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Biocontrol of *Listeria monocytogenes* by lactic acid bacteria isolated from brewer's grains used as feedstuff in Argentina

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A B S T R A C T

Brewer's grains are the most important by-product of the brewery industry and it is mostly used as a protein and energy source in animal nutrition. *Listeria monocytogenes* is a food-borne pathogen can cause invasive diseases such as meningoencephalitis, sepsis, abortion, and gastroenteritis in humans and several animal species. The aim of this work was to study the antilisterial activity of lactic acid bacteria (LAB) isolated from brewer's grains. The incidence of *Listeria* spp. in brewer's grains was 3.12%. Twenty-one LAB inhibited the growth of the eight strains of *L. monocytogenes*. The mean inhibition halo of cell free supernatants of LAB ranged between 11.5 and 24.5 mm. The isolation of lactic acid bacteria with antilisterial activity from brewer's grains is promising based on their capacity to produce antimicrobial compounds. The production of antimicrobial metabolites by LAB in the substrate would generate an unfavorable environment for the growth of the pathogenic bacterium under study.

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1. Introduction

Spoilage of animal feed by bacteria is a problem worldwide. They can cause extensive damage such as unpleasant smell, taste, or appearance. Another important aspect involved in contamination of feed by bacteria is the presence of potential pathogenic species, which cause a great risk for animal health (Broberg et al., 2007). *Listeria monocytogenes* is a Gram positive, non-sporulating bacillus that has been isolated from a range of sources including vegetables, processed foods, dairy products, silage and soils (Albano et al., 2007; Alsheikh et al., 2012). This facultative intracellular pathogen can cause invasive diseases such as meningoencephalitis, sepsis, abortion, and gastroenteritis in humans and several animal species, including farm animals such as cows, sheep, pigs, and goats (Buchrieser et al., 2011; Mohammed et al., 2010). The risks for human health occur as a result of direct contact with infected animals or after ingestion of contaminated animal products (Dimitrijevic et al., 2006; Oevermann et al., 2010; Schuppler et al., 2010). The main virulence factor of *L. monocyogenes* is listeriolisin O (LLO). This is a cholesterol-dependent toxin and is encoded by the *hly* gene. It belongs to a large family of pore-forming toxins, the cytolysins, typical of Gram-positive bacteria. This cytolysin is the main factor responsible for the hemolytic activity of *L. monocytogenes* (Pushkareva and Ermolaeva, 2010).

Brewer's grains are the most important by-product of the brewery industry and it is mostly used as a protein and energy source in animal nutrition (Gerbaldo et al., 2012; Gupta et al., 2010; Jovanka et al., 2010). Previous studies carried out by our research group demonstrated the humid and acidic conditions of this alternative feedstuff for swine production (Asurmendi et al., 2013, 2014). Such conditions could allow the development of *L. monocytogenes* since this microorganism is well known for its resistance to different environmental conditions, including acid pH, high NaCl concentration, microaerophilia and refrigeration temperatures (Dimitrijevic et al., 2006; Ivy et al., 2012; Jeyaletchumi et al., 2010).

In spite of technological advances, the preservation of feeds is still a debated issue. Processed foods with minimal food additives and thermal treatment are an increasing trend among consumers (Vásquez et al., 2009). Recently, the concept of biopreservation has emerged. It refers to the use of non-pathogenic microorganisms and/or their metabolites to extend the life of food and improve its microbiological quality (Gaggia et al., 2011; García et al., 2010). The antagonistic properties of lactic acid bacteria (LAB) and the security provided by these microorganisms in fermented foods makes them attractive for use as biopreservatives. The fermentative metabolism that lowers the pH, the competition for nutrients and space, the accumulation of D-amino acids, the reduction of the redox potential

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and the production of antimicrobial metabolites such as bacteriocins and hydrogen peroxide, among others, contribute to the inhibitor effect of these microorganisms (Parada et al., 2007; Pascual et al., 2007a,b; Ruiz et al., 2009). The aim of this work was to study the antilisterial activity of LAB isolated from brewer's grains.

