ISSN: 2230-9926

Available online at http://www.journalijdr.com

IJDR

International Journal of Development Research
Vol. 11, Issue, 06, pp. 47714-47720, June, 2021
https://doi.org/10.37118/ijdr.21975.06.2021

RESEARCH ARTICLE OPEN ACCESS

CHEMIOPREVENTIVE EFFECT OF PROBIOTICS ON EXPERIMENTAL CARCINOGENESIS INDUCED BY 1,2-DIMETHYHTHRAZINE IN RATS

Marceli Pitt Coser[a], Conrado Gamba de Oliveira[b], Jane Mary Lafayette Neves Gelinski[c] and Claudriana Locatelli[d]

[a]Master's in Science and Biotechnology. Nutritionist. Undergraduate professor. Department of Life Sciences and Health. University of West Santa Catarina – Campus Videira. Paese Street 180, Videira-SC, Brazil; [b]Veterinary pathologist at the Veterinary Research and Diagnosis Institute, Brazil; [c]Program in Science and Biotechnology, University of WestSanta Catarina – Campus Videira. Paese Street 180, Videira-SC, Brazil; [d]Program in Development and Society, University Alto Vale do Rio do Peixe – UNIARP, Campus – Caçador, Victor BaptistaAdami Street 800, Caçador-SC, Brazil

ARTICLE INFO

Article History:
Received 20th March, 2021
Received in revised form
18th April, 2021
Accepted 17th May, 2021
Published online 26th June, 2021

Key Words:

Microbiota. Dysbiosis. Colorectal cancer. Probiotics. Chemoprevention.

Corresponding author: Marceli Pitt Cosera

ABSTRACT

The disorder between beneficial microorganisms and pathogens generates dysbiosis. Probiotics balance the environment and demonstrate efficacy in the prevention and treatment of neoplasms. This study aimed to evaluate the effect of the probiotics *Lactobacillus helveticus* and *Bifidobacterium bifidum*, isolated or combined, on the carcinogenic effect induced by 1,2-dimethylhydrazine in rats. Six different experimental groups were used, containing six animals each, and the treatment occurred for six weeks. Body mass gain, pro-carcinogenic enzyme activity, quantification of aberrant crypts, measurement of intestinal macroscopic changes, and colorectal histological evaluation were evaluated. Through the analysis of the results, it can be observed that the probiotics were shown to be effective in decreasing the colorectal toxicity induced by 1,2-dimethylhydrazine, and promising chemopreventive on intestinal lesions.

Citation: Marceli Pitt Coser, Conrado Gamba de Oliveira, Jane Mary Lafayette Neves Gelinski and Claudriana Locatelli. 2021. "Chemiopreventive effect of probiotics on experimental carcinogenesis induced by 1,2-dimethyhthrazine in rats", *International Journal of Development Research*, 11, (06), 47714-47720.

INTRODUCTION

Colorectal cancer (CRC) is the third most common cancer in the world, represents one of the biggest causes of mortality among different types of cancer, and is among the biggest health problems in industrialized countries (AN; HA, 2017), being one of the most common causes of death among men and women (SIEGEL; MILLER; JEMAL). The intestinal tract has approximately 100 trillion (10^{14}) microorganisms, mostly residing in the colon, in an environment rich in nutrients. The microbiota and the host form a complex "superorganism" in which relationships provide benefits to the host in many key aspects of life (SCHWABE; JOBIN, 2013). When a microbial community in a specific area of the body changes, it leads to a condition called "dysbiosis". The balance in the microbiota is related to health, while an imbalance or dysbiosis is related to health problems (VANDENPLAS; HUYS; DAUBE, 2015). Scientific evidence indicates a key role of the bacterial microbiota in the prevention or development of colorectal carcinogenesis (SCHWABE; JOBIN, 2013). In order to neutralize harmful effects on the intestinal microbiota, probiotic strains have been developed to improve the health of the intestine, especially to restore the impaired intestinal barrier (BARZ, et al., 2015).

At least eight probiotic mechanisms of action potentials are known, and include competitiveness for food ingredients used as growth substrates; bioconversion of, for example, sugars into fermentation products with inhibitory properties; production of growth substrates, such as EPS (exopolysaccharides) or vitamins, for other bacteria; direct antagonism by bacteriocins; competitive exclusion for connecting sites; improved activity in barrier function; reduction of inflammation, thus altering intestinal properties to facilitate colonization and permanence; stimulating the innate immune response by unknown underlying mechanisms (O`TOOLE; COONEY, 2008). The first isolated and characterized microorganism from the human intestine was *Bifidobacterium bifidum*, since then several laboratory studies have been applied among the different strains, and properties of potential clinical significance have been identified as immunomodulatory, metabolic, antibacterial and antiviral. In humans, clinical trials have shown positive effects on diarrhea, mucositis and inflammation of the gastrointestinal tract (QUIGLEY, 2017). It was in 1900 that the researcher Tissier first described the genus *Bifidobacterium* isolating *Bacillus bifidus comunis* from children's feces (FERREIRA, 2018). The genus *Bifidobacterium* belongs to the domain of bacteria of the phylum firmicutes, class Actinobacteria, order Bifidobacteriales, family Bifidobacteriaceae. Bifid bacteria are anaerobic, gram positive, and

almost always catalase negative, and in the presence of blood / hematin, they produce true catalase. They are sensitive to acidic environments, usually at a pH below 5. They exhibit pleomorphism and may present themselves in the form of Y and V, in addition to several elongated and coccoid forms, with protuberance at one end. They differ from other bacteria in the lactic group in that they have activity of the enzyme fructose-6-phosphate phosphocetolase (FERREIRA, 2018). *Lactobacillus* are microorganisms - bacteria - strictly fermentative from the phylum Firmicutes, usually found in several environmental niches with high levels of nutrients, especially sugars and organic nitrogen. The genus *Lactobacillus* comprises several different species, where most can be grouped according to their phylogenetic relationships (HAMMES; HERTEL, 2006). Currently, 170 species and 27 subspecies are described (FERREIRA, 2018). It was in 1919, isolated from Emmental cheese, that *L. helveticus* was first described by Orla-Jensen (NASSER et al., 2006). L. *helveticus* is part of the group of lactic acid bacteria. Its consumption is recognized as safe, being commonly used in the manufacture of dairy products, and used as a health-promoting probiotic food for its potential to produce bioactive peptides or bacteriocins (GIRAFFA, 2014). *L.helveticus* is part of the obligatory homofermentative bacteria, gram-positive, presenting in bacillary form (FERREIRA, 2018). Bacilli can vary between long and thin, even curved and small, or in the form of coccobacilli, with the formation of a chain being common. The size of the bacilli and the degree of curvature is dependent on the age of the culture. Irregular shapes can be observed in the growth of a symbiotic medium. They react to Gram stain and methylene blue (MELLO et al., 2011). It has good growth at a temperature of 40-45ºC and at most 50- 2ºC, not growing at temperatures below 15ºC (FURTADO, 1990).

