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CONTENT

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EDITORIAL INTRODUCTION SPECIAL ISSUE "PROBIOTIC, PREBIOTIC, AND MICRONUTRIENTS IN IMMUNE SYSTEM"

Alpha Fardah Athiyyah[1*], Liviu Giurgiulescu[2]

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Significant progress in the field of food sciences and technology has been observed over the years. It applies the used of probiotics not only in basic research but also in clinical studies. In this conference are divided the research interest in several areas about probiotic, prebiotic and synbiotic, effect of of micronutrient in immune system and clinical studies.The first group on this papers is microbiology in clinical studies, there are 7 papers used microbiology like probiotic, prebiotic and synbioticc to prevent disease especially on children and learn about the pathway of immunity to help the healing process. One interesting *in vivo* experimental studies paper is presented how supernatans cell free of antimicobial *Lactobacillus achidophilus* in inhibiting *Salmonella Typhi* growth. In another paper is about probiotics effect on structure of the intestinal examined by scanning electron microscope (SEM),on the other papers are probiotic effecting pathway of immunology respons in mouse model with different organ target examination among of them is blood, liver, ileum. Beside on *in vivo* and immunological systems studies we are conduct probiotic studies in children, on the other paper we had incidance rate of acute respiratory infection between healthy children group who given formula milk contain probiotic, prebiotic, and synbiotic. And next paper shown of effect heat killed probiotic complex on acute diarrhoea with clinical trial but there were no significant result of until the last day of observation.Secondly of interest studies is discuss about nutrients administration effect child development and proven theraphy on animal model, in one paper is experimental studied proven if there are significant result on animal model given suplement zinc with healing proses intestinal mucous and another paper shown that zinc administration can increased immune system on respiratory tract. The other paper analyzing immunoglobulin levels after exposuring cow's milk protein as allergen. Next paper can proven how extract pomegranate affect the initial process of fibrogenesis. Furthemore the last group related to the other condition concern about how dietary effect baby, children and adolescent. One of paper present how variance of dietary intake can affect the inflamatory biomarkers in adolescents with obesity. Next paper comparing obesity within carotid intima media thickness(CMIT). On next paper comparing tradional spoon feeding with led weaning to risk iron defeciency anemia. Another paper is about how early parenteral nutrition effect on gaining weight pattern from premature infants with low birth weight. And the last one paper is analyzed baby who given human milk and human milk fotified effect gain weight velocity.

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THE INHIBITION OF *SALMONELLA TYPHI* GROWTH BY THE CELL FREE SUPERNATANS OF *LACTOBACILLUS ACIDOPHILUS* CULTURES

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ABSTRACT

Fever caused by *Salmonella enterica serovar typhi* is presently a major public health concern in developing countries. The extensive use of recommended antibiotics such as ciprofloxacin has resulted in the reduction of its efficacy in the elimination of *Salmonella typhi*. *Lactobacillus acidophilus* secretes antimicrobial compounds against pathogenic bacteria. The aim of this study was to evaluate the antibacterial activity of cell-free supernatants (CFS) of *Lactobacillus acidophilus* against the growth of *Salmonella typhi.* The efficacy of organic acids and other compounds from *L. acidophilus* CFS was tested against *Salmonella typhi* using macro dilution test and time-kill study. The results were reported descriptively. Four-fold dilutions of organic acids and two-fold dilutions of other compounds from *L. acidophilus* CFS were found to inhibit the growth of *S. typhi*. It was observed that *L. acidophilus* CFS can completely inhibit the growth of *S. typhi*. The CFS of *Lactobacillus acidophilus* especially due to its organic acid content showed an inhibitory effect against *S. typhi* growth.

1.Introduction

Typhoid fever is an ongoing health concern over the world, especially in developing countries (Zaki, 2011). Typhoid fever is an acute infectious disease caused by *Salmonella enterica* serovar *typhi* (Zaki, 2011). *S. typhi* is a gram-negative, rod-shaped, facultatively anaerobic, and motile bacterium that is pathogenic to humans. Transmission of these bacteria occurs through the ingestion of food and water contaminated with infected human waste or via carriers of the disease. An estimated 17.8 million (6.9–48.4 million) new cases of typhoid fever have been reported per year. The Central, South and Southeast Asia have a high incidence of typhoid (Garcia-Fernandez *et al*., 2014; Garcia *et al*., 2015; Warren, *et al*., 2017). The endemic nature of typhoid in developing countries is closely associated with poor hygiene and sanitation (Eng, *et al.*, 2015). Serious complications that could occur in typhoid fever are intestinal bleeding, perforation, and even death (Eng, *et al.,* 2015).

The first line of antibiotics used to eliminate *S. typhi* includes chloramphenicol, ampicillin, and cotrimoxazole. However, some strains of *S. typhi* are presently resistant to these antibiotics (Zaki, 2011). Ciprofloxacin is now used to treat infections caused by multidrug resistant strains. However, various studies in Peru, Italy, Africa, and Southeast Asia have also reported that the antimicrobial activity of ciprofloxacin in

inhibition of multidrug-resistant strains has also declined, probably due to its extensive use (Garcia-Fernandez *et al*., 2014; Garcia *et al*., 2015; Schellack, 2018). In Cambodia, 90% of the *S. typhi* isolates have shown a decrease in susceptibility to ciprofloxacin and azithromycin (Vlieghe *et al*.,2012). This has resulted in an urgent situation requiring the development of novel therapeutic strategies to inhibit the occurrence of antibiotic resistance in *S. typhi*

Probiotics are microorganisms that have potential health benefits to human, when ingested in an appropriate quantity (Bermudez-Brito *et al*.,2012). *Lactobacillus* spp. and *Bifidobacterium* spp. have been extensively studied, as they are the predominant groups of the human gastrointestinal microflora (Bermudez-Brito *et al*., 2012). The research on probiotics under *in vitro* and *in vivo* conditions has concluded that probiotics can improve the response to inflammation due to microbial infection, through various mechanism of action (Bermudez-Brito *et al*., 2012). Probiotics have been known to enhance the first line of defense by limiting the attachment and proliferation of microbial pathogens (Bermudez-Brito *et al*., 2012). Certain probiotics produce antimicrobial compounds and modulate the immune system, though the exact mechanism of action has not yet been fully understood (Bermudez-Brito *et al*., 2012). Lactic acid bacteria form a group of probiotics that are able to produce lactic acid. *Lactobacillus acidophilus* is classified as a lactic acid bacterium. *L. acidophilus* is a gram-positive, catalase-negative rod that produces lactic acid through carbohydrate fermentation (Bull *et al*., 2013). *L. acidophilus* can be isolated as a normal flora from various parts of the human body, such as the buccal cavity, intestinal tract, colon and vagina. *L. acidophilus* has potential benefits for human health and exerts therapeutic effects. It is known that *L. acidophilus* produces antibacterial substances, maintains barrier function, ameliorates inflammation due to infection by *Helicobacter* sp*.,* and offers immunomodulation (Bull *et al*., 2013; Coconnier *et al*., 1998; Sengupta *et al*., 2013). The aim of this study was to evaluate the inhibitory potential of cell free supernatants of *L. acidophilus* on the growth of *S. typhi.*

2. Materials and methods

2.1. Microorganisms

The strain of *Salmonella typhi* used in this study was isolated from a hospitalized patient that was diagnosed with typhoid fever at the Immanuel Hospital, Bandung, Indonesia. The isolate was identified using standard bacteriological methods. The purified strain was grown on Trypticase Soya Agar (Oxoid) and sub cultured in Trypticase Soya Broth (Oxoid) at 37 ^{o}C for 24 h. Before the use in the experiment, the culture was inoculated in Tryptic Soya Agar at 37 ^{o}C for 24 hours. Bacterial density was measured by suspending 5–10 colonies in 0.9% NaCl (Sigma-Aldrich-Merck) to the equivalent of the 0.5 McFarland standard.

Lactobacillus acidophilus ATCC 4356 was purchased from Microbiologics^{TM} Fisher scientific. The strain was identified using gram staining and biochemical tests (Branch,2015). The culture was stored in de man Rogosa Sharpe slant agar (Oxoid) at 4–8^{o}C and subcultured in MRS broth at 37 °C for 24 h before the use in the experiment.

2.2. Preparation of Organic Acid Cell free supernatant (OACFS)

L. acidophilus ATCC 4356 was anaerobically cultured in de man Rogosa Sharpe broth at 37 ^{o}C for 24 h. CFS was obtained by centrifuging the bacterial culture at 6,000 rpm and 4 °C for 15 minutes (Eppendorf, Centrifuge 5424 R, Germany). The supernatant was filter-sterilized using a 0.2-micron filter (Minisart, Sartorius Stedim biotech). The filtrate was then placed in UV light Biosafety Cabinet (Telstar Bio II Advance) for 40 minutes.

2.3. Preparation of Other Compunds Cell Free Supernatant (OCCFS)

CFS from the *L. acidophilus* was obtained as described above and was neutralized to pH 7.0 using 2N NaOH (Sigma-Aldrich-Merck) and

sterilized using 0.2-micron filter (Minisart, Sartorius Stedim biotech) (Kaur, 2015).

2.4. Determination of Minimal Inhibitory Concentration (MIC).

The MIC values were determined by the tube macro dilution technique. Two-fold serial dilutions of ciprofloxacin, OACFS, and OCCFS (crude bacteriocin) were prepared volumetrically in Mueller-Hinton (MH) broth (Oxoid). The bacterial suspension was added to the broth containing the dilutions and incubated at 37 °C for 24 h. MIC was defined as the lowest concentration of the compound that showed no visible growth of *S. typhi,* demonstrated by the absence of turbidity in the broth.

2.5. Time-kill Study

The Time-kill curves of *S. typhi* were constructed by evaluating the reduction in the number of colony forming units (CFU)/mL following exposure to CFS in MH broth at 37°C for 48 h. An inoculum of 10^8 CFU/mL of *S. typhi* was used for this test. The samples were collected from the culture of the test bacteria at time intervals of 0, 4, 8,12, 24, 28, 32, 36 and 48 h, diluted serially, and cultured in triplicate in Plate Count Agar at 37 °C for 24 h (Del, 2017). The numbers of viable cells were counted as CFU/mL.

3.Results and discussions

The strain of *L. acidophilus* used in this study was tested for its ability to produce lactic acid and other compounds which inhibited the growth of *S. typhi*. Upon inoculating *L. acidophilus* on de man Rogosa Sharpe Agar (MRSA) plates containing $CaCO_3$, zones of inhibition were obtained around the colonies, which indicated that *L. acidophilus* could produce lactic acid. OACFS and OCCFS were prepared from the culture of *L. acidophilus* and their antimicrobial activity was evaluated. The pH of OACFS was 4.1. The antimicrobial activity of CFS was compared with the recommended antibiotics.

Two-fold serial dilutions of ciprofloxacin, OACFS and OCCFS were prepared for the broth macro dilution method. The MIC was 0.2 µg/mL of ciprofloxacin (Table 1), 4-fold dilution of OACFS, and 2-fold dilution of OCCFS (Table 2). For the time-kill study, normal *S. typhi* growth curves were compared with that in the OACFS, OCCFS, and ciprofloxacin at their respective MIC (figure 1).

Table 1. Minimal Inhibitory Concentration (MIC) of ciprofloxacin

	Concentration (µg/ml)									
Ciprofloxacin	25	12.5	6.25	3.12	1.56	0.78	0.39	0.2	0.1	0.05
	No growth	No growth	No growth	No growth	No growth	No growth	No growth	No growth	Growth	Growth

Table 2. Minimal Inhibitory Concentration (MIC) of organic acid cell-free supernatant (OACFS) and other compounds cell-free supernatant (OCCFS)

	Concentration of CFS dilution				
	2-fold	4-fold	8-fold	16-fold	32-fold
Organic acid	No growth	No growth	Growth	Growth	Growth
Other Compounds	No growth	Growth	Growth	Growth	Growth

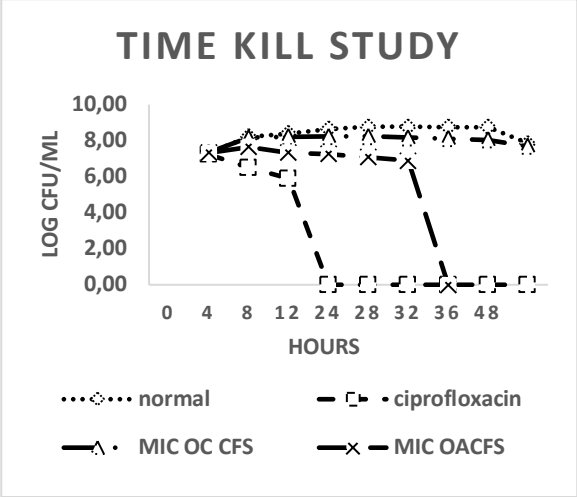

Figure 1. The comparison of *S. typhi* growth in the presnce of ciprofloxacin, OACFS, and OCCFS.

Lactobacillus spp. are probiotics that can be found in the gastrointestinal tract. *L. acidophilus* is used extensively in research to develop probiotics as a complementary therapy. In this study, OACFS showed the bactericidal effect after 36 hours of incubation. The possible mechanism of the bactericidal effect of these organic acids especially acetic acid and lactic acid involves their ability to enter the bacterial cell and disrupt cytoplasm (Bermudez-Brito *et al*., 2012). *S. typhi* is an acid-sensitive bacterium. Organic acids with low pH can directly lead to the death of *S. typhi*. The bactericidal effect of organic acids appears to accelerate the typhoid fever recovery process. In this study, the antimicrobial activity of OCCFS appears to be limited against *S. typhi*. The inhibition of the OCCFS may be attributed to the presence of bacteriocin and other peptides (having antimicrobial activity) that are produced by the lactic acid bacteria. Bacteriocin is an antimicrobial substance that could disrupt the bacterial cell membrane permeability, which may cause perforation in the bacterial cell wall (Gogineni et al., 2013). Further studies by using other isolation OCCFS method is needed to evaluate its *in vitro* effect in inhibition the growth of *S. typhi*.

Ciprofloxacin is a quinolone antibiotic, which acts by inhibiting the bacterial DNA gyrase and topoisomerase IV during bacterial replication (Abde-daim *et al*., 2013). The isolate studied in this work was sensitive to ciprofloxacin based on recent CLSI MIC recommendation (sensitive: ≤ 1 µg/mL, Intermediate: 2 µg/mL, Resistant ≥ 4 µg/mL (Scellack *et al*., 2018). However, this strain required a higher concentration of ciprofloxacin to show growth inhibition.

The decrease in susceptibility to ciprofloxacin is defined when an isolate of *Salmonella* showed an MIC of 0.12–2 µg/mL (Inical *et al*., 2011). Further studies are required to ascertain the decrease in susceptibility of this strain to ciprofloxacin.

4. Conclusions

Our study found that the cell-free supernatants of *L. acidophilus* especially those containing organic acids have an inhibitory effect in the growth curves of *S. typhi*.It appears that *L. acidophilus* exerts an antimicrobial effect on *S. typhi* and it may offer a promising therapeutic approach for the elimination of *S. typhi*

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THE EFFECTS OF LACTOBACILLUS PLANTARUM IS-10506 ON ACCELERATING THE REGENERATION OF THE INTESTINAL MUCOSA STRUCTURE EXAMINED BY THE SCANNING ELECTRON MICROSCOPE

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ABSTRACT

Lactobacillus plantarum IS-10506 (LIS-10506) is a native Indonesian probiotic derived from fermented milk originating from Sumatera. Intestinal mucosa damage results from gastrointestinal tract infections. Using Scanning Electron Microscopy (SEM), the intestinal mucosa structure, following mucosal damage, was evaluated. The objective of this study to evaluate effect of *Lactobacillus plantarum* IS-10506 on accelerating the regeneration of the intestinal mucosa structure, using SEM. This study conducted on sixty-four Sprague-Dawley rats were divided into 4 groups: control group (K_1); the group that received Lipopolysaccharide (LPS) *Escherichia coli* O55:B5 (K_2); the curative group, which received LPS *Escherichia coli* O55:B5 then probiotics (K_3); the preventive group, which received probiotics six days before LPS *Escherichia coli* O55:B5 and continued probiotics (K_4). The probiotics used were LIS-10506. Necroscopy was performed on days 3, 4, 6 and 7, and the ileum was analysed. SEM was conducted to evaluate the structure of the intestinal mucosa. SEM showed that the K_4 group experienced the fastest mucosal regeneration on the fourth day. On the seventh day, examination of the ileum using SEM showed improvement in the intestinal villi of all groups. The conclusion, SEM showed that all groups underwent regeneration following mucosal damage. Probiotics accelerated the regeneration of the intestinal mucosa, and the best results were observed in the preventive group.

