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Prevention of DNA damage and anticarcinogenic activity of Activia® in a preclinical model

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ABSTRACT. Colorectal cancer is a global public health issue. Studies have pointed to the protective effect of probiotics on colorectal carcinogenesis. Activia® is a lacto probiotic product that is widely consumed all over the world and its beneficial properties are related,

mainly, to the lineage of traditional yoghurt bacteria combined with a specific bacillus, DanRegularis, which gives the product a proven capacity to intestinal regulation in humans. The aim of this study was to evaluate the antigenotoxic, antimutagenic, and anticarcinogenic proprieties of the Activia product, in response to damage caused by 1,2-dimethylhydrazine (DMH) in Swiss mice. Activia does not have shown antigenotoxic activity. However, the percent of DNA damage reduction, evaluated by the antimutagenicity assay, ranged from 69.23 to 96.15% indicating effective chemopreventive action. Activia reduced up to 79.82% the induction of aberrant crypt foci by DMH. Facing the results, it is inferred that Activia facilitates the weight loss, prevents DNA damage and pre-cancerous lesions in the intestinal mucosa.

Key words: Probiotic; Chemoprevention; Colorectal cancer

INTRODUCTION

According to GLOBOCAN (2012), 17.113.588 new cancer cases are estimated for 2020 when 1,678,127 people will be diagnosed with colorectal cancer. The high incidence of this disease makes it an important worldwide public health problem.

Colorectal cancer is originated from polyps, benign lesions that grow on the inner walls of the large intestine which in most cases when detected early can be treated (Tariq and Ghias, 2016).

The most important risk factor for this type of neoplasia in human beings is the family history of colon and rectal cancer as well as the genetic predisposition for developing chronic bowel diseases (adenomatous polyposis). Alcohol intake, cigarettes, and diet habits (low intake of natural products and excess quantity of processed meat) may also increase the risk of colorectal cancer (Stevens et al., 2007).

Based on the above, there is a clear correlation between an inadequate diet and an increased incidence of colorectal cancer (Chen and Kong, 2004; Thomasset et al., 2007). In contrast, high intake of fiber (prebiotics) can reduce the risk of colon cancer (Ishii et al., 2011; Pesarini et al., 2013; Mauro et al., 2013; Cantero et al., 2015; Navarro et al., 2015). Besides prebiotics and probiotics (Cui et al., 2013), the symbiotic relationship between immune cells and gut microbiota (Yamamoto and Matsumoto, 2016) in the prevention of this disease also needs to be highlighted. Foods with the above mentioned benefits are directly associated with the prevention of diseases or are able to beneficially contribute to the individual's health, being called functional foods (Oliveira et al., 2006, 2007; Ogunremi et al., 2015). Among commercialized probiotic products, we can refer to the industrialized product Activia® (Danone), which is consumed worldwide. Its composition is basically fermented milk with bacterium strains of traditional yogurt combined with a specific bacillus (DanRegularis) promoting benefits to the human intestinal transit (Waitzberg et al., 2006).

Several studies indicate the anticarcinogenic activity of probiotics and the protective mechanism that, although not fully described, would be due to the reduction of pathogenic bacteria of the intestinal flora and bile acid the increase of short chain fatty acid production, and the modulation of the inflammatory response and acceleration and/or regulation of

intestinal transit, which would diminish the contact of the intestinal mucosa with xenobiotics and carcinogens (Durko and Malecka-Panas, 2014). Some studies have shown an association between antigenotoxicity/antimutagenic and colon cancer prevention (Navarro et al., 2015; Cantero et al., 2015; Pattananandecha et al., 2016); however, there is no description in the literature related to the effects of Activia® product or DanRegularis lineage regarding the prevention of DNA damage and its possible correlation with the prevention of aberrant crypt foci, an important bioindicator of colorectal cancer.

Considering the above, the present study evaluated the antigenotoxic, antimutagenic, and anticarcinogenic effects of the Activia® product on chemical carcinogenesis induced by 1,2-dimethylhydrazine (DMH).

