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Profile of Bacteria and Short Chain Fatty Acids of Caecal Digesta in Malnourished Rat Fed Goat Milk Yoghurt

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Abstract The intestinal microbiota is an important determinant for general health of the human body, and disturbance of the proper balance of microbiota is involved in several pathologies. The profile of gastrointestinal microbiota can be influenced by nutritional factors and or health status of individuals. This study aimed to determine the effect of goat milk yogurt on the profile of caecal bacteria and short chain fatty acid (SCFA) in malnourished rats. Yoghurt was prepared by using of pasteurized goat milk with adding the Lacto-B powder containing *Lactobacillus acidophilus, Sterptococcus thermophilus* and *Bifidobacterium longum.* Male malnourished Wistar rats 3 weeks old were created using standard feed restriction up to 50% of normal rats for 21 d. After 21 d, the rats continued to restricted feeding and supplemented with goat milk yoghurt for 7 d. The rats were killed and analyzed the profile of caecal bacteria and SCFA. There were no significantly differences in the lactic acid bacteria (LAB) and bifidobacteria in both of caecal digesta of malnourished or normal rats fed yoghurt and control rats. However, the amount of *E. coli* was higher in malnourished rats than the normal rats. The acetic acid of caecal digesta was lower in the rats fed goat milk yoghurt than the control rats, whereas the butyric acid was higher in the caecal digesta of normal rats compared to the malnourished rats. The pH and moisture of caecal digesta in rats fed yoghurt were not significantly different from the control. In conclusion, goat milk yoghurt supplemented up to 2.0 ml/100 g body weight for 7 d had no effect on profile of caecal bacteria and physical properties and could not increase of caecal SCFA in malnourished and normal rats. Malnourished condition could increase the number of *E. coli*, decrease the butyric acid and weight of caecal.

Keywords: *Goat milk yoghurt, Caecal bacteria, SCFA, Malnourished rat*

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1. Introduction

The gastrointestinal tract is a complex and dynamic network where an intricate and mutualistic symbiosis modulates the relationship between the host and the microbiota in order to establish and ensure gut homeostasis [1]. The cecum itself is part of gastrointestinal tract, a pouch that connects the large and small intestines. Food is temporarily stored in the cecum while helpful bacteria digest the cellulose found in plant cells. Most herbivores such as the rat have a large cecum [2].

Normally, the gastrointestinal tract of mammals is colonized with an extraordinarily large number of highly diverse microorganisms termed the commensal microbiota, predominantly the colon, harbors the greatest number and diversity of organisms, primarily bacteria [3,4]. Intestinal microbiota interact with the host digestive and immune systems [5], provides positive or negative effects on the health of the host [6].

The positive effects of gut microbiota including playing a pivotal role in nutrient digestion and energy recovery, as a source of vitamins [7], SCFA (especially butyrate) production [5], protect the intestine against colonization by exogenous pathogens [8], and regulating the balance and homeostasis of different helper T cell populations in the lamina propria and further emphasize the critical role that the microbiota play in the development of the immune system [4]. Protective effects of commensal bifidobacteria were attributed primarily to the production of acetate that improves intestinal defence mediated by epithelial cells and thereby protects the host against lethal infection [9]. Furthermore, butyrate is the primary energy source for colonocytes, is mainly produced by Clostridium cluster IV and XIVa [10]. Negative effects of the gut microbiota including production of putrefactive compound, such as volatile basic -N, ammonia-N, indole, p-cresole, and sulfide [11].

Protein malnutrition disrupts the normal ecology of the microflora affecting strictly anaerobes, impairs host immune response and antibacterial defenses, enhances the susceptibility to infection, and leads to mucosal atrophy, impaired child development, increased mortality rate and individuals who come to function in suboptimal ways [12,13]. According to [14], the mild malnutrition or first degree when the weight deficit reached 10–25%; moderate or second degree when the weight deficit reached 25–40%; and severe or third degree when the weight deficit was greater than 40% of that of age-matched control rats.

