Using kefir glucomannan to modify the diversity and composition of cecum bacterial in Sprague Dawley rats with metabolic syndrome

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Abstract. This study aimed to determine the effect of kefir and kefir glucomannan (Kefir-GM) to the diversity and composition cecum bacteria and bodyweight and feed intake in rat metabolic syndrome. Research carried out used four group’s treatment, namely (1) control, normal rat (2) rats with metabolic syndrome (3) diet kefir in rats with metabolic syndrome, and (4) diet kefir-GM in rats with metabolic syndrome. After 4 weeks, cecum was taken for analysis of the diversity and composition of bacterial. Kefir and Kefir-GM in rats metabolic syndrome did not significantly affect the diversity at p<0.05, but increased Lactobacillus 14.61% and Bifidobacterium 2.2%, and decreased Clostridium 38.15% and Bacteroides 22.51%. Bodyweight and feed intake did not significantly different at p<0.05. Based on this research, kefir and Kefir-GM did not affect diversity but it is able to modify composition bacteria on cecum and also can keep the bodyweight and feed intake in rats.

1. Introduction
Diet and lifestyle nowadays are the important factor that leading to the rapid occurrence of metabolic syndrome. Former research was finding that this regarding with the function of gut microflora [1]. The changes composition gut microflora because bad diet effect to increasing gain energy in gastrointestinal tract and become easy gain weight [2]. Moreover, gut microflora has function related to the health of the host.

Research on obese and non-obese patients showed that those who were obese have a poor diversity microbiome compare with non-obese [3]. In the contrary, research by Nakayama et al [4] showed that the high Shannon Weiner’s diversity index in 7-10 years old school-age children in Asia does not related to the good bacterial community. The results showed that Japanese children with the smallest microbial diversity index actually have greater abundance of Bifidobacterium compare with Indonesian children who have higher diversity index. This showed that food influences the diversity of gut microbiome.
Therefore, many researchers assume that the administration of probiotics, prebiotics and symbiotic as dietary supplements can increase the microflora which is beneficial to the health of the host.

Kefir is the traditional fermented milk using kefir grain that is consists of a polysaccharide composed of a complex microbial association among bacteria and yeasts. Kefir is widely known as an excellent source of probiotics with potential health benefits [5] and is produced based on cow milk or goat milk. Satir and Guzel-seydim [6] reported that kefir based goat milk consist of abundant microbe (Lactobacillus spp., Lactococcus spp., Lactobacillus acidophilus, Bifidobacterium and Yeast) and antioxidant compound (phenolic) higher compare with kefir based cow milk. Furthermore, this research showed that goat milk kefir contain bioactive peptide that act as immunomodulatory, antioxidant, and hypercholesteremic substance [7]. Moreover, Likotrafiti et al. [8] research showed that Lactobacillus kefiri isolated from kefir grain promoted the stabilization of gut microbiome.

To enhance the efficacy of probiotics and promote the growth of autochthonous beneficial bacteria, prebiotics were introduced as potential food ingredients. Glucomannan has potential as prebiotic that promoted the growth of good microflora. Research by Chen et al. [9] showed that mice feeding with glucomannan increased fecal Bifidobacteria count and suppressed Clostridium perfringens counts. Moreover Harmayani et al. [10] research showed that diet supplemented with porang glucomannan enhanced the production of total SCFA that promoted decreased cholesterol content in blood, inhibited the growth of Escherichia coli, and was not significantly supported growth of the Bifidobacteria and Lactobacilli.

In this current research, we produced Kefir-GM product based on goat milk that supplementation glucomannan from porang (Amorphopallus oncophylus) using kefir grain as starter. This Kefir-GM to be expected has good effect to the metabolic syndrome especially the effect to the gut microbiome because of the product contains of prebiotic and probiotic that is called symbiotic product. Therefore, the aim of this research is to evaluate the effect of Kefir-GM supplementation on the diversity and composition bacteria in the rats Sprague Dawley with the metabolic syndrome.

