J Ind Microbiol Biotechnol (2008) 35:1387–1395
DOI 10.1007/s10295-008-0438-2

ORIGINAL PAPER

Advances in the design of probiotic products for the prevention of major diseases in dairy cattle

M. Elena Fátima Nader-Macías · M. Claudia Otero · M. Carolina Espeche · Natalia C. Maldonado

Received: 22 March 2008 / Accepted: 30 July 2008 / Published online: 30 August 2008

Abstract Probiotics are frequently used for multiple objectives and the majority of applications are performed both in human beings and in different animals. The specificity of species showed by the members of the indigenous microbiotia led us to isolate lactic acid bacteria (LAB) from their natural bovine ecosystems and to select those with probiotic potentiality. The beneficial probiotic LAB were isolated from the vaginal tract and mammary gland of adult heifers, faeces and oral cavity of newborn calves. Bacterial strains and their numbers varied in each tract under study. Their surface properties, evaluated using the MATH test, revealed that the higher number of low hydrophobic strains were isolated from vaginal tract, mammary gland or faeces. The capability of autoaggregation also differed with higher degree in those isolated from vagina, whereas those that originated in the mammary gland or faeces showed low autoaggregation characteristics. The production of antagonistic substances showed that the production of hydrogen peroxide was higher in the strains isolated from the vaginal tract, while those able to produce organic acid were isolated from all the environments. A few strains were able to produce bacteriocins and were isolated only from the mammary gland and faeces, but not from the vaginal tract. Further studies are being performed to complete the functional characterization of the strains in order to evaluate their survival in the environmental conditions of each tract. Finally, the selected strains could be combined for the design of probiotic products to be used both in adult cows and in neonates, based on the host-specificity, due to their autochthonous origin and their potential use in the tract where they were isolated.

Keywords Probiotic lactic acid bacteria · Veterinary product · Health status · Metritis · Mastitis · Calf diarrhea

M. E. F. Nader-Macías (✉) · M. C. Otero · M. C. Espeche · N. C. Maldonado
CERELA-Centro de Referencia para Lactobacilos-CONICET, Chacabuco 145, 4000 San Miguel de Tucumán, Argentina
e-mail: fnader@cerela.org.ar

Introduction

Mastitis, endometritis, and calf diarrhea are the most common diseases in dairy cattle, and affect the productivity parameters as well as the fertility degree: calving to conception intervals, first service, and conception rate. They cause the main economic losses in farms and involve both adults and neonates. They are also associated with: decreasing average milk production (kg), average fat and protein content (%), and increasing calf mortality rate (%) and lowering calf birth weight (kg) [10, 13].

Traditional veterinary medicine is focused on diagnostics and antibiotic therapeutic applications that produce residues in animal food products and cross resistance between zoonotic pathogens and human colonizing microorganisms. The new concept of health management has been defined as the promotion of health, improvement of productivity, and prevention of diseases in animals within the economic framework. It also includes the animal's welfare, food safety, public health, and environmental sustainability [8, 12]. For the above reasons, there is a wide interest to apply alternative therapies that includes the use of probiotics (defined as live microorganisms that, when administered in adequate amounts confer a health benefit on the host) to prevent infections in humans and other animals, which have been mainly studied in the gastrointestinal and urogenital tracts [14]. Based on the host specificity exerted by the

indigenous microbiota, in previous works the isolation and identification of the dominant microorganisms from different ecological niches (bovine urogenital tract, mammary gland and newborn oral cavity-faeces) were carried out [9, 17, 21]. The indigenous microbiota that colonize the cow vaginal mucosa is stable and include: Gram positive cocci (*Staphylococcus* spp., *Streptococcus* spp. and *Enterococcus* spp.) and *Lactobacillus* spp. [21]. Also, spore forming *Bacillus* spp. was isolated from the teat canal and intestinal tract. Some specific and beneficial characteristics were evaluated in all the (LAB) strains, and published previously. As the host-specificity phenomenon is being demonstrated for specific strains that are part of the indigenous microbiota [30, 32, 33], we have previously isolated strains from all the environments under study [9, 17, 21].

Our research group is involved in the design of veterinary probiotic products to restore the ecological balance of different tracts for the prevention of infections in dairy cows and calves. Our hypothesis is to first promote the colonization of the vaginal mucosa of pregnant cows with specific strains for the prevention of post-partum metritis. If continuously administered to pregnant cows, these vaginal probiotics would increase the first contact of these microorganisms with the sterile, unprotected neonate intestinal tract, during the passage through the vaginal tract at birth. Later, the calf would be in contact with the mammary gland during the first days of life, when the intestinal tract-isolated probiotic could also be administered to stimulate the immature immune system. Infections of the mammary gland could also be prevented by specific strains. With all this considerations, the objective of the present work was to perform a comparative evaluation of the beneficial properties of selected beneficial LAB isolated from three different bovine niches (digestive tract, vagina and teat canal).

