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Bioactive Compounds of Lactic Acid Bacteria. Case Study: Evaluation of Antimicrobial Activity of Bacteriocin-producing Lactobacilli Isolated from Native Ecological Niches of Ecuador

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Additional information is available at the end of the chapter

Abstract

Food preservation through natural methods represents one of the concerns world-wide to solve economic losses due to microbial decomposition of raw materials and foodstuffs. However, public concern over the emergence of strains resistant to many antibiotics, particularly pathogens such as *E. coli* and *Salmonella* sp. draw much attention as new challenge in food industry is to find new alternative quality-control methods of food products. In Ecuador, the lack of quality control, bad storage condition, and insufficient preservation against spoilage bacteria had at higher extent repercussions on food safety and security. The most frequent pathogens detected in fresh meat and drinks along with traditional local food products, represent a serious problem producing sizable food damage and associated diseases. The capacity of lactobacilli to inhibit pathogens has been recently exploited to prevent microbial spoilage. Here we briefly review the principal biopeptides (i.e., bacteriocins) of lactic acid bacteria, their main mode of action, the classification, and its biotechnological applications. Moreover, we discussed the preliminary results on the evaluation of antimicrobial activity of some native lactic acid bacteria isolated from microbiota of Ecuador against frequent contaminants found in the local market.

Keywords: lactic acid bacteria, biopreservation, bacteriocins, food pathogens, probiotic

1. Introduction

Lactic acid bacteria (LAB) are among the most favorable microorganisms known for their probiotic properties and for the ability to produce antimicrobial compounds (i.e., bacteriocin, organic acids, diacetyl, hydrogen peroxide) with inhibitory action of harmful bacteria growth along with their critical role in food protection and health maintenance [1–3].

Nowadays, one of the biggest issues faced by the food-processing industry is contamination with pathogens caused by poor maintenance and unhygienic sanitary behavior and insufficient attention to the handling and preservation, contributing greatly to decrease the quality of products and also increase consummators foodborne illness in the population [4–6]. Thus, the preservation through natural methods represents one of the main concerns at the global level to solve economic losses due to microbial decomposition of raw materials and foodstuffs.

With concomitant expansion of the research, commercial, food industry and medical sectors, the field of biopreservation using probiotic bacteria is developing rapidly with accumulation of many data about their benefits. The complete genome sequencing as well as the identification of functional properties will further contribute to the reinforcement of most powerful products with improved biotechnological characteristics. Although many bacteria produce antimicrobial substances, the benefits of those produced by LAB is of particular interest because of their Generally Recognized as Safe (GRAS) status, which acts as natural biopreservative and natural flavor enhancers [3, 7–9]. Hence, the majority of antimicrobial peptide-producing LAB are ideally suited to food applications. Therefore, the production of bacteriocins by LAB is not only advantageous to the bacteria themselves but could also be exploited as a tool of food industry to control undesirable bacteria in a natural manner, and be allowable to the consumer.

As the main source of knowing LAB is represented by the human microflora and fermented milk products, it would be more valuable to search for other sources of probiotic microorganism, which might possess powerful properties and beneficial for either human health or food preservation. During the last decade, extensive progress has been made with respect to the isolation of LAB with highly antimicrobial properties as well as comprehension of bacteriocin structure and function, regulation, and immunity. Further investigations may help to develop new methods for food preservation by direct comparisons between strains bacteriocin producers and non-produced isogenic strains. In this context, bacteriocin of LAB would offer several benefits such as the use reduction of chemical compounds in food preservation. In this chapter, we will briefly review the main information about the role of bacteriocin of LAB in food preservation, their classification and mode of action along with their biotechnological benefits. Moreover, we shall present the preliminary results on the evaluation of antimicrobial activity of some native lactic acid bacteria isolated from microbiota of Ecuador against frequent contaminants found in the local food market.

2. Bacteriocins of lactic acid bacteria and their biotechnological applications

Antimicrobial heterogeneous compounds (i.e., bacteriocine) are ribosomally synthesized polypeptide or low-molecular-weight proteins (composed of 20–60 amino acid residues), which, in case of LAB, are generally recognized as safe compounds [9]. They bind to the receptor of the target cell, and their mode of action included pore formation, degradation of cellular DNA, disruption through specific cleavage of 16S rRNA, and inhibition of peptido-glycan synthesis [10, 11]. Bacteriocins being proteinaceous agents differ from most antibiotics because they are rapidly digested by proteases in the digestive tract.

2.1. Types of bacteriocins

More than three hundred different bacteriocins have been described for the genera *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Enterococcus*. These peptides are colorless, odorless, and tasteless, and according to their molecular mass, thermo stability, enzymatic, and sensitivity, the presence of posttranslational modified amino acids and their mode of action are classified into four major groups [10–13].

Bacteriocins of Class I: They, known as lantibiotics, are small peptides of <5 kDa, heat stable that acting on the membrane structure, and contain the thio-ester amino acids lanthionine and methyllanthionine as well as other modified amino acids such as dehydrated serine and threonine. From this class, the most studied bacteriocin is nisin produced by *Lactococcus lactis* subsp. *lactis* and discovered since 1928 as being the first bioactive compound used in food system as biopreservative [12]. According to their structural similarities, the lantibiotics were divided into two subclasses. *Subclass Ia*, comprising positively charged peptides (i.e., nisin), generally acts by forming pores in the cytoplasmic membrane of the target species. *Subclass Ib* are peptides either negatively charged or no net charged, more rigid in their structure which exert their action by interfering with enzymatic reactions of sensitive bacteria. The most studied bacteriocins of class I are Nisin Z and Q, Enterocin W, and Nukacin ISK-1 [14–16].

