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Evaluation of the role of environmental factors in the human gastrointestinal tract on the behaviour of probiotic cultures of *Lactobacillus casei* Shirota and *Lactobacillus casei* LC01 by the use of a semi-dynamic *in vitro* model

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Abstract - This study evaluated the influence of gastrointestinal environmental factors (pH, digestive enzymes, food components, medicaments) on the survival of *Lactobacillus casei* Shirota and *Lactobacillus casei* LC01, using a semi-dynamic *in vitro* model that simulates the transit of microorganisms through the human GIT. The strains were first exposed to different simulated gastric juices for different periods of time (0, 30, 60 and 120 min), and then to simulated intestinal fluids for zero, 120, 180 and 240 min, in a step-wise format. The number of viable cells was determined after each step. The influence of food residues (skim milk) in the fluids and resistance to medicaments commonly used for varied therapeutic purposes (analgesics, antiarrhythmics, antibiotics, antihistaminics, proton pump inhibitors, etc.) were also evaluated. Results indicated that survival of both cultures was pH and time dependent, and digestive enzymes had little influence. Milk components presented a protective effect, and medicaments, especially anti-inflammatory drugs, influenced markedly the viability of the probiotic cultures, indicating that the beneficial effects of the two probiotic cultures to health are dependent of environmental factors encountered in the human gastrointestinal tract.

Key words: *Lactobacillus casei* Shirota; *Lactobacillus casei* LC01; probiotic; gastrointestinal tract.

INTRODUCTION

Lactic acid bacteria (LAB) are commonly used as probiotic organisms. They may offer a safe and practical means of modulating the function and metabolic activity of the human intestinal microbiota, excluding pathogens and helping to keep the gut homeostasis by influencing the mucosal immune system (Morita *et al.*, 2006). Lactobacilli, particularly certain selected strains with immunomodulatory properties, can modify the responses of the host, thereby inducing beneficial effects (Ezendam and van Loveren, 2008; Shida and Nanno, 2008). Recent studies suggest that probiotics can be used for treatment of diseases and allergic disorders (He *et al.*, 2001; Ezendam and van Loveren, 2008; Ghadimi *et al.*, 2008; Shida and Nanno, 2008).

The normally wide accepted definition of probiotic is the following: probiotics are live microorganisms which when administered in adequate amounts confer a health benefit on the host (FAO/WHO, 2001). Probiotic lactobacilli encounter various environmental conditions upon ingestion by the host and during transit in the gastrointestinal tract (GIT) (Leeber *et al.*, 2008). Their viability may be affected by the harsh conditions of the stomach and the deleterious activity of bile and pancreatic juices which contain acids and digestive enzymes (Kimoto *et al.,* 2000; Vinderola and Reinheimer, 2003; Todorov and Dicks, 2008). Presence of non-antibiotic medications in the GIT may also induce a stress to these microorganisms (Todorov *et al.*, 2007, 2008; Botes *et al.,* 2008). Several studies indicate that survival of LAB in this environment is variable and strain-dependent (Charteris *et al.,* 1998a; Prasad *et al.*, 1998; Norikatsu *et al.*, 1999; Vinderola and Reinhheimer, 2003; Botes *et al.*, 2008; Todorov *et al*., 2008) and strongly influenced by the nutrients of the diet (Kos *et al.,* 2000, Souza and Saad, 2009).

Proper evaluation of survival capability of LAB and probiotic bacteria in the human GIT is a challenge. Results of evaluations based on exposure to acid solutions and digestive enzymes should be interpreted with care, as the movement of bacteria along the gastrointestinal tract is not taken into account (Botes *et al.*, 2008). Several models comprised by single or multiple vessels simulating the physiological transit through the colon were developed, but microorganisms did not reach physiological densi-

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ties and microbial metabolites accumulated with time (Saarela *et al.*, 2000; Botes *et al.,* 2008; Todorov *et al.,* 2009). Other models included addition of a food matrix to simulate conditions of ingestion and digestion (Mainville *et al.,* 2005). More recently, computer controlled systems were able to better simulate conditions of the large intestine due to peristaltic mixing and/or constant removal of metabolites and water (Egert *et al.,* 2006; Botes *et al.*, 2008).

