Inhibitory Effect of Substances Produced by Native *Lactococcus lactis* Strains of Tropical Fruits towards Food Pathogens

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ABSTRACT: The use of peptides produced by lactic acid bacteria (LAB) as antimicrobial agents in food emerged from the increasing need of replacing chemicals with natural substances to ensure their safety and quality. A total of 30 LAB belonging to the genus *Lactococcus* sp. (10) and *Enterococcus* sp. (20) were isolated from native fruits of Ecuador subtropical rainforest. Among *Lactococcus* species, the isolates assigned Gt28, Gt29, and Ella8, identified as *Lactococcus lactis* subsp. *lactis* with 99% identity, showing highly inhibitory potential against four food pathogens were further characterized. The treatment of cell-free supernatant with proteolytic enzymes indicated the protein nature of released components, which displayed a broad antimicrobial activity against Gram-positive and -negative bacteria. Polymerase chain reaction analysis indicated the presence of lacticin 3147 gene in all isolates, lactococcin M gene in Gt28 and Gt29 but not in Ella8 and lactococcin A gene in Gt28 only. The antimicrobial activity was not linked to the presence of structural nisin gene as no amplification product was obtained. Treatment of *Salmonella enterica* ATCC 51741 and *Escherichia coli* ATCC 25922 at both vegetative and exponential phase of growth with the cell-free supernatant of Gt28 resulted in complete inactivation upon 3 h suggesting its bactericidal mode of action. An increment on inhibitory activity occurred when partial purified bacteriocin Gt28 was combined with ethylenediaminetetraacetic acid rather than bacteriocin only, indicating that the cells were sensitized *in vitro* by the chelator agent acting synergistically to induce the killing of pathogenic cells.

Keywords: antimicrobial substances, *Lactococcus lactis*, preservation, food pathogens, bacteriolytic

INTRODUCTION

Targeting spoilage or pathogenic food bacteria using chemicals (organic compounds and antibiotics) or acidification and thermal treatment of food products has been applied in different food products for the purpose of conservation (1). Although these methodologies have been successfully used worldwide, consumers are consistently concerned about their possible adverse effects. To control the pathogen microorganism growth in foods remains a dispute because of their rapid expanding, higher ability to grow and persistence in adverse environments (2). A recent report published by European Food Safety Authority (3) draws attention to the excessive use of antibiotics within the European Union (EU) as well as the concern for the higher occurrence of *Campylobacter* in broiler meat and the presence of *Salmonella* in poultry meat (3). In addition, yersiniosis was reported as the third most common zoonosis in the EU. Listeriosis and Shiga toxins produced by *Escherichia coli* were also reported in fishery products and soft cheeses. Hence, to fulfill consumer demands on food safety, the innovative technologies including the use of biological systems are developing. Conceding that subsequent food processing incorporates antimicrobial treatments, a reduction of spoilage microorganism viability might occur.

The presence of pathogenic microorganisms in ready-to-eat artisanal food was reported (4). Daily consumed artisanal products (e.g. mote, chocho, and chicha) are sold in unsecure and uncontrolled local markets, beyond inappropriate storage and improperly manipulated by the vendors. Although the national normative for food products is well established and aligned to the international one, they are deficiently applied (5), and therefore, the exposure to contaminants is higher compromising the quality of the products. Lactic acid bacteria (LAB) enabling the productions of antimicrobial peptides (i.e. bacteriocins) and other organic acids (lactic acid, diacetyl, and hydrogen peroxide) are qualified for human consumption due to their generally recognized as safe (GRAS) status and represents an attractive mechanism for preservation (6,7). LAB include different genera such as *Lactococcus*,

Lactobacillus, Streptococcus, and *Enterococcus* species traditionally used as starter cultures or probiotics in the food industry or pharmaceutical companies (8-12). Usually the antimicrobial substances produced by LAB exhibit antagonistic activity against microorganisms closely related to the producing strain and only few can control the growth of food spoilage bacteria (6,8). Although many species are reported as bacteriocin producers, nisin from *Lactococcus lactis,* remains the only approved bacteriocin by the Food and Agriculture Organization. As a food additive, nisin suppresses the post-germination spores and toxin formation by *Clostridium botulinum* and *Listeria monocytogenes* in pasteurized processed cheese (11). Bacteriocins of *L. lactis* belong to the clade I of lanthionine small peptides (<5 kDa), and nisin production is encoded by a cluster of genes proposed to be organized as *nisABTCIP, nisRK,* and *nisFEG* (13). Along with nisin A, there are genes encoding for immunity, transport, and processing peptides. Previous studies indicate that nisin interacts with the precursor of peptidoglycan and lipid II on the bacterial cell wall and inserts into the bacterial cell membrane, forming pores that successively cause leakage of essential cellular components resulting in cell death (14). Unluckily, nisin is inactive at alkaline pH, higher temperature, and its activity depends on several external factors. For example, organic acids are considered as bacteriocin enhancers facilitating their translocation through the cell wall of pathogenic bacteria upon increase of peptide net charge (15). Not all identified *L. lactis* strains harbor the structural nisin gene; however, several species contain the two peptides system lacticin 3147, two peptide component lactococcin G and M (class IIc) as well as lactococcus specific bacteriocins (class IId) such as lactococcin A (9).