Bacteriocins of Class II: They, known as non-lantibiotics, are heat-stable bacteriocins of variable molecular weight, <10 kDa, containing in their composition regular amino acids. This class was subdivided into four subclasses. *Subclass IIa*, comprising Pediocin PA-1 and Sakacin P, are known for their antimicrobial activity against *Listeria*. Members of pediocin-like peptides have a high degree of homology (40–60%), particularly at the N-terminal domain, containing "pediocine box" or homologous region YGNGVXCXXXXCXV, with two residues of cysteine forming a disulfide bridge. Other known bacteriocins of *subclass IIa* are Enterocin NKR-5-3C [17, 18], Enterocin A [15], Munditicin [19], and Leucocin A [15]. *Subclass IIb*, comprising distinct peptides with little or no activity, refers to two-component bacteriocins that require two peptides to work synergistically. In this group are enclosed Lactacin F and Lactococcin G. *Subclass IIc* are small peptides, heat stable, and transported by leader peptides, comprising Diverginin A and Acidocin B. *Subclass IId* includes sec-dependent bacteriocins, and leaderless bacteriocins are Lacticin Q [20], Z [21], Weissellicin Y and M [22], and Leucocin Q and N [15].

Bacteriocins of Class III: They are larger peptides, about 430 kDa, heat liable comprising Helveticins J and V, Acidofilicin A, and Lactacins A and B.

Bacteriocins of Class IV: They contain modified peptides with either lipid or carbohydrate components, or they form large complexes with other chemical moieties, lipids, or carbohydrates.

Regardless of many biotechnological applications, *nisin* remain the only commercial bacteriocin approved by World Health Organization Expert Committee on Food Additives and by the US Food and Drug for its use in food industry [23]. Nisin is a 34 amino acid long peptide of 5-kDa molecular weight, and its synthesis is a complex mechanism involving processes of transcription, transduction, posttranscriptional modifications, secretion, and signs of transduction [24]. There are two forms of nisin, A and Z known for their action against *Bacillus* and *Clostridium* in processed cheese. Its lethal activity is close related with two important properties, cationic and hydrophobicity. However, small-size bacteriocins are active at different ranges of pH (i.e., from 3.0 up to 9.0), and their high isoelectric point allows the interaction with the anionic surface at the bacterial membrane at physiological pH values. Another feature is heat stability related to the monosulfide and disulfide intramolecular bonds, which maintain stable the secondary structure by reduction of the possible unfolded structures. This property explains the high resistance to autoclaving conditions of some LAB bacteriocins [25]. For example, Helveticin J is inhibited after 1- to 15-min incubation at 60–100°C, but can be easily recovered from bacterial culture. On the other hand, nisin has higher antimicrobial activity at pH of 2.0–4.0, and has heated stability at 100°C for 10 min of incubation while at pH 7.0 it is inactivated making this bacteriocin useful for food preservation [25].

Early studies showed that bacteriocins overcome different functions of the living cells, such as transcription, translation, and replication, due to their variation in the chemical structure, but most of them are acting by forming membrane channels or pores that destroy the energy of sensitive cells [25]. Regarding their mode of action, it has been shown that they are effective against Gram-positive bacteria and might be inefficient to inhibit Gram-negative organisms [24, 26–28]. Have been proposed numerous mechanisms of action such as the inhibition of spore germination as well as inactivation of anionic carriers through the formation of selective and non-selective pores and alteration of enzymatic activity [26, 27]. The effect on sensitive cells could be bactericidal or bacteriostatic depending on the dosage, degree of purification, and physiological state on the indicator cells along with experimental working conditions [24]. They bind to the cell cytoplasmic membrane with harmful effects in different ways. *Subclass Ia* bacteriocins are associated electrostatically with the negatively charged membrane phospholipids, which allowed the interaction with the cytoplasmic membrane of the target cell generating unspecific ionic channels. Inhibitory activity of *subclass IIa* is related to the presence of the sequence YGNGV at their N-terminus region. According to previous studies, some non-lanthionine bacteriocins are more active at the lower pH [24, 26]. In case of *subclass IIc*, the mechanism of action is controlled by the presence or absence of intramolecular disulfide bonds. For example, in case of lactococcin A, a bacteriocine without cysteine residues, the activity is related to the pore formation on sensitive cell membranes, while, in cerein 7/8, activity

decreases the osmolarity of growth culture suggesting that this bacteriocin acts at the membrane level [25].

