Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 9 (4): 189-198, 2014
ISSN 1816-4935 / DOI: 10.3923/jm.2014.189.198

Impact of Tempeh Supplementation on Gut Microbiota Composition in Sprague-Dawley Rats

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ABSTRACT

Tempeh is a popular Indonesian fermented food made from soybean that can be a good source of dietary fibers for human health. Dietary fibers have the ability to modulate gut microbiota composition in order to improve human health. In this study, Sprague-Dawley (SD) rats were fed a standard diet supplemented with cooked and de-hulled soybean or tempeh for 28 days. The specific bacterial groups in fecal samples were quantified using real-time polymerase chain reaction with 16S rRNA gene-targeted, group-specific primers. Populations of *Bacteroidetes*, *Bacteroides fragilis*, *Firmicutes* and *Clostridium leptum* increased when supplemented with tempeh. In addition, the ratio of *Firmicutes/Bacteroidetes* was lower in groups fed with either raw or cooked tempeh compared to the soybean group. Previous studies showed that obese hosts have higher ratios of *Firmicutes/Bacteroidetes* compared to the lean hosts. Increased *Bacteroidetes* populations after tempeh supplementation indicated that tempeh might modulate the composition of gut microbiota toward a healthier gut.

Key words: Tempeh, gut microbiota, real-time PCR, *Firmicutes*/*Bacteroidetes* ratio

INTRODUCTION

Recent studies have shown that trillions of bacteria that normally reside within the human gastrointestinal tract (referred to as the gut microbiota) can affect nutrient acquisition and energy regulation. This suggests that obese and lean people have different gut microbiota (Ley *et al*., 2005). These findings showed the important role of gut microbiota in human health through their effect on the gut defense barrier, immune development and nutrient utilization. Comparative studies using 16S rRNA sequencing have shown that the intestinal microbiota in mice and humans is very similar in composition at the division level (i.e., = 80% of both mouse and human microbiota is dominated by two phyla, the *Firmicutes* and the *Bacteroidetes*) (Sekirov *et al*., 2010). However, composition of a microbial community is host specific. It evolves throughout an individual's lifetime and is susceptible to both exogenous and endogenous modifications. The composition of human gut microbiota is mainly influenced by maternal colonization but is further influenced by

diet, environmental exposures and antimicrobial therapies. Diverse disorders, such as antibiotic-associated diarrhea, Crohn's disease, ulcerative colitis and obesity have been correlated with large-scale imbalances in the gastrointestinal microbiota, demonstrating the importance of commensal microorganisms in maintaining gastrointestinal balance.

Tempeh is a well-known Indonesian fermented food made from soybean (*Glycine max*). It is gaining popularity in the world, especially in the diets of vegetarians. A diverse group of microorganisms including molds, yeasts, lactic acid-producing bacteria and some Gram-negative bacteria plays an important role during the fermentation process (Steinkraus *et al*., 1983). Many studies also have reported that tempeh is a good source of protein (Astuti *et al*., 2000), vitamin B_{12} (Okada, 1989), phytochemicals (Murakami *et al*., 1984) and other bioactive substances. Stachyose and raffinose are non-digestible galactooligosaccharides in soybean found in tempeh, although it has been reported that the concentrations of oligosaccharides in soybeans are reduced during the fermentation process (Ruiz-Teran and Owens, 1999; Egounlety and Aworh, 2003). Since these dietary components are undigested by human enzymes, the human colonic bacteria play important roles in the digestion of dietary fiber through the fermentation process (Scott *et al*., 2008). This fermentation process will further lead to the formation of some acidic compounds, such as acetate, lactate, butyrate and propionate. These short-chain fatty acids will be released and cause further decrease in pH value (Scott *et al*., 2008; Binns, 2013). The benefits of a lower pH in the gut intestine include enhanced multiplication and survival of organisms that prefer acidic conditions and general inhibition of some pathogens. There are many health benefits associated with dietary fiber intake, not including effects on satiety (Samra and Anderson, 2007) and fecal bulking. Therefore, many countries are attempting to increase the recommended dietary fiber intake in an effort to prevent some of the diseases afflicting modern society.

The objective of this study was to evaluate the impact of tempeh supplementation on gut microbiota composition in Sprague-Dawley (SD) rats. We hypothesized that tempeh might modulate the composition of gut microbiota toward a healthier gut.

