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FULL PAPER

A diet high in protein and fiber changes the gut microbiota of colorectal cancer rat model

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Abstract

Emerging evidence has suggested that gut microbiota dysbiosis may have a role in colorectal cancer (CRC) progression. Dietary intake high in plant protein and fiber has been associated with reduced risk of this cancer through modulation of gut microbiota. This study aimed to evaluate the effect of the soy protein and fiber diet on gut *Lactobacillus* and *Bifidobacterium* of azoxymethane/dextran sodium sulfate (AOM/DSS)-induced rat. A total of twenty-five Wistar rats aged eight weeks were randomly assigned into five groups: the normal control group (NC), the AOM/DSS group, the high protein diet +AOM/DSS group (HP), the high fiber diet +AOM/DSS group (HF), and the combination soy protein and fiber diet+ AOM/DSS group (PF). CRC was induced by injecting 12 mg/kg BW per week of AOM and adding 2% DSS into the drinking water. After four weeks, the rats were sacrificed, and the total DNA was isolated from the cecum. The relative abundance of *Lactobacillus* and *Bifidobacterium* was analyzed using quantitative real-time PCR. The fecal cecum samples analysis demonstrated that rats receiving a high soy protein diet (HP), high fiber diet (HF), or both diets (PF) have significantly higher *Lactobacillus* count compared with the AOM/DSS group that only received a standard diet. In the case of *Bifidobacterium,* both HP and PF diets showed higher abundance relative to AOM/DSS group, though it was not significant. In conclusion, dietary intake consisting of high soy protein, fiber, or a combination of the two diets modulates the gut microbiota of AOM/DSS-treated rats.

1. Introduction

Colorectal cancer (CRC), also called colon cancer, is a disease in which incidence and mortality are included in the top 10 most common types of cancer globally (Bray *et al.,* 2018). CRC is the most common type of cancer and the deadliest malignancy in both men and women. The number of new cases and deaths keeps increasing for adults under 50 years old (Recio-Boiles and Cagir, 2020). Dysbiosis of gut microbiota was suggested as one of the originators of colorectal cancer (Fan *et al.,* 2021).

A gastrointestinal microbiota imbalance can cause dysbiosis (Jahani-Sherafat *et al.,* 2018). Dysbiosis conditions could trigger certain bacteria to produce carcinogens. These bacteria activate immune cells to release pro-mitogenic and proangiogenic cytokines (Ogunrinola *et al.,* 2020). The progression of colon cancer mediated by gut microbiota could occur via different mechanisms, such as Inflammatory response, DNA damage, tumorigenic-immunity-response, and cell proliferation (Fan *et al.,* 2021). Activation of inflammatory pathways has been found to increase the risk of colon cancer (Fan *et al.,* 2021; Artemev *et al.,* 2022). Furthermore, it may imply the prognosis and response to a particular systemic therapy. Some bacteria associated with colon cancer are *Bacteroides fragilis*, *Escherichia coli*, *Enterococcus faecalis* and *Streptococcus galloticus* (Rebersek, 2021). Those bacteria serve as pro-carcinogenic factors in different

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ways. In contrast, several beneficial bacteria can prevent colon cancer. *Lactobacillus* and *Bifidobacterium* are beneficial and could prevent colon cancer and other disease linked to the gastrointestinal tract (Irecta-Nájera *et al*., 2017; Parisa *et al*., 2020).

Prior study demonstrated that both *Lactobacillus acidophilus* and *Lactobacillus plantarum* might protect against polyps or colon cancer (Zinatizadeh *et al.,* 2018). *Lactobacillus* is a gut microbiota that helps reduce the symptoms of inflammatory gut diseases by controlling the immune system and altering the gut microbiota (Jang *et al.,* 2019). On the other hand, *Bifidobacterium* is a typical inhabitant of a healthy human intestine. Some species of *Bifidobacteria* can reduce carcinogen-induced DNA damage, pre-neoplastic lesions, and colon cancers in rats (Hidalgo-Cantabrana *et al*., 2016; Faghfoori *et al*., 2021). Faghfoori *et al.* (2021) also showed that *Bifidobacterium* could prevent colon cancer development by regulating the anti- and pro-apoptotic genes.

