein of *Bacillus subtilis*. *Antimicrob. Agents Chemother.* 53, 651–661. doi: 10.1128/aac.01189-08

Dunlap, C. A., Schisler, D. A., Price, N. P., and Vaughn, S. F. (2011). Cyclic lipopeptide profile of three *Bacillus subtilis* strains; antagonists of *Fusarium* head blight. *J. Microbiol.* 49, 603–609. doi: 10.1007/s12275-011-1044-y

Effmert, U., Kalderás, J., Warnke, R., and Piechulla, B. (2012). Volatile mediated interactions between bacteria and fungi in the soil. *J. Chem. Ecol.* 38, 665–703. doi: 10.1007/s10886-012-0135-5

Eshita, S. M., Roberto, N. H., Beale, J. M., Mamiya, B. M., and Workman, R. F. (1995). Bacillomycin Lc, a new antibiotic of the iturin group: isolations, structures, and antifungal activities of the congeners. *J. Antibiot.* 48, 1240–1247. doi: 10.7164/antibiotics.48.1240

Eustáquio, A. S., McGlinchey, R. P., Liu, Y., Hazzard, C., Beer, L. L., Florova, G., et al. (2009). Biosynthesis of the salinosporamide A polyketide synthase substrate chloroethylmalonyl-coenzyme A from *S*-adenosyl-L-methionine. *Proc. Natl. Acad. Sci. U.S.A.* 106, 12295–12300. doi: 10.1073/pnas.0901237106

Even, S., Burguière, P., Auger, S., Soutourina, O., Danchin, A., and Martin-Verstraete, I. (2006). Global control of cysteine metabolism by CymR in *Bacillus subtilis*. *J. Bacteriol.* 188, 2184–2197. doi: 10.1128/jb.188.6.2184-2197.2006

Fan, B., Blom, J., Klenk, H.-P., and Borriss, R. (2017). *Bacillus amyloliquefaciens*, *Bacillus velezensis,* and *Bacillus siamensis* form an "operational group *B. amyloliquefaciens*" within the *B. subtilis* species complex. *Front. Microbiol.* 8:22. doi: 10.3389/fmicb.2017.00022

Fickers, P., Guez, J.-S., Damblon, C., Leclère, V., Béchet, M., Jacques, P., et al. (2009). High-level biosynthesis of the anteiso-C17 isoform of the antibiotic mycosubtilin in *Bacillus subtilis* and characterization of its candidacidal activity. *Appl. Environ. Microbiol.* 75, 4636–4640. doi: 10.1128/aem.00548-09

Fisch, K. M. (2013). Biosynthesis of natural products by microbial iterative hybrid PKS–NRPS. *RSC Adv.* 3, 18228–18247. doi: 10.1039/C3RA42661K

Fisher, A. J., Rosenstiel, T. N., Shirk, M. C., and Fall, R. (2001). Nonradioactive assay for cellular dimethylallyl diphosphate. *Anal. Biochem.* 292, 272–279. doi: 10.1006/abio.2001.5079

Fu, L.-H., Hu, K.-D., Hu, L.-Y., Li, Y.-H., Hu, L.-B., Yan, H., et al. (2014). An antifungal role of hydrogen sulfide on the postharvest pathogens *Aspergillus niger* and *Penicillium italicum*. *PLoS One* 9:e104206. doi: 10.1371/journal.pone.0104206

Fuchs, S. W., Jaskolla, T. W., Bochmann, S., Kötter, P., Wichelhaus, T., Karas, M., et al. (2011). Entianin, a novel subtilin-like lantibiotic from *Bacillus subtilis* DSM 15029T with high antimicrobial activity. *Appl. Environ. Microbiol.* 77, 1698–1707. doi: 10.1128/aem.01962-10

Furuta, Y., Harima, H., Ito, E., Maruyama, F., Ohnishi, N., Osaki, K., et al. (2018). Loss of bacitracin resistance due to a large genomic deletion among *Bacillus anthracis* strains. *mSystems* 3:e00182-18. doi: 10.1128/mSystems.00182-18

Gao, L., Han, J., Liu, H., Qu, X., Lu, Z., and Bie, X. (2017). Plipastatin and surfactin coproduction by *Bacillus subtilis* pB2-L and their effects on microorganisms. *Antonie Van Leeuwenhoek* 110, 1007–1018. doi: 10.1007/s10482-017-0874-y

Gao, Z., Zhang, B., Liu, H., Han, J., and Zhang, Y. (2017). Identification of endophytic *Bacillus velezensis* ZSY-1 strain and antifungal activity of its volatile compounds against *Alternaria solani* and *Botrytis cinerea*. *Biol. Control* 105, 27–39. doi: 10.1016/j.biocontrol.2016.11.007

Gautam, N., and Sharma, N. (2009). Bacteriocin: safest approach to preserve food products. *Indian J. Microbiol.* 49, 204–211. doi: 10.1007/s12088-009-0048-3

Geraldine, A. M., Lopes, F. A. C., Carvalho, D. D. C., Barbosa, E. T., Rodrigues, A. R., Brandão, R. S., et al. (2013). Cell wall-degrading enzymes and parasitism of sclerotia are key factors on field biocontrol of white mold by *Trichoderma* spp. *Biol. Control* 67, 308–316. doi: 10.1016/j.biocontrol.2013.09.013

Gerik, J. S. (2005). Evaluation of soil fumigants applied by drip irrigation for liatris production. *Plant Dis.* 89, 883–887. doi: 10.1094/PD-89-0883

Gershenzon, J., and Dudareva, N. (2007). The function of terpene natural products in the natural world. *Nat. Chem. Biol.* 3, 408–414. doi: 10.1038/nchembio.2007.5

Giorgio, A., De Stradis, A., Lo Cantore, P., and Iacobellis, N. S. (2015). Biocide effects of volatile organic compounds produced by potential biocontrol rhizobacteria on *Sclerotinia sclerotiorum*. *Front. Microbiol.* 6:1056. doi: 10.3389/fmicb.2015.01056

Gomaa, E. Z. (2012). Chitinase production by *Bacillus thuringiensis* and *Bacillus licheniformis*: their potential in antifungal biocontrol. *J. Microbiol.* 50, 103–111. doi: 10.1007/s12275-012-1343-y

Gong, A.-D., Li, H.-P., Yuan, Q.-S., Song, X.-S., Yao, W., He, W.-J., et al. (2015). Antagonistic mechanism of iturin A and plipastatin A from *Bacillus amyloliquefaciens* S76-3 from wheat spikes against *Fusarium graminearum*. *PLoS One* 10:e0116871. doi: 10.1371/journal.pone.0116871

Gong, Q., Zhang, C., Lu, F., Zhao, H., Bie, X., and Lu, Z. (2014). Identification of bacillomycin D from *Bacillus subtilis* fmbJ and its inhibition effects against *Aspergillus flavus*. *Food Control* 36, 8–14. doi: 10.1016/j.foodcont.2013.07.034

González, J. E., and Keshavan, N. D. (2006). Messing with bacterial quorum sensing. *Microbiol. Mol. Biol. Rev.* 70, 859–875. doi: 10.1128/mmbr.00002-06

Gordon, R., Haynes, W., Pang, C., and Smith, N. (1973). *The Genus Bacillus*. Washington, DC: United States Department of Agriculture, 109–126.

Gotor-Vila, A., Teixidó, N., Di Francesco, A., Usall, J., Ugolini, L., Torres, R., et al. (2017). Antifungal effect of volatile organic compounds produced by *Bacillus amyloliquefaciens* CPA-8 against fruit pathogen decays of cherry. *Food Microbiol.* 64, 219–225. doi: 10.1016/j.fm.2017.01.006

Gu, Y.-Q., Mo, M.-H., Zhou, J.-P., Zou, C.-S., and Zhang, K.-Q. (2007). Evaluation and identification of potential organic nematicidal volatiles from soil bacteria. *Soil Biol. Biochem.* 39, 2567–2575. doi: 10.1016/j.soilbio.2007.05.011

Guan, Z., Xue, D., Abdallah, I. I., Dijkshoorn, L., Setroikromo, R., Lv, G., et al. (2015). Metabolic engineering of *Bacillus subtilis* for terpenoid production. *Appl. Microbiol. Biotechnol.* 99, 9395–9406. doi: 10.1007/s00253-015-6950-1

Guder, A., Wiedemann, I., and Sahl, H.-G. (2000). Posttranslationally modified bacteriocins—the lantibiotics. *Biopolymers* 55, 62–73. doi: 10.1002/1097-0282(2000)55:1<62::AID-BIP60>3.0.CO;2-Y

Guo, Q., Dong, W., Li, S., Lu, X., Wang, P., Zhang, X., et al. (2014). Fengycin produced by *Bacillus subtilis* NCD-2 plays a major role in biocontrol of cotton seedling damping-off disease. *Microbiol. Res.* 169, 533–540. doi: 10.1016/j.micres.2013.12.001

Gustafson, K., Roman, M., and Fenical, W. (1989). The macrolactin, a novel class of antiviral and cytotoxic macrolides from a deep-sea marine bacterium. *J. Am. Chem. Soc.* 111, 7519–7524. doi: 10.1021/ja00201a036

Haidar, R., Roudet, J., Bonnard, O., Dufour, M. C., Corio-Costet, M. F., Fert, M., et al. (2016). Screening and modes of action of antagonistic bacteria to control the fungal pathogen *Phaeomoniella chlamydospora* involved in grapevine trunk diseases. *Microbiol. Res.* 192, 172–184. doi: 10.1016/j.micres.2016.07.003

Hammami, I., Jaouadi, B., Bacha, A. B., Rebai, A., Bejar, S., Nesme, X., et al. (2012). *Bacillus subtilis* bacteriocin Bac 14B with a broad inhibitory spectrum: purification, amino acid sequence analysis, and physicochemical characterization. *Biotechnol. Bioprocess Eng.* 17, 41–49. doi: 10.1007/s12257-010-0401-8

Hammer, K. A., Carson, C. F., and Riley, T. V. (2003). Antifungal activity of the components of *Melaleuca alternifolia* (tea tree) oil. *J. Appl. Microbiol.* 95, 853–860. doi: 10.1046/j.1365-2672.2003.02059.x

Hamoen, L. W., Venema, G., and Kuipers, O. P. (2003). Controlling competence in *Bacillus subtilis:* shared use of regulators. *Microbiology* 149, 9–17. doi: 10.1099/mic.0.26003-0

Heinzmann, S., Entian, K.-D., and Stein, T. (2006). Engineering *Bacillus subtilis* ATCC 6633 for improved production of the lantibiotic subtilin. *Appl. Microbiol. Biotechnol.* 69, 532–536. doi: 10.1007/s00253-005-0023-9

Herrera-Estrella, A., and Chet, I. (1999). Chitinases in biological control. *EXS* 87, 171–184. doi: 10.1007/978-3-0348-8757-1_12

Hertweck, C. (2009). The biosynthetic logic of polyketide diversity. *Angew. Chem. Int. Ed. Engl.* 48, 4688–4716. doi: 10.1002/anie.200806121

Horsman, G. P., Van Lanen, S. G., and Shen, B. (2009). Chapter 5 Iterative type I polyketide synthases for enediyne core biosynthesis. *Methods Enzymol.* 459, 97–112. doi: 10.1016/S0076-6879(09)04605-9

Howell, C., Beier, R., and Stipanovic, R. (1988). Production of ammonia by *Enterobacter cloacae* and its possible role in the biological control of *Pythium* preemergence damping-off by the bacterium. *Phytopathology* 78, 1075–1078. doi: 10.1094/Phyto-78-1075

Hsieh, F.-C., Lin, T.-C., Meng, M., and Kao, S.-S. (2008). Comparing methods for identifying *Bacillus* strains capable of producing the antifungal lipopeptide iturin A. *Curr. Microbiol.* 56, 1–5. doi: 10.1007/s00284-007-9003-x

Hussein, A., and Al-Janabi, S. (2006). Identification of bacitracin produced by local isolate of *Bacillus licheniformis*. *Afr. J. Biotechnol.* 5, 1600–1601.

Inoue, Y., Shiraishi, A., Hada, T., Hirose, K., Hamashima, H., and Shimada, J. (2004). The antibacterial effects of terpene alcohols on *Staphylococcus aureus* and their mode of action. *FEMS Microbiol. Lett.* 237, 325–331. doi: 10.1111/j.1574-6968.2004.tb09714.x

Insam, H., and Seewald, M. S. A. (2010). Volatile organic compounds (VOCs) in soils. *Biol. Fertil. Soils* 46, 199–213. doi: 10.1007/s00374-010-0442-3

Jacques, P. (2011). "Surfactin and other lipopeptides from *Bacillus* spp," in *Biosurfactants: From Genes to Applications*, ed. G. Soberón-Chávez (Berlin: Springer), 57–91. doi: 10.1007/978-3-642-14490-5_3

Janiak, A. M., and Milewski, S. (2001). Mechanism of antifungal action of kanosamine. *Med. Mycol.* 39, 401–408. doi: 10.1080/mmy.39.5.401.408

Jaruchoktaweechai, C., Suwanborirux, K., Tanasupawatt, S., Kittakoop, P., and Menasveta, P. (2000). New Macrolactins from a Marine *Bacillus* sp. Sc026. *J. Nat. Prod.* 63, 984–986. doi: 10.1021/np990605c

Jenny, K., Käppeli, O., and Fiechter, A. (1991). Biosurfactants from *Bacillus licheniformis*: structural analysis and characterization. *Appl. Microbiol. Biotechnol.* 36, 5–13. doi: 10.1007/bf00164690

Johnson, B. A., Anker, H., and Meleney, F. L. (1945). Bacitracin: a new antibiotic produced by a member of the *B. subtilis* group. *Science* 102, 376–377. doi: 10.1126/science.102.2650.376

Julsing, M. K., Rijpkema, M., Woerdenbag, H. J., Quax, W. J., and Kayser, O. (2007). Functional analysis of genes involved in the biosynthesis of isoprene in *Bacillus subtilis*. *Appl. Microbiol. Biotechnol.* 75, 1377–1384. doi: 10.1007/s00253-007-0953-5

Justicia, J., Oltra, J. E., Barrero, A. F., Guadaño, A., González-Coloma, A., and Cuerva, J. M. (2005). Total synthesis of 3-hydroxydrimanes mediated by titanocene(III) – evaluation of their antifeedant activity. *Eur. J. Org. Chem.* 2005, 712–718. doi: 10.1002/ejoc.200400634

Kai, M., Haustein, M., Molina, F., Petri, A., Scholz, B., and Piechulla, B. (2009). Bacterial volatiles and their action potential. *Appl. Microbiol. Biotechnol.* 81, 1001–1012. doi: 10.1007/s00253-008-1760-3

Kawulka, K. E., Sprules, T., Diaper, C. M., Whittal, R. M., McKay, R. T., Mercier, P., et al. (2004). Structure of subtilosin A, a cyclic antimicrobial peptide from *Bacillus subtilis* with unusual sulfur to (-carbon cross-links: formation and reduction of (-Thio-(-Amino Acid derivatives. *Biochemistry* 43, 3385–3395. doi: 10.1021/bi0359527

Kenig, M., and Abraham, E. P. (1976). Antimicrobial activities and antagonists of bacilysin and anticapsin. *Microbiology* 94, 37–45. doi: 10.1099/00221287-94-1-37

Kenig, M., Vandamme, E., and Abraham, E. P. (1976). The mode of action of bacilysin and anticapsin and biochemical properties of bacilysin-resistant mutants. *Microbiology* 94, 46–54. doi: 10.1099/00221287-94-1-46

Kim, H.-S., Yoon, B.-D., Lee, C.-H., Suh, H.-H., Oh, H.-M., Katsuragi, T., et al. (1997). Production and properties of a lipopeptide biosurfactant from *Bacillus subtilis* C9. *J. Ferment. Bioeng.* 84, 41–46. doi: 10.1016/S0922-338X(97)82784-5

Kim, P. I., Ryu, J., Kim, Y. H., and Chi, Y.-T. (2010). Production of biosurfactant lipopeptides iturin A, fengycin and surfactin A from *Bacillus subtilis* CMB32 for control of *Colletotrichum gloeosporioides*. *J. Microbiol. Biotechnol.* 20, 138–145. doi: 10.4014/jmb.0905.05007

Klaenhammer, T. R. (1988). Bacteriocins of lactic acid bacteria. *Biochimie* 70, 337–349. doi: 10.1016/0300-9084(88)90206-4

Kleerebezem, M. (2004). Quorum sensing control of lantibiotic production; nisin and subtilin autoregulate their own biosynthesis. *Peptides* 25, 1405–1414. doi: 10.1016/j.peptides.2003.10.021

Konz, D., Klens, A., Schörgendorfer, K., and Marahiel, M. A. (1997). The bacitracin biosynthesis operon of *Bacillus licheniformis* ATCC 10716: molecular characterization of three multi-modular peptide synthetases. *Chem. Biol.* 4, 927–937. doi: 10.1016/S1074-5521(97)90301-X

Kopp, F., and Marahiel, M. A. (2007). Macrocyclization strategies in polyketide and nonribosomal peptide biosynthesis. *Nat. Prod. Rep.* 24, 735–749. doi: 10.1039/B613652B

Korpi, A., Järnberg, J., and Pasanen, A.-L. (2009). Microbial volatile organic compounds. *Crit. Rev. Toxicol.* 39, 139–193. doi: 10.1080/10408440802291497

Kosuge, T., and Kamiya, H. (1962). Discovery of a pyrazine in a natural product: tetramethylpyrazine from cultures of a strain of *Bacillus subtilis*. *Nature* 193:776. doi: 10.1038/193776a0

Kugler, M., Loeffler, W., Rapp, C., Kern, A., and Jung, G. (1990). Rhizocticin A, an antifungal phosphono-oligopeptide of *Bacillus subtilis* ATCC 6633: biological properties. *Arch. Microbiol.* 153, 276–281. doi: 10.1007/bf00249082

Kumar, P., Dubey, R. C., and Maheshwari, D. K. (2012). *Bacillus* strains isolated from rhizosphere showed plant growth promoting and antagonistic activity against phytopathogens. *Microbiol. Res.* 167, 493–499. doi: 10.1016/j.micres.2012.05.002

Kwon, J. W., and Kim, S. D. (2014). Characterization of an antibiotic produced by *Bacillus subtilis* JW-1 that suppresses *Ralstonia solanacearum*. *J. Microbiol. Biotechnol.* 24, 13–18. doi: 10.4014/jmb.1308.08060

Landy, M., Warren, G. H., RosenmanM, S. B., and Colio, L. G. (1948). Bacillomycin: an antibiotic from *Bacillus subtilis* active against pathogenic fungi. *Proc. Soc. Exp. Biol. Med.* 67, 539–541. doi: 10.3181/00379727-67-16367

Larroche, C., Besson, I., and Gros, J.-B. (1999). High pyrazine production by *Bacillus subtilis* in solid substrate fermentation on ground soybeans. *Process Biochem.* 34, 667–674. doi: 10.1016/S0032-9592(98)00141-1

Leclere, V., Bechet, M., Adam, A., Guez, J. S., Wathelet, B., Ongena, M., et al. (2005). Mycosubtilin overproduction by *Bacillus subtilis* BBG100 enhances the organism's antagonistic and biocontrol activities. *Appl. Environ. Microbiol.* 71, 4577–4584. doi: 10.1128/aem.71.8.4577-4584.2005

Lee, D. W., and Kim, B. S. (2015). Antimicrobial cyclic peptides for plant disease control. *Plant Pathol. J.* 31, 1–11. doi: 10.5423/PPJ.RW.08.2014.0074

Lee, H., Churey, J. J., and Worobo, R. W. (2008). Purification and structural characterization of bacillomycin F produced by a bacterial honey isolate active against *Byssochlamys fulva* H25. *J. Appl. Microbiol.* 105, 663–673. doi: 10.1111/j.1365-2672.2008.03797.x

Lee, S., Peterson, C. J., and Coats, J. R. (2003). Fumigation toxicity of monoterpenoids to several stored product insects. *J. Stored Prod. Res.* 39, 77–85. doi: 10.1016/S0022-474X(02)00020-6

Lee, S.-C., Kim, S.-H., Park, I.-H., Chung, S.-Y., and Choi, Y.-L. (2007). Isolation and structural analysis of bamylocin A, novel lipopeptide from *Bacillus amyloliquefaciens* LP03 having antagonistic and crude oil-emulsifying activity. *Arch. Microbiol.* 188, 307–312. doi: 10.1007/s00203-007-0250-9

Leejeerajumnean, A., Duckham, S. C., Owens, J. D., and Ames, J. M. (2001). Volatile compounds in *Bacillus*-fermented soybeans. *J. Sci. Food Agric.* 81, 525–529. doi: 10.1002/jsfa.843

Lemfack, M. C., Gohlke, B.-O., Toguem, S. M. T., Preissner, S., Piechulla, B., and Preissner, R. (2018). mVOC 2.0: a database of microbial volatiles. *Nucleic Acids Res.* 46, D1261–D1265. doi: 10.1093/nar/gkx1016

Li, L., Ma, M., Huang, R., Qu, Q., Li, G., Zhou, J., et al. (2012). Induction of chlamydospore formation in *Fusarium* by cyclic lipopeptide antibiotics from *Bacillus subtilis* C2. *J. Chem. Ecol.* 38, 966–974. doi: 10.1007/s10886-012-0171-1

Li, N., Shi, Z., Tang, Y., Chen, J., and Li, X. (2008). Recent progress on the total synthesis of acetogenins from Annonaceae. *Beilstein J. Org. Chem.* 4:48. doi: 10.3762/bjoc.4.48

Linnett, P. E., and Strominger, J. L. (1973). Additional antibiotic inhibitors of peptidoglycan synthesis. *Antimicrob. Agents Chemother.* 4, 231–236. doi: 10.1128/aac.4.3.231

Liu, W. W., Mu, W., Zhu, B. Y., Du, Y. C., and Liu, F. (2008). Antagonistic activities of volatiles from four strains of *Bacillus* spp. and *Paenibacillus* spp. against soil-borne plant pathogens. *Agric. Sci. China* 7, 1104–1114. doi: 10.1016/S1671-2927(08)60153-4

Liu, Y., Chen, Z., Ng, T. B., Zhang, J., Zhou, M., Song, F., et al. (2007). Bacisubin, an antifungal protein with ribonuclease and hemagglutinating activities from *Bacillus subtilis* strain B-916. *Peptides* 28, 553–559. doi: 10.1016/j.peptides.2006.10.009

Loeffler, W., Tschen, J. S.-M., Vanittanakom, N., Kugler, M., Knorpp, E., Hsieh, T. F., et al. (1986). Antifungal effects of bacilysin and fengymycin from *Bacillus subtilis* F-29-3 a comparison with activities of other *Bacillus* antibiotics. *J. Phytopathol.* 115, 204–213. doi: 10.1111/j.1439-0434.1986.tb00878.x

Loiseau, C., Schlusselhuber, M., Bigot, R., Bertaux, J., Berjeaud, J.-M., and Verdon, J. (2015). Surfactin from *Bacillus subtilis* displays an unexpected anti-*Legionella* activity. *Appl. Microbiol. Biotechnol.* 99, 5083–5093. doi: 10.1007/s00253-014-6317-z

Lu, X. L., Xu, Q. Z., Shen, Y. H., Liu, X. Y., Jiao, B. H., Zhang, W. D. et al. (2008). Macrolactin S, a novel macrolactin antibiotic from marine *Bacillus* sp. *Nat. Prod. Res.* 22, 342–347. doi: 10.1080/14786410701768162

Luo, C., Liu, X., Zhou, X., Guo, J., Truong, J., Wang, X., et al. (2015). Unusual biosynthesis and structure of locillomycins from *Bacillus*

subtilis 916. *Appl. Environ. Microbiol.* 81, 6601–6609. doi: 10.1128/aem.01639-15

Lupetti, A., Danesi, R., Campa, M., Tacca, M. D., and Kelly, S. (2002). Molecular basis of resistance to azole antifungals. *Trends Mol. Med.* 8, 76–81. doi: 10.1016/S1471-4914(02)02280-3

Ma, Z., Hu, J., Wang, X., and Wang, S. (2013). NMR spectroscopic and MS/MS spectrometric characterization of a new lipopeptide antibiotic bacillopeptin B1 produced by a marine sediment-derived *Bacillus amyloliquefaciens* SH-B74. *J. Antibiot.* 67, 175–178. doi: 10.1038/ja.2013.89

Mahlstedt, S. A., and Walsh, C. T. (2010). Investigation of anticapsin biosynthesis reveals a four-enzyme pathway to tetrahydrotyrosine in *Bacillus subtilis*. *Biochemistry* 49, 912–923. doi: 10.1021/bi9021186

Majumdar, S. K., and Bose, S. K. (1958). Mycobacillin, a new antifungal antibiotic produced by *B. subtilis*. *Nature* 181, 134–135. doi: 10.1038/181134a0

Malfanova, N., Franzil, L., Lugtenberg, B., Chebotar, V., and Ongena, M. (2012). Cyclic lipopeptide profile of the plant-beneficial endophytic bacterium *Bacillus subtilis* HC8. *Arch. Microbiol.* 194, 893–899. doi: 10.1007/s00203-012-0823-0

Marquez-Villavicencio, M. D. P., Weber, B., Witherell, R. A., Willis, D. K., and Charkowski, A. O. (2011). The 3-Hydroxy-2-Butanone pathway is required for *Pectobacterium carotovorum* pathogenesis. *PLoS One* 6:e22974. doi: 10.1371/journal.pone.0022974

Marx, R., Stein, T., Entian, K.-D., and Glaser, S. J. (2001). Structure of the *Bacillus subtilis* peptide antibiotic subtilosin A determined by 1H-NMR and matrix assisted laser desorption/ionization time-of-flight mass spectrometry. *J. Protein Chem.* 20, 501–506. doi: 10.1023/a:1012562631268

May, J. J., Wendrich, T. M., and Marahiel, M. A. (2001). The *dhb* operon of *Bacillus subtilis* encodes the biosynthetic template for the catecholic siderophore 2,3-Dihydroxybenzoate-Glycine-Threonine trimeric ester bacillibactin. *J. Biol. Chem.* 276, 7209–7217. doi: 10.1074/jbc.M009140200

McAuliffe, O., Ross, R. P., and Hill, C. (2001). Lantibiotics: structure, biosynthesis and mode of action. *FEMS Microbiol. Rev.* 25, 285–308. doi: 10.1111/j.1574-6976.2001.tb00579.x

McIntosh, J. A., Donia, M. S., and Schmidt, E. W. (2009). Ribosomal peptide natural products: bridging the ribosomal and nonribosomal worlds. *Nat. Prod. Rep.* 26, 537–559. doi: 10.1039/B714132G

McNeal, K. S., and Herbert, B. E. (2009). Volatile organic metabolites as indicators of soil microbial activity and community composition shifts. *Soil Sci. Soc. Am. J.* 73, 579–588. doi: 10.2136/sssaj2007.0245

Mhammedi, A., Peypoux, F., Besson, F., and Michel, G. (1982). Bacillomycin F, a new antibiotic of iturin group: isolation and characterization. *J. Antibiot.* 35, 306–311. doi: 10.7164/antibiotics.35.306

Milewski, S., Chmara, H., and Borowski, E. (1986). Antibiotic tetaine — a selective inhibitor of chitin and mannoprotein biosynthesis in *Candida albicans*. *Arch. Microbiol.* 145, 234–240. doi: 10.1007/bf00443651

Mojid Mondol, M. A., Kim, J. H., Lee, H.-S., Lee, Y.-J., and Shin, H. J. (2011). Macrolactin W, a new antibacterial macrolide from a marine *Bacillus* sp. *Bioorg. Med. Chem. Lett.* 21, 3832–3835. doi: 10.1016/j.bmcl.2010.12.050

Moldenhauer, J., Chen, X.-H., Borriss, R., and Piel, J. (2007). Biosynthesis of the antibiotic bacillaene, the product of a giant polyketide synthase complex of the trans-AT family. *Angew. Chem. Int. Ed. Engl.* 46, 8195–8197. doi: 10.1002/anie.200703386

Moyne, A.-L., Shelby, R., Cleveland, T. E., and Tuzun, S. (2001). Bacillomycin D: an iturin with antifungal activity against *Aspergillus flavus*. *J. Appl. Microbiol.* 90, 622–629. doi: 10.1046/j.1365-2672.2001.01290.x

Müller, S., Strack, S. N., Hoefler, B. C., Straight, P., Kearns, D. B., and Kirby, J. R. (2014). Bacillaene and sporulation protect *Bacillus subtilis* from predation by *Myxococcus xanthus*. *Appl. Environ. Microbiol.* 80, 5603–5610. doi: 10.1128/aem.01621-14

Mulligan, C. N., Yong, R. N., and Gibbs, B. F. (2001). Heavy metal removal from sediments by biosurfactants. *J. Hazard. Mater.* 85, 111–125. doi: 10.1016/S0304-3894(01)00224-2

Nagao, T., Adachi, K., Sakai, M., Nishijima, M., and Sano, H. (2001). Novel macrolactins as antibiotic lactones from a marine bacterium. *J. Antibiot.* 54, 333–339. doi: 10.7164/antibiotics.54.333

Naruse, N., Tenmyo, O., Kobaru, S., Kamei, H., Miyaki, T., Konishi, M., et al. (1990). Pumilacidin, a complex of new antiviral antibiotics. *J. Antibiot.* 43, 267–280. doi: 10.7164/antibiotics.43.267

Nes, I. F., Diep, D. B., and Holo, H. (2007). Bacteriocin diversity in *Streptococcus* and *Enterococcus*. *J. Bacteriol.* 189, 1189–1198. doi: 10.1128/jb.01254-06

Nicholson, W. L. (2002). Roles of *Bacillus* endospores in the environment. *Cell. Mol. Life Sci.* 59, 410–416. doi: 10.1007/s00018-002-8433-7

Oman, T. J., and van der Donk, W. A. (2009). Follow the leader: the use of leader peptides to guide natural product biosynthesis. *Nat. Chem. Biol.* 6, 9–18. doi: 10.1038/nchembio.286

Ongena, M., Duby, F., Jourdan, E., Beaudry, T., Jadin, V., Dommes, J., et al. (2005). *Bacillus subtilis* M4 decreases plant susceptibility towards fungal pathogens by increasing host resistance associated with differential gene expression. *Appl. Microbiol. Biotechnol.* 67, 692–698. doi: 10.1007/s00253-004-1741-0

Ongena, M., and Jacques, P. (2008). *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. *Trends Microbiol.* 16, 115–125. doi: 10.1016/j.tim.2007.12.009

O'Sullivan, L., Ross, R. P., and Hill, C. (2002). Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. *Biochimie* 84, 593–604. doi: 10.1016/S0300-9084(02)01457-8

Owens, J. D., Allagheny, N., Kipping, G., and Ames, J. M. (1997). Formation of volatile compounds during *Bacillus subtilis* fermentation of soya beans. *J. Sci. Food Agric.* 74, 132–140. doi: 10.1002/(SICI)1097-0010(199705)74:1<132::AID-JSFA779>3.0.CO;2-8

Paik, S. H., Chakicherla, A., and Hansen, J. N. (1998). Identification and characterization of the structural and transporter genes for, and the chemical and biological properties of, sublancin 168, a novel lantibiotic produced by *Bacillus subtilis* 168. *J. Biol. Chem.* 273, 23134–23142. doi: 10.1074/jbc.273.36.23134

Parisot, J., Carey, S., Breukink, E., Chan, W. C., Narbad, A., and Bonev, B. (2008). Molecular mechanism of target recognition by subtilin, a class I lanthionine antibiotic. *Antimicrob. Agents Chemother.* 52, 612–618. doi: 10.1128/aac.00836-07

Patel, P. S., Huang, S., Fisher, S., Pirnik, D., Aklonis, C., Dean, L., et al. (1995). Bacillaene, a novel inhibitor of procaryotic protein synthesis produced by *Bacillus subtilis*: production, taxonomy, isolation, physico-chemical characterization and biological activity. *J. Antibiot.* 48, 997–1003. doi: 10.7164/antibiotics.48.997

Pattnaik, P., Kaushik, J. K., Grover, S., and Batish, V. K. (2001). Purification and characterization of a bacteriocin-like compound (Lichenin) produced anaerobically by *Bacillus licheniformis* isolated from water buffalo. *J. Appl. Microbiol.* 91, 636–645. doi: 10.1046/j.1365-2672.2001.01429.x

Pedersen, P. B., Bjørnvad, M. E., Rasmussen, M. D., and Petersen, J. N. (2002). Cytotoxic potential of industrial strains of *Bacillus* sp. *Regul. Toxicol. Pharmacol.* 36, 155–161. doi: 10.1006/rtph.2002.1574

Peñuelas, J., Asensio, D., Tholl, D., Wenke, K., Rosenkranz, M., Piechulla, B., et al. (2014). Biogenic volatile emissions from the soil. *Plant Cell Environ.* 37, 1866–1891. doi: 10.1111/pce.12340

Podile, A. R., and Prakash, A. P. (1996). Lysis and biological control of *Aspergillus niger* by *Bacillus subtilis* AF 1. *Can. J. Microbiol.* 42, 533–538. doi: 10.1139/m96-072

Preecha, C., Sadowsky, M. J., and Prathuangwong, S. (2010). Lipopeptide surfactin produced by *Bacillus amyloliquefaciens* KPS46 is required for biocontrol efficacy against *Xanthomonas axonopodis* pv. Glycines. *Kasetsart J. Nat. Sci.* 44, 84–99.

