Research in Veterinary Science 92 (2012) 342–349

Contents lists available at ScienceDirect

Research in Veterinary Science

journal homepage: www.elsevier.com/locate/rvsc

Lactic acid bacteria isolated from young calves – Characterization and potential as probiotics

Natalia C. Maldonado [a], Clara Silva de Ruiz [b], María Claudia Otero [a], Fernando Sesma [a], María Elena Nader-Macías [a,*]

[a] Centro de Referencia para Lactobacilos (CERELA-CONICET), Chacabuco 145, 4000 San Miguel de Tucumán, Argentina
[b] Facultad de Bioquímica, Química y Farmacia, Universidad Nacional de Tucumán, Ayacucho 491,4000 San Miguel de Tucumán, Argentina

ARTICLE INFO

Article history:
Received 12 January 2011
Accepted 7 March 2011

Keywords:
Calves
Lactic acid bacteria
Probiotic
Prevention
Diarrhea

ABSTRACT

Lactic acid bacteria (LAB) are widely used as probiotics in humans and animals to restore the ecological balance of different mucosa. They help in the physiological functions of newborn calves that are susceptible to a variety of syndromes. The criteria for the selection of strains for the design of probiotic products are not available. Based in the host-specificity of the indigenous microbiota, 96 LAB isolates from faeces and oral cavity of calves were obtained. The surface properties were screened showing a small number of highly hydrophobic or autoagglutinating isolates. Also, a group produced H_2O_2 and were able to inhibit pathogens, and two strains were bacteriocin-producers. Some grew at very low pH and high bile concentrations.

The strains sharing some of the specific properties evaluated were identified genetically, assayed their compatibility and exopolysaccharide production. The results allow going further in the establishment of criteria to select strains to be included in a multi-strain-probiotic-product to be further assayed in animals.

1. Introduction

The gastrointestinal microbiota of farm animals is of fundamental importance and can affect the host in the digestion of fibre (in ruminants) and act as a barrier against pathogens and toxic substances, mainly in young or neonates and those experiencing stress and unfavourable environmental conditions. The indigenous microbiota established after birth interacts with the digestive and immune systems of the host. The colonization of the different compartments of the intestinal tract by specific commensal bacteria is facilitated initially by their association with the mucus layer or epithelial cells of the mucosal surfaces to produce a first barrier against invading microorganisms (Nousiainnen et al., 2004). Lactic acid bacteria (LAB) and related microorganisms are microbial groups that initially colonise the intestinal tract of newborns, coming from the vaginal tract during parturition, and later from the mammary gland and the environment (Long and Swenson, 1977).

In healthy animals, each segment of the intestine is colonized by a typical microbiota, which are adapted to grow in symbiosis with the host. The indigenous microbiota is stable for some months of life, and can be modified by exogenous and endogenous factors, exert many different effects in the host, and some scientists suggest that it acts as a "different organ" interacting permanently with the host. Also, microbial imbalances during growth might permit the colonization by pathogens and the appearance of many different syndromes with deleterious effects on the host (Bauer et al., 2006; O'Hara and Shanahan, 2006; Guarner and Malagelada, 2003). Diarrhoea is one and causes the calf death and financial loss in dairy farms (Torsein et al., 2011; Gulliksen et al., 2009; Morrell et al., 2008; Virtala et al., 1996; Azzam et al., 1993; Bellinzoni et al., 1990; Bellows et al., 1987). Probiotic microorganisms have been proposed as adjuvant to promote health status in newborn calves (Ewaschuk et al., 2004; Magalhães et al., 2008; Timmerman et al., 2005). Probiotics are defined as "live microorganisms administered to the host to produce a beneficial physiological effect" (Reid et al., 2003) and are recognized as GRAS (generally recognized as safe) microorganisms. Many probiotic products are available in the market for different purposes, but the bacteria they contain are sometimes uncharacterized (Saxelin, 2008; Brink et al., 2005).

One of the objectives of our research group was to identify the lactic acid bacteria to administer to newborn calves, isolated from this group of animals. We initially isolated bacteria classified as GRAS from faeces and oral cavity of newborn calves, supported by the host specificity phenomenon described and exerted by members of the indigenous microbiota (Kotarski and Savage 1979; Zoetendal et al., 2006). Screening of properties relating to

* Corresponding author. Tel.: +54 381 4311720x141; fax: +54 381 4005600.
E-mail address: fnader@cerela.org.ar (M.E. Nader-Macías).

0034-5288/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.rvsc.2011.03.017

beneficial characteristics (potential to adhere to the intestinal tract or production of inhibitory substances) was performed based in previous experience (Ocaña et al., 1999; Ocaña and Nader Macías, 2002; Otero et al., 2006). The resistance to the intestinal tract conditions was also evaluated. Selected bacteria were identified by molecular techniques, included in an international Culture Collection and assessed for suitability as probiotics for newborn calves.

2. Materials and methods

2.1. Animals, samples collection and enumeration of aerobic population

Fifty-one samples were obtained from the oral cavities and rectums of 42 healthy young calves (less than four months of age) from dairy and beef farms in Tucumán and Jujuy provinces (Argentina). Animals sampled were clinically healthy (based on body weight gain, daily intake, natural behavior, lack of diarrhoea symptoms) and had not received antibiotics for the last month prior to sample collection.

The samples were collected with sterile swabs in MRS 1% agar pH 4.5 (De Man et al., 1969) (Merck, Darmstadt, Germany) and also in LAPT (1% yeast extract, 1.5% peptone, 1% tryptone, 1% glucose, 0.1% Tween 80; Fournaud et al., 1964) medium supplemented with 0.7% agar (w./vol.) MRS agar was stored at 4 °C.

The swabs collected in LAPT medium were resuspended (10^{-1} dilution) in peptone water, followed by serial dilutions. Aliquots of each dilution were plated onto LAPTg (1% yeast extract, 1.5% peptone, 1% tryptone, 1% glucose, 0.1% Tween 80), MacConkey agar (Britania, Buenos Aires, Argentina) and MRS agar, in order to quantify enterobacteria and LAB members, respectively.

2.2. Isolation and partial identification of lactic acid bacteria strains

For the enrichment and isolation of LAB, MRS agar pH 4.5 was added with 1 ml of MRS broth and incubated 24 h at 37 °C. After incubation, the swabs were spread onto MRS agar and LBS agar (Lactobacilli Selective Media; Rogosa et al., 1951) (Merck, Darmstadt, Germany). Plates were incubated in a 5% CO_2 atmosphere for 48 h at 37 °C. One to three isolated colonies were randomly selected and transferred to MRS broth for 12 h at 37 °C and later spread onto MRS agar to assess the purity of the isolated microorganisms. The isolates were stored in milk yeast extract (13% non fat milk, 1% yeast extract) containing 20% glycerol (vol./vol.) at −20 °C.

The presumptive identification of the microorganisms was performed by Gram stain, morphology and catalase reaction, reduction of nitrate and indole production. Gram positive isolates that resulted negative for catalase reaction, indole and nitrate reduction were selected for further evaluation and included in the LAB group. In order to identify the isolates at the metabolic group level, the gas production from glucose or gluconate was determined. The gas production from glucose was evaluated in Gibson medium (Gibson and Abd-El-Malek, 1945). The gas production from gluconate was performed in gluconate medium and sealed with a layer of vaseline-paraffin (1:1). Gibson and gluconate media inoculated with lactobacilli were incubated for 48 h at 37 °C.