MATERIAL AND METHODS

Induction of colorectal carcinogenesis

For the induction of aberrant crypt foci (ACF), DMH (Sigma, USA, CAS No. 306-37-6) was utilized in a concentration of 20 mg/kg body weight (b.w.) administered twice a week for two consecutive weeks, intraperitoneally (*ip*) (Ishii et al., 2011; Mauro et al., 2013; Cantero et al., 2015; Navarro et al., 2015). During the use, DMH was diluted in EDTA solution (0.37 mg/mL) with the same solution used as a vehicle for the control group treatment (Ishii et al., 2011; Mauro et al., 2013; Pesarini et al., 2013; Cantero et al., 2015; Navarro et al., 2015). DMH is an indirect trigger of colorectal carcinogenesis andone of the most used drugs in experimental models (Jucá et al., 2014).

Probiotic

The probiotic used was the commercial product Activia® at a dosage of 0.1 mL/10 g b.w. administered by gavage (*vo*).

Animals

A total of 70 Swiss (*Mus musculus*) male, sexually mature mice, with an average weight of 40 g were used. The animals were from the Center for Nutrition and Genetic Toxicology- CENUGEN and experiments were carried out in the vivarium of small mammals of the Universidade Estadual de Londrina - PR. The animals were kept in polypropylene boxes, covered with wood shavings that were changed daily. The diet consisted of basal diet (Nuvital®) and filtered water, *ad libitum*. Temperature and light were controlled using a 12-h photoperiod (12-h light/12-h dark) at a constant temperature of 22° ± 2°C and humidity of 55 ± 10%. The Experiment respected the goals of the Universal Declaration of the Rights of Animals and was approved by the Ethics Committee on Animal Use of Universidade Federal de Mato Grosso do Sul under protocol No. 454/2012.

Experimental design

The animals were divided into seven experimental groups (N = 10) and were treated for 12 weeks. The collection of blood samples (caudal puncture) was carried out on 24 h (T1)

to evaluate the (anti) genotoxicity by the comet assay and 24, 48, and 72 h, corresponding to T1, T2, and T3, respectively, to evaluate the (anti) mutagenicity using the micronucleus test. At the end of the experimentation period (12 weeks) the animals were euthanized for evaluation of (anti) carcinogenicity through the foci of aberrant crypts (Mauro et al., 2013; Pesarini et al., 2013; Navarro et al., 2015). The control group received distilled water by gavage every day until the 12th week. In the 3rd and 4th weeks they were administered four doses, two in each week, of EDTA solution (0.1 mL/10 g b.w., *ip*). The DMH group received distilled water by gavage every day until the 12th week. In the 3rd and 4th weeks, they were administered a total of four doses of DMH (20 mg/kg b.w., *ip*), two doses each week. The Activia® group received the Activia® product by gavage (0.1 mL/10 g b.w., *vo*) every day until the 12th week. In the 3rd and 4th weeks, four doses of EDTA solution (0.1 mL/10 g b.w., *ip*) were administered, two each week. Pre-treatment group (Activia®) received the Activia® product (0.1 mL/10 g b.w., *vo*) during the first 2 weeks. In the subsequent 2 weeks it was administered distilled water (*vo*) and two doses of DMH (*ip*), twice a week. After the DMH administration, the animals were given distilled water up to the 12th week (*vo*). The simultaneous group (Activia®) received distilled water (*vo*) during the first 2 weeks. In the two subsequent weeks, it was administered Activia® product (*vo*) and two doses of DMH (*ip*) twice a week. From the 5th week on it was given distilled water (*vo*). The post-treatment group (Activia®) received distilled water (*vo*) in the first 4 weeks, and in the 3rd and 4th weeks, DMH was administered (*ip*) twice, each week. In the following eight weeks it was administered Activia® (*vo*). The pre + continuous group (Activia®) received Activia® product during the 12-week experiment. DMH (*ip*) was given twice a week during the 3rd and 4th weeks.

