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Human gastrointestinal tract resistance of *Lactobacillus* strains isolated from infant faeces

Resistência ao trato gastrointestinal humano de linhagens de *Lactobacillus* isoladas de fezes de crianças

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Abstract

The probiotic bacteria survival during the gastrointestinal transit is primordial, and implies in the ability of microorganisms to survive at the stomach acidity and bile, so they can exert their beneficial effects on the host. The aim of this study was to evaluate, "in vitro", *Lactobacillus* strains originated from one year old children fecal material in the selection of probiotic microorganisms. Two commercial strains, *L. casei* (Lc 01™) and *L. acidophilus* (La-05™) were used as controls. The first screening resulted in 75 colonies and they were isolated from six samples faeces. Isolates were Gram positive, mostly rod shaped (*cocobacilli*, long and thin rods) and rarely *cocci*. They were submitted to catalase test and evaluated for the presence of spores, resulting in 30 pre-selected strains. Among those strains, eight strains: L4, L5, L12, L19, L20, L22, L23, L24 were the most resistant to Oxgall (bile salts) concentration (0.3 w/v). These eight strains were also resistant to acid conditions (pH 3.0) and all strains were able to grow in the presence of 0.3 w/v of phenol. The results of treatments were compared to the Neuman Keuls Student test at 5% of probability, with regression analyses made at different times for tolerance to intestinal conditions. The results demonstrated that all these strains were able to survive under gastrointestinal stress condition, indicating potential use as probiotics. The high survival rate of probiotic strains, in conditions that simulate the gastrointestinal transit, is strain dependent and thus, a proper selection of strains in the development of dairy probiotic products is vital.

Key words: Biological barriers, oxgall inhibition, phenol tolerance, acidity resistance

Resumo

A sobrevivência de bactérias probióticas durante o trânsito no trato gastrointestinal é fundamental, e implica na capacidade de sobrevivência dos microrganismos à acidez do estômago e a bile para que elas possam exercer os seus efeitos benéficos sobre o hospedeiro. O objetivo deste estudo foi avaliar, "in vitro", cepas de *Lactobacillus* originadas de material fecal de crianças de um ano de idade para o processo de seleção de microrganismos probióticos. Linhagens comerciais de *L. casei* (Lc 01™) e *L. acidophilus* (La-05 ™) foram utilizadas como controle. A primeira triagem resultou no isolamento de 75 colônias provenientes de seis amostras de fezes. Os isolados foram apresentados como Gram

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positivos, principalmente bacilos (cocobacilos, bacilos longos e finos), e raramente cocos. Estes foram submetidos a testes de catalase e avaliados quanto à presença de esporos, resultando em 30 amostras pré-selecionadas. Entre estas cepas, oito linhagens: L4, L5, L12, L19, L20, L22, L23, L24 foram as mais resistentes à inibição por Oxgall. Estas oito cepas foram também resistentes às condições ácidas (pH 3,0) e todas as cepas foram capazes de crescer na presença de fenol. Os resultados dos tratamentos foram comparados através do teste Student Neuman Keuls a 5% de probabilidade, com análises de regressão feitas em diferentes tempos, para verificar a tolerância às condições do trato intestinal. Os resultados demonstraram que as oito cepas estudadas foram capazes de sobreviver às condições de estresse gastrointestinal, indicando potencial para utilização como probióticos. A alta taxa de sobrevivência das cepas probióticas, em condições que simulam o trânsito no trato gastrintestinal depende da cepa utilizada e, assim, é vital conduzir uma boa seleção de cepas para o desenvolvimento de produtos lácteos probióticos.
Palavras-chave: Barreiras biológicas, inibição por Oxgall, tolerância a fenol, resistência a acidez

Introduction

Probiotics are currently defined as living organisms that promote health benefits to the host when administered in adequate amounts, promoting health benefits to the host (FAO/WHO, 2001). Probiotics have their efficacy established as dietary adjuncts providing benefits to consumers, but the selection of probiotics before incorporation in diet requires close scrutiny in the in vitro form as well as in vivo tests (MISHRA; PRASAD, 2005). Their pharmacology is more complex than that found in the inert drugs, but is now being studied in detail. Some strains have a high survival capacity until they reach the faeces, whereas others are rapidly killed by acid and bile (a characteristic that can be used for the delivery of active intracellular components) (MARTEAU; SHANAHAN, 2003).

