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ORIGINAL ARTICLE

Probiotic properties of vaginal lactic acid bacteria to prevent metritis in cattle

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Abstract

Aims: The isolation of bovine vaginal lactic acid bacteria (LAB) and the screening of their beneficial properties to select those that could be used as probiotics in the prevention of bovine metritis were performed.
Methods and Results: Out of 76 *Lactobacillus* sp. and seven *Streptococcus* sp. strains, a small number showed high- and medium hydrophobicity when the microbial adhesion to hydrocarbons method (MATH) was applied. In the agar plate diffusion test, a large number of strains inhibited vaginal bovine *Escherichia coli* 99/14 and human *E. coli*. This inhibition was due to acid. Only a few strains inhibited *Actinomyces pyogenes* 96/393, a pathogen isolated from bovine metritis. This inhibition remained after neutralization. The taxonomic identification of the selected strains was carried out by an amplified ribosomal DNA restriction analysis (ARDRA). Most of the strains were identified as *Lactobacillus fermentum*, a few as *Lactobacillus gasseri* and one as *Lactobacillus rhamnosus*.
Conclusions: Bovine vaginal lactobacilli strains have differential surface properties. The strains selected are capable of inhibiting specific metritis pathogens.
Significance and Impact of the Study: Our results can be applied for future studies to design a probiotic product to prevent metritis in dairy postpartum cows.

Introduction

Uterine infections are one of the main causes of infertility in postpartum cows (Rajala-Schultz and Gröhn 1999). These infections increase the interval from calving to first recorded oestrous and the rate of services to conception. These variables reduce the reproductive performance in the dairy herd (Lewis 1997). The pathogenic micro-organisms associated with postpartum metritis are *Actinomyces pyogenes* (Griffin *et al.* 1974), *Escherichia coli*, *Fusobacterium necrophorum* and *Bacteroides melaninogenicus* (Lewis 1997; Bondurant 1999).

The traditional antimicrobial treatment applied for metritis may lack efficacy and does not improve the reproductive performance of the treated animals (Gilbert and Schwark 1992); it can also leave residues in the milk, which must then be discarded (Lowder 1993).

The economic and health-related problems described led us to study the probiotic effect of lactic acid bacteria (LAB) in the vaginal tract of cows. The objective of the present work was to isolate *Lactobacillus* and *Streptococcus* strains from the bovine vagina and to perform the screening and selection of these micro-organisms with beneficial characteristics to be potentially used as vaginal probiotics for the prevention of bovine metritis. The beneficial effects of lactobacilli on the human vagina have been associated with the adhesion ability and production of antagonistic substances against pathogens (Lepargneur and Rousseau 2002). Screening was performed taking into account these two probiotic characteristics: adhesion properties and inhibition of pathogenic micro-organisms specific for metritis. The first is a consequence of the bacterial surface characteristics, which could be predicted by two different *in vitro* assays: hydrophobicity and haemagglutination

(HA) capabilities (Marshall and Cruickshank 1973; Rosenberg and Doyle 1990; Reid and Bruce 2001). Antagonistic effects were studied against two common pathogens capable of producing metritis: *Act. pyogenes* and *E. coli*.

In this paper, we present the screening and the genetic identification of bovine vaginal *Lactobacillus* strains selected by using the amplified ribosomal 16S-DNA restriction analysis (ARDRA) standardized by Ventura *et al.* (2000).

Materials and methods

Bacterial strains and culture conditions

Lactobacillus and *Streptococcus* strains were isolated from bovine vaginal samples and subcultured in MRS (de Man *et al.* 1960) broth at 37°C for 12–14 h. The cultures were stored in milk-yeast extract (13% nonfat milk, 1% yeast extract) at −20°C.