Biological assays

Comet assay

For the comet assay, pre-covered slides were prepared with normal agarose (5%) where it was deposited on 30 μL peripheral blood and 120 μL agarose LMP (1.5%) at 37°C. The slides were covered with coverslip glass and cooled to 4°C for 20 min. Coverslips were removed and slides immersed in solution composed of 89 mL lysis stock (2.5 M NaCl, 100 mM EDTA, 10.0 mM Tris, pH 10, corrected with solid NaOH, 890 mL distilled water and 1% sodium lauroyl sarcosinate), 1.0 mL Triton X-100 and 10 mL DMSO, for 1 h, protected from light and at 4°C. Then, the slides were placed in a electrophoresis tank with buffer of pH > 13.0 (300 mM NaOH and 1 mM EDTA, prepared from a stock solution of 10 N NaOH and 200 mM EDTA, pH 10.0) for 20 min at 4°C for DNA denaturation. The electrophoresis was performed at 25V and 300 mA (1.25V/cm) for 20 min and the neutralization with pH 7.5 buffer (0.4M Tris-HCl) during 3 cycles of 5 min. After the neutralization, the slides were air dried and fixed in absolute ethanol for10 min, and subsequently stored until the moment of analysis. The Comet analysis was performed under epifluorescence microscopy (Bioval®) at 40X magnification with 420-490-nm excitation filter and 520-nm barrier filter, the slides were previously subjected to staining with ethidium bromide 100 mL (20 mg/mL).The analyzed cells, a total of 100 cells per slide, were classified as Class 0 - nucleoids that are not damaged and do not have tail; Class 1 - nucleoid tail shorter than the diameter of the nucleoid; Class 2 - nucleoids with tail size between 1 to 2 times the diameter of the nucleoid; Class 3 - nucleoids with tail 2 times longer than the diameter of the nucleoid. The nucleoids of apoptotic cells,

which showed totally fragmented, were not counted (Kobayashi et al., 1995). The total score was calculated by summing the resulting values from the multiplication of the total cells observed in each lesion class. The statistical analysis was performed using ANOVA or the Tukey test ($P < 0.05$) (Oliveira et al., 2015).

Micronucleus assay

The micronucleus assay is important to identify clastogenic and aneugenic agents and was carried out according to Hayashi et al. (1990) with the modifications suggested by Oliveira et al. (2009). Glass slides were covered with a layer of 20 μL acridine orange (1.0 mg/mL) and after dried, 50 μL peripheral blood was added to the slide followed by a coverslip with subsequently cooling (-20°C) for a minimum period of 7days. After the storage period, the analysis was carried out with an epifluorescence microscope (Bioval®) at 40X magnification with 420- 490-nm excitation filter and 520-nm barrier filter (2000 cells/animals were analyzed). The statistical analysis was obtained by using ANOVA or the Tukey test.

Aberrant crypt focus essay

The euthanasia of the animals was done by cervical dislocation at the end of the 12th week of treatment. The heart, lung, liver, and kidneys were weighed, then the colon was collected and fixed in 10% formalin-buffered solution. For analysis, the colon segment was colored with 0.1% methylene blue solution for 10 min. After overnight fixation, the intestines were arranged on a glass slide with the luminal surface facing up. The analysis took place in bright field microscopy (D.B.G.®) at 10X magnification. All the mucosa was assessed for identification and quantification of aberrant crypt foci, based on the criteria described by Bird (1987), with modifications. Statistical analysis was performed among the groups, comparing the average of focus number. Data concerning the total number of ACF, aberrant crypts per foci, and the relation of crypt/foci were also analyzed. Statistical analysis was done by ANOVA or the Tukey test ($P < 0.05$) (Ishii et al., 2011; Mauro et al., 2013; Pesarini et al., 2013; Cantero et al., 2015; Navarro et al., 2015).

Percentage of damage reduction (%DR)

The %DR of the DMH Activia® product was calculated by the number of cells with Comet / micronucleus / aberrant crypt foci observed in the DMH treatment minus the number of cells with Comet / micronucleus / crypt foci aberrant observed in associated groups, divided by the number of cells with comet / micronucleus / aberrant crypt foci observed in the DMH treatment minus the cells with comet / micronucleus / aberrant crypt foci observed in the control group. The result was multiplied by 100 (Ishii et al., 2011; Mauro et al., 2013; Pesarini et al., 2013; Cantero et al., 2015; Navarro et al., 2015).

RESULTS

Biometric parameters

Biometric data demonstrated that animals had similar weights at the beginning of the

experiment. However, at the end of the experiment, there was a reduction ($P<0.05$) in the weight of the animals from all groups except for the simultaneous treatment group. When the weight gain was evaluated, significant difference was noted only between the control group and pre + continuous treatment protocol (Table 1). Regarding the absolute organ weight, statistically significant difference was only observed between Activia® treatments when compared with pre-and post-treatment groups. There was no difference ($P > 0.05$) in the relative weight of organs (Table 2).

Table 1. Mean values for initial weight, final weight and weight gain of the animals during the experimental period.

