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GENOMICS, TRANSCRIPTOMICS, PROTEOMICS

Synbiotic VSL#3 and yacon-based product modulate the intestinal microbiota and prevent the development of pre-neoplastic lesions in a colorectal carcinogenesis model

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Abstract

Colorectal cancer is a public health problem, with dysbiosis being one of the risk factors due to its role in intestinal inflammation. Probiotics and synbiotics have been used in order to restore the microbiota balance and to prevent colorectal carcinogenesis. We aimed to investigate the effects of the probiotic VSL#3® alone or in combination with a yacon-based prebiotic concentrate on the microbiota modulation and its influence on colorectal carcinogenesis in an animal model. C57BL/6J mice were divided into three groups: control (control diet), probiotic (control diet + VSL#3®), and synbiotic (yacon diet + VSL#3®). The diets were provided for 13 weeks and, from the third one, all animals were subjected to induction of colorectal cancer precursor lesions. Stool samples were collected to evaluate organic acids, feces pH, β-glucuronidase activity, and microbiota composition. The colon was used to count pre-neoplastic lesions and to determine the cytokines. The microbiota composition was influenced by the use of probiotic and synbiotic. Modifications were also observed in the abundance of bacterial genera with respect to the control group, which confirms the interference of carcinogenesis in the microbiota. Pre-neoplastic lesions were reduced by the use of the synbiotic, but not with the probiotic. The protection provided by the synbiotic can be attributed to the modulation of the intestinal inflammatory response, to the inhibition of a pro-carcinogenic enzyme, and to the production of organic acids. The modulation of the composition and activity of the microbiota contributed to beneficial changes in the intestinal microenvironment, which led to a reduction in carcinogenesis.

Key points

- *Synbiotic reduces the incidence of colorectal cancer precursor lesions.*
- *Synbiotic modulates the composition and activity of intestinal microbiota.*
- *Synbiotic increases the abundance of butyrate-producing bacteria.*

Keywords Gut microbiota · Probiotic · Prebiotic · Synbiotic · Colorectal cancer

Introduction

Colorectal cancer (CRC) is a public health problem worldwide, with 1.8 million new cases and almost 861,000 deaths reported in 2018 (IARC 2019). Colorectal carcinogenesis is characterized by a series of morphological changes of the intestinal epithelium, with the formation of precursor lesions of CRC, such as aberrant crypt foci (ACF), which can progress to invasive adenomas and carcinomas (Calderwood et al. 2016; Armaghany et al. 2012). The ACF study has become an important marker for understanding the pathogenesis of CRC and represents a challenge for cancer screening and surveillance in the early stages, in addition to being proposed to identify new chemopreventive agents (Newell and Heddle 2004).

Sporadic CRC, that is, not associated with heredity, is diagnosed in approximately 70 to 87% of the cases, indicating the existence of other important risk factors for the

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development of the disease (Frank et al. 2017; Johnson et al. 2013). The intestinal microbiota, defined as a complex community of microorganisms that coexist in close association with the host, has gained prominence in the pathogenesis of CRC (Mori et al. 2018; Jie et al. 2017; Cougnoux et al. 2014).

Although there is no consensus on the composition of the intestinal microbiota during the development and progression of CRC, the percentage of some bacterial species such as *Streptococcus bovis*, *Bacteroides fragilis*, *Enterococcus faecalis*, *Fusobacterium nucleatum*, and *Escherichia coli* was related to CRC occurrence (Gagnière et al. 2016). According to the "passenger-driver" hypothesis, intestinal bacteria called "drivers" induce damage to epithelial DNA, cause inflammation, increase cell proliferation, and produce genotoxic substances that contribute to tumorigenesis. Then, "passenger" bacteria begin to colonize the intestine, with growth advantages in the tumor microenvironment. Both have roles in CRC progression (Tjalsma et al. 2012).

On the other hand, several studies have investigated chemopreventive agents that could restore the intestinal microbiota balance and thereby reduce the CRC risk. Probiotics and prebiotics have been used for this purpose, alone or combined (Hill et al. 2014; Verma and Shukla 2014). Synbiotic is defined as the association between probiotic and prebiotic components (e.g., fructooligosaccharides and inulin) that work synergistically improving the colonization and survivability of beneficial microorganisms (Shinde et al. 2020; Kearney and Gibbons 2018). It has been reported that the greatest effects of synbiotic supplementation in the gastrointestinal (GI) tract include local inflammation reduction, modulation of the immune response, improvement of the intestinal barrier, compound production with anticarcinogenic activity, and oxidative stress improvement (Cruz et al. 2020).

Several bacterial species are considered probiotic, with lactic acid bacteria (LAB), in particular members of the genera *Lactobacillus* and *Bifidobacterium*, being the most used ones. VSL#3® is a mix of probiotic bacteria, which includes strains of distinct taxa such as *Lactobacillus*, *Bifidobacterium*, and *Streptococcus*, widely used in studies of inflammatory bowel diseases (Liu et al. 2019; Chung et al. 2017; Rossi et al. 2014; Arthur et al. 2013; Uronis et al. 2011; Reiff et al. 2009). Based on these studies, VSL#3® demonstrated an interesting anti-inflammatory activity and the ability to increase beneficial bacterial species in the colon; however, there is no information on the power to prevent colorectal carcinogenesis.

Yacon (*Smallanthus sonchifolius*) is a tuberous root, considered a prebiotic food due to its high content of fructooligosaccharides (FOS) and inulin, in addition to being rich in phenolic compounds (Russo et al. 2015). Notwithstanding the protective effects of yacon intake on colorectal carcinogenesis having been demonstrated in previous experimental studies (Grancieri et al. 2017; Moura et al. 2012), special attention was given to pre-neoplastic lesion development and short-chain fatty acid (SCFA) production. In the present study, the effects of yacon concentrate combined with the probiotic VSL#3®, an unprecedented synbiotic formulation, were evaluated in terms of gut microbial composition and immunological response, as well as organic acid production and their influence on CRC development.

Here, we hypothesize that the prophylactic administration of the probiotic VSL#3® alone or combined with a yacon-based prebiotic concentrate (PBY) could be able to modulate the composition and metabolism of the intestinal microbiota and, consequently, reduce the CRC risk at the early stages. Thus, the present study aimed to investigate the effects of the probiotic and synbiotic product on the intestinal microbiota modulation and its influence on the development of precursor lesions of CRC in an experimental animal model.

Materials and methods

Probiotics

The commercial probiotic VSL#3® (Sigma-Tau Pharmaceuticals, Gaithersburg, USA) was used. VSL#3®, according to the company's claim, contains eight bacterial strains, namely *Streptococcus thermophilus* BT01, three bifidobacteria (*Bifidobacterium breve* BB02, *Bifidobacterium animalis* subsp. *lactis* BL03, *Bifidobacterium animalis* subsp. *lactis* BI04), and four lactobacilli (*Lactobacillus acidophilus* BA05, *Lactobacillus plantarum* BP06, *Lactobacillus paracasei* BP07, *Lactobacillus helveticus* BD08). The commercial lyophilized formulation, in sachets containing 450 billion viable bacteria, was used. The product was kept refrigerated and reconstituted in distilled water immediately before administration.

The animals (probiotic and synbiotic groups) received 0.1 mL of the probiotic by orogastric gavage, in the morning, for 5 days a week, for 13 weeks (Arthur et al. 2013). The animals of the control group received 0.1 mL of autoclaved tap water. The volume was adjusted to provide a daily supply of 2.25×10^9 colony-forming units (CFU)/animal, based on the daily intake of about 10^9 CFU for an adult of 70 kg (Uronis et al. 2011; Zavisic et al. 2012; Dai et al. 2013).

Yacon-based product and synbiotic

The processing of yacon-based product (PBY) cannot be detailed due to ongoing patent application (PI 1106621-0). PBY sufficient to provide 6% of FOS and inulin (Paula et al. 2012) was added to the standard diet of the AIN-93M rodents (Reeves et al. 1993), in order to provide the benefits of fructan consumption without promoting toxicity or adverse effects. Considering that 100 g of PBY contains 23.6 g of FOS and inulin, 25.4 g of PBY was added to every 100 g of diet.

Carbohydrate, protein, and fiber contents were adjusted so that the experimental diets had a similar composition. The diets were prepared as pellets and stored at − 20 °C. The synbiotic was composed of the probiotic VSL#3® and the PBY, source of the prebiotics, FOS, and inulin.

Animals, diets, and experimental design

Forty-five male C57BL6/J mice, healthy, 8 weeks old, and with a body weight of approximately 22 g were obtained from the Central Bioterium at the Biological Sciences and Health Center at Federal University of Viçosa, Minas Gerais, Brazil. The animals were collectively allocated in polypropylene cages, containing five mice each. The animals were kept under controlled conditions, at a temperature of 22 ± 2 °C and humidity of 60–70% with a 12-h light/dark cycle.

After the acclimation period (1 week) during which they received a commercial diet and water, the animals were randomly assigned to three experimental groups:

1. Control group (CON, n = 15): AIN-93M diet and water (0.1 mL), via orogastric gavage, for 13 weeks
2. Probiotic group (PRO, n = 15): AIN-93M diet and probiotic VSL#3® (2.25×10^9 CFU/0.1 mL), via orogastric gavage, for 13 weeks
3. Synbiotic group (SYN, n = 15): modified AIN-93M diet containing PBY (6% FOS and inulin) and probiotic VSL#3® (2.25×10^9 CFU/0.1 mL), via orogastric gavage, for 13 weeks

Diet and water were provided ad libitum throughout the experimental period. From the third experimental week, the protocol for the induction of pre-neoplastic lesions (ACF) in the colon was introduced for all. The colon carcinogen 1,2-dimethylhydrazine (DMH) (Sigma-Aldrich, Saint Louis, USA) was prepared in 0.9% saline solution containing 1 mM ethylenediamine tetraacetic acid (EDTA) and 10 mM sodium citrate, pH 8 (Newell and Heddle 2004). A dose of 20 mg/kg body weight was injected intraperitoneally (0.1 mL), once a week, for eight consecutive weeks (Gomides et al. 2014).

At the end of the experimental period (13 weeks), the animals were anesthetized with 3% isoflurane (Isoflorine®, Cristalia, Itapira, Brazil) and blood samples collected from the retro-orbital sinus. The mice were sacrificed by cervical dislocation. The colon was resected, washed with cold 0.1 M phosphate-buffered saline (PBS, pH 7.2), sliced into small fragments, snap-frozen in nitrogen liquid, and then stored at − 80 °C.

All experimental procedures using animals were performed following the Directive 2010/63/EU, in compliance with the ethical principles for animal experimentation. The study protocol was approved by the Ethics Committee of the Federal University of Viçosa (CEUA/UFV, protocol n° 08/2017; approval: May 9, 2017).