2. Materials and methods

2.1. Sampling

Samples of brewer's grains were collected from a factory located in Córdoba, Argentina. Samples (3 kg each) were obtained from July 2010–June 2012. Primary samples were homogenized and quartered to obtain laboratory samples of 1 kg each.

2.2. Isolation of Listeria spp.

The isolation of *Listeria* spp. from brewer's grains was performed according to the double enrichment method (non-selective enrichment and selective enrichment) followed by plate isolation procedures described in the Bacteriological Analytical Manual of the USA-FDA (Hitchins, 1998), with modifications in the media and selective agents used.

Twenty five grams of each sample were homogenized in 225 mL of tryptic soy broth (TSB) and incubated at 37° C for 24 h (non-selective enrichment). Aliquots of 1 mL of primary enrichment were transferred to 10 mL of TSB supplemented with tripaflavine and ceftazidime and incubated at 4 °C for 24 h and then at 37° C for 24 h (selective enrichment). Subsequently, aliquots of 0.1 mL of secondary enrichments were plated in duplicate in Palcam agar (Merck®, Germany) supplemented with selective agents. Typical colonies selected by Gram staining were subcultured in tryptic soy agar (TSA) for further biochemical characterization.

2.3. Hemolytic activity of L. monocytogenes

The hemolytic activity of *Listeria* strains was determined by using a procedure developed by Gallego et al. (2007). The isolates were inoculated in brain hearth infusion (BHI) and incubated at 37° C for 18 h. Serial twofold dilutions were made by mixing 50 μL of each bacterial suspension with 0.85% ClNa in microtitle plates with U-form wells. Then, 100 μL of a 3% suspension of human erythrocytes, previously washed with a solution containing 0.85% ClNa, 0.01% gelatin and 0.43% NaN_3, was added to each dilution. The microplates were incubated at 37 °C for 4 h. The hemolytic activity titer was expressed as complete hemolysis units (CHU: the reciprocal of the highest dilution at which a 100% hemolysis was observed) and minimum hemolysis units (MHU: the reciprocal of the highest dilution at which hemolysis was detected).

2.4. Bacterial strains

LAB strains were isolated from brewer's grains, according to the methodology proposed by The International Commission on Microbiological Specification for Foods (ICMSF, 1996). LAB were maintained at −20 °C in Man Rogosa Sharpe broth (MRS) containing 30% (w/v) glycerol. Eight strains of *L. monocytogenes* were used in this study. *L. monocytogenes* LM1 was obtained from brewer's grains samples. *L. monocytogenes* LM2, LM5, LM12, LM15, LM17, LM25 and LM26 belong to the culture collection of the Bacteriology Laboratory of Universidad Nacional de Río Cuarto, Córdoba. These bacteria were grown in TSB at 37° C for 24 h. All strains were stored at −20 °C in TSB broth containing 30% (w/v) glycerol.

2.5. Identification of bacterial strains

LAB isolates were identified using the following tests, Gram stain, production of catalase and cytochrome oxidase, CO_2 production from glucose, growth at different temperatures (10 and 45 °C), growth at different pH values (4.4 and 9.6), growth at different NaCl concentrations (6.5 and 18%) and production of acid from different carbon sources (glycerol, erythritol, D-arabinose, L-arabinose, D-xylose, D-adonitol, D-galactose, D-glucose, D-fructose, D-mannose, L-rhamnose, Dulcitol, Inositol, D-mannitol,D-celobiose, D-maltose, D-lactose, D-melibiose, D-saccharose, D-trehalose, D-rafinose and starch.) (Holt et al., 1994).

Morphologically typical colonies of *Listeria* spp. in Palcam agar were confirmed by Gram's staining, catalase reaction, oxidase test, motility at 25 °C, methyl red test, Voges–Proskauer test, CAMP test and fermentation of sugars (D-glucose, rhamnose, mannitol, α-methyl D-mannoside and xylose) (Holt et al., 1994).

