Abstract. The present study investigated the relationship between intestinal bifidobacteria and intake of commercial dietary oils of different fatty acids compositions on the development of aberrant crypt foci (ACF). Wistar rats were grouped according to diet and treatment with dimethylhydrazine (DMH): standard diet (CN), canola oil (CAN), olive oil (OLI), corn oil (COR), standard diet and DMH (CNDMH), canola oil and DMH (CANDMH), olive oil and DMH (OLIDMH) and corn oil and DMH (CORDMH). Diets and DMH did not alter the amount of bifidobacteria, fecal pH and serum total cholesterol level. DMH-treated groups had lower serum triglyceride levels compared to respective controls without DMH. Olive and corn oil diets resulted in higher hepatic cholesterol levels than standard diet under treatment with DMH. The numbers of ACF/field and cell proliferation were lower under treatment with CANDMH and OLIDMH, suggesting a protective effect of these oils on colorectal carcinogenesis.

Diet is one of the most important factors influencing colorectal cancer. The incidence of this type of cancer has very different patterns in different parts of the world, suggesting a strong influence of local environmental factors (1). Among dietary risk factors, high consumption of fat and calories associated with low intake of fiber and vegetables increases the risk of colorectal cancer. However, the amount and composition of dietary fat should be considered (2). Experimental and epidemiological studies have shown that diets rich in ω-3 polyunsaturated fatty acids (PUFA) reduce colorectal carcinogenesis (3-9), while diets rich in ω-6 PUFA or saturated fatty acids promote it (2, 3, 6, 10). Several mechanisms have been proposed for the effects of fatty acids on colorectal carcinogenesis, including oxidative stress, influence on lipid, glycemic and bile acid metabolism, membrane properties, in cell-signaling, and immune and inflammatory responses (1, 11-16). In addition, it is suggested that fatty acids may influence the metabolism of intestinal microbiota (17).

Bifidobacteria are able to minimize gastrointestinal disorders and diseases (18, 19) through restoration of intestinal biota after anti-microbial therapy, reduction of serum cholesterol by degradation and absorption of bile acids, reduction of the pH, thereby inhibiting the growth of pathogenic bacteria, showing immunomodulatory and antitumor activity, and improving resistance to pathogens (20-23). Furthermore, studies also support the hypothesis that supplementation with bifidobacteria has beneficial effects on colonic tumorigenesis associated with the reduction of aberrant crypt foci (ACF) (24-27). However, there are no studies to demonstrate whether initiation of the carcinogenic processes affects bifidobacteria regarding intestinal microbiota.

Moreover, is not possible to conclude whether fatty acids act as co-initiators, promoters, or protectors in formation of ACF through these studies. Therefore, the objective of the present study was to investigate the influence of intake of canola, olive, and corn oil during the initiation of ACF development in rats. We also investigated whether proliferation of colonic epithelium or modification in colon microbiota could explain the effects of these oils on the development of ACF.

Materials and Methods

Animals and experimental design. Sixty-four male Wistar rats (Rattus norvegicus, variety Albinus, Rodentia) were randomly allocated into eight groups (n=8): standard diet (CN), canola oil (CAN), olive oil (OLI), corn oil (COR), standard diet and DMH (CNDMH), canola oil and DMH (CANDMH), olive oil and DMH (OLIDMH), and corn oil and DMH (CORDMH).
(OLIDMH) and corn oil and DMH (CORDMH). The rats received a standard diet (Nutrilabor®) and ad libitum water for a one-week acclimation period. After this period, treatment was initiated with different diets and intra-peritoneal administration of DMH (Sigma-Aldrich, St. Louis, MO, USA) in a single dose of 150 mg/kg body weight. After eight weeks of treatment, fecal samples were collected to determine bifidobacteria content, fecal cholesterol, and fecal pH. Blood samples were also collected. Animals were euthanized, and their colons removed for histological analysis. Livers were also removed, weighed, and frozen to determine hepatic cholesterol. This project was approved by the Animal Research Committee of Federal University of Santa Catarina.