1.Introduction

Probiotics have been shown to shorten the duration of diarrhoea in children with infectious diarrhoea (Allen *et al.*, 2011). Recovery from diarrhoea is closely related to damaged intestinal epithelial repair. *Lactobacillus plantarum* IS strains 10506 and 20506 have demonstrated a regenerative effect on the intestinal brush border protein, which was represented by the expression of Galectin-4, Myosin-1a, Occludin and ZO-1 (Ranuh, 2008). Research conducted by Ciorba found that the administration of probiotics can affect mesenchymal stem cells by invoking their migration towards the crypts, and consequently increasing crypt survival (Ciorba *et al.*, 2012).

Lactobacillus plantarum IS-10506 is a native Indonesian probiotic derived from curd, fermented milk from Sumatera (Collado *et al.*, 2007; Collado *et al.*, 2016). Exposure of the intact epithelium to probiotics activates Hsp27 and IL-10. Hsp27 and IL-10 affect intestinal

stem cell niche, as IL-10 will activate the JAK/STAT pathway, and both Hsp27 and IL-10 will activate stromal cells, paneth cells and intestinal stem cells. Signalling pathways stimulate proliferation of activate stem cells, followed by differentiation and maturation. Other studies have shown that *L. plantarum* IS-10506 increases the expression of Lgr-5 and Bmi1. *L. plantarum* IS-10506 also induces activation of intestinal stem cell pool by increasing ERK and β-catenin expression, reducing intestinal mucosal injury caused by inflammation. These findings suggest that *L. plantarum* IS-10506 is a potentially effective therapy, especially when used as prophylaxis for gastrointestinal diseases (Athiyyah *et al.*, 2018).

Therefore, a study is needed to show the regeneration of intestinal mucosa by the probiotic *Lactobacillus plantarum* IS-10506 using Scanning Electron Microscopy (SEM) to evaluate the surface structure of the ileum.

2. Materials and methods

2.1. Lipopolysaccharides (LPS)

LPS used in this study came from *Escherichia coli* bacteria serotype 055:B5. LPS was diluted with NaCl 0.9%, at a ratio of 10:1, and administered at a dose of 250 µg/kg bw through a gastric tube on day 1 of the study to groups K_2, K_3 and K_4.

2.2. Probiotic

Microencapsulated *Lactobacillus plantarum* IS-10506 (*GeneBank accession* n° DQ860148) was dissolved in 1.5 ml of sterile water and administered daily, through a gastric tube, at a of dose 2.86 x 10^{10} CFU/day. The probiotic was given for 6 days to the K_3 group and 13 days to the K_4 group.

2.3. Animals

This research was approved by the Ethics Committee (Animal Care and Use Committee) of Veterinary Medicine School, Universitas Airlangga (Surabaya, Indonesia). Subjects of this study were male Sprague-Dawley rats (*Rattus norvegicus*) obtained from the animal management unit of the veterinary faculty of Bogor Agricultural Institute (12 weeks old; 100–120 grams; n = 64), randomised into 4 groups: K_1 (control group); K_2 (Lipopolysaccharide (LPS) *Escherichia coli* O55:B5 group); K_3 (LPS *Escherichia coli* O55:B5 + *Lactobacillus plantarum* IS-10506 group); K_4 (*Lactobacillus plantarum* IS-10506 + LPS *Escherichia coli* O55:B5 + *Lactobacillus plantarum* IS-10506 group). K_1 was given the placebo via tube for 14 days, which consisted of sterile water. LPS *Escherichia coli* O55:B5 was administered at 250 µg/kg bw/day, starting on day 1, of the study for all groups except group K_1. *Lactobacillus plantarum* IS-10506 were administered via tube every day at a dose of 2.86 x 10^{10} CFU; group K_3 received this probiotic for 6 days and group K_4 received it for 13 days. This methods is part of a study on intestinal mucose (Athiyyah *et al.*, 2018). After observation and treatment, the subjects were sacrificed on days 3, 4, 6 and 7 by guillotine and the ileum was analysed. The anatomical structure of the ileum was determined by electron microscopy.

2.4. SEM

For the organic SEM process, fresh samples should be observed. The ileum was fixed in a 2% glutaraldehyde solution for 2–3 hours at 4 °C. The samples were washed with pH 7.4 phosphate buffer solution (PBS) (SIGMA, USA) 3 times, 5 minutes each time, at 4 °C. The PBS was replaced with a 1% post fixation osmic acid solution for 1–2 hours at 4 °C. The samples were then washed with pH 7.4 PBS 3 times, 5 minutes each time, at 4 °C. The samples were gradually dehydrated with alcohol: 30%, 50%, 70%, 80%, 90% and absolute, 2 times each, for 15–20 minutes. Dehydration using 30%–70% alcohol was performed at 4 °C, and dehydration using 80%–absolute alcohol was performed at room temperature. The alcohol was then replaced with absolute amyl acetate (MERCK, D-6100 Darmstadt, F.R. Germany), as a preservative to wait for drying time. The

samples were dried using the Critical Point Drying (CPD) procedure (Samdri®-780), with a pressure of 1000 psi, at 45 °C. Each sample was attached to the stub (holder) using Araldite (Araldite®) and coated with pure gold using a vacuum evaporator (JEOL, JEE-4X Thermal Evaporator Operation OPER-001). Finally, the samples were observed and photographed using SEM (JEOL, JSM-T100 Scanning Microscope).

3.Results and discussions

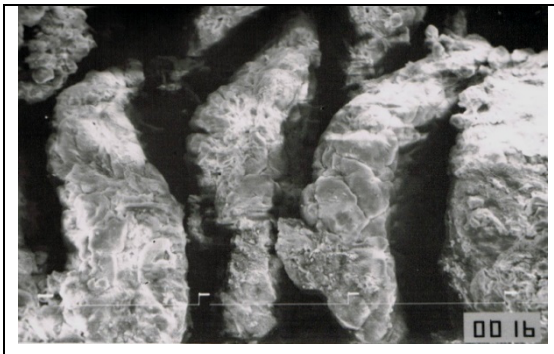
The ileum surface structure was visualised by SEM, and the results of the structure on the third day can be seen in Figure 1 for each group. At 350x magnification, an intact surface epithelial structure was observed in the K_1 group. For the K_2 group, the villi structure was damaged and the epithelium had an irregular arrangement. The damage observed in the K_3 group was more severe compared to the K_2 group. Damaged villi and epithelium were also observed in the K_4 group, but not as severe as that observed in the K_2 and K_3 groups.

On the fourth day (Figure 2), a slight improvement in the structure of the epithelial villi was observed in the K_2 group, but the structure was still irregular. The improvement observed in the K_3 group was better than that observed in the K_2 group, and the greatest improvement was observed in the K_4 group.

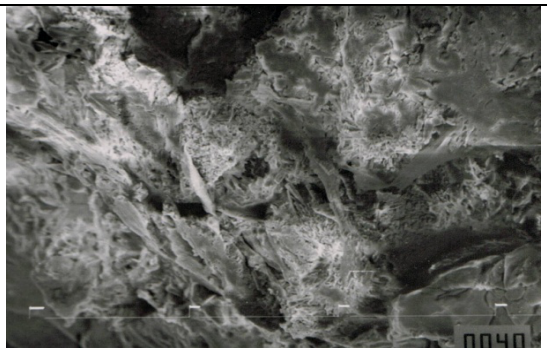
On the sixth day (Figure 3), the ileum structure of K_3 and K_4 appeared like normal intestinal villi, with a good villous structure and well-arranged epithelium; although, the K_3 group had no improvement in the overall structure of the surface.

On the seventh day (Figure 4), an improvement in the intestinal villi was observed in all groups, as determined by SEM examination of the ileum.

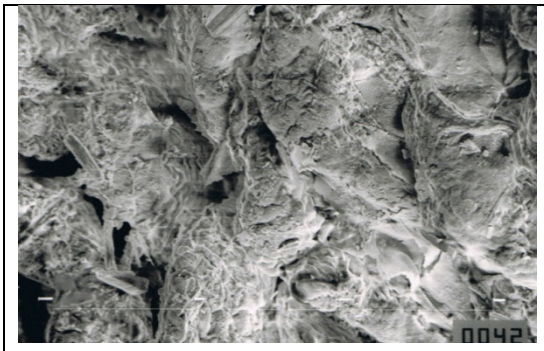
SEM aims to evaluate the mucous structure of the ileum. Mucosal damage was found in the groups that received LPS *Escherichia coli*, K_2, K_3 and K_4 groups. This result is in line with research conducted by Nikaido, which demonstrated that the administration of LPS parenterally can induce clinical manifestation in some species. In the gastrointestinal tract, bacterial endotoxins induce damage to the intestinal mucosa (Nikaido, 1996).



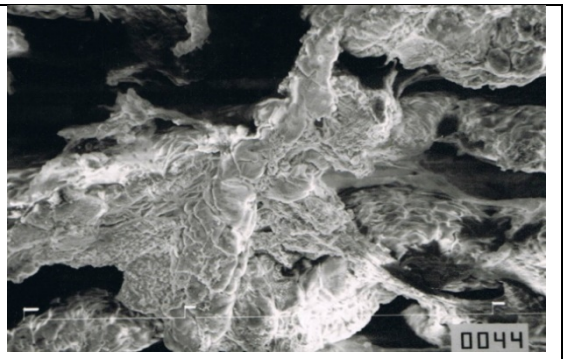
A. K_1 group, day 3



B. K_2 group, day 3



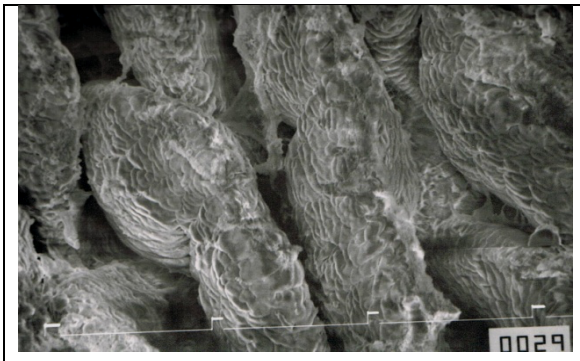
C. K_3 group, day 3



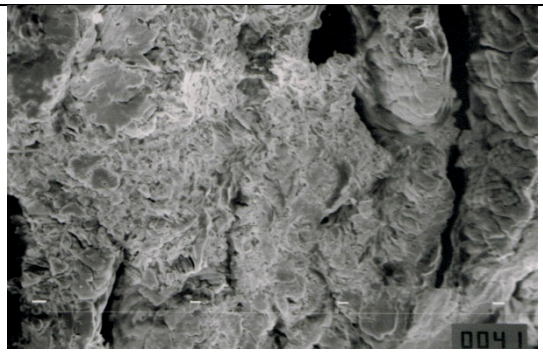
D. K_4 group, day 3

Figure 1. Images of the ileum of Sprague-Dawley rats on the third day in the K_1, K_2, K_3 and K_4 groups obtained using SEM, with 350x lens magnification. K_1 (control group); K_2 (Lipopolysaccharide (LPS) *Escherichia coli* O55:B5 group); K_3 (LPS *Escherichia coli* O55:B5 + *Lactobacillus plantarum* IS-10506 group); K_4 (*Lactobacillus plantarum* IS-10506 + LPS *Escherichia coli* O55:B5 + *Lactobacillus plantarum* IS-10506 group).

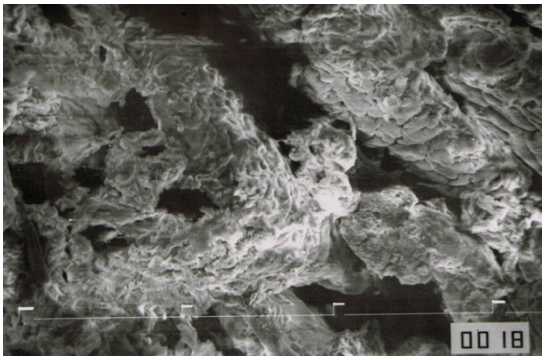
SEM was carried out two days after the administration of LPS, which was considered the third day of this study. The probiotic, *Lactobacillus plantarum* IS-10506, was given on the second day. On the third day, damage was observed in the ileum of the K_4 group, but this damage was not as severe as the damage observed in the K_2 and K_3 groups. The most severe damage was detected in the K_2 group (Figure 1). Based on research conducted by Khonyoung, heat-killed *Lactobacillus plantarum* L-137 can increase protuberant cells in all intestinal segments, especially at doses of 2 and 4 mg/kg compared to the control and a dose of 1 mg/kg. Thus, *Lactobacillus plantarum* L-137 can preserve intestinal function by preventing infection and disease in the intestinal mucosa. (Khonyoung and Yamauchi, 2012). The fastest intestinal repair was seen in the K_4 group, followed by the K_3 group and the K_2 group, respectively, on the fourth day (Figure 2). On the seventh day, improvement in the structure of the intestinal mucosa was observed in all study groups. These results suggest that the administration of probiotics can accelerate the repair of the intestinal mucosa (Figure 4). The results of this study are in line with research conducted by Dock in 2004, which found there was mucous damage in the ileum of Wistar rats given protein-free food. Ileal mucosal damage was seen in the villi length, depth and thickness of the intestinal wall crypt. Probiotic administration (*Streptococcus thermophilus* and *Lactobacillus helveticus*) accelerated the repair of the intestinal mucosal damage ('Probiotics enhance the recovery of gut atrophy in experimental', 2004). Other studies also reported the same results, namely *Lactobacillus casei* MTCC 1423 accelerated repair in malnourished Balb/c mice infected with *Giardia lamblia*. The *Lactobacillus casei* MTCC 1423-mediated repair was characterised by reduced Giardia trophozoites and improved histological morphology observed with haematoxylin eosin staining (Shukla and Sidhu, 2011).



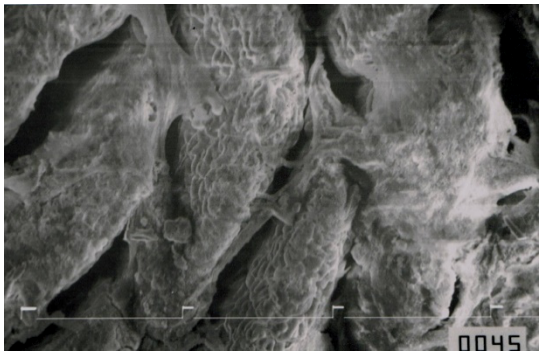
A. K_1 group, day 4



B. K_2 group, day 4

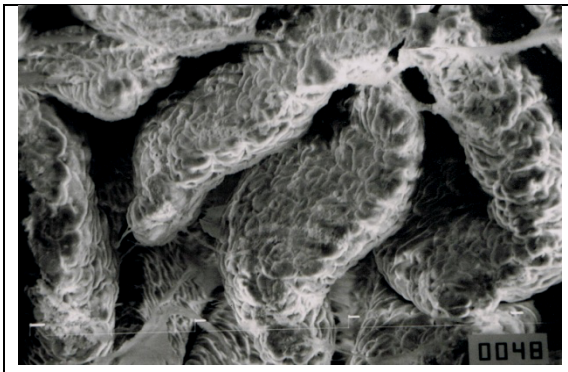


C. K_3 group, day 4

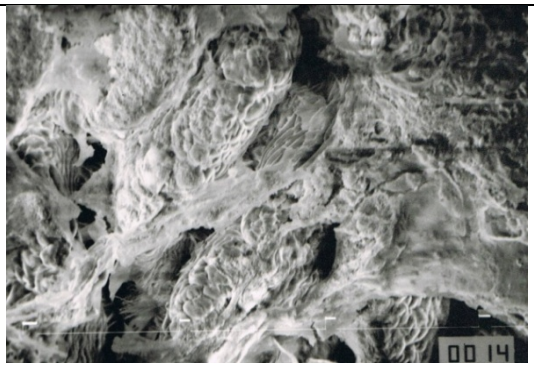


D. K_4 group, day 4

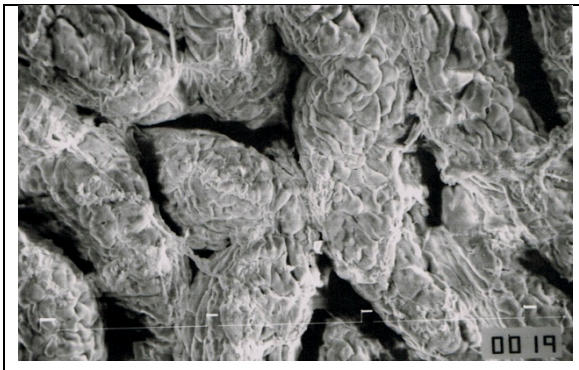
Figure 2. Images of the ileum of Sprague-Dawley rats on the fourth day in the K_1, K_2, K_3 and K_4 groups obtained using SEM, with 350x lens magnification.