Vaginal samples

Samples were obtained from 15 nulliparous heifers and 15 adult cows (Nelore × Hereford and Criolla breed). The animals that had no history of metritis infection were selected. They were fed *ad libitum* in the Campo Experimental Leales (Leales Experimental Field) of INTA (National Agricultural and Livestock Technology Institute) in the province of Tucumán, Argentina. The vulvar area was washed with povidone–iodine and water and a disposable speculum was inserted into the vagina to swab the posterior area. Samples were collected in LBS (lactobacilli selective media) broth (Rogosa and Sharpe 1960) and LAPT (1% yeast extract, 1·5% peptone, 1% tryptone, 0·1% Tween 80; Raibaud *et al.* 1973) broth.

Isolation and identification of micro-organisms

Lactobacilli were enriched in LBS broth and then grown on LBS agar (Rogosa and Sharpe 1960) and LAPTg agar (Raibaud *et al.* 1973). The LAPT samples were plated on LBS and MRS agars. The plates were incubated under aerobic conditions with the exception of LBS plates, which were incubated in a 5% CO_2 atmosphere for 24–48 h at 37°C. Identification to genus level was performed by morphological characteristics, Gram staining, catalase reaction, NO_3 reduction and indol production. The identification of micro-organisms was carried out according to Bergey's Manual of Systematic Bacteriology (Kandler and Weiss 1986).

Pathogenic micro-organisms

Escherichia coli 99/14 and *Act. pyogenes* 96/393 were isolated from bovine clinical samples of metritis. They were kindly supplied by INTA, Balcarce, Argentina. Human *E. coli*, isolated from a urogenital infection, was provided by the Instituto de Microbiología de la Universidad Nacional de Tucumán, Argentina. *Escherichia coli* strains were cultured in BHI (brain heart broth) and LAPTg, and *Act. pyogenes* in BHI agar enriched with vitamin K (1 mg ml^{-1}) and haemin (0·5 mg ml^{-1}) sterilized by filtration. Both micro-organisms were stored in 30% glycerol at −70°C.

Surface characteristics

For the assessment of the degree of surface hydrophobicity, the microbial adhesion to hydrocarbons method (MATH) described by Rosenberg *et al.* (1983) and modified by Geertsema *et al.* (1993), was used with three different hydrophobic solvents: hexadecane, xylene and toluene. Each test was performed in triplicate and the means of the results were calculated.

HA ability

Bacterial suspensions of 10^9 CFU ml^{-1} (colony-forming units per millilitre) were prepared and serially diluted (1/2, 1/4, 1/8, 1/16) in round-bottom microplates. Bovine red blood cells obtained by venipuncture were centrifuged, washed twice with saline, adjusted to 2% (v/v) and added to the wells. The plates were mixed and incubated at 37°C for 30 min and then at 4°C for 6 h. The HA titre was visually determined. The results were expressed as the inverse of the highest dilution of bacteria producing red blood cell agglutination.

Detection of antagonistic substances against pathogenic micro-organisms

The plate diffusion technique was used as described by Jack *et al.* (1995). The inhibitory effect of the supernatants of 83 LAB strains on the growth of the selected pathogens was tested. The supernatant fluids of LAB grown in MRS broth were filter sterilized (millipore filters, pore of 0·22 μm and diameter of 25 mm), and aliquots were neutralized with NaOH (2 mol l^{-1}). The pure and neutralized aliquots were placed in 4-mm holes, which were punched into BHI plates containing $1{\cdot}5 \times 10^7$ to $1{\cdot}5 \times 10^8$ CFU pathogenic micro-organisms. *Escherichia coli* 99/14 plates were incubated for 24 h at 37°C and *Act. pyogenes* 96/393 for 48 h at 37°C under a 5% CO_2 atmosphere, and the diameters of the inhibition halos were measured. The negative controls were performed with fresh MRS medium and no halos were observed around them. The positive controls were

obtained with inhibitory strains previously isolated from other environments.

Taxonomic identification of selected *Lactobacillus* strains by ARDRA

The PCR–ARDRA method was used for the genetic identification of the species of *Lactobacillus* isolated from bovine vagina. The ability to produce CO_2 from glucose, to grow in MRS-Vancomycin (12 μg ml^{-1}) and to ferment ribose (in MRS fermentation broth with 10-g l^{-1} ribose) was tested to classify the micro-organisms according to their metabolic group.

