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Beneficial effects of fermented vegetal beverages on human gastrointestinal microbial ecosystem in a simulator

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ABSTRACT

The aim of this study was to evaluate the effects of four beverage formulations (prebiotic — fructooligosaccharide, probiotic — *Lactobacillus casei* Lc-01, synbiotic — fructooligosaccharide and *L. casei* Lc-01 and placebo) based on aqueous extracts of soy and quinoa, towards the human intestinal microbiota using the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®), a dynamic model of the human gut. To monitor the effects on microbial community composition, plate counts on specific growth media and a PCR–DGGE analysis were performed on samples from all colon compartments — ascending, transverse and descending. To verify the effects on microbial metabolism, we analyzed the ammonium and short chain fatty acids (SCFAs) concentrations. The synbiotic beverage showed the best microbiological results in the ascending colon compartment, stimulating the growth of *Lactobacillus* spp. and *Bifidobacterium* spp., and reducing *Clostridium* spp., *Bacteroides* spp., enterobacteria and *Enterococcus* spp. populations in this compartment. A larger reduction ($p < 0.05$) of ammonia ions in the ascending colon was observed during the synbiotic beverage treatment. No statistical difference was observed in SCFA production among the treatments and the basal period. Plate count and DGGE analysis showed the survival of *L. casei* Lc-01 in the colon. DGGE analysis also showed higher richness and diversity of the *Lactobacillus* spp. community during the treatment with synbiotic beverage, with higher accentuation in the ascending colon.

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1. Introduction

The intestinal microbiota is a complex community of microorganisms that colonizes the gastrointestinal tract. About 10^{14} intestinal microbes belonging to >1000 different species-level phylogenetic types are distributed along the human gastrointestinal tract, with the highest densities reach in the colon (Frick & Autenrieth, 2013; Payne, Zihler, Chassard, & Lacroix, 2012; Rajilic-Stojanovic, Smidt, & De vos, 2007).

Although the colonic microbiota is relatively stable throughout adult life, age-related changes in the gastrointestinal (GI) tract, as well as changes in diet and immune system reactivity, inevitably affect population composition (Woodmansey, 2007). Many efforts have focused on the modulation of the colonic microbiota and its metabolic activities in search of the community balance. The inclusion of foods containing prebiotics and probiotics in the daily diet can help to improve the gut health by increasing the number of beneficial bacteria and reducing the harmful ones, in addition to alleviate diarrhea symptoms (Possemiers, Marzorati, Verstraete, & Van de Wiele, 2010).

However, 16S rRNA-based cataloguing of gut microbiota via high-throughput sequencing platforms does not provide information on the functionality of any species identified. Unraveling the complexity of microbe–microbe interactions and identifying niches central to gut fermentation depend on functional studies. In vitro gut fermentation models represent an innovative technological platform consisting of multiple model designs, which permit investigations on the existence of gut microbial species (Payne et al., 2012).

The Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) is an in vitro system proven to be a very useful model for nutrition studies, in terms of analysis of the intestinal microbial community composition (Kontula et al., 2002; Molly, Vandewoestyne, Desmet, & Verstraete, 1994; Possemiers et al., 2010; Sivieri et al., 2013). It is a five-stage sequential reactor system simulating the different parts of the gastrointestinal tract, based on the model developed by Molly et al. (1994), optimized by De Boever, Deplancke, and Verstraete (2000) and validated by Possemiers, Verthé, Uyttendaele, and Verstraete (2004). The SHIME harbors a stable microbial community, representative of the

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human gut microbiota, both in fermentation activity and in composition (Molly et al., 1994).

The beverages used in this study, prepared with aqueous extracts of quinoa and soy, were acidified by probiotic microorganisms and/or with added fructooligosaccharide (FOS). These beverages may contribute to the intestinal microbiota health, influenced by the probiotic and prebiotic action and the bioactive constituents of soy and quinoa. Besides having all the essential amino acids and fatty acids of high quality (ω6, ω3 and ω9), quinoa is an excellent example of functional food that aims at lowering the risk of various diseases (Ando et al., 2002; Vega-Gálvez et al., 2010). In addition, it is free from cholesterol, lactose and gluten, as the soybean. The synbiotic beverage, based on aqueous extract of soy and quinoa, presents proper physicochemical composition, and good acceptability and provides high viability to the probiotic microorganism during storage. However, its action in the human intestinal microbiota has not been studied and proven yet (Bianchi et al., unpublished data). Therefore, the aim of this study was to investigate the capacity of the synbiotic, prebiotic, probiotic and placebo beverages, based on aqueous extract of soy and quinoa, to temporarily modulate the intestinal microbiota after oral administration using the SHIME® reactor system. This paper also evaluates the viability of *L. casei* Lc-01 after the passage through simulated stomach and duodenum conditions.

2. Material and methods

2.1. Production of quinoa and soy extracts

Quinoa grains were washed and rubbed manually in water to remove the antinutritional components (saponins) and, consequently, reduce the bitter aftertaste. They were immersed in water (55 g/L) and boiled for 10 min. Later, the grains and water were blended to a homogeneous mixture, which was filtered on a 250 mu-mesh screen to obtain the extract.

The aqueous extract of soybean was produced by the Soy Derivative Development and Production Unit, UNIVERSOJA, at the Faculty of Pharmaceutical Sciences of UNESP (Araraquara, Brazil), as described by Rossi, Vendramine, Carlos, Pei, and Valdez (1999).

2.2. Production of synbiotic, prebiotic, placebo and probiotic beverages

The four beverages were produced with 30% aqueous extracts of quinoa and 70% soy extract (89.5% of the extracts mixture), 6% sucrose (União, Brazil), 0.8% soybean oil (Liza, Brazil), 1% food grade lactose (Purac, Brazil), 0.14% Recodan TM RS-B stabilizer (Danisco, Brazil) and 2.5% milk powder (Molico, Brazil). All beverages were elaborated according to Rossi, Reddy, and Silva (1984), differing only in acidification form and FOS content (Table 1).

All beverages were pasteurized prior to microorganism inoculation or acidification. Lactic acid PA (85%) was used to acidify the placebo and prebiotic beverage to pH 4.8. In formulations with added FOS, 3% of fructooligosaccharides (FOS-SKL Pharma, Brazil) was used after the pasteurization.

A commercial lyophilized culture of *Lactobacillus casei* (Lc-01) was activated in a milk medium (10% milk, 1% glucose, 0.5% yeast extract) (2% v/v) and kept at 37 °C for 15 h (until the stationary phase) under aerobic conditions. Subsequently, 2% (v/v) of activated culture (10^8 UFC/mL) was added to the synbiotic and probiotic beverages and incubated at 37 °C until reaching pH 4.8.

Table 1
Differences in the preparation of synbiotic, prebiotic, probiotic and placebo beverages.

Beverage	FOS	Acidification
Synbiotic (TS)	+ FOS	Acidified with *L. casei* Lc-01
Prebiotic (Tpe)	+ FOS	Artificially acidified
Probiotic (Tpo)	− FOS	Acidified with *L. casei* Lc-01
Placebo (Tpa)	− FOS	Artificially acidified

2.3. Long-term SHIME run

The SHIME (registered tradename from Ghent University and ProDigest) is a simulator of the human intestinal microbial ecosystem (Molly et al., 1994) in which environmental conditions (pH, residence time and temperature) are controlled (Table 2). It consists of five double-jacketed vessels simulating the stomach, the duodenum, and the ascending (R3), transverse (R4) and descending colon (R5) (Fig. 1).

