AN ALTERNATIVE METHOD FOR EVALUATION OF RESISTANCE TO LOW pH AND BILE SALTS IN PROBIOTIC CHARACTERIZATION OF *Lactobacillus reuteri* STRAINS. A TECHNICAL NOTE

Un Método Alternativo para la Evaluación de Resistencia a pH Ácido y Sales Biliares dentro de la Caracterización Probiótica de Cepas de *Lactobacillus reuteri*. Nota Técnica

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ABSTRACT

A considerable amount of attention has been focused on the use of lactobacilli as probiotics. The use of microorganisms as probiotics must meet certain criteria, among these; the resistance to low pH and bile salts is of great importance. Different procedures have been designed to evaluate tolerance to gastrointestinal transit. Since resistance to low pH and bile salts is critical in the evaluation of probiotic strains, the aim of this study was to compare resistance to bile salts for 20 strains of porcine *Lactobacillus reuteri* that had been isolated from healthy pigs, using two different procedures based on exposure to low pH. Statistical analysis revealed no difference in resistance of the strains to bile salts whether pH was low or not. The use of low pH prior to exposure to bile salts therefore more closely represents physiological conditions that should be used for the evaluation of potential porcine probiotic lactobacilli. Further studies using digestive enzymes are required to evaluate resistance to gastrointestinal transit, since it is an important factor in the evaluation of strains with potential probiotic activity.

Key words: Probiotic, pig, lactobacilli, bile resistance, low pH resistance.

RESUMEN

Existe un interés creciente en el uso de lactobacilos como probióticos. El uso de microorganismos como probióticos, requiere que éstos cubran ciertos requisitos, dentro de los cuales la resistencia a pH ácido y a sales biliares, se consideran de suma importancia. Actualmente existen distintos procedimientos que permiten evaluar la tolerancia al tránsito gastrointestinal. Debido a que la resistencia a pH ácido y a sales biliares es una condición crítica dentro de la evaluación de cepas probióticas, la finalidad del presente trabajo consistió en comparar la resistencia a sales biliares de 20 cepas de *Lactobacillus reuteri* aisladas de cerdos sanos, mediante dos procedimientos diferentes basados en la exposición a pH ácido. El análisis estadístico no mostró diferencias en la resistencia de las cepas a sales biliares cuando se realizó una exposición previa a pH ácido. Así, la exposición a pH ácido previo a la incubación con sales biliares, asemeja más a las condiciones fisiológicas que el microorganismo encontrará de manera natural y es una buena alternativa en la evaluación probiótica de lactobacilos de origen porcino. Se requieren estudios que incluyan enzimas digestivas, con el fin de evaluar la resistencia al tránsito gastrointestinal, dado que es uno de los factores más importantes en la evaluación de cepas con potencial actividad probiótica.

Palabras clave: Probiótico, cerdos, lactobacilos, resistencia a sales biliares, resistencia a pH ácido.

INTRODUCTION

Recently, the use of probiotics has improved the food industry. For poultry, alternatives to the use of growth-promoting antibiotics are required, and probiotic bacteria, including *Lactobacillus* and *Bifidobacterium* strains offer a possible solution [7]. A critical aspect of the characterization of probiotic strains is their capacity to avoid biological barriers during digestion [4]. Therefore, the effectiveness of these microorganisms at colonizing the intestinal tract might be related to their ability to withstand the low pH in stomach, pancreatic enzymes and intestinal bile salts [8].

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Several procedures have been designed to simulate biological barriers. In most of those procedures, used in probiotic characterization, the capacity of the organism to tolerate both, stomach acidity and bile salts are evaluated separately [4, 5]. Some simulations of the gastrointestinal tract have been used, in lactobacilli and bifidobacteria strains with potential probiotic activity for humans [2, 9]. However, when some of these procedures evaluated the effect of low pH and bile salts consecutively, the stressing effect of bile salts was limited and the authors did not compare the effect of prior exposure to low pH on the patterns of resistance to bile salts [1, 10].

In this work, a new approach for simulating physiological conditions in an *in vitro* assay, by first exposing probiotic strains to low pH, and then to bile salts is proposed.

