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EFFECTIVENESS OF A COMMERCIAL SYMBIOTIC IN AN EXPERIMENTAL MODEL OF ULCERATIVE COLITIS

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ABSTRACT

To verify the effects of a commercial symbiotic in an experimental model of ulcerative colitis. For this, male Wistar rats (5 weeks old) were used. The induction of Ulcerative Colitis was performed via intracolonic route with trinitrobenzenesulfonic acid (TNBS) (10mg TNBS/0.75ml ethanol/50%). The animals were distributed in: sham, treated with ultrapure water (CT); colitis, treated with ultrapure water (TNBS CT); colitis, treated with mesalazine (TNBS MES); and colitis, treated with the symbiotic (TNBS SYM). The following were evaluated: weight, food intake, and the food efficiency coefficient (FEC). After 7 days, euthanasia was performed by removing tissue from the colon for macroscopic and microscopic analysis. After treatment, the TNBS SYM group presented similar body weight to the CT group, but with lower food intake ($p<0.001$), as well as lower FEC. In the macroscopic analysis, the TNBS SYM group presented lower signs of inflammation and extension of the ulcerations, however without statistical difference in the microscopic analysis. It was observed that under the experimental conditions of the study, the use of the symbiotic maintained body weight and macroscopically reduced the extent of ulceration and tissue aggression, with similar effect to drug use, however, without statistical difference in the microscopic evaluation.

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INTRODUCTION

Inflammatory bowel diseases are considered one of the major problems of the modern population, and although incidence varies considerably, a significant increase has occurred in the last decades, especially in industrialized countries (Wilson, 2001). Among the most common inflammatory bowel diseases, Crohn's disease and Ulcerative Colitis represent a serious health problem, since they cause clinical forms of high severity, with important repercussions on the quality of life of the patients (Ferraz, 2016).

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Ulcerative colitis is manifested by a diffuse inflammation of the mucosa, reaching the terminal region of the large intestine and extending to the rectum, presenting variable extent and severity (Fell, 2011). Patients suffering from this disease have weight loss, diarrhea with blood and/or mucus, fever, abdominal pain, and shortening of the colon. Another factor associated with the development of this pathogenesis would be the increase of the immune response against the commensal microbiota in genetically susceptible individuals (Ferraz, 2016). The immunological factor responsible for loss of recognition of luminal antigens includes an overactivity of effector lymphocytes and proinflammatory cytokines, and failure of regulatory lymphocytes and anti-inflammatory cytokines in controlling inflammation (Molodecky *et al.*, 2011). Currently, there is a range of pharmacological

treatments for inflammatory bowel diseases, such as aminosalicylates, immunosuppressants, and immunomodulators (Yamomoto-Furusho, 2007). The aminosalicylates (salicylic derivatives) include sulfasalazine and mesalazine, responsible for modulating the secretion of proinflammatory cytokines, thus inhibiting the production of leukotrienes and prostaglandins, activating the peroxisome proliferator-activated receptors (PPAR) involved in the control of inflammation, in cell proliferation, and in apoptosis (Peppercorn, 1994). Notwithstanding, there are several side effects, such as headaches, nausea, anorexia, allergic reactions, fever, hemolysis, neutropenia, and anemia (Ransford and Langman, 2002), which may also evolve to hypersensitivity reactions, among them hepatitis, pancreatitis, pneumonia, pericarditis, and peripheral neuropathies (Alonso *et al*., 2009). Glucocorticoids or corticosteroids, which are used in severe cases of ulcerative colitis, act on intracellular receptors by controlling gene transcription, promoting the formation of dimers that migrate to the cell nucleus, binding to DNA (Rogler, 2010). These drugs also affect the function of various cells involved in the inflammatory process (cytokines, chemokines, kinins, and their respective receptors), causing cell adhesion such as nitric oxide and cyclooxygenase to be impaired, decreasing the recruitment of macrophages, and the production of IL-1, IL-2, and TNF-α (Witaicenis, 2010). Hence, despite the advances in the development of more effective treatments and medications, there is still an important side effect, becoming a factor that hinders adherence to treatment (McLoughlin *et al*., 2017). According to a study by Rossi *et al*. (2016), manipulation of the intestinal microbiota has been shown to be an important strategy in the maintenance of colonic homeostasis, since adequate modification of the intestinal ecosystem is considered a viable and timely therapy for the treatment of inflammatory bowel diseases.

