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An exploratory study on the influence of orange juice on gut microbiota using a dynamic colonic model

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ABSTRACT

The aim of this research was to evaluate the influence of fresh orange juice (FOJ) and pasteurized orange juice (POJ) on gut microbiota using the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®) in a long-term experiment. SHIME® vessels were used to investigate orange juice fermentation throughout the colon and to assess changes in microbial composition and fermentation metabolites (short-chain fatty acids, or — SCFA, and ammonium). Antioxidant activity of the SHIME® vessels and juice was also evaluated. The FOJ increased ($p \leq 0.05$) *Lactobacillus* spp., *Enterococcus* spp., *Bifidobacterium* spp., and *Clostridium* spp. and reduced ($p \leq 0.05$) enterobacteria. The POJ increased ($p \leq 0.05$) *Lactobacillus* spp. and reduced ($p \leq 0.05$) enterobacteria. The PCR-DGGE analysis showed a reduction in total bacteria population richness values. The FOJ and POJ increased ($p \leq 0.05$) butyric, acetic, and propionic acid concentrations, whereas ammonium production was reduced. High values of antioxidant activity were observed as a result of the FOJ and POJ treatments. Principal component analysis indicated that both POJ and FOJ juices had a positive influence on gut microbiota. The FOJ and POJ were found to exhibit selective prebiotic activity, particularly in terms of gut microbiota. This finding is in agreement with increases in both SCFAs and commensal bacteria, as well as with decreases in ammonium levels, though total bacteria richness values were reduced.

1. Introduction

Gut microbiota represents a complex and diversified system of microorganisms that colonize the gastrointestinal (GI) tract, particularly in the colon region within the large intestine. The favorable conditions of this part of the GI tract include slow gut transit time, the availability of nutrients, and favorable pH (Frick & Autenrieth, 2013; Payne, Zihler, Chassard, & Lacroix, 2012). There are approximately 10^{12} bacterial cells per gram of luminal content inhabiting the colon. Estimates of the number of bacterial species present in the gut microbiota vary from 500 to 1150 (Frick & Autenrieth, 2013).

The gut microbiota has a direct impact on host's health, since this system plays an important role in immunological, physiological, and metabolic processes in the human body (Gerritsen, Smidt, Rijkers, & De Vos, 2011). This microbiota is involved in the synthesis of vitamins B and K, in the resistance of the colonization of pathogenic microorganisms, and in the synthesis of fermentation products that supply energy to the epithelium of the colon, such as short-chain fatty acids, or SCFAs (Davila et al., 2013; Gerritsen et al., 2011; Sekirov, Russell, Antunes, & Finlay, 2010). The gut microbiota is also responsible for regulating the immune system by promoting the maturation of immune cells and by maintaining the motor functions of the gastrointestinal tract (Clemente, Ursell, Parfrey, & Knight, 2012; Round & Mazmanian, 2009).

Both the composition and the metabolism of the gut microbiota are strongly influenced by diet. Many studies indicate that nutritional intervention may selectively modify certain groups of bacteria (Russell et al., 2011; Scott, Gratz, Sheridan, Flint, & Duncan, 2013; Walker et al., 2011). Gut microbiota can be modulated using probiotic microorganisms, prebiotic fibers, or symbiotic combinations (Bianchi et al., 2014; Chaikham & Apichartsrangkoon, 2014; Costabile et al., 2015; Pereira-Caro, Oliver, et al., 2015). However, few studies have been performed to evaluate the impact of fruits or fruit juices on the modulation of gut microbiota (Mosele et al., 2015; Sánchez-Patán et al., 2015).

The importance of orange juice consumption has long been established. It is a source of vitamin C, flavonoids, and carotenoids and also contains folic acid, potassium, and fibers (Stella, Ferrarezi, Santos, & Monteiro, 2011; Stinco et al., 2012). Orange juice consumption has been associated with a reduced risk of chronic diseases, largely because of the presence of bioactive compounds such as ascorbic acid, carotenoids, and flavonoids (Aptekmann & Cesar, 2013; Ghanim et al., 2010;

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Morand et al., 2011). It also contributes to vascular health and to reduce in atherogenesis. Ascorbic acid is considered the main antioxidant compound of orange juice (Ness, Khaw, Bingham, & Day, 1996; Simon, 1992). Flavonoids — particularly hesperidin and narirutin — also exhibit antioxidant activity (Tripoli, La Guardia, Giammanco, Di Majo, & Giammanco, 2007), anti-inflammatory properties (Milenkovic, Deval, Dubray, Mazur, & Morand, 2011), lipid-lowering properties (Monforte et al., 1995), and anticarcinogenic properties (Birt, Hendrich, & Wang, 2001; Yang, Landau, Huang, & Newmark, 2001). Carotenoids exhibit provitamin A activity and also reduce the risk of developing macular degeneration (Krinsky & Johnson, 2005).

The bioactive compounds present in orange juice — particularly polyphenols — may also be associated with the metabolism of gut microbiota (Laparra & Sanz, 2010; Pereira-Caro, Oliver, et al., 2015; Pereira-Caro, Borges, et al., 2015). Some studies have shown that gut microbiota transforms phenolic compounds into bioactive metabolites. It therefore contributes to intestinal homeostasis, stimulates the growth of beneficial bacteria (*Lactobacillus* spp. and *Bifidobacterium* spp.), and inhibits pathogenic bacteria. In these ways, it has a prebiotic effect (Dueñas et al., 2015; Guglielmetti et al., 2013; Parkar, Trower, & Stevenson, 2013).

The objective of this study was to evaluate the influence of fresh orange juice (FOJ) and pasteurized orange juice (POJ) on gut microbiota using the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®). The SHIME® is an *in vitro* model that dynamically simulates the human gastrointestinal tract and has proven useful for nutrition studies that analyze the composition and activity of the gut microbial community (De Boever, Deplancke, & Verstraete, 2000; Possemiers, Marzorati, Verstraete, & Van de Wiele, 2010; Sivieri et al., 2013; Van de Wiele, Boon, Possemiers, Jacobs, & Verstraete, 2007).

2. Material and methods

2.1. Orange juice

The FOJ and POJ were prepared from Pêra-Rio orange, a Brazilian variety, responsible for most of the exportation. The FOJ was extracted using a JBT 391B extractor at the JBT FoodTech pilot plant in Araraquara, SP, Brazil. The POJ was supplied by a citrus industry from Araraquara region, SP, Brazil. This juice was pasteurized at 95 °C for 30 s (Bisconsin-Junior, Rosenthal, & Monteiro, 2014). The juices were frozen and kept at −18 °C until use.

2.2. Physicochemical characteristics of the orange juice

Soluble solids, pH, titratable acidity, ascorbic acid, reducing sugars, and total sugars were determined following the methods described in the AOAC (2012). All analyses were performed in triplicate.

2.3. Simulator of Human Intestinal Microbial Ecosystem (SHIME®)

The SHIME® (registered trademark from Ghent University and ProDigest) is a simulator of the human intestinal microbial ecosystem in which environmental conditions (pH, retention time, and temperature) are controlled. The SHIME® consists of five double-jacketed vessels representing the stomach, small intestine and ascending colon (AC), the transverse colon (TC) and the descending colon (DC) of the human gastrointestinal tract (Molly, Woestyne, De Smet, & Verstraete, 1994). The overall retention time of the last three vessels, simulating the large intestine, was 76 h. The pH, retention time, and volumetric capacity corresponding to each vessel are based on the methodology described by Possemiers, Verthé, Uyttendaele, and Verstraete (2004).

