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Natalia C. Maldonado and María Elena Fátima Nader-Macías*

Reference Center for Lactobacilli (CERELA-CONICET). 145. Chacabuco 4000 San Miguel de Tucuman. Argentina

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***Corresponding author:** Dr. Mary E. Fàtima Nader - Macias, Reference Center for Lactobacilli, Chacabuco 145, 4000. Tucuman, Argentina, Tel: 54-381.4311720; Ext 141; Fax: 54-381-4005600; E-mail: fnader@cerela.org.ar

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Research Article

Functional Properties (Acid and Bile Tolerance) and Antibiotic Susceptibility of Lactic Acid Bacteria Isolated from Newborn Calves for the Design of a Probiotic Product

Abstract

Diarrhea in young calves in dairy farms is one of the main causes of economic losses, morbidity and mortality. The use of probiotic products as feed additives or complements is a novel alternative for the prevention of intestinal syndromes. In order to include beneficial bacteria in the design of a probiotic product, their functional and safety characteristics must be studied. The aim of this work is to evaluate the behavior of the strains in some "*in vitro*" gastrointestinal conditions such as acid stress and bile salts in the specific physiological concentration of young calves. The antibiotic susceptibility of a group of lactic acid bacteria from calves which were identified due to their beneficial properties was also studied. The strains, genetically identified and used for the resistance assays were: *Lactobacillus johnsonii* CRL1692, CRL1693, CRL1699, CRL1700, CRL1701 and CRL1706; *L. amylovorus* CRL1697; *L. murinus* CRL 1695 and CRL1705; *L. mucosae* CRL1696 and CRL1698; *L. salivarius* CRL1694 and CRL1702; and *Enterococcus faecium* CRL1703. The results of gut resistance assays showed that all the strains were resistant to pH 4 and to a bile salts concentration of less than 0.5%. However, some of them were sensitive to pH 2. The most pH-sensitive strains were found to be *L. johnsonii* and *L. amylovorus*, and enterococci. However, pre-treatment at low pH increased the growth rate of the *L. salivarius* strains. The minimal inhibitory concentration showed that the strains were sensitive to Tetracycline, Erythromycin, Chloramphenicol and Ampicillin, while most of them were resistant to Kanamycin. The results allowed the selection of the most adequate strains to be included in a probiotic product that can be utilized most successfully in young calves.

Introduction

One of the main causes of mortality in newborn calves of dairy farms is neonatal diarrhea, which causes severe economic losses [1-4]. As a novel alternative in this field, the use of probiotics as feed additives or complements is being proposed for the prevention of intestinal infections by restoring the balance of the microbiome. Probiotics are defined as "*live microorganisms which when administered in adequate amounts confer a beneficial health on the host*" [5]. In a previous work, fourteen Lactic Acid Bacteria (LAB) strains were selected for their beneficial properties to include some of them in the design a new probiotic product for newborn calves [6]. Later, the evaluation of the technological properties of most strains was also performed [7]. At the same time, functional properties and others related to the safe status of the strains must be determined for the final design of such product [8]. As the probiotic supplement is going to be administered orally, the bacterial constituents should be resistant to the conditions of the gastrointestinal tract (GIT). Moreover, the number of live organisms that reach the gut should be large enough to produce their beneficial effect on the host [9]. On the other hand, LAB can be reservoirs of resistance genes, which could be transferred to other bacteria and even to human pathogens [10], which is why some of their safety-related characteristics should be studied. The probiotic product on which we are working is directed to newborn calves, recognized as *non-ruminant* up to the time when the rumen becomes functional, which means that the digestive process is developed in a different way than in young animals. In calves, gastric juices are secreted daily by the stomach, resulting in the destruction of most of the microorganisms ingested with the food. Then, resistance to the acid conditions of the stomach is one of the selection criteria applied in the selection of probiotic bacteria [11].

Bile salts, which act as detergents that emulsify and solubilize fats, have been shown to exert a bactericidal effect. Consequently, the capability of LAB to resist bile is essential to maintain their viability in the intestine [12,13].

In the last decade the evaluation of the antibiotic susceptibility of LAB has grown because of their potential to spread resistance genes to other microorganisms by horizontal transfer resistance [14-17]. The evaluation of the antimicrobial susceptibility of microorganisms can be performed using different methods, including agar diffusion, E-test, macro and micro dilution [18-20]. Also, techniques included in the International Organization of Standardization/International Dairy Federation (ISO/IDF) Standard and Clinical and Laboratory Standards Institute (CLSI) guidelines [21], for Antimicrobial Susceptibility Testing of Lactobacilli have often been applied. For

the determination of minimal inhibitory concentration (MIC), the EFSA (European Food Safety Authority) Panel on Additives and Products or Substances used in Animal Feed (FEEDAP) has proposed epidemiological breakpoints to evaluate and identify phenotypic resistance. Moreover, the European Union has strongly recommended the study of the antibiotic susceptibility of bacteria included in feed additives for veterinary use and later used in the Food Chain.

The aim of this work was to evaluate some of the functional (acid and bile tolerance) and safety (antibiotic susceptibility) related properties of a LAB strains group previously selected for their beneficial characteristics to be used in the design of a probiotic product to prevent diarrhea in newborn calves.

Materials and Methods

Microorganisms and growth conditions

LABs were isolated from young calves' feces and selected by their beneficial properties [6]. They were genetically identified as: *Lactobacillus johnsonii* CRL1692, CRL1693, CRL1699, CRL1700 and CRL1701 CRL1706, *Lactobacillus amylovorus* CRL1697 *Lactobacillus murinus* CRL 1695 and CRL1705, *Lactobacillus mucosae* CRL1696 and CRL1698, *Lactobacillus salivarius* CRL1694 and CRL1702, and *Enterococcus faecium* CRL1703. The strains were stored in milk yeast extract (13% nonfat milk, 1% yeast extract) containing 20% glycerol (vol/vol) at –20°C. For the daily experiments, the strains were cultured in MRS broth [22] (Merck, Darmstadt, Germany) at 37°C for 24 h, and sub-cultured twice in the same media at 37°C for 12 h and 16 h respectively.

Resistance to gastrointestinal conditions

The strains were washed twice and the pellets were re-suspended in saline solution at neutral pH 7.0 (control) or at pH 2 or pH 4 adjusted with 1N HCl (Anedra, San Fernando, Argentina) and incubated for 90 min at 37°C (the estimated time that the liquid food remains in the abomasum during the calves' gastric digestion) [23]. After incubation, the bacterial cells were washed three times in standard saline solution (0.9% NaCl, Anedra, San Fernando, Argentina). These cultures were adjusted to 0.9-1 Optical Density at 560nm and later 2 µl corresponding to 5 x 10^8 CFU/mL) were inoculated into polystyrene microplates (Deltalab SL, Barcelona, Spain) containing MRS (Merck, Darmstadt, Germany) broth supplemented with different bovine ox-bile concentrations (0.1%, 0.3%, 0.5% and 1%) (Fluka-70168, Sigma-Aldrich, St. Louise, USA) and also into MRS pH 4 (adjusted with 1N HCl). The bacterial growth was determined by changes in the Optical Density (OD) at 560nm in a microplate reader (VersaMax Tunable Microplate reader, Sunny Valley, USA) at different time periods (3, 5, 7, 12, 20 and 24 h). Final ΔOD was calculated as the increase in OD between OD_0 (OD_0 is OD at t=0) and OD_{24} (OD_{24} is OD at t=24). The growth curves were performed from the OD of each time period. The growth rate was calculated as: µ/h (growth rate); µ = ln2/g; generation time $g = (t_2 - t_1)\ \log 2 / (\log OD_2 - \log OD_1)$.