Cancer prevention strategies through lifestyle improvements, especially diet, can be an option for some people. The individual's diet affects the composition and relative abundance of the gut microbiota (Grosso *et al.,* 2017). Dietary intake consisting of high protein components from different sources impacted gut microbiota homeostasis (Wu *et al.,* 2022). Previous studies have shown that a diet high in plant-based protein promotes gut microbiota development and protects against obesity (Kiilerich *et al.,* 2016). Tempeh is a rich source of beneficial compounds. In each 100 g, tempeh contains protein (20.8 g), dietary fiber (1.4 g), potassium (234 mg), zinc (1.7 mg), antioxidants, phytochemicals, and other beneficial compounds (Astuti *et al*., 2000; Romulo and Surya, 2021). It also has a beneficial effect in improving colon health and enhancing the *Bifidobacteria* population in the human gut (Stephanie *et al.,* 2019).

In addition to a high-protein diet, a high-fiber diet also benefits the gut microbiota. A meta-analysis study indicated the necessity to maintain adequate fiber intake. Calculating an individual's daily dietary fiber intake is an effective measure of preventing colon cancer progression (Masrul and Nindrea, 2019). Dietary intake, which is high in protein composition, could improve gut microbiota dysbiosis and inflammatory markers (Fritsch *et al.,* 2021). Fiber reduces the contact time of carcinogens in the gut lumen and modulates the gut microbiota. Dietary fiber intake may increase short-chain fatty acid production, thereby modulating anti-inflammatory bioactive substances (Zeng, 2014). This study aimed to investigate the effect of high-soy protein, high-fiber, and a combination of both diets in affecting the population of *Lactobacillus* and *Bifidobacterium* in the AOM/DSS-induced colorectal cancer rat model.

2. Materials and methods

2.1 Materials

Small and Medium Enterprise (SME) "Kusuka Ubiku" Bantul-Yogyakarta, Indonesia, provided the tempeh flour used in this study. A high-protein diet was prepared by adding tempeh flour to the standard food AIN-93M (Reeves *et al*., 1993). The fiber was obtained from commercial jelly flour made from seaweed (Swallow Globe).

2.2 Animal study

Eight-week-old male Wistar rats were obtained from the Inter-University Center (IUC) at Gadjah Mada University, Yogyakarta. The animals were allowed to eat *ad libitum* with free access to water. Twenty-five male rats were randomly divided into five groups of five rats each. The first group was given standard feed only (normal control, NC), the second group was given standard feed and AOM/DSS induction (AOM/DSS), the third group was given a high soy protein diet and AOM/DSS induction (HP), the fourth group was given a high fiber diet with AOM/DSS induction (HF), and the fifth group was given a combination of both diets equally (PF). The dose of AOM used in this study was 12 mg/kg BW, administered intraperitoneally once a week for four consecutive weeks. The DSS dose was given to rats' drinking water with a solution concentration of 3% DSS (w/v). Rats in the HP group received an extra 1.6 g/200 g BW tempeh flour to the standard food. In the HF group, rats received 0.54 g/200 g BW Seaweed flour in addition to the standard food. Cecum samples of all groups were collected after 28 days of treatment (Figure 1).

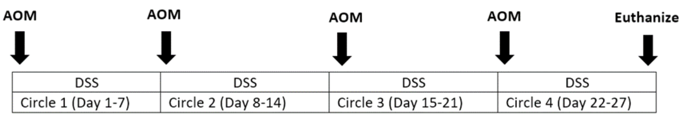

Figure 1. Schematic timeline for AOM/DSS induced inflammatory carcinogenesis.

2.3 Total cecum bacterial DNA extraction

Cecum samples were initially homogenized and followed by bacterial DNA extraction using the Favor PrepTM Stool DNA isolation Kit (Favorgen Biotech, Ping-Tung, Taiwan, China). The protocol was adjusted according to the kit instructions. The total DNA concentration was calculated using a nanodrop machine (Maestrogen).

2.4 Construction of standard curve

Lactobacillus bacteria (concentration of 10^7 CFU/g)

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and *Bifidobacterium* (concentration of 10^7 CFU/g) were gradually diluted up to 10^2 CFU/g. Each tube of diluted cells was centrifuged at 3000 RPM for 5 mins at 4°C to obtain a precipitate, then washed with 10 mL of NaCl 0.9%. After that, it was vortexed and centrifuged again to form a precipitate. Tris Lysozyme solution (200 µL) was added to each tube, followed by vortexing the solution and incubating it for 30 mins at 37°C in a water bath. Subsequently, 300 µL of lysis buffer was added and vortexed again until mixed. The solution was transferred into a labeled 2 mL tube.

