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Effects of intramammary inoculation of *Lactobacillus perolens* CRL1724 in lactating cows' udders

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Bovine mastitis is the most important infectious disease on dairy farms. Conventional antibiotic therapy is often unsatisfactory and alternative treatments are continually under investigation. *Lactobacillus (Lb.) perolens* CRL 1724 and *Lactobacillus plantarum* CRL 1716 were previously isolated from milk of dairy cows and selected according to their potential probiotic properties. In the present work the in-vitro capacity of *Lactobacillus* strains to adhere to bovine teat canal epithelial cells (BTCEC) and to inhibit and co-aggregate 14 mastitis-causing pathogens (MCPs) was investigated. The effect of *Lb. perolens* CRL 1724 after intramammary inoculation in lactating cows was evaluated through determination of clinical signs of mastitis, milk appearance, somatic cell counts and *Lb. perolens* CRL 1724 recovery from milk. *Lb. perolens* CRL 1724 was able to inhibit 12 of 14 MCPs (85·7%) in vitro, especially those considered to be major pathogens. In addition, *Lb. perolens* CRL 1724 co-aggregated with all of them. *Lb. plantarum* CRL 1716 was able to inhibit 7 of 14 MCPs (50%) in vitro and showed co-aggregation ability similar to *Lb. perolens* CRL 1724. *Lb. perolens* CRL 1724 showed a higher efficacy of adhesion to BTCEC (values of percentage of adhesion and adhesion index of 75% and 14·4, respectively) than *Lb. plantarum* CRL 1716 (37% and 7·4, respectively). *Lb. perolens* CRL 1724 was recovered from all mammary quarters and no clinical signs or teat damage were observed after the inoculation of 10^6 cfu/ml. The udders presented a normal aspect and there were no changes in the appearance of the milk. The results obtained will serve as the basis for further trials to evaluate the potential of *Lb. perolens* CRL 1724 to be included in a non-antibiotic formulation for the prevention of bovine mastitis.

Keywords: Bovine mastitis, probiotic, *Lactobacillus perolens*, *Lactobacillus plantarum*, milk.

Mastitis, defined as an inflammation of the mammary gland, is one of the most expensive diseases in dairy farming, affecting the net earnings of milk producers all over the world (Fetrow, 2000). In Argentine herds, the main causative agents are *Staphylococcus (Staph.) aureus, Streptococcus (Str.) dysgalactiae* and *Streptococcus uberis* (Acuña et al. 2001; Calvinho & Tirante, 2005). These bacteria are very important, especially because they infect the udder shortly after drying off and before calving, when immunosuppression of cows increases the incidence of mastitis compared with the incidence during lactation (Sordillo, 2005). The conventional methods of controlling mastitis are based upon adoption of preventive control strategies including diagnosis, segregation of the animals and the use of improved hygiene and therapeutic protocols. Even though these current management practices contribute to the decrease in the occurrence of the disease, the treatment for bovine mastitis relies heavily on the use of antibiotics.

During the last three decades, long-acting intramammary antibiotics have been used routinely for the treatment of existing infections and also for preventing new infections mainly at drying off (Calvinho et al. 1991). However, while dry-cow antibiotic therapy has helped to reduce the incidence of mastitis, the emergence of antibiotic-resistant

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pathogens has been increasingly problematic (McDougall et al. 2009).

In order to reduce antibiotic residues in dairy products and in agreement with global pressures to limit their use in dairy cattle, research has been focused on enhancing cows' natural defence mechanisms through the development of innovative methods for the treatment and the prevention of bovine mastitis (Ryan et al. 1999; Meaney et al. 2001; Crispie et al. 2008; McDougall et al. 2009; Pellegrino et al. 2010).

The use of probiotic bacteria, live microorganisms that when administered in adequate amounts confer a beneficial effect to the host (FAO & WHO, 2008), has been widely studied as a novel approach to prevent infections in animals, especially in the gastrointestinal and vaginal tract (Otero & Nader-Macías, 2007; Walsh et al. 2008). Probiotics may exert their beneficial effects on the health of the host by different and several mechanisms: adhesion to epithelial cells, colonization, biofilm formation, production of biosurfactants, aggregation and co-aggregation, production of antagonistic metabolites (organic acids, hydrogen peroxide, bacteriocins), competition for nutrients, production of enzymes and/or immune system modulation. It is likely that microorganisms may exert their effects as a result of one or more of these mechanisms (Espeche et al. 2009). In a previous study, Espeche et al. (2009) isolated several lactic acid bacteria (LAB) from bovine milk with the aim of studying their properties for the design of a probiotic product. Two of these strains, *Lactobacillus perolens* CRL 1724 and *Lactobacillus plantarum* CRL 1716 were selected for further studies to determine their beneficial properties against bovine mastitis.