Body weight and dietary intake

To evaluate the physiological effects of the administration of probiotics and synbiotic in terms of weight loss or gain, the animals were weighed weekly on a digital semi-analytical scale. Dietary intake was measured daily and was calculated by the difference from the amount of diet offered (g) and the remaining amount (g) in the successive day.

Feces collection and feces characteristics

The feces of each animal (n = 15 mice/group) was harvested four times: *t0*, end of the first experimental week; *t1*, fourth experimental week, after the first ACF induction; *t2*, tenth experimental week, last ACF induction; and *t3*, last experimental week. To obtain samples, individual cages were previously cleaned and sanitized and a single mouse was kept there until a sufficient amount of feces was spontaneously expelled. Samples were kept at − 80 °C for further analysis.

Fecal samples collected during the last week (*t3*) were used to evaluate pH and fecal scores. For each animal, an aliquot of feces was diluted in distilled water (1:10) and homogenized, and the pH was measured with a duly calibrated digital pH meter (Ultra Basic UB-10®, Hexis, Jundiaí, Brazil) in a temperature-controlled room after an adequate amount of time for pH stabilization (Bedani et al. 2011).

To assess the fecal score, a visual inspection was carried out, and each sample received a score according to its characteristics. The following ranking was considered: (1) firm or normal feces consistency; (2) viscous non-diarrhea feces; and (3) watery feces characteristic of diarrhea (De Freitas et al. 2006, with modifications).

ACF count

Colonic ACF were analyzed using the method proposed by Bird and Good (2000). The colon was removed, cut along the longitudinal axis, and flushed with saline solution 0.1 M, pH 7 (n = 7 mice/group). Each colon was fixed in Karnovsky solution for 24 h and transferred to ethyl alcohol solution (70%) until analysis. The colon was cut into three sections with equal length (namely proximal, middle, and distal) and stained with 0.1% methylene blue solution (Vetec®, Duque de Caxias, Brazil) for 4 min. Then, ACF were observed using a light microscope (Zeiss®, Primo Star, Oberkochen, Germany). ACF were identified as elevated focal lesions with multiple aberrant crypts (AC), with a thickened lining of epithelium and an increased luminal opening relative to normal adjacent mucosa (Bird and Good 2000). Counting was performed by two trained evaluators.

Analysis of cytokine profile

Pro- and anti-inflammatory cytokines were simultaneously determined by the Cytometric Bead Array (CBA) mouse Th1/Th2/Th17 Cytokine Kit (BD Biosciences, San Jose, USA) in a BD FACSVerse Flow Cytometry, following the manufacturer's instructions. Colon samples (100 to 200 mg; n = 6 or 7 mice/group) were ground using a tissue homogenizer (T10 basic UltraTurrax, IKA®, Rio de Janeiro, Brazil) in PBS buffer (pH 7.0) and centrifuged (10,000g, for 10 min at 4 °C) and the supernatant recovered. The beads were diluted with the diluent solution and distributed in microtubes. Twenty-five microliters of the sample and 17 μL of the detector solution were added to each microtube, followed by incubation for 2 h. Subsequently, 1 mL of the washing solution was added, followed by centrifugation (1800g, for 5 min at 4 °C), and part of the supernatant discarded (approximately 800 μL). The remaining volume was used for the measurement on the flow cytometer. The standard curve was built from the most concentrated solution (top standard). The data was processed using the FCAP Array 3.0 (FCAP Array Software, BD Biosciences, San Jose, CA, USA), and the results were expressed in picograms per gram of tissue.

Evaluation of β-glucuronidase activity

To evaluate the β-glucuronidase enzyme activity, stool samples collected in the last experimental week (*t3*) were used. The enzymatic activity was evaluated according to de Moreno de LeBlanc and Perdigon (2005), with the modifications described below. For the preparation of enzymatic extract, stool samples (n = 7 mice/group) were homogenized in distilled water (1:30) with the aid of a homogenizer (T10 basic UltraTurrax, IKA®, Rio de Janeiro, Brazil), for 1 min, on ice. Then, samples were sonicated (Branson 1210 Ultrasonic, Marshall Scientific LLC, Hampton, USA), on ice, by 3 bursts of 2 min each (1-min interval between each burst) and then centrifuged (10,000g, for 15 min at 4 °C). The supernatant was collected. All enzymatic assays were carried out in phosphate buffer, 200 mM, pH 6.5, at 37 °C in triplicate, and the mean values calculated. Relative standard deviations of measurements were below 5%. The enzymatic reaction contained 65 μL buffer, 25 μL ρ-nitrophenyl β-D-glucuronide 4 mM (ρNPG, Sigma-Aldrich, Saint Louis, USA), and 10 μL enzymatic extract. The samples were incubated at 37 °C for 3 h. Absorbance was measured at 410 nm and the amount of ρ-nitrophenol released assessed by a standard curve. One enzyme activity unit (U) was defined as the amount of enzyme which released a micromole of the ρ-nitrophenol per hour under assay conditions.

Fecal SCFA quantification

The concentration of SCFA was determined in stool samples (n = 7 mice/group) collected at four times (*t0*, *t1*, *t2*, and *t3*), as described above. Quantification was performed according to Smiricky-Tjardes et al. (2003) with some modifications. Approximately 50 mg of feces was vortexed with deionized water (950 μL) and incubated in ice for 30 min, followed by homogenization in vortex every 5 min. Samples were centrifuged at 10,000g, for 30 min at 4 °C three times, and the supernatant was collected and filtered through a 0.45-μm membrane filter. The SCFA were measured by high-performance liquid chromatography (HPLC) (Shimadzu®, Quito, Japan) using an Aminex HPX 87H column at 32 °C, with acidified water (H_2SO_4, 0.005 M) as the eluent at a flow rate of 0.6 mL/min. The products were detected and quantified by an ultraviolet detector (SPD-20A VP) at 210 nm. Standard curves of acetic, propionic, and butyric acid were constructed (Supelco®, Darmstadt, Germany). Results were expressed in micromoles per gram of feces.

DNA extraction

Mice fecal samples (250 mg; n = 5 mice/group) were harvested at the end of the first experimental week (*t0*) and after the intervention period (*t3*). Total genomic DNA (gDNA) was extracted using the PowerSoil DNA isolation kit (Mo Bio, Carlsbad, USA) following the manufacturer's instructions. Total genomic DNA (gDNA) concentration and purity were determined via 260/280 and 260/230 absorbance ratios measured on the NanoDrop 2000c (Thermo Fisher Scientific, Waltham, USA).

DNA sequencing

After stool DNA extraction, samples of five different animals composing each experimental group at the time points *t0* and *t3* (30 samples) were randomly collected and sent for next-generation sequencing (NGS) at Eurofins (Eurofins Genomics Germany GmbH, Ebersberg, Germany). To assess the gut microbial profile, the hypervariable region V3–V5 of the bacterial 16S rRNA genes was PCR amplified, amplicon libraries generated using the Nextera XT DNA Library Preparation Kit (Illumina Inc, San Diego, USA) and sequenced with the Illumina MiSeq desktop sequencer producing 300-bp paired-end (PE) reads.

Bioinformatic analyses

Sequence data were analyzed with the CLC Genomics Workbench software (v.12.0.2, QIAGEN Bioinformatics, Hilden, Germany) using the microbial genomics module

plugin as described by Treu et al. (2018). Briefly, quality filtering, operational taxonomic unit (OTU) clustering, taxonomical assignment (Greengenes v13_8 database), and biodiversity indicator (alpha and beta diversity indices) determination were performed using default parameters. When appropriate, the OTU consensus sequence of the most relevant taxa was manually verified using MEGABLAST (database: 16S ribosomal RNA sequences) to improve the taxonomical assignment. Raw reads were deposited in the Sequence Read Archive (SRA) database (http://www.ncbi.nlm.nih.gov/sra) under the BioProject PRJNA625308.

For diversity analysis, data were rarefied to the minimum library size (18,039 sequences) when appropriate. The Shannon, Chao 1, and Simpson indices were calculated for each experimental group (CON, PRO, and SYN) at the time points *t0* and *t3* and compared using ANOVA with Tukey's multiple-comparison test in GraphPad Prism 7 (GraphPad Software LLC, La Jolla, CA). In terms of bacterial community structure (beta diversity analysis), gut microbial dissimilarities among the groups were assessed by PERMANOVA with 99,999 permutations using unweighted and weighted UniFrac diversity metrics. Principal coordinate analysis (PCoA) was chosen as the ordination method to explore and visualize the data.

The abundance and comparison of specific taxa were carried out with STAMP (Parks et al. 2014) using Kruskal-Wallis *H* test analysis and Benjamini-Hochberg FDR as a multiple test correction method. Lastly, canonical correspondence analysis (CCA) was performed with PAST v3.25 (Hammer et al. 2001).

Statistical analysis

Statistical processing and analysis were performed using the Statistical Package for the Social Sciences 20.0 (SPSS Software IBM, Chicago, USA), and graphs were constructed using GraphPad Prism 7 (GraphPad Software LLC, La Jolla, CA). The normality of variables was determined by the Shapiro-Wilk test. The mean values of the three groups (CON, PRO, SYN) were compared by one-way analysis of variance (ANOVA) followed by Bonferroni multiple-comparison post hoc test, for parametric data. For non-parametric data, the Kruskal-Wallis test was applied, complemented by Dunn's multiple-comparison test. The paired *t* test or Wilcoxon test was used to compare the pre- and post-treatment. Correlations between continuous variables were determined by Pearson's (parametric data) or Spearman (non-parametric data) correlation. Differences were considered significant at $p < 0.05$. All results were expressed as mean ± standard deviation (SD).

Results

Effect of probiotics and synbiotic on mice body weight and dietary intake

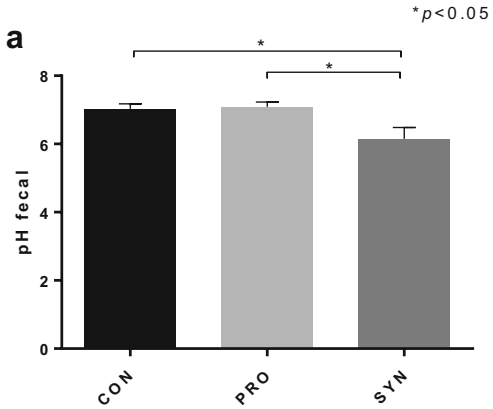
The mice groups started the experimental period with homogeneous body weight, and after 13 weeks, there were no significant differences among the experimental groups. This result indicates that the consumption of the probiotic VSL#3® and synbiotic VSL#3® + PBY did not interfere with body weight gain. Similarly, there were no significant differences among the groups concerning dietary intake regardless of the experimental week. However, when the total dietary intake per week was taken into consideration (i.e., CON + PRO + SYN), a significant reduction in dietary consumption in the third experimental (after DMH injection) week was observed and was significant when compared to the 1st (2.73 g ± 0.15 g × 4.06 g ± 0.05 g; $p = 0.000$), 2nd (2.73 g ± 0.15 g × 3.46 g ± 0.15 g; $p = 0.018$), 11th (2.73 g ± 0.15 g × 3.70 g ± 0.17 g; $p = 0.000$), and 12th (2.73 g ± 0.15 g × 3.73 g ± 0.20 g; $p = 0.000$) experimental weeks (Supplemental Fig. S1).

