APPLICATION OF PROTECTIVE CULTURES AND BACTERIOCINS FOR FOOD BIOPRESERVATION

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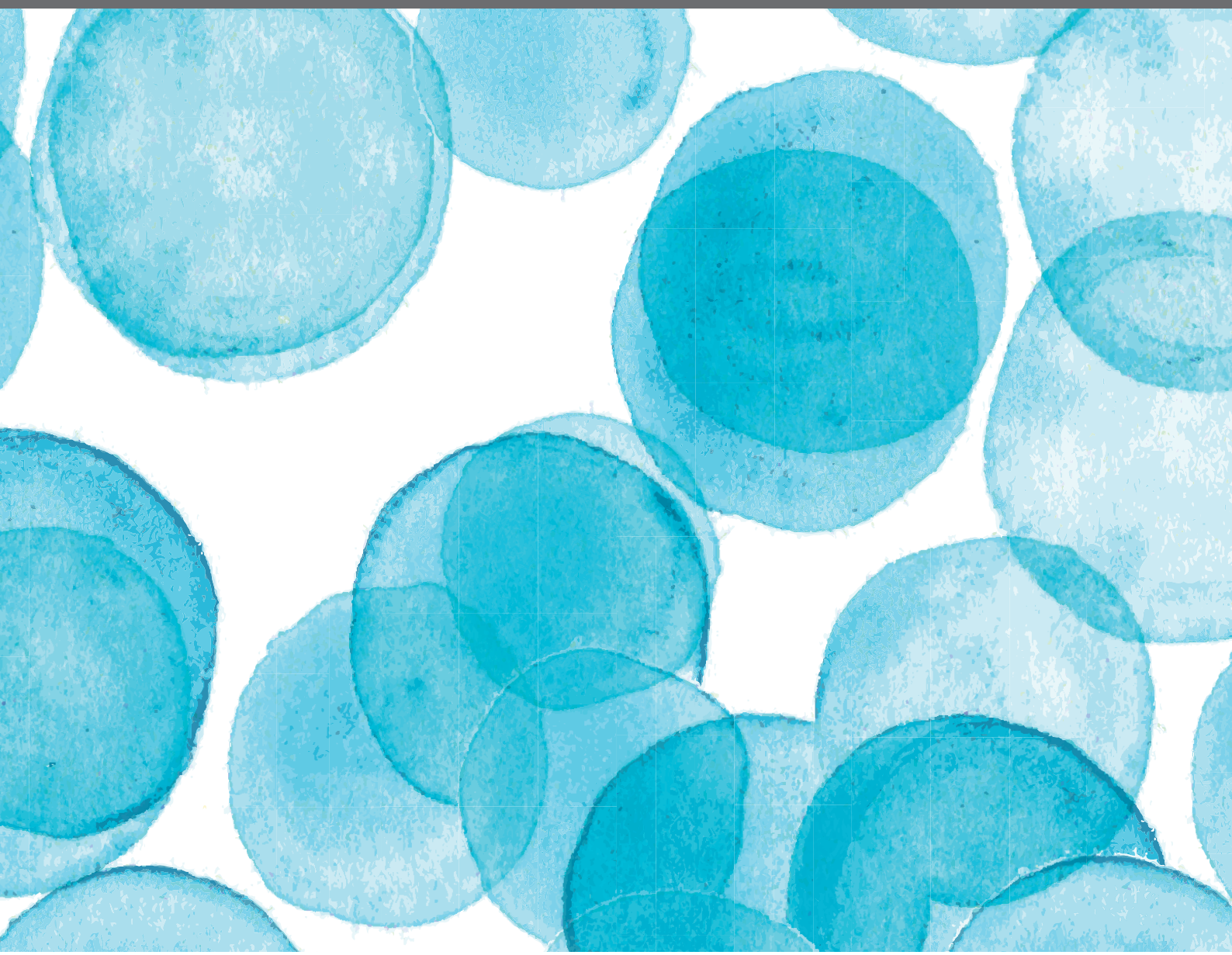

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APPLICATION OF PROTECTIVE CULTURES AND BACTERIOCINS FOR FOOD BIOPRESERVATION

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The use of microorganisms and their metabolites for the preservation of foods began in prehistory. Lactic acid bacteria are generally recognized as safe (GRAS) for this purpose. They produce organic acids, diacetyl, acetoin, hydrogen peroxide, reuterin, reutericyclin and bacteriocins, all of which inhibit foodborne pathogens and spoilage microorganisms. Bacteriocins and the strains that produce them are particularly effective as bio-preservatives in cheese, meat and vegetables. They hold the promise of ensuring the quality and safety of ready-to-eat, extended-shelf-life, fresh-tasting and minimally processed foods without chemical preservatives. This Research Topic provides an overview of bacterial cultures, bacteriocins and other metabolites that have shown promise for use as antimicrobial bio-preservatives in foods in general. Articles describing novel analytical technologies, strategies to reduce or eliminate pathogens in food systems or emerging technologies for the production or use of protective cultures or their bacteriocins are presented.

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EDITORIAL
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Editorial: Application of Protective Cultures and Bacteriocins for Food Biopreservation

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Keywords: protective cultures, bacteriocins, food biopreservation, Lactic acid bacteria, foodborne pathogens, food microbiota

Editorial on the Research Topic

Application of Protective Cultures and Bacteriocins for Food Biopreservation

The use of microorganisms and their metabolites for the preservation of foods began in prehistory. Lactic acid bacteria (LAB) are generally recognized as safe for this purpose. They produce an array of antimicrobial substances such as organic acids, diacetyl, acetoin, hydrogen peroxide, reuterin, reutericyclin, and bacteriocins, all of which inhibit foodborne pathogens and spoilage microorganisms. The efficacy of bacteriocins as well as their producing strains for inhibiting several bacterial pathogens has been shown in different food matrices including cheese, meat and vegetables. Nowadays, Protective cultures and bacteriocins are considered as promising alternative. They hold the promise of ensuring the quality and safety of ready-to-eat, extended-shelf-life, fresh-tasting, and minimally processed foods without chemical preservatives. This editorial provides comprehensive overview of bacterial cultures, bacteriocins, and other metabolites that have shown promise for use as antimicrobial bio-preservatives in foods in general. Articles describing novel analytical technologies, strategies to reduce or eliminate pathogens in food systems or emerging technologies for the production or use of protective cultures or their bacteriocins are presented.

The number of bacteriocins and bacteriocin-producing LAB reported to inhibit foodborne pathogens in dairy products including milk, yogurt, and cheeses has grown steadily since the approval of nisin and pediocin for commercial use in foods. In this Research Topic, Silva et al. review the most recent trends in their use in dairy products and discuss critically the efficacy and the pros and cons of their application.

Elimination of foodborne *Listeria monocytogenes* is the focus of *in vitro* studies by Saraoui et al., who are investigating the antagonistic effect of *Lactococcus piscium* CNCM I-4031 against several strains of the pathogen in co-culture or in HT-29 cell culture. Teixeira et al. are studying the impact of pressure and RTE meat microbiota with or without nisin and rosemary oil on the survival of *Listeria* during storage at refrigerator temperatures. Two articles on a similar subject discuss the role of microbial communities as a potential major influence on bacteriocin efficacy and pathogen survival in RTE foods (Ortega Blázquez et al.; Teixeira et al.).

One of the more recent developments included in this Research Topic is the incorporation of bacteriocins into polymer films and coatings applied directly onto food surfaces and packaging materials, also reviewed by Silva et al. The impact of activated plastic and chitosan films loaded with enterocin AS-48 or divergicin M35 on the dynamics of commensal bacteria and the survival of pathogens in sea bream filets and cold smoked salmon is evaluated in two of the articles included (Benabbou et al.; Ortega Blázquez et al.).

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The broad-spectrum antimicrobial compound known as reuterin (3-hydroxypropionaldehyde, produced by *Lactobacillus reuteri* from glycerol) is used as a preservative on pre-washed and fresh-cut vegetables (lettuce) in a study by Asare et al., also included in this topic.

Of particular interest are the modes of action of bacteriocins, which differ markedly from those of antibiotics. The inhibitory potential and associated mechanisms of LAB strains in co-culture with enterohemorrhagic *E. coli* (EHEC) in a meat-based medium are presented in a recent article (Orihuel et al.). These authors show also that the activity of the bacteriocin produced by *Enterococcus mundtii* CRL35 is due to an inhibitory mechanism that is more robust. A cell-to-cell contact mechanism is described in studies by Orihuel et al.; Saraoui et al., and novel transcriptomic data relating to a mechanism that regulates hydrogen peroxide production by *Lactococcus garvieae* are presented in an article on inhibition of *Staphylococcus aureus* (Delpech et al.). Most bacteriocins interact specifically with bacterial membranes and kill microbial cells by causing leakage of cellular contents. Wayah and Philip have described pentocin MQ1, a novel bacteriocin produced by a strain of *Lactobacillus pentosus* CS2 and having a bactericidal mode of action mediated by pore formation. The application of pentocin MQ1 to preserving fresh bananas is also presented. Zhang et al. have examined the role of oxidative stress in the efficacy of the yeast *Cryptococcus laurentii* as an inhibitor of the fruit-spoiling fungus *Penicillium expansum*. The authors report an oxidative stress response that appears to activate an antioxidant enzyme system (catalase and superoxide dismutase). Using glutathione to enhance ROS scavenging, they show that *C. laurentii* viability under conditions of oxidative stress can be improved.

The discovery and full characterization of novel bacteriocins with little or no homology to known bacteriocins involves lengthy research. Yi et al. have combined complete genome and peptidome data in an effective approach to discovering novel bacteriocins. They describe the bacteriocin BM1122 from *L. crustorum* MN047 as proof of concept.

An important focus in this Research Topic is the use of emerging technologies for the large-scale production of bacteriocins, a major obstacle to the commercial viability of using these natural preservatives in food or pharma applications. Mesa-Pereira et al. review the different expression systems for bacteriocin production using *E. coli* as host and identify the most important features to guarantee successful production of a range of bacteriocins. Bédard and Biron have summarized synthetic approaches that have been developed for the large-scale production of class IIa bacteriocins and S-linked glycopeptides obtained from LAB. They also highlight the recent advances in peptide engineering and synthesis that have made the chemical synthesis of bacteriocins more cost-competitive.

In summary, the articles included in this Research Topic provide an overview of the current and future trends in food industrial applications of bacteriocins and bacteriocin-producing bacteria.

AUTHOR CONTRIBUTIONS

RH wrote the manuscript with support from IF and AC. All authors read and approved the final manuscript.

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REVIEW
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Application of Bacteriocins and Protective Cultures in Dairy Food Preservation

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In the last years, consumers are becoming increasingly aware of the human health risk posed by the use of chemical preservatives in foods. In contrast, the increasing demand by the dairy industry to extend shelf-life and prevent spoilage of dairy products has appeal for new preservatives and new methods of conservation. Bacteriocins are antimicrobial peptides, which can be considered as safe since they can be easily degraded by proteolytic enzymes of the mammalian gastrointestinal tract. Also, most bacteriocin producers belong to lactic acid bacteria (LAB), a group that occurs naturally in foods and have a long history of safe use in dairy industry. Since they pose no health risk concerns, bacteriocins, either purified or excreted by bacteriocin producing strains, are a great alternative to the use of chemical preservatives in dairy products. Bacteriocins can be applied to dairy foods on a purified/crude form or as a bacteriocin-producing LAB as a part of fermentation process or as adjuvant culture. A number of applications of bacteriocins and bacteriocin-producing LAB have been reported to successful control pathogens in milk, yogurt, and cheeses. One of the more recent trends consists in the incorporation of bacteriocins, directly as purified or semi-purified form or in incorporation of bacteriocin-producing LAB into bioactive films and coatings, applied directly onto the food surfaces and packaging. This review is focused on recent developments and applications of bacteriocins and bacteriocin-producing LAB for reducing the microbiological spoilage and improve safety of dairy products.

Keywords: bacteriocins, lactic acid bacteria, dairy products, biopreservation, edible coatings

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INTRODUCTION

Bacteriocins are generally defined as peptides or proteins ribosomal synthesized by bacteria that inhibit or kill other related or unrelated microorganisms (Leroy and De Vuyst, 2004; Cotter et al., 2005). Bacteriocins may have a narrow spectrum, by inhibiting bacteria taxonomically close, or a broad spectrum, by inhibiting a wide variety of bacteria (Cotter et al., 2005; Mills et al., 2011).

For the past years, bacteriocins have attracted considerable interest for their use as safe food preservatives, as they are easily digested by the human gastrointestinal tract (Mills et al., 2011). The use of bacteriocins as natural food preservatives fulfills consumer demands for high quality and safe foods without the use of chemical preservatives. However, the application of bacteriocins as food additives can be limited for various reasons, such as effectiveness of pathogen elimination or its high price (Chen and Hoover, 2003). Nevertheless, research interest in bacteriocins has continued over the past years, as investigators continuing to search for new and more effective bacteriocins to address both biologic and economic concerns.

The application of bacteriocins for biopreservation of foods usually includes the following approaches: inoculation of food with the bacteriocin-producer strain; addition of purified or semi-purified bacteriocin as food additive; and use of a product previously fermented with a bacteriocin-producing strain as an ingredient in food processing (Chen and Hoover, 2003).

An increasingly number of bacteriocins have been isolated and identified from Gram-positive and Gram-negative microorganisms. As a result, databases have been created to compile the information that can be used for the automated screening of bacteriocin gene clusters (Blin et al., 2013; van Heel et al., 2013).

Classification of Bacteriocins

For the past years, several classifications of bacteriocins have been proposed taking into consideration the first classification proposed by Klaenhammer (1993). Recently, in order to classify novel bacteriocins, Alvarez-Sieiro et al. (2016) proposed an adjusted classification scheme based on the biosynthesis mechanism and biological activity in accordance with other proposals (Arnison et al., 2013). They propose three major classes: Class I – small post-translationally modified peptides; Class II – unmodified bacteriocins; and Class III – larger peptides (>10 kDa, thermo-labile), being each one subdivided into subclasses.

Mode of Action

Bacteriocins have distinct mechanisms of action and can be divided into those that promote a bactericidal effect, with or without cell lysis, or bacteriostatic, inhibiting cell growth (da Silva Sabo et al., 2014). Most of the bacteriocins produced from LAB, in particular those inhibiting Gram-positive bacteria, exert their antibacterial effect by targeting the cell envelope-associated mechanisms (Cotter et al., 2013). Several lantibiotics and some class II bacteriocins target Lipid II, an intermediate in the peptidoglycan biosynthesis machinery within the bacterial cell envelope and, by this way they inhibit peptidoglycan synthesis (Breukink and de Kruijff, 2006). Other bacteriocins use Lipid II as a docking molecule to facilitate pore formation resulting in variation of the cytoplasm membrane potential and ultimately, cell death (Machaidze and Seelig, 2003). Nisin, the most studied lantibiotic, is capable of both mechanisms (Cotter et al., 2005). Some bacteriocins damage or kill target cells by binding to the cell envelope-associated mannose phosphotransferase system (Man-PTS) and subsequent formation of pores in the cell membrane (Cotter et al., 2013). Other bacteriocins can kill their target cells by inhibition of gene expression (Parks et al., 2007; Vincent and Morero, 2009) and protein production (Metlitskaya et al., 2006).

BACTERIOCINS PRODUCED BY LAB

Although there are several microorganisms that produce bacteriocins, those produced by the lactic acid bacteria (LAB) are of particular interest to the dairy industry (Egan et al., 2016). LAB have long been used in a variety of food fermentations by converting lactose to lactic acid, as well as producing additional antimicrobial molecules such as other organic acids, diacetyl, acetoin, hydrogen peroxide, antifungal peptides, and bacteriocins (Egan et al., 2016). As a result of their extensive use in traditional fermented products, most of the LAB are Generally Regarded as Safe (GRAS), granted by the American Food and Drug Agency (FDA). The European Food Safety Authority (EFSA) also granted the Qualified Presumption of Safety (QPS) status to most of the LAB genera, such as *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Pediococcus*, and some *Streptococcus* (EFSA, 2007). Nevertheless, species of the genus *Enterococcus* and some *Streptococcus* are pathogenic, thus, they do not have GRAS status and were not proposed for QPS status (EFSA, 2007).

Lactic acid bacteria bacteriocins are often active across a range of pH values, resistant to high temperatures and active against a range of food pathogenic and spoilage bacteria (Ahmad et al., 2017). In addition, LAB bacteriocins are sensitive to digestive proteases such as pancreatin complex, trypsin and chymotrypsin, and thus do not impact negatively on the gut microbiota (Egan et al., 2016).

APPLICATION OF PURIFIED/SEMI-PURIFIED BACTERIOCINS TO DAIRY PRODUCTS

Bacteriocins have been used in the biopreservation of various foods, either alone or in combination with other methods of preservation, known as hurdle technology (De Vuyst and Leroy, 2007; Perez et al., 2014). Although results obtained from culture media may show that bacteriocins inhibit target organisms, the application of bacteriocins into foods must be tested to confirm their effectiveness. Many studies showed the potential of applying bacteriocins or bacteriocin-producing strains into foods, such as meat, dairy products, fish, alcoholic beverages, salads, and fermented vegetables (O'Sullivan et al., 2003; Ramu et al., 2015). To date, only nisin (Nisaplin, Danisco) and pediocin PA1 (Microgard^{TM}, ALTA 2431, Quest) have been commercialized as food preservatives (Simha et al., 2012). Although other LAB bacteriocins offered promising perspectives to be used as biopreservatives, as for instance the enterocin AS-48 (Sánchez-Hidalgo et al., 2011) or lacticin 3147 (Suda et al., 2012), no other bacteriocin has been proposed for industrial application. The screening of bacteriocins to be applied to foods requires the fulfillment of some important criteria. Producing strains should be food grade (GRAS or QPS), exhibit a broad spectrum of inhibition, present high specific activity, have no associated health risks, present beneficial effects (e.g., improve safety, quality, and flavor of foods), display heat and pH stability, and optimal solubility and stability for a particular food (Cotter et al., 2005; Leroy and De Vuyst, 2010). Various authors have reported that inactivation of several foodborne pathogens by bacteriocins may differ greatly depending on the food matrix used (Muñoz et al., 2007). Therefore, the effectiveness of different bacteriocins to foodborne pathogens must be tested in all food systems.

Recent applications of bacteriocins into dairy foods to control food-borne pathogens included the inoculation of food with LAB that produce bacteriocins (**Table 1**) or the addition of purified or semi-purified bacteriocins directly to food (**Table 2**). Applying bacteriocin-producing LAB strains as antibacterial starter cultures and protective cultures may confer an advantage over the use of semi-purified/purified bacteriocins. In most cases, bacteriocins are adsorbed into food matrices and are easily degraded, which results in a loss of antibacterial activity. Therefore, an alternative method is the incorporation of bacteriocins into food packaging films/coatings, which improve their activity and stability in complex food systems (Salgado et al., 2015). Many researchers reported the potential of using immobilized bacteriocins in the development of antimicrobial packaging films to control food-borne pathogenic bacteria, such as *L. monocytogenes* (Sánchez-González et al., 2013; Ibarguren et al., 2015; Narsaiah et al., 2015). In fermented foods, the contamination with *L. monocytogenes* is a major concern. Several listeriosis outbreaks have been linked with the consumption of dairy products, in particular soft cheeses, causing a problem to dairy industry and public health authorities (Melo et al., 2015). Despite most dairy products, in particular cheeses, being made from pasteurized milk, contamination with *Listeria* still occurs. Cheeses are ready to use products and are usually conserved at refrigeration temperatures that allow the survival and growth of psychrotrophic bacteria, such as *L. monocytogenes*. Therefore, contamination can occur in later stages of dairy product processing (Carpentier and Cerf, 2011; Melo et al., 2015). Consequently, *Listeria* active bacteriocins emerge as an ideal solution for preventing the growth of this pathogen after cooking or packaging (Cotter et al., 2005). Bacteriocins can also be used to control adventitious non-starter flora, such as non-starter lactic acid bacteria (NSLAB) in cheese and wine, and in this way, contribute to the quality of the final product (Oumer et al., 2001; O'Sullivan et al., 2003). In addition, bacteriocins can also be used to enhance food fermentation, accelerating cheese ripening, and even improve its flavor (Oumer et al., 2001).

Over 230 bacteriocins produced by LAB have been isolated and reported, but only half of them were identified at the protein or DNA levels (Alvarez-Sieiro et al., 2016). Moreover, a limited number of purified or semi-purified bacteriocins have been tested in food systems, especially in dairy foods.

Nisin

The bacteriocin nisin is classified as a class-Ia bacteriocin or lantibiotic and is the most characterized and commercially important bacteriocin (Ross et al., 2002). Nisin is licensed as a food preservative (E234) and is recognized to be safe by the Joint Food and Agriculture Organization/World Health Organization (FAO/WHO) Expert Committee on Food Additives (FAO and WHO, 2006; Favaro et al., 2015). To date, eight types of nisin variants were discovered and characterized: nisins A, Z, F, and Q produced by *Lactococcus lactis* and nisins U, U2, P, and H produced by some *Streptococcus* strains (O'Connor et al., 2015). The commercially available form of nisin for use as a food preservative is Nisaplin^{TM}, with the active ingredient nisin A (2.5%) and other ingredients such as NaCl and non-fat dry milk (Chen and Hoover, 2003).

Nisin has antimicrobial activity against numerous Gram-positive bacteria, including LAB, pathogens such as *Listeria* and *Staphylococcus*, and the spore forming bacteria, *Bacillus* and *Clostridium* (Chen and Hoover, 2003). One of the earliest applications of nisin was to prevent late blowing in cheese caused by gas-producing *Clostridium* spp. (Galvez et al., 2008).

Nisin has been widely applied in cheese and pasteurized cheese spreads to replace nitrate for preventing the outgrowth of clostridia spores (Abee et al., 1995; Chen and Hoover, 2003). Nisin has also been shown to be effective in the control of different pathogens such as *L. monocytogenes* and *Staphylococcus aureus* in dairy products (Sobrino-López and Martín-Belloso, 2008). In a study of Arqués et al. (2011), nisin was shown to reduce *L. monocytogenes* and *S. aureus* in milk stored at refrigeration temperatures. Several studies also tested the addition of nisin to diverse cheese types (cottage cheese, cheddar, and ricotta-type cheeses) and show an effective reduction of *L. monocytogenes* growth, although limited to 1–3 log cycles (Chen and Hoover, 2003). Several reports indicate that nisin A is not very active against *L. monocytogenes*, but its anti-listerial effect is enhanced by the reduction of pH and addition of NaCl (Chen and Hoover, 2003; Khan and Oh, 2016).

In Minas Frescal cheese, *S. aureus* counts were reduced by approximately 1.5 log cycles after addition of nisin (Felicio et al., 2015). In processed cheese, *Bacillus cereus* and *Bacillus subtilis* were also inhibited by nisin (Sobrino-López and Martín-Belloso, 2008). The use of nisin as an antimicrobial treatment extended the shelf-life of a Greek soft acid–curd cheese (Galotyri) by >21 days (Kykkidou et al., 2007). Incorporation of nisin (at a level of 2.5 mg l^{-1}) has also shown to increase shelf-life of Ricotta-type cheese by inhibiting the growth of *L. monocytogenes* for >8 weeks (Davies et al., 1997). Moreover, a high level of retention of nisin was observed over the 10-week storage period, with only 10–32% of nisin loss. Ferreira and Lund (1996) also studied the effect of nisin on survival of the most resistant strains of *L. monocytogenes* in cottage cheese and showed a reduction to approximately 3 log cycles in 3 days. This bacteriocin was also tested in other pasteurized dairy products, such as chilled desserts, flavored milk, clotted cream, and canned evaporated milks, and was shown to reduce post process contaminating bacteria such as *L. monocytogenes* (Galvez et al., 2008). The efficacy of a combination of nisin and bovicin HC5 against *L. monocytogenes* and *S. aureus* in fresh cheese was studied by Pimentel-Filho et al. (2014). They observed a reduction of *L. monocytogenes* to undetected levels after 9 days of storage at 4°C, although the combination of bacteriocins did not prevent the growth of *S. aureus*.

Several studies showed that the antimicrobial activity of nisin is affected by several factors including pH, temperature, composition, structure, and natural microbiota of food (Zhou et al., 2014). Proteolysis in cheese-making process may also affect the activity of nisin and limit its antimicrobial efficacy. Nevertheless, some authors described a limited loss in nisin activity (10–32%) in Ricotta cheese after 10 weeks of storage

TABLE 1 | Applications of bacteriocin-producing LAB in dairy products (2000–present).

Bacteriocin-producing strain	Application technique	Product	Features	Reference
Staphylococcus equorum WS 2733	Cheese was inoculated with bacteriocin-producing strains	Soft cheese	*L. monocytogenes* growth was completely inhibited at low contamination levels and was reduced by 1-2 log at higher contamination levels.	Carnio et al., 2000
Lactococcus lactis IFPL359 (transconjugant)	Starter culture	Goat's milk cheese	Acceleration of cheese ripening.	Martinez-Cuesta et al., 2001
Lactococcus lactis CNRZ481	Adjunct culture	Cheddar cheese	Greater enzyme release through lacticin 481-induced lysis and reduction in the growth of NSLAB by 5 log.	O'Sullivan et al., 2003
Enterococcus faecalis A-48-32	Adjunct culture	Skimmed milk and non-fat hard cheese	*Bacillus cereus* was reduced in milk to non-detectable levels after 72 h. In cheese, *B. cereus* decreased progressively, reaching 5.6 log after 30 days.	Muñoz et al., 2004
Lactococcus lactis subsp. *lactis* INIA 415	Adjunct culture	Hispánico cheese	Acceleration of cheese ripening.	Avila et al., 2005
Enterococcus faecium M241 and M249	Inoculation with bacteriocin-producing strains	Skimmed milk	Co-culture with *E. faecium* showed a delay in the growth *L. monocytogenes* of 6 h and reduced this pathogen by 2 log.	Cocolin et al., 2007
Lactococcus lactis CL1 and CL2 (transconjugants, Nis^+, Ped^+)	Adjunct to commercial starter culture	Cheese	*L. monocytogenes* was reduced by 2.97 (CL1) and 1.64 log (CL2).	Rodriguez et al., 2005
Enterococcus faecium F58	Inoculation with bacteriocin-producing strain	Jben (traditional fresh cheese)	*L. monocytogenes* was reduced to below detection limits after 7 days of storage at 22°C.	Achemchem et al., 2006
Enterococcus faecalis A-48-32	Inoculation with bacteriocin-producing strain	Skimmed milk and fresh cheese	Reduced *Staphylococcus aureus*, but efficacy was lower in cheese *as S. aureus* remained 1 log lower than control throughout storage.	Muñoz et al., 2007
Lactococcus lactis	Starter culture	Cottage cheese	Reduction of *L. monocytogenes* growth after 2 days, but a re-growth on days 5 and 7.	Dal Bello et al., 2012
Enterococcus mundtii CRL35 and *Enterococcus faecium* ST88Ch	Inoculation with bacteriocin-producing strains	Fresh Minas cheese	*E. mundtii* CRL35 displayed a bacteriostatic effect on *L. monocytogenes* up to 12 days at 8°C. *E. faecium* ST88Ch was less effective to control *L. monocytogenes.*	Pingitore et al., 2012
Lactococcus lactis L3A21M1 and *Enterococcus faecalis*	Inoculation with bacteriocin-producing strains	Fresh cheese	A combination of strains of *E. faecalis* reduced *L. monocytogenes* by 4 to 5 log after 7 days at 4°C. *Enterococcus* spp. were more effective in reducing *L. monocytogenes* in cheese.	Coelho et al., 2014
Enterococcus durans E204	Adjunct culture	Jben	*L. monocytogenes* throughout the storage period and was undetectable after 6–8 days.	Khay et al., 2014
Lactococcus lactis ssp. *lactis CCMM / IAV/ BK1*	Starter culture	Jben	*L. monocytogenes* was reduced to below the detectable level during storage at 7°C from days 1 to 6.	Benkerroum et al., 2002
Lactobacillus sakei subsp. *sakei* 2a	Cheese was inoculated with bacteriocin-producing strain	Cheese spread	*L. monocytogenes* was reduced at 4°C and decreased to below detection limit at 15°C, after 22–28 days	Martinez et al., 2015
Lactobacillus plantarum CCDM 1078	Inoculation with bacteriocin-producing strains	Cheese and quark based spreads	*L. monocytogenes* was reduced by 1 log in both spreads.	Patrovský et al., 2016
Staphylococcus equorum SE3	Inoculation with bacteriocin-producing strains	Cheese model	Inhibition of *L. monocytogenes* growth by 7 log units after 24 h.	Bockelmann et al., 2017
Enterococcus faecium	Cheese was inoculated with bacteriocin-producing strains	Fresh whey cheese	*L. monocytogenes* was reduced by 4 log after 6 days of storage at 4°C and was undetectable by the 9th day.	Aspri et al., 2017
Enterococcus faecium KE82	Adjunct culture to commercial starter culture	Raw and sterile milk	*L. monocytogenes* was reduced by 4 to 5 log after 72 h at 22°C.	Vandera et al., 2017
Lactococcus lactis (nisin producer)	Starter culture	Fresh cheese	*L. monocytogenes* was reduced by 2 log after 7 days of storage at 4°C	Kondrotiene et al., 2018

TABLE 2 | Applications of purified/semi-purified bacteriocins to control food-borne pathogens in dairy products (2000–present).

Bacteriocin, producer	Product	Features	Reference
Nisin Z, *Lactococcus lactis* W8	Skim and whole-fat milk	Reduced *L. monocytogenes* (5 log CFU ml^{-1}) to undetectable levels in both skim and fat milk within 16 h at 8°C.	Mitra et al., 2011
Nisin Z and A and lacticin 481, *Lactococcus lactis*	Cottage cheese	Cell-free supernatant (CFS) from the bacteriocin producing strains show weak ability to reduce *L. monocytogenes*	Dal Bello et al., 2012
Nisin (commercial preparation, Nisaplin®)	Galotyri PDO cheese	Extended the shelf life of fresh Galotyri cheese stored at 4°C by 7 days.	Kykkidou et al., 2007
Nisin A (Nisaplin®)	Milk pudding	Nisin A was effective to control spore-forming bacteria and extend shelf life. Reduced heat treatments to improve the flavor and aroma without compromising food safety.	Oshima et al., 2014
Nisin (Nisaplin®)	Minas Frescal cheese	Nisin (500 IU·mL^{-1}) reduced *S. aureus* in curd and whey, but the effect was minor during cheese storage.	Felicio et al., 2015
Nisin, *Lactococcus lactis* N5764	Cow milk	Combined nisin and phenolic compounds have a bacteriostatic effect on *Staph. aureus* and *L. monocytogenes* growth	Alves et al., 2016
Lacticin 3147, *Lactococcus lactis* subsp. *lactis* DPC3147	Yogurt and cottage cheese	Reduced *L. monocytogenes* to undetectable levels in yogurt within 10 min. In cottage cheese reduced 85% of *L. monocytogenes* contamination after 120 min.	Morgan et al., 2001
Lacticin 481, *Lactococcus lactis* L3A21M1	Model fresh cheese	Application of purified lacticin 481 reduced *L. monocytogenes* by 3 log after 3 to 7 days at 4°C.	Ribeiro et al., 2016
Lactococcin BZ, *L. lactis* spp. *lactis* BZ and enterocin KP, *E. faecalis* KP	Skim, half and full fat UHT milks	Lactococcin BZ reduced *L. monocytogenes* in all milks to undetectable levels at 4°C or 20°C. Enterocin KP antilisterial result was affected by fat content.	Yildirim et al., 2016
Leucocin K7, *Leuconostoc mesenteroides* K7	UHT whole-fat milk	Combined leucocin K7 at 80 AU/ml and glycine at 5 mg/ml completely inhibited the growth of *L. monocytogenes* over 7 days	Shi et al., 2016
Reuterin, *Lactobacillus reuteri* INIA PRO 137	UHT skim milk	Combined reuterin and nisin reduced *L. monocytogenes* and *S. aureus* to undetectable levels after 12 days at refrigeration temperatures (4° and 8°C).	Arqués et al., 2011
Variacin, *Kocuria varians* NCC 1482	Dairy food models	Variacin (1% of fermented ingredient) was inhibitory to the growth of *Bacillus cereus* at 8°C, but this inhibition weakened as storage temperature increased.	O'Mahony et al., 2001
Bovicin HC5, *Streptococcus bovis* HC5, and nisin	Minas Frescal cheese	Combined bovicin at and nisin at 600 AU g^{-1} completely inhibited the growth of *L. monocytogenes* after 9 days of storage at 4°C.	Pimentel-Filho et al., 2014
Gassericins A and T, *Lactobacillus gasseri* LA39 and LA158	Custard cream	The combined use of bacteriocins and glycine inhibited the growth of *B. cereus.*	Arakawa et al., 2009
Aureocin A70, *Staphylococcus aureus* A70	UHT skim milk	A partially purified aureocin preparation (16 AU mL^{-1}) inhibited *L. monocytogenes* by 5.5 log at 4°C after 7 days.	Fagundes et al., 2016
Enterocin CCM 4231, *Enterococcus faecium* CCM 4231	Saint-Paulin cheese	Reduced *L. monocytogenes* by 4 log, but difference decreased to 1 log after 6 weeks of ripening.	Lauková et al., 2001
Enterocin AS-48, *Enterococcus faecalis* A-48-32	Skimmed milk	Combined with sub-lethal heat treatment (65°C for 5 min) reduced *Staphylococcus aureus* below detection limits.	Muñoz et al., 2007
Ent35-MccV (hybrid bacteriocin), *Escherichia coli* BL21	Skim milk	A complete elimination of *E. coli* was observed after 10 h at 37°C and 4 days at 4°C. *L. monocytogenes* was initially reduced but regrowth after 48 h at 37°C. At refrigeration (4°C), *L. monocytogenes* was reduced by 3 log after 10 days.	Acuña et al., 2015
Enterocins, *Enterococcus faecalis* L3B1K3	Model fresh cheese	Semi purified enterocin (536 μg/g) reduced *L. monocytogenes* to undetectable levels in cheese within 6 h.	Ribeiro et al., 2017

(Cleveland et al., 2001). Moreover, nisin activity was not affected by proteases in a study on Emmental cheese (Favaro et al., 2015).

The application of nisin in dairy products is also limited to pH values lower than 7, as nisin greatly loses the activity at higher pH (de Arauz et al., 2009). Other studies also mention several limitations on the use of nisin in dairy products, due to its interaction with fat and other components in the food matrix (Favaro et al., 2015). However, the role of fat in the activity of nisin is not entirely clear, as studies on heat-treated cream show the inhibition of *B. cereus* growth by low concentrations of nisin (Nissen et al., 2001). Additionally, nisin was found to prevent spoilage bacteria and extend shelf-life in high-fat milk-based pudding (Oshima et al., 2014). In contrast, homogenization of milk was show to reduce the anti-listerial effects of nisin, demonstrating that the treatment of foods may play an important role in the efficacy of bacteriocins such as nisin (Bhatti et al., 2004).

The wide spectrum of inhibition associated with nisin includes LAB themselves (Abee et al., 1995). Therefore, in dairy foods which require LAB for fermentation processes, the application of nisin presents a great limitation. An alternative consists in employing other bacteriocins with a highly specific activity range (Abee et al., 1995). As a result, there has been a great interest in the search for new bacteriocins with a widespread range of antibacterial activity, stability in different food environments, tolerance to heat, and resistance to proteolytic enzymes.

Pediocins

Pediocins are a class IIa bacteriocins produced by *Pediococcus* spp. and are commercially available under the name Alta 2341^{TM} or Microgard^{TM} (Garsa et al., 2014). This bacteriocin has been shown to be more effective than nisin against some food-borne pathogens such as *L. monocytogenes* and *S. aureus* (Cintas et al., 1998; Eijsink et al., 1998) and Gram-negative organisms such as *Pseudomonas* and *Escherichia coli* (Jamuna and Jeevaratnam, 2004). The potential application of pediocins to dairy products is further enhanced by its stability in aqueous solutions, its wide pH range, and high resistance to heating or freezing (Sobrino-López and Martín-Belloso, 2008). Despite this high potential, few studies have investigated the addition of pediocins to milk or dairy foods. Pediocin (PA-1) was found to reduce *L. monocytogenes* counts in cottage cheese, cream, and cheese sauce (Pucci et al., 1988). The anti-listerial effect was noticeable over a wide temperature and pH ranges and was particularly effective at low initial *L. monocytogenes* contamination (10^2 cfu ml^{-1}). Recently, Verma et al. (2017) described the production of food-grade pediocin from supplemented cheese whey medium. This semi-purified pediocin containing fermented cheese whey was shown to be effective in reducing *S. aureus* counts and enhancing shelf-life of raw buffalo milk (Verma et al., 2017).

Lacticins

Lacticins are produced by certain strains of *Lc. lactis* and comprise lacticin 3147 and lacticin 481 (Piard et al., 1992; McAuliffe et al., 1998).

Lacticin 3147 was isolated from an Irish kefir grain used for making buttermilk and is a two-component lantibiotic, requiring both structural proteins to give full biological activity (McAuliffe et al., 1998). This bacteriocin exhibit antimicrobial activity against a wide range of food pathogenic and food spoilage bacteria in addition to other LAB (Sobrino-López and Martín-Belloso, 2008; Martínez-Cuesta et al., 2010). A lacticin 3147 powder preparation was shown to be effective for the control of *Listeria* and *Bacillus* in infant milk formulation, natural yogurt, and cottage cheese (Morgan et al., 2001). However, the high concentrations of lacticin powder used, which represent 10% of product weight, were considered impracticable and non-economic by the authors (Morgan et al., 2001).

Lacticin 481 is a single-peptide lantibiotic that exhibits a medium spectrum of inhibition, mainly active against other LAB, *Clostridium tyrobutyricum* (O'Sullivan et al., 2003) and *L. monocytogenes* (Ribeiro et al., 2016). The use of non-purified lacticin 481 was show to have a mild bacteriostatic activity in milk stored at refrigeration temperatures (Arqués et al., 2011). Yet, the application of semi-purified lacticin 481 to fresh cheeses stored at refrigeration temperatures reduced *L. monocytogenes* by 3 log cycles in 3–7 days (Ribeiro et al., 2016). Nevertheless, the application of lacticins in food systems is not likely to ensure complete elimination of pathogens such as *L. monocytogenes.*

Enterocins

Enterocins are produced by *Enterococcus* species and comprise a diverse group of bacteriocins, both in terms of their classification and inhibitory spectrum (Egan et al., 2016).

Although most LAB have the GRAS status and can be used safely in food applications, bacteriocinogenic enterococci raise some safety concerns (EFSA, 2007). Enterococci are among the pathogens that have been associated with a number of infections in humans (Moreno et al., 2006). In addition, some enterococci may harbor virulence determinants and antibiotic resistance genes (EFSA, 2007). While food enterococci have fewer virulence determinants than clinical strains, they are known for their capacity to exchange genetic information (Eaton and Gasson, 2001). Considering the safe concerns of using bacteriocinogenic enterococci, the use of purified enterocins may be considered more suitable for food consumption.

Enterocin AS-48 is produced by *Enterococcus faecalis* and is a class IIc cyclic bacteriocin that is active against a number of *Bacillus* and *Clostridium* sp. (Egan et al., 2016). This is one of most studied bacteriocins, showing high stability to pH and heat, which makes a great candidate for application in food. Muñoz et al. (2007) tested the efficacy of the enterocin AS-48 for controlling staphylococci in skimmed milk. They observed a bactericidal effect proportional to the bacteriocin concentration (10–50 μg ml^{-1}), but complete elimination of staphylococci was not achieved for any of the concentrations tested. This apparently lower effectiveness of AS-48 in milk when compared to culture medium could be attributed to a higher retention of the bacteriocin molecules by milk components and slower diffusion (Muñoz et al., 2007).

Yildirim et al. (2016) investigated the antimicrobial effects of enterocin KP toward *L. monocytogenes* in skim, half fat, and

full fat milks. Enterocin KP had a high anti-listerial effect, but this bactericidal effect decreased as both the fat content of milk and inoculation amount of *L. monocytogenes* increased. Also, Lauková and Czikková (1999) reported an inhibitory effect of purified enterocin CCM 4231 (3200 AU ml^{-1}) on the growth of *S. aureus* and *L. monocytogene*s in skimmed milk and yogurt. Still, the antagonistic effect of enterocin on *S. aureus* in yogurt was less influential after 24 h.

Usually, enterocins are active against foodborne pathogens, such as *Listeria* spp. and *Clostridium* spp., but their application in food systems is not likely to prevent the re-growth of pathogens throughout the storage time (Zacharof and Lovitt, 2012; de Souza Barbosa et al., 2015). However, Arqués et al. (2011) observed the reduction of *L. monocytogenes* counts in milk below the detection limit after 4 and 24 h by the combined effect of two bacteriocins, reuterin and enterocin AS-48.

Recently, Ribeiro et al. (2017) reported the efficacy of a semi-purified enterocin produced by an *E. faecalis* strain, in reducing the contamination of *L. monocytogenes* in fresh cheese in a dose-dependent manner. Moreover, the highest dose applied to cheeses (approximately 2000 AU g^{-1} of cheese) resulted in reduction of this pathogen below detection levels and this effect remained for all the storage time (72 h).

Other Bacteriocins

Lactococcin BZ is produced by some strains of *Lc. lactis* spp. (lactis BZ) and has a wide antibacterial activity against Gram-positive and Gram-negative bacteria (Şahingil et al., 2011). This bacteriocin also displayed strong anti-listerial activity in milk. The partially purified lactococcin BZ (400–2500 AU ml^{-1}) reduced *L. monocytogenes* counts to an undetectable level in both skim and full-fat milk during storage at 4 and 20°C (Yildirim et al., 2016). In addition, this anti-listerial activity was stable until the end of the storage period (25 days) and was not adversely affected by milk fat content.

Aureocin A70 is a class II bacteriocin produced by a *S. aureus* strain isolated from pasteurized commercial milk (Fagundes et al., 2016). This bacteriocin exerted a bactericidal effect on a wide range of Gram-positive bacteria, including *L. monocytogenes*, and was tested in UHT-treated skimmed milk by Fagundes et al. (2016). Aureocin A70 caused a time-dependent reduction in *L. monocytogenes* counts (5.51 log units) up to 7 days of incubation, but was insufficient to achieve the total elimination of the pathogen, resulting in <0.001% survivors (Fagundes et al., 2016).

Genetically engineered bacteriocins have been proposed to overcome the narrow range of activity of most bacteriocins. Acuña et al. (2012) reported the construction of a chimerical bacteriocin named Ent35-MccV. This hybrid bacteriocin combines in a single molecule the anti-listerial activity of enterocin CRL35 and the anti-*E. coli* activity of microcin V. This hybrid wide-spectrum bacteriocin was active against pathogenic strains of *L. monocytogenes* and *E. coli* O157:H7 and was effective in controlling the growth of both pathogens in skim milk (Acuña et al., 2015).

APPLICATION OF BACTERIOCIN-PRODUCING BACTERIA TO DAIRY PRODUCTS

Despite the recent advances in bacteriocin research for food applications, the use of purified bacteriocins in the dairy industry remains limited. Frequently, the application of a bacteriocin alone does not provide sufficient protection against microbial contamination of dairy products. The high cost of bacteriocin isolation and purification also limits the commercial exploration of new bacteriocins. In addition, the restrictive food legislation of the health regulatory authorities (FDA and EFSA) limits the approval of new bacteriocins as food preservatives and, as a consequence, only two bacteriocins (nisin and pediocin) are currently commercially available.

The use of bacteriocin-producing bacteria to control contamination microorganisms is an alternative for the use of purified bacteriocins as food additives. Many LAB genera and species have a long history of apparent safe use and they have been granted the GRAS and QPS status. In this regard, incorporation of such bacteria into foods offers a viable solution for controlling contamination microorganisms (**Table 2**). In addition, LAB are commonly used as starter cultures in food fermentations. Thus, researchers have explored the *in situ* production of bacteriocins by adding protective cultures that may grow and produce bacteriocins during the manufacture and storage of dairy foods. Many studies have also focused on the selection and development of bacteriocinogenic cultures as cell lysis-inducing agents to improve cheese maturation and flavor (Beshkova and Frengova, 2012). In addition, the use of bacteriocin-producing LAB has been proposed to prevent late blowing occurring in cheeses. Late blowing defect is a major cause of spoilage in ripened cheeses, resulting in the appearance of texture and flavor defects, due to the ubiquitous presence of *Clostridium* spores (Gómez-Torres et al., 2015). The most common strategies to reduce *Clostridium* spores are often not sufficient to prevent late blowing in cheeses (Garde et al., 2011) and the use of bacteriocinogenic LAB emerges as an alternative strategy (**Table 3**).

Among the various LAB species/strains producing bacteriocins, *Lactococcus* sp. has gained a particular interest in the biopreservation of dairy foods. Benkerroum et al. (2002) tested the effect of *in situ* bacteriocin production of *L. lactis* ssp. *lactis* against *L. monocytogenes*, in a traditional fermented milk (lben). They found that *L. monocytogenes* decreased to below the detectable level within 24 h of storage at 7°C in lben fermented with the bacteriogenic starter culture. Moreover, the pathogen was efficiently inactivated from contaminated samples despite the high level of contamination (10^7 cfu ml^{-1}) for up to 6 days of storage at 7°C (Benkerroum et al., 2002).

The application of nisin-producing *Lactococcus* sp. in dairy foods which requires LAB starters presents a problem, because the wide spectrum of inhibition associated with nisin includes LAB themselves (Abee et al., 1995). Still, Yamauchi et al. (1996) produced a yogurt by incorporating a nisin-producing strain, *L. lactis* subsp. *lactis*, in raw milk. The bacteriocin-producing

TABLE 3 | Applications of bacteriocin-producing LAB to prevent gas blowing in cheese caused by *Clostridium* spp. (2000–present).

Bacteriocin-producing strain	Application technique	Product	Features	Reference
Lactobacillus plantarum TF711	Adjunct to commercial starter culture	Cow milk cheese	Reduction of 2-2 log units of *Clostridium* spores.	González and Zárate, 2015
Lactobacillus reuteri INIA P572	Adjunct to commercial starter culture together with glycerol (required for reuterin production).	Cow milk cheese	Controlled the growth of *Clostridium* and prevented the development of late blowing.	Gómez-Torres et al., 2014
Lactobacillus gasseri K7	Adjunct to commercial starter culture	Semi-hard cheese	Failed to reduced *Clostridium* spores, but delayed blowing of cheeses.	Matijasic et al., 2007
Lactococcus lactis IFPL 3593	Adjunct to commercial starter culture	Semi-hard cheeses	5 log g^{-1} reduction in the numbers of spores.	Martínez-Cuesta et al., 2010
Lactococcus lactis ssp. *lactis* IPLA 729	Adjunct to starter culture	Vidiago cheese	Reduced *C. tyrobutyricum* by 3 log during ripening	Rilla et al., 2003
Lactococcus lactis subsp. *lactis* INIA 415	Starter culture	Ovine milk cheese	Outgrowth inhibition of *Clostridium* spores without altering sensory characteristics.	Garde et al., 2011
Streptococcus macedonicus ACA-DC 198	Adjunct to starter culture	Kasseri cheese	Reduced *C. tyrobutyricum* by 1.4 log during ripening	Anastasiou et al., 2009
Streptococcus thermophilus 580	Starter culture	Milk curd	No gas production for up to 20 days.	Mathot et al., 2003

LAB were killed before the addition of the traditional yogurt cultures, and this resulted in increased storage life of the yogurts by preventing the growth of spoilage bacteria (Yamauchi et al., 1996).

Recently, Kondrotiene et al. (2018) reported a reduction in *L. monocytogenes* when three nisin A producing *Lc. lactis* strains were added to fresh cheese. However, the reduction on *Listeria* contamination was limited to 2 log units within 7 days of cheese storage. Likewise, the use of a nisin A-producing *Lactococcus diacetylactis* in a mixed starter culture was not effective in inhibiting *L. innocua* growth in Cheddar cheese (Benech et al., 2002). Maisnier-Patin et al. (1992) examined the inhibitory effect of another nisin-producing *Lc. lactis* on *L. monocytogenes* in Camembert cheese. In the presence of nisin-producing starter, the numbers of the pathogen decreased until the end of the second week, leading to a reduction of 3 log cfu g^{-1}, but a regrowth of *Listeria* was observed in cheeses throughout ripening (6 weeks).

A reduction of *L. monocytogenes* counts was observed in raw milk cheese by the use of two nisin-producing *Lactobacillus lactis* subsp. *lactis* as starter cultures (Rodrígez et al., 2001). In contrast, the use of nisin-producing strains to control *E. coli* and *L. monocytogenes* in Feta and Camembert cheeses has shown limited results (Ramsaran et al., 1998). *E. coli* O157:H7 survived the manufacturing process of both cheeses and was present in cheese made with nisin-producing strains at the end of 75 days of storage in greater numbers than the initial inoculum. The Feta cheese that contained nisin was the only cheese in which *L. monocytogenes* remained at the level of the initial inoculum after 75 days of storage. The use of bacteriocinogenic pediococci in dairy products also exhibited limited results due to their inability to ferment lactose (Renye et al., 2011).

Mills et al. (2011) tested a plantaricin-producing *Lactobacillus plantarum* strain as an anti-listerial adjunct in the presence and absence of nisin-producing starters for the manufacture of cheeses. The combination of *Lb. plantarum* strain (at 10^8 cfu ml^{-1}) with a nisin producer reduced *Listeria* to undetectable levels by day 28. Moreover, they found that *Lb. plantarum* was much more effective at inhibiting *Listeria* than the nisin producer alone.

Carnio et al. (2000) studied the anti-listerial potential of a food-grade strain *Staphylococcus equorum*, producing a bacteriocin named as micrococcin P1, in soft cheese. A remarkable reduction of *L. monocytogenes* growth was achieved, but this effect was dependent on the contamination level. In addition, a regrowth of the viable *Listeria* could be observed after 10–16 days of maturation. Likewise, Dal Bello et al. (2012) detected a reduction of *L. monocytogenes* by 3 log units in cottage cheese, after incubation with a lacticin-481-producing *Lc. lactis* strain. However, after an initial period, *L. monocytogenes* counts increased to values comparable to the control.

In addition to natural bacteriocinogenic strains, the heterologous production of bacteriocin by genetically engineered LAB was tested in dairy foods (Leroy and De Vuyst, 2004). A lacticin 3147 transconjugant generated via conjugation of

bacteriocin-encoding plasmids was used successfully against *L. monocytogenes* in cottage cheese (McAuliffe et al., 1999). Reductions of 99.9% were seen in cottage cheese held at 4°C after 5 days. In another study, a lacticin 3147 transconjugant presented also a protective effect when applied to the cheese surface, reducing *L. monocytogenes* by 3 log units (Ross et al., 2000). In contrast, this protective effect was not evident when a nisin-producing culture was used, possibly due to pH instability (Ross et al., 1999).

The use of *Enterococcus* spp. in foods may represent a risk for consumers and requires a safety assessment by the European food authorities (EFSA, 2007). However, enterococci are found naturally in some dairy foods, as they are used as starter cultures and are often part of the microbiota of artisanal cheeses (Domingos-Lopes et al., 2017). In addition, some strains of bacteriocinogenic enterococci were show to lack many of the virulence determinants (Jaouani et al., 2015). De Vuyst et al. (2003) suggested that *Enterococcus* species could be safely used in food if virulence genes were absent. Consequently, several studies have also employed enterocin-producing enterococcus in food systems.

The inhibitory effect of enterocin-producing enterococci against *L. monocytogenes* and *S. aureus* in dairy foods such as milk and cheeses was demonstrated by several authors (Giraffa, 1995; Giraffa and Carminati, 1997; Nunez et al., 1997; Lauková et al., 1999a,b: Lauková et al., 2001). Many enterococcal strains producing enterocins were isolated and found to be effective in controlling contamination in cheeses, without compromising the acid-producing activity of the starter and the organoleptic characteristics of the final product (Khan et al., 2010). Bacteriocinogenic *E. faecalis* strain was show to reduce *L. monocytogenes* counts in Manchego cheese by 1 and 2 log units after 7 and 60 days, respectively (Nunez et al., 1997). Although inoculation of milk with *E. faecalis* strain reduced the rate of acid production in the curd, the flavor and bitterness of the final product were not influenced.

Other bacteriocinogenic enterococci have also been reported to reduce *L. monocytogenes* in dairy foods. Pingitore et al. (2012) investigated two bacteriocinogenic enterococci strains (*Enterococcus mundtii* CRL35 and *Enterococcus faecium* ST88Ch) isolated from cheeses. Growth of *L. monocytogenes* was inhibited in Minas cheeses containing *E. mundtii* up to 12 days of storage at 8°C. This bacterium displayed a bacteriostatic effect since *Listeria* counts remained similar to the initial inoculum. *E. faecium* strain was found to be less effective, as the bacteriostatic affect occurred only after 6 days at 8°C.

Further studies also demonstrated the inhibitory effect of enterocin-producing enterococci strains against *L. monocytogenes* in raw milk. Vandera et al. (2017) investigated the use of a multiple enterocin-producing bacterial strains possessing the structural entA, entB, and entP enterocin genes. Some strains exhibited a bacteriostatic effect on *L. monocytogenes* in raw milk incubated at 37°C for 6 h. When raw milk cultures were further incubated at 18°C, viable populations of the pathogen were reduced slightly (by 0.2–0.4 log cfu ml^{-1}) after 24 h and up to 72 h.

Achemchem et al. (2006) studied the effectiveness of an *E. faecium* strain in controlling *L. monocytogenes* in goat's milk and goat's traditional cheese (jben). Coculture experiments of *E. faecium* and *L. monocytogenes* in milk demonstrated that the pathogen was not eliminated, but when the bacteriocinogenic strain was previously inoculated in whole milk and left to grow for 12 h before contamination, *Listeria* was undetectable after 130 h of coculture. Moreover, addition of the bacteriocinogenic strain to jben cheese contaminated with *L. monocytogenes* prior to packaging, reduced the number of viable *Listeria* to undetectable levels, after 1 week of storage at 22°C.

Coelho et al. (2014) examined the inhibitory effect of enterocin-producing *E. faecalis* strains against *L. monocytogenes* in fresh cheese. Inoculation of milk with bacteriocinogenic *E. faecalis* strains was shown to reduce *L. monocytogenes* counts by 3–4 log units in fresh cheese compared to the control. The combination of two enterocin producers optimized the reduction of *Listeria* counts in fresh cheese, decreasing this pathogen by 4 log cfu g^{-1} in the first 3 days of storage and by 5 log cfu g^{-1} on day 7.

Bacteriocin-producing LAB strains have also been assessed to improve cheese maturation and flavor. These LAB cultures may induce controlled lysis of starter and/or non-starter LAB (NSLAB) and subsequent intracellular release of proteinases and peptidases, resulting in rapid onset of proteolysis and cheese ripening. *Lc. lactis* producing lacticin 3147 was shown to accelerate cheese ripening and also prevent late blowing in cheese by the inhibition of clostridia growth (Martínez-Cuesta et al., 2010).

Various bacteriocin-producing strains were shown to possess a lytic effect on starter cultures. The use of bacteriocin-producing *Lc. lactis* ssp. *cremoris* as a starter adjunct in Cheddar cheese manufacture was show to increase the rate of starter lysis. Cheese manufactured with the bacteriocinogenic adjunct exhibited increased cell lysis and higher concentrations of free amino acids, with associated higher sensory evaluation scores (Morgan et al., 1997). Another bacteriocin-producing starter *Lc. lactis* (a lacticin 3147 producer) was tested for controlling the proliferation of undesirable microorganisms during cheese manufacture. Cheeses made with lacticin 3147-producing starters exhibited significantly lower levels of NSLAB that remained constant over 6 months of ripening (Ryan et al., 1996).

Some strains of lacticin 481-producing *Lc. lactis* were also tested in cheese production and showed to cause partial lysis of starter lactococcal cultures, which continue to grow at a slower rate (O'Sullivan et al., 2003). As a direct result of starter lysis with concomitant enzyme release in the cheese matrix, these strains may be employed to increase cheese ripening. Additionally, a three-strain starter system was tested on cheese by Morgan et al. (2002). This system comprised a bacteriocin producer which causes the lysis of a second strain (sensitive to bacteriocin) and a third strain resistant to bacteriocin activity, for acid production during cheese manufacture. The experimental cheese made with this three-strain starter system showed an increase in lysis and decrease in bitterness compared to cheeses manufactured without the bacteriocin-producing adjunct (Morgan et al., 2002).

COMBINING BACTERIOCINS WITH OTHER HURDLES

One of the approaches to improve the protective action of bacteriocins is the combination with other hurdles such as chemical additives (such as EDTA, sodium lactate, potassium diacetate, and others), heating, and high-pressure treatments (Egan et al., 2016).

Narayanan and Ramana (2013) observed that the use of pediocin in combination with eugenol incorporated into polyhydroxybutyrate films worked in synergized form and provided an effective hurdle preventing food contamination. Other researchers used successfully the mixture of bacteriocins and EDTA in the sensitization of Gram-negative bacteria (Prudêncio et al., 2015). Gram-negative bacteria become sensitive to bacteriocins if the permeability of their outer membrane is compromised with chelating agents, such as EDTA (Chen and Hoover, 2003). Zapico et al. (1998) demonstrated a synergistic effect of the combined use of nisin and the lactoperoxidase system (LPS) to control *L. monocytogenes* in skim milk. A listericidal effect (5.6 log units lower than the control milk) was observed in treatments containing nisin (10 or 100 IU ml^{-1}) with LPS, after 24 h at 30°C. Moreover, when the two preservatives were added in two steps (LPS was added after 3 h and nisin after 5 h of growth), the difference in *L. monocytogenes* counts increased by 7.4 log units.

Several authors also observed the synergistic effect of bacteriocins after temperature treatments (Prudêncio et al., 2015). Kalchayanand et al. (1992) exposed bacteriocin-resistant bacteria to sub-lethal stresses, either at low or high temperatures, and treated with nisin and pediocin. They found that both bacteriocins were effective in reducing the cell viability. This synergistic effect was also observed in Gram-negative bacteria, which are normally insensitive to these bacteriocins (Boziaris et al., 1998).

High pressure processing is a common technique for inactivating microorganisms at room temperature, but this treatment does not ensure the complete inactivation of microorganisms (Prudêncio et al., 2015). Several studies have demonstrated the synergistic effect of bacteriocins such as nisin with high pressure processing on the inactivation of food microorganisms (Garriga et al., 2002; Zhao et al., 2013). It is well documented that the use of bacteriocins in combination with these processing techniques enhances bacterial inactivation (Chen and Hoover, 2003). As an example, Rodriguez et al. (2005) demonstrated the efficacy of the application of reduced pressures combined with bacteriocin-producing LAB to improve cheese safety.

INCORPORATION OF BACTERIOCINS IN ANTIMICROBIAL FILMS AND COATINGS

A common strategy for preservation of foods that are eaten raw or without further cooking is the application of edible films or coatings containing antimicrobial substances. The incorporation of antimicrobial compounds such as bacteriocins in edible coatings and films presents as an interesting alternative for ensuring the control of pathogenic microorganisms in food products (Valdés et al., 2017).

Edible coatings and films are composed of thin layers of biopolymers that modify the surrounding atmosphere of foods, forming a barrier between the food and the environment, improve the safety, quality, and functionality of food products without changing organoleptic and nutritional properties (Han, 2003; Valdés et al., 2017). The use of purified bacteriocins or bacteriocin-producing bacteria in the packaging system may be more effective in the inhibition of the growth of pathogenic and/or deterioration microorganisms throughout the extent of the latency phase (Balciunas et al., 2013). Hydrocolloids (proteins and polysaccharides) are the most extensively investigated biopolymers in edible coatings and films applied to cheese. They facilitate the incorporation of functional compounds such as bacteriocins and bacteriocin-producing bacteria and allow an increase in the stability, safety, and shelf-life of dairy foods (Scannell et al., 2000a).

To date, few studies have investigated the effectiveness of incorporating bacteriocins and/or bacteriocin-producing LAB in coatings and films applied to dairy products. Some authors observed an inhibition of the growth of pathogenic microorganisms in foods packed with coatings and films containing antimicrobial metabolites synthesized by LAB (Cao-Hoang et al., 2010; da Silva Malheiros et al., 2010; Ercolini et al., 2010; Aguayo et al., 2016; Malheiros et al., 2016) or containing viable LAB in the film/coating matrix (Concha-Meyer et al., 2011; Barbosa et al., 2015).

Studies of the effectiveness of incorporating purified bacteriocins in edible coatings show a limited reduction of pathogens such as *L. monocytogenes*. Cheeses, particularly fresh cheeses, are highly perishable due to their high content in caseins, lipids, and water. The complexity of cheese composition and its manufacture support the development of pathogenic and deteriorating microorganisms that increase the risk of foodborne illness and reduce cheese quality and acceptability (Ramos et al., 2012). By acting as additional hurdle, the application of edible coatings and films with incorporation of bacteriocins may overcome problems associated with post-process contamination, therefore enhancing the safety and extending the shelf-life of the cheese.

Cao-Hoang et al. (2010) incorporated nisin in films of sodium caseinate applied in semi-soft cheese and observed a small reduction in *L. innocua* counts (1.1 log cfu g^{-1}) after a week of storage at 4°C. In another study, the incorporation of nisin and lacticin in cellulose coatings applied to Cheddar cheese reduced levels of *L. innocua* by 2 log cycles, and *S. aureus* by 1.5 log cycles (Scannell et al., 2000b). However, other studies have shown an effective reduction on pathogen growth. In Ricotta cheese coated with galactomannan and nisin, the growth of *L. monocytogenes* was prevented for 7 days at 4°C (Martins et al., 2010). The application of a coating in Port Salut cheese, consisting of tapioca starch combined with nisin and natamycin, reduced *L. innocua* counts above 10 cfu ml^{-1} during storage, acting as a barrier to post-process contamination (Resa et al., 2014).

Recently, Marques et al. (2017) used a biodegradable film incorporated with cell-free supernatant (CFS) containing bacteriocin-like substances of *Lactobacillus curvatus* P99, to control the growth of *L. monocytogenes* in sliced "Prato" cheese. These films containing the bactericidal concentration of CFS were able to control *L. monocytogenes* for 10 days of storage at 4°C.

CONCLUSION

This review highlights the most recent trends in the use of bacteriocins and bacteriocin-producer bacteria in dairy foods. Bacteriocins, either *per se* or produced by live bacteria, can be successfully incorporated into dairy products to assure safety, extending shelf-life and preserve quality. The use of purified and concentrated bacteriocins as food additives has been the preferred method due to more efficacy, compared to direct application of bacteriocinogenic cultures. However, the effectiveness of bacteriocins in food systems is often low due to several factors such as adsorption to food components, enzymatic degradation, poor solubility, or uneven distribution in the food matrix. On the other hand, the application of live bacteriocin-producing bacteria into dairy foods may overcome the limitations on the use of purified bacteriocins. The bacteriocinogenic LAB added to dairy products such as yogurts and cheeses will ensure continuous production of bacteriocins throughout maturation and storage, and may be included as starter/adjunct cultures in fermentation. The main difficulty associated with this application is the lack of compatibility between the bacteriocin-producing strain and other cultures required in the fermentation of dairy foods.

Studies on application of bacteriocins and/or bacteriocin producers have been focused mainly on cheeses because most discovered LAB bacteriocins were effective against *Listeria monocytogenes*. This foodborne pathogen is often a great concern in traditional cheeses made from raw milk and as a post-processing contaminant in cheeses made from pasteurized milk. Cheeses are particularly vulnerable to the contamination of *L. monocytogenes* because they provide the appropriate growth conditions for this pathogen. Listeriosis out-breaks linked to the consumption of contaminated cheeses have been reported worldwide. Therefore, the application of bacteriocins as natural preservatives for improving cheese safety has attracted significant research interest in recent years. In contrast, very limited research has been focused on the application of bacteriocins to the preservation of milk, cream, yogurts, and other dairy products.

Although most efforts have been devoted to discover novel bacteriocins with unique properties, more studies are necessary for effective application of bacteriocins in dairy foods in order to understand bacteriocin performance in the complex environment of food matrices. In addition, bacteriocins may be combined with other protection tools as part of hurdle technologies to ensure bio-preservation and shelf-life extension of dairy foods. Their use may lie in the combination with other preservation techniques, or in the incorporation in biofilms and active packaging. In the latter case, further studies are necessary to ensure the adaptation of edible coatings and films to bacteriocin activity and efficacy in dairy products.

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All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Bioprotective Effect of *Lactococcus piscium* CNCM I-4031 Against *Listeria monocytogenes* Growth and Virulence

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Listeria monocytogenes is a Gram-positive pathogen occurring in many refrigerated ready-to-eat foods. It is responsible for foodborne listeriosis, a rare but severe disease with a high mortality rate (20–30%). *Lactococcus piscium* CNCM I-4031 has the capacity to prevent the growth of *L. monocytogenes* in contaminated peeled and cooked shrimp and in a chemically defined medium using a cell-to-cell contact-dependent mechanism. To characterize this inhibition further, the effect of *L. piscium* was tested on a collection of 42 *L. monocytogenes* strains. All strains were inhibited but had different sensitivities. The effect of the initial concentration of the protective and the target bacteria revealed that the inhibition always occurred when *L. piscium* had reached its maximum population density, whatever the initial concentration of the protective bacteria. Viewed by scanning electron microscopy, *L. monocytogenes* cell shape and surface appeared modified in co-culture with *L. piscium* CNCM I-4031. Lastly, *L. monocytogenes* virulence, evaluated by a plaque-forming assay on the HT-29 cell line, was reduced after cell pre-treatment by the protective bacteria. In conclusion, the bioprotective effect of *L. piscium* toward *L. monocytogenes* growth and virulence was demonstrated, and a hypothesis for the inhibition mechanism is put forward.

Keywords: biopreservation, *Lactococcus piscium*, *Listeria monocytogenes*, co-culture, cell ratio, scanning electron microscopy, virulence

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INTRODUCTION

Listeria monocytogenes is a human pathogenic Gram-positive bacterium, which is responsible for foodborne listeriosis generally associated with a high mortality rate (20–30%). All human population groups can be infected and particularly newborn infants, pregnant women, elderly people, and immuno-compromised patients (Lecuit et al., 2015). This species constitutes a major problem in refrigerated ready-to-eat (RTE) foods (Rocourt et al., 2003). According to the Codex Alimentarius Commission, RTE products are "any food which is normally eaten in its raw state or any food handled, processed, mixed, cooked, or otherwise prepared into a form which is normally eaten without further listericidal steps" (CAC, 2007). *L. monocytogenes* differs from most other food-borne pathogens in that it is ubiquitous and can grow or survive in most conditions

encountered in the food chain and food-processing procedures (Buchanan et al., 2017). Among foodstuffs, fishery products recorded the highest level of non-compliance with EU safety criteria in 2015 (EFSA, 2016) while seafood products were implicated in 15% of *L. monocytogenes* outbreaks reported in Europe in the last 3 years. Its survival ability makes the control of this microorganism in food products, especially RTE foods, a major challenge. The lactic acid bacteria (LAB), generally recognized as safe (Salminen et al., 1998), are also present and dominant in RTE foods such as meat and seafood products stored under vacuum or modified atmosphere packaging. Selected LAB can limit the development of *L. monocytogenes* and are thus recognized as efficient bioprotective agents in food systems (Brillet et al., 2004; Nilsson et al., 2005; Tomé et al., 2006; Vermeiren et al., 2006a; Unlu et al., 2015). The competition between LAB and *L. monocytogenes* involves various bactericidal or bacteriostatic mechanisms such as (i) competition for nutrients (Nilsson et al., 2005); (ii) production of one or more antimicrobial active metabolites such as bacteriocins (Richard et al., 2003; Dortu et al., 2008; Martinez et al., 2015), reuterin (El-Ziney et al., 1999), organic acids (Amezquita and Brashears, 2002), and hydrogen peroxide (Ito et al., 2003; Batdorj et al., 2007). *Lactococcus piscium* CNCM I-4031 is an efficient bioprotective strain for seafood products isolated from raw salmon stored under modified atmosphere packaging (Matamoros et al., 2009a). It improves the sensory quality of cooked shrimp by preventing the growth of *Brochothrix thermosphacta* (Fall et al., 2012). *L. piscium* CNCM I-4031 can also limit the growth of *L. monocytogenes* RF191 during the storage of cooked shrimp (Fall et al., 2010). The inhibition mechanism has not yet been entirely elucidated but using a chemically defined medium (MSMA) to mimic the shrimp matrix, it has been suggested that cell contact is required for the inhibition of *L. monocytogenes* RF191 by *L. piscium* CNCM I-4031 (Saraoui et al., 2016).

The aim of this study was to characterize the further inhibitory effect of *L. piscium* CNCM I-4031 against *L. monocytogenes* species. First, the inhibitory activity of the CNCM I-4031 strain was evaluated on a collection of 42 *L. monocytogenes* strains. Then, the effect of the protective strain CNCM I-4031 on the growth, morphological shape, and virulence of *L. monocytogenes* was investigated.

MATERIALS AND METHODS

Bacterial Strains, Culture Media, and Conditions

Lactococcus piscium CNCM-I 4031 was isolated from fresh salmon steak packed under modified atmosphere packaging (Matamoros et al., 2009b). The *L. monocytogenes* strains used in this study are listed in **Table 1**. All strains were stored in aliquots of 250 µl at −80°C in a final concentration of 10% (v/v) of glycerol. For all experiments, an aliquot of the strain was subcultured in Elliker broth (Biokar Diagnostic, Beauvais, France) for 24 h at 26°C for *L. piscium*, and in Brain Heart Infusion supplemented by 2% NaCl (mBHI) (Biokar Diagnostic, Beauvais, France) for 24 h at 20°C for *L. monocytogenes*. The cultures were diluted in their culture medium to obtain appropriate initial cell concentrations. The chemically defined medium MSMA used for the bacterial interaction observation

TABLE 1 | List of *L. monocytogenes* strains used in this study.

Strain	Origin	Country	Collection
ScottA	Milk (incriminated in listeriosis)	United States	CIP 103575
EGD-e	Rabbit (incriminated in listeriosis)	England	ATCC BAA-679
EU2208	Cod croquette	Spain	AZTI
EU2169	Fresh trout	Spain	AZTI
EU2170	Fresh trout	Spain	AZTI
EU2171	Fresh trout	Spain	AZTI
EU2148	Shrimp	Iceland	MATIS
EU2209	Smoked cod	Spain	AZTI
EU2158	Smoked salmon production plant	France	ASEPT
EU2159	Smoked salmon production plant	France	ASEPT
EU2160	Smoked salmon production plant	France	ASEPT
EU2161	Smoked salmon production plant	France	ASEPT
EU2162	Smoked salmon production plant	France	ASEPT
EU2163	Smoked salmon production plant	France	ASEPT
EU2164	Smoked salmon production plant	France	ASEPT
RF101	Smoked salmon production plant	France	ASEPT
RF102	Smoked salmon production plant	France	ASEPT
RF103	Smoked salmon production plant	France	ASEPT
RF104	Smoked salmon production plant	France	ASEPT
RF105	Smoked salmon production plant	France	ASEPT
RF106	Smoked salmon production plant	France	ASEPT
RF96	Smoked salmon production plant	France	ASEPT
RF97	Smoked salmon production plant	France	ASEPT
RF98	Smoked salmon production plant	France	ASEPT
RF99	Smoked salmon production plant	France	ASEPT
RF113	Smoked salmon production plant	France	ASEPT
RF115	Smoked salmon production plant	France	ASEPT
RF116	Smoked salmon production plant	France	ASEPT
RF118	Smoked salmon production plant	France	ASEPT
RF120	Smoked salmon production plant	France	ASEPT
RF122	Smoked salmon production plant	France	ASEPT
RF123	Smoked salmon production plant	France	ASEPT
RF124	Smoked salmon production plant	France	ASEPT
RF125	Smoked salmon production plant	France	ASEPT
RF133	Smoked salmon production plant	France	ASEPT
RF135	Smoked salmon production plant	France	ASEPT
RF138	Smoked salmon production plant	France	ASEPT
RF142	Smoked salmon production plant	France	ASEPT
RF152	Smoked salmon production plant	France	ASEPT
RF92	Smoked trout	France	Aqualande
RF93	Smoked trout	France	Aqualande
RF166	Taramasalata	France	Biocéane
RF191	Tropical cooked peeled shrimp	France	PFI Nouvelles Vagues

ATCC, American Type Culture Collection; AZTI, Derio, Spain; MATIS, Reykjavik, Iceland; ASEPT, Le Mans, France; Aqualande, Roquefort, France; Biocéane, Nantes, France; PFI Nouvelles Vagues, Boulogne-sur-Mer, France.

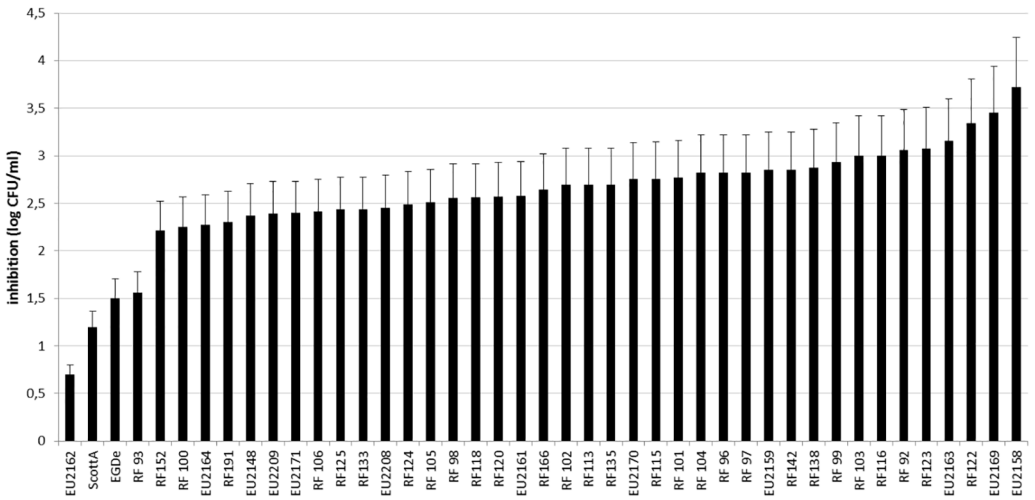

FIGURE 1 | Inhibition of 42 *L. monocytogenes* strains after a 30-h culture with *L. piscium* CNCM I-4031 in MSMA.

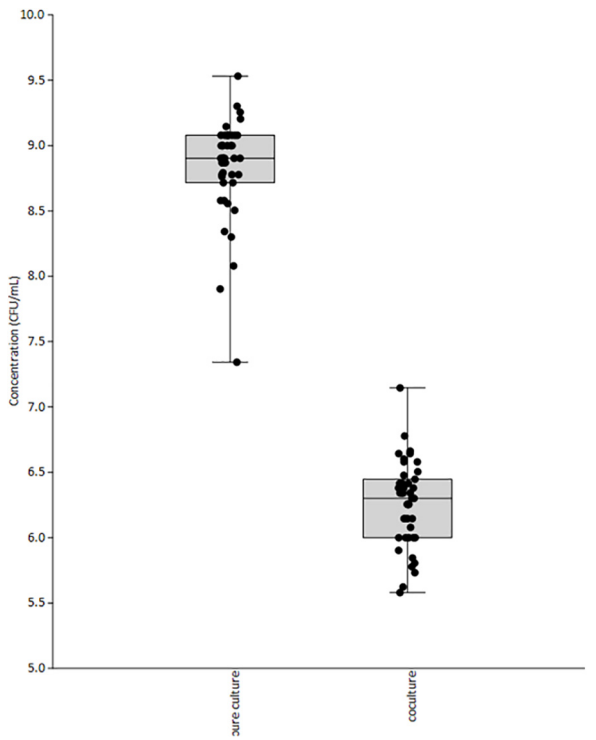

FIGURE 2 | Final concentration of 42 *L. monocytogenes* strains in pure and mixed culture with *L. piscium* CNCM I-4031, after 30 h in MSMA medium.

was prepared as previously described by Saraoui et al. (2016). *L. piscium* was enumerated by spreading 100 µl of 10-fold serial dilutions on Elliker agar plates incubated at 8°C for 5 days under anaerobiosis (co-cultures) or at 26°C for 48 h (pure cultures). *L. monocytogenes* was enumerated by spread-plating 100 µl (classic enumeration) or 5 µl (microenumeration method) of 10-fold serial dilutions on mBHI agar incubated at 37°C for 24 h.

Biodiversity of *L. monocytogenes* Sensitivity to *L. piscium* CNCM I-4031

After subculture, *L. piscium* CNCM I-4031 and each of the 42 *L. monocytogenes* strains (**Table 1**) were co-cultured in a 96-well microplate filled with 200 µl of MSMA at an initial concentration of 10^6 and 10^3 CFU/ml, respectively. The microplate was incubated at 26°C for 30 h without shaking. Controls consisted of monocultures of each *L. monocytogenes* strain inoculated in the same conditions. *L. monocytogenes* strains were enumerated in CFU/ml using the microenumeration method described in section "Bacterial Strains, Culture Media, and Conditions." The inhibition was calculated by the difference in the log-concentration of *L. monocytogenes* strain in pure culture and in co-culture. The standard deviation was estimated according to five independent replicates of the inhibition tests for *L. monocytogenes* RF191 and *L. piscium* CNCM-4031.

Effect of Co-culture Ratios on the Inhibition of *L. monocytogenes* by *L. piscium* CNCM I-4031

Lactococcus piscium CNCM I-4031 and *L. monocytogenes* RF191 were co-inoculated in 250-ml flasks of MSMA medium, without shaking. Depending on the experiments, the initial ratios between *L. piscium* and *L. monocytogenes* were $10^3/10^3$ (A), $10^5/10^3$ (B), and $10^6/10^6$ (C) CFU/ml, respectively. The co-cultures were incubated at 26°C, and the growth of both strains was monitored during 48 to 72 h by the classic enumeration method described in section "Bacterial Strains, Culture Media, and Conditions." Controls consisted of monocultures of each strain in MSMA at 26°C at the same inoculation levels. All the cultures were performed in triplicate.

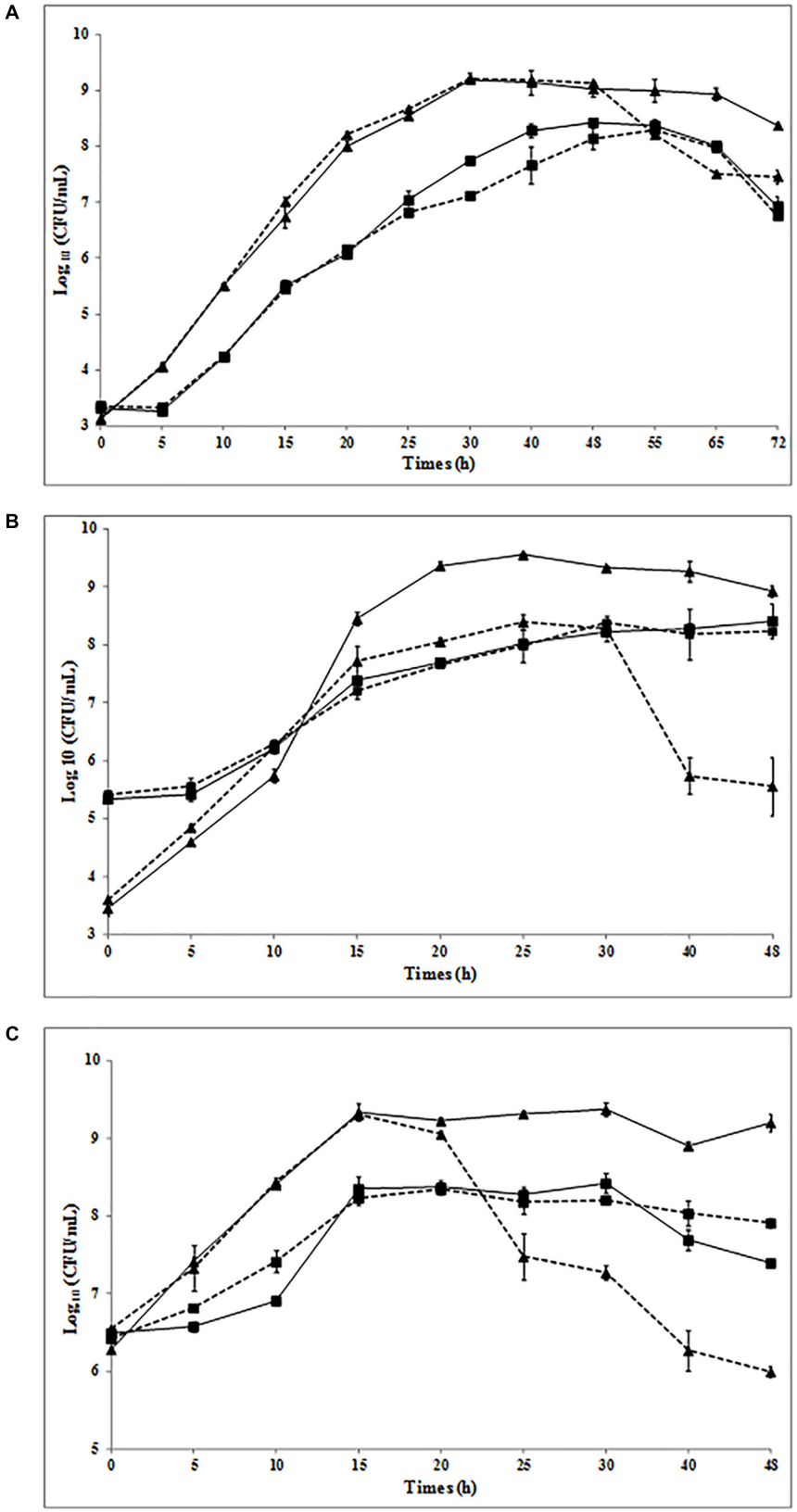

FIGURE 3 | Growth of *L. piscium* CNCM I-4031 (■) and *L. monocytogenes* RF191 (▲) in pure culture (solid line) and in co-culture (dotted line) in MSMA at 26°C. Initial concentrations of *L. piscium*/*L. monocytogenes*: **(A)** $10^{3}/10^{3}$ CFU/ml, **(B)** $10^{5}/10^{3}$ CFU/ml, and **(C)** $10^{6}/10^{6}$ CFU/ml.

TABLE 2 | Maximal population density and growth rate of *L. piscium* CNCM I-4031 (Lp) and *L. monocytogenes* RF191 (Lm) in pure (PC) or co-culture (CC) in MSMA medium at 26°C.

	***Lactococcus piscium* CNCM I-4031**						***Listeria monocytogenes* RF191**					
Initial ratio UFC/ml (Lp/Lm)	(A) $10^3/10^3$		(B) $10^5/10^3$		(C) $10^6/10^6$		(A) $10^3/10^3$		(B) $10^5/10^3$		(C) $10^6/10^6$	
Culture specifications	PC	CC	PC	CC	PC	CC	PC	CC	PC	CC	PC	CC
Maximal population density (log CFU/ml) ± SD	8.28 ± 0.12	8.14 ± 0.2	8.01 ± 0.01	7.98 ± 0.27	8.35 ± 0.15	8.23 ± 0.09	9.19 ± 0.04	9.21 ± 0.09	9.36 ± 0.03	8.05 ± 0.07	9.33 ± 0.11	9.31 ± 0.05
Growth rate (h^{-1})	0.43	0.40	0.45	0.37	0.40	0.32	0.60	0.63	0.76	0.66	0.51	0.63
Final population density (log CFU/ml) ± SD	6.92 ± 0.02[a]	6.75 ± 0.02[a]	8.40 ± 0.29[a]	8.23 ± 0.03[a]	7.39 ± 0.04[b]	7.91 ± 0.05[a]	8.37 ± 0.11[a]	7.46 ± 0.04[b]	8.92 ± 0.09[a]	5.55 ± 0.50[b]	9.20 ± 0.10[a]	5.99 ± 0.06[b]

SD, standard deviation. [a,b] Represent groups determined with LSD test, a same letter indicates values not significantly different (p-value < 0.05 by one-way ANOVA) through one condition.

Observation of Cells in Co-culture by Scanning Electron Microscopy

Pure cultures and co-cultures (ratio $10^6/10^3$ CFU/ml) of *L. piscium* CNCM I-4031 and *L. monocytogenes* RF191 were cultivated in 10 ml of MSMA medium at 26°C for 24 h. Then, 1 ml (10^8 cells) of the suspension was filtered on a Nuclepore® polycarbonate membrane with a 0.22-μm pore size and 13-mm diameter (Whatman International Ltd., Maidstone, United Kingdom). In order to observe the bacterial interaction on a solid medium (Dubey and Ben-Yehuda, 2011), another filter membrane was placed on an MSMA agar (15 g/l) plate and spotted with 10 μl of co-culture and incubated for 6 h at 26°C. All membranes containing the cells were fixed with 2.5% (v/v) glutaraldehyde (diluted in sodium cacodylate 0.1 M, pH 7.2) (Sigma Aldrich, Saint-Quentin Fallavier, France) for 48 h at 4°C. The fixing solution was renewed twice. Then, the samples were washed using a solution of sodium cacodylate (0.2 M, pH 7.2) and dehydrated in serial concentrations of ethanol (60, 70, 80, 90, 95%), 10 min for each concentration, followed by three times/20 min in 100% ethanol. The membranes were transferred to a critical point dryer, and the samples were subsequently sputter-coated and observed with a scanning electron microscope (Jeol JSM 6301F) at the CMEBA platform (Rennes, France).

Analysis of the Virulence of *L. monocytogenes* Co-cultivated With *L. piscium*

Lactococcus piscium CNCM I-4031, *L. monocytogenes* RF191, and *L. monocytogenes* Scott A strains were cultivated in MSMA medium at 26°C for 24 h in triplicates. The cultures were centrifuged, re-suspended in phosphate buffered-saline (PBS, Eurobio, Courtaboeuf, France), and then diluted to obtain appropriate cell concentrations for the cell line infection.

The human adenocarcinoma cells (line HT-29) (European Collection of Animal Cell Cultures, Salisbury, United Kingdom) were routinely grown in 75-cm^2 flasks (Sigma) in a complete medium, DMEM (Dulbecco's modified Eagle's medium), with 10% (v/v) fetal calf serum (SCF), and 1% amphotericin B 250 μg/ml (Eurobio). Gentamicin 100 μg/ml (Sigma) was added to the culture medium. Cells were kept in the humidified atmosphere of a 5% CO_2 incubator at 37°C. One hundred microliter of HT-29 cell suspension (2×10^4 cells) was deposited per well in a 96-well tissue culture plate (Sigma). The plates were incubated for 4 days with antibiotics followed by incubation for 24 h without antibiotics to obtain confluent monolayers.