The aim of the present study was to investigate the in-vitro capacity of *Lactobacillus* to adhere to bovine teat canal epithelial cells (BTCEC) and to inhibit and co-aggregate mastitis-causing pathogens (MCPs). The effect of *Lb. perolens* CRL 1724 after intramammary inoculation in lactating cows, through the determination of udder clinical signs, milk appearance, somatic cell counts (SCC) and recovery of *Lb. perolens* CRL 1724 in milk, was also evaluated.

Materials and Methods

Bacterial strain and culture conditions

Lactobacillus strains used in this study were isolated from milk of healthy Holstein cows from Tucumán, Argentina, and genetically identified by 16S rRNA gene sequencing as *Lb. perolens* CRL 1724 and *Lb. plantarum* CRL 1716. These strains were selected as potentially probiotic because of their high hydrophobicity index, moderate autoaggregation ability and ability to produce organic acid (Espeche et al. 2009).

Lb. perolens CRL 1724 and *Lb. plantarum* CRL 1716, resistant to streptomycin, were grown in Man, Rogosa and Sharpe (MRS, Britania) broth at 37 °C for 18 h, and stored in milk yeast extract (MYE) (10 g low-fat milk, 0·5 g yeast extract and 1 g glucose per 100 ml) with 12% glycerol at − 20 °C. Before performing additional studies, bacterial were subcultured three times, every 12–14 h at 37 °C in MRS broth.

The following indicator bacteria were used to assess antagonistic activity and co-aggregation: *Str. agalactiae* ATCC27956, *Str. dysgalactiae* ATCC27957, *Str. uberis* 102, *Str. uberis* ATCC27958, *Staph. hyicus* 112249, *Str. bovis* ATCC27960, *Enterococcus (Ec.) faecalis* 19433, *Ec. faecium* 35667, *Escherichia (Esch.) coli* ATCC35218, *Klebsiella (K.) pneumoniae* ATCC10031 and *Staph. epidermidis* ATCC14990 provided by Dr Odierno (Universidad Nacional de Río Cuarto, Argentina). *Staph. aureus* RC108, *Esch. coli* 345 and *Pseudomonas* spp. 224 were isolated from milk of cows with bovine mastitis and identified in our laboratory. All these strains were cultivated on trypticase soy agar (TSA, Britania) at 37 °C for 18 h and stored in trypticase soy broth (TSB, Britania) with 20% glycerol at − 20 °C.

Antimicrobial activity

Antimicrobial activity of *Lactobacillus* strains against MCPs in vitro was assayed by the streak line method (Hütt et al. 2006). The inhibitory effect was estimated as the width of the inhibition zone and ranked as high (> 25 mm), intermediate (13–25 mm), low (1–12 mm) and no inhibition (0 mm). The assay was performed in duplicate.

Co-aggregation assay

To assess the interaction between *Lactobacillus* strains and MCPs, the method described by Reid et al. (1990) was used. A suspension of *Lactobacillus* spp. adjusted to a concentration of 10^9 cfu/ml in 1m-phosphate-buffered saline (PBS) (pH 6·2) was mixed with 500 µl of 10^8 cfu/ml of each MCP and incubated at 37 °C in an orbital shaker at 2 ***g*** for 4 h. Suspensions, in duplicate, were Gram-stained and observed under an optical microscope. Pure cultures were used as negative controls.

Adhesion to BTCEC

Adhesion of *Lactobacillus* strains to BTCEC was determined by the methodology described previously by Otero & Nader-Macías (2007) with modifications. Overnight cultures of *Lactobacillus* strains were centrifuged and the bacterial pellet was washed twice with saline solution (0·8% NaCl), once with Eagle's minimum essential media (MEM; Gibco; pH 7·0), and finally suspended in MEM to obtain a concentration of 10^7 cfu/ml.

To isolate BTCEC, cows' udders were obtained from an abattoir. At the laboratory, udders were washed with water and the teat orifice was disinfected with 70% ethanol. BTCEC were obtained by scraping the teat canal wall with a Medibrush XL (Medical Engineering Co.) and suspended immediately in 1 ml MEM (pH 7·0). The suspension was

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centrifuged for 10 min at 120 ***g*** and the pellet washed three times with 10 ml MEM and finally suspended in MEM at 10^5 cells/ml. Differential cell counts were carried out microscopically by using Wright stained smears. Cell viability was determined by the trypan blue exclusion method. The results were expressed as follows: (number of viable cells/number of total cells) × 100. BTCEC were stored refrigerated until the adhesion assay. This assay was performed in triplicate to ensure the test reproducibility.