For this purpose, prebiotics and probiotics can be used. The former are foods that do not undergo digestion by the human gastrointestinal tract, which can stimulate the growth of some species of bacteria living in this environment, conferring a series of benefits to the organism (Kinross *et al*., 2013). On the other hand, probiotics are described as living microorganisms that, when administered in adequate amounts, provide advantages to the health of the host; the action of these products must be demonstrated for each strain. Among their effects, the following are highlighted: microbiota normalization, decreased intestinal permeability, protection against pathogenic invaders, aid in reestablishment after antibiotic therapy, and stimulation of the immune system (Denipote *et al*., 2010). In this sense, symbiotics are considered foods that have a combined probiotic and prebiotic formulation. Joint administration of a probiotic with a specific prebiotic may favor the development of probiotics, enhancing their survival and establishment in the gastrointestinal tract. This occurs through the selective stimulation of growth and metabolism activation of a limited number of health-promoting microorganisms, due to their substrate being available for fermentation (Park and Floch, 2007; Guarner *et al*., 2011).
For this reason, it is important to verify the effect of symbiotics in an experimental model of ulcerative colitis, to investigate new therapeutic approaches that do not present intense side effects.

MATERIALS AND METHODS

The research was carried out at the Central Bioterium of the Federal University of Mato Grosso do Sul (UFMS), Campo Grande, Mato Grosso do Sul, Brazil. The project was approved by the Ethics Committee on Animal Use (CEUA) of UFMS (Protocol No. 839/2017). Male Wistar rats (n=40), 5 weeks old (160-200g), were used. Prior to initiating the experiments, the animals were kept in a period of adaptation to the test environment for 5 days. The temperature was maintained around 22 °C ± 2 in a light-dark cycle of 12h, and the animals were fed commercial feed (Nuvital®) and filtered water *ad libitum*. The ulcerative colitis model was reproduced by intracolonic administration of trinitrobenzenesulfonic acid (TNBS) (Sigma® - St. Louis, MO) (10 mg of TNBS dissolved in a volume of 2 ml of 50% ethanol/water) (Hee *et al*., 2011). The animals were fasted for 12h and anesthetized with xylazine and ketamine (10mg/kg:75mg/kg); then, intracolonic injection was performed using a cannula of 8 cm in length and 0.76 mm in diameter (Chen *et al*., 2015; Choi *et al*., 2016). After 48 hours, the following were evaluated according to Gupta *et al*. (2015): weight loss, feces consistency, and blood in feces, which characterized the effective induction of experimental colitis.

With the induction of experimental colitis, the animals were redistributed in the experimental groups so that body weight did not present statistical difference in the comparison between groups, being: control, without colitis (sham) (n=10); TNBS colitis, treated by gavage with ultrapure water (1 ml) (n=10); TNBS colitis, treated by gavage with mesalazine (dose of 25 mg/kg) (n=10); and TNBS colitis, treated by gavage with commercial symbiotic (Simbioflora®) (Table 1) (1 ml of the product - 2g being diluted in 5 ml of ultrapure water) (n=10).
Using an electronic scale (Camry®), all animals had their food intake measured daily, with values presented in g/animal, as well as their body weight, whose values were expressed in grams. The food efficiency coefficient (FEC) was calculated by the equation: FEC = [weight gain (g)] / [food intake (g)] (Almeida *et al*., 2011). After 7 days of treatment, the animals were fasted for 12 hours and euthanized after xylazine and ketamine anesthesia (10mg/kg:75mg/kg), with confirmation of death in a CO_2 chamber.

Table 1. Composition of the symbiotic (Simbioflora®) after dilution with ultrapure water, supplied to the animals of the group with ulcerative colitis treated with the symbiotic (TNBS SYM) (n=10)

Strains	Quantity
Lactobacillus paracasei	10^9CFU
Lactobacillus rhamnosus	10^9CFU
Lactobacillus acidophilus	10^9CFU
Bifidobacterium lactis	10^9CFU
Fructooligosaccharides (FOS)	5.5g

Source: Simbioflora®.