Statistical analyses

Growth determinations were performed in triplicate. Data express the mean ±SD. The experimental results were used to create the Main Effects Plot using the Minitab 16 Statistical Software.

Antibiotic susceptibility

The strains selected to determine the antibiotic susceptibility were those expressing beneficial properties: surface characteristics (hydrophobicity degree and autoaggregation patterns), antagonistic activity (production of hydrogen peroxide, organic acids, or bacteriocins) and functional properties (resistance to acid and bile salts). In all cases, only one of each of the species of *Lactobacillus* under study was included in these assays.

Disk diffusion: The Kirby-Bauer disk diffusion susceptibility test was carried out according to the Performance Standards for Antimicrobial Disk Susceptibility Tests of CLSI with minor modifications [24]. The technique was performed in three different culture media: MRS agar pH 6.2; LAPTg agar (15 g/L peptone, 10 g/L tryptone, 10 g/L glucose, 10 g/L yeast, 1 ml/L Tween 80, 15 g agar, distilled water 1L), LAPTg agar pH 6.5 and LSM agar [25], (LAB susceptibility test medium). This last medium is formulated with 90% Müller Hinton agar (Britania, Buenos Aires, Argentina) and 10% MRS agar. The antibiotics assayed were: Sulfamethoxazole+Trimethoprim (TMS) 25 µg, Clindamycin (CLIN) 2 µg, Erythromycin (ERY) 15 µg, Gentamicin (GEN) 10 µg, Ampicillin (AM) 10 µg, Vancomycin (VAN) 30 µg, Nalidixic Acid (NAL) 30 µg, Cephalexin (CEF) 30 µg, Ciprofloxacin (CIP) 5 µg, Ampicillin Sulbactam (AMS) 30µg, Teicoplamin (T) 30 µg, Rifampicin (RFA) 5 µg, and Minomicyn (MIN) 30 µg. All disks were obtained from Britania (Buenos Aires, Argentina). For the inoculum preparation, LABs were spread on MRS agar and incubated at 37°C for 48 h, and isolated colonies were suspended to McFarland standard 1. Later, the strains were spread onto the different media where the antibiotic disks were added. The plates were incubated for 48 h and the diameter of the inhibition zone was determined after incubation [26].

Minimum inhibitory concentration (MIC): The Minimum Inhibitory Concentration (MIC) was determined by the microdilution method in solid media in LSM agar (LAB Susceptibility test medium) as the culture medium [25]. The bacteria assayed at this stage were: *L. salivarius* CRL1694, *L. amylovorus* CRL1697, *L. johnsonii* CRL1693, *L.mucosae* CRL1696 and *L. murinus* CRL1695. The following antibiotics and concentration ranges were assayed: Vancomycin (0.25-128 µg/mL), Rifampin (0.25-128 µg/mL), Ciprofloxacin (0.25-128 µg/mL), Ampicillin (0.25-128 µg/mL), Chloramphenicol (0.12-64 µg/mL), Tetracycline (0.12-64 µg/mL), Oxytetracycline (0.12-64 µg/mL), Lincosamide (0.12-64 µg/mL), Kanamycin (2-1024 µg/mL), (Sigma-Aldrich, St Louis, USA) and Erythromycin (0.25-128 µg/mL) (ICN Biomedicals, Santa Ana, USA).

Antibiotics were stored at -20°C until the preparation of stock solutions, re-suspending antibiotics in distilled water or methanol (Cicarrelli, San Lorenzo, Argentina) for those not soluble in water (Chloramphenicol, Erythromycin, Rifampicin), and dilutions were performed in twofold series according to CLSI specifications [27]. Agar plates were prepared with 1 mL of each of the antibiotic solutions and 9 mL of LSM agar melted and cooled to 45±5°C. The bacterial inoculum was adjusted to 0.16 to 0.2 OD at 625nm,

corresponding to approximately 3x10^8 CFU/mL and to McFarland standard 1, following the procedures proposed by ISO 10932/IDF 233 described in Shao et al. [28]. In order to determine the effect of different bacterial concentrations (10^5 CFU/mL, 10^6 CFU/mL and 10^7 CFU/mL) in the susceptibility assays, serial dilutions in saline solution were prepared and added to the plates. All the plates were inoculated with 2 µL of bacterial suspensions and incubated for 48 h in microaerophilic conditions. MIC was calculated from the lowest antibiotic concentration that caused inhibition of the microorganism. The interpretation of the results was performed was performed on the basis of the EFSA document [15], according to the results obtained with the highest microorganism concentration. The *L plantarum* ATCC14917 strain was also used as quality control. All the antibiotic assays were performed in triplicate.

Results

Resistance of beneficial LAB strains to gastrointestinal conditions (acid and bile salts)

The resistance of beneficial LAB strains to bile salts after pre-treatment at pH 4 and pH 2 was determined in all the strains used. Growth curves were plotted to compare the behavior of each strain, and also those from the same species in a combined figure. The standard deviation was not included in the graphs, except for *L. salivarius* CRL1702 in order to allow a better interpretation of the results. The resistance of the two *L. salivarius* (CRL1694 and CRL1702) and *L. mucosae* (CRL1696 and CRL1698) strains to the acid conditions and bile salts was similar for each species (Figures 1a,1b). However, in the case of *L. murinus* (CRL 1695 and CRL1705), the two strains showed a different behavior: CRL 1705 grew at pH 4 and 1% bile salts, but both strains proved to be more resistant after pretreatment at pH 2. In the *L. salivarius* strains, acid pretreatment improved their growth at all bile concentrations used (with the exception of 1% where the strains failed to grow) and also at pH 4. The two *L. mucosae* strains grew in all conditions assayed when compared with controls; however, their growth was lower than all the other species (Figure 1c). The growth curves of *Enterococcus* and *L. amylovorus* strains are shown in Figures 2a,1b. The six *L. johnsonii* strains exhibited a different behavior, with two different patterns or profiles. The strains CRL 1692, CRL1693 and CRL1700 pretreated at pH 4 showed a higher growth, in contrast with CRL 1966, CRL1701 and CRL 1706, which were stimulated when the acid pretreatment was at pH 2, as indicated in Figure 3.

All the strains assayed increased the lag phase length in bile salts after the pretreatments at low pH, either 2 or 4.