The vessels operated at 37 °C and were stirred continuously using a magnetic stirrer. The inside of each vessel was kept in anaerobiosis through the daily injection of N_2 for 30 min, and the pH of each portion of the tract was automatically adjusted with the addition of 0.5 N NaOH or 0.5 N HCl (Molly et al., 1994; Possemiers et al., 2004).

2.3.1. Carbohydrate-based medium composition

The carbohydrate-based medium used in the SHIME® was prepared in distilled water. It was made of 3 g/L of starch (Unilever, Brazil), 2 g/L of pectin (Sigma, USA), 4 g/L of type III mucin from porcine stomach (Sigma, USA), 1 g/L of xylan (Sigma, USA), 1 g/L of peptone (Acumedia, USA), 1 g/L of arabinogalactan (Sigma, USA), 0.4 g/L of glucose (Synth, Brazil), 3 g/L of yeast extract (Acumedia, USA), and 0.5 g/L of L-cysteine (Sigma, USA) following Possemiers et al. (2004).

2.3.2. Fecal inoculum

The fecal inoculum was prepared using a fecal sample from an adult volunteer who had not taken antibiotics for 2 years prior to the experiment. A 20-gram fecal sample was collected. The sample was diluted in 200 mL of phosphate buffer containing 0.05 mol/L of Na_2HPO_4, 0.05 mol/L of NaH_2PO_4, and 0.1% sodium thioglycolate ($C_2H_3NaO_2S$); pH was 6.5. The diluted sample was homogenized in a sample homogenizer (Model No. 130, Nova Ética, Brazil) for 10 min and centrifuged for 5 min at 3000 rpm. Next, 40 mL of supernatant were added to each of the three simulated colon vessels (AC, TC, and DC) and each vessel was then filled with the carbohydrate-based medium (Possemiers et al., 2010).

2.3.3. Experimental protocol

The SHIME® experiment was performed continually for 8 weeks. During the control period, the carbohydrate-based medium (210 mL) was added to the system twice a day for 14 days so that the microbial community could be adapted to the physicochemical and nutritional conditions that dominate the different parts of the colon, and also so that a stable microbial community could be formed (Van de Wiele et al., 2007). After two weeks of adaptation, the treatment periods were initiated (FOJ and POJ). The microbial communities present in the last three vessels of the culture system were fed, twice a day, with the carbohydrate-based medium (105 mL) and juices (105 mL). The treatments lasted for 14 days. At the end of each treatment, a seven-day washout period was initiated. During this period, the carbohydrate-based medium (210 mL) was added into the SHIME®. As shown in Fig. 1, the experimental conditions used in the SHIME® were consistent with the methodology described by Chaikham and Apichartsrangkoon (2014).

2.4. Microbiological analysis

The number of colony forming units log CFU/mL SHIME® fluid was determined by plating serial dilutions of samples of sterile 0.1% peptone water on selective culture media according previously described by Bianchi et al. (2014). Numbers of total aerobic bacteria and facultative anaerobic bacteria were determined by plating on Standard Methods agar and incubation at 37 °C/48 h aerobically or anaerobically, respectively. MRS agar with incubation at 37 °C/48 h, anaerobically, was used to determine the number of lactobacilli. BIM-25 agar with incubation at 37 °C/72 h, anaerobically, was used to determine the number of *Bifidobacterium* spp. *Clostridium* spp. was enumerated anaerobically using Reinforced Clostridial agar at 37 °C/48 h. *Enterococcus* spp. was enumerated aerobically using KF Streptococcus agar at 37 °C/48 h. MacConkey agar was used as selective media for enterobacteria with incubation at 37 °C/48 h, anaerobically.

2.4.1. Analysis using Polymerase Chain Reaction-Denaturing Gradient Gel Electrophoresis (PCR-DGGE)

The PCR-DGGE of the total bacteria was performed in order to determine the effect of the treatments on the microbial community of the colon. The DNA was extracted from the simulated colon vessels samples (AC, TC, and DC) using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol, though with changes to the initial sample quantity (200 mg per 2 mL) and an ATE

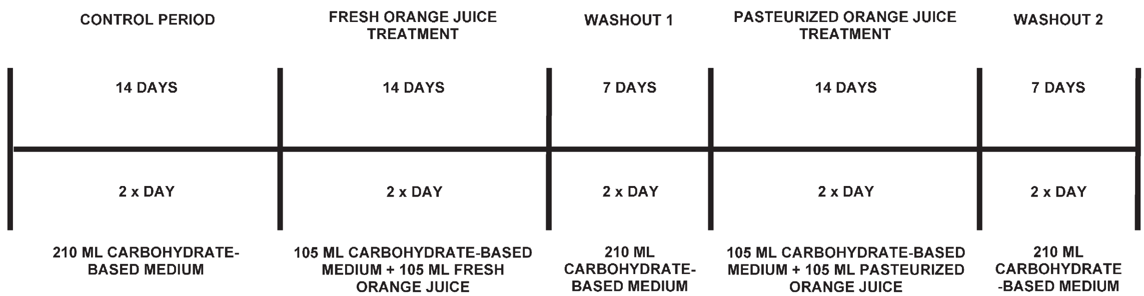

Fig. 1. Experimental protocol using the Simulator of the Human Intestinal Microbial Ecosystem (SHIME®).

buffer (200 μL per 50 μL). The DNA was quantified using a NanoVue™ Plus spectrophotometer (GE Healthcare, USA).

The primers used to replicate the DNA were 968FGC (5′-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA GAA CCT TAC-3′) and 1401R (5′-CGG TGT GTA CAA GAC CC-3′) (Nübel et al., 1996). DNA polymerization was performed using GoTaq® Green Master Mix (Promega, USA). The samples were amplified in a thermal cycler (Applied Biosystems, USA) using the following conditions: initial denaturation at 95 °C for 7 min; 35 denaturation cycles at 94 °C for 45 s, annealing at 56 °C for 45 s, extension at 72 °C for 1 min, and final extension at 72 °C for 10 min, followed by cooling at 4 °C.

Electrophoresis was performed using 8% polyacrylamide gel with a 45–65% denaturing gradient for 16 h at 75 V in a 1× TAE buffer at constant temperature of 60 °C, as described by Reis, Carosia, Sakamoto, Varesche, and Silva (2015). The gels were stained using ethidium bromide following Sanguinetti, Dias Neto, and Simpson (1994), scanned (400 dpi), and analyzed using the BioNumerics software, version 6.0 (Applied Maths, Belgium).

The distance matrices for each DGGE were based on the Jaccard similarity coefficient. These matrices were used for the cluster analysis. The analysis was performed using the BioNumerics software, version 6.0.