Equal volumes (500 µl) of BTCEC and lactobacilli suspensions were mixed and incubated under low agitation conditions at 37 °C for 1 h. A tube with MEM was used as control. To remove non-adherent bacteria, tubes were centrifuged for 10 min at 120 ***g*** and the pellet was washed four times in 1 ml MEM. Bacterial binding to BTCEC were examined by optical microscopy (Gram stain) and results expressed as (1) percentage of adhesion: (number of BTCEC with bacteria adhered/total number of BTCEC) × 100; and (2) adhesion index: (total number of bacteria attached to BTCEC/total number of cells with bacteria adhered). The application of the index allowed us to evaluate the efficiency of adhesion.

Scanning electron microscopy

Scanning electron microscopy was applied to illustrate the adhesion capabilities of *Lb. perolens* CRL 1724 to BTCEC and the bacterial aggregation. The adhesion assay was performed as previously described and the methodology utilized for scanning electron microscopy was described previously by Otero & Nader-Macías (2007) with slight modifications. Briefly, after adhesion assay pellets were fixed with 3·16% glutaraldehyde in 0·1 mol/l phosphate buffer (pH 7·4), incubated for 4 h at 4 °C and homogenized. Fixed samples were centrifuged for 10 min at 300 g, washed twice with phosphate buffers (pH 7·2) and the pellet treated with 1% OsO_4 buffer. The samples were dehydrated with increasing acetone-ethanol concentrations, critically point dried mounted and mineralized. Samples were examined in a Joel JSM35CF scanning electron microscope.

Intramammary inoculation of Lb. perolens *CRL 1724*

Five Holando-Argentino lactating cows were used for the assays. Cows were clinically healthy, free of milk major MCPs and with somatic cell counts (SCC) in individual quarters < 200 000 cells/ml. Before intramammary inoculation, the animals were removed from the herd and maintained separately during the rest of the trial. The bacterial inoculum was prepared as follows: a culture of the strain (10^9 cfu/ml) incubated for 18 h at 37 °C in MRS broth was centrifuged and the bacterial pellet was washed twice with saline solution (0·8% NaCl). Cells were suspended in 5 ml of saline solution to obtain a concentration of 10^9 cfu/ml. The concentrated preparation was serially diluted in saline solution to 10^3 cfu/ml and 10^6 cfu/ml. The inocula were fractionated and stored at 4 °C until inoculation was performed (a period no longer than 2 h). All animals were inoculated after evening milking. Before inoculation, udders were cleaned with 70% ethanol and allowed to dry. Bacterial suspensions were infused directly into the teat via the streak canal to a depth of 17 mm using a syringe with a blunted smoothed tip to prevent injury to the teat.

Two cows were first used to determine the maximum bacterial concentration that did not produce udder inflammation. Three quarters of each cow were infused with 1 ml containing 10^3, 10^6 or 10^9 cfu of *Lb. perolens* CRL 1724. The remain quarter was used as control. To minimize animal handling and conform to animal welfare best practices, no infusion was made in the control quarter.

Another three lactating cows were used to evaluate the effect of intramammary inoculation through the determination of udder clinical signs, milk appearance, SCC and recovery of *Lb. perolens* CRL 1724 in milk. Three quarters of each cow were infused once on day 0 (D0), with 1 ml of the maximum bacterial concentration that did not produce udder inflammation selected as described in the former assay. One quarter was used as control.

Sampling and bacterial recovery

Before inoculation, foremilk samples were collected from each quarter according to the National Mastitis Council procedure (National Mastitis Council, 2004) immediately before milking. Milk samples were transported refrigerated (a period no longer than 2 h) to the laboratory and immediately 10 µl was plated onto blood-agar (TSA with 5% of sheep blood) and incubated at 37 °C for 24 h. Bacteria were characterized by standard biochemical tests (Bergey & Holt, 1994). SCC were determined with a Somacount 300 (Bentley) according to the revised protocol of the 148A method C, fluoro-opto-electronic (International Dairy Federation Laboratory, 1995). Milk samples were collected 2 d before infusion (D2), immediately prior to infusion (D0) and post-infusion, as outlined below.

Serial dilutions of milk in saline solution were streaked on MRS agar plates in duplicate and incubated at 37 °C for 24–48 h under microaerophilic conditions (5% CO_2, 95% air) for *Lactobacillus* isolation. The isolated colonies were identified as *Lb. perolens* CRL 1724 by phenotypic tests (Gram stain, morphology, catalase activity, nitrate reduction, indole production) and by streptomycin resistance determination.