2.2. Genetics and biotechnological potential of LAB bacteriocins

Recent studies showed that almost all genetic determinants of bacteriocins are clustered in *operons* or *regulons* and its production is controlled by the presence of extrachromosomal elements such as plasmids [25]. Genes encoding for bacteriocins are located on the chromosome (e.g., subtilin), plasmids (e.g., divergicin A), or transposons (e.g., nisin). In general, lantibiotic operons are more complex than non-lantibiotic ones because they need additional genes encoding enzymes for posttranscriptional modifications. In case of nicin, the genetic determinants are located on the conjugative transposon Tn5276 within the bacterial chromosome. Gene *nisA* has been sequenced and found as been part of a polycistronic operon [24]. Other genes presented in the nisin operon are *nisB, nisI, nisR*, and *nisP. NisB* contains several putative transmembrane helical regions and appears to bind to artificial phospholipid vesicles suggesting that the nisin synthesis occurs at the cytoplasmic region, while *nisP* appears to be involved in the regulation of nisin biosynthesis. Another bacteriocin, lacticin 481, produced by *Lactococcus lactis* had the genes on the transposon Tn5721 located on a 70-kb plasmid [24].

Most of the genetically characterized class II bacteriocin gene clusters are composed of three gene modules: a module that includes the structural and immunity genes, a transport gene module, and a regulatory gene module. The structural gene for the bacteriocin is cotranscribed with the corresponding immunity gene located downstream, although there are exceptions to this genetic organization. For example, in case of the non-lantibiotic bacteriocin, carnobacteriocin BM1 produced by *Carnobacterium piscicola*, while its structural gene is located on the bacterial chromosome, its expression is dependent on the presence of a 61-kb plasmid, which carries some of the genes required for the export and the immunity.

Pediocin-like bacteriocins of *subclass IIa* have a very complex structure, containing double-glycine leader peptide, and are transported by ABC transporter. Among this class, few bacteriocins pediocin such as PA-1, AcH, and sakacin A were most characterized [5]. Pediocin PA-1 and pediocin AcH were produced by strains of *Pediococcus acidilactici*, possessing plasmids with sizes 9.4 and 8.9 kb respectively, and Sakakin A was determined by a 60-kb *Lactobacillus sakei* plasmid.

Although the expression of bacteriocin genes is regulated by external induction factors, bacteriocins' production depends upon environmental conditions (temperature, pH, etc.). Their use in food preservation offers several benefits: among them, it reduces the use of chemical preservatives and decreases the elongation of heated treatments. Bacteriocins can be produced *in situ* by the inoculation of the producer strain or can be produced *ex situ* and added to the food as antimicrobial additives. However, the composition of the food matrix and the interaction with other preservation factors affect its production and its activity.

In food industry, numerous control measurements to prevent or minimize pathogen contamination, including good manufacturing practices, effective sanitation, and hygiene measures, have been developed [29]. Nevertheless, despite these safety measures, foodborne outbreaks

do occur frequently with particular concern on consumers health. Among food pathogens, *L. monocytogenes* is extremely strong, surviving refrigeration temperatures and high salt concentration. Other pathogens such as *Salmonella* sp. and *E. coli* are also frequently detected in processed or fresh foods. Nowadays, many investigations are focused on discovering novel bacteriocins for controlling the undesirable bacteria in food products [25, 29]. There is a need to attract consumer attention to natural substances rather than conventional synthesis of chemical one as protector against pathogens. As probiotics has been accepted in the market for their beneficial properties, and in the same way, the bacteriocin-producing probiotic strains should become attractive especially to natural food preservation.

Continued research on bacteriocins will undoubtedly lead to our increased understanding, and with the emergence of new bacteriocins, new potential biopreservatives.

3. Antimicrobial activity of LAB strains isolated from native microbiota of Ecuador

The presence of pathogens in many food products has become a serious problem worldwide. During the last decade, several laboratories have worked towards the identification of novel probiotic strains with better performance benefits such as novel attractive alternative antimicrobial methods to conventional ones [30–36].

Ecuador is known as country with large diversity of native unexploited resources. Some regions were included recently in the governmental policy as important resources to be exploited as reservoirs of unknown microorganisms that could become as potential areas of highly interest for biotechnology research, food sovereignty, and security. The lack of quality control, bad storage condition, and lack of preservation against spoilage bacteria had effect on food safety and security. Among the most food pathogens worldwide due to the considerable human rates of illness reported, *Salmonella* and *E. coli* remain the wide species detected in the local food market in Ecuador. Most produced traditional foods, such as mote (a fermented maize dish), handmade chees, and milk containing drinks, maintained in defective storage conditions appear to pose significant number of pathogens; therefore, the risk of developing diseases associated with food born pathogens is elevated. In this context, the aforementioned problems identifying new alternatives for food biopreservation have become an attractive approach to be considered. Some native wild plants and fruits derived have been recently screened for the presence of probiotic LAB [37]. Preliminary investigation reveled the presence of LAB showing probiotic potential (submitted manuscript). Probiotic bacteria, although not a new concept, draw the attention of the scientific community for their highly potential to act as natural food preservative. However, in this study, we present the results on the antimicrobial activity of ten LAB strains to select those with promising potential in biopreservation. A preliminary characterization of the bacteriocin of selected LAB is also described.

