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Original/*Cancer*

Prebiotics prevent the appearance of aberrant crypt foci (ACF) in the colon of Balb/C mice for increasing the gene expression of p16 protein

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Abstract

Studies have shown fibers to be effective in reducing the appearance of aberrant crypt foci (ACF) in rodents. *Objective:* The goal of this study was to investigate the preventive effects of fructooligosaccharide (FOS) and inulin prebiotics on the appearance of ACF in mice.

***Materials and Methods:* The techniques used were: RT-PCR to evaluate the gene expression of p16, p21, p54, cyclin D1 and cyclin E in the distal colon; the quantification of Number of aberrant crypt foci (ACF) and measurement of catalase activity in the liver and distal colon. The animals were divided into five treatments (n=8); C-: AIN93M diet without fibers + DMH (1,2-dimethylhydrazine); INL: AIN93M diet with inulin; INLCA: AIN93M diet with inulin + DMH; FOS: AIN93M diet with FOS; FOSCA: AIN93M diet with FOS + DMH, during 15 weeks.**

***Results:* Inulin prevented the appearance of ACF in the proximal, middle and distal colon, compared to the control without fibers. In the middle and distal colon, FOS was also effective in preventing the incidence of ACF. This effectiveness may be attributed to the increased gene expression of p16 following FOS treatment. Both prebiotics also decreased catalase activity in the distal colon, thus suggesting an antioxidant effect.**

***Conclusion:* These results suggesting an antioxidant effect prebiotics that may be attributed to the increased gene expression of p16.**

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Key words: *aberrant crypt, Prebiotics, prevention colon, p16 protein*

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LOS PREBIÓTICOS PREVIENEN LA APARICIÓN DE FOCOS DE CRIPTA ABERRANTES (FCA) EN EL COLON DE RATONES BALB/C PARA AUMENTAR LA EXPRESIÓN GÉNICA DE LA PROTEÍNA P16

Resumen

Existen estudios que demuestran la eficacia de fibras para reducir la aparición de focos de cripta aberrantes (FCA) en roedores. *Objetivo:* El objetivo de este estudio consistió en investigar los efectos preventivos de los fructooligosacáridos (FOS) y el prebiótico inulina sobre la aparición de FCA en ratones.

***Materiales y métodos:* Las técnicas empleadas fueron: RT-PCR para evaluar la expresión génica de p16, p21, p54, ciclina D1 y ciclina E en el colon distal; la cuantificación del Número de FCA y la medición de la actividad de la catalasa en el hígado y el colon distal. Los animales fueron divididos en cinco tratamientos (n=8); C-: dieta AIN93M sin fibra + DMH (1.2-dimetilhidrazina); INL: dieta AIN93M con inulina; INLCA: dieta AIN93M con inulina + DMH; FOS: dieta ANIN93M con FOS; FOSCA: dieta AIN93M con FOS + DMH, durante 15 semanas.**

***Resultados:* La inulina previno la aparición de FCA en el colon proximal, medio y distal, comparado con el control sin fibras. En el colon medio y distal, FOS también fue efectiva para prevenir la incidencia de FCA. Esta efectividad podría ser atribuida al aumento de la expresión génica de p16 tras el tratamiento con FOS. Ambos prebióticos también disminuyeron la actividad de la catalasa en el colon distal, lo que sugiere un efecto antioxidante.**

***Conclusión:* Estos resultados sugieren un efecto antioxidante de los prebióticos que podría atribuirse a un aumento de la expresión génica de p16.**

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Palabras clave: *cripta aberrante, prebióticos, prevención colon, proteína p16*.

Introduction

Aberrant crypt foci (ACF) are preneoplastic lesions localized in the mucosa and exhibit morphological changes such as dysplasia and abnormal proliferation. ACF can be observed and quantified microscopically, acting as a marker for the risk of developing colorectal cancer[1,2]. With time, the lesions become more noticeable and less differentiated, acquiring the phenotype of a cancer with metastases[3].

Prebiotics are defined as a non-digestible food ingredient that benefits the host by selective stimulation, growth and activity of a limited number of bacteria in the colon, thus improving the host's health. In animals and humans, prebiotics are selectively used by bifidobacteria to improve the intestinal microbiota leading for a composition of beneficial microorganisms. Prebiotics also decrease the oxidative stress and they increase the catalase activity in the bowel, after producing butyrate, which is a short-chain fatty acid[4].

