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Bacteriocin Producing Lactobacilli Strains as a Biological Strategy to Control Listerial Growth

Research Article

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Abstract

Listeria monocytogenes causes listeriosis in humans and animals, and is predominantly transmitted by ingestion of contaminated food. Its severity and high mortality rate make listeriosis a relevant foodborne disease. Currently, biological strategies using probiotic lactobacilli to prevent and control infectious diseases are being globally investigated. The biocontrol exerted by certain *Lactobacillus* spp. is mainly due to the production of a variety of antimicrobial substances. The aims of this work were (1) to investigate the presence of *Listeria* spp. in raw cow milk and (2) to determine the inhibitory activity of bacteriocin-like inhibitory substances (BLIS-es) produced by *Lactobacillus fermentum* L23 and *Lactobacillus rhamnosus* L60 on *L. monocytogenes* and other listerial species isolated from food and clinical samples. The presence of *Listeria* spp. in raw milk was evaluated in 814 samples from 238 cows. The listerial prevalence in cattle was 0.84% and the isolates were identified as *L. innocua* (LI1) and *L. welshimeri* (LW1). A total of 29 listerial strains were used as indicator microorganisms to evaluate the antimicrobial activity of BLIS-es L23 and L60. Bioactive metabolites produced by these lactobacilli strains were able to inhibit the listerial growth. This biological activity was mainly attributed to the BLIS-es L23 and L60 which, even at low concentration, were active on 100% of listerial strains. This study reveals a strong potential for the biotechnological use of these bacteriocin producing lactobacilli as a biostrategy against *Listeria* spp.

Keywords: Antilisterial Activity; Bacteriocins; *Lactobacillus* spp.; *Listeria monocytogenes*; Raw Cow Milk.

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Introduction

Listeria monocytogenes causes listeriosis in humans and animals, and is predominantly transmitted by ingestion of contaminated food. This microorganism can also be transmitted transplacentally from mother to child, during passage through the colonized birth canal, or after direct contact with infected animals. The major groups at risk of invasive listeriosis include pregnant women, neonates, elderly people and immunocompromised individuals. Clinical manifestations such as neonatal infections, abortions, bacteremia, meningitis and rhombencephalitis have been the most severe infections [1, 2]. Listeriosis is one of the most relevant foodborne diseases with an elevated socio-economical impact due to severity of invasive infections associated with a high mortality rate [3]. *Listeria* spp. are widely distributed in the environment and different foods, including dairy products. Recently, several outbreaks of listeriosis associated to consumption of dairy products have been reported worldwide [4]. *L. monocytogenes* represents an important concern for food safety due to its ability to resist extreme conditions (low pH, high salt concentrations and refrigeration temperatures) which are commonly used as preservation procedures and thus may survive in food for long periods of time [2, 5]. Other listerial species, such as *L. ivanovii*, *L. seeligeri*, *L. welshimeri* and *L. innocua*, have been reported to cause infections in humans. Some of these species have been associated with bacteremia, acute meningitis, coagulation disorders, and multiple-organ dysfunction, leading to the death of the patients [6].

Although listeriosis is a notifiable disease in many developed countries, there has not been a reduction in its incidence [7, 8]. During the last years, the rate of *L. monocytogenes* infection has varied between 0.29 and 1.8 cases per 100,000 inhabitants [9, 10]. For this reason, other strategies of sanitary control against *L. monocytogenes* are being investigated. The application of lactobacilli exhibits a promising approach for the control of pathogenic microorganisms in both, the food industry as well as the biomedical field. The biocontrol exerted by *Lactobacillus* spp. is mainly due to the production of antimicrobial substances such as organic acids, hydrogen peroxide and bacteriocins [5, 11-13]. Bacteriocins are defined as proteinaceous antimicrobial substances, produced by bacteria that inhibit growth of related or unrelated bacterial spe-