A real guide for the probiotics classification was developed because few strains commonly meet this criterion, since there are few published clinical studies demonstrating their beneficial effect on the host (FAO/WHO, 2002). For probiotics used in this way, their survival during the transit through the gastrointestinal tract is important, which implies the ability of microorganisms to survive at the stomach acidity and bile, so they can exert their beneficial effects on the host (COLLINS; THORNTON; O'SULLIVAN, 1998).

In vitro tests for selection of new probiotic bacteria presents a challenge for scientists and technicians of dairy companies. Presently, most probiotics are assessed using assays focused on their ability to survive in, and subsequently colonize, the gastrointestinal environment (MORELLI, 2007). Microorganisms ingested with food begin their trajectory in the intestinal tract through the mouth, being exposed during their transit to successive stressful factors that will influence their survival rate. At the beginning, they should resist to enzymes from the oral cavity, including lysozyme. Later, the next hostile environment is the stomach, considering that the time of entry and release in the stomach is approximately 90 minutes (BERRADA; LEMELAND; LAROCH, 1991).

The fermented products available in the market nowadays, are mostly in the form of liquid or semisolid formulations, exhibiting low cell viability after oral administration. This happens mainly because the bacteria do not survive the harsh conditions in the stomach (KLAYRAUNG; VIERNSTEIN; OKONOGI, 2009). The survival of bacteria to the gastric juice depends on their ability to tolerate low pH values. The pH of the hydrochloric acid secreted in the stomach is 0.9, whereas in the presence of food, it increases to 3.0 (ERKKILA; PETAJA, 2000).

About 2.5 liters of gastric juice with pH 2.0 are secreted in the stomach, causing the destruction

of most microorganisms. In this context, the resistance to the gastric transit in human is an important criterion for the selection of probiotic microorganisms, considering that the ability of probiotic bacteria to survive the passage through the stomach is variable and strain-dependent (CHARTERIS; KELLY; MORELLI, 1998).

The bile secreted in the liver and released in the small intestine, reduces the survival of bacteria by destroying their cell membrane, whose main components are lipids and fatty acids (GILLILAND; SPECK, 1987). The bile secretion rate and concentration depend mainly on the type of ingested food (LANKAPUTHRA; SHAH, 1995); and the bile concentration in the human gastrointestinal tract, in a given time, is variable and difficult to predict (CONWAY; GORBACH; GOLDEN, 1987).

Bile salts are synthesized in the liver from cholesterol, stored in the gallbladder, and released in the duodenum after the intake of fatty foods, having detergent function (ERKKILA; PETAJA, 2000) However, some microorganisms are able to reduce this detergent effect through the ability of hydrolyzing bile salts and reducing their solubility, through the bile salts hydrolase enzyme (BSH). The BSH activity has been found in many genera, including *Lactobacillus*. The resistance to bile salts differs widely between *Lactobacillus* species and also among strains, which mechanism is still unknown (PENNACHIA et. al., 2004).

There is no correlation between the potential of the bacterium for bile salts deconjugation and its ability to withstand the effect of bile. Also, the physiological function of this hydrolytic activity is not clear yet. It seems that the reabsorption of primary/secondary bile salts in the enterohepatic circulation is reduced after their deconjugation. Moreover, due to the fact that the increase in the bile acid concentration affects the carcinogenesis in the large intestine, the discussion about the risks and benefits of bile salts disjointing in the human health, remains contradictory (HALLER; COLBUS; GANZLE, 2001).