Prebiotics may also reduce the risk of the development of the colon cancer, by direct and indirect mechanisms. The direct mechanisms influence on the growth of lactic acid bacteria and bifidobacteria providing inhibitory effect in many bacteria, which produce carcinogenic enzymes. Indirect effects include the production of protective metabolites, such as butyrate[5,6]. The Prebiotics of proven efficacy and commercially available are inulin and fructooligosaccharide (FOS), both considered as conventional food, food ingredients or fibers in different countries[7].

Objective

Given the proven beneficial effects of the use of prebiotics as a component of diet, this study was aimed to investigating the preventive effect of soluble fibers, fructooligosaccharide (FOS) and Inulina in mice on the appearance of ACF in mice treated with DMH.

Material and Methods

Animals and diets

Forty 12-wekk old, male Balb/C mice weighing approximately 25 grams, from the Federal University of Viçosa (UFV) were used. During the experiment, the animals were allocated in individual cages that the cleaned daily and maintained in environment with temperature (22°C±2°C), humidity (60-70%) and illumination (12-h light/dark cycles) controlled. The animals were divided into five treatments (n=8); C-: AIN93M diet without fibers + DMH; INL: AIN93M diet with inulin; INLCA: AIN93M diet with inulin + DMH; FOS: ANIN93M diet with FOS; FOSCA: AIN93M diet with FOS + DMH. Animals were given *ad libitum* water and modified AIN93M diet[8], with 10% FOS prebiotics or inulin replacing cellulose and fiber-free diet during 15 weeks. The experiment was conducted according to the Guiding Principles in the Use of Animals Ethics Committee of the Department of Veterinary Medicine of Universidade Federal de Viçosa (registration 169/2009/DVT).

Induction of the appearance of ACF with the administration of 1,2-dimethylhydrazine (DMH) and material collection

Pre-neoplasic colorectal lesions were induced by intraperitoneal injection once a week of 20 mg/kg body weight DMH for 8 weeks. DMH was dissolved in 0.9% saline solution containing 1 mM EDTA and 10 mM sodium citrate, pH 8. Seven weeks after the last DMH administration, the animals were euthanized by CO_2 asphyxiation[9]. The large intestine of the animals was removed from the cecum to the anus for counting ACF. Catalase activity was measured in the distal colon and liver of the animals for evaluation of the oxidative stress caused by the administration of DMH. The mRNA for cyclin-D1, cyclin-E, p16, p21, and p53 was also quantified in the distal colon to evaluate the gene expression of these proteins.

Tissue preparation for ACF counting

After removal, the large intestine was washed in phosphate-buffered saline (PBS) solution, opened along the mesenteric margin, placed in paraffin plates with the mucous facing the top of the plate, and fixed in Carson's formalin for 24 hours. Once set, the large intestine was measured and divided into three equal fragments, called proximal, middle, and distal, in relation to the cecum. Then, the fragments were stained with 0.1% methylene blue solution for 2 minutes. The assessment of ACF was performed using a BX-60® light microscope (Olympus, Tokyo, Japan) at 40x and 100x magnification. The ACF were counted across the mucosal surface of the large intestine, cecum to rectum, by two observers in a double-blind manner. ACF categorization was based on the number of aberrant crypts per focus: foci with fewer than or equal to three crypts (ACF≤3) and foci with more than three crypts (ACF>3)[10].

Catalase activity in the liver and distal colon

For the analysis of catalase activity (CAT), the liver and distal colon of mice were macerated in phosphate buffer, resulting in an homogenate that was packaged in an Eppendorf tube, and centrifuged at 6000 g at 4 ºC for 10 minutes. Then, the supernatant was collected. Subsequent to centrifugation, the homogenate of the liver and distal colon was used to measure CAT. CAT was evaluated by measuring the rate of hydrogen peroxide

decomposition[11]. Total protein levels were measured using the Bradford method[12].