Effect of probiotic and synbiotic on pH and feces characteristics

Feces characteristics were evaluated in order to verify the possible interferences of probiotic and synbiotic on feces production and intestinal transit. The use of the synbiotic reduced the fecal pH compared to the control diet (Fig. 1a). Fecal pH was positively correlated to the total ACF ($r = 0.584$; $p = 0.011$). Predominantly, the fecal score of the synbiotic group was classified as 1 (33.3%; feces with normal consistency) and 2 (40.0%; viscous non-diarrhea feces) (Fig. 1b).

Effects of probiotic and synbiotic on colonic ACF in mice

ACF incidence was identified in all the experimental groups. The foci consisted predominantly of up to three aberrant crypts (AC) each, which indicates an early stage of precancerous lesions. The AC were wider and exhibited slit-like apertures compared to the circular appearance of normal crypts (Fig. 2). Synbiotic administration reduced the incidence of total ACF by 38.1% compared to the CON group (Table 1). In the segment analysis of the colon, we observed that the SYN group presented a lower ACF count in the proximal and medial regions, compared to the CON group. In the distal colon, there was no difference among the groups. These results demonstrate that the synbiotic was able to reduce the development of pre-neoplastic lesions in the colon of mice. The mean number of AC was significantly higher in the CON group, indicating a greater multiplicity of pre-neoplastic lesions. The mortality rate in the groups was 0%.



b

Group	Fecal Score (%)					
	Score 1	%	Score 2	%	Score 3	%
CON	14	93.3	1	6.7	0	0
PRO	15	100.0	0	0	0	0
SYN	5	33.3	6	40.0	4	26.7

Score 1: firm or normal feces consistency; score 2: viscous non-diarrhea feces; score 3: watery feces characteristic of diarrhea.

Fig. 1 Effect of probiotic and synbiotic on **a** fecal pH and **b** fecal score in C57BL/6J mice. Data are expressed as the mean ± SD (n = 15/group). Statistical differences between groups were analyzed by ANOVA test. (*) $p < 0.05$. CON, AIN-93M diet; PRO, AIN-93M diet and probiotic VSL#3®; SYN, AIN-93M diet with PBY and probiotic VSL#3®

Effects of probiotic and synbiotic on cytokine profile in colon tissue

The cytokines IL-2, IL-4, IL-6, IL-10, IL-17, TNF, and IFN-ɣ were measured in colon tissue by flow cytometry (Fig. 3). Animals receiving the synbiotic displayed an increase of interleukin (IL)-2 in colonic homogenates compared to the control group (Fig. 3a), while an increase in interleukin (IL)-4 levels was observed in both PRO and SYN groups (Fig. 3b). Lastly, the levels of the pro-inflammatory cytokine, tumor necrosis factor (TNF), were significantly lower in the SYN group when compared to the other groups (Fig. 3f). There were no significant differences among the levels of IL-6, IL-10, IL-17, and IFN-ɣ (Fig. 3c–e, g).

Effects of probiotic and synbiotic on β-glucuronidase enzyme activity

Animals receiving the synbiotic displayed a significant reduction in β-glucuronidase enzyme activity when compared to the control group (Fig. 4). The PRO group activity did not differ from the other groups.

Effects of probiotic and synbiotic on the fecal concentration of SCFA

The SCFA profile of acetate, propionate, and butyrate was evaluated in fecal samples collected at time *t0*, *t1*, *t2*, and *t3*. SCFA concentrations varied widely between groups (Fig. 5). In general, the inter-group analysis showed that the animals in the SYN group had higher concentrations of acetic, propionic, and butyric acids at all times (*t0*, *t1*, *t2*, *t3*), compared to the CON and PRO groups (except for propionic acid at *t2*, in which there were no significant differences between groups) (differences not indicated in the graphs). The CON and PRO groups did not differ from each other.

We emphasize that when intra-group analysis (*t0* × *t3*) was performed, a reduction in acetic and butyric acid levels in the CON and PRO groups was observed throughout the experiment (Fig. 5a, c), as well as a reduction of propionic acid in the PRO group (Fig. 5b). On the other hand, the SYN group was able to maintain the concentrations of all SCFA throughout the experimental period. Total ACF incidence was negatively correlated to the concentration of total SCFA ($r = -0.797$; $p = 0.000$).

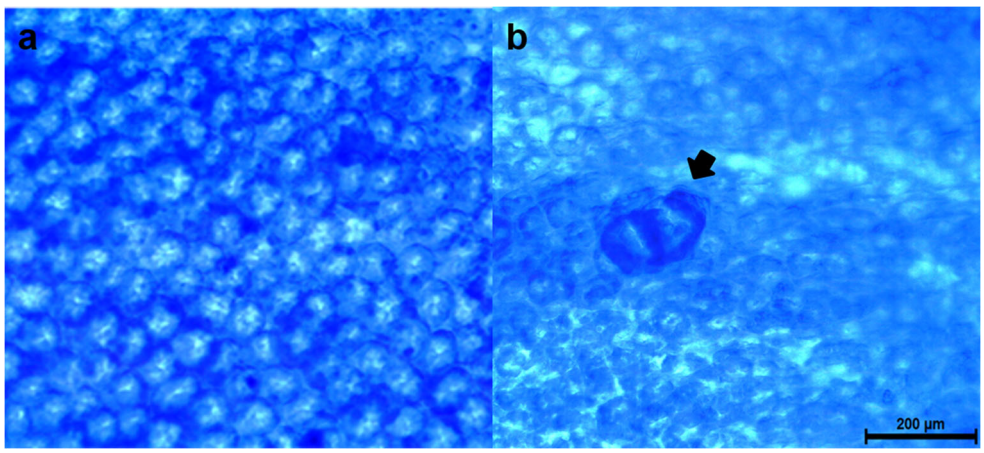


Fig. 2 Photomicrograph of the colon of C57BL/6J mice induced to colorectal carcinogenesis with DMH, stained with 0.1% methylene blue. **a** Normal crypts; **b** ACF with two aberrant crypts (indicated by the arrow)

Table 1 Effects of probiotic and synbiotic on DMH-induced ACF in the colon of C57BL/6 mice

Number of ACF	CON	PRO	SYN	*p*
ACF total	48.7 ± 9.3[a]	39.1 ± 5.1[a,b]	30.1 ± 5.6[b]	0.001[#]
Proximal colon	18.8 ± 5.8[a]	14.3 ± 3.6[a,b]	10.7 ± 5.7[b]	0.029[*]
Medial colon	17.0 ± 7.5[a]	11.1 ± 3.5[a,b]	7.4 ± 4.1[b]	0.013[*]
Distal colon	12.8 ± 5.3	13.7 ± 4.5	12.0 ± 5.1	0.817[*]
AC total	68.3 ± 10.8[a]	50.6 ± 4.6[b]	41.1 ± 7.7[b]	0.000[*]
AC/ACF	1.36 ± 0.14	1.35 ± 0.09	1.42 ± 0.23	0.681[*]

Data are expressed as the mean ± SD (n = 7/group). Statistical difference between groups was analyzed by ANOVA[*] test or Kruskal-Wallis test[#], with $p < 0.05$. Different letters in the same line indicate statistical difference

ACF aberrant crypt foci, *AC* aberrant crypt, *CON* AIN-93M diet, *PRO* AIN-93M diet and probiotic VSL#3®, *SYN* AIN-93M diet with PBY and probiotic VSL#3®

Bioinformatic analysis and microbiota profiling

Conducting a deep amplicon sequencing of the V3–V5 hypervariable region of the 16S rRNA genes, a total of 1,181,027 sequences with a length of 254 bp were obtained. After the removal of low-quality and chimeric sequences from 30 data sets (6 groups, n = 5), a total of 725,924 high-quality reads, with an average of 24,197 (± 2876) sequences for each sample, were assigned to 2275 predicted OTUs (≥ 97% similarity). The number of OTUs per sample ranged from 307 to 597 (Supplemental Table S1).

Considering the analysis of alpha diversity, Shannon's entropy and phylogenetic diversity curves reached a plateau and were used to estimate the depth of sequencing in this study (Supplemental Table S2). These results suggest that sequencing covered most of the microbial diversity and that the majority of bacterial phylotypes were sampled.

Alpha and beta diversity

Alpha diversities were compared among the groups by Shannon and Simpson indices, whereas the Chao 1 index was used to evaluate bacterial richness (Fig. 6a–c). There was no statistically significant difference in terms of bacterial diversity among the groups before probiotics or synbiotic administration ($p > 0.05$). However, after the intervention (*t3*), the Shannon index of SYN*t3* (5.97 ± 0.54) was significantly higher than that of PRO*t3* (4.77 ± 0.45; p = 0.0242). With regard to bacterial richness, there was no significant difference among the groups ($p > 0.05$).

The fecal bacterial community structures of animals fed with probiotic or synbiotic before and after colon carcinogenesis induction with DMH were assessed by the unweighted and weighted UniFrac distance metrics. As depicted in Fig. 7a, b, a scatter plot of the principal coordinate analysis (PCoA) using both distance metrics revealed that SYN groups significantly clustered separately from CON and PRO (PERMANOVA weighted UniFrac: p = 0.00001, pseudo-f statistic = 8.97; PERMANOVA unweighted UniFrac: p = 0.00001, pseudo-f statistic = 8.21). In fact, after performing pairwise comparisons to evaluate microbiota composition similarities among the groups, it was observed that, except for CON*t0* vs. PRO*t0* (PERMANOVA unweighted UniFrac: p = 0.10, pseudo-f statistic = 2.35; PERMANOVA weighted UniFrac: p = 0.39, pseudo-f statistic = 0.91) and CON*t3* vs. PRO*t3* (PERMANOVA weighted UniFrac: p = 0.22, pseudo-f statistic = 1.48), all the groups showed significant differences ($p < 0.05$) in the bacterial community structure (Supplemental Table S3). In total, unweighted and weighted UniFrac components (PCoA 1 and PCoA 2) accounted, respectively, for 61% and 71% of the total variance.

