Viability and Resistance of Lactobacilli Isolated from Cocoa Fermentation to Simulated Gastrointestinal Digestive Steps in Soy Yogurt

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Abstract: To study the potential probiotic characteristics such as decrease of pH, microbial viability, and tolerance to simulated digestive steps of fermented soy beverage ("soy yogurt") produced with lactobacilli isolated from cocoa fermentation (*Lactobacillus fermentum* TcUESC01 and *Lactobacillus plantarum* TcUESC02) during fermentation and refrigerated storage. The sensory acceptance of the yogurts was also tested. Samples of soy yogurt produced with *L. fermentum* TcUESC01 or *L. plantarum* TcUESC02 were collected during fermentation (0, 4, 8, and 12 h) and refrigerated storage (1, 9, 18, and 27 d), and submitted to pH and bacterial viability determinations. Tolerance to simulated digestion steps was done with refrigerated storage samples at 9 °C. Simulated digestion was performed in 3 successive steps: exposure to pepsin-HCl solution, bile shock, and simulated small intestinal juice. During storage, a decrease in pH and lactobacillus viability was observed. *L. fermentum* TcUESC01 showed to be more resistant than *L. plantarum* TcUESC02 to simulated gastrointestinal digestion. All soy yogurts showed acceptable hedonic scores (greater than 5 in a 9-point hedonic scale ranging from "like extremely" to "dislike extremely") in sensory evaluation for flavor, aroma, color, consistency, and overall impression. *L. plantarum* TcUESC02 and, especially, *L. fermentum* TcUESC01 showed potential probiotic characteristics when considering pH, cell viability, and tolerance to simulated digestive steps and did not affect the sensory characteristics when supplemented to soy yogurt during storage.

Keywords: *Lactobacillus*, microbial survival, probiotics, sensory analysis, soy yogurt

Practical Application: Using a sequential test procedure for *in vitro* verification of microbial viability barriers of the gastrointestinal tract and sensory evaluation, strains of lactobacilli isolated from cocoa fermentation showed promissory results for the development of new functional foods.

Introduction

Isolation and characterization of novel strains of lactobacilli from uninvestigated sources could have the double advantage of revealing taxonomic characteristics and obtaining strains with interesting functional traits that may be useful for biotechnological and/or probiotic applications (Ortu and others 2007). A large number of novel species have been described in recent years from different sources as a result of investigations searching for new strains for food application, mainly in the field of probiotics (Dellaglio and Felis 2005; Todorov and others 2008). The search for greater diversification of sources of isolation of potentially probiotic microorganisms, including traditional fermented foods of different cultures and geographical settings, is a current trend in different fields of microbial biotechnology (Mahasneh and Abbas 2010).

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Cocoa is the most important agricultural product of southern Bahia in Brazil (Schroth and others 2011). The cocoa pulp is a rich medium for microbial growth and successful cocoa bean fermentation requires a succession of specific microbial activities. At the onset of cocoa bean fermentation, yeasts are the dominating microorganisms, creating ideal conditions for the posterior growth of lactic acid bacteria (Lefeber and others 2010). According to Nielsen and others (2005) and Camu and others (2007), the species *Lactobacillus plantarum* and *Lactobacillus fermentum* are the first to dominate the cocoa lactic fermentation.

The food matrix to which probiotics are added has a decisive role on their functionality (Ranadheera and others 2010). For this reason, in the development of functional foods containing probiotic bacteria, research is required to select the right vehicle to ensure that microbial cells remain viable throughout the shelf life and overcome the physical and chemical barriers encountered in the gastrointestinal tract (Vinderola and Reinheimer 2000; Piano and others 2006). Although yogurt produced from bovine milk is the most popular type of fermented milk in the world, the demand for alternatives to bovine milk products is growing, primarily because of the increasing incidence in the population of allergy to bovine milk proteins and the growing market of vegetarian consumers (Wang and others 2003). Soy-based foods could provide additional health benefits to the consumers because of their hypolipidemic, anticholesterolemic, and antiatherogenic properties; they could also reduce the risk of hormone-associated health disorders (Lopez-Lazaro and Akiyama 2002). However, only few

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studies have been carried out in order to determine the effects of vegetarian foods on the gastric transit tolerance of lactic acid bacteria.

