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ORIGINAL PAPER

Evaluation of the probiotic potential and effect of encapsulation on survival for *Lactobacillus plantarum* ST16Pa isolated from papaya

Svetoslav D. Todorov · Jean Guy LeBlanc · Bernadette D. G. M. Franco

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Abstract Capability to produce antilisterial bacteriocins by lactic acid bacteria (LAB) can be explored by the food industry as a tool to increase the safety of foods. Furthermore, probiotic activity of bacteriogenic LAB brings extra advantages to these strains, as they can confer health benefits to the consumer. Beneficial effects depend on the ability of the probiotic strains to maintain viability in the food during shelf-life and to survive the natural defenses of the host and multiply in the gastrointestinal tract (GIT). This study evaluated the probiotic potential of a bacteriocinogenic *Lactobacillus plantarum* strain (*Lb. plantarum* ST16Pa) isolated from papaya fruit and studied the effect of encapsulation in alginate on survival in conditions simulating the human GIT. Good growth of *Lb. plantarum* ST16Pa was recorded in MRS broth with initial pH values between 5.0 and 9.0 and good capability to survive in pH 4.0, 11.0 and 13.0. *Lb. plantarum* ST16Pa grew well in the presence of oxbile at concentrations ranging from 0.2 to 3.0%. The level of auto-aggregation was 37%, and various degrees of co-aggregation were observed with different strains of *Lb. plantarum*, *Enterococcus* spp., *Lb. sakei* and *Listeria*, which are important features for probiotic activity. Growth was affected negatively by several medicaments used for human therapy, mainly anti-inflammatory drugs and antibiotics. Adhesion to Caco-2 cells was within the range reported for other probiotic strains, and PCR analysis indicated that the strain harbored the adhesion genes *mapA*, *mub* and *EF-Tu*. Encapsulation in 2, 3 and 4% alginate protected the cells from exposure to 1 or 2% oxbile added to MRS broth. Studies in a model simulating the transit through the GIT indicated that encapsulated cells were protected from the acidic conditions in the stomach but were less resistant when in conditions simulating the duodenum, jejunum, ileum and first section of the colon. To our knowledge, this is the first report on a bacteriocinogenic LAB isolated from papaya that presents application in food biopreservation and may be beneficial to the consumer health due to its potential probiotic characteristics.

Keywords *Lactobacillus plantarum* · Papaya · Probiotic · Encapsulation · Bacteriocin

S. D. Todorov (✉) · B. D. G. M. Franco
Departamento de Alimentos e Nutrição Experimental, Faculdade de Ciências Farmacêuticas, Laboratório de Microbiologia de Alimentos, Universidade de São Paulo, Av. Prof. Lineu Prestes 580 Bloco 14, São Paulo, SP 05508-000, Brazil
e-mail: slavi310570@abv.bg

J. G. LeBlanc
Centro de Referencia Para Lactobacilos (CERELA-CONICET), Chacabuco 145, C.P. T4000ILC San Miguel de Tucumán, Tucumán, Argentina

Introduction

Lactic acid bacteria (LAB) present many important properties in food manufacturing, such as production of lactic acid, improvement of organoleptic and physical characteristics and reduction of lactose content. In addition, many LAB are used in the food industry, mainly dairy industries, as starter cultures and also as probiotics (Todorov and Dicks 2006; Todorov 2009). Probiotics are defined as live microorganisms which when administered in adequate amounts (10^6–10^7 CFU per gram of food) confer health benefits to the host (Bertazzoni-Minelli et al. 2004; FAO/WHO 2001). Probiotic benefits include suppression of growth of pathogens, control of serum cholesterol level, modulation of the immune system, improvement of lactose digestion, synthesis of vitamins, increase in bio-availability

of minerals and possible anti-carcinogenic activity (Chan and Zhang 2005; Gomes and Xavier 1999; Kailasapathy and Chin 2000; Shah 2000). Beneficial effects depend on the ability of the probiotic strains to survive the natural defenses of the host and multiply in the gastrointestinal tract (GIT) (Brink et al. 2006; Gilliland 1989; Todorov et al. 2008). Besides these effects, production of antimicrobial compounds such as bacteriocins by probiotic LAB may contribute to colonization of the GIT, providing them competitive advantage over other bacteria (Garriga et al. 1993; Todorov 2009).

Most products currently on the market that contain probiotic bacteria have a very short shelf-life, even when stored at low temperature (0–4°C). Some do not have the required viable cell counts necessary to exert a probiotic effect and thus should not be marketed as probiotic foods (Kailasapathy and Chin 2000). After consumption, the numbers of viable cells lowers significantly due to the acidic environment and bile secretions in the GIT. One method to protect microorganisms from these harsh conditions is their encapsulation in hydrocolloid beads, prepared with alginate, alginate-chitosan or other polymeric compounds (Krasaekoopt et al. 2003). Several studies have shown that microencapsulation of probiotic strains in alginate improved their survival in foods and protected them during passage through the human GIT (Chandramouli et al. 2004; Jankowski et al. 1997; Kailasapathy 2002; Liserre et al. 2007; Mandal et al. 2006; Mollendorff 2008; Sheu and Marshall 1991, 1993; Sheu et al. 1993; Suita-Cruz and Goulet 2001).

This study evaluated the probiotic potential of a bacteriocinogenic *Lactobacillus plantarum* strain (*Lb. plantarum* ST16Pa) isolated from papaya fruit (Todorov et al. 2011) and studied the effect of encapsulation of this strain in alginate on survival in conditions simulating the human GIT. These features are important when selecting a starter culture for food biopreservation that also presents potential beneficial properties to the consumer health.

Materials and methods

Cultures

The study was conducted with *Lactobacillus plantarum* ST16Pa, a bacteriocinogenic strain isolated from papaya fruit (Todorov et al. 2011). Other microbial cultures used in the study were *Lb. rhamnosus* GG, *Lb. plantarum* ST202Ch, *Lb. plantarum* ST69BZ, *Enterococcus faecium* ST62BZ, *Enterococcus faecalis* ATCC 19443, *Lb. sakei* subsp. *sakei* 2a, *Listeria ivanovii* subsp. *ivanovii* ATCC 19119 and *Listeria innocua* 2030C. Pure cultures were stored at −20°C with 20% glycerol.

