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Original Article

Effects of probiotic on gut microbiota in children with acute diarrhea: a pilot study

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Abstract

Background Acute diarrhea is a common health problem in Indonesia. During acute diarrhea, changes in gut microbiota are marked by decrease beneficial microbes *Bifidobacterium* and *Lactobacillus*, and increased pathogenic bacteria *Enterobacter* and *Clostridium*. Such microbial imbalances are known as dysbiosis. Treatment with probiotics may help repair dysbiosis, quicken healing time, and decrease complications.

Objective To assess for dysbiosis during acute diarrhea, and determine if it can be normalized by probiotic treatment.

Methods This placebo-controlled, unblinded clinical trial was performed in Budhi Asih District Hospital, Jakarta, from January to March 2018. Twenty-four children age 6-24 months with acute diarrhea and 12 healthy children were enrolled. First fecal specimen was collected for all subjects and analyzed using non-culture real time PCR to count the population of *Lactobacillus, Bifidobacterium, Enterobacter, Clostridium*, and all bacteria. Children with diarrhea were assigned to probiotic or placebo treatment for 5 days and the second fecal specimen was analyzed two weeks after the diarrhea subsided.

Results Prior to treatment, significant higher amounts of *Lactobacillus* were observed in children with acute diarrhea than in healthy controls [median (interquartile range/IR): $1.52x10^3$ (1.22x104) *vs.* 6.87x10 (2.41x102), respectively; proportion in percentage (from total bacteria population): 0.044% *vs.* 0.003%, respectively]. However, median (IR) *Clostridium* was significantly higher in healthy controls than in children with acute diarrhea [$2.37x10^2$ ($4.64x10^3$) *vs.* 4.67 ($1.50x10^2$), respectively, with proportion of 0.01% *vs.* 0.0001%, respectively]. Children who received probio tics had significantly higher count of *Bifidobacterium* compared to the placebo group [$1.94x10^4$ ($4.97x10^4$) *vs.* $1.74x10^3$ ($2.08x10^7$), respectively, with proportion of 0.394% *vs.* 0.081%, respectively].

Conclusion This pilot study do not find evidence of dysbiosis in children with acute diarrhea. Group who received probiotic has higher *Bifidobacterium* count compared towards those who received placebo. **[Paediatr Indones. 2020;60:83-90; doi: http://dx.doi.org/10.14238/pi60.2.2020.83-90]**.

Keywords: *acute diarrhea; dysbiosis; gut microbiota; gastroenteritis; probiotic*

Diarrhea is one of the most common problems in Indonesian children, ranking third in children under 5 years' mortality, after neonatal death and pneumonia.[1] Current diarrhea treatments are based on 5 principles, also known as the 5 pillars of diarrheal management, which are: rehydration, adequate nutrition, zinc therapy, antibiotics when indicated, and education.[2] Despite these principles, probiotics are also given for the treatment of diarrhea. However, the evidence is conflicting,[3,4] and Indonesian data is lacking about the benefit of probiotics for acute diarrhea, particularly regarding microbiological changes in the gut.

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Under normal conditions, gut microbiota of children is dominated by Gram-positive anaerobic bacteria from the phyla *Firmicutes* and *Bacteroides*, such as genus *Lactobacillus* and *Bifidobacterium*. Such equilibrium is known as eubiosis.[5] Pathogenic invasion of pathogen to the intestinal mucosa damages the mucosal layer, administers toxins, and alters gut microbiota.[5,6] Gut microbiota changes occur when there is decreased commensal bacteria and increased pathogenic bacteria. This condition is known as dysbiosis.[5,6] For example, dysbiosis could occur when the commensal bacteria mentioned above decline, and Gram-negative bacteria from the *Actinobacteria* phylum (genus *Enterobacter* and *Clostridium*) increase.[6]

Probiotics are commonly used in children with diarrhea, because it can reduce the frequency of loose stools, repair stool consistency, and reduce the number of sick days.[7] On the other hand, the microbiological changes in children with acute diarrhea have not been thoroughly evaluated. There is conflicting evidence about the benefits of probiotics in acute diarrhea, particularly regarding the repair of gut microbiota composition.[8,9] To our knowledge, no study has been done in Indonesian on administration of probiotic and microbiotic changes in acute diarrhea in children. As such, we aimed to assess for dysbiosis during acute diarrhea, and evaluate microbiota alterations after probiotic administration in children.