In addition to bile salts, there is also the presence of toxic compounds such as phenol in the intestine. This compound is produced by the intestinal microbiota, since this is formed by different bacterial species, and where exists the conversion of various substances into products both beneficial and harmful to the host (MITSUOKA, 1996). Intestinal bacteria, such as enterobacteriaceae, peptostreptococcus, clostridia and eubacteria, produce urease that hydrolyses urea into potentially toxic substances such as ammonia, phenol, pharmacologically active substances and indole amines. The liver normally detoxifies those compounds before excretion in urine and feces (RASIC; KURMANN, 1983).

In this context, the objective of this work was to select lactobacilli strains isolates containing probiotic characteristics, from infant faeces, testing their resistance to the gastrointestinal tract conditions “in vitro”.

Material and Methods

This work was conducted at the Food Technology Laboratory - Londrina State University and at the Microbiology and Biochemistry Laboratory - Western Parana State University.

Faeces samples were collected from six children of both genders who attend to the day nursery center of the Londrina State University, with up to one year of age, during the months of November and December 2004 using the swab technique (KONEMAN; ALLENS; DOWELL, 1997). The Ethic Research Committee of the University Hospital-HURNP-Londrina-PR approved the procedure.

The swab was placed in 10 mL of Rogosa broth

(LBS, Difco, Detroit) and incubated at 37°C for 24 hours. For the isolation it was used MRS agar (De Man, Rogosa and Sharpe, Himedia, India) (DE MAN, ROGOSA; SHARPE, 1960), pH 5.4 added of acetate (1.5% w/v, Merck, Darmstadt) and bromocresol purple (0.045% w/v, Merck, Darmstadt), incubated at 37°C for 48 hours (BRASIL, 1981).

The 75 isolated colonies, from six samples children faeces, were mostly Gram positive, rods (cocobacillus, long and thin rods) and rarely cocci. The predominance of Gram positive rod-shaped bacillus confirmed the effectiveness of the culture medium used, LBS. Thirty isolates have been previously selected, and showed isolated bacilli or rods morphology, negative reaction for catalase test, no gas and non spores producers (HARRIGAN; McCANCE, 1976). All isolates used were kept in MRS broth and stored at -20°C, being later thawed and reactivated.

For the resistance to bile salts determination, after three reactivations in MRS broth, the 30 isolates were inoculated at 1% (v/v) in MRS broth and MRS broth containing 0.3% w/v of bile (Oxgall, Merck, Darmstadt). The absorbance of the culture was determined in spectrophotometer (Femto) at 620 nm, at times 0, 1, 2, 3, 4, 5, 6, 10, 12, 15, 18, 21 and 24 hours. The counting of the isolates submitted to treatments, with and without Oxgall, was also performed by plating tenfold diluted (10^{-8}) on MRS agar, under pour plate aerobic incubation (GILLILAND; STALEY; BUSH, 1984).

The bacteria considered resistant to bile were selected for the following tests and compared with standard strains of *L. casei* (Lc 01™) and *L. acidophilus* (La -05™).

To evaluate the tolerance to acidic conditions, an experiment in factorial scheme 3X4 was conducted, with factors represented by pH (MRS broth adjusted to pH 2.0, 3.0 and 6.5 by adding HCl 0.1 N, Merck) and by time 0, 1, 2 and 3 hours of incubation. The viable cells counting was determined on MRS agar after incubation at 37 º C for 2 to 3 days, in time 0, 1, 2 and 3 hours (LANKAPUTHRA; SHAH, 1995).

The tolerance to phenol was verified, inoculating 1% v/v of active culture in 100 mL reconstituted skimmed milk (RSM, Nestlé, Switzerlad) at 10%, containing 0.3%w/v of phenol (Vetec, Brazil, 35ºC/03days), with determinations of pH and acidity (PAULO, 1991; GARCIA, 1999). The experimental design was random blocks and the experimental unit was represented by the isolates, with three repetitions.

Statistical analyses

The data were analyzed by the Sistema para Análises Estatísticas e Genéticas, SAEG (UFV, 1999). The treatments were compared through the Student Neuman Keuls (SNK) test at 5% of probability, with regression analyses being made at different times for tolerance to acidic conditions data.

