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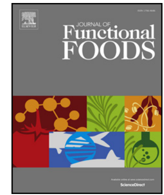

A soy-based probiotic drink modulates the microbiota and reduces body weight gain in diet-induced obese mice

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A R T I C L E I N F O

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A B S T R A C T

This work investigates the effect of a soy-based probiotic drink (*Enterococcus faecium* CRL 183 and *Bifidobacterium longum* ATCC 15707) on the fecal microbiota composition, body weight and inflammatory parameters in diet-induced obese mice. The probiotic group had a lower body weight until 9th week of the study, reduced area and diameter of adipocytes, and showed a significant increase of IL-6 and IL-10 compared to the obesite non-treated group. The intake of a high-fat diet results in an increase of *Lactobacillus* spp. while the probiotic drink positively modulates the intestinal microbiota by maintaining the population of microorganisms belonging to the phylum *Bacteroidetes* and increases *Bifidobacterium* spp. Our study finds that the regular intake of this probiotic drink is able to reduce body weight gain and the size of adipocytes while modulating the fecal microbiota and the immune profile of animals, therefore acting in a beneficial manner in the control of obesity.

1. Introduction

In recent decades, several studies indicate changes related to the understanding of the risks and the genesis of various non-communicable diseases such as cardiovascular diseases, type 2 diabetes mellitus (T2DM) or non–insulin-dependent diabetes mellitus (NIDDM), insulin resistance, and obesity. The development of such diseases is related mainly to genetic predisposition, dietary habits and lifestyle to which individuals are subject. However, the etiology of these diseases is multifactorial, which complicates treatment and leads to high morbidity as well as mortality rates worldwide (Eid et al., 2017; WHO, 2014).

Among these non-communicable diseases, obesity – abnormal or excessive accumulation of body fat that causes a health risk to an individual (WHO, 2016) – has emerged in developed and developing countries (Bhurosy & Jeewon, 2014; Ng, Fleming, Robinson, Thomson, & Graetz, 2014). The etiology of obesity is represented by endogenous and exogenous factors, which can act at the same time or in isolation. These etiological factors contribute to an increase in adiposity that result in serious health conditions (Eid et al., 2017; Sabin, Werther & Kiess, 2011).

The intestinal microbiota composition is one of these etiological factors involved in the development of obesity and insulin resistance, as it influences fat storage, energy capture and it can triggers a systemic inflammation as well as metabolic disorders (Zhang et al., 2012). This complex ecosystem plays an important role in the proper function and homeostasis of the digestive system, and on the overall health of the human body (Koliada et al., 2017).

Evidence suggests that obese individuals exhibit a shift in the microbiota composition, displaying a higher population of the phylum *Firmicutes* with a minor population of *Bacteroidetes,* and *Firmicutes/Bacteroidetes* ratio tend to decrease with weight loss. This imbalance in the composition of the intestinal microbiota, known as dysbiosis, seems to promotes an increase in energy absorption capacity from the diet,

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modulating the individual's immune response and leading to a state of obesity (Koliada et al., 2017; Krajmalnik-Brown, Ilhan, Kang, & DiBaise, 2012; Sweeney & Morton, 2013). Some studies indicate that a shift in diversity of intestinal microbiota and specific microrganism genera are also associated with the development of obesity (Carlucci, Petrof, & Allen-Vercoe, 2016; Le Chatelier et al., 2013; Million et al., 2012; Million, Lagier, Yahav, & Paul, 2013).

Consequently, the ingestion of probiotics, defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002; Hill et al., 2014), has been used as a strategy to positively modulate the microbiota and thereby reduce the risk of, or aid in the treatment of different diseases, including obesity (Kobyliak et al., 2016). Some species of *Lactobacillus* spp. benefit obese mice in addition to humans, demonstrating an influence on both lipid metabolism and body weight. A similar effect was verified when *Bifidobacterium* spp. strains were incorporated into the regular diet (Alard et al. 2016; Marchesi et al., 2016).

Previous studies showed that a soy-based product fermented with *Enterococcus faecium* CRL 183 and *Lactobacillus helveticus* 416 is capable of positively modulating inflammatory markers, the intestinal microbiota, and adipocyte circumference as well as deposits of body fat (Cavallini et al., 2011; Manzoni et al., 2005). However, the relationship between the ingestion of the this specific probiotic product, modulation of microbiota, and obesity has not yet been studied.

Considering the above, the aim of the present study was to verify the effect of a soy-based product, fermented with *E. faecium* CRL 183 and *L. helveticus* 416 with the addition of *Bifidobacterium longum* ATCC 15707, on the composition of fecal microbiota in mice fed a high-fat diet and its relationship with inflammatory parameters and body weight variation.

2. Material and methods

2.1. Material

The soy based probiotic product was fermented by a mixed inoculum of *E. faecium* CRL 183 (Center of reference to *Lactobacillus* - CERELA, Tucumán, Argentina) and *L. helveticus* 416 (Institute of food technology - ITAL, Campinas, SP, Brazil) with the addition of *B. longum* ATCC 15707 (American Type Culture Collection, USA).

2.2. Methods

2.2.1. Probiotic and placebo products

Probiotic and respective placebo products were obtained from UniverSoja – a unit that develops and produces soy products at the Faculty of Pharmaceutical Sciences, UNESP-Araraquara, Brazil. The fermented product was obtained according to the methodology proposed by Rossi, Vendramini, Carlos, Pei, and de Valdez (1999), with modifications (Celiberto et al., 2017), using the starting cultures *E. faecium* CRL 183 (probiotic - 1.5% v/v) and *L. helveticus* 416 (technological purposes - 1.5% v/v). The fermentation was carried out at 37 °C and when the product reached a pH of 4.5, the strain of *B. longum* ATCC 15707 (probiotic) was added in sufficient amount to achieve 8 logCFU ml^{-1} in the final product. The strains were propagated in milk medium (10% of skimmed-milk powder, 1% glucose, 0.5% yeast extract) overnight at 37 °C before being used in the preparation of the products. The viability of each strain was determined immediately after the preparation of the probiotic product (T0) and after seven days of storage at 5 °C (T7). For CFU counts, *L. helveticus* 416, *E. faecium* CRL 183 and *B. longum* ATCC 15707 were plated in Lactobacilli Man Rogosa Sharpe agar – MRS medium (Difco, France), M17 agar (Difco, France), and BIM-25 agar (Reinforced Clostridium agar Difco, France - with the addition of nalidixic acid, polymyxin B sulfate, kanamycin sulfate, iodoacetic acid and triphenyl tetrazolium chloride), respectively. The plates of MRS and M17 were incubated under aerobic conditions for 48 h at 37 °C and BIM-25 plates were incubated under anaerobic conditions for 72 h at 37 °C. The placebo product (unfermented) had identical composition compared to fermented product, without the bacterial cultures. The product was acidified by adding lactic acid food grade (Purac Phytochemical, Brazil) in sufficient amount to reach a pH of 4.5, similar to the fermented product. The products were manufactured weekly and stored at 5 °C ± 1 °C until their administration in the experiment.

2.2.2. Study in animal model

Adult Swiss male mice (Unib: SW) (8 weeks old), Specific Pathogen Free (SPF), were purchased from the Multidisciplinary Centre for Biological Research in the area of science in laboratory animals (CEMIB - UNICAMP, Brazil). The experimental procedures were performed at São Paulo State University (UNESP - Araraquara, Brazil) and the protocol employed was approved by the Sao Paulo State University's Animal Care Committee (CEUA) under the protocol 05/2016.