As the effectiveness of antimicrobial components is related to the producer strain, the identification of new bacteria releasing components with a larger spectrum against several food pathogenic/spoilage bacteria are of interest. The presence of LAB in native wild-type fruits of tropical rainforest in Ecuador exhibiting elevated bacteriocinogenic potential was reported (16). The use of wild tropical fruits is limited to the local tribes for daily consumption or used in traditionally medicine (16); however, its microbiota might carry microorganisms with greater antimicrobial performance. The application of these strains or their active components in food products for the control of spoilage microorganism might be successful considering their genetic variation and capacity to survive along with other pathogenic microorganisms in the same environment (unpublished data). Even if the dominant genus associated with the wild-fruits was *Lactobacillus,* some cocci colonies were selected for further analysis (16). In this study, a total of 30 cocci isolates belonging to the genus *Lactococcus* sp. (10) and *Enterococcus* sp. (20)

were characterized. Among them, three isolates showing highly inhibitory potential were evaluated for the presence of antimicrobial substances along with their mode of action *in vitro* for further developing an efficient system to control and prevent food contamination.

MATERIALS AND METHODS

Bacterial strain isolation and identification
Samples consisting of tropical rainforest wild-type fruits were collected aseptically from three regions of Ecuador: humid mesothermal region of Santo Domingo de Los Tsachilas province (43 km away from Quito), subtropical rainforest of Sucumbios (province located in the Amazon region), and Esmeraldas province, a forest of semiarid climate in a costal area. The samples were transferred in sterile plastic bags and processed as previously reported (17). The purified cocci colonies (>100 colonies/fruit) were Gram stained and tested for mobility, indole-, catalase-production, spore formation, and gas production from glucose. Thirty colonies were selected and stored at −80°C in 20% glycerol.

Screening for isolates with antimicrobial capacity
To select for bacteriocin producing strains, the purified *Lactococcus* isolates were tested for their capacity to inhibit the indicator bacteria: *E. coli* ATCC 25922, *Salmonella enterica* ATCC 51741, *E. coli* UTN Ec1, and *Salmonella* UTN Sm2 (two strains isolated from local fresh cheese) were used in agar-well diffusion method (16). Briefly, the LAB strains grown in De Man, Rogosa, and Sharpe (MRS) broth at 37°C for 20 h were used to isolate the cell-free supernatant (CFS) containing active substances by centrifugation at 13,000 g for 20 min at 4°C followed by filtration using 0.22 μm porosity syringe filter. To rule out the possible inhibition activity of organic acids, the CFS was heated at 80°C for 10 min, the pH adjusted at 6.0 [(neutralized cell-free supernatant (NFS)], and the activity was determined. The indicator strain (100 μL) grown in broth medium (7 log CFU/mL) was mixed with 3.5 mL of soft MRS agar (0.75%), overlaid on the nutrient agar plates, and incubated at 37°C for 2 h. The NFS (100 μL) was transferred onto the wells (6 mm) on overlaid agar, incubated at 37°C, and subsequently examined for inhibition zones at 48 h. The experiments were conducted in triplicates, and the residual activity was determined. Eight isolates showing elevated inhibitory activity against all four indicator strains were selected.

Identification of species
The identification of species was performed using the carbohydrate fermentation API50CHL kit (cat. # 50300, bioMérieux, Marcy-l'Étoile, France) and 16S rRNA gene

sequencing (Macrogen Inc., Seoul, Korea) as described by Garzón et al. (16). BLAST was conducted to search for homology of the sequences (http://www.ncbi.nlm.nih.gov/BLAST).

Spectrum of antimicrobial activity

The isolates Gt28, Gt29, and Ella8 showing grater activity towards the four indicator bacteria mentioned above were considered the promising bacteriocin-producers, and their inhibitory spectrum was evaluated against several Gram-positive and -negative bacteria listed in Table 1. The residual activity was evaluated as described above.

Enzymatic sensitivity, heat, acidity, and chemicals effect on inhibitory activity

Sensitization of released components of selected isolates was determined as described by Garzón et al. (16), using proteinase K, pepsin, lysozyme, and α-chymotrypsin (Sigma-Aldrich Co., St. Louis, MO, USA) at the final concentration of 1 mg/mL. Aliquots of CFS were incubated for 10, 30, 60, and 75 min at 60, 80, 90, and 100°C and 15 min at 121°C (autoclaving temperature). Aliquots of CFS were adjusted to pH 2.0, 4.0, 6.0, and 10.0, and incubated for 3 h at room temperature. Moreover, the effect of several chemicals such as Triton X-100 (BDH Chemicals, Poole, UK), ethylenediaminetetraacetic acid (EDTA) (Merck, Kenilworth, NJ, USA), sodium dodecyl sulphate (SDS) (Sigma-Aldrich Co.), and Tween 20 (Sigma-Aldrich Co.) at the final concentration of 10 mg/mL was evaluated. The experiments were conducted in triplicates using *E. coli* ATCC 25922 and *S. enterica* ATCC 51471 as indicator strains. As positive and negative controls untreated CFS and sterile MRS were used, respectively, followed by the termination of residual antimicrobial activity. *Lactobacillus fermentum* CNCM1-2998 (Lac) recovered from an available commercial probiotic, Lacteol Fort (Lactobacillus LB, Axcan Pharma, Houdan, France) was used as a reference (17).

