Antimicrobial effect of bacteriocin from *Lactobacillus fermentum* isolated from goat milk on perishable foods. San Luis. Argentina

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Abstract— *Strain selected for this study was isolated and named as sl36, from samples of goat milk collected from stainless steel drums in a dairy farm (San Luis, Argentina). The LAB strain was biochemically typified as Lactobacillus fermentum and designated as L. fermentum sl36. This identification was confirmed by 16S rRNA full sequences. The selected strain showed antimicrobial activity against food-borne pathogens, Listeria monocytogenes, Listeria innocua, Staphylococcus aureus, Enterococcus faecalis, Pseudomonas aeruginosa and Escherichia coli. The inhibitory activity was lost after treatment with trypsin, which indicates that this activity is due to a protein nature substance compatible with bacteriocins produced by Gram positive bacteria.The inhibitory substance was stable at different pH and temperatures. Perishable food samples (semi-hard cheese, cream, cooked pork shoulder) were treated with cell free supernatant from studied strain and then with indicator S. aureus and E. faecalis bacteria suspensions. Lactobacillus fermentum sl36 caused the inhibition of the growth of E. faecalis and S. aureus in the treated foods. Our work shows that it is possible to increase the safety of food perishable directly using the bacteriocins produced by the LAB strains, difference from the more frequent practice of using the bacteria themselves as probiotics.*

Keywords— Antimicrobial activity, goat milk, Lactic Acid Bacteria, Lactobacillus fermentum, perishable foods.

I. INTRODUCTION

There is an increasing demand on preservatives from natural sources due to potential toxicity of food chemical preservatives. In the last years, numerous studies have been focused on the reduction of the use of chemical preservatives; additionally, new preservation techniques by physical methods or by using natural antimicrobials have been developed. [1]

Lactic acid bacteria (LAB) have been used for centuries in food processing and are very important for their contribution to the value of products. Due to several metabolic properties, LAB play an important role in the food industry, for their contribution to flavour, smell, texture, sensory characteristics, therapeutic properties and nutritional value of food products. The use of LAB and its metabolites in food preservation is accepted as natural by consumers [2] and integrate an important group of generally recognized as safe (GRAS) for the American Food and Drug Administration (FDA). [3]

These microorganisms are found in very diverse environments, including resident microbiote of humans, as well as in cereals, fruits and vegetables, milk and meat, playing an essential role in the fermentation of these substrates and for the manufacture of many fermented foods and beverages[4].

The protective role of LAB lies in their capability to decrease pH and synthesis of bacteriostatic and bactericidal substances. These substances include hydrogen peroxide, lactic acid, carbon dioxide and bacteriocins which are defined as peptides produced by bacteria that inhibit or kill other related and unrelated microorganisms. In these years, a lot of studies have focused on the inhibition, by using bacteriocins, of food spoilage and of human pathogens associated with vegetable foods and beverages, and bacteriocin application has appeared as an interesting alternative to chemical compounds and antibiotics. [5], [6]

The bacteriocins produced by LAB exhibit properties that make them suitable for food preservation: recognized as GRAS, nontoxic and have no activity against eukaryotic cells, inactivated by digestive proteases, having little influence on the gut microbiota, are stable in wide ranges of pH and temperature, have a relatively broad antimicrobial spectrum against many food-borne pathogenic and spoilage bacteria, generally have a bactericidal mechanism and no cross resistance with antibiotics; and their genetic determinants are usually plasmid encoded, facilitating genetic manipulation, including the transfer of the gene clusters involved in their production to other food grade bacteria.[7]

Bacteriocins have emerged as an alternative antimicrobial treatment; consequently it is important to continue investigating about bacteriocin-producer strains and their spectrum of antimicrobial activity.