In each well, HT-29 cells were infected with 10^8 *L. piscium* CNCM I-4031 and incubated for 1 h at 37°C. *L. piscium* cells were then removed by suction, and the HT-29 cells were infected with 100-μl suspensions from 10^8 to 10^3 CFU/ml of *L. monocytogenes* per well and incubated for 2 h at 37°C. *L. monocytogenes* cells were then removed by suction. The HT-29 cells were covered with 100 μl of DMEM 10% SCF with 100 μg/ml of gentamicin and incubated for 1.5 h at 37°C to eliminate bacterial cells from the plates. Each well was then overlaid with DMEM 10% SCF with 100 μg/ml of gentamicin containing 0.47% of indubiose to prevent cell starvation. Incubation was carried out

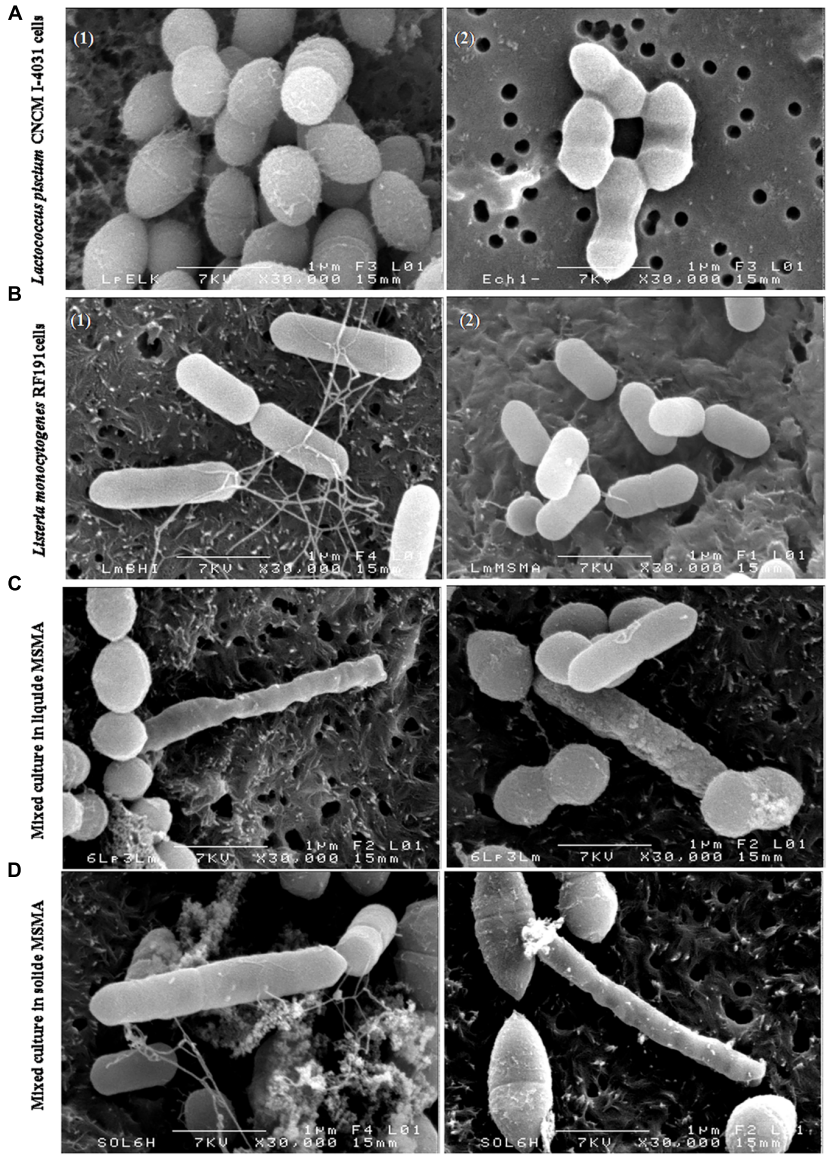

FIGURE 4 | *L. piscium* CNCM I-4031 and *L. monocytogenes* RF191 cells in pure and co-culture viewed using scanning electron microscopy on polycarbonate membranes. Magnification: × 30,000. **(A)** *L. piscium* in pure culture (1) in Elliker; (2) in MSMA, 24 h at 26°C. **(B)** *L. monocytogenes* RF191 in pure culture (1) in mBHI; (2) in MSMA, 24 h at 26°C. **(C)** Co-culture of *L. piscium* CNCM I-4031 and *L. monocytogenes* RF191 in liquid MSMA. **(D)** Co-culture of *L. piscium* CNCM I-4031 and *L. monocytogenes* RF191 on MSMA plate.

at 37°C for 24 to 48 h. The virulence of the bacterial cells was evaluated by plaque-forming assay (PFA) in the cell monolayers using an optical microscope (VWR, Pennsylvania, United States). Controls consisted of HT-29 cells infected with serial dilutions of 10^8 to 10^2 UFC of *L. piscium* CNCM I-4031 or *L. monocytogenes* RF191 or ScottA per well. The whole experiment was repeated three times (different weeks) corresponding to nine independent tests.

Statistical Analyses

Statistical analyses on bacterial counts concentrations were performed using R software (R Core Team, 2014) by the analysis of one-way analysis of factor variance (ANOVA) followed by least significant difference (LSD) test.

RESULTS AND DISCUSSION

Inhibition Capacity of *L. piscium* CNCM I-4031 Strain Toward Various *L. monocytogenes* Strains

A total of 42 *L. monocytogenes* strains from diverse sources, and geographical origins were selected (**Table 1**). Most of them were isolated from seafood except the reference strains

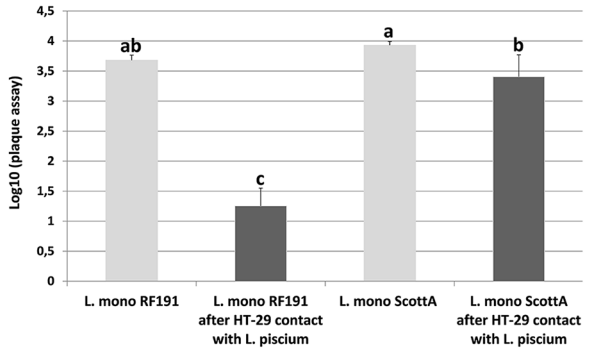

FIGURE 5 | *L. monocytogenes* plaque-forming assay (PFA) using HT-29 epithelial cells infected with strains ScottA and RF191 directly or after 1 h of pre-incubation with *L. piscium* CNCM I-4031. Plaques were counted in HT-29 cells infected with 10^5 UFC/ml of *L. monocytogenes*. No plaque was observed when *L. piscium* CNCM I-4031 was inoculated alone. Error bars indicate standard deviations from at least three independent experiments. ^{a,b,c}Represent groups determined with LSD test, a same letter indicates values not significantly different (p-value < 0.05 by one way-ANOVA).

ScottA (Fleming et al., 1985) and EGD-e (Murray et al., 1926) isolated during a listeriosis outbreak from human and animal tissue, respectively. The inhibition of *L. monocytogenes* strains was measured after 30 h of co-culture on microplates with *L. piscium* CNCM I-4031, in MSMA liquid medium. In these miniaturized experimental conditions, *L. monocytogenes* RF191 displayed an inhibition of 2.40 ± 0.35 log CFU/ml, which is lower than that previously described in larger culture volumes with an inhibition of 3–4 log CFU/ml (Saraoui et al., 2016). However, this screening test showed that *L. piscium* CNCM I-4031 could inhibit all *L. monocytogenes* strains, whatever their origin (**Figure 1**). The inhibition yield was strain-dependent, varying between 0.69 log CFU/ml for EU2162 and 3.72 log CFU/ml for EU2158. These values can be correlated with the low growth in MSMA medium of EU2262 in pure culture (7.3 log CFU/ml) and, in contrast, the strong growth of EU2158 (9.3 log CFU/ml). In mixed culture, after 30 h of incubation, the concentration of *L. monocytogenes* ranged between 5.6 and 7.1 log CFU/ml when cultivated alone and 7.3 and 9.5 in co-culture (**Figure 2**).

Influence of the Initial Ratio of *L. piscium* CNCM I-4031/*L. monocytogenes*

Previous studies have shown that *L. piscium* CNCM I-4031 can inhibit the growth of *L. monocytogenes* RF191 from 3 to 4 log units with an inoculum ratio of $10^6/10^3$ CFU/ml (*L. piscium*/*L. monocytogenes*) in shrimp at 8°C and in MSMA medium at 26°C (Fall et al., 2010; Saraoui et al., 2016). Considering that the concentration of pathogenic bacteria in food at the beginning of storage is low, protective bacteria are usually added at high concentrations to food products (Ananou et al., 2005; Brillet et al., 2005). In order to determine whether the inhibition was linked with these initial concentrations, three different initial ratios of *L. piscium*/*L. monocytogenes* RF191 were analyzed (**Figure 3**): $10^3/10^3$ CFU/ml (A), $10^5/10^3$ CFU/ml (B), and $10^6/10^6$ CFU/ml (C).

The growth kinetics of *L. piscium* in pure and co-culture were similar whatever the initial ratios. The maximum population density (MPD) of approximately 10^8 CFU/ml was reached after 40–48 h in (A), 25 h in (B), and 15 h in (C) with a growth rate between 0.32 to 0.45 h^{-1} (**Table 2**). *L. monocytogenes* growth started with an exponential phase and reached the MPD ($\sim 10^9$ CFU/ml) at 30 h in (A), 20 h in (B), and 15 h in (C) (**Table 2**). In each co-culture, the *L. monocytogenes* population increased to reach 10^8 to 10^9 CFU/ml until *L. piscium* achieved the MPD, then decreased (**Figure 3**). These results indicate that, regardless of the inoculum ratio of the two strains, *L. monocytogenes* was always significantly inhibited in co-cultures when *L. piscium* reached its MPD. This inhibition was proportional to the initial concentration of *L. piscium* and ranged from 1.42 to 3.37 log CFU/g (**Table 2**) with a higher inhibitory effect when the protective strain was inoculated at 10^5–10^6 CFU/ml. These observations are in accordance with previous studies with other bioprotective LAB isolated from seafood, such as a non-bacteriocin-producing *Carnobacterium piscicola* A9b (Nilsson et al., 2005) and *Lactobacillus sakei* 10A (Vermeiren et al., 2006b). Nevertheless, contrary to *L. sakei* 10A, which required an initial concentration of up to 10^5 CFU/g to inhibit *L. monocytogenes* effectively, *L. piscium* showed an inhibition with a low initial concentration of 10^3 CFU/ml.

The competition between LAB species and other populations in food and mixed cultures by one single "dominant" strain and when LAB have reached their maximum level is described in the literature as the "Jameson effect" (Jameson, 1962; Gimenez and Dalgaard, 2004). The Jameson effect is considered a race between species in order to maximize their growth by exploiting the environmental nutrients. Thus, the species that first reaches its MPD inhibits the growth of the other species (Mellefont et al., 2008; Cornu et al., 2011). However, in our experiments, we showed that the inhibition of *L. monocytogenes* by *L. piscium* occurred even when the concentration of the pathogenic bacteria was higher than that of the protective bacteria (**Figures 3A,C**). In these conditions, *L. monocytogenes* reached its MPD before *L. piscium* but no inhibition of *L. piscium* occurred. The same results were found when *L. monocytogenes* ScottA was co-inoculated with *Escherichia coli* whereas in co-culture with *Lactobacillus plantarum* or *Pseudomonas fluorescens*, the first strain reaching its MPD stopped the growth of the other one (Mellefont et al., 2008).

In a previous study, we demonstrated that the amount of lactic acid produced by *L. piscium* CNCM I-4031 in co-culture conditions was not responsible for the inhibition (Saraoui et al., 2016). Moreover, the supplementation of the co-culture with nutrients did not restore the ability of *L. monocytogenes* to grow, and no inhibition was observed when both cultures were separated by a 0.45-μm membrane. The present study evidenced that *L. piscium* could limit the growth of *L. monocytogenes* when it reached its maximum density, whatever its initial level.

Inhibition phenomena linked to maximum cellular concentration have been shown in other LAB, and some authors have suggested that they could involve quorum sensing (Kuipers et al., 2000; Risøen et al., 2000; Rohde and Quadri, 2006; Moslehi-Jenabian et al., 2011; Rizzello et al., 2014). In our case, the mechanism remains unknown and has still to be investigated.

Observation of Cells in Co-culture by Scanning Electron Microscopy

With the aim of investigating the behavior of the pathogenic bacteria in co-culture at the microscopic scale, the cell morphology of *L. piscium* CNCM I-4031 and *L. monocytogenes* RF191 was compared in monoculture in MSMA medium with co-culture conditions at the time of inhibition. The results presented in **Figure 4** show that *L. piscium* in monocultures were spherical (Elliker) or ovoid (MSMA) cells between 0.5 and 1 μm in diameter and appeared in pairs or short chains. *L. monocytogenes* were rod-shaped cells between 0.5 μm (MSMA) and 1.5 μm (mBHI) in length and ∼0.5 μm in width, appearing individually or in pairs (**Figure 4B**). For co-cultures, strains were observed in either liquid or solid media (**Figures 4C,D**). Co-cultures on solid media have already revealed the presence of nanotubes, which are extensions of the cytoplasmic membrane used by bacteria to exchange their cellular compounds in cell contact interactions (Dubey and Ben-Yehuda, 2011). These authors described intra-species nanotubes between cells of *Bacillus subtilis*, as well as inter-species connections between cells of *B. subtilis*, *S. aureus*, and *E. coli* using scanning electron microscopy. In the conditions of our experiments, such nanotubes were not observed between the cells. However, the pictures show that in the presence of *L. piscium*, *L. monocytogenes* cells appeared to be more elongated (**Figures 4C,D**). The elongation of *Listeria* cells in stress conditions (low pH, high salt concentration) has been reported in previous studies (Besnard et al., 2000; Bereksi et al., 2002). *Listeria* cells can divide without septation, leading to the modification of their surface properties with the presence of filamentous structures. Our observations also suggest that the surface of *L. monocytogenes* cells was damaged or completely altered in the presence of LAB (**Figures 4C,D**). These effects on *L. monocytogenes* morphology have been reported in previous studies dealing with the mechanism of action of anti-*Listeria* components (Dieuleveux et al., 1998).

Effect of *L. piscium* CNCM I-4031 on the Virulence of *L. monocytogenes*

As an important pathogen, *L. monocytogenes* encompasses a large spectrum of strains with varying virulence effects (Buchanan et al., 2017). In order to examine another aspect of the bioprotective effect of *L. piscium*, the impact on *L. monocytogenes* virulence was investigated. For this purpose, the virulence of two selected *L. monocytogenes* strains, RF191 isolated from seafood and ScottA known to be a highly virulent strain (Lindback et al., 2010), were tested using an HT-29 cell PFA, in the presence or absence of *L. piscium*. On an HT-29 monolayer pre-treated with 10^8 *L. piscium*, no lysis plates were detected after 24 or 48 h confirming that *L. piscium* has no pathogenic activity. Plaques were counted in the well containing HT-29 cells infected by 5 log CFU/ml of *L. monocytogenes* ScottA or RF191 strains. The ScottA strain formed large deep plaques whereas the RF191 strain formed small shallow ones. The mean log PFA values were 3.93 ± 0.06 and 3.68 ± 0.08, respectively (**Figure 5**). According to the study of Roche et al. (2001), the RF191 strain that forms more than 3.34 log plaques should be considered virulent, even though it remains less virulent than the ScottA strain. When HT-29 cells were infected by *L. monocytogenes* strains after being treated by *L. piscium*, the mean log PFA values were 3.40 ± 0.36 and 1.25 ± 0.29 for ScottA and RF191 strains, respectively (**Figure 5**). A significant effect was found for the RF191 strain that had lost its virulence after pre-treatment with *L. piscium* CNCM I-64031. A slight but a significant decrease in virulence was also observed for the ScottA strain, suggesting that the effect of the protective bacteria on *L. monocytogenes* virulence is strain-dependent. The effect on virulence reduction by LAB has already been described for foodborne pathogens such as *L. monocytogenes* (Garriga et al., 2015; Pilchova et al., 2016) or *Campylobacter* (Alemka et al., 2010). The effect is usually investigated as one of the probiotic properties of bacteria; however, such additional properties also increase the bioprotective value of strains and their safety assessment.

CONCLUSION

In this study, we have demonstrated the ability of *L. piscium* CNCM I-4031 to inhibit a large collection of *L. monocytogenes* strains. This inhibition occurs whatever the initial concentration of the protective strain when *L. piscium* has reached its MPD. The inhibition mechanism is still under investigation but our different sets of results suggest that it requires the proximity of cells and affects the cellular surface of the targeted bacteria. In addition to inhibiting *L. monocytogenes* growth, *L. piscium* decreased *L. monocytogenes* virulence with a variable effect according to the strain. This study provides additional knowledge about the inhibitory activity of a non-bacteriocin-producing LAB toward *L. monocytogenes* and significant information for the potential use of *L. piscium* CNCM I-4031 to control *L. monocytogenes* in seafood products.

AUTHOR CONTRIBUTIONS

M-FP and FL designed the work. TS, DP, and FC performed the experiments. TS and FL assisted with the scanning electron microscopy analysis. TS, M-FP, FL, DP, and J-MC analyzed the data. TS drafted the paper. MF-P, FL, and DP wrote the final version of the manuscript, which was read and approved by all authors.

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Effect of Pressure, Reconstituted RTE Meat Microbiota, and Antimicrobials on Survival and Post-pressure Growth of *Listeria monocytogenes* on Ham

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Pressure treatment of ready-to-eat (RTE) meats extends the shelf life and reduces risks associated with *Listeria monocytogenes*. However, pressure reduces numbers of *Listeria* on ham by less than 5 log (CFU/g) and pressure effects on other meat microbiota are poorly documented. This study investigated the impact of pressure and RTE meat microbiota, with or without nisin and rosemary oil, on survival of *Listeria* after refrigerated storage. Ham was inoculated with a 5-strain cocktail of *L. monocytogenes* alone or with a cocktail of RTE meat microbiota consisting of *Brochothrix thermosphacta*, *Carnobacterium maltaromaticum*, *Leuconostoc gelidum*, and *Lactobacillus sakei*. Products were treated at 500 MPa at 5°C for 1 or 3 min, with or without rosemary extract or nisin. Surviving cells were differentially enumerated after pressure treatment and after 4 weeks of refrigerated storage. After 4 weeks of storage, products were also analyzed by high throughput sequencing of 16S rRNA amplicons. Pressure treatment reduced counts of *Listeria* by 1 to 2 log (CFU/g); inactivation of RTE meat microbiota was comparable. Counts of *Listeria* increased by 1–3 log (CFU/g) during refrigerated storage. RTE meat microbiota did not influence pressure inactivation of *Listeria* but prevented growth of *Listeria* during refrigerated storage. Rosemary extract did not influence bacterial inactivation or growth. The combination of nisin with pressure treatment for 3 min reduced counts of *Listeria* and meat microbiota by >5 log (CFU/g); after 4 weeks of storage, counts were below the detection limit. In conclusion, pressure alone does not eliminate *Listeria* or other microbiota on RTE ham; however, the presence of non-pathogenic microbiota prevents growth of *Listeria* on pressure treated ham and has a decisive influence on post-pressure survival and growth.

Keywords: high pressure processing, nisin, *Listeria monocytogenes*, *Leuconostoc gelidum*, *Lactobacillus sakei*, meat microbiota, antimicrobials, ready-to-eat meat

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INTRODUCTION

Processing of packaged RTE meats products with high pressure is used by the meat industry to eliminate *Listeria monocytogenes*. Pressure processing does not alter the quality of RTE meats quality and is thus considered an attractive alternative to chemical preservatives. High pressure treatment disrupts bacterial membranes, and when used after packaging can be an effective method to reduce the overall microbial load and to extend the storage life of RTE

products (Chen et al., 2012). Pressure treatment of RTE meat does generally not achieve a 5 log reduction of cell counts of *L. monocytogenes*, the most significant food safety concern associated with RTE meats. Treatment at 450 MPa reduced cell counts of *L. monocytogenes* by 1–3 log CFU/g (Chung et al., 2005; Morales et al., 2006). Treatments on ham at 500 or 600 MPa reduced cell counts by 3–4 log (CFU/g) when the product was tempered to refrigeration temperature prior to pressure treatment (Teixeira et al., 2016). Moreover, the decrease in microbial viability after pressure treatments is partially compensated by recovery of sublethally injured *L. monocytogenes* during post-pressure refrigerated storage (Bull et al., 2005; Marcos et al., 2008; Juck et al., 2012; Muñoz-Cuevas et al., 2013; Teixeira et al., 2016).

Listeria monocytogenes can recover even after the application of more than 600 MPa, the upper pressure limit of current commercial equipment for pressure treatment (Marcos et al., 2008; Jofré et al., 2010). Therefore, the combination of high pressure processing with other hurdles for microbial growth in the food product is needed to warrant a sufficient pathogen reduction and shelf life extension. The use of natural antimicrobials with high pressure processing provides additional food safety assurance while providing a clean label product. Essential oils and bacteriocins from protective cultures have been used in combination with high pressure for improved control of *L. monocytogenes* on RTE meats (Jofré et al., 2008; Marcos et al., 2008; for review, see de Oliveira et al., 2015); however, even the application of hurdle technologies may not reduce cell counts of *L. monocytogenes* by more than 5 log, or prevent re-growth during storage (Marcos et al., 2008; Hereu et al., 2012; de Oliveira et al., 2015). Moreover, past studies did not account for the presence of other meat microbiota that may contribute to spoilage, or to prevention of growth of *L. monocytogenes* during post-treatment refrigerated storage.

Microbiota of refrigerated meat and vacuum-packaged RTE are dominated by *Brochothrix thermosphacta*, *Carnobacterium* spp. and other lactic acid bacteria including psychrotrophic lactobacilli and *Leuconostoc* spp. (Susiluoto et al., 2003; Leisner et al., 2007; Miller et al., 2015). Because RTE products are cooked prior to slicing, package and storage, the microbiota of commercial vacuum packaged RTE products typically has a low diversity (Miller et al., 2015). The extension of the refrigerated storage life of RTE meats by pressure processing requires the control of psychrotrophic spoilage microbiota, or a shift toward growth of microorganisms that do not negatively affect product quality. Reproducible data on pressure effects on non-pathogenic meat microbiota requires experimentation with defined, controlled and reproducible inocula; however, pressure effects on defined strains of meat spoilage organisms or protective cultures *in vitro* or *in situ* are poorly documented (Ulmer et al., 2000; Liu et al., 2012). Moreover, competitive meat microbiota may inhibit growth of *L. monocytogenes*. Inhibition of the growth of *L. monocytogenes* by lactic acid bacteria was often attributed to the production of bacteriocins (Schillinger et al., 1991) but organic acid production by lactic acid bacteria may suffice for inhibition of growth (Bredholt et al., 1999). The role of competitive meat microbiota on post-pressure growth and survival of *L. monocytogenes* has not been systematically explored. Therefore, this study aimed to investigate the impact of pressure, reconstituted non-pathogenic meat microbiota, and antimicrobials on the survival and post-pressure growth of *L. monocytogenes* on RTE ham.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions

A cocktail of strains containing *L. monocytogenes* FSL J1-177, FSL C1-056, FSL N3-013, FSL R2-499, and FSL N1-227 was used as the "human disease cocktail" recommended for challenge studies in food (Fugett et al., 2006). A cocktail of non-pathogenic RTE meat microbiota contained *B. thermosphacta* FUA3558, *Carnobacterium maltaromaticum* FUA3559, *Leuconostoc gelidum* FUA3560 and FUA3561, and *Lactobacillus sakei* FUA3562. These strains were previously isolated from RTE meats (Miller et al., 2015) and are a reasonable representation of the composition and diversity of microbiota that is recovered from vacuum-packaged and refrigerated RTE meats. *L. monocytogenes* were streaked from −80°C stock cultures onto Tryptic Soy (TS) agar (Difco, Becton Dickinson, Sparks, MD, United States), followed by an overnight subculture in TS broth (TSB), and a second sub-culture with 1% (v/v) inoculum and 16 h incubation. *Listeria* were routinely incubated at 37°C. RTE meat microbiota were prepared in the same manner but grown on All Purpose Tween (APT) agar or broth and incubated at 25°C. For preparation of cocktails, an equal volume of each individual culture was mixed to form a 5-strain cocktail of *L. monocytogenes* or meat microbiota. Strain cocktails were harvested by centrifugation and resuspended in saline (0.85% NaCl) to achieve an optical density at 600 nm of 1.0.

Preparation of Ham and Antimicrobials

The study used cooked ham with a NaCl concentration of 3% (w/w). The ham was produced experimentally using a product composition and ingredients that match commercial practice in Canada and the United States (Teixeira et al., 2016) Stock solutions of a commercial rosemary extract (NatureGuard^{TM}, Newly Weds Foods, Edmonton, AB, Canada) and a 2.5% nisin preparation (>1,000,000 IU/g Sigma-Aldrich, St. Louis, MO, United States) for determination of the minimum inhibitory concentrations were prepared in 50% ethanol and 0.02 N hydrogen chloride (HCl), respectively, to a final concentration of 10% (v/v) and 0.05% (w/v), respectively. Stock solutions of rosemary extract and the 2.5% nisin preparation for surface inoculation of ham were prepared in 0.5% ethanol and 0.02 N HCl, respectively, for a final concentration of 3.3% (v/v) and 175 mg/L, respectively.

Determination of the Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentrations (MBCs)

Critical dilution assays were used to determine the minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of rosemary extract and nisin

against strains of *L. monocytogenes, B. thermosphacta*, *C. maltaromaticum*, *L. gelidum,* and *L. sakei*. In brief, 100 μl of TS or APT broth was added to each well of a 96 well microtiter plate. Rosemary and nisin stock solutions (100 μl) were added to separate wells and serially diluted across the plate to obtain concentrations ranging from 0.003 to 3% (rosemary extract) and 0.00002 to 0.02% (nisin). Stationary phase cultures of indicator strains were 10-fold diluted in TS or APT broth to a final concentration of about 10^8 CFU/mL, and microtiter plates were inoculated with 50 μL of diluted cultures. Plates were incubated at 37 (*Listeria*) or 25°C (RTE meat microbiota) for 24 h. To determine the MBC, 100 μl TS or APT broth was pipetted into all wells of a sterile 96 well microtiter plate. After 24 h of incubation of the MIC plates, 10 μl of each well were transferred to a well on the MBC plate. The plates were incubated at the same conditions as the MIC plates. For a better visualization of the bacteria growth in MIC and MBC plates, 40 μl of 0.2 mg/mL p-iodonitrotetrazolium violet (INT; Sigma-Aldrich) was added to each well and incubated for 3 h at 37 (*Listeria* strains) or 25°C (RTE meat microbiota). In the wells that remained colorless after incubation with INT, no bacterial growth was detectable (Eloff, 1998). Experiments were performed in triplicate.

Sample Preparation and Inoculation for Pressure Treatment

Cooked ham was cut to 20-mm-thick slices with a surface area of 50 cm^2, vacuum packaged, and stored at 0°C until use. Ham samples were shaped, inoculated and heat-sealed as described (Teixeira et al., 2016). All handling of ham was performed under sterile conditions to prevent microbial contamination, Ham was inoculated with the cocktail of *L. monocytogenes* strains and/or the cocktail of RTE meat microbiota to achieve cell counts of about 10^7 CFU *Listeria*/g and/or 10^8 CFU RTE meat microbiota/g. Experimental groups were categorized as follows: (i) *L. monocytogenes*, (ii) RTE meat microbiota, and (iii) *L. monocytogenes* combined with RTE meat microbiota. After inoculation, the ham was placed in Tygon tubing (Tygon S3™ E-3603 Flexible Tubings, Fisherbrand™, Pittsburgh, PA, United States) as described (Teixeira et al., 2016). Each of the three experimental groups were treated with antimicrobials and a solvent control by addition of 20 μL of rosemary extract or 20 μL of nisin to achieve a volumetric concentration exceeding the MIC values against the five strains of *L. monocytogenes* 5- to 20-fold. Antimicrobials were added into the Tygon tubing, the tubing was then heat-sealed and then massaged. Because the ham was surface-inoculated with bacterial strains and the antimicrobial were also added to the surface of the ham, the concentration of antimicrobials was expressed relative to the surface area rather than the volume or weight of the samples. The addition of antimicrobials as described above provided a final surface concentration of ∼325 μg/cm^2 of rosemary or ∼2 μg/cm^2 of nisin preparation on the ham samples. To assess the impact of the solvents used for addition of rosemary oil and nisin, solvent controls were prepared by addition of either 20 μL 0.5% ethanol or 20 μL 0.02 N HCl. Samples were maintained at ambient temperature until pressure treatment; after placement of samples in the pressure vessel, the temperature was equilibrated to the treatment temperature for 10 min prior to compression.

Pressure Treatment

Pressure treatments were carried out in a 2.2 mL high pressure vessel immersed in a temperature-controlled water bath (Teixeira et al., 2016). Initial temperature in the vessel was 5°C and the temperature increase in the pressure vessel during compression was less than 5°C. Ham samples were treated at 500 MPa at 5°C for 1 or 3 min (Teixeira et al., 2016). Detection of surviving cells was determined by surface plating as described below. Additionally, treated samples were stored for 4 weeks at 4°C after treatment, and the presence of viable cells was detected as outlined below. Experiments were performed in triplicate.

Detection of Surviving Cells

The presence or absence of *L. monocytogenes* and/or RTE microbiota was monitored immediately after pressure treatment and after storage for 4 weeks at 4°C. Untreated samples were analyzed to determine the initial cell count. Un-inoculated ham samples were prepared and stored for 4 weeks at 4°C to ensure the absence of contaminating microbiota from the meat prior the experiment and after storage. Samples were opened aseptically, and the contents were transferred to a sterile 1.5 mL Eppendorf tube and diluted with sterile saline (0.85% NaCl). Ham samples were homogenized for 60 s prior to serial dilutions.

Surviving cells were enumerated by surface plating on selective PALCAM agar (Becton Dickinson) (*L. monocytogenes* plus RTE meat microbiota) and on non-selective TS (*L. monocytogenes*) or APT agar (RTE meat microbiota and *L. monocytogenes* plus RTE meat microbiota). Appropriate dilutions were plated and incubated at 37°C (PALCAM and TS agar) or 25°C (APT agar) for 48 h.

Extraction of Total DNA and Amplification of Genes Coding for 16S rRNA

DNA was isolated from approximately 20 mg ham by washing of samples with 1 mL saline (0.85% NaCl), followed by centrifugation (5000 × *g* for 10 min) to collect bacterial cells. DNA was extracted from the pellet using DNeasy Blood and Tissue Kit (Qiagen, Toronto, ON, Canada) following the Gram-positive bacteria protocol provided by the manufacturer. Amplicons of the V5–V6 region of 16S rRNA genes obtained with the barcoded primer pair F (5′-GTGCCAGCMGCCGCGGTA A-3′) and R (5′-GGACTACHVGGGTWTCTAAT-3′) (Caporaso et al., 2012) were sequenced on the Illumina MiSeq 2 × 300 bp platform (University of Minnesota Genomics Center; UMGC). Raw sequences were quality filtered with the FastQC tool. Subsequently, the USEARCH pipeline (v9.0.2132, Edgar, 2010) was used to trim and merge pair-end reads, and to sort the sequences into operational taxonomic units (OTUs). OTUs accounting for less than 0.05 % of the total sequences were discarded. Taxonomic classification for each OTU was determined with Ribosomal Database Project (RDP) Classifier 11.1 tool and NCBI database. The relative abundance of bacterial

taxa was calculated from three independent replicate samples as percent proportions relative to the total number of sequences in each sample.

Statistical Analysis

One-way analysis of variance (ANOVA) was performed on SigmaPlot Software to test for significant differences in inhibitory activities of rosemary extract and nisin, and the bactericidal effect of pressure treatments. Significance was assessed with an error probability of 5% ($P \leq 0.05$). Principle component analysis (PCA) was performed using JMP software (version 13.1.0, SAS Institute Inc., Cary, NC, United States) to examine correlations between gene copies of bacterial groups, pressure treatments and antimicrobials.

RESULTS

Inhibitory Activity of Rosemary Extract and Nisin Against *L. monocytogenes* and Competitive Microbiota

Treatments with rosemary extract and nisin were standardized with respect to the antimicrobial activity of these two inhibitors. Therefore, the inhibitory and bactericidal concentrations of rosemary extract and nisin against *Listeria* and RTE meat microbiota were determined. The bactericidal concentration of rosemary extract and nisin was equal or 2- to 3-fold higher than the inhibitory concentration (**Table 1**). Rosemary extract was inhibitory at levels ranging from 1 to 10 g/l; the MIC of nisin ranged from 0.1 to 10 mg/l, corresponding to 2.5–250 μg/l pure nisin. *L. monocytogenes* was significantly more resistant to nisin than other organisms (**Table 1**). Lactic acid bacteria exhibited the highest resistance to rosemary extract and *B. thermosphacta* was most sensitive (**Table 1**).

Effect of Pressure and Reconstituted Microbiota on Survival and Post-pressure Growth of *L. monocytogenes* on Ham

All pressure treatments and storage experiments in this study were performed in aseptically prepared ham containing 3% NaCl. Plating of ham without inoculum confirmed that bacterial contaminants were absent before and after storage (data not shown). The lethality of pressure and antimicrobials was assessed on ham inoculated only with a 5-strain cocktail containing *L. monocytogenes*, a 5-strain cocktail of RTE meat microbiota, or with a combination of both strain cocktails.

Interactions of Competitive Microbiota and Rosemary Extract on the Lethality of Pressure Against *L. monocytogenes*

The effect of addition of rosemary extract in 0.5% ethanol on the lethality of pressure was compared to addition of a 0.5% ethanol solution as solvent control (**Figure 1**). Cell counts of control samples containing 0.5% ethanol were comparable to cell counts of control sample without any additives (**Supplementary Figures S1**, **S2**). *L. monocytogenes* counts were reduced by about 1 and 2.5 CFU/g after treatment at 500 MPa for 1 and 3 min, respectively, when ham was inoculated with the *L. monocytogenes* cocktail only (**Figure 1A**). The pressure inactivation of RTE meat microbiota was comparable (**Figure 1B**). Counts of *L. monocytogenes* on pressure-treated samples did not increase during refrigerated storage, while the RTE meat microbiota grew to high cell counts of 10^8–10^9 CFU/g during post-pressure refrigerated storage (**Figure 1**).

The addition of rosemary extract did not affect cell counts or growth of *Listeria* or other organisms (**Figure 1**). In combination with pressure at 1 or 3 min, and after 4 weeks of storage at 4°C, rosemary did not affect cell counts of *Listeria* or meat microbiota compared to samples that were subjected to pressure without antimicrobial (**Figure 1**). After 4 weeks of refrigerated storage, all of the samples inoculated with RTE meat microbiota had high cell counts suggesting that rosemary was ineffective in preventing the growth of microorganisms during refrigerated storage (**Figure 1B**).

The interaction of RTE meat microbiota and antimicrobials on the lethality of pressure was further investigated on ham that was inoculated with both strain cocktails, the *Listeria* cocktail and the RTE meat microbiota. The total cell counts of ham that was inoculated with both cocktails and treated at 500 MPa were comparable to the cell counts of pressure treated ham with RTE meat microbiota only (**Figures 1B**, **2A**). After 4 weeks of refrigerated storage, RTE microbiota grew to high cell counts (**Figure 2A**) irrespective of the addition of rosemary extract. Growth of RTE meat microbiota inhibited the growth of *L. monocytogenes* regardless of the presence of rosemary extract (**Figure 2B**). Treatment of ham with 500 MPa for 3 min in presence of RTE meat microbiota reduced cell counts of *Listeria* to levels below the detection limit after 4 weeks of storage (**Figure 2B**). Of note, the enumeration of *L. monocytogenes* on selective PALCAM agar immediately after pressure treatment may not accurately reflect total cell counts because sublethally injured cells are not accounted for. However, 4 weeks of refrigerated storage suffice to repair sublethal injury in the psychrotrophic *L. monocytogenes* and cell counts on selective PALCAM agar after 4 weeks of storage thus accurately reflect total cell counts (Teixeira et al., 2016).

Interactions of RTE Meat Microbiota and Nisin on the Lethality of Pressure

The effect of nisin in 0.02 N HCl on the lethality of pressure was compared to addition of 0.02 N HCl as solvent control (**Figure 3**). Cell counts of control samples containing 0.02 N HCl were comparable to cell counts of control samples containing 0.5% ethanol (**Figure 1**) or samples without any additive other than the inoculum (**Supplementary Figure S1**).

The combined effect of pressure and nisin was evaluated by treatments at 500 MPa for 1 and 3 min. Nisin reduced cell counts by 2 log (CFU/g) without pressure treatment but did not prevent growth of *L. monocytogenes* during refrigerated storage (**Figure 3A**). In combination with pressure treatment for 3 min, nisin increased the inactivation of *L. monocytogenes*

TABLE 1 | MIC and MBC of nisin and rosemary against *L. monocytogenes* and RTE meat microbiota.

Microorganisms	Rosemary extract		Nisin[1]	
	MIC (g/l)	MBC (g/l)	MIC (mg/l)	MBC (mg/l)
L. monocytogenes FSL J1-177	3.65 ± 1.04^{a}	6.94 ± 2.41^{a}	10.42 ± 0.00^{a}	10.42 ± 0.00^{a}
L. monocytogenes FSL R2-499	2.08 ± 0.00^{ab}	4.17 ± 0.00^{a}	5.21 ± 0.00^{ab}	10.42 ± 0.00^{a}
L. monocytogenes FSL C1-056	2.08 ± 0.00^{ab}	4.17 ± 0.00^{a}	6.94 ± 3.01^{ab}	10.42 ± 0.00^{a}
L. monocytogenes FSL N1-227	1.82 ± 0.52^{b}	5.56 ± 2.41^{a}	8.68 ± 3.01^{ab}	10.42 ± 0.00^{a}
L. monocytogenes FSL N3-013	2.60 ± 1.04^{ab}	4.17 ± 0.00^{a}	3.47 ± 1.50^{b}	13.89 ± 6.01^{a}
Brochothrix thermosphacta FUA3558	1.04 ± 0.00^{B}	1.39 ± 0.60^{C}	1.52 ± 0.99^{AB}	0.87 ± 0.38^{B}
Carnobacterium maltaromaticum FUA3559	4.17 ± 0.00^{A}	3.47 ± 1.20^{B}	0.33 ± 0.00^{B}	0.87 ± 0.38^{B}
Leuconostoc gelidum FUA3560	4.17 ± 0.00^{A}	4.17 ± 0.00^{B}	2.60 ± 0.00^{AB}	4.34 ± 1.50^{A}
Leuconostoc gelidum FUA3561	4.17 ± 0.00^{A}	4.17 ± 0.00^{B}	3.47 ± 1.50^{A}	3.47 ± 1.50^{AB}
Lactobacillus sakei FUA3562	4.17 ± 0.00^{A}	8.33 ± 0.00^{A}	0.87 ± 0.38^{B}	1.52 ± 0.99^{AB}

Data are shown as means ± standard deviations of triplicate independent experiments. Within each column, means with different letters are significantly different ($P < 0.05$); small letters within Listeria strains, capital letters within competitive organisms. MIC, minimum inhibitory concentrations; MBC, minimum bactericidal concentration.
[1]Indicated in the concentration of the nisin preparation containing 2.5% nisin.

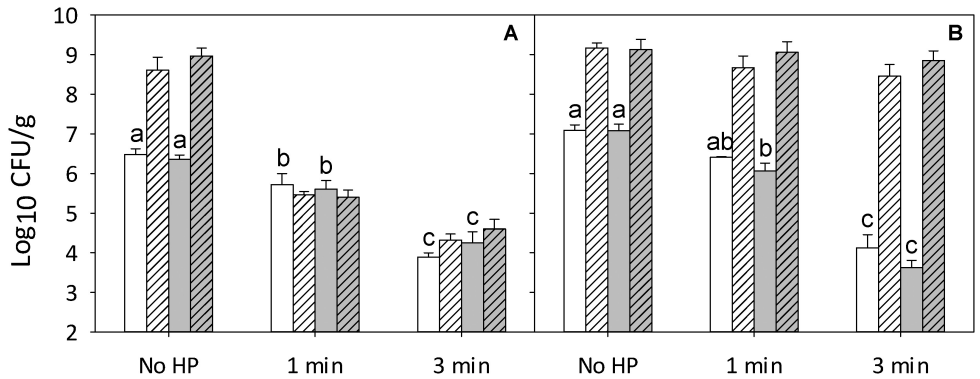

FIGURE 1 | Effect of pressure on survival and post-pressure growth of *L. monocytogenes* and RTE meat microbiota on ham treated with rosemary extract or an equivalent volume of 0.5% ethanol as solvent control. Shown are cell counts of ham inoculated with a 5-strain cocktail of *L. monocytogenes* **(A)** or RTE meat microbiota **(B)** after treatment at 500 MPa for 1 or 3 min without (white) or with (gray) rosemary extract at ∼325 μg/cm^2. Cell counts of *L. monocytogenes* and other strains were monitored immediately after pressure treatment (solid columns) and after storage for 4 weeks at 4°C (hatched columns). Surviving cells were enumerated by surface plating on non-selective TS (*L. monocytogenes*) or APT agar (RTE meat microbiota). Data are shown as means ± standard deviations of triplicate independent experiments. Treatment means (solid columns) within each panel with different letters are significantly different ($P < 0.05$). The interception point of abscissa and ordinate represents the highest detection limit.

by more than 2 log (CFU/g) compared to samples subjected to pressure alone, and reduced counts of *L. monocytogenes* to levels below the detection limit of 2 log (CFU/g) after 28 days of storage at 4°C (**Figure 3A**). RTE meat microbiota were more resistant to nisin than *L. monocytogenes* (**Figure 3B**). After 4 weeks of refrigerated storage, untreated samples inoculated with RTE meat microbiota had high cell counts, suggesting that nisin alone did not prolong the storage life (**Figure 3B**). In combination with a 3 min pressure treatment, nisin reduced cell counts of RTE meat microbiota to levels below the detection limit of 2 log (CFU/g) after 28 days of storage at 4°C (**Figure 3B**).

The interaction of competitive microbiota and nisin on the lethality of pressure was further investigated on ham surface-inoculated with *Listeria* and RTE meat microbiota. The cocktail of RTE meat microbiota alone prevented growth of *Listeria* refrigerated storage even without pressure treatment or addition of nisin (**Figure 4B**). Pressure effects on RTE meat microbiota were comparable to those effects that were observed with ham containing RTE meat microbiota only (**Figures 3B**, **4A**). After 4 weeks of refrigerated storage, total cell counts exceeded 8 log (CFU/g) (**Figure 4A**). Pressure treatment of ham for 1–3 min eliminated *Listeria* after 4 weeks of storage when RTE meat microbiota were also present (**Figure 4B**). The effect of nisin on total cell counts of ham containing both strain cocktails was comparable to the effect of nisin ham inoculated with RTE meat microbiota only (**Figures 3B**, **4B**). Nisin in combination with pressure reduced cell counts to less than 3 log (CFU/g) and prevented growth during refrigerated storage (**Figure 4A**). Combination of nisin with 1 or 3 min pressure treatment in presence of RTE meat microbiota reduced cell counts of *L. monocytogenes* to levels below the detection limit after 4 weeks of refrigerated storage (**Figure 4B**). The presence of non-pathogenic meat microbiota thus not only inhibited growth

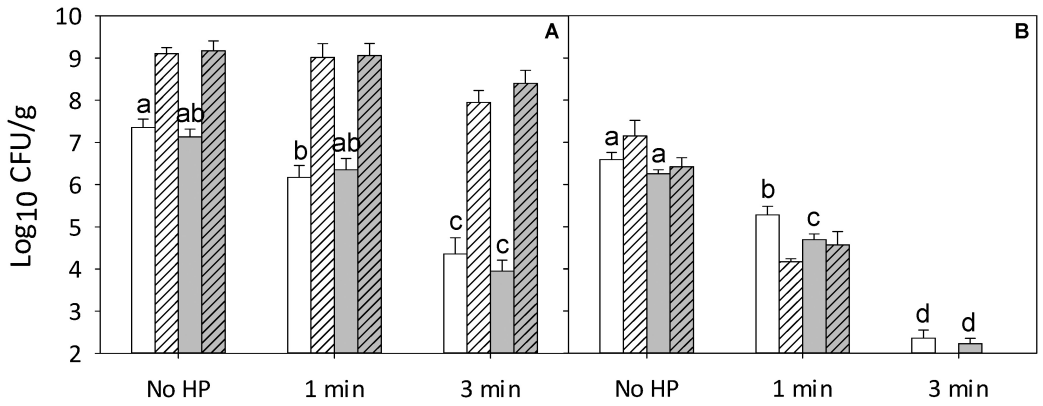

FIGURE 2 | Effect of pressure and RTE meat microbiota on survival and post-pressure growth of *L. monocytogenes* and RTE meat microbiota on ham treated with 0.5% ethanol (solvent control) or an equivalent volume of rosemary extract in 0.5% ethanol. Shown are total cell counts **(A)** on non-selective agar and cell counts of *L. monocytogenes* **(B)** enumerated on selective PALCAM **(B)** agar. Samples were treated at 500 MPa for 1 or 3 min without (white) or with (gray) rosemary at ~325 μg/cm^2. Cell counts were enumerated immediately after pressure treatment (solid columns) and after storage for 4 weeks at 4°C (hatched columns). Data are shown as means ± standard deviations of triplicate independent experiments. Treatment means (solid columns) within each panel with different letters are significantly different ($P < 0.05$). The interception point of abscissa and ordinate represents the highest detection limit.

of *L. monocytogenes* even without pressure or antimicrobials, but also increased the efficacy of pressure and antimicrobial compounds.

Composition of Ham Microbiota Assessed by High Throughput Sequencing of 16S rRNA Sequence Tags

The cocktail of RTE meat microbiota contains species that differ with respect to their resistance to pressure and antimicrobials, and with respect to their impact on product quality. To characterize the composition of ham microbiota after pressure processing and refrigerated storage, 16S rRNA tags were sequenced by paired end Illumina sequencing. The average and median number of reads per sample after quality control and trimming was 34,000 reads per sample; the relative abundance of bacterial taxa is reported as average ± standard deviation of three independent replicates (**Table 2**). The microbiota of ham without additions matched the microbiota of control ham with addition of 0.5% ethanol or 0.02 N HCl (**Table 2** and **Supplementary Table S1**). Sequences matching species present in the inoculum accounted for more than 99.9% of sequences in most samples, demonstrating that the aseptic preparation of ham achieved control of ham microbiota throughout sample processing and storage (**Table 2** and **Supplementary Table S1**). The only exceptions pertain to ham that was pressure treated in presence of nisin. In these samples, total cell counts were below 3 log (CFU/g) and sequencing of 16S rRNA sequence tags likely also accounted for DNA of dead bacterial cells that carried over from raw material used for ham processing. In keeping with the cell count data, sequences matching *L. monocytogenes* were below 1% of sequences in all samples. Again, exceptions pertain to ham that was pressure treated in presence of nisin; here, the relative abundance of bacterial DNA reflects the proportion of species in the inoculum and sequences matching *L. monocytogenes* accounted for about 10% of sequences (**Table 2**).

The predominant species in untreated ham after 4 weeks were *B. thermosphacta, C. maltaromaticum*, and *L. gelidum*; *L. sakei* was less abundant. The use of rosemary extract did not influence the composition of meat microbiota. Pressure treatment increased the relative abundance of *B. thermosphacta* and nisin shifted the composition of ham microbiota toward a higher abundance *L. gelidum*.

Multivariate Data Analysis of the Composition of Ham Microbiota After Storage

The relationship between the composition of meat microbiota, pressure treatment, and addition of antimicrobials was further assessed using principal component analysis (PCA) (**Figure 5**). The loading plot separated samples into four main clusters comprising pressure treated ham, untreated ham, ham prepared with nisin, and pressure treated ham prepared with nisin (**Figure 5**). The loading plot of individual bacterial taxa demonstrated that the separate clustering of pressure treated ham related to the high abundance of *B. thermosphacta;* separate clustering of nisin-treated samples related to a high abundance of *L. gelidum*. The abundance of bacterial species in pressure treated ham containing nisin essentially represents the inoculum prior to pressure treatment and their location in the linear discriminant analysis plot thus relates to *Carnobacterium*, *Lactobacillus*, and *Listeria*.

DISCUSSION

Pressure processing of RTE meats at cold or ambient temperatures does not consistently result in a 5-log reduction of *L. monocytogenes* and surviving cells are able to grow during

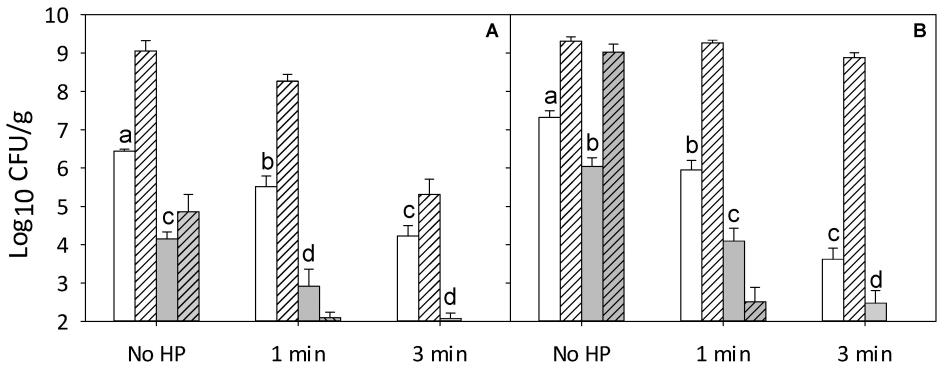

FIGURE 3 | Effect of pressure on survival and post-pressure growth of *L. monocytogenes* and RTE meat microbiota on ham. Ham was inoculated with a 5-strain cocktail of *L. monocytogenes* **(A)** or RTE meat microbiota **(B)** and treated at 500 MPa for 1 or 3 min with 0.02 N HCl (solvent control) or an equivalent volume of a nisin preparation in 0.02 N HCl to achieve a concentration of 2 μg/ cm^2, corresponding to 0.05 μg/cm^2 pure nisin. Cell counts of *L. monocytogenes* and RTE meat microbiota were monitored immediately after pressure treatment (solid columns) and after storage for 4 weeks at 4°C (hatched columns). Surviving cells were enumerated on non-selective TS (*L. monocytogenes*) or APT agar (other strains). Data are shown as means ± standard deviations of triplicate independent experiments. Treatment means (solid columns) within each panel with different letters are significantly different ($P < 0.05$). The interception point of abscissa and ordinate represents the highest detection limit.

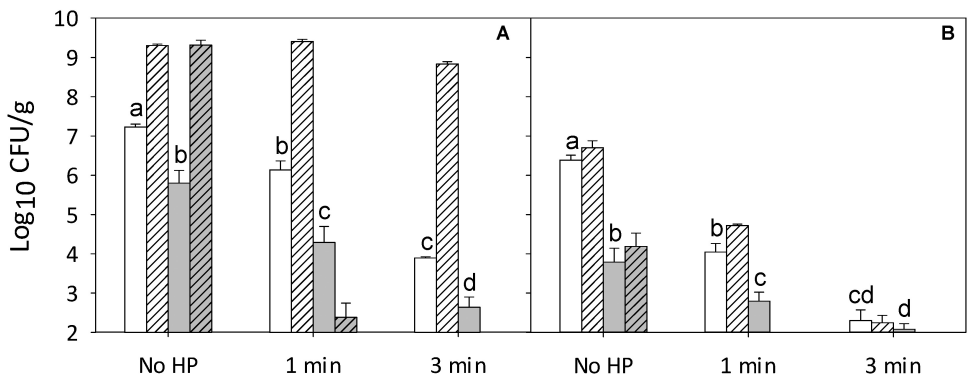

FIGURE 4 | Effect of pressure and RTE meat microbiota on survival and post-pressure growth of *L. monocytogenes* and RTE meat microbiota on ham treated with 0.02 N HCl (solvent control) or an equivalent volume of a nisin preparation in 0.02 N HCl to achieve a concentration of 2 μg/cm^2, corresponding to 0.05 μg/cm^2 pure nisin. Shown are total cell counts **(A)** on non-selective agar and cell counts of *L. monocytogenes* **(B)** enumerated on selective PALCAM **(B)** agar. Samples were treated at 500 MPa for 1 or 3 min without (white) or with (gray) nisin. Cell counts ere enumerated immediately after pressure treatment (solid columns) and after storage for 4 weeks at 4°C (hatched columns). Data are shown as means ± standard deviations of triplicate independent experiments. Treatment means (solid columns) within each panel with different letters are significantly different ($P < 0.05$). The interception point of abscissa and ordinate represents the highest detection limit.

subsequent refrigerated storage (Jofré et al., 2010; Teixeira et al., 2016). This is not satisfactory for the requirements of the industry to guarantee product safety and extended storage life. An alternative approach to inhibit *L. monocytogenes* in RTE meats comprises the combination of high pressure and natural antimicrobials (Marcos et al., 2008; Hereu et al., 2012; de Oliveira et al., 2015). This study assessed the impact of pressure, RTE meat microbiota, and antimicrobials on survival and growth of *L. monocytogenes* on ready-to-eat ham. The ham was custom produced for use in challenge studies to obtain a product that matches commercial RTE ham but is free of preservatives or other antimicrobials (Liu et al., 2014; Teixeira et al., 2016). Aseptic preparation of ham slices for pressure treatment allowed control of microbiota throughout treatment and 4 weeks of storage. Products were inoculated with a 5 strain cocktail of non-pathogenic ham microbiota containing *B. thermosphacta*, *L. gelidum*, *C. maltaromaticum* and *L. sakei* to obtain a defined and controlled inoculum, and to match the diversity and composition of microbiota on vacuum packaged RTE meat products that are stored at refrigeration temperature (Susiluoto et al., 2003; Leisner et al., 2007; Miller et al., 2015). After storage, sampled contained only those organisms that were used as inoculum, i.e., *L. monocytogenes* and/or RTE meat microbiota. In addition to culture dependent analysis of ham microbiota after pressure treatment and storage, microbiota were analyzed by high throughput sequencing of 16S rRNA sequence tags, which has become a valuable tool for analysis of meat microbiota (Pothakos et al., 2014; Fougy et al., 2016; Säde et al., 2017). Taken together, the experimental approach allowed control of product composition,

TABLE 2 | Relative abundance (%) of 16S rRNA gene sequences from DNA isolated from ham after treatment at 500 MPa for 1 or 3 min, followed by storage for 4 weeks at 4°C.

Controls	**0.5% EtOH**						**HCl**					
Samples	**Meat microbiota**			***Listeria* and meat microbiota**			**Meat microbiota**			***Listeria* and meat microbiota**		
Treatments	**No HP**	**1 min**	**3 min**	**No HP**	**1 min**	**3 min**	**No HP**	**1 min**	**3 min**	**No HP**	**1 min**	**3 min**
Organism (%)												
Brochothrix	45.1 ± 3.5	72.3 ± 11.5	81.5 ± 14.7	47.0 ± 1.0	75.9 ± 10.1	93.6 ± 4.0	51.6 ± 2.7	71.7 ± 16.2	75.6 ± 4.7	50.5 ± 5.2	58.4 ± 8.8	76.8 ± 10.0
Carnobacterium	18.7 ± 0.2	3.1 ± 2.6	12.6 ± 10.6	17.5 ± 4.1	2.7 ± 2.0	0.68 ± 0.34	14.6 ± 1.7	3.45 ± 1.28	9.17 ± 2.84	14.0 ± 0.83	4.65 ± 4.88	8.81 ± 6.29
Leuconostoc	23.8 ± 0.26	23.0 ± 13.2	1.28 ± 0.83	21.9 ± 11.0	19.6 ± 12.3	4.8 ± 4.8	25.5 ± 4.7	23.2 ± 14.6	11.4 ± 3.3	26.4 ± 5.5	31.0 ± 10.0	10.5 ± 4.5
Lactobacillus	12.1 ± 3.5	1.61 ± 0.86	4.51 ± 3.30	9.44 ± 1.28	1.26 ± 0.62	0.56 ± 0.30	8.11 ± 0.84	1.54 ± 0.56	3.70 ± 1.16	8.21 ± 0.39	1.93 ± 1.86	3.40 ± 2.02
Listeria	0.08 ± 0.01	0.02 ± 0.01	0.05 ± 0.01	0.64 ± 0.25	0.42 ± 0.36	0.26 ± 0.14	0.04 ± 0.01	0.03 ± 0.01	0.03 ± 0.02	0.80 ± 0.23	0.49 ± 0.26	0.32 ± 0.26
Others	–	–	–	–	–	–	–	–	–	–	–	–
Antimicrobials	**Rosemary extract**						**Nisin**					
Samples	**Meat microbiota**			***Listeria* and meat microbiota**			**Meat microbiota**			***Listeria* and meat microbiota**		
Treatments	**No HP**	**1 min**	**3 min**	**No HP**	**1 min**	**3 min**	**No HP**	**1 min**	**3 min**	**No HP**	**1 min**	**3 min**
Organism (%)												
Brochothrix	30.7 ± 12.6	56.2 ± 23.7	68.1 ± 24.0	37.0 ± 5.9	74.8 ± 30.2	84.9 ± 15.1	4.8 ± 2.9	21.1 ± 4.4	20.3 ± 3.1	7.1 ± 5.4	15.9 ± 2.0	20.9 ± 1.7
Carnobacterium	22.6 ± 1.9	3.64 ± 3.18	3.33 ± 3.00	24.3 ± 5.0	1.85 ± 1.97	1.82 ± 2.01	3.68 ± 1.42	19.5 ± 0.7	19.3 ± 0.8	4.90 ± 3.46	16.5 ± 1.9	16.2 ± 1.1
Leuconostoc	32.6 ± 11.5	38.1 ± 24.8	26.6 ± 26.0	25.3 ± 4.0	21.4 ± 26.2	11.3 ± 11.6	85.8 ± 6.00	21.1 ± 3.3	27.4 ± 6.3	75.0 ± 16.2	22.8 ± 7.3	19.8 ± 5.3
Lactobacillus	13.7 ± 2.4	1.69 ± 0.56	1.75 ± 0.93	12.7 ± 1.1	1.11 ± 1.24	1.45 ± 1.71	3.44 ± 1.65	22.9 ± 5.0	18.6 ± 4.1	5.32 ± 4.12	18.3 ± 5.2	17.3 ± 5.2
Listeria	0.03 ± 0.02	0.05 ± 0.03	0.02 ± 0.01	0.59 ± 0.09	0.62 ± 0.85	0.38 ± 0.06	0.01 ± 0.00	0.21 ± 0.15	0.06 ± 0.00	3.11 ± 1.93	10.8 ± 4.5	12.8 ± 6.5
Others	–	–	–	–	–	–	1.96 ± 0.10	13.6 ± 4.9	12.9 ± 7.5	3.72 ± 1.71	13.8 ± 7.8	10.9 ± 2.4

Data are shown as means ± standard deviations of triplicate independent experiments.

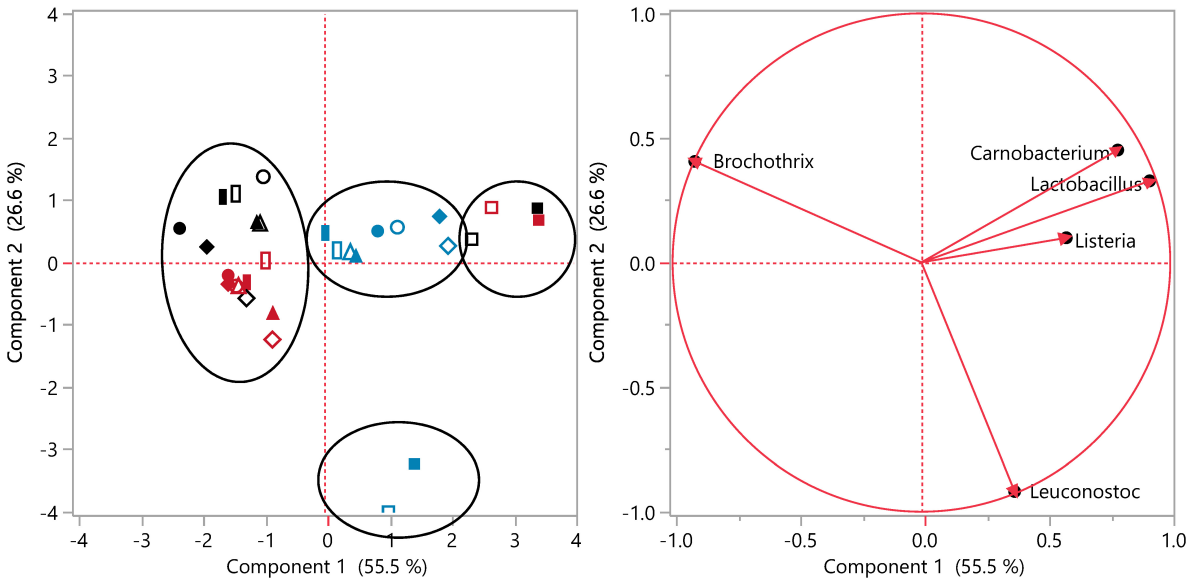

FIGURE 5 | Principal component analysis (PCA) of the % abundance of 16S rRNA sequences on ham containing a nisin preparation, rosemary extract, or without additions after storage for 4 weeks at 4°C. Ham was inoculated with 5-strain cocktails of *L. monocytogenes* and RTE meat microbiota, treated for 1 or 3 min at 500 MPa, or left untreated. The percentage of variation explained by the plotted principal coordinates is indicated on the axes. Each symbol represents a type of sample and each color represents a treatment: no addition (■), or controls with 0.5% ethanol (●) or 0.02N HCl (▲), rosemary extract (◆), nisin (■); no pressure treatment (blue), 1 min at 500 MPa (red), 3 min at 500 MPa (black).

process parameters, and microbiota throughout processing and storage.

Rosemary essential oil is used commercially in meat products to prevent lipid oxidation (Estévez and Cava, 2006); rosemary essential oil also has antimicrobial effects against *L. monocytogenes* and demonstrated synergistic effects with pressure application in buffer (Espina et al., 2013; Abdollahzadeh et al., 2014). Our findings confirmed antimicrobial activity of rosemary essential oil against *L. monocytogenes* but rosemary oil did not affect growth and survival of *L. monocytogenes* in ham or pressure treated ham during refrigerated storage. The meat matrix may protect bacteria against the inhibitory activity of spice extracts (Zhang et al., 2009). The strain-dependent variation of nisin sensitivity of observed in this study was consistent with prior reports that employed 200 strains of *L. monocytogenes* and reported an MIC range of 0.002–0.8 mg pure nisin/L (Katla et al., 2003), corresponding to 0.08–32 mg/L of the nisin preparation employed in the present study. Also consistent with prior reports, the combination of nisin and pressure in RTE ham displayed an additive effect against *L. monocytogenes* and prevented post-pressure resuscitation of sublethally injured cells (Jofré et al., 2008; Hereu et al., 2012). Surface treatment of ham, however, may not be economically feasible and nisin is inactivated in raw meat (Rose et al., 1999). Other bacteriocins of lactic acid bacteria, particularly Listeria-active class IIa bacteriocins, have a comparable mode of action, are compatible with use on raw or processed meats, and exert a comparable synergistic effect with high pressure on *L. monocytogenes* in ham (Garriga et al., 2002; Marcos et al., 2008).

Competitive meat microbiota consisting of lactic acid bacteria inhibit growth of *L. monocytogenes* in meat products; however, their resistance to pressure is poorly documented (Bredholt et al., 1999; Liu et al., 2012). Differential inactivation of lactic acid bacteria and *Listeria* on meat by pressure and/or antimicrobials may enhance growth of *L. monocytogenes* if the antibacterial intervention inactivates competitive microbiota while few cells of *L. monocytogenes* survive. To date, differential inactivation of lactic acid bacteria and *Listeria* has only been evaluated with cheese starter cultures (Arqués et al., 2005). Our study demonstrated that reconstitution with a 5-strain cocktail of RTE meat microbiota inhibited growth of *L. monocytogenes* during refrigerated storage. The pressure resistance of RTE meat microbiota was equivalent to *L. monocytogenes*; remarkably, the combination of reconstituted meat microbiota with pressure treatment of 3 min reduced levels of *L. monocytogenes* by more than 5 log (CFU/g). This results contrasts recovery and growth of *L. monocytogenes* after pressure treatment without competitive microbiota (this study, Jofré et al., 2008; Hereu et al., 2012) and may allow the design of pressure processes for control of *Listeria* with protective cultures but without use of additional antimicrobial hurdles.

Meat microbiota are differentially inactivated by bacteriocins of lactic acid bacteria (Balay et al., 2017); this may impact product quality because different organisms differentially affect the sensory properties of meat products. *B. thermosphacta* causes off-odors already at relatively low cell counts (Vermeiren et al., 2005), *L. gelidum* and other psychrotrophic *Leuconostoc* spp. cause spoilage through production of CO_2, and by exopolysaccharide production from sucrose

(Vermeiren et al., 2005; Pothakos et al., 2014). In comparison, growth of *L. sakei* or *C. maltaromaticum* on RTE meats has only limited impact on sensory properties (Vermeiren et al., 2005; Leisner et al., 2007). *L. sakei* and *C. maltaromaticum* were least resistant to treatments with pressure and/or nisin. The addition of nisin consistently selected for growth of *L. gelidum* while pressure treatments favored growth of *B. thermosphacta*. Antimicrobial interventions aiming at control of *L. monocytogenes* also shifted the composition of other meat microbiota and may result in a more prominent spoilage phenotype (Vermeiren et al., 2005; Leisner et al., 2007) unless microbiota are controlled by process hygiene and/or protective cultures. *C. maltaromaticum*, which is used commercially as a protective culture on meat products and seafood (Balay et al., 2017; Saraoui et al., 2017), may not be suitable for use in combination with pressure treatment.

CONCLUSION

This study evaluated the use of pressure in combination with antimicrobials for control of *L. monocytogenes* in a meat system with controlled and reconstituted meat microbiota. Consistent with prior reports, pressure alone was insufficient for control of *L. monocytogenes*. Pressure treatment in presence of reconstituted meat microbiota; however, reduced cell counts of *L. monocytogenes* by more than 5 log (CFU/g) and cell counts were reduced further to levels below the detection limit after 4 weeks of refrigerated storage. This finding demonstrates that competitive meat microbiota inhibit recovery of sub-lethally injured *L. monocytogenes* after pressure treatment. Rosemary essential oil did not improve control of *Listeria* by pressure and/or competitive meat microbiota but nisin additionally decreased cell counts of *L. monocytogenes*. Competitive organisms displayed higher resistance to nisin than *L. monocytogenes* and nisin alone did not extend the storage life of products. Nisin and pressure application, however, resulted in characteristic shifts of meat microbiota which may alter the spoilage phenotype of products. Overall, the study supports earlier reports (Arqués et al., 2005; Balay et al., 2017) that competitive microbiota are an important determinant of the survival of *L. monocytogenes* in ready-to-eat foods and their presence should be considered in challenge trials aiming at improved *Listeria* control.

AUTHOR CONTRIBUTIONS

JT, MG, and LM designed the experiments. JT and LR conducted the experiments. JT and MG drafted the figures and tables. JT, MG, and LM wrote the manuscript.

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SUPPLEMENTARY MATERIAL

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Treatment With High-Hydrostatic Pressure, Activated Film Packaging With Thymol Plus Enterocin AS-48, and Its Combination Modify the Bacterial Communities of Refrigerated Sea Bream (*Sparus aurata*) Fillets

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The aim of this study was to determine the impact of activated plastic films with thymol and enterocin AS-48 and high-hydrostatic pressure (HP) treatment on the bacterial load and bacterial diversity of vacuum-packaged sea bream fillets under refrigerated storage for 10 days. The activated film and the HP treatment reduced aerobic mesophiles viable counts by 1.46 and 2.36 log cycles, respectively, while the combined treatment achieved a reduction of 4.13 log cycles. HP and combined treatments resulted in longer delays in bacterial growth. *Proteobacteria* were the dominant phyla in sea bream fillets. The relative abundance of *Firmicutes* increased by the end of storage both in controls and in samples treated by HP singly or in combination with the activated films. The predominant operational taxonomic units (OTUs) found at time 0 in control samples (*Listeria, Acinetobacter, Pseudomonas, Enterobacteriaceae, Chryseobacterium*) rapidly changed during storage (with an increase of *Vibrio, Photobacterium,* and *Shewanella* together with *Cloacibacterium* and *Lactobacillales* by the end of storage). The activated film and the HP treatment induced drastic changes in bacterial diversity right after treatments (with *Comamonadaceae, Methylobacterium, Acidovorax,* and *Sphingomonas* as main OTUs) and also induced further modifications during storage. Bacterial diversity in activated film samples was quite homogeneous during storage (with *Vibrio, Photobacterium,* and *Shewanella* as main OTUs) and approached control samples. HP treatments (singly or in combination with activated films) determined a high relative abundance of *Acinetobacter* (followed by *Pseudomonas* and *Shewanella*) during early storage as well as a higher relative abundance of lactic acid bacteria by the end of storage. The results indicate that the complex dynamics of bacterial populations in the refrigerated sea bream fillets are markedly influenced by treatment and antimicrobials applied.

Keywords: fish fillets, thymol, bacteriocin, high-hydrostatic pressure, biodiversity

INTRODUCTION

Fresh fish is a convenient protein ready for preparation of many dishes, but at the same time it is highly perishable. The main cause of deterioration of fresh fish is the metabolic activity of spoilage seafood microorganisms that provoke degradation of organic molecules and fish tissue, loss of essential fatty acids, fat-soluble vitamins and protein functionality, production of biogenic amines, and formation of off-odors (Gram and Dalgaard, 2002).

Natural antimicrobials and non-thermal treatments such as high-hydrostatic pressure (HP), singly or in combination are attractive candidates for preservation of fish and fish products. Previous studies have addressed possible applications of bacteriocins for food preservation, including seafoods (Galvez et al., 2014; Johnson et al., 2017). Enterocin AS-48 is a broad-spectrum circular bacteriocin with a generally-recognized as safe (GRAS) status (Grande Burgos et al., 2014). Immersion in an enterocin AS-48 solution for 1 min delayed bacterial growth and reduced biogenic amine production in sardines during refrigerated storage (Ananou et al., 2014). Spray-application of an enterocin AS-48 solution reduced viable counts of a cocktail of *Listeria monocytogenes* strains on raw hake and salmon fillets as well as on smoked salmon, an effect that was potentiated by bacteriophage P100 (Baños et al., 2016).

Essential oils and their antimicrobial compounds have been studied for preservation of different types of foods (Hyldgaard et al., 2012; Patel, 2015; Pandey et al., 2017). Addition of thymol in combination with other hurdles improved the preservation of fresh packed plaice fillets (Altieri et al., 2005), sea bream, fresh cod, and fresh blue fish burgers (Corbo et al., 2008, 2009; Del Nobile et al., 2009). The antibacterial activity of enterocin AS-48 can be potentiated by essential oils and phenolic compounds (Grande Burgos et al., 2014). Thymol in combination with enterocin AS-48 improved inactivation of *L. monocytogenes* in salads (Cobo Molinos et al., 2009). Combinations of enterocin AS-48 and phenolic compounds such as thymol could also offer new possibilities for fish preservation.

Bacteriocins and essential oils or their antimicrobial compounds have been incorporated on different coating materials with the purpose of preserving fish products, as exemplified by nisin (Neetoo et al., 2008; Lu et al., 2010). However, the only use of natural antimicrobial compounds to inhibit or delay bacterial growth in fish products may require addition of too high concentrations having a negative impact on food flavor. The efficacy of natural antimicrobials incorporated on coating materials could be improved in combination with other hurdles such as HP. HP can be applied on seafood products with several purposes such as inactivation of foodborne pathogens, reduction of biogenic amine production, improving the product shelf life, product texturization, and recovery of fish meat (Murchie et al., 2005; Campus, 2010; Wang et al., 2016). However, pressure treatment of fresh fish flesh formed products whose color deteriorated (cooked appearance) with increasing pressure as well as holding time (Campus, 2010; Wang et al., 2016). Decreasing the intensity of HP treatment reduces the impact on the food properties, but it also decreases its effects on inactivation of microorganisms. Application of HP treatments of low intensity in combination with natural antimicrobials would presumably have a greater effect on inactivation of microorganisms, improving the preservation of fish products.

There is also a growing interest on understanding the complex changes in the food microbiota that may occur during product shelf life as influenced by several factors such as storage temperature, atmosphere, and added antimicrobials. High throughput sequencing (HTS) technology is a powerful tool for studying microbial communities in food systems (Ercolini, 2013; Kergourlay et al., 2015). HTS has proven to be useful in exploring bacterial communities in fish intestine (Ghanbari et al., 2015; Standen et al., 2015; Song et al., 2016) and seafood products (Chaillou et al., 2015; Zhang et al., 2017). In spite of the many studies carried out on the synergistic activities of natural antimicrobials and treatments such as HP, there is still limited information on the effect of such treatments on bacterial populations in fish products. The aim of the present study was to determine the impact of selected treatments (natural antimicrobials thymol and enterocin AS-48 applied on an activated plastic film and HP treatment) on the bacterial load and bacterial diversity of sea bream fillets under refrigerated storage.

MATERIALS AND METHODS

Sample Preparation

Fresh sea bream (*Sparus aurata*) fillets with skin were purchased from a local supermarket and kept under refrigeration until use (for not longer than 24 h). Fillets were cut into 5 g portions with a sterile knife, and the portions were sealed under vacuum in polyethylene–polyamide bags activated or not with antimicrobials as described below.

Bags (10 × 15 cm) prepared from polyethylene–polyamide film were activated by addition of 1 ml 0.5% thymol (Sigma, Madrid, Spain) plus 1 ml of partially-purified bacteriocin enterocin AS-48 (0.8 mg/ml) prepared as described elsewhere (Abriouel et al., 2003). Briefly, bacteriocin from cultured broths of the producer strain *Enterococcus faecalis* A-48-32 were concentrated by cation exchange chromatography. Bacteriocin concentrates were dialyzed with distilled water for 24 h by using 2,000 molecular weight cut-off benzoylated dialysis tubing (Sigma-Aldrich, Madrid) and filtered through 0.22 μm pore size low protein binding filters (Millex GV; Millipore Corp., Belford, MA, USA) under sterile conditions. The concentrations of antimicrobials used for the study were the ones that achieved highest number of log reductions in viable cell counts while having lowest impact (aromatic odor) on the sensory properties of sea bream fillets among preliminary tests carried out using combinations of 1 ml thymol solutions at 0.25, 0.5, and 0.75% and 1 ml bacteriocin solutions at 0.4 and 0.8 mg/ml. Bags were rubbed by hand to ensure mixing and homogeneous distribution of antimicrobial solutions and incubated for 60 min at ambient temperature to facilitate adsorption of antimicrobials. Then, excess liquid was removed and the bags were allowed to dry for 60 min on filter paper in a biosafety cabinet (Telstar, Madrid,

Spain) under UV irradiation. Activated bags were used within 24 h of preparation. Bags not activated with antimicrobials were also kept for 60 min under UV irradiation in a biosafety cabinet.

High-Hydrostatic Pressure Treatment

The vacuum-packed sea bream fillets were treated by high-HP at low intensity (300 MPa for 5 min at ambient temperature). This treatment was selected among trials at 200, 300, and 400 MPa (5 min each) because it achieved highest number of log reductions while having lowest impact on the natural color of fillets. HP treatment was applied by using a Stansted Fluid Power LTD high pressure Iso-Lab system (model FPG9400B711; SFP, Essex, UK). Come-up speed was 75 MPa/min. Decompression was immediate. Pressurization fluid was water with added 10% propylenglycol. The temperature inside the vessel during treatments ranged between 23 and 27°C. The temperature of samples was 24.5°C at the end of treatment. The samples were placed on ice right after treatment.

The following treatments were applied (two replicates, each one in triplicate): C, controls packed in bags not activated with antimicrobials. AF, samples packed in bags activated with antimicrobials. HP, samples packed in bags not activated with antimicrobials, treated by HP. AF-HP, samples packed in bags activated with antimicrobials, treated by HP. Samples were stored for 10 days at 5°C. At desired incubation times, bags (in triplicate) were removed and the food content was pooled and homogenized with 10 ml sterile saline solution. The resulting homogenate was serially diluted in sterile saline solution and plated in triplicate on trypticase soya agar (TSA; Scharlab, Barcelona, Spain) for determination of total aerobic mesophiles. The average number of colonies obtained after 24–48 h incubation of the plates at 37°C was used to calculate the viable cell concentration [expressed as Log_{10} colony forming units (CFU)/g].

DNA Extraction

For each sampling point, aliquots (5 ml) from food homogenates were mixed in sterile 50 ml test tubes and centrifuged at 600 × *g* for 5 min in order to remove solids. An aliquot (1.5 ml) of the resulting supernatant was then transferred to an Eppendorf test tube and centrifuged at 13,500 × *g* for 5 min to recover microbial cells. The pellet was resuspended in 0.5 ml sterile saline solution each. Then, Propidium Monoazide (PMA™, GenIUL, S.L, Barcelona, Spain) was added to block subsequent PCR amplification of the genetic material from dead cells (Nocker et al., 2007) as described by Elizaquivel et al. (2012). DNA from PMA-treated cells was extracted by using a GenElute™ Bacterial Genomic DNA Kit (Sigma-Aldrich), following instructions provided by the manufacturer. The resulting DNA from the two batch replicates and same sampling point was pooled into a single sample. DNA concentration and quality were measured with a NanoDrop spectrophotometer (Thermo Scientific, United Kingdom).

DNA Sequencing and Analysis

The sequence of the V3–V4 region of 16S rRNA gene was used as the taxonomic basis to estimate bacterial populations present in the samples (Caporaso et al., 2011) using Illumina technology essentially as described elsewhere (Grande Burgos et al., 2017). DNA template was adjusted to 10–12 ng in each PCR reaction. The amplicon sequencing protocol targets the V3 and V4 regions of the 16S genes with the primers designed surrounding conserved regions (Klindworth et al., 2013). Following the Illumina amplicon libraries protocol, DNA amplicon libraries were generated using a limited cycle PCR (KAPA HiFi HotStart ReadyMix, KK2602; KAPA Biosystems, Wilmington, MA, USA), then Illumina sequencing adaptors and dual-index barcodes were added to the amplicon. Libraries were then normalized and pooled prior to sequencing. The sample containing indexed amplicons was then loaded onto the MiSeq reagent cartridge v3 (MS-102-3003; San Diego, CA, USA) and onto the instrument along with the flow cell. Automated cluster generation and paired-end sequencing with dual indexes reads was performed (2 × 300 bp run). After demultiplexing, paired end reads were joined together with the fastq-join program (https://expressionanalysis.github.io/ea-utils/). Only reads that had quality value (QV) scores of ≥20 for more than 99% of the sequence were extracted for further analysis. All sequences with ambiguous base calls were discarded. After filtering, sequence reads were assigned to operational taxonomic units (OTUs) based on sequence similarity for each read to 16S rRNA genes from the NCBI nt database by using BLASTN function. Each read was assigned to the taxon corresponding to the Best Blast Hit over a threshold of similarity (e<1E-15). The sequencing output files were deposited in the Sequence Read Archive (SRA) service of the European Bioinformatics Institute (EBI) database under Accession Number PRJEB22751.

Statistical Analysis

Data on viable cell counts for the different treatments and storage times were analyzed with *t*-test and two-way ANOVA at $P < 0.05$ (Microsoft Excel). Diversity indices Shannon Wiever (H′), Simpson (D) and Chao1 were calculated using the statistical software packages Paleontological Statistics (PAST). Euclidean distance matrix was obtained with SPSS software.

RESULTS

Effect of Treatments on Bacterial Inactivation

When control sea bream fillets packed in plastic bags without antimicrobials were stored under refrigeration, viable cell counts (total aerobic mesophiles) increased gradually and were 1.14 log cycles higher by the end of storage period (**Table 1**). Microbial inactivation was significantly influenced ($P < 0.05$) by activated film packaging, HP treatment, and the combination of activated film and HP. For samples packed in the activated plastic bags, viable cell counts were reduced significantly ($P < 0.05$) by 1.45 log cycles at time 0. However, viable counts obtained during storage were not significantly lower ($P > 0.05$) than untreated controls. The HP treatment applied to samples packed in films without activation with antimicrobials reduced viable cell counts significantly ($P < 0.05$) by 2.36 log cycles. Furthermore, viable counts in the HP-treated samples also remained significantly

TABLE 1 | Viable cell counts for total aerobic mesophiles in sea bream fillets stored under refrigeration.

	Storage time (days)				
	0	**2**	**5**	**7**	**10**
Control	5.97 ± 0.09^{a}	6.22 ± 0.15^{a}	6.58 ± 0.11^{a}	6.55 ± 0.07^{a}	7.11 ± 0.15^{a}
Activated film (AF)	4.51 ± 0.05^{b}	6.09 ± 0.31^{a}	6.09 ± 0.31^{a}	6.10 ± 0.32^{a}	6.93 ± 0.08^{a}
HP	3.61 ± 0.19^{b}	3.01 ± 0.09^{b}	3.96 ± 0.12^{b}	5.64 ± 0.38^{a}	6.81 ± 0.25^{a}
AF-HP	1.84 ± 0.09^{c}	1.30 ± 0.25^{c}	3.91 ± 0.18^{b}	4.92 ± 0.19^{b}	7.16 ± 0.36^{a}

Data (Log10 CFU/g) are the average of two replicates ± standard deviation.
^{a,b,c} down columns indicate significantly different ($P < 0.05$) values.

($P < 0.05$) lower than controls by 3.21 and 2.62 log cycles for storage times 2 and 5, respectively. However, a remarkable increase in viable counts was noticed by day 7 and after. Application of HP treatment to samples packed in films activated with antimicrobials provided best results, achieving a significant ($P < 0.05$) reduction of 4.13 log cycles. Although viable cell counts for the combined treatment increased during late storage (days 5, 7, and 10), they still remained significantly ($P < 0.05$) lower than the untreated controls for storage times 2, 5, and 7 (by 4.92, 2.67, and 1.63 log cycles, respectively). Compared with the activated film alone, microbial inactivation was influenced significantly ($P < 0.05$) by the combined treatment, which achieved additional and significant ($P < 0.05$) reductions of viable counts (2.67, 4.79, 2.18, and 1.18 log cycles for storage times 0, 2, 5, and 7, respectively). Compared with the single HP treatment, the combined treatment with activated films achieved additional and significant ($P < 0.05$) reductions of viable counts of 1.77 and 1.71 log cycles at storage times 0 and 2, respectively.

Effect of Treatments on Bacterial Diversity

The numbers of reads assigned to OTUs was comprised between 77,780 and 190,124 (**Table 2**). Nevertheless, in spite of the in-depth coverage provided by Illumina sequencing, differences in copy number of the 16S rRNA gene sequencing need to be taken into consideration as well as the variability that may occur between samples. Control samples showed higher alpha diversity values compared with samples packed in the activated films or with samples treated by HP alone (**Table 2**). In samples from the combined treatments, alpha diversity values decreased at days 2 and 5 but then increased again during storage. *Proteobacteria* were the predominant phylum in the controls as well as in most of the treated samples, with relative abundances ranging from 54.06 up to 99.79% (**Figure 1**). In control samples, the relative abundance of *Proteobacteria* decreased toward the end of storage, while other phyla (*Firmicutes*, *Bacteroidetes*, *Actinobacteria*) increased. For the HP treated samples, the relative abundance of *Firmicutes* increased toward the end of storage, reaching 71.91% at day 10 (HP10). This sample had the lowest relative abundance of *Proteobacteria* (27.58%). For the combined treatments, *Firmicutes* had higher relative abundances both after treatment (AF-HP0, 29.30%) and by the end of storage (AF-HP7, 33.79%; AF-HP10, 29.95%). The rest of phyla (19 in total) had very low relative abundances, and altogether accounted for <1.3% OTUs.

TABLE 2 | Number of sequences (reads) and observed diversity for 16S rRNA amplicons for the different samples analyzed in this study.

Sample	N° of reads	Shannon index	Simpson index	Chao 1 index
UNTREATED CONTROLS				
C0	77,780	2.59	0.88	150.6
C2	83,091	1.79	0.77	77.5
C5	190,124	1.91	0.79	116.1
C7	120,747	3.22	0.91	208.2
C10	94,357	2.42	0.86	126
ACTIVATED FILM				
AF0	106,044	2.79	0.88	131.1
AF2	92,738	1.64	0.74	113
AF5	83,014	1.92	0.79	108
AF7	84,833	1.98	0.75	142.5
AF10	78,491	1.73	0.74	110.8
HIGH HYDROSTATIC PRESSURE				
HP0	99,732	0.99	0.78	107.6
HP2	94,189	1.96	0.71	123
HP5	89,368	1.72	0.73	150.1
HP7	98,761	1.72	0.74	110.5
HP10	81,864	1.94	0.74	132.5
ACTIVATED FILM PLUS HIGH HYDROSTATIC PRESSURE				
AF-HP0	114,530	3.34	0.94	172
AF-HP2	90,700	1.68	0.68	132.3
AF-HP5	98,905	1.96	0.76	142.6
AF-HP7	96,927	2.30	0.84	150.8
AF-HP10	97,467	3.44	0.94	157.8

Comparison of the relative abundances of OTUs clustered at genus level indicated important differences between controls and treated samples that depended on treatment and also on storage time (**Figure 2**). For the control samples, OTUs assigned to genus *Listeria* (mainly *Listeria seeligeri*) showed highest relative abundance at time 0 (C0, 24.90%). OTUs assigned to *Acinetobacter, Pseudomonas,* and *Enterobacteriaceae* were also relevant (with relative abundances comprised between 12 and 13%), and to a less extent *Chryseobacterium* (8.88%). The bacterial diversity of control samples changed markedly during storage. By day 2, the main bacterial groups detected at time 0 were displaced by other groups, mainly *Vibrio* (mostly *Vibrio rumoiensis*), *Photobacterium* (mostly *Photobacterium angustum*),

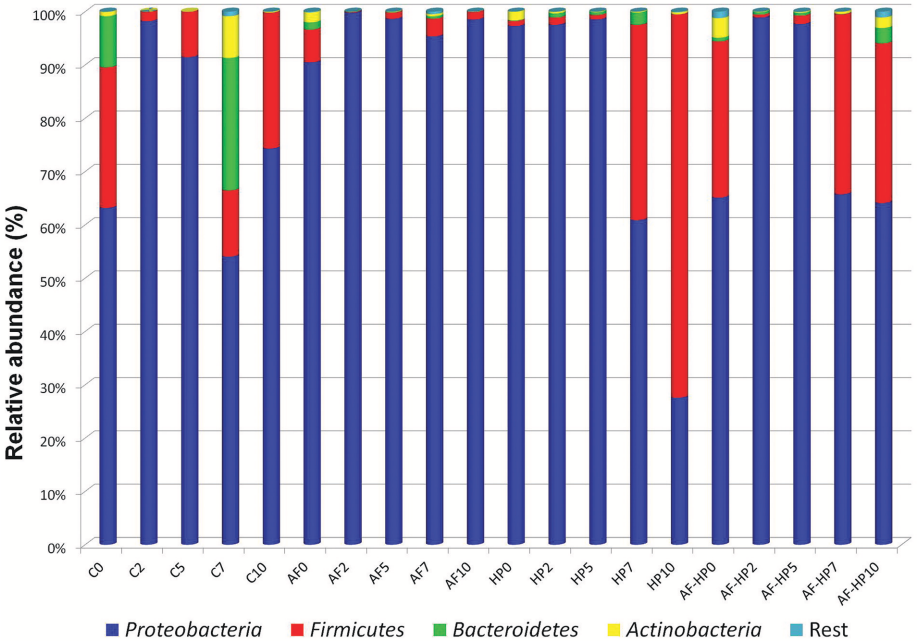

FIGURE 1 | Relative abundance of OTUs based on paired-end 16S rRNA gene sequencing analysis of DNA from refrigerated sea bream fillets. Controls packed in films without activation with antimicrobials (C); samples packed in films activated with thymol plus enterocin AS-48 (AF); samples packed in films without activation and treated by high-hydrostatic pressure (HP); samples packed in activated films and treated by high-hydrostatic pressure (AF-HP). Sampling was performed at days 0, 2, 5, 7, and 10. OTUs were grouped at Phylum level.

and *Shewanella*. The first two groups were still important at the end of storage, but other groups (*Comamonadaceae*, *Lactobacillales,* and *Cloacibacterium*) also became relevant at some points during mid to late storage.

Activated film packaging induced an early, transient change in the microbiota of samples. The main OTUs detected at time 0 (AF0) belonged to *Comamonadaceae* (23.19%), *Methylobacterium* (21.37%), *Acidovorax* (7.62%), and *Sphingomonas* (6.57%) (**Figure 2**). However, the relative abundances of these bacterial groups decreased markedly by day 2. Furthermore, the main OTUs detected for the activated film samples during storage (AF2 to AF10) belonged to *Vibrio, Photobacterium,* and *Shewanella*, resembling the bacterial diversity of control samples.

The HP-treated samples (HP0) showed a similar profile as AF0 samples, with *Comamonadaceae* (29.07%), *Methylobacterium* (33.88%), *Acidovorax* (9.86%), and *Sphingomonas* (8.76%) as main OTUs (**Figure 2**). As in the activated films, these bacterial groups also decreased rapidly in relative abundance during early storage of HP-treated samples. However, *Vibrio* and *Photobacterium* had very low relative abundances during storage. Instead, *Acinetobacter* (mainly *Acinetobacter guillouiae* and *Acinetobacter johnsonii*) showed very high relative abundances at days 2 and 5 (HP2, 50.5%; HP5, 43.03%). *Pseudomonas* and *Shewanella* were also relevant in the HP-treated samples during mid-storage. Furthermore, *Lactobacillales* and *Carnobacterium* became the predominant OTUs during late storage (HP7, HP10).