Search for the presence of bacteriocin-encoding genes

Polymerase chain reaction (PCR) amplification of selected LAB total genomic DNA (PureLink™Genomic DNA minikit, #K1820-00, Invitrogen, Carlsbad, CA, USA) targeting several bacteriocin encoding genes (lactococcin A, lactococcin M, lacticin 3147, nisin A, and nisin G) was performed (9). The primer sequences: LacA-forward (5'-GAA GAG GCA ATC AGT AGA G-3') and LacA-reverse (5'-GTG TTC TAT TTA TAG CTA ATG-3') corresponding to lactococcin A; LacM-forward (5'-GAA GAG GCA ATC AGT AGA G-3') and LacM-reverse (5'-GTG TAT GGT CTA GCA TAA G-3') corresponding to lactococcin M; Lac3147-forward (5'-GTC TTT GTG TTG TTT GGA GAT G-3') and Lac3147-reverse (5'-CAA CTC CCG AAA TAA ATC ATC G-3') corresponding to lacticin 3147; NisA-forward (5'-GAT AGT ATC CAT GTC TG-3') and NisA-reverse (5'-CAA TGA TTT CGT TCG AAG-3') for nisin A; NisG-forward (5'-CTA TGA AGT TGC GAC GCA TCA-3') and NisG-reverse (5'-CAT GCC ACT GAT ACC CAA GT-3') for nisin G were used (9). PCR reaction was performed in a Multigene Thermal Cycler (Labnet International, Inc., Edison, NJ, USA) with a Taq Platinum DNA kit (Invitrogen) in a total volume of 50 μL with the following amplification conditions: denaturation step 5 min at 94°C, followed by 35 cycles of 1 min (denaturation) at 94°C, 1 min (annealing) at 47°C for LacM and NisA genes, 50°C for LacA, and 52°C for NisG and

Table 1. Antimicrobial spectrum of *Lactococcus lactis* isolates

Indicator strains	% residual activity		
	Gt28	Gt29	Ella8
Gram (+)			
Bifidobacterium breves ATCC 15700	97.50	93.33	90.00
Streptococcus thermophiles ATCC 19258	97.00	90.00	87.00
Lactobacillus acidophilus ATCC 4356	95.00	90.00	90.00
Gram (−)			
Escherichia coli ATCC 25922	94.00	89.00	90.91
Shigella UTN Shg1	88.33	83.33	83.33
Escherichia coli UTN Ec1	87.00	77.29	78.00
Salmonella enterica subsp. *enterica* ATCC 51741	86.00	76.76	78.66
Staphylococcus aureus ATCC 1026	85.00	75.62	77.66
Escherichia coli ATCC 10536	83.50	73.33	78.33
Salmonella enterica subsp. Abaetetuba ATCC 35640	80.00	69.25	79.25
Salmonella UTN Sm2	80.00	64.71	74.66
Enterobacter UTN En1	78.00	67.89	77.33
Shigella sonnei ATCC 25931	75.00	67.14	77.66

Data represent the residual activity (%) of neutralized cell-free supernatant (NFS). The results of the assay are from three experiments, each with triplicate samples. Residual activity in % was calculated as ratio between the inhibition zone of NFS and inhibition zone of cell-free supernatant (no treatment) counterpart multiplied with 100.

Lac3147, and 1.5 min (extension) at 72°C, and 1 cycle of 10 min (final step extension) at 72°C. The PCR products were separated by electrophoresis on 1.2% agarose gels in 1× Tris-borate EDTA (TBE, pH 8.0) buffer (Sigma-Aldrich Co.). Gels were stained in TBE buffer containing 0.5 μg/mL ethidium bromide (Sigma-Aldrich Co.).

Effect of bacteriocin producing Gt28 strain on indicator cells viability

The indicator bacteria *E. coli* ATCC 25922 and *S. enterica* ATCC 51741 were grown independently in tubes containing LB (Luria Bertani, Difco, Detroit, MI, USA) and nutrient agar medium (19). The CFS (20 mL) was added to the indicator strain culture (100 mL) independently at early [optical density (OD)$_{605}$=0.2] and exponential phase growth (OD$_{605}$=0.7) followed by incubation at 37°C for 7 and 9 h measuring the optical density (OD$_{605}$) every h using a spectrophotometer (Nova60, Merck) followed by the plate-agar method to determine the viable cell counts. The experiments were repeated three times, and the untreated indicator strain culture was used as a control.