The animals were opened and had their colon removed, which was also opened longitudinally and washed with saline for macroscopic evaluation, by means of scoring, according to the methodology of Bell *et al*. (1995). The colon pieces were then maintained in 10 ml of 10% formalin. For fixation, the specimens were dehydrated in ethanol and xylol batteries, embedded in paraffin, cut into a microtome section (5 µm thick each), stained with hematoxylin-eosin, and subjected to microscopic evaluation, according to the methodology adopted in the work of Arribas *et al*. (2010). For presentation of weight and food intake, the results were expressed as mean ± standard deviation. For multiple comparison of parametric results, Analysis of Variance (ANOVA) was used, followed by

Tukey's post-test. For the statistical analysis, the software Jandel Sigma-Stat, version 3.5 (Systal software, Inc., USA) was used. Kruskal-Wallis nonparametric test was used for the comparison of the experimental groups in the variables related to tissue histological analysis, followed by Dunn's post-test, since the data were ordinal, and the samples did not pass the Shapiro-Wilk normality test. Statistical analysis was performed using the statistical program SigmaPlot, version 12.5. In all analyses, the level of significance was 5%.

RESULTS

After the period of adaptation to the environment, the average daily food intake of the animals was evaluated throughout the experiment. It was observed that all groups with colitis had a reduction in food intake.

In the macroscopic evaluation (Figure 2), it was observed that although there were changes in the intestinal colon tissue of animals treated with the symbiotic, there was a lower mucosal involvement and a lower extent of ulceration when compared to the TNBS CT group, with similar visual effects to the TNBS MES group. The microscopic evaluation of colonic tissue (Figure 3) for the colitis groups showed ulcerative inflammatory process with variable extension in the intestinal wall layers, in addition to the presence of mitosis in the epithelium of the portions closest to the intestinal crypt lumen, decrease in the population of goblet cells, and luminal dilation of greater intensity. In the stroma corresponding to the ulcer, it was observed the formation of granulation tissue, consisting of fibrosis, reactive capillary vascular proliferation, and leukocyte infiltration with lymphocytes, neutrophils, and histiocytes.

Table 2. Weight prior to induction, at the start of treatment and at the end of treatment (g), and weight gain (g) of animals from the control group (CT) and the group with ulcerative colitis treated with ultrapure water (TNBS CT), with mesalazine (TNBS MES), and with the symbiotic (TNBS SYM)

Groups	Weight prior to induction (g)	Weight at the start of treatment (g)	Weight at the end of treatment (g)	Weight gain during treatment (g)
CT	199.4±18.4	258.5±12.1	269.8±17.9	12.5±9.5[b]
TNBS CT	187.6±15.3	154.0±21.8*	189.2±23.7**	37.2±19.3[a]
TNBS MES	204.4±15.8	189.5±14.4*	194.1±45.7***	12.9±-30.7
TNBS SYM	216.3±22.9	183.4±16.1*	210.2±57.7	28.7±47.4[a]

Values expressed as mean ± standard deviation. In the columns: * for p<0.05; ** for p<0.01; *** for p<0.001, relative to the control group (CT). In the lines: [a] for p<0.01; [b] for p<0.001, weight gain during treatment, relative to the weight at the end of treatment and the weight at the start of treatment. ANOVA followed by Tukey's post-test. n = 10.

Thus, the TNBS CT group and the TNBS SYM group presented a statistical difference in relation to the CT group (p<0.001), as well as the TNBS MES group (p<0.01) (Figure 1). When the weight of the animals was evaluated, these were distributed in their respective groups, with similar mean weight, without statistical difference between them (Table 2). Subsequently, colitis was induced, and at the time of confirmation of the disease, in addition to the presence of diarrhea and blood in the feces, the animals were weighed again, and the colitis groups presented weight loss, with statistical difference in relation to the control group (p<0.001). At the end of treatment, all groups had lower values in weight compared to the weight prior to induction, as can be seen in Table 2, except for the control group, without induction of colitis. However, the TNBS group treated with ultrapure water and with medication presented lower body weight when compared to the CT group.

