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The Effect of Probiotic Biscuit on Faecal Microbiota in Malnourished Children

(Kesan Biskut Probiotik kepada Mikrobiota Najis dalam Kalangan Kanak-kanak Kurang Nutrisi)

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ABSTRACT

A human intervention study on the effect of 90 days supplementation of creamed biscuit with the potential of containing probiotic strain *E. faecium* IS-27526 at a dose of 10^8cfu/day on Bifidobacterium in the faecal microbiota of malnourished children under 5 years was conducted. The study was a Randomized Double Blind Placebo Controlled Trial for children under five years old. There were 5 groups with 6 children each: P0 = control biscuit with control cream; P1 = fish and soy protein isolate biscuit with control cream; P2 = control biscuit with probiotic cream; P3 = fish and soy protein isolate biscuit with probiotic cream (every day) and P4 = fish and soy protein isolate biscuit with probiotic cream (every two days). PCR was used to detect Bifidobacterium and *Enterococcus faecium* in faeces samples. It was concluded after 90 days of supplementation, only 25% of children in the controlled group (P0) harboured Bifidobacterium. In treated groups P1 and P4, the percentage of children positive for Bifidobacterium was approximately 60%, while in groups P2 and P3 the prevalence was 100%. However, the increase of 10% was only demonstrated by P3 as 100% of Bifidobacterium content has been shown in group P2 at the pre-treatment condition. In the controlled group (P0), *E. faecium* could not be identified either pre or post-intervention, in contrast to the treated groups which showed an increase in some treatments with the highest of 66.7% (group P3). No adverse effect was observed during 90 days of supplementation to children. In conclusion, supplementation of fish and soy protein isolate biscuit and/or probiotic cream every day at a dose of 10^8cfu/day is considered safe for children under five years old and even increased the number of samples with the presence of bifidobacteria and *E. faecium* in the fecal microbiota.

Keywords: Bifidobacterium; *Enterococcus faecium;* malnourished; microbiota; probiotic cream

ABSTRAK

Kajian campur tangan manusia terhadap kesan suplemen 90 hari biskut berkrim dengan potensi mengandungi strain probiotik *E. faecium* IS-27526 pada dos 10^8cfu/hari pada Bifidobakteria dalam mikrobiota najis kanak-kanak kurang zat makanan di bawah 5 tahun telah dijalankan. Kajian ini ialah Percubaan Rawak Terkawal Dwibuta Plasebo untuk kanak-kanak di bawah umur lima tahun. Terdapat 5 kumpulan dengan 6 kanak-kanak setiap satu: P0 = biskut kawalan dengan krim kawalan; P1 = biskut pencilan protein ikan dan soya dengan krim kawalan; P2 = biskut kawalan dengan krim probiotik; P3 = biskut pencilan protein ikan dan soya dengan krim probiotik (setiap hari) dan P4 = biskut pencilan protein ikan dan soya dengan krim probiotik (setiap dua hari). PCR digunakan untuk mengesan Bifidobakteria dan *Enterococcus faecium* dalam sampel najis. Kesimpulannya, selepas 90 hari suplemen, hanya 25% kanak-kanak dalam kumpulan terkawal (P0) memendam Bifidobacterium. Dalam kumpulan rawatan P1 dan P4, peratusan kanak-kanak positif Bifidobakteria adalah kira-kira 60% manakala dalam kumpulan P2 dan P3 prevalens adalah 100%. Walau bagaimanapun, peningkatan sebanyak 10% hanya ditunjukkan oleh P3 kerana 100% kandungan Bifidobakteria telah ditunjukkan dalam kumpulan P2 pada keadaan pra-rawatan. Dalam kumpulan kawalan (P0), *E. faecium* tidak dapat dikenal pasti sama ada sebelum atau selepas intervensi, berbeza dengan kumpulan yang dirawat yang menunjukkan peningkatan dalam beberapa rawatan dengan tertinggi sebanyak 66.7% (kumpulan P3). Tiada kesan sampingan diperhatikan semasa 90 hari pemberian suplemen kepada kanak-kanak. Kesimpulannya, penambahan biskut pencilan protein ikan dan soya dan/atau krim probiotik setiap hari pada dos 10^8cfu/hari dianggap selamat untuk kanak-kanak di bawah umur lima tahun malah menambah bilangan sampel dengan kehadiran bifidobakteria dan *E. faecium* dalam mikrobiota najis.

