Beneficial Microbes, 2017; 8(4): 589-596

Wageningen Academic Publishers

Bovine mastitis prevention: humoral and cellular response of dairy cows inoculated with lactic acid bacteria at the dry-off period

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Received: 25 October 2016 / Accepted: 4 March 2017

RESEARCH ARTICLE

Abstract

The use of lactic acid bacteria (LAB) in animal feed, constitute an alternative tool for bovine mastitis prevention. Previously, two LAB strains were isolated from bovine milk and selected for their probiotics properties. So far, immune response of inoculating LAB in bovine udders at dry-off period has not been investigated. The immunoglobulin isotype levels and memory cell proliferation in blood and milk of animals inoculated with *Lactobacillus lactis* subsp. *lactis* CRL1655 and *Lactobacillus perolens* CRL1724 at dry-off period was studied. Ten animals were inoculated intramammarily with 10^6 cells of each LAB (IG) and 2 animals used as control (NIG). Milk and blood samples were taken before inoculation and 1, 2, 4, 6, 12 and 24 h and 7 and 14 days after inoculation. Somatic cell count (SCC) in milk, the presence of bovine mastitis pathogens, the levels of antibodies and lymphocyte proliferation were determined. In the IG, the SCC was <250,000 cells/ml up to 4 h after intramammary inoculation. Six and 12 h after inoculation, the SCC increased up to 600,000 and 2,000,000 cells/ml, respectively. In the NIG, the SCC reached the maximum value 7 days after inoculation. Microbiological analysis showed that all samples were negative for major bovine mastitis pathogens after 24-48 h of incubation. In general, LAB inoculation increased the amount of IgG isotypes in blood and milk, and these antibodies were able to recognise *Staphylococcus aureus* epitopes. Lymphocytes proliferation was significantly higher in the IG at all time points assayed, following LAB or *S. aureus* stimulation. The lymphocytes of animals inoculated with LAB do not react *in vitro* to the presence of *S. aureus* antigen.. The results showed that probiotic microorganisms could be a natural and effective alternative in the prevention of bovine mastitis at dry-off period and act as immunomodulatory stimulating local and systemic defence lines.

Keywords: bovine mastitis, probiotics, lactic acid bacteria, *Staphylococcus*, *Lactobacillus*

1. Introduction

In the last years there has been an increasing tendency from the regulation organs on the requirement to apply preventive strategies in veterinarian and human diseases (Nader-Macías *et al.*, 2011; Pellegrino *et al.*, 2016). Supported by these regulations, the use of probiotic microorganisms constitute a very interesting and novel alternative (Espeche *et al.*, 2012) Probiotics are 'live microorganisms which, when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO, 2002; Nader-Macías *et al.*, 2011). Lactic acid bacteria (LAB), one of the main probiotic groups, has been included in the GRAS (generally regarded as safe) or food-grade microorganisms (FGM) categories. Probiotics may exert their beneficial effects on the host´s health through different mechanisms: adhesion to epithelial cell, aggregation and coaggregation, biofilm formation, colonisation, production of biosurfactants, and/or

antagonistic metabolites (organic acids, hydrogen peroxide, bacteriocins), competition for nutrients and/or production of enzymes (Espeche *et al.*, 2009; Frola *et al.*, 2011). The protective function also includes immunomodulation, which means that probiotics modulate immune homeostasis by affecting the development, differentiation and effector function of a wide variety of immune cell subsets (Hardy *et al.*, 2013) and also in epithelial cells (Bouchard *et al.*, 2015). Most of the studies published have focused mainly on the evaluation of probiotics for the prevention of infections in the gastrointestinal tract. However, all the mucosal sites or ecosystems of the body colonised by an indigenous or normal microbiota are potential targets for specific probiotics (Ouwehand *et al.*, 2002). The use of beneficial microorganisms in animal feed is substantially increased because of the progressive exigencies of regulatory agencies for a decrease in the use of antibiotics as growth promoters. Probiotics may improve the health status, increase the weight gain and inhibit pathogens (Ewaschuk *et al.*, 2004; Nader-Macías *et al.*, 2008). Lately, the target of probiotic formula was extended to other ecosystems, including the mammary gland to prevent mastitis. Defined as the inflammation of the mammary gland, mastitis is one of the most prevalent diseases in dairy herds (Bogni *et al.*, 2011; Rollin *et al.*, 2015) and more than 80% of the cases are caused by microorganisms (Dieser *et al.*, 2014; Raspanti *et al.*, 2016). Dairy cows are highly susceptible to mastitis, mainly during the dry-off period (Frola *et al.*, 2012) when antibiotics are currently administered to eliminate subclinical cases and to prevent the establishment of new intramammary infections (Bogni *et al.*, 2011). Antibiotics for prophylactic treatment are being subjected to considerable discussion because of the perceived connection with the emergence of antibiotic resistance in bacteria (Dalton, 2006; Martins 2014). In order to reduce antibiotic residues in dairy products, and in agreement with global requirements 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 prevention of bovine mastitis (Bogni *et al.*, 2011; Pellegrino *et al.*, 2010).

