



Isolation and identification of potentially probiotic lactic acid bacteria for Holstein calves in the Mexican Plateau



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Abstract:

Neonate calves are continuously exposed to a wide range of microorganisms in the environment, including diarrhea-causing enteropathogens. Lactic acid bacteria (LAB) was isolated from the oral mucosa of calves, and colostrum and milk from Holstein cows, the strains identified and their resistance to acid pH and bile salts tested. Isolation was done on plated de Man-Rogosa-Sharpe agar. Once decontaminated, the LAB colonies were morphologically and biochemically characterized. Sixteen of the isolated bacterial strains were selected: 12 from oral mucosa, 2 from milk and 2 from colostrum. After testing for resistance to an acid environment (pH 4 and 4.5) and bile salts (0.3 and 1.5 g), the five most resistant species (pH 4 and 1.5 g bile salts) were identified with the API 50 CHL system:

Leuconostoc mesenteroides, *Pediococcus pentosaceus*, *Lactobacillus plantarum*, *Lactobacillus crispatus* and *Lactococcus lactis*. These strains have probiotic potential in calves.

Key words: Calves, Probiotics, Isolation, Resistance.

Received: 01/06/2017

Accepted: 27/02/2018

Introduction

A number of problems can arise when breeding heifers for replacement, including poor colostrum supply, feeding with low quality milk substitutes and sudden changes in ration⁽¹⁾. These substandard breeding practices can lead to diarrhea, caused mainly by enteropathogens, with mortality rates exceeding 10 % during the first weeks of life⁽²⁾. Antibiotics are used to reduce mortality, but many pathogenic strains have developed resistance, negatively affecting animal health^(3,4). Several veterinary pharmaceutical laboratories now promote the use of probiotics containing lactic acid bacteria (LAB) and claim benefits such as prevention and reduction of diarrhea, and improved weight gain. However, to qualify as efficient probiotics these products must comply with certain requirements. For example, the minimum number of microorganisms required in a calf's intestine to generate adequate health is 10⁶ colony-forming units (CFU)/ml⁽¹⁾.

Clinical trials done over the last ten years have found that 45% of probiotics on the market contain LAB with null efficiency in the prevention of diarrhea in heifers. Some even seemed to aggravate diarrhea incidence and severity^(4,5), and provided no improvements in daily weight gain and feed conversion^(6,7). The same still holds true for the probiotics marketed to dairy cattle production units: low viability probiotic microorganisms are used, and bacteria species other than those on the label have been identified⁽⁸⁾. Some strains come from different geographical regions and/or other animal species, which causes low viability and probiotic activity⁽⁴⁾. The present study objective was to isolate and identify bacteria with probiotic potential (i.e. resistance to acid pH and bile salts) in Holstein cattle in the Plateau region of Mexico.

Material and methods

Isolation of bacteria from oral mucosa and colostrum

The experimental animals consisted of five lactating calves (30 d of age) and five multiparous adult cows in lactation, all Friesian Holstein from College of Postgraduates (Colegio de Postgraduados, Campus Montecillo) installations. Colostrum samples were taken from five newly-calved Holstein cows on the private Xalapango ranch. Both sites are located in the Texcoco Valley, in the State of Mexico, Mexico (18°21' and 20°17' N; 98°36' and 100°36' W)⁽⁹⁾.

Sampling

Oral mucosa: Duplicate exudate samples were taken of the oral mucosa from each lactating calf, by rubbing the mucosa for 3 sec with a swab (3MTMSwab-sampler) prior to the morning feeding. Each swab was placed inside a sterile tube with 10 ml buffered peptone water (RS96010BPW).

Colostrum and milk: Before sampling, the cows' nipples were cleaned, disinfected and pulled down, and 5 ml of colostrum and 10 ml of milk collected per group of cows. Samples were deposited in sterile vials, kept at 4 °C and immediately transferred to the laboratory for analysis following standardized procedures⁽¹⁰⁾.

