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ORIGINAL ARTICLE

Effect of gastric and small intestinal digestion on lactic acid bacteria activity in a GIS1 simulator

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Abstract The selection of probiotic strains resistant to gastrointestinal transit is an important stage when developing supplements that contain viable biomass. A total of six strains belonging to different genotypes were tested and compared with both a positive and negative control (*Lactobacillus plantarum* 5s). Significant differences were found between strains as a result of gastrointestinal transit using the *in vitro* GIS1 static simulator. The *Lactobacillus rhamnosus* 428ST strain showed maximum viability as a result of *in vitro* transit, featuring a survival capacity value, Cs, of over 50 ± 0.01%. The remaining genotypes that were tested showed significant reductions in the enzymes and bile salts at the time of action. The value of the survivability capacity was directly correlated with the synthesis of exopolysaccharides and lactic acid. The test results of the GIS1 system have been compared with those of other studies on gastrointestinal transit resistance that used dynamic models.

1. Introduction

The digestion process is essential for maintaining one's health status, as components of ingested food become assimilated. This process begins in the mouth, continues in the stomach and the small intestine, and is finalized in the colon, both by the absorbed substances and by the action of the microbiota that are developed at this level (Guerra et al., 2012). Thus, according to the degree of resistance to this transit process, and to the effects of the interaction between the microbiota that already exist in the colon of each person, positive health-related effects can be achieved. These effects are manifested by a high tolerance to lactose, the correction of episodes of diarrhoea, a reduction in the frequency of bloating, or an inhibition of pathogenic bacterial strains (Hertzler and Clancy, 2003).

The use of functional supplements that are based on the biomass of some probiotic strains is widespread today, even though the *in vivo* effect of these agents is poorly known. Their administration is usually performed according to the results of an *in vitro* test, which is based on each agent's degree

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of resistance in various conditions given that it partly stimulates the gastrointestinal environment (either by affecting the pH level or bile salt concentration) (Pitino et al., 2010). Currently, one particular method that is employed to test these strains is the use of *in vitro* simulators; this accurately simulates the physiological conditions associated with gastrointestinal transit. These models, dynamic or static, precisely reproduce the time of retention, pH level, bile salt concentration, and quantity of the digestive enzymes. The systems can be controlled in real time and show the exact changes that appear following the specific action of each tested strain (Hur et al., 2011; Vamanu et al., 2012).

This study determined the viability of six strains belonging to types *Weissella* and *Lactobacillus*, which were used to formulate a probiotic product; we subsequently compared these strains to positive and negative controls. The two controls served to accurately analyse the data. Freeze-dried biomass was used because it is regarded as the best preservation method for probiotic microbial strains (Rathnayaka, 2013). In fact, the final form of the tested probiotic strains will be freeze-dried.

Prior research has used a static system to achieve gastrointestinal simulation *in vivo*, otherwise known as GIS1. Survival capacity (Cs) was determined to highlight the influence of gastrointestinal transit *in vitro* on the viability of these probiotic strains, as well as to assess its influence on secondary parameters – namely, viscosity, lactic acid synthesis, and exopolysaccharides.

2. Materials and methods

2.1. Samples

The *Weissella paramesenteroides* FT1a, *Lactobacillus* sp. 34.1, *Lactobacillus rhamnosus* E 4.2, *Lactobacillus* sp. 18.1, *Lactobacillus fermentum* 428ST, and *Lactobacillus plantarum* FCA3 were obtained from the collection of the Faculty of Biotechnology, Bucharest, Romania. The *L. plantarum* 5s strain was used as a positive control (Vamanu et al., 2006), while the *Escherichia coli* CBAB 2 strain was used as a negative control; these were obtained from the collection of the Faculty of Biotechnology, Bucharest, Romania (Vamanu et al., 2006; Vamanu, 2014). The bacterial strains were maintained in MRS (Oxoid, Basingstoke, Hampshire, UK) and LB broth (Sigma–Aldrich Co., St. Louis, MO, USA), and they were freeze-dried in a Christ-Alpha 1–2 LD freeze dryer (Vamanu et al., 2012).

