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Characterization of Bacteriocin-Producing Lactic Acid Bacteria Isolated from Native Fruits of Ecuadorian Amazon

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Abstract

Tropical, wild-type fruits are considered biodiverse "hotspots" of microorganisms with possible functional characteristics to be investigated. In this study, several native lactic acid bacteria (LAB) of Ecuadorian Amazon showing highly inhibitory potential were identified and characterized. Based on carbohydrate fermentation profile and 16S rRNA gene sequencing, seven strains were assigned as *Lactobacillus plantarum* and one strain as *Weissella confusa*. Using agar-well diffusion method the active synthetized components released in the neutralized and hydroxide peroxide eliminated cell-free supernatant were inhibited by proteolytic enzymes, while the activity was maintained stable after the treatment with catalase, lysozyme, α-amylase and lipase suggesting their proteinaceous nature. The inhibitory activity was stimulated by acidic conditions, upon exposure to high heat and maintained stable at different ranges of sodium chloride (4–10%). The DNA sequencing analysis confirmed the presence of *plw* structural gene encoding for plantacirin W in the selected *L. plantarum* strains. Moreover, we showed that the active peptides of Cys5-4 strains contrast effectively, in a bactericidal manner, the growth of food borne *E. coli* UTNEc1 and *Salmonella* UTNSm2, with about tree fold reduction of viable counts at the early stage of the target cell growth. The results indicated that the bacteriocin produced by selected native lactic acid bacteria strains has elevated capacity to suppress several pathogenic microorganisms implying their potential as antimicrobial agents or food preservatives.

Key words: antimicrobial substances, bacteriocins, foodborne pathogens, lactic acid bacteria (LAB)

Introduction

Worldwide, the presence of spoilage bacteria in food represents a serious problem with repercussion on consumer safety and security, therefore, identifying new alternatives for natural food preservation is of priority. Numerous studies demonstrated the capacity of probiotic microorganisms to inhibit pathogens through different mechanisms such the production of antimicrobial agents (*e.g.* bacteriocin or bacteriocin-like peptides), organic acids (*e.g.* lactic acid, hydrogen peroxidase) or throughout competing with other microbes on binding target cell sites and receptors (Danielsen and Wind, 2003; Collado *et al.*, 2007; Longdet *et al.*, 2011; O'Shea *et al.*, 2011; Zendo, 2013; da Silva Sabo *et al.*, 2014). Bacteriocins of lactic acid bacteria (LAB) are ribosomal synthetized, extracellular and antimicrobial peptides or a complex of proteins biologically active with low molecular weight that reach into the target cells by binding to their surface receptors, cell lyses, pore formation or degradation of cellular DNA (Atrih *et al.*, 2001; Corsetti *et al.*, 2004; Nes and Johnsborg, 2004; Deegan *et al.*, 2006). The LAB bacteriocin attracted significant attention for food industry due to their GRAS status (Generally Considered Safe) and potential use in bio-preservation (Reis *et al.*, 2012). As the inhibitory effect is strain related, in the last few years, the research in many laboratories was centered on identification and selection of novel lactobacilli able to produce bioactive compounds with stronger inhibitory activity against numerous foodborne pathogens to be further aggregated in aliments as preservatives (Todorov, 2008; Todorov *et al.,* 2011; Todorov *et al.*, 2013; da Silva Sabo *et al.*, 2014). In Ecuador, Provence of Imbabura, considerable human illness related to food contaminants such as salmonellosis, shigellosis *etc.* were reported by the Ministry of Health (Gaona, 2013). Most artisanal minimally processed foods, typical dishes (*i.e.* mote) and natural fruit or cereals fermented drinks (*i.e.* chicha), maintained in defective storage conditions or manipulated incorrectly appears to pose significant number of pathogens, therefore the risk of developing

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diseases is elevated. Consequently, increasing attention at the policymaker's level was assumed to increase the control and protection of the consumer by preventing contamination, improving communication about safety with producers, packers, processors and distributors by facilitating relevant research on food preservation.

Previously, we reported the probiotic potential of some lactic acid bacteria and their capacity to inhibit pathogenic microorganisms (Benavidez *et al*., 2016, Tenea and Yépez, 2016). Since the identification of microorganisms from natural sources has been considered a powerful mean for obtaining useful and genetically stable strains in this study, we explored the native fruits of Amazon rainforest with respect to identifying indigenous bacteria producers of bioactive substances with possible use as antimicrobial agents for further control and prevention of food contamination.

Experimental

Materials and Methods

Isolation and selection of LAB strains with antimicrobial activity. Fruits of native plant species: *Chrysophyllum cainito, Solanum stramofolium*, *Cheilocostus specious* and *Theobroma grandiflorum*, were initially collected from Sucumbíos rainforest (Provence located at the northeast of Ecuador, in Amazon Region), transferred into Erlenmeyer flasks (500 ml) containing sterile water (100 ml) and incubated statically for up to 5 days at room temperature. MRS agar (De Man *et al*., 1960) plates were inoculated and incubated under anaerobic conditions (37°C for 72 hours) and individual colonies were randomly selected and purified. The purified colonies (< 100 colonies/ each sample) were Gram stained, tested for mobility, indole, catalase-production, spore formation and gas production from glucose. Thirty selected LAB isolates were further screened for antimicrobial activity using agar-well diffusion assay against the following indicator strains: *E. coli* ATCC 25922, *Salmonella enterica* subsp. *enterica* ATCC 51741, *E. coli* O157:H7 UTNEc1 (isolated from local fresh cheese) and *Salmonella typhimurium* UTNSm2 (isolated from local cooked chicken) (Benavidez *et al*., 2016). Although all thirty isolates displayed antimicrobial activity against at least one indicator strain, eight strains showing broad range activity towards all indicator bacteria were further characterized for the presence of bacteriocin-like substances (BLIS).