Amplification and restriction analysis of 16S-rRNA gene
Colonies were picked from cultures in plates and suspended in 20 μl of micro-LYSIS reagent (LABOGEN SRL). Suspensions were treated with two cycles of 5 min at 65°C, 2 min at 96°C, 4 min at 65°C, 1 min at 96°C, 1 min at 65°C and 30 s at 96°C.

DNA amplification was carried out with 2 μl of lysis product and 48 μl of Mega Mix reagent (LABOGEN SRL) with P_0 and P_6 primers added (Di Cello and Fani 1996) under the following conditions: 3 min at 94°C of initial denaturation, 30 cycles consisting of 30 s at 94°C, 30 s at 58°C and 2 min at 72°C and a final extension at 72°C for 7 min.

The PCR products were electrophoresed in 0·8% agarose gels. Then they were digested with four restriction enzymes (*Sau* 3AI, *Hinf* I, *Hinc* II and *Dra* I). The DNA digested was analysed on 2·5% agarose gel using a 50 bp DNA Ladder (Gibco, BRL) as a molecular-weight marker. These profiles were compared with those obtained by computer analysis of the restriction sites in the 16S rDNA sequences.

Statistical analysis

All the experiments were performed in triplicates and the means of the data were calculated. The statistical analysis of the data was performed using the software MINITAB (version 14). *t*-test was applied to determine statistically significant differences between the two groups of animals studied. The correlation between the probiotic properties was performed by calculating the Pearson coefficient.

Results

Isolation and identification of micro-organisms

Seventy-six strains of *Lactobacillus* sp. and seven strains of *Streptoccus* sp. were isolated from the two groups of sampled animals. They were later studied for their probiotic properties.

Surface hydrophobicity

The Pearson coefficients calculated from hydrophobicity values (hexadecane–xylene 0·96; hexadecane-toluene 0·95 and xylene–toluene 0·97) show a high correlation between the results obtained with the three solvents employed (Fig. 1). The majority of the strains showed low hydrophobicity indexes. The mean was 6.3 and 5.9 for strains isolated from nulliparous heifers and stimulated cows, respectively. No statistically significant differences were found in the hydrophobicity indexes measured in the strains isolated from the two groups of animals ($P > 0{\cdot}05$). However, the hydrophobicity values of the strains isolated from nulliparous heifers were more evenly distributed.

HA reaction

None of the strains tested showed a positive HA reaction with bovine red blood cells.

Detection of antimicrobial substances against pathogenic micro-organisms

The screening of the production of antagonistic substances by LAB culture supernatants on *E. coli* growth revealed a high degree of inhibition of this pathogen. This inhibition can be attributed to organic acid production, as it was completely lost after the treatment with NaOH (2 mol l^{-1}). No statistically significant differences between both groups were observed when the diameters of the inhibition halos on the three pathogens were analysed ($P > 0{\cdot}05$). Only six of the strains that inhibit bovine *E. coli* were unable to inhibit the human *E. coli* strain.

A small number of strains were able to inhibit *Act. pyogenes* 96/393. This effect was not produced by organic acids, because the inhibition halos were not modified by the neutralization of the supernatant.

The low values of the Pearson coefficients obtained from the analysis of the probiotic properties showed that there is no correlation between these properties. These results agree with the matrix plot represented in Fig. 2. The majority of the strains showed a low hydrophobicity index and *E. coli* inhibition capability; only a few strains with high hydrophobicity exhibited inhibitory activity against *E. coli*. From the latter group, some potentially probiotic strains were selected. Six other strains showing inhibitory effects against the three pathogens assayed were also selected (Fig. 2).

