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Use of the synbiotic VSL#3 and yacon-based concentrate attenuates intestinal damage and reduces the abundance of Candidatus Saccharimonas in a colitis-associated carcinogenesis model

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ABSTRACT

Individuals with inflammatory bowel disease are at high risk of developing colitis-associated cancer; thus, strategies to inhibit disease progression should be investigated. The study aimed to explore the role of the synbiotic (probiotic VSL#3® and yacon-based concentrate) in a colitis-associated carcinogenesis model. IL-10$^{-/-}$ mice were induced to carcinogenesis with 1,2-dimethylhydrazine and divided into two experimental groups: control and synbiotic. Manifestations of colitis, colon histology, expression of antioxidant enzymes, production of organic acids and intestinal microbiota were evaluated. The use of the synbiotic showed benefits, such as the preservation of intestinal architecture, increased expression of antioxidant enzymes and the concentration of organic acids, especially butyrate. It was also observed different microbial community profiles between the groups during the study. Together, these factors contributed to mitigate the manifestations of colitis and improve intestinal integrity, suggesting the potential benefit of the synbiotic in intestinal diseases.

1. Introduction

Individuals with inflammatory bowel disease (IBD), which includes ulcerative colitis (UC) and Crohn's disease (CD), are at higher risk of developing colorectal cancer (CRC) (Zheng & Jiang, 2016). Colitis-associated carcinogenesis (CAC) is the main complication of IBD in humans (Richard et al., 2018; Wang, Hua, et al., 2019; Wang, Li, et al., 2019) and is directly related to chronic inflammation (Potack & Itzkowitz, 2008). The risk for developing CRC is particularly relevant in patients with long-standing IBD involving at least 1/3 of the colon, and starts approximately 7 years after diagnosis, increasing linearly thereafter (Clarke & Feuerstein, 2019; Zhou et al., 2019). For the UC, the cumulative risk of CRC is 2% at 10 years, 8% at 20 years and 18% at 30 years. The overall prevalence of CRC is shown to be 3.7% in patients with UC and 5.4% in patients with pancolitis (Eaden, Abrams, & Mayberry, 2001).

Although the mechanisms of association between IBD and carcinogenesis are not completely understood, it is believed that the malignant process is enhanced by the time, extent and severity of tissue inflammation (Herszenyi, Barabás, Miheller, & Tulassay, 2015). Chronic inflammation is responsible for the increase of a variety of pro-inflammatory cytokines, such as tumor necrosis factor (TNF),

Abbreviations: IBD, inflammatory bowel disease; CRC, colorectal cancer; CAC, colitis-associated cancer; PBY, yacon-based product; DSS, dextran sulfate sodium; TNBS, trinitrobenzene sulfonic acid; FOS, fructooligosaccharides; HPLC, high performance liquid chromatography; DMH, 1,2-dimethylhydrazine; CFE, food efficiency; SCFA, short chain-fatty acid; DAI, disease activity index; TNF, tumor necrosis factor; UC, ulcerative colitis; CD, Crohn's disease; MDA, malondialdehyde; CP, carbonyls protein.

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interleukin 1β and interleukin 6, which stimulate cell proliferation and inhibit apoptosis, contributing to carcinogenesis (De Almeida, De Camargo, Russo, & Amedei, 2019; Francescone, Hou, & Grivennikov, 2016; Grivennikov, 2013). On the other hand, interleukin 10 (IL-10) is a critical immunosuppressive cytokine, associated with CAC inhibition (Mantovani, Allavena, Sica, & Balkwill, 2008; Uronis et al., 2009).

In particular, the presence of dysbiosis in individuals with IBD is among the possible factors that explain the association between intestinal inflammation and the potential risk of cancer (Arthur, 2012; Kostic, 2013; Rubinstein, 2013). It is estimated that the colon hosts 70% of the human microbiome, being the most colonized site of the gastrointestinal tract (Sekirov, Russell, Antunes, & Finlay, 2010). It is also the most prone place for the development of cancer, with a 12-fold higher incidence compared to the small intestine (Gagniere, 2016). Thus, a plausible hypothesis is that colonization by pathogenic microorganisms or the imbalance in the microbiota metabolic activity promotes a more inflammatory microenvironment, favorable to tumor development (Tanaka, 2012; Wong et al., 2017).

Knowing specific microbial signatures and their relationship with the origin and progression of CAC is useful for generating tools for screening and discovering specific bacterial genera for therapeutic purposes (Villéger et al., 2018). Recent studies have demonstrated changes in the microbiota associated with IBD and CRC (Guo, Liu, & Hao, 2020; Kim et al., 2020). However, little is known about the structure and function of the microbiota in the progression of CAC so far.

To understand the pathogenesis of CAC, experimental models of drug-induced colitis are used, such as dextran sulfate sodium (DSS) and trinitrobenzene sulfonic acid (TNBS) or even genetically modified animals, such as interleukin-10-knockout mice (IL-$10^{-/-}$) (Rothemich & Arthur, 2019). These animals have defects in immune regulation and spontaneously develop chronic enterocolitis (Hale, Chichlowski, Trinh, & Greer, 2010; Mizoguchi, Mizoguchi, Takedatsu, Blumberg, & Bhan, 2002). When exposed to chemical carcinogens, inflammation-dependent colorectal mutagenesis and tumorigenesis occurs, driven by the composition of the intestinal microbiota (Rothemich & Arthur, 2019).

In this context, the use of probiotics, prebiotics and, or synbiotics has gained support in the scientific community (Lynch & Pedersen, 2016; Montalban-Arques & Scharl, 2019; Schmidt, Raes, & Bork, 2018). In a recent systematic review, the protective effect of probiotics and synbiotics on colorectal carcinogenesis and on the progression of colitis-associated cancer was demonstrated by different mechanisms, such as modulation of the intestinal microbiota and immune response, reduction of inflammation, biosynthesis of compounds with antitumor activity and improvement in the antioxidant system (Cruz et al., 2020).

The probiotic VSL#3® has a high bacterial concentration, and has been proved significantly effective in the treatment of inflammatory bowel diseases, in clinical and experimental studies (Bibiloni et al., 2003; Mimura, Rizzello, & Helwig, 2004; Kim, Camilleri, & McKinzie, 2003). Rats with colitis who received VSL#3® had lower macroscopic and microscopic damage in the colon, reduction of macrophage infiltration, reduction of serum cytokine levels, and restoration of colonic transcript levels for anti-inflammatory, and barrier proteins (Wang, Li, et al., 2019; Isidro et al., 2017; Kumar, Kissoon-Singh, Coria, Moreau, & Chadee, 2017). However, the effects of probiotic VSL#3® in models of colitis-associated colorectal carcinogenesis are controversial (Appleyard, 2011; Arthur, 2013; Talero et al., 2015).

Yacon (*Smallanthus sonchifolius*) is a tuberous root rich in phenolic compounds and considered a prebiotic food due to its high soluble fibers contents (Russo, Valentão, Andrade, Fernandez, & Milella, 2015). The yacon-based product (PBY) is a concentrate rich in fructooligosaccharides (FOS) and inulin. The evaluation of the effects of PBY on colitis-associated carcinogenesis is unprecedented, however, in colorectal carcinogenesis model, the use of PBY intensified fecal short-chain fatty acid (SCFA) production, increased the number of regulatory T cells, and downregulated the expression of RORγt transcription factor in the colon, improving anti-inflammatory immune responses (De Nadai Marcon et al., 2019).

The yacon powder decrease IFN-γ levels and improve the healing of intestinal mucosa by increasing the number of goblet cells in TNBS-induced colitis model (Umizah, Wasityastuti, Widasari, & Setyo, 2020). In colorectal carcinogenesis induced with DMH, the animals that received yacon powder had a percentage reduction of aberrant crypt foci (preneoplastic lesions) in more than 40%, lower intestinal permeability, higher concentrations of SCFA and increase in the depth and number of colonic crypts (Grancieri et al., 2017).

The concomitant administration of the probiotic VSL#3® and the prebiotic PBY is an unprecedented synbiotic formulation. Previous studies that used synbiotics in CRC or colitis obtained additional results when compared to the use of probiotic and prebiotic alone (Moura, 2012; Sheng et al., 2020). In these studies, the efficacy of synbiotics was explained by the additive combination of the direct anti-inflammatory effects of the probiotic and prebiotic components and their ability to fortify colonic epithelial barrier integrity. Our hypothesis is that the prophylactic administration of the synbiotic could be able to modulate the composition and metabolism of the intestinal microbiota and, consequently, reduce the manifestations of CAC.