Our research group is focused on the prevention of bovine mastitis by restoring the balance in the indigenous microbiota of the mammary gland (Espeche *et al.*, 2009, 2012; Frola *et al.*, 2011, 2012). The evaluation of LAB present in the mammary gland microbita was performed with the aim of restoring the ecological homeostasis with a probiotic product. Based on the host specificity, 102 LAB from foremilk, stripping milk and scrapped teat canal were isolated and the production of antagonistic substances and their surface properties, were evaluated. Safety aspects as virulence traits and antibiotic resistance were also considered. Finally, two strains, *Lactococcus lactis* subsp. *lactis* CRL1655 and *Lactobacillus perolens* CRL1724, were selected (Espeche *et al.*, 2009, 2012). So far, the immune response that is targeted when inoculating LAB in bovine udders at dry-off period has not been investigated. In order to determine the immunomodulatory effect of LAB, the immunoglobulin isotype concentration and memory cell proliferation in blood and milk of animals inoculated with *L. lactis* subsp. *lactis* CRL1655 and *L. perolens* CRL1724 at dry-off period were studied in this work.

2. Materials and methods

Selection of animals

Milk quarter samples were collected from 16 lactating Holstein cows next to dry-off period. Animals belonged to a commercial dairy farm operated under conventional management practices. To determine the health status of the animals, milk quarter samples were taken one week before the trial and the microbiological analyses were carried out by the National Mastitis Council Microbiological Procedures (2004). The Somatic Cell Count (SCC) was determined according to the revised protocol of the standard fluoro-opto-electronic method (method C, IDF 148A). Animals with the following characteristics were selected for the trial: SCC <250,000 cells/ml, devoid of the major bovine mastitis pathogens in at least 3 of the 4 quarters and without clinical signs of bovine mastitis.

Twelve cows with the characteristics described above, were randomly divided into two groups. One group (n=10) was inoculated intramammarily at the dry-off period with the selected LAB (inoculated group, IG) and other group (n=2) was used as control (non-inoculated group, NIG). We selected only two animals in the NIG taking into account previous studies (Frola *et al.,* 2011) and unpublished data where we observed homogeneous results among them. The animals were between parity 1 and 4 and were in late lactation. Cows were milked twice daily and produced an average of 14 kg milk/d before interruption of lactation.

Bacterial strains and culture conditions

Lactobacillus perolens CRL1724 and *Lactococcus lactis* sub. *lactis* CRL1655 were previously selected as potentially probiotic (Espeche *et al.*, 2009, 2012). *L. perolens* CRL1724 and *L. lactis* sub. *lactis* CRL1655 were grown in De Man, Rogosa and Sharpe (MRS, Britania, Buenos Aires, Argentina) broth at 37 °C for 18 h, and stored in milk yeast extract (Britania) (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, bacteria were subcultured three times, every 12-14 h at 37 °C in MRS broth.

Staphylococcus aureus RC108 isolated from bovine subclinical mastitis (Bogni *et al.*, 1998) was cultivated on trypticase soy agar (TSA, Britania) at 37 °C for 18 h and

stored in trypticase soy broth (Britania) with 20% glycerol at -20 °C.