Clinical observations and animal care

Clinical signs were monitored throughout the experiment by a veterinarian, every 8 h during the first 24 h, and subsequently every time the cows were milked. General attitude and appetite were observed. The udders were palpated for soreness, swelling, hardness and heat and the appearance of milk was assessed visually for clots and changes in colour or composition every time the cows were milked. All animals involved in this investigation were cared for in accordance

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Table 1. Antimicrobial activity of *Lactobacillus perolens* CRL 1724 and *Lactobacillus plantarum* CRL 1716 against 14 mastitis-causing pathogens (MCP)

	Lactic acid bacteria	
	Lactobacillus perolens CRL 1724	*Lactobacillus plantarum* CRL 1716
Mastitis-causing pathogens	Inhibition zone, mm†	
Staphylococcus aureus RC108	+	−
Staphylococcus hyicus 112249	+	+
Streptococcus agalactiae ATCC27956	+	−
Streptococcus dysgalactiae ATCC27957	++	++
Streptococcus uberis 102	+	−
Streptococcus uberis ATCC27958	+	−
Streptococcus bovis ATCC27960	+	−
Enterococcus faecalis 19433	−	−
Enterococcus faecium 35667	−	−
Pseudomonas spp. 224	+	++
Escherichia coli ATCC35218	+	+
Escherichia coli 345	+	+
Klebsiella pneumoniae ATCC10031	+	+
Staphylococcus epidermidis ATCC14990	+	+

†Interpretation of zone diameter of inhibition: −, no inhibition; +, 1–12 mm; ++, 13–25 mm; +++, >25 mm

with The International Guiding Principles for Biomedical Research Involving Animals (1985).

Statistical analysis

Differences among Ln-SCCs and means of recovered *Lb. perolens* CRL 1724 were analysed using the software INFOSTAT (2004). Treatment and days of sampling were the initial variables included in the model for each analysis. Means were compared by analysis of variance (ANOVA). Differences were considered significant at P value $< 0{\cdot}05$.

Results

Antagonistic activity

The antagonistic activity of *Lactobacillus* strains against 14 MCPs was evaluated through the growth inhibition values. *Str. dysgalactiae* ATCC27957, *Pseudomonas* spp.224, *Esch. coli* ATCC35218, *Esch. coli* 345, *Str. epidermidis* ATCC14990, *Str. hyicus* 112249 and *K. pneumoniae* ATCC10031 were inhibited by both *Lactobacillus* strains, albeit with different growth inhibition values (Table 1). *Lb. perolens* CRL 1724 was able to inhibit 12 of 14 MCPs (85·7%) in vitro, especially those considered to be major pathogens; whereas *Lb. plantarum* CRL 1716 was able to inhibit 7 of 14 MCPs (50%) in vitro. *Ec. faecalis* 19433 and *Ec. faecium* 35667 were not inhibited by either strain.

Co-aggregation

Lb. perolens CRL 1724 showed co-aggregation with all of the MCPs assayed. A similar co-aggregation of MCPs was observed with *Lb. plantarum* CRL 1716 except that no co-aggregation was observed against *Pseudomonas spp.*224 and *Esch. coli* 345 (data not shown).

Adhesion capacity of lactobacilli

A high number of epithelial cells could be isolated from the bovine teat canal with the method set up in our laboratory, and no bacterial contaminants were observed after Gram staining. The two strains of lactobacilli were able to adhere to BTCEC. The percentages of adhesion and the adhesion index were different for the strains. *Lb. perolens* CRL 1724 showed a higher capability of adhesion (75% and 14·4 respectively) than *Lb. plantarum* CRL 1716 (37% and 7·4, respectively). *Lb. perolens* CRL 1724 aggregated to BTCEC as can be seen in Fig. 1. Figures 1B and 1C show different numbers of bacterial adherent to the surface of epithelial cells, showing an irregular pattern of distribution on the cell surface. Figure 1D demonstrate auto-aggregative pattern and adherence of lactobacilli as clusters on the cell surface.

The microphotographs obtained by scanning microscopy illustrate the adhesion and aggregation of *Lb. perolens* CRL 1724 on the surface of BTCEC (Figs 2A, 2B and 2C) without producing morphological or ultrastructural modifications of the epithelial cells. Also the scanning electron microscopy shows *Lb. perolens* CRL 1724 aggregated and adhered on the surfaces of the eukaryotic cells but not on keratin (Fig. 2D).

Intramammary inoculation of Lb. perolens *CRL 1724*

To evaluate the in-vivo performance of *Lb. perolens* CRL 1724, the tolerance of udders to the inoculation of different