When all the strains were pretreated at pH 2, 4 or 6.5 and later transferred to evaluate their growth at pH 4, *Lactobacillus johnsonii* and *L. murinus* CRL1695 strains or enterococcus did not grow, in contrast to both *L. salivarius* and *L. mucosae* strains, and *L. murinus* CRL1705, which grew in acid conditions, as indicated in Table 1. The strains that were able to grow at higher bile salts concentrations were *L. mucosae* CRL1696 and CRL1698, *L. amylovorus* CRL 1697, *E. faecium* CRL 1703 and *L. murinus* CRL 1705; the other strains grew at a bile concentration of 0.5% or less. Most of the strains treated in acid conditions (pH 4) and later transferred to MRS-0.1%, 0.3% and 0.5% bile did not modify their growth, but in some cases their growth rates were lower (*L. murinus* CRL 1695 *L. mucosae* CRL1696 and CRL1698), as shown in Table 2. In addition, the growing and growth rate of *L. murinus* CRL 1695, *L. salivarius*, *L. amylovorus* CRL1697 and enterococcus strains pretreated at pH 2 were similar to those observed after pH 4 pretreatment. The growth rates of *L. salivarius* and *L. murinus* increased in MRS-bile salts pretreated at pH 2.

The application of the main effect analysis to the growth and growth rate data of all LAB strains used, analyzing strain, bile salt concentrations, acid pretreatment and growth at different pH values, resulted in the plot in Figure 4. *L. salivarius* CRL1694 and CRL1702 proved to be the strains with higher growth in all conditions studied. In contrast, *L. mucosae* CRL1696 and CRL1698, *L. johnsonii* CRL1699 and *L. johnsonii* CRL1701 showed a lower growth. Pretreatment in acid conditions (pH 2-pH 4) did not affect growth at pH 4 or in bile salts of all the strains. The highest bile concentration exerted a higher negative effect on the growth of all the strains.

With respect to the growth rate, the same pattern was obtained for all the strains (data not shown).

Antimicrobial susceptibility testing

In this paper, the evaluation of the most suitable media to determine the antibiotic susceptibility or resistance of beneficial LAB isolated from calves' feces was carried out. The disk diffusion assay showed that Gentamicin, Ciprofloxacin and Cephalexin were the antibiotics showing different patterns according to the culture media, because different inhibitions zone diameters were obtained in MRS, LAPTg and LSM. Also, Rifampicin, Erythromycin and Ampicillin Sulbactam showed different diameters of the inhibition zone. Minomycin was the antibiotic that caused the highest inhibition in all strains assayed. There were no inhibition halos for Sulfamethoxazole+Trimethoprim and Nalidixic Acid, as shown in Figure 5. Considering the results for each strain, *L. mucosae* CRL1696 grew poorly in LMS medium, *L. amylovorus* CRL1697 was the most sensitive, and *L. johnsonii* CRL1693 and *L. amylovorus* were inhibited by Vancomycin. These results are summarized in Table 3.

The MIC values obtained in solid media were compared with those suggested by EFSA [15], as shown in Table 4. All the strains were sensitive to Erythromycin, Ampicillin and Chloramphenicol but were resistant to Kanamycin (except for *L. mucosae* CRL1696). The MIC values of all the strains were slightly higher for Oxytetracycline than for Tetracycline, except for *L. johnsonii* CRL1693l. The MIC assays using different concentrations of the inoculum performed with the macro dilution agar technique indicated differences of one dilution, mainly in Kanamycin and Oxytetracycline.

When comparing the two methods, the resistance to Vancomycin of those strains carrying intrinsic resistance to this antibiotic showed similar patterns. No inhibition zones were observed in the disk assay and MIC values indicated that the cut-off points were higher than those suggested by EFSA [15].

Discussion

Over the last few decades probiotics have been used in the gut, skin, respiratory or urogenital mucosa of both humans and animals [29-31]. One of the main objectives of these feed additives

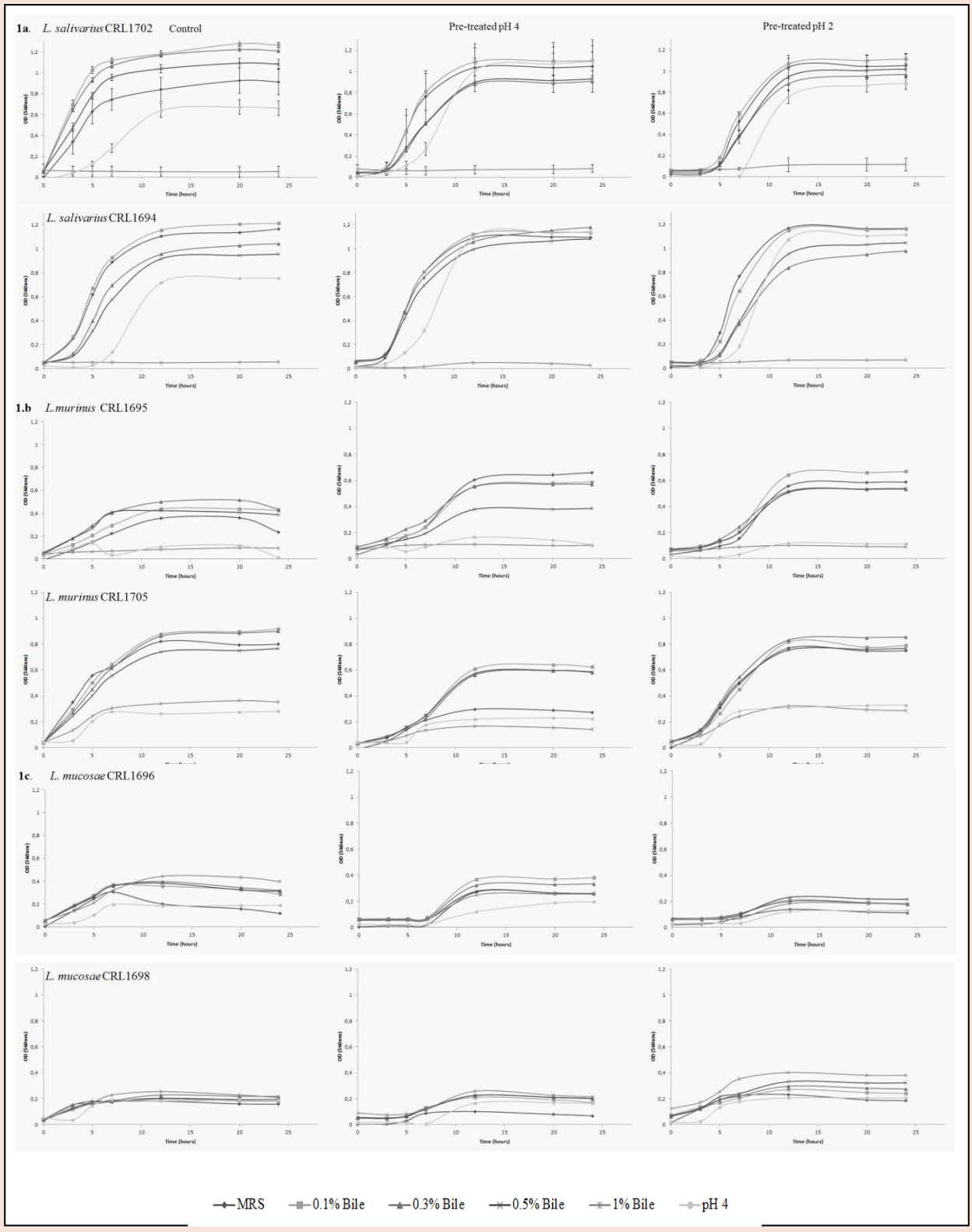

Figure 1: Growth pattern of different *Lactobacillus* strains: *Lactobacillus salivarius*, *L. murinus* and *L. mucosae*, and resistance to acid pretreatment and bile salts. The conditions are described in Materials and Methods.