Experimental group	Initial weight (g)	Final weight (g)	Weight gain (g)
Control	41.70 ± 0.59^{a}	50.00 ± 0.45^{c}	8.30 ± 0.73^{b}
DMH	41.00 ± 0.66^{a}	$48.80 \pm 0.97^{b,d,e}$	$7.80 \pm 0.64^{a,b}$
Activia®	40.00 ± 0.14^{a}	$46.20 \pm 0.49^{a,b}$	$6.20 \pm 0.41^{a,b}$
Pre-treatment	40.30 ± 0.21^{a}	$47.00 \pm 0.49^{a,b,d}$	$6.70 \pm 0.53^{a,b}$
Simultaneous	40.90 ± 0.23^{a}	$49.30 \pm 0.65^{c,d,e}$	8.40 ± 0.56^{b}
Post-treatment	41.00 ± 0.25^{a}	$48.70 \pm 0.59^{a,b,e}$	$7.70 \pm 0.55^{a,b}$
Pre + continuous	41.00 ± 0.25^{a}	$46.60 \pm 0.49^{a,b,e}$	5.60 ± 0.47^{a}

g - grams; Control - negative control - EDTA 0.1 mL/10 g b.w. (body weight); DMH - DMH (dimethylhydrazine) 20 mg/kg b.w. for 2 weeks (positive control); Activia® - Activia® 0.1 mg/10 g b.w. for 12 weeks; Pre-treatment - Activia® 0.1 mg/10 g b.w. during the first 2 weeks + DMH 20 mg/kg b.w. for 2 weeks; Simultaneous - simultaneous treatment - Activia® 0.1 mg/10 g b.w. + DMH 20 mg/kg, b.w. for 2 weeks; Post-treatment - DMH 20 mg/kg b.w. during the first 2 weeks + Activia® 0.1 mg/10 g b.w. for the following 10 weeks; Pre + Continuous treatment - Activia® 0.1 mg/10 g b.w. for 12 weeks + DMH 20 mg/kg b.w. for 2 weeks. Different letters indicate statistically significant differences ($P < 0.05$; ANOVA/Tukey).

Table 2. Mean values of absolute and relative weights with regard to the means for the other organs of the animals after of experimentation period.

Experimental group	Maternal weight (g)				Relative organ weight (g)			
	Heart	Lungs	Kidneys	Liver	Heart	Lungs	Kidneys	Liver
Control	0.2606 ± 0.0119^{a}	0.2934 ± 0.0068^{b}	0.6749 ± 0.0184^{a}	$2.1724 \pm 0.0581^{a,b}$	0.0062 ± 0.0002^{a}	0.0070 ± 0.0001^{a}	0.0162 ± 0.0005^{a}	0.0520 ± 0.001^{a}
DMH	0.2930 ± 0.0102^{a}	0.2430 ± 0.0130^{a}	0.6840 ± 0.0297^{a}	$2.1920 \pm 0.0565^{a,b}$	0.0071 ± 0.0003^{a}	0.0059 ± 0.0003^{a}	0.0166 ± 0.0006^{a}	0.0535 ± 0.001^{a}
Activia®	0.2602 ± 0.0056^{a}	$0.2559 \pm 0.0057^{a,b}$	0.6616 ± 0.0086^{a}	2.3813 ± 0.0884^{b}	0.0065 ± 0.0001^{a}	0.0063 ± 0.0001^{a}	0.0165 ± 0.0002^{a}	0.0595 ± 0.002^{a}
Pre-treatment	0.2800 ± 0.0214^{a}	0.2830 ± 0.0120^{b}	0.6430 ± 0.0195^{a}	2.0120 ± 0.0928^{a}	0.0069 ± 0.0005^{a}	0.0070 ± 0.0003^{a}	0.0159 ± 0.0004^{a}	0.0499 ± 0.002^{a}
Simultaneous	0.2700 ± 0.0126^{a}	$0.2770 \pm 0.0076^{a,b}$	0.6790 ± 0.0235^{a}	2.4950 ± 0.1068^{b}	0.0066 ± 0.0002^{a}	0.0067 ± 0.0001^{a}	0.0165 ± 0.0005^{a}	0.0610 ± 0.002^{a}
Post-treatment	0.2720 ± 0.0134^{a}	$0.2680 \pm 0.0071^{a,b}$	0.6330 ± 0.0213^{a}	2.0080 ± 0.1145^{a}	0.0066 ± 0.0003^{a}	0.0065 ± 0.0001^{a}	0.0154 ± 0.0005^{a}	0.0489 ± 0.002^{a}
Pre + continuous	0.2800 ± 0.0051^{a}	$0.2550 \pm 0.0093^{a,b}$	0.6580 ± 0.0218^{a}	$2.2690 \pm 0.0610^{a,b}$	0.0070 ± 0.0001^{a}	0.0062 ± 0.0002^{a}	0.0160 ± 0.0005^{a}	0.0553 ± 0.001^{a}

