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Naturally Acquired Lactic Acid Bacteria from Fermented Cassava Improves Nutrient and Anti-dysbiosis Activity of Soy Tempeh

Rio Kusuma[1,2]*, Jaka Widada[3], Emy Huriyati[1,2], Madarina Julia[2,4]

[1]Department of Nutrition and Health, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia; [2]Doctorate Program of Medicine and Health, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia; [3]Department of Agricultural Microbiology, Faculty of Agriculture, Universitas Gadjah Mada, Yogyakarta, Indonesia; [4]Department of Child Health, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia

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***Correspondence:** Rio Kusuma, Doctorate Program of Medicine and Health, Department of Nutrition and Health, Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia.
E-mail: riojatikusuma@gmail.com
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Abstract

BACKGROUND: Dysbiosis of the gut microbiota has been associated with impaired glucose metabolism. Tempeh is a traditional fermented soy food that can stimulate the growth of beneficial bacteria. In Indonesia, the process of making tempeh involved adding an acidifier that contains lactic acid bacteria. This process may affect the nutritional and anti-dysbiosis activity of tempeh.

AIM: The objective of the study was to evaluate the effects of acidifiers on the nutrient and gut microbiota profile of a diabetic animal model.

METHODS: Modified tempeh was prepared by adding water extract from fermented cassava. Standard tempeh and modified tempeh were subjected to proximate and dietary fiber analysis. Diabetic animals were fed a standard tempeh or modified tempeh diet for 4 weeks, replacing 15% or 30% of the protein in the diet, respectively. At the end of the experiment, the contents of the appendix were collected. The short-chain fatty acids (SCFAs) and microbiota composition were analyzed by 16s rDNA next-generation sequencing.

RESULTS: There is a significant difference ($p < 0.05$) in fat, protein, water, and fiber content between regular soy tempeh and modified tempeh. There is a significant difference ($p < 0.05$) between the groups in serum glucose and SCFA composition. The diabetic animal has a low ratio of *Firmicutes*/*Bacteroidetes*. The addition of both tempehs increases bacterial diversity, *Firmicutes*/*Bacteroidetes* ratio, and SCFA-producing bacteria.

CONCLUSION: The addition of naturally occurring lactic acid bacteria from fermented cassava during tempeh processing improved both nutrient and microbiota compositions in the gut of diabetes mellitus.

Introduction

Diabetes mellitus is a group of metabolic diseases caused by a disturbance in insulin secretion, insulin action, or both, manifested by chronic hyperglycemia or high blood glucose levels [1]. Diabetes mellitus is an emerging disease with a rapidly increasing incidence worldwide. It is estimated that 451 million people worldwide had diabetes in 2017, and this number will increase to 473 million in just 2 years [2], [3]. The increasing rise in obesity and overweight is factors associated with the sharp rise in diabetes incidence worldwide [4].

Numerous studies have reported the role of gut microbiota alterations and diversity in the development of many metabolic diseases such as diabetes mellitus [5], [6], [7]. In people with diabetes mellitus, alterations in the composition and diversity of the gut microbiota or gut dysbiosis have been found to be associated with disease. Diabetics tend to have fewer bacteria that produce short-chain fatty acids (SCFAs) and increased levels of Gram-negative bacteria such as *Bacteroidetes*, which promote inflammation and impaired glucose metabolism [8], [9], [10], [11]. The goal of normalizing the composition and diversity of the gut microbiota in people with diabetes mellitus has been associated with improved glycemic status in diabetic patients [12].

Tempeh is a traditional fermented soy food from Indonesia that has anti-dysbiotic activity. Several studies have reported the ability of tempeh to inhibit the growth and adhesion of pathogenic bacteria and promote the growth of beneficial bacteria such as *Lactobacillus*, *Bifidobacterium*, and *Akkermansia muciniphila* [13], [14], [15], [16]. Interestingly, the anti-dysbiosis activity of tempeh was affected by its processing method as previously reported by Huang *et al*. [17]. Conventionally,

tempeh was produced by soaking soybeans with water or lactic acid bacteria to lower the pH of soy. Some tempeh producers in Indonesia used lactic acid bacteria during the souring process, which may affect the diversity of microbiota and mold composition in tempeh [18], [19], [20].