Contamination of raw milk and cheese during ripening process has been reported with *Listeria monocytogenes, Escherichia coli* and members of the family Enterobacteriaceae causing alterations in taste and flavors and affecting human health. [8], [9]

L. monocytogenes, the bacterial agent of listeriosis, is of particular concern to the food industry since it is a difficult foodborne pathogen to control due to its ubiquitous distribution, tolerance to high levels of salt, and its ability to grow at a relatively low pH and at low refrigeration temperatures. Other pathogens as *Staphylococcus aureus* can also cause food-borne diseases. [10]

In recent years, the genus *Enterococcus* has acquired great importance because of its high incidence in nosocomial diseases and the resistance to antimicrobials. In addition, they are considered indicators of food contamination and faecal contamination of the waters, due to their wide distribution and to their high resistance to adverse conditions. [11]

Many studies are aimed to selection and development of protective bacteriocinogenic cultures for food applications. Although a large body of data concerning bacteriocin-producing LAB has been reported, strains prevalent in San Luis region has not been studied much, as well as which of them are producers of bacteriocins that might be used in food preservation. [12]

The purposes of this study were to evaluate the antimicrobial activity of a LAB strain isolated in this region from raw goat milk, to characterize the inhibitory substance, and also to study its effect on the conservation of perishable foods. The antimicrobial activity was tested against foodborne pathogens such as *Listeria monocytogenes*, *Listeria innocua*, *Staphylococcus aureus, Pseudomonas aeruginosa*, *Escherichia coli* and *Enterococcus faecalis.*

II. MATERIALS AND METHODS

2.1 Bacterial strain and growth conditions

Strain selected for this study was isolated and named as sl36, from samples of goat milk collected from stainless steel drums in a dairy farm (San Luis, Argentina). 10-fold dilutions of samples were made in 0.1 % peptone water and plated onto Man, Rogosa and Sharpe agar medium (MRS) and incubated at 37° C for 48 h in microaerophilic conditions.

2.2 Identification and characterization of LAB strain

The isolated strain was characterized as LAB by Gram stain, catalase and oxidase tests, and was biochemically typified by identification system API 50 CHL (BioMerieux) The identification of the microorganism was performed by using the software Apilab plus (Biomerieux).Confirmation of identification was obtained by amplifying the genomic DNA based on 16S rRNA full sequences (Macrogen, Inc, Seoul).

2.3 Obtaining cell-free supernatant

sl36 strain was propagated, in microaerophilic conditions, twice in MRS broth at 37°C for 24h and then propagated in MRS broth at 37ºC for 16h, incubation time in which a maximum of antimicrobial activity is detected (results not shown).After that, cell free supernatant (CFS) was obtained by centrifugation (8000 g, 20 min, and 4 °C) and filtered through a 0.2 µm pore-size cellulose acetate membrane. For some tests CFS was neutralized (NCFS) by adjusting pH to 7.0 with 4N NaOH.

2.4 Sensitive strains

The indicators organisms selected to be used in antimicrobial test were *Listeria monocytogenes CLIP 74902*, *Listeria innocua CLIP 74915*, *Staphylococcus aureus* (Microbiology Laboratory collection, San Luis National University), *Enterococcus faecalis ATCC 29212*, *Pseudomonas aeruginosa ATCC 27853*, *Escherichia coli* (Microbiology Laboratory collection, San Luis National University) which were grown in Trypticase Soy agar (TSA) at 37°C for 24 h.

2.5 Antimicrobial Activity

The antimicrobial activity was determined by liquid medium method described by Cabo M.L. et al [13]. To 1 ml of sensitive strains suspension (6×10^8 UFC.ml^{-1}) was added 0.5 ml of CFS and 0.5 ml of medium Trypticase Soy Broth (TSB). The mix was incubated for 6 h a 37°C. Absorbance measurements were made at 700 nm. The inhibition percentage (%I) was calculated according to the formula $I= 1-A_s/A_c$, considering A_s and A_c as sample absorbance and control absorbance

respectively. In control assay, the CFS was replaced by MRS broth. Each trial was done in triplicate and the average value was reported.