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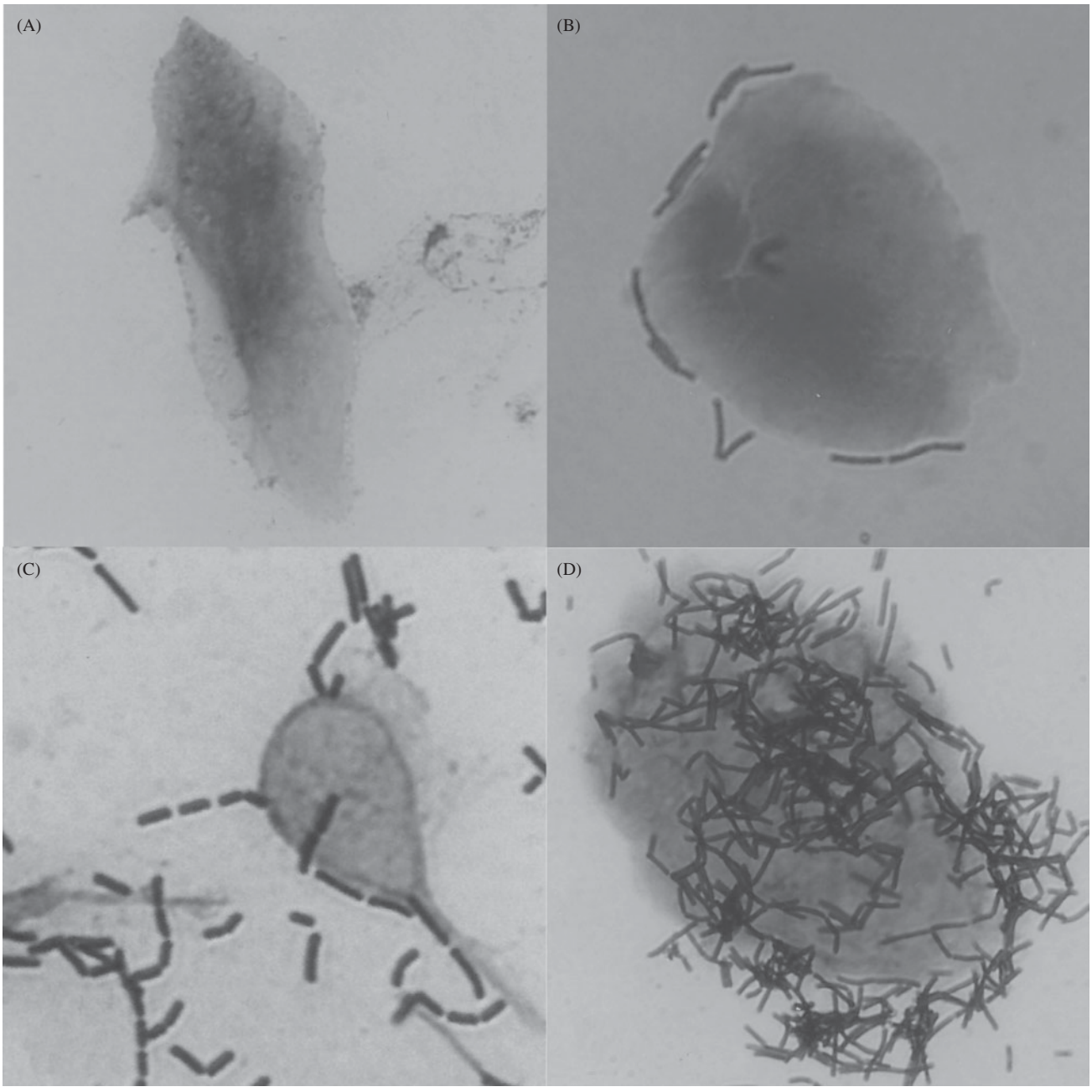

Fig. 1. Light photomicrographs showing Gram-stained *Lactobacillus perolens* CRL 1724 adherent to bovine teat canal epithelial cells. (A) Control. (B) and (C) Different numbers of bacterial adherent to the surface of epithelial cells, showing an irregular pattern of distribution on the cell surface. (D) Autoagreggative pattern and adherence of lactobacilli as clusters on the cell surface (1000×).

concentrations of lactobacilli was first determined in two cows. Concentrations of 10^3 and 10^6 cfu/ml of lactobacilli were well tolerated by the animals. No clinical signs or teat damage were observed in the inoculated quarters and the udders presented a normal aspect. The appearance of the milk from these inoculated animals was normal, without clots, lumps, blood or any changes in the colour. After the inoculation of 10^9 cfu/ml, changes in the appearance of the milk (clots and lumps) were observed. These changes disappeared 48 h after inoculation.

SCC in milk samples from cows inoculated with 10^3 and 10^6 cfu/ml increased 2-fold with respect to the control quarters after 24 h of intramammary inoculation, decreasing to normal values (2×10^5 cells/ml) after day 2 and remaining low until the end of the assay (data not shown). The greatest SCC (6×10^6 cells/ml) was observed in milk samples inoculated with 10^9 cfu/ml on day 1 after inoculation. *Lb. perolens* CRL 1724 was recovered until the end of the assay and from all inoculated quarters. The highest bacterial recovery value (10^3 cfu/ml) was obtained 24 h after