Processing of the spleen to quantify CD4 and CD8 cells

After the mice's euthanasia, the spleens were collected, macerated in PBS, and centrifuged for 10 minutes at 1600 g at 4°C. Then, the supernatant was discarded and the pellets containing the splenocytes and red blood cells were diluted in cold lysis buffer (0.16M NH4Cl; 0.7M Tris HCl, pH 7.5; 2 mM EDTA) and incubated at room temperature for 4 minutes in the dark to lyse the red blood cells. After this step, 8 ml of incomplete RPMI-16 mean with 2 mM of EDTA was added and the cellular suspension was centrifuged for 10 minutes at 1600 g at 4°C. After centrifugation, pellets of splenocytes were resuspended in 3 ml of complete RPMI-1640 and the cells were counted in a Neubauer chamber. Flow cytometry was used to quantify the CD4 and CD8 cells. The antibodies used were acquired from BD Pharmingen, NJ USA (anti-CD44-PE Cy5.5) and Caltag Laboratories, Thailand (anti-CD4-FITC and anti-CD8-FITC). Cells extracted from the spleens of animals from all treatments were incubated with a mixture of antibodies containing anti-CD4+ or anti-CD8+ to evaluate the expression of CD4 and CD8 cells, respectively. After 2 hours of incubation, the samples were centrifuged at 1500 g for 10 minutes and resuspended in PBS with 1% formaldehyde. Samples were analyzed in a GUAVA flow cytometer (Guava EasyCyte Mini System, Millipore, Billerica, MA) and data were analyzed using the Guava CytoSoft 4.2.1 software[13].

Organ weights

After seven weeks from the last DMH application, when animals were euthanized, we weighed the liver, heart and kidney.

Quantification of Bifidobacterium spp., Clostridium spp. and total anaerobes

Bifidobacterium spp. was determined in gar MRS + NPML[14] and total anaerobes were determined on Wilkins-Chalgren Agar. The plated samples containing total anaerobes and *Bifidobacterium spp*. were incubated at 37 ºC in an anaerobic chamber (Gas Pak System – BBL, Sparks, USA) for 48 hours and 72 hours, respectively. For counting *Clostridium spp*., we used Reinforced Clostridial Medium (RCM) agar. The plated samples were incubated at 37ºC in an anaerobic chamber (Gas Pak System – BBL, Sparks, USA) for 48 hours[15]. The analyses were conducted in duplicate and the results for counting *Bifidobacterium spp*. colonies, total anaerobes, and *Clostridium spp*. were expressed in Log_{10} CFU/g.

RT-PCR:

Extraction of mRNA and quantitative analysis of gene expression of cyclin-E, cyclin-D1, p16, p21, and p53 proteins

RNA was extracted after the maceration of the distal colon in an Eppendorf tube using TRIzol® (Invitrogen, CA, USA). We briefly homogenized 500 mL of the sample with 750 mL TRIzol® and the mixture was incubated at room temperature for 5 minutes. Then, we added 300 mL of chloroform, manually agitating the tubes for 15 minutes and incubating them at room temperature for 2 more minutes. The samples were centrifuged at 12,000 g for 15 minutes at 4ºC. The transparent aqueous phase was subsequently separated and transferred to another tube. We then added 350 mL of isopropanol and the samples were incubated at room temperature for 10 minutes to promote the precipitation of RNA and then, they underwent centrifugation for 10 minutes at 4ºC. The pellets were washed with 750 mL ethanol (75%), centrifuged for 10 minutes at 7,500 g and dried at room temperature for 30 minutes. When dried, pellets were resuspended in 20 mL diethylpyrocarbonate (DEPC)-treated water. Approximately 10 mg of each total RNA was used to synthesize the first cDNA strands, using primers for cyclin-D1, cyclin-E, p16, p21, and p53. After the extraction of total RNA from the samples, we performed the synthesis of the first cDNA strand for cyclin-D1, cyclin-E, p16, p21, and p53 using the Superscript™ kit (Invitrogen©, New York, USA).

Synthesis of cDNA

After the extraction of total RNA from the samples, we performed the synthesis of the first cDNA strand for cyclin-D1, cyclin-E, p16, p21 and p53, using the Superscript™ kit (Invitrogen©, New York, USA). 1ml random primer ($pd(N)_6$) (Amersham-Pharmacia, Buckinghamshire, England) was added to approximately 2mg RNA of the colon, then heated for 10 minutes under 70ºC. After the extraction, a mixture containing the reverse transcriptase buffer, DTT, reverse transcriptase and dNTPs was prepared. Then the mixture was heated and added to the sample. Then, this solution was incubated for 1 hour under 42ºC, and for 1 minute under 70ºC.

Quantification of mRNA for cyclin-D1, cyclin-E, p16, p21, and p53 by real-time polymerase chain reaction (RT-PCR).