cies. Bacteriocin-like inhibitory substance (BLIS) is an acronym used to refer to those bacteriocins whose amino-acid sequences have not yet been elucidated [13]. Our research group has identified and characterized two lactobacilli strains, *Lactobacillus fermentum* L23 and *Lactobacillus rhamnosus* L60, which were selected for their bacteriocinogenic and probiotic properties. Furthermore, extensive investigations have demonstrated the ability of these strains to inhibit the growth of several pathogenic microorganisms[13-18]. Currently, researchers have become increasingly interested in the search for natural antimicrobial substances, such as bacteriocins, to develop biotechnological products for food industry applications as alternative to reduce chemical and thermal preservation methods [11, 19].

The aims of this work were (1) to investigate the presence of *Listeria* spp. in raw cow milk and (2) to determine the inhibitory activity of BLIS-es produced by *Lactobacillus fermentum* L23 and *Lactobacillus rhamnosus* L60 on *L. monocytogenes* and other listerial species isolated from food and clinical samples.

Materials and Methods

Isolation and identification of *Listeria* spp.

Raw milk samples were collected between August 2012 and April 2014 from Villa María, Córdoba. 814 samples from different quarters of the mammary gland of 238 cows were analyzed for the presence of *Listeria* spp.

The isolation of *Listeria* spp. from milk samples was performed by means of the double enrichment method followed by a plate isolation procedure. The first selective enrichment of *Listeria* spp. was done in tryptic soy broth (TSB) (Britania, Argentina) added with ceftazidime (0.002% w/v) and trypaflavine neutral (0.25% w/v). Cultures were incubated for 48 h at 37°C. The enrichment broths were kept at 4°C for 48 h as a second selective enrichment. Each culture broth was seeded on Oxford agar plates (Britania, Argentina) and incubated at 37°C for 24-48 h. Typical colonies were identified by biochemical tests [20]. Other *L. monocytogenes* strains previously isolated from food and clinical samples were provided by the Bacteriology laboratory of Universidad Nacional de Río Cuarto, Argentina. They were seeded on tryptic soy agar (TSA) plates (Britania, Argentina) and incubated at 37°C for 24 h. Strains were stored at -80°C in TSB containing 30% (v/v) glycerol. All *Listeria* spp. from different origins were used as indicator microorganisms to evaluate the antimicrobial activity of the bioactive substances produced by *L. fermentum* L23 and *L. rhamnosus* L60.

Lactobacilli and culture media condition

Two human *Lactobacillus* strains, *L. fermentum* L23 and *L. rhamnosus* L60, were previously identified by standard biochemical tests, the API 50 CHL system (BioM`erieux, Inc., France) and 16S rRNA analysis. The 16S rRNA sequences of both lactobacilli were deposited in the GenBank under the accession numbers GQ 455406 and EF 495247 to *L. fermentum* L23 and *L. rhamnosus* L60, respectively [18]. Lactobacilli strains were grown in De Man Rogosa Sharpe (MRS) broth or agar (Britania, Argentina) at 37°C under a 5% CO_2 atmosphere for 24 h. They were stored at -80°C in MRS broth containing 30% (v/v) glycerol.

Both *Lactobacillus* strains produce different antimicrobial compounds such as organic acids, bacteriocins and, in the case of *L. rhamnosus* L60, also hydrogen peroxide. The BLIS-es L23 and L60 were previously purified by at least three-step procedure developed for class II bacteriocins [14-16].

Test of antimicrobial activity

The antilisterial activity of *L. fermentum* L23 and *L. rhamnosus* L60 was tested by the streak diffusion method described by Asurmendi *et al.* [12]. On the other hand, the inhibitory activity of cell free supernatant (CFS) and treated or neutralized CFS (NCFS), containing the BLIS-es L23 or L60, was evaluated against *Listeria* spp. strains by well diffusion test, on agar plates [18]. *L. fermentum* L23 and *L. rhamnosus* L60 were cultured in MRS broth and incubated at 37°C under a 5% CO_2 atmosphere for 20h. Then, the supernatants were removed by centrifugation (4,000xg at 4°C for 20 min). These fractions with biological activity were neutralized with NaOH 1 mol ml^{-1} to eliminate the inhibitory effects of the organic acids. TSA plates were seeded with a *Listeria* spp. culture (1.5×10^8 CFU ml^{-1}) and wells were made into agar plates. 100 µl of CFS and NCFS from each *Lactobacillus* strain were added to different wells. Plates were incubated for 24 h at 37°C and inhibition halos were measured.