Evaluation of the probiotic potential

Effect of pH and oxbile on growth of *Lb. plantarum* ST16Pa

This experiment was performed according to Todorov et al. (2008). *Lb. plantarum* ST16Pa was grown in MRS broth (Difco, USA) adjusted to pH 3.0, 4.0, 5.0, 6.0, 7.0, 9.0, 11.0 and 13.0. Resistance to oxbile salts was tested by cultivating the strain in MRS broth containing 0.2, 0.4, 0.6, 0.8, 1.0, 2.0 and 3.0% (w/v) oxbile (Sigma–Aldrich). All tests were conducted in sterile flat-bottom 96-well micro titer plates (TPP, Switzerland). Each well was filled with 180 µl of tested MRS broth and 20 µl of the culture with OD_{650nm} adjusted to 0.2. Optical density at 600 nm were recorded every hour for 12 h. Cultures grown in MRS broth at pH 7.0 and without oxbile served as controls. Experiments were done in triplicate.

Adhesion of *Lb. plantarum* ST16Pa to Caco-2 cells

Caco-2 cells were grown in wells of flat-bottom 96-well microtiter plates (TPP, Switzerland) containing 100 µl of Eagle Minimal Essential Medium (MEM) (Merck) supplemented with 10% (v/v) fetal bovine serum (Sigma–Aldrich), 100 U/ml penicillin (Sigma–Aldrich) and 100 U/ml streptomycin (Sigma–Aldrich) for 24 h at 37°C in humidified atmosphere containing 5% CO_2 (Todorov et al. 2008). *Lb. plantarum* ST16Pa was grown in MRS for 24 h at 37°C. The wells containing the Caco-2 cells were inoculated with 100 µl of the bacterial suspension (ca. 1×10^6 CFU/ml) and after 2 h at 37°C the plates centrifuged at $300 \times g$ for 2 min at 4°C. The wells were washed twice with 1 ml sterile PBS and the Caco-2 cells lysed by adding 1 ml 0.5% (v/v) Triton X-100. Appropriate decimal dilutions were then plated onto MRS agar. The percentage of adhesion of *Lb. plantarum* ST16Pa to Caco-2 cells was calculated based on the initial number of viable cells, number of cells in the supernatant and number of cells in the lysate after treatment with Triton X-100. Experiments were done in triplicate. *Lb. rhamnosus* GG was used as control of adhesion to Caco-2 cells.

Confirmation of the presence of genes encoding MapA and Mub adhesion proteins and EF-Tu elongation factor in *Lb. plantarum* ST16Pa

DNA from *Lb. plantarum* ST16Pa was isolated according to Dellaglio et al. (1973). Primers Mub423F (5′-GTA GTT ACT CAG TGA CGA TCA ATG-3′), Mub423R (5′-TAA TTG TAA AGG TAT AAT CGG AGG-3′), Map423F (5′-TGG ATT CTG CTT GAG GTA AG -3′), Map423R (5′-GAC TAG TAA TAA CGC GAC CG-3′), EFTu423F

(5′-TTC TGG TCG TAT CGA TCG TG-3′) and EFTu423R (5′-CCA CGT AAT AAC GCA CCA AC-3′) were used for amplification of genes *MapA, Mub* and *EF-Tu* by PCR, as described by Todorov and Dicks (2008).

Determination of cell surface hydrophobicity in *Lb. plantarum* ST16Pa

Cell surface hydrophobicity was measured as described by Doyle and Rosenberg (1995), and modified by Todorov et al. (2008). In summary, *Lb. plantarum* ST16Pa was grown in MRS broth at 37°C for 18 h. Cells were harvested (6,700×*g*, 4°C, 6 min), washed twice with sterile saline solution (pH 6.5), re-suspended in the same solution and the optical density (OD_{580nm}) determined. The cell suspension (1.5 ml) was mixed with equal volume of n-hexadecane (Sigma–Aldrich) and vortexed for 2 min. After 30 min at room temperature for separation of the two phases, 1 ml of the aqueous phase was removed and the optical density (OD_{580nm}) determined. The experiment was repeated and the average optical density value determined. The percentage hydrophobicity was calculated as follows: % hydrophobicity = $[(OD_0-OD_{30})/OD_0] \times 100$, where OD_0 and OD_{30} refer to the initial OD and OD measured after 30 min, respectively. Experiments were conducted in triplicate.

Effect of commercial drugs and antibiotics on growth of *Lb. plantarum* ST16Pa

The experiments were performed as described by Carvalho et al. (2009). Commercial drugs listed in Table 1 were purchased from a local drugstore, and solubilized in sterile water to achieve the desired concentration. *Lb. plantarum* ST16Pa was inoculated into 10 ml MRS broth and incubated at 37°C for 18 h and mixed into MRS soft agar (1.0%, w/v) to achieve a population of 10^6 CFU/ml. After solidification of the agar, 10 µl of each medicament 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). For this test, serial two-fold dilutions of each medicament were prepared in sterile water and 10 µl spotted onto the surface of MRS soft agar plates, previously inoculated with *Lb. plantarum* ST16Pa (10^6 CFU/ml). 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.

In a similar experimental approach, the susceptibility of *Lb. plantarum* ST16Pa to antibiotic disks listed in Table 2 (CEFAR, Sao Paulo, SP, Brazil) was tested. The inhibitory effect of the antibiotics was expressed in millimeters of the inhibition zones (Carvalho et al. 2009).

Aggregation properties of *Lb. plantarum* ST16Pa

The experiment was performed according to Basson et al. (2008) with modifications (Todorov et al. 2008). *Lb. plantarum ST16Pa* was grown in MRS broth for 24 h at 37°C, harvested (7,000×*g*, 10 min, 20°C), washed, re-suspended in sterile saline solution (pH 6.5) and diluted to $OD_{660nm} = 0.3$. One ml of the cell suspension was transferred to a 2 ml sterile plastic cuvette and the OD_{660nm} recorded over 60 min by using a spectrophotometer. After 60 min the cell suspension was centrifuged (300×*g*, 2 min, 20°C) and the OD_{60} of the supernatant determined.

Auto-aggregation was determined using the following equation: % Auto-aggregation = $[(OD_0-OD_{60})/OD_0] \times 100$, where OD_0 and OD_{60} refer to the initial OD and OD measured after 60 min, respectively.