The gene expression of cyclin-D1, cyclin-E, p16, p21, and p53 was evaluated in the distal colon. After 24 hours, the colon was cleared by centrifugation at 10,000 g for 2 minutes and subjected to RNA extraction and synthesis of complementary DNA, as previously described. The evaluation of gene expression was performed using the

RT-PCR technique, in which the expression of these mRNAs was compared with the expression of the mRNA of b-actin, a constitutive gene. To this end, approximately 2 mg of total cDNA from the distal colon was used for the RT-PCR, in a 50 ml reaction that contained 0.45 nmol of sense and antisense primers suitable for each gene[16,17], 20 U/ml of RNAse inhibitor, and 12.5 ml of SYBR Green PCR Master Mix (Life Technologies do Brasil Ltda, São Paulo, SP). The cycling conditions were 95°C for 10 minutes followed by 40 cycles of 94°C for 1 minute, 56°C for 1 minute, and 72°C for 2 minutes. The quantification of transcriptional gene expression was performed using the Gene Amp^{Ò} 5700 Sequence Detection System Version 1.3 software (Applied Biosystems, São Paulo, SP). The quantification of the transcriptional gene expression was performed using the Gene Amp^{Ò} 5700 Sequence Detection System Version 1.3 software (Applied Biosystems, São Paulo, SP)[17].

The quantification of the transcriptional gene expression was performed using the Gene Amp^{Ò} 5700 Sequence Detection System Version 1.3 software (Applied Biosystems).

Statistical analysis

Results are expressed as measures of central trend means, and standard deviations (mean±SD). All tests were performed using the Statistical Analysing System 9.1 software USA). Comparisons between three or more independent groups were performed by variance analysis (ANOVA) for data with a normal distribution. If there were statistical differences, we used the Tukey's multiple comparison tests and Lilliefors non-parametric test to detect groups that differed. The value (p) was fixed at 5% to obtain 95% of reliability in the comparisons for Tukey's test

Results

Count of aberrant crypt foci (ACF) in the proximal, middle and distal colon

In treatments (INL and FOS) where did not receive the inoculated by DMH, it was not observe ACF in any segments of the intestine, significantly differing on control for both FCA≤3 and FCA>3. In the proximal colon, only the FOSCA treatment presented FCA≤3 significantly lower when compared to the control ($p<0.05$). In the middle and distal colon, the INLCA treatment presented FCA≤3 significantly lower when compared to FOSCA and Control group. The FOSCA group also presented lower values when compared to control group ($p<0.05$). In relation of FCA>3 in the middle colon INLCA and FOSCA groups presented lower values compared to control Group ($p<0.05$). In the distal colon, the FOSCA treatment presented lower values when compared to INLCA and Control group ($p<0.05$). The INLCA group also presented lower values when compared to control group ($p<0.05$).

Catalase activity in the liver and distal colon and Organs weights

The catalase activity in the liver did not differ in any treatment analyzed regarding control (C-) ($p>0.05$) (Data not shown). However, this activity in the distal colon for treatments INL, INLCA and FOSCA was lower, differing significantly from the control (C-) ($p<0.05$). In the liver, it was observed greater weight for the INLCA, FOS and FOSCA treatments, differing from the C- ($p<0,05$) treatment, and in the kidney, it was observed a higher weight for the INL, INLCA, FOS and FOSCA treatments, differing from the C- ($p<0.05$) treatment. In the heart and spleen, no differences were observed for any treatment ($p>0.05$). (Data not shown).

Flow cytometry of spleen cells

The count of CD4 cells was not different in any treatment compared to control (C-) (Lilliefors' non-parametric test, $p=0.01$). For CD8 cells, the count also did not differ in any treatment compared to the control ($p>0.05$) (Turkey's test) and the CD4/CD8 ration was

Table I

Genes	*Primers*
Ciclina D1(Rajkumar et al.; 2007)	Sense: 5'-CATCAAGTGTGACCCGGACTG-3' Antisense: 5'-CCTCCTCCTCAGTGGCCTTG-3'
Ciclina-E (Nakade, et al.; 2009)	Sense: 5'-AGACCCACACCAACAGCTTG-3' Antisense: 5'-TCATTCTGTCTCCTGCTCGC-3'
p16 (Muddhasani, et al.; 2008)	Sense: 5'- ATCTGGAGCAGCATGGAGTC-3' Antisense: 5'- TCGAATCTGCACCGTAGTTG-3'
p21 (Muddhasani, et al.; 2008)	Sense: 5'-GTCAGGCTGGTCTGCCTCCG-3' Antisense:5'-CGGTCCCGTGGACAGTGAGCAG-3'
p53 (Hailfinger, et al.; 2007)	Sense: 5'- GGAGACATTTTCAGGCTTATGG -3' Antisense:5'-GAAGGGACAAAAGATGACAGG-3'

Table II

Number of aberrant crypt foci (ACF) with three or less crypts in the proximal, middle and distal colon and number of ACF with more than three crypts in the middle and distal colon of Balb/C mice exposed to DMH (20mg/Kg) for eight weeks.

