Journal of Functional Foods 55 (2019) 333–342

Contents lists available at ScienceDirect

Journal of Functional Foods

journal homepage: www.elsevier.com/locate/jff

Yacon (*Smallanthus sonchifolius*)-based product increases fecal short-chain fatty acids and enhances regulatory T cells by downregulating *RORγt* in the colon of BALB/c mice

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ARTICLE INFO

Keywords:
Yacon
Fructooligosaccharides
Prebiotic
Treg cell
Short-chain fatty acids
RORγt

ABSTRACT

Although yacon (*Smallanthus sonchifolius*) is a known source of prebiotics (fructooligosaccharides (FOS) and inulin) and phenolic compounds beneficial to gut microbiota and intestinal immune response modulation, its regulatory mechanisms still remain unclear. Therefore, we investigated whether the consumption of a yacon-based product (PBY) modulates the population of intestinal lymphocytes as well as transcription factors that drive host adaptive immune responses. For this purpose, BALB/c male mice were fed either a standard AIN-93M diet or AIN-93M diet supplemented with PBY (6.0% FOS + Inulin) for 8 weeks. We found that PBY consumption in mice reduced food intake, improved fecal humidity and viscosity, intensified fecal short-chain fatty acid production, increased the number of regulatory T cells, and downregulated the expression of RORγt transcription factor in the colon. Thus, it can be inferred from the findings that PBY consumption improves satiety and mucosal integrity, and possibly favors anti-inflammatory immune responses in the colon.

1. Introduction

Yacon (*Smallanthus sonchifolius*) is a tuberous root rich in phenolic compounds and considered prebiotic due to its high fructooligosaccharides (FOS) and inulin content (Choque Delgado, Thomé, Gabriel, Tamashiro, & Pastore, 2012; Russo, Valentão, Andrade, Fernandez, & Milella, 2015). FOS and inulin are fructose oligomers composed mainly of α (2 → 1) or β (2 → 6) linkages which are not hydrolyzed by human digestive enzymes thus remain intact in the colon (Fernández et al., 2013). As a result, these fructans serve as fermentable substrates for probiotic bacteria (Gibson & Roberfroid, 1995).

The consumption of yacon has been reported in the literature to increase the number of *Bifidobacteria* and *Lactobacilli* (de Souza Lima Sant'Anna, Rodrigues, Araújo, de Oliveirado Carmo Gouveia Peluzio, & de Luces Fortes Ferreira, 2015; Utami et al., 2013) and concurrently inhibit the growth of pathogenic bacteria (Sant'Anna et al., 2018; Veiga et al., 2014). Interestingly, the number of probiotic bacteria directly reflects the production of fecal short-chain fatty acids (SCFA) (Grancieri et al., 2017; Sant'Anna et al., 2018; Utami et al., 2013; Vaz-Tostes et al., 2014). Yacon consumption also improves intestinal transit (de Souza Lima Sant'Anna et al., 2015; Geyer, Manrique, Degen, & Beglinger, 2008; Sant'Anna et al., 2018), satiety and body weight (Genta et al., 2009; Gomes da Silva et al., 2017). In addition, it confers antiobesity properties by inhibiting adipogenesis (Honoré, Grande, Gomez Rojas, & Sánchez, 2018). Other metabolic benefits have been commonly reported such as glycemia control (Honoré et al., 2018; Scheid, Genaro, Moreno, & Pastore, 2014), decrease in total cholesterol and triglycerides levels, as well as improvement in high-density lipoprotein-

Abbreviations: PBY, yacon-based product; FOS, fructooligosaccharides; SCFA, short-chain fatty acids; RORγt, Retinoic acid receptor (RAR)-related orphan receptor gamma

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https://doi.org/10.1016/j.jff.2019.02.039
Received 12 November 2018; Received in revised form 20 February 2019; Accepted 21 February 2019
Available online 02 March 2019
1756-4646/

cholesterol levels (Habib, Honoré, Genta, & Sánchez, 2011; Habib, Serra-Barcellona, Honoré, Genta, & Sánchez, 2015; Roselino et al., 2012).

Yacon consumption is capable of improving intestinal architecture and cellularity despite regulating the number and depth of intestinal crypts (Lobo, Colli, Alvares, & Filisetti, 2007; Sant'Anna et al., 2018), which in turn increases the intestinal absorption of calcium, magnesium and iron, and calcium deposition in bones (Lobo et al., 2007; Lobo, Gaievski, De Carli, Alvares, & Colli, 2014; Rodrigues et al., 2012). Regarding immunological benefits, yacon intake increases secretory immunoglobulin A (IgA) production in feces (Choque Delgado et al., 2012; Grancieri et al., 2016), serum cytokines related to anti-inflammatory processes, such as interleukin (IL)-10 and IL-4 (Bonet et al., 2010; Grancieri et al., 2017; Vaz-Tostes et al., 2014), and phagocytic activity of macrophages (Paredes et al., 2018). Furthermore, it has been reported that yacon consumption promotes the regression of pre-neoplastic lesions in experimental models of colorectal carcinogenesis (de Moura et al., 2012; Grancieri et al., 2017), and it presents a high in vivo antioxidant activity (Habib et al., 2015).

Evidently, the health benefits of yacon consumption are mainly attributed to the modulation of the intestinal microbiota (de Souza Lima Sant'Anna et al., 2015; Utami et al., 2013) and intestinal immune response (Bonet et al., 2010), however the regulatory mechanisms involved in these events remain unclear and unexplored. Studies have shown that probiotic bacteria may induce anti-inflammatory responses through the activation of regulatory T cells (Treg cells) (Roselli et al., 2009) and the attenuation of a pro-inflammatory microenvironment through reduced IL-17 and IL-23 production (Ghadimi, Helwig, Schrezenmeir, Heller, & de Vrese, 2012; Ichiyama et al., 2008; Tanabe, 2013).

Treg cells play an important role in immune response regulation owing to the release of cytokines and immune modulating factors that prevent exacerbated and auto immune responses (Josefowicz, Lu, & Rudensky, 2012). Alternatively, T-helper-17 (Th17) cells seem to release pro-inflammatory cytokines, such as IL-17A, IL-17F, IL-21, IL-22, tumor necrosis factor α (TNF-α), and IL-6, associated with inflammatory bowel disease and colorectal cancer progression (Cătană et al., 2015; De Simone et al., 2015). The immune cell phenotype is targeted by specific transcription factors such as FOXP3, the key transcription factor for Treg cells differentiation and RORγT, the crucial Th17-lineage transcription factor. Moreover, two important adaptive immunity pathways are generated according to specific transcription factors: T-bet, a hallmark of the Th1 cell lineage development, is responsible for interferon-gamma regulation (IFN-γ), IL-2, and TNF-α genes (Szabo et al., 2000); and GATA-3, a Th2 cell lineage-promoter factor, produces cytokines, such as IL-4 e IL-5, IL-6, IL-9 and IL-13 (Szabo et al., 2000; Zhang, Cohn, Ray, Bottomly, & Ray, 1997; Zheng & Flavell, 1997).

It is hypothesized that the consumption of PBY, a source of FOS and inulin, is able to modulate the intestinal microbiota of mice and consequently increase the production of SCFA in feces. In addition, we speculate that the modulation of the microbiota and the fermentation products generated by the consumption of PBY could alter intestinal immune response pathways, favoring an increase in the number of Treg cells and attenuating the expression of immune response transcription factors responsible for the activation of pro-inflammatory responses. Given the above, we aimed to investigate the immunomodulatory response triggered by PBY consumption in BALB/c male mice.