As these new investigation resources are not available in many research laboratories, the aim of the present study was to use a simple semi-dynamic *in vitro* model that simulates the transit of microorganisms through the human GIT to evaluate the behavior of *Lactobacillus casei* Shirota and *L. casei* LC01 in this environment. The influence of medicaments and milk components on the survival of these two strains was also investigated.

MATERIALS AND METHODS

Bacterial cultures. Lyophilized cultures of *L. casei* LC01 and *L. casei* Shirota were provided by Chr. Hansen S/A (Valinhos-SP) and Yakult S/A, respectively. Cultures, maintained at −18 °C until used, were reactivated in MRS Broth (Oxoid Ltd., Basingstoke, OK) at 37 °C for 18 h, and submitted to enumeration of viable cells (CFU/ml) by pour-plating in MRS agar pH 5.4, in duplicate, incubated at 37 °C for 48 h. Before use, both cultures were tested for purity using Gram staining and proper biochemical tests (API50CHL, bioMérieux, France). For dilutions, sterile 0.5% NaCl solution (w/v) was used throughout the study.

Evaluation of survival of *Lactobacillus casei* Shirota and *Lactobacillus casei* LC01 in the semi-dynamic *in vitro* model. The method of Kos *et al.* (2000), with minor modifications, was used. *L. casei* Shirota and *L. casei* LC01 were grown in MRS broth for 24 h, and washed three times by centrifugation (Hettich Zentrifugen, model Mikro 22R, Germany) at 2810 *x g*, at 4 °C for 10 min, using the same volume of sterile 0.5% NaCl solution to re-suspend the pellets. Two ml aliquots of the washed cell suspension were transferred to sterile flasks containing 10 ml of simulated gastric juice, composed of sterile 0.5% NaCl solution at pH 1.5, 2.0, 2.5 or 3.0, adjusted with 1 M chloridric acid, and added of pepsin (3 g/l, Sigma Chemical Co, Inc., USA). After incubation at 37 °C in a rotary shaker (150 rpm), one ml aliquots were withdrawn from each flask at times 0, 30, 60 and 120 min, and submitted to enumeration of viable cells as described previously. In the next step, the cultures in each gastric juice were washed by centrifugation at 2810 *x g* for 10 min, suspended in 5 ml of 0.5% NaCl solution, and mixed with 10 ml of simulated intestinal fluids, composed of sterile 0.5% NaCl solution pH 8.0 containing bile (10 g/l, Sigma Chemical Co) and pancreatin (1 g/l, Sigma Chemical Co). The mixture was incubated at 37 °C in a rotary shaker (150 rpm) and one ml aliquots were withdrawn after 0, 60, 120 and 240 min and submitted to enumeration of viable cells as described previously. Each experiment was repeated three times and results were expressed as mean log CFU/ml counts.

Evaluation of the effect of addition of skim milk to the gastric juice on the survival of *Lactobacillus casei* Shirota and *Lactobacillus casei* LC01. The evaluation of survival of *L. casei* Shirota and *L. casei* LC01 in the semi-dynamic *in vitro* model, described previously, was repeated using gastric juice at pH 1.5, 2.0, 2.5 and 3.0 and pepsin (3 g/l), supplemented with 10% skim milk, prepared reconstituting powdered skim milk (Molico, Nestle, Brazil) to 10% in sterile water. Each experiment was repeated three times and results were expressed as mean log CFU/ml counts.

Evaluation of the effect of medicaments on survival of *Lactobacillus casei* Shirota and *Lactobacillus casei* LC01. Commercial medicaments (see Table 3) were purchased in a local drugstore, and solubilized in sterile water to achieve the concentrations indicated in Table 3. *Lactobacillus casei* Shirota and *L. casei* LC01 were inoculated, separately, into 10 ml MRS broth (Difco), incubated at 37 °C for 18 h and mixed into MRS soft agar (1.0%, w/v, Difco), in order to achieve a population of 10^6 CFU/ml. After solidification of the agar, each medicament (10 µl) was spotted onto the surface of the plates, and incubated at 37 °C for 24 h. The plates were examined for the presence of inhibition zones around the spotted medicament, and those presenting inhibition zones larger than 2 mm diameter were subjected to the determination of the minimal inhibition concentration (MIC). Serial two-fold dilutions of the medicaments were prepared in sterile water and 10 µl spotted onto the surface of MRS soft agar plates, previously inoculated with *L. casei* Shirota or *L. casei* LC01. The plates were incubated at 37 °C for 24 h and examined for the presence of inhibition zones around the spots. The MIC corresponded to the highest dilution that resulted in inhibition halos of at least 2 mm diameter.