Thus, the present study aimed to evaluate the effects of the synbiotic, composed of the probiotic VSL#3® and the concentrated product based on yacon (PBY), in the manifestations of CAC, in the oxidative stress, in the intestinal microbiota composition and in the production of short-chain fatty acids (SCFA) an experimental model of colitis-associated carcinogenesis.

2. Material and Methods

2.1. Synbiotic

The probiotic VSL#3® (Sigma Tau Pharmaceuticals, Inc.; acquired in 2016, valid 04/2018, lot number 604094) was selected based on studies that demonstrated its benefits in the treatment of IBD (Chen et al., 2019; Isidro et al., 2017; Liu, Yu, & Zou, 2019). This is a mixture of eight bacterial species, including *Bifidobacterium breve*, *Bifidobacterium infantis*, *Bifidobacterium longum*, *Lactobacillus acidophilus*, *Lactobacillus bulgaricus*, *Lactobacillus casei*, *Lactobacillus planatarum*, and *Streptococcus thermophilus*. The probiotic was obtained lyophilized, in sachets containing 450 billion viable bacteria. Reconstitution was performed daily in distilled water immediately before administration.

The description of PBY processing has been safeguarded due to the requirements of the patent application (PI 1106621-0). In order to define the diet composition containing PBY, it was necessary to determine its centesimal composition. Therefore, the concentrations of total carbohydrates, sugars, proteins, lipids, total fiber, ash and moisture (AOAC, 1997), and the concentrations of FOS and inulin by high performance liquid chromatography (HPLC) were analyzed. PBY was added to the diet in sufficient quantity to provide 6% FOS and inulin (Paula et al., 2012).

The control and PBY diets were based on the AIN-93 M diet as recommended by the American Institute of Nutrition (Reeves, Nielsen, & Fahey, 1993), and they had the contents of carbohydrates, proteins and fibers adjusted so that both had similar composition. The diets were prepared in the form of pellets and stored at −20 °C.

2.2. Animals and experimental design

Interleukin-10-knockout mice (IL-$10^{-/-}$), male, with eight weeks of age and body weight of approximately 25 g, from the Central Bioterium of the Federal University of Alfenas (UNIFAL), Minas Gerais, Brazil, were used. The IL-$10^{-/-}$ mice were generated from the C57BL/6J background. Under conventional housing conditions, they develop chronic colitis at six weeks of age (Sturlan et al., 2001).

The animals were housed collectively in polypropylene boxes covered with metal gratings, in a controlled temperature environment

(22 ± 2 °C) and a 12-hour light/dark cycle. After a week of acclimatization with free access to the commercial diet and water, the animals were randomly divided into two experimental groups: control group (KOCON, n = 8), AIN-93 M diet and gavage with distilled water, and synbiotic group (KOSYN, n = 7), AIN-93M diet with PBY (6% FOS and inulin) and gavage with probiotic VSL#3®.

The animals in the KOSYN group received the probiotic in a volume of 0.1 mL by orogastric gavage in the morning, five days a week (Arthur, 2012). The KOCON group received gavage with distilled water in the same volume. The amount of probiotic administered was adjusted to ensure a daily supply of 2.25×10^9 CFU, based on an approximate intake of 10^9 CFU/day for a 70 kg adult (Dai et al., 2013; Zavisic et al., 2012). Diets and water were provided *ad libitum* for 13 weeks (Fig. 1).

In the 3rd experimental week, the protocol for inducing precursor lesions of the CRC was started and all animals received an intraperitoneal injection (0.1 mL) of the drug 1,2-dimethylhydrazine (DMH) (Sigma-Aldrich®), at a dose of 20 mg/kg of body weight, once a week, for eight consecutive weeks (Gomides et al., 2015). DMH was prepared in 0.9% saline, with 1 mM EDTA and 10 mM sodium citrate, pH 8 (Newell & Heddle, 2004).

At the end of 13 weeks, the animals were anesthetized with isoflurane 3% (Isoflorine®, Cristalia), followed by total exsanguination by the retro orbital sinus. After dissection, the tissues were washed with phosphate-buffered solution, weighed in a semi-analytical balance, placed in a container with liquid nitrogen and stored at −80 °C until the time of analysis.

2.3. Body weight and dietary intake

To evaluate the weight loss or gain of the animals, individual body weight was recorded weekly using a digital weighing scale. Dietary intake was measured daily and was calculated by the difference in the amount of diet offered (g) and the remaining amount in the day after. This quantification was done during all experimental period using a digital weighing scale. The coefficient of food efficiency (CFE) was calculated by the equation: CFE = weight gain (g) / total dietary intake (g).

2.4. Analysis of serum biomarkers

The blood was centrifuged at 1190*g* for 10 min at 4 °C. The serum markers albumin, creatinine, and urea were assessed by specific colorimetric assays following manufacturer's recommendations (Bioclin®, Brazil) using a clinical chemistry analyzer BS-200 (Mindray®).

2.5. Assessment of colitis symptoms and disease activity index

The disease activity index (DAI) was evaluated from the first application of DMH (third experimental week). Mice were monitored weekly for body weight loss, compared to baseline weight (score 0–4), fecal consistency (score 0–4) and blood in stool (score 0–4). The DAI score was assessed as the combined score of the above three criteria (Hamamoto et al., 1999).

2.6. Colonic tissue processing, morphometry and histopathological score

After washing, fragments of the colon were fixed with Carson's formalin for 24 h (Carson, Martin, & Lynn, 1973) and then transferred to a 70% ethanol solution. Subsequently, they were dehydrated in an increasing gradient of ethanol, diaphanized in xylol and included in paraffin. Cross sections of 5 μm thick were obtained on a rotary microtome for the preparation of slides.

The slides were stained with hematoxylin-eosin (HE) and the images were obtained directly from the light microscope (Leica Microsystems®, Inc.) to assess intestinal morphometry and histopathological score. Intestinal morphometry was performed using the Image Pro Plus 4.5 software in which the crypt depth, thickness of the submucosa, muscular and external muscular layers were measured. The crypt length measure was taken after the identification of the base and the apex of the crypt. Only the straight aspect crypts were included in this assessment. It was prepared slides of seven animals per group, each one with ten non-consecutive cuts. It was taken about 20 photos of each slide.

The histopathological score was calculated by adding the combined score of three criteria: damage to the colonic crypt (score 0: none; 1: basal 1/3; 2: basal 2/3; 3: only surface epithelium intact; 4: complete loss of crypt and epithelium), presence of inflammatory infiltrate (score 0: none; 1: slight; 2: moderate; 3: severe) and infiltration depth (score 0: none; 1: mucosal; 2: mucosal and submucosal; 3: trasmural) (Dieleam et al., 1994). The assessment was carried out by two examiners, independently.

2.7. Determination of oxidation products and hepatic enzyme expression

The liver samples were weighed (150 mg) and properly homogenized in cold potassium phosphate buffer (1.5 mL, pH 7.4) using an Ultra-Turrax homogenizer (IKA T10 basic). The homogenate was centrifuged at 10,000*g* for 10 min at 4° C. The supernatant was then pipetted into eppendorf tubes and used for hepatic enzyme analysis, catalase (Dieterich, Bieligk, Beulich, Hasenfuss, & Prestle, 2000), superoxide dismutase (Aebi, 1984; Buege & Aust, 1978) and glutathione-S-transferase (Habig & Jakoby, 1981); and for oxidation biomarkers assessment, malondyaldeide (Wallin, Rosengren, Shetzer, & Cameja, 1993) and carbonyls protein (Levine, 1990). The results were normalized by total protein concentration of supernatant (Lowry, Rosebrough, Farr, & Randall, 1951).

2.8. 16S rRNA gene sequencing

The feces were collected on the first (t0) and last (t1) experimental week. To obtain those samples, individual cages were previously cleaned and sanitized and mice kept there until a sufficient amount of feces have been spontaneously expelled. Samples were kept at −80 °C until processing. The DNA extraction was performed as previously described (Zhang, Li, Ma, & Wei, 2006). The concentration and integrity of bacterial DNA were assessed using Qubit and agarose gel electrophoresis 1.8%, respectively. The DNA sequencing for microbial analysis was performed by Macrogen (Seoul, Korea). The V3-V4 hypervariable

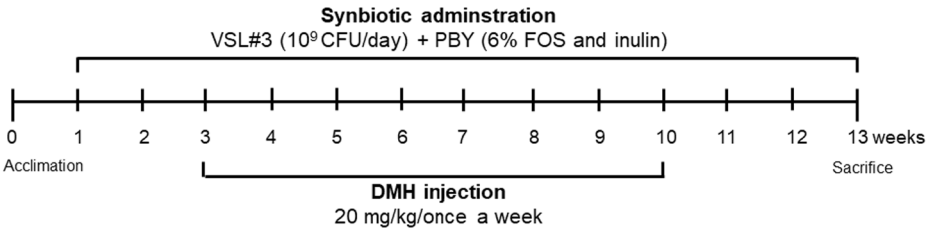

Fig. 1. Experimental timeline for colitis-associated colorectal carcinogenesis model with synbiotic administration. Carcinogenesis was initiated with eight injections of DMH (20 mg/kg), once a week. The VSL#3® was administered via gavage (10^9 CFU/day) and the PBY added to the standard diet throughout the experiment. After thirteen weeks, IL-10-deficient mice were sacrificed and tissues and feces were harvested.

region of the 16S rRNA gene was initially amplified using the primers Bakt_341F (CCTACGGGNGGCWGCAG) and Bakt_805R (GACTACHVGGGTATCTAATCC). The PCR products were sequencing in MiSeq plataform (Illumina, USA).