2. Material and methods

2.1. Animals and experimental design

Thirty-four BALB/c male mice were obtained from the Central Bioterium (Health and Biology Science Center) of Universidade Federal de Viçosa, Brazil. The animals were housed at the Experimental Nutrition lab in a temperature-controlled room (22 ± 2 °C) with a 12-hour light/dark cycle and had *ad libitum* access to water and food. The animal protocol was approved by the Ethics Committee on Animal Experimentation of Universidade Federal de Viçosa, Brazil, under the process number 30/2016. Upon arrival, the animals were randomly assigned to two experimental groups: CC, control diet (n = 16), and CY, the group fed diet supplemented with PBY (n = 18) (Table 1). The diets were offered *ad libitum* for 8 weeks. Subsequently, the mice were anesthetized using 3% isoflurane and blood samples were collected from the *retro*-orbital sinus. The mice were euthanized by cervical dislocation and their tissues and feces were harvested for analysis.

Table 1
Composition of AIN-93 M diet for control and PBY diet.

Ingredients (g/100 g)	Control diet (g/kg)	PBY diet (g/kg)
Casein	155.5	145.3
Dextrinized starch	155	148.2
Sucrose	100	0
Soybean oil	40	40
Fiber (microfine cellulose)	67	0
Mineral mix	35	35
Vitamin mix	10	10
L-Cystine	1.8	1.8
Choline bitartrate	2.5	2.5
Cornstarch	303	263.6
*PBY	0	353.6
Distilled water	130.2	0
Total weight (g)	1000	1000
Total energy (kcal)	3214	3064

* Centesimal composition and digestible content of carbohydrate, inulin and FOS on PBY in 100 g of product: Fructose: 15.25 g; Glucose: 8.59 g; Sucrose: 6.35 g; FOS: 12.81 g; Inulin: 4.16 g; Total carbohydrate: 45.49 g; Fibers: 1.99 g; Humidity: 36.82 g; Ashes: 3.39 g; Lipids: 0.21 g; Protein: 2.89 g. PBY, yacon-based product.

2.2. Yacon-based product and experimental diet

Yacon was purchased from a local market in Viçosa – Minas Gerais, Brazil. The yacon-based product was processed according to the methodology proposed by Rodrigues et al. (2012) currently undergoing a patent application process (PI 1106621-0). The chemical composition of PBY (carbohydrates, proteins, fats, fiber, ash and humidity) was determined according to the AOAC methodology (AOAC, 1997). FOS and inulin content of the PBY diet was determined by High Performance Liquid Chromatography (HPLC) using a BIO-RAD brand HPX-87p column (lead stationary phase) whose mobile phase is purified water.

The experimental purified diets were based on the AIN93-M diet, as recommended by the American Institute of Nutrition (Reeves, Nielsen, & Fahey, 1993). PBY diet was supplemented with 6.0% FOS + Inulin from PBY (Table 1), as suggested by Paula et al. (2012). Casein, sucrose, dextrinized starch, starch, and fiber in the control and PBY diets were adjusted, aiming to obtain similar amounts of carbohydrates, lipids, proteins, fibers and calories. The diets were made in pellets and stored at −20 °C for a maximum period of thirty days before consumption.

The human equivalent amount of dietary PBY consumed by the BALB/c male mice was calculated using the body surface area normalization method as previously described (Reagan-Shaw, Nihal, & Ahmad, 2007). PBY supplementation was 353,565 g PBY/kg of diet. In our study, the average daily consumption was 6 g per mouse. This is equivalent to 2121 mg of PBY daily for an adult mouse of 45 g, which approximately corresponds to 47 g PBY/kg body mass/day. Taking into account that the average weight of an adult human is 60 kg, the equivalent average daily consumption per day for humans is 229 g.

2.3. Body weight and dietary intake

To evaluate the weight loss/gain of the animals, individual body

weight was recorded weekly using a digital weighing scale. Dietary intake was determined based on the diet offered (g) minus diet waste. This quantification was done every 3 to 5 days during the 8 week dietary intervention period using a digital weighing scale. The data represent diet consumption per cage (7 to 10 animals/cage). Diet consumption (g) was corrected by humidity loss (fresh diet weight (g)/ diet weight (g) in the cage per day).

2.4. Fecal characteristics

Fresh excreted feces were harvested and stored at −80 °C for pH determination. For each animal, an aliquot of feces was diluted in distilled water (1:10), homogenized, and the pH was measured with a duly calibrated digital pH meter (Hexis ultra Basic UB-10®) in a temperature-controlled room for an adequate amount of time for pH stabilization (Bedani et al., 2011).

Fecal humidity was determined using approximately 110 mg of moist feces. The feces were weighed in petri dishes, previously dried in an oven for 24 h at 105 °C. Afterwards, the material (petri dish + feces) was placed in a desiccator until it reached ambient temperature, and was later weighed for the determination of humidity using Eq. (1) (Cecchi, 2007).

$$\text{humidity}(\%) = (\text{Initial Weight}^* - \text{Final Weight}^*) \times 100/(\text{Sample Weight}) \quad (1)$$

*Initial and final weight: weight of the dishes containing the samples, before and after drying, respectively.

In the last week of the experiment, fresh feces were harvested and used for fecal score. The following scale was considered: 1. Firm or normal feces consistency; 2. Viscous non-diarrhea feces; 3. Watery feces characteristic of diarrhea (De Freitas et al., 2006, with modifications).

2.5. Anatomical characteristics

After euthanasia, the organs (liver, spleen, small intestine, cecum, colon, abdominal adipose tissue and kidney) were harvested, washed in phosphate-buffered saline and weighed using a semi-analytical weighing scale. Hepatosomatic index was obtained by dividing liver weight by body weight of the animal. Colon length was measured from the end of the cecum to the end of the rectum on a flat surface using a millimeter ruler.

2.6. Analysis of serum biomarkers

After euthanasia, the blood of the animals was collected from the *retro*-orbital sinus and centrifuged at 1190 × g/10 min/4 °C. The serum markers: total cholesterol, triglycerides, gamma-glutamyl transferase (GGT), aspartate aminotransferase (AST) alanine aminotransferase (ALT), albumin, alkaline phosphatase, creatinine, and urea were assessed by specific colorimetric assays (Bioclin®, Brazil) using a clinical chemistry analyzer BS-200 (Mindray®). The results are expressed as mean ± SEM.

2.7. Fecal SCFA quantification

SCFA quantification was performed according to the method of Smiricky-Tjardes, Grieshop, Flickinger, Bauer, and Fahey (2003) with some modifications. 50 mg of frozen feces, which was previously weighed and thoroughly vortexed with deionized water (950 µL) was used. While being incubated on ice for 30 min, the samples were homogenized every 5 min for 2 min. The samples were centrifuged (10,000*g*, 30 min, 4 °C) three times and the supernatants were collected. The final supernatant from each sample was filtered through a 0.45 µm membrane and transferred to vials. SCFA were measured by high performance liquid chromatography - HPLC (Shimadzu®) using an Aminex HPX 87H column (300 × 7,8 mm, Bio-rad®, Rio de Janeiro, Brazil) at 32 °C with acidified water (0.005 M H2SO4) as eluent at a flow rate of 0.6 mL/minute. The products were detected and quantified by an ultraviolet detector (model SPD-20A VP) at 210 nm. Standard curves of formic, acetic, propionic, isobutyric, butyric, isovaleric, valeric, isocaproic and caproic acids (SUPELCO®) were constructed. The results were expressed as µmol SCFA/g feces.