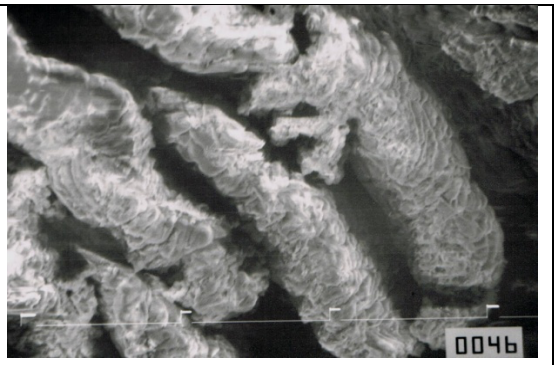
A. K_1 group, day 6



B. K_2 group, day 6



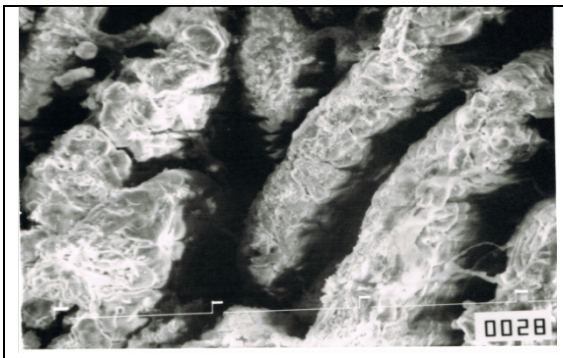
C. K_3 group, day 6



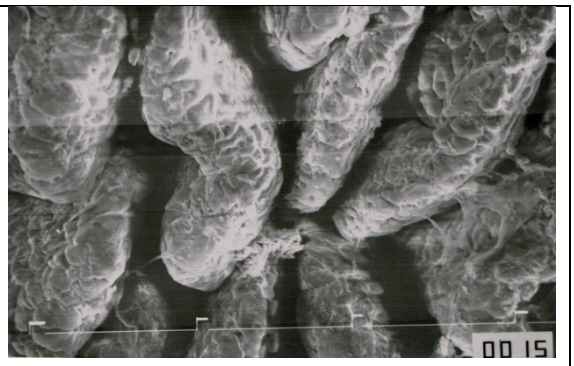
D. K_4 group, day 6

Figure 3. Images of the ileum of Sprague-Dawley rats on the sixth day in the K_1, K_2, K_3 and K_4 groups obtained using SEM, with 350x lens magnification.

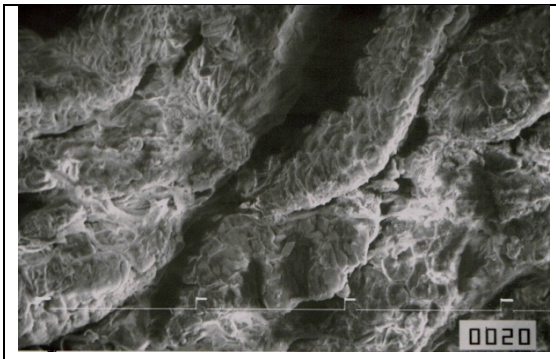
Probiotics (*Bacillus subtilis* Bs964, *Candida utilis* BKM-Y74 and *Lactobacillus acidophilus* LH1F) can enhance the number lymph and lymphatic cells of the intestinal epithelium (IEL) in chicken caecal tonsils, based on research conducted by Yurong, which found an increase in the density and length of microvilli on the surface of the tonsil caecal villi (Yurong *et al.*, 2005). Another study stated that not all Lactobacillus strains adhere to enterocytes. Lactobacillus has been shown to inhibit *in vitro* growth and adhesion of various Salmonella strains, as well as the production of antimicrobial substances (Kankaanpää *et al.*, 2004).



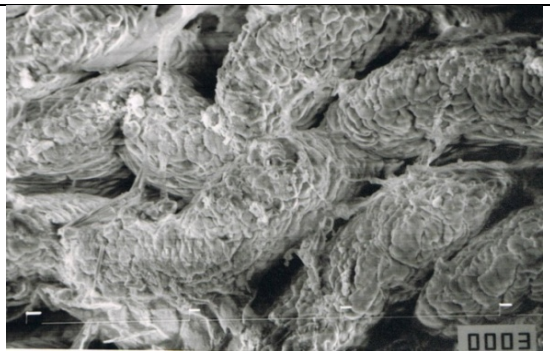
A. K_1 group, day 7



B. K_2 group, day 7



C. K_3 group, day 7



D. K_4 group, day 7

Figure 4. Images of the ileum of Sprague-Dawley rats on the seventh day in the K_1, K_2, K_3 and K_4 groups obtained using SEM, with 350x lens magnification.

4. Conclusions

SEM showed that all groups experienced regeneration following mucosal damage. On the third day, damage was observed in the K_4 group, but the damage was not as severe as that in the K_2 and K_3 groups. The regenerative process was most accelerated in the preventive group (K_4), which showed improvement on day 4 of the study.

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CORRELATION BETWEEN MUCOSAL AND SYSTEMIC ADAPTIVE IMMUNE RESPONSE AFTER PROBIOTIC ADMINISTRATION IN MOUSE MODEL

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ABSTRACT

Background: Several previous studies were conducted to explain probiotic mechanism of action, especially associated to immune system. Probiotic was proved to induce adaptive immune response in mucosa, providing a hope if it presents evidence to affect the systemic immune response.

Objective: To investigate the correlation of adaptive immune response in intestinal mucosa to systemic immune response after probiotic administration.

Methods: Thirty-two male Balb/c mice were divided into 4 groups of treatment, including: LPS + probiotic, LPS, probiotic and control group. LPS were tested in the first day, while probiotic was administered in later 7 days. The ileum and blood were collected and analyzed to measure the number of cells that produce various cytokine indicating the T_H subset.

Results: Significant findings were found in all treatment groups, indicating similar patterns of cytokine-produced cell detection, found more in mucosal ileum than in blood. In the control group, the pattern was irregular. There was no correlation between immune response evoked in mucosal ileum and systemic immune response. Probiotics presents various mechanisms to modulate adaptive immune response. Mixture of probiotics could increase all subsets of T_H, indicating the variety. In short period, cell number of each subset was higher in intestine mucosa than in blood, negating that immune modulation effect of probiotics only acts locally on intestinal mucosa, not on systemic immune response.

Conclusions: Probiotics was found to have immunomodulation effect to adaptive immune response on intestinal mucosa. In sum, there is insignificant correlation between the two.

1. Introduction

In recent years, probiotics has gained popularity due to its beneficial attribute, utilized by healthy person as prevention, and also by unhealthy person as adjuvant therapy. Probiotic has been consumed as part of treatments, specifically for gastrointestinal diseases, such as: diarrhea, infection, inflammatory bowel disease, irritable bowel syndrome, and for other diseases including: allergic treatment and atopic dermatitis (Canani *et al*, 2007; Floch *et al*, 2008). Protection effect of probiotic in gastrointestinal lumen has well known and explained from several mechanisms of pathways, such as: to increase antimicrobial activity, to decrease pH of gut's lumen, secreting antimicrobial peptide, inhibit bacterial infection, blockage bacterial adhesion on epithelial wall, to increase the barrier defence by enhancing mucous production (bacteriosin / defensin), to modulate immune system, etc (Isolauri *et al*, 2001; Galdeano *et al*, 2006; Kim *et al*, 2006;

Saavendra *et al*, 2007; Hart *et al,* 2009). Probiotic also modulate innate and adaptive immunity by producing cytokine pro and anti-inflammation (Delcenserie *et al*, 2007; Galdeano *et al*, 2007; Dharma *et al*, 2009; Iskandar *et al*, 2009).

In addition to clinical research, several supporting studies were also conducted related to in-vitro research on immune response after oral probiotic administration at animal study in some research (Isolauri *et al*, 1995; Perdigon *et al*, 2001; Perdigon *et al*, 2002; Asahara *et al*, 2004; Bauer *et al*, 2004; Rastall *et al*, 2005; Madsen, 2006; Corthesy *et al*, 2007). Previous studies also present attempt to reveal the immune response after probiotic treatment from gastrointestinal, innate, adaptive, celluler or even humoral immune response, from blood serum. Thus this raises questions proving: any relationship between mucosal gastrointestinal immunity and systemic immune response inside the blood; the "connection" or "link" between mucosal immune response inside gastrointestinal and systemic immune response inside the blood,; and the rational connection from scientific research approach. The relationship may be explained by the patterns of mucosal and systemic immune respond, after administration of probiotic. It opens into other research interests: (1) if probiotic treatment could necessarily activate immune response simultaneously, identically with gastrointestinal mucosa and blood serum, or (2) if immune response at gastrointestinal will start series of the next systemic response, or (3) if the immune response in gastrointestinal will inhibit systemic immune response.

The aim of this research is to compare between adaptive immune response inside gastrointesinal mucosa and immune response outside gastrointestinal, after administration of probiotic by using mice (*Mus musculus Balb/C*) as experimental animal.

2. Materials and methods

The method of experimental *Randomized Post Test to Control Group Design* is utilized to discover any correlation between immune response in mucosal ileum and in blood by induction of probiotic. Immune response was measured by detecting cells producing certain biomarker such as cytokine by flowcytometry procedure. This research was conducted at Biomedical Laboratory and pharmacology Laboratory in Faculty of Medicine, at Brawijaya University Malang.

2.1.Research Sample

Thirty two white mice *Mus musculus* (BALB/c mice) which age 10-12 weeks with weight of between 30-40 grams and male gender were applied in this research, divided into 4 groups, including: LPS + probiotic, LPS, probiotic, and control group. The mice were taken from Veterinarian Centre Farma at Ahmad Yani Street Surabaya.

Research sample will be excluded if the tested animal was found sick tracked from change of activity (change of food/drink pattern, and animal activity) and other important clinical signs (decrease of body weight, breathing pattern, diarrhea, vomitting, and so forth). The tested animal which dies due to physical or mental stress, and damage to organ or tissue was also applied for sampling to examination of flowcytometry. The mice underwent acclimatization for a week before the treatment was started.

All the protocols in this research has already been approved by the Ethical Committee of Faculty of Medicine, Brawijaya University, Malang Indonesia.

2.2.Lipopolysacharide (LPS) administration

LPS is derived from *Escherichia coli serotype 055:B5* bacteria, with dosage of 250 µg/kg BB, thus each mice will get average of 7,5 µg. LPS will be dilluted with NaCl of 0,9% with comparation of 10:1, and will be given with orogastric tube at the first day of treatment for LPS and LPS+probiotic group mice.

2.3.Probiotic administration

Probiotic is derived from Mix bacteria with the composition of *Lactobacillus casei, Lactobacillus rhamnosus, Lactobacillus*

acidophilus, Lactobacillus bulgaricus, Bifidobacteria breve, Bifidobacteria infantis, and *Streptococcus thermophilus*, in which every sachet got the amount of living bacteria (*total viable count*) of 1,00x10^9 CFU. Probiotic is derived from dosage of 10^9/kgBB/day, thus each mice will get average dosage of 3 x 10^7 CFU. Probiotic will then be dilluted in D5% media with volume of 0.5 cc administered with orogastric tube (once a day) for 7 days. In LPS+probiotic group mice, the probiotic solution was administered in the following day after LPS induction.

2.4.Collection of samples

Samples of mucosal ileum and blood were taken from each mice. After all the treatment was completed, each mice underwent euthanasia by ether, to withdraw sample of ileum and blood. The ileum mucosa was homogenized, then diluted and analyzed by flowcytometry procedure. The plasma was separated from the cell by centrifugation to be analyzed by flowcytometry procedure. The number of mucosal and blood cells that expressed specific cytokine (IL-2 , IFN-γ, IL-4, IL-5, TGF-β, IL-10, IL-17, IL-22) were detect and counted by flowcytometry machine.

2.5.Flowcytometry

Flowcytometry was performed to measure the amount of cell producing cytokine to mark the subsets of immune response, including: IL-2 & IFN-γ indicate T_H1 subset, IL-4 & IL-5 indicate T_H2 subset, IL-10 & TGF-β indicate Treg subset, and IL-17 & IL-22 indicate T_H17 subset. Each cell which produced certain cytokine was detected by antibody of each cytokine from the flowcytometry kit (BioLegend, USA), according to manufacturer protocol.

2.4.Collection of samples

Data are presented in mean ± SD. Paired t-test was performed to reveal the differences between mucosa and systemic. If the data was not distributed normally, Wilcoxon test would be applied. Pearson Correlation test was performed to find correlation between adaptive and systemic immune response, taken from intestine mucosa and blood. If the data was not distributed normally, Spearman test will be utilized. The statistical calculation was performed by employing SPSS 21 software (SPSS Inc.). The differences is considered statistically significant at $p \leq 0.05$.

3. Results and discussions

In the first group (LPS + probiotic), there were some significantly different results between mucosal and systemic immune response, which was IL-2 with P=0.002, IFN-γ with P=0.000, IL-5 with P=0.000, TGF-β with P=0.004, IL-10 with P=0.000, and IL-22 with P=0.000. In contrast, IL-4 and IL-17 presented no significant difference (P=0.078 and 0.159 respectively) of adaptive immune response in ileum mucosa compared to systemic, as depicted in figure 1.

In the second group (LPS only), some significantly different results were found from adaptive immune response in ileum mucosa, which was IL-2 with P=0.05, IFN-γ with P=0.000, IL-5 with P=0.000, TGF-β with P=0.012, IL-10 with P=0.000, IL-17 with P=0.000, and IL-22 with P=0.000. Meanwhile, IL-4 presents insignificant difference (P=0.843) of adaptive immune response in ileum mucosa compared to systemic, as illustrated in figure 2.

In the third group (probiotic only), there were significantly different results of all cytokine level from adaptive immune response between in ileum mucosa and in systemic immune response, which was IL-2 with P=0.001, IFN-γ with P=0.001, IL-4 with P=0.003, IL-5 with P=0.001, TGF-β with P=0.002, IL-10 with P=0.000, IL-17 with P=0.008, and IL-22 with P=0.000 as depicted in figure 3.

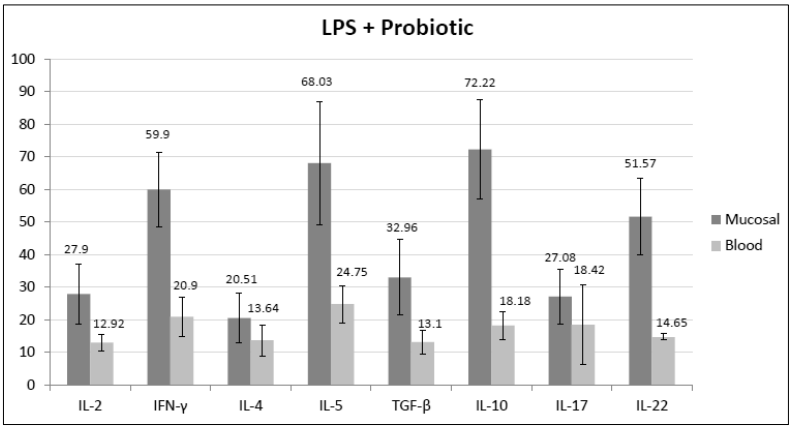

Figure. 1. Mean response of adaptive immune system in ileum mucosa and in systemic immune response in mice, administered with probiotic and LPS. The number of all mucosal cell that expressed specific cytokines taken from intestinal mucosa were significantly higher than blood cell, except for IL-4 and IL-17

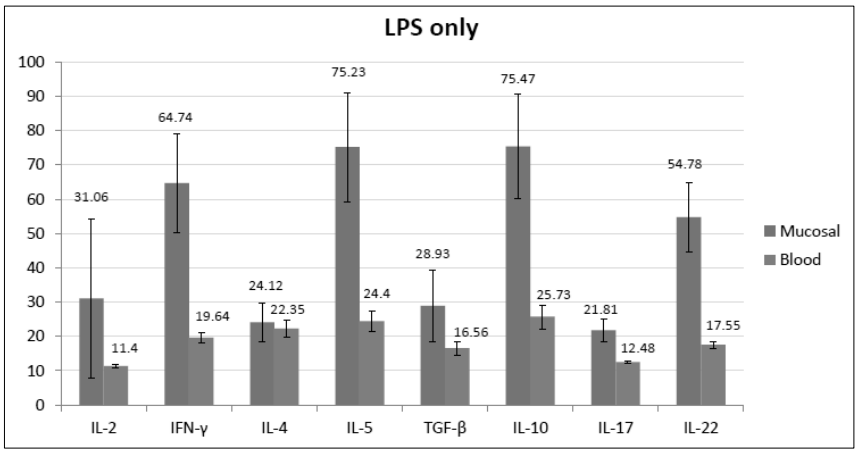

Figure. 2. Mean response of adaptive immune system in ileum mucosa and in systemic immune response in mice administered with LPS. Overall, the number of all mucosal cell that expressed specific cytokines taken from intestinal mucosa were significantly higher than blood cell, except for IL-4