2.6 Characterization of the inhibitory substance

2.6.1 Determination of peptide nature of the inhibitory substance

The peptide nature of the antimicrobial agent in CFS was verified by treatment CFS aliquots with the following enzymes: Trypsin (Fluka, optimal conditions: pH 8, temperature 37°C, final concentration: 5 $mg.ml^{-1}$), proteinase K (Productos Bio-Lógicos, optimal conditions, pH 7,0, temperature 37ºC, final concentration: 0,1 $mg\ ml^{-1}$) and Pepsin (optimal conditions: pH 2, temperature 37°C, Fluka,final concentration:1 $mg\ ml^{-1}$). The experiments were conducted at optimal conditions for each enzyme and were incubated for 2 h at 37ºC. [14] Supernatants treated with proteases were adjusted to pH 6.8 with 4 N NaOH and then the antimicrobial activity against *E. faecalis* was determined by liquid medium method.

2.6.2 Effect of pH on antimicrobial activity

The effect of pH variation on bacteriocin stability was measured by rising from 2.0 to 10.0, varying every 2 pH units with 1 N HCl or 4 N NaOH. The samples were incubated for 2 h at 37ºC. After incubation, pH of each aliquot was adjusted to 6.8 and assays of antimicrobial activity against *E.faecalis* were performed.

2.6.3 Effect of temperature on antimicrobial activity

Aliquots of CFS neutralized were preserved for one week at 4°C and at -20°C and then were treated at 50ºC, 100ºC and 121ºC for 15 min. Residual antimicrobial activity was determined by the liquid medium method using *E. faecalis* as sensitive strain.

2.7 Application of antimicrobial peptides produced by BAL as preservatives of perishable foods

Perishable food samples (semi-hard cheese, cream, cooked pork shoulder) were treated with CFS from studied strain and then with indicator *S. aureus* and *E. faecalis* bacteria suspensions.

2.7.1 Treatment of semi-hard cheese.

Cheese aliquots of 1g were treated with CFS or with MRS broth (controls). Samples were incubated at 4°C for 6h. At a later stage, the aliquots were submerged in tubes containing 10^8 $CFU.ml^{-1}$ of each indicator and maintained at 4°C for 30 min. After that, samples were placed in sterile Petri dishes and incubated at 37 °C for 24 h, at the end of the incubation period were placed in tubes with 10 ml of sterile distilled water and mixed for 2 min, 1 ml of 10-fold serial dilution was poured on Petri dishes using 1.5% TSA medium. The plates were incubated at 37°C for 24h. *S. aureus* and *E. faecalis* counts in samples were determined according to National Standard Test Methods for Food Microbiology [15].

2.7.2 Treatment of cream

Aliquots of 8 ml of milk cream were treated with 1 ml of CFS or 1 ml MRS broth (controls) and 1 ml of indicator strain suspension (10^8 $CFU.ml^{-1}$). Aliquots were incubated at 37 °C for 12 h, 1 ml of 10-fold serial dilution was poured on a Petri dish using 1.5% TSA medium. The plates were incubated at 37°C for 24h. *S. aureus* and *E. faecalis* counts in samples were determined according to National Standard Test Methods for Food Microbiology.[15]

2.7.3 Treatment of cooked pork shoulder.

Sample aliquots of 1 g were treated with CFS or with MRS broth (controls). Samples were maintained at 4°C for 6 hours. Then, the aliquots were submerged in tubes containing 10^8 $CFU.ml^{-1}$ of each indicator and maintained at 4°C for 30 min. The samples were then placed in sterile Petri dishes and incubated at 37°C for 24 h. At the end of the incubation period were placed in tubes with 10 ml of sterile distilled water and mixed for 2 min, 1 ml of 10-fold serial dilution was poured on Petri dishes using 1.5% TSA medium. The plates were incubated at 37°C for 24h. *S. aureus* and *E. faecalis* counts in samples were determined according to National Standard Test Methods for Food Microbiology [15].