For evaluation of co-aggregation of *Lb. plantarum* ST16Pa with *Lb. plantarum* ST202Ch, *Lb. plantarum* ST69BZ, *E. faecium* ST62BZ, *E. faecalis* ATCC 19443, *Lb. sakei* subsp. *sakei* 2a, *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *L. innocua* 2030C, the lactobacilli were grown in MRS broth for 24 h at 37°C and the other cultures were prepared in BHI for 24 h at 37°C. The experimental protocol for study of co-aggregation was the same used for auto-aggregation, except that one milliliter of *Lb. plantarum* ST16Pa and one milliliter of the co-aggregation partner were coupled.

Effect of encapsulation on survival of *Lb. plantarum* ST16Pa in conditions simulating the gastrointestinal tract

Encapsulation of *Lb. plantarum* ST16Pa

The encapsulation method of Sheu et al. (1993) was used. *Lb. plantarum* ST16Pa was grown in 10 ml MRS broth to an optical density (OD_{600nm}) of 3.0 (approximately 1×10^9 CFU/ml) and harvested (2,000×*g*, 10 min, 4°C). The cells were washed twice with 10 ml sterile peptone water (0.1%, w/v) and resuspended in 5 ml of the same solution. For encapsulation, the *Lb. plantarum* ST16Pa cell suspension was mixed with 25 ml of sterile 2, 3 and 4% sodium alginate solutions prepared in distilled water. After vortexed to homogeneity, the mixtures were transferred to sterile syringes fitted with a 0.45 mm diameter needle and slowly ejected and allowed to drop into sterile 100 ml 0.05 M $CaCl_2$ supplemented with 0.1% (v/v) Tween 80. After 30 min for stabilization, the beads were harvested

Table 1 Effect of commercial drugs on the growth of *Lactobacillus plantarum* ST16Pa

Brazilian commercial name	Concentration (mg/ml)	Active substance	Medicament class	Inhibition zone (mm)[a] [MIC (mg/ml)]
AAS	20	Acetylsalicylic acid	Analgesic/antipyretic	
Amoxil	**100**	**Amoxicillin**	**β-Lactam antibiotic (penicilin)**	**35 (<0.2)**
Antak	30	Ranitidine hydrochloride	Histamine H2-receptor antagonist that inhibits stomach acid production (proton pump inhibitor)	
Arotin	**4**	**Paroxetine**	**Selective serotonin reuptake inhibitor (SSRI) antidepressant**	**17 (1.0)**
Aspirina	**100**	**Acetylsalicylic acid**	**Analgesic/antipyretic**	
Atlansil	**40**	**Amiodarone**	**Antiarrhythmic**	**12 (1.25)**
Cataflam	**10**	**Diclofenac potassium**	**Non-steroidal anti-inflammatory drug (NSAID)**	**11 (5.0)**
Celebra	40	Celecoxib	NSAID	
Clorana	5	Hydrochlorothiazide	Diuretic	
Coristina R		Acetylsalicylic acid, pheniramine maleate, phenylephrine hydrochloride, cafein	Analgesic/antipyretic/antihistaminic/decongestant	
Diclofenac potassico[b]	**10**	**Diclofenac potassium**	**NSAID**	**15 (5.0)**
Diclofenaco potassico[b]	**10**	**Diclofenac potassium**	**NSAID**	**12 (5.0)**
Dorflex	**10**	**Orphenadrine citrate, metamizole sodium, cafein**	**Analgesic**	**8 (5.0)**
Doxuran	0.8	Doxazosin	Antihypertensive/treatment of prostatic hyperplasia	
Dramin	20	Dimenhydrinate	Antiemetic	
Fenergan	**5**	**Promethazine hydrochloride**	**Antihistaminic**	**10 (5.0)**
Fluimucil	8	Acetylcysteine	Mucolitic agent	
Flutec	30	Fluconazole	Antifungal	
Higroton	10	Chlorthalidone	Thiazide diuretic	
Omeprazole	4	Omeprazole	Proton pump inhibitor	
Neosaldina	60	Metamizole sodium, isometheptene mucate, cafein	Analgesic	
Nimesulida	20	Nimesulide	NSAID	
Nisulid	20	Nimesulide	NSAID	
Redulip	3	Sibutramine hydrochloride monohydrate	Anorexiant/sympathomimetic	
Seki	3.54	Cloperastine	Antitussives (central and periferic mode of action)	
Spidufen	**120**	**Ibuprofen arginine**	**NSAID**	**25 (15.0)**
Superhist	80	Acetylsalicylic acid, pheniramine maleate, phenylephrine hydrochloride	Analgesic/antipyretic/antihistaminic/decongestant	
Tylenol	150	Paracetamol	Analgesic/antipyretic	
Tylex	6	Paracetamol, Codein	Analgesic	
Urotrobel	**80**	**Norfloxacin**	**Antibiotic**	**8 (5.0)**
Yasmin	0.6	Ethinylestradiol, drospirenone	Contraceptive	
Zestril	4	Lisinopril	Antihypertensive (Angiotensin-converting enzyme (ACE) inhibitor)	
Zocor	2	Simvastatin	Hypolipidemic	
Zyrtec	2	Cetirizine hydrochloride	Antihistaminic	

[a] Average diameter of inhibition zones of the test microorganism

[b] Produced by two different companies

In bold drugs with an inhibitory effect on *L. plantarum*

Table 2 Effect of antibiotics on the growth of *Lactobacillus plantarum* ST16Pa, presented as diameter of inhibition zones in millimeters