The five reactor vessels were continuously stirred by means of a magnetic stirrer and the temperature was kept at 37 °C. The system was maintained anaerobically through a daily N_2 flushing of 30 min. The pH culture of vessels 3, 4 and 5 was automatically adjusted by the addition of NaOH 1 M or HCl 0.1 M and HCl 1 M for the stomach region (Molly et al., 1994; Possemiers et al., 2004). Peristaltic pumps a–d (Fig. 1) worked semi-continuously, while the remainder (e–g) worked continuously, simulating the gastrointestinal tract.

2.4. Intestinal microbiota stabilization and experimental protocol

The reactor setup and the composition of the liquid feed (Table 3), which entered the system three times per day, were previously described by Possemiers et al. (2004).

The three colon vessels of the SHIME system were inoculated with bacteria from a fecal sample of a healthy 26-year-old adult female with no history of antibiotic treatment six months prior to the study. Aliquots (20 g) of fresh fecal samples were diluted and homogenized with 100 mL of sterilized phosphate buffer (0.1 M/L, pH 6.5), containing 1 g/L sodium thioglycolate as the reducing agent. The microbial inoculum was stabilized over two weeks (basal period) on a carbohydrate-based medium (liquid feed) and allowed to adapt to the specific environmental conditions of the ascending, transverse and descending colon, in terms of pH range, retention time and available carbon sources (Molly et al., 1994). After two weeks of stabilization, treatments with synbiotic, prebiotic, placebo and probiotic beverages were performed. The beverages were added along with the basal feed in a proportion equivalent to 10^8 CFU/mL in the final mixture. The protocol began with two weeks of basal period followed by four weeks of treatment with synbiotic beverage (TS), two weeks of post treatment (PT) (when the passage of beverages was ceased, with only the liquid feed running in the system), two weeks of prebiotic beverage treatment (TPe), two weeks of treatment with the placebo beverage (TPa), ending with two weeks of probiotic beverage treatment (TPo). The complete protocol, based on Kontula et al. (2002), is shown in Fig. 2.

2.5. Viability of the probiotics in the stomach and duodenum

The viability of *L. casei* in the stomach and duodenum passages was assessed in a sequential batch experiment designed to simulate the conditions and residence time in these organs. The beverages were added along with the liquid feed, a liquid start matrix based on a complex feed, which is normally dosed to the SHIME in a proportion equivalent to 10^8 CFU/mL in the final mixture (Possemiers et al., 2004).

Table 2
Values of volume, residence time and pH established in each of the reactors of the SHIME system.
Source: Possemiers et al. (2004).

Reactor	Volume (mL)	Residence time (h)	pH
R1: stomach	200	2.5	2.2–2.4
R2: duodenum	200	4.0	–
R3: ascending colon	500	20.0	5.6–5.9
R4: transverse colon	800	32.0	6.1–6.9
R5: descending colon	600	24.0	6.6–6.9

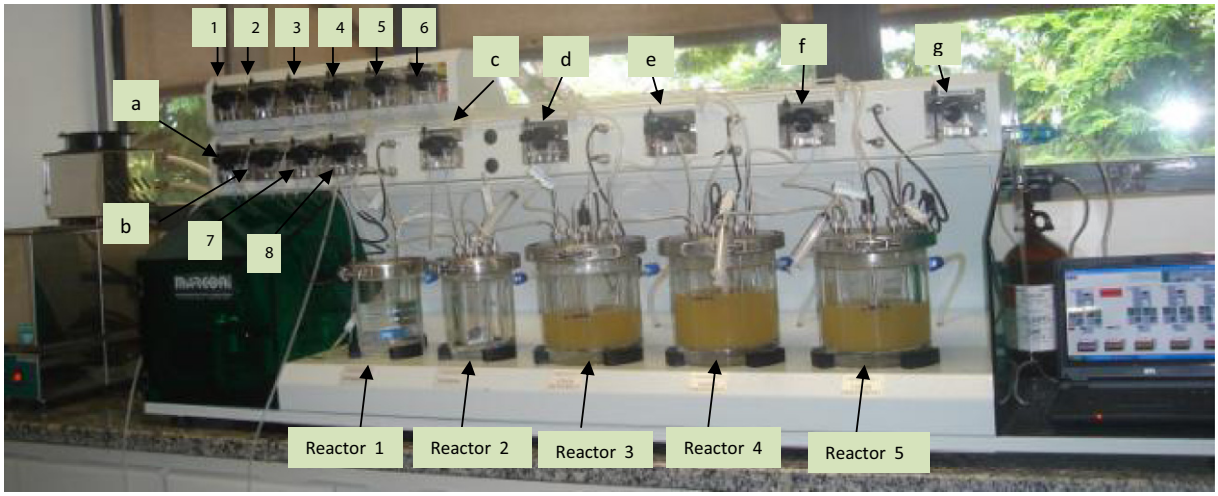

Fig. 1. Photography of Simulator of the Human Intestinal Microbial Ecosystem (SHIME®). Reactor 1 = stomach; reactor 2 = duodenum; reactor 3 = ascending colon; reactor 4 = transverse colon; reactor 5 = descending colon; a = pump that takes the sample to the stomach; b = pump that takes the pancreatic juice to the duodenum; c = Transfer pump of the stomach to the duodenum; d = Transfer pump of the duodenum to ascending colon; e = Transfer pump of the ascending colon to transverse colon; f = transfer pump of the transverse colon to descending colon; g = transfer pump of the descending colon to the final discard; 1 = control pump of reactor 3 pH (acid); 2 = control pump of reactor 3 pH (base); 3 = control pump of reactor 4 pH (acid); 4 = control pump of reactor 4 pH (base); 5 = control pump of reactor 5 pH (acid); 6 = control pump of reactor 5 pH (base); 7 = control pump of stomach pH (base); 8 = control pump of stomach pH (acid).

The stomach environment was simulated at pH 2 with 1 g/L pepsin, under microaerophilic conditions for 1.5 h at 37 °C. Pancreatic solution (per liter: 12.5 g $NaHCO_3$, 6 g Oxgall and 0.9 g pancreatin) was added and incubated under anaerobic conditions for 2 h at 37 °C to simulate the duodenum environment. Before the start of the experiment and after half and complete incubation under respective stomach and duodenum conditions, a sample was taken for plate count analysis to assay the strain viability. One mL of each sample was suspended in 9 mL of peptone water. Serial dilutions were prepared and cultured on MRS Agar (Himedia, India) at 37 °C/48 h under anaerobic conditions.

The evaluation of *L. casei* Lc-01 survival was also carried out using the culture-independent molecular method: polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE). The used protocol is explained below.

2.6. Microbiological analyses

2.6.1. Plate counts

At weekly intervals, throughout the entire experimental period (basal, treatments and post-treatment), 5 mL samples were collected from the reactors for plate counts. The analysis of the intestinal microbiota composition was based on the enumeration of total aerobic and anaerobic bacteria, *Enterococcus* spp., *Lactobacillus* spp., *Bifidobacterium* spp., enterobacteria, *Bacteroides* spp. and *Clostridium* spp. One mL of a sample taken from each reactor was suspended in 9 mL of peptone water. Serial dilutions were prepared and inoculated into selective culture media, as shown in Table 4.

Table 3
Ingredients (g) employed for each liter of the liquid feed used in the SHIME reactor. Source: Payne et al.(2003).