MATERIALS AND METHODS

Tempeh preparation: Tempeh was collected from two different locations in Bogor, Indonesia. Empang (EMP) and Warung Jambu (WJB) tempeh collected in April 2013 were used for this study. These tempeh samples have been previously explored by Barus *et al*. (2008) and Seumahu *et al*. (2012). Cooked samples of tempeh were prepared by steaming tempeh for 10 min in a steamer. The raw and cooked samples of soybean tempeh were further sliced, freeze-dried and powdered. Soybean tempeh powder was then mixed with the animal feed. Cooked and de-hulled soybean from EMP and WJB tempeh were added to the feed for the control group.

Animal study: Six-week-old female Sprague-Dawley (SD) rats were obtained from the rodent facility at Bimana Indomedical in Bogor, Indonesia and allowed to feed *ad libitum* with free access to water. Thirty female SD rats were randomly divided into six groups of five rats. The first and second groups were supplemented with cooked and raw EMP tempeh. The third group was the control EMP tempeh group which was supplemented with cooked and de-hulled EMP soybean. The fourth and fifth groups were supplemented with cooked and raw WJB tempeh. The sixth group was a control WJB tempeh group which was supplemented with cooked and de-hulled WJB soybean.

Table 1: 16S rRNA gene-targeted, group-specific primers (Matsuki *et al*., 2004; Guo *et al*., 2008)

Target bacterial group	Primer	Sequence	Size (bp)	Annealing temperature (°C)
Bacteroidetes	Bact934F	GGARCATGTGGTTTAATTCGATGAT	126	60
	Bact1060R	AGCTGACGACAACCATGCAG		
Bacteroides fragilis	g-Bfra-F	ATAGCCTTTCGAAAGRAAGAT	495	50
	G-Bfra-R	CCAGTATCAACTGCAATTTTA		
Firmicutes	Firm934F	GGAGYATGTGGTTTAATTCGAAGCA	126	60
	Firm 1060R	AGCTGACGACAACCATGCAC		
Clostridium leptum	Sg-Clep-F	GCACAAGCAGTGGAGT	239	50
	Sg-Clep-R	CTTCCTCCGTTTTGTCAA		
All bacteria	Eub338F	ACTCCTACGGGAGGCAGCAG	220	60
	Eub518R	ATTACCGCGGCTGCTGG		

Tempeh added 10% protein to the standard basal diet containing 18% protein, 3% crude fat and 18% crude fiber. Fecal samples were collected before and after 28 days of treatment. All animal procedures were approved by Ethical Commission of Primate Research Center, Bogor Agricultural University, Bogor, Indonesia.

Total fecal bacterial DNA extraction: Total fecal bacterial DNA was extracted using QIAamp® DNA Stool Minikit (Qiagen, Hilden, Germany) with a 1 min bead-beating modification (Furet *et al*., 2009). To verify genomic DNA, electrophoresis was performed with 5 μL DNA in a 1%-agarose gel run at 60 V for 90 min, stained with ethidium bromide and visualized using UV light.

Construction of standard curve: The Polymerase Chain Reaction (PCR) master mix contained 1 μL of DNA template, 12.5 μL GoTaq Green® Master Mix (Promega, Madison, WI, USA), 1 μL of each primer (10 pmol μL^{-1}) (Table 1) (Matsuki *et al*., 2004; Guo *et al*., 2008) and nuclease-free water for a total volume of 25 μL. The PCR reaction conditions for amplification of DNA were 94°C for 5 min; 30 cycles of 94°C for 30 sec, 50°C or 60°C for 30 sec (Table 1) and 72°C for 1 min and final extension at 72°C for 10 min.

The amplified products were purified using the QIAquick® PCR Purification Kit (Qiagen, Valencia, CA, USA), cloned into pGEM-T Easy vectors (Promega, Madison, WI, USA) and transformed into DH5α competent *Escherichia coli* cells. The plasmid containing 16S rRNA gene sequences of the targeted group was extracted using the Wizard®Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA) with DNA concentration determined by spectrophotometry. The standard curve was constructed using triplicate tenfold dilutions of the plasmid DNA with a minimum of five standard concentrations between 10^4-10^{10} DNA copy per reaction. The copy numbers of the target group for each reaction were calculated from the standard curves.