g - grams; Control - negative control - EDTA 0.1 mL/10 g b.w. (body weight); DMH - DMH (dimethylhydrazine) 20 mg/kg b.w. for 2 weeks (positive control); Activia® - Activia® 0.1 mg/10 g b.w. for 12 weeks; Pre-treatment - Activia® 0.1 mg/10 g b.w. during the first 2 weeks + DMH 20 mg/kg b.w. for 2 weeks; Simultaneous - simultaneous treatment - Activia® 0.1 mg/10 g b.w. + DMH 20 mg/kg, b.w. for 2 weeks; Post-treatment - DMH 20 mg/kg b.w. during the first 2 weeks + Activia® 0.1 mg/10 g b.w. for the following 10 weeks; Pre + Contínuous treatment - Activia® 0.1 mg/10 g b.w. for 12 weeks + DMH 20 mg/kg b.w. for 2 weeks. Different letters indicate statistically significant differences ($P < 0.05$; ANOVA/Tukey).

Toxicogenetics and chemopreventive evaluation

The frequency of damaged cells in the comet assay increased in 18.61 and 4.21X when animals were treated with DMH and Activia®, respectively. DMH associated with Activia® demonstrated %DR of 9.06, 6.98, -0.65, and 16.59% for the pre-treatment, simultaneous, post-treatment, and pre + continuous protocols, respectively (Table 3).

Table 3. Means ± standard deviation of the frequency of damaged cells, distribution among the classes of DNA damage and scores with regard to the score in the tests for genotoxicity and antigenotoxicity (comet assay) of Activia®.

Experimental group	Damaged cells	Damage classes				Score
		0	1	2	3	
Genotoxicity						
Control	5.20 ± 0.95[a]	94.80 ± 0.95	3.50 ± 0.80	1.50 ± 0.37	0.20 ± 0.13	7.20 ± 1.20[a]
DMH	96.80 ± 0.74[e]	3.2 ± 0.74	8.10 ± 0.70	45.00 ± 2.17	43.10 ± 1.58	227.40 ± 3.05[f]
Activia®	21.90 ± 1.37[b]	78.10 ± 1. 37	16.00 ± 1.31	4.90 ± 1.02	1.00 ± 0.25	28.80 ± 2.03[b]
Antigenotoxicity						
Pre-treatment	88,50 ± 0,90[d]	11,50 ± 0.90	46.50 ± 1.86	24.80 ± 1.34	17.20 ± 0.86	147.70 ± 2.32[c]
Simultaneous	90.40 ± 0.74[d]	9.60 ± 0.74	48.60 ± 2.37	33.20 ± 1.94	8.60 ± 0.93	140.80 ± 3.29[c]
Post-treatment	97.40 ± 0.67[e]	2.60 ± 0.67	29.80 ± 0.85	44.4 ± 1.66	23.20 ± 1.50	188.20 ± 1.23[e]
Pre + continuous	81.60 ± 0.79[c]	18.40 ± 0.79	27.7 ± 0.70	29.60 ± 1.74	24.30 ± 1.81	159.80 ± 2.59[d]

g - grams; Control - negative control - EDTA 0.1 mL/10 g b.w. (body weight); DMH - DMH (dimethylhydrazine) 20 mg/kg b.w. for 2 weeks (positive control); Activia® - Activia® 0.1 mg/10 g b.w. for 12 weeks; Pre-treatment - Activia® 0.1 mg/10 g b.w. during the first 2 weeks + DMH 20 mg/kg b.w. for 2 weeks; Simultaneous - simultaneous treatment - Activia® 0.1 mg/10 g b.w. + DMH 20 mg/kg, b.w. for 2 weeks; Post-treatment - DMH 20 mg/kg b.w. during the first 2 weeks + Activia® 0.1 mg/10 g b.w. for the following 10 weeks; Pre + Continuous treatment - Activia® 0.1 mg/10 g b.w. for 12 weeks + DMH 20 mg/kg b.w. for 2 weeks. Different letters indicate statistically significant differences ($P < 0.05$; ANOVA/Tukey).