Sample processing

The samples were pre-enriched (to favor LAB growth) in liquid culture medium⁽¹¹⁾. Duplicate samples (1 ml) of the oral mucosa bacterial suspension were taken and placed in tubes containing 5 ml de Man/Rogosa/Sharp (MRS) broth. The inoculated tubes were divided into two random groups: one under aerobic conditions and the other under anaerobic conditions, both were incubated at 37 °C for 18 h. In the colostrum and milk samples, 200 µl were taken in duplicate and deposited in tubes containing 5 ml MRS broth, and kept in a desiccator under an anaerobic environment (induced by a burning candle) for 18 h at 37 °C. Samples were then taken from the tubes with an inoculation loop and sown in Petri dishes containing MRS agar⁽¹²⁾, and incubated at 37 °C for 48 h under anaerobic and aerobic conditions. A strain of *Lactobacillus casei* ATCC was used as a positive control and one of *E. coli* O42 as a negative control (both donated by the Universidad Autónoma de Querétaro).

Bacteria selection

During the sowing process the cultures were seriated according to sample duplication in the solid MRS medium. The result was a total of 54 colonies with LAB characteristics based on colony size, shape, surface, elevation, edge and color⁽¹³⁾. Strain characterization was done by the Gram stain test, cell morphology, spore staining and the catalase test⁽¹²⁾. Indole production and motility tests were done in SIM (hydrogen sulfide, indole, motility) culture medium; gelatin hydrolysis and nitrate reduction tests were also done⁽¹⁴⁾. A second selection of the isolated colonies was made based on the best scores and ideal coccobacilli and bacilli morphology. A total of 27 colonies were identified which were cultured in 5 ml MRS broth for 18 h for later evaluation as probiotic bacteria. Duplicate 800 µl samples were taken from each bacterial suspension and transferred to Eppendorf tubes containing 800 µl sterile 50% glycerol as a cryoprotectant. These were stored at -20 °C for 3 h and subsequently at -80 °C indefinitely.

Resistance and survival of selected strains under gastrointestinal conditions

Resistance to acid pH

Inoculum preparation: A further selection was made of the 27 colonies obtained in the isolation process; 16 were chosen for having well-defined coccobacilli and bacilli morphology. Of these sixteen, twelve were isolates from oral mucosa, two from milk and two from colostrum. All selected colonies were reactivated by raising storage temperature to $-20\text{ }^{\circ}\text{C}$ and then to room temperature. Each colony was then transferred into tubes containing 5 ml MRS broth and incubated at $37\text{ }^{\circ}\text{C}$ under anaerobic conditions for 24 h. A 1 ml sample of each bacterial suspension ($10^6\text{ Log}_{10}\text{ CFU/ml}$) was added to tubes containing 9 ml MRS broth and incubated another 18 h. These final bacterial suspensions were centrifuged at $2,056\text{ xg}$ for 10 min, and the cellular packages resuspended in 10 ml sterilized ($110\text{ }^{\circ}\text{C}$ for 15 min) skim milk (Alpura[®] 2000[®]) for the resistance test at pH 4.5 and 4.0. The milk functioned as a protective medium and a vehicle for probiotic microorganisms⁽¹⁵⁾, following the protocol described by Fernández de Palencia *et al*⁽¹⁶⁾. Resistance to acid pH conditions was assessed by reducing the pH to which the bacterial cells were exposed. Reduction of pH was done with controlled HCl aliquots. When PH stabilized at 4.5 and 4.0, samples were incubated at $37\text{ }^{\circ}\text{C}$ for 10 min. Subsequently, 1 ml of each suspension was taken to make serial dilutions. From each dilution, 100 μl was taken and sown in MRS agar to estimate bacterial cell viability. The colonies evaluated at pH 4.5 and 4.0 were sown at 10^{-6} and 10^{-7} in a milk suspension.