Quantification of bacterial specific-group from feces: RT-PCR amplification and detection were performed with the iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, Palo Alto, CA, USA). A triplicate PCR reaction was performed on a 20 μL (total

volume) mixture of 10 μL SsoFast™ EvaGreen® Supermix (Bio-Rad, Hercules, CA, USA), 1 μL each of the specific primers at a concentration of 10 pmol μL^{-1}, 1 μL template DNA and nuclease-free water. The amplification reaction conditions were 94°C for 5 min, 40 cycles of 94°C for 20 sec, 50 or 60°C for 20 sec (Table 1) and 72°C for 50 sec (Matsuki *et al*., 2004; Guo *et al*., 2008).

Standard curve: Standard curves were generated using serial dilutions of plasmids containing the 16S rRNA bacterial-specific group sequence with known concentration. All of the RT-PCR assays showed a linear relationship between C_t value and the log of plasmid DNA copy number (R^2>0.95) in all cases which allowed for determination of the concentration of the unknown sample based on their C_t values.

Statistical analysis: Statistically significant differences before and after intervention in each group were analyzed using paired-samples t-tests ($p<0.05$) using SPSS Statistics 20.0 software (SPSS Inc., Chicago, IL, USA). Significant differences among ratios of *Firmicutes/Bacteroidetes*, the control group and the groups fed with tempeh were calculated by one way Analysis of Variance (ANOVA) followed by the Least Significant Difference (LSD) test. Effects were considered statistically significant at $p<0.05$.

RESULTS

Quantification of all bacteria and bacterial specific-group: In this study, Real-time PCR (RT-PCR) of gene-targeted, group-specific 16S rRNA was done to quantify and analyze *Bacteroidetes*, *Bacteroides fragilis*, *Firmicutes*, *Clostridium leptum* and all bacteria. This method had been applied to microbiota analysis as the most sensitive and rapid method (Matsuki *et al*., 2004). The specificity of each primer was previously assayed by Matsuki *et al*. (2004) and Guo *et al*. (2008).

The results of this study showed that in the control group supplemented with EMP and WJB soybean, the population of all bacteria, *Firmicutes* and *C. leptum* increased slightly while the population of *Bacteroidetes* and *B. fragilis* decreased (Fig. 1a and 2a). *Firmicutes* and *C. leptum* populations increased significantly after rats were supplemented with WJB soybean for 28 days; only *C. leptum* increased significantly in the group supplemented with EMP soybean. The populations of all bacteria, *Bacteroidetes*, *Firmicutes*, *B. fragilis* and *C. leptum* increased after rats were supplemented with EMP or WJB tempeh for 28 days (Fig. 1b, c and 2b, c).

The populations of *Firmicutes*, *C. leptum* and all bacteria increased significantly after rats were supplemented with raw EMP tempeh for 28 days (Fig. 1b). Conversely, the populations of *Bacteroidetes*, *B. fragilis* and *C. leptum* increased significantly in rats supplemented with cooked EMP tempeh (Fig. 1c). In the group supplemented with raw WJB tempeh, the populations of *Bacteroidetes* and *C. leptum* increased significantly after 28 days of treatment (Fig. 2b). Also, the populations of all bacteria and *C. leptum* increased significantly after rats were supplemented with cooked WJB tempeh (Fig. 2c).

