Probiotics 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 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 is to investigate the preventive role of *Propionibacterium freudenreichii* and *Faecalibacterium prausnitzii* probiotics against CRC in rats induced with azoxymethane (AOM). Microscopical examination of rat colons after methylene blue staining showed that the total number and multiplicity of ACF were significantly lower in the probiotic groups ($p < 0.05$) than the AOM control group. Histological examination of the colon showed increased severe hyperplasia and dysplasia of the ACF in the AOM control group compared to the treatment groups. Probiotics administration also reduced the levels of malondialdehyde (MDA) in the colon of the 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

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 is diagnosed at the advanced stages which leads to the increased mortality associated with CRC [2].

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

Probiotics are live microorganisms that offer health benefits to consumers when provided in sufficient quantities. [6, 7].The anticancer benefits of bacterial probiotics such as *Lactobacillus* and

Bifidobacteria genera are widely described in several studies [8-12], creating a gap for more novel probiotics to be investigated for their role in 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 [13]. *P. freudenreichii* has been discovered to help minimize the risk of cancer, due to its ability to bind to cancer-causing compounds like aflatoxin, concanavalin A and mycotoxins [14].

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 [15].

In this study we investigated the preventive role of *P. freudenreichii* and *F. prausnitzii* probiotics against CRC initiation in rats treated with azoxymethane (AOM).

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, Germany.

2.2. Animals and Colon ACF induction

10 weeks old male Sprague Dawley rats (n = 30) were obtained from FOM-Experimental Animal Unit, University of Malaya., To induce carcinogenesis in the rat colon, azoxymethane (AOM, Sigma-Aldrich, country) was used. The AOM 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 animals were divided into six groups of five animals each after one week of acclimatization the rats were randomly distributed as follows: normal control and AOM control groups received the vehicle, standard drug group received intraperitoneal injections of 5- fluorouracil (35mg/kg), 3 times per week for 5 weeks, Propionibacterium group received oral daily dose of 1x 10^9 CFU/ml of *P. freudenreichii* for 5 weeks, Faecalibacterium group received oral daily dose of 1x 10^9 *F. prausnitzii* CFU/ml for 5 weeks and probiotics mixture group received oral daily dose of 1x 10^9 CFU/ml mixture of *P. freudenreichii* + *F. prausnitzii* for 5 weeks.

2.4. ACF Counting

Finally, all rats were anesthetized, sacrificed and the ACF was counted to assess the degree of colonic ACF formation and multiplicity. 0.5% methylene blue solution 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 [16].

2.5. Histological Assay

The colon was harvested and cut into 1 cm × 1 cm square, which were then preserved in 10% buffered formalin and histologically processed [17]. The tissues were stained with H & E stain and examined under microscope.

2.6. Malondialdehyde (MDA) assay

This assay was carried out using the thiobarbituric acid reactive substances (TBARS) assay. A commercial kit (Cayman Chemical, USA) was used to test MDA levels in colon tissue lysates. The MDA amount, that reflects lipid peroxidation strength is determined by the TBARS assay according to the manufacturer's protocol [18].

3. Results

3.1. ACF Frequency and Microscopical findings

ACF was used as a biomarker to evaluate preliminary stage of AOM-induced colon cancer in rats to analyse the effect of probiotics on mitigating colon carcinogenesis. Methylene blue staining was used to examine the occurrence of aberant 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 less ACF (Figure 1). The groups that obtained AOM produced identifiable ACF in the colon after being stained with methylene blue. The rats in the normal control group had no irregular crypts in their colons (Figure 2).
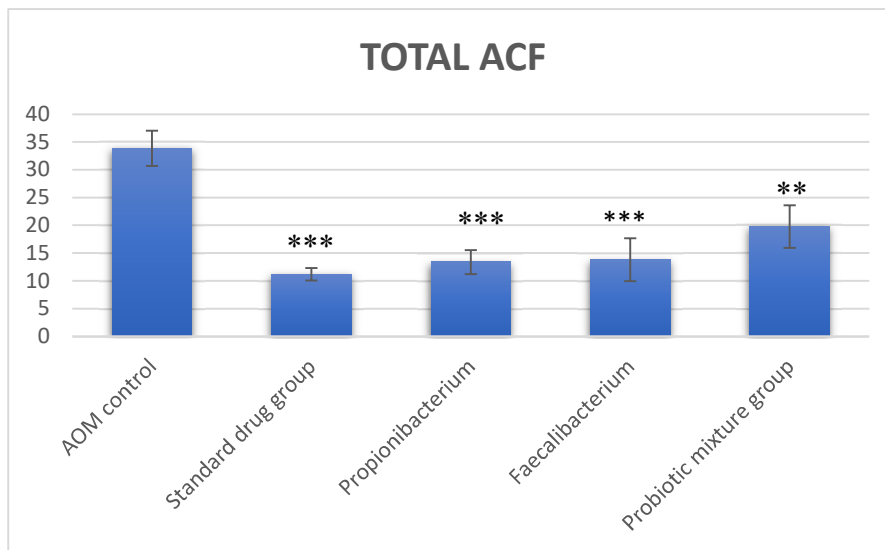