4. Materials and methods

4.1. Bacteria and sampling source of isolation

Sampling material consisting of native fruits and flowers has been collected without no specific ethic permits. The reservation was located on subtropical humid mesothermal region of Santo Domingo de Los Tsachilas Provence at 43 km away from Quito, the capital city. At the location, the GPS points have been recorded and the location map was designed using the ArcGIS software (a complete platform of GIS to create, analyze, store, and disseminate geographic data, models, and maps) in order to track each sample in case of cross-contamination. Approximately ten grams of wild orange, immature and mature berries, guayusa, strawberry, achiote and flower inflorescence (*Heliconia* sp., *Fucsia* sp., *Bromelia* sp.) collected aseptically were transferred in Erlenmeyer flasks (500 ml) containing sterile water (100 ml) and incubated statically for up to 5 days at the room temperature. MRS agar [38] plates were used for the inoculation, the samples were incubated under anaerobic conditions at 37°C for 72 h, and isolated individual colonies were randomly selected and purified by replating on same medium. The purified colonies (>100 colonies/each sample) were Gram stained and tested for the mobility, indole production, catalase production, spore formation, and production of gas from glucose. Cells morphology and colonial characteristics on MRS agar were examined, and based on these results the colonies were preliminary classified as follows: (i) presumptive lactococci, gram positive, coccal morphology, catalase negative, non-motile, and gas production from glucose, and (ii) gram positive, with morphological aspect of rods, catalase negative, non-motile, with and without production of gas from glucose, and presumptive lactobacilli, stored at −80°C in 20% glycerol. Moreover, the API 50CH strips (Biomerieux, Marcy l'Etoile France, cat # 50300) were used for the metabolic characterization of the each isolate and tentatively identified at genus level. Furthermore, the isolates selected for their probiotic performance (bile tolerance, survival under acidic conditions, antibiotic tolerance, and salt tolerance) were analyzed for their antimicrobial activity. As reference strain, *Lactobacillus fermentum* CNCM 1-2998 (API50CH, 80% identity) recuperated from an available commercial probiotic Lacteol Forte (Axcan Pharma, France) has been used.

4.2. Pathogens isolation

Food products consisting of chicken and cheese were purchased from the local market, and standard bacterial culture media were used to screen and isolate the contaminants. However, *Salmonella* sp. and *Escherichia coli* were identified in each food sample. The isolated and purified bacterial cultures were further purified and used as indicator strain.

4.3. Antimicrobial activity of selected isolates

Antimicrobial activity was performed against both *E. coli* and *Salmonella* sp., using agar well diffusion method under anaerobic conditions [1]. The LAB isolates were grown in MRS broth at 37°C for 16 h, and the supernatants were collected by centrifugation at 13000×*g* for 20 min sterilized using 0.22 μm porosity filter. The indicator strains (100 μl) grown in broth medium

(7 log CFU/ml) were mixed with 1.5 ml of soft MRS agar (0.75%), were overlaid on the nutrient agar plates, and incubated at 37°C for 2 h. The cell-free supernatant (100 μl) was spotted onto the wells (7 mm) made on overlaid agar, incubated at 37°C, and subsequently examined for inhibition zones at different intervals of time (18–24–36–48 h). The experiments were run in triplicate, and the mean values of zone of inhibition were estimated. We considered that the isolates had higher inhibitory activity when the diameter of zone of inhibition was higher than >15 mm, intermediary activity when the zone of inhibition was 10–15 mm, and lower activity when the diameter of zone of inhibition was lower than 7 mm.

4.4. The effect of different pH, heat, and detergents on antimicrobial activity

The pH of supernatant was adjusted to 3.0, 4.0, and 7.0 and then kept at room temperature for 4 h. To test heat sensitivity, 100 μl of culture supernatant was heated for 30 min at 30, 45, 60, 75, and 90°C. Residual activity of each isolate for different pHs and temperature was determined by the agar well diffusion method as described above for both indicator strains. The resistant culture supernatants were further heated for 10, 30, and 60 min at 100°C. Another batch of cell-free supernatants treated with 1, 2 and 5% Triton X-100 (BDH Chemicals Ltd, Poole, England) and the same concentration of EDTA (Merck) were incubated for 30 min at 30°C. The activity was measured using agar well diffusion method [1].

4.5. Effect of chloroform on antimicrobial activity

To test the effect of chloroform on inhibitory activity, the culture supernatant of each sample was mixed with an equal volume of chloroform and kept at room temperature for 4 h before antimicrobial activity testing.

4.6. Statistical analysis

Statistical analysis was carried out by one-way analysis of variance, the means were separated by Tukey post-hoc test, and the results were considered statistically significant at the $p < 0.05$ level (SPSS version 10.0.6, USA).

5. Results and discussions

5.1. Screening of LAB isolates

Regardless of numerous probiotic strains presented in the market, there is an ongoing need for the improvement of LAB strains to be used as starter cultures or to develop new natural method for biopreservation; thus, LAB isolated from their natural environment (e.g., native fruits, flowers) might possess unusual characteristics including phenotypic differences and intraspecific variability compared to the known ones. In this investigation, we assumed that acid-tolerant bacteria might be detected as the fermentation of raw material reached at about pH 3.5. **Figure 1** shows the distribution of biological material used as source of initial screening of LAB.