Ingredients	Quantity (g/L)
Starch (Maizena, Brasil)	3.0
Pectin (Vetec, Brasil)	2.0
Mucin (Sigma, USA)	4.0
Xylan (Sigma, USA)	1.0
Peptone (Acumedia, USA)	1.0
Arabinogalactan (Sigma, USA)	1.0
Glucose (Synth, Brasil)	0.4
Yeast extract (Acumedia, USA)	3.0
L-cysteine (Sigma, USA)	0.5
Sterile distilled water	Amount required to complete 1 L

2.6.2. Behavior and diversity of Lactobacillus spp. in the colon

Molecular methods (PCR–DGGE) were used to analyze the behavior and diversity of the *Lactobacillus* spp. community through the entire experimental period.

The QIAamp DNA Stool Mini Kit (Qiagen, Germany) was employed to extract DNA from each reactor vessel, at each period of study and from the lyophilized culture of *L casei* Lc-01. "Isolation of DNA from Stool for Human DNA" (QIAGEN, 2010) was the protocol used in this study with modifications in the amount of the initial sample (220 mg to 2 mL) and AE buffer (200 mu to 50 mu). DNA yield was quantified using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, USA). The specific primers used as starting point for DNA replication were Lab159f (5′-GGAAACAGATGCTAATACCG-3′) and Lab677GCr (5′GCCCGGGGCGCGCCCCGGGCGGG GCGGGGGCACGGGGGGGCACCG CTACACATGGAG-3′) (Heilig et al., 2002). PCR was performed using the Taq polymerase (Invitrogen, Brazil). Samples were amplified in a Veriti® 96-well thermal cycler (Applied Biosystems, USA) by using the following program: initial denaturation at 94 °C for 2 min, 35 cycles of denaturation at 94 °C for 30 s, annealing of primer at 56 °C for 40 s, elongation at 72 °C for 1 min and extension at 72 °C for 5 min, followed by a final cooling to 4 °C.

Electrophoresis was carried out as previously described (Heilig et al., 2002) in an 8% polyacrylamide gel with a denaturant gradient of 30–50% for 16 h at 85 V in a 0.5× TAE buffer at a constant temperature of 60 °C. Gels were stained with ethidium bromide according to Sanguinetti, Dias Neto, and Simpson (1994), scanned at 400 d.p.i., and further analyzed by the BioNumerics 6.0 software (Applied Maths, Kortrijk, Belgium).

The distance matrices of each DGGE based on the Jaccard similarity coefficient to cluster the samples, were analyzed using the BioNumerics 6.0 software (Applied Maths, Kortrijk, Belgium).

2.6.3. Ecological interpretation of fingerprint of Lactobacillus spp. population

Ecological interpretation of fingerprint (range-weighted richness and functional organization) was conducted as suggested by Marzorati, Wittebolle, Boon, Daffonchio, and Verstraete (2008).

The range-weighted richness (*Rr*) is correlated with the distribution of the bands in the DGGE pattern and the percentage denaturant

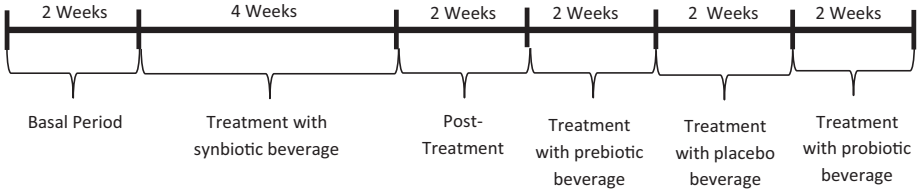

Fig. 2. Experimental SHIME® protocol.

gradient of the gel needed to represent the sample's total diversity (within the limits of the technique). This concept can be mathematically expressed by defining an *Rr* index = ($N^2 \times Dg$), where N represents the total number of bands in the pattern, and Dg the denaturing gradient comprised between the first and the last band of the pattern (Marzorati et al., 2008).

The functional organization (Fo) is the result of the action of the microorganisms that are most fitting to the ongoing environmental–microbiological interactions (Marzorati et al., 2008). Pareto–Lorenz distribution curves were created to graphically represent the uniformity of *Lactobacillus* spp. communities, based on DGGE profile and evaluated on the 20% level of the x axis, as previously described (Lorenz, 1905; Mertens, Boon, & Verstraete, 2005; Wittebolle, Vervaeren, Verstraete, & Boon, 2008).

2.7. Ammonium analysis

Samples were collected once a week from the vessels for ammonium analysis, throughout the entire experimental period (basal, treatments and post-treatment). The ammonia content was determined using a selective ion meter (710A model, Orion) coupled to an ammonia selective-ion electrode (Orion 95–12). The apparatus was calibrated using 0.1 M standard ammonium chloride solutions, at 10, 100, and 1000 mg/L of ammonia. A total of 0.5 mL ISA solution (Ionic Strength Adjuster, Orion), a pH-adjusting and ionic force solution, was added to every 25 mL of sample. All measurements were carried out at 25 °C (Bedani, 2008). The analyses were performed in triplicate.

2.8. Analysis of short-chain fatty acids (SCFAs)

Samples were collected weekly from the reactors for SCFA analysis throughout the entire experimental period (basal, treatments and post-treatment). The analyses were carried out in triplicate and the samples were frozen at −20 °C. The SCFAs were determined using a gas chromatograph equipped with a flame-ionization gas detector, a capillary split/splitless injector and a HP-INNOWAX column with a 30 m × 0.25 mm × 0.25 μm inlet (Shimadzu GC2010). Hydrogen was the carrier gas at a flow rate of 1.56 mL/min. The temperatures of the column, injector and detector were 170, 250 and 280 °C, respectively (Van de Wiele, Boon, Possemiers, Jacobs, & Verstraete, 2007).

2.9. Statistical analysis

The significance of all results was investigated with one-way ANOVA, and individual means were compared using the Tukey's test ($p < 0.05$), using the Sigma Stat 5.0 software (Systat Software Inc., San Jose, California).

3. Results and discussion

3.1. Viability of the probiotic in the stomach and duodenum

Plate counts showed an enhanced protection of *L. casei* Lc-01 with the synbiotic beverage. There was no decrease in *L. casei* Lc-01 viability under stomach conditions during the treatment with the synbiotic beverage, although a reduction of 1 log CFU/mL was observed under duodenum conditions (Fig. 3). In the treatment with the probiotic beverage, a significant reduction in the viability of *L. casei* was observed under stomach and duodenum conditions, indicating that the association between the probiotic microorganism and prebiotic FOS provides a positive effect on *L. casei* Lc-01 survival under acid conditions (Fig. 3).

Malolactic fermentation and histidine accumulation were revealed as important features of acid adaptation in *L. casei*. Malolactic enzyme was upregulated 16-fold and 7-fold following 5 and 20 min of acid exposure, respectively. This enzyme functions to decarboxylate L-malate to L-lactate and CO_2, thus contributing to cytoplasm alkalization (Mills, Stanton, Fitzgerald, & Ross, 2011).

Guergoletto, Magnani, Martin, Andrade, and Garcia (2010) affirm that *L. casei* Lc-01 has good resistance to the gastric and enteric juices. According to Mishra and Prasad (2005), the ability to tolerate acid and pancreatic juice can vary from one strain to another. Pozza, Miglioranza, Garcia, Garcia, and Pozza (2011) studied different *Lactobacillus* strains isolated from feces of children and confirmed eight strains resistant to acidic conditions and bile salts.