2.6. Determination of the antimicrobial activity of LAB

Sensitivity to inhibitory substances from LAB was tested by the streak-diffusion method described by Barberis and co-workers. An overnight culture in MRS broth of the LAB strain to be tested for production of antimicrobial compounds was centrally streaked on MRS broth added with 1.2% of agar. The agar plate was incubated at 37 °C for 18 h under microaerobic conditions. Growth was sterilized by exposure to chloroform vapors for 20 min and air for 10 min. Then, five LAB strains were streaked perpendicularly to central growth and plates were re-incubated at 37 °C for 18 h under microaerobic conditions. Plates were examined for inhibition zones.

2.7. Detection of antilisterial activity

Antilisterial activity by LAB was performed following the methodology proposed by Barberis et al. (2002), as described above. The indicator strains were eight different strains of *L. monocytogenes* (LM1, LM2, LM5, LM12, LM15, LM17, LM25 and LM26). The inhibition of bacterial growth around the central LAB was measured.

2.8. Preparation of cell free supernatant and well diffusion method

In order to obtain cell free supernatants (CFS), each LAB strain was cultivated in MRS broth at 37 °C for 24 h with 5% CO_2. CFS were obtained by centrifuging the cultures at 8078× g in the cold (4 °C) for 20 min followed by exposure to chloroform for 20 min. In order to eliminate the inhibitory effects attributed to the organic acids, these fractions with biological activity were neutralized by the addition of 1 mol l^{-1} sodium hydroxide. All strains that showed antilisterial activity were tested by the well diffusion assay. TSA was first shown with the indicator organisms and then wells of 8 mm of diameter were made. Then, 100 μl of CFS and neutralized CFS from each LAB were poured into different wells. The diameters of inhibition zones around each well were measured after incubation at 37° C for 24 h with 5% CO_2. All the experiments were carried out in duplicate (Ruíz et al., 2012).

2.9. Statistical analysis

The antimicrobial activity of LAB were statistically analyzed using InfoStat software through an ANOVA. Means antilisterial inhibition halo were compared by Bonferroni test in streak-diffusion method and Tukey test in well diffusion method to determine the significant difference between the different strains ($p \leq 0.05$) (Di Rienzo et al., 2011).

3. Results

3.1. Isolation of Listeria spp and hemolytic activity

Of the total brewer's grains samples studied (n = 32), *Listeria* spp. was isolated from one sample (3.12%) that corresponded to the November–December 2010 sampling period. In Palcam agar, colonies were grayish-green with a brown–black surrounding halo caused by cleavage of esculin. The strain was identified as *L. monocytogenes* (LM1) after biochemical tests proposed by the Bergey's Manual of Determinative Bacteriology. *L. monocytogenes* isolated from brewer's grains showed values of hemolytic activity of 2 CHU and 8 MHU.

3.2. Determination of the antimicrobial activity of LAB

The study of antimicrobial activity between LAB allowed the selection of the strains with the highest inhibitory activity. Eighty strains, previously isolated from brewer's grains, were assayed for their antagonistic activity against other LAB. Fifty nine strains showed inhibition halos that ranged between 2.5 and 19.5 mm. Of these, eight strains (13.6%) exhibited inhibition halos lower than 5 mm, 26 (44%) showed halos between 5 and 10 mm and 25 strains had inhibition halos higher than 10 mm (42.4%). Those LAB which showed inhibition halos greater than 10 mm were selected for further studies (Table 1).