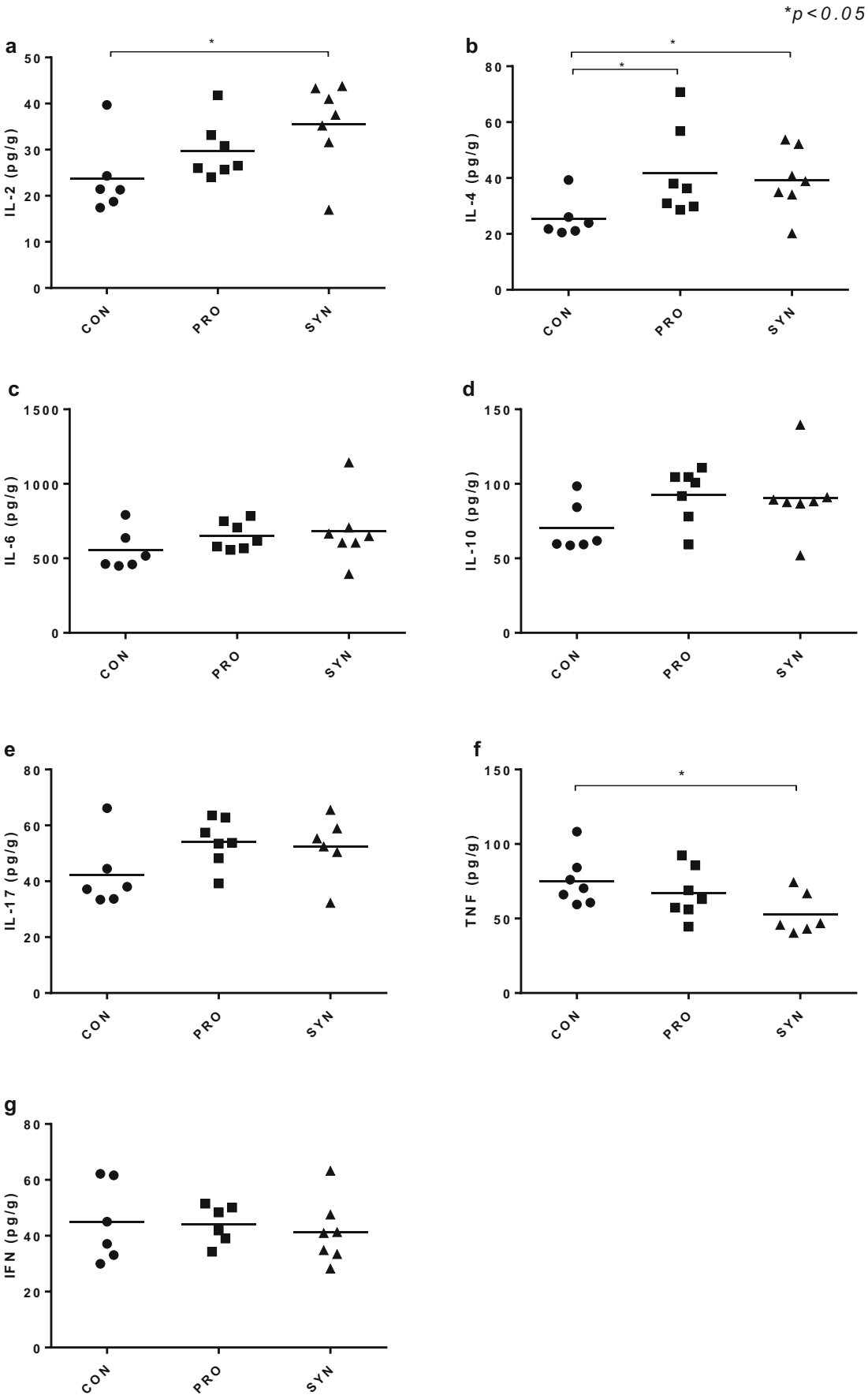
Bacterial community before and after the use of probiotics and synbiotic

Phylum level

The relative distributions of bacteria at the phylum, family, and genus levels identified by 16S rRNA gene amplicon sequencing are reported in Fig. 8a–c. The prevalent bacterial phyla among all groups were *Firmicutes* (75%), *Proteobacteria* (11%), *Bacteroidetes* (6%), *Deferribacteres* (2%), and *TM7* (1%) (Fig. 8a; Supplemental Fig. S2). At the end of the experimental period (*t3*), the phylum *Firmicutes* was significantly more abundant in CON*t3* ($p < 0.01$; 0.3-fold) and PRO*t3* ($p < 0.01$; 0.3-fold) when compared to CON*t0* and PRO*t0* groups, respectively. There was no significant difference in the relative abundance of *Firmicutes* between SYN*t0* and SYN*t3* (p = 0.52.), however, a significant reduction of this taxon was observed in SYN*t3* when compared to CON*t3* ($p < 0.001$; 0.2-fold). With regard to *Bacteroidetes*, a significantly lower abundance of this phylum was observed after intervention in all groups when compared to CON*t0* (CON*t3*: $p < 0.05$, 1.5-fold; PRO*t3*: $p < 0.02$, 1.8-fold; SYN*t3*: p = 0.05, 1.3-fold). Except for the group CON*t3*, a significant reduction was also observed for the phylum *Proteobacteria* in relation to CON*t0* after the intervention (PRO*t3*: p = 3.47 e-3, 1.5-fold; SYN*t3*: p = 0.012, 0.8-fold). The ratio of *Firmicutes* to *Bacteroidetes* (*F*/*B*) was also calculated, and an augmented *F*/*B* ratio was observed between CON*t0* and PRO*t3* (ANOVA, Dunn; p = 0.030) (Supplemental Fig. S3).

Family level

At the family level, 50 bacterial taxa were identified in total. After considering only the most abundant OTUs (relative abundance higher than 0.05% in at least one sample), 23


*p < 0.05
a
IL-2 (pg/g)
b
IL-4 (pg/g)
c
IL-6 (pg/g)
d
IL-10 (pg/g)
e
IL-17 (pg/g)
f
TNF (pg/g)
g
IFN (pg/g)
CON
PRO
SYN

◀ **Fig. 3** Effect of probiotic and synbiotic on colon cytokine profile in C57BL/6J mice. Data are expressed as the mean ± SD (n = 6 or 7/group). Statistical differences between groups were analyzed by ANOVA test. (*) $p < 0.05$. CON, AIN-93M diet; PRO, AIN-93M diet and probiotic VSL#3®; SYN, AIN-93M diet with PBY and probiotic VSL#3®

families accounted for more than 99.6% of the total sequences in each group. Among them, *Lactobacillaceae* was the most abundant family in the CON and PRO groups, whereas *Lachnospiraceae* was the most prevalent in the SYN groups (Fig. 8b; Supplemental Fig. S4).

Concerning the microbial changes after the intervention, a significant increase in the relative abundance of the family *Lachnospiraceae* was observed in the group SYNt3 when compared to CONt3 ($p < 0.01$; 1.7-fold) and PROt3 ($p < 0.01$, 1.8-fold) (Supplemental Fig. S4a).

Regarding *Clostridiaceae* and *Turicibacteraceae*, these families were found to be significantly more abundant in CON*t3* and PRO*t3* (*Clostridiaceae*, $p < 0.01$; *Turicibacteraceae*, $p < 0.001$) in comparison to CONt0 and PROt0, respectively (Supplemental Fig. S4b and S4c). Interestingly, the relative abundance of *Clostridiaceae* increased proportionally in both groups (~ 1.5-fold), whereas the abundance of *Turicibacteraceae* increased by 5- and 6.7-fold in CON*t3* and PRO*t3*, respectively.

Lastly, except for SYNt3, the relative abundance of the bacterial taxon *Desulfovibrionaceae* significantly diminished in all groups after the intervention when compared to their corresponding groups at the time point *t0* (CON*t3*, $p < 0.05$, 1-fold; PRO*t3*, $p < 0.01$, 1.5-fold) (Supplemental Fig. S4d).

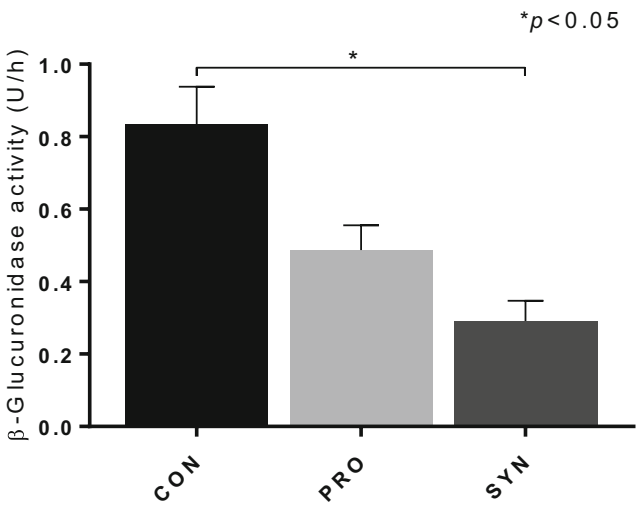


Fig. 4 Effect of probiotic and synbiotic on bacterial enzyme activity β-glucuronidase in C57BL/6J mice. Data are expressed as the mean ± SD (n = 7/group). Statistical differences between groups were analyzed by ANOVA test. (*) $p < 0.05$. CON, AIN-93M diet; PRO, AIN-93M diet and probiotic VSL#3®; SYN, AIN-93M diet with PBY and probiotic VSL#3®

With regard to the family *Helicobacteraceae*, a significant decrease of abundance was noticed in the groups PRO*t3* ($p < 0.05$, 2-fold) and SYNt3 ($p < 0.05$, 1.1-fold) when compared to their corresponding groups at the time point *t0* (Supplemental Fig. S4e).

Genus level

In total, 66 bacterial taxa were assigned to the genus level. After defining the most abundant genera (relative abundance higher than 0.05% in at least one sample), 27 OTUs were selected and accounted for more than 99.3% of the total sequences in each group. *Lactobacillus* was the most abundant microorganism in the groups CON*t3*, PRO*t3*, and SYN*t3*, whereas *Kineothrix* dominated the group SYN*t0* (Fig. 8c). In order to understand the microbial changes before and after the use of probiotics and synbiotic, a differential abundance analysis was conducted. As depicted in Fig. 9, the PRO group presented the highest number of microorganisms among the groups in which the relative abundance changed significantly (fold change ≥ 2; FDR $p < 0.05$). Common to all groups involved in this study, a higher relative abundance of *Turicibacter* with a concomitant reduction of *Lactococcus* was observed after the intervention.

