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Synbiotic preparation with Lactic acid bacteria and inulin as a functional food: *In vivo* evaluation of microbial activities, and preneoplastic aberrant crypt foci

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Abstract

Host microbiome and metabolome are associated with the incidence of colorectal cancer (CC), one of the major health problems in developed countries. The pro and prebiotic supplementation helps to improve the host health. Inulin is one such prebiotic used for the enhancement of naïve probiotic bacterial population. This paper explains the impact of inulin (PRE) extracted from *Jerusalem artichoke* (JA), *Lactobacillus plantarum* HII11 (PRO), and synbiotic (SYN; inulin + *L. plantarum* HII11) preparation on Azoxymethane mediated CC induced rat model with respect to changes in microbial load, microbial enzymes, and preneoplastic aberrant crypt foci. The results suggested that the PRE and SYN supplementation effectively reduced the selected pathogenic bacteria (*Salmonella* spp., and *Escherichia coli*), microbial enzymes and increased the probiotic load. The intervention of SYN significantly reduced the colonic ACF in CC model. The study results revealed that the supplementation of SYN diet (inulin and *L. plantarum* HII11) protects the AOM-mediated colon cancer induced host.

Keywords: inulin; *L. plantarum*; probiotic; prebiotic; synbiotic.

Practical Application: The study results help to develop functional food to improve the human health.

1 Introduction

Colorectal cancer (CC) is one of the major health problems with the vast incidence in developed countries. Currently, the incidence and mortality of CC increased rapidly in Asian countries (Pourhoseingholi, 2012). Diet, environment and lifestyle play a significant role in carcinogenesis. The increase in CC incidence is parallel with economic development and adoption of a western way of life (Center et al., 2009). Epidemiological studies suggested that consumption of high-fat diet, red meat, and processed meat, could be associated with the contribution of CC risk (Huxley et al., 2009; Chan et al., 2011). However, fiber-rich diet appeared to be protective against CC (Riboli & Norat, 2003). Scientific evidences revealed that colonic microflora have a significant role in the cause of CC by altering the metabolome and the transcriptome of the host (Louis et al., 2014; Ohtani, 2015).

Live microbes with a beneficial impact on host system upon consumption are known as probiotics. Most of the probiotics are lactic acid bacteria in nature such as *Lactobacillus*, *Leuconostoc*, *Pediococcus*, *Lactococcus*, and *Streptococcus*, etc. Several probiotic based clinical trials were conducted to address the advantageous role of probiotics in pathological conditions like lactose intolerance, antibiotic-induced diarrhea, gastroenteritis, constipation favorable against diseases caused by an imbalance of the colonic microflora, and anti-colon tumor effect (Iannitti & Palmieri, 2010). The prebiotics, naturally occurring non-digestible food compounds, are responsible for the favorable changes in host system by positively regulating

the growth and/ or activity of probiotic bacteria, resisting especially in colon region (Gibson et al., 2010). Inulin, existing in the form of oligo- and polysaccharides has been recognized as a prebiotic with desirable traits like stability at gastric acidity, accessibility to mammalian enzymes and gut microbes. Inulin extracted from *Jerusalem artichoke* (JA), exhibited a prebiotic activity, especially for *Lactobacillus plantarum* and showed the antimicrobial response against *Escherichia coli* and *Salmonella enterica* serovar Typhi *in vivo* (Pattananandecha et al., 2015). We also demonstrated the prebiotic and anti-colon cancer nature of JA-inulin in azoxymethane (AOM)-induced rat model. In detail, inulin administration positively regulated the population of representative probiotic strains and suppresses the pathogenic strains (Pattananandecha et al., 2016).

The retaining of microbial balance in the human gut plays a significant role in preventing diseases (Guinane & Cotter, 2013; Slavin, 2013). It is known that appropriate pre and probiotic supplementation enhance the survival and activities of a distinct group of microbes both *in-vitro* and *in-vivo* (Anandharaj et al., 2014). Pro and prebiotic candidates aids the suppression of harmful bacterial growth and releases of genotoxic, and carcinogenic substances (phenol, *p*-cresol, and phenolic compounds) (Burns & Rowland, 2000) and CC associated bacterial enzymes (β -glucuronidase, nitroreductase, and azoreductase) (Rowland et al., 1998).