2.4.2. Ecological analysis of the total bacteria population

The DGGE fingerprinting technique is commonly used to represent the genetic structure and diversity of a microbial community from a specific environment (Marzorati, Wittebolle, Boon, Daffonchio, & Verstraete, 2008; Muyzer & Smalla, 1998). The ecological analysis (richness and functional organization) was performed following Marzorati et al. (2008). Richness (Rr) is correlated with band distribution patterns in the DGGE and with the percentage of the gel denaturing gradient necessary to represent the total diversity of the sample. This concept may be expressed mathematically when the Rr index is defined as ($N^2 \times Dg$), where N represents the total number of standard bands and Dg represents the denaturing gradient between the first and last band of the pattern.

The Pareto–Lorenz distribution curves were created in order to graphically represent the structure of bacterial communities based on DGGE profiles (Lorenz, 1905; Mertens, Boon, & Verstraete, 2005; Wittebolle, Vervaeren, Verstraete, & Boon, 2008). For each DGGE lane, the respective bands are ranked from high to low, based on their intensities. The cumulative normalized number of bands is used as *x*-axis, and their respective cumulative normalized intensities represent the *y*-axis. The more the PL curve deviates from the 45° diagonal (the theoretical perfect evenness line), the less evenness can be observed in the structure of the studied community. The latter means that a smaller fraction of the different species is present in dominant numbers (Dejonghe, Boon, Seghers, Top, & Verstraete, 2001; Mertens et al., 2005; Wittebolle et al., 2008).

2.5. Ammonium analysis

Ammonium levels were determined using a specific ion meter (Model No. 710A, Orion) coupled with the ammonia electrode ion selective meter (Model No. 95-12, Orion). The samples from the colon vessels (25 mL) were added to 0.5 mL of an ammonia pH adjusting ionic strength adjuster solution (Orion), and the ammonium level was measured three times (Bianchi et al., 2014).

2.6. Short-Chain Fatty Acid (SCFA) analysis

Samples were collected weekly from the simulated colon vessels (AC, TC, and DC) and were stored at −20 °C. The samples (2 mL) were centrifuged at 14000 rpm for 5 min, and 100 μL of the supernatant was diluted in 1.900 μL of ultrapure water. Afterwards, 1 g of NaCl was added, as well as 100 μL of crotonic acid, 70 μL of isobutanol, and 200 μL of 2 M H_2SO_4. The SCFAs were analyzed using a 2010-Model gas chromatograph (Shimadzu, Japan) equipped with a split/splitless injector, a flame ionization detector, and a CombiPAL automated sampler for headspace analysis. The SCFAs were separated using a HP-INNOWAX column (30 m × 0.25 mm × 0.25 μm) (Agilent Technologies, USA). The carrier gas was hydrogen and the flow rate was 1.45 mL/min. The temperature of both the injector and detector was 240 °C (Adorno, Hirasawa, & Varesche, 2014).

2.7. Antioxidant activity

2.7.1. Extraction of antioxidant compounds from orange juice

The antioxidant compounds were extracted from the orange juice following the method described by Asami, Hong, Barrett, and Mitchell (2003). Orange juice (5 mL) and a methanol:water solution (80:20, v/v) (10 mL) were vortexed for 1 min, placed in an ultrasonic bath at room temperature for 15 min, and centrifuged at 9000 rpm for 20 min. The supernatant was filtered (Whatman filter paper, No. 1), and the extraction was repeated using the precipitate, under the same conditions. The supernatants were collected and combined in a volumetric flask. Juice extraction was performed in triplicate.

2.7.2. Preparing the samples from the SHIME®

Samples from the simulated colon vessels (AC, TC, and DC) (20 mL) were collected in each period of analysis and centrifuged at 5000 rpm for 5 min. The supernatant was filtered (Whatman filter paper, No. 1) and stored in a freezer at −20 °C until use.

2.7.3. $ABTS^+$ assay

The antioxidant activity of the juices and the antioxidant activity of the samples from the simulated colon vessels were determined using $ABTS^+$ assay, based on the method described by Rufino et al. (2010). ABTS radical cation ($ABTS^+$) was obtained from the reaction of 5 mL of ABTS solution (7 mM) with 88 μL of potassium persulfate solution (140 mM). The solution was left in the dark for 16 h to guarantee the complete formation of the $ABTS^+$. The $ABTS^+$ solution was diluted with ethanol until an absorbance of 0.66 ± 0.01 at 750 nm. The orange juice extracts and the samples from colon vessels (AC, TC, and DC) were used to prepare diluted solutions. Three orange juice:ethanol solutions (1:5; 3:5 v/v, and whole extract), and three colon vessel sample:water solutions (1:3; 2:3 v/v, and whole supernatant) were used to determine antioxidant activity. The orange juice and colon vessel sample solutions (30 μL) were added to 3.0 mL of the diluted $ABTS^{+}$ solution. After 6 min, the decrease in absorbance was read at 750 nm against a blank (ethanol) using an Evolution 220 spectrophotometer (Thermo Scientific, USA). Calibration curves were built with Trolox (100–600 μmol/L). The analyses were performed in triplicate.

2.8. Statistical analysis

Analysis of variance (ANOVA), Tukey's test, and Student's t-test were applied to the data ($p \leq 0.05$) using the StatGraphics Centurion 15.0 software (StatPoint Inc., USA). For Principal Component Analysis (PCA), SCFA and ammonium production, antioxidant activity, and *Lactobacillus* spp., *Bifidobacterium* spp., and enterobacteria counts were fixed in columns (variables), and the experimental periods were organized into lines (cases). The data was standardized before the analyses. The PCA analysis was performed with correlation matrix and without factor rotation using the Statistica 10.0 software (StatSoft Inc., USA).

3. Results and discussion

3.1. Physicochemical characteristics of orange juice

The physicochemical characteristics of the FOJ and POJ are in Table 1. The physicochemical characteristics of the FOJ and POJ were in accordance with the requirements of the Brazilian law (Brasil, 2000). The results were consistent with those from ten brands of ready-to-drink orange juice and nectar, as reported by Stella et al. (2011), and those of fresh orange juice, described by Bisconsin-Junior et al. (2014).

3.2. Microbiological analysis

In the FOJ treatment, 1 log CFU increase in *Lactobacillus* spp. and *Enterococcus* spp. populations was observed ($p \leq 0.05$) in all the three regions of the colon. The largest *Lactobacillus* spp. count was observed in the simulated AC (Table 2). According to Sivieri, Bianchi, Tallarico, and Rossi (2011), the pH of the ascending colon is between 5.6 and 5.9, a range that favors the growth of this genus. In addition, the greatest amount of saccharolytic bacteria (such as *Lactobacillus* spp.) occurs in this part of the colon due to the greater quantity of carbohydrates available in this compartment (Besten et al., 2013). 1 log CFU increase ($p \leq 0.05$) in *Bifidobacterium* spp. population was observed in the simulated TC and DC. In addition, 1 log CFU increase ($p \leq 0.05$) in *Clostridium* spp. was found in the simulated AC and TC. Total aerobes and facultative anaerobes were found to have increased by 1 log CFU ($p \leq 0.05$) in the simulated AC. 1 log CFU reduction in the enterobacteria population ($p \leq 0.05$) was observed in the AC relative to the control period. In the POJ treatment, there was 1 log CFU increase ($p \leq 0.05$) in the *Lactobacillus* spp. population and in total aerobes in the simulated DC. Meanwhile, there was 2 log CFU reduction in the enterobacteria population in the AC and 1 log CFU reduction in the TC relative to washout 1 (Table 2).