Xanthopoulos, Litopoulou-Tanetaki, and Tzanetakis (2000) affirm that bile salt resistance tends to vary between the lactic acid bacteria and among strains of same species. According to Gilliland (1987) and Saarela, Mogensen, Fondén, Matto, and Mattila-Sandholm (2000), bile salts are toxic to cells because they disrupt the cellular membrane structure and, therefore, bile salt tolerance is considered one of the required characteristics for the survival of lactic acid bacteria in the duodenum. Despite the reduction of *L. casei* Lc-01 population (1 log CFU/mL) after the passage of the microorganism through the duodenum, in both synbiotic and probiotic beverages, *L. casei* preserved the viability

Table 4
Culture medium and culture conditions used in plate count analysis.

Genus	Culture medium	Brand	Time/temperature	Oxygen condition	Reference
Lactobacillus	MRS agar	Himedia (India)	37 °C/48 h	Anaerobiosis	Yoshioka, Iseki, and Fujita (1983)
Bifidobacterium	Bifido Medium BIM-25	Difco (France)	37 °C/72 h	Anaerobiosis	Munoa and Pares (1988)
Clostridium	RCA agar	Difco (France)	37 °C/48 h	Anaerobiosis	Marzotto et al. (2006)
Enterobacteia	MacConkey agar	Acumedia (USA)	37 °C/48 h	Anaerobiosis	Brigidi, Vitali, Swennen, Bazzocchi, and Matteuzzi (2001)
Enterococcus	KF *Streptococcus* agar	Acumedia (USA)	37 °C/48 h	Aerobiose	Edlund et al. (2000)
Bacteroides	BE agar	Acumedia, (USA)	37 °C/120 h	Anaerobiosis	Livingston, Kominos, and Yee (1978)
Total anaerobes	Standard methods agar	Acumedia (USA)	37 °C/48 h	Anaerobiosis	Yoshioka et al. (1983)
Facultative aerobes	Standard methods agar	Acumedia (USA)	37 °C/48 h	Aerobiose	Yoshioka et al. (1983)

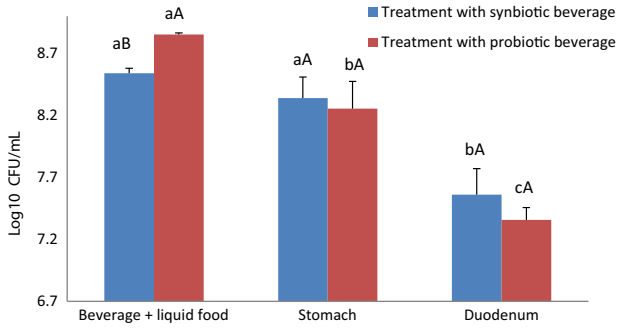

Fig. 3. *L. casei* population (log CFU/mL) in synbiotic and probiotic beverages with added liquid feed and under stomach and duodenum conditions. Different lowercase letters represent a significant difference ($p \leq 0.05$) in *L. casei* population to the same treatment between the two compartments and the liquid feed added to beverage, whereas different uppercase letters represent a significant difference ($p \leq 0.05$) in *L. casei* population between the treatments.

upon entering the first colon compartment, maintaining an average of 7.45 ± 0.14 log CFU/mL.

DGGE fingerprints for lactobacilli (Fig. 4) indicated that *L. casei* Lc-01 survived the stomach and duodenum conditions and, subsequently, reached the colon during the experiment period. In the last two weeks of the treatment with synbiotic beverages (TS3 and TS4), there was an intensification of the bands that ran in the same position of the pure *L. casei* Lc-01 culture (Fig. 4a). The inverse occurred in the post-treatment (PT1 and PT2), when the passage of synbiotic beverage was ceased. Fig. 4b shows that the appearance of equivalent bands to *L. casei* Lc-01 (control) is observed only when the treatment was performed with the probiotic beverages (TPo). When the prebiotic (Tpe) and placebo (TPa) beverages were analyzed, it is clear that few or no band is visualized running in the same position of pure *L. casei* Lc-01 culture. According to Capela, Hay, and Shah (2006), the prebiotics provide extra solids, which tend to protect cells from injury. Oligosaccharides used in this study are hydrocolloids, which play a protective role towards probiotic microorganisms (Desai, Powell, & Shah, 2004). This way, the microorganism can provide its probiotic beneficial effects more effectively.

3.2. *L. casei* Lc-01 capacity to temporarily colonize the colon in the SHIME experiment

As reflected in the plate count data (Table 5), the administration of the synbiotic beverage to the system induced a significant increase ($p < 0.05$) in lactobacilli counts, with a concentration increase of at least 1 log CFU/mL in the ascending colon compartment. There was an increase of *Bifidobacterium* spp. and a decrease of *Clostridium* spp., *Bacteroides* spp., enterobacteria and *Enterococcus* spp. populations. Regarding the other beverages, a decrease in *Enterococcus* spp., *Clostridium* spp. and enterobacteria was observed. However, the results were more significant ($p < 0.05$) in the treatment with synbiotic beverage, both in relation to the increase of beneficial bacteria and the reduction of pathogenic ones.

DGGE analysis was used to monitor qualitative changes in the composition and structure of the microbial communities in the three compartments simulating colon conditions. The dendrograms from the ascending (R3), transverse (R4) and descending colon (R5) showed

