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Proceeding Paper

Probiotic Administration Ameliorate Azoxymethane Induced-Carcinogenesis by Reducing the Formation of Aberrant Crypt Foci and Modulation Oxidative Stress in Rats †

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Abstract: Aberrant crypt foci (ACF) are early lesions in the neoplastic induction found in the rat colon of a carcinogenic model. ACF are one of the first changes in the colon that lead to colorectal cancer (CRC). Probiotics and fermented foods are well known for their beneficial role in gut health and previous studies showed their therapeutic effects on gastrointestinal diseases. The aim of this study was to investigate the preventive role of *Propionibacterium freudenreichii* and *Faecalibacterium prausnitzii* probiotics against CRC in rats induced with azoxymethane (AOM). Microscopical examination of the rat's colon stained with methylene blue showed that the total number and multiplicity of ACF were significantly lower in the probiotic treated rats ($p < 0.05$) than in the AOM control group. Histological examination of the colon revealed severe hyperplasia and dysplasia of the ACF in the colon of the AOM control group compared to the treatment groups. Probiotics administration also reduced the levels of malondialdehyde (MDA) in the colon of treated rats compared to the rats in the AOM control group. These results suggest that probiotics play a preventive role in CRC initiation and development by slowing down ACF formation, reducing the severity of ACF lesions and reducing lipid peroxidation levels in the colon.

Keywords: probiotics; colorectal cancer; *Propionibacterium freudenreichii*; *Faecalibacterium prausnitzii*; oxidative stress

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1. Introduction

Colorectal cancer (CRC) currently is known to be one of the leading causes of cancer-related deaths worldwide [1]. This is mainly because most CRC patients are diagnosed at the advanced stages, which leads to the increased mortality associated with CRC [2].

The earliest neoplastic lesions in CRC development are aberrant crypt foci (ACF). Most ACF are thought to develop into a malignant phenotype and become CRC, possibly bypassing polyp formation [3–5].

Probiotics are live microorganisms that offer health benefits to consumers when provided in sufficient quantities [6,7]. In the past few years, it has been observed that the gut microbiota make-up is associated with the occurrence of CRC [8,9]. The quality of the microbiota in the intestine can affect many aspects of the health of the intestine, including metabolism, cellular characteristics, development, gut physiology and immune homeostasis [10]. Some experimental findings have also shown that the makeup of the microbiota in the intestines of people ailing from CRC is different from the intestinal microbiota makeup of healthy individuals [11]. Consequently, it has been noticed that positive

alteration of the make-up of the gut microbiome can happen when certain probiotics are consumed in sufficient amounts may avoid the onset of CRC and also help alleviate CRC symptoms in patients already living with the disease because these probiotics may regulate the gut microbiota and typically provide health advantages to the consumer. The anticancer benefits of bacterial probiotics such as *Lactobacillus* and *Bifidobacteria* genera have been widely described in several studies [12–16], creating a gap for more novel probiotics to be investigated for their role in the prevention of CRC development and proliferation.

Propionibacterium freudenreichii is a dairy probiotic that has long been used as a cheese ripening agent, but its probiotic properties have not been largely investigated [17]. The beneficial role of *P. freudenreichii* to minimize the risk of cancer could be attributed to its ability to bind to cancer-causing compounds like aflatoxin, concanavalin A and mycotoxins [18].

Faecalibacterium prausnitzii is one of the gut's main butyrate sources, which has been shown to have a powerful anti-inflammatory effect in the treatment of inflammatory bowel disease, Crohn's disease, and CRC. Butyrate inhibits the initiation of the NF-κB transcription factor, which reduces inflammation in the gastrointestinal tract [19].

Although it is known that *P. freudenreichii* and *F. prausnitzii* may exhibit promising preventive effects against CRC, there are limited studies investigating the anti-cancer beneficial effects of these two probiotics against CRC. Hence, this study aimed to investigate the preventive role of *P. freudenreichii* and *F. prausnitzii* probiotics against CRC initiation in azoxymethane (AOM)-induced colon carcinogenesis in rats.

2. Materials and Methods

2.1. Preparation of Probiotic Strains

Propionibacterium freudenreichii (DSM 2027) and *Faecalibacterium prausnitzii* (DSM 17677) were purchased from the Leibniz Institute DSMZ, Braunschweig, Germany.