During the orange juice treatments, an increase in *Lactobacillus* spp. and *Bifidobacterium* spp. populations and a decrease in enterobacteria population were observed, showing the effect on the gut microbiota.

Table 1
Physicochemical characteristics of fresh orange juice (FOJ) and pasteurized orange juice (POJ).

Parameter	FOJ	POJ
Soluble solids (°Brix)	12.56 ± 0.05	10.93 ± 0.05
Titratable acidity (g of citric acid/100 mL)	0.70 ± 0.02	0.47 ± 0.00
pH	3.22 ± 0.02	3.62 ± 0.01
Reducing sugars (g glucose/100 mL)	4.32 ± 0.08	3.53 ± 0.02
Total sugars (g glucose/100 mL)	8.47 ± 0.10	7.36 ± 0.08
Ascorbic acid (mg/100 mL)	43.13 ± 0.44	34.18 ± 0.25

Average ± standard deviation (n = 3).

Table 2
Microbial counts (log CFU/mL) of different groups of bacteria from the vessels mimicking the ascending colon (AC), the transverse colon (TC), and the descending colon (DC) as a result of treatments with fresh orange juice (FOJ) and pasteurized orange juice (POJ).

Microbial groups	Experimental period	AC	TC	DC
Lactobacillus spp.	Control	$7.51^{d} \pm 0.15$	$7.45^{c} \pm 0.02$	$7.46^{c} \pm 0.05$
	FOJ	$8.84^{a} \pm 0.02$	$8.60^{a} \pm 0.19$	$8.51^{a} \pm 0.03$
	Washout 1	$7.81^{c} \pm 0.03$	$7.95^{b} \pm 0.05$	$7.39^{c} \pm 0.09$
	POJ	$8.58^{b} \pm 0.08$	$8.52^{a} \pm 0.01$	$8.50^{a} \pm 0.10$
	Washout 2	$7.92^{c} \pm 0.22$	$7.39^{c} \pm 0.09$	$7.70^{b} \pm 0.01$
Bifidobacterium spp.	Control	$7.52^{c} \pm 0.03$	$7.15^{c} \pm 0.01$	$7.23^{c} \pm 0.01$
	FOJ	$7.84^{b} \pm 0.05$	$8.30^{a} \pm 0.04$	$8.21^{a} \pm 0.07$
	Washout 1	$7.90^{b} \pm 0.04$	$8.00^{b} \pm 0.01$	$7.38^{b} \pm 0.02$
	POJ	$8.10^{a} \pm 0.04$	$8.34^{a} \pm 0.04$	$8.25^{a} \pm 0.08$
	Washout 2	$7.59^{c} \pm 0.11$	$6.35^{d} \pm 0.35$	$7.15^{c} \pm 0.15$
Clostridium spp.	Control	$7.74^{e} \pm 0.03$	$7.30^{d} \pm 0.10$	$7.53^{d} \pm 0.06$
	FOJ	$8.81^{a} \pm 0.03$	$8.38^{b} \pm 0.02$	$8.46^{ab} \pm 0.03$
	Washout 1	$8.44^{c} \pm 0.02$	$8.57^{a} \pm 0.01$	$8.11^{c} \pm 0.03$
	POJ	$8.61^{b} \pm 0.02$	$8.44^{b} \pm 0.04$	$8.40^{b} \pm 0.08$
	Washout 2	$8.20^{d} \pm 0.05$	$8.06^{c} \pm 0.02$	$8.52^{a} \pm 0.09$
Enterococcus spp.	Control	$5.17^{d} \pm 0.09$	$5.16^{d} \pm 0.01$	$5.26^{e} \pm 0.06$
	FOJ	$6.85^{c} \pm 0.03$	$6.77^{c} \pm 0.03$	$6.33^{c} \pm 0.03$
	Washout 1	$7.39^{b} \pm 0.09$	$7.39^{b} \pm 0.39$	$7.00^{b} \pm 0.02$
	POJ	$8.14^{a} \pm 0.04$	$7.38^{b} \pm 0.09$	$7.66^{a} \pm 0.05$
	Washout 2	$7.39^{b} \pm 0.07$	$8.72^{a} \pm 0.07$	$6.00^{d} \pm 0.00$
Enterobacteria	Control	$5.74^{a} \pm 0.44$	$6.59^{a} \pm 0.04$	$6.53^{d} \pm 0.03$
	FOJ	$4.35^{b} \pm 0.05$	$5.92^{c} \pm 0.05$	$6.47^{d} \pm 0.05$
	Washout 1	$5.63^{a} \pm 0.15$	$6.51^{b} \pm 0.01$	$6.90^{c} \pm 0.04$
	POJ	$3.39^{c} \pm 0.09$	$5.18^{d} \pm 0.07$	$7.10^{b} \pm 0.03$
	Washout 2	$2.87^{d} \pm 0.03$	$5.01^{e} \pm 0.01$	$7.68^{a} \pm 0.14$
Total Aerobes	Control	$7.75^{e} \pm 0.04$	$7.61^{c} \pm 0.02$	$7.59^{c} \pm 0.01$
	FOJ	$8.83^{b} \pm 0.04$	$8.46^{a} \pm 0.06$	$8.37^{a} \pm 0.07$
	Washout 1	$9.00^{a} \pm 0.07$	$8.19^{b} \pm 0.11$	$7.39^{d} \pm 0.09$
	POJ	$8.47^{c} \pm 0.02$	$8.34^{ab} \pm 0.06$	$8.44^{a} \pm 0.05$
	Washout 2	$7.87^{d} \pm 0.09$	$7.15^{d} \pm 0.15$	$7.74^{b} \pm 0.04$
Facultative Anaerobes	Control	$7.68^{e} \pm 0.04$	$7.64^{c} \pm 0.09$	$7.57^{c} \pm 0.01$
	FOJ	$8.77^{a} \pm 0.01$	$8.12^{b} \pm 0.05$	$8.29^{a} \pm 0.01$
	Washout 1	$8.04^{c} \pm 0.04$	$8.01^{b} \pm 0.17$	$7.76^{b} \pm 0.07$
	POJ	$8.41^{b} \pm 0.03$	$8.43^{a} \pm 0.01$	$8.32^{a} \pm 0.04$
	Washout 2	$7.90^{d} \pm 0.02$	$7.15^{d} \pm 0.15$	$7.69^{bc} \pm 0.21$

Average ± standard deviation (n = 3).
Averages with different lower-case letters in the same column differ significantly according to the Tukey's test ($p \leq 0.05$).

Studies involving gut microbiota and the ingestion of orange juice are still rare; however, some authors have suggested a relationship between a diet rich in polyphenols and an increase in *Lactobacillus* spp., *Bifidobacterium* spp., and *Enterococcus* spp. diversity (Cueva et al., 2013; Molan, Liu & Kruger, 2010; Valdés et al., 2015; Viveros et al., 2011).

