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Article

Production and evaluation of probiotic milk inocula obtained from the digestive tract of piglets (*Sus scrofa domesticus*) proposed for pig feed

Carmen Rojas Mogollón [a]

Gloria Ochoa Mogollón [b]

Rubén Alfaro Aguilera [c]

Javier Querevalú Ortiz [d]

Héctor Sánchez Suárez [e*]

[a] Universidad Nacional de Tumbes. Facultad de Agronomía, Escuela de Agroindustria. Perú.

[b] Universidad Nacional de Tumbes. Facultad de Ciencias de la Salud. Laboratorio de Biología Molecular. Perú.

[c] Facultad de Ciencias de la Salud. Laboratorio de Biotecnología. Biodes- Jefatura de práctica. Perú.

[d] Universidad Nacional de Tumbes. Facultad de Agronomía. Escuela de Agroindustria. Laboratorio de procesamiento de alimentos. Perú.

[e] Universidad Nacional de Tumbes. Facultad Ciencias Agrarias. Escuela de Medicina Veterinaria y Zootecnia. Perú.

[*] Corresponding author: hsanchezs@untumbes.edu.pe

Abstract:

In order to evaluate a probiotic milk inoculum (MI), from lactic acid bacteria (LAB) native to the piglet for use as feed (yogurt) in piglets, samples were taken from the final part of the digestive tract (excreta) of ten piglets raised in the backyard and sown in selective medium (MRS agar with aniline blue). To verify its purity, biochemically characterized and probiotic capacity, tests were performed (low pH tolerance, high bile salts, and NaCl, oxidase, catalase, gas production, and antagonism tests), molecular

identification by standard method CTAB-DTAB. For the elaboration of MI (yogurt), with selected, reactivated and homogenized probiotic strains (OD from 1 to 600 nm), each LAB was activated in pasteurized milk (1 ml/100 ml) obtaining mixture one and two with the strains 1, 5, 2 and 1, 5, 6 respectively. They were evaluated every 5 days for 15 days in refrigeration. The following bacteria were molecularly identified: 01 *Lactobacillus reuteri*, 04 *Enterococcus faecium* (LAB), and *Escherichia fergusonii*, *Shigella flexneri* (pathogenic). The LAB were selected by tolerance as probiotics: $2.3x10^4$ CFU/ml in pH 3.5, $7.00x10^3$ CFU/ml in 5% bile salt, and $2.80x10^4$ CFU/ml in 13% NaCl. In viability of the milk inoculum (yogurt) was obtained according to the Peruvian technical norm NTP 202.092:2014 and to the norm INEN 710: 1996, stored in refrigeration for 15 days; mixture one turned out to be better, and mixture two, acceptable, with counts of 10^6 CFU/ml and 10^7 CFU/ml of probiotic cells. Therefore, they are both suitable as probiotic milk inocula to be provided orally to piglets.

Key words*:* Piglets, LAB, Probiotics, Lactic Inoculum, Safe food, Antagonism.

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Introduction

Pigs are born without bacterial flora in their digestive tract; they become infected as maternal antibodies disappear, installing a pattern of microorganisms and production of digestive enzymes that adapt to each stage of digestion, thus avoiding microbiological imbalance. The intestinal bacterial flora native to the piglet is changing[1,2], colonizes, is replaced or lost according to age, type of feed and changes in the environment. When the microbiological balance is broken, the diarrhea syndrome related to weaning is generated[3-6].

Lactic acid bacteria (LAB), are part of the normal intestinal microbiota of many animals and act as probiotics. They share common morphological, physiological and metabolic characteristics; they are cocci or Gram-positive, non-sporulated, immobile, anaerobic, microaerophilic or aerotolerant bacilli and they are oxidase and catalase negative. Likewise, as the main product of carbohydrate fermentation, they generate lactic acid[2,7]; they grow at different temperatures and high salt concentration; they tolerate acid or alkaline pH, and they are the main microorganisms used as probiotics[3,8,9].