Effect of EDTA on partial purified Gt28 activity

To obtain precipitated peptides (PP), the CFS of Gt28 was treated with 80% ammonium sulfate, incubated overnight in a refrigerator without stirring and centrifuged at 8,000 *g* for 30 min at 4°C. The PP were recovered in 25 mM ammonium acetate (pH 6.5), desalted by using a midi dialysis kit (cat # PURD10005-1KT, Sigma-Aldrich Co.), pre-equilibrated with phosphate buffer (pH 7.0), and stored at −20°C before use in antimicrobial assays. The effect of EDTA (20 mM) on bacteriocin activity was tested as previously described by Chopra et al. (20). Briefly, the PP (final concentration 6,400 AU/mL) combined with 20 mM EDTA was added to the indicator bacteria cell culture (*E. coli* ATCC 25922 and *S. enterica* ATCC 51741) at early (OD$_{605}$=0.2) and exponential phases (0.7). Log reduction was calculated as the difference between log 10 (CFU) of the untreated cells (no bacteriocin, no EDTA) and the treated cells (bacteriocin added, EDTA or a combination thereof). Log reduction of <1 was considered insignificant. Titer estimated as AU/mL was defined as the highest dilution that inhibited the growth of the indicator strain (21). The experiments were repeated three times, and the untreated indicator strain culture was used as control.

Statistical analysis

All experiments were performed in triplicates, from three independent experiments and expressed as mean±standard deviation. Analysis of variance was applied with least significant difference (LSD with Bonferroni correction) to determine significant differences between the means

(SPSS version 15.0, SPSS Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Bacteriocinogenic strains identification

Bacteriocins produced by LAB are known for the antimicrobial potential towards food spoilage bacteria (9,22-24). Although *L. lactis* species have a higher inhibitory activity towards pathogenic species such as *Listeria* and *Clostridium* (25) due to the presence of nisin, the overall inhibitory activity remains relatively narrow and depends in part by the performance of the producer strain and the type of harbored bacteriocin(s). The presence of *Lactobacillus plantarum* as a dominant specie in wild-type fruits of the Amazon region showing high inhibitory potential against some food-borne pathogenic bacteria has been reported (16). In this study, 30 cocci isolates of wild-type fruits of three geographical region of Ecuador were characterized knowing the importance of *Lactococcus* genus and its components for natural preservation. According to the morphological and biochemical profiles, the selected and purified isolates were affiliated to *Lactococcus* sp. (10 isolates) and *Enterococcus* sp. (20 isolates) groups. Initial screening for bacteriocinogenic strains indicated that the isolates of the *Lactococcus* group exhibited grater inhibitory capacity than the *Enterococcus* group; therefore, they were selected for further analysis (data not shown). Among the *Lactococcus* group, five isolates were from fruits originated in Esmeraldas, four originated in Sucumbios, and only one isolate originated in Santo Domingo de Los Tsachilas. Based on carbohydrate profiles, the isolates were identified as *L. lactis* subsp. *lactis* with 99% identity. The 16S rRNA sequencing analysis confirmed the *L. lactis* species for seven isolates while three isolates failed identification and were discarded for further analysis. Based on multiple alignment of the 16S rRNA gene partial sequences analysis (Jalview version 2.10.1) (26), the isolates were grouped according with the percentage of sequence identity, revealing their larger genetic variability (Fig. 1). Moreover, the results from the agar-well diffusion assay showed that the isolates assigned Gt28, Gt29, and Ella8 displayed elevated activity against all four-indicator species (the average inhibition zone varied between 16∼19 mm), while the other isolates inhibited only one or two indicator strains (data not shown). These isolates were considered for further analysis and were deposited at the NCBI gene bank database with the accession number MG675576.1 (*L. lactis* Gt28), MG675577.1 (*L. lactis* Gt29), and MG675578.1 (*L. lactis* Ella8). BLAST analysis with other species from GenBank database showed a similarity of 99% with *L. lactis* strain NBRC 100933 (accession number: NR_113960.1).
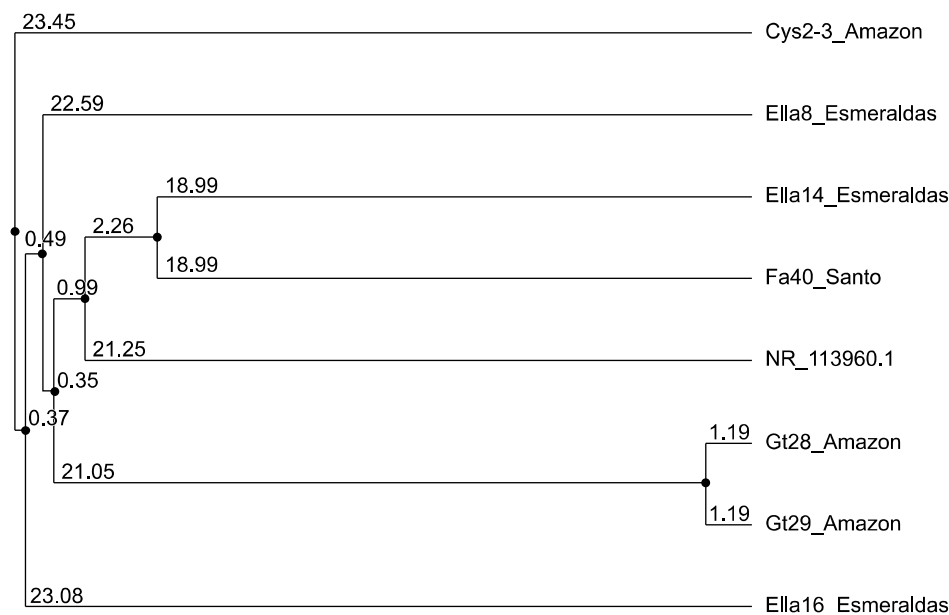
Fig. 1. Average distance tree based on the percentage of 16S rRNA gene partial sequence identity of *Lactococcus* isolates. The percentage identity between two sequences at each alignment position is shown. NR_113960.1, *Lactococcus lactis* strain NBRC 100933 (NCBI).