Priest, F. G., Goodfellow, M., Shute, L. A., and Berkeley, R. C. W. (1987). *Bacillus amyloliquefaciens* sp. nov., nom. rev. *Int. J. Syst. Evol. Microbiol.* 37, 69–71. doi: 10.1099/00207713-37-1-69

Qi, G., Zhu, F., Du, P., Yang, X., Qiu, D., Yu, Z., et al. (2010). Lipopeptide induces apoptosis in fungal cells by a mitochondria-dependent pathway. *Peptides* 31, 1978–1986. doi: 10.1016/j.peptides.2010.08.003

Raaijmakers, J. M., De Bruijn, I., Nybroe, O., and Ongena, M. (2010). Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. *FEMS Microbiol. Rev.* 34, 1037–1062. doi: 10.1111/j.1574-6976.2010.00221.x

Ramarathnam, R., Bo, S., Chen, Y., Dilantha Fernando, W. G., Xuewen, G., and Kievit, T. (2007). Molecular and biochemical detection of fengycin- and bacillomycin D-producing *Bacillus* spp., antagonistic to fungal pathogens of canola and wheat. *Can. J. Microbiol.* 53, 901–911. doi: 10.1139/w07-049

Rapp, C., Jung, G., Katzer, W., and Loeffler, W. (1988). Chlorotetain from *Bacillus subtilis*, an antifungal dipeptide with an unusual chlorine-containing amino acid. *Angew. Chem. Int. Ed. Engl.* 27, 1733–1734. doi: 10.1002/anie.198817331

Raubitschek, F., and Dostrovsky, A. (1950). An antibiotic active against dermatophytes, derived from *Bacillus Subtilis*. *Dermatology* 100, 45–49. doi: 10.1159/000257151

Raza, W., Ling, N., Yang, L., Huang, Q., and Shen, Q. (2016). Response of tomato wilt pathogen *Ralstonia solanacearum* to the volatile organic compounds produced by a biocontrol strain *Bacillus amyloliquefaciens* SQR-9. *Sci. Rep.* 6:24856. doi: 10.1038/srep24856

Riley, M. A., and Wertz, J. E. (2002). Bacteriocins: evolution, ecology, and application. *Annu. Rev. Microbiol.* 56, 117–137. doi: 10.1146/annurev.micro.56.012302.161024

Rohr, J. (1992). Comparison of multicyclic polyketides by folding analysis: a novel approach to recognize biosynthetic and/or evolutionary interrelationships of the natural products or intermediates and its exemplification on hepta-, octa-, and decaketides. *J. Org. Chem.* 57, 5217–5223. doi: 10.1021/jo00045a040

Romano, A., Vitullo, D., Senatore, M., Lima, G., and Lanzotti, V. (2013). Antifungal cyclic lipopeptides from *Bacillus amyloliquefaciens* strain BO5A. *J. Nat. Prod.* 76, 2019–2025. doi: 10.1021/np400119n

Romero, D., de Vicente, A., Rakotoaly, R. H., Dufour, S. E., Veening, J.-W., Arrebola, E., et al. (2007). The iturin and fengycin families of lipopeptides are key factors in antagonism of *Bacillus subtilis* Toward *Podosphaera fusca*. *Mol. Plant Microbe Interact.* 20, 430–440. doi: 10.1094/MPMI-20-4-0430

Romero-Tabarez, M., Jansen, R., Sylla, M., Lünsdorf, H., Häußler, S., Santosa, D. A., et al. (2006). 7-*O*-Malonyl macrolactin A, a new macrolactin antibiotic from *Bacillus subtilis* active against methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant *Enterococci*, and a small-colony variant of *Burkholderia cepacia*. *Antimicrob. Agents Chemother.* 50, 1701–1709. doi: 10.1128/aac.50.5.1701-1709.2006

Ryu, C.-M. (2015). "Bacterial volatiles as airborne signals for plants and bacteria," in *Principles of Plant-Microbe Interactions: Microbes for Sustainable Agriculture*, ed. B. Lugtenberg (Cham: Springer International Publishing), 53–61.

Ryu, C.-M., Farag, M. A., Hu, C.-H., Reddy, M. S., Kloepper, J. W., and Paré, P. W. (2004). Bacterial volatiles induce systemic resistance in *Arabidopsis*. *Plant Physiol.* 134, 1017–1026. doi: 10.1104/pp.103.026583

Ryu, C.-M., Farag, M. A., Hu, C.-H., Reddy, M. S., Wei, H.-X., Paré, P. W., et al. (2003). Bacterial volatiles promote growth in *Arabidopsis*. *Proc. Natl. Acad. Sci. U.S.A.* 100, 4927–4932. doi: 10.1073/pnas.0730845100

Sabaté, D. C., and Audisio, M. C. (2013). Inhibitory activity of surfactin, produced by different *Bacillus subtilis* subsp. subtilis strains, against *Listeria monocytogenes* sensitive and bacteriocin-resistant strains. *Microbiol. Res.* 168, 125–129. doi: 10.1016/j.micres.2012.11.004

Saggese, A., Culurciello, R., Casillo, A., Corsaro, M., Ricca, E., and Baccigalupi, L. (2018). A marine isolate of *Bacillus pumilus* secretes a pumilacidin active against *Staphylococcus aureus*. *Mar. Drugs* 16:E180. doi: 10.3390/md16060180

Schmidt, E. W. (2010). The hidden diversity of ribosomal peptide natural products. *BMC Biol.* 8:83. doi: 10.1186/1741-7007-8-83

Schmidt, R., Cordovez, V., de Boer, W., Raaijmakers, J., and Garbeva, P. (2015). Volatile affairs in microbial interactions. *ISME J.* 9, 2329–2335. doi: 10.1038/ismej.2015.42

Scholz, R., Molohon, K. J., Nachtigall, J., Vater, J., Markley, A. L., Süssmuth, R. D., et al. (2011). Plantazolicin, a novel microcin B17/Streptolysin S-Like natural product from *Bacillus amyloliquefaciens* FZB42. *J. Bacteriol.* 193, 215–224. doi: 10.1128/jb.00784-10

Scholz, R., Vater, J., Budiharjo, A., Wang, Z., He, Y., Dietel, K., et al. (2014). Amylocyclicin, a novel circular bacteriocin produced by *Bacillus amyloliquefaciens* FZB42. *J. Bacteriol.* 196, 1842–1852. doi: 10.1128/jb.01474-14

Schulz, S., and Dickschat, J. S. (2007). Bacterial volatiles: the smell of small organisms. *Nat. Prod. Rep.* 24, 814–842. doi: 10.1039/b507392h

Scortichini, M., and Rossi, M. P. (1991). Preliminary *in vitro* evaluation of the antimicrobial activity of terpenes and terpenoids towards *Erwinia amylovora*. *J. Appl. Bacteriol.* 71, 109–112. doi: 10.1111/j.1365-2672.1991.tb02963.x

Shafi, J., Tian, H., and Ji, M. (2017). *Bacillus* species as versatile weapons for plant pathogens: a review. *Biotechnol. Biotechnol. Equ.* 31, 446–459. doi: 10.1080/13102818.2017.1286950

Shatalin, K., Shatalina, E., Mironov, A., and Nudler, E. (2011). H2S: a universal defense against antibiotics in bacteria. *Science* 334, 986–990. doi: 10.1126/science.1209855

Shelburne, C. E., An, F. Y., Dholpe, V., Ramamoorthy, A., Lopatin, D. E., and Lantz, M. S. (2007). The spectrum of antimicrobial activity of the bacteriocin subtilosin A. *J. Antimicrob. Chemother.* 59, 297–300. doi: 10.1093/jac/dkl495

Shrivastava, G., Ownley, B. H., Augé, R. M., Toler, H., Dee, M., Vu, A., et al. (2015). Colonization by arbuscular mycorrhizal and endophytic fungi enhanced terpene production in tomato plants and their defense against a herbivorous insect. *Symbiosis* 65, 65–74. doi: 10.1007/s13199-015-0319-1

Siewert, G., and Strominger, J. L. (1967). Bacitracin: an inhibitor of the dephosphorylation of lipid pyrophosphate, an intermediate in the biosynthesis of the peptidoglycan of bacterial cell walls. *Proc. Natl. Acad. Sci. U.S.A.* 57, 767–773. doi: 10.1073/pnas.57.3.767

Smith, S., and Tsai, S.-C. (2007). The type I fatty acid and polyketide synthases: a tale of two megasynthases. *Nat. Prod. Rep.* 24, 1041–1072. doi: 10.1039/B603600G

Snelders, E., Huis in 't Veld, R. A., Rijs, A. J., Kema, G. H. J., Melchers, W. J. G., and Verweij, P. E. (2009). Possible environmental origin of resistance of *Aspergillus fumigatus* to medical triazoles. *Appl. Environ. Microbiol.* 75, 4053–4057. doi: 10.1128/aem.00231-09

Sourabié, A. M., Spinnler, H.-E., Bourdat-Deschamps, M., Tallon, R., Landaud, S., and Bonnarme, P. (2012). S-methyl thioesters are produced from fatty acids and branched-chain amino acids by brevibacteria: focus on l-leucine catabolic pathway and identification of acyl–CoA intermediates. *Appl. Microbiol. Biotechnol.* 93, 1673–1683. doi: 10.1007/s00253-011-3500-3

Spieß, T., Korn, S. M., Kötter, P., and Entian, K.-D. (2015). Autoinduction Specificities of the Lantibiotics Subtilin and Nisin. *Appl. Environ. Microbiol.* 81, 7914–7923. doi: 10.1128/aem.02392-15

Stein, T. (2005). *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. *Mol. Microbiol.* 56, 845–857. doi: 10.1111/j.1365-2958.2005.04587.x

Stein, T., Borchert, S., Conrad, B., Feesche, J., Hofemeister, B., Hofemeister, J., et al. (2002). Two different lantibiotic-like peptides originate from the ericin gene cluster of *Bacillus subtilis* A1/3. *J. Bacteriol.* 184, 1703–1711. doi: 10.1128/JB.184.6.1703-1711.2002

Steinborn, G., Hajirezaei, M.-R., and Hofemeister, J. (2005). bac genes for recombinant bacilysin and anticapsin production in *Bacillus* host strains. *Arch. Microbiol.* 183, 71–79. doi: 10.1007/s00203-004-0743-8

Sugawara, E., Ito, T., Odagiri, S., Kubota, K., and Kobayashi, A. (1985). Comparison of compositions of odor components of natto and cooked soybeans. *Agric. Biol. Chem.* 49, 311–317. doi: 10.1271/bbb1961.49.311

Sumi, C. D., Yang, B. W., Yeo, I.-C., and Hahm, Y. T. (2015). Antimicrobial peptides of the genus *Bacillus*: a new era for antibiotics. *Can. J. Microbiol.* 61, 93–103. doi: 10.1139/cjm-2014-0613

Switzer Blum, J., Burns Bindi, A., Buzzelli, J., Stolz, J. F., and Oremland, R. S. (1998). *Bacillus arsenicoselenatis*, sp. nov., and *Bacillus selenitireducens*, sp. nov.: two haloalkaliphiles from Mono Lake, California that respire oxyanions of selenium and arsenic. *Arch. Microbiol.* 171, 19–30. doi: 10.1007/s002030050673

Tagg, J. R., Dajani, A. S., and Wannamaker, L. W. (1976). Bacteriocins of gram-positive bacteria. *Bacteriol. Rev.* 40, 722–756.

Tahir, H. A. S., Gu, Q., Wu, H., Niu, Y., Huo, R., and Gao, X. (2017). *Bacillus* volatiles adversely affect the physiology and ultra-structure of *Ralstonia solanacearum* and induce systemic resistance in tobacco against bacterial wilt. *Sci. Rep.* 7:40481. doi: 10.1038/srep40481

Tanaka, K., Ishihara, A., and Nakajima, H. (2014). Isolation of anteiso-C17, iso-C17, iso-C16, and iso-C15 bacillomycin D from *Bacillus amyloliquefaciens* SD-32 and their antifungal activities against plant pathogens. *J. Agric. Food Chem.* 62, 1469–1476. doi: 10.1021/jf404531t

Thasana, N., Prapagdee, B., Rangkadilok, N., Sallabhan, R., Aye, S. L., Ruchirawat, S., et al. (2010). *Bacillus subtilis* SSE4 produces subtulene A, a new lipopeptide antibiotic possessing an unusual C15 unsaturated (-amino acid. *FEBS Lett.* 584, 3209–3214. doi: 10.1016/j.febslet.2010.06.005

Thimon, L., Peyoux, F., Maget-Dana, R., and Michel, G. (1992). Surface-active properties of antifungal lipopeptides produced by *Bacillus subtilis*. *J. Am. Oil Chem. Soc.* 69, 92–93. doi: 10.1007/bf02635884

Um, S., Fraimout, A., Sapountzis, P., Oh, D.-C., and Poulsen, M. (2013). The fungus-growing termite *Macrotermes natalensis* harbors bacillaene-producing

Bacillus sp. that inhibit potentially antagonistic fungi. *Sci. Rep.* 3:3250. doi: 10.1038/srep03250

van Straaten, K. E., Ko, J. B., Jagdhane, R., Anjum, S., Palmer, D. R. J., and Sanders, D. A. R. (2013). The structure of NtdA, a sugar aminotransferase involved in the kanosamine biosynthetic pathway in *Bacillus subtilis*, reveals a new sub-class of aminotransferases. *J. Biol. Chem.* 288, 34121–34130. doi: 10.1074/jbc.M113.500637

Velivelli, S. L. S., Kromann, P., Lojan, P., Rojas, M., Franco, J., Suarez, J. P., et al. (2015). Identification of mVOCs from andean rhizobacteria and field evaluation of bacterial and mycorrhizal inoculants on growth of potato in its center of origin. *Microb. Ecol.* 69, 652–667. doi: 10.1007/s00248-014-0514-2

Wang, B., Yuan, J., Zhang, J., Shen, Z., Zhang, M., Li, R., et al. (2013). Effects of novel bioorganic fertilizer produced by *Bacillus amyloliquefaciens* W19 on antagonism of Fusarium wilt of banana. *Biol. Fertil. Soils* 49, 435–446. doi: 10.1007/s00374-012-0739-5

Wang, D., Rosen, C., Kinkel, L., Cao, A., Tharayil, N., and Gerik, J. (2009). Production of methyl sulfide and dimethyl disulfide from soil-incorporated plant materials and implications for controlling soilborne pathogens. *Plant Soil* 324, 185–197. doi: 10.1007/s11104-009-9943-y

Wang, H., Fewer, D. P., Holm, L., Rouhiainen, L., and Sivonen, K. (2014). Atlas of nonribosomal peptide and polyketide biosynthetic pathways reveals common occurrence of nonmodular enzymes. *Proc. Natl. Acad. Sci. U.S.A.* 111, 9259–9264. doi: 10.1073/pnas.1401734111

Wang, T., Liang, Y., Wu, M., Chen, Z., Lin, J., and Yang, L. (2015). Natural products from *Bacillus subtilis* with antimicrobial properties. *Chin. J. Chem. Eng.* 23, 744–754. doi: 10.1016/j.cjche.2014.05.020

Wang, T., Wu, M.-B., Chen, Z.-J., Lin, J.-P., and Yang, L.-R. (2016). Separation, determination and antifungal activity test of the products from a new *Bacillus amyloliquefaciens*. *Nat. Prod. Res.* 30, 1215–1218. doi: 10.1080/14786419.2015.1048246

Wang, Y., Yang, Q., Tosa, Y., Nakayashiki, H., and Mayama, S. (2005). Nitric oxide-overproducing transformants of *Pseudomonas fluorescens* with enhanced biocontrol of tomato bacterial wilt. *J. Gen. Plant Pathol.* 71, 33–38. doi: 10.1007/s10327-004-0157-0

Willey, J. M., and Donk, W. A. V. D. (2007). Lantibiotics: peptides of diverse structure and function. *Annu. Rev. Microbiol.* 61, 477–501. doi: 10.1146/annurev.micro.61.080706.093501

Williams, T. H. (1975). Naphthomycin, a novel ansa macrocyclic antimetabolite. Proton NMR spectra and structure elucidation using lanthanide shift reagent. *J. Antibiot.* 28, 85–86. doi: 10.7164/antibiotics.28.85

Wise, C., Falardeau, J., Hagberg, I., and Avis, T. J. (2014). Cellular lipid composition affects sensitivity of plant pathogens to fengycin, an antifungal compound produced by *Bacillus subtilis* strain CU12. *Phytopathology* 104, 1036–1041. doi: 10.1094/PHYTO-12-13-0336-R

Wong, J. H., Hao, J., Cao, Z., Qiao, M., Xu, H., Bai, Y., et al. (2008). An antifungal protein from *Bacillus amyloliquefaciens*. *J. Appl. Microbiol.* 105, 1888–1898. doi: 10.1111/j.1365-2672.2008.03917.x

Wright, S. J. L., and Thompson, R. J. (1985). *Bacillus* volatiles antagonize cyanobacteria. *FEMS Microbiol. Lett.* 30, 263–267. doi: 10.1111/j.1574-6968.1985.tb01093.x

Wu, L., Wu, H., Chen, L., Lin, L., Borriss, R., and Gao, X. (2015a). Bacilysin overproduction in *Bacillus amyloliquefaciens* FZB42 markerless derivative strains FZBREP and FZBSPA enhances antibacterial activity. *Appl. Microbiol. Biotechnol.* 99, 4255–4263. doi: 10.1007/s00253-014-6251-0

Wu, L., Wu, H., Chen, L., Yu, X., Borriss, R., and Gao, X. (2015b). Difficidin and bacilysin from *Bacillus amyloliquefaciens* FZB42 have antibacterial activity against *Xanthomonas oryzae* rice pathogens. *Sci. Rep.* 5:12975. doi: 10.1038/srep12975

Wu, L., Wu, H., Chen, L., Xie, S., Zang, H., Borriss, R., et al. (2014). Bacilysin from *Bacillus amyloliquefaciens* FZB42 has specific bactericidal activity against harmful algal bloom species. *Appl. Environ. Microbiol.* 80, 7512–7520. doi: 10.1128/aem.02605-14

Wu, S., Jia, S., Sun, D., Chen, M., Chen, X., Zhong, J., et al. (2005). Purification and characterization of two novel antimicrobial peptides subpeptin JM4-A and subpeptin JM4-B produced by *Bacillus subtilis* JM4. *Curr. Microbiol.* 51, 292–296. doi: 10.1007/s00284-005-0004-3

Xiu, P., Liu, R., Zhang, D., and Sun, C. (2017). Pumilacidin-like lipopeptides derived from marine bacterium *Bacillus* sp. strain 176 suppress the motility of *Vibrio alginolyticus*. *Appl. Environ. Microbiol.* 83:e00450-17. doi: 10.1128/aem.00450-17

Xue, C., Tian, L., Xu, M., Deng, Z., and Lin, W. (2008). A new 24-membered lactone and a new polyene (-lactone from the marine bacterium *Bacillus marinus*. *J. Antibiot.* 61, 668–674. doi: 10.1038/ja.2008.94

Yakimov, M. M., Timmis, K. N., Wray, V., and Fredrickson, H. L. (1995). Characterization of a new lipopeptide surfactant produced by thermotolerant and halotolerant subsurface *Bacillus licheniformis* BAS50. *Appl. Environ. Microbiol.* 61, 1706–1713.

Yoo, J.-S., Zheng, C.-J., Lee, S., Kwak, J.-H., and Kim, W.-G. (2006). Macrolactin N, a new peptide deformylase inhibitor produced by *Bacillus subtilis*. *Bioorg. Med. Chem. Lett.* 16, 4889–4892. doi: 10.1016/j.bmcl.2006.06.058

Yu, X., Ai, C., Xin, L., and Zhou, G. (2011). The siderophore-producing bacterium, *Bacillus subtilis* CAS15, has a biocontrol effect on *Fusarium* wilt and promotes the growth of pepper. *Eur. J. Soil Biol.* 47, 138–145. doi: 10.1016/j.ejsobi.2010.11.001

Yuan, J., Li, B., Zhang, N., Waseem, R., Shen, Q., and Huang, Q. (2012a). Production of bacillomycin- and macrolactin-type antibiotics by *Bacillus amyloliquefaciens* NJN-6 for suppressing soilborne plant pathogens. *J. Agric. Food Chem.* 60, 2976–2981. doi: 10.1021/jf204868z

Yuan, J., Raza, W., Shen, Q., and Huang, Q. (2012b). Antifungal activity of *Bacillus amyloliquefaciens* NJN-6 volatile compounds against *Fusarium oxysporum* f. sp. cubense. *Appl. Environ. Microbiol.* 78, 5942–5944. doi: 10.1128/aem.01357-12

Zhang, B., Dong, C., Shang, Q., Han, Y., and Li, P. (2013). New insights into membrane-active action in plasma membrane of fungal hyphae by the lipopeptide antibiotic bacillomycin L. *Biochim. Biophys. Acta* 1828, 2230–2237. doi: 10.1016/j.bbamem.2013.05.033

Zhao, P., Quan, C., Wang, Y., Wang, J., and Fan, S. (2014). Bacillus amyloliquefaciens Q-426 as a potential biocontrol agent against *Fusarium oxysporum* f. sp. spinaciae. *J. Basic Microbiol.* 54, 448–456. doi: 10.1002/jobm.201200414

Zhao, Z., Wang, Q., Wang, K., Brian, K., Liu, C., and Gu, Y. (2010). Study of the antifungal activity of *Bacillus vallismortis* ZZ185 in vitro and identification of its antifungal components. *Bioresour. Technol.* 101, 292–297. doi: 10.1016/j.biortech.2009.07.071

Zheng, M., Shi, J., Shi, J., Wang, Q., and Li, Y. (2013). Antimicrobial effects of volatiles produced by two antagonistic *Bacillus* strains on the anthracnose pathogen in postharvest mangos. *Biol. Control* 65, 200–206. doi: 10.1016/j.biocontrol.2013.02.004

Zhu, B.-F., Xu, Y., and Fan, W.-L. (2010). High-yield fermentative preparation of tetramethylpyrazine by *Bacillus* sp. using an endogenous precursor approach. *J. Ind. Microbiol. Biotechnol.* 37, 179–186. doi: 10.1007/s10295-009-0661-5

Zimmerman, S., Schwartz, C., Monaghan, R., Pelak, B., Weissberger, B., Gilfillan, E., et al. (1987). Difficidin and oxydifficidin: novel broad spectrum antibacterial antibiotics produced by *Bacillus subtilis*. I. Production, taxonomy and antibacterial activity. *J. Antibiot.* 40, 1677–1681. doi: 10.7164/antibiotics.40.1677

Zuber, P., Nakano, M. M., and Marahiel, M. A. (1993). "Peptide antibiotics," in *Bacillus Subtilis and Other Gram-Positive Bacteria*, eds A. L. Sonenshein, J. A. Hoch, and R. Losick (Washington, DC: America Society for Microbiology), 897–916.

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 05 March 2019
doi: 10.3389/fmicb.2019.00389

Over 2000-Fold Increased Production of the Leaderless Bacteriocin Garvicin KS by Increasing Gene Dose and Optimization of Culture Conditions

*Amar A. Telke, Kirill V. Ovchinnikov, Kiira S. Vuoristo, Geir Mathiesen, Tage Thorstensen and Dzung B. Diep**

Faculty of Chemistry, Biotechnology and Food Science, Norwegian University of Life Sciences, Ås, Norway

The leaderless bacteriocin Garvicin KS (GarKS) is a potent antimicrobial, being active against a wide range of important pathogens. GarKS production by the native producer *Lactococcus garvieae* KS1546 is, however, relatively low (80 BU/ml) under standard laboratory growth conditions (batch culture in GM17 at 30°C). To improve the production, we systematically evaluated the impact of different media and media components on bacteriocin production. Based on the outcomes, a new medium formulation was made that increased GarKS production about 60-fold compared to that achieved in GM17. The new medium was composed of pasteurized milk and tryptone (PM-T). GarKS production was increased further 4-fold (i.e., to 20,000 BU/ml) by increasing the gene dose of the bacteriocin gene cluster (*gak*) in the native producer. Finally, a combination of the newly composed medium (PM-T), an increased gene dose and cultivation at a constant pH 6 and a 50–60% dissolved oxygen level in growth medium, gave rise to a GarKS production of 164,000 BU/ml. This high production, which is about 2000-fold higher compared to that initially achieved in GM17, corresponds to a GarKS production of 1.2 g/L. To our knowledge, this is one of the highest bacteriocin production reported hitherto.

Keywords: garvicin KS, leaderless bacteriocins, bacteriocin production, antimicrobial production, lactic acid bacteria, *Lactococcus garviae*, growth media

OPEN ACCESS

Edited by:
José E. Barboza-Corona, Universidad de Guanajuato, Mexico

Reviewed by:
Koshy Philip, University of Malaya, Malaysia
Ming Sun, Huazhong Agricultural University, China

****Correspondence:***
Dzung B. Diep
dzung.diep@nmbu.no

Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: *05 October 2018*
Accepted: *13 February 2019*
Published: *05 March 2019*

Citation:
Telke AA, Ovchinnikov KV, Vuoristo KS, Mathiesen G, Thorstensen T and Diep DB (2019) Over 2000-Fold Increased Production of the Leaderless Bacteriocin Garvicin KS by Increasing Gene Dose and Optimization of Culture Conditions. Front. Microbiol. 10:389. doi: 10.3389/fmicb.2019.00389

INTRODUCTION

The decreasing effectiveness of antibiotics has become a serious worldwide problem due to the emergence of multidrug-resistant bacteria (Bush et al., 2011; Laxminarayan et al., 2016). Despite that, the number of new commercially available antibiotics is dwindling. This is partly due to the fact that developing new antibiotics is a costly process (Holmes and Mauer, 2016), and most biopharma companies are therefore reluctant to invest large money in new antibiotics that soon may be useless because of resistance development. Consequently, there is an urgent need of cost-effective and efficient antimicrobial agents with different killing mechanisms to overcome multidrug-resistant bacteria.

Bacteriocins are ribosomally synthesized antibacterial peptides produced by bacteria, probably as a means to compete for nutrients and habitats (Cotter et al., 2005). So far, hundreds of bacteriocins have been isolated and characterized. Most of them have narrow-spectrum activity, but some are active against a broad-spectrum of bacteria including food-spoiling bacteria as well as important pathogens (Chikindas et al., 1993; de Arauz et al., 2009). Bacteriocins produced by lactic acid bacteria (LAB) are particularly interesting due to LAB's safe status as they are commonly found in our foods (Grosu-Tudor et al., 2014; Henning et al., 2015) and the gastrointestinal tract of man (Millette et al., 2008) and animals (O'Shea et al., 2009). Most bacteriocins are membrane-active peptides, killing sensitive bacteria by membrane disruption after selective interaction with specific membrane receptors (Oscariz and Pisabarro, 2001; Hasper et al., 2006; Diep et al., 2007; Nissen-Meyer et al., 2009; Tymoszewska et al., 2017). This mode of action is different from most antibiotics, which often act as enzyme-inhibitors (Davis, 1987; Bush and Jacoby, 2010). For this reason, antibiotic-resistant pathogens are often sensitive to bacteriocins, thus making the latter very attractive as alternative or complementary drugs for therapeutic use, especially to fight antibiotic resistance. Nevertheless, poor production is often a bottleneck in large-scaled production of bacteriocins. Previous studies have shown that bacteriocin production can be increased by optimization of growth conditions such as cultivation temperature, pH, aeration and growth medium (Biswas et al., 1991; Parente and Ricciardi, 1994; Aasen et al., 2000; Cabo et al., 2001; Nel et al., 2001; Guerra and Pastrana, 2002; Penna and Moraes, 2002; Tafreshi et al., 2010). In addition, various heterologous expression systems have been reported for increased bacteriocin production (Horn et al., 2004; Kong and Lu, 2014; Jimenez et al., 2015; Jiang et al., 2016; Mesa-Pereira et al., 2017).

Recently, we have reported the identification and characterization of a novel three-peptide bacteriocin called garvicin KS (GarKS), produced by *L. garvieae* KS1546, a strain isolated from raw bovine milk in Kosovo (Ovchinnikov et al., 2016). A gene cluster (*gak*) containing the three structural genes (*gakABC*) and genes likely involved in immunity (*gakIR*) and transport (*gakT*) has been identified in the genome (Ovchinnikov et al., 2016). GarKS is active against a broad spectrum of bacteria such as *Listeria, Staphylococcus*, *Bacillus*, *Streptococcus* and *Enterococcus* (Ovchinnikov et al., 2016). Despite its great potential, production of GarKS is relatively moderate under standard laboratory growth conditions. To overcome this problem, we conducted a multi-factorial optimization study that resulted in over 2000-fold increased bacteriocin production. This approach includes medium optimization, increased gene dose and cultivation optimization.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

All bacterial strains and plasmids used in this study are listed in **Table 1**. Unless otherwise stated, the native bacteriocin producer *L. garvieae* KS1546 was grown in M17 broth supplemented with 0.5% glucose (GM17) under static condition at 30°C. NEB® 10-beta *E. coli* (New England Biolabs, Beverly, MA, United States) was grown in Luria-Bertani (LB) broth with shaking (200 rpm) at 37°C. Bacterial culture media and supplements were obtained from Oxoid Ltd. (Hampshire, United Kingdom). When necessary, erythromycin (Sigma-Aldrich Inc., St. Louis, MO, United States) was added at 200 μg/ml for *E. coli* and at 5 μg/ml for LAB strains.

TABLE 1 | Bacterial strains, plasmids and primers used in this study.

Strain, plasmid or Primer	Description	Source/ reference
Strains		
L. garvieae KS1546	Wild type strain, native GarKS bacteriocin producer	[31]
L. garvieae KS1546-pA2T	*L. garvieae* KS1546 containing the recombinant plasmid pA2T	This study
L. lactis IL 1403-pA2T	*L. lactis* 1403 containing the recombinant plasmid pA2T	This study
L. garvieae KS1546-pMG	*L. garvieae* KS1546 containing the empty plasmid pMG36e	This study
L. lactis IL 1403-pMG	*L. lactis* IL 1403 containing the empty plasmid pMG36e	This study
Escherichia coli NEB 10-beta	Subcloning host strain	New England Biolab
Plasmids		
pMG36e	Em^R, *E. coli-Lactococcus* shuttle vector	[48]
pABC	pMG36e containing the structural genes *gakABC*, Em^R	This study
pA2T	pMG36e containing the entire *gak* cluster; Em^R	This study
Primers		
gakF	5′-CGTAATTCGAGCTCCACCTC TGCTGTTTTTC-3′	This study
gakR	5′-AGACTTTGCAAGCTTGCAAT ATTACGTTTGTGGG-3′	This study
gakR1	5′-AGACTTTGCAAGCTTTTAATCC TGACTCATCAGATATTC-3′	This study
gakSeqF	5′-GTACATAGTACCTCAAAATTAT TTGAGC-3′	This study
gakseqF1	5′-GCAGAGCTTTAGTGTGGGAT-3′	This study
gakseqF2	5′-CGCTATTGCTTCTGAATATATA GTGGAC-3′	This study
gakseqF3	5′-GGCACTTTTACAAGAAATAGG ACT-3′	This study
gakseqR	5′-AGTAATTGCTTTATCAACTGCT GC-3′	This study
pMGF	5′-CATCCTCTTCGTCTTGGTAGC-3′	This study
pMGR	5′-GGCAGCTGATCTCAACAATG-3′	This study

Growth Media for GarKS Production

The influence of different growth media on GarKS production was assessed in batch cultures under static condition at 30°C. Following commercial complex media were used: GM17, deMan, Rogosa and Sharpe (MRS), Todd-Hewitt (TH) and Brain Heart Infusion (BHI). To make new milk-based medium formulations, skim milk (5%, w/v) or pasteurized skim milk was combined with an equal volume of GM17, MRS, TH, and BHI, or with tryptone (10% w/v). Skim milk (SM) was prepared by using milk powder (Oxoid, United Kingdom) while pasteurized milk (PM) was obtained from a dairy company in Norway, Q-milk.

DNA Manipulation

The *gak* cluster responsible for production of GarKS was amplified from genomic DNA of *L. garvieae* KS1546 using Phusion High-fidelity DNA polymerase (New England Biolabs, United Kingdom) and the primers gakF and gakR1 (**Table 1**). The genes *gakABC* encoding the three peptides constituting GarKS were amplified using the primers gakF and gakR (**Table 1**). Restriction sites SacI and HindIII were introduced at the 5′end of forward and reverse primers. NEBuilder HiFi DNA assembly cloning kit (New England Biolabs) was used to assemble the PCR fragments into the plasmid pMG36e (van de Guchte et al., 1989). Plasmid DNA was amplified in *E. coli* NEB® 10-beta before being transferred into *L. garvieae* KS1546 or *L. lactis* IL1403 cells using a Gene Pulser™ (Bio-Rad Laboratories, Hercules, CA, United States). Primers used in this study were obtained from Life Technologies AS (Thermofisher Scientific, Oslo, Norway). The integrity of all recombinant plasmids was confirmed by Sanger DNA sequencing (GATC Biotech AG; Constance, Germany), which were sequenced using primers gakseqF, gakseqF1, gakseqF2, gakseqF3, gakseqR, pMGF, and pMGR (**Table 1**).

Optimization of Bacteriocin Production in Bioreactor Conditions

The effects of pH and aeration on GarKS production were tested at various constant pHs (5, 6, and 7), and at controlled aeration in a fully automated 2.5 L Minifors 1 bioreactor (Infors AG, Switzerland). The pH was controlled by automatic addition of 5 M HCl or 5 M NaOH. The aeration was maintained by purging sterile air into culture medium. Temperature (30°C) and agitation speed of 150 rpm were maintained constant for all experiments. Samples of 2 ml were withdrawn aseptically every 2 h for determination of bacteriocin production and cell growth (see below).

Determination of Bacteriocin Production and Cell Growth

Bacteriocin activity was measured from heat-inactivated (100°C for 10 min) cell-free culture supernatants. Bacteriocin activity was quantified using a microtiter plate assay as previously described (Jimenez et al., 2015; Ovchinnikov et al., 2016). One bacteriocin unit (BU) was defined as the minimum amount of the bacteriocin that inhibited at least 50% of growth of the indicator (*L. lactis* IL103) in a 200 μl culture volume. Growth curve was determined by measuring turbidity of culture at OD_{600} every 30 min for 24 h or by counting colony forming units (CFU) from serially diluted bacterial cultures on agar plates. Synthetic GarKS peptides were purchased from Pepmic Co., LTD., China, with GarA of about 85% purity and GarB and GarC of at least 95% purity. (Higher purity of GarA could not be synthesized due to constant problems occuring during synthesis/purification). GarKS composed of these synthetic peptides in equal amounts (1:1:1, w/v), has a specific activity of 130–140 BU/μg. This specific activity was used to estimate the amount of GarKS produced in cultures.

RESULTS

GarKS Production in Complex Media

Two different ways were used to describe the production of garvicin KS in a culture: production per ml expressed as bacteriocin unit per ml (BU/ml), and production per 10^8 cells expressed as BU/10^8 cells, the latter being referred to as specific production. *L. garvieae* KS1546 (hereafter shortened as KS1546) was routinely grown in the complex medium GM17 at 30°C without agitation, and GarKS production was typically of 80 BU/mL after 7–12 h growth. To examine whether the level of production was medium-dependent, KS1546 was grown in different complex media (MRS, BHI, and TH). Highest production was found between 7–12 h of growth in all tested media except for TH where bacteriocin production appeared constantly low for all time-points tested (**Figure 1A**). Relative to GM17, GarKS production increased 2 to 4-fold in MRS, while it was about 2 to 4-fold less in BHI and TH (**Figure 1A**). Cell growth was best in GM17 (30×10^8 cells/ml) but poorest in MRS (10×10^8 cells/ml) after 24 h at 30°C (**Table 2**). The growth in MRS gave highest specific production, 32 BU/10^8 cells, which is about 12 fold higher than that obtained when cells were grown in GM17 (2.7 BU/10^8 cells).