Methods

Non-randomized, placebo-controlled, unblinded clinical trial was performed in Budhi Asih District Hospital, Jakarta. Subjects were children aged 6-24 months who visited the emergency unit, inpatient, or outpatient clinic with acute diarrhea (defined as loose stool category 5-7 in the Bristol stool chart,[10] frequency of more than 3 times a day, total duration of diarrhea less than 14 days, and no blood in stool),[11] had normal nutritional status [defined as weight for length/height between Z+2 and Z-2 (*World Health Organization 2007* curve)][12] and whose parents provided informed consent.

Children with severe malnutrition (defined as weight for length/height below Z-3 *WHO 2007* curve), persistent or chronic diarrhea, cow's milk allergy, or immune deficiency such as human immunodeficiency virus (HIV) infection, intake of high-dose steroids or chemotherapy, intake of antibiotics or probiotics within the preceding 2 months were excluded from this study. Allocation to the placebo or probiotic group was performed using consecutive sampling until the minimum required sample size was achieved. This pilot study is the first in Indonesia and we use a sample size of 12 per group (12 healthy children and 24 children with diarrhea, further divided into 12 probiotic and 12 placebo).[13]

Stool microbiota analysis was performed for *Bifidobacterium* and *Lactobacillus*, the healthy bacteria, and for *Enterobacter* and *Clostridium*, the potentially pathogenic bacteria. Total bacteria count in feces was measured as total bacteria. Absolute count is presented in copy number DNA/200 mg of feces and proportion (median of a specific bacteria divided by median total bacteria).

For normal subjects, fecal specimens were collected only once. In the diarrhea group, the first fecal specimen from the time of recruitment was referred to as T1, and second fecal specimen (2 weeks after the diarrhea subsided) was referred to as T2. The diarrhea placebo group received 20 mg zinc/day for 10 days, oral rehydration solution (ORS), and education. The probiotic group received *Probiotic Lacto-B®* from *Novell®* Pharmaceutical (content: *Lactobacillus acidophilus sp, Bifidobacterium longum sp* and *Streptococcus thermophilus sp*) twice per day for 5 days, zinc 20 mg/day for 10 days, and ORS. Diarrhea frequency, consistency, and stool characteristics were recorded every day by parents. Side effects were monitored and reported, as well as any severe adverse events (SAE). Participants in this study were not insured. Fecal specimens were delivered promptly within 2 hours stored in packaged box with cold packs to the laboratory (*Molecular Biology Laboratory*, Pediatric Gastrohepatology Division, Universitas Indonesia, Jakarta).

Fecal specimen was subjected to spin column method to isolate bacterial deoxyribonucleic acid (DNA) of *Lactobacillus, Bifidobacterium, Enterobacter, Clostridium* and all bacteria from the feces. Isolated DNA was identified using quantitative real-time polymerase chain reaction (PCR) (in total copy number DNA/200 mg of feces). Final data were presented in absolute count and proportion. Equipment used: Fast 7500 Real-time PCR (*Applied Biosystems*), computer

(*DELL optiplex 960*), mini centrifuge (*Profuge 6K*), centrifuge (*Hettich Zentrifugen*), laminar air flow cabinet (*Streamline*), splash free support base (*Micro-Amp®*), rubber base, micro pipette (*Eppendorf*), vortex (*Heidolph*), microfuge tube rack, digital scale (*AND GF-600*), freezer -20°C (*ARDO*), freezer -80°C (*New Brunswick Scientific*), nanodrop spectrophotometer (*Thermo Scientific*) and spatula.

Clinical parameters of subjects with diarrhea were converted into numerical data and compared. Statistical data analysis was performed using independent samples Kruskal-Wallis test. Changes in microbiota composition between groups were analyzed using unpaired non-parametric test Mann-Whitney or independent-samples Kruskal-Wallis test (if the data distribution was not normal). Paired data were analyzed using non-parametric T-test for normal data distribution, and Wilcoxon signed rank test for non-normal data distribution. Results were deemed significant if $P<0.05$. Statistical analyses were performed using *SPSS version 23.0* software. This study was approved by the Ethics Committee at the Universitas Indonesia Medical School.