Identification of selected LAB isolates. The carbohydrate fermentation API50CHL strips were used following the manufacturer guidelines (Biomerieux, Marcy l'Etoile France, cat # 50300). The results obtained after incubation of strips for 48 hours at 37°C were analyzed using the API software. Moreover, the bacteriocin-producing species were identified based on 16S rRNA gene sequencing (Macrogen Inc., Korea, custom-service). Briefly, the primers 27F 5' (AGA GTT TGA TCM TGG CTC AG) 3' and 1492R 5' (TAC GGY TAC CTTGTT ACG ACT T) 3' were used for the PCR amplification. The PCR reaction was performed with 20 ng of genomic DNA in a 30 µl reaction mixture by using EF-Taq (SolGent, Korea) as follows: activation of Taq polymerase at 95°C for 2 minutes, 35 cycles of 95°C for 1 minute, 55°C and 72°C for 1 minute, finishing with 10 minutes at 72°C. The amplification products were purified with a multiscreen filter plate (Millipore Corp., Bedford, MA, USA). Sequencing reaction was performed using a PRISM BigDye Terminator v3.1 Cycle sequencing Kit. The DNA samples containing the extension products were added to Hi-Di formamide (Applied Biosystems, Foster City, CA). The mixture was incubated at 95°C for 5 minutes, followed by 5 minutes on ice and then analyzed by ABI Prism 3730XL DNA analyzer (Applied Biosystems, Foster City, CA). A homology search of the sequences was conducted using BLAST program at the NCBI database (http://www.ncbi.nlm.nih.gov/BLAST).

Antimicrobial activity. The agar-well diffusion assay was used (Benavidez *et al*., 2016). Briefly, LAB strains were grown in MRS broth at 37°C for 20 hours and the supernatants were collected by centrifugation at 13,000 × g for 20 minutes, 4°C. The CFS (crude extract supernatant) was recovered and filtered using 0.22 µm porosity syringe filter. The indicator strain (100 µl) grown in broth medium (7 log CFU/ml) were mixed with 3.5 ml of soft MRS agar (0.75%), overlaid on the nutrient agar plates and incubated at 37°C for 2 hours. The CFS of each strain (100 µl) were transferred onto the wells (6 mm) on overlaid agar, incubated at 37°C and subsequently examined for inhibition zones at different intervals of time (24–48 hours). To rule out the possible inhibition activity of organic acids, the CFS was heated at 80°C for 10 minutes, the pH adjusted at 6.0 (TFS – neutralized CFS pH 6.0) and the activity was determined. The experiments were run in triplicate and the mean value of the inhibition zone was determined. As control, *Lactobacillus plantarum* ATCC 8014 has been used.

Enzymatic sensitivity. To investigate the chemical nature of antimicrobial substances, filtered CFS were submitted to different treatments and the activity was evaluated using well-diffusion assay followed by determination of bacteriocin titer expressed as arbitrary units per ml (AU/ml). One arbitrary unit was defined as the highest dilution showing about 2 mm of inhibition zone on the indicator lawn (Todorov, 2008). Briefly, aliquots of CFS were buffered with 7% solution of calcium carbonate and the activity was determined in compari-

son with untreated CFS. When the inhibition zone was determined around the wells the inhibitory effect was assumed to be as results of bacteriocin or hydrogen peroxidase (Jimenez-Diaz *et al*., 1993). In other batch TFS was treated with catalase enzyme (1 mg/ml) to prevent the possible inhibitory of hydrogen peroxidase (NCFS – neutralized CFS and hydrogen peroxide eliminated). Moreover, NCFS was independently treated with proteinase K, trypsin, pepsin, lysozyme, α-amylase and lipase (Sigma-Aldrich Corporation, USA) at the final concentration of 1 mg/ml, incubated for 2 hours at 37°C and 5 minutes at 100°C to enzyme inactivation. All experiments were run in triplicate using *E. coli* O157:H7 UTNEc1 and *S. typhimurium* UTNSm2 as indicator strains. The control for all experiments was sterile MRS medium.

Effect of heat, pH, detergents and sodium chloride on bacteriocin activity. Aliquots of CFS were incubated for 10, 30, 60 and 75 minutes at 60, 80, 90, 100°C as well as 15 minutes at 121°C (autoclaving). In other batch, aliquots of the CFS were adjusted at the pH 2.0, 4.0, 6.0 and 10.0, incubated for 3 hours at room temperature. The effect of Triton X-100 (BDH Chemicals Ltd, Poole, England), EDTA (Merck, US), SDS (Sigma-Aldrich Corporation, US) and Tween 20 (Sigma-Aldrich Corporation, US) at the final concentration 10 mg/ml was also evaluated. Furthermore, aliquots of CFS were treated independently with 1%, 4%, 7% and 10% sodium chloride, incubated for 1 hour at 30°C. All experiments were run in triplicates using *E. coli* O157:H7 UTNEc1 and *S. typhimurium* UTNSm2 as indicator strains. The control for all experiments was sterile MRS medium.