2.3. Bacterial surface properties

The hydrophobic nature of the cell surface was studied by the method of microbial adhesion to hydrocarbons, as described by Ocaña et al. (1999) in all the isolates. The cell suspensions with n-hexadecane (Merk, Darmstadt, Germany) were gently agitated for three minutes. After the separation of the two phases, the optical density of the aqueous phase was determined. The index of hydrophobicity was the result of the decrease of turbidity of the aqueous phase calculated by the following expression: Hydrophobicity (%) = [(OD final − OD initial)/OD final] × 100.

The extent of bacterial auto-aggregation was assessed according to the technique described by Ocaña et al. (1999). Briefly, the OD_{600nm} variation of cellular suspensions in phosphate buffered saline (PBS) solution pH 7 was monitored for two hours. The degree of auto-aggregation was calculated using the same expression as hydrophobicity.

The score of hydrophobicity and auto-aggregation applied was: high (60–100%), medium (30–60%) and low (0–30%).

2.4. Screening of antagonistic activity against pathogens

2.4.1. H_2O_2 production

The H_2O_2 production was qualitatively determined for isolates by the plate method described by Juárez Tomás et al. (2004) by employing horseradish peroxidase (Sigma Chemical Co, St. Louise, MO, USA) incorporated in tetramethyl-benzidine (TMB) (Sigma Chemical Co, St. Louise, MO, USA) agar medium. The microorganisms were inoculated in the TMB-MRS plates. A score was assigned according to the intensity of blue colour of LAB colonies in the plates: − (negative), + (weakly positive), ++ (moderately positive) and +++ (strongly positive).

2.4.2. Inhibitory activity

The pathogenic microorganisms used for the detection of antagonistic substances were: *Salmonella dublin* MP/07 (isolated from a bovine clinical sample in our Laboratory, MP states for Microbiología Preventiva Laboratory), *Yersinia enterocolitica* 1845/00 provided by ANLIS (Administración Nacional de Laboratorios e Institutos de Salud "Dr. Carlos Malbrán"; Buenos Aires, Argentina); *Escherichia coli* 3511AD, *Streptococcus uberis* MP/06 and *Streptococcus dysgalactiae* 05/84 provided by INTA Rafaela, (Instituto Nacional de Tecnología Agropecuaria; Santa Fé, Argentina) and *Klebsiella* sp. MP/05 (a biofilm producer strain).

The plate diffusion technique was used to evaluate the production of inhibitory substances in the supernatant fluid of the isolates (Jack et al., 1995). Briefly, LAPTg 1% agar plates with 10^6–10^7 CFU/ml of each pathogen were prepared. Standardized aliquots (35 μl) of cell-free supernatant obtained from the third subculture of the microorganisms grown in LAPTg broth were placed into holes (4 mm diameter) of pathogens plates. The plates were incubated for 2 h at room temperature and then 24 h at 37 °C. An inhibition zone of at least 6 mm diameter was considered positive. Positive supernatants were neutralised with sterile 1 N NaOH, and later treated with catalase (1000 U/ml) (Sigma–Aldrich, St. Louise, MO, USA) to determine the nature of the inhibitory substances (organic acids, hydrogen peroxide or bacteriocin).

2.5. Resistance to bile salts

MRS plates with bovine ox-bile (Fluka, Signa-Aldrich, St. Louise, MO, USA) at concentrations of 0.5%, 1% and 2% (wt./vol.) were prepared. Samples of the third subcultures of LAB (2 μl corresponding to 5×10^8 CFU/ml) were spotted onto MRS- ox-bile plates and incubated aerobically for 48 h at 37 °C.

2.6. Criteria for selection of the isolates

The selection of isolates with beneficial properties was performed based on their surface pattern (high values of hydrophobicity and self-aggregation), expression of antagonistic activity against calf pathogens (production of hydrogen peroxide, lactic acid or bacteriocin) and functional properties such as resistance to high bile salts concentration.

2.6.1. Genetic identification of the selected strains

Those LAB isolates sharing some of the screened properties were further identified by molecular biology using 16S ribosomal RNA gene sequencing. Amplifications of DNA were carried out by colony PCR. Microorganisms were cultured on LAPTg agar. The reaction was performed with PCR Buffer (1x) (Invitrogen, Carlsbad, CA, USA), 2.5 mM $MgCl_2$ (Invitrogen, Carlsbad, CA, USA), 0.2 mM dNTPs (Invitrogen, Carlsbad, CA, USA), 1 μM MLB16 (5′GGCTGCTGGCACGTAGTTAG) and PLB16 (5′AGAGTTTGATCCTGGCTCAG) primers (Hébert et al., 2000), Taq DNA polymerase (2.5 U) (Invitrogen, Carlsbad, CA, USA) and Milli-Q water. The final volume was 50 μl. The PCRs were performed in a Biorad MyCycle thermal cycler (BioRad Laboratories, Hercules, CA, USA) under the following conditions: 4 min at 94 °C of initial denaturation, 30 cycles consisting of 30 s at 94 °C, 45 s at 52 °C and 45 s at 72 °C and a final extension at 72 °C for 7 min. The PCR products were electrophoresed in 0.8% agarose gels, purified and sequenced using the DNA sequencing service of RURALEX FAGOS (Capital Federal, Buenos Aires, Argentina). Identification was performed by comparing our 16S rDNA sequences with those deposited in Genbank database by using the BLAST algorithm.

2.6.2. Complementary evaluation of selected strains

2.6.2.1. Bacterial aggregation and exopolysaccharide (EPS) production. The agglutination of the selected LAB strains that left a clear supernatant after incubation was further studied. The autoaggregation test previously described was modified by the addition of 10% (vol./vol.) of the spent culture supernatant of each microorganism (Schachtsiek et al., 2004).

The production of EPS by LAB strains was evaluated in 16 h culture in skim milk (10%, w./vol.) at 37 °C, according to the technique described by Mozzi et al. (2004). Capsular polysaccharides formation was determined by the Indian ink negative staining technique (Mozzi et al., 2004).

2.6.2.2. Inhibitory activity against different pathogens. Different pathogens were used to further evaluate the inhibitory spectrum of the selected LAB strains: *Staphylococcus aureus* MP/08, and *Listeria* sp. MP/08 (isolated from dairy products) and *Salmonella thyphimurium* MP/08, *Salmonella infantis* 1533/00 and *Staphylococcus epidermidis* 3267, isolated from clinical samples were evaluated.

2.6.2.3. Compatibility between LAB strains. The screening assay to evaluate the compatibility between different lactobacilli was applied only in six LAB strains by using the plate diffusion technique (Jack et al., 1995). Aliquots (35 μl) of cell-free supernatant obtained from early stationary phase of the third subculture of the microorganisms grown in MRS broth were placed into holes (4 mm diameter) of MRS 1% agar plates with 10^9 CFU/ml, 10^7 CFU/ml and 10^5 CFU/ml with the indicator lactobacilli strains. The plates were incubated for 2 h at room temperature and then 48 h at 37 °C, when inhibition was assessed.