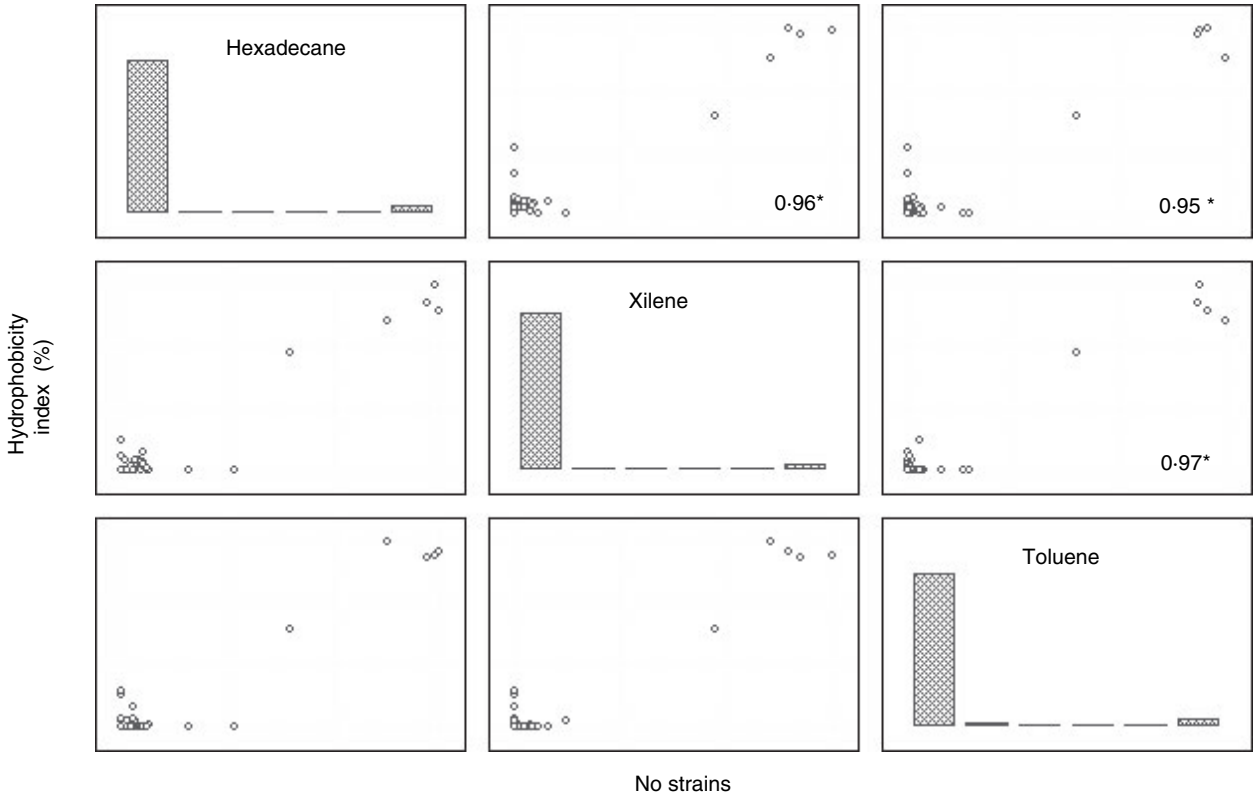

Figure 1 Correlation between the degree of hydrophobicity (%) tested by microbial adhesion to hydrocarbons method with hexadecane, xylene and toluene. Pearson coefficient values are represented by the numbers included in the boxes *, Pearson correlation.

Taxonomic identification of selected *Lactobacillus* strains by ARDRA

Most of the digestion profiles obtained from the amplified 16S rDNA gene of the strains selected agreed with the theoretical profile corresponding to *Lactobacillus fermentum* (Fig. 3a). The facultative heterofermentative strain was identified as *Lactobacillus rhamnosus* (Fig. 3b). Four strains of homofermentative lactobacilli showed a restriction profile that corresponded to *Lactobacillus gasseri* and only one could not be identified by this system (Fig. 3b).