Detection of bacteriocin encoding gene. PCR amplification of DNA genomic (PureLink™Genomic DNA minikit, #K1820-00, Invitrogen) of selected LAB was performed using published primer sequences to target plantacirin W gene (20). The primers sequences were: plwFW 5' (GCG CTT GCC AAT GAA CAA AT) 3' and plwRW 5' (TAT CTT CTC CCC AAA CTC AC) 3'. The reaction was realized in a Thermocycler device (MultiGene, Labnet International Inc.) with a Taq Platinum DNA Kit (Invitrogen) in a total volume of 50 µl. The amplification profile was as follows: 1 cycle of 5 min at 94°C; 35 cycles of 1 min at 94°C, 1 min at 52°C, and 1.5 min at 72°C; and 1 cycle of 10 min at 72°C. The amplification products (591 bp) were electrophoresed in a 1.2% agarose gel in 1X TBE (Tris-Borate-EDTA) buffer at 100 V for 1.5 h, using a 100 base-pair ladder as a fragment size marker (Trackit Invitrogen) and visualized by ethidium bromide staining. Moreover, the PCR fragments were sequenced (Macrogen Inc., Korea, custom-service). A homology search of the amino acid sequences was conducted using BLASTX program at the NCBI database (http://www.ncbi.nlm.nih.gov/BLASTX).

Effect of bacteriocin producing Cys5-4 strain on target viability. Five hundred milliliter of CFS containing bacteriocin-producing Cys5-4 was precipitated with 60% ammonium sulfate, incubated overnight at 4°C and centrifuged at 8000 × g for 30 minutes. The precipitated peptides were recovered in ammonium acetate 25 mM and stored at (–) 20°C before use. Ten milliliter of Cys5-4 bacteriocin was added to 90 ml of 3 hours old culture ($OD_{605} = 0.2$) of indicator strains (Mahrous *et al*., 2013). Incubation was performed at 37°C for 9 hours and OD_{605} was measured every hour using spectrophotometer UV-VIS (Nova60, Millipore, Merck) followed by plate-agar method to determine the viable cell counts. As control, the untreated indicator strain culture have been used. The same procedure was applied when adding the bacteriocin in the stationary-phase of the indicator cell growth.

Statistical analysis. For the effect of pH, temperature and sodium chloride treatments, the ANOVA with split-split-plot experimental design was performed. Then, Duncan's multiples range tests and LSD (Least Significant Difference with Bonferroni correction) were applied to determine significant differences between the means. The statistical significance used was $P < 0.05$ (SPSS version 10.0.6, USA and Excel).

Results and Discussion

Identification and characterization of bacteriocinogenic LAB strains. Tropical, wild-type fruits offer a new source of microorganisms with possible biotechnological characteristics to be identified. From nutritional or medicinal point of view, the consumption of those fruits is limited to the local tribes; however, the bacterial microbiota of those fruits is not well investigated. It is believed that the microorganisms from this region might provide a newly source of functional compounds to be for further exploited industrially. The lactic acid bacteria are known for extensive use as probiotics and "natural" preservatives to control harmful microorganisms in food (Georgieva *et al*., 2009; Arena *et al*., 2016). Previous studies suggested that antimicrobial components production depends on the LAB species and several exogenous factors such as medium composition, growth factors, organic and inorganic salt treatments, *etc.* (Arena *et al*., 2016). In this study, eight novel indigenous LAB strains were isolated, identified and characterized for the presence of antimicrobial substances. Based on carbohydrate profiles and confirmed by 16S rRNA sequencing, the isolates assigned Cys2-1 (GenBank accession no. KY041683), Cys4-1 (GenBank accession no. KY041685), Cys5-4 (GenBank accession no. KY041686), Cys7-1 (GenBank accession no. KY041687), Gt2 (GenBank accession

Table I
LAB identification and the residual activity (%) of neutralized cell-free supernatant

Code isolate	Species / GenBank Accession Number*	Residual activity (%)			
		E. coli UTNEc1	*S. typhimurium* UTNSm2	*E. coli* ATCC 25922	*S. enterica* subsp. *enterica* ATCC 51741
Cys2-1	*L. plantarum* / KY041683	94.78	92.67	87.80	87.80
Cys2-2	*W. confusa* / KY041684	92.30	90.26	86.95	86.95
Cys4-1	*L. plantarum* / KY041685	97.32	92.49	88.23	88.23
Cys5-4	*L. plantarum* / KY041686	97.69	86.55	90.17	90.17
Cys7-1	*L. plantarum* / KY041687	97.38	85.73	87.65	88.65
Gt2	*L. plantarum* / KY041688	97.32	94.78	85.20	85.16
Gt4	*L. plantarum* / KY041689	83.74	90.71	97.87	97.88
Gt6	*L. plantarum* / KY041690	90.26	89.67	90.18	90.18
Control	*L. plantarum* ATCC8014	92.85	89.10	88.73	88.73

* Assigned number by NCBI (https://www.ncbi.nlm.nih.gov).
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 TFS and inhibition zone of CFS counterpart multiplied with 100.