2.8. Determination of leukocytes by immunophenotyping

Leukocytes were quantified and characterized in the mucosa of the large intestine as previously described (Belkaid, Jouin, & Milon, 1996) with some modifications. The colon was removed and washed in ice-cold PBS (NaCl: 0.85%, NaH_2PO_4: 0.023%, $NaHPO_4 2H_2O$: 0.15%, pH 7.2), cut into small fragments and incubated in cell culture medium, DMEN, pH 7.2 (Sigma-aldrich ™) for 90 min at 37 °C, the suspension was centrifuged three times at 42*g* for 5 min in order to harvest the supernatant, and lastly at 543*g* for 10 min. After the last centrifugation, the remaining pellet was resuspended with PBS buffer (100 µL, pH 7.2). Cell viability was assessed with Trypan blue exclusion and the cells were counted in a Neubauer chamber. The obtained leukocytes were incubated with the following antibodies, according to manufacturer's instructions: anti-CD4 (PeCy5), anti-CD25 FITC-conjugated, anti-CD196 (anti-CCR6) PE-conjugated, anti-CD49b (anti-PanNK) APC-conjugated, anti-CD8 PECy7-conjugated (Biolegend, San Diego, CA, USA). The leukocytes (1×10^4 events) were acquired (FACSVerse™ and BD FACSuite software; BD Biosciences PharMingen San Jose, CA, USA) according to size (forward scatter) and granularity (side scatter). One or two stains were used to identify TCD4 lymphocytes ($CD4^+$), TCD8 lymphocytes ($CD8^+$), regulatory T cell ($CD4^+CD25^+$), Th17 lymphocytes ($CD196^+$) and *Natural Killer* cells ($CD49b^+$). The results are expressed as mean ± SEM of the percentage of each subpopulation of antibodies specifically colored within the gated cells.

2.9. Real-time PCR

Total RNA was extracted from the whole colon using Trizol reagent (Invitrogen™, Carlsbad, CA, USA) according to manufacturer's instructions. cDNA was synthesized using 5 µg of RNA through an RT reaction in a specific kit (GoScript™ Reverse Transcription System, Promega, Madison, WI, USA). Random primer and RNAse free water were added to the sample and heated for 5 min at 70 °C. Subsequently, a mixture containing the reverse transcriptase dNTPs and ribonuclease inhibitor was added to the sample and heated for 60 min at 37 °C. Real-time PCR quantitative mRNA analyses were performed in an Applied Biosystems® 7500 Real-Time PCR System. Sybr green (Ludwig, Biotec), sense primer, and antisense primer (400 nm/reação), DNase free water and the sample (250 ng cDNA/µl) were added to each reaction. Standard PCR conditions were used during the reading (7500 software V2.3, Applied Biosystems®). The sequences of murine primers (Integrated DNA Technologies®) were as follows: FOXP3, sense: 5′-AGG AGC CGC AAG CTA AAA GC- 3′, antisense: 5′-TGC CTT CGT GCC CAC TGT-3′; RORγt, sense: 5′-GGA GCT CTG CCA GAA TGA CC-3′, antisense: 5′-CAA GGT TCG AAA CAG CTC CAC-3′; Tbet, sense: 5′-AGC AAG GAC GGC GAA TGT T-3′, antisense: 5′-GGG TGG ACA TAT AAG CGG TTC-3′; GATA3, sense: 5′-CAA TCT GAC CGG GCA GGT-3′, antisense: 5′-CAG AGA CGG TTG CTC TTC CG-3′; GAPDH, sense: 5′-TCA ACA GCA ACT CCC ACT CTT CCA-3′, antisense: 5′-ACC CTG TTG CTG TAG CCG TAT TCA-3′.mRNA values were calculated according to the constitutive GAPDH gene on the basis of the ΔΔCt algorithm.

2.10. Statistical analysis

The results were expressed as mean ± SEM. The data were analyzed using GraphPad Prism (version 6.0). The means were evaluated by the Kolmogorov-Smirnov normality test, and the groups with a

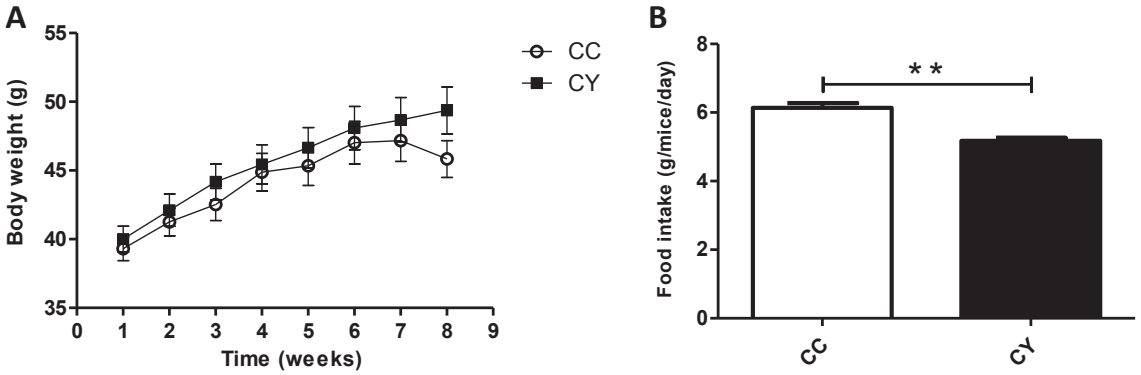
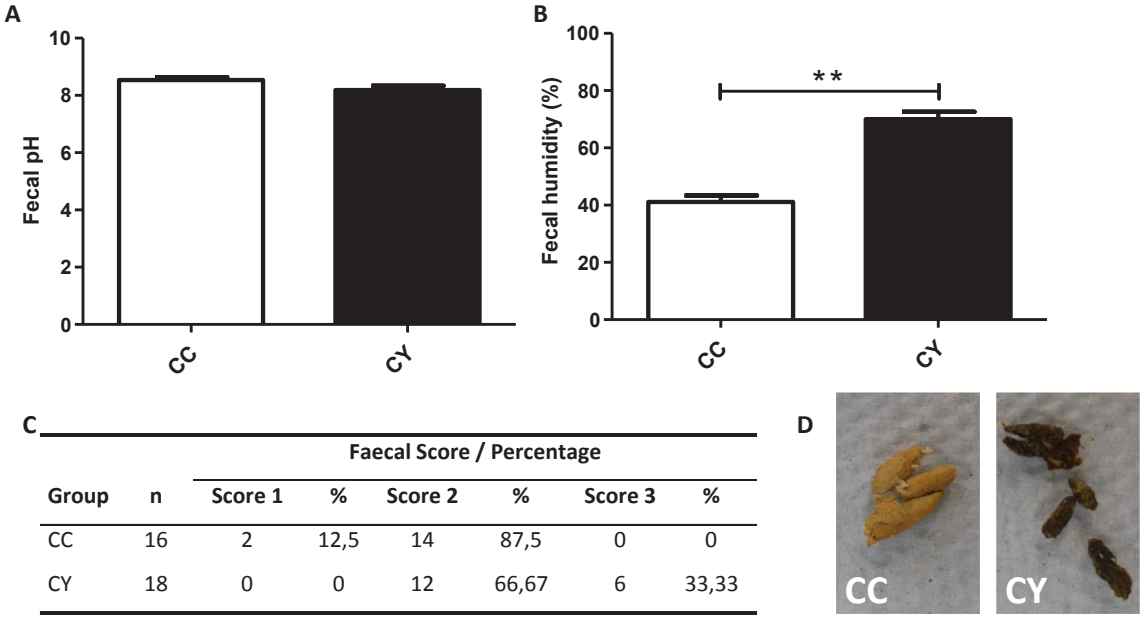