2.9. Microbial bioinformatics analysis

The raw data (fastq format) were filtered to remove low quality reads with Phred Quality score smaller than 30 using the program Trimmomatic v0.36 (Bolger, Lohse, & Usadel, 2014). The high quality reads were inputted in DADA2 package version 1.8 (Callahan et al., 2016) implemented in R plataform version 3.6.1. Chimeric sequences were identified and deleted. The representative OTU sequences were taxonomically classified using the Silva 16S rRNA Database release 138 (Quast et al., 2013).

To estimate the diversity of the microbial community of the sample, we calculated the within-sample alpha-diversity using the Shannon and Simpson index, and Chao-1 for richness assessment using the phyloseq package (McMurdie & Holmes, 2012). Beta-diversity was estimated by computing Jaccard distance and visualized by multidimensional scaling (MDS) plot using R platform. Bacterial abundance profiles were calculated at taxonomic levels from phylum to species in percent abundance and compared using a paired Student's *t*-test or Wilcoxon test for dependent samples and Student's *t*-test or Mann-Whitney test for independent samples (IBM SPSS Statistics 20).

2.10. Fecal short chain-fatty acids quantification

Mice fecal samples were obtained at the end of the experimental period for SCFA assessment. The concentration of acetate, propionate, butyrate and total SCFA were evaluated by high-performance liquid chromatography (HPLC) according to the method described by Smiricky-Tjardes, Grieshop, Flickinger, Bauer, and Fahey Jr (2003), with some modifications. Approximately 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 (10,000g, 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 acetic, propionic and butyric acids (Supelco®) were constructed. Results are expressed in μmol/g feces.

2.11. Statistical analyses

Data were analyzed using Software Social Package Statistical Science for Windows-SPSS (20 version, IBM® SPSS, Chicago, USA). The means were evaluated by the Shapiro-Wilk normality test and the groups with a 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. $p < 0.05$ was considered to be statistically significant and the data are expressed as the mean ± standard deviation (SD). The graphics were built using Graphpad Prism (version 7.0).

3. Results

The composition of the experimental diets was described in Table 1. Considering that 100 g of PBY contains 23.6 g of FOS and inulin, to meet the dose of 6%, 25.4 g of PBY was added for each 100 g of diet.

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

Ingredients (g 100 g^{-1})	Control Diet	Synbiotic Diet
Cornstarch	33.20	28.55
Casein	16.50	16.40
Dextrinized starch	15.50	15.50
Sucrose	10.00	5.20
Soybean oil	4.00	4.00
Microfine cellulose	6.40	0.00
PBY*	0.00	25.40
Mineral mix	3.50	3.50
Vitamin mix	1.00	1.00
L-Cystine	0.18	0.18
Choline bitartrate	0.25	0.25
Energy density (kcal/g)	3.37	3.19

*Centesimal composition and digestible content of carbohydrate. inulin and FOS on PBY (100 g of product): fructose: 9.4 g; glucose: 6.45 g; sucrose: 3.05 g; FOS: 17.65 g; inulin: 5.95; total carbohydrate: 42.49 g; fibers: 1.64 g; humidity: 37.20 g; ashes: 1.55 g; lipids: 0.04 g; protein: 2.51 g.

3.1. Synbiotic reduces dietary intake and body weight without change the CFE

Dietary intake was lower in the KOSYN group at the 5th, 6th and

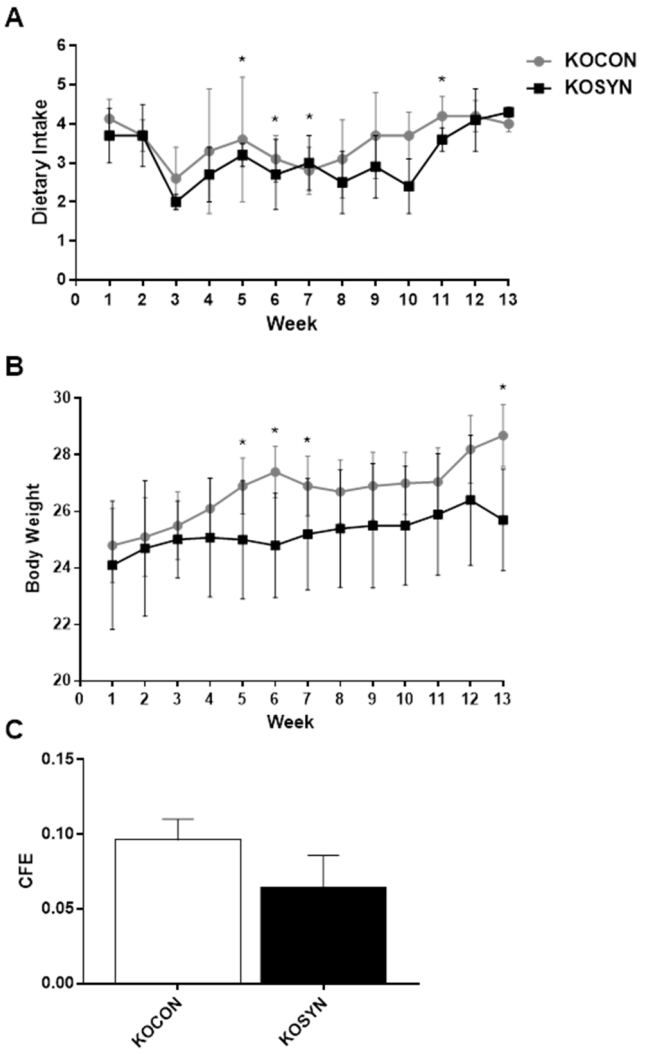

Fig. 2. Effect of synbiotic on (A) dietary intake, (B) body weight and (C) food efficiency (CFE) in colitis-associated carcinogenesis model. *p-value lesser than 0.05 using Student's *t*-test between control (KOCON) and synbiotic-treated group (KOSYN).

11th experimental weeks (Fig. 2A). Consequently, a reduction in the average body weight was observed in this group (Fig. 2B). Only at the 7th week, the ingestion of the KOSYN group increased without, however, promoting weight gain. We point out that at the 3rd experimental week, when the carcinogenesis induction protocol with DMH was started, the lowest mean food intake was observed in both groups. There was no significant difference on the CFE (Fig. 2C).

The weight of the liver (1.17 g ± 0.03 × 1.21 g ± 0.03; p = 0.531), the weight of the colon (0.22 g ± 0.01 × 0.25 g ± 0.01; p = 0.059) and the length of the colon (6.42 cm ± 0.19 × 6.07 cm ± 0.22; p = 0.261) did not differ between the KOCON and KOSYN groups. On the other hand, the animals that received the synbiotic showed an increase in the weight of the cecum (0.26 g ± 0.02 × 0.44 g ± 0.03; p = 0.000).

3.2. Synbiotic does not alter the biomarkers of liver function and albumin

Serum biomarkers were evaluated in order to verify alterations in kidney function, and albumin production. No change was observed between the groups KOCON and KOSYN in relation to serum levels of urea (31.90 mg ± 1.43 × 40.47 mg ± 1.17; p = 0.460), creatinine (0.31 mg ± 0.01 × 0.30 mg ± 0.01; p = 0.673), and albumin (2.37 g ± 0.14 × 2.58 g ± 0.04; p = 0.168).

3.3. Synbiotic attenuates the manifestations of colitis in CAC model

The experimental groups showed significant differences in the symptomatic parameters of colitis (% of weight loss, stool consistency and presence of visible blood in the stool) only in the seventh experimental week (Fig. 3). The mortality rate was 20% in the KOCON group and 10% in the KOSYN group. Two animals in the KOSYN group showed rectal prolapse and were excluded.

