Proteinaceous Postbiotic Metabolite Produced by *Lactobacillus plantarum* I-UL4 Exhibits Anti-colorectal cancer and Immunomodulatory Properties in Azoxymethane-induced Rats

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Abstract

This study determined the anti-cancer properties of proteinaceous postbiotic metabolite (PPM) produced by *Lactobacillus plantarum* I-UL4, against colorectal cancer model in rats and elucidated the immunomodulatory activities involved. The rats were subjected to injection with azoxymethane for two subsequent weeks to induce colorectal cancer, and subsequently fed with PPM (0.25%, 0.5% and 0.75% (w/v)) added into drinking water, at week 26, once daily for 12 weeks. Cancer severity and immunomodulatory properties of PPM were determined by histopathology analysis and level of cytokines (IFN-γ, TNF-α, IL-12 and IL-5) in serum, spleen and thymus cell suspensions, respectively. Treatment with PPM significantly ($p<0.05$) reduced the total number of aberrant crypt foci (ACF) and crypt multiplicity, incidence of tumor, adenoma and adenocarcinoma, with decreased expression of ß-catenin in a dose-independent manner. The level of all cytokines, except for IL-12 in serum, increased significantly ($p<0.05$) in both spleen and thymus cell suspensions after treatment with PPM compared to control. PPM produced by *Lactobacillus plantarum* I-UL4 exhibited anti-colorectal cancer properties and enhanced the level of several cytokines that play crucial roles in the inhibition of colon carcinogenesis.

Keywords: Proteinaceous postbiotic metabolite, *Lactobacillus plantarum* I-UL4, colorectal cancer, immunomodulation

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Introduction

Globally, it is estimated that over 1.8 million new colorectal cancer (CRC) cases and more than 800,000 death in 2018 which accounting for approximately 1 in 10 cancer cases and death [1]. The American Cancer Society estimated approximately 141,210 people diagnosed with CRC and about 49,380 people died of the disease in the US in 2016 [2]. Colorectal carcinogenesis is a multistep process involving multiple genetic alterations. Pathologically, the earlier phases of colorectal tumorigenesis are initiated in the normal mucosa, with a generalized disorder of cell replication, and with the appearance of clusters of enlarged crypts (aberrant crypts) showing proliferative, biochemical and biomolecular abnormalities [3]. CRC development is commonly initiated by activation of mutations of the *adenomatous polyposis coli (APC), β-catenin* [4,5] and/or *K-ras* [6] that play roles in the Wnt/ β-catenin and RAS(ERK) pathways.

There are several established drugs for standard first line therapy for CRC. Nevertheless, the drugs have various adverse side effects including loss of appetite, hair loss, fatigue and weakening of the immune system. Therefore, much attention has been directed towards the development of natural product-based anti-cancer agents that are believed to have fewer side effects. Probiotics including lactic acid bacteria (LAB) was claimed to promote health by enhancing host immunity [7] and can be a potential preventive and therapeutic agent [8,9]. LAB was found to improve gastrointestinal microbiology [10], exert antimicrobial activities [11,12] and anti-carcinogenic properties [13,14]. Probiotics and their metabolites, postbiotics, have been proposed as food supplements to promote health and intestinal composition, as well as therapeutic agent for inflammatory bowel disease (IBD) [15].

Recently, metabolites of LAB such as bacteriocins were shown to exert probiotic effects. Bacteriocins are ribosomally synthesised antimicrobial peptides (30-60 amino acids) or complex protein metabolites secreted by various Gram-positive and Gram-negative bacteria [16] to prevent the growth of competing microorganisms in a particular biological niche. LAB bacteriocins are commonly utilized as food natural preservatives, particularly for meat and dairy products, due to their high potency against various pathogenic bacteria and food spoilage microbes and their resistance towards proteolytic enzymes [17,18]. Bacteriocins also possessed anti-microbial, anti-viral [12,19] and anti-neoplastic activities [19].

Nisin, azurin, pyocin, colicins, pediocin and microcin are among the metabolites (bacteriocins) from LAB with inhibitory properties toward various types of cancer including colon cancer, breast cancer, human cervical adenocarcinoma and skin cancer [20-23]. In addition, bacteriocins have a potential to act synergistically with the conventional cancer drugs [12]. Azurin, for instance, is a well-characterized anti-cancer bacteriocin with proven cytostatic and pro-apoptotic effect to inhibit the growth of human breast cancer and prostate cancer cells [22,24]. It is a multi-target anti-cancer agent that inhibited ubiquitin mediated degradation of p53 thus interfering the p53 signaling pathway and disturbing the non-receptor tyrosine kinases signaling pathway [22]. In addition, colicins produced by *Escherichia coli*, will only target and cause death to tumor cells through recognition of antibodies that are specific to the outer membrane proteins of the cells [25,26] suggesting their specificity towards tumor cells [27]. Nisin, a polycyclic peptide consists of 34 amino acids, which is the fermentation product by *Lactococcus lactis*, can be considered as an anti-cancer peptide due to its ability to induce cancer cell death through intrinsic apoptotic pathway in CRC cells [21]. In another study, nisin has been shown to induce selective toxicity and decrease the invasion and proliferation in melanoma cells [28]. Colicins exerted *in vitro* anti-cancer activities against breast, colon and bone cancer cells via apoptotic pathway [29].

PPM is a metabolite produced by *Lactobacillus plantarum* I-UL4 isolated from fermented tapioca, *Manihot esculenta*. PPM encouraged the growth and increased the population of indigenous LAB in the intestine and faeces of rats and decreased the faecal pH and faecal *Enterobacteriaceae* counts. Modler et al. [30] suggested that large bowel cancer could be directly controlled by reducing the intestinal pH, hence, preventing the growth of putrefactive bacteria. PPM also reduced the plasma cholesterol [31-33]. PPM was cytotoxic towards breast cancer cells (MCF-7) [34].