no. KY041688), Gt4 (GenBank accession no. KY041689) and Gt6 (GenBank accession no. KY041690) were identified as *L. plantarum* and the isolate assigned Cys2-2 (GenBank accession no. KY041684) was identified as *Weissella confusa* (Table I). *L. plantarum* is known as one of the most versatile LAB species with a broad range of antimicrobial activity against Gram-positive and Gram-negative bacteria (Ali and Musleh, 2015). Recently, *W. confusa* A3 isolated from dairy products, with capacity to inhibit pathogenic bacteria as result of dextran, was reported (Goh and Philip, 2015). Although a slightly decrease in residual activity was observed when removal of organic acids the eight selected LAB strains exhibited homogeneous antimicrobial activity (above 85%) towards all four indicator strains suggesting the presence of active compounds (Table I). Besides, the organic acids in CFS, locally produced in the bacterial growth, might establish the appropriate micro environmental condition to activate the antagonistic mechanism of produced peptides against harmful microorganisms founded in the same niche. Additionally, the presence of organic acids might be beneficial if use CFS in food due to their enzymatic resistance and higher solubility compared with the bacteriocin-like peptides. The effectiveness of inhibitory activity was not influenced by the addition of calcium and removal of hydrogen peroxide (NCFS), while the treatment with proteinase K, trypsin and pepsin completely abolished the activity (Table II). Similar characteristics were observed in case of *Enterococcus mundtii* QU2, isolated from soybean, when bacteriocin production was negatively regulated albeit calcium was added to the bacterial culture medium, while treatment of crude bacterial extract with calcium the activity was still maintained (Zendo *et al.*, 2005). After lysozyme treatment the activity was maintained stable indicating that the protein might be glycosylated, while treatment with lipase and α-amylase explain the lack of carbohydrate or lipid moiety. The sensibility to enzymes might be considered an advantage in case of incorporate in food meaning that upon ingestion will not alter the digestive tract (Ali and Musleh, 2015). Overall, our data indicated the presence of antimicrobial substances (bacteriocin-like) in the bacterial crude extract of selected native LAB strains with elevated capacity to inhibit pathogenic bacteria.

Table II
Effect of enzymes and pH on bacteriocin activity of selected native LAB

Treatment		Activity (AU/ml)
Enzymes (1 mg/ml)	NCFS + Proteinase K	–
	NCFS + Trypsin	–
	NCFS + Pepsin	–
	NCFS + Lysozyme	6400
	NCFS + Lipase	6400
	NCFS+ α-amylase	6400
	NCFS	6400
	TFS	6400
pH	2.0	12800
	4.0	6400
	6.0	3200
	10.0	800
	CFS	6400

The results of the assay are from three experiments, each with triplicate samples.
CFS-crude extract supernatant; TFS-neutralized CFS (pH 6.0);
NCFS: neutralized CFS and hydrogen peroxide eliminated.

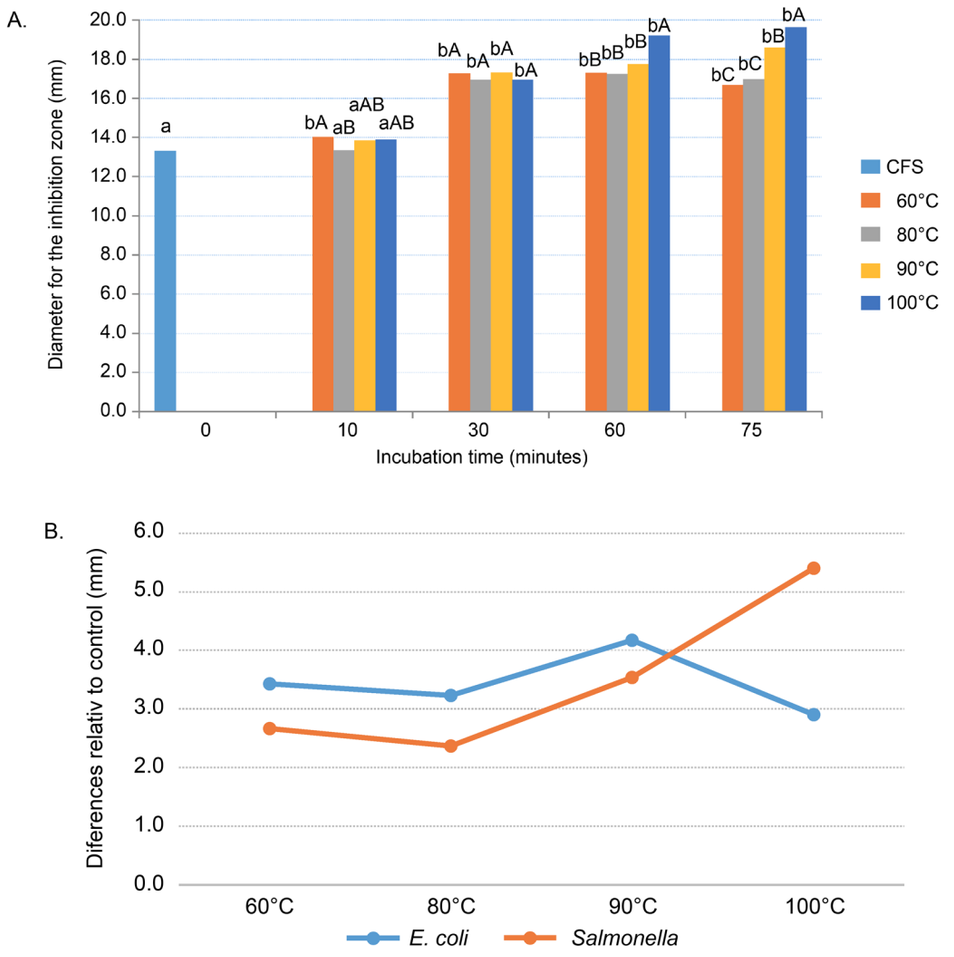

Fig. 1. Effect of heat on bacteriocin activity.