L. fermentum TcUESC01 and *L. plantarum* TcUESC02 were isolated in our laboratory during cocoa fermentation and preliminary results showed their probiotic potential in a model of colitis in mice (recent unpublished data). In this study, these strains were tested for their ability to be used as starter to produce soy yogurt and for the evaluation of viability and resistance to simulated gastrointestinal digestion during storage.

Materials and Methods

Bacterial strains and culture conditions

L. fermentum TcUESC01 and *L. plantarum* TcUESC02 were isolated and identified from the fermentation process of cocoa by Santos (2010) and were kept frozen at –80 °C in soy extract supplemented with glycerol (20% w/v). The strains were reactivated by 3 successive transfers (37 °C, 16 h) in soy extract before their use in the production of soy yogurt.

Preparation of fermented soy beverage (yogurt)

Soy yogurt was prepared with the BRS257 soybean variety, with low lipoxygenase supplied by Sementes Paraná (Ponta Grossa, Paraná, Brazil). The water extract was extracted from soys (150 g/L) with water using a Sojamac SQ930 machine (Sojamac, São Paulo, Brazil) following the manufacturer's instructions. Soy extract was separated from insoluble residues by filtration through a nylon 100 μm mesh filter. The soy extract was supplemented with 3.33% (w/v) sucrose to obtain 9 °Bx and autoclaved at 115 °C for 15 min. The soy extract supplemented was cooled to 40 °C and aseptically divided into 2 batches. Each batch was inoculated (4% v/v) with *L. fermentum* TcUESC01 or *L. plantarum* TcUESC0. Inoculated soy extract supplemented was poured into 100 mL sterile transparent plastic cups with lids (60 mL per cup) and incubated at 37 °C until reaching pH 4.60 (approximately 12 h). Soy yogurts were stored at 4 °C for 28 d for further analysis. As a reference for the sensory analysis, soy yogurt was also produced using a commercial starter (BioRich®, Chr. Hansen, Brazil) containing *Bifidobacterium animalis* subsp. *lactis* BB-12, *Lactobacillus acidophilus* LA-5 and *Streptococcus thermophilus.* All yogurts were flavored after fermentation with 20% (w/v) strawberry jelly and 4% (w/v) of wild strawberry aroma, both obtained from Duas Rodas Industrial (Jaraguá do Sul, Santa Catarina, Brazil).

pH and titratable activity

pH and titratable acidity (TA) were determined during fermentation (0, 4, 8, and 12 h) and storage (1, 9, 18, and 27 d). TA was determined by titrating a diluted sample (1:10 in distilled water) with 0.1 N NaOH to an end point indicated by phenolphthalein (pH 8.4). TA was calculated on the basis of lactic acid as the predominant acid and was expressed as percent (w/v) of lactic acid at 25 °C. Measurements were performed in triplicate.

Cell counts

The viability of *L. plantarum* TcUESC02 and *L. fermentum* TcUESC01 was determined along fermentation and storage at the same time described earlier. A sample of 10 g was diluted 10-fold in 0.85% (w/v) sterile saline solution. Serial dilutions were made in the same diluent and 0.1 mL were spread onto MRS (Difco, Sparks, Maryland, U.S.A.) agar and incubated at 37 °C for 48 h in aerobiosis.