Kata kunci: Bifidobakteria; *Enterococcus faecium;* krim probiotik; kurang zat; mikrobiota

Introduction

Protein-energy deficiency remains a public health problem in Indonesia, as reflected by a high prevalence of underweight, which is mainly shown in poverty pockets. The national prevalence of underweight among children under five years old was 19.6% in 2013 and it decreased to 17.7% in 2018, but it is higher than the target set by Health Ministry: 15.5% (Indonesia Ministry of Health 2018, 2013). Underweight is defined by a weight-for-age z-score (WAZ) less than -2. Dietary deficiency, whether in macronutrients or micronutrients combined with the risk of a high infection are the main underlying causes of child malnutrition, including underweight. Indonesia's Basic Health Research in 2018 reported that a higher prevalence of acute respiratory infection (12.8%) and diarrhoea (12.3%) were found among children under five years old, with diarrhoea as one of the main causes of mortality (Indonesia Ministry of Health 2013).

Malnutrition is the common cause of immune deficiency in developing countries. Protein deficiency leads to immunity impairment which is characterized by a weakened systemic and mucosa immunity (Khan et al. 2017). Children under five years old are more susceptible to infections compared to adults due to their immature immune systems (Rana et al. 2019).

Several traditional fermented foods which might contain probiotics are widely spread in Indonesia, with one of them is *dadih*. *Dadih* not only contains potential probiotics but is also high in protein (around 38%) which contains almost all essential amino acids required for growth and is beneficial for human health. *Dadih* also contains vitamin B-complex and vitamin K (Arnold, Rajagukguk & Gramza-Michalowska 2021; Harnavi et al. 2020; Harnentis et al. 2019; Wirawati et al. 2017). The health benefits of probiotic-containing food products are not only produced by the contents of the nutrients, but also beneficial effects of probiotics on the gastrointestinal environment and immune system to maintain or improve health, or to reduce the risk of exposure to diseases (Maldonado et al. 2019). The microbiota is essential to human health (Chung et al. 2018; Nkamga et al. 2017). Microbiota in the gastrointestinal tract of healthy individuals is different from those in unhealthy ones. The composition of microbiota in the faeces might indicate a person's health condition (Dieterich et al. 2018).

Fermentation process of *dadih* includes Gram positive bacteria organisms such as *Lactobacillus plantarum, L. brevis, Streptococcus agalactiae, S. uberis, Bacillus cereus*, and Gram negative bacteria microbes such as *Escherichia coli* and *Klebsiella* sp. Lactic acid organisms such as *L. plantarum* included in maturation of *dadih* are among the probiotic bacteria. The main ingredient in making *dadih* is buffalo drain matured in a bamboo tube, secured with plastic or banana leaves without any starter. Lactic acid organism ferment mature buffalo milk in bamboo tubes (Roza et al. 2022). Clumping happens in the *dadih* due to the nearness of organisms starting from bamboo and banana leaves so that it produces a form that's wrapped and yellowish white and features a characteristic smell (Roza et al. 2022).

Protein Energy Malnutrition (PEM) and infections among children under five years old are serious problems which require urgent treatment for they might cause a serious impact on the quality of human resources in the future. Therefore, an intervention in the form of providing nutritious supplementary foods as well as strengthening the immune system would be essential to prevent children under five years old of underweight and various disease problems.