However, preliminary phenotypic analysis suggested the relatedness of the bacterial isolates from wild-type fruits and mature inflorescence of several tropical flowers (>100 colonies/ sample) with LAB, which were affiliated to two larger groups: *Lactococcus* (54%) and *Lactobacilli* (46%) genera. Furthermore, carbohydrate profiles conducted on ten randomly selected isolates related to each type of biological material (sample of origin) assigned the selected isolates as follows: UTNFa38, UTNFa40, and UTNFa41 were identified as *Lactococcus lactis* ssp. *lactis*, with identity of 90–99%, the isolate UTNFa37, as *Lactobacillus collinoides* (99%), UTNFa39, as *Lactobacillus brevis* 3 with 98% identity, while UTNFa19 and UTNFa23 were identified as *Lactobacillus paracasei* ssp. *paracasei* 1 with 99.7 and 98.2%, respectively. The isolates UTNFa33 and UTNFa17.2 were identified as *Lactobacillus paracasei* ssp. *paracasei* 3 with 99.6 and 97.9% identity, and UTNFa8.2 was identified as *Lactobacillus pentosus* with 98.3%. **Table 1** presents the classification of isolates on the basis of morphological, physiological and metabolic properties. Similar to our study, numerous lactobacilli species (i.e., *L. paracasei, L. pentosus)* were identified in different fruits and vegetables [39].

Strain code	Cell form/cellular arrangement	Specie assignation	% of identity based on API 50 CHL
UTNFa19	Coccus/single	*Lactobacillus paracasei* ssp. *paracasei* 1	99.70
UTNFa38	Bacilli/rods/single	*Lactococcus lactis* ssp. *lactis*	98.00
UTNFa17.2	Bacilli/rods/single	*Lactobacillus paracasei* ssp. *paracasei* 3	97.90
UTNFa23	Bacilli/rods/single	*Lactobacillus paracasei* ssp. *paracasei* 1	98.20
UTNFa8.2	Bacilli/rods/single	*Lactobacillus pentosus*	98.30
UTNFa33	Bacilli/rods/single	*Lactobacillus paracasei* ssp. *paracasei* 1	99.60
UTNFa39	Bacilli/rods/single	*Lactobacillus brevis* 3	98.00
UTNFa40	Coccus/single	*Lactococcus lactis* ssp. *lactis*	90.00
UTNFa41	Coccus/single	*Lactococcus lactis* ssp. *lactis*	99.00
UTNFa37	Bacilli/rods/single	*Lactobacillus collinoides*	99.00

Table 1. Classification of LAB isolates.

The antimicrobial activity of the selected strains was evaluated against two Selected foodborn pathogens using agar-well assay. The zone of inhibition was easily visualized, and the mean value of the inhibition zone was determined. The cell-free supernatants were considered as crude bacteriocin. Among tested isolates, most of them showed elevated inhibitory activity for both pathogen tested. Nonetheless, results from enzyme inactivation analysis demonstrated that antimicrobial activity was lost or unstable after treatment with proteolytic enzymes such proteinase K and trypsin, whereas catalase treatment did not affect the activity of antimicrobial substance produced by the tested isolates, confirming its protein status. The sensitivity of the found substance to proteolytic enzymes is a proof of its proteinaceous nature, which allows considering as bacteriocin.
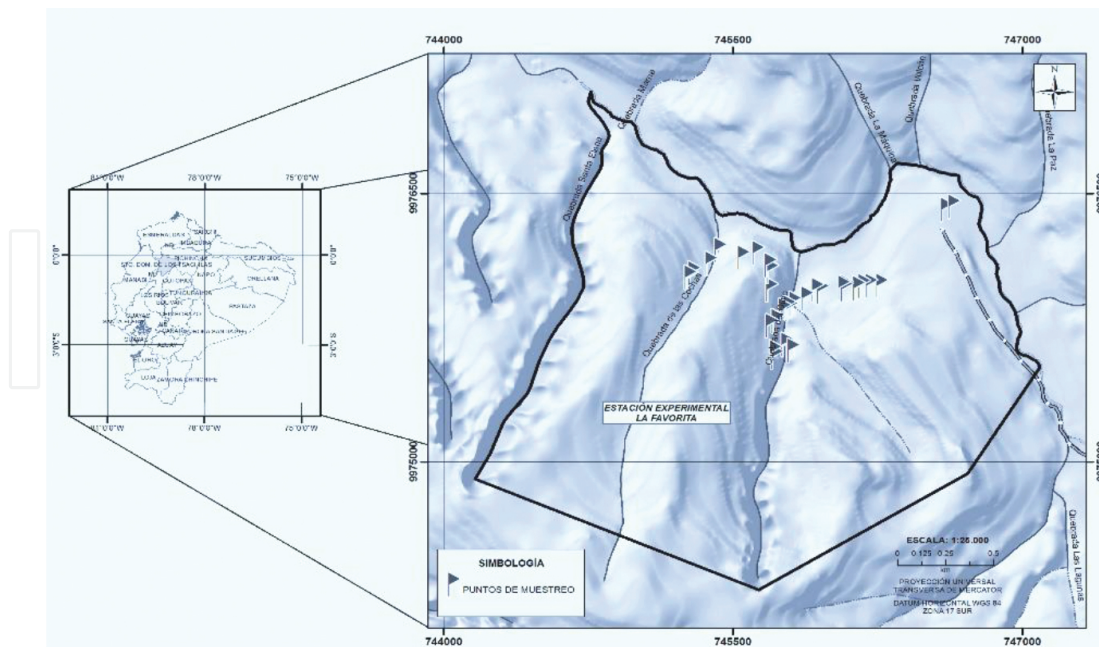
Figure 1. Origin of sampling (geographical distribution according with ArcGIS software).