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Synbiotic, the combination of pre and probiotic, has synergistic effects on promoting the growth of existing beneficial strains in the colon, and improving the survival, attaching and growth of newly introduced probiotic strains. Synbiotic intervention showed improved health benefits to the host compared to either unaided probiotic or prebiotic supplementations (Liong, 2008). To the best of the authors' knowledge, there are no detailed reports on the beneficial effect of a synbiotic preparation containing JA-inulin and *L. plantarum* HII11. Thus, we executed the experiments with prebiotics (Inulin), probiotic (*L. plantarum* HII11), and synbiotic (Inulin + *L. plantarum* HII11) preparations to assess the effect on AOM-induced colon cancer in a rat model.

2 Materials and methods

2.1 Bacterial strain and chemicals

The probiotic bacterium, *L. plantarum* HII11 was acquired from Health Innovation Institute, Chiang Mai, Thailand. Azoxymethane (AOM) was purchased from Wako Pure Chemical Industries, Ltd. Osaka, Japan. *p*-Nitrophenyl- β -D-glucuronide, Trisodium 3-hydroxy-4-(4'-sulphonatonaphthylazo) naphthalene-2,7-disulphonate (Amaranth), *p*-Nitrobenzoic acid, ammonium sulfamate and N-(1-Naphthyl) ethylenediamine dihydrochloride (NEDD) were bought from Sigma-Aldrich Co. LLC, St. Louis, MO, USA. Hydrolyzed inulin was obtained from Health Innovation Institute, Chiang Mai, Thailand.

2.2 Animals and diets

The National Laboratory Animal Center (NLAC), Mahidol University, Bangkok, Thailand provided the sum of 72 Sprague-Dawley male rats at the age of 4-5 weeks. The lab acclimatized (2 weeks) animals were randomly divided into six groups of 12 rats each (3 control groups & 3 AOM treated groups). All the rats were maintained at 21-25 °C with 12 hours of light-dark cycles and water and diets were given *ad libitum*. After 2 weeks of normal diet (Mouse and rat diet No. 082, NLAC, Thailand), animals were fed with normal diet (Control), diet containing 10% of inulin (PRE), diet containing 10^8 - 10^9 CFU/g of probiotic strain (PRO), and diet containing 10^8 - 10^9 CFU/g probiotic strain with 10% (w/w) inulin (SYN) for 17 weeks. All rats in the AOM-treated group were subjected to subcutaneous injection of AOM (15.0 mg/kg body weight) at week 8 and 9 of the experimental period. Body mass and feed intakes were measured weekly. Animal experiments were performed as per the regulations and approval of an ethical committee of Faculty of Pharmacy, Ubon Ratchathani University, Ubon Ratchathani, Thailand (24/2009/Research).

2.3 Measurement of cecal content weight and cecal pH

All the animals were sacrificed after 17 weeks of experimental diets, and the whole cecum were excised and weighed. Then the cecal pH was measured (Metrohm- 691) after slitting the cecum open.

2.4 Bacterial enumeration and detection of microbial enzymes

The representative fecal bacterial (*Lactobacillus* spp., *Bifidobacteria* spp, *Escherichia coli* and *Salmonella* spp.) load was determined using plate count method as detailed in our previous study (Pattananandecha et al., 2016) and the results were represented as log difference between before and after the treatments. Bacterial enzymes such as β -glucuronidase, azoreductase, and nitroreductase were determined by the spectrophotometric method as described previously (Pattananandecha et al., 2016).

2.5 Examination of putrefactive compounds and organic acids

The content of organic acids (lactic, acetic, propionic, and butyric acids) and putrefactive compounds (indole, phenol, and *p*-cresol) in the rat feces were estimated using high-performance liquid chromatography (HPLC) as described previously (Pattananandecha et al., 2016).

2.6 Microscopy

The colon samples were collected after sacrificing the experimental animals at the end of study period. Then the number of ACF were counted by staining with 0.2% methylene blue using the microscope (Olympus/Bx60) as reported previously (Pattananandecha et al., 2016).

2.7 Statistical analysis

All the analyses were performed in triplicates and the results were represented as mean \pm SD. The values were analyzed by one-way ANOVA and Duncan studentized range test using SPSS version 17 (Chicago, SPSS Inc, U.S.A). A significant difference was calculated at $p < 0.001$ -0.05.