Alternatively, there are several sources of naturally acquired lactic acid bacteria that can be used to reduce the acidity of soy during the soaking phase. One of them is fermented cassava or gaplek which contain several beneficial lactic acid bacteria [21], [22]. Our preliminary study found that the addition of water extract of fermented cassava can significantly lower the pH of soaking water up to 4.7 ± 0.02 and promote the growth of *Rhizopus* molds. However, little is known about the effects of this modified process on the nutrient content and anti-dysbiotic activity of tempeh. Therefore, we aimed to investigate how the addition of naturally acquired lactic acid bacteria from fermented cassava affects the proximate and dietary fiber composition of tempeh, as well as the gut microbiota composition of a diabetic animal model.

Methods

Preparation of the water extract of fermented cassava

The fermented cassava tuber was obtained from the traditional market. The fermented cassava tuber was cleaned, washed, and cut into small pieces. The fermented cassava tuber was immersed in distilled water for 2 h at room temperature, the ratio of fermented cassava tuber to water was 1:5. The water was removed and another distilled water was added and incubated in a closed flask at room temperature for another 10 h. After 10 h, the mixture was filtered with a sterile cheesecloth to obtain the water extract of fermented cassava tuber.

Preparation of modified tempeh

Soybean was obtained from the local market. Tempeh was developed with modifications according to our previous study [23]. Briefly, the soybean was cleaned, washed, and immersed in distilled water for 2 h. The soybean was boiled for 15 min and dehulled. The water extract of fermented cassava was added in the ratio of 1:5 and incubated in a closed vessel for 10 h. The water extract was removed and the dehulled soybean was boiled in sterile distilled water for another 15 min. The soybean was air dried and 2 g of a commercial tempeh mold (Raprima)/kg soybean was added. The soybean was aerobically incubated for 72 h to obtain modified tempeh. The modified tempeh was dried, milled, and sieved through 70 mesh sieves to obtain modified tempeh flour. Regular tempeh (Kadar) from the local market was also subjected to lipoylation using a similar method to that used for modified tempeh. This manufacturer was selected because it uses acidifiers from cooked soybeans in the second soaking of tempeh. Both tempeh flours were stored at 4°C before use.

Proximate and dietary fiber analysis of tempeh

Proximate analysis of tempeh was carried out according to Cempaka *et al*. [24]. Total protein was analyzed using Kjeldahl with a protein conversion factor of 5.71. Fat was determined by Soxhlet method using ether as extracting agent. Moisture content was determined by thermogravimetric method. Dietary fiber was analyzed by the enzymatic gravimetric method using AOAC 991.43 [25]. All analyses were performed in triplicate.

Animal and diet

Thirty (30) male Wistar rats aged 8 weeks were purchased from the Centre of Nutrition and Food Science, PAU, Universitas Gadjah Mada, Indonesia. These numbers were obtained from Arifin and Zahirudin [26] equation. Rats were housed in groups (5 rats/cage) for 5 days during the acclimation period. The cage was 1800 cm^2 wide and 24 cm high. The rats were given food (AIN −93M standard diet) and water *ad libitum*. Room temperature was set at 22°C, humidity at 70%, and lighting on a 12 h dark-light cycle.