Figure 1: Total ACF count for the different groups. Values were expressed as mean± S.E.M, values with *p <0.05, **p <0.01 and ***p < 0.001 are significant when compared to AOM group.
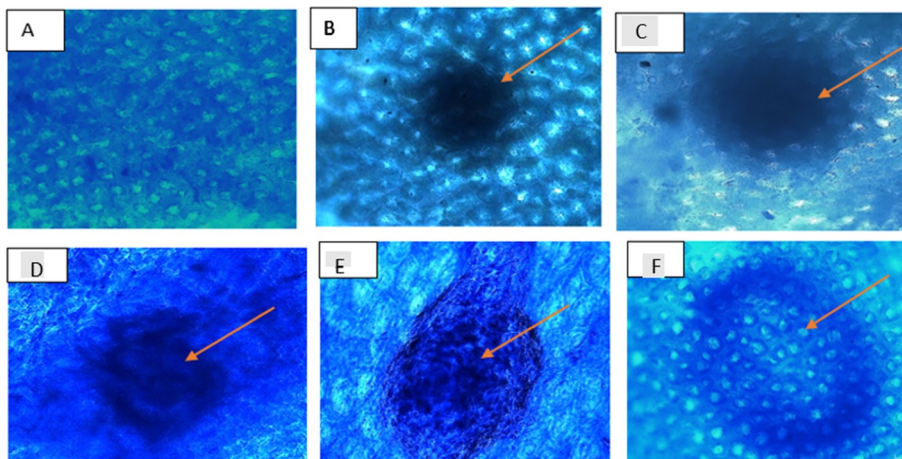
Figure 2: A topographic view shows the ACF found in the colon of the different groups. (**A**) Normal colon crypts from rats in the normal control group treated with PBS (pH 7.4) (**B**) ACF from AOM control group treated with 7 mg/kg AOM (**C**) Standard drug group treated with 35 mg/kg 5-flouroacil + 7 mg/kg AOM (**D**) Propionibacterium group treated with 1x 10^9 CFU/ml *P. freundreichii* + 7 mg/kg AOM (**E**) Faecalibacterium group treated with 1x 10^9 CFU/ml *F. prausnitzii* + 7 mg/kg AOM (**F**) Probiotics mixture group treated with a mixture of 1x 10^9 CFU/ml of *P. freundreichii* 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 histological analysis of the colon cells (Figure 3).
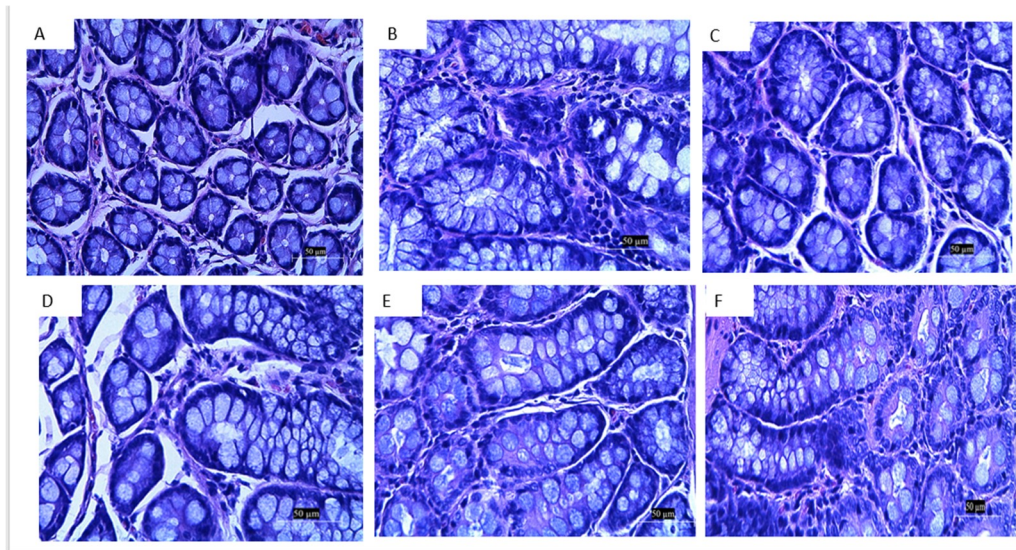
Figure 3: A topographic view of H&E stained colon cells shows the ACF found in the colon of the different groups. (**A**) Normal colon crypts from rats in the normal control group treated with PBS (pH 7.4) (**B**) ACF from AOM control group treated with 7 mg/kg AOM (**C**) Standard drug group treated with 35 mg/kg 5-flouroacil + 7 mg/kg AOM (**D**) Propionibacterium group treated with 1x 10^9 CFU/ml *P. freundreichii* + 7 mg/kg AOM (**E**) Faecalibacterium group treated with 1x 10^9 CFU/ml *F. prausnitzii* + 7 mg/kg AOM (**F**) Probiotics mixture group treated with a mixture of 1x 10^9 CFU/ml of *P. freundreichii* and *F. prausnitzii* + 7 mg/kg AOM.