GarKS Production Increased in Milk-Based Media

It is well known that bacteria are ecologically adapted to the environments where they normally thrive. Since the producer KS1546 was isolated from raw milk (Ovchinnikov et al., 2016), we examine the possibility to use skim milk (SM) as growth medium. Bacteriocin production was increased 2-fold in SM (160 BU/ml) compared to GM17 (**Figure 1B**). However, cell growth was remarkably poor in skim milk (2×10^8 cells/ml) (**Table 2**), resulting in a relatively high specific production, 80 BU/10^8 cells. The poor growth suggests that some growth factors were present in complex media but absent in SM. Therefore, we tested the mixtures (50:50; v/v) of skim milk and complex media (GM17, MRS, BHI, and TH). As a result, the bacteriocin production was increased 16 times in skim milk combined with TH (SM-TH) and 8 times in SM-GM17, compared to the production in skim milk (SM) (**Table 1** and **Figure 1B**). The bacteriocin production in SM-TH and SM-GM17 was 2600 BU/ml and 1280 BU/ml after 9 h of incubation, respectively. On the other hand, no significant increase of GarKS in SM-MRS (320 BU/ml) and SM-BHI (160 BU/ml) was found in all time points (**Figure 1B**). All medium formulations gave similar cell densities, i.e., between 28×10^8–30×10^8 cells/ml (**Table 1**). In terms of specific production, SM-TH gave the highest while SM-BHI gave the lowest, 90 BU/10^8 cells and 5.5 BU/10^8 cells, respectively.

The results above indicate that bacteriocin production was significantly influenced by some specific factor(s)/nutrient(s), which are present in TH and GM17, but absent in MRS and BHI. Tryptone, a tryptic digest of milk protein casein (Oh et al., 1995), is one of the nutrients found in GM17 and TH, but not in MRS and BHI. The final concentration of tryptone in GM17 and TH broth is 0.5 and 2%, respectively. To examine whether

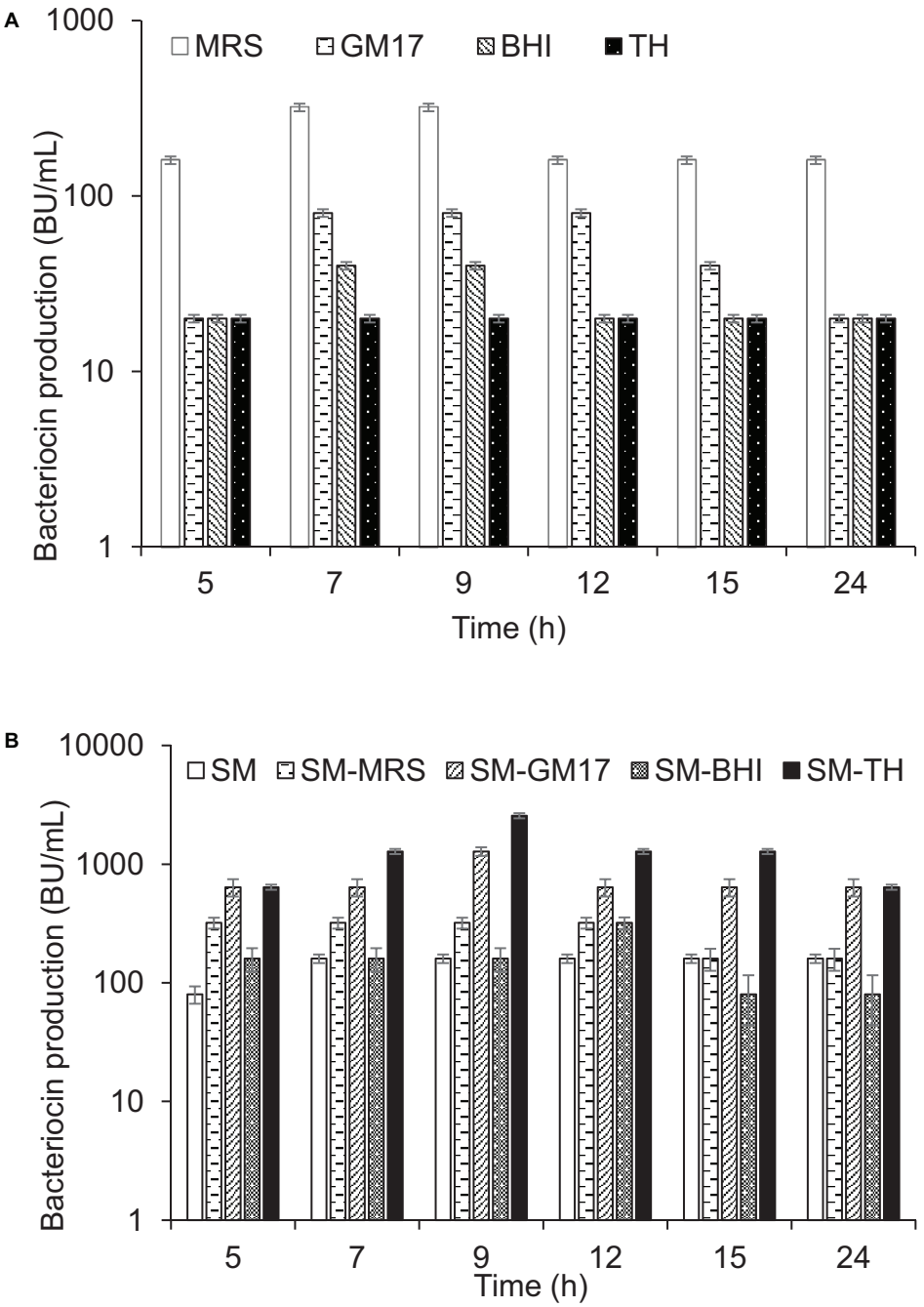


FIGURE 1 | GarKS production by the native producer in different complex growth media **(A)**, and in skim milk (SM) combined with complex growth media **(B)**. Each culture was started by adding 1% (v/v) culture inoculum to 5 ml growth medium and then incubated at 30°C without shaking. Bacteriocin activity was measured at different time points. Standard deviations were based on triplicate assays.

tryptone could improve bacteriocin production in combination with SM, we made formulations with different v/v ratios of SM and 10% tryptone (w/v). Highest bacteriocin production (about 2,600 BU/ml) was achieved when they were mixed in equal volumes (50%; v/v); this mixture had a final concentration of tryptone at 5% (w/v) (**Figure 2**). Under these circumstances, final cell density was comparable to that in GM17, i.e., about 30×10^8 cells/ml (**Table 2**), giving a relatively high specific production, 87 BU/10^8 cells, which is comparable to that in SM-TH (90 BU/10^8 cells). The formulation composed of SM (50%; v/v) and a final 5% of tryptone (w/v) is hereafter called SM-T.

Yeast extract is a rich source of vitamins, minerals, and amino acids, which often improves bacterial growth. We examined the

TABLE 2 | Influence of growth media, increased gene dose and culture conditions on bacteriocin production.

Strain	Growth medium	Bacteriocin production (BU/ml)[c]	Cell growth ($\times 10^8$ cells/ml)[c]	Specific activity (BU/10^8 cells)[d]
Native producer *L. garvieae* KS1546	GM17[a]	80 (1)	30 (1)	2.7 (1)
	MRS[a]	320 (4)	10 (0.3)	32 (12)
	BHI[a]	20 (0.25)	15 (0.5)	1.3 (0.5)
	TH[a]	20 (0.25)	20 (0.7)	1.0 (0.4)
	SM[b] (10%, w/v)	160 (2)	2 (0.1)	80 (30)
	Tryptone[a] (10%, w/v)	80 (1)	3 (0.1)	27 (10)
	SM-TH[b]	2600 (32.5)	29 (1)	90 (33)
	SM-GM17[b]	1280 (16)	30 (1)	43 (16)
	SM-MRS[b]	320 (4)	28 (0.9)	11 (4.2)
	SM-BHI[b]	160 (2)	29 (1)	5.5 (2)
	SM-T[b]	2600 (32.5)	30 (1)	87 (32)
	SM-T-YE[b]	1300 (16)	30 (1)	43 (16)
	PM-T[b]	5100 (64)	35 (1.2)	146 (54)
The recombinant producer *L. garvieae* KS1546-pA2T	PM-T[b] (uncontrolled pH)	20,000 (259)	35 (1.2)	570 (210)
	PM-T[b] (constant pH 5)	2600 (32,5)	32 1.1)	81 (30)
	PM-T[b] (constant pH 6)	82,000 (1025)	70 (2.3)	1170 (430)
	PM-T[b] (constant pH 7)	41,000 (512)	65 (2.2)	630 (230)
	PM-T[b] (constant pH 6 and aeration)	164,000 (2050)	100 (3.3)	1640 (610)

The bacteriocin activity and cell growth from complex growth media (a) and milk based media (b) were measured after 7 and 9 h of incubation, respectively. [c]: Values are average of triplicate assays, numbers in parentheses are fold increased or decreased in relation to the value in GM17. [d]: numbers in parentheses are fold increased or decreased in relation to the value in GM17.

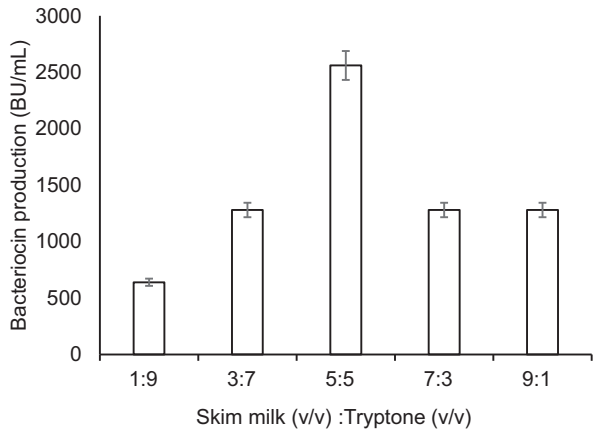


FIGURE 2 | Bacteriocin production in a medium composed of skim milk and tryptone. Different ratios of skim milk and tryptone were made in the formulation by mixing an increasing portion of skim milk (10%; w/v; from 1 volume to 9 volumes) with a corresponding decreasing portion of tryptone (10%, w/v; 9 volumes to 1 volume). For growth conditions, see legend in **Figure 1**. The bacteriocin activity was measured after 9 h of culture incubation. Standard deviations were based on triplicate assays.

effect of yeast extract (YE) in combination with SM-T. The resulting formulation, SM-T-YE (SM-T containing 1% (w/v) yeast extract) yielded the same cell density as in SM-T (30×10^8 cells/ml), but bacteriocin production was reduced by 50% (**Table 2**). Yeast extract was therefore excluded from the growth medium.

Although SM-T appeared as a good medium for the producer, we constantly encountered the problem associated with caramelization of milk sugars in skim milk during autoclaving, which might have detrimental effects on nutrition value. To avoid this problem, the autoclaved skim milk in SM-T was replaced with an equal amount of pasteurized skim milk, resulting in a new medium termed pasteurized milk–tryptone (PM-T). The content in pasteurized milk (Q-milk) according to the manufacturer (Q-Meieriene AS, Bergen, Norway) is, g/l: fat, 5; carbohydrate, 45; protein, 35; salt, 1; calcium, 1.3; vitamin B_2, 0.001; and vitamin B_{12}, 0.7×10^{-5}. Indeed, cell growth in PM-T was slightly increased from 30×10^8 cells/ml to 35×10^8 cells/ml, and GarKS production was increased two-fold in comparison to that in SM-T (**Table 2**).

Taken together, compared to other media analyzed so far, PM-T gave the best results in all aspects assessed: highest production (5100 BU/ml), highest cell density (35×10^8 cells/ml) and highest specific production (146 BU/10^8 cells).

GarKS Production Increased by Higher Gene Dose

The three structural genes (*gakABC*) encoding the three peptides that constitute GarKS are clustered with genes probably involved in immunity (*gakIR*) and transport (*gakT*). First we explored the possibility to increase bacteriocin production by increasing only the gene dose of structural genes *gakABC* in the native producer. The recombinant plasmid pABC carrying structural genes *gakABC* was constructed to deliver high gene dose in the native producer (**Table 1**). However, we failed to get any transformants even after several attempts. Similar negative result (i.e., no transformants) was obtained when we attempted to transfer pABC into the heterologous host *L. lactis* IL1403 (data not shown). Probably, increased gene dose of the structural genes might override the immunity or/and the transporter in the native producer, leading to toxicity to cells. Consequently, the plasmid pA2T carrying the entire *gak* locus including the genes involved in immunity and transport was constructed pA2T (**Table 1**). The resulting plasmid was first transferred into *L. lactis* IL1403 to assess the functionality of the locus. As expected, transformation was successful and bacteriocin production was detected in transformed cells (data not shown), confirming the functionality of the *gak* locus. Next, the plasmid was transferred into the native KS1546 and the clone (KS1546-PA2T) was assessed for bacteriocin production. Using PM-T as growth medium, GarKS production by the recombinant producer KS1546-pA2T was found to increase to 20,000 BU/mL, which is about 4 times more than the production without increased gene dose (native KS1546 in PM-T), and about 250-fold more than that initially obtained in GM17 (native KS1546 in GM17) (**Table 2**).

To compare their growth profiles, the native and recombinant producers were grown in MRS under similar growth conditions and their growth was measured spectrophotometerically. (The medium PM-T was not used due to the turbidity of milk

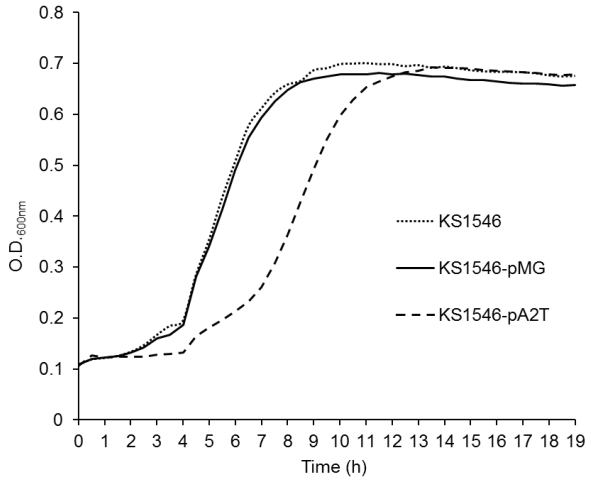


FIGURE 3 | Temporal growth profile of the recombinant producer (KS1546-pA2T), and the native producer with empty plasmid (KS1546-pMG) or without plasmid (*L. garvieae* KS1546). Data were acquired from triplicate assays. Standard deviations are within a range ± 0.01 to ± 0.05.

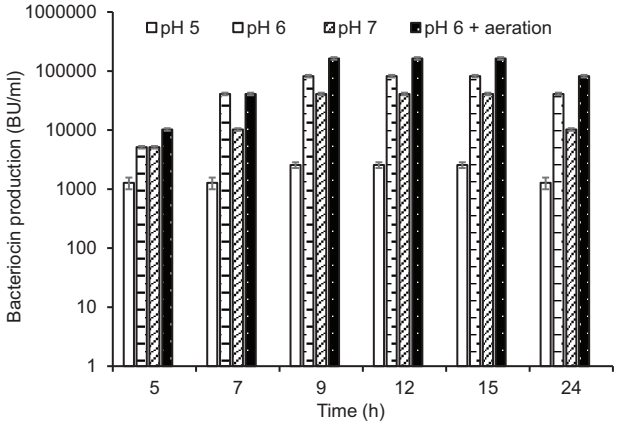


FIGURE 4 | GarKS production of the recombinant producer (KS1546-pA2T) in cultivation at constant pH (pH at 5, 6, or 7) or at constant pH 6 and aeration (50–60% dissolved oxygen). Each culture was started by adding 2% (v/v) culture inoculum in 1.5 l of PM-T medium containing erythromycin at final concentration of 5 μg/ml. Standard deviations were based on triplicate assays.

particles causing problem for spectrophotometric reading). The recombinant producer KS1546-pA2T showed a prolonged lag growth phase compared to the native GarKS producer or the native GarKS producer containing the empty plasmid. Nevertheless, KS1546-pA2T reached eventually about the same high cell density as the wild type control cells when it entered stationary growth phase (see **Figure 3**).

The plasmid map of pABC **(A)** and pA2T **(B)**, which were used to increase the gene dose of the structural genes (*gakABC*) and the *gak* cluster in the native producer, respectively.

Optimization of Culture Conditions in a Bioreactor Increased GarKS Production

The initial pH at 7 was declined to 4.8 when the recombinant producer KS1546-pA2T was grown in PM-T for 6–7 h at 30°C (data not shown). To examine whether pH reduction could have a negative impact on cell growth and bacteriocin production, we grew the recombinant producer (KS1546-pA2T) in PM-T in a bioreactor with constant pH at 5, 6, or 7. Indeed, pH had a great impact on cell growth and bacteriocin production. Highest cell growth (70×10^8 cells/ml) and bacteriocin production (82,000 BU/ml) were found at constant pH 6 (**Table 2**). Bacteriocin production measured at all time-points was also highest at constant pH 6 (**Figure 4**). Cell growth and bacteriocin production were lowest at constant pH 5. The impact of constant pH also reflects in specific production, that amounted to 1170 BU/10^8 cells at pH 6 but 81 BU/10^8 cells at pH 5.

Aeration is defined as dissolved oxygen (DO) percentage in a culture medium. We observed that the initial DO level at 50–60% was declined to 10% after 2 hours of cell growth in PM-T medium and at constant pH 6. The effect of aeration on GarKS production was therefore examined by purging the atmospheric sterile air into the growth medium. With aeration kept at 50–60% and constant pH at 6, highest cell growth (100×10^8 cells/ml) as well as highest bacteriocin production (164,000 BU/ml) and highest specific production (1640 BU/10^8 cells) were obtained (**Table 2** and **Figure 4**). This level of bacteriocin production (164,000 BU/ml) was about 2000-fold more than the initial production in GM17 which was 80 BU/ml, and about 600-fold more in terms of specific production (1649 BU/10^8 cells vs. 2.7 BU/10^8 cells, respectively).

We have previously shown that synthetic GarKS is functionally comparable to the biologically produced counterpart (Ovchinnikov et al., 2016). Synthetic GarKS has a specific activity of 130–140 BU/μg. Hence, the production of 164,000 BU/ml is equivalent to 1.2 g GarKS per liter which is a level of commercial importance.

DISCUSSION

GarKS is potent against a set of important pathogens including *Staphylococcus*, *Bacillus*, *Listeria, Streptococcus,* and *Enterococcus*, making it very attractive in diverse antimicrobial applications from food to medicine. Unfortunately, as also for many other bacteriocins, GarKS is produced at relatively low levels under normal laboratory growth conditions (Ovchinnikov et al., 2016). The low production by the native producer can dramatically hamper potential applications of GarKS as industrial use of bacteriocins requires high and cost-effective production. We have shown in this study that optimization of bacteriocin production by a bacterial strain is a multi-factorial process, which involves a systematic evaluation of nutritional ingredients and growth conditions e.g., temperature, pH, and aeration. The type of growth medium is probably one of the key factors

in bacteriocin production (Guerra et al., 2005). The complex media e.g., GM17, MRS, BHI, and TH have been used in cultivation of LAB because they give relatively good cell growth under laboratory conditions but not necessary for bacteriocin production (Mataragas et al., 2004). This was also illustrated in our study: GarKS production was best in MRS (320 BU/ml) but poorest in BHI and TH (both 20 BU/ml) while the cell growth appeared about in the same range in these media ($10-20 \times 10^8$ cells/ml).

To choose the optimal medium for bacteriocin production is often an empirical matter. The components from complex media influencing bacteriocin production are often elusive and the outcomes might vary significantly dependent on the type of producers. Nevertheless, some media components have been shown to enhance bacteriocin production by inducing stress conditions due to nutrient limitation (Verluyten et al., 2004) or stabilizing the bacteriocin molecules (Herranz et al., 2001). The use of commercial complex media (e.g., MRS) is not a cost-effective approach for large-scale bacteriocin production. For instance, culture medium could account for up to 30% of the total production cost in commercial biomolecule production (Rivas et al., 2004). Accordingly, high costs of complex media will reduce attractiveness of bacteriocins for commercial application. Our bacteriocin producer is a strain of *L. garvieae* isolated from raw milk and it has the capacity to ferment milk-associated sugars such as lactose and galactose while another strain of *L. garvieae* isolated from intestine of Mallard duck can not (Ovchinnikov et al., 2016). Milk is a low-cost product relative to complex media and could be an ideal medium for GarKS producer. However, the native producer appeared to grow poorly in sole skim milk. Skim milk is enriched in lactose and galactose as carbon source but does not contain easily accessed nitrogen-containing components for bacteria. Thus, the combination of tryptone and pasteurized skim milk, which was found best for cell growth, was in line with the notion that tryptone serves as an enriched source of nitrogen. Further, this formula also increased bacteriocin production over 30 fold compared to the growth in GM17.

Increase of gene dose is another means to enhance the production of biomolecules (Nijland et al., 2010). In the present study, we observed a 4-fold increase in bacteriocin production when a plasmid carrying the entire *gak* locus was introduced into the native producer. Interestingly, when we attempted to increase gene dose by introducing the structural genes only (using the plasmid pABC), no transformed cells were obtained. One possible explanation for this negative outcome is that expression of genetic determinants involved in bacteriocin biosynthesis is often highly fine-tuned to secure immunity and efficient export. The extra gene dose of the structural genes alone might override either immunity and/or transporter proteins, leading to toxicity and cell death. It is worth mentioning that most bacteriocins are expressed with a leader sequence which is necessary not only for export but also to keep the bacteriocins in an inactive form before export. For leaderless bacteriocins, such as GarKS, they are produced in mature active forms before export, therefore an intracellular dedicated protection mechanism (immunity) is crucial for cell survival.

We and others have observed that bacteriocin production by a certain strain is unstable, and dependent on the culture conditions applied (Diep et al., 2000; Criado et al., 2006). Consequently, different growth parameters were examined to optimize the production of GarKS. LAB are well known for reducing culture pH due to lactic acid production (Bartkiene et al., 2015) and this is also true for the GarKS producer. We found that culture conditions with constant pH 6 favors the cell growth and a high level of GarKS production. Similarly, optimal nisin production has been reported at constant pH 6.5 (Gonzalez-Toledo et al., 2010). The availability of oxygen also has a great influence on microbial cell growth and metabolic activities (Garcia-Ochoa and Gomez, 2009). Microorganisms vary with respect to their requirements and tolerance toward molecular oxygen. *L. garvieae* is a facultative anaerobic microorganism and its metabolic activities have been reported to differ between aerobic and anaerobic conditions (Delpech et al., 2017). We observed that the controlled aeration had a positive effect on the cell growth and bacteriocin production. Similar results have also been observed for other bacteriocins. For example, nisin A production by *L. lactis* UL719 was enhanced with aeration (Amiali et al., 1998). On the other hand, aeration has also been reported to be antagonistic to the production of lactosin S (Mortvedt-Abildgaard et al., 1995) and LIQ-4 bacteriocin (Kuhnen et al., 1985), suggesting that the effect of aeration on bacteriocin production is strain-dependent.

In terms of cost-effectiveness, the medium PM-T contained tryptone which is a relatively costly component; therefore we are searching for alternatives to replace tryptone. In preliminary studies, we have tested a chicken hydrolysate (processed from a waste product from meat industry) as an alternative low-cost protein source to produce GarKS. We found that the recombinant producer grew well in a medium based on Pasteurized milk and chicken hydrolysate (PM-CH), yielding a cell density of 30×10^8 cells/ml. However, although GarKS production in PM-CH was 8 times better than in the complex media GM17, the production was 8 times less than in PM-T. Thus, further studies are necessary to optimize a PM-CH-based medium in order to achieve high and cost-effective bacteriocin production.

Low bacteriocin production is often a bottle-neck in large-scaled production of bacteriocins for commercial use. Optimization of bacteriocin production is therefore an important research field to better exploit the antimicrobial potential of bacteriocins, especially with regard to the decreasing effects of antibiotics in infection treatments due to the global emergence of antibiotic resistance. In the present study we have achieved a very high level of GarKS production, amounting to 164,000 BU/ml, by combining medium optimization, increased gene dose and culture condition optimization. This amount is about 2,000 times higher compared to the initial production in GM17 (80 BU/ml). A production of 164,000 BU/ml is equivalent to 1.2 g GarKS per liter. This estimation was based the activity of synthetic GarKS peptides which have an activity comparable to the biological ones (Ovchinnikov et al., 2016). Ideally, the activity should be purified and quantified directly

by chromatography or immuno-detection approach. However, purification was a challenging task due to their relatively small sizes (32-34 aa), an inherent high hydrophobicity (especially GarA), the multi-peptide property, and not the least, the presence of milk particles in growth medium (PM-T). In fact, purification of GarKS in a previous work was heavily assisted by genetic data to help identity the peptides of GarKS (Ovchinnikov et al., 2016). Immuno-detection was also difficult due to their small sizes and lack of antigenic property (i.e., too hydrophobic). However, given that this estimation is correct, the production of 1.2 g GarKS per liter is, to our knowledge, one of the highest bacteriocin production achieved so far. In comparison, nisin production has been reported to 0.40–0.80 g/L by *L. lactis* grown in a medium composed of equal volume of skim milk and complex media GM17 (de Arauz et al., 2009). Finally, our study and others' have shown that optimization of bacteriocin production is an empirical and multi-factorial process and that it is highly strain-dependent. Only by systematic evaluation of different aspects influencing growth and gene regulation one can find conditions suitable for high levels of production.

AUTHOR CONTRIBUTIONS

AT and KO were involved in experimental design, conducted experiments, collected data and drafted the manuscript. KV was involved in fermentation in bioreactors, GM in cloning, TT in project guidance, DD in experimental design, project leadership and paper writing.

FUNDING

This study was financed by the Research Council of Norway, Project no. 254784.

ACKNOWLEDGMENTS

We thank Linda Godager and Line Degn Hansen for technical assistance, and David Lapena Gomez for providing chicken hydrolysate. This work has appeared as a preprint in bioRxiv with doi number: https://doi.org/10.1101/298489 (Diep et al., 2018).

REFERENCES

Aasen, I. M., Moretro, T., Katla, T., Axelsson, L., and Storro, I. (2000). Influence of complex nutrients, temperature and pH on bacteriocin production by *Lactobacillus sakei* CCUG 42687. *Appl. Microbiol. Biotechnol.* 53, 159–166. doi: 10.1007/s002530050003

Amiali, M. N., Lacroix, C., and Simard, R. E. (1998). High nisin Z production by *Lactococcus lactis* UL719 in whey permeate with aeration. *World J. Microbiol. Biotechnol.* 14, 887–894. doi: 10.1023/A:1008863111274

Bartkiene, E., Krungleviciute, V., Juodeikiene, G., Vidmantiene, D., and Maknickiene, Z. (2015). Solid state fermentation with lactic acid bacteria to improve the nutritional quality of lupin and soya bean. *J. Sci. Food Agric.* 95, 1336–1342. doi: 10.1002/jsfa.6827

Biswas, S. R., Ray, P., Johnson, M. C., and Ray, B. (1991). Influence of growth-conditions on the production of a bacteriocin, pediocin Ach, by *Pediococcus acidilactici* H. *Appl. Environ. Microbiol.* 57, 1265–1267.

Bush, K., Courvalin, P., Dantas, G., Davies, J., Eisenstein, B., Huovinen, P., et al. (2011). Tackling antibiotic resistance. *Nat. Rev. Microbiol.* 9, 894–896. doi: 10.1038/nrmicro2693

Bush, K., and Jacoby, G. A. (2010). Updated functional classification of beta-lactamases. *Antimicrob. Agents Chemother.* 54, 969–976. doi: 10.1128/AAC.01009-09

Cabo, M. L., Murado, M. A., Gonzalez, P., and Pastoriza, L. (2001). Effects of aeration and pH gradient on nisin production. A mathematical model. *Enzyme Microb. Technol.* 29, 264–273. doi: 10.1007/s00253-009-2217-z

Chikindas, M. L., Garcia-Garcera, M. J., Driessen, A. J., Ledeboer, A. M., Nissen-Meyer, J., Nes, I. F., et al. (1993). Pediocin PA-1, a bacteriocin from *Pediococcus acidilactici* PAC1.0, forms hydrophilic pores in the cytoplasmic membrane of target cells. *Appl. Environ. Microbiol.* 59, 3577–3584.

Cotter, P. D., Hill, C., and Ross, R. P. (2005). Bacteriocins: developing innate immunity for food. *Nat. Rev. Microbiol.* 3, 777–788. doi: 10.1038/nrmicro1273

Criado, R., Gutierrez, J., Martin, M., Herranz, C., Hernandez, P. E., and Cintas, L. M. (2006). Immunochemical characterization of temperature-regulated production of enterocin L50 (EntL50A and EntL50B), enterocin P, and enterocin Q by *Enterococcus faecium* L50. *Appl. Environ. Microbiol.* 72, 7634–7643. doi: 10.1128/AEM.00983-06

Davis, B. D. (1987). Mechanism of bactericidal action of aminoglycosides. *Microbiol. Rev.* 51, 341–350.

de Arauz, L. J., Jozala, A. F., Mazzola, P. G., and Penna, T. C. V. (2009). Nisin biotechnological production and application: a review. *Trends Food Sci. Technol.* 20, 146–154. doi: 10.1016/j.tifs.2009.01.056

Delpech, P., Rifa, E., Ball, G., Nidelet, S., Dubois, E., Gagne, G., et al. (2017). New insights into the anti-pathogenic potential of *Lactococcus garvieae* against *Staphylococcus aureus* based on RNA sequencing profiling. *Front. Microbiol.* 8:359. doi: 10.3389/fmicb.2017.00359

Diep, D. B., Axelsson, L., Grefsli, C., and Nes, I. F. (2000). The synthesis of the bacteriocin sakacin A is a temperature-sensitive process regulated by a pheromone peptide through a three-component regulatory system. *Microbiology* 146, 2155–2160. doi: 10.1099/00221287-146-9-2155

Diep, D. B., Skaugen, M., Salehian, Z., Holo, H., and Nes, I. F. (2007). Common mechanisms of target cell recognition and immunity for class II bacteriocins. *Proc. Natl. Acad. Sci. U.S.A.* 104, 2384–2389. doi: 10.1073/pnas.0608775104

Diep, D. B., Telke, A. A., Ovchinnikov, K. V., Vuoristo, K., Mathiesen, G., and Thorstensen, T. (2018). Over 2000-fold increased production of the leaderless bacteriocin garvicin KS by genetic engineering and optimization of culture conditions. *bioRxiv* [Preprint]. doi: 10.1101/298489

Garcia-Ochoa, F., and Gomez, E. (2009). Bioreactor scale-up and oxygen transfer rate in microbial processes: an overview. *Biotechnol. Adv.* 27, 153–176. doi: 10.1016/j.biotechadv.2008.10.006

Gonzalez-Toledo, S. Y., Dominguez-Dominguez, J., Garcia-Almendarez, B. E., Prado-Barragan, L. A., and Regalado-Gonzalez, C. (2010). Optimization of nisin production by *Lactococcus lactis* UQ2 using supplemented whey as alternative culture medium. *J. Food Sci.* 75, M347–M353. doi: 10.1111/j.1750-3841.2010.01670.x

Grosu-Tudor, S. S., Stancu, M. M., Pelinescu, D., and Zamfir, M. (2014). Characterization of some bacteriocins produced by lactic acid bacteria isolated from fermented foods. *World J. Microbiol. Biotechnol.* 30, 2459–2469. doi: 10.1007/s11274-014-1671-7

Guerra, N. P., Agrasar, A. T., Macias, C. L., and Pastrana, L. (2005). Modelling the fed-batch production of pediocin using mussel processing wastes. *Process Biochem.* 40, 1071–1083. doi: 10.1016/j.procbio.2004.03.014

Guerra, N. P., and Pastrana, L. (2002). Nisin and pediocin production on mussel-processing waste supplemented with glucose and five nitrogen sources. *Lett. Appl. Microbiol.* 34, 114–118. doi: 10.1046/j.1472-765x.2002.01054.x

Hasper, H. E., Kramer, N. E., Smith, J. L., Hillman, J. D., Zachariah, C., Kuipers, O. P., et al. (2006). An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II. *Science* 313, 1636–1637. doi: 10.1126/science.1129818

Henning, C., Vijayakumar, P., Adhikari, R., Jagannathan, B., Gautam, D., and Muriana, P. M. (2015). Isolation and taxonomic identity of bacteriocin-producing lactic acid bacteria from retail foods and animal sources. *Microorganisms* 3, 80–93. doi: 10.3390/microorganisms3010080

Herranz, C., Martinez, J. M., Rodriguez, J. M., Hernandez, P. E., and Cintas, L. M. (2001). Optimization of enterocin P production by batch fermentation of *Enterococcus faecium* P13 at constant pH. *Appl. Microbiol. Biotechnol.* 56, 378–383. doi: 10.1007/s002530100656

Holmes, P., and Mauer, J. (2016). Antimicrobial resistance and new antibiotics. *Health Aff.* 35:1935. doi: 10.1377/hlthaff.2016.1105

Horn, N., Fernandez, A., Dodd, H. M., Gasson, M. J., and Rodriguez, J. M. (2004). Nisin-controlled production of pediocin PA-1 and colicin V in nisin- and non-nisin-producing *Lactococcus lactis* strains. *Appl. Environ. Microbiol.* 70, 5030–5032. doi: 10.1128/AEM.70.8.5030-5032.2004

Jiang, H., Li, P., and Gu, Q. (2016). Heterologous expression and purification of plantaricin NC8, a two-peptide bacteriocin against *Salmonella* spp. from *Lactobacillus plantarum* ZJ316. *Protein Expr. Purif.* 127, 28–34. doi: 10.1016/j.pep.2016.06.013

Jimenez, J. J., Diep, D. B., Borrero, J., Gutiez, L., Arbulu, S., Nes, I. F., et al. (2015). Cloning strategies for heterologous expression of the bacteriocin enterocin A by *Lactobacillus sakei* Lb790, Lb. *plantarum NC*8 and Lb. casei CECT475. *Microb. Cell Fact.* 14:166. doi: 10.1186/s12934-015-0346-x

Kong, W., and Lu, T. (2014). Cloning and optimization of a nisin biosynthesis pathway for bacteriocin harvest. *ACS Synth. Biol.* 3, 439–445. doi: 10.1021/sb500225r

Kuhnen, E., Sahl, H. G., and Brandis, H. (1985). Purification and properties of Liq 4, an antibacterial substance produced by Streptococcus-Faecalis var Liquefaciens-K4. *J. Gen. Microbiol.* 131, 1925–1932.