2.6.2.4. Organic acids quantification. To determine the concentration of the lactic acid produced, the supernatants of the third subculture incubated in LAPTg for 16 h at 37 °C were analyzed by using HPLC. A Knauer Smartline System chromatographer (Knauer, Berlin, Germany) with a column for organic acids (BioRad HPX-87H 300 × 7.8 mm) was employed at 41 °C. The flow-rate was 0.6 ml/min. The eluent used was 5 mM H_2SO_4 (pH 2) and the detection was carried out with an RI detector Knauer K-2301.

2.6.2.5. Bacteriocin characterization and genetic identification. From the screened isolates, one strain that produced a bacteriocin-like substance was obtained, and its chemical nature was studied: the effect of some enzymes on the activity was evaluated: proteinase K (10 mg/ml) (Sigma–Aldrich, St. Louise, MO, USA), α-chymiotrypsin (10 mg/ml) (Sigma–Aldrich, St. Louise, MO, USA) and trypsin (10 mg/ml) (Sigma–Aldrich, St. Louise, MO, USA) by the plate diffusion technique. Briefly, the protease solutions were placed in holes next to the bacteriocin and the modifications in the size of the halos were considered as sensitive to the proteases.

The identification of the bacteriocin structural genes was performed by PCR amplification by using primers designed for enterocin A (Aymerich et al., 1996), enterocin L50, enterocin P, enterocin B, mundticin KS or enterocin CRL 35 (Saavedra et al., 2004) and LanB and LanC primers that were able to amplify lantibiotic biosynthesis genes (Wirawan et al., 2006). Amplicons of the expected size were cloned or directly sequenced. *Enterococcus mundii* CRL35, *Enterococcus faecium* ATCC 19434 and *Lactobacillus lactis* ATCC11454 were used as positive controls for mundticin, enterocin A and nisin A genes, respectively.

2.7. Statistical analysis

The Kruskal–Wallis test was applied to determine the statistical significance of the mean data obtained for the different aerobic population of the faecal samples.

3. Results

3.1. Isolation of microorganisms

The mesophilic bacteria recovered from faecal samples of calves of different age was from 10^7 to 10^9 CFU/g, without any significant differences between them ($p \leqslant 0.05$, Kruskal Wallis test) as shown in Fig. 1. There were differences statistically significant between the numbers of LAB and enterobacteria isolated from newborn calves (from 0 to 1 month age) and those from older animals (2–4 months age) as indicated in the same Fig. 1.

Ninety-six isolates were identified as LAB: 81% of the bacilli and cocci were isolated from well-nourished calves; the majority of cocci (30 isolates) being isolated from animals on dairy farms, while bacilli were isolated from both dairy (27 isolates) and beef farms (26 isolates) (data not shown). Most of the bacilli and cocci were isolated from rectal swabs (94%); cocci were isolated from faecal (70%) and oral samples (30%). Oral cocci were isolated mainly from dairy-farms, while only few bacilli were isolated from the oral cavity. The majority of the bacilli from beef and dairy farms were isolated from faeces. The isolates classified as LAB were also separated by their metabolic activities. Most of the LAB were facultative heterofermentative (48%) or obligate homofermentative (40%) and only a few were obligate heterofermentative. Bacilli

Fig. 1. Bacteria isolated from rectal swabs of newborn calves of different ages. The data are expressed as log CFU/g: ■, total mesophilic bacteria; ■, LAB; and ■, enterobacteria. Different superscripts represent statistical differences ($p < 0.05$) between the CFU/g of each bacterial populationat different ages.

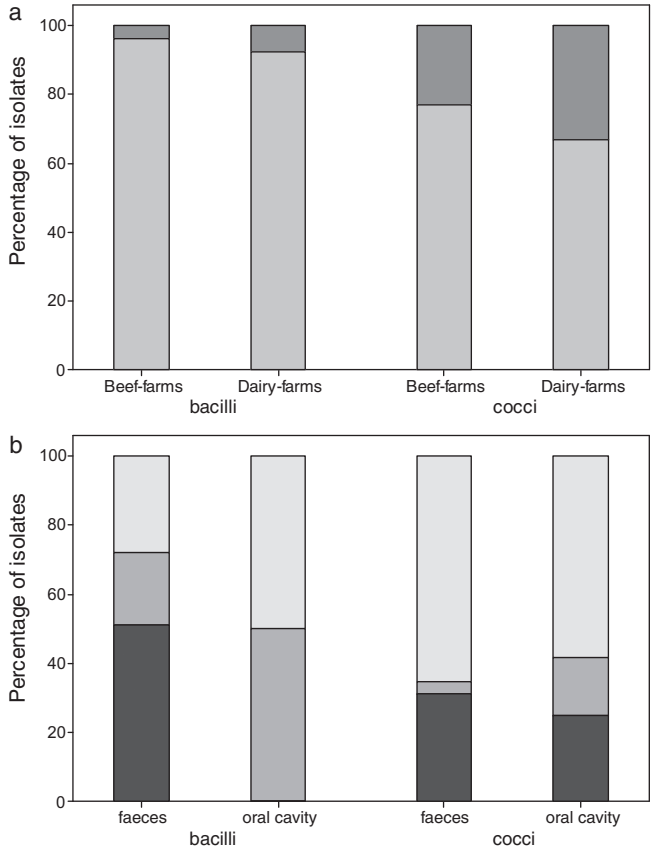

Fig. 2. Characteristics and distribution of the population of the BAL isolates according to the farm, source of isolation and metabolic group. 2a ▒, faeces and ▒, oral cavity; 2b ▒, facultative heterofermentative, ▒, obligate heterofermentative and ■, homofermentative.

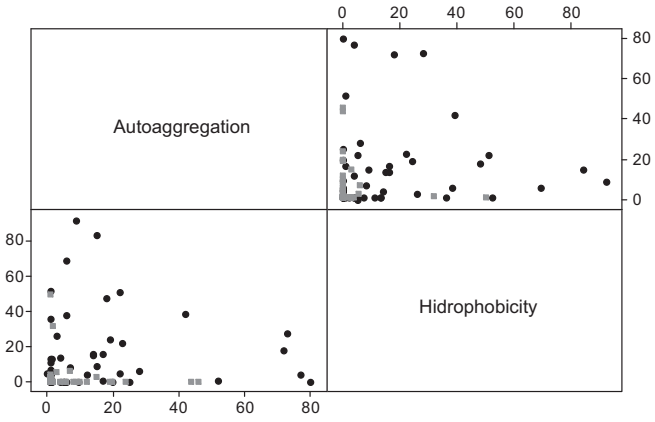

Fig. 3. Matrix plot of auto-aggregation and hydrophobicity index of BAL isolates (bacilli, ● and cocci, ▒) from calves.

showed a similar distribution between heterofermentative and homofermenative groups in faeces, and there were no homofermentative bacilli from the oral cavity. Most of the isolated cocci from oral and fecal samples belong to the heterofermentative group (Fig. 2).

