Brazilian Journal of Microbiology (2020) 51:1645–1654
https://doi.org/10.1007/s42770-020-00369-z

BIOTECHNOLOGY AND INDUSTRIAL MICROBIOLOGY - RESEARCH PAPER

Soymilk fermentation: effect of cooling protocol on cell viability during storage and in vitro gastrointestinal stress

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Received: 11 April 2020 / Accepted: 21 August 2020 / Published online: 31 August 2020

Abstract

This work covers soymilk fermentation by starter and probiotic cultures and explores the influence of cooling protocol on cell viability, organic acid production, sugar consumption, fatty acid profile, and cell survival to in vitro gastrointestinal stress. After fermentation at 37 °C by mono- or co-cultures of *Streptococcus thermophilus* (St), *Lactobacillus bulgaricus* (Lb), and *Lactobacillus paracasei* (Lp), fermented soymilk was cooled directly at 4 °C for 28 days or cooled in two phases (TPC), i.e., by preceding that step by another at 25 °C for 8 h. Soybean milk fermentation by Lb alone lasted longer (15 h) than by StLb or StLbLp (9 h). In ternary culture, TPC increased Lp viability, linoleic, and lactic acid concentrations by 3.8, 22.6, and 96.2%, respectively, whereas the cooling protocol did not influence Lp and St counts after in vitro gastrointestinal stress.

Keywords Probiotic · Starter culture · Fermentation · Soymilk · Cooling · Metabolic activity

Introduction

Soymilk is a vegetable product with high potential in the development of new beverages, mainly because of its chemical and nutritional characteristics that qualify it as functional food [1]. However, its acceptance by consumers is limited by sensory characteristics similar to those of raw beans, production of low-molecular-weight volatile compounds by lipoxygenases, and flatulence resulting from metabolization of oligosaccharides by the intestinal microbiota [2].

Soymilk sensorial quality can be improved by sugar fermentation by lactic acid bacteria, which is already used to produce tofu and different fermented beverages [3–5].

Responsible Editor: Rosane Freitas Schwan

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Probiotics are commonly used in food fermentations because of their health benefits, being able to regulate the intestinal microbiota, adjuvate the absorption of certain nutrients, and modulate the immune system [6]. Among them, several species and strains belonging to the genera *Lactobacillus* and *Bifidobacterium* stand out. Particularly, *Lactobacillus paracasei* Lpc-37 is a strain known for its ability to adhere to human intestinal mucosa [6] and its inhibitory action against *Clostridium difficile* [7].

Refrigeration is an important final step to preserve fermented products, because microbial metabolic activity is slow at temperatures around 10 °C. However, the thermal shock caused by a sudden drop in temperature can reduce cell viability [8, 9]; therefore, alternative protocols ensuring intermediate cooling conditions may be useful. They are generally divided into fermentation (30–45 °C), intermediate cooling (22–28 °C), and final shelf life (4–10 °C) that varies according to the intermediate cooling temperature or the time of exposure (2–12 h) [8, 10].

To date, there is no report in the literature on the intermediate cooling to preserve a fermented soybean product. In this context, a soymilk product, obtained by fermentation with a probiotic (*L. paracasei* Lpc-37) and starter cultures (*Streptococcus thermophilus* and *Lactobacillus bulgaricus*) followed by intermediate cooling, would be an innovative proposal in the search for new fermented functional foods.

Therefore, the objectives of this study were to test the application of three different microbial mixtures and two different types of intermediate cooling, as well as to check the impact of these conditions on cell viability, fatty acid profile, organic acid production, and survival to in vitro simulated gastrointestinal digestion.

Materials and methods

Microorganisms and preparation of inocula

Lyophilized strains of *Streptococcus thermophilus* TA040 (St), *Lactobacillus delbrueckii* spp. *bulgaricus* LB340 (Lb) (Danisco-Dupont, Sassenage, France), and *Lactobacillus paracasei* Lpc-37 (Lp) (Danisco-Dupont, Madison, WI, USA) were used. The inoculum medium was prepared by dissolving 24 g of soybean powder (Soymilke, Olvebra, Eldorado do Sul, RS, Brazil), containing 19.6 ± 0.14 g/L of sucrose, in 200 mL of distilled water, and autoclaving for 15 min at 121 °C. Subsequently, 300 mg of St, 500 mg of Lb, or 1000 mg of Lp (dry cells) were added to vials, each containing 50 mL of reconstituted and sterilized soybean powder, hereinafter referred to as "soymilk." Flasks containing the strains and the reconstituted sterile soymilk were set at 37 °C for 30 min so that the strains could be reactivated. After this time, the inocula contained about 10^8 colony-forming units per milliliter (CFU/mL).

Soymilk preparation

To prepare the culture medium, 240 g of soybean powder were dissolved in 2.0-L of filtered drinking water and then pasteurized in a Thermomix (Vorwek & Co. KG, TM31, Wuppertal, Germany) at 90 °C for 5 min. About 500-mL aliquots of this medium were then transferred to flasks, which were cooled in ice bath and stored at 4 °C.

Fermentations and acidification profiles

Aliquots (approximately 1.4 mL) of each inoculum suspension were transferred into flasks containing 500 mL of pasteurized soymilk in order to obtain a starting viable cell count of roughly 10^6 CFU/mL. Fermentations were carried out in triplicate using the following microbial combinations: (i) Lp (monoculture), (ii) StLb (binary co-culture), and (iii) StLbLp (ternary co-culture), being the amount of inoculum added according to the microbial combinations. Flasks were kept in a 37 °C water bath and monitored by the CINAC® system (Ysebaert, Frépillon, France) until reaching pH 4.5. The CINAC® system allowed determining the following parameters: (i) maximum acidification rate (V_{max}) expressed in pH units per minute (upH/min), (ii) time to reach V_{max} (t_{Vmax}) expressed in hour, (iii) pH at t_{Vmax} (pH_{Vmax}), and (iv) times to reach pH 4.5 ($t_{pH4.5}$), 5.0 ($t_{pH5.0}$), and 5.5 ($t_{pH5.5}$) expressed in hour.