Materials and methods

Isolation of LAB strains

The isolation and identification of LAB from the different bovine ecosystem were described previously [9, 17, 21, 23], and taken from vagina and teat canal of adult heifers, oral cavity and rectum of young health calves (0–4 months of age) and colostrum. The samples were collected in MRS 1% agar pH 4.5 [7] and in 0.7% agar LAPT [29] as transport medium. Also milk samples were taken for the isolation of LAB from mammary gland. Strains were stored in milk yeast extract at −20 °C. In some specific cases, the quantification of different microbial populations was performed by using selective culture media.

Taxonomical identification of strains

The microorganisms were taxonomically identified in genus by phenotypic tests (Gram stain, morphology, catalase activity, nitrate reduction, and indole production) and in some cases in species by API CHL50 system. In order to determine the metabolic group of the isolated microorganisms, the gas production from glucose [11] (Gibson test) and gluconate was evaluated. The cocci identification from faeces and oral cavity of newborn calves was performed by the standard tests described in the Bergey's Manual of Determinative Bacteriology [1]. Microscopic disposition, growth in 40% bilis, esculin hydrolysis, growth at 45 °C, 10 °C, and 6.5% NaCl, presence of pyrolidonyl arylaminase and leucinoaminopeptidase enzymes were assayed to identify this group.

Genetic identification

The cocci isolated from bovine vagina were identified by biochemical tests. Identification of the species of *Lactobacillus* isolated from bovine vagina was performed by API 50 CHL system (Biomerieux, France) and then by an amplified ribosomal DNA restriction analysis (ARDRA) [23]. Colonies were picked and amplification was performed with P_0 and P_6 primers. PCR products were electrophorated in 0.8% agarose gels. Then they were digested with four restriction enzymes (*Sau* 3AI, *Hin*f I, *Hin*c II, and *Dra* I). The digested DNA 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.

The strains isolated from mammary gland and newborn oral cavity-faeces were identified by sequencing the 16S ribosomal gene. Only strains pre-selected for their probiotic properties were genetically identified. Colonies were picked and amplification was performed with MLB and PLB primers. The obtained amplicons were sequenced and then identified through the Genbank database by using the BLAST algorithm.

Bacterial surface properties

The microbial adhesion to hydrocarbons was performed as described previously [27] and was used to determine the hydrophobic nature of the cell surface. The degree of hydrophobicity was quantified through the index and the strains classified as high (H), middle (M) or low (L) hydrophobic strains.

The quantification of the autoaggregation was assessed according to the technique described by Ocaña and Nader-Macías [19], by monitoring the OD_{600} nm modifications of

cellular suspensions in PBS buffer for 2 h, and through the auto-aggregation index.

Screening of antagonistic activity

H_2O_2 production

The H_2O_2 production was qualitatively determined according to the previously published technique [16]. The plate method employing horseradish peroxidase incorporated in tetramethyl-benzidine (TMB) agar medium was used as a first screening of H_2O_2 production. A score was assigned based in the intensity of blue colour of colonies in TMB-MRS plates: HP (high producers), P (producers), LP (low producers), and NP (non producers).

Antagonistic activity

The inhibitory effect of the culture supernatant was assayed by the plate diffusion technique [15]. Briefly, LAPTg agar (1% agar) plates with 10^6–10^7 CFU $ml^{-1}$of each pathogen were prepared, as described previously [16]. Standardized aliquots (25 µl) of cell-free supernatant obtained from the third subculture of the LAB strains grown in LAPTg or MRS broth were placed into wells made into the agar pathogens plates. The inhibition properties were studied with different pathogens that are related or responsible for infections or isolated from each one of the tracts under study.

Pathogenic microorganisms

The pathogens used through this study were: *Escherichia coli* 99/14 and *Act. pyogenes* 96/393 isolated from bovine clinical samples of metritis (supplied by INTA-National Agricultural and Livestock Technology Institute, Balcarce, Argentina), and human *E. coli*, isolated from an urogenital infection (provided by the Instituto de Microbiología, Universidad Nacional de Tucumán), for LAB from vaginal tract.

For LAB from mammary glands, the pathogens assayed were: *Staphylococcus aureus* Newbould, *S. agalactiae* ATCC 27956, *S. dysgalactiae* ATCC 27957, *E. coli*, *Streptococcus uberis*, *S. dysgalactiae* (provided by Dr. Luis Calvinho INTA-Rafaela), *S. aureus* isolated from bovine mastitis (provided by Dr. Cristina Bogni (UNRC). Coagulase negative S*taphylococcus* was isolated in our laboratory from bovine mastitis and *Listeria monocytogenes* Scott A was provided by Dr. Sergio Pasteris.

For strains isolated from newborn faeces, *Salmonella* Dublin (isolated from a calf clinical sample in our laboratory), *Yersinia enterocolitica (*provided by Instituto Malbran), *E. coli*, *Staphylococcus aureus*, *Staphylococcus aureus* Newbould *S. uberis*, *Streptococcus dysagalactiae* (isolated from bovine mastitis, provided by INTA-Rafaela*)*, *Klebsiella* sp. (a biofilm producer strain) were studied. The activity against *Salmonella thyphimurium*, *Salmonella infantis*, and *Listeria* sp. was also evaluated (these pathogens were provided by the Instituto de Microbiologia "Luis Verna" of the University of Tucumán, Argentina).