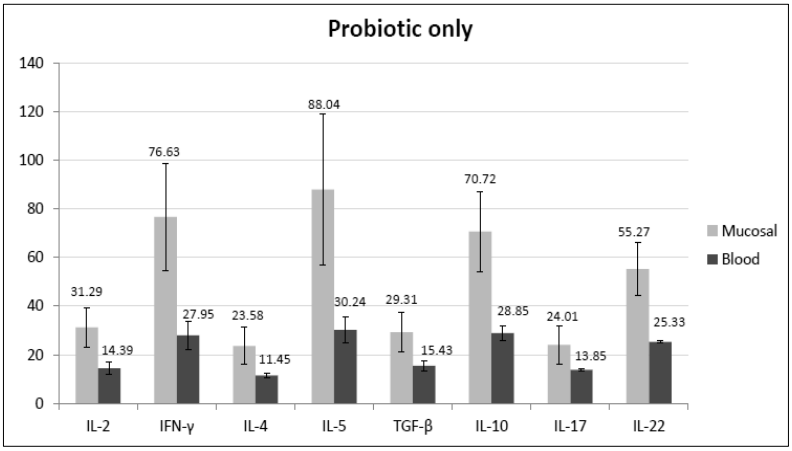

Figure. 3. Mean response of adaptive immune system in ileum mucosa and in systemic immune response in mice administered with probiotic. The number of all mucosal cell that expressed specific cytokines taken from intestinal mucosa were significantly higher than blood cell

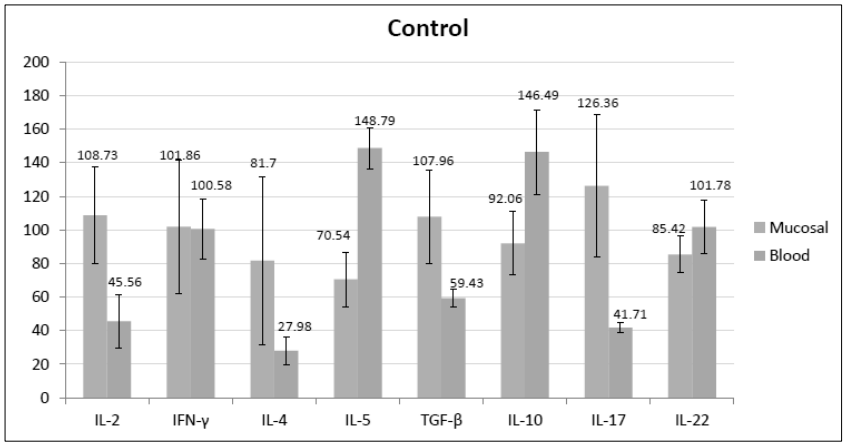

Figure. 4. Mean response of adaptive immune system in ileum mucosa and in systemic immune response in control group mice. The number of most mucosal cell that expressed specific cytokines taken from intestinal mucosa were significantly higher than blood cell, but there higher number count on cell that expressed IL-5, IL-10, and IL-22 obtained from blood than from mucosal ileum.

Table 1. Correlation of adaptive immune response in ileum mucosa and blood serum. There was no significant relationship in adaptive immune response in ileum mucosa and in systemic immune response at number of cell that expressed IL-2, IFN-γ, IL-4, IL-5, IL-10, TGF-β, IL-17, and IL-22

Parameter	Mucosal ileum	Blood	p	Correlation Coefficient	P
IL-2	27.9 ± 9.18	12.92 ± 2.47	0.002	0.213	0.613
IFN-γ	59.9 ± 11.54	20.9 ± 5.96	0.000	-0.192	0.648
IL-4	20.51 ± 7.67	13.64 ± 4.74	0.078	-0.102	0.810
IL-5	68.03 ± 18.95	24.75 ± 5.64	0.000	-0.055	0.897
TGF-B	32.96 ± 11.58	13.10 ± 3.67	0.004	-0.425	0.294
IL-10	72.22 ± 15.33	18.18 ± 4.39	0.000	0.551	0.157
IL-17	27.08 ± 8.4	18.42 ± 12.23	0.159	-0.103	0.808
IL-22	51.57 ± 11.75	14.65 ± 0.95	0.000	-0.106	0.803

Table 2. Correlation of adaptive immune response in ileum mucosa and blood serum. There was no significant relationship in adaptive immune response in ileum mucosa and in systemic immune response at number of cell that expressed IL-2, IFN-γ, IL-4, IL-5, IL-10, TGF-β and IL-22. There was only significant relationship in IL-17

Parameter	Mucosal ileum	Blood	P	Correlation Coefficient	P
IL-2	31.06 ± 23.33	11.4 ± 0.4	0.05	-0.551	0.157
IFN-γ	64.74 ± 14.39	19.64 ± 1.38	0.000	-0.155	0.713
IL-4	24.12 ± 5.56	22.35 ± 2.57	0.843	0.210	0.618
IL-5	75.23 ± 15.99	24.4 ± 3.08	0.000	0.514	0.193
TGF-B	28.93 ± 10.51	16.56 ± 1.93	0.012	0.106	0.803
IL-10	75.47 ± 15.14	25.73 ± 3.49	0.000	0.427	0.292
IL-17	21.81 ± 3.19	12.48 ± 0.36	0.000	-0.741	0.035

IL-22	54.78 ± 10.18	17.55 ± 0.98	0.000	0.144	0.734

Table 3. Correlation of adaptive immune response in ileum mucosa and blood serum. There was no significant relationship in adaptive immune response in ileum mucosa and in systemic immune response at number of cell that expressed IL-2, IFN-γ, IL-4, IL-5, IL-10, TGF-β, IL-17, and IL-22

Parameter	Mucosal ileum	Blood	P	Correlation Coefficient	P
IL-2	31.29 ± 8.15	14.39 ± 2.58	0.001	-0.166	0.695
IFN-γ	76.63 ± 22.18	27.95 ± 5.68	0.001	-0.165	0.696
IL-4	23.58 ± 7.73	11.45 ± 0.8	0.003	0.274	0.512
IL-5	88.04 ± 31.07	30.24 ± 5.32	0.001	-0.020	0.963
TGF-B	29.31 ± 8.21	15.43 ± 2.1	0.002	0.178	0.674
IL-10	70.72 ± 16.54	28.85 ± 2.9	0.000	-0.323	0.436
IL-17	24.01 ± 7.84	13.85 ± 0.31	0.008	0.116	0.785
IL-22	55.27 ± 10.94	25.33 ± 0.5	0.000	0.073	0.864

Table 4. Correlation of adaptive immune response in ileum mucosa and blood serum. There was no significant relationship in all adaptive immune response in ileum mucosa and in systemic immune response at number of cell that expressed specific cytokines.

Parameter	Mucosal ileum	Blood	P	Correlation Coefficient	P
IL-2	108.73 ± 28.68	45.56 ± 15.82	0.000	-0.166	0.695
IFN-γ	101.86 ± 39.67	100.58 ± 18.04	0.91	-0.165	0.696
IL-4	81.7 ± 49.92	27.98 ± 8.43	0.026	0.274	0.512
IL-5	70.54 ± 16.22	148.79 ± 12.15	0.000	-0.020	0.963
TGF-B	107.96 ± 27.72	59.43 ± 5.37	0.002	0.178	0.674
IL-10	92.06 ± 18.83	146.49 ± 25.24	0.001	-0.323	0.436
IL-17	126.36 ± 42.37	41.71 ± 2.97	0.001	0.116	0.785
IL-22	85.42 ± 10.97	101.78 ± 16.1	0.05	0.073	0.864

In the fourth group (control group), there were significant differences of almost all cytokine levels from adaptive immune response between ileum mucosa and systemic, but the pattern was random, as presented in figure 4. There was significant higher concentration on mucosal ileum of IL-2, IL-4, TGF-β & IL-17. In the other hand, higher concentration of IL-5, IL-10, and IL-22 obtained from blood than from mucosal ileum.

In this study, probiotic treatment could induce all of the subset of T_H cell, whether preceded by induction of LPS or standing alone. This result is consistent with previous statement of variety effect on T_H cell by probiotics. The amount of all cytokine illustrate the same pattern, which was cytokine produced locally in intestinal mucose had a higher level compared to cytokine level in serum. This result definitely shows that probiotic had a significant effect of immunomodulation locally, not systemic. LPS known to be an inducer of innate immunity, by attached to TLRs of macrophage or dendritic cell. Then, macrophage as an APC produces some cytokine that could induce all of T_H subset, according to its environment (Abbas et al, 2007).

Previous result from Nunez et al. showed that effect of probiotic to immune response dominantly on intestinal environment. Probiotic treatment could improve vili condition, increase IgA mucosal production, and maintain activity of macrophage intestinal whether probiotic couldn't increase IgG in the serum, which indicate locally scope of probiotic effect (Nunez *et al*, 2014).

Probiotic used as a prevention of intestinal pathogen, which could be shown from result that probiotic could increase the amount of cell producing IL-2 and IFN-γ significantly, indicate activation of T_H1 subset. Previous studies reported that *L.casei* in probiotic could increase activation of transcription factor NF-κB in macrophage induced by LPS. This activation lead to increased IL-12 production, which has the activity to induce differentiation form T_H naïve to T_H1 (Ishida *et al*, 2007). Probiotic also could potentiate semi-mature dendritic cell, to produce more pro-inflammatory cytokine, such as IL-6 and TNF-α to fortify intestinal lining from pathogen invasion (Rizzello *et al*, 2011).

Regarding T_H17 subset, it was shown that IL-17 production on intestinal mucosa in this study, was not significantly differs from serum. Source of IL-17 dominantly from T_H17 and a very little amount from γδT cell, so it needs significant activation of T_H17 to increase IL-17 production significantly, which couldn't achieved by short term induction of probiotics (Jin and Dong, 2013). But, as consistent to previous statement, probiotic could induce development of T_H1/T_H17 subset locally on intestinal mucosa.

Activation of macrophage could also induce T_H2 subset, and this effect could be increased by certain type of microorganism. Increased number of cell producing IL-4 and IL-5 indicate activation of T_H2 to further activate B lymphocyte to produce Immunoglobulin. This result consistent with previous studies that report probiotic (*L.reuteri, B. longum*) could reduce TNF-α and increase IL-4 production by LPS-induced macrophage (Rodes *et al*, 2013). The variety of immune modulation by probiotic not only in the scene of pro-inflammatory T_H subset, even in Treg development. Another study of probiotic, using mixture of probiotic showed that probiotic could interact with regulatory Dendritic Cell (rDC) to produce IL-10 and TGF-β (Kwon *et al*, 2009). These two cytokine are necessary for differentiation of naïve T_H cell to Treg. Similar result gained from our study that probiotic also increase number of cell to produce IL-10 and TGF-β. This result completely shows us again that probiotics have a variety effect to immune system. One probiotics, which contain *Lactobacillus reuteri*, could induce IL-10 production by macrophage and dendritic cell to activate Treg subset (Hemarajata, 2013). Another probiotics which contain *Lactobacillus rhamnosus*, could induce production of TNF-α, IL-12, and IL-6 by dendritic cell that induce activation of T_H1 and T_H17 subset (Evrard *et al*, 2011).

Probiotic well reported as a prevention of allergy, although the evidences still not convincing. This indicate that probiotic preferential is to suppress T_H2 subset, which mainly causes allergy reaction (Cuello *et al*, 2015). In this study, on the contrary, the difference between IL-4 in mucosal ileum and blood seems to be significant on probiotic only group. This result likely indicate that probiotic actually activating T_H2 subset response. This contrary can be explained by the real amount of cells that produce cytokine. In probiotic only group, it was actualy the amount of cytokine produced by decreasing, made the differences to significant statistically. This decrease due to absence of LPS induction which can elicit systemic immune response. The mean amount of cell producing IL-4 was similar between all three treatment group, indicate the action of probiotic less dominant than the LPS induction. There were any similar pattern between treatment group, which was any cytokine produced much higher in mucosal ileum than systemically produced, which indicate dominance of immune response occurred locally, whether elicited by LPS or probiotic. In control group, the pattern was inconsistent between mucosal ileum and blood.

Correlation analysis performed to measure if there are any correlation between effect of probiotic on mucosal immunity to the systemic one in circulation. From the correlation result, it was showed that no significant correlation performed by probiotics. It means that the effect of probiotic to modulate immune system only happened locally on intestinal mucosa. This result similarly consistent with previous study by Galdeano et al. when probiotic was given as an adjuvant to re-nutrition diet on protein-energy malnutrition mice. Probiotic effectively induce local immunity, shown by increase of DC and various cytokine such as IFN-γ, IL-2, and IL-6. In systemic scope, IgG production increased after probiotic and re-nourishment therapy, which indicate that probiotic doesn't has direct correlation effect to systemic immunity (Galdeano *et al*, 2011).

4. Conclusions

The importance of this study is to clarify usefulness of probiotic to various disease on local or systemic base. The result from present study showed that probiotic has a variety effect on intestinal mucosa and systemic immunity, whether it is pro- or anti-inflammation. There are significant difference number of cell that produce cytokines taken from intestinal mucosa compare with cell that taken from blood. The number of cell that taken from intestinal mucosa significantly higher than blood. Furthermore, using correlation test, there were no correlations between the changing number of cell in ileum and blood. It indicates that adaptive immune respon after administrastion of probiotics, in short periode, seems only act locally on intestinal mucose, but not systemically. There is no correlation between probiotic treatment with activation of systemic immune response. Further necessarily studies of probiotic to identify how commensal bacteria interact with immune cell on the molecular base.

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RESPONSES OF DENDRITIC AND NK CELLS AFTER MULTISPECIES PROBIOTIC ADMINISTRATION IN BALB/C MICE

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ABSTRACT

Dendritic and Natural killer (NK) cells play important roles in the innate immune response. The administration of probiotics is known to affect the immune response. The study aims to assess the effects of multiple probiotic species on the activities of dendritic and NK cells after gastrointestinal damage induced by bacterial lipopolysaccharide (LPS). Male Balb/c mice (n=24) were randomized into four groups: the K-I group (LPS and probiotics), K-II group (LPS only), K-III group (probiotics only), or K-IV group (no intervention). LPS was produced by *Escherichia coli* O5:B55 cells, while the probiotics were a combination of *Lactobacillus acidophilus* PXN 35, *L. casei* subsp. *casei* PXN 37, L. rhamnosus PXN 54, L. bulgaricusPXN 39, Bifidobacterium breve PXN 25, B. infantis PXN 27, and *Streptococcus thermophilus* PXN 66. LPS was administered on day 15, while probiotics were administered for 21 consecutive days. After 21 days, the mice were sacrificed and the numbers of dendritic and NK cells were determined by immunohistochemical staining of the ileum. Comparisons with the independent samples *t*-test showed that as compared to the control group, probiotic administration had significantly increased the numbers of dendritic cells, but not NK cells. Meanwhile, in the presence of LPS, there was a significant difference in the number of dendritic cells between the probiotic-LPS and the LPS only groups, but not NK cells. Multiple probiotic species can regulate the innate immunity response through dendritic cells, but not NK cells, in Balb/c mice.

1.Introduction

Pathogenic bacteria, including those that cause diarrhea, induce the release of various cytokines (Sheil *et al.*, 2006; Kuo, Merhige and Hagey, 2013)2), which will lead to an imbalance in the immune response (Saavedra, 2007). Macrophages are the first line of defense against microbial invasion. Secreted cytokines can recruit polymorphonuclear cells to the area of inflammation in the lamina propria. In the immature phase, dendritic cells are also phagocytes that can process both soluble and particulate antigens. The most significant functions of dendritic cells are the processing and presentation of antigens, especially naive T cells (Mannon, 2005; Shi *et al.*, 2017). Dendritic cells can recognize antigens by monitoring extracellular areas of the lamina propria, by phagocytosis of apoptotic epithelial cells, or hand interdigitation through tight junctions (to prevent damaging to junction

integrity) in order to directly recognize antigens in the lumen (Mannon, 2005; Delcenserie *et al.*, 2008).

Natural killer (NK) cells also participate in the innate immune response in the intestinal mucosa and play important roles against bacterial infections. Despite being derived from T cells as precursors, NK cells do not react to adaptive antigens. Nevertheless, NK cells recognize class I major histocompatibility complex (MHC) molecules through the binding of the surface receptors, which then inhibits the release of perforin and ganzyme proteins. Cells that do not express MHC class I molecules, such as virus-infected or tumor cells, are subjected to the activities of NK cells (Mannon, 2005).