2.2. In vitro simulation system

All *in vitro* experiments were performed using the GIS1 system (www.gissystems.ro). The *in vitro* system consisted of a 1000 mL Duran bottle with a removable screw cap; the bottle was made of borosilicate glass. The Duran bottle screw cap had four entry points: the first entry point was used for the sample and gastric juice (pump 1) and HCl (Fluka Biochemica, Switzerland) 1 N solutions (pump 2); a second entry was used for the $NaHCO_3$ (Fluka Biochemica, Switzerland) 1 N solutions (pump 3), as well as for the bile salts and pancreatin solution (pump 4); a third entry point was used for the pH electrode, 3 mm in diameter, from Hanna Benchtop pH Meters HI 2211; and the fourth entry point was used for the thermometer, which maintained the contents at a temperature of 37 °C. To enter the system, four laboratory peristaltic pumps (Behrotest, Type PLP 33; flow rate: 0.4–2.0 L h^{-1}) were used. The Duran vessel was placed on the magnetic stirrer with a ceramic heating plate (IKA C-MAG HS 7) to constantly shake the contents at 50 rpm (Vamanu et al., 2012).

2.3. Viable counting

Cell viability was determined by serial dilution in physiological saline solution. The highest dilutions were then plated on MRS/LB agar and evaluated by an automated colony counter (ColonyQuant; Schuett-biotec GmbH, Göttingen, Germany) using the corresponding software (Vamanu et al., 2012; Vamanu, 2014). To compare bacterial survival at the gastrointestinal level, the Cs was calculated (Vamanu et al., 2012, 2013).

2.4. Determination of lactate concentrations

Lactate concentrations were measured *in vitro*, during gastrointestinal digestion, using the Lactate Assay Kit (Sigma–Aldrich Co., St. Louis, MO, USA) (Curtis et al., 2000). The reaction mix consisted of 1000 µL of phosphate buffer (pH 7.5), 100 µL of lactate dehydrogenase, and 100 µL of the sample. The mix was kept in the dark at the room temperature for 30 min. The absorbance was read at 570 nm with a Helios λ spectrophotometer. The amount of lactate from the samples was determined from the standard curve.

2.5. Determination of exopolysaccharides

Exopolysaccharide synthesis was determined by precipitation (24 h, at 4–6 °C) with cold absolute ethanol after the bacterial cells were removed (3500*g*, for 30 min and at 9 °C) by centrifugation at 3000*g* for 15 min (Salazar et al., 2008; Polak-Berecka et al., 2015; Wasko et al., 2013).

2.6. Determination of viscosity

Viscosity (cP) was measured on an Ubbelohde viscometer. The analysed sample was introduced via capillary laboratory equipment. The sample was maintained at 20 °C, and the flow time of the liquid sample between the two parts of the equipment was determined. Viscosity was calculated using the following formula:

$$\eta = kt\rho, \quad (1)$$

where η = dynamic viscosity; k = viscometer constant; t = flow time (seconds); and ρ = density at 20 °C (Frengova et al., 2002; Polak-Berecka et al., 2015).

2.7. Statistical analysis

All of the parameters were assessed in triplicate, and the results were expressed as the mean ± standard deviation (SD) of the three observations ($P < 0.05$). Mean values and standard deviations were calculated using the Excel program from the Microsoft Office 2010 software package.

3. Results and discussion

The viability of the strains used during gastric and intestinal transit is shown in Fig. 1, in parallel with that of the two controls, *L. plantarum* 5s and *E. coli* CBAB 2. After 1 h in the presence of an average pH of 2–3, a significant decrease in cell viability was observed; this was mainly found for the two controls, although it was also noted for the *L. plantarum* FCA3 and *W. paramesenteroides* FT1a strains. No significant change was observed in the Cs of the *L. rhamnosus* E 4.2 and *Lactobacillus* sp. 18.1 strains as a result of the gastric transit. The most significant decrease in viability following digestion at the gastric level was noted for the *W. paramesenteroides* FT1a and *L. plantarum* 5s strains, which lost approximately 50 ± 0.01% of the number of viable cells, particularly during the first hour of action in the presence of pepsin and hydrochloric acid. The negative control was stronger by approximately 10 ± 0.1%.

The *W. paramesenteroides* FT1a, *L. plantarum* 5s, and *L. rhamnosus* E 4.2 strains showed the most significant decrease in Cs by approximately 12 ± 0.5% during the last hour of digestion at the gastric level ($P < 0.1$). Strains of the *Lactobacillus* sp. 18.1 and *Lactobacillus* sp. 34.1 showed constant Cs values. The remaining strains did not exhibit variations in viability greater than 5 ± 0.01% during the final stage of gastric digestion; thus, the general loss in cell viability was barely 3 ± 0.001% for the *Lactobacillus* sp. 18.1 between the initial and final moments in the gastric digestion process.