Probiotics are live microorganisms that, when supplied in the diet, benefit the development of microbial flora in the intestine, stimulate the protective functions of the

digestive system, and are biotherapeutic, bioprotective or bioprophylactic[7,8,10], capable of producing antimicrobial compounds. Their reproduction time is short; they have the ability to cross the gastric barrier (secretions from the stomach and duodenum), and they must be stable during the manufacturing and marketing process, so that they can reach the intestine alive. They also act by preventing the adhesion of pathogenic bacteria in the receptors of the intestinal epithelium[10], neutralizing toxic metabolites[7,9,11] and they adapt to a particular region of the intestine according to the age of the piglet[3,12,13].

In pig nutrition, probiotics help establish beneficial microbiota and inhibit the enteropathogens *Escherichia coli* and *Salmonella typhimurium*[3]. Also, *Lactobacillum plantarum* has probiotic potential in piglets[14], and *Rhodopseudomonas* spp, *Lactobacillus* spp and *Saccharomyces* spp inhibit the growth of *Salmonella tiphymurium*, *L. acidophilus* SS80 and *Streptococcus thermophilus*[15,16,17]. In addition, probiotics are also present in saliva, and it is recommended that they be used in the same host species from which they were isolated [9,10].

These probiotics can be supplied as MI (yogurt), which is a product without excessive acidification, where the LAB are viable to incubation and storage; there are time tolerant strains such as *L. acidophilus* and others that deteriorate rapidly in refrigeration when *Lactobacillus bulgaricus* is used[18,19,20]. Most of the microorganisms used to manufacture yogurt are commercial. Cow's and goat's milk are used to prepare yogurt, which has good syneresis, viability of LAB and probiotic characteristics[17,18,20]; its consumption improves food efficiency and avoids contracting gastrointestinal diseases[5,19,20], being of little use for animal nutrition.

In this context, it was proposed to produce a dairy inoculum with native LAB isolated from the pig's digestive tract, phenotypically and genotypically characterized, innocuous and with probiotic properties for the feeding of piglets.

Material and methods

Population and sample

Ten lactating backyard piglets aged 35 days fed with antibiotic-free diets, coming from the El Limón farm, located in the Pampas district of Hospital, department of Tumbes (3°43'35" S, 80°26'38" W), were used. The sample included lactic acid bacteria isolated from the final part of the digestive tract (excrements).

Sample collection

From each piglet, excrements were collected with sterile swabs (scraping) and placed individually in sterile and airtight plastic tubes, to be transported in a cold chain[19] to the Molecular Biology Laboratory located at the Faculty of Health Sciences of the National University of Tumbes.

Microbiological analysis

Each sample was immersed in a sterile physiological saline solution at 0.85% (diluent); the dilutions were homogenized for 5 to 10 seconds, and 1 ml was transferred for the following tubes with 9 ml of MRS Broth, 10^{-1} to 10^{-5} dilutions for bacterial colony counting. The seeding was carried out by the surface method, taking 25 µl of the decimal dilutions in plates with selective agar for LAB (MRS agar + Aniline Blue 0. 13%); selecting the colonies stained with blue (from the surface of the agar), purified in MRS agar by the striae method, and verifying the population of LAB. The macroscopic characterization was carried out according to size, shape, color, density, consistency, and Gram staining for identification[21,22]. The LAB were preserved in tubes with MRS agar tilted at an angle of 20º, sown by the striae method, kept at 4 ºC, and cryopreserved in TSB medium with 30% glycerol at -20 ºC, after refrigeration[21,22].

For the pathogenic samples, the sowing was done by the exhaustion method, in specific media such as SS agar (salmonella, shiguella) and EMB agar (methylene blue eosin)[22]. The strains of the samples, obtained from the fluid excretions of piglets, were isolated, purified, identified and preserved.

Biochemical analysis and tolerance tests

The isolated and purified LAB strains were tested for selection as probiotics: oxidase test, using paper strips impregnated with the reagent para-amino-N-dimethylaniline, which in the presence of the cytochrome enzyme C-oxidase changes its color, considered as positive or negative. Catalase test: the capacity was observed to split H_2O_2 at 30%, in water and oxygen; it was verified with the intense bubbling that can be determined as positive or negative (attributed to the catalase enzyme)[16,23]. Gas (CO_2) was generated by the metabolic fermentative process. For the tolerance tests, the selected LAB strains were used, and cultivated in MRS Broth at 37 °C for 24 h; their growth was measured by optical density (OD=1) at 600 nm in a UV spectrophotometer, and one ml of LAB was used for each test. Viability was evaluated by counting bacteria on MRS agar before and after