Resistance to simulated gastrointestinal digestion

Twenty milliliters of soy yogurt were mixed with the same volume of a simulated saliva-gastric solution (step I). Saliva-gastric solution contained $CaCl_2$ (0.22 g/L), NaCl (16.2 g/L), KCl (2.2 g/L), $NaHCO_3$ (1.2 g/L), and 0.3% (w/v) bovine pepsin (Sigma-Aldrich, St. Louis, Miss., U.S.A.) (Marteau and others 1997). A 1 mL sample was removed for cell counts immediately after mixture and pH was quickly lowered to 3.0 and 2.5, with 5 N and 0.1 N HCl. Samples were brought to 37 °C in a water bath and maintained there for 90 min. Aliquots (1 mL) were taken periodically and serial dilutions were plated for cell counts as described above. After 90 min of simulated saliva-gastric digestion, a volume was centrifuged (4000 *g*, 5 min, 5 °C) (Eppendorf Centrifuge mod. 5810R, Hamburg, Germany) The supernatant was removed, the pellet was washed twice with phosphate buffered saline (PBS) buffer (pH 7.4), and resuspended to the original volume in 1% (w/v) bovine bile (Sigma-Aldrich) at pH 8.0. A sample was removed for cell viability assessment and the remaining cell suspension was incubated in a water bath for 10 min at 37 °C (bile shock—step II). After this incubation, a sample was collected for cell viability assessment. Again, a volume was centrifuged (4000 *g*, 5 min, 5 °C), the supernatant removed, and the pellet was washed twice with PBS buffer (pH 7.4) and resuspended to the original volume in 0.3% (w/v) bovine bile (Sigma-Aldrich) plus 0.1% (w/v) pancreatin (Sigma-Aldrich) (step III) at pH 8.0. Aliquots (1 mL) were taken before and after an incubation period of 180 min at 37 °C to assess cell viability. The test was performed in triplicate.

Sensory evaluation

The sensory properties of the manufactured soy yogurt were evaluated by an untrained panel of 90 assessors recruited among students and staff members of the State Univ. of Santa Cruz (Ilhéus, Bahia, Brazil). The products were evaluated at days 1 and 21 of storage at 4 °C. The samples were served at 7 to 10 °C in plastic cups and were coded with 3-digit numbers. Water and crackers were supplied for panel members. A test form comprising 5 sensory attributes (flavor, aroma, color, consistency, and overall impression), was given to each assessor. A structured 9-point hedonic scale ranging from "like extremely" to "dislike extremely," was used to numerically describe the sensory properties.

Statistical analysis

Most of the data were analyzed by the 2-tailed Student's *t*-test. All statistical tests were performed using the GraphPad Prims 4.0 software. A *P* value below 0.05 was considered to be statistically significant.

Results and Discussion

Fermentation characteristics and viability of *L. fermentum* TcUESC01 and *L. plantarum* TcUESC02 during soy yogurt fermentation and refrigerated storage

TA, pH, and cell counts of *L. fermentum* TcUESC01 and *L. plantarum* TcUESC02 in soy yogurt along fermentation and storage are shown in Table 1. During fermentation, a significant decrease in pH and a consequent increase in TA were observed for both samples of soy yogurt. By the end of the storage period (27 d), postacidification phenomena were observed, causing a further reduction in pH by both bacteria *L. fermentum* TcUESC01 and *L. plantarum* TcUESC02 (decreasing 10.15% and 9.14%, respectively), indicating that they were able to produce acid(s) under refrigeration. Despite the decrease in pH, final values

Table 1–Cell counts (log CFU/mL) of *L. plantarum* TcUESC02 (*L.p.*) and *L. fermentum* TcUESC01 (*L.f.*), titratable acidity (TA), pH during the manufacture (fermentation) and storage of soy yogurt. The values are means (±SD) of 3 replicates for each bacterial strain.

	Fermentation (hours)											
	0 h			4 h			8 h			12 h		
Strain	Counts	TA	pH	Counts	TA	pH	Counts	TA	pH	Counts	TA	pH
L.f.	6.62 ± 0.02^{a}	0.123 ± 0.003^{a}	6.35 ± 0.00^{a}	7.73 ± 0.06^{b}	0.213 ± 0.003^{b}	5.84 ± 0.02^{b}	8.47 ± 0.07^{c}	0.325 ± 0.002^{c}	5.05 ± 0.04^{c}	8.72 ± 0.04^{d}	0.456 ± 0.003^{d}	4.63 ± 0.04^{d}
L.p.	6.62 ± 0.02^{a}	0.120 ± 0.00^{a}	6.35 ± 0.00^{a}	7.7 ± 0.02^{b}	0.213 ± 0.003^{b}	5.80 ± 0.01^{b}	8.44 ± 0.07^{c}	0.32 ± 0.002^{c}	5.03 ± 0.02^{c}	8.79 ± 0.04^{d}	0.450 ± 0.025^{d}	4.6 ± 0.01^{d}
	Storage (days)											
	1 d			9 d			18 d			27 d		
Strain	Counts	TA	pH	Counts	TA	pH	Counts	TA	pH	Counts	TA	pH
L.f.	8.72 ± 0.03^{a}	0.456 ± 0.003^{a}	4.63 ± 0.04^{a}	8.65 ± 0.04^{a}	0.540 ± 0.009^{b}	4.45 ± 0.04^{b}	8.54 ± 0.05^{b}	0.583 ± 0.007^{c}	4.35 ± 0.06^{b}	8.40 ± 0.02^{c}	0.666 ± 0.005^{d}	4.16 ± 0.02^{c}
L.p.	8.79 ± 0.05^{a}	0.454 ± 0.003^{a}	4.6 ± 0.01^{a}	8.68 ± 0.02^{a}	0.513 ± 0.016^{b}	4.47 ± 0.03^{b}	8.53 ± 0.06^{b}	0.562 ± 0.009^{c}	4.33 ± 0.03^{c}	8.46 ± 0.02^{c}	0.642 ± 0.007^{d}	4.18 ± 0.03^{d}