Samples packed in the activated film and then treated by high-HP (AF-HP0) showed lower relative abundances of *Methylobacterium* (12.00%), *Comamonadaceae* (11.58%), *Acidovorax* (3.54%), and *Sphingomonas* (2.91%) compared to the single HP treatment, and higher relative abundances of *Enterococcus* (12.56%), *Enterobacteriaceae* (7.19%), *Lactobacillaceae* (5.68%), *Xanthomonadaceae* (4.88%), and *Escherichia* (3.50%) (**Figure 2**). However, the relative abundances of these groups decreased rapidly during storage while other groups resembling the microbiota of the single HP treatment increased. Thus, the predominant OTUs detected in samples from the combined treatment at days 2 and 5 belonged to *Acinetobacter* (AF-HP2, 52.88%; AF-HP5, 40.65%) followed by *Shewanella* and *Pseudomonas* in lower proportions. There was also a change in the microbiota during late storage, with increases in the relative abundances of OTUs assigned to *Photobacterium*, *Vibrio*, *Shewanella,* and *Enterobacteriaceae* at day 7, *Comamonadaceae* and *Methylobacterium* at day 10, and *Lactobacillales* at both days 7 and 10.

Principal coordinates analysis indicated that most of the activated film samples (AF2 to AF10) as well as control samples C2 and C5 clustered together, as they had high relative abundances for OTUs assigned to *Vibrio*, *Photobacterium,* and *Shewanella* (**Figure 3**). Samples just treated by the activated film (AF0), HP (HP0), or both (AF-HP0) formed a separate cluster (with *Comamonadaceae*, *Methylobacterium*, *Acidovorax,* and *Sphingomonas* as main OTUs in common). Samples treated by HP and samples from the combined treatment also clustered

	Controls					Activated film					High hydrostatic pressure					Activated film + high hydrostatic pressure				
	C0	C2	C5	C7	C10	AF0	AF2	AF5	AF7	AF10	HP0	HP2	HP5	HP7	HP10	AF-HP0	AF-HP2	AF-HP5	AF-HP7	AF-HP10
Listeria	24.90	0.00	0.00	0.00	0.79	0.00	0.00	0.03	0.00	0.09	0.00	0.15	0.08	0.01	0.01	0.59	0.06	0.46	3.17	0.42
Acinetobacter	12.49	2.31	2.09	0.39	0.11	2.59	5.99	10.78	0.09	0.04	0.79	50.50	43.03	0.35	0.25	0.94	52.88	40.65	0.04	0.59
Vibrio	0.06	24.59	20.56	1.89	11.79	0.00	29.41	17.33	14.75	39.84	0.00	2.97	0.11	0.04	0.58	0.49	0.38	0.19	10.18	1.94
Photobacterium	0.02	33.44	36.20	3.85	20.50	0.01	37.60	35.63	42.86	14.29	0.00	5.47	0.21	1.03	1.41	0.52	0.25	0.32	21.85	5.62
Pseudomonas	12.08	8.23	8.83	0.97	7.36	6.26	2.92	3.42	2.88	6.37	1.11	8.55	18.06	37.39	2.75	2.72	10.72	14.98	6.96	2.02
Shewanella	2.82	19.17	12.54	1.29	9.67	0.64	16.26	18.04	16.50	24.98	0.01	9.45	21.52	0.22	0.88	0.33	13.44	19.43	9.73	2.01
Fam. *Comamonadaceae* (Other)	4.18	0.05	0.03	10.24	4.25	23.19	0.05	0.14	1.63	0.10	29.07	1.22	0.28	0.49	6.72	11.58	0.73	0.93	1.11	10.93
Methylobacterium	2.87	0.01	0.01	7.05	3.68	21.37	0.03	0.21	1.74	0.09	33.88	0.82	0.17	0.26	6.16	12.00	0.36	0.84	0.88	9.27
Fam. *Enterobacteriaceae* (Other)	12.91	4.26	5.24	0.55	6.70	4.34	2.87	6.28	6.62	5.39	0.00	1.30	3.57	9.76	0.92	7.19	0.70	5.02	7.52	3.08
Psychrobacter	3.73	0.02	0.01	0.01	0.03	0.20	0.01	0.00	0.00	0.01	0.00	0.14	0.13	9.99	0.27	0.08	0.04	0.00	0.02	0.00
Acidovorax	0.86	0.00	0.00	3.27	1.33	7.62	0.00	0.03	0.43	0.02	9.86	0.27	0.06	0.09	2.17	3.54	0.14	0.21	0.44	3.25
Sphingomonas	0.60	0.01	0.00	1.36	0.67	6.57	0.01	0.05	0.43	0.03	8.76	0.12	0.04	0.05	1.10	2.91	0.12	0.25	0.17	1.91
Fam. *Pseudomonadaceae* (Other)	1.30	0.15	0.18	2.76	0.35	4.01	0.06	0.13	0.19	0.23	0.07	0.34	0.69	0.46	0.68	5.24	0.33	0.71	0.26	5.95
Stenotrophomonas	0.43	0.04	0.08	0.01	0.18	0.20	0.17	0.81	0.06	0.08	0.14	3.84	5.65	0.05	0.19	0.22	6.98	6.30	0.08	0.15
O. *Lactobacillales* (Other)	0.04	1.11	7.44	0.35	22.65	0.14	0.00	0.04	0.03	0.07	0.00	0.06	0.05	1.71	30.12	0.53	0.01	0.09	26.73	13.52
Carnobacterium	0.06	0.47	0.55	0.12	0.52	0.00	0.05	0.87	1.93	0.90	0.00	0.03	0.32	31.75	39.82	0.34	0.00	0.00	1.24	0.53
Fam. *Lactobacillaceae* (Other)	0.06	0.00	0.00	0.15	0.12	0.84	0.00	0.02	0.12	0.02	0.00	0.09	0.03	0.00	0.00	5.68	0.02	0.14	0.06	1.43
Enterococcus	0.01	0.01	0.01	1.44	0.03	1.36	0.02	0.03	0.03	0.02	0.00	0.54	0.08	0.01	0.06	12.56	0.31	0.20	0.09	3.64
Cloacibacterium	0.02	0.00	0.00	22.93	0.00	0.77	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.00	0.00	0.00	0.00	0.00	0.04	0.09
Agrobacterium	0.20	0.14	0.19	0.47	0.00	0.88	0.38	0.86	0.08	0.01	1.25	8.50	2.73	0.02	0.17	0.48	7.73	4.63	0.04	0.61
Corynebacterium	0.19	0.00	0.00	5.72	0.01	0.34	0.00	0.01	0.01	0.00	0.00	0.01	0.03	0.00	0.00	0.59	0.00	0.04	0.31	0.30
Chryseobacterium	8.88	0.03	0.02	0.00	0.01	0.36	0.04	0.03	0.00	0.02	0.00	0.55	0.12	2.28	0.01	0.54	0.12	0.08	0.02	0.10
Enhydrobacter	5.19	0.01	0.00	0.04	0.00	0.86	0.01	0.00	0.00	0.00	0.00	0.10	0.01	0.02	0.00	0.14	0.01	0.00	0.00	0.42
Fam. *Xanthomonadaceae* (Other)	0.21	0.01	0.01	1.14	0.05	1.03	0.01	0.03	0.08	0.06	0.00	0.10	0.12	0.06	0.47	4.88	0.11	0.28	0.10	4.41
Escherichia	0.00	0.00	0.00	0.00	0.01	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.00	0.00	0.06	3.50	0.00	0.01	0.01	0.95
Fam. *Bacillaceae* (Other)	0.07	0.00	0.00	4.24	0.08	0.27	0.01	0.01	0.07	0.05	0.00	0.04	0.05	0.04	0.08	2.05	0.02	0.07	0.14	4.23
Bradyrhizobium	0.31	0.00	0.00	0.87	0.48	1.84	0.00	0.03	0.28	0.01	4.24	0.08	0.01	0.02	0.54	0.99	0.03	0.12	0.11	0.82
Serratia	0.03	0.11	0.35	0.00	2.68	0.00	0.03	0.42	0.67	2.59	0.00	0.01	0.00	0.01	0.10	0.07	0.00	0.01	2.72	1.34
Comamonas	0.07	0.06	0.05	0.02	0.00	0.02	0.10	0.17	0.02	0.01	0.03	2.12	0.75	0.00	0.02	0.01	2.35	1.17	0.01	0.01
Rest (<2%)	5.38	5.76	5.59	28.87	5.93	14.29	3.99	4.58	8.51	4.67	10.78	2.58	2.05	3.90	4.46	19.29	2.16	2.87	5.99	20.47

FIGURE 2 | Heatmap of bacterial diversity in sea bream samples based on the relative abundance at genus level. The values represent the relative abundance of OTUs clustered at the Genus level. OTUs not assigned to genus were computed in the nearest higher taxonomic level. Color scale: 0, white; 100, dark magenta. Controls packed in films without activation with antimicrobials (C); samples packed in films activated with thymol plus enterocin AS-48 (AF); samples packed in films without activation and treated by high-hydrostatic pressure (HP); samples packed in activated films and treated by high-hydrostatic pressure (AF-HP). Sampling was performed at days 0, 2, 5, 7, and 10.

together for days 2 and 5 (HP2, HP5, AF-HP2, AF-HP5) as they shared in common high relative abundances of *Acinetobacter* followed by *Pseudomonas* and *Shewanella*. The treated samples from late storage HP10 and AF-HP10 also clustered closely, and shared high relative abundances of *Lactobacillales*. Control samples from late storage also had a high relative abundance of *Lactobacillales*, but they clustered more closely to samples from the combined treatment AF-HP7 because they had similar relevant relative abundances of *Vibrio* and *Photobacterium*.

DISCUSSION

Results presented in this study indicate that reduction of the initial microbial load in sea bream fillets and retardation of its growth during refrigerated storage can be improved considerably by application of combined treatments such as films activated with a mixture of thymol plus the bacteriocin enterocin AS-48 and a mild high-HP treatment. The combined treatment had the advantage of increasing microbial inactivation at the beginning, and still kept viable cell counts in the packed fillets 1.2 log cycles lower than the untreated controls at day 7 of storage. Compared to other methods for bacteriocin addition such as dipping or spraying with bacteriocin solutions, activated films provide the advantage of a slow release of antimicrobials into the medium while at the same time can also provide some protection against bacteriocin degradation by tissue proteases or by complex formation with food components (Gálvez et al., 2007). Although bacteriocins can show synergistic effects with other natural antimicrobials and with non-thermal food processing treatments such as HP (Galvez et al., 2014; Mathur et al., 2017), there are no previous studies on the combined effect of activated packaging with thymol plus enterocin AS-48 and HP treatment. Inability to repair multiple damages caused by the combined action of different antimicrobials most likely results in energy exhaustion and cell death. Furthermore, damage to the outer membrane of Gram-negative bacteria caused by some antimicrobials and/or treatments results in cell permeabilization and faster diffusion of bacteriocins such as enterocin AS-48 to its main cellular target, the bacterial cytoplasmic membrane (Gálvez et al., 1991).

Considering that foods are complex ecosystems containing a mixture of bacterial species that may change in relative abundance during storage depending of different environmental factors such as storage temperature and the presence of antimicrobial substances, among others, it is important to study

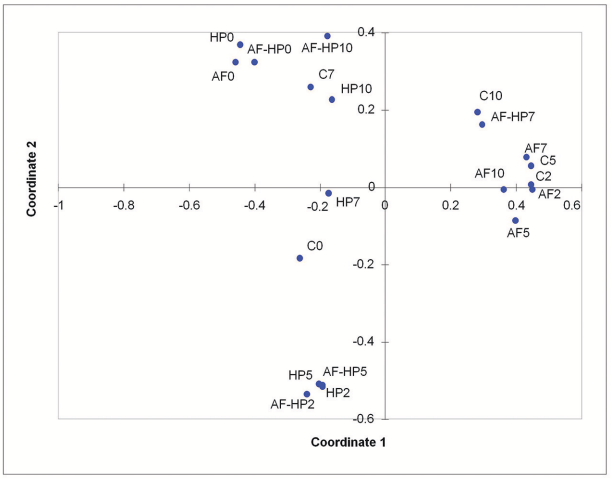

FIGURE 3 | Principal Coordinates analysis (PCoA) of the dissimilarities between controls and treated sea bream samples during refrigerated storage. Controls packed in films without activation with antimicrobials (C); samples packed in films activated with thymol plus enterocin AS-48 (AF); samples packed in films without activation and treated by high-hydrostatic pressure (HP); samples packed in activated films and treated by high-hydrostatic pressure (AF-HP). Sampling was performed at days 0, 2, 5, 7, and 10.

the changes in the bacterial diversity of foods after application of food preservation methods. The data obtained by HTS can throw light on the behavior of potentially pathogenic and spoilage bacteria in foods during storage. Nevertheless, the influence of the method used to assess the biodiversity on the results needs to be taken into consideration. For example, differences in copy number of the 16S rRNA gene can cause quantification problems. Furthermore, results will also depend on sample variability. Considering these limitations, the results obtained in the present study by HTS revealed that the main bacterial groups detected in the fresh fillets after being packed in vacuum (*Listeria*, *Acinetobacter*, *Pseudomonas*, *Chryseobacterium,* and members of *Enterobacteriaceae*) were rapidly displaced by other bacterial groups during storage (mainly members of *Vibrio, Photobacterium,* and *Shewanella* together with other groups such *Cloacibacterium, Lactobacillales,* and *Comamonadaceae* appearing during late storage). Previous studies reported that *Shewanella putrefaciens* was found to be very important for spoilage of packed cod (Jorgensen et al., 1988) as well as *Photobacterium phosphoreum*, *Pseudomonas* spp., *Achromobacter* spp., *Acinetobacter* spp., *Flavobacterium* spp., and *Aeromonas* spp. (Dalgaard et al., 1993; Dalgaard, 1995). It is worth mentioning that OTUs for *Listeria* detected in the present study were assigned to *L. seeligeri* and that *L. monocytogenes* was not detected in the sea bream fillets. While isolates of the species *L. seeligeri* are typically hemolytic, this species is generally considered non-pathogenic (Orsi and Wiedmann, 2016). Furthermore, OTUs assigned to *Listeria* had very low relative abundance in all treated samples. *L. monocytogenes* is very sensitive to enterocin AS-48, but it is also known for its high barotolerance and its wide pressure resistance variation between strains (Bruschi et al., 2017). Compared to *L. monocytogenes,* there are scarce data on pressure resistance variations between strains of *L. seeligeri*.

Packing in the activated film induced drastic changes on the sea bream microbiota. All relevant groups in control samples (C0) had reduced relative abundances in samples packed in the activated films (AF0), where OTUs assigned to *Comamonadaceae* and *Methylobacterium* (and to a less extent also *Acidovorax* and *Shewanella*) became predominant. Members of fam. *Comamonadaceae* have been described as part of the culturable microbiota of zebra fish (Cantas et al., 2012), and *Methylobacterium* has been described as a commensal bacterium from the skin of the salmonid brook charr (*Salvelinus fontinalis*) (Boutin et al., 2014). During storage, the bacterial communities of sea bream fillets packed in the activated films seemed to become quite stable, and the main groups detected (*Vibrio*, *Photobacterium,* and *Shewanella*) were the same as in the untreated controls. These results would be expected since the activated films did not prevent bacterial growth during storage. A possible explanation for the observed low efficacy of activated films during storage could be that added antimicrobials decreased below their minimum inhibitory concentrations upon diffusion from the activated film to the fish tissue, together with perhaps partial degradation of bacteriocin molecules by tissue proteases. The results also suggest that these bacterial groups are less sensitive to the antimicrobials employed, thymol and enterocin AS-48 and/or have a greater capacity to survive in the fish fillets under stress conditions. Since enterocin AS-48 acts on the bacterial cytoplasmic membrane (Gálvez et al., 1991), this bacteriocin has much lower activity against Gram-negative bacteria. Nevertheless, large differences in sensitivity to the bacteriocin have also been reported among Gram-negatives (Gálvez et al., 1989). By contrast, thymol can freely cross the cell wall and therefore inhibit the growth of both Gram-positive and Gram-negative bacteria by interacting with the phospholipid bi-layer of cell membranes, resulting in metabolite dissipation (Hyldgaard et al., 2012). The possible species-dependence of the synergistic action of thymol with enterocin AS-48 deserves to be investigated in further studies.

The HP-treated samples had much lower relative abundances of OTUs assigned to *Vibrio* and *Photobacterium* compared with controls (C2, C5, C10) and with activated film packaging samples (AF2 to AF10). This may suggest a higher sensitivity of these bacterial groups to HP treatment. Compared to the single HP treatment, the combined treatment (activated film-HP) decreased the relative abundance at time 0 of *Comamonadaceae* and *Methylobacterium* and increased that of *Firmicutes* (mainly *Enterococcus* and *Lactobacillaceae*). However, samples from both treatments had similar bacterial compositions during storage. These results could be explained because the activated film alone only had limited effects on bacterial diversity during storage as discussed above. It is also worth mentioning that the main bacterial group detected during early storage of samples treated by HP (singly or in combination with the activated film) was *Acinetobacter*. Genus *Acinetobacter* has been reported as a member of the bacterial community of sea bream (*S. aurata*) larvae early after hatching (Califano et al., 2017) and also as one

of the dominant genera of the intestinal microbiota of Antarctic fish (Song et al., 2016). *Acinetobacter* was also predominant in salted bighead carp (*Aristichthys nobilis*) refrigerated fillets (Liu et al., 2017), in farmed sea bream (*S. aurata*) (Parlapani et al., 2013) and in grass carp (*Ctenopharyngodon idellus*) packed in sealed polyvinyl chloride bags during the first 6 days of chill storage (Wang et al., 2014). *Acinetobacter* was also detected in common carp (*Cyprinus carpio*) fillets packed in air or under vacuum and stored under refrigeration (Zhang et al., 2015). In the present study, most OTUs included in this group were assigned to the environmental species *A. guillouiae* and to *A. johnsonii*, which rarely causes human infections (Montaña et al., 2016). Nevertheless, further studies need to be carried out in order to determine the potential risks of *Acinetobacter* in the sea bream fillets as well as resistance of the bacterium to inactivation by HP. *Acinetobacter*, together with *Pseudomonas* and *Shewanella* seemed to be the main spoilage bacteria during early storage in the HP-treated samples and also in the samples from the combined treatment (AF-HP). The observed higher relative abundances of these bacterial groups could be explained by several factors such as higher capacity to repair cellular damage induced by HP treatment, a higher capacity for utilization of available nutrients, ammensalism, or inactivation of competitors by HP treatment.

Carnobacterium and *Lactobacillales* were detected at high relative abundances in the HP-treated samples during late storage. *Lactobacillales* were also the most relevant group during late storage of samples from the combined treatment (AF-HP7, AF-HP10). These results would suggest that HP had selected lactic acid bacteria. Similar results have been reported by high-throughput sequencing studies in mango pulp (Pérez Pulido et al., 2017) and by culture-dependent methods in meat products treated by HP (Garriga et al., 2004; Diez et al., 2009; Han et al., 2011).

Altogether, results from the present study reveal the complexity of bacterial populations in sea bream fillets and how these can be influenced by application of different preservation methods and storage time. The activated film and the HP treatment distinctively selected for different bacterial communities during food storage. While the HP treatment (applied singly or in combination) was most clearly associated with a predominance of OTUs assigned to lactic acid bacteria toward the end of storage, the high relative abundance of OTUs assigned to *Acinetobacter* in the HP-treated samples deserves further investigation.

AUTHOR CONTRIBUTIONS

IO carried out sample preparation, microbiological analysis and DNA extraction for biodiversity studies. MG participated in supervision of the experimental work and interpretation of data. RP-P contributed with high-HP processing, data analysis and preparation of graphical material for the manuscript. RL was responsible for planning and supervision of the study. RL and AG carried out global analysis of the results and wrote the manuscript.

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The Impact of Chitosan-Divergicin Film on Growth of *Listeria monocytogenes* in Cold-Smoked Salmon

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The aim of this study was to evaluate the impact of chitosan film, with bacteriocin divergicin 35 incorporate, on growth of *Listeria monocytogenes* in Cold smoked salmon. The simples of Cold-smoked wild salmon were inoculated with *L. monocytogenes* and treated with chitosan (100 kDa, 94.7% de-acetylated) and divergicin M35 was stored for 3 weeks at 4–8°C. The compounds were applied to the fish flesh in the form of solution or dried film. The film reduced *L. monocytogenes* to below the detection limit (<50 cfu/g) and kept total counts below 10^4 cfu per g compared to 10^9 cfu per g in control samples while the effectiveness of the solution was very limited. The inhibitory activity of the film lasted for 3 weeks, while the solution had no effect on *L. monocytogenes* counts measured on day 14. The film provided a better preservation of fish color (redness) and firmness than others treatments, while the solution had little impact on these parameters. It kept the volatile basic nitrogen (17.5 mg N/100 g) below the control value 29.9 mg N/100 g. Divergicin-loaded chitosan film thus may represent an interesting alternative for the bio-preservation of cold-smoked fish.

Keywords: ***Listeria monocytogenes*, divergicin M35, chitosan, chitosan-divergicin film, cold-smoked wild salmon**

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INTRODUCTION

Listeria monocytogenes is a foodborne human pathogen responsible for an estimated 28% of food-related deaths each year in the United States (Mead et al., 1999). On August 2008, Ready to eat products were responsible or a multi-state outbreak in Canada that resulted in 57 cases of listeriosis have been confirmed (mostely in Ontario), and 22 confirmed deaths (Public Health Agency of Canada, 2008). Compared to other foodborne pathogens, the mortality rate associated with *L. monocytogenes* infection is relatively high (∼20% compared to <1% for *Salmonella* or *Escherichia coli* O157) (Crerar et al., 1996; De Valk et al., 2005; Scallan et al., 2011). This pathogen is widespread in the environment and can grow in many food products (Alhogail et al., 2016).

Cold-smoked salmon is a good substrate for *L. monocytogenes* growth (Pelroy et al., 1994; Duffes, 1999; Katla et al., 2001; Brillet et al., 2005) because it offers favorable proliferation conditions such as, neutral pH and high water activity, which allow this organism to grow even at refrigeration temperatures. The prevalence of *L. monocytogenes* has been reported in several studies to be as

high as 34–43% in cold-smoked salmon (Jorgensen and Huss, 1998). Ben Embarek (1994) reported values between 0 and 75% with an overall prevalence of 10%, and Gombas et al. (2003) reported 4.31% in smoked seafood in Maryland and northern California in 2000 and 2001. The cold smoked salmon contamination with *L. monocytogenes* can be related to several factors such as cleaning and sanitation practices applied during cold smoked salmon production (Jorgensen and Huss, 1998; Johansson et al., 1999) the plant environment, the refrigerated storage and absence of guidance on the fundamental principles of microbiology, including testing for *L. monocytogenes* (Rotariu et al., 2014).

Bio-preservation has been proposed as means of improving food quality and safety. This approach is based on using protective bacterial cultures or antimicrobial compounds obtained there from, in particular bacteriocins, to control spoilage and pathogenic microorganisms such as *L. monocytogenes* (Nilsson et al., 1997; Tahiri et al., 2009). Different strategies have been described for the incorporation of bacteriocins into food matrices. These include direct inoculation with the producing strain, adding purified or semi-purified bacteriocin and immobilizing antimicrobial agents on solid supports such as polymer coatings or films (Fu et al., 2016). This latter approach offers several advantages over the other two, notably better protection of the active compound from inhibitors by decreasing its interaction with the food matrix, maintenance of a high concentration on the food surface, slow and continuous release during food storage and synergistic effects with antimicrobial properties of the polymer support (Coma et al., 2001, 2002; Cutter et al., 2001; Pranoto et al., 2005; Elsabee and Abdou, 2013).

Chitosan is a polymer obtained by deacetylation of chitin obtained from crustacean shells and fungi such as *Aspergillus niger*, *Mucor rouxii,* and *Penicillium notatum* (Tan et al., 1996; No et al., 2002; Qin et al., 2006). Chitosan is now widely recognized as non-toxic, biocompatible and biodegradable (Ye et al., 2008a). The Japanese department of health classified chitin and its derivatives as functional food ingredients in 1992. More recently (2001), the US FDA approved the GRAS status of chitosan. The capacity of chitosan to form films with excellent mechanical and physicochemical properties (Begin and Van Calsteren, 1999; Srinivasa et al., 2004; Suyatma et al., 2004) and to inhibit a wide variety of microorganisms (Shahidi et al., 1999; Agullo et al., 2003; Rinaudo, 2006; Moreira et al., 2011) makes it an interesting polymer for the development of films as antimicrobial delivery systems. Chitosan films containing added natural antimicrobial agents such as organic acids, essential oils, potassium sorbate, lysostaphin, polyphenols, sodium lactate, and bacteriocins have been shown to inhibit several pathogens in foods (Ouattara et al., 2000; Cha et al., 2003; Pranoto et al., 2005; He et al., 2016; Schelegueda et al., 2016; Nithya et al., 2018). Several studies have demonstrated the antimicrobial effect of chitosan films on pathogens in simple models with pure cultures (Pranoto et al., 2005; Li et al., 2006). A few more publications have used chitosan-base packaging films in food such as meat, poultry and fish (Beverlya et al., 2008; Higueras et al., 2013). The intrinsic activity of low-molecular-weight chitosan alone in combination with added antimicrobial compounds has rarely been investigated. To the best of our knowledge, no study has been published on the feasibility of using chitosan based film containing bacteriocin to improve the preservation of cold-smoked salmon during storage.

Divergicin M35 is a class IIa bacteriocin produced by a strain of *Carnobacterium divergens* M35 isolated by our group from frozen mussels (Tahiri et al., 2004). In a previous study, we showed synergetic inhibition by divergicin M35 and low-molecular-weight chitosan against *L. monocytogenes* LSD532 (Benabbou et al., 2009). A bio-ingredient containing a mixture of *C. divergens* and divergicin M35 has been developed and found to be a strong inhibitor of *Listeria* on smoked salmon. Health Canada recently has approved this bio-ingredient as a new additive for smoked fish (Public Health Agency of Canada, 2016).

The aim of the present study was to evaluate the potential of chitosan-divergicin M35 film as an inhibitor of *L. monocytogenes* on cold-smoked salmon stored for 3 weeks at refrigerator temperatures and the impact of the antimicrobial film on the physical and chemical properties of this seafood product. The impact of chitosan-divergicin M35 film on growth of *L. monocytogenes* was compared with treatment using chitosan-divergicin M35.

MATERIALS AND METHODS

Materials

Chitosan (100 kDa, 94.7% de-acetylated) was obtained from DNP Canada inc. (Granby, QC, Canada). It was provided in 1% (v/v) acetic acid and stored at −20°C until use.

Divergicin M35 was produced and purified using a protocol similar to that previously described by (Tahiri et al., 2004) but adapted for higher culture supernatant volume. Purified divergicin M35 was obtained from supernatant of *C. divergens* M35 MRS culture. Supernatant (500 ml) was heated in a water bath at 100°C for 10 min and then passed through an SP-Sepharose Fast Flow Cation Exchange Column (Amersham, Pharmacia Biotech, Uppsala, Sweden) at flow rate of 3 ml/min. The column was then washed and equilibrated with 1 l of ammonium acetate buffer (5 mM, pH 5). The bacteriocin was eluted with 250 ml of 1.5% (w/v) sodium chloride in ammonium acetate buffer, loaded onto a Sep-Pack C18 Cartridge micro-column (Waters, Milford, MA, United States) previously equilibrated with 5 mM of HCl in HPLC grade water and eluted from the Sep-Pack with 60 ml of 50% (v/v) acetonitrile in water. Acetonitrile was removed using a rotary evaporator. Bacteriocin was concentrated under vacuum with a Speed-Vac concentrator (Thermo Savant Instruments Inc., NY, United States) and kept at −80°C until use. Antimicrobial activity of Divergicin M35 during the steps of purification against *L. innocua* HPB13 was confirmed using the agar diffusion method (Tagg et al., 1976).

Carnobacterium divergens M35 was grown in de Man, Rogosa and Sharpe (MRS) broth (De Man et al., 1960) obtained from Rosell Institute (Montreal, PQ, Canada) containing 0.1% (v/v) Tween 80 and incubated aerobically at 30°C. *L. monocytogenes* strain LSD532 (*L. monocytogenes*) was obtained from the Canadian Food Inspection Agency Laboratory Services Division

(Ottawa, ON, Canada). *L. innocua* HPB13 (*L. innocua*, used as an indicator) was obtained from Health Protection Branch, Health and Welfare Canada (Ottawa, ON, Canada). Both strains were maintained as 20% glycerol stock at −80°C. *L. innocua* and *L. monocytogenes* were grown aerobically at 30°C and 37°C, respectively, in tryptic soy broth (TSB, Difco Laboratories, Sparks, MD, United States) supplemented with 0.6% (w/v) yeast extract (YE, Difco).

Film-Forming Solution and Film Preparation

Chitosan-divergicin M35 (C-M35) solution was prepared by blending stock solutions to obtain 0.125 mg/ml divergicin M35 and 6.25 mg/ml chitosan in 1% (v/v) acetic acid.

Chitosan-divergicin M35 film was prepared by pouring 145 ml of solution into Petri dishes to a height of 1 cm and drying the liquid in a laminar flow hood for 72 h at ambient temperature. The resulting film was peeled and used directly for each experiment. Chitosan film was made also without divergicin M35 for comparison.

Preparation of *L. monocytogenes* Inoculum

Listeria monocytogenes was grown in TSBYE at 37°C and cells were harvested by centrifugation at 7,500 × *g* for 15 min at 4°C, washed twice with sterilized phosphate buffer saline: PBS (0.01 M phosphate, pH 7.2) and re-suspended in 10 ml of PBS. Buffer was added to obtain a final concentration of approximately 10^5 cfu/ml. Dilutions were plated on TSBYE plates and incubated aerobically at 37°C for 24 h before determining viable cell counts.

Assays With Cold-Smoked Salmon

Cold-smoked wild Pacific sockeye salmon (hereinafter 'fish') was provided by Fumoir Grizzly Inc. (St-Augustin, Québec, Canada) and used immediately upon reception. The flesh was cut into squares (3 cm, 4 g) and divided into six groups corresponding to the following treatments:

Treatment A: control (untreated) samples.
Treatment B: 100 μl of *L. monocytogenes* suspension (10^5 cfu/ml) spread on one side of each square (2.5×10^3 cfu/g).
Treatment C: 100 μl of C-M35 solution spread on the side inoculated with *L. monocytogenes*.
Treatment D: inoculated squares covered with 9 cm^2 of C-M35 film.
Treatment E: un-inoculated squares coated with 100 μl of C-M35 solution.
Treatment F: un-inoculated squares covered with 9 cm^2 of C-M35 film.

The experiments were performed in duplicate and each simple was analyzed twice. Simples were dried for 10 min in a laminar-flow biological safety cabinet, wrapped in oxygen permeable film obtained from Fumoir Grizzly Inc., and incubated for 21 days under the conditions suggested by AFNOR (Association Française de Normalisation NF V 45-065, 1997): 14 days at 4°C followed by 7 days at 8°C. These conditions are designed to simulate changes in temperature during storage and handling in commercial and domestic environments.

Color, texture and total volatile basic nitrogen (TVBN) were determined at 1, 7, 14, and 21 days of storage. Microbial composition, pH and antimicrobial activity were determined at 1, 3, 7, 14, and 21 days of storage.

Microbiological Analysis

The whole 4 g sample was placed in a sterile filtering stomacher bag (Seward Medical, London, United Kingdom) with peptone water (0.1% w/v) to obtain a 1/10 dilution (i.e., 36 ml). For treatment D, the C-M35 film was removed first and the films were processed the same way (in 36 ml of peptone water) for measurement of the residual inhibition activity. Bag contents were homogenized for 3 min using a Stomacher 400 circulator (Seward, Therfford, Norfolk, United Kingdom). The filtered homogenate was serially diluted 10-fold in peptone water and dilutions were spread-plated in duplicate on appropriate selective media. For *L. monocytogenes*, CM0856 *Listeria* selective medium supplemented with SR140 *Listeria* selective supplement (Oxoid Ltd., Basingstoke, Hampshire, England) was used and plates were incubated aerobically at 37°C for 48–72 h. Total lactic acid bacteria (LAB) were enumerated on nitrite actidion polymyxin (NAP) agar (Davidson and Cronin, 1973). NAP medium consists of APT agar (Difco Laboratories) supplemented with Polymyxin B (0.003 g/l), cycloheximide (0.01 g/l) and $NaNO_2$ (0.6 g/l), all obtained from Sigma-Aldrich (Oakville, Ontario, Canada). Plates were incubated aerobically at 25°C for 48 h. For the determination of the total viable bacterial counts, dilutions were spread-plated on plate count agar (PCA, Difco Laboratories, Sparks, MD, United States) and incubated aerobically at 30°C for 48 h.

Antimicrobial Activity

Residual inhibitory activity in fish and film in treatments C and D was determined by centrifuging homogenate (7,500 × *g* for 15 min) at 4°C and filtering the supernatant using sterile syringe filters (0.45 μm, Corning, Germany). The agar diffusion method and a quantitative critical dilution micro-method (Tahiri et al., 2004) were used with *L. innocua* HPB13.

Physical and Chemical Analysis

Color parameters L, 'a' and 'b' (brightness, redness and yellowness) were measured using a Minolta Chroma Meter CR-300 (Minolta Camera Co., Ltd., Osaka, Japan) with the CIE color system (CIE, 1996). Measurements were made after standardizing the instrument with light source D and the measuring head was rotated 90° between duplicate measurements at each position. Two fish samples per treatment (A, E, and F only) were measured four times each and the mean of the 8 measurements was used for statistical analyses.

Total volatile basic nitrogen (TVBN) production was determined by a distillation procedure (Pearson, 1976) and the pH of the homogenate was measured.

Texture profile analysis (TPA) involved using a Texture Analyzer TA.XT2 (Stable Micro Systems, Texture Technologies

Corps, Scarsdale, NY, United States) with double compression. Firmness was defined as the peak force of the first compression and cohesiveness was defined as the ratio of the total energy required for the second compression to that of the first compression. The probe (a flat-ended cylinder 4 mm in diameter) descended at a constant speed of 1 mm/s. Four measurements were performed on each of two samples per treatment and the mean value was used for statistical analyses.

Statistical Analyses

Statistical analyses were performed based on duplicate and each simple was analyzed twice. Statistical analyses were performed using STATGRAPHICS plus 4.1 (Manugistics Inc., Rockville, MD, United States). Significant differences among the treatment means of each parameter were tested by analysis of variance. Treatment comparisons were performed using Fisher's least-significant differences (LSD) test with a P-value of ≤ 0.05 considered significant.

RESULTS

Microbiological Analysis

The antimicrobial activity of divergicin M35 during the steps of purification was confirmed using the agar diffusion test (**Figure 1**). A clear zone of inhibition of *L. innocua* HPB 13 was obtained around the well, confirming anti-listerial activity.

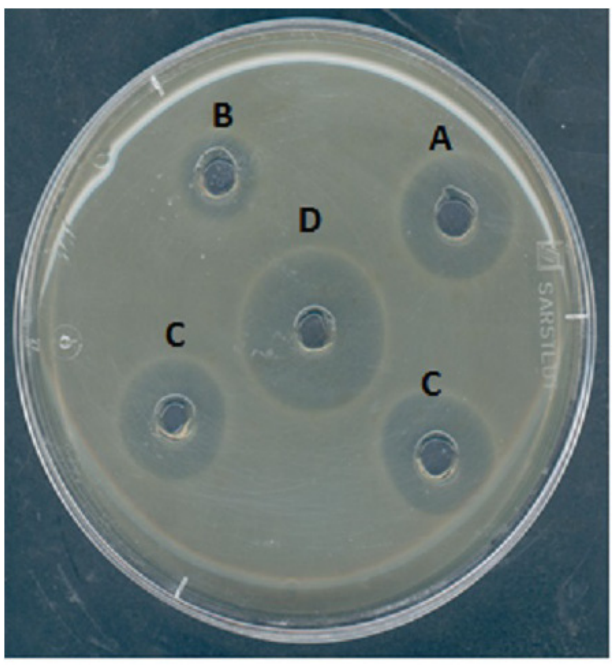


FIGURE 1 | Inhibition of *Listeria innocua* HPB13 by divergicin M35 from *Carnobacterium divergens* M35 culture supernatant **(A)**, eluate from SP-Sepharose column **(B)**, eluate from Sep-Pack C18 column **(C)** and purified divergicin M35 **(D)**.

Figure 2 represents the antimicrobial activity of C-M35 solution and film against *L. monocytogenes* in fish flesh stored at refrigerator temperatures. By day 3, the viable count was about 1 log lower in fish coated with inhibitor solution than in the control, and this difference increased only slightly throughout the storage. There was no reduction in viable count after 14 days of storage. However, the film reduced the count to below the detection limit (<50 cfu/g) from the 1st day of storage.

Total aerobic counts on these samples are shown in **Figure 3**. The chitosan-divergicin M35 solution had no effect on the total aerobic count. In contrast, the film had a strong effect, differing from the control by 1.8 log and 5.8 log cycles, respectively, on days 3 and 21.

The effects of C-M35 solution and film on total lactic acid bacteria are illustrated in **Figure 4**. For all treatments except film, the total lactic acid bacteria counts increased progressively during the 21 days of storage, while counts were below the detection limit (<50 cfu/g) throughout storage with the film.

Counts of *L. monocytogenes*, total aerobes and lactic acid bacteria in the film homogenate were below the detection limit (<50 cfu/g), indicating that no detectable viable cells remained attached to the film (data not shown).

Residual Antimicrobial Activity in Fish and Chitosan Films

Residual inhibitory activity in the fish treated with C-M35 solution and film and in the C-M35 film after storage is shown in **Table 1**. For the entire 21-day period, no activity was obtained from fish treated with solution. However, residual activity was detected in fish treated with the film, decreasing significantly over days 1–14 and ending up at 2.3×10^3 on day 21. A residual activity was observed in the film removed after 1 day of storage, decreasing progressively over 14 days and then stabilizing at 4.61×10^3 through to day 21. Fish treated with chitosan solution or film without bacteriocin contained no inhibitory activity, confirming that the residual activity detected was due to divergicin M35 (results not shown).

Physical and Chemical Analysis of Fish Samples

The effect of C-M35 solution and film on the color values of un-inoculated fish is shown in **Table 2**. Differences in lightness (L) between the control and the treatment with solution were significant after 14 days of storage, although no significant difference was observed after 21 days. Application of the film did not produce any significant difference in the L-value during the 1st week, although a significant increase was observed thereafter. The redness (a) values of control and solution-treated fish were the same after 1 week but were significantly different after 14 days of storage. For fish treated with film, the redness values were higher than those of the control. The C-M35 solution and film, respectively, resulted in decreased and increased yellowness (b) compared to the control until 21 days of storage. **Figure 5** shows the overall appearance and difference in color between the treatments after 21 days of storage. The difference in redness matches the measured (a) values.

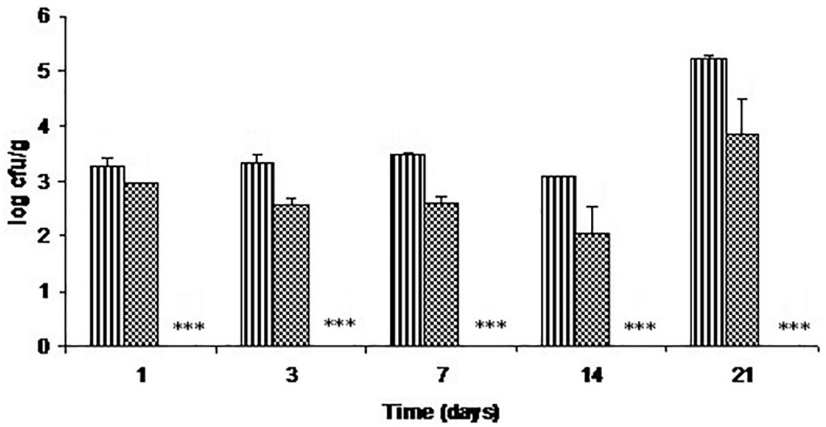


FIGURE 2 | Growth of *L. monocytogenes* on cold-smoked wild salmon during storage at 4°C for 14 days then at 8°C for 7 days: stripe pattern represents untreated fish, checker pattern represents treatment with C-M35 solution; values for the C-M35 film treatment (***) were below the detection limit (<50 cfu/g).

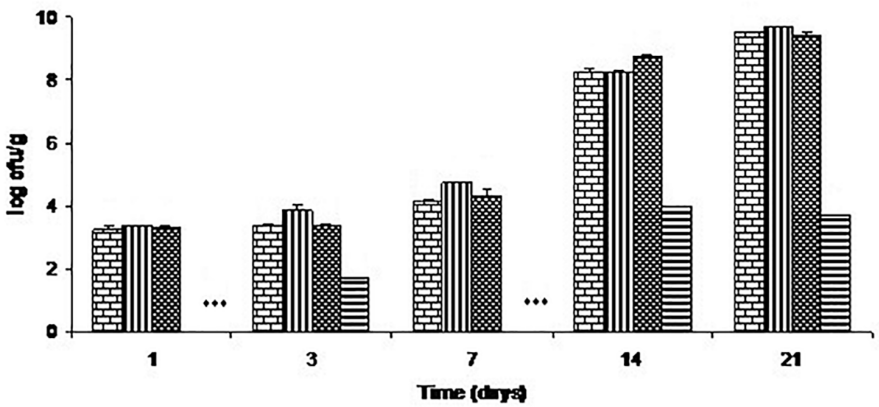


FIGURE 3 | Growth of total aerobes on cold-smoked wild salmon during storage at 4°C for 14 days then at 8°C for 7 days: brick pattern represents the control, striped represents fish inoculated with *L. monocytogenes*, tight weave represents the C-M35 solution treatment and horizontal stripe represents the C-M35 film treatment. *** Values were below the detection limit (<50 cfu/g).

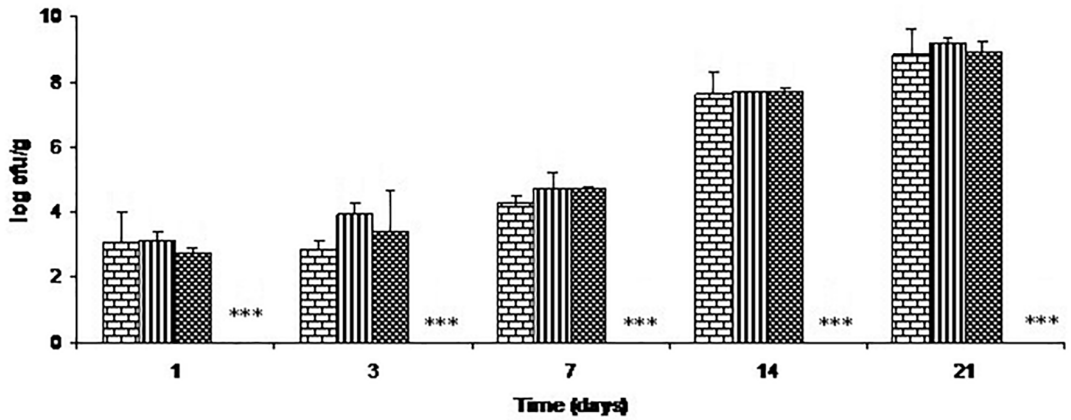
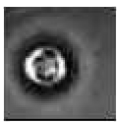
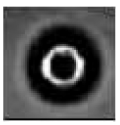
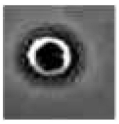
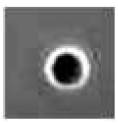


FIGURE 4 | Growth of lactic acid bacteria on cold-smoked wild salmon during storage at 4°C for 14 days then at 8°C for 7 days: brick pattern represents the control, stripe represents fish inoculated with *L. monocytogenes*, tight weave represents treatment with C-M35 solution; values for fish covered with C-M35 film (***) were below the detection limit (<50 cfu/g).

TABLE 1 | Residual divergicin activity (arbitrary units) in homogenate of cold-smoked wild salmon and its chitosan-divergicin M35 film covering after 14 days at 4°C followed by 7 days at 8°C, determined by the agar diffusion test (a) and the critical dilution micro-method (b).

Day	1		3		7		14		21	
Method	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**	**a**	**b**
1		4.61×10^{3a}		2.30×10^{3c}		2.30×10^{3c}		5.76×10^{2b}		2.30×10^{3c}
2		3.69×10^{4a}		1.84×10^{4b}		9.22×10^{3c}		4.61×10^{3d}		4.61×10^{3d}
3		0		0		0		0		0

1: In fish treated with chitosan-divergicin film. 2: In chitosan-divergicin film. 3: In fish treated with chitosan-divergicin solution. Means with different letters within the same row are significantly different ($P < 0.05$).

TABLE 2 | Color parameters L, a, and b for cold smoked wild salmon during storage at 4°C for 14 days then 8°C for 7 days, A: control, E: fish coated with chitosan-divergicin solution, F: fish covered with chitosan-divergicin film.

Color parameter	Day	Treatment		
		A	***E***	***F***
L	1	48.01 ± 1.12^{hg}	47.01 ± 1.07^{h}	48.81 ± 2.37^{fg}
	7	49.89 ± 0.91^{def}	50.42 ± 0.87^{cde}	49.17 ± 2.69^{efg}
	14	48.80 ± 1.12^{fg}	52.74 ± 0.97^{a}	51.31 ± 0.63^{bc}
	21	49.86 ± 1.84^{def}	50.67 ± 1.15^{bcd}	52.05 ± 0.48^{ab}
a	1	32.99 ± 2.03^{bcd}	32.46 ± 1.31^{cd}	34.63 ± 1.58^{a}
	7	33.07 ± 1.73^{bcd}	31.65 ± 1.03^{d}	35.15 ± 0.60^{a}
	14	34.86 ± 1.82^{a}	29.00 ± 1.40^{e}	34.15 ± 0.65^{ab}
	21	32.95 ± 2.22^{bcd}	28.15 ± 2.13^{e}	33.86 ± 0.71^{abc}
b	1	32.38 ± 1.55^{drf}	29.71 ± 1.33^{g}	34.39 ± 2.27^{abc}
	7	34.02 ± 1.12^{bc}	31.76 ± 1.31^{f}	34.73 ± 2.11^{abc}
	14	33.75 ± 1.63^{cd}	31.89 ± 1.06^{ef}	35.90 ± 1.03^{a}
	21	33.43 ± 2.70^{cde}	28.83 ± 0.96^{g}	35.47 ± 0.82^{ab}

Means ± std with different letters for the same color parameter are significantly different ($P < 0.05$).

Figure 6 shows the firmness (A) and cohesiveness (B) profiles of the stored fish samples. Firmness increased in samples treated with solution, although no significant difference was observed compared to the control. For fish treated with film, there was a significant difference throughout the storage period in the firmness value compared to the control. The solution did not affect cohesiveness significantly during the storage. The cohesiveness value of fish treated with film was the same after 1 week of storage compared to the control and decreased significantly after 14 days of storage.

The pH of fish treated with C-M35 solution was stable, ranging between 5.9 and 6 during the 21 days of storage, and no significant difference was observed compared to the control (data not shown). Application of the film resulted in a decrease to 5.5 after 1 day of storage and this value was maintained throughout the 3 weeks of storage. TVBN production increased from 10.3 to 30.4 mg N/100 g in solution-treated samples over the 21 days, which was similar to the control range of 12.4–29.9 mg N/100 g (results not shown). Application of the film slowed TVBN production significantly to 17.5 mg N/100 g at the end of the 21 days.

DISCUSSION

The efficacy of bacteriocins as bio-preservatives is influenced by various food matrix parameters and by the form in which they are applied. One of the most promising strategies proposed to enhance efficacy is incorporation into polymer films. Polymer-based films allow direct contact with the food surface and therefore continuous release of the antimicrobial compound, allowing the maintenance of inhibitory activity on food surfaces (Quintavalla and Vicini, 2002; Ture et al., 2011; Fu et al., 2016).

The combination of a natural polymer such as chitosan (by itself somewhat inhibitory) with a natural soluble inhibitor such as divergicin M35 is a plausible strategy for suppressing bacterial growth in ready-to-eat products and one that may be acceptable to consumers. Our work has shown clearly that this combination does inhibit *L. monocytogenes* on cold-smoked salmon and much more when applied as a film, in which case it can keep viable counts below the detection limit (<50 cfu/g) for 21 days. Since *L. monocytogenes* did not infiltrate the film, we can conclude that inhibition was complete. This was no doubt due to a continuous release of divergicin M35. Previous studies have shown that antimicrobial effects of active films in various food systems depend on the nature and concentration of the antimicrobial agent used. Ye et al. (2008b) increased the anti-*L. monocytogenes* activity of chitosan-coated plastic film on cold-smoked salmon by incorporating

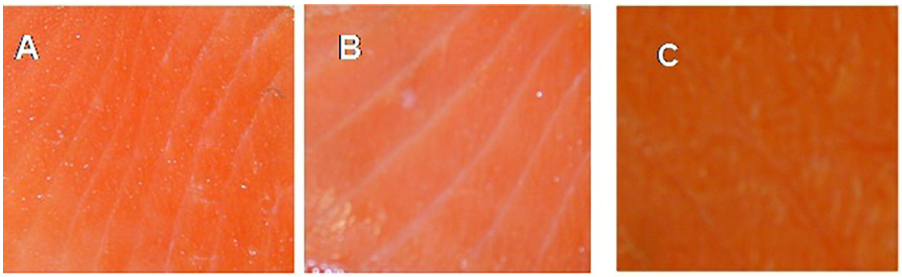


FIGURE 5 | Appearance of cold-smoked wild salmon flesh at the end of 21 days of storage (14 days at 4°C then 7 days at 8°C): control **(A)**, coated with C-M35 solution **(B)**, covered with C-M35 film **(C)**.

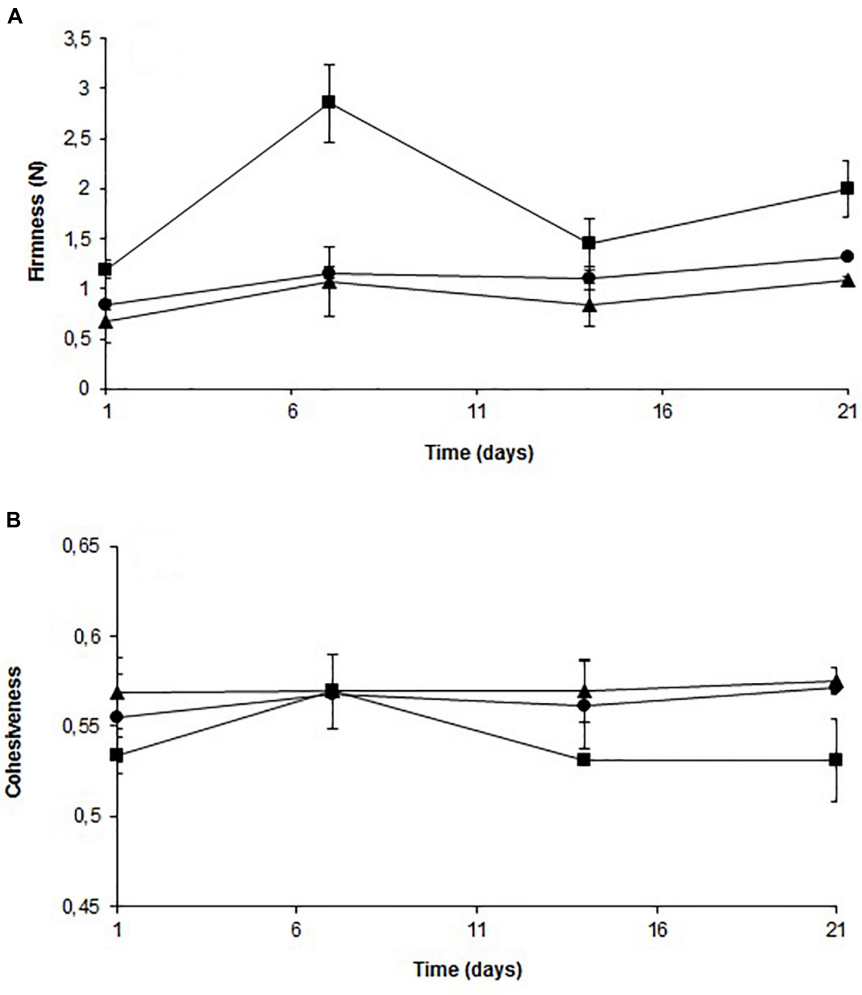


FIGURE 6 | Evolution of firmness **(A)** and cohesiveness **(B)** of cold-smoked wild salmon during storage at 4°C for 14 days followed by 8°C for 7 days: triangle represents control, octagon represents treatment with C-M35 solution and square represents treatment with C-M35 film.

sodium lactate alone or with nisin or potassium sorbate. They obtained similar results on ham steaks by incorporating with these compounds as well as sodium diacetate or sodium benzoate Ye et al. (2008a). Sodium lactate was the most effective in this case. Marcos et al. (2007) found that alginate was superior to zein and polyvinyl alcohol as an enhancer of *L. monocytogens* inhibition by enterocin at 200 or 2,000 units per cm^2.

The weak anti-listerial activity and absence of residual activity of the C-M35 solution in the sample homogenate is likely due to

interactions between the bacteriocin and food components such as lipids as well as partial inactivation by endogenous proteases produced *in situ* by other microorganisms (Katla et al., 2001; Vaz-Velho et al., 2005). Aasen et al. (2003) showed that more than 80% of the sakacin P added to cold-smoked salmon and chicken was adsorbed to proteins. The antimicrobial activity of inhibitory substances in cold-smoked salmon may also be affected by added salt. Devlieghere et al. (2004) observed that adding NaCl to a medium decreased the antimicrobial activity of chitosan, due to interaction between Cl^- ions and positive charges on the chitosan and to competition of Na^+ for the negative charges on the microbial cell surface, and that antimicrobial activity also depended on pH and the isoelectric point of proteins in the food matrix. These phenomena probably affect bacteriocin activity, whether free or in film. However, continuous release of divergicin M35 during storage may minimize such losses. Our results showed that the fish and the film covering contained residual activity of divergicin M35 during the 21 days of storage. The difference in activity added and activity recovered in the film and fish is probably due to the phenomena mentioned above.

The total aerobic and lactic acid bacteria counts as well as TVBN production suggest that the antimicrobial solution was practically ineffective while the antibacterial film was quite effective. Since initial levels of bacteria in this salmon product were low, the bacteriocin-loaded chitosan film reduced these to below the limit of detection. Ye et al. (2008a) also obtained a substantial reduction in total and anaerobic counts using their chitosan-coated plastic film loaded with antimicrobial agents. The microbiological results obtained in our study demonstrate the potential of divergicin-loaded chitosan film for extending the shelf life of cold-smoked salmon. Previous reports that chitosan inhibits the growth of a wide variety of microorganisms (Shahidi et al., 1999; Jeon et al., 2002; Agullo et al., 2003; Rinaudo, 2006; Yingyuad et al., 2006). However, and to the best of our knowledge, no study has been published on application of chitosan based film containing bacteriocin to improve the preservation of cold-smoked salmon during storage.

In the present study, we were also interested in the impact of chitosan-divergicin M35 film on the physical and chemical characteristics of the fish product. The increase in firmness during the 1st week of storage was likely due to moisture loss, while changes during the 2nd week could be due to proteases produced by spoilage microorganisms (Morzel et al., 1997) or to autolytic enzymes in the fish flesh (Stoknes and Rustad, 1995). The significant differences obtained with film were probably related to the strong affinity of chitosan NH_2 groups for water molecules (Wu et al., 2004). The increase in the firmness of film-covered fish during the last week of storage may be due to the onset of film breakdown. The cohesiveness of these samples decreased slightly during the last week of storage, which was consistent with the changes in the muscle strength retention capacity after the first compression.

Increased brightness (L) and decreased redness and yellowness (a and b) values of C-M35 solution-treated compared to untreated fish are consistent with previous observations. Jo et al. (2001) observed similar changes in brightness and redness for sausage containing chitosan as a preservative. Tahiri et al. (2009) reported a significant decrease of the yellowness value of divergicin-M35-treated cold-smoked salmon stored for 21 days at 4°C. The increased in redness of film-covered samples is probably due to decreased breakdown of carotenoids because of the low oxygen permeability of chitosan film (Xu et al., 2005). The loss of water from the sample may also result in increased pigment concentration (Choubert et al., 1992).

For all treatments, the TVBN concentration was below the acceptable maximum proposed by various authors (Civera et al., 1995; Leroi et al., 2001). The C-M35film-covered samples thus showed a higher hygienic quality compared to the control and solution-treated samples. TVBN concentration is considered a major quality index for cold-smoked salmon (Leroi et al., 2001; Brillet et al., 2005). These low concentrations could be a result of reduced counts of spoilage bacteria due the effect of the C-M35 film. Jeon et al. (2002) reported reductions in TVBN of 33–50% and 26–51% in chitosan-glycerol-coated cod and herring, respectively, at the end of 12 days of storage at 4°C.

CONCLUSION

In this study, the combination of chitosan and divergicin M35 was more effective for inhibiting *L. monocytogenes* in cold-smoked wild salmon when applied as a film rather than as a solution. The film reduced *L. monocytogenes* to below detection limit (<50 cfu/g). The film was shown to maintain acceptable color and texture of this fish product during 3 weeks of storage at refrigerator temperatures. This represents an extension of product shelf life. Additional studies using sensory evaluation panels should be conducted before considering commercial application of such films.

AUTHOR CONTRIBUTIONS

RB was the Ph.D. student in charge of performing all the laboratory work. RB also was in charge of the preparation of the manuscript. MS was in charge of the laboratory work related to the preparation and characterization of divergicin-chitosan films. MD was involved in the preparation and study of the antimicrobial activity of divergicin M35 and chitosan. IF supervised the entire work and was in charge of the microbiological aspects of the work.

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Decontamination of Minimally-Processed Fresh Lettuce Using Reuterin Produced by *Lactobacillus reuteri*

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Over the last years the demand for pre-washed, fresh-cut, and minimally-processed (MP) produce has increased. MP fresh vegetable are rapidly spoiled, whereas there is consumers' concern about chemical disinfection treatments such as with chlorine. A promising antimicrobial is reuterin, a broad-spectrum-antimicrobial compound produced by food-grade *Lactobacillus reuteri* from glycerol. In aqueous solution, reuterin is a dynamic system consisting of 3-hydroxypropionaldehyde (3-HPA), its hydrate, its dimer as well as acrolein, which was recently identified as the main antimicrobial component of the system. Here, we tested the use of reuterin containing similar 3-HPA levels but different acrolein concentrations for decontaminating and preserving fresh-cut lettuce. Crude reuterin (CR) was produced by biotransformation of 600 mM glycerol using *L. reuteri* DSM 20016T. CR preparations were further incubated for 16 h at 50°C to produce enhanced reuterin (ER) with raised concentration of acrolein. Fresh-cut iceberg lettuce (*Lactuca sativa)* was washed using CR (1.5–1.9 mM acrolein) and ER (7.2–21.9 mM acrolein) solutions at 4°C, or sodium hypochloride (250 mg/L) and tap water, and compared with unwashed lettuce. Washed lettuce samples were packed under modified atmosphere (2% O_2, 5% CO_2, and 93% N_2) and stored for 13 days at 4°C. Application of ER containing 12.1, 20.9, or 21.9 mM acrolein reduced the initial viable plate counts of *Enterobacteriaceae* (by 2.1–2.8 log CFU/g), and yeasts and molds (by 1.3–2.0 log CFU/g) when compared with unwashed samples. In contrast, reuterin solutions containing 7.2 mM acrolein, sodium hypochlorite and tap water only showed very limited and transient, or no effects on the cell loads of lettuce after washing and during storage. Visual assessment of leaves washed with ER showed acrolein concentration-dependent discoloration noticeable already after 3 days of storage for the highest acrolein concentrations. Discoloration became severe for all ER treatments after 7 days, while the other treatments preserved the aspect of washed lettuce. Our data show the predominant role of acrolein as the main antimicrobial component of the reuterin system for food biopreservation. Reuterin preparations with enhanced acrolein concentration of 12.1 mM and higher were effective to reduce plate counts of *Enterobacteriaceae* and yeasts and molds washed lettuce until day 7 but induced pronounced discoloration of lettuce.

Keywords: reuterin, biopreservation, minimal processing, *Lactobacillus reuteri*, acrolein

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INTRODUCTION

The market of pre-washed, fresh-cut and minimally-processed (MP) vegetable produce is rising worldwide. MP fresh vegetable produce contain complex bacterial communities which may include spoilage microbes, e.g., fluorescent *Pseudomonas* spp. and *Erwinia carotovora,* and pathogens (Ragaert et al., 2007; Barth et al., 2009). The consumption of these produce has been associated with foodborne outbreaks by *Salmonella enterica*, pathogenic *Escherichia coli*, *Shigella* spp., *Campylobacter* spp., *Listeria monocytogenes, Staphylococcus aureus, Yersinia* spp. and *Bacillus cereus* (Harris et al., 2003; FAO/WHO, 2008; Critzer and Doyle, 2010; Fatica and Schneider, 2011). Therefore, suppliers are increasingly interested in implementing natural antimicrobial compounds for decontaminating the produce and providing high-quality and safe products.

Post-harvest microbial contamination rather than contamination in the field is a major cause of human pathogenic bacteria on MP fresh produce (Jensen et al., 2013). Tissue damages associated with peeling, cutting and slicing of MP fresh produce reduces the defense against pathogens (Allende et al., 2004). Internalization of fresh produce by enteric pathogens through cut surfaces reduces their exposure to sanitizing agents (Erickson, 2012). Moreover, microorganisms can adhere to the surface of freshly harvested fruits and vegetables and may survive decontamination steps due to the formation of biofilms (Allende et al., 2008). The main decontaminating steps in the processing chain of MP fruits and vegetables are washing and disinfection. Washing removes soil, insects and other debris and has an added advantage of reducing microbial loads (Gil et al., 2009). However, washing water can serve as a vehicle for dispersal of microorganisms (Holvoet et al., 2012). Thus, sanitation of produce is pivotal for guaranteeing quality and safety for human consumption.

Chlorine is the most widely used washing and sanitizing agent in the processing of fruits and vegetables (FAO/WHO, 2008). However, because of the increasing public health concerns about possible formation of chlorinated organic compounds, European countries such as Germany, The Netherlands, Denmark, and Belgium, as well as Switzerland have banned the use of chlorine in fresh-cut produce (Betts and Everis, 2005). Several alternatives to chlorine such as chlorine dioxide, ozone, organic acids, peracetic acid (PAA), and hydrogen peroxide have been gaining interest in recent years but none of them were found to have the expected requirements (Ölmez and Kretzschmar, 2009). Among alternative preservation technologies, a particular attention has been paid to biopreservation, which is defined as the use of naturally-produced compounds for decontamination and preservation (Lacroix, 2011). A promising broad spectrum biopreservative agent is the bacterial metabolite reuterin, produced by *Lactobacillus reuteri* with antimicrobial activity against Gram-positive and Gram-negative bacteria, yeasts and molds (Cleusix et al., 2007; Stevens et al., 2010).

Reuterin is produced by certain strains of *L. reuteri* during the anaerobic fermentation of glycerol. In aqueous solution, reuterin is a dynamic system consisting of 3-hydroxypropionaldehyde (3-HPA), its hydrate 1,1,3-propanetriol, its dimer 2-(2-hydroxyethyl)- 4-hydroxy-1,3-dioxane, and acrolein (Vollenweider and Lacroix, 2004; Engels et al., 2016). The 3-HPA can be further converted to 1,3-propanediol (1,3-PD) in the presence of glucose, but large amounts of reuterin are excreted into the medium when glucose is low (Stevens et al., 2013). Acrolein was recently shown to be the main component responsible for the antimicrobial activity of reuterin (Engels et al., 2016). 3-HPA and acrolein interconversion does not occur at 4°C and pH 4, while at higher temperatures the equilibrium is shifted toward acrolein (Engels et al., 2016). Minimal inhibitory concentrations (MIC) and bactericidal concentrations (MBC) of reuterin (determined as 3-HPA) were measured in the ranges 0.5–15 mM for *Listeria* spp., 35 mM for *Bacillus subtilis*, 0.3–50 mM for Gram-negative *E. coli* and *P. aeruginosa*, and 0.15–0.98 mM for yeasts (Stevens et al., 2010).

The aim of the present work was to investigate whether biotechnology-produced reuterin can be used for decontamination of fresh lettuce, and the role of acrolein as the main antimicrobial compound of reuterin in a food system. The washing efficacy of crude reuterin (CR) and reuterin with enhanced acrolein concentrations (ER) was tested in reducing the microbial population of MP fresh-cut lettuce during 13 days of refrigerated storage (4°C) under modified atmosphere. Reuterin treatments were compared with washing with tap water or chlorinated water (250 mg/L), and with unwashed produce.

MATERIALS AND METHODS

Bacterial Strain and Reuterin Stock Production

Lactobacillus reuteri DSM 20016T was obtained from the DSM strain collection (Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany) and used for the production of reuterin using a two-step process, as described previously (Stevens et al., 2013). *L. reuteri* was routinely cultivated in Man, Rogosa and Sharpe medium (MRS, Biolife, Milan, Italy) at 37°C. Briefly, *L. reuteri* was grown overnight at 37°C in 10 mL MRS broth. One percentage (1%) of the overnight culture was added to filter sterilized MRS supplemented with 20 mM of glycerol (Sigma-Aldrich, Buchs, Switzerland) and incubated to an OD_{600} of approximately 8.0, representing the early stationary growth phase. To obtain cells with comparable metabolic activity among the trials, 10 mM of glucose and 20 mM of glycerol (final concentrations) were added to this early stationary phase culture and cells were reactivated for 30 min at 37°C. Subsequently, cells were harvested (4,000 × g, 10 min, room temperature) and re-suspended in sterile 600 mM glycerol solution. Conversion of glycerol to reuterin was conducted at 25°C for 3 h. Reuterin-containing supernatant was recovered by centrifugation (12,000 × *g*, 5 min, 4°C) and sterile filtered (0.2 μm). Several batches of reuterin were produced over a 10 day period, pooled together, and stored at 4°C for the washing trails. The freshly produced reuterin stock solution contained 433 mM 3-HPA and 6 mM acrolein. The same reuterin stock was used for all four washing trials, which were carried out over a total period of 7 months.

HPLC-RI and IC-PAD Analysis of Reuterin Solutions

Glycerol, 1,3-PD, 3-HPA concentrations were determined by HPLC with refractive index detector (Hitachi LaChrome, Merck, Dietikon, Switzerland) on an Aminex HPX-87H column (300 mm × 7.8 mm, Bio Rad, Reinach, Switzerland) as described previously (Cleusix et al., 2008). Solutions for HPLC analysis were diluted in phosphate buffer (20 mM, pH 4.0) as previously recommended by Engels et al. (2016). Sulfuric acid (10 mM) was used as eluent and isocratic conditions were applied at a flow rate of 0.6 mL/min for 30 min at 40°C. The injection volume was 40 μL. Quantification was performed with external standards of glycerol and 1,3-PD (Sigma-Aldrich GmbH, Buchs, Switzerland). Purification of 3-HPA used as standard was done according to Vollenweider et al. (2003). HPA was diluted with distilled water to about 10 M solution, which was stable for at least 6 months during storage at 4°C.

Acrolein concentration was determined using IC-PAD as previously described (Engels et al., 2016). Hydroquinone (2%) was added to the samples to stabilize acrolein (Kächele et al., 2014). To minimize acrolein evaporation, airtight 0.7 ml PP Crimp/Snap LC vials (BGB Analytik, Boeckten, Switzerland) were used for analysis. Briefly, IC-PAD was performed on a Thermo Scientific (Reinach, Switzerland) ICS-5000+ system equipped with a quaternary gradient pump, a thermostated autosampler and an electrochemical detector with a cell containing a Ag/AgCl reference electrode and a disposable thin-film platinum working electrode tempered at 25°C. Analytes were separated with a Thermo Scientific IonPac ICE-AS1 4 × 250 mm ion-exclusion column with a guard column, operated at 30°C. The solvent system was isocratic 0.1 M methanesulfonic acid at 0.2 mL/min for 36 min. The injection volume was 10 μL. Electrochemical data were obtained after modification and optimization of the triple-potential waveform consisting of regeneration/detection, oxidation and reduction potentials. Commercial pure acrolein (>99%, stabilized with 0.2% hydroquinone) was purchased from Sigma-Aldrich GmbH (Buchs, Switzerland) and used as external standard. Acrolein is a volatile and toxic compound, hence, all safety measures were observed.

Preparation of Lettuce Washing Solutions

Three different washing solutions (i) sodium hypochloride (NaOCl), (ii) enhanced reuterin (ER) and (iii) crude reuterin (CR) were prepared using autoclaved tap water. Sterile tap water washed (TW) and not washed (NW) lettuce were included as controls. To prepare NaOCl solution (2.5 L), liquid sodium hypochloride (Sigma-Aldrich) was added to autoclaved tap water to a final concentration of 250 mg/L free chlorine. On the day of washing, 2.5 L of CR washing solution was freshly prepared by mixing 580 mL reuterin stock to autoclaved tap water to achieve a final concentration of 100 mM 3-HPA. This solution (CR) was then cooled down on ice to 4°C and used immediately. For preparing ER, 2.5 L of CR solution was prepared as presented above and incubated for 16 h at 50°C in a dry oven (Cleanroom drying oven UF750plus, Memmert GmbH + Co. KG, Schwabach, Germany) to shift the reuterin equilibrium from 3-HPA to acrolein. The ER solution was cooled down on ice to 4°C and immediately used for washing.

Preparation and Processing of Romaine Lettuce Samples

Romaine lettuce (*Lactuca sativa*) from Switzerland (Zürich area) which had not been prewashed was purchased from a local retailer on the same day of arrival from the grower. Trial 1, 2, and 3 were performed at intervals of 14 days with lettuce obtained during the winter season of 2016, whereas trial 4 was performed with lettuce obtained in the spring season of 2017 (7 months after trial 1). The lettuce heads were transported under cold conditions to ETH Zürich and immediately processed and washed in conditions simulating continuous MP process in industry, according to the flow diagram described in **Figure 1**. Soiled, damaged outer leaves, and stem were removed and leaves were cut (approximately 2 × 2 cm) using disinfected knives.

For each treatment, 250 g of lettuce were washed by hand with gentle mixing in a 5 L beaker containing 2.5 L of solution (1:10 w/v) for 1 min. Following the washing process, the products were placed in a sieve for 30 s to allow the washing water to drain off. The products were then rinsed with 2.5 L of autoclaved tap water to remove residue treatment on the product and a salad spinner was used to manually centrifuge the washed lettuce. Lettuce was packed into polyethylene sterile bags (18 × 12 cm, 50 g each) under modified atmosphere (2 ± 1% O_2, 5 ± 1% CO_2, and 93 ± 1% N_2), as previously described by Pereira et al. (2014). For packing, a gas exchange device with a vacuum packaging machine (Multivac C200, Sepp Haggenmüller GmbH & Co. KG, Wolfertschwenden, Germany) and a mixing station (Gas mixer KM 100-3M, Witt-Gasetechnik GmbH & Co. KG, Written, Germany) were used.

Five milliliter of the washing solution were collected before and after lettuce washing to measure the concentrations of glycerol, 3-HPA, 1, 3-PD and acrolein, and the pH (Metrohm 780 pH Meter, Metrohm AG, Herisau, Switzerland).

Bacteria, Yeasts and Molds Counts During Storage

The population of aero-tolerant microbes was enumerated in duplicate after 1, 3, 7, and 13 days of storage at 4°C. At each sampling point, the content of a package (50 g) was transferred into a stomacher bag (Stomacher® 400 Classic Bags, Seward, West Sussex, United Kingdom), combined with 200 mL of 0.1% peptone and macerated in a stomacher (BagMixer® 400 P, Interscience, Saint Nom, France) at high speed for 5 min. Serial dilutions were made in 0.1% peptone and surface plated (0.1 mL) in duplicate onto selective and non-selective media.

Total aerobic mesophilic bacteria were enumerated in duplicate on Luria-Bertani (LB) agar (Becton Dickinson, Allschwil, Switzerland), and *Enterobacteriaceae* were grown using ENDO agar (Sigma-Aldrich, Buchs, Switzerland). LB and ENDO agar were incubated at 37°C for 48 h. Yeasts and molds were enumerated using Yeast extract Glucose Chloramphenicol (YGC)

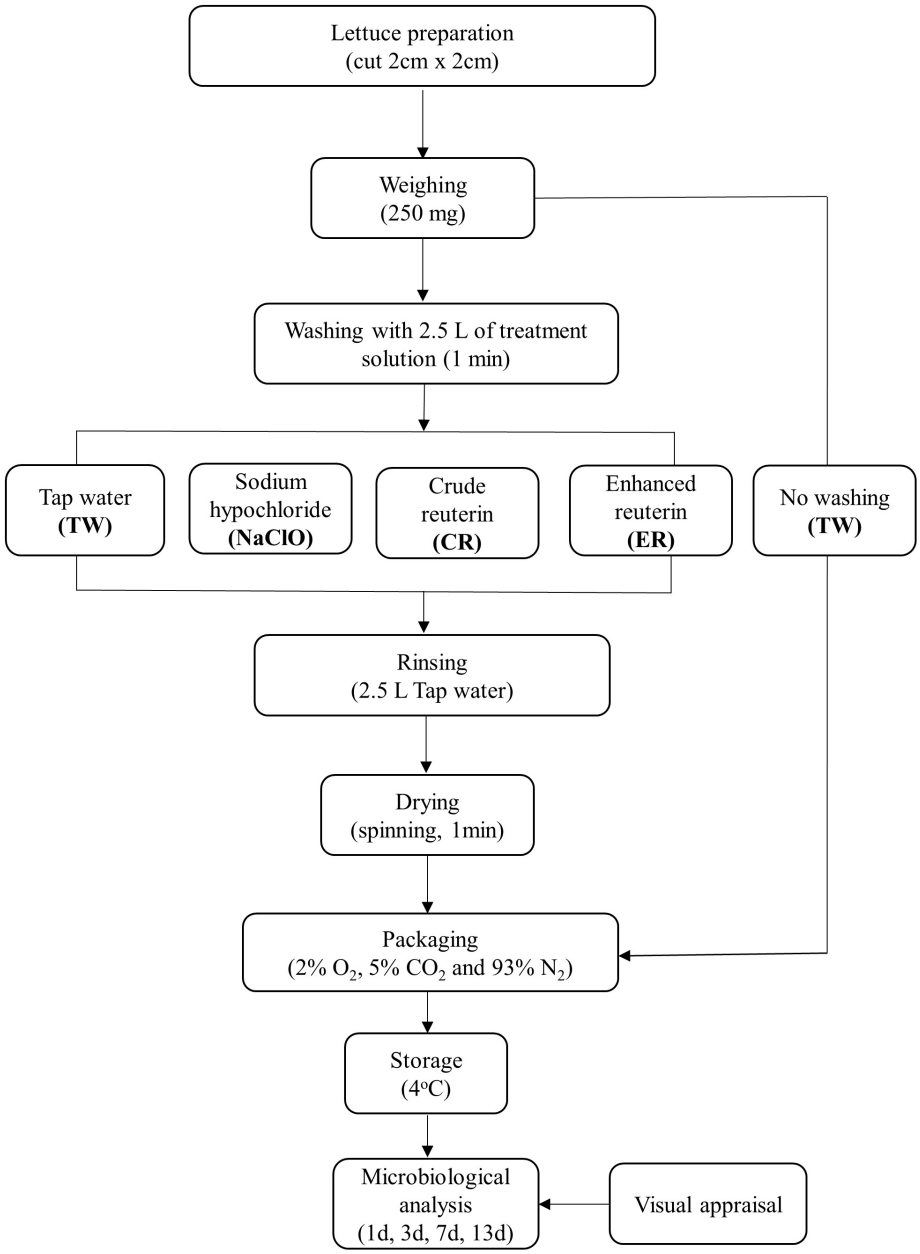

FIGURE 1 | Experimental set up of the washing trials of minimally-processed (MP) lettuce using different treatments.

agar (Merck KGaA, Darmstadt, Germany) further supplemented with 10 μg m/L chloramphenicol after autoclaving, and the plates were incubated at 30°C for 48 h.

Acrolein Quantification in Lettuce Washed With Reuterin

To estimate the residual acrolein concentration in reuterin washed lettuce, 2 mL of macerated lettuce prepared above was centrifuged at 4°C at 5000 × *g* for 5 min. The supernatant was passed through a 0.45 μm filter (Infochroma AG, Switzerland), and acrolein concentration was determined using IC-PAD.

Visual Evaluation of Lettuce Leaves

The appearance of fresh-cut lettuce was assessed visually after 1, 3, 7, and 13 days after treatment. The focus was on discoloration, browning of cut edges and appearance of brown spots on the leaves.

Data Analysis

The experiments were carried out with four blocks using the same reuterin stock, with each block including washing treatments with TW, CR, ER, and NaClO compare to un-washed lettuce. Plate counts were performed in duplicate, and

mean data were reported as colony-forming units per gram lettuce (CFU/g). The decimal reduction of a washing treatment was calculated at each time point by the difference between the treatment and unwashed lettuce count. To assess the dose dependent effects of acrolein on cell counts, a regression analysis was used with CR and ER data after 1 and 13 days of storage, using SigmaPlot 12.5 (Systat Software, San Jose, CA, United States).

RESULTS

Fate of Reuterin Components During Washing

A single stock of reuterin was produced for the four trials carried out over a total period of 7 months. It was observed that the composition of CR prepared from the stock solution changed over time, with a steady decrease of 3-HPA concentration, from 98.5 in trial 1 to 87.1 mM in trial 4. However, the acrolein concentration of 1.8 ± 0.2 mM in the CR stock did not change with time (**Table 1**). The concentrations of 3-HPA and acrolein in the solution collected after washing the lettuce were slightly lower than before washing for CR treatments. Between 0.6–1.3 mM 3-HPA and 0.1–0.7 mM acrolein were not recovered in the CR washing solution after washing.

Acrolein was recently shown to be the main component for antimicrobial activity of reuterin (Engels et al., 2016). Therefore we incubated the CR reuterin solutions at 50°C for 16 h to produce ER solutions containing an increased acrolein concentration. The 3-HPA concentration in ER decreased from trial 1 (92.5 mM) to trial 4 (75.8 mM), consistent with the decreased concentration in CR observed over time. The acrolein titres in ER solutions for the four trials were in the range of 7.2–21.9 mM, which corresponded to between 4- and 12-fold increase compare to the corresponding CR solutions, but with no time effect. The variation in acrolein synthesis in ER solution was due to the oven not able to control the set temperature during incubation. In case where 7.2 and 12.1 mM acrolein was obtained, the temperature of the oven as measured by external thermometer was 5°C less than the set temperature. Between 2–4 mM 3-HPA and 0.6–1.6 mM acrolein were not recovered in the ER washing solution after washing (**Table 1**).

Antimicrobial Effect of Washing Solutions

We investigated the impact of different washing solutions (CR, ER, NaClO, and TW) on the viable cell counts of *Enterobacteriaceae*, yeasts and molds, and total aerobic mesophilic bacteria of lettuce after washing and during storage for 13 days at 4°C (**Figure 2**). The effectiveness of treatments was also expressed by the decimal reduction of the counts for a treatment at a defined time point compare to the unwashed lettuce NW (Supplementary Figure 1).

Fresh-cut lettuce washed with 250 mg/L of NaClO showed a decimal reduction of the *Enterobacteriaceae* population between 0.3 and 1.8 log CFU/g compare to NW (5.6 ± 0.2 log CFU/g, $n = 4$) after day 1 of storage. The counts of *Enterobacteriaceae* steadily increased with storage time to reach similar levels to the unwashed lettuce at day 13 (6.7 ± 0.1 and 6.7 ± 0.3, respectively). Washing with TW, CR, and ER with 7.2 mM acrolein (trial 4) resulted in less than 1 log CFU/g decimal reduction after 1 day compare to NW, followed by regrowth of *Enterobacteriaceae* on lettuce during storage to reach similar levels as for NW after 13 days. In contrast, washing with ER containing 12.1 (trial 1), 20.9 (trial 3), or 21.9 mM acrolein (trial 2) strongly reduced the initial counts of *Enterobacteriaceae* between 2.1 and 2.8 log CFU/g, after the first day of treatment compare to NW. The decimal reductions induced by ER treatments (12.1 mM or more acrolein) were maintained until day 7 (between 1.4 and 2.0 log CFU/g), while a pronounced regrowth of *Enterobacteriaceae* occurred after day 3. At the end of storage, *Enterobacteriaceae* counts in active ER treatments were similar to the other washing treatments and NW lettuce (**Figures 2D,G,J**).

The yeasts and molds counts (NW, 4.7 ± 0.9 log CFU/g, $n = 4$) was reduced by less than 1 log unit after washing with TW, CR, and ER with 7.2 mM acrolein after 1 day of storage compared to NW. Washing with NaClO resulted in a 0.6–1.2 log CFU/g decimal reduction of yeasts and molds after the first day of storage. ER washing treatments containing 12.1, 20.9, and 21.9 mM acrolein reduced the initial counts of yeasts and moulds between 1.3 and 2.0 log CFU/g after day 1 of treatment. The ER inhibition effect was maintained until day 3. At day 7, while yeasts and molds started to regrow, the decimal reduction remained between 1.0 and 1.8 log CFU/g compared to unwashed lettuce. At day 13, the yeasts and molds counts on lettuce washed with ER containing acrolein 12.1 mM and

TABLE 1 | 3-HPA and acrolein concentrations in washing solution quantified by HPLC and IC-PAD. Acrolein loss during washing was estimated by the difference of concentration in the solution before and after washing.

Washing solution	Washing step	Trial 1 [mM]		Trial 2 [mM]		Trial 3 [mM]		Trial 4 [mM]	
		3-HPA	Acrolein	3-HPA	Acrolein	3-HPA	Acrolein	3-HPA	Acrolein
Crude reuterin, CR	Before washing	98.5	1.5	95.5	1.9	93.9	1.7	87.1	1.9
	After washing	97.2	1.4	94.8	1.7	92.9	1.0	86.5	1.8
	Loss	1.3	0.1	0.7	0.2	1.0	0.7	0.6	0.1
Enhanced reuterin, ER	Before washing	92.5	12.1	86.7	21.9	85.7	20.9	75.8	7.2
	After washing	90.7	11.5	85.2	20.3	83.2	20.2	71.8	6.4
	Loss	1.8	0.6	1.5	1.6	2.5	0.7	4.0	0.8

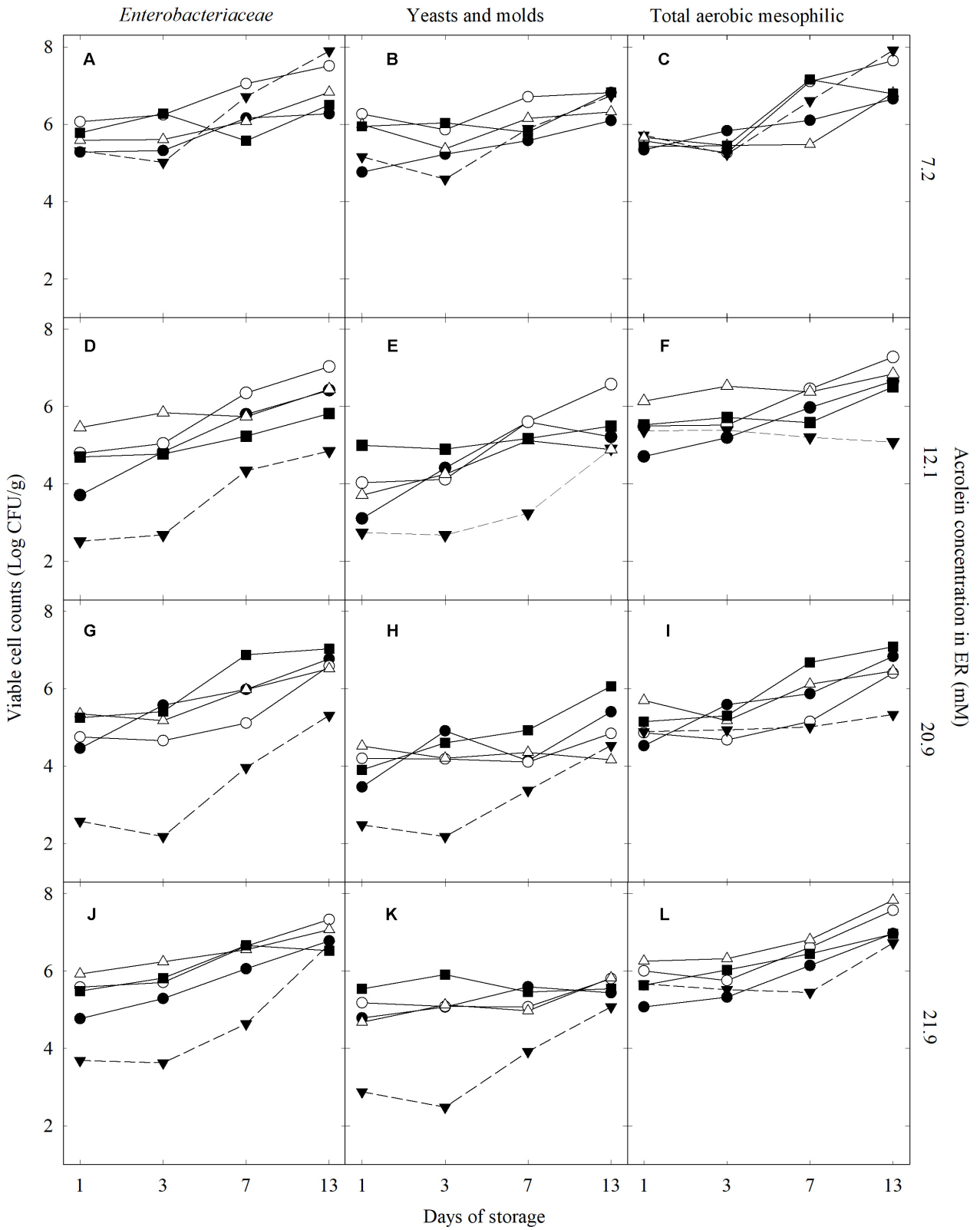

FIGURE 2 | *Enterobacteriaceae*, yeasts and molds, and total aerobic mesophilic viable cell counts in lettuce washed with different treatments and stored for 13 days at 4°C under protective atmosphere: crude (-○-) and enhanced (-▼-) reuterin, NaClO (-●-), water (-■-), and unwashed (-△-). Data was grouped based on the acrolein concentration in the ER washing solution: 7.2 mM (**A–C**; trial 4), 12.1 mM (**D–F;** trial 1), 20.9 mM (**G–I;** trial 3), and 21.9 mM (**J–L;** trial 2).