Animals were housed in polypropylene cages on ventilated shelf (Alesco E-520, Brazil), at 22 °C ± 2 °C with controlled photoperiod (12 h light/12 h dark). During the acclimatization period the animals received only standard diet specific to rodents (Presence® - Labina), with nutritional composition according to specifications of the American Institute of Nutrition (AIN-93M) (Reeves, Nielsen & Fahey, 1993) - total energy value (TEV): 3.6 Kcal/g; protein: 26% of TEV; lipid: 11% of TEV; carbohydrate: 63% Kcal of TEV - and autoclaved water *ad libitum*. After this period, the animals were randomly assigned into four groups (n = 10):

- Control (C): animals that received standard diet;
- Obese (OB): animals that received high-fat diet (HFD);
- Obese + fermented probiotics (OBF): animals that received HFD plus the probiotic fermented product;
- Obese + placebo (OBP): animals that received HFD plus the placebo product (unfermented and without addition of probiotic cultures).

The control group (C) continued to receive the standard diet, while the OB, OBF and OBP groups began to receive HFD (TEV: 5.15 Kcal/g; protein: 14.48% of TEV; lipids: 61.01% of TEV; carbohydrates: 24.51% of TEV), from Prag Bioscience ® Solutions (Jaú, Brazil) (Table 1). The composition of the diet has been adapted from Lenquiste et al. (2015), taking into consideration what is required by AIN-93M (Reeves et al., 1993), with additional animal fat to increase the caloric content of the diet and induce weight gain in the animals. All groups received the diets *ad libitum* during the 70 days of the experimental protocol. Water and food intake as well as body weight were monitored daily before the

Table 1
Nutritional composition of high-fat diet administered to animals under study.

Ingredients	High-fat diet (HFD)		
	Quantity (%)	Kcal/g	Kcal (%)
Corn starch	4.43	0.18	3.44
Ground soybean meal	41.00	1.32	25.75
Dextrinizado starch	5.00	0.20	3.89
Sucrose	8.00	0.32	6.22
Lard	30.20	2.72	52.80
Soybean oil	4.00	0.36	6.99
Microcrystalline cellulose	2.54	–	–
L-cystine	0.18	0.01	0.14
Choline bitartrate	0.15	–	–
Butylated hydroxytoluene	0.00	–	–
Mineral mix AIN 93	3.50	–	–
Vitamin mix AIN 93	1.00	0.04	0.78
Total	100.00	5.15	100.00

High-fat diet (HFD): 18.63% of proteins (14.48% of VET); 34.9% of lipids (61.01% of VET) e 31.55% of carbohydrates (24.51% do VET).
Kcal/g: kilocalorie/gram.
Composition of the diet adapted from Lenquiste et al. (2015).

Fig. 1. Experimental design.

treatments and then weekly until euthanasia of the animals.

The probiotic and placebo products were administered daily by oral gavage. The volume administered was 1.0% of body weight, in order to respect the animal's anatomy and gastric capacity, and to ensure the intake of at least 8.0 logCFU ml^{-1} of probiotic microorganisms (OBF group) (Diehl et al., 2001; Turner, Brabb, Pekow, & Vasbinder, 2011). The administration of the probiotic or placebo products (OBF and OBP groups, respectively) and the high-fat diet was carried out concurrently. The animals of the control (C) and obese (OB) groups, which did not receive the probiotic or placebo products, were orally gavaged daily with the same amount of sterile water. On the 70th day of the experimental protocol the animals were euthanized in a carbon dioxide (CO_2) chamber. Subsequently the organs and tissues of interest were removed for later analysis (liver, retroperitoneal fat and epididymal fat) (Fig. 1).

2.2.3. Determination of the Feed Efficiency Ratio

The Feed Efficiency Ratio (FER), calculated by the ratio of the weight gain to the amount of chow consumed, was used to determine the feed efficiency of the diets (Chan, Zhao & Heng, 2008).

$$FER = (FW - IW)/TF$$

where

FW: final body weight (g) of the animal (end of the experiment)
IW: initial body weight (g) of the animal
TF: total amount of food (rodent chow - g) ingested in the experimental period

2.2.4. Determination of fasting blood glucose

In the ninth week of the experiment (T63) a fasting glucose test was done after a 6-h fasting period. The test was performed using a tail-vein blood sample and glucose levels were measured in a portable glucometer (G-tech Free NoCode System, Rebublic of Korea) (Ayala et al., 2010).

2.2.5. Determination of the Lee index

The Lee index was calculated at the end of the experiment, using the ratio of the cube root of body weight (g) and the nasoanal length (LNA - cm) of each animal, multiplied by 1000 (Lei et al., 2007).

2.2.6. Collection of tissues and organs of interest

After euthanasia of mice, laparotomy was performed to remove organs and tissues of interest. The depositions of intra-abdominal adipose tissue including retroperitoneal and epididymal fat were observed *in situ* and then removed. Subsequently, the liver was dissected. The collected samples were then weighed (semi-analytical balance SHIMADZU, BL 3200H) and processed further and/or stored for histological (epididymal fat) as well as immune (retroperitoneal fat) analysis.

2.2.7. Morphometric analysis of adipocytes

Epididymal fat samples were immediately fixed by immersion in 10% formaldehyde for 24 h. After fixation, the samples were dehydrated in alcohol and xylene, embebed in paraffin and cuts of 5 µm thickness were mounted on glass slides and stained with hematoxylin and eosin (H&E). Fat cells were observed using a light microscope (Olympus BX51-Olympus Optical, Tokyo Japan) coupled to a CCD camera-Iris (Tokyo Japan-Olympus) and an image analyzer system (UTHSCSA Image Tool 3.0® software, The University of Texas Health Science Center) was used to determine the area in addition to the diameter of the fat cells, in which case 200 adipocytes per animal were analyzed to obtain a estimation of adipocyte size (Parlee, Lentz, Mori, & MacDougald, 2014).

2.2.8. Detection of cytokine mRNA expression

For the analysis of the cytokines profile, retroperitoneal white adipose tissue samples were placed in microtubes of 2 mL containing a solution to conserve the RNA: 350 µL of lysis buffer (RA1-RNAspin Mini kit-GE Healthcare, UK) and 3.5 µL of β-mercaptoethanol (β) (Sigma, St. Louis, USA) and frozen at −80 °C (IULT ultrafreezer 335D, Brazil). Subsequently, the tissue was processed using T10 Basic Ultra Turrax Homogenizer (IKA® - Staufen, Alemanha) and extraction of total RNA from white adipose tissue was performed (RNAspin Mini kit-GE Healthcare, UK). The concentration and purity of mRNA samples were measured by determining the ratio of 260/280 nm ($A_{260/280}$ 2.2–1.8) with the Epoch™ microplate spectrophotometer (BioTek multimedia, Winooski, US) running Gen 5 software (BioTek multimedia™). The synthesis of complementary DNAs (cDNAs) was performed by reverse transcriptase reaction (iScript cDNA kit-BioRad, USA).

Expression of interleukin 1 beta (IL-1β), interleukin 6 (IL-6), interleukin 10 (IL-10), transforming growth factor beta (TGF-β) and tumor necrosis factor alpha (TNF-α) mRNA was determined by quantitative polymerase chain reaction (qPCR) using SYBR Green PCR Master Mix (Thermo Fisher Scientific). The specificity was confirmed by the dissociation curve. Each qPCR reaction was performed in duplicate, and the expression was determined by taking the average value of the two replicates in relation to endogenous gene, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), mRNA.