[a]Values in rows, for the same parameter (cell counts, TA or pH), with different superscripts are significantly different ($P < 0.05$).

corresponded to recommended values desirable in terms of sensory characteristics, which are between 4.0 and 4.6 (Ronka and others 2003). The drop in pH is often the cause of losses in viability during storage (Vinderola and others 2011b). It has not been ascertained if this small decrease in viability was specifically linked to pH, or another factor such as oxygen, but it can be argued that the cultures are quite resistant to low pH in light of the minimal viability loss. The variation in TA values of soy milk during storage may indicate that soy yogurt has a lower pH buffering capacity compared to bovine milk, which might be attributed to the protein composition of soy extract and to the physicochemical properties of these proteins (Wang and others 2009).

Both strains showed significant growth (approximately 2 log cycles), with no difference between them during fermentation and storage steps ($P < 0.001$) (Table 1), indicating that soy extract was a good growth medium for both *L. fermentum* TcUESC01 and *L. plantarum* TcUESC02. High numbers of viable cells of probiotic cultures by the end of fermentation is one of the prerequisites to maintain satisfactory levels of functional cells during storage (Lourens-Hattingh and Viljeon 2001). Chang and others (2010) showed cell counts of more than 8 log CFU/g for *L. acidophilus* and *B. brevis* in fermented soy milk during 15 d of refrigerated storage. Counts of *L. plantarum* TcUESC02 and *L. fermentum* TcUESC01 found in soy yogurt in this study are in the range of the results obtained by other authors (Donkor and others 2007; Pyo and Song 2009; Yeo and Liong 2010). Generally, it has been suggested that fermented milk products must contain at least 10^7 to 10^8 CFU/mL of probiotics to exert health effects (Ouwehand and Salminen 1998; Champagne and Gardner 2005). Donkor and Shah (2008) showed that during storage of fermented soy beverage, at 4 °C for 28 d, soy extract fermented by *L. acidophilus* L10 and *B. lactis* B94 exhibited an increase of population by 20% and 14%, respectively. However, growth of cells during storage is not always desirable because it may adversely modify the sensory characteristics of food (Vinderola and others 2011b). In this study, we observed that *L. plantarum* TcUESC02 and *L. fermentum* TcUESC01 showed satisfactory ability to grow in soy extract and cell viability was maintained in the samples at approximately 8 log orders during the time of refrigerated storage. Although there has been growth of the strains tested in soy extract and a desirable maintenance cell viability, more research is necessary using these lactobacilli, seeking to decrease the fermentation time in order to make them more attractive to industry, perhaps associating them with the use of prebiotic such as raffinose or inulin or a combination of glucose and raffinose.

Resistance to exposure to simulated gastrointestinal conditions during refrigerated storage

To be considered a probiotic, the strain/product has to be able to resist harsh conditions found in the gastrointestinal tract (gastric acidity, bile salts, pepsin, pancreatin, and other enzymes and antimicrobial compounds). Food matrix can have a protective effect for probiotics during passage through the stomach (Charteris and others 1998; Vinderola and Reinheimer 2000; Mishra and Prasad 2005). Therefore, a methodology for the screening of tolerance to gastrointestinal conditions of potential probiotic strains should consider these aspects. Even if *in vitro* tests do not predict real gastrointestinal resistance, they are still useful for screening purposes and to preliminary explore the impact of some technological factors, such as storage, on the gastrointestinal resistance of probiotic bacteria (Vinderola and others 2011a).