2.8 Statistical analysis

Statistical analyses were carried out with InfoStat software using Friedmann nonparametric variance testA $p<0.05$ was considered to be significant.

III. RESULTS AND DISCUSSION

3.1 Identification and characterization of LAB strain

The isolated strain was characterized as LAB,bacilli Gram positive, catalase and oxidase tests negative.

The LAB strain was biochemically typified as *Lactobacillus fermentum* and designated as *L. fermentum sl36*. This identification was confirmed by 16S rRNA full sequences. (Fig.1)

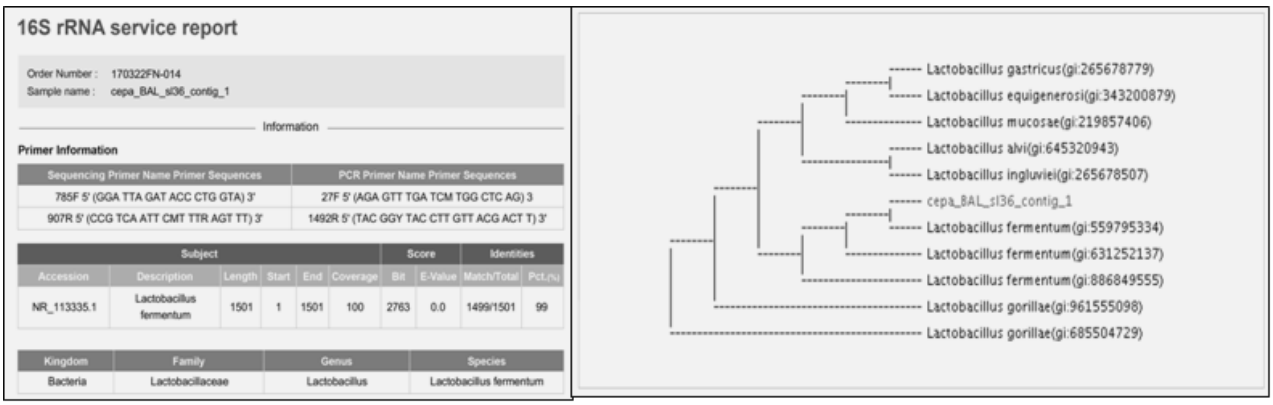

FIGURE 1: IDENTIFICATION BY AMPLIFYING THE GENOMIC DNA BASED ON 16S rRNA FULL SEQUENCES

3.2 Antimicrobial activity

Spectrum of inhibitory activity as inhibition average percentage (%I) against the microorganisms selected as sensitive strains is shown in Table 1.

TABLE 1
INHIBITORY ACTIVITY SPECTRUM FROM *L fermentum sl36*.

Sensitive Strains	Inhibition Percentage	
	CFS	NCFS
P. aeruginosa ATCC 27853	96.50%	27.0%
E. coli	94.00%	34.0%
S. aureus	96.75%	37.0%
E. faecalis ATCC 29212	94.40%	41.0%
L. monocytogenesCLIP 74902	97.75%	38.0%
L. innocuaCLIP 74915	96.75%	43.0%

CFS antimicrobial activity was higher than 90% against all the microorganisms tested and no significant differences were found (p = 0.8587) when comparing the inhibition against all the indicators used. The antimicrobial activity of NCFS showed percentages of inhibition between 27% and 43%. By analysis of CFS versus NCFS, a significant difference was found (p = 0.0008). The inhibition percentage decreases considerably when NCFS is analyzed, which shows that part of the inhibitory effect is due to the production of acids by *L. fermentum sl36*.The results agree with those obtained by Kang et al (2017) [16], Andreeva et al, (2016) [17] and Gong et al (2014) [18], where the production of lactic acid is presented as one of the mechanisms antimicrobials of lactobacilli. It is important to note that CFS and NCFS inhibited the growth of *E. coli* and *P. aeruginosa* that are Gram-negative strains.