5.2. Effect of pH on inhibitory activity

The antimicrobial effect exerted by LAB strains is related to the production of lactic acid, reduction of pH, and inhibitory compounds [39], has attracted much attention, and attributed as important selection criteria of a probiotic microorganism [2, 33]. An elevated antimicrobial activity against both food pathogens was observed at the pH 3.0 with the mean range value of inhibition zone 15.25 mm (±0.5) of the supernatant of tested isolates. In **Figure 2A**, we showed the mean value of inhibition zone displayed by each isolate at different pH towards *Salmonella* sp. Although at the pH 4.0 no significant difference between the mean values of inhibition zone was recorded, the mean range of inhibition zone was 13.58 mm (±1.24) for *E. coli* and 12.09 mm (±2.04) for *Salmonella* sp., after 48 h of incubation. With the increase of the pH, we observed a gradually reduction of the antimicrobial activity as no activity was recorded at the pH 7.0 for all selected isolates as well as the reference probiotic. **Figure 2B** showed the clear inhibition zone of two isolates UTNFa40 and UTNFa41 at the pH 3.0 and 4.0, and no zone formation at pH 7.0.

Overall all selected isolates, in particular two isolates, UTNFa40 and UTNFa41, displayed elevated inhibitory activity in comparison with the reference strain. Of course, the efficiency and the nature of this antimicrobial activity have to be investigated. Recent studies showed the importance of bacteriocin produced by the *Lactobacillus pentosus* ST712BZ strain isolated from boza in the preservation of beverage products [3]. In other investigation, *L. pentosus*, a bile-resistant strain, displayed bacteriocin activity against a wide range of spoilage and pathogen bacteria [32]. In agreement with the studies, we showed that the isolate UTNFa8.2
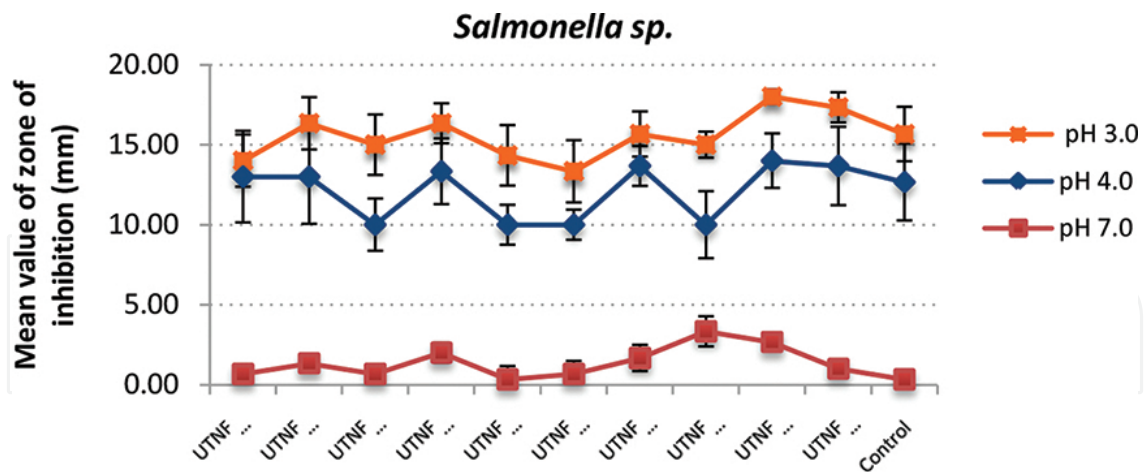
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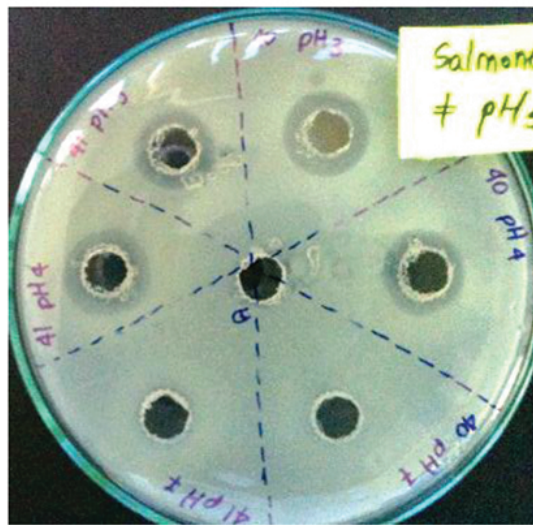
B.

Figure 2. Antimicrobial activity towards *Salmonella* sp. (A) Mean value of zone of inhibition in mm recorded at tested pHs (bars represent the means ± SD). (B) The visualized clear zone of inhibition at pH 3.0, 4.0, and 7.0 of UTNFa41 and UTNFa40.

assigned as *L. pentosus*, a bile, and acid-resistant strain displayed elevated antimicrobial activity, which will further allow us to explore its biotechnological properties.