2. Materials and methods

2.1. Kefir and kefir-GM preparation
Kefir in this research was made with two different formulations, without glucomannan and with the addition of glucomannan 0.3% b/v. Kefir was made based goat milk and was added with whey 0,1% (v/v). The solution was pasteurized in 75°C for 15 minutes, and cooled at room temperature. The mixture was added with 2% (b/v) kefir grain, and then was incubated in 28°C for 20 hours. After incubation, the kefir grain was separated with the kefir used sterilized filter [11].

2.2. Animal study
The group of male Sprague Dawley rats age of 8-12 weeks old were divided into 4 groups (each group used 6 rats): 1) Normal control (negative control rats) that received standard diet only, 2) rat with metabolic syndrome (fed high-fat/high-fructose (HFHF)) (positive control), 3) rat with metabolic syndrome fed HFHF supplemented with kefir, 4) rat with syndrome metabolic fed HFHF supplemented with Kefir-GM. The dose of kefir was 3.6 mL/200 g body weight/ day, for 4 weeks. Before treatment, the rats were acclimated with standard diet AIN-93 for 1 week, and then fed high fat and high fructose for 2 weeks. The dose of kefir was 3.6 mL/200 g body weight/ day, for 4 weeks. Before treatment, the rats were acclimated with standard diet AIN-93 for 1 week, and then fed high fat and high fructose for 2 weeks. The rats were then divided into 4 groups as above. High fat and high fructose diet were administered until the end of experiment (4 weeks). The composition of standard diet and high-fat/high-fructose diet were formulated according to Reeves et al. [12] and de Castro et al [13]. Every week the body weight of the rat was weighted and every day the leftover feed not consumed by rat was weighted to. The sample from digesta was collected from cecum in the end period of the experiment. All procedures related to animal experiment in this study were approved by Medical and Health Research.
Ethics Committee (MHREC), Faculty of Medicine Universitas Gadjah Mada, Indonesia (Approval Number: KE/FK/95/EC/2015).

2.3. Analyze the Diversity and Composition Cecum Bacteria Using TRFLP (Terminal Restriction Fragment Length Polymorphism)

2.3.1. DNA isolation, PCR amplification, and restriction enzyme digestion. Isolation DNA bacteria was used Favor Prep™ Stool DNA Isolation Mini Kit. After that, the isolate DNA was amplification gen 16s rRNA using universal primer 27F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492R (5’-GGTTACCTTGTTACGACTT-3’) that has been added with FAM dye at the forward primer [14]. Amplification 16S rRNA was used Gotaq Green Mixture with total reaction 25µl (12.5µl Gotaq Green Mix, 1µl forward primer (27FFAM), 1µl reverse primer (1492R), 1µl sample DNA (250ng), and 9.5µl nuclease free water). PCR program cycle condition: an initial denaturing step at 95°C for 2 min, 30 cycles of denaturing step 95°C for 1 min, annealing at 56°C for 30 s and extension at 72°C for 1 min. The final extension was performed at 72°C for 5 min. The PCR product was digested with MspI (Thermo Scientific) and HhaI (GeneMark enzyme) [14]. Formulation for digested product PCR was used 1.5µl buffer tango, 12 µl DNA, 1 µl enzyme and 0.5µl nuclease free water. This was incubated in 37°C for 4 hours.

2.3.2. Identification product restriction enzyme. The fragment that was produced from digested by restriction enzyme was identification used Capillary Electrophoresis Applied Biosystems 3130 series Genetic. This was performed by 1st Base. The profile of the fragment was obtained in this process was analyzed next.

2.3.3. Analyzed the TRFLP data. The profile of the fragment from the identification process was analyzed using Software Peak Scanner™ v1.0 (Thermo scientific). The length of fragment T-RFs was selected between 50bp until <500bp. To reduce the noise data, the sample was selected again. The T-RFs must have value of peak area was >1% (by dividing the value of the peak area with the total peak area of the T-RFs) [15]. The selected T-RFs analyzed using T-align program in http://inismor.ucd.ie/~talign/. The data was obtained was binomial data for the clustered analysis. Analysis clustered was performed using NTSYSpc version 2.1. Dendrogram was performed using underweight pair group with arithme average (UPGMA). The diversity and distribution frequency was performed using Shannon Weinner and Shimpson index [16]. Prediction bacteria was performed using web-based program MICA III (mica.ibest.uidaho.edu), RDP 16S bacterial rRNA database, and also database NCBI 16S rRNA that has been simulation cutting in silico using Bio-edit.