Refrigeration was performed either in a single step (direct cooling, DC) at 4 °C for 28 days, placing the fermented soymilk in an ice bath and then in a refrigerator, or in two steps (two-phase cooling, TPC), by preceding the above step by an additional intermediate cooling at 25 °C for 8 h. To perform cooling at 4 °C, the content of each flask was aseptically transferred to 50-mL polypropylene plastic pots covered with aluminum foil, sealed using a Selopar equipment (BrasHolanda, Pinhais, SP, Brazil) and stored at 4 °C for later analyses.

Analyses were performed either during refrigerated storage 1, 14, and 28 days after fermentation or at the start of storage (controls at 0 day). Since control samples were never statistically different from those after 1 day, they were disregarded in this study.

Microbiological analyses during storage

To count cells, 1.0-mL aliquots of fermented soymilk were homogenized with 9.0 mL of 0.1% (w/v) peptone water and serially diluted in the same medium. Media used to evaluate the viability of cells during 1, 14, and 28 days of storage (4 °C) were M17 agar medium (Difco, Detroit, MI, USA) supplemented with 10% (w/v) lactose for St, De Man, Rogosa, Sharpe medium (MRS, Difco) acidified to pH 5.4 with acetic acid [11] for Lb and MRS agar medium (Difco) supplemented with 0.5 mg/mL vancomycin [12] for Lp. The pour plate method was used, and the results were expressed in log CFU/mL of fermented soymilk.

Determination of fatty acid profile

The fatty acid profile of fermented soymilk was analyzed after 1, 14, and 28 days of storage, after lipid extraction [13] and subsequent fatty acid transesterification with methanol [14].

Fatty acid methyl esters were quantified in deproteinized soymilk with a gas chromatograph, model 430-GC (Varian, Darmstadt, Germany), using a fused silica column (100 mm × 0.25 mm × 0.2 μm). Injector and detector temperatures were set at 250 and 280 °C, while the column was initially maintained at 140 °C for 5 min, then heated to 240 °C at a rate of 4 °C/min and left at that temperature for 20 min. The volume of injected samples was 1.0 μL, and helium was used as carrier gas [15].

Sample composition was determined by comparing the peak retention times with those of the respective fatty acid standards by the Galaxie Chromatography Data System software, version 1.9 (Agilent Technologies, Santa Clara, CA, USA). Mass percentages were determined by area normalization [16]. All determinations were done in triplicate.

Determinations of pH, organic acids, and sugars

pH of fermented soymilk was determined after 1, 14, and 28 days of storage at 4 °C using a pH meter, model Q-400 M1 (Quimis, São Paulo, SP, Brazil).

Lactic and acetic acids and sugars (sucrose, glucose, and fructose) present in the fermented soymilk were also determined after the same storage times. After sample centrifugation at 14,462.5 *g* and 10 °C for 10 min, the supernatant was filtered through membranes with 0.45-μm pore diameter (Merck Millipore, Cork, Ireland) and stored at − 80 °C until analyses. Concentrations of glucose, sucrose, fructose, lactic acid, and acetic acid were determined by a high-performance liquid chromatograph, model LC-20A Prominence (Shimadzu, Kyoto, Japan), equipped with two pumps (LC-20AD), a degassing unit (DGU-20A), a self-injector (SIL-20ACHT), a column oven (CTO-20 AC), a refractive index detector (RI-210) (Shodex, Kawasaki, Kanagawa, Japan), and a 300 mm × 7.8 mm column, model HPX-87H (Aminex, Bio-Rad, Hercules, CA, USA). Analyses were performed at 50 °C using 0.003 M sulfuric acid as the mobile phase at a flow rate of 0.6 mL/min [17].

Survival of microorganisms to in vitro simulated gastrointestinal conditions

Survival of microorganisms in fermented soymilk submitted to in vitro simulated gastrointestinal stress was checked after the entire refrigerated storage period (28 days). For this purpose, we used a static digestion model [4] divided into three phases, namely, gastric phase, enteric phase I, and enteric phase II, each lasting 2 h. At the end of each phase, aliquots were withdrawn for cell enumeration using selective culture media as described in the "Microbiological analyses during storage" section. Samples, prepared in triplicate, were 1:10 (v/v) diluted in 0.85% (w/v) NaCl, and 10 mL of the diluted suspensions were transferred to 50-mL flasks.

To simulate the gastric phase, we added 1.0 N HCl (Labsynth, São Paulo, SP, Brazil) up to pH 2.4–2.7, 3.0 g/L pepsin (Pepsina, Henrifarma Produtos Químicos e Farmacêuticos, São Paulo, SP, Brazil), and 0.9 mg/L lipase (Amano lipase G, from *Penicillium camemberti*, Sigma-Aldrich, St. Louis, MO, USA). Flasks were incubated for 2 h in a shaker bath (Maxi-Shake, Heto-Holten, Allerød, Denmark) at 37 °C and 150 rpm. In the enteric phase I, the pH was increased to 6.0–6.3 using an alkaline solution of 10 g/L bile (Porcine bile, Sigma-Aldrich) and 1.0 g/L pancreatin (Pancreatina 3 NF, Henrifarma Produtos Químicos e Farmacêuticos). Flasks were again incubated at 37 °C under stirring for 2 h. In the enteric phase II, the pH was adjusted to 7.2–7.4 using the same solution as in the enteric phase I, and the flasks were again incubated at 37 °C under agitation for 2 h.