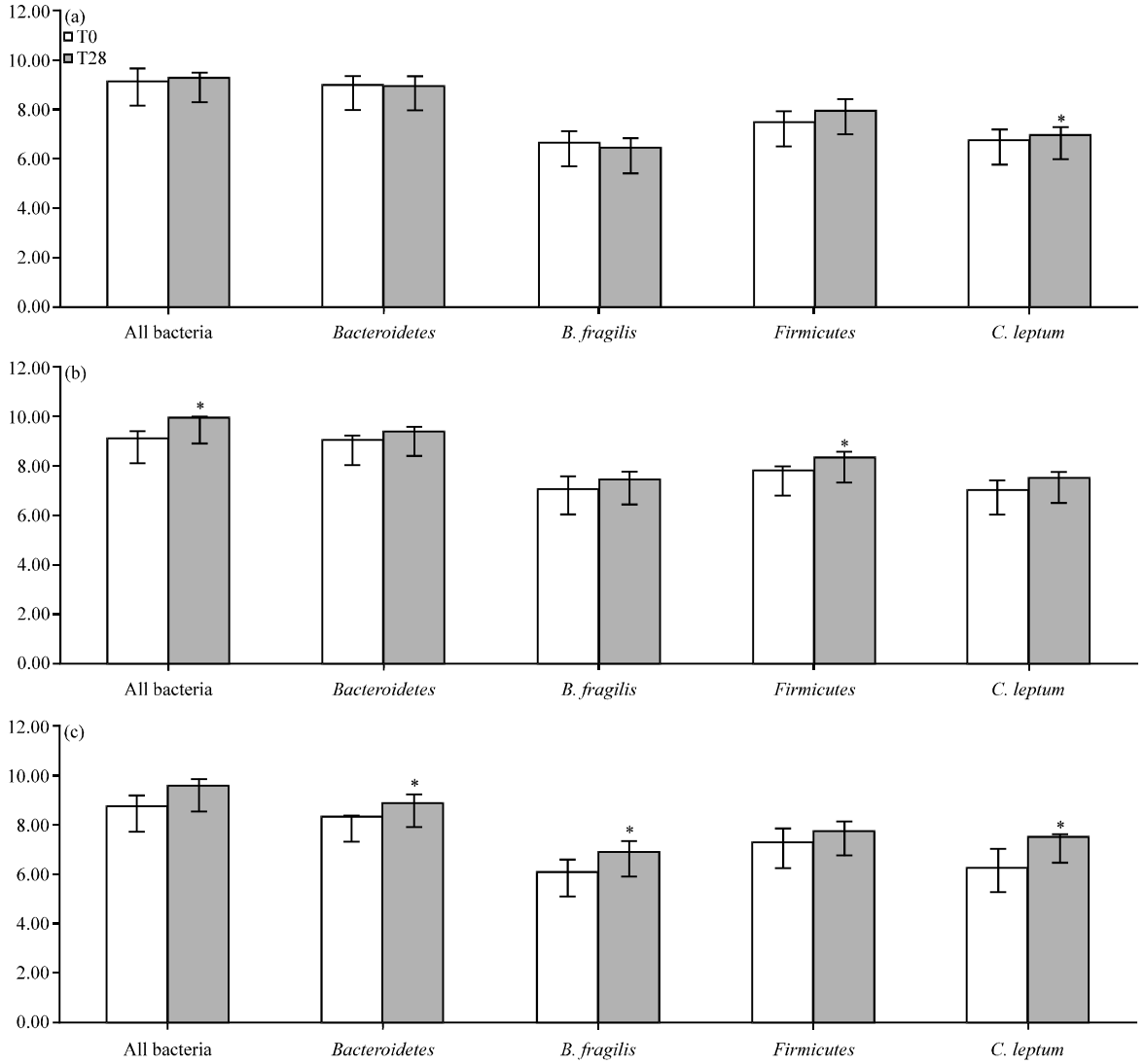


Fig. 1(a-c): Total all bacteria, *Bacteroidetes*, *Bacteroides fragilis*, *Firmicutes* and *Clostridium leptum* fecal microbiota in Sprague-Dawley rats before (T0) and after (T28) supplemented with, (a) Cooked and de-hulled Empang (EMP) soybean, (b) Raw EMP tempeh and (c) Cooked EMP tempeh. Values are in $\log_{10}$ copies per gram of wet weight of feces±SEM (n = 5 group). *Significant differences at $p<0.05$ vs. group after intervention

The differences of bacterial copy numbers before and after EMP and WJB tempeh supplementation are shown in Fig. 3 and 4, respectively.

Ratio of *Firmicutes/Bacteroidetes*: The ratio of *Firmicutes/Bacteroidetes* in the groups supplemented with either EMP or WJB tempeh is not significantly different compared to the control group (Table 2 and 3). However, supplementation of raw and cooked EMP tempeh reduced the ratio of *Firmicutes/Bacteroidetes*.