Minimum inhibitory concentration of the bacteriocin like inhibitory substances on the listerial growth

The minimum inhibitory concentration (MIC) values of each BLIS, L23 and L60, were evaluated using a modification of the well diffusion test on agar plates [16]. To obtain the BLIS L23, the CFS of *L. fermentum* L23 was neutralized with NaOH 1 mol ml^{-1}. In the case of BLIS L60, the NCFS of *L. rhamnosus* L60 was also treated with 0.1 mg ml^{-1} peroxidase (Sigma) to eliminate the inhibitory effects attributed to the hydrogen peroxide. A suspension of *Listeria* spp. in TSB broth (1.5×10^8 CFU ml^{-1}) was seeded on TSA plates. 100 µl of two fold serial dilutions of each BLIS were spotted on different agar wells and plates were incubated. MIC of both BLIS-es was defined as the reciprocal of the highest dilution which produced complete inhibition of the indicator growth, and was expressed as activity units per milliliter (AU ml^{-1}) [21].

Statistical Analysis

All tests were performed in triplicate, and mean ± SD were expressed. Differences in inhibitory activities between bacteriocin producing strains and their different bioactive supernatants were analyzed by ANOVA ($P<0.05$) using InfoStat Software. A P value of <0.05 was considered statistically significant.

Results and Discussion

In the present study, a total of 814 raw milk samples from 238 cows were examined for the presence of *Listeria* spp. Two samples, obtained from different cows, were positive for *Listeria* spp. The listerial prevalence percentage in cattle was 0.84%. According to the biochemical identification, the isolates were identified as *L. innocua* (LI1) and *L. welshimeri* (LW1). Our results agreed with a previous report by Aygun and Pehlivanlar [22], who found a low prevalence value in raw cow milk. In contrast, a significantly higher listerial prevalence has been reported in the US (23%) and Iran (22.5%) during the last years [4, 23]. The listerial species isolated in this work coincided with recent works reported by Jamali *et al.* [4] and Rahimi *et al.* [24] who recovered *L. innocua* and *L. welshimeri*

among other listerial species.

Bacteria that belong to *Listeria* genus are indirect indicators of the potential presence of *L. monocytogenes* in food because all listerial species occur in similar environmental niches [25]. Since *L. innocua* and *L. welshimeri* have been reported to cause infections in humans, the presence of these *Listeria* spp. found in this work, would consequently imply that the consumption of raw or improperly pasteurized milk could represent a potential risk for human health. Moreover, the main causes of contamination for these bacteria in raw milk are associated with fecal and environmental sources. Such contaminations could occur during milking, storage or transport, or directly from infected animals in dairy farms [26]. Since the listerial species isolated in the present study are non-pathogenic for cows, we suggest that the presence of these microorganisms could be associated to environmental contaminations during milking practices, which are related with a poor hygienic quality of milk. In Argentina, the information about the prevalence of *L. monocytogenes* and other *Listeria* spp. in milk, is still extremely limited, bearing in mind, the detection of these microorganisms in milk is not regulated by the Código Alimentario Argentino [27]. To our knowledge, the only previous work carried out in our country to determine the presence of *Listeria* spp. in raw milk was reported by Laciar *et al.* [28]. These authors isolated *L. monocytogenes*, *L. innocua* and *L. welshimeri*. Considering that Argentina is an important producer of milk worldwide, the detection of listerial species, such as the one carried out in this work, shows that it is essential to search for these bacteria in milk in order to ensure its safety.