After the acclimation period, 25 rats were injected with nicotinamide 230 mg/kg body weight followed by streptozotocin 65 mg/kg body weight after 15 min of the first injection [27]. Five rats served as control group. Blood was drawn for serum glucose pre-test 4 days after injection after overnight fasting. The diabetic rats were randomly divided into five diet groups: Rats were fed a standard diet (negative control), a modified standard diet in which 15% and 30% of protein were replaced with tempeh (TP −15 and TP −30), and a modified standard diet in which 15% and 30% of protein were replaced with modified tempeh (TG-15 and TG-30). The composition of the animal diets is shown in Table 1. Randomization was performed using the Microsoft Excel program.

After 4 weeks of treatment, blood was drawn from the overnight fasting rats for post-test analysis of serum glucose level. The rats were euthanized by injection of ketamine (100 mg/kg body weight) and xylazine (10 mg/kg body weight) followed by cervical dislocation. The appendix was removed from each rat and immediately used for DNA isolation. All animal experiments were approved by the Ethics Committee of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada, Yogyakarta, Indonesia, under ethics number KE/FK/0918/EC/2020.

Table 1: Animal diet composition

Composition (g/kg)	Standard diet	TP-15 diet	TP-30 diet	TG-15 diet	TG-30 diet
Corn starch	62.07	56.18	48.86	57.72	52.41
Casein	14.0	11.9	9.80	11.9	9.80
Sucrose	10.0	10.0	10.0	10.0	10.0
Cellulose	5.00	1.80	-	2.02	-
Soybean oil	4.00	2.86	17.12	28.52	17
AIN-93 vitamin mix	1.00	1.00	10	10	10
AIN-93 mineral mix	3.50	3.50	35	35	35
L-Sistine	0.18	0.18	0.18	1.8	1.8
Choline bitartrate	0.25	0.25	0.25	2.5	2.5
Tempeh	-	12.35	24.70	-	-
Modified tempeh	-	-	-	10.58	21.16

TP-15: Diabetic rats received regular tempeh replacing 15% of protein in the diet, TP-30: Diabetic rats received regular tempeh replacing 30% of protein in the diet, TG-15: Diabetic rats received modified tempeh replacing 15% of protein in the diet, TG-30: Diabetic rats received modified tempeh replacing 30% of protein in the diet.

Cecal SCFA

About 100 mg of cecal content were homogenized with 1 ml of deionized water. The mixture was sonicated and centrifuged at 14,000 × g for 10 min. The supernatant was collected and injected into the Thermo Scientific Trace 1310 gas chromatography (GC) coupled to the Thermo Scientific ISQ LT single quadrupole mass spectrometer (MS). The injector temperature was set to 260°C, the ion source to 230°C, the quadrupole to 150°C, and the GC/MS interphase to 280°C. Helium was used as the carrier gas.

Cecal total DNA isolation

Total DNA from the cecum was isolated using the modified FavorPrep Stool DNA Isolation Mini Kit (Favorgen, Taiwan). Briefly, 100 mg of cecum was weighed and homogenized in lysis buffer using Precellys homogenizer (Bertin instrument, France). Approximately 100 μl of 1 mg/ml lysozyme (Sigma-Aldrich, Singapore) was added to the mixture and incubated at 37°C for 2 h in a water bath. Proteinase K was added according to the instructions in the kit and incubated at 55°C for 8 h. Samples were processed according to the manufacturer's instructions. The quality and quantity of DNA was checked using a nanodrop (MaestroNano Pro MN -913A, Taiwan).

Amplicon generation

16S rRNA from the V3-V4 region was barcoded amplified with specific primers (forward: 5-CCTAYGGGRBGCASCAG-3 and reverse: 5-GGACTACNNGGGTATCTAAT-3). All polymerase chain reaction (PCR) reactions were performed using Phusion High Fidelity PCR Master Mix (New England Biolabs) according to the manufacturer's instructions. The PCR products were run in a 2% agarose gel and the samples with a bright major band between 450 and 470 bp were selected for further experiments. The gel was purified using Qiagen Gel Extraction Kit (Qiagen, Germany) and libraries were prepared using NEBNEXT Ultra TM DNA Library Preparation Kit for Illumina. The purified amplicons were sequenced in Illumina NovaSeq 6000 in pairs.