A. Diameter of the inhibition zone (mm) at different temperatures and incubation time. Bars are the means ± standard error. Values with different letters are significantly different $P < 0.05$. Small letters show the difference between temperature-incubation time and control (LSD with Bonferroni correction); Capital letters indicate the differences within incubation time (Duncan's test). B. The influence of pathogen in the inhibitory activity. The differences relative to control in the inhibition zone are shown (mm). The significant different is observed at 100°C. CFS: cell-free supernatant without incubation.

Effectiveness of inhibitory activity upon heat, pH and detergents exposure. Heat resistance is one important characteristic to be considered when selecting BLIS (bacteriocin-like inhibitory substances) producing strains to be used as preservation agent in processed food. Previous studies indicated that bacteriocins of *L. plantarum* or *W. confusa* species showed stability after heat and lower pH exposure (Goh and Philip, 2015; Arena *et al*., 2016). In this study, the statistical analysis revealed that the effectiveness of inhibitory activity was influenced by both temperature-time and pathogen-temperature interactions. Analysis from split-split-plot design (where main plot: pathogen; sub-plot 1: temperature; sub-plot 2: incubation time) indicated that the activity was maintained at the same level as untreated control after 10 minutes of incubation at all four temperatures tested, while a significant increase was recorded after 30, 60 and 75 minutes incubation (LSD with Bonferroni correction) (Fig. 1A). An increase in antimicrobial activity was recorded at the highest temperatures (90 and 100°C) and after more than 60 minutes incubation time. We hypothesized that the enhanced inhibitory activity after heat exposure throughout time might follow the same pathway as heat-treatment-induced chemical reaction between active components as amino- and carbonyl groups, known as Maillard Reaction (Hiramoto *et al*., 2004). Early studies reported the beneficial role of Maillard reaction products on pathogenic inhibition stating that high-molecular weight proteins might develop antimicrobial activity when binding chemical elements such iron, copper or zinc, known for their essential role in the growth of pathogenic microorganism (Hiramoto *et al*., 2004; Trang *et al*., 2013). Likewise, a statistically

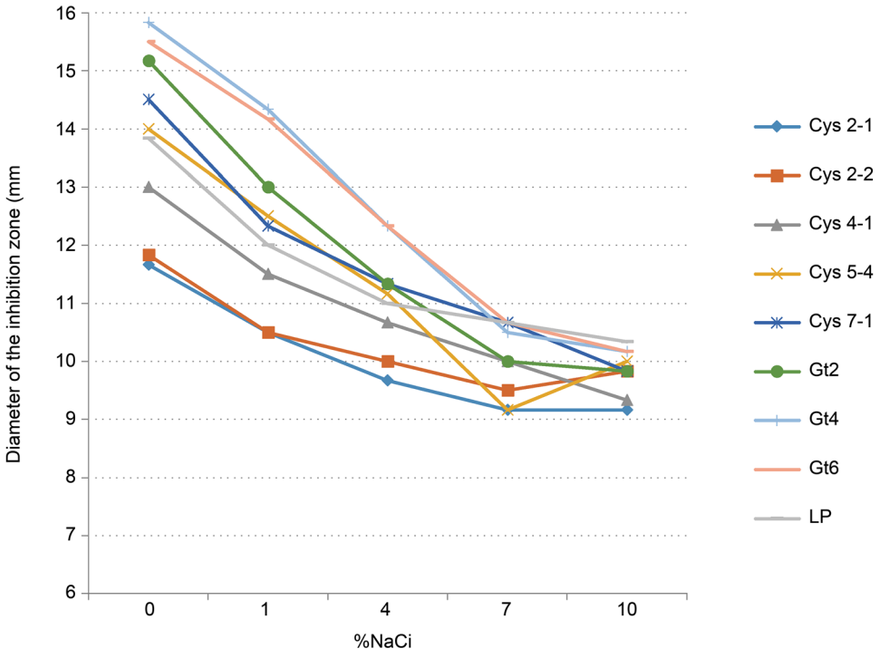

Fig. 2. Stability of bacteriocin activity upon NaCl treatment. Data represent the mean and standard error of the mean. LP: *L. plantarum* ATCC8014; 0-CFS without treatment

significant difference between both pathogens within same temperature level was observed at 100°C (Fig. 1B). Moreover, after 15 minutes autoclaving the activity remained stable for all selected LAB (*e.g.* inhibition zone of 12.00 ± 0.0 mm towards *E. coli* and 12.36 ± 0.5 mm towards *Salmonella)* indicating the advantage of those active molecules if tested as preservatives in combination with thermal processing food. Considering tropical niches origin of the raw material, this might be a significant finding as other studies did not mention such property of heat-time inducing inhibitory activity of lactic acid bacteria.

A statistically significant increase ($P < 0.05$) in activity was observed in highly acidic conditions (pH 2.0) with titer of 12800 AU/ml towards both indicator strains (Table II). At the pH 4.0 and 6.0 the titer was 6400 AU/ml while a significant decrease was register at the pH 10.0 (800 AU/ml). It is likely that effectiveness of antimicrobial activity is pH dependent. After 24 hours of incubation of bacterial culture the pH range between 3.8–4.5, however, the maximum activity was exhibited at pH 2.0. Previously, it was suggested that under acidic conditions the enhanced activity might be attributed to the increase of bacteriocin solubility or 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 (Banerjee *et al.*, 2013). In addition, a significant increase in antimicrobial activity ($P < 0.05$) relative to untreated counterpart was observed when adding EDTA and SDS (12800 AU/ml) for all selected LAB strains. On the other hand, a slightly decrease in activity was observed when CFS was treated with Triton-X100 (3200 AU/ml), while no changes occur after treatment with Tween 20 (6400 AU/ml). As previously described, the exposure to several detergents might enhance activity through penetration of outer membrane of Gram-negative strains beyond extracting cations ($Ca^{2+}Mg^{2+}$) thus allowing bacteriocins to reach the cytoplasmic membrane (Galvez *et al.*, 2007). Alike, in this study the effectiveness of inhibitory activity was positively influenced by the treatments with SDS and chelating EDTA agent. In conclusion, our results indicated that efficiency of bacteriocin-like substances of selected LAB is positively regulated by acidic condition and heat.