3 Results and discussion

3.1 Body weight, feed intake, cecal weight, and pH

Body weight gain (21 weeks) in rats fed with PRO (319 ± 25.88 g), PRE (325 ± 36.25 g) and SYN (320 ± 28.28 g) diets were increased compared to rat fed with the control diet (305 ± 25.07 g; $p < 0.05$) and the weight of AOM-treated control rats (280 ± 22.80 g) was significantly lower than the naive rats (305 ± 25.07 g; $p < 0.05$). PRO and SYN supplementation significantly ($p < 0.05$) improved the body mass of the AOM-treated rats. Moreover, PRE supplementation improved the weight of AOM-treated rats (313 ± 22.52 g) than PRO and SYN intervention (283 ± 15.06 g and 296 ± 23.26 g, respectively; Table 1).

All tested interventions (PRO, PRE, and SYN) did not affect the feed intake among the experimental animals. The cecal weight of the rats fed with PRE, and SYN diets were significantly higher compared to respective controls ($p < 0.001$) and PRO rats. The cecal pH of the rats fed with PRE, and SYN diets were significantly low than respective controls ($p < 0.001$) and PRO rats (Table 1).

The results showed that PRE and SYN diet increased the cecal weight and reduced the cecal pH in AOM (Table 1), which was due to the colonic bacterial fermentation of inulin resulting in the release of organic acids and increased biomass (Kolida et al., 2002; Rossi et al., 2005). The fermentation of prebiotic inulin by newly added probiotic strain and the existing beneficial strains may modulate the population of bacteria in the gut (Kolida & Gibson, 2011; Raman et al., 2013), which may also influence the microbial balance in the colon. Thus, we estimated the representative probiotic, and pathogenic bacterial genus upon various supplementations.

3.2 Bacterial enumeration

The changes in the selected fecal bacterial load are shown in Figure 1. The results are represented as log difference between before and after the treatments. A significant level of increase

in the population of *Lactobacillus* spp. and *Bifidobacterium* spp. was observed in PRO ($p < 0.01$), PRE ($p < 0.001$), and SYN ($p < 0.001$) diets compared to control in both AOM-treated and non-treated groups. Notably, PRE and SYN diets considerably increased the selected probiotic strains than PRO diet in both AOM-treated and non-treated groups. The log CFU values of representative pathogenic strains *E. coli* and *Salmonella* spp. were found to be significantly ($p < 0.001$) reduced upon PRO, PRE, and SYN supplementation in AOM-treated and non-treated groups (Figure 1).

The results suggested that all studied supplements significantly ($p < 0.01$ - 0.001) promoted the growth of *Lactobacillus* spp., and *Bifidobacteria* in both naïve (0.10-0.56 and 0.42-1.43 log CFU/ g of feces, respectively), and AOM-treated (0.14-0.38 and 0.34-1.05 log CFU/ g of feces, respectively) animals compared to controls (-0.45- -0.20 and -0.60-0.03 log CFU/ g of feces, respectively) (Figure 1A, B). The selected pathogenic strains,

Table 1. Influence of probiotic, and synbiotic on weight gain, feed intake, cecal weight and cecal pH of rats.

Group	Weight gain (g)	Feed intake (g/d)	Cecal weight (g)	Cecal pH
Control	305 ± 25.07	22.7 ± 0.26	2.45 ± 0.74	7.49 ± 0.22
Control + AOM	280 ± 22.80	22.6 ± 0.13	2.81 ± 0.45	7.64 ± 0.16
PRO	319 ± 25.88	22.8 ± 0.50	2.81 ± 0.39	7.44 ± 0.19
PRO + AOM	283 ± 15.06	22.5 ± 0.24	2.82 ± 0.83	7.55 ± 0.22
PRE	325 ± 36.25	22.6 ± 1.10	5.96 ± 0.85***	6.51 ± 0.22***
PRE+AOM	313 ± 22.52†	22.6 ± 0.80	5.56 ± 0.98†††	6.61 ± 0.13†††
SYN	320 ± 28.28	22.2 ± 0.77	6.10 ± 0.89***	6.48 ± 0.12***
SYN + AOM	296 ± 23.26	21.9 ± 0.65	5.31 ± 0.49†††	6.42 ± 0.11†††

All the values are mean ± SD (n = 12 per group). *** $p < 0.001$ represents the significantly differed from naïve control group. † $p < 0.05$. ††† $p < 0.001$ represents the significantly differed from AOM treated- control group.