3.4. Synbiotic alters colonic morphometry and improves histopathological score

The use of the synbiotic caused changes in layers thickness that make up the colonic tissue. An increase was observed in the crypts depth and in the thickness of the external muscular layer (Fig. 4A and D), which, besides the trophic effect of the synbiotic, demonstrates the possible benefit regarding the integrity of the intestinal barrier. Measurements of the colon layers are shown in Fig. 4E.

The total histopathological score, calculated based on the cumulative scores of the crypts damage parameters, the presence of inflammatory infiltrate and the infiltration depth, was significantly higher in the KOCON group (Fig. 5A). Similarly, when the aforementioned parameters were evaluated in isolation, a higher score was also observed in the KOCON group (Fig. 5B), with emphasis on the presence of damaged crypts and infiltration of immune cells involving the submucosa and the muscular layer (Fig. 5C).

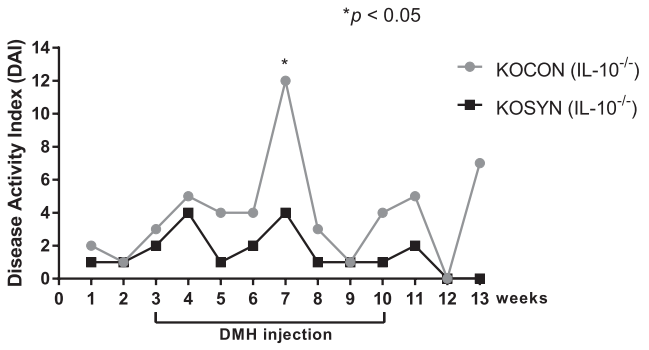

Fig. 3. Effect of synbiotic on disease activity index (DAI) in colitis-associated carcinogenesis model. *p-value lesser than 0.05 using Student's *t*-test between control (KOCON) and synbiotic-treated group (KOSYN).

3.5. Synbiotic positively modulates hepatic antioxidants enzymes expression

The effects of supplementation with the synbiotic on biomarkers of oxidative stress were evaluated by measuring the products of lipid and protein oxidation, malondialdehyde (MDA) and carbonyls protein (CP), respectively. The enzymes involved in the endogenous antioxidant defense system (SOD, CAT, and GST) were measured. The concentrations of the MDA and CP were not altered with the use of the synbiotic (Fig. 6A,B). However, according to our results, increased liver expression of SOD and CAT was observed, when compared to the KOCON (Fig. 6C,D). GST enzyme expression has not been altered (Fig. 6E).

3.6. Bioinformatics analysis and microbiota profiling

Conducting a deep amplicon sequencing of the V4-V6 hypervariable regions of the 16S rRNA gene, a total of 222,228 to 328,252 sequences with a length of 100 to 301 bp were obtained. After the removal of low quality and chimeric sequences, a total of 161,035 to 237,864 high-quality sequences, with an average of 188,280 sequences for each sample that were assigned to 2,415 OTUs (≥97% similarity). The number of OTU per sample ranged from 124 to 306.

The global fecal bacterial community structures of animals fed with synbiotic before and after colon carcinogenesis induction with DMH were assessed by the multidimensional scaling (MDS). As depicted (Fig. 7A), scatter plot of the MDS revealed that KOSYN groups did not clustering separated from KOCON group.

Alpha diversities were compared among the groups by Shannon and Simpson indices, whereas Chao 1 index was used to evaluate bacterial richness. There was no statistically significant difference in terms of bacterial diversity among the groups before or after synbiotic use (Fig. 7B,C). With regards to the bacterial richness, also there was no significant difference among the groups (Fig. 7D).

The relative distributions of bacteria at the phylum, family and genus level identified by 16S rRNA gene amplicon sequencing are depicted in Fig. 8. The most prevalent bacterial phyla among all groups were Patescibacteria (68.2%), Proteobacteria (14.2%), Firmicutes (11.0%), Actinobacteria (4.8%), and Bacteroidetes (1.5%).

In the intra-group comparison (t0 × t1), a reduction in the phylum Patescibacteria in the KOSYN group (1.2-fold) and an increase in the KOCON group (1.3-fold) were observed. Similarly, the phylum Firmicutes decreased in the KOSYN group (1.6-fold) and increased in the KOCON group (2.1-fold). On the other hand, there was an increase in Proteobacteria after the synbiotic use (2.7-fold) and a reduction in the control group (3.8-fold). The use of the synbiotic also resulted in a reduction in the relative abundance of the phylum Bacteroidetes for both groups (KOSYN = 2.0-fold; KONCON = 1.9-fold) and a concomitant increase in Actinobacteria (KOSYN = 2.9-fold; KONCON = 0.1-fold).

At the family level, 10 bacterial taxa were identified in total with 4 families accounted for more than 90% of the relative abundance in all groups. Among them, the family Saccharimonadaceae was the most abundant between all groups and in both times (*t0 × t1*). Regarding microbial changes at the family level before and after the intervention (t0 × t1), there was a Saccharimonadaceae reduction in the KOSYN group (1.2-fold) and an increase in the KOCON group (1.3-fold), following the same profile of the corresponding phylum (phylum Patescibacteria). Two families of the phylum Proteobacteria were identified: Desulfovibrionaceae and Legionellaceae. In both families, an increase in relative abundance in the KOSYN group (2.7-fold and 0.5-fold, respectively) and a reduction in the KOCON group (3.9-fold and 1.7-fold) were identified after intervention. The Ruminococcaceae, Lachnospiraceae and Erysipelotrichaceae families, belonging to the phylum Firmicutes, presented different profiles of changes compared to time zero. There was a Ruminococcaceae reduction in the KOSYN group (3.3-fold) and an increase in the KOCON group (6.6-fold); a