Studies in animal models indicate that goat milk might have beneficial effects on malabsorption disorders and inflammatory bowel diseases [15]. In the previous study, administration of fermented milk maintained the concentrations of the total SCFAs at the same levels as before administration 5- fluoracil into mice, and suggest that the protective effect of oral administration of fermented milk against the endogenous *E. coli* infection resulted from prevention of any abnormal increase in *E. coli* levels via normalization of the 5-fluoracil-induced disruption of the intestinal environment [16].

Lacto-B is a non dairy powder-containing multi-species probiotic consists of *Bifidobacterium longum*, *Lactobacillus acidophilus*, and *Streptococcus thermophilus*, which in Indonesia known as treatment for diarrhea. So far the Lacto-B powder was never used in the production of yoghurt. Multi-strain and multi-species probiotics have improved functionality as compared to single strain [17]. The growth of probiotic will increase if there is source of prebiotic. Acording to [18], goat milk showed rich in sources of prebiotic oligosaccharides that resemble breast milk oligosaccharides [19], and in contrast to other prebiotics such as inulin or frukto short-chain oligosaccharides [20]. The aim of the present study, therefore, was to determine the effect of goat milk yoghurt prepared by Lacto-B powder on the profil of caecal bacteria and SCFA in malnourished rats.

2. Materials and Methods

2.1. Yoghurt Preparation

Yoghurt was prepared from goat milk of Etawah Crossedbred from Indonesia and culture of Lacto-B powder. Goat milk was pasteurized at 85°C for 30 min and cooled to 43-45°C. After cooled, pasteurized goat milk was inoculated directly with 3% Lacto-B powder (Novell Pharmaceutical Laboratories) containing *Streptococcus thermophilus, Lactobacillus acidophilus and Bifidobacterium longum* and incubated at 45°C for 6 h [21].

2.2. Animals Experimental

Three week old male Wistar rats weighing 25.5-41.0 g, were individually caged and housed. During 7 d the rats fed unrestricted amounts of a standard laboratory diet AIN-93G [22] and then randomly assigned into two groups: one maintained in the same conditions (control) and another group fed 50% of the intake of the control's (restricted or malnourished) [23] for 21 d. Both groups were allowed to drink water ad libitum. After 21 d, the rat were assigned to four groups: 1) Normal rats (NC), 2) Normal rats with goat milk yoghurt (NY), 3) Malnourished rats (MC), and 4) Malnourished rats with goat milk yoghurt (MY). MC and NC groups (control without yoghurt) were given aquadest with a volume equal to the volume of treated yoghurt. Aquadest as control or yoghurt were given orally with force feeding. The dose of goat milk yoghurt was 2.0 ml/100 g body weight/day, whose consumption is equivalent to the children consumption of 100 ml yoghurt/day [24]. Each group of rat using 7 replications (n= 7 rats). All of groups were treated for 7 d, and then were killed and sampled. Caecum of the rats was taken for analysis of profile bacteria, SCFA concentration, pH and moisture content. Design of the experimental groups in this study showed on Figure 1. All procedures related to animal experiment were conducted following the recommendation of Medical and Health Research Ethics Committee (MHREC) Faculty of Medicine Universitas Gadjah Mada, Indonesia (KE/FK/753/EC).