Inhibitory spectrum and identification of antimicrobial substances

The results from agar-well diffusion assay measurements indicated that the components produced by the Gt28, Gt29, and Ella8 isolates were active towards both Gram-positive and Gram-negative strains (Table 1). Early studies on *Lactococcus* species suggested that the increment on the inhibitory activity might be attributed to the presence of fatty acids or hydrogen peroxidase as well as the antimicrobial peptides; most species inhibit Gram-positive bacteria from the same genera and only few inhibit pathogenic bacteria (9). In general, the effectiveness of bacteriocin is affected by the sensitiveness to proteolytic enzymes. Contrary with the previous study on bacteriocin produced by *L. plantarum* when the activity was completely inactivated by proteolytic enzymes (16), the activity of the crude-extract of the selected *Lactococcus* isolates was not influenced by the removal of NFS and neutralized CFS and hydrogen peroxide eliminated showing the same activity as CFS; a 50% reduction was registered after the treatment with proteinase K, pepsin, and α-chymotrypsin (Table 2). The activity was stable when lysozyme was applied on the crude-extract indicating that the protein might be glycosylated. The activity was partially inhibited by enzymes implying that the released bacteriocins of the selected isolates might possess specific characteristics and bounded by lipids or carbohydrates. The activity was stable overtime after heat treatment of both indicator strains demonstrating their resistance. A 50% loss of inhibitory activity was found when autoclaving. The bacteriocin produced by Gt28 isolate showed a significant increase ($P<0.05$) in activity compared to the untreated control (Fig. 2). Based on multiple comparisons (temperature, time and indicator strain) of the estimated marginal means of the inhibition zone, Gt28 dis-

Table 2. Effect of enzymes, acidity and surfactants on bacteriocin activity

Treatment	Activity (AU/mL)	
	Escherichia coli ATCC 25922	*Salmonella enterica* ATCC 51741
Enzymes (1 mg/mL)		
NHFS+Proteinase K	1,600	1,600
NHFS+α-chymotripsin	1,600	1,600
NHFS+Pepsin	3,200	3,200
NHFS+Lysozyme	6,400	9,600
NHFS	6,400	6,400
NFS	6,400	6,400
pH		
2.0	12,800	12,800
4.0	6,400	9,600
6.0	3,200	6,400
10.0	800	1,600
Ionic/anionic surfactants (10 mg/mL)		
Tween20	1,600	3,200
EDTA	9,600	9,600
SDS	9,600	9,600
Triton-X100	1,600	3,200
CFS (no treatment)	6,400	6,400

The results of the assay are from three independent experiments. CFS, crude extract supernatant; NFS, neutralized CFS (pH 6.0); NHFS, neutralized CFS and hydrogen peroxide eliminated; EDTA, Ethylenediamine tetraacetic acid; SDS, sodium dodecyl sulphate.