BLIS-producing LAB stability at higher sodium chloride. The activity of bacteriocin produced by *L. plantarum* isolates was maintained stable after treatment with sodium chloride indicating that the inorganic salts might have a synergistic effect on bacteriocin efficacy (Fig. 2). Similar study on bacteriocin produced by *L. plantarum* LPCO10 strain showed that the activity was stable at 4% NaCl (Jimenez-Diaz *et al.*, 1993), while 10% NaCl abolished the activity (Mahrous *et al.*, 2013). In contrary, in the current study the bacteriocin activity was maintained stable in the presence of higher salt suggesting that no changes might occur in protein conformation thus demonstrating its advantage if using as preservative as interaction with higher salt content

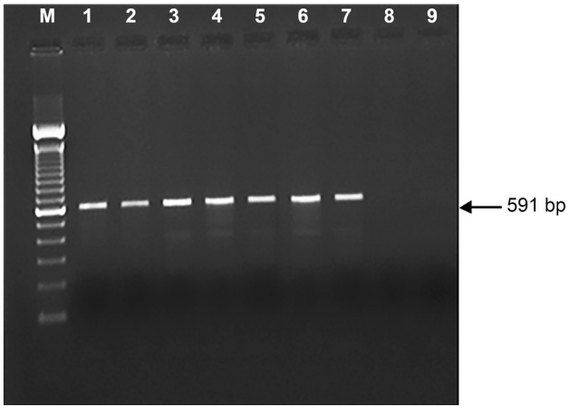

Fig. 3. Amplification fragment of 591 bp corresponding to *plw* gene detected in agarose gel electrophoresis.
M: 100 bp molecular marker, samples 1–7: DNAg isolated from *L. plantarum* isolates, 8: Cys2-2 isolate; 9-negativ control (PCR mix without DNA).

food would not have totally inhibiting their activity. Taken together, these results correlates with our previous findings about the growth tolerance of selected LAB at lower pH (2.0), in culture medium containing higher concentration of NaCl (6%) as well as high temperature of growth (40°C) (data not shown).

Detection of bacteriocin encoding *plw* structural gene. The primers used for the amplification of 591 bp fragment were complementary to sequences occurring proximal to the 3' and 5' ends targeting the structural *plw* gene (Halo *et al*., 2001). The expected fragment was identify in *L. plantarum* strains while no amplification was detected for the Cys2-2 meaning that the strain is not producing plw (Fig. 3). The nucleotide sequence homology of the selected *L. plantarum* strains except Cys4-1 was matching 100% the *plw* gene locus of *L. plantarum* RS5. The amino acid sequence analysis showed 100% identity with plantacirin W alpha and beta precursors of *L. plantarum* LMG2379. In addition, the propeptide plwα showed 41% identity with a hypothetical protein of *Streptococcus aureus* and 53% with a lantibiotic mersacidin of *Bacillus liqueniformis*. In previous study, it has been shown that both plantacirin peptides containing leader sequences, once processed gave rise to mature peptides of 32 and 29 amino acids that might act synergistically to exert their inhibitory activity (Halo *et al*., 2001). No similarity at nucleotide level with other bacteriocins has been found for Cys4-1, although the translation to amino acids sequence revealed 49% identity of with plantacirin W beta precursor.

Effect of Cys5-4 bacteriocin on indicator cells viability. Bacteriocins act as bactericidal or bacteriostatic manner depending on the dose, degree of purification and the physiological stage of the growth of indicator cells. In this study, the addition of bacteriocin at the early stage of growth resulted in decreasing of the target cell viability after 2 hours of incubation and maintained up to 9 hours of measurement suggesting that the Cys5-4 bacteriocin might follow a bactericidal pathway. The viability of *E. coli* decreased from 6.16 to 2.32 log CFU/ml (Fig. 4) and from 6.28 to 3.53 log CFU/ml, respectively, for *Salmonella*. Similarly, when the bacteriocin was added at the late phase (OD 0.7) the viability was reduced but not at the same level as in the early stage of target growth (data not shown). This complies with early studies, which revealed that bacteriocin-like inhibitory substances were active during the exponential to early stationary phase of growth (Hernandez *et al*., 2005). The indicator cells were smaller than those seen in the positive control cells suggesting that the active bacteriocin Cys5-4 might bind the cell-wall leading to their destabilization, thus exerting its bactericidal mode of action. Those findings must be validated *ex vitro* knowing that the food act as complex ecosystem in which interactions between active components with target cells and other metabolic components of food matrix might influence the inhibitory mechanism.