Even before tumor development, changes in the architecture of the microbiota in the colon and rectum are observed. The first author who described aberrant crypts was Bird in 1987 and called them FCA (foci of aberrant crypts), in addition to recognizing them as precocious and precursor lesions of the CRC after exposing murines to carcinogens (BIRD, 1987). When submitting the mucosa to methylene blue staining, the FCA can be seen, as the areas that have FCA contrast with the intact mucosa, which can be precancerous lesions, and can also serve as a valid biomarker of subsequent adenoma and formation of colorectal cancer, since they are precursor lesions in both animal and human CRC (HURLSTONE; CROSS, 2005). The role of probiotics in the prevention and / or treatment of cancer, especially colorectal, must be realized, since the role of the microbiota seems to be entirely linked to the condition of health or disease, demonstrating the urgency of further research. The aim of the present study was to evaluate the effect of the probiotics *Lactobacillus helveticus* and *Bifidobacterium bifidum*, isolated or associated, on the toxic effect on colorectal tissue induced by 1,2-dimethylhydrazine in rats.

MATERIALS AND METHODS

Animals, habitat and diet: Thirty-six five-week-old rats were randomly divided into six groups of six animals each, and housed in polyethylene cages (two or three rats per cage). They received a standard diet and water on demand. The light / dark cycles were 12 h each. The shaving bed was changed every two days. Before starting the experiments, the animals were adapted to the laboratory conditions for one week. The animals were kept in accordance with the principle and guidelines of the Ethics Committee on Animal Care, after approval of the experimental protocol by CEUA / UNOESC under opinion number 04/2018.

Induction of carcinogenesis: The colorectal tumor was induced as proposed by Zang et al. (2015), through the administration of 30 mg / kg of 1,2-dimethylhydrazine (DMH) in E.D.T.A. (ethylenediamine tetra-acetic acid) 15%, subcutaneously, once a week for four weeks. The administration of DMH started after five days of the administration of the antibiotic ampicillin at a dose of 75 mg / kg to eliminate possible pathogens and these do not compromise the response to tumor induction (KUUGBEE et al., 2016).

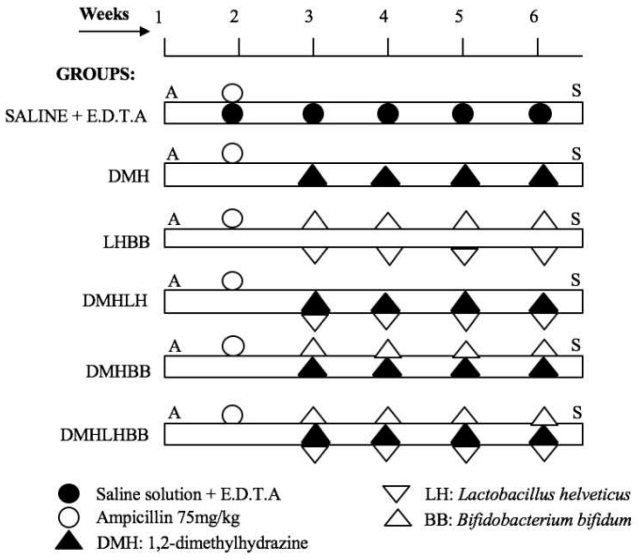
Preparation of probiotics: The probiotic strain *Lactobacillus helveticus* 140Bi / Gr from Belgium, marketed and distributed by Pharmanostra, and *Bifidobacterium bifidum*, marketed and distributed by Embrafarma, were used in this study, both without contraindications and / or reports of adverse reactions. The dosage of probiotic formulas was calculated according to the suppliers' recommendations and the dose of humans to rodents was converted by the formula suggested by Kuugbee et al. (2016):

d_{mice} (miligrams [mg] / kilograms [kg]) = d_{human} (mg / kg) x (k_{mice} / k_{human})

where k = conversion factor, K_{mice} = 1; k_{human} = 0.16.

The conversion was performed based on the dosage used for an adult individual of 70 kg. After conversion, the following dosages were obtained for each kg of rat: L. *helveticus* 12 mg (1.7x106 CFU / day), B. *bifidum* 67 mg (6.7x108 CFU / day). The formulations were packed in sterile sachets, using microcrystalline cellulose as sufficient quantity for (q.s.p). The storage followed the manufacturers' recommendations.

Experimental design: The administration of the 0.9% saline solution, and the probiotic (s) was by gavage once a day, daily, except on the day of administration of the carcinogen inducing drug DMH (30 mg / kg) as suggested by Liboredo et al. (2013), until the end of the experiment. E.D.T.A. 15% was administered subcutaneously on the same day as the tumor was induced. The probiotics were diluted in sterile saline. The division of groups was configured as follows: Group 1: saline and E.D.T.A; Group 2: DMH; Group 3: *L.helveticus* + B. *bifidum*; Group 4: DMH and *L.helveticus*; Group 5: DMH and *B.bifidum*; Group 6: DMH and *L.helveticus* + *B.bifidum*. After four doses of DMH and six weeks of treatment with probiotic (s), the animals were sacrificed with an overdose of ketamine/xilasine (> 150 mg / kg /> 16 mg / kg) i.p. (intraperitoneally) and analyzes of body mass, intestinal microbiota, activity of pro-carcinogenic enzymes in the feces, presence of aberrant crypts and histological analysis of the colorectal. The experimental scheme can be seen in Figure 1.



Saline + E.D.T.A: saline solution and ethylenediamine tetra-acetic acid; DMH: 30 mg / kg 1,2-dimethylhydrazine; LHBB: *L. helveticus* + *B.bifidum*; DMHLH: 30 mg / kg DMH + *L.helveticus*; DMHBB: 30 mg / kg DMH + *B.bifidum*; DMHLHBB: 30 mg / kg DMH + *L.helveticus* + *B.bifidum*; S: sacrifice after 4 doses of DMH; A: adaptation before the start of interventions.

Figure 1. Experimental design for the treatment of animals

Analysis of body mass: Weighing the animals was performed at the beginning once a week and throughout the experiment using a *Precision*® PR1000 semi-analytical scale. The initial weight was considered that of the day before the first intervention with antibiotics and the final weight, that immediately before sacrifice.

Microbiological analysis of feces: After four doses of DMH, fresh stool samples from each experimental group were subjected to homogenization and sonication, followed by serial dilution for each sample. The samples were made in duplicate and incubated on plates containing MRS medium and agar agar (Agar De Man, Rogosa and Sharpe) for lactic acid bacteria, and MC medium (MacConkey Agar) for enterobacteria, and grown in an oven at 37ºC for 48h (MRS medium) or 72h (MC medium). The total number of colonies forming units of each medium in each group was counted and recorded. To evaluate the morphological characterization, after macroscopically evaluating the colony-forming units, the samples were subjected to the GRAM stain test.