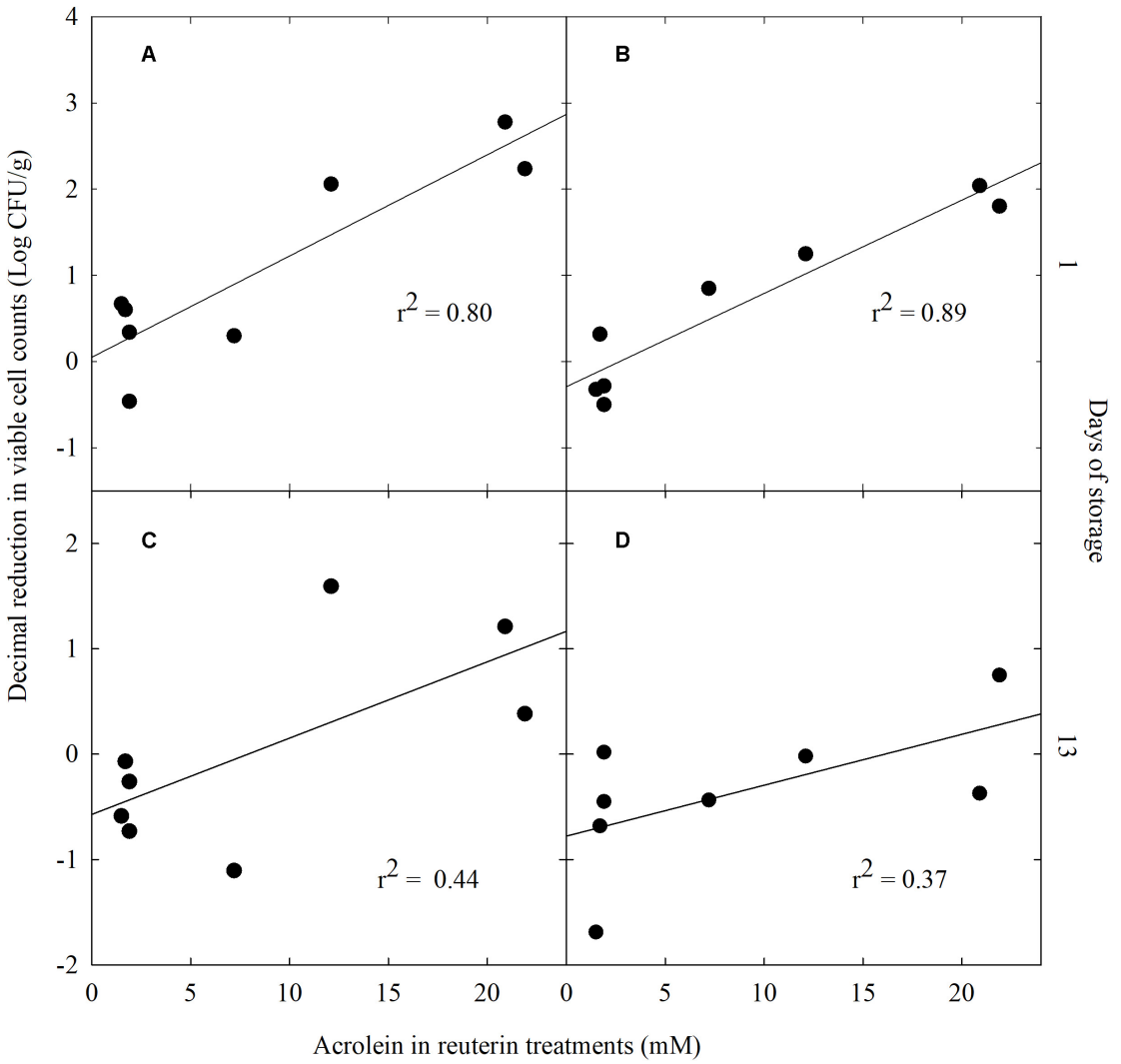

FIGURE 3 | Linear regression analysis of decimal reduction of *Enterobacteriaceae* **(A,C)** and yeasts and molds **(B,D)** viable cell counts versus acrolein concentration in crude reuterin (CR) and enhanced reuterin (ER) treatments after washing and storage for day 1 **(A,B)** and day 13 **(C,D)**.

above were similar to unwashed lettuce (5.3 ± 0.9 log CFU/g). For NaClO, TW, and CR treatments, growth of yeasts and molds were already noticeable at day 3, and at day 13, the counts of yeasts and molds on the stored lettuce were 5.5 ± 0.4, 5.8 ± 0.3, and 6.0 ± 0.4 log CFU/g (n = 4), respectively.

The total aerobic mesophilic bacteria counts were reduced between 0.3 and 1.4 log CFU/g on day 1 after NaClO washing compare to NW lettuce (5.9 ± 0.3 log CFU/g, n = 4). TW, CR, and ER treatments resulted in a limited reduction of total aerobic mesophilic bacteria, less than 1 log after 1 day of storage compare to NW. At day 7, washing with ER containing 12.1 mM or more acrolein still reduced the growth total aerobic mesophilic bacteria between 1.1 and 1.4 log CFU/g compared to unwashed lettuce. This effect was maintained until the end of storage in trail 2 (12.1 mM) and 3 (20.9 mM) (**Figures 2F,I**). The counts of total aerobic mesophilic bacteria of TW, CR, and NaClO were similar to unwashed lettuce at the end of the 13 day storage (**Figures 2F,I,L**), except in trial 4 (**Figure 2C**).

Relationship Between Acrolein Concentration and Antimicrobial Effect

A regression analysis was applied to test the dose dependent effect of acrolein on the decimal reduction of *Enterobacteriaceae* and yeasts and molds which were most affected compare to NW lettuce. A significant linear dependency was calculated between the decimal reduction of *Enterobacteriaceae* (r^2 = 0.80, $P < 0.05$) and of yeasts and molds (r^2 = 0.89; $P < 0.05$) after 1 day and acrolein concentration of the washing treatment (**Figures 3A,B**, respectively). On day 13 of storage there was no significant correlation between acrolein concentration and decimal reduction of *Enterobacteriaceae* (r^2 = 0.40, $P > 0.05$) and of yeasts and molds (r^2 = 0.37; $P > 0.05$) (**Figures 3C,D**, respectively).

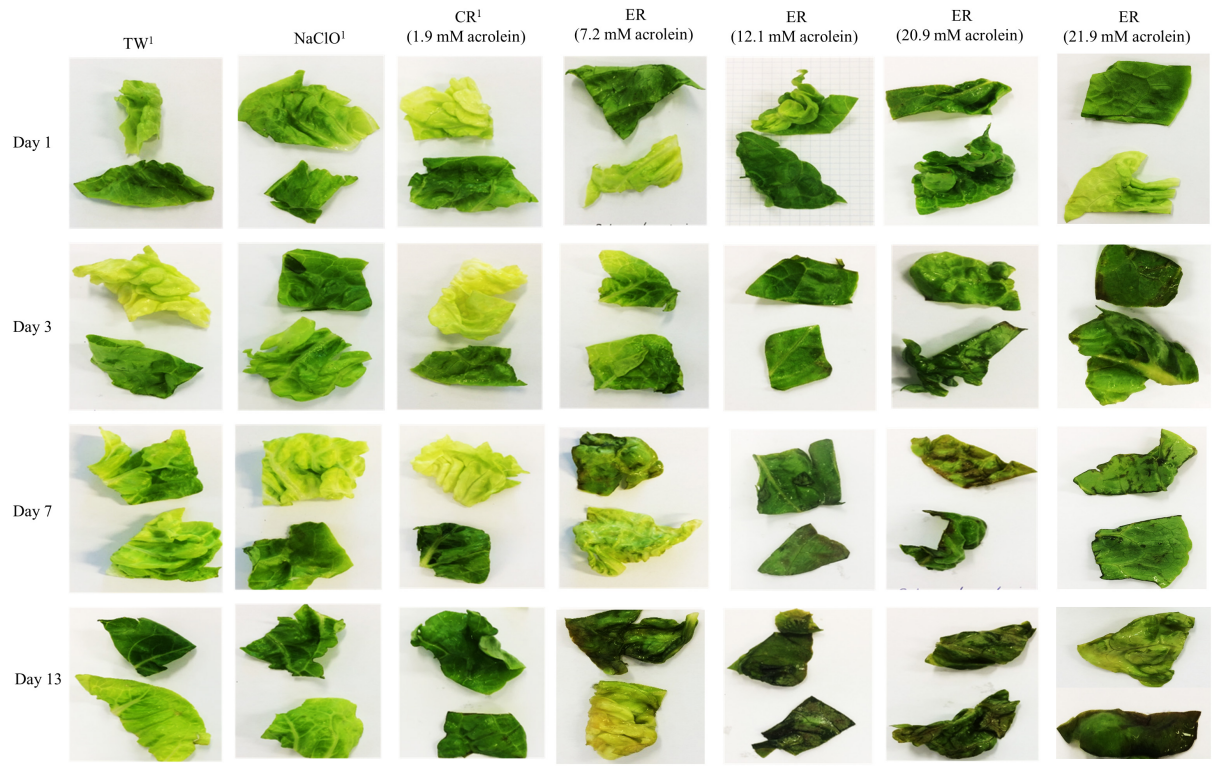

FIGURE 4 | Visual evaluation of lettuce after washing with different treatments and during 13 days of storage under modified atmosphere. Washed with water (TW), NaClO, ER, and CR. [1]Images obtained from trial 2. Selected images of damaged leaves illustrating spoilage of different treatments. The image may not be representative of the entire lettuce.

Visual Appearance of Washed Lettuce During Storage

Differences in the visual appearance of lettuce washed with ER solutions were observed starting on day 3 for lettuce washed with ER containing 20.9 and 21.9 mM acrolein. At 7-days storage at 4°C, marked discoloration were observed for lettuce washed with reuterin treatment containing 7.2, 12.1, 20.9, and 21.9 mM acrolein. The effect was slightly more pronounced with increasing acrolein concentration (**Figure 4**). Lettuce washed with NaClO, TW, and CR showed similar visual quality in all four trials, and hence, we present images from trial 2 to represent all four trials.

DISCUSSION

Acrolein Is the Main Antimicrobial Compound in Reuterin Washed Lettuce

The mechanistic basis of reuterin's antimicrobial activity has been proposed to be an imbalance in cellular redox status resulting from reactions of 3-HPA with free thiol groups, causing the depletion of glutathione and modification of proteins, including functional enzymes within the cells (Schaefer et al., 2010; Vollenweider et al., 2010). We recently showed that acrolein is the compound of the reuterin system responsible for antimicrobial activity on indicator strains in simple buffered media (Engels et al., 2016). Using lettuce as a model vegetable, we confirmed the activity of acrolein as the main antimicrobial of the reuterin system in a complex food matrix, with a dose-dependent activity on *Enterobacteriaceae* and on yeasts and molds.

The antimicrobial potential of CR as a food biopreservative was previously investigated against different food-borne pathogens like *S. aureus, L. monocytogenes*, *E. coli* O157:H7, or *S. enteritidis* in different food matrices, including cold-smoked salmon (Montiel et al., 2014, 2016), milk and dairy products (Arqués et al., 2004, 2008a,b). However, no significant antimicrobial effect was observed when CR was applied at concentrations ranging from 2 to 8 AU/g or mL in food stored at refrigeration temperatures (4–10°C). Interestingly, bactericidal activity of reuterin (8 AU/ml) against *L. monocytogenes* and *S. aureus* in milk was reported after 24 h of incubation at 37°C (Arqués et al., 2004). In line with the observation that acrolein is the main antimicrobial component of the reuterin system (Engels et al., 2016), it may be speculated that incubation of milk at 37°C promoted the continuous formation of acrolein from 3-HPA leading to the bactericidal activity of reuterin against *L. monocytogenes* and *S. aureus*. In our study, a simple heat treatment of CR at 50°C for 16 h was used to cause a 4–12 folds increase in acrolein concentration in enhanced reuterin preparation.

Most investigations carried out with reuterin in food systems did not provide information on purity of reuterin, and the

exact concentrations of 3-HPA and acrolein were not disclosed. Instead reuterin activity was determined using arbitrary activity tests which does not allow comparing results between different studies. Therefore the exact composition of reuterin, and more specifically the concentration of acrolein in the conditions of the test should be established for rigorous testing of reuterin as a disinfectant or preservative in food.

Acrolein Inhibits Enterobacteriaceae, Yeasts and Molds in Washed Lettuce

The sanitation of vegetable produce is intended to reduce the natural microbial load to increase shelf life and ensure safety of raw consumed vegetables. The effect of decontamination solutions used in MP vegetable produce on the natural lettuce microbiota (total aerobic mesophilic count) was usually reported smaller than that obtained with artificially inoculated bacteria (Oliveira et al., 2015). Our results showed that the population of initial total aerobic mesophilic bacteria was less affected by all the treatments applied than *Enterobacteriaceae*, and yeasts and molds. This may be partly due to biofilm formation by native lettuce microbiota, which may result in a protective effect from the action of antimicrobial compounds (Hunter et al., 2010; Vorholt, 2012). Pathogenic bacteria from the *Enterobacteriaceae* family such as *Salmonella, Shigella*, and *E. coli* O157:H7 mostly from facal origin have been involved in outbreaks of foodborne diseases associated with the consumption of fresh-cut produce (Ramos et al., 2013). Therefore, the counts of *Enterobacteriaceae* are of special importance to assess the efficacy of a microbial-reducing process in respect of food safety. The application of enhanced reuterin washing with acrolein concentration of 12.1 mM and higher on fresh-cut lettuce resulted in a strong reduction of *Enterobacteriaceae* for at least 7 days compared to unwashed lettuce and all the other tested conditions, including CR, ER with 7.2 mM acrolein, TW and NaClO. Our results are in agreement with previous finding suggesting that Gram-negative bacteria are more sensitive to reuterin than Gram-positive bacteria (Chung et al., 1989; El-Ziney et al., 1999; Arqués et al., 2004; Stevens et al., 2010).

The role of yeasts and molds in fresh-cut vegetables spoilage and mycotoxin production was previously reported by Tournas (2005). In our study, between 1.3 and 2.0 log unit reductions in the count of yeasts and molds were observed after 1 day storage of lettuce washed with ER treatments containing between 12.1 and 21.9 mM acrolein. The MIC of reuterin on several yeasts and filamentous fungi were previously reported between 0.15 and 3 mM (Chung et al., 1989; Stevens et al., 2010). Sanitizing agents of fresh-cut produce may be considerably more effective against yeasts and molds than against aerobic mesophilic microbiota, because the later include several Gram-positive spore-forming bacteria of the *Bacillus* genus, known to be more resistant to chemical sanitizing agents (Brackett and Splittstoesser, 2001).

After an initial reduction after washing with ER, *Enterobacteriaceae*, and yeasts and molds counts increased steadily, eventually reaching equal levels of the other treatments and the unwashed produce at the end of the 13 day storage period. Several authors have demonstrated that the washing steps commonly used in MP produce were only bacteriostatic as microbial populations recover to prewashed numbers at the end of storage (Gómez-López et al., 2008; Tirpanalan et al., 2011). This also emphasizes that a decontamination process may not be aimed at increasing the shelf-life of the product but rather enhance its safety during the normal storage life.

Many studies have identified that chlorine is the most popular disinfection method for fresh produce (Shen et al., 2012; Van Haute et al., 2013). Sodium hypochlorite (NaClO) is the source of chlorine commonly used by the food industry for sanitizing both products and equipments (Gil et al., 2009). For efficacy and stability, the pH should be kept in the range of 6.5–7.5 (Suslow, 1997). Using 50–200 ppm of hypochloride and 1–2 min contact time at this pH results in a maximum of 1–2 log reduction of the initial total aerobic microbes in many commodities (Parish et al., 2003; Gonzalez et al., 2004; Allende et al., 2008). Pezzuto et al. (2016) recently report that NaClO (200 mg/L) was able to reduce *Salmonella* counts in leafy green vegetable by 2 log units, but only in the case of a high initial contamination (7 log CFU/g). In our study the NaClO washing solution at uncontrolled pH of approximately 9.0 led to a reduction between 0.9 and 1.8 log of *Enterobacteriaceae*, total aerobic mesophilic bacteria and yeasts and molds counts after washing, except for trial 4 which showed no effect of chlorine. The lack of efficiency could be associated with difference in microbiota composition of the lettuce used in trial 4, evident by high numbers of initial counts of yeasts and molds in trial 4, compare to other trials.

Since the ban of the use of chlorine for decontamination of fresh produce in some European countries, most fresh-cut producers currently use tap water for washing. Our results indicate that washing fresh-cut produce with tap water alone was not sufficient to significantly reduce the initial microbial load on the fresh-cut lettuce (0.3–0.6 log reduction). Similar results were obtained by Gonzalez et al. (2004) who found that washing shredded carrots with water reduced total aerobic microbial counts by only 0.3–0.4 log units, regardless of the water quality used. Additionally, Luo (2007) did not find differences in the aerobic mesophilic growth of Romaine lettuce after washing with potable tap water.

In our study, washing fresh-cut produce with reuterin treatments containing acrolein in the range between 12.1 and 21.9 mM resulted in a reduction of *Enterobacteriaceae*, yeasts and molds in the range from 1.3 to 2.8 log units, well above the effects observed with chlorine and tap water washing.

Reuterin Washing Causes No Detectable Residual Acrolein in the Produce but Lead to Discoloration of Fresh-Cut Lettuce During Storage

The fate of reuterin and its degradation product is of importance for predicting the safety of the produce after washing (Fernández-Cruz et al., 2016). The main antimicrobial component of

the reuterin system, acrolein, is highly volatile, colorless, and may be present in many food, sometimes at high levels of more than 4.0 ng/g such as in potato chips fried in corn oil (Watzek et al., 2012). Acrolein is considered a highly cytotoxic compound after a single exposure, hence a tolerable daily intake of 0.75 mg/kg body weight/day was suggested (Abraham et al., 2011; Fernández-Cruz et al., 2016; Zhang et al., 2018). In our study, between 0.6–1.6 mM and 0.1–0.7 mM of acrolein was lost during washing with ER and CR, respectively. We therefore tested acrolein in the rinsing water and in washed lettuce using the high sensitive IC-PAD method developed by Engels et al. (2016). We did not detect acrolein in the macerated lettuce and rinsing water after washing with ER solutions, with the detection limit of the method of 4.4 μM. Because acrolein is a volatile compound, we speculate that part of unrecovered acrolein in the wash solution may have been lost by volatization. It was previously reported that because of its volatility, acrolein was not observed after 1 day in lettuce grown on soils irrigated with MAGNACIDE@ H Herbicide which contains at least 92% acrolein (Nordone et al., 1997). Aside volatilization and reaction with microbes and plant components, reversible, first-order hydration of acrolein to 3-HPA which is enhanced at low temperatures may be a significant pathway for the elimination (Engels et al., 2016). However, little is known about reactions and degradation products of acrolein which is a very reactive component (Engels et al., 2016; Zhang et al., 2017, 2018). Taken together, the absence of detectable acrolein in the treated lettuce suggest safety of reuterin washing at effective acrolein concentrations, but additional testing may be needed to identify potential by-products of acrolein.

Quality preservation is, after safety, the most important attribute of minimally processed vegetables, since purchasing decisions often depends on consumers satisfaction in terms of visual, textural and flavor quality of the product (Allende et al., 2008; Barrett et al., 2010). In this study, we observed increased brown discoloration of the fresh-cut lettuce after washing with ER containing acrolein concentrations in the range from 7.2 to 21.9 mM and during subsequent storage. The dark green color of the cut-lettuce started to decrease from day 3 and continued to decline throughout the remaining storage period. Interestingly, the point of discoloration of lettuce after washing with ER corresponds to regrowth of *Enterobacteriaceae* and yeast and molds. We speculate that these microbes were able to multiply faster once the tissue has been damage. Unwashed and NaClO washed samples maintained fresh appearance during 13 day storage. This is in agreement with previous studies where washing fresh-cut lettuce with chlorinated water reduced browning (Baur et al., 2004). The major enzyme controlling oxidative discolouration of cut lettuce has been reported to be polyphenol oxidase (PPO) (Hunter et al., 2017). Acrolein induces oxidative stress in cells, due to the oxidation of phenolic compounds (Schaefer et al., 2010). We speculate that this deterioration of produce washed with enhanced reuterin is due to acrolein oxidation of the phenolic compounds in the cut lettuce. Further studies are required to understand the phenomenon for the increase browning on lettuce washed with enhance reuterin on a molecular level.

CONCLUSION

Our study shows that in food system as for laboratory test conditions, acrolein is the main antimicrobial component of the reuterin system because only the application of ER containing between 12.1 and 21.9 mM acrolein reduced initial counts of *Enterobacteriaceae,* yeasts and molds on fresh-cut lettuce. From the microbiological point of view, the use of reuterin with enhanced acrolein ensured the acceptability of modified atmosphere packaged lettuce for up to 7 days of storage at 4°C and reduced the risks associated with putative pathogenic genera' of the *Enterobacteriaceae* family. However, at effective acrolein concentration, reuterin may not be used to extend the shelf-life of fresh-cut lettuce, due to chemical reactions changing the visual appearance. The application of reuterin for the decontamination and biopreservation of other vegetable less sensitive to oxidative stresses than lettuce, such as carrot, apples, beets, and radish, should be tested. Furthermore the reuse and recycling ER washing solutions containing high levels of acrolein should be investigated to limit environmental impact.

AUTHOR CONTRIBUTIONS

PA, AG, CS, MJS, and CL designed the study. PA, AG, CS, and MS conducted the experiments and analyzed the data. PA, AG, and CL drafted the manuscript. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01421/full#supplementary-material

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Differential Proteomic Analysis of Lactic Acid Bacteria—*Escherichia coli* O157:H7 Interaction and Its Contribution to Bioprotection Strategies in Meat

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Human infection by Enterohemorrhagic *Escherichia (E.) coli* (EHEC) occurs through the ingestion of contaminated foods such as milk, vegetable products, water-based drinks, and particularly minced meats. Indeed EHEC is a pathogen that threatens public health and meat industry. The potential of different Lactic Acid Bacteria (LAB) strains to control EHEC in a meat-based medium was evaluated by using a simple and rapid method and by analyzing the growth kinetics of co-cultures (LAB-EHEC) in a meat-based medium. The activity of LAB toward EHEC in co-cultures showed variable inhibitory effect. Although, LAB were able to control EHEC, neither the produced acid nor bacteriocins were responsible of the inhibition. The bacteriocinogenic *Enteroccus (Ent.) mundtii* CRL35 presented one of the highest inhibition activities. A proteomic approach was used to evaluate bacterial interaction and antagonistic mechanisms between *Ent. mundtii* and EHEC. Physiological observations, such as growth kinetics, acidification ability and EHEC inhibitory potential were supported by the proteomic results, demonstrating significant differences in protein expression in LAB: (i) due to the presence of the pathogen and (ii) according to the growth phase analyzed. Most of the identified proteins belonged to carbohydrate/amino acid metabolism, energy production, transcription/translation, and cell division. These results contribute to the knowledge of competition strategies used by *Ent. mundtii* during its co-culture with EHEC setting new perspectives for the use of LAB to control this pathogen in meat.

Keywords: Lactic acid bacteria (LAB), bioprotective cultures, enterohemorrhagic *Escherichia coli* (EHEC), meat safety, bacterial interaction, proteomics

INTRODUCTION

Contamination with Shiga toxin-producing *Escherichia (E.) coli* (STEC) and related enteric pathogens is among the main causes of concern and fresh meat product recalls. In the European Union STEC prevalence on hides is estimated at 44%, before falling to 0.4% on carcasses, and 1.2% in raw beef meat. In addition, in the United States, the Centers for Disease Control and Prevention (CDC) have estimated that STEC infections cause 73,000 illnesses, 2,200 hospitalizations, and 60 deaths yearly. The annual cost of illness due to STEC was 405 million dollars, including lost productivity, medical care, and premature deaths (Lim et al., 2010). High economic losses in meat industry and the high cost of the illness evidence the necessity of additional efforts to control this pathogen. Within the STEC pathotype, the *E. coli* enterohemorrhagic (EHEC) subgroup is important because of its impact on Public Health. Human infection by EHEC occurs through the ingestion of contaminated foods such as milk, vegetable products, water-based drinks, and particularly, minced meats (Colello et al., 2016). Moreover, 5–10% of the patients infected with EHEC develop the more severe hemolytic-uremic syndrome (HUS). HUS is the most common cause of acute renal failure and the second cause of chronic renal failure and renal transplantation in children. Therefore, STEC/EHEC constitutes a serious threat to public health and a major concern for the sustainability of the meat industry as well as for its entire production chain. Presently, consumers assumed a crucial role requiring safer and healthier foods. This context highlights the need to provide the meat industry with sustainable and eco-friendly solutions to limit and prevent future risks surrounding this problematic.

Lactic acid bacteria (LAB), naturally present in meat, are of technological interest due to their inhibitory potential on spoilage, toxin production or food poisoning microorganisms in foodstuffs (Vignolo et al., 2015). Their antagonism toward spoilage bacteria is due to the direct competition for nutrients and/or production of different antimicrobial metabolites, such as organic acids, hydrogen peroxide, and bacteriocins (Woraprayote et al., 2016). In particular, by producing lactic acid and thus lowering the pH, LAB inhibit the growth of bacterial pathogens and even kill them (Atassi and Servin, 2010). Moreover, some of them produce bacteriocins, ribosomally synthesized peptides with antibacterial activity toward closely related strains, playing an important role in food preservation. Some type of LAB bacteriocins are specifically active toward Gram positive spoilage and pathogenic microorganisms such as *Listeria monocytogenes* and *Brochothrix termosphacta* (Woraprayote et al., 2016). Due to these properties, the use of LAB is an interesting substitute for chemical and/or physical preservatives. Moreover, LAB are generally regarded as safe (GRAS) and usually fit all recommendations for food usage (Wessels et al., 2004). These characteristics make LAB ideal candidates for the development of bioprotective agents, providing a good antagonistic activity toward target organisms (Chikindas et al., 2017). It is known that most of LAB bacteriocins are not effective against Gram negative microorganisms such as *E. coli*, although they can became active in association with agents such as EDTA or organic acids, affecting membrane integrity of the target organisms (Belfiore et al., 2007). Even though, no bioprotective LAB culture capable of inhibiting EHEC in meat is available on the market so far (Varsha and Nampoothiri, 2016).

On this basis, it is proposed that certain LAB could control and/or inhibit the growth of EHEC in meat through direct or indirect interaction with the pathogen. In order to proceed toward an efficient bioprotective culture as strategy of EHEC control for meat preservation, it is necessary to have a highly competitive strain to fight the pathogen. The knowledge of the mechanisms by which both microorganisms interact is therefore of paramount importance. On this context, the objective of this work was to evaluate the potential of LAB for inhibiting EHEC. The assayed LAB strains were examined for antagonistic activity toward EHEC by using a simple and rapid method and by analyzing the growth kinetics of co-cultures (LAB-EHEC) in a meat-based medium. A comparative proteomic approach was used to identify the underlying mechanisms involved in the antagonistic action carried out by the selected LAB strain.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Lactobacillus curvatus CRL705, *Lactobacillus plantarum* CRL681 isolated from artisanal fermented sausages and *Enterococcus mundtii* CRL35 of cheese origin, belonging to CERELA culture collection were used. They were selected for this study, due to their well-studied biochemical, bioprotective activity toward *Listeria monocytogenes* and/or their technological features (Fadda et al., 1998, 1999, 2010; Saavedra et al., 2004; Salvucci et al., 2007).

Fresh cultures were obtained from freeze-dried stocks and transferred twice in MRS (Merck, Buenos Aires, Argentina) (De Man et al., 1960) incubated at 30°C for 24 h and used for further inoculation. The stock culture was stored at −80°C in milk yeast extract medium (10% w/v skim milk, 0.5% w/v yeast extract) containing 10% (v/v) glycerol as cryo-protectant.

The atoxigenic *Escherichia coli* O157:H7 NCTC12900 (National Type Culture Collection, Colindale, London) was selected as the pathogen model to evaluate LAB-EHEC interaction. *E. coli* NCTC12900 was isolated in Austria in 1992 and does not produce enterotoxins Stx1 nor Stx2 (Best et al., 2003). This strain was kept at −80°C in LB (Luria Bertani) medium in the presence of 20% (v/v) glycerol as cryo-protectant. To obtain fresh cultures, the strain was transferred twice in LB broth and incubated at 37°C for 8 h, in the first transfer, and for 16 h in the second transfer.

E. coli Growth Inhibition Assay

The inhibitory capacity of the strains was evaluated by the well-diffusion assay according to Salvucci et al. (2007) with some modifications. Briefly, 5 µl of each treatment of LAB culture were spotted onto a plate containing MRS agar. The indicator lawn was prepared by adding 100 µl of an overnight culture of EHEC to 10 ml of LB soft agar (0.7%); poured on top of MRS agar inoculated with each strain. The plates were incubated at 30°C for 24 h. In order to evaluate, the mechanisms of inhibition

toward EHEC, different conditions were assayed for each LAB strain: (1) intact/viable cells, removing the effects of soluble factors from the supernatant. Cell suspensions were washed with physiological solution and spotted onto MRS agar; (2) non-viable cells, cell suspensions from the overnight culture washed with distilled water and heated 15 min at 95°C; (3) cells collected, washed with physiological solution and resuspended in 1 mg/ml lysozyme solution incubated 2 h and spotted onto MRS agar, to evaluate if cell wall is involved in *E. coli* inhibition; (4) overnight LAB culture in MRS was directly spotted onto MRS agar, to evaluate all components (viable cells plus all metabolic products) present in the medium; (5) cell–free supernatant of the overnight culture heated (5 min, 95°C) and spotted onto the MRS agar, to evaluate bacterial inhibition due to acid, bacteriocins, and other heat stable compounds; (6) cell-free supernatant of the overnight culture heated (5 min, 95°C), and neutralized to pH 7 with 1N NaOH and spotted onto MRS agar, to neutralize the acids produced; (7) untreated cell-free supernatant was spotted onto the MRS agar, to evaluate additional soluble factors that could inhibit the pathogen; (8) 4% lactic acid solution was spotted as a control of the acid effect.

Sarcoplasmic Model System

The sarcoplasmic model system was used as culture medium and prepared according to Fadda et al. (1998) with some modifications. Briefly, 10 g of bovine *semimembranosus* muscle were homogenized with 100 ml of deionized water for 8 min in a Stomacher 400 blender (Stomacher, London, UK). The homogenate was centrifuged (14,000 g, 20 min at 4°C). The supernatant containing sarcoplasmic proteins was filtered through Whatman paper, filter-sterilized through a 0.22 μm-pore-size filter (Steritop GP, Biopore, Buenos Aires, Argentina) and supplemented with 0.5% (w/v) glucose and 0.01% (v/v) Tween 80. The sterility of the system was confirmed by plating in Plate Count Agar (PCA).

LAB—EHEC Co-cultures in Sarcoplasmic Model System. Focus on LAB Inhibitory Potential

Co-cultures of each LAB strain with *E. coli* NTCC12900 were carried out in the sarcoplasmic model to evaluate the performance of both microorganisms in co- and individual culture. Fifty ml of the sarcoplasmic model was inoculated with 10^6 CFU/ml of LAB and 10^4 CFU/ml of *E. coli* and incubated under gentle stirring at 30°C for 96 h. In addition, each microorganism was grown individually under the same conditions (30°C, 96 h; same inoculum) to evaluate the behavior of each strain without competition.

Samples were taken at 0, 3, 6, 8, 24, 48, 72, and 96 h to analyze pH and viability of both microbial groups using selective agar media. For bacterial enumeration, decimal dilutions were prepared and plated on the corresponding medium, MRS agar for LAB and Mac Conkey agar (Britania, Buenos Aires, Argentina) for *E. coli*, and incubated at 30°C for 48 and 24 h, respectively. Measurements of pH were determined by using pHmeter Altronix TPX I (New York, USA).

Three independent cultures were carried out for each mixed and independent cultures.

Proteomic Study

For differential proteomic analysis, one strain, *Ent. mundtii* CRL35, was selected to evaluate LAB – *E. coli* interaction in the sarcoplasmic model system by means of two dimensional electrophoresis (2DE). Two different time periods during the growth were evaluated: T6, corresponding to 6 h of growth when both microorganisms (LAB and *E. coli* NTCC 12900) were in the exponential phase of growth, both in single or co-cultures, and T30 corresponding to 30 h when the stationary phase was achieved by LAB (in both single and co-culture), while *E. coli* in co-culture was already in the death phase. However, when *E. coli* grew alone 96 h was taken as the second sampling time, this corresponding to the death phase for the pure culture of *E. coli*.

Cells Recovery for Proteomic Analyses

Ent. mundtii CRL35 was incubated as individual and co-culture with *E. coli* O157:H7 NTCC12900, as described before, in 100 ml of the sarcoplasmic model system to achieve sufficient amount of cells for proteomic analysis. Cells from co-cultures were harvested at 6 h (T6) and 30 h (T30). Cells from single cultures of *Ent. mundtii* were also harvested at 6 h (T6) and 30 h (T30). As mentioned before, cells from *E. coli* growing alone were collected at 6 h (T6) and 96 h (T96). Cells from different cultures were harvested by centrifugation at 8,000 × g for 10 min at 20°C and twice washed with 40 ml 0.1M Tris-HCl buffer, pH 7.5 and centrifuged at 8,000 × g for 10 min at 20°C. The resulting pellets were stored at −20°C until lysis for protein extraction. Three independent biological replicates were performed for each condition.

Preparation of Cell Free Protein Extracts for Proteomic Analyses

The cell pellets from the co-cultures were mixed with glass beads (150 ± 212 μm diameter, Sigma-Aldrich Co., St. Louis, MO, USA) and further re-suspended in 0.1 M Tris-HCl buffer, pH 7.5 in a 1:2:1 (cell:buffer:bead) ratio. Then, cells were disrupted using a Mini-BeadBeater-8 cell disrupter (Biospec Products Inc., Bartlesville, OK, USA) at maximum speed for 10 min (10 cycles of 1 min each, with 1-min intervals on ice among cycles). To remove cell debris, unbroken cells and glass beads, samples were centrifuged (14,500 × g, 5 min, 15°C). The supernatant constituted the cell free extract. The protein concentration of this extract was estimated according to Bradford assay using bovine serum albumin as a standard. The whole process is described in **Figure 1**. Aliquots of 600 μg of protein were finally stored at −80°C, until further analysis.

Ent. mundtii and *E. coli* cell pellets from individual cultures at T6 were mixed before lysis and the same was performed for T30/T96 (see schematics in **Figure 1**). This procedure was carried out to standardize and avoid differences in cell lysis efficiency between mono and co-cultures, as well as problems with differences in protein enrichment of each microorganism in 2DE gels, that could affect proteome comparisons. The cell proportions of each microorganism used for T6 and T30

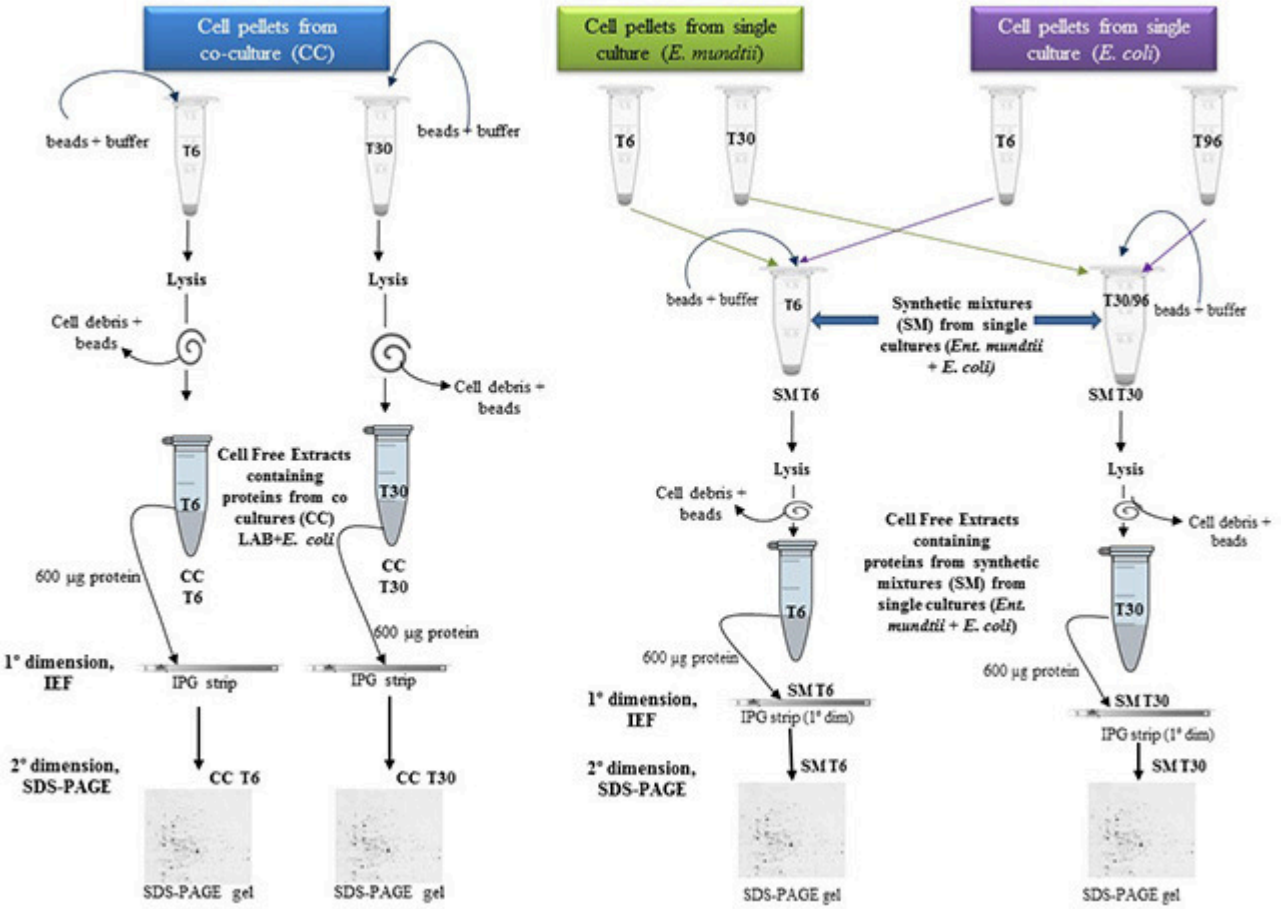


FIGURE 1 | Schematic description of cell free extracts preparation for proteomic analysis.

mixtures coming from single cultures were established according to the cell counts obtained in the respective co-culture in order to have in the mix a similar ratio between the two microorganisms. This way, assuring to have 600 µg of proteins placed in the IPG strip, with the same protein proportion of each microorganism than in IPG strips with samples from the co-culture. Each of these synthetic mixtures (SM) will constitute the respective controls (single culture) at T6 and T30 respectively. The SM were mixed with glass beads and subjected to lysis as previously described for cell pellets originating from co-cultures (see **Figure 1** for details).

Two-Dimensional Gel Electrophoresis

Sample preparation and 2DE gels were carried out according to Bustos et al. (2015). Isoelectrofocusing (IEF) was performed in IPGphor (GE Healthcare, Uppsala, Sweden) at 53,500 Vh, using the immobilized pH gradient (IPG) strips (Immobiline DryStrip Gels, linear pH 4–7, 18 cm, GE Healthcare; Uppsala, Sweden). For the second dimension, IEF strips were equilibrated at room temperature in 6 M urea, 2% (w/v) SDS, 30% (w/v) glycerol, 50 mMTris-HCl, pH 8.0, containing alternatively 50 mM DTT (15 min) and then 400 mM iodoacetamide (15 min in the dark). Second dimension was performed on homogeneous 12.5% (w/v) polyacrylamide gels at the constant current of 15 mA/gel at 15°C (∼16 h) using an Ettan DALTsix Large Vertical System (GE Healthcare, Uppsala, Sweden). Gels were stained with colloidal Coomassie blue Stain according to Candiano et al. (2004), distained with distilled water. The 2DE maps were digitalized using Image Scanner III LabScan 6.0 (GE Healthcare, Uppsala, Sweden).

Image Acquisition and Data Analysis

Volume spot quantization and normalization were performed on digitalized gel images (600 dpi) using the software Prodigy SameSpots version 1.0.3400.25570 (Totallab, Newcastle, UK). The volume of each spot was calculated and normalized by referring the values to the sum of total spot volumes within each gel. Student test for unpaired samples was applied. A protein was considered differentially abundant if the mean normalized spot volume varied at least 1.5-fold between compared spots. The effect was confirmed by analysis of variance at a significance level of $p < 0.05$. Protein spots showing significant variation between studied conditions were manually excised from the gels using a scalpel blade and identified using Mass Spectrometry.

Mass Spectrometry Protein Identification

Selected spots were excised from the corresponding gel, digested with trypsin and, submitted to tryptic digestion and then to mass spectrometry analyses as previously described (Nally et al., 2017). Tryptic peptides were subsequently ionized using α-cyano-4-hydroxycinnamic acid as matrix. Mass spectrometric analysis of the peptide solutions was carried out on a MALDI-TOF/TOF tandem mass spectrometer ABI 4700 proteomics analyzer (Applied Biosystems, Foster City, USA) according to Grosu-Tudor et al. (2016) or on a MALDI 5800 (Sciex, Foster City, USA) and performed at CEQUIBIEM (Facultad de Ciencias Exactas y Naturales, UBA, Buenos Aires, Argentina) and at LIST - Luxembourg Institute of Science and Technology "Environmental Research and Innovation" (ERIN), respectively. MASCOT search engine (Matrix Science Inc., Boston, MA; http://www.matrixscience.com/search_form_select.html) was used to identify proteins from peptide mass fingerprint data based on the annotated genome of *Ent. mundtii* CRL35 (https://www.ncbi.nlm.nih.gov/nuccore/JDFT00000000). All proteins were identified using BLASTp in NCBI database (Altschul et al., 1990).

Functional Analysis and Interaction of Proteins

The functional study of identified proteins and their classification into functional categories were performed using the databases Universal Protein Resource (UniProt) (UniProt Consortium, 2015) and COGNITOR to identify the Clusters of Orthologous Groups of proteins (COGs) (Galperin et al., 2015).

To explore the interactions between the proteins that have shown differential expression, we conducted an *in-silico* analysis using the publicly available STRING version 10.05 (database Search Tool for the Retrieval of Interacting Genes/Proteins) (Szklarczyk et al., 2015). For the differentially over expressed proteins, the number of protein-protein interactions documented in the database were determined. For visualization purposes, a graph was constructed linking proteins represented by nodes with known interactions with the identified proteins. All available prediction methods on STRING were used and 0.4 was select as confidence level (Szklarczyk et al., 2015).

Statistical Analyses

All experiments (growth inhibition, growth kinetics and differential protein expression assays) were done three times, and the values and the standard error were calculated from the data with three repetitions. One-way analysis of variance with *t*-test was conducted, and a *p* value of less than 0.05 was considered to indicate a statistically significant difference. The hypergeometric distribution was assayed to evaluate the enrichment of COG categories of the proteins encoded by *Ent. mundtii* CRL35 related to the ones differentially expressed by it in co-culture or alone (CC T30 - SM T30) and in co-culture during the time (CC T6 – CC T30).

RESULTS

E. coli Growth Inhibition Assay

The *E.coli* growth inhibition assay was performed as a first approach to determine the antagonistic potential of LAB strains toward the pathogen (**Figures 2A–C**). By means of this fast and simple method the inhibitory capacity of each LAB strain on *E. coli* NTCC12900, as well as the nature of the inhibition effect were evaluated. The three assayed LAB showed a similar inhibitory pattern, although they presented low variability in their inhibition halos. In general, halos of inhibition were registered in those conditions where LAB cells remained viable and not seriously damaged; regardless of the presence of the culture supernatant (spot #1 and 4). When cells were treated with lysozyme, minor inhibition halos were present (spot #3). In contrast, no inhibition were observed when lysed cells or cell free supernatant (untreated, heated or heated and neutralized) containing metabolites such as organic acids, heat stable bacteriocins and different soluble factors were spotted (spot #5, 6 and 7). The absence of inhibitory halos when 4% lactic acid solution was spotted (spot #8) confirmed the known acid resistance of *E. coli* (**Figure 2**).

Performance of LAB and *E. coli* NCTC 12900 When Grown Individually Or in Co-culture in Sarcoplasmic Model System

When grown individually, LAB presented an adequate growth in the sarcoplasmic model system, achieving a maximal growth approximately at 24–48 h depending on the strain (2.0×10^8 – 9.8×10^8 CFU/ml) (**Figures 3A–C**). They reached the exponential growth between 3 and 8 h and the stationary growth phase around 24 h. Afterwards, LAB strains maintained approximately initial bacterial counts until 96 h of incubation (1×10^6 CFU/ml). On the other hand, a significant pH drop was observed in all LAB strains growing alone. When *E. coli* was inoculated alone, it also achieved an optimal growth presenting a traditional sigmoid kinetic curve, the exponential growth was attained between 4 and 6 h with the maximal cell viability at 24 h during the stationary phase (1.8×10^8 CFU/ml) (**Figures 3A–C**). When LAB-*E. coli* co-cultures were analyzed, a different growth kinetic was achieved by both type of microorganisms compared to its individual growth. A decreased growth rate of LAB in the presence of *E. coli* was observed, achieving 1–2 logarithmic units less of cell viability than in the single culture condition, depending on the strain. However during co-culturing, all LAB were able to keep the steady state until the end, with a population almost similar to the beginning. It is worth noting that the acidifying potential of LAB was not affected by the presence of the pathogen, reaching similar values than those observed in pure cultures (final pH between 4.5 and 3.7) (**Figures 3A–C**). On the other hand, the growth of *E. coli* in co-culture was affected considerably by LAB, specifically after the first 8 h. For instance *L. curvatus* CRL705 (bacteriocin producer), showed a slight decrease of *E. coli* population, ∼0.6 log units (**Figure 3A**). Whereas the bacteriocinogenic strain *Ent. mundtii* CRL 35 (**Figure 3C**) and *L. plantarum* CRL 681 (non-bacteriocin

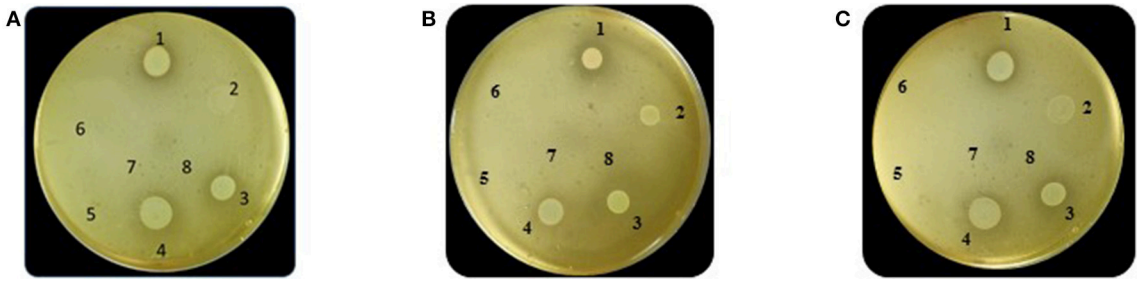


FIGURE 2 | Results of the Plate inhibition assay of LAB toward EHEC. Studied conditions of *Ent. mundtii* **(A)**, *L. plantarum* CRL 681 **(B)** and *L. curvatus* CRL 705 **(C)** toward *E. coli* NCTC12900: 1- LAB cell suspension in physiological solution, 2- LAB cell suspension in distilled water and heated 15 min at 95°C, 3- LAB cells in 1 mg/ml lysozyme solution, 4- direct overnight LAB culture in MRS, 5- heated supernatant (5 min, 95°C), 6- heated (5 min, 95°C), and neutralized supernatant, 7- intact supernatant, 8- 4% lactic acid.

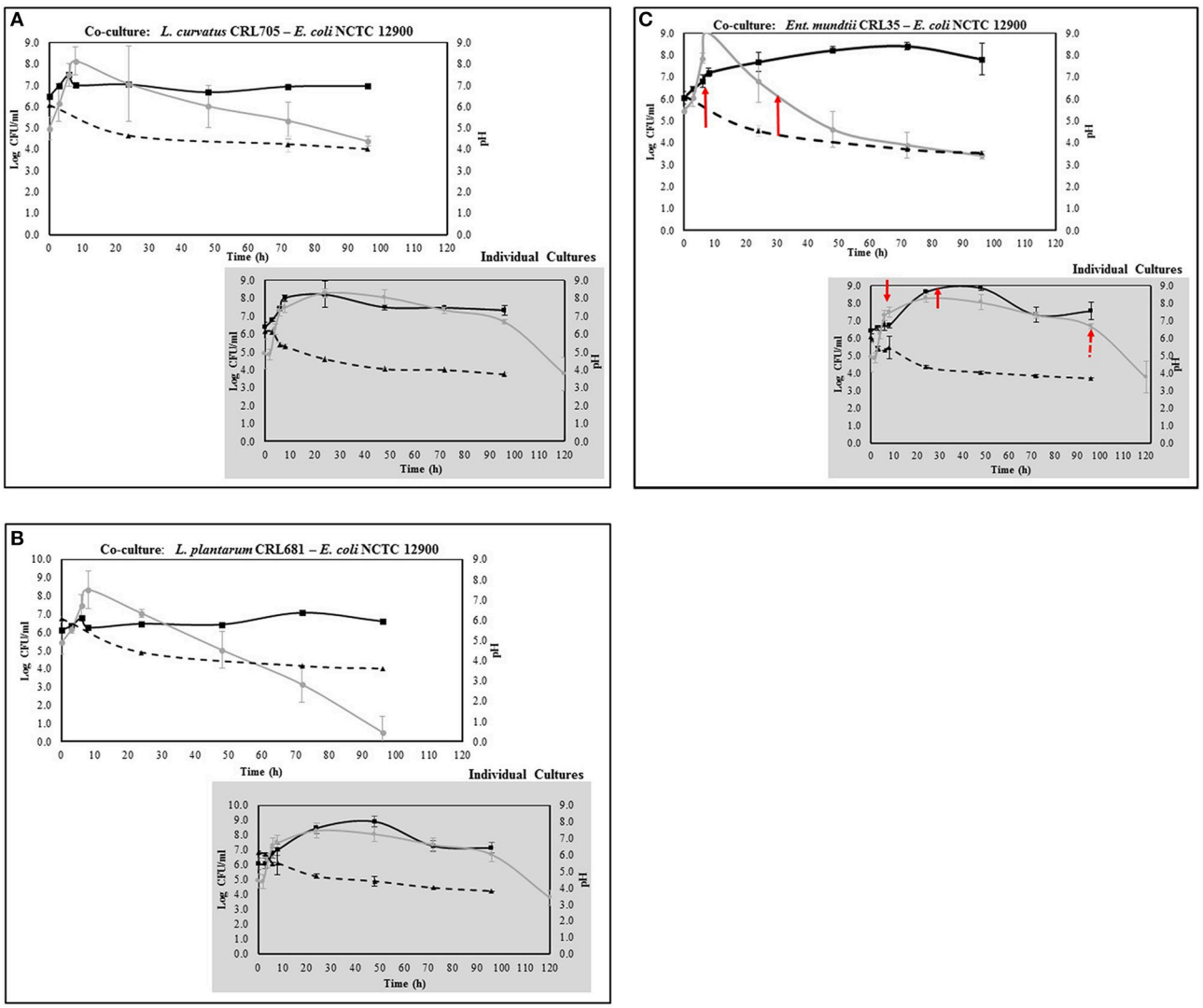


FIGURE 3 | Kinetics of LAB (black line) and *E. coli* (gray line) growth (Log CFU/ml) in co- and individual culture in the sarcoplasmic model system at 30°C. The pH is represented with dashed line. **(A)** Co-culture and individual culture of *L. curvatus* CRL705, **(B)** Co-culture and individual culture of *L. plantarum* CRL681, **(C)** Co-culture and individual culture of *Ent. mundtii* CRL35. The red arrows indicate the time of sampling for proteomic assays.

producer) (**Figure 3B**), showed higher inhibitory effect. In fact, their presence produced a significant reduction of *E. coli* viability after 8 h of growth, accelerating the entrance of *E. coli* into the death phase. At 96 h a significant decrease of *E. coli* counts was observed reaching more than 2 and 3.5 logarithmic units of decrease in the co-cultures containing *Ent. mundtii* CRL35 and *L. plantarum* CRL681, respectively (**Figures 3B,C**).

Differential Protein Expression of *Ent. mundtii* CRL35 in Co-culture With *E. coli* NCTC12900

L. plantarum CRL681 and *Ent. mundtii* CRL35 resulted the most effective strains to fight against *E. coli* in co-cultures. However, we decided to select *Ent. mundti* CRL35 for the proteomic analyses due to the availability of its genome, partially sequenced and annotated, which is essential for protein identification during proteomic studies. Additionally, this strain is able to produce the Enterocin CRL35, a very effective bacteriocin toward *Listeria monocytogenes*. The well-established bioprotective activity of *Ent. mundtii* CRL35, offers an extended range of bioprotection, not only against *E. coli* O157:H7 but also against other food-borne pathogens of concern to meat industry (Salvucci et al., 2007). Finally, *Ent. mundtii* CRL35 possess complete biochemical and genetic studies that guarantee its technological and bioprotective features that make this strain an interesting candidate to be used as adjunct culture in food (Saavedra et al., 2004; Pingitore et al., 2012). Nevertheless interaction studies between *E. coli* and *L. plantarum* CRL681, as well as, the effect of both LAB toward *E. coli* O157:H7 are focus of future experiments.

In order to study the interaction between *Ent. mundtii* CRL35 and *E. coli* O157:H7 NCTC12900 and to identify the molecular mechanisms underlying the antagonistic action carried out by *Ent. mundtii* in a meat environment, differential protein expression during the growth of *Ent. mundtii* alone or in co-culture was evaluated by 2DE. Two key moments of the growth were analyzed: 6 h (T6), when both microorganisms grew exponentially, and 30 h (T30), when *Ent. mundtii* achieved the stationary phase and *E. coli* entered in death state during co-culture (**Figure 3C**). Differential protein expression was evaluated according to the following comparisons: (i) *Ent. mundtii* growing in co-culture vs. *Ent. mundtii* growing individually at T6 (CC T6 - SM T6); (ii) *Ent. mundtii* growing in co-culture vs. *Ent. mundtii* growing individually at T30 (CC T30 - SM T30) and iii) *Ent. mundtii* growing in co-culture at T6 vs. *Ent. mundtii* growing in co-culture at T30 (CC T6 - CC T30). Representative 2DE maps of the bacterial proteomes when grown alone or in co-cultures are depicted in **Figure 4**.

In the three proteomic analyses, the most significant differentially expressed proteins ($p < 0.05$, fold > 1.5), 106 spots, were submitted to MS identification. Of these, a total of 91 proteins were successfully identified, 50 belonged to *E. coli* and 41 to *Ent. mundtii*, according with protein databases. In the present work, we focused in proteins related to *Ent. mundtii* CRL35 proteome (**Tables 1**, **2**).

Differential Protein Expression of *Ent. mundtii* CRL35 Growing Alone Or in Co-culture

When proteomes from *Ent. mundtii* CRL35 grown in co-culture were compared with *Ent. mundtii* growing alone in the sarcoplasmic model system, a significant protein over expression in co-culture was obtained at both analyzed times (T6 and T30). The identified proteins were successfully assigned to different functional categories. Specifically, at the first 6 h, 4 proteins related to carbohydrate (spot #1, enolase) (25%), amino acid metabolism (carbamoyl phosphate synthase large subunit, methionine ABC transporter substrate-binding Protein) (50%) and cell division (cell division protein FtsZ) (25%), resulted significantly over expressed by *Ent. mundtii* in co-culture (**Table 1**, **Figure 5**). On the other hand, after 30 h of growth in co-culture, 16 identified proteins resulted over expressed with a significant difference between 2.1 and 4.7 fold change. These proteins were involved in carbohydrate metabolism (phosphoglycerate kinase, fructose-bisphosphatealdolase, 6-phosphofructokinase, fructose-bisphosphatealdolase and enolase) (31.25%), energy production and conversion (pyruvate dehydrogenase E1 subunit alpha, L-lactate dehydrogenase, 2-oxoisovalerate dehydrogenase subunit beta) (18.75%), transcription (DNA-directed RNA polymerase subunit alpha) (6.25%), cell division (Cell division protein DivIVA oxidoreductase) (6.25%), cell wall biosynthesis (choloylglycine hydrolase) (6.25%), amino acid metabolism (hypothetical protein AK89_04275) (6.25%), folding and protein processing (molecular chaperone DnaK) (6.25%), ribosomal structure (30S ribosomal protein S1) (6.25%) and stress (stress response regulator Gls24) (6.25%) (**Table 1**; **Figure 5**). These results indicate that the proteome of *Ent. mundtii* was affected by the presence of *E. coli* at 6 and 30 h although in a different way. In fact, a higher number of proteins were over produced at 30 h when the LAB achieved the stationary growth phase and the pathogen began its death cycle (**Table 1**; **Figure 3C**). Also, by performing a hypergeometric distribution, the probabilities of obtaining a certain COG category in our sample in relation with the ones encoded by the whole cell were analyzed. Whilst it was more probable to find one protein related with the metabolism and transport of carbohydrates (Figure S1A) we find five of them in our study (**Table 1**). This means that this category might be enriched by the obtained proteins. This also occurs with energy conversion and production, folding and protein processing and cell wall biosynthesis categories. For transcription, stress, ribosomal structure, and amino acid metabolism we found one of each as expected by the hypergeometric distribution. However, for the oxidoreductase (spot # 15) that includes three COG categories, was more probable to find two, and we found only one. This could imply an impoverishment of these categories (Figure S1A and **Table 1**).

Differential Protein Expression in *Ent. mundtii* CRL35 Growing in Co-culture at 6 and 30 h

Regarding the differential protein expression when *Ent. mundtii* grew in co-culture at T6 vs. T30, a total of 21 proteins were successfully identified. Twenty spots

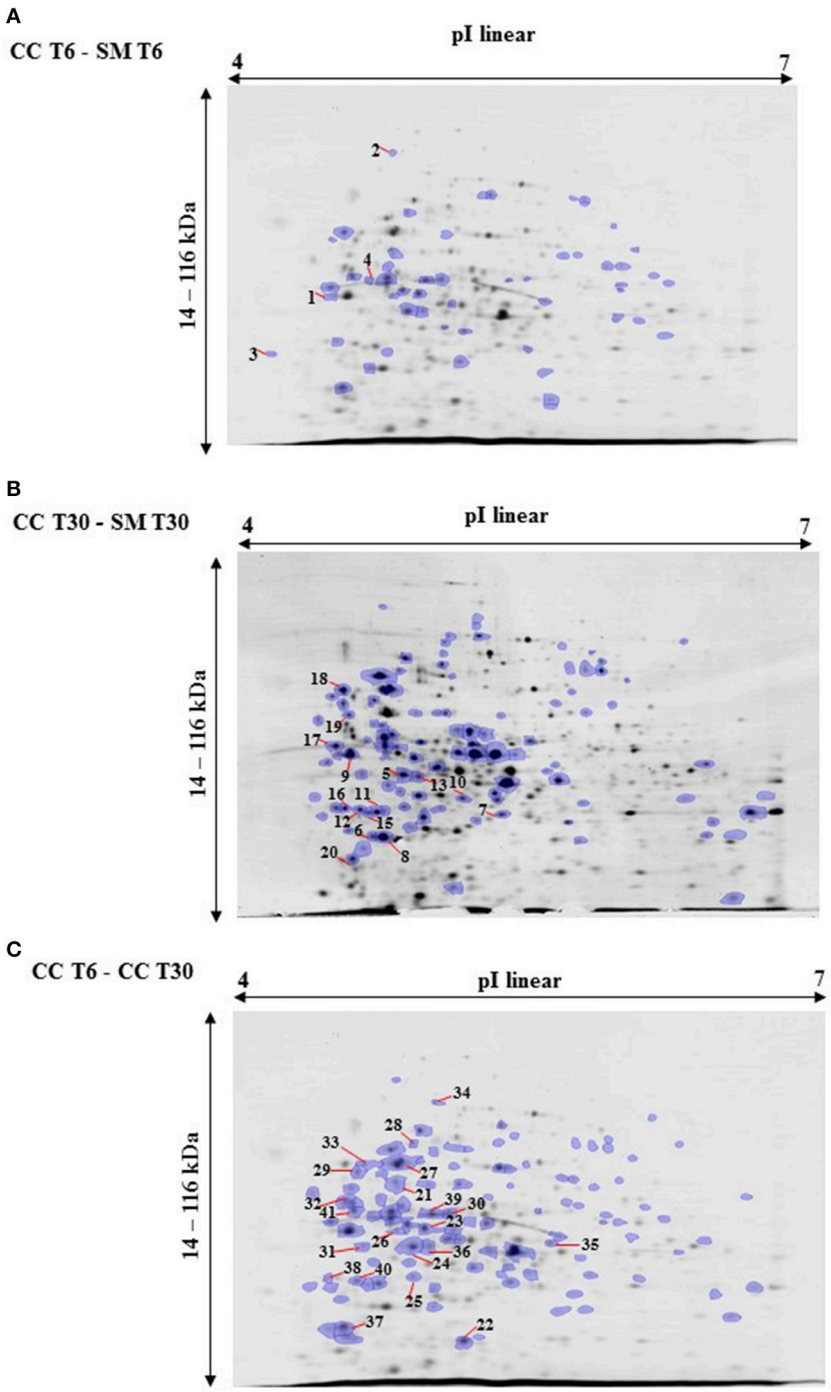


FIGURE 4 | 2DE gels showing the proteins differentially expressed of both microorganisms. The differentially expressed and successfully identified proteins of *Ent. mundtii* are numbered. **(A)** Co-culture T6 vs. individual growth T6 (CC T6 - SM T6). **(B)** Co-culture T30 vs. individual growth T30 (CC T30 - SM T30). **(C)** Co-culture T6 vs. co-culture T30 (CC T6 - CC T30).

presented higher abundances at T6 than at T30 and one, the phosphoglucomutase/phosphomannomutase, was under expressed at T6. The overexpressed proteins participate of different functional categories, namely carbohydrate metabolism (phosphoglycerate mutase 1 family, glucose-6-phosphate isomerase, phosphoglycerate kinase, phosphopyruvate hydratase, phosphogluconate dehydrogenase, type I glyceraldehyde-3-phosphate dehydrogenase, transketolase) (40%), amino acid metabolism (glutamine synthetase, aminopeptidase, dipeptidase PepV and peptidase M13) (15%); energy production and

TABLE 1 | Identified overexpressed proteins during *Enterococcus mundtii* CRL35 growth in co-culture respect to its individual growth at T6 (6 h) and T30 (30 h) in the sarcoplasmic model system at 30°C.

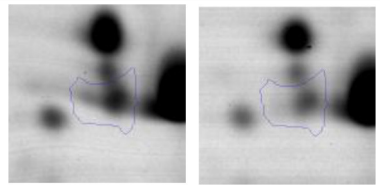
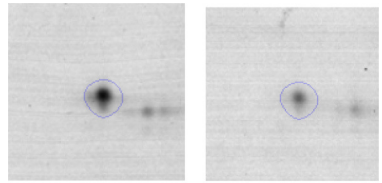
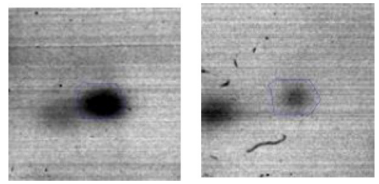
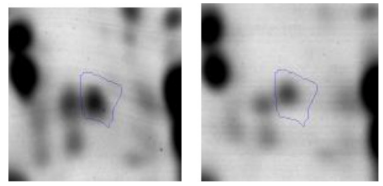
Function	COG[a]	Spot[b]	Protein name	Accession N°[c]	Score[d]	Gene	Theoretical MM[e]	pI[f]	Overexp/Fold change[g]	Detailed 2DE gels showing differentially expressed spot in: Co-culture/ Individ.growth
T6										
Carbohydrate Metabolism	G	1	Enolase	gi|602619948	1120	eno	46496	4.60	1.9	
Amino acid metabolism	E F	2	Carbamoyl Phosphate Synthase Large Subunit	gi|602619378	1170	AK89_04630	117592	4.82	1.8	
	P	3	Methionine ABC Transporter Substrate-Binding Protein	gi|602620029	668	AK89_00090	30407	4.36	2.1	
Cell division	D	4	Cell Division Protein FtsZ	gi|602618182	367	ftsZ	44517	4.73	2.2	

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TABLE 1 | Continued

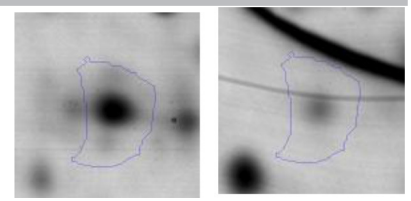
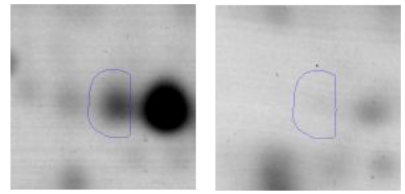
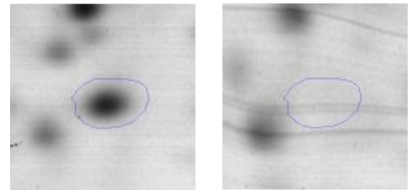
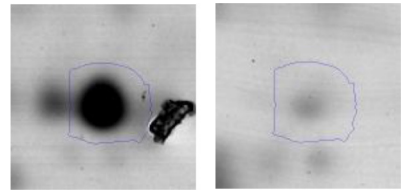
Function	COG[a]	Spot[b]	Protein name	Accession N°[c]	Score[d]	Gene	Theoretical MM[e]	pI[f]	Overexp/Fold change[g]	Detailed 2DE gels showing differentially expressed spot in: Co-culture/ Individ.growth
T30										
Carbohydrate Metabolism	G	5	Phosphoglycerate kinase	gi|602619950	1040	Pgk	42045	4.94	2.1	
	G	6	Fructose-bisphosphate aldolase	gi|602618838	707	AK89_07810	30955	4.77	4.0	
	G	7	6-phosphofructokinase	gi|602619230	1320	pfkA	34342	5.32	4.7	
	G	8	Fructose-bisphosphate aldolase	gi|602618838	626	AK89_07810	30955	4.77	4.6	

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TABLE 1 | Continued

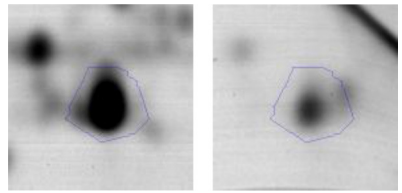
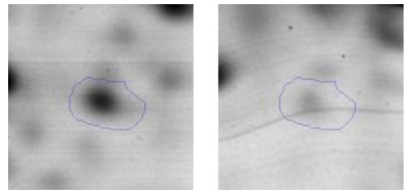
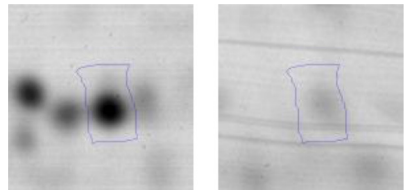
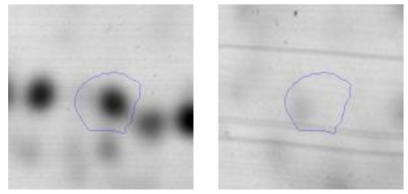
Function	COG[a]	Spot[b]	Protein name	Accession N°[c]	Score[d]	Gene	Theoretical MM[e]	pI[f]	Overexp/Fold change[g]	Detailed 2DE gels showing differentially expressed spot in: Co-culture/ Individ.growth
	G	9	Enolase	gi|602619948	1460	Eno	46496	4.60	2.0	
Energy production and conversion	C	10	Pyruvate dehydrogenase E1 subunitalpha	gi|602618669	938	AK89_08920	41004	5.14	2.6	
	C	11	L-lactate dehydrogenase	gi|602619124	1230	Ldh	35809	4.74	3.1	
	C	12	2-oxoisovalerate dehydrogenase subunit beta	gi|602618668	1240	AK89_08915	35393	4.70	2.7	

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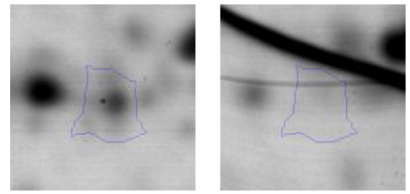
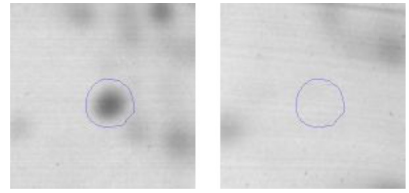
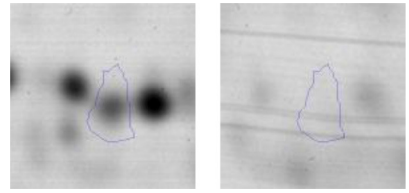
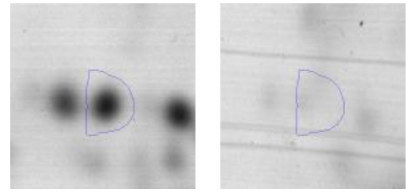
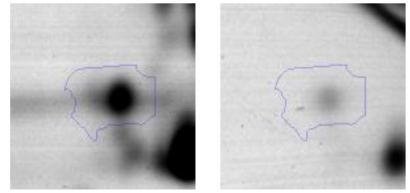
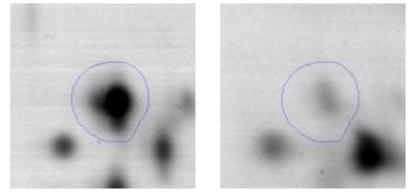
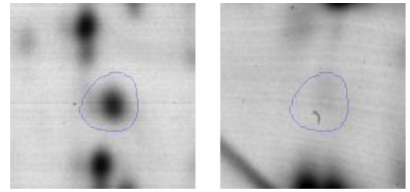
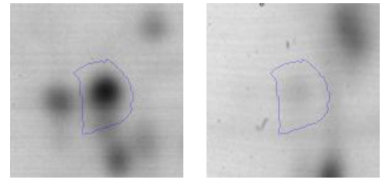
TABLE 1 | Continued

Function	COG[a]	Spot[b]	Protein name	Accession N°[c]	Score[d]	Gene	Theoretical MM[e]	pI[f]	Overexp/Fold change[g]	Detailed 2DE gels showing differentially expressed spot in: Co-culture/ Individ.growth
Transcription	K	13	DNA-directed RNA polymerase subunit alpha	gi|602618530	445	rpoA	35276	4.98	2.3	
Cell division	D	14	Cell division protein DivIVA	gi|602618177	549	AK89_10945	26893	4.61	4.0	
Metabolism	I Q R	15	Oxidoreductase	gi|602619024	1020	AK89_06445	31882	4.77	3,5	
Cell wall biosynthesis	M	16	Choloylglycine hydrolase	gi|602619043	951	AK89_06560	36948	4.62	3.2	

(Continued)

TABLE 1 | Continued

Function	COG[a]	Spot[b]	Protein name	Accession N°[c]	Score[d]	Gene	Theoretical MM[e]	pI[f]	Overexp/Fold change[g]	Detailed 2DE gels showing differentially expressed spot in: Co-culture/ Individ.growth
Amino acid metabolism	E	17	Hypothetical protein AK89_04275	gi|602619580	938	AK89_04275	49541	4.58	2.6	
Folding and protein processing	O	18	Molecular chaperone DnaK	gi|602618370	1360	dnaK	65585	4.63	2.5	
Ribosomal structure	J	19	30S ribosomalprotein S1	gi|602619573	929	AK89_04240	44564	4.66	2.5	
Stress	S	20	Stress response regulator Gls24	gi|602619010	873	AK89_06350	20148	4.63	2.3	

[a] *Functional category according to COG database. One letter abbreviations for the COG functional categories: G, carbohydrate metabolism and transport; C, energy production and conversion; K, transcription; D, cell division and chromosome partitioning; I, lipid metabolism; Q, secondary metabolites biosynthesis, transport, and catabolism; R, general functional prediction only; M, cell wall structure and biogenesis and outer membrane; E, amino acid transport and metabolism; O, molecular chaperones and related functions; J, translation, including ribosome structure and biogenesis; S, no functional prediction.*

[b] *Spot designations correspond to those of the gels shown in* ***Figure 4****.*

[c] *Accession number in the NCBI database.*

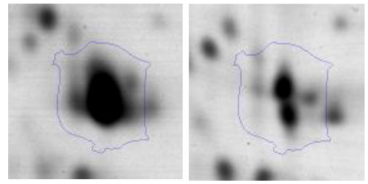
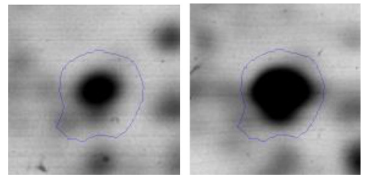
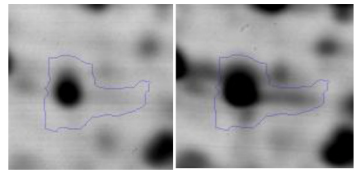
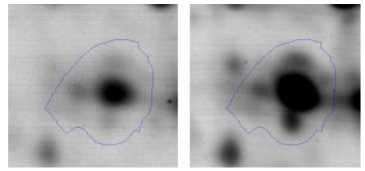
[d] *Protein Score is −10*Log(P), where P is the probability that the observed match is a random event. Protein scores larger than 81 are considered significant ($P < 0.05$).*

[e] *Molecular Mass (Da).*

[f] *Calculated isoelectric point.*

[g] *Relative Fold change: Normalized Volumes of protein spot in co-culture/Normalized Volumes of protein spots in individual growth.*

TABLE 2 | Identified overexpressed proteins during *Enterococcus mundtii* CRL35 growth in co-culture at T6 (6 h) respect to their growth in co-culture at T30 (30 h) in the sarcoplasmic model system at 30 °C.

Function	COG[a]	Spot[b]	Protein name	Accession N°[c]	Score[d]	Gene	TheoreticalMM[e]	pI[f]	Overexp./Fold change[g]	Detailed 2DE gels showing the differentially expressed spot in: Co-cultureT6/Co-cultureT30
Carbohydrate Metabolism	G	21	Phosphoglucomutase/ phosphomannomutase	gi|498429168	108	UAC_01121	63509	4.96	–2.2	
	G	22	Phosphoglycerate mutase 1 family	gi|498428689	84	gpmA	33999	6.35	2.0	
	G	23	Glucose-6-phosphate isomerase	gi|736681785	138	pgi	49574	4.99	1.9	
	G	24	Phosphoglyceratekinase	gi|736681519	186	pgk	42045	4.94	1.9	

(Continued)

TABLE 2 | Continued

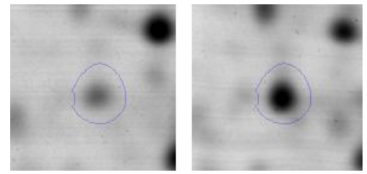
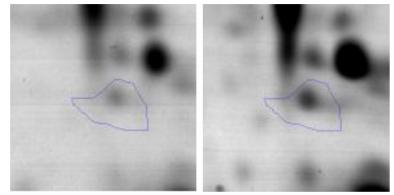
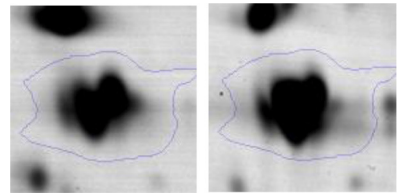
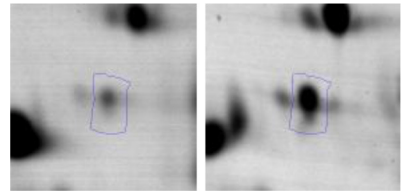
Function	COG[a]	Spot[b]	Protein name	Accession N°[c]	Score[d]	Gene	TheoreticalMM[e]	pI[f]	Overexp./Fold change[g]	Detailed 2DE gels showing the differentially expressed spot in: Co-cultureT6/Co-cultureT30
	C	25	Phosphopyruvate hydratase	gi|498429079	134	eno	46496	4.60	1.9	
	G	26	Phosphogluconate dehydrogenase (NADP(+)-dependent, decarboxylating)	gi|498430058	134	AK89_05050	52733	4.81	2.0	
	G	27	Type I glyceraldehyde-3-phosphate dehydrogenase	gi|736681521	270	AX758_08405	35827	5.08	1.5	
	G	28	Transketolase	gi|602617512	147	AK89_14370	49060	5.04	2.4	

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TABLE 2 | Continued

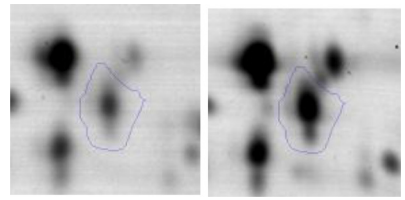
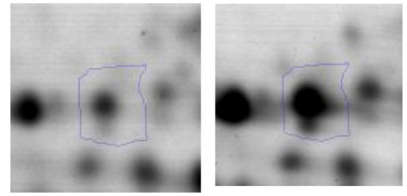
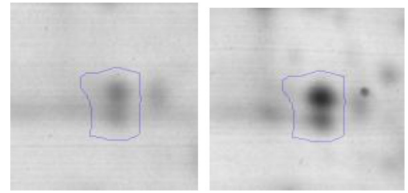
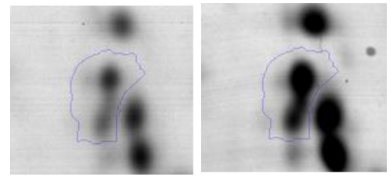
Function	COG[a]	Spot[b]	Protein name	Accession N°[c]	Score[d]	Gene	TheoreticalMM[e]	pI[f]	Overexp./Fold change[g]	Detailed 2DE gels showing the differentially expressed spot in: Co-cultureT6/Co-cultureT30
Sugar Transport	G	29	Phosphoenolpyruvate–protein phosphotransferase	gi|736682554	121	AK89_05300	63384	4.69	2.0	
Amino acid metabolism	E	30	Glutamine synthetase	gi|498430289	109	AK89_07820	50870	5.06	2.4	
	E	31	Aminopeptidase	gi|558689945	227	pepT	45091	4.58	1.9	
	E	32	DipeptidasePepV	gi|736683314	154	AK89_08025	51939	4.62	2.0	

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TABLE 2 | Continued

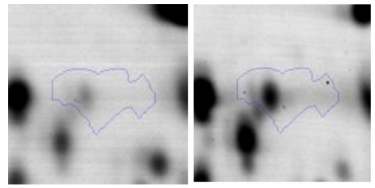
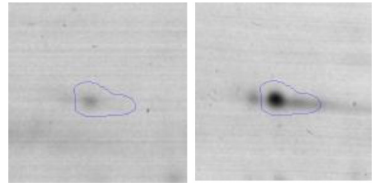
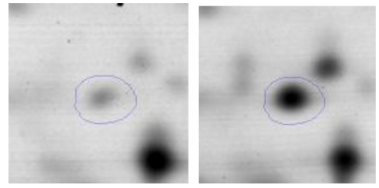
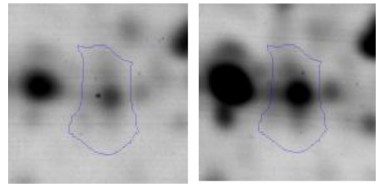
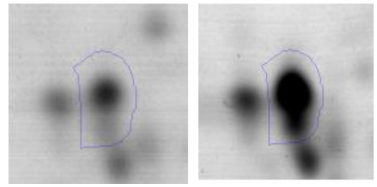
Function	COG[a]	Spot[b]	Protein name	Accession N°[c]	Score[d]	Gene	TheoreticalMM[e]	pI[f]	Overexp./Fold change[g]	Detailed 2DE gels showing the differentially expressed spot in: Co-cultureT6/Co-cultureT30
	O	33	Peptidase M13	gi|498430418	111	AK89_07270	72189	4.71	2.9	
Translation	J	34	Leucine–tRNA ligase	gi|736683966	130	leuS	92016	4.99	3.4	
Nucleotides metabolism	F	35	Adenylosuccinate Synthase	gi|736683880	168	purA	48097	5.49	2.7	
Transcription	K	36	DNA-directed RNA polymerase subunit alpha	gi|492544374	83	rpoA	35276	4.99	1.9	
Stress	S	37	General stress protein	gi|498429607	98	AK89_02030	20130	9.19	3.1	

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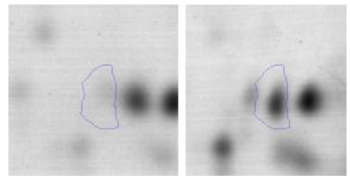
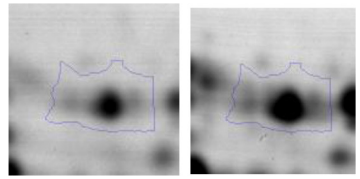
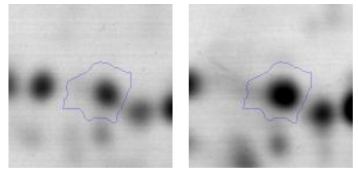
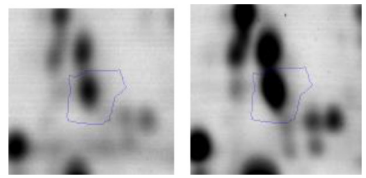
TABLE 2 | Continued

Function	COG[a]	Spot[b]	Protein name	Accession N°[c]	Score[d]	Gene	TheoreticalMM[e]	pI[f]	Overexp./Fold change[g]	Detailed 2DE gels showing the differentially expressed spot in: Co-cultureT6/Co-cultureT30
Cell wall biosynthesis	M	38	Choloylglycine hydrolase	gi|736682868	111	AK89_06560	36948	4.62	3.2	
Energy production and conversion	C	39	ATP synthase subunit alpha	gi|63091861	106	atpA	56590	5.01	2.0	
	C	40	2-oxoisovalerate dehydrogenase	gi|736683518	243	AK89_08915	35393	4.70	1.9	
	C	41	F0F1 ATP synthase subunit beta	gi|736681478	84	atpD	51170	4.72	2.0	

[a] *Functional category according to COG database. One letter abbreviations for the COG functional categories: G, carbohydrate metabolism and transport; C, energy production and conversion; K, transcription; D, cell division and chromosome partitioning; I, lipid metabolism; Q, secondary metabolites biosynthesis, transport, and catabolism; R, general functional prediction only; M, cell wall structure and biogenesis and outer membrane; E, amino acid transport and metabolism; O, molecular chaperones and related functions; J, translation, including ribosome structure and biogenesis; S, no functional prediction.*

[b] *Spot designations correspond to those of the gels shown in* ***Figure 4****.*

[c] *Accession number in the NCBI database.*

[d] *Protein Score is −10*Log(P), where P is the probability that the observed match is a random event. Protein scores larger than either 81 are considered significant ($P < 0.05$).*

[e] *Molecular Mass (Da).*

[f] *Calculated isoelectric point.*

[g] *Relative Fold change: Normalized Volumes of protein spot in co-culture/Normalized Volumes of protein spots in individual growth.*

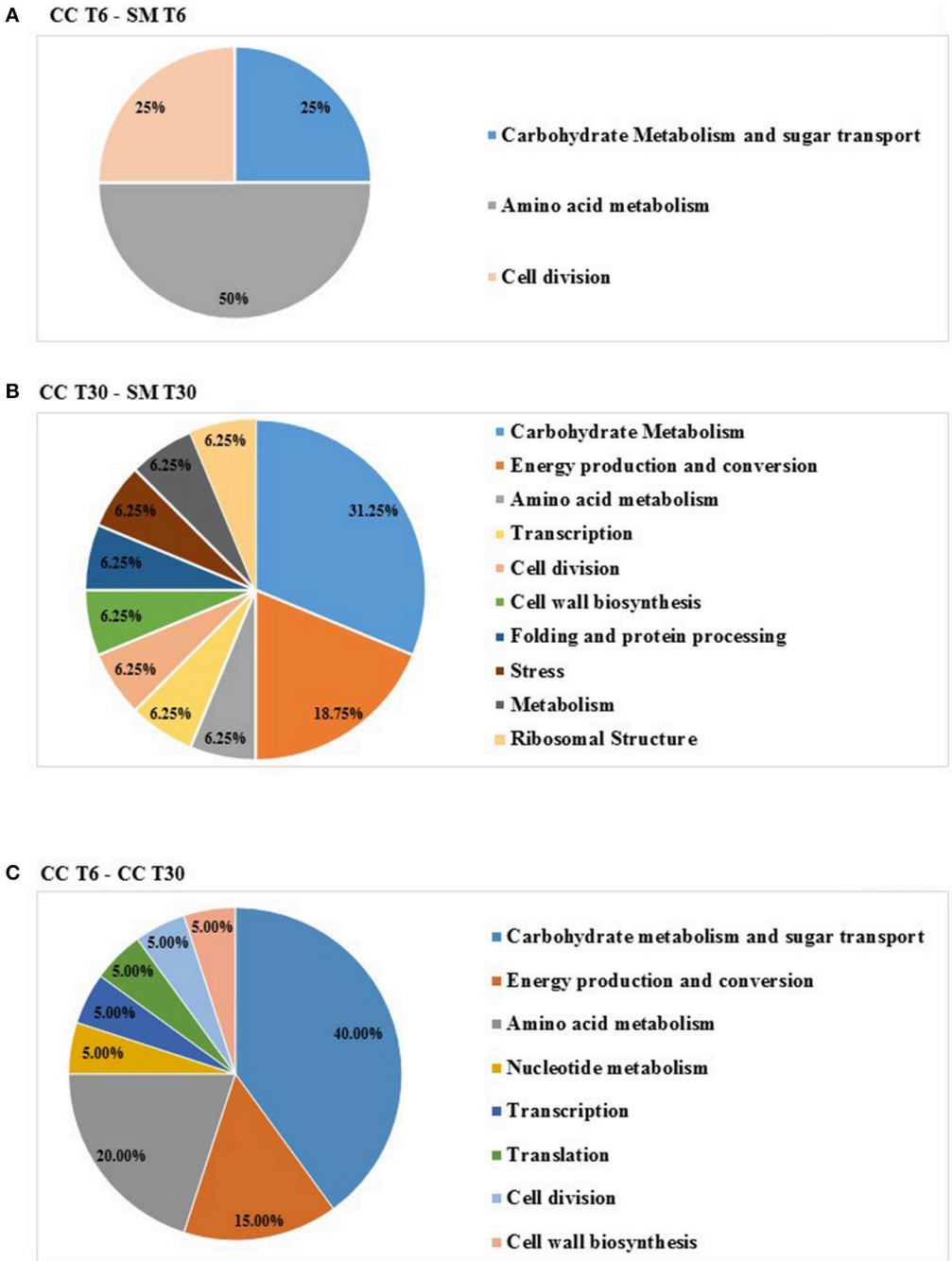


FIGURE 5 | Relative abundance (%) of *Ent. mundtii* identified proteins, grouped according to their functional category, differentially expressed in: **(A)** Co-culture 6 h vs. individual growth 6 h (CC T6 - SM T6); **(B)** Co-culture 30 h vs. individual growth 30 h (CC T30 - SM T30; **(C)** Co-culture 6 h vs. Co-culture 30 h (CC T6 - CC T30).

conversion (ATP synthase subunit alpha, 2-oxoisovalerate dehydrogenase and F0F1 ATP synthase subunit beta) (20%), transcription (DNA-directed RNA polymerase subunit alpha) (5%), nucleotide metabolism (adenylosuccinate synthase) (5%), translation (leucine–tRNA ligase) (5%), stress (general stress protein) (5%), cell wall biosynthesis (choloylglycine hydrolase) (5%) (**Table 2**, **Figure 5**). Moreover, according to the hypergeometric distribution, the categories related with carbohydrate metabolism, energy production and conversion, amino acid transport and metabolism, nucleotide transport and metabolism are enriched. While the categories of translation, transcription, cell wall biogenesis, and of unknown function were obtained as expected (Figure S1B).

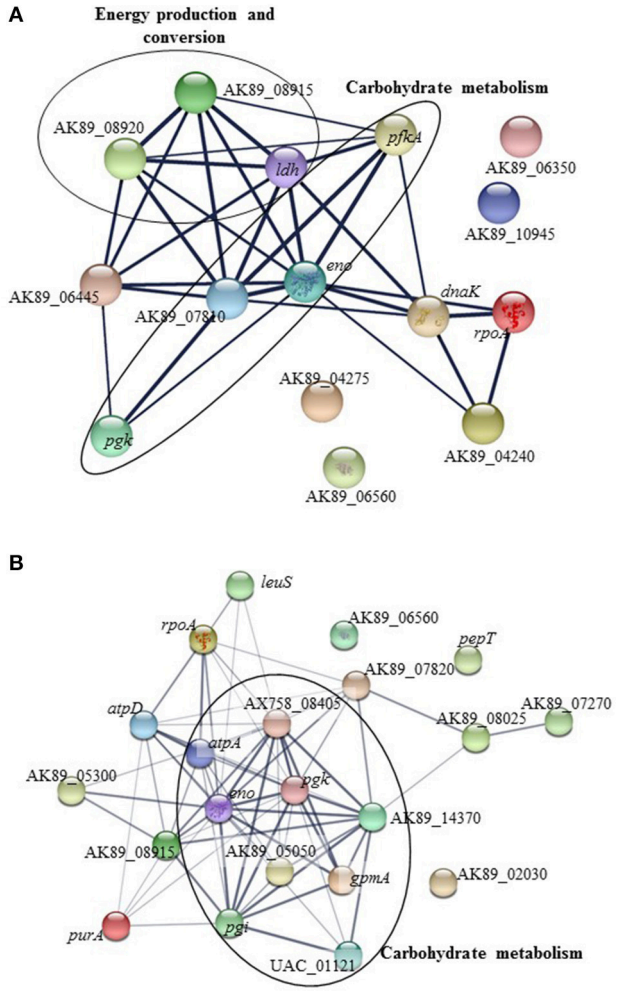


FIGURE 6 | Protein-protein interaction network of overexpressed *Ent. mundtii* proteins. The circle highlights the proteins related with carbohydrate metabolism and energy production and conversion. The proteins are represented by nodes whereas their interactions by edges. Strength of the different interactions is represented by the thickness of the lines. The network was constructed with STRING v10.05. **(A)** Interaction network of *Ent. mundtii* proteins overexpressed in co-culture with respect to their individual grown at T30. **(B)** Interaction network of proteins overexpressed at T6 with respect to T30, when *Ent. mundtii* grew in co-culture with *E. coli*.