Several types of food that nutritionally have good protein content is such as fish and soy. According to research conducted by Adeyeye et al. (2017) showed that there was an increase in the protein quality of cookies by adding ISP. In addition, study conducted by Diah Ikasari, Ema Hastarini and Theresia Dwi Suryaningrum (2020) on the addition of fish protein concentrate into cookies showed an increase in the value and quality of cookies, especially in terms of protein content and texture. Functional biscuits enriched with fish and soy protein isolate with a cream containing potential probiotic *E. faecium* IS-27526 at a dose of 10^8cfu/day could be a beneficial intervention. *E. faecium* IS-27526 is a bacteria isolated from traditional *dadih* fermented milk (Horison & Surono 2020). Therefore, it is essential to study the efficacy of these functional biscuits in improving the profile of 'good bacteria' in underweight children under five years old. The biscuits might improve the children's energy and protein intake. Some of the benefits that lead to increased use of prebiotics, probiotics and dietary modifications are to modulate the gut microbiota in improving nutrition and health (Umu, Rudi & Diep 2017). The role of the children's gut microbiota in growth depends on the microbial composition obtained (Robertson et al. 2019).

Functional properties of the potential probiotic in the biscuits might also give benefit to the integrity of intestinal mucosa and improve the children's immunity, hence might lower their susceptibility to disease attacks.

Research conducted by Agustina et al. (2013) showed the role of probiotic foods on weight gain in children with nutritional problems. Likewise, Onubi et al. (2015) in their research explained that there was a positive relationship in children with nutritional problems when probiotic food interventions were given. Siti Helmyati et al. (2021) in their study also mentions the role of probiotics that can objectively increase growth in children. In addition, there are not many studies that explain the effect of probiotic intake with changes in children's height in order to reduce stunting problems, where this problem cannot go down in just a short time, but in a short time.

This paper describes the effect of 90 days supplementation of functional fish and soy protein isolate biscuits with a cream containing probiotic *E. faecium* IS-27526 at a dose of 10^8cfu/day on the faecal microbiota of malnourished children.

Materials and Methods

SUBJECTS

Subjects were underweight under-five children (WAZ <-2) aged 2-5 years old, from 3 different areas in sub-district Sukabumi, West Java, Indonesia: Kadudampit (representing high-land area), Warungkiara (low-land area) and Cikakak (coastal area). Subjects were selected from children aged 2-5 years old who met the inclusion and exclusion criteria. Children aged 2-5 years old were less than -2 on their WAZ (Regulation of Indonesian Minister of Health 2, 2020). The sample size was calculated using the sample size formula according to Lwanga and Lemeshow (1991) with a 95% confidence interval and 7.67% margin of error, and an estimated 4.9% proportion of underweight children under five from 2,001 children under five in Sukabumi (Sukabumi Department of Health 2012). Furthermore, with a design effect of 1.00 and response rate of 90%, the sample size required was 30 childrens. Other criteria consisted of anthropometric assessment, clinical check, and interview about the inclusion and exclusion criteria. Subjects who were excluded from the criteria: 1) congenital defects; 2) consumption of antibiotics and/or laxative substances (within 4 weeks before the study); 3) acquired similar supplementary foods from other studies; 4) disapproval of the informed consent, and 5) participation in other studies. After the selection of 30 children per area, the random allocation was applied to allocate 6 subjects per treated group.

PREPARATION OF FISH AND SOY FUNCTIONAL BISCUIT

The composition of both control and functional biscuits was: chicken eggs, sugar, butter, and milk (Adi 2010; Kusharto et al. 2009). Meanwhile, for functional biscuits, they are substituted with *clarias* sp. fish flour (meat and bone flour); and soy protein isolate (Table 1). Organoleptic test and statistical analysis using paired t-test showed that no significant difference ($p<0.05$) was present between functional biscuits and control biscuits in term of acceptance (data not shown). Based on the storage test, the biscuits can be preserved for up to 6 months. According to *in vitro* enzymatic methods, the digestibility of the protein was 89.34%, which was considered moderate, since the value was similar to the digestibility rate of nuts, legumes and beans.

TABLE 1. Composition of functional biscuits and control biscuits

Nutrient content	Control biscuits	Functional biscuits with fish and soy isolate
Fat %	23.20	23.78
Protein %	9.07	23.08
Carbohydrate %	60.92	46.76
Energy (kcal/100 g)	488.80	489.40

COMPOSITION OF THE PROBIOTIC CREAM

The compositions of cream containing the potential probiotic *E. faecium* IS-27526 are butter (*unsalted*), margarine, sugar, milk, and *E. faecium* IS-27526 (Table 2). *E. faecium* IS-27526 was microencapsulated by using *Fluid Bed Drier* (FBD) method to retain the viability of the probiotics from extreme environments: baking process, digestive tract and storage conditions (Adi 2010). The encapsulation process was done to retain the quality and the nutrition of the biscuits.