Probiotics are combinations of microorganisms that have demonstrated or thought to have some beneficial effect when consumed (Kearney and Gibbons, 2018), such as reducing the pH of the intestinal lumen, secretion of antimicrobial peptides, inhibition of bacterial adhesion and invasion of epithelial cells, improving barrier function by increasing mucus production, increasing barrier integrity, and improving immunomodulation of epithelial cells, dendritic cells, monocytes/macrophages, and lymphocytes (B lymphocytes, NK cells, T cells) (Floch and Montrose, 2005; Ng *et al.*, 2009). Studies of mice and humans have confirmed that probiotics can induce an immune response and accelerate the healing of various gastrointestinal disorders both acute and chronic (Isolauri *et al.*, 2001; Marteau *et al.*, 2001; Guarino, Vecchio and Canani, 2009; Ritchie and Romanuk, 2012).

These findings of previous studies present an opportunity to explain the mechanisms of probiotics in the prevention of diarrhea through the role of dendritic and NK cells. The mechanism are further explained through experiments that are designed to analyze the relationship between innate immunity activation, especially dendritic and NK cells, in healthy versus pathogen-exposed mice.

2. Materials and methods

2.1. Animals

Male Balb/c mice (n = 24; age, 10–12 weeks; body weight, 30–40 g) were obtained from the Farma Veterinary Center (Surabaya, Indonesia) and acclimated for 1 week prior to experimentation. The study protocol was approved by the Animal Care and Use Committee of the Veterinary Medicine School of Universitas Airlangga (Surabaya, Indonesia). The mice were fed standard feed with free access to water at all times. After acclimation, the mice were randomly allocated to one of four groups: the K-I group, which received probiotics and lipopolysaccharide (LPS), the K-II group, which received LPS only, the K-III group, which received probiotics only, or the K-IV group, which received no intervention. LPS was administered only once on day 15, while probiotics were administered for 21 consecutive days. Each experimental group consisted of six mice. All mice were examined daily for morbidity and other symptoms of illness, such as reduced activity level, abnormal evacuation, and decreased body weight. At the end of the experiment, the ileum was dissected for analysis.

2.2. Probiotics and LPS

The probiotics used in this study contained 1×10^9 colony-forming units of a combination of bacteria *Lactobacillus acidophilus* PXN 35, *L. casei* subsp. *casei* PXN 37, *L. rhamnosus* PXN 54, *L. bulgaricus* PXN 39, *Bifidobacterium breve* PXN 25, *B. infantis* PXN 27, and *Streptococcus thermophilus* PXN 66. The probiotics in powder form were dissolving in 1.5 mL of sterile water and administered to mice in groups K-I and K-III via a gastric tube once daily for 21 consecutive days.

LPS, as a representative bacterial endotoxin, was produced by Escherichia coli O55:B5 cells (L2880; Sigma-Aldrich Corporation, St. Louis, MO, USA). LPS was dissolved in 0.9% non-pyrogenic sterile NaCl (10:1 ratio) and administered orally through a gastric tube on day 15 at dose of 250 μg/kg

body weight. LPS was orally administered via a gastric tube on day 15 of the study to the mice in both groups.

2.3. Histological analysis and detection of immunoglobulin-producing cells

On day 22 (at the end of the experiment), the abdomens of Balb/c mice in all groups were opened under ether anesthesia. After cleaning, 10% formalin buffer solution was used to fix the ileum sections. This process was followed by dehydration, clearing, and embedding. Tissue sections were probed with mouse monoclonal antibodies against follicular dendritic cells (F3803; Sigma-Aldrich Corporation) and NK cells (MA1-70100; Thermo Fisher Scientific, Waltham, MA, USA). The samples were observed under a light microscope (CX21; Olympus, Tokyo, Japan) and photographed with an ILCE6000 camera (Sony, Tokyo, Japan). The number of immunopositive cells was determined by counting the mean number of cells in 20 random fields at 450× magnification. The results are expressed as the number of cells in fields of vision.

2.4. Statistical analysis

Differences between groups were analyzed with the independent sample *t*-test for normally distributed data or the Mann–Whitney test for abnormally distributed data. A probability (*p*) value of < 0.05 was considered statistically significant

3.Results and discussions

The aim of this study was to analyze ability of probiotics to modulate the mouse immune responses, as represented by dendritic and NK cells, in response to exposure to LPS. The mean values of dendritic and NK cell counts for each group are presented in Figure 1 and the baseline characteristic has shown on table 1.

Table 1. Baseline characteristic

GROUP	WEIGHT(G)	AGE(W)
	N=6 Mean ±6SD	N=6 Mean ±6SD
1 (probiotic + LPS)	31,61±1,59	11,50±0,83
2 (LPS)	33,92±3,27	10,66±0,81
3(Probiotic)	31,61±1,59	11,00±082
4 (none)	32,73±2,75	10,83±0,75
Total	32,46±2,18	10,99±0,80

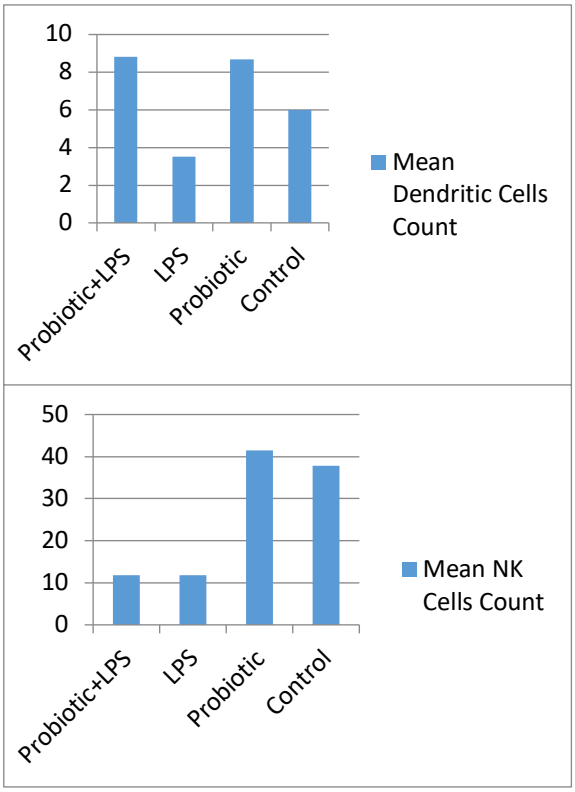

Figure 1. Mean numbers of (a) dendritic cells and (b) NK cells.

The numbers of both dendritic and NK cells were normally distributed; therefore, the independent sample t-test was performed to compare cell counts between the probiotic and control (no treatment) groups. The results revealed significant differences in the numbers of dendritic cell, but not NK cells, between the groups

Table 2. Comparisons of dendritic and NK cell counts in the probiotic and control groups

Variables	Probiotic		Control		*P*
	n	Mean (SD)	n	Mean (SD)	
Dendritic cells	6	8.67 (2.07)	6	6 (1.41)	0.026
NK cells	6	41.5 (13.23)	6	37.83 (20.87)	0.724

The results of the independent sample t-test between the probiotic-LPS (probiotics for 21 consecutive days and LPS on day 15) and LPS groups revealed significant differences in the numbers of dendritic cells, but not NK cells.

Table 3. Comparison between dendritic and NK cell counts of the probiotic+LPS and LPS groups

Variables	Probiotic-LPS		LPS		*P*
	n	Mean (SD)	n	Mean (SD)	
Dendritic	6	8.83 (1.72)	6	3.5 (1.05)	0.000
NK cell	6	11.83 (3.82)	6	11.83 (9.67)	1.000

3.1. Effects of Probiotics on Innate Immunity

Defense of the intestinal mucosa involves a combination of immunological and non-immunological processes, both of which can strengthen resistance of the intestinal mucosa (Blum and Schiffrin, 2003). Probiotic-induced enhancement of the immune system has been confirmed by evidence-based studies (Saavedra, 2007; Shi *et al.*, 2017). Various studies of probiotics mention the benefits of modulating the immune system both in vitro and in vivo (Cross *et al.*, 2004).

3.2. Effects of Probiotics on Dendritic Cells

The innate immune status of the probiotic group, as determined by the number of dendritic cells, was significantly increased, as compared to that of the control group, demonstrating that the administration of probiotics improves the innate immune response. Therefore, it can be concluded that defense mechanisms and the immune response were improved in the probiotic-treated intestinal mucosa, as compared to non-treated controls. This finding is consistent with the theory that synbiotics stimulate both the innate and adaptive immune responses in the mucosa (Saavedra, 2007). Communication occurs between probiotic bacteria as normal flora and the host's immune system (Corthésy, Gaskins and Mercenier, 2007; Barzegari *et al.*, 2014). Probiotics are potential immunomodulators that increase the amount and intensify the maturation of dendritic cells in the form of antigen-presenting cells (Mohamadzadeh *et al.*, 2005), which can recognize pathogen-associated molecular patterns through toll-like receptors (TLRs) (Corthésy, Gaskins and Mercenier, 2007). Therefore, dendritic cells have the capacity to "drive" a T cell subset based on intestinal microflora composition (Christensen, Frøkiær and Pestka, 2002) of both normal and pathogenic microflora (Foligne, 2007).

In this study, there were significantly increased numbers of dendritic cells in the probiotic-LPS group, as compared to the LPS-only group. Past studies have reported that the increased immunological resistance of the host fights against diarrhea-causing pathogens. This concept is considered more reasonable, considering that the main target is to improve the immune response in the mucosa. Also, several randomized controlled studies and meta-analysis have found that the administration of probiotics can effectively prevent gastroenteritis (Guarino, Vecchio and Canani, 2009).

LPS administration will induce an inflammatory response in healthy mice, as demonstrated by the upregulated responses of factors of the innate and adaptive immune systems. Since probiotic exposure, which can increase activation and the number of dendritic cells through TLR-2 expression, was absent

(Dogi, Galdeano and Perdigón, 2008) in the LPS-only group, a lower number of dendritic cells was obtained. In this group, there was also a decrease in TLR-4 expression caused by CD14 deficiency, so that the immunological process was leaning toward TH2 cells and there was a disruption of stimulation of TLR-4 expression, which inhibited dendritic cell activation (Mohamadzadeh *et al.*, 2005; Dogi, Galdeano and Perdigón, 2008). In contrast, mice that were first administered probiotics had statistically significant higher numbers of dendritic cells, as compared to the LPS-only group, which further reinforces the theory that probiotics have anti-inflammatory activities. The most marked anti-inflammatory effect was shown by bifidobacterial species, which upregulated interleukin (IL)-10 production by dendritic cells and decreased expression of the costimulatory molecules CD80 and CD40. These effects of probiotic bacteria on dendritic cells may underlie their anti-inflammatory activities.

Effects of Probiotics on NK Cells

No previous study has used NK cells to evaluate the status of innate immunity. NK cells are natural defensive components against viral infections and tumor cells (Delves *et al*., 2006). Increased NK cell activity is thought to be part of the capability of the immune response to inhibit malignancy. Upon activation, NK cells respond to bacterial infection in two ways: first, by removing perforin and granzyme enzymes, which induce the apoptosis of infected cells, and, second, by producing interferon (IFN)-γ, which activates macrophages and increases the ability to kill and devour bacteria. Macrophages release IL-12, a potent cytokine that re-activates NK cells (Abbas, Lichtman and Pillai, 2012; Baratawidjaja and Rengganis, 2009).

Many studies have revealed that the administration of probiotic bacteria will affect the overall nonspecific immune response by increasing pathogen phagocytosis through increased macrophage activation and subsequent cytokine production (Erickson and Hubbard, 2000). Another study of 50 adults found that the addition of the probiotic B. lactis HN019 to milk could increase the activities of polymorphonuclear cells and NK cells, as compared to the control group (Chiang *et al.*, 2000). Similarly, another study found that the administration of Lactobacillus casei ssp. with dextran significantly increased the activities of NK cells in the spleen of Balb/c mice (Ogawa *et al.*, 2005). Also, the addition of the probiotics L. gasseri and L. coryniformis to yogurt had significantly increased the number of NK cells in the blood of healthy adults by 21% (Olivares *et al.*, 2006). This is in line with the results of a present study of an increase in the number of NK cells, although this increase was not significant. However, another study of 20 healthy young female subjects reported that the consumption of L. casei Shirota fermented milk for 4 weeks had no influence NK cell activity (Spanhaak, Havenaar and Schaafsma, 1998). Therefore, further studies are needed to determine the most effective strains and doses of probiotics for the stimulation of NK cells.

The results of the in vivo study showed that the number of NK cells in the innate cell immune was decreased after the administration of LPS. Different results were obtained in another study that found that LPS can stimulate NK cell proliferation, secrete IFN-γ, and increase toxicity of NK cells to in vitro target cells in human blood (Nedvetzki *et al.*, 2007). This difference can explained by the differences in observation methods (in vivo vs. in vitro). The decrease in NK cell number and activities in the in vivo studies can occur due to natural microbiota in the intestine that act as probiotics.

Probiotic administration increases the activity and production of NK cells, which are cytotoxic lymphocytes, the main component of the innate immune system, are increased in number along with increased production of IL-12. This finding is in line with the results of previous studies, which stated that administration of probiotics could increase the numbers of cells expressing IL-1, IL-2, and IL-12. IL-12 and IL-18 are produced by Th1 and

NK cells and act as synergistic stimulators of IFN-γ and enforce the probiotic response toward mononuclear cells. LPS will increase the activities of NK cells through mechanisms that involve IFN-γ. Hence, NK cells become more efficient and toxic (Salata *et al.*, 1984). However, in our in vivo study, the number of NK cells had continued to decrease after LPS exposure, even though probiotics were administered beforehand. The NK cell count did not significantly differ between the probiotic-LPS and LPS-only groups.

4. Conclusions

The results of this study showed that multiple probiotic species can regulate the innate immune response through dendritic cells, but not NK cells, in Balb/c mice.

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EFFECT OF PROBIOTIC ON INNATE IMMUNE RESPONSE IN THE LIVER OF *MUS MUSCULUS* BALB/C

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ABSTRACT

Microflora in the gastrointestinal tract plays an important role in the hepatocyte function from the gut-liver axis. However, the effect of probiotic in innate immune response especially in Kupffer cells is still unclear. This study aimed to examine the effect of probiotic on the innate immune response in the liver. This study is a randomized posttest-only control group experimental animal study using white mice (*Mus musculus* BALB/c). The inclusion criteria are 10–12 weeks old, male, and weighing 30–40 mg. Samples were randomized and divided into two groups: probiotic and placebo. The probiotic group was given multispecies probiotic. Probiotic and placebo were administrated for 21 days via a gastric tube. On day 22, necropsy was performed, and liver was obtained for immunohistochemical examination at the Laboratory of Biochemistry, University of Brawijaya, Malang. Number of Kupffer cells and cells which expressing NF-κB p105 and p65 were examined. A total of 16 mice met the inclusion criteria. A significant increase in the number of Kupffer cells ($p<0.001$) and NF-κB p105 ($p=0.001$) was observed after administration of probiotic. No significant differences were observed in NF-kB p65 ($p=0.236$). Administration of probiotics affects the innate immune response (NF-κB p105 and Kupffer cell) in the liver tissue but not in NF-κB p65.

1.Introduction

In Indonesia, the prevalence of liver disease in children is still relatively high, although the actual prevalence is still unknown (Hadi, 2000). The incidence of liver disease in infants is 1 from 2,500 live births with atresia biliary, metabolic abnormalities, and neonatal hepatitis. Thus, older children experience metabolic disorder, intrahepatic chronic cholestasis, and obesity-related steatohepatitis (Arya and Balistreri, 2002).

Microflora in the gastrointestinal tract has an important role in hepatocyte cell function (Gratz et al., 2010; Iacono et al., 2011; Jonkers and Stockbrügger, 2007). A symbiotic relationship exists between the liver and the digestive tract, known as the gut-liver axis (Imani Fooladi et al., 2013; Jonkers and Stockbrügger, 2007). The functional relationship between the intestines with the liver includes the balance of immunological responses (Imani Fooladi et al., 2013; Lata et al., 2011; Miyake and Yamamoto, 2013).

Probiotics is known to have a protection effect in the gastrointestinal tract and is explained through a variety of mechanisms (Boirivant and Strober, 2007; Pagnini et al., 2010; Yan and Polk, 2011).

Probiotic effects in animal studies have demonstrated increasing innate immune response through enhancement of TLR-2, TLR-4, and transcription factors NF-kB p65 and p105 (Hegazy and El-Bedewy, 2010; Petrof et al., 2004; Yao et al., 2017).

The role of probiotics through epitope lipoteichoic acid will be captured by lipoteichoic-binding protein that will be recognized by TLR-2 and TLR-4 in dendritic cells, which migrate through the lymphatic tract. Dendritic cells will interact with Kupffer cells in the liver, therefore resulting in innate immune response (Boirivant and Strober, 2007; Thomas and Versalovic, n.d.; Trivedi and Adams, 2012; Yan and Polk, 2011).