The increase in *Bifidobacterium* spp. and *Lactobacillus* spp. populations in the colon provides benefits to intestinal health, including the regulation of the immune system and the production of short-chain fatty acids. These populations can also exhibit antimicrobial capacities through bacteriocin production, which enables the reduction of pathogenic bacteria populations (Jacobsen et al., 1999; Mitsuoka, 1990).

The reduction in the enterobacteria population is also considered beneficial to the body; when present in high quantities, enterobacteria exhibit greater pathogenic potential (Montesi et al., 2005). Sánchez-Patán et al. (2015) also observed a significant reduction in the enterobacteria population after *in vitro* batch incubations with cranberry and grape seed extracts in the ascending and descending colons, showing that fruits rich in polyphenols may inhibit some genera of colonic microbiota such as enterobacteria.

Elevated *Clostridium* spp. counts are not necessarily associated with negative effects on health. Some species of the genus *Clostridium* are associated with the production of short-chain fatty acids, which are beneficial to health (Possemiers et al., 2010; Sivieri et al., 2013). However, other species are involved in the inflammatory processes of gastrointestinal disorders because of their metabolic activity and pathogenic nature (Guarner & Malagelada, 2003; Montesi et al., 2005).

3.2.1. PCR-DGGE

The analysis of the cluster generated by the DGGE band profile revealed that the samples from the simulated AC vessel were separated into three different clusters (Fig. 2A). Cluster 1 (C1) was represented by the total bacteria in the treatments with FOJ and POJ (50% similarity), Cluster 2 (C2) included the total bacteria in washout periods 1 and 2 (50% similarity), and Cluster 3 (C3) was represented by the total bacteria in the control period. The same cluster division was observed in the simulated TC, though with different similarity values: the clusters were formed by FOJ and POJ (56% similarity), washout 1 and washout 2 (74% similarity), and the control period (Fig. 2B). In the case of the simulated DC, total bacteria in the control period and washout 1 formed one cluster with 73% similarity and another cluster with 77% similarity between the two juice treatments and washout 2 (Fig. 2C).

In the simulated AC and TC vessels, the treatments with FOJ and POJ were clustered separately from the control and washout periods, respectively, indicating that there were changes in total bacteria composition induced by the orange juice treatments. This result is supported by the decrease in the number of PCR-DGGE bands during the orange juice treatments; however, when the juice administration was suspended (the washout periods), an increase in the number of bands was observed. Similar results were observed by Kemperman et al. (2013), who reported a reduction in the number of bands representing total bacteria after treatment with black tea extract and red wine grape extract using the SHIME® model. One of the advantages of PCR-DGGE analysis is the ability to visually monitor the profile and changes that occur in the microbial community when different treatments are applied (Chaikham, Apichartsrangkoon, Jirarattanarangsri, & Van de Wiele, 2012; Muyzer & Smalla, 1998).

3.2.2. Ecological analysis of the total bacteria population

The richness values of the simulated AC vessel in the treatments with FOJ and POJ were 18.58 and 19.01, respectively; after the treatments, there was an increase in richness in the washout 1 (58.21) and the washout 2 (42.77). The simulated TC vessel was found to have a higher richness value in the POJ treatment (58.21). The richness values of the simulated DC vessel in the two juice treatments and in washout 1 were very similar (39.20). The highest value observed in this colon vessel was in the control period (48.40) (Fig. 3).

According to Marzorati et al. (2008), richness values between 10 and 30 correspond to a microbial community of average richness, while richness values above 30 represent a highly rich microbial community typical of very habitable environments and high diversity. Thus, it can be said that the simulated TC and DC provide a healthier environment than the simulated AC, which results in a large number of total bacteria species in these colon vessels. Meanwhile, the richness values of the simulated AC decreased in the juice treatments, a finding which likely reflects colonization by fewer species. This result suggests that, in the simulated AC, the juice treatments had a reducing effect on the total bacteria.

Kemperman et al. (2013) observed that the polyphenols present in black tea and red wine grape extracts affected microbial composition and promoted a reduction in richness values in the three colon vessels that represented the ascending, transverse, and descending colons in the SHIME® model. The authors attributed these results to the selective effect of the polyphenols and suggested that dominant species may express metabolic pathways for degradation of polyphenols or may be resistant to the antimicrobial effect of the phenolic compounds.

Another parameter used to evaluate the structure and functionality of the total bacteria population was functional organization through the construction of a Pareto–Lorenz (PL) curve. The treatments with FOJ and POJ were not found to influence the functionality of the total bacteria population in the three regions of the colon. The average functionality coefficient of the total bacteria population was 32%, close to the 25% Pareto–Lorenz curve (Fig. 4). In ecological terms, this represents high community uniformity, but it does not present a well-defined internal structure in

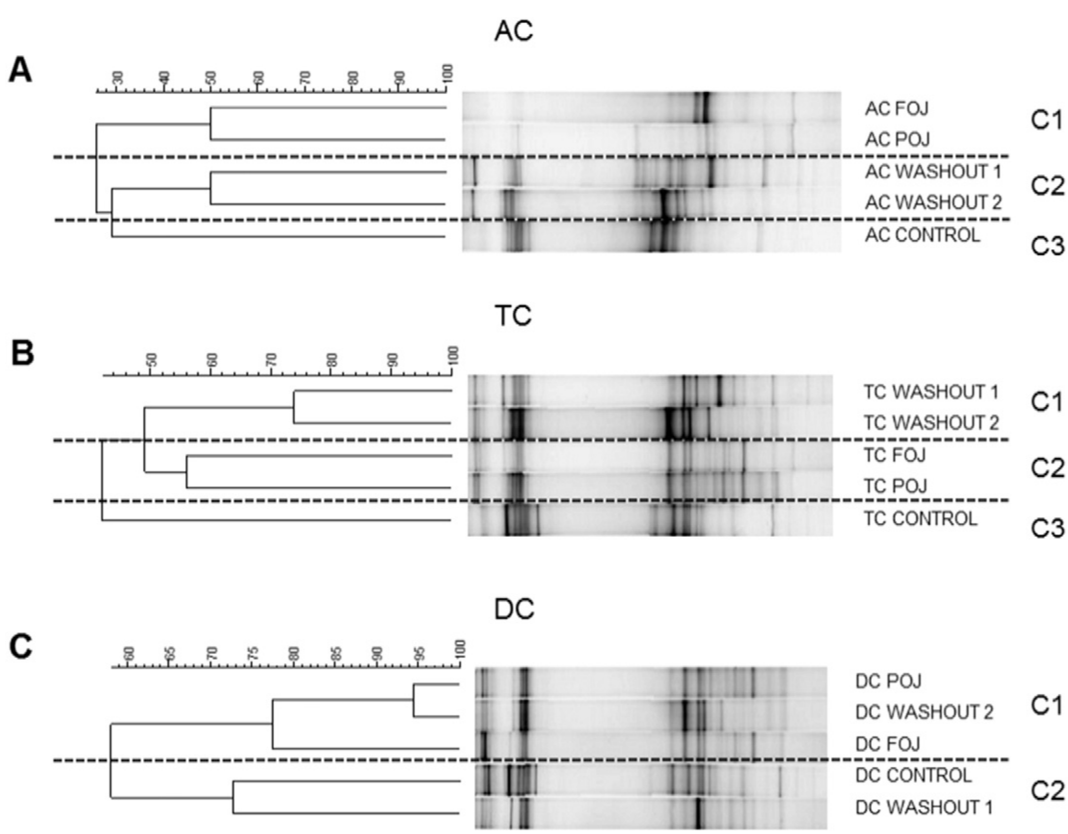

Fig. 2. Cluster analysis of the DGGE profiles of total bacteria from the vessels mimicking the ascending colon (AC), the transverse colon (TC), and the descending colon (DC) as a result of treatments with fresh orange juice (FOJ) and pasteurized orange juice (POJ). C1: Cluster 1; C2: Cluster 2; C3: Cluster 3.

