Antimicrobial Activity of Lactococcus and Lactobacillus Species Isolated from Raw Goat Milk. San Luis Argentina

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Abstract— *The goat milk and their products are used to human consumption with health benefits. The lactic acid bacteria (LAB) have ability to produce bacteriocins that inhibit the growth of spoilage microorganisms or pathogens present in food. In this study, bacteriocins-producing strains were isolated from goat milk samples. The isolates were able to inhibit the growth of Listeria monocytogenes ATCC 74902, Enterococcus faecalis ATCC 29212, Escherichia coli and/or Pseudomonas aeruginosa strains with an average inhibition halo between 8 and 12 mm. The strains were identified as Lactococcus lactis ssp lactis 2 (gmSL3, gmSL9, gmSL11, gmSL12, gmSL15 and gmSL40), Lactobacillus fermentum (gmSL28), Lactobacillus paracasei ssp paracasei 1 (gmSL5, gmSL20 and gmSL23). The antimicrobial substances showed high stability at temperatures and/or pH extremes. The tested strains inhibit the growth of more than one pathogen, of both bacteria Gram (+) and Gram (-). By RT-PCR, one band of 136 bp was found, whose size corresponds to Nisin A.*

Keywords— antimicrobial peptides, bacteriocins, Lactic Acid Bacteria (LAB), raw goat milk.

I. INTRODUCTION

Goat milk and cheese are foods that offer certain advantages over the cow milk and derivatives; the higher content of short chain fatty acids and small globules of fat promotes the digestion. In addition the goat milk contains more calcium, phosphorous and potassium as that human milk and cow milk. Furthermore, the high content of retinol and alpha-tocoferol, the goat's milk can replace milk cow in people with allergy to the protein present in the last one [1- 3]. Goat's milk and its derivatives are usually present in the diet of the population in different regions of the Argentine Republic. In the State of San Luis several dairy farms distribute their products to regions away from the production site, they should be consumed in a short period of time to avoid the loss of its nutritional values, so it is necessary to find new methods of conservation to prolong the useful life of these foods.

It has also been observed contamination of raw milk and the process of maturation of the cheese with spoilage microorganisms or pathogens such as *Listeria monocytogenes*, *E.coli* and other members of the family *Enterobacteriaceae* [4, 5]. These microorganisms can cause changes in the taste of food or diseases that affect the health of the consumer.

It is important to have safe methods for the preservation of food to prevent food poisoning that can cause damage to the health of the population. In the last ten years studies in this field have multiplied, aimed at reducing the use of chemical preservatives and at developing new conservation techniques employing physical methods and/or use of natural antimicrobials synthesized by bacteria present in various foods such as milk, fermented products and processed foods. [4-12]

Lactic Acid Bacteria have been used as fermentation starters in food and beverage industries because they contribute to taste and flavor development, and to prevent food spoilage. LAB's protective role is attributed to their capacity to decrease pH, and to synthesize bacteriostatic and bactericidal substances. These substances include hydrogen peroxide, lactic acid, carbon dioxide and bacteriocins, or other similar compounds. LAB have been isolated from different sources such as meat, meat products, cow dairy products, fish, etc. Recently, many research studies are aimed at finding new strains of bacteriocin-producing LAB that could be used in foods preservation processes. Most studies on this topic have used cow milk and its derivatives as a LAB source; however, goat milk has been less studied. Studies by Guesas and Kihal [9] on LAB isolates of raw goat milk indicate that the predominant bacteria are: *Lactococcus sp*, *Streptococcus thermophilus*, *Leuconostoc sp.* and *Lactobacilli*. Eva Rodriguez et al [13], who isolated LAB from raw milk of ewe goats and cows, published similar results. Other authors have also isolated LAB from goat's raw milk and cheese, some of which are bacteriocin producers, named: *Lactococcus lactis*, *Lactobacillus curvatus*, *Lactobacillus paracasei subsp. paracasei*, *Enterococcus faecalis* and

Leuconostoc mesenteroides ssp [4, 5, 7, 8]. Caridi A. reported that two strains of *Lactobacillus paracasei subsp. paracasei* isolated from goat cheese showed an inhibitory capacity against strains of *Escherichia coli* [14]. Cocolin, L. et al. found that two bacteriocin-producing strains of *Enterococcus faecium* isolated from raw goat milk were able to inhibit the growth of *Listeria monocytogenes* and *Clostridium butyricum* [8]. Therefore, the aim of our study has been to isolate bacteriocin-producing LAB from goat milk samples and determine their activity spectrum against foodborne pathogens and determinate the expression of bacteriocins by these strains.