The plates were incubated for 2 h at room temperature and then 24 h at 37 °C. An inhibitory zone of at least 2-mm diameter of around the agar plate holes was defined as a positive result.

The pathogens were grown in their specific culture media, and subcultured three times before being used. Pathogens used for the screening of the strains isolated from mammary gland, metritis, and calves digestive tract were subcultured in LAPTg or Brain Heart Infusion broth (Britania, Buenos Aires, Argentina) at 37 °C (except *L. innocua* that was incubated at 30 °C), and stored in BHI-glycerol at −20 °C. *A. pyogenes* was growth in BHI agar enriched with K vitamin (1 mg/ml) and haemin (0.5 mg/ml) sterilized by filtration and stored in 30% glycerol at −70 °C.

Statistical analysis

The experiments were performed in duplicate. The statistical analysis of the data was performed by using the software MINITAB (version 14). The correlation between the surface properties (hydrophobicity and autoaggregative capability) was determined by calculating the Pearson coefficient.

Results

Identification of the strains

A total of 281 bovine LAB strains were isolated from different bovine tracts: 82 lactobacilli strains were isolated from vaginal swabs, 87 cocci and 6 bacilli from milk, 7 cocci and 2 bacilli from teat canal. Ninety-seven strains were isolated from newborn calves: 13 strains from oral cavity (11 cocci and 2 bacilli) and 84 strains isolated from faeces (50 bacilli and 34 cocci). Three strains were also isolated from calostrum. All of these strains were identified by phenotypical tests, and classified into the lactic acid bacteria group [9, 17, 21, 23].

Genetic identification

Most of the vaginal pre-selected strains were *Lactobacillus fermentum* (80%). The others microorganisms identified were *L. gasseri*, *L. rhamnosus*, and *L. delbrueckii* subsp. *delbruekii* (identified by API CHL50) [23].

Most of the strains isolated from the mammary gland and pre-selected for their beneficial properties were identified as *Streptococcus bovis* (38.1%), *Weissella paramesenteroides* (14.3%) and *Lactococcus lactis* subsp. *lactis* (9.5%). The other isolated species were *Enterococcus hirae*, *Enterococcus mundtii*, *Enterococcus saccharominimus*, *Streptococcus agalactiae*, *Lactobacillus brevis*, *Enterococcus faecium*, *Enterococcus saccharolyticus*, *Lactobacillus mucosae*, *Lactobacillus plantarum*, *Lactobacillus reuteri*, and *Lactococcus garvieae* [9].

The genetic identification of the pre-selected bacilli isolated from newborn calves was: *Lactobacillus johnsonii* (40%), *Lactobacillus mucosae* (13%), *Lactobacillus murinus* (13%), *Lactobacillus salivarius* (13%) and *Lactobacillus amylovorus* (6%). The pre-selected cocci were identified as *Enterococcus faecium* and *S. bovis* [17].

Results of the quantification in different tracts in cattle and newborn calves showed the heterogeneity of the population and the number of microbial cells in those ecosystems. In the mammary gland the LAB counts in MRS agar plates were between 10^0 and 10^3 CFU/ml. The isolation of the viable cells of total aerobic bacteria from faecal samples was at levels between 10^7 and 10^9 CFU/samples. LAB and Enterobacteria were present at 10^6–10^8 CFU/samples. In the vaginal tract of healthy cows the aerobic bacteria reached 10^5 CFU/samples and the LAB ranged between 10^1 and 10^2 [9, 17, 21].

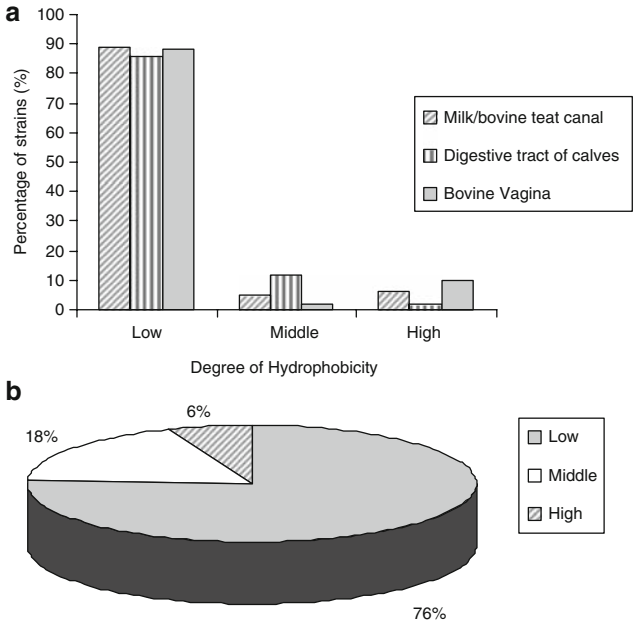

Fig. 2 **a** Distribution of the strains according to their degree of hydrophobicity (MATH in hexadecane) and the origin of isolation. **b** Distribution of the 281 isolated strains according to their degree of hydrophobicity. Low: 0–33.33%. Middle: 33.34–66.66%. High: 66.67–100%

Screening of surface properties

The screening of the surface characteristics of each individual strain, as degree of hydrophobicity by MATH and bacterial aggregation, are summarized in Fig. 1. Most of the strains (80–90%), independently from their origin, showed a low degree of hydrophobicity (≤30%), that suggests that this is a characteristic of the microbiota that colonize the cow mucosa, as shown in Fig. 2. Only 7% of the microorganisms expressed an aggregative phenotype, as shown in Fig. 3. There was no correlation between both surface properties (Pearson correlation coefficient: 0.145).