The primers used for gene expression quantification of pro-and anti-inflammatory cytokines were: *IL-6*: Foward (F): 5′-TTCCATCCAGTTG CCTTCTTG-3′ and Reverse (R): 5′-AGGTCTGTTGGGAGTGGTATC-3′ (McGuire et al., 2016); *IL-1β*: F: 5′-CATCCAGCTTCAAATCTCGCAG-3′ and R: 5′-CACACACCAGCA GGTTATCATC-3′ (Liu et al., 2017); *IL-10*: F: 5′-CCCTTTGCTAT GGTGTCCTTTC-3′ and R: 5′-GATCTCCCTGGTTTCT CTTCCC-3′ (Darling, Toth, Arthur, & Clark, 2017); *TGF-β*: F: 5′-TGAC GTCACTGGAGTTGTACGG-3′ and R: 5′-GGTTC ATGTCATGGATGG TGC-3′ (Xu et al., 2017); *TNF-α*: F: 5′-CATCTTCTCAAAAT TCGAGTGA CAA-3′ and R: 5′-TGGGAGTAGACAAGGTACAACCC-3′ (Nikolaidis et al., 2010); GAPDH: F: 5′-AACTTTGGCATTGTGGAAGG-3′ and R: 5′-ACACATTGGGGGTAGGAACA-3′ (Liu, Guan & Ma, 2007). The conditions of the reaction were 50 °C for two minutes, 95 °C for 10 min and 40 repetitions of 95 °C for 15 s and 60 °C for one minute. The analyses of the melting curves were performed on ramp of 65 °C heating to 95 °C. Fluorescence readings were performed by the equipment CFX96 real-time PCR TouchTM Detection System (BioRad) connected to CFX Manager 3.1 software (BioRad). For calculation of relative quantification of the studied sample, the comparative method $2^{-\Delta Cq}$ was applied. The average quantification cycle (Cq) of each gene of interest was subtracted from the average endogenous Cq (GAPDH), resulting in a ΔCq. To calculate gene expression, the ΔCq value obtained was added to formula $2^{-\Delta Cq}$.

2.2.9. Determination of fecal microbiota composition

Fresh stool pellets were collected at the beginning of the experiment (T0) and after euthanasia/dissection (T70) (feces from distal colon). The samples were placed in sterile containers and immediately frozen at −80 °C (IULT ultrafreezer 335 d, Brazil) for further analysis of fecal

Table 2
Primers and reaction conditions for the bacterial genera analyzed by Real-Time Polymerase Chain Reaction.

Microorganisms	Primers	Conditions of PCR*, denaturation/annealing/extension	References
Bifidobacterium spp.	F: 5′-GGGTGGTAATGCCGGATG-3′ R: 5′-CCACCGTTACACCGGGAA-3′	94 °C, 30 s/62 °C, 30 s/72 °C, 45 s	Ryz et al. (2015)
Lactobacillus spp.	F: 5′-AGCAGTAGGGAATCTTCCA-3′ R: 5′-CACCGCTACACATGGAG-3′	94 °C, 30 s/56 °C, 30 s/72 °C, 45 s	Harley et al. (2013) and Morampudi et al. (2016)
Bacteroides spp.	F: 5′-GAGAGGAAGGTCCCCCAC-3′ R: 5′-CGCTACTTGGCTGGTTCAG-3′	94 °C, 30 s/60 °C, 30 s/72 °C, 45 s	Morampudi et al. (2016) and Pachikian et al. (2011)
Clostridium spp.	F: 5′-ATGCAAGTCGAGCGA(G/T)G-3′ R: 5′-TATGCGGTATTAATCT(C/T)CCTTT-3′	94 °C, 30 s/55 °C, 30 s/72 °C, 45 s	Morampudi et al. (2016) and Rinttilä, Kassinen, Malinen, Krogius, and Palva (2004)
Eubacteria (*primer* universal)	F: 5′- ACTCCTACGGGAGGCAGCAGT -3′ R: 5′-ATTACCGCGGCTGCTGGC-3′	94 °C, 30 s/63 °C, 30 s/72 °C, 45 s	Hartman et al. (2009)

All PCR analysis had an initial denaturation step for 3–5 min at 95 °C.
F, *Forward*; R, *Reverse*.

microbiota composition by qPCR. The DNA was extracted using QIAamp DNA Stool Mini Kit (Qiagen) according to manufacturer's recommendations. The concentration and purity of DNA samples were confirmed using the Epoch™ microplate spectrophotometer (BioTek multimedia, Winooski, US) running the Gen 5 software (BioTek multimedia™). The purity was evaluated by the 260/280 nm ratio ($A_{260/280}$ 2.2–1.8). The qPCR was carried out in a *Quant Studio™ 6 Flex System* machine from Applied Biosystems™ (Life Technologies) using SYBR Green PCR Master Mix (Thermo Fisher Scientific).

The total extracted DNA was normalized to the concentration of 2 ng/µL and used for amplification of the 16S ribosomal RNA gene region (16S rRNA) by qPCR. The total volume of reaction was of 20 µL (5 µL DNA template, 10 µL of SYBR-Green, 0.6 µL of the forward primer, 0.6 µL of reverse primer - Sigma Aldrich and 3.8 µL of pyrogen free sterile water - Farmace). As described in Table 2, specific primers and conditions were used for each genus investigated. Universal primer Eubacteria was used to determine the total of 16S rRNA bacteria in each sample. The relative abundance of each genus was determined by calculating the average Cq value relative to Cq value for total bacteria, normalized to each primer's determined efficiency (Ryz et al., 2015).

2.2.10. Statistical analysis of the results

Data obtained from the experiments are shown as means ± standard deviations (SD). The normality of the distribution of the data was checked by the Shapiro-Wilk test. The parametric data were subjected to analysis of variance (ANOVA) followed by the Tukey's post-test and nonparametric data were analyzed by Kruskal-Wallis followed by the Dunn's post-test. Microbiota variation were analyzed by two-tailed independent *t*-test. Differences were considered significant when $p < 0.05$.

3. Results

3.1. Control of microorganism viability in fermented probiotic product

Throughout the experimental protocol, the average viable cells of the probiotic microorganisms used in the probiotic product (*E. faecium* CRL 183 and *B. longum* ATCC 15707) remained between 8.63 and 8.85 logCFU mL^{-1}.

3.2. Weight variation

Weekly evaluation of mice body weight indicated a continuous increase, and the average weight of the obese group (OB) fed a high-fat diet was statistically higher from the control group (C) fed a standard diet from the second week onwards ($p < 0.05$). On the other hand, the weights of the obese mice fed a placebo drink (OBP) and obese mice fed a fermented probiotic drink (OBF) differed statistically from group C at

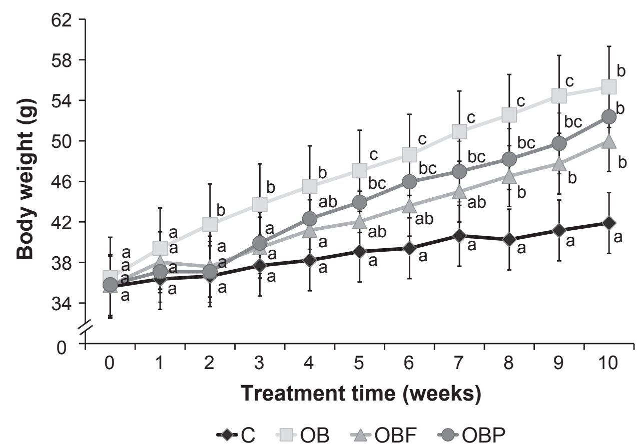

Fig. 2. High-fat diet and soy-based probiotic drink effect on weekly body weight gain (g). [abc]Different letters at the same point represent significant difference by ANOVA and Tukey's post-test ($p < 0.05$). n = 10. C = animals that received standard diet; OB = animals that received high-fat diet (HFD); OBF = animals that received HFD plus the probiotic fermented product; OBP = animals that received HFD plus the placebo product.

the 5th and 8th week of treatment, respectively ($p < 0.05$). Moreover, the OBF group receiving the probiotics drink exhibited a lower average weight gain compared to the OB group until the 9th week of the study ($p < 0.05$). The placebo group (OBP) presented average weight gain similar to the fermented group (OBF), with no difference when compared to the obese group (OB) between the 4th and 9th week ($p < 0.05$) (Fig. 2).