Antibiotic (μg/disk)	Classification	Inhibition zone (mm)[a]
Amicacin 30	Aminoglycoside (inhibits protein synthesis)	**0**
Ampicilin 10	Penicillins/β-lactam (interferes with bacteria cell wall synthesis)	30
Bacitracin 10	Cyclic polypeptide (inhibits bacteria cell wall synthesis)	19
Cefazolin 30	1st generation cephalosporin/β-lactam (interferes with bacteria cell wall synthesis)	25
Cefepime 30	4th generation cephalosporin/β-lactam (interferes with bacteria cell wall synthesis)	25
Cefotaxim 30	2nd generation cephalosporin/β-lactam (interferes with bacteria cell wall synthesis)	27
Ceftazidim 30	3th generation cephalosporin/β-lactam (interferes with bacteria cell wall synthesis)	20
Ceftriaxon 30	3th generation cephalosporin/β-lactam (interferes with bacteria cell wall synthesis)	22
Cefuroxim 30	β-Lactam (interferes with bacteria cell wall synthesis)	20
Ciprofloxacin 5	Fluoroquinolones (inhibits the bacterial topoisomerase II)	8
Clindamicin 2	Licosamides (inhibits protein synthesis)	37
Chloramphenicol 30	Prevents peptide bond formation—inhibits protein synthesis)	30
Erytromicin 15	Macrolide (inhibits protein synthesis)	26
Furazolidon 10	Antibiotic/antiparasitic	18
Gentamicin 10	Aminoglycoside (inhibits protein synthesis)	14
Imipenem 10	Carbapenem/β-lactam (interferes with bacteria cell wall synthesis)	40
Kanamicin 30	Aminoglycoside (inhibits protein synthesis)	**0**
Metronidazol 50	Nitroimidazole antibiotic (acts on DNA of microorganisms ameba, and protozoa)	**0**
Nalidixic acid 30	Synthetic quinolone antibiotic (acts on DNA gyrase)	**0**
Neomicin 30	Aminoglycosides (inhibit protein synthesis)	14
Nitrofurantoin 300	Nitrofuran derivative (nucleic acid inhibitor)	25
Ofloxacin 5	Licosamide (inhibits protein synthesis)	17
Oxacilin 1	β-Lactam (interferes with bacteria cell wall synthesis)	**0**
Penicillin G 10	β-Lactam (interferes with bacteria cell wall synthesis)	15
Rifampicin 30	Semisynthetic compound derived from *Amycolatopsis rifamycinica*	22
Rifampicin 5	Semisynthetic compound derived from *Amycolatopsis rifamycinica*	19
Streptomicin 10	Aminoglycoside (inhibits protein synthesis)	18
Tetraciclin 30	(inhibits protein synthesis)	25
Tobramicin 10	Aminoglycoside (inhibits protein synthesis)	**0**
Trimetoprim 5	(Inhibits folate synthesis)	25
Vancomicin 30	Glycopeptide (inhibits bacteria cell wall synthesis)	**0**

[a] Average diameter of inhibition zones of the test microorganism

In bold antibiotics not affecting the growth of *L. plantarum* ST16Pa

(350×*g*, 10 min, 4°C), washed three times with sterile peptone water and collected by filtration through sterile Grade 1 Whatman filter paper. The average diameter of the alginate beads was 1 mm. The beads were stored in sterile peptone water at 4°C for a maximum of 2 days.

Effect of pH and oxbile on survival of encapsulated *Lb. plantarum* ST16Pa

One gram of alginate beads containing entrapped cells of *Lb. plantarum* ST16Pa was transferred to 10 ml of 0.08 M HCl supplemented with 0.2% (w/v) NaCl (pH 1.6), gently mixed and incubated at 37°C in an orbital shaker (60 rpm). After 1 and 3 h of incubation, the beads were harvested by filtration through sterile Grade 1 Whatman filter paper and mixed with 10 ml phosphate buffer (0.1 M KH_2PO_4, pH 7.4) for depolymerization and release of cells. The cell suspension was submitted to ten-fold dilutions in sterile distilled water and plated onto MRS agar and incubated at 37°C for 48 h. The experiment was repeated by transferring one gram of alginate beads to 10 ml of 0.1 M KH_2PO_4 (pH 7.4). Samples were taken after 30 min, 1, 2 and 3 h and viable counts determined as described before. In both experiments, viable cell numbers were also determined immediately after encapsulation. The same experiments were carried out with non-encapsulated *Lb. plantarum* ST16Pa. The experiments were performed in triplicate.

The effect of oxbile on survival of encapsulated *Lb. plantarum* ST16Pa was measured adding one gram of the beads to 10 ml 1 and 2% (w/v) oxbile (Oxoid). After 0, 3, 6

and 12 h of incubation at 37°C, the beads were depolymerized and the number of viable cells determined by plating onto MRS agar, as described before. The experiments were also carried out with non-encapsulated *Lb. plantarum* ST16Pa, adding free cells to the oxbile solutions. The experiments were performed in triplicate.

Release of encapsulated *Lb. plantarum* ST16Pa at conditions simulating different sections of the gastrointestinal tract

Ten grams of the alginate beads containing *Lb. plantarum* ST16Pa were transferred to 250 ml of a sterile solution simulating the stomach condition (12.5 g MRS, 0.78 g NaCl, 0.28 g KCl, 0.03 g $CaCl_2$ and 0.15 g $NaHCO_3$ per liter, pH adjusted to 4.0 with 1 M HCl), prepared according to Mollendorff (2008). After 2.5 h at 37°C, the pH of the solution was adjusted to 3.0 with sterile 1 M HCl and the number of viable *Lb. plantarum* ST16Pa determined as described before. The beads were then removed from this solution, washed twice with sterile 0.1% (m/v) peptone water and transferred to a mixture prepared with 250 ml of the stomach simulating solution and 125 ml of sterile pancreatic solution [1.5 g $NaHCO_3$, 0.11 g Pancreatin (Sigma–Aldrich), 0.08 g Oxgall (Oxoid)], prepared according to Mollendorff (2008). The pH of the mixture was adjusted to 6.5 with sterile 1 N NaOH. After 4 h at this pH, simulating the conditions in the duodenum and ileum, the pH was reduced to 6.0 with sterile 1 N HCl and kept at this value for 2.5 h to simulate conditions in the jejunum and first section of the colon. All incubations were performed at 37°C. Samples (1 ml) were withdrawn after 2 h (simulating the duodenum), 4 h (simulating the jejunum), 6 h (simulating the ileum) and 6.5 h (simulating the first section of the colon), and submitted to counts of *Lb. plantarum* ST16Pa. The percentage of cell release from the beads and the percentage of free cells survival were calculated as described before. The experiment was repeated with non-encapsulated cells of *Lb. plantarum* ST16Pa to serve as control. All experiments were performed in triplicates.