Fig. 1. Effect of PBY diet on weight gain and food intake in BALB/c mice during 8 weeks of dietary supplementation. (A) Average weight gain of animals over the weeks of dietary supplementation. The data are expressed as mean ± SEM (n = 16 to 18 mice/group). (B) Mean food intake (g/mice/day) of PBY diet or control diet consumed by BALB/c. The data are expressed as mean ± SEM of each experimental group (means referring to the group/cage consumption pool (4 pools/week). Statistical difference between groups were analyzed by the unpaired Student's *t*-test or Mann Whitney, (*) $p < 0.05$, (**) $p < 0.001$. CC, control diet; CY, PBY diet.



Group	n	Score 1	%	Score 2	%	Score 3	%
CC	16	2	12,5	14	87,5	0	0
CY	18	0	0	12	66,67	6	33,33

Fig. 2. Effect of PBY diet on fecal characteristics in BALB/c mice during 8 weeks of dietary supplementation. (A) Fecal pH; (B) Fecal humidity percentage; (C) Fecal Score (Score 1: normal to firm consistency; Score 2: viscous non-diarrheal consistency; Score 3: watery consistency characteristic of diarrhea). The results of fecal score are represented as number of animals in each score and their percentage into the group (n = 16 to 18/group); (D) Feces color after dehydration. The data of fecal pH and fecal humidity are expressed as mean ± SEM (n = 10 mice/group). Statistical difference between groups were analyzed by the unpaired Student's *t*-test or Mann Whitney, (*) $p < 0.05$, (**) $p < 0.001$. CC, control diet; CY, PBY diet.

normal distribution were tested using the unpaired Student's *t*-test. The samples that did not follow a normal distribution were tested by the Mann-Whitney test. Statistical differences were considered for $p < 0.05$ (*) or $p < 0.001$ (**).

3. Results

3.1. PBY diet reduces food intake without weight gain

During the 8 week dietary intervention, there was no difference in the body weight gain of the animals (Fig. 1A). However, food intake was lower in the groups that received the PBY diet ($p < 0,001$) (Fig. 1B), suggesting a possible satiety in the animals who received this diet.

3.2. PBY diet increases fecal humidity and viscosity

Fecal characteristics were evaluated in order to verify the possible interferences of PBY diet on feces production and intestinal transit. There was no difference in the fecal pH of the animals treated with the PBY diet when compared to the control diet group (Fig. 2A). However, fecal humidity was higher in animals fed the PBY diet ($p < 0,001$) (Fig. 2B). Predominantly, fecal score of the PBY group was classified as stage 2 (viscous non-diarrhea feces), and stage 3 (watery feces characteristic of diarrhea) (Fig. 2C).

3.3. PBY diet increases intestine weight without changing the anatomical characteristics of visceral organs

Colon length and weight were higher in the PBY diet group ($p < 0,001$) (Fig. 3A and B). The same was observed for the weight of the cecum ($p < 0,001$), and the small intestine ($p < 0,05$) (Fig. 3C and D). No differences were found in the weight of the liver, spleen, abdominal adipose tissue and kidneys. The same was observed for the hepatosomatic index (Fig. 3E–I).