3.3. Food and water consumption and Feed Efficiency Ratio (FER)

The group that received standard diet (Group C) presented a higher daily food intake compared to the high-fat diet fed groups (OB, OBF, OBP) ($p < 0.01$). Meanwhile, the high-fat diet group that received the probiotic product (OBF) showed lower food intake compared to the OB ($p = 0.04$) and OBP groups ($p < 0.01$). The groups that ingested a high-fat diet (OB, OBF and OBP) presented FER higher than the group fed with standard diet (C) ($p < 0.05$) (Table 3).

3.4. Determination of fasting blood glucose

The control group (C) presented the lowest fasting blood glucose level (Mean ± SD = 163.5 ± 24.3 mg dL^{-1}) and differed significantly from the high-fat diet groups ($p < 0.05$). Among the induced to obesity groups, the OBF exhibited the smallest value of fasting blood glucose

Table 3
Food and water consumption, Feed Efficiency Ratio (FER), total mass of the epididymal fat, retroperitoneal fat and liver, Lee index, area and diameter of adipocytes of the different groups.

Group	Daily food consumption (g)	Daily water consumption (g)	FER (g/g)	Epididymal fat (g)	Retroperitoneal fat (g)	Liver (g)	Lee index (g/cm^3)	Area of adipocytes (μm^2)	Diameter of adipocytes (μm)
C	6.30 ± 0.74^c	12.16 ± 3.43^{a,b}	0.016 ± 0.009^a	0.79 ± 0.33^a	0.24 ± 0.13^a	2.05 ± 0.25^a	323.44 ± 7.80^a	23.50 ± 13.80^a	5.81 ± 1.05^a
OB	4.64 ± 0.83^b	12.63 ± 3.54^b	0.067 ± 0.025^b	2.80 ± 0.74^b	0.72 ± 0.19^b	2.27 ± 0.33^a	339.29 ± 11.79^b	67.48 ± 31.10^c	11.98 ± 2.44^c
OBF	3.98 ± 0.52^a	8.51 ± 1.79^a	0.059 ± 0.016^b	2.68 ± 0.77^b	0.66 ± 0.22^b	2.21 ± 0.45^a	336.92 ± 10.92^{a,b}	56.22 ± 27.92^b	9.19 ± 1.53^b
OBP	4.92 ± 0.47^b	8.94 ± 3.29^{a,b}	0.055 ± 0.011^b	2.29 ± 0.70^b	0.62 ± 0.18^b	2.15 ± 0.32^a	343.04 ± 17.96^b	70.39 ± 35.39^c	10.96 ± 2.39^c

Results presented as mean ± SD. n = 10.
C = animals that received standard diet; OB = animals that received high-fat diet (HFD); OBF = animals that received HFD plus the probiotic fermented product; OBP = animals that received HFD plus the placebo product. Different letters in the same column (comparison between groups) represent significant difference by ANOVA and Tukey's post-test or by Kruskal-Wallis and Dunn's post-test (only for FER) ($p < 0.05$).

(Mean ± SD = 258.6 ± 20.7 mg dL^{-1}), but with no significant difference in comparison with the OB (Mean ± SD = 287.5 ± 66.3 mg dL^{-1}) and OBP (Mean ± SD = 308.9 ± 39.7 mg dL^{-1}) groups.

3.5. Evaluation of the adipose tissue, liver and Lee index

All groups fed with the high-fat diet demonstrated an increase in intra-abdominal white fat content (epididymal and retroperitoneal) compared to control group (C) ($p < 0.01$), although without displaying significant differences among themselves. In addition, there was no significant difference in the weight of the livers among groups ($p = 0.538$). The Lee index from the control group (C) was significantly lower compared to the diet-induced obese groups (OB, $p < 0.05$ and OBP, $p < 0.01$) and the intake of the probiotic product (OBF group) contributed to the improvement of this parameter (Table 3).

3.6. Morphometric assessment of adipocytes

The adipocytes in the diet-induced obese mice showed significantly higher area and average diameter in addition to macrophage accumulation compared to the control group. Among the diet-induced obese mice, the probiotic group (OBF) presented with a smaller area and diameter of adipocytes ($p < 0.05$) (Table 3 and Fig. 3).

3.7. Analysis of cytokine expression profile in adipose tissue

The probiotic (OBF) and placebo (OBP) groups showed a higher level of IL-10 compared to control and obese groups as well as elevated IL-6 when compared to the control group (Fig. 4B/C). The expression of TGF-β was higher in the placebo group (OBP) ($p < 0.05$) when compared to negative (C) and positive (OB) controls (Fig. 4D). However, there was no difference in IL-1β expression between the groups C, OB and OBF ($p < 0.05$) (Fig. 4A). Nonetheless, the placebo group (OBP) showed significantly higher levels of IL-1β, without difference to control group (C). TNF-α levels were similar ($p < 0.05$) between all groups (C, OB, OBF and OBP) (Fig. 4E).

3.8. Determination of the composition of fecal microbiota

Regarding the four genera of bacteria analyzed before and after the induction of obesity and the product administration, there was a prevalence of the genus *Bacteroides* spp. at the baseline level (T0) in samples of all the experimental groups (C = 99.73%; OB = 99.72%; OBF = 96.76% and OBP = 86.97%) (Fig. 5A). Similarly, at the end of the experimental protocol (T70) this same genus was prevalent in all groups, together with an increase in the population of *Lactobacillus* spp. in diet-induced obese animals, especially in the OB and OBP groups. The percentage of *Bacteroides* spp. was 99.78% in Group C, 91.47% in OBF group, 74.47% in Group OB and 51.61% in Group OBP. On the other hand, the proportion of *Lactobacillus* spp. was 48.39%, 25.53%, 8.53% and 0.22% in the OBP, OB, OBF and C groups, respectively (Fig. 5B).

At the end of the experiment the population of *Bifidobacterium* spp. was equivalent to 0.0020% in Group C, 0.0014% in OBF group, 0.0008% in Group OBP and 0.0005% in Group OB. A similar result was verified for the genus *Clostridium* spp. with proportions of 0.0018% (OBP), 0.0006% (OBF group), 0.0004% (OB) and 0.0003% (Group C) (Fig. 5B). Although the genera *Bifidobacterium* spp. and *Clostridium* spp. were present in very small proportions in all groups studied, the intragroup comparison-initial (T0) and final (T70) time indicated a significant increase in the relative abundance of *Bifidobacterium* spp. in the OBF (63.22% - $p < 0.05$) and *Clostridium* spp. in the OBP (83.25% - $p < 0.05$) (Fig. 5D, F).