A significant difference was found for most strains tested between the start of digestion and the end of the *in vitro* simulation. The differences in viability were generally over 50 ± 0.01%, meaning that there was a significant decrease in the number of viable cells. The *L. fermentum* 428ST strain and the negative control (*E. coli* CBAB 2) were exceptions to this finding; they showed an average loss of approximately 40 ± 0.01% (Fig. 1).

In general, the various strains showed the most significant decrease in viability after 2–3 h of digestion at the duodenal level. The greatest loss was found for *Lactobacillus* sp. 18.1, which had lost 50 ± 0.01% of its viable cells during the last hour of digestion, resulting in a final Cs value of 0.34 ± 0.02. In contrast, *L. fermentum* 428ST had lost only 6 ± 0.001% of its viable cells during the same period. The remaining strains showed losses in viability during the last 2 h of development at the duodenal level between 25% and 46 ± 0.01%. Lower losses were observed for *W. paramesenteroides* FT1a, which exhibited a reduction of approximately 12%, while losses of 13 ± 0.03% were found for the negative control (*E. coli* CBAB 2) (Vamanu, 2014).

The results highlight the fact that the functional products used in this study hold promise in the formulation designed herein, especially with respect to the *L. fermentum* 428ST, *Lactobacillus* sp. 34.1, *L. rhamnosus* E 4.2, *Lactobacillus* sp. 18.1, and *L. plantarum* FCA3 strains. Comparative studies carried out with the two controls demonstrated that cell viability is a specific parameter that can be accurately assessed using the *in vitro* simulator, GIS1. The synthesis of secondary metabolites (lactic acid and/or exopolysaccharides) is a direct indicator of the capacity of strains tested in unfavourable environments at the gastric and small-intestine levels (Cock et al., 2013). These findings were confirmed by previous *in vitro* studies of *Lactobacillus acidophilus johnsonii*, *Lactobacillus casei immunitas*, and *Lactobacillus casei shirota* (Lo Curto et al., 2011). The exact role of exopolysaccharides on the viability of lactic acid bacteria is not well known. It is accepted that it favours colonization and the capacity to remain at the gastrointestinal level, which is evident based on its ability to maintain viability and by its prominent role in human health (Darilmaz et al., 2011). With respect to *L. fermentum* 428ST, a significant quantity of carbon dioxide appeared to be released during simulation. Moreover, the synthesis of polysaccharides was reduced during simulation, decreasing throughout the various stages of gastrointestinal digestion. Thus, by the end of the digestion process, the quantity of polysaccharides was 0.53 ± 0.005 μg/mL, and during the last 2 h of digestion in the intestine, the value decreased

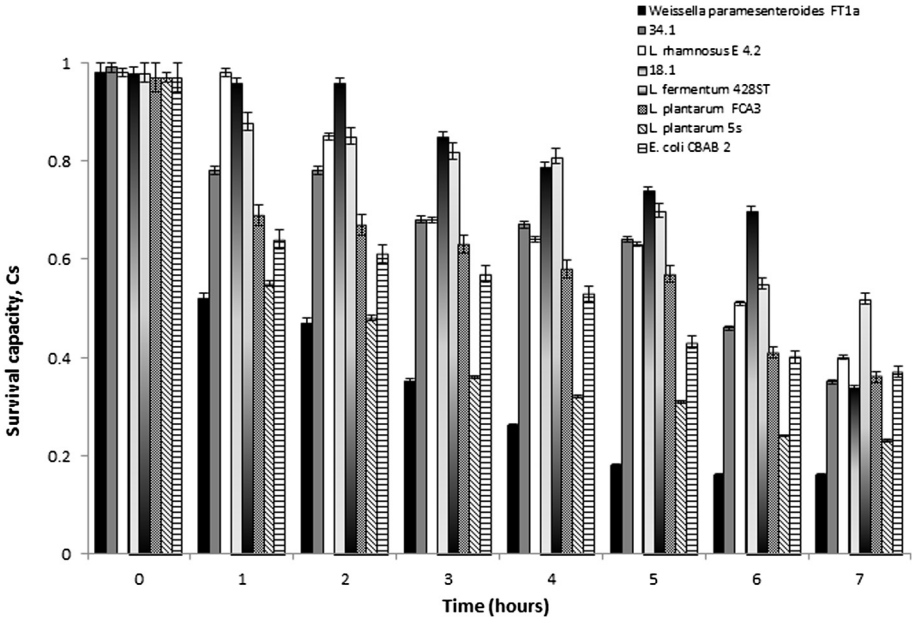

Figure 1 Survival capacity of lactic acid bacterial strains during simulated gastrointestinal digestion.