RESULTS AND DISCUSSION

As shown in Table 1, pepsin had little, if any, influence on the survival of *L. casei* Shirota and *L. casei* LC01 in the simulated gastric juice at pH 3.0. The differences in the counts were not statistically significant ($p < 0.05$). The same was observed after exposure to enteric juice at pH 8.0 containing bile or pancreatin (Table 2).

The survival of *L. casei* Shirota and *L. casei* LC01 in simulated gastric juices at pH 1.5, 2.0, 2.5 and 3.0 is presented in Fig. 1A and 1B, respectively. For both strains, the deleterious effect of the acidic environment was pH and time-dependent. At pH 1.5, after 15 min a reduction of almost 5 log was observed and no viable cells could be detected after 30 min. At pH 2.0, a better survival could be observed: little reduction (< 1 log) in 15 min, 4 log reduction after 30 min and no viable cells after 60 min. The scenario changed at pH 2.5, with both strains resisting well for up to 60 min, but decreasing 4 log in 120 min. At pH 3.0, good survival was observed in the gastric juice, with practically the same viable counts after 15, 30, 60 and 120 min of exposure.

The protective effect of skim milk on the survival of both probiotic strains in the simulated gastric juices can be seen in Fig. 1C and 1D, referring to *L. casei* Shirota and *L. casei* LC1, respectively. For both cultures, the intensity of the protection was more evident at pH 1.5 and 2.0.

The numbers of viable cells of *L. casei* Shirota and *L. casei* LC01 in the simulated enteric juices after having passed through the plain gastric juices at pH 1.5, 2.0, 2.5 and 3.0 for 120 min are shown in Fig. 2A and 2B. The transfer from the harsh gastric conditions to the less harmful environment encountered in the intestine increased the number of viable cells. Skim milk protected

TABLE 1 – Effect of pepsin (3.0 g/l) on the survival of *Lactobacillus casei* Shirota and *Lactobacillus casei* LC01 in simulated gastric juice (pH 3.0)

Time (min)	*Lactobacillus casei* Shirota (log CFU/ml)		*Lactobacillus casei* LC01 (log CFU/ml)	
	With pepsin	Without pepsin	With pepsin	Without pepsin
Zero	9.43 ± 0.09	9.58 ± 0.07	9.54 ± 0.07	9.59 ± 0.05
15	9.53 ± 0.03	9.61 ± 0.06	9.93 ± 0.02	9.66 ± 0.05
30	9.45 ± 0.02	9.51 ± 0.02	9.73 ± 0.03	9.84 ± 0.06
60	9.37 ± 0.03	9.81 ± 0.03	9.89 ± 0.02	9.34 ± 0.02
120	9.38 ± 0.08	9.36 ± 0.04	9.67 ± 0.06	9.59 ± 0.05

TABLE 2 – Effect of bile (10.0 g/l) and pancreatin (1.0 g/l) on the survival of *Lactobacillus casei* Shirota and *Lactobacillus casei* LC01 in simulated enteric fluid (pH 8.0)

Time (min)	*Lactobacillus casei* Shirota (log CFU/ml)		*Lactobacillus casei* LC01 (log CFU/ml)	
	Without bile and pancreatin	With bile and pancreatin	Without bile and pancreatin	With bile and pancreatin
Zero	9.99 ± 0.04	9.08 ± 0.05	9.49 ± 0.04	9.89 ± 0.06
120	9.20 ± 0.03	9.11 ± 0.02	9.63 ± 0.05	9.41 ± 0.04
180	9.95 ± 0.06	9.94 ± 0.03	9.94 ± 0.04	9.98 ± 0.05
240	9.30 ± 0.04	9.78 ± 0.04	9.93 ± 0.03	9.49 ± 0.04

the probiotic cells (Fig. 2C and 2D), probably due to presence of protective milk proteins in addition to the buffering capacity of the milk. These results are important, since babies and infants are on high milk-containing diets, and milk is part of the diet of most adults, except for lactose intolerant individuals.