The intra-group comparison revealed a lower abundance of *Enterococcus* (6-fold) and *Acinetobacter* (49-fold) in CON*t3*, whereas the relative abundance of *Dehalobacterium* was enhanced by 96-fold. In PRO*t3*, an impressive increase in the relative abundance of beneficial microorganisms such as *Bifidobacterium* (20-fold), *Roseburia* (29-fold), and *Blautia* (157-fold) was noticed. On the contrary, a reduced abundance of *Coprococcus* (2-fold), *Dorea* (3-fold), *Flexispira* (3-fold), *Oscillospira* (2-fold), *Ruminococcus* (2-fold), *Butyrivibrio* (8-fold), and *Sutterella* (27-fold) was observed. Regarding the SYN*t3* group, the taxon *Lactobacillus* increased by 3-fold.

The taxa *Gemella*, *Helicobacter*, and *02d06* increased in CON*t3* by 80-, 2-, and 29-fold, respectively, whereas *Odoribacter* was reduced by 2-fold. The higher relative abundance of *Gemella* (16-fold) and *02d06* (15-fold) was also observed in PRO*t3*; however, *Odoribacter* (2-fold) and *Helicobacter* (3-fold) were less abundant in this group. An increase of *Clostridium* (CON*t3*, 6-fold; SYN*t3*, 88-fold) was followed by a reduction of *Brachyspira* (CON*t3*, 197-fold; SYN*t3*, 36-fold). Lastly, a mutual increase in the abundance of *Allobaculum* (PRO*t3*, 12-fold; SYN*t3*, 16-fold) and *Streptococcus* (PRO*t3*, 21-fold; SYN*t3*, 24-fold) was detected.

Correlation analysis

Canonical correspondence analysis (CCA) was used to investigate relationships between bacterial community structure at

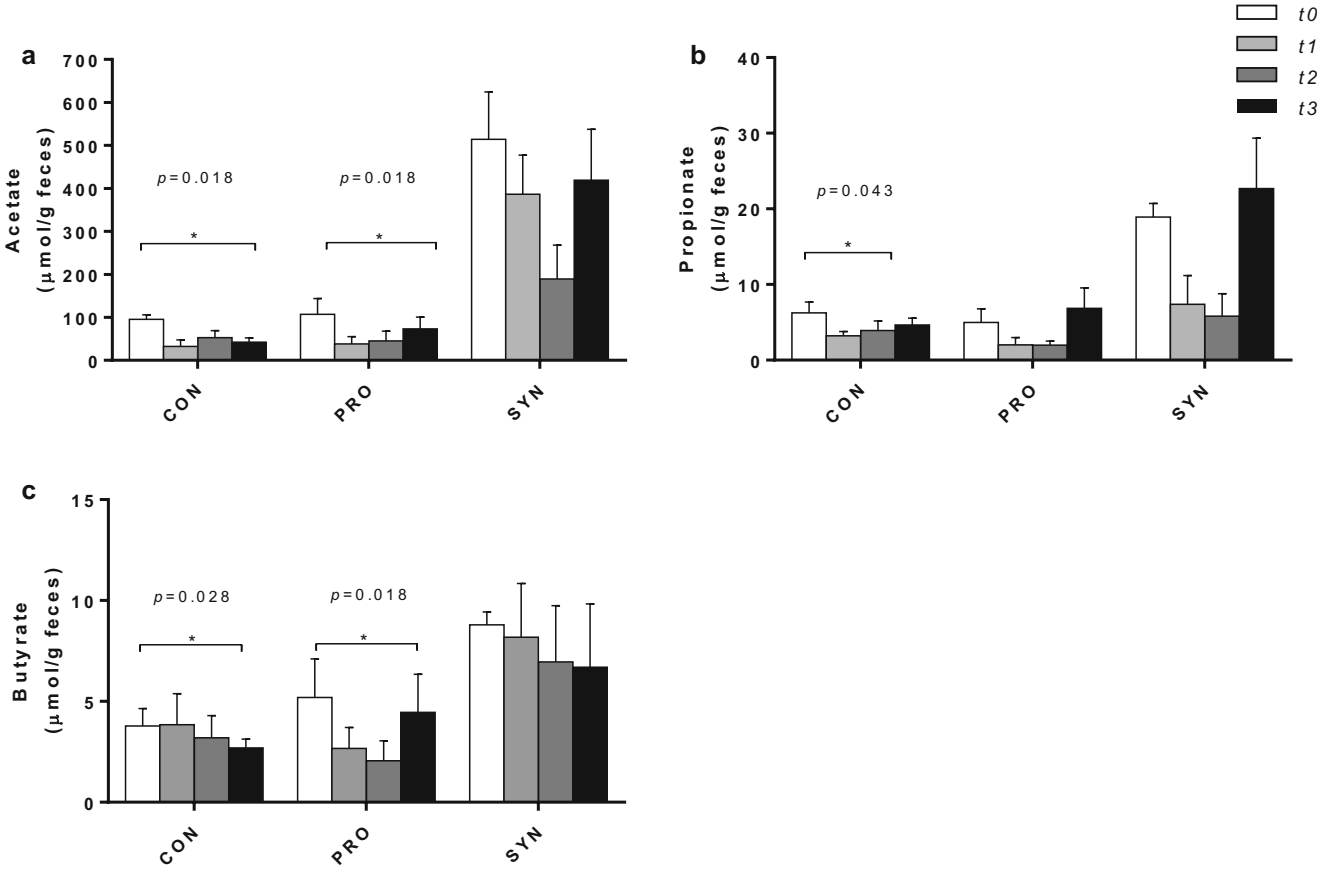


Fig. 5 Effect of probiotic and synbiotic on fecal concentrations of short-chain fatty acids, **a** acetate, **b** propionate, and **c** butyrate, before (*t0*), during (*t1*, *t2*), and after (*t3*) intervention

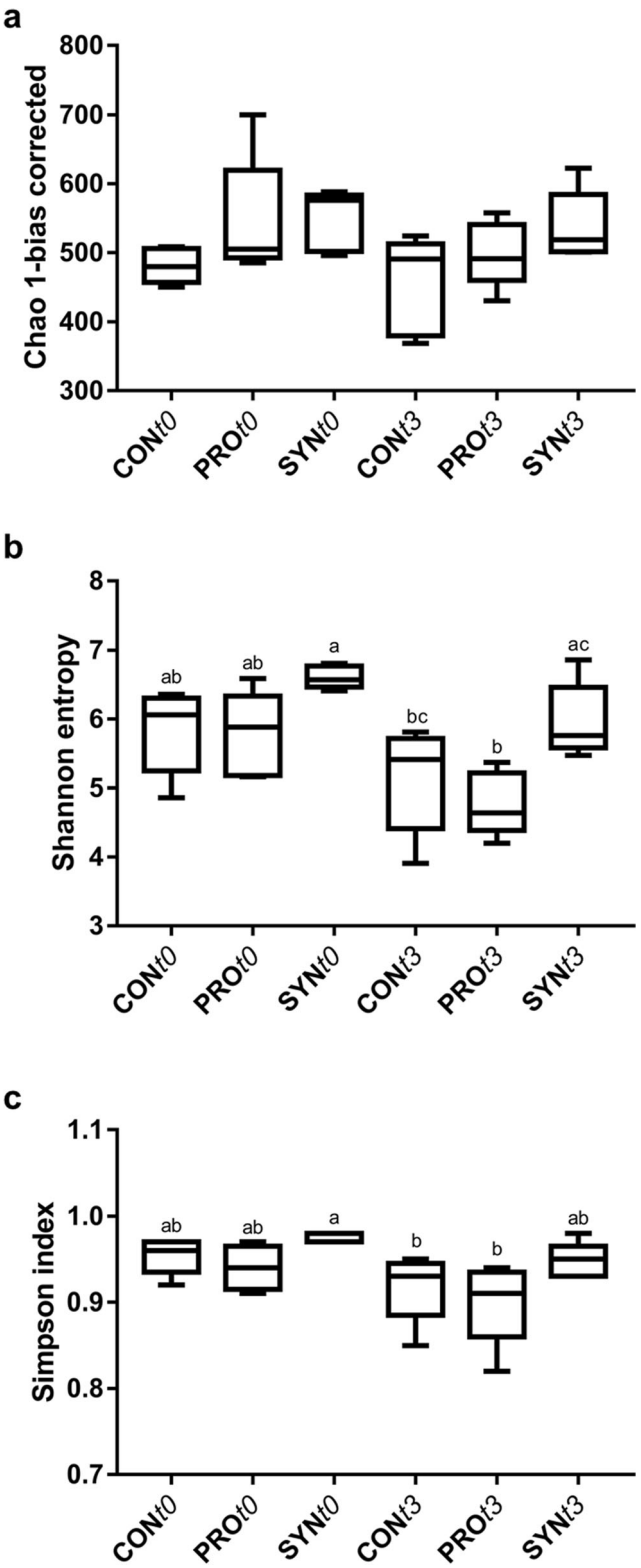
the family and genus levels; the amount of the short-chain fatty acids (SCFA) acetate, butyrate, and propionate; and fecal pH. A negative correlation was observed between the production of propionate ($r = -0.69$, $p = 0.0056$) and butyrate ($r = -0.76$, $p = 0.0015$), and fecal pH. At the family level, *Lachnospiraceae* (propionate: $r = 0.56$, $p = 0.0340$; butyrate: $r = 0.59$, $p = 0.0223$; acetate: $r = 0.63$, $p = 0.0141$), *Veillonellaceae* (propionate: $r = 0.82$, $p = 0.0003$; butyrate: $r = 0.82$, $p = 0.0003$; acetate: $r = 0.61$, $p = 0.0174$), and *Alcaligenaceae* (propionate: $r = 0.61$, $p = 0.0185$; butyrate: $r = 0.82$, $p = 0.0003$; acetate: $r = 0.75$, $p = 0.0019$) were positively correlated with SCFA production (Fig. 10a). The family *Erysipelotrichaceae*, a known butyrate-producing group, was positively correlated with butyrate ($r = 0.62$, $p = 0.0152$) and acetate (r = 0.59, $p = 0.0242$) production.

On the contrary, the taxa *Clostridiaceae* (propionate: $r = -0.63$, $p = 0.0139$; butyrate: $r = -0.68$, $p = 0.0063$; acetate: $r = -0.72$, $p = 0.0033$), *Peptostreptococcaceae* (propionate: $r = -0.56$, $p = 0.0308$; butyrate: $r = -0.83$, $p = 0.0003$; acetate: $r = -0.67$, $p = 0.0078$), and *Turicibacteraceae* (propionate: $r = -0.60$, $p = 0.0197$; butyrate: $r = -0.74$, $p = 0.0021$; acetate: $r = -0.75$, $p = 0.0020$) were negatively correlated with SCFA production (Fig. 10a).