**Diet.** Standard diet (chow) consisted of crude protein (min. 22%), moisture (max. 12.5%), ether extract (max. 4.5%), phosphor (min. 0.8%), vitamin A (12,000 IU), vitamin D3 (1,800 IU), vitamin E (30 mg), vitamin K3 (3 mg), vitamin B1 (5 mg), vitamin B2 (6 mg), vitamin B6 (7 mg), vitamin B12 (20 μg), niacin (60 mg), pantenonic acid (20 mg), folic acid (1 mg), antioxidants (100 mg), mineral matter (max. 10%), fibrous matter (max. 8%), calcium (max. 1.4%), iron (50 mg), zinc (60 mg), copper (10 mg), iodine (2 mg), manganese (60 mg), selenium (0.05 mg), cobalt (1.5 mg), lysine (100 mg), methionine (300 mg), biotin (0.05 mg), and choline (600 mg) per kilogram of product. For the experimental diet, canola oil (Bunge®), olive oil (d’Aguiar®) or corn oil (Bunge®) was added to standard chow at 10% (w/w).

Total weight gain was calculated as the difference between final and initial weight. Fatty acid profile of dietary oils was analyzed by gas chromatography using the method described by Hartman and Lago (28) with modifications.

**Fecal pH and bifidobacteria content.** Feces of each animal were weighed and diluted in sterile deionized water for pH determination. Each sample was also weighed and diluted in 9 ml of 0.31 mM phosphate buffer. From this dilution (10⁻¹), serial dilutions were performed up to 10⁻⁷ and 100 μl of each dilution was spread-plated on selective medium for bifidobacteria BIM-25 (29). The plates were incubated at 37°C for 72 h under anaerobic conditions (Anaerobic system, Probac, São Paulo, São Paulo, Brazil). At a later stage, the bacterial population (bifidobacteria) count was calculated as colony-forming units (CFU) per gram of fecal sample. Gram stain, catalase test and fructose-6-phosphate phosphoketolase (F6PPK) test according to Orban and Patterson (30) were performed to confirm *Bifidobacterium* spp. genus.

**Determination of biochemical parameters.** Hepatic and fecal cholesterol measurements were according to Melo et al. (31). Serum triglycerides, total cholesterol and high-density lipoprotein (HDL)-cholesterol levels in each animal were measured using commercial enzymatic kits (Labtest, Montes Claros, Minas Gerais, Brazil).

**Histopathological analysis.** Colons were removed, rinsed in 0.9% saline, fixed in 10% buffered formalin, and dehydrated, diaphanized, and embedded in paraffin. Sections (3-μm thick) were cut transversally and longitudinally on glass slides, mounted, and rehydrated. Transversal sections of crypts were stained with Harris hematoxylin/eosin (H&E) for identification and quantification of ACF per microscopic field. Twenty microscopic fields/section/animal were analyzed at ×400 magnification. The presence of two or more characteristics (increased size, thicker and more stained epithelial layer than adjacent normal crypts, larger lumen with altered form) was used to define an aberrant crypt (32, 23). Multiplicity was evaluated by the number of aberrant crypts per focus. To estimate colonic cell proliferation in all animals, the longitudinal sections were immunostained for proliferative cellular nuclear antigen (PCNA) using an antibody against PCNA (Thermo Scientific, Pittsburgh, Pennsylvania, USA). The labeling index (LI) was determined as the ratio of the number of stained nuclei and total number of nuclei along each crypt. Ten crypts were counted per section/animal (×400). Rat colon (Wistar, male) specimens were used as a positive control and primary antibody suppression in the reaction was used as negative control.

**Statistical analysis.** Data analyses were performed using the mean±standard error (SE). Verification of symmetric distribution of data was performed using Levene’s normality (Shaprio-Wilk test). Analysis of variance (ANOVA) was used to compare groups at a significance level of 95% (p<0.05 Tukey’s post hoc test).