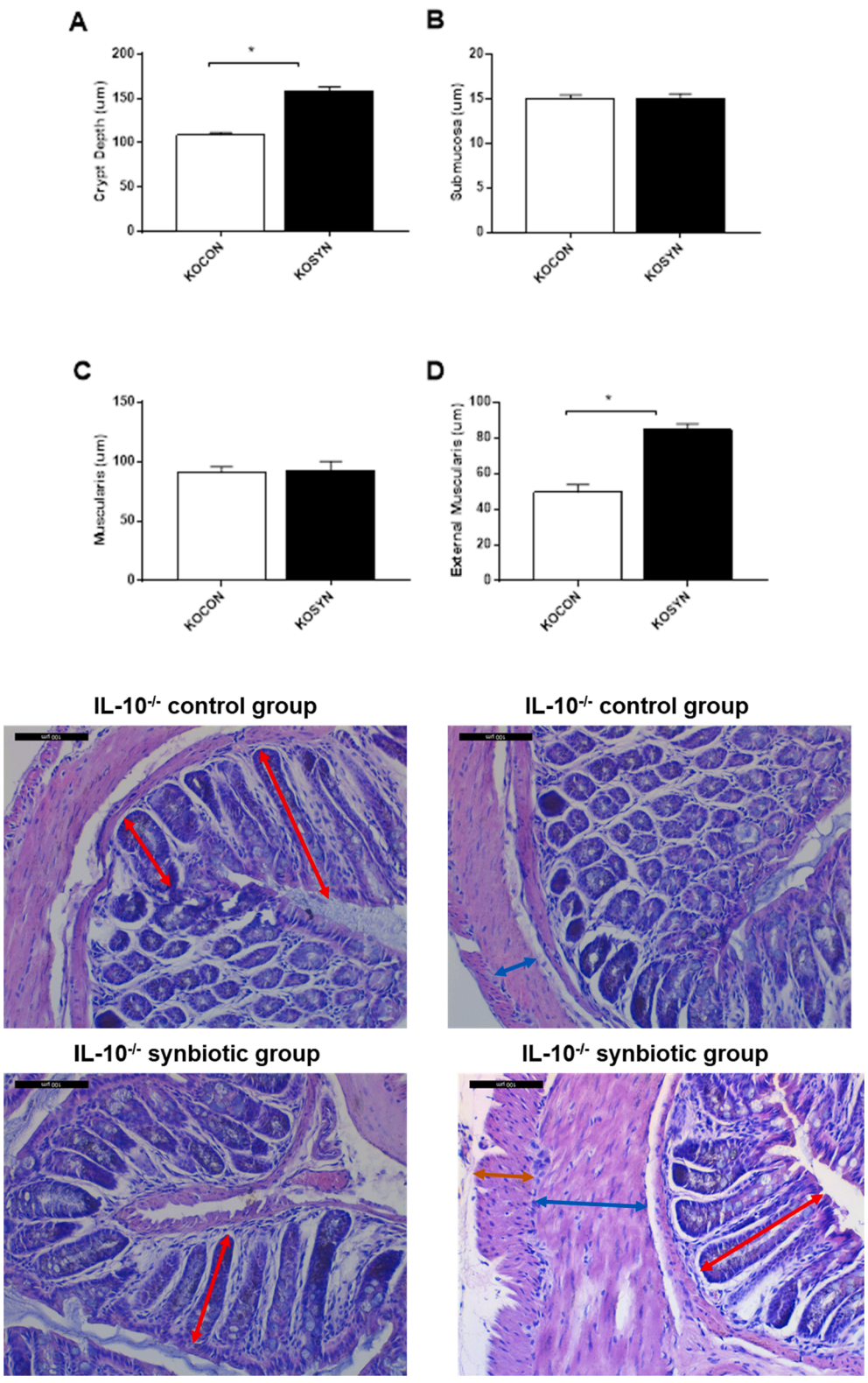

Fig. 4. Effect of synbiotic on intestinal morphometry in colitis-associated carcinogenesis model. (A) crypt depth, (B) submucosa, (C) muscularis, (D) external muscularis, and (E) illustrative photomicrography of the intestinal morphometry. Red arrow: measure of crypt depth; blue arrow: muscularis layer; orange arrow: external muscularis layer (scale bars, 100 μm). *p-value lesser than 0.05 using Student's *t*-test between control (KOCON) and synbiotic-treated group (KOSYN). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Lachnospiraceae reduction in both groups (KOSYN = 2.9-fold; KONCON = 1.3-fold); and an Erysipelotrichaceae increase in the KOSYN group (2.7-fold) and reduction in the KOCON group (3.9-fold).

In total, 14 bacterial taxa were assigned to the genus level. After defining the most abundant genera (relative abundance higher than 4%), 4 OTUs accounted 93.1% of the total sequences of groups. Candidatus Saccharimonas was the most abundant genus in all groups. The intra-group (*t0* × *t1*) comparison revealed a less abundance of Candidatus Saccharimonas (1.2-fold; $p = 0.002$), Ruminococcaceae_UCG-014 (3.5-fold; $p < 0.0001$), Aloprevotella (1.1-fold), Lachnospiraceae_UCG-006 (0.2-fold), and Tyzzerella_3 (0.4-fold) in KOSYN. In contrast, there was an increase of Coriobacteriaceae_UCG-002 (2.6-fold), Dubosiella (1.9-fold), Legionella (0,4-fold), and Olsenella (2.7-fold).

In KOCON*t1* group, a decrease in the relative abundance of

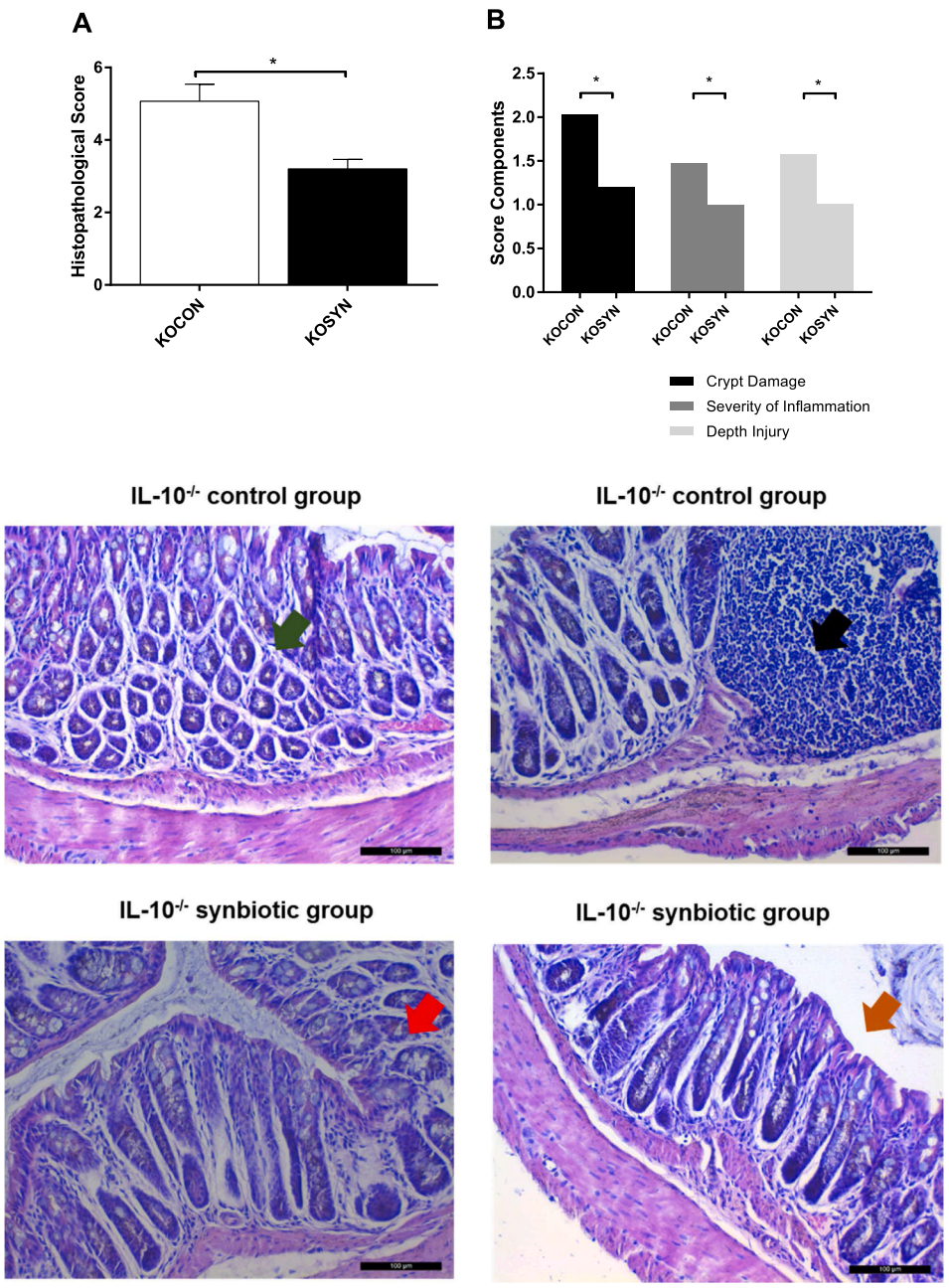

Fig. 5. Effect of synbiotic on histopathological score in colitis-associated carcinogenesis model. (A) histopathological score, (B) components of histopathological score, and (C) illustrative photomicrography of the colon of IL-10-deficient mice, stained with Hematoxylin-Eosin (HE). Orange arrow: preserved crypts and epithelium; black arrow: infiltration in the submucosa; red arrow: preserved colonic crypts and damaged crypt transition area; green arrow: extensive area of damaged crypts (scale bars, 100 μm). *p-value lesser than 0.05 using Student's *t*-test between control (KOCON) and synbiotic-treated group (KOSYN). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Desulfovibrio (6.2-fold; $p = 0.003$), Lacchnoclostridium (3-fold; $p = 0.043$), Acetatifactor (1.4-fold), Lachnospiraceae_NK4A136-group (2.2-fold), Legionella (1.7-fold), and Rikenellaceae_RC9-group (1.9-fold) was observed. There was an increase of Candidatus Saccharimonas (1.4-fold), Lachnospiraceae_UCG-006 (27.9-fold) and Olsenella (0.2-fold).

The comparison between groups after intervention (*t1*) revealed a significant difference ($p < 0.0001$) in abundance of Desulfovibrio genus between KOSYN (17%) and KOCON (3%) groups.

3.7. Synbiotic increases the fecal short-chain fatty acids

Intestinal bacteria activity was evaluated by fecal SCFA concentration. The KOSYN group presented higher levels of acetic ($p = 0.010$), propionic ($p = 0.013$), butyric ($p = 0.001$), and total fatty acids ($p = 0.009$) compared to the KOCON group (Table 2).

4. Discussion

Chronic intestinal inflammation that occurs in IBD induces increased mucosal permeability and persistent damage throughout the gastrointestinal tract, playing an important role in the development of CRC (McConnell & Yang, 2009). The manipulation of the intestinal microbiota with probiotics and prebiotics use has been shown to be effective in the regulation of intestinal homeostasis, by suppressing the growth of pathogenic bacteria and the production of beneficial metabolites (Israel, 2000).

The present study evaluated the influence of the synbiotic VSL#3® + PBY in a CAC model. In general, the use of the synbiotic showed benefits, such as the attenuation of CAC manifestations in the seventh experimental week, improvement of the histopathological score and intestinal morphometry, increased expression of antioxidant enzymes and concentration of SCFA, and alteration in the general composition of the intestinal microbiota.