In the three proteome comparisons, higher number of differentially expressed proteins were related with carbohydrate, energy production, and amino acid metabolism (**Figure 5**).

Functional Analysis and Interaction of Proteins

The interactions among the over expressed proteins from *Ent. mundtii* were obtained using the STRING v10.05 database. This analysis contributes to understand microbial performance in the meat-based medium. Two protein-protein interaction networks were constructed: (i) containing proteins over expressed by *Ent. mundtii* in co-culture with respect to their individual growth at T30 (**Figure 6A**). A network for T6 was not built as only four proteins at this time were over expressed and (ii) containing proteins over expressed at T6 with respect to T30 when *Ent. mundtii* was grown in co-culture with *E. coli* (**Figure 6B**). As shown in **Figure 6A**, 4 out of the 15 proteins over expressed at T30 with respect to the single culture at T30 have no interactions in between each other. However 11 proteins were related showing interactions (32 edges). Four proteins are related with sugar carbohydrate metabolism and four of them with energy production and conversion, which shows very strong interactions in between each other. This also support the fact that carbohydrates metabolism (G) through glycolysis is enriched at T30 in the presence of *E. coli*.

The second network (**Figure 6B**) corresponds to the overexpressed proteins of *Ent. mundtii* CRL35 grown in co-culture at T6 respect to their growth in co-culture at T30. Three out of 20 proteins were not included in the network as no interactions were found in the STRING database. In this network sixty eight interactions were obtained among the proteins overexpressed by *Ent. mundtii*, the thickness of lines between the nodes indicating the degree of interaction. As mentioned before, the overexpression of these 20 proteins in co-culture at T6 respect to T30 could be explained by the logarithmic growth phase of *Ent. mundtii* CRL35 at 6 h. Moreover, as it can be observed in **Figure 6B**, the main interactions found in the network are related with carbohydrate metabolism (9 proteins) in particular these involved in glycolysis and pentose phosphate pathway. This network support the activation and interaction of proteins related with carbohydrate metabolism in the exponential growth phase which is in agreement with the hypergeometric distribution test.

DISCUSSION

Performance of LAB and *E. coli* NCTC 12900 in a Meat-Based Medium. Physiological Results

The interaction and antagonistic activities of LAB with different pathogenic microorganisms were the focus of a number of studies. For instance, Atassi and Servin (2010) investigated the killing activity of *Lactobacillus* strains against *Salmonella enterica* serovar Typhimurium in co-cultures; Angmo et al. (2016) evaluated different LAB strains as biocontrol agents against *Yersinia enterocolitica* using agar spot tests as screening method. Thereafter, the growth of *Y. enterocolitica* in mixed cultures co-inoculated with two selected *Lactobacillus* strains was investigated. Also Yang et al. (2017) studied mixed cultures of bifidobacteria with *Listeria monocytogenes* to detect the changes in their growth pattern after mutual interaction by applying a proteomic approach. The present study is the first where the LAB inhibitory potential against *E. coli* O157:H7 is evaluated in co-cultures using physiological and proteomic approaches. The first objective of this study was to evaluate the inhibitory potential of three lactic acid bacteria strains toward *E. coli* O157:H7 NCTC12900. By a simple and rapid method such as the well-diffusion assay, a variable LAB inhibitory activity was

evidenced. Then, the kinetic of growth in a meat model system in the presence of *E. coli*, was analyzed. The three LAB strains affected negatively the growth of EHEC in the meat environment after 8 h, evidencing a higher inhibitory potential of *L. plantarum* CRL681 and *Ent. mundtii* CRL35, which produced a significant decrease of *E. coli* counts at 96 h. It seems therefore that LAB trigged *E. coli* death, due to mechanisms other than acid effect or bacteriocin activity. In fact during the spot on lawn assay, no inhibitory halos were detected by supernatants containing acids or bacteriocins, they not triggering *E. coli* inhibition. Contrarily, Angmo et al. (2016) concluded that low pH and production of lactic acid were the main factors for inhibition of growth of *Y. enterocolitica* (Angmo et al., 2016). Moreover, even if all LAB strains were able to acidify the sarcoplasmic medium, they presented different inhibitory activity toward *E. coli*. According to this, EHEC has three powerful systems of resistance to acid stress; these including an acid-induced oxidative system, an acid-induced arginine-dependent system and a glutamate-dependent system (Bearson et al., 2009). These three systems of resistance to acids have different requirements, allowing an overlapping between them, ensuring that at least one of them will always be active to protect the cell in an acid environment (Bearson et al., 2009). These properties of EHEC contribute to low infectious doses by allowing small numbers of microorganisms to pass through the gastric acidity barrier. Therefore, its acid resistance ability is an important virulence factor and explains the absence of growth inhibition halos by culture supernatants observed herein. As regards to *E. coli* inhibition by bacteriocin action, it is known that these peptides do not act on Gram negative microorganisms unless they are combined with a treatment to damage the cell wall to allow bacteriocin entrance into the cell (Castellano et al., 2011). Therefore, our results, showing absence of inhibition due to *Ent. mundtii* CRL35 and *L. curvatus* CRL705 bacteriocins, suggest that EHEC antagonistic action involves other mechanisms such as competition for nutrients, quorum sensing, or a close cell-cell relationship where the bioprotective culture must preserve its vitality to cope with EHEC. In a similar work, Rios-Covian et al. (2015) reported a delayed growth of *Bacillus fragilis* by the presence of *Bifidobacterim longun* in co-culture during the first 14 h. They observed an improved growth of bifidobacteria compared to the corresponding mono-culture. Our results showed that *E. coli* affects slightly to moderately the maximal cell densities achieved by LAB. *Ent. mundtii* CRL35 showed an earlier exponential phase and the stabilization of the stationary phase with a slight viability decrease after 40 h compared to its growth in mono-culture. Yang et al. (2017) studying the co-incubation of *Bifidobacterium bifidum* with *Listeria monocytogenes,* have also reported the earlier entrance into the logarithmic growth phase suggesting a mutual growth promoting effect during the co-cultivation.

Differential Protein Expression Analyses

Ent. mundtii CRL35 in Co-culture vs. *Ent. mundtii* in Mono-Culture

In this work, 2DE was employed to analyze differential expression induced by the interaction between *Ent. mundtii* CRL35 and *E. coli* NCTC12900, focusing on the LAB proteome. This approach allowed us to investigate the molecular basis of this interaction and the relation with the physiological changes undergoing during co-cultivation in the meat-based medium. Slight proteome variations were observed in *Ent. mundtii* during the first hours of co-culture with *E. coli* with respect to its growth as mono-culture at 6 h. One ABC transporter for methionine and the carbamoyl phosphate synthase large unit are among the over expressed proteins. They are related to amino acid biosynthesis and metabolism. One enzyme related to glycolysis, the enolase (spot #9), and one related to cell division (protein FtsZ, spot #14) resulted over expressed at 6 h and at 30 h indicating that LAB activated glycolysis and cell division to cope the presence of *E. coli*. It should be mentioned that enolase is also known as a moonlighting protein. These are proteins that display additional functions other than their major described biochemical catalytic activity. In general, these cytoplasmic/cell surface moonlighting proteins can be important in infection, virulence, or immune responses (Jeffery, 2015). For example enolase is also associated with epithelial cell binding (Castaldo et al., 2009). In fact, Peng et al. (2014) reported enolase as one of the actin-binding proteins in *Enterococcus faecalis*. It could therefore be suggested that *Ent. mundtii* can up-regulate enolase during co culturing with *E. coli* as an additional strategy to compete with *E. coli* for actin binding during adhesion to meat. On the other hand, *Ent. mundtii* proteome was much more affected in co-culture at 30 h than when it grew alone at the same time. In fact 16 proteins resulted over expressed, including some spots also up-regulated at 6 h (enolase and cell division protein FtsZ). Protein-protein network showed interaction in 12 of these proteins, mainly related with carbohydrate metabolism. The 31.25% of differentially over expressed proteins were involved in glycolysis. The 18.75% of proteins synthesized in higher amounts were related to other pathways, also involved in energy production and conversion, thus indicating that co-culturing with *E. coli* exerted more effective activation of these pathways at 30 h of co-culturing than during the first hours of the growth. In addition, physiological results indicate that at 30 h, *E. coli* is dying in co-culture, suggesting that *E. coli* viability decrease resulted convenient for *Ent. mundtii* which persisted in the stationary phase. *Ent. mundtii* during co-culture, resulted even more stable at stationary phase than when it grew alone. This fact is consistent with the over-expression of many proteins from sugar metabolism, energy production, transcription, cell division, and amino acid metabolism indicating an active metabolism of *Ent. mundtii* which allowed its persistence in the meat-based medium. It should also be highlighted the up regulation of proteins related to folding/processing and stress such as the chaperone DnaK and the stress response regulator Gls24 that could contribute to the satisfactory resistance of *Ent. mundtii* to stressful conditions dominating the microbial environment at 30 h in conjunction with a low pH (close to 4.0). There are some studies demonstrating the interaction of certain microorganisms during its growth in mixed cultures. Yang et al. (2017) proposed that the growth of *Bifidobacterium bifidum* WBBI03 and *Listeria monocytogenes* together promotes the growth of each other, resulting in earlier entry into the logarithmic phase, and the expression of proteins mostly tended

to be up regulated at the translational and transcriptional level. While Rios-Covián et al. (2016) reported the stimulation of the growth of *Bifidobacterium longum* in co-culture while retarding the growth of *Bacteroides fragilis*, with concomitant changes in the production of some proteins and metabolites of both bacteria. In the present work a different interaction seems to occur between *Ent. mundtii* CRL35 and *E. coli* O157:H7. In fact, a positive effect of *E. coli* on the fitness of the LAB could occur, while the latter triggered the pathogen death after 8 h of co-culture.

Ent. mundtii CRL35 in Co-culture: T6 vs. T30

When comparing protein expression of co-cultures along the time (CC T6 vs. CC T30), 20 proteins resulted over expressed during the first hours (6 h). This is in relation to the exponential growth phase going through the microorganism, in which the general metabolism is activated as reported by other (Cohen et al., 2006; Koistinen et al., 2007). Those results are also supported by the observed protein network, where proteins related to carbohydrate metabolism presented stronger interactions. Among up regulated proteins, 7 spots were identified as belonging to carbohydrate metabolism and 1 to sugar transport. Five enzymes were related to glycolysis (spots #22, 23, 24, 25 and 27) and two involved in the pentose phosphate pathway (spots #26 and 28) (**Figure 7**). The over expression of phosphoenolpyruvate–protein phosphotransferase, involved in carbohydrate transport, could facilitate glucose entrance into the cell, as a consequence of a more efficient competition with the pathogen for sugar uptake, thus contributing to cope with *E. coli* presence which is also in the logarithmic growth state. Only one glycolytic enzyme, the phosphoglucomutase (spot #21) was under expressed at 6 h. This could be related to the up regulation of the PTS phosphoenolpyruvate-protein phosphotransferase (PTS system) involved in glucose transport by the generating glucose-6P which enters directly into the Embden-Meyerhorff-Parnas pathway, explaining the under expression of phosphoglucomutase which produces glucose-6P from glucose-1P coming from other pathways such as glycogen hydrolysis (Bonacina, 2017) (**Figure 7**). Glycogen metabolism would be less active than glucose during the first hours. In fact glucose is added to the meat-based medium which could be chosen firstly as primary energy source. On the other hand, two enzymes related to the pentose phosphate/phospohoketolase (PKP) pathway resulted over expressed in *Ent. mundtii* at 6 h, the 6-P gluconate dehydrogenase and transketolase, indicative also of the active metabolism of ribose, one of the sugars present in meat (Chaillou et al., 2005) (**Figure 7**). Concurring with our findings, Koistinen et al. (2007) reported that proteins preferentially expressed by *L. plantarum* in the early exponential phase were related to sugar consumption and biomass increase. Four enzymes related to amino acid metabolism were also up regulated by *Ent. mundtii* in co-culture at 6 h. Among them glutamine synthetase, a key enzyme of nitrogen metabolism that catalyzes the incorporation of ammonium into glutamate and is related to arginine biosynthesis, alanine, glutamate, and aspartate metabolism among other (Magasanik and Rothstein, 1980). In addition, some other peptidases and aminopeptidases resulted up regulated also at 6 h, indicating an active peptidolytic metabolism during the first hours of co-culturing. Also the adenylosuccinate synthase resulted 2.7-fold over expressed at 6 h with respect to T30 by *Ent. mundtii* in co-culture, this enzyme plays an important role in the *de novo* pathway of purine nucleotide biosynthesis, it catalyzing the first committed step in the biosynthesis of AMP from IMP, also indicating a more active metabolism of this LAB during the first hours of co-culture. Also, proteins involved in transcription and translation such as DNA-directed RNA polymerase subunit alpha, leucine-tRNA ligase resulted up regulated by this LAB strain during the first hours of co-culturing. In accordance, Yang et al. (2017), reported the up regulation of enzymes related to transcription and translation when mixed cultures of *Bifidobaterium bifidum* and *Listeria monocytogenes* were evaluated.

One general stress protein presented also higher amounts at T6, as well as, the choloylglycine hydrolase involved in lipid metabolism and cell wall biosynthesis. This pattern can be related with the exponential growth and the consequent active cellular division. Two ATP synthases (alpha and beta subunits) were found increased in this condition. They are the prime producers of ATP, using the proton gradient generated by oxidative phosphorylation. Finally, the 2-oxoisovalerate dehydrogenase beta subunit was 1.9 fold over expressed at 6 h during co-culture. This enzyme belongs to the oxidoreductase family, being also implicated in Phe, Tyr, Trp, Leu, Ile, Val, Asp, and Asn degradation. The branched-chain alpha-ketodehydrogenase complex catalyzes the overall conversion of alpha-keto acids to acyl-CoA and CO_2. Summarizing, differential protein expression by *Ent. mundtii* in co-culture at two different growth phases correlated with the observed physiological behavior. The up regulation of many enzymes implicated in sugar and nitrogen metabolism, transcription, translation and energy production was in relation with the metabolism and the physiological state crossing LAB during the first hours of growth. A direct consequence of this active global metabolism contributed to the competition with *E. coli* at this specific moment when the pathogen was going through the exponential phase, while at 30 h, *Ent. mundtii* CRL35 reaching the stationary phase did not have to face a strong competition with *E. coli* since it had already entered into its death phase.

According with the obtained results, on can postulate the positive effect of *E. coli* on the fitness of the LAB, while a negative impact exerted the LAB on the pathogen by triggering its death after 8 h of co-culture. On the other hand, differential overexpression of *Ent. mundtii* proteins was higher in co-culture with *E. coli* than when it grew alone at 30 h. Concomitantly, physiological results indicated that at 30 h, *E. coli* was dying in co-culture, suggesting that the decrease of *E. coli* viability resulted convenient for *Ent. mundtii* which persisted in the stationary phase. This fact is consistent with the over-expression of many proteins from sugar metabolism, energy production, transcription, cell division and amino acid metabolism. This fact indicating the active *Ent. mundtii* metabolism allowed its persistence over the pathogen in the meat medium. It should also be highlighted the up regulation of proteins related to

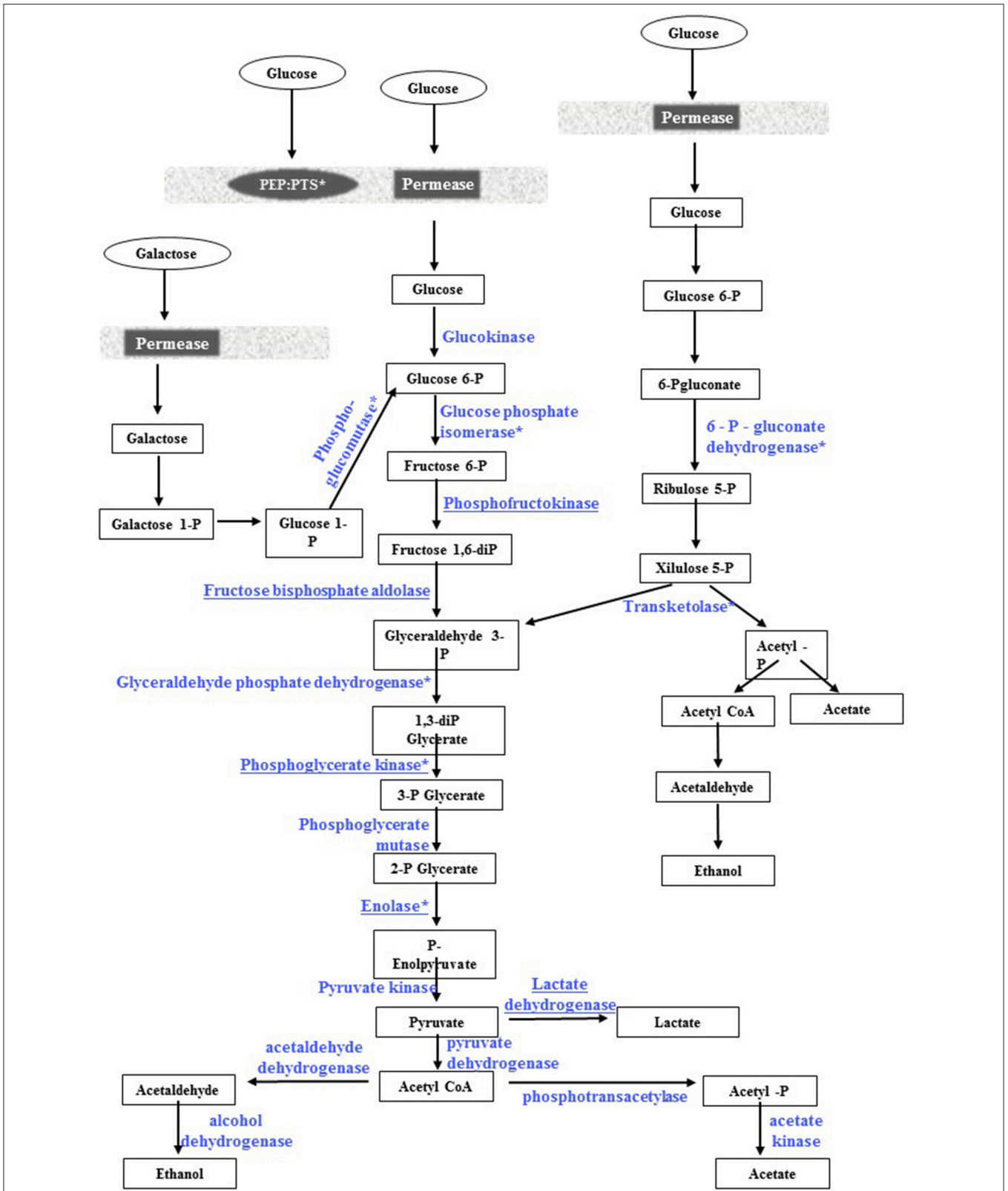


FIGURE 7 | Glycolytic pathway in *Ent. mundtii* CRL35. Enzymes overexpressed at 30 h in co-culture with respect to their individual growth are underlined. With asterisks are presented the proteins overexpressed in co-culture at 30 h with respect to 6 h.

folding/processing and stress such as the chaperone DnaK and the stress response regulator Gls24 that could contribute to the satisfactory resistance of *Ent. mundtii* to stressful conditions at 30 h, such as the low pH. When comparing protein expression of *Ent. mundtii* in co-culture along the time (CC T6 *vs* CC T30), the higher number of proteins over expressed during the first 6 h, was in relation with the metabolism and the physiological state crossing LAB during the first hours of co-culture. A direct consequence of this active global metabolism contributed to the competition with *E. coli* which was going through the exponential phase. Whereas at 30 h, *Ent. mundtii* was reaching the stationary phase and *E. coli* had already entered into its death phase. Indeed the LAB did not have to face a strong competition, accordingly, less differential protein expression was achieved at 30 h. Finally, mechanisms involved in this interaction, such as competition for nutrients, quorum sensing, or a close cell-cell relationship are suggested. The detailed study of these mechanisms is focus of ongoing investigations.

CONCLUSION

Current results have demonstrated the true inhibitory potential of LAB against a pathogen of great concern such as *E. coli* O157:H7. Such inhibition was not due to acid or bacteriocin production but instead to a more complex relationship during the microbial interaction. The proteomic results herein presented supported physiological observations, demonstrating significant differences in protein expression in LAB (i) due to the presence of the pathogen and (ii) according to the growth phase analyzed. Even when more studies have to be performed in fresh meat to confirm *in vitro* observations, these results lay the foundations of the molecular basis for understanding the interaction between *Ent. mundtii* CRL35 and *E. coli* O157:H7 NCTC12900, as well as on the strategies of competition applied by both microorganisms. This work finally opens new perspectives for the application of this bioprotective LAB to control *E. coli* O157:H7 in meat products.

AUTHOR CONTRIBUTIONS

AO carried out the experiments, analyzed the results and wrote the paper. SF conceived the idea of the project, coordinated the study, analyzed and discussed the results and wrote the paper. MS coordinated the study, discussed the results and wrote the paper. LT performed the *in silico* analysis of the identified proteins (STRING, COGNITOR) and the analysis of the hypergeometric distribution. JR and AA contributed in mass spectrometric analysis (MALDI-TOF/TOF) and discussed the paper. GV contributed to the discussion of the paper. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.01083/full#supplementary-material

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New Insights into the Anti-pathogenic Potential of *Lactococcus garvieae* against *Staphylococcus aureus* Based on RNA Sequencing Profiling

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The bio-preservation potential of *Lactococcus garvieae* lies in its capacity to inhibit the growth of *staphylococci*, especially *Staphylococcus aureus,* in dairy products and *in vitro*. *In vitro*, inhibition is modulated by the level of aeration, owing to hydrogen peroxide (H_2O_2) production by *L. garvieae* under aeration. The *S. aureus* response to this inhibition has already been studied. However, the molecular mechanisms of *L. garvieae* underlying the antagonism against *S. aureus* have never been explored. This study provides evidence of the presence of another extracellular inhibition effector *in vitro*. This effector was neither a protein, nor a lipid, nor a polysaccharide, nor related to an L-threonine deficiency. To better understand the H_2O_2-related inhibition mechanism at the transcriptome level and to identify other mechanisms potentially involved, we used RNA sequencing to determine the transcriptome response of *L. garvieae* to different aeration levels and to the presence or absence of *S. aureus*. The *L. garvieae* transcriptome differed radically between different aeration levels mainly in biological processes related to fundamental functions and nutritional adaptation. The transcriptomic response of *L. garvieae* to aeration level differed according to the presence or absence of *S. aureus*. The higher concentration of H_2O_2 with high aeration was not associated with a higher expression of *L. garvieae* H_2O_2-synthesis genes (*pox*, *sodA*, and *spxA1*) but rather with a repression of *L. garvieae* H_2O_2-degradation genes (*trxB1*, *ahpC*, *ahpF*, and *gpx*). We showed that *L. garvieae* displayed an original, previously undiscovered, H_2O_2 production regulation mechanism among bacteria. In addition to the key factor H_2O_2, the involvement of another extracellular effector in the antagonism against *S. aureus* was shown. Future studies should explore the relation between H_2O_2-metabolism, H_2O_2-producing LAB and the pathogen they inhibit. The nature of the other extracellular effector should also be determined.

Keywords: *Lactococcus garvieae*, *Staphylococcus aureus*, hydrogen peroxide, transcriptome, growth inhibition, anti-pathogenic interactions

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INTRODUCTION

Interest in Lactic Acid Bacteria (LAB) as bio-preservation agents against foodborne pathogens has been growing for the last 15 years (Ito et al., 2003; Batdorj et al., 2007; Charlier et al., 2009; Adesokan et al., 2010). Considering their beneficial properties, the dairy industry could find it useful to employ *Lactococcus garvieae* strains as starter or adjunct cultures, provided the strains are safe (Fernández et al., 2010). Aquatic strains of *L. garvieae* are frequently referenced as fish pathogens (Eldar and Ghittino, 1999; Vendrell et al., 2006) but other strains are regarded as opportunistic pathogens for humans (Aguado-Urda et al., 2011; Ortiz et al., 2014). Despite its ubiquity in foods such as milk (Devriese et al., 1999; Villani et al., 2001; Callon et al., 2007) and dairy products of various origins (Flórez and Mayo, 2006; Fortina et al., 2007; Alomar et al., 2008b; El-Baradei et al., 2008; Jokovic et al., 2008; Alegría et al., 2009; Sip et al., 2009; Monfredini et al., 2012; Pangallo et al., 2014; Morandi et al., 2015), to our knowledge, *L. garvieae* has never been involved in a foodborne disease outbreak. *L. garvieae* is of interest for bio-preservation owing to its ability to inhibit the growth of staphylococci, particularly *Staphylococcus aureus*, as has been observed in milk, in cheese and *in vitro* (Alomar et al., 2008a,b; Delbes-Paus et al., 2010; Delpech et al., 2015). The transcriptome response of *S. aureus* to this inhibition has already been explored (Delpech et al., 2015). It is associated with a repression of the stress response (especially H_2O_2 response) and of cell division genes and with modulation of the expression of virulence genes (particularly *agrA*, *hld,* and enterotoxin-encoding genes) genes. However, the molecular mechanisms of *L. garvieae* underlying the antagonism against *S. aureus* have never been explored.

Since *L. garvieae* produces low amounts of acetic and lactic acid in milk (Alomar et al., 2008a,b; Nouaille et al., 2009; Sip et al., 2009; Delbes-Paus et al., 2010; Delpech et al., 2015; Morandi et al., 2015), its antagonism against *S. aureus* is not associated with acidification. It is also unlikely to be associated with nutritional competition (Alomar et al., 2008a). With high aeration, *S. aureus* inhibition is mainly associated with hydrogen peroxide (H_2O_2) production by *L. garvieae*, as already observed *in vitro* (Delbes-Paus et al., 2010; Delpech et al., 2015). With low aeration, the inhibition is weaker and H_2O_2 is not detected. The addition of catalase (an H_2O_2-degrading enzyme) partly suppressed the inhibition (Delbes-Paus et al., 2010). Delbes-Paus et al. (2010) therefore suggested that at least one other molecule from *L. garvieae* must be involved in the residual inhibition of *S. aureus* in the absence of H_2O_2. The role and nature of this molecule have not yet been determined.

In Gram-positive bacteria, the aeration level and the presence of H_2O_2 generally strongly affect the expression of H_2O_2 metabolism genes. The expression of major H_2O_2 degradation genes (*ahpC*, *ahpF*, *gshR*, *gpo*, *trxA*, and *trxB*) and their related proteins is strongly induced in *L. lactis* (Pedersen et al., 2008) under aeration and in *Bacillus subtilis* in the presence of H_2O_2 (Mostertz et al., 2004). As regards the major H_2O_2 synthesis genes, *sodA* is generally regulated in the same way as H_2O_2-degradation genes while the pyruvate oxidase (*pox*) gene is not affected by these parameters. Biological functions of *L. lactis* related to aerobiosis, i.e., O_2 response, menaquinone metabolism and stress response, are variably affected by the presence of *S. aureus* (Nouaille et al., 2009). However, little is known about the H_2O_2-metabolism of *L. garvieae* as an anti-pathogenic process.

In this study, we aimed to characterize the antagonism of *L. garvieae* against *S. aureus in vitro* in greater depth. Firstly, we investigated the presence of potential antagonist molecules in H_2O_2-free *L. garvieae* supernatants and their impact on *S. aureus* growth. Secondly, the transcriptome of *L. garvieae* with different aeration levels and in different biotic environments (absence or presence of *S. aureus*) was determined through RNA sequencing, an accurate and efficient method for revealing bacterial transcriptome profiles (Pinto et al., 2011). The resulting data led us to a better understanding of *L. garvieae* hydrogen peroxide metabolism and to some initial hypotheses on the nature of a new inhibition effector.

MATERIALS AND METHODS

Strains and Culture Conditions

Lactococcus garvieae N201 and *S. aureus* SA15, isolated from raw milk, were obtained from the INRA UR545 collection (Alomar et al., 2008a). Both strains were aerobically grown in Brain-Heart Infusion broth ("BHI", Biokar Diagnostic, Pantin, France) for 20 h, at 30°C for *L. garvieae* and at 37°C for *S. aureus*. They were then inoculated separately or in co-culture at 10^6 cells.mL^{-1} for *S. aureus* and 10^7 cells.mL^{-1} for *L. garvieae* into BHI buffered at pH = 7 with phosphate buffer KH_2PO_4, $3H_2O/K_2HPO_4$ at 0.1 mol.L^{-1} (KH_2PO_4, $3H_2O$, Riedel-de-Haen, Honeywell GmbH, Seelze, Germany; K_2HPO_4, Merck KGaA, Darmstadt, Germany) previously equilibrated at 30°C. Pure cultures and co-cultures of both strains were performed with either a high or a low aeration depending on the experiment. Low aeration cultures were set in static, fully filled and sealed, 50-mL Nunc EZ Flip conical centrifuge tubes (Sigma–Aldrich, St. Louis, MO, USA). High aeration was obtained by a mechanical shaking at 150 rpm on 50-mL cultures in 250-mL Erlenmeyers. All cultures were incubated at 30°C for 24 h in an Infors HT Minitron (Infors AG, Bottmingen, Switzerland). The cultivable cell counts were determined after plating for each sampling time as described by Delpech et al. (2015).

Impact of H_2O_2-Free Co-culture Supernatants on *S. aureus* Growth

The potential presence of other antistaphylococcal molecules in *L. garvieae* N201 and *S. aureus* SA15 co-culture supernatant and their impact on *S. aureus* planktonic growth were investigated. After adding catalase at 400 U.mL^{-1}, a pure culture of *S. aureus* SA15, a co-culture of *S. aureus* SA15 and *L. garvieae* N201 and plain BHI were incubated with low aeration as described above. After 6 h of incubation, 40 mL of each tube were centrifuged at 9,600 × *g* for 10 min at 4°C and supernatants stored at 4°C until utilization. Four milliliters of the remaining *S. aureus* culture were centrifuged at 7,500 × *g* for 10 min. The cell pellet was resuspended at a concentration of ∼10^6 cells.mL^{-1} in 20 mL of

fresh 2X-concentrated BHI buffered at pH = 7. The supernatants were filtered through a cellulose acetate membrane (pore size 0.45 μm; GVS S.p.A., Zola Predosa, Italy). They were either treated with proteinase K (AMRESCO LLC, Solon, OH, USA, ref: 0706-100MG) and pronase E (Merck KGaA, ref: 537088), i.e., with two proteases, or treated with lipase (Sigma–Aldrich, ref: L3126-100G), or treated with α-amylase (Sigma–Aldrich, ref: A3176-500KU), or not treated at all. Each enzyme was added at 0.2 $mg.mL^{-1}$ from stock solutions at 10 $mg.mL^{-1}$ prepared in 200 mM of phosphate buffer at pH = 7. Treatment with proteases consisted of a first incubation of the supernatants with proteinase K at 50°C for 2 h 15 and a second incubation with pronase E at 37°C for 2 h 15. Treatments with lipase and α-amylase consisted of an incubation of the supernatants with the enzyme at 37°C for 5 h. After each incubation step, the enzyme was inactivated by heating (95°C during 10 min) and all supernatants were filtered again through a cellulose acetate membrane (pore size 0.45 μm; GVS S.p.A.). Prepared supernatants and *S. aureus* culture in 2X-BHI were then distributed at 1:1 volume ratio (total volume = 1.5 mL) in a CytoOne 24-well cell plate covered with a lid (ref: CC7672-7524; STARLAB, Hambourg, Germany). The cell plate was incubated for 25 h in a SAFAS Xenius XC spectrophotometer (SAFAS Monaco, Monaco) and thermostated at 30°C with a Julabo CryoStat (JULABO Gmbh, Seelbach, Germany). *S. aureus* growth in each well was determined by measuring the OD_{600} every 15 min for 1 h and then every 30 min for 24 h. Before each OD_{600} measurement, the cell plate was shaken at 5 Hz for 20 s with an orbital diameter of 6 mm. The whole experimental design was repeated three times. To identify sample means that were significantly different from each other, statistical analyses were performed on values at 9, 12, 15, 18, 21, and 24 h, using Statistica software (StatSoft) with a single-factor analysis of variance (ANOVA) followed by a Newman–Keuls *post hoc* test.

Sample Preparation for RNA Analysis

Pure cultures and co-cultures of *L. garvieae* N201 and *S. aureus* SA15 were grown with high or low aeration, as specified above. After 3, 6, 9, and 24 h of incubation, 40 mL of each culture were centrifuged at 9,600 × *g* for 10 min at 4°C. Hydrogen peroxide concentrations and pH values were determined on the supernatants by enzymatic reaction and spectrophotometry as described by Delbes-Paus et al. (2010). The cell pellets were immediately frozen in an ethanol bath and stored at −80°C. Extraction of total RNA from the frozen cell pellets was performed as described by Delpech et al. (2015). For each sample of total RNA obtained with one culture condition, a first part of the aliquot was used for RNA sequencing after rRNA depletion and a second part of the aliquot was used for RT-qPCR analyses. The whole experimental design was repeated three times.

Determination of *L. garvieae* Transcriptome Changes by RNA Sequencing

Ribosomal RNAs were removed from the total RNA (2 × 5 μg of RNA by sample) using a RiboZero Magnetic Kit for Gram Positive Bacteria (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. The quality and concentration of RNA in each sample were assessed using a RNA 6000 pico kit (Agilent Technologies, Santa Clara, CA, USA). The following steps were performed by the MGX Platform (Montpellier GenomiX, CNRS, Montpellier, France) using Illumina kits and devices (Illumina Inc.): construction of the mRNA library using a TruSeq Stranded mRNA Sample Preparation Kit; cluster generation with the cBot system using a Cluster Generation Kit; hybridization of the sequencing primer on the flow-cell; 50-bp single-read sequencing using a HiSeq 2000 device with SBS technology; informatic pretreatments, i.e., image analysis with the HiSeq Control Software and Real-Time Analysis component, base-calling with the RTA software and demultiplexing with CASAVA (Illumina). The RNAseq data are available from NCBI GEO datasets under the accession number GSE74030.

The quality scores across all bases of all reads and the N (non-attributed bases) content across all bases were determined for each condition with the FastQC software from the Babraham Institute[1]. Both analyses showed good quality values (data not shown). The reads were next aligned simultaneously on reference genomes, i.e., *L. garvieae* N201 (unpublished, GOLD project ID = Gp0034836 and NCBI BioProject ID = 184287) and *S. aureus* MW2 (RefSeq number = NC_003923.1), using the BWA package (Li and Durbin, 2009) with a seed of 32 bases and a maximum of two mismatches tolerated on the seed. Using the Samtools suite (Li et al., 2009) we excluded from further analyses those reads with low alignment quality scores (MAPQ index < 20). Reads which mapped on multiple sites (between 0.3 and 3.2% depending on the sample) and reads which did not map on any site considering the stringency applied (between 8.7 and 15.5% depending on the sample) were excluded from further analysis (see Supplementary Table 1). Reads overlapping genes were counted with the HTSeq Count software in Union mode (Anders et al., 2015). Differentially expressed genes were identified using the Bioconductor R[2] packages EdgeR, DESeq and DESeq2 (Anders and Huber, 2010; Robinson et al., 2010; Love et al., 2014). Genes with less than 15 reads (from the three biological replicates of two compared samples) were filtered and thus removed from the analysis. Data were normalized using the Relative Log Expression (RLE) normalization factor for EdgeR and the DESeq normalization factor for DESeq and DESeq2. Gene expression changes with adjusted *p*-value of less than 0.05 (by the FDR method from Benjamini–Hochberg) were declared differentially expressed. Differentially expressed genes highlighted by at least one of the three packages were considered for further investigation.

Genes were sorted into two lists: one of genes differentially expressed in both pure culture and co-culture and one of genes differentially expressed only in pure culture or only in co-culture. These lists were separately subjected to a

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

[2]http://www.R-project.org/

Blast2Go analysis (Conesa et al., 2005). Blast2Go analysis consisted first of a BlastX[3] on the nr database with 20 hits and a maximum *E*-value of $1.0E^{-15}$. The resulting data were enriched with an InterPro scan analysis (Zdobnov and Apweiler, 2001). Blast hits of each sequence were then mapped with Gene Ontology terms (Gene Ontology Consortium, 2008) annotated with a maximum *E*-value of $1.0E^{-6}$, a cut-off of 55 and a GO weight of 5. The number of genes involved in each Biological Process GO category was calculated from combined graphs with no filter and a score alpha of 0.6. Since more than 450 different biological processes were identified for each category of genes, we excluded biological processes involving less than nine genes. When several biological processes involved the same genes and were associated with comparable functions, we considered only the one most relevant to our scientific hypotheses.

For a deeper analysis of *S. aureus* direct effect on *L. garvieae* gene expression regardless of aeration level, data mining was undertaken using the regression functions in Microsoft excel. Expression values were systematically plotted for each pair of samples (co-culture versus pure culture), a standard residual value was determined from the regression analysis for each gene across multiple pair wise comparisons and the mean value was determined. This mean standard residual value was used to rank the genes and identify the strongest gene expression differences. A standard residual cut-off of 5 was used as the threshold for significance to account for false discovery using a Bonferroni correction and approximating the standard residual to an equivalent *p*-value for the number of comparisons.

Determination of Gene Expression by RT-qPCR

Total RNA was retro-transcribed using a High Capacity cDNA Reverse Transcription kit (Invitrogen, Life Technologies, Carlsbad, CA, USA) following the supplier's instructions. Ct of genes of interest (see Supplementary Table 2) were determined by RT-qPCR as described by Delpech et al. (2015). All primers were designed using PrimerExpress® software (Applied Biosystems®, Life Technologies). By comparison with the Ct of the *tufB* reference gene stable in our conditions (data not shown), expression of a gene of interest ("goi") was calculated using the formula introduced by Pfaffl (2001).

The influence of two experimental factors was studied: the presence of *S. aureus* (with high or low aeration, gene expression in co-culture divided by gene expression in pure culture) and aeration level (in presence or absence of *S. aureus*, gene expression in shaken condition divided by gene expression in static condition). Statistical analyses were performed using Statistica software (StatSoft, Inc., StatSoft France, Maisons-Alfort, France) by single-factor ANOVA followed by a Newman–Keuls *post hoc* test.

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

RESULTS

Effect of Enzyme-Treated Co-culture Supernatants on *S. aureus* Planktonic Growth

Since the inhibition of *S. aureus* may not be related solely to hydrogen peroxide, we looked for another possible anti-staphylococcal molecule produced by *L. garvieae* in the culture supernatants. We monitored *S. aureus* growth (OD_{600}) over 24 h in either non-inoculated BHI or in supernatants prepared from H_2O_2-free cultures (presence of catalase) with low aeration (either pure culture of *S. aureus* or co-culture of *L. garvieae* and *S. aureus*). In order to avoid any nutritional competition, BHI at a final concentration of 1X was added to each culture.

Growth of *S. aureus* in supernatant from *S. aureus* pure culture was comparable to that in plain BHI (data not shown). During the stationary phase, the OD_{600} measured in *S. aureus* cultures in supernatant from co-culture was lower than that in supernatant from *S. aureus* pure culture (see Data Sheet 1). This inhibition was still observed when these supernatants were treated with proteases, lipase or α-amylase, the main macromolecule-degrading enzymes. pH values remained between 6.9 and 7.1 in all cultures (data not shown).

Determination of Aeration and *S. aureus* Effects on *L. garvieae* Transcriptome by RNA Sequencing

To identify the best conditions for studying the transcriptome of *L. garvieae* N201 with respect to its capacity to inhibit *S. aureus* SA15, we followed the growth of the two bacteria in pure cultures and co-cultures in BHI for 24 h under high or low aeration conditions (**Table 1**).

The growth of *L. garvieae* was not affected by *S. aureus*. The growth of *S. aureus* was inhibited by *L. garvieae* at both aeration levels. With high aeration, maximal inhibition was observed from 9 to 24 h (difference with pure culture of 4 and 4.5 log CFU/ml, respectively, at 9 and 24 h). At 9 h, H_2O_2 concentration reached a peak concomitant with the lowest *S. aureus* concentration. With low aeration, inhibition was weaker than with high aeration and was observed later, at 24 h. Concomitantly, H_2O_2 was not detected in these cultures. pH values remained between 6.9 and 7.1 in all cultures (data not shown).

Considering these data, we analyzed the *L. garvieae* transcriptome in pure culture and in co-culture with *S. aureus* after 9 h of incubation with high and the low aerations. RNA sequencing generated a number of reads per sample, ranging from 12,365,133 to 15,670,131 depending on the sample after the initial quality filter (see Supplementary Table 1). Between 83.1 and 91.0% of these reads mapped correctly onto the reference genomes. Considering the direct effect of *S. aureus* on *L. garvieae* gene expression, analysis of the mapped reads using the Bioconductor R packages failed to identify expression differences (data not shown). Additional regression analyses highlighted 39 genes differentially

TABLE 1 | Cell counts and H_2O_2 concentration over 24 h in cultures of *Lactococcus garvieae* N201 and *Staphylococcus aureus* SA15 with high or low aeration levels.

Aeration level	Culture	*L. garvieae* cell concentration (log [CFU.mL^{-1}])					*S. aureus* cell concentration (log [CFU.mL^{-1}])					Hydrogen peroxide concentration (mM)				
		0 h	3 h	6 h	9 h	24 h	0 h	3 h	6 h	9 h	24 h	0 h	3 h	6 h	9 h	24 h
High	N201	6.6^{a}	7.3^{a}	8.4^{a}	9.0^{a}	8.6^{a}	NT	NT	NT	NT	NT	ND	NT	0.5^{a}	1.7^{a}	1.5^{a}
	SA15	NT	NT	NT	NT	NT	5.6^{a}	5.2^{ab}	6.1^{a}	7.4^{a}	9.0^{a}	ND	NT	ND	ND	ND
	N201 + SA15	6.5^{a}	7.4^{a}	8.5^{a}	8.6^{a}	8.7^{a}	5.6^{a}	4.8^{a}	3.9^{b}	3.4^{b}	4.5^{b}	ND	NT	0.5^{a}	1.6^{a}	1.5^{a}
Low	N201	6.6^{a}	7.4^{a}	8.4^{a}	8.7^{a}	8.9^{a}	NT	NT	NT	NT	NT	ND	NT	ND	ND	ND
	SA15	NT	NT	NT	NT	NT	5.6^{a}	6.1^{c}	7.0^{a}	7.3^{a}	8.0^{c}	ND	NT	ND	ND	ND
	N201 + SA15	6.5^{a}	7.3^{a}	8.8^{a}	8.8^{a}	8.9^{a}	5.6^{a}	5.6^{bc}	6.2^{a}	6.5^{a}	6.2^{d}	ND	NT	ND	ND	ND

In the same column, letters (a, b, and c) indicate homogeneous statistical groupings (p-value < 0.05 by Newman–Keuls method). ND, non-detectable values (below the spectrometry detection limit). NT, not tested.

expressed in presence of *S. aureus*, of which 4 genes under low aeration and 35 genes under high aeration conditions (see Supplementary Table 3). The expression of ∼18% of *L. garvieae* genes, i.e., 358 genes, differed between the two aeration levels (see Supplementary Table 4). Among these 358 genes, the expression of 181 genes differed regardless of the presence or absence of *S. aureus* (i.e., similarly in pure culture and in co-culture). The expression of 177 *L. garvieae* genes responded differently to different aeration levels, depending on the presence or absence of *S. aureus*: 88 gene expressions differed only in pure culture and 89 gene expressions differed only in co-culture.

Effect of Aeration Level on *L. garvieae* Biological Processes

After RNA sequencing data treatments, 22 biological processes (named according to Gene Ontology termes) related to *L. garvieae* genes differentially expressed depending on aeration level in both pure culture and co-culture were identified using Blast2Go (**Figure 1**). Changes in gene expression are shown in Supplementary Table 4.

Most of the biological processes affected were related to fundamental growth functions (**Figures 1A,B**) and nutrition (**Figure 1C**). With high aeration, two genes (*ilvA*, LCGN_1922) related to "threonine metabolism" were repressed while two genes (LCGN_1919, LCGN_1920) were over-expressed.

Fourteen genes related to "transport" processes were differently expressed depending on aeration level both in pure culture and co-culture, including genes involved in the transport of metals (lead, cadmium, zinc, copper and/or mercury) and vitamins (riboflavin and folate).

Genes and GO biological processes related to O_2 and H_2O_2 metabolism were also affected. "Oxidation-reduction process" and "response to stress" biological processes involved more repressed genes (17 and 8, respectively) than over-expressed genes (6 and 1, respectively). Three genes related to H_2O_2 metabolism (*ahpF*, *pox,* and *spxA1*) and one gene related to O_2 consumption (*lox*) were repressed with the high aeration level. Several stress response genes (*hrcA*, *groES*, *groEL*, *dnaK*, *dnaJ*, *grpE*, *clpB,* and five genes belonging to the universal stress protein family) were repressed. Genes related to peroxide resistance (*ohrA* and *ohrR*) were strongly over-expressed under high aeration conditions. Electron Transport Chain (ETC) genes (*cydB*, *menH,* and *ubiE*) were over-expressed.

Modulation by *S. aureus* of *L. garvieae* Response to Aeration

We identified 27 Gene Ontology biological processes related to genes differentially expressed depending on aeration level either in pure culture or in co-culture (**Figure 2**).

Changes in gene expressions are shown in Supplementary Table 4. Most of the biological processes affected were related to fundamental growth functions (**Figures 2A,B**) or nutrition (**Figure 2C**). Under high aeration conditions, four lysine metabolism genes (LCGN_0575, LCGN_0576, LCGN_0577, LCGN_0578) and two threonine metabolism genes (LCGN_0576 and LCGN_0577) were over-expressed exclusively in pure culture while four galactose metabolism genes were over-expressed exclusively in co-culture (LCGN_1809, LCGN_1810, LCGN_1811, and LCGN_1812). Eight genes involved in pyruvate and carbohydrate metabolism and the citrate cycle (*glmS*, *glmM, galE*, PTS-Man-EIIC and EIID *pdhA, pdhB*, DLAT) were repressed by *S. aureus*. With low aeration, the enolase encoding gene was repressed by *S. aureus.*

Four biological processes related to transport functions were affected (**Figure 2D**): "transport," "transmembrane transport," "organic substance transport" and "ion transport." In pure culture, genes involved in the transport of cobalt/zinc/cadmium (LCGN_1867) and an unspecified monosaccharide (LCGN_0332) were over-expressed with high aeration while a gene involved in copper transport (LCGN_1427) was repressed. Two genes of the *fur* regulon (ferrous iron transport) were over-expressed with high aeration, one of them in pure culture (*feoA*) and one in co-culture (*feoB*).

With low aeration, the H_2O_2 synthesis gene *sodA* was up-regulated by *S. aureus.* The H_2O_2 degradation gene *ahpC* was repressed by high aeration in co-culture but not in pure culture. In pure culture, two genes related to O_2 consumption, noxE and LCGN_0208, were repressed and six genes related to ETC, *cydA*, *cydC, menB*, *menC*, *menD,* and LCGN_0364, were over-expressed.

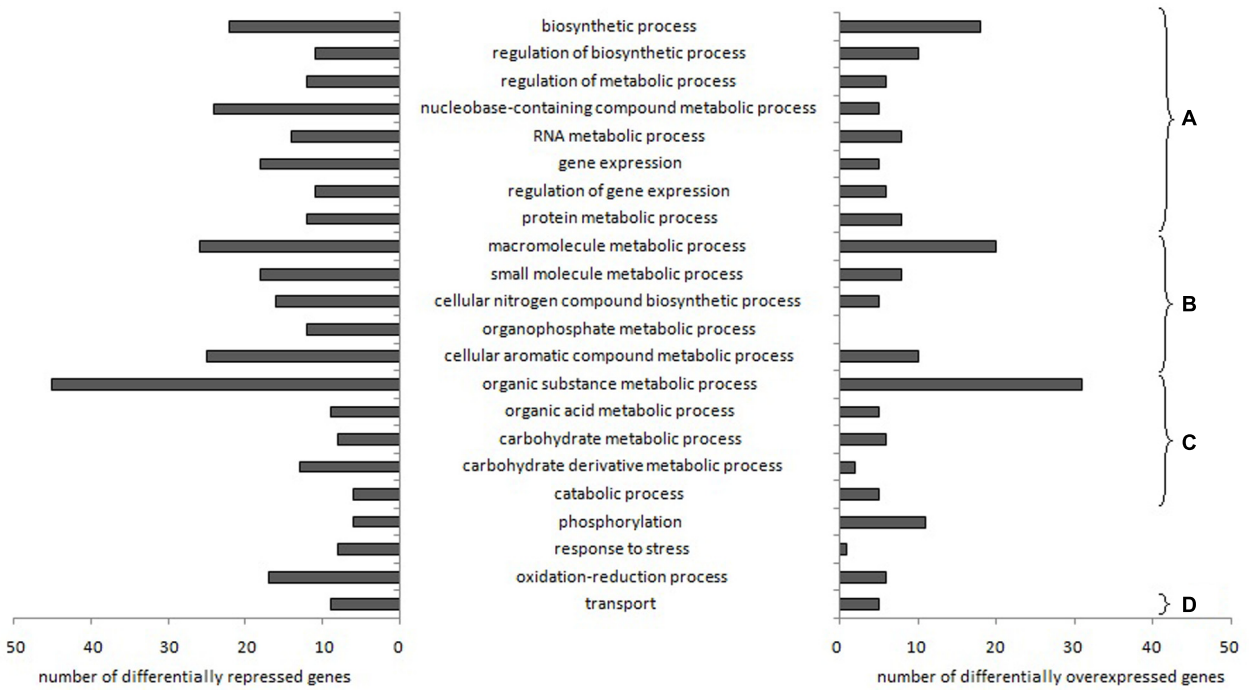

FIGURE 1 | *Lactococcus garvieae* biological processes involving genes differentially expressed depending on aeration level in both pure culture and co-culture. Bars represent the number of genes significantly overexpressed (left) or repressed (right) with high aeration compared to low aeration, according to at least one of the three R packages used (EdgeR, DESeq, and DESeq2). After the Blast2Go analysis and data filtering, biological processes were manually sorted into several categories: **(A)** processes related to fundamental growth function, i.e., global cellular, metabolic and biosynthetic processes, (post-)transcriptional and (post-)translational functions, **(B)** processes potentially associated with fundamental growth functions but also potentially associated with other metabolisms, **(C)** nutrition-related processes and **(D)** transport processes.

Impact of Aeration on the Expression of *L. garvieae* Genes Potentially Involved in the Antagonism against *S. aureus*

To complete the RNA-seq data obtained only at 9 h, we determined the expression of ten genes from the cultures previously used for RNA-seq analyses, at 6, 9, and 24 h by RT-qPCR. These genes, potentially involved in the antagonism mechanisms, were involved in O_2 consumption (*noxE* and *lox*), H_2O_2 synthesis (*pox* and *sodA*), H_2O_2 degradation (*ahpC*, *ahpF*, *gpx,* and *trxB1*) and resistance to other peroxides (*ohrA* and *ohrR*).

The presence of *S. aureus* induced only slight changes in their expression (data not shown). With high aeration, *ahpC* and *pox* were over-expressed 2.4-fold and 2.0-fold in the presence of *S. aureus* at 6 h. With low aeration, *S. aureus* induced a 3.1-fold over-expression of *trxB1* at 9 h and a 3.3-fold repression of *gpx* at 24 h.

Conversely, RT-qPCR results showed major differences in H_2O_2-metabolism gene expression between the two aeration levels (**Table 2**). The expression of *ohrA* was strongly induced under high aeration conditions in both pure culture and co-culture at 9 h. While RNA sequencing identified *trxA2* as up-regulated with high aeration in both pure culture and co-culture at 9 h (Supplementary Table 4), RT-qPCR identified other H_2O_2-degradation genes (*ahpC*, *ahpF*, *gpx,* and *trxB1*) as repressed at 6, 9, or 24 h. The lactate mono-oxygenase gene *lox* seemed slightly repressed with high aeration but this modification was significant only at 9 h in co-culture. In pure culture with high aeration, the expression of *noxE* reached a peak at 6 h when it was 11.0 times higher than with low aeration. With low aeration, *noxE* expression gradually increased over time. No significant difference in the expression of H_2O_2-synthesis genes (*pox* and *sodA*) according to aeration level was observed.

DISCUSSION

This study aimed to improve understanding of the mechanisms underlying the antagonism of *L. garvieae* against *S. aureus*, especially as regards H_2O_2-related pathways.

The high aeration level and the resulting high H_2O_2 concentration were associated with stronger inhibition of *S. aureus*, confirming previous observations under the same conditions (Alomar et al., 2008a; Delbes-Paus et al., 2010; Delpech et al., 2015). It was already known that this inhibition is associated with a modulation of the expression of *S. aureus* virulence genes and a repression of the H_2O_2 response, stress response and cell division genes of *S. aureus* by *L. garvieae* and with high aeration (Delpech et al., 2015). However, the transcriptome adaptation of *L. garvieae* to prevailing aeration conditions during this interaction had not been explored until

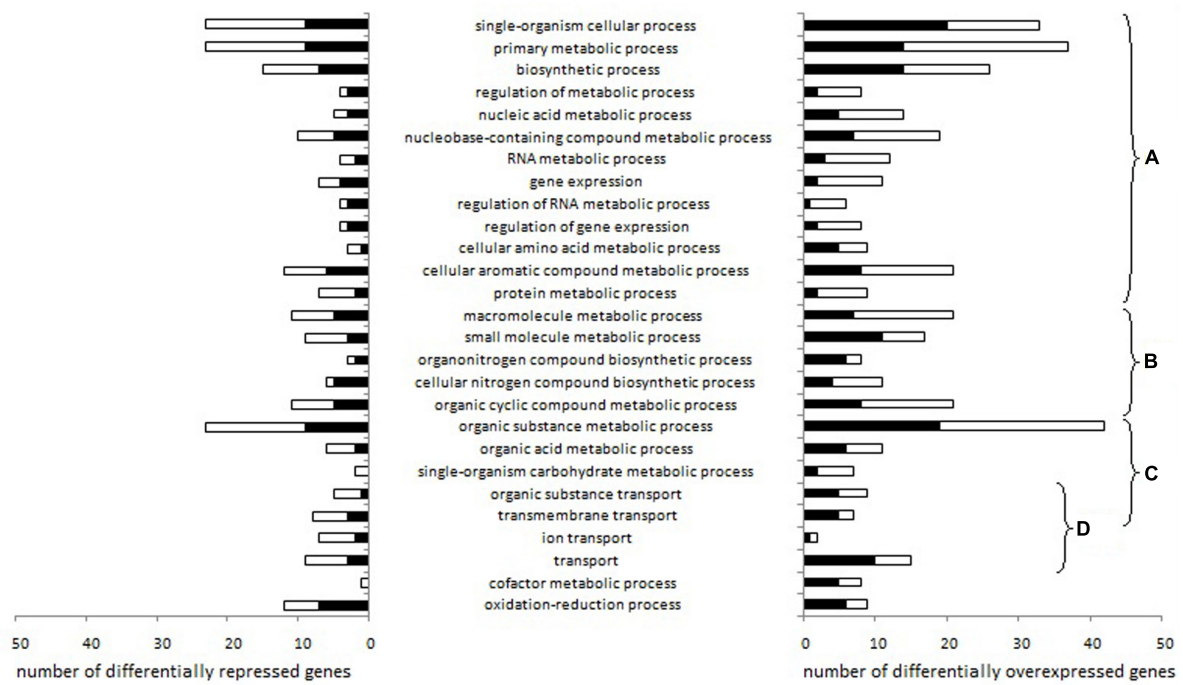

FIGURE 2 | *Lactococcus garvieae* biological processes involving genes differentially expressed depending on aeration level exclusively in pure culture or exclusively in co-culture. Stacked bars represent the number of genes significantly overexpressed (left) or repressed (right) with high aeration compared to low aeration in pure culture only (black bars) or in co-culture only (white bars), according to at least one of the three R packages used (EdgeR, DESeq, and DESeq2). After the Blast2Go analysis and data filtering, biological processes were manually sorted into several categories: **(A)** processes related to fundamental growth function, i.e., global cellular, metabolic and biosynthetic processes, (post-)transcriptional and (post-)translational functions, **(B)** processes potentially associated with fundamental growth functions but also potentially associated with other metabolisms, **(C)** nutrition-related processes and **(D)** transport processes.

this study. It is known that difference in aeration is associated with drastic modifications of the *L. lactis* transcriptome (Pedersen et al., 2008; Dijkstra et al., 2014) and consequent metabolic adaptations (Jensen et al., 2001; Nordkvist et al., 2003; Pedersen et al., 2008; Dijkstra et al., 2014; Larsen et al., 2016a,b). In accordance with these findings, we found that different aeration levels were associated with significant differences in the *L. garvieae* transcriptome in biological processes related to fundamental growth functions, nutritional and metabolic adaptations and transport functions. Moreover, *L. garvieae* genes involved in fundamental growth functions were slightly repressed by *S. aureus* under both aeration levels suggesting an impact of *S. aureus* on *L. garvieae* metabolism. While *L. garvieae* is a catalase-negative bacterium, the main *S. aureus* enzyme involved in H_2O_2 dismutation is the O_2-forming catalase KatA (Cosgrove et al., 2007). The *S. aureus* catalase may affect the transcriptomic response of *L. garvieae* to different aeration conditions by modulating O_2 and H_2O_2 concentrations. It is known that the *katA* gene of *S. aureus* SA15 is repressed by *L. garvieae* in high aeration conditions but not in low aeration conditions (Delpech

TABLE 2 | Effect of high aeration level on the expression of *L. garvieae* genes in pure culture and co-culture, as determined by RT-qPCR.

		In pure culture[a]			In co-culture[a]		
	Gene	**6 h**	**9 h**	**24 h**	**6 h**	**9 h**	**24 h**
Degradation of H_2O_2	*ahpC*		0.6*		0.6*	0.4**	
	ahpF		0.3**	NT		0.4*	NT
	gpx			0.2*			0.4*
	trxB1			0.2**			0.2**
Degradation of other peroxides	*ohrA*		16.7**	NT	7.0*	20.2*	NT
O_2 consumption/H_2O synthesis	*noxE*	11.0**	0.4*	0.1*			
	lox			NT		0.4**	NT

[a]*Indicated values, at 6, 9, or 24 h in pure culture or in co-culture, corresponds to the ratios of gene expressions with high aeration to gene expressions with low aeration. Only ratios that are significant according to the Newman–Keuls test are shown (*p-value < 0.1, **p-value < 0.05). NT, not tested.*

et al., 2015). This may explain why the presence of *S. aureus* (*via* its catalase production) modulated the *L. garvieae* transcriptome response under different aeration conditions. Indeed, with low aeration, the *L. garvieae* H_2O_2 synthesis gene *sodA* expression was slightly higher in presence of *S. aureus*. With high aeration, the main enzymes involved in O_2 consumption of *L. lactis* (NADH-oxidase NoxE and ETC enzymes (Tachon et al., 2010), were repressed only in *L. garvieae* pure culture. The expression of the *L. garvieae noxE* reached a peak at 6 h (beginning of exponential growth) and then decreased until 24 h. This suggests that *L. garvieae* NoxE is the main enzyme responsible for O_2 consumption at the beginning of exponential growth with high aeration, as observed for *L. lactis* NoxE (Lopez de Felipe and Hugenholtz, 2001; Tachon et al., 2010). The induction of most of the *L. garvieae* ETC genes (*cydA*, *cydB*, *cydC*, *menB*, *menC*, *menD*, *menH,* and LCGN_0364) under high aeration conditions at 9 h (beginning of stationary phase) suggested that ETC may be involved in O_2 consumption by *L. garvieae* during the stationary phase, as already shown with *L. lactis* (Tachon et al., 2009, 2010).

Previous studies have showed that hydrogen peroxide production by *L. garvieae* depends on aeration level and plays a key role in *S. aureus* inhibition. As regards *S. aureus*, Delpech et al. (2015) suggested that the stronger inhibition with a high aeration level may be caused by the higher concentration of H_2O_2 associated with the *L. garvieae*-induced repression of *S. aureus* genes involved in the H_2O_2 response (*katA* and *sodA* at 6 and 9 h) and cell division (*mraZ*, *mraW* and potentially the *dcw* cluster). As regards *L. garvieae*, our RNA-sequencing and RT-qPCR analyses showed an overexpression of the H_2O_2-degradation genes *ahpC*, *ahpF*, *gpx,* and *trxB1* under low aeration conditions compared to high aeration, while the expression of H_2O_2 synthesis genes *pox* and *sodA* remained stable. The expression of the main H_2O_2 degradation genes of Gram-positive bacteria is generally induced more under aeration and in the presence of H_2O_2 (Mostertz et al., 2004; Pedersen et al., 2008). Also, H_2O_2 metabolism may be very different in *L. garvieae* compared to other Gram-positive bacteria. Although the difference in H_2O_2 concentration between the two aeration levels was probably primarily conditioned by the availability of O_2, our transcriptome results suggest that it was also associated with a control of H_2O_2 degradation by *L. garvieae* rather than with a control of H_2O_2 synthesis. Since the AhpCF peroxy-redoxin system was repressed, the OhrAR system was probably essential for the resistance of *L. garvieae* to ROS (other than H_2O_2) under the high aeration conditions. The widespread organic hydroperoxide detoxifying system *ohrAR*, known to be over-expressed under high O_2 conditions (Mongkolsuk et al., 1998; Fuangthong et al., 2001; Chuchue et al., 2006; Oh et al., 2007; Atichartpongkul et al., 2010; da Silva Neto et al., 2012; Clair et al., 2013), was consistently induced in *L. garvieae* at the high aeration level.

The fact that *S. aureus* population levels were lower in the stationary phase in H_2O_2-free supernatant from a co-culture of *S. aureus* and *L. garvieae* revealed the presence of a new molecule involved in this inhibition. This effector is extracellular, is produced by *L. garvieae* during its exponential growth phase and can reduce the population level of *S. aureus* during its stationary growth phase. In view of the results of our enzymatic treatments on supernatants, the inhibitory gap during the stationary phase was probably caused neither by hydrogen peroxide, nor by a protein, nor by a lipid, nor by a polysaccharide. The only putative bacteriocin identified in the *L. garvieae* N201 genome was homologous to garvieaecin Q (GarQ, data not shown), a class IId bacteriocin (Tosukhowong et al., 2012). Class IId bacteriocins are generally sensitive to protease treatments as stringent as the one we used in this study (Kuo et al., 2013; Song et al., 2014), suggesting that garvieacin Q is unlikely to be the effector we are seeking. This effector may instead be related to several genes identified by RNA seq as being regulated by aeration level, such as genes involved in metal homeostasis (e.g., siderophores (Hannauer et al., 2015), chemical and ionic equilibrium (Doyle et al., 1975; Chudobova et al., 2015), transport of vitamin-related compounds (Schlievert et al., 2013) and export of unknown proteins. For example, the differential expression of ferrous ion transport encoding genes has already been observed in *L. lactis* under oxidative stress in milk (Larsen et al., 2016b). It may also be related to signaling molecules (stress, quorum sensing). It is known that *L. garvieae* can modify the expression of several *S. aureus* genes involved in environment-sensing systems like the *agr* system, CodY or two-component systems SaeRS and SrrAB (Delpech et al., 2015).

RNA-seq revealed variations in the expression of the *codY* gene (involved in nutritional adaptation (Guédon et al., 2001; Ercan et al., 2015), and of several nutritional-related metabolisms (lysine, threonine, mannitol, aspartate, ribose, fructose, and galactose). This suggests that *L. garvieae* adapts its nutritional behavior to the prevailing aeration level and the presence or absence of *S. aureus*. In a rich medium like BHI, there should be little nutritional competition. It is known that *L. garvieae* can consume all the L-threonine in micro-filtered milk in less than 3 h (Alomar et al., 2008b) and that *S. aureus* growth could be inhibited by L-threonine depletion (Pohl et al., 2009). However, we showed that the antagonism of *L. garvieae* against *S. aureus* was not associated with nutritional competition for L-threonine in micro-filtered milk (see Supplementary Table 5).

CONCLUSION

RNA sequencing analyses revealed a *L. garvieae* transcriptome adaptation to aeration level. This adaptation differed depending on the presence or absence of *S. aureus*. Our findings show that the control of autogenic H_2O_2 levels by *L. garvieae* was probably carried out by H_2O_2 degradation genes rather than H_2O_2 synthesis genes. Our study also leads us to suggest that an unidentified effector was involved in the inhibition of *S. aureus* in the stationary phase. The potential inhibitory role of metals, siderophores and signal molecules (e.g., stress signal, quorum sensing) generated by *L. garvieae* should be investigated. In order to promote the use of H_2O_2-producing bacteria as bio-preservation agents, future studies should explore the relation between H_2O_2-metabolism, H_2O_2-producing LAB and the pathogen they inhibit.

AUTHOR CONTRIBUTIONS

PD carried out all the experiments, excluding preparation of cDNA libraries and RNA sequencing, and drafted the manuscript helped by CD and SB. ER, ED, and GB analyzed RNA sequencing data. SN performed the RNA sequencing (preparation of libraries and the sequencing itself). GG, M-CM, CD and SB conceived the study. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00359/full#supplementary-material

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Pentocin MQ1: A Novel, Broad-Spectrum, Pore-Forming Bacteriocin From *Lactobacillus pentosus* CS2 With Quorum Sensing Regulatory Mechanism and Biopreservative Potential

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Micrococcus luteus, *Listeria monocytogenes,* and *Bacillus cereus* are major food-borne pathogenic and spoilage bacteria. Emergence of antibiotic resistance and consumer demand for foods containing less of chemical preservatives led to a search for natural antimicrobials. A study aimed at characterizing, investigating the mechanism of action and regulation of biosynthesis and evaluating the biopreservative potential of pentocin from *Lactobacillus pentosus* CS2 was conducted. Pentocin MQ1 is a novel bacteriocin isolated from *L. pentosus* CS2 of coconut shake origin. The purification strategy involved adsorption-desorption of bacteriocin followed by RP-HPLC. It has a molecular weight of 2110.672 Da as determined by MALDI-TOF mass spectrometry and a molar extinction value of 298.82 M^{-1} cm^{-1}. Pentocin MQ1 is not plasmid-borne and its biosynthesis is regulated by a quorum sensing mechanism. It has a broad spectrum of antibacterial activity, exhibited high chemical, thermal and pH stability but proved sensitive to proteolytic enzymes. It is potent against *M. luteus*, *B. cereus,* and *L. monocytogenes* at micromolar concentrations. It is quick-acting and exhibited a bactericidal mode of action against its targets. Target killing was mediated by pore formation. We report for the first time membrane permeabilization as a mechanism of action of the pentocin from the study against Gram-positive bacteria. Pentocin MQ1 is a cell wall-associated bacteriocin. Application of pentocin MQ1 improved the microbiological quality and extended the shelf life of fresh banana. This is the first report on the biopreservation of banana using bacteriocin. These findings place pentocin MQ1 as a potential biopreservative for further evaluation in food and medical applications.

Keywords: bacteriocin, cell-wall associated bacteriocin, quorum sensing, *Lactobacillus pentosus*, bactericidal, pore formation, broad-spectrum bacteriocin, biopreservation

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INTRODUCTION

Consumer's requisition for food products containing less chemical preservatives (Barbosa et al., 2017) and emergence of antibiotic resistance among pathogenic and food spoilage bacteria prompted the search for novel antimicrobials (Berendonk et al., 2015; Jia et al., 2017). Bacteriocins are an attractive class of natural antimicrobials with potential for future use as a synergistic

combination with antibiotics or complete replacement of antibiotics (Behrens et al., 2017; Collins et al., 2017) and currently used chemical preservatives (Kaškoniene et al., 2017; Wiernasz et al., 2017). This is due to their ability to inhibit some drug-resistant pathogens (Mathur et al., 2017). These interesting antimicrobial peptides are of bacterial origin and are ribosomally synthesized (Langa et al., 2017). They have narrow or broad spectrum of inhibitory activity (Hanchi et al., 2017).

Bacteriocins are a highly diverse group of antimicrobial peptides. They have variations in molecular weight, inhibitory spectrum, mode of action, mechanism of biosynthesis and externalization, and self-protection mechanism (Salazar et al., 2017). They are part of the inherent defense system of bacteria and play other roles such as niche colonization, direct killing of competing strains and signaling (cross-talk and quorum sensing) within bacterial communities (Dobson et al., 2012; Inglis et al., 2013; Yang et al., 2014). Bacteriocins are commonly classified into two groups namely class I (undergo post-translational modification) and class II (unmodified). In less popular classification schemes class III (high molecular weight and heat-sensitive bacteriocins) (Alvarez-Sieiro et al., 2016) and class IV (bacteriocins with carbohydrate or lipid moieties) (Kaškoniene et al., 2017) were introduced. Bacteriocins are produced by lactic acid bacteria (LAB) and non-lactic acid bacteria (Mechoud et al., 2017). LAB bacteriocins are given more attention because they are generally recognized as safe (GRAS) facilitating their use *in situ* and *ex situ* in preservation of foods (Bali et al., 2016; Castro et al., 2017; Hu et al., 2017). Moreover, they are inactivated by gut proteases, heat-stable, active at various pH, potent even at nanomolar concentration and their biosynthetic gene cluster is often plasmid-borne, facilitating the use genetic engineering approaches in improving production (Cotter et al., 2013; Lakshminarayanan et al., 2013; Messaoudi et al., 2013; Woraprayote et al., 2016).

Biopreservation involves the use of microorganisms or their products and other natural bio-products to enhance safety and extend shelf life of food. This is achieved either by killing or reduction of the load of food spoilage microorganisms (Johnson et al., 2017; Saraoui et al., 2017). The concept of biopreservation of food has recently intensified due to growing consumer inclination toward foods containing biopreservatives or less synthetic chemical preservatives, fear of side effects of currently used chemical preservatives, demand for fresh-tasting and less processed food (Kashani et al., 2012; Woraprayote et al., 2016; Barbosa et al., 2017). Fruits and vegetables are one the major reservoirs of minerals, vitamins, and fiber and are consumed worldwide. Fresh fruits such as banana have short shelf life due to their high moisture content (Joardder et al., 2014). Preservation of banana is a huge task especially if required fresh. Moreover, eating them fresh exposes consumers to food-borne pathogens (Berger et al., 2010; Tian et al., 2012).

Abbreviations: CFS, Cell-free supernatant; FU, Fluorescence unit; HIC, Hydrophobic interaction chromatography; LAB, Lactic acid bacteria; MALDI-TOF, Matrix-assisted laser desorption ionization time-of-flight; MIC, Minimum inhibitory concentration; MRS, De Man, Rogosa and Sharpe; RP-HPLC, Reversed-phase high performance liquid chromatography; SDS, Sodium dodecyl sulfate.

It has been suggested that live cultures of LAB can be used to prevent the growth of food spoilage and pathogenic bacteria on the surfaces of fruits and vegetables (Trias et al., 2008). *In situ* biopreservation of foods has not been quite successful due to two main reasons. Firstly, difficulty in getting the LAB strain adapted and established to the new environment. Secondly, difficulty in the production of an effective concentration of bacteriocin required to control spoilage and food-borne pathogens. However, a few breakthroughs have been reported. *Leuconostoc mesenteroides* was effective at inhibiting the growth of *Listeria monocytogenes* on fresh apples and lettuce (Trias et al., 2008). The Safety and shelf life of minimally processed lettuce and apples were improved by the inoculation of two strains of *Lactobacillus plantarum* (Siroli et al., 2015). A bacteriocinogenic strain of *Lactococcus lactis* inhibited the growth of yeasts and *L. monocytogenes* in minimally processed apples. Furthermore, extension of shelf life was observed (Siroli et al., 2016). Growth of *L. monocytogenes* and Salmonella on minimally processed pear was controlled by the inoculation of *Lactobacillus rhamnosus* GG (Iglesias et al., 2017). Addition of *L. rhamnosus* GG to fresh pear was an effective strategy in controlling the growth of *L. monocytogenes* (Iglesias et al., 2018).

Bacteriocin was first discovered in early 1925 when antagonistic activity was observed among strains of *Escherichia coli* (Ghazaryan et al., 2014). The discovery of colicin as the first bacteriocin was closely followed by that of nisin (1928), the first LAB bacteriocin (Shin et al., 2016). Despite the long history of LAB bacteriocins only nisin and pediocin PA-1/AcH have gained approval for preservation of selected foods (Saraniya and Jeevaratnam, 2014; Barbosa et al., 2017). The potential of bacteriocins in the biopreservation of fresh fruits or minimally processed fruits has been highly underexploited. Combined application of nisin-EDTA and chlorine was effective at reducing the surface microbial load of whole melon (Ukuku and Fett, 2002). Application of nisin, hydrogen peroxide, citric acid and sodium lactate effectively reduced the transfer of pathogens from the surface of melons to freshly cut pieces (Ukuku et al., 2005). Load of pathogens on the surfaces of minimally processed mangoes was controlled by packaging in nisin films (Barbosa et al., 2013). Enterocin AS-48 was effective at controlling contamination of raw fruits by *L. monocytogenes* (Molinos et al., 2008). Enterocin 416K1 inhibited the growth of *L. monocytogenes* on apples and grapes (Anacarso et al., 2011). The potential of preserving minimally processed papaya by applying alginate coatings containing pediocin has been demonstrated (Narsaiah et al., 2015). Biopreservation of fresh banana using bacteriocin has not been investigated.

Although *Lactobacillus pentosus* has been isolated from various sources, bacteriocinogenic strains are rare (Liu et al., 2008). A bacteriocin-producing strain of *L. pentosus* with probiotic potential has been reported (Aarti et al., 2016). Bacteriocins of *L. pentosus* origin have not been adequately studied. Pentocins have been poorly characterized and their regulatory mechanisms have not been sufficiently investigated. Their modes of action are unknown. Moreover biopreservation of fresh banana using *L. pentosus*-derived bacteriocins (commonly called pentocins) has not been studied. In this

study a novel bacteriocin (pentocin MQ1) from *L. pentosus* CS2 of coconut shake origin was purified to homogeneity and characterized. Its regulatory mechanism and mode of action was investigated. Finally, its ability to preserve fresh banana was studied *in-vivo*.

MATERIALS AND METHODS

Bacterial Strains and Culture Media

Streptococcus pyogenes, *Enterococci*, *Bacillus cereus*, *Micrococcus luteus,* and *L. lactis* were obtained from American Type Culture Collection (ATCC). *L. monocytogenes* NCTC 10890 was obtained from National Collection of Type Culture (NCTC). *Staphylococcus aureus* RF122, *Streptococcus mutans* GEJ11, *Pseudomonas aeruginosa* PA7, *Corynebacterium* spp. GH17, *E. coli* UT181, *L. plantarum* K25, and *L. pentosus* CS2 were taken from the culture collection of Microbial Biotechnology Laboratory, Division of Microbiology, Institute of Biological Science, Faculty of Science, University of Malaya, Kuala Lumpur, Malaysia. *L. plantarum* K25 and *L. pentosus* CS2 were maintained on MRS agar (Merck, Darmstadt, Germany). *S. pyogenes* ATCC 12344 and *S. mutans* GEJ11 were maintained on Todd-Hewitt agar (Difco, Le Pont de Claix, France). *M. luteus* ATCC 10240, *B. cereus* ATCC 14579, *S. aureus* RF122, *P. aeruginosa* PA7, *Corynebacterium* spp. GH17 and *E. coli* UT181 were maintained on Mueller-Hinton agar (Merck, Darmstadt, Germany). *Enterococcus faecium* ATCC BAA-2127 and *E. faecium* ATCC 349 were maintained on Tryptic soy agar (Merck, Darmstadt, Germany) while other enterococcal strains and *L. monocytogenes* NCTC 10890 were maintained on Brain-heart infusion agar (Merck, Darmstadt, Germany). *L. lactis* ATCC 11454 was maintained on M17 agar (Merck, Darmstadt, Germany) supplemented with 5% glucose (Merck, Darmstadt, Germany).

Isolation and Screening of LAB for Bacteriocin Production

Indigenously sourced coconut shake was inoculated into freshly prepared De man Rogose and Sharpe (MRS) broth and incubated at 37°C for 24 h. The culture was serially diluted in peptone water and LAB was isolated by growing on MRS agar plate (Merck Germany) at 37°C. MRS broth was inoculated with single colonies from a 24 h old MRS agar LAB culture and incubated aerobically at 37°C for 24 h. Screening of LAB for bacteriocin production was carried out using well diffusion assay in which cell-free supernatant (CFS) was tested for inhibitory activity against *M. luteus* ATCC 10240, *L. monocytogenes* NCTC 10890, *B. cereus* ATCC 14579, and *S. aureus* RF122. MRS agar used for well diffusion assay was supplemented with 0.1% $CaCO_3$ (Friedemann Schmidt Chemical, Germany) to neutralize acidity due to organic acids.

Molecular identification of LAB was conducted by amplifying 16S rRNA gene via PCR using the universal primers 27F [5′-AGAGTTTGATC(A/C)TGGCTCAG-3′] and 1492R [5′-ACGG(C/T)TACCTTGTTACGACTT-3′]. The 16S rRNA gene was sequenced and similarity search was performed using NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

Purification, Determination of Molecular Weight, and Molar Extinction Coefficient

Bacteriocin was purified using adsorption-desorption approach followed by reversed-phase high performance liquid chromatography (RP-HPLC). A 24-h old culture of *L. pentosus* CS2 was subcultured in freshly prepared MRS broth in a bioreactor (Sartorius Stedim, Germany). The Bioreactor was set up (agitation at 150 rpm, temperature at 37°C) and run for 20 h after which the culture was collected and the pH was adjusted to 5.8 and allowed for 1 h. The culture was centrifuged (9,000 × g, for 20 min at 4°C) and the cell pellet re-suspended in 95% methanol (Merck, Darmstadt, Germany) with pH-value adjusted to 2. The cell suspension was stirred overnight at 4°C and subsequently centrifuged (9,000 × g for 30 min at 4°C) to obtain the supernatant which was filtered using 0.22 μm sterilized cellulose membrane (Millipore). The clear supernatant was evaporated to dryness at 40°C using a water bath and the crude bacteriocin was reconstituted in ultrapure water. Inhibitory activity was tested using well diffusion assay and crude bacteriocin was subjected to RP-HPLC containing SemiPrep RP-18e 100-10 mm column. The mobile phase consisted of two solvents: A (95% Mili-Q water Millipore, USA) and 5% acetonitrile (Merck, Germany) and B (100% acetonitrile). Elution was done using a biphasic gradient of 20–80% acetonitrile at a flow rate of 1 ml/min over 65 min. Fractions were collected and evaporated using a vacuum evaporator. Antibacterial activity of HPLC fractions were was tested. Molecular weight of the bacteriocin was determined by subjecting the active HPLC fraction to MALDI-TOF mass spectrometry. To ascertain the molar extinction coefficient, 2-fold dilutions of the bacteriocin were prepared and bacteriocin concentration was expressed in molar units. Absorbance at 280 nm was measured and a standard curve was generated from which the molar extinction coefficient was determined.

Antibacterial Spectrum

This experiment was done to ascertain the inhibitory spectrum of pentocin MQ1. Bacteriocin producer was grown in MRS broth for 20 h and CFS (400 AU/ml) was used in well diffusion assay to test antibacterial activity against selected targets. All agar plates were supplemented with 0.1% $CaCO_3$ (Friedemann Schmidt Chemical, Germany) to neutralize acidity.

Bacteriocin-Cell Wall Association Assay

An investigation was done to assess the association of pentocin MQ1 with the cell wall of its producer. Overnight broth culture of bacteriocin producer was centrifuged (9,000 × g for 20 min) at 4°C. Antibacterial activity of CFS was tested using well diffusion assay. The cell pellet was re-suspended in 95% methanol (Merck, Darmstadt, Germany) adjusted to pH = 2 and stirred overnight at 4°C on a magnetic stirrer. The cell suspension was centrifuged (9,000 × g for 30 min at 4°C) and the supernatant was filtered using a Millipore filter (0.22 μm). This was followed by evaporation of methanol on a water bath at 40°C. The cell extract was reconstituted in ultrapure water and antibacterial activity was tested.

Bacteriocin Stability Test

In order to ascertain the stability of pentocin MQ1, bacteriocin preparation was exposed to different temperatures: 40, 60, and 80°C for 40 min; 100 and 121°C for 15 min. Samples were cooled to room temperature before testing antibacterial activity. Stability of bacteriocin to different enzymes (Sigma-Aldrich, St. Louis, USA) namely: proteinase K, lysozyme, pepsin, lyticase, catalase, trypsin, α-chymotrypsin, protease, proteinase, and hyaluronidase was tested. This was achieved by adding different enzyme preparations to a final enzyme concentration of 1 mg/ml and incubating for 1 h at 37°C. Inhibitory activity was tested afterwards. Bacteriocin was adjusted to various pH (2, 3, 5, 8, and 10) and incubated for 2 h at room temperature. Antibacterial activity was tested. Stability of bacteriocin upon exposure to different chemicals viz: 1% (v/v) Tween 80, 1% (v/v) Tween 20, 1% (w/v) sodium dodecyl sulfate (SDS) (Fisher scientific, New Jersey, USA) and 1% (v/v) triton X-100 was investigated. These chemicals were added to the bacteriocin and incubated for 2 h at room temperature after which antibacterial activity was tested.

Plasmid Isolation

To investigate if plasmids harbor the bacteriocin structural gene or not, plasmid isolation was carried out. This was done using easy pure® plasmid miniprep kit (TransGen Biotech, Beijing) according to manufacturer's instruction.

Regulatory Mechanism

This experiment was done to understand the regulatory system of pentocin MQ1 production. The bacteriocin was semi-purified using ammonium sulfate precipitation. This was achieved by the following procedure. An overnight MRS broth culture of the bacteriocin-producing phenotype of *L. pentosus* CS2 was centrifuged (9,000 × g, for 20 min at 4°C). The supernatant was collected and filtered using 0.22 μm sterilized cellulose membrane (Millipore) to obtain CFS. The CFS was subjected to 80% ammonium sulfate precipitation after which it was centrifuged at the same condition to obtain the precipitate. The resulting active ammonium sulfate precipitate (semi-purified bacteriocin) was dissolved in minimum ultrapure water. Another semi-purified form of the bacteriocin was obtained through hydrophobic interaction chromatography (HIC) by the following method. Active CFS from an overnight culture of the bacteriocin-producing phenotype of *L. pentosus* CS2 was subjected to HIC. Acetonitrile (Merck, Darmstadt, Germany) gradient (20, 40, 60, and 80% v/v) was used for elution of the bacteriocin adsorbed onto the surfaces of amberlite XAD-16 particles (Sigma-Aldrich, St. Louis, USA) packed in a glass column. Fractions were evaporated and antibacterial activity was determined using well diffusion assay. The active fraction from HIC was used in the next experiment. In order to investigate the regulatory mechanism of pentocin MQ1 production a bacteriocin-negative (bac^-) phenotype was generated using the following procedure. Ten milliliters (10 ml) of fresh MRS broth was inoculated with colonies from an overnight culture of the bacteriocin-producing phenotype of *L. pentosus* CS2 and incubated at 37°C for 20 h. Cell pellet was collected by centrifugation at 2,000 rpm for 5 min. It was re-suspended in saline solution (0.85%) and washed three times at the same condition to produce a bac^- phenotype of *L. pentosus* CS2. Thereafter, 100 μl of this bacterial suspension was added to 900 μl of fresh MRS broth in 2 ml Eppendorf tube. This was followed by the addition of 50 μl of 0.21 μM pentocin MQ1, active ammonium sulfate precipitate (8 AU/ml), and the active fraction from HIC (8 AU/ml). These tubes were marked as "induced" while tubes that do not contain the bacteriocin were marked as "control." All tubes were incubated at 37°C for 20 h after which 50 μl of 0.21 μM pentocin MQ1was added to the control tube. CFS from all tubes was tested for antibacterial activity. Induction of pentocin MQ1 production was said to occur if CFS from an induced tube produced inhibition zone while the control tube did not.

Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) was determined by employing the broth microdilution assay as described by Mota-Meira et al. (2000) with little modifications. Two-fold dilutions of bacteriocin were prepared in adequate media and 10 μl for pentocin MQ1 was added to 96-well microtiter plate. Overnight culture of indicator bacteria was diluted (1×10^8 CFU/ml) and added to 150 μl of each bacteriocin preparation. Wells containing indicator without pentocin MQ1 were used as positive control while wells containing only the media were used as blank. Incubation was done at 37°C and optical density at 600 nm was monitored with a multiskan GO microplate reader (Multiskan GO, Thermo Scientific) over a period of 24 h. MIC was defined as the bacteriocin preparation which caused growth reduction by more than 90% compared with the positive control.

Mode of Action

Time-Killing

This assay was done to investigate the mode and speed of action of pentocin MQ1. Indicators were grown for 10 h and centrifuged (2,000 rpm for 5 min) to collect cell pellet. Each cell pellet was re-suspended in ice-cold 5 mM sodium phosphate buffer (pH 7.2) and washed twice. The cell suspension was mixed at a ratio of 1:1 with the bacteriocin preparation (5 X MIC) and incubated at 37°C. Control consisted of bacterial suspension without the addition of bacteriocin. Experiments were done in triplicates. Growth was monitored over a period of 120 min.

Membrane Permeabilization

Pore formation assay was done to understand the mechanism of action of pentocinMQ1. *M. luteus* was grown in Mueller-Hinton broth until $OD_{600nm} = 0.45$ after which 5 μM SYTOX green dye (Invitrogen, USA) was added. Ninety microliters of stained bacteria was added to MicroAmp Fast Optical 96-well reaction plate (Applied Biosystems, Life Technologies, USA). After a stable line base was attained, 10 μl of pentocin MQ1 (5 X MIC) was added to the stained bacteria. Sodium phosphate buffer (5 mM) and nisin (Sigma-Aldrich, USA) were added to stained bacteria in different wells to serve as negative and positive controls, respectively. Experiments were done in triplicates. Fluorescence as a result of binding of SYTOX green to leaked

intracellular DNA was monitored using Real-Time PCR (Applied Biosystems, USA).

Biopreservation of Banana

To investigate the biopreservative potential of pentocin MQ1, a bacteriocin preparation (66.4 μM) was topically applied to mature, fresh banana samples. This was achieved by soaking sterile cotton swab in the bacteriocin preparation and gently rubbing the surfaces of banana samples with it. Some pentocin MQ1-treated banana samples were kept at ambient condition while others were refrigerated. Control samples consisted of non-pentocin MQ1-treated banana samples kept at ambient condition and others refrigerated. Samples were monitored for morphological changes. At the onset of deterioration of control samples, sterile cotton swabs were used to collect surface microflora of both control and pentocin MQ1-treated banana samples and bacterial count (CFU/ml) was measured. The experiment was allowed to proceed until the onset of deterioration of bacteriocin-treated banana samples. Shelf-life was measured. Experiments were done in triplicates.

RESULTS

Isolation and Screening of LAB for Bacteriocin Production

Sixteen different strains of LAB were isolated and identified based on 99% sequence homology. The 16S rRNA gene sequences of all isolated LAB strains were deposited in the NCBI database. *L. pentosus* CS2 with accession number MG976651 exhibited the strongest antibacterial activity and broadest antibacterial spectrum. The remaining 15 LAB strains and their accession numbers are *Lactobacillus fermentum* HFCS1(MG966463), *Weissella cibaria* BavoCS3 (MG976765), *Weissella confusa* PBCS4 (MG976757), *W. confusa* CS5 (MG976685), *W. cibaria* CS6 (MG976766), *W. confusa* CS7 (MG976686), *Lactobacillus nagelii* CS8SB (MG976652), *W. confusa* CS9 (MG980308), *L. fermentum* CS10 (MH032758), *L. fermentum* CS11 (MG966465), *L. fermentum* CS12 (MG966459), *L. fermentum* CS13 (MG966468), *L. fermentum* CS14 (MG980307), *W. cibaria* CS15 (MG982483), and *L. fermentum* CS16 (MG980073).

Purification, Determination of Molecular Weight, and Molar Extinction Coefficient

Purification of bacteriocin by a combination adsorption-desorption method and RP-HPLC proved successful. Bacteriocin was obtained at a retention time of 31–33 min (**Figure 1**). The purified bacteriocin resulting from RP-HPLC had a purification yield of 1.6% (**Table 1**). MALDI-TOF mass spectrometry revealed that the molecular weight is 2110.672 Da (**Figure 2**). A molar extinction coefficient of 298.82 M^{-1} cm^{-1} was obtained.