incubation[15,16,23]. Tolerance to low pH: in 15 ml falcon tubes: 10 ml of MRS broth adjusted to pH 2.5, 3.5, 4.5 with HCl were added. Tolerance to bile salts: in 15 ml capacity falcon tubes, 10 ml of MRS broth enriched with 1 g (1% w/v), 5 g (5% w/v) and 10 g (10% w/v) of Ox-Bilis[15,16,23] were added. Tolerance to NaCl concentrations, in 15 ml falcon tubes, 10 ml of MRS Broth enriched with 5 g (5% w/v), 9 g (9% w/v) and 13 g (13% w/v) of NaCl were added[15,16,23].

Inhibitory activity against pathogenic microorganisms of the piglets

It consists in the confrontation of each of the selected LAB strains against each pathogenic strain of the piglets (*E. fergusonii* and *S. flexneri*). Cells and supernatants were used according to the proposed method; the observation of the halo was considered as a positive inhibitory activity[2,15,16]. LAB and pathogenic bacteria (homogenized DO=1, at 600 nm) were preserved in tubes with PCA agar slants, activated at 37 °C during 24 h for their use.

Direct or contact method. The LAB strain was sown in Petri dishes, on MRS agar, using the swab technique; at the same time, 25 µl of the pathogenic strain were sown in Mueller Hinton agar, by surface technique. Circular bits with a diameter of 6 mm were extracted from the plate with LAB and placed on the plate with the pathogen[15,16].

Non-neutralizing dish method. The LAB strain was sown in MRS broth at 37 °C during 24 h, the pH was determined, and 1 ml of the broth was added in 1.5 microtubes for centrifugation at 16,800 xg during 10 min, in order to obtain the supernatant to perform the antagonism tests. Sowing in parallel 25 µl of the pathogenic strain in Mueller Hinton agar by the surface method, on which cylindrical perforations of a 6 mm diameter were made, where 35 to 40 µl of the LAB supernatant were added[15,16].

Neutralizing dish method. The procedure was the same as for the non-neutralizing dish method; the supernatant changed, having been neutralized by adjusting it to pH 7 with a 1N NaOH solution[15,16].

Molecular analysis

LAB bacteria from healthy piglets and pathogenic bacteria from piglets with diarrhea were molecularly identified, adapting the DNA extraction by Gustincich's standard CTAB-DTAB method for bacterial cells[15,24]. For PCR (Polymerase Chain Reaction), the amplification of the 16S rRNA gene, the universal primers 8F (5' AGA GTT TGA TCC TGG CTC AG 3') and 1510R (5' GGT TAC CTT GTT ACG ACT T 3') described by

Weisburg for bacterial phylogenetic studies were used; the electrophoresis was performed in 1% agarose gel. For the sequencing of the PCR products, 10 µl were used; 5 µl portions of each universal primer for the 16S rRNA gene were deposited in 0.2 ml microtubes, which were then packaged and sent for sequencing to Macrogen company in Korea[15]. The DNA sequences were aligned using the free software MEGA 7 and compared with the 16S rRNA sequences, which are in the GenBank public access database using the free software BLAST (Basic Local Alignment Search Tool)[15,24].

Preparation of the milk inoculum (MI)

The elaboration and evaluation of the MI were carried out according to the norms of the INEN[25]. The stages were: reception of fresh milk; organoleptic inspection; sieving; mechanical homogenization[21,26,27]; pasteurization, carried out at 75 °C during 10 min; cooling, and incubation, at a temperature ranging between 40 and 45 °C[26,27].

Milk inoculum mixture. Selected LABs with probiotic capacity were activated[28-29] on MRS agar and incubated at 37 ºC for 48 to 72 h, depending on the strain; an aliquot was sown in 10 ml MRS broth and incubated at 37 ºC for 48 h, to be used it was homogenized at OD= 1; at 600 nm[26,27,28]. 100 ml of pasteurized milk and 1 ml of MRS broth with a selected LAB strain were placed in 250 ml sterile flasks and incubated at 32 ºC during 12 h. For the preparation of the final mixture of MI (yogurt), 50 ml of each previous inoculum (per strain) were extracted and mixed (strains 1, 2 and 5 and strains 1, 5 and 6) in half a liter of pasteurized milk, incubated at 32 ºC during 12 h and kept at 4 ºC[27, 28,].