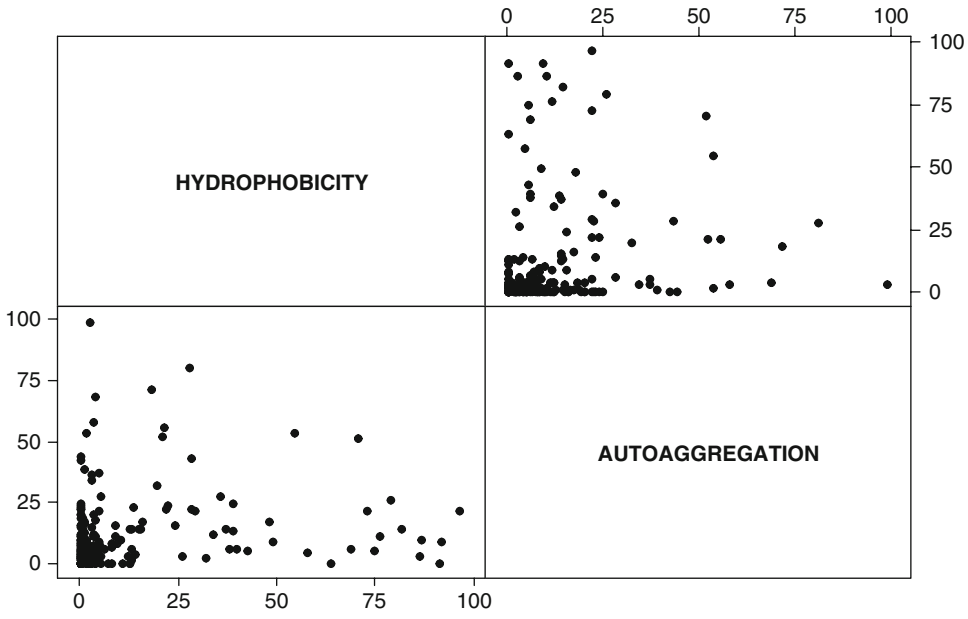

Fig. 1 Matrix plot of the relationship between hydrophobicty and autoaggregation of the lactic acid bacteria isolated from vaginal tract and mammary gland of adult heifers, faeces, and oral cavity of newborn calves. There was no correlation between these two properties (Pearson correlation coefficient: 0.145)

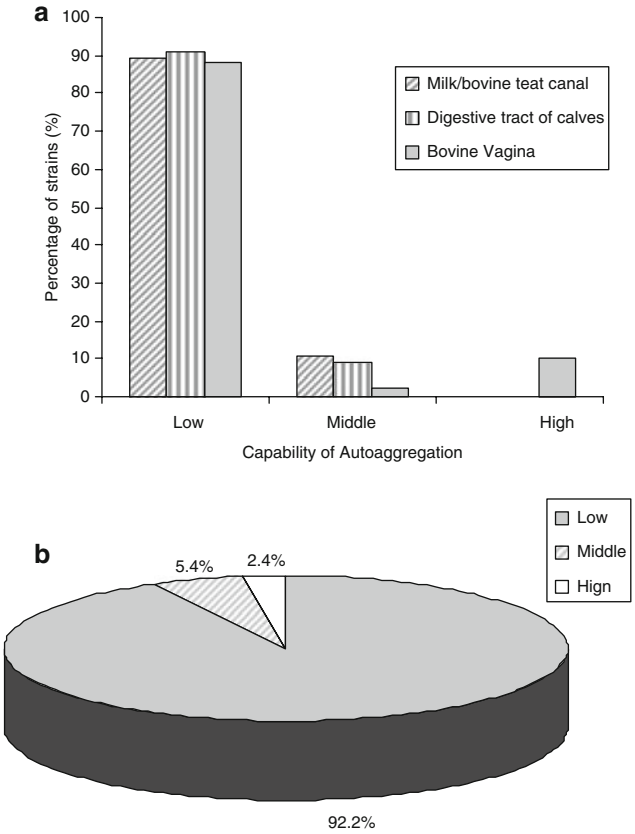

Fig. 3 **a** Distribution of the strains according to their degree of autoaggegation and their origin. Most of the isolated strains showed low autoaggregative capability. **b** Distribution of the 281 isolated strains according to their autoaggregative capability. Low: 0–33.33%. Middle: 33.34–66.66%. High: 66.67–100%