2.4. Statistical analysis.
The body weight rat, daily feed intake, and diversity index data was analyzed using ANOVA and if there was significant different data was analysis next using Duncan.

3. Results and discussions

3.1. Diversity and prediction composition bacteria in cecum
Following this a dendrogram of the results of running TRFs data through the Group Average clustering algorithm was showed in Figure 1.
Figure 1. Similarity dendrogram composition bacteria according TRFs that digested using HhaI and MspI. In which: Normal rat (1.1-1.3), metabolic syndrome rat (2.1-2.3), diet kefir in metabolic syndrome rat (3.1-3.3), diet kefir-gm in metabolic syndrome rat (4.1-4.3).

Figure 1 shows that the similarity TRFs of Kefir and Kefir-GM were the highest compare with TRFs bacteria in gut rat treatment was 0.93. Composition bacteria in 3.3 group (rat with metabolic syndrome diet kefir) and 4.1 group (rat with metabolic syndrome diet kefir-gm) has 0.86 similarity with kefir and kefir-gm. Normal rat (1.1 and 1.3) group has similarity score 0.79 with kefir and kefir-gm. Group 3.2 and 3.3 (rat with metabolic syndrome diet kefir) showed similarity score 0.78 and 0.76 with kefir and kefir-gm. Group 4.2 (rat with metabolic syndrome diet kefir-gm) showed similarity score 0.74. It is all indicated that composition bacteria in kefir and kefir-gm similar with the composition bacteria in cecum of rat with metabolic syndrome that was supplemented with kefir and kefir-gm. This similarity indicated that kefir and kefir-gm could modify microflora of cecum in rat with metabolic syndrome.

Number of species, diversity index Shannon Weiner, and distribution frequency bacteria in cecum rat was showed in table 1. Statistical analysis diversity index bacteria in all group treatment rats showed was not significantly different.

| Community TRFs | Normal rat | Metabolic syndrome rat | Metabolic syndrome + Kefir | Metabolic syndrome + Kefir-GM |
|----------------|-----------|------------------------|--------------------------|-----------------------------|
| Parameter      | 1.1       | 1.2                    | 1.3                      | 2.1                         |
|                | 2.1       | 2.2                    | 2.3                      | 3.1                         |
|                | 3.1       | 3.2                    | 3.3                      | 4.1                         |
|                | 4.1       | 4.2                    | 4.3                      |
| $S^a$          | 20        | 32                     | 14                       | 17                          |
|                | 17        | 24                     | 26                       | 21                          |
|                | 26        | 22                     | 22                       | 18                          |
|                | 18        | 19                     | 38                       |
| $H^b$          | 1.86      | 2.39                   | 0.97                     | 1.71                        |
|                | 0.97      | 2.40                   | 2.43                     | 2.20                        |
|                | 2.43      | 2.20                   | 2.07                     | 1.78                        |
|                | 1.78      | 1.36                   | 2.13                     |
| $H/H_{max}^c$  | 0.62      | 0.69                   | 0.37                     | 0.61                        |
|                | 0.61      | 1.00                   | 0.77                     | 0.75                        |
|                | 1.00      | 0.77                   | 0.68                     | 0.58                        |
|                | 0.77      | 0.68                   | 0.58                     | 0.62                        |
|                | 0.68      | 0.58                   | 0.46                     | 0.59                        |

a: number of species, it was count based on number different TRFs in the group
b: diversity index, Shannon Wiener
c: frequency distribution that was count from Shannon Wiener