Statistical analysis

Results were expressed as means ± standard deviations and submitted to analysis of variance (ANOVA) using the Statistica 10.0 software (TIBCO, Palo Alto, CA, USA). Mean values were compared using the Tukey's test ($p < 0.05$). Differences between TPC and DC were analyzed for each microbial combination during the 28 days of storage.

Results and discussion

Acidification profile

As expected, the cultivation time was inversely related to the maximum acidification rate (V_{max}) (Table 1), whose mean values in the binary *Streptococcus thermophilus* and *Lactobacillus bulgaricus* co-culture (StLb) (9.1 upH/min) and ternary *S. thermophilus*, *L. bulgaricus*, and *Lactobacillus paracasei* co-culture (StLbLp) (10.8 upH/min) were significantly higher than in the *L. paracasei* monoculture (Lp) (5.8 upH/min). StLbLp showed in skim milk V_{max} values at 42 °C (20.0 upH/min) [18] and 36 °C (9.6 upH/min) [19] 46% higher and 11% lower, respectively, than that obtained in the present work in soymilk (10.8 ± 0.4 upH/min). These results suggest that an increase in temperature could have accelerated the metabolism of the thermophilic St species more than the presence of an easily-metabolizable sugar such as lactose in milk.

Regarding the acidification profiles of soymilk fermentation at 37 °C by lactic acid bacteria, the Lp monoculture took longer (15.2 h) to reach pH 4.5 than StLb (9.0 h), as expected by the well-known synergism between these last two species [18–21].

Viability of probiotic and starter cultures

Table 2 lists the cell counts of *L. paracasei*, *S. thermophilus*, and *L. bulgaricus* in Lp monoculture, StLb, and StLbLp in fermented soymilk after direct cooling (DC) or two-phase cooling (TPC). After culturing alone, Lp grew stably throughout storage, reaching counts between 9.38 and 9.50 log CFU/mL after DC and between 9.70 and 9.80 log CFU/mL after TPC. Looking at the combined effects of cooling protocol and type of microbial cocktail, Lp showed in ternary co-culture significantly lower counts after any storage time (8.59–9.25 log CFU/mL) than in monoculture (9.38–9.80 log CFU/mL) and higher counts after TPC (9.18–9.80 log CFU/mL) compared with DC (8.59–9.50 log CFU/mL). TPC may have been able to prevent such a microorganism from suffering a shock related to too rapid cooling. On the other hand, the cooling time seemed to have no significant effect, except on ternary culture in which Lp counts were very low after DC especially

Table 1 Acidification parameters of *L. paracasei* (Lp) monoculture, *S. thermophilus* and *L. bulgaricus* (StLb) binary culture, and *S. thermophilus*, *L. bulgaricus*, and *L. paracasei* (StLbLp) ternary culture in fermented soymilk at 37 °C until pH 4.5

Fermented soymilk	$t_{pH5.5}$ (h)	$t_{pH5.0}$ (h)	$t_{pH4.5}$ (h)	V_{max} (mUpH/min)	t_{Vmax} (h)	pH_{Vmax}
Lp	10.7 ± 0.3 a	12.4 ± 0.2 a	15.2 ± 0.1 a	5.8 ± 0.1 c	12.2 ± 0.3 a	5.0 ± 0.2 c
StLb	4.9 ± 0.3 b	6.1 ± 0.1 c	9.0 ± 0.1 c	9.1 ± 0.1 b	3.0 ± 0.1 c	6.1 ± 0.1 a
StLbLp	5.3 ± 0.1 b	6.6 ± 0.1 b	9.6 ± 0.3 b	10.8 ± 0.4 a	5.0 ± 0.5 b	5.7 ± 0.2 b

$t_{pH5.5}$ = time to reach pH 5.5 (representative of early exponential phase); $t_{pH5.0}$ = time to reach pH 5.0 (representative of central exponential phase); $t_{pH4.5}$ = time to complete the fermentation (representative of stationary phase); V_{max} = maximum acidification rate; t_{Vmax} = time to reach V_{max}; pH_{Vmax} = pH at V_{max}. Different letters in the same column mean statistically different values according to the Tukey's test ($p < 0.05$)

after 1 day of storage. These results taken together not only demonstrate that TPC was more effective in preserving Lp viability during storage than DC but also suggest that the simultaneous presence of two other microorganisms in the ternary culture could have impaired its metabolism or more simply competition for the same substrate occurred, thereby resulting in lower counts of this strain compared with the monoculture.

In general terms, St counts were almost stable throughout storage, and in ternary culture they were on average higher after TPC (9.26 log CFU/mL) than DC (8.77 log CFU/mL), similarly to Lp, and higher than in binary culture (8.74 log CFU/mL). The accelerated growth of St likely slowed that of Lp. Counts of St in ternary culture after DC (8.62–9.04 log CFU/mL) were very close to that observed in fermented milk (9 log CFU/mL) after a similar cooling protocol [21].

Lb viability in binary culture after either DC or TPC was on average 47.0 and 29.1% lower ($p < 0.05$) than in ternary culture, which suggests a stimulating action of Lp on Lb growth. Additionally, and unlike what was observed for the two other bacteria, there was a progressive reduction ($p < 0.05$) in Lb viability during storage in binary culture, especially that submitted to DC (about 3 log CFU/mL reduction). A possible explanation of Lb counts as low as 0.64 log CFU/mL in StLb-fermented samples submitted to TPC may be a progressive adaptation during exposure to intermediate temperature

Table 2 Viable cell counts (log CFU/mL) throughout storage (4 °C) in soymilk fermented by *L. paracasei* (Lp) monoculture, *S. thermophilus* + *L. bulgaricus* (StLb) binary culture, and *S. thermophilus* + *L. bulgaricus* + *L. paracasei* (StLbLp) ternary culture, using two different cooling protocols