3.3. Probiotics administration reduced MDA levels

The administration of P. freundreichii and F. prausnitzii led to a lower MDA levels in the tratment group even though not statistically significant when compared to the AOM control group (Figure 4).
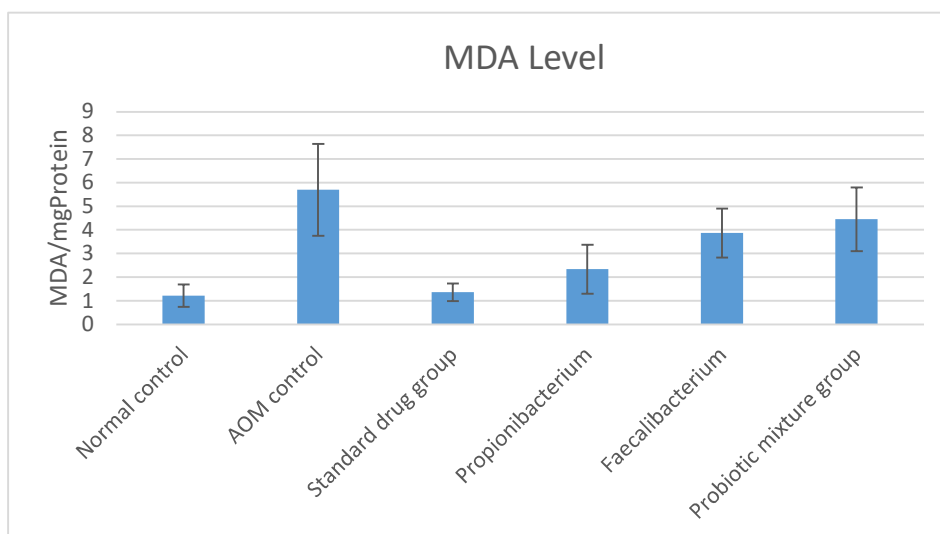
Figure 4: Lipid peroxidation level in treated and untreated groups. All values were expressed as mean ±SEM

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 in this study which is similar to the findings of several other studies, where it was found that Lactobacillus inhibited DMH-induced ACF in in Sprague Dawley rats [19-21]. In 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. MDA levels were lower in the probiotic groups than in the AOM control group, but the differences were not statistical significantl. Elevated lipid peroxidation levels in AOM group is a sign of acute colonic cell injury. This finidng is in agreement to a study by Obuagu et al., where probiotics reduced the malondehyde levels in animal model[22].

5. Conclusion

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. freundreichii and F. prausnitzii against CRC.

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