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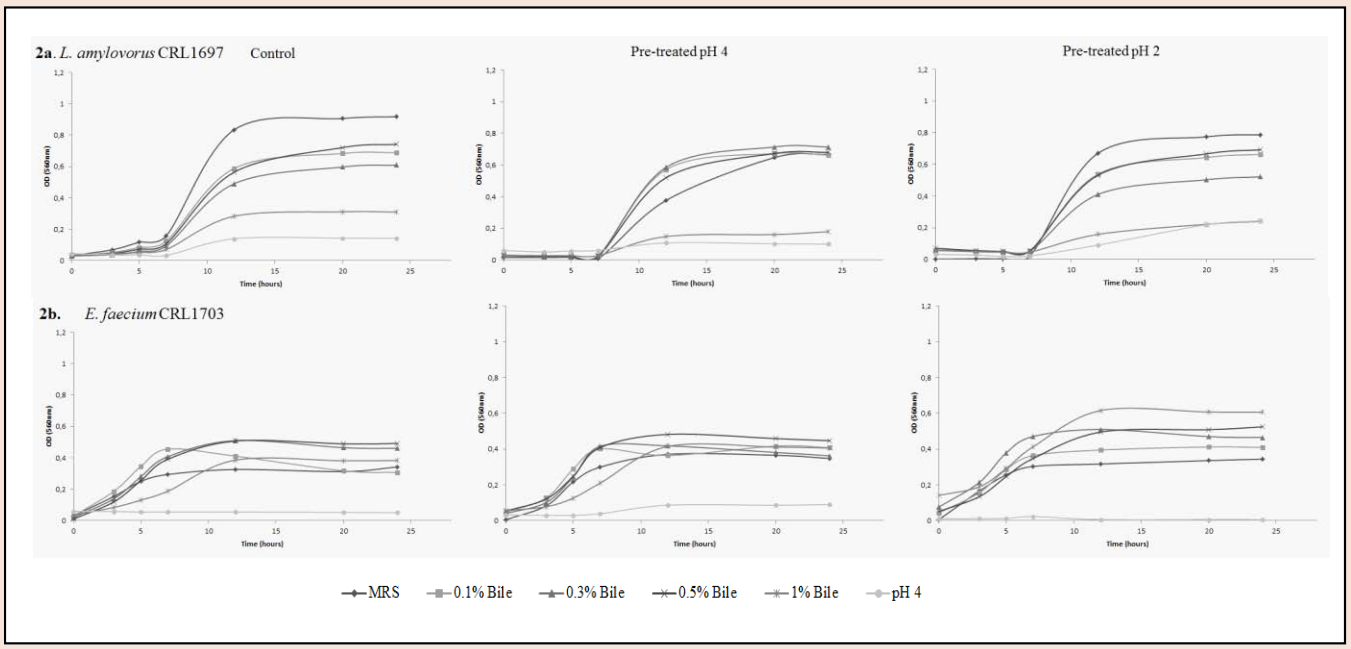

Figure 2: Growth curves of different *Lactobacillus* strains: *Lactobacillus amylovorus*, *E. faecium* and resistance to acid pretreatment and bile salts. The conditions are described in Materials and Methods.

or complements is the restoration of the indigenous microbiome of different tracts, supported by the host-specificity evidenced some years ago [32-34]. Some authors suggest that the autochthonous microbiota of the intestine could be better adapted to gastrointestinal conditions, and also that the bacterial resistance profiles of some of the microorganisms could be influenced by the region of the tract [13].

A basic characteristic to be evaluated when working in the design of a probiotic adjunct or complement to a specific host is its resistance to the conditions where it will be applied, the so-called functional properties [11]. On the other hand, viable bacteria in high numbers should be able to exert their beneficial effect on the target organ or mucosae. The microorganisms should cross over several biological barriers through the GIT, including gastric acid, enzymes, secretions and bile salts [12,13]. The digestive system of newborn calves is different from that of adult; in fact, calves behave as monogastrics up to the development of rumen in older animals.

The capability of the bacteria to survive the passage through the GIT is variable and strain dependent [35]. Lactic Acid Bacteria have several mechanisms that confer resistance in acidic conditions [36]. According to our results, each strain showed a different resistance profile and different sensitivity to acid in the presence of bile. However, those strains that produce higher concentrations of lactic acid [6], were the most sensible to these conditions. Similar results were obtained by other authors, where resistance to gastrointestinal conditions varied with the microorganism [37,38].

Lactic Acid Bacteria can also induce stress tolerance responses [39]. Our results suggest that the growth of microorganisms previously treated in acid conditions was higher in bile. All the strains should increase the lag phase length as observed in the growth curves. This behavior could suggest an adaptation of the strain; Burns et al. [35], evaluated the pre-adaptation and cross-resistance mechanisms to gastric conditions submitting the strains to sub-lethal acid stress. The resistance patterns observed in this group of strains could support a better adaptability of certain bacteria that should be further studied. The beneficial properties evaluated in a previous work [6], indicated that there is no relation between hydrophobicity or auto aggregation and resistance profiles.

The antibiotic susceptibility of LABs should be determined to prevent the incorporation of multi-resistance strains into the Food Chain or feed additives for animals, as stated before [40]. The standardization of the method for the assessment of antibiotic susceptibility and its interpretation was determined by different research groups [16,19,21,25,28,41,42], in order to generate a data base to establish and compare susceptibility profiles in different hosts and areas. Moreover, the selection of the technique is essential to determine the resistance criteria, as suggested by Mayrhofer et al. [21], who compared the procedures of CLSI and ISO/ IDF Standard guides. The disk diffusion method was applied for LAB against antibiotics of different groups. The LSM media was compared with two frequent media use for lactobacilli, which are nutritionally demanding bacteria. The diffusion of the antibiotic can be modified by the composition or pH of the media and is of major importance to obtain reproducible results [24]. In the case of LABs, different media were used [42-44]. In this work, the LSM media proposed by Klare et al. [25], and studied by others authors was included [16,42], supporting the growth of most of the strains, with the exception of *L. mucosae* strains. Higher inhibitions zones were observed in LSM, which could indicate a better diffusion compared with LAPTg media.