Strains	Cooling protocol	Time (days)		
		1	14	28
L. paracasei				
Lp	DC	9.38 ± 0.08^{Ab}	9.43 ± 0.06^{Ab}	9.50 ± 0.06^{Aa}
	TPC	9.76 ± 0.05^{Aa}	9.80 ± 0.09^{Aa}	9.70 ± 0.15^{Aa}
StLbLp	DC	8.59 ± 0.04^{Ad}	8.99 ± 0.11^{Bd}	8.91 ± 0.07^{Bc}
	TPC	9.18 ± 0.03^{Ac}	9.21 ± 0.05^{Ac}	9.25 ± 0.08^{Ab}
S. thermophilus				
StLb	DC	8.88 ± 0.05^{Ac}	8.76 ± 0.11^{Ab}	8.59 ± 0.29^{Bab}
	TPC	9.00 ± 0.06^{Ab}	8.76 ± 0.29^{Ab}	8.47 ± 0.42^{Bb}
StLbLp	DC	8.62 ± 0.06^{Ac}	8.65 ± 0.05^{Ab}	9.04 ± 0.12^{Bab}
	TPC	9.26 ± 0.09^{Aa}	9.29 ± 0.04^{Aa}	9.23 ± 0.11^{Aa}
L. delbrueckii spp. *bulgaricus*				
StLb	DC	7.79 ± 0.15^{Ac}	5.72 ± 0.14^{Bc}	4.46 ± 0.21^{Bc}
	TPC	7.32 ± 0.10^{Ad}	7.27 ± 0.09^{Ab}	6.68 ± 0.05^{Bb}
StLbLp	DC	8.62 ± 0.01^{Ab}	8.84 ± 0.01^{Aab}	8.95 ± 0.15^{Aa}
	TPC	9.22 ± 0.06^{Aa}	9.25 ± 0.09^{Aa}	8.99 ± 0.16^{Aa}

The results are expressed as means ± standard deviations. Different superscript capital letters in the same line indicate a significant effect of the time of cold storage on counts of a given bacterium ($p < 0.05$). Different superscript lowercase letters in the same column indicate a significant combined effect of cooling protocol (DC or TPC) and microbial cocktail (mono-, binary or ternary cultures) on counts of a given bacterium ($p < 0.05$)

DC direct cooling, *TPC* two-phase cooling

[22]. Moreover, consistently with the well-known Lb ability to stimulate St growth through a synergistic relationship, Lb counts in binary culture were several orders of magnitude lower than those of St.

Likewise, both St and Lb showed improved viability when used together with a probiotic to ferment soybean extract or milk [3, 19, 21].

Post-acidification and concentrations of organic acids and sugars

Post-acidification profiles in fermented soymilk, as well as the concentrations of organic acids produced and sugars consumed by the different cultures, are listed in Table 3. In general, a significant reduction of pH ($p < 0.05$) took place during storage either by DC or TPC in almost all fermented samples.

The ternary culture, as well as the monoculture subjected to TPC, showed a significant pH reduction (0.32 pH units) just after 1 day of storage, likely due to the favorable temperature employed in the former step of TPC (25 °C) that still allowed bacterial metabolic activity [23]. On the other hand, StLbLp-fermented soymilk submitted to DC showed a significant pH decrease (0.65 pH units) only at the end of storage, consistently with the slowness of metabolic activities under these conditions. Likewise, *L. paracasei* showed low post-acidification capacity in fermented soybean, with a pH decrease of only 0.2 units after 28 days of refrigerated storage [24].

The results of Table 3 also show that the above post-acidification profiles were directly associated with the production of organic acids, especially lactic acid. In soymilk fermented by Lp alone and submitted to DC and TPC, lactic acid concentration at the end of storage was about 10 and 19 g/L, respectively. However, it was much lower in soymilk fermented by the binary culture, with values as low as about 3 and 7 g/L, respectively. So, lactic acid production by StLb was independent of the type of cooling, pointing out that even in refrigerated storage (4 °C) these bacteria were able to slowly metabolize soymilk sugars [17, 24].

Lp-fermented soymilk had higher acetic acid concentration throughout the whole storage period compared with that fermented by the ternary culture, consistently with Lp heterofermentative metabolism. Moreover, concentrations of both lactic and acetic acids in both cultures subjected to TPC were significantly higher than in the directly cooled ones, due to more favorable fermentation conditions.

Consistently with the above observation, at the end of storage, lactic acid concentration in StLb-fermented soymilk submitted to DC (3.4 g/L) was less than half of that submitted to TPC (7.0 g/L). However, contrary to what was observed in Lp-fermented soymilk, it did not show any statistically

Table 3 Values of pH and concentrations of acidic products and sugars in soymilks fermented by a monoculture of *L. paracasei* (Lp), a binary culture of *S. thermophilus* e *L. bulgaricus* (StLb) and a ternary culture of *S. thermophilus*, *L. bulgaricus*, and *L. paracasei* (StLbLp), after 1, 14, and 28 days of storage at 4 °C