Chemical analysis of the milk inoculum. The pH, titratable acidity, syneresis and colony count were evaluated at 0, 5, 10 and 15 days in refrigeration at 4 °C. The pH value of the MI was measured according to method 981.12 (AOAC, 1990), using the digital potentiometer, calibrated. 40 to 45 ml of the MI were placed in a container; the pH electrode was introduced, and the reading was recorded. In order to determine the titratable acidity, 5 g of sample were taken, and three drops of phenolphthalein were homogenized and titrated with NaOH 0.1N, until a persistent pale pink color was obtained (lactic acid formula factor 0.09)[29,30,31]. For the evaluation of syneresis, 10 g of sample were used, placed in falcon tubes, and centrifuged for 20 min at 4,200 xg; after centrifugation, the weight of the supernatant was obtained, and the percentage of syneresis (w/w) was calculated based on the relationship between the weight of the supernatant and the weight of the sample multiplied by 100[31,32,33].

Microbiological analysis of the milk inoculum. This analysis was carried out taking into account the bacterial identity for yogurt, utilized by NTP 202.092:2014. The ISO 7889:2003 method (IDF 117:2003) was used according to the enumeration of characteristic microorganisms with the technique of counting colonies at 37 °C[27,28,30,34].

Results and discussion

Evaluation of microbiological analysis

Ten strains with LAB characteristics were found after discarding in MRS agar + aniline blue and purification on MRS agar and confirmed by method validation[16,23,29]. LAB are stained an intense blue in the selective medium by the presence of colony metabolites reacting with aniline blue[23,24,29]; the literature also confirms that LAB are Gram positive and can include different forms of bacilli, coconut and cocobacilli[16,19,29], as shown in Table 1, where the growth of LAB is also exhibited, being greater the 05 strain[2]. 80 x 10^4 CFU/ml), followed by the 01 strain (2.60 x 10^4 CFU/ml). The 04, 09, 07 and 02 strains had similar values; however, the 03, 06, 08 and 10 strains presented less growth (1.00 x 10^4 to 1.20 x 10^4 CFU/ml), understanding that the reproduction capacity of the LAB strains is variable according to the temperature and environment conditions[29,35,36].

Table 1: Initial evaluation of isolated strains for determining the characteristics of LAB

LAB	Size (mm)	Sh	Ele	Mar	Col	Den	Con	Group	Shape	Size CFU/ml
01 Strain	P 1.72	C	Convex	w	W	O	Viscous	Gram +	Bacilli	2.60x10^4
02 Strain	P 1.82	C	Convex	w	W	O	Viscous	Gram +	Coccobacillus	1.70x10^4
03 Strain	M 2.44	C	Flat	w	W	O	Viscous	Gram +	Bacilli	1.10x10^4
04 Strain	P 1.28	C	Flat	w	W	O	Viscous	Gram +	Bacilli	2.20x10^4
05 Strain	P 1.51	C	Convex	w	W	O	Viscous	Gram +	Cocci	2.80x10^4
06 Strain	M 3.54	C	Convex	w	W	O	Viscous	Gram +	Coccobacillus	1.10x10^4
07 Strain	M 3.54	C	Convex	w	W	O	Viscous	Gram +	Cocci	1.70x10^4
08 Strain	D 0.48	C	Convex	w	W	O	Viscous	Gram +	Bacilli	1.20x10^4
09 Strain	D 0.48	C	Flat	w	W	O	Viscous	Gram +	Bacilli	1.90x10^4
10 Strain	D 0.48	C	Flat	w	W	O	Viscous	Gram +	Bacilli	1.00x10^4

LAB= lactic acid bacteria; Sh= shape; Ele= elevation; Mar= margin; Col= color; Den= density; Con= consistency.
C= circular, w= whole, W= white, o= opaque.