**Results.**

**Fatty acid profile of oils used in the preparation of the chow.** The fatty acid composition of oils added to standard chow was analyzed by gas chromatography. Table I shows the percentage of each fatty acid in relation to total fatty acids identified in oils. For the calculation of this profile, fatty acids representing less than 1% of composition were ignored. Once these oils were added at a concentration of 10% to standard chow, diets with canola, olive, and corn oil contained 0.8%, 1.8% and 1.4% of saturated fat; 6.4%, 6.7% and 3.7% of ω-9 MUFA; and 2.8%, 1.5% and 4.9% of ω-6 PUFA, respectively.

The average daily intake of feed, per animal, in groups overall was 22.5±1.9 g, which corresponds to a daily ingestion of 0.18%, 1.8% and 1.4% of saturated fat; 1.4%, 1.5% and 0.9% of MUFA; and 0.6%, 0.3% and 1.1% of PUFA in groups treated with canola, olive, and corn oil, respectively.

**Body weight gain.** The animals were weighed at the beginning and at the end of the treatment to evaluate weight gain during treatment (Table II). Oil-treated groups had a significantly higher weight gain than the control group under standard diet. DMH treatment did not affect weight gain in animals when compared to their respective counterparts with diet without DMH.

**Bifidobacteria and fecal pH.** Colonies with ivory, wine and light pink colors and with creamy and smooth surfaces were observed on macroscopic analysis. All colonies were positive in the catalase test and F6PPK and showed gram-positive rods with bifurcated extremities, short, and coccoid rods on microscopic morphology, therefore belonging to the *Bifidobacterium* spp. genus.
Fecal bifidobacteria in CN group were found at 4.3×10^{10}±3.4×10^{10} CFU/g of feces. Canola, olive, and corn oil had 7.1×10^{10}±3.9×10^{10}, 4.5×10^{10}±1.8×10^{10}, and 7.5×10^{10}±3.5×10^{10} CFU/g of feces, respectively. CNDMH, CANDMH, OLIDMH, and CORDMH had 8.4×10^{10}±3.1×10^{10}, 2.1×10^{10}±1.1×10^{10}, 7.7×10^{10}±2.4×10^{10} and 5.0×10^{10}±2.8×10^{10} CFU/g of feces, respectively. No significant differences were observed among groups.

The average fecal pH in CN, CAN, OLI, and COR groups was 7.0±0.3, 6.5±0.2, 7.1±0.1 and 6.7±0.5, respectively. In DMH groups, pH was 7.2±0.3, 7.0±0.2, 6.6±0.2 and 6.4±0.1 in CNDMH, CANDMH, OLIDMH, and CORDMH, respectively. No significant difference was observed among the different groups.

Biochemical parameters. Serum levels of total cholesterol, triglycerides, and HDL-cholesterol are presented in Figure 1. No alterations were detected in total cholesterol levels among groups. In contrast, triglyceride levels in DMH-treated groups were significantly lower than the respective groups treated with the same diet without DMH. The CORDMH group had significantly lower HDL-cholesterol levels when compared to the COR group.

Fecal cholesterol levels in the CN, CAN, OLI, and COR groups was 34±5, 35±3, 26±3, and 27±3 mg/dl, respectively. In the CNDMH, CANDMH, OLIDMH, and CORDMH groups was 24±3, 28±3, 38±5, and 27±2 mg/dl, respectively. There was no statistically significant difference in fecal cholesterol levels among the groups treated with different diets and DMH or not.

As can be seen in Figure 2, the COR group had significantly higher levels of hepatic cholesterol than other groups not treated with DMH. In addition, the OLI group had significantly higher levels than the CN and the CAN groups. Among the groups treated with DMH, OLIDMH and CORDMH groups had significantly higher hepatic cholesterol levels than CNDMH and CANDMH groups. The CANDMH group had a significantly higher hepatic cholesterol level than the CAN group.