Focusing on the genus level, seven genera were found to be positively correlated with increased levels of SCFA (Fig. 10b). Family *Lachnospiraceae*: *Dorea* (propionate: $r = 0.61$, $p = 0.0175$; butyrate: $r = 0.62$, $p = 0.0164$; acetate: $r = 0.78$, $p = 0.0010$), *Coprococcus* (propionate: $r = 0.52$, $p = 0.0470$; butyrate: $r = 0.65$, $p = 0.0105$; acetate: $r = 0.78$, $p = 0.0012$), and *Kineothrix* (acetate: $r = 0.60$, $p = 0.0202$); family *Alcaligenaceae*: *Sutterella* (propionate: $r = 0.66$, $p = 0.0106$; butyrate: $r = 0.80$, $p = 0.0004$; acetate: $r = 0.83$, $p = 0.00004$); and family *Erysipelotrichaceae*: *Coprobacillus* (butyrate: $r = 0.75$, $p = 0.0020$; acetate: $r = 0.76$, $p = 0.0011$). Although the taxon *Ruminococcaceae* was not significantly associated with SCFA production, taking into consideration the relative abundance of the most abundant families as above mentioned,

Fig. 6 Box and whisker plots comparing species richness (**a**) and diversity (**b** and **c**) between the different groups (CON, PRO, and SYN) before (*t0*) and after (*t3*) their respective intervention. Horizontal bold lines show the median values. The bottom and top of the boxes show the 25th and the 75th percentiles, respectively. The whiskers extend up to the most extreme points within 1.5 times the interquartile ranges (IQR). Different letters indicate significant differences between groups (Tukey's test, $p < 0.05$)

Oscillospira (propionate: $r = 0.62$, $p = 0.0161$) and *Ruminococcus* (propionate: $r = 0.54$, $p = 0.0418$; butyrate: $r = 0.55$, $p = 0.0370$; acetate: $r = 0.65$, $p = 0.0109$) were found to be positively correlated with increased values of SCFA. As depicted in Fig. 10b, both taxa were positively correlated with the SCFA measured in the group SYN*t1*.



Discussion

The use of probiotics isolated or associated to prebiotics (synbiotic) to promote intestinal health has gained prominence in the scientific literature (Tandon et al. 2019). Previous studies demonstrated the protective effect of probiotics and varied combinations of synbiotics in preventing colorectal cancer and other intestinal diseases (Lee et al. 2016; Gavresea et al. 2018). Current evidence suggests that the composition and metabolic activity of the intestinal microbiota are a key variable in this process (Wu et al. 2013; Yu 2018).

It was observed that the use of the synbiotic increased fecal humidity and viscosity, with alteration in fecal pH. The presence of soluble fibers present in the yacon retains water, increasing the humidity in the feces. In addition, the bacterial fermentation favors the reduction of fecal pH, leading to water retention in the intestinal lumen in order to preserve intraluminal osmotic pressure (De Nadai Marcon et al. 2019; Le Blay et al. 1999). Thus, we believe that the change in the consistency of feces is probably related to the synbiotic administration and not to the induction of carcinogenesis.

The aberrant crypt foci (ACF) were used as a morphological marker of colorectal carcinogenesis, as previously described (Islam and Gallaher 2015). The present study demonstrated a 38.1% reduction in ACF in the SYN group compared to the CON group ($p = 0.001$). As expected, the total count of aberrant crypt (AC) was significantly higher in the CON group compared to the others.

The ACF in the PRO and SYN groups were predominantly composed of one or two aberrant crypts (data not shown). The ACF containing a single crypt are classified as quiescent or senescent. These ACF can reenter the proliferation cycle and develop bifurcations (proliferative ACF) or disappear via apoptosis (Tsukamoto et al. 1999). The use of probiotic and synbiotic limited the development of proliferative ACF (with multiple crypts), suggesting a possible action in apoptosis or even in the reversal of ACF into normal crypts since the ACF are potentially reversible lesions (Bird and Good 2000).

The regulation of gene expression involved in cell proliferation, differentiation, and apoptosis is one of the mechanisms suggested to be responsible for the antitumor effect of probiotics and synbiotics (Cruz et al. 2020; Reis et al. 2017), although it is not completely clear how each probiotic and synbiotic acts specifically on gene expression. We hypothesize that the additional protection afforded by the synbiotic can be explained, at least in part, by the increase in butyrate production in this group. Butyrate is a potential modulator of the Wnt/β-catenin signaling pathway; in colonic tumorigenesis, the Wnt pathway is constitutively activated, resulting in

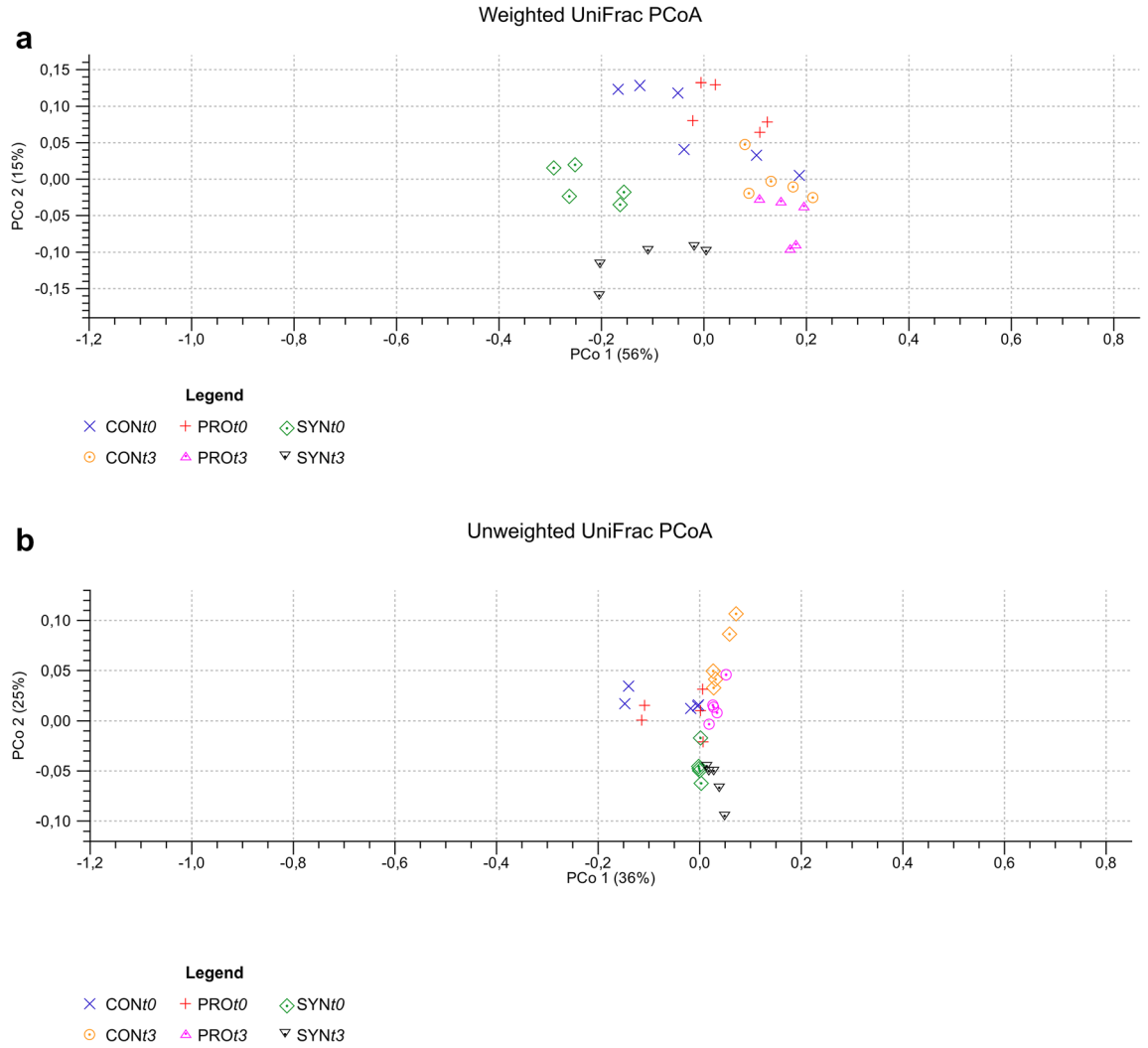


Fig. 7 Principal coordinate analysis (PCoA) based on **a** weighted and **b** unweighted UniFrac distances for CON, PRO, and SYN before (*t0*) and after (*t3*) their respective intervention. PERMANOVA with 99,999 permutations was used to detect significant differences between microbial communities (dissimilarity) of different experimental groups

the overexpression of several oncogenes (Cheng et al. 2019; Uchiyama et al. 2016).

Tumor development and progression are favored in chronically inflamed tissues, in which an increase in the secretion of pro-inflammatory cytokines is observed, such as IL-1β, IL-6, IL-8, IL-17, IL-12, and TNF (tumor necrosis factor), along with a reduction of anti-inflammatory cytokines, such as IL-10 and TGF-β (transforming growth factor beta) (Reis et al. 2017; Zhu et al. 2013; Pagès et al. 2010). This fact justifies, for example, the increased risk of CRC in individuals with inflammatory bowel diseases. During carcinogenesis, the tissue inflammatory response reflects an attempt by the immune system itself to eradicate the tumor (cancer immunoediting), which seems paradoxical, since inflammation stimulates tumor progression (Hanahan and Weinberg 2000; Fouad and Aanei 2017; Qian and Pollard 2010; Karnoub and Weinberg 2007).

In this sense, the probiotic VSL#3® and the synbiotic VSL#3® + PBY were used to attenuate the inflammatory process initiated with the induction of carcinogenesis and, thus, to mitigate tumor development. Microorganisms and their metabolites interact with cells of the immune system, through binding to receptors, such as Toll-like (TLR) and NOD-like (NLR). As a consequence, immune and intestinal cells begin to secrete cytokines to regulate the innate and adaptive immune response, with an increase in the anti-inflammatory response (Corthésy et al. 2007; Delcenserie et al. 2008; Maldonado Galdeano et al. 2015; Igarashi et al. 2017).

In the present study, a significant increase in IL-2 was observed in the SYN group compared to the CON group. A similar result was demonstrated by Štofilová et al. (2015), after administration of the synbiotic *L. plantarum* and inulin in an experimental model of DMH-induced carcinogenesis.