Strains	Time (days)	Type of cooling	pH	Lactic acid (g/L)	Acetic acid (g/L)	Sucrose (g/L)	Glucose (g/L)	Fructose (g/L)
Lp	1	DC	4.06 ± 0.02^{cd}	4.76 ± 0.16^{bc}	0.52 ± 0.06^{d}	16.48 ± 0.10^{h}	nd	nd
		TPC	3.97 ± 0.01^{bc}	9.64 ± 0.73^{fg}	0.83 ± 0.11^{ef}	3.53 ± 0.70^{b}	2.38 ± 0.30^{d}	nd
	14	DC	3.69 ± 0.03^{a}	9.55 ± 0.22^{gh}	0.55 ± 0.14^{d}	13.39 ± 0.30^{e}	nd	$1.1 \pm 0{,}10^{bc}$
		TPC	3.66 ± 0.01^{a}	15.43 ± 0.40^{j}	0.91 ± 0.04^{fg}	5.39 ± 0.10^{c}	5.07 ± 0.20^{f}	nd
	28	DC	3.63 ± 0.04^{a}	10.83 ± 0.00^{hi}	0.70 ± 0.03^{e}	13.40 ± 0.30^{e}	1.12 ± 0.20^{c}	$2.9 \pm 0{,}50^{e}$
		TPC	3.60 ± 0.01^{a}	19.74 ± 0.79^{k}	1.01 ± 0.01^{g}	6.21 ± 0.00^{d}	6.27 ± 0.00^{g}	$1.7 \pm 0{,}30^{cd}$
StLb	1	DC	4.34 ± 0.01^{ef}	2.38 ± 0.04^{a}	nd	16.53 ± 0.10^{h}	0.75 ± 0.00^{b}	nd
		TPC	4.38 ± 0.01^{efg}	6.29 ± 0.23^{cde}	nd	0.86 ± 0.00^{a}	nd	nd
	14	DC	4.31 ± 0.01^{e}	2.71 ± 0.17^{a}	nd	17.96 ± 0.10^{i}	nd	nd
		TPC	4.42 ± 0.01^{efg}	6.94 ± 0.14^{de}	nd	3.13 ± 0.00^{b}	nd	nd
	28	DC	4.43 ± 0.05^{efg}	3.44 ± 0.09^{ab}	nd	21.53 ± 0.20^{k}	nd	nd
		TPC	4.47 ± 0.01^{fg}	7.04 ± 0.10^{de}	nd	3.26 ± 0.00^{b}	nd	nd
StLbLp	1	DC	4.49 ± 0.02^{g}	2.42 ± 0.35^{a}	nd	14.42 ± 0.20^{f}	nd	nd
		TPC	4.18 ± 0.01^{d}	7.48 ± 0.49^{ef}	0.38 ± 0.01^{bc}	0.97 ± 0.00^{a}	0.43 ± 0.00^{a}	nd
	14	DC	3.98 ± 0.08^{c}	5.75 ± 0.02^{cd}	0.20 ± 0.02^{a}	17.51 ± 0.30^{i}	nd	$0.7 \pm 0{,}30^{ab}$
		TPC	4.01 ± 0.05^{c}	10.24 ± 0.53^{gh}	0.42 ± 0.00^{bcd}	2.82 ± 0.00^{b}	2.48 ± 0.10^{d}	nd
	28	DC	3.85 ± 0.09^{b}	6.04 ± 0.62^{cde}	0.31 ± 0.00^{ab}	15.45 ± 0.30^{g}	nd	$1.0 \pm 0{,}10^{bc}$
		TPC	3.95 ± 0.08^{bc}	11.85 ± 0.93^{i}	0.45 ± 0.01^{cd}	3.23 ± 0.10^{b}	3.59 ± 0.10^{e}	$0.6 \pm 0{,}00^{ab}$

Different letters in the same column mean statistically significant differences according to the Tukey's test ($p < 0.05$)

nd not detected, *DC* direct cooling, *TPC* two-phase cooling

significant change during storage ($p > 0.05$) using either cooling protocol. On the other hand, acetic acid production was negligible, consistently with the homolactic nature of both St and Lb and contrary to some observations [24, 25].

Even in ternary culture, higher concentrations of lactic (7.5–11.8 g/L) and acetic (0.4 g/L) acids were observed in samples submitted to TPC (2.4–6.0 g/L) rather than to DC (≤ 0.2 g/L), but in all cases, they were always lower than using the Lp monoculture. These results taken together are consistent with the acetic acid released by *L. paracasei*, which was also observed when soymilk was fermented by another strain of this species [24].

Sugar concentrations are also listed in Table 3. In general, sucrose consumption was about 15 times higher in fermented soymilk submitted to TPC rather than to DC, regardless of both storage time and type of culture, confirming that a slighter decrease in temperature provided more favorable conditions to complete fermentation during storage. Glucose and fructose concentrations progressively increased in fermented soymilk submitted to either cooling protocol (Table 3), probably because at 4 °C lactic acid bacteria, and especially the thermophilic St, failed to quickly ferment these sugars resulting from sucrose hydrolysis [25].

Fatty acid profile in fermented soymilk

Fatty acid profile of fermented soymilk either before or during storage is illustrated in Figs. 1 and 2.

Concentrations of palmitic acid (C16:0) (10.7 g/100 g) and stearic acid (C18:0) (4.3 g/100 g) practically coincided with those reported by Peñalvo et al. [26] and did not show any significant difference depending on storage time, cooling protocol, and type of culture, or compared with unfermented soymilk taken as control. Unlike what was observed for palmitic and stearic acids, oleic acid (C18:1n9c) content after TPC (25.9 g/100 g) was, on average throughout the storage