Raw tags were merged and filtered using FLASH (version 1.2.7) and QIIME (version 1.7.0) to obtain high-quality clean tags. The tags were compared with the reference database (Gold Database) using UCHIME algorithm and chimeras were removed to get effective tags. The effective tags were analyzed using Uparse software to obtain OTU. For each representative sequence, Mothur software was used to obtain a species annotation for each taxonomic rank. The phylogenetic relationship of all OTUs was determined using MUSCLE.

Data analysis

Proximate, dietary fiber, and antioxidant activity of tempeh were analyzed using the independent samples t-test when normality of the data was met according to Kolmogorov–Smirnov test. The Friedman and Wilcoxon non-parametric tests were used to analyze the diversity index and relative abundance of major bacterial phyla and families among groups [28]. Principal component analysis (PCA) was performed to discriminate the differences of the gut microbiome between groups. Analysis of molecular variance (AMOVA) was performed to analyze the differences in gut microbiota between groups. Significant was set at $p < 0.05$. All statistical analyses were performed in R.

Results

Proximate, dietary fiber, and antioxidant activity of tempeh

There is a significant difference ($p < 0.001$) between regular tempeh and modified tempeh in water content, protein, fat, and antioxidant activity. There were also differences ($p = 0.002$) in dietary fiber between tempeh, with the highest dietary fiber content found in modified tempeh (8.20 ± 0.19%). Modified tempeh has higher fat, protein, and fiber content compared to normal tempeh (Table 2).

Effect of tempeh supplementation on serum glucose

There is a significant difference ($p < 0.001$) in fasting serum glucose before and after treatment. After induction of diabetes mellitus, blood glucose was significantly increased and was above 200 mg/dl in all rats, indicating successful induction of diabetes mellitus in the animals (Figure 1).

Effect of tempeh supplementation on SCFA

There are significant differences ($p < 0.001$) in the composition of SCFA (acetate, propionate, and

Table 2: Proximate, dietary fiber, and antioxidant activity of tempeh*

Variable	Groups		*p*-value
	Regular tempeh	Modified tempeh	
Moisture	63.17 ± 0.08	57.34 ± 0.13	<0.001
Crude protein	17.03 ± 0.02	19.85 ± 0.02	<0.001
Fat	9.27 ± 0.01	10.85 ± 0.06	<0.001
Dietary fiber	5.92 ± 0.02	8.20 ± 0.19	0.002

*Data were presented in mean±standard deviation (*n*=3).

butyrate) between the groups. Acetate and propionate in the caeca were significantly higher in diabetic rats treated with modified tempeh replacing 30% of the protein in the diet (TG-30) than in the other groups. However, the cecal butyrate of this group was not statistically different from that of the healthy control group, the group with modified tempeh or the group with normal tempeh replacing 15% of the protein in the diet (Table 3).

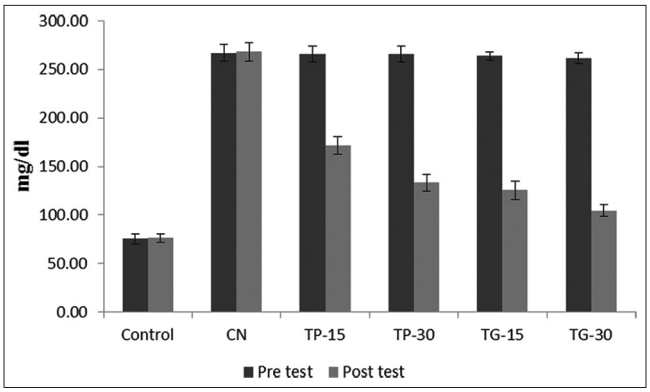

Figure 1: Effect of tempeh supplementation on fasting serum glucose before and after 4 weeks of treatment. Control: Healthy control group, CN: Diabetic control group, TP-15: Diabetic rats received regular tempeh diet replacing 15% of protein in the diet, TP-30: Diabetic rats received regular diet replacing 30% of protein in the diet, TG-15: Diabetic rats received modified tempeh diet replacing 15% of protein in the diet, TG-30: Diabetic rats received modified tempeh diet replacing 30% of protein in the diet