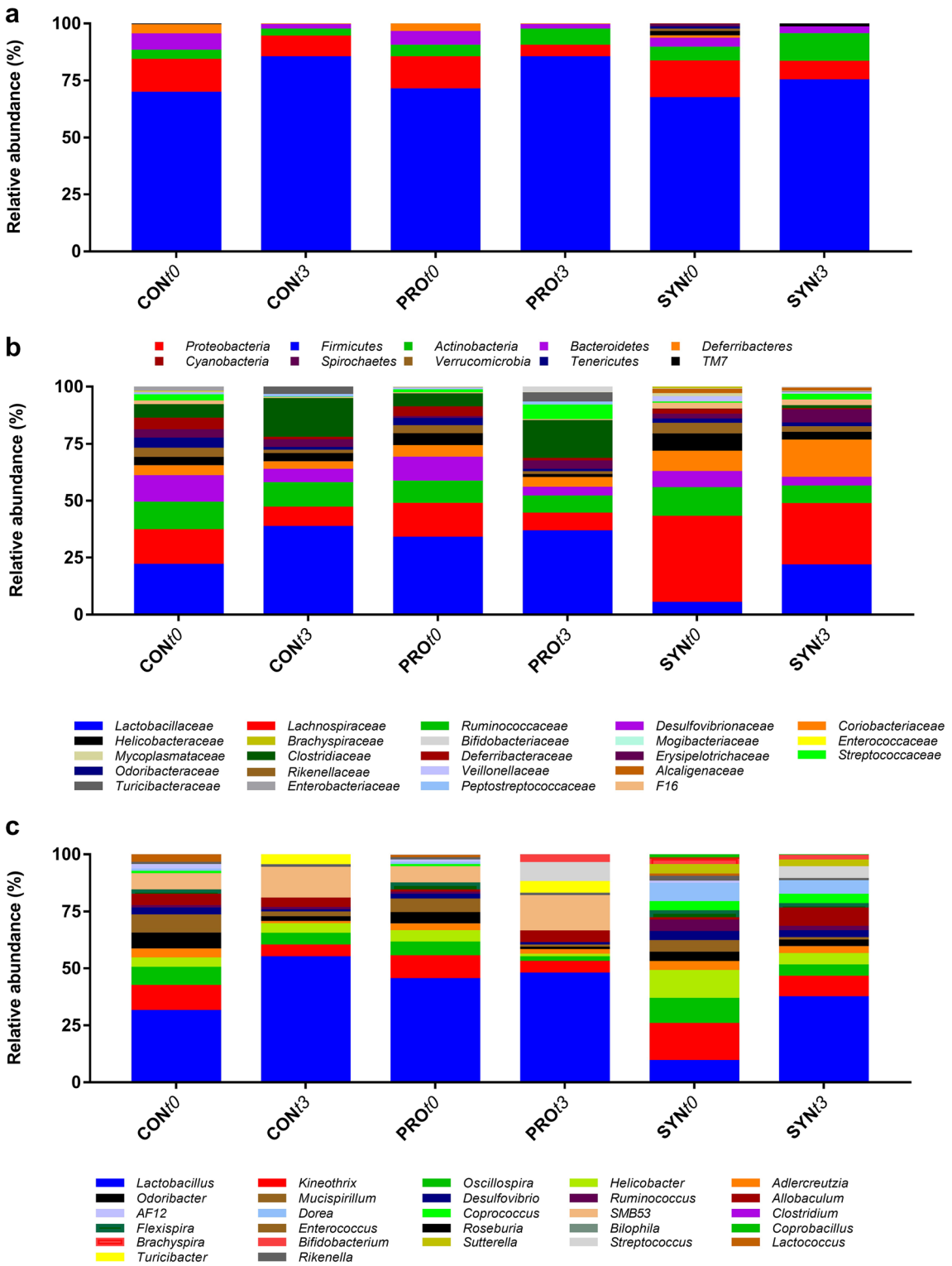


Fig. 8 Relative abundance distribution of major phyla (**a**), families (**b**), and genera (**c**) across the groups CON, PRO, and SYN in two different time points (*t0* and *t3*). Only families and genera with relative abundance greater than 0.05% were shown. The taxa were sorted by the decreasing order of average relative abundance

IL-2 has effects on the regulation of immune cells, and its concentration is inversely correlated with tumor size (Jacouton et al. 2019). *Lactobacillus casei* appears to have an important effect on IL-2 secretion (Hoyer et al. 2008).

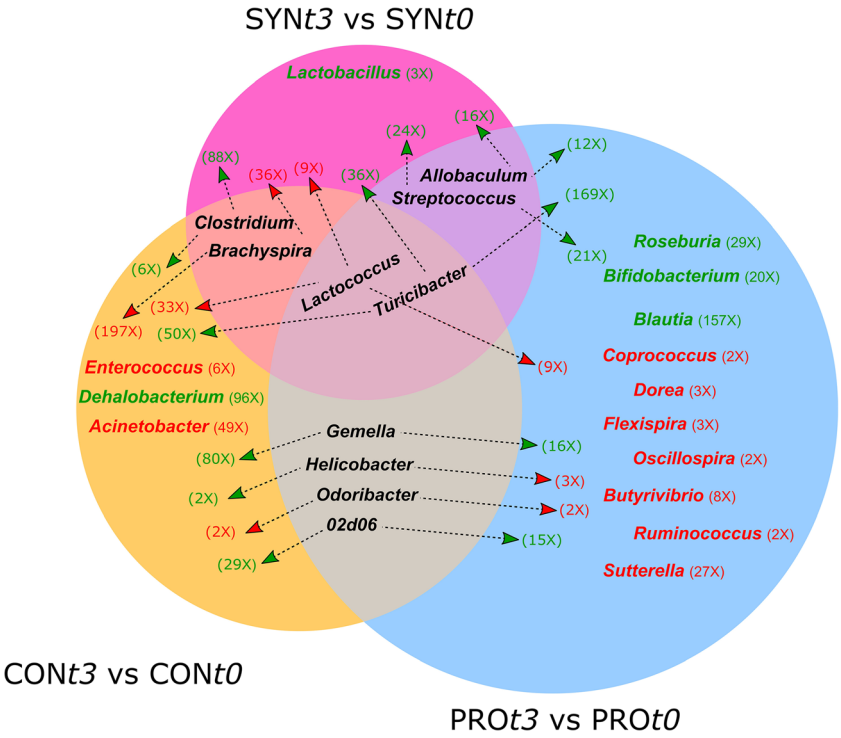


Fig. 9 A Venn diagram showing the number of differentially abundant genera (fold change ≥ 2; FDR $p < 0.05$) associated with each group (CON, PRO, and SYN) at the time points *t0* and *t1*. Green and red values indicate, respectively, an increase or a reduction in the relative abundance of a certain genus observed after the intervention in the different groups

Interestingly, after the consumption of VLS#3® + PBY, there was a 3-fold increase in the abundance of members of the genus *Lactobacillus*.

We also observed an increase in IL-4 (Th2 pattern) in the PRO and SYN groups. Studies using probiotic (Sivieri et al. 2008) and yacon (Delgado et al. 2012; Velez et al. 2013) supplementation have also obtained similar results in experimental models. The increase in IL-4 occurs concomitantly with the TLR4 expression, which results in the improvement of the innate immune response and antitumor defense (Velez et al. 2013; Nakamura et al. 2004).

TNF is another cytokine that plays an important role in carcinogenesis and that was influenced by the use of the synbiotic VSL#3® + PBY (significantly reduced compared to the CON group). Studies that evaluated TNF concentrations show discordant results, with either an increase or decrease in TNF after the use of probiotic or synbiotic (Sivieri et al. 2008; Bassaganya-Riera et al. 2012; Talero et al. 2015; Lee et al. 2015; Štofilová et al. 2015).

Although TNF can activate apoptosis pathways, which would be interesting in controlling tumorigenesis, high concentrations of TNF have been associated with a higher prevalence of colorectal adenomas (Cabal-Hierro and O'Dwyer 2017; Park et al. 2014; Engstrom et al. 2014; Kim et al. 2008). Thus, we believe that changes in cytokine concentrations, promoted mainly by the synbiotic, may have contributed to an early antitumor response.

The assessment of bacterial enzyme activity is often used to demonstrate changes related to dietary intervention in the colon and to provide additional information on its effect on the intestinal microbiota modulation (De Preter et al. 2008). Bacterial enzymes such as β-glucuronidase, nitroreductase, and azoreductase are involved in the conversion of pro-carcinogens into carcinogens in the colon, with the release of cytotoxic and genotoxic metabolites (Chandel et al. 2019; Uccello et al. 2012). Therefore, interventions that result in the lower activity of these enzymes may be a strategy for preventing colorectal carcinogenesis.

In this study, we used the DMH drug to induce precursor lesions of CRC. DMH is classified as a pro-carcinogen, which needs metabolic activation to become an active carcinogen. Activation occurs mainly in the liver, where DMH is oxidized to azomethane and later to azoxymethane, which in turn is N-hydroxylated to methylazoxymethanol (Štofilová et al. 2015; Perše and Cerar 2011). In the liver, methylazoxymethanol can be conjugated with glucuronic acid and secreted through the bile duct into the intestine. The intestinal bacteria that show β-glucuronidase activity are able to hydrolyze the complex formed in the liver, with the release of azoxymethane (an active carcinogen), triggering carcinogenesis in the colon (Zhu et al. 2013; Arthur and Jobin 2011).

Bacterial strains commonly used as probiotics, such as organisms belonging to the lactic acid bacteria group, must not produce β-glucuronidase or show a low enzymatic activity (Son et al. 2017). For instance, microorganisms classified into the genera *Bifidobacterium* and *Lactobacillus* have displayed a minimal β-glucuronidase activity, unlike the obligate