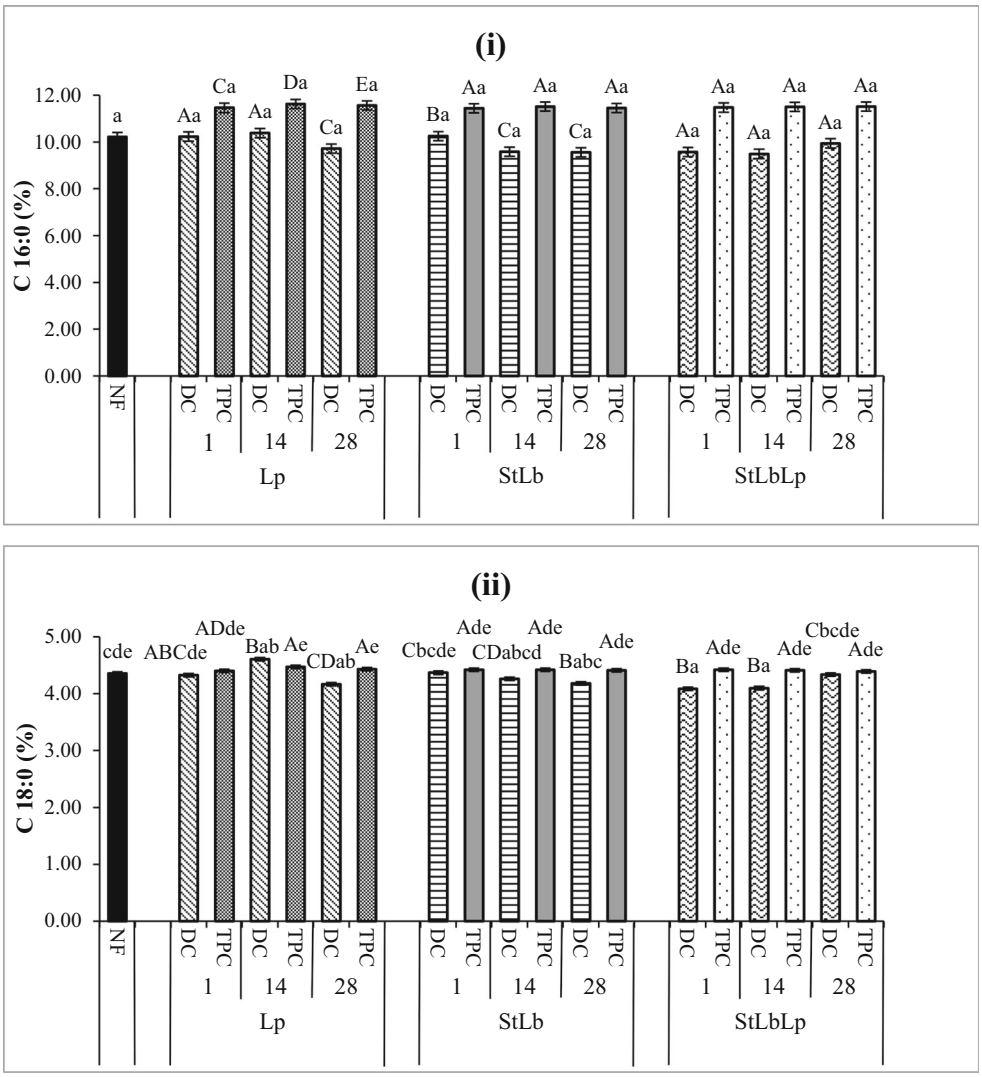

Fig. 1 Profile of saturated fatty acids (g/100 g of total fatty acids) in soymilk fermented by the monoculture of *L. paracasei* (Lp), binary culture of *S. thermophilus* and *L. bulgaricus* (StLb), and ternary culture of *S. thermophilus, L. bulgaricus* and *L. paracasei* (StLbLp) after 1, 14, and 28 days of storage at 4 °C. Palmitic acid C 16:0 (i), stearic acid C18:0 (ii). NF non-fermented soymilk taken as a control. Different superscript capital letters indicate statistically significant differences for a given culture ($p < 0.05$). Different superscript lowercase letters indicate statistically significant differences for all cultures taken as a whole ($p < 0.05$)