Laxminarayan, R., Matsoso, P., Pant, S., Brower, C., Rottingen, J. A., Klugman, K., et al. (2016). Access to effective antimicrobials: a worldwide challenge. *Lancet* 387, 168–175. doi: 10.1016/S0140-6736(15)00474-2

Mataragas, M., Drosinos, E. H., Tsakalidou, E., and Metaxopoulos, J. (2004). Influence of nutrients on growth and bacteriocin production by *Leuconostoc mesenteroides* L124 and *Lactobacillus curvatus* L442. *Antonie Van Leeuwenhoek* 85, 191–198. doi: 10.1023/B:ANTO.0000020291.01957.a2

Mesa-Pereira, B., O'Connor, P. M., Rea, M. C., Cotter, P. D., Hill, C., and Ross, R. P. (2017). Controlled functional expression of the bacteriocins pediocin PA-1 and bactofencin A in *Escherichia coli*. *Sci. Rep.* 7:3069. doi: 10.1038/s41598-017-02868-w

Millette, M., Dupont, C., Shareck, F., Ruiz, M. T., Archambault, D., and Lacroix, M. (2008). Purification and identification of the pediocin produced by *Pediococcus acidilactici* MM33, a new human intestinal strain. *J. Appl. Microbiol.* 104, 269–275.

Mortvedt-Abildgaard, C. I., Nissenmeyer, J., Jelle, B., Grenov, B., Skaugen, M., and Nes, I. F. (1995). Production and pH-Dependent bactericidal activity of lactocin-S, a lantibiotic from Lactobacillus-Sake-L45. *Appl. Environ. Microbiol.* 61, 175–179.

Nel, H. A., Bauer, R., Vandamme, E. J., and Dicks, L. M. T. (2001). Growth optimization of *Pediococcus damnosus* NCFB 1832 and the influence of pH and nutrients on the production of pediocin PD-1. *J. Appl. Microbiol.* 91, 1131–1138. doi: 10.1046/j.1365-2672.2001.01486.x

Nijland, J. G., Ebbendorf, B., Woszczynska, M., Boer, R., Bovenberg, R. A. L., and Driessen, A. J. M. (2010). Nonlinear biosynthetic gene cluster dose effect on penicillin production by Penicillium chrysogenum. *Appl. Environ. Microbiol.* 76, 7109–7115. doi: 10.1128/AEM.01702-10

Nissen-Meyer, J., Rogne, P., Oppegard, C., Haugen, H. S., and Kristiansen, P. E. (2009). Structure-function relationships of the non-lanthionine-containing peptide (class II) bacteriocins produced by gram-positive bacteria. *Curr. Pharm. Biotechnol.* 10, 19–37. doi: 10.2174/138920109787048661

Oh, S., Rheem, S., Sim, J., Kim, S., and Baek, Y. (1995). Optimizing conditions for the growth of Lactobacillus-casei Yit-9018 in tryptone-yeast extract-glucose medium by using response-surface methodology. *Appl. Environ. Microbiol.* 61, 3809–3814.

Oscariz, J. C., and Pisabarro, A. G. (2001). Classification and mode of action of membrane-active bacteriocins produced by gram-positive bacteria. *Int. Microbiol.* 4, 13–19.

O'Shea, E. F., Gardiner, G. E., O'Connor, P. M., Mills, S., Ross, R. P., and Hill, C. (2009). Characterization of enterocin- and salivaricin-producing lactic acid bacteria from the mammalian gastrointestinal tract. *FEMS Microbiol. Lett.* 291, 24–34. doi: 10.1111/j.1574-6968.2008.01427.x

Ovchinnikov, K. V., Chi, H., Mehmeti, I., Holo, H., Nes, I. F., and Diep, D. B. (2016). Novel group of leaderless multipeptide bacteriocins from Gram-positive bacteria. *Appl. Environ. Microbiol.* 82, 5216–5224. doi: 10.1128/AEM.01094-16

Parente, E., and Ricciardi, A. (1994). Influence of pH on the production of enterocin-1146 during batch fermentation. *Lett. Appl. Microbiol.* 19, 12–15. doi: 10.1111/j.1472-765X.1994.tb00891.x

Penna, T. C. V., and Moraes, D. A. (2002). Optimization of nisin production by *Lactococcus lactis*. *Appl. Biochem. Biotechnol.* 98, 775–789. doi: 10.1385/ABAB:98-100:1-9:775

Rivas, B., Moldes, A. B., Dominguez, J. M., and Parajo, J. C. (2004). Development of culture media containing spent yeast cells of *Debaryomyces hansenii* and corn steep liquor for lactic acid production with *Lactobacillus rhamnosus*. *Int. J. Food Microbiol.* 97, 93–98. doi: 10.1016/j.ijfoodmicro.2004.05.006

Tafreshi, S. Y. H., Mirdamadi, S., Norouzian, D., Khatami, S., and Sardari, S. (2010). Optimization of non-nutritional factors for a cost-effective enhancement of nisin production using orthogonal array method. *Probiotics Antimicrob. Proteins* 2, 267–273. doi: 10.1007/s12602-010-9051-6

Tymoszewska, A., Diep, D. B., Wirtek, P., and Aleksandrzak-Piekarczyk, T. (2017). The non-lantibiotic bacteriocin garvicin Q targets man-PTS in a broad spectrum of sensitive bacterial genera. *Sci. Rep.* 7:8359. doi: 10.1038/s41598-017-09102-7

van de Guchte, M., van der Vossen, J. M., Kok, J., and Venema, G. (1989). Construction of a lactococcal expression vector: expression of hen egg white lysozyme in *Lactococcus lactis* subsp. lactis. *Appl. Environ. Microbiol.* 55, 224–228.

Verluyten, J., Leroy, F., and de Vuyst, L. (2004). Influence of complex nutrient source on growth of and curvacin A production by sausage isolate *Lactobacillus curvatus* LTH 1174. *Appl. Environ. Microbiol.* 70, 5081–5088. doi: 10.1128/AEM.70.9.5081-5088.2004

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 18 March 2019
doi: 10.3389/fmicb.2019.00527

GABA-Producing Natural Dairy Isolate From Artisanal Zlatar Cheese Attenuates Gut Inflammation and Strengthens Gut Epithelial Barrier *in vitro*

Svetlana Sokovic Bajic, Jelena Djokic, Miroslav Dinic, Katarina Veljovic, Natasa Golic, Sanja Mihajlovic and Maja Tolinacki*

Laboratory for Molecular Microbiology, The Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Belgrade, Serbia

OPEN ACCESS

Edited by:
Rubén Salcedo-Hernández, Universidad de Guanajuato, Mexico

Reviewed by:
Suleyman Yildirim, Istanbul Medipol University, Turkey
Enrica Pessione, University of Turin, Italy

***Correspondence:**
Maja Tolinacki
maja_tolinacki@imgge.bg.ac.rs

Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: *23 July 2018*
Accepted: *01 March 2019*
Published: *18 March 2019*

Citation:
Sokovic Bajic S, Djokic J, Dinic M, Veljovic K, Golic N, Mihajlovic S and Tolinacki M (2019) GABA-Producing Natural Dairy Isolate From Artisanal Zlatar Cheese Attenuates Gut Inflammation and Strengthens Gut Epithelial Barrier in vitro. Front. Microbiol. 10:527. doi: 10.3389/fmicb.2019.00527

Probiotic bacteria are recognized for their health-promoting properties, including maintenance of gut epithelial integrity and host immune system homeostasis. Taking into account the beneficial health-promoting effects of GABA, the presence of the *gadB* gene, encoding glutamate decarboxylase that converts L-glutamate to GABA, was analyzed in Lactic Acid Bacteria (LAB) natural isolates from Zlatar cheese. The results revealed that 52% of tested *Lactobacillus* spp. and 8% of *Lactococcus* spp. isolates harbor the *gadB* gene. Qualitative and quantitative analysis of GABA production performed by thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) revealed the highest GABA production by *Lactobacillus brevis* BGZLS10-17. Since high GABA-producing LAB natural isolates are the most valuable source of naturally produced GABA, the probiotic properties of BGZLS10-17 were characterized. This study demonstrated high adhesion of BGZLS10-17 strain to Caco-2 cells and the ability to decrease the adhesion of *Escherichia coli* ATCC25922 and *Salmonella enterica* C29039. Treatment of differentiated Caco-2 cells monolayer with BGZLS10-17 supernatant containing GABA alleviated inflammation (production of IL-8) caused by IL-1β and significantly stimulated the expression of tight junction proteins (zonulin, occludin, and claudin 4), as well as the expression of *TGF*-β cytokine leading to the conclusion that immunosuppression and strengthening the tight junctions can have significant role in the maintenance of intestinal epithelial barrier integrity. Taken together the results obtained in this study support the idea that using of GABA producing BGZLS10-17 probiotic strain could be a good strategy to modulate immunological response in various inflammatory diseases, and at the same time, it could be a good candidate for adjunct starter culture for production of GABA-enriched dairy foods and beverages offering new perspectives in designing the novel functional foods.

Keywords: GABA, lactobacilli, artisanal food, antimicrobial, anti-inflammatory activity

INTRODUCTION

γ-amino butyric acid (GABA) is the major inhibitory neurotransmitter in the mammalian central nervous system, directly affecting the personality and the stress management, and has hypotensive, tranquilizing, diuretic and antidiabetic effects (Li and Cao, 2010; Dhakal et al., 2012). The protective role of GABA is demonstrated in many autoimmune diseases such as type 1 diabetes (Tian et al., 2004), experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis (Bhat et al., 2010), collagen-induced arthritis (Kelley et al., 2008; Tian et al., 2011), and contact dermatitis (Nigam et al., 2010). This GABA effect is usually interpreted through the inhibitory effect of this molecule on the immune system. On the other hands, different human and experimental autoimmune models have been characterized by intestinal tight junction dysfunction (Visser et al., 2009). Disruption of intestinal homeostasis and increased intestinal permeability have been shown as an early and immune-mediated event in EAE (Nouri et al., 2014), inflammatory bowel disease (Suenaert et al., 2002), and celiac disease (Vogelsang et al., 1998). In addition, different infectious agents affect intestinal permeability in order to penetrate into deeper tissues (Wang et al., 2018), while entry of unwanted antigens, due to the leaky intestinal barrier, can lead to systemic inflammatory response syndrome, characterized by a whole body inflammatory state, and multiple organ failure (Liu et al., 2005). Thus, intestinal permeability represents a potential therapeutic target in treatment/prevention of MS and other autoimmune and inflammatory diseases.

Many drugs and bioactive components targeting GABA receptors are widely used in pharmaceutical and food industry. It is believed that GABA of extra brain origin, except through the pituitary gland, cannot pass the blood-brain barrier, although the results of studies dealing with this issue are inconsistent (Kakee et al., 2001; Powers et al., 2008; Boonstra et al., 2015). GABA contributes to gut-brain signaling through different pathways including enteric neurons, entero-endocrine cells and immune cells (Mazzoli et al., 2017). The microbial biosynthesis of GABA occurs in simple, highly efficient, and environmentally friendly reaction (Dhakal et al., 2012.). The biosynthesis of GABA occurs in a single step. Glutamate decarboxylase (GAD) (EC 4.1.1.15), a pyridoxal 5′-phosphate-dependent enzyme, catalyzes the irreversible α-decarboxylation of L-glutamate to GABA (Ueno, 2000).

Lactobacilli known for their beneficial effects on human and animal health, represent the most important group of GABA producers (Cho et al., 2007; Lebeer et al., 2008). GABA production was detected in *Lactobacillus brevis*, *Lb. paracasei*, *Lb. delbrueckii* subsp. *bulgaricus*, *Lb. buchneri*, *Lb. plantarum*, *Lb. helveticus*, and *Lb. rhamnosus*, mostly isolated from traditional fermented foods such as cheese, kimchi and sour dough (Cho et al., 2007; Siragusa et al., 2007; Komatsuzaki et al., 2008; Seok et al., 2008; Kim et al., 2009; Sun et al., 2009). Our previous results showed considerable diversity among LAB natural isolates from artisanal dairy products in Western Balkan with great technological and probiotic potential (Golić et al., 2013; Uroić et al., 2014). Due to increased public awareness of the use of natural food compounds, higher attention has been made on the use of metabolites produced by LAB isolates from artisanal products. In this study, natural isolates originating from Zlatar cheese were used. Zlatar cheese is an artisanal cheese manufactured in the remote households on the highlands of the nature reserve, mountain Zlatar, Serbia. Natural dairy LAB isolates from Zlatar cheese produce antimicrobial compounds with broad inhibitory spectrum, exopolysaccharides (EPS) with specific immunomodulatory activity and various other bioactive compounds with bile salt hydrolase activity and ability of cholesterol assimilation (Veljovic et al., 2007).

Although the immunomodulatory activity of GABA on different immune cells have been repeatedly proven, there is no references about the effects of GABA on enterocytes and intestinal integrity in inflammatory condition. Just a few references investigated the effect of GABA on enterocytes and intestinal integrity in different condition. Braun et al. (2015) proposed that the positive effects of glutamine on intestinal integrity are partly attributable to the promoting effects of its metabolite GABA on the expression of protective mucin in enterocytes. El-Hady et al. (2017) showed that administrated GABA have a role in improving histopathological and biochemical disturbances in the rat's small intestine following gamma radiation. Considering protective effects of GABA on stressed enterocytes showed in these studies as well as repeatedly proven positive effect of different *Lactobacillus* strains, we aimed to investigate the effects of GABA-producing *Lactobacillus* strain on enterocytes exposed to inflammatory condition *in vitro*. Additionally, the other health-promoting effects of GABA-producing LAB natural isolates from Zlatar cheese was investigated in different *in vitro* settings.

MATERIALS AND METHODS

Bacterial Strains, Media and Growth Conditions

Bacterial strains used in this study are presented in **Table 1**. The *Lactobacillus* strains were grown in De Man-Rogosa-Sharpe (MRS) medium (Merck GmbH, Darmstadt, Germany), while *Lactococcus* strains were grown in M17 medium (pH 7.2) (Merck GmbH) supplemented with 0.5% (w/v) glucose (GM17). Bacteria were grown at 30 or 37°C, under aerobic or anaerobic conditions, depending on the strain. Anaerobic conditions were achieved by using the Anaerocult A (Merck GmbH) in anaerobic jars. Solid medium was prepared by adding agar (1.5%, w/ v) (Torlak, Belgrade, Serbia) to the broth medium. The strains were maintained at −80°C in an appropriate medium (GM17 or MRS) supplemented with 15% (v/v) glycerol.

DNA Manipulation and Identification of GABA-Producing Strains

The total DNA of lactobacilli strains was extracted using the QIA DNA Mini Kit (Qiagen GmbH, Hilden, Germany). The PCR amplification of 16S rDNA, with UNI16SF (5′-GAGAGTTTGATCCTGGC-3′) and UNI16SR (5′-AGG

TABLE 1 | The list of strains used in this study.

Species	*gadB*-negative strains	*gadB*-positive strains	GABA producing strains
Lactobacillus brevis	BGZLS30-23	BGZLS45-36	BGLMM10, BGLMM11, BGZLS10-17, BGZLS30-2
Lactobacillus paracasei subsp. *paracasei*	BGZLS10-6, BGZLS45-50, BGZLS60-32	BGZLS10-1, BGZLS20-1, BGZLS45-25, BGZLS60-50	BGZLS45-49
Lactobacillus paraplantarum	BGZLS60-58		
Lactobacillus plantarum	BGZLS60-59	BGZLS60-43	BGZLS20-20, BGZLS30-41
Lactobacillus rhamnosus	BGZLS10-2		
Lactococcus lactis		BGZLS10-33	
Lactococcus lactis subsp. *lactis*	BGZLS1-11, BGZLS10-35	BGZLS10-34	

AGGTGATCCAGCCG-3′) primers was performed as described by Jovčić et al. (2009). To amplify highly conserved region of *gad* gene, CoreF (5′-CCTCGAGAAGCCGATCGCTTAGTTCG-3′) and CoreR (5′-TCATATTGACCGGTATAAGTGATGCCC-3′) primers were used as described by Siragusa et al. (2007). Briefly, 25 μl reaction contained 500 ng/μl of DNA, 0.5 U of Q5 High-Fidelity DNA Polymerase (New England Biolabs, Inc., Ipswich, MA, United States), 200 μM of each deoxynucleoside triphosphate, 0.5 μM of both primers and 1x Q5 Reaction Buffer. The obtained PCR amplicons were purified (Qiagen) and sequenced (Macrogen, Amsterdam, the Netherlands). Sequence annotation and the database searches for sequence similarities were performed with the BLAST tool available online[1].

Analysis of GABA Production

The strains were incubated in MRS or GM17 medium supplemented with various concentrations of monosodium glutamate-MSG (Acros organics, Morris Plains, NJ, United States) (0.5–4%) for 48 h. The cells were harvested by centrifugation (4500 × *g* for 15 min at 4°C) and 1 ml of the supernatant was evaporated up to 200 μl and diluted 2-fold by 7% acetic acid. The samples were centrifuged at 8100 × *g* for 15 min at room temperature. Obtained supernatants were used for thin-layer chromatography (TLC) analysis (Park et al., 2014.) and high-performance liquid chromatography (HPLC) (Holdiness, 1983). Briefly, 1 μl of supernatant was spotted on a silica gel $60F_{254}$ TLC plate (Macherey-Nagel, Düren, Germany) and developed with *n*-butanol, acetic acid, and water (4:1:1 v/v/v). Upon development was complete the plate was dried and visualized with 0.2% ninhydrin reagent (Sigma, Chemical Co., St. Louis, MO, United States). The conversion rate of MSG to GABA was analyzed using ImageJ software. The aliquots of 100 μl [bacterial supernatants and GABA standard (Sigma)] were filtrated through 0.22 μm filters and derivatized to phenylthiocarbamyl-GABA (Rossetti and Lombard, 1996). The derivatized samples were dissolved in 200 μl of initial mobile phase, solution A (138 mM sodium acetate, pH 6.3, 6% acetonitrile, 0.05% triethylamine). HPLC separation was performed on the instrument of Thermo scientific 3000 equipped with a Hypersil gold column (Thermo Fisher Scientific, Waltham, MA, United States 150 × 4.6, 5 μm). The elution solvent system comprised of solution A, solution B (acetonitrile) and solution C (water). The elution program is shown in **Table 2**. The amount of GABA production was calculated from GABA standard curve.

[1]http://www.ncbi.nlm.nih.gov/BLAST

The Cumulative Effect of Simulated Gastrointestinal Transit on Survival of Selected Strains

The survival of LAB strains during the passage through gastrointestinal tract (GIT) was monitored in an *in vitro* model that simulates the physiological conditions as described by Sánchez et al. (2010). *Lactobacillus* isolates from 10 ml of overnight cultures in MRS medium were harvested by centrifugation (4500 × *g*, 10 min), washed in 0.85% NaCl and resuspended in gastric, duodenal and intestinal juice(s) (Sánchez et al., 2010) at pH 2.0 and pH 8.0 supplemented with 10% reconstituted skimmed milk. The aliquots were taken at 0, 90, and 180 min and serial 10× dilutions in 0.85% NaCl were plated on MRS agar plates. The plates were incubated anaerobically at 37°C for 48 h. The results were expressed as Log colony forming units (cfu) ml^{-1} and the survival rate was calculated from the viable cell count with respect to initial cell counts. The experiments were performed in triplicates.

Gastric Tolerance at pH 2.0 in the Presence of Skim Milk, Milk Proteins, and Mucin

One mg ml^{-1} of milk powder (Mlekara d.o.o., Pancevo, Serbia), β-lactoglobulin, and mucin (Sigma) were resuspended in gastric juice at pH 2.0 (125 mM NaCl, 7 mM KCl, 45 mM $NaHCO_3$ and 0.3% pepsin). Skim milk was prepared according to the manufacturer's instructions at 110 mg ml^{-1}. Lactobacilli strains from an MRS overnight culture were harvested by centrifugation (4500 × *g*, 10 min), washed twice in 0.85% NaCl (Merck) and resuspended in 10 ml of an appropriate gastric juice containing milk proteins, β-lactoglobulin, skim milk or mucin. The mixtures were incubated anaerobically for 2 h at 37°C. The tolerance of the tested strains was calculated as described in previous section.

TABLE 2 | Elution program of HPLC.

Time (min)	Solution A (%)	Solution B (%)	Solution C (%)	Flow (ml/min)
0	60	12	28	0.6
6	60	12	28	0.6
6.1	20	13.5	66.5	0.4
22	20	13.5	66.5	0.4
25	60	12	28	0.6
40	60	12	28	0.6

Antibiotic Susceptibility Testing

Determination of the minimal inhibitory concentration (MIC) was performed by microdilution tests in Iso-Sensitest Broth (Oxoid, Hampshire, United Kingdom). The MIC breakpoints of eight antibiotics (ampicillin, gentamicin, kanamycin, streptomycin, erythromycin, clindamycin, tetracycline, and chloramphenicol) were determined in accordance to European Food Safety Authority recommendations (EFSA, 2008). Appropriate cell culture was added in wells of the microtiter plate containing increasing concentrations of antibiotics dissolved in 180 μL ISO medium. Cell density was monitored by OD_{600} measurements after 24 h of incubation at 37°C in a microtiter plate reader (Tecan Austria GmbH, Grödig, Austria). The lowest concentration of antibiotic at which no growth of bacteria was detected was taken as MIC. Experiments were done in triplicate.

Hemolytic Activity

Lactobacillus brevis strains were cultured in MRS broth and were streaked on Columbia agar plates containing 5% of sheep blood (Oxoid). The plates were incubated for 48 h at 30°C. According to Argyri et al. (2013), blood agar plates were examined for signs of β-haemolysis (clear zones around colonies), α-haemolysis (green-hued zones around colonies) or γ-haemolysis (no zones around colonies).

Gelatinase Activity

The phenotypic assay was performed as described by Su et al. (1991). Briefly, the lactobacilli strains were grown on agar plates containing 3% gelatine (Oxoid) at 37°C for 48 h and flooded with a saturated solution of ammonium sulfate (Centohem, Stara Pazova, Serbia). A transparent halo around cells and gelatine precipitates indicated gelatinase producers. As a positive control *E. faecalis* V583 (Paulsen et al., 2003) was used.

Antimicrobial Activity

All *Lb. brevis* strains and their supernatants were tested in triplicates for their antimicrobial activity against following pathogen strains: *Bacillus cereus* ATCC 11778, *Bacillus spizizeni* ATCC 6633, *Citrobacter freundii* ATCC 43864, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Listeria innocua* ATCC 33090, *Listeria ivanovii* ATCC 19119, *Listeria monocytogenes* ATCC 19111, *Proteus hauseri* ATCC 13315, *Proteus mirabilis* ATCC 12453, *Pseudomonas aeurginosa* PAOI, *Rhodococcus equi* ATCC 6936, *Salmonella enterica* C2 9039, *Salmonella typhimurium* ATCC 14028, *Shigela sonnei* ATCC 29930, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, and *Yersinia enterocolitica* ATCC 27729. For production of antimicrobial compounds, *Lb. brevis* strains were inoculated (1%, v/v) in MRS broth and incubated for 16 h. Both, the overnight cultures and the supernatants obtained after centrifugation (12,000 × *g*, 5 min) were tested for antimicrobial activity by the agar-well diffusion assay against indicator pathogen strains. Soft LB and BHI soft agar (0.7% w/v) containing pathogenic strains, was overlaid onto LB and BHI agar plates, respectively. Wells were made in the lawn of hardened soft agars. Aliquots (50 μl) of supernatants and overnight cultures were poured into the wells. A clear zone of inhibition around the well was taken as a positive signal for antimicrobial activity.

Adherence to Caco-2 Cells

The colonocyte-like cell lines Caco-2, was used to determine the adhesion ability of bacterial strains. Caco-2 was purchased from the European Collection of Cell Cultures (ECACC No. 86010202). The culture and maintenance of the cell line was carried out following standard procedures (Sánchez et al., 2010) using Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U ml^{-1} penicillin and 100 mg ml^{-1} streptomycin and 2 mM l-glutamine. Media and reagents were purchased from Thermo Fisher Scientific. Caco-2 cells were seeded in 24-well plates and cultivated until monolayers formed with no further visible differentiation. For adhesion experiments, 13 ± 1 day-old cellular monolayers were used. Overnight bacterial cultures (24 h) were washed twice with PBS solution. The pellets were resuspended in the corresponding cell-line media without antibiotics at a concentration of about 10^8 cfu ml^{-1}. Cellular monolayers were also carefully washed and bacterial suspensions were added at a ratio of 10:1 (bacteria : eukaryotic cell). Following co-incubation for 1 h at 37°C and 5% CO_2 the cells were gently washed and lysed with 0.25% Trypsin–EDTA solution (Sigma). Serial dilutions of samples, before and after adhesion, were diluted in PBS and plated on MRS-agar plates. The adherence (expressed as a percentage) was calculated as: cfu adhered bacteria/cfu added bacteria. Experiments were performed in two replicated plates and in each plate three wells were used per sample.

Competitive Exclusion Assay

Exclusion of pathogen strains *Escherichia coli* ATCC25922 and *Salmonella enterica* C29039 was done to Caco-2 cell line by *Lactobacillus brevis* strains as described previously (Živkovic et al., 2016). Briefly, bacterial cultures were washed twice with PBS and resuspended in DMEM without antibiotics at a concentration of $\sim 1 \times 10^7$ CFU ml^{-1}. The bacterial suspensions containing pathogen or a combination of pathogen strain and lactobacilli (ratio 1:1) were independently added to the Caco-2 monolayers at a ratio of 10:1 (bacteria : eukaryotic cells) and incubated at 37°C, with 5% CO2 for 1 h. Afterward, the monolayers were gently washed twice with PBS and lysed with 0.25% Trypsin–EDTA solution (Sigma). Associated *E. coli* and *S. enterica* strains were counted by plating the serial 10-fold dilutions of the suspension on LA plates. The percentage of *E. coli* and *S. enterica* strains association was calculated as follows: 100 × CFU ml^{-1} bacteria associated/CFU ml^{-1} bacteria added. Each combination was tested in triplicate. To determine the capability of the lactobacilli to decrease the association of pathogen strains to Caco-2 monolayers, data were referred to that obtained with the *E. coli* and *S. enterica* strains alone, respectively (i.e., 100% association).

Epithelial Integrity Analysis, Inflammation Induction and Treatments

Lactobacillus brevis BGZLS10-17, the best GABA producing strain, was used to determine the expression of tight junction proteins (zonulin, occludin, and claudin 4) and cytokine production (IL-8 and TGF-β) in differentiated Caco-2 cells. Caco-2 cells were seeded in 24 well plate and cultivated until monolayers formed with further differentiation for 21 days. Cell culture medium was changed every second day during the 21-day differentiation period.

Bacterial culture was grown in MRS medium and according to HPLC analysis there was no GABA production. To stimulate GABA production by BGZLS10-17, MRS medium was supplemented with 0.6% of MSG (Acros organics). Differentiated Caco-2 cells were treated with BGZLS10-17 supernatants in concentrations of 0.625 %, 1.25 % and 2.5 %, with 1 mM, 2 mM and 4 mM GABA respectively, determined by HPLC. Supernatant from the bacterial culture grown in MRS (the final concentration of 2.5%) was used as control. This supernatant with addition of artificial GABA (Sigma) in final concentration of 4 mM was used as an additional control in order to correlate the effect of supernatant with GABA produced by bacteria. Supernatant was obtained from 48 h bacterial culture and neutralized to pH about 7. Additionally, cells were treated with recombinant human IL-1β beta (R&D systems, Minneapolis, MN, United States), as a trigger of inflammation, and it was monitored whether a supernatant containing 4 mM GABA can alleviate the induced inflammation. All treatment lasted 24 h.

Cytotoxicity Assay

The level of cytotoxicity in the cell cultures was measured by lactate dehydrogenase (LDH) Cytotoxicity Assay Kit (Thermo Fisher Scientific) which detects LDH released from dead cells. After treatments, supernatants were collected and LDH activity was determined by following the manufacturer's instructions. The absorbance was measured at 450 nm on a microplate reader (Tecan).

Quantitative Real-Time PCR

Total RNA was extracted from Caco-2 as previously described by Lukic et al. (2013) with slight modifications. Cells were lysed in denaturing solution (4 M guanidine thiocyanate, 25 mM sodium citrate, 0.1 M *b*-mercaptoethanol, 0.5% [wt/vol] *N*-lauroylsarcosinate sodium salt) followed by acid phenol (pH 4) extractions and isopropanol precipitation. cDNA was generated from 0.5 μg total RNA according to the reverse transcriptase manufacturer's protocol (Thermo Fisher Scientific). Quantitative PCR was carried out on 7500 real-time PCR system (Applied Biosystems, Waltham, MA, United States) using KAPA SYBR Fast qPCR Kit (Kapa Biosystems, Wilmington, MA, United States) under the following conditions: 3 min at 95°C activation, 40 cycles of 15 s at 95°C and 60 s at 60°C. For β-actin cDNA amplification were used β-actin forward (5′TTGCTGACAGGATGCAGAAGGAGA3′), and reverse (5′TCAGTAACAGTCCGCCTAGAAGCA3′) (Li et al., 2015). Following primers were used for the claudin cDNA amplification CLDN 4 forward (5′ACAGACAAGCCTTA CTCC3′) and reverse (5′GGAAGAACAAAGCAGAG3′), occludin cDNA amplification OCLN forward (5′TCAG GGAATATCCACCTATCACTTCAG3′) and reverse (5′CAT CAGCAGCAGCCATGTACTCTTCAC3′) and for zonulin cDNA amplification ZO-1 forward (5′AGGGGCAGTGGTG GTTTTCTGTTCTTTC3′) and reverse (5′GCAGAGGTCAAA GTTCAAGGCTCAAGAGG3′) (Elamin et al., 2012). For expression of IL-8 were used cIL-8 forward (5′GGCA CAAACTTTCAGAGACAG3′) and reverse (5′ACA CAGAGCTGCAGAAATCAGG3′) primers (Angrisano et al., 2010). For the TGF-β cDNA expression were used TGF-β_F forward (5′AAGGACCTCGGCTGGAAGTGG3′) and reverse (5′CCGGGTTATGCTGGTTGTACAG3′) (Dragicevic et al., 2017). Expression of the tight junction proteins and cytokines mRNAs was normalized against β-actin mRNA expression. All used primers were purchased from Thermo Fisher Scientific.

Western Blot

Proteins were isolated from Caco-2 cells using RIPA buffer and subsequently subjected to Western blot analysis as described by Dinić et al. (2017). Briefly, the extracted proteins (10 μg) were separated on 12% SDS–PAGE and transferred to 0.2 mm nitrocellulose membrane (GE Healthcare, Chicago, IL, United States) using Bio-Rad Mini trans-blot system (Bio-Rad, Hercules, CA, United States). The membranes were incubated for 2 h with anti-claudin (CLDN-4) antibody (1:1000; Novus Biologicals, United States) and anti-β-actin (1:1000; Thermo Fisher Scientific). The membranes were washed and incubated with appropriate HPR-conjugated secondary antibodies (goat anti-rabbit; 1:10000; Thermo Fisher Scientific) for 1 h at room temperature. Proteins were detected by enhanced chemiluminescence (Immobilon Western, Merck Millipore).

Statistical Analysis

All data are presented as mean values ± standard error (SD). One-way ANOVA with the Tukey's *post hoc* test was used to compare multiple groups. Values at $p < 0.05$ or less were considered statistically significant. All experiments were repeated at least three times. Statistical analysis was carried out using SPSS 20.0 for Windows.

RESULTS AND DISCUSSION

"Probiotics are live microorganisms that confer health benefits to the host when ingested in adequate amounts" (WHO-FAO, 2006). Various mechanisms staying behind the health-promoting activity of probiotic bacteria have been proposed, including antimicrobial activity, positive influence on gut microbiota composition, competitive adhesion to gut mucosa and intestinal epithelial cells (IEC), modulation of the immune response, strengthening the gut epithelial barrier and producing of various important bioactive molecules, including vitamins, amino acids and GABA, among others (Bermudez-Brito et al., 2012). The aim of this study was the characterization of GABA-producing natural

LAB isolates in order to be eventually used in formulation of added-value fermented foods with GABA.

Evaluation of GABA Producing LAB Strains

In order to select natural LAB isolates with the highest GAD activity, LAB strains previously isolated from Zlatar cheese (Veljovic et al., 2007) were screened for presence of the *gadB* gene by PCR amplification. Out of 25 LAB isolates from Zlatar cheese 15 strains (60%), including 13 lactobacilli (52%) and two lactococci (8%) were positive for presence of the *gadB* gene (**Table 1**). The production of GABA was determined by TLC analysis in seven out of 15 analyzed lactobacilli strains (28% in total from 25 LAB strains) (**Figure 1A**). In comparison, Siragusa et al. (2007) revealed 61 GABA-producing isolates out of 440 randomly taken gram-positive, catalase-negative, non-motile, and acidifying isolates (13.86%) from various Italian cheeses, while Franciosi et al. (2015) determined 68 out of the 97 GABA-producing isolates (70.10%) from alpine raw milk "*Nostrano*-cheeses."

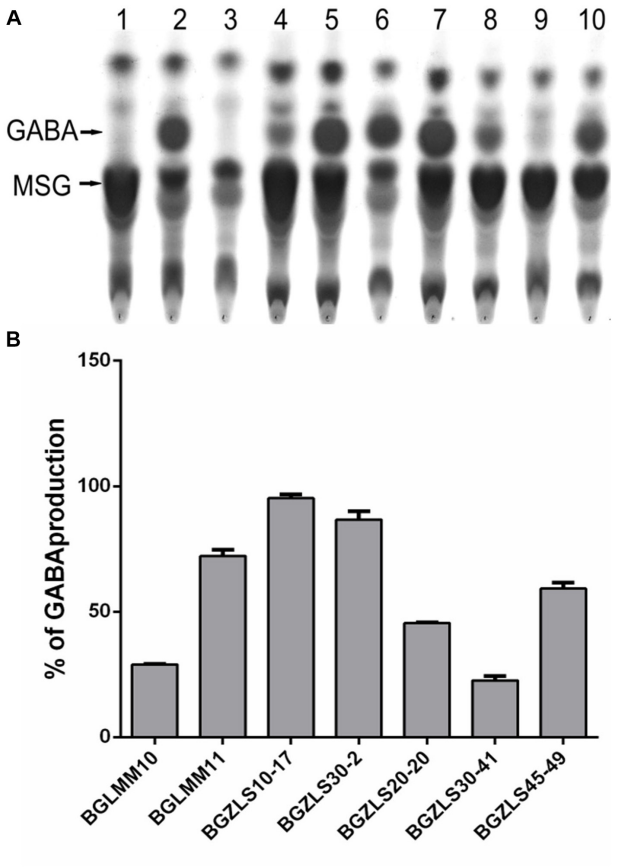


FIGURE 1 | (A) TLC chromatogram of GABA production. Lane 1, MSG in MRS; Lane 2, GABA in MRS; Lane 3. MRS; Lane 4, BGLMM10; Lane 5, BGLMM11; Lane 6, BGZLS10-17; Lane 7, BGZLS30-2; Lane 8, BGZLS20-20; Lane 9, BGZLS30-41; Lane 10, BGZLS45-49. Arrows indicate MSG and GABA. **(B)** Conversion of MSG to GABA. The percent of MSG conversion to GABA is indicated on *y*-axis. The bars represent GABA production of *Lactobacillus* isolates. Data from three independent experiments are expressed as mean ± SD.