4. Discussion

The present study was undertaken to verify the anti-obesity effect of a probiotic soy beverage and its relationship with gut microbiota composition in an animal model of diet-induced obesity.

Probiotic microorganisms are able to confer many health benefits that assist in the positive modulation of intestinal microbiota and in the control of diseases related to dysbiosis, such as obesity (Alard et al., 2016; Park et al., 2013; Park, Oh, & Cha, 2014). The beneficial effects of probiotics is due to the intrinsic feature of each strain and the amount of viable cells ingested daily. In accordance to the recommendations of the International Scientific Association for Probiotics and Prebiotics (ISAPP), the probiotic product must contain enough viable cells to induce the beneficial effect, which must be proven using *in vivo* studies (Hill et al., 2014). In this study the probiotic soy product used contained viable cells of *E. faecium* CRL 183 and *B. longum* ATCC 15,707 higher than 8 logCFU mL^{-1}, which is similar to other studies that used probiotic strains (Lee, Lo & Pan, 2013; Ya-Ni, Qiong-Fen, Nian, Xiao-Wei, & Fang-Gen, 2010).

Overall, the proportion of lipids derived from obesity-inducing diets in mice and rats ranges from 30 to 78 percent of TEV. This energy is given by the increased percentage of fat (saturated or unsaturated) or by the addition of food with a high content of lipids (cafeteria diet) (Bourgeois, Alexiu & Lemonnier, 1983; Huang, Xin, McLennan, & Storlien, 2004). In the present study, the percentage of energy derived from the lipid fraction of the diet, mainly as saturated fatty acids (lard), corresponded to 61.01%. This percentage was sufficient to induce high-weight gain in the animals to a degree approaching severe obesity (weight gain of approximately 40% compared to the control group) (Hariri & Thibault, 2010).

Various experiments in murine animal models indicate that palatable and nutritionally complete food with high levels of fat and sugar induces binge eating disorders and excessive body weight gain (Rolls, Van Duijvenvoorde & Rowe, 1983). However, other studies have shown that animals exposed to a diet with high levels of fat or energy, reduce food consumption as a way to compensate for elevated energy intake

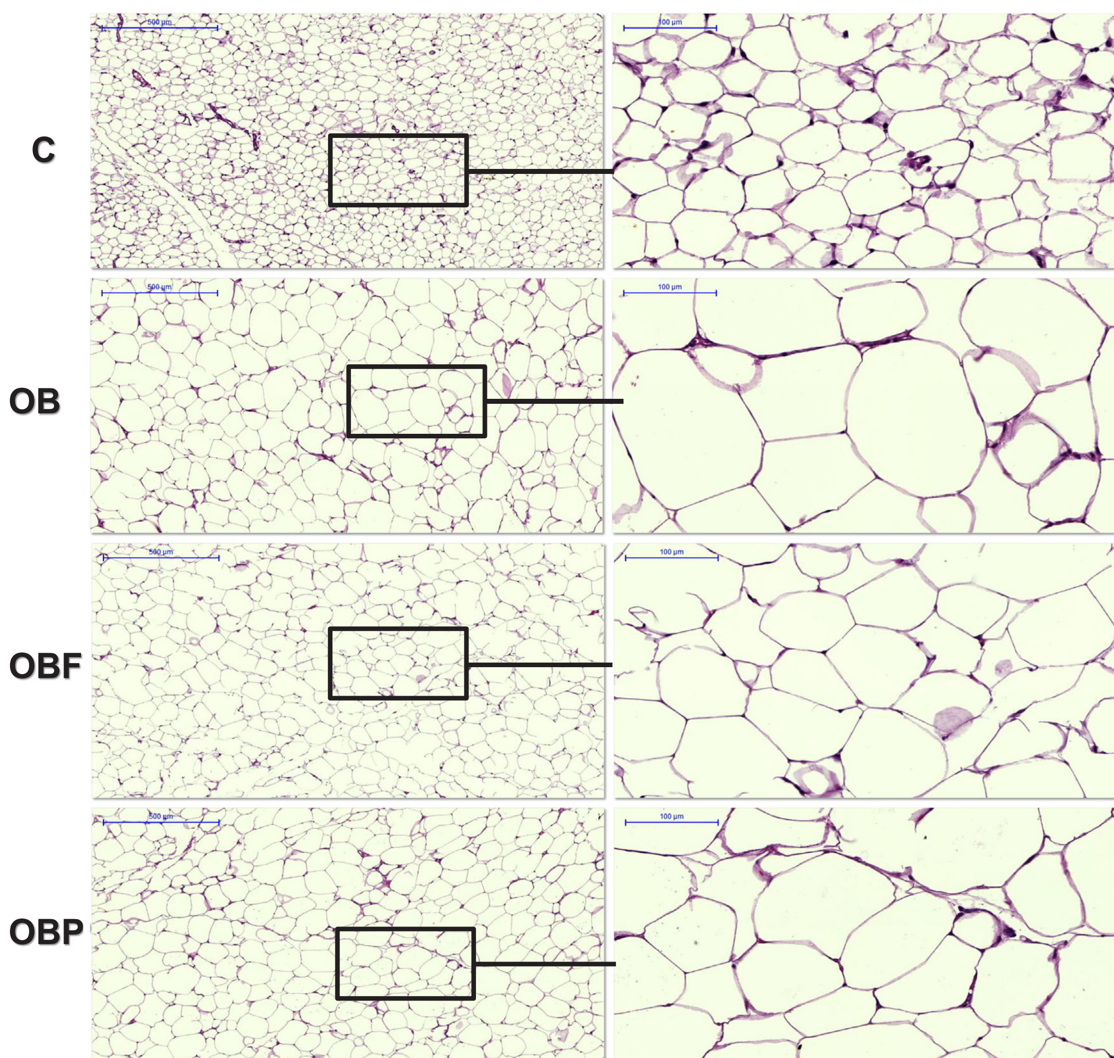

Fig. 3. Representative photomicrograph of H&E stained epididymal fat sections. C = animals that received standard diet; OB = animals that received high-fat diet (HFD); OBF = animals that received HFD plus the probiotic fermented product; OBP = animals that received HFD plus the placebo product.

(La Fleur, Van Rozen, Luijendijk, Groeneweg, & Adan, 2010; Mercer & Archer, 2008).

We observed that animals receiving a hyperlipid diet (OB, OBF, and OBP) exhibited lower food intake during the experimental protocol (Table 3). One possible explanation for this result would be a body compensatory mechanism to maintain energy balance, since the high-fat diet is hypercaloric (5.15 Kcal/g) compared to the standard diet (3.6 Kcal/g). The Feed Efficiency Ratio (FER) assess the ability of the animal to convert each gram of food consumed into body weight (Timon & Eisen, 1970). Although obese-induced animals consumed a lower amount of food during the experimental protocol, they showed increased body-weight gain (g) and a higher FER (Table 3), suggesting that the high energy supply, especially in the form of lipids, contributed to the accumulation of adipose tissue. This indicates that the development of obesity in animals fed a HFD was due to a higher feed efficiency and not to hyperphagia (Harada et al., 2016).