2.3. Analysis of Caecal Bacteria

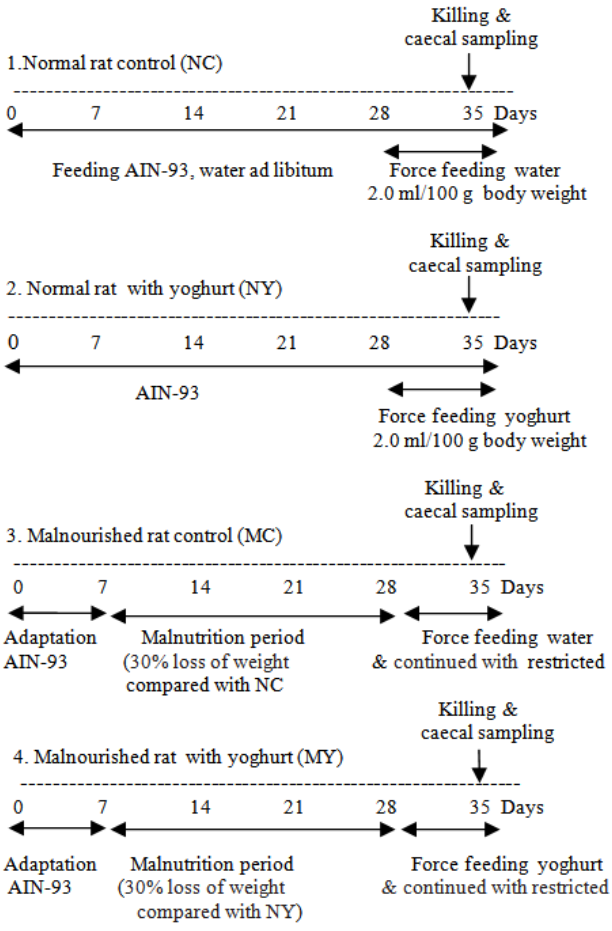

Figure 1. Design of the experimental groups under study (1.NC, 2.NY, 3. MC, 4. MY)

The caecal contents were aseptically collected. The samples for bacterial analysis were diluted in a 10-fold series with 9 ml physiological NaCl. About 0.1 ml of 10^{-5} and 10^{-6} dilutions (appropriate decimal dilutions) were spread on the surface of each agar plate. This was subjected to incubation at 37°C for 48 h for LAB and Bifidobacteria, and 24 h for *E. coli*. To determine the total LAB, Bifidobacteria and *E. coli* populations, the colonies formed were counted and expressed as log CFU/g wet

weight of caecal digesta [25]. Lactic acid bacteria was determined on modified deMan, Rogosa and Sharpe (MRS) agar (Merck) containing 100 ppm NaN_3 [26] and 100 ppm $CaCO_3$ [27]. Lactic acid is strong enough to dissolve calcium carbonate and that can be seen as a clear area in the agar. Thus, in the isolation process clear areas indicate lactic acid bacteria [28]. Bifidobacteria population was determined on MRS agar supplemented with 0.05% of L-cysteine- HCl and 0.2% of bile salts [29]. Tryptone Bile X- glucoronic medium (TBX) used to determine *E. coli* (Oxoid, Code: CM0945).

2.4. pH, SCFA and Moisture Analysis

Measurement of pH and SCFA concentrations were determined according to the procedure of [25]. For pH determination, the caecal contents was diluted 1:3 in aquabidest and immediately after sampling, measured with a pH- meter (HANNA-HI 98103). For analysis of SCFA concentrations, frozen caecal samples was thawed, and centrifuged at 3,500 r.p.m. for 20 min and the supernatans was analysed with a Shimadzu Model GC-8A, Kyoto, Japan [25]. Condition of GC: Capillary column with 10 & SP-1200: 1 mol/L H_3PO_4 in 80/100 mesh Chromosorb WAW (Supelco), and nitrogen as a gas carrier at flow rate 75 ml/min. Temperature of oven, detector and injector was 125°C, 175°C and 180°C, respectively. Caecal moisture contents was analyzed according to [30].

2.5. Statistical Analysis

All data were analyzed by Two Way ANOVA (Normal and Malnourished rat x Control and Goat milk yoghurt).

3. Results and Discussion

Shown on Table 1, goat milk yoghurt prepared from Lacto-B powder had no effect on the profile of bacteria of caecal digesta in malnourished and normal rats. This may be due the dose of goat milk yoghurt was too low and duration of this feeding period was also too short. Although oligosaccharides present in goat milk yoghurt but not enough doses to support probiotics. Different from the other study which using enough doses and feeding period of probiotic and prebiotics by [31], the daily supplementation with 10^9 (cfu) *L acidophilus* and fructooligosaccharides (3 g) for 4 - 8 ws to healthy humans increased fecal LAB by wk 6 – 8. According to [32], intake of low (1.5 g/kg) and high (7.5 g/kg) dosage of probiotics and prebiotics (inulin from chicory) mixture (synbiotic powder) for 8 wk significantly improved the ecosystem of the intestinal tract by increasing the probiotics population and digestive enzyme activities in rats. In addition, the mean fecal lactobacilli and bifidobacterium counts also increased and enterobacteriaceae decreased after 3 wk daily consumption of regular yoghurt, synbiotic yoghurt and traditional fermented sobya [33]. Differences in the survival of lactic acid bacteria in *in vivo* conditions, depending on the combination of bacteria in the fermented milk. The ingestion of lactic acid bacteria influences the composition and/or metabolism of endogenous microbiota. The new association of *L. casei* with yogurt cultures has specific effects of its own that differ from the simple addition of the effects of *L. casei*–fermented milk and yogurt. These effect may offer potential benefits for health [34].