Results

The 30 pre-selected isolates were tested to determine its resistance to bile (Table 1). Comparing the growth results with the absorbance values obtained, it was found that the isolates L4, L19, L20, L22, L23 and L24 could be classified as resistant, but they had absorbance values above 0.3 only after 10 hours of incubation.

The strain L12 was selected like tolerant, once it did not grow quickly in the MRS medium containing bile, when compared with the control broth. Strains L1, L2, L3, L7, L9, L10, L11, L13, L14, L15, L21, L25, L26, L27, L28 and L29 were selected as low bile salts tolerance and the Oxgall exerted inhibitory effect on strains L6, L8, L16, L17, L18 and L30 (SARON, 2003).

Table 1. Isolates resistance to bile salts (0.3% w/v of Oxgall).

	MRS			MRS + Oxgall		
Isolate	**Time 0**	**Time 24**	**Growth**	**Time 0**	**Time 24**	**Growth**
L1	6.63	8.56	1.93	6.51	6.70	0.19
L2	6.58	8.79	2.21	6.35	7.14	0.79
L3	6.48	8.85	2.37	6.50	6.35	-0.15
L4	7.40	9.12	1.72	6.88	8.74	1.86
L5	6.82	9.07	2.25	6.77	7.67	0.90
L6	6.52	8.71	2.19	6.22	6.42	0.20
L7	6.60	8.64	2.04	6.54	6.27	-0.27
L8	6.38	8.77	2.39	6.18	6.10	-0.08
L9	6.50	8.93	2.43	6.60	6.68	0.08
L10	6.69	8.97	2.28	6.49	6.51	0.02
L11	6.61	8.81	2.20	6.47	6.72	0.25
L12	6.51	8.56	2.05	6.50	6.99	0.49
L13	6.60	8.82	2.22	6.47	6.41	-0.06
L14	6.83	8.91	2.08	6.77	6.56	-0.21
L15	6.81	8.69	1.88	6.69	6.59	-0.10
L16	6.46	8.42	1.96	6.11	5.61	-0.50
L17	6.64	8.89	2.25	6.61	1.75	-4.86
L18	7.12	9.20	2.08	5.00	3.48	-1.52
L19	7.31	8.52	1.21	7.15	8.15	1.00
L20	7.30	9.12	1.82	7.06	8.57	1.51
L21	7.31	8.90	1.59	5.08	4.95	-0.13
L22	7.35	8.83	1.48	7.10	8.08	0.98
L23	7.22	8.88	1.66	7.07	8.28	1.21
L24	7.25	9.20	1.95	7.17	8.68	1.51
L25	7.47	9.38	1.91	4.93	5.19	0.26
L26	8.67	8.48	0.19	6.18	5.77	-0.41
L27	6.87	8.68	1.81	6.43	6.49	0.06
L28	6.80	9.11	2.31	6.66	7.43	0.77
L29	6.87	8.80	1.93	6.46	6.27	-0.19
L30	6.71	8.76	2.05	6.72	6.45	-0.27
L. casei	8.52	9.06	0.54	8.37	7.34	-1.03

Average results with values expressed in Logarithms of the number of colony forming units (CFU/ml). Growth = $\log_{10}$ (final population) - $\log_{10}$ (initial population).

It was demonstrated the inhibitory effect of 0.3% w/v Oxgall over the strain L24 in MRS medium (Figure 1).