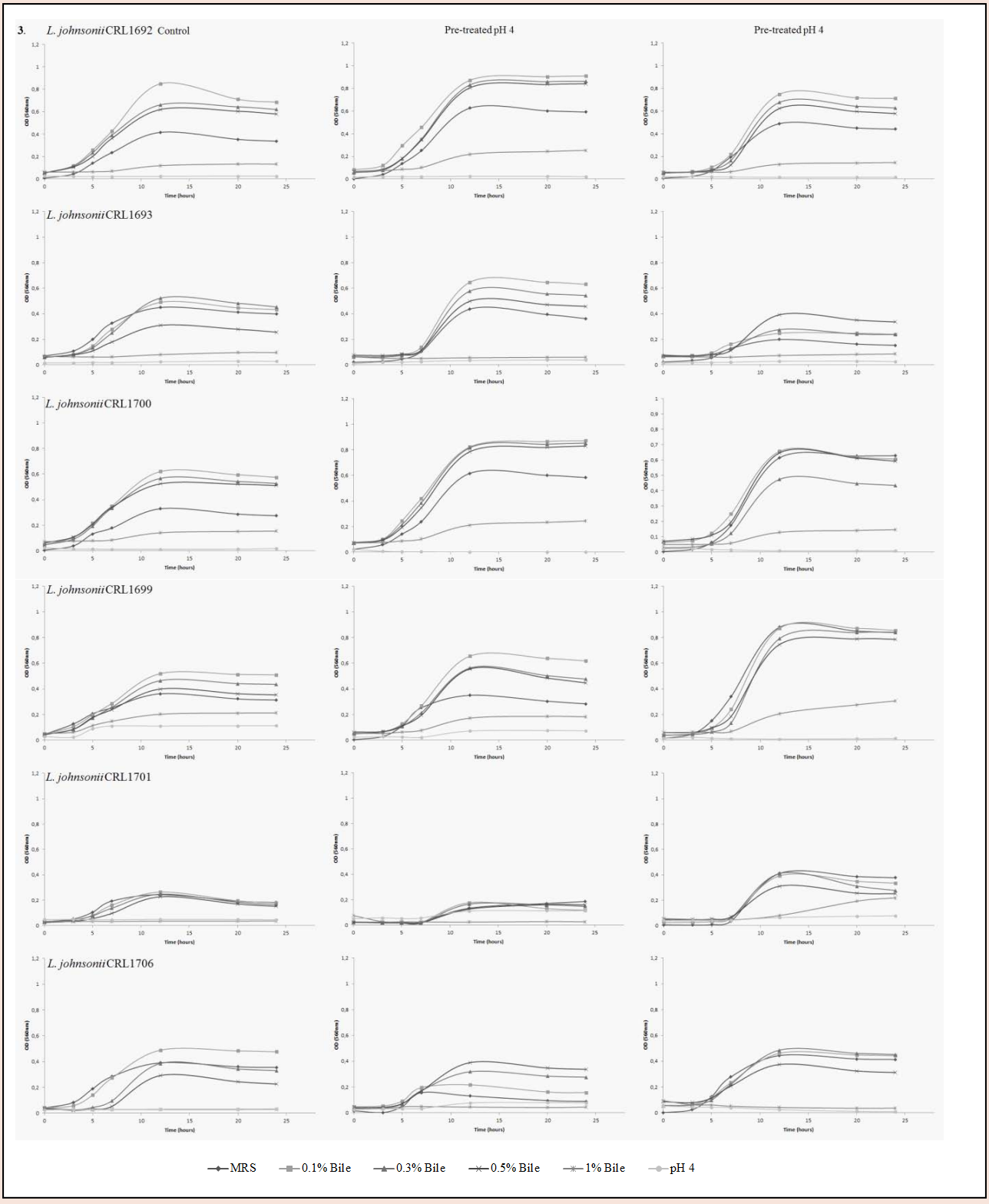

Figure 3: Growth curves of *Lactobacillus johnsonii* strains and resistance to acid pretreatment and bile salts. The conditions are described in Materials and Methods.

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Table 1: Acid and bile resistance of Lactic Acid Bacteria strains isolated from newborn calves.