3.3. Antilisterial activity of selected LAB

The antimicrobial activity of 21 selected LAB on the growth of 8 *L. monocytogenes* strains were evaluated by the streak-diffusion method. The LAB strains were identified as *Lactobacillus brevis* B20, *Lactobacillus plantarum* B29, *Lactobacillus paracasei* B38, *Lactobacillus* sp. B49, *L. plantarum* B54, *L. plantarum* B57, *Pediococcus pentosaceus* B65, *L. brevis* B72, *L. brevis* B78, *P.* acidilactici B82, *P.* acidilactici B83, *P. pentosaceus* B86, *Lactococcus lactis* subsp. *lactis* B87, *L. brevis* B131, *L. brevis* B132, *L. brevis* B133, *L. brevis* B134, *Lactobacillus cellobiosus* B143, *Lactobacillus* sp. B144, *L. brevis* B146 and *L.* cellobiosus B149. All LAB inhibited the growth of the 8 strains of *L. monocytogenes.* Table 2 shows the mean value of inhibition zones. The statistical analysis (ANOVA) of mean inhibition halos values showed significant differences between the strain *Lactobacillus* sp B49, which had the lowest value (6.40 mm) and LAB B83, B133, B143 and B146, which showed the highest values (23.50, 23.64, 23.13 and 23.75 mm, respectively).

The antilisterial activity of CFS and neutralized CFS from the 21 LAB strains listed above were tested by the well diffusion method on the growth of *L. monocytogenes* LM1. A 100% of CFS showed antimicrobial activity against *L. monocytogenes* LM1 with statistically significant differences ($P \leq 0.05$). The highest antimicrobial activity was observed with the CFS of B87. The inhibition halo obtained was of 24.5 mm. The strain B146 showed the lowest antimicrobial activity, with an inhibition halo of 11.5 mm. On the other hand, the antilisterial activity of B29, B38, B72, B83, B86, B131 and B146 was absent when CFS were neutralized. There was also a decrease in the inhibitory activity of the rest of the LAB after neutralization (Fig. 1).

Table 1
Antimicrobial activity between lactic acid bacteria strains isolated from brewer's grains.

LAB strains	Inhibition halo (mm) ± SD	LAB strains	Inhibition halo (mm) ± SD
B20	14.5 ± 2.1	B82	10.2 ± 1.6
B26	12.0 ± 3.2	B83	10.9 ± 2.3
B27	10.5 ± 0.7	B86	10.5 ± 2.4
B29	11.2 ± 6.4	B87	11.0 ± 3.4
B49	10.2 ± 1.8	B89	19.5 ± 3.9
B50	13.5 ± 10.6	B131	10.8 ± 4.8
B51	11.2 ± 7.9	B132	11.0 ± 1.0
B52	12.5 ± 7.8	B133	18.5 ± 5.7
B54	10.4 ± 2.0	B143	14.6 ± 2.6
B57	10.0 ± 2.9	B144	13.2 ± 2.3
B59	11.6 ± 5.9	B146	16.8 ± 1.8
B72	10.0 ± 0.4	B149	10.0 ± 0.2
B78	10.6 ± 0.9		

Table 2
Inhibitory activity of lactic acid bacteria against *Listeria monocytogenes* strains.

LAB strains	Morphology and Gram stain	LAB identification	Media inhibition halo (mm) ± SD
B20	BG(+)[b]	*Lactobacillus brevis*	17.53 ± 4.4 ab[a]
B29	BG(+)	*Lactobacillus plantarum*	18.03 ± 1.8 ab
B38	BG(+)	*Lactobacillus paracasei*	17.38 ± 2.7 ab
B49	BG(+)	*Lactobacillus* sp.	6.40 ± 3.3 c
B54	BG(+)	*Lactobacillus plantarum*	19.00 ± 4.6 ab
B57	BG(+)	*Lactobacillus plantarum*	20.00 ± 1.6 ab
B65	BG(+)	*Pediococcus pentosaceus*	15.03 ± 0.9 b
B72	BG(+)	*Lactobacillus brevis*	21.11 ± 2.5 ab
B78	BG(+)	*Lactobacillus brevis*	22.67 ± 3.2 c
B82	CG(+)[c]	*Pediococcus acidilactici*	18.25 ± 2.0 ab
B83	CG(+)	*Pediococcus acidilactici*	23.50 ± 3.0 a
B86	CG(+)	*Pediococcus pentosaceus*	18.81 ± 4.4 ab
B87	CG(+)	*Lactococcus lactis* subsp. *lactis*	20.25 ± 4.1 ab
B131	BG(+)	*Lactobacillus brevis*	19.50 ± 2.5 ab
B132	BG(+)	*Lactobacillus brevis*	21.25 ± 0.9 ab
B133	BG(+)	*Lactobacillus brevis*	23.64 ± 2.2 a
B134	BG(+)	*Lactobacillus brevis*	21.19 ± 3.6 ab
B143	BG(+)	*Lactobacillus cellobiosus*	23.13 ± 1.7 a
B144	BG(+)	*Lactobacillus* sp.	21.47 ± 4.4 ab
B146	BG(+)	*Lactobacillus brevis*	23.75 ± 1.2 a
B149	BG(+)	*Lactobacillus cellobiosus*	17.38 ± 2.9 ab