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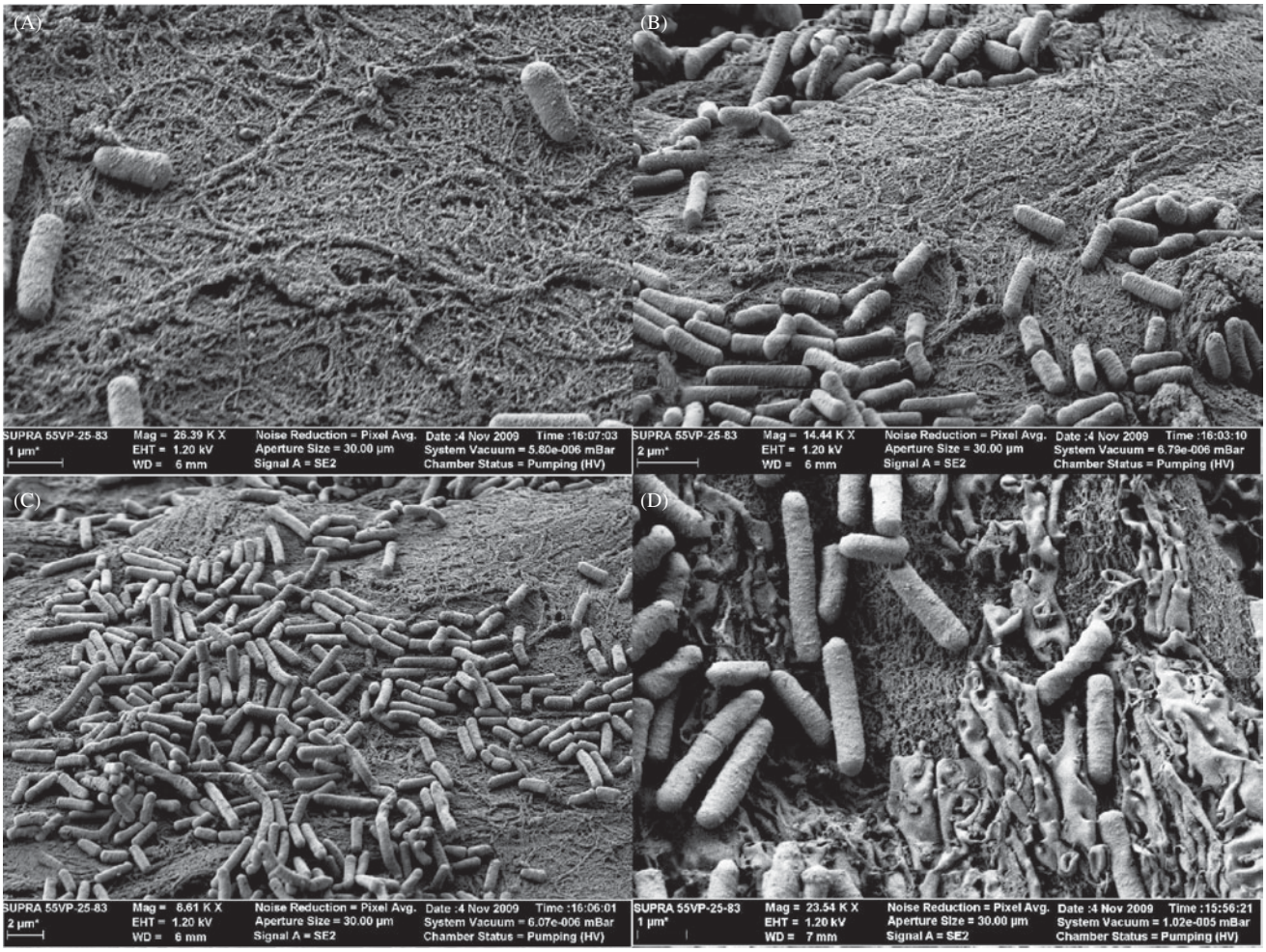

Fig. 2. Adhesion of *Lactobacillus perolens* CRL 1724 to epithelial cells isolated by teat canal wall scraping observed by scanning electron microscopy. (A), (B) and (C) Bacilli adherent to and aggregated on the surface of epithelial cells (26 390 ×, 14 440 × and 8610 ×, respectively). (D) Bacilli adherent to and aggregated on the surface of epithelial cells and surrounded by keratin (23 540 ×).

intramammary inoculations in the quarters inoculated with 10^9 cfu/ml. All quarters inoculated were negative for MCP isolation after the 7-d trial.

Taking into account the results obtained above, 10^6 cfu/ml of *Lb. perolens* CRL 1724 were inoculated into nine quarters of three lactating cows. There was a significant increase ($P<0{\cdot}05$) in the SCC of all inoculated quarters 24 h after inoculation (Fig. 3). This increase was observed until 48 h post inoculation. After this, SCCs decreased to the control value (2×10^5 cells/ml) at day 5. No significant differences were observed between the SCCs of inoculated and control (not inoculated) quarters during the trial. *Lb. perolens* CRL 1724 was recovered during the 15-d trial from 88·9%, 77·8% and 55·6% of the inoculated quarters on days 1, 2 and 7, respectively. At the end of the trial (D15), 22·2% of the inoculated quarters continued to shed the lactobacilli inoculated. Recovery of *Lb. perolens* CRL 1724 on day 1 showed a significant increase ($P<0{\cdot}05$) with respect to the other days. No MCPs were isolated from milk during the trial.