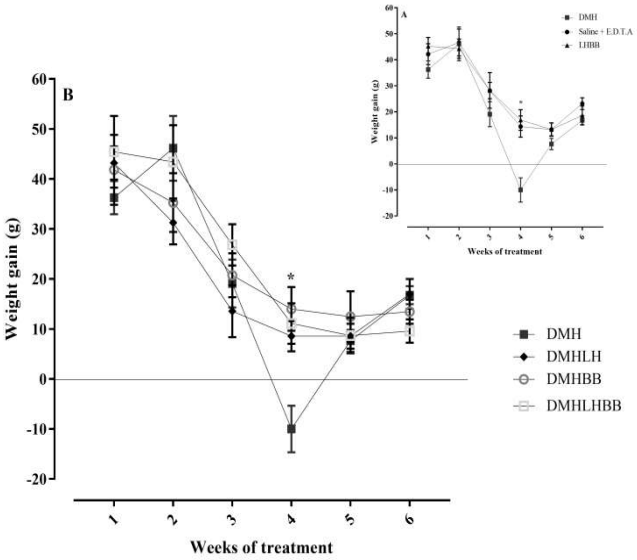
Activity of pro-carcinogenic enzymes in feces: β-glucuronidase and β-glucosidase: The activities of the enzymes β-glucuronidase and β-glucosidase were carried out in the feces supernatant of each animal group after the preparation of a suspension obtained by sonication at 4°C for 3 minutes and cold centrifugation for 15 minutes. The activity of the enzymes β-glucuronidase and β-glucosidase were determined spectrophotometrically using a wavelength of 540 nm and 450 nm respectively. The method was based on the color reaction between the substrate and the enzyme analyzed, according to the methodology described by Goldin and Gorbach (1976), using β-D-glucuronide phenolphthalein (Sigma) for β-glucuronidase and p-nitrophenyl- β-D-glycopyranoside (Sigma) for β-glucosidase. The unit of activity adopted was equal to the amount of phenolphthalein (β-glucuronidase) and p-nitrophenol (β-glucosidase) expressed in µmol / mg of protein, which was released during the reaction in 1 hour at 37ºC, calculated by 1 mg of protein present in the stool. The total protein concentration in the supernatant was determined using the Lowry method (LOWRY et al., 1951).

Quantification of aberrant crypts and measurement of intestinal macroscopic changes: The intestines were removed, washed with saline, and opened longitudinally. Afterwards, they were stretched on a flat surface and the internal structures were examined for the presence of macro and microscopic lesions. Macroscopic lesions were observed in terms of incidence, multiplicity, and total number per group. The size of these lesions was assessed using vernier forceps with a 0.1 mm graduation from the *Cescorf*® brand. The preventive response for the presence of macroscopic lesions was assessed based on their incidence and multiplicity, calculated as follows: Injuryincidence = Determination of the number of animals with injuries. Multiplication = number of lesions counted ÷ number of animals, and the Total number of lesions = sum of macroscopic lesions per group. Microscopic topographic analysis of the colorectal mucosa was performed after 24 h of fixation in 10% neutral formaldehyde (BIRD, 1987). The colon and rectum were stained with a 0.2% methylene blue solution for 10 min. The mucous side of the intestine was exposed on a microscopic slide and observed under a light microscope for counting aberrant crypts (CA).

Histological analysis of the intestine: The necropsy was performed, and colonic fragments were collected, fixed in 10% neutral formaldehyde, embedded in paraffin, and microtomized in 4 µm thick sections stained with hematoxylin and eosin. The samples were evaluated according to the evaluation system described by Del Carmen et al. (2017), by optical microscopy. In this System, the following criteria are considered: 1) Loss of mucosal architecture (0: absent; 1: medium; 2: severe); 2) Cell infiltration (0: absent; 1: in muscularis mucosa; 2: in lamina propria; 3: in serosa); 3) Muscle thickness (0: ½ the thickness of the mucosa; 1: muscle = ½ - ¾ of the thickness of the mucosa; 2: muscle = thickness of the mucosa; 3 = or thicker than the mucosa); 4) Depletion of goblet cells (0: absent; 1: present); 5) Crypt abscess formation (0: absent; 1: present); and 6) Tumor (0: absent; 1: present). The scores of the variables were added to obtain the final score for each sample. The sum of points was exposed in Multiple Colorectal Lesions (MLs / colorectal) after four doses of 1,2-dimethylhydrazine - DMH. The samples were observed by the pathologist under an optical microscope in a "blind" manner with a magnification of 400 x. This trial aimed to verify the colorectal damage induced by 1,2-dimethylhydrazine and the ability to prevent colorectal damage by probiotics.

OUTCOMES

When evaluating the weight gain in grams (g) between the control groups (Fig. 2A), it is observed that the second group (DMH) obtained weight gain lower than the others, being significant after the second dose of the tumor-inducing drug, where Group Saline and EDTA gained an average of 14.33 g ± 8.14, the DMH group lost an average of 10.00 g ± 9.33, and LHBB gained 16.88 g ± 7.95.

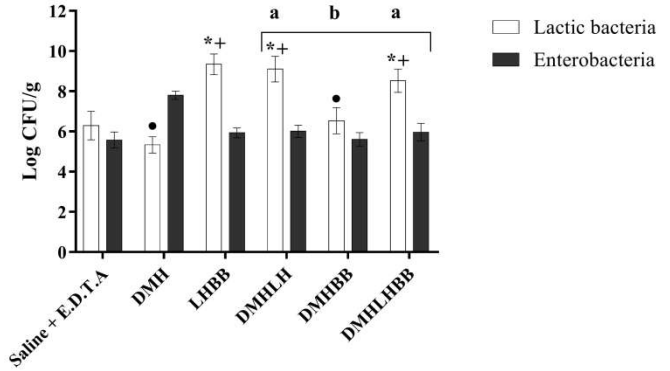


Average weight gain in grams per group during the treatment period. E.D.T.A.: ethylenediamine tetra-acetic acid. DMH: 1,2-dimethylhydrazine. LH: *Lactobacillus helveticus*. BB: *Bifidobacterium bifidum*. (*) Weeks of treatment where there were statistical differences in relation to the DMH Positive Control Group. **(A)** Average weight gain in grams between Saline and E.D.T.A., DMH, and LHBB groups. Statistical differences in the fourth week (2 doses of DMH) p <0.001 by Student's t test. Between the groups Saline and E.D.T.A. vs LHBB there was no statistical difference at any stage of treatment p> 0.05 by Student's t test. **(B)** Weight variation per week during the treatment period, comparing the DMH and DMH group associated with probiotic (s). (*) Treatment week where there was statistical difference (in the fourth week after two doses of DMH) in relation to the DMH Positive Control Group, where p <0.001 by the Tukey test.

Figure 2. Effect of probiotics on the body weight gain of male *Wistar* rats submitted to treatment with 4 doses of 1,2-dimethylhydrazine 30 mg / kg of weight

Regarding weight gain in g between all groups (Fig. 2B), it is noted that there was greater weight gain among the groups treated with DMH associated with the probiotic (s), differing statistically after the second dose of DMH in relation to the group only DMH. At the end of the treatment, in the groups treated simultaneously with DMH and a probiotic, they presented higher final weight. Lactic acid and enterobacteria counts were performed after four doses of the tumor-inducing drug 1,2-dimethylhydrazine. In the control group Saline + E.D.T.A, the lactic acid bacteria count remained stable during the analysis time.