The immunomodulatory properties of bacteriocins and postbiotics are believed to play a role in reducing cancer severity. Bacteria and their metabolites were shown to enhance immune systems by several mechanisms including activation of inflammasome pathway and TNF-α innate immune system [23]. Anti-cancer properties of LAB on colon cancer were activating by immune response as well as the metabolic activity of the cancer cells [35]. In addition, prebiotics such as inulin and oligofructose have reported to reduce risk of colorectal carcinogenesis [36] by enhancement of the systemic immunity via an increased level of cytokines such as interferon gamma (IFN-γ) and interleukin 12 (IL-12) [37]. IFN-γ, tumor necrosis factor alpha (TNF-α), IL-12 and IL-5 are the cytokines that have been shown to mediate tumor regression and inhibit tumor development [23,38-40]. This study determined the anti-colorectal cancer and immunomodulatory properties of PPM in chemically-induced colon cancer model *in vivo*.

Materials and Methods

Compound

Proteinaceous postbiotic metabolite (PPM) was kindly supplied by Professor Dr. Foo Hooi Ling from the Department of Bioprocess Technology, Faculty of Biotechnology and Biomolecular Sciences, Universiti Putra Malaysia. Method of extraction was described previously (Raha et al., 2015).

Chemicals and Reagents

Azoxymethane (AOM), chloroform, sodium chloride, Tween-20, RNase A, Tris base, sodium citrate trisodium salt dehydrate, EDTA, d-biotin and Tris EDTA base were purchased from Sigma (St. Louis, MO, USA); absolute alcohol, diethyl ether and xylene (Systerm, USA), eosin and hematoxylin (Surgipath, USA), phosphate-buffered saline, fetal bovine serum, Dulbecco's Modified Eagle Medium (DMEM) culture medium (Gibco®), trypsin EDTA, penicillin-streptomycin and bovine serum albumin were from PAA (Austria); Enzyme-linked Immunoassay (ELISA) kits were from eBioscience, BenderMedSystem, Austria and Uscnlife, China; K2-EDTA coated vacutainer tubes were from BD Biosciences Pharmingen (Franklin Lakes, NJ, USA).

Induction of colorectal cancer and treatment with PPM

A total of 75 male *Sprague dawley* rats of approximately 90-100 g were placed at the Animal Experimental Unit, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia. The rats were acclimatized with free access to water and basal diet (Gold Coin Rat Chow, Australia) for a week prior to the induction of colorectal cancer. The rats were housed in individual cages with woodchip bedding in a well-ventilated room at 29-32^{0}C; 50-60% relative humidity; 12-hour light and darkness cycle. The experiment was approved by the Animal Care and Use (Ethics) Committee of the Faculty of Medicine

and Health Sciences, Universiti Putra Malaysia, Serdang, Selangor (Reference Number: UPM/FPSKPADS/F01-00181).

The rats were subjected to subcutaneous injection of AOM once a week for 2 subsequent weeks (15 mg/kg/week). Following induction, the rats were randomly divided into 5 groups (15 rats per group), which were (1) positive control (injected with AOM, unfed with PPM); (2) fed with 0.25% of PPM (w/w); (3) fed with 0.5% of PPM; (4) fed with 0.75% of PPM and (5) the negative control group (not injected with AOM, unfed with PPM). After 24 weeks of induction, each rat in group (2) to (4) was administered daily with freshly prepared PPM added into drinking water, for 12 weeks.

Sample collection

Body weight of the rats was recorded weekly until the end of experiment. Prior to termination, 10 mL of blood was collected via cardiac puncture. The blood was mixed well in an EDTA tube and the serum was kept at -80°C prior to use. The animals were sacrificed, and the colons were excised, weighed and fixed in RCL2® solution. The samples were kept at 4ºC until further use. For the *ex-vivo* study, thymocytes and spleenocytes were cultured under sterile condition.

Aberrant crypt foci scoring and histological classification

The colon was processed using an automated tissue processor machine (Leica TP 1020, Germany) for 14 hours. Subsequently, colon was embedded in paraffin wax using an embedding machine (Leica EG 1160, Germany) and sectioned (4 µm thickness) by using a microtome (Leica RM 2145). The sections were subjected to hematoxylin and eosin (H&E) staining. Aberrant crypt foci (ACF) were counted in 6 different sections of colon for each rat [41]. The aberrant crypts are stained more darkly, are larger and have a thicker epithelial lining and a larger pericryptal zone than normal crypts [42]. The number of crypts present in each focus represents the growth feature and is referred to as "crypt multiplicity". ACF from each group were also assessed based on the presence and degree of dysplasia. ACF have been categorized into three classifications; hyperplasia without dysplasia, mild to moderate dysplasia and moderate to severe dysplasia [43].

Tumor assessment

Tumor was assessed based on the incidence and multiplicity. Tumor incidence is defined as the percentage of total animals with adenoma/adenocarcinoma. Tumor multiplicity is described as an average number of tumors per tumor-bearing rat [44].

Immunohistochemical analysis of the expression of β-catenin

The paraffin-embedded colon tissues were cut using a microtome (Leica RM2145) and placed on slides coated with poly-L-lysine. The paraffin-embedded sections were heated at 65°C for 30 minutes, deparaffinized in xylene, and rehydrated through graded alcohol at room temperature. Next, the samples were pretreated with 10 mmol/L of citrate buffer (pH 6.0) in a microwave oven for 20 minutes. The sections were then immersed in peroxidase blocking reagent (DAKO) for 5 minutes. The sections were treated with 3% bovine serum albumin for 1 hour at room temperature to reduce the non-specific antibody binding, and subsequently incubated with a primary antibody against β-catenin (dilution

1:2000, Abcam Inc., USA) for 1 hour at room temperature. After incubation with the primary antibody, the sections were washed with Tris-buffered saline (TBS) and were then incubated with rabbit Envision Plus horseradish peroxidase reagent (DAKO) at room temperature for 30 minutes. After the sections were washed with TBS, the staining was then completed by incubation with liquid DAB (3,3-diamino-benzidine; DAKO).