Discussion

During the last two decades, several studies of disease prevention by normal microbiota manipulation have been studied in domestic animals (gastrointestinal of pigs, chickens and turkeys). More relevant to these studies, *Corynebacterium bovis* has been used to colonize the teat canal for protection against mastitis. The mechanism of defence in this case is thought to be due to increased somatic cell count rather than to direct bacterial inhibition (Brooks & Barnum, 1984).

The intramammary immune system's ability to eliminate infections naturally depends on a rapid and competent response to pathogens (Burvenich et al. 1994) and the primary phagocytic cells of the bovine mammary gland, polymorphonuclear (PMN) and macrophages, comprise the first line of defence against invading bacteria (Crispie et al. 2008). Indeed, impairment of the immune response is associated with increased susceptibility to mastitis infection (Burvenich et al. 1994). In this sense, the use of a product capable of

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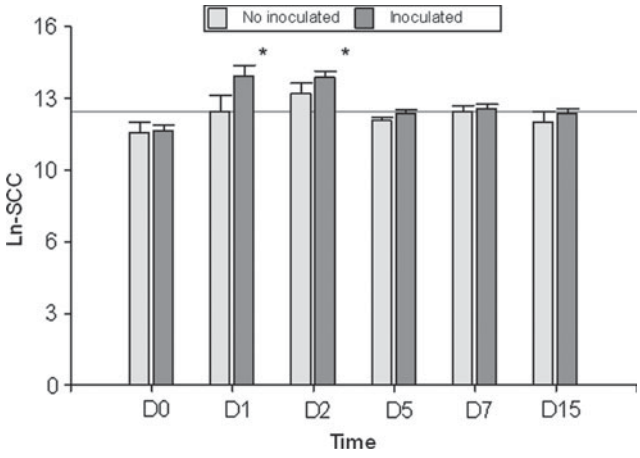

Fig. 3. Mean Ln-somatic cell counts (Ln-SCC) recovered after inoculation of lactating cows with 10^6 cfu/ml *Lactobacillus perolens* CRL 1724. Cut-line: 200 000 cells/ml. D, days. * $P < 0{\cdot}05$.

eliciting a rapid immune response can provide host protection against mastitis infection (Crispie et al. 2008).

Among the parameters to take into account in designing a probiotic, the origin of the strains, based on the host specificity of the indigenous microbiota (Kotarsky & Savage, 1979), the capability to produce antagonistic substances, adhesion to host tissues and colonization to different sites of the host surfaces are the most important features to exert a beneficial effect (Nader-Macias et al. 2008; Espeche et al. 2009).

In a previous report (Espeche et al. 2009), 102 LAB strains were isolated from the teat canal and milk samples of healthy cows. The strains were selected according to high hydrophobicity index, moderate auto-aggregation and organic acid production. Two *Lactobacillus* strains were chosen to conduct further studies.

It is well known that *Lactobacillus* strains are able to inhibit pathogenic microorganisms by organic acids, hydrogen peroxide and bacteriocins (Chaimanee et al. 2009). In the present work *Lb. perolens* CRL 1724 was able to inhibit 85·7% of the MCP assayed, especially those considered major pathogens as *Staph. aureus, Str. agalactiae, Str. dysgalactiae* and *Esch. coli*. In addition, a co-aggregation effect of *Lb. perolens* CRL 1724 with all of them was observed. *Lb. plantarum* CRL 1716 showed a lower percentage of inhibition (50%) but a similar co-aggregation effect compared with *Lb. perolens* CRL 1724. Soleimani et al. (2010) showed that different strains of *Lactobacillus* are capable of co-aggregation with *Staph. aureus* strains causing bovine mastitis. Also Soleimani et al. (2010) suggest that the co-aggregation assay is a reliable method to evaluate the close interaction between lactobacilli and pathogenic bacteria and that many surface proteins are found in lactobacilli which are predicted to promote binding to environmental surfaces like other bacteria surface. Co-aggregation may be beneficial to *Lactobacillus* that produces antimicrobial compounds, as it would force the cells into closer contact (Reid & McGroarty, 1988). Pascual et al. (2008) proposed that co-aggregation could be an important factor in maintaining health, because it produces an area around the pathogen where the concentration of antimicrobial substances produced by lactobacilli is increased.