In this work, we report the *in vitro* antimicrobial activity of two probiotic and bacteriocinogenic strains, *L. fermentum* L23 and *L. rhamnosus* L60, on a total of 29 listerial strains (27 strains of *L. monocytogenes* isolated from food and clinical samples, and *L. innocua* LI1 and *L. welshimeri* LW1 recovered from raw cow milk). The streak diffusion method performed served as a preliminary technique for screening of listerial susceptibility to antimicrobial substances produced by these lactobacilli strains. The results showed that lactobacilli inhibited 100% of the tested *Listeria* spp. (data not shown). The inhibition zone produced by *L. fermentum* L23 showed a mean value of 20.64 ± 4.92 mm, whereas that of *L. rhamnosus* L60 was of 19.67 ± 4.94 mm. There was no statistical difference between these inhibition values ($P<0.05$). These results showed that both *Lactobacillus* strains had a strong biological activity to inhibit the listerial growth. Previous studies have demonstrated the antimicrobial power of different lactobacilli strains against certain species of *Listeria* spp. [29]. Nevertheless, in comparison with our work, *L. fermentum* L23 and *L. rhamnosus* L60 proved to have a wider range of antilisterial activity because they inhibited all *Listeria* spp. tested, independently from where they were originally isolated.

The antilisterial activity of the CFS and NCFS containing the BLIS-es, L23 or L60, from *L. fermentum* L23 or *L. rhamnosus* L60, respectively, was tested by the well diffusion method. The mean inhibition halos obtained on each listerial strain are shown in table 1. Results showed that both CFS containing all the substances with antimicrobial activity inhibited all the tested *Listeria* spp. The mean inhibition halos produced by the CFS of *L. fermentum* L23 and *L. rhamnosus* L60 on *Listeria* spp. were 21.10 ± 1.94 and 21.08 ± 2.35, respectively. There was no statistical difference between these inhibition values ($P<0.05$). After treatment with NaOH 1 mol ml^{-1}, both NCFS maintained a high antimicrobial activity on listerial growth. The mean inhibition halos produced by the NCFS of *L. fermentum* L23 and *L. rhamnosus* L60 on listerial growth were 17.90 ± 1.74 mm and 17.73 ± 1.93 mm, respectively. Antimicrobial activities found with each NCFS against *Listeria* spp. growth did not show significant differences ($P<0.05$). The higher percentage of antilisterial activity remained in the NCFSs of both lactobacilli strains. In the case of *L. fermentum* L23 this biological activity was due to the BLIS L23 while for *L. rhamnosus* L60, the NCFS contains the BLIS L60 as well as hydrogen peroxide. Our research group have previously demonstrated that hydrogen peroxide produced by *L. rhamnosus* L60 had a weak antimicrobial activity on a wide variety of bacterial genera [13, 15, 21]. On this regards, it was assumed that the antimicrobial effect of both NCFSs was due to the BLIS-es L23 and L60, which were the metabolites responsible of the main antilisterial effect. Indeed, both bacteriocins inhibited the growth of 100% of *Listeria* spp. Only in the case of *L. monocytogenes*, similar results were found by Altuntas *et al.* [11], who demonstrated the susceptibility of those strains to the non-treated CFS containing a bacteriocin produced by *Pediococcus* spp. In a similar study, Vera Pingitore *et al.* [30] studied the antimicrobial activity of two NCFS containing different bacteriocins and found different levels of resistance among *L. monocytogenes*, *L. innocua*, *L. welshimeri* and *L. seeligeri* strains. Furthermore, Dortu *et al.* [31] reported a high sakacin G resistance level (40%) for *L. monocytogenes* strains. At this point, our findings demonstrate the relevant antilisterial activity of these two bacteriocins based on both, the large number of susceptible strains and the high sizes of inhibition halos produced by them.