Effect of tempeh supplementation on gut microbiota diversity

There is a significant difference ($p < 0.05$) between the groups in terms of Shannon, Simpson, and phylogenetic diversity indices (pd) for the whole tree. There are no significant differences between groups in Chao1 index. Diabetic rats in TP30 and TG30 groups have similar Shannon and Simpson diversity index. However, TG30 was significantly different ($p < 0.05$) from healthy control group in pd whole tree diversity index (Table 4).

Gut microbiota composition

There is a significant difference ($p = 0.039$) in the composition of the gut microbiota between the groups as shown by the AMOVA test. There is no significant difference in the relative abundance of each bacterium between the groups, either at phylum or family level. At phyla level, the gut microbiota of diabetic rats consisted mainly of *Bacteroidetes* (36.31%) followed by *Firmicutes* (23.72%) and *Proteobacteria* (17.35%). The ratio of *Firmicutes*/*Bacteroidetes* was 0.65 in the diabetic group. Treatment of diabetic rats with normal tempeh or modified tempeh increased the abundance of *Firmicutes* (29.47–46.49%) and *Actinobacteria* (11.58–27.33%). Both tempeh supplements also decreased the abundance of *Bacteroidetes* (22.55–32.31%) and *Proteobacteria* (7.5–14.29%). Under these conditions, the ratio of *Firmicutes*/*Bacteroidetes* increased, with the highest ratio found at TG-30 (1.93).

At family level, the diabetic animals had higher abundance of *Muribaculaceae* (17.27%), *Bacteroidaceae* (13.63%), and *Fusobacteria* (11.67%). Both tempeh supplementations increased the abundance of *Prevotellaceae* (8.06–17.07%) and *Lachnospiraceae* (10.40–17.77%), while decreased the abundance of *Fusobacteria* (0.3–5.3%) and *Bacteroidaceae* (1.26–3.99%). The composition of the gut microbiota is shown in Table 5.

The relative abundance of each bacterium is shown in Figure 2. The composition of microbiota between groups was analyzed and compared based on the relative abundance of OTUs using PCA. The first two results of PCA, PC1 and PC2, can explain 15.35% and 11.86% of the variance, respectively (Figure 3). Among the treatment groups, the rats fed with normal tempeh or modified tempeh replacing 30% of the protein in the diet formed one group and were separated from the other groups.

Discussion

This is the first study to investigate the effects of using lactic acid bacteria during tempeh processing on nutrient, SCFA, and gut microbiota composition in diabetes mellitus. Our study shows that modified tempeh has higher dietary fiber content compared to normal tempeh. Interestingly, although modified tempeh has higher nutrient and fiber composition than normal tempeh, both tempehs can increase the diversity of the gut microbiota of diabetic animal model. Both tempehs also show a similar trend in gut microbiota composition, that is, less *Bacteroidetes* and *Proteobacteria* and more *Firmicutes* and *Actinobacteria* compared to diabetic rats.

In the past, soaking soybeans with acidifiers such as acetic or lactic acid has been reported to increase the quality of tempeh by inhibiting the growth of pathogenic bacteria such as *Listeria monocytogenes*, *Bacillus cereus*, *Salmonella infantis*, *Staphylococcus aureus*, and *Escherichia coli* [29]. In addition, soaking soybeans in water can lower the pH and promote the growth of *Enterococcus*,

Table 3: Cecal short-chain fatty acid composition (mmol/g)