The micronucleus assay demonstrated that DMH is mutagenic an dincreased ($P < 0.05$) the frequency of DNA damage during the time points evaluated. Activia® is not mutagenic. Antimutagenicity protocols showed a reduction ($P < 0.05$) in the frequency of micronuclei, for all protocols and time points evaluated. The %DRs of the pre-treatment, simultaneous treatment, post-treatment, and pre + continuous protocols were 81.56, 106.38, 80.85, and 95.03% for T1; of 87.17, 92.30, 78.63, and 77.77 for T2; and 69.23, 75.64, 83.33, and 96.15% for T3, respectively (Table 4).

Table 4. Total frequency, mean values ± standard deviation and %DR related to mutagenicity and antimutagenicity tests (micronucleus assay) in peripheral blood of mice treated with Activia®.

Experimental group	Total frequency of micronuclei			Means ± SD			%DR		
	T1	T2	T3	T1	T2	T3	T1	T2	T3
Mutagenicity									
Control	78	40	18	7.8 ± 0.84[a,b]	4 ± 0.39[a]	1.8 ± 0.29[a]	-	-	-
DMH	219	157	96	21.9 ± 0.85[c]	15.7 ± 0.51[c]	9.6 ± 0.79[c]	-	-	-
Activia®	104	60	31	10.4 ± 0.83[b]	6 ± 0.49[a,b]	3.1 ± 0.48[a,b]	-	-	-
Antimutagenicity									
Pretreatment	104	55	42	10.4 ± 0.79[b]	5.5 ± 0.84[a,b]	4.2 ± 0.59[b]	81.56	87.17	69.23
Simultaneous	69	49	37	6.9 ± 0.56[a]	4.9 ± 0.58[a,b]	3.7 ± 0.51[a,b]	106.38	92.30	75.64
Post-treatment	105	65	31	10.5 ± 0.71[b]	6.5 ± 0.40[b]	3.1 ± 0.40[a,b]	80.85	78.63	83.33
Pre + Continuous	85	66	21	8.5 ± 0.86[a,b]	6.6 ± 0.52[b]	2.1 ± 0.45[a,b]	95.03	77.77	96.15

%DR - percent reduction of DNA damage; g - grams; Control - negative control - EDTA 0.1 mL/10 g b.w. (body weight); DMH - DMH (dimethylhydrazine) 20 mg/kg b.w. for 2 weeks (positive control); Activia® - Activia® 0.1 mg/10 g b.w. for 12 weeks; Pre-treatment - Activia® 0.1 mg/10 g b.w. during the first 2 weeks + DMH 20 mg/kg b.w. for 2 weeks; Simultaneous - simultaneous treatment - Activia® 0.1 mg/10 g b.w. + DMH 20 mg/kg, b.w. for 2 weeks; Post-treatment - DMH 20 mg/kg b.w. during the first 2 weeks + Activia® 0.1 mg/10 g b.w. for the following 10 weeks; Pre + Contínuous treatment - Activia® 0.1 mg/10 g b.w. for 12 weeks + DMH 20 mg/kg b.w. for 2 weeks. Different letters indicate statistically significant differences ($P < 0.05$; ANOVA/Tukey).

Evaluation of colorectal anticarcinogenesis

DMH has induced an average of 22.80 ± 1.26 ACFs. In control groups and Activia® treatments, ACF were absent. Anticarcinogenicity protocols showed a reduction ($P < 0.05$) of

the ACF frequency in all treatments and the %DR were 79.82, 74.12, 75.43, and 69.73% for the pre-treatment, simultaneous treatment, post-treatment, and pre + continuous protocols, respectively (Table 5).

Table 5. Number, distribution and reduction in damage with regard to aberrant crypt foci (ACF) in the colon of male mice.