Strains	Pre-treatment	Bacterial Growth					
		MRS	MRS 0.1% bile	MRS 0.3% bile	MRS 0.5% bile	MRS 1% bile	MRS-pH 4
***L. johnsonii* CRL1692**	Control	0.337±0.03	0.685±0.06	0.620±0.06	0.580±0.18	0.132±0.06	0.026±0.01
	pH4	0.563±0.09	0.909±0.09	0.862±0.05	0.841±0.09	0.253±0.05	0.020±0.01
	pH 2	0.419±0.06	0.742±0.06	0.627±0.06	0.578±0.18	0.145±0.09	0.015±0.02
***L. johnsonii* CRL1693**	Control	0.456±0.14	0.430±0.05	0.458±0.18	0.256±0.19	0.025±0.09	0.043±0.01
	pH4	0.316±0.11	0.587±0.28	0.507±0.25	0.439±0.23	0.036±0.06	0.050±0.01
	pH 2	0.117±0.11	0.293±0.06	0.237±0.28	0.339±0.25	0.026±0.23	0.053±0.06
***L. salivarius* CRL1694**	Control	1.132±0.12	1.069±0.16	0.930±0.12	0.856±0.11	0.004±0.04	0.782±0.05
	pH4	1.172±0.09	1.105±0.09	0.934±0.02	0.985±0.02	0.121±0.07	1.116±0.10
	pH 2	1.078±0.06	1.120±0.06	1.186±0.05	1.124±0.03	0.067±0.03	1.091±0.06
***L. murinus* CRL1695**	Control	0.631±0.15	0.352±0.07	0.323±0.11	0.400±0.18	0.065±0.04	0.015±0.01
	pH4	0.649±0.06	0.450±0.09	0.308±0.03	0.222±0.11	0.080±0.02	0.070±0.02
	pH 2	0.543±0.06	0.614±0.03	0.481±0.11	0.490±0.02	0.056±0.02	0.043±0.02
***L. mucosae* CRL1696**	Control	0.207±0.08	0.211±0.12	0.221±0.18	0.268±0.12	0.294±0.16	0.156±0.16
	pH4	0.226±0.07	0.290±0.12	0.287±0.18	0.223±0.17	0.230±0.09	0.139±0.11
	pH 2	0.079±0.09	0.132±0.12	0.126±0.03	0.175±0.04	0.145±0.03	0.075±0.03
***L. amylovorus* CRL1697**	Control	0918±0.15	0.689±0.12	0.609±0.17	0.741±0.07	0.310±0.03	0.142±0.01
	pH4	0.667 ±0.01	0.648±0.06	0.697±0.12	0.671±0.00	0.215±0.03	0.100±0.01
	pH 2	0.757±0.07	0.630±0.04	0.486±0.05	0.645±0.10	0.199±0.01	0.031±0.08
***L. mucosae* CRL1698**	Control	0.264±0.09	0.234±0.11	0.267±0.11	0.236±0.11	0.317±0.14	0.269±0.09
	pH4	0.168±0.01	0.207±0.05	0.232±0.01	0.287±0.02	0.333±0.03	0.150±0.01
	pH 2	0.157±0.04	0.227±0.02	0.246±0.04	0.307±0.02	0.406±0.04	0.201±0.01
***L. johnsonii* CRL1699**	Control	0.314±0.07	0.509±0.05	0.437±0.06	0.353±0.02	0.252±0.08	0.113±0.06
	pH4	0.283±0.10	0.619±0.19	0.476±0.23	0.446±0.25	0.182±0.21	0.074±0.01
	pH 2	0.839±0.03	0.855±0.05	0.841±0.19	0.786±0.19	0.307±0.08	0.014±0.02
***L. johnsonii* CRL1700**	Control	0.274±0.03	0.568±0.02	0.526±0.01	0.555±0.15	0.154±0.04	0.035±0.03
	pH4	0.534±0.03	0.870±0.02	0.853±0.01	0.830±0.03	0.244±0.15	0.002±0.04
	pH 2	0.611±0.03	0.607±0.02	0.434±0.01	0.593±0.15	0.146±0.04	0.008±0.02
***L. johnsonii* CRL1701**	Control	0.163±0.04	0.178±0.14	0.165±0.02	0.151±0.03	0.035±0.03	0.047±0.01
	pH4	0.191±0.26	0.107±0.04	0.134±0.01	0.158±0.00	0.026±0.00	0.112±0.00
	pH 2	0.376±0.01	0.343±0.09	0.271±0.01	0.250±0.03	0.205±0.30	0.006±0.01
***L. salivarius* CRL1702**	Control	1.115±0.14	1.160±0.09	1.136±0.05	1.049±0.15	0.007±0.037	0.734±0.12
	pH4	1.070±0.09	1.075±0.09	0.979±0.03	0.915±0.07	0.024±0.102	0.813±0.09
	pH 2	0.897±0.12	1.089±0.12	0.948±0.02	0.999±0.04	0.111±0.082	1.056±0.12
***E. faecium* CRL 1703**	Control	0.342±0.03	0.306±0.01	0.394±0.07	0.461±0.02	0.416±0.06	0.039±0.07
	pH4	0.378±0.00	0.408±0.15	0.363±0.09	0.448±0.05	0.406±0.13	0.090±0.01
	pH 2	0.344±0.13	0.409±0.15	0.465±0.09	0.524±0.05	0.606±0.02	0.053±0.05
***L. murinus* CRL 1705**	Control	0.763±0.05	0.730±0.22	0.610±0.36	0.647±0.28	0.564±0.34	0.241±0.05
	pH4	0.363±0.10	0.374±0.03	0.346±0.04	0.373±0.03	0.156±0.11	0.300±0.01
	pH 2	0.192±0.03	0.161±0.04	0.174±0.03	0.208±0.11	0.213±0.04	0.254±0.01
***L. johnsonii* CRL1706**	Control	0.389±0.12	0.476±0.10	0.330±0.23	0.226±0.14	0.020±0.030	0.054±0.01
	pH4	0.086±0.05	0.124±0.05	0.252±0.01	0.317±0.03	0.042±0.022	0.122±0.02
	pH 2	0.439±0.32	0.442±0.07	0.438±0.04	0.312±0.03	0.036±0.011	0.009±0.013

Bacterial Growth was determined by optical density

Similar results were obtained by Ocaña et al. [43], using MRS and LATPg.

The selection of antibiotics for MIC assays was performed according to EFSA 2012 and Klare et al. [16,25]. Microorganism concentration, incubation time and atmospheric conditions must be defined. Egervärn et al. [42], observed that high inoculum concentrations and longer incubation periods in the microdilution test increase MIC values. Our results showed that some resistances profiles were affected by the inoculum of the bacteria and supported the importance of standardization of the inoculum to compare results. MIC values were compared with others authors, Kanamicyn resistance were also observed in lactobacillus isolated from pigs, human GUT and food [45-47]. The intrinsic resistance of heterofermentative lactobacilli to Vanomicyn, which was phenotypically determined in the strains, does not present the risk of gene transfer [14,18,40].

Conclusions

According to our results, the behavior of LAB in acid conditions and bile salts cannot be predicted based on bacterial species and should be evaluated in each of the strains. Although some of the strains showed similar resistant patterns, since all the strains were be affected by the acid pretreatment, each strain was affected in a

Table 2: Acid and bile tolerance of Beneficial Lactic Acid Bacteria strains isolated from newborn calves. Maximal growth rate of bacteria in gut related conditions.