Several studies have shown that probiotic cultures were strongly affected by the exposure to simulated gastric fluids, depending on the strain, pH and time of exposure, but were resistant to small intestine transit (Charteris *et al.*, 1998b; Kos *et al.*, 2000). Mainville *et al.* (2005) have shown that only 0.1% of cells of *Lactobacillus rhamnosus* GG survived in a stomach reactor, whereas *Lactobacillus johnsonii* La1 showed 76% survival. However, previous studies showed that high cell numbers of *L. rhamnosus* GG reached the colon *in vivo* (Siitonen *et al.*, 1990; Goldin *et al.*, 1992; Mainville *et al.*, 2005). Furthermore, in human trial studies *L. casei* Shirota and *L. johnsonii* La1 survived conditions in the GIT the best (Salminen *et al.*, 1998; Spanhaak *et al.*, 1998; Holzapfel *et al*., 2001; Mainville *et al.*, 2005).

Several studies have shown that proteins, especially from milk, may protect the cultures. Recently, Botes *et al.* (2008) evaluated the behaviour of two probiotic candidates *Lactobacillus plantarum* 423 and *Enterococcus mundtii* ST4SA in a computerized simulated gastro-intestinal model (GIM) using two commercial infant milk formulations, and reported that concentrations up to 10^9-10^{10} CFU/ml could be detected after 15 h. These authors also evaluated the survival of *L. casei* Shirota, *Lactobacillus acidophilus* La5 and *L. rhamnosus* R-11 in the two formulations observing that *L. casei* Shirota decreased from 1.0 x 10^8 CFU/ml to 2.0 x 10^7 CFU/ml in the stomach, but increased to 1.0 x 10^8 CFU/ml in the ileum. Viable counts of *L. acidophilus* La5 and *L. rhamnosus* R-11 decreased two log cycles in the presence of acid and bile and were present at 10^6 CFU/ml in the ileum.

When the pH of the stomach in individuals with very low food ingestion is low (pH < 2.0), intake of foods containing probiotic bacteria may result in rapid destruction of these microorganisms, and the expected beneficial effects will not occur. In these cases, probiotic bacteria should be ingested with food containing components with buffering capacity such as milk, yoghurt or other protein-rich foods.

Bile salts in the intestinal fluids also affect the behavior of probiotic cultures (Kos *et al*., 2000). It is well known that non-intestinal bacteria such as *Lactobacillus bulgaricus* and *Lactococcus lactis* are more sensitive to bile compared to the natural GIT microflora (Gilliland and Speck, 1977; Vinderola and Reinheimer, 2003). However, more recent studies have observed that certain bacteriocinogenic strains of *L. plantarum*, *Enterococcus faecium*, *Leuconostoc mesenteroides* subsp. *mesenteroides* and *L. lactis* subsp. *lactis* can resist to high concentrations of ox-bile and low pH (Pinto *et al.*, 2006; Mathara *et al.*, 2008; Todorov and Dicks, 2008; Todorov *et al.*, 2008; Pan *et al.,* 2009). The ability to produce bacteriocins may be important to ensure longer shelf-life and safety of probiotic foods, but the interactions of these microorganisms with the normal intestinal microbiota need to be better understood.

Patients taking probiotics are often treated for other illnesses. It is thus important to determine the effect of medicaments on the survival of probiotic strains. As shown in Table 3, both *L. casei* Shirota and *L. casei* LC01 were inhibited by non-steroidal anti-inflammatory drugs (NSAID) containing diclofenac potassium or ibuprofen arginine as well as by the two antibiotics tested. In addition, *L. casei* Shirota was affected by selective serotonin reuptake inhibitors (SSRI) antidepressant containing paroxetine and antiarrhythmic medication containing amiodarone. *Lactobacillus casei* LC01 was inhbited by hypolipidemic medication containing simvastatin. The interference of anti-inflammatory drugs containing diclofenac on the viability of LAB detected in this study (Table 3) was also reported in other studies. Todorov and Dicks (2008) observed that sodium diclofenac inhibited the growth of *L. plantarum* ST8KF and ST341LD, *E. faecium* ST311LD and *L. mesenteroides* subsp. *mesenteroides* ST33LD. In another study, potassium diclofenac and ibuprofen inhibited