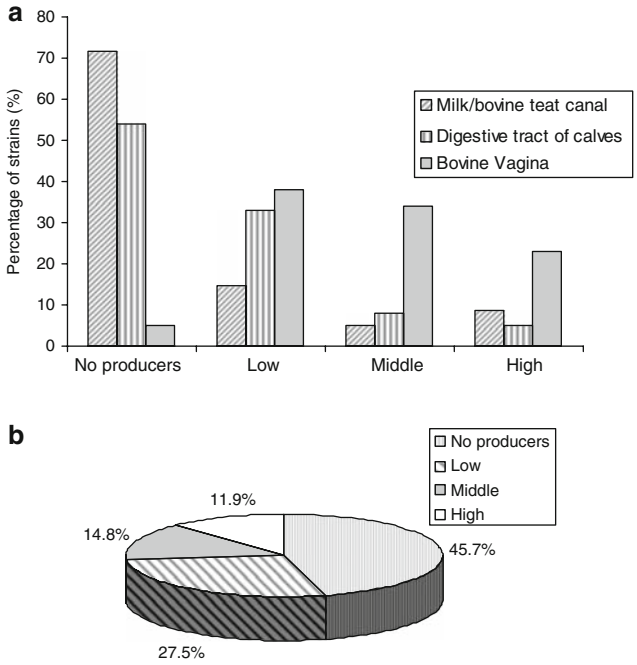

Fig. 4 **a** Distribution the strains according to their H_2O_2-production and their original niche of isolation. Strains were classified according to the intensity of their colour in TMB-agar plates. **b** Distribution of the 281 isolated strains according to their degree of H_2O_2-production

Production of antagonistic substances

Hydrogen peroxide

The production of antagonistic substances (lactic acid, hydrogen peroxide and bacteriocin) and pathogen growth inhibition (20 strains of pathogens that cause specific infections to each tract) showed that the spectrum of antagonistic substances produced varied according to the ecological origin of the micro organisms: 95% of vaginal LAB and 28.4% udder strains showed different degree of H_2O_2 production, while only 21% of digestive strains expressed this characteristic [9, 17, 25]. These results are showed in Fig. 4.

Lactic acid production

More than 80% of the LAB isolated from vagina and digestive tract produce lactic acid, whereas only 18.6% from the teat canal released these metabolites in high amounts [9, 17, 23]. The organic acids are mainly responsible for pathogen inhibition in the vaginal and digestive tracts. The lactic acid production of the strains isolated from teat canal and mammary gland is variable between different bacteria; however, the assayed pathogens were sensitive to the acid produced by the strains isolated from this ecological niche. The correlation between the concentration of organic acids produced and the final pH in broth is summarized in Table 1.

Bacteriocin production

Three strains isolated from the teat canal were able to produce bacteriocin like substances against the following pathogens: *Staphylococcus aureus*, *Staphylococcus aureus* Newbould, *Streptococcus agalactiae* ATCC 27956, *S. uberis*, coagulase negative S*taphylococcus* and *Listeria monocytogenes* Scott A [9]. In all the assays performed, some LAB were able to inhibit the growth of pathogenic strains specific from the original isolation niches. One strain isolated from newborn faeces produces a substance characterized as bacteriocin, active against *Listeria* sp., *Yersinia enterocolitica*, and *S. uberis* [17]. The summary of the inhibitory substances produced, and the pathogen inhibited are summarized in Table 2.

Discussion

The design of a probiotic product to be applied in a tract or animal must include strains with specific characteristics.

Table 1 Inhibition of pathogenic microorganisms by production of organic acids in strains isolated from different bovine tracts

Origin of strains	Percentage of strains that inhibit pathogens by organic acid[a]	Inhibited pathogens	Lactic acid [g/ml] (intervals)	pH of the supernatant (intervals)
Vaginal tract	94.4	*E. coli*, *A. pyogenes*	6–10[b]	3.59–4.2
Mammary gland	18.6	*S. dysgalactiae* ATCC 27957, *L. monocytogenes* Scott A, *S. uberis*, *S. dysgalactiae*, *E. coli*, *S. agalactiae* ATCC 27956	1.38–10.02[c]	4.14–6.0
Newborn oral cavity-faeces	66.6	*E.coli*, *Y. enterocolitica, Sal.* Dublin, *Klebsiella* sp., *S. uberis*, *S. dysgalactiae*, *S. aureus*	6.38–16.39[c]	3.61–5.25

[a] Analyzed by the plate diffusion technique [15]

[b] Determined by UV-Test (Roche, Germany)

[c] Determined by HPLC technique in strains isolated from mammary gland and newborn oral cavity-faeces, as described in the text

The first criterion to be used is the specificity of ecological area, and animal host, described some years ago by pioneer researchers in the area [30] and lately by some recent publications [32, 33]. Even though the reviewed literature describes applications in human beings and other animals, there are many strains frequently isolated from food origin included in probiotic products. Recent studies have shown that the capability of colonization of mucosal surfaces can be facilitated by the autochthonous origin of the strains. The host-specificity demonstrated for strains isolated from different animals, and the increased possibilities to colonize specific mucosal and tracts led our research group to isolate LAB from different bovine tracts. Other basic criterion was that LAB are members of the GRAS (generally regarded as safe) group of bacteria. Then, many strains from vaginal tract, mammary gland or newborn faeces or oral cavity were available in our laboratory, and were screened for their beneficial or probiotic properties. They were first identified at the group level, as LAB. Only strains showing some of the screened properties were later taxonomically identified at the species level using molecular biology or genetically based techniques, mainly because the objective of our studies was not taxonomical.