Experimental group	Total of ACF		%RD	Total of AC	Absolute values of AC/Foci				Relation AC/Foci
	Absolute values	Means ± SEM			1AC/Foci	2AC/Foci	3AC/Foci	4AC/Foci	
Carcinogenicity									
Control	0.00	0[a]	-	0.00	0.00	0.00	0.00	0.00	0.00
DMH	228	22.80 ± 1.26[c]	-	438	95	75	39	19	1.92
Activia®	0.00	0[a]	-	0.00	0.00	0.00	0.00	0.00	0.00
Anticarcinogenicity									
Pretreatment	46	4.60 ± 0.60[b]	79.82	73	27	11	8	0	1.58
Simultaneous	59	5.90 ± 0.75[b]	74.12	90	36	15	8	0	1.52
Post-treatment	56	5.60 ± 0.30[b]	75.43	106	23	16	17	0	1.89
Pre + Continuous	69	6.90 ± 1.11[b]	69.73	116	35	22	11	1	1.68

g - grams; Control - negative control - EDTA 0.1 mL/10 g b.w. (body weight); DMH - DMH (dimethylhydrazine) 20 mg/kg b.w. for 2 weeks (positive control); Activia® - Activia® 0.1 mg/10 g b.w. for 12 weeks; Pre-treatment - Activia® 0.1 mg/10 g b.w. during the first 2 weeks + DMH 20 mg/kg b.w. for 2 weeks; Simultaneous - simultaneous treatment - Activia® 0.1 mg/10 g b.w. + DMH 20 mg/kg, b.w. for 2 weeks; Post-treatment - DMH 20 mg/kg b.w. during the first 2 weeks + Activia® 0.1 mg/10 g b.w. for the following 10 weeks; Pre + Continuous treatment - Activia® 0.1 mg/10 g b.w. for 12 weeks + DMH 20 mg/kg b.w. for 2 weeks. Different letters indicate statistically significant differences ($P < 0.05$; ANOVA/Tukey).

DISCUSSION

There is considerable evidence that the intestinal bacterial flora is involved in the development of colon/rectal cancer. This fact increased interest in diets containing agents that can influence the microflora and reduce the potential risk of colon cancer (Durko and Malecka-Panas, 2014). This caused an increase in consumption of probiotics, mainly due to the capability of reducing the number of fecal putrefactive bacteria such as coliforms and at the same time increase the number of commensal bacteria, such as lactobacilli (Shoaib et al., 2015). Despite of various studies about the beneficial effects of intestinal microflora on the development of colorectal cancer and the prevention of DNA damage (Brennan and Garrett, 2016), no record in the literature about the (anti)carcinogenic, (anti)genotoxic or (anti)mutagenic potential of Activia® and DanRegularis strains was found, which makes this a pioneering study. Thus, for the first time it was described that this commercial product is capable of preventing DNA damage and prevent aberrant crypt foci, which is an important biomarker for colorectal carcinogenesis.

In a systematic analysis, biometric parameters do not indicate excessive weight loss that would correlate to toxicity (Magosso et al., 2016). Also, no changes were observed in the behavior or in variations in feed intake or water. According to Christian (2001), toxicity can also be inferred by the presence of the bristly, hypo- or hyperactivity, presence of sialorrhea, diarrhea, and/or death, for example.

Regarding weight loss it should be considered that Activia® and Pre + Continuous groups, which received the probiotic for a longer period, presented a greater weight reduction. Thus, it is suggested that the Activia® intake being able to regulate the intestinal transit (Katan, 2008), can contribute to weight loss.

Regarding the absolute and relative weight of organs, no notable change was recorded and this confirms the absence of toxicity in the experiment. Other studies also use these two

parameters to prove the absence of toxicity (Mauro et al., 2013; Cantero et al., 2015; Navarro et al., 2015; Martello et al., 2016).

When evaluated the comet assay, it was observed that the Activia® caused an increase of DNA damage frequency. However, the damage was probably repaired since the product showed no mutagenic potential. However, this kind of result requires further studies so that we can understand the genotoxic effect found. Besides, literature did not show any previous report that could help us to understand these data. However, the fact that the damage was not fixed on the cellular genome, in the form of micronuclei, supports the use of the product. Evaluation of antigenotoxicity showed that there was low %DRs in the pre-treatment, simultaneous and pre +continuous protocols and absence in the post-treatment protocol. However, the antimutagenicity effect showed %DRs near or above 70% and also values reaching more than 100%. These data demonstrate an important chemopreventive activity and also suggest that Activia® can prevent basal damage. Examples of functional food (prebiotic) with basal damage prevention capacity were also recorded by Karnjanapratum et al., (2016).