Treatments	Less or Equal to 3 ACF			More than 3 ACF	
	Proximal colon	Middle colon	Distal colon	Middle colon	Distal colon
C-	3.5±0.7[a]	30.5±7.7[a]	39.5±6.3[a]	5.0±1.4[a]	8.0±1.4[a]
INL	0.0±0.0[b]	0.±0.0[b]	0.0±0.0[b]	0.0±0.0[b]	0.0±0.0[b]
INLCA	2.5±0.7[a]	5.5±0.8[c]	8.5±0.7[c]	1.5±0.3[c]	3.0±0.0[c]
FOS	0.0± 0.0[b]	0.0±0.0[b]	0.0±0.0[b]	0.0±0.0[b]	0.0±0.0[b]
FOSCA	1.5±0.5[c]	16.5±2.1[d]	24.5±2.3[d]	1.5±0.5[c]	1.7±0.5[d]

Data are reported as mean ± S.D. C-: AIN93M without fibers+DMH; INL: AIN93M with inulin; INLCA: AIN93M with inulin+DMH; FOS: AIN93M with FOS (fructooligosaccharide); FOSCA: AIN93M with FOS + DMH. a,b,c,d Averages followed by the same letter in the column do not significantly differ in the Tukey's test at 5% probability. Not significant according to Tukey's test at 5% probability.

Table III

Levels of hepatic and distal colon Catalase – CAT (U/mg protein). Liver and kidney weight of Balb/C mice exposed to DMH (20mg/kg for eight week).

Treatment	Catalase (U/mg proteín)		Organ weights/g	
	CAT distal colon	CAT Liver	Liver	Kidney
C-	0.91±0.78[a]	0.92±0.37[a]	1.23±0.16[a]	0.21±0.05[a]
INL	0.04±0.03[b]	0.34±0.15[a]	1.31±0.17[a,b]	0.45±0.06[b]
INLCA	0.05±0.02[b]	0.67±0.65[a]	1.64±0.12[b]	0.51±0.02[b]
FOS	0.18±0.07[ab]	0.72±0.31[a]	1.66±0.25[b]	0.53±0.08[b]
FOSCA	0.02±0.03[b]	0.88±0.42[a]	1.63±0.21[b]	0.50±0.06[b]

[a,b]Different letters indicate statistical significances between the groups ($p<0.05$), according to the Anova one-way. Data are reported as mean±S.D. C-:AIN93M without fibers+DMH; INL: AIN93M with inulin; INLCA: AIN93M with inulin+DMH; FOS: AIN93M with FOS (fructooligosaccharide); FOSCA: AIN93M diet with FOS + DMH.

not different for any treatment compared to the control (C-) (Lilliefors's non-parametric test, $p=0.01$). (Data not shown).

Quantification of Bifidobacterium spp., Clostridium ssp., total anaerobes and measurement of fecal pH.

The count of *Clostridium spp.*, *Bifidobacterium spp.* and total anaerobes did not differ compared to the control (C-), for any evaluated treatment ($p>0.05$). The pH of colon stool also did not differ for any treatment.

RT-PCR

There was no significant difference compared to control (C-) regarding any treatment for the gene expression of p53, p21 and cyclin-E proteins ($p>0.05$). The FOSCA treatment differed from the control (C-), with a higher gene expression of p16 protein ($p<0.05$). The INL treatment differed from the control (C-), INLCA and FOSCA, with a higher gene expression of cyclin-D1 protein ($p<0.05$).

Discussion

Inulin prebiotics have been observed to exert preventive effects on ACF formation in the proximal, middle and distal colon, while FOS has also been reported to have the same effect in the middle and distal colon. ACF are morphologically characterized by abnormal crypts in the surface mucous of the colon and may undergo further development into colorectal cancer (CRC) [6]. DMH and azoxymethane (AOM) are the most commonly used drugs in ACF induction, as they elicit the proliferation of these foci in the colon in great numbers, mainly in the sigmoid portion[18].