Discussion

Bovine uterine disorders during puerperal periods reduce the reproductive efficiency of dairy cows, increase herd health costs, reduce feed consumption and cause a reduction in milk production. Moreover, some treatments contaminate the milk. Such situations force producers to cull cows that would otherwise be productive and remain in the herd, increasing costs (Lewis 1997).

The use of probiotic products in the urogenital tract could prevent the undesirable consequences of the antibiotics routinely used in the treatment of infections (Reid and Bruce 2001). Except for the report of Kummer *et al.* (1997), who described the stimulation of the cell defence mechanisms of the bovine endometrium by inoculation of dairy *Lactobacillus* strains, there is no available literature concerning the role of LAB in the bovine reproductive tract.

We reported the dynamics of the bovine vaginal microflora during the oestrous cycle (Otero *et al.* 1999) and during the growth of heifers (Otero *et al.* 2000). The *Lactobacillus* population increases under hormonal influence, particularly oestrogens. Our research group demonstrated the urogenital protective effect of lactobacilli against a pathogen challenge in a mouse experimental model (Silva de Ruiz *et al.* 2001). In the present work, a screening of the LAB strains with probiotic potentialities was performed on bacteria isolated from the same ecological niche, where they will be potentially applied on the basis of the fact that host specificity is an important characteristic for the colonization of the indigenous microflora (Savage and Kotarsky 1979; Huey *et al.* 1984). To evaluate the probiotic properties of the isolated strains, three *in vitro* assays were used to study their