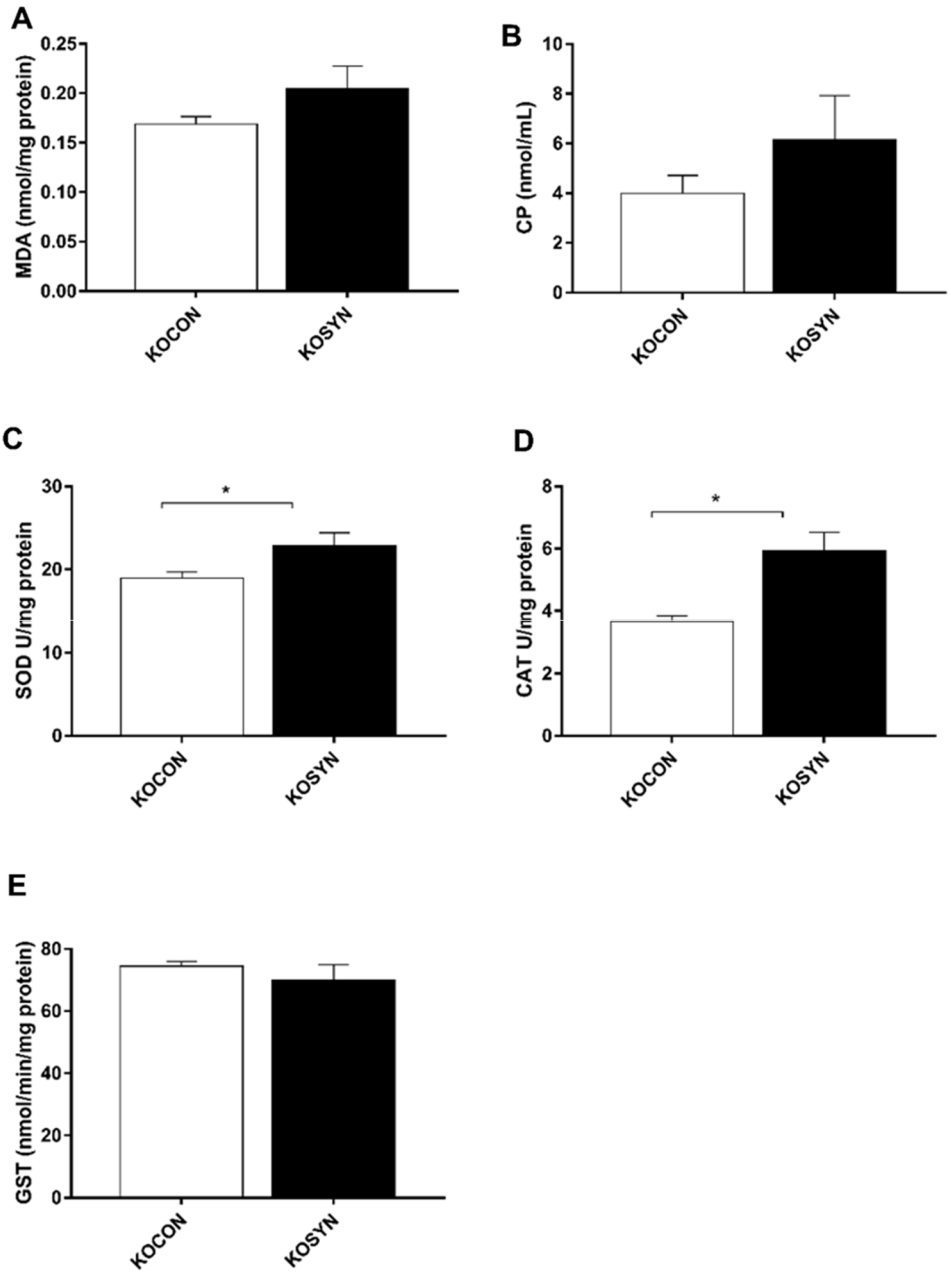

Fig. 6. Effect of synbiotic on liver concentrations of (A) MDA, malondyaldeide, and (B) CP, carbonylated protein, and on antioxidants enzymes expression (C) SOD, superoxide dismutase, (D) CAT, catalase, and (E) GST, glutathione-S-transferase in colitis-associated carcinogenesis model. *p- value lesser than 0.05 using Student's *t*-test between control (KOCON) and synbiotic-treated group (KOSYN).

This is a prevention study, therefore, the synbiotic was administered prophylactically, 3 weeks before the induction of preneoplastic lesions. The pre-treatment period is variable in the literature, and studies were found that started the intervention 1 week before or even months before the induction of injury. It is expected that the prophylactic use of synbiotic is able to modulate the intestinal microenvironment, altering the responses of the host to inflammatory and carcinogenic stimuli. Modulation of the composition and metabolism of the intestinal microbiota, with consequent improvement of the innate immune system and intestinal barrier function, is the key mechanism of synbiotic action (Appleyard, 2011; Bassaganya-Riera, Viladomiu, Pedragosa, De Simone, & Hontecillas, 2012; Chung et al., 2017; Fong, Li, & Yu, 2020; Leu, Hu, Brown, Woodman, & Young, 2010).

Studies using the CAC model show contradictory results in relation to the weight gain in animals, in which some authors identify weight gain in groups that receive synbiotic (Kumar, Singh, & Sinha, 2010; Urbanska, Bhathena, Cherif, & Prakash, 2012) and others find no difference (Chang, Shim, Cha, Reaney, & Chee, 2012; Leu et al., 2010). In our study, a lower average body weight was observed in the KOSYN group in some weeks, possibly due to the lower intake of the PBY diet, which, being rich in soluble fibers, promotes greater satiety. A similar result was observed in the study by De Nadai Marcon et al. (2019), in which mice fed a diet supplemented with PBY (6% FOS + inulin) had lower food intake. In addition, it is known that SCFA (identified in higher concentrations in the KOSYN group), can influence satiety mechanisms through the production of leptin (Xiong, 2004). However, it is worth mentioning that there was no significant difference in the coefficient of feeding efficiency (CFE) final average between the groups.

The synbiotic administration significantly reduced the DAI score only at the 7th experimental week, even though the mean values of the

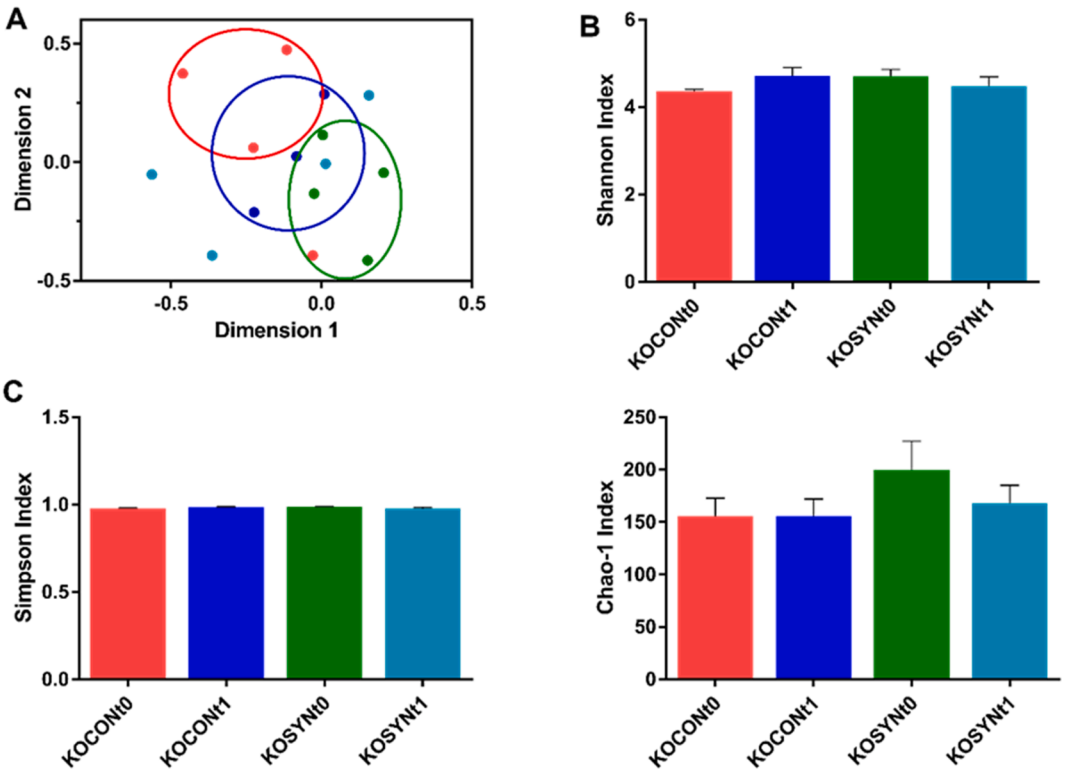

Fig. 7. Microbiome characterization before (*t0*) and after (*t1*) using the synbiotic in colitis-associated carcinogenesis model. (A) Multidimensional scaling (MDS), (B) Shannon Index, (C) Simpson Index, and (D) Chao-1 Index. Red and blue colors represent the control group before and after treatment, respectively. Green and cyan colors represent the synbiotic group before and after the treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

score have remained lower in almost every period evaluated. It is worth mentioning that the presence of visible blood in the stool was identified exclusively in the KOCON group (data not shown). In a similar study, Shinde et al. (2020) found that the synbiotic administration was able to reduce the DAI over the entire experimental period, which, however, was considerably lower than in our study (14 days). The short period of exposure to the drug that induces intestinal inflammation may explain, at least partially, the differences observed.