II. MATERIALS AND METHODS

2.1 Samples and Isolation of Lactic Acid Bacteria

The samples of goat milk were collected from stainless steel drums at a local dairy (San Luis, Argentina).The samples taken from the top and bottom of the dairy drum in triplicate. Selective isolation of LAB performed using a MRS (Britania) medium. Isolation of bacteria was carried out from appropriate 10-fold dilutions by incubation on MRS agar, at 37°C for 48 h in microaerophilic conditions. To obtain pure cultures, each isolate was grown in MRS agar plates. The strain was called gmSL (goat milk from San Luis).

2.2 Identification and characterization of LAB strains

The test kit API 50 CHL method used by Melinda A. Boyd [15], it consists of 50 standardized biochemical tests and a database was used for biochemical identification.

To achieve genetic level identification of the different strains previously characterization by the API 50 CHL, the intergenic regions of the fractions 16S-23S rRNA using specific primers were studied, according Atul Kumar Sing and Aiyagari Ramesh method [16] modified.

2.3 Determination of antimicrobial activity

The bacteriocin activity of the isolated strains were determined by the well diffusion method [17] using *Listeria monocytogenes ATCC* 74902 (bought at Pasteur Institute in Paris), *Enterococcus faecalis ATCC 29212*, *Escherichia coli sp* and *Pseudomonas aeruginosa sp* (strain collection from Microbiology Laboratory of San Luis National University) as indicator strains. All bacteria used as indicator organisms in sensitivity tests grew in Trypticase Soy Agar (Britania) at 37°C for 24h. LAB strains were grown in MRS Broth (Britania) at pH 7.0 for 48 h at 37°C in microaerophilic conditions. Cell-free supernatant (SNlc) were obtained by centrifugation the cultures at 10,000 *xg* during 15 min at 4°C, and adjusting pH to 7.0 with 1 mol l^{-1} NaOH. Each supernatant was filtered through a 0.2 µm pore-size cellulose acetate membrane, and the remaining activity was determined by the well diffusion method. Plates were incubated in aerobic conditions at 37°C, and finally, antimicrobial activity was tested for the presence of clear zones around the wells. Halos were considered positive at above 6 mm.

The quantification of the antimicrobial activity in the liquid medium was carried out by the method described by Cabo M.L. et al [18], 1 ml of suspension of pathogens (6×10^8 UFC.ml^{-1}) was added 0.5 ml of supernatant and 0.5 ml of medium TSC. The mix was incubated for 6 h a 37°C and was read the absorbance at 700nm. The inhibition percentage (% I) was calculated according to the formula I= 1-Am/Ao. Taking Am and Ao as the absorbance sample and control respectively. In control assay, the supernatant is replacement by MRS broth.

2.4 Sensitivity of antimicrobial compounds to enzymes, pH and temperature

Isolated strains were grown in MRS Broth for 48 h at 35ºC, cells were harvested by centrifugation at 10,000 *x g*, for 15 min, at 4ºC. Each supernatant was filtered through a 0.2 µm cellulose acetate membrane. Cell-free supernatants were used in the following tests: i) Proteases action was tested by incubation for 2 h at 37º C in the presence of 1 mg.ml^{-1} trypsin, chymotrypsin and pepsin (Sigma, USA) under different pH conditions, trypsin at pH 7,2 chymotrypsin, at pH 8, and pepsin, at pH 2.0. Antimicrobial activity of supernatants later of treated with proteases and neutralized, was determined using the well diffusion method described above. ii) The effect of pH on antimicrobial activity was tested by adjusting cell-free supernatants from pH 3.0 to 11.0, at increments of two pH units with sterile 1 N HCl or 1 N NaOH during 2 h at 37ºC. Neutralization was performed at pH 6.5 and activity tests were finally carried out. iii) The effect of temperature on antimicrobial activity was tested by incubating cell-free supernatants (adjusted to pH 6.5) at -20ºC for 30 and 60 min, and at 100ºC, for 15 and 60 min. The temperatures were selected for use in food preservation and pasteurization processes. Residual antimicrobial activity was evaluated using the agar well diffusion method in all the cases.