When trying to select strains with higher colonization capabilities, to increase and facilitate their permanence in the tract, either to form a protective biofilm, or to stimulate the immune system of the host, some surface characteristics have to be assayed. These are the reasons for which the degree of surface hydrophobicity and autoaggregative patterns were assayed in all the isolated strains. The hydrophobicity index can be used to predict the adhesion potentiality of the strains, by facilitating their contact with the hydrophobic surface of the eucaryotic epithelial cells, or with the hydrophilic nature of the mucus that covers the epithelial surface in some specific tracts. The majority of the strains isolated from the three ecosystems were shown to be non-hydrophobic. These results are different from those obtained in strains isolated from human vaginal tract [18], or oral cavity [4]. These differences could indicate some characteristic of specific ecological niches or tracts, but further studies should be performed to increase the knowledge in the specific bovine host.

Autoaggregation can also be used to predict the capability of the strains to form a protective biofilm at the different mucosal areas. The differential behaviour of the planktonic cells and those that are part of a biofilm and their "quorum sensing" capabilities are widely known. Results in the intestinal tract published by the Morelli group [2] showed that a non-aggregative mutant was not able to protect against a chemical-induced diarrhea in mice, while the aggregative native strain was highly effective. The use of bacterial cells with surface properties that could facilitate the biofilm formation should be highly recommended when selecting probiotic bacteria. In the results obtained throughout this paper, most of the strains showed a non-aggregative phenotype that could indicate that these strains might not adhere strongly to the protective mucosa in each specific site. But, again, the assayed properties are not demonstrated in "in vivo" bovine environments or tracts.

The production of inhibitory substances is the next specific requirement for the probiotic-potential strains, mainly because the release of such metabolites, such as hydrogen peroxide, organic acids, or bacteriocins, can participate in the antagonistic effect against pathogens in the specific site of action. In this sense, most of the strains isolated from the three ecological niches are able to produce high levels of organic acids that inhibit many of the assayed pathogens. The pathogens selected for the experiments were those isolated from each ecological niche, or responsible for specific infections in them. One remarkable characteristic is that the strains isolated from the vaginal tract are those that are able to produce the highest detectable level of hydrogen peroxide. These results are similar to those obtained for human vaginal strains [20], supported by the epidemiological results of other scientists that showed a correlation between the presence of H_2O_2 producer strains and a healthy vaginal tract [3, 28].

Table 2 Lactic acid bacteria strains selected by their beneficial properties from different bovine environments

Strain[a]	Hydrophobicity [percentage][b]	Autoaggregation capability [percentage][c]	Epithelial cells adhesion[d]	Inhibited pathogens[e]	Lactic acid [g/l][g]	Production of H_2O_2	Bacteriocin[j]
Strains isolated from bovine vagina							
Lactobacillus gasseri CRL1412	78.0	0.0	22.4 ± 5 bact/cell	*E. coli* 99/14, *Actinomyces pyogenes* 96/393	–	P[h] 0.041 mmol/10^7CFU[i]	–
L. gasseri CRL1421	49.5	0.0	43.9 ± 4.5 bact/cell	*E. coli* 99/14, *A. pyogenes* 96/393, *S. aureus*[f]	10.00	M[h] 1517.92 mmol/10^7CFU[i]	–
L. delbrueckii Subsp. *delbrueckii* CRL1461	74.7	25.0	67.5 ± 8.2 bact/cell	*E. coli* 99/14	7.30	NP[h]	–

Strain[k]	Hydrophobicity [percentage][b]	Autoaggregation capability [percentage][c]	Inhibited pathogens[e]	Lactic acid [g/l][g]	Production of H_2O_2[h]	Bacteriocin[j]
Strains isolated from mammary gland						
Lactococcus lactis Subsp. *lactis* CRL 1655	13.5	14.4	*S. aureus* Newbould, *S. uberis*, Coagulase-negative *Staphylococcus*, *S. aureus*, *S. agalactiae* ATCC 27956	6.06	P	+
Enterococcus mundtii CRL 1656	0.0	11.1	*L. monocytogenes* Scott A	6.49	P	+
E. faecium CRL 1657	4.3	7.8	*L. monocytogenes* Scott A	5.69	LP	+
Strains isolated from faeces						
L. johnsonii CRL 1693	82.0	22.8	*E. coli*, *Sal.* Dublin, *Sal. thyphimurium*, *S. aureus*	10.56	H	–
L. salivarius CRL 1694	73.2	14.5	*E. coli*, *Sal. thyphimurium*, *S. aureus*	–	P	–
E. faecium CRL 1692	0.0	3.0	*Listeria* sp., *Y. enterocolitica*, *S. uberis*	7.79	NP	+
L. murinus CRL 1695	18.2	72.0	*Sal. thyphimurium*	6.38	P	–

NP no producer, *LP* low producer, *P* producer, *HP* high producer

[a] Identified by PCR-ARDRA [23]

[b] Determined by MATH in hexadecane [27]

[c] Autoaggregative capability [19]

[d] Adhesion to vaginal bovine epithelial cells [26]