3.2. Evaluation of potentially beneficial properties

3.2.1. Surface properties

When evaluating the surface characteristics of the LAB isolates, most showed a low degree of hydrophobicity and autoaggregation. In the lactobacilli group only a few isolates expressed high or mean hydrophobicity. Autoaggregation was observed in lactobacilli more commonly than in cocci. Overall, there was no correlation between the two surfaces properties evaluated, which was in agreement with the Pearson correlation factor (0.148) obtained. Nevertheless, there was a group of three LAB isolates that expressed a hydrophobicity value higher than 60% and eight isolates showing autoaggregation index higher than 30%. Fig. 3 summarizes the individual values of all the isolates and their relationships.

3.2.2. Hydrogen peroxide production

The 20% of bacilli and 8% of cocci were strong and moderate producers of hydrogen peroxide.

3.2.3. Inhibition of pathogens by BAL supernatants

The BAL isolates from faeces showed a wider spectrum of inhibition compared with those from the oral cavity (data not shown). The 80–100% of BAL with inhibitory activity against *E. coli* 3511AD, *Y. enterocolitica* 1845/00, *S. dublin* MP/07 and *Klebsiella* sp. MP/05 were bacilli isolated from faecal samples (Table 1). Moreover, some bacteria of this group showed inhibition of Gram positive cocci: *S. dysgalactiae* and *S. uberis* MP/06. On the other hand, most of the cocci ($n = 44$) did not show inhibitory activity against the enteropathogenic microorganisms evaluated. 13% of the isolates with activity against *Y. enterocolitica* 1845/00 and 10% of those active against *E. coli* 3511AD were cocci from faecal samples. 7% of the BAL isolates active on *Y. enterocolitica* 1845/00 were cocci (Table 1).

The inhibitory substances produced by the bacilli were acid in nature. However, two cocci isolated from the oral cavity and faeces were able to produce bacteriocin-like substances, active against different bovine and food-borne pathogens. The isolates that showed the widest inhibitory spectrum and the supernatants with lower pH were bacilli; however, only three isolates of this group were able to inhibit the growth of all the pathogens assayed. Fig. 4 includes the final pH and the antagonist activity of all the isolates.

3.2.4. Characterization and genetic identification of bacteriocin

The bacteriocin produced by *E. faecium* was inactivated by proteinase K, α-chymiotrypsin and trypsin, as shown in Fig. 5. The genetic identification of the bacteriocin produced by *E. faecium* CRL 1692 showed identity with the class IIa peptide enterocin A (Aymerich et al., 1996).

Table 1
Inhibitory activity of LAB isolates from newborn calves against pathogens responsible of infections in cattle, by the plate-diffusion technique. The samples were taken from faecal swabs and oral cavity.

	Escherichia coli 3511AD (%)	*Yersinia enterocolitica* 1845/00 (%)	*Klebsiella* sp. MP/05 (%)	*Salmonella dublin* MP/07 (%)	*Streptococcus dysgalactiae* 05/84 (%)	*Streptococcus uberis* MP/06 (%)
Fecal bacilli	90	80	100	100	22	26
Oral bacilli	–	–	–	–	12	–
Fecal cocci	10	13	–	–	44	52
Oral cocci	–	7	–	–	22	22

The numbers indicate de percentage of isolates from each group of LAB that show inhibition against the pathogens. (–) No inhibition detected.

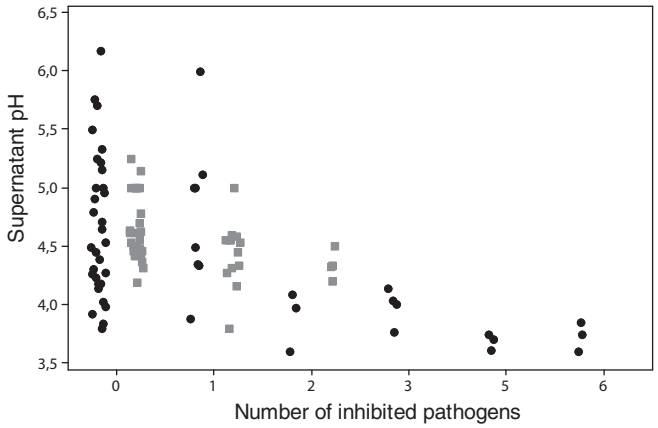

Fig. 4. Antagonistic activity (number of inhibited pathogen strains) and pH of the LAB (bacilli, ● and cocci, ■) supernatants. The pH was determined in 16 h-cultures in LAPTg broth. The pathogenic microorganisms were: *Salmonella dublin* MP/07, *Y. enterocolitica, Escherichia coli* 3511AD, *Streptococcus uberis* MP/06 and *S. dysgalactiae* 05/84 provided and *Klebsiella* sp. MP/05.

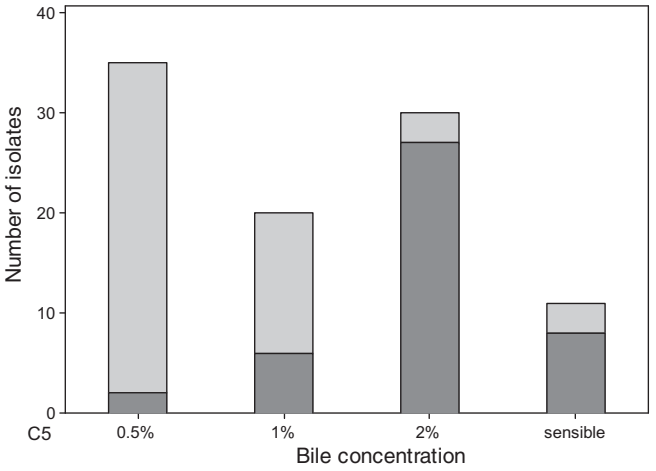

Fig. 6. LAB resistance to different concentration of bile. The symbols indicate bacilli, ■ and cocci, ■.

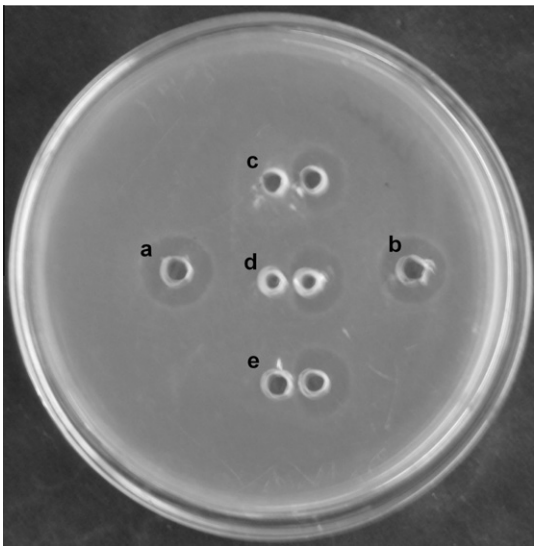

Fig. 5. Agar plate showing the inhibitory activity of *Enterococcus faecium* CRL 1703 against *Listeria sp.*: (a) *E. faecium* cell free supernatant and (b) neutralized supernatant. In the centre of the plate the effect of proteinase *K* (c), α-chimiotrypsin (d) and trypsin (e) is shown. The modified holes on the right contain *Enterococcus faecium* CRL 1703 neutralized supernatant.