It is possible to observe that in the DMH group there was a significant decrease in lactic acid bacteria, and inversely proportional, an increase in enterobacteria. The induction of carcinogenesis concomitant with the administration of probiotic (s) favored the modulation of a microbiota prevalent in lactic acid bacteria (Figure 3).



Log: Logarithm. UFC: Colony Forming Units. g: grams. Bac: bacteria. E.D.T.A.: ethylenediamine tetra-acetic acid. DMH: 1,2-dimethylhydrazine. LH: *Lactobacillus helveticus*. BB: *Bifidobacterium bifidum*. Demonstration of the count of lactic acid bacteria and enterobacteria after administration of four doses of DMH. Differences between the counts of lactic acid bacteria: (*) statistical difference in relation to the positive control group DMH p <0.001 by the Tukey test. (+) statistical difference in relation to the negative control group Saline + E.D.T.A. p <0.001 by the Tukey test. (●) Statistical difference in relation to the positive control group LHBB p <0.001 by the Tukey test. Letters compare differences between groups treated with DMH + probiotic (s). Letter alone differs statistically from the other p <0.05 by the Tukey test. Equal letters do not differ statistically from each other p> 0.05 by Tukey's test. Values expressed as mean ± standard error of the mean.

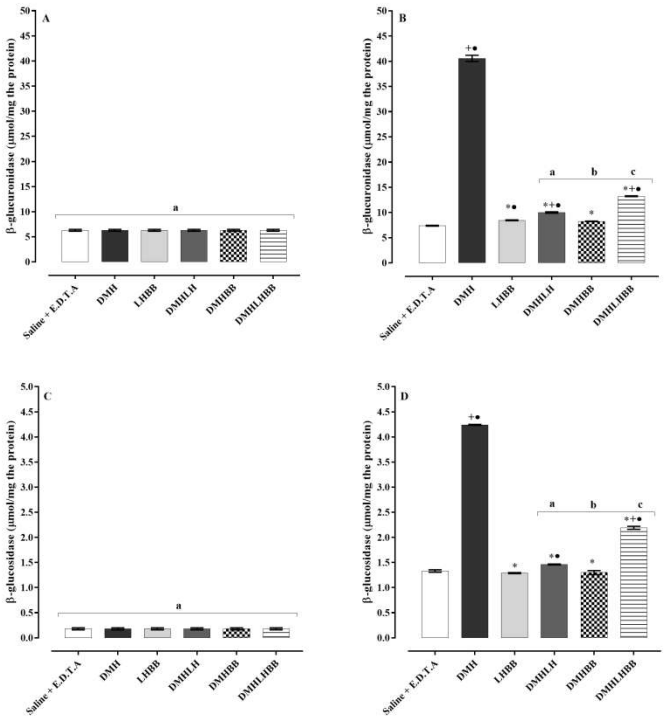
Figure 3. Counting of lactic acid bacteria and enterobacteria in the feces of male *Wistar* rats submitted to treatment with 4 doses of 1,2-dimethylhydrazine 30 mg / kg of weight

Table 1. Development, multiplicity and number of macroscopic changes observed in the colorectal of male *Wistar* rats after four doses of DMH (30mg / kg)

GROUPS	Injury development (%)	Multiplicity of colon lesions (n/a)	Number of changes (n)
1 – Saline + E.D.T.A	0%	0	0
2 - DMH	100%	5,25	21
3 - LHBB	0%	0	0
4 - DMHLH	100%	4,00	16
5 - DMHBB	100%	4,25	17
6 - DMHLHBB	100%	4,25	17

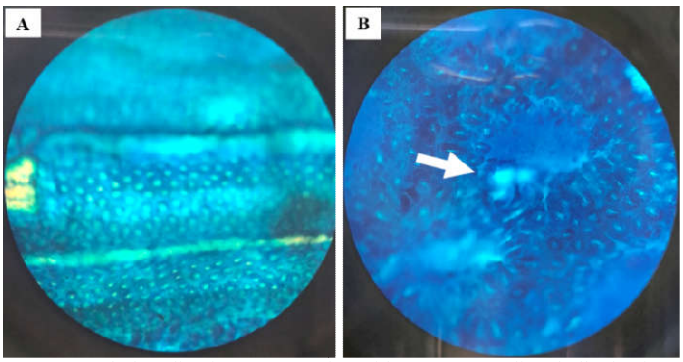
% percentage. (n / a) Number of changes divided by the number of animals. (n) Number. E.D.T.A.: ethylenediamine tetra-acetic acid. DMH: (1,2-dimethylhydrazine). LH: *Lactobacillus helveticus*. BB: *Bifidobacterium bifidum*. Injury development =% of animals that developed changes in the intestine. Multiplicity of changes in the colon = number of changes observed in the intestine divided by the number of animals. Number of changes = sum of the number of lesions observed in all animals in the group.

Regarding the enzymatic activity of β-glucuronidase (Fig. 4A and 4B), and of β-glucosidase in feces (Fig. 4C and 4D), after the induction of tumurogenesis, its activity was high (Fig. 4B and 4D) when comparing with the period prior to toxic exposure (Fig. 4A and 4C), and the increase was statistically greater in the group that received only DMH. Data on the development, multiplicity and number of macroscopic lesions (Table 1) demonstrate that all animals treated with DMH had lesions in the intestine after four doses of DMH. When analyzing the multiplicity of these lesions, it was higher in the group that received only DMH. When comparing the group only DMH, and DMH with probiotic (s), there was a decrease of -23.81% in the number of lesions in the group that received L. helveticus concomitantly, and of -19.05% in the groups with B. bifidum and in the group treated with the two probiotics. When analyzing the incidence (n) of macroscopic lesions (Fig. 5A, Table 1), there is no significant difference between the groups treated with probiotic (s) and DMH, and the one that received only DMH. However, when the size of these lesions was evaluated (Fig. 5B), they were significantly smaller in all groups where, in addition to DMH, treatment with probiotic (s) was administered. This was also observed in the incidence of aberrant crypts (Fig. 5C).



E.D.T.A.: ethylenediamine tetra-acetic acid. DMH: 1,2-dimethylhydrazine. LH: *Lactobacillus helveticus*. BB: *Bifidobacterium bifidum*. Activity of the enzyme β-glucuronidase and β-glucosidase in the feces of animals. (A) Activity of the β-glucuronidase enzyme after treatment with antibiotics and before starting treatment with DMH and / or probiotic (s). (B) Activity of the β-glucuronidase enzyme after three doses of DMH. (C) Activity of the β-glucosidase enzyme after antibiotic treatment and before starting treatment with DMH and / or probiotic (s). (D) Activity of the β-glucosidase enzyme after three doses of DMH. (*) statistical difference in relation to the positive control group DMH p <0.001 by the Tukey test. (+) statistical difference in relation to the negative control group Saline + E.D.T.A. p <0.001 by the Tukey test. (●) Statistical difference in relation to the positive control group LHBB p <0.05 by the Tukey test. Letters compare differences between groups treated with DMH + probiotic (s). Letter alone differs statistically from the other p <0.001 by the Tukey test. Equal letters do not differ statistically from each other p> 0.05 by Tukey's test. Values expressed as mean ± standard error of the mean.