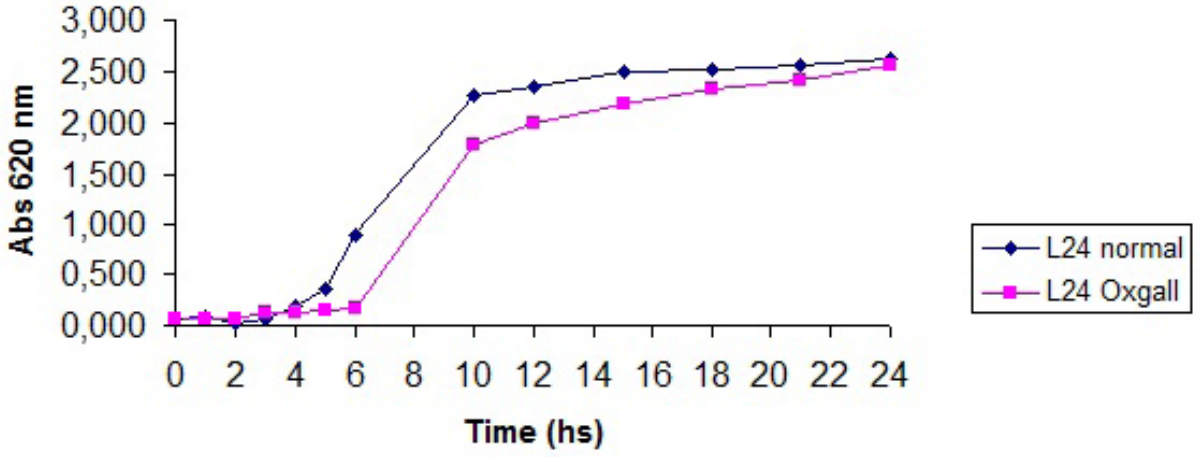

Figure 1. Isolate L24 growth in MRS broth containing 0.3% w/v Oxgall.

Isolates that had greater resistance at pH 2.0 were L20 and L22, which lost their viability only after 3 incubation hours.

After 1 hour of incubation, it was verified a difference in averages obtained at pH 2.0 compared to pH 3.0 and control, except for L22 isolate and *L. casei*, which counts differed statistically ($P<0.05$) in the studied pH values (pH 6.5; 3.0 and 2.0). After 2 and 3 hours, it was found that the counts differed statistically ($P<0.05$) at pH 2.0, 3.0 and control in all isolates. The exception was for L20 and L23 isolates, which obtained averages statistically similar between pH 3.0 and control, therefore presenting less influence from low pH values (pH 3.0) over counts.

It was demonstrated also the acidity resistance of selected isolates (Table 2).

The selected isolates were tested to verify their phenol tolerance (Table 3).

L19, L22 and L24 isolates showed a slow milk acidification during the incubation period, changing the pH values to 5.17, 5.20 and 5.25, respectively. Therefore, these isolates showed a lower acidity reduction percentage.

Table 2. Growth of isolates exposed up to 3 hours at 37ºC in hydrochloric acid (HCl 0.1 N).