On the other hand, the group treated with the symbiotic presented no statistical difference in body weight when compared to the CT group. When observing weight gain during the treatment, it was found that the control group maintained the weight, since they were adult animals, for which large variations in body weight as a function of age are not really expected. TNBS SYM presented an increase in body weight from the start of symbiotic supplementation, with a statistical difference (p<0.01), showing to be more effective than the group treated with medication, which presented weight gain, however without statistical difference when evaluating the weight before and after treatment. According to the food efficiency coefficient, TNBS SYM was the one that presented the greatest weight loss at the time of induction; with the treatment, it showed weight recovery even with lower food intake when compared to the other groups (Table 3), that is, it made better use of what was ingested.

In the evaluation of the morphological scores, it was observed that when assessing both the extent of mucosal ulceration and damage of the crypts and the infiltration and edema of the submucosal and muscular layers of the colon, the groups TNBS CT, TNBS MES, and TNBS SYM presented statistical difference, p<0.001, when compared to CT (Table 4), but there was no significant difference between the treated groups.

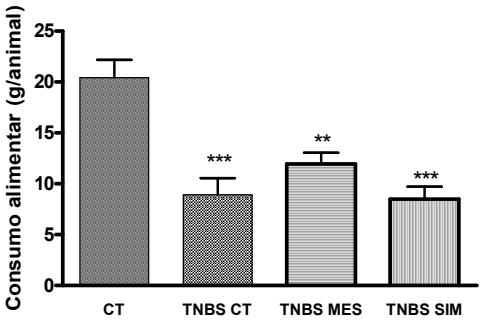

Figure 1 – Daily food intake (g/animal) of animals from the control group (CT) and the group with ulcerative colitis treated with ultrapure water (TNBS CT), with mesalazine (TNBS MES), and with the symbiotic (TNBS SYM). Values expressed as mean ± standard deviation. ** p < 0.01; *** p < 0.001. ANOVA followed by Tukey's post-test. n = 10.

Table 3 – Food efficiency coefficient (FEC) of animals from the control group (CT) and the group with ulcerative colitis treated with ultrapure water (TNBS CT), with mesalazine (TNBS MES), and with the symbiotic (TNBS SYM)

Groups	Food efficiency coefficient
CT	0.6±0.4
TNBS CT	4.2±0.9*
TNBS MES	1.2±0.8
TNBS SYM	3.6±0,2***

Values expressed as mean ± standard deviation. In the columns: * for p<0.05; *** for p<0.001, relative to the control group (CT). ANOVA followed by Tukey's post-test. n = 10.

Table 4. Comparison between the experimental groups in relation to the scores obtained in the tissue histological analysis

Variable	Experimental Group				P value
	CT	TNBS CT	TNBS MES	TNBS SYM	
		Mucosal ulceration			
Linear extension	0 (0 to 0)b	2 (1 to 2)a	2 (1 to 3)a	2 (1 to 2)a	<0.001
Vertical extension	0 (0 to 0)b	3 (2 to 4)a	3.5 (2 to 4)a	3 (2 to 4)a	<0.001
		Crypts			
Mitotic activity	0 (0 to 0)b	1 (0 to 2)a	1 (0 to 2)a	1 (0 to 2)a	<0.001
Glandular luminal dilation	0 (0 to 0)b	2 (1 to 3)a	1 (1 to 3)a	1 (0 to 3)a	<0.001
Depletion of goblet cells	0 (0 to 0)b	2 (1 to 3)a	1 (1 to 3)a	2 (0 to 2)a	<0.001
		Submucosa			
Polymorphonuclear cell infiltrate	0 (0 to 0)b	3 (2 to 3)a	2 (1 to 3)a	3 (1 to 3)a	<0.001
Mononuclear cell infiltrate	1 (1 to 1)b	3 (2 to 3)a	3 (2 to 3)a	3 (1 to 3)a	<0.001
Edema	0 (0 to 0)b	2 (2 to 3)a	3 (1 to 3)a	2.5 (1 to 3)a	<0.001
Vascularization	0 (0 to 0)b	3 (3 to 3)a	3 (1 to 3)a	3 (2 to 3)a	<0.001
		Muscle layer			
Polymorphonuclear cell infiltrate	0 (0 to 0)b	3 (1 to 3)a	2 (1 to 3)a	3 (1 to 3)a	<0.001
Mononuclear cell infiltrate	0 (0 to 0)b	3 (2 to 3)a	2 (1 to 3)a	3 (1 to 3)a	<0.001
Edema	0 (0 to 0)b	2 (1 to 3)a	2 (0 to 3)a	2.5 (1 to 3)a	<0.001
Serous infiltration	0 (0 to 0)b	3 (1 to 3)a	3 (1 to 3)a	3 (1 to 3)a	<0.001
Total Score	1 (1 to 1)b	31 (25 to 35)a	28.5 (17 to 35)a	32 (13 to 35)a	<0.001