Survival of GABA Producers in Simulated Gastrointestinal Conditions

According to WHO-FAO (2006) the survival in simulated gastrointestinal (GI) conditions is an important criterion for selection of probiotic strains. It is particularly important for GABA-producing strains since they could synthesize GABA only if remain viable through GI passage. Survival of seven strains that effectively produce GABA was tested in simulated conditions of the gastrointestinal tract. Our results revealed that among seven GABA-producers only *Lb. brevis* strains BGLMM10, BGLMM11, BGZLS10-17 and BGZLS30-2 successfully survived after 220 min of incubation in chemically simulated GI transit (8.7–8.9 log CFU ml^{-1}), when applied in one of the protecting carriers such as milk, skim milk, β-lactoglobulin, or mucin (**Figures 2A,B**). All tested carriers showed significant ($p < 0.001$; about two log cycles) improvement of survival, compared to survival of the strains applied in gastric juice (**Figure 2B**). The relatively high survival degree in the presence of bile salts in simulated small intestinal juice indicate resistance of some chosen strains to bile salts which could be ascribed to bile salts hydrolase (BSH) activity. BSH catalyzes the deconjugation of bile salts. Free, deconjugated bile salts have lower solubility at low pH because deconjugation increases their pKa values and precipitate as result of the fermentative metabolism of LAB (Begley et al., 2006).

The Safety Status of the *Lb. brevis* GABA-Producing Strains

The absence of virulence factors (e.g., hemolytic and/or gelatinase activity) as well as acquired or transmissible antibiotic resistance is a safety prerequisite for selection of potential probiotic strains (WHO-FAO, 2002). The results revealed that none of the strains exhibited hemolytic and/or gelatinase activity (data not shown). Besides, in line with EFSA recommendations (EFSA, 2008) the susceptibility of the isolates to different antibiotics groups was evaluated. Cell wall inhibitors (ampicillin) and protein synthesis inhibitors (gentamicin, kanamycin, streptomycin, erythromycin, chloramphenicol, clindamycin, and tetracycline) were tested in order to confirm that the strains do not contain transferable resistance genes. The results showed that only the strain *Lb. brevis* BGZLS10-17 was susceptible to all tested antibiotics, while the strain *Lb. brevis* BGLMM11 was resistant to kanamycin (64 $\mu g ml^{-1}$), BGLMM10 to kanamycin (64 $\mu g ml^{-1}$) and tetracycline (16 $\mu g ml^{-1}$), and the strain and BGZLS30-2 to ampicillin (4 $\mu g ml^{-1}$), kanamycin (64 $\mu g ml^{-1}$) and tetracycline (16 $\mu g ml^{-1}$) (**Table 3**). Nevertheless, according to previous studies it is also important to differentiate the transferable resistance from natural intrinsic non-transmissible resistance (Danielsen and Wind, 2003; Argyri et al., 2013). The resistance to kanamycin has been confirmed for most *Lactobacillus* species and is considered intrinsic among lactobacilli due to the absence of cytochrome-mediated electron transport that mediates drug uptake (Temmerman et al., 2003; Argyri et al., 2013). The resistance to kanamycin and tetracycline was shown

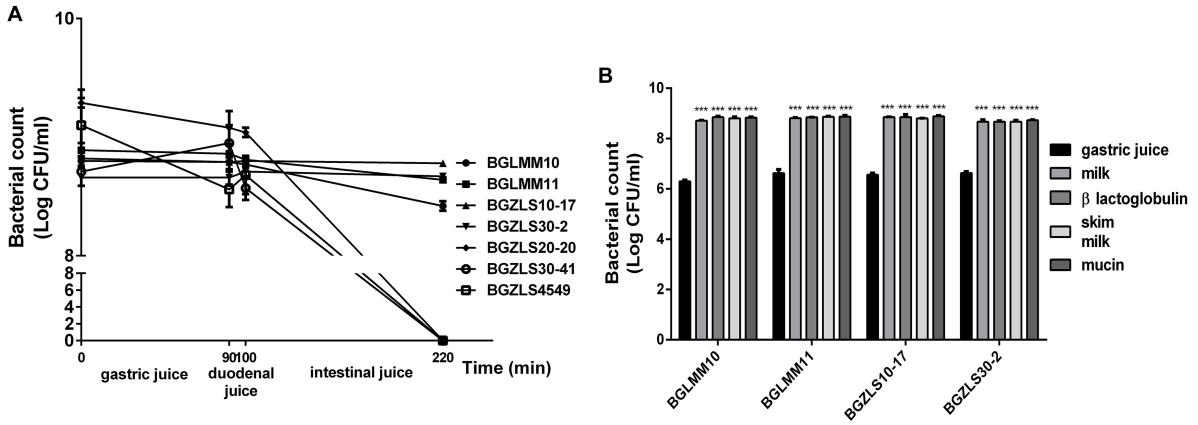


FIGURE 2 | (A) Resistance to simulated gastrointestinal conditions. Survival of selected *Lactobacillus* isolates, as indicated on the right, under gastric conditions (0–90 min), duodenal (90–100 min) and intestinal conditions (100–220 min). The values are the averages of three replicates, and standard deviations are indicated by vertical bars. **(B)** Gastric tolerance at pH 2.0 in the presence of skim milk, milk proteins and mucin. All values are reported as a mean ± SD of 3 replicates. Statistical significance of gastric tolerance enhancement is shown (***$p < 0.001$).

TABLE 3 | List of the tested antibiotics and MICs of four selected *L. brevis* isolates.

	Ampicillin	Vancomycin	Gentamicin	Kanamycin	Streptomycin	Erythromycin	Clindamycin	Tetracycline	Chloramphenicol
BGLMM10	1^S	n. r	8^S	64^R	64^S	0.5^S	0.5^S	16^R	2^S
BGLMM11	1^S	n. r.	8^S	64^R	64^S	0.5^S	0.5^S	8^S	2^S
BGZLS10-17	1^S	n. r.	8^S	32^S	64^S	0.5^S	0.5^S	4^S	2^S
BGZLS30-2	4^R	n. r.	8^S	64^R	64^S	0.5^S	0.5^S	16^R	4^S

^{S}, susceptible; ^{R}, resistant; n. r., not required.

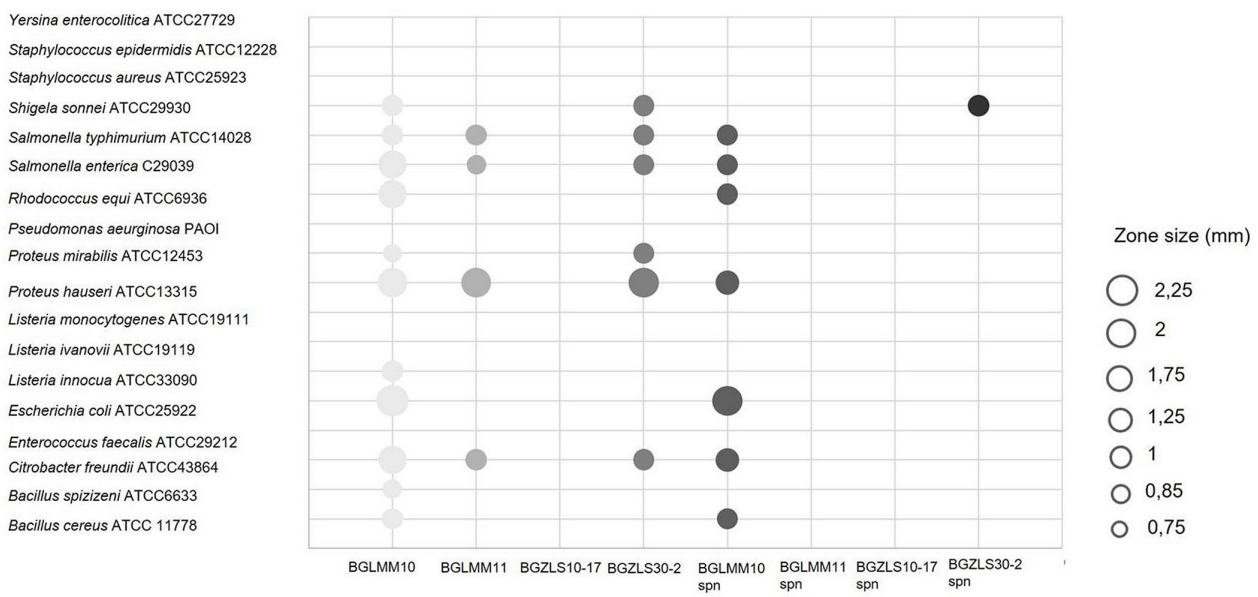


FIGURE 3 | Antimicrobial activity of four *Lb. brevis* strains and their supernatants against clinically relevant pathogens. The zone of inhibition is represented by circles whereas the diameter of circle (mm) represents the size of inhibition.

in *Lactobacillus paracasei* subsp. *paracasei* (Argyri et al., 2013). On the other hand, the results of Zhou et al. (2005) showed that *Lactobacillus* strains were susceptible to β-lactam antibiotics (penicillin, ampicillin, and cephalothin), but the frequency of transmission among LAB seems to be low because statistically only few LAB are beta lactam resistant. Therefore, the antibiotic

resistances detected in *Lactobacillus* strains, could be considered intrinsic or natural resistances and, hence, non-transmissible.

Quantification of GABA Production

The GABA produced by the strains *Lb. brevis* BGLMM10, BGLMM11, BGZLS10-17 and BGZLS30-20 was quantified by HPLC analysis. It appeared that the isolate *Lb. brevis* BGZLS10-17 produces the highest amount of GABA among all tested strains (6.4 ± 0.2 $mgml^{-1}$ [62 ± 1.94 mM] at 1% MSG; the conversion rate of MSG to GABA was 95%) (**Figure 1B**). Interestingly, the strain *Lb. paracasei* NFRI 7415, isolated from traditional fermented crucians (tuna-sushi) in Japan, produced similar amount (60 mM) but after 144 h (6 days) cultivation (Komatsuzaki et al., 2005). The isolates *Lb. brevis* BGZLS30-2, BGLMM11 and BGLMM10 produced 5.8 ± 0.8 $mgml^{-1}$ [56.2 ± 7.76 mM], 5.6 ± 0.6 $mgml^{-1}$ [54.3 ± 5.82 mM], and 3.7 ± 0.2 $mgml^{-1}$ [35.88 ± 1.94 mM] in MRS containing 1% MSG, respectively. The conversion rate of MSG to GABA was the lowest (30%) by the isolate *Lb. brevis* BGLMM10 while *Lb. brevis* BGZLS30-2 and BGLMM11 converted 86 and 72% of MSG to GABA, respectively (**Figure 1B**). On the other hand, various authors revealed that the GABA production could be enhanced by optimizing the culture conditions. For example, the strain *Lb. buchneri* MS, isolated from kimchi, produced GABA at a concentration of 251 mM with a 94% GABA conversion rate, under optimized conditions (Cho et al., 2007), while *Lactobacillus brevis* NCL912 in the optimized fermentation medium produced GABA in concentration of 345.83 mM (Li et al., 2010). Interestingly, although *Lc. lactis* NCDO218 produces more GABA with the higher glutamate supplementation, addition of arginine to the cell culture medium even more improves GABA production. Arginin stimulates glutamate decarboxylation, and the highest GABA production (8.6 mM) was observed when cell culture medium was supplemented together with glutamate and arginin (Laroute et al., 2016). Hence, the aim of our future work will be to optimize the cultivation conditions in order to further increase the GABA yield by the tested isolates.

Antimicrobial Activity

Antimicrobial activity has been highly appreciated as a key property for selection of probiotic LAB as an alternative to antibiotics to fight against clinical pathogens (O'Shea et al., 2012). The antimicrobial activity of *Lb. brevis* strains against 18 pathogenic strains was determined (**Figure 3**). The *Lb. brevis* BGLM10, BGLM11, and BGZLS30-2 strains showed various degrees of antagonistic effects against number of clinically relevant pathogens (**Figure 3**). Interestingly, supernatant of the strain *Lb. brevis* BGLMM10 showed antagonistic effect against seven of 18 bacterial strains (*Salmonella typhimurium* ATCC14028, *S. enterica* C29039, *Rhodococcus equi* ATCC6936, *Proteus hauseri* ATCC13315, *Escherichia coli* ATCC25922, *Citrobacter freundii* ATCC43864, *Bacillus cereus* ATCC11778), while supernatant of the strain BGZLS30-2 showed antimicrobial activity against *Shigella sonnei* ATCC 29930. The negative results obtained by using pronase E revealed that antimicrobial activity is not of proteinaceous nature. The strain *Lb. brevis* BGZLS10-17 did not exhibit antimicrobial activity.

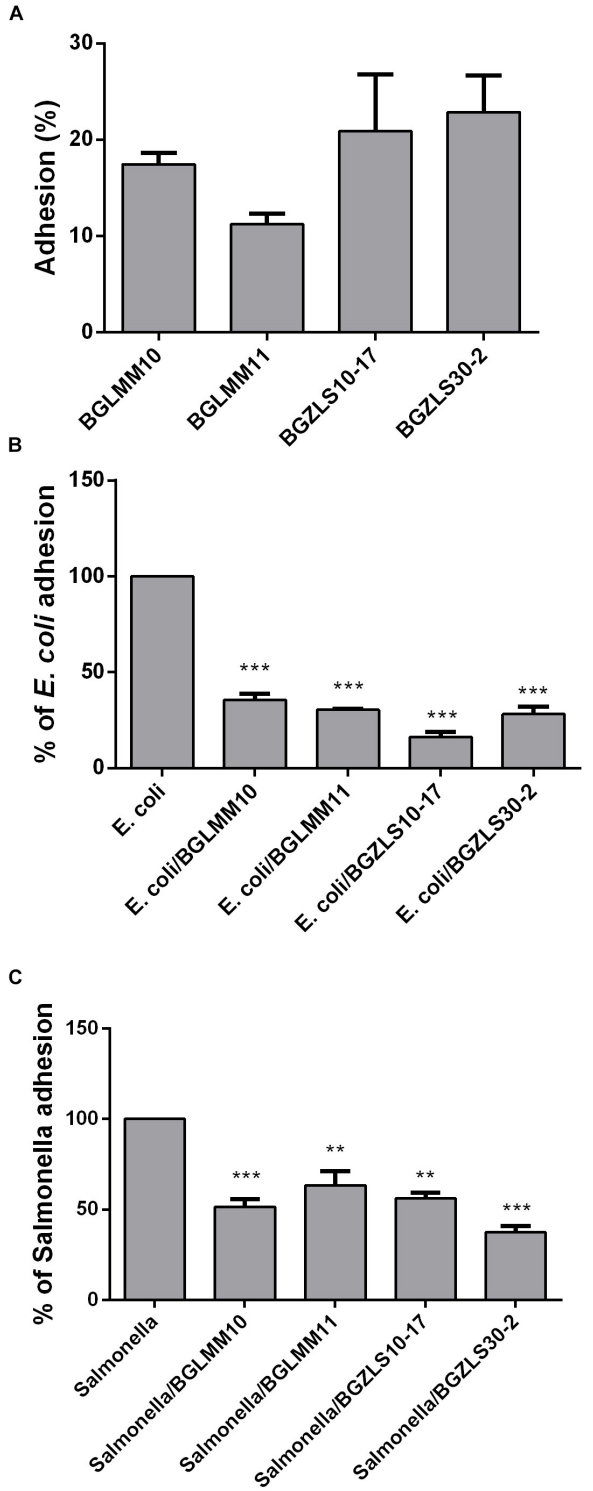


FIGURE 4 | Adhesion of *Lb. brevis* BGLMM10, BGLMM11, BGZLS10-17 and BGZLS30-2 on Caco-2 cells **(A)** and association of *E. coli* ATCC25922 **(B)** and *Salmonella enterica* C29039 **(C)** to Caco-2 cells in the presence of *Lb. brevis* BGLMM10, BGLMM11, BGZLS10-17, and BGZLS30-2 strains; the adhesion of *E. coli* ATCC25922 and *S. enterica* C29039 in the absence of lactobacilli strains was inferred as 100%. Statistical significance of adhesion is shown (**$p < 0.01$; ***$p < 0.001$).

Adhesion to Caco-2 Intestinal Epithelial Cells

The ability of probiotic strains to adhere to IEC is an important criterion proposed by FAO/WHO guidelines for the selection of probiotic strains (WHO-FAO, 2006). Adhesion ability allows the strain to colonize intestinal mucosa and to persist in the intestine (Muñoz-Provencio et al., 2009). This is particularly important for the GABA-producers, since the maximal GABA production in these strains occurs after 48 h, hence this feature might be partly dependent on the persistence of the strain in the intestine and adhesion to mucosal surfaces. All four *Lb. brevis* strains were able to adhere to Caco-2 cells, although the adhesion varied among the strains (**Figure 4A**). The strain BGZLS30-2 exhibited the highest adhesion (22%), while the strain BGLMM11 showed the lowest adhesion to Caco-2 (11%) (**Figure 4A**).

In addition, the adhesive properties of probiotic strains are important health promoting property in term of their capability to competitively counteract the negative effects of pathogenic bacteria (WHO-FAO, 2006). This characteristic is shown to be strain dependent and attributed to the various cell surface components such as cell surface associated proteins (Varma et al., 2010), proteinaceous S-layer macromolecules (Zhang et al., 2010) aggregation factors (Miljkovic et al., 2015), as well as EPS (Zivkovic et al., 2015; Živkovic et al., 2016). The capability of the *Lb. brevis* strains to decrease the adhesion of *E. coli* ATCC25922 and *S. enterica* C29039 to the intestinal epithelium in the presence and absence of lactobacilli was tested. The results demonstrated high reduction of *E. coli* ATCC25922 and *S. enterica* C29039 adhesion in the presence of four tested *Lb. brevis* isolates (**Figures 4B,C**). The reduction of *E. coli* ATCC25922 adhesion to Caco-2 was 64, 70, 80 and 72%, while the reduction of *S. enterica* C29039 adhesion was 50, 37, 44, and 63% in the presence of BGLMM10, BGLMM11, BGZLS10-17 and BGZLS30-2 strains, respectively (the adhesion of *E. coli* ATCC25922 and *S. enterica* C29039 in the absence of lactobacilli strains was inferred as 100%) (**Figures 4B,C**).

Effect of *Lactobacillus brevis* BGZLS10-17 on the Expression of Tight Junction Proteins and Cytokine Production

Finally, the important probiotic feature is the maintenance of gut barrier integrity (Bron et al., 2017) and immunomodulatory ability (Živkovic et al., 2016). There are evidences that reveal the GABA signaling system is involved in maintenance of immune system homeostasis (Jin et al., 2013). IL-1β has a central role in promoting intestinal inflammation, partially by stimulating IEC s to produce IL-8, the potent neutrophil and T-lymphocyte chemoattractant (Schuerer-Maly et al., 1994). This proinflammatory cascade is implicated in different intestinal inflammatory diseases. The epithelium in inflamed intestinal segments is characterized by a reduction of tight junction strands (Schulzke et al., 2009). Inflammatory bowel disease (IBD) patients demonstrate a loss of tight junction barrier function, increased proinflammatory cytokine production, and immune dysregulation (Edelblum and Turner, 2009). Regarding this,

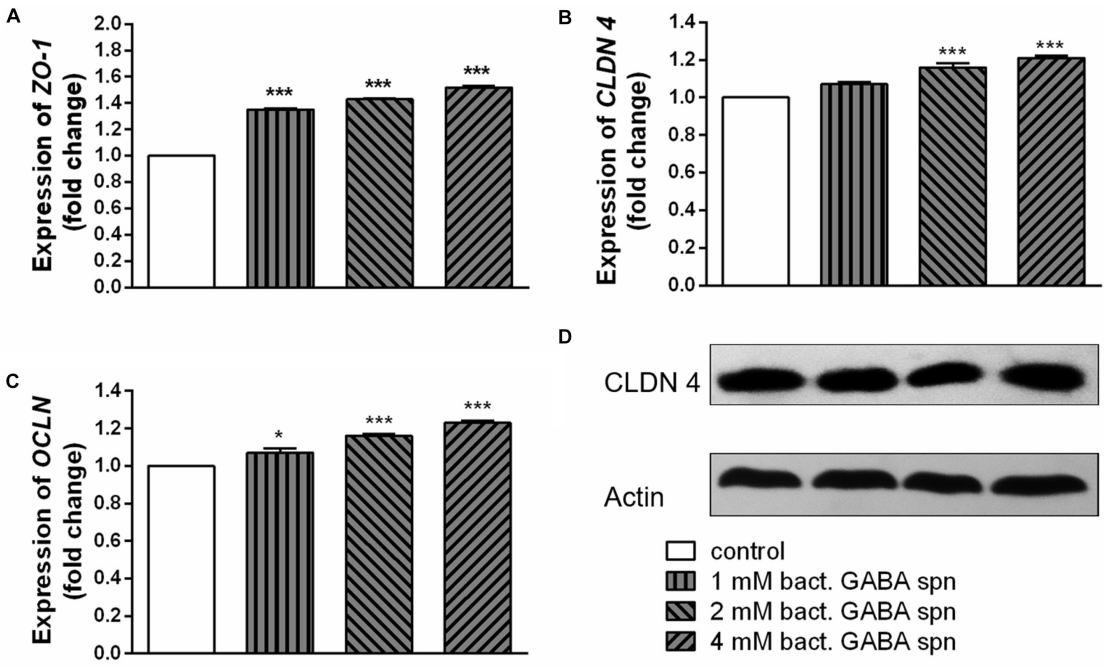


FIGURE 5 | Effect of BGZLS10-17 supernatant containing bacterial GABA in three concentrations (1, 2, and 4 mM) on the expression of tight junction proteins. mRNA expression levels of *zonulin* **(A)**, *occludin* **(B),** and *claudin 4* **(C)** and representative western blot showing claudin 4 protein expression **(D)** in differentiated Caco-2 cells. The statistical significance of mRNAs expression is shown (*$p < 0.05$; ***$p < 0.001$); bact., bacterial.

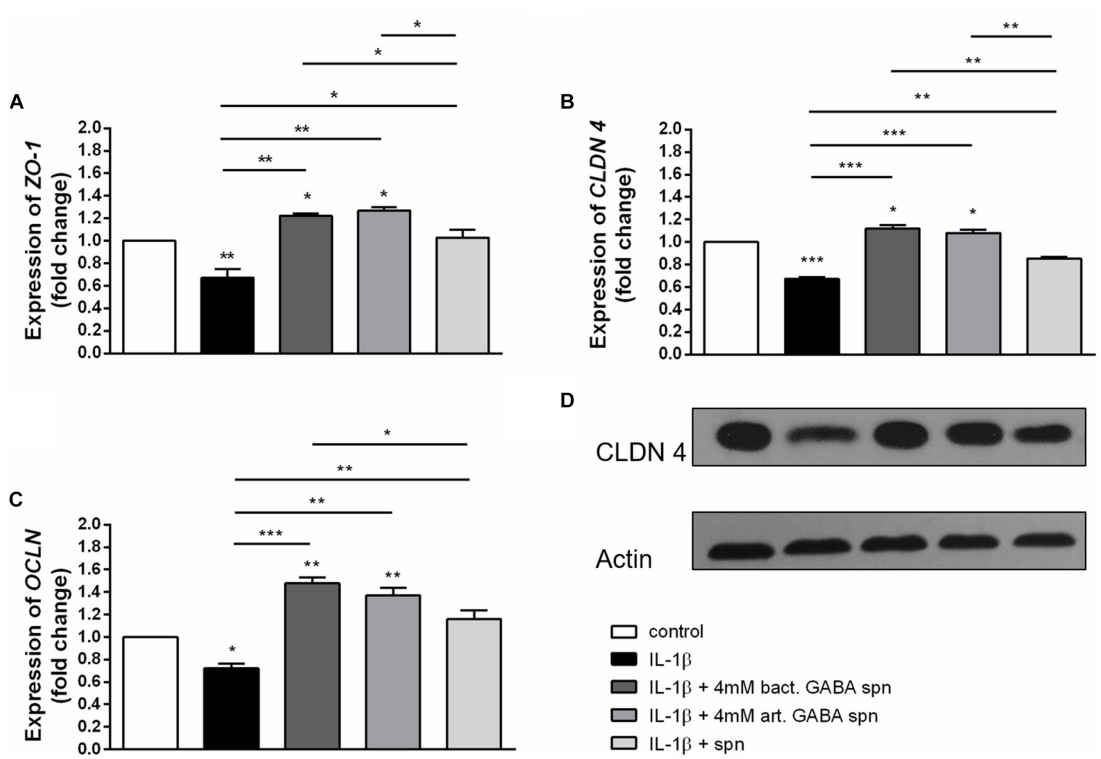


FIGURE 6 | Correlation between IL-1β and protective effect of BGZLS10-17 supernatant without GABA, supernatant containing 4 mM bacterial GABA, or supernatant with 4 mM artificial GABA mRNA expression levels of *zonulin* **(A)**, *occludin* **(B)** and *claudin 4* **(C)** and representative western blot showing claudin 4 protein expression **(D)** in differentiated Caco-2 cells treated with IL-1β together with the BGZLS10-17 supernatant without GABA, supernatant containing 4 mM bacterial GABA, or supernatant with 4 mM artificial GABA; The statistical significance of mRNAs expression is shown (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$); bact., bacterial; art., artificial.

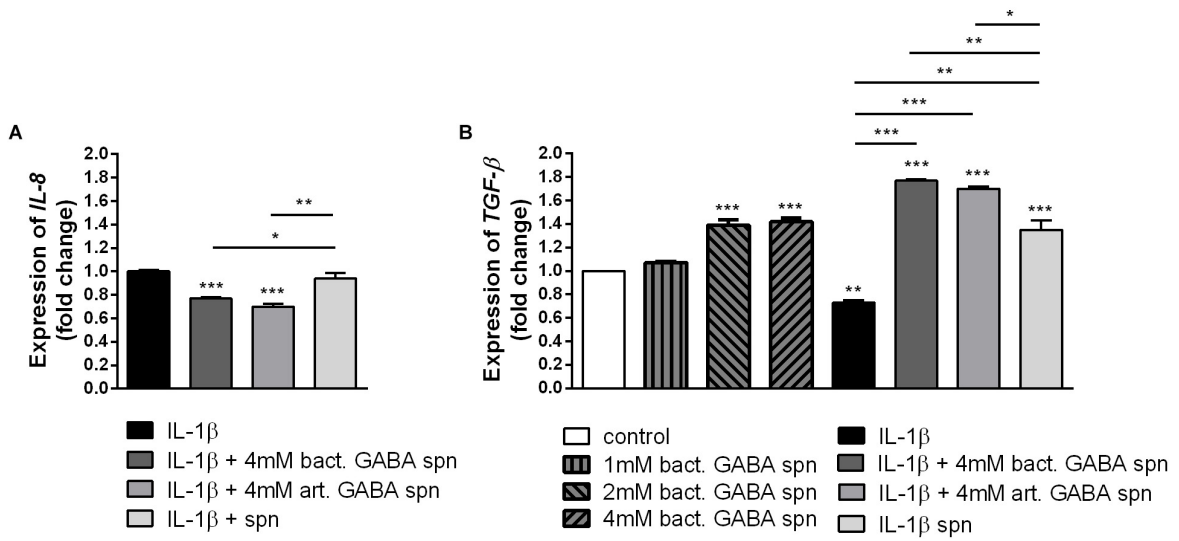


FIGURE 7 | The effect of BGZLS10-17 supernatant on the cytokine production. mRNA expression levels of *IL-8* stimulated by IL-1β with and without BGZLS10-17 supernatant without GABA, 4 mM bacterial GABA in supernatant, and 4 mM artificial GABA in supernatant **(A)** and *TGF*-β expression levels in presence of BGZLS10-17 supernatant containing bacterial GABA in three concentrations (1, 2, and 4 mM) and expression of *TGF*-β in presence of IL-1β, or IL-1β together with the BGZLS10-17 supernatant containing 4 mM bacterial GABA, and supernatant with addition of 4 mM artificial GABA **(B)** in differentiated Caco-2 cells. The statistical significance of mRNAs expression is shown (*$p < 0.05$; **$p < 0.01$, ***$p < 0.001$), bact., bacterial; art., artificial.

we investigated the effects of BGZLS10-17, the best GABA-producing strain supernatant containing different (1 mM, 2 mM and 4 mM) non-toxic (data not shown) concentrations of GABA on the expression of tight junction proteins in differentiated Caco-2 cells monolayer. Interestingly, supernatants containing all three GABA concentrations significantly stimulated the expression of *ZO-1* and *OCLN* mRNA (**Figures 5A,B**), while supernatants containing 2 and 4 mM GABA significantly stimulated the expression of *CLDN 4* (**Figures 5C,D**). Ct values are presented in the **Supplementary Table 1**. In addition to promoting inflammatory cascade, IL-1β has been shown to induce increase in intestinal epithelial tight junction permeability, the mechanism shown to be an early event in the development of different inflammatory conditions (Al-Sadi et al., 2008). In that sense, we investigated whether these treatments can alleviate deleterious effects of IL-1β on tight junction proteins. The treatment of differentiated Caco-2 cells monolayer with IL-1β significantly reduced the expression of all tight junction mRNAs (**Figures 6A–C**), as well as claudin protein (**Figure 6D**), while addition of either of these treatments reverted significantly all these effects of IL-1β on tight junction. It is interesting that the supernatants containing GABA have more significant protective effect on tight junction proteins in comparison to supernatant without GABA. Such pronounced potential of the supernatant to protect tight junction proteins from deleterious effect of inflammation is in accordance with the recent results related to GABA potential to improve the gut barrier function, acting through selective up-regulation of Mucin-1 protein in isolated pig jejunum (Braun et al., 2015). Additionally, El-Hady et al. (2017) demonstrated that GABA administration reduced the degenerative changes in the jejunal epithelial cells and significantly improved the survival of villi and crypts in gamma-irradiated rats. Considering this we further investigated whether BGZLS10-17 supernatant with highest GABA concentration (4 mM), supernatant with addition of 4mM artificial GABA, and supernatant without GABA, may have affect on IL-1β induced IL-8 production by differentiated Caco-2 cells monolayer. The results are very promising, disclosing that treatment of Caco-2 by supernatant containing GABA produced by BGZLS10-17, as well as supernatant containing artificial GABA, significantly decreased *IL-8* production by Caco-2 induced by IL-1β in this experimental setting. Interestingly, treatment with supernatant without GABA had no modulatory effects on *IL-8* mRNA expression by Caco-2 cells (**Figure 7A**). TGF-β has significant role in the maintenance of epithelial barrier integrity as well as restriction of unrestrained inflammation (Planchon et al., 1994). In that sense, looking for a possible mechanism on preventing the disruption of tight junction proteins expression in IL-1β treated Caco-2 cells, we analyzed the modulatory effects of the treatments on TGF-β production by Caco-2 cells. The treatments with supernatants containing 2 and 4 mM bacterial GABA significantly stimulated the expression of *TGF-β* (**Figure 7B**). Importantly, all supernatants prevented the decreasement of *TGF*-β expression by Caco-2 cells induced by IL-1β (**Figure 7B**). Interestingly, the supernatants containing GABA (bacterial or artificial) have more significant effect on prevention of IL-1β induced decreasement in TGF-β expression by Caco-2 cells. All these results point to the potential of supernatants obtained from BGZLS10-17 culture in different media to prevent the deleterious effects of IL-1β on Caco-2 cells. Interestingly, the presence of GABA (bacterial or artificial) in supernatant have shown significant additional protective effect on IL-1β treated Caco-2 cells. Almost identical modulatory effect of supernatant containing bacterial GABA and artificial GABA in same concentration additionally supports the hypothesis that GABA is an important molecule that contributes to the protective effect of BGZLS10-17 supernatant on IL-1β induced disruption of the intestinal barrier. Additionally, these results together with the adhesive properties of BGZLS10-17 and its capability to decrease the adhesion of important gut pathogens point to the promising role of BGZLS10-17 in the treatment of chronical gut infections characterized by aggravated inflammation and disrupted epithelial integrity.

CONCLUSION

The results of this study support the idea that using GABA producing BGZLS10-17 probiotic strain could be a good strategy to modulate immunological response in various inflammatory diseases, and at the same time, BGZLS10-17 stands out as a promising candidate for adjunct starter culture for production of innovative added-value GABA-enriched dairy products and offers new perspectives in designing the novel functional foods.

AUTHOR CONTRIBUTIONS

SSB performed main work, analyzed and interpreted the data, and drafted the work. JD conceived and designed the experiments, performed part of the experiments, analyzed and interpreted the data, and critically revised the manuscript. MD performed part of the experiments and analyzed and interpreted the data. KV performed part of the experiments and analyzed and interpreted the data. NG supervised the work, analyzed and interpreted the data, and drafted the work. SM conceived and designed the work, performed part of the experiments, and analyzed and interpreted the data. MT conceived and designed the experiments, supervised the work, analyzed and interpreted the data, and critically revised the manuscript. All authors finally approved the version to be published and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

FUNDING

This work was funded by the Ministry of Education, Science and Technological Development of the Republic of Serbia, grant OI 173019.