	Time (h)				
Isolate	**0**	**1**	**2**	**3**	**CV %**
L4 pH 6,5	6.88 ± 0.14[a]	6.95± 0.04[a]	7.11 ±0.16[a]	7.46± 0.10[a]	2.80
L4 pH 2.0	6.30 ±0.20[b]	3.78 ±0.01[b]	0.00 ±0.00[c]	0.00 ±0.00[c]	
L4 pH 3.0	6.84 ±0.11[a]	6.85 ±0.15[a]	6.44 ±0.31[b]	6.28 ±0.22[b]	
L5 pH6,5	6.95 ±0.06[a]	6.90 ±0.10[a]	7.11 ±0.08[a]	7.54 ±0.12[a]	9,48
L5 pH 2.0	6.47 ±0.65[a]	0.00 ±0.00[b]	0.00 ±0.00[c]	0.00 ±0.00[c]	
L5 pH 3.0	6.87 ±0.06[a]	6.73 ±0.21[a]	6.08 ±1.00[b]	5.39 ±1.09[b]	
L12 pH6,5	6.59 ±0.08[a]	6.71 ±0.15[a]	6.73 ±0.11[a]	7.35 ±0.31[a]	9.96
L12 pH 2.0	5.28 ±1.11[b]	4.88 ±0.05[b]	0.00 ±0.00[c]	0.00 ±0.00[c]	
L12 pH 3.0	6.41 ±0.15[a]	6.27 ±0.41[a]	4.5 ±1.17[b]	5.05 ±0.02[b]	
L19 pH6,5	7.26 ±0.17 [a]	7.30 ±0.20 [a]	7.43 ±0.17 [a]	7.45 ±0.12 [a]	4.28
L19 pH 2.0	6.39 ±0.53 [b]	0.00 ±0.00 [b]	0.00 ±0.00 [c]	0.00 ±0.00 [c]	
L19 pH 3.0	7.16 ±0.13 [a]	7.14 ±0.15 [a]	6.36 ±0.40 [b]	5.87 ±0.01 [b]	
L20 pH6,5	7.22 ±0.38 [a]	7.21 ±0.33 [a]	7.31 ±0.33 [a]	7.38 ±0.35 [a]	7.47
L20 pH 2.0	6.55 ±0.09 [a]	4.45 ±0.03 [b]	3.04 ±0.02 [b]	0.00 ±0.00 [b]	
L20 pH 3.0	7.17 ±0.41 [a]	6.97 ±0.64 [a]	6.86 ±0.78 [a]	6.84 ±0.81 [a]	
L22 pH6,5	7.36 ±0.25 [a]	7.33 ±0.20 [a]	7.36 ±0.22 [a]	7.49 ±0.26 [a]	6.03
L22 pH 2.0	6.21 ±0.40 [b]	4.63 ±0.01 [c]	2.34 ±0.01 [c]	0.00 ±0.00 [c]	
L22 pH 3.0	6.25 ±0.55 [b]	6.23 ±0.63 [b]	6.05 ±0.36 [b]	6.01 ±0.38 [b]	
L23 pH6,5	6.99 ±0.07 [a]	7.18 ±0.06 [a]	7.22 ±0.05 [a]	7.38 ±0.10 [a]	7.76
L23 pH 2.0	6.14 ±1.24 [b]	0.00 ±0.00 [b]	0.00 ±0.00 [b]	0.00 ±0.00 [b]	
L23 pH 3.0	7.04 ±0.04 [a]	7.04 ±0.05 [a]	7.33 ±0.56 [a]	6.89 ±0.32 [a]	
L24 pH6,5	7.25 ±0.17 [a]	7.29 ±0.20 [a]	7.33 ±0.14 [a]	7.51 ±0.28 [a]	3.33
L24 pH 2.0	7.17 ±0.15 [a]	0.00 ±0.00 [b]	0.00 ±0.00 [b]	0.00 ±0.00 [c]	
L24 pH 3.0	7.00 ±0.09 [a]	7.17 ±0.09 [a]	7.26 ±0.18 [a]	7.09 ±0.39 [b]	
L.casei pH6,5	7.23 ±0.31 [a]	7.26 ±0.23 [a]	7.32 ±0.28 [a]	7.42 ±0.27 [a]	4.31
L. casei pH 2.0	7.08 ±0.42 [a]	1.81 ±0.01 [c]	0.00 ±0.00 [b]	0.00 ±0.00 [c]	
L. casei pH 3.0	7.15 ±0.41 [a]	6.18 ±0.06 [b]	6.30± 0.01 [c]	6.80± 0.04 [b]	

* Average values from 3 repetitions, expressed as Log (CFU / ml).
Averages followed by same letter, in columns, do not differ by Neuman Keuls Student test at 5% probability.

Table 3. % Acidity Reduction of isolates in 10% reconstituted skimmed milk (RSM), for 3 days at 35ºC, with and without (control) 0.3% w/v phenol.