THE MICROENCAPSULATION PROCESS OF *E. faecium* IS-27526

E. faecium IS-27526 culture (2.5 g) and filler Avicel pH 101 (89.5 g) were first layer material Na-alginate (1.5 g in 100 mL) solidified with $CaCl_2$ 0.1M (1.1 g in 100 mL) and skim milk as the prebiotic substance (9 g). The encapsulation process was done by using the air suspension method. The encapsulated bacteria were evaluated for viability using a plating test for viable lactic acid bacteria and showed 7.4×10^{10} cfu/day. As the daily intake and the minimum dose effective for therapy of the probiotic is 10^9 cfu/day (Zucko et al. 2020), 1.4 g microencapsulates/kg cream was used to give a potential health benefit (10^8cfu/day).

TABLE 2. Composition of probiotics cream and control cream per 100 g

Composition	Control cream	Probiotic cream
Probiotic	None	*Enterococcus faecium* IS-27526 (0.14 g)
Colour	White-yellow	White-yellow
Energy (Kcal/100 g)	435.5	435.5
Fat %	2.33	2.33
Protein %	0.65	0.65
Carbohydrate %	91.21	91.21

STUDY DESIGN AND INTERVENTION

The study was performed using a Randomized Double Blind Placebo Controlled Trial. There were five groups namely: P0 = control biscuit with control cream; P1 = fish and soy protein isolate biscuit with control cream; P2 = control biscuit with probiotic cream; P3 = fish and soy protein isolate biscuit with probiotic cream (fed every day) and P4 = fish and soy protein isolate biscuit with probiotic cream (fed every two days). Each group consists of 6 children. At the end of the intervention, 6 children were drop out, therefore the final number of subjects was 24 with 3-6 children per group. However, only 24 subjects can be analysed for the faecal PCR. In the intervention study, supplementation of functional biscuits made from fish and soy protein isolate combined with cream containing *E. faecium* IS-27526 at a dose of 10^8cfu/day was given for 90 days to underweight under-five children in Sukabumi, West Java.

Biscuit packages were given to toddlers with a recommended consumption of 1 serving consists of biscuits (50 grams) and 15 grams of cream (per pack of 4 pieces) per day. In fulfilling the 90-days intervention period, each formula was made in three batches, which distributed in three stages or once a month during visitation. Visitation activities were not only for distributing biscuits but also for monitoring the adherence of each toddler. The distribution of biscuits was carried out directly from the researcher to toddlers to prevent the possibility of sharing among the subjects, in addition, collaboration with local *posyandu* cadres was carried out

to assist in monitoring and reminding the consumption of biscuits in order to maintain consumption compliance through recording using a monitoring card.

DETECTION OF THE BIFIDOBACTERIUM AND *Enterococcus faecium*

Detection of bacteria through the feces of toddlers, where mothers/caregivers of toddlers were asked to take samples of feces that were stored in feces containers/pots and collected directly from researchers. Feces collection was carried out twice, namely pre and post intervention. Toddler defecation time is expected in the morning or following the toddler's natural time. Furthermore, the researchers stored all the feces samples in a special freezer to maintain the fecal content. The analysis of the faeces using the PCR method was done in a microbiology laboratory in Balivet, Cimanggu, Bogor. Amounts of beneficial bacteria (bifidobacteria and *Enterococcus faecium*) during the intervention were determined from fecal-derived DNA samples using quantitative PCR (qPCR) (Jeong et al. 2017; Kim et al. 2017a, 2017b, 2015b). To amplify the Bifidobacterium and *E. faecium*, the primers *g-Bifid* and EM1A-EM1B were used respectively (Matsuki et al. 2004). Extraction of the faeces was done using the *QIAmp Stool Mini Kit* (Qiagen, Jerman).