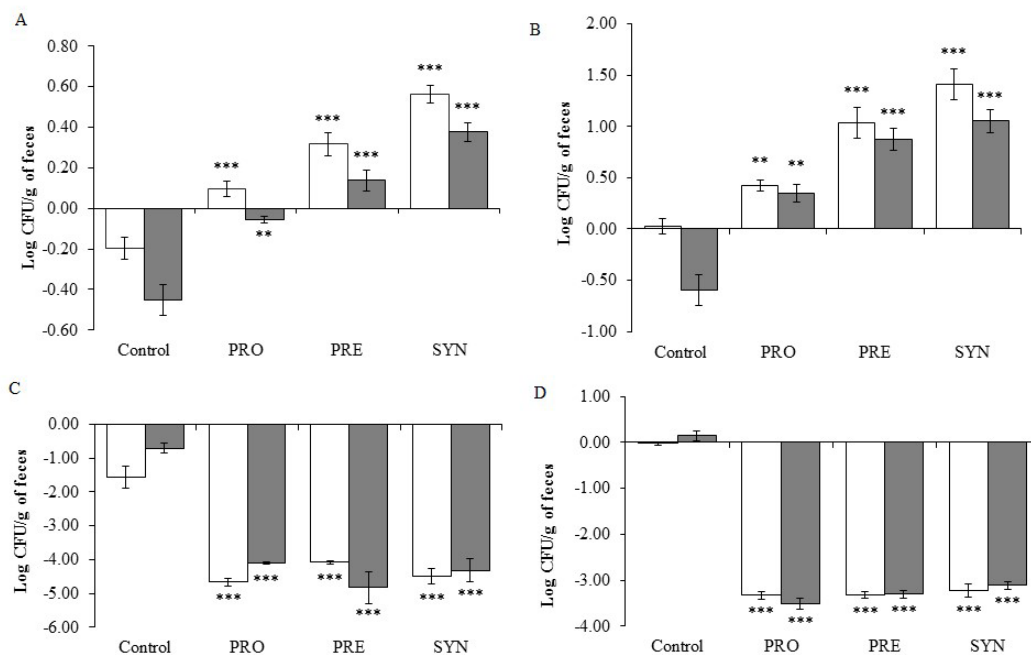


Figure 1. Changes in the bacterial population (End-Start) upon various treatments (CFU/g of feces). Naïve (□) and AOM-treated (■). *Lactobacillus* spp. (A), *Bifidobacteria* (B), *Escherichia coli* (C) and *Salmonella* spp. (D). Values are the mean ± SD. ** $p < 0.01$ vs control group; *** $p < 0.001$ vs control group.

E. coli, and *Salmonella* spp., were drastically reduced after the PRO, PRE, and SYN supplementation in naïve (-4.67- -4.08 and -3.33- -3.23 log CFU/ g of feces, respectively), and colon cancer induced (-4.82- -4.11 and -3.51- -3.07 log CFU/ g of feces, respectively) animals compared to controls (-1.57 - -0.71 and -0.02 - 0.14 log CFU/ g of feces, respectively) (Figure 1C, D).

3.3 Organic acids and putrefactive compounds

The inhibitory action of probiotic on pathogenic bacteria is possibly by the production of short-chain fatty acids (SCFA), lactic acid, antimicrobial compounds, and the competitive nature of probiotic bacteria for nutrients and physical attachment sites. Moreover, probiotics help to activate and enhance the host immune system, which subsequently suppresses the growth of potentially pathogenic bacteria such as *E. coli*, *S. Typhi* and *C. perfringens* (Cummings et al., 2001; Callaway et al., 2012). SCFAs and lactate are the end-products of microbial fermentation of polysaccharides and oligosaccharides (Pan et al., 2009). These acids play an important role in the maintenance of gut health, intestinal morphology and function (Ruemmele et al., 2003; Fung et al., 2011). Lactate improves the gut health by inducing

the cell migration and cell clusters (Hirschhaeuser et al., 2011). Butyrate is a preferred energy source for colonocytes, lowering the luminal pH, (Raman et al., 2013), involved in cell cycle processes like proliferation, differentiation, and apoptosis (Siavoshian et al., 2000; Le Leu et al., 2003; Pool-Zobel et al., 2005; Comalada et al., 2006; Waldecker et al., 2008). The reports revealed that propionate and acetate can induce apoptosis in human colorectal carcinoma cell lines through the loss of mitochondrial transmembrane potential, generation of ROS, caspase-3-processing, and nuclear chromatin condensation (Hosseini et al., 2011).