Of note, OBF mice showed a greater reduction in food intake compared to the rest of the groups and lower weight gain compared to the obese group (OB) up to the ninth week of study (Fig. 2), demonstrating the suppressive role of the probiotic drink in diet-induced obesity. Strain specific effects of probiotics on body weight has been evidenced by several studies. Ya-Ni et al. (2010) evaluated the effect of four strains of *Bifidobacterium* spp. (L66-5, L75-4, M13-4 and FS31-12) in the weight gain of rats fed with a high-fat diet (16.52% protein; 25.17% fat and 56.66% carbohydrate) for six weeks. When compared to the control group, the animals that received the strain M13-4 experienced significant increased weight gain and strain L66-5 rather induced a reduction in weight gain. However, the other two strains evaluated did not influence the weight variation. Forssten et al. (2013) investigated the relationship among intake of fermented milks containing different species of *Lactobacillus* spp., production of satiety hormones, and food intake in Wistar rats. Dependent on the given strain, the authors found a significant lower consumption of food, reduction in weight gain and modified production of satiety hormones. Alard et al. (2016) also confirmed the strain-specific effect of probiotics, with animals given *Lactobacillus salivarius* Ls33 displaying a lack of obesity control, while *Lactobacillus rhamnosus* LMG S-28148 and *Bifidobacterium animalis* subsp. *lactis* LMG P-28149 (mix) were able to control obesity induced by a high-fat diet. The beneficial effect was mainly supported by LMG P-28149 strain. Kefir, a beverage composed of different species of lactic acid bacteria, acetic acid bacteria and yeasts, also has proven anti-obesity effects. Kim et al. (2017) observed that dairy intake of kefir for 12 weeks resulted in a reduction in weight gain and liver injury, positive modulation of the gut microbiota and up-regulation of genes related to fatty acid oxidation in mice fed with a high-fat diet.

Obesity is strongly associated to insulin resistance, which is an important risk factor in development of type 2 diabetes (Meissburger et al., 2011). In our study, the OBF group showed a lower increase in

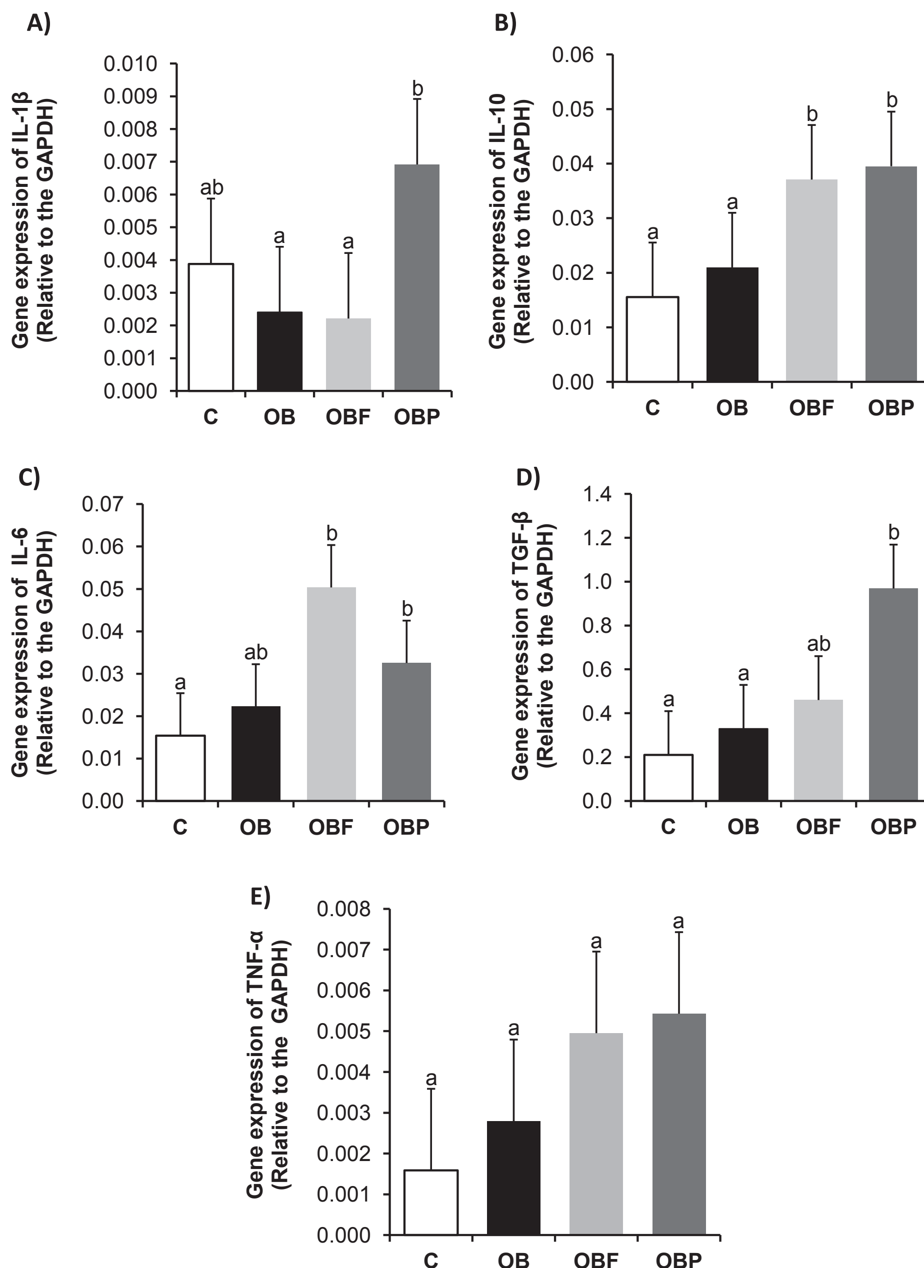

Fig. 4. Relative expression of cytokines in relation to endogenous gene GAPDH in white adipose tissue (retroperitoneal fat). (A) relative expression of interleukin 1 beta (IL-β 1); (B) relative expression of interleukin 10 (IL-10), (C) relative expression of Interleukin 6 (IL-6); (D) relative expression of transforming growth factor beta (TGF-β); (E) relative expression of tumor necrosis factor alpha (TNF-α. C = animals that received standard diet; OB = animals that received high-fat diet (HFD); OBF = animals that received HFD plus the probiotic fermented product; OBP = animals that received HFD plus the placebo product. ^{ab}Different letters represent significant difference by ANOVA and Tukey's post-test ($p < 0.05$). n = 10.

fasting blood glucose (58.17%) compare to control group, but this effect was not significant compared to OB and OBP groups (increase of 75.85% and 88.93%, respectively). Manzoni et al. (2005) showed that the regular intake of a similar probiotic beverage (*E. faecium* CRL 183) supplemented with isoflavones (50 mg isoflavones/100 g of fermented soy product), reduced the blood glucose level in hypercholesterolemic animals compared to the group fed only cholesterol-enriched diet. Despite using a probiotic beverage fermented with the same probiotic strain, the product composition, diets, and experimental protocols were different, which may explain the discrepancy among the results.

Experimental diets with high lipid content (> 40% of TEV) and with low or moderate carbohydrate content (9–30% from the TEV) contribute to the increase in fatness index related to the proportion of epididymal and retroperitoneum white fat deposits (Ghibaudi, Cook, Farley, van Heek, & Hwa, 2002). As evidenced by the total mass of epididymal and retroperitoneal fat, regular ingestion of the probiotic beverage did not alter fat deposits (Table 3). Similar results were obtained from Park et al. (2014), who found no significant reduction in the amount of epididymal fat in mice fed with a high-fat diet (60% Kcal from the TEV) and supplemented with 10^7 CFU of *L. plantarum* LG42, compared to the group that received only a high-fat diet. However, a higher intake of the same probiotic (10^9 CFU) resulted in a significant decrease in epididymal fat deposit ($p < 0.05$), highlighting the importance of the dose effect of the administered strain. Savcheniuk et al. (2014) concluded that the ingestion of a mixture of probiotics (*Lactobacillus casei* 2:1:1 IMVB-7280, *Bifidobacterium animalis* VKL, *B. animalis* VKB) showed a protective effect in the development of monosodium glutamate (MSG)-induced obesity in rats, since the Lee index values and visceral fat weight were significantly lower than non-treated group.