MATERIALS AND METHODS

A total of 20 wild-type strains of *Lactobacillus* previously isolated from the gastrointestinal tract of healthy adult *Landrace* crossed with *Large White* pigs (*Sus scrofa*), were used in this study. DNA was extracted and purified with the phenol-chloroform procedure [3]. The first domain of the 16S rDNA was amplified by polymerase chain reaction (PCR) using the universal primers 27F and 519R [11], in a 480 thermocycler (Perkin-Elmer, Wellesley, MA, U.S.A.) with the following program: 96°C for 5 min, followed by 36 cycles of 94°C for 1 min, 50°C for 1 min and 72°C for 1 min, and a final extension of 72°C for 5 min. Amplified PCR products were purified using GFX columns (GE Healthcare, Piscataway, NJ, U.S.A.); and sent to the Arizona Research Laboratories (University of Arizona), to obtain the complete sequence. Sequences were aligned with those available at GeneBank using BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

In order to determine resistance to bile salts, strains were grown in MRS broth (Difco, Sparks, MD, U.S.A.) at pH 6 supplemented with 0.05% (w/v), cysteine (JT Baker, Phillipsburg, NJ, U.S.A.), for 48 h at 37ºC in a candle jar. After incubation, serial tenfold dilutions in 0.85% (w/v) NaCl were done, and each dilution was plated on MRS agar and MRS agar with 0.5% (w/v) porcine bile salts (Sigma Chemical Co, San Luis MO, U.S.A.), cysteine 0.05%, pH 6. All plates were incubated for 48 h at 37°C in a candle jar. Resistance to bile salts was expressed as the percentage of surviving colony forming units (CFU) on MRS agar in the presence of porcine bile salts with respect to the CFU on the MRS agar control [6].

Resistance to low pH was evaluated as follows: strains were grown as described, and were then separated into two 5 ml aliquots. One of these was plated on MRS agar and the other was harvested by centrifugation at 2,600 x *g* for 10 min. The pellet was resuspended in MRS broth supplemented with cysteine 0.05% (w/v), pH 3, followed by 1 hour of incubation under aerobic conditions. Viable counts were determined by plating serial dilutions in 0.85% (w/v) NaCl on MRS agar. Resistance to low pH was expressed as the percentage of surviving CFU after incubation at pH 3 for 1 hour [6].

To evaluate resistance to low pH and bile salts, the strains were grown as described, and then divided into two 5 ml aliquots, as shown in FIG. 1. One aliquot was used to make viable counts, by plating serial dilutions in 0.85% (w/v) NaCl on MRS agar. The other aliquot was used to determine resistance to low pH, as described above. The same dilutions were plated on MRS agar supplemented with cysteine 0.5 % (w/v), porcine bile salts 0.5%, pH 6, to determine resistance to bile salts. All the dilutions were plated in duplicate and only those plates with 25 to 300 CFU were taken into account.

Data were analyzed using a paired *t*-test, on a commercial statistical package (NCSS 2001, Kaysville, UT, U.S.A.). A statistical significance of $P<0.05$ in two-tailed test was used as the criterion.

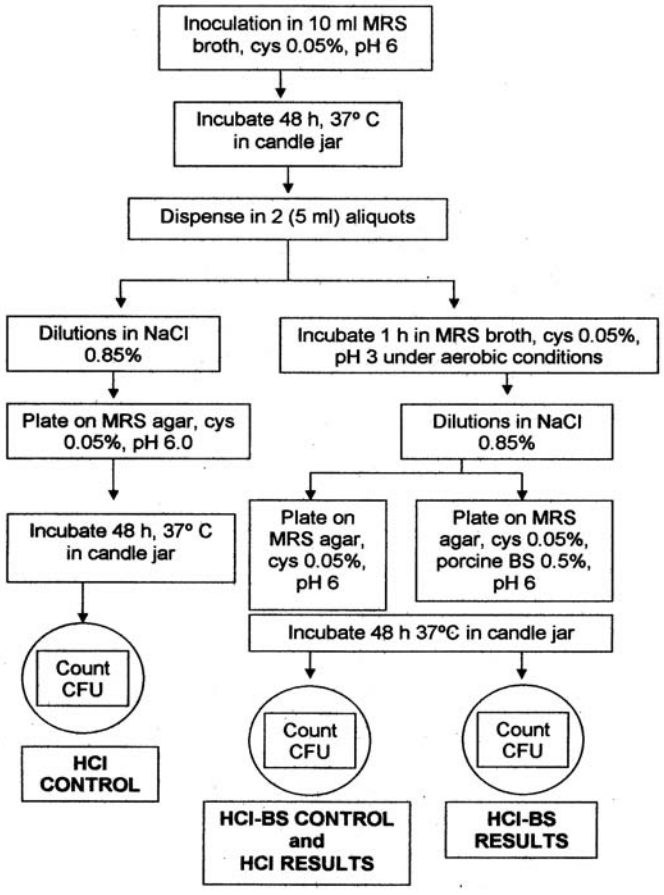

FIGURA 1. FLOW CHART FOR THE RECOMMENDED PROCEDURE OF THE EVALUATION OF TOLERANCE TO LOW pH AND BILE SALTS / DIAGRAMA DE FLUJO RECOMENDADO PARA LA EVALUACIÓN DE TOLERANCIA A pH ÁCIDO Y SALES BILIARES.