Strains	Pre-treatment	Maximal growth rate (μ max) in acid and bile salts conditions					
		MRS	MRS 0.1% bile	MRS 0.3% bile	MRS 0.5% bile	MRS 1% bile	MRS-pH4
***L. johnsonii* CRL 1692**	Control	0.486±0.06	0.312±0.03	0.304±0.01	0.269±0.03	0.059±0.01	-
	pH4	0.532±0.13	0.251±0.01	0.239±0.03	0.209±0.03	0.066±0.01	-
	pH 2	0.506±0.08	0.301±0.01	0.207±0.05	0.188±0.02	0.067±0.00	-
***L. johnsonii* CRL 1693**	Control	0.264±0.05	0.298±0.02	0.272±0.01	0.209±0.04	-	-
	pH4	0.304±0.07	0.202±0.01	0.154±0.01	0.110±0.01	-	-
	pH 2	0.303±0.05	0.207±0.01	0.161±0.03	0.113±0.01	-	-
***L. salivarius* CRL 1694**	Control	0.614±0.01	0.495±0.04	0.437±0.02	0.372±0.03	-	0.243±0.05
	pH4	0.688±0.08	0.427±0.03	0.393±0.03	0.362±0.01	-	0.497±0.05
	pH 2	0.672±0.03	0.505±0.11	0.486±0.09	0.452±0.13	-	0.586±0.23
***L. murinus* CRL 1695**	Control	0.313±0.01	0.282±0.03	0.223±0.07	0.288±0.02	-	-
	pH4	0.357±0.03	0.135±0.01	0.185±0.01	0.153±0.01	-	-
	pH 2	0.254±0.05	0.136±0.01	0.201±0.02	0.191±0.04	-	-
***L. mucosae* CRL 1696**	Control	0.539±0.15	0.310±0.05	0.304±0.05	0.298±0.04	0.343±0.10	0.342±0.10
	pH4	0.237±0.01	0.122±0.08	0.042±0.01	0.042±0.01	0.070±0.08	0.067±0.01
	pH 2	0.301±0.14	0.144±0.01	0.106±0.01	0.141±0.03	0.043±0.02	0.041±0.02
***L.amylovorus* CRL 1697**	Control	0.353 ±0.02	0.298±0.09	0.303±0.04	0.310±0.05	0.067±0.06	-
	pH4	0.166±0.08	0.188±0.03	0.292±0.09	0.265±0.07	0.120±0.02	-
	pH 2	0.357±0.04	0.219±0.01	0.171±0.01	0.218±0.02		-
***L. mucosae* CRL 1698**	Control	0.353±0.03	0.353±0.06	0.285±0.01	0.329±0.05	0.325±0.06	0.412±0.01
	pH4	0.579±0.01	0.261±0.01	0.22±0.01	0.165±0.08	0.083±0.01	0.412±0.09
	pH 2	0.462±0.10	0.164±0.01	0.235±0.03	0.227±0.05	0.48±0.08	0.483±0.00
***L. johnsonii* CRL 1699**	Control	0.359±0.02	0.351±0.14	0.285±0.04	0.290±0.02	0.129±0.08	-
	pH4	0.479±0.10	0.348±0.02	0.299±0.01	0.275±0.02	0.106±0.05	-
	pH 2	0.488±0.02	0.342±0.07	0.272±0.04	0.273±0.03	0.161±0.09	-
***L. johnsonii* CRL 1700**	Control	0.487±0.07	0.333±0.01	0.281±0.04	0.284±0.03	-	-
	pH4	0.363±0.07	0.326±0.05	0.283±0.05	0.299±0.05	-	-
	pH 2	0.537±0.04	0.241±0.09	0.328±0.06	0.209±0.01	-	-
***L. johnsonii* CRL 1701**	Control	0.291±0.03	0.343±0.01	0.281±0.01	0.267±0.03	-	-
	pH4	-	0.200±0.02	0.203±0.02	0.170±0.02	-	-
	pH 2	0.309±0.03	0.205±0.002	0.187±0.01	0.150±0.01	-	-
***L. salivarius* CRL 1702**	Control	0.592±0.01	0.553±0.08	0.487±0.10	0.426±0.12	-	0.514±0.08
	pH4	0.698±0.08	0.429±0.02	0.353±0.03	0.382±0.010	-	0.419±0.13
	pH 2	0.752±0.01	0.550±0.02	0.640±0.14	0.432±0.14	-	0.187±0.23
***E. faecium* CRL 1703**	Control	0.466±0.04	0.505±0.01	0.537±0.01	0.667±0.01	0.259±0.01	-
	pH4	0.322±0.04	0.363±0.02	0.362±0.01	0.305±0.01	0.161±0.01	-
	pH 2	0.880±0.20	0.386±0.01	0.326±0.01	0.315±0.01	0.154±0.06	-
***L. murinus* CRL 1705**	Control	0.377±0.04	0.524±0.02	0.437±0.02	0.428±0.08	0.349±0.05	0.403±0.06
	pH4	0.327±0.07	0.306±0.03	0.282±0.01	0.255±0.01	0.208±0.02	0.131±0.07
	pH 2	0.784±0.33	0.342±0.07	0.365±0.08	0.370±0.02	0.225±0.01	0.713±0.31
***L. johnsonii* CRL 1706**	Control	0.280±0.02	0.229±0.01	0.320±0.03	0.211±0.04	-	-
	pH4	-	0.332±0.07	0.383±0.01	0.366±0.02	-	-
	pH 2	0.615±0.04	0.317±0.02	0.323±0.06	0.251±0.05	-	-

The growth rate was determined as described in materials and methods.

different way, and in some cases the strain became more resistant. On the other hand, all the strains proved to be resistant to 0.5% bile salts after acid pretreatment.

The antibiotic resistance profile of the strains indicated that the culture media affected the inhibition zone in the disk diffusion technique for most of the antibiotics assayed. Also, almost all the strains grew in LSM media, proposed by the ISO/IDF Standard guidelines. The phenotypic studies of microbial resistance of the strains according to the EFSA breakpoints indicated they are sensitive to the antibiotics evaluated, except for Kanamicyn. Some of these strains are being included in the design of a probiotic product for calves to prevent diarrhea (Table 5).

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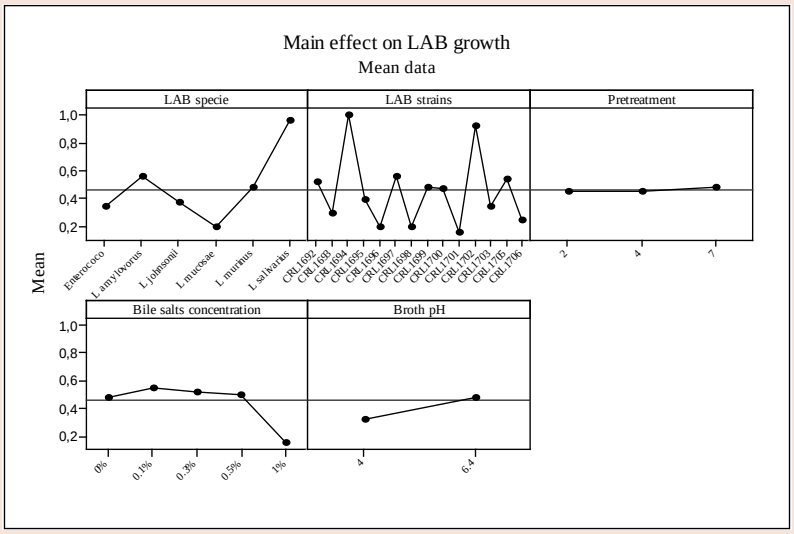

Figure 4: Plot of the Main Effects on the Growth Lactic Acid Bacteria strains.

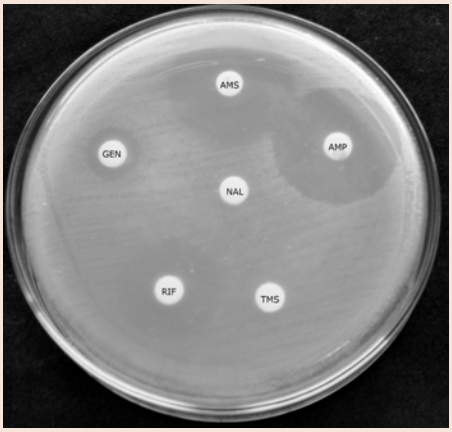

Figure 5: Inhibition zones in the disk diffusion technique of *Lactobacillus salivarius* CRL1702 in LSM media for: Ampicillin (AMP); Ampicillin Sulbactam (AMS); Sulfamethoxazole+Trimethoprim (TMS); Rifampicin (RIF); Gentamicin (GEN); Nalidixic Acid (NAL).

Table 3: Antibiotic sensitivity of Beneficial Lactic Acid Bacteria from new born calves by Disk Diffusion Assay.