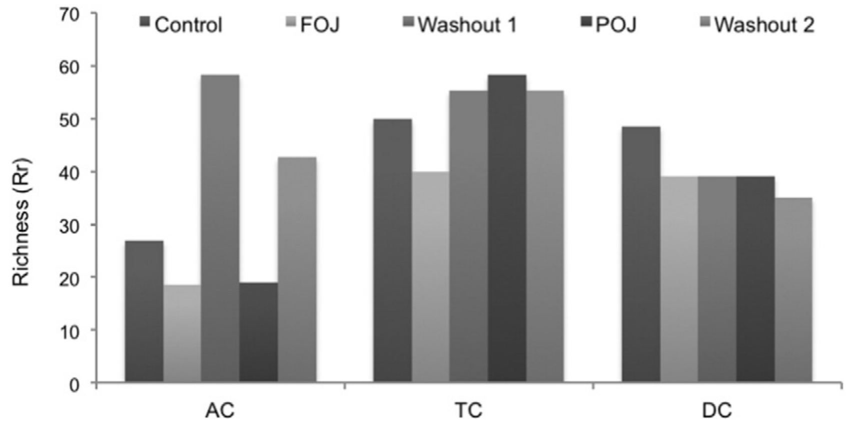

Fig. 3. Richness level of the total bacteria population from the vessels mimicking the ascending colon (AC), the transverse colon (TC), and the descending colon (DC) as a result of treatments with fresh orange juice (FOJ) and pasteurized orange juice (POJ).

terms of dominant species. This community is characterized as a community with low functional organization (Marzorati et al., 2008).

3.3. Ammonium

There was a reduction ($p \leq 0.05$) in ammonium production in the treatments with FOJ and POJ (Table 3). This reduction was positive, since ammonium correspond to one of the products of the protein degradation performed by the gut bacteria, which is considered a metabolite that negatively affects intestinal health (Davila et al., 2013; Montalto, D'onofrio, Gallo, Cazzato, & Gasbarrini, 2009). When present at high concentrations, ammonium have the capacity to change the morphology of epithelial cells of the gut and to act as tumor promoters in the colon (Hughes, Magee, & Bingham, 2000; Scott et al., 2013).

Similar results were found by Chaikham and Apichartsrangkoon (2014), who observed a reduction in the concentation of ammonium in all of the colon vessels in the SHIME® model in a treatment with pasteurized longan juice.

When the colon vessels were compared to each other, an increase ($p \leq 0.05$) in the ammonium concentration was observed along the simulated colon vessels (AC to DC); the experimental period as a whole was considered except for washout 2 (Table 3). According to Macfarlane, Gibson, and Cummings (1992), the ammonium concentration in the intestinal lumen increases progressively from the ascending colon to the descending colon due to the higher rate of protein fermentation in the descending colon relative to the ascending colon. However, Smith and Macfarlane (1998) attributed the lower ammonium production in the ascending colon to low pH and high availability of carbohydrates in the region.

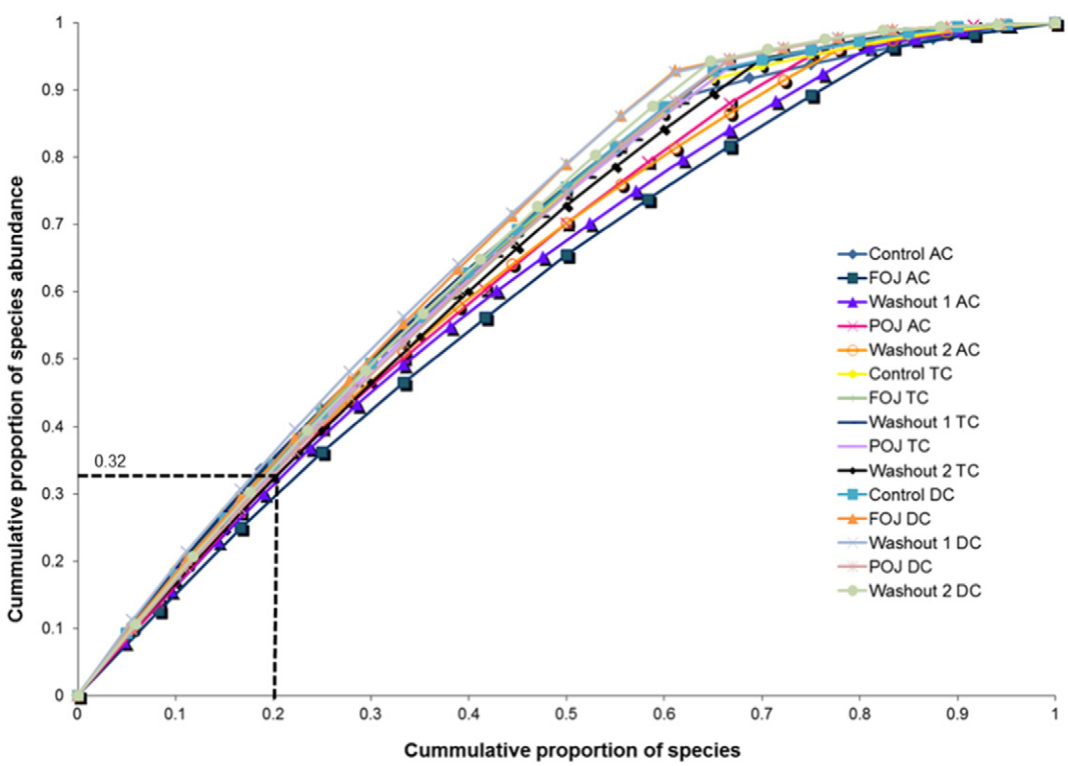

Fig. 4. Pareto–Lorenz curve from the vessels mimicking the ascending colon (AC), the transverse colon (TC), and the descending colon (DC) as a result of treatments with fresh orange juice (FOJ) and pasteurized orange juice (POJ).

Table 3
Ammonium concentration (mmol/L) from the vessels mimicking the ascending colon (AC), the transverse colon (TC), and the descending colon (DC) as a result of treatments with fresh orange juice (FOJ) and pasteurized orange juice (POJ).