2.2. Induction of Aberrant Crypt Foci in the Colon of Rats

10 weeks old male Sprague Dawley rats (n = 30) were obtained from the FOM-Experimental Animal Unit, University of Malaya. To induce carcinogenesis in the colon of rats, azoxymethane (AOM, Sigma-Aldrich, St. Louis, MO, USA) was prepared in PBS and administered to the rats subcutaneously once a week for three weeks at a dose of 7 mg/kg body weight.

2.3. Experimental Design and Grouping

The animal experimental protocol was approved by the Faculty of Medicine Institutional Animal Care and Use Committee (FOM-IACUC) at the University of Malaya with an IACUC reference number: 2020-221205/PHARM/R/MAM (2019367). The animals were housed and cared for according to the animal experimental unit standard guidelines. The rats were housed in groups of 3 per IVC cage and kept in a standard condition (temperature of 22 °C, humidity 75%, and 12 h of light/dark cycle). The rats were given Altromin rat pellets and RO water ad libitum. All rats received regular human care and attention and were weighed weekly throughout the study. The animals were randomly divided into six groups of five animals each. After one week of acclimatization the rats were treated for five weeks as follows: normal control and AOM control groups received the vehicle, standard drug group received intraperitoneal injections of 5-fluorouracil (35 mg/kg, 3 times per week), *Propionibacterium* group received an oral daily dose of 1×10^9 CFU/mL of *P. freudenreichii*, *Faecalibacterium* group received oral daily dose of 1×10^9 *F. prausnitzii* CFU/mL and probiotics mixture group received an oral daily dose of 1×10^9 CFU/mL mixture of *P. freudenreichii* + *F. prausnitzii*.

2.4. ACF Counting

After five weeks of treatment, all rats were anesthetized and sacrificed and the ACF were counted to assess the degree of colonic ACF formation and multiplicity. Methylene blue solution (0.5%) was used to stain the proximal and distal portions of the fixed colons

in similar lengths. Topographic analysis was performed under a light microscope after washing away the excess stain to evaluate the total number of ACF as well as the number of crypts in each focus [20].

2.5. Histological Assay

The colon was harvested and cut into 1 cm × 1 cm squares, which were then preserved in 10% buffered formalin and histologically processed [21]. The tissues were stained with H & E stain and examined under a microscope.

2.6. Malondialdehyde (MDA) Assay

This assay was carried out using the thiobarbituric acid reactive substances (TBARS) assay. A commercial kit (Cayman Chemical, Ann Arbor, MI, USA) was used to test MDA levels in colon tissue lysates. The MDA amount that reflects lipid peroxidation level was determined by the TBARS assay according to the manufacturer's protocol [22].

2.7. Statistical Analysis

The statistical significance of the data was analyzed using a One-way analysis of variance (ANOVA) with Tukey's multiple comparisons post hoc test. All the data were expressed as mean ± standard error mean (SEM) of 5 rats. Statistical analyses were performed with SPSS, version 27.0 for Windows. The values of $p < 0.05$ were considered as significant.

3. Results

3.1. ACF Analysis and Microscopical Findings

ACF were used as a biomarker to evaluate the preliminary stage of AOM-induced colon carcinogenesis in rats and to investigate the preventive effects of probiotics against colon cancer. Methylene blue staining was used to examine the occurrence of aberrant crypt foci on the distal and proximal sections of the colon mucosa shortly after the animals were sacrificed. When compared to the AOM-control group, the rats treated with both probiotics had significantly ($p < 0.01$) less ACF compared to AOM control (Figure 1). The rats that were induced with AOM produced identifiable ACF in the colon after being stained with methylene blue while the rats in the normal control group had no irregular crypts in their colons (Figure 2).

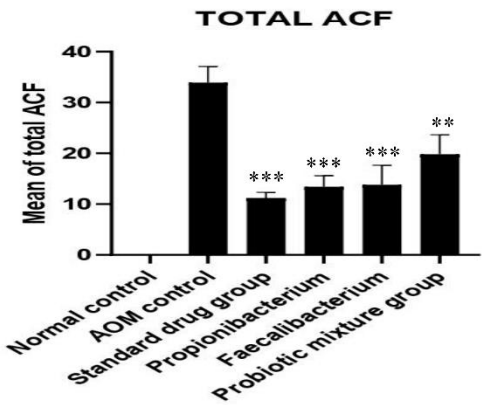

Figure 1. Total ACF count for the different groups. Values were expressed as mean ± S.E.M, values with ** p <0.01 and *** $p < 0.001$ are significant when compared to AOM group. All samples were assed in triplicates.