Antibacterial Spectrum

Pentocin MQ1 displayed strong inhibitory activity toward *L. monocytogenes* NCTC 10890, *M. luteus* ATCC 10240 and *B. cereus* ATCC 14579. It was also inhibitory albeit to a less extent toward *S. pyogenes* ATCC 12344, *S. aureus* RF122, *P. aeruginosa* PA7, *E. faecium* ATCC 19434, *E. faecium* ATCC 27270, *E. faecium* ATCC 27273, *E. faecium* ATCC BAA-2318, *E. faecium* ATCC BAA-2127, *E. faecium* ATCC 6569, *E. faecium* ATCC 25307, and *E. faecium* ATCC 349 but was not active against *S. mutans* GEJ11, *L. lactis* ATCC 11454 and *Corynebacterium* spp. GH17 (**Table 2**).

Bacteriocin-Cell Wall Association Assay

This experiment was designed to know whether pentocin MQ1 abounds in the supernatant or cell wall of the producer. Of the total activity of 6.9×10^4 AU, 66.67% (4.6×10^4 AU) was detected in the cell extract while 33.33% (2.4×10^4 AU) was found in the CFS (**Table 3**).This shows the cell-wall adhering characteristic of pentocin MQ1.

Bacteriocin Stability Test

Stability of pentocin MQ1 under different conditions of heat, enzyme and pH are shown in **Table 4**. Its stability when exposed to different chemicals (1% Tween 80, Tween 20, SDS and triton X-100) are not shown in **Table 4** because it retained 100% residual activity. Residual activities of 99.82, 97.99, 91.32, 90.78, and 83.11% were obtained after heating at 40, 60, 80, 100, and 121°C revealing its high thermal stability. Proteinase K, pepsin, and proteinase significantly reduced its activity (**Table 4**). There was a complete loss of activity when it was treated with trypsin, α-chymotrypsin, and protease. Pentocin MQ1 retained its activity after exposure to lyticase, catalase, and hyaluronidase (**Table 4**). pH variation had effect on its activity. It had higher activity in the pH range of 2–5 than at pH-value of 8. There was no activity at pH-value of 10 (**Table 4**).

Plasmid Isolation

This experiment was done to ascertain if genes encoding pentocin MQ1 production are plasmid-borne. After agarose gel electrophoresis, clear bands were observed for the 1 kb molecular ladder but no band was seen for *L. pentosus* CS2. This indicates the absence of plasmids in *L. pentosus* CS2 (Supplementary Figure 1).

Regulatory Mechanism

This assay was done to investigate the regulatory mechanism of pentocin MQ1 production by *L. pentosus* CS2. A bacteriocin-negative (bac^-) phenotype of *L. pentosus* CS2 was produced by washing off pentocin MQ1 from the cell wall of the producer. Addition of inducible concentrations (quorum) of active ammonium sulfate precipitate, active HIC fraction and pure pentocin MQ1 to the bac^- phenotype of *L. pentosus* CS2 restored pentocin MQ1 production. This was made evident by the presence of inhibition zones for the induced tubes but the absence of zone of inhibition for the non-induced (control) tubes (Supplementary Figure 2).

Minimum Inhibitory Concentration

Pentocin MQ1 exhibited strong inhibitory effect against *L. monocytogenes* NCTC 10890, *M. luteus* ATCC 10240, and *B. cereus* ATCC 14579. MIC-value for *M. luteus* and *L. monocytogenes* and *B. cereus* were 1.66, 1.66, and 3.32 μM, respectively.

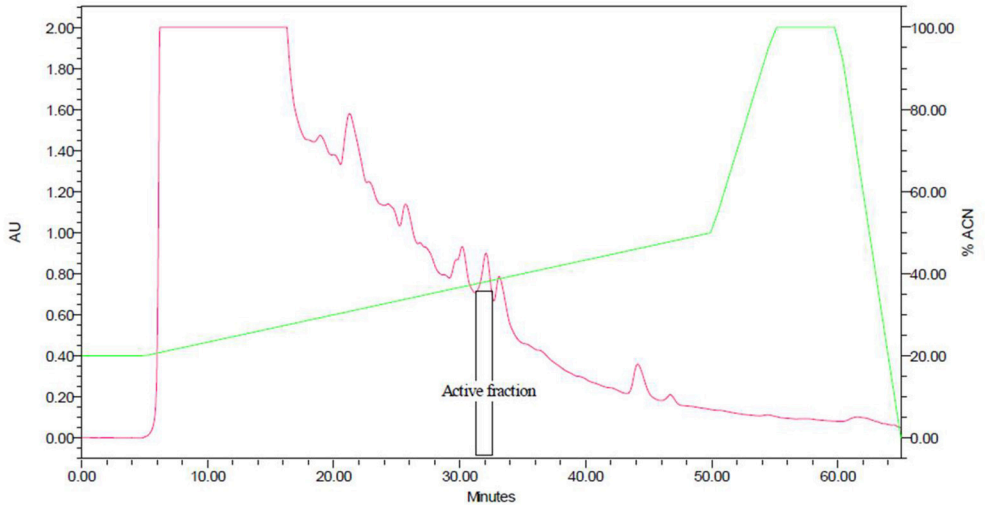

FIGURE 1 | Reversed-phase HPLC chromatogram of crude cell extract from *Lactobacillus pentosus* CS2. Bacteriocin released into MRS broth was adsorbed onto the cell wall of the producer by increasing the pH of the medium to 5.8 followed by desorption from the cell wall by lowering the pH to 2. Total crude bacteriocin obtained was subjected to RP-HPLC. The vertical lines indicate the retention time.

TABLE 1 | Purification of bacteriocin using adsorption-desorption followed by RP-HPLC.

Bacteriocin preparation	Volume (ml)	Activity (AU/ml)	Total activity (AU)	Yield (%)
CFS	1,000	800	800,000	100
Cell extract	10	3,200	32,000	4.0
Purified bacteriocin	2	6,400	12,800	1.6

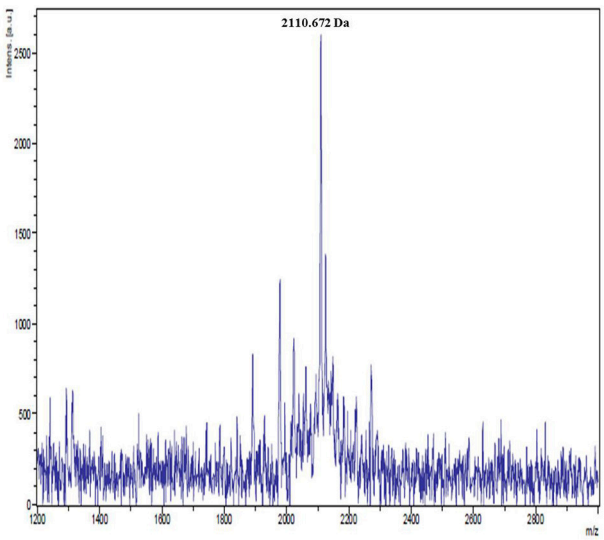

FIGURE 2 | MALDI-TOF mass spectrum of purified pentocin MQ1. Purified pentocin MQ1 was subjected to MALDI-TOF mass spectrometry.

Mode of Action

Time-Killing

Pentocin MQ1 caused a decline in the Log_{10} CFU/ml of *L. monocytogenes* and *B. cereus* (**Figure 3**). After 120 min the Log_{10} viable cell count for *L. monocytogenes* had decreased from 10.27 to 1.80 (82.47% reduction) while that of *B. cereus* had decreased from 9.27 to 3.10 (66.56% reduction).

Membrane Permeabilization

Treatment of *M. luteus* with pentocin MQ1 caused an increase in fluorescence intensity over the course of the study indicating pore formation. Similar observation was made for nisin although higher fluorescence intensity was observed. Fluorescence intensity of the untreated bacterial cells remained stable (**Figure 4**).

Biopreservation of Banana

Total surface bacterial count and LAB count of 4.00×10^7 CFU/ml and 2.10×10^3 CFU/ml (0.005% of total bacterial count), 3.70×10^5 CFU/ml and 9.40×10^2 CFU/ml (0.254% of total bacterial count), 2.14×10^4 CFU/ml and 1.76×10^3 CFU/ml (8.22% of total bacterial count), 7.30×10^2 CFU/ml and 3.20×10^2 CFU/ml (43.84% of total bacterial count) were obtained for nonbacteriocin-treated sample stored at ambient condition, nonbacteriocin-treated sample stored at refrigeration condition, pentocin MQ1-treated sample stored at ambient condition and pentocin MQ1-treated sample stored at refrigeration condition, respectively (**Table 5**). The shelf life of nonbacteriocin-treated sample stored at ambient condition, nonbacteriocin-treated sample stored at refrigeration condition, pentocin MQ1-treated sample stored at ambient condition and pentocin MQ1-treated sample stored at refrigeration condition are 3, 5, 7, and 11 days, respectively. Total surface bacterial count and shelf had a Pearson correlation coefficient (r) value

TABLE 2 | Antibacterial Spectrum of pentocin MQ1.

Indicator strain	Zone of inhibition
Streptococcus pyogenes ATCC 12344	+++
Streptococcus mutans GEJ11	–
Lactococcus lactis ATCC 11454	–
Staphylococcus aureus RF122	+++
MRSA	+
Listeria monocytogenes NCTC 10890	++++
Bacillus cereus ATCC 14579	+++
Pseudomonas aeruginosa PA7	+++
Corynebacterium spp. GH17	–
Escherichia coli UT181	++
Micrococcus luteus ATCC 10240	++++
Enterococcus faecium ATCC 19434	++
Enterococcus faecium ATCC 27270	++
Enterococcus faecium ATCC 27273	++
Enterococcus faecium ATCC BAA-2318	++
Enterococcus faecium ATCC BAA-2127	++
Enterococcus faecium ATCC 6569	++
Enterococcus faecium ATCC 25307	+++
Enterococcus faecium ATCC 349	++
Lactobacillus plantarum K25	++

++++ Inhibition zone >20mm, +++ Inhibition zone 15–20 mm, ++ Inhibition zone <15mm, – No inhibition.

TABLE 3 | Pentocin MQ1 recovered from the cell-free supernatant and cell extract of *Lactobacillus pentosus* CS2.

Bacteriocin preparation	Activity (AU)	Activity (%)
Cell-free supernatant	2.3×10^4	33.33
Cell extract	4.6×10^4	66.67
Total	6.9×10^4	100.00

TABLE 4 | Stability tests for pentocin MQ1.

Test	Zone of inhibition (mm)	Residual Activity (%)
HEAT		
Control	15.95	100.00
40	15.93	99.82
60	15.73	97.99
80	15.00	91.32
100	14.94	90.78
121	14.17	83.11
ENZYME		
Control	15.18	100.00
Proteinase K	9.13	40.28
Lysozyme	15.18	100
Pepsin	7.87	28.19
Lyticase	14.95	97.74
Catalase	15.18	100.00
Trypsin	0.00	0.00
α-Chymotrypsin	0.00	0.00
Protease	0.00	0.00
Proteinase	10.23	51.38
Hyaluronidase	15.18	100.00
pH		
Control	15.80	100.00
2	16.60	107.40
3	16.53	106.76
5	16.00	101.85
8	9.10	37.96
10	0.00	0.00

of −0.779 indicating a strong inverse relationship between the two parameters. An *r*-value of 0.863 was obtained for Pearson correlation analysis between percentage of LAB and shelf life of banana suggesting a strong direct relationship between the two parameters. Changes in organoleptic characteristics of nonbacteriocin-treated banana occurred much earlier than in bacteriocin-treated samples (**Figure 5**).These results show that treatment of banana with pentocin MQ1 extended its shelf. The microbiological quality and shelf life of pentocin MQ1-treated banana stored at refrigeration condition was better than that of pentocin MQ1-treated banana stored at ambient condition.

DISCUSSION

Bacteriocin-producing LAB confer various beneficial effects (such as improvement of quality and shelf life extension) on dairy products (Sultan et al., 2017). As such, the presence of *L. pentosus* CS2 in coconut shake suggests it has bioprotective role. Although *L. pentosus* CS2 has been isolated from vagina (Okkers et al., 1999), fermented Xuan-Wei ham (Zhang et al., 2009) and fermented shrimp (Watthanasakphuban et al., 2016), this is the first report of its isolation from coconut shake considered as a dairy product. Purification of pentocin MQ1 by sequential use of adsorption-desorption method and RP-HPLC proved successful. At low pH bacteriocins are released into the culture medium but when pH is increased to around 5.8–6.0 they become adsorbed onto the producer cells. This phenomenon was observed in this study due to the fact that no activity was detected in the CFS after the adsorption process. Adsorption-desorption approach has been used previously in the purification of some bacteriocins (Siying et al., 2000). Adsorption-desorption method has some advantages over traditional approaches such ammonium sulfate precipitation. These include reduced time of processing, purer crude bacteriocin and cheap running cost (Mu-xu and Zhi-jiang, 2009; Jia-qi et al., 2011). Based on the retention time of pentocin MQ1 which corresponds to high concentration of acetonitrile (high hydrophobicity), it can be deduced that it contains slightly more hydrophobic amino acid residues than polar or hydrophilic ones.

MALDI-TOF mass spectrometry revealed that the molecular weight of pentocin MQ1 is 2110.672 Da. There are only a few reports on purification of bacteriocin from *L. pentosus*. To date pentocins that have been successfully purified to homogeneity

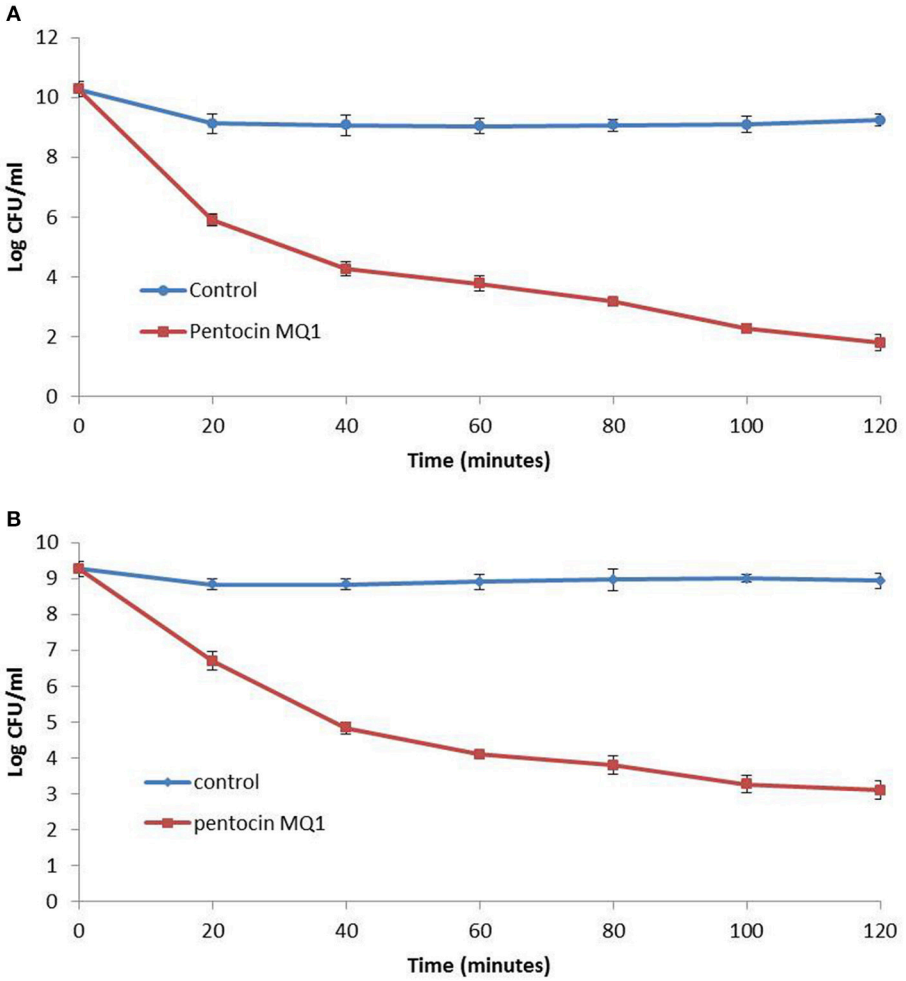

FIGURE 3 | Time-killing assay for pentocin MQ1 **(A)** against *Listeria monocytogenes* NCTC 10890 **(B)** against *Bacillus cereus* ATCC 14579**.** Different preparations of pentocin MQ1 (5 X MIC) was added to cultures of bacterial targets. Bars indicate standard deviation.

and molecular weight accurately determined are pentocin TV35b (3929.63 Da) (Okkers et al., 1999), pentocin 31-1 (5592.225 Da) (Zhang et al., 2009), and bacteriocin K2N7 (2.017 kDa) (Watthanasakphuban et al., 2016). The molecular weight of pentocin MQ1 does not match with any of the reported pentocins. Hence, it is a novel pentocin. Molar extinction coefficient is an important biophysical parameter that can facilitate the quantitation and future industrial application of pentocin MQ1.

Pentocin MQ1 displayed a broad spectrum of antibacterial activity. This attribute has been observed in cerein 7 (Oscáriz et al., 1999), enterocin P (Cintas et al., 1997), and enterocin LR/6 (Kumar and Srivastava, 2010). It was reported that pentocin TV35b is not inhibitory toward *B. cereus* (Okkers et al., 1999). Liu et al. (2008) also reported that pentocin 31-1 is a broad spectrum bacteriocin with inhibitory activity against *L. monocytogenes*, *B. cereus*, *S. aureus* and *E. coli*. Watthanasakphuban et al. (2016) reported that bacteriocin K2N7 has a narrow spectrum of antibacterial activity and was not inhibitory against *L. monocytogenes*, *B. cereus*, *S. aureus*, *E. coli,* and *E. faecium*. Pentocin MQ1 is different from pentocin TV35b, pentocin 31-1 and bacteriocin K2N7 in that in addition to the aforementioned bacterial targets it is also inhibitory against *M. luteus*, *S. pyogenes*, *P. aeruginosa,* and *E. faecium*. It also showed inhibitory activity against closely related species *L. plantarum* K25. Bacteriocins from many LAB strains have been found to inhibit the growth of both closely related and distantly-related bacterial strains (Müller et al., 2009). Broad spectrum of antibacterial activity is one of the important criteria for selection of bacteriocin for use in the biopreservation of foods (Johnson et al., 2017; Kaškoniene et al., 2017). The broad antibacterial spectrum of pentocin MQ1 well positions it as a good candidate for preservation of various types of foods.

Investigating the association of pentocin MQ1 with the cell wall of the producer is important because it can reveal whether the bacteriocin abound in the supernatant or on the cell wall. In this study more activity was detected on the cell wall than

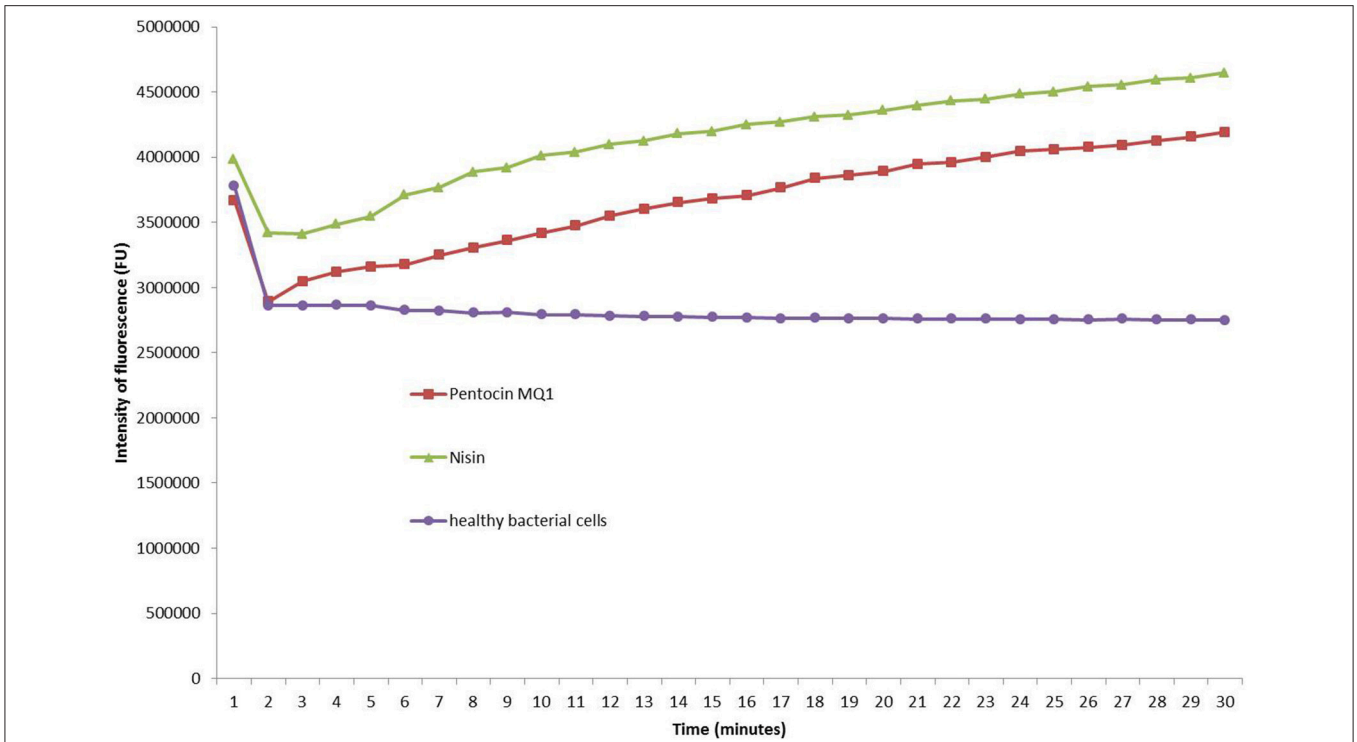

FIGURE 4 | Pore-formation in the cell membrane of *Micrococcus luteus* ATCC 10240 by pentocin MQ1**.** Pentocin MQ1 was added to *M. luteus* stained with SYTOX green dye and increase in fluorescence as a result of leakage of intracellualr DNA was monitored using Real-Time PCR.

TABLE 5 | Effect of bacteriocin application on surface bacterial count and shelf-life of banana.

Bacteriocin	Bacterial count (CFU/ml) after 5 days of storage				Shelf-life (days)	
	A		R		A	R
	Total	LAB	Total	LAB		
P	2.14×10^4	1.76×10^3	7.30×10^2	3.20×10^2	7	11
C	4.00×10^7	2.10×10^3	3.70×10^5	9.40×10^2	3	5

P, pentocin MQ1; C, negative control; A, ambient condition; R, refrigerated.

the supernatant indicating the cell wall-binding characteristic of pentocin MQ1. This finding shows that for a better recovery of pentocin MQ1 produced by *L. pentosus* CS2, an adsorption-desorption approach facilitated by pH modifications should be employed. In this study, an adsorption-desorption approach suitable for purification of pentocin MQ1 was demonstrated. Association of bacteriocin with the cell wall of the producer is thought to enhance niche competition. Cell-wall associated bacteriocins have also been described in *Lactobacillus crispatus*, *Streptococcus salivarius,* and *Streptococcus bovis* HC5 (Tahara and Kanatani, 1997; Mantovani et al., 2002; Barbour and Philip, 2014).

Pentocin MQ1 was highly stable to all chemical treatments investigated. This is evidenced by its retention of 100% residual antibacterial activity. It exhibited high thermal and pH stability. Higher activity was detected in the acidic pH range (2–5) while moderately alkaline pH (pH 8) caused a drastic reduction in activity. Lack of activity at pH 10 (high alkaline pH) indicates severe denaturing of pentocin MQ1. Pentocin MQ1 is a proteinaceous biomolecule due to its susceptibility to proteinases. Retention of high antibacterial activity after exposure to lyticase, catalase and hyaluronidase provides more evidence on its proteinaceous nature. Pentocin TV35b was active in the pH range of 1–10 and after heating at 60–100°C (Okkers et al., 1999). Pentocin 31-1 was active at pH 2–10 and at 60–121°C but sensitive to SDS (Liu et al., 2008). Bacteriocin K2N7 retained activity at pH 2–12 but unlike pentocin MQ1 it was inactive at 121°C (Watthanasakphuban et al., 2016). The combined attributes of chemical, pH and thermal stability of pentocin MQ1 favors its future application in food systems subjected to harsh processing conditions (Hemu et al., 2016; Yi et al., 2016). Its sensitivity to proteases is a desirable characteristic in that its chances of inhibiting beneficial components of the gut microbiota is reduced thereby, enhancing its safety (Zacharof and Lovitt, 2012; Hemu et al., 2016). Moreover, degradation of bacteriocin by proteases reduces the time of interaction between fragments of a given bacteriocin and its target thereby decreasing the possibility of resistance development (Perez et al., 2014). Its application in the treatment of gut infection would require encapsulation in nanoparticles or bioengineering to make it resistant to protease of the gut (Zhang L. et al., 2010; Arthur et al., 2014; Cavera et al., 2015).

FIGURE 5 | Appearance of banana after 5 days of storage at different conditions. **(A)** Nonbacteriocin-treated stored at ambient condition **(B)** nonbacteriocin-treated stored at refrigeration condition **(C)** pentocin MQ1-treated stored at ambient condition **(D)** pentocin MQ1-treated stored at refrigeration condition.

Genetic element harboring *L. pentosus*-derived bacteriocin has not been reported. Genes encoding bacteriocin production have been detected on plasmids and chromosomes (Garcia et al., 2010). Absence of plasmids in *L. pentosus* CS2 suggests that genes encoding pentocin MQ1 production are chromosome-borne. It is thought that chromosome-encoded bacteriocin genes are more stable than plasmid-encoded bacteriocin genes because plasmids, being small and mobile genetic elements can be lost by leaking out of bacterial cells (Sengupta and Austin, 2011). Hence, bacteriocin-producing LAB strains harboring chromosome-borne bacteriocin genes have an edge over those with plasmid-borne bacteriocin genes. Thus, *L. pentosus* CS2 is genetically stable. Chromosome-borne bacteriocins include enterocin A (Aymerich et al., 1996) and ABP-118 (Flynn et al., 2002) and acidocins LF221 (Majhenič et al., 2003).

Pentocin MQ1 production was restored when active ammonium sulfate precipitate, active HIC fraction and pure pentocin MQ 1 were added separately to the bac$^-$ phenotype of *L. pentosus* CS2. This shows that pentocin MQ1 production is auto-inducible suggesting its regulation by a two-component quorum sensing mechanism involving an inducing peptide (the bacteriocin), a histidine protein kinase (HPK) and a response regulator (RR). Pentocin MQ1 acts as the inducing peptide. Upon attaining an inducible concentration (quorum) the bacteriocin binds to HPK and activates it. Activated HPK phosphorylates RR which in turn binds to the promoter of bacteriocin genes and upregulates bacteriocin production (Chanos and Mygind, 2016). Nisin production is regulated by a similar mechanism (Dobson et al., 2012). Pentocin 31-1 production is also controlled by quorum sensing (Zhang et al., 2012). Regulation of bacteriocin production via quorum sensing mechanism is commonly found among class II bacteriocins. (Straume et al., 2007; Di Cagno et al., 2010, 2011).

Pentocin MQ1 was strongly inhibitory against *L. monocytogenes* NCTC 10890, *M. luteus* ATCC 10240, and *B. cereus* ATCC14579 at micromolar concentrations. High activity at low concentration is a desirable property of natural biopreservatives (Bali et al., 2016). MIC-values for nisin A against *Micrococcus* spp. and *Bacillus* spp. (Mota-Meira et al., 2000) are lower than that of pentocin MQ1. However, the broad antibacterial spectrum of pentocin MQ1 suggests wide food and medical applications. Pentocin MQ1 exhibits a bactericidal mode of action against *L. monocytogenes* and *B. cereus*. After 120 min the viable cell count for *L. monocytogenes* and *B. cereus* had been reduced significantly. This shows the quick-acting characteristic of pentocin MQ1 against these pathogens. Pentocin 31-1 was also shown to exert a bactericidal effect against *L. monocytogenes* (Liu et al., 2008). Pentocin TV35b had a bactericidal activity against *Listeria innocua* (Okkers et al., 1999).

The two main modes of action of LAB bacteriocins against Gram-positive bacteria are pore formation and inhibition of cell wall synthesis (Cotter et al., 2013). Inhibition of cell wall biosynthesis of *M. luteus* by salivaricin B did not cause an increase in fluorescence within 30 min. But within the same time frame nisin, a known pore former caused an increase in fluorescence intensity (Barbour et al., 2016). This implies that pore formation is a more rapid killing mechanism. Since increase in fluorescence intensity was observed for the pentocin MQ1-treated *M. luteus* within 30 min it can be concluded that it was due to membrane permeabilization. Pentocin MQ1 caused membrane permeabilization of *M. luteus* leading to leakage of intracellular DNA and consequently death of the bacteria. This is the first report on membrane permeabilization as a mechanism of action of *L. pentosus*-derived bacteriocins. It is thought that pore formation also led to loss of other valuable intracellular molecules such as ATP contributing to the rapid death of the bacterial target. Pentocin MQ1 was quick-acting against its target. It is thought that resistance to a quick-acting antimicrobial agent is less likely to occur compared to a slow-acting one. Pore formation has been reported for several LAB bacteriocins (Perez et al., 2014; Snyder and Worobo, 2014).

Banana is one of the most consumed fruit in the tropics and subtropics (Huang et al., 2014). It is a good source of antioxidants, carbohydrates, calcium, and potassium (Mohapatra et al., 2011). As a perishable and climacteric crop it has a short shelf life. Preserving fresh banana is quite challenging (Mohapatra et al., 2010). Various chemical and physical approaches are employed in the preservation of banana (Zaman et al., 2007; Kudachikar et al., 2011; Mohapatra et al., 2011). However, consumer inclination toward foods containing biopreservatives and less chemical preservatives triggered the search for natural products that can be used for biopreservation (Barbosa et al., 2017). In a recent study, combined application of phenylurea and gibberellins was effective at extending the shelf life of banana (Huang et al., 2014). Although the bioprotective capabilities of several bacteriocins have been reported (Galvez et al., 2008; Abriouel et al., 2010; Bhatia et al., 2016), no report has been made for banana. Only one report has been made on biopreservation of food using pentocin. In that study, the potential of pentocin 31-1 for preserving pork meat was demonstrated (Zhang J. et al., 2010). Topical application of pentocin MQ1 extended the shelf of banana in this study. Shelf life extension was due to decrease in total bacterial count

and increase in the percentage of LAB compared to the other microflora (**Table 5**). It can be deduced that pentocin MQ1 decreased the population of pathogenic and spoilage bacteria on the surface of banana. Moreover, it had a positive effect on the population dynamics of the surface microflora such that decrease in spoilage bacteria enhanced the growth of beneficial LAB strains leading to shelf life extension. These results reveal the biopreservative potential of pentocin MQ1. Furthermore, bacteriocin treatment and refrigeration had a synergistic effect on the microbiological quality of banana resulting in extension of shelf life. These findings pave the way for future *ex situ* application of pentocin MQ1 in the biopreservation of banana.

In conclusion, this is the first report on the presence of bacteriocinogenic strain of *L. pentosus* in coconut shake. *L. pentosus* CS2 produces a novel bacteriocin (pentocin MQ1) with a broad spectrum of antibacterial activity, high chemical, thermal and pH stability but sensitive to proteolytic enzymes. It is cell-wall associated and possesses a bactericidal mode of action. Pentocin MQ1 acted against its target through pore formation. Genes encoding pentocin MQ1 production are not plasmid-borne. Its biosynthesis is regulated by a quorum sensing mechanism. Its ability to preserve fresh banana was demonstrated in this study. The characteristics of pentocin MQ1 show its potential for the preservation of food.

AUTHOR CONTRIBUTIONS

KP designed, supervised execution of the experiments, and wrote the manuscript; KP also edited the manuscript; SW designed the experiments, performed it, and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2018.00564/full#supplementary-material

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Influence of Oxidative Stress on Biocontrol Activity of *Cryptococcus laurentii* against Blue Mold on Peach Fruit

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The limitations of chemical fungicides for the control of postharvest diseases have recently become more apparent. The utilization of antagonistic microorganisms is a promising alternative to that of fungicides to control postharvest decay. In previous studies, the antagonistic yeast *Cryptococcus laurentii* has shown excellent effects of biocontrol and great potential for practical application. Adverse conditions, such as oxidative stress, limit the practical application of antagonistic yeast. In this study, we investigated the oxidative stress tolerance of *C. laurentii* and the associated mechanisms. The results indicated that exogenous oxidative stress has a significant effect on the viability and biocontrol efficiency of *C. laurentii*. H_2O_2-induced oxidative stress led to the accumulation of reactive oxygen species. The results of flow cytometric analysis suggested that apoptosis is responsible for the reduced survival rate of *C. laurentii* under oxidative stress. Using tests of antioxidant activity, we found that *C. laurentii* could employ enzymatic systems to resist exogenous oxidative stress. The addition of exogenous glutathione, a non-enzymatic antioxidant, to the media can significantly enhance oxidative tolerance and biocontrol efficiency of *C. laurentii*.

Keywords: *Cryptococcus laurentii*, biocontrol, oxidative stress, apoptosis, antioxidant systems, glutathione

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INTRODUCTION

Postharvest diseases of fruits and vegetables cause considerable economic losses worldwide, and account for more than 25% of total production in developed countries and more than 50% in developing countries (Nunes, 2012). The application of chemical fungicides is currently the primary means of controlling postharvest disease. Nevertheless, the excessive use of fungicides has led to several negative effects, e.g., drug resistance of pathogens, environmental pollution, and the subsequent harm to human health (Janisiewicz and Korsten, 2002; Droby et al., 2009; Jamalizadeh et al., 2011). Therefore, the quest for safe and effective alternatives to fungicides is crucial. Antagonistic yeasts, such as *Cryptococcus laurentii*, *Rhodotorula glutinis*, and *Pichia membranifaciens*, have been exploited as promising alternatives to synthetic fungicides, and have

Abbreviations: CAT, catalase; GSH, reduced glutathione; GSSG, oxidized glutathione; NADPH, nicotinamide adenine dinucleotide phosphate; PBS, phosphate buffered saline; PI, propidium iodide; ROS, reactive oxygen species; SOD, superoxide dismutase; YPD, yeast peptone dextrose.

been gradually receiving considerable attention (Fan and Tian, 2000; Qin et al., 2003, 2004; Li and Tian, 2006).

During their application, biocontrol agents are subjected to many adverse stresses that affect their survival and performance (Sui et al., 2015). Yeasts are commonly subjected to oxidative stress (Li and Tian, 2007; Macarisin et al., 2010). After pathogenic attack in the host, an oxidative burst, which is associated with increased levels of H_2O_2 and O_2^-, can be generated in the area surrounding the infection site, and serves as an early resistance response to pathogenic invasion (Segal, 2005; Temme and Tudzynski, 2009). Furthermore, antagonistic yeasts could also act as an elicitor that triggers ROS signaling in host tissue and thereby activates host defenses (Chan and Tian, 2006; Xu et al., 2008; Macarisin et al., 2010). Excessive ROS can affect the viability and biocontrol efficacy of antagonistic yeasts. However, the ability of antagonistic yeasts to withstand oxidative stress varies among different species. Liu et al. (2011a,b, 2012) examined the responses of *Metschnikowia fructicola*, *Candida oleophila*, and *Cystofilobasidium infirmominiatum* to oxidative stress. They found that *C. infirmominiatum* was sensitive and *M. fructicola* was relatively tolerant to oxidative stress. The antagonistic yeast *C. laurentii* has been widely studied and has shown excellent biocontrol efficacy against many postharvest diseases of apples, strawberries, mangoes, and sweet cherries (Tian et al., 2004; Bautista-Rosales et al., 2014; Navarta et al., 2014; Zhang et al., 2015). A previous study has indicated that oxidative stress tolerance of an antagonistic yeast species is closely associated with its biocontrol performance in postharvest application (Castoria et al., 2003). Although many studies have reported on oxidative stress resistance of antagonistic yeasts, further discovery regarding the mechanisms of action by which oxidative stress regulates their viability and biocontrol efficacy remain unknown.

The present study aimed to evaluate the tolerance of *C. laurentii* to oxidative stress and elucidate the antioxidative mechanism. Moreover, the mechanisms by which oxidative stress is used to regulate survival and biocontrol efficacy of *C. laurentii* were investigated, using flow cytometric analysis. Methods to improve oxidative stress resistance and biocontrol performance were also exploited.

MATERIALS AND METHODS

Yeast and Pathogens

Cryptococcus laurentii was isolated from the surfaces of apple fruits in a previous experiment (Qin et al., 2004) and grown in YPD broth (10 g yeast extract, 20 g peptone, and 20 g dextrose in 1 L water). Yeast cultures with an initial concentration of 1×10^5 cells/mL were incubated at 26°C on a rotary shaker at 200 rpm for 17 h to reach the mid-log phase. *Penicillium expansum* was isolated from naturally infected apple fruits. It was routinely cultured on potato dextrose agar plates for 14 days at 25°C. Fungal spores were harvested by flooding the surface of the culture with sterile distilled water, followed by filtration through four layers of sterile cheesecloth. The number of spores in the resulting suspension was calculated using a hemocytometer. Before inoculation, the spore concentration in sterile distilled water was adjusted to 1×10^4/mL.

Fruit

Peach fruits (*Prunus persica* L. Batsch) at commercial maturity were harvested from an orchard in Beijing and immediately transported to the laboratory. Fruits without blemishes or rot were selected based on uniformity of size. Selected fruits were surface-disinfected with 2% (v/v) sodium hypochlorite for 2 min, rinsed with tap water, and air-dried prior to further use.

Oxidative Stress Tolerance Assays

The median lethal concentration of H_2O_2 for *C. laurentii* was determined according to the methods of Chen et al. (2015). Cells in the mid-log phase were obtained by centrifugation. After being washed twice with sterile distilled water, yeast cells were resuspended in fresh YPD medium to a final concentration of 5×10^7 cells/mL. H_2O_2 was added to each yeast culture to final concentrations of 0, 100, 200, 300, and 400 mM. Following incubation for 90 min (150 rpm, 26°C), yeast cells of each sample were collected and adjusted to 1×10^6 cells/mL. To analyze survival rates, a 50 μL yeast sample was spread on a YPD solid plate. The plates were subsequently observed under a light microscope (Carl Zeiss, Oberkochen, Germany). The effects of treatment time with H_2O_2 on yeast viability were determined using a plate assay according to the methods of Liu et al. (2011b). Yeast cell viability was expressed as a percentage of the colony number following H_2O_2 treatment, relative to that without treatment. For each treatment, there were three replicates and the experiment was performed twice.

Detection of Intracellular ROS

Intracellular ROS was detected using a 10 μM 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) oxidant-sensitive probe (Molecular Probes, Eugene, OR, USA). DCFH-DA was added to the yeast suspension and incubated in the dark at 37°C for 30 min. After being washed twice with PBS, yeast cells were examined under a microscope (Zeiss Axioskop, Oberkochen, Germany) using a 485-nm excitation and 530-nm emission filter combination. Three independent experiments were performed.

The fluorescence intensity of *C. laurentii* cells was determined using a fluorescence microplate reader (Synergy H4, BioTek, Winooski, VT, USA). Yeast samples were washed twice with *N*-2-hydroxyethylpiperazine-*N*-2'-ethanesulfonic acid (HEPES) buffer (pH 7.0) and incubated with 10 μM DCFH-DA at 37°C for 30 min. The samples were then washed twice with HEPES buffer and diluted to an optical density (OD) at 600 nm of 1.4. Yeast samples (200 μL/well) were then added to a 96-well dark microplate, and fluorescence was analyzed using a fluorescence microplate reader with an excitation wavelength of 492 nm and an emission wavelength of 527 nm. Three replicate wells were analyzed for each treatment, and the experiment was performed twice.

Biocontrol Analysis of *C. laurentii*

Biocontrol performance of *C. laurentii* against *P. expansum* was determined on peach fruits. *C. laurentii* cells at mid-log phase were either treated with 300 mM H_2O_2 for 90 min as described above or left untreated. Peach fruits were punctured at the equatorial line (three wounds per fruit) using a sterile nail, and first inoculated with 10 μL *C. laurentii* cell suspension (5×10^7 cells/mL) and then with 10 μL *P. expansum* spore suspension (1×10^4 spores/mL). Treated fruits were placed in plastic boxes. Each tray was enclosed with a polyethylene bag to maintain high humidity (about 95% relative humidity), and stored at 25°C. Disease incidence and lesion diameters of the fruits were recorded after 3, 4, and 5 days. Each treatment comprised three replicates with ten fruits per replicate, and the experiment was performed twice.

Analysis of Apoptosis of *C. laurentii* Cells under Oxidative Stress

To discriminate between viable, necrotic, and apoptotic cells, flow cytometric measurements of Hoechst 33342/PI double stained yeast cells were carried out using a MoFlo XDP Cell Sorter (Beckman Coulter, Brea, CA, USA). Stained cells were analyzed using laser-based flow cytometry systems. Yeast cells at the mid-log phase were collected and treated with 300 mM H_2O_2 for 90 min. After being washed twice with PBS, the treated and untreated yeast cells were incubated with 10 μg/mL PI and 5 μg/mL Hoechst 33342 for 20 min in the dark. PI-positive yeast cells indicated damaged plasma membranes and the presence of necrotic cells. PI-negative and Hoechst 33342-positive yeast cells were considered apoptotic (Hong et al., 2007). The cell density of each sample was maintained at approximately 1×10^6 cells/mL. The sample flow rate was 700 cells/s. A total of 20,000 cells were measured for each sample.

Assays of CAT and SOD Activity

Rhodotorula glutinis, an oxidative stress-sensitive yeast, was used as a positive control in the analysis of antioxidant systems. For the enzyme activity assay, yeast cells were collected by centrifugation at specific intervals (0, 0.5, 1, 2, and 3 h) after being treated with a moderately lethal concentration of H_2O_2 and washed twice with PBS. The yeast cells were disintegrated with glass beads through vibration on a vortex mixer. SOD and CAT were extracted with 50 mM PBS (pH 7.0, 1 mM EDTA, and 2 mM phenylmethanesulfonyl fluoride). The reaction mixture (3 mL) with SOD contained 50 mM PBS, 13 mM methionine, 75 μM nitroblue tetrazolium, 10 μM EDTA, 2 μM riboflavin, and 50 μL enzyme extract. The mixtures were illuminated by light (4000 lx) for 20 min, and the absorbance was determined at 560 nm. Identical solutions held in the dark served as blanks. One unit of SOD activity was defined as the amount of enzyme causing 50% inhibition in the nitroblue tetrazolium reduction. The reaction mixture (1.5 mL) with CAT consisted of 1.4 mL H_2O_2 (40 mM) and 100 μL enzyme extract. The decomposition of H_2O_2 was determined based on the decline in absorbance at 240 nm. One unit of CAT activity was defined as the decomposition of 1 μM H_2O_2 per min. The activity of both enzymes was expressed as U/mg protein.

Assays of Total Glutathione

Extracts for the total glutathione assay were prepared according to the methods of Ellman (1959), with minor modifications. Cells of *R. glutinis* and *C. laurentii* at the mid-log phase were collected and treated with 30 and 300 mM H_2O_2 for 90 min, respectively. The cells were then harvested by centrifugation at 8000 × *g* for 3 min, washed three times with sterile distilled water, resuspended in PBS (pH 7.0), and extracted by vortexing with glass beads. The extracts were used for the total glutathione assay. Total glutathione content was determined using the GSH and GSSG Assay Kit S0053 (Beyotime, Shanghai, China) and 5, 5′-dithio-bis-nitrobenzoic acid. GSSG was reduced to GSH by glutathione reductase and NADPH. The absorbance was monitored at 412 nm and the results were expressed as mM/g.

Exogenous GSH Treatment

The effects of exogenous GSH on yeast cell viability following H_2O_2 treatment were determined according to the methods of Liu et al. (2011b), with slight modifications. Yeast cells with an initial concentration of 1×10^5 cells/mL were supplemented with GSH to yield final concentrations of 1 and 10 mM. After overnight cultivation, yeast cells at the mid-log phase were harvested by centrifugation at 8000 × *g* for 3 min and washed three times with fresh YPD to remove any residual GSH. Yeast cells were then resuspended in fresh YPD medium to a final concentration of 5×10^7 cells/mL, and *C. laurentii* cells were treated with 300 mM H_2O_2 for 90 min. Cell viability was evaluated by the aforementioned methods.

Statistical Analysis

All statistical analyses were performed using the SPSS version 13 software (SPSS Inc., Chicago, IL, USA). Data were analyzed using one-way ANOVA, and comparisons between means were performed using the Duncan's multiple range test. Differences at $P < 0.05$ were considered significant.

RESULTS

Survival of *C. laurentii* under H_2O_2-Induced Oxidative Stress

Cell viability was measured following exposure of *C. laurentii* to increasing doses of H_2O_2 (ranging from 0 to 400 mM) and treatment intervals in YPD liquid media. Oxidative stress induced by H_2O_2 significantly inhibited cell viability in a dose- and time-dependent manner, and exposure to 300 mM H_2O_2 over 90 min was moderately lethal to cells (about 50% inhibitory) (**Figures 1** and **2**). Based on these results, 300 mM H_2O_2 over 90 min was selected as the appropriate concentration and interval to promote oxidative stress for subsequent studies.

Measurement of ROS Production

Intracellular ROS production was measured by detection of fluorescence by DCFH-DA, which could be converted to highly

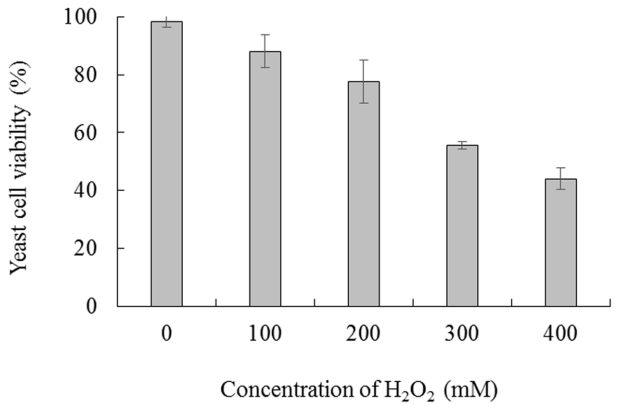

FIGURE 1 | Viability of *Cryptococcus laurentii* under oxidative stress. *C. laurentii* cells were treated with a series of concentrations of H_2O_2 over 90 min. Vertical bars represent standard errors of the mean.

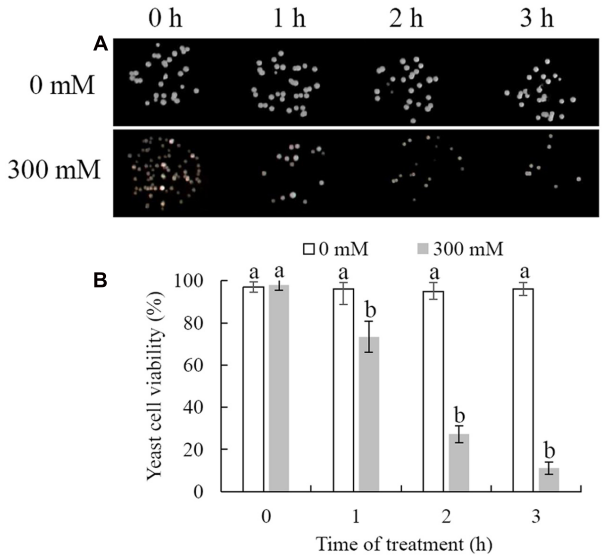

FIGURE 2 | Survival of *Cryptococcus laurentii* following treatment with 300 mM H_2O_2. (A) Spotting assay of viability of *C. laurentii* following treatment with 300 mM H_2O_2. **(B)** Survival rate of *C. laurentii* following treatment with 300 mM H_2O_2. Vertical bars represent standard errors of the mean. Columns with different letters indicate significant differences ($P < 0.05$).

fluorescent dichlorofluorescein in the presence of intracellular ROS. The results showed that H_2O_2 treatment could significantly induce the accumulation of intracellular ROS in *C. laurentii* (**Figure 3A**). Under conditions of oxidative stress induced by 300 mM H_2O_2, the percentage of ROS-positive cells was 46.6%. In contrast, only 5.6% of *C. laurentii* cells that were not subjected to exogenous oxidative stress showed visible ROS accumulation (**Figure 3B**). The results obtained in the analysis of fluorescence intensity were consistent with these findings. Treatment with 300 mM H_2O_2 significantly increased the intensity of dichlorofluorescein fluorescence in *C. laurentii* cells (**Figure 3C**).

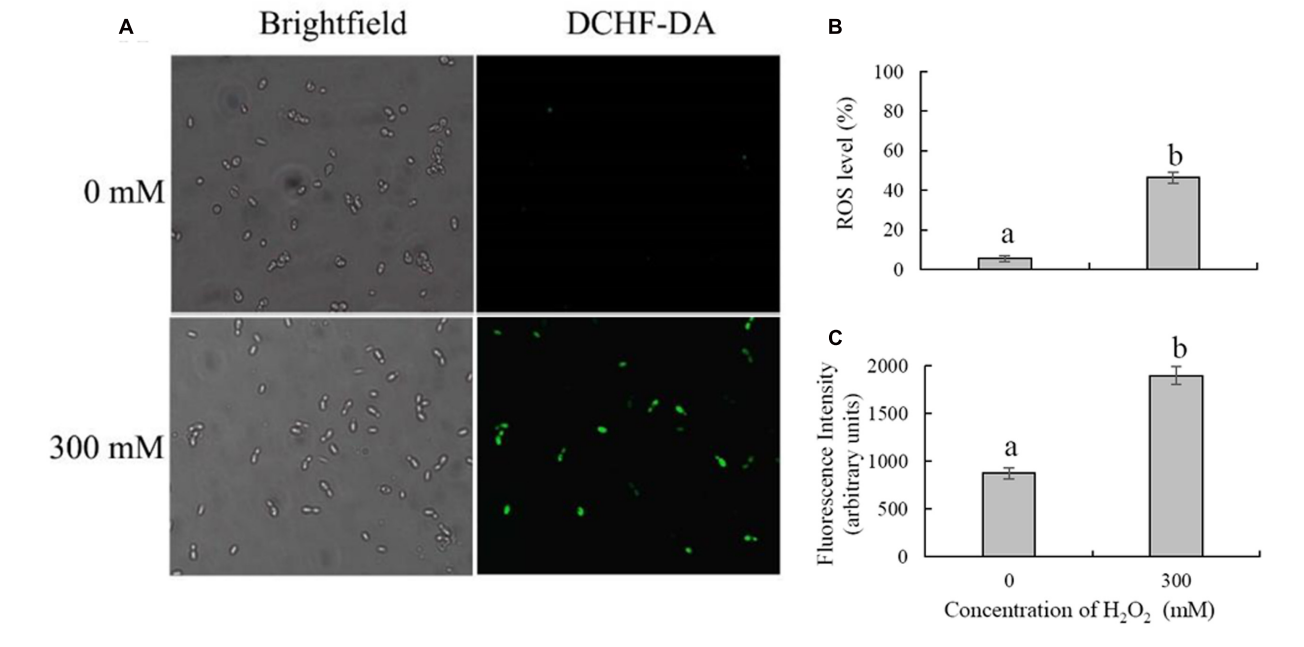

FIGURE 3 | Detection of intracellular ROS in *Cryptococcus laurentii* following treatment with and without exogenous H_2O_2. (A) Microscopic images of *C. laurentii* cells stained with 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA). **(B)** Percentage of *C. laurentii* cells exhibiting visible ROS accumulation. **(C)** Statistical analysis of the fluorescence intensity of *C. laurentii* cells. Vertical bars represent standard errors of the mean of three independent experiments. Columns with different letters indicate significant differences ($P < 0.05$).

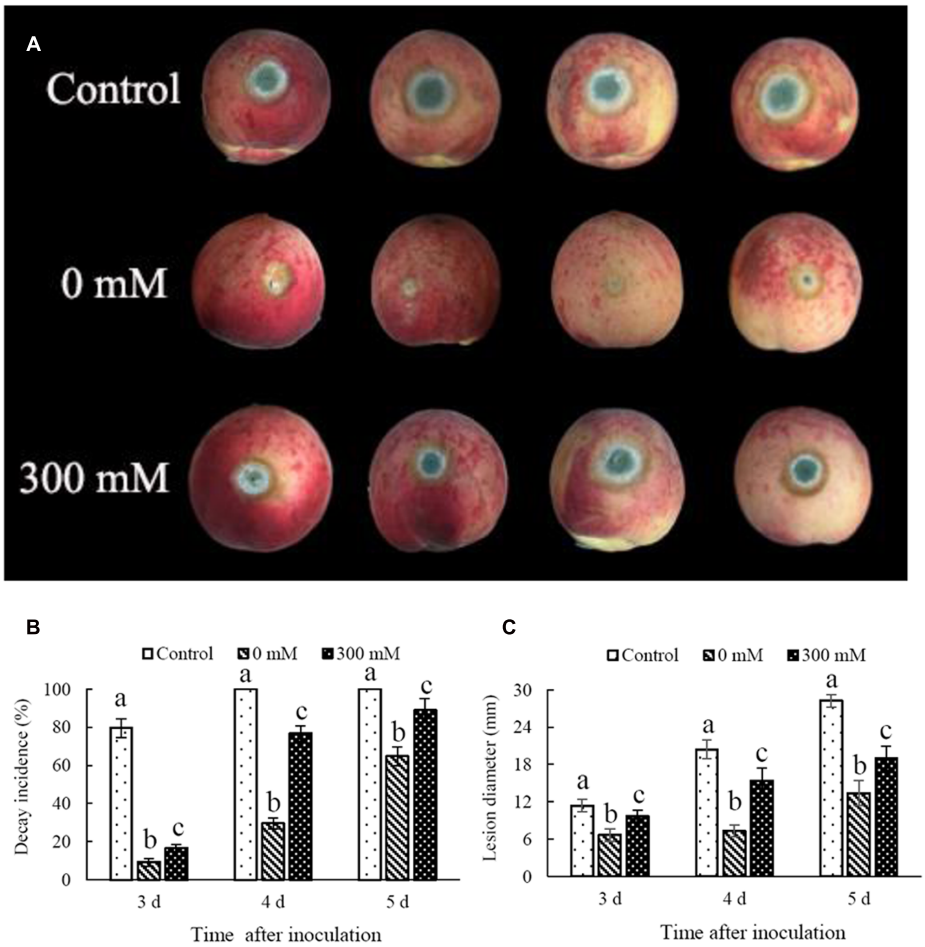

FIGURE 4 | Effect of oxidative stress on biocontrol efficiency of *Cryptococcus laurentii* against *Penicillium expansum*. (A) Biocontrol performance of H_2O_2-treated *C. laurentii* cells and non-treated *C. laurentii* cells against *P. expansum* on peach fruits (5 days post inoculation). **(B)** Statistical analysis of decay incidence on peach fruits 3, 4, and 5 days post inoculation. **(C)** Statistical analysis of lesion diameters on peach fruits 3, 4, and 5 days post inoculation. Vertical bars represent standard errors of the mean. Columns with different letters indicate significant differences ($P < 0.05$).

Biocontrol Efficacy of *C. laurentii*

In comparison with the control, non-H_2O_2-treated *C. laurentii* cells effectively inhibited postharvest decay caused by *P. expansum* on peach fruits (**Figure 4A**). Notably, the addition of 300 mM H_2O_2 significantly reduced biocontrol activity of *C. laurentii*. After 4 days, disease incidence in peach fruits treated with *C. laurentii* cells that had not been subjected to oxidative stress was 30%; whereas disease incidence in H_2O_2-treated samples was as high as 76% (**Figure 4B**). Moreover, the efficiency of *C. laurentii* cells in inhibiting lesion expansion was also significantly suppressed under exogenous oxidative stress (**Figure 4C**). These results suggest that exogenous oxidative stress could exert significant influence on biocontrol efficiency of *C. laurentii*. Thus, improving oxidative stress tolerance in *C. laurentii* is essential to enhancing its biocontrol efficiency.

Cell Membrane Integrity and Apoptosis

Necrosis and apoptosis of *C. laurentii* cells were demonstrated using Hoechst 33342 and PI staining. Necrotic yeast cells with a damaged plasma membrane were detectable, based on their high red fluorescence due to PI uptake (**Figure 5A**). Following exposure to 300 mM H_2O_2 for 90 min, the integrity of the plasma membrane of *C. laurentii* cells was reduced to 75%, and the number of stained apoptotic yeast cells was increased to 29% (**Figure 5B**). However, damage to the plasma membrane and apoptosis were not significant in *C. laurentii* cells that had not been subjected to H_2O_2 treatment (**Figures 5A,B**).

For further analysis, necrosis and apoptosis in *C. laurentii* cells exposed to H_2O_2 were examined by flow cytometric analysis. On the dot plots, each cell is represented by a single dot (**Figure 5C**). When cells were stained with a combination of Hoechst 33342 and PI, three cell populations were observed: viable (R1), apoptotic (R2), and necrotic (R3) (**Figure 5C**). In the absence of H_2O_2-induced oxidative stress, the vast majority of *C. laurentii* cells was viable; however, following treatment with 300 mM H_2O_2, the percentage of viable cells was reduced to 46%, and the percentage of apoptotic and necrotic cells was 35 and 16%, respectively. These results are consistent with those

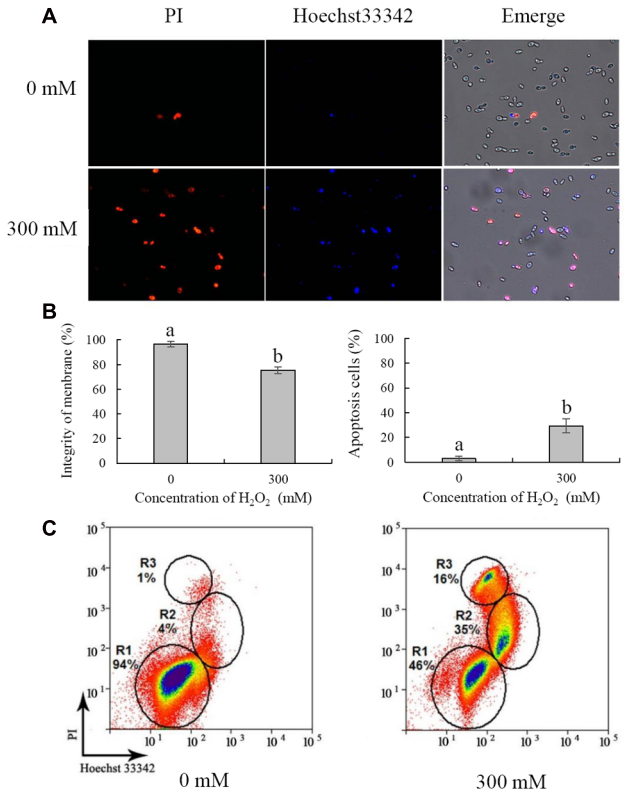

FIGURE 5 | Necrosis and apoptosis analysis of *Cryptococcus laurentii* cells under oxidative stress. (A) Fluorescence microscopic image of *C. laurentii* cells treated with and without exogenous H_2O_2 and double stained with PI/Hoechst 33342. **(B)** Percentage of *C. laurentii* cells with plasma membrane integrity and percentage of apoptotic cells. **(C)** Flow cytometric analysis. R1 represents viable cells, R2 represents apoptotic cells, and R3 represents necrotic cells. Vertical bars represent standard errors of the means of three independent experiments. Columns with different letters indicate significant differences ($P < 0.05$).

of the survival analysis of *C. laurentii* under oxidative stress. These findings suggest that exogenous oxidative stress induced by 300 mM H_2O_2 could lead to apoptosis of *C. laurentii* cells that is primarily responsible for the decline in viability under oxidative stress.

Antioxidant Enzyme and Total Glutathione Assays

To resist the effects of oxidative stress and maintain cellular homeostasis, cells have evolved two sophisticated antioxidant systems, the enzymatic (e.g., CAT and SOD) and non-enzymatic antioxidant (e.g., glutathione and vitamins) defense systems. CAT and SOD are two major antioxidant enzymes involved in the enzymatic antioxidant defense system. Previous studies have shown that treatment with 30 mM H_2O_2 for 90 min has a moderately lethal effect on *R. glutinis* cells (about 50% inhibitory). In contrast, the same concentration of H_2O_2 has no significant effects on the viability of *C. laurentii* (data not shown), indicating that in comparison to *R. glutinis*, *C. laurentii* shows greater resistance to exogenous oxidative stress. To determine the reasons for this higher oxidative tolerance of *C. laurentii*, the enzyme activities of CAT, SODe and total glutathione of *R. glutinis* and *C. laurentii* under moderately lethal oxidative stress (*R. glutinis*: 30 mM H_2O_2; *C. laurentii*: 300 mM H_2O_2), were assessed. CAT activity in *C. laurentii* was strongly induced by H_2O_2 and maintained at a higher level than it was in *R. glutinis*. The activity level in *C. laurentii* was approximately eightfold that in *R. glutinis* (**Figure 6A**). Similarly, H_2O_2 treatment was able to induce SOD activity in *C. laurentii*. In contrast, exogenous oxidative stress showed slight inhibitory effects on SOD activity in *R. glutinis* (**Figure 6B**). These data indicate that enzymatic antioxidants are major contributors to the overall antioxidant capacity of *C. laurentii* under exogenous oxidative stress.

Glutathione also plays an important role in cellular antioxidant defenses. More than 90% of the total glutathione pool was in the reduced form (GSH) and the remainder was in the oxidized form (glutathione disulfide, GSSG). As shown in **Figure 6C**, total glutathione levels in *C. laurentii* cells remained relatively stable under H_2O_2-induced oxidative stress. However, this form of oxidative stress showed an obvious inductive effect on total glutathione levels in *R. glutinis*. In terms of resistance to oxidative stress, the difference between *C. laurentii* and *R. glutinis* might be dependent on the various antioxidant systems.

GSH Treatment Improves the Viability and Biocontrol Efficiency of *C. laurentii*

Glutathione is considered the main ROS scavenger in cells (Schafer and Buettner, 2001). We evaluated the effects of exogenous GSH on the cell viability and biocontrol performance of *C. laurentii* under exogenous oxidative stress. Adding GSH to the culture medium could suppress the accumulation of intracellular ROS and enhance the tolerance of *C. laurentii* to H_2O_2-induced oxidative stress. When treated with 10 mM GSH, the percentage of *C. laurentii* cells exhibiting visible ROS staining was reduced by 23% (**Figures 7A,B**). When 10 mM GSH was added to YPD medium, the cell viability of *C. laurentii* was improved by 19% under moderately lethal oxidative stress (**Figure 7C**). Furthermore, we validated the beneficial effects of exogenous GSH on the biocontrol efficiency of *C. laurentii* against blue mold on peach fruits. Treatment with 1 mM GSH improved biocontrol efficiency of *C. laurentii* at the earlier intervals following inoculation (**Figures 8A,B**), whereas 10 mM GSH improved biocontrol efficacy of *C. laurentii* throughout the entire experiment. On days 4 and 5 post inoculation, the lesion diameters were reduced by 24 and 13%, respectively, following treatment with 10 mM GSH (**Figures 8A,B**).

DISCUSSION

In this study, we investigated the influence of exogenous oxidative stress on the viability and biocontrol efficiency of *C. laurentii* and the putative mechanisms of action. Furthermore, the methods by which the oxidative resistance of *C. laurentii* can be improved were explored.

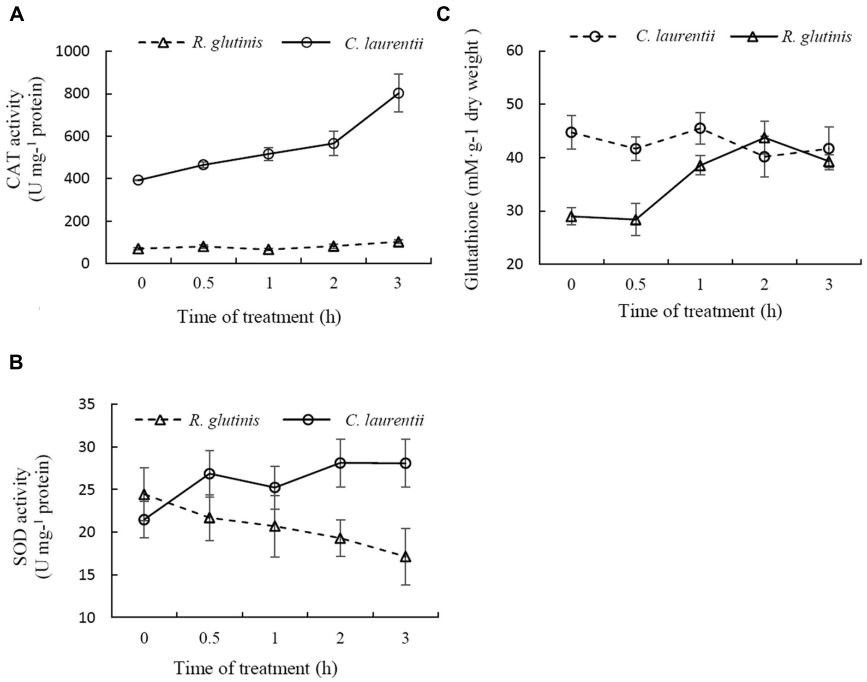

FIGURE 6 | Comparison between antioxidative reactions of *Cryptococcus laurentii* and *Rhodotorula glutinis* following treatment with moderately lethal concentrations of H_2O_2. (A) Determination of CAT activities of *C. laurentii* and *R. glutinis* cells following treatment with 300 mM and 30 mM H_2O_2, respectively. **(B)** Determination of SOD activities of *C. laurentii* and *R. glutinis* cells following treatment with 300 mM and 30 mM H_2O_2, respectively. **(C)** Determination of glutathione activity of *C. laurentii* and *R. glutinis* cells following treatment with 300 mM and 30 mM H_2O_2, respectively. Vertical bars represent standard errors of the means of three independent experiments.

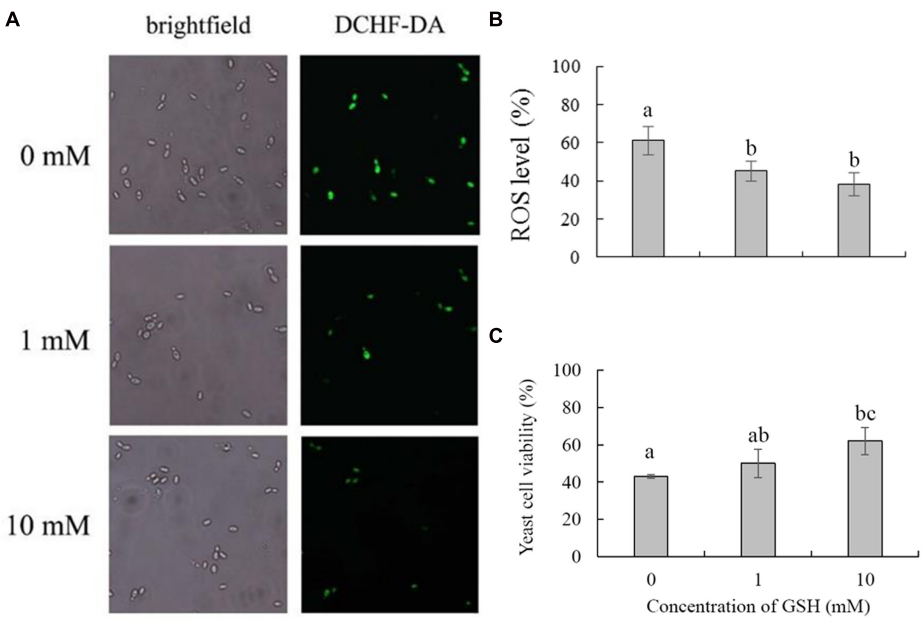

FIGURE 7 | Effects of GSH treatment on oxidative stress tolerance of *Cryptococcus laurentii*. (A) ROS accumulation in *C. laurentii* cells under oxidative stress following treatment with different concentrations of GSH. Detection of intracellular ROS was facilitated by staining with DCHF2-DA. **(B)** Percentage of *C. laurentii* cells exhibiting visible ROS accumulation under oxidative stress following treatment with GSH. **(C)** Viability of *C. laurentii* cells under oxidative stress following treatment with GSH. Vertical bars represent standard errors of the means of three independent experiments. Columns with different letters indicate significant differences ($P < 0.05$).

Over the past few decades, numerous laboratory studies have been conducted to evaluate the biocontrol performance of antagonistic yeasts (Droby et al., 2009; Sharma et al., 2009; Nunes, 2012; Liu et al., 2013). However, only a few yeast-based biocontrol products are presently available commercially. Various stresses in the natural environment that have significant effects on yeast viability and product stability make the use of commercial antagonistic yeasts as biocontrol agents particularly challenging (Chen et al., 2015; Sui et al., 2015). Oxidative stress is one of the major challenges posed to antagonistic yeasts in the control of pre- and postharvest diseases (Li and Tian, 2007; Macarisin et al., 2010). Enhancing the tolerance of antagonistic yeasts to oxidative stress is an effective way to improve their biocontrol ability.

The results of the present study indicate that *C. laurentii* possesses higher levels of adaptability to oxidative stress in comparison with other antagonistic yeasts, such as *R. glutinis* and *C. infirmominiatum*. Treating *C. laurentii* with 300 mM H_2O_2 for 90 min was moderately lethal to the cells (**Figure 1**), whereas the median lethal concentration of H_2O_2 for *R. glutinis* was 30 mM (Chen et al., 2015). After 20 min of incubation, the survival of *C. infirmominiatum* in 20 mM H_2O_2 was 23% (Liu et al., 2011b). Previous studies have indicated that competition for space and nutrients is a major factor that affects the resistance of antagonistic yeasts to postharvest fungal pathogens (Chan and Tian, 2005; Bautista-Rosales et al., 2014). These fungal pathogens infect the host tissue mainly through wounds inflicted during harvest, transportation, packinghouse operations, and storage processes (Barkai-Goland, 2001). Therefore, wound competence of yeasts is important in their mechanisms of antagonism against pathogens, even as they compete for space and nutrients. Castoria et al. (2003) suggested that resistance to oxidative stress represents a pivotal mechanism of action involved in wound competence of antagonistic yeasts, which is closely associated with their biocontrol activity. The *C. laurentii* isolate LS-28 showed higher biocontrol activity, compared with the *R. glutinis* isolate LS-11, a finding that could be attributed to the higher tolerance of *C. laurentii* to oxidative stress, as compared with *R. glutinis* (Castoria et al., 2003). In the present study, we also found that the biocontrol efficiency of *C. laurentii* was significantly suppressed under oxidative stress (**Figure 4**).

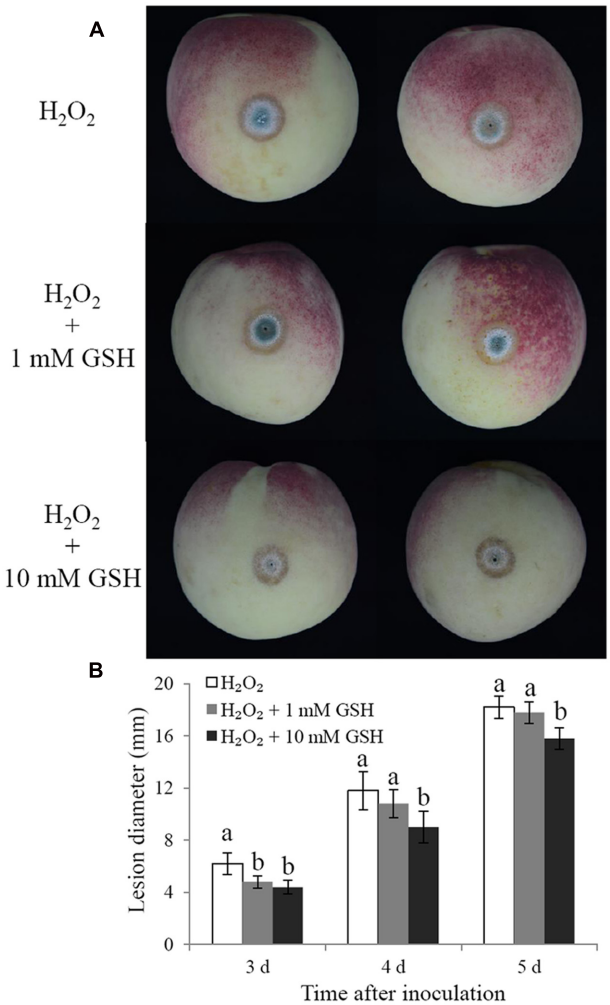

FIGURE 8 | Effects of GSH treatment on the biocontrol efficiency of *C. laurentii*. **(A)** Signs of disease on peach fruits at 5 days post inoculation. **(B)** Statistical analysis of lesion diameters on peach fruits at 3, 4, and 5 days post inoculation. Vertical bars represent standard errors of the mean. Columns with different letters indicate significant differences ($P < 0.05$).

As signal molecules, ROS can regulate senescence, apoptosis, and the stress response (Perrone et al., 2008). Low concentrations of ROS can activate a variety of antioxidant systems in yeast cells, and delay cell division that relies on the transcription factors Yap1p and Msn2/4p, thereby enhancing the resistance of yeast cells to subsequent lethal stress (Collinson and Dawes, 1992; Temple et al., 2005). However, excessive oxidative stress can cause a series of injuries to cellular components, including the cell membrane, proteins, lipids, and nucleic acids, resulting in compromised cell function or loss of viability (Reverter-Branchat et al., 2004; Branduardi et al., 2007).

Apoptosis and necrosis are two common forms of cell death that are associated with the viability of yeast cells. Apoptosis is a highly regulated form of programmed cell death that is characterized by nuclear DNA fragmentation, condensed chromatin, and inversion of the plasma membrane (Madeo et al., 1999; Poljak et al., 2003). The difference between apoptosis and necrosis is mainly manifested in the integrity of the cell membrane. When apoptosis occurs, the cell membrane remains intact, whereas in the necrotic cell, the membrane is broken down. The cell dye Hoechst 33342 has strong cell membrane permeability. However, PI cannot permeate the intact cell membrane. Flow cytometric analysis combined with Hoechst 33342-PI double staining is usually used to distinguish between viable, apoptotic, and necrotic cells (Sriram et al., 1992; Vermes et al., 2000). Thus, we used this method to investigate the mechanisms whereby H_2O_2 causes cell death in *C. laurentii*. The results of flow cytometry suggested that exogenous oxidative stress primarily triggered apoptosis in *C. laurentii* cells, resulting in the eventual suppression of viability. This indicated that the main mechanism associated with exogenous oxidative stress on *C. laurentii* cells was both systematic and progressive.

Both enzymatic and non-enzymatic antioxidant defense systems exist in yeasts (Jamnik and Raspor, 2005). In enzymatic antioxidant defense systems, SOD and CAT are two important components (Scandalios, 1993; Lee and Lee, 2000). SOD catalyzes the superoxide radical to H_2O_2, and H_2O_2 is then converted to H_2O and O_2, via the action of CAT. GSH, a small antioxidant molecule that is ubiquitous in plants and animals, plays a vital role in maintaining the antioxidant status of organisms (Cnubben et al., 2001; Herouart et al., 2002). *C. laurentii* cells subjected to treatment with exogenous H_2O_2, mainly employ enzymatic antioxidant defense systems against oxidative stress. In contrast, the ROS-sensitive yeast strain, *R. glutinis* tends to use the non-enzymatic antioxidant glutathione to resist oxidative stress. This might explain the disparity observed between *C. laurentii* and *R. glutinis* in their tolerance to oxidative stress.

Methods for improving stress resistance of biocontrol yeasts include preadaptation to stress, physiological manipulation, and the addition of anti-stress compounds to the medium. Previous reports have shown that combining biocontrol yeasts with exogenous chemical compounds, such as calcium (Tian et al., 2002), salicylic acid (Qin et al., 2003), sodium bicarbonate (Yao et al., 2004), and trehalose (Li and Tian, 2006) are effective ways to enhance their biocontrol performance. GSH has strong antioxidant capacity, and can be easily absorbed by cells. In the present study, we first validated the application of exogenous GSH as an effective method to improve oxidative stress tolerance in *C. laurentii*. In addition, the protective effect of GSH on the biocontrol efficiency of *C. laurentii* was confirmed on altered peach fruits. These results provide us with a potential alternative to enhancing the environmental adaptability and biocontrol performance of antagonistic yeasts.

CONCLUSION

We found that oxidative stress could induce apoptosis in *C. laurentii* that further leads to a reduction in cell viability and biocontrol efficiency. The enzymatic defense system might play a significant role in the antioxidative effects of *C. laurentii*. The addition of the non-enzymatic compound GSH to the culture media is an effective method to improve the oxidative stress resistance and biocontrol efficiency of *C. laurentii*.

AUTHOR CONTRIBUTIONS

ST conceived and designed the experiments. ZZ, JC, CH, and YC performed the experiments. ZZ analyzed the data. ZZ and BL drafted the manuscript. All authors read and approved the final manuscript.

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Efficient Exploitation of Multiple Novel Bacteriocins by Combination of Complete Genome and Peptidome

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Backgroud: The growing emergence of antibiotic-resistant pathogens including the most dangerous superbugs requires quick discovery of novel antibiotics/biopreservatives for human health and food safety. Bacteriocins, a subgroup of antimicrobial peptides, have been considered as promising alternatives to antibiotics. Abundant novel bacteriocins are stored in genome sequences of lactic acid bacteria. However, discovery of novel bacteriocins still mainly relies on dubious traditional purification with low efficiency. Moreover, sequence alignment is invalid for novel bacteriocins which have no homology to known bacteriocins in databases. Therefore, an efficient, simple, universal, and time-saving method was needed to discover novel bacteriocins.

Methods and Results: Crude bacteriocins from both cell-related and culture supernatant of *Lactobacillus crustorum* MN047 fermentation were applied to LC-MS/MS for peptidome assay, by which 131 extracellular peptides or proteins were identified in the complete genome sequence of *L. crustorum* MN047. Further, the genes of suspected bacteriocins were verified by expressed in *Escherichia coli* BL21 (DE3) pLysS. Thereafter, eight novel bacteriocins and two nonribosomal antimicrobial peptides were identified to be broad-spectrum activity against both Gram-positive and Gram-negative bacteria, including some multidrug-resistant strains. Among them, BM1556 located within predicted bacteriocin gene cluster. The most active bacteriocin BM1122 had low MIC values of 13.7 mg/L against both *Staphylococcus aureus* ATCC29213 and *E. coli* ATCC25922. The BM1122 had bactericidal action mode by biofilm-destruction, pore-formation, and membrane permeability change.

Conclusions: The combination of complete genome and peptidome is a valid approach for quick discovery of novel bacteriocins without/with-low homology to known ones. This method will contribute to deep exploitation of novel bacteriocins in genome of bacteria submitted to GenBank.

Keywords: novel bacteriocins, genome, peptidome, cloning and expression, antibiotic-resistance

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INTRODUCTION

Since the discovery of penicillin in 1929, antibiotics have played an indispensable role in human medicine and food production. Unfortunately, antimicrobial resistance is found in all microorganisms, whether bacteria, fungi, virus or parasites (Sabtu et al., 2015). Moreover, many bacteria are resistant to not only single but multiple antibiotics. In 2014, 480,000 new cases of

multidrug-resistant tuberculosis (MDR-TB) were estimated by WHO. Among MDR-TB, 9.7% were extensively drug-resistant tuberculosis (XDR-TB), which was identified in 105 countries. *Klebsiella pneumoniae* resistant to carbapenems, which is usually the last line of available treatment, was reported in all WHO regions (Band et al., 2018). According to "global PPL" published by WHO in 2017, the most important resistant bacteria had resistance to carbapenem, 3rd generation cephalosporin, vancomycin, methicillin, clarithromycin, etc. Antibiotic resistance makes the existing drugs become increasingly ineffective or even invalid. Consequently, many common infections are becoming risky or untreatable, leading to longer illnesses and higher mortality, like returning to the pre-antibiotic era. The Centers for Disease Control and Prevention (CDC) estimated that antimicrobial-resistance had caused at least two million illnesses and 23,000 deaths each year in the United States alone (Leal et al., 2017), leading to health and economic burden up to $55 billion directly or indirectly. Undoubtedly, antibiotic resistance has become one of the biggest threats to global health in the twenty-first century (Arango-Argoty et al., 2018). However, the discovery of new antimicrobial drugs lags behind the emergence of superbugs. Recently, bacteriocins have attracted much attention because they are not only used as food preservatives but also looked as promising alternatives to antibiotics (Cotter et al., 2013). Bacteriocins are ribosomally synthesized antimicrobial peptides or proteins produced by bacteria with narrow or broad antibacterial spectrum (Cotter et al., 2005). Many bacteriocins are produced by lactic acid bacteria which are generally recognized as safe (GRAS) (Alvarez-Sieiro et al., 2016).

Although hundreds of bacteriocins have been discovered, only nisin has been widely used as food preservative in more than fifty countries. The limitation of bacteriocins as preservatives and antimicrobials in the market mainly derives from narrow-spectrum inhibitory activity and high cost of their commercial production (Fahim et al., 2016). Thus, exploitation of novel bacteriocins with broad-spectrum activity is vital for the development of antibiotic substitutes. Generally, novel bacteriocins are obtained and characterized through traditional purification process combining with mass spectrum (Stern et al., 2006). Okubo et al. (2012) and Ngoc Hieu et al. (2016) purified the proteins, and then applied them to LC-MS/MS to *de novo* sequencing. Kuyama et al. (2015) purified a human basic fetoprotein, which was subsequently identified by N/C-terminal sequencing. However, the traditional method is time-consuming, most importantly, the uncertainty of novelty makes the research easy to become meaningless. Further, more than one kind of bacteriocins can be produced by lactic acid bacteria, which adds the difficulty by traditional purification. In addition, processes of traditional purification vary with different samples as the component diversity.

Comparing with traditional purification, bacteriocin gene sequence alignment is much more simple, clear and, convenient (Porto et al., 2017). Moreover, another sequence search way of pattern-matching, such as profile Hidden Markov Models (profile-HMM) and regular expressions (REGEX) (Porto et al., 2017), had higher efficiency and higher precision. Lactic acid bacteria (LAB, e.g., *Lactococcus*, *Enterococcus*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Leuconostoc,* and *Lactobacillus* species) are the richest sources for bacteriocin production (Makarova et al., 2006). Many genome data of these LAB were stored in GenBank of NCBI. For example, only for *Lactobacillus*, complete or draft genome sequences of 173 species were submitted. Bacteriocin-coding genes can be identified according to their identity with known bacteriocins in databases. However, the novel bacteriocins, which are possible promising, will be overlooked by sequence alignment if they are not very similar to known genes (Tatusova et al., 2016). Therefore, an efficient, simple, universal and time-saving method was needed to discover more novel bacteriocins.

In the study of Dong et al. (2014), a LC-MS/MS-based *de novo* sequencing assisted database search was used to identified phosphopeptides, which simplified the whole analysis. However, the used database gathering abundant species limited specificity and accuracy. In this study, with experiments-based function confirmation, a combined method of complete genome of bacteriocin-producer itself and peptidome was used to specifically discover novel bacteriocins produced by probiotic *L. crustorum* MN047. The antimicrobial activities of multiple bacteriocins against pathogens were investigated, including against antibiotic-resistant isolates. Then, action mode of the most activity bacteriocin was preliminarily studied.

MATERIALS AND METHODS

Function Annotation

Complete genome of *L. crustorum* MN047 was sequenced in our previous study (Yi et al., 2017). Function annotation was performed using local alignment with databases of NR (Non-Redundant Protein Database), Swiss-Prot, TrEMBL, COG (the Clusters of Orthologous Groups of proteins), KEGG (the Kyoto Encyclopedia of Genes and Genomes), and GO (Gene Ontology). Genes potentially related with bacteriocin were identified using BAGEL4 (http://bagel4.molgenrug.nl/) and antiSMASH (http://antismash.secondarymetabolites.org) (Liu et al., 2016).

Peptidome

The *L. crustorum* MN047 was statically incubated in MRS medium (2 L) at 30°C for 60 h, after which cell suspension was divided into two equal parts to prepare crude bacteriocin samples. One was prepared by ammonium sulfate precipitation as previous study (Yi et al., 2016). The other was made by pH-mediated cell adsorption-desorption method (Yang et al., 1992). For the latter, adsorption and desorption pH value was 5.85 and 2.10, respectively. Briefly, cell suspension was heated at 70°C for 30 min to kill cells and inactivate enzymes. Then, pH of cell suspension was adjusted to 5.85 and slowly stirred at 4°C for 120 min. Cells were harvested by centrifugation and washed by citric acid-phosphate buffer (pH 5.85) for 3 times. Thereafter, pH of cell suspension was adjusted to 2.10. Supernatant was obtained by centrifugation and its pH value was adjusted to 7.0, namely, crude bacteriocin was acquired.

In order to identify potential bacteriocins produced by *L. crustorum* MN047, the two samples were respectively applied to LC-MS/MS, a Q Exactive mass spectrometer that was coupled to LC-20AD (Shimadzu, Japan). Samples were pretreated using Amicon Ultra centrifugal Filters (10 K, Millipore, USA). Filtered component was loaded onto a Trap column at 8 μL/min, followed by a LP-C18 analytical column (180 mm length × 76 μm i.d., 3 μm) at 300 nL/min under gradient elution. MS and MS/MS data were acquired and switched under DDA (data dependent acquisition) mode. Scan of mass-to-charge ratio was 350–1,800 m/z at resolution of 70,000 for MS and HCD (high-energy collisional dissociation) fragmentation was used at resolution of 17,500 for MS/MS. Raw files of MS/MS spectra were applied to Maxquant 1.5.2.8 (Cox and Mann, 2008) and MASCOT 2.2 (Koenig et al., 2008) against database of complete genome sequence of *L. crustorum* MN047, as well as databases of antimicrobial peptide APD3 (Wang et al., 2016) (http://aps.unmc.edu/AP/) and CAMP (Waghu et al., 2016) (http://www.camp3.bicnirrh.res.in/). Function annotation of suspicious bacteriocins was conducted by InterPro (http://www.ebi.ac.uk/interpro/) (Jones et al., 2014).

Heterologous Expression of Bacteriocins

Escherichia coli expression system was used to obtain each one of suspicious bacteriocins. The pET-30a (Novagen, Germany) and *E. coli* BL21 (DE3) pLysS (Trans, China) were used as expression vector and expression host, respectively. Genome of *L. crustorum* MN047 was extracted and used as template to amplify genes of 8 hypothetical bacteriocins with 8 pairs of primers shown in **Table 1**. All PCR reactions ran for 35 cycles under corresponding annealing temperature of each primer pair and PCR products were purified using Gel Extraction Kit (OMEGA, USA). After which, PCR products were inserted into pET-30a by digested with *FlyCut*^{TM} *Nde* I and *FlyCut*^{TM} *Hind* III and ligated with T4 Ligase (all from Trans, China). Specifically, there were no encoding genes on genome for nonribosomal antimicrobial peptide EP-20 and GP-19, so their encoding genes were directly synthesized (Sangon, China) (**Table 1**) according to amino acid sequence. These recombinant plasmids with specific bacteriocin gene were separately transformed into *E. coli* BL21 (DE3) pLysS competent cells by heat shock at 42°C. The transformants with correct insertion of bacteriocin genes were confirmed by PCR amplification and sequencing.

Escherichia coli BL21(DE3) pLysS carrying recombinant vector was incubated in 100 mL LB broth containing kanamycin to an OD_{600} of 0.6 at 37°C (shaking at 150 rpm), and then induced by isopropyl β-D-thiogalactoside (IPTG) over night at 25°C (shaking at 180 rpm). Cells were harvested by centrifugation (4,000 ×g, 4°C, 15 min), washed by PBS (pH 7.2) for three times, and resuspended in 20 mM Tris-HCl (pH 6.68). Cells were repeated 5 freeze-thaw cycles to disrupt cells and heated at 80°C to kill cells and inactivate enzymes. The supernatants (1 mL) were collected by centrifugation (16,000 ×g, 4°C, 15 min) and applied to antimicrobial activity test by agar well diffusion method as previous study (Lü et al., 2014) using *S. aureus* ATCC29213 as indicator. The concentrations of kanamycin and IPTG of each bacteriocin were optimized until an obvious antibacterial activity was gotten. The same treatment of each bacteriocin transformant without inducement was used as control in antimicrobial activity test. One arbitrary unit (AU) was defined as the reciprocal of the highest dilution of the bacteriocin showing a visible clear zone (Jabrane et al., 2002).