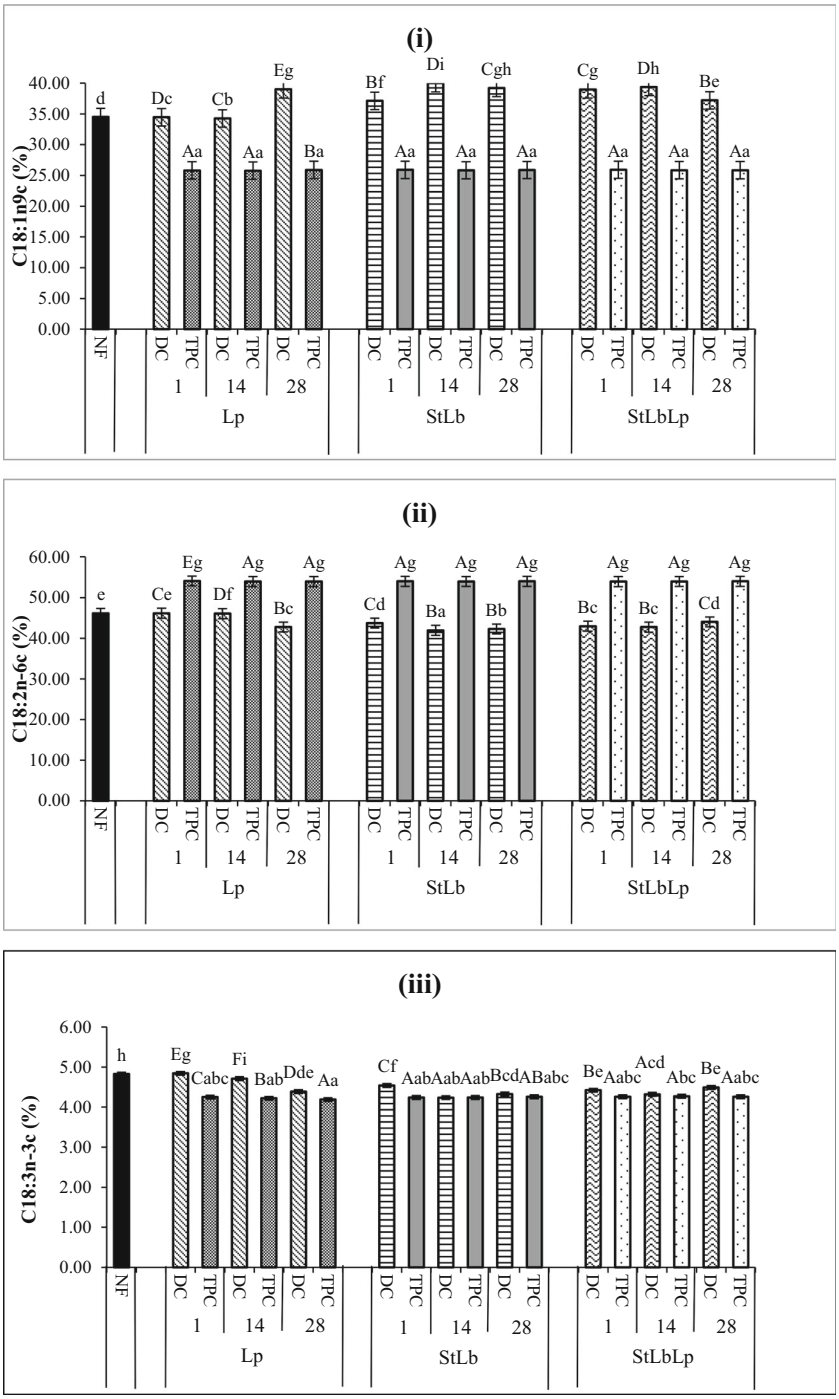

Fig. 2 Profile of unsaturated fatty acids (g/100 g of total fatty acids) in soymilk fermented by the monoculture of *L. paracasei* (Lp), binary culture of *S. thermophilus* and *L. bulgaricus* (StLb), and ternary culture of *S. thermophilus, L. bulgaricus* and *L. paracasei* (StLbLp) after 1, 14, and 28 days of storage at 4 °C. Oleic acid C18:1n-9c (i), linoleic acid C18:2n-6c (ii), and α-linolenic acid C18:3n-3c (iii). NF non-fermented soymilk taken as a control. Different superscript capital letters indicate statistically significant differences for a given culture ($p < 0.05$). Different superscript lowercase letters indicate statistically significant differences for all cultures taken as a whole ($p < 0.05$)

period and regardless of the type of culture, 31% lower than after DC and 25% lower than in unfermented soymilk. Conversely, linoleic acid (C18:2n-6c) content after TPC (54.0 g/100 g) was about 17% higher than in unfermented soymilk and 28% higher, on average, than after DC, without any significant influence of the storage time. Lee et al. [27] reported similar linoleic acid contents (32.9–59.9 mg/g) in some soybean powders. The α-linolenic acid (C18:3n-3c) content in samples submitted to TPC was not significantly influenced by the storage time or culture type, being on average 4.3 g/100 g, while an almost negligible decrease (of up to 0.3 g/100 g) was observed during DC of soymilk fermented by Lp and StLb.

These results suggest that the lactic acid bacteria employed in this study did not require a rearrangement of relative proportions of fatty acids in soymilk as those observed throughout milk fermentation and subsequent product storage [8, 15, 20].

Survival of microorganisms under in vitro gastrointestinal conditions

Lp either in monoculture or in binary or ternary co-cultures with St and Lb was submitted to in vitro simulated gastrointestinal stress to investigate the survival ability of each microorganism. It can be seen in Fig. 3 (i), which illustrates Lp survival before this treatment (0 h) or after the gastric phase (2 h), the enteric phase I (4 h), or the enteric phase II (6 h), that there was a reduction of only 3.6 log CFU/mL in its population in soymilks fermented by either the monoculture or the ternary culture, whereas cold storage did not have any statistically significant effect ($p > 0.05$). This resistance to acidic conditions agrees with the results of Reale et al. [23] and Bengoa et al. [28], who observed satisfactory Lp survival after treatment for 24 h at pH 3.5 or with a bile salt solution at pH 3.0.

As shown in Fig. 3 (ii), St viability in binary culture suffered, in the gastric phase, greater decrease (3.4 log CFU/mL) in samples submitted to DC than to TPC (2.0 log CFU/mL), confirming the positive influence of milder cooling on bacterial survival. On the other hand, the enteric phase I (4 h) led to