STATISTICAL ANALYSIS

Data analysis to evaluate the efficacy (influence) of functional biscuits supplementation on the profile of microbiota in children within each group (before-after) was performed by using the binomial test, while analysis of the difference in the parameters (adherence to the consumption of biscuits) within groups (before and after) was done by using Paired sample T-test. One-way ANOVA test was performed to analyse the difference in parameters (adherence to the consumption of biscuits) among groups. Statistical analysis was performed by using SPSS 15.0 for windows. A p-value < 0.05 was considered to be significant.

ETHICS STATEMENT

Ethical approval of this study was obtained from the National Institute of Health Research and Development, Indonesian Ministry of Health, code LB.03.04/KE/1008. Written informed consent approval was obtained from caregivers of all subjects.

RESULTS AND DISCUSSION

ACCEPTANCE AND ADHERENCE TO CONSUMPTION OF THE BISCUITS

The consumption of the biscuits during intervention study was obtained from the records of the monitoring card, with the results as shown in Table 3. As described in the Table, no significant difference was found in the average biscuit consumption among treated groups ($p>0.05$).

TABLE 3. Average daily consumption of biscuits during the intervention

Average consumption	Treated groups				
	P0 (n=4)	P1 (n=5)	P2 (n=3)	P3 (n=6)	P4 (n=6)
Piece					
Per day	3.34 ± 0.81	3.07 ± 1.14	3.27 ± 0.86	3.61 ± 0.54	3.29 ± 0.69
Per 90 days	300.3 ± 72.6	276.0 ±102.8	294.1± 77.4	325.3 ± 48.4	296.5 ± 61.7

There was a slightly lower trend in the adherence to consuming the biscuits from the 1st to 3rd month in all intervention groups (Figure 1). Paired t-test showed that this was not significant ($p>0.05$). ANOVA test also indicated that the adherence to consumption of the biscuits in the first, second, and third month were not significantly different ($p>0.05$).

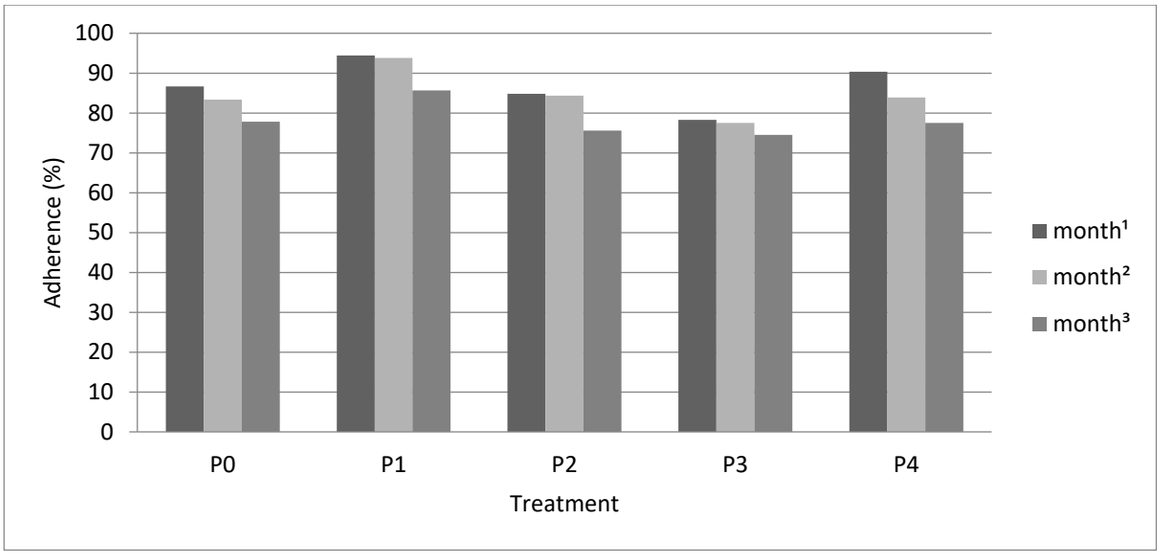

FIGURE 1. Adherence to consumption of functional biscuits in the first to third month showed that there was a tendency of slightly lower adherence in all groups of intervention