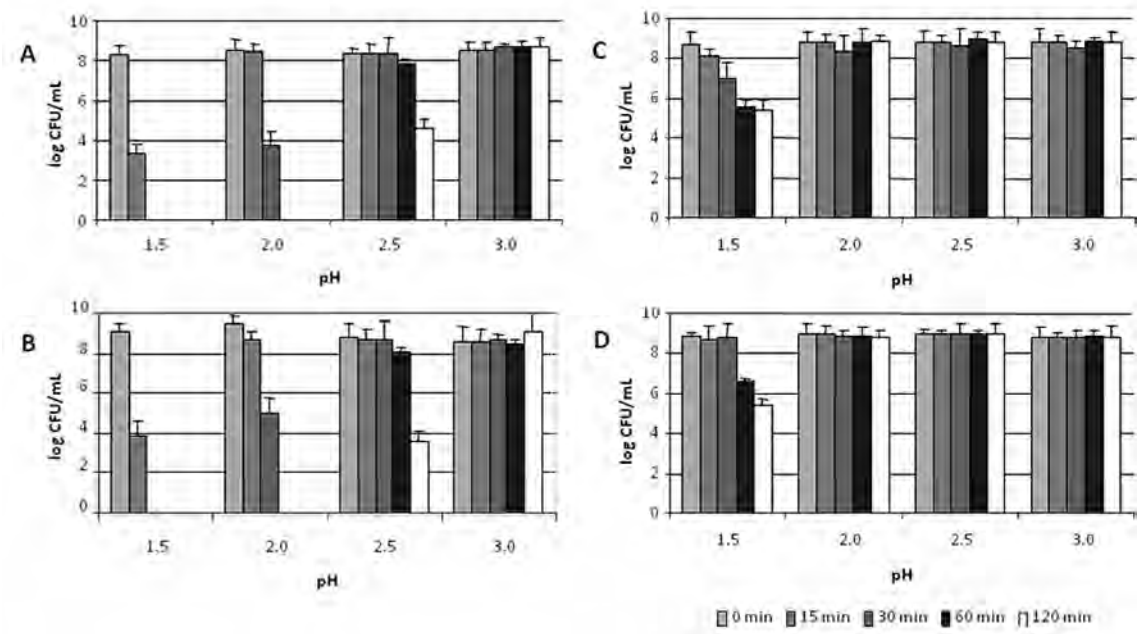

FIG. 1 - Survival of *Lactobacillus casei* Shirota and *Lactobacillus casei* LC01 in simulated gastric juice (A and C, respectively) and in simulated gastric juice added of skim milk (B and D, respectively) at various pH.