Assessment of the biochemical analysis of LAB

Test for oxidase, hydrogen peroxide, gas generation and tolerance to pH, NaCl and bile salts. Table 2 shows that the 10 strains were negative oxidase (they do not produce the cytochrome enzyme C-oxidase in their breathing process). They are not aerobic; therefore, they do not need oxygen in their cell membrane. Furthermore, they exhibited negative catalase reaction (not reacting with H_2O_2) and did not produce CO_2[7,16,24] (except 04, 08 and 10). In the experiment, strains 03, 04, 08, 09 and 10 did not achieve tolerance to the concentrations of pH, NaCl, or bile salts, which are characteristic of probiotic cells[19,23,29]; therefore, they were definitely discarded.

Table 2: Biochemical and tolerance evaluation of LAB strains as a probiotic

Strain	Oxi	pH	GG	Tolerance to pH			Tolerance to NaCl, %			Tolerance to bile Salt, %		
				2.5	3.5	4.5	5	9	13	1	5	10
01	-	-	-	-	+	+	+	+	+	+	+	-
02	-	-	-	-	+	+	+	-	-	+	+	-
03	-	+	-	-	-	-	-	-	-	-	-	-
04	-	-	+	-	-	-	-	-	-	-	-	-
05	-	-	-	-	+	+	+	+	+	+	+	-
06	-	-	-	-	+	+	+	+	+	+	+	-
07	-	-	-	-	+	+	+	+	-	+	+	-
08	-	-	+	-	-	-	-	-	-	-	-	-
09	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	+	-	-	-	-	-	-	-	-	-

Oxi= oxidasa; pH= hydrogen peroxide; GG= gas generation;
Positive test: + Negative test: -

Table 3 shows the initial and final amount, in CFU/ml, of the strains submitted to different tolerance concentrations for selection purposes. The 1st strain was the one that presented the highest final growth, followed by strains 2, 5 and 6 in pH 4.5 and 3.5, which are sufficient for selection[3,16,35]; however, they were all susceptible to the highly acidic culture medium (pH 2.5). The same table shows the tolerance to NaCl, where stumps 5, 1 and 6 evidenced greater tolerance in all the concentrations, while stumps 2 and 7 were susceptible to the highest concentration (w/v). Also, tolerance to bile salts is observed in all the stumps at a maximum concentration of 5%, which is a reason for their selection[24,29,35]; of these, stump 5 exhibited the greatest growth –7.0 x 10^3 CFU/ml–, followed by the stumps 6, 1, 2 and 7.

Table 3: Evaluation of the final count of LAB colonies (CFU/ml), according to the tolerance of strains as probiotics

Strain		pH tolerance			NaCl tolerance, %			Bile salt tolerance, %		
		2.5	3.5	4.5	5	9	13	1	5	10
1	Baseline	6.80x10^3	7.90x10^3	1.30x10^4	2.85x10^3	3.75x10^3	3.25x10^3	2.10x10^4	9.40x10^3	6.50x10^3
	Final	-	2.30x10^4	3.80x10^4	1.05x10^4	1.19x10^4	5.25x10^3	6.40x10^3	4.90x10^3	-
2	Baseline	6.30x10^3	7.20x10^3	9.20x10^3	2.50x10^3	2.55x10^3	2.90x10^3	1.00x10^4	8.60x10^3	2.90x10^3
	Final	-	1.90x10^4	2.90x10^4	9.73x10^3	-	-	6.30x10^3	5.00x10^3	-
5	Baseline	6,40x10^3	7.50x10^3	1.30x10^4	5.03x10^4	1.50x10^4	7.56x10^3	1.70x10^4	1.40x10^4	1.70x10^4
	Final	-	1.80x10^4	2.30x10^4	1.13x10^4	2.80x10^4	2.80x10^4	1.10x10^4	7.00x10^3	-
6	Baseline	4.70x10^3	6.50x10^3	1.30x10^4	4.67x10^3	5.20x10^3	3.25x10^3	1.20x10^4	7.10x10^3	6.20x10^3
	Final	-	1,80x10^4	2.30x10^4	9.80x10^3	1,00x10^4	5.25x10^3	7.50x10^3	5.80x10^3	-
7	Baseline	4.50x10^3	5.50x10^3	1.00x10^4	4.50x10^3	4.80x10^3	3.00x10^3	7.10x10^3	4.50x10^3	5.00x10^3
	Final	-	9.50x10^3	2.00x10^4	7.50x10^3	5.50x10^3	-	5.30x10^3	3.80x10^3	-

Negative test: - ; LAB colony forming unit = CFU/ml.