The proportion of antimicrobial effect of the main biometabolites was estimated based on the sizes of inhibition halos produced by CFS and NCFS of each *Lactobacillus* strain. The average sizes of inhibition zones produced by CFS and NCFS of *L. fermentum* L23 strain were significantly different ($P<0.05$), due to the joint action of organic acid and the BLIS L23, in comparison with the inhibition found by the BLIS alone. Thus, the proportion of biological activity on *Listeria* spp. produced by BLIS L23 and organic acids were 85% and 15%, respectively. The same differences were observed between CFS and NCFS of *L. rhamnosus* L60. In this case, 84% of inhibition was mainly attributed to BLIS L60 and 16% to organic acids.

Table 2 shows the MIC values of BLIS-es L23 or L60 on all *Listeria* spp. MICs of both BLIS-es ranged between 40 and 160 AU ml^{-1} for all strains tested of *Listeria* spp. The BLIS L23 inhibited the growth of 19 strains of *L. monocytogenes* (70.37%) with a very low MIC value. In the case of the BLIS L60, 23 strains of *L. monocytogenes* (85.18%) were inhibited with MIC values ranged between 40-80 AU ml^{-1}. On the other hand, both BLIS-es showed the same MIC value to inhibit the growth of *L. innocua* LI1 and *L. welshimeri* LW1. The low MIC values for each BLIS, in comparison with their maximum activity (640 AU ml^{-1}), showed that even when they are highly diluted, they remained active against all *Listeria* spp. tested. These results differed from those by Kaur *et al.* [32], who evaluated pediocin 34 and enterocin FH99, whose MIC values were higher in comparison with the reported here for L23 and L60. These findings could serve as an biotechnological advantage for the development of novel products, potentially useful, to minimize the presence of *Listeria* spp. in food industry.

Conclusion

This study demonstrated that *Listeria* species contaminated raw cow milk in an important milking area of Argentina. In addition,

Table 1. Antimicrobial activity of non-treated and treated cell free supernatants of *Lactobacillus fermentum* L23 and *Lactobacillus rhamnosus* L60 on the growth of different *Listeria* spp.