Results are presented in median (minimum to maximum). P value in the Kruskal-Wallis test. Different letters in the line indicate difference between the experimental groups (Dunn's post-test, $p<0.05$). Mucosal ulceration - Linear extension: 0 = absent, 1 = <50% of the mucosal surface sampled, 2 = >50% of the mucosal surface sampled; Mucosal ulceration - Vertical extension: 0 = absent, 1 = mucosa, 2 = submucosa, 3 = muscle layer, 4 = serous (transmural); Crypts - Mitotic activity: 1 = lower third, 2 = middle third, 3 = upper third; Other variables: 0 = none, 1 = discrete, 2 = moderate, 3 = accentuated.

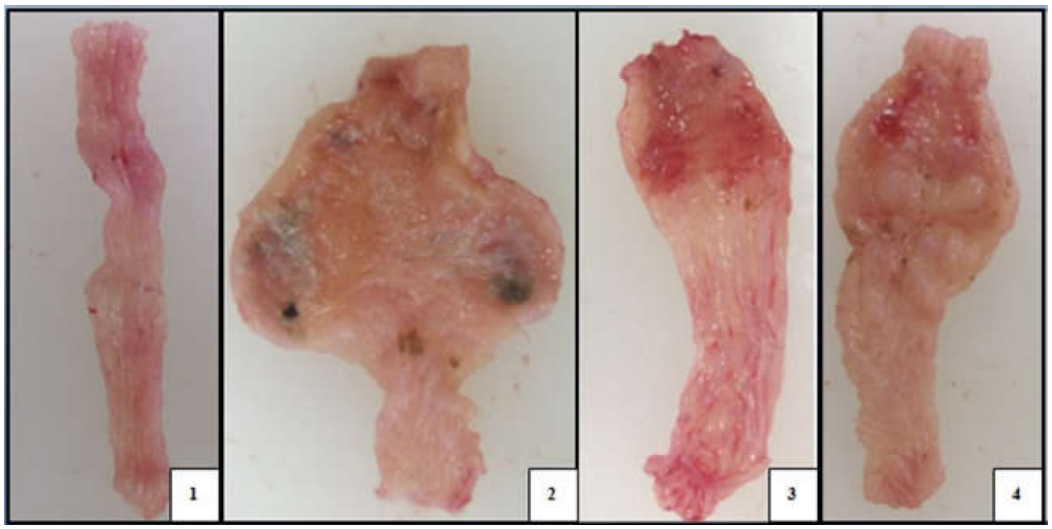

Figure 2 – Macroscopic analysis of the colon of animals from the groups: CT - without ulcerative colitis, treated with ultrapure water (1) / TNBS CT - with ulcerative colitis, treated with ultrapure water (2) / TNBS MES - with ulcerative colitis, treated with mesalazine) (3) / and TNBS SYM - with ulcerative colitis, treated with the symbiotic (4), for 7 days. 1) CT - compatible with normal intestinal colon tissue; 2) TNBS CT - presence of ulceration and dilatation; 3) TNBS MES - presence of inflammatory process with ulceration, dilatation, and irregularity with lower intensity; 4) TNBS SYM - presence of inflammatory process with ulceration, dilatation, and luminal irregularity with lower intensity

Notwithstanding, the variation of the total score of the group treated with the symbiotic (13-35) presented a behavior similar to that of the medication (17-35), showing that the symbiotic may have presented a protective effect on the mucosa of the intestinal colon.