The LABs found in the study exhibited probiotic characteristics evaluated according to tolerance to low pH concentrations, as stated by most authors, who consider 3 to 3.4 as survival pH values, and 3.5 as an optimal pH[14,35,36]. They also exhibited tolerance to high concentrations of bile salts and NaCl similar to those of other researches[7,35,36] –conditions considered to be mandatory as probiotics–; thus, the LAB strains (5, 1, 2 and 6) were found to exhibit viability for their selection as probiotics according to the methodology carried out by other researchers[16,37,38].

Evaluation of the molecular analysis. DNA sequencing

Table 4 presents the molecular identification of the LAB strains and pathogenic bacteria of the work with a high percentage of identity (99 %).

Table 4: Molecular identification by the 16S rRNA gene of strains extracted from the final part of the piglet's gastrointestinal tract

Strains	Sequence size (pb)	Identified species	Identity (%)	Accession Number
LAB 01	1371	*Lactobacillus reuteri*	99	NR075036.1
LAB 02	1328	*Enterococcus faecium*	99	NR113904.1
LAB 05	1344	*Enterococcus faecium*	99	NR113904.1
LAB 06	1366	*Enterococcus faecium*	99	NR113904.1
LAB 07	1383	*Enterococcus faecium*	99	NR113904.1
(A)	1352	*Escherichia fergusonii*	99	NR074902.1
(B)	1359	*Shigella flexneri*	99	NR026331.1

The *E. faecium* and *L. reuteri* LAB strains detected and molecularly identified are present as native microorganisms of the pigs' digestive tract and have an antagonistic effect against *Escherichia*, similar to that against *E. faecium* NCIMB 10415 and *E. faecium* NCIMB 11181[38,39,40], as well as against *L. reuteri* I5007 and *L. reuteri* KT260178, *Lactobacillus* sp, and *L. acidophilus*, used as probiotics in swine production[29,41,42].

Samples of pathogenic bacteria from piglets, (Table 4) are reported to be most prevalent in pig breeding[39,40].

Evaluation of the inhibitory activity of LABs against pathogenic bacteria

When comparing the three methods in order to determine the inhibitory activity (Table 4), it can be seen that the direct method and the neutralized dish method show less inhibition than the non-neutralizing dish method, given that the latter has an acidic pH due to the organic acids present in it, which have bactericidal activity[29,35,41].

E. faecium and *L. reuteri* had greater antagonistic activity against *E. fergunsonii*, which is more susceptible, than against *S. flexneri*, as reported by others[12,38,39]. Also, *Eschericha* is susceptible to most lactic acid bacteria, such as *Lactobacillus* spp strains extracted from lactating calves, *L. plantarum* isolated from creole pigs, and *L. lactis* isolated from piglets[40,41,42].

Direct method. The results of Table 5 show the inhibitory effect of LAB through direct contact. Strains 5, 6 and 7 showed inhibition against the two pathogenic strains with larger halos against *E. fergunsonii*, the most prominent of which is strain 5, with halo of 8.46 ± 3.02. Strains 1 and 2 showed less halos than the pathogenic ones[38,41].

Table 5: Halo size of LAB inhibition tests against the pathogens of *Shigella flexneri* and *Escherichia fergusonii*

Strains	*Escherichia fergusonii*			*Shigella flexneri*		
	Direct	**Un-neutralized**	**Neutralized**	**Direct**	**Un-neutralized**	**Neutralized**
01	6.52 ± 0.132	7.54 ± 0.43	6.85 ± 036	6.00	6.30 ± 0.56	6.00
02	6.94 ± 0.44	7.90 ± 0.078	7.10 ± 0.60	6.00	7.34 ± 0.05	6.00
05	8.46 ± 3.02	8.86 ± 0.62	8.76 ± 3.80	6.9 ± 0.45	10.34 ± 0.13	7.34 ± 0.89
06	8.33 ± 2.70	9.72 ± 1.88	8.52 ± 3.17	6.72 ± 0.25	9.84 ± 0.40	6.84 ± 0.35
07	8.25 ± 2.53	9.24 ± 0.60	8.65 ± 3.51	6.24 ± 0.02	10.10 ± 2.0	7.10 ± 0.60

Negative test: 6.00

Non-neutralizing dish method. The test was performed using the supernatant of the LAB culture, with an average pH of 4.486 ± 0.001. Table 5 shows that all the stumps exhibit inhibition halos in the confrontation against *E. fergusonii*; the most prominent stumps were Nos. 6 and 7, followed by stump 5 and, finally, by stumps 2 and 1. The halos formed in the presence of *S. flexneri* were of a larger size than those formed with the other pathogen, the stumps (in order of size from the largest to the smallest) were 5, 7, 6, 2, and 1 respectively.