played a stable inhibitory activity towards both *E. coli* and *Salmonella* at 60°C and 90°C, while at 100°C, the inhibitory activity was enhanced with the increasing time of incubation (60 min). Previously, we showed that the crude-extract of some *Lactobacillus plantarum* strains displayed higher inhibitory activity after heat exposure, suggesting a pathway similar to the Millard reaction when heat-induced products inferred with antimicrobial activ-
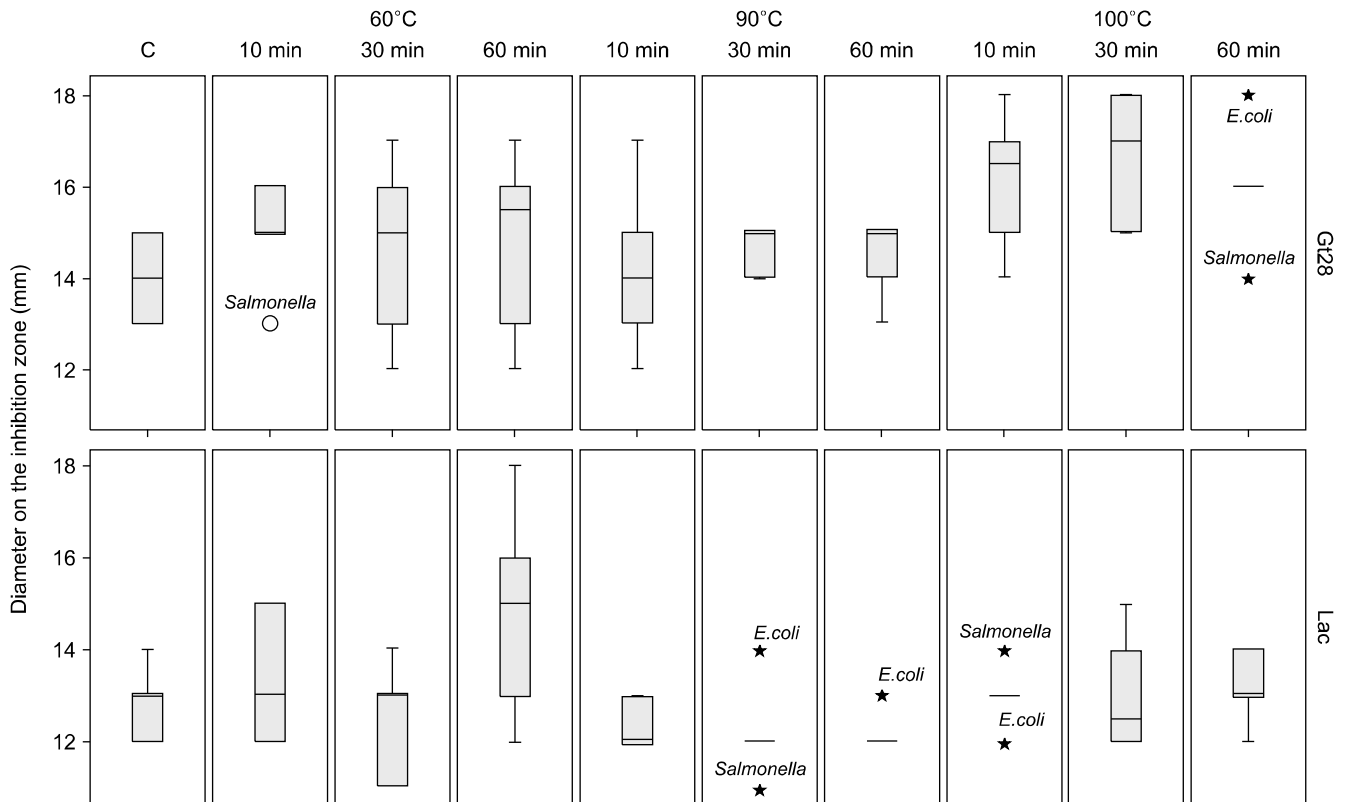
Fig. 2. Difference on antimicrobial activity towards *Escherichia coli* and *Salmonella* after heat treatment of Gt28 and Lac (standard) and different incubation time. Boxplots show the median, interquartile range, outliers (★) of individual variables. C–untreated CFS.

ity (16). No such increase was presented by the reference strain (Lac); the activity varies upon pathogen and incubation time, but in the same range as the control (without incubation) at 90°C and 100°C. A slight increase was noted at 60°C (Fig. 2). Likewise, a significant difference ($P<0.05$) between both pathogens within the same temperature level was observed at 100°C for Gt28 and both 90°C and 100°C for the reference strain. Early studies on bacteriocin producing *L. lactis* subsp. *lactis* KT2W2L reported a considerable decrease in activity after heat treatment (27). Similarly, the activity of bozacion B14, a bacteriocin of *L. lactis* subsp. *lactis* B14 was inhibited after incubation for 10 min at 90°C (28), while a complete inhibition of activity when autoclaving (6). No such resistance to high-heat was observed in the case of isolates originated from fruits of Santo Domingo de Los Tsachilas or Esmeraldas (data not shown). Considering that Gt28 was isolated from tropical climate of the Amazon might explain its resistance at higher temperatures. Likewise, the results indicated that the inhibitory activity was enhanced about 50% in acidic conditions (pH 2), while a significant reduction was noted at pH 10 (80% decrease). Similarly, nisin produced by *L. lactis* subsp. *lactis* WNC20 was inactivated by autoclaving and pH 7.0 but not at the pH of 3.0 (29). We suggest that the increment of activity in the acidic environment might be attributed to the microclimate origin of the isolates, as the fruit samples were relatively acid (pH 4.0). A previous study indicated that

the activity might be linked to the intensification of bacteriocin solubility or due to the ability of acids to pass beyond the target cell membranes acidifying the cytoplasm and increasing its permeability, thus leading to inhibition of pathogen growth (16). The same trend was found by Fatima and Mebrouk (30) when they studied the inhibitory activity of bacteriocin produced by *L. plantarum* and *Pediococcus pentaceus*. Inconsistent with these results, the inhibitory activity of bacteriocin produced by *L. lactis* subsp. *lactis* A15 and *Enterococcus faecium* A15 decreased at pH 2, while the activity was maintained at pH 5, 8, and 10 (6). Additionally, the results indicated a significant increment on inhibitory activity ($P<0.05$) when adding EDTA and SDS, while a slightly decrease was observed when CFS was treated with Triton-X100 and Tween 20 ($1,600 \sim 3,200$ AU/mL towards both *E. coli* and *Salmonella* (Table 2). Similarly, the treatment with SDS, Triton-X100, Tween 20, urea, and EDTA had no effect on the bacteriocin activity produced by *Lactobacillus sakei* (31). From a safety point of view, the only food additive approved by the EU regulation no. 2008-1333 is EDTA (E365); however, the combination of bacteriocin with EDTA pursue the regulation as both are GRAS considered. Tween 20 or polysorbate 20 is used as a wetting agent in flavored mouth drops such as ice drops, which helps to provide a spreading feeling to other ingredients like mint flavor according with the data from World of Chemicals (www.worldofchemicals.com). The World