In this study, the resistance of strains to simulated gastrointestinal digestion during storage in soy yogurt differed depending on the strain and on the pH considered (Figure 1 and 2). At days 1 and 9 of storage, cell counts diminished by less than 1 log order at the end of simulated gastrointestinal digestion in all gastric resistance assessed (Figure 1 and 2). By day 18, cell counts decreased approximately 2 log orders for both strains at pH 2.5. By day 27, cell counts decreased approximately 3 and 2 log cycles for *L. plantarum* TcUESC02 (Figure 1A) and *L. fermentum* TcUESC01 (Figure 2A), respectively. Interestingly, after the step I (simulated saliva-gastric exposure), in general, no further reduction in cell counts were observed for any of the strains, instead a plateau against time was observed. When saliva-gastric exposure was carried out at pH 3.0 (Figure 1B and 2B), a reduction of about 0.5 to 1 log order was observed by the end of the experiment for *L. plantarum* TcUESC02 at days 1, 9, and 18 of storage. By day 27, at pH 3.0, cell counts decreased approximately 3 log orders for this strain (Figure 1B). *L. fermentum* TcUESC01 showed higher resistance than *L. plantarum* to the simulated gastrointestinal digestion under these conditions, maintaining its population profile during storage and losing only approximately 1 log order of cell viability (Figure 2B). Wang and others (2009) reported the same reduction in gastric resistance of *Lactobacillus casei* during the refrigerated storage of experimental fermented bovine and soy milks, with no significant changes in cell viability. According to these authors, there was no loss of cell viability during storage, but a decrease of cell resistance to gastric acidity was observed, as well as the presented work (Figure 1 and 2).

Usman and Hosomo (1999) suggested that survival at 0.1% (w/v) of bile salts is considered important for probiotic organisms. In our study, *L. plantarum* TcUESC02 and *L. fermentum* TcUESC01 resisted at 1% (w/v) bovine bile salts, hence showing a satisfactory bile tolerance (Figure 1 and 2). Jacobsen and others (1999) and Sanni and others (2002) have also reported that lactic acid bacteria such as *L. fermentum* isolated from African-fermented cereal are able to survive to physiological levels of acid and bile in probiotic selection.

Small intestine tolerance is perhaps more important than gastric survival, since with the development of new delivery systems and the use of specific foods, acid-sensitive strains can be buffered through the stomach (Huang and Adams 2004).