The difference in the percentage of reducing DNA damage, i.e., the difference in frequency of micronuclei and comets, can be elucidated when thinking that the two tests evaluate different kinds of damage. The micronucleus test quantifies the cytogenetic damage, mutational events already set in the cell genome (Salvadori et al., 2003). On the other hand, the comet assay indicates genotoxic damage, which may or may not become mutations as they are subject to repair (Oliveira et al., 2007). Antimutagenic agents can prevent the initiation or suppress carcinogenesis by reverting the promotion and/or progression of cancer, presumably affecting decisive factors that control the differentiation, cell proliferation, cell senescence, and/or apoptosis (CWG, 1999). Also, there are two basic types of DNA protective effects: desmutagenic substances, which are capable of blocking damage-inducing agents mainly by adsorbing them and largely actind in the extracellular environment, and bioantimutagenic substances, which are capable of preventing damage or working at the level of DNA repair, acting within the cell (Kada and Shimoi, 1987). Antimutagenic agents can also act as modulators of repair and replication of the DNA; stimulating the error-free repair or inhibiting the repair systems subject to error (De Flora, 1998).

From the different protocols proposed in the literature, this study utilized the pre-treatment, simultaneous, and post-treatment protocols. It was also utilized as an alternative protocol of pre+continuous proposed by Ishii et al. (2011), Mauro et al. (2013), Pesarini et al. (2013), and Navarro et al. (2015). According to these authors it was considered that pre-treatment protocols, simultaneous treatment, and pre + continuous indicate both the desmutagenesis and bioantimutagenesis activity and the post-treatment act exclusive by bioantimutagenesis. Thus, it is inferred that the Activia® can act as both desmutagenesis and bioantimutagenesis, since the %DR were high for all protocols and time points. It is assumed then that this probiotic modes of action can be either by chemical inactivation and/or enzymatic (intercepting the carcinogen) as the stimulation of machinery DNA repair. It can also be assumed that the probiotic microorganisms can remove the source of pro-carcinogens or putrefactive bacteria involved in the compounds' conversion from pro-carcinogens to carcinogens (Saber et al., 2016). Antimutagenicity results also stimulate the anticarcinogenicity evaluation since there is a direct correlation between mutagenesis and carcinogenesis (Ribeiro, 2003), and thus, establishes a relationship between antimutagenesis and anticarcinogenesis. The biomarker chosen for indirect evaluation of anticarcinogenesis was the ACF induced by DMH (Tawfik and Mohamed, 2016). Based on this context Activia®

can be indicated as an anticancer product since it reduced the incidence of aberrant crypt foci around 70 to 80%. This compound was still able to reduce the relation crypt/foci, which strengthen its anticarcinogenic action as indicated by Pattananandecha et al. (2016). Besides the modes of action mentioned above, it is important to highlight that the DMH is hydrolyzed in the liver and generates methylazoxymethanol, which in conjunction with β-glucuronic acid is transported to the intestinal lumen where the β-glucuronidase bacterial enzyme releases the active metabolite of the DMH, the azoxymethane (LaMont and O'Gorman, 1978). This metabolite triggers an inflammatory reaction and an increase in cell proliferation in the rodent colonic mucosa culminating in development of ACF and cause GC → AT mutations in specific genes related to colorectal cancer such as β-catenin and K-ras (Krutovskikh and Turosov, 1994). Thus, the reduction of bacteria capable of converting these pro-carcinogens, indirectly reduces mucosal damage and inflammation. This reduction of unwanted bacteria can be achieved through the increased resistance. The increased resistance against pathogens is the most promising feature in the probiotic development. The use of probiotic cultures excludes potentially pathogenic microorganisms and enhances the natural mechanisms of the body's defense (Puupponen-Pimia et al., 2002). The modulation of the intestinal microbiota by the probiotic microorganisms occurs through a mechanism called competitive exclusion. This mechanism prevents the colonization of the mucosa by potentially pathogenic microorganisms, by competition for adhesion sites, competition for nutrients and/or production of antimicrobial compounds, and by connecting the final carcinogen promoting their removal via faeces (Durko and Malecka-Panas, 2014). Facing that, it is considered that the Activia® product favors the weight reduction, prevents DNA damage and precancerous lesions in the intestinal mucosa.

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