Intramammary inoculation at dry-off period

The bacterial inoculum was prepared as described by Frola *et al.* (2012) with some modifications: a culture of the each LAB strains (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 suspension was diluted in saline solution to 10^6 cfu/ml. Inoculums of LAB were combined, aliquoted and stored at 4 °C until inoculation (a period no longer than 2 h). Animals of the IG were inoculated in all quarters after morning milking. Before inoculation, udders were cleaned with 70% ethanol and allowed to dry. One ml of the bacterial suspensions was infused into the teat canal according to Frola *et al.* (2011). To minimise animal handling and following animal welfare best practices, no infusion was made in the control animals (NIG).

Clinical observations and animal care

Clinical signs were monitored throughout the experiment by a veterinarian every 8 h during the first 24 h and daily until the end of the assay. General attitude and appetite of cows were observed. The udders were palpated for soreness, swelling, hardness and heat, and the appearance of milk and dry-off secretion was assessed macroscopically for clots and changes in colour or composition.

The implementation of the trial was approved by Ethics Committee of the University of Río Cuarto (Exp. 54/11). All animals involved in this investigation were cared for in accordance with The International Guiding Principles for Biomedical Research Involving Animals (2012).

Milk sampling and analysis

Samples of quarter milk or dry-off period secretion samples were collected according to the National Mastitis Council procedure (2004) before the intramammary inoculation and at 1, 2, 4, 6, 12 and 24 h and 7 and 14 days after inoculation. For ELISA assay, milk or dry-off secretion samples obtained before the intramammary inoculation and 6, 12 and 24 h and 7 and 14 days after inoculation were centrifuged at 3,700×*g* for 15 min and the serum stored at -20 °C.

Bacteriological assays were performed according to the criteria of the National Mastitis Council (2004). The presence of bacterial strains of the *Staphylococcus* genus was determined using 10 µl of milk plated onto blood-agar (TSA with 5% of sheep blood) and incubated at 37 °C for 24 h. The Gram-positive cocci were characterised by standard biochemical tests (Rampone *et al.*, 1993). The SCC was performed with a Somacount 300 (Bentley Instruments, Chaska, MN, USA) according to the revised protocol of the standard fluoro-opto-electronic method (method C, IDF 148A; IDF, 1995).

Blood sampling

For ELISA assay, blood samples were collected before the intramammary inoculation and 6, 12 and 24 h and 7 and 14 days after inoculation. 20 ml of blood were obtained from the tail vein and placed in sterile tubes, maintained at room temperature, centrifuged at 1,200×*g* for 10 min and the sera collected and stored at -20 °C. For the lymphocyte proliferation assay, heparinised whole blood samples were obtained from the jugular vein (approximately 30 ml) before inoculation and 1, 7 and 14 days after inoculation. To avoid blood coagulation, syringes were rotated slowly and immediately processed in the laboratory for lymphocytes isolation.

Detection of antibodies

The levels of specific antibodies against LAB (total immunoglobulin G (IgG), IgG_1 and IgG_2) in blood, milk and dry-off secretion were determined by the enzyme-linked immunosorbent assay (ELISA) as described by Pellegrino *et al.* (2010). To determine cross reaction of specific LAB antibodies with *S. aureus*, the strain RC108 was fixed on 96-well plates (Nunc-Immuno Plate Maxi Sorb™; Thermo Scientific, Waltham, MA, USA) and the same samples of blood, milk and dry-off secretion were assayed.

Lymphocyte proliferation assay

Lymphocytes from whole blood were obtained from IG and NIG. The proliferation response was determined as described by Pellegrino *et al.* (2016) and calculated as a stimulation index (SI). The average count per minute (CPM) for five wells cultured with the whole heat-killed bacteria was divided by the average CPM for the five wells cultured with completed lymphocyte medium (Pellegrino *et al.*, 2016).

Statistical analysis

For SCC, antibody levels and lymphoproliferation assays, comparisons among treatments at each time point were evaluated using the mixed model analysis of variance (ANOVA) for repeated measurements (SAS Institute, Cary, NC, USA). A P-value <0.05 was considered indicative of a statistically significant difference.