Table 1. Profile of bacteria (Log CFU/g) of caecal digesta in malnourished and normal rats treated with goat milk yoghurt

	Goat milk yoghurt	Control	Average
LAB			
Malnourished	8.09±0.53	8.18 ± 0.27	8.13±0.40
Normal	8.04±0.69	7.75 ± 0.73	7.89±0.69
Average	8.06±0.58	7.96 ± 0.57	8.01±0.57 ^{ns}
Bifidobacteria			
Malnourished	7.65±0.55	8.00 ± 0.64	7.82±0.60
Normal	7.76±0.73	7.12 ± 0.57	7.44±0.71
Average	7.70± 0.62	7.56 ± 0.74	7.63±0.67^{ns}
Escherichia coli			
Malnourished	4.27± 0.88	4.88 ± 0.30	4.55±0.72^{a}
Normal	3.72 ± 0.83	3.51 ± 1.30	3.61±1.06^{b}
Average	4.02 ± 0.86 ^{a}	4.13 ± 1.18 ^{a}	4.08±1.01

ns: not significant
a, b: the different letter in the same column or the same row indicates significantly different (P<0.05)

The result of this study (Table 1) indicated that malnourihed rats have more *E. coli* than the normal rats, because according to epidemiological and experimental observations have proven that malnourished children are more susceptible to infectious disease; therefore, protein calorie malnutritiom is considered a strong risk factor for higher morbidity and mortality rates in infectious disease [13].

Goat milk yoghurt prepared from Lacto-B powder (Table 2) had no effect on propionic and butyric acid, but the acetic in rat caecal decreased significantly ($p<0.05$). Since goat milk yoghurt in this study could not increase the number of caecal LAB and bifidobacteria, so that no increase in the metabolites produced (including SCFA). The dose of goat milk yoghurt in this study not enough to increase the total number of LAB and bifidobacteria in the caecal that produce SCFA. *Streptococcus thermophilus*, *Lactobacillus acidophilus* and *Bifidobacterium longum* combination in Lacto-B powder which was used to make yoghurt, tending to be negative effect (i.e. decreased caecal acetic acid). Similar to the previous study, consumed *Lactobacillus acidophillus* (LAC) $1x10^9$ cfu for 4 wk had negative effect in healthy humans (i.e.increased fecal protein catabolites), whereas fructooligosaccharides (FOS) tending to be beneficial (decreased fecal protein catabolites) [31]. In the other study, the fecal SCFA concentrations in male subjects were also unchanged following the dietary intervention for 3 wk with regular yoghurt, synbiotic yoghurt, sobya and placebo. This result was interpreted the negative response to short duration of intervention or to the need to use new combinations of LAB strains [33]. However, according to the study by [35], that probiotic bacteria affect both the carboxylic acid pattern and the site of carboxylic acid release in the hindgut of rats. The indigestible carbohydrate had more pronounced effects on both the profiles and the caecal concentrations and pools of carboxylic acid. Thus, based on several studies mentioned above, can be explained that the differences in the subject, a combination of probiotics, duration of interventions and dose of probiotics may influence the production of caecal SCFA. Possibility variations in human diet causes a given probiotic effect is not significant. Related to insignificant caecal SCFA in this study, thought to be caused by low doses of yoghurt

and in short time interventions. This is understandable because this study uses the rat subject were given the same standard diets, so as to reduce the effect of variation of diets other than the treatment given.