3.3. Functional properties

3.3.1. Resistance to bile

Most of the BAL isolates were able to grow in media added with 0.5%, 1% and 1.5% bile and only 10 were sensitive to the three concentrations of bile evaluated. While the bacilli were able to resist 0.5% bile concentration, most of the cocci grew at the highest concentration (1.5%) (Fig. 6).

3.3.2. Strains Selected by beneficial properties

Fourteen BAL strains sharing beneficial properties (hydrophobicity, autoaggregation) were selected and identified by genetic methods. The lactobacilli included in this group were genetically identified as: *Lactobacillus johnsonii*,*Lactobacillus mucosae, Lactobacillus murinus, Lactobacillus salivarius* and *Lactobacillus amylovorus.* The strains that produced the bacteriocin were identified as *E. faecium* and *Streptococcus bovis*. *S. bovis* was excluded from the group of selected strains to be used as a probiotic, since it may produce ruminal acidosis in calves. The results of the individual properties of each LAB strain are included in Tables 2a and 2b.

Among the selected strains, only *L. murinus* CRL 1695 showed a high autoaggregative pattern. Low or medium autoaggregation index strains, but able to settle down after long periods of incubation, were further studied. They were subjected to a second autoaggregation test, where 10% (vol./vol.) of their depleted culture supernatants were added to PBS. *L. johnsonii* CRL 1693, *L. johnsonii* CRL 1701 and *L. johnsonii* CRL 1706 showed an increased aggregative index by this technique.

L. *johnsonii* CRL 1692, *L. johnsonii* CRL 1693, *L. johnsonii* CRL 1694, *L. johnsonii* CRL 1701 and *L. salivarius* CRL 1702 showed hydrophobic properties. On the other hand, *E. faecium* CRL 1703 did not exhibit autoaggregation or hydrophobicity.

The concentration of lactic acid produced in LAPTg broth after 16 h incubation showed that the strains identified as *L. johnsonii* and *L. amylovorus* produced the highest amounts of lactic acid, in agreement with their low pH values and inhibitory spectrum (Table 2b). *L. johnsonii* CRL 1692, *L. johnsonii* CRL 1699 and *L. johnsonii* CRL 1700 inhibited the growth of all the *Salmonella* strains, *Y. enterocolitica* 1845/00, *Klebsiella* sp. MP/05 and some Gram positive cocci tested.

Although all the bacilli selected produced hydrogen peroxide in low amounts, only *L. mucosae* CRL 1696, *L. mucosae* CRL 1698, *L. johnsonii* CRL 1692 and *L. johnsonii* CRL 1706 were strong producers.

Among the selected strains, *L. johnsonii* CRL 1692 produced ropiness in liquid media, while capsular exopolisaccharides were observed in *L. salivarius* CRL 1694, *L. amylovorus* CRL 1697, *L. murinus* CRL 1705, *L. johnsonii* CRL 1700 and *L. johnsonii* CRL 1693.

Only the *L. murinus* strain was inhibited by *L. salivarius*, *L. mucosae, L. johnsonii* and *L. amylovorus* in the compatibility assay. The inhibition was observed as a partial decrease of the growth around the spot inoculated with 10^7 and 10^5 CFU/ml of *L. murinus* CRL 1695.

4. Discussion

The increased use of uncharacterised probiotics in animals to modify weight gain and resistance to infections led us to study the characteristics of LAB strains that might be included in the design of a probiotic for calves. This product could increase the health status of this group of animals, and also might be used for the

Table 2a
Origin of isolation and surface properties of selected strains of LAB.

Selected strains		Source of isolation	Hydrophobicity index (%)[a]	Autoaggregation score[b]	Autoaggregation index in PBS (%)[c]	Autoaggregation index in depleted supernatant (%)[d]
Lactobacillus johnsonii	CRL 1692	Rectal swabs	69	–	6	0
Lactobacillus johnsonii	CRL 1693	Rectal swabs	82	+++	15	60
Lactobacillus salivarius	CRL 1694	Rectal swabs	67	++	22	ND
Lactobacillus murinus	CRL 1695	Rectal swabs	18	+++	72	64
Lactobacillus mucosae	CRL 1696	Rectal swabs	19	+	14	26
Lactobacillus amylovorus	CRL 1697	Rectal swabs	9	–	15	23
Lactobacillus mucosae	CRL 1698	Rectal swabs	19	+	16	18
Lactobacillus johnsonii	CRL 1699	Rectal swabs	11	–	0	ND
Lactobacillus johnsonii	CRL 1700	Rectal swabs	15	–	4	ND
Lactobacillus johnsonii	CRL 1701	Rectal swabs	48	+++	18	73
Lactobacillus salivarius	CRL 1702	Rectal swabs	38	–	6	0
Enterococcus faecium	CRL 1703	Oral cavity	1	–	1	0
Lactobacillus murinus	CRL 1705	Rectal swabs	5	+	22	ND
Lactobacillus johnsonii	CRL 1706	Rectal swabs	25	+++	14	73

ND, no determined.
[a] The index was determined by MATH in hexadecane (Ocaña et al., 1999).
[b] The autoaggregation score was defined as the visual observation of the culture after the incubation. The score for the strain was: –, no aggregative; +, weak; ++, mean; +++, strong.
[c] The autoaggregation index was determined as described by Ocaña and Nader Macías (2002).
[d] The autoaggregation index was performed in a PBS solution added with 10% of the strain depleted supernatant.

Table 2b
Inhibitory substances produced by selected LAB strains isolated from newborn calves.