Listeria spp.	Mean of inhibition halo in mm (mean ± SD)			
	Lactobacillus fermentum L23		*Lactobacillus rhamnosus* L60	
	CFS	NCFS	CFS	NCFS
L. monocytogenes LM1	23.75±2.50[a]	20.00±2.45[a]	21.00±4.18[a]	20.33±3.01[a]
L. monocytogenes LM2	23.00±1.00[a]	18.00±0.00[b]	25.00±2.00[a]	18.67±1.15[b]
L. monocytogenes LM3	19.33±1.15[a]	17.75±1.26[a]	20.00±1.00[a]	17.33±1.53[a]
L. monocytogenes LM4	19.60±3.58[a]	18.00±2.83[a]	18.00±1.82[a]	17.67±0.58[a]
L. monocytogenes LM5	19.33±1.53[a]	15.00±1.00[b]	20.00±1.73[a]	16.67±1.15[a]
L. monocytogenes LM6	22.50±3.56[a]	20.83±1.04[a]	21.67±0.58[a]	21.00±2.64[a]
L. monocytogenes LM7	21.33±0.58[a]	19.67±0.58[b]	19.00±1.00[a]	16.00±1.00[b]
L. monocytogenes LM8	23.17±1.33[a]	20.00±1.87[b]	24.50±1.22[a]	20.60±1.34[b]
L. monocytogenes LM9	22.33±0.58[a]	19.00±0.00[b]	20.00±0.00[a]	17.67±1.53[a]
L. monocytogenes LM11	20.80±2.68[a]	16.20±1.64[b]	19.30±0.67[a]	16.60±1.14[b]
L. monocytogenes LM12	19.67±0.58[a]	15.00±1.00[b]	21.00±2.65[a]	16.33±0.58[b]
L. monocytogenes LM14	25.67±0.58[a]	19.67±0.58[b]	27.67±0.58[a]	19.00±1.00[b]
L. monocytogenes LM15	21.00±1.00[a]	17.67±1.15[b]	20.67±1.53[a]	18.33±1.53[a]
L. monocytogenes LM16	20.50±3.70[a]	18.67±1.15[a]	17.25±0.95[a]	17.00±0.00[a]
L. monocytogenes LM17	23.67±2.31[a]	17.75±3.86[a]	19.50±1.80[a]	18.50±0.71[a]
L. monocytogenes LM18	21.00±2.16[a]	20.00±1.00[a]	21.50±3.11[a]	15.57±0.58[b]
L. monocytogenes LM19	18.00±0.00[a]	17.00±1.41[a]	21.20±1.09[a]	19.00±0.82[b]
L. monocytogenes LM20	21.67±2.31[a]	20.00±1.00[a]	22.33±0.58[a]	18.33±0.58[b]
L. monocytogenes LM21	23.33±1.53[a]	17.67±0.58[b]	24.33±0.58[a]	21.67±1.53[b]
L. monocytogenes LM22	19.33±0.58[a]	19.00±1.00[a]	22.50±2.38[a]	18.67±1.15[a]
L. monocytogenes LM23	19.67±1.37[a]	15.75±2.22[b]	22.40±1.95[a]	18.00±1.00[b]
L. monocytogenes LM24	20.13±6.00[a]	15.17±5.00[b]	18.60±4.27[a]	14.00±2.34[a]
L. monocytogenes LM25	24.00±1.73[a]	18.33±0.58[b]	24.00±0.00[a]	18.67±1.15[b]
L. monocytogenes LM26	21.67±1.53[a]	18.00±1.00[b]	20.33±0.51[a]	14.67±0.58[b]
L. monocytogenes LM30	21.00±1.00[a]	19.00±1.00[a]	19.00±1.00[a]	17.33±0.58[a]
L. monocytogenes LM31	19.33±0.58[a]	16.67±0.58[b]	20.33±1.15[a]	18.00±2.00[a]
L. monocytogenes LM32	20.00±1.15[a]	18.67±1.53[a]	22.00±0.00[a]	19.33±0.58[b]
L. innocua LI1	17.67±1.15[a]	15.00±1.41[b]	20.00±1.00[a]	14.75±0.50[b]
L. welshimeri LW1	19.33±0.58[a]	15.67±1.15[b]	18.33±0.58[a]	15.33±0.58[b]
Total mean ± SD	21.10 ± 1.94[a]	17.90 ± 1.74[b]	21.08 ± 2.35[a]	17.76 ± 1.91[b]

References: CFS: cell free supernatant, NCFS: cell free supernatant neutralized with NaOH 1 mol ml^{-1}. Inhibition halos with different letters indicate significant difference between CFS and NCFS of each lactobacilli strains ($P< 0.05$).

Table 2. Minimum inhibitory concentrations of bacteriocin like inhibitory substances L23 and L60 produced by probiotic lactobacilli on all susceptible strains of *Listeria* spp.

Susceptible *Listeria* spp. (n=29)	MIC (AU ml^{-1})	% sensitive strains	
		BLIS L23	BLIS L60
L. monocytogenes	160	11.11	14.82
	80	18.52	40.74
	40	70.37	44.44
L. innocua	160	100	100
L. welshimeri	160	100	100

References: BLIS: Bacteriocin-like inhibitory substance; MIC: Minimum inhibitory concentration; AU^{-1} ml: Activity units per milliliter

L. fermentum L23 and *L. rhamnosus* L60 proved to have meaningful antimicrobial activity on all of the tested listerial strains. This antilisterial effect was mainly due to bacteriocins L23 and L60, which were still active at very low concentrations. These findings are promising as a biological strategy to prevent or reduce the risk of acquiring severe infections by the main pathogen, *L. monocytogenes*, through food transmission to human. Future studies shall be needed to evaluate the application of these bacteriocin producing strains in milk and/or dairy food.

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