The sections were counterstained with hematoxylin and mounted on glass slides. The slides were viewed and scored under light microscope at 40X magnification. A scoring system was used to evaluate the expression of specific antibody for immunohistochemical staining as described previously [45]. Briefly, staining was scored semi-quantitatively for staining intensity (0 = no expression; 1 = weak; 2 = moderate; 3 = strong) and for the percentage of positive cells (1 = 0–24%; 2 = 25–49%; 3 = 50–74%; and 4 = 75–100%).

Preparation of rat spleen and thymus cell suspension

The spleen and thymus were removed and quickly washed with HANK'S Balance Salt (HBSS) (Sigma, USA). Subsequently, the spleen and thymus were pressed with a rubber syringe plunger and pushed through a sterile nylon mesh screen in phosphate-buffered saline (PBS). The cell suspension was washed three times with PBS-BSA-EDTA (PBS-137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4)-0.1% bovine serum albumin (BSA)-2 mg/mL EDTA buffer and resuspended in Dulbecco's Modified Eagle Medium (DMEM), Gibco® (Flowlab, Australia) supplemented with 100 IU/mL of penicillin, 100 µg/mL of streptomycin (Flowlab, Australia) and 10% foetal calf serum (v/v) (PAA, Austria). Red blood cells in spleen cell suspension were removed by using lysis buffer (8 g NH_4Cl, 1 g Na_2EDTA, 0.1 g KH_2PO_4, pH 7.4). Subsequently, the cell suspension was washed three times with PBS-BSA-EDTA buffer and resuspended in RPMI-1640 media (Flowlab, Australia) supplemented with 100 IU/mL of penicillin, 100 µg/mL of streptomycin (Flowlab, Australia) and 10% FCS (v/v) (PAA, Austria). Cell number and viability of spleenocytes and thymocytes were determined by using a hemacytometer and trypan blue dye exclusion method, respectively.

Determination of level of cytokines in serum and culture supernatants

The spleen and thymus cells were counted using a haemocytometer. Approximately, $2x10^6$ of the cells/mL were resuspended in DMEM, supplemented with 10% of heat-inactivated FBS. The cells were distributed into a 6-well tissue culture plate, incubated with mitogen (1 µg/mL concanavalin A (ConA) for IFN-γ and TNF-α, and 5 µg/mL lipopolysaccharide (LPS) for IL-5 and IL-12) at 37°C in a humidified 5% CO_2 incubator. The culture supernatant was collected after 24 hours of incubation for determination of level of cytokines. The level of cytokines in the serum and culture supernatant was measured using Rat IFN-γ ELISA Ready-SET-Go (eBioscience), Rat TNF ELISA Kit II (BD OptEIA), Rat IL-5 ELISA Kit (Uscnlife, China) and Rat IL-12 ELISA Kit (Uscnlife, China), respectively, according to the manufacturer's instructions.

Data analysis

Statistical analysis was performed using Statistical Package for Social Science Version 20.0 and significance was set at $p<0.05$. Data were analyzed using ANOVA. The value was expressed as mean ± SEM, and means were separated using Duncan Multiple Range Test.

Results

Effects of PPM on the crypt multiplicity, total number, regional distribution and morphology of aberrant crypt foci

The crypt multiplicity and total number of aberrant crypt foci (ACF) are depicted in Figure 1. Treatment with PPM at all doses showed a reduced number of ACF with more than 4 crypts and in the total number of ACF compared to the positive control group ($p<0.05$). Distribution of ACF along the colon is summarized in Table 1. ACF were found in all rats injected with AOM but not in the negative control group, being highly distributed in the proximal part of the colon. The highest number of ACF was observed in the positive control group (165.80 ± 7.26). The total number of ACF was significantly lower in the rats treated with all percentages of PPM as compared to the positive control group ($p<0.05$). The lowest total number of ACF was observed in the groups treated with 0.75% of PPM (63.40 ± 2.26), followed by 0.5% of PPM (83.40 ± 2.70) and 0.25% of PPM (97.60 ± 3.47). Nevertheless, the difference in the total number of ACF among the three PPM-treated groups was insignificant ($p>0.05$).

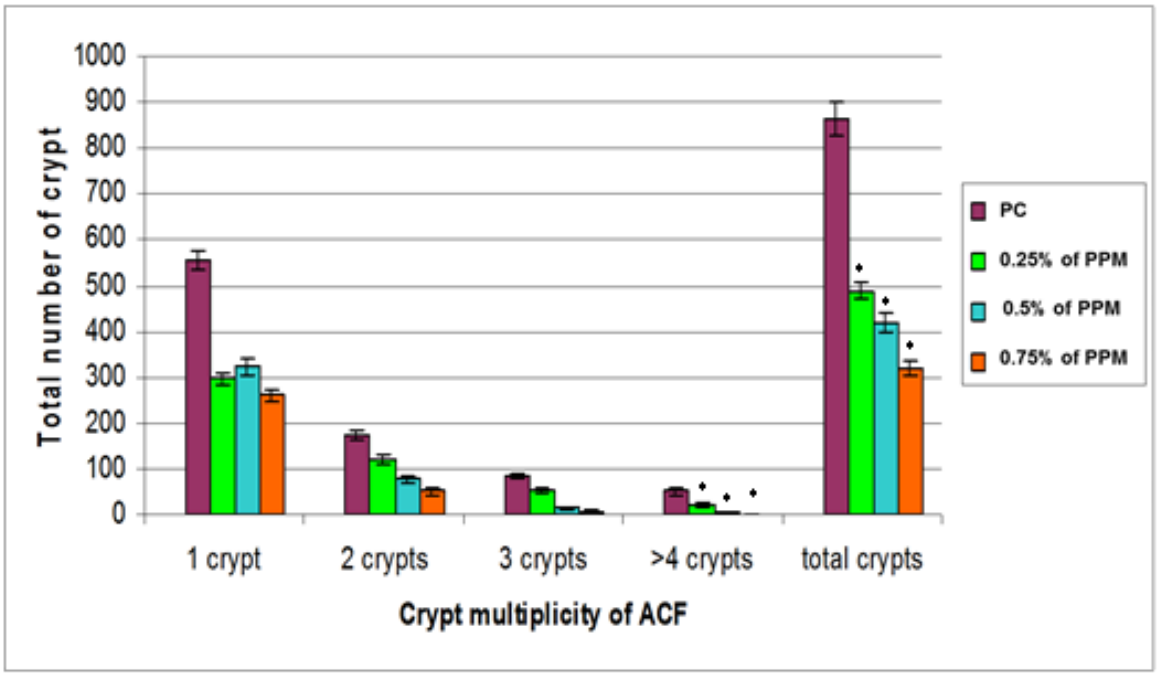

Figure 1: Effects of PPM on the crypt multiplicity and total number of aberrant crypt foci of *Sprague dawley* rats induced with AOM. Each value is expressed as mean ± SEM (n=6). Values with different superscript indicate significant difference by Duncan test ($p<0.05$). Note: PC = positive control group. The value $p<0.05$ was considered significant.