DISCUSSION

There are currently a range of pharmacological treatments for inflammatory bowel diseases, including aminosalicylates, immunosuppressants and immunomodulators, glucocorticoids or corticosteroids, and anti-inflammatories (Yamomoto-Furusho, 2007). However, they have several side effects, such as headaches, nausea, anorexia, allergic reactions, fever, hemolysis, neutropenia, and anemia (Ransford and Langman, 2002), which may also evolve to hypersensitivity reactions, including hepatitis, pancreatitis, pneumonia, pericarditis, and peripheral neuropathies (Alonso *et al*., 2009).Thus, despite the advances in the development of more effective treatments and medications, there is still an important side effect, becoming a factor that hinders adherence to treatment (McLoughlin *et al*., 2017). Studies such as that by Rossi *et al*. (2016) argue that manipulation of the intestinal microbiota may be an important strategy in maintaining colonic homeostasis. Moreover, when providing a suitable modification, manipulation has been considered a viable and timely therapy for the treatment of inflammatory bowel diseases. In this sense, the symbiotic product selected in this study contains in its composition strains of the genera *Lactobacillus* and *Bifidobacterium*, in addition to fructooligosaccharides (FOS). It presents a great variety of species of bacteria capable of adhering, colonizing, and inducing effects on the human organism, such as in cases of individuals with acquired immunodeficiency syndrome,