Short-chain fatty acids	Groups						*p*-value
	Control	Negative control	TP-15	TP-30	TG-15	TG-30	
Acetate	26.23 ± 2.74[a]	13.5 ± 3.17[b]	12.13 ± 1.23[b]	26.2 ± 3.03[a]	41.4 ± 2.62[c]	64.98 ± 8.96[d]	<0.001
Propionate	11.1 ± 4.57[a]	5.1 ± 0.83[ab]	3.84 ± 2.01[b]	11.4 ± 3.84[abc]	13.78 ± 2.75[ac]	21.58 ± 3.15[d]	<0.001
Butyrate	2.83 ± 1.00[a]	0.5 ± 0.24[ab]	4.93 ± 1.35[ac]	2.18 ± 0.89[abd]	2.53 ± 0.74[abc]	4.58 ± 1.63[ac]	<0.001

Data are presented in mean±standard deviation. [abc]Different annotation indicating significant different (p<0.05) based on Tukey-Kramer *post hoc* test. Notes: Control: Non-diabetic control group, negative control: Diabetic control group, TP-15: Diabetic rats received tempeh replacing 15% of protein in the diet, TP-30: Diabetic rats received tempeh replacing 30% of protein in the diet, TG-15: Diabetic rats received modified tempeh replacing 15% of protein in the diet, TG-30: Diabetic rats received modified tempeh replacing 30% of protein in the diet.

Table 4: Microbiota diversity of diabetic rats treated with tempeh*

Diversity index	Group					
	Control	Negative control	TP-15	TP-30	TG-15	TG-30
Shannon	4.92 ± 1.25[a]	5.90 ± 0.64[a]	6.38 ± 0.20[a]	6.76 ± 0.23[b]	5.77 ± 0.46[a]	6.95 ± 0.49[b]
Simpson	0.87 ± 0.05[a]	0.93 ± 0.03[a]	0.96 ± 0.02[b]	0.97 ± 0.01[bc]	0.94 ± 0.02[a]	0.98 ± 0.01[c]
Chao1	823.96 ± 202.14[a]	1111.99 ± 201.47[a]	1015.69 ± 17.95[a]	1266.08 ± 348.19[a]	951.97 ± 123.52[a]	1033.41 ± 68.55[a]
Phylogenetic Diversity (PD) whole tree	68.05 ± 12.48[a]	89.08 ± 15.95[ab]	77.54 ± 4.67[ab]	87.33 ± 13.66[ab]	94.15 ± 17.96[ab]	102.42 ± 9.73[b]

*Data are presented in mean±standard deviation (n=2). [abc]Different annotation indicated P<0.05 according to Wilcoxon test. Control: Non-diabetic control group, negative control: Diabetic control group, TP-15: Diabetic rats received tempeh replacing 15% of protein in the diet, TP-30: Diabetic rats received tempeh replacing 30% of protein in the diet, TG-15: Diabetic rats received modified tempeh replacing 15% of protein in the diet, TG-30: Diabetic rats received modified tempeh replacing 30% of protein in the diet.

Lactococcus, *Pediococcus*, *Weissella*, and *Enterobacter* in the soaked water [30], [31]. These conditions have been reported to affect the growth of tempeh mold and the nutritional composition of