2.5 Relationship between antimicrobial activity and the growth of LAB strains in study stage

The growth curve was based on a standard curve built with the dry weights of culture broths after 48 h incubation. Changes in optical density readings (at 735 nm) were taken every hour for 24 h. Each time, cell-free supernatants were obtained to determine the antimicrobial activity against *Enterococcus faecalis* as indicator strain, using the well diffusion technique describe above.

2.6 Identification of bacteriocins genes

This study was conducted with strains that showed inhibitory activity against both Gram (+) and Gram (-) bacteria. Genes encoding for the bacteriocins were targeted by PCR using the specific primers for Nisin A, Lactococin A and Lacticin 481. RNA was isolated, cDNA was obtained, and genes were amplified. Finally, a gel electrophoresis was run, and characteristic bands were identified.

2.6.1 RNA isolation and Reverse Transcriptase (RT) reaction

Total RNA was extracted from a bacteria pellet obtained by centrifugation of 3 ml of a 48-h culture in MRS medium. Cells were lysated using 1 ml of Trizol reagent and then centrifuged at 12.000 *x g*, at 4ºC, for 10 min. The precipitate was discarded and the supernatant was used for RNA isolation. This was performed by adding 200 µl of chloroform followed by vigorous shaking for 15 seconds in a capped tube. The extract was then incubated at room temperature for 3 min and centrifuged at 12,000 *x g* at 4°C during 15 min. Following that, RNA precipitation was performed by adding 450 □l of isopropanol to 450 µl of the upper phase from the previous centrifugation step and by incubation at room temperature for 15 min. The extract was then centrifuged at 12,000 *x g* at 4°C during 10 min. Supernatant was discarded and RNA pellet was washed with 1 ml of 75% ethanol and centrifuged at 7,500 *x g* at 4ºC for 5 min. The washed RNA pellet was then left to dry for 3-5 minutes, avoiding complete drying. Finally, total RNA was dissolved in 100 µl of nuclease-free water (Biodynamics SRL) and incubated at 60°C for 10 min to improve dilution. The yield and purity of total RNA were determined spectrophotometrically at 260 and 280 nm. Gel electrophoresis and Gel-Red staining (0.01%) confirmed the integrity of RNA. Three micrograms of total RNA were reverse-transcribed with 200 units of MMLV RT (Promega Inc.) using random primer hexamers in a 25 µl reaction mixture, following the manufacturer's instructions.

2.6.2 PCR amplification

The presence of Nisin A, Lacticin 481, Lactococcin A bacteriocins transcripts was determined by PCR using specific primers. Bacteriocin fragments were amplified by PCR in 50 ul of reaction solution containing 0.2 mM dNTPs, 1.5 mM $MgCl_2$, 1.25 U of Taq polymerase, 50 pmol of each specific oligonucleotide primer, and RT-generated cDNA (1/5 of RT reaction). The sequences of specific primers, as well as fragment sizes are shown in Table 1. Samples were heated in a thermalcycler (My Cycler, T1 BioRad) at 94ºC for 2 min, followed by 40 cycles of: (1) denaturation, at 94ºC for 30 s; (2) annealing, at 59ºC during 1 min; (3) extension, at 72ºC for 1 min. After 40 reaction cycles, the extension reaction continued for another 5 min. PCR products were later electrophoresed on 2% (w/v) agarose gel with 0.01% (w/v) of Gel Red dye. The amplified fragments were visualized under ultraviolet transillumination and photographed using a Cannon PowerShot A75 3.2MP digital camera.