While several studies described numerous lactobacilli isolated from human, animal or vegetable with antimicrobial activity (Zambou *et al*., 2013), up to now,

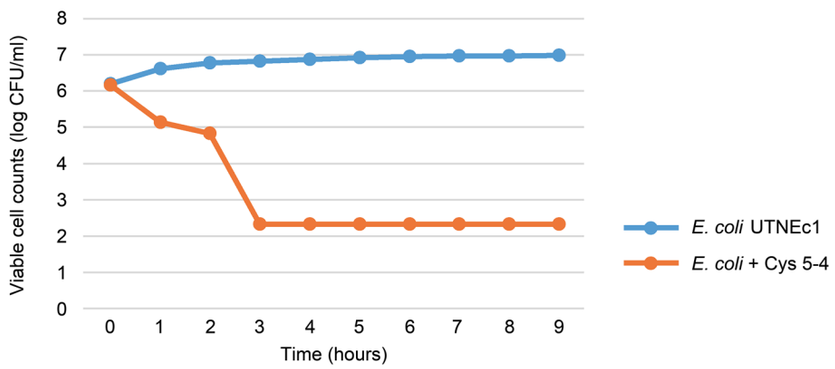

Fig. 4. Effect of bacteriocin Cys5-4 on the viability of *E. coli* UTNEc1.

no study about the presence of bacteriocinogenic LAB in native fruits of Ecuadorian Amazon rainforest was shown. Early investigation on *L. plantarum* PMU33 strain isolated from a fermented fish food reported the presence of two peptide bacteriocin plwα and plwβ with tolerance to heat and acidity (Noonpakdee *et al*., 2009). Here, we showed that the bacteriocin activity of selected strains is enhanced upon heat exposure throughout time demonstrating their valuable potential as used in processed food. Furthermore, we reported for the first time the presence of a native *W. confusa* and *L. plantarum* strains that produced inhibitory substances with strongest capacity to suppress the growth of foodborne pathogens founded in the local market implying further investigation to demonstrate their promising potential as antimicrobial agents.

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Conflict of interest

The authors declare that they have no conflict of interest in the present publication.

Literature

Ali W.S. and R.M. Musleh. 2015. Purification and characterization of plantacirin vgw8, a bacteriocin produced by *Lactobacillus plantarum* VGW8. *J. Biol*. 5(1): 147–152.

Arena M.P., A. Silvain, G. Normanno, F. Grieco, D. Drider, G. Spano, and D. Fiocco. 2016. Use of *Lactobacillus plantarum* strains as a bio-control strategy against food-borne pathogenic microorganisms. *Front. Microbiol.* 7(464): 1–10.

Atrih A., N. Rekhif, A.J.G. Moir, A. Lebrihi and G. Lefebvre 2001. Mode of action, purification and amino acid sequence of plantacirin C19, an anti-*Listeria* bacteriocin produced by *Lactobacillus plantarum* C19. *Int. J. Food Microbiol*. 68: 93–109.

Banerjee S.P., K.C. Dora and S. Chowdhury. 2013. Detection, partial purification and characterization of bacteriocin produced by *Lactobacillus brevis* FPTLB3 isolated from freshwater fish. *J. Food Sci. Technol*. 50: 17–25.

Benavidez A.B., M. Ulcuango, L. Yepez, and G.N. Tenea. 2016. Assessment of the *in vitro* bioactive properties of lactic acid bacteria isolated from native ecological niches of Ecuador. *Rev. Argent. Microbiol*. 48: 236–244.

Collado M., J. Meriluoto, and S. Salminen. 2007 Role of commercial probiotic strains against human pathogen adhesion to intestinal mucus. *Lett. Appl. Microbiol*. 45: 454–460.

Corsetti A., L. Settanni and D. van Sinderen. 2004. Characterization of bacteriocin-like inhibitory substances (BLIS) from sourdough lactic acid bacteria and evaluation of their *in vitro* and *in situ* activity. *J. Appl. Microbiol.* 96: 521–534.

Danielsen, M. and A. Wind A. 2003. Susceptibility of *Lactobacillus* spp. to antimicrobial agents. *Int. J. Food Microbiol*. 82: 1–11.

da Silva Sabo S., M. Vitolo, J.M.D. Gonzalez and R.P. de Souza Oliveira. 2014. Overview of the *Lactobacillus plantarum* as a promising bacteriocin producer among lactic acid bacteria. *Food Res. Int*. 64: 527–536.

Deegan L.H., P.D. Cotter, C. Hill and P. Ross. 2006. Bacteriocins: Biological tools for bio-preservation and shelf-life extension. *Int. Dairy J*. 16: 1058–1071.

De Man J.C., M. Rogosa and E. Sharpe. 1960. A medium for the cultivation of lactobacilli. *J. Appl. Bacteriol*. 23: 130–155.

Deraz S.F., E.N. Karlsson, A.A. Khalil and B. Mattiasson. 2007. Mode of action of acidocin D20079, a bacteriocin produced by the potential probiotic strain, *Lactobacillus achidophilus* DSM 20079. *J. Ind. Micobiol. Biotechnol.* 34: 34373–379.

Galvez A., H. Abriouel, R.L. Lopez and B.N. Omar. 2007. Bacteriocin-based strategies for food biopreservation. *Int. J. Food Microbiol.* 120: 51–70.

Gaona J. 2013. Ministerio de Salud Pública del Ecuador. https://public.tableau.com/profile/javier.gaona#!/vizhome/MORBILIDAD_RDACAA_2013_0/Presentacin.

Georgieva R., I. Iliev, T. Haertle, J.M. Chobert, I. Ivanova and S. Danova. 2009. Technological properties of candidate probiotic *Lactobacillus plantarum* strains. *Int. Dairy J.* 19: 696–702.

Goh H.F. and K. Philip. 2015. Purification and characterization of bacteriocin produced by *Weissella confusa* A3 of dairy origin. *PLOS One*. 10: e0140434.