Results

During the study period from January to March 2018, 56 patients fulfilled the inclusion criteria. However, 3 patients declined to participate in the study, and

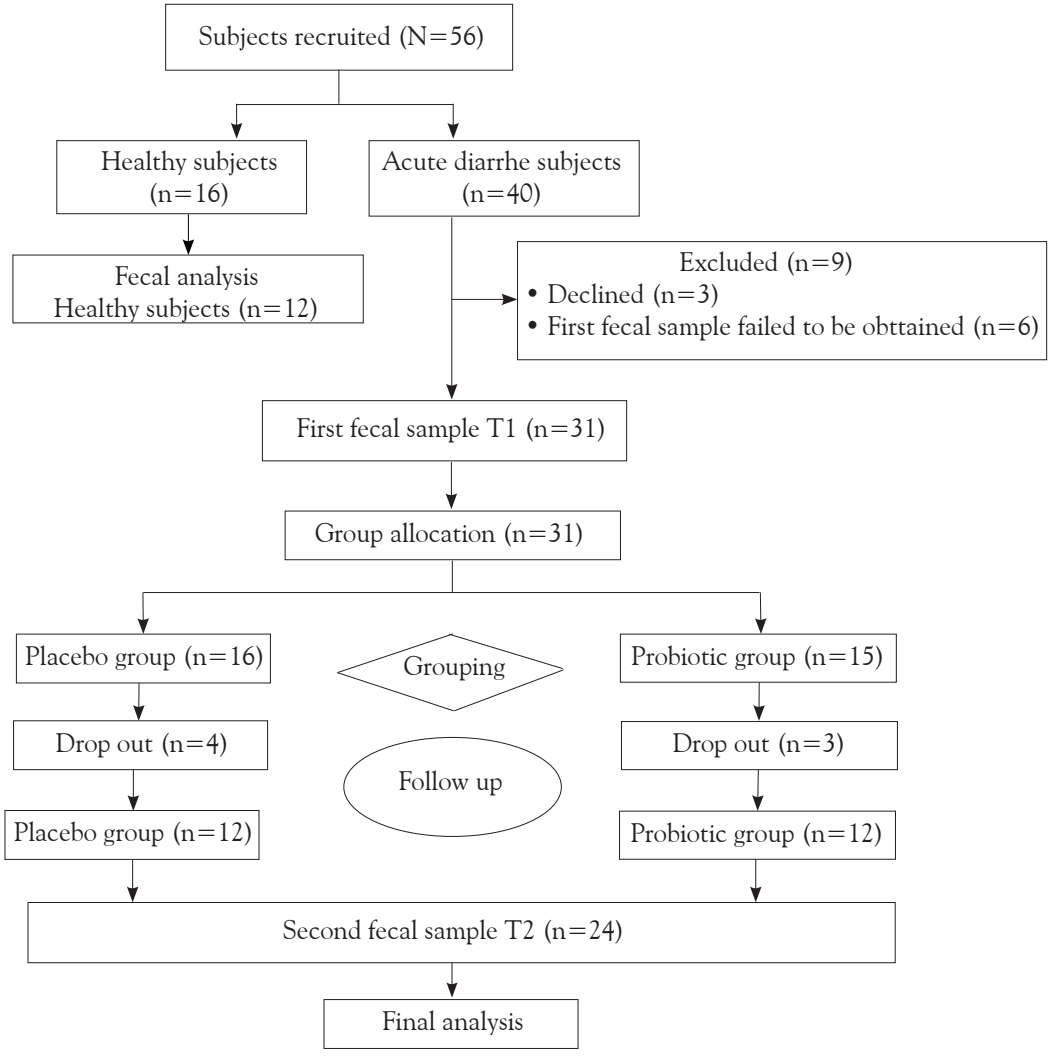

Figure 1. Study flow chart

6 patients were excluded because the initial fecal specimen was not collected. Sixteen healthy children were recruited from the children of hospital healthcare personnels. Of these, 12 children were randomly chosen for the final fecal analysis. Thirty-one patients with acute diarrhea were recruited from the outpatient department (2 patients) and the inpatient ward (29 patients). Of these, 7 patients did not provide the second fecal specimen, and therefore, were excluded from the final analysis. Hence, 24 subjects with acute diarrhea were allocated into two groups, placebo or probiotic, and included in the final analysis. A total of 36 subjects were included in the final analysis. The detailed recruitment is showed in **Figure 1**. Characteristics of subjects by group are shown in **Table 1**.

Clinical characteristics of acute diarrhea patients at the time of recruitment are provided in **Table 2**. There were no significant differences between the probiotic and placebo groups with regards to the characteristics of acute diarrhea, other accompanying symptoms, and intake of breastmilk. All the diarrhea subjects fell into the mild-to-moderate dehydration category.