Adhesion of lactobacilli to the epithelium is the first step in the formation of a barrier to prevent undesirable microbial colonization and has consequently been defined as an essential characteristic when selecting probiotic strains (Havenaar et al. 1992; Reid et al. 2003). In the present work, a percentage of adhesion and adhesion index of 75% and 14·4, respectively, demonstrated the high efficacy of adhesion of *Lb. perolens* CRL 1724 to BTCEC. Lower values were observed for *Lb. plantarum* CRL 1716 (37% and 7·4, respectively). The study of the inhibitory effect of lactobacilli against the mastitis pathogens using teat-canal cells should give relevant information. Future studies of co-inoculation (BAL/MCPs) will be conducted before performing in-vivo protection assays. Interestingly, a great pool of viable BTCEC was isolated with the method set up in our laboratory, and this allowed the development of an easy and rapid method to evaluate adherence in vitro. To our knowledge, this is the first report that demonstrated adhesion of lactobacilli to BTCEC. Similar results were obtained by Otero & Nader-Macías (2007) in epithelial cells from the bovine vagina.

Several studies have suggested that *Lactobacillus* adherence is mediated by proteins associated with the external protein S-layer (Wadström et al. 1987; Henriksson et al. 1991; Frece et al. 2005), while others have suggested a role for lipoteichoic acid and carbohydrate (Fuller, 1975); further studies need to be conducted to determine the chemical nature of the structures involved in adhesion to BTCEC. The adhesion of *Lb. perolens* CRL 1724 to BTCEC was confirmed by scanning electron microscopy. No evidence of morphological or structural modifications of BTCEC due to the adhesion of lactobacilli was observed through any of the scanning electron microscopy observations. The adherence of lactobacilli to epithelial cells, even after treatment employed to scanning electron microscopy preparations, suggests the adhesion efficacy of the strain to the epithelial cells.

Lb. perolens CRL 1724 was selected for udder inoculations because of its elevated percentage of inhibition and co-aggregation of MCPs and their major capability of adhesion to BTCEC. The tolerance of the udders to different concentration of *Lb. perolens* CRL 1724 was determined. The results showed that 10^3 and 10^6 cfu/ml were well tolerated by the udder, but 10^9 cfu/ml was not tolerated because of milk alterations and udder inflammation. A concentration of 10^6 cfu/ml was the dose selected for the intramammary inoculation assay because it was the highest lactobacilli concentration that did not produce long-term udder inflammation or alteration of milk.

The nine quarters inoculated with 10^6 cfu/ml showed that there were no adverse clinical signs in the udders, which remained free of clinical mastitis during the 15-d trial period. On the other hand, with the concentration used, there was a

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short-term significant increase in SCC 2 d post inoculation, returning to normal values at the end of the trial. The short-term significant increase observed in SCC is a normal reaction of the udder against inoculation and it cannot be due to any change or internal damage caused in the mammary glands by the lactobacilli inoculated. In this sense Crispie et al. (2008) concluded that the mechanism by which the live culture can provide host protection against mastitis infection may be associated with its ability to elicit a rapid immune response, inducing substantial recruitment of PMN, lymphocytes and localized production of acute phase proteins, which together can subsequently clear the gland of the infecting pathogen. Although no infusion was administered to the control quarter, nonetheless, these quarters exhibited a negligible increase in SCC (Crispie et al. 2008). These increases were most likely due to cross-talk between quarters. Previous and repeated trials by our research team have shown that infusion of sterile water into the control quarter does not cause irritation or inflammation.

Several reports showed that the intramammary application of probiotic bacteria (Greene et al. 1991) or bacteriocin (Ryan et al. 1999) resulted in a short term increase in SCC. The results obtained in the present work are similar to those of Crispie et al. (2008), who observed an increase in the values of PMN leucocytes in the first 2 d after the inoculation of 10^9 cfu/ml of *Lactococcus lactis*, and a decrease on days 5 and 7 post inoculation. Interestingly, *Lb. perolens* CRL 1724 could be recovered during the 15 d of the assay. This indicates that the strain persisted in the udder, even though the inoculation was done in lactating cows, where milking favours the elimination of bacteria. Beecher et al. (2009) recovered *Lc. lactis* for 2 d post inoculation.

Taking in account the high susceptibility of dairy cows to bovine mastitis during the dry period, the intramammary application of lactobacilli in cows during this period will also be the subject of a further study. The effect of the lactobacilli on milk also requires investigation, but it is fairly unlikely that bacteria would still be found after the dry period and calving. The results obtained will serve as the basis for further studies on the generation of non-antibiotic formulations for the prevention of mastitis in dairy cows.

Conclusions

The results obtained from this work demonstrate the in-vitro capacity of two *Lactobacillus* strains to adhere to BTCEC and to inhibit and co-aggregate MCPs. The in-vitro method of obtaining BTCEC, set up in the laboratory constitutes an easy and rapid method to evaluate adherence in vitro. In vivo, *Lb. perolens* CRL 1724 resulted in a short-term increase in SCC and was recovered from all quarters inoculated during the 15 d of the trial without producing clinical signs in the udder.

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