Selected strains	H_2O_2 production[a]	Lactic acid (g/l)[b]	$DO_{(560nm)}$[c]	pH Supernatant[d]	Inhibited pathogens[e]
Lactobacillus johnsonii CRL 1692	+++	15.95	1.53 ± 0.04	3.70 ± 0.02	Escherichia coli 3511AD, Salmonella typhimurium MP/08, S. dublin MP/07, S. infantis 1533/00, Klebsiella sp. MP/05, Yersinia enterocolitica 1845/00, Staphylococcus aureus MP/08, S. epidermidis 3267
Lactobacillus johnsonii CRL 1693	+	10.56	1.36 ± 0.15	3.99 ± 0.13	*Escherchia coli* 3511AD, *Salmonella dublin* MP/07, *Staphylococcus aureus* MP/08
Lactobacillus salivarius CRL 1694	+	8.41	1.15 ± 0.13	4.09 ± 0.08	*Escherichia coli* 3511AD, *Salmonella typhimurium* MP/08, *Staphylococcus aureus* MP/08
Lactobacillus murinus CRL 1695	+	6.38	0.88 ± 0.16	4.23 ± 0.09	*Salmonella typhimurium* MP/08
Lactobacillus mucosae CRL 1696	+++	5.7	0.55 ± 0.05	5.25 ± 0.16	NI
Lactobacillus amylovorus CRL 1697	+	12.87	1.45 ± 0.07	3.60 ± 0.32	*Escherichia coli* 3511AD, *Salmonella typhimurium* MP/08, S. dublin MP/07, *S. infantis* 1533/00, *Streptococcus dysgalactiae* 05/84
Lactobacillus mucosae CRL 1698	+++	5.9	0.60 ± 0.05	4.98 ± 0.14	NI
Lactobacillus johnsonii CRL 1699	+	14.56	1.5 ± 0.15	3.75 ± 0.02	*Escherichia coli* 3511AD, *Salmonella typhimurium* MP/08, S. dublin MP/07, *S. infantis* 1533/00, *Yersinia enterocolitica* 1845/00, *Klebsiella* sp. MP/05, *Staphylococcus aureus* MP/08, *S. epidermidis* 3267
Lactobacillus johnsonii CRL 1700	++	16.39	1.63 ± 0.16	3.79 ± 0.15	*Escherichia coli* 3511AD, *Salmonella typhimurium* MP/08, S. dublin MP/07, *S. infantis* 1533/00, *Yersinia enterocolitica* 1845/00, *Klebsiella* sp. MP/05, *Staphylococcus aureus* MP/08, *Streptococcus uberis* MP/06, *S. epidermidis* 3267
Lactobacillus johnsonii CRL 1701	++	10.67	1.36 ± 0.15	3.97 ± 0.10	*Salmonella dublin* MP/07, *Yersinia enterocolitica* 1845/00
Lactobacillus salivarius CRL 1702	+	8.12	0.93 ± 0.10	4.18 ± 0.25	*Salmonella typhimurium* MP/08
Enterococcus faecium CRL 1703	–	7.79	0.9 ± 0.07	4.34 ± 0.03	*Listeria* sp. MP/08, *Streptococcus uberis* MP/06
Lactobacillus murinus CRL 1705	++	6.52	0.7 ± 0.19	4.26 ± 0.16	NI
Lactobacillus johnsonii CRL 1706	+++	8.18	1.30 ± 0.10	4.07 ± 0.28	NI

NI, no inhibition was observed.
[a] The production of H_2O_2 in TMB-plates was performed according to Juárez Tomás et al. (2004). A score was assigned as the intensity of the blue colour of the colonies was increasing: –, negative; +, weak positive; ++, moderate and +++, strong.
[b] The lactic acid production was obtained by HPLC in a 16 h culture in LAPTg broth.
[c] The Optical density $DO_{(560nm)}$ was measured in a 16 h culture in LAPTg medium.
[d] The pH of the supernatant was determined by using a pH meter.
[e] Analyzed by the plate diffusion method described by Juárez Tomás et al. (2004).

prevention of diarrhoea responsible for high mortality and morbidity in neonates (Morrell et al., 2008; Azzam et al., 1993; Bellinzoni et al., 1990; Bellows et al., 1987). The beneficial use of probiotics for calves has been reported by groups investigating the use of potential probiotic bacteria in swine and poultry (Ross et al., 2010; Frizzo et al., 2008; De Angelis et al., 2006; Timmerman et al., 2005, 2006; Patterson and Burkholder, 2003; Abe et al., 1995). However, there are no data available to document the characteristics of effective probiotic isolates. Additionally, strains must be classified as GRAS (Generally Regarded as Safe) and FGM

(Food-Grade Microorganisms), groups where most of the lactic acid bacteria are included.

Based on the host-specificity showed by members of the indigenous microbiota (Kotarski and Savage, 1979; Zoetendal et al., 2006), the isolation of LAB from the same ecological niche where they will be applied was carried out as a first step, taking into consideration the homologous host. As there are no established criteria to select the suitable strains to be included in the design of a probiotic or beneficial product, some characteristics assayed in strains from different hosts and origins were applied. Hydrophobicity and autoaggregation, as surface properties, were assessed for all isolates, based on the principle that adhesion to the epithelial surface is the first step required for colonization of probiotic microorganisms, to allow the later formation of a biofilm (Ocaña and Nader Macías, 2002; An et al., 2000). Only a few isolates showed these characteristics. Similar results were also reported by other researchers in strains isolated from the intestinal tract of calves and piglets (Iñiguez Palomares et al., 2007; Frizzo et al., 2006) or in bovine vaginal samples (Otero et al., 2006). Regarding the hydrophobicity and autoaggregation indices, some isolates or strains identified into the same specie did not exhibit the same degree or index, evidencing again that these properties are present in specific strains, but not in all the strains of the same specie. When evaluating the expression of the two properties, it is possible to determine that they are not exhibited by the same strain. Similar findings have been reported by Espeche et al., 2009 in bovine strains. On the other hand, the probiotic adhesion of the strains to intestinal mucus has been correlated with the ability to produce exopolysaccharides (Ruas-Madiedo et al., 2007). The capsular polysaccharide was only observed in a few of selected strains and only one showed the ropy characteristic.

The production of inhibitory substances is an *in vitro* assay used widely to select beneficial bacteria from different origins. The nature of the inhibitory substances can be organic acids, hydrogen peroxide or bacteriocin. The lactic acid produced and the low pH are inhibitory to susceptible microorganisms such as enterobacteria (Rodriguez-Palacios et al., 2009; Vandenbergh, 1993). The results indicate that the LAB isolates inhibited the pathogens mainly by production of lactic acid, bacilli showing the highest degree. Only two strains morphologically identified as cocci were shown to produce a bacteriocin like substance.

The production of high levels of hydrogen peroxide was detected in a small number of isolates. The strains identified as *L. mucosae* were the highest producers. The same specie was also recently isolated from healthy calves (Busconi et al., 2008), but the beneficial characteristics in this particular ecological niche were not reported. This property was also studied in many strains isolated from bovine vaginal environments (Otero et al., 2006), where they may have ecological function in the maintenance of a healthy vaginal tract, or in bovine mammary gland (Espeche et al., 2009). The significance of the bactericidal effect from "*in vitro*" assays was not determined in the gastrointestinal tract of animals.

In the screening performed to determine the resistance to bile, some isolates were able to grow, including cocci, at very high concentrations. All the microorganisms assayed were able to grow at 0.5% bile concentration which is physiological in calves (Frizzo et al., 2006; Timmerman et al., 2005).

According to the molecular identification of the selected strains, some of these LAB species are used or proposed as probiotic bacteria for humans and farm animals. For example, *L. johnsonii* NC533 (formerly *Lactobacillus acidophilus* La1 with completely sequenced DNA), a human isolate that showed inhibitory properties, immunomodulation and adhesion to epithelial cells (Pridmore et al., 2004) is used as a probiotic bacterium. On the other hand, previous studies in pigs indicated that *L. amylovorus*, a highly acid-producer strain, showed inhibitory properties against diarrhoeal pathogens (Klose et al., 2010). *L. salivarius* DSPV 315T, a bovine microorganism, was also proposed as a probiotic product for administration during the feeding of calves by Frizzo et al. (2006).

Bacteriocin-producing strains were identified as *E. faecium* and *S. bovis*. Some strains of the genus *Enterococcus* are being evaluated as probiotics, which opens a new possibility, mainly in the production of bacteriocins (enterocins) as inhibitory substances in veterinary medicine (Diez-Gonzalez, 2007; Strompfová et al., 2006; Veir et al., 2007).