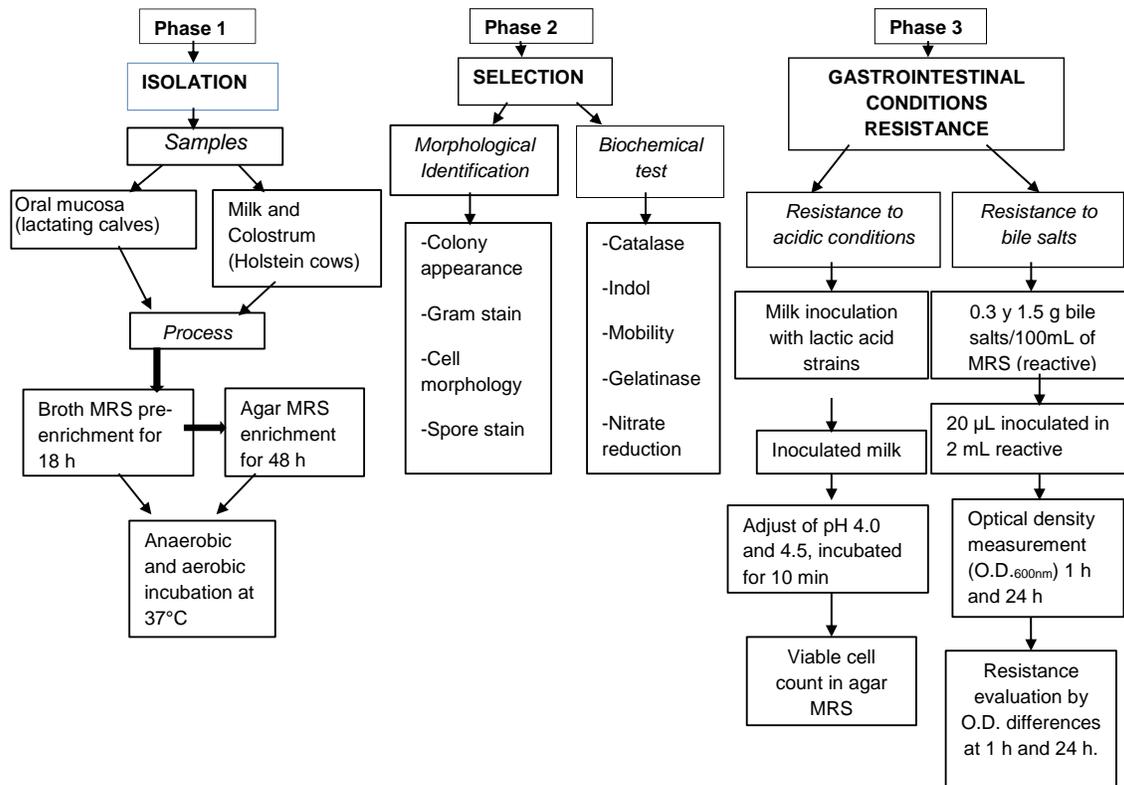
Resistance to bile salts exposure in microtitre plates

The selected colonies were exposed to bile salts (BS) in microtitre plates (BD Primaria[™]) with 3.5 ml wells. One plate was used per concentration. Before beginning the test, two flasks were prepared containing 100 ml MRS broth: one with 0.3 g bovine BS and the other with

1.5 g bovine BS (Oxgall Difco™)^(17,18). Two negative controls were run: MRS with BS and without bacteria, and MRS without BS and with bacteria. The BS resistance test was run in triplicate and each colony occupied six plate wells. In addition, 2 ml BS solution (MRS with BS and without bacteria) and 20 µl (1:10 v/v) of bacterial suspension were incubated for 18 h. One hour after inoculation and before the plates were incubated, the optical density (OD) of each suspension was measured at 600 nm using a spectrophotometer (GENESYS 10 UV/Thermo Spectronic). The OD reading was taken again once the plates had been incubated under anaerobic conditions at 37 °C for 24 h.