[e] Plate diffusion technique [15]

[f] Associative cultures [25]

[g] Determined by HPLC technique as described in the text

[h] Production of H_2O_2 in TMB-plates [16]

[i] Determined by a spectrophotometric method [16]

[j] Plate diffusion technique [15]

[k] Identified by sequencing the 16S ribosomal gene

With respect to the production of bacteriocin-like substances, three strains from mammary gland and one from newborn calves were able to produce this type of substances. However, no strains from the vaginal tract were bacteriocin-producers. Bacteriocin-producer strains were also isolated from the mammary gland of cows by other scientists, and later tested as a teat sealer [6, 31]. Recently, the group of Hill [5] has demonstrated that bacteriocin-producing strains protect against *Listeria monocytogenes* in a mice model, which could support the use of these strains able to produce bacteriocins or bacteriocin-like substances for the design of a probiotic product.

Based on the results obtained from the different screening assays carried out in this study, specific strains were selected for further studies. They are summarized in Table 2, which includes all their beneficial or probiotic characteristics.

These characteristics could help to select strains with higher potentialities to be used in a specific host; however, additional tests should also be performed, including the

study of functional and technological properties. Some of these trails are currently being studied in our laboratory. The evaluation of the functional properties of the different LAB strains is supported by their potential applicability in each tract, some of them previously published and others are still being studied. The selected vaginal strains were tested for their adhesion capability to epithelial cells [26] and resistance to the most frequent antibiotic therapies [24]. Some of the selected intestinal strains were assayed for their resistance to the gastrointestinal conditions [17].

Also, some technological properties (freeze drying and storage conditions) were evaluated in vaginal strains with specific characteristics [22], and are under study for the other selected strains.

The selected microorganisms could be potentially included in a wide range of veterinary probiotic products to be applied in different ecological bovine mucosa: reproductive and digestive tracts, and mammary gland. They could improve the health of the animal and promote their productivity. The main reason to support the use of the combined strains is that they could be administered to the vaginal tract of pregnant cows before the calf birth, as a way to prevent urogenital infections of the adult cows, and also as a way to facilitate the colonization of the intestinal tract of the newborn when they pass through the birth-canal. Afterwards, these same or other strains could be administered to the newborn calf to prevent neonatal diarrhea, a cause of high morbidity and mortality rates in this specific age-group. And finally, some strains could be used to prevent mastitis when administered to the mammary gland, as a way to promote at the same time the colonization of the newborn intestinal tract.

In this way, these results contribute to increasing knowledge of some strains, which will be also assayed in animal models, as suggested by ISAPP.

Acknowledgments This work was supported by PICT 13730 from ANPCYT (Agencia Nacional de Promoción Científica y Tecnológica) and PIP 6248 from CONICET.