Histological analysis. Histological analysis showed that groups not treated with DMH did not have any changes in colonic tissue on spontaneous development of ACFs. Figure 3 illustrates an ACF with three aberrant crypts (indicated by black arrows), representative of ACFs observed in colon of rats treated with DMH. Morphologically, ACFs were similar in all groups treated with DMH.

CANDMH and OLIDMH groups had significantly fewer ACFs/field when compared to the CNDMH group. *p<0.05 compared to corresponding group treated with same diet and DMH. **p<0.01 compared to CNDMH and MILDMH.

Results are expressed as the mean value±SE. *p<0.05 compared to CN group. **p<0.01 compared to CNDMH group. Standard diet (CN); canola oil (CAN); olive oil (OLI); corn oil (COR); standard diet and DMH (CNDMH); canola oil and DMH (CANDMH); olive oil and DMH (OLIDMH) and corn oil and DMH (CORDMH).

### Table I. Composition of fatty acids (%) in canola, olive and corn oils.

<table>
<thead>
<tr>
<th>Fatty acids</th>
<th>Canola oil</th>
<th>Olive oil</th>
<th>Corn oil</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturated</td>
<td>8.0</td>
<td>18.1</td>
<td>14.0</td>
</tr>
<tr>
<td>Monounsaturated</td>
<td>64.4</td>
<td>67.1</td>
<td>36.7</td>
</tr>
<tr>
<td>Polyunsaturated</td>
<td>27.6</td>
<td>14.8</td>
<td>49.2</td>
</tr>
</tbody>
</table>

### Table II. Mean weight (g) of rats prior to and after treatment.

<table>
<thead>
<tr>
<th>Group</th>
<th>Initial weight</th>
<th>Final weight</th>
<th>Weight gain</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN</td>
<td>180±6</td>
<td>314±9</td>
<td>134±7</td>
</tr>
<tr>
<td>CAN</td>
<td>169±5</td>
<td>371±14</td>
<td>212±15*</td>
</tr>
<tr>
<td>OLI</td>
<td>149±5</td>
<td>358±9</td>
<td>210±6*</td>
</tr>
<tr>
<td>COR</td>
<td>155±6</td>
<td>362±7</td>
<td>215±10*</td>
</tr>
<tr>
<td>CNDMH</td>
<td>166±7</td>
<td>297±16</td>
<td>149±7</td>
</tr>
<tr>
<td>CANDMH</td>
<td>165±7</td>
<td>345±8</td>
<td>185±8**</td>
</tr>
<tr>
<td>OLIDMH</td>
<td>143±3</td>
<td>340±8</td>
<td>203±9**</td>
</tr>
<tr>
<td>CORDMH</td>
<td>161±6</td>
<td>345±9</td>
<td>191±6**</td>
</tr>
</tbody>
</table>

Results are expressed as the mean value±SE. *p<0.05 compared to CN group. **p<0.01 compared to CNDMH group. Standard diet (CN); canola oil (CAN); olive oil (OLI); corn oil (COR); standard diet and DMH (CNDMH); canola oil and DMH (CANDMH); olive oil and DMH (OLIDMH) and corn oil and DMH (CORDMH).

### Table III. Number of aberrant crypt foci (ACF) per field, number of aberrant crypts (AC) per focus, and proliferative cellular nuclear antigen (PCNA)-labeling index (LI) in groups treated with different dietary oils and treated with dimethylhydrazine (DMH) or not.