Biochemical identification and collection of strains with probiotic potential

Colonies with LAB characteristics were identified using the APICHL system (BioMerieux SA, France). In this procedure the colonies were reactivated in 5 ml MRS broth under anaerobic conditions at 37 °C for 18 H, adding 50CH diluent (supplied with the gallery: API50CH) following manufacturer instructions (see Figure 1 for summary of procedure). The prepared suspension was added to 50 microtubes in the gallery, and the domes of these microtubes filled with sterile mineral oil to generate anaerobic conditions. The inoculated galleries (one per colony) were kept at 37 °C for 48 h to establish each colony's biochemical profile. Results interpretation was done based on color change in the API50CHL medium of each microtube: blue is negative, and yellow and black indicate positive values (plate safety sheet). Results were analyzed with the Apiweb® computer system.

Figure 1: Probiotic bacteria isolation and selection procedure

Statistical Analyses

When analyzed with Kolmogorov–Smirnov test the data exhibited a normal distribution, and a Levene test showed variance homogeneity. Means were compared with an ANOVA and a Tukey test; significance level was 0.05%. All analyses were run with the SPSS ver. 15 statistics package⁽¹⁹⁾.

Results and discussion

Colony isolation and growth

The colonies cultured in the anaerobic environment exhibited better growth than those cultured under aerobic conditions. Based on morphology, the selected colonies were Gram positive coccobacilli and bacilli, with no spores, catalase negative, no motility, no indole or gelatinase production and nitrate reduction negative.

The colonies from the oral mucosa samples had an average size of 2 to 4 mm in diameter with homogeneous morphological characteristics; circular shape, convex elevation, complete edge, smooth surface and white color without pigments. Of the milk samples only 20 % supported bacterial colony growth, which had an average size of 2.5 mm diameter. Those colonies isolated from the colostrum were beige in color and varied in size from 1 to 5 mm diameter. Probiotic bacteria have generally been isolated from the oral, vaginal, and intestinal mucosa of healthy calves and from milk samples^(11,20). Lactobacilli colonies isolated from the oral mucosa and milk have the capacity to adapt and survive⁽¹³⁾. This is due to the presence of the hemin group, which allows them to activate the respiratory chain with oxygen as the electron recipient⁽²¹⁾. The different LAB genera share morphological, metabolic and physiological characteristics such as shape, elevation, edge, color and biochemical reactions⁽¹³⁾. For the purpose of probiotic strain selection, their cell morphology and biochemical tests have been reported as basic^(5,22), although it is recommended that selection be complemented with molecular studies⁽²³⁾.

API biochemical strain identification

Colony identification based on carbohydrate fermentation profile (API50CHL-BioMerieux) produced a 96 to 99 % effectiveness interval (Table 1). Identified colonies from the oral

mucosa included six *Lactobacillus*, five *Leuconostoc* and one *Pediococcus*, while those from the colostrum included *Leuconostoc* and *Lactobacillus*.

Strain viability based on resistance to acid pH

Analysis of colony population resistance to different pH levels (Table 1), found growth at the control pH (6.5) to average $9.07 \log_{10}$ CFU/ml. At pH 4.0 growth decreased ($P < 0.001$) to $5.09 \log_{10}$ CFU/ml. The two colonies from the milk samples did not grow at pH 4 and were not included in the final strains. Resistance to acid pH is relevant because to reach the action site and remain viable probiotic bacteria must withstand acid pH and the presence of BS in the duodenum⁽²⁴⁾. Several authors have developed methodologies to evaluate probiotic strain resistance under gastrointestinal conditions^(7,25). The present results coincide with a study of *L. plantarum* and *L. acidophilus* in which these strains grew and remained viable at pH 5.0, but became inactive at pH 4.0 and 3.0⁽¹⁶⁾. Strain sensitivity may be related to the acid tolerant response or acquired resistance. For example, in a study comparing a control of *L. casei* cells grown at pH 6.0 to acid adapted cells at pH 4.5 for 10 and 20 min, viability decreased up to $4.0 \log_{10}$ CFU/ml at 10 min adaptation, and from 0.7 to $2.4 \log_{10}$ CFU/ml at 20 min⁽²⁶⁾. Cell adaptation to an acid environment caused changes in membrane lipid composition, with a dramatic increase in saturated and unsaturated fatty acids, as well as malolactic fermentation and intracellular histidine accumulation. The ability of probiotic bacteria to survive the stomach's acid environment varies by strain^(27,28,29), which would explain the differences in resistance between LAB strains observed here at pH 4.0. Lactobacilli commonly grow better at pH 4.0 than at pH 3.0⁽³⁰⁾, and at pH 3.0 only four of 200 known LAB strains survive⁽³¹⁾.