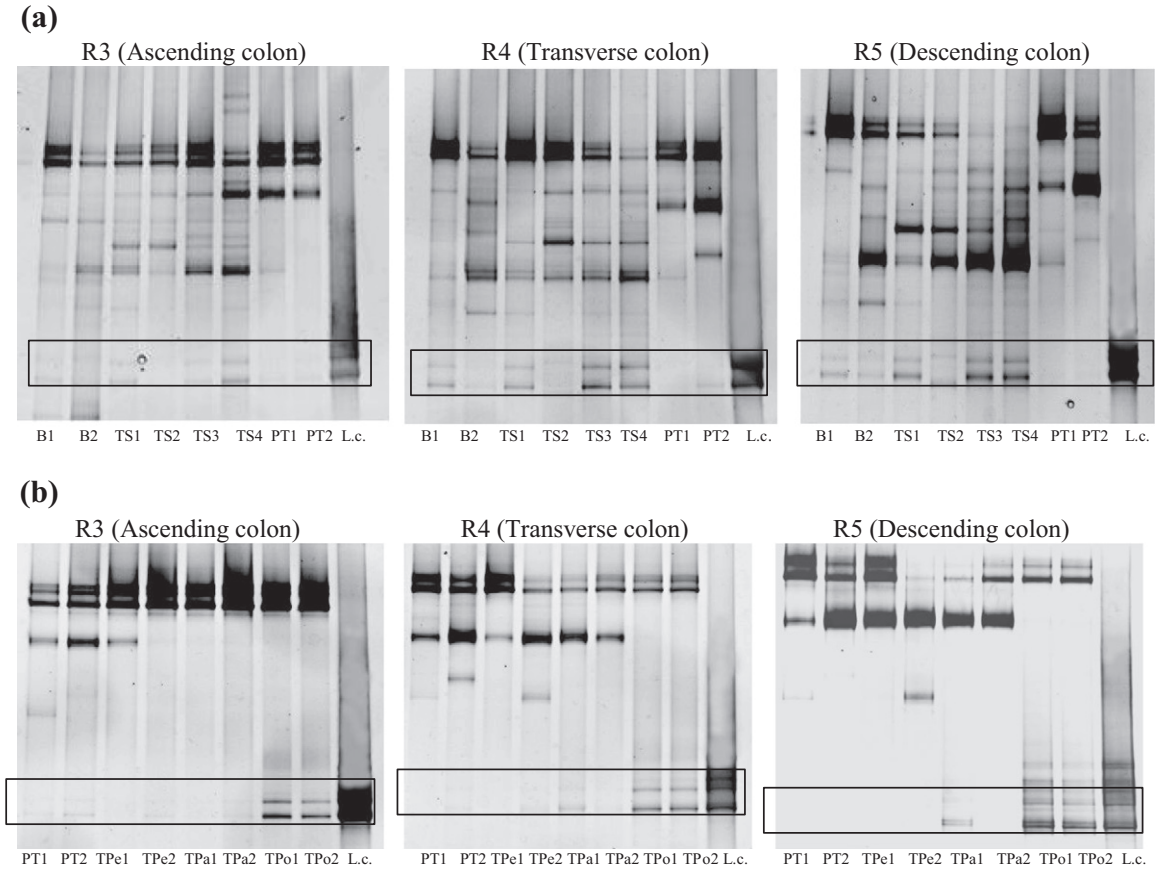

Fig. 4. *L. casei* survival in the three simulating colon regions by denaturing gradient gel electrophoresis (DGGE) during the treatments with synbiotic (a), prebiotic, placebo and probiotic beverages (b). B1: basal period (week 1); B2: basal period (week 2); TS1: synbiotic treatment (week 1); TS2: synbiotic treatment (week 2); TS3: synbiotic treatment (week 3); TS4: synbiotic treatment (week 4); PT1: post-treatment (week 1); PT2: post-treatment (week 2); L.c. pure culture of *L. casei* Lc-01; TPe1: prebiotic treatment (week 1); TPe2: prebiotic treatment (week 2); TPa1: placebo treatment (week 1); TPa2: placebo treatment (week 2); TPo1: probiotic treatment (week 1); TPo2: probiotic treatment (week 2).

Table 5
Average plate count measurements (±SEM), expressed in log CFU/mL, for the different microbial groups, SHIME® compartments and periods.

	B	TS	PT	Tpe	TPa	TPo
R3 (Ascending colon)						
Lactobacillus spp.	7.88 ± 0.06^{C}	8.39 ± 0.02^{A}	8.40 ± 0.03^{A}	7.29 ± 0.08^{D}	8.04 ± 0.02^{B}	8.11 ± 0.07^{B}
Bifidobacterium spp.	7.75 ± 0.00^{C}	8.21 ± 0.01^{B}	8.44 ± 0.02^{A}	7.48 ± 0.01^{D}	7.31 ± 0.10^{E}	7.82 ± 0.06^{C}
Clostridium spp.	8.44 ± 0.04^{A}	8.02 ± 0.00^{BC}	8.35 ± 0.01^{AB}	7.01 ± 0.01^{D}	8.66 ± 0.01^{A}	7.97 ± 0.01^{C}
Enterococcus spp.	7.45 ± 0.21^{B}	4.73 ± 0.07^{D}	8.77 ± 0.01^{A}	6.66 ± 0.08^{C}	7.86 ± 0.09^{B}	6.53 ± 0.28^{C}
Enterobacteria	9.23 ± 0.07^{A}	7.45 ± 0.03^{C}	7.61 ± 0.06^{B}	7.09 ± 0.00^{D}	7.19 ± 0.07^{D}	5.79 ± 0.04^{E}
Total aerobes	8.20 ± 0.07^{A}	7.74 ± 0.03^{B}	8.39 ± 0.03^{A}	7.68 ± 0.05^{B}	7.27 ± 0.02^{C}	7.80 ± 0.02^{B}
Facultative anaerobes	8.13 ± 0.05^{C}	7.68 ± 0.05^{D}	8.89 ± 0.03^{A}	7.23 ± 0.11^{E}	8.59 ± 0.05^{B}	8.06 ± 0.02^{C}
Bacteroides	4.94 ± 0.05^{B}	2.58 ± 0.11^{D}	4.43 ± 0.02^{B}	5.46 ± 0.05^{A}	5.47 ± 0.12^{A}	4.04 ± 0.16^{C}
R4 (Transverse colon)						
Lactobacillus spp.	7.82 ± 0.02^{A}	7.04 ± 0.03^{BC}	6.91 ± 0.02^{C}	7.40 ± 0.33^{B}	6.36 ± 0.10^{D}	7.01 ± 0.01^{C}
Bifidobacterium spp.	7.56 ± 0.00^{A}	7.02 ± 0.02^{B}	7.10 ± 0.03^{B}	6.82 ± 0.09^{C}	5.06 ± 0.11^{E}	6.30 ± 0.02^{D}
Clostridium spp.	8.24 ± 0.04^{A}	7.26 ± 0.13^{CD}	7.40 ± 0.06^{C}	7.11 ± 0.05^{D}	7.93 ± 0.04^{B}	6.85 ± 0.04^{E}
Enterococcus spp.	7.09 ± 0.08^{B}	4.61 ± 0.04^{E}	7.98 ± 0.07^{A}	6.90 ± 0.22^{B}	6.16 ± 0.15^{C}	5.42 ± 0.12^{D}
Enterobacteria	7.69 ± 0.13^{A}	6.19 ± 0.03^{D}	7.09 ± 0.04^{B}	7.46 ± 0.10^{A}	6.40 ± 0.08^{D}	6.62 ± 0.05^{C}
Total aerobes	7.76 ± 0.09^{A}	7.77 ± 0.08^{A}	7.52 ± 0.00^{A}	7.15 ± 0.14^{B}	7.44 ± 0.24^{AB}	6.68 ± 0.01^{C}
Facultative anaerobes	7.82 ± 0.02^{A}	6.87 ± 0.01^{BC}	7.90 ± 0.09^{A}	6.87 ± 0.22^{BC}	5.75 ± 0.02^{D}	6.66 ± 0.05^{C}
Bacteroides	7.39 ± 0.01^{B}	7.65 ± 0.07^{B}	7.30 ± 0.09^{B}	8.68 ± 0.26^{A}	8.63 ± 0.27^{A}	7.73 ± 0.27^{B}
R5 (Descending colon)						
Lactobacillus spp.	7.90 ± 0.06^{A}	6.50 ± 0.03^{B}	6.40 ± 0.09^{B}	6.23 ± 0.15^{B}	5.62 ± 0.29^{C}	6.29 ± 0.08^{B}
Bifidobacterium spp.	7.44 ± 0.07^{A}	7.00 ± 0.01^{B}	6.63 ± 0.00^{C}	6.10 ± 0.13^{D}	5.22 ± 0.16^{F}	5.54 ± 0.27^{E}
Clostridium spp.	8.24 ± 0.04^{A}	7.26 ± 0.13^{C}	7.69 ± 0.01^{B}	6.73 ± 0.13^{D}	8.14 ± 0.24^{A}	6.47 ± 0.08^{D}
Enterococcus spp.	7.21 ± 0.28^{A}	4.94 ± 0.16^{EC}	6.50 ± 0.01^{B}	5.59 ± 0.38^{CD}	5.48 ± 0.20^{DC}	5.02 ± 0.21^{DE}
Enterobacteria	7.73 ± 0.11^{A}	6.77 ± 0.06^{B}	6.50 ± 0.22^{BD}	5.40 ± 0.41^{C}	6.14 ± 0.09^{D}	6.49 ± 0.09^{BD}
Total aerobes	7.65 ± 0.13^{A}	7.27 ± 0.64^{B}	7.47 ± 1.03^{A}	6.97 ± 0.29^{C}	6.23 ± 0.97^{E}	6.60 ± 0.11^{D}
Facultative anaerobes	6.79 ± 0.08^{A}	6.79 ± 0.05^{A}	6.88 ± 0.00^{A}	6.97 ± 0.10^{A}	6.85 ± 0.02^{B}	6.99 ± 0.07^{A}
Bacteroides	7.78 ± 0.05^{BC}	8.00 ± 0.11^{B}	7.35 ± 0.02^{C}	8.14 ± 0.06^{B}	8.45 ± 0.28^{A}	8.14 ± 0.34^{B}