<table>
<thead>
<tr>
<th>Group</th>
<th>ACF/Field</th>
<th>AC/ACF</th>
<th>PCNA-LI (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CN</td>
<td>0</td>
<td>0</td>
<td>5.8±0.3*</td>
</tr>
<tr>
<td>CAN</td>
<td>0</td>
<td>0</td>
<td>5.5±0.2*</td>
</tr>
<tr>
<td>OLI</td>
<td>0</td>
<td>0</td>
<td>6.9±0.14*</td>
</tr>
<tr>
<td>COR</td>
<td>0</td>
<td>0</td>
<td>7.0±0.3*</td>
</tr>
<tr>
<td>CNDMH</td>
<td>0.97±0.06</td>
<td>1.77±0.18</td>
<td>16.2±1.7</td>
</tr>
<tr>
<td>CANDMH</td>
<td>0.60±0.07</td>
<td>1.57±0.11</td>
<td>11.7±0.7**</td>
</tr>
<tr>
<td>OLIDMH</td>
<td>0.74±0.03</td>
<td>1.51±0.07</td>
<td>9.3±0.5**</td>
</tr>
<tr>
<td>CORDMH</td>
<td>0.78±0.02</td>
<td>1.49±0.07</td>
<td>20.1±0.7</td>
</tr>
</tbody>
</table>

Results are expressed as the mean value±SE. *p<0.01 compared to the CNDMH group. **p<0.05 compared to corresponding group treated with same diet and DMH. ***p<0.01 compared to CNDMH and MILDMH. Standard diet (CN); canola oil (CAN); olive oil (OLI); corn oil (COR); standard diet and DMH (CNDMH); canola oil and DMH (CANDMH); olive oil and DMH (OLIDMH) and corn oil and DMH (CORDMH).
groups had PCNA-LI of 5.8±0.3%, 5.5±0.2%, 6.9±0.1%, and 7.0±0.3%, respectively. LIs in CNDMH, CANDMH, OLIDMH and CORDMH groups were 16.2±1.7%, 11.7±0.7%, 9.3±0.5%, and 20.1±0.7%, respectively. Groups not treated with DMH had a significantly lower PCNA-LI than groups treated with their respective diet and DMH. Among the groups treated with DMH, CANDH and OLIDMH had significantly lower PCNA-LI than CNDMH and CORDMH groups. Furthermore, the CORDHM group showed a tendency for greater proliferation (p=0.053) (Table III).

Discussion

The influence of dietary lipids on colonic tumor depends on the amount and type of fatty acids in the diet (15, 34). Gas chromatographic analysis of the fatty acid composition of commercial oils used in chow production revealed high amounts of saturated, fatty acids, MUFAs and PUFAs, similar to those reported in product labeling, according to information disclosed by the oil manufacturers. However, according to the manufacturer, canola oil had 6.7% of ω-3 PUFA, not found in our analysis. The MUFA and PUFA percentage should be, at least 80% of the value stated on the label. Thus, according to the analytical method used to determine total lipids, and consequently for isolated fatty acids, it may be subject to fluctuation (35).

Although animals consumed the same amount of chow (results not shown), groups treated with oils gained more weight than the control group whether they received DMH or not. Higher weight gain is possible even though the amount of food eaten is similar because the nutrient...
composition can alter the efficiency of feed utilization (36-40). A probable mechanism for the effects of different fatty acids on the gain of body weight may be due to the saturated fatty acid being little used as an energy source, thus being stored in adipose tissue, while MUFAs and PUFAs are easily used as an energy source and therefore less often stored (37). In addition, saturated fatty acids have lower oxidation rates in relation to PUFAs, taking more time to enter the beta-oxidation stage, favoring its deposition in adipose tissue (38).

Even though oil-treated groups gained more weight, there were no significant differences in serum triglyceride levels when compared to groups treated with standard diet. However, serum triglyceride levels were lower in DMH-treated groups when compared to groups that had received the same diet and were not treated with DMH, as observed by Nauss et al. (40). The authors observed that serum triglyceride levels in animals treated with diets rich in lipids decreased in the first weeks after treatment with DMH, but increased in advanced stages of tumorigenesis (10 months after treatment). Although the mechanism by which DMH reduces serum triglycerides is unknown, it may result from hepatic metabolism, oxidative stress and hepatic necrosis that can be caused by DMH (41).