Health Organization has suggested acceptable daily intake levels of 0~25 mg of polyoxyethylene sorbitan esters per kg body weight and is recognized with E432 in the EU regulation 2008-1333. On the other hand, SDS a GRAS ingredient for food use according to the guidelines published by the Food and Drug Administration (FAD) (Code of Federal Regulations 21 CFR 172.822) but not allowed according to the EU regulation 2008-1333. Triton X-100, a non-ionic detergent, is considered a mild surfactant as it breaks protein-lipid, lipid-lipid associations, but not protein-protein interactions. Triton-X is not recognized by neither EU regulations nor by the FDA as a food additive. When proteins are isolated from their native form are partially inactive—but they solubilize with surfactants (32). When the crude or precipitated bacteriocin with ionic or anionic surfactants is incorporated in foods, the food producer must be aware of national and international safety legislations.

Targeting bacteriocin encoding genes

Several bacteriocin encoding gene of *L. lactis* species were previously characterized (9). The PCR amplification showed positive results for some genes encoding bacteriocins of *L. lactis*. The lactococcin A (600 bp) was detected only in Gt28 isolated (Fig. 3A), the lactococcin M (300 bp) was detected in both Gt28 and Gt29 nor Ella8 isolates (Fig. 3B), while positive amplification of lacticin 3147 was obtained in all isolates (Fig. 3C). Instead, no amplification products were obtained for nisin A and nisin G (data not shown). Therefore, we suggest that the antimicrobial activity of theses strains is not linked to the presence of nisin encoding genes. Based on previous research, *Lactococcus* species are divergent in the type of harbored encoding bacteriocin. For example, the PCR analysis confirmed the presence of nisin but not lacticin 3147, lactococcin A, lactococcin M, lactococcin G, and lactococcin Q in *L. lactis* 69 (33). Another report suggested that the antimicrobial activity of *L. lactis* strains is

not linked to the presence of structural nisin genes (34). Likewise, from the biochemical profile, the selected isolates were able to ferment sucrose although they do not harbor nisin structural gene. Opposite to nisin, lacticin 3147 is a two peptide lantibiotic that works synergistically and exhibits a broad spectrum activity against Grampositive targets (35). A recent study indicated that the lacticin 3147 harbors seven lanthionine bridges across its two peptides, Ltna and Ltnb, and their presence might contribute to protecting the peptides from thermal or proteolytic degradation (36). The antimicrobial activity of nisin producer strains was completely retained at pH 2.0 or autoclaving and completely missing after 30 min at 63°C and pH 11 (37). In our study, the antimicrobial activity of bacteriocins produced by *Lactococcus* strains was largely linked to the high heat or acidic conditions. The analysis should be further confirmed by sequencing in order to differentiate between the selected isolates in relation to their functionality.

Bacteriocins produced by Gt28 reduced the viability of pathogenic bacteria *in vitro*

The addition of CFS Gt28 to the early logarithmic phase of *E. coli* resulted in a total inhibition at 3 h of incubation, while 6 h was sufficient to totally inhibit *Salmonella* cells. Although the optical density was maintained in the same range over 9 h, the viable cell counts reduced considerable (Fig. 4A). The correlation of optical density measurements with the bacteriostatic mode of action of some bacteriocins was previously suggested (38). This might be an imprecise result since the viability of the indicator cells was not determined. In this study, complete inactivation of *E. coli* ATCC 25922 cells occurred at 3 h (no viable cells detected), suggesting its bactericidal mode of action. Similarly, a 2-fold reduction on the viability was recorded for *S. enterica* ATCC 51741 at 3 h with total inhibition at 6 h (Fig. 4A). The results indicated that the bacteriocin Gt28 was effective against both pathogenic
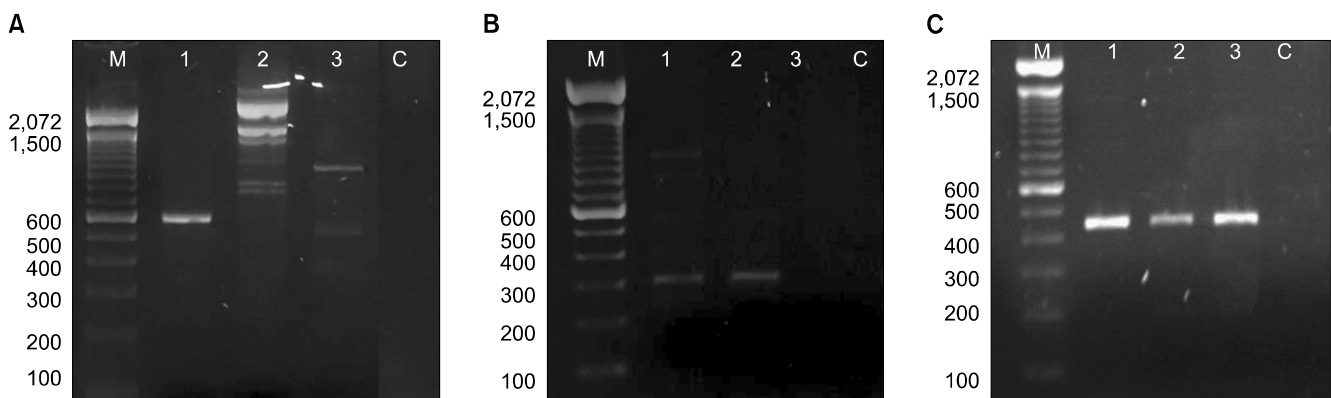