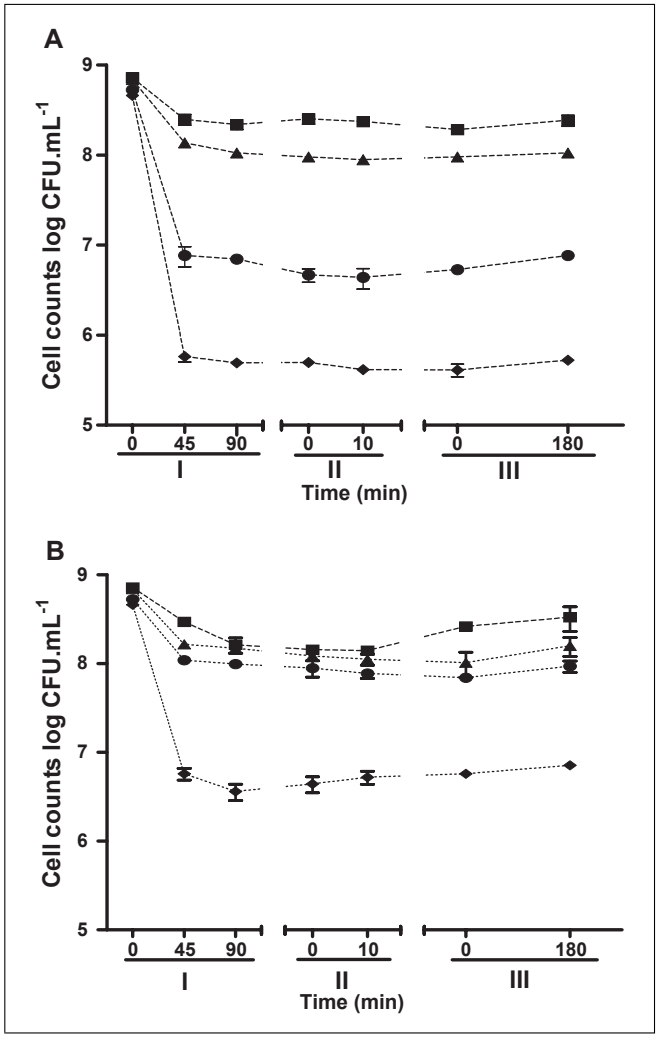

Figure 1–Cell counts of *L. plantarum* TcUESC02 soy yogurt during simulated gastric digestion at pH 2.5 (A) and 3.0 (B), in saliva-gastric exposure (I), bile shock (II), and simulated intestinal digestion (bile + pancreatin) (III) after 1 (■), 9 (▲), 18 (●), and 27 (♦) d of storage at 5 °C. Values are means (±SD) of 3 replicates.

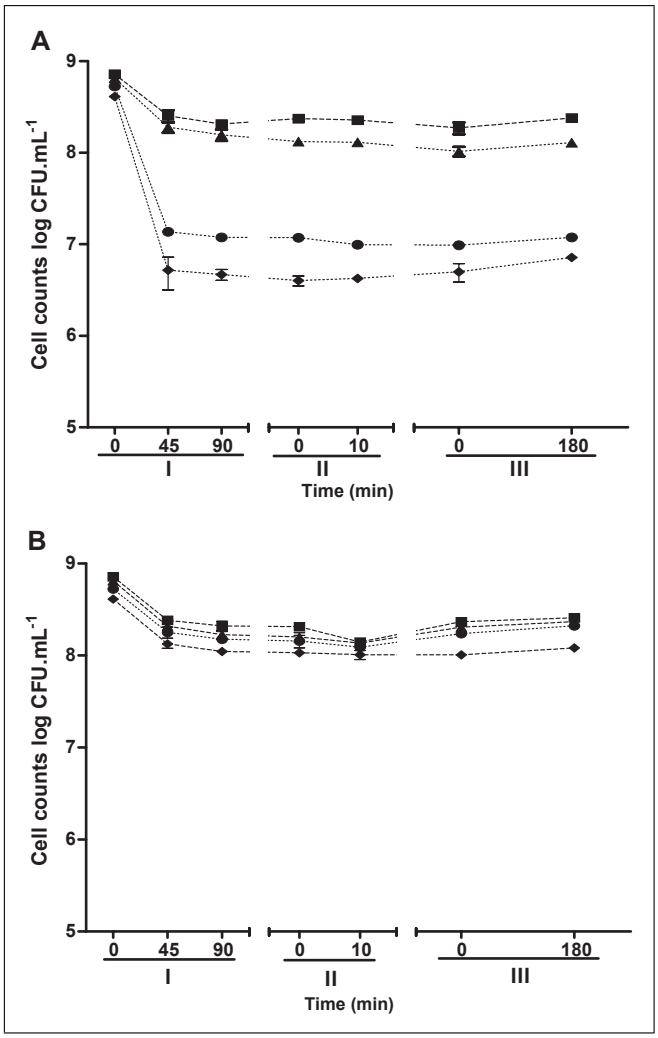

Figure 2–Cell counts of *L. fermentum* TcUESC01 in soy yogurt during simulated gastric digestion at pH 2.5 (A) and 3.0 (B), in saliva-gastric exposure (I), bile shock (II), and simulated intestinal digestion (bile + pancreatin) (III) after 1 (■), 9 (▲), 18 (●), and 27 (♦) d of storage at 5 °C. Values are means (±SD) of 3 replicates.

Table 2–Sensory evaluation of soy yogurt with *L. plantarum* TcUESC01 or *L. fermentum* TcUESC02 compared to the reference product (soy yogurt with a commercial starter containing *Bifidobacterium animalis* subsp. *lactis* BB-12, *L. acidophilus* LA-5, and *S. thermophilus*) during storage at 4 °C.