Table 1: Colony counts in de Man-Rogosa-Sharpe (MRS) agar immediately after exposure to acid pH, and biochemical strain identifications

Strain count	T1 pH 6.5	T2 pH 4.5	T3 pH 4.0	Biochemical strain identifications*
~	9.40	9.21	5.74	
Mucosa				
1	8.77	8.40	5.08	<i>Leuconostoc mesenteroides</i>
2	9.46	8.43	5.49	<i>Leuconostoc mesenteroides</i>
3	9.02	9.36	5.09	<i>Pediococcus pentosaceus</i>
4	9.06	7.23	4.83	<i>Lactobacillus plantarum</i>
5	8.28	7.67	5.06	<i>Lactobacillus plantarum</i>
6	8.78	8.47	4.01	<i>Lactobacillus salivarius</i>
7	8.71	8.49	6.43	<i>Leuconostoc mesenteroides</i>
8	9.36	8.39	6.47	<i>Lactobacillus crispatus</i>
9	9.39	9.11	5.44	<i>Leuconostoc mesenteroides</i>
10	9.37	8.68	NG	<i>Lactobacillus brevis</i>
11	9.36	8.84	NG	<i>Lactobacillus brevis</i>
12	8.82	8.48	3.33	<i>Leuconostoc mesenteroides</i>
Milk				
13	9.02	8.14	NG	<i>Lactobacillus brevis</i>
14	9.15	8.31	NG	<i>Lactobacillus brevis</i>
Colostrum				
15	9.32	9.14	5.34	<i>Lactococcus lactis</i>
16	9.32	8.95	4.52	<i>Leuconostoc mesenteroides</i>
Mean	9.07±0.33 ^a	8.50±0.54 ^b	5.09±0.89 ^c	

* Identification done with API system (API 50CHL). Treatment ~ corresponds to positive control strain *L. casei*. Data are the mean of three replicates and correspond to log₁₀ CFU/ml. NG: No growth.

^{a,b} Different superscript letters in the mean value ± standard deviation indicate significant difference.

Viability under bile salts exposure

When exposed to high BS concentrations, the LAB tested in the present study continued to grow at concentrations as high as 1.5 g. Lactic acid bacteria resistance to and growth under exposure to BS has been tested at concentrations from 0.1 to 4.0 %^(32,33); this is an important parameter for microorganisms in commercial products^(34,35), but one rarely tested. In another study⁽³⁶⁾, *L. plantarum* resistance was exposed to four concentrations of porcine BS (0.01, 0.05, 0.10 and 0.15 g), and strain growth monitored for 24 h via OD measurements. The highest growth rate was observed at the lowest BS concentration, and, at the final density and 0.10 g BS, this strain's growth rate was three times lower than in the control. This is higher growth inhibition at lower BS concentrations than observed in the present study: final colony OD was only 2.5 times lower at 0.3 g BS than in the control treatment. There are reports of resistance to BS at concentrations from 0.3 to 1% BS in LAB (*Streptococcus thermophilus*, *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactococcus lactis*) and probiotic bacteria (*L. acidophilus*, *L. casei*, *L. rhamnosus* and *Bifidobacterium*)^(37,38). In these studies, *S. thermophilus* was the most sensitive LAB strain (growth inhibition at 0.5 g BS), *L. lactis* was the most resistant LAB (growth inhibition at 1 g BS), and all the probiotic strains exhibited resistance to 1.5 g BS. Resistance to BS exposure may differ between *Lactobacillus* species based on their ability to colonize and rapidly stabilize, as has been tested in the intestine of heifers⁽⁷⁾. For example, in an *in vivo* study in which *L. acidophilus* was administered to heifers, the total lactobacilli count in the jejunum increased from 13 to 39 %, but strains of *L. plantarum* and *Lactococcus acidilactici* exhibited better growth at pH 4.0 and 0.3 g BS⁽⁵⁾. In the present results, the LAB were more tolerant of BS exposure than of acid pH levels (4.0). However, their relatively good resistance to prolonged exposure to acid pH and very good resistance to high BS concentrations are effective indicators of their survival and colonization capacity during intestinal transit^(28,39).