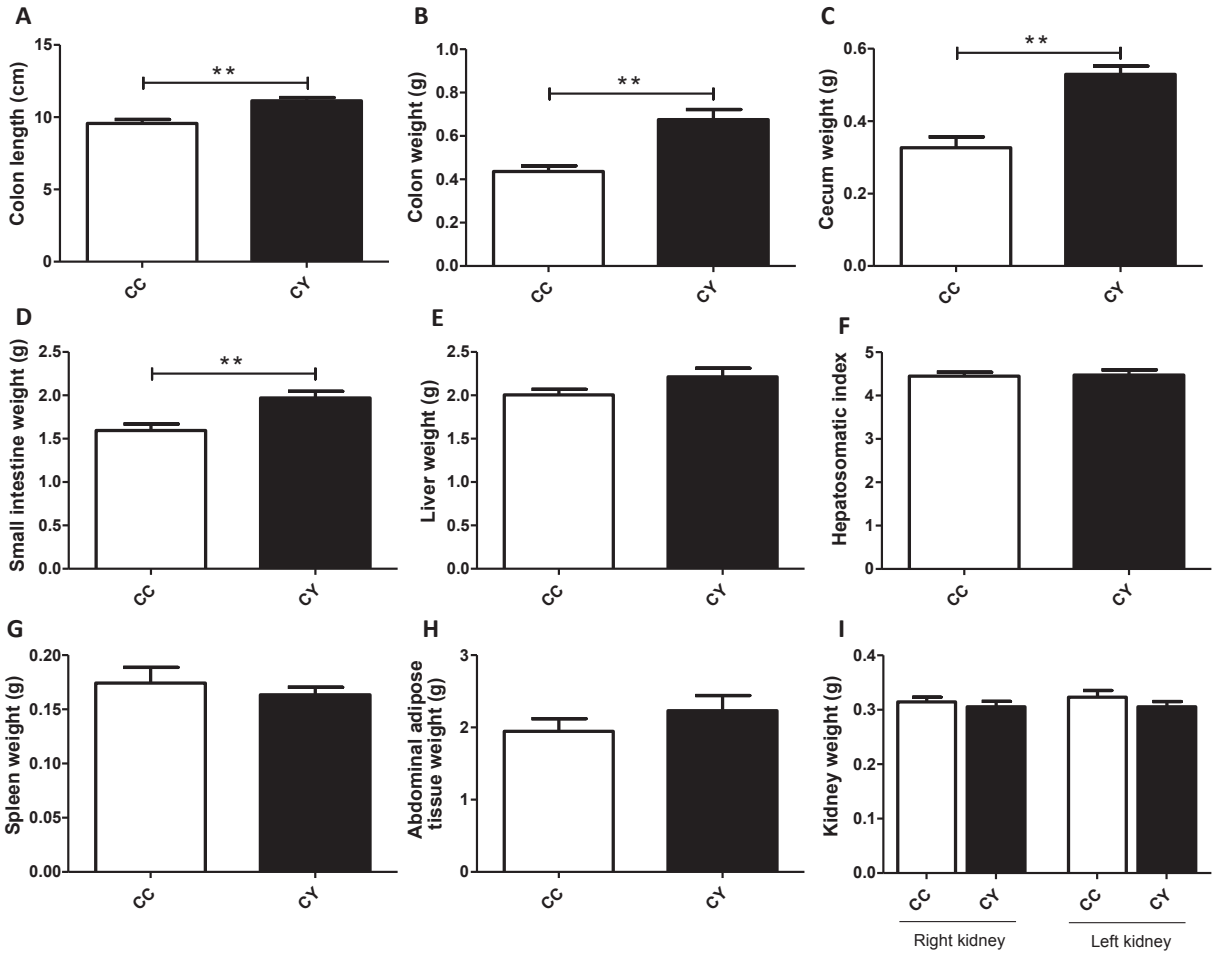


Fig. 3. Effect of PBY diet on the anatomical characteristics of visceral organs of BALB/c mice during 8 weeks of dietary supplementation. (A) colon length (cm); (B) colon weight (g); (C) cecum weight (g); (D) small intestine weight (g); (E) liver weight (g); (F) hepatosomatic index; (G) spleen weight (g); (H) abdominal adipose tissue weight (g); (I) kidney weight (g). The data are expressed as mean ± SEM (n = 16 to 18 mice /group). Statistical difference between groups were analyzed by the unpaired Student's *t*-test or Mann Whitney, (*) $p < 0.05$, (**) $p < 0.001$. CC, control diet; CY, PBY diet.

3.4. PBY diet does not alter the biomarkers of liver function; however, it increases serum triglycerides

Serum biomarkers were evaluated in order to verify alterations in liver and kidney functions, and lipid profile. There were no changes between the groups in relation to serum levels of GGT, AST, ALT, creatinine, alkaline phosphatase, and total cholesterol (Fig. 4). Nonetheless, an increase in triglycerides and albumin was found as well as a reduction in serum urea of the group fed the PBY diet ($p < 0,05$).

3.5. PBY diet increases the fecal short-chain fatty acids (SCFA)

Intestinal bacteria activity was evaluated by fecal SCFA concentration The PBY diet group presented higher levels of acetic ($p < 0,001$), propionic ($p < 0,05$), butyric ($p < 0,001$), isovaleric ($p < 0,05$), valeric ($p < 0,05$) e caproic ($p < 0,05$) fatty-acids compared to the control group (Fig. 5).

3.6. PBY diet increases Treg cell number in the colon

Immunophenotyping of immune cells in the colon was performed to evaluate the profile of intestinal lymphocytes. The analysis showed an increase in the percentage of Treg cells ($CD4^+CD25^+$) in the PBY diet group ($p < 0,05$) compared to the control diet group (Fig. 6). There was no significant difference among the groups in relation to the percentage of $CD4^+$ and $CD8^+$ cells, $CD8^+/CD4^+$ ratio, NK cells, and Th17 lymphocytes.

3.7. PBY diet reduces RORγt expression in the colon

Specific transcription factors of adaptive immune response were evaluated to verify the intestinal immune response pattern. A lower expression of RORγt transcription factor was detected in the colon of animals fed the PBY diet compared to the control diet group ($p < 0,05$) (Fig. 7). There was no significant difference in FOXP3, GATA-3, and T-bet transcription factors in the colon of the mice.

4. Discussion

Yacon has been associated with intestinal microbiota modulation due to its high soluble fiber content, FOS, and inulin. The benefits are related to the increment of *Bifidobacterium* and *Lactobacillus*, and bacterial metabolites, such as SCFA (Caetano et al., 2016). Furthermore, yacon consumption has been demonstrated to attenuate colon cancer in experimental models (de Moura et al., 2012; Grancieri et al., 2017). Although the immunomodulatory role of yacon is suspected to favor increased secretory fecal IgA production (Grancieri et al., 2016), inflammatory cytokine reduction (Choque Delgado et al., 2012), increased phagocytic activity of macrophages (Paredes et al., 2018), as well as possibly decreasing the number of pathogenic bacteria in colon (Sant'Anna et al., 2018), its immune response profile in the intestine is still unknown.

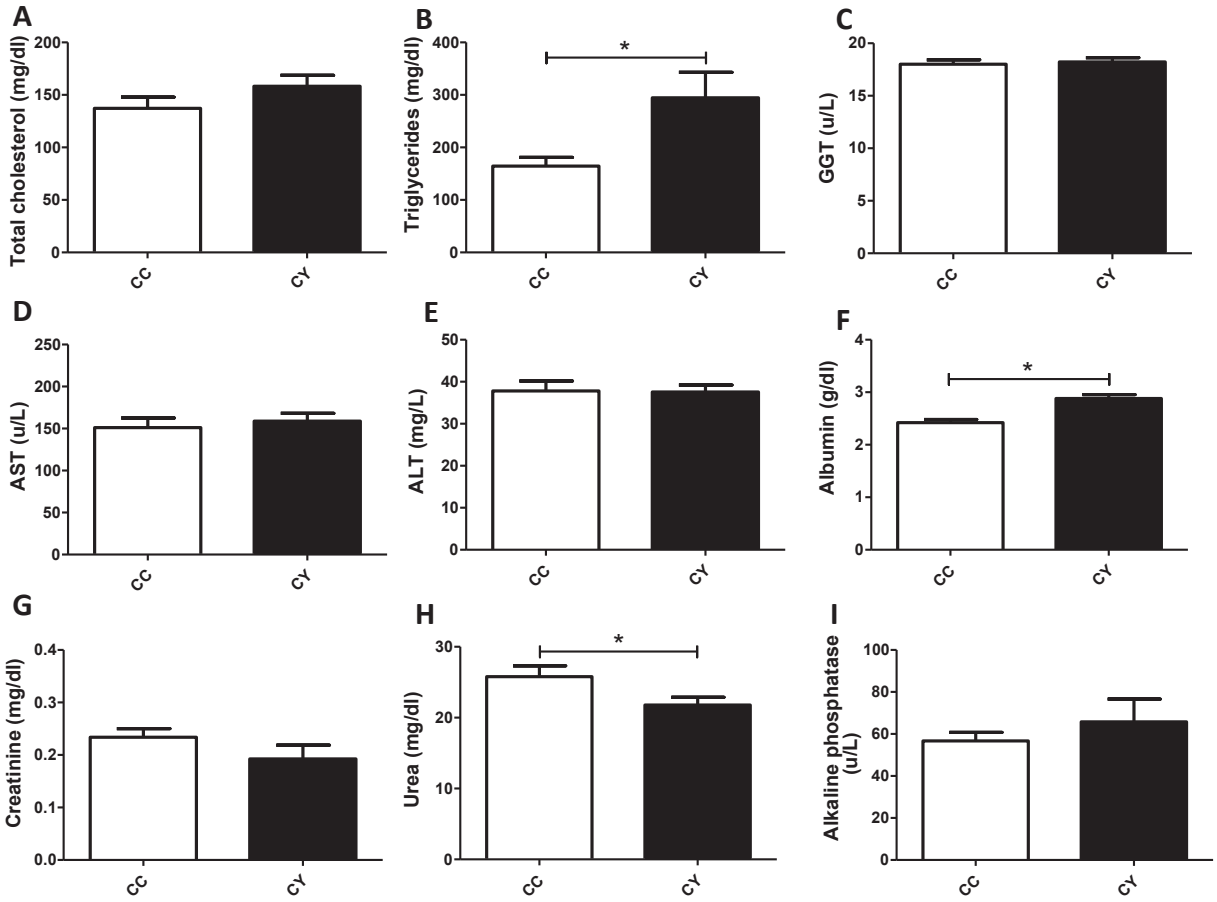


Fig. 4. Effect of PBY diet on serum biomarkers level in BALB/c mice during 8 weeks of dietary supplementation. (A) total cholesterol (mg/dl); (B) triglycerides (mg/dl); (C) GGT (gamma-glutamyl transferase) (u/L); (D) AST (aspartate aminotransferase) (u/L); (E) ALT (alanine aminotransferase) (mg/dl); (F) albumin; (G) creatinine; (H) urea; (I) alkaline phosphatase. The data are expressed as mean ± SEM (n = 10 mice/group). Statistical difference between groups were analyzed by the unpaired Student's *t*-test or Mann Whitney, (*) $p < 0.05$, (**) $p < 0.001$. CC, control diet; CY, PBY diet.