Antimicrobial Spectrum

Some Gram-positive and Gram-negative foodborne pathogenic bacteria including multidrug-resistant strains (**Table 2**) were used to investigate the inhibitory spectrum of 8 bacteriocins and 2 nonribosomal antimicrobial peptides. Supernatant of cell disruption was used and antimicrobial activity toward all indicators was measured by the agar well diffusion assay.

Purification of BM1122

Crude bacteriocin BM1122 was dialyzed in a dialysis tube with MW 8,000. Then, the sample within tube passed through an Amicon Ultra centrifugal Filter (10 K). The permeate was concentrated and purified on an AKTA system (AKTA Purifier 100, GE, Sweden) equipped with an UV detector and an automatic collector. Sample was loaded on a HiTrap Q FF anion-exchange column at 1.5 mL/min. Citric acid-phosphate buffer (pH 6.0) was used as equilibrium buffer. Sample was gradiently eluted with 1 M NaCl in equilibrium buffer. Then, the active fraction was purified by analytical RP-HPLC (Waters 1525, USA) equipped with an Agilent ZORBAX 300SB-C18 column (250 × 4.6 mm, 5 μm). The mobile phase A (80% H_2O, 20% acetonitrile and 0.05% TFA) and mobile phase B (100% acetonitrile) were used in gradient elution. The purified BM1122 was digested by trypsin (37°C, 16 h), and then desalted by a manual Pierce C18 Tips (USA, Thermo Scientific). Subsequently, the sample was applied to LC-MS/MS as above to identify the BM1122.

MIC Value

The BM1122 from RP-HPLC was concentrated and applied to measurement of MIC value as described method (Bhattacharyya et al., 2017) in 96 well plate (Costar, Corning, USA). *S. aureus* ATCC 29213 and *E. coli* ATCC 25922 were used as indicators. Specifically, indicators were cultured at 37°C to log-phase and diluted using fresh LB broth to about 10^6 CFU/mL. The initial concentration of BM1122 was adjusted to 2 g/L using sterile PBS buffer, and then it was double diluted to 2^{-1}, 2^{-2}, 2^{-3}, 2^{-4}, 2^{-5}, 2^{-6}, 2^{-7}, 2^{-8}, 2^{-9}, and 2^{-10}. Ten microliter of each BM1122 dilution and 90 μL indicator dilution were mixed in wells, and then incubated at 37°C for 24 h. MIC was defined as the lowest concentration at which no growth of indicator was observed. Meanwhile, protein concentration of each diluted bacteriocin was measured again by BCA assay in case of deviations of concentrations during serial dilution and BSA was used as standard.

Time-Kill Curve

Foodborne pathogen *L. monocytogenes* CMCC54004 and *C. sakazakii* ATCC29544 were used as indicators. Indicators were cultured at 37°C to logarithmic-phase (10^6–10^8 CFU/mL), and then sample BM1122 was added to a final concentration of 4 × MIC. Indicators were continuously incubated at 37°C,

TABLE 1 | Oligonucleotide primers used in PCR amplification and synthesized genes in this study.

Primers	Sequence (5′ → 3′)	Restriction site
BM173F	GGGAATTCCATATGTCACAAAATACACATAAAGG	*Nde* I
BM173R	CCCAAGCTTTTATTTAGATTCTTTAGTATCACGG	*Hind* III
BM797F	GGGAATTCCATATGAGATATAAAGTTACTTTAG	*Nde* I
BM797R	CCCAAGCTTCTATGCACTCATTTTCAATAAT	*Hind* III
BM1029F	GGGAATTCCATATGGATAAACCAAATATTGCTGAAAT	*Nde* I
BM1029R	CCCAAGCTTTTATTGATGCAATTTGATATATTCTTGA	*Hind* III
BM1122F	GGGAATTCCATATGGCAAATAAAGCTGAACTTATTG	*Nde* I
BM1122R	CCCAAGCTTTTATTTAACTGAGTCCTTCAAAGCCTTA	*Hind* III
BM1556F	GGGAATTCCATATGAAAAGAATATTGTTAAAGTC	*Nde* I
BM1556R	CCCAAGCTTTTAATGCCAGCGTTGATCAAT	*Hind* III
BM1829F	GGGAATTCCATATGGCAGAAGTAGATCCATCAAAGAT	*Nde* I
BM1829R	CCCAAGCTTTTATTTTTTAAGGTTTTTTTCGACAAAATCACG	*Hind* III
BMP11F	GGGAATTCCATATGAGTATAAATAAGCAAAAAATTTCG	*Nde* I
BMP11R	CCCAAGCTTTTATTTTCCCTTTTTAATGGTCTTAATGA	*Hind* III
BMP32F	GGGAATTCCATATGACAGTCACAGATCCGCGTAGTCCG	*Nde* I
BMP32R	CCCAAGCTTTCAAGCGATCATCGCGCCACTACTCGTT	*Hind* III
EP-20	GGGAATTCCATATGGAAGGTCCGGTTGGTCTGGCAGA TCCGGATGGTCCGGCAAGCGCACCGCTGGGTGCACCG TAA*AAGCTT*GGG	*Nde* I, *Hind* III
GP-19	GGGAATTCCATATGGGTCCGGTTGGTCTGCTGAGCAG CCCGGGTAGCCTGCCGCCGGTTGGTGGTGCACCGTAA *AAGCTT*GGG	*Nde* I, *Hind* III

F, forward primer; R, reverse primer; the underlined sequence is restriction enzyme site.

and 0.5 mL bacterial suspensions were taken immediately at appointed times (0.5, 1, 1.5, and 2 h). Cell suspensions were 10-fold serially diluted using sterile PBS buffer (pH 7.2). Cells of two appropriate serial dilutions were spread on plates of LB medium with triplicate. Bacterial colonies were counted after cultivation at 37°C for 24 h. Indicators without bacteriocin were used as controls.

Scanning Electron Microscope and Transmission Electron Microscope

Electron microscopes were used to visualize action mode of BM1122. Foodborne pathogen *L. monocytogenes* CMCC54004 and *C. sakazakii* ATCC29544 were used as indicators. Exponential-phase indicators were treated by a concentration of 2 × MIC bacteriocin at 37°C for 0.5 and 2 h, respectively. After washing, fixation and dehydration as described method (Yi et al., 2016), cells were dried by CO_2 and coated with gold. Ultrastructure of indicators was observed using a high resolution Nova NanoSEM 450 scanning electron microscope (SEM) (FEI, USA).

Pretreatment of indicators for transmission electron microscope (TEM) was the same with that for scanning electron microscope. After post-fixed by osmic acid, cells were dehydrated using alcohol and permeated using white resin. Embedding was performed by roasting at 55°C for 48 h. Seventy nanometer thin sections were prepared on copper grids and stained with lead citrate and uranyl acetate. Ultrastructure observation was conducted on a Tecnai G2 Spirit Bio-Twin TEM (FEI, USA).

Data Availability

All sequence data that support the findings of this study have been deposited in GenBank under accession numbers: CP017996 (chromosome), CP017997 (plasmid MN047p1), and CP017998 (plasmid MN047p2).

RESULTS

Function Annotation

Lactobacillus crustorum MN047, firstly isolated from koumiss, which could produce at least three bacteriocins in our previous study (Yi et al., 2016). Complete genome sequence was investigated to analyze genes related to antimicrobial activity. There were 2218 protein coding genes after prediction of its complete genome sequence. A total of 2,176 genes were assigned a putative function by elaborate annotation based on local alignment using six databases. A gene cluster of bacteriocin (locus_tag: BI355_1567-BI355_1581) (**Figure 1**) was identified using secondary metabolite-specific database antiSMASH. Among the 15 putative *orf*s, homologous proteins of the proteins encoded by *orf*6, *orf*9, and *orf*10 were widely found in other bacteriocin biosynthetic gene clusters (Smokvina et al., 2013; Toh et al., 2013). The structure of bacteriocin gene cluster predicted in *L. crustorum* MN047 was similar to that of multipeptide leaderless bacteriocin family (Ovchinnikov et al., 2016), in which a metal resistance protein gene was included beside bacteriocin export/regulation related genes. Bacteriocin immunity gene was not included in the predicted bacteriocin gene cluster, which was a common feature among

TABLE 2 | The inhibitory spectrum of 8 novel bacteriocins and 2 nonribosomal antimicrobial peptides.

Indicator strains	Diameter of inhibition zone (mm)									
	BM173	**BM797**	**BM1029**	**BM1122**	**BM1556**	**BM1829**	**BMP11**	**BMP32**	**EP-20**	**GP-19**
GRAM-POSITIVE										
Staphylococcus aureus ATCC29213	21.4 ± 0.3	20.0 ± 0.7	20.8 ± 0.2	23.1 ± 0.8	15.9 ± 0.1	18.8 ± 0.2	22.0 ± 0.6	19.7 ± 0.6	15.3 ± 0.2	19.2 ± 0.7
S. aureus ATCC25923	21.6 ± 0.4	19.9 ± 0.1	20.9 ± 0.4	24.4 ± 0.7	16.0 ± 1.1	18.8 ± 0.1	22.8 ± 0.4	19.0 ± 0.1	17.4 ± 0.6	19.1 ± 0.5
Enterococcus faecalis ATCC29212	12.6 ± 1.4	0	16.0 ± 0.2	12.9 ± 0.2	0	11.0 ± 0.3	13.6 ± 0.5	15.2 ± 0.4	10.2 ± 1.1	10.3 ± 0.3
Listeria monocytogenes CMCC54004	19.5 ± 0.5	17.5 ± 0.4	19.1 ± 1.0	21.7 ± 0.8	12.8 ± 0.5	17.9 ± 0.3	21.8 ± 0.6	16.7 ± 0.5	12.1 ± 1.6	17.2 ± 0.9
Antibiotic-resistant *S. aureus* 1[a]	12.7 ± 0.6	17.5 ± 0.2	0	13.2 ± 0.7	0	0	17.3 ± 0.6	0	0	0
Antibiotic-resistant *S. aureus* 2[b]	0	17.6 ± 0.6	0	0	0	0	9.8 ± 0.5	0	0	0
Antibiotic-resistant *S. aureus* 3[c]	11.2 ± 0.5	18.1 ± 1.7	0	10.6 ± 0.7	0	0	13.7 ± 0.5	0	0	0
Antibiotic-resistant *S. aureus* 4[d]	0	15.6 ± 0.7	0	11.6 ± 1.0	0	0	13.1 ± 0.7	0	0	0
Antibiotic-resistant *S. aureus* 5[e]	0	13.0 ± 0.8	0	0	0	0	12.0 ± 1.2	0	0	0
GRAM-NEGATIVE										
Escherichia coli ATCC25922	20.2 ± 0.4	14.4 ± 0.7	19.1 ± 0.5	20.9 ± 0.4	13.0 ± 0.6	17.0 ± 1.0	20.7 ± 0.9	17.4 ± 0.5	12.3 ± 0.7	17.0 ± 1.6
Salmonella CMCC 50071	26.7 ± 0.7	14.6 ± 0.3	22.7 ± 1.5	27.3 ± 1.5	17.6 ± 1.4	17.7 ± 0.6	23.6 ± 1.3	20.6 ± 0.8	12.1 ± 0.4	18.7 ± 0.5
Cronobacter sakazakii ATCC29544	18.7 ± 1.0	10.8 ± 0.4	19.3 ± 0.4	22.7 ± 0.7	16.1 ± 0.3	19.4 ± 0.3	21.5 ± 0.1	17.6 ± 0.5	15.0 ± 1.1	18.0 ± 0.7
Antibiotic-resistant *Salmonella* 36T[f]	0	0	0	0	0	0	0	0	0	0
Antibiotic-resistant *Salmonella* 87T4[g]	0	0	0	0	0	0	0	0	0	0
Antibiotic-resistant *Salmonella* 557D[h]	19.7 ± 0.7	9.6 ± 0.3	17.7 ± 1.1	22.0 ± 0.9	13.1 ± 0.4	13.9 ± 0.6	21.5 ± 0.4	18.6 ± 1.7	12.0 ± 1.1	15.6 ± 1.4
Antibiotic-resistant *Salmonella* 798D[i]	0	0	0	0	0	0	0	0	0	0
Antibiotic-resistant *Salmonella* 1006D[j]	0	0	0	0	0	0	0	0	0	0
Antibiotic-resistant *C. sakazakii* 6–12 (1)[k]	19.5 ± 0.3	10.4 ± 1.0	20.5 ± 0.4	22.3 ± 0.5	15.4 ± 0.9	18.0 ± 0.3	22.9 ± 0.9	18.4 ± 0.3	14.6 ± 0.3	16.9 ± 1.0
Antibiotic-resistant *C. sakazakii* 6–12 (2)[l]	20.3 ± 0.6	10.8 ± 0.5	17.8 ± 0.6	21.9 ± 0.9	15.7 ± 1.2	17.4 ± 0.3	22.7 ± 0.4	19.3 ± 1.0	14.9 ± 0.9	18.4 ± 0.4
Antibiotic-resistant *C. sakazakii* 11–18 (2)[m]	17.9 ± 0.7	10.9 ± 0.4	20.0 ± 0.2	21.5 ± 0.4	16.0 ± 1.2	18.3 ± 0.5	21.6 ± 1.3	18.5 ± 0.4	15.1 ± 0.2	17.4 ± 0.4
Antibiotic-resistant *C. sakazakii* 14–18 (2)[n]	22.3 ± 0.7	9.9 ± 0.3	20.4 ± 0.7	23.5 ± 0.9	16.8 ± 0.6	18.3 ± 0.2	23.2 ± 0.9	21.2 ± 0.6	15.7 ± 0.6	19.4 ± 1.2
Antibiotic-resistant *C. sakazakii* 18–15 (2)[o]	20.3 ± 0.6	9.4 ± 0.3	20.1 ± 0.8	21.8 ± 0.2	15.0 ± 0.2	17.8 ± 0.4	22.4 ± 0.2	17.5 ± 0.3	18.1 ± 0.8	17.7 ± 0.1

[a] *Resistant to cephalothin, ciprofloxacin, clarithromycin, cefuroxime, cefoxitin, gentamicin, levofloxacin, tobramycin, ofloxacin, oxacillin, piperacillin, vancomycin.*

[b] *Resistant to ciprofloxacin, cefoxitin, gentamicin, levofloxacin, minocycline, tobramycin, ofloxacin, penicillin, piperacillin, vancomycin.*

[c] *Resistant to ciprofloxacin, cefoxitin, gentamicin, levofloxacin, minocycline, tobramycin, ofloxacin, vancomycin.*

[d] *Resistant to ciprofloxacin, cefoxitin, gentamicin, levofloxacin, minocycline, tobramycin, ofloxacin, vancomycin.*

[e] *Resistant to ciprofloxacin, cefoxitin, gentamicin, levofloxacin, minocycline, tobramycin, ofloxacin, oxacillin, vancomycin.*

[f] *Resistant to ampicillin, amoxicillin, amikacin, chloramphenicol, ciprofloxacin, cefoperazone, ceftriaxone, gatifloxacin, gentamicin, kanamycin, levofloxacin, nalidixic acid, trimethoprim-sulfamethoxazole, tetracycline.*

[g] *Resistant to ampicillin, amoxicillin, amikacin, chloramphenicol, ciprofloxacin, cephalothin, cefoperazone, ceftriaxone, gatifloxacin, gentamicin, kanamycin, levofloxacin, nalidixic acid, streptomycin, trimethoprim-sulfamethoxazole, tetracycline.*

[h] *Resistant to ampicillin, amoxicillin, amikacin, chloramphenicol, cefoxitin, gatifloxacin, gentamicin, kanamycin, levofloxacin, nalidixic acid, trimethoprim-sulfamethoxazole, tetracycline.*

[i] *Resistant to ampicillin, amoxicillin, amikacin, chloramphenicol, ciprofloxacin, cephalothin, ceftriaxone, cefoxitin, gatifloxacin, gentamicin, kanamycin, levofloxacin, nalidixic acid, streptomycin, trimethoprim-sulfamethoxazole, tetracycline.*

[j] *Ampicillin, amoxicillin, chloramphenicol, gentamicin, kanamycin, nalidixic acid, trimethoprim-sulfamethoxazole, tetracycline.*

[k] *Cefoxitin, rifampin.*

[l] *Chloramphenicol, rifampin, tetracycline.*

[m] *Ciprofloxacin, rifampin, streptomycin.*

[n] *Ciprofloxacin, rifampin, streptomycin.*

[o] *Ampicillin, amoxicillin, chloramphenicol, nalidixic acid, rifampin, streptomycin, tetracycline.*

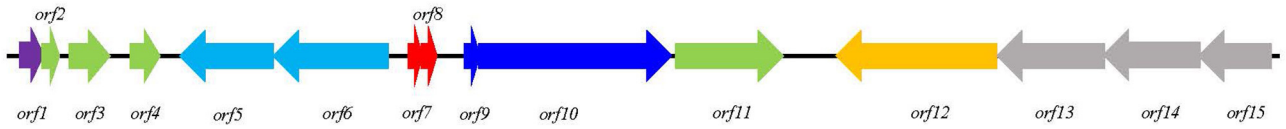

FIGURE 1 | Schematic overview of the bacteriocin gene cluster predicted in *L. crustorum* MN047 (drawn in scale). Orf1, hypothetical protein (transcriptional regulator, 47%); orf2, hypothetical protein (IS5/IS1182 family transposase, 94%); orf3, hypothetical protein (IS5/IS1182 family transposase, 55%); orf4, hypothetical protein (IS5/IS1182 family transposase, 63%); orf5, hypothetical protein (ABC transporter permease, 46%); orf6, bacteriocin ABC transporter ATP-binding protein; orf7, hypothetical protein; orf8, hypothetical protein; orf9, hypothetical protein (bacteriocin ABC transporter, 58%); orf10, bacteriocin ABC transporter; orf11, IS30 family transposase; orf12, Magnesium transporter; orf13, RluA family pseudouridine synthase; orf14, NAD kinase; orf15, GTP pyrophosphokinase. Putative bacteriocin structure genes (red), ABC transporter genes (blue), transport-related genes (light blue), regulatory related genes (green), transcriptional regulator (purple), metal resistance gene (orange), and other genes (gray).

TABLE 3 | Information statistics of targeted suspicious bacteriocins.

NO.	Name[a]	Sequences	Function annotation[b]
1	BM173	MSQNTHKGMTGHRRPVNQKNGAEKRAKTQAVLDFLRSRDTKESK	hypothetical protein
2	BM797	MRYKVTLDTKQQLFTVFDKKNTRVSACGKSIEEAMNKLLKMSA	hypothetical protein
3	BM1029	MDKPNIAEMIIQYEKNKDMTDTQFAFESHLSVERVHNLKSGDYEPTA DEIKTVQEYIKLHQ	putative λ repressor-like DNA-binding protein
4	BM1122	MANKAELIDSVASKTGLTKKDATSAVDAVFETIQENLSEGNKVQLIGFGN FEVRQRAARKGRNPQTGEEIKIPASKVPAFKPGKALKDSVK	DNA-binding protein
5	BM1556	MKRILLKSDRTLDDSELAKVIGGGFFEGIGRWIDQRWH	putative bacteriocin
6	BM1829	MAEVDPSKMADAAIAKEPEVLNLKMSEAFDWSDDDTVVRDAIWD YFMENNNHDTVKTEEAEKPFLDMKDEEVRDFVEKNLKK	hypothetical protein
7	BMP11	MSINKQKISRNKVLNLLTLFQLLISLYQVIKTIKKGK	putative transmembrane protein
8	BMP32	MTVTDPRSPLTTWIFFCSKKTTPPLKGAVVMPNSGLSRHLHYLRLSSR CLSNSRNTPTSSGAMIA	hypothetical protein

[a] *BM is the abbreviation of bacteriocin MN047.*
[b] *Predicted by InterPro (http://www.ebi.ac.uk/interpro/).*

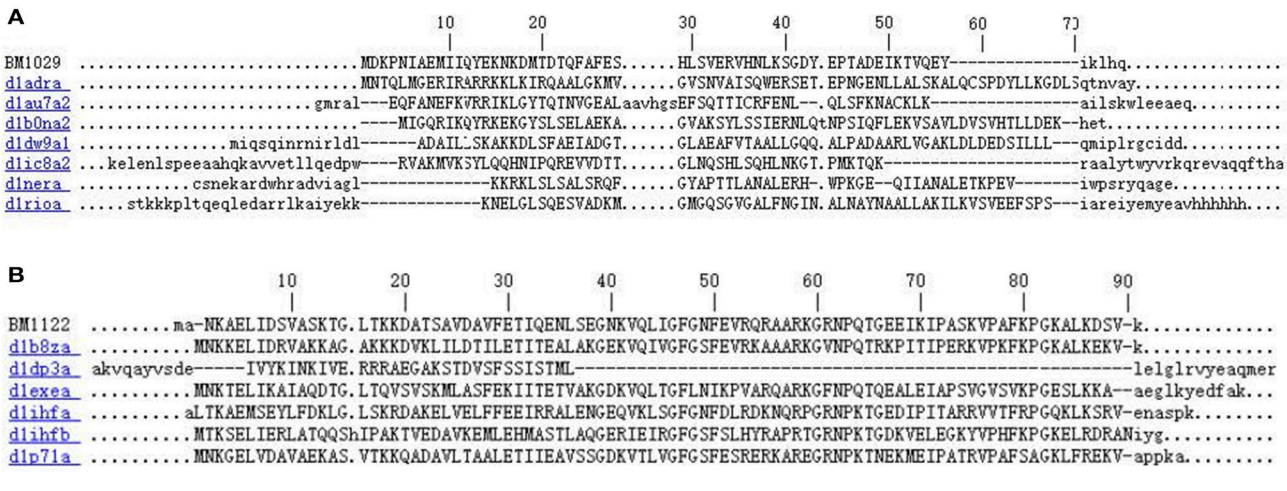

FIGURE 2 | Sequence alignments of BM1029 **(A)** and BM1122 **(B)** with DNA-binding domain subfamily proteins by InterPro.

leaderless bacteriocins (Alvarez-Sieiro et al., 2016). One or both proteins from *orf7* and *orf8* were putative bacteriocin structural gene, which should be verified further. Although lack of genes encoding immunity protein in the bacteriocin gene cluster, four genes of bacteriocin immunity protein (locus_tag: BI355_0201, BI355_0202, BI355_2153, and BI355_2161) were found on chromosome far from the bacteriocin gene cluster. The multiple bacteriocin immunity proteins may be related to the multiple bacteriocins produced by *L. crustorum* MN047. In addition, a CvpA family protein (colicin V production protein, locus_tag:

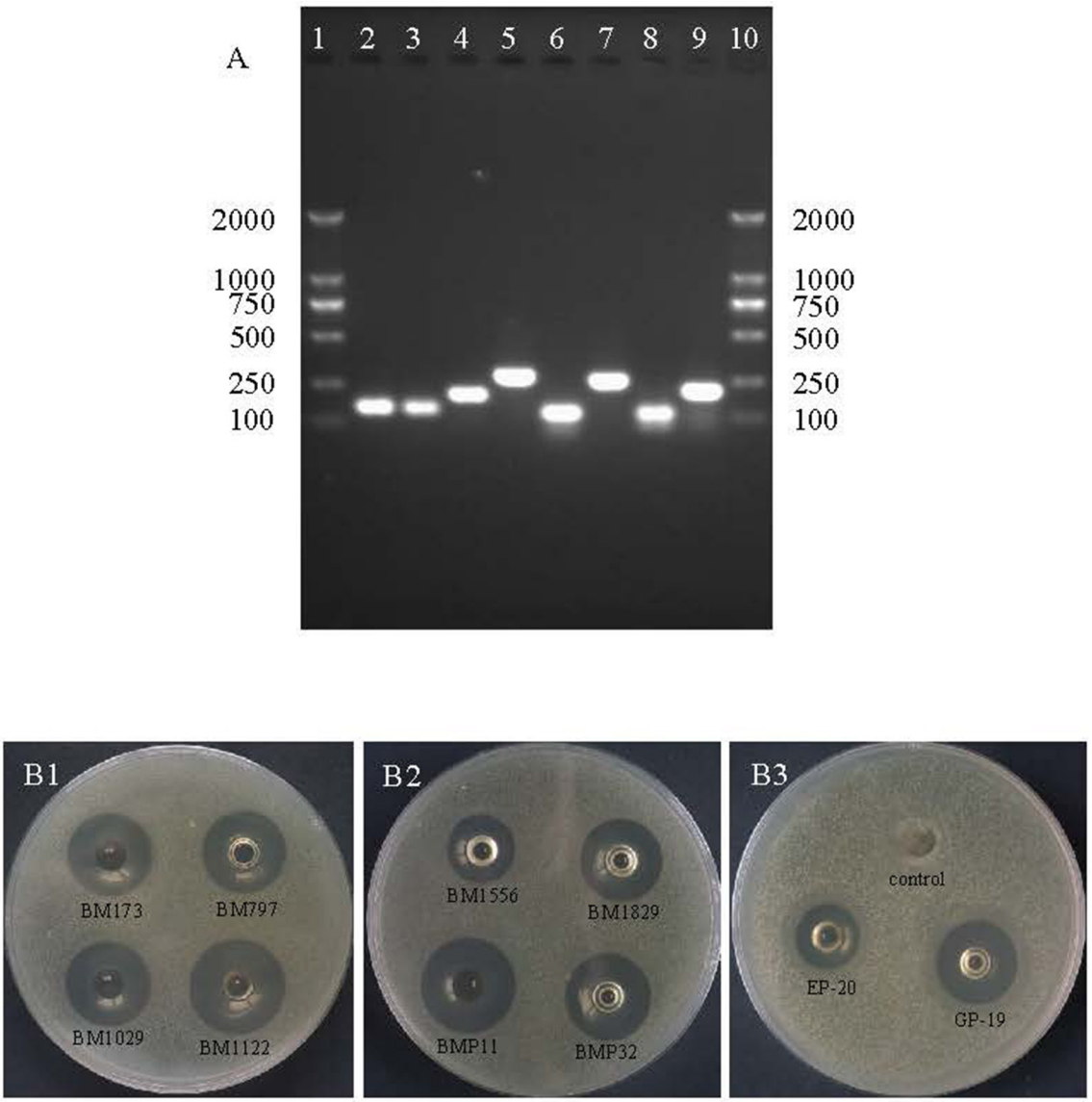

FIGURE 3 | DNA agarose gel electrophoresis of PCR products of 8 hypothetical bacteriocins and inhibition zones against *S. aureus* ATCC29213. **(A)** Lane 1 and 10 are DNA marker (Novagen); lane 2 is gene of BM173; lane 3 is gene of BM797; lane 4 is gene of BM1029; lane 5 is gene of BM1122; lane 6 is gene of BM1556; lane 7 is gene of BM1829; lane 8 is gene of BMP11; lane 9 is gene of BMP32. **(B1)** Inhibition zones of BM173, BM797, BM1029, and BM1122; **(B2)** inhibition zones of BM1556, BM1829, BMP11, and BMP32; **(B3)** inhibition zones of EP-20, GP-19 and control without induction.

BI355_0565) was located on chromosome which might involve in bacteriocin biosynthesis.

However, no peptide or protein was annotated to be bacteriocin by database BAGEL3 and antiSMASH that no one had identity more than 50% with known bacteriocins in databases. Also, no encoding gene of bacteriocin BMA identified in our previous study (Yi et al., 2016) was found. Therefore, the BMA might be a nonribosomal antimicrobial peptide. Bacitracin is a nonribosomally synthesized peptide, two bacitracin export ATP-binding proteins (locus_tag: BI355_1593 and BI355_2140) were located on the chromosome of *L. crustorum* MN047. Moreover, an ABC transporter ATP-binding protein (locus_tag: BI355_1814) and four ABC transporter permeases (locus_tag: BI355_1140, BI355_1815, BI355_1888, and BI355_2139) of ABC-type antimicrobial peptide transport system were contained. They could be responsible for the transportation of nonribosomally synthesized peptides produced by *L. crustorum* MN047.

LC-MS/MS

It is an obvious contradiction that multiple bacteriocins were found according to traditional purification process in our previous study while no related genes were annotated in the complete genome. There is only one possible reason that bacteriocins produced by *L. crustorum* MN047 are novel without any record in databases. Bacteriocins are extracellular

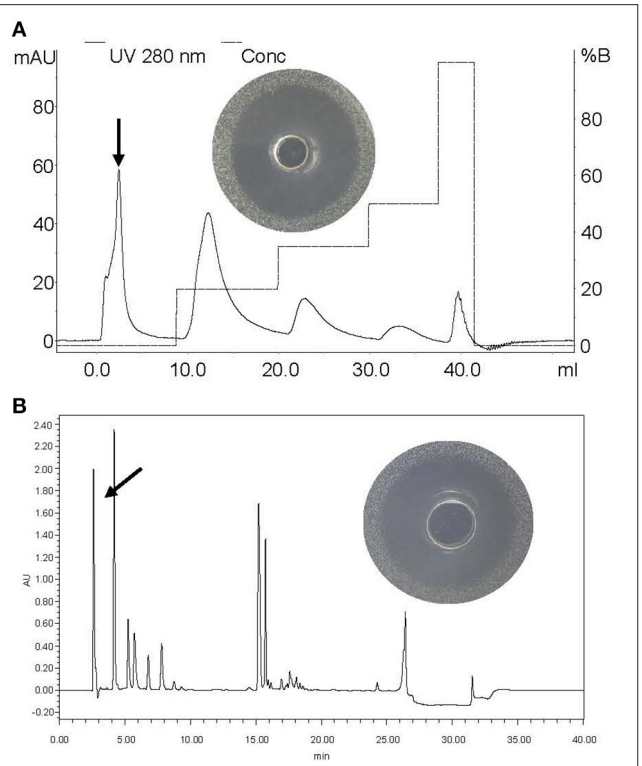

FIGURE 4 | Purification of BM1122 by chromatographic columns. **(A)** Anion-exchange column and inhibition zone of arrowed peak. **(B)** RP-HPLC and inhibition zone of arrowed peak. The y-axis in **(B)** is the absorbance value (arbitrary units, AU) under 215 nm. Solid line is 280 nm, dotted line is conc.

antimicrobial peptides or proteins existed in both culture supernatant and cell associated form (Dündar et al., 2015). Ammonium sulfate precipitation can concentrate bacteriocins in culture supernatant. Moreover, pH-mediated cell adsorption-desorption method is good for cell associated bacteriocins. In this study, two methods above were used simultaneously to prepare crude bacteriocins.

MS/MS data were matched to complete genome sequence of *L. crustorum* MN047, by which 66 and 170 peptide sequences were identified from ammonium sulfate precipitation sample and pH-mediated cell adsorption-desorption sample, respectively. They were fragments of 131 extracellular peptides or proteins of *L. crustorum* MN047 on chromosome or plasmid. The identified peptides with molecular mass <10,000 and indefinite function annotation were focused. Finally, eight peptides were selected as suspicious bacteriocins (**Table 3**). Among them, the BM1556 was one of the putative structural genes of bacteriocin gene cluster above. The BM1029 was predicted to be putative λ repressor-like DNA-binding protein by InterPro according to structure similarity despite of a low sequence identity (**Figure 2A**). The BM1122 was annotated to be DNA-binding protein with a high sequence identity (**Figure 2B**). For the eight peptides, BM797 was from crude bacteriocin of ammonium sulfate precipitation, others were from pH-mediated cell adsorption-desorption sample. On the other hand, two nonribosomal antimicrobial peptides (EP-20 and GP-19) were also identified from the sample of ammonium sulfate precipitation after sequence alignment search in databases of APD3 and CAMP. The two antimicrobial peptides were previously found in symbiotic bacteria *Xenorhabdus budapestensis* NMC-10 (Xiao et al., 2012). In this study, a combined method of complete genome and peptidome was used to identify bacteriocins, in which the bacteriocins and nonribosomal antimicrobial peptides were not purified. Namely, the samples used in peptidome analysis also contained a mass of undesirable proteins or peptides from metabolites of *L. crustorum* MN047 and MRS medium (MRS medium contained peptone, beef extract and yeast extract). Therefore, the native masses of bacteriocins and nonribosomal antimicrobial peptides were not displayable by MS analysis as their very low abundance. All MS/MS data of bacteriocins and nonribosomal antimicrobial peptides were showed in Figure S1.

Heterologous Production and Functional Expression of Bacteriocins

Non-fusion heterologous expression was used to verify these identified bacteriocin encoding genes. After PCR amplification using genome DNA of *L. crustorum* MN047 as template, genes of 8 hypothetical bacteriocins were all amplified with single band (**Figure 3A**). *E. coli* BL21 (DE3) pLysS was used as expression host to reduce the toxicity of bacteriocins or nonribosomal antimicrobial peptides under background expression.

After optimization of kanamycin and IPTG concentration (Table S1), the production and functional expression of bacteriocins were confirmed by antimicrobial activity test using agar well diffusion method. Consequently, the 8 hypothetical bacteriocins and 2 nonribosomal antimicrobial peptides all showed antimicrobial activity compared with control as shown in **Figure 3B**. The nonribosomal antimicrobial peptide EP-20 had the weakest antibacterial activity (320 AU/mL), the activity of bacteriocin BM797, BM1556, BM1829, and the other nonribosomal antimicrobial peptide GP-19 was 2-fold higher. Other bacteriocins BM173, BM1029, BM1122, BMP11, and BMP32 were 4-fold higher.

Antimicrobial Spectrum

Antimicrobial spectrum of 8 bacteriocins and 2 nonribosomal antimicrobial peptides was measured as shown in **Table 2**. All of which had broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative bacteria. These peptides had no remarkable preference between Gram-positive and Gram-negative wild bacteria except for BM797 that the BM797 seemed to be more effective toward Gram-positive strains. The approximate order of inhibitory competence was BM1122 > BMP11 > BM173 > BM1029 > BM797 > BMP32 > GP-19 > BM1829 > BM1556 > EP-20. Moreover, they also showed antibacterial activity against multidrug-resistant strains. Strains of multidrug-resistant *S. aureus* used in this study were formidable with resistance to different kinds of antibiotics containing multiple lethal modes. The BM797 was specifically more powerful toward multidrug-resistant *S. aureus* than other bacteriocins. The 8 bacteriocins and 2 nonribosomal antimicrobial peptides revealed same antibacterial activity

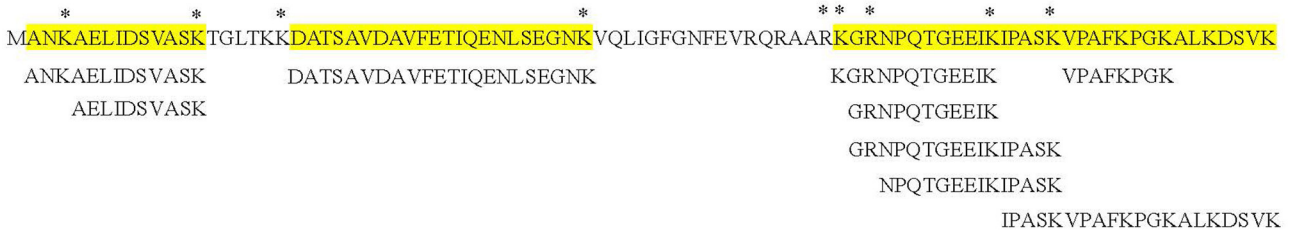

FIGURE 5 | Covered residues of BM1122 by LC-MS/MS (highlight in yellow). * is the digestion sites of trypsin.

against strains of standard and resistant *Cronobacter sakazakii*. Among these bacteriocins and nonribosomal antimicrobial peptides, BM1122 exhibited superior activity, which was further studied.

Purification of BM1122

Crude BM1122 was firstly dialyzed, after which the sample was purified by an anion-exchange column as shown in **Figure 4A**. Five isolated peaks were obtained for BM1122 and the active peak (arrow in **Figure 4A**) was collected and applied to RP-HPLC. Subsequently, nine clear peaks were gotten (**Figure 4B**). The summit of active peak (arrow in **Figure 4B**) was collected. BM1122 was digested by trypsin which acted on arginine (R) or lysine (K). After analysis by LC-MS/MS, 9 fragments of BM1122 (Figure S2) were identified with a coverage of 73.63%. Sequences of both ends and middle were covered (**Figure 5**), which indicated that the active BM1122 was the core bacteriocin without any remove of N/C-terminal. The unidentified fragments of TGLTK and VQLIGFGNFEVR might attribute to loss during filtration and elution using manual C18 Tips to desalt after digestion.

MIC Value

Staphylococcus aureus ATCC 29213 and *E. coli* ATCC 25922, a representative of Gram-positive and Gram-negative bacteria, respectively, were used as indicators. As a result, the MIC value of BM1122 against both indicators was 13.7 mg/L.

Action Modes of BM1122

Foodborne pathogen *L. monocytogenes* CMCC54004 and *C. sakazakii* ATCC29544 were used as indicators to investigate action mode of BM1122. Treatment with BM1122 for 1 h resulted in 2.9 $\log_{10}$ and 3.16 $\log_{10}$ reduction for *L. monocytogenes* and *C. sakazakii* (**Figure 6**), respectively. Viable cells further decreased with time, then all treatment caused more than 3 $\log_{10}$ (99.9%) reduction. Therefore, bacteriocin BM1122 was bactericidal (Kalia et al., 2009) at concentration of 4 × MIC.

Scanning electron microscope (SEM) and TEM visualized changes of ultrastructure treated by bacteriocin. *L. monocytogenes* (**Figure 7A0**) and *C. sakazakii* (**Figure 7B0**) without treatment showed plump cell profiles with integrated biofilm in SEM. By TEM, cytoplasmic substances of both untreated indicators uniformly distributed (**Figures 8A0, B0**). However, after treated by bacteriocin, great changes of ultrastructure for both indicators have taken place. For

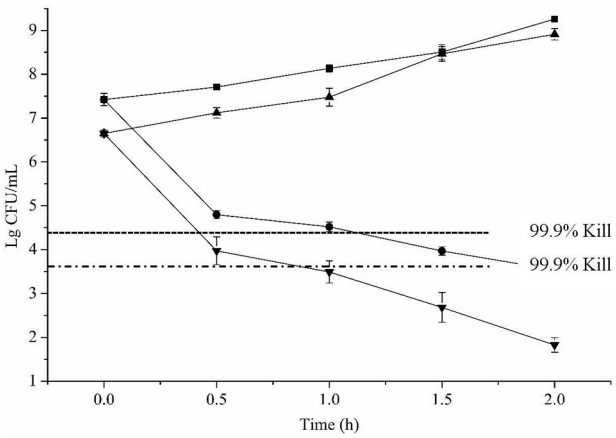

FIGURE 6 | Time-kill cures. ■ is *L. monocytogenes* for control, • is *L. monocytogenes* treated by BM1122 at 4 × MIC, ▲ is *C. sakazakii* for control, ▼ is *C. sakazakii* treated by BM1122 at 4 × MIC.— — — – is 99.9% (3$\log_{10}$ CFU/mL) kill of *L. monocytogenes* by bacteriocin;— - — - — is 99.9% kill of *C. sakazakii* by bacteriocin.

L. monocytogenes, treatment by bacteriocin BM1122 for 0.5 h caused distorted profiles, biofilm-destruction, and pore-formation (**Figure 7A1**). Prolonged exposure time deteriorated cellular damage, the deformation was aggravated and pores became holes (**Figure 7A2**). Deformation may be a result of cytoplasm concentrated around cytomembrane and rarefied in the center (**Figure 8A1**). The nonuniform distribution of cytoplasm substance intensified at 2 h (**Figure 8A2**). The falling biofilm dispersed beside cells (arrows in **Figures 8A1, A2**). The appearance of *C. sakazakii* by BM1122 was different from *L. monocytogenes*. More pores were formed, as well as deformation was milder (**Figure 7B1**). The number and size of pores increased with treatment time (**Figure 7B2**). The abundant pores could pave more passages for outflow of cytoplasmic substances. So, the attenuated cytoplasm was more uniform (**Figure 8B1**). With longer treatment time, cell envelope was broken and cytoplasm substance was observed outside cells (arrows in **Figure 8B2**). In addition, BM1122 had very high sequence identity with DNA-binding proteins and conserved structure of histone-like DNA-binding protein. Antimicrobial peptide APP has been reported to have inhibitory activity due to efficient cell-penetrating efficiency, significant physiological

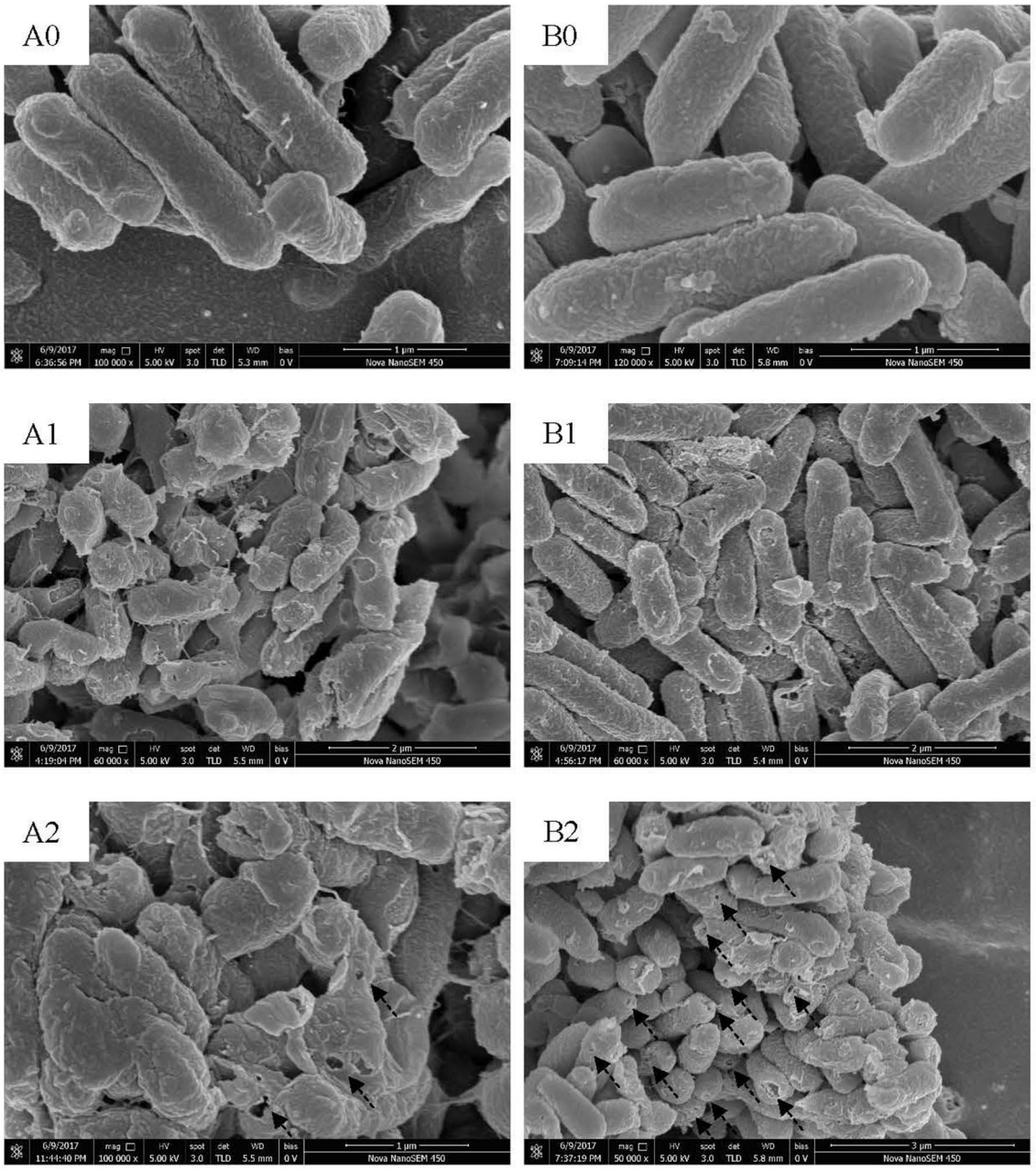

FIGURE 7 | SEM images of *L. monocytogenes* [control **(A0)**; treated by BM1122 for 0.5 h **(A1)**; treated by BM1122 for 2 h **(A2)**] and *C. sakazakii* [control **(B0)**; treated by BM1122 for 0.5 h **(B1)**; treated by BM1122 for 2 h **(B2)**].

changes and strong DNA-binding affinity (Li et al., 2016). Antimicrobial activity of BM1122 may also partly derive from DNA-binding action like Antimicrobial peptide APP (Li et al., 2016) and MBP-1 (Sousa et al., 2016), which needs to be further verified.

DISCUSSION

The *L. crustorum* MN047 strain, a poorly studied species, was isolated from koumiss. Koumiss is traditional fermented mare's milk and used as functional food for medical purposes (Wang et al., 2008; Vimont et al., 2017). A bacteriocin gene cluster was identified on the chromosome of *L. crustorum* MN047. In addition, multiple bacteriocin immunity proteins dispersed on chromosome far from the bacteriocin gene cluster. Moreover, multiple components of antimicrobial peptide transport system were found for *L. crustorum* MN047. These indicated that the *L. crustorum* MN047 might be able to produce multiple bacteriocins or antimicrobial peptides as our previous study on this strain (Yi et al., 2016). However,

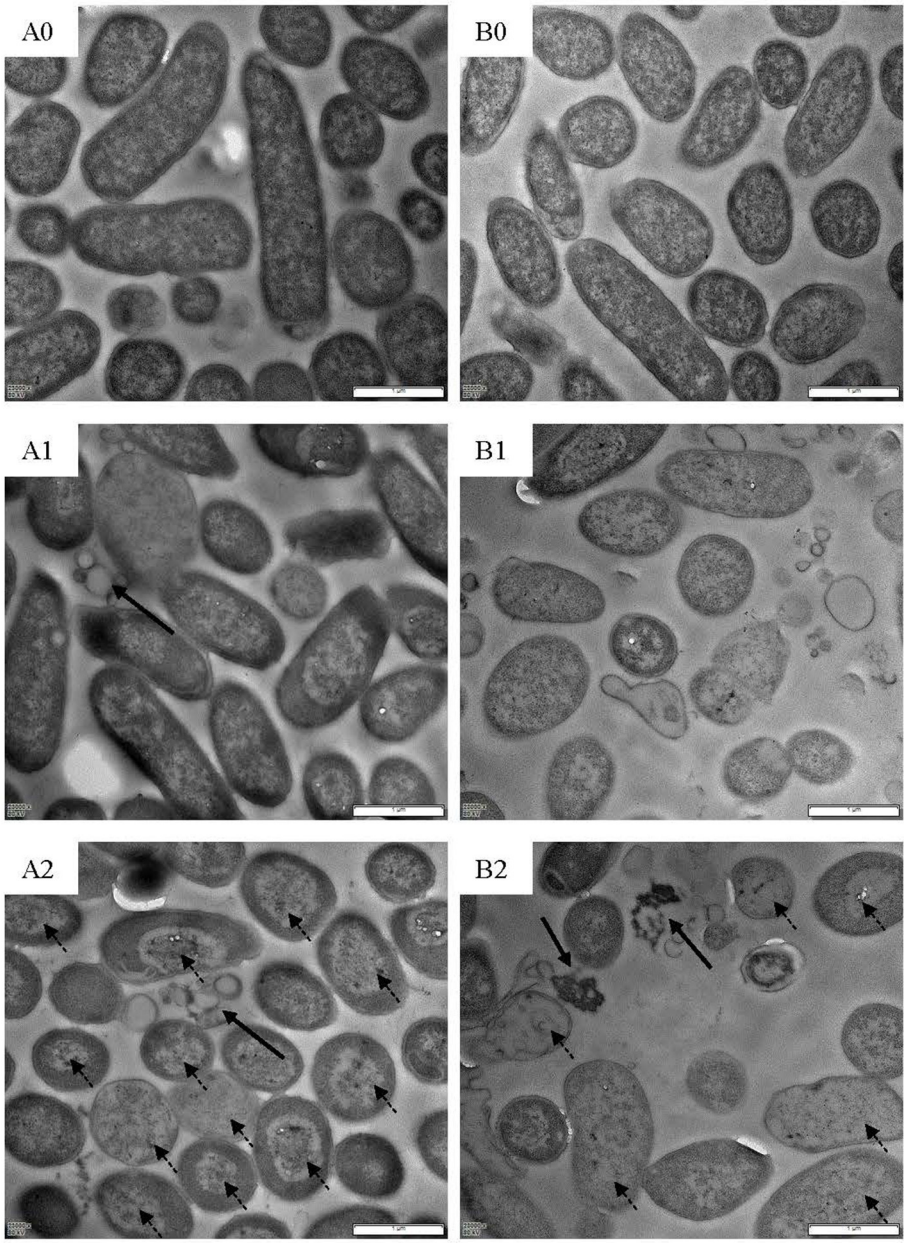

FIGURE 8 | TEM images of *L. monocytogenes* [control **(A0)**; treated by BM1122 for 0.5 h **(A1)**; treated by BM1122 for 2 h **(A2)**] and *C. sakazakii* [control **(B0)**; treated by BM1122 for 0.5 h **(B1)**; treated by BM1122 for 2 h **(B2)**].

no exact bacteriocin encoding genes were found after identification in databases of BAGEL3 and antiSMASH. Therefore, bacteriocins produced by the *L. crustorum* MN047 were novel. After further investigation by combining complete genome and peptidome information, encouragingly eight novel bacteriocins produced by *L. crustorum* MN047 were identified. The function of eight novel bacteriocins was verified by cloning and heterologous expression. It proved that LC-MS/MS-based peptidome analysis combining complete genome is an efficient way to discover new bacteriocins.

Among the 8 novel bacteriocins, the BM1122 was originally annotated as "DNA-binding protein" in the NR database. The remarkable activity as bacteriocin in this study renovated its function annotation. The BM1029 had λ repressor-likeDNA-binding domains and the BMP11 had transmembrane region by InterPro analysis. The BM1556 was one of the putative bacteriocin structural genes in bacteriocin gene cluster. The other 4 novel bacteriocins were all originally annotated as "hypothetical protein." Therefore, it is the first time to give a definite functional illustration for them. Biosynthesis mechanisms of other 7 bacteriocins in *L. crustorum* MN047 were mysterious. These

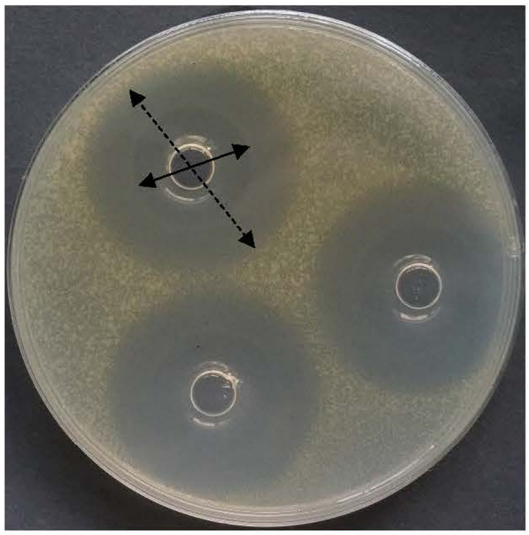

FIGURE 9 | Inhibitory zone of bacteriocin BM1122 against antibiotic-resistant *Sakazakii*. Solid arrow is totally killed by the bacteriocin, dotted arrow is partially killed by the bacteriocin.

bacteriocins might share the gene cluster of BM1556 because the core genes of biosynthesis and transport were flanked by four insertion sequence (IS) elements (Quintiliani and Courvalin, 1996). This composite transposon was mobile genetic element, which would facilitate the intracellular movement of carried genes (Hochhut et al., 2001). Namely, the composite transposon could play a key role in regulating multiple bacteriocins biosynthesis. As broad-spectrum antimicrobial activity, although these peptides were expressed in *E. coli*, the self-toxicity had prevented the production of bacteriocin beyond a limit.

Nisin is the only widely used bacteriocin as biopreservative. However, it has a weakness of feeble activity to Gram-negative bacteria. Because of drug efflux pump mechanism of Gram-negative bacteria, it is a common phenomenon for many antimicrobials. Excitingly, the 10 peptides all showed broad-spectrum activity against not only Gram-positive but also Gram-negative bacteria. Both *Salmonella* and *C. sakazakii* were Gram-negative bacteria, and their multidrug-resistant strains were studied in this work. As shown in **Figure 9**, an obvious partial killing zone (dotted arrow) was found for Gram-negative pathogens. All data used in this study were total killing zone (solid arrow in **Figure 9**). Except for antibiotic-resistant *Salmonella* 557D, other resistant *Salmonella* strains were all resistant to the ten peptides. However, the resistance-spectrum of 557D contained all detected resistance-spectrum of 1006D. Sensitivity of multidrug-resistant *C. sakazakii* strains to the ten peptides was the same as that of wild strain. These indicate that antimicrobial mechanism of these peptides may be different from these prevalent antibiotics used in this study.

According to the study of Xiao et al. (2012), the EC_{50} value of EP-20 to *Phytophthora capsici* was 3.14 mg/L, that of GP-19 against *Verticillium dahlia* was 17.54 mg/L. EP-20 was antifungal with weak antibacterial activity while GP-19 had anti-Gram-positive, anti-Gram-negative and antifungal activity (Xiao et al., 2012). It is in accordance with the order of inhibitory competence in this study. However, the inhibitory activity of EP-20 and GP-19 was far less than that of BM1122.

The bacteriocin BM1122 had bactericidal action mode, which was powerful in controlling foodborne pathogens. The source of probiotic lactic acid bacteria and proteinaceous nature of BM1122 endow the potential as food preservative. According to SEM and TEM, action mechanisms of BM1122 against Gram-positive and Gram-negative bacteria were different. Action mechanisms of bacteriocins include cell envelope-associated mechanisms (e.g., pore formation, targeting lipid II) and intracellular mechanisms (interfering with DNA, RNA, and protein metabolism) (Cotter et al., 2013). The transmembrane region of BMP11 (**Table 3**) analyzed by InterPro may contribute to its antibacterial activity. For intracellular mechanism, DNA binding also is one approach, such as BM1029 and BM1122 analyzed by InterPro. How much contribution of the DNA binding of BM1029 and BM1122 to antibacterial activity needs to be verified in future study. However, the BM1122 also induced pore-formation (dotted arrows in **Figure 7**) and permeabilization of membrane according to thinned cytoplasm substance (dotted arrows in **Figure 8**). The pore-formation and membrane permeabilization could not be the results of DNA-binding. The difference and diversity of action mode contribute to antibacterial activity against broad pathogens, especially antibiotic-resistant strains.

In conclusion, combination of complete genome and peptidome was an excellent method in discovery of novel bacteriocins which were overlooked by general genome annotation. By which, eight novel bacteriocins and two antimicrobial peptides were identified from probiotic *L. crustorum* MN047. It is much more efficient than traditional ways in identification of bacteriocins.

AUTHOR CONTRIBUTIONS

LY and XL designed the experiments. LY and LL performed the experiments and analyzed the experimental data. LY wrote this paper. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

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REVIEW
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Heterologous Expression of Biopreservative Bacteriocins With a View to Low Cost Production

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Bacteriocins, a heterogenous group of antibacterial ribosomally synthesized peptides, have potential as bio-preservatives in in a wide range of foods and as future therapeutics for the inhibition of antibiotic-resistant bacteria. While many bacteriocins have been characterized, several factors limit their production in large quantities, a requirement to make them commercially viable for food or pharma applications. The identification of new bacteriocins by database mining has been promising, but their potential is difficult to evaluate in the absence of suitable expression systems. *E. coli* has been used as a heterologous host to produce recombinant proteins for decades and has an extensive set of expression vectors and strains available. Here, we review the different expression systems for bacteriocin production using this host and identify the most important features to guarantee successful production of a range of bacteriocins.

Keywords: bacteriocins, heterologous expression, *E. coli*, vectors, strains

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INTRODUCTION

Bacteriocins are antimicrobial peptides produced by Gram-negative and Gram-positive bacteria. These molecules have attracted considerable interest, especially those produced by GRAS (Generally Recognized As Safe) microorganisms, as natural food preservatives in the food industry. They also represent potential alternatives to traditional antibiotics in the treatment of infections in humans and food-producing animals (Cotter et al., 2005; Desriac et al., 2010; Svetoch and Stern, 2010) and, in some cases, show promise as drugs for cancer treatment (reviewed in Kaur and Kaur, 2015).

Since the identification of colicin in 1925 (Gratia, 1925), many bacteriocins have been described (BAGEL4 database, http://bagel4.molgenrug.nl). However, only nisin (e.g., Nisaplin®) and pediocin PA-1 (e.g., ALTA®-2431) have been commercialized to any significant extent and these mainly as food biopreservatives. This may be explained by the multitude of studies that are required before a bacteriocin can be considered commercially viable, including characterization, potent antimicrobial activity, product stability, mechanism of action, mode of delivery, toxicity and assessment of their applications and industrial-scale production (Ingham and Moore, 2007).

The most important prerequisite for these studies is the production of high levels of biologically active bacteriocin. Although these peptides can be purified from their native producing strains, the process is time-consuming and laborious, and bacteriocin yields are often low (Rodríguez et al., 2003). Synthetic production is one alternative in some cases, but the complexity of some of the bacteriocins and the cost of the process limit the synthesis of large quantities (Chen et al., 2012). Therefore, attempts to increase the production of bacteriocins using alternative

hosts such as *Lactococcus lactis* and other lactic acid bacteria (LAB) have been investigated (reviewed in Cintas et al., 2011). These strains, which are food-grade organisms, offer a safer choice for industrial food products, and provide the genetic and secretory machinery for efficient LAB bacteriocin production. However, the expression strains available are highly specific and the results in terms of yield are still disappointing at industrial level, restricting the variety and quantity of bacteriocin produced (Rodríguez et al., 2003). Given these limitations *E. coli*, the most commonly used organism for heterologous protein production, is an attractive option for the heterologous bacteriocin expression due to its rapid growth on inexpensive media, its extensive genetic characterization and the availability of versatile cloning tools, expression systems and strains (Mergulhao et al., 2004; Rosano and Ceccarelli, 2014; Jia and Jeon, 2016). This could facilitate the functional characterization and establishment of a production process in bacteriocins from sources that are difficult to cultivate, in addition to those bacteriocins discovered by data mining from sequenced bacterial genomes (Kuo et al., 2013), increasing their potential for manufacture and commerialization by food and pharmaceutical industries (Ongey and Neubauer, 2016). However, this approach is not without obstacles which may arise during the expression, secretion or processing of these peptides in *E. coli* (Choi and Lee, 2004).

In order to provide a guide for a design of a successful expression system for bacteriocin production in this host, the present review focuses on the different systems currently available for bacteriocin production in *E. coli*.

GENERAL CONSIDERATIONS BEFORE THE EXPRESSION SYSTEM SELECTION

Bacteriocin

The choice of an expression system is not simple. Firstly, the characteristics of the bacteriocin should be taken into account, such as the presence of post-translational modifications and disulfide bonds, because these may affect its heterologous production.

According to Cotter et al. (2012), LAB bacteriocins are classified into those which are either post-translationally modified (class I) and unmodified or minimally modified peptides (class II). Class I can be subdivided into lantibiotics (with lanthionine bridges), linaridins, proteusins, linear azole- or azoline-containing peptides, cyanobactins, thiopeptides, lasso peptides, sactibiotics (contain sulfur-α-carbon linkages), bottromycins, glycocins, and modified microcins that do not belong to other subgroups. Class II is further divided into class IIa (the pediocin-like bacteriocins), IIb (the two-peptide bacteriocins), IIc (circular bacteriocins), IId (unmodified, linear, non-pediocin-like, single-peptide bacteriocins that do not belong to other subclasses), and IIe (the microcin E492-like bacteriocins). In this classification, the large (>10 kDa), heat-labile antimicrobial peptides bacteriolysins (formerly class III bacteriocins) were removed from the bacteriocin category.

Similarly, bacteriocins from Gram-negative organisms can be divided into small peptides, such as microcins [class I (presence of modifications) or II (unmodified)], and large peptides such as colicins (Drider and Rebuffat, 2011).

Genes Required for Bacteriocin Expression

In general, the production of bacteriocins in the native host requires several genes including a structural gene that encodes the prepeptide (or two structural genes for the two peptide bacteriocins). Other genes encode an immunity protein, specialized secretion machinery and in many cases proteins capable of performing modifications and regulatory sequences (Nes et al., 1996). Therefore, different strategies are required depending on the characteristics of each bacteriocin to ensure production (see **Table 1**).

In most cases, the expression of the structural gene or its mature sequence is enough to produce the active bacteriocin. Some examples include carnobacteriocin B2 (Jasniewski et al., 2008), divercin AS7 and V41(Richard et al., 2004; Ingham et al., 2005; Yildirim et al., 2007; Olejnik-Schmidt et al., 2014), epidermicin NI01 (Sandiford and Upton, 2012), gassericin A (Kawai et al., 2003), or sakacin P (Chen et al., 2012) (see **Table 1**). However, the transporter gene is also necessary for the synthesis of some bacteriocins, for example in the production of pediocin PA-1 and bactofencin A (Bukhtiyarova et al., 1994; Mesa-Pereira et al., 2017). In other cases, the co-expression of the structural gene with the genes involved in post-translational modifications on the same or different plasmids are required for the heterologous expression of lantibiotics such as lichenicidin (Caetano et al., 2011a,b; Kuthning et al., 2015), nukacin ISK-1 (Nagao et al., 2005), prochlorosin, haloduracin, nisin (Shi et al., 2011), suicin (Wang et al., 2014), and the sactibiotic subtilosin A (Himes et al., 2016), amongst others.

Toxicity

The potential toxicity to *E. coli* due to the overexpression of the mature peptides or components of the secretion machinery and other bacterial integral membrane proteins (Fath and Kolter, 1993; Miller et al., 1993) must also be considered as these could interfere with the growth and viability of *E. coli*, limiting bacteriocin production (Bentley et al., 1990; Mccormick et al., 1996; Biet et al., 1998; Gutiérrez et al., 2005; Ingham et al., 2005; Moon et al., 2005; Masias et al., 2014; Mesa-Pereira et al., 2017).

PLASMIDS FOR BACTERIOCIN EXPRESSION IN *E. COLI*

General Features

Expression vectors require various components to carry out their functions, including; (i) an origin of replication; (ii) a selection marker (generally genes encoding resistance to antibiotics); (iii) a promoter region for gene transcription initiation; and (iv) multiple unique restriction enzyme sites arranged in a polylinker region after the promoter to facilitate the cloning (referred to as multiple cloning sites; MCS). In some cases, two or more MCS are available in commercial plasmids (i.e., Duet vectors and pRSFDuet^{TM}-1) for cloning several genes of interest without the need to use multiple plasmids. In addition, the MCS can additionally provide fusion tags to facilitate the purification of the

TABLE 1 | Bacteriocins heterologously produced by *E. coli*.

Bacteriocin	Native host[a]	*E. coli* strain[b]	Vector[c]	Location[d]	Culture conditions[e]	References
Ala(0)actagardine (Class I, Lantibiotic)	*A. garbadinensis* ATCC 31049	BL21 (DE3)	pRSFDuet-1-*garA-garM* pCDFDuet-1-2X*garO*	SCF	LB, 0.2 mM IPTG 20 h, 18°C	Shi et al., 2012
BacR1 (Class II)	*Sta. aureus* UT0007	BL21 (DE3)	pSuV1- *bacR1**[C]	M	LB, 2 mM IPTG ON, RT	Ingham et al., 2005
Bactofencin A (Class IId)	*Lb. salivarius* DPC6502	Tuner (DE3)	pETcoco-2-*bfnAbfnI DLSL0052DLSL0053*	M	LB, 0.05–0.1 mM IPTG 3 h, 37°C	Mesa-Pereira et al., 2017
Bovicin HJ50 (Class I, Lantibiotic, SS bond)	*Str. bovis* HJ50	BL21 (DE3)	pET28a-*bovAM*, pET28a-*bovT150*	SCF	BovA expression: LB, 0.5 mM IPTG 20 h, 18°C BovT150 expression: LB, 0.5 mM IPTG 12 h, 20°C	Lin et al., 2011
	Str. bovis HJ50	BL21 (DE3)	pET28a-*bovAM* pACYC-Duet-*bovT* or $bovT_{150}$	SCF	LB, 0.5 mM IPTG 5, 10, 20 h, 37°C	Wang et al., 2016
	Str. bovis HJ50	C43 (DE3)	pET28a-*bovAM* pACYC-Duet-*bovT* or $bovT_{150}$	M	LB, 0.5 mM IPTG 10-20 h, 37°C	Wang et al., 2016
Carnobacteriocin B2 (Class IIa)	*C. piscicola* LV17B	–	pMALc-*CbnB2** pMALc-*CbnB2P*	TE	Rich broth, 0.3 mM IPTG 3 h, 37°C	Quadri et al., 1997
	C. maltaromaticum CP5	BL21 (DE3)	pET32a-*CbnB2**	TE	TB, 0.55 mM IPTG or 14.6 mM lactose 3 h, 37°C	Jasniewski et al., 2008
Carnobacteriocin BM1 (Class IIa)	*C. maltaromaticum* CP5	BL21 (DE3)	pET32a-Cbn*BM1** pET32a-Cbn*BM1M41V**	TE	TB, 0.55 mM IPTG or 14.6 mM lactose 3 h, 37°C	Jasniewski et al., 2008
ColA-43864 (Colicins)	*Cit. freundii* ATC43864	S17-1	pMQ124-*colA-43864*	SCF	LB, 0.2% L-arabinose 3 h, 37°C	Shanks et al., 2012
Colicin V (Colicins)	*E. coli*	KS300/pMS421	pHLZ01 (pBR322)- *cvaC**	TE (periplasm)		Zhang et al., 1995
Divercin AS7 (Class IIa, two SS bonds)	*C. divergens* AS7	BL21 (DE3) pLys	pET28b-*AS7*	SCF	LB, 0.1 mM IPTG 24 h, 37°C	Olejnik-Schmidt et al., 2014
Divercin V41 (Class IIa)	*C. divergens* V41	Origami (DE3) pLysS	pET32b- *DvnV41*c*	SCF	TB, 1 mM IPTG 3 h, 37°C	Richard et al., 2004
	C. divergens V41	BL21 (DE3)	pSuV1- *DivV41**[C]	M	SOC, 2 mM IPTG ON, RT	Ingham et al., 2005
	C. divergens V41	Origami (DE3) pLysS/pCR03	pET32b- *DvnV41**	SCF	TB or M9, 0-2 mM IPTG 3 h, 30°C	Yildirim et al., 2007
Divergicin A (Class IIc)	*C. divergens LV13*	MC4100/pHk22	pMG36e-Leucocin A leader-divergicin A pMG36e-ColV leader-divergicin A	M	YT and 0.2mM 2,2′-dipyridyl. 37°C	Van Belkum et al., 1997
	C. divergens LV13	BL21 (DE3)/pHk22	pT7-1-Lactococcin A leader-divergicin A	TE	YT and 0.2 mM 2,2′-dipyridyl, 0.4 mM IPTG 2 h, 37°C	Van Belkum et al., 1997
E50-52 (Class IIa)	*Ent. faecium* NRRL B-30746	BL21 (DE3)	pET SUMO-*rb50-52*	SCF	LB, 1.5 mM IPTG 5 h, 37°C	Wang et al., 2013
Enterocin A (Class IIa)	*Ent. faecium* ATB 197a	BL21 (DE3)	pET37b-*entA*	TE SCF M	LB, 0.1 mM IPTG 1-4 h, 37°C	Klocke et al., 2005
Enterocin B (Class II)	*Ent. faecium* ATB 197a	BL21 (DE3)	pET37b-*entB*	TE	LB, 0.1 mM IPTG 1–4 h, 37°C	Klocke et al., 2005
Enterocin CRL35 (Class IIa, one SS bond)	*Ent. mundtii* CRL35	Rosetta (DE3) pLysS	pET22b-*munA*	M	LB or M9, 0.5 mM IPTG 1 h, 37°C	Masias et al., 2014
	Ent. mundtii CRL35	BL21 (DE3) pLysS	pET22b-*munA*	M	LB, 0.5 mM IPTG 1 h, 37°C	Masias et al., 2014
	Ent. mundtii CRL35	C41 (DE3) pLysS	pET22b-*munA*	M	LB, 0.5 mM IPTG 1 h, 37°C	Masias et al., 2014

(Continued)

TABLE 1 | Continued

Bacteriocin	Native host[a]	*E. coli* strain[b]	Vector[c]	Location[d]	Culture conditions[e]	References
	Ent. mundtii CRL35	C43 (DE3) pLysS	pET22b-*munA*	M	LB, 0.5 mM IPTG 1 h, 37°C	Masias et al., 2014
	Ent. mundtii CRL35	Rosetta –gami 2 (DE3)	pET22b-*munA*	M	LB, 0.5 mM IPTG 1 h, 37°C	Masias et al., 2014
	Ent. mundtii CRL35	Origami (DE3)	pET22b-*munA*	M	LB, 0.5 mM IPTG 1 h, 37°C	Masias et al., 2014
	Ent. mundtii CRL35	BL21 (DE3)	p8760 (PBAD24)-EtpM-*munA* pACYCDuet-1-*munC*	TE (membrane)	Ent35: LB, 0.6% arabinose 5 min MunC: LB, 0.02% lactose 1 h, 37°C	Barraza et al., 2017
Enterocin P (Class IIa)	*Ent. faecium*	BL21 (DE3)	pTYB1-*EntP**[c]	M	LB, 2 mM IPTG ON, RT	Ingham et al., 2005
	Ent. faecium P13	Tuner (DE3) pLacI	pETBlue-1-*entP* pETBlue-1-*entP-entiP*	M SCF IB	M9, 0-1 mM IPTG 3 h, 37°C	Gutiérrez et al., 2005
Epidermicin NI01 (Class IId)	*Sta. epidermidis* 224	BL21 (DE3)	pET29a-*edcA*	SCF	2 × YT broth, 0.05 mM IPTG ON, 17°C	Sandiford and Upton, 2012
Gassericin A (class IIc)	*Lb. gasseri* LA39	JM109	PinPoint Xa-1-gassericinA*	SCF	LB, 2 μmol biotin 8 h, 37°C	Kawai et al., 2003
Haloduracin (Class I, two-peptide lantibiotic)	*B. halodurans*	BL21 (DE3)	pRSFDUET-1-*HalA1*-Xa-*HalM1* pRSFDUET-1-*HalA2*-Xa-*HalM2*	SCF	LB, 0.5 mM IPTG 18 h, 18°C	Shi et al., 2011
Lactococcin G (Class IIb, two-peptides)	*L. lactis* LMGT 2081	BL21 RIL (DE3) pLysS	pGEV-*LcnG*-α or *LcnG*-β	IB	M9, 1 mM IPTG 6-8 h, 37°C	Rogne et al., 2008
Lactococcin K	*L. lactis* MY23	BL21 (DE3)	pEMBP- *lcnK* pET21c- *lcnK*	SCF IB	LB, 1 mM IPTG 12 h, 37°C	Kim et al., 2006
Lichenicidin (Class I, two-peptide lantibiotic)	*B. licheniformis* 189	BLic5	pCC2FOS™- *lic* operon	M	Medium M 24 h, 37°C	Caetano et al., 2011a,b
	B. licheniformis 189	BL21 gold (DE3)	pRSFDuet-1_*TM1A1* and pRSFDuet-1_*TPM2A2*	M	Medium M, 2 × YT, SB, TB and LB-Kelly 0.5 mM IPTG 24 h, 30°C	Kuthning et al., 2015
LSEI_2163 (Class IId, one SS bond)	*Lb. casei* ATCC334	Origami (DE3) pLysS	pAB-238- *LSEI_2163*	TE	LB, 1 mM IPTG 5 h, 37°C	Kuo et al., 2013
LSEI_2386 (Class II)	*Lb. casei* ATCC334	Origami (DE3) pLysS	pAB-238- *LSEI_2386*	TE	LB, 1 mM IPTG 5 h, 37°C	Kuo et al., 2013
Mersadicin (Class I, Lantibiotic)	*B. licheniformis* MKU3	M15/pRep4	pQE-30UA-*lanA*	SCF	LB, 0.4 mM IPTG 4 h, 37°C	Kayalvizhi et al., 2016
Mesentericin Y105 (Class IIa)	*Leu. mesenteroides* Y105	DH5α	pBluescript SKII+- *dvnA* leader- *mesY*-mesI* pBluescript SKII+- *dvnA* leader- *mesY-mesI*	M	LB, 1 mM IPTG 37°C	Biet et al., 1998
Microcin B	*Ps. syringae* pv. *glycinea* B076	BL21 (DE3)	pBAD His/B- *mcb*	SCF	M9, 10 mM Arabinose 24 h, 30°C	Metelev et al., 2013
Nisin (Class I, Lantibiotic)	*L.lactis*	BL21 (DE3)	pRSFDUET-1-*nisA*- *nisB* pACYCDUET-1-*nisC*	SCF	LB, 0.5 mM IPTG 15 h, 18°C	Shi et al., 2011
Nukacin ISK-1 (Class I, Lantibiotic)	*Sta. warneri* ISK-1	BL21 (DE3)	pET14b-*nukAM*	SCF	2 xYT, 1 mM IPTG 20 h, 20°C or 3 h, 37°C	Nagao et al., 2005
Pediocin AcH (Class IIa, two SS bonds)	*P. acidilactici* H	E609L	pPR682-*pap**	M	LB, 1 mM IPTG 3 h, 37°C	Miller et al., 1998
	P. acidilactici LB42-923	JM109	pHPS9- *pap* operon	M	LB, 37°C	Bukhtiyarova et al., 1994

(Continued)

TABLE 1 | Continued

Bacteriocin	Native host[a]	*E. coli* strain[b]	Vector[c]	Location[d]	Culture conditions[e]	References
Pediocin PA-1 (Class IIa, two SS bonds)	*P. acidilactici* PAC1.0	V850	pSRQ11- *ped* operon pSRQ11.2- *ped* operon pBR322- *ped* operon	M	M9 supplemented with 1% yeast extract and 1% Hy Case ON, 37°C	Marugg et al., 1992
	P. acidilactici F	DH5α	pPC418- *ped* operon pHPS9- *ped* operon	M	LB, ON, 37°C	Coderre and Somkuti, 1999
	P. acidilactici	BL21 (DE3)	pSuV1- *PedPA-1**[c]	M	LB, 2 mM IPTG ON, RT	Ingham et al., 2005
	P. acidilactici K10	M15/pRep4	PQE-30 Xa-*pedA**	ET	LB, 1 mM IPTG 4 h, 37°C	Moon et al., 2005
	P. acidilactici K10	M15/pRep4	PQE-40-*pedA**	ET IB	LB, 1 mM IPTG 4 h, 37°C	Moon et al., 2006
	P. acidilactici PAC1.0	Origami (DE3)	pET32b- *pedA**	SCF	LB, 0.02 mM IPTG 4 h, 37°C	Beaulieu et al., 2007
	P. acidilactici PA003	BL21 (DE3)	pET32b- *pedA** pET20b- *pedA**	IB SCF	LB, 0.02 mM IPTG 4 h, 37°C	Liu et al., 2011
	P. acidilactici LMG2351	Tuner (DE3)	pETcoco2-*ped* operon	M	LB, 0.05-0.1 mM IPTG 3 h, 37°C	Mesa-Pereira et al., 2017
Piscicolin 126 (Class IIa, one SS bond)	*C. piscicola* JG126	AD494 (DE3)	pET32- *pisA**	TE	LB, 0.1 mM IPTG 4 h, 37°C	Gibbs et al., 2004
	C. piscicola	BL21 (DE3)	pSuV1- *pisA**[c]	M	LB, 2 mM IPTG ON, RT	Ingham et al., 2005
Plantaricin E (Class IIb, two-peptide bacteriocin)	Soil metagenome	BL21 (DE3)	pET32a-*plnE**	SCF	LB, 1 mM IPTG 5 h, 16, 25, 30 and 37°C	Pal and Srivastava, 2014
	Lb. plantarum LR/14	BL21 (DE3)	pET32a-*plnE,plnG* or *plnH.* pET28a-*plnE,plnG* or *plnH.*	SCF IB (PlnG and PlnH)	LB, 1 mM IPTG 16 h, 22°C	Pal and Srivastava, 2015a
	Soil metagenome	BL21 (DE3)	pET32a-*plnE*	SCF	Small scale: LB, TB 0.5 mM IPTG 5 and 9 h at 22°C Large scale: 12 L LB 0.5 mM IPTG 3 h, 25 and 30°C	Pal and Srivastava, 2015b
	Lb. plantarum 163	BL21 (DE3)	pET32a-*plnEm**	SCF	LB, 0.5 mM IPTG 8 h, 25°C	Meng et al., 2017
Plantaricin EF (Class IIb, two-peptide bacteriocin)	*Lb. plantarum* C11	BL21 RIL (DE3) pLysS	pGEV2- *plnE* pGEV2- *plnF*	IB	M9, 1 mM IPTG 4 h, 37°C	Fimland et al., 2008
	Lb. plantarum	BL21 (DE3)	pET32a-*plnE* pET32a-*plnF*	SCF	LB, 1 mM IPTG 6 h, 16, 20, 25, 30**,** 37°C	Tang et al., 2018
Plantaricin F	Soil metagenome	BL21 (DE3)	pET32a-*plnF**	SCF	LB, 1 mM IPTG 5 h, 16, 25, 30 and 37°C	Pal and Srivastava, 2014
Plantaricin J	Soil metagenome	BL21 (DE3)	pET32a-*plnJ**	SCF	LB, 1 mM IPTG 5 h, 16, 25, 30 and 37°C	Pal and Srivastava, 2014
Plantaricin JK (Class IIb, two-peptides)	*Lb. plantarum* C11	BL21 RIL (DE3) pLysS	pGEV2- *plnJ* pGEV2- *plnK*	IB	LB or M9, 1 mM IPTG 3-4 h, 37°C	Rogne et al., 2009
Plantaricin K	Soil metagenome	BL21 (DE3)	pET32a-*plnK**	SCF	LB, 1 mM IPTG 5 h, 16, 25, 30 and 37°C	Pal and Srivastava, 2014
Plantaricin NC8 (Class IIb, two-peptide bacteriocin)	*Lb. plantarum* ZJ316	BL21 (DE3)	pET32a- *PLNC8α* pET32a- *PLNC8β*	SCF	LB 0.1, 0.2, 0.5, 1 mM IPTG 5,10,16, 20 h 16, 20, 25, 30 and 37°C	Jiang et al., 2016
Plantaricin Pln1 (Class II)	*Lb. plantarum* 163	BL21 (DE3)	pET32a-*pln1*	TE	LB 0.5, 1, 2, 4 mM IPTG 4, 6, 8, 10 h 20, 25, 30 and 37°C	Meng et al., 2016

(Continued)

TABLE 1 | Continued

Bacteriocin	Native host[a]	*E. coli* strain[b]	Vector[c]	Location[d]	Culture conditions[e]	References
Plantaricin S34 (Class II)	*Lb. plantarum* S34	BL21 (DE3) pLysS	pET32a-*plnF** pET32a-*plnE**	SCF	LB, 0.5 mM IPTG 5 h, 22°C	Mustopa et al., 2016
Prochlorosin 1.7, 2.11 and 3.3 (Class I, Lantibiotics)	*Prochlorococcus*	BL21 (DE3)	pRSFDUET-1-*procA-procM*	SCF	LB, 0.1 mM IPTG 20 h, 18°C	Shi et al., 2011
Pyocin S4	*Ps. aeruginosa* PAO	BL21 (DE3) pLysS	pET15b-S4imm	SCF	LB, 1 mM IPTG ON, 28°C	Elfarash et al., 2012
Sakacin P (class IIa)	*Lb. sakei*	BL21 (DE3)	pET28a–sakP*	IB	LB, 0.8 mM IPTG 3 h, 20 or 37°C	Chen et al., 2012
Subtilosin A (Sactipeptide)	*B. subtilis* 168	BL21 (DE3)/pPH151	pETDuet-*sboA-albA*	IB	LB, 0.5 mM IPTG 22–24 h, 18°C	Himes et al., 2016
Suicin (Lantibiotic, SS-bond)	*Str. suis* serotype 2	BL21 (DE3)	pET28a-*suiAM* pET28a-*suiTR*	IB	LB, 0.5 mM IPTG 20 h 16°C	Wang et al., 2014
Warnericin RK (Class II)	*Sta. warneri* RK	M15/pREP4	pQE30-*war*^{C} pQE70-*war*^{C}	TE	LB or M9, 1 mM IPTG 6 h, 37°C	Verdon et al., 2013

[a]*Native hosts: A., Actinoplanes; B., Bacillus; C., Carnobacterium; Cit., Citrobacter; E., Escherichia, Ent., Enterococcus; Str., Streptococcus; L., Latococcus; Lb., Lactobacillus; Ps., Pseudomonas; Sta., Staphylococcus; P., Pediococcus.*

[b]*E. coli strains containing plasmids: pCR03, derivative pET-32 plasmid; pHK22, contains the structural gene and the immunity gene for colicin V as well as the genes encoding the two inner-membrane transport proteins, CvaA and CvaB, for colicin V; pMS421, pSC101 with lacI^{Q}; pPH151, containing the E. coli suf ABCDSE genes that facilitate the proper assembly and repair of the Fe– S cluster; pREP4, contains lacI gene for regulating expression from PQE vectors. pLysS and pLacI information is listed in the text.*

[c]**Mature sequence (without leader peptide),* [c]*codon optimized genes.*

[d]*Location: IB, Inclusion bodies; SCF, Soluble Cellular Fraction (soluble fraction after cell pellet sonication); M, culture medium (cell-free supernatants), TE, Total cell extract.*

[e]*Culture medium: Luria broth (LB) medium, Terrific broth (TB), 2 × Yeast extract-Tryptone broth (2 × YT). ON, overnight; RT, Room Temperature.*

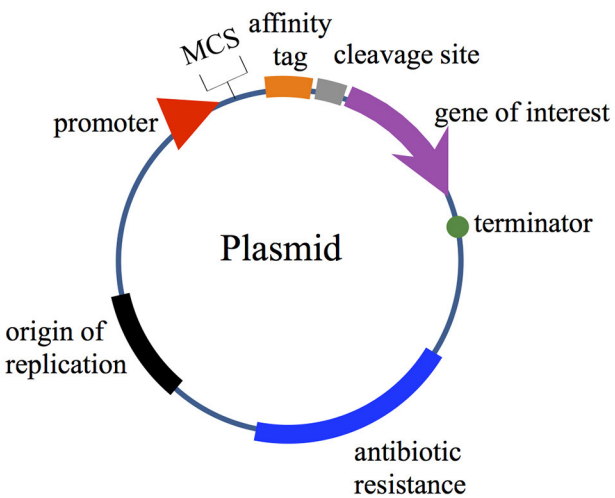

FIGURE 1 | Schematic plasmid map showing the major features present in common expression vectors.

expressed bacteriocins. Plasmids must also contain one or several terminators to ensure an efficient transcriptional termination and prevent the transcription downstream of the coding sequence of interest (**Figure 1**). In terms of translational features, the plasmid must include a ribosome binding site (RBS) with a Shine-Dalgarno sequence (UAAGGAGG) located 5–13 bases upstream of the start codon for the interaction with the 3′ end of rRNA during translation initiation (reviewed in Mergulhao et al., 2004; Terpe, 2006; Durani et al., 2012; Rosano and Ceccarelli, 2014).

Plasmid Copy Number

A replicon consists of one origin of replication with associated control elements and is involved in plasmid replication and copy number control (del Solar and Espinosa, 2000). The number of copies per cell can vary between one and approximately one hundred. Theoretically, the higher the copy number of a plasmid, the higher the expression of the gene of interest. However, this often results in aggregation, misfolding or protein degradation in *E. coli* (Tolia and Joshua-Tor, 2006), and could also cause cellular toxicity when the overexpression of secretion machinery and other integral membrane proteins are required for bacteriocin production. For this reason, low and middle copy vectors (15–20 copies per cell) based on ColE1, pMB1 replicons, including the pBR322 and pET vector systems and pACYC plasmids with an origin replication derived from p15A, have been successfully used for bacteriocin expression (**Table 2**). In addition, the use of two plasmids with compatible origins (e.g., ColE1 with p15A, pMB1 with p15A) has allowed for dual expression of proteins for functional characterization (**Table 1**). In this regard, the expression vector pETcoco-2 could be a useful tool for studying whole operons since it enables the control of copy number by arabinose induction, facilitating the optimization of bacteriocin expression (Sektas and Szybalski, 2002; Mesa-Pereira et al., 2017).

Promoter Region

A careful balance of promoter strength and gene copy number is necessary for the optimization of the bacteriocin expression level. Since bacteriocins could be toxic for the host, the promoter strength should be adequate in order to minimize the metabolic burden on *E. coli* prior to the production phase and prevent the saturation of the host transport machinery (Rosenberg, 1998;

TABLE 2 | Features of expression vectors used for bacteriocin production in *E.coli*.

Vector[a]	Size (bp)	Promoter[b]	Selection[c]	Tags and fusion partners[d]	Protease cleavage sites[e]	Origin	Supplier/References
pAB-238	5,800	T7*lac*	Amp	N-Trx	Thr	pBR322	Kuo et al., 2013
pACYCDuet-1*	4,008	T7*lac*	Cm	N-His S	None	P15A	Novagen
pBAD His/B	4,100	*araBAD*	Amp	N-His	EK	pUC	Invitrogen
pBAD-24	4,542	P_{BAD}	Amp	None	None	pBR322	Invitrogen
pBluescript SKII+	2,961	*lac*	Amp	None	None	pUC	Stratagene
pBR322	4,361	None	Amp, Tet	None	None	pMB1	NEB
pCC2FOS™	8,181	T7	Cm	None	None	*oriV*/*oriS*	Epicentre Biotechnologies
pCDFDuet-1*	3,781	T7*lac*	Str/Spe	N-His S	None	pCloDF13	Novagen
pEMBP		T7	Amp	MBP	EK	pBR322	Bioprogen
pETBlue-1	3,476	T7*lac*	Amp	C-His	None	pUC	Novagen
pETcoco-2	12,417	T7lac pBAD	Amp	N-His S C-HSV tag	EK	Mini-F/RK2	Novagen
pETDuet-1	5,420	T7	Amp	N-His S	None	pBR322	Novagen
pET SUMO	5,643	T7*lac*	Kan	N-His N-SUMO	SUMO protease	pBR322	Invitrogen
pET-14b	4,671	T7	Amp	N-His	Thr	pBR322	Novagen
pET-15b	5,708	T7	Amp	N-His	Thr	pBR322	Novagen
pET-20b (+)	3,716	T7	Amp	Signal sequence C-His	None	pBR322	Novagen
pET-21c	5,441	T7*lac*	Amp	C-His	None	pBR322	Novagen
pET-22b (+)	5,493	T7*lac*	Amp	Signal sequence C-His	None	pBR322	Novagen
pET-28a,b	5,369	T7*lac*	Kan	N-His C- His	Thr	pBR322	Novagen
pET-29a	5,371	T7*lac*	Kan	C-His Stag	Thr	pBR322	Novagen
pET-32a,b	5,900	T7*lac*	Amp	N-Trx Internal His C-His	Thr EK	pBR322	Novagen
pET-37b (+)	–	T7*lac*	Kan	Signal sequence N-CBD_{cenA} C-His	Thr Xa	pBR322	Novagen
pGEV2	>5,443	T7*lac*	Amp	N-GB1 domain C-His	Thr Xa	pBR322	Huth et al., 1997
PinPoint	3,331	T7 or *tac*	Amp	Biotin	Xa	ColE1	Promega
pHPS9**	5,700	P59	Em Cm	None	None	pMB1 pTA1060	ATCC
pMG36e	3,700	P32	Em	None	None	pWV01	van de Guchte et al., 1989
pMQ124	7,621	P_{BAD}	Gm	None	None	ColE1/pRO1600	Shanks et al., 2009
pPC418**	9,135	STP_{2201}	Amp Em	None	None	–	Coderre and Somkuti, 1999
pPR682 (pMAL-c2x)	6,645	*tac*	Amp	N-MBP	Xa	ColE1	NEB
pQE-30 UA	3,504	T5*lac*	Amp	N-His	None	ColE1	Quiagen
pQE-30 Xa	3,500	T5*lac*	Amp	N-His	Xa	ColE1	Quiagen
pQE-40	4,031	T5*lac*	Amp	N-His N-DHFR	None	ColE1	Quiagen
PQE-70	3,426	T5*lac*	Amp	C-His	None	ColE1	Quiagen
pRSFDuet-1*	3,829	T7*lac*	Kan	N-His S	None	RSF1030 (NTP1)	Novagen

(Continued)

TABLE 2 | Continued

Vector[a]	Size (bp)	Promoter[b]	Selection[c]	Tags and fusion partners[d]	Protease cleavage sites[e]	Origin	Supplier/References
pT7-1	2,400	T7	Amp	None	None	ColE1	Tabor and Richardson, 1985
pTYB12	7,417	T7	Amp	N-VMA intein CBD	None	pBR322	NEB
pSRQ11***	9,400	–	Em	–	None	–	Gonzalez and Kunka, 1987
pSuV1	7,332	T7	Amp	*pelB* signal sequence C-VMA intein CBD	Self-cleavage	ColE1	Ingham et al., 2005

[a]*Vectors containing two MCS (Multiple Cloning Site). **Shuttle vectors: pHPS9 E.coli-Bacillus subtilis shutle vector; pPC418, E. coli–St. thermophiles shuttle vector. ***pSRQ11 PA-1 pediocin plasmid.*
[b]*Promoters information is listed in the text. constitutive p32 and p59 promoter from L. lactis subsp. cremoris Wg2, STP2201 promoter from S. thermophilus ST128.*
[c]*Antibiotic resistance markers: Amp, ampicillin; Cm, chloramphenicol; Em, erythromycin; Gm, gentamicin; Kan, kanamycin; Tet, tetracycline; Str, streptomycin; Spe, spectinomycin.*
[d]*CBD, chitin binding domain; DHFR, Dehydrofolate reductase; GB1 domain: immunoglobulin- DNA binding domain of streptococcal protein G.*
[e]*EK, enterokinase; Thr, thrombin.*

Mergulhão et al., 2003; Mergulhao et al., 2004). This might explain the failure to generate successful expression plasmids under the control of constitutive promoters (Mccormick et al., 1998; McCormick et al., 1999), while the use of inducible promoters enables stable bacteriocin expression (Gutiérrez et al., 2005). Thus, an appropriate promoter must be strong, have a low basal expression level (i.e., be highly repressible) and a cost-effective induction system.