ACKNOWLEDGMENTS

Ian Paulsen, Macquarie University, Sidney, NSW, Australia is acknowledged for the kind supply of *E. faecalis* V583.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00527/full#supplementary-material

REFERENCES

Al-Sadi, R., Ye, D., Dokladny, K., and Ma, T. Y. (2008). Mechanism of IL-1β-induced increase in intestinal epithelial tight junction permeability. *J. Immunol.* 180, 5653–5661. doi: 10.4049/jimmunol.180.8.5653

Angrisano, T., Pero, R., Peluso, S., Keller, S., Sacchetti, S., Bruni, C. B., et al. (2010). LPS-induced IL-8 activation in human intestinal epithelial cells is accompanied by specific histone H3 acetylation and methylation changes. *BMC Microbiol.* 10:172. doi: 10.1186/1471-2180-10-172

Argyri, A. A., Zoumpopoulou, G., Karatzas, K. A., Tsakalidou, E., Nychas, G. J., Panagou, E. Z., et al. (2013). Selection of potential probiotic lactic acid bacteria from fermented olives by *in vitro* tests. *Food Microbiol.* 33, 282–291. doi: 10.1016/j.fm.2012.10.005

Begley, M., Hill, C., and Gahan, C. G. M. (2006). Bile salt hydrolase activity in probiotics. *Appl. Environ. Microbiol.* 72, 1729–1738. doi: 10.1128/AEM.72.3.1729-1738.2006

Bermudez-Brito, M., Plaza-Díaz, J., Muñoz-Quezada, S., Gómez-Llorente, C., and Gil, A. (2012). Probiotic mechanisms of action. *Ann. Nutr. Metab.* 61, 160–174. doi: 10.1159/000342079

Bhat, R., Axtell, R., Mitra, A., Miranda, M., Lock, C., Tsien, R. W., et al. (2010). Inhibitory role for GABA in autoimmune inflammation. *Proc. Natl. Acad. Sci. U.S.A.* 107, 2580–2585. doi: 10.1073/pnas.0915139107

Boonstra, E., de Kleijn, R., Colzato, L. S., Alkemade, A., Forstmann, B. U., and Nieuwenhuis, S. (2015). Neurotransmitters as food supplements: the effects of GABA on brain and behavior. *Front. Psychol.* 6:1520. doi: 10.3389/fpsyg.2015.01520

Braun, H. S., Sponder, G., Pieper, R., Aschenbach, J. R., and Deiner, C. (2015). GABA selectively increases mucin-1 expression in isolated pig jejunum. *Genes Nutr.* 10:47. doi: 10.1007/s12263-015-0497-8

Bron, P. A., Kleerebezem, M., Brummer, R. J., Cani, P. D., Mercenier, A., MacDonald, T. T., et al. (2017). Can probiotics modulate human disease by impacting intestinal barrier function? *Br. J. Nutr.* 117, 93–107. doi: 10.1017/S0007114516004037

Cho, Y. R., Chang, J. Y., and Chang, H. C. (2007). Production of γ-aminobutyric acid (GABA) by *Lactobacillus buchneri* isolated from kimchi and its neuroprotective effect on neuronal cells. *J. Microbiol. Biotechnol.* 17, 104–109.

Danielsen, M., and Wind, A. (2003). Susceptibility of *Lactobacillus* spp. to antimicrobial agents. *Int. J. Food Microbiol.* 82, 1–11. doi: 10.1016/S0168-1605(02)00254-4

Dhakal, R., Bajpai, V. K., and Baek, K. (2012). Production of GABA (γ-Aminobutyric acid) by microorganisms: a review. *Braz. J. Microbiol.* 43, 1230–1241. doi: 10.1590/S1517-83822012000400001

Dinić, M., Lukić, J., Djokić, J., Milenković, M., Strahinić, I., Golić, N., et al. (2017). *Lactobacillus fermentum* postbiotic-induced autophagy as potential approach for treatment of acetaminophen hepatotoxicity. *Front. Microbiol.* 8:594. doi: 10.3389/fmicb.2017.00594

Dragicevic, S., Milosevic, K., Nestorovic, B., and Nikolic, A. (2017). Influence of the polymorphism C-509T in the TGFB1 gene promoter on the response to montelukast. *Pediatr. Allergy Immunol. Pulmonol.* 30, 239–245. doi: 10.1089/ped.2017.0770

Edelblum, K. L., and Turner, J. R. (2009). The tight junction in inflammatory disease: communication breakdown. *Curr. Opin. Pharmacol.* 9, 715–720. doi: 10.1016/j.coph.2009.06.022

EFSA (2008). Technical guidance prepared by the panel on additives and products or substances used in animal feed (FEEDAP) on the update of the criteria used in the assessment of bacterial resistance to antibiotics of human or veterinary importance. *EFSA J.* 732, 1–15.

Elamin, E., Jonkers, D., Jutti-Uusitalo, K., Van Uzendoorn, S., Troost, F., Duimel, H., et al. (2012). Effects of ethanol and acetaldehyde on tight junction integrity: *in vitro* study in a three-dimensional intestinal epithelial cell culture model. *PLoS One* 7:e35008. doi: 10.1371/journal.pone.0035008

El-Hady, A. M. A., Gewefel, H. S., Badawi, M. A., and Eltahawy, N. A. (2017). Gamma-aminobutyric acid ameliorates gamma rays-induced oxidative stress in the small intestine of rats. *J. Basic Appl. Zool.* 78:2. doi: 10.1186/s41936-017-0005-3

Franciosi, E., Carafa, I., Nardin, T., Schiavon, S., Poznanski, E., Cavazza, A., et al. (2015). Biodiversity and γ-aminobutyric acid production by lactic acid bacteria isolated from traditional alpine raw cow's milk cheeses. *Biomed Res. Int.* 2015:625740. doi: 10.1155/2015/625740

Golić, N., Čadež, N., Terzić-Vidojević, A., Šuranská, H., Beganović, J., Lozo, J., et al. (2013). Evaluation of microbial diversity in traditional white-pickled and fresh soft cheeses from mountain regions in Serbia and low laying regions in Croatia. *Int. J. Food Microbiol.* 166, 294–300. doi: 10.1016/j.ijfoodmicro.2013.05.032

Holdiness, M. R. (1983). Chromatographic analysis of glutamic acid decarboxylase in biological samples. *J. Chromatogr.* 14, 1–24. doi: 10.1016/S0378-4347(00)84819-7

Jin, Z., Mendu, S. K., and Birnir, B. (2013). GABA is an effective immunomodulatory molecule. *Amino Acids* 45, 87–94. doi: 10.1007/s00726-011-1193-7

Jovčić, B., Begović, J., Lozo, J., Topisirović, L. J., and Kojić, M. (2009). Dynamics of sodium dodecyl sulfate utilization andantibiotic susceptibility of strain *Pseudomonas* sp. ATCC19151. *Arch. Biol. Sci.* 61, 159–164. doi: 10.2298/ABS0902159J

Kakee, A., Takanage, H., Terasaki, T., Naito, M., Tsuruo, T., and Sugiyama, Y. (2001). Efflux of a suppressive neurotransmitter, GABA, across the blood-brain barrier. *J. Neurochem.* 79, 110–118. doi: 10.1046/j.1471-4159.2001.00540.x

Kelley, J. M., Hughes, L. B., and Bridges, S. L. Jr. (2008). Does gamma-aminobutyric acid (GABA) influence the development of chronic inflammation in rheumatoid arthritis? *J. Neuroinflammation* 5:1. doi: 10.1186/1742-2094-5-1

Kim, J. Y., Lee, M. Y., Ji, G. E., Lee, Y. S., and Hwang, K. T. (2009). Production of gamma-aminobutyric acid in black raspberry juice during fermentation by *Lactobacillus brevis* GABA100. *Int. J. Food Microbiol.* 130, 12–16. doi: 10.1016/j.ijfoodmicro.2008.12.028

Komatsuzaki, N., Nakamura, T., Kimura, T., and Shima, J. (2008). Characterization of glutamate decarboxylase from a high gamma-aminobutyric acid (GABA)-producer, *Lactobacillus paracasei*. *Biosci. Biotechnol. Biochem.* 72, 278–285. doi: 10.1271/bbb.70163

Komatsuzaki, N., Shimaa, J., Kawamotoa, S., Momosed, H., and Kimurab, T. (2005). Production of γ-aminobutyric acid (GABA) by *Lactobacillus paracasei* isolated from traditional fermented foods. *Food Microbiol.* 22, 497–504. doi: 10.1016/j.fm.2005.01.002

Laroute, V., Yasaro, C., Narin, W., Mazzoli, R., Pessione, E., Cocaign-Bousqet, M., et al. (2016). GABA production in *Lactococcus lactis* is enhanced by arginine and co-addition of malate. *Front. Microbiol.* 7:1050. doi: 10.3389/fmicb.2016.01050

Lebeer, S., Vanderleyden, J., and De Keersmaecker, S. C. (2008). Genes and molecules of lactobacilli supporting probiotic action. *Microbiol. Mol. Biol. Rev.* 72, 728–764. doi: 10.1128/MMBR.00017-08

Li, H., and Cao, Y. (2010). Lactic acid bacterial cell factories for gamma-aminobutyric acid. *Amino Acids* 39, 1107–1116. doi: 10.1007/s00726-010-0582-7

Li, H., Qiu, T., Hang, G., and Cao, Y. (2010). Production of gamma-aminobutyric acid by *Lactobacillus brevis* NCL912 using fed-batch fermentation. *Microb. Cell Fact.* 9:85. doi: 10.1186/1475-2859-9-85

Li, X., Xu, M., Liu, M., Ji, Y., and Li, Z. (2015). TNF-alpha and IL-6 inhibit apolipoprotein A-IV production induced by linoleic acid in human intestinal Caco2 cells. *J. Inflamm.* 12:22. doi: 10.1186/s12950-015-0069-0

Liu, Z., Li, N., and Neu, J. (2005). Tight junctions, leaky intestines, and pediatric diseases. *Acta Paediatr.* 94, 386–393. doi: 10.1111/j.1651-2227.2005.tb01904.x

Lukic, J., Strahinic, I., Milenkovic, M., Golic, N., Kojic, M., Topisirovic, L., et al. (2013). Interaction of *Lactobacillus fermentum* BGHI14 with rat colonic mucosa: implications for colitis induction. *Appl. Environ. Microbiol.* 79, 5735–5744. doi: 10.1128/AEM.01807-13

Mazzoli, R., Riedel, K., and Pessione, E. (2017). Editorial: bioactive compounds from microbes. *Front. Microbiol.* 8:392. doi: 10.3389/fmicb.2017.00392

Miljkovic, M., Strahinic, I., Tolinacki, M., Zivkovic, M., Kojic, S., Golic, N., et al. (2015). AggLb is the largest cell-aggregation factor from *Lactobacillus paracasei* subsp. *paracasei* BGNJ1-64, functions in collagen adhesion, and pathogen exclusion *in vitro*. *PLoS One* 10:e0126387. doi: 10.1371/journal.pone.0126387

Muñoz-Provencio, D., Llopis, M., Antolín, M., de Torres, I., Guarner, F., Pérez-Martínez, G., et al. (2009). Adhesion properties of *Lactobacillus casei* strains to resected intestinal fragments and components of the extracellular matrix. *Arch. Microbiol.* 191, 153–161. doi: 10.1007/s00203-008-0436-9

Nigam, R., El-Nour, H., Amatya, B., and Nordlind, K. (2010). GABA and GABA(A) receptor expression on immune cells in psoriasis: a pathophysiological role. *Arch. Dermatol. Res.* 302, 507–515. doi: 10.1007/s00403-010-1052-5

Nouri, M., Bredberg, A., Weström, B., and Lavasani, S. (2014). Intestinal barrier dysfunction develops at the onset of experimental autoimmune encephalomyelitis and can be induced by adoptive transfer of auto-reactive T cells. *PLoS One* 9:e106335. doi: 10.1371/journal.pone.0106335

O'Shea, E. F., Cotter, P. D., Stanton, C., Ross, R. P., and Hill, C. (2012). Production of bioactive substances by intestinal bacteria as a basis for explaining probiotic mechanisms: bacteriocins and conjugated linoleic acid. *Int. J. Food Microbiol.* 152, 189–205. doi: 10.1016/j.ijfoodmicro.2011.05.025

Park, J. Y., Jeong, S. J., and Kim, J. H. (2014). Characterization of a glutamate decarboxylase (GAD) gene from *Lactobacillus zymae*. *Biotechnol. Lett.* 36, 1791–1799. doi: 10.1007/s10529-014-1539-9

Paulsen, I. T., Banerjei, L., Myers, G. S., Nelson, K. E., Seshadri, R., Read, T. D., et al. (2003). Role of mobile DNA in the evolution of vancomycin-resistant *Enterococcus faecalis*. *Science* 299, 2071–2074. doi: 10.1126/science.1080613

Planchon, S. M., Martins, C. A., Guerrant, R. L., and Roche, J. K. (1994). Regulation of intestinal epithelial barrier function by TGF-beta 1. Evidence for its role in abrogating the effect of a T cell cytokine. *J. Immunol.* 153, 5730–5739.

Powers, M. E., Yarrow, J. F., McCoy, S. C., and Borst, S. E. (2008). Growth hormone isoform responses to GABA ingestion at rest and after exercise. *Med. Sci. Sports Exerc.* 40, 104–110. doi: 10.1249/mss.0b013e318158b518

Rossetti, V., and Lombard, A. (1996). Determination of glutamate decarboxylase by high-performance liquid chromatography. *J. Chromatogr. B Biomed. Appl.* 681, 63–67. doi: 10.1016/0378-4347(96)88202-8

Sánchez, B., Fernandez-Garcia, M., Margolles, A., de los Reyes-Gavilan, C. G., and Ruas-Madiedo, P. (2010). Technological and probiotic selection criteria of a bile-adapted *Bifidobacterium animalis* subsp. *lactis* strain. *Int. Dairy J.* 20, 7078–7085. doi: 10.1016/j.idairyj.2010.06.004

Schuerer-Maly, C. C., Eckmann, L., Kagnoff, M. F., Falco, M. T., and Maly, F. E. (1994). Colonic epithelial cell lines as a source of interleukin-8: stimulation by inflammatory cytokines and bacterial lipopolysaccharide. *Immunology* 81, 85–91.

Schulzke, J. D., Ploeger, S., Amasheh, M., Fromm, A., Zeissig, S., Troeger, H., et al. (2009). Epithelial tight junctions in intestinal inflammation. *Ann. N. Y. Acad. Sci.* 1165, 294–300. doi: 10.1111/j.1749-6632.2009.04062.x

Seok, J. H., Park, K. B., Kim, Y. H., Bae, M. O., Lee, M. K., and Oh, S. H. (2008). Production and characterization of kimchi with enhanced levels of gamma-aminobutyric acid. *Food Sci. Biotechnol.* 17, 940–946.

Siragusa, S., De Angelis, M., Di Cagno, R., Rizzello, C. G., Coda, R., and Gobbetti, M. (2007). Synthesis of γ-aminobutyric acid by lactic acid bacteria isolated from a variety of Italian cheeses. *Appl. Environ. Microbiol.* 73, 7283–7290. doi: 10.1128/AEM.01064-07

Su, Y. A., Sulavik, M. C., He, P., Makinen, K. K., Makinen, P. L., Fiedler, S., et al. (1991). Nucleotide sequence of the gelatinase gene (*gelE*) from *Enterococcus faecalis* subsp. *liquefaciens*. *Infect. Immun.* 59, 415–420.

Suenaert, P., Bulteel, V., Lemmens, L., Noman, M., Geypens, B., Van Assche, G., et al. (2002). Anti-tumor necrosis factor treatment restores the gut barrier in Crohn's disease. *Am. J. Gastroenterol.* 97, 2000–2004. doi: 10.1111/j.1572-0241.2002.05914.x

Sun, T. S., Zhao, S. P., Wang, H. K., Cai, C. K., Chen, Y. F., and Zhang, H. P. (2009). ACE-inhibitory activity and gamma-aminobutyric acid content of fermented skim milk by *Lactobacillus helveticus* isolated from Xinjiang koumiss in China. *Eur. Food Res. Technol.* 228, 607–612. doi: 10.1007/s00217-008-0969-9

Temmerman, R., Pot, B., Huys, G., and Swings, J. (2003). Identification and antibiotic susceptibility of bacterial isolates from probiotic products. *Int. J. Food Microbiol.* 81, 1–10. doi: 10.1016/S0168-1605(02)00162-9

Tian, J., Lu, Y., Zhang, H., Chau, C. H., Dang, H. N., and Kaufman, D. L. (2004). Gamma-aminobutyric acid inhibits T cell autoimmunity and the development of inflammatory responses in a mouse type 1 diabetes model. *J. Immunol.* 173, 5298–5304. doi: 10.4049/jimmunol.173.8.5298

Tian, J., Yong, J., Dang, H., and Kaufman, D. L. (2011). Oral GABA treatment downregulates inflammatory responses in a mouse model of rheumatoid arthritis. *Autoimmunity* 44, 465–470. doi: 10.3109/08916934.2011.571223

Ueno, H. (2000). Enzymatic and structural aspects on glutamate decarboxylase. *J. Mol. Catal. B Enzym.* 10, 67–79. doi: 10.1016/S1381-1177(00)00114-4

Uroić, K., Nikolić, M., Kos, B., Leboš Pavunc, A., Beganović, J., Lukić, J., et al. (2014). Probiotic properties of lactic acid bacteria isolated from Croatian fresh soft cheese and Serbian white pickled cheese. *Food Technol. Biotechnol.* 52, 232–241.

Varma, P., Dinesh, K. R., Menon, K. K., and Biswas, R. (2010). *Lactobacillus fermentum* isolated from human colonic mucosal biopsy inhibits the growth and adhesion of enteric and foodborne pathogens. *J. Food Sci.* 75, 546–551. doi: 10.1111/j.1750-3841.2010.01818.x

Veljovic, K., Terzic-Vidojevic, A., Vukasinovic, M., Strahinic, I., Begovic, J., Lozo, J., et al. (2007). Preliminary characterization of lactic acid bacteria isolated from Zlatar cheese. *J. Appl. Microbiol.* 103, 2142–2152. doi: 10.1111/j.1365-2672.2007.03450.x

Visser, J., Rozing, J., Sapone, A., Lammers, K., and Fasano, A. (2009). Tight junctions, intestinal permeability, and autoimmunity: celiac disease and type 1 diabetes paradigms. *Ann. N. Y. Acad. Sci.* 1165, 195–205. doi: 10.1111/j.1749-6632.2009.04037.x

Vogelsang, H., Schwarzenhofer, M., and Oberhuber, G. (1998). Changes in gastrointestinal permeability in celiac disease. *Dig. Dis.* 16, 333–336. doi: 10.1159/000016886

Wang, L., Li, L., Lv, Y., Chen, Q., Feng, J., and Zhao, X. (2018). *Lactobacillus plantarum* restores intestinal permeability disrupted by *Salmonella* infection in newly-hatched chicks. *Sci. Rep.* 8:2229. doi: 10.1038/s41598-018-20752-z

WHO-FAO (2002). *Guidelines for the Evaluation of Probiotics in Food. Report of a Joint FAO/WHO Working Group on Drafting Guidelines for the Evaluation of Probiotics in Food*. London: WHO.

WHO-FAO (2006). *Probiotics in foods. Health and nutritional properties and guidelines for evaluation*. FAO Food and Nutritional Paper No. 8592-5-105513.

Zhang, Y., Zhang, L., Tuo, Y., Guo, C., Yi, H., Li, J., et al. (2010). Inhibition of *Shigella sonnei* adherence to HT-29 cells by lactobacilli from Chinese fermented food and preliminary characterization of S-layer protein involvement. *Res. Microbiol.* 161, 667–672. doi: 10.1016/j.resmic.2010.06.005

Zhou, J. S., Pillidge, C. J., Gopal, P. K., and Gill, H. S. (2005). Antibiotic susceptibility profiles of new probiotic *Lactobacillus* and *Bifidobacterium* strains. *Int. J. Food Microbiol.* 98, 211–217. doi: 10.1016/j.ijfoodmicro.2004.05.011

Zivkovic, M., Hidalgo-Cantabrana, C., Kojic, M., Gueimonde, M., Golic, N., and Ruas-Madiedo, P. (2015). Capability of exopolysaccharide-producing *Lactobacillus paraplantarum* BGCG11 and its non-producing isogenic strain NB1, to counteract the effect of enteropathogens upon the epithelial cell line HT29-MTX. *Food Res. Int.* 74, 199–207. doi: 10.1016/j.foodres.2015.05.012

Živkovic, M., Miljkovic, M. S., Ruas-Madiedo, P., Markelic, M. B., Veljovic, K., Tolinacki, M., et al. (2016). EPS-SJ exopolisaccharide produced by the strain *Lactobacillus paracasei* subsp. *paracasei* BGSJ2-8 is involved in adhesion to epithelial intestinal cells and decrease on *E. coli* association to Caco-2 cells. *Front. Microbiol.* 7:286. doi: 10.3389/fmicb.2016.00286

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Microbiology

MINI REVIEW
published: 29 March 2019
doi: 10.3389/fmicb.2019.00463

Bioactive Products From Plant-Endophytic Gram-Positive Bacteria

*María J. Ek-Ramos[1], Ricardo Gomez-Flores[1], Alonso A. Orozco-Flores[1], Cristina Rodríguez-Padilla[1], Guadalupe González-Ochoa[2] and Patricia Tamez-Guerra[1]**

[1] Departamento de Microbiología e Inmunología, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, San Nicolás de los Garza, Mexico, [2] Departamento de Ciencias Químico Biológicas, División de Ciencias e Ingeniería, Universidad de Sonora, Navojoa, Mexico

Endophytes constitute plant-colonizing microorganisms in a mutualistic symbiosis relationship. They are found in most ecosystems reducing plant crops' biotic and abiotic stressors by stimulating immune responses, excluding plant pathogens by niche competition, and participating in antioxidant activities and phenylpropanoid metabolism, whose activation produces plant defense, structural support, and survival molecules. In fact, metabolomic studies have demonstrated that endophyte genes associated to specific metabolites are involved in plant growth promotion (PGP) by stimulating plant hormones production such as auxins and gibberellins or as plant protective agents against microbial pathogens, cancer, and insect pests, but eco-friendly and eco-safe. A number of metabolites of Gram-positive endophytes isolated from agriculture, forest, mangrove, and medicinal plants, mainly related to the Firmicutes phyla, possess distinctive biocontrol and plant growth-promoting activities. In general, Actinobacteria and *Bacillus* endophytes produce aromatic compounds, lipopeptides, plant hormones, polysaccharides, and several enzymes linked to phenylpropanoid metabolism, thus representing high potential for PGP and crop management strategies. Furthermore, Actinobacteria have been shown to produce metabolites with antimicrobial and antitumor activities, useful in agriculture, medicine, and veterinary areas. The great endophytes diversity, their metabolites production, and their adaptation to stress conditions make them a suitable and unlimited source of novel metabolites, whose application could reduce agrochemicals usage in food and drugs production.

Keywords: metabolites, amylases, chitinases, endoglucanases, esterases, proteases, plant hormones, toxins

OPEN ACCESS

Edited by:
José E. Barboza-Corona, Universidad de Guanajuato, Mexico

Reviewed by:
Ashutosh Sharma, Tecnologico de Monterrey, Mexico
Tapan Kumar Adhya, KIIT University, India

***Correspondence:**
Patricia Tamez-Guerra
patamez@hotmail.com;
patricia.tamezgr@uanl.edu.mx

Specialty section:
This article was submitted to Microbiotechnology, Ecotoxicology and Bioremediation, a section of the journal Frontiers in Microbiology

Received: *29 October 2018*
Accepted: *21 February 2019*
Published: *29 March 2019*

Citation:
Ek-Ramos MJ, Gomez-Flores R, Orozco-Flores AA, Rodríguez-Padilla C, González-Ochoa G and Tamez-Guerra P (2019) Bioactive Products From Plant-Endophytic Gram-Positive Bacteria. Front. Microbiol. 10:463. doi: 10.3389/fmicb.2019.00463

INTRODUCTION TO ENDOPHYTES

Endophytes are facultative or obligate symbiotic microorganisms, mainly bacterial and fungal species, that live in apparently healthy internal plant tissues, without causing disease (Schulz and Boyle, 2006). The most studied ones are bacterial and fungal species.

The purpose of this minireview is to highlight the importance of previously reported endophytic Gram-positive bacteria bioactive products. The International Union for Conservation of Nature and Natural Resources estimates that there are about 297,326 species of plants (Monocotyledons, Dicotyledons, Gymnosperms, Ferns and allies and Mosses), but only a few of them have been studied for their endophyte

microbiota (Strobel and Daisy, 2003; Aitken, 2004). Endophytic microorganisms are known to influence plant physiology and development, among which, Gram-positive bacteria are important in such activities as bioremediation, biocontrol, plant growth, symbiotic-mutualistic, commensalistic, trophobiotic interactions, control of soil-borne pathogens, and support of host plant defense against environmental stress (Ryan et al., 2008). An endophytic community is complex and several factors may affect its structure, such as plant-microbe and microbe-microbe interactions and environmental conditions (Ryan et al., 2008). For bacterial endophytes diversity analysis, cultivation-based and culture-independent methods are used. In regard to cultivation studies, a great number of bacteria, mostly Proteobacteria, have been reported as endophytes, being the most frequent from Actinobacteria, Bacteroidetes, and Firmicutes phyla (Rosenblueth and Martínez-Romero, 2006).

The most abundant metabolite producing Gram-positive bacteria endophytes found within diverse environments are *Bacillus* and *Streptomyces* species (Reinhold-Hurek and Hurek, 2011; Frank et al., 2017; **Figures 1–3**).

Endophytes are found in plants of most ecosystems and are of agricultural importance since they help to improve crops yields, by stimulating plants growth and immune response, excluding plant pathogens by niche competition, as well as actively participating in phenylpropanoid metabolism and antioxidant activities (Pandey et al., 2018). Among plant microbiota, endophytic bacteria can be found in most plant species and be recovered from roots, leaves, stems, and a few from flowers, fruits, and seeds (Lodewyckx et al., 2002); they have the potential to produce a variety of secondary metabolites with application in agriculture and pharmaceutical and industrial biotechnology (Lodewyckx et al., 2002; Strobel and Daisy, 2003; Ryan et al., 2008). Bacterial endophytes live within cell walls and xylem vessels intercellular regions and they may colonize seeds (Cankar et al., 2005; Johnston-Monje and Raizada, 2011), fruits (de Melo-Pereira et al., 2012), and flowers (Compant et al., 2011), among other tissues. It is known that endophytic bacteria are located in the apoplast (Koskimäki et al., 2015), and plant roots are proposed to be the entry point (Paungfoo-Lonhienne et al., 2010). It is also suggested that they are transmitted using an alternative vertical strategy due to their presence in flowers and seeds (Tamosiune et al., 2017). The potential explanation for their ubiquitous presence into plant tissues is the diversity of positive effects on plant growth and fitness they have shown, by stimulating the host phenylpropanoid pathway or by producing several linked-metabolites to the plants' metabolism (Brader et al., 2014; Haidar et al., 2016; Lòpez-Fernàndez et al., 2016; Alaimo et al., 2018). Many reports indicate that bacterial endophytes help to provide nutrients as plant growth-promoters, and induce tolerance/resistance against biotic and abiotic stress conditions (Ryan et al., 2008).

In addition, several metabolites produced by microbial endophytes act as antimicrobial agents against human, animal, and plant pathogens. Whereas the antimicrobial effect against phytopathogens will have the positive effect on the host plant, the efficacies of endophyte metabolites may show a great clinical potential for medical and veterinary treatments. Indeed, nature-occurring antibiotics are low-molecular-weight products made by microbes that inhibit the growth or kill phytopathogens, bacteria, fungi, viruses, and protozoans, that cause human and animal diseases (Demain, 1981; Jakubiec-Krzesniak et al., 2018; Tripathi et al., 2018). Some important antibiotics producers have been recently found as endophytes in different plant species (Eljounaidi et al., 2016).

It is known that immunocompromised individuals (AIDS, cancer, and organ transplant patients) are at high risk for developing opportunistic microbial infections by *Aspergillus* sp., *Candida albicans*, *Clostridium difficile*, *Coccidioides immitis*, *Cryptococcus neoformans*, *Cryptosporidium*, *Mycobacterium avium* complex, *M. tuberculosis*, *Pneumocystis jirovecii*, *Pseudomonas aeruginosa*, *Salmonella* sp., *Staphylococcus aureus*, *Streptococcus pneumoniae*, and *S. pyogenes*, as well as parasitic infections caused by *Cryptosporidium* spp., *Encephalitozoon* spp., *Isospora belli*, *Leishmania* spp., *Plasmodium falciparum*, *Toxoplasma gondii*, and *Trypanosoma cruzi*. The urgent need for human diseases prevention and treatment, has promoted the discovery and development of novel and efficient therapeutic agents to which resistance has not been produced (Strobel and Daisy, 2003; Chinedum, 2005). For instance, drug resistance is a recognized phenomenon that disease-causing microbial agents develop against pharmaceutical therapy. Infectious diseases and cancer share similarities in the mechanisms of resistance to drugs, such as drug efflux, which is evolutionarily conserved (Housman et al., 2014). Therefore, in this review, the antibacterial, antifungal, antiviral, and antitumor activities of metabolites produced by specific Gram-positive bacteria endophytes are highlighted, as well as their potential use as plant growth promoters (PGP).

GRAM-POSITIVE ENDOPHYTES AGAINST HUMAN/ANIMAL PATHOGENS

Among Actinobacteria, Actinomycetes are Gram-positive, filamentous bacteria with great potential as biocontrol agents, which produce approximately two-thirds of natural antibiotics, where 75% derived from *Streptomyces* species (de Lima Procópio et al., 2012; **Figures 1**, **3**). Actinomycetes, including the *Actinomyces*, *Actinoplanes*, *Amycolatopsis*, *Micromonospora*, *Saccharopolyspora*, and *Streptomyces* genera, are recognized as bioactive secondary metabolites producers, not only showing antimicrobial, but also insecticidal and antitumoral activities (Renu et al., 2008; Kekuda et al., 2010).

Lipopeptides are among the most important classes of secondary metabolites produced by endophytic bacteria, which are formed by cyclic or short linear peptides linked to a lipid tail or lipophilic molecules. Lipopeptides may show antimicrobial, cytotoxic and surfactant activities; they are synthesized by non-ribosomal peptide synthetases (NRPS), or polyketide synthase (PKS) and have great structural diversity based on a hydrophobic fatty acid acyl chain of 13 to 17 carbons, linked to a hydrophilic peptide of 7–25 aminoacids. Lipopeptides are important for both, their antibiotic activity

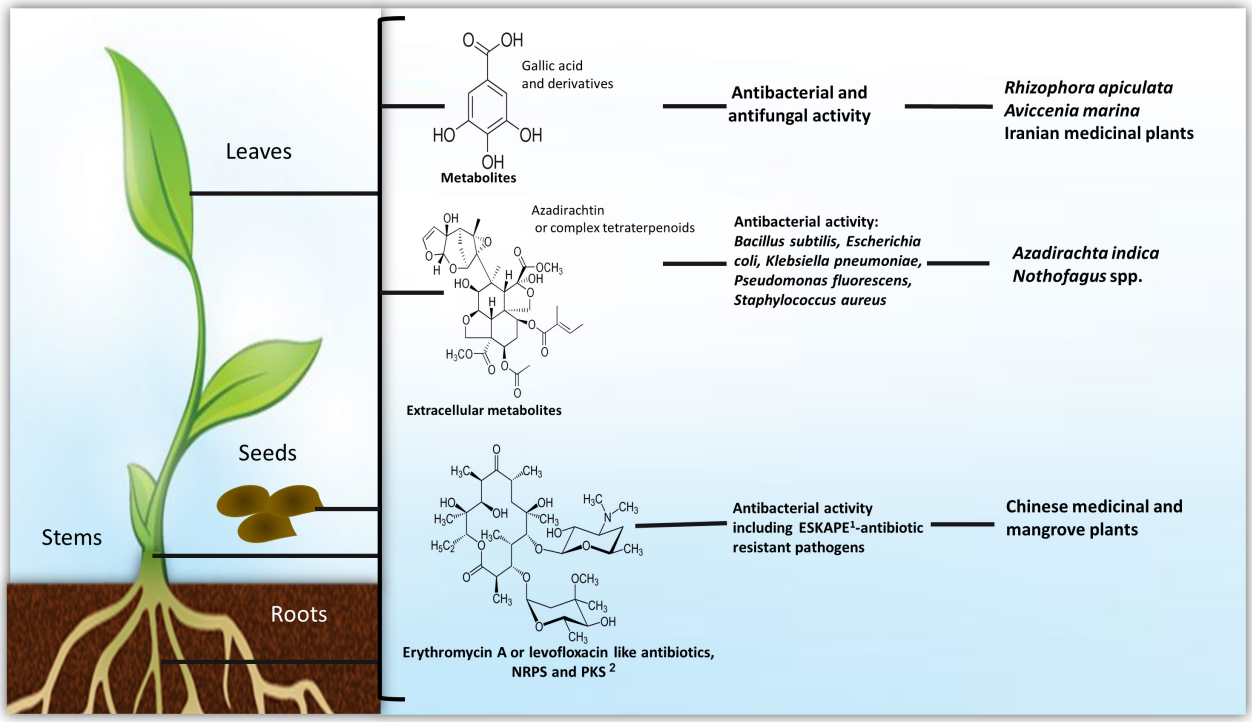


FIGURE 1 | Production of metabolites by endophytes among plant tissues. [1]The "ESKAPE" bacterial pathogens (*Acinetobacter baumannii*, *Enterobacter* spp., *Enterococcus faecium*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*) are the leading nosocomial infectious agents throughout the world. [2]Metabolites synthesized by non-ribosomal peptide synthetases (NRPS) or polyketide synthase (PKS). Detailed information on antimicrobial metabolites against animal/human pathogens are in **Supplementary Table S1**, against plant pathogens and insect pests in **Supplementary Table S2**, as plant growth stimulant in **Supplementary Table S3** and as anti-cancer agent in **Supplementary Table S4**.

and for inducing plant defense mechanisms (Stein, 2005; Raaijmakers et al., 2010). One bacterial strain may synthesize several polypeptide isoforms. *Bacillus* and *Paenibacillus*-related lipopeptides are the most studied ones (Villarreal-Delgado et al., 2018), whereas several *Bacillus amyloliquefaciens* strains have been recognized as higher lipopeptides producers (**Figure 2A**; Ongena and Jacques, 2008). In addition to lipopeptides, *B. subtilis* produces NRPS lantibiotics (lanthionine-containing antibiotics) (Stein, 2005); lipopeptides are responsible for biofilm and swarming development, whereas lantibiotics play as pheromones in quorum-sensing (Stein, 2005). *B. subtilis* also produces compounds such as polyketides, an aminosugar, and a phospholipid; polyketides include bacillomycin, fengycin, iturin, lichenysin, mycosubtilin, plipastatin, pumilacidin, and surfactin (**Figure 2B**); whereas *Paenibacillus polymyxa* synthesizes polymyxins (cyclic cationic lipopeptides) (Stein, 2005; Grady et al., 2016; **Supplementary Tables S1–S4**).