Normal rat (1.1-1.3), metabolic syndrome rat (2.1-2.3), diet kefir in metabolic syndrome rat (3.1-3.3), diet kefir-gm in metabolic syndrome rat (4.1-4.3)
Figure 2. Composition bacteria in cecum normal rat (a), metabolic syndrome rate (b), metabolic syndrome rat + diet kefir (c), and metabolic syndrome rat + diet kefir-gm (c) based on TRFs.
Figure 2 displays the bacterial composition in cecum sample based on peak area of electrogram. Result showed that *Lactobacillus*, *Bifidobacterium*, *Bacteroides*, *Lactococcus*, *Clostridium*, and *Proteobacterium* were found in the cecum rat. Previously, Kovatcheva-datchary and Arora [2] reported that microflora *Lactobacillus*, *Bifidobacterium*, *Bacteroides* and *Clostridium* were found in human gut. Diverse microbiota composition in human gut is strongly related with human diet. *Campylobacter* was found in normal rat, metabolic syndrome rat, and metabolic syndrome rat that was supplemented with kefir. *Flavobacterium* was found in cecum normal rat, metabolic syndrome supplemented with kefir and kefir-gm.

Average percentage of *Lactobacillus* in cecum rat with metabolic syndrome that supplemented kefir or kefir-gm was increase until 14.61% compare with metabolic syndrome rat. Percentage *Lactobacillus* in cecum rat with metabolic syndrome that was supplemented with kefir was higher than that was supplemented with kefir. Average percentage of *Bifidobacterium* in rat with metabolic syndrome that was supplemented kefir and kefir-gm also was increase 3.7%. These finding was indicated that supplementation kefir and kefir-gm could increase composition the beneficial bacteria in gut. *Lactobacillus* and *Bifidobacterium* according Hema明智aya *et al.* [17] were genera that recognize as probiotics to help and to build up the beneficial bacterial flora in the intestine and completely exclude the pathogenic bacteria. Lactobacillus as probiotic has been shown to exhibit a mannose specific adhesion by which it can adhere to human colonic cells. Once the probiotic adheres to the cell, various biological activities take place, which primarily include the release of cytokines and chemokines. These then exert their secondary activity such as stimulation of mucosal and systemic host immunity.

Supplementation kefir and kefir-gm to the rat with the metabolic syndrome could decrease percentage *Clostridium* and *Bacteroides* compare with the metabolic syndrome rat was not supplemented with kefir. The average percentage *Clostridium* was decrease 38.15%, for the diet kefir 54.11% and 22.19% for the diet kefir-gm, respectively. The average percentage *Bacteroides* in metabolic syndrome rat with diet kefir and kefir-gm was decrease 22.51%, for diet with kefir 45.01% and diet with kefir-gm 37.63%, respectively. According to Gibson and Roberfroid [18], *Bacteroides* and *Clostridium* dominated microflora in gastrointestinal in rat and human. These bacteria were commensal that some was beneficial to support the health of the host and much become pathogen.

The low increase of *Bifidobacterium* in cecum rat with metabolic syndrome with diet kefir-gm was associated with the decrease of the *Clostridium*. *Clostridium* was needed to degrade the glucomannan by the enzyme that they produced. Enzyme glucanase and 1,4-D mannose was performed to degrade glucomannan become oligosaccharide and monosaccharide that could be utilized by *Lactobacillus* and *Bifidobacterium*. This process used Embden Meyerhoff Parnas pathway. In the other hand, *Bifidobacterium* produced glycoside hydrolase that actively degrades polysaccharide in plant [10].