		Strain				
Antibiotics	**Media**	***L. salivarius* CRL1694**	***L. amylovorus* CRL1697**	***L. johnsonii* CRL1693**	***L. murinus* CRL1695**	***L. mucosae* CRL1696**
		Diameters of Inhibition zone (mm)				
TMS	LSM	<6	<6	<6	<6	-
	MRS	<6	<6	<6	-	<6
	LAPTg	<6	<6	<6	<6	<6
CLIN	LSM	28	>39	38	28	-
	MRS	27	>39	36	-	>39
	LAPTg	27	>39	33	29	>39
ERY	LSM	30	>39	37	30	-
	MRS	29	>39	37	-	>39
	LAPTg	29	>39	34	28	>39
GEN	LSM	15	20	24	10	-
	MRS	<6	-	11	-	8
	LAPTg	8	10	8	6	10

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AM	LSM	37	>39	33	18	-
	MRS	34	36	>39	-	>39
	LAPTg	32	37	>39	20	>39
VAN	LSM	<6	27	25	<6	-
	MRS	<6	27	22	-	<6
	LAPTg	<6	26	23	<6	<6
NAL	LSM	<6	<6	<6	<6	-
	MRS	<6	-	<6	-	<6
	LAPTg	<6	<6	<6	<6	<6
CEF	LSM	>39	32	>39	28	-
	MRS	33	38	>39	-	>39
	LAPTg	31	37	>39	20	30
CIP	LSM	19	7	-	22	-
	MRS	19	<6	<6	-	<6
	LAPTg	17	12	11	18	<6
AMS	LSM	35	>39	>39	18	-
	MRS	36	>39	>39	-	>39
	LAPTg	32	38	>39	18	>39
T	LSM	8	>39	-	<6	-
	MRS	<6	30	23	-	<6
	LAPTg	<6	30	25	<6	<6
RFA	LSM	26	24	-	20	-
	MRS	25	24	>39	-	>39
	LAPTg	21	22	29	15	>39
MIN	LSM	>39	>39	>39	>39	-
	MRS	>39	>39	>39	-	>39
	LAPTg	35	>39	>39	>39	>39

TMS: Sulfamethoxazole Trimethoprim; CLIN: Clindamycin ; ERY: Erythromycin; GEN: Gentamicin; AM: Ampicillin; VAN: Vancomycin; NAL: Nalidixic Acid; CEF: Cephalexin ; CIP: Ciprofloxacin; AMS: Ampicillin Sulbactam ; T: Teicoplanin; RFA Rifampicin; MIN: Minomicyn

Table 4: Antibiotic susceptibility of lactic acid bacteria isolated from newborn calves calves.

Strains	Bacterial Inoculum	Antibiotics															
		Antibiotics proposed by EFSA with microbial breakpoints												Others Antibiotics			
		VAN		ERY		AMP			CLOR		KAN		TET		OXITET	RIF	CIP	LIN
	CFU/mL	MIC	I	MIC	I	MIC	I		MIC	I	MIC	I	MIC	I	MIC			
***L. salivarius* CRL1702**	105	>128		Nr	<0.25	S	<0.25	S	1	S	64	R	4	S	4	<0.25	2	<0.12
	106	>128			<0.25		<0.25		1		128		4		4	<0.25	2	<0.12
	107	>128			<0.25		<0.25		1		128		4		16	<0.25	2	<0.12
***L. mucosae* CRL1696**	105	>128		Nr	<0.25	S	<0.25	S	0.25	S	2	S	4	S	16	1	16	<0.12
	106	>128			<0.25		<0.25		0.25		4		4		16	1	32	<0.12
	107	>128			<0.25		<0.25		0.25		4		4		16	1	32	<0.12
***L. murinus* CRL1695**	105	>128		Nr	<0.25	S	2	S	2		128	R	2	S	4	2	1	<0.12
	106	>128			<0.25		2		2		254		4		4	2	1	<0.12
	107	>128			<0.25		2		2		254		4		8	4	1	<0.12
***L. johnsonii* CRL1693**	105	0.5		S	<0.25	S	<0.25	S	1	S	16	R	2	S	1	<0.25	8	<0.12
	106	0.5			<0.25		<0.25		1		32		4		4	<0.25	.	<0.12
	107	0.5			<0.25		<0.25		2		32		4		4	<0.25	.	<0.12
***L. amylovorus* CRL1697**	105	<0.25		S	<0.25	S	<0.25	S	<0.12	S	4	R	1	S	2	2	64	<0.12
	106	<0.25			<0.25		<0.25		<0.12		16		2		4	2	64	<0.12
	107	<0.25			<0.25		0.5		<0.12		32		2		4	4	64	<0.12

I: antibiotic susceptibility interpretation. Nr: not required. S (Susceptible): the strain is inhibited at a concentration of a antimicrobial equal or lower than the established cut-off value (S ≤ x mg/L). R (Resistant): the strain is not inhibited at a concentration of a antimicrobial higher than the established cut off value (R > x mg/L) according to EFSA [15].
VAN: Vancomycin; ERY: Erythromycin; AMP:Ampicillin; CLOR: Chloramphenicol; KAN: Kanamycin; TET: Tetracycline; OXITET: Oxytetracycline; RIF: Rifampin; CIP: Ciprofloxacin; LIN: Lincosamide.

Citation: *Maldonado NC, Nader-Macías MEF (2015) Functional Properties (Acid and Bile Tolerance) and Antibiotic Susceptibility of Lactic Acid Bacteria Isolated from Newborn Calves for the Design of a Probiotic Product. Int J Vet Sci Res 1(1): 011-022.*

Table 5: Beneficial, functional and safety properties of LAB selected for the design of a probiotic product for calves.

	Superficial properties		Production of Antagonic Substances			Functional Properties	Safety properties
Strains	**Auto aggregat**	**Hydro-phobicity**	**Inhibition of pathogens**	**Peroxidase production**	**Production of lactic acid**		
***L. salivarius* CRL1702**	Negative	High	*Salmonella typhimurium* MP/08	Positive	Low	Bile tolerant- Acid resistant	S: AMP; ERY; TET; CHLO
***L. mucosae* CRL1696**	Negative	Low	-	Positive	Low	Bile tolerant-Acid resistant	S: AMP; ERY; TET; KAN; CHLO
***L. murinus* CRL1695**	Positive	Low	*S. typhimurium* MP/08	Positive	Low	Bile tolerant	S: AMP; ERY; TET; VAN; CHLO
***L. johnsonii* CRL1693**	Positive	High	*Escherichia coli* 3511AD, *S. dublin* MP/07, *Staphylococcus aureus* MP/08	Positive	High	Bile tolerant	S: AMP; ERY; TET; CHLO
***L. amylovorus* CRL1697**	Low	Low	*E. coli* 3511AD, *S. typhimurium* MP/08, *S. dublin* MP/07, *S.infantis* 1533/00, Strept. *dysgalactiae* 05/84	Positive	Low	Bile tolerant Acid resistant	S: AMP; ERY; TET; VAN; CHLO

Superficial properties and production of antagonistic substances were previously determined in Maldonado et al. [6].
S: Sensitive. AMP (Ampicillin); ERY (Erythromycin); TET (Tetracycline); KAN (Kanamycin); CHLO (Choranfenicol); VAN (Vancomycin).

strains were included in a patent form presented by CONICET at INPI.

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