The expansion of adipose tissue is linked to adipocyte hypertrophy and hyperplasia, an increase in macrophage accumulation, and production of pro-inflammatory cytokines that characterize the systemic inflammation observed in obesity (Apovian et al., 2008; Caesar et al., 2012; Oh et al., 2017). In this study, the intake of a high-fat diet resulted in an increased adipocyte size along with an elevated presence of macrophages. Of note, probiotic intake prevented the increase in area

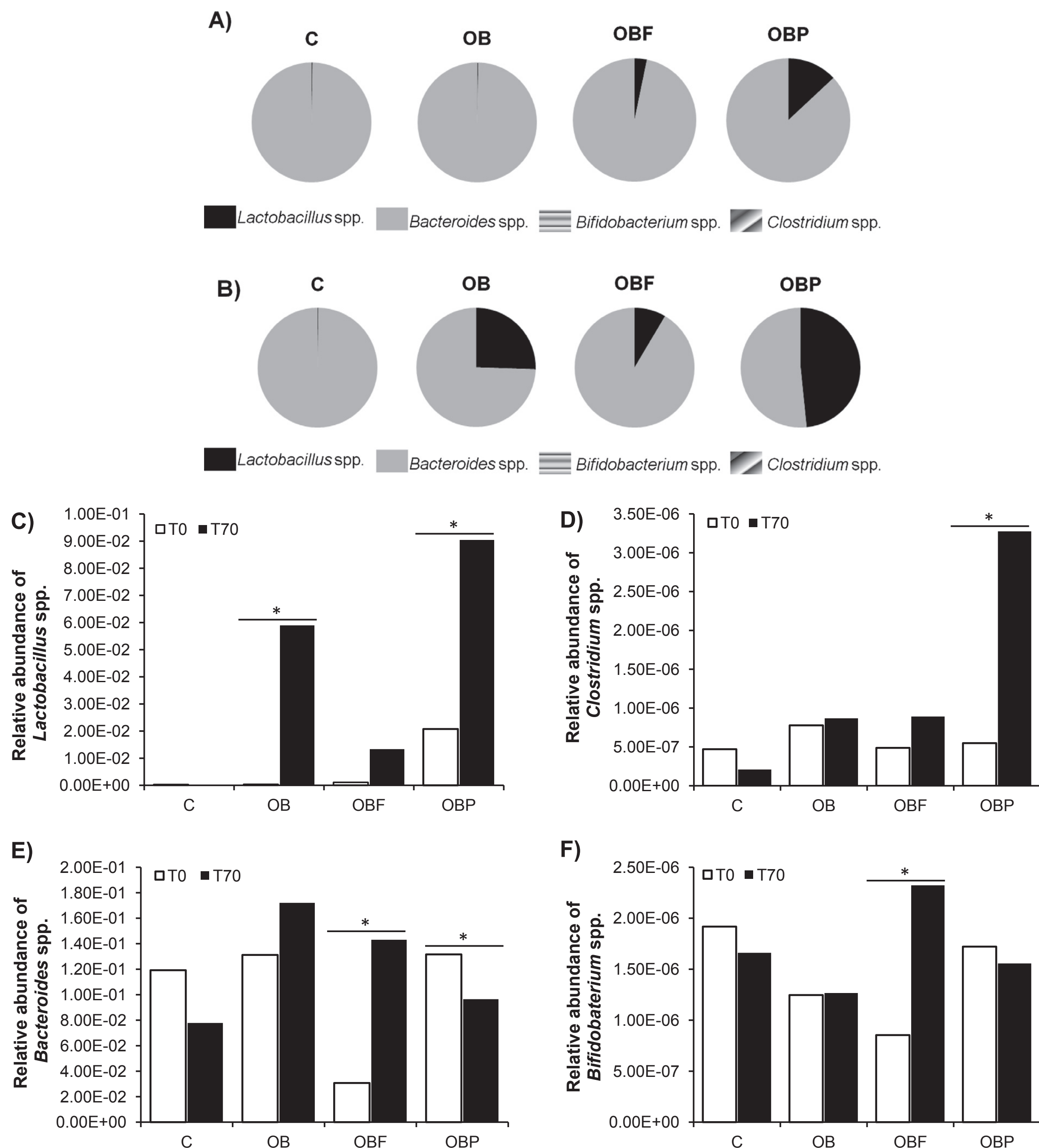

Fig. 5. High-fat diet and soy-based probiotic drink effect on the microbiota composition at the genus level. (Proportion of *Lactobacillus* spp., *Bacteroides* spp., *Bifidobacterium* spp and *Clostridium* spp. determined at (A) initial time (T0) and at (B) end time (T70). Abundance of *Lactobacillus* spp. (C), *Clostridium* spp. (D), *Bacteroides* spp. (E) and *Bifidobacterium* spp. (F) relative to total bacterial (two-tailed independent *t*-test – $p < 0.05$). C = animals that received standard diet; OB = animals that received high-fat diet (HFD); OBF = animals that received HFD plus the probiotic fermented product; OBP = animals that received HFD plus the placebo product.

and diameter of adipocytes compared to OB and OBP groups (Table 3, Fig. 3). The *E. faecium* CRL 183 strain had already been found to maintain the adipocyte morphology in rats induced to hyperlipidemia (Manzoni et al., 2005), confirming its effect on the control of obesity.

Resident macrophages in adipose tissue are classified into two subtypes: M1 (classically activated), characterized by production of high levels of pro-inflammatory cytokines, and M2 (alternatively activated), which produce anti-inflammatory mediators and are dominant in lean adipose tissue (Darling et al., 2017; Lumeng, Bodzin, & Saltiel, 2007; Xu, 2013). In adipose tissue, obesity causes a change in the macrophage profile resulting in the prevalence of the M1 phenotype and accordingly, the production of inflammatory cytokines (TNF, IL-1, IL-6). Thus, the balance between M1 macrophages (pro-inflammatory) and M2 (anti-inflammatory) play an important role in inflammation of adipose tissue and are associated with complications, such as in insulin resistance (Zhang et al., 2015).

Our data show that adipose tissue from animals treated with probiotics (OBF) expressed higher expression of IL-10 and IL-6 compared to control group (C) (Fig. 4). A recent study has shown the ability of different strains of lactic acid bacteria to modulate the secretion of adipokines in adipose tissue using an *in vitro* technique, and it was observed that lactic acid bacteria exert different effects on the production

of TNF-α, IL-6, IL-10 and MCP-1 in adipocytes. *Lactococcus lactis* CRL1434 strain increases TNF-α, MCP-1, and IL-10 expression in comparison to other strains, while the *Lactobacillus plantarum* CRL350 strain induced higher levels of IL-6 (Fabersani et al., 2017).