Table 1: Effects of PPM on the regional distribution of colonic ACF and its morphology in *Sprague dawley* rats induced with AOM. Each value is expressed as mean ± SEM (n=6). Values in the same column with different superscripts indicate significant difference by Duncan test (p<0.05).

	Proximal	Medial	Distal	Total	Hyperplasia without dysplasia	Mild to moderate dysplasia	Moderate to severe dysplasia
Negative control	0^{a}	0^{a}	0^{a}	0^{a}	0	0	0
Positive control	69.40 ± 16.67^{c}	35.60 ± 7.59^{c}	60.80 ± 8.00^{c}	165.80 ± 7.26^{c}	63.6 ± 9.29^{d}	58.8 ± 9.10^{c}	65.6 ± 18.66^{b}
0.25% of PPM	46.00 ± 5.92^{bc}	22.00 ± 1.58^{bc}	29.60 ± 3.68^{b}	97.60 ± 3.47^{b}	32 ± 4.34^{c}	27.2 ± 4.42^{b}	15.8 ± 2.29^{a}
0.5% of PPM	27.40 ± 3.75^{ab}	24.60 ± 3.20^{bc}	31.40 ± 6.80^{b}	83.40 ± 2.70^{b}	14.8 ± 1.77^{b}	10.4 ± 1.33^{a}	6.2 ± 0.58^{a}
0.75% of PPM	24.20 ± 3.77^{ab}	15.20 ± 2.78^{b}	24.00 ± 4.19^{b}	63.40 ± 2.26^{b}	9.0 ± 1.04^{ab}	7.4 ± 1.54^{a}	6.0 ± 1.30^{a}

The morphology of ACF was further evaluated based on the established histological criteria. Effects of PPM on the morphology of ACF are depicted in Table 1, and ACF with different degree of dysplasia are shown in Figure 2. The number of ACF of hyperplasia without dysplasia, mild to moderate dysplasia and moderate to severe dysplasia was lower in the group treated with 0.25%, 0.5% and 0.75% of PPM compared to the positive control group ($p<0.05$). The lowest number of ACF within the three different degrees of dysplasia was observed in the group treated with 0.75% of PPM. There was a significant difference in the number of ACF of hyperplasia without dysplasia in the group treated with 0.75% of PPM compared to the other 2 PPM-treated groups (0.25% and 0.5%) ($p<0.05$). The difference in number of ACF of mild to moderate dysplasia between the group of 0.75% and 0.25% of PPM was found to be significant ($p<0.05$).