Histopathological evaluation also showed a beneficial result with the use of synbiotic. Greater preservation of the intestinal crypts architecture was observed, with limited damage to the epithelial surface, compared to the KOCON group. In addition, the areas of inflammatory infiltrate were more extensive in the control group, as well as the infiltration depth, which reached areas of the submucosa and mucosa. The benefits of using the probiotic VSL#3® alone in reducing chronic inflammation scores had already been demonstrated in IL-10$^{-/-}$ mice induced to colorectal carcinogenesis and inflammatory diseases such as colitis and ileitis (Fitzpatrick, 2007; López-Posadas, 2011; Pagnini, 2010; Reiff, 2009; Soo, 2008).

Similarly, the effect of isolated dietary supplementation of PBY (6% FOS and inulin) in the treatment of precursor lesions of CRC in mice has been previously assessed (De Nadai Marcon et al., 2019). The authors observed an increase in regulatory T cells and downregulation in the transcription factor RORγt expression in the colon of the animals, suggesting the potential of PBY in improving the anti-inflammatory and immune response.

One of the possible mechanisms attributed to the synbiotic in the control of CAC is the ability to stimulate the endogenous antioxidant defenses, that can mitigate the damage caused by the chemical carcinogen and the effects of intestinal inflammation on the liver.

The biotransformation of DMH into its active metabolite occurs predominantly in the liver, with formation of a large amount of free radicals occurs, involved in the genesis and progression of intestinal diseases (Jackson, O'Connor, Cooper, Margison, & Povey, 2003). Additionally, dysbiosis and increased intestinal permeability will result in bacterial products in the portal blood, and the cells of the liver will be first to be exposed. Liver cells have receptors, such as TLR 4, in which the presumed ligand is a bacterial product. The LPS produced by gram negative bacteria, for example, is a ligand for TLR4 associated with liver injury (Brenner, Paik, & Schnabl, 2015). Thus, the determination of the antioxidant enzyme expression aimed to assess whether the changes in the gut microenvironment triggered by the synbiotic could minimize the oxidative stress induced by DMH/intestinal inflammation on the liver.

The antioxidant potential of probiotics has been reported for decades (Lin & Yen, 1999) and is attributed to its ability to eliminate and inhibit the formation of free radicals in the intestine, chelate metal ions, inhibit the autoxidation of ascorbate (Azcárate-Peril, Sikes, & Bruno-Bárcena, 2011), and induce the transcription of genes involved in the synthesis of glutathione by the intestinal mucosa (Lutgendorff, 2009) and in pancreatic cells (Lutgendorff, 2008).

In addition, yacon has in its composition flavonoids and phenolic compounds that protect biomolecules against damage caused by free radicals (Simonovska, Vovk, Andrensek, Valentová, & Ulrichová, 2003). Protein and lipid oxidation products were identified in both groups, which was expected since they were IL-10$^{-/-}$ mice that received injections with the DMH carcinogen. Although the concentrations of these products did not differ between groups, there was an increase in the expression of the antioxidant enzymes SOD and CAT in the KOSYN group.

Although the indexes of richness and diversity have not changed significantly with the use of the synbiotic, the relative abundance of some microorganisms at the end of the intervention (*t1*) differed significantly from the initial profile (*t0*) (intra-group analysis). Studies have indicated that perturbed gut microbiota, for example, reduced

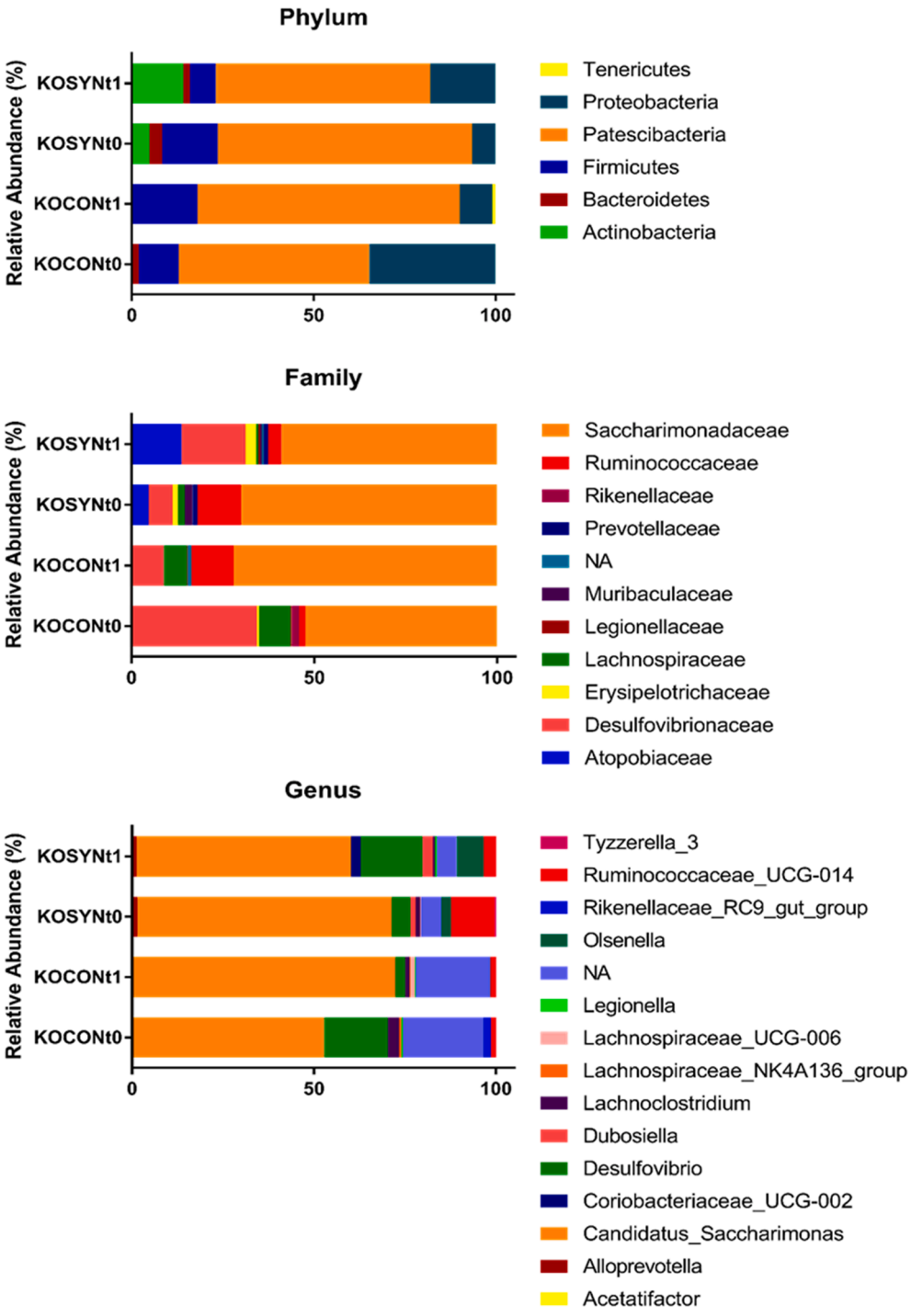

Fig. 8. Relative abundance (%) of Phylum, Family and Genus levels before (*t0*) and after (*t1*) use the synbiotic in colitis-associated carcinogenesis model.

Table 2
Effect of synbiotic on the production of faecal short-chain fatty acids in colitis-associated colorectal cancer model.