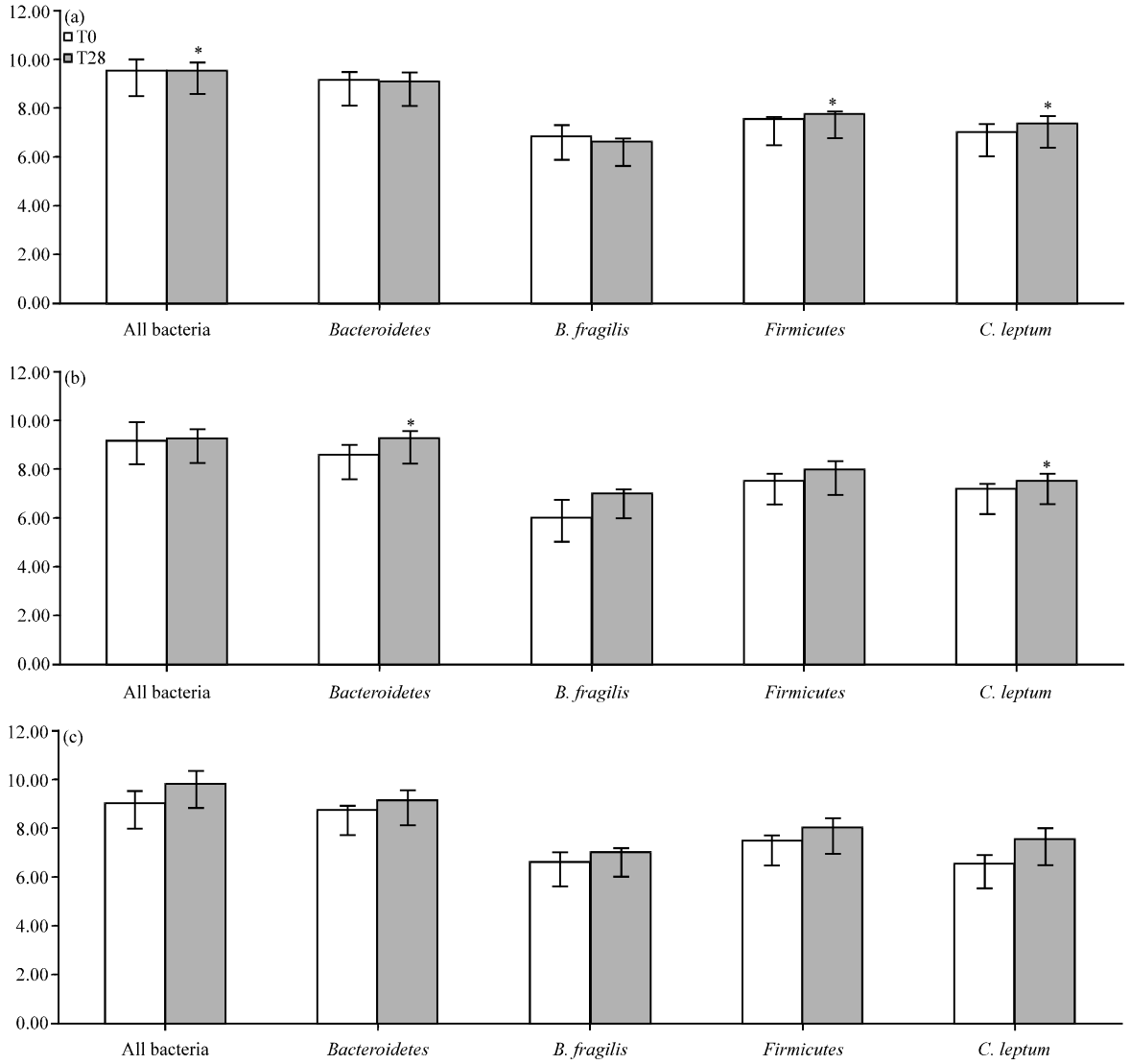


Fig. 2(a-c): Total all bacteria, *Bacteroidetes*, *Bacteroides fragilis*, *Firmicutes* and *Clostridium leptum* fecal microbiota in Sprague-Dawley rats before T_0 and after T_{28} supplemented with, (a) Cooked and de-hulled Warung Jambu (WJB) soybean, (b) Raw WJB tempeh and (c) Cooked WJB tempeh. Values are in $\log_{10}$ copies per gram of wet weight of feces±SEM (n = 5 group), *Significant differences at $p<0.05$ vs group after intervention

Table 2: Ratio of *Firmicutes/Bacteroidetes* in the three groups following supplementation with Empang (EMP)-tempeh for 28 days