Real-time Polymerase Chain Reaction (RT-PCR) conditions for DNA amplification were set as follows: 50°C for 2 mins, 95°C for 10 mins, followed by 40 cycles for 15 s at 95°C and 1 min at 60°C. The standard curve was constructed using triplicate tenfold dilutions of the DNA with a minimum of five standard concentrations between 10^4-10^{10} DNA copies per reaction. The copy numbers of the target group for each reaction were calculated from the standard curves.

2.5 Quantification of bacterial by RT PCR

RT-PCR amplification and detection were performed in triplicate using the CFX96 Real-Time System. A PCR mixture was composed of 10 µL Thunderbird SYBR Green Real-Time PCR Master Mix (TOYOBO Co. Ltd., Osaka, Japan), 2 µL of 0.3 µM forward and reverse primers (Table 1), DNA template (100 ng/µL), and nuclease-free water adjusted to a final volume of 20 µL. The temperature settings used for amplification were 50° C for 2 mins, 95°C for 10 mins, followed by 40 cycles for 15 s at 95°C and 1 min at 60°C.

2.6 Statistical analysis

The One-Way ANOVA test was used to examine the study's findings with a 95% confidence level. The Post-hoc Tuckey test was used following the alternative test to evaluate group differences. SPSS Version 16 will be used to analyze each piece of data.

2.7 Ethical approval

The experiments in this study were compiled with the bioethical research established by the Health Research Ethics Commission, Faculty of Medicine of Diponegoro University (No. 113/EC/H/FK-UNDIP/IX/2021).

3. Results

The standard curve was generated by plotting the logarithmic concentration of the control bacteria culture against the number of threshold cycles (Cq, the number of cycles required for the fluorescence signal to reach the set threshold in each reaction tube). Standard curves were found to be as follows: *Lactobacillus* spp.: Y = -2.907X + 39.9438 (R^2 = 0.9956) and *Bifidobacterium* spp.: Y = -1.9074X + 33.401 (R^2 = 0.9856); Within the dilution range, the template displayed an excellent linear relationship, and the detection line was 1×10^2 - 1×10^7 copies/L. copies/µL.

AOM/DSS treated rats demonstrated a lower abundance of the gut microbiota *Lactobacillus* spp. and *Bifidobacterium* spp. than the healthy rats (NC). Modifying the rats' standard diet by increasing protein (HP) or increasing fiber (HF), or a combination of the two diets (PF), could significantly improve ($p < 0.05$) the *Lactobacillus* spp. population (Figure 2). Combining high protein and high fiber intake has similar effects on rats receiving only high protein or high fiber alone ($p >$

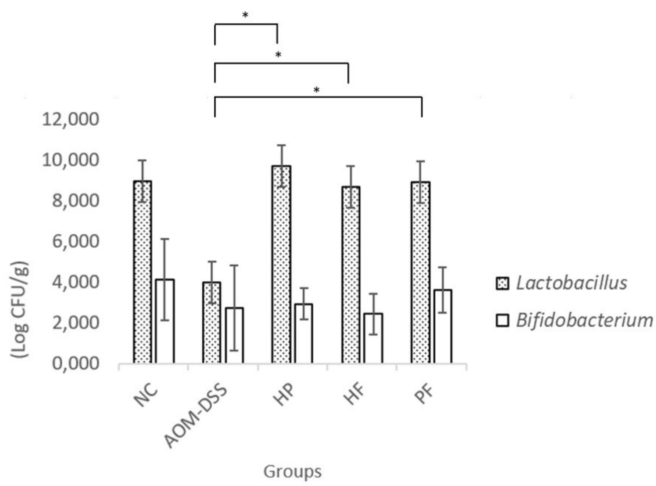

Figure 2. The effect of different diets on the abundance of gut *Lactobacillus* and *Bifidobacterium*. NC (normal control with standard feed); AOM/DSS (standard feed with AOM/DSS induction); HP (high protein diet group, 1.6 g/200 g BW tempeh flour and AOM/DSS induction); HF (0.54 g/200 g BW jelly flour and AOM/DSS induction); and PF (diet group, 50:50 protein fiber combination). The mean and standard deviation were displayed within the data. One-way ANOVA was used to calculate the *p*-Value and continue with the Tukey HSD posthoc test. (*) AOM/DSS-significant ($p < 0.05$).

Table 1. Primers-targeted.