Results and discussion

A previous study indicated that *Lb. plantarum* ST16Pa produces a large spectrum bacteriocin capable of inhibiting many food spoilage bacteria and foodborne pathogens, including *Listeria monocytogenes,* a psychrotrophic foodborne pathogen of increasing importance (Todorov et al. 2011). Further research evaluating potential probiotic characteristics of this strain is important as this additional beneficial property would lead to a broader application of the strain in foods, both for improvement of quality and safety of food products and for conferring beneficial health effects to the consumer.

Effect of pH and oxbile on growth of *Lb. plantarum* ST16Pa

Capability to grow and survive at low pH and high concentrations of oxbile salts, as those encountered in the human GIT, is a major characteristic of a strain with potential probiotic activity (Vinderola and Reinheimer 2003). Results reported in this study indicate that *Lb. plantarum* ST16Pa grew well in MRS broth with initial pH values of 5.0, 6.0, 7.0 and 9.0 (Fig. 1A), however, at pH 4.0 the growth rate was much lower. Previous studies indicated that pH affects the growth of bacteriocinogenic *Lactobacillus* spp. in different degrees. Growth of several strains of *Lb. plantarum*, *Lb. rhamnosus*, *Lb. pentosus* and *Lb. paracasei* was suppressed at pH 3.0 and 4.0, and variable results were recorded for pH of 11.0 and 13.0, but poor growth recorded for strains *Lb. paracasei* ST242BZ and ST284BZ, *Lb. rhamnosus* ST462BZ, *Lb. plantarum* ST664BZ and *Lb. pentosus* ST712BZ at pH 13.0 (Todorov et al. 2008).

Lactobacillus plantarum ST16Pa grew well in the presence of oxbile at concentrations ranging from 0.2 to 3.0% (Fig. 1B), suggesting that this strain would be able to

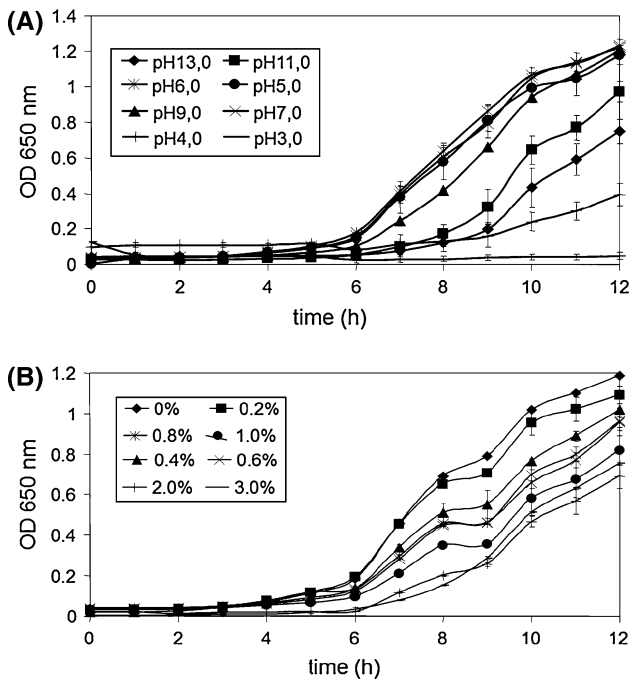

Fig. 1 Growth of *Lactobacillus plantarum* ST16Pa in MRS broth at pH 3.0–13.0 (**a**) and MRS broth supplemented with 0–3.0% ox-bile (**b**). Each *result* represents an average of three independent experiments

survive in the human GIT. Bile salts have different effects in different *Lactobacillus* strains. Growth of strains *Lb. plantarum* ST194BZ, ST414BZ and ST664BZ, *Lb. rhamnosus* ST462BZ, and *Lb. pentosus* ST712BZ were less affected by the presence of 0.3% bile, compared to strains *Lb. paracasei* ST242BZ and ST284BZ and *Lb. rhamnosus* ST461BZ (Todorov et al. 2008). Bile at 0.6% (w/v) suppressed the growth of *Lb. plantarum* ST194BZ and ST441BZ, *Lb. paracasei* ST242BZ and ST284BZ and *Lb. rhamnosus* ST462BZ, but not *Lb. rhamosus* ST461BZ, *Lb. plantarum* ST664BZ and *Lb. pentosus* ST712BZ. Bile levels above 0.6% (w/v) suppressed the growth of all tested strains (*Lb. plantarum* ST194BZ, ST441BZ and ST664BZ, *Lb. paracasei* ST242BZ and ST284BZ, *Lb. rhamnosus* ST461BZ and ST462BZ and *Lb. pentosus* ST712BZ) (Todorov et al. 2008).

Similar effects of oxbile and pH on *Lb. plantarum* 423, *Lb. salivarius* 241 and *Lb. curvatus* DF38 were reported by Brink et al. (2006). In another study, Haller et al. (2001), reported that as many as 10% of *Lb. plantarum* cells, but less than 0.001% of *Lb. sakei* and *Lb. paracasei* cells, survived when exposed to HCl (pH 2.0) and bile salts.

Adhesion of *Lb. plantarum* ST16Pa to Caco-2 cells

Adhesion of probiotic strains to intestinal cells such as Caco-2 cells is believed to be a critical factor to increase the possibility of colonizing the GIT and survive in this hostile environment (Tuomola and Salminen 1998). *Lb. plantarum* ST16Pa adhered to Caco-2 cells at a rate similar to that recorded for the well-known probiotic reference strain *Lb. rhamnosus* GG (9.5 and 11.3%, respectively) (data not shown). These values are similar to those reported by Todorov et al. (2008) for several bacteriocins producing LAB isolated from boza (0.26–9.0%) and by Tuomola and Salminen (1998) for some probiotic and dairy *Lactobacillus* strains (3.2–14.4%). Bertazzoni-Minelli et al. (2004) reported much lower adhesion rates for probiotic *Lactobacillus casei* strains (0.08–0.74%).