Isolates	pH control	pH with phenol	% of lactic acid without phenol	% of latic acid with phenol	% Acidity Reduction
L4	4.84±0.04	5.86±0.02	0.570±0.02	0.284±0.02c	50.17
L5	4.66±0.12	5.49±0.16	0.624±0.09	0.348±0.06c	44.23
L12	4.63±0.19	5.16±0.18	0.670±0.09	0.481±0.04b	28.20
L19	5.17±0.35	5.73±0.13	0.407±0.08	0.304±0.03 c	25.31
L20	4.62±0.02	5.72±0.21	0.648±0.07	0.293±0.05 c	54.78
L22	5.20± 0.45	5.60±0.15	0.422± 0.11	0.321±0.04 c	23.93
L23	4.80±0.29	5.63±0.32	0.559±0.15	0.338±0.05 c	39.53
L24	5.25±0.48	5.95±0.10	0.396±0.12	0.268±0.04 c	32.32
L. casei	3.87±0.20	5.91±0.49	1.163±0.15	0.294±0.08 c	74.72
L.acidophilus	3.54±0.05	4.04±0.07	1.749±0.19	1.039±0.07a	40.59

*Average values from 3 repetitions.
% Reduction = % acidity without phenol - % acidity with phenol /% acidity without phenol.
Averages followed by same letter, in columns, do not differ through the Student Neuman Keuls test at 5% probability.

Discussion

The differentiated tolerance of lactobacilli in the presence of bile salts was also reported by Gardiner et al. (2002), which evaluated the tolerance of *L. fermentum* and *L. rhamnosus* in different Oxgall concentrations. They observed that the viability of both species was not reduced at Oxgall 0.3 and 0.5% w/v concentrations, after 48 hours of anaerobic incubation, concluding that such bacteria could be considered as bile tolerant, since the concentration of 0.3 % is considered physiologically relevant.

The comparison of 30 strains of lactobacilli in MRS broth, with and without 0.3%w/v Oxgall, revealed considerable growing ability variation among strains. L4, L5, L19, L20, L22, L23 and L24 were the most resistant strains. It was found that the growth curves were similar, with an increase in the absorbance values only after 10 hours of incubation, except for L5 isolate, which obtained in 10 hours, the absorbance value of 0.208 and after 12 hours achieved absorbance value of 0.434, without observable reduction on viability (Table 1). Strain L12 was considered tolerant.

In this experiment, the eight most resistant strains were selected, which represents 27% of total isolates.

Eleven strains of lactobacilli were screened for their bile salt deconjugation ability, bile salt hydrolase activity (BSH) and co-precipitation of cholesterol with deconjugated bile. Bile salt deconjugation, as determined by the release of cholic acid, showed that it was liberated more cholic acid from the sodium glycocholate deconjugation than sodium taurocholate, and *Lactobacillus acidophilus* strains had higher deconjugation ability than *L. casei* strains (LIONG; SHAH, 2005).

BSH activity (BEGLEY; HILL; GAHAN, 2006) has been detected in *Lactobacillus, Bifidobacterium, Enterococcus, Clostridium and Bacteroides* spp. A better understanding of the role of BSH may be exploited in the selection and rational design of probiotic strains. Since it is likely that BSH significantly contributes to the bile tolerance, as well as to the survival and persistence of strains

in the intestinal tract, it may be desirable to select probiotic bacteria that possess these enzymes.

In a study with seven *L. acidophilus* strains, Gupta, Mital and Garg (1996), used a dietary supplement with 0.3% w/v Oxgall concentration, and found that three strains were completely inhibited, while others showed 44 to 82% of inhibition. No isolate was able to grow in pH 2.0, when hydrochloric acid was added to the MRS broth. Similar data were also obtained by Hood and Zottola (1988), however, Conway, Gorbach and Golden (1987) observed better survival rate of *L. acidophilus* when compared to *L. bulgaricus* and *Streptococcus thermophilus* at low pH values.

In the present study, there were significant interactions between pH and time, and the variation coefficients ranged from 3.33 to 9.96 % (Table 2).

It was found that in the time 0, there was no significant difference in counts through the SNK test ($P>0.05$) between the studied pH levels (pH 2.0; 3.0 and control). However, at pH 2.0, the isolates L4, L12, L19 and L23 showed lower counts in relation to those observed at pH 3.0 and control, and these two pH values were similar for all isolates except for isolate L22, which presented the same average found in pH 2.0 and 3.0.