In our study, the diet supplemented with PBY, containing soluble fibers, increased fecal humidity and viscosity, without altering fecal pH. It is known that bacterial fermentation favors the reduction of fecal pH, leading to water retention in the intestinal lumen in order to preserve intraluminal osmotic pressure (Le Blay, Michel, Blottière, & Cherbut, 1999). Furthermore, the osmotic effect provided by the fructans increases the absorption of Ca and Mg due to the optimization of paracellular transport. This transport is favored by increased solubilization of minerals attributed to high amount of fluid in the colon (Bongers & Van den Heuvel, 2003; Lobo et al., 2007). However, we did not observe any reduction in fecal pH, this is probably related to the reabsorption of fatty-acids along the colon, resulting in less acidic feces.

FOS and inulin are initially fermented in the cecum (Roberfroid, 2007), leading to the production of bacterial metabolites, which stimulate cell proliferation. Among these metabolites, butyrate is highlighted, which is the main source of energy for the colonocytes, helping in the maintenance and integrity of the mucosa (Le Blay et al., 1999). The trophic effect in the mucosa, reflects an increase in the weight and length of the intestinal tissue. An increase in the weight of the cecum, colon and small intestine, and the length of the colon was verified in our study, suggesting a potent trophic action caused by the PBY diet, which may be linked to the higher production of fecal SCFA, also observed in our results.

Regarding serum biomarkers, there were no alterations in the levels of GGT, AST, ALT, creatinine, FA, or total cholesterol between the groups. However, higher concentration of TG in the PBY diet group was found. This increase may be attributed to free fructose present in the PBY diet. The increase of TG, total lipids, and low-density lipoproteins in the blood may occur after the consumption of diets with fructose when compared to diets with complex carbohydrates and other sugars since greater activity of lipogenic enzymes in the liver can increase the synthesis of glycerol and fatty-acids in the same (Barreiros, Bossolan, & Trindade, 2005). It is possible that the amounts of fructose ingested by the animals in the PBY group may have deviated their metabolism for lipid synthesis.

The reduction of serum urea in the PBY diet group may be related to the reduced ingestion of proteins, as this group presented a significant reduction in dietary intake. Moreover, fiber in the PBY diet might favor the lower absorption of proteins due to increased intestinal transit. This fact may be confirmed by the feces consistency of the animals fed the PBY diet, classified as viscous - watery feces.

PBY diet also induced an increase in the concentration of acetic, propionic, butyric, isovaleric, valeric, and caproic fatty-acids in the feces of the animals in the PBY diet group. This increase in SCFA may be attributed to the prebiotic role of PBY, capable of stimulating *Lactobacillus* and *Bifidobacterium* growth, and consequently generating these acids as end products of fermentation. According to the literature, the SCFA produced in the largest quantity by the fecal microbiota is acetic acid, followed by propionate, and butyrate (Cummings, Pomare, Branch, Naylor, & Macfarlane, 1987). However, in the present study this trend was only relevant in the feces of animals fed the control diet, while in the PBY diet group, a higher production of butyric acid, followed by acetic, and propionic acid, was verified respectively. Carbohydrates which are not absorbed in the small intestine can be used by the intestinal microbiota to generate acetate, being the main route by which the body obtains energy from undigested carbohydrates