Fig. 3. Ethidium bromide-stained agarose gel (1.2%) of PCR amplification products targeting genes encoding bacteriocins of *Lactococcus* isolates. (A) Lactococcin A (600 bp), (B) Lactococcin M (300 bp), and (C) Lacticin 3147 (490 bp). Line M, 100 bp molecular weight marker; 1, Gt28; 2, Gt29; 3, Ella8; C, negative control.
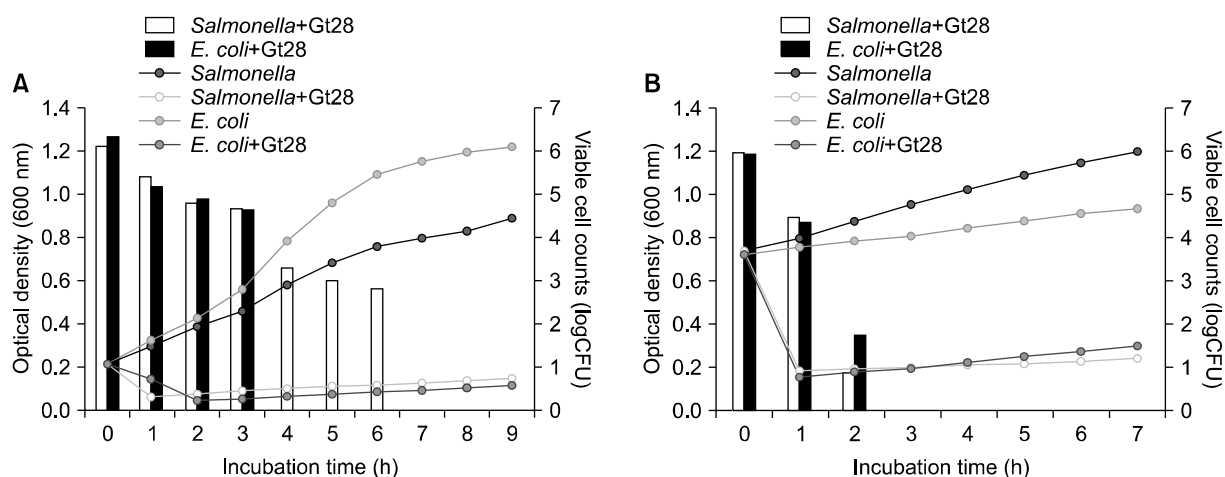
Fig. 4. Effect of bacteriocin Gt28 on the growth of *Escherichia coli* ATCC 25922 and *Salmonella enterica* ATCC 51741. (A) Early growth and (B) exponential phase. Bars represent the viable cells counts determined after the treatment with bacteriocin Gt28 overtime.

microorganisms but the difference might arise from their mode of action during incubation as more time was needed to completely inhibit *Salmonella* growth. A previous study on the mode of action of bacteriocins produced by *L. sakei* strains indicated a complete inhibition of *E. faecium* ATCC 19433 after 10 h of incubation (31). At the exponential phase (OD$_{600}$=0.7) the viability of *Salmonella* was reduced at 3 h but a total inhibition occurred at 7 h after applying Gt28 (Fig. 4B). Similarly, the viability of *E. coli* decreased from 5.47 log to 2 log after 3 h followed by total inhibition at 7 h. Bacteriocin Gt28 seems more effective for killing both pathogens at the exponential phase of growth rather than the early exponential counterpart. The effectiveness of bacteriocin Gt28 as the partial purified form to the pathogen growth was marginal compared with the CFS counterpart (1.2 log reduction). The application of EDTA does not show any changes on the inhibitory activity while an increment was obtained when combining bacteriocin with EDTA (2.3 log reduction) indicating that the reduction of the bacterial population is facilitated by a the synergy between bacteriocin and the chelating agent (data not shown). Recent investigations indicated that lacticin 3147 act synergistically with the antibiotic polymyxin to inhibit Gram-negative bacteria, suggesting that polymyxin might increase the outer membrane permeability allowing the lacticin 3147 to gain access to the cytoplasmic membrane and its lipid II target (39). Similar findings were reported when purified nisin, inhibited the activity of *E. coli*, *Salmonella*, and *Pseudomonas* in combination with EDTA (40). Our results are in compliance with previous findings revealing that EDTA, SDS, freezing, heat, and acidity might induce alterations of the cell wall of target bacteria facilitating the interaction between bacteriocin and the cytoplasmic membrane (20).

Altogether, the results showed the potential of three native *L. lactis* strains to produce bacteriocin substances exhibiting a bactericidal effect against food pathogens *in vitro*; however, the application of these bacteriocin-producing strains directly on a food system matrix is compulsory to determine their efficiency as natural preservatives.

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AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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