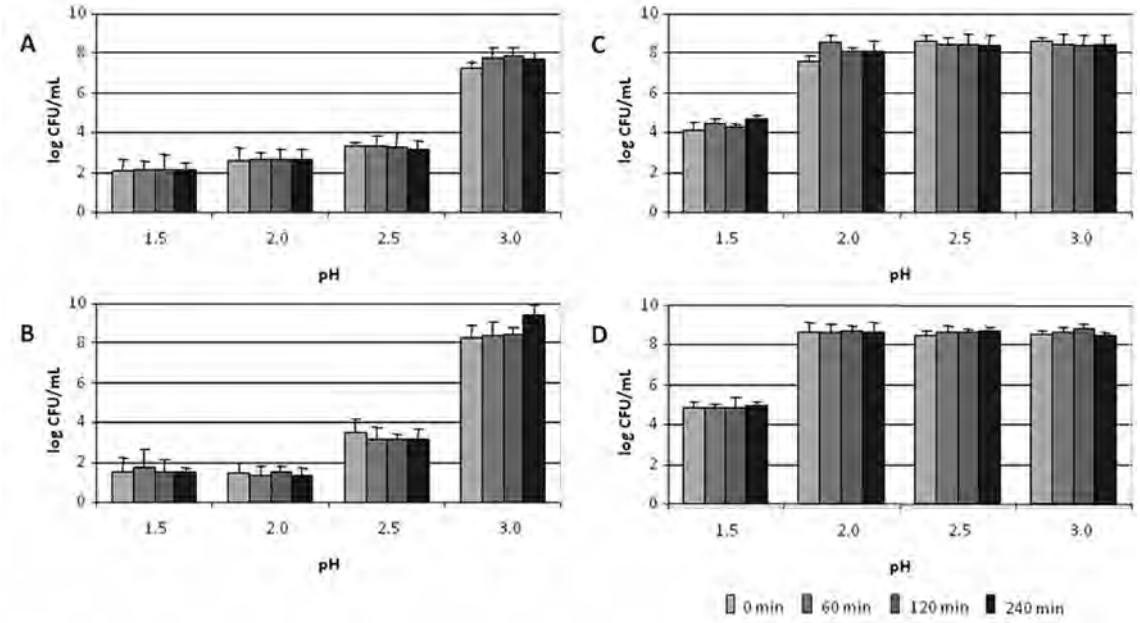

FIG. 2 - Survival of *Lactobacillus casei* Shirota and *Lactobacillus casei* LC01 in simulated enteric fluid, after passage through gastric juice at pH 1.5, 2.0, 2.5 and 3.0 for 120 min (A and C, respectively) and in simulated enteric fluid, after passage through gastric juice at pH 1.5, 2.0, 2.5 and 3.0, added of skim milk, for 120 min (B and D, respectively).

the growth of *L. lactis* subsp. *lactis* HV219 (Todorov *et al.*, 2007). Anti-inflammatory medicaments, moderate diuretic and neuroleptic containing potassium or sodium diclofenac, ibuprofen, triamterene hydrochlorothiaziden and thioridazine hydrochlorid acted as inhibitors of *L. plantarum*, *L. rhamnosus*, *L. paracasei* and *L. pentosus* strains isolated from boza and evaluated as a probiotic (Todorov *et al.*, 2008).

Dimenhydrinate inhibited the growth of *L. rhamnosus* ST462BZ and *L. plantarum* ST664BZ (Todorov *et al.*, 2008). It is, however, important to mention that the concentration of these substances in the GIT is critical for their action against the potential probiotic bacteria (Todorov *et al.*, 2007, 2008). Botes *et al.* (2008) reported that *L. casei* Shirota was inhibited by several commercial antibiotics (ciprofloxacin, amoxicillin, cefadroxil, roxithromycin, doxycycline and norfloxacin). Anti-inflammatory drugs containing meloxican (Coxflam), ibuprofen (Dolocyl, Adco-Ibuprofen), potassium diclofenac (Cataflam) and prednisolone (Preflam) also inhibited the growth, in a lesser extent. Pinmed, that contains paracetamol, codeine phosphate and promethazine HCl, misclassified as analgesic instead of antitussive agent, was also inhibitory to *L. casei* Shirota. Same authors also reported the inhibitory effect of Pynmed (Botes *et al.*, 2008), which is more likely due the presence of alcohol in the formulation than to the drug

TABLE 3 - Effect of medicaments on the growth of *Lactobacillus casei* Shirota and *Lactobacillus casei* LC01