The results of this work provide a preliminary study directed to establish valid criteria that may be applied for the selection of isolates that might be used as probiotics in newborn calves. They can be further studied as single or combined strains in colonization assays, or for the stimulation of the immature newborn immune system in animal models. They should be also tested to detect the production of adverse effects, and also the prevention of diarrhoeal disease.

Acknowledgements

This work was supported by PICT 0543 from ANPCYT (Agencia Nacional de Promoción Científica y Tecnológica Agencia, Argentina) and PIP 0632 from CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas, Argentina)

We thank Lic. Elena Bru for the statistical evaluation of the data.

References

Abe, F., Ishibashi, N., Shimamura, S., 1995. Effect of administration of bifidobacteria and lactic acid bacteria to newborn calves and piglets. Journal of Dairy Science 78, 2838–2846.

An, Y.H., Blair, B.K., Martin, K.L., Friedman, R.J., 2000. Macromolecule surface coating for preventing bacterial adhesion. In: An, Y.H., Friedman, R.J. (Eds.), Handbook of Bacterial Adhesion: Principles, Methods, and Applications. Humana Press Inc, Nueva Jersey, USA, pp. 609–626.

Aymerich, T., Holo, H., Håvarstein, L.S., Hugas, M., Garriga, M., Nes, I.F., 1996. Biochemical and genetic characterization of enterocin A from *Enterococcus faecium*, a new antilisterial bacteriocin in the pediocin family of bacteriocins. Applied and Environmental Microbiology 62, 1676–1682.

Azzam, S., Kinder, J., Nielsen, M., Werth, L., Gregory, K., Cundiff, L., Koch, R.M., 1993. Environmental effects on neonatal mortality of beef calves. Journal of Animal Science 71, 282–290.

Bauer, E., Williams, B.A., Smidt, H., Verstegen, M.W., Mosenthin, R., 2006. Influence of the gastrointestinal microbiota on development of the immune system in young animals. Current Issues in Intestinal Microbiology 7, 35–52.

Bellinzoni, R.C., Blackhall, J., Terzolo, H.R., Moreira, A.R., Auza, N., Mattion, N., Micheo, G.L., La Torre, J.L., Scodeller, E.A., 1990. Microbiology of diarrhoea in young beef and dairy calves in Argentina. Revista Argentina de Microbiología 22, 130–137.

Bellows, R., Patterson, D., Burfening, P., Phelps, D., 1987. Occurrence of neonatal and postnatal mortality in range beef cattle. II. Factors contributing to calf death. Theriogenology 28, 573–586.

Brink, M., Senekal, M., Dicks, L.M., 2005. Market and product assessment of probiotic/prebiotic-containing functional foods and supplements manufactured in South Africa. South Africa Medical Journal 95, 114–119.

Busconi, M., Reggi, S., Fogher, C., 2008. Evaluation of biodiversity of lactic acid bacteria microbiota in the calf intestinal tracts. Antonie van Leeuwenhoek 94, 145–155.

De Angelis, M., Siragusa, S., Berloco, M., Caputo, L., Settanni, L., Alfonsi, G., Amerio, M., Grandi, A., Ragni, A., Gobbetti, M., 2006. Selection of potential probiotic lactobacilli from pig feces to be used as additives in pelleted feeding. Research in Microbiology 157, 792–801.

De Man, J.C., Rogosa, M., Sharpe, E., 1969. A medium for cultivation of lactobacilli. Journal of Applied Bacteriology 23, 130–135.

Diez-Gonzalez, F., 2007. Applications of bacteriocins in livestock. Current Issues in Intestinal Microbiology 8, 15–23.

Espeche, M.C., Otero, M.C., Sesma, F., Nader Macías, M.E., 2009. Screening of surface properties and antagonistic substances production by lactic acid bacteria isolated from the mammary gland of healthy and mastitic cows. Veterinary Microbiology 30, 346–357.

Ewaschuk, J.B., Naylor, J.M., Chirino-Trejo, M., Zello, G.A., 2004. *Lactobacillus rhamnosus* strain GG is a potential probiotic for calves. The Canadian Journal of Veterinary Research 68, 249–253.

Fournaud, J., Raibaud, P., Mocquot, G., 1964. Etude de la réduction des nitrites par une souche de *Lactobacillus lactis*. Mise en évidence de ce métabolisme chez dautres bactéries du genre Lactobacillus. Annales de ÍInstitut Pasteur 15, 213–224.

Frizzo, L.S., Soto, L.P., Bertozzi, E., Sequeira, G., Marti, L.E., Rosmini, M.R., 2006. Evaluación *in vitro* de las capacidades probióticas microbianas orientadas al diseño de inóculos probióticos multiespecie para ser utilizados en la crianza de terneros. Revista Fave: Sección Ciencias Veterinarias 5, 69–80.

Frizzo, L.S., Bertozzi, E., Soto, L.P., Zbrun, M.V., Sequeira, G.J., Dalla Santina, R., Rodriguez Armesto, R., 2008. The effect of supplementation with three lactic acid bacteria from bovine origin on growth performance and health status of group calves. Journal of Animal and Veterinary Advances 7, 400–408.

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. Journal of Dairy Research 14, 35–38.

Guarner, F., Malagelada, J.R., 2003. Gut flora in health and disease. Lancet 361, 512–519.

Gulliksen, S.M., Jor, E., Lie, K.I., Hamnes, I.S., Løken, T., Akerstedt, J., Osterås, O., 2009. Enteropathogens and risk factors for diarrhea in Norwegian dairy calves. Journal of Dairy Science 92, 5057–5066.

Hébert, E.M., Raya, R.R., de Giori, G.S., 2000. Nutritional requirements and nitrogen-dependent regulation of proteinase activity of *Lactobacillus helveticus* CRL 1062. Applied and Environmental Microbiology 66, 5316–5321.

Iñiguez Palomares, C., Pérez Morales, R., Acedo, F.E., 2007. Evaluation of probiotic properties in *Lactobacillus* isolated from small intestine of piglets. Revista Latinoamericana de Microbiología, 46–54.

Jack, R.W., Tagg, J.R., Ray, B., 1995. Bacteriocins of gram positive bacteria. Microbial Reviews 59, 171–200.

Juárez Tomás, M.S., Otero, M.C., Ocaña, V.S., Nader-Macías, M.E., 2004. Production of antimicrobial substances in lactic acid bacteria I. Determination of hydrogen peroxide. In: Spencer, J.F.T., Ragout de Spencer, A.L. (Eds.), Public Health Microbiology: Methods and Protocols, vol. 268. Humana Pres Inc, New Jersey, pp. 337–346.

Klose, V., Bayer, K., Bruckbeck, R., Schatzmayr, G., Loibner, A.P., 2010. *In vitro* antagonistic activities of animal intestinal strains against swine-associated pathogens. Veterinary Microbiology 144, 515–521.

Kotarski, S.F., Savage, D.C., 1979. Models for study the specificity by which lactobacilli adhere to murine gastric epithelium. Infection and Immunity 26, 966–973.

Long, S.S., Swenson, R.M., 1977. Development of anaerobic fecal flora in healthy newborn infants. Journal of Pediatric 91, 298–301.