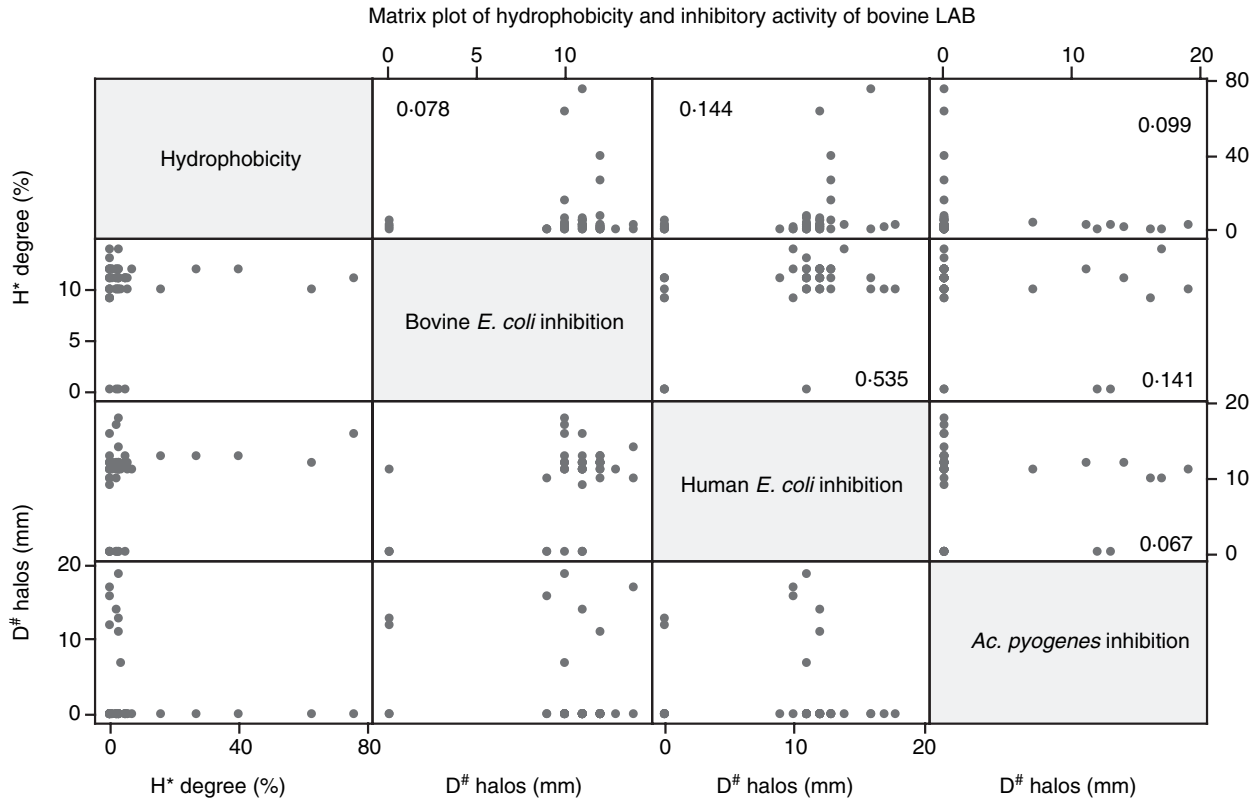

Figure 2 Relationship between the probiotic properties measured by hydrophobicity degree (*) and the diameters of the inhibition halos (#) on pathogens specific to bovine metritis. The Pearson coefficients included in the boxes suggest the lack of correlation between these properties.

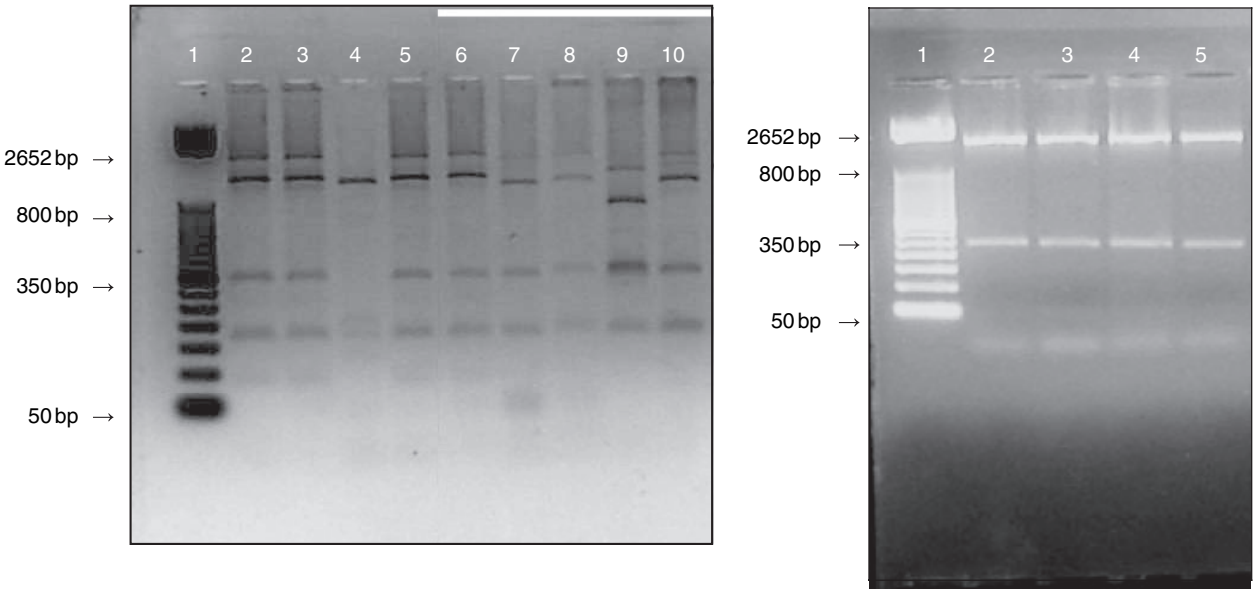

Figure 3 Amplified ribosomal DNA restriction analysis (ARDRA). (a) Restriction profiles of the 16S-rRNA gene obtained using *Sau* 3AI. *Lactobacillus gasseri* CRL1412 (lane 2); *L. gasseri* CRL1421 (lane 3); CRL1461 (lane 4); *L. gasseri* CRL1460 (lane 5); *L. gasseri* 1556 (lane 6); *Lactobacillus fermentum* CRL1573 and CRL1565 (lanes 7 and 8); *Lactobacillus rhamnosus* CRL1557 (lane 9) and *L. fermentum* CRL1577 (lane 10). (b) Restriction profiles of the 16S-rRNA gene obtained using *Dra* I of *L. gasseri* CRL1412, 1460, 1421 and 1556 (lanes 2, 3, 4 and 5, respectively).