3. Results

Animal´s selection and inoculation

The immunological response of cows to intramammary LAB inoculation was investigated in this work. Sixteen cows (64 quarters) were analysed (SCC and bacteriology) to determine the udder health status. The majority of samples, 81% (52 samples), showed a SCC <250,000 cells/ml and were bacteriologically negative for the *Staphylococcus* genus. 13% (8 samples) showed elevated SCC (>400,000 cells/ml) and were positive for the *Staphylococcus* genus. 6% of the samples (4 samples) were positive for *Staphylococcus* genus but showed a low SCC (<250,000 cells/ml). *Staphylococcus* coagulase negative and other bacteria not belonging to *Staphylococcus* genus were isolated in 50% each one from the bacteriologically positive samples. *S. aureus*, the main bovine mastitis pathogen, was not isolated from the analysed samples. Based on these results, 12 cows were selected and divided in two groups: an inoculated group (IG, n=10) and a non-inoculated group (NIG, n=2). The animals from IG were inoculated with the two LABs at the dry-off period. Cows did not show any abnormal sign of sensitivity to the LAB. No clinical signs or teat damage were observed in inoculated quarters, and the udders showed a normal aspect. The appearance of the milk from these inoculated animals was regular, without clots, lumps, blood or any changes in colour.

Somatic cell count and detection of bacteria

In the IG, the SCC was <250,000 cells/ml up to 4 h after intramammary inoculation. Six and 12 h after inoculation, the SCC increased up to a value of approximately 600,000 and 2,000,000 cells/ml, respectively (Figure 1). In the NIG, the SCC reaches the maximum value 7 days after the start of the trial. After 12 and 24 h, the difference between groups was significant ($P<0.05$) and no significant differences ($P>0.05$) were detected between groups at days 7 and 14. All samples were bacteriologically negative after 24-48 h of incubation, as recommended by the National Mastitis Council (2004). Bacterial growth was observed in 60% of the samples only after 48-72 h of incubation. *Micrococcus* spp., *Corynebacterium* spp. and coagulase negative *Staphylococcus* were isolated in 26, 39 and 35% of the samples respectively. Coliforms, *Streptococcus* spp. and *Staphylococcus aureus* were not found.

Detection of antibodies

The inoculation of LAB to the bovine mammary gland increased significantly the amount of specific antibodies in blood and milk. In the IG, a significant increase in anti-LAB specific IgG was observed ($P<0.05$) in blood, 6 h after intramammary inoculation. This difference was maintained until the end of the assay. 12 h after inoculation, specific antibodies in the IG reached the highest level compared to samples taken at previous times ($P>0.05$) (Figure 2A). Similar results were observed in milk, where a significant increase in specific IgG was observed in the IG ($P<0.05$) at all the times assayed (Figure 2B). Specific antibodies reached the highest level 24 h after inoculation, but this

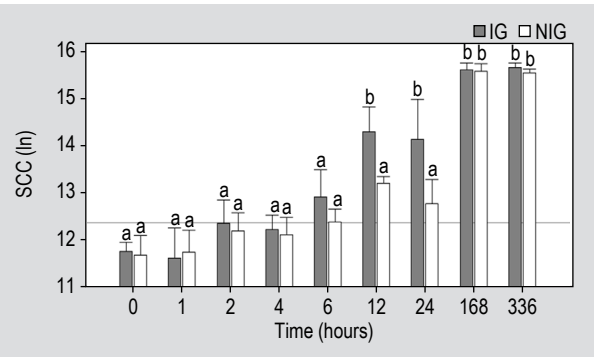

Figure 1. Somatic cell count (SCC) of milk samples from inoculated (IG) and non-inoculated (NIG) groups obtained before inoculation and 1, 2, 4, 6 12, 24, 168 and 336 h after inoculation. Data are expressed as mean ± standard error values of ln of the SCC. Differences between groups were considered significant at $P<0.05$ and are indicated by distinct letters.