[a] The media inhibition zones with different letters indicate significant difference according to Bonferroni test ($p \leq 0.05$).
[b] Bacilli Gram positive.
[c] Cocci Gram positive.

4. Discussion

This is the first report on the isolation of *L. monocytogenes* from brewer's grains used for swine feed. This finding is important because we were able to isolate this microorganism despite the acidic conditions of the fermented substrate. Adaptation of *L. monocytogenes* to acidic environments involves mechanisms that maintain intracellular pH homeostasis by directing H^+ ions out of the cell and by consumption of internal H^+ through decarboxylation reactions, generation of ammonium ions, and macromolecule repair by heat shock proteins (Ivy et al., 2012).

Currently, there is no available information on the isolation of *L. monocytogenes* from brewer's grains. *L. monocytogenes* is pathogenic to animals and humans and often contaminates food and feed, leading to losses in pig production. *L. monocytogenes* also represents an indirect risk to healthy people that consume contaminated pork meat. In a related study, Belceil et al. (2003) isolated *L. monocytogenes* in 6 (14%) samples of wet feedstuff for swine production. This result differs from that found in the present work. In addition, Oliveira et al. (2008a) found a higher incidence of *L. monocytogenes* (15%) in silage samples from the north of Portugal using microbiological methods. This substrate shares some similarities with brewer's grains as both suffer fermentation processes and are used for animal nutrition. However, the incidence of *L. monocytogenes* found in this study is coincident with that presented by Konosonoka et al. (2012), who obtained a percentage of 4.7 from silage samples destined for feeding pigs.

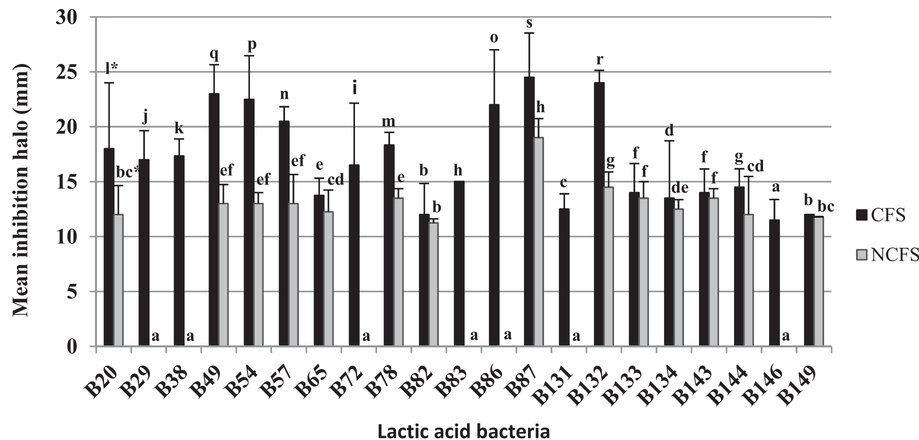

Fig. 1. Inhibition of *L. monocytogenes* LM1 growth by different lactic acid bacteria strains. CFS: Cell Free Supernatant. NCFS: Neutralized Cell Free Supernatant with NaOH 1 N. l*: The media inhibition zones of CFS of different LAB with different letters indicate significant difference according to Tukey test ($p \leq 0.05$). bc*: The media inhibition zones of NCFS of different LAB with different letters indicate significant difference according to Tukey test ($p \leq 0.05$).