In addition to biologically active secondary metabolites, bacterial endophytes also produce important antimicrobial enzymes, mainly by Bacilli class members (**Figure 2**). In a study looking for highly producing enzymes endophytes, in the mangrove in Thailand, Khianngam et al. (2013) found that Gram-positive bacteria showed more hydrolytic activity compared with that of Gram-negative ones. Testing endophytes in hosts from the Rhizophoraceae family, results showed amylase, cellulase, and lipase activity by *B. infantis* and *B. granadenis*; and amylase, cellulase, lipase, lipolytic and proteinase activity by *B. safensis*. Similarly, cellulase, lipase, and proteinase activities by *Paenibacillus* sp. and *S. warneri* were detected in Acanthaceae family endophytes. Endophytes isolated from Brazilian mangrove plants showed high enzymatic activity; among these isolates, *Bacillus* sp. (MCR2.56) was reported to show particularly high amylase and esterasic activities; six *Bacillus* isolates (MCR2.51, MCA2.42, MCA2.51, MBR2.4, MBA2.33, and MBA2.4) high endocellulolytic activity, whereas the actinobacteria *Microbacterium* sp. (MCA2.54) and *Curtobacterium* sp. (MBR2.20) showed high endoglucanase and protease activity, respectively (Castro et al., 2014; **Figure 2** and **Supplementary Tables S1–S3**).

Bioactive endophytic Streptomycetes can be isolated from plants worldwide (Castillo et al., 2007; **Figure 3**). Medicinal plants have been used for centuries as an alternative therapy for disease treatment. Interestingly, Chinese medicinal plants-endophytic actinomycetes were reported to have antibacterial activity against *E. coli* and *S. aureus* (Zhao et al., 2011; **Figure 1**). Recently, endophytic actinomycetes were reported in several Chinese mangrove plants to exhibit antibacterial activity against *Acinetobacter baumannii*, *Enterococcus faecalis*, *E. coli*, *Klebsiella pneumoniae*, *P. aeruginosa*, and *S. aureus*, some of which are resistant to the vancomycin, methicillin,

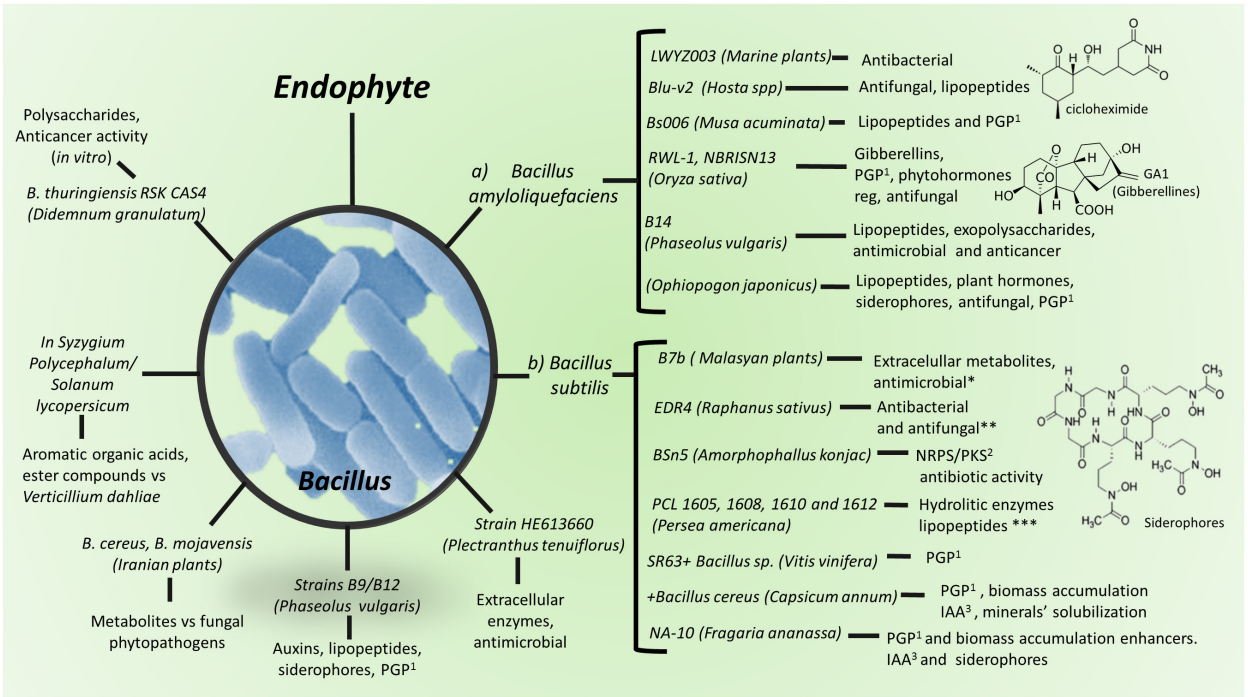


FIGURE 2 | Production of metabolites by *Bacillus* spp. and strains as plant endophytes. (a) *Bacillus amyloliquefaciens*; (b) *Bacillus subtilis*. [1]PGP, plant growth promoting. [2]Metabolites synthesized by NRPS or PKS. [3]IAA, indol acetic acid. Detailed information on antimicrobial metabolites against animal/human pathogens are in **Supplementary Table S1**, against plant pathogens and insect pests in **Supplementary Table S2**, as plant growth stimulant in **Supplementary Table S3** and as anti-cancer agent in **Supplementary Table S4**.

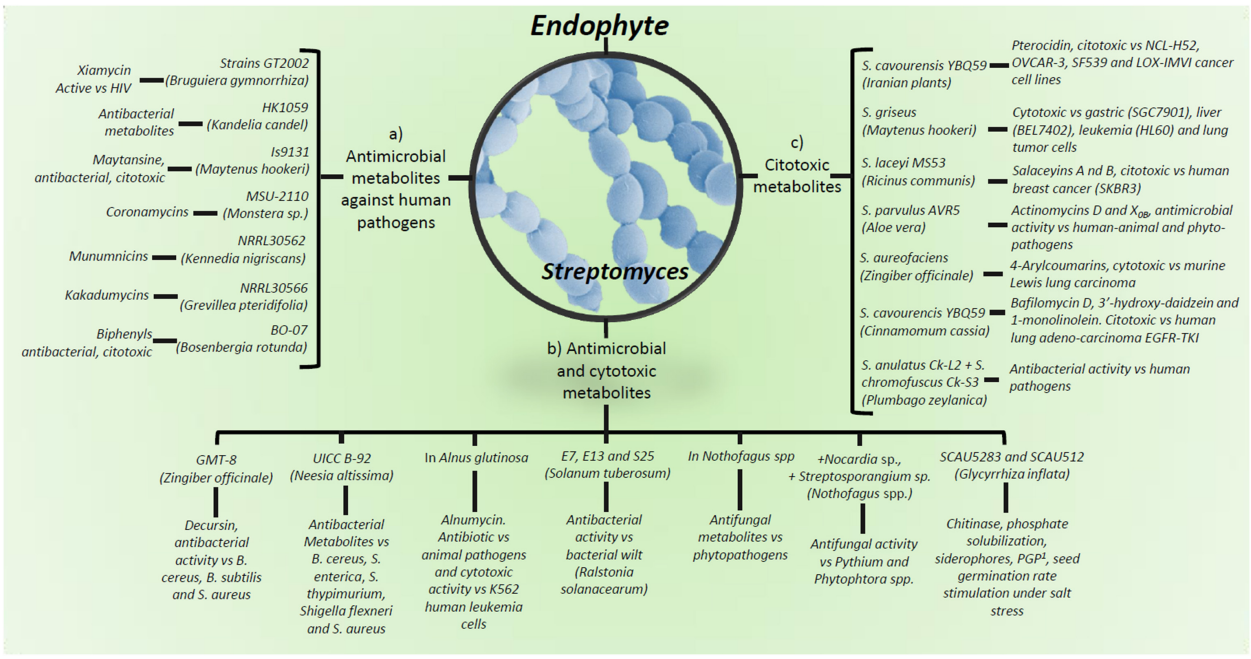


FIGURE 3 | Production of metabolites by *Streptomyces* spp. and strains as plant endophytes. (a) Antimicrobial metabolites against human pathogens; (b) metabolites showing antimicrobial and cytotoxic activity; (c) cytotoxic metabolites against several tumor cell lines. [1]PGP, plant growth promote. Detailed information on antimicrobial metabolites against animal/human pathogens are in **Supplementary Table S1**, against plant pathogens and insect pests in **Supplementary Table S2**, as plant growth stimulant in **Supplementary Table S3** and as anti-cancer agent in **Supplementary Table S4**.

and carbapenem antibiotics (Jiang et al., 2018; **Figures 1**, **3**). Several metabolites from endophytic *Streptomyces* species have been characterized and associated with antibiotic activity, including kakadumycins, munumbicins, *p*-aminoacetophenonic acids, and xiamycins (Castillo et al., 2002, 2003; Guan et al., 2005); antimalarial (coronamycin, munumbicin D) (Castillo et al., 2002; Ezra et al., 2004); and antifungal (munumbicin D) (Shimizu et al., 2000; Ezra et al., 2004) activities (**Figure 3** and **Supplementary Table S1**). Similarly, Iranian medicinal plants-endophytic actinomyctes exhibited antimicrobial activity against the pathogenic bacteria *B. cereus*, *B. subtilis*, *E. coli*, *Citrobacter freundii*, *K. pneumoniae*, *Proteus mirabilis*, *Shigella flexneri*, and *S. aureus* (Beiranvand et al., 2017; **Figures 1**, **3**), whereas Malaysian plants-endophytic *B. subtilis* possessed antibacterial activity against *S. aureus*, methicillin resistant *S. aureus*, and *P. aeruginosa* (Fikri et al., 2018; **Figure 2B**). The African *Combretum molle*-endophytic *Bacillus* and *Lysinibacillus* species exhibited antibacterial activity against *B. cereus*, *E. coli*, *P. aeruginosa*, and *S. aureus* (Diale et al., 2018; **Supplementary Table S1**).

Kennedia nigriscans-endophytic *Streptomyces* sp. strain NRRL 30562 produces munumbicins A, B, C, and D, active against growth of *B. anthracis*, *E. faecalis*, vancomycin-resistant *E. faecalis*, multiple-drug-resistant (MDR) *M. tuberculosis*, *S. pneumoniae*, *S. aureus*, and methicillin-resistant *S. aureus*; furthermore, munumbicins have been shown to be more effective than chloroquine to kill the malaria-causing agent *P. falciparum* (Castillo et al., 2002, 2006; Christina et al., 2013; **Figure 3A**). Similarly, kakadumycins have antibacterial and antimalarial activities comparable with those of munumbicins (Waring and Wakelin, 1974; Katagiri et al., 1975; Castillo et al., 2003; **Figure 3A** and **Supplementary Table S1**).

Streptomyces sp. strain SUK06, isolated from the Malasian medicinal plant *Thottea grandiflora*, commonly used as an alternative mean to heal wounds and treat skin infections and fever, produces secondary antimicrobial metabolites against *B. cereus*, *B. subtilis*, *Plesiomonas shigelloides*, *P. aeruginosa*, and methicillin-resistant *S. aureus* (Ghadin et al., 2008). Similarly, metabolites and cell wall-degrading enzymes from *Panax ginseng*- and *Plectranthus tenuiflorus*-endophytic *Bacillus* sp., *Micrococcus* sp., and *P. polymyxa*, were reported to possess antibacterial activity against *E. coli*, *K. pneumoniae*, *P. mirabilis*, *Salmonella enterica* subsp. *enterica* serovar Typhi, *S. aureus*, and *S. agalactiae* (El-Deeb et al., 2013; **Figure 2** and **Supplementary Table S1**).

Secondary metabolites spinosyn A and D, are produced by the soil actinomycete *Saccharopolyspora spinosa*, highly effective against lepidopteran and dipteran pests, among others, which commercial product named spinosad, has been commercialized for ∼250 countries and adopted in integrated pest management programs worldwide. Furthermore, there are reports of endophytic *Saccharopolyspora* species, although their potential as bioinsecticide has not yet been elucidated (Qin et al., 2011).

There are many medical and agricultural applications for the *Azadirachta indica* (known as neem) produced compounds (**Figure 1**). *Macrococcus caseolyticus* (ALS-1), a member of the Firmicutes, has been reported to produce free radical scavenging compounds. This strain was isolated from *Aloe vera* in an effort to cultivate bacterial endophytes that could be related to this plant curative and therapeutic uses (Akinsanya et al., 2015). In fact, 80% of the *A. vera* bacterial endophytes produced 1,1-diphenyl-2-picrylhydrazyl, showing over 75% scavenging properties. The *Raphanus sativus* (young radish) leaf and root endophytic *B. subtilis*, *Sphingobacterium siyangensis*, and *P. polymyxa* were shown to inhibit the growth of *B. cereus*, *E. coli*, *P. aeruginosa*, and *S. aureus*, in addition to *Salmonella*, *Shigella*, and *Listeria* species (Liu et al., 2010). *Zingiber officinale* roots-endophytic *Streptomyces* sp. was shown to possess antimicrobial activity against *B. cereus*, *B. subtilis*, and *S. aureus* (Taechowisan et al., 2013; **Figure 3B** and **Supplementary Table S1**).

GRAM-POSITIVE ENDOPHYTES AGAINST PLANT PATHOGENS

Endophytes found in grapevine (*Vitis vinifera*) may represent one interesting example of a widely studied crop system. Metatranscriptoma analysis of vineyards prokaryotic microbiome confirmed that two out of three main bacterial phyla detected (Actinobacteria and Firmicutes) belonged to the Gram-positive group, thus reflecting bacterial metabolic assessments to become symbionts (either epiphytes or endophytes) and be distributed along plant tissues. This study demonstrated that the abundance and richness balance between beneficial microorganisms was critical for phytopathogens biocontrol and grapevine management, where the microbiota stability relied on environmental physicochemical conditions; being the soil type, geography and climate crucial factors to favor this crop. Moreover, detection of a specific strain reflected its ability to be established under the host microclimatic conditions, where *Bacillus* spp. were widely spread in flowers, leaves and grapes (Alaimo et al., 2018). In addition, Haidar et al. (2016) found that *B. pumilus* conferred systemic resistance against this pathogen, after studying the antagonistic bacteria modes of action for the phytopathogen *Phaeomoniella chlamydospora* biocontrol in grapevine.

The biological control of plant phytopathogens by endophytes was reported in late 50s where, a *Micromonospora* isolate from tomato showed antagonistic activity against *Fusarium oxysporum* f.sp. *lycopersici* (Manikprabhu and Li, 2016). As previously stated, the most abundant Gram-positive bacterial endophytes found within diverse environments are *Bacillus* and *Streptomyces* species (Reinhold-Hurek and Hurek, 2011; Frank et al., 2017), both exhibiting secondary metabolites showing antimicrobial activity also against plant pathogens. In fact, there have been proposed *Bacillus* spp. endophytes for crop management (Aloo et al., 2018; **Figure 2**). Similarly, *Streptomyces* spp. endophytes are widely reported as phytopathogens biocontrol agents (**Figures 1**, **3**). For example, *K. nigriscans*-endophytic *Streptomyces* sp. strain NRRL 30562 were recently reported to produce antibiotics as munumbicins A, B, C, and D, active against plant pathogenic bacteria and fungi (Castillo et al., 2002). Leguminose plants-endophytic

Streptomyces caeruleatus was reported to be effective against the soybean pathogen *X. campestris* pv. *glycine* (Mingma et al., 2014; **Figure 3B**); whereas metabolites from *A. indica*- and *Nothofagus* spp.-endophytic actinomyctes inhibited the plant pathogenic fungi *Mycosphaerella fijiensis*, *Sclerotinia sclerotiorum*, and *Rhizoctonia solani*, and *Pythium* and *Phytophthora* species (Castillo et al., 2007; Verma et al., 2009; **Figures 1**, **3B**). Indeed, Castillo et al. (2007) reported *Streptomyces* spp. endophytic of *Nothofagus* spp. in southern Patagonia, where the same strain characterized as *Streptomyces seoulensis* (based on molecular sequenciation and biological activity) was isolated from two different plants (strains coded C2 and C4, respectively); their antifungal activity was then proposed to elucidate the native plants survival mechanisms against plant pathogens within that area (**Figures 1**, **3B**). Similarly, metabolites produced by roots' endophytic actinomycetes previously described (Matsumoto and Takahashi, 2017), inhibited *Kocuria rhizophila* strain KB-212, *Mucor racemosus* strain KF-223, and *Xanthomonas campestris* pv. *oryzae* strain KB-88 growth (**Figure 1** and **Supplementary Table S2**).

Zea mays seeds-endophytic *B. amyloliquefaciens* and *B. subtilis* were observed to inhibit *F. moniliforme* fungus growth by producing lipopeptides (Gond et al., 2015); similarly, *Bruguiera gymnorrhiza* (L.) Lam-endophytic *B. amyloliquefaciens* was shown to be antagonistic to various bacterial (*Ralstonia solanacearum*, *P. syringae*, and *X. campestris*), and fungal (*Colletotrichum musae*, *F. oxysporum*, *Phytophthora capsici*, and *R. solani*) pathogens of plants and to be effective in the biocontrol of *Capsicum* bacterial wilt in pot and field trials (Hu et al., 2010).

Oryza sativa-endophytic *B. cereus* and *B. mojavensis* were observed to exhibit antimicrobial activity against the fungal rice pathogens *F. fujikuroi*, *F. proliferum*, *F. verticillioides*, *Magnaporthe grisea*, and *M. salvinii* (Etesami and Alikhani, 2017; **Supplementary Table S2**).

Young radish-endophytic *B. subtilis*, *Brachybacterium*, and *P. polymyxa* were reported to possess antifungal activity against *F. oxysporum*, *Pythium ultimum*, *Phytophthora capsici*, and *R. solani* (Seo et al., 2010). An antifungal protein from the wheat-endophytic *B. subtilis* strain EDR4 inhibited *B. cinerea*, *F. graminearum*, *F. oxysporum* f.sp. *vasinfectum*, *G. graminis* var. *tritici*, *Macrophoma kuwatsukai*, and *R. cerealis* growth (Liu et al., 2010; **Supplementary Table S2**).

METABOLITES FROM GRAM-POSITIVE ENDOPHYTES AS PLANT GROWTH-PROMOTERS

Endophytic bacteria use to protect crops from microbial diseases is relevant, for their potential to promote host growth and antimicrobial activity (Safiyazov et al., 1995; Berg et al., 2005). Plant growth promotion (PGP) and, in most cases, abiotic stress tolerance and disease protection properties induction, are associated with endophytic bacteria potential to produce different compounds. Plants acclimate to environmental stresses by altering their physiology to be able to overcome stress factors such as dehydration, mechanical injury, nutrient deficiency, high solar radiation, or biotic/abiotic factors. It has been observed that plant inoculation with endophytic bacteria leads to accumulation of "protective" compounds, such as proline, carbohydrates, and antioxidants, in addition to antibiotics and fungal cell-wall lytic enzymes, which can inhibit growth of plant pathogens (Brader et al., 2014) or prime plant response to pathogens by induced systemic resistance (ISR) mechanisms (Pieterse et al., 2014).

Bacterial endophytes PGP potential is explained through several proposed mechanisms. Several of which help to increase accessibility to nutrients, e.g., nitrogen and phosphorus or metals, or produce metabolites that could regulate plant growth, development and defense responses, such as the well-known phytohormones abscisic acid, auxins, brassinosteroids, cytokinins, ethylene, gibberellins, jasmonates, and strigolactones (Reinhold-Hurek and Hurek, 2011; Brader et al., 2014; Santoyo et al., 2016; Shahzad et al., 2016; **Figures 1–3**).

Some examples of Gram-positive PGP bacterial endophytes are *B. pumilus* strain E2S2, whose treatment increased roots and shoots length and fresh and dry biomass, as compared with untreated sorghum plants, and helped to augment cadmium uptake (Luo et al., 2012). *B. amyloliquefaciens* strain NBRI-SN13 (SN13) isolated from an alkaline soil of Banthara, Lucknow, India, showed several PGP attributes and to induce solubilization of tricalcium phosphate more efficiently, when inoculated as endophyte (Nautiyal et al., 2013; **Figure 2A**). Plants treated with *B. atrophaeus* strain EY6, *B. sphaericus* GC subgroup B EY30, *B. subtilis* strain EY2, *S. kloosii* strain EY37 and *K. erythromyxa* strain EY43 as endophytes, have been shown to increase strawberry fruit growth and yield (Karlidag et al., 2011; **Figure 2**).

Interestingly, *C. botulinum* strain 2301 has been shown to have a significant PGP effect on clover in field experiments (Zeiller et al., 2015); whereas *Exiguobacterium acetylicum* 1P strain MTCC 8707, a cold tolerant bacterial strain from the Uttarakhand Himalayas, promotes wheat seedlings growth (Selvakumar et al., 2010; **Supplementary Table S3**).

Brevibacillus brevis strain SVC(II)14 exerted beneficial PGP on cotton crop (Nehra et al., 2016). *Bacillus* spp. strains CPMO6 and BM17, actinobacteria isolates ACT01 and ACT07, and lactic acid bacteria strain BL06 induce phosphate solubilization more efficiently when present as endophytes in citrus (Giassi et al., 2016).

In recent years, it has also been demonstrated that the entomopathogenic bacteria *B. thuringiensis* can have PGP attributes. Armada et al. (2016) tested an autochthonous isolate in interaction with native arbuscular mycorrhizal fungi (single or mixture) and found stimulating plant growth, nutrition and drought tolerance responses.

Siderophores production by endophytes improves plant growth by binding to available iron, competing for this element with phytopathogens and protecting the host plant from their infection (**Figures 2**, **3**; Sabaté et al., 2018). *B. subtilis* strain B26 has been shown to induce drought tolerance in *Brachypodium distachyon* grass. This was correlated to augmentation of starch, fructose, glucose and total soluble carbohydrates content. However, increase of raffinose-related

family carbohydrates (well-known stress response metabolites) was not observed in control and treated plants (Gagné-Bourque et al., 2015). A proline accumulation stimulating effect by endophytic strains of the actinobacteria *Arthrobacter* sp. and the Firmicutes *Bacillus* spp. were reported in pepper (*Capsicum annuum* L., Solanales: Solanaceae) plants *in vitro*, where their synthesis was related to osmotic stress responses (Sziderics et al., 2007). In addition, plants inoculated with bacterial endophytes, could tolerate abiotic stresses by increasing enzymatic activity. *B. cereus* strain CSR-B-1, *B. marisflavi* strain CSR-G-4, *B. pumilus* strain CSR-B-2, *B. saffensis* strain CSR-G-5, *B. subtilis* strain CSR-G-1, and *B. thuringiensis* strain CSRB-3, induced increment of superoxide dismutase, phenylalanine lyase, catalase, and peroxidase enzymes activity in gladiolus plants under sodium high concentration conditions (Damodaran et al., 2014; **Supplementary Table S3**).

Tolerance to low temperatures and growth promotion by endophytic activity has been reported as well. Verma et al. (2015), found *Bacillus* and *Bacillus* derived genera as wheat (*Triticum aestivum*) endophytes from the northern hills zone of India, among others. Phosphate and potassium are major essential macronutrients, but soluble phosphate and potassium concentrations in soil for plant intake are usually very low. Plants need zinc at low concentration since it is toxic at high concentration, thus zinc solubilization by endophytes dosifies the plant intake amount in response to plant and microbial nutritional requests. The most efficient phosphate solubilizing Gram-positive bacteria (PSB) belong to the genera *Bacillus*. Besides, it has been reported that *B. amyloliquefasciens*, *B. megaterium*, and *Bacillus* sp., exhibit phosphorus, potassium, and zinc solubilization (**Figure 2**; Verma et al., 2015).

ANTICANCER ACTIVITY OF GRAM-POSITIVE ENDOPHYTIC BACTERIA

Cancer prevails as one of the leading causes of death worldwide, in spite of therapy advances (Global Cancer Observatory [GLOBOCAN], 2008; Chen et al., 2013). Conventional chemotherapy and radiotherapy have important disadvantages including drug resistance and serious side effects, which has prompted the search for new antitumor agents with high therapeutic efficacy and marginal or null detrimental effects.

Many endophytic actinomycete compounds were isolated and have found application not only as antimicrobial agents but also as cytotoxic agents against tumor cells (**Figures 1**, **3**). Some members of the Gram-positive bacteria group have been recently found as endophytes in different plant species (Eljounaidi et al., 2016). Endophyte extracts have demonstrated to be a better choice versus chemotherapy agents due to their antitumor activity efficacy and lower side-effects, since they are less toxic to normal cells and more effective against several drug resistant microorganisms. As a consequence, the natural endophyte-derived metabolites have attracted peculiar attention with the purpose of being human cancer-chemopreventive compounds and anticancer chemotherapeutic drugs (Cardoso-Filho, 2018; **Figures 3B,C**). Endophytic Gram-positive bacterial natural products have emerged as one of the most reliable alternative treatment sources (Gutierrez et al., 2012; Chen et al., 2013), including antitumor agents such as anthracyclines, anthraquinones, aureolic acids, β-glucans, carzinophilin, coumarins, enediynes, flavonoids, glycopeptides, macrotetrolides, mitomycins, naphthoquinones, polysaccharides, and quinoxalines (Waring and Wakelin, 1974; Igarashi et al., 2007; Taechowisan et al., 2007; Chen et al., 2013; Cardoso-Filho, 2018; **Figures 3B,C** and **Supplementary Table S4**).

Actinomycetes, including the genera *Actinomyces*, *Actinoplanes*, *Amycolatopsis*, *Micromonospora*, *Saccharopolyspora*, and *Streptomyces* are recognized as producers of bioactive metabolites with not only antimicrobial, but also antitumor potential (Renu et al., 2008; Kekuda et al., 2010; **Figure 3C** and **Supplementary Table S4**). In this concern, *Ophiopogon japonicus*-endophytic *B. amyloliquefaciens* sp. exopolysaccharides were reported to possess antitumor activity against the human gastric carcinoma cell lines MC-4 and SGC-7901 (Chen et al., 2013; **Figure 2A**). Furthermore, *Maytenus hookeri*-maytansine-producing endophytic *Streptomyces* sp. strain Is9131 inhibited human SGC7901 gastric, HL60 leukemia, BEL7402 liver, and A-549 lung tumor cell lines growth (Lu and Shen, 2003; Zhao et al., 2005; **Figure 3B** and **Supplementary Table S4**). *Alnus glutinosa*-endophytic *Streptomyces* alnumycin was reported to inhibit the growth of K562 human leukemia cells (Bieber et al., 1998; **Figure 3C**), whereas *Ricinus communis*-endophytic salaceyins-producing *Streptomyces laceyi* strain MS53 was observed to be cytotoxic against the human breast cancer cell line SKBR3 (Kim et al., 2006; **Figure 3B**). In addition, herbaceous and arbor plants-pterocidin-producing endophytic *Streptomyces hygroscopicus* strain TP-A0451 was reported to inhibit human cancer cell lines NCI-H522, OVCAR-3, SF539, and LOX-IMVI growth (Igarashi et al., 2006; Qin et al., 2011), and *Z. officinale* 4-arylcoumarins-producing-endophytic *Streptomyces aureofaciens* strain CMUAc130 was shown to be cytotoxic against murine Lewis lung carcinoma (Taechowisan et al., 2007; Qin et al., 2011; **Figure 3C** and **Supplementary Table S4**).

Many plant growth promoter compounds have shown cytotoxicity against tumor cells. Nodules of *Lupinus angustifolius*-endophytic anthraquinones-producing *Micromonospora* sp. actinomycete significantly inhibited invasion of murine colon 26-L5 carcinoma cells (Igarashi et al., 2007; Qin et al., 2011). Recently, Taechowisan et al. (2017) reported anticancer activity of *Boesenbergia rotunda*-endophytic biphenyls-producing *Streptomyces* sp. strain BO-07 against human HepG2 and Huh7 liver, and HeLa cervical tumor cell lines (**Figure 3B**). Furthermore, *Cinnamomum cassia*-endophytic *Streptomyces cavourensis* strain YBQ59 was shown to inhibit human lung adenocarcinoma EGFR-TKI-resistant cells A549 and H1299 growth (Vu et al., 2018; **Figure 3C** and **Supplementary Table S4**).

CONCLUSION AND PERSPECTIVES

Since the first reports of the industrial potential use of secondary metabolites produced by endophytes, there is more evidence that endophytic Gram-positive bacteria are one of the most important sources of novel compounds that have proven potential for either agriculture, medical and/or pharmaceutical application, thanks to their PGP, antimicrobial and anticancer activities. Indeed, endophytic Gram-positive bacteria help plants to better survive under biotic and abiotic stress conditions. It is not random that many endophytic Gram-positive have been isolated from medicinal plants from all over the world. In fact, many reports of endophytic Gram-positive bacteria showing such activities have been isolated from mangrove and under extreme environment conditions growing plants, like crops from salty soils and crops and trees from cold areas, where phylogenic analysis of native strains demonstrate they contain genes to produce different metabolites that, all together, are helping the host plant, not just to survive, but also to improve the plant adaptation to these extreme environmental conditions. In general, *Bacillus* class endophytes have been reported to produce aromatic compounds, lipopeptides, plant hormones, polysaccharides, and several enzymes, thus representing higher potential in agriculture for PGP and crop management strategies (Villarreal-Delgado et al., 2018). Similarly, Actinobacteria class endophytes are being found to produce antimicrobial- and antitumor-like activity metabolites, thus representing high potential for agriculture, medical, and veterinary application. In this minireview, we presented sources and specific isolated strains information, with the aim to provide current research highlights and perspectives for their future applications. These fascinating microorganisms are diverse and greatly adaptable to extreme stress conditions, making them excellent novel metabolites sources, whose application would be important for environmentally friendly and sustainable food and drugs production.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

FUNDING

This work was supported by Consejo Nacional de Ciencia y Tecnología, Grant No. 5109 to PTG and by Laboratorio de Inmunología y Virología del Departamento de Microbiología e Inmunologia (LIV-DEMI) de la FCB-UANL.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00463/full#supplementary-material

REFERENCES

Aitken, G. (2004). *A New Approach to Conservation: The Importance of the Individual through Wildlife Rehabilitation*. London: Routledge.

Akinsanya, M. A., Goh, J. K., Lim, S. P., and Ting, A. S. Y. (2015). Diversity, antimicrobial and antioxidant activities of culturable bacterial endophyte communities in Aloe vera. *FEMS Microbiol. Lett.* 362:fnv184. doi: 10.1093/femsle/fnv184

Alaimo, S., Marceca, G. P., Giugno, R., Ferro, A., and Pulvirenti, A. (2018). Current knowledge and computational techniques for grapevine meta-omics analysis. *Front. Plant Sci.* 8:2241. doi: 10.3389/fpls.2017.02241

Aloo, B. N., Makumba, B. A., and Mbega, E. R. (2018). The potential of bacilli rhizobacteria for sustainable crop production and environmental sustainability. *Microbiol. Res.* 219, 26–39. doi: 10.1016/j.micres.2018.10.011

Armada, E., Probanza, A., Roldán, A., and Azcón, R. (2016). Native plant growth promoting bacteria *Bacillus thuringiensis* and mixed or individual mycorrhizal species improved drought tolerance and oxidative metabolism in *Lavandula dentata* plants. *J. Plant Physiol.* 192, 1–12. doi: 10.1016/j.jplph.2015.11.007

Baldan, E., Nigris, S., Populin, F., Zottini, M., Squartini, A., and Baldan, B. (2014). Identification of culturable bacterial endophyte community isolated from tissues of Vitis vinifera "Glera". *Plant Biosys.* 148, 508–516. doi: 10.1080/11263504.2014.916364

Beck, H. C., Hansen, A. M., and Lauritsen, F. R. (2003). Novel pyrazine metabolites found in polymyxin biosynthesis by *Paenibacillus polymyxa*. *FEMS Microbiol. Lett.* 220, 67–73. doi: 10.1016/S0378-1097(03)00054-5

Beiranvand, M., Amin, M., Hashemi-Shahraki, A., Romani, B., Yaghoubi, S., and Sadeghi, P. (2017). Antimicrobial activity of endophytic bacterial populations isolated from medical plants of Iran. *Iran. J. Microbiol.* 9, 11–18.

Berg, G., Krechel, A., Ditz, M., Sikora, R. A., Ulrich, A., and Hallmann, J. (2005). Endophytic and ectophytic potato-associated bacterial communities differ in structure and antagonistic function against plant pathogenic fungi. *FEMS Microbiol. Ecol.* 51, 215–229. doi: 10.1016/j.femsec.2004.08.006

Bieber, B., Nüske, J., Ritzau, M., and Gräfe, U. (1998). Alnumycin a new naphthoquinone antibiotic produced by an endophytic *Streptomyces* sp. *J. Antibiot.* 51, 381–382. doi: 10.7164/antibiotics.51.381

Brader, G., Compant, S., Mitter, B., Trognitz, F., and Sessitsch, A. (2014). Metabolic potential of endophytic bacteria. *Curr. Opin. Biotechnol.* 27, 30–37. doi: 10.1016/j.copbio.2013.09.012

Cai, X. C., Liu, C. H., Wang, B. T., and Xue, Y. R. (2017). Genomic and metabolic traits endow bacillus velezensis CC09 with a potential biocontrol agent in control of wheat powdery mildew disease. *Microbiol. Res.* 196, 89–94. doi: 10.1016/j.micres.2016.12.007

Cankar, K., Kraigher, H., Ravnikar, M., and Rupnik, M. (2005). Bacterial endophytes from seeds of Norway spruce (*Picea abies* L. Karst). *FEMS Microbiol. Lett.* 244, 341–345. doi: 10.1016/j.femsle.2005.02.008

Cardoso-Filho, J. A. (2018). "Endophytic microbes as a novel source for producing anticancer compounds as multidrug resistance modulators," in *Anticancer Plants: Natural Products and Biotechnological Implements*, eds M. Akhtar and M. Swamy (Singapore: Springer), 343–381.

Castillo, U., Harper, J. K., Strobel, G. A., Sears, J., Alesi, K., Ford, E., et al. (2003). Kakadumycins, novel antibiotics from Streptomyces sp. NRRL 30566, an endophyte of *Grevillea pteridifolia*. *FEMS Microbiol. Lett.* 224, 183–190. doi: 10.1016/S0378-1097(03)00426-9

Castillo, U. F., Browne, L., Strobel, G. A., Hess, W. M., Ezra, S., Pacheco, G., et al. (2007). Biologically active endophytic streptomycetes from *Nothofagus* spp. and other plants in Patagonia. *Microb. Ecol.* 53, 12–19. doi: 10.1007/s00248-006-9129-6

Castillo, U. F., Strobel, G. A., Ford, E. J., Hess, W. M., Porter, H., Jensen, J. B., et al. (2002). Munumbicins, wide-spectrum antibiotics produced by *Streptomyces* NRRL30562, endophytic on *Kennedia nigriscans*. *Microbiology* 148, 2675–2685. doi: 10.1099/00221287-148-9-2675

Castillo, U. F., Strobel, G. A., Mullenberg, K., Condron, M. M., Teplow, D. B., Folgiano, V., et al. (2006). Munumbicins E-4 and E-5: novel broad-spectrum antibiotics from *Streptomyces* NRRL 3052. *FEMS Microbiol. Lett.* 255, 296–300. doi: 10.1111/j.1574-6968.2005.00080.x

Castro, R. A., Quecine, M. C., Lacava, P. T., Batista, B. D., Luvizotto, D. M., Marcon, J., et al. (2014). Isolation and enzyme bioprospection of endophytic bacteria associated with plants of Brazilian mangrove ecosystem. *SpringerPlus* 3:382. doi: 10.1186/2193-1801-3-382

Cazorla, F. M., Romero, D., Pérez-García, A., Lugtenberg, B. J. J., Vicente, A. D., and Bloemberg, G. (2007). Isolation and characterization of antagonistic Bacillus subtilis strains from the avocado rhizoplane displaying biocontrol activity. *J. Appl. Microbiol.* 103, 1950–1959. doi: 10.1111/j.1365-2672.2007.03433.x

Chandrakar, S., and Gupta, A. K. (2017). Antibiotic potential of endophytic actinomycetes of medicinal herbs against human pathogenic bacteria. *Proc. Natl. Acad. Sci. India Biol. Sci.* 87, 905–915.