Based on this idea, we studied the effects of probiotic to the innate immune response in the liver.

2. Materials and methods

This study used randomized posttest-only control group design conducted in Biochemistry Laboratories Universitas Airlangga, Surabaya, from February until May 2008. Ethical approval was issued by the Veterinary Faculty Research Ethical Committee with ethics certificate number 034-KE/ II/ 2008.

2.1. Samples

We used the animal study model obtained from Pusat Veterinaria Farma Surabaya. Sixteen *Mus musculus* (BALB/c) mice aged 10–12 weeks old, male, and approximately 30–40 gr were adopted for 1 week before starting treatment (Figure 1). The mice were divided into two groups: probiotic and placebo. The probiotic group was given multispecies probiotic for 21 days. The placebo group was given a placebo for 21 days via a gastric tube. Necropsy was performed on the probiotic and placebo groups on day 22. The liver was dissected for analysis.

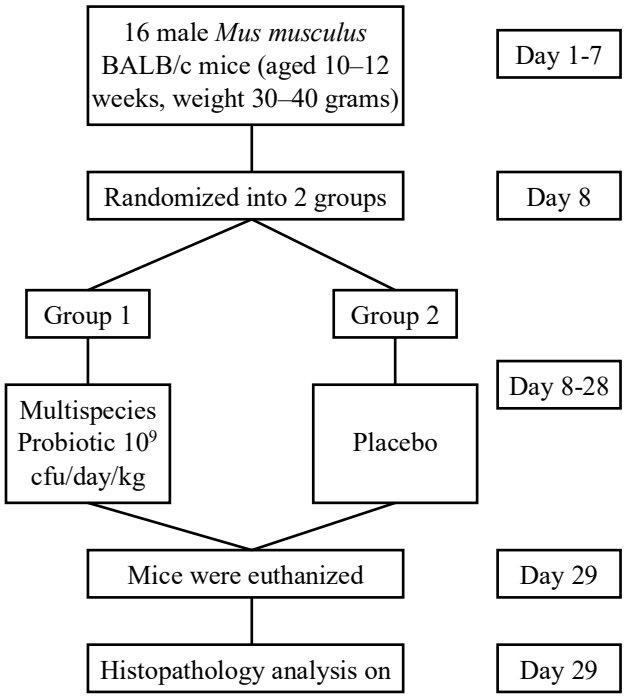

Figure 1. Animal treatment

2.2. Probiotic

The probiotic group used multispecies probiotic with composition *Lactobacillus casei* PXN 37, *Lactobacillus rhamnosus* PXN 54, *Lactobacillus acidophilus* PXN 35, *Lactobacillus bulgaricus* PXN 39, *Bifidobacteria breve* PXN 25, *Bifidobacteria infantis* PXN 27, *Streptococcus thermophilis* PXN 66, and fructooligosaccharide packed in an aluminum foil sachet. Probiotic was administrated by dissolving in 0.5 ml D5% and dose of 10^9 cfu/day/kg animal weight via a gastric tube once daily for 21 days.

2.3. Immunohistochemistry

The liver section was cleaned in a 10% formalin buffer solution, followed by dehydration, clearing, impregnating, and embedding. Immunohistochemistry was performed to determine the innate immune response. The liver section was probed with NF-κB p65 monoclonal antibody (33-9900; Thermo Fisher Scientific, Waltham, MA, USA) and p105 monoclonal antibody (GTX60465; GeneTex Inc., Irvine, CA, USA). The Kupffer cell was probed with CD68 monoclonal antibody. Number of Kupffer

cells and cells which expressing NF-κB p65 and p105 were counted the mean number of cells within 20 random fields under a light microscope (CX21; Olympus, Tokyo, Japan) at 1000x magnification.

2.4. Statistical analysis

Data were analyzed using SPSS version 22 software. Descriptive analysis was conducted to determine the immune response profile in each group and difference in profile changes from the probiotic and control groups. Differences between groups were analyzed by the t-test for variables that were normally distributed and the Mann–Whitney test for variables that were not normally distributed.

3.Results and discussions

A total of 16 mice were included in this study, all of which were male. They were divided into two groups. The characteristics of the subject are described in Table 1.

A significant difference was observed between the Kupffer cell count and NF-κB p105 in the probiotic group compared with the control group. By contrast, no significant difference was observed between NF-κB p65 in the probiotic group and that in the control group, as shown in Table 2. The liver section that had been stained with immunohistochemistry, which showed number of Kupffer cells and Kupffer cells that expressed NF-κB p105 and NF-κB p65 in mice is shown at the figure 2, figure 3 and figure 4.
Innate immune response in the liver is a defense mechanism because it is a physical barrier and stimulates the adaptive immune system. The Kupffer cell is the main macrophage in the liver and plays an important role in normal physiology and homeostasis. It participates in the acute and chronic response in the liver with toxic substances and acts as a liver protector (Roberts et al., 2006).

Innate immune response activation also stimulates the Kupffer cell to produce hepatoprotective cytokine, IL-6, and IL-10 during alcoholic liver disease and express TLRs and main cytokine producer pro- and anti-inflammation (Gao, 2012; Szabo et al., 2007).

Similar to a previous study by Neuman using the probiotic *Lactobacillus acidophilus* UFV-H2b20, this *Lactobacillus acidophilus* can survive in stressful conditions in the gut. Furthermore, the probiotic group had two times more Kupffer cells that are responsible for bacterial clearance and can stimulate nonspecific immune response (Neumann et al., 1998).

Corbitt et al. (2013), in their study, showed a strong correlation between probiotic with the Kupffer cell maturation status and its function. Gut bacteria will release unique composition *microbe-associated molecular patterns* in the circulation, which can upregulate LSEC ICAM-I expression, that influence the number and function of Kupffer cells in the liver.

Giving probiotic in mice significantly increased NF-κB p105 compared with the control group. Miyoshi et al. (2001), studied three rats as cholestasis animal models showing that NF-κB is activated in the hepatocyte. The NF-κB activation function decreases hepatocyte apoptosis and liver damage. The ability of bile acids to become potentially toxic in activating NF-κB might cause pathological adaptation that helps survival and continues the hepatocyte function.

The NF-κB function bridges the innate immune system and the adaptive immune system by releasing an inflammatory mediator (Luedde and Schwabe, 2012; Seki and Schnabl, 2012).

However, no significant difference in NF-κB p65 was observed between the treatment and the control group. This result was caused by the fact that the animal models in this study were a healthy sample not found in the inflammation model. Proteolysis NF-κB p105 becomes NF-κB p65, and NF-κB p50 is triggered by inflammation (Baud and Derudder, 2011; Oeckinghaus and Ghosh, 2009).

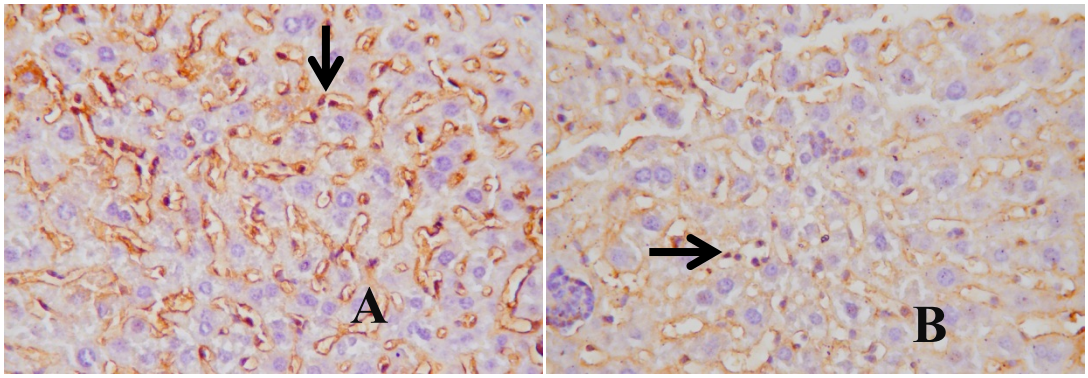

Figure 2. The liver section stained with immunohistochemistry method with monoclonal antibody anti mouse CD68 with 1000x magnification. The arrows pointed the Kupffer cells with expressed CD68 as the antibody marker for Kupffer cells.
A. Probiotic group; **B.** Control group.

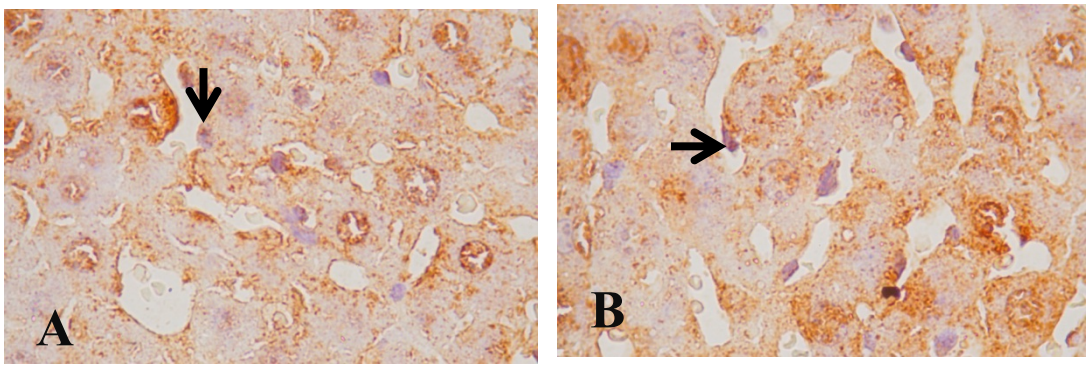

Figure 3. The liver section stained with immunohistochemistry method with monoclonal antibody anti mouse NF-kB p105 with 1000x magnification. The arrows pointed the Kupffer cells with expressed NF-kB p105 as the antibody marker for Kupffer cells.
A. Probiotic group; **B.** Control group

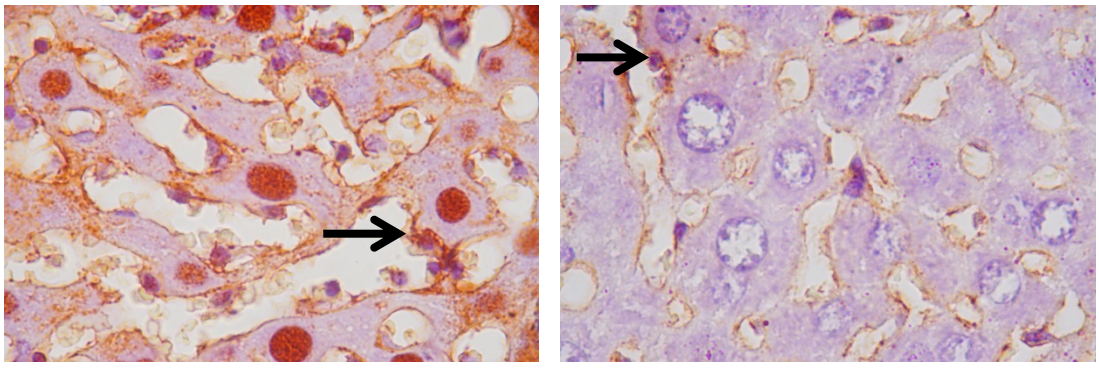

Figure 4. The liver section stained with immunohistochemistry method with monoclonal antibody anti mouse NF-kB p65 with 1000x magnification. The arrows pointed the Kupffer cells with expressed NF-kB p65 as the antibody marker for Kupffer cells.
A. Probiotic group; **B.** Control group.

This result did not agree with previous studies on the gut due to the difference in organ and immune response. Moreover, probiotic was administrated through the gastrointestinal tract, so the stronger immune response occurred in the gut and the weaker immune response occurred in the liver (Darma et al., 2009; Trivedi and Adams, 2016).Previous studies showed that giving the probiotic group with LPS increased TLR-2 and TLR-4 activation and NF-κB p50 and p65 activation. Administration of probiotic decreased TLR-2 expression and NF-κB p50 activation caused by LPS, which was expected to decrease the inflammation reaction (Luh Putu HM et al., 2011).

Expressing the protein NF-κB can give specificity in responding to some stimulus. NF-κB p50 and p65 play an important role in IL-6 production in synovial fibroblast and are closely involved in inflammation gene activation with IL-1 or TNF-α in human monocytes (Seki and Schnabl, 2012). However, inhibition of NF-κB can reduce the viability of hepatocytes besides beneficial results (Luedde and Schwabe, 2012).

Xu et al. (2011), in his animal model research, suggested administration of probiotic as a safe and cheap therapy for nonalcoholic fatty liver disease because oral probiotic supplementation has been proven to decrease liver fat accumulation.

This study has limitations because mice were used, although mice share many similarities with humans in the immunologic aspect (Mestas and Hughes, 2004) and need pathogen as an inflammation inducer. Further studies are needed to describe the probiotic mechanism in the immunological aspect.

4. Conclusions

Administration of probiotic influences the innate immune response in the liver with increasing Kupffer cells and number of Kupffer cells that express NF-κB p105 in mice. However, no influence was observed in the number of Kupffer cells that express NF-κB p65 in mice.

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THE EFFECT OF PROBIOTICS, PREBIOTICS AND SYNBIOTICS IN FORMULA MILK TOWARDS THE INCIDENCE OF ACUTE RESPIRATORY INFECTION ON 1-5 YEARS OLD HEALTHY CHILDREN IN DAY CARE CENTRES

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ABSTRACT

The incidence and the transmission of acute respiratory infection (ARI) in children, especially those at daycare centres (DCCs), are still high. The aim of this study is to investigate whether probiotics, prebiotics, or synbiotics in formula milk can lower the incidence and duration of ARI in healthy children at DCCs. This randomized, double-blind, and placebo-controlled clinical study was performed in 12 DCCs. Healthy children aged 1-5 years were recruited. The subjects were divided into four groups (probiotic, prebiotic, synbiotic and control); the intervention lasted for 26 weeks. Statistical analysis included analysis of variance, Fisher's exact test, chi-squared, Relative Risk Reduction (RRR), Absolute Risk Reduction (ARR) and Number Needed to Treat (NNT). Chi-squared analysis for ARI incidence and duration for the probiotic and synbiotic groups showed significantly different results compared to the control group ($p < 0.05$). The NNT calculation showed that synbiotics provided the best prevention from ARI (NNT = 6.25). The use of probiotics, and synbiotics decreased the incidence of ARI in healthy children, but the best prevention for ARI was conferred by synbiotics.

1.Introduction

Acute respiratory infection (ARI) in children, especially 1-5 year olds, is still a dominant disease on multiple levels: primary health care (Puskesmas), hospitals and referral hospitals (Kementerian Kesehatan Republik Indonesia, 2007). In RSUD Dr Soetomo, ARI patients were recorded at 12.7% in 2007, and this incidence increased to 13.4% in 2008 (Unit Rawat Jalan RSUD Dr Soetomo, 2008). Meanwhile, according to Wald, the risk of contracting ARI in daycare centres (DCCs) is 1.5-3-times higher than those being cared for at home (Wald, Guerra and Byers, 1991). Participation in DCCs is a great infection risk factor for children (Louhiala *et al.*, 2001). Often, exposure to infection sources, antibiotic treatments (incorrect according to the indication) and decrease in body immunity are causes of the high ARI incidence (Wantania, Naning and Wahani, 2008). This problem drives scientists to find new alternatives for decreasing ARI risk, namely by increasing body resistance. Thus, the increased interest towards probiotic bacteria is quite understandable.

There are several clinical studies that tested the effect of probiotics in respiratory infection in

healthy subjects. These studies concluded that probiotics can reduce the severity of respiratory infections and the incidence of lower respiratory tract infection (Hattaka, 2001; Cobo Sanz, Mateos and Munoz-Conejo, 2006). Meanwhile, microflora begins to spread starting from when a baby is born. There are four phases of normal intestinal flora development (Mack *et al.*, 1999): early inception of external microbes (1st and 2nd week of life), the breastfeeding period, complimentary food and breastfeeding cessation and finally conversion to an adult microbiota pattern. During the third and fourth phases, exposure to the environment is very high, and the child will be more prone to respiratory infections, especially those who are cared for in DCCs, where infectious diseases are common. Therefore, there are three strategies to improve the colonisation of normal microflora: elevate the number of normal microflora (probiotic), increase the nutritional substances that will elevate the growth of probiotic bacteria (prebiotic) and combine both microflora and nutritional substrates (symbiotic; Markowiak and Śliżewska, 2017).

Nevertheless, evidence regarding the clinical benefits of these supplements are still sparse, and therefore the effect of prebiotics, probiotics and synbiotics towards preventing ARI is not yet clearly known and requires better evidence through clinical trials. This research's objective was to examine the effects of prebiotics, probiotics and synbiotics contained in formula milk towards preventing ARI for 1-to-5-year-old children attending DCCs, with the ultimate goal of reducing the ARI incidence in DCCs.