Table 2. SCFA concentration (mMol) of wet caecal digesta in malnourished and normal rats treated with goat milk yoghurt

	Goat milk yoghurt	Control	Average
Acetic acid			
Malnourished	40.03±3.77	57.45 ±15.59	48.74±14.16[a]
Normal	47.30±5.69	49.64 ±11.44	48.47±8.76[a]
Average	43.66±5.98[a]	53.54 ±13.75[b]	48.60±11.55
Propionic acid			
Malnourished	10.29±1.55	14.70 ± 4.96	12.49±4.21
Normal	12.59±3.24	13.68 ± 5.45	13.13±4.35
Average	11.44± 2.72	14.19 ± 5.04	12.81±4.21[ns]
Butyric acid			
Malnourished	2.77± 2.18	2.29 ± 0.68	2.55±1.62[a]
Normal	3.97 ± 1.70	5.27 ± 2.24	4.73±2.06[b]
Average	3.27 ± 2.00[a]	3.89 ± 2.25[a]	3.59±12.12

ns: not significant

a, b: the different letter in the same column or the same row indicates significantly different (P<0.05).

Propionic and butyric acids affect metabolic parameters, low-grade systemic inflammation, insulin resistance and obesity. The formation of propionic and butyric acids in the caecum is reflected by increased concentrations in the aortic blood [36].

Malnourished rats in this study showed lower butyric acid compared to normal rats (Table 2) due the malnourished rats have more number of *E.coli* which cause infection and may cause inflammation. This condition would change the ratio of gut microbiota. Since the number of LAB and bifidobacteria in the caecal of malnourished rats did not increase, then the resulting SCFA was also not increase and even decreasing butyric. At the intestinal level, butyrate plays a regulatory role on the transepithelial fluid transport, ameliorates mucosal inflammation and oxidative status, reinforces the epithelial defense barrier, and modulates visceral sensitivity and intestinal motility [37]. Butyrate is a major metabolite in colonic lumen arising from bacterial fermentation of dietary fiber and has been shown to be a critical mediator of the colonic inflammatory response [38]. Butyrate is mostly used by colonocytes as an energy source [39] could be metabolized by colonic epithelium, was shown to decrease the risk of mucosal damage. In contrast to butyrate, propionic and acetic acid may have similar but less prominent effects. These acids are associated mainly with metabolic effects [40]. This butyrate possesses both preventive and therapeutic potential to counteract inflammation-mediated ulcerative colitis (UC) and colorectal cancer. One mechanism underlying butyrate function in suppression of colonic inflammation is inhibition of the IFN-γ/STAT1 signaling pathways at least partially through acting as a histone deacetylase (HDAC) inhibitor [38]. The role of butyrate differs between normal and cancerous cells. This is known as the "butyrate paradox". Butyrate inhibits colonic tumor cells, and promotes healthy colonic epithelial cells [41]. As for the level of SCFAs also differs remarkably between the faecal samples of healthy subjects and these of Inflammatory Bowel Disease (IBD) patients [42].

Similar to the previous study by [25], that total SCFA concentrations were reduced remarkably in proportion to the food restriction rate, especially butyrate which decreased strikingly compared with the other SCFAs. The results means that food restriction may influence the physiological processes through alterations in the gut microbiota and its metabolic products. In addition, the other study in fecal sample of IBD showed decreased dramatically in butyric, and also acetic and propionic acids compared to the healthy subjects [42].

The were no significantly differences of pH value and moisture content of caecal digesta in malnourished and normal rats fed goat milk yoghurt (Table 3). Because no increase of SCFA produced in the rat caecal by the administration of yogurt then it did not decrease the caecal pH. This result in this study similar to the study by [43], that yoghurt supplemented with *B. longum* at 250 ml per day for 2 wk to healthy volunteers was not change the fecal pH (diversity of diet may be the reason), eventhough the SCFA production was increase. When ingestion of this yoghurt was stopped, then levels of SCFA declined slowly. As a result of increasing concentrations of acidic fermentation products, the luminal pH in the proximal colon is lower. This pH seems to boost the formation of butyrate, as mildly acidic pH values allow butyrate-producing bacteria to compete against Gram-negative carbohydrate-utilizing bacteria, such as *Bacteroides spp.* [37]. In addition, yoghurt is a low pH food and SCFA accumulation in caecal leads to acidic pH [44].