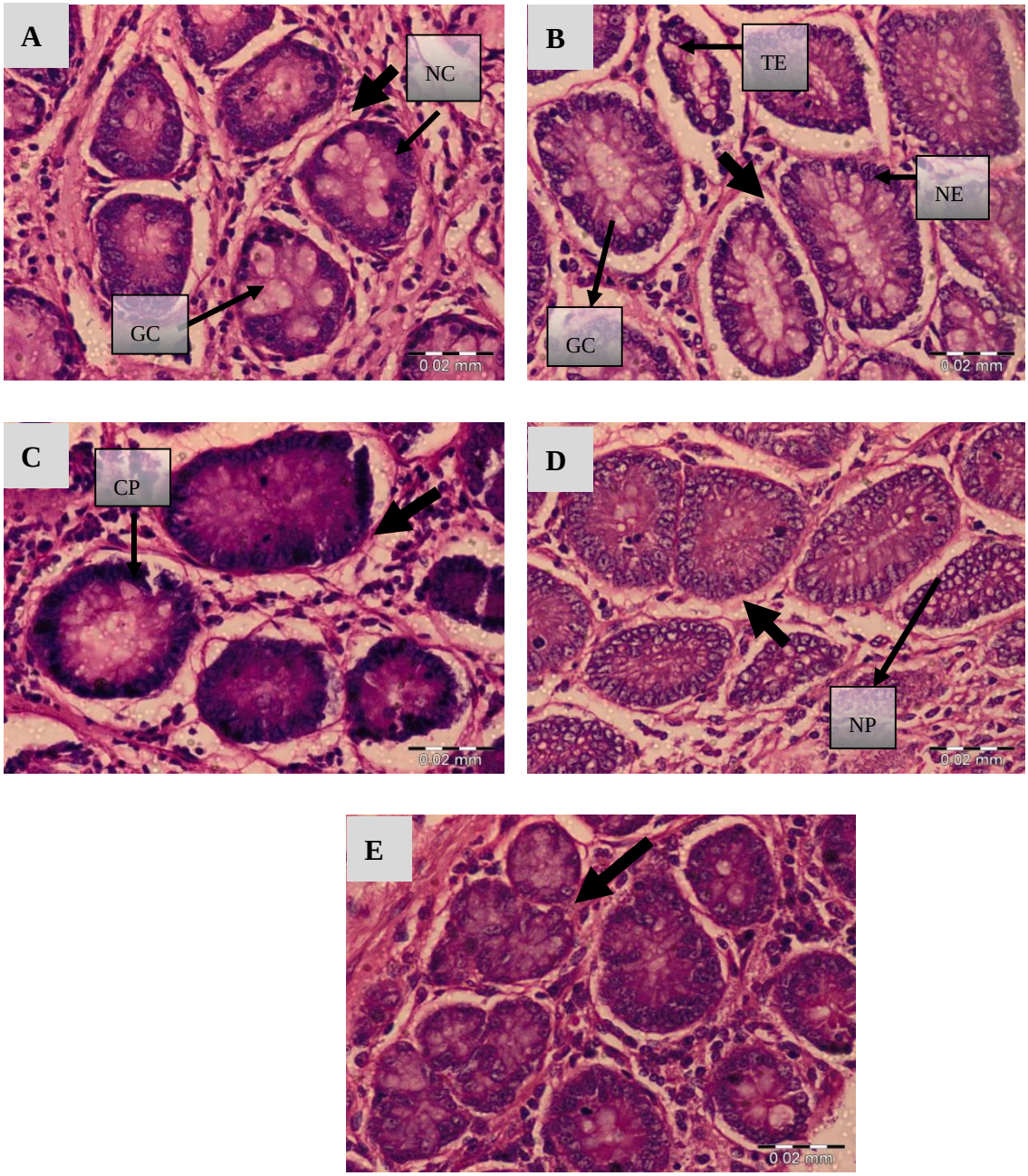

Figure 2: Aberrant crypt foci (ACF) with different degree of dysplasia. (A) ACF with hyperplasia without dysplasia with goblet cell (GC) formation and relatively normal nuclear (NC) morphology; (B) ACF with mild to moderate dysplasia with lack of goblet cell differentiation, thickening of the epithelium (TE), nuclear elongation and stratification (NE); (C) and (D) ACF with severe dysplasia with nuclear stratification and elongation as well as nuclear pleomorphism (NP) and loss of cell polarity (CP); (E) ACF with variable histological classification of the crypts, ranging from hyperplasia to dysplasia (400X magnification). Arrows indicate ACF.

Effects of PPM on the number of total tumors, adenoma and adenocarcinoma and expression of β-catenin

The incidence of total tumor, adenoma and adenocarcinoma is summarized in Table 2. The highest incidence of tumors was noted in the positive control group (66%) followed by the groups of 0.25% of PPM (50%), and 0.5% and 0.75% of PPM (33%). None of the animals in the negative control group developed colon tumors. There was a lower incidence of adenoma and adenocarcinoma in the groups treated with 0.25% and 0.5% of PPM compared to the positive control group. In the group treated with 0.75% of PPM, adenocarcinoma was absent. The expression of β-catenin significantly decreased ($p<0.05$) in the groups treated with 0.5% and 0.75% of PPM as compared to the positive control group. The lowest mean score was from the groups treated with 0.75% of PPM (9.1) followed by 0.5% (13.4) and 0.25% of PPM (17.5) (Table 2).

Table 2: Effects of PPM on the number of total tumors, adenoma and adenocarcinoma and expression of β-catenin in *Sprague dawley* rats induced with AOM. Each value is expressed as mean ± SEM (n=6). Values with different superscript indicate significant difference by Duncan test, $p<0.05$.

Treatment	Percentage (%)			B-catenin expression (Mean score)
	Tumor	Adenoma	Adenocarcinoma	
Negative control	0	0	0	3.0 ± 1.0^{a}
Positive control	66	33	33	22.0 ± 5.5^{d}
0.25% of PPM	50	33	17	17.5 ± 6.1^{c}
0.5% of PPM	33	17	17	13.4 ± 2.3^{c}
0.75% of PPM	33	33	0	9.1 ± 3.6^{b}

Effects of PPM on the level of serum IFN-γ, TNF-α, IL-5 and IL-12

Effects of PPM on the level of cytokines (IFN-γ, TNF-α, IL-5 and IL-12) in the serum of rats induced with AOM are summarized in Figure 3. In general, all the groups treated with PPM showed higher level of all the 4 cytokines as compared to the positive control group ($p<0.05$). The level of IFN-γ increased significantly only in the group treated with 0.75% of PPM as compared to the positive control group ($p<0.05$). The difference in the level of IFN-γ was also noted between the group treated with 0.75% of PPM with the other two UL4-treated groups (0.25% and 0.5%) ($p<0.05$). The level of TNF-α increased significantly in the groups treated with 0.25% and 0.5% of PPM compared to both positive and negative control groups ($p<0.05$). The difference in the level of TNF-α among the three PPM-treated groups was insignificant ($p>0.05$). Meanwhile, the level of IL-5 increased in the groups treated with

0.5% and 0.75% of PPM as compared to the positive control group ($p<0.05$). In addition, the group treated with 0.75% of PPM showed the highest IL-5 level among the treatment groups. However, there was an insignificant difference in the level of IL-12 between all the PPM-treated groups compared to the positive control group ($p>0.05$).