LAB 5, 7 and 6 (*E. faecium*) exhibited larger halos in the presence of *S. flexneri*, and similar and smaller halos to those obtained in the test with *Lactobacillus* spp, compared to pathogens of the pig; halos ranging between 11.24 ± 0.03 and 32.62 ± 0.04 have been reported in the presence of *Salmonella* sp[19,36,41]. In tests using the bacterial supernatant without neutralization, it has exhibited a greater inhibition action, due to the effect of the organic acids, according to the antagonism tests[29,35,40].

Neutralizing dish method. In this case, the supernatant of the LAB culture was adjusted to pH 7.00 (neutralized with sodium hydroxide) in order to exclude the inhibition of organic acids. All the strains exhibited halos (Table 5) against *E. fergusonii*, but of a smaller size than in the test without neutralizing, the largest halos being for strains 5, 7 and 6 in the test with *S. flexneri*. In this method, since there is no acid action, the antimicrobial action is attributed to the presence of non-acid metabolites. Reportedly, LAB produce peptide substances that have a bactericidal or bacteriostatic mode of action[16,42], which is also referred to the activity of protein metabolites or complex lipid molecules or carbohydrates[11].

With all three methods, the assessed strains 5, 6 and 7 (*E. faecium*) exhibited the largest halos against *E. fergunsonii*, the non-neutralizing dish method being the one that generated the largest halos, as previously reported[11,16]. The antagonism of LAB is influenced by several factors, such as the type of bacterium, the place where it was obtained, the host species, the temperature, and the incubation time[14,15,41]. The LAB with

probiotic activity exhibited antagonism against pig pathogens, and its action is compared with the majority of probiotics obtained from bacteria *Lactobacillus* ssp. *L. acidophilus*, *L. plantarum*, (*L. casei* and *L. brevis*)[14,19,29], which act against the pathogenic bacteria *E. coli* ATCC 25922 and *S. typhimurium*[14].

Evaluation of the milk inoculum

Physical-chemical evaluation (pH, acidity and syneresis). Strains 1, 2, 5 and 6 were selected from the evaluated LAB as probiotics. Two mixtures were prepared with them as MI (yogurt); the first mixture utilized strains 1, 2, and 5, and the second one, strains 1, 5 and 6 (1 ml of activated strain in 100 ml of milk). 50 ml of each strain were used, according to the mixture, in order to evaluate its viability, at 0, 5, 10 and 15 days of storage until its use as MI (yogurt). Table 6 shows that mixture one exhibited better stability; in it, the pH values were inversely proportional to the acidity, which decreases according to the number of days of refrigeration. The pH at 15 days was pH of 4.53 for mixture one, and pH 4.78 for mixture two; these values are within the acceptable parameters of stability and useful life, related to the time of degradation of lactose to lactic acid. The results obtained are acceptable, comparable to those obtained with pH 4.65 in the manufacture of yogurt with goat's or cow's milk using commercial fermenting microorganisms and symbiotic yogurts[26,27]; besides, they comply with the Codex standard STAN 243-2003[43], which states that all yogurts must have a pH of ≤ 4. 6 to 4.90 –values similar to those of yogurts and non-traditional milk products[25,27,30]. The pH obtained was similar to that of milk ferments for pigs, ensilaged with milk products that maintain pH values of 3 to 4.9[18,35,44]. Despite the fact that mixture two presented slightly higher pH, this is also within the technical norms, NTP 202.092:2014 and Norm INEN 710 of 1996[20]; the pH is modified to cover a greater range when incorporating wheat fiber and other grains into Mexican artisanal yogurts[18,32,33]. Although the yogurt is refrigerated, the growth of LAB strains ceases. However, the acidity proceeds slowly, due to its residual activity[26,30,31]; its shelf life is increased by incorporating acid fruits and pectin shakes[27,34], and its quality and flavor are also improved by adding fruits (lucuma, banana, mango, and others) with functional components[34,45]; therefore, the pH in the incubation and storage of MI determines its acceptability for use[31].