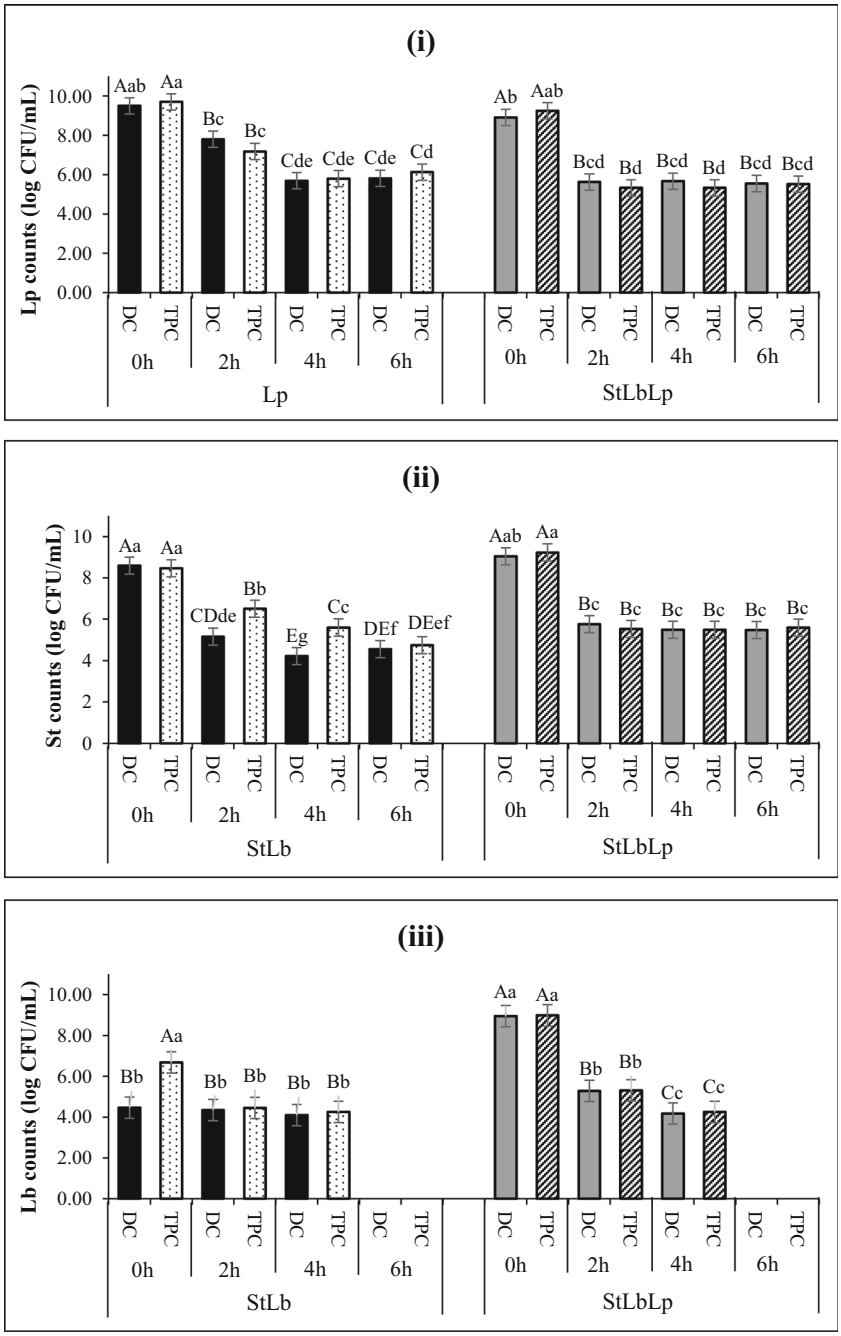

Fig. 3 Survival of *L. paracasei* (i), *S. thermophilus* (ii), and *L. bulgaricus* (iii) in soymilks fermented by a monoculture of *L. paracasei* (Lp), binary culture of *S. thermophilus* and *L. bulgaricus* (StLb), and ternary culture of *S. thermophilus, L. bulgaricus* and *L. paracasei* (StLbLp) after 28 days of storage at 4 °C, before (0 h) and during exposure to in vitro-simulated conditions of gastric phase (2 h), enteric phase I (4 h), and enteric phase II (6 h). The absence of any histogram means that the counts were < 100 CFU/mL. Different superscript capital letters indicate statistically significant differences for a given culture ($p < 0.05$). Different superscript lowercase letters indicate statistically significant differences for all cultures taken as a whole ($p < 0.05$)

an almost equal decline in viability (about 0.9 log CFU/mL) using either protocol, while no significant difference in counts was detected at the end of the enteric phase II. Counts in ternary culture showed a mean decrease of 3.6 log CFU/mL in the gastric phase, after either DC or TPC, and remained stable at around 5.5 log CFU/mL until the end of the enteric phase II. In this case, not even the type of cooling protocol influenced St survival. These results compare with those reported by Uriot et al. [29], who isolated three St strains from dairy products, used a similar digestion model and observed counts of about 5–6 log CFU/mL for all.

Counts of Lb in binary culture did not show statistically significant differences ($p > 0.05$) in DC-stored samples, whereas in those submitted to TPC there was a 2.43 log CFU/mL decrease at the end of the enteric phase I. On the other hand, Lb counts in ternary culture suffered an average drop of 4.74 log CFU/mL at the end of the enteric phase I, without any significant influence ($p > 0.05$) either of the treatment time or the cooling protocol (Fig. 3, iii). Moreover, such a microorganism completely lost its viability at the end of the enteric phase II. Likewise, Hou et al. [30], testing Lb resistance to pH 2 and bile salts in milk, detected viable cells only until 4 h after the start of simulation.

Despite the decline in bacterial viability during the in vitro gastrointestinal digestion, in general, the use of two-phase cooling did not significantly influence bacterial survival. Although using a different probiotic, Bedani et al. [4] reported a mean population of 4 log CFU/mL of *L. acidophilus* La-5 after the enteric phase II after 28 days of refrigerated storage of a fermented soy-based product.

Conclusions

The addition of *Streptococcus thermophilus* and *Lactobacillus bulgaricus* to a *Lactobacillus paracasei* culture accelerated soymilk fermentation. Two-phase cooling rather than direct cooling of soymilk fermented by a cocktail of these microorganisms showed a positive influence on their viability, giving counts around 9 log CFU/mL for all of them, even after 28 days of storage at 4 °C. The two-phase cooling protocol promoted substantial increases in the concentrations of linoleic and lactic acids compared with direct cooling. However, no influence of the cooling protocol was observed on bacterial survival to in vitro simulated gastrointestinal conditions, with both *S. thermophilus* and *L. paracasei* showing counts in the range 4.5–5.6 log CFU/mL after 6 h in the ending phase of this treatment. These results suggest that a cocktail of the tested microorganisms could viably reach the gastrointestinal tract and exert its benefits on the health of the host during most of its crossing. However, in vivo studies are needed to prove this hypothesis. Based on these results it can be concluded that the use of ternary cultures and two-stage cooling can significantly improve fermentation, production of organic acids, and viability of the strains, mainly *L. paracasei*; therefore, they may be considered new methodologies for obtaining fermented soy products.

Acknowledgments The authors thank Danisco-DuPont for the bacterial cultures.

Funding information This study was financed in part by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior—Brasil (CAPES)—Finance Code 001, the National Council for Scientific and Technological Development (CNPq), and the São Paulo Research Foundation (FAPESP process no. 2019/19054-0).

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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