Resistance to BS was also quantified by comparing strain average OD at two BS concentrations (0.3 and 1.5 g) (Table 2). Average OD in all the evaluated strains increased 3.1 times ($P<0.05$) after 24 h incubation at 0.3 g SB, compared to the initial reading, but when exposed to 1.5 g BS for 24 h, it increased 2.7 times. Optical density (OD) dropped significantly ($P<0.023$) as BS concentration increased, but it still increased ($P<0.0001$) from 1 to 24 h.

Table 2: Average optic density (OD) of growth in lactic bacteria strains at two bile salts (BS, %) concentrations at 1 and 24 hours' incubation

I	Treatment					Main Effects					
	0.3% BS		1.5% BS		MSE	0.3% BS	1.5% BS	MSE	1h	24h	MSE
	1h	24h	1h	24h							
OD	0.33	1.02	0.31	0.84	0.03	0.68	0.57	0.02	0.32	0.93	0.02
P>F	0.008		0.008			0.023			0.0001		

MSE = Mean standard error.

Evaluations of the benefits of probiotic bacteria in heifers have been contradictory, perhaps due to a lack of diversity in probiotics for specific geographical regions and sale of unviable strains. This makes it difficult to find experimental sequences utilizing the same strain⁽⁴⁰⁾. The marketing of probiotic products for heifers in Latin America is limited and dubious. Most suppliers offer only *L. acidophilus* strain KA1-A 8 (3 trillion CFU/dose) and *L. casei* (3 billion CFU/dose), without specifying their use in heifers. Others promote products containing *Lactobacillus rhamnosus* and *Bifidobacterium lactis* (1×10^{11} CFU/ dose or 50 units for direct application in 1,000 L milk). But they do not specify strain origin, leaving open the possibility that they have been isolated from other animal species or food, making them suboptimum options for use in heifers. For example, when human probiotic strains are administered to livestock the microorganisms cannot resist gastrointestinal conditions or colonize the intestine due to interspecies differences in physiology and food^(41,42). This is why the strains isolated in the present study were taken from the species in which they are intended for use and in the region they are to be applied; their probiotic potential can therefore be stated to be for Holstein cattle in the study area. Currently, the most widely used probiotic bacteria genera in livestock are *Lactobacillus*, *Enterococcus*, *Bifidobacterium*, *Lactococcus* and *Leuconostoc*⁽⁴³⁾; *Lactobacillus plantarum*, *L. acidophilus*, *L. casei*, *L. salivarius* and *Lactococcus lactis*^(5,44,45); and *L. fermentum* VC3B-08, *W. hellinica* V1V-30 and *L. farciminis* B4F-06⁽²⁰⁾.

Conclusions and implications

Sixteen lactic acid bacteria colonies were selected from the oral mucosa, milk and colostrum of Holstein cattle. *Lactobacillus brevis* isolated from samples of the oral mucosa and milk did not grow in acid pH (4.0). Based on their relative resistance to acid pH and good

resistance to bile salts, five strains were selected: *Leuconostoc mesenteroides*, *Pediococcus pentosaceus*, *Lactobacillus plantarum*, *Lactobacillus crispatus* and *Lactococcus lactis*. These strains have broad probiotic potential in heifers and require further direct *in vivo* evaluation in the gastrointestinal tract.

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