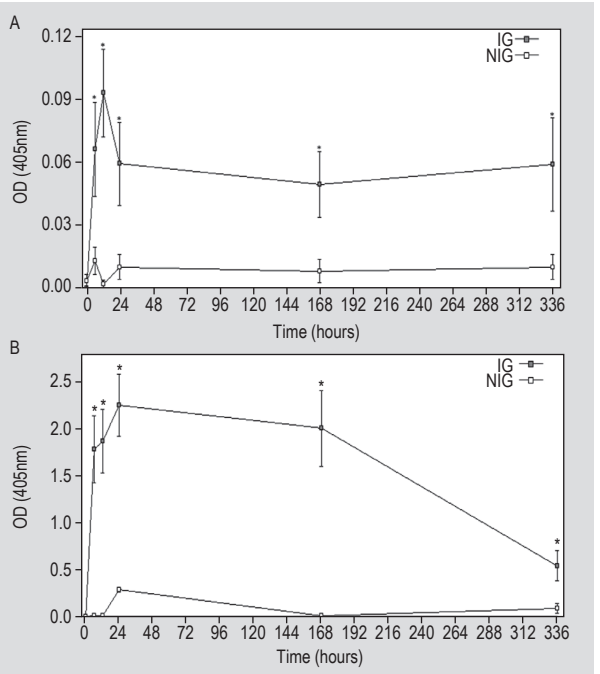

Figure 2. Lactic acidic bacteria antigen specific immunoglobulin (Ig)G response in (A) blood and (B) milk as determined by ELISA. Samples were diluted 1/80 and 1/10 for blood and milk, respectively. Data are expressed as mean ± standard error of the optical density (OD) values read at 405 nm. IG = inoculated group, NIG = non-inoculated group. Differences between groups were considered significant at $P<0.05$ and are indicated by an asterisk.

difference was not significant ($P>0.05$) compared with previous times in the IG.

Significant increase ($P<0.05$) in both, IgG_1 and IgG_2, was observed in blood of the IG 6 days after inoculation. The level of IgG_2 was significantly higher ($P<0.05$) than IgG_1 at 12, 24, 168 (7 days) and 336 h (14 days) after inoculation. Although the highest IgG_2 level was detected 12 after inoculation, this value was not significant ($P>0.05$) with respect to 14 d after inoculation (Figure 3A). Similar results were obtained in milk samples, where IgG_2 was the main isotype detected at 12, 24, 168 (7 days) and 336 h (14 days) after inoculation (Figure 3B). IgG_1 and IgG_2 levels were significantly higher ($P<0.05$) at all the time points assayed in the blood and milk of the IG.

With the aim of determining cross reaction of specific LAB antibodies in blood and milk with *S. aureus*, an additional assay was performed using the whole cells antigens of this pathogen to fix the ELISA wells-plates. The results showed that specific LAB antibodies, present in blood and milk samples, were able to recognise *S. aureus* epitopes, indicating the existence of some type of cross reaction. A significant increase in anti-staphylococcal antibody levels was observed in the first hours after inoculation in blood and milk, followed by a slight drop extending up to 168 h (7 days). The level of antibodies increased again at the end of the trial (14 days) when the values significantly higher ($P<0.05$) from the initial ones were reached. Significant IgG levels ($P<0.05$) were found in the IG at all time points (Figure 4A and B). The antibody kinetic curve obtained in this experiment was similar to the one obtained for LAB.

Lymphocyte proliferation assay

Lymphocytes isolated from blood samples obtained from animals assigned to IG or NIG groups at 1, 7 and 14 days after inoculation, were exposed to LAB and *S. aureus* and the lymphocyte proliferation was expressed as Stimulation Index (SI). One day after inoculation, similar values of SI were obtained after stimulation with LAB and *S. aureus* ($P>0.05$). For LAB, the SI increased over the time and reached the highest level 14 days after inoculation. At this time, significant differences ($P<0.05$) were detected in the SI in relation to previous days (1 and 7 days) and in comparison with *S. aureus*. On the other hand, results for *S. aureus* showed a decrease at days 7 and 14 referred to day 1. Fourteen days after inoculation, the SI was three fold higher for LAB compared with those for *S. aureus* ($P<0.05$) (Figure 5). The SI was significantly higher ($P<0.05$) in the