It is known that inulin and FOS decrease the appearance of ACF in mice induced with AOM by stimulating the growth of intestinal microbiota[19,20]. In the present study, inulin treatment led to a significant reduction in ACF with three or less crypts in the proximal, middle and distal colon, as well as a significant decrease in the number of ACF with more than three crypts in the middle and distal colon. FOS was efficient in reducing the occurrence of ACF (containing any number of crypts) only in the middle and distal colon.

Table IV
Average count of bacteria (Log_{10} UFC/g) and the pH of feces in the colon of mice undergoing different treatments.

Treatment	Average count of bacteria			
	Bifidobacterium spp.	*Clostridium spp.*	*Total anaerobes PH*	
C-	7.99 ± 0.63*	8.81 ± 0.06AB	8.73±0.795*	6.93±0.51*
INL	7.93 ± 0.60*	8.91 ± 0.18AB	8.85±0.07*	6.94±0.66*
INLCA	8.27 ± 0.06*	8.81 ± 0.13AB	8.86±0.18*	7.33±0.05*
FOS	8.87 ± 0.87*	9.36 ± 0.39AB	9.57±0.14*	6.62±0.63*
FOSCA	8.16 ± 0.02*	9.12 ± 0.06AB	9.55±0.21*	7.28±0.12*

Data are reported as mean ± S.D. C-: AIN93M without fibers+DMH; INL: AIN93M with inulin; INLCA: AIN93M with inulin+DMH; FOS: AIN93M with FOS (fructooligosaccharide); FOSCA: AIN93M with FOS + DMH. A,B Averages followed by the same letter in the column do not significantly differ in the Tukey's test at 5% probability. *Not significant according to Tukey's test at 5% probability.

Table V
RT-PCR for quantification of p53, p21, p16, Cyclin E and Cyclin D1 mRNA in the distal colon of Balb/C mice exposed to DMH (20mg/Kg) for eight weeks.

Treatment	*P53/ β actin*	*P21/ β actin*	*P16/ β actin*	*Cyclin E/β actin*	*Cyclin D1/β actin*
C-	0.25±0.04a	0.88±0.90a	0.07±0.04a	0.30±0.21b	0.04±0.21b
INL	0.72±0.40a	0.45±0.17a	0.01±0.00 a	0.64±0.21ab	0.48±0.36a
INLCA	0.21±0.16a	0.29±0.20a	0.00±0.00 a	0.54±0.36ab	0.24±0.18 b
FOS	1.04±0.60ab	0.45±0.29a	0.01±0.00 a	0.36±0.31b	0.32±0.28 ab
FOSCA	1.28±0.58ab	0.23±0.10a	0.67±0.31b	0.32±0.29b	0.01±0.00 b

a,bDifferent letters indicate statistical significances between the groups ($p<0.05$), and groups with the same letter do not differ statistically (one-way ANOVA). Data are reported as mean±S.D. C-:AIN93M without fibers+DMH; INL: AIN93M with inulin; INLCA: AIN93M with inulin+DMH; FOS: AIN93M with FOS (fructooligosaccharide); FOSCA: AIN93M diet with FOS + DMH.

Catalase (CAT) is one of the main systems of defense against oxidative stress, since it rapidly converts H_2O_2 into H_2O and O_2 [21]. In the process of biological oxidation, CAT plays an important role in protecting the liver and other organs from the toxic effects of many xenobiotics, thereby representing a cellular defense mechanism against reactive species[23]. Here, treatment with DMH and no fibers (C-) induced a greater number of ACF, which justified the high levels of catalase observed in this treatment group, since this enzyme represents an important defense mechanism in the body (Novaes et al. 2012). The mice groups treated with inulin or FOS showed lower levels of catalase and ACF number. We believe that in the distal colon, a decrease in catalase activity and a probable decrease in oxidative stress in the presence of prebiotics are important in preventing the appearance of ACF when mice are treated with DMH.