5.3. Effect of the heat, detergents, and chloroform on inhibitory activity

The inhibitory activity was not significantly reduced in case of the heat treatment. The mean value of zone on inhibition varied at the incubation temperature of 30°C from 19 mm (±2.34) towards *Salmonella* sp. and, respectively, from 20.18 mm (±3.72) towards *E. coli*. At the 60°C, it varies from 16.33 mm (±2.92) towards *Salmonella* sp. and from 17.5 mm (±3.17) towards *E. coli*, and at the 75°C it varies from 14.83 mm (±3.05) towards *Salmonella* and from 15.5 mm (±3.27) in case of *E. coli*. The increase of temperature of 90°C showed a reduction of the

inhibition zone was observed for both pathogens. **Figure 3** shows the mean values of the inhibition zone recorded after 30-min incubation at different temperature. At 100°C, after 30 min of incubation, two isolates were resistant and maintain its inhibitory activity.
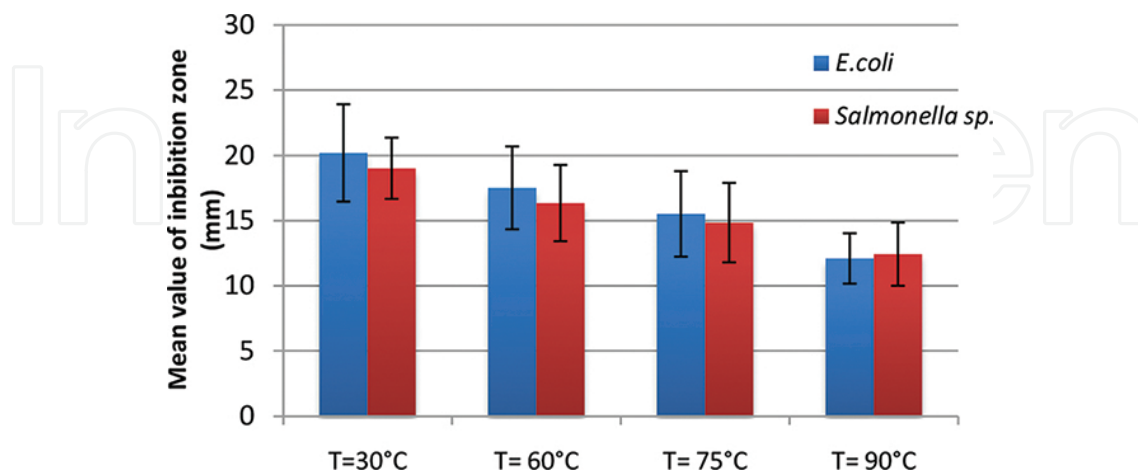

Figure 3. Mean values of zone of inhibition at different temperature of cell-free supernatant towards *E. coli* and *Salmonella* sp. (bars represent the means ± SD).

The heat stability could be an advantage when the strains are intended to be used as biopreservative of processed foods. Similarly, Todorov and col., showed that some bacteriocins remain stable after incubation at 100°C for 120 min [34]. In other study, bacteriocin-like substance of *Lactobacillus fermentum* KN02 was strongly influenced by the pH and temperature. The strain has the maximum productivity at the pH 2.0 and was resistant to heat at 100°C [40].

Due to their resistance to temperature and low pH, the bacteriocins would be digested by human and animal peptidases, thus avoiding resistance and problems associated with the presence of residues in feed and food [35]. However, at the treatment of the selected cell-free supernatants with Triton-X 100 and EDTA, an increase in the inhibitory activity was recorded. An increase with 5% of both Triton-X 100 and EDTA results in an increase of inhibitory activity for some of the isolates. For example, **Figure 4** shows the mean values of the zone of inhibition recorded towards *E. coli* and *Salmonella* sp. after the treatment with 1, 2 and 5% Triton-X 100 for each strain tested. Similar studies showed that the heat does not have any effect on cell-free supernatants activity as well as no effect on the inhibitory activity of the bacteriocins of *Lactobacillus sakei* isolated from the fermented meat was observed after the treatment with several detergents including EDTA and Triton-X 100 [35].