TABLE 1
PRIMER PAIRS USED FOR RT-PCR

Gene name	GenBank accession	Forward primer 5´–3´	Reverse primer 5´–3´	Fragment size
Nisin A	AF465351	5'-GATTTTAACTTGGATTTGGTATCTGTTTC-3'	5'-GACAAGTTGCTGTTTTCATGTTACAA-3'	136 bp;
Lactococin A	M90969	5'-TCAATTAAATTTTAATATTGTT-3'	5'-CTCAATGGTGCAACCCGAAACC-3'	220 bp
Lacticin 481	X71410	5'-TCTGCACTCACTTCATTAGTTA-3'	5'-AAGGTAATTACACCTCTTTTAT-3'	366 bp

III. RESULTS AND DISCUSSION

3.1 Isolation, identification and determination of the antimicrobial activity of LAB strains

The colony count of an average of 11 milk samples observed in the plates was 8-16 x $CFU.10^3$ $.ml^{-1}$. We observed the presence of cocci, coccobacilli and bacilli Gram (+), catalase (-) and oxidase (-). All trials were conducted in triplicate. Forty-six (46) isolates were tested for antimicrobial activity, twenty-one (21) of which presented antibacterial activity against *Listeria monocytogenes* 74902 and/or *Enterococcus faecalis*, *Escherichia coli sp* and *Pseudomonas aeruginosa sp* (average inhibition halo, 6.0 to 12 mm). Subsequent research were selected the isolates that showed inhibition zones between 8 and 12

mm. This strains were identified as *Lactococcus lactis ssp lactis 2 (gmSL3, gmSL9, gmSL11, gmSL12, gmSL15 and gmSL40), Lactobacillus fermentum (gmSL28), Lactobacillus paracasei ssp paracasei 1 (gmSL5, gmSL20 and gmSL23)*. (Table 2 and Fig 1).

TABLE 2
INHIBITION OF VARIOUS INDICATOR ORGANISMS BY LAB STRAINS ISOLATES

Strain gmSL Nº	Organism	L monocytogenes *74902*	*E. faecalis*	*E. coli*	*P. aeruginosa*
3	*L. lactis ssp lactis 2*	8,5	12	8	-
5	*L. paracasei ssp paracasei 1*	7	8	-	-
9	*L. lactis ssp lactis 2*	-	10	9,5	-
11	*L. lactis ssp lactis 2*	-	8	9,5	-
12	*L. lactis ssp lactis 2*	9,5	7	8,5	-
15	*L. lactis ssp lactis 2*	-	10	-	-
20	*L. paracasei ssp paracasei 1*	8	8	7	-
23	*L. paracasei ssp paracasei 1*	8	7	9	7
28	*L. fermentum*	8	9	8	-
40	*L. lactis ssp lactis 2*	8	8	8	9

The results are expressed in mm of inhibition halo, average of 3 trials.