Figure 4. Activity of the enzymes β-glucuronidase and β-glucosidase in the feces of male *Wistar* rats submitted to treatment with 4 doses of 1,2-dimethylhydrazine 30 mg / kg of weight in different phases of the experiment



E.D.T.A.: ethylenediamine tetra-acetic acid. DMH: 1,2-dimethylhydrazine. LH: *Lactobacillus helveticus*. BB: *Bifidobacterium bifidum*. (A) Ordering of intestinal cells observed in Groups without induction of carcinogenesis (Saline + E.D.T.A. and LHBB). (B) Aberrant crypt focus observed in groups where there was induction of carcinogenesis with DMH, with or without treatments with probiotic (s). Observation under an optical microscope after staining with methylene blue on the 100x objective.
Source: Data from research.

Photo 1. Cell ordination and foci of aberrant crypts found in the intestinal mucosa of male *Wistar* rats after sacrifice

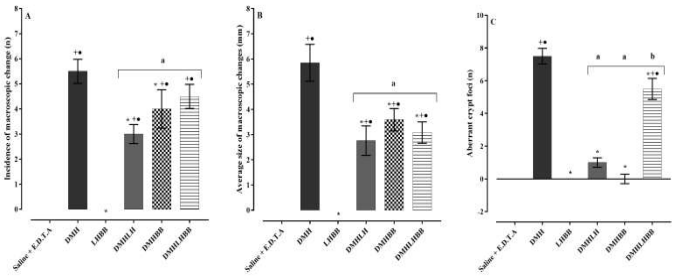
In Photos 1A and 1B, records of the intestinal mucosa can be observed in the analysis of aberrant crypts of the control and DMH animals respectively after administration of 4 doses of DMH 30 mg /

kg of weight. Regarding the results of the histological analysis of the intestine (Table 2), when observing the scores for multiple lesions (sum of the amount of mucosal architecture, cell infiltration, thickness of muscles, calciform cells, and crypt abscess of the animals analyzed from each group), there were no significant differences between the DMH *vs* DMH group associated with the probiotic (s) after the four doses of DMH (p> 0.05 by the Tukey test). When assessing the prevalence of intestinal tumors, 50% of the animals developed it in group 2 (DMH), also in group 6 (DMHLHBB), however in the groups treated with isolated probiotics, group 4 (DMHLH) and 5 (DMHBB) there was a -50% decrease.

Table 2. Presence of multiple lesions and tumors observed microscopically in the colorectal segment by histological analysis of the intestines of male *Wistar* rats after four doses of DMH (30 mg / kg)

GROUPS	MLs/colorectal[#]	Tumors (n)[##]
1 – Saline + E.D.T.A	2,00 ± 0,00*	0/6
2 - DMH	4,00 ± 2,00	3/6
3 - LHBB	4,00 ± 0,00	0/6
4 - DMHLH	4,00 ± 2,00[a]	0/6
5 - DMHBB	5,00 ± 2,00[a]	0/6
6 - DMHLHBB	5,00 ± 1,00[a]	3/6

MLs: Multiple lesions (sum of the amount of mucosal architecture, cell infiltration, thickness of muscles, calciform cells, and crypt abscess of the animals analyzed in each group). (n) Number of animals with tumors. E.D.T.A.: ethylenediamine tetra-acetic acid. DMH: (1,2-dimethylhydrazine). LH: *Lactobacillus helveticus*. BB: *Bifidobacterium bifidum*. (*) statistical difference in relation to the DMH Positive Control Group p <0.05 by the Tukey test. (+) statistical difference in relation to the Negative Control Group Saline + E.D.T.A. p <0.01 by the Tukey test. (●) Statistical difference in relation to the LHBB Positive Control Group p <0.05 by the Tukey test. Letters compare differences between groups treated with DMH + probiotic (s). Letter alone differs statistically from the other p <0.05 by the Tukey test. Equal letters do not differ statistically from each other p> 0.05 by Tukey's test. [#]: Each value in the column represents the average of MLs counted in the colorectal segment of the intestine. Results are expressed as mean (total sum of points) and ± SD (standard deviation of the mean). [##]: Each value in the column shows the number of mice that developed a tumor out of the total number of mice after four doses of DMH. Observation: *in situ*, tumors were considered.



E.D.T.A.: ethylenediamine tetra-acetic acid. DMH: 1,2-dimethylhydrazine. LH: *Lactobacillus helveticus*. BB: *Bifidobacterium bifidum*. (n): number. (mm): millimeters. Incidence reports the mean (n) of tumors between the different groups and time of treatment. (A) Incidence of macroscopic changes (n). (B) Average size of macroscopic changes (mm). (C) Aberrant crypt foci (n). (*) statistical difference in relation to the positive control group DMH p <0.05 by the Tukey test. (+) statistical difference in relation to the negative control group Saline + E.D.T.A. p <0.001 by the Tukey test. (●) Statistical difference in relation to the positive control group LHBB p <0.05 by the Tukey test. Letters compare differences between groups treated with DMH + probiotic (s). Equal letters do not differ statistically from each other p> 0.05 by Tukey's test. Values expressed as mean ± standard error of the mean.

Figure 5. Variation in mean incidence (number), mean size (millimeters) of macroscopic changes, and outbreaks of aberrant crypts in male *Wistar* rats submitted to treatment with 4 doses of 1,2-dimethylhydrazine 30 mg / kg in weight

DISCUSSIONS

Probiotics have been used more and more worldwide in order to maintain a healthy intestine and relieve gastrointestinal diseases including cancer. To verify the effect of the intervention of probiotics *L. helveticus* and *B. bifidum* on the development of colorectal cancer, the DMH-induced colorectal cancer model was used, since this model provides data on the chemopreventive potential of probiotics. In the present study, there was a reduction in the weight of the animals that were treated with the tumor-inducing drug (DMH), while in the groups that received the drug associated with the probiotics, there was no significant reduction in weight gain during the treatment time. Corroborating the work of Walia et al. (2015), that when inducing colorectal cancer with DMH concomitant with doses of *L. plantarum*(AdF10) and *L.rhamnosus*GG (LGG), in isolation at the dose of 10^{10} CFU, found that animals treated only with DMH had lower gain of body weight in the 8th and 16th week of the experiment. In another study where *L.acidophillus*was administered with *B.bifidum* at a dose of $2x10^9$ CFU, the authors observed greater weight gain compared to those exposed only to DMH (MOHANIA et al., 2014). On the other hand, when the performance of *L. delbrueckii*, *B. animalis*, and *Saccharomyces boulardii* was investigated, in the dose of $3x10^8$ CFU, in animals under tumor induction with DMH, there were no differences in weight in any of the groups, either treated with isolated or combined probiotics (LIBOREDO et al., 2013). Several bacteria usually present in the microbiota are associated with the development of colorectal cancer (RCC), such as *Streptococcus galloyticus, Bacterioidetes*and *Escherichia coli*. Some are frequently increased, such as *Phylum Fusobacteria, Porphyromonadaceae, Coriobacteridae* and *Staphylococcaceae*, and others decreased such as *Bifidobacterium, Lactobacillus, Ruminococcus*and *Treponema* (SANTOS; ANDRADE; LOPES, 2017). In this experiment, it is possible to observe a predominance of lactic bacteria over enterobacteria in the groups where carcinogenesis was induced and were concomitantly treated with probiotics. Shin, Whon and Bae (2015), when reviewing studies that explore the association between an abnormal expansion of *Procteobacterias*, showed that they compromise the balance of the microbiota, and suggest that their increased prevalence is a potential diagnosis of dysbiosis and risk of disease. Supplementation with microorganisms beneficial to the health of their host, called probiotics, can be a tool for rebalancing or maintaining the intestinal structure (SHIDA, 2017).