Den-Besten *et al.* [19] described the degradation process of glucomannan in which the microbiota hydrolyze non digestible carbohydrates into oligosaccharides and monosaccharides with fermentation in the anaerobic environment inside the gut. Major bacterial metabolic routes are the Embden-Meyerhof-Parnas pathway (glycolysis, for six-carbon sugars) and the pentose-phosphate pathway (for five-carbon sugars), which convert monosaccharides into phosphoenolpyruvate (PEP). Subsequently, PEP is converted into fermentation products such as organic acids or alcohols. At the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) the electron carrier NADH is formed. An-aerobically, there are three types of pathways to get rid of excess reducing equivalents. Major end products of the described fermentation pathways are the SCFAs (Acetate, Propionate, and Butyrate) in abundant number. Prediction bacteria in cecum rat with metabolic syndrome that was supplemented kefir and kefir-gm showed existence of *Pediococcus acidilactici*. *P. acidilactici* is lactic acid bacteria that acid and bile salt resistant. This bacteria also has a role to fermented carbohydrate [20]. *P. acidilactici* is a type of probiotics typically found as a member of normal flora of alimentary tract, oral cavity gastrointestinal tract and has been commonly used in the food industry [21]. *P. acidilactici* was produced pediocin that including bacteriocin that has a role as antimicrobial [22]. Furthermore *P. acidilactici* could stand in the gastrointestinal tract temporary. Moreover supplementation *P. acidilactici* with FOS in rat did not significantly showed differences in metabolic parameter [20]. Previous study showed that
supplementation *P. acidilactici* strain M76 in the hyper cholesterol rat could decrease cholesterol total in blood serum and liver through the mechanism forming exopolysaccharide that effected reducing cholesterol contain in the body [23]. Moreover, other study also showed that administration of *P. acidilactici* R037 in mice and rat lowered serum triglycerides, possibly through suppressing intestinal absorption and potentiating lipolytic pathways [24], indicating suppression of intestinal digestion and absorption of food, and suppression of appetite.

3.2. Effect diet kefir and kefir-GM on body weight and feed consumed in metabolic syndrome (MS) rats

The body weight of the rat was shown in figure 3. Analysis body weight was performed using the difference of body weight before and after diet with kefir and kefir-GM. Statistical analysis showed did not significantly different between group of treatment in p>0.05. It was indicated that diet kefir or kefir-gm could stabilize body weight. This result same with the research result by Judiono *et al.* [25] that was supplemented clear kefir in the hyperglycemic rat.

![Figure 3](image-url) The body weight of the rat during the treatments.

The body weight gain of the rat in contrast with the average feed intake every day (Figure 4). It was shown that there were no significant differences between groups. Supplementation of konjac glucomannan, porang, inulin, and normal feed in the group treatment of the rat in the research by Harmayani *et al.* [10] was shown the same trend that the difference body weight and the average feed intake of the rat was not significantly different.

![Figure 4](image-url) The average feed consumed per day of group treatment.
The difference body weight and the average food intake did not significantly different could be indicated that supplementation kefir and kefir-gm could stabilize body weight gain with inhibited the rat to increase the feed consume. Bogsan et al. [26] declared that supplementation probiotic such as *L. plantarum*, *L.rhamnosus*, and *Propionibacterium L66-5* in human and rat could promote satiety effect. Flint et al [27] assumed that complex carbohydrate has the low energy density. Then the low energy density affected the rate of stomach emptying so then effect the feeling of satiety. In the same satiety, consumed complex carbohydrate affected low energy intake to the body. This is because of diet complex carbohydrate could increase the energy expenditure by feces. Protein, lipid or carbohydrate from food that we consumed could be trap by complex carbohydrate that it could not degraded in intestine. Kaats et al. [28] assumed that glucomannan has ability to quickly and profoundly absorb water suggests that it may lead to a swelling in the stomach, resulting in consequent feelings of fullness and decreased appetite.

St-Onge et al. [29] was recommendation minimal number microbe in kefir was $10^{10}$CFU/ml. This number of microbes could support the health of the hyperlipidemia patient. Furthermore in 2010 European Food Safety Authority (EFSA) declared that glucomannan could decrease the body weight in overweight adult if it was consumed $\geq 3$g per day. The doses was 1g for 3 times and addition with consumed 1-2 glass of water before eat [30].

4. Conclusions
Kefir and Kefir-GM did not affect diversity but it is able to modify microbiota composition on digesta by increasing *Lactobacillus* and *Bifidobacterium* and suppressing *Clostridium* and *Bacteroides*. Kefir and Kefir-GM can keep the weight body and feed intake in rats with metabolic syndrome.

Conflict of interest
The authors have no conflict of interest to state regarding this manuscript.

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