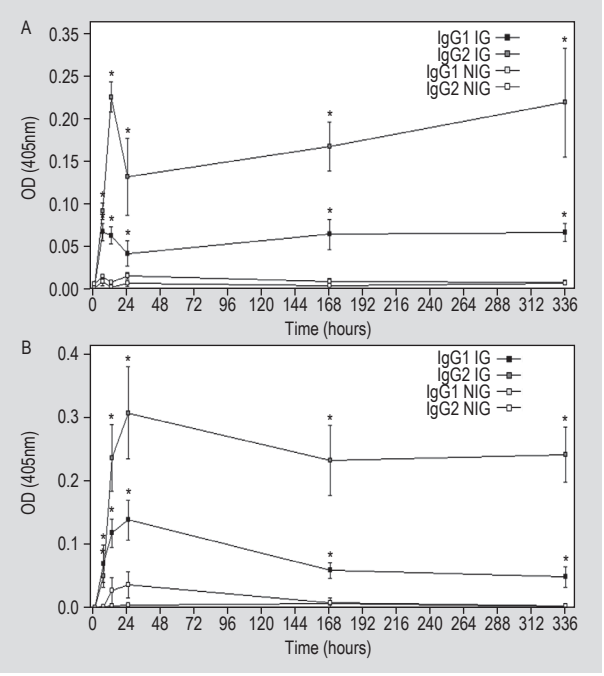

Figure 3. Lactic acidic bacteria specific antibody levels of immunoglobulin (Ig)G1 and IgG2 in (A) blood and (B) milk from inoculated (IG) and non-inoculated (NIG) groups determined by ELISA. Samples were diluted 1/80 and 1/10 for blood and milk, respectively. Data are expressed as mean ± standard error of the optical density (OD) values read at 405 nm. Differences between groups were considered significant at *P*<0.05 and are indicated by an asterisk.

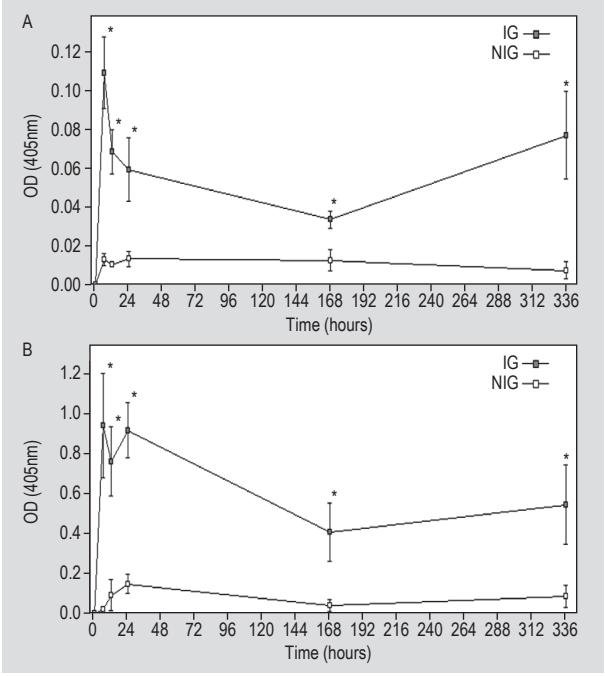

Figure 4. Total specific antibody levels against *Staphylococcus aureus* in (A) blood and (B) milk from inoculated (IG) and non-inoculated (NIG) groups determined by ELISA. Samples were diluted 1/80 and 1/10 for blood and milk, respectively. Data are expressed as mean ± standard error of the optical density (OD) values read at 405 nm. Differences between groups were considered significant at *P*<0.05 and indicated by an asterisk.

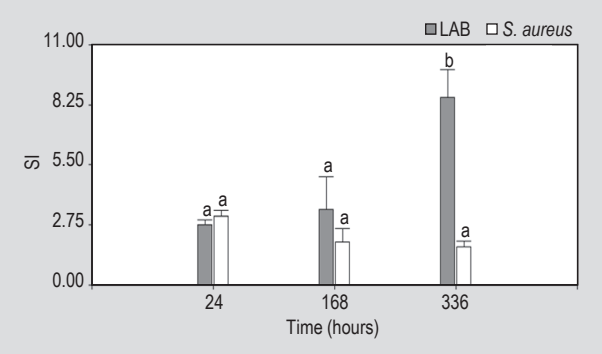

Figure 5. Stimulation index (SI) of blood lymphocytes isolated from cows intramammarlly inoculated with lactic acid bacteria (LAB), and then stimulated with LAB or *Staphylococcus aureus*. Differences between groups were considered significant at *P*<0.05 and indicated with distinct letters.