RESULTS AND DISCUSSION

All of the strains evaluated in the present study were identified as *Lactobacillus reuteri*, with homologies of 97 to 100% in both strands, according to the 16S RNA sequence of *L. reuteri* DST 2707. The GeneBank accession numbers for each of the sequences are shown in TABLE I.

The procedure described allowed the comparison of two procedures. One of them is commonly used for probiotic characterization of lactobacilli strains. The other one (FIG. 1) allows for the consecutive evaluation of resistance to low pH and porcine bile salts. TABLE I shows the survival rates at low pH and in the presence of bile salts. As expected, percentage survival in bile salts was lower for most of the strains after exposure to low pH. The results for two of the strains, 2 and 1729 are quite different and unexpected. It was surprising that these two strains were more resistant to bile salts when they had previously been exposed to low pH conditions. No statistical difference was found between the procedures in the evaluation of resistance to bile salts (TABLE II).

The evaluation of resistance to bile salts, following exposure to low pH, offers a more realistic representation of physiological conditions than traditional methods do. Although some studies have evaluated resistance to bile salts after exposure to low pH, those studies differ considerably from this report. Some evaluated the growth rate using turbidimetry in MRS broth, rather than by a plate count in MRS agar [1, 9], so their results are qualitative or semiquantitative because CFU were not counted. Another study exposed the strains to low pH and bile salts followed by plating on MRS agar without stressing agents [10], limiting the time of the effect of stress from bile salts to the moment of plating on MRS. Additionally, it has been evaluated the effect of both low pH and bile salts simultaneously [2], but not in a consecutive manner as occurs in the gastrointestinal tract.

Previous studies only evaluated one stressing condition, and the results obtained from one study cannot be compared with the others. This study directly compares methodologies used in characterizing probiotics and offers an alternative for the evaluation of resistance to low pH and bile salts, simulating the series of events that the bacteria will be exposed to in the natural gut environment after being ingested by the host.

TABLE I

PERCENTAGE SURVIVAL FOR 20 STRAINS OF *Lactobacillus reuteri* EXPOSED TO LOW pH AND BILE SALTS /

PORCENTAJES DE SOBREVIVENCIA A pH ÁCIDO Y SALES BILIARES EN 20 CEPAS DE *Lactobacillus reuteri*.

Strain	GeneBank accession number	HCl*	BS†	HCl-BS‡
2	EF437169	33	84	98
30	EF437170	61	80	78
32	EF437171	2	98	79
119	EF437172	92	70	64
124	EF437173	6	72	56
169	EF437174	100	8	11
676	EF437175	87	1	0
703	EF437176	11	9	4
1415	EF437177	32	81	79
1447	EF437178	18	88	42
1703	EF437179	46	58	35
1704	EF437180	96	68	67
1705	EF437181	73	1	1
1715	EF437182	53	17	7
1717	EF437183	92	64	18
1722	EF437184	89	9	2
1723	EF437185	52	17	22
1725	EF437186	100	72	70
1726	EF437187	73	5	3
1729	EF437188	66	66	88

* Percentage survival after exposure low pH.
† Percentage survival after exposure to porcine bile salts.
‡ Percentage survival on exposure to porcine bile salts, after exposure to low pH.

TABLE II

RESULTS OF THE PAIRED *t*-TEST FOR THE EVALUATION OF THE PROCEDURES SIMULATING GASTROINTESTINAL TRANSIT / ANÁLISIS ESTADÍSTICO MEDIANTE PRUEBA DE *t*-PAREADA, PARA LA EVALUACIÓN DE PROCEDIMIENTOS QUE SIMULAN EL TRÁNSITO GASTROINTESTINAL.

Comparison	Mean	S	SE	*p*
BS*	48.4	34.83	7.79	0.068
HCl-BS†	41.2	34.47	7.71	

* Percentage survival after exposure to porcine bile salts.
† Percentage survival on exposure to porcine bile salts, after exposure to low pH.
S: Standard deviation.
SE: Standard error.

CONCLUSIONS AND IMPLICATIONS

Although there is great variability in the survival rates to low pH and bile salts in porcine lactobacilli, no statistical difference was found between the procedures in the evaluation of resistance to bile salts. The evaluation of resistance to bile salts after exposing the bacterium to low pH would be more representative of physiological conditions. Further studies using digestive enzymes are required to evaluate resistance to gastrointestinal transit, since it is an important factor in the evaluation of strains with potential probiotic activity.

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