A large number of promoter systems have been described for protein production in *E. coli* (reviewed in Terpe, 2006; Durani et al., 2012; Rosano and Ceccarelli, 2014). In terms of bacteriocin expression, the L-arabinose inducible *araBAD* (P_{BAD}) promoter has been used in some studies (Shanks et al., 2012; Metelev et al., 2013; Barraza et al., 2017), while most bacteriocins have been expressed using *lac*-derived promoters inducible by lactose or its non-hydrolyzable analog isopropyl β-D-thiogalactopyranoside (IPTG), including *tac* promoter (Miller et al., 1998; Kawai et al., 2003), T5*lac* (Moon et al., 2005, 2006; Verdon et al., 2013; Kayalvizhi et al., 2016) and the most widely used T7 and T7*lac* promoters (**Table 2**).

In T7 promoter systems, the gene of interest is cloned behind a promoter recognized by the phage T7 RNA polymerase that is provided by another plasmid or in the bacterial genome in a prophage (λDE3). T7 RNA polymerase is under the transcriptional control of a *lac*UV5 promoter inducible by lactose or IPTG. Basal expression can be controlled by the introduction of a mutated promoter of the *lacI* gene, called $lacI^Q$, that increases the expression of the *lac* promoter repressor LacI. Additionally, the co-expression of T7 lysozyme provided in a compatible plasmid (pLysS or pLysE) can inhibit the transcription of T7 RNA polymerase. In the case of the T7*lac* promoter, this also includes a *lac*O operator downstream of the promoter that avoids basal expression (reviewed in Rosano and Ceccarelli, 2014).

Protein Targeting-Signal Secretion Sequences

Since *E. coli* is a Gram-negative bacterium, bacteriocin production can be confined to the cytoplasm where it can accumulate in a soluble form or aggregate in insoluble inclusion bodies, or can also be secreted into the periplasmic space or into the culture medium. Several factors including protein size, amino acid composition, and the type of leader peptide can affect bacteriocin translocation to these locations (reviewed in Mergulhao et al., 2004).

Most bacteriocins use a dedicated secretion machinery in their natural hosts to export the bacteriocin to the extracellular media. However, the production of active bacteriocins directly to the culture medium by cloning their whole operons in *E. coli* has been reported on a limited number of occasions (**Figure 2**) (Caetano et al., 2011a; Mesa-Pereira et al., 2017). Generally, the overexpression of cloned native genes leads to bacteriocin accumulation in inclusion bodies in the cytoplasm. At first glance, the formation of inclusion bodies could be advantageous as the expressed bacteriocins are inactive and protected against host proteases, facilitating their purification and a high protein yield. However, this aggregation can affect the host metabolism and additional steps (inclusion body isolation, solubilization of the aggregates, and protein refolding) are required for their purification, limiting their large-scale production (Mergulhao et al., 2004).

The processing and the correct folding of bacteriocins as well as their recovery can be simplified when the peptide is secreted into the *E. coli* periplasm or into the culture medium. To achieve that, signal sequences of proteins recognized by a general protein secretory pathway (*sec* pathway), such as the maltose-binding protein *malE* (Miller et al., 1998), the pectase lyase secretion signal *pelB* (Ingham et al., 2005), and the outer membrane protein *ompA* (Zhang et al., 1995), have been fused to the bacteriocin sequence for targeting their secretion to the periplasm. In addition, the bacteriocin divergicin A signal peptide has been used to direct the expression of mesentericin Y105 in *E. coli* in the absence of their dedicated secretion machinery (Biet et al., 1998). This relies on the fact that divergicin A, as well as other bacteriocins such as hiracin JM79 (Sánchez et al., 2007), acidocin B (Leer et al., 1995), and enterocin P (Cintas et al., 1997; Gutiérrez et al., 2005), can be exported by the *E. coli sec* pathway. Subsequently, the peptides can be

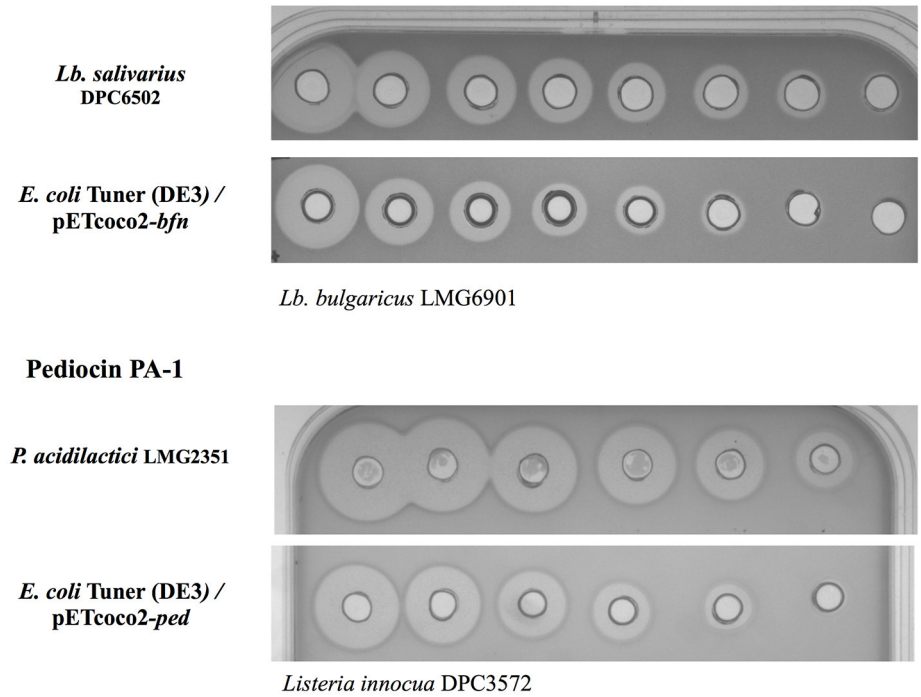

FIGURE 2 | Bacteriocin activity of 2-fold serial dilutions of cell-free supernatants. Antimicrobial activity of bactofencin A against *Lb. bulgaricus* LMG 6901 produced by *Lb. salivarius* DPC6502 (natural producer) and *E. coli* Tuner (DE3) carrying the vector expressing the whole bactofencin A operon (*bfn*) and pediocin PA-1 against *Listeria innocua* DPC3572 produced by *P. acidilactici* LMG2351 (natural producer) or by *E. coli* Tuner (DE3) carrying the plasmid with the pediocin PA-1 operon (*ped*).

secreted into the culture medium by osmotic shock or cell wall permeabilization or, alternatively, be released directly into the medium using a periplasmic leaky *E. coli* host (E609L, Miller et al., 1998).

It is important to mention that bacteriocins containing disulfide bonds are normally accumulated in the periplasm where disulfide binding proteins catalyze the oxidation process (Miller et al., 1998) or alternatively overexpressed in the cytoplasm by thioreductase-deficient (*trxB*) and glutathione reductase (*gor*) deficient strains (Terpe, 2006).

Affinity Tags and Other Fusion Partners

Given the small size of bacteriocins, the incorporation of affinity tags, such as poly-His-tags, facilitates their detection and allows for one-step affinity purification. In addition, the use of fusion protein partners can increase the expression level, enhance protein solubility and assist in correct folding and disulfide bond formation (Ingham and Moore, 2007). Common fusion partners used for bacteriocin production include the cellulose binding domain (CBD_{cenA}) (Klocke et al., 2005), maltose-binding protein (MBP) (Quadri et al., 1997; Kim et al., 2006), thiorredoxin (Trx) (Gibbs et al., 2004; Richard et al., 2004; Beaulieu et al., 2007; Yildirim et al., 2007; Jasniewski et al., 2008; Caetano et al., 2011a; Liu et al., 2011; Pal and Srivastava, 2014, 2015a,b; Jiang et al., 2016; Mustopa et al., 2016; Meng et al., 2017; Tang et al., 2018) and the small ubiquitin-related modifier SUMO (Wang et al., 2013).

Since secretion in the native hosts involves the cleavage of the signal sequence, such fusions often lack antimicrobial activity until chemical (e.g., cyanogen bromide cleaves proteins on the C-terminal side of methionine residues) or enymatic cleavage occurs. The cyanogen bromide (CNBr) chemical cleavage strategy has been used to release the mature PlnE and PlnF (Fimland et al., 2008) and PlnJ and PlnK (Rogne et al., 2009), as has also been described for production of carnobacteriocin B2 and BM1 (Jasniewski et al., 2008) and piscicolin 126 (Gibbs et al., 2004). However, the most common approach to release recombinant bacteriocins is to include a sequence, between the signal peptide and the bacteriocin, recognized by Factor Xa (Quadri et al., 1997; Kawai et al., 2003; Klocke et al., 2005; Moon et al., 2006; Ingham and Moore, 2007; Rogne et al., 2008; Shi et al., 2011), trypsin (Shi et al., 2012; Himes et al., 2016), thrombin (Klocke et al., 2005), enterokinases (Beaulieu et al., 2007; Jasniewski et al., 2008; Liu et al., 2011; Pal and Srivastava, 2014, 2015a,b; Jiang et al., 2016; Meng et al., 2016; Tang et al., 2018), and SUMO proteases (Wang et al., 2013). Alternatively, the use of intein fusions has been described for the cloning and expression of self-cleaving fusion forms of unmodified bacteriocins under appropriate buffer conditions (Ingham et al., 2005).

E. COLI STRAINS FOR BACTERIOCIN EXPRESSION

Since bacteriocins can easily be degraded in the expression strain (Chen et al., 2012), *E. coli* BL21 (DE3) and its derivates are most frequently used for bacteriocin expression (**Tables 1**, **3**) as they are deficient in the Lon protease and the outer membrane protease OmpT (Gottesman, 1996). Tuner™ (DE3) strains (Novagen) are *lacZY* deletion mutants of BL21. The lac permease mutation (*lacY*) allows uniform entry of IPTG into all cells in the population, which enables the regulation of the levels of protein expression by adjusting the concentration of the inductor IPTG. Other B related strains such as C41 (DE3) and C43 (DE3), described specially for the production of toxic proteins (Terpe, 2006), have been used successfully for the expression of the bacteriocin bovicin HJ50 while the use of *E. coli* BL21 (DE3) did not produce this peptide (Wang et al., 2016).

The designation DE3 indicates that the host is a lysogen of λDE3 which carries a chromosomal copy of the T7 RNA polymerase under the *lac*UV5 promoter required for the expression of genes under a T7 promoter (Rosano and Ceccarelli, 2014). To suppress the basal expression of T7 RNA polymerase prior to induction, especially important for toxic protein expression that affect cell growth and viability of the host, pLysS hosts carry a plasmid that encodes the T7 lysozyme, an inhibitor of T7 RNA polymerase. The pLacI designation is given to hosts bearing a plasmid that encodes the *lac* repressor.

K-12 derivatives have also been used for bacteriocin expression. *E. coli* JM109, which is *lon* protease deficient, has been used for gassericin A expression (Kawai et al., 2003). Divercin V41, enterocin CRL35, LSE_2163 and LSE_2386 and pediocin PA-1 that require disulphide bond formation for proper folding have been expressed in Origami™ (DE3) strains (Beaulieu et al., 2007; Yildirim et al., 2007; Kuo et al., 2013; Masias et al., 2014), which carry a double mutation in thioredoxin reductase (*trxB*) and glutathione reductase (*gor*) genes with an oxidative cytoplasmic environment that allows disulfide bond formation. The same *trxB/gor* mutations are carried by a derived Tuner™ strain, Origami B, combining the characteristics of BL21 and Origami hosts in one strain (Novagen). Other combinations, including Rosetta-gami™ (K-12 derivative) and Rosetta-gami™ B (BL21 derivative), facilitate the expression of eukaryotic proteins containing rare codons and cytoplasmic disulphide bond formation at the same time. **Table 3** summarizes the features of commercial *E. coli* strains most used for bacteriocin production.

TABLE 3 | Features of commercial *E. coli* strains commonly used for bacteriocin expression.

Strain[a]	Strain background	$lacI^q$	$ompT^-$	lon^-	$trxB^-$	gor^-	$lacY^-$	Expression of toxic proteins	Rare codons tRNAs	pLysS	pLacI	dcm^-	Antibiotic resistance[b]	Supplier[c]
AD494 (DE3)	K12	•			•								Kan	N
BL21	B		•	•								•		N
BL21 (DE3)	B		•	•								•		N
BL21 gold (DE3)	B		•	•								•	Tet	AT
BL21 (DE3) pLys	B		•	•						•		•	Cam	N
BL21 (DE3) RIL (DE3) pLysS	B		•	•					•	•		•	Cam	S
C41 (DE3) pLysS	B		•	•				•		•		•	Cam	L
C43 (DE3) pLysS	B		•	•				•		•		•	Cam	L
ER2566	K12		•	•								•		NEB
M15[pRep4]	K12	•											Kan	Q
Origami (DE3)	K12	•			•	•							Cam, Kan, Str, Tet	N
Origami (DE3) pLysS	K12	•			•	•				•			Cam, Kan, Tet	N
Rosetta (DE3) pLysS	B		•	•			•		•	•		•	Cam	N
Rosetta-gami 2 (DE3)	K12	•			•	•			•				Cam, Str, Tet	N
Tuner (DE3)	B		•	•			•					•		N
Tuner (DE3) pLacI	B		•	•			•				•	•	Cam	N

$lacI^q$ (constitutive expression of the lac repressor), $ompT^-$ (mutation in outer-membrane protease), lon^- (Inactivation of Lon protease), $trxB^-$ (mutation in thioredoxin reductase), gor^- (mutation in gluthatione reductase), $lacY^-$ (lactose permease activity abolished), pLysS (encodes T7 lysozyme), pLacI (encodes lac repressor), dcm^- (blocks cytosine methylation). Toxic proteins include many membrane proteins, some cytoplasmic proteins, and nucleases.

[a] Gold: provide increased transformation efficiency and produce high-quality miniprep DNA. RIL contain extra copies of the argU, ileY, and leuW TRNA genes.

[b] Antibiotic resistance: Cam, chloramphenicol; Kan, kanamycine; Tet, Tetracycline; Str, Streptomycin.

[c] Supplier: AT, Agilent Technologies; L, Lucigen; N, Novagen; NEB, New England Biolabs, Q, Quiagen, B, Stratagene.

Codon Bias

Major differences between the codon usage of *E. coli* and the overexpressed protein may be an obstacle for bacteriocin production, especially in the case of rare codons. Rare codons are defined as codons used by *E. coli* at a frequency <1% (Kane, 1995), which means the tRNA for these codons are rare or lacking in the expression host. To overcome this problem, two strategies have been used: codon optimization of the mature sequences of bacteriocins (Richard et al., 2004; Ingham et al., 2005; Verdon et al., 2013) or increasing the availability of underrepresented tRNAs by supplying pRIL or pRARE plasmids. pRIL vector provides extra genes for the tRNAs for Arg (AGG/AGA), Ile (AUA), and Leu (CUA) while pRARE encodes tRNA genes for all the above-mentioned codons plus Gly (GGA) and Pro (CCC). pRIL or pRARE plasmids are supplied in the BL21 derivatives, BL21 (DE3) Codon Plus strain (Stratagene), and Rosetta (DE3) strains (Novagen), respectively. However, it is important to mention that although these strains can improve the level of production, they sometimes can cause a decrease in protein solubility (Rosano and Ceccarelli, 2014) or even decrease the growth rate of *E. coli* significantly (Masias et al., 2014).

Selecting a Suitable *E. coli* Strain

Given that the expression level of bacteriocins can vary in different *E. coli* strains (**Figure 3**), it is recommended to test different strains, chosen to reflect the properties of the bacteriocin (e.g., disulfide bonds, rare codons, etc.) to select the best host for the heterologous expression. A clear example is described by Masias et al. (2014), who showed the expression of enterocin CRL35 in *E. coli* BL21, C41, C43, Origami, and Rosetta-gami 2. In this study, the expression of enterocin CRL35 was lower in *E. coli* C41 and C43 than in other *E. coli* strains. The best strains for enterocin CRL35 expression were *E. coli* Rosetta and *E. coli* Rosetta-gami 2 since they are able to synthesize proteins despite the presence of rare codons. In addition, this study showed that *E. coli* Rosetta produced an additional enterocin CRL35 variant. Therefore, the correct choice of the strain is critical for bacteriocin expression.

CULTURE CONDITIONS FOR BACTERIOCIN EXPRESSION

Both culture media composition and culture conditions are important for optimizing the heterologous expression of bacteriocins and must be optimized for each bacteriocin in each expression system as reviewed in **Table 1**. Even two peptides of the same bacteriocin might require different conditions, for example the maximum soluble fraction of PLNC8α was observed under 0.5 mM IPTG induction for 16 h at 20°C, while for PLNC8β it was 0.2 mM IPTG for 20 h at 16°C (Jiang et al., 2016). Bacteriocin production can be also increased with different production strategies such as batch and fed-batch cultivation (Gibbs et al., 2004; Yildirim et al., 2007). Although it is hard to generalize, there are some observations that can be taken into account in terms of culture conditions to facilitate this trial-and-error process.

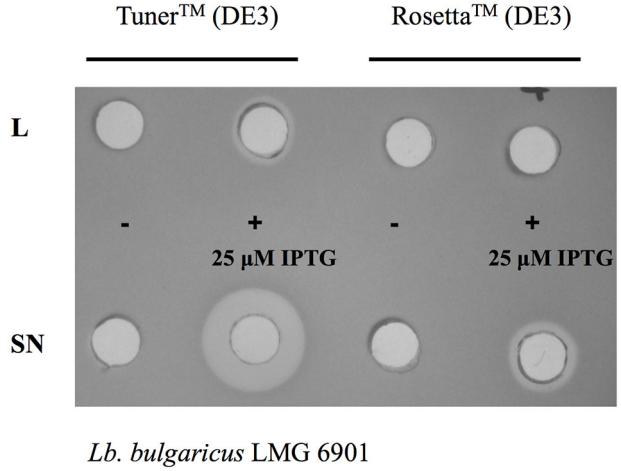

FIGURE 3 | Differences in antimicrobial activity of cell-free supernatants (SN) and lysates (L) of *E. coli* Tuner™ (DE3) and Rosetta™ (DE3) cells carrying the plasmid encoding the whole operon of pediocin PA-1 without induction (–) and after 3 h of 25 μM IPTG induction (+).

Growth Media

Luria Broth (LB) is the most commonly used medium for culturing *E. coli* for bacteriocin expression as it is easy to make and it is nutritionally rich. However, the cell density obtained with this medium is low, affecting bacteriocin yield. To overcome this problem, there are media superior to LB available for reaching higher cell densities such as 2 × yeast extract tryptone (YT), Terrific Broth (TB), and Super Broth (SB) (Rosano and Ceccarelli, 2014). 2 × YT medium has been used for expression of nukacin ISK-1 (Nagao et al., 2005) and epidermicin NI01 (Sandiford and Upton, 2012), while TB has been used for divercin V41 (Richard et al., 2004), carnobacteriocin Cbn1 and CbnB2 production (Jasniewski et al., 2008). Other bacteriocins have been produced using minimal medium (Rogne et al., 2008; Metelev et al., 2013). Therefore, there are no general rules. Pal and Srivastava (2015b) found higher Plantaricin E yield in LB than in TB, while Kuthning et al. (2015) found the best-producing conditions for Bliα and Bliβ lichenicidin peptides was medium M compared to 2 × YT, SB, TB, and LB-Kelly.

Alternatively, M9 medium supplementation with 10 mM of EDTA and 0.05% Tween 20 at the time of induction could also increase the final yield (Masias et al., 2014). In addition, the pH of the culturing media has also an impact on peptide yields either promoting bacteriocin expression or increasing peptide stability as described for lichenicidin expression which increased at pH 6.5 compared to pH 8 (Kuthning et al., 2015).

Inducer Agents

Bacteriocin expression levels can be tuned by varying the inducer concentration (Gutiérrez et al., 2005; Yildirim et al., 2007; Masias et al., 2014; Jiang et al., 2016), but use of a high concentration to fully induce the promoter does not necessarily lead to maximal

expression due to the metabolic burden and toxicity of the inducer to the cells (Glick, 1995).

Although the IPTG inducible *lac* expression system of *E. coli* is the most used for bacteriocin expression, IPTG is expensive and toxic and therefore not suitable for large-scale production. This problem could be solved by replacing IPTG by lactose, which is not toxic, and has resulted in an increase in the production yield of some bacteriocins such as the carnobacteriocins Cbn BM1 and Cbn B2 (Jasniewski et al., 2008).

Temperature

Temperature is one of the most important factors for the expression of functional proteins (Sambrook and Russell, 2001). Chen et al. (2012) showed that the expression level of sakacin P was higher when *E. coli* BL21 (DE3) carrying pET28a-sakP was induced at 20°C than at 37°C. Similar results were observed when the expression of plantaricin E was induced at 25°C rather than at 37°C (Pal and Srivastava, 2015b). Although the optimum growth temperature for *E. coli* is around 37°C, the cell growth and the protein synthesis are slowed down at lower temperatures, which provides the peptides the time and optimal environment to fold into their native conformation (Sambrook and Russell, 2001; Peng et al., 2004), decreasing the aggregation and increasing the expression of soluble protein. In addition, lowering the temperature, in combination with the time after induction, might shift the codon usage bias in *E. coli* sufficiently to solve some codon-usage based expression problems (Terpe, 2006). Therefore, it is essential to determine the optimal induction temperature in each case to improve the amount of the soluble fraction. When inclusion bodies formation is a problem, it is recommended to express the protein in the range of 15–25°C (Rosano and Ceccarelli, 2014).

CONCLUSION

The development of heterologous expression systems to improve bacteriocin yield may facilitate their characterization and broaden their applications in food and pharmaceutical industries. Currently *E. coli* is the most popular recombinant protein expression platform. However, choosing the perfect combination of expression vector and strain for bacteriocin production in *E. coli* is not possible *a priori* due to the many variables that can affect bacteriocin production. This review covers different strategies used for the bacteriocin expression in *E. coli* to help the process of choosing the best expression system and the conditions for any particular bacteriocin with a view to producing bacteriocins economically for both food and pharmaceutical applications.

AUTHOR CONTRIBUTIONS

BM-P, MR, PC, CH, and RR wrote the manuscript and approved its final version.

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REVIEW
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Recent Progress in the Chemical Synthesis of Class II and S-Glycosylated Bacteriocins

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A wide variety of antimicrobial peptides produced by lactic acid bacteria (LAB) have been identified and studied in the last decades. Known as bacteriocins, these ribosomally synthesized peptides inhibit the growth of a wide range of bacterial species through numerous mechanisms and show a great variety of spectrum of activity. With their great potential as antimicrobial additives and alternatives to traditional antibiotics in food preservation and handling, animal production and in veterinary and medical medicine, the demand for bacteriocins is rapidly increasing. Bacteriocins are most often produced by fermentation but, in several cases, the low isolated yields and difficulties associated with their purification seriously limit their use on a large scale. Chemical synthesis has been proposed for their production and recent advances in peptide synthesis methodologies have allowed the preparation of several bacteriocins. Moreover, the significant cost reduction for peptide synthesis reagents and building blocks has made chemical synthesis of bacteriocins more attractive and competitive. From a protein engineering point of view, the chemical approach offers many advantages such as the possibility to rapidly perform amino acid substitution, use unnatural or modified residues, and make backbone and side chain modifications to improve potency, modify the activity spectrum or increase the stability of the targeted bacteriocin. This review summarized synthetic approaches that have been developed and used in recent years to allow the preparation of class IIa bacteriocins and *S*-linked glycopeptides from LAB. Synthetic strategies such as the use of pseudoprolines, backbone protecting groups, microwave irradiations, selective disulfide bridge formation and chemical ligations to prepare class II and *S*-glycosylsated bacteriocins are discussed.

Keywords: bacteriocins, antimicrobial peptides, peptide synthesis, solid-phase peptide synthesis, native chemical ligation, microwave-assisted synthesis

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INTRODUCTION

Antimicrobial resistance to antibiotics has become a major challenge in the treatment of infectious diseases, chronic illnesses and immune disorders (Bush et al., 2011; Lewis, 2013; Michael et al., 2014; Ferri et al., 2017). This public health problem is very costly both in care and life and often faces limited treatment options. The unregulated use and misuse of antibiotics in the food and animal production industry as well as veterinary and human medicine led to the emergence of multi-resistant pathogenic strains (Center for Disease Controls and Prevention: U.S. Department of Health and Human Services, 2013; Cotter et al., 2013; Van Boeckel et al., 2015).

In this context, new highly effective antimicrobial agents for which resistance cannot be readily acquired are sorely needed in order to maintain the ability of modern medicine to treat bacterial infections. Among the most promising alternatives to conventional antibiotics, bacteriocins show very attractive antimicrobial properties and their potential use as food preservatives, bio-controlling agents or therapeutics has been widely studied in the last decades (Papagianni, 2003; Bali et al., 2016; López-Cuellar et al., 2016; Ahmad et al., 2017; Mathur et al., 2017).

Produced by a wide variety of bacteria to fight other microorganisms in their competitive environments, bacteriocins form a heterogeneous group of peptides with great variations in size, structure and mode of action. Hundreds of these ribosomally synthesized peptides have been identified and characterized over the years and are now described in detail in various databases (Hammami et al., 2007; van Heel et al., 2013). Several approaches have been developed to classify bacteriocins and the classification used in this review is based on the system used to classify the bacteriocins of lactic acid bacteria (LAB) (Cotter et al., 2005, 2013) on their structural characteristics with respect to the nomenclature proposed for ribosomally-synthesized post-translationally modified peptides (RiPPs) (Arnison et al., 2013). Bacteriocins produced by Gram-positive bacteria, such as those from LAB, are divided into three major classes: the heat stable post-translationally modified peptides (class I), the low-molecular weight (<10 kDa) heat stable unmodified peptides (class II) and the heat labile high-molecular-weight proteins (class III). In Gram-negative bacteria, most characterized bacteriocins have been isolated from *Escherichia coli* and other *enterobacteria*, and they are often referred to as microcins (small peptides) or colicins (larger proteins).

The most important appeal of bacteriocins as antibacterial agents and antibiotic substitutes is their multiple advantages over other antimicrobial agents commonly used in food preservation and handling, animal production and in human and veterinary medicine. For example, bacteriocins have been shown to be: (i) safe for consumption since they are completely digested in the gastrointestinal tract (Kheadr et al., 2010; Fernandez et al., 2016), (ii) highly potent (10^3 to 10^6 times more than several other antimicrobials including conventional antibiotics); (iii) resistant to common thermal treatments for pasteurization or even sterilization (De Vuyst and Leroy, 2007; Keymanesh et al., 2009; Abriouel et al., 2010). Moreover, several bacteriocins are recognized as GRAS (generally recognized as safe) substances by the United States Food and Drug Administration (FDA) and the European legislation for pharmaceutical and food industry uses. However, despite their great potential and attractive efficacy, the use of bacteriocins remains limited due largely to high production costs usually associated with low production yields and onerous technological requirements. More research and development as well as new approaches are needed in order to make the use of bacteriocins as antimicrobial agents feasible on a larger scale, whether in the food industry or in human health and veterinary medicine.

Chemical synthesis has been proposed for the large-scale production of active bacteriocins. However, very few bacteriocins have been successfully prepared in satisfying yields using such means. Several challenging features that are essential for their bioactivity, such as lasso structure, large macrocycles, and presence of long hydrophobic segments, lanthionines, glycosylated side chains or complicated peptide motifs can make the task very daunting. Beside their production on a large scale, access to bacteriocins by chemical synthesis would allow further molecular engineering to enhance the potency, improve pharmacological properties, increase the stability and modify the spectrum of activity. As a result, new approaches were urgently needed to overcome synthetic pitfalls and efficiently prepare bioactive bacteriocins by chemical synthesis. Fortunately, several developments in peptide synthesis methodologies have been successfully used to prepare several bacteriocins. Because class III bacteriocins are complex large proteins currently inaccessible by chemical approaches and the synthesis of class I lanthibiotic bacteriocins has already been reviewed by Tabor et *al.* (Tabor, 2011), this review will focus on class I *S*-linked glycopeptides and class II bacteriocins produced by Gram-positive bacteria and discuss the recent advances made for their synthesis.

GENERAL CHEMICAL SYNTHESIS STRATEGIES

A wide variety of technologies including chemical synthesis, recombinant DNA technology, cell-free expression systems, transgenic plants or enzymatic synthesis have been developed to produce peptide-based compounds. Generally the choice of the most suitable technology to produce a peptide is based on its size and the chemical synthesis approach is more appropriate and efficient for low-molecular weight peptides (<6 kDa). With the possibility to use unnatural amino acids, introduce pseudo-peptide bonds and perform side chain modifications, chemical synthesis offers access to a more important chemical diversity than peptides produced by recombinant methodologies. With the development of solid-phase peptide synthesis (SPPS), large-scale chemical synthesis has become a viable approach for the production of small- and medium-sized peptides ranging from approximately 5 to 50 residues, and the chemical way is now often a better option than the biotechnological methods of recombinant DNA or biocatalysis for the synthesis of medium-sized peptides. Compared to conventional synthesis in solution where a purification is required after each step, the SPPS approach is considerably more convenient and efficient since the growing peptide chain is attached to an insoluble polymeric support and allows the use of larger amount of reagents to favor reaction completion, simple removal of excess reagents by filtration and washing, and a single purification step once the peptide sequence is completed and removed from the solid support. In the SPPS methodology, N^{α}-protected amino acids are attached to the N-terminal amine of the growing peptide chain on the solid support followed by deprotection of the amino group (**Figure 1A**). This two-step cycle (coupling and deprotection) is repeated until the peptide sequence is completed and the desired peptide can be obtained after its release from the solid support and removal of the side-chain protecting groups.

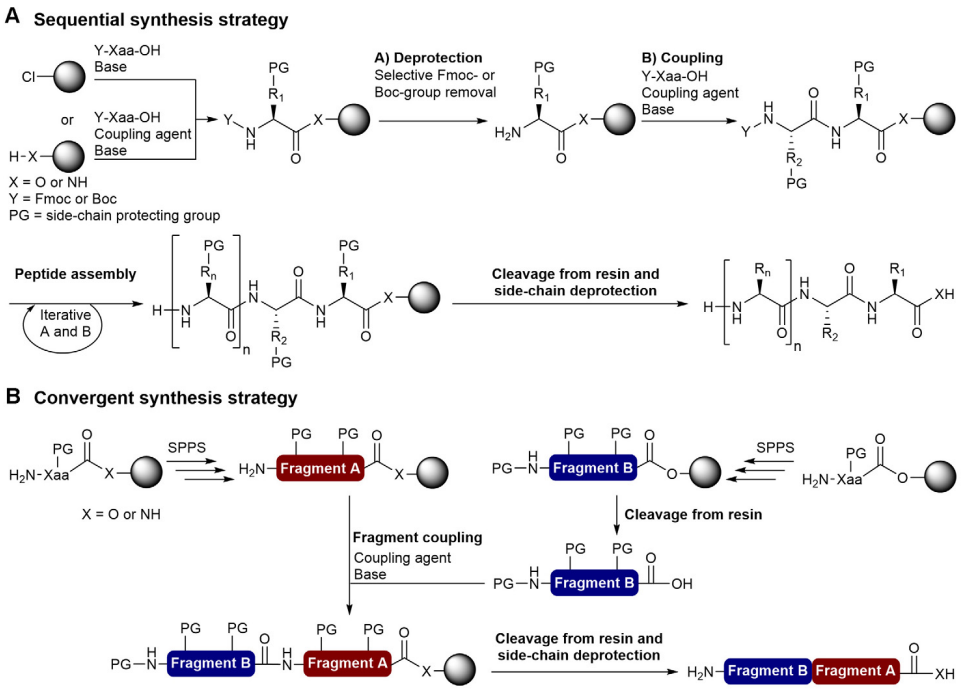

FIGURE 1 | Solid-phase peptide synthesis strategies. **(A)** Stepwise synthesis using the Fmoc/tBu or Boc/Bzl strategy and **(B)** Convergent synthesis.

The chemical synthesis of peptides has remarkably progressed since the first work of Bruce Merrifield on SPPS (Merrifield, 1963). A great number of synthetic improvements including more efficient coupling reagents (Valeur and Bradley, 2009; El-Faham and Albericio, 2011), solid supports (Kates et al., 1998; Rademann et al., 1999; García-Martín et al., 2006; Rapp, 2007), linkers (Boas et al., 2009; Góngora-Benítez et al., 2013), orthogonal protecting groups (Isidro-Llobet et al., 2009), and the use of microwave (MW) irradiations (Collins et al., 2014) have emerged to overcome difficulties associated with SPPS and access a wide variety of peptides (Guan et al., 2015; Behrendt et al., 2016; Paradis-Bas et al., 2016), including bacteriocins.

The two most important SPPS strategies are the linear and convergent synthesis. The linear (sequential) synthesis approach involves the stepwise addition of amino acids until the desired peptide is achieved (**Figure 1A**). Currently, peptides prepared by stepwise amino acid additions via standard SPPS are generally limited to approximately 50 residues (Chen et al., 2015; Guan et al., 2015). Convergent synthesis involves the independent solid-phase synthesis of peptide fragments that are then cleaved from the polymer and linked by condensation on solid support or in solution with standard coupling reagents or chemoselective reactions (chemical ligation) (**Figure 1B**). The convergent approach is often the most appropriate way to synthesize peptides that contain >50 amino acid residues and the development of several chemical ligation methods allowed the preparation of long peptide chains and small proteins (Harmand et al., 2014; Thapa et al., 2014; Chen et al., 2015; Bondalapati et al., 2016). While fragment condensation with standard coupling reagents requires N- or C-terminal and side-chain protected fragments, chemical ligation methods such as the native chemical ligation (NCL) (Dawson et al., 1994), α-ketoacid-hydroxylamine ligation (KAHA) (Bode et al., 2006; Pusterla and Bode, 2015; Bode, 2017), salicylaldehyde (SAL) ester-mediated ligation (Zhang et al., 2013) and traceless-Staudinger ligation (Nilsson et al., 2000; Saxon et al., 2000) are compatible with unprotected peptide fragments.

For both sequential and convergent synthesis approaches, the solid support plays a critical role in peptide assembly and should be mechanically stable, show good swelling properties in commonly used organic solvents, and be compatible with the selected synthetic methodology. The most frequently used solid supports for peptide synthesis are the classic cross-linked polystyrene (PS) resins, polyethylene glycol (PEG)-PS composite resins (e.g., TentalGel^{TM}) (Rapp, 2007) and cross-linked PEG resins (e.g., ChemMatrix®) (García-Martín et al., 2006). Compared to PS resins, PEG-containing resins are compatible with polar solvents and they were proposed to more suitable for the synthesis of large peptides as they are able to form aggregation-disrupting interactions with growing peptide chains (García-Martín et al., 2006). The Boc/Bzl and Fmoc/tBu synthetic methodologies are the two most common SPPS strategies, and the strategy utilized should be considered when choosing the appropriate type of resin linker (Sewald and Jakubke, 2009; Góngora-Benítez et al., 2013). Several systems for the automated synthesis of peptides covering scales ranging from 1.5 mg to 5 kg and compatible with Boc/Bzl (Boc-SPPS) and Fmoc/tBu SPPS (Fmoc-SPPS) are now available from several companies. Based on the mild acidic conditions for final deprotection and

the commercial availability of a wide variety of orthogonally protected amino acids, the Fmoc/tBu approach has been the most commonly used strategy to prepare bacteriocins by chemical synthesis. This review describes the different strategies that have been reported to overcome synthetic pitfalls and access bioactive bacteriocins.

SYNTHESIS OF CLASS II BACTERIOCINS

Class II bacteriocins are heat stable small peptides containing from 25 to 70 amino acid residues that are largely unmodified, with the exception of disulfide bridges, head-to-tail macrocyclization and N-terminus formylation. LAB are frequently found as producers of class II bacteriocins and members of this class can be further divided into subgroups, including the class IIa, IIb, IIc, and IId covered in this review. Showing variations in size and structure, a great variety of synthetic strategies have been used to overcome pitfalls and successfully prepare members of this class.

Class IIa Bacteriocins

Also called pediocin-like bacteriocins, members of the class IIa are well-known for their strong antilisterial activity and have been widely studied. Characterized by an N-terminal consensus YGNGV sequence, they contain from 35 to 50 amino acid residues and generally a minimum of two Cys residues involved in a disulfide bond. Several class IIa bacteriocins and analogs thereof have been successfully prepared by chemical synthesis. Total synthesis for leucocin A, pediocin PA-1, sakacin P, curvacin A, mesentericin Y105, enterocin CRL35 and lactococcin MMFII have been reported (**Table 1**). Several studies reported the use of a synthetic class IIa bacteriocin but complete details about their synthesis are unfortunately often missing. The first described total synthesis of class IIa bacteriocins was reported by Fimland et al. (1996) to determine the role of the C-terminal region in bacterial strain specificity. In their study, sakacin P, curvacin A, leucocin A and pediocin PA-1 were prepared by stepwise standard Boc-SPPS. After their release from the resin (not described), the deprotected peptides were obtained with crude purities ranging from 1 to 10%. While sakacin P required two HPLC runs to obtain a 70–80% purity, the purification of pediocin PA-1, curvacin A and leucocin A needed a cation-exchange chromatography and three HPLC runs to reach >80% purity. In this case, the yields obtained were relatively low and found to be about 10% for sakacin P, 3% for curvacin A and leucocin A and about 1% for pediocin PA-1 (**Table 1**).

In order to overcome synthetic pitfalls and increase the yields, researchers have introduced several SPPS strategies, including the use of pseudoprolines, specialized resins, heating during coupling and NCL. The case of leucocin A, a bacteriocin of 37 amino acids from *Leuconostoc gelidium*, is particularly interesting since several strategies have been used to achieved higher yields and develop bioactive analogs (**Table 1**). To produce leucocin A by standard Fmoc-SPPS, Fleury et al. (1996) used a polyamide/kiesel guhr resin bearing the 4-hydroxymethylphenoxyacetic acid linker (HMPA) and were able to isolated the bacteriocin in 16% crude yield before purification. In another study, Yan et al. prepared the enantiomer of leucocin A (composed of D-amino acids) by stepwise Fmoc-SPPS with D-amino acids on Wang resin using the coupling reagent 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU) for the 20 first residues and 2-(7-aza-1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HATU) at 50°C for the remaining positions (Yan et al., 2000). This simple modification in the synthetic pathway allowed the synthesis of all-D leucocin A in a 6% overall yield after cleavage from the resin and purification by HPLC (**Table 1**). In other reports from the same group, several unnatural analogs of leucocin A, such as [9,14]-dicarba-, [9,14]-diallyl-, [C9S, C14S]-, and [C9F, C14F]-leucocin A analogs were prepared on NovaSyn TGA resin (HMPA-PEG-PS) by incorporating pseudoproline dipeptides at positions 14/15 [Cys-Ser($\Psi^{Me,Me}$Pro)] and/or 22/23 [Phe-Ser($\Psi^{Me,Me}$Pro)] in the sequence to minimize on-resin aggregation of the growing peptide chain (Derksen et al., 2006; Derksen et al., 2008). Unfortunately, low overall yields have been obtained for these leucocin A analogs (**Table 1**). More recently, Bodapati et al. (2013) used a convergent NCL strategy to prepare bioactive leucocin A (**Figure 2**). In their approach, the Cys at position 14 was selected as the ligation site (**Figure 2A**) and the two fragments have been prepared by standard Fmoc-SPPS on 2-chlorotrityl resin. After its release from the resin, the protected N-terminal fragment (fragment 1 [1–13]) was submitted to thioesterification with ethyl 3-mercaptopropionate in presence of *N*,*N*′-diisopropylcarbodiimide (DIC), *N*-hydroxybenzotriazole (HOBt) and *N*,*N*-diisopropylethylamine (DIPEA) and precipitated in cold diethyl ether. The protecting groups were removed under acidic conditions in presence of trifluoroacetic acid (TFA) and the fragment 1 thioester obtained in 33% yield after purification by HPLC. On the other hand, the C-terminal fragment (fragment 2 [14–37]) was isolated in 55% yield after cleavage from the resin, side chain deprotection and purification by HPLC. Next, the two fragments were coupled by NCL in phosphate buffer (pH 7.6) containing 6 M guanidinium chloride (GdnHCl) and thiophenol (4%)/benzyl mecaptan (4%) as catalysts (**Figure 2B**). Monitoring of the reaction showed that the ligation was completed in 24 h. The ligation product was purified by HPLC and isolated in 98% yield. Finally, the disulfide bond was formed by air oxidation in Tris buffer (pH 8.4) containing DMSO (20%) for 48 h and the cyclic product subjected to HPLC purification to afford pure leucocin A in 70% yield. The overall yield for the synthesis of leucocin A using the NCL approach was 12% [fragment 1 (33%) × fragment 2 (55%) × NCL (98%) × cyclization (70%)] (**Table 1**).

Pediocin PA-1, a bacteriocin of 44 amino acid residues from *Pediococcus acidilactici* containing two disulfide bonds between Cys9-Cys14 and Cys24-Cys44, is another very interesting case since the problems associated with its production by recombinant technologies and chemical synthesis continue to limit its applicability and delay regulatory approval. The very high sensitivity of the methionine at position 31 to aerobic oxidation is the first challenge for the production of this bacteriocin. Pediocin PA-1 oxidized at this position is 100

TABLE 1 | Reported synthetic class IIa bacteriocins and methods used for their synthesis.

Bacteriocin	AA	Sequence	Method	Yield[a]
Sakacin P	43	KYYGNGVHCGKHSCTVDWGTAIGNIGNNAAANWATGGNAGWNK	Boc-SPPS	10%[b]
Curvacin A	41	ARSYGNGVYCNNKKCWVNRGEATQSIIGGMISGWASGLAGM	Boc-SPPS	3%[b]
Leucocin A	37	KYYGNGVHCTKSGCSVNWGEAFSAGVHRLANGGNGFW	Boc-SPPS Fmoc-SPPS + couplings at 50°C NCL	3%[b] 6%[c] 12%[d]
Leucocin A analogs	37	KYYGNGVHXTKSGXSVNWGEAFSAGVHRLANGGNGFW	Fmoc-SPPS + Pseudoproline	<1–3%[e,f]
Pediocin PA-1	44	KYYGNGVTCGKHSCSVDWGKATTCIINNGAMAWATGGHQGNHKC	Boc-SPPS	1%[b]
[M31Nle]-Pediocin PA-1 and its analogs	44	KYYGNGVTXGKHSXSVDWGKATTCIINNGAZAWATGGHQGNHKC	Fmoc-SPPS + couplings at 50°C Fmoc-SPPS + Pseudoproline	3.8%[g] <1%[f]
Mesentericin Y105	37	KYYGNGVHCTKSGCSVNWGEAASAGIHRLANGGNGFW	Fmoc-SPPS	n.r[h]
Enterocin CRL35	43	KYYGNGVSCNKKGCSVDWGKAIGIIGNNSAANLATGGAAGWKS	Fmoc-SPPS	n.r.[i]
Lactococcin MMFFII	37	TSYGNGVHCNKSKCWIDVSELETYKAGTVSNPKDILW	Fmoc-SPPS	n.r.[j]

n.r.: not reported; X = allylglycine, Ser, Phe, norvaline, or Cys; Z = Norleucine (Nle). [a]Overall isolated yield for purified bacteriocins; [b]sequence analysis revealed 85–95% purity for pediocin PA-1 and leucocin A and 70–80% purity for sakacin P and curvacin A (Fimland et al., 1996); [c]leucocin A enantiomer (all-D leucocin A) and amino acids after position 20 were coupled with HATU at 50°C in DMF (Yan et al., 2000); [d]calculated overall yield based on yields obtained for each step (fragment 1 (33%) × fragment 2 (55%) × NCL (98%) × cyclization (70%)) (Bodapati et al., 2013); [e]overall yields based on resin loading (Derksen et al., 2006); [f]overall yields based on resin loading (Derksen et al., 2008); [g]yield for [31Nle]-pediocin PA-1 (Kaur et al., 2004); [h](Fleury et al., 1996; Castano et al., 2005); [i](Masias et al., 2017); [j](Ferchichi et al., 2001).

times less active (Fimland et al., 1996) and no currently used methods for disulfide bond formation have been able to prevent Met31 oxidation. To avoid this problem, Kaur et al. (2004) replaced the methionine at position 31 by norleucine. The peptide was prepared by stepwise standard Fmoc-SPPS on Wang resin and by coupling the amino acids with HBTU in DMF for the first 15 residues and HATU at 50°C in *N*-methylpyrrolidone (NMP) for the remaining positions. After cleavage from the resin and side chain deprotection with a TFA cocktail, the linear precursor was purified by HPLC. The Acm protecting groups on Cys9 and Cys14 were removed along with disulfide bond formation with I_2 in MeOH (0.1 M) for 2 h followed by quenching with aqueous ascorbic acid (1 M). A final HPLC purification afforded [M31Nle]-pediocin PA-1 in 3.8% overall yield (**Table 1**). Antimicrobial activity assays showed that the synthetic [M31Nle]-pediocin PA-1 was equally potent as the natural pediocin PA-1 against *Listeria innocua* and *Carnobacterium divergens* (Kaur et al., 2004). In another study from the same group, several [M31Nle]-pediocin PA-1 analogs were prepared on 2-chlorotrityl resin by incorporating pseudoproline dipeptides at positions 21/22 and 34/35 [Ala-The($\Psi^{Me,Me}$Pro)] in each case and 14/15 [Phe-Ser($\Psi^{Me,Me}$Pro)] for [9,14]-diPhe and 7/8 [Val-Thr($\Psi^{Me,Me}$Pro)] for [9,14]-diallyl analogs (Derksen et al., 2008). Unfortunately, after their release from the resin, side chain deprotection and purification by HPLC, the [M31Nle]-pediocin PA-1 analogs have been isolated in <1% overall yields, based on resin loading (**Table 1**).

While several reports on the synthesis of leucocin A and pediocin PA-1 and analogs thereof have been published, detailed total syntheses for other class IIa bacteriocins are substantially rarer. The synthesis of mesentericin Y105, a class IIa bacteriocin from *Leuconostoc mesenteroides* containing 37 amino acids, was achieved by standard Fmoc-SPPS on HMPA-PEG-PS resin using DIC or HBTU as coupling reagent (Fleury et al., 1996; Castano et al., 2005). Similarly, a bacteriocin of 43 amino acids from *Enterococcus mundtii* CRL35 and named enterocin CRL35 has been prepared on Rink amide resin using standard Fmoc-SPPS (Saavedra et al., 2004; Emilse et al., 2015; Masias et al., 2017). Other class IIa bacteriocins such as the lactococcin MMFII from *Lactococcus lactis* (Ferchichi et al., 2001) have been synthesized but detailed information about their preparation and yield are not reported in the published study. Carnobacteriocin B2 from *Carnobacterium piscicola* has been prepared with its leader sequence in <1% overall yield (Sprules et al., 2004).

Class IIb Bacteriocins

Class IIb bacteriocins, also known as two-peptide bacteriocins, act by the complementary action of two different peptides. Compared to pediocin-like bacteriocins, members of the class IIb have been less studied and reports on their synthesis are rarer. Among the few reported studies on class IIb bacteriocins, we were able to find only one that uses a synthetic bacteriocin. In this case, the complementary peptides lactocin 705α (GMSGY IQGIPDFLKGYLHGISAANKHKKGRLGY) and lactocin 705β (GFWGGLGYIAGRVGAAYGHAQASANNHHSPING) from *Lactobacillus casei* CRL705 were prepared by standard Fmoc-SPPS (Cuozzo et al., 2000; Castellano et al., 2007). Unfortunately, the peptides were prepared by an external company and information about their preparation and isolated yields could not be found.

Class IIc Bacteriocins

Class IIc bacteriocins are head-to-tail cyclic bacteriocins containing from 35 to 70 amino acids. The exceptionally large size of the macrocycle and the high content of hydrophobic residues found in several members of this class pose serious synthetic challenges for their preparation. Several macrocyclization

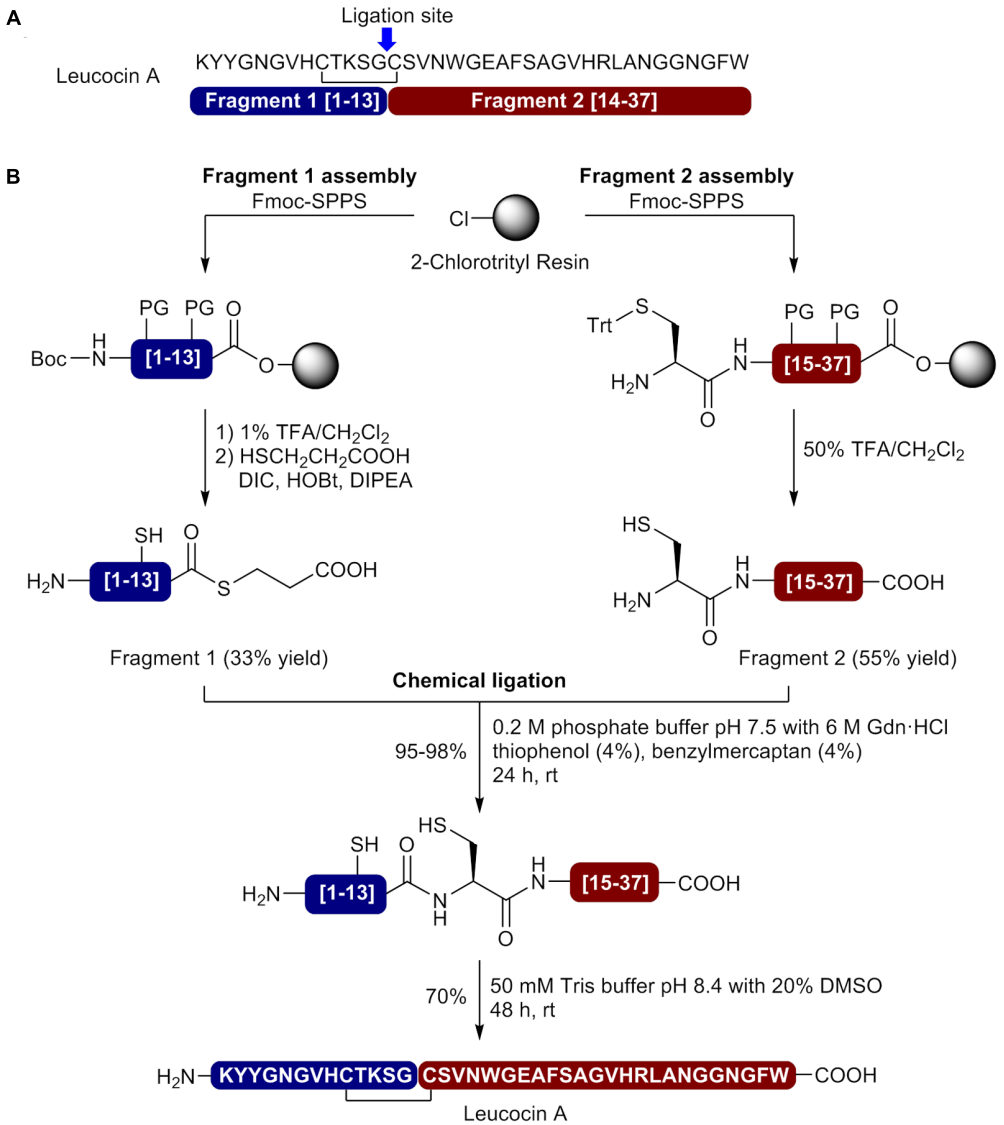

FIGURE 2 | Synthesis of leucocin A using native chemical ligation. **(A)** Leucocin A sequence showing the selected ligation site and identification of the fragments. **(B)** Assembly of the N- and C-terminal fragments by Fmoc-SPPS, native chemical ligation and oxidative cyclization (Bodapati et al., 2013).

methodologies, including a thia-zip cyclization/desulfurization combination have been attempted for the final ring closure but none were able to yield the final circular bacteriocin.

The first successful total synthesis of circular bacteriocins has been achieved by Hemu et al. (2016) using a chemoenzymatic approach. In this breakthrough study, the enzyme butelase I, an Asp/Asn specific ligase isolated from the leguminous plant *Clitoria ternatea*, was used to perform the final head-to-tail macrocyclization (Nguyen et al., 2014, 2016). Butelase I recognizes the tripeptide NHV motif in a precursor and eliminates the HV dipeptide in the ligated product (Cao et al., 2015; Nguyen et al., 2015, 2016). It also displays a broad specificity for the incoming sequence with no preference for the N-terminal P1 position and favoring hydrophobic residues (Leu, Val, Ile) at the P2 position (Nguyen et al., 2014, 2016). The most attractive advantages of the reported butelase-mediated macrocyclization are the absence of extra sequence in the ligation product (traceless reaction) and the possibility to add polar amino acids in the recognition signal to increase aqueous solubility of the linear precursor. The only unavoidable requirement is the presence of an Asn or Asp residue in the peptide sequence. In the described study, the butelase-mediated macrocyclization was applied to prepare the circular bacteriocins AS-48, uberolysin and garvicin ML, each containing at least one Asn (**Figure 3A**). Linear precursors containing a C-terminal NHV recognition signal were first assembled on PEG-PS resin bearing the 5-(3,5-dimethoxy-4-(aminomethyl)phenoxy)pentanoic acid (PAL) linker by stepwise MW-assisted Fmoc-SPPS in only 6 h using DIC and ethyl 2-cyano-2-(hydroxyimino)acetate (Oxyma) as coupling reagents (**Figure 3B**) (Collins et al., 2014). A supplementary

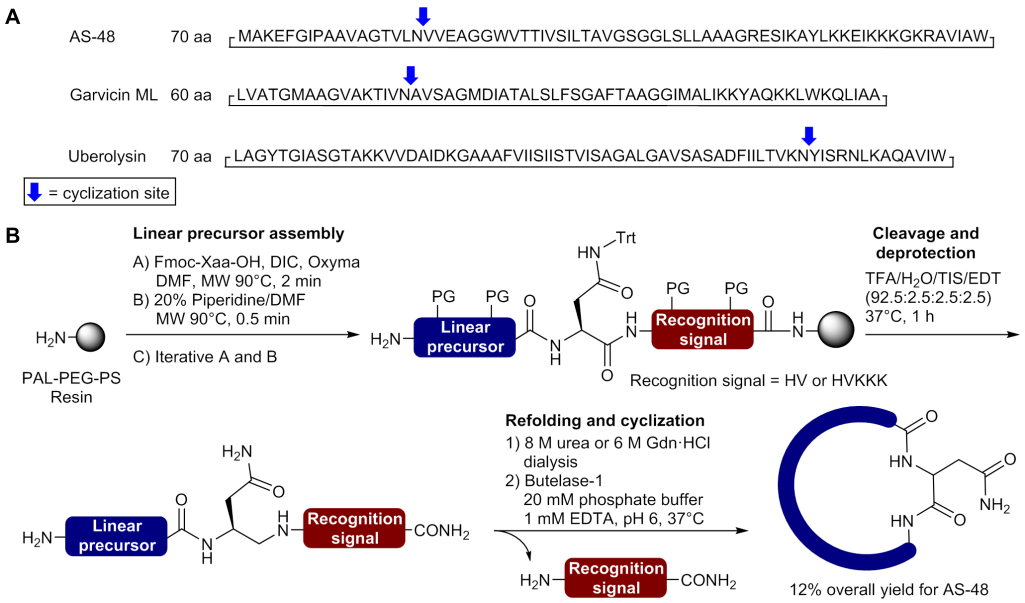

FIGURE 3 | Synthesis of circular bacteriocins using the butelase chemoenzymatic approach. **(A)** Sequences and selected macrocyclization sites. **(B)** Preparation of the linear precursor and butelase-mediated macrocyclization (Hemu et al., 2016).

C-terminal KKK sequence was added on AS-48 and uberolysin linear precursors to increase aqueous solubility of highly hydrophobic segments found in their sequence and facilitate butelase-mediated cyclization. After their release from the resin with a TFA cocktail, the linear precursors were purified by HPLC. Direct macrocyclization of the unfolded linear precursors by the traceless butelase-mediated ligation was unsuccessful. To overcome this setback and possibly brings the N and C termini in close proximity, the linear precursors were refolded by dissolution in 8 M urea or 6 M GdnHCl at 50–100 mM followed by dialysis for folding (**Figure 3B**). To perform the butelase-mediated macrocyclization, Hemu et *al.* used a peptide concentration of 50 μM in a sodium phosphate buffer (20 mM Na_2HPO_4, 1 mM EDTA, pH 6) with a peptide: enzyme ratio of 100:1 and observed that the ring closing reaction at 37°C was completed in 1 h for AS-48, 0.5 h for garvicin ML and 24 h for uberolysin with yields of 85%, 90 and 93%, respectively. To accelerate the procedure, the authors performed a one-pot synthesis of AS-48 where the purification of the linear precursor was bypass. With only a final purification required, the entire process for the synthesis of AS-48 was completed in less than 24 h (6 h for the linear precursor synthesis, 5 h for refolding, 1 h for the macrocyclization and 1 h for the purification) and the circular bacteriocin obtained with an excellent 12% overall yield. This chemoenzymatic approach developed by Tam and coworkers is really promising and will certainly be applied for the synthesis of unusually large cyclic peptides and circular proteins. Unfortunately butelase is not readily available and is produced by extraction from pods of *Clitoria ternatea* (Nguyen et al., 2014), as it cannot be produced by recombinant technologies at the present time. The development of new methodologies to produce recombinant ligases will certainly promote the use of enzyme-mediated ligation and cyclization in the synthesis of bacteriocins, complex peptides and proteins (Yang et al., 2017).

More recently, Rohrbacher et al. (2017) reported the preparation of a homoserine-mutant of AS-48 by using a convergent synthesis with α-ketoacid-hydroxylamine (KAHA) ligations for linear precursor assembly and final macrocyclization. Compatible with organic and aqueous solvents, the KAHA ligation relies on the chemoselective reaction of ketoacids with hydroxylamines to generate an amide bond and is well-suited for assembling hydrophobic peptides and proteins (Harmand et al., 2014; Rohrbacher et al., 2015; Bode, 2017). In the reported approach, the authors use two fragments bearing an *N*-terminal (*S*)-5-oxaproline as cyclic hydroxylamine to generate homoserine residues at the ligation and macrocyclization sites after reaction with C-terminal α-ketoacids and *O*- to *N*-acyl transfers (**Figure 4A**). An important property of the KAHA approach is the formation of depsipeptide bonds (ester bonds) as primary ligation products leaving free polar amino groups at the reaction site (**Figure 4B**). This interesting feature can facilitate the assembly of hydrophobic sequences before the *O*- to *N*-acyl transfer and final folding. For the synthesis of the circular bacteriocin AS-48, the selection of V25-T26 as the ligation site and E49-S50 as the cyclization sites was based on a suitable hydrophobicity distribution for each fragment and the lowest impact of homoserine residues on the bioactivity (**Figure 4A**). Moreover, in order to minimize handling and avoid tedious purification of the hydrophobic linear precursor, the authors used photolabile protecting groups for the C-terminal α-ketoacid of fragment 1 and N-terminal 5-oxaproline of fragment 2. A valine α-ketoacid for fragment 1 and a photoprotected glutamic acid α-ketoacid for fragment 2

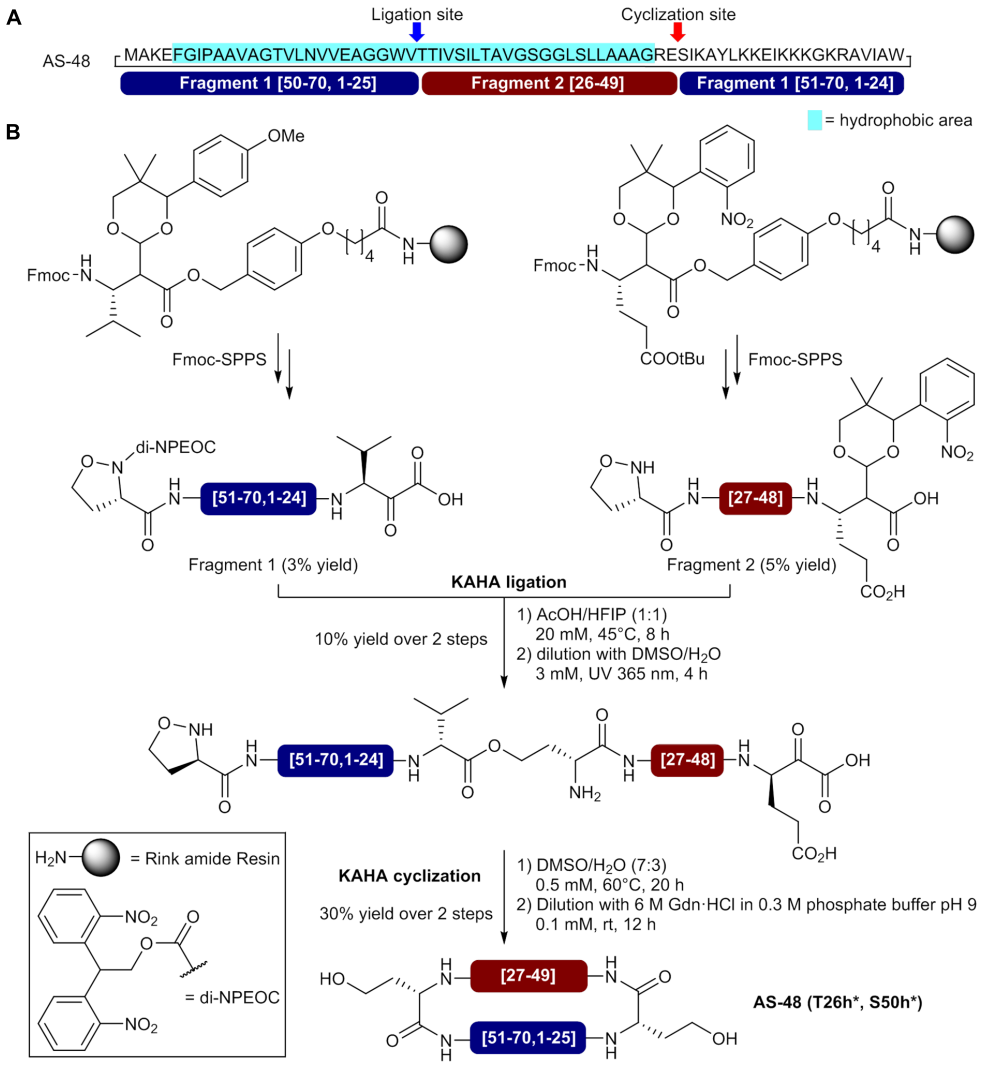

FIGURE 4 | Synthesis of a homoserine-mutant of the circular bacteriocin AS-48 by sequential KAHA ligation and macrocyclization. **(A)** Sequence of AS-48 showing the selected ligation and macrocyclization sites and composition of the fragments. **(B)** Preparation of the N- and C-terminal fragments and circular bacteriocin synthesis by KAHA ligation and macrocyclization (Rohrbacher et al., 2017). h* = homoserine.

were attached to Rink amide resins via a Wang-type linker and both fragments were assembled by standard Fmoc-SPPS using *O*-(6-chlorobenzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HCTU) and HATU as coupling reagents (**Figure 4B**) (Thuaud et al., 2016). Following cleavage from the resin and side chain deprotection with a TFA cocktail, the fragments were purified by HPLC and obtained in 3–5% yields. Fragments 1 and 2 were then subjected to KAHA ligation in a mixture of acetic acid (AcOH) and hexafluoroisopropanol (HFIP) (1:1) at 45°C and the reaction was completed in 8 h. After N- and C-termini deprotection under UV irradiations at 365 nm, the linear precursor was purified by HPLC and obtained in 10% yield. The KAHA macrocyclization was performed with the linear precursor in aqueous DMSO (0.5 mM) at 60°C and completed in 20 h. Finally, the mixture was diluted with 0.3 M phosphate buffer containing 6 M GdnHCl to induce the *O*- to *N*- acyl shifts. After 12 h, both rearrangements were completed and HPLC purification afforded pure homoserine mutant AS-48 (T26h*, S50h*) in 30% yield for the entire macrocyclization process. Surprisingly, the synthetic cyclic bacteriocin showed lower antibacterial activity (MIC >10 mM) than the reported value of 0.5 μM for the natural peptide. While incomplete folding was observed in circular dichroism spectra, the authors showed that the synthetic circular bacteriocin was able to adopt correct folding and recover full activity after storage for 1 month at 4°C in buffer at pH 3. Using the KAHA ligation for fragments condensation and macrocyclization, the authors were able to obtain 1 mg of bioactive homoserine-mutant AS-48 in 0.005% overall yield, based on the loading of preloaded resins. Despite the low yield, the results obtained by Rohrbacher et *al.* demonstrated the utility of the KAHA ligation for the synthesis of hydrophobic proteins via the formation of depsipeptide bonds

and its compatibility with acidic conditions in the presence of organic solvents such as AcOH/HFIP that can efficiently dissolve difficult sequences. The KAHA ligation approach is really promising and will certainly be used again for the synthesis of unusually large cyclic peptides and circular proteins.

Class IId Bacteriocins

Finally, the class IId includes linear non-pediocin-like one-peptide bacteriocins. As observed with class IIb bacteriocins, very few syntheses for members of the class IId have been described in details. Among the few reported studies using synthetic class IId bacteriocins, the 43 amino acids durancin A5-11a (MGAIAKLVTKFGWPLIKKFYKQIMQFIGQGWTIDQIEKW LKRH) and the 44 amino acids durancin A5-11b (MGAIAKL VAKFGWPIVKKYYKQIMQFIGEGWAINKIIEWIKKHI) from *Enterococcus durans* A5-11 have been successfully prepared using MW-assisted Fmoc-SPPS on Rink amide resin (Belguesmia et al., 2013). In another study, the 22 amino acids bactofancin A (KRKKHRCRVYNNGMPTGMYRWC) has also been synthesized by MW-assisted Fmoc-SPPS but using the cross-linked PEG resin ChemMatrix® bearing the 4-(4-hydroxymethyl-3-methoxyphenoxy)-butyric acid (HMPB) linker (O'Shea et al., 2013; Guinane et al., 2016). Unfortunately detailed information about their preparation and isolated yields are not reported in the published studies. The use of synthetic epidermicin NI01 (Gibreel and Upton, 2013), a *N*-terminal formylated bacteriocin from *Staphylococcus epidermis* 224 containing 51 amino acid residues (*N*-formyl-MAAFMKLIQFLATKGQKYVSLAWKHKGTILKWINAGQSFE WIYKQIKKLWA), has also been reported but the peptide was prepared by an external company and information about its synthesis could not be found (Sandiford and Upton, 2012).

SYNTHESIS OF S-GLYCOSYLATED BACTERIOCINS

Glycocins are class I bacteriocins containing at least one carbohydrate moiety linked to a Cys residue via its side chain. In some reports, they can also be found in the class IV as complex bacteriocins containing a carbohydrate moiety. These *S*-linked glycopeptides are rare and only three *S*-glycosylated bacteriocins have been isolated and characterized until now; namely, glycocin F (Stepper et al., 2011), sublancin 168 (Wang and van der Donk, 2011) and thurandacins A and B (Wang et al., 2014). While the *in vitro* reconstitution of the biosynthesis of thurandacins A and B using recombinant technologies has been reported (Wang et al., 2014), glycocin F and sublancin 168 were recently successfully prepared by chemical synthesis.

Sublancin 168 is a glycocin of 37 amino acids produced by *Bacillus subtilis* 168 containing a β–*S*-linked glucose moiety on the Cys22 residue and two disulfide bonds between Cys7-Cys36 and Cys14-Cys29 (Oman et al., 2011; Wang and van der Donk, 2011). The first total synthesis of sublancin 168 was achieved by Katayama et al. (2011) using a thioglycosylamino acid unit (Fmoc-Cys(Glc(OAc)$_4$)-OH) in stepwise Fmoc-SPPS (Katayama et al., 2011). The linear precursor was first assembled on Wang resin by coupling the amino acids at 50°C with *N*,*N*′-dicyclohexylcarbodiimide (DCC) and HOBt in NMP for 1 h. Moreover, a pseudoproline dipeptide (Gly-The($\Psi^{Me,Me}$Pro)) was incorporated at positions 18/19 during peptide elongation. After cleavage from the resin and side chain deprotection with a TFA cocktail, a first disulfide bond was formed between Cys7 and Cys37 in a solution of 6 M urea/5% hydrazine/10% DMSO. The monocyclic product was purified by HPLC and isolated in 5% yield. The Acm protecting groups on Cys14 and Cys29 were removed along with disulfide bond formation using I_2 in MeOH (20 mM) for 1 h at 40°C followed by quenching with aqueous ascorbic acid (1 M). A final HPLC purification afforded sublancin 168 in 20% yield for the cyclization step. By using a stepwise synthesis strategy, the authors were able to produce sublancin 168 with a 1% overall yield based on resin loading.

More recently, Hsieh et al. used a convergent NCL approach to prepare sublancin 168 and *S*-glycosylated analogs (Hsieh et al., 2012). In their approach, the Cys at position 14 was selected as the ligation site and two segments have been prepared by MW-assisted Fmoc-SPPS (**Figure 5A**). The N-terminal fragment (fragment 1 [1–13]) was assembled on Rink amide resin bearing a side chain anchored Fmoc-Glu-Oallyl (**Figure 5B**). This side chain anchoring strategy allowed on-resin selective demasking of the C-terminal carboxylate with Pd(PPh$_3$)$_4$ and thioesterification using ethyl-3-mercaptopropionate, DIC, HOBt and DIPEA as base. The peptide thioester was released from the resin and the side chains deprotected by treatment with a TFA cocktail. Fragment 1 was obtained in 15% yield after purification by HPLC. The C-terminal fragment (fragment 2 [14–37]) was prepared on Wang NovaPEG resin and the beforehand prepared thioglycosylamino acid Fmoc-Cys(Glc(OAc)$_4$)-OH introduced at position 22 during peptide synthesis (**Figure 5B**). Moreover, 2,4-dimethoxybenzyl (Dmb) derived dipeptides Fmoc-Gly-(Dmb)Gly-OH were incorporated at positions 17/18 and 23/24 to prevent aggregation during peptide elongation. After cleavage from the resin and side chain deprotection by acidolysis, the *O*-acetate protecting groups on the D-glucose moiety were removed with aqueous hydrazine. Purification of the resulting product afforded fragment 2 in 8% yield. Next, the two segments were subjected to NCL in presence of mercaptophenylacetic acid (MPAA) as thiol catalyst and tris(2-carboxyethyl)phosphine (TCEP) as reductant in 6 M GdnHCl/phosphate buffer at pH 7.2 (**Figure 5B**). The reaction was completed after 16 h and the ligated product isolated in 81% yield after HPLC purification. Finally, the linear precursor was cyclized using oxidized and reduced glutathione (GSH) and purification by HPLC afforded sublancin 168 in 95% yield. In this case, the use of a convergent NCL approach allowed the production of sublancin 168 with a 1% overall yield.

Glocycin F, a 43 amino acid glycopeptide from *Lactobacillus plantarum* KW30, contains two β–linked *N*-acetylglucosamine (GlcNAc) moieties and two disulfide bonds between Cys5-Cys28 and Cys12-Cys21 (**Figure 6A**) (Stepper et al., 2011; Venugopal et al., 2011). While one GlcNAc unit is attached to the sulfur atom of Cys43, the other is linked to side chain oxygen of Ser18. Both GlcNAc moieties have been shown to be essential for glycocin F activity. The first total synthesis

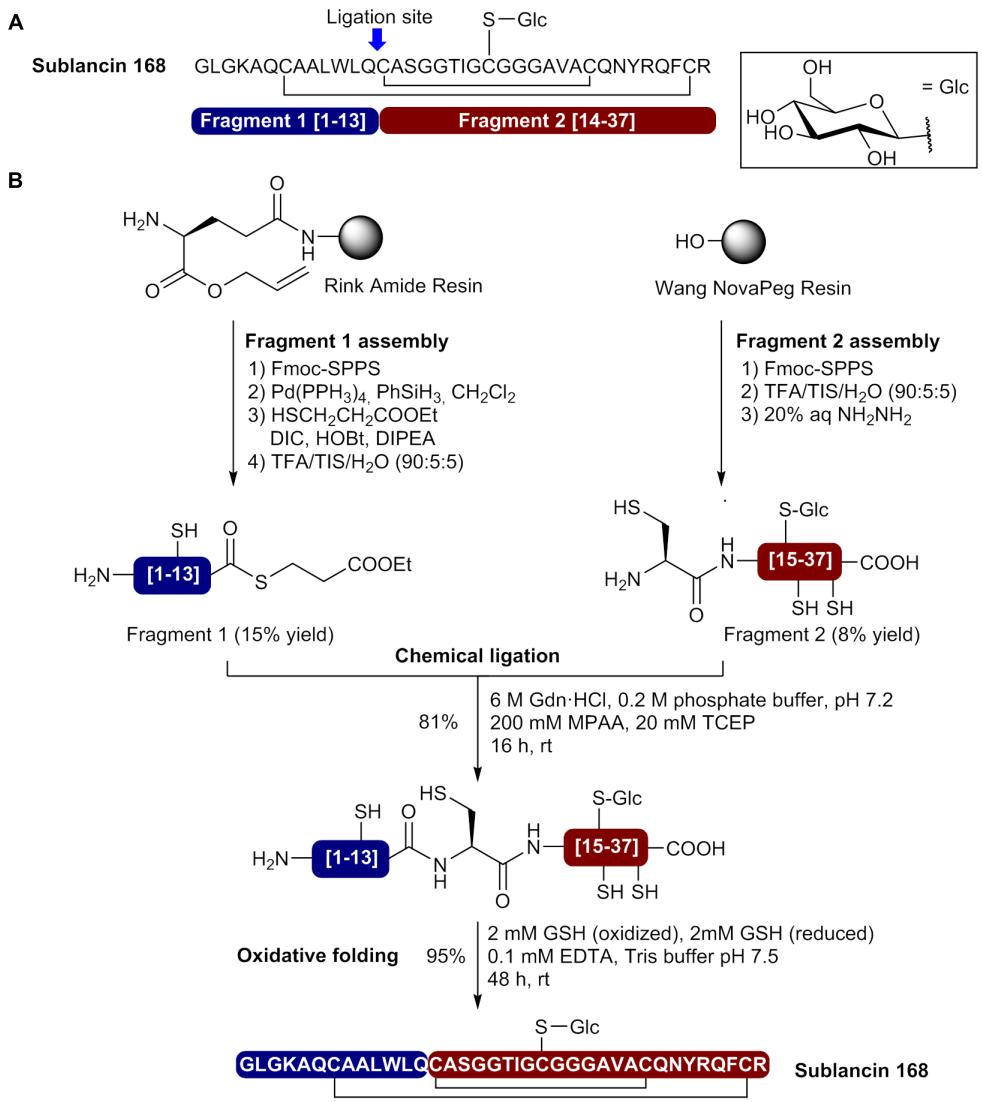

FIGURE 5 | Synthesis of sublancin 168 by a convergent native chemical ligation approach. **(A)** Sequence of sublancin 168 showing the selected ligation site and composition of the fragments. **(B)** Preparation of the N- and C-terminal fragments and bacteriocin synthesis by native chemical ligation and oxidative folding (Hsieh et al., 2012).

of glycocin F was recently reported by Brimble et al. (2015) using a convergent NCL approach. This case is very interesting since several synthetic strategies such as Boc- and Fmoc-SPPS, MW irradiations, incorporation of a pseudoproline dipeptide and N-terminal thiazolidine (Thz) for Cys masking have been combined to successfully achieve the synthesis of bioactive glycocin F. In the described approach, ligation sites at positions 12 and 28 have been selected and involved the use of three fragments to perform two ligation steps and reach the full length peptide (**Figure 6**). First, the side chain glycosylated amino acids Fmoc-Cys(GlcNAc(OAc)$_3$)-OH and Fmoc-Ser(GlcNAc(OAc)$_3$)-OH were beforehand prepared in solution and introduced in their respective segments during peptide elongation by SPPS. The C-terminal fragment (fragment 3 [28–43]) was assembled on Rink amide resin using MW-assisted Fmoc-SPPS and the *S*-glycosylated amino acid Fmoc-Cys(GlcNAc(OAc)$_3$)-OH introduced as the first residue (**Figure 6B**). A pseudoproline dipeptide [Ser(tBu)-Ser($\Psi^{Me,Me}$Pro)] was incorporated at positions 35/36 during peptide elongation. Fragment 3 (H-Cys28-Cys43(GlcNAc(OAc)$_3$)-NH_2) was obtained in 21% yield after resin cleavage and protecting groups removal with a TFA cocktail containing 2,2′-(ethylenedioxy)diethane thiol (DODT) and HPLC purification. The central fragment (fragment 2 [12–27]) was prepared on PS resin bearing the acid labile HMPB linker by MW-assisted Fmoc-SPPS and the *O*-glycosylated amino acid Fmoc-Ser(GlcNAc(OAc)$_3$)-OH introduced position 18 during peptide elongation (**Figure 6B**). The fragment has been capped with an N-terminal thiazolidine residue as a latent Cys to allow the second NCL step. The fully protected peptide fragment was release from the resin using 1% TFA in CH_2Cl_2 and

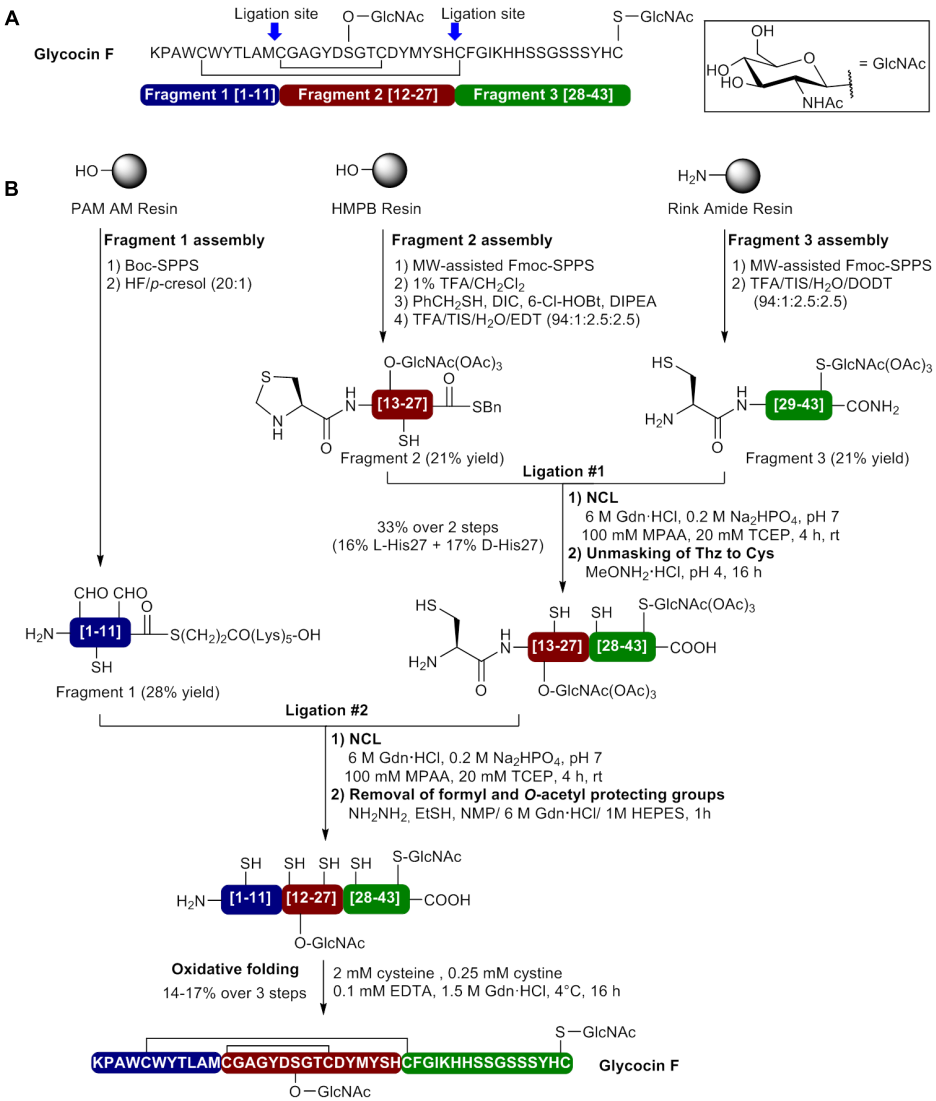

FIGURE 6 | Synthesis of glycocin F by a convergent native chemical ligation approach. **(A)** Sequence of glycocin F showing the selected ligation sites and composition of the fragments. **(B)** Preparation of the three fragments and bacteriocin synthesis by sequential native chemical ligation and oxidative folding (Brimble et al., 2015).

the C-terminal carboxylate thioesterified using benzyl mercaptan in presence of DIC, 6-Cl-HOBt and DIPEA as base. Treatment of the protected peptide thioester with a TFA cocktail containing ethylene dithiol (EDT) and purification by HPLC afforded segment 2 thioester (Thz12- Ser18(GlcNAc(OAc)$_3$)His27-SBn) in 21% yield. Fragments 2 and 3 were then coupled under NCL conditions in phosphate buffer containing 6 M GdnHCl, MPAA and TCEP at pH 7 and the reaction was completed in 4 h to afford the first ligation product Thz12-Ser18(GlcNAc(OAc)$_3$)-Cys43(GlcNAc(OAc)$_3$)-NH$_2$. At this point, LC-MS analyses showed the presence of two ligation products in a 1:1 ratio having identical mass spectra. As NCL has been shown to proceed with stereochemical integrity (Lu et al., 1996; Kent, 2009; Thapa et al., 2014), the authors hypothesized that complete racemization of the His27 on the C-terminus of segment 2 took place during the thioesterification step with benzyl mercaptan in presence of DIC, 6-Cl-HOBt and DIPEA (Jones et al., 1980). After the unmasking of Thz12 to Cys12 using methoxyamine·HCl at pH4, the diastereomers were successfully separated during HPLC purification and obtained in 16.6 and 17.5% yield. As the L-His27 and D-His27 epimers could not be unambiguously assigned, both diastereomers were used in the next steps. The N-terminal fragment (fragment 1 [1–11]) bearing a C-terminal S(CH$_2$)$_2$CO-(Lys)$_5$-OH thioester tail was assembled on 4-(hydroxymethyl)phenylacetamidomethyl resin (PAM AM resin) using standard Boc-SPPS. After cleavage from the resin with anhydrous HF/p-cresol (20:1), the fragment H-Lys1-Met11-COS(CH$_2$)$_2$CO-(Lys)$_5$-OH containing N_{in}-formyl-protected Trp4 and Trp6 was purified by HPLC and isolated in 28% yield. Next, the ligation products were separately subjected to NCL

with fragment 1 using the same conditions as the first ligation and the reaction was completed in 4 h. Removal of the formyl and *O*-acetyl protecting groups on the separated epimeric products was achieved with a mixture of hydrazine and 2-mercaptoethanol in NMP/GdnHCl/HEPES to give the epimeric linear precursors H-Lys1-Ser18(GlcNAc(OAc)$_3$)-Cys43(GlcNAc(OAc)$_3$)-NH$_2$. Oxidative folding was performed with a redox couple containing 2 mM cysteine, 0.25 mM cystine and 0.1 mM EDTA in 1.5 M GdnHCl (pH 8.2) at 4°C for 16 h and HPLC purification afforded glycocin F epimers in 14 and 17% yield over three steps. The HPLC profiles of the synthetic glycocin F epimers were compared to the natural product and the second epimer was identified as the one containing L-His27 since it showed the same retention time as the natural Glycocin F. Furthermore, the second epimer was 3 times more active than the synthetic D-His27 glycocin F. By using a convergent NCL approach, the authors were able to produce glycocin F with a <1% overall yield.

CONCLUSION AND FUTURE PERSPECTIVES

There has been considerable progress over recent years in peptide synthesis and previously inaccessible bacteriocins such as *S*-glycopeptides and circular bacteriocins can now be prepared by chemical synthesis. The syntheses described herein show that the methodologies and approaches that have been used until now to prepare bacteriocins are very diverse. A wide variety of solid supports, linkers, coupling reagents and solvents have been involved in the chemical synthesis of bacteriocins and the Fmoc-SPPS approach has emerged as the most frequently used strategy. While the synthesis of some small bacteriocins was performed straightforwardly by standard stepwise SPPS, other bacteriocins could not be accessed by conventional peptide synthesis methodologies. The most frequently encountered synthetic challenge during the preparation of bacteriocins is certainly the presence of hydrophobic segments that can lead to peptide self-aggregation during elongation and hinder the coupling of residues toward the N-terminus. Fortunately, several strategies have been developed to prevent or disrupt self-aggregation and overcome this synthetic pitfall. Among them, PEG-based resins, turn-inducing residues like pseudoprolines and backbone protecting groups, special solvent mixtures and couplings at higher temperature with conventional heating or microwave irradiations have been used in several syntheses described herein for the preparation of bacteriocins. Gaining a lot of popularity in peptide synthesis, the use of higher temperature and microwave-assisted synthesis have emerged as very efficient methods to significantly reduce the required time for peptide assembly, perform difficult couplings and achieve long peptide sequences. The convergent approach using chemical ligations has also experienced tremendous progress in recent years and will certainly allow the synthesis of several other and larger bacteriocins in the coming years. At present time, the most important setback to the production of bacteriocins by chemical synthesis on an industrial scale is the low obtained yields. It is important to note that the syntheses described herein were not developed for large-scale production but to obtain pure bacteriocins and their analogs in sufficient quantity for antimicrobial assays, structural determination by NMR and mechanistic studies. Optimization of the different steps involved in their synthesis would undoubtedly allow the production of bacteriocins with better yields and on a large-scale. The most important appeal of chemical synthesis to produce bacteriocins is the possibility to easily perform molecular engineering to enhance the potency, improve pharmacological properties, increase the stability and even generate chimeras of different classes. Complementary to recombinant technologies, the chemical synthesis approach will certainly help fulfill the needs for bioactive bacteriocins and analogs thereof in the food industry, animal production and veterinary and human medicine.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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