3.3 Characterization of the inhibitory substance

3.3.1 Determination of peptide nature of the inhibitory substance.

The inhibitory activity of NCFS was lost after treatment with trypsin. This indicates that the antimicrobial activity is due to a peptide nature substance compatible with bacteriocins produced by Gram positive bacteria.

3.3.2 Effect of pH on antimicrobial activity

The pH stability of the active compound present in NCFS was studied in the range of pH 2.0–10.0. It was observed that the inhibitory substance was active at pH values from 2.0 to 6.0 but reduced at pH 8.0 and 10.0. *L. fermentum sl36* produces antimicrobial compounds that remain stable over a wide pH range, losing part of their inhibitory capacity at alkaline pH values.

TABLE 2
EFFECT OF PH TREATMENT ON CFS PRODUCED BY *L fermentum sl36*

NCFS treatment pH values	pH: 2,0	pH:4,0	pH:6,0	pH:8,0	pH:10
Average Inhibition Percentage	94.0 %	93.7 %	94.6 %	41.9 %	33.0 %

3.3.3 Effect of temperature on antimicrobial activity

Antibacterial activity was not altered by the storage for one week at 4ºC and at -20ºC. Identically the inhibitory activity was not modified by heat treatment as shown in Table 3.

TABLE 3
EFFECT OF HEAT TREATMENT ON CFS PRODUCED BY *L fermentum sl36*

HEAT TREATMENT	CFS preserved at 4°C	CFS preserved at -20 ° C
CFS Without Heat Treatment	94.00%	86.20 %
NCFS Without Heat Treatment	40.85 %	41.00 %
50ºC 15’	40.75 %	42.20 %
100ºC 15’	40.40 %	42.6 %
121ºC 15’	40.20 %	40.70 %

3.4 Application of antimicrobial peptides produced by BAL as preservatives of perishable foods

The inhibitory substance present in the CFS of the culture of *Lactobacillus fermentum sl36* caused the inhibition of the growth of *E. faecalis* and *S. aureus* in the treated foods, when compared with the growth of the indicator strains in the controls (aliquots of samples treated with MRS broth), since the average values of the counts expressed as $CFU.ml^{-1}$ in the

controls were significantly higher than the counts in the aliquots of foods treated with CFS. The CFS inhibits the development of the two indicators with similar efficacy. The figure 2 show that *L. fermentum sl36* has a lower inhibitory effect against *E. faecalis* and *S. aureus* when cream was treated. It is possible to establish, when analyzing the development of the indicators in each food separately, that there is a significant difference (<0.0001) between the counts of the controls and those treated with CFS (Friedman test).