Hospital length of stay [median (interquartile range)] for acute diarrhea patients in the placebo group was 4 (4) days and 3 (1) days in the probiotic group (P=0.287). Daily follow-up on loose stool frequency (defined as category 5-7 in the Bristol stool chart) was recorded (**Table 3**). After 6 days of treatment, all subjects who received probiotics showed no loose stool, whereas some subjects in the placebo group still had loose stool. (P=0.048).

Microbiota comparison between healthy and acute diarrhea subjects is provided in **Table 4**. Absolute bacteria count is presented in median (interquartile range) and proportion in %

The comparison of microbiotic composition in acute diarrhea subjects before and after intervention is shown in **Table 5**. Higher amounts of Lactobacillus were observed in children with acute diarrhea than in healthy controls [median

Table 2. Clinical characteristics of acute diarrhea at recruitment

Clinical characteristics	Diarrhea	
	Placebo group (n=12)	Probiotic group (n=12)
Median frequency of defecation (IQR)	6 (8)	8 (4)
Start of diarrhea, no. of days before*	1 (2)	2 (2)
Mucus present, n	4	3
Blood present, n	1	0
Fever present, n	8	7
Vomiting present, n	8	5
ORS treatment at home, n	4	8
Received breastmilk, n	5	4
Mild-to-moderate dehydration, n	12	12

* Results are provided in median (interquartile range). There was no significant difference (P<0.05) between characteristics in all groups.

Table 1. Characteristics of subjects

Characteristics	Healthy subjects	Diarrhea	
		Placebo group	Probiotic group
Number of subjects	12	12	12
Gender, n			
Male	8	9	8
Female	4	3	4
Age group, n			
6-12 months	7	4	3
13-18 months	2	1	3
19-24 months	3	7	6
Median age (IQR), months	12 (10)	20.5 (14)	17.5 (12)
Normal nutritional status, n	12	12	12
Median body weight (IQR), kg	9.3 (1.8)	9.2 (4.6)	10 (4.2)
Previoius rotavirus immunization, n	4	4	3

IQR=interquartile range

(IR): $1.52x10^3$ ($1.22x10^4$) *vs.* 6.87x10 ($2.41x10^2$), respectively, proportion: 0.044% *vs.* 0.003%, respectively]. However, median (IR) *Clostridium* was significantly higher in healthy controls than in children with acute diarrhea [$2.37x10^2$ ($4.64x10^3$) *vs.* 4.67 ($1.50x10^2$), respectively, with proportion of 0.01% *vs.* 0.0001%, respectively]. Children who received probiotics had significant higher count of Bifidobacterium compared to the placebo group [$1.94x10^4$ ($4.97x10^4$) *vs.* $1.74x10^3$ ($2.08x10^7$), respectively (P<0.05), with proportion of 0.394% *vs.* 0.081%, respectively (P<0.05)]. Both the placebo and probiotic groups had significantly higher *Clostridium* absolute count and proportion compared to their corresponding T1 specimens (P<0.05 for all).

Figure 2 shows a bar chart to better visualize the proportions of bacteria in healthy controls,

Table 3. Daily follow-up of loose stool frequency

Variables	Frequency of loose stool*						
	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Placebo	5 (7)	4 (5)	3 (3)	1.5 (3.8)	0 (3)	0 (3)	0 (2)
Probiotic	4.5 (4)	3.5 (3)	2.5 (1)	0 (3)	0 (2)	0 (0)	0 (0)
P value	0.294	0.305	0.253	0.251	0.281	0.048#	0.247

* Frequency of loose stool is presented in median (interquartile range).

Statistical analysis was performed using independent-samples Kruskal-Wallis test and P<0.05 was deemed significant.

Table 4. Microbiota composition in the initial fecal specimens from healthy and acute diarrhea subjects

Variables	Healthy subjects (n=12)	Acute diarrhea (n=24) First fecal sample (T1)	P value
Total bacteria	2.33×10^6 (1.05×10^7)	8.35×10^6 (1.77×10^7)	0.008^
Bifidobacterium	2.19×10^3 (3.42×10^4)	1.14×10^4 ($3.55x10^4$)	0.209
Proportion	0.094%	0.110%	0.347
Lactobacillus	6.87 x 10 (2.41×10^2)	1.52×10^3 (1.22×10^4)	0.012^
Proportion	0.003%	0.044%	0.034^
Enterobacter	8.79×10^3 (3.69×10^4)	9.73×10^3 (4.29×10^4)	0.023^
Proportion	0.377%	0.146%	0.388
Clostridium	2.37×10^2 (4.64×10^3)	4.67 (1.5×10^2)	0.041^
Proportion	0.01%	0.0001%	0.019^

Bacterial count is presented in median (interquartile range).