Chandrakar, S., and Gupta, A. K. (2018). Actinomycin-producing endophytic *Streptomyces parvulus* associated with root of aloe vera and optimization of conditions for antibiotic production. *Probiotics Antimicrob. Prot.* doi: 10.1007/s12602-018-9451-6 [Epub ahead of print].

Chatterjee, P., Samaddar, S., Niinemets, Ü., and Sa, T. M. (2018). Brevibacterium linens RS16 confers salt tolerance to Oryza sativa genotypes by regulating antioxidant defense and H+ ATPase activity. *Microbiol. Res.* 215, 89–101. doi: 10.1016/j.micres.2018.06.007

Chen, Y. T., Yuan, Q., Shan, L. T., Lin, M. A., Cheng, D. Q., and Li, C. Y. (2013). Antitumor activity of bacterial exopolysaccharides from the endophyte Bacillus amyloliquefaciens sp. Isolated from *Ophiopogon japonicus*. *Oncol. Lett.* 5, 1787–1792. doi: 10.3892/ol.2013.1284

Chinedum, I. E. (2005). Microbial resistance to antibiotics. *Afr. J. Biotechnol.* 10, 1606–1611.

Christina, A., Christapher, V., and Bhore, S. J. (2013). Endophytic bacteria as a source of novel antibiotics: an overview. *Pharmacog. Rev.* 7, 11–16. doi: 10.4103/0973-7847.112833

Compant, S., Mitter, B., Colli-Mull, J. G., Gangl, H., and Sessitsch, A. (2011). Endophytes of grapevine flowers, berries, and seeds: identification of cultivable bacteria, comparison with other plant parts, and visualization of niches of colonization. *Microb. Ecol.* 62, 188–197. doi: 10.1007/s00248-011-9883-y

Damodaran, T., Rai, R. B., Jha, S. K., Kannan, R., Pandey, B. K., Sah, V., et al. (2014). Rhizosphere and endophytic bacteria for induction of salt tolerance in gladiolus grown in sodic soils. *J. Plant Interact.* 9, 577–584. doi: 10.1080/17429145.2013.873958

de Lima Procópio, R. E., da Silva, I. R., Martins, M. K., de Azevedo, J. L., and de Araújo, J. M. (2012). Antibiotics produced by streptomyces. *Braz. J. Infect. Dis.* 16, 466–471. doi: 10.1016/j.bjid.2012.08.014

de Melo-Pereira, G. V., Magalhães, K. T., Lorenzetii, E. R., Souza, T. P., and Schwan, R. F. (2012). A multiphasic approach for the identification of endophytic bacterial in strawberry fruit and their potential for plant growth promotion. *Microb. Ecol.* 63, 405–417. doi: 10.1007/s00248-011-9919-3

Demain, A. L. (1981). Industrial microbiology. *Science* 214, 987–994. doi: 10.1126/science.6946560

Deng, Y., Zhu, Y., Wang, P., Zhu, L., Zheng, J., Li, R., et al. (2011). Complete genome sequence of Bacillus subtilis BSn5, an endophytic bacterium of *Amorphophallus konjac* with antimicrobial activity for the plant pathogen *Erwinia carotovora* subsp. Carotovora. *J. Bacteriol.* 193, 2070–2071. doi: 10.1128/JB.00129-11

Diale, M. O., Ubomba-Jaswa, E., and Serepa-Dlamini, M. H. (2018). The antibacterial activity of bacterial endophytes isolated from Combretum molle. *Afr. J. Biotechnol.* 17, 255–262. doi: 10.5897/AJB2017.16349

Ding, L., Maier, A., Fiebig, H. H., Lin, W. H., and Hertweck, C. (2011). A family of multicyclic indolosesquiterpenes from a bacterial endophyte. *Organ. Biomol. Chem.* 9, 4029–4031. doi: 10.1039/c1ob05283g

Ding, L., Münch, J., Goerls, H., Maier, A., Fiebig, H. H., Lin, W. H., et al. (2010). Xiamycin, a pentacyclic indolosesquiterpene with selective anti-HIV activity from a bacterial mangrove endophyte. *Bioorg. Med. Chem. Lett.* 20, 6685–6687. doi: 10.1016/j.bmcl.2010.09.010

El-Deeb, B., Fayez, K., and Gherbawy, Y. (2013). Isolation and characterization of endophytic bacteria from Plectranthus tenuiflorus medicinal plant in Saudi Arabia desert and their antimicrobial activities. *J. Plant Interact.* 8, 56–64. doi: 10.1080/17429145.2012.680077

Eljounaidi, K., Lee, S. K., and Bae, H. (2016). Bacterial endophytes as potential biocontrol agents of vascular wilt diseases – Review and future prospects. *Biol. Control* 103, 62–68. doi: 10.1016/j.biocontrol.2016.07.013

Etesami, H., and Alikhani, H. A. (2017). Evaluation of gram-positive rhizosphere and endophytic bacteria for biological control of fungal rice (*Oryzia sativa* L.) pathogens. *Eur. J. Plant Pathol.* 147, 7–14. doi: 10.1007/s10658-016-0981-z

Ezra, D., Castillo, U. F., Strobel, G. A., Hess, W. M., Porter, H., Jensen, J. B., et al. (2004). Coronamycins, peptide antibiotics produced by a verticillate Streptomyces sp. *(MSU-*2110) endophytic on Monstera sp. *Microbiology* 150, 785–793. doi: 10.1099/mic.0.26645-0

Ferrigo, D., Causin, R., and Raiola, A. (2017). Effect of potential biocontrol agents selected among grapevine endophytes and commercial products on crown gall disease. *BioControl* 62, 821–833. doi: 10.1007/s10526-017-9847-3

Fikri, A. S. I., Rahman, I. A., Nor, N. S. M., and Hamzah, A. (2018). "Isolation and identification of local bacteria endophyte and screening of its antimicrobial property against pathogenic bacteria and fungi," in *Proceedings of AIP Conference*, Vol. 1940, (Melville, NY: AIP Publishing), 020072. doi: 10.1063/1.5027987

Frank, A., Saldierna-Guzmán, J., and Shay, J. (2017). Transmission of bacterial endophytes. *Microorganisms* 5:E70. doi: 10.3390/microorganisms5040070

Gagné-Bourque, F., Mayer, B. B. F., Charron, J.-B. B., Vali, H., Bertrand, A., and Jabaji, S. (2015). Accelerated growth rate and increased drought stress resilience of the model grass *Brachypodium distachyon* colonized by Bacillus subtilis B26. *PLoS One* 10:e130456. doi: 10.1371/journal.pone.0130456

Gamez, R., Cardinale, M., Montes, M., Ramirez, S., Schnell, S., and Rodriguez, F. (2019). Screening, plant growth promotion and root colonization pattern of two rhizobacteria (*Pseudomonas* fluorescens Ps006 and *Bacillus amyloliquefaciens* Bs006) on banana cv. Williams (*Musa acuminata Colla*). *Microbiol. Res.* 220, 12–20. doi: 10.1016/j.micres.2018.11.006

Gayathri, P., and Muralikrishnan, V. (2013). Isolation and characterization of endophytic actinomycetes from mangrove plant for antimicrobial activity. *Int. J. Curr. Microbiol. Appl. Sci.* 2, 78–89.

Ghadin, N., Zin, N. M., Sabaratnam, V., Badya, N., Basri, D. F., Lian, H. H., et al. (2008). Isolation and characterization of a novel endophytic Streptomyces SUK 06 with antimicrobial activity from Malaysian plant. *Asian J. Plant Sci.* 7, 189–194. doi: 10.3923/ajps.2008.189.194

Giassi, V., Kiritani, C., and Kupper, K. C. (2016). Bacteria as growth-promoting agents for citrus rootstocks. *Microbiol. Res.* 190, 46–54. doi: 10.1016/j.micres.2015.12.006

Global Cancer Observatory [GLOBOCAN] (2008). *Section of Cancer Information WHO*. Available at: http://www.who.int/mediacentre/factsheets/fs297/en/ [accessed August 05, 2018].

Gond, S. K., Bergen, M. S., and Torres, M. S. (2015). Endophytic bacillus spp. Produce antifungal lipopeptides and induce host defence gene expression in maize. *Microbiol. Res.* 172, 79–87. doi: 10.1016/j.micres.2014.11.004

Gowtham, H. G., Murali, M., Singh, S. B., Lakshmeesha, T. R., Murthy, K. N., Amruthesh, K. N., et al. (2018). Plant growth promoting rhizobacteria *Bacillus amyloliquefaciens* improves plant growth and induces resistance in chilli against anthracnose disease. *Biol. Control* 126, 209–217. doi: 10.1016/j.biocontrol.2018.05.022

Grady, E. N., MacDonald, J., Liu, L., Richman, A., and Yuan, Z. C. (2016). Current knowledge and perspectives of paenibacillus: a review. . *Microb. Cell Fact* 15:203. doi: 10.1186/s12934-016-0603-7

Guan, S. H., Sattler, I., Lin, W. H., Guo, D. A., and Grabley, S. (2005). p-Aminoacetophenonic acids produced by a mangrove endophyte: *Streptomyces griseus* subspecies. *J. Nat. Prod.* 68, 1198–1200. doi: 10.1021/np0500777

Gutierrez, R. M., Gonzalez, A. M., and Ramirez, A. M. (2012). Compounds derived from endophytes: a review of phytochemistry and pharmacology. *Curr. Med. Chem.* 19, 2992–3030. doi: 10.2174/092986712800672111

Haidar, R., Roudet, J., Bonnard, O., Dufour, M. C., Corio-Costet, M. F., Fert, M., et al. (2016). Screening and modes of action of antagonistic bacteria to control the fungal pathogen *Phaeomoniella chlamydospora* involved in grapevine

trunk diseases. *Microbiol. Res.* 192, 172–184. doi: 10.1016/j.micres.2016.07.003

Housman, G., Byler, S., Heerboth, S., Lapinska, K., Longacre, M., Snyder, N., et al. (2014). Drug resistance in cancer: an overview. *Cancers* 6, 1769–1792. doi: 10.3390/cancers6031769

Hu, H. Q., Li, X. S., and He, H. (2010). Characterization of an antimicrobial material from a newly isolated *Bacillus amyloliquefaciens* from mangrove for biocontrol of Capsicum bacterial wilt. *Biol. Control* 54, 359–365. doi: 10.1016/j.biocontrol.2010.06.015

Igarashi, Y., Miura, S. S., Fujita, T., and Furumai, T. (2006). Pterocidin, a cytotoxic compound from the endophytic *Streptomyces hygroscopicus*. *J. Antibiot.* 59, 193–195. doi: 10.1038/ja.2006.28

Igarashi, Y., Trujillo, M. E., Martínez-Molina, E., Yanase, S., Miyanaga, S., Obata, T., et al. (2007). Antitumor anthraquinones from an endophytic actinomycete *Micromonospora lupine* sp, nov. *Bioorg. Med. Chem. Lett.* 17, 3702–3705. doi: 10.1016/j.bmcl.2007.04.039

Inahashi, Y., Iwatsuki, M., Ishiyama, A., Namatame, M., Nishihara-Tsukashima, A., Matsumoto, A., et al. (2011a). Spoxazomicins A-C, novel antitrypanosomal alkaloids produced by an endophytic actinomycete. Streptosporangium oxazolinicum K07-0460T. *J. Antibiot.* 64, 303–307. doi: 10.1038/ja.2011.16

Inahashi, Y., Matsumoto, A., Mura, S., and Takahashi, Y. (2011b). Streptosporangium oxazolinicum sp. nov., a novel endophytic actinomycete producing new antitrypanosomal antibiotics, spoxazomicins. *J. Antibiot.* 64, 297–302. doi: 10.1038/ja.2011.18

Indrawati, I., Rossiana, N., and Hidayat, T. R. (2018). "Antibacterial activity of bacterial endophytes from Kupa plant (Syzygium polycephalum Miq. (Merr & Perry) against pathogenic bacteria," in *Proceedings of the IOP Conference Series: Earth and Environmental Science*, Vol. 166, (Bristol: IOP Publishing), 012013. doi: 10.1088/1755-1315/166/1/012013

Jakubiec-Krzesniak, K., Rajnisz-Mateusiak, A., Guspiel, A., Ziemska, J., and Solecka, J. (2018). Secondary metabolites of actinomycetes and their antibacterial, antifungal and antiviral properties. *Pol. J. Microbiol.* 67, 259–272. doi: 10.21307/pjm-2018-048

Jiang, Z., Tuo, L., Huang, D., Osterman, I. A., Tyurin, A., Liu, S., et al. (2018). Diversity, novelty and antimicrobial activity of endophytic actinobacteria from mangrove plants in Beilun estuary national nature reserve of Guangxi. China. *Front. Microbiol.* 9:868. doi: 10.3389/fmicb.2018.00868

Johnston-Monje, D., and Raizada, M. N. (2011). Conservation and diversity of seed associated endophytes in Zea across boundaries of evolution, ethnography and ecology. *PLoS One* 6:e20396. doi: 10.1371/journal.pone.0020396

Karlidag, H., Esitken, A., Yildirim, E., Donmez, M. F., and Turan, M. (2011). Effects of plant growth promoting bacteria on yield, growth, leaf water content, membrane permeability, and ionic composition of strawberry under saline conditions. *J. Plant Nutr.* 34, 34–45. doi: 10.1080/01904167.2011.531357

Katagiri, K., Yoshida, T., and Sato, K. (1975). "Quinoxaline antibiotics," in *Antibiotics Mechanism of Action of Antimicrobial and Antitumour Agents*, eds J. W. Corcoran and F. E. Hahn (Heidelberg: Springer-Verlag), 234–251.

Kekuda, T. P., Shobha, K. S., and Onkarappa, R. (2010). Fascinating diversity and potent biological activities of actinomycete metabolites. *J. Pharm. Res.* 3, 250–256.

Khianngam, S., Techakriengkrai, T., Raksasiri, B. V., Kanjanamaneesathian, M., and Tanasupawat, S. (2013). "Isolation and screening of endophytic bacteria for hydrolytic enzymes from plant in mangrove forest at Pranburi, Prachuap Khiri Khan, Thailand," in *Proceedings of the 5th International Symposium on Plant Protection and Plant Health in Europe: Endophytes for plant protection: the state of the art*, (Berlin: Humboldt University Berlin).

Kim, N., Shin, J. C., Kim, W., Hwang, B. Y., Kim, B. S., Hong, Y. S., et al. (2006). Cytotoxic 6-alkylsalicylic acids from the endophytic Streptomyces laceyi. *J. Antibiot.* 59, 797–800. doi: 10.1038/ja.2006.105

Koskimäki, J. J., Pirttilä, A. M., Ihantola, E. L., Halonen, O., and Frank, A. C. (2015). The intracellular scots pine shoot symbiont *Methylobacterium extorquens* DSM13060 aggregates around the host nucleus and encodes eukaryote-like proteins. *mBio* 6:e39–15. doi: 10.1128/mBio.00039-15

Li, H., Soares, M. A., Torres, M. S., and Bergen, M. (2015). Endophytic bacterium, *Bacillus amyloliquefaciens*, enhances ornamental hosta resistance to diseases and insect pests. *J. Plant Interact.* 10, 224–229. doi: 10.1080/17429145.2015.1056261

Liu, B., Huang, L., Buchenauer, H., and Kang, Z. (2010). Isolation and partial characterization of an antifungal protein from the endophytic Bacillus subtilis strain EDR4. *Pesticide Biochem. Physiol.* 98, 305–311. doi: 10.1016/j.pestbp.2010.07.001

Liu, Z., Wang, Y., Jia, X., and Lu, W. (2018). Isolation of secondary metabolites with antimicrobial activities from *Bacillus amyloliquefaciens* LWYZ003. *Trans. Tianjin Univ.* 25, 38–44. doi: 10.1016/j.jgar.2017.08.008

Lodewyckx, C., Vangronsveld, J., Porteous, F., Moore, E. R. B., Taghavi, S., Mezgeay, M., et al. (2002). Endophytic bacteria and their potential applications. *Crit. Rev. Plant Sci.* 21, 583–606. doi: 10.1080/0735-260291044377

Lòpez-Fernàndez, S., Compant, S., Vrhovsek, U., Bianchedi, P. L., Sessitsch, A., Pertot, I., et al. (2016). Grapevine colonization by endophytic bacteria shifts secondary metabolism and suggests activation of defense pathways. *Plant Soil* 405, 155–175. doi: 10.1007/s11104-015-2631-1

Lu, C., and Shen, Y. (2003). A new macrolide antibiotic with antitumor activity produced by Streptomyces sp. CS, a commensal microbe of *Maytenus hookeri*. *J. Antibiot.* 56, 415–418. doi: 10.7164/antibiotics.56.415

Luo, S., Xu, T., Chen, L., Chen, J., Rao, C., Xiao, X., et al. (2012). Endophyte-assisted promotion of biomass production and metal-uptake of energy crop sweet sorghum by plant-growth-promoting endophyte Bacillus sp. SLS18. *Appl. Microbiol. Biotechnol.* 93, 1745–1753. doi: 10.1007/s00253-011-3483-0

Manikprabhu, D., and Li, W. J. (2016). "Antimicrobial agents from actinomycetes Chemistry and Applications," in *Antimicrobials Synthetic and Natural Compounds*, eds D. Dhanasekaran, N. Thajuddin, and A. Panneerselvam (Milton Park: Taylor & Francis Group), 99–115.

Matsumoto, A., and Takahashi, Y. (2017). Endophytic actinomycetes: promising source of novel bioactive compounds. *J. Antibiot.* 70, 514–519. doi: 10.1038/ja.2017.20

Mingma, R., Pathom-aree, W., Trakulnaleamsai, S., Thamchaipenet, A., and Duangmal, K. (2014). Isolation of rhizospheric and roots endophytic actinomycetes from leguminosae plant and their activities to inhibit soybean pathogen. Xanthomonas campestris pv. glycine. *World J. Microbiol. Biotechnol.* 30, 271–280. doi: 10.1007/s11274-013-1451-9

Mohamad, O. A., Li, L., Ma, J. B., Hatab, S., Xu, L., Guo, J. W., et al. (2018). Evaluation of the antimicrobial activity of endophytic bacterial populations from Chinese traditional medicinal plant licorice and characterization of the bioactive secondary metabolites produced by *Bacillus atrophaeus* against *Verticillium dahliae*. *Front. Microbiol.* 9:924. doi: 10.3389/fmicb.2018.00924

Nautiyal, C. S., Srivastava, S., Chauhan, P. S., Seem, K., Mishra, A., and Sopory, S. K. (2013). Plant growth-promoting bacteria *Bacillus amyloliquefaciens* NBRISN13 modulates gene expression profile of leaf and rhizosphere community in rice during salt stress. *Plant Physiol. Biochem.* 66, 1–9. doi: 10.1016/j.plaphy.2013.01.020

Nehra, V., Saharan, B. S., and Choudhary, M. (2016). Evaluation of Brevibacillus brevis as a potential plant growth promoting rhizobacteria for cotton (Gossypium hirsutum) crop. *SpringerPlus* 5:948. doi: 10.1186/s40064-016-2584-8

Ongena, M., and Jacques, P. (2008). Bacillus lipopeptides: versatile weapons for plant disease biocontrol. *Trends Microbiol.* 16, 115–125. doi: 10.1016/j.tim.2007.12.009

Pandey, P. K., Singh, S., Singh, M. C., Singh, A. K., Yadav, S. K., Pandey, A. K., et al. (2018). "Diversity, ecology, and conservation of fungal and bacterial endophytes," in *Microbial Resource Conservation*, eds S. K. Sharma and A. Varma (New York, NY: Springer International Publishing), 393–430.

Paungfoo-Lonhienne, C., Rentsch, D., Robatzek, S., Webb, R. I., Sagulenko, E., Näsholm, T., et al. (2010). Turning the table: plants consume microbes as a source of nutrients. *PLoS One* 5:e11915. doi: 10.1371/journal.pone.0011915

Peña-Yam, L. P., Ruíz-Sánchez, E., Barboza-Corona, J. E., and Reyes-Ramírez, A. (2016). Isolation of mexican bacillus species and their effects in promoting growth of chili pepper (*Capsicum annuum* L. cv Jalapeño). *Indian J. Microbiol.* 56, 375–378. doi: 10.1007/s12088-016-0582-8

Pieterse, C. M. J., Zamioudis, C., Berendsen, R. L., Weller, D. M., Van Wees, S. C. M., and Bakker, P. A. H. M. (2014). Induced systemic resistance by beneficial microbes. *Ann. Rev. Phytopathol.* 52, 347–375. doi: 10.1146/annurev-phyto-082712-102340

Posada, L. F., Álvarez, J. C., Romero-Tabarez, M., de-Bashan, L., and Villegas-Escobar, V. (2018). Enhanced molecular visualization of root colonization and growth promotion by bacillus subtilis EA-CB0575 in different growth systems. *Microbiol. Res.* 217, 69–80. doi: 10.1016/j.micres.2018.08.017

Pratiwi, R. H., Hanafi, M., Artanti, N., and Pratiwi, R. D. (2018). Bioactivity of antibacterial compounds produced by endophytic actinomycetes from *Neesia altissima*. *J. Trop. Life Sci.* 8, 37–42. doi: 10.11594/jtls.08.01.07

Qin, S., Xing, K., Jiang, J. H., Xu, L. H., and Li, W. J. (2011). Biodiversity, bioactive natural products and biotechnological potential of plant-associated endophytic actinobacteria. *Appl. Microbiol. Biotechnol.* 89, 457–473. doi: 10.1007/s00253-010-2923-6

Raaijmakers, J. M., de Bruijn, I., Nybroe, O., and Ongena, M. (2010). Natural functions of lipopeptides from Bacillus and *Pseudomonas*: more than surfactants and antibiotics. *FEMS Microbiol. Rev.* 34, 1037–1062. doi: 10.1111/j.1574-6976.2010.00221.x

Rado, R., Andrianarisoa, B., Ravelomanantsoa, S., Rakotoarimanga, N., Rahetlah, V., Fienena, F. R., et al. (2015). Biocontrol of potato wilt by selective rhizospheric and endophytic bacteria associated with potato plant. *Afr. J. Food Agric. Nutr. Dev.* 15, 9762–9776.

Ramamoorthy, S., Gnanakan, A., Lakshmana, S. S., Meivelu, M., and Jeganathan, A. (2018). Structural characterization and anticancer activity of extracellular polysaccharides from ascidian symbiotic bacterium *Bacillus thuringiensis*. *Carbohydrate Pol.* 190, 113–120. doi: 10.1016/j.carbpol.2018.02.047

Reinhold-Hurek, B., and Hurek, T. (2011). Living inside plants: bacterial endophytes. *Curr. Opin. Plant Biol.* 14, 435–443. doi: 10.1016/j.pbi.2011.04.004

Renu, S., Monisha, K., and Lal, R. (2008). Bioactive compounds from marine actinomycetes. *Indian J. Microb.* 48, 410–431. doi: 10.1007/s12088-008-0052-z

Rosenblueth, M., and Martínez-Romero, E. (2006). Bacterial endophytes and their interactions with hosts. *Mol. Plant Microbe Interact.* 19, 827–837. doi: 10.1094/MPMI-19-0827

Ryan, R. P., Germaine, K., Franks, A., Ryan, D. J., and Dowling, D. N. (2008). Bacterial endophytes: recent developments and applications. *FEMS Microbiol. Lett.* 278, 1–9. doi: 10.1111/j.1574-6968.2007.00918.x

Sabaté, D. C., Brandan, C. P., Petroselli, G., Erra-Balsells, R., and Audisio, M. C. (2018). Biocontrol of Sclerotinia sclerotiorum (Lib.) de Bary on common bean by native lipopeptide-producer Bacillus strains. *Microbiol. Res.* 211, 21–30. doi: 10.1016/j.micres.2018.04.003

Safiyazov, J. S., Mannanov, R. N., and Sattarova, R. K. (1995). The use of bacterial antagonists for the control of cotton disease. *Field Crops Res.* 43, 51–54. doi: 10.1016/0378-4290(95)00038-R

Santoyo, G., Moreno-Hagelsieb, G., del Orozco-Mosqueda, M. C., and Glick, B. R. (2016). Plant growth-promoting bacterial endophytes. *Microbiol. Res.* 183, 92–99. doi: 10.1016/j.micres.2015.11.008

Schulz, B., and Boyle, C. (2006). What are endophytes? *Soil Biol.* 9, 1–14. doi: 10.1007/3-540-33526-9_1

Selvakumar, G., Kundu, S., Joshi, P., Nazim, S., Gupta, A. D., and Gupta, H. S. (2010). Growth promotion of wheat seedlings by exiguobacterium acetylicum 1P (MTCC 8707) a cold tolerant bacterial strain from the uttarakhand himalayas. *Indian J. Microbiol.* 50, 50–56. doi: 10.1007/s12088-009-0024-y

Seo, W. T., Lim, W. J., Kim, E. J., Yun, H. D., Lee, Y. H., and Cho, K. M. (2010). Endophytic bacterial diversity in the young radish and their antimicrobial activity against pathogens. *J. Korean Soc. Appl. Biol. Chem.* 53, 493–503. doi: 10.3839/jksabc.2010.075

Shahzad, R., Waqas, M., Khan, A. L., Asaf, S., Khan, M. A., Kang, S. M., et al. (2016). Seed-borne endophytic *Bacillus amyloliquefaciens* RWL-1 produces gibberellins and regulates endogenous phytohormones of *Oryza sativa*. *Plant Physiol. Biochem.* 106, 236–243. doi: 10.1016/j.plaphy.2016.05.006

Shimizu, M., Nakagawa, Y., Yukio, S. A. T. O., Furumai, T., Igarashi, Y., Onaka, H., et al. (2000). Studies on endophytic actinomycetes (I) Streptomyces sp. Isolated from rhododendron and its antifungal activity. *J. Gen. Plant Pathol.* 66, 360–366. doi: 10.1007/PL00012978

Stein, T. (2005). Bacillus subtilis antibiotics: structures, syntheses and specific functions. *Mol. Microbiol.* 56, 845–857. doi: 10.1111/j.1365-2958.2005.04587.x

Strobel, G. A., and Daisy, B. (2003). Bioprospecting for microbial endophytes and their natural products. *Microbiol. Mol. Biol. Rev.* 67, 491–502. doi: 10.1128/MMBR.67.4.491-502.2003

Sziderics, A. H., Rasche, F., Trognitz, F., Sessitsch, A., and Wilhelm, E. (2007). Bacterial endophytes contribute to abiotic stress adaptation in pepper plants (*Capsicum annuum* L.). *Can. J. Microbiol.* 53, 1195–1202. doi: 10.1139/W07-082

Taechowisan, T., Chaisaeng, S., and Phutdhawong, W. S. (2017). Antibacterial, antioxidant and anticancer activities of biphenyls from Streptomyces sp. BO-07: an endophyte in *Boesenbergia rotunda* (L.) Mansf A. *Food Agric. Immunol.* 28, 1330–1346. doi: 10.1080/09540105.2017.1339669

Taechowisan, T., Chanaphat, S., Ruensamran, W., and Phutdhawong, W. S. (2013). Antibacterial activity of decursin from streptomyces sp. GMT-8; an endophyte in zingiber officinale rosc. *J. Appl. Pharm. Sci.* 3, 74–78.

Taechowisan, T. C., Lu, C. H., Shen, Y. M., and Lumyong, S. (2007). Antitumor activity of 4-arylcoumarins from endophytic *Streptomyces aureofaciens* CMUAc130. *J. Cancer Res. Ther.* 3, 86–91. doi: 10.4103/0973-1482.34685

Tamosiune, I., Baniulis, D., and Stanys, V. (2017). "Role of endophytic bacteria in stress tolerance of agricultural plants: diversity of microorganisms and molecular mechanisms," in *Probiotics in Agroecosystem*, eds V. Kumar, M. Kumar, S. Sharma, and R. Prasad (Singapore: Springer).

Tian, B., Zhang, C., Ye, Y., Wen, J., Wu, Y., Wang, H., et al. (2017). Beneficial traits of bacterial endophytes belonging to the core communities of the tomato root microbiome. *Agric. Ecosys. Environ.* 247, 149–156. doi: 10.1016/j.agee.2017.06.041

Tripathi, V. C., Satish, S., Horam, S., Raj, S., Arockiaraj, J., Pasupuleti, M., et al. (2018). Natural products from polar organisms: structural diversity, bioactivities and potential pharmaceutical applications. *Polar Sci.* 18, 147–166. doi: 10.1016/j.polar.2018.04.006

Tsurumaru, H., Okubo, T., Okazaki, K., Hashimoto, M., Kakizaki, K., Hanzawa, E., et al. (2015). Metagenomic analysis of the bacterial community associated with the taproot of sugar beet. *Microbes Environ.* 30, 63–69. doi: 10.1264/jsme2.ME14109

Verma, P., Yadav, A. N., Khannam, K. S., Panjiar, N., Kumar, S., Saxena, A. K., et al. (2015). Assessment of genetic diversity and plant growth promoting attributes of psychrotolerant bacteria allied with wheat (Triticum aestivum) from the northern hills zone of India. *Ann. Microbiol.* 65, 1885–1899. doi: 10.1007/s13213-014-1027-4

Verma, V. C., Gond, S. K., Kumar, A., Mishra, A., Kharwar, R. N., and Gange, A. C. (2009). Endophytic actinomycetes from Azadirachta indica A. Juss.: isolation, diversity, and anti-microbial activity. *Microb. Ecol.* 57, 749–756. doi: 10.1007/s00248-008-9450-3

Villarreal-Delgado, M. F., Villa-Rodríguez, E. D., Cira-Chávez, L. A., Estrada-Alvarado, M. I, Parra-Cota, F. I., and de los Santos-Villalobos, S. (2018). The genus bacillus as a biological control agent and its implications in the agricultural biosecurity. *Mex. J. Phytopathol.* 36, 95–130.

Vu, H. N. T., Nguyen, D. T., Nguyen, H. Q., Chu, H. H., Chu, S. K., Van Chau, M., et al. (2018). Antimicrobial and cytotoxic properties of bioactive metabolites produced by *Streptomyces cavourensis* YBQ59 isolated from cinnamomum cassia prels in yen bai province of vietnam. *Curr. Microbiol.* 75, 1247–1255. doi: 10.1007/s00284-018-1517-x

Waring, M. J., and Wakelin, L. P. (1974). Echinomycin: abifunctional intercalating antibiotic. *Nature* 252, 653–657. doi: 10.1038/252653a0

Wei, W., Zhou, Y., Chen, F., Yan, X., Lai, Y., Wei, C., et al. (2018). Isolation, diversity, and antimicrobial and immunomodulatory activities of endophytic actinobacteria from tea cultivars Zijuan and Yunkang-10 (Camellia sinensis var. *assamica)*. *Front. Microbiol.* 9:1304. doi: 10.3389/fmicb.2018.01304

Xia, Y., DeBolt, S., Dreyer, J., Scott, D., and Williams, M. A. (2015). Characterization of culturable bacterial endophytes and their capacity to promote plant growth from plants grown using organic or conventional practices. *Front. Plant Sci.* 6:490. doi: 10.3389/fpls.2015.00490

Yuan, J., Wu, Y., Zhao, M., Wen, T., Huang, Q., and Shen, Q. (2018). Effect of phenolic acids from banana root exudates on root colonization and pathogen suppressive properties of Bacillus amyloliquefaciens NJN-6. *Biol. Control* 125, 131–137. doi: 10.1016/j.biocontrol.2018.05.016

Zeiller, M., Rothballer, M., Iwobi, A. N., Böhnel, H., Gessler, F., Hartmann, A., et al. (2015). Systemic colonization of clover (Trifolium repens) by clostridium botulinum strain 2301. *Front. Microbiol.* 6:1207. doi: 10.3389/fmicb.2015.01207

Zhao, K., Li, J., Zhang, X., Chen, Q., Liu, M., Ao, X., et al. (2018). Actinobacteria associated with *Glycyrrhiza inflata* Bat. Are diverse and have plant growth promoting and antimicrobial activity. *Sci. Rep.* 8:13661. doi: 10.1038/s41598-018-32097-8

Zhao, K., Penttinen, P., Guan, T., Xiao, J., Chen, Q., Xu, J., et al. (2011). The diversity and anti-microbial activity of endophytic actinomycetes isolated from medicinal plants in Panxi Plateau. *Chin. Curr. Microbiol.* 62, 182–190. doi: 10.1007/s00284-010-9685-3

Zhao, P. J., Fan, L. M., Li, G. H., Zhu, N., and Shen, Y. M. (2005). Antibacterial and antitumor macrolides from Streptomyces sp. Is9131. *Arch. Pharm. Res.* 28, 1228–1232. doi: 10.1007/BF029782031

Conflict of Interest Statement: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Advantages of publishing in Frontiers

OPEN ACCESS
Articles are free to read for greatest visibility and readership

FAST PUBLICATION
Around 90 days from submission to decision

HIGH QUALITY PEER-REVIEW
Rigorous, collaborative, and constructive peer-review

TRANSPARENT PEER-REVIEW
Editors and reviewers acknowledged by name on published articles

Frontiers
Avenue du Tribunal-Fédéral 34
1005 Lausanne | Switzerland

Visit us: www.frontiersin.org
Contact us: frontiersin.org/about/contact

REPRODUCIBILITY OF RESEARCH
Support open data and methods to enhance research reproducibility

DIGITAL PUBLISHING
Articles designed for optimal readership across devices

FOLLOW US
@frontiersin

IMPACT METRICS
Advanced article metrics track visibility across digital media

EXTENSIVE PROMOTION
Marketing and promotion of impactful research

LOOP RESEARCH NETWORK
Our network increases your article's readership