Table 3. Value of pH and moisture content of caecal digesta in malnourished and normal rats treated with goat milk yoghurt

	Goat milk yoghurt	Control	Average
pH			
Malnourished	7.22±0.38	7.12 ± 0.32	7.17±0.34
Normal	7.28±0.29	7.13 ± 0.36	7.20±0.33
Average	7.25±0.33	7.12 ± 0.33	7.18±0.33[ns]
Moisture (%)			
Malnourished	83.47±2.27	85.11 ± 1.31	84.29±1.97
Normal	84.37±1.24	84.59 ± 0.93	84.48±1.06
Average	83.92± 1.82	84.85 ± 1.12	84.38±1.56[ns]

ns: not significant

Administration of goat milk yoghurt in this study did not affect the caecal moisture content in malnourished and normal rats (Table 3), due the caecal SCFA was not increase. In the previous study, the fecal moisture content tended to increase when either yoghurt supplemented with *Bifidobacterium longum* or standard yoghurt was ingested in healthy volunteer. The increase in moisture is considered to reflect the increase in organic acid levels and it may facilitate excretion. Consumption of yoghurt supplemented with *B. longum* may be expected to alleviate constipation [37]. According to [45], there is weak positive correlations between faecal water content and the major SCFAs, acetic, propionic and butyric acids. However, in other study showed that probiotic fermented milk beverage that contains *Lactobacillus casei* at 40 billion bacterial cells/bottle for 4 wk (probiotics, 1 bottle/day), has an intestine-conditioning effect by improving the stool quality (hardened) and increasing the intrinsic bifidobacteria in healthy individuals with soft stool and also the water content of the stools was lower in the probiotic group than in the placebo group [46]. This means the kind and dose of probiotics can affect the quality and moisture contents of stool.

Shown on Table 4, caecal weight of malnourished rat was lower (p<0.05) than normal rats, and the caecal weight of rat treated with yoghurt was also lower than the control rat.

Restriction feeding 50% intake from the normal feeding in this study could decrease the caecal weight, and feeding yoghurt during 7 d after restriction feeding could not increase the caecal weight. This result similar to the study by [47], that feeding *B.longum* without inulin for 1 wk did not increase caecal weight nor did it decrease pH of the caecal contents. This may be the dose of yoghurt was too low and also short period of yoghurt feeding. In the previous study by [44], caecal weight of albino rats fed plain yoghurt, xylooligosaccharide enriched yoghurt and xylooligosaccharide during 21 d was higher than the control rats. The other study showed that administration of fermentable carbohydrates such as inulin had a marked influence on both caecal weight and caecal pH [48]. Thus, it can be interpreted that caecal weight depends on the dose and duration of feeding the microbial or probiotic combinations and also depends on prebiotics or it combination with probiotics.

Table 4. Caecal weight in malnourished and normal rats treated with goat milk yoghurt

	Goat milk yoghurt	Control	Average
Caecal weight (g)			
Malnourished	1.42±0.14	1.67 ±0.33	1.54±0.27[a]
Normal	1.97±0.40	2.32 ±0.36	2.14±0.40[b]
Average	1.70±0.40 [a]	1.99 ±0.47 [b]	1.84±0.45

a, b: the different letter in the same column or the same row indicates significantly different (P<0.05).

Probiotic yoghurt and prebiotic diet leading towards production of SCFA in the large intestine, and these SCFA serve as energy source to increase cell density along with regularized cell proliferation [37,44]. This acid has a trophic effect on the intestinal epithelium, by increasing the blood flow and stimulating mucosal proliferation [40]. Thus, the caecal weight could increase, and this is not occur in malnourished rats.

4. Conclusion

Goat milk yoghurt by using Lacto-B powder given at a low dose (2 ml/100 g body weight) and short time period (7 d) had no effect on the profile of caecal bacteria, caecal physical properties and could not increase of SCFA in malnourished rats. Furthermore, malnourished rats have more number of *Escherichia coli*, so that resulting in lower of caecal butyrate and caecal weight than normal rats.

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