The amount of canola, olive and corn oil added to the standard diet was not sufficient to alter serum total cholesterol and HDL-cholesterol levels significantly in the evaluated period of time. Similarly, treatment with DMH also did not cause modification in serum cholesterol levels in different groups. Our results are in agreement with Dauqan et al. (42) who observed that rats treated with 15% of corn or coconut oil for four and eight weeks presented no difference in serum total cholesterol when compared to treatment with standard diet.

Completing the analysis of the effect of different diets on lipid metabolism, we also recorded higher hepatic cholesterol concentrations in corn- and olive oil-treated groups. The ratio of fatty acids [PUFA+MUFAs/saturated] has been previously used to evaluate the influence of dietary lipids on lipid metabolism, where high ratios are associated with health benefits (43). According to this hypothesis, canola oil has a ratio of 11.5/1, olive oil 4.5/1 and corn oil, 6.1/1. The lowest ratios observed for olive and corn oils may explain the higher hepatic cholesterol levels, but had no influence on serum and fecal levels.

Furthermore, as expected, dietary oils did not affect the amount of bifidobacteria in feces, since these bacteria use mostly short chain oligosaccharides such as galactooligosaccharides and fructooligosaccharides as substrate (44). However, this result was the opposite of what was reported by Hekmatdoost et al. in a 4-week study with diets supplemented with 20% fish, canola, or safflower oil on cecal microbiota, inflammatory markers, and biochemical parameters in experimental colitis (45). These researchers observed that groups treated with oils rich in ω-3 PUFA (fish and canola oils) had a greater amount of bifidobacteria when compared with these on the standard-diet group.

Challa et al. suggested that the beneficial effect of bifidobacteria on colorectal carcinogenesis is possibly due to a reduction of cecal pH (46). No change was observed in fecal pH in our study, in line with data of other authors (46, 47).

Regarding the effects of oils on the development of pre-cancerous lesions induced by DMH, we observed that groups not treated with chemical carcinogens had no aberrant crypts. These results indicate that lipids, at the concentration added, did not initiate colonic carcinogenesis by themselves. Among the DMH-treated groups, those treated with canola and olive oil had fewer ACF, but without any difference in the number of crypts per focus, and less cell proliferation (assessed by PCNA-LI) than the group treated with standard diet and DMH, corroborating with findings of Fujise et al. (2), Cho et al. (49) and Bathia et al. (50).

Bhatia et al. reported that canola oil reduced the azoxymethane-induced ACF number and multiplicity when compared to treatment with corn oil, possibly due to an increase of ω-3 PUFA and lower cicloxygenase-2 (COX) expression. As we did not detect ω-3 PUFA in the chromatographic analyses of canola oil, the highest ratio [PUFAs+MUFAs/saturated] should be related to beneficial effects on the development of pre-cancerous lesions and colorectal cancer. Additionally, canola and olive oils had higher amounts of ω-9 MUFA than corn oil, and this MUFA has been associated with the reduction of colonic tumorigenesis (7). In addition, olive oil also contained minor compounds (squalene, tocopherols, and phenolic compounds) which have chemopreventive effects on cancer (51).

From the results observed in this study, we suggest that the mechanism by which dietary oils and DMH influence colorectal carcinogenesis does not occur through modulation of intestinal microbiota. Moreover, we may conclude that canola and olive oils have a protective effect against the development of DMH-induced ACFs and cell proliferation, although leading to greater weight gain (obesity). In this context, we suggest that the type of fatty acid consumed influences the development of Pre-cancerous lesions more than obesity itself. Therefore, more studies are required in defining the optimal amount of functional foods, ideal timing of dietary intervention, as well as further analysis are required regarding the relationship of intestinal microbiota with colorectal cancer risk and interactions of biota with nutrients, nutrients with pre-cancerous lesions and microbiota with pre-cancerous lesions, in order to develop specific recommendations for dietary modifications to prevent formation of colonic cancer.

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202