The relatively higher acceptance of adherence to consumption of P3 functional biscuits from the beginning to the end of intervention probably was not only influenced by the sensory characteristics of the biscuits, portion size, and familiarity of the children with the biscuits, but also due to the benefits after the consumption. Food taste is fundamental, and food culture is an essential factor in the process of acceptance of new products (Siegrist & Hartmann 2020). New products will be accepted more easily if they suit the concepts of what the consumers are already familiar with, and technically can be applied in the targeted area. The adherence to consumption of the functional biscuits remained high and relatively unchanged during the three months of intervention. The highest adherence (100%) was mainly found among the under-five years old children whose caregivers stated that the children liked the biscuits, so they obtained the health and nutrition benefits after consumption.

EFFECT OF PREVALENCE ON BIFIDOBACTERIA

The gut microbiota is an important organ intimately related to metabolic health and is also crucial for determining the efficacy of therapeutic diets (Wang et al. 2019). The effect of supplementation during the study period on the prevalence of bifidobacteria was determined from faecal analysis, where the presence of Gram-positive bacteria in the feces indicated that these bacteria also live in the intestines (regardless of the number) which then plays a role in improving digestibility and health. As shown in Table 4, only 25% of the samples identified as positive with bifidobacteria. Moreover, in the treated groups P1 and P4, the percentage of samples positive with bifidobacteria was 60%, and the percentage in the groups P2 and P3 was 100%. For the treated group P2, the level of bifidobacteria after and before the treatments remained constant at 100%. In contrast, the treated group P3 was able to increase bifidobacteria content from 84% to 100% by the end of the intervention. Despite this increment, the binomial test showed no significant difference before and after the intervention.

Although faecal samples were only measured in a subpopulation of the participating children, supplementation of functional biscuits containing probiotic cream, both administered every day or once every other day, may increase bifidobacteria in the digestive tract. Bifidobacterium is part of microbiota in healthy individuals and has an important role in fighting hazardous bacteria and other microorganisms, stimulating the immune system, and supporting the digestion process and absorption of nutrients (Lin et al. 2022), so it does help modulate the adaptive immune response in mice (Mahooti et al. 2019). It was striking to notice that some children did not have detectable

TABLE 4. Distribution of subjects based on PCR of bifidobacterial

Results of PCR test	Treated groups									
	P0		P1		P2		P3		P4	
	n	%	n	%	n	%	n	%	n	%
Start of intervention										
- Negative	4	100	1	20	0	0	1	16.0	3	50.0
- Positive	0	0	4	80	3	100	5	84.0	3	50.0
Total	4	100	5	100	3	100	6	100	6	100
End of intervention										
- Negative	3	75.0	2	40.0	0	0	0	0	2	33.3
- Positive	1	25.0	3	60.0	3	100	6	100	4	66.7
Total	4	100	5	100	3	100	6	100	6	100

amounts of bifidobacteria (Table 4). The increment of the microbiota such as bifidobacteria population is influenced by several factors such as diet, antibiotics, and stress (Rinninella et al. 2019). At first, microbes begin to colonize the newborn while in the uterus, then Gram-positive probiotics (in mothers vaginal and fecal) including bifidobacterium have access to interact with the baby (Dudley 2020; Freitas & Hill 2018). In the growth period, bifidobacteria population in the digestive tract can be increased by providing good nutrition, stabilizing the environment (Lu & Walker 2001), and adding more proportion of probiotics (Piiraine et al. 2008; Surono 2006), to increase the chance of these beneficial bacteria to reproduce, compete with pathogenic bacteria, and reduce the ability of pathogen bacteria to dominate the digestive tract. Probiotics are also likely to enhance the barrier function of naive epithelial cells so that they will not get exposed to any pathogen (EFSA Panel et al. 2018), probiotic bacteria also modulating intestinal microbiota to stimulate immune cells such as Th1, Th2, Th17, Treg cells and B cells (Dargahi et al. 2019). The consumption of probiotic yogurt by malnourished children might accelerate the restoration of gut function (Cano, Aguero & Perdigon 2002). As well as any other publication results, addition of microencapsulated *E. faecium* IS-27526 in pasta cream form has been proven to increase body weight and fecal lactic acid bacteria in mice (Harianti 2009). Hence, we reported here that supplementation of the diet with biscuits enriched with *E. faecium* IS 27526 increased the prevalence of bifidobacteria.