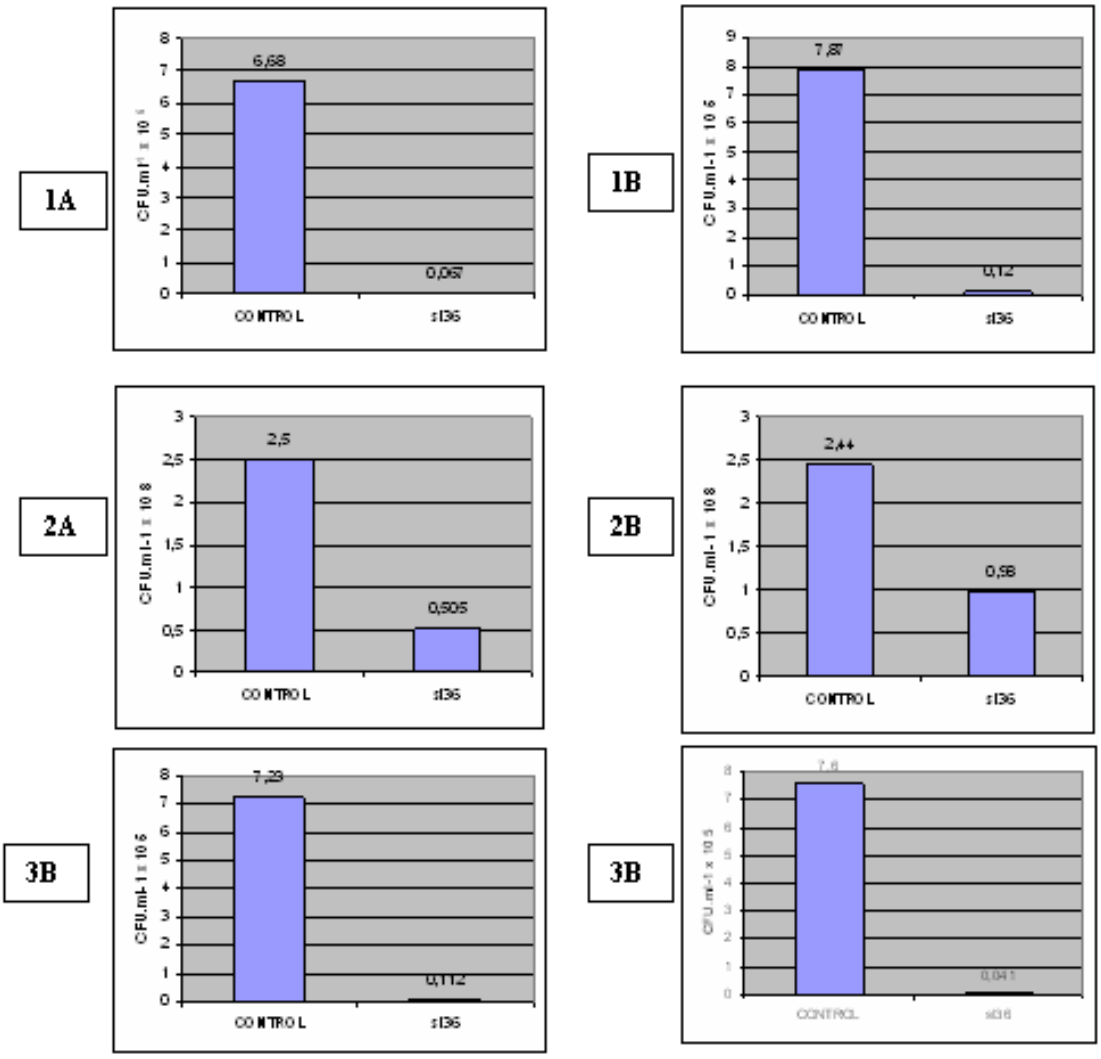

FIGURE 2: Counts of *E. faecalis* and *S. aureus* in perishable foods treated with CFS of *Lactobacillus fermentum sl36*. 1A.semi-hard cheese inoculated with *E. faecalis*. 1B.semi-hard cheese inoculated with *S. aureus*. 2A cream inoculated with *E. faecalis*. 2B cream inoculated with *S. aureus*. 3A.cooked pork shoulder inoculated with *E. faecalis*. 3B cooked pork shoulder inoculated with *S. aureus*

Antimicrobial activity of *Lactobacillus spp* strains isolated of food has been studied for many investigators. Azizi et al (2017) [19] showed that *Lactobacillus spp.* strains isolated from Iranian raw milk Motal cheese inhibited growth of *Staphylococcus aureus* from ATCC, *Escherichia coli* and *Listeria innocua* strains. In accordance of this, Macaluso et al (2016) [20 found lactobacilli strains isolated from Sicilian cheese with antimicrobial activity for *Listeria monocytogenes*, nevertheless the same strains did not inhibit the *Staphylococcus aureus*, *Escherichia coli* and *Salmonella enteritidis* growth.