References

1. Bergey DH, Holt JG (1994) Bergey's manual of determinative bacteriology. Lippincott Williams & Wilkins, Baltimore, MD, USA
2. Castagliuolo I, Galeazzi F, Ferrari S, Elli M, Brun P, Cavaggioni A, Tormen D, Sturniolo GC, Morelli L, Palù G (2005) Beneficial effect of auto-aggregating *Lactobacillus crispatus* on experimentally induced colitis in mice. FEMS Immunol Med Microbiol 43:197–204
3. Cherpes TL, Hillier SL, Meyn LA, Busch JL, Krohn MA (2008) A delicate balance: risk factors for acquisition of bacterial vaginosis include sexual activity, absence of hydrogen peroxide-producing lactobacilli, black race, and positive herpes simplex virus type 2 serology. Sex Transm Dis 35:78–83
4. Colloca ME, Ahumada MC, López ME, Nader-Macías ME (2000) Surface properties of lactobacilli isolated from healthy subjects. Oral Dis 6:227–233
5. Corr SC, Li Y, Riedel CU, O'Toole PW, Hill C, Gahan CG (2007) Bacteriocin production as a mechanism for the antiinfective activity of *Lactobacillus salivarius* UCC118. Proc Natl Acad Sci USA 104(18):7617–7621
6. Crispie F, Twomey D, Flynn J, Hill C, Ross P, Meaney W (2005) The lantibiotic lacticin 3147 produced in a milk-based medium improves the efficacy of a bismuth-based teat seal in cattle deliberately infected with *Staphylococcus aureus*. J Dairy Res 72:159–167
7. De Man JC, Rogosa M, Sharpe ME (1960) A medium for the cultivation of lactobacilli. J Appl Bacteriol 23:130–135
8. Enevoldsen C, Hindhede J, Kristensen T (1996) Dairy herd management types assessed from indicators of health, reproduction, replacement, and milk production. J Dairy Sci 79:1221–1236
9. Espeche MC, Otero MC, Sesma F, Nader-Macias ME (2008) Screening of surface properties and antagonistic substances production by lactic acid bacteria isolated from the mammary gland of healthy and mastitic cows. Vet Microbiol. Submitted
10. Fourichon C, Seegers H, Bareille N, Beaudeau F (1999) Effects of disease on milk production in the dairy cow: a review. Prev Vet Med 41:1–35
11. Gibson T, Abd-El-Malek Y (1945) The formation of carbon dioxide by lactic acid bacteria and *Bacillus licheniformis* and a cultural method of detecting the process. J Dairy Res 14:35–38
12. Gunnarsson S (2006) The conceptualisation of health and disease in veterinary medicine. Acta Vet Scand 47:71
13. Ingvartsen KL, Dewhurst RJ, Friggens NC (2003) On the relationship between lactational performance and health: is it yield or metabolic imbalance that cause production diseases in dairy cattle? A position paper. Livest Prod Sci 83:277–308
14. International Scientific Association for Probiotics and Prebiotics. http://www.isapp.net/
15. Jack RW, Tagg JR, Ray B (1995) Bacteriocins of Gram positive bacteria. Microbiol Rev 59:171–200
16. Juárez Tomás MS, Otero MC, Ocaña VS, Nader-Macías ME (2004) Production of antimicrobial substances in lactic acid bacteria I. Determination of hydrogen peroxide. In: Spencer JFT, Ragout de Spencer AL (eds) Methods in molecular biology. Public health microbiology: methods and protocols, vol 268. Humana Press Inc, Totowa, New Jersey, pp 337–346
17. Maldonado NM, Silva de Ruiz C, Otero MC, Nader-Macias ME (2008) Isolation, selection and beneficial properties of lactic acid bacteria isolated from young calves. J Dairy Res. Submitted
18. Ocaña VS, Bru E, De Ruiz Holgado AA, Nader-Macias ME (1999) Surface characteristics of lactobacilli isolated from human vagina. J Gen Appl Microbiol 45:203–212
19. Ocaña VS, Nader-Macías ME (2002) Vaginal lactobacilli: self and co-aggregation. Br J Biomed Sci 59:183–190
20. Ocaña VS, Pesce de Ruiz Holgado AA, Nader-Macías ME (1999) Selection of vaginal H_2O_2-generating *Lactobacillus* species for probiotic use. Curr Microbiol 38:279–284
21. Otero C, Silva de Ruiz C, Ibáñez R, Wilde O, Ruiz Holgado A, Nader-Macías ME (1999) Lactobacilli and Enterococci isolated from the bovine vaginal during the estrous cycle. Anaerobe 5:305–307
22. Otero MC, Espeche MC, Nader-Macías ME (2007) Optimization of the freeze-drying media and survival throughout storage of freeze-dried *Lactobacillus gasseri* and *Lactobacillus delbrueckii* subsp.*delbrueckii* for veterinarian probiotic applications. Process Biochem 42:1406–1411
23. Otero MC, Morelli L, Nader-Macías ME (2006) Probiotic properties of vaginal lactic acid bacteria to prevent metritis in cattle. Lett Appl Microbiol 43:91–97

24. Otero MC, Nader-Macías M.E (2008) Susceptibility of probiotic bovine vaginal *Lactobacillus* to antimicrobial agents J Anim Vet Adv. Submitted
25. Otero MC, Nader-Macias ME (2006) Inhibition of *Staphylococcus aureus* by H_2O_2-producing *Lactobacillus gasseri* isolated from the vaginal tract of cattle. Anim Reprod Sci 96:35–46
26. Otero MC, Nader-Macías ME (2007) *Lactobacillus* adhesion to epithelial cells from bovine vagina. In: Méndez-Vilas A (ed) Communicating current research and educational topics and trends in applied microbiology. Microbiology series N 1. Badajoz, Spain, pp 749–757
27. Otero MC, Ocaña VS, Nader-Macías ME (2004) Bacterial surface characteristics applied to the selection of probiotic microorganisms. In: Spencer JFT, Ragout de Spencer AL (eds) Methods in molecular biology. Public health microbiology: methods and protocols, vol 268. Humana Press Inc, Totowa, New Jersey, pp 435–440
28. Patterson JL, Girerd PH, Karjane NW, Jefferson KK (2008) Effect of biofilm phenotype on resistance of *Gardnerella vaginalis* to hydrogen peroxide and lactic acid. Am J Obstet Gynecol 197:170.e1–170.e7
29. Raibaud P, Galpin JV, Duclezeau R, Mocquot G, Oliver G (1973) Le Genre *Lactobacillus* dans le tube digestif du rat. II. Caracters de souches heterofermentaires isolates de rats. "Holo" et "Gnotoxeniques". Ann Microbiol 124:2223–2235
30. Savage DC, Kotarski SF (1979) Models for study the specificity by which lactobacilli adhere to murine gastric epithelium. Infect Immun 26:966–973
31. Twomey DP, Wheelock AI, Flynn J, Meaney WJ, Hill C, Ross RP (2000) Protection against *Staphylococcus aureus* mastitis in dairy cows using a bismuth-based teat seal containing the bacteriocin, lacticin 3147. J Dairy Sci 83:191–198
32. Vaughan EE, Heilig HG, Ben-Amor K, De Vos WM (2005) Diversity, vitality and activities of intestinal lactic acid bacteria and bifidobacteria assessed by molecular approaches. FEMS Microbiol Rev 29:477–490
33. Zoetendal EG, Vaughan EE, de Vos WM (2006) A microbial world within us. Mol Microbiol 59:1639–1650