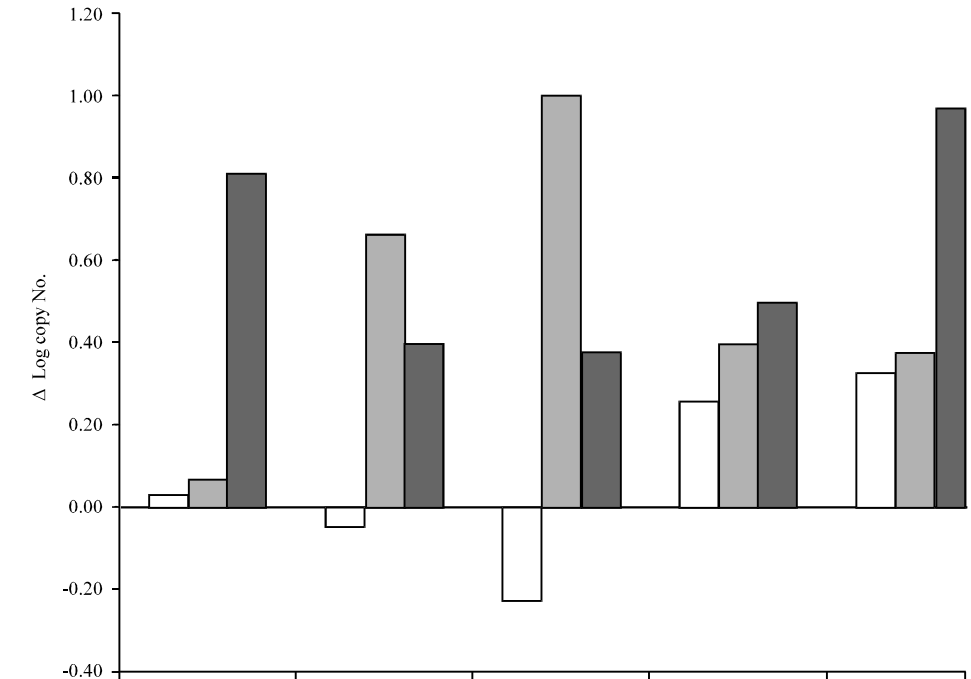
Group	*Firmicutes/Bacteroidetes* ratio
EMP soybean	0.90±0.09
Raw EMP tempeh	0.88±0.03
Cooked EMP tempeh	0.87±0.07
p-value*	0.871

*Based on one-way analysis of variance (ANOVA) test ($\alpha = 0.05$)

Table 3: Ratio of *Firmicutes/Bacteroidetes* in the three groups following supplementation with Warung Jambu (WJB)-tempeh for 28 days

Group	*Firmicutes/Bacteroidetes* ratio
WJB soybean	0.86±0.03
Raw WJB tempeh	0.86±0.04
Cooked WJB tempeh	0.88±0.03
p-value*	0.641

*Based on one-way analysis of variance (ANOVA) test ($\alpha = 0.05$)

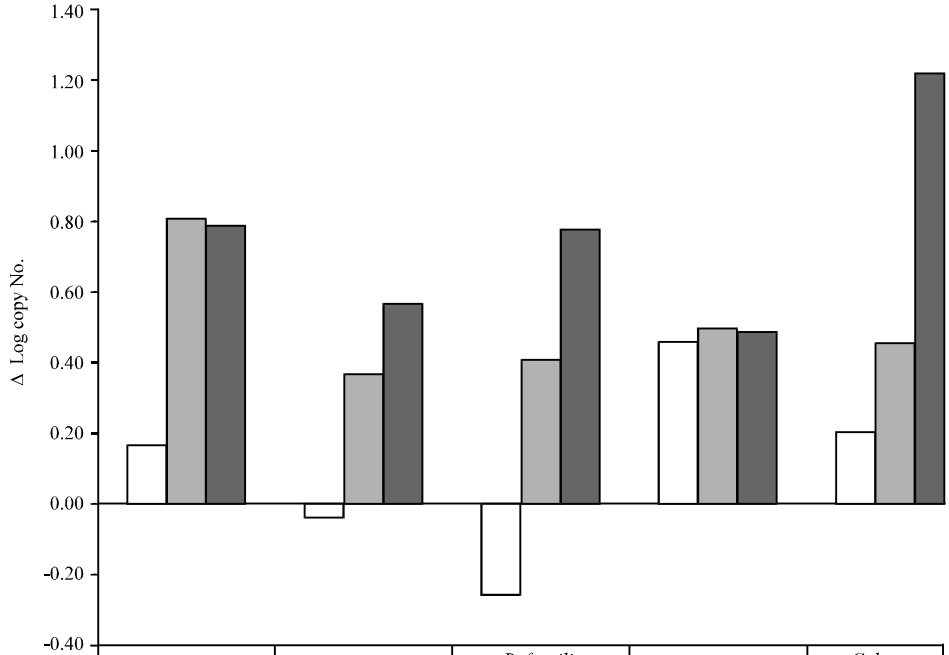


	All bacteria	*Bacteroidetes*	*B. fragilis*	*Firmicutes*	*C. leptum*
Control	0.03	-0.05	-0.23	0.26	0.33
Raw	0.07	0.67	1.00	0.40	0.38
Cooked	0.81	0.40	0.38	0.50	0.97