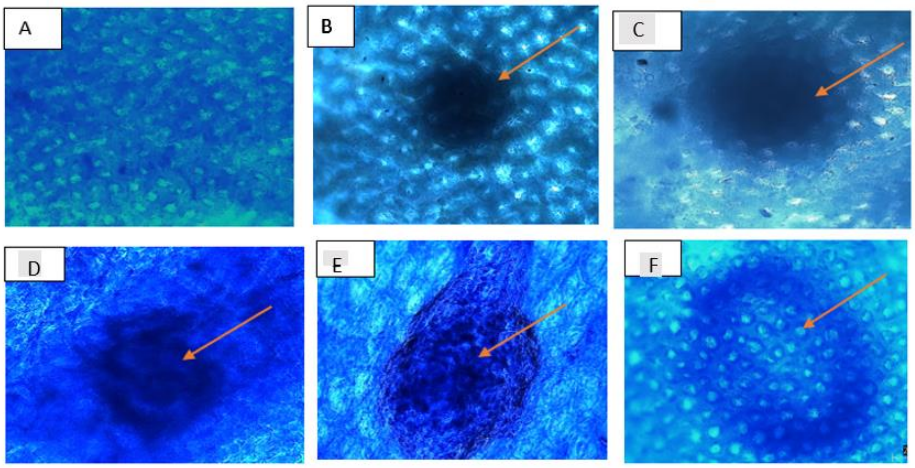

Figure 2. A topographic view shows the ACF found in the colon of the different groups. (**A**) Normal colon crypts from rats in the normal control group treated with PBS (pH 7.4); (**B**) ACF from AOM control group treated with 7 mg/kg AOM; (**C**) Standard drug group treated with 35 mg/kg 5-fluorouracil + 7 mg/kg AOM; (**D**) Propionibacterium group treated with 1×10^9 CFU/mL *P. freudenreichii* + 7 mg/kg AOM; (**E**) Faecalibacterium group treated with 1×10^9 CFU/mL *F. prausnitzii* + 7 mg/kg AOM; (**F**) Probiotics mixture group treated with a mixture of 1×10^9 CFU/mL of *P. freudenreichii* and *F. prausnitzii* + 7 mg/kg AOM.

3.2. Histological Findings

ACF of the rats in the AOM control group showed bigger and longer mucosal lining, clear degradation of the cell, increased inflammation throughout the cell, crowding of the nuclei, depletion of goblet cells, and loss of polarity. According to the histological analysis of the colon tissues, rats treated with probiotics showed less multiplicity and number of ACF compared to the AOM group (Figure 3).