Mainville, Arcand and Farnworth (2005) studied the behavior of some probiotic strains and found that, despite *Lactobacillus rhamnosus* counts reduces 2 log cycles after 15 minutes at pH 2.0 (using hydrochloric acid), this strain could reach the colon in sufficient numbers to show beneficial effects to host, arguing that only highly resistant bacteria would survive the acidic conditions. So, when conventional exclusion criteria are employed, many strains could be discarded, despite having desirable properties for the health maintenance.

According to Erkkila and Petaja (2000), the decrease in the survival rate of probiotic strains under conditions that simulate the transit through the gastrointestinal tract depends on the strain used, and thus, a proper selection of strains in the development of dairy probiotic products is vital.

Ding and Shah (2007), improved the survival of probiotic bacteria, by microencapsulating before submit to acidic conditions, bile salts, and mild heat treatment. Eight strains of probiotic bacteria, including *Lactobacillus rhamnosus, Bifidobacterium longum, L. salivarius, L. plantarum, L. acidophilus, L. paracasei* and *B. lactis* were studied for their acid, bile, and heat tolerance. Results indicated that the microencapsulated probiotic bacteria survived better ($P < 0.05$) than free probiotic bacteria in MRS containing HCl. When free probiotic bacteria were exposed to Oxgall, the viability was reduced 6.51-log CFU/mL, whereas only 3.36-log CFU/mL was lost in microencapsulated strains.

The pH values of the isolates L19, L22 and L24 are below the limits considered desirable for a fermented product. According to Ronka, Malinen and Saarela (2003), they should be in a range from 4.4 to 4.6. Similarly, Arici et al. (2004) found that the milk pH values of lactobacilli strains from children were lower than 5.50. In the present study, the isolates L5, L12 and L20 showed higher acidification ability in milk.

The viability of *Lactobacillus acidophilus, Bifidobacterium lactis* and *L. paracasei* and their proteolytic activities were assessed in yoghurt at different final pH: 4.45, 4.50, 4.55 and 4.60 during 28 days of storage at 4 °C (DONKOR, et. al., 2006). Lactobacilli strains showed a good cellular stability maintaining constant concentration throughout storage period regardless of final pH.

Ninety strains of *L. fermentum*, which were isolated from traditional dairy products, were evaluated for probiotic potential and resistance to low pH (BAO et al., 2010). The results showed that 35 strains grew well at acid condition (pH 3.0). Also, eleven strains were further screened out from 35 strains demonstrating high tolerance to the simulated gastric juice (pH 2.5, 3 h of incubation). While in simulated gastric juice (pH 2.0), only the isolate F6 could survive to a rate of 53.7% and

showed good tolerance to bile salt. They concluded that this strain had potential application in functional foods and health-associated products.

It was found a significant difference among various isolates with respect to the acidity, in the presence of phenol, through the SNK test ($P<0.05$). *L. acidophilus* and the L12 isolate presented higher acidity values ($P<0.05$) and it was also different from other bacteria, which had similar behavior on average acidity values. However, it should be noted that all isolates grew in the presence of 0.3% w/v phenol concentration. Paulo (1991) found tolerance at 0.3% w/v and full inhibition of lactobacilli in the presence of 0.5% w/v phenol concentration. Xanthopoulos, Litopoulou-Tanetaki and Tzanetakis (2000) found a bacteriostatic effect on lactobacilli in the presence of 0.4% w/v phenol.

Although no testing was done to evaluate the enzymes (e.g. lysozyme) resistance, as well as adherence to epithelial cells and hydrophobicity, these strains possess desirable characteristics for use in fermented products containing multistrains, according to the results presented earlier.

Conclusion

The potential to be used as probiotic was demonstrated in eight isolates: L4, L5, L12, L19, L20, L22, L23 and L24. These species were able to grow in the presence of phenol, and furthermore had good performance under acidic conditions (hydrochloric acid) and bile salts (Oxgall in MRS medium). Technologically, the selected strains may be used in the future, particularly as starter cultures for development of fermented dairy products.

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