Halo H., Z. Jeknic, M. Daeschel, S. Stevanovic and I.F. Nes. 2001. Plantacirin W from *Lactobacillus plantarum* belongs to a new family of two-peptide lantibiotics. *Microbiology* 147: 643–651.

Hernandez D., E. Cardell and V. Zarate. 2005. Antimicrobial activity of lactic acid bacteria isolated from Tenerife cheese: initial characterization of plantaricin TF711, a bacteriocin-like substance produced by *Lactobacillus plantarum* TF711. *J. Appl. Microbiol*. 99: 77–84.

Hiramoto S., K. Itoh, S. Shizuuchi, Y. Kawachi, Y. Morishita, M. Nagase, Y. Suzuki, Y. Nobuta, O. Nakamura, I. Kagaya and others. 2004. Melanoidin, a food protein-derived advanced Maillard reaction product, suppresses *Helicobacter pylori in vitro* and *in vivo*. *Helicobacter*. 9: 429–435.

Jimenez-Diaz R., R.M. Rios-Sanchez, M. Desmazeaud, J.L. Ruiz-Barba and J.C. Piard. 1993. Plantaricins S and T, two new bacteriocins produced by *Lactobacillus plantarum* LPCO10 isolated from a green olive fermentation. *Appl. Environ. Microbiol.* 59: 1416–1424.

Longdet I.Y., R.J. Kutdhik and I.G. Nwoyeocha. 2011. The probiotic efficacy of *Lactobacillus casei* from human breast milk against shigellosis in albino rats. *Advances Biotech. Chem. Proc.* 1: 12–16.

Mahrous H., A. Mohamed, M. Abd El-Mongy, A.I. El-Batal and H.A. Hamza. 2013. Study bacteriocin production and optimization using new isolates of *Lactobacillus* ssp. isolated from some dairy products under different culture conditions. *Food Nutr. Sci.* 4: 342–356.

Nes I.F. and O. Johnsborg. 2004 Exploration of antimicrobial potential of LAB by genomics. *Curr. Opin. Biotech.* 15: 100–104.

Noonpakdee W., P. Jumriangrit, K. Wittayakom, J. Zendo, J. Nakayama, K. Sonomoto, and S. Panyim. Two-peptide bacteriocin from *Lactobacillus plantarum* PMU 33 strain isolated from som-fak, a Thai low salt fermented fish product. *As. Pac. J. Mol. Biol. Biotech.* 17: 19–25.

O'Shea, E., P. Cotter, C. Stanton, R. Ross and C. Hill. 2011. Production of bioactive substances by intestinal bacteria as a basis for explaining probiotic mechanism: bacteriocins and conjugated linoleic acid. *Int. J. Food Microbiol.* 152: 189–205.

Reis J.A., A.T. Paula, S.N. Casarotti, and A.I.B. Penna. 2012. Lactic acid bacteria antimicrobial compounds: characteristics and applications. *Food Eng. Rev.* 4: 124–140.

Tenea G.N. and L. Yépez. 2016. Bioactive Compounds of Lactic Acid Bacteria. Case Study: Evaluation of Antimicrobial Activity of Bacteriocin-Producing Lactobacilli Isolated from Native Ecological Niches of Ecuador, pp. 147–169. In: Rao V. and L.G. Rao (eds.) *Probiotics and prebiotics in human nutrition and health*, InTech.

Todorov S.D. 2008. Bacteriocin production by *Lactobacillus plantarum* AMA-K isolated from amasi, a Zimbabwean fermented milk product and study of the adsorption of bacteriocin AMA-K to *Listeria* sp. *Brazilian J. Microbiol.* 39: 178–187.

Todorov S.D., C. Rachman, A. Fourrier, L.M.T. Dicks C.A. van Reenen, H. Prevost and X. Dousset. 2011. Characterization of a bacteriocins produced by *Lactobacillus sakei* R1333 isolated from smoked salmon. *Anaerobe* 17: 23–31.

Todorov S.D., M. Vaz-Velho, G. de Melo, B.D. Franco and W.H. Holzapfel. 2013. Partial characterization of a bacteriocin produced by three strains of *Lactobacillus sakei* isolated from salpicao, a fermented meat product from North-West of Portugal. *Food Control*. 30: 111–121.

Trang V.T., V.H. Son, L.X. Thanh, S. Sarter, T. Shimamura, H. Uked. and H. Takeurchi. 2013. Functional properties of Maillard reaction products in food: Antimicrobial activity of aminoreductone against pathogenic bacteria. *Food Sci. Technol. Res.* 19: 833–841.

Yi H., L. Zhang, Y. Tuo, X. Han and M. Du. 2010. A novel method for rapid detection of class IIa bacteriocin-producing lactic acid bacteria. *Food Control*. 21: 426–430.

Zambou N.F., P.M. Kaktcham, A.H.N. Tiogo and W.R.E. Guetiya. 2013. Antimicrobial activity of a bacteriocin produced by *Lactobacillus plantarum* 29V and strain's viability in palm kernel oil. *Int. J. Nutri. Food Sci.* 2: 102–108.

Zendo T. 2013. Screening and Characterization of novel bacteriocins from lactic acid bacteria. *Biosci. Biotechnol. Biochem*. 77: 839–899.

Zendo T., N. Eungruttanagorn, S. Fujioka, Y. Tashiro, K. Nomura, Y. Sera, G. Kobayashi, J. Nakayama, A. Ishizaki, and K. Sonomoto. 2005. Identification and production of a bacteriocin from *Enterococcus mundtii* QU 2 isolated from soybean. *J. Appl. Microbiol.* 99: 1181–1190.