A "healthier" microbial composition is believed to have a higher proportion of bifidobacteria and lactobacilli (BINNS, 2013). Fecal bacterial enzymes β-glucuronidase and β-glucosidase are biomarkers of the harmful action of the intestinal microbiota and are known to mediate the development of CRC (NOWAK; ŚLIŻEWSKA, 2014). B-glucuronidase activity is stimulated by contact with toxic substances, tobacco smoke or carcinogenic substances (ŻÓŁTASZEK et al., 2008), catalyzes the hydrolysis of β-D-glucuronic acid residues (LAMPE et al., 2002), both exogenous naturally occurring in diets and drugs, and endogenous produced in the liver by glucuronosyltransferases, being one of the main xenobiotic detoxification pathways. B-glucosidase acts on secondary compounds of glycosidic and xenobiotic plants that enter the colon, potentially implying in the health of the host. The colorectal segment of the intestine is involved in the metabolism and activation of xenobiotics derived from dietary compounds (GLOUX et al., 2011). Fecal bacterial enzymes β-glucuronidase and β-glucosidase are biomarkers of the harmful action of the intestinal microbiota and are known to mediate the development of colorectal cancer (CRC) (NOWAK; ŚLIŻEWSKA, 2014). The results of the present study demonstrated that the activities of the pro carcinogenic enzymes β-glucuronidase and β-glucosidase were attenuated when the induction of carcinogenesis was concomitant with the administration of probiotics, either in isolation (one strain), or combined (two strains). Corroborating this study, the administration of *L.acidophillus* ($2x10^9$ CFU) concomitant with the use of the DMH carcinogen, also reduced the β-glucuronidase and β-glucosidase activity after 10 weeks of treatment (CHANG et al., 2012); and the administration of *L. plantarum* and prebiotics (Inulin and *Aesculushippocastanum L.*) with the carcinogen DMH, associated with a high-fat diet, decreased the performance of these two enzymes after 6 weeks of experiment (BERTKOVA et al., 2010). Affirming our results on the decrease in the incidence, size or number of macroscopic lesions, which may be tumors or pre-tumors, other studies that used DMH to induce

colorectal carcinogenesis although sometimes in different doses, exposure time, and / or types probiotics, showed that, when administering LBB (a commercial product) containing *L.acidophillus*, *B.bifidum* and *B.infantum*, there was a decrease in the incidence and size of the tumors (KUUGBEE et al., 2016); with *L.salivarius Ren* decrease in the incidence of colon cancer in the range of 25% to 87.5% (ZHANG et al., 2015); isolated administration of *L. plantarum*(AdF10) and *L. rhamnosus*GG (LGG) decreased the incidence from 60% to 65%, and the size of the tumors decreased (WALIA et al., 2015); with 10^9CFU of *L.plantarum* there was a 42.13% decrease in incidence and 36.12% in tumor size (KUMAR et al., 2012). Aberrant crypts are considered precocious lesions and precursors to CRC (colorectal cancer) (HURLSTONE; CROSS, 2005). When administering *L.salivarius Ren* (ZHU et al., 2014), the association of *B.bifidum* and *L.acidophillus*(MOHANIA et al., 2014), *L.acidophillus*, *L. rhamnosus*, *L.casei*, *L.plantarum*, and *B.bifidum* (VERMA; SHUKLA, 2013), the authors observed a reduction in the incidence of this marker of colorectal damage. These data are compared with the present study. In contrast, another study, when administering *B.longum*, the authors observed an increase in their incidence (BOLOGNANI et al., 2001). Toxicity has become a major problem for the cause of many types of cancer (PHARMACEU et al., 2017). More than 50% of primary tumors originate from the gastrointestinal tract, especially the colon and rectum (LIU et al., 2015). Antitumor activity is an effect attributed to fermented and probiotic foods (DE MORENO DE LEBLANC; PERDIGÓN, 2005). In agreement with the result of this study, where the prevalence of animals with tumors was similar among those treated with DMH and some probiotics, and lower in others, Del Carmen et al. (2017), when inducing a tumor with DMH (20 mg / kg) for 10 weeks, found an increase in the number of animals with tumors treated with DMH and *Lactococcuslactis subsp. cremoris*, and decreased prevalence with *Streptococcus thermophilus*CRL807, compared to the DMH group. The authors associated the already known anti-inflammatory capacity of *Streptococcus thermophillus*as a possible cause of the positive response only of that probiotic in the prevention of carcinogenesis. In other experiments that analyzed the role of probiotics under the prevention of DMH-induced carcinogenesis, in the study by Zhu et al. (2014), treatment with *L.salivarius Ren* decreased the levels of PCNA cells (nuclear cell proliferation antigen), a marker of cell proliferative activity correlated with carcinoma. Decreased PCNA was also verified by Foo et al. (2011), when administering a free standard diet, and *L.gasseri*and / or *B.longum*probiotics in the average doses of $1x10^{11}$ CFU and $5x10^9$ CFU. In addition, *B.longum*showed greater phagocytic activity, and in the group treated with *L.gasseri*they had a higher number of macrophages. Mohania et al. (2014), when administering *B. bifidum* and *L.acidophillus*associated and DMH, in addition to the decrease in PCNA cells, observed smaller foci of mucins (associated with cancer), compared to the group that received only DMH. Antitumor activity is an effect attributed to fermented and probiotic foods (DE MORENO DE LEBLANC et al., 2005). Therefore, it can be inferred from the results of this study, that the probiotics administered in isolation obtained the best results on the development of tumors at this time of analysis, considering that the presence of multiple lesions was equal or higher in the groups where concomitant carcinogenesis was induced with probiotic (s), when compared with the DMH control group. This correlation may suggest a delayed action in the carcinogenic process, since the number of tumors was null when administered probiotic in isolation. As for the association of probiotics having not shown potential activity, a possible explanation may be for the antagonistic effect among microorganisms. Although probiotics are promising in maintaining and / or recovering health, more studies need to be carried out to better understand the doses, treatment times and combinations that are effective (MEIER; HAWARY, 2018).