Table 6: Evaluation of pH, % acidity, degree of syneresis and viable LAB count of MI (yogurt) at different days of storage at 4 °C

Mixture 01					Mixture 02			
Days	**pH**	**Acidity %**	**Syneresis %**	**CFU/g**	**Ph**	**Acidity %**	**Syneresis %**	**CFU /g**
0	4.65	0.80	0.36	$4.8x10^6$	4.94	0.47	0.39	$2.8x10^4$
5	4.61	0.86	0.49	$1.5x10^7$	4.88	0.50	0.45	$7.4x10^4$
10	4.57	0.90	0.55	$2.7x10^7$	4.82	0.55	0.53	$9.6x10^4$
15	4.53	0.93	0.61	$3.9x10^7$	4.78	0.58	0.58	$1.1x10^6$

The acidity value (%) is a function of the content of lactic acid, reaching up to 0.93 and 0.58 %, considered acceptable in a dairy product according to the standard for the preparation of yogurt as established by Codex STAN 243-2003[44], to the Standard INEN 710 of 1996. It has a final range of 0.6 to 1.5 % during refrigeration[20], and the percentage of acidity obtained is accepted in fermented foods and silage[28,35,44]. The degree of measured syneresis of MI (yogurt) increased slowly during storage, an effect caused by loss of stability, water retention, and its components. Table 6 shows the values of the evaluation of the degree of syneresis during storage; at 15 d of refrigeration, it exhibited an acceptable range of 0.36 to 0. 61 % (mixture one and two) –results similar to those obtained in yogurt and goat milk shake with fruits[26,27,34], but higher than those obtained for modified yogurts, because of the addition of commercial stabilizers, microcapsules of gum Arabic and maltodextrin (0.12 to 0.1 %)[27,30], and fiber that helps to prevent the separation of whey[32,33].

Microbiological evaluation. Table 6 shows the viability of LAB as probiotics in the MI (yogurt). Mixture one shows greater increase of probiotic microorganisms than mixture two, during its evolution in refrigeration. The LAB count in both mixtures is similar to that recommended by NTP 202.092:2014 for the preparation of yogurt with the ISO 7889:2003 (IDF 117:2003) method[33], which considers – at least for total number of lactic bacteria microorganisms in yogurt during its useful life– a concentration of 10^7 CFU/g. Mixtures one and two attained this concentration (3. 9 x 10^7 CFU/g and 1.1 x 10^6 CFU/g, respectively) at 15 d of refrigeration. Thereby, the processed product was guaranteed to contain and preserve its viability and probiotic activity, as in the manufacture of the different yogurts, in which goat or cow milk, commercial fermenting bacteria, flavorings, fruits, and fiber are utilized, with a concentration of 10^7 CFU/g to 10^6 CFU/g of viable probiotic cells in the first 16 d[30,34,46]. Currently the consumer demands less processed and more natural, functional foods (antioxidant fruits)[33,45]; therefore, it also seeks to improve their life span by providing antimicrobial qualities using beneficial native bacteria[17,18,42]. Reuterine, produced by *L. reuteri*, is an antibacterial that can be used as a biopreservative with potential probiotic controller of *Salmonella* spp. and *E. coli* in food for people or animals. The trend is the use of native LAB and its bacterial extracts as isolated probiotic potentials used in the same animal species[12,38,39].

Conclusions and implications

The biochemical characterization, the tolerance tests, and the antagonistic effect of the selected LAB were of great importance for the growth and survival of four probiotic strains (three *Enterococcus faecium* and one *Lactobacillus reuteri* strain). The production of organic acids presents in the non-neutralized supernatants stood out for their antimicrobial activity, and there is a non-acid action by metabolic modifiers in neutralized supernatants with bactericidal effect. It was possible to elaborate a lactic inoculum (yogurt) with acceptable, viable and innocuous characteristics with the isolated stumps for it to be considered as a potential probiotic for oral administration, with 15 d of useful life.

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