Magalhães, V.J.A., Susca, F., Lima, F.S., Branco, A.F., Yoon, I., Santos, J.E.P., 2008. Effect of feeding yeast culture on performance, health, and immunocompetence of dairy calves. Journal of Dairy Science 91, 1497–1509.

Morrell, E.L., Moore, D.P., Odeón, A.C., Poso, M.A., Odriozola, E., Cantón, G., Paolicchi, F., Malena, F., Leunda, M.R., Morsella, C., Campero, C.M., 2008. Retrospective study of bovine neonatal mortality: cases reported from INTA Balcarce, Argentina. Revista Argentina de Microbiología 40, 151–157.

Mozzi, F., Torino, M.I., de Valdez, G.F., 2004. Identification of exopolysaccharide-producing lactic acid bacteria. A method for the isolation of polysaccharides in milk cultures. In: Spencer, J.F.T., Ragout de Spencer, A.L. (Eds.), Food Microbiology Protocols, Methods in Biotechnology, vol. 14. Humana Press Inc, New Jersey, USA, pp. 183–190.

Nousiainnen, J., Javanainen, P., Setälä, J., 2004. Lactic acid bacteria as aimal probiotics. In: Lactic Acid Bacteria Microbiological and Functional Aspects, third ed. Marcel Dekker, Inc, New York, USA, pp. 547–580.

Ocaña, V., Nader Macías, M.E., 2002. Vaginal lactobacilli: self and coaggregation ability. British Journal of Biomedical Science 59 (4), 183–190.

Ocaña, V.S., Bru, E., de Ruiz Holgado, A.P., Nader-Macías, M.E., 1999. Surface characteristics of lactobacilli isolated from human vagina. The Journal of General and Applied Microbiology 45, 203–212.

O'Hara, A.M., Shanahan, F., 2006. The gut flora as a forgotten organ. EMBO Reports 7, 688–693.

Otero, M.C., Morelli, L., Nader Macías, M.E., 2006. Probiotic properties of vaginal lactic acid bacteria to prevent metritis in cattle. Letters in Applied Microbiology 43, 91–97.

Patterson, J.A., Burkholder, K.M., 2003. Application of prebiotics and probiotics in poultry production. Poultry Science 82, 627–631.

Pridmore, R.D., Berger, B., Desiere, F., Vilanova, D., Barretto, C., Pittet, C.A., Zwahlen, M.C., Rouvet, M., Altermann, E., Barrangou, R., Mollet, B., Mercenier, A., Klaenhammer, T., Arigoni, F., Schell, M.A., 2004. The genome sequence of the probiotic intestinal bacterium *Lactobacillus johnsonii* NCC 533. Proceedings of the National Academy of Science 101, 2512–2517.

Reid, G., Sanders, M.E., Gaskins, H.R., Gibson, G.R., Mercenier, A., Rastall, R., Roberfroid, M., Rowland, I., Cherbut, C., Klaenhammer, T.R., 2003. New scientific paradigms for probiotics and prebiotics. Journal of Clinical Gastroenterology 37, 105–118.

Rodriguez-Palacios, A., Staempfli, H.R., Duffield, T., Weese, J.S., 2009. Isolation of bovine intestinal *Lactobacillus plantarum* and *Pediococcus acidilactici* with inhibitory activity against *Escherichia coli* O157 and F5. Journal of Applied Microbiology 106, 393–401.

Rogosa, M., Mitchel, J.A., Wisemann, A., 1951. A selective medium for the isolation of oral and faecal lactobacilli. Journal of Bacteriology 62, 132–133.

Ross, G.R., Gusils, C., Oliszewski, R., de Holgado, S.C., González, S.N., 2010. Effects of probiotic administration in swine. Journal of Bioscience and Bioengineering 109, 545–549.

Ruas-Madiedo, P., Moreno, J.A., Salazar, N., Delgado, S., Mayo, B., Margolles, A., de los Reyes-Gavilán, C., 2007. Screening of Exopolysaccharide-Producing *Lactobacillus* and *Bifidobacterium* Strains Isolated from the Human Intestinal Microbiota. Applied and Environmental Microbiology 73, 4385–4388.

Saavedra, L., Minahk, C., de Ruiz Holgado, A.P., Sesma, F., 2004. Enhancement of the enterocin CRL35 activity by a synthetic peptide derived from the NH_2-terminal sequence. Antimicrobial Agents and Chemotherapy 48, 2778–2781.

Saxelin, M., 2008. Probiotic formulations and applications, the current probiotics market, and changes in the marketplace: a European perspective. Clinical Infectious Diseases 46 (2), S76–S79. S144–S151.

Schachtsiek, M., Hammes, W.P., Hertel, C., 2004. Characterization of *Lactobacillus coryniformis* DSM 20001T surface protein Cpf mediating coaggregation with and aggregation among pathogens. Applied and Environmental Microbiology 70, 7078–7085.

Strompfová, V., Marciňáková, M., Simonová, M., Gancarčíková, S., Jonecová, Z., Sciranková, L., Koščová, J., Buleca, V., Čobanová, K., Lauková, A., 2006. *Enterococcus faecium* EK13 – an enterocin A-producing strain with probiotic character and its effect in piglets. Anaerobe 12, 242–248.

Timmerman, H.M., Mulder, L., Everts, H., van Espen, D.C., van der Wal, E., Klaassen, G., Rouwers, S.M.G., Hartemink, R., Rombouts, F.M., Beynen, A.C., 2005. Health and growth of veal calves fed milk replacers with or without probiotics. Journal of Dairy Science 88, 2154–2165.

Timmerman, H.M., Veldman, A., van den Elsen, E., Rombouts, F.M., Beynen, A.C., 2006. Mortality and growth performance of broilers given drinking water supplemented with chicken-specific probiotics. Poultry Science 85, 1383–1388.

Torsein, M., Lindberg, A., Sandgren, C.H., Waller, K.P., Törnquist, M., Svensson, C ., 2011. Risk factors for calf mortality in large Swedish dairy herds. Preventive Veterinary Medicine. doi:10.1016/j.prevetmed.2010.12.001.

Vandenbergh, P.A., 1993. Lactic acid bacteria, their metabolic products and interference with microbial growth. FEMS Microbiology Reviews 12, 221–238.

Veir, J.K., Knorr, R., Cavadini, C., Sherrill, S.J., Benyacoub, J., Satyaraj, E., Lappin, M.R., 2007. Effect of supplementation with *Enterococcus faecium* (SF68) on immune functions in cats. Veterinary Therapeutics: Research in Applied Veterinary Medicine 8, 229–238.

Virtala, A.M., Mechor, G.D., Grohn, Y.T., Erb, H.N., 1996. Morbidity from nonrespiratory diseases and mortality in dairy heifers during the first three months of life. Journal of the American Veterinary Medical Association 208, 2043–2046.

Wirawan, R.E., Klesse, N.A., Jack, R.W., Tagg, J.R., 2006. Molecular and genetic characterization of a novel nisin variant produced by *Streptococcus uberis*. Applied Environmental Microbiology 72, 1148–1156.

Zoetendal, E.G., Vaughan, E.E., de Vos, W.M., 2006. A microbial world within us. Moleculary Microbiology 59, 1639–1650.