CONCLUSIONS

The alteration of the intestinal microbiome induces immunological imbalance, consequently chronic inflammation, and cellular dysfunctions. The administration of probiotics was able to prevent weight loss, decrease the activity of pro-carcinogenic enzymes, the size of macroscopic lesions of the intestinal mucosa and the incidence of aberrant crypts. In the histological analysis of the intestine - microscopic observation - the presence of multiple lesions did not present significant differences between groups, but in relation to the number of tumors, where the administration of probiotic *B.bifidum* and *L.helveticus*, in isolation, proved to be effective in prevention of carcinogenesis. Through the data obtained, it can be concluded that the probiotics studied were effective in decreasing colorectal carcinogenic lesions induced by 1,2-dimethylhydrazine, and colonization of the intestine with a greater number of lactic bacteria on the enterobacteria was correlated with the positive results in prevention of colorectal cancer. It is worth mentioning that in this study, the combined performance of probiotics did not show better results than their isolated administration, demonstrating that there may be antagonism or competition between microorganisms. Therefore, more studies need to be carried out to elucidate these underlying mechanisms. It should be considered that further studies are needed to determine the dose, and ideal treatment times for each disease and / or maintenance of a healthy microbiota. And yet, more studies applied in humans are essential, especially in the treatment of cancer, especially colorectal cancer, given that research in animals suggests efficacy in this pathology. It can be inferred that, although positive results may depend on the time of exposure to the carcinogenic agent, the probiotics studied were shown to be effective in decreasing colorectal toxicity induced by DMH.

Acknowledgment

To the Research Support Fund of the Alto Vale do Rio do Peixe University - UNIARP, for the assistance with a scientific initiation scholarship. The University of the West of Santa Catarina - UNOESC for the release of laboratories and equipment to carry out the research. The Coodination for the Impovement of Higher Education Personnel - CAPES, which, through the Graduate Program in Science and Biotechnology, enabled the development of research and completion of the master's degree.

REFERENCES

AN, J.; HA, E. Combination Therapy of Lactobacillus plantarum Supernatant and 5-Fluouracil Increases Chemosensitivity in Colorectal Cancer Cells. Journal Microbiology and Biotechnology, Coréia, v. 26, n. 8, p. 1490-1503, maio. 2016. doi: 10.4014/jmb.1605.05024.

BERTKOVA, I. et al. The effect of probiotic microorganisms and bioactive compounds on chemically induced carcinogenesis in rats. Neoplasma, [S.l.], v. 57, n. 5, p. 422-428, mar. 2010. doi: 10.4149/neo_2010_05_422.

BINNS, N. Probióticos, prebióticos e a microbiota intestinal. International Life Sciences Institute (ILSI), Belgica, [S.v, S.n.], p. 1-144, 2013.

BIRD, R. P. Observation and quantification of aberrant crypts in the murine colon treated with a colon carcinogen: Preliminary findings. Cancer Letters, [S.l.], v. 37, n. 2, p. 147–151, out. 1987. doi: 10.1016/0304-3835(87)90157-1.

BOLOGNANI, F. et al. Effect of lactobacilli, bifidobacteria and inulin on the formation of aberrant crypt foci in rats. European Journal of Nutrition, [S.l.], v. 40, n. 6, p. 293-300, dez. 2001. doi: 10.1007/s394-001-8359-7.

CHANG, J.-H. et al. Effect of Lactobacillus acidophilus KFRI342 on the development of chemically induced precancerous growths in the rat colon. Journal of Medical Microbiology, GrãBetanha, v. 61, n. 3, p. 361-368, out. 2012. doi: 10.1099/jmm.0.035154-0.

DE MORENO DE LEBLANC, A.; PERDIGÓN, G. Reduction of beta-glucuronidase and nitroreductase activity by yoghurt in a murine colon cancer model. Biocell, [S.l.], v. 29, n. 1, p. 15-24, abr. 2005.

DEL CARMEN, S. et al. Anti-cancer effect of lactic acid bacteria expressing antioxidant enzymes or IL-10 in a colorectal cancer

mouse model. International Immunopharmacology, [S.l.], v. 42, p. 122–129, 2017. doi: 10.1016/j.intimp.2016.11.017.

FERREIRA, C. L. L. Prebióticos e Probióticos: atualização e prospecção. 2. ed. Rio de Janeiro: Rubio, 2018.

FOO, N.-P. et al. Probiotics Prevent the Development of 1,2-Dimethylhydrazine (DMH)-Induced Colonic Tumorigenesis through Suppressed Colonic Mucosa Cellular Proliferation and Increased Stimulation of Macrophages. Journal of Agricultural and Food Chemistry, Taiwan, v. 59, n. 24, p. 13337-13345, dez. 2011. doi: 10.1021 / jf203444d.

FURTADO, M. M. Isolamento de bactériasláticas de leite cru e soro de queijo de leite cru da região do Serro, Minas Gerais. 1990. 95 p. Dissertação (Mestrado em Ciência e Tecnologia de Alimentos) - Universidade Federal de Viçosa, Viçosa, 1990.

GIRAFFA, G. Lactobacillus helveticus: importance in food and health. Frontiers in microbiology, Itália, v. 5, p. 338, jul. 2014. doi: 10.3389 / fmicb.2014.00338.

GLOUX, K. et al. A metagenomic β-glucuronidase uncovers a core adaptive function of the human intestinal microbiome. National Academy of Sciences, EUA, v. 108 Suppl 1, n. Supplement 1, p. 4539–4546, 2011. doi: 10.1073/pnas.1000066107.

GOLDIN, B. R.; GORBACH, S. L. The relationship between diet and rat fecal bacterial enzymes implicated in colon cancer. Journal of the National Cancer Institute, [S.l], v. 57, n. 2, p. 371-375, ago. 1976.

HAMMES, W. P.; HERTEL, C. The Prokaryotes: The Genera Lactobacillus and Carnobacterium. Nova Iorque: Springer, 2006.

HURLSTONE, D. P.; CROSS, S. S. Role of aberrant crypt foci detected using high-magnification-chromoscopic colonoscopy in human colorectal carcinogenesis. Journal of Gastroenterology and Hepatology, [S.l.], v. 20, n. 2, p. 173–181, fev. 2005. doi: 10.1111/j.1440-1746.2004.03433.x.

KUMAR, R. S. et al. Lactobacillus plantarum AS1 Isolated from South Indian Fermented Food Kallappam Suppress 1,2-Dimethyl Hydrazine (DMH)-Induced Colorectal Cancer in Male Wistar Rats. Applied Biochemistry and Biotechnology, [S.l.], v. 166, n. 3, p. 620-631, fev. 2012. doi: 0.1007 / s12010-011-9453-2.

KUUGBEE, E. D. et al. Structural Change in Microbiota by a Probiotic Cocktail Enhances the Gut Barrier and Reduces Cancer via TLR2 Signaling in a Rat Model of Colon Cancer. Digestive Diseases and Sciences, [S.l.], v. 61, n. 10, p. 2908-2920, out. 2016.

LAMPE, J. W. et al. Serum β-Glucuronidase Activity Is Inversely Associated with Plant-Food Intakes in Humans. The Journal of Nutrition, [S.l.], v. 132, n. 6, p. 1341–1344, 2002.