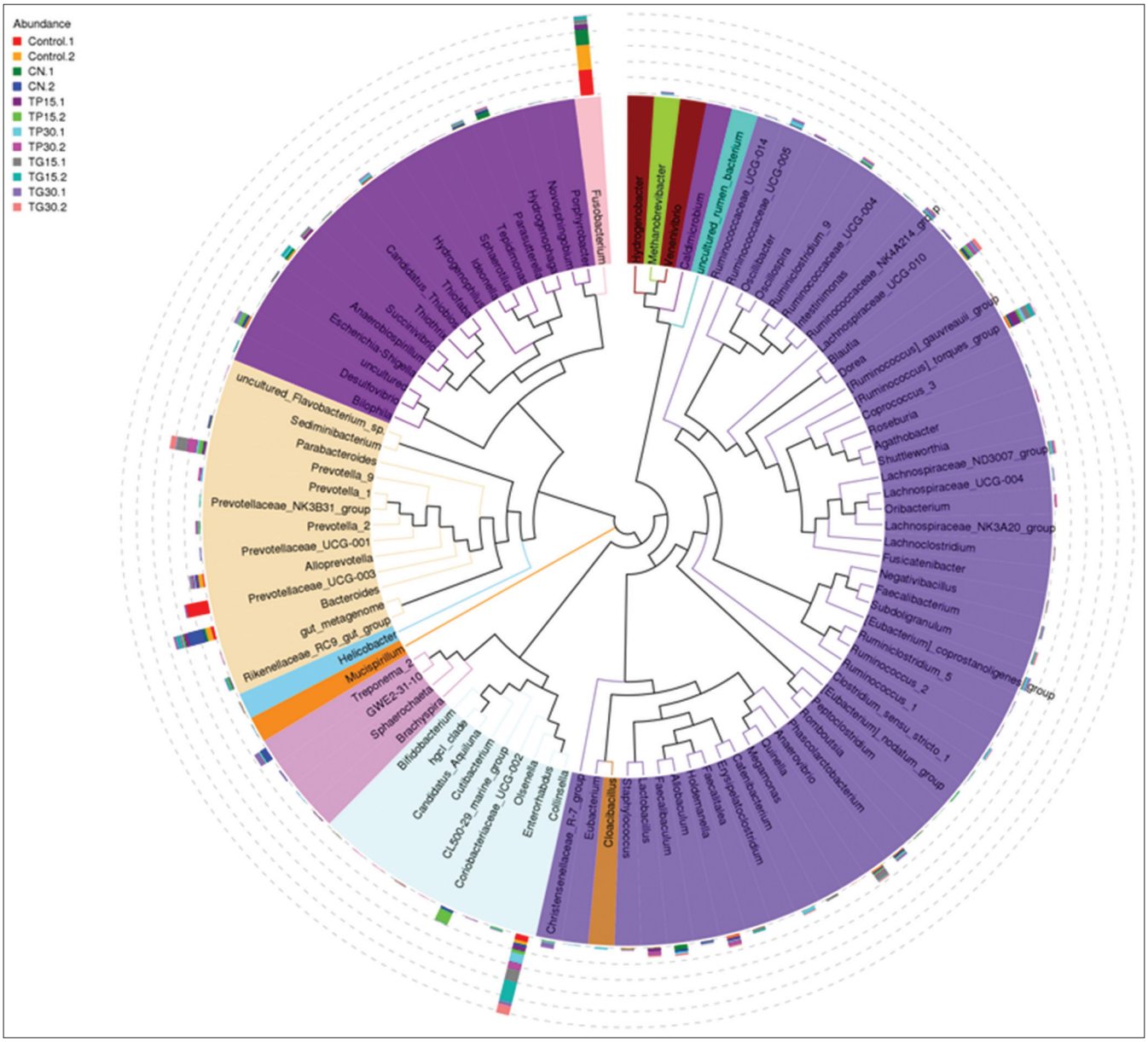

Figure 2: Relative abundance of 100 genera in response to tempeh treatment. Different colors of the branch inside the circle represent different phyla. Relative abundance of each genus in each group was displayed outside the circle.