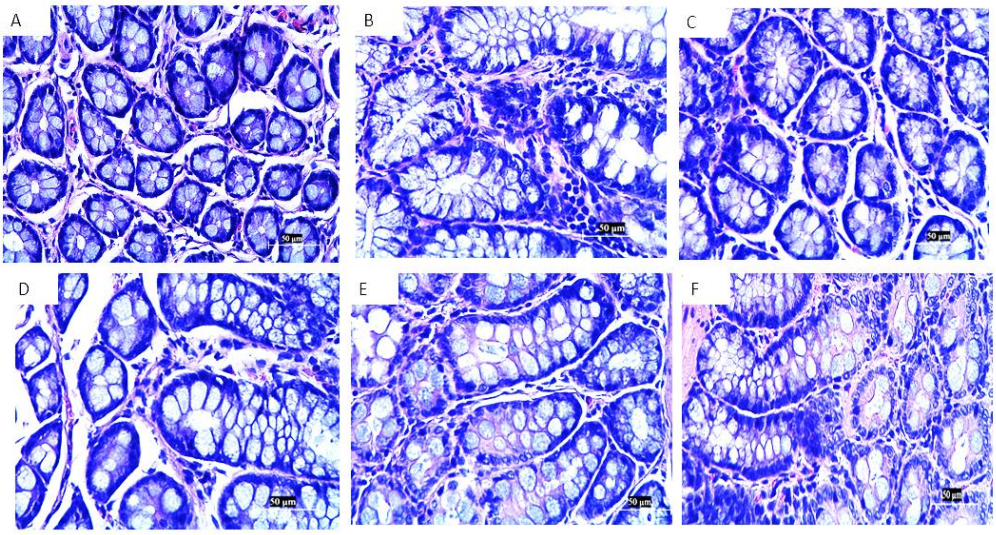

Figure 3. A topographic view of H&E stained colon tissues shows the ACF found in the colon of the different groups. (**A**) Normal colon crypts from rats in the normal control group treated with PBS (pH 7.4); (**B**) ACF from AOM control group treated with 7 mg/kg AOM; (**C**) Standard drug group treated with 35 mg/kg 5- fluorouracil + 7 mg/kg AOM; (**D**) Propionibacterium group treated with 1×10^9 CFU/mL *P. freudenreichii* + 7 mg/kg AOM; (**E**) Faecalibacterium group treated with 1×10^9 CFU/mL *F. prausnitzii* + 7 mg/kg AOM; (**F**) Probiotics mixture group treated with a mixture of 1×10^9 CFU/mL of *P. freudenreichii* and *F. prausnitzii* + 7 mg/kg AOM.

3.3. Probiotics Administration Reduced MDA Levels

The administration of *P. freudenreichii* and *F. prausnitzii* probiotics to rats for five weeks resulted in a lower MDA level in the colon of treated groups compared to AOM-induced rats, although the difference was not statistically significant (Figure 4).

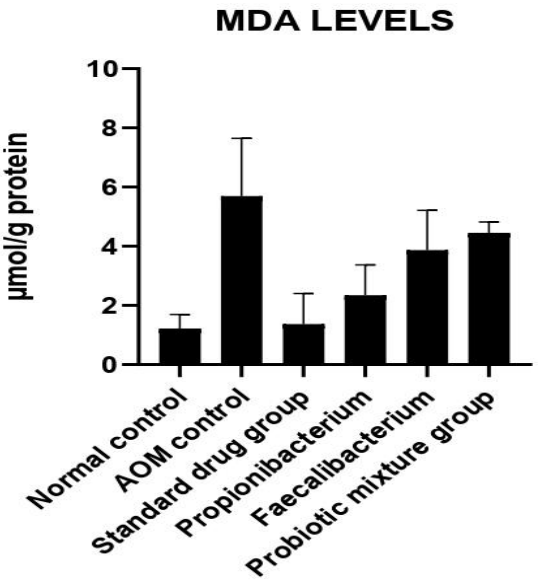

Figure 4. Lipid peroxidation level in treated and untreated groups. All values were expressed as mean ± SEM.

4. Discussion

The probiotic microorganisms were found to be effective in preventing the development of ACF and reducing the number of aberrant multi crypt and preneoplastic lesions in the rat colon, which is similar to the findings of several other probiotics studies, where it was found that *Lactobacillus* inhibited DMH-induced ACF in Sprague Dawley rats [23–25]. In the AOM control group, the presence of overcrowding and elongation of the nuclei, atypical epithelial cells, and architectural atypia indicated that the majority of the ACF were dysplastic aberrant crypts, which are precursor lesions. However, probiotics were able to reduce the number and multiplicity of ACF in treated rats. Elevated lipid peroxidation levels in AOM-induced rats, as evident by increased MDA levels, are a sign of acute colonic cell injury. MDA levels were reduced in the probiotic groups compared to the AOM control group, but the differences were not statistically significant. This finding is in agreement with a study by Obuagu et al., where probiotics reduced the MDA levels in animal models [26]. The limitations and challenges associated with this study include the small number of rats per group and the difficulty of culturing *F. prausnitzii* as it is extremely oxygen-sensitive. In addition, it would be good if the duration of the treatment can be extended for a longer time to explore the long-term exposure to these probiotics. The findings of this study can be translated clinically by adding the two species as part of a well-standardized probiotic formula and encouraging the general population, especially older people and people at risk of developing CRC, to increase their intake of probiotic supplements containing *P. freudenreichii* and *F. prausnitzii*. However, more studies and clinical trials need to be carried out to confirm their beneficial effects on humans.

5. Conclusions

The findings from this study suggest that probiotics can help prevent CRC by slowing the progression of ACF, reducing the incidence of ACF lesions, and decreasing lipid peroxidation in the colon. Since the inhibition of ACF development in the mixture group was lower than in the two individual probiotic groups, we infer that there is no synergistic effect between *P. freudenreichii* and *F. prausnitzii* against CRC.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ECB2021-10255/s1.

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