The concentration of fecal organic acids in normal and AOM-treated rats after PRO, PRE, and SYN diets are shown in Figure 2. All tested organic acids (acetic, propionic, butyric, and lactic acid) significantly ($p < 0.001-0.05$) increased in rats fed with PRE, and SYN preparation. However, PRO diet did not affect the acid content; there was no significant difference between control, and AOM-treated rats. PRE, and SYN supplementation significantly ($p < 0.001$) decreased the amount of fecal indole, phenol and *p*-cresol compared to control and PRO diet groups in both AOM-treated and non-treated groups (Figure 3).

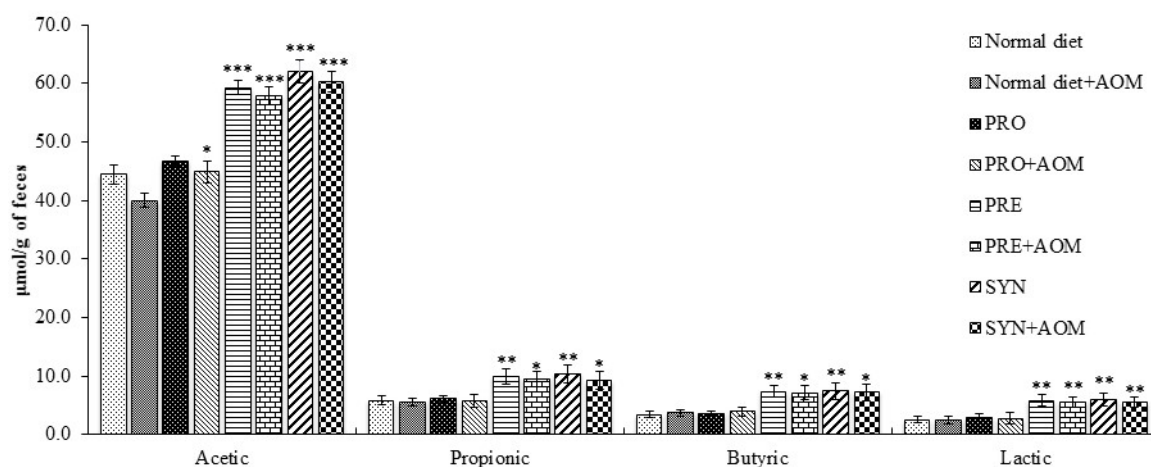


Figure 2. Profiling of organic acid content ($\mu\text{g/g}$ of feces). Values are represented as mean \pm SD. * $p < 0.05$ vs control group; ** $p < 0.01$ vs control group; *** $p < 0.001$ vs control group.

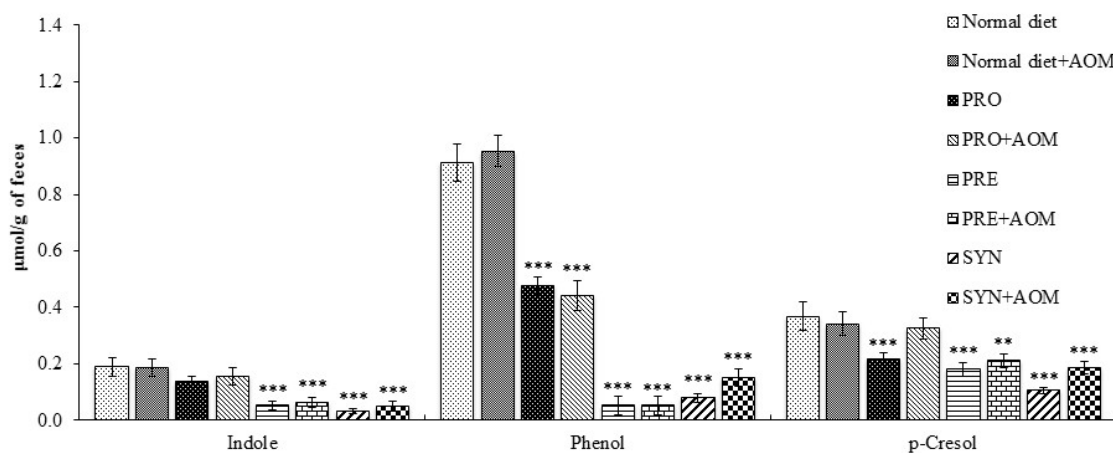


Figure 3. Concentration of putrefactive compounds among the experimental rats ($\mu\text{mol/g}$ of feces). Values are the mean \pm SD. ** $p < 0.01$ vs control group; *** $p < 0.001$ vs control group.