Different uppercase letters in the same line represent statistical difference ($p < 0.05$) between the treatments for the same reactor. B: basal period. TS: treatment with synbiotic beverage, PT: Post-treatment, Tpe: treatment with prebiotic beverage, Tpo: treatment with probiotic beverage and TPa: treatment with placebo beverage.

the formation of two major groups: from the synbiotic treatment (ST) and other from the prebiotic, probiotic and placebo treatments, which formed group I (Fig. 5). This division into two major groups with low similarity (~20%) shows different *Lactobacillus* species in the SHIME reactor (Marzorati et al., 2010) during the different treatments and, therefore, changes in the *Lactobacillus* spp. community, particularly in the synbiotic beverage treatment. The synbiotic beverage stimulated the growth of several species of *Lactobacillus* in all reactors, which did not occur with the other treatments. Fig. 4a shows an increase in the number of bands in the three reactors along the treatment with synbiotic beverage. In contrast, there was a decrease in the number of bands during the post-treatment and treatment with other beverages (Fig. 4b). Similar results were found by Sivieri, Bianchi, and Rossi (2011) and Van de Wiele, Boon, Possemiers, Jacobs and Verstraete (2004), who reported beneficial effects of probiotics and prebiotics, respectively, in the three colon regions, limited to the treatment period, thus requiring continuous consumption of the studied component to obtain the beneficial effects. In addition, effect of probiotics on the growth of colon lactobacilli was elucidated by a study of Chaikham and Apichartsrangkoon (2014), who showed that the oral administration of the encapsulated *Lactobacillus acidophilus* LA5 along with pasteurized longan juice stimulated a significant increase of lactobacilli (≥2 log cycle) in all colon compartments in SHIME reactor. Molly, De Smet, Nollet, De Woestyne, and Verstraete (1996) showed that the quantities of colon lactobacilli significantly increased by 10^8 CFU/day after feeding probiotics to the SHIME reactor.

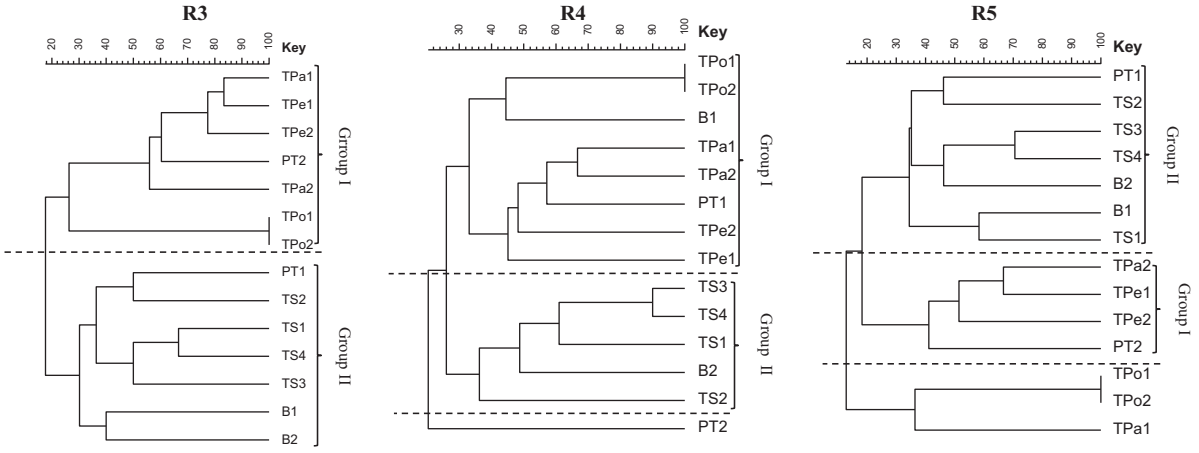

Fig. 5. Dendrogram illustrating the correlation between the different denaturing gradient gel electrophoresis (DGGE) profiles from the *Lactobacillus* community samples obtained from SHIME® compartments (ascending: R3, transverse: R4 and descending colon: R5). B1: basal period (week 1); B2: basal period (week 2); TS1: synbiotic treatment (week 1); TS2: synbiotic treatment (week 2); TS3: synbiotic treatment (week 3); TS4: synbiotic treatment (week 4); PT1: post-treatment (week 1); PT2: post-treatment (week 2); TPe1: prebiotic treatment (week 1); TPe2: prebiotic treatment (week 2); TPa1: placebo treatment (week 1); TPa2: placebo treatment (week 2); TPo1: probiotic treatment (week 1); TPo2: probiotic treatment (week 2).

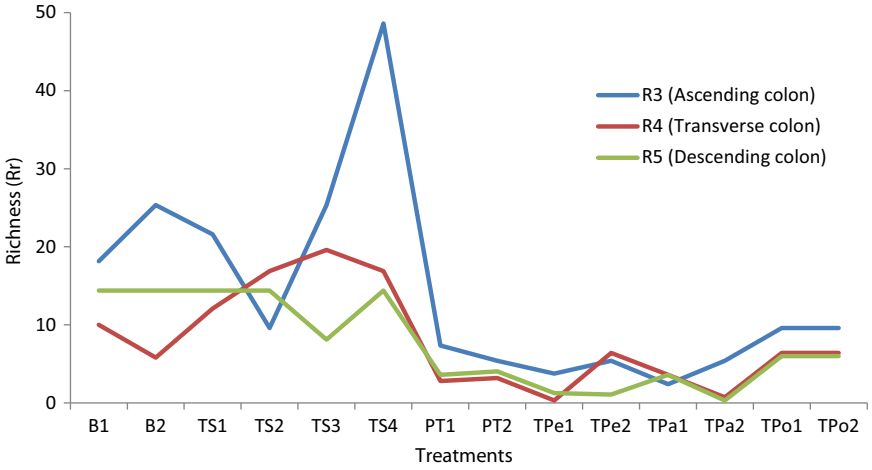

Fig. 6. Levels of richness in reactors R3, R4 and R5, which simulate the ascending transverse and descending colon regions in SHIME® system throughout the experimental period. B1: basal period (week 1); B2: basal period (week 2); TS1: synbiotic treatment (week 1); TS2: synbiotic treatment (week 2); TS3: synbiotic treatment (week 3); TS4: synbiotic treatment (week 4); PT1: post-treatment (week 1); PT2: post-treatment (week 2); TPe1: prebiotic treatment (week 1); TPe2: prebiotic treatment (week 2); TPa1: placebo treatment (week 1); TPa2: placebo treatment (week 2); TPo1: probiotic treatment (week 1); TPo2: probiotic treatment (week 2).