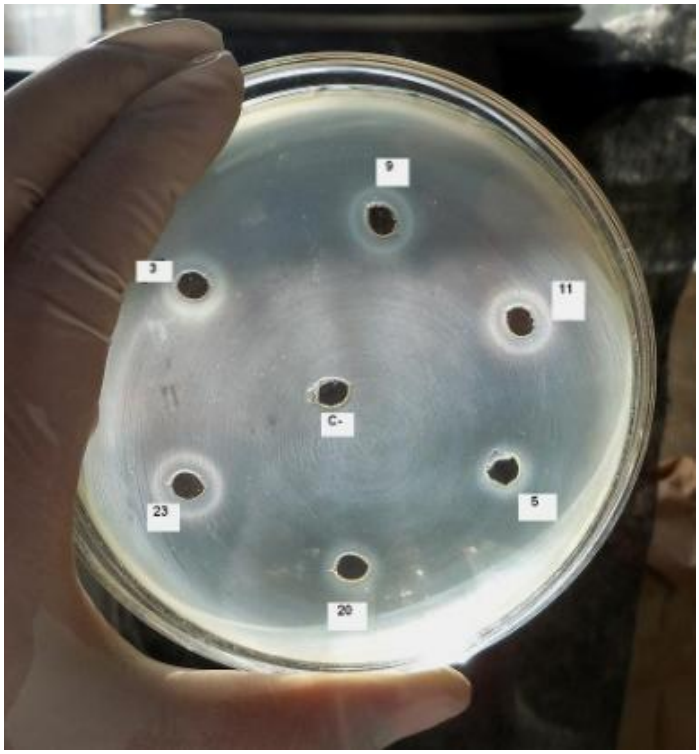

FIGURE 1: ANTIBACTERIAL ACTIVITY. INHIBITION HALOS, gmSL3, gmSL5, gmSL9, gmSL11, gmSL20 and gmSL23 STRAINS

3.2 Determination of peptide nature of the antimicrobial substances

Cell-free supernatants treated with pepsin (pH 2.0), trypsin (pH 7,2) and chymotrypsin (pH 8) showed no antimicrobial activity against *L. monocytogenes* and *E. faecalis.* Absence of antimicrobial activity after treatment revealed the protein nature of the antimicrobial substance.

3.3 Stability of bacteriocins at different pH and temperatures.

Supernatants were tested at different pH values against indicator organisms *L. monocytogenes* or *E. faecalis*. At pH 3, all strains showed a higher or equal antimicrobial activity than that presented at pH 7 against one of the two pathogens under study. Strains *gmSL11* and *gmSL5* at pH 3 showed the highest antibacterial activity against *E. faecalis*. Strains *gmSL3* and *gmSL5* showed antimicrobial activity against *L. monocytogenes* at pH 8 and 10, and were the only strains capable of producing antimicrobial peptides which remained stable at alkaline pH.

When subjected to thermal treatments, supernatants were tested at different temperatures against *L. monocytogenes* or *E. faecalis.* The extracellular extracts of strains *gmSL9, gmSL20, gmSL28 and gmSL 40*, all showed stability at pH 3 and at temperatures of -20° C and 100° C. Strains *gmSL9* and *gmSL20* are able to synthesize antimicrobial substances stable at extreme temperatures (-20°C and 100°C) for each period of time. (Table 3).

TABLE 3
BACTERIOCINS STABILITY AT EXTREME TEMPERATURE

Indicator organisms	Strains	-20°C		100°C		SN untreated
		30 min	60 min	15 min	60 min	
L. monocytogenes	gmSL3	-	-	+	+	+
	gmSL4	-	-	+	+	+
	gmSL5	-	-	+	-	+
	gmSL20	+	+	+	+	+
	gmSL23	-	-	+	-	+
Enterococcus faecalis	gmSL9	+	+	+	+	+
	gmSL11	-	-	+	+	+
	gmSL12	-	-	+	+	+
	gmSL28	+	-	+	-	+
	gmSL40	+	-	+	-	+

Only strains indicate that retained antimicrobial activity against indicator organisms.
(+) retained, (-) unretained, SN: untreated supernatant.

3.4 Relationship between antimicrobial activity and the growth of LAB strains in study stage

The *LAB* strains that showed the highest activity against Gram-positive and Gram-negative bacteria were tested: *gmSL3, gmSL12 and gmSL40*. Antimicrobial activity was observed after 8 h of incubation in all strains and the highest antimicrobial activity was determined at 16h, 14h and 16h of incubation respectively. The growth curves showed that the stationary phase begins around 14 h of incubation (Fig.2). The highest bacteriocins activity was achieved at the beginning of the stationary phase (8 and 9 mm inhibition halos). This indicates that the bacteriocins are primary metabolites. Recent studies have shown that increased antimicrobial activity occurs primarily between the end of the log phase and the beginning of the stationary phase [19].