Experimental period	AC	TC	DC
Control	$13.77^{cC} \pm 0.09$	$19.98^{cB} \pm 0.09$	$23.78^{bA} \pm 0.17$
FOJ	$0.38^{dC} \pm 0.01$	$3.51^{dB} \pm 0.05$	$4.60^{dA} \pm 0.03$
Washout 1	$30.18^{aC} \pm 0.26$	$34.85^{aB} \pm 0.38$	$38.65^{aA} \pm 0.23$
POJ	$0.39^{dC} \pm 0.01$	$0.62^{eB} \pm 0.02$	$3.42^{eA} \pm 0.03$
Washout 2	$24.76^{bB} \pm 0.28$	$29.16^{bA} \pm 0.17$	$20.21^{cC} \pm 0.54$

Average ± standard deviation (n = 3).
Averages with different lower-case letters in the same column differ significantly according to the Tukey's test ($p \leq 0.05$).
Averages with different upper-case letters in the same line differ significantly according to the Tukey's test ($p \leq 0.05$).

3.4. Short-Chain Fatty Acids (SCFAs)

In the FOJ treatment, a substantial increase ($p \leq 0.05$) in acetic acid and butyric acid was observed in all of the colon vessels, as well as an increase ($p \leq 0.05$) in propionic acid in the simulated TC relative to the control period (Table 4). In the POJ treatment, an increase ($p \leq 0.05$) in acetic acid was observed in all of the colon vessels, as well as an increase ($p \leq 0.05$) in propionic acid and butyric acid in the simulated colon vessels TC and DC relative to washout 1. Total SCFA production in the FOJ treatment was greater ($p \leq 0.05$) than in the POJ treatment in the simulated AC and DC vessels, but no difference was observed in the simulated TC ($p > 0.05$) (Table 4).

The acetate, the most abundant SCFA in the colon, may increase cholesterol synthesis after absorption. Meanwhile, the propionate may reduce lipogenesis and inhibit serum cholesterol synthesis (Hijova & Chmelarova, 2007; Hosseini, Grootaert, Verstraete, & Van de Wiele, 2011). The butyrate is considered the main energy substrate used by the epithelial cells of the colon; it supplies 70% of these cells' energy needs (Montalto et al., 2009). The butyrate also reduces the risk of colon cancer, intensifies water and sodium absorption, and stimulates blood flow to the intestinal mucosa (Mortensen & Clausen, 1996; Velázquez, Lederer, & Rombeau, 1996).

Table 4
Short-chain fatty acid (SCFA) concentration (mmol/L) from the vessels mimicking the ascending colon (AC), the transverse colon (TC), and the descending colon (DC) as a result of treatments with fresh orange juice (FOJ) and pasteurized orange juice (POJ).

SCFA	Experimental period	AC	TC	DC
Acetic acid	Control	$27.18^{d} \pm 0.39$	$64.98^{c} \pm 0.58$	$69.77^{c} \pm 8.29$
	FOJ	$155.76^{a} \pm 10.64$	$184.14^{a} \pm 9.74$	$179.73^{a} \pm 16.19$
	Washout 1	$63.13^{c} \pm 23.38$	$97.20^{b} \pm 2.23$	$95.60^{b} \pm 11.04$
	POJ	$124.00^{b} \pm 16.92$	$153.28^{a} \pm 39.78$	$150.87^{a} \pm 27.20$
	Washout 2	$56.22^{c} \pm 5.74$	$91.85^{b} \pm 26.13$	$102.05^{b} \pm 10.59$
Propionic acid	Control	$7.32^{c} \pm 0.22$	$12.18^{c} \pm 0.75$	$12.75^{d} \pm 0.00$
	FOJ	$4.66^{d} \pm 0.75$	$15.57^{b} \pm 1.60$	$13.24^{d} \pm 0.55$
	Washout 1	$14.59^{a} \pm 1.95$	$10.60^{c} \pm 0.34$	$18.30^{b} \pm 0.81$
	POJ	$14.40^{a} \pm 1.25$	$20.82^{a} \pm 2.07$	$22.89^{a} \pm 0.97$
	Washout 2	$10.16^{b} \pm 0.28$	$16.48^{b} \pm 0.76$	$16.50^{c} \pm 1.29$
Butyric acid	Control	$6.45^{d} \pm 0.84$	$11.90^{c} \pm 1.48$	$12.13^{d} \pm 2.98$
	FOJ	$21.62^{a} \pm 0.68$	$74.21^{a} \pm 14.49$	$81.77^{a} \pm 3.35$
	Washout 1	$15.31^{b} \pm 1.28$	$6.89^{c} \pm 0.09$	$10.18^{d} \pm 0.00$
	POJ	$11.76^{c} \pm 0.24$	$63.36^{ab} \pm 5.02$	$65.79^{b} \pm 1.92$
	Washout 2	$2.35^{e} \pm 0.18$	$55.62^{b} \pm 1.63$	$48.59^{c} \pm 4.64$
Total SCFA	Control	$40.95^{d} \pm 0.95$	$89.06^{d} \pm 1.94$	$94.65^{d} \pm 8.94$
	FOJ	$182.05^{a} \pm 11.60$	$273.92^{a} \pm 12.04$	$274.75^{a} \pm 17.99$
	Washout 1	$93.02^{c} \pm 24.48$	$114.69^{c} \pm 1.98$	$124.09^{d} \pm 11.85$
	POJ	$150.16^{b} \pm 17.31$	$237.45^{a} \pm 36.56$	$239.55^{b} \pm 25.62$
	Washout 2	$68.73^{c} \pm 6.18$	$163.94^{b} \pm 25.23$	$167.14^{c} \pm 14.72$

Average ± standard deviation (n = 3).
Averages with different lower-case letters in the same column differ significantly according to the Tukey's test ($p \leq 0.05$).

SCFA production may be influenced by the number and species of microorganisms present in the colon, as well as the substrate available (Macfarlane & Macfarlane, 2003). In the current study, increases in *Lactobacillus* spp., *Bifidobacterium* spp., and *Clostridium* spp. populations were observed in the treatments with FOJ and POJ. Some studies have shown that these genera are involved in SCFA production and that they play an important role in both the physiology of the colon and metabolism (Hijova & Chmelarova, 2007; Possemiers et al., 2010). An increase in SCFAs may also be attributed to carbon sources from the orange juice, since carbon serves as a substrate in the fermentation of gut bacteria and effectively participates in the SCFA production in the colon (Chaikham et al., 2012). In addition, orange juice flavanones — particularly hesperidin and narirutin metabolized into hesperitin and naringenin — are responsible for the production of propionic acid and acetic acid (Pereira-Caro, Borges, et al., 2015). According to Pereira-Caro, Borges, et al. (2015) colonic microbiota affect the overall bioavailability of orange juice (poly)phenols through the production of hydroxy- and methoxyphenylpropionic acids.

Chaikham and Apichartsrangkoon (2014) evaluated the influence of pasteurized longan juice on gut microbiota using the SHIME® model and observed an increase ($p \leq 0.05$) in SCFA concentration (acetate, propionate, and butyrate) in all of the colons in their juice treatment. Meanwhile, in the study performed by Costabile et al. (2015), there were no changes in SCFA concentrations in the case of a reconstituted orange juice treatment using a three-stage *in vitro* colonic model system.

SCFA and ammonium analyses are typically used to evaluate microbial metabolism. Thus, the results of this study show that the treatments with FOJ and POJ stimulated microbial metabolism, with a significant decrease in ammonium and an increase in SCFA. These results suggest a positive influence on gut microbiota.