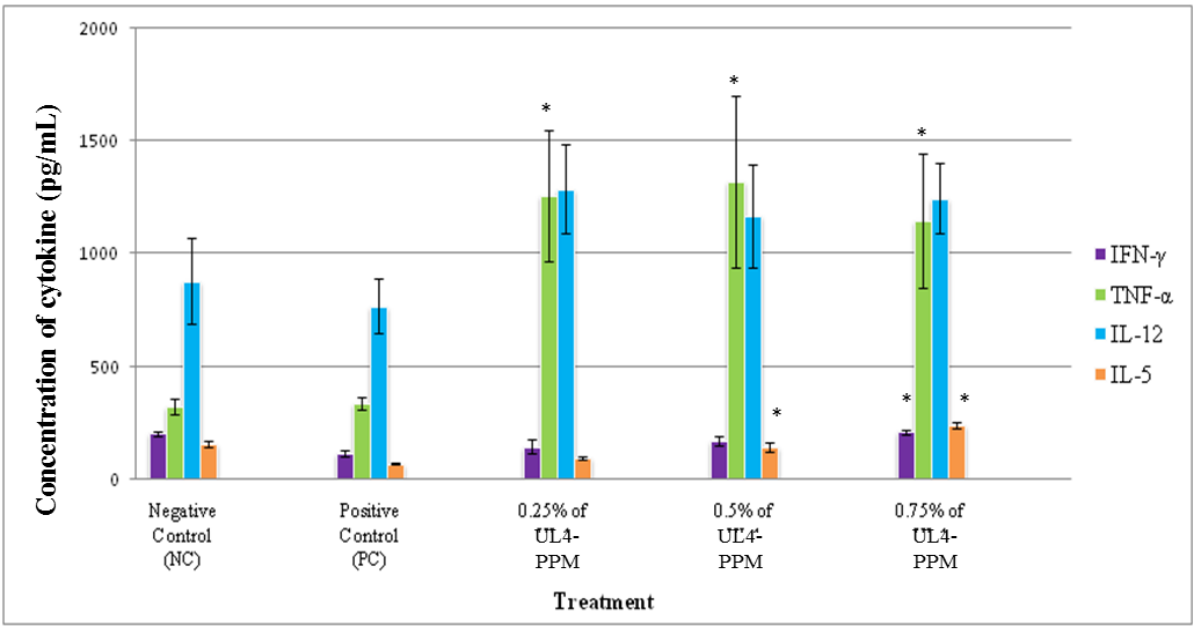

Figure 3: Effects of PPM on the level of serum TNF-α, IFN-γ, IL-5 and IL-12. The value of $p<0.05$ was considered significant. Each value is expressed as mean ± SEM (n=6). Values with different superscript indicate significant difference by Duncan test, $p<0.05$.

Effects of PPM on the level of IFN-γ, TNF-α, IL-5 and 1l.-12 in spleen cell suspensions

Figure 4 shows the effects of PPM on the level of IFN-γ, TNF-α, IL-5 and IL-12 in the spleen cell suspensions at 24 hours of incubation time. The level of TNF-α was higher ($p<0.05$) in the group treated with 0.75% of PPM as compared to the other two PPM-treated groups (0.25% and 0.5%). Increase in the level of IFN-γ was also observed in all the PPM-treated groups however insignificant ($p>0.05$).

Effects of PPM on the level IFN-γ, TNF-α, IL-5 and IL-12 in thymus cell suspensions

Figure 5 shows the effects of PPM on the level of IFN-γ, TNF-α, IL-5 and IL-12 in the thymus cell suspensions at 24 h of incubation time. The level of IFN-γ and IL-5 increased significantly in the thymus cells suspension treated with 0.75% of PPM as compared to the positive control group ($p<0.05$). Similarly, the level of IL-12 also increased in the group treated with 0.75% of PPM.

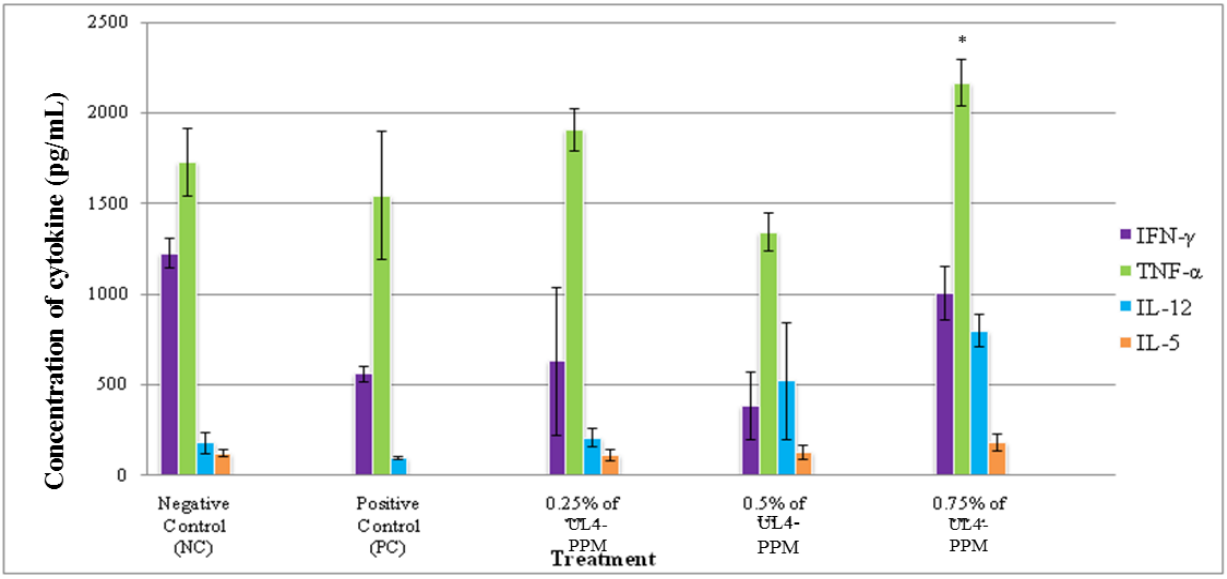

Figure 4: Effects of PPM on the level of TNF-α, IFN-γ, IL-5 and IL-12 in spleen cell suspensions at 24 hours incubation. The value of p<0.05 was considered significant. Each value is expressed as mean ± SEM (n=6). Values with different superscript indicate significant difference by Duncan test, p<0.05.