There are reports in the literature on the benefits of prebiotic treatment on adenomas and tumors of the colon. Studies in mice using the prebiotics inulin and FOS separately[24,25] or in combination[26] demonstrated their inhibitory effects on the formation of ACF[19,24,25], through the activation of lymphocytes. In a study with mice, the authors revealed a decrease in the CD4/CD8 cell ratio in the spleen and mesenteric lymph nodes following inulin treatment. CD8 cells protect against the appearance of adenomas in the colon, thus suggesting that the prebiotic effects could involve these immune cells[24]. Our results did not show a decrease in the CD4/CD8 ratio in the spleen, although treatment with prebiotics was efficient in preventing the appearance of ACF in the colon of mice.

We observed a decline in the weight of the liver in C- and INL-treated mice and of the kidney in C-treated animals, suggesting a change in organ homeostasis caused by DMH. These results indicate that inulin and FOS may protect the liver and kidney from changes in homeostasis. In a study with mice using FOS and inulin and evaluating the microbiota of the cecum and colon, no differences were observed in the weights of the liver, kidney, spleen and heart [26].

An increase in bifidobacteria in the intestinal microbiota has been shown to improve the health of patients with chronic gastrointestinal diseases and the elderly. Bifidobacteria suppress many bacteria that produce carcinogenic enzymes[27]. Studies in animals suggest that prebiotics may have a preventive effect against the development of tumors in the colon; however, data in humans are still inconsistent. In this study, no differences were observed in the quantification of *Bifidobacterium spp*. for any of the treatments evaluated. Thus, it is not possible to suggest that these bacteria may be involved in protecting the colon of mice administered

DMH. Studies have shown that supplementation with prebiotics can reduce fecal pH by acting as a bactericidal [28,29]. In this study, pH did not produce any differences for any of the treatments evaluated.

We also examined the gene expression of the tumor suppressors, p53, p21 and p16, and those controlling cell cycle, cyclin-E and cyclin-D1. It is known that mutations in the *TP53* gene, which encodes the p53 protein, are found in about half of all human cancers and is strongly linked to CCR[30]. In this study, p53 gene expression was not significantly affected in any of the treatments, implying that the prebiotics FOS and inulin do not have preventive effects on the distal colon of mice as they increase the gene expression of p53.

Damaged DNA increases the concentration and activity of p53, which actives the expression of a Ckd inhibitor (CdKI), the p21 protein. p21 halts the cell cycle at G_1, allowing the cell time to repair the damaged DNA before replicating and can also bind to several proteins involved in the cell cycle and DNA repair, thereby inhibiting the progression of the cell cycle[31]. In this study, we did not observe an increase in p21 expression in any of the treatment, indicating that prebiotic treatment did not act through p21 to prevent the effects of DMH in the distal colon. One of the reasons for the lack of change in the expression of p21 could be due to the low expression of p53. We also observed that p21 had no influence on the decreased expression of cyclin-E in any of the treatments. An increase in cyclin-E expression suggests the progression of cell cycle [32,33,34].

Mutations in CdKIs could also lead to uncontrolled cell cycle through the unregulated activation of cyclins and CdKs. For instance, the CDKN2A gene that encodes the p16 protein is an extremely common target for deletion or mutational inactivation in human tumors. Activated p16 inhibits the phosphorylation of Rb by binding to CdK-4 and blocking the formation of the cyclin-D/CdK4 complex and consequently, the expression of cyclin-E[34]. Here, we found a higher expression of p16 in the FOSCA treatment, but this was not enough to reduce the expression of cyclin-D1, since the expression of this cyclin did not differ among the treatments. However, p16 could inhibit CdK-4 (gene not measured in this study) and impede the formation of the cyclin-D1/Cdk-4 complex and consequently the phosphorylation of the retinoblastoma protein, which could be involved in preventing the appearance of ACF in the distal colon of FOSCA–treated mice.

Conclusion

The results of this study allowed us to conclude that inulin is efficient in preventing the appearance of ACF≤3 and ACF>3 in the proximal, middle and distal colon; however, a low expression of the tumor suppressors p53 and p21 was also observed. These data suggest that more studies on this prebiotic should be conducted to gain better understanding of the mechanisms underlying the prevention of ACF occurrence in the distal colon of mice. Fructooligosaccharide was efficient in reducing the numbers of ACF≤3 and ACF>3 in the middle and distal colon. This effectiveness may be attributed to the increased expression of p16. Both prebiotics also decreased catalase activity in the presence of DMH, thus suggesting lower oxidative stress in the distal colon. Considering these results, more studies are required to better understand the preventive effects of prebiotics on the incidence of ACF.

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