The results of the current study indicated that both prebiotic and synbiotic interventions possibly increased the organic acids concentration (Figure 2), which led to the reduction of colonic pH, stimulation of beneficial bacteria, and inhibition of pathogenic bacteria. Intake of fructooligosaccharide (FOS) and galacto-oligosaccharide (GOS) improved the concentrations of total cecal SCFAs in mouse bowel (Pan et al., 2009). Noack et al. (2013) reported that the *in vitro* fermentation of inulin results in a high concentration of total SCFA. Femia et al. (2002) reported that the synbiotic preparation (inulin + *L. rhamnosus* + *Bifidobacterium lactis*) have anti-tumorigenic properties with respect to the caecal SCFA concentration. Moreover, synbiotic preparations are more efficient than probiotic supplements, attributed to the total SCFA concentration (Le Leu et al., 2010).

High-protein diets increase the availability of protein in the colon, which facilitates oncogenesis through the generation of toxic products (Le Leu et al., 2007). Those products include, ammonia, phenolic compounds and hydrogen sulfide (Windey et al., 2012). The phenolic compounds are formed following the bacterial degradation of aromatic amino acids such as phenylalanine, tyrosine and tryptophan (Hughes et al., 2000). Phenol and *p*-cresol affect the cell permeability; thereby they enhance the accessibility of a broad range of carcinogens to the colonic mucosa (Cerini et al., 2004; McCall et al., 2009; Windey et al., 2012). Indole acts as colon cancer promoter and also enhances nitrosation (Nowak & Libudzisz, 2006). The supplementation of inulin reduced the fecal phenols (Propst et al., 2003), and fructooligosaccharide (FOS) lowered the total fecal indole and phenol concentrations in dogs (Swanson et al., 2002). An *in vitro* study suggested that the inulin and *Clostridium difficile* have the ability to reduce the concentration of phenol, indole, and *p*-cresol in the colon (Van et al., 2003). Synbiotic containing oligofructose-enriched inulin (OF-IN) and *Bifidobacterium breve* also reduces the phenol in healthy humans (De Preter et al., 2007a).

In this study, the concentration of phenol was reduced in PRO, PRE and SYN supplemented groups, while the concentration of *p*-cresol and indole were reduced only in PRE and SYN groups (Figure 3). The results indicated that the supplementation of inulin reduced the putrefactive compounds by selective stimulation of growth of *Lactobacillus* spp. and *Bifidobacteria*, which further leads to the inhibition of proteolytic bacterial growth in the colon.

3.4 Microbial enzymes

The effect of PRO, PRE, and SYN diets on microbial enzyme activity are shown in Table 2.

All the supplements significantly reduced the activity of tested microbial enzymes such as β -glucuronidase (1.5-4.2 fold; $p < 0.001$), azoreductase (1.1-1.9 fold; $p < 0.001-0.05$), and nitroreductase (2.1-2.5 fold; $p < 0.001$) in naive and AOM-treated groups when compared to respective controls.