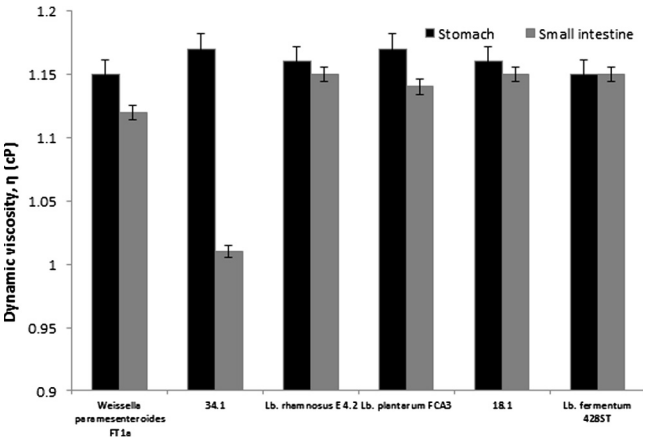

Fig. 2 Dynamic viscosity during simulated gastrointestinal digestion.

by approximately 30 ± 0.01%. In contrast, the strains of *W. paramesenteroides* FT1a had the lowest level of exopolysaccharide synthesis, with a value close to that of the control, *L. plantarum* 5s; the value of *W. paramesenteroides* FT1a reached a maximum of 0.2 ± 0.0005 μg/mL. The most significant quantity was found for *L. rhamnosus* E 4.2, which was an average of four times greater than that for *L. fermentum* 428ST throughout the entire *in vitro* simulation process. It was further demonstrated that polysaccharide synthesis was correlated with the lack of carbon dioxide released.

In terms of viscosity, there was an evolution proportional to exopolysaccharide synthesis. A decrease was observed in relation to passage from one compartment in the digestion process to another. The most significant decrease, by approximately 10 ± 0.60%, was calculated for the *Lactobacillus* sp. 34.1 strain; otherwise, the η value decreased by an average of 5 ± 0.40%. The *L. fermentum* 428ST strain appeared to be the exception to this finding, as a constant average value was calculated (1.15 ± 0.06 cP) during the entire period of *in vitro* simulation (Fig. 2).

The Cs value directly correlated with the synthesis of lactic acid, which was interpreted to be a direct indicator of viable cell metabolism. During the *in vitro* transit process, the lactic acid level decreased, particularly during the intestinal stage, for strains *W. paramesenteroides* FT1a, *Lactobacillus* sp. 34.1, and *L. plantarum* FCA3. The average value determined at the end of the intestinal stage was of 25 ± 0.20 mM. In contrast, the *L. fermentum* 428ST strains showed double the quantity of lactic acid at the end of the intestinal transit. The correlation between the Cs value and lactic acid synthesis yielded significant values during gastric transit, 0.7–0.86 ± 0.02; this was lower during transit at the intestinal level (0.34–0.48 ± 0.10; $P < 0.1$). Furthermore, the correlation between cell viability and the dynamic viscosity values was significant (0.85–0.97 ± 0.07; $P < 0.1$).

The most resistant strain, *L. fermentum* 428ST, presented with an average viability of 4.73 ± 0.24 log CFU/mL when compared to controls, whose average viability decreased by approximately 25% following transit. When compared with the other strains that were extremely sensitive to the action of pancreatin and bile salts during the last 2 h of digestion, *L. fermentum* 428ST maintained a constant decrease, not exceeding an average of 0.5 ± 0.03 log CFU/mL. The remaining strains that were tested even showed losses that reached 1 log CFU/mL (*L. plantarum* FCA3). In this study, the loss of viability for some probiotic strains reached a maximum of 60 ± 0.19%. The results obtained herein were expressed in terms of the Cs value, which fell within the limits reported in the literature for all strains ($P < 0.05$); the only exception to this finding was observed for *W. paramesenteroides* FT1a. Previous studies have shown that viability can be improved by the presence of a prebiotic (Vamanu et al., 2012).

In conclusion, this study showed that the value of cell viability was directly correlated with the synthesis of lactic acid and exopolysaccharides, as well as with the value of viscosity during the *in vitro* simulation of gastrointestinal transit in the GIS1 system. Further studies are needed to determine the Cs in the colon using, in parallel, a static and a dynamic model for the *in vitro* simulation of segments of the human colon.

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