3.3. Effects of the long-term treatment on the lactobacilli community structure

There was a greater enrichment of lactobacilli population when the treatment was carried out with the synbiotic beverage, both in the ascending and transverse colons. There is no difference in the descending colon when compared to the basal period (Fig. 6). According to Marzorati et al. (2008), *Rr* values below 10 indicate a low-richness population, whereas *Rr* values between 10 and 30 and above 30 indicate medium and high-richness populations, respectively, in terms of entire community. The latter is characterized as a typical population of very healthy environments, with a high microbial diversity (Marzorati et al., 2008). This way, it is possible to say that R3 provided a healthier environment when treated with synbiotic beverage than other reactors, resulting in a high-richness of lactobacilli and changing the diversity of this population in a positive way. R4 also resulted in a richness increase

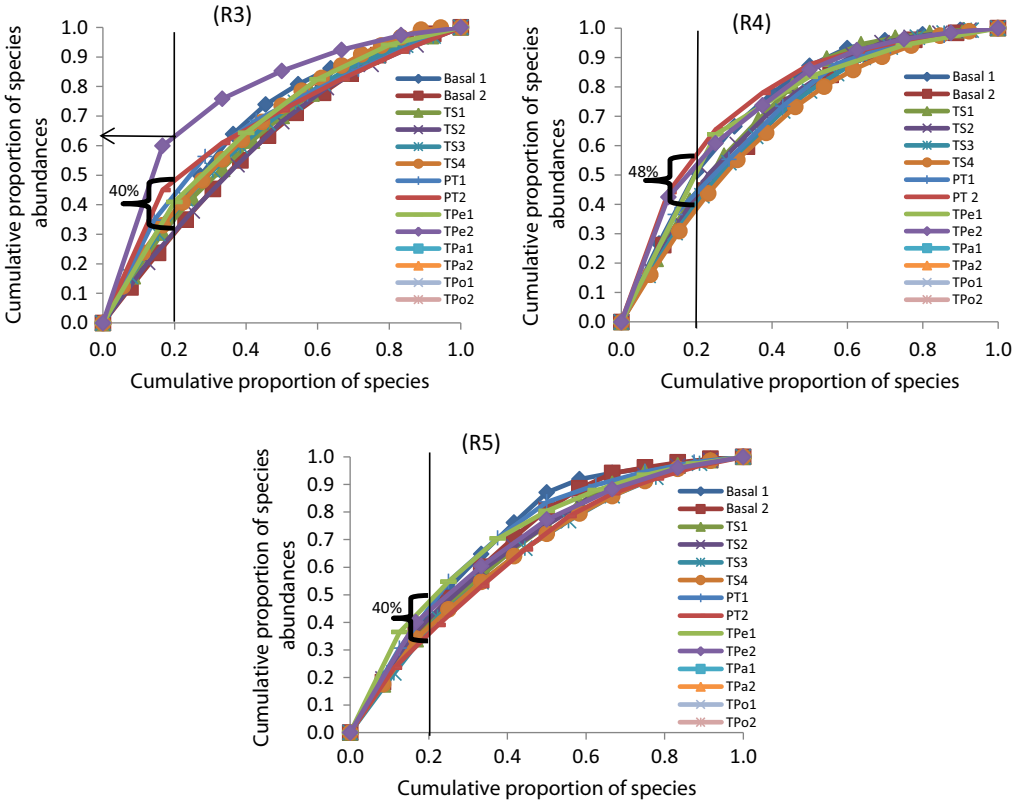

Fig. 7. Pareto–Lorenz curves of reactors R3 (ascending colon), R4 (transverse colon) and R5 (descending colon) from the SHIME® system, during the different treatments. Basal 1: basal period (week 1); basal 2: basal period (week 2); TS1: synbiotic treatment (week 1); TS2: synbiotic treatment (week 2); TS3: synbiotic treatment (week 3); TS4: synbiotic treatment (week 4); PT1: post-treatment (week 1); PT2: post-treatment (week 2); L.c.: pure culture of *L. casei* Lc-01; TPe1: prebiotic treatment (week 1); TPe2: prebiotic treatment (week 2); TPa1: placebo treatment (week 1); TPa2: placebo treatment (week 2); TPo1: probiotic treatment (week 1); TPo2: probiotic treatment (week 2).

Table 6
Average ammonium ion production (ppm) in SHIME® run during the different treatments.

	Ascending colon	Transverse colon	Descending colon
Basal period	580.91 ± 109.71^{A}	614.91 ± 60.22^{A}	680.58 ± 49.38^{A}
Synbiotic treatment	65.30 ± 14.05^{C}	311.13 ± 25.15^{B}	434.41 ± 44.43^{B}
Post-treatment week 1	95.20 ± 7.46^{C}	252.00 ± 6.24^{B}	360.66 ± 8.32^{B}
Post- treatment week 2	508.66 ± 20.30^{A}	593.00 ± 17.57^{A}	663.3 ± 15.27^{A}
Prebiotic treatment	255.83 ± 140.24^{B}	565.50 ± 101.12^{A}	639.17 ± 28.05^{A}
Placebo treatment	277.66 ± 12.66^{B}	664.00 ± 17.34^{A}	732.33 ± 24.17^{A}
Probiotic treatment	332.66 ± 0.70^{B}	588.33 ± 16.50^{A}	693.50 ± 46.90^{A}

Different uppercase letters in the same column represent statistical difference ($p < 0.05$) between the different treatments.

of lactobacilli species, but this contribution was lower than that provided by R3. No changes were observed in R5 richness during the treatment with synbiotic beverage.

These results were expected because the pH in the colon (between 5.6 and 5.9) favors the growth of *Lactobacillus* spp., which explains the higher richness of *Lactobacillus* spp. in the R3 reactor (Sivieri et al., 2011).

In addition to the analysis on *Lactobacillus* spp. community richness, a Pareto–Lorenz (PL) curve was also built, seeking a better understanding of lactobacilli population behavior and its functionality in the different treatments.

Except for the treatment with the prebiotic beverage at week 2 (TPe2) in R3, which showed a distance point from the other treatments, there was an average of functionality coefficient value of 43% between the reactors. There were no great changes in the functionality of a reactor to another and from one treatment to another (Fig. 7).

According to Marzorati et al. (2008) and Carballa, Smits, Etchebehere, Boon, and Verstraete (2011), in terms of entire community, low (20–25%) and high (>80%) PL curves indicate a highly uniform and specialized community, respectively. Consequently, a long lag phase could be needed to counteract a sudden stress. A PL curve between 45 and 60% represents balanced communities, which can potentially deal with changing environmental conditions while preserving their functionality.

In view of this, it is possible to classify the lactobacilli population in the three colon regions during different treatments, as a balanced community, capable of surviving at different environmental conditions while maintaining its functionality. It indicates that even with the increase or decrease of *Lactobacillus* species (proven by dependent and independent methods of cultivation) and other bacterial genera, in accordance with the used treatment, there were no major changes in the *Lactobacillus* spp. functional organization. Therefore, this population remained stable and balanced throughout the study.

3.4. Ammonium ions

A significant reduction in ammonia ions production in all reactors during the treatment with synbiotic beverage, with greater intensity in ascending colon, was observed. These values remained low until the first week after this treatment, with a large increase only in the second week of post-treatment, but no higher than the control period value (Table 6). This result is considered beneficial because ammonia can alter the morphology and the intermediary metabolism of intestinal cells by increasing DNA synthesis and promoting colon carcinogenesis (Ichikawa & Sakata, 1998).