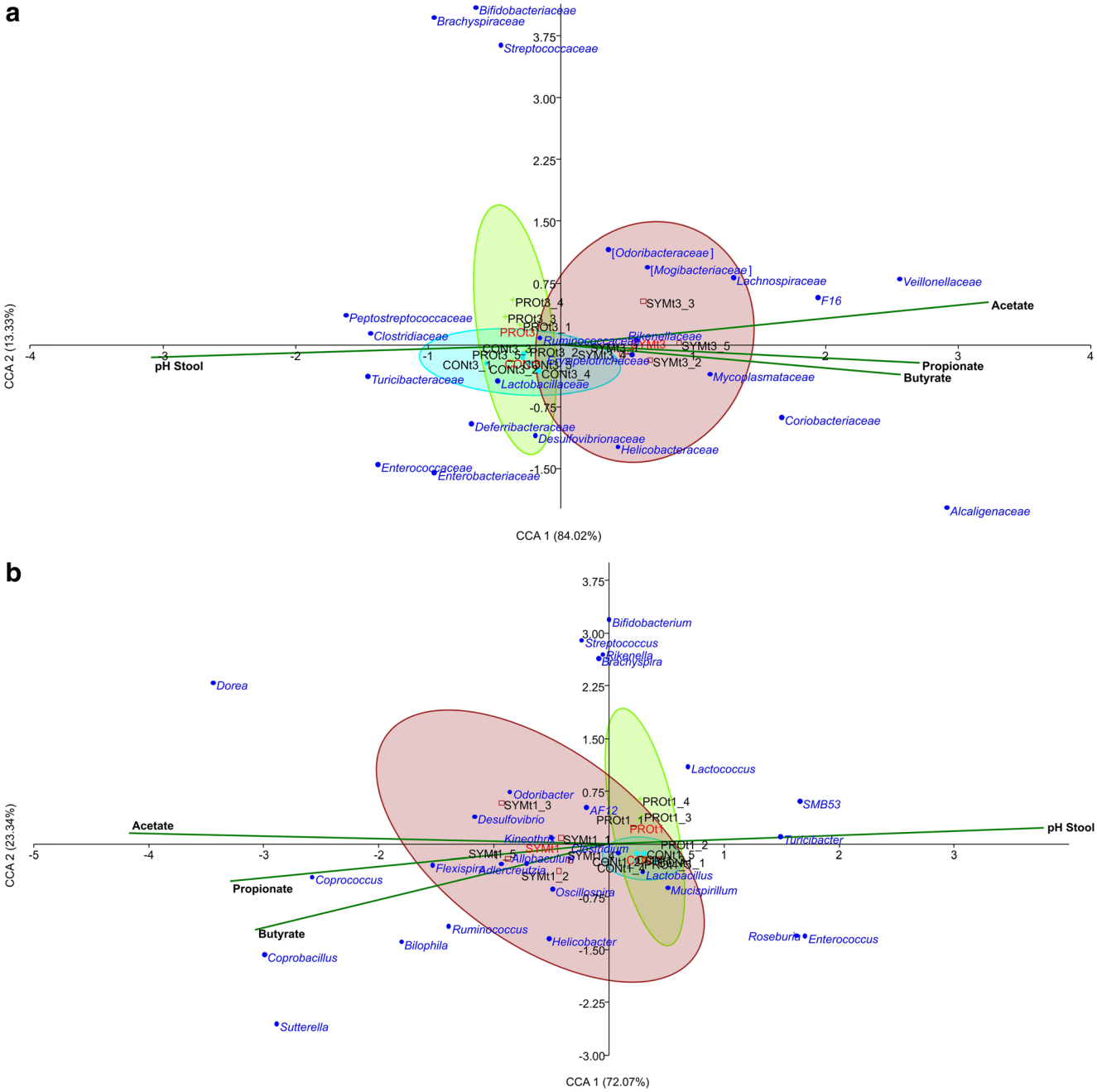


Fig. 10 Canonical correspondence analysis (CCA) performed on the most abundant OTUs (relative abundance ≥ 0.05%) at the family (**a**) and genus (**b**) levels, short-chain fatty acids (SCFA) (acetate, butyrate, and propionate), and fecal pH. Green lines indicate the direction and magnitude of measurable variables (SCFA and fecal pH) associated with community structures. Ellipses, colored according to the group, assume a bivariate normal distribution and estimate a region where 95% of population points are expected to fall

anaerobes *Bacteroides* spp., *Eubacterium* spp., and *Clostridium* spp.) (Anuradha and Rajeshwari 2005; Nakamura et al. 2002).

Reduction of enzyme activity after the use of probiotic and synbiotic has been demonstrated in experimental models and studies with humans (Mohania et al. 2013; Verma and Shukla 2013; Chang et al. 2012; Dominici et al. 2014; Villarini et al. 2008; Hatakka et al. 2008). In our study, β-glucuronidase activity decreased in the PRO group; however, a significant difference was observed only in the SYN group. The presence of fructooligosaccharides and inulin in the synbiotic possibly contributed to these results, since these substrates selectively stimulate the growth of bifidobacteria (Roberfroid 2005; Hijova et al. 2014; De Preter et al. 2011).

The changes in the microbiota after the use of the probiotic and synbiotic corroborate our findings. An increase in the genus *Bifidobacterium* was observed in the PRO and SYN groups, as well as a significant enrichment of the genus *Lactobacillus* in the SYN group. Additionally, β-glucuronidase-producing bacteria such as *Flavobacterium* spp., *Bacteroides* spp., and *Corynebacterium* spp. (Tryland and Fiksdal 1998) were not identified in this study by using 16S rRNA gene amplicon sequencing.

Dysbiosis associated with CRC is characterized by the depletion of SCFA-producing bacteria (Wu et al. 2013). Bacteria metabolize fibers, resistant starch, and fructooligosaccharides to SCFA, which strengthen the intestinal barrier through the production of mucins, antimicrobial peptides, and cell junction proteins (Scheppach and Weiler 2004; Lin et al. 2018). It is common to observe an inverse correlation between SCFA concentrations and the incidence of precursor lesions and tumors in the colon (Worthley et al. 2009), as also demonstrated in this study. Butyrate-producing bacteria receive special attention, since butyrate acts as an inhibitor of histone deacetylase, regulating the expression of oncogenes, and stimulates the secretion of anti-inflammatory cytokines (Farrokhi et al. 2019; Kumar et al. 2012; Sokol et al. 2009).

The acetate, propionate, and butyrate concentrations varied throughout the experiment. In general, the lowest concentrations of SCFA were observed during the period of DMH administration, between the third and tenth experimental weeks (*t1* and *t2*). These results suggest that in addition to the availability of fermentable substrates, other factors, such as the administration of the carcinogen, may have influenced the intestinal microbiota metabolism. However, we emphasize that the SYN group was the only one capable of maintaining the concentrations of all SCFA compared to the initial time (*t0* × *t3*).

Previous studies have shown the reduction of butyrate-producing microorganisms in individuals with inflammatory bowel diseases and CRC, such as the species *Faecalibacterium prausnitzii*, *Clostridium butyricum*, and *Butyrivibrio fibrisolvens*; the genera *Roseburia* and *Eubacterium*; and the families *Lachnospiraceae* and *Ruminococcaceae* (Wu et al. 2013; Wang et al. 2011; Balamurugan et al. 2008; Prosberg et al. 2016; Chen et al. 2014; Ohkawara et al. 2005). Our results are in agreement with previous findings, and it is worth mentioning that genera positively correlated to SCFA production were depleted in the CON and PRO groups; however, a higher relative abundance of these genera was observed in the SYN group.

Based on the canonical correspondence analysis (CCA), a positive correlation between the concentration of SCFA and the abundance of the families *Lachnospiraceae*, *Veillonellaceae*, *Alcaligenaceae*, and *Erysipelotrichaceae* was observed. On the contrary, an inverse correlation between the families *Clostridiaceae*, *Peptostreptococcaceae*, and *Turicibacteraceae* and SCFA levels was noticed. Although commonly found as a low-abundant taxon in the intestinal microbiota, the genus *Clostridium* (family *Clostridiaceae*) is an important butyrate producer (Chen et al. 2020) and had its abundance considerably increased after the synbiotic intervention.

At the genus level, there was a positive correlation between *Dorea*, *Coprococcus*, *Kineothrix*, *Sutterella*, *Coprobacillus*, *Oscillospira*, *Ruminococcus*, and the SCFA levels. All these genera were identified in greater proportions in the SYN group, which explains the higher concentrations of SCFA total in this group.

It is also suggested that SCFA protects against CRC indirectly by decreasing the intestinal pH. The correlation between CRC risk and fecal pH has been demonstrated previously (Chandel et al. 2019; Chang et al. 2012). The acidification of the colonic content limits the colonization of pathogenic bacteria. In the present study, we observed a positive correlation between the incidence of ACF and fecal pH, and an inverse correlation between propionate and butyrate concentrations and fecal pH.

Given the probable involvement of the intestinal microbiota in the origin and progression of the CRC, and the advances in culture-independent microbial profiling techniques, several studies have been seeking specific microbial signatures that can assist in the screening and surveillance of the CRC. Bacterial species such as *Streptococcus gallolyticus*, *F. nucleatum*, *E. coli*, *B. fragilis*, and *E. faecalis* have a high prevalence in individuals with CRC compared to the healthy population, while the genera such as *Roseburia*, *Clostridium*, *Faecalibacterium*, and *Bifidobacterium* may be depleted (Saus et al. 2019; Goodwin et al. 2011; Wu et al. 2009).

The *Firmicutes*/*Bacteroidetes* (*F*/*B*) ratio can be considered an important marker of intestinal dysbiosis. In general, individuals with pre-neoplastic lesions or tumors present a decrease in the proportion of *F*/*B*, and *Actinobacteria*, concomitantly to the expansion of the phylum *Proteobacteria* (Mori et al. 2018; Lu et al. 2016). Although the *Firmicutes*/*Bacteroidetes* ratio apparently increased at the end of the experiment (*t3*), a significant difference was identified only between the CON*t0* and PRO*t3* groups.

In general, the use of the probiotic VSL#3® and the synbiotic VSL#3® + PBY promoted remarkable changes in gut microbiota composition and might be a consequence of the availability of fermentable substrates, as demonstrated in this study. There were an increase and maintenance throughout the experiment in the abundance of the genera present in a mix of probiotic used (*Lactobacillus*, *Bifidobacterium*, and *Streptococcus*), unlike other genera. Changes in the metabolic activity of the microbiota, such as the increase in the production of organic acids, were also observed. We emphasize that the relative abundance of some bacterial genera was also altered in the CON group, which indicates that exposure to the chemical carcinogen is capable of influencing the microbiota composition.

In conclusion, the synbiotic VSL#3® in combination with PBY showed additional benefits compared to the use of VSL#3® alone, which culminated in a significant reduction in the precursor lesions of CRC. We hypothesize that this result is linked to changes in the composition and metabolic activity of the intestinal microbiota. The modulation of the intestinal inflammatory response, the inhibition of pro-carcinogenic enzymes, and the production of SCFA can be considered important targets of synbiotics in CRC prevention. The enrichment of potentially pathogenic microorganisms and the reduction of SCFA-producing species may represent a specific microbial signature of CRC. Understanding the dynamic changes of the microbiota from health to disease can assist in the development of diagnostic tools based on the fecal microbial structure.

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Author contribution statement BCSC, MCGP, and CLLFF conceived and designed the research. BCSC, VSD, and LSF conducted the experiments. AG, VC, VMG, SOP, and MCGP contributed new reagents or analytical tools. BCSC, VSD, and LSF analyzed data. BCSC wrote the manuscript. All authors read and approved the manuscript.

Compliance with ethical standards

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

Ethical approval All experimental protocols were approved by the Ethics Committee in Animal Experimentation of the Federal University of Viçosa (n° 08/2017, date of approval: May 09, 2017), under the guidelines of the European Community (Directive 2010/63/EU).

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