The microbial enzymes such as β -glucuronidase, nitroreductase, and azoreductase are commonly produced by *Bacteroides*, *Clostridium*, and *Enterobacteriaceae*, which mediates the carcinogenesis (Raman et al., 2013). These enzymes have been associated with colon cancer through the formation of genotoxic and carcinogenic products (Schwabe & Jobin, 2013; Uccello et al., 2012). The enzyme, β -glucuronidase hydrolyzes the glucuronide, which generates the potentially toxic and carcinogenic compounds (Moreno de LeBlanc & Perdígón, 2005). Nitroreductase reduces the aromatic and heterocyclic nitro compounds to potentially mutagenic and carcinogenic N-nitroso and N-hydroxy compounds before converting them into aromatic amines (Hughes & Rowland, 2000; Lee & Lee, 2011; Moreno de LeBlanc & Perdígón, 2005) while azoreductase reduces the azo compounds to mutagens (Rafii et al., 1990). Some *Lactobacilli* and *Bifidobacteria* have been reported for their microbial enzyme lowering activity (Yoon et al., 2000; Wollowski et al., 2001; Ouwehand et al., 2002). *L. acidophilus* 74-2 and FOS mediated simulation of human intestinal microbiota decreases the β -glucuronidase in the intestine and diminishes cancer risk (Gmeiner et al., 2000). The inulin supplementation can reduce the β -glucuronidase production (Rowland et al., 1998; Hijova et al., 2014). The individual intervention of oligofructose-enriched inulin, *L. casei*, and *B. breve* significantly decreased the β -glucuronidase activity, but supplementation of synbiotic formulation did not appear to be more beneficial than either compound alone (De Preter et al., 2007b). Whereas, the current study revealed that SYN diet significantly ($p < 0.001$) reduced the selected microbial enzymes production in AOM-treated rats, even though PRO diet also showed a significant ($p < 0.001$) reduction in the enzymes (Table 2). The results suggested that the inulin-containing synbiotic formulation was greatly influenced by the strain of probiotic bacteria in it.

Table 2. Profile of microbial enzyme activities of rat feces.

Group	β -glucuronidase ($\mu\text{mol nitrophenol h}^{-1} \text{g}^{-1}$ feces)	Azoreductase ($\mu\text{mol Amaranth metabolised h}^{-1} \text{g}^{-1}$ feces)	Nitroreductase ($\mu\text{mol 4-Aminobenzoic acid h}^{-1} \text{g}^{-1}$ feces)
Control	0.338 \pm 0.052	0.447 \pm 0.025	0.269 \pm 0.028
Control + AOM	0.484 \pm 0.009	0.445 \pm 0.026	0.299 \pm 0.032
PRO	0.100 \pm 0.008***	0.376 \pm 0.062*	0.128 \pm 0.027***
PRO + AOM	0.315 \pm 0.052†††	0.397 \pm 0.048†	0.135 \pm 0.038†††
PRE	0.089 \pm 0.042***	0.348 \pm 0.088***	0.119 \pm 0.041***
PRE+AOM	0.253 \pm 0.008†††	0.388 \pm 0.037†††	0.127 \pm 0.035†††
SYN	0.080 \pm 0.016***	0.238 \pm 0.022***	0.116 \pm 0.038***
SYN + AOM	0.195 \pm 0.018†††	0.275 \pm 0.027†††	0.118 \pm 0.024†††

All the values are mean \pm SD (n = 12 per group). * $p < 0.05$. *** $p < 0.001$ represents the significantly differed from naive control group. † $p < 0.05$. ††† $p < 0.001$ represents the significantly differed from AOM treated- control group.