Identification of genes encoding MapA and Mub adhesion proteins and EF-Tu elongation factor in *Lb. plantarum* ST16Pa

The expression of mucus adhesion proteins, such as those encoded by the *mub* and *mapA* genes, and of GTP-binding EF-Tu protein has been shown to be critical in adhesion of probiotic strains to human intestine cells (Ramiah et al. 2008). Results of PCR amplification using specific primers and sequence analysis of the PCR amplicons indicated that *Lb. plantarum* ST16Pa contains the *mub, mapA* and *EF-Tu* genes (Fig. 2). Most probably presence of these genes is species dependent and not related with the ecological

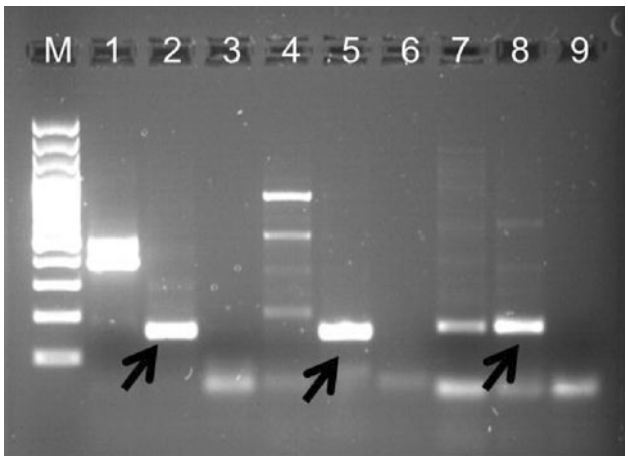

Fig. 2 Agarose gels showing amplified fragments for genes *MapA* (*lanes 1*, *2* and *3*), *Mub* (*lanes 4*, *5* and *6*) and *EF-Tu* (*lanes 7*, *8* and *9*) amplified by PCR. *Lanes 2*, *5* and *8* correspond to *Lactobacillus plantarum* ST16Pa and *lanes 1*, *4* and *7* to *Enterococcus faecium* ST88Ch. *Lanes 3*, *6* and *9* contain no DNA and *lane M* corresponds to 100 bp molecular weight marker

origin of the strain/s. However, same genes were recorded in other *Lb. plantarum* strains isolated from plants (Ramiah et al., 2008). The presence of these adhesion genes in this *Lb. plantarum* strain is not surprising due to the high adhesive properties of this microbial species. Whole genome sequence data of *Lb. plantarum* WCFS1, a human isolate, revealed the presence 223 extracellular proteins, most of them involved in the adhesion of the cell to its environment (Kleerebezem et al. 2003). The domain composition of *Lb. plantarum* WCFS1 proteins predicted to be associated with adhesion indicated the presence of seven proteins to be involved with mucus. Some of these possible mucus binding proteins contain the Mub domain, a trait unique to LAB (Boekhorst et al. 2006). The presence of these adhesive proteins in lactic acid bacteria is imperative for probiotic strains to colonize the GIT.

Determination of cell surface hydrophobicity in *Lb. plantarum* ST16Pa

Bacterial cells with a high hydrophobicity usually form strong interactions with mucosal cells. The interactions between microbial and host cells are non-specific, but in probiotic strains, there is a good correlation between surface hydrophobicity and the ability to adhere to the intestinal mucosa (Wadström et al. 1987). The initial interaction may be weak, is often reversible and precedes subsequent adhesion processes mediated by more specific mechanisms involving cell-surface proteins and lipoteichoic acids (Granato et al. 1999; Rojas et al. 2002; Roos and Jonsson 2002). Hydrophobicity varies among even genetically closely related species and even among strains of the same

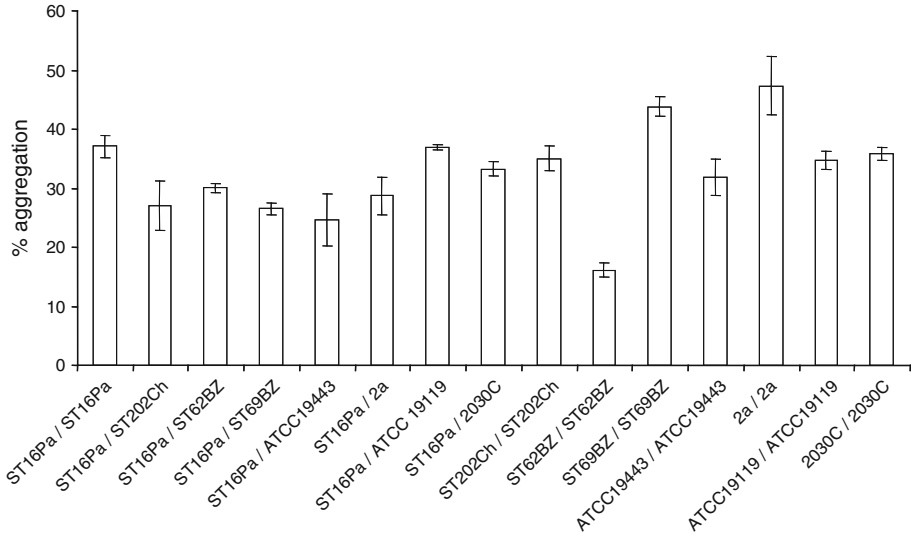

Fig. 3 Auto-aggregation and co-aggregation of *Lactobacillus plantarum* ST16Pa with *Lactobacillus plantarum* ST202Ch, *Lactobacillus plantarum* ST69BZ, *Enterococcus faecium* ST62BZ, *Enterococcus faecalis* ATCC 19443, *Lactobacillus sakei* subsp. *sakei* 2a, *Listeria ivanovii* subsp. *ivanovii* ATCC 19119 and *Listeria innocua* 2030c. Each *result* represents an average of three experiments

species (Schar-Zammaretti and Ubbink 2003). Although hydrophobicity may assist in adhesion, it is not a prerequisite for strong adhesion to human intestinal cells.

The hydrophobicity recorded for *Lb. plantarum* ST16Pa was higher than that recorded for the reference probiotic *Lb. rhamnosus* GG strain (68.7 and 53.3%, respectively). Similar results were observed by Todorov et al. (2008), for strains *Lb. rhamnosus* ST461BZ, *Lb. rhamnosus* ST462BZ and *Lb. plantarum* ST664BZ (75–80%) and *Lb. rhamnosus* GG (55%).