IG for all time points assayed and for both, LAB and *S. aureus* (data not shown).

4. Discussion

Immunomodulatory strategies for the control of bovine mastitis during the dry-off period, constitute a novel alternative approach to antibiotics therapy. The probiotic characteristics of two LAB isolated from bovine milk were described previously (Espeche *et al.*, 2009, 2012; Frola *et al.*, 2011, 2012). *L. perolens* inhibited mastitis pathogens and showed an interesting pattern of adhesion to bovine teat canal epithelial cells (Frola *et al.,* 2011). *L. lactis* is a bacteriocinogenic strain that produces nisin Z (Espeche *et al.*, 2009). This work describes, for the first time, the immunomodulatory effect in cows inoculated intramammarily with probiotics strains at the dry-off period.

We determined the effect of LAB inoculation on SCC and bacterial content. The SCC is considered an important tool to evaluate the udders health status in dairy herds. This parameter is constantly monitored by the dairy companies and the price of the milk varies depending of the SCC value (Nielsen *et al.*, 2010). The intramammary lactobacilli inoculation was performed at the dry-off period, in which animals are not milked and the milk production is low. Therefore, the SCC in this period is worthless and is not monitored frequently. In the IG, a progressive increase in the SCC was observed from 6 h after lactobacilli inoculation. This increase could be a first sign of a local acute inflammation of the udder in which neutrophils, the predominant cell type in milk, are recruited from the lymph nodes to the udder (Alnakip *et al.*, 2014). At 7 and 14 days, no significant differences were found between IG and NIG. The white cells recruitment originated by LAB inoculation kept an immunologically active udder during that period. Neutrophils in milk and mammary gland secretion in cows at dry-off period could be optimising the udder remodelling (Yu *et al.*, 2011) and even enhancing self-healing of infected or damaged glands and facilitate animals going to the next lactation healthy and at its highest production level. Finally, we must consider that the gland stops producing milk gradually when it begins to dry, thus becoming a more watery secretion, lumpy or coagulated with a high number of white cell conglomerations. These changes cause a higher concentration of the somatic cells and the increase of the SCC. Some studies have reported a fast accumulation of soluble plasmin and specific proteins in the mammary secretion of dry cows with a parallel increase in the SCC (Yu *et al.*, 2011). Regarding the bacteriological analysis, the National Mastitis Council requires that results must be recorded within 24-48 h of incubation. Microorganisms were isolated after 48-72 h incubation in blood agar plates at 37 °C. At this time, only environmental bacteria such as *Micrococcus* spp., *Corynebacterium* spp. and coagulase negative *Staphylococcus,* were isolated. Coliforms, *Streptococcus* spp. and *S. aureus* were isolated neither from IG nor from NIG. In this study, we were focused to the major mastitis pathogens only.

At present there are no studies that describe the effect of the inoculation of LAB in bovine udders at the dry-off period and their effect on humoral immune response. In this work, an increase in total IgG in blood and milk was observed within 6 h after inoculation. This rapid response may be due to the fact that LAB are part of the indigenous microbiota of cattle and the intramammary inoculation could act as a booster against antigenic epitopes of the strains of this group, thus causing an acute response. In blood, the higher level of antibodies was observed 12 h after inoculation. Later, they decreased to similar values as those detected at 6 h after inoculation. This decrease is correlated with the increase on the level of antibodies observed in milk, which reached the higher level 24 h after inoculation. As previously described (Pellegrino *et al.*, 2016), the antibodies present in milk after an antigenic stimulus, as a consequence of the natural entrance of a pathogen or a local administration of an antigen can be synthesised locally in low levels or transported from the blood. The later process is the most effective, since in the peripheral lymphoid organs most of the antibody-producing lymphocytes are present. Since in this work, an increase in the number of cells in milk at 12-24 h after inoculation was observed, the highest antibody levels detected in milk could be explained partially to the presence of these white cells in the udder. In the available literature, there are no trials describing the immunological effect of LAB intramammary inoculated in cows at the dry-off period as reported in this work. Nevertheless, similar results were observed in most of the experiments on inoculation in bovines reported previously (Lee *et al.*, 2005; Pellegrino *et al.*, 2008, 2010, 2016). Previous results obtained by our research group demonstrated that the LAB inoculated at

the dry-off period were isolated from the udder up 14 days after inoculation (Frola *et al.*, 2012). This fact could explain the decrease in the antibody levels observed in milk 14 days after inoculation, where the local stimuli produced by LAB disappeared. Although blood and milk antibody levels decreased at the end of the trial, the levels were significantly higher in the IG.