SCFA (*μmol/g feces*)	KOCON	KOSYN	*p*
Acetic acid	137.6 ± 52.4	561.2 ± 180.1	0.010*
Propionic acid	2.2 ± 2.2	9.9 ± 5.93	0.013*
Butyric acid	6.3 ± 2.8	12.6 ± 2.3	0.001*
Total SCFA	146.2 ± 56.2	583.7 ± 179.0	0.009*

SCFA: short-chain fatty acids. Data are expressed as the mean ± SD (n = 7 or 8/group). Statistical difference between groups were analyzed by Student's t-test with $p < 0.05$. KOCON. control diet; KOSYN. synbiotic diet.

microbial diversity is associated with several diseases including gastrointestinal disorders (Gong, Gong, Wang, Yu, & Dong, 2016; Mosca, Leclerc, & Hugot, 2016). Changes in the indexes of richness and diversity with the use of probiotics and synbiotics may not be observed, although there are variations in the abundance of bacterial species (Jacouton, Chain, Sokol, Langella, & Bermúdez-Humarán, 2017).

Significant differences were observed for Candidatus Saccharimonas (KOSYN*t0* × KOSYN*t1*), Desulfovibrio (KOCON*t0* × KOCON*t1* and KOCON*t1* × KOSYN*t1*), Lacchnoclostridium (KOCON*t0* × KOCON*t1*) and Ruminococcaceae (KOCON*t0* × KOCON*t1* and KOSYN*t0* × KOSYN*t1*) after the intervention. Therefore, it is likely that the results obtained cannot be attributed solely to changes in the composition of the

intestinal microbiota.

Furthermore, we highlight that the microbiota of the experimental model used was characterized by a restricted biodiversity, with a limited number of bacterial genera representing the total microbial community. This finding has been consistently demonstrated in cases of cancer in experimental models and in humans, and in IBD patients (Richard et al., 2018; Saraggi et al., 2017; Sartor & Wu, 2017).

The evidence on the microbiota profile in IBD is limited, especially when associated with carcinogenesis. Some studies have shown a reduction in the phylum Firmicutes (Roseburia and Ruminococcaceae) and in the Fusobacterium genus; an increase in classes of the phylum Proteobacteria, such as Gammaproteobacteria and Deltaproteobacteria; and an increase of the Enterobacteriacae family, of the genus Sphingomonas, and of the species Ruminococcus gnavus (Morgan, 2012; Richard et al., 2018).

Candidatus Saccharimonas was the main genus identified in our study, in both groups (KOCON*t0* = 52.4%, KOCON*t1* = 72.0%, KOSYN*t0* = 69.9%, KOSYN*t1* = 58.9%). There is little data in the literature concerning this genus, and its genome was sequenced relatively recently (Abrams, 2012; Albertsen et al., 2013). This microorganism belongs to the superphylum Patescibacteria, formerly known as TM7. It is an important member of the oral microbiome (Bor, Bedree, Shi, McLean, & He, 2019), and is also found throughout the gastrointestinal tract, skin and genital tract (Dewhirst, 2010; Fredricks, Fiedler, & Marrazzo, 2005; Grice & Segre, 2011).

Although incipient, human and animal studies have indicated that oral microbiota can translocate into the intestine and alter its microbiota and possibly the immune defense (Olsen & Yamazaki, 2019). Association of oral microbiota with the CRC was previously demonstrated (Flemer, Warren, & Barrett, 2018). Several OTUs were shared between oral swabs and stool samples of patients with CRC, amongst them *Parvimonas micra*, *Peptostreptococcus stomatitis* and *Fusobacterium nucleatum,* a species commonly enriched in tumor tissues (Sun et al., 2019). We speculate whether the oral translocation of Candidatus Saccharimonas could contribute to the high abundance of this genus in the intestinal microbiota and for intestinal inflammation.

The expansion of the Candidatus Saccharimonas has been associated with inflammatory disease such as gingivitis and other periodontal dysfunctions (Camelo-Castillo, 2015). Apparently, can also play a role in other clinical conditions, having an increase identified, for example, in a study of obesity induced by high-fat diet (Lin, An, Hao, Wang, & Tang, 2016).

The relationship between Candidatus Saccharimonas, IBD, CRC or CAC has not been established previously, however, it is a microorganism globally distributed and often associated with inflammatory mucosal diseases in humans. Its ability to suppress the production of TNF in macrophages has been demonstrated, suggesting a potential capacity for immune suppression (He et al., 2015). We emphasize that the use of the synbiotic reduced the abundance of Candidatus Saccharimonas by 1.2-fold, while in the KOCON group there was an increase of 1.4-fold. It is speculated whether Candidatus Saccharimonas could directly influence the inflammatory response in the initiation and progression of CAC or have its growth favored by the presence of an inflammatory condition.

SCFA produced in the colon are active metabolites that act to reduce inflammatory mediators and increase the function of the intestinal barrier (Fernández et al., 2016). These are important indicators of dysbiosis in IBD and CRC, since the depletion of SCFA-producing bacteria in these situations is common (Levy, Thaiss, & Elinav, 2016). Low concentrations of SCFA, especially butyrate, have been shown to have a direct effect on intestinal homeostasis, resulting in intestinal barrier defects and in aberrant immune response activation (Geier, Butler, & Howarth, 2006; van der Beek, Dejong, Troost, Masclee, & Lenaerts, 2017).

The most abundant SCFA in the colon are acetate, propionate and butyrate, produced by the intestinal microbiota through the fermentation of non-digestible fibers, with butyrate being the preferred energy source for colonocytes (Koh, De Vadder, Kovatcheva-Datchary, & Bäckhed, 2016). In this study, it was observed that the synergistic administration of the probiotic VSL#3® and the prebiotic PBY was able to increase the production of SCFA. These results possibly mediated beneficial trophic effects on CAC, resulting in improved colon morphometry, integrity of the barrier with increased intestinal crypts depth, increased cecum weight and attenuation of CAC manifestations. Similar results were observed in the study by Shinde et al. (2020), with the use of the synbiotic composed of the prebiotic based on resistant starch and the probiotic *Bacillus coagulans* MTCC 5856 in an IBD model.

The increase in the acetate and propionate metabolites, observed with the synbiotic supplementation, also benefits the integrity of the intestinal barrier through modulation of the immune response and binding to G protein-coupled receptors, such as GPR43 and GPR109A (Tedelind, Westberg, Kjerrulf, & Vidal, 2007). Thus, the joint offer of fermentable substrates, such as FOS and inulin, which directly or indirectly influence the production of SCFA and probiotic bacteria, can be an advantageous strategy in the progression of CAC.

In the present study, genera producing SCFA were identified, such as Dubosiella, Ruminococcaceae_UCG-014 (ordem Clostridia) and Lachnoclostridium (Gutiérrez & Garrido, 2019; Li et al., 2020; Takahashi et al., 2016), that can justify the increase in SCFA in the synbiotic group. Some genus of Ruminococcaceae can consume hydrogen to produce acetate, which is subsequently used by other bacteria to produce butyrate (Wang, Hua, et al., 2019; Wang, Li, et al., 2019). Moreover, the prebiotics are being researched to stimulate beneficial bacterial species, such as butyrate producers. Of particular interest are prebiotics that cause a bifidogenic and butyrogenic effect, such as inulin-type fructans (Rivière, Gagnon, Weckx, Roy, & DeVuyst, 2015). The increase in SCFA after administration of the yacon-based product (PBY), rich in fructooligosaccharides and inulin, has been previously demonstrated (De Nadai Marcon et al., 2019).

5. Conclusion

The synbiotic combination of VSL#3® and PBY showed potential benefits in the CAC model. Although we have identified changes in the microbial community associated with the use of the synbiotic, the causal relationship between these changes and the attenuation of CAC manifestations cannot be directly established at this time. The predominance of the Candidatus Saccharimonas genus, previously related to inflammatory mucosal diseases, may be a key component of CAC that needs to be further investigated. The use of the synbiotic reduced the abundance of the Candidatus Saccharimonas genus in the treated group when compared to the control one. It is speculated whether Candidatus Saccharimonas could directly influence the inflammatory response in the initiation and progression of CAC. The increase in the antioxidant enzymes expression and the concentration of SCFA in the synbiotic group may also partially explain our findings. Together, these changes contributed especially to the trophism and improvement of the intestinal mucosa integrity.

6. Ethics statement

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 of the European Community (Directive 2010/63/EU).

CRediT authorship contribution statement

Bruna Cristina dos Santos Cruz: Conceptualization, Methodology, Formal analysis, Investigation, Writing - original draft. **Lisiane Lopes da Conceição:** Investigation, Formal analysis, Writing - review & editing. **Tiago Antônio de Oliveira Mendes:** Methodology, Formal analysis, Writing - review & editing. **Célia Lúcia de Luces Fortes Ferreira:**

Conceptualization, Methodology. **Reggiani Vilela Gonçalves:** Conceptualization, Methodology, Formal analysis. **Maria do Carmo Gouveia Peluzio: . :** Conceptualization, Methodology, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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