3.5. Antioxidant activity

The antioxidant activity of the FOJ was 3014.70 μmol of Trolox/L and the POJ, 3024.00 μmol of Trolox/L, similar to those reported by Bisconsin-Junior, Alvarenga, Rosenthal, and Monteiro (2015) for fresh orange juice, and higher than those found by Stella et al. (2011).

Fig. 5 shows the antioxidant activity of the samples from the simulated colon vessels (AC, TC, and DC) during the treatments with FOJ and POJ. In all regions of the colon, the antioxidant activity of samples from the treatments with FOJ and POJ was higher than that of the control period and washout 1, respectively. A difference was observed ($p \leq 0.05$) in antioxidant activity resulting from the two orange juice treatments in the DC vessel, while there was no difference in the AC or TC vessels ($p > 0.05$) (Fig. 5).

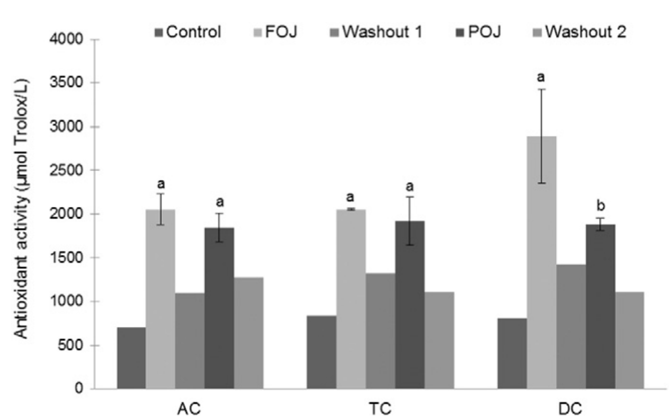

Fig. 5. Antioxidant activity from the vessels mimicking the ascending colon (AC), the transverse colon (TC), and the descending colon (DC) as a result of treatments with fresh orange juice (FOJ) and pasteurized orange juice (POJ). Averages with different letters differ significantly according to the Student's t-test ($p \leq 0.05$).

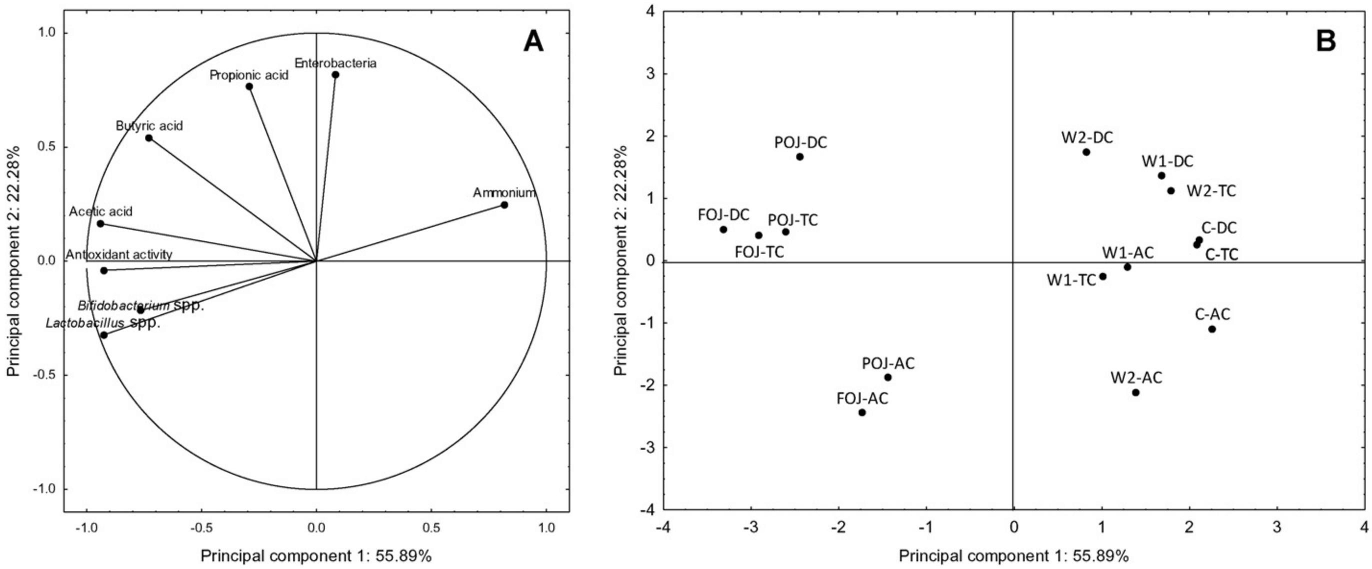

Fig. 6. Principal component analysis (projection of the variables (A); projection of the samples (B)) in the SHIME® model. AC: ascending colon. TC: transverse colon. DC: descending colon. C: control. FOJ: fresh orange juice. W1: washout 1. POJ: pasteurized orange juice. W2: washout 2.

There was a slight reduction in antioxidant activity in the samples from the simulated colon vessels during the orange juice treatments relative to the antioxidant activity of the juices. Even so, the antioxidant activity of the samples from the simulated colon vessels remained high, suggesting that much of the phenolic compound content in the orange juice is not absorbed in the duodenum; it is therefore bioavailable after fermentation by gut microbiota (Gil-Izquierdo, Gil, & Ferreres, 2002).

3.6. Principal Component Analysis (PCA)

The PCA revealed similarities and differences during the experimental period in which the SHIME® was used, explaining 78.17% of the total variation of the data (Fig. 6). Principal component 1 explained 55.89% of the variation in the data, which was described by the *Lactobacillus* spp. and *Bifidobacterium* spp. populations, the antioxidant activity, acetic acid production, butyric acid production, and ammonium level. Principal component 2 explained 22.28% of the variation in the data and was itself explained by propionic acid levels and by the enterobacteria population (Fig. 6A). The control and washout periods samples (C-AC, C-TC, C-DC, W1-AC, W1-TC, W1-DC, W2-AC, W2-TC, and W2-DC) were described by the increase in ammonium production. The two juice treatments in the respective colon vessels (FOJ-AC, FOJ-TC, FOJ-DC, POJ-AC, POJ-TC, and POJ-DC) were described by the increase in *Lactobacillus* spp. and *Bifidobacterium* spp. populations, the antioxidant activity, acetic acid production, and butyric acid production (Fig. 6B). These results suggest that both fresh and pasteurized orange juice had a positive effect on gut microbiota through the improved metabolic activity of the microbiota in different regions of the colon.

4. Conclusions

The long-term experiment in the SHIME® showed that both the fresh and pasteurized orange juice treatments changed the composition of the microbial community by increasing *Lactobacillus* spp. and *Bifidobacterium* spp. populations and reducing the enterobacteria population. On the other hand, the PCR-DGGE analysis indicated a reduction in total bacteria population richness values, though not necessarily with a negative impact on gut microbiota: increases in both SCFA production and antioxidant activity were observed, as well as decreases in ammonium levels. These results indicate that orange juice has a selective and prebiotic effect on gut microbiota. This exploratory study on the influence of orange juice over gut microbiota using SHIME® model should be deepen. Further studies should be performed to better understand the effects of interactions among the bioactive compounds from orange juice and their metabolites on human gut microbiota.

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