^ Significant (P<0.05) results were obtained using independent-samples Kruskal-Wallis test.

Data distribution was assessed using Shapiro-Wilk test.

Table 5. Microbiota composition in the initial fecal specimens from healthy and acute diarrhea subjects

Variables	Placebo			Probiotic			T2 placebo *vs.* probiotic P value
	T1 (n=12)	Ts (n=12)	P value	T1 (n=12)	T2 (n=12)	P value	
Total bacteria	$1.82x10^7$($4.17x10^7$)	$6.06x10^6$($5.12x10^6$)	0.019^	$7.23x10^6$($5.37x10^6$)	$3.06x10^6$($2.08x10^7$)	0.937	0.433
Bifidobacterium	$1.14x10^4$($1.66x10^4$)	$1.74x10^3$($2.08x10^7$)	0.099	$1.36x10^4$($4.33x10^4$)	$1.94x10^4$($4.97x10^4$)	0.695	0.006^
Proportion	0.110%	0.081%	0.480	0.306%	0.394%	0.308	0.015^
Lactobacillus	$5.37x10^4$($1.15x10^4$)	$3.13x10^2$($4.33x10^3$)	0.099	$5.57x10^2$($2.84x10^4$)	8.79x10 ($3.34x10^3$)	0.117	0.433
Proportion	0.078%	0.127%	0.041^	0.008%	0.025%	0.695	0.433
Enterobacter	$4.01x10^4$($1.50x10^5$)	$2.03x10^4$($2.83x10^4$)	0.136	$5.34x10^3$($8.76x10^3$)	$4.71x10^3$($1.14x10^4$)	0.754	0.084
Proportion	0.235 %	0.370 %	0.530	0.051 %	0.110 %	0.209	0.388
Clostridium	4.04 (4.34x10)	$2.49x10^2$($2.89x10^2$)	0.003^	1.78x10 ($2.36x10^2$)	$1.73x10^3$($4.07x10^3$)	0.005^	0.012^
Proportion	0%	0.003%	0.023^	0.001%	0.034 %	0.008^	0.136

Bacterial count is presented in median (interquartile range).

^ Significant (P<0.05) result was obtained using Wilcoxon signed rank test.

Data distribution was measured using Shapiro-Wilk test.

initial diarrhea fecal specimens (T1), and final fecal specimens after intervention (T2), since this was a pilot study with small sample size.

Discussion

This study investigated 36 children, 12 healthy and 24 with acute diarrhea, from ages 6-24 months in Jakarta. Other probiotic studies in Indonesia recruited 40-90 subjects in Jakarta,[14-15] Semarang,[16] Bali,[17] and Manado,[18] aged 3 months until 14 years. There were no significant differences in subjects' characteristics between the healthy and both treatment groups. Probiotic supplementation in children with acute diarrhea has been associated with shorter hospital stay, improved stool consistency, and overall health.[14-18] We noted that the probiotic group had shorter hospital stay than the placebo group (3 *vs.* 4 days, respectively), but this difference was not significant. After 6 days of treatment, all subjects who received probiotics showed no loose stool, whereas some subjects in the placebo group still had loose stool (P=0.048).

The first objective of this study was to assess acute diarrhea patients for dysbiosis, by comparing microbiota composition in healthy and diarrhea subjects (T1). Dysbiosis can be identified by changes in microbiota composition, namely increased potentially pathogenic bacteria (*Enterobacter* and *Clostridium*), and decreased commensal bacteria (*Lactobacillus* and *Bifidobacterium*).[5,6] The initial fecal specimen (T1) had significantly higher medial total bacteria, higher median *Lactobacillus*, and higher *Lactobacillus* proportion in the diarrhea group than in the healthy group. There were no significant differences between absolute count and proportion of *Bifidobacterium*. However, *Enterobacter* was significantly higher in the diarrhea group than in the healthy group, although the proportion was not significantly different. *Clostridium* counts and proportions were significantly lower in the acute diarrhea group than in the healthy group. This result was contradictory to our current hypothesis of dysbiosis in patients with diarrhea.