The isolated *L. monocytogenes* strain demonstrated hemolytic activity. López et al. (2006) suggested that between 8 and 21% of the food and environmental isolates of *L. monocytogenes* have attenuated virulence or are completely avirulent. *In vitro* assays that detect phenotypic markers, such as hemolytic activity, provide information about virulence. The hemolytic activity found in the isolated strain evidenced an active virulence. Thus, it represents a risk for pigs consuming these feeds.

The antimicrobial activity between LAB remains a significant criteria for the selection of the most promising strains to be employed in later studies with other microorganisms. Our results are in agreement with a previous study presented by Oliveira et al. (2008b). However, different results to those found in this study were presented by Saidi et al. (2011), who tested homologous antimicrobial activity between LAB and observed that only 22.93% of them had an inhibitory effect on the growth of other lactic bacteria. This percentage is much lower than that observed in this study with LAB isolated from brewer's grains (59%).

Listeriosis in animals is mainly related to the consumption of feeds contaminated with *L. monocytogenes*. Preservation methods such as drying or using chemical preservatives can prevent microbial growth. Nevertheless, these types of solutions have many drawbacks such as the alteration of the organoleptic and nutritional properties of foods, among other. Currently, alternative methods are being developed. LAB have been recognized as beneficial microorganisms and it is well known that produce many antimicrobial metabolites such as organic acids, bacteriocins, hydrogen peroxide, among others. The present study shows that LAB isolated from brewer's grains produce antilisterial compounds which inhibit *L. monocytogenes* growth. As in this study, Teixeira de Carvalho et al. (2006) and Kivanc et al. (2011) found that all tested lactic bacteria inhibited a strain of *L. monocytogenes*. However, the number of indicator strains was higher in our work. On the other hand, Albano et al. (2007) and Singh and Prakash (2009) obtained results that do not coincide with those reported in this study. They found that certain strains of LAB did not inhibit the growth of *L. monocytogenes*.

The CFS of all of the LAB strains assayed showed antilisteria activity on LM1. By comparison, some strains showed a lower antagonistic effect while others showed none when LAB supernatants were neutralized. These results agree with those recently reported by Aguilar and Klotz (2010). They reported antimicrobial activity of CFS from *Lactobacillus* species and found a higher inhibitory effect with untreated supernatants, as compared to neutralized supernatants. Similar findings were obtained by Sica et al. (2010), who stated that all CFS from LAB showed antimicrobial activity on *L. monocytogenes*, and only some supernatants maintained their inhibitory activity after being neutralized with 5 N sodium hydroxide. The authors explained that the loss of antimicrobial activity after neutralizing the supernatant was due to the fact that the strains were only capable of producing organic acids. In contrast, Botina et al. (2008) found that the supernatants of 11 LAB did not show antimicrobial activity on *L. monocytogenes* when tested by the well diffusion method. Also, Mezaini et al. (2009) demonstrated that only 2 LAB supernatants inhibited the growth of the pathogen. In a recent study with lactic bacteria from fish, Marguet et al. (2011) showed that 7 of 74 CFS (9.45%) presented antilisterial activity. While the percentage found by these authors is very low, our results are in agreement because CFS of some strains maintained their activity on *Listeria* spp. after being neutralized.

In conclusion, the isolation of lactic acid bacteria with antilisterial activity from brewer's grains is promising based on their capacity to produce antimicrobial compounds and not only organic acids. The production of antimicrobial metabolites by LAB in the substrate would generate an unfavorable environment for the growth of the pathogenic bacterium under study. Further studies are needed in order to determine the potential efficacy of these bacteria as antilisterial agents.

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