IgG_2 is the major opsonic antibody present in blood and milk. An increase in the level of IgG_2 stimulates phagocytosis by neutrophils and could streamline the removal of the pathogens present in the udder (Alnakip *et al.*, 2014). In addition, neutrophils, which are the main cells responsible for phagocytosis, express on the surface a large number of specific receptors for IgG_2 antibody. It is important to know the kinetics and concentration of both Igs isotypes in immunological assays to determine how effective the inoculation to stimulate the immune response is. The results obtained in this study show that the IG_2 is the main isotype detected in both, blood and milk, of LAB inoculated animals. These results would indicate that intramammary inoculation of LAB at the dry-off period in dairy cattle stimulates in some way the immune system of the host, triggering, among other factors, the production of specific antibodies, mainly opsonic IgG_2 isotype. The production of high levels of specific antibodies could have the ability to protect the udder against further bacterial invasions in this period when animals are highly susceptible to mastitis (Sordillo and Streicher, 2002). Furthermore, an increase in the milk cells, mainly neutrophils, between 12-24 h after inoculation was observed. *In vitro* studies have shown that *S. aureus* is able of both internalising itself within host cells and surviving in macrophages and alveolar cells isolated from chronically infected cows with mastitis (Hensen *et al.*, 2000). Internalisation and survival of *S. aureus* could explain why humoral immune response alone is inefficient and antibiotic treatments partially fail to eliminate this pathogen (Wei *et al.*, 2014). An immunomodulatory effect able to awake a cellular immune response and opsonic antibodies could be a very effective tool for the prevention of bovine mastitis.

To determine if LAB were able to induce cellular response and to evaluate lymphocyte proliferation in the presence of whole cells of *S. aureus* antigens, an ex-vivo assay was performed. The increase of the lymphocyte proliferation observed after LAB inoculation indicates that lymphocytes isolated from this group proliferate after the second *in vitro* exposure to the same antigen. This response could be related to the ability of lymphocytes to acquire immunological memory. A proved advantage in using live strains for the prevention of various diseases is the ability to immunomodulate both, humoral and cellular responses by activating B and T cells. This allows the antigen presentation for long term period (Pellegrino *et al.*, 2010, 2016). These features enable those T lymphocytes from immunised animals to respond quickly and effectively to the contact with LAB strains. These findings may explain the significant increase in the SI observed at all assayed times. On the other hand, although no differences on SI were detected between LAB and *S. aureus* 24 h after inoculation, results related to *S. aureus* show a decrease at days 7 and 14 in comparison to those obtained at day 1. The SI observed 24 h after inoculation may be explained as a collateral effect of the acute inflammation observed at this time. At the end of the inflammation, the decrease in lymphocyte proliferation can be seen, which corresponds to the absence of memory. The lymphocyte populations of animals inoculated with the probiotic do not react *in vitro* to the presence of *S. aureus* antigen for a long time. This would indicate the high specificity that the acquired immune response has. The effect of LAB inoculation on the cellular response to *S. aureus* was observed within 24 h after inoculation and this could demonstrate the proliferation of lymphocytes in presence of this pathogen in a short time after udder stimulation and it could show a partial cross reaction with LAB epitopes.

This is the first work that describes the immunological effect of LAB on cows intramammary inoculated at dry-off period. The results show that probiotic microorganisms would be a natural and effective alternative in the prevention of bovine mastitis at dry-off period. These bacteria could act as immunomodulatory on the host udder immune system and stimulate both, local and systemic defence lines.

Acknowledgements

This work was supported by grants from the Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT-FonCyT) and the Secretaría de Ciencia y Técnica de la Universidad Nacional de Río Cuarto. M. Pellegrino and M.E.F. Nader-Macías are Career Investigator of the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET) and N. Berardo is recipient of a fellowship from CONICET.

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