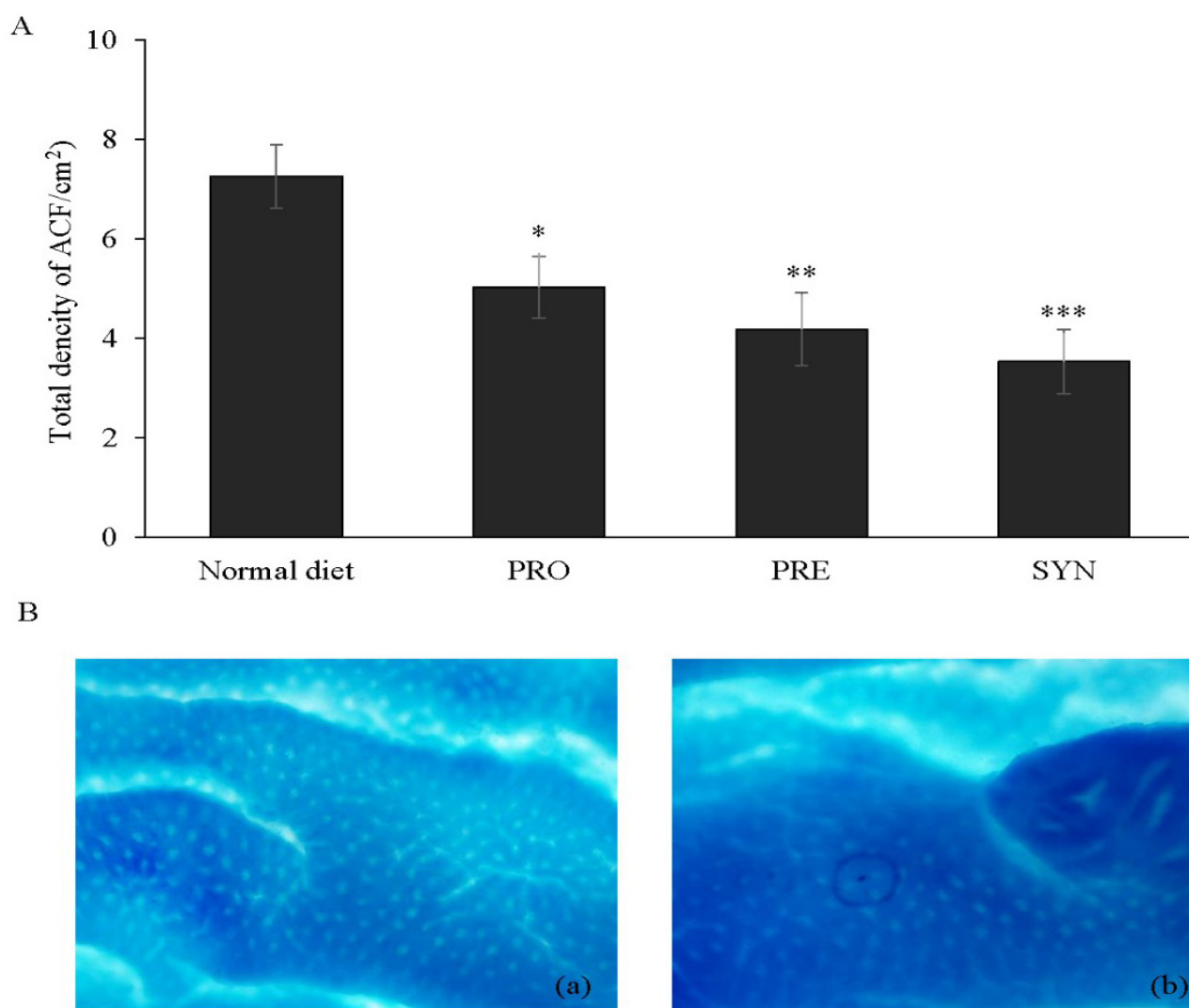


Figure 4. (A) Changes in the density of ACF among the experimental rats. Values are represented as mean \pm SD. * $p < 0.05$ vs control group; ** $p < 0.01$ vs control group; *** $p < 0.001$ vs control group. (B) Representative images showing colon of naive (a) and AOM treated (b) rat's tissue samples stained with 0.2% methylene blue.

3.5 Aberrant crypt foci

ACF is one of the markers for colon cancer development in rodents and human, they are the earliest identifiable lesions and are highly correlated with eventual tumor incidence (Ochiai et al., 2014). *L. acidophilus* showed inhibitory effect on ACF formation in AOM-induced rats (Arimochi et al., 1997). Inulin has been reported to inhibit the formation of ACF (Reddy et al., 1997; Pattananandecha et al., 2016).

The intervention of PRO ($p < 0.05$), PRE ($p < 0.01$) and SYN ($p < 0.001$) were significantly reduced the colonic ACF in AOM-induced rats compared to control. The total density of ACF in control, PRO, PRE, and SYN fed rats were 7.25, 5.02, 4.18 and 3.53 ACF/cm², respectively (Figure 4). Apparently, the SYN diet decreases the colonic damages more efficiently than other tested reagents in terms of number of ACF.

Synbiotic preparations with oligofructose-enriched inulin + *L. rhamnosus*, and resistant starch + *B. lactis* more significantly

reduced the formation of ACF than separate prebiotic treatment (Femia et al., 2002; Le Leu et al., 2010). Current study results suggested that the combination of inulin and *L. plantarum* HII11 suppressed the occurrence of ACF induced by AOM when compared to the administration of either probiotic or prebiotic alone (Figure 4).

4 Conclusions

The results of this study revealed that the supplementation of SYN diet (inulin and *L. plantarum* HII11) protected the AOM-mediated colon cancer induced host by increasing the probiotic population, and organic acids, reducing the pathogen load, microbial enzymes, putrefactive compounds, and ACF formation than either PRO or PRE diets. Moreover, the PRE diets are superior to PRO diets with respect to the assessed parameters. The study results helped to develop a functional food to improve the human immunity and general health, especially against cancerous conditions.

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