Effect of commercial drugs and antibiotics on growth of *Lb. plantarum* ST16Pa

Probiotic consumers may be under treatment for a variety of illnesses, and the beneficial effects of the probiotic strain may be hampered by possible interactions with the medicaments used by these consumers. As shown in Table 1, there were several drugs that reduced the growth of *Lb. plantarum* ST16Pa, especially non-steroidal anti-inflammatory drugs that contain potassium diclofenac or ibuprofen arginine, promethazine hydrochloride (antihistaminic), orphenadrine citrate and sodium metamizole (analgesic), paroxetine (antidepressant) and amiodarone (antiarrhythmic). The negative effect of sodium diclofenac on growth of potential probiotic strains was also observed in other studies (Botes et al. 2008; Carvalho et al. 2009; Todorov et al. 2007, 2008; Todorov and Dicks 2008). Dimenhydrinate inhibited the growth of *Lb. rhamnosus* ST462BZ and *Lb. plantarum* ST664BZ (Todorov et al. 2008).

The majority of the antibiotics tested in this study inhibited the growth of *Lb. plantarum* ST16Pa (Table 2). The strain was resistant to amicacin, tobramicin, vancomicin, oxacilin, kanamicin, metronidazol and nalidixic acid. Resistance of potential probiotic LAB to antibiotics is a controversial subject, as these strains may be reservoirs of antibiotic resistance genes, and can be transferred horizontally to other in the human GIT (Dicks et al. 2009). Resistance may be inherent to a bacterial genus or species, but may also be acquired through exchange of genetic material, mutations and the incorporation of new genes (Ammor et al. 2007; Levy and Marshall 2004; Salyers et al. 2004; Teuber 1999).

It should be emphasized that the interaction between medicaments or antibiotics and probiotic bacteria in the GIT depends on their concentration in this environment (Todorov et al. 2007, 2008), so that the Minimal Inhibitory Concentration (MIC) values play an important for the proper evaluation of these interactions. As an example, spidufen reduced the growth of *Lb. plantarum* ST16Pa in MRS, but the MIC for this drug was as high as 15.0 mg/ml (Table 1), indicating that the recommended daily dose (600 mg) will hardly affect the survival of *Lb. plantarum* ST16Pa in the GIT. More important are the drugs for treatment of chronic diseases, such as 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 prolonged treatments, which presented MIC of 1.25 and 1.0 mg/ml, respectively. Due to their long-term application, they may accumulate in the gastrointestinal tract and affect the viability of *Lb. plantarum* ST16Pa.

Aggregation properties of *Lb. plantarum* ST16Pa

Aggregation is an important feature for biofilm formation. *Lb. plantarum* has a number of genes encoding for surface proteins responsible for recognition of or binding to components present in the environment. Several of these genes are homologous to proteins with predicted functions, such

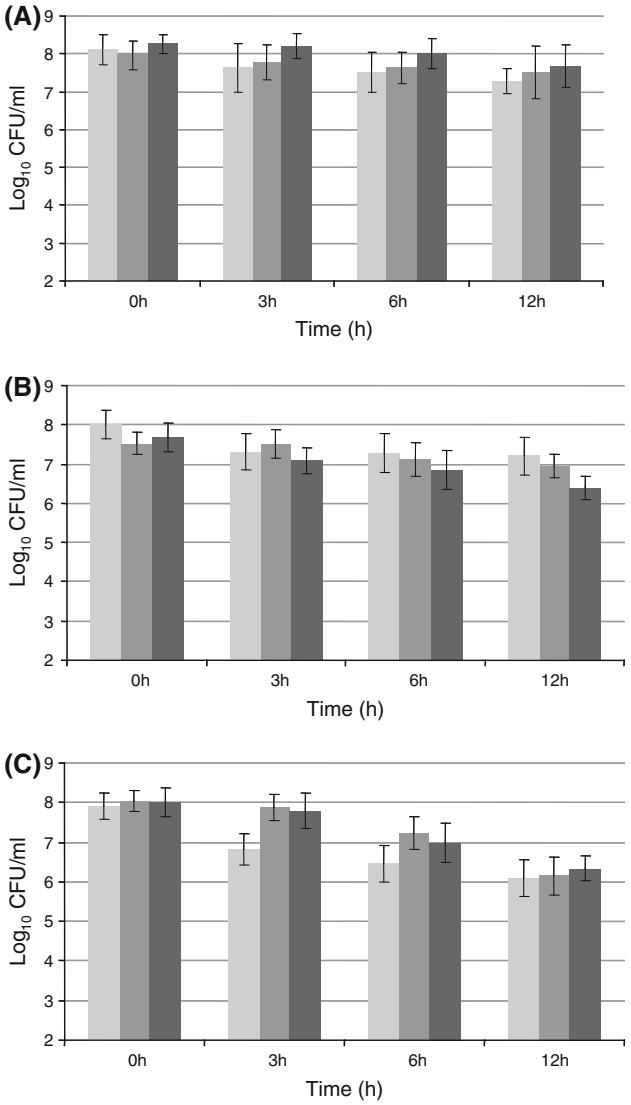

Fig. 4 **a** Survival of encapsulated *L. plantarum* ST16Pa in MRS with no added oxbile (encapsulation in 2% alginate—*light grey bars*, in 3% alginate—*dark grey bars* and in 4% alginate—*black bars*). **b** Survival of encapsulated *L. plantarum* ST16Pa in MRS containing 1% oxbile (encapsulation in 2% alginate—*light grey bars*, in 3% alginate—*dark grey bars* and in 4% alginate—*black bars*). **c** Survival of encapsulated *L. plantarum* ST16Pa in MRS containing 2% oxbile (encapsulation in 2% alginate—*light grey bars*, in 3% alginate—*dark grey bars* and in 4% alginate—*black bars*)

as mucus-binding, aggregation-promotion and intracellular adhesion (Kleerebezem et al. 2003).

Aggregation properties need to be discussed taking in account the bacteriocin spectrum of activity. If the bacteriocin produced by *Lb. plantarum* ST16Pa does not inhibit the co-aggregation partner, the two strains can co-exist and facilitate biofilm formation. In opposite, if the bacteriocin is active against the co-aggregation partner, high co-aggregation will facilitate the bactericidal mode of action and easier elimination of the co-aggregation partner from the system.