EFFECT OF PREVALENCE ON *Enterococcus faecium*

Besides bifidobacteria, the effect of supplementation was also observed on *Enterococcus faecium* (Table 5). As presented in the Table, *Enterococcus faecium* was not identified in the treated group (P0), neither in pre and post-intervention. Whereas among the treated groups (P1, P2, P3, P4), *Enterococcus faecium* was not detected at the start of the intervention. However, it then appeared in some samples after the treatment, in which the increment percentage of groups P3 and P4 were, respectively, 66.7% and 100%.

TABLE 5. Distribution of subjects based on PCR of *Enterococcus faecium*

Results of PCR test	Treated groups									
	P0		P1		P2		P3		P4	
	n	%	n	%	n	%	n	%	n	%
Start of intervention										
- Negative	4	100	5	100	3	100	6	100	6	100
- Positive	0	0	0	0	0	0	0	0	0	0
Total	4	100	5	100	3	100	6	100	6	100
End of intervention										
- Negative	4	100	3	60.0	2	66.7	2	33.3	0	0
- Positive	0	0	2	40.0	1	33.3	4	66.7	6	100*
Total	4	100	5	100	3	100	6	100	6	100

*Binomial test (p=0.031, p<0.05)

The presence of *E. faecium* in the samples' faeces resulted from functional biscuit supplementation either with or without *E. faecium* IS 27526. Only the controlled biscuits did not lead to the detection of *E. faecium.* This indicated that the presence of *E. faecium* in the stool as a reflection of the undernourished children's digestive tract was the effect of giving functional biscuits with probiotic *E. faecium* IS 27526 both daily and between one day. This study specifically analyzed the presence of *E. faecium* in feces without identifying the amount, because its presence in feces reflected that this bacterium had previously been present in the intestine. Components in the functional biscuits, soy protein isolate, and *Clarias* sp. fish flour were proven before to give a prebiotic effect that can support the viability of probiotic *E. faecium* IS-27526. This finding is in line with other research (Harianti 2009), which showed that functional biscuits were not only able to increase the body weight of mice but also able to increase lactic acid bacteria (LAB) content in the faeces. Prebiotic and probiotic known to have synbiotic term that implies synergy to give benefit to the host by improving the survival and existence of beneficial microorganism (Cencic & Chingwaru 2010; Skalkam et al. 2016; Surono 2016).

DETECTION OF ADVERSE EFFECT OF BISCUITS IN THE CHILDREN

The safety aspects of foods are an essential requirement that should be fulfilled. The safety parameter measured in this study was based on the health-related complaints after consuming the biscuits (nausea, diarrhea). As shown in Table 6, according to the interview data of the child's caregivers, no side effect and complaint after 90 days of consuming the biscuits were reported by 96.6% of the caregivers. The rest of 3.3% of the caregivers stated the evident of slight diarrhea (more like watery defecation) at the beginning of consuming the biscuits but no differences among the treated groups.

TABLE 6. Detection of the adverse effect during intervention

Adverse effect	P0		P1		P2		P3		P4		Jumlah	
	n	%	n	%	n	%	n	%	N	%	N	%
Never	18	100	15	100	15	93.8	17	94.4	16	100	81	96.6
Ever (diarrhea), once at the beginning	0	0	0	0	0	0	1	5.6	0	0	1	1.2
Ever, sometimes nauseous	0	0	0	0	1	6.2	0	0	0	0	1	1.2
Total	18	100	15	100	16	100	18	100	16	100	83	100

Conclusion

In conclusion, supplementation of fish and soy protein isolate biscuits combined with a probiotic cream every day or once every two days at a dose of 0.5 - 1.0*10^8cfu/day was considered safe for undernourished children under 5 years old based on the detection of the adversed effect and modulated the microbiota as demonstrated by increasing the number of samples with the presence of bifidobacteria and *E. faecium* in the feces in several categories (formulas).

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