On the contrary, in our study, we observed an increase in the concentration of either EDTA or Triton-X 100, and the inhibitory activity was elevated for most of the isolates. **Figure 5A** shows the inhibitory activity towards *Salmonella* sp. by the appearance of the clear zone after treatment of cell-free supernatant with different concentration of EDTA. In **Figure 5B**, the effect of EDTA on antimicrobial activity towards *Salmonella* is shown. We observed an increase in the inhibitory activity with the increase of the concentration of EDTA. However, a positive effect of detergents in the antimicrobial activity of each isolate has been detected.
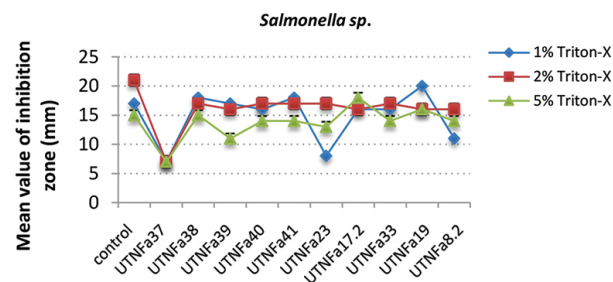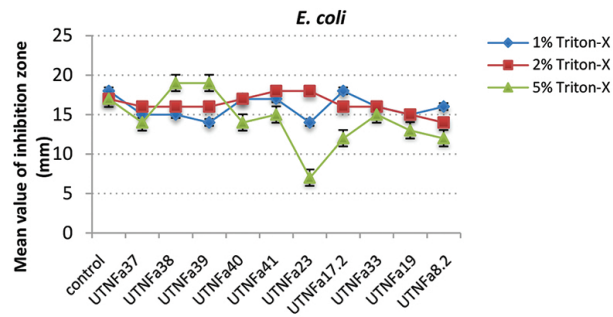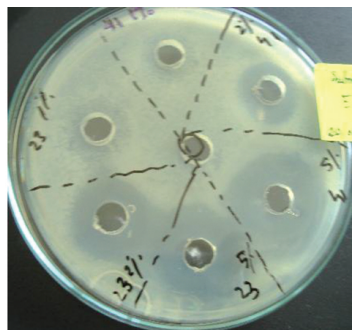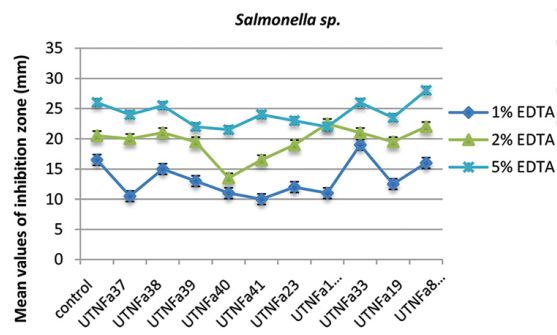
Figure 4. The inhibition activity of the isolated strains towards *Salmonella* sp. (A) and *E. coli* (B) after the treatment with Triton-X 100.

Figure 5. (A) The appearance of the clear inhibition zone at different concentration of EDTA of isolates UTNFa23 and UTNFa41 towards *Salmonella* sp. (B). The antimicrobial activity recorded as mean value of inhibition zone of LAB after the treatment with 1, 2, 5% EDTA towards *Salmonella* sp.

The antimicrobial activity of most of the isolates was lost in case of chloroform treatment of the cell-free supernatants. Among analyzed strains, the isolate UTNFa38 and isolate UTNFa41 remained active towards *E. coli* as well as *Salmonella* sp., after the treatment with chloroform. The mean value of inhibition zone was 10 mm for UTNFa38 and respectively, 11 mm for UTNFa41 towards *E. coli*, while the mean value of inhibition zone was 9 mm for UTNFa38 and 12 mm for UTNFa41, towards *Salmonella* sp. The resistance to chloroform treatment and boiling demonstrates the nature of *low-molecular, non-lipid-containing bacteriocins*. Eight isolates were identified as lipid-containing bacteriocins because of their sensitivity to chloroform. Similar studies showed the broad spectrum of inhibitory activity of *Lactobacillus paracasei* subsp. *paracasei* isolated from natural homemade cheese [41]. Besides several *Lactobacillus* strains from different species, the bacteriocin from *L. paracasei* ssp. *paracasei* also inhibits the growth of various pathogenic bacteria such as *Streptococcus*, *Staphylococcus*, *Shigella*, *Listeria*, and *Pseudomonas*.

The stability of crude cell supernatant of each selected LAB to different conditions reflects that these compounds would remain effective in the processing of foods [42]. Recent investigation showed the broad spectrum of inhibitory activity towards *Pseudomonas* of some bacilli isolated from onion and fresh-cut salads [43]. In other work, it has been demonstrated the antimicrobial activity against spoilage pathogens of some LAB isolated from mango pulp [44]. The six isolated strains had inhibitory effects on sensitive bacteria including *E. coli*, demonstrating the potential of usage of this compound as a preservative in mango or fruit pulp industry. In similar work, several LAB isolated from foods and spoilage halotolerant bacteria isolated from charqui, a Brazilian fermented, salted meat product. The bacteriocin of *Lactococcus lactis* subsp. *lactis* (*L. lactis* 69) inhibited, *in vitro*, *Listeria monocytogenes*, *S. aureus* [45]. In our study, the resulted data revealed a wide spectrum of inhibitory activity against two food pathogens of some LAB isolated from natural microbiota of Ecuador, and shall further characterize and determine its molecular size and mode of action, as well as its effectiveness as a biopreservative in different food products as such or in combination with other methods.

6. Conclusions

Bacteriocins produced by genera *Lactobacillus* or other genera have been reported. Nevertheless, the studies in the field of natural food biopreservation are conducted to an increasing extent. As consumers are more concern about the food quality along with their refusal of chemical additives, there is a growing demand for alternative antimicrobial treatments and bioactive compounds such as bacteriocins from lactic acid bacteria are well-accepted natural means of selective microbial inhibition.

However, characterization of specific microbiota would further contribute substantially to gain better knowledge for the improvement of current commercial probiotic strains. The studies conducted up to date indicate that interest on bacteriocins will be high. Thus, all the studies carried out on novel bacteriocins are important to propose new alternatives in food preservation.

Acknowledgements

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