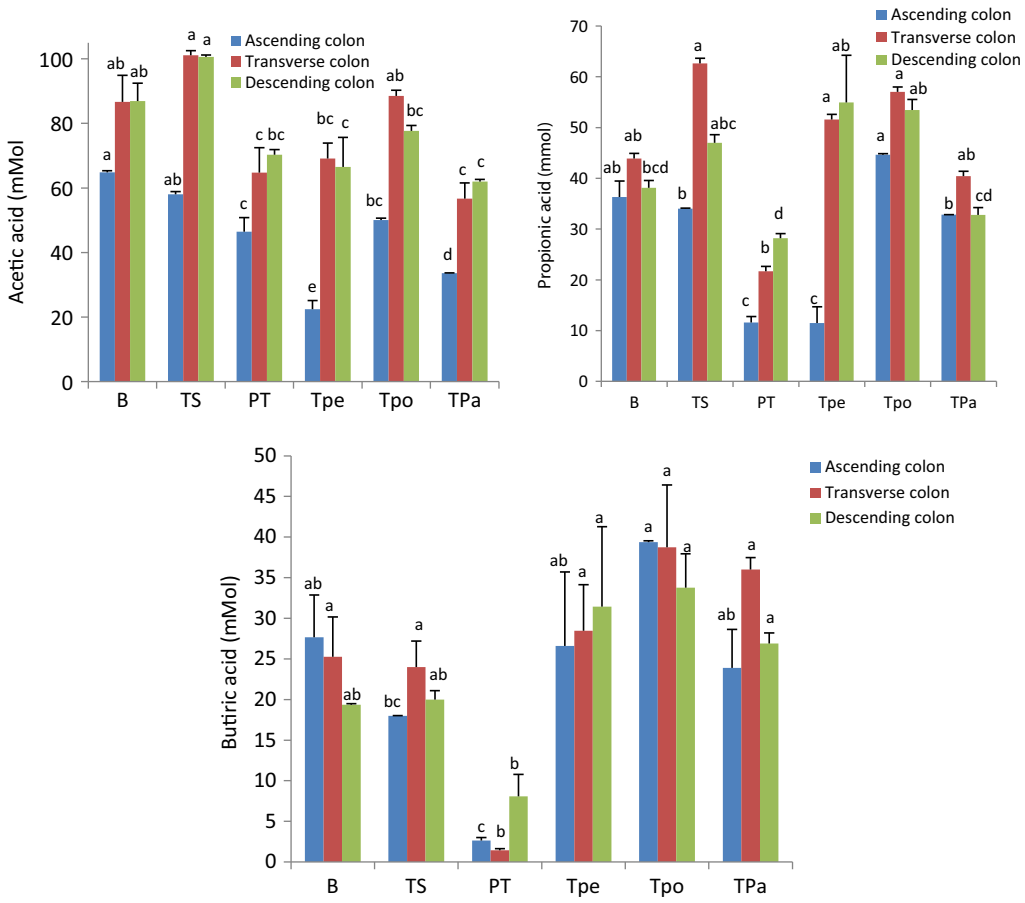

Fig. 8. Metabolic activity SCFA: acetic acid, propionic acid and butyric acid in reactor three, four and five from the different treatments in the SHIME®. Statistically significant differences among the samples were investigated with one-way ANOVA. Samples with the same letter on the top of the bar are not statistically different. B: basal period. TS: treatment with synbiotic beverage, PT: post-treatment, Tpe: treatment with prebiotic beverage, Tpo: treatment with probiotic beverage and TPa: treatment with placebo beverage.

Regarding the other beverages, there was a significant reduction in ammonia ions only in R3; however, this reduction was significantly lower when compared to the synbiotic beverage. There was no statistical difference among the prebiotic, probiotic and placebo beverages in any of the three compartments, but they were all statistically different from the synbiotic beverage. Kontula et al. (2002) found similar results when evaluating the effect of lactulose on *Lactobacillus rhamnosus* survival using the SHIME system, suggesting changes in the metabolic activity of the SHIME microbiota. In addition, similar effects with *L. acidophilus* LA5 in pressurized longan juice induced lower ammonia by 52.7, 59.5 and 50.4% in ascending, transverse, and descending colons, respectively, in comparison with the controls (Chaikham & Apichartsrangkoon, 2014).

In this study, R3 and R5 showed the lowest and highest amounts of ammonia ions, respectively, independently of experimental period, corroborating Kontula et al. (2002), Possemiers et al. (2004) and Sivieri et al. (2011). According to Macfarlane, Gibson, and Cummings (1992), the concentration of ammonia in the intestinal lumen increases progressively from the ascending to the descending colon, which is related to a higher rate of protein fermentation in the descending colon compared to the ascending colon (Davila et al., 2013). Smith and Macfarlane (1998) attributed the lower production of ammonia from the ascending colon to low pH and high carbohydrate availability in this compartment.

3.5. Analysis of short-chain fatty acids (SCFAs)

No significant increase in the SCFA production in the colon compartments was observed during treatments compared to the basal period (Fig. 8). Although the FOS and probiotic *L. casei* Lc-01 stimulated (in combination or not) the growth of other *Lactobacillus* species, this stimulation was not sufficient to increase the production of SCFA, because the reactors had a limited amount of nutrients. *Lactobacillus* are saccharolytic microorganisms and produce lactate, ethanol and succinate as fermentation products, which are intermediates in the global fermentation process in the microbiota, being metabolized to SCFA by cross-feeding species in the ecosystem. Generally, these SCFAs do not accumulate in the intestine due to low or insufficient production (Macfarlane & Macfarlane, 2003). These microorganisms cannot continue their normal cycle in the lack of nutrients, thereby reducing SCFA accumulation.

SCFA concentrations are a temporary state, because the majority of SCFA formed during the fermentation will be immediately used by colonocytes or other intestinal bacteria (Topping & Clifton, 2001). According to Macfarlane and Gibson (1994), the formation of SCFA in the gut depends on various factors. From a microbiological viewpoint, the chemical composition, physical form and amount of substrate available affect bacterial fermentation reactions, which are also dependent on the types and numbers of different bacterial populations found in the gut, catabolite regulatory mechanisms, the availability of inorganic electron donors, as well as competitive and cooperative interactions between different species in the microbiota (Allison & Macfarlane, 1988; Macfarlane & Gibson, 1994).

4. Conclusions

The new beverage, based on aqueous extracts of quinoa and soy and fortified by *L. casei* Lc-01 and the prebiotic FOS, had the greatest beneficial action on the intestinal microbiota. The synbiotic beverage showed more significant effects in the ascending colon, increasing *Lactobacillus* spp. and *Bifidobacterium* spp. populations and reducing detrimental genera to the host, such as *Bacteroides* and *Clostridium* spp. It also significantly reduced production of ammonia ions in this compartment, and improved the diversity and richness of the *Lactobacillus* spp. community without affecting its functionality. Although the prebiotic and probiotic beverages had good results regarding the increase of commensal bacteria, the reduction of ammonia ions and pathogenic bacteria, these results were not as satisfactory as those provided by the synbiotic beverage. Therefore, the synbiotic beverage is a good matrix candidate for the intestinal delivery of probiotics, which helps the indigenous microbiota to improve human health.

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