	Fermented soy milk		
Attribute	**Reference**	***L. fermentum***	***L. plantarum***
Day 1			
Mouth feel	6.13 ± 1.72^{a}	5.84 ± 1.79^{a}	6.15 ± 1.70^{a}
Flavor	6.93 ± 1.65^{a}	$6.34 \pm 1.74^{a,b}$	6.21 ± 1.53^{b}
Appearance (color)	6.53 ± 1.94^{a}	6.71 ± 1.70^{a}	6.61 ± 1.80^{a}
Consistency	5.83 ± 2.33^{a}	6.36 ± 1.97^{a}	5.99 ± 2.09^{a}
Overall acceptability	6.17 ± 1.93^{a}	6.00 ± 1.78^{a}	6.21 ± 1.69^{a}
Day 21			
Mouth feel	7.31 ± 1.49^{a}	6.17 ± 1.62^{b}	6.31 ± 1.62^{b}
Flavor	6.96 ± 1.65^{a}	6.44 ± 1.76^{a}	7.06 ± 1.51^{a}
Appearance (color)	7.13 ± 1.30^{a}	7.06 ± 1.19^{a}	7.00 ± 1.19^{a}
Consistency	7.24 ± 1.70^{a}	7.13 ± 1.48^{a}	7.03 ± 1.48^{a}
Overall acceptability	7.40 ± 1.30^{a}	6.74 ± 1.28^{a}	6.66 ± 1.20^{a}

[a]Values in rows with different superscripts are significantly different ($P < 0.05$).
9 = like extremely; 8 = like very much; 7 = like moderately; 6 = like slightly; 5 = neither like nor dislike; 4 = dislike slightly; 3 = dislike moderately; 2 = dislike very much; 1 = dislike extremely.

However, to exert a positive effect on the health and well being of a host, probiotics need to survive and, at least transiently, to adhere and colonize the small intestine (Havenaar and others 1992). The stressful conditions of this environment may be an essential selection criterion for future probiotics (Huang and Adams 2004). Although the strains under study gradually lost resistance to simulated gastrointestinal digestion during storage, their survival remained within the same order of magnitude throughout the 180 min in which they were exposed to the bovine bile salts and pancreatin (Figure 1 and 2).

Sensory evaluation

The sensory properties of the soy yogurt were evaluated by an untrained panel of 90 assessors recruited among students and staff members of the State Univ. of Santa Cruz. Significant differences compared to reference soy yogurt were found only for attributes such as flavor and mouth feel (Table 2). Mouth feel scores were similar for products manufactured with *L. fermentum* TcUESC02 and *L. plantarum* TcUESC02, but not for the reference product that obtained higher scores. It is likely that the mixed cultures of the reference soy yogurt could have exerted a synergistic interaction during fermentation and thus led to significantly different values for this feature. Soy yogurt containing *L. plantarum* TcUESC02 received lower scores for the attribute flavor than those of the reference yogurt and the product containing *L. fermentum* TcUESC01. This may be partly due to the objectionable beanie flavor or taste of soy extract, which was more pronounced in products containing *L. plantarum* TcUESC02. The color, consistency, and general acceptance remained stable throughout the storage period, obtaining mean scores that ranged from "like slightly" to "like moderately." Overall, based on the mean acceptability scores, all soy yogurt received high scores, indicating that consumers liked the product and storage time did not significantly affect organoleptic properties. However, unpleasant features of artisanal fermented milks can always be positively masked by the use of natural or synthetic aroma and color agents, commonly used in the dairy industry (Potter and others 2007). In our case, this issue deserves more attention and future research.

Conclusion

The results from this study showed a promising future for the further characterization and use of microorganism isolated from cocoa fermentation with potential industrial application. Both strains showed good fermentative capacity in soy yogurt as well as satisfactory cell viability along storage and capacity to overcome, at least *in vitro*, the physiological barriers found along digestion. More studies should be done with these strains since they are interesting candidates for application in the use of soy yogurts as functional food.

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Conflict of Interest

The authors declare no conflict of interest.

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