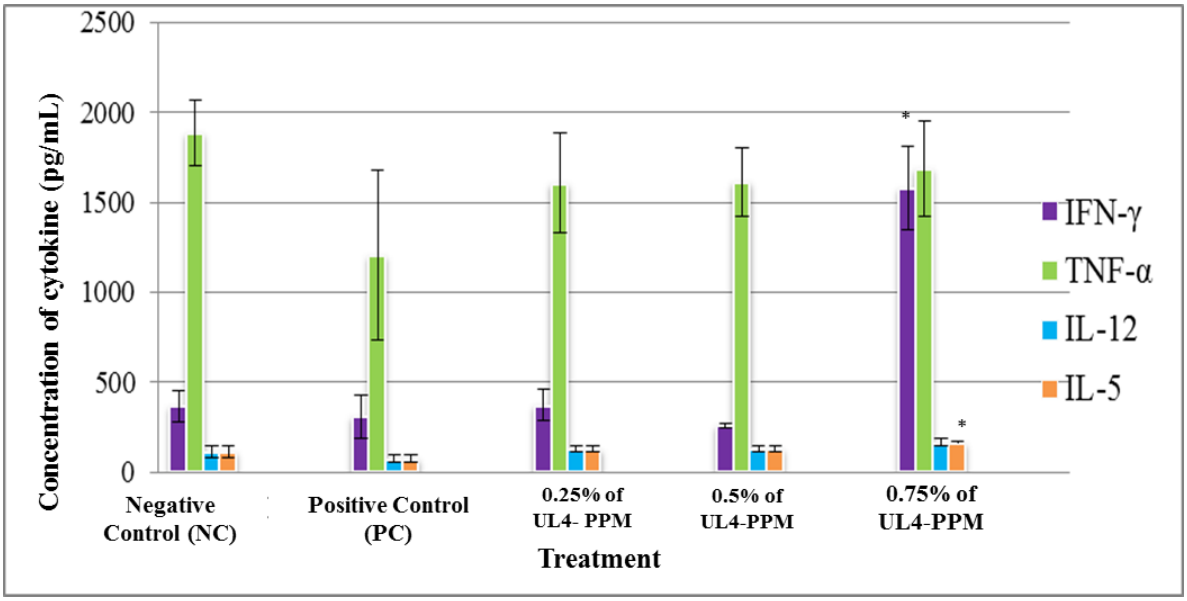

Figure 5: Effects of PPM on the level of TNF-α, IFN-γ, IL-5 and IL-12 in thymus cell suspensions for 24 h of incubation. The value of p<0.05 was considered significant. Each value is expressed as mean ± SEM (n=6). Values with different superscript indicate significant difference by Duncan test, p<0.05.

Discussion

Novel and more specific treatments for cancer are in demand due to the limitations of currently available treatments. The developments of natural or natural product-based anti-cancer agents such as probiotics and their metabolites, and prebiotics, that are believed to have fewer side effects, are of priority. Consumption of probiotics have been associated with reduced risk of CRC [46,47], but the anti-colon cancer properties of their metabolites, in particular bacteriocins, are not being extensively studied as yet. In this study, we investigated the anti-colon cancer properties of PPM, the post-biotic compound produced by *L. plantarum*. The anti-tumor promoting activities of PPM have been discovered in our previous studies (51). It is important to note that not much discussion and comparison can be made on our findings of PPM since not many studies have been done on post-biotic compound produced by LAB. So far, only one type has been studied for its potential in cancer treatment. Bacteriocin from *E. coli* i.e. colicins have been previously shown to specifically inhibit the proliferation of cancer cells [27]. The anti-cancer properties of LAB mediated by activation of immune response and metabolic activity in cancer cells have been reported in colon cancer [13,14,35]. In fact, probiotics have also been shown to prevent intestinal carcinogenesis by suppressing bacterial growth associated with tumor development by altering composition of intestinal microbiota [48].

Animal models are extremely valuable in understanding human cancers such as colorectal and breast cancer due to the similarities in histopathology, molecular and genetic lesion during stages of carcinogenesis [49]. The azoxymethane-induced *Sprague dawley* rats used in this study was found to be a very good animal model of CRC [43,50,51] because all the animals developed ACF after 6 months. Furthermore, ACF, the preneoplastic lesions of rodents and human share similar features [52]. The ACF system has been used extensively to identify modulators of colon carcinogenesis as it is the only endpoint, which provides a quantitative approach to assess the disease process, and the underlying cellular and molecular events [53]. The system is also able to predict the ability of a test agent, PPM in this study, to affect tumor outcome in a consistent manner [54].

In this study, the number of ACF and crypt multiplicity was used as indicators of anti-colon cancer properties of PPM. This is in accordance with [43] and [55], that the ACF system is an important tool to study colon carcinogenesis. Treatment with 0.75% of PPM exhibited the best anti-cancer activities among other doses with the lowest number of ACF and crypt multiplicity and total crypts. Reduction in crypt multiplicity especially of 4 and more crypts shows that PPM is capable in inhibiting colon carcinogenesis as they have a high tendency to progress to real cancer or malignancy (adenocarcinoma) [55]. Suppression of ACF with lower crypt multiplicity (1, 2 and 3 crypts) by PPM could possibly be one of the reasons of reduction in the formation of 4 and more crypts. As time progresses, many foci of aberrant crypts contain more than one crypt. At a later time point, a colon may contain a large number of ACF exhibiting a spectrum of growth features as determined by the number of crypts present in each focus (crypt multiplicity). A single aberrant crypt expands by crypt branching or multiplication. As the ACF develop, majority of them exhibit varying grades of dysplasia i.e. hyperplasia without dysplasia, mild to moderate dysplasia and moderate to severe dysplasia [41]. Treatment with PPM reduced the number of ACF of all the three grades of dysplasia. The reduction of ACF by PPM of moderate to severe dysplasia that are highly related to tumorigenesis and can lead to malignancy indicates its anti-colon cancer properties [53].

Besides ACF, reduced incidence of tumor, adenoma and adenocarcinoma by PPM was also noted but the mechanisms are unknown. There are few well-characterized anti-cancer bacteriocins (colicins, nisin and azurin) with established anti-cancer mechanisms, which PPM may mimic, as follows: Bacteriocins have been shown to exert a direct cell lysis and pore formation via specific or non-specific receptor binding lead to cell death [12,56]. Bacteriocins are preferentially bound to cancer cell membranes than to the normal cell membranes as membranes of the cancer cells commonly carry a negative charge [12]. Colicins have been postulated to kill tumor cells with the same mechanisms of action used to kill closely related strains of bacteria, which include depolarization of the cytoplasmic membrane [57], non-specific DNase activity [58], a highly specific RNase activity [59], inhibition of protein synthesis [60] and by formation of pore lead to cell wall degradation [12]. Absence of colicins within the bowel has been claimed to be one of the factors in the development of colorectal carcinoma [61]. Nisin was found to attack the cytoplasmic membrane in combination with EDTA through chelating effect [12]. Azurin was shown to have a cytotoxic effect via endocytosis process by entering cancer cells through receptor-mediated endocytosis, which is highly expressed on the surface of cancer cells [62]. It is also being suggested that azurin may interfere in key independent signaling pathways that are associated with cancer progression including promoting cancer cell apoptosis in p53 signaling pathway, inhibiting cancer cell growth through the receptor tyrosine kinase EphB2-mediated signaling pathway and reducing angiogenesis by reducing VEGFR-2 tyrosine kinase activity [23].