2. Materials and methods

This double-blind, randomised and controlled trial examined healthy 1-5-year-old children at DCCs, each of whom were given prebiotics, probiotics, synbiotics or placebo (control). This research was ethically approved by the Ethical Commission of Airlangga University Faculty of Medicine, Surabaya, and all respondents were entitled to health protection from health insurance, which covered the maintenance of outpatient and inpatient care during the research and 6 months after it finished. The parents of all participants provided consent for their participation in the study.

2.1. Samples

Subjects were recruited at 12 DCCs: Soetomo A DCC, Soetomo B DCC, Airlangga DCC, Ngagel DCC, BPPLSP DCC, Taman Ceria DCC, Telkom DCC and Harapan Aisyah DCC (Surabaya); Intan Citra DCC, Intan Tamasa DCC and Miftahul Jannah DCC (Sidoarjo); and Aisyah DCC (Gresik). The treatment was performed over 6 months (26 weeks); all participants met the inclusion criteria. Subjects were excluded if they were sick since the beginning of the research, lactose intolerant, had a disability/hereditary disease that interferes with respiratory, cardiovacular or digestive system, were allergic to cow's milk and/or had a history of consuming probiotics/prebiotics or food/drinks that contain them during the prior 2 weeks. All samples were collected from the 12 DCCs and were made in order according to the participation start date. The subjects were then randomised to determine groups (A through D). Randomisation was performed by *block random sampling*; there were four groups: prebiotic (P1), probiotic (P2), synbiotic (P3) and formula milk-only or control (P4).

2.2. Probiotic, Prebiotic, Synbiotic, Control

The probiotic treatment contained *Bifido* BB12 and *Lactobacillus casei* CRL 431, 1 x 10^9 colony forming units (CFU)/feeding (within 30 g formula/150 mL water). The prebiotic treatment contained galactooligosaccharide (GOS); as much as 840 mg was given per feeding (within 400 mg formula/100 mL water). The synbiotic treatment contained *Bifido* BB12, *L. Casei* CRL 431 and GOS, 1 x 10^9

CFU/feeding (within 30 g formula milk/150 mL water). The control contained only formula milk (30 g formula/150 mL water).

The treatments were given seven days a week. Each day, the subject consumed a minimum of 600 mL milk (within the composition of 1 part/30 mL). Milk was given three times at the daycare and the rest at home as needed for the day. If milk consumption was less than 600 mL per day, the participant was excluded.

2.3. Statistical analysis

All subjects completed a daily form, which contained information about body weight, milk consumption, temperature and ARI signs and symptoms during the 26-week study. The research team called to check if there were any complaints. Once a week, a medical check-up was performed by the research medical team. Data was compiled and analysed using SPSS. The statistical methods included chi-squared, analysis of variance (ANOVA), Mann-Whitney U test, Kruskall-Wallis test and Fisher's exact test.

Fisher's exact test, the independent t-test and ANOVA were used to test the differences in the incidence of ARI infections. ANOVA was used to test for differences in ARI episode length (in days) in the probiotic, prebiotic, synbiotic and control groups. Relative risk reduction (RRR), absolute risk reduction (ART) and number needed to treat (NTT) were also calculated. RRR is the percentage difference in event rates between treatment and control groups, and thus it shows the possibility of ARI events in the treatment group in proportion to the probability of ARI events in the control group. ARR is the arithmetic difference between event rates over a fixed period of time, while NNT is the number of patients who must be treated during a fixed period of time in order to prevent an event from happening.

3.Results and discussions

At the beginning of the study, there were a total of 267 children from 12 DCCs in Surabaya, Sidoarjo and Gresik. Twenty-three children were unable to continue participating due to the exclusion criteria (13 children were allergic to cow's milk, 6 children moved out of town and 4 children rarely drank milk). Two weeks of adaptation were performed before randomisation. During the adaptation period, the formula milk usually consumed by the subjects was gradually substituted with the new formula milk used for the research (without adding any substance). The formula milk's cover and brand were sealed. After the adaptation period, 28 children were unable to continue participating in the research due to several reasons (12 children did not like the milk's taste, 5 children had prolonged diarrhea, 5 children moved out of town and 6 children's parents refused to continue participating).

The remaining children were randomised and divided into four groups: 55 children in the prebiotic group, 53 children in the probiotic group, 54 children in the synbiotic group and 54 children in the control group. During the 26-week treatment, 3 children were unable to continue participating. Thus, only 213 children completed the research. The charcteristics of the 213 subjects are presented in Table 1.

Table 1. The characteristics of study subjects by group.

Characteristic	Prebiotic (P1)	Probiotic (P2)	Synbiotic (P3)	Control (P4)	p-value
Age (Months)	38.57 ± 11.8	35.75 ± 13.7	34.57 ± 12.3	36.75 ± 12.9	0.423
Male Female	31 (58.5%) 22 (41.5%)	26 (49.1%) 27 (50.9%)	23 (42.6%) 31 (57.4%)	21 (39.6%) 32 (60.4%)	0.214
Good Nutrition Mild Malnutrition	48 (90.5%) 5 (9.5%)	47 (88.7%) 6 (11.3%)	46 (85.2%) 8 (14.8%)	47 (88.7%) 6 (11.3%)	0.856
Weight (kg)	13.62 ± 3.5	13.04 ± 3.3	13.20 ± 3.7	12.77 ± 3.1	0.450
Siblings ≤ 1	40 (75.5%)	41 (77.4%)	40 (74%)	38 (71.7%)	0.997
> 1	13 (24.5%)	12 (22.6%)	14 (26%)	15 (28.3%)	
Smoker at home Yes No	31 (58.5%) 22 (41.5%)	27 (51%) 26 (49%)	33 (61%) 21 (39%)	30 (56.6%) 24 (43.4%)	0.385
History of ARI ≤ 2 >2	39 (73.6%) 14 (26.4%)	32 (60.4%) 21 (39.6%)	33 (61%) 21 (39%)	36 (68%) 17 (32%)	0.332
Milk Consumption (average mL/day)	1322.38 ± 527.33	1359.13 ± 610.45	1276.67 ± 520.67	1170.51 ± 532.28	0.320

Table 2. Comparison of ARI incidence rate, RRR, ARR, NNT and average length (in days) of ARI episodes in probiotic, prebiotic, synbiotic and control groups.

	Prebiotic (P1)	Probiotic (P2)	Synbiotic (P3)	Control (P4)	p
N	53	53	54	53	-
Incidence Rate	0,036[a]	0,030[b]	0,026[c]	0,044	0,0429
RRR*	8%	25%	27%	-	-
ARR*	5%	15%	16%	-	-
NNT*	20	6,6	6,25	-	-
Days/episode	2,62±2,92[d]	2,26±3,23[e]	1,78±2,26[f]	5,85±8,28	0,041

[a] *p = 0.247*
[b] *p = 0.045*
[c] *p = 0.0079*
[d] *p = 0.157*
[e] *p = 0.031*
[f] *p = 0.010*

From the table 2, the ARI incidence in prebiotic, probiotic, synbiotic and control groups showed significant p result among those four groups (p=0,0429), Chi Square test was further performed to compare between groups. From the chi square test results between Probiotic and Control groups, and Synbiotic and Control groups, significantly different results were obtained (p<0,05), while the chi square test result between Prebiotic and Control groups obtained unsignificantly different result (p>0,05).

The ARI incidence was significantly different among the treatment groups ($p = 0.0429$; Table 2). A chi-squared test was performed to compare between groups; the probiotic compared to control and synbiotic compared to control were significantly different ($p < 0.05$), but there was no difference between the prebiotic and Control groups ($p > 0.05$; Table 2).In the prebiotic group, the RRR was 8%, a value that indicates there will be a reduction in ARI events in as many as 8% of subjects after prebiotic treatment. The prebiotic group ARR was 5%, which results in an NNT of 20. These results imply that we should treat 20 healthy children for 6 months to prevent ARI from occuring. In the probiotic group, the RRR was 25%; there would be a reduction in ARI events as many as 25% of subjects after probiotic treatment. The probiotic group ARR was 15% and the NNT was 6.6; thus, we should treat 6.6 healthy children for 6 months to prevent ARI. Finally, the synbiotic group RRR was 27%, a value that indicates there will be a reduction in ARI events in as many as 27% of subjects after synbiotic treatment. The synbiotic group ARR was 16% and the NNT was 6.25, so we should treat 6.25 healthy children for 6 months to prevent ARI from occuring.

The length per ARI episode was 2.62 (standard deviation [SD] 2.92) days for the prebiotic group, 2.62 (SD 3.23) days for the probiotic group, 1.78 (SD 2.26) days for the synbiotic group and 5.85 (SD 8.28) days for the control group. There was a significant difference in this measure among the groups, where the control group value was higher than the other three groups ($p < 0.05$). Chi-squared test results showed significant differences between the probiotic and control groups and the synbiotic and control groups ($p < 0.05$), but no difference between the prebiotic and control groups (Table 2). Thus, the probiotic and synbiotic group were more effective in reducing the ARI duration. To evaluate the homogeneity of the sample, we analysed numerous variables between the treatment and control groups before the study began. Variables analysed included: subject characteristics (age, gender, weight and nutritional status), environmental factors (number of siblings, smokers in the house and ARI and allergy history from the last 3 months) and random factors such as antibiotic usage. There were no differences between the treatment (probiotic, prebiotic and synbiotic) and control groups. Thus, there were no specific conditional differences among the groups before the study commenced.

There was a decrease in ARI cases in the prebiotic compared to the control group, but this difference was not significant. Further, the length per ARI episode between the prebiotic and control groups was not significantly different. Thus, prebiotic exposure did not affect ARI incidence or length. This finding may be related to the subjects' age (11-59 months with an average of 38.57 ± 11.8 months). At that age, the amount of *endogenous* normal flora has begun to decline, and thus the administration of prebiotic (nutrition for endogenous normal flora) at that age would be heavily dependent on the remaining amount of endogenous normal flora. If the amount is still adequate, then the resulting mucosa colonisation will be strong and provide mucosal protection and adequate adaptive immunity, which will ultimately provide protection from ARI (Fijan, 2014).

Research on the effect of prebiotics towards ARI was previously done using a randomised

double-blind study with 259 aterm babies with a history of an atopic parent. Half (129 babies) were given fructooligosaccharides (FOS) and GOS supplements while the remaining 130 babies comprised the control group. The treatment was administered from the first 2 weeks to 6 months of life. Infectious disease, ARI, urinary tract infection and the relapse of infectious disease in both groups was evaluated. The prebiotic group had fewer ARI cases, reduced ARI relapse and less antibiotics usage compared to the control group (Arslanoglu *et al.*, 2008).

The probiotic group showed better results than the prebiotic group. The ARI incidence of 0.030 represented a 25% decrease when compared to the control group. The probiotic group would require treating 6.6 children to prevent 1 child from contracting ARI. This finding is in line with a previous study that showed probiotics significantly decrease symptom severity, common cold duration and fever length, although there are no apparent effect on the overall incidence of respiratory tract infections (de Vrese *et al.*, 2006).

The synbiotic group showed an incidence of 0.026, which, when compared to the control group, represented a 27% decrease in ARI cases. The synbiotic group would require treating 6.25 children to prevent 1 from contracting ARI. Research on the effect of synbiotics towards ARI was examined in a previous randomised, double-blind clinical study. The trial, performed between November 2000 and March 2003 with 1,018 pregnant mothers with a history of atopy, were given synbiotic supplementation, consisting of four probiotics (*Lactobacillus rhamnosus* GG and LC705*, Bifidobacterium breve* Bb99 and *Propionibacterium freudenreichii* ssp) and GOS for 4 weeks before giving birth and during the first 6 months of the baby's life. The symbiotic group exhibited fewer ARI cases and reduced antibiotic prescriptions compared to the control group (Kukkonen *et al.*, 2006). These results show that both prebiotic and probiotic exposure are beneficial to prevent ARI, but the prebiotic effects in this study were statistically insignificant. This data fits the theory of a common mucosal immune system (CMIS) pathway to boost the immune response, by treatment in the intestinal mucosa, and the resulting immune response on other mucosa, namely the respiratory mucosa.

Although the probiotics and synbiotics were more effective in boosting the immune response through the CMIS pathway compared to prebiotics, even when the mild probiotic protective effects against ARI are overcome by the infection, the remaining immune response is still effective in decreasing the length of the ARI episode. This phenomenon may be explained by previous research where administration of fermented milk that contained the probiotic *L. casei* DN 114001 elevated natural killer (NK) cell activity and increased monocyte capacity compared to the control group (Parra *et al.*, 2004). Meanwhile, another study that involved consuming milk that contained *Bifidobacterium lactis* HN019 showed increased phagocyte capacity on peripheral polymorphonuclear leucocytes compared to placebo (Arunachalam, Gill and Chandra, 2000). Also, consumption of formula milk with *Lactobacillus reuteri* or *B. lactis* BB-12 for 12 weeks did not decrease the average number of respiratory tract disease, but did reduce the illness episode, absence from daycare and antibiotic prescription for babies age 4-10 months (Weizman, Asli and Alsheikh, 2005).

4. Conclusions

ARI incidence in children aged 1 to 5 years at DCCs decreased by 8%, 25% and 27% after receiving formula with probiotics, prebiotics and synbiotics, respectively, compared to the control group. ARI duration length (days per episode of ARI) was significantly reduced with the addition of probiotics and synbiotics but not with prebiotics in formula milk. The best

prevention for ARI, shown by NNT, is provided by synbiotics.

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EFFECTS OF HEAT-KILLED PROBIOTIC COMPLEX ON ACUTE DIARRHOEA IN CHILDREN

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ABSTRACT

Probiotics are known in paediatrics as a new alternative treatment for diarrhoea. Some studies have found that a heat-killed probiotic complex (HKPC) has the same potential effects as active probiotics on diarrhoea in children. However, there is little research on this subject. We conducted a study to examine the HKPC effect on duration of illness and degree of recovery from acute diarrhoea in 6–24-month-old children. This study is a randomised, double-blind, controlled clinical trial with children aged 6–24 months with acute diarrhoea. The children were divided into two groups: one received HKPC and the other placebo. Stool samples were collected prior to treatment to determine the presence of rotavirus and the evidence of fat and/or lactose malabsorption. All the children were observed regarding the duration of diarrhoea, stool frequency, and stool consistency until they recovered. A total of 98 children met the selection criteria. Rotavirus was found in more than half of the stool samples (53%). Fat malabsorption was present in 46% of the samples, yet lactose malabsorption was detected in only 8% of the samples. The HKPC group had a shorter recovery time (3 days) than the placebo group (4 days), which is not statistically different ($p=0.100$). There were no significant differences in recovery levels from the first day until the last day of observation ($p=0.487$). According to the result, administration of HKPC has no significant effect on duration of illness and level of recovery in 6–24-month-old children with acute diarrhoea.

1. Introduction

Diarrhoea is the main cause of child morbidity in developing countries. The mortality rate of diarrhoea in children is 1.4 million per year globally, with the highest prevalence in developing countries such as India, Nigeria, Congo, Pakistan, and China (Black *et al.*, 2010). The incidence rate of diarrhoea is high in children under the age of 2, with the peak rate in children aged 6–24 months who start to eat other foods and reduce breast milk intake (Soeparto *et al.*, 1999; Ansari *et al.*, 2012). The most common causes of diarrhoea associated with high mortality are rotavirus, followed by *Cryptosporidium* spp and *Shigella* spp (Troeger *et al.*, 2017). Cohort studies have shown that nearly all children suffer at least one rotavirus infection before the age of 5, independent of their socioeconomic status (WHO, 2009). Acute diarrhoea caused by infection can reduce lactose absorption. It damages the small intestinal mucosa and decreases the lactase enzyme, which leads to decreased lactose absorption. This, along with decreased fat absorption, leads to increased lactose and fat in the stool (Khani *et al.*, 2012).

Probiotics are known in paediatrics as a new alternative treatment for diarrhoea, due to their beneficial functions in the gastrointestinal tract.

They produce bacteriocin, which acts as a competitive inhibitor, decreasing the bacterial growth and strengthening the tight junctions in the brush border. Some studies have shown that probiotics could favour the recovery phase in virus-associated diarrhoea by enhancing pathogen-specific secretory IgA production and inhibiting viral multiplication (Ohland and MacNaughton, 2010).

A heat-killed probiotic complex (HKPC) is a preparation with inactive probiotic bacteria or killed probiotics. It is more stable in heat and prolonged storage. Though it is inactive, the complex still contains bacteriocin, lactase, and the deoxyribonucleic acid (DNA) of the probiotic bacteria. These have the function of decreasing inflammation and stopping cell necrosis. It has been stated that HKPCs have the same beneficial potential as living probiotics (Ng *et al.*, 2009). A study by Supriatmo (2006) demonstrated that HKPCs are better than living probiotics at shortening the duration of diarrhoea in children.