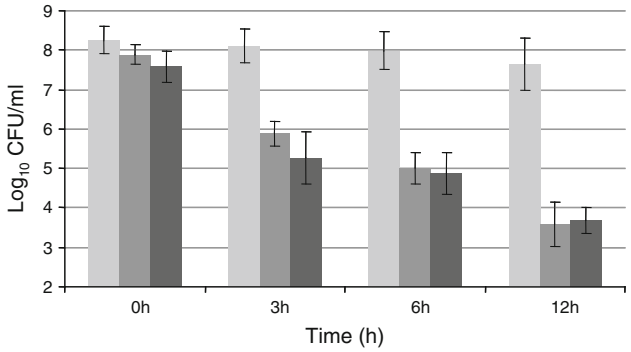

Fig. 5 Effect of oxbile on survival of free cells of *L. plantarum* ST16Pa (0% oxbile—*light grey bars*, 1% oxbile—*dark grey bars* and 2% oxbile—*black bars*)

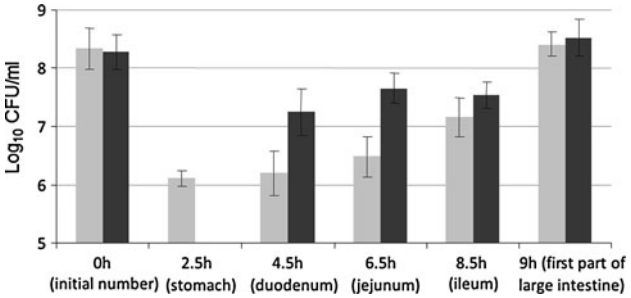

Fig. 6 Release and survival of encapsulated (*black bars*) and non-encapsulated (*grey bars*) *L. plantarum* ST16Pa in a model simulating the gastrointestinal tract (encapsulation in 2% alginate). *Time zero* corresponds to the encapsulated *L. plantarum* ST16Pa

Auto-aggregation is a strain-specific trait (Todorov and Dicks 2008). *Lb. plantarum* ST16Pa was determined to have an auto-aggregation level of 37.05% (Fig. 3), which is lower than the levels reported by Todorov et al. (2008) for other lactobacilli: *Lb. pentosus* ST712BZ (67%) and *Lb. paracasei* ST284BZ (99%). The co-aggregation of *Lb. plantarum* ST16Pa with *Lb. plantarum* ST202Ch, *Lb. plantarum* ST69BZ, *E. faecium* ST62BZ, *E. faecalis* ATCC 19443, *Lb. sakei* subsp. *sakei* 2a, *L. ivanovii* subsp. *ivanovii* ATCC 19119 and *L. innocua* 2030C also varied according to the strain (Fig. 3), but the lowest levels were observed for *Listeria* and *Enterococcus* strains. Low levels of co-aggregation may play an important role in preventing the formation of biofilms, and in this way preventing the persistence of pathogenic species in the GIT.

Effect of pH and oxbile on survival of encapsulated *Lb. plantarum* ST16Pa

Encapsulation of *Lb. plantarum* ST16Pa in alginate protected the cells from the acidic pH, regardless the concentration of alginate used in the encapsulation process.

The counts of *Lb. plantarum* ST16Pa after exposure to simulated gastric conditions (pH 1.6) for 3 h were 1.2 log lower than the initial count, while for non-encapsulated cell a five-log reduction was observed (data not shown).

The effects of oxbile on the survival of encapsulated and free *Lb. plantarum* ST16Pa are shown in Figs. 4a–c and 5, respectively. In the absence of oxbile, survival of encapsulated (Fig. 4a) and non-encapsulated *Lb. plantarum* ST16Pa (Fig. 5) in MRS up to 12 h was similar, regardless the percentage of alginate used for encapsulation. However, exposure to 1 and 2% oxbile for 12 h caused less than two log reduction in the counts of *Lb. plantarum* ST16Pa when encapsulated in 2, 3 or 4% alginate (Fig. 4b, c), while the number of non-encapsulated cells decreased five logs (Fig. 5). There was not a direct correlation between the concentration of alginate in the encapsulation process and the number of viable cells after exposure to 1 and 2% oxbile. Similar results were reported by Mollendorff (2008) in encapsulation of two strains of *Lb. plantarum*. However, Mandal et al. (2006) observed an increase in viability of *Lb. casei* NCDC-298 with the increase in alginate concentration, similarly to Lee and Heo (2000), who reported a decrease in the death rate of cells of *Bifidobacterium longum* with the increase in alginate concentration. Strain specific interactions between bacterial species and alginate or variations in the encapsulation methodology could explain these differing results.

Release of encapsulated *Lb. plantarum* ST16Pa at conditions simulating different sections of the gastrointestinal tract

Counts of free and encapsulated *Lb. plantarum* ST16Pa after exposure to conditions simulating the transit through the GIT are shown in Fig. 6. After 2.5 h in the solution simulating the stomach (pH 3.0), no counts of *Lb. plantarum* ST16Pa were obtained when they were encapsulated in alginate, indicating that the cells remained inside the capsules and not affected by the low pH. In counterpart, the counts of free cells after the same treatment were 2 log lower than the initial counts. When transferred from the solution simulating the stomach due to low pH to the new stomach solution added of pancreatic components (digestive enzymes and bile salts), the protection given by the encapsulation weakened along exposure to this new environment as the number of released cells increased gradually with time. However, the number of viable cells obtained for non-encapsulated *Lb. plantarum* ST16Pa was always lower than that recorded for their encapsulated counterparts. This result indicates that protection given by encapsulation is more evident in conditions simulating the stomach than in conditions simulating the duodenum, jejunum, ileum and the first part of the large intestine. Similar results were reported for whey protein-based microencapsulated cells of bifidobacteria when tested at simulated intestinal conditions (Picot and Lacroix 2004). In contrast, Trindade and Grosso (2000) reported a decrease in viability of immobilized *Bifidobacterium bifidum* and *Lb. acidophilus* after exposure to 2% (w/v) and 4% (w/v) bile salts.

Based on the results reported in this study, *Lb. plantarum* ST16Pa presents high potential of application by the food industry as probiotic since it not only produces a bacteriocin, but also possesses many characteristics responsible for survival and possible reproduction in the harsh conditions of the gastrointestinal tract. Studies run with *Lb. plantarum* ST16Pa encapsulated in alginate indicate that encapsulation can be used to provide extra protection to the cells. Further studies on survival of the encapsulated bacteria in foods and on the delivery of viable cells to the consumer are necessary to elucidate the complimentary probiotic activity of this strain. To our knowledge, this is the first report on a bacteriocinogenic LAB isolated from fruit that presents application in food biopreservation and may be beneficial to the consumer health due to its potential probiotic characteristics.

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