surface characteristics and their inhibitory effect on some pathogens responsible for metritis. Bacterial adhesion to tissues is considered the first and key step in the colonization of tissues by micro-organisms and may be influenced through hydrophobic nonspecific interactions (Ofek and Doyle 1994). Thus, probiotic micro-organisms can prevent pathogen access either by steric interactions or specific blockage of cell receptors (McGroarty 1993). Bacterial adhesion has been associated with the attachment to a variety of substrata, and the hydrophobicity of the bacterial cell surface is used to predict adhesion (Rosenberg and Doyle 1990). In the bovine LAB population studied, the number of strains with high hydrophobicity was lower than that reported for human vaginal lactobacilli (Ocaña *et al.* 1999). The micro-organisms isolated from adult animals, which received hormonal treatment, did not show surface properties different from those of the heifers. On the whole, no differences were found between the hydrophobicity degrees obtained with the three solvents tested (Fig. 1), which demonstrates that the assay can be performed with any of them.

The HA reaction is used to monitor bacterial adhesion as the erythrocyte surface shares the ontogenetic origin of the cells from other tissues (Ofek and Doyle 1994), particularly the epithelial tissue cells (Madigan *et al.* 1997). Andreu *et al.* (1995) studied the HA capability of human vaginal lactobacilli. Based on these reports, HA was included in the present screening, but none of the LAB strains reacted with bovine erythrocytes. Hynönen *et al.* (2002) reported a *Lactobacillus brevis* strain harbouring a high capability to adhere to epithelial cell lines and lacking HA properties. Further assays will be performed to know if the hydrophobic strains that are unable to haemagglutinate can adhere to the bovine vaginal epithelial cells or not.

Another important property that accounts for the *in vitro* selection of vaginal probiotic strains is the inhibition of pathogenic micro-organisms (Lepargneur and Rousseau 2002). The pathogenic micro-organisms employed in this study are associated with acute (bovine *E. coli*) and chronic (*Act. pyogenes*) endometritis in cows (Torres *et al.* 1994; Bondurant 1999; Zerbe *et al.* 2001); the source of both strains used was clinical material from metritis.

Our results showed that the majority of the strains were able to inhibit *E. coli* 99/14, mainly by acid production. Alakomi *et al.* (2000) reported that the outer membrane of *E. coli* was disrupted by lactic acid and that this permeabilization of the gram-negative cell wall increased its susceptibility to other antimicrobial substances. Only a few strains inhibited *Act. pyogenes* 96/393; this inhibition remaining after the neutralization step. Future studies will be focussed on the evaluation of the chemical nature of this inhibition.

Accurate taxonomic characterization is required for probiotic micro-organisms (Reid *et al.* 2003). In the present paper, the ARDRA system, a rapid and easy molecular method, was applied to identify a great number of strains in only a few assays. It was also used by Ventura *et al.* (2000) to identify human vaginal and intestinal lactobacilli. However, although this is not an epidemiological study, there was an increased prevalence of the *fermentum* species among the *Lactobacillus* identified. Different results were obtained by Juárez Tomás *et al.* (2005) who, using ARDRA, identified human probiotic vaginal *Lactobacillus*, *L. gasseri* being the predominant species.

Although the selection of probiotic micro-organisms must be initially performed through the application of *in vitro* characteristics, the definitive application and clinical evidence of their *in vivo* effects should be evaluated through the use of animal models or specific hosts, which is the next step in our work. The final objective of our group is the inclusion of these strains in a pharmaceutical product to be used in cows for the potential prevention of metritis in the postpartum.

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