Target Bacteria	Primer	Sequence (5' – 3')	Targeted DNA (bp)	Tm (°C)
Lactobacillus spp.	F	AGCAGTAGGGAATCTTCCA	341 bp	58
(Rinttilä *et al.*, 2004)	R	CACCGCTACACATGGAG		
Bifidobacterium spp.	F	GGGTGGTAATGCCGGATG	278 bp	59
(Langendijk *et al.*, 1995)	R	TAAGCGATGGACTTTCACACC		

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0.05). All of the dietary type interventions (HP, HF, or PF) on AOM/DSS treated rats returned the *Lactobacillus* population almost to its normal level, as seen in the NC group ($p > 0.05$). On the other hand, *the Bifidobacterium* spp. population tended to be higher in HP and PF groups compared to the AOM/DSS group but without statistical differences. It is also observed that the population of *Bifidobacterium* was lower than that of *Lactobacillus*.

4. Discussion

Numerous recent studies have shown how important gut bacteria are for maintaining human health (Zhang *et al.*, 2015). *Lactobacillus (*Ghorbani *et al.,* 2022) and *Bifidobacterium* (Parisa *et al.,* 2020) are two crucial bacteria that could modulate colorectal cancer development and progression. Compared to healthy subjects, individuals with polyps or colon cancer have a lower population of *Lactobacillus acidophilus*. The copy number of this bacterium was also lower in individuals with polyps compared to healthy individuals (Zinatizadeh *et al.,* 2018). The present study was in line with prior studies, where rats with AOM/DSS-induced colorectal cancer have significantly lowered *Lactobacillus* spp. A similar trend was noticed in humans; the number of *Lactobacillus* bacteria is significantly lower in colon cancer patients than in healthy people (Huang *et al*., 2022). Similar trends were observed for the *Bifidobacterium* population, though the difference was not significant. Previous studies have shown that the abundance of *Bifidobacterium* is sometimes also lower than *Lactobacillus* (Parisa *et al.,* 2020).

Tempeh is a fermented soybean product that originates from Indonesia and is consumed daily by Indonesian people as a main dish or snack food. Besides being popular, tempeh has been studied for its various health benefits. Due to its high nutritional quality and excellent health effects, this mold-fermented soybean has been an essential diet for ages (Romulo and Surya, 2021). This study confirmed that dietary intake of fermented soybean (tempeh) flour for 28 days could significantly enhance *Lactobacillus* spp. populations of AOM/DSS-treated rats. The bioactive compounds from soybean showed anti-inflammatory, antioxidant, and protective actions against intestinal permeability in animal models of inflammatory bowel disease (Basson *et al*., 2021).

Previous studies have demonstrated that tempeh could increase the number of good gut bacteria, *Lactobacillus,* and suppress the growth of harmful bacteria such as *Enterobacteriaceae* (Yang *et al.,* 2018). *Lactobacillus* could reduce the severity of colitis cases by suppressing the production of pro-inflammatory cytokines such as IL-23, IL-6 and IL-1 (Silveira *et al.,* 2020). *L. acidophilus* is thought to improve colitis by suppressing the function of Th17 cells and TNF-α (Ogunrinola *et al*., 2020). *Lactobacillus* could also improve the immune system by increasing Immunoglobulin A (IgA) (Yang *et al*., 2018).

Isoflavones and polyphenols are bioactive compounds found in soybean. The nutritional content of fermented soybean (tempeh) is easier to absorb, thus eventually improving the bioavailability of isoflavone (Kuligowski *et al.,* 2017; Jang *et al.,* 2020). Isoflavone aglycones and equol could modulate the overall population and proportions of gut microbiota (Vázquez *et al.,* 2017). Tempeh can also increase the concentration of short chain fatty acid (SCFA) in the gut system, such as propionate and acetate, and promote the population of *Bifidobacterium* and *Lactobacillus* (Yang *et al.,* 2018). Moreover, the resistant carbohydrates contained in tempeh, such as raffinose and galactooligosaccharides, are responsible for intensifying the growth of *Bifidobacterium* (Huang *et al.,* 2016).

This study also showed that a high-fiber diet intake in AOM/DSS-treated rats significantly increased the gut *Lactobacillus* population. This result is in accordance with previous research that suggested a diet enriched with fiber can help minimize inflammation, modulate the immune response, restore gut microbiota and prevent colorectal cancer. In addition, fiber can also help improve general health (Fritsch *et al*., 2021). Seaweed is a food that is easy to apply in food technology because of its ease of combining with other food sources such as milk, fish, and meat to increase its sensory value and increase the benefits of its nutritional content (Zeng, 2014).

5. Conclusion

In conclusion, this study showed that dietary intake of high soy protein from tempeh or high in fiber could significantly modulate the *Lactobacillus* spp. population in the colorectal cancer rat's model.

Conflict of interest

The authors declare no conflict of interest.

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