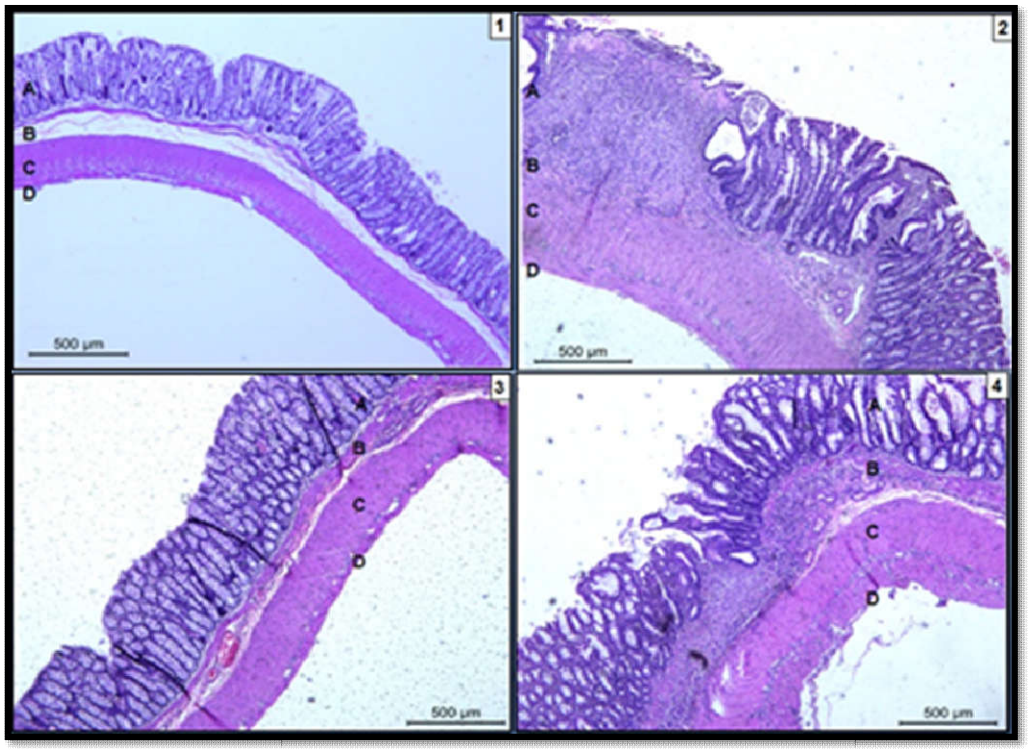

Figure 3. Histopathological analysis of the colon stained with hematoxylin and eosin (H&E - 10x) in animals from the groups: CT - without ulcerative colitis, treated with ultrapure water / TNBS CT - with ulcerative colitis, treated with saline solution / TNBS MES – with ulcerative colitis, treated with mesalazine / and TNBS SYM – with ulcerative colitis, treated with the symbiotic, for 7 days. 1) CT - compatible with normal segment, in which the layers are identified by A - Mucosa, B - Submucosa, C – Muscle Layer, and D - Serous; 2) TNBS CT - presence of inflammatory process with ulceration, dilatation, and luminal irregularity; 3) TNBS MES - presence of inflammatory process with ulceration, dilatation, and luminal irregularity; 4) TNBS SYM - presence of inflammatory process with ulceration, dilatation, and luminal irregularity

leukemia, and in the preoperative period of gastrointestinal surgeries (Chalas *et al*., 2016). Its effect was further observed in the treatment of inflammatory bowel disease, although with limited results that do not guarantee sufficient safety to assert that its use will bring benefits in the presence of inflammatory diseases (Viswanathan *et al*., 2016). The present study reproduced all the signs and symptoms described in the literature when inducing ulcerative colitis with TNBS, since all animals showed a decrease in food intake followed by weight loss with statistical difference in relation to the CT group, thus showing that the experimental model for induction of ulcerative colitis was effective (Hee *et al*., 2011). After the treatment period, only the group treated with the symbiotic showed a greater weight recovery; however, it was also the group that consumed the least amount of feed.

This evolution can relate to the fact that in the macroscopic analysis, the animals with colitis presented characteristics which demonstrate involvement of luminal tissue with great extension, thus contributing to weight gain (Repka *et al*., 2004). On the other hand, we can extrapolate this benefit in relation to body weight, relating it to the total score, since the lowest adjacent value was observed exactly in this group, that is, the maintenance of mucosal tissue allowed less body stress, allied to a better intestinal absorption, requiring, therefore, lower food intake to generate good results, verified when observing the results of the food efficiency coefficient. Ivanovska *et al*. (2017), under the same conditions of our study, that is, animals with ulcerative colitis induced with TNBS, treated with a symbiotic for 7 days, showed that when using the combination *Lactobacillus casei* + oligofructose-enriched inulin, the inflammatory process and the occurrence of ulcerations were reduced, and the amount of lactobacilli in the feces was increased. In addition, there was a significant decline in myeloperoxidase (MPO), which is the enzyme responsible for the production of reactive oxygen species (ROS), having as main function the cellular signaling, proving to be an advantageous approach in the prevention and treatment of ulcerative colitis. In turn, Winkler *et al*. (2007) and Lara-Villoslada *et al*. (2006), who chemically induced ulcerative colitis with sodium dextran sulfate - SDS and TNBS, respectively, showed attenuation in the inflammatory process in both the macroscopic and microscopic evaluation after administration of fructooligosaccharides, bifidobacteria, and lactobacilli. Likewise, when evaluating the same strains in transgenic mice, who spontaneously develop ulcerative colitis, beneficial effects were observed in the prevention and treatment of the disease. There are studies that have found benefits, and studies that showed no effects (Winkler *et al*., 2007; Lara-Villoslada *et al*., 2006) of the use of symbiotics on the prevention and treatment of ulcerative colitis, which may be related to the absence of standardization of the drug concentration for induction, as well as in the interpretation of clinical improvement regarding the macroscopic and microscopic evaluation by means of scores, and in the concentration and combination of the strains, thus making it difficult to know what is the real effect of the use of symbiotics in ulcerative colitis (Laurell and Sjöberg, 2017).

Conclusion

In conclusion, under the conditions of this study, it was observed that the use of the symbiotic maintained the body weight, and there was a reduction in the extent of ulcerations and tissue aggression, with behavior similar to the medication.

However, further studies are needed, especially investigating the coadjuvant effect of the symbiotic drug associated with the drug currently available for the treatment of ulcerative colitis.

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