β-catenin plays an important role in colon carcinogenesis [5]. Mutations of *β-catenin* (up-regulated) are observed in the majority of colon cancers in humans and rodents [63]. In this study, β-catenin was found to be expressed only in the colonic cytoplasm and nucleus of animals with cancer. The highest expression of β-catenin was noted in the untreated positive control group but being down regulated following treatment with PPM. These findings correlate well with the incidence of ACF, tumor, adenoma and adenocarcinoma. The PPM-treated groups with lower incidence of ACF, tumor, adenoma and adenocarcinoma were found to have higher expression of β-catenin compared to the positive control group.

It is claimed that generation and development of a tumor may occur in immunocompromised people. Thus, stimulation of the immune system may enhance host defense toward tumor cells and be one of the most important mechanisms to treat the disease. In this study, besides the anti-colorectal properties, the immunomodulatory potential of PPM was also being given attention. Indeed, protection of colon carcinogenesis by the metabolites of probiotics has been reported to be closely related to their immunomodulatory potential [64]. Cytokines are one of the most important components of an immune system [36,39,65-68]. TNF-α, IFN-γ, IL-5 and IL-12 are crucially involved in tumor development [36,39,67,68]. In general, the level of all the four cytokines in serum and, spleen and thymus cell suspensions was significantly enhanced following treatment with PPM compared to the positive control group.

The higher level of TNF-α in both serum and spleen cells following treatment PPM is in accordance with the lower incidence of ACF, tumor, adenoma and adenocarcinoma. With the increase in the level of TNF-α, the incidence decreases. A lot of evidence supports that TNF exerts antineoplastic activity where there is a cascade of molecular events underlying TNF-mediated tumor regression observed *in vivo*. The antineoplastic effects comprise of direct cytotoxic effects, tumor vasculature and synergism with conventional antineoplastic agents [65]. The direct toxic

effects of TNF can be seen if TNF-R1 is expressed on the malignant cells and subsequently sensitized them to TNF-driven cell death by over expression of cathepsin-β [69]. Vascular effects are critical for TNF antitumor, in which blood vessels are the primary targets [70]. In addition, in cancer, TNF can mediate tumor suppression in mice by increasing the production of cytokines such as IL-6 and IL-8 and also cytotoxic factors, nitric oxide (NO) and reactive oxygen species (ROS) by macrophages [71,72]. Recently, bacteria and their metabolites were found to enhance immunity by activating inflammasome pathways and TNF-α innate immune system resulting in significant release of inflammatory cytokines such as IL-1β, TNF-α and IL-18 which enhances tumor suppression [73]. Therefore, this study suggests that PPM induces immune response via increasing the level of TNF-α that is essential in reducing cancer severity.

In general, the level of IFN-γ was also elevated in the serum (0.75% of PPM) but not in the spleen and thymus cells. The reasons behind the difference in the effects are unclear. IFN-γ is a cytokine that plays an important role both immunologically and non-immunologically in tumor development [67]. It is produced by natural killer (NK) cells, the primary effector cells that play a role in tumor cell elimination [40]. IFN-γ involves in inhibition of tumor growth via several mechanisms such as induction of IFN-γ-inducible protein 10 (IP-10) and induces apoptosis in endothelial cells that limit angiogenesis [74]. It also stimulates the production of nitric oxide by tumor and/or host cells, which can inhibit tumor growth (48). These findings suggest that PPM could regulate the level of important cytokines that leads to tumor suppression. IFN-γ also has an ability to control tumor development in carcinogen-treated mice, which depends on the direct effect of IFN-γ on tumor cells itself [70]. In addition, the level of IFN-γ was significantly increased in animals treated with combination therapy of 5-FU/ celecoxib [69]. In this process, the immune system and specific lymphocytes are capable to recognize and eliminate transformed cells during primary tumor development [67].

IL-12 has antiangiogenic effect, as well as antitumor and anticancer agent [75]. This study shows that there was a significant increase in the level of IL-12 in spleen and thymus cell suspensions of animals treated with 0.75% of PPM as compared to the positive control group. The antitumor activity of IL-12 has been extensively reported in mouse cancer models, where it has been shown to inhibit tumorigenesis and induce regression of established tumors [39]. Suppression of angiogenesis by IL-12 was dependent on its ability to induce IFN-γ expression and depending on IFN-γ receptors that are expressed in neoplastic cells [36]. The immunomodulatory properties of PPM are strengthened by an increased level of IL-5. IL-5 plays indirect roles in inhibition of tumor development. A study has shown that IL-5 transgenic mice has enhanced level of circulating eosinophils that effectively eradicates transplantable tumors [76].

In short, it is believed that the immunomodulatory properties of PPM contribute to its anti-colorectal cancer activities. Nevertheless, the actual mechanisms warrant further investigation. *L. plantarum* that produces PPM has been reported to exhibit immunomodulatory effects on human colon cancer cells [77].

Conclusions

PPM exhibited the anti-colorectal cancer and immunomodulatory properties in *Sprague dawley* rats induced with azoxymethane. The present findings suggest that PPM can be considered as a potential alternative of anti-colorectal cancer agent with the advantage of having immunomodulatory properties that can help to enhance immune response of cancer patients to fight the disease.

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Author Contributions

Conceptualization, N.M.Z.; L.S.Y.; N.B.M.A.; R.A.R; Materials (PPM compound), F.H.L.; Data Analysis, N.M.Z.; S.D.; Histopathological Analysis, N.M.Z.; M.H.B.; Manuscript preparation, N.M.Z.; L.S.Y.; S.A.A.G.

Disclosure of Conflict of Interest

The authors declare no conflict of interest.

Compliance with Ethical Standards

The work is compliant with ethical standards.

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