The use of bacteriocins in the food industry is proposed by many investigators. The control of the development of pathogen microorganisms in cheese using bacteriocin-producing LAB strains has been effective for the treatment of dairy products [21]. Castellano et al (2018) [22] described bacteriocins produced by *Lactobacillus curvatus* and *Lactobacillus sakei* strains able to prevent the growth of *L. monocytogenes* in frankfurters stored at 10 °C during 36 days. In the same way, Mills et al (2017) [23] generated a suite of single- and double-bacteriocin-producing starter cultures capable of produce two classes of bacteriocins, and demonstrated an increment in the control of the *Listeria innocua* growth in laboratory-scale cheeses. On the

other hand, Scatassa et al (2017) [24] demonstrated that bacteriocin-like inhibitory substances produced by lactic acid bacteria prevented the growth of *L. monocytogenes* in tradicional Sicilian Cheeses, nevertheless this effect was not observed neither in raw nor in pasteurized milk. In our research, growth inhibition of *S. aureus* and *E. faecalis* in cheese and cream was studied by applying the supernatants of cultures containing the antimicrobials, which avoids possible alterations in the food that could cause the use of LAB on foods. With regard to meat products treatment, the results of Beristain Bauza (2012) [25] and Camargo Peralta et al (2009) [26] coincide with our results what refers to the evident inhibition of *S. aureus* and other pathogens.

The antimicrobial properties of bacteriocin isolated in our research, in combination with their sensitivity to the digestive proteases as trypsin, as well as their stability at different temperatures and over a wide range of pH values, converts them into prospective agents destined to be applied to the bio-conservation of food.

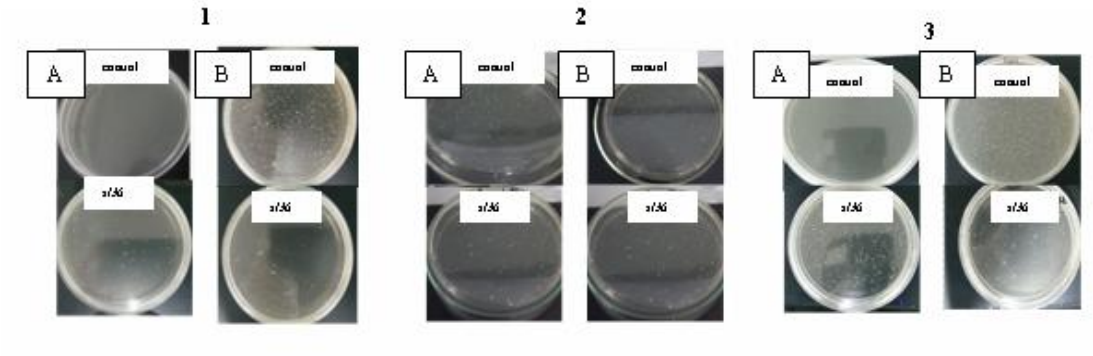

FIGURE 3. PERISHABLES FOOD TREATED WITH CFS OF *LACTOBACILUS FERMENTUM* (s136).

1. **Semi-hard cheese, 1A. inoculated with *E. faecalis*, 1B inoculated with *S. aureus.***
2. **Cream, 2A inoculated with *E. faecalis*. 2B inoculated with *S.aureus*.**
3. **Coocked park shoulder, 3A inoculated with *E. faecalis*. 3B inoculated with *S.aureus*.**

IV. CONCLUSION

The strain of *Lactobacillus fermentum sl36* isolated in this region from raw goat milk showed antimicrobial activity that remain stable at different pH values and at different heat treatment. Our work demonstrates that it is possible to increase the safety of perishable foods by utilization of bacteriocins produced by LAB strains, unlike the using the bacteria as probiotics which is most frequent practice. The development of biotechnological alternatives that use bacteriocins, such as smart packaging and aerosols for application on surfaces, would improve the conditions of storage and preservation of food by controlling microorganisms. The discovery of new bacteriocins represents an opportunity in the production of substances with preservative qualities.

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