In this study, we examined the effect of HKPC on the duration of illness and the degree of recovery from acute diarrhoea in 6–24-month-old children.

2. Materials and methods

2.1. Materials

2.1.1.Samples

All 6–24-month-old children with acute diarrhoea (described as watery diarrhoea occurring more than 3 times per day, for less than 3 days before hospitalisation) in the Child Health Department of Dr Soetomo Hospital from April 2008 to August 2008 were included in this study. Children with a history of probiotic, antibiotic, or zinc administration prior to hospitalisation and with severe comorbidities or malnutrition were excluded from this study. The study design described in figure 1.

The duration of illness was determined by the mean duration of diarrhoea in days after being hospitalised until recovery. Recovery level was determined based on frequency of defecation and consistency of stool, and was classified as the following:

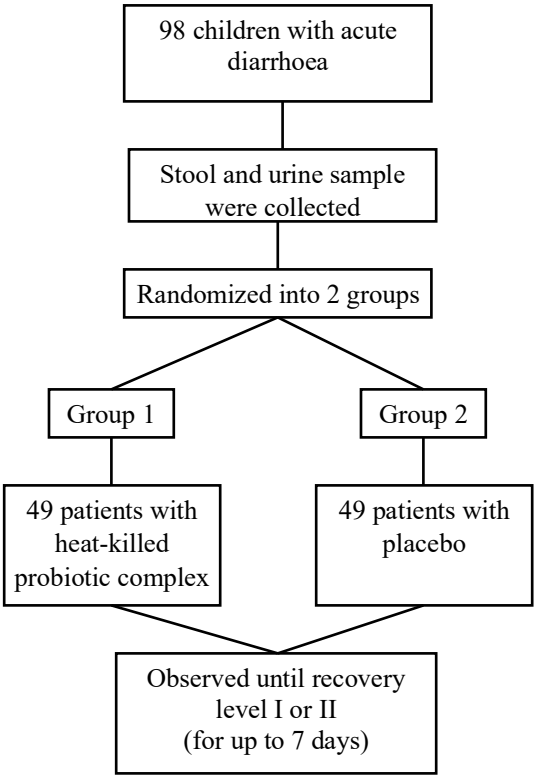

Figure 1. Subject treatment

(1) recovery level I: solid stool consistency no more than 3 times per day; (2) recovery level II: mushy stool consistency no more than 3 times per day; (3) recovery level III: watery stools with lumps no more than 3 times per day; (4) no recovery: watery diarrhoea more than 3 times per day until the seventh day of diarrhoea with standardised treatment. Stool consistency was observed according to the Bristol stool chart.

2.1.2.Heat-Killed Probiotic Complex

In this study, we used a manufactured heat-killed probiotic complex (Dialac®) with tyndallized lyophilisate of *Lactobacillus acidophilus* as an inactive probiotic at a dose of $3x10^{10}$ CFU/day. This HKPC also contains bacteriocin, lactase enzyme, bacterial CpG-DNA, vitamins B2, B3, B6 and C, thiamine, zinc, calcium, sweetener and fruit flavouring. The product is packaged in sachets with a net weight of 1 gram. The control group received a similar sachet, but it contained only 300 mg of Saccharum lactis (placebo).

2.2. Methods

The Ethical Research Commission of Dr Soetomo General Hospital in Surabaya approved this study. This study is a randomised, double-blind, controlled clinical trial in which randomisation was done by a third party. Each child meeting the selection criteria was examined and a stool sample was collected for laboratory analysis. The children were then randomised into two groups: an HKPC group and a control group. They received standard treatment for diarrhoea and were given a sachet of HKPC or placebo twice a day until they reached recovery level I or II. All the patients were evaluated every day in terms of defecation frequency and faecal consistency for up to 7 days until they reached recovery level I or II.

The stool and urine examination was done only once before the HKPC or placebo administration. The urine samples were collected using the sterile urine collector for at least 5 ml. The stool samples were collected using a sterile pot. Approximately 5 grams of stool sample, taken from the middle part of the stool, was sent inside the sterile pot to the laboratory for the examinations within 3 hours. A rotavirus kit (Meridian Bioscience, Immuno*Card* STAT!® Rotavirus Devices 750030, USA) was used to detect rotavirus antigen in the stool using the qualitative immunochromatic assay method. Approximately 0.25 µL (if liquid or semi-solid) or 2 mm^3 (if solid) of the stool sample was diluted with 350 µL of sample diluent (a buffer containing 0.1% sodium azide) in a 12x75 mm glass test-tube and homogenised with vortex mixer (QL System, MX-2500 Vortex Mixer, UK) for ten seconds. The diluted sample (as much as 150 µL) was transferred to the sample port then incubated for ten minutes at room temperature (25 to 27°C). The result was obtained by visually reading the control and test zones for the presence or absence of a line at the end of the incubation period. The test result was positive if the test and control lines were both visually detectable.

Every stool sample was also analysed using the Clinitest® tablet (Bayer HealthCare, Mexico) to detect any lactose malabsorption by detecting unabsorbed sugars in the stool. Approximately 2 grams of the stool was diluted with twice its volume of distilled water (RPI, Distilled Water W20525, USA) in a 12x100 mm glass test-tube, then mixed with a vortex mixer. Fifteen drops of this mixture were transferred to a test-tube, a Clinitest tablet was added, then boiled and wait for 15 seconds after boiling. The result was observed at the end of 15 seconds and compared to the Clinitest® colour chart. The amount of reducing substance present was rated as 0%, 0.25%, 0.5%, 0.75%, 1%, or 2%. A result of 0.5% or more indicated the presence of an abnormal amount of sugar in the stool.

For every stool sample, the floating test was done to detect any fat malabsorption. 3 grams of the stool sample was gently placed in a 100 mL glass flask containing 30 mL of distilled water (RPI, Distilled Water W20525, USA) at room temperature (25 to 27°C), and its floating or sinking position was observed within 30 seconds. If the stool floated, it indicated the presence of steatorrhoea.

Both the stool and urine samples were examined for bacterial identification. The urine and stool samples were cultured in MacConkey agar (Merck Millipore, MacCONKEY Agar 105465, Germany) for 18-24 hours to isolate the bacteria colony. Gram staining was done to confirm and choose the right panel for bacteria identification. The colony then suspended into the Phoenix™ ID broth (BD Diagnostic, Phoenix™ ID broth 246001, USA) with inoculum density 0,5 McFarland (BD Diagnostic, BD PhoenixSpec™ nephelometer 440910, USA). The bacteria identification was using BD Phoenix™ Automated Microbiology System (BD Diagnostic, Phoenix™ 100 instruments 448100, USA) with the panel for gram positive bacteria is PMIC/ID-55 and gram negative bacteria is NMIC/ID-4.

The collected data was analysed statistically using SPSS Windows Release 12.00 software. Descriptive analysis was carried out to compare the duration of illness, frequency of defecation, stool consistency, and recovery levels between the two groups. Lactose and fat malabsorption

tests and the rotavirus test were carried out for the sample median, modus, and proportion. The differences between the two groups were analysed using Chi Square for normally distributed variables and the Mann–Whitney U test for non-normally distributed variables.

3. Results and discussions

A total of 98 eligible patients were included in the study. They were divided randomly into equally sized HKPC and control groups (49 patients each). None of the patients met the exclusion criteria or withdrew from the study. The average age of the selected patients were 11 months old, with almost equal proportions of males (53%) and females (46%). The nutritional status of all the patients in this study was predominantly mild malnutrition (47%). The baseline data is shown in Table 1.

Table 1. Baseline data

	HKPC group (%)	Control group (%)
Age (median)	11	11
Gender		
Male	26 (53%)	27 (55%)
Female	23 (47%)	22 (45%)
Nutritional status		
Overweight	6 (28%)	6 (4%)
Normal	24 (49%)	16 (33%)
Mild malnutrition	19 (39%)	27 (55%)
Dehydration states		
Without dehydration	1 (2%)	1 (2%)
Mild dehydration	9 (18%)	9 (18%)
Moderate dehydration	39 (80%)	39 (80%)
Breast feeding		
Receive breast feeding	28 (57%)	26 (53%)
Not breast feeding	21 (43%)	23 (47%)

The history of diarrhoea prior to hospitalisation is shown at table 2. In this study, most children with diarrhoea were hospitalised after one-day period of diarrhoea with approximately 6 times diarrhoea per day. More than half of the total children had at least once diarrhoea episode within the last 3 months (73% for both HKPC and control group). The feature of diarrhoea mostly watery with lumpy stool (55% for HKPC group and 65% for control group), none are followed with blood and mucus (78% for HKPC group and 71% for control group), and other symptoms that followed are vomiting, fever and flu-like syndrome.

Table 2. Clinical characteristic

	HKPC group	Control group
Mode duration of diarrhoea (range)	1 (1-3)	1 (0-3)
Mode frequency of diarrhoea	6 (2-11)	6 (1-20)
Diarrhoea consistency		
Entirely liquid	10 (20%)	13 (27%)
Watery with lumpy stool	27 (55%)	32 (65%)
Mushy stool	1 (2%)	0
Diarrhoea characteristic		
Precense of mucus	10 (20%)	13 (27%)
Precense of blood and mucus	1 (2%)	1 (2%)
No blood and mucus	38 (78%)	35 (71%)
Comorbidities		
Vomitting (n)	41	36
Flu-like syndrome (n)	28	36
Fever (n)	28	27
Convulsion (n)	0	0
Episode of diarrhoea in the last 3 months		
One time	36 (73%)	36 (73%)
Two times	7 (14%)	7 (14%)
Three times	2 (5%)	5 (10%)
Four times	4 (8%)	1 (3%)

Data from the stool examination (Table 3) shows that rotavirus was found in more than half of the total samples (53%). This is consistent with the study of Sudarmo *et al.* (2015), which found rotavirus in 80.7% of children aged 6–24 months with diarrhoea. An observation conducted in Jakarta showed that rotavirus prevalence was high among hospitalised children aged 6–23 months with acute diarrhoea,

and it was even more prevalent in the dry season (Soenarto *et al.*, 2009).

Table 3. Stool examinations data

	HKPC group	Control group
Rotavirus test		
Positive	23 (47%)	29 (59%)
Negative	26 (53%)	20 (41%)
Lactose malabsorption		
Positive	46 (94%)	44 (90%)
Negative	3 (6%)	5 (10%)
Fat malabsorption		
Positive	26 (53%)	27 (55%)
Negative	23 (6%)	22 (45%)

Fat malabsorption in acute diarrhoea is more prevalent than lactose malabsorption (46% vs. 8%). Although uncommon in this study, in research done by Nyeko *et al.* (2010), 68% of 3–12-month-old malnourished children with acute diarrhoea displayed lactose malabsorption. This malabsorption is caused by decreased intraluminal digestion due to a reduced absorptive surface area and disturbed enterocyte metabolism. The undigested fat substrate is then hydrolysed by the normal flora in the colon into free fatty acids (Thapar and Sanderson, 2004).

Table 4. Result of the urine and stool culture

	HKPC group (%)	Control group (%)
Urine culture		
No bacterial growth	32 (65%)	27 (55%)
E. colli	13 (27%)	17 (35%)
Proteus	1 (2%)	0
Klebsiella pneumoniae	2 (4%)	2 (4%)
Klebsiella oxytoca	1 (2%)	1 (2%)
Enterobacteriaceae	0	2 (4%)
Stool culture		
No bacterial growth	45 (92%)	46 (94%)
Pathogenic E. colli	0	1 (2%)
Klebsiella pneumonia	4 (8%)	2 (4%)

The table 4 shows the result of urine and stool culture. From the urine culture, although mostly for both the two groups found no bacterial growth (65% for HKPC group and 55% for control group), but E. colli found in 27% HKPC group and 35% in control group. In this study, the stool culture result showed that it is less likely to had bacterial co-infection in children acute diarrhoea (92% for HKPC group and 94% for control group).

3.1.Duration of illness

Between the two groups, the control group needed a longer time to recover (4 days) than the HKPC group (3 days) with range duration for HKPC group is 2-8 days and for control group 2-7 days, but this was not statistically different (p=0.100). A similar result was found in another study, which compared the effects of HKPC and active probiotics administration on the duration of illness in children with acute diarrhoea (Indriyani, Juffrie and Setyati, 2016).

Previous studies, in which a higher dosage of heat-killed probiotics than live probiotics was administered to treat acute diarrhoea, found that heat-killed probiotics reduced the duration of acute watery diarrhoea and shortened the length of hospitalisation (Applegate *et al.*, 2013; Supriatmo, 2006). This result showed that HKPCs are as beneficial as live probiotics because they have developed an ability to adhere to the mucus of the human intestine and inhibit the process of diarrheal infection (Supriatmo, 2006).

3.2. Level of Recovery

The recovery level data is shown in Table 5. There were no significant differences in recovery levels between the HKPC and control groups from the first day until the last day of observation (p=0.487). This result shows that heat-killed probiotics failed to improve the level of recovery in treating acute diarrhoea in 6–24-month-old children.

Table 5. Recovery level between the HKPC group and control group

	HKPC group	Control group	*P* value
Recovery level I	12	12	0.487
Recovery level II	31	43	

Many clinical trials on probiotics have also focussed on reduced severity in addition to level of recovery. Severity was represented as stool frequency a few days after administration of probiotics (Applegate *et al.*, 2013). Supriatmo (2006) found that heat-killed probiotics did shorten the duration, but not the stool frequency, of acute diarrhoea in children. Previous studies by Hatta *et al.* (2011) have assessed recovery levels based on the frequency (times/day) and duration of diarrhoea (hours). They found it difficult to assess stool consistency, and this could introduce bias (Hatta *et al.*, 2011). However, in this study, we used the Bristol stool chart to prevent a biased stool consistency assessment.

There are only a few studies of recovery levels based on stool frequency and consistency. Further studies are needed on recovery levels for HKPC administration in acute diarrhoea.

4. Conclusions

Administration of HKPC does not make any significant difference on the duration of illness or recovery levels in 6–24-month-old children with acute diarrhoea, whether or not associated with rotavirus or malabsorption.

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EFFECT OF PREVENTATIVE ZINC SUPPLEMENTATION ON DAMAGE TO INTESTINAL INTEGRITY CAUSED BY ESCHERICHIA COLI LIPOPOLYSACCHARIDE ADMINISTRATION: EXPERIMENTAL STUDY IN AN ANIMAL MODEL

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ABSTRACT

Zinc is widely used as a therapy for gastrointestinal diseases and as a food supplement. Research has suggested beneficial effects of zinc supplementation; however, there is limited knowledge on the effect of zinc on intestinal integrity. The aim of this study to investigate the effect of zinc on damage to the intestinal integrity induced by treatment with *Escherichia coli*-derived lipopolysaccharide (LPS). Thirty-two male Wistar rats were randomised into eight experimental groups that consumed either a normal diet or zinc-deficient diet, comprising a control group, LPS group (parenteral LPS administration on day 36), zinc group (parenteral zinc administration for 14 days), and zinc + LPS group (parenteral zinc administration for 14 days with additional LPS on day 36). All groups were sacrificed on day 43, and the ileum was removed for histological analysis. The villi length and number of enterocytes were measured on histological slices of the ileum. The results of this study, in the normal diet group treated with LPS, zinc supplementation improved the villi length ($p<0.0001$) and enterocyte number ($p<0.0001$). Zinc supplementation of rats in the deficient diet group treated with LPS showed improved villi length ($p<0.0001$) and enterocyte number ($p<0.0001$). In rats fed a normal diet, there were significant differences in villi length and enterocyte number in LPS-treated rats compared to those that were not exposed to LPS. In animals fed a zinc-deficient diet and treated with LPS, zinc supplementation improved villi length and enterocyte number.

1. Introduction

Data indicate that 21% of the global population suffers from zinc deficiency. This is an important problem worldwide, but especially in developing countries (Brown *et al.*, 2002). Indonesia is a high-risk country for zinc deficiencies, as more than 25% of the Indonesian population have suffered from zinc deficiencies (Samman, 2007).

In 2004, the World Health Organization (WHO) and UNICEF published guidelines for diarrhoea therapy with zinc and oralit for a period of 10–14 days, where administration of zinc can decrease the incidence of diarrhoea for the next 2–3 months, preventing an estimated 90% of diarrhoea-related morbidity (WHO/UNICEF, 2004). Zinc is a component of many enzymes that are essential for basic cellular function during all stages of the cell cycle. It is also essential for normal growth (WHO, 2006), as it acts as an antioxidant, improves intestinal absorption (Wapnir, 2000)

and immune response (Duggan *et al*., 2002), and also