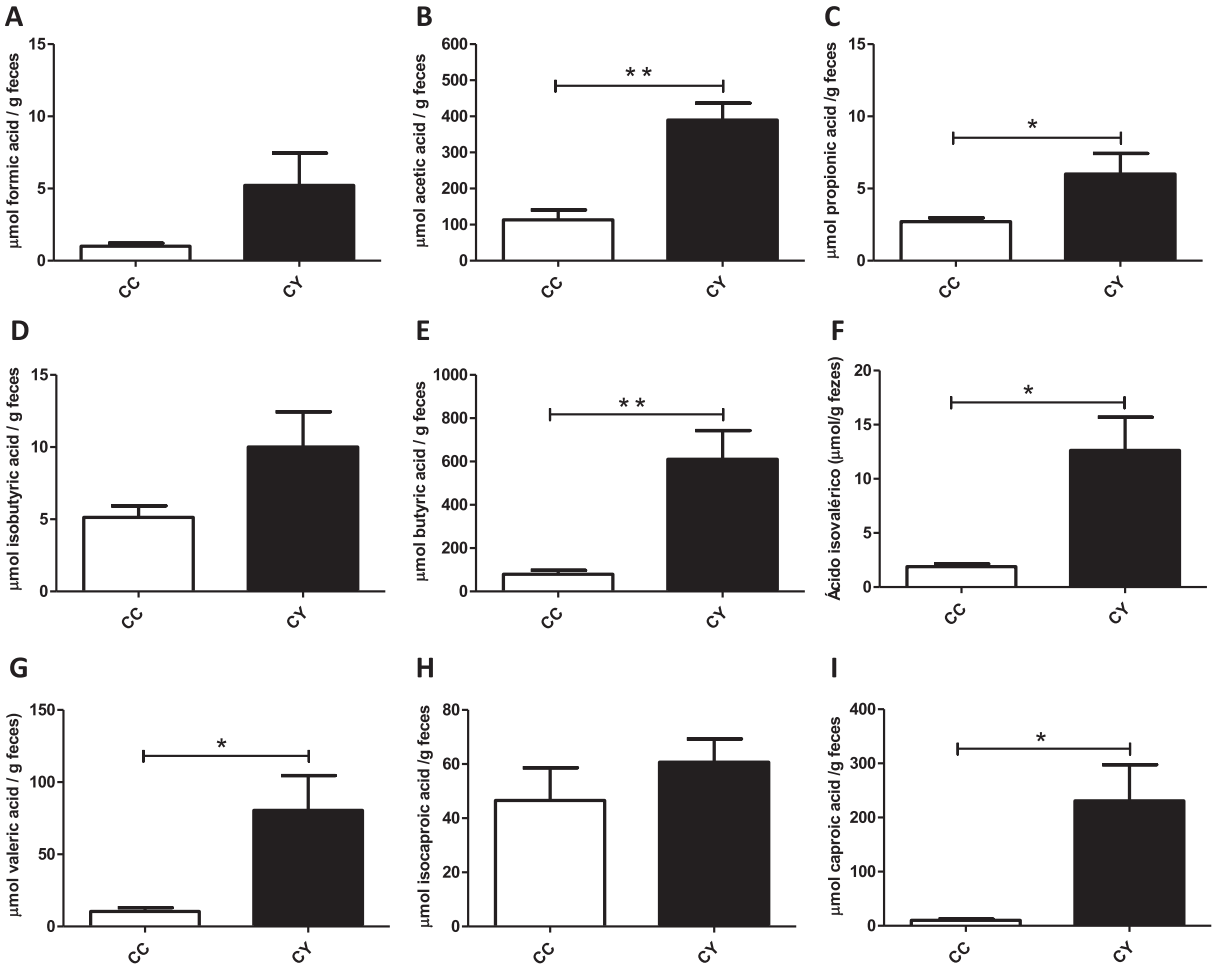


Fig. 5. Effect of PBY diet on fecal SCFA concentration in BALB/c mice during 8 weeks of dietary supplementation. (A) formic acid (µmol); (B) acetic acid (µmol); (C) propionic acid (µmol); (D) isobutyric acid (µmol); (E) butyric acid (µmol); (F) isovaleric acid (µmol); (G) valeric acid (µmol); (H) isocaproic acid (µmol); (I) caproic acid (µmol). The data are expressed as mean ± SEM (n = 4 to 10 mice/group). Statistical difference between groups were analyzed by the unpaired Student's *t*-test or Mann Whitney, (*) $p < 0.05$, (**) $p < 0.001$. CC, control diet; CY, PBY diet.

(Salminen et al., 1998). Almost all the heterotrophic anaerobic eubacteria in the intestine can produce acetate (Flint, 2006). However, butyrate is considered the most significant SCFA in relation to human health, because it may promote a trophic action in the mucosa, and an apoptotic action in cancer cells (Williams, Coxhead, & Mathers, 2003), promoting the reduction of pre-neoplastic lesions. Caproic acid is also capable of reducing the viability of *in vitro* HCT-116 cancer cells to up to 80% (Narayanan, Baskaran, Amalaradjou, & Venkitanarayanan, 2015).

Branched-chain fatty acids (BCFA), such as isobutyrate and isovalerate acids are formed through the fermentation of amino acids, valine and leucine, respectively (Macfarlane & Gibson, 1995). It is worth mentioning that protein fermentation is greater than carbohydrate fermentation in the descending colon, due to the availability of substrate, and increased pH. Therefore, the quantity of BCFA in the descending colon is higher than that of SCFA (Cummings et al., 1987). As observed in our study, PBY diet consumption promoted a significant increase of isovaleric acid, but not significant for isobutyric acid. This fact may be attributed to the possible increase of proteins and amino acids in the colon, which were not absorbed in the small intestine.

Furthermore, SCFA have been demonstrated to influence satiety, due to their interference in the production of leptin, a hormone released by the white adipose tissue, whose signaling pathway defects are associated with severe obesity, hyperphagia, infertility, and immune system disorders (Hoyles & Wallace, 2010). Studies using mice showed that SCFA (C2 – C6), and BCFA (C4 – C6) act in the production of leptin in the adipocytes through the G protein-coupled receptor GPR41, shown to be the agonists of this receptor (Xiong et al., 2004). Thus, the consumption of the PBY diet may be directly related to the increased production of SCFA (acetic (C2), propionic (C3), butyric (C4), isovaleric, and valeric (C5)), which might modulate the production of leptin in the animals.

In this study, a significant increase in the percentage of Treg cells in the colon of animals fed the PBY diet was found, as well as a reduction in *RORγt* expression. Treg cells produce IL-10 as a mechanism to modulate immune response, which promotes inflammatory response control, inhibiting hyperactivation of cells that could cause tissue damage (Laidlaw et al., 2015; Zhu & Paul, 2009). Furthermore, modulation of the immune response by the Treg cells indicates that there are no stimuli for Th17 cells differentiation (Korn, Bettelli, Oukka, & Kuchroo, 2009), as verified in our study. During the early stages of $TCD4^+$ cells differentiation there is the expression of both *RORγt and FOXP3* (Ichiyama et al., 2008). However, Treg or Th17 cells differentiation are affected by the amounts of TGF-β and pro-inflammatory cytokine milieu, on one hand, high IL-6 and TGF-β promote *RORγt* expression, and on the other hand, low pro-inflammatory cytokines and high TGF-β, induce *FOXP3* expression (Zhu & Paul, 2009). Therefore, an anti-inflammatory environment promotes an immune response appropriate for Treg cells differentiation.

In the present study, *RORγt* reduction might explain the lack of alteration in the Th17 cells. Despite the increase of Treg cells in the PBY diet group, we did not verify an increase in the FOXP3 transcription