Commercial name	Concentration (mg/ml)	Active substance	Medicament class	*L. casei* Shirota		*L. casei* LC01	
				Inhibition (mm)	MIC (mg/ml)	Inhibition (mm)	MIC (mg/ml)
AAS	20	Acetylsalicylic acid	Analgesic / Antipyretic	0		0	
Amoxil	100	Amoxicillin	β-Lactam antibiotic (Penicilin)	36 ± 2	< 0.4	40 ± 2	< 0.4
Antak	30	Ranitidine hydrochloride	Histamine H2-receptor antagonist that inhibits stomach acid production (Proton pump inhibitor)	0		0	
Arotin	4	Paroxetine	selective serotonin reuptake inhibitor (SSRI) antidepressant	10 ± 1	1.0	0	
Aspirina	100	Acetylsalicylic acid	Analgesic / Antipyretic	0		0	
Atlansil	40	Amiodarone	Antiarrhythmic	15 ± 1	1.25	0	
Cataflam	10	Diclofenac potassium	Non-steroidal anti-inflammatory drug (NSAID)	12 ± 1	5.0	17 ± 2	2.5
Celebra	40	Celecoxib	NSAID	0		0	
Clorana	5	Hydrochlorothiazide	Diuretic	0		0	
Coristina R		Acetylsalicylic acid, Pheniramine maleate, Phenylephrine hydrochloride, Cafein	Analgesic / Antipyretic / Antihistaminic / Decongestant	0		0	
Diclofenac potassico*	10	Diclofenac potassium	NSAID	11 ± 1	5.0	10 ± 1	2.5
Diclofenac potassico*	10	Diclofenac potassium	NSAID	12 ± 1	5.0	19 ± 2	2.5
Dorflex	10	Orphenadrine citrate, Metamizole sodium, Cafein	Analgesic	0		0	
Doxuran	0.8	Doxazosin	Antihypertensive / Treatment of prostatic hyperplasia	0		0	
Dramin	20	Dimenhydrinate	Antiemetic	0		0	
Fenergan	5	Promethazine hydrochloride	Antihistaminic	0		0	
Fluimucil	8	Acetylcysteine	Mucolitic agent	0		0	
Flutec	30	Fluconazole	Antifungal	0		0	
Higroton	10	Chlorthalidone	Thiazide diuretic	0		0	
Medley	4	Omeprazole	Proton pump inhibitor	0		0	
Neosaldina	60	Metamizole sodium, Isometheptene mucate, Cafein	Analgesic	0		0	
Nimesulida	20	Nimesulide	NSAID	0		0	
Nisulid	20	Nimesulide	NSAID	0		0	
Redulip	3	Sibutramine hydrochloride monohydrate	Anorexiant / Sympathomimetic	0		0	
Seki	3.54	Cloperastine	Antitussives (central and periferic mode of action)	0		0	
Spidufen	120	Ibuprofen arginine	NSAID	21 ± 2	40.0	20 ± 2	40.0
Superhist	80	Acetylsalicylic acid, Pheniramine maleate, Phenylephrine hydrochloride	Analgesic / Antipyretic / Antihistaminic / Decongestant	0		0	
Tylenol	150	Paracetamol	Analgesic / Antipyretic	0		0	
Tylex	6	Paracetamol, Codein	Analgesic	0		0	
Urotrobel	80	Norfloxacin	Antibiotic	18 ± 2	20.0	21 ± 2	10.0
Yasmin	0.6	Ethinylestradiol, Drospirenone	Contraceptive	0		0	
Zestril	4	Lisinopril	Antihypertensive (Angiotensin-converting enzyme (ACE) inhibitor)	0		0	
Zocor	2	Simvastatin	Hypolipidemic	0		10	1.0
Zyrtec	2	Cetirizine hydrochloride	Antihistaminic	0		0	

* Produced by two different companies.

itself. An important point is that in the study of Botes *et al*. (2008) the MIC of the active drugs were not determined, hampering the correct evaluation of their activity against *L. casei* Shirota in the human body, especially when used on a daily basis by patients with chronic diseases.

The correct evaluation of possible interactions between medicaments and probiotic bacteria depends on the determination of MIC of these medicaments. As shown in Table 3, the MIC for Spidufen, an anti-inflamatory and anti-rheumatic drug, was 40 mg/ml for both *L. casei* Shirota and *L. casei* LC01. Considering that the daily dose for this medicament is 600 mg (Zambon Laboratórios Farmacêuticos Ltda, www.zambon.com.br), the MIC value associated to the volume of the human GIT indicate that the recommended daily dose will hardly affect the survival of the probiotic bacteria. More important are the medicaments for treatment of chronic diseases, such as Zocor, an anti-lipemic drug used for the reduction of the body lipids, Atlansil, an anti-arrhytmic drug normally used in long course treatments and Arotin, a drug from the group of the anti-depressants with neuroleptic effect, also used in long-term treatments, which presented a MIC of 1.0, 1.25 and 1.0 mg/ml, respectively. Due to their long-term application, they can accumulate in the gastrointestinal tract and affect the viability of the probiotic cultures.

Among the tested medicaments, the anti-inflammatory drugs were the ones that affected *L. casei* Shirota and *L. casei* LC01 more significantly. These results agree with other studies, run with other probiotic LAB and gastrointestinal tract related bacteria (Todorov *et al.*, 2007, 2008; Botes *et al.*, 2008; Todorov and Dicks, 2008). Their inhibitory activity may be a consequence of the increased concentration of potassium ions in the gastric content as a result of the dissolution of potassium diclofenac in the stomach. The excess of potassium ions in the environment is incompatible with microbial cell viability. Other potassium-based medications may cause a similar negative effect. Individuals under permanent therapy with drugs should be aware that these drugs may reduce the beneficial effects of the probiotic bacteria.

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