Two weeks after the diarrhea was cured, stool specimens were collected (T2) and analyzed for microbiota composition. The probiotic group had significantly higher *Bifidobacterium* count and proportion compared to the placebo group. Plausible explanation for this result is because the diarrhea probiotic group received probiotics which contains *Bifidobacterium longum sp*, a beneficial microbe which promote proliferation of other *Bifidobacterium* species. In addition, both the placebo and probiotic groups had significantly higher *Clostridium* absolute count

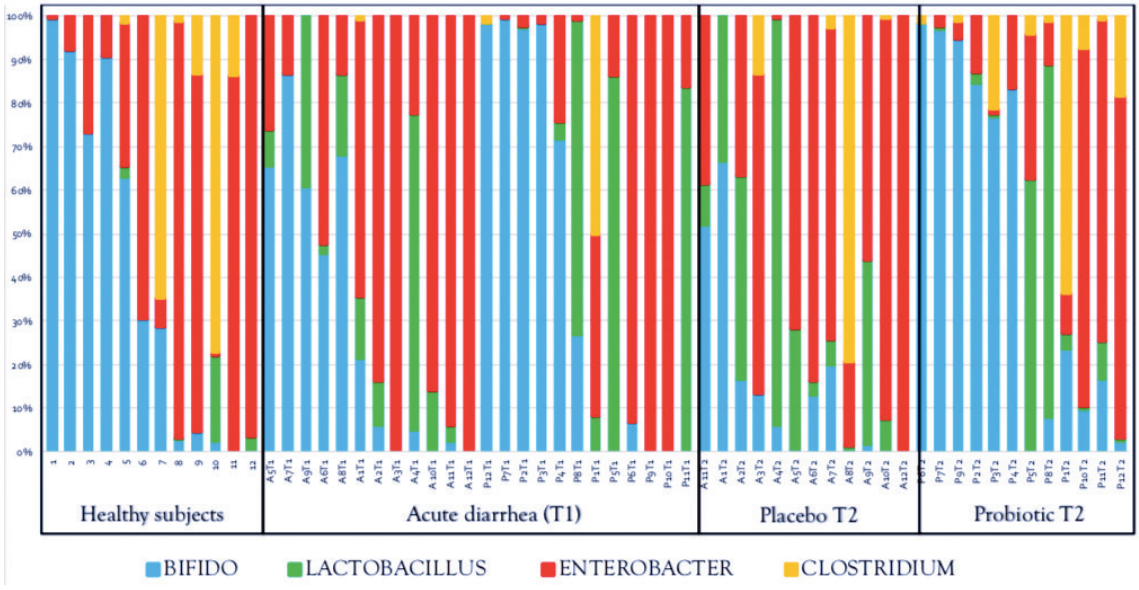

Figure 2. Bar chart of microbiota proportions in healthy subjects and acute diarrhea subjects by group, T1 and T2. This bar chart was based from proportion of each bacteria, totaling to 100%. Each bar represents bacterial composition of a specific subject in the corresponding group.

and proportion compared to their corresponding T1 specimens (**Table 5**). Based from our hypothesis that *Clostridium* rises during acute phase of diarrhea, we do not know how long this surge last, and our follow up of 2 weeks showed that *Clostridium* level is still higher than the first specimen (T1). We concluded that microbial changes after acute diarrhea might persist more than two weeks after the diarrhea ceased. A further study is needed to prove this claim. We could not prove dysbiosis in children with acute diarrhea in this study because we did not find increased potentially pathogenic bacteria (*Enterobacter* and *Clostridium*), and decreased commensal bacteria (*Lactobacillus* and *Bifidobacterium*).

To our knowledge, this is the first study to evaluate stool microbiota of children with diarrhea in Indonesia. However, we can only identify less than 1% proportion of microbiota in the stool. A previous study achieved a higher proportion (around 20%) stool microbiota identification up to the phylum level using a more advanced method known as next generation sequencing, massively parallel or deep sequencing, which was also used for the *Human Genome Project and Human Microbiome Project*.[19] Our findings are not comparable to other studies outside of Indonesia, because of differences in subjects' diets, gut profiles, and lifestyles. Furthermore, most studies had an inadequate sample size, and we could not find a single, good, randomized controlled trial (RCT) to evaluate dysbiosis in children with acute diarrhea.

There were several strengths of this study. This was the first, prospective microbiota study in Indonesian children aged 6-24 months which aimed to assess dysbiosis in acute diarrhea, as well as the effect of probiotics on dysbiosis. Also, follow-up was done 2 weeks after diarrhea ceased, and healthy comparisons were provided. The limitations of our study were the small sample size was (12 subjects for each group), the lack of randomization (consecutive sampling), the unblinded study design, and that only four genuses of gut microbiota were analyzed.

Conflict of Interest

The probiotics used for this pilot study was provided by Novell Pharmaceutical Laboratories, which also provided partial funding for this study.

Acknowledgements

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