LE BARZ, M. L. et al. Probiotics as complementary treatment for metabolic disorders. Diabetes & Metabolism Journal, Canadá, v. 4, n. 39, p. 291-303, ago. 2015. doi: 10.4093/dmj.2015.39.4.291.

LIBOREDO, J. C. et al. Effect of probiotics on the development of dimethylhydrazine-induced preneoplastic lesions in the mice colon. ActaCirurgicaBrasileira, São Paulo, v. 28, n. 5, p. 367-372, maio. 2013. doi: 10.1590/S0102-86502013000500008.

LIU, Z. et al. Positive regulatory effects of perioperative probiotic treatment on postoperative liver complications after colorectal liver metastases surgery: a double-center and double-blind randomized clinical trial. BMC gastroenterology, [S.l.], v. 15, p. 1-13, mar. 2015. doi: 10.1186/s12876-015-0260-z.

LOWRY, O. H. et al. Protein measurement with the folin phenol reagent*. Jounal of Biological Chemistry, [S.l.], [S.v.], n. 193, p. 265-275, maio. 1951.

MEIER, R.; HAWARY, R. Avanços em nutriçãoclínica: prebióticos e simbióticosnapráticaclínica. In: SAWAYA, A. L.; LEANDRO, C. G.; WAITZBERG, D. L. (Ed.). Fisiologia da nutriçãonasaúde e nadoença: da biologia molecular aotratamento. 2. ed. Rio de Janeiro: Atheneu, 2018. cap. 43, p. 709-722.

MELO, R.T. et al. Lactobacillus helveticus e suaimportânciana indústria de laticínios. PUBVET, Londrina, v. 5, n. 9, p.1-24, 2011.

MOHAMED, S. et al. Genotoxicity: Mechanisms, Testing Guidelines and Methods. Global Journal of Pharmacy & Pharmaceutical Sciences, Índia, v. 1, n. 15, p. 1-5, mar. 2017. doi: 10.19080/GJPPS.2017.01.555575.

MOHANIA, D. et al. Probiotic Dahi Containing Lactobacillus acidophilus and Bifidobacterium bifidum Modulates the Formation of Aberrant Crypt Foci, Mucin-Depleted Foci, and Cell Proliferation on 1,2-Dimethylhydrazine-Induced Colorectal Carcinogenesis in Wistar Rats. Rejuvenation Research, [S.l.], v. 17, n. 4, p. 325-333, ago. 2014. doi: 10.1089/rej.2013.1537.

NASER, S. M. et al. Lactobacillus suntoryeusCachat and Priest 2005 is a later synonym of Lactobacillus helveticus (Orla-Jensen 1919) Bergey et al. 1925 (Approved Lists 1980). International Journal of Systematic and Evolutionary Microbiology, [S.l.], v. 56, n. 2, p. 355-360, fev. 2006. doi: 10.1099/ijs.0.64001-0.

NOWAK, A.; ŚLIŻEWSKA, K. β-Glucuronidase and β-glucosidase activity and human fecal water genotoxicity in the presence of probiotic lactobacilli and the heterocyclic aromatic amine IQ in vitro. Environmental Toxicology and Pharmacology, [S.l.], v. 37, n. 1, p. 66–73, 2014. doi: 10.1016/J.ETAP.2013.10.014.

O'TOOLE, P. W.; COONEY, J. C. Probiotic bacteria influence the composition and function of the intestinal microbiota. Interdisciplinary perspectives on infectious diseases, [S.l.], v. 2008, [S.n.], p. 1-9, set. 2008. doi: 10.1155 / 2008/175285.

QUIGLEY, E. M. M. The Microbiota in Gastrointestinal Pathophysiology: Implications for human health prebiotics, probiotics, and dysbiosis. 1 ed. EUA: Elsevier, 2017.

SANTOS, L. M.; ANDRADE, A. C. M. M.; LOPES, M. E. M. Microbioma e câncer: correlaçõeslocais ou sistêmicas?. In: FAINTUCH, J. (Org.). Microbioma, disbiose, probióticos e bacterioterapia. São Paulo: Manole, 2017. cap. 4, p. 31-36.

SCHWABE, R. F.; JOBIN, C.. The microbiome and câncer. Nature Reviews Clinical Oncology, EUA, v. 13, n.11, p.800-812, nov. 2013. doi: 10.1038/nrc3610.

SHIDA, K. Probióticos, imunomodulação e saúde: resultadosatuais e perspectivas. In: FAINTUCH, J. (Org.). Microbioma, disbiose, probióticos e bacterioterapia. São Paulo: Manole, 2017. cap. 10, p. 73-81.

SHIN, N.-R.; WHON, T. W.; BAE, J.-W. Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends in biotechnology, [S.l.], v. 33, n. 9, p. 496–503, 2015.

SIEGEL, R.; MILLER, K.. D.; JEMAL, A. Cancer statistics, 2017. CA: A Cancer Journal for Clinicians, EUA, v. 67, n. 1, p. 7-30, jan./fev. 2017. doi: 10.3322/caac.21387.

VANDENPLAS, Y.; HUYS, G.; DAUBE, G. Probiotics: an update. Jornal de Pediatria, Rio de Janeiro, v. 91, n. 1, p. 6-21, jan./fev. 2015. doi: 10.1016/j.jped.2014.08.005.

VERMA, A.; SHUKLA, G. Probiotics Lactobacillus rhamnosusGG , Lactobacillus acidophilus Suppresses DMH-Induced Procarcinogenic Fecal Enzymes and Preneoplastic Aberrant Crypt Foci in Early Colon Carcinogenesis in Sprague Dawley Rats. Nutrition and Cancer, [S.l.], v. 65, n. 1, p. 84-91, jan. 2013. doi: 10.1080/01635581.2013.741746.

WALIA, S. et al. Cyclooxygenase as a Target in Chemoprevention by Probiotics During 1,2-Dimethylhydrazine Induced Colon Carcinogenesis in Rats. Nutrition and Cancer, [S.l.], v. 67, n. 4, p. 603-611, mar. 2015. doi: 10.1080 / 01635581.2015.1011788.

ZHANG, M. et al. Effects of Lactobacillus salivarius Ren on cancer prevention and intestinal microbiota in 1, 2-dimethylhydrazine-induced rat model. Journal of Microbiology, [S.l], v. 53, n. 6, p. 398-405, jun. 2015. doi: 10.1007 / s12275-015-5046-z.

ZHU, J. et al. Lactobacillus salivarius Ren prevent the early colorectal carcinogenesis in 1, 2-dimethylhydrazine-induced rat model. Journal of Applied Microbiology, [S.l.], v. 117, n. 1, p. 208-216, jul. 2014. doi: 10.1111 / jam.12499.

ŻÓŁTASZEK, R. et al. Biologicznarolakwasu D-glukarowegoijegopochodnych; potencjalnezastosowanie w medycynie.PostepyHig Med Dosw, [S.l., S.v.], n. 62, p.451-462, 2008.

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