Table 5: Relative abundance (%) of top 5 gut microbiota in the cecal of rats

Microbiota	Group						*p*-value
	Control	CN	TP-15	TP-30	TG-15	TG-30	
Phylum							
Firmicutes	13.85	23.72	37.07	41.58	29.47	46.49	0.08
Bacteroidetes	34.18	36.31	27.48	32.31	22.55	23.99	0.68
Fusobacteria	36.46	11.68	3.73	0.30	5.26	0.40	0.11
Proteobacteria	6.22	17.35	12.71	7.76	14.28	7.48	0.13
Actinobacteria	7.86	4.11	17.02	14.79	27.34	11.58	0.23
Ratio F/B	0.41	0.65	1.35	1.29	1.31	1.94	0.19
Family							
Fusobacteria	36.46	11.67	3.72	0.28	5.26	0.41	0.11
Prevotellaceae	20.96	2.23	8.06	17.07	10.85	7.68	0.51
Coriobacteriaceae	6.57	1.79	5.28	1.18	24.45	9.19	0.14
Bacteroidaceae	6.09	13.63	3.99	1.26	3.15	2.12	0.43
Lachnospiraceae	5.62	8.09	17.77	15.03	10.40	17.31	0.10

Ratio F/B: Ratio of *Firmicutes*/*Bacteroidetes*. Control: Healthy control group; CN: Diabetic control group; TP-15: Regular tempeh replacing 15% of protein in the diet; TP-30: Regular tempeh replacing 30% of protein in the diet; TG-15: Modified tempeh replacing 15% of protein in the diet; TG-30: Modified tempeh replacing 30% of protein in the diet.

tempeh, in particular the moisture, fat, carbohydrate, and fiber content [32], [33], [34].

The lactic acid bacteria in tempeh are also an important aspect for the enhancement of aglycone isoflavones due to the presence of the enzyme β-glucosidase [35]. It has been reported that aglycone isoflavone stimulates the growth of Gram-positive bacteria such as *Firmicutes*, *Lactobacillus*, *Bifidobacteria*, *Lachnospiraceae,* and *Coriobacteriaceae* [15], [36], [37], which was also found in this study. The high number of *Lachnospiraceae* in the tempeh-fed group was associated with high production of SCFA from fermentation of carbohydrates [38], while high number of *Coriobacteriaceae* indicated high equol production from isoflavones [39].

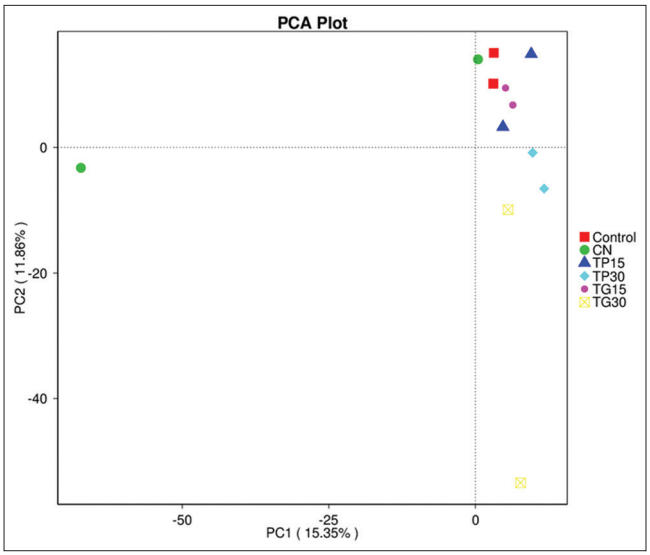

Figure 3: Principal component analysis plot of cecal microbiome samples. The first component (PC1) explained 15.35% of the total variance while the PC2 – 11.86%. The same labels indicate samples from similar group

In addition, consumption of tempeh also increased the abundance of *Prevotella*, which was previously reported by another group [17]. High abundance of *Prevotella* was found in the intestines of people who consumed a high proportion of plant foods [40], [41], [42], and this effect was associated with the anti-inflammatory and antidiabetic effects of plant foods [43], [44].

Conclusion

Modifying the preprocessing of tempeh has implications for the nutrients, fiber, and antioxidants of tempeh. Consumption of tempeh favors the growth of the gut microbiota, which was important for the antidiabetic effect of tempeh. Further studies are needed to investigate the effects of tempeh supplementation on the composition and function of the gut microbiota in a human study.

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