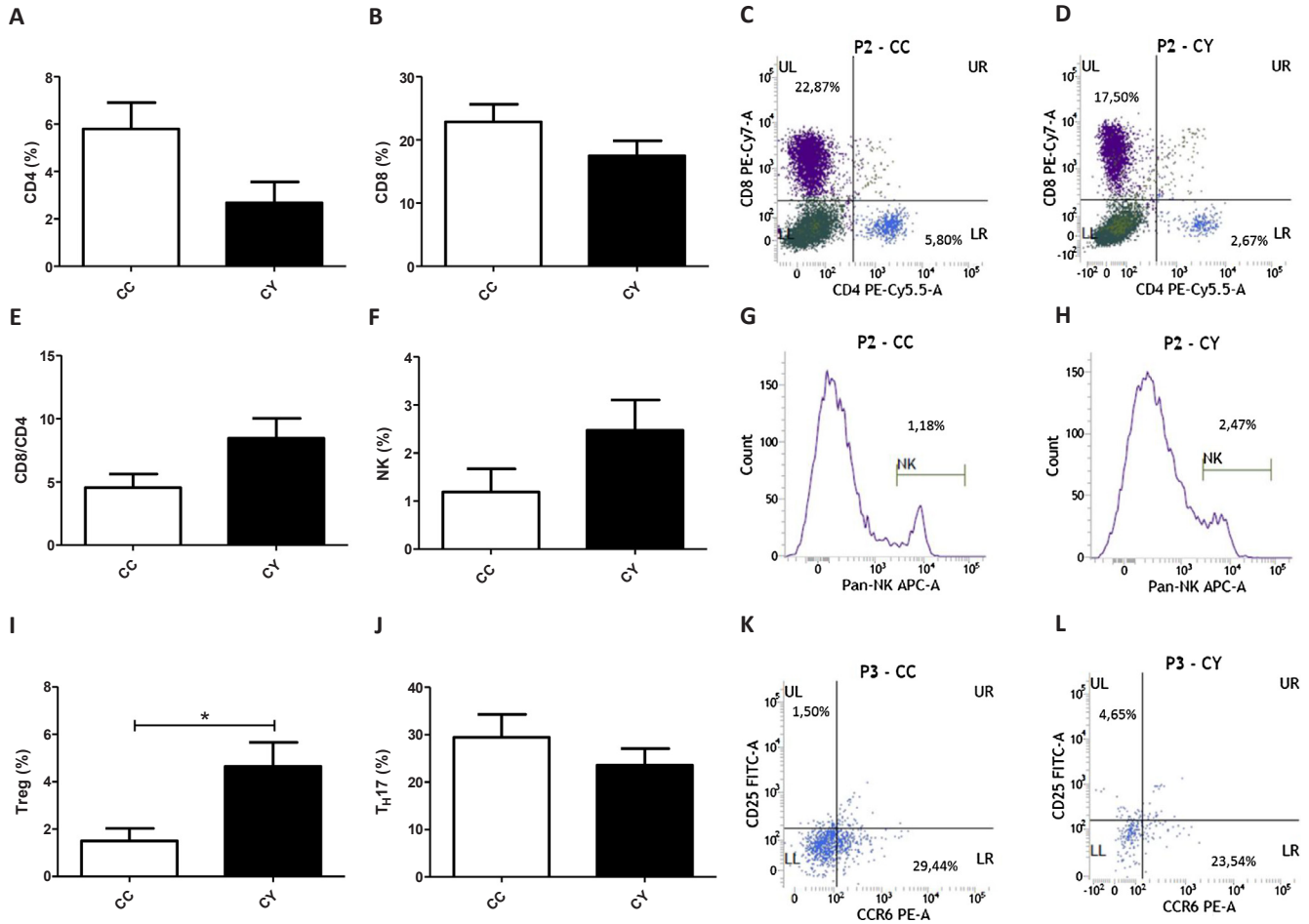


Fig. 6. Effect of PBY diet on the phenotypic profile of lymphocytes in the colon of BALB/c mice during 8 weeks of dietary supplementation. (A) % $CD4^+$ cells; (B) % $CD8^+$ cells; Flow cytometry plots from forward scatter/side scatter-gated lymphocytes cells marked with anti-CD8 (PE-Cy7) on the upper left side quadrants (% CD8 cells) and anti-CD4 (PE-Cy5) on the lower right side quadrants (% CD4 cells) on CC (C) and CY groups (D); (E) % $CD8^+/CD4^+$ ratio; (F) % NK cells (Pan NK^+ cells); Histogram plots of gated lymphocytes cells marked with anti-Pan-NK (APC) on CC (G) and CY groups (H); (I) % Treg cells ($CD4^+CD25^+$ cells); (J) % Th17 cells ($CD4^+CCR6^+$). Flow cytometry plots from gated $CD4^+$ cells marked with anti-CD25 (FITC) on the upper left side quadrants (% Treg cells), and anti-CCR6 (PE) on the lower right side quadrants (% Th17 cells) on CC (K) and CY groups (L). The results are represented as mean ± SEM of each experimental group (n = 5 to 6/group). Statistical difference between groups were analyzed by the unpaired Student's *t*-test or Mann Whitney, (*) $p < 0.05$, (**) $p < 0.001$. CC, control diet; CY, PBY diet.

factor. Furthermore, there were no changes in T-bet and GATA-3 expression, which suggest a local immune balance. Therefore, we propose a possible mechanism underlying the health benefits of PBY. PBY, a prebiotic source of FOS and inulin, increases the proliferation of probiotic bacteria which enhances SCFA production through fermentation. These SCFA might modulate transcription factors responsible for adaptive immune responses in the colon, downregulating RORγt and, therefore, reducing the stimuli required for Th17 cell differentiation, which favors Treg cell induction. Higher Treg cells conduce to reduced activation of pro-inflammatory cells, and consequent formation of an anti-inflammatory microenvironment. Thus, PBY supposingly prevents and attenuates inflammatory processes by increasing SCFA concentration, negatively modulating RORγt and increasing Treg cells in the colon.

5. Conclusion

PBY diet reduced food intake in the animals, suggesting its important and beneficial role in satiety due to soluble fiber content and SCFA production, which possibly interfere with leptin production. PBY diet also increased fecal humidity and viscosity, favoring intestinal transit. Furthermore, the observed increase in Treg cells and reduction in RORγt transcription factor, indicate that PBY is beneficial for the modulation of inflammatory processes and immune cells. Therefore, the consumption of a diet containing PBY may favor the regulation of satiety, improve intestinal transit, maintain the integrity of the intestinal tissues, and increase Treg cells differentiation by downregulating *RORγt* expression which consequently reduces inflammatory processes.

6. Ethics statement

All the trials and experimental protocols were approved by the Ethics Committee on Animal Experimentation of Universidade Federal de Viçosa, Brazil, under process number 30/2016 and were performed according to the ethical guidelines of the European Community (Directive 2010/63/EU).

Acknowledgement

The study was supported by the National Council for Scientific and Technological Development - CNPq, Coordination for the Improvement of Higher Education Personnel - CAPES, Minas Gerais Research Foundation - FAPEMIG, and Microscopy and Microanalysis Division (NMM) of Universidade Federal de Viçosa - UFV.

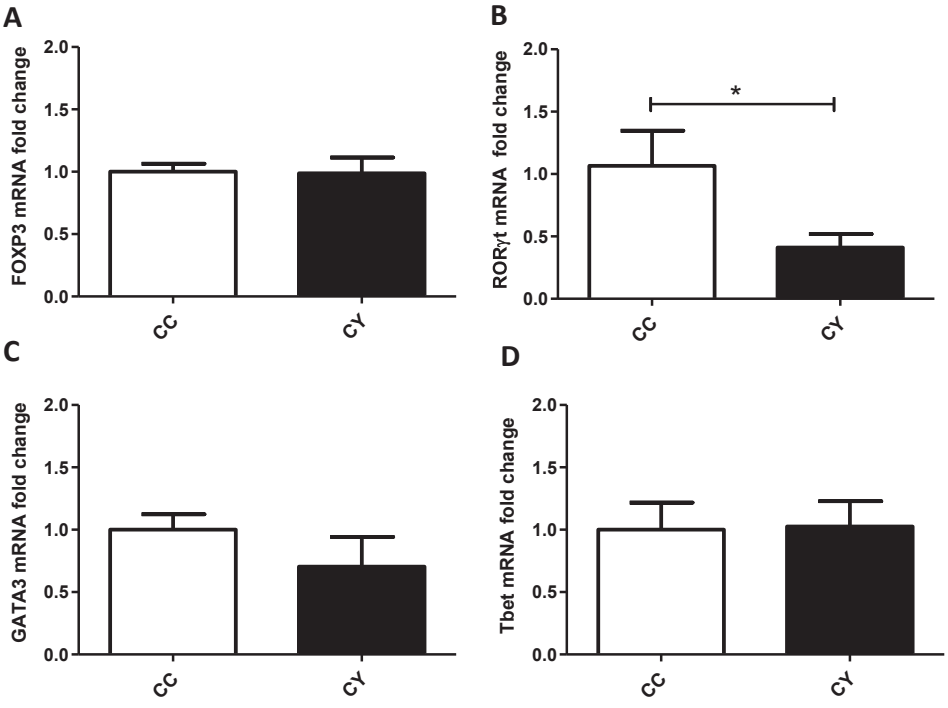


Fig. 7. Effect of PBY diet on the transcription factor expression of immune response on the colon of BALB/c mice during 8 weeks of dietary supplementation. Real-time PCR analysis of the colonic gene expression of FOXP3 (A), RORγt (B), GATA-3 (C) and T-bet (D). Gene expression was calculated in relation to the constitutive GAPDH gene and presented as a relative variation of the control group. The data are expressed as mean ± SEM (n = 4 to 6 mice/group). Statistical difference between groups were analyzed by the unpaired Student's *t*-test or Mann Whitney, (*) $p < 0.05$, (**) $p < 0.001$. CC, control diet; CY, PBY diet.

Conflict of interest

There is no conflict of interest.

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