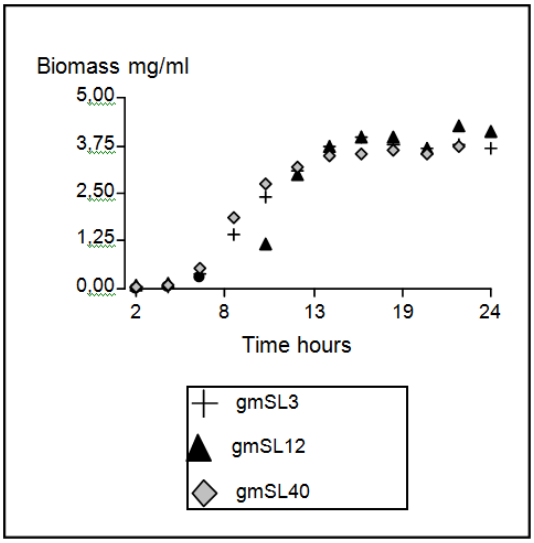

FIGURE 2: GROWTH CURVE OF gmSL3, gmSL12 and gmSL40 STRAINS

3.5 Quantification of inhibitory activity

gmSL3 strain showing inhibitory activity against at least three of the four strains indicators, was selected for trials of quantification of inhibitory activity in liquid medium. The strain caused an 80% inhibition of growth of *E. faecalis* and *L. monocytogenes* while the inhibition was 58% against E. coli.

3.6 Bacteriocin gene expression LAB strains showing antimicrobial activity against Gram (+) and Gram (-) microorganisms

Considering the results derived from inhibition spectrum of these strains and stability of antimicrobial peptides present in the supernatants, messenger RNA expression was investigated on five isolated LAB strains *(gmSL3, gmSL4, gmSL5, gmSL20, and gmSL23).* By using molecular biology techniques and specific primers for known bacteriocin gene sequences, we investigated the presence of Nisin A, Lacticin 481 and Lactococin A transcripts in the isolated LAB strains. We found a 136 bp band that matches the expected fragment size for Nisin A only in strain *gmSL3* (Fig 3). Lacticin 481 and Lactococcin A transcripts were not found in any of the studied strains (results are not shown).

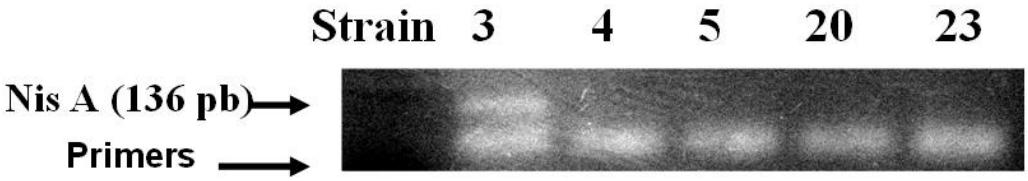

FIGURE 3: RESULTS OF AGAROSE GEL ELECTROPHORESIS OF cDNA

IV. CONCLUSION

According to the results presented above, we can conclude that raw goat milk obtained from a dairy located in the Province of San Luis (Argentina) has bacteriocin-producing LAB strains in its microbiota, which can inhibit the growth of food pathogens and spoiling organisms, *L. monocytogenes* and/or *E. faecalis* and/or *E. coli* and/or *P. aeruginosa*. The fact that the first two pathogens are Gram(+) strains, and that the latter are Gram(-) broadens the action spectrum of these bacteriocins. Some strains can inhibit more than one pathogen, which makes them interesting to focus in future studies. Todorov S.D and Dicks isolated lactobacillus bacteriocin-producer, stable a pH values between 2.0 and 10,0 and at 100ºC active against Gram-negative bacteria [20]. Our results are similar in most of the bacteriocins produced by the isolated, activity at low pH and temperature extremes, some of them are active at alkaline pH, allowing its use in different environmental conditions. These results lead us to continue our studies in search of new LAB strains with broad-spectrum inhibitory against disruptive or pathogenic microorganisms in food. The tests performed at the molecular level showed a band of bp 136 that matches the pattern of band of nisin in one of the five strains studied. Therefore, these strains can be useful to produce this particular bacteriocin, which is the only one authorized for the Food and Agriculture Organization of the United Nations (FAO) to add to food [21]. Given the interest that exists in the use of these peptides in food preservation or to control pathogens human, it would be interesting to purify and identify these and other bacteriocins in future studies.

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