Fig. 3: Difference of total fecal bacteria before and after Empang (EMP)-tempeh supplementation for 28 days

DISCUSSION

Many recent studies showed that gut microbiota play an important role in maintaining human health. Gut microbiota can affect host immunity and be a physical barrier from other pathogens. Furthermore, gut microbiota composition can be manipulated through diet. Tempeh is an Indonesian fermented food that contains some resistant carbohydrates, such as stachyose, raffinose and galactooligosaccharides (Wang *et al*., 2007; Yogo *et al*., 2011). Resistant carbohydrates are defined as carbohydratest hat cannot be hydrolyzed by endogenous enzymes in the small intestine of humans. However, these carbohydrates play an important role in modulating gut microbiota composition which results in positive impacts on human health (Scott *et al*., 2008).



	All bacteria	*Bacteroidetes*	*B. fragilis*	*Firmicutes*	*C. leptum*
Control	0.17	-0.04	-0.26	0.46	0.21
Raw	0.81	0.37	0.41	0.50	0.46
Cooked	0.79	0.57	0.78	0.49	1.22

Fig. 4: Difference of total fecal bacteria before and after Warung Jambu (WJB)-tempeh supplementation for 28 days

In this study, EMP and WJB tempeh was obtained from local sources in the Bogor area and used as feed supplement for Sprague-Dawley rats. Previous metagenomics studies have shown that EMP tempeh had a higher number of bacterial cells than WJB tempeh. *Acetobacter indonesiensis*, *Klebsiella pneumoniae*, *Bacillus subtilis* and *Flavobacterium* sp. were the dominant bacteria found in EMP tempeh; *Klebsiella* sp. and *Pseudomonas putida* were the dominant bacteria found in WJB tempeh (Barus *et al*., 2008). Moreover, EMP tempeh had a higher bacterial biodiversity compared to WJB tempeh. The differences in bacteria communities between both tempeh will further influence the nutrients in tempeh. Changes in the composition of gut microbiota in response to the source of resistant carbohydrates from EMP and WJB tempeh might occur because different bacterial species are better at utilizing different substrates. For example, *Firmicutes* was previously reported to comprise the dominant bacterial communities in tempeh (Barus *et al*., 2008).

Tempeh is consumed when cooked and served as a side dish, often boiled, fried, or steamed. However, several communities consume tempeh in its raw form. The results of this study indicate that compositional changes in fecal microbiota after intervention with cooked and raw tempeh showed the same results. Since raw tempeh may contain several pathogenic microorganisms (Barus *et al*., 2008), consuming cooked tempeh will eliminate these microorganisms.

A lower ratio of *Firmicutes*/*Bacteroidetes* might further indicate that tempeh can be a new strategy for a low-diet plan and positively impact human health (Mariat *et al*., 2009). Comparison

of gut microbiota of obese and lean mice and obese and lean humans showed a statistically significant reduction in the relative abundance of *Bacteroidetes* and a significantly greater proportion of *Firmicutes* in obese mice and humans (Ley *et al*., 2005; Turnbaugh *et al*., 2009). Moreover, the abundance of *Bacteroidetes* can impact other factors, such as altering host Gastrointestinal Tract (GIT) which leads to morphological and functional changes. *Bacteroidetes* can stimulate the activation of T-cell-mediated responses and limit GIT colonization by pathogenic bacteria. *Bacteroidetes* typically produce butyrate acid which was shown to have an antineoplastic properties and a role in maintaining a healthy gut (Thomas *et al*., 2011).

The results of this study indicate that tempeh might modulate the composition of gut microbiota through the effect of resistant carbohydrates and may offer a new strategy to improve human health. However, different types of tempeh may offer variable health benefits due to its unique microorganism composition that plays an important role during the fermentation process.

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