IL-6 is secreted by adipocytes primarily in sites that present with acute or chronic inflammation, such as by the visceral adipose tissue (Castoldi, De Souza, Saraiva Câmara, & Moraes-Vieira, 2016). However, some studies indicate that IL-6 signaling during inflammation or metabolic disorders like obesity and diabetes mellitus type 2, presents a homeostatic anti-inflammatory role (Carey et al., 2006; Kelly, Gauthier, Saha, & Ruderman, 2009; Matthews et al., 2010; Petersen & Pedersen, 2005; Stolarczk, 2017; Timper et al., 2017). IL-6 can increase IL-10, IL-1 receptor antagonist, and soluble TNF receptors (Petersen & Pedersen, 2005; Steensberg, Fischer, Keller, Moller, & Pedersen, 2003). Also, IL-6 appears to have an influence on the regulation of appetite, energy expenditure and body composition (Pascoal et al., 2017; Ropelle et al., 2010). Matthews et al. (2010) found that IL-6-deficient mice (IL-6$^{-/-}$) consuming a normal diet showed obesity, liver inflammation and insulin resistance compared to control animals (IL6$^{+/+}$), without a change in food consumption. IL-6-deficient animals exposed to a high-fat diet demonstrated increased liver inflammation and insulin resistance when compared to wild type (WT) mice. Moreover, no differences were found in body weight, fat mass and ectopic lipid deposition in the liver between the two groups. The presence of IL-10 in the microenvironment promotes inhibition of cytokines TNF-α as well as IL-1β production, and the development of obesity is associated with a reduction in IL-10 levels (Opal & DePalo, 2000). IL-10 and TGF-β (anti-inflammatory cytokines) also can be secreted as a way to control the chronic obesity-related inflammation (Pereira et al., 2014).

During obesity, the pro-inflammatory cytokines IL-1β and TNF-α can interfere directly with insulin signaling in adipocytes (Liang et al., 2018). We observed that IL-1β expression in the OBF group did not differ from C and OB groups, while TNF-α levels were similar between all groups (C, OB, OBF and OBP) (Fig. 4). These results were not expected, since a higher expression of IL-1 β and TNF-α (proinflammatory cytokines) is generally observed during inflammatory states, such as obesity. Considering the positive effect of probiotics in the OBF group regarding weight gain reduction compared to the obese group (OB) until the 9th week, future studies are needed to evaluate the effect of this particular probiotic product in the modulation of pro and anti-inflammatory cytokines. It is important to understand the effect of this probiotic in different phases of obesity, considering that in the present study the cytokine profile was analyzed in the 10th week when weight gain had similar levels compared to obese animals (OB) not receiving probiotics.

The intestinal microbiota is the name given to the microorganism living in the intestine, and it comprises primarily of the phyla *Bacteroidetes* and *Firmicutes* with a lower proportion of *Proteobacteria, Actinobacteria and Verrumicrobia*. The phylum *Bacteroidetes* is mainly composed of the genera *Bacteroides* spp., *Prevotella* spp., *Parabacteroides* spp., *Porphyromonas* spp., *Alistipes* spp., while the phylum *Firmicutes* includes the genera *Clostridium* spp., *Lactobacillus* spp., *Peptostreptococcus* spp, *Ruminococcus* spp., among other Gram-positive bacteria (Clarke et al., 2012; Hakansson & Molin, 2011).

Excessive consumption of foods with a high energy content and a lower nutritional quality, consisting mainly of lipids and refined carbohydrates, may cause intestinal dysbiosis, metabolic disorders and immune imbalance (Haque & Haque, 2017; Myles, 2014). Several studies show that obesity-induced animals as well as obese individuals exhibit greater relative abundance of *Firmicutes* and lower abundance of *Bacteroidetes* compared to eutrophic controls (Million et al., 2013; Xiao et al., 2017). In contrast, other studies have failed to prove a robust relationship between the *Bacteroidetes: Firmicutes* ratio and obesity (Park et al., 2013; Schwiertz et al., 2010), indicating that the differences in genera and/or species that make up such phyla could offer a better explanation. At lower taxonomic levels, an increase in *Lactobacillus* spp. (Armougom, Henry, Vialettes, Raccah, & Raoult, 2009; Million et al., 2012) and a reduction in *Bifidobacterium* spp. (Million et al., 2012) was associated with weight gain and obesity.

There are some non-exclusive mechanisms involved in the relationship between intestinal microbiota composition and obesity. Among them is the production of short chain fatty acids (SCFA – mainly butyrate, propionate, and acetate) by intestinal bacteria that promote changes in intestinal motility while facilitate the uptake of nutrients and energy from the diet; the low-grade chronic inflammation due to lipopolysaccharides (LPS) from Gram-negative bacteria; and the deregulation of the fasting-induced adipose factor (FIAF) by intestinal microorganisms that leads to the deposition of triglycerides in adipocytes (Boulangé, Neves, Chilloux, Nicholson, & Dumas, 2016; Louis & Flint, 2009; Lu et al., 2016).

Positive modulations in gut microbes by probiotics have been associated with maintenance of the intestinal barrier, and inflammation reduction in overweight or obese individuals (Kobyliak et al., 2016). However, the results obtained are often contradictory since the microbiota composition is influenced by several factors such as diet, age, and genetics (Richards, Yap, McLeod, Mackay, & Mariño, 2016). Alard et al. (2016) concluded that a probiotic mixture (*Lactobacillus rhamnosus* LMG S-28148 and *B. animalis* subsp. lactis LMG P-28149) reduced the adiposity, insulin resistance and positively modulated the production of butyrate and propionate. The authors suggested that the microbiome modulation, with restoration in the abundance of *Akkermansia muciniphila* as well as *Rikenellaceae,* and reduction in *Lactobacillaceae* was involved in the beneficial effects.

In this study, we observed that a soy probiotic drink positively modulates the fecal microbiota by raising the population of *Bifidobacterium* spp. in addition to maintaining a balance between the relative abundance of *Lactobacillus* spp. and *Bacteroides* spp. (Fig. 5), genera comprised in the phyla *Firmicutes* and *Bacteroidetes*, respectively. This result is important because an increase in the *Firmicutes/Bacteroidetes* ratio and a decrease in *Bifidobacterium* spp. seems to be involved in obesity-associated dysbiosis (Ignacio et al., 2015; Koliada et al., 2017; Krajmalnik-Brown et al., 2012; Million et al., 2012; Sweeney & Morton, 2013). A previous study that evaluated the effect of the same probiotic drink on the development of DSS-induced colitis, found an increase in the fecal population of *Bifidobacterium* spp., and elevation in acetate and propionate levels in the colon of C57BL6 mice (Celiberto et al., 2017). The genus *Bifidobacterium* spp. is known to produce propionate and acetate from non-digestible carbohydrates, and acetate serves as co-substrate in the production of butyrate (Rivière, Selak, Lantin, Leroy, & De Vuyst, 2016). Despite a lack of consensus, acetate, proprionate and butyrate appear to attenuate body weight to a similar extent, and the microbiota composition directly interferes with the production of such metabolites (Lin et al., 2012; Lu et al., 2016). Future determination of the microbiota composition at more specific levels (species), as well their metabolites, may help to confirm the exact mechanisms involved in the observed effect.

5. Conclusion

In conclusion, the regular intake of the soy probiotic drink was able to reduce weight gain, the degree of adipocyte hypertrophy, and to modulate cytokine expression related to the obesity control in diet-induced obese mice. We speculate that the beneficial effects verified in the OBF group could be related to the positive modulation of intestinal microbiota composition, which consequently influences the production of different metabolites and expression of cytokines involved in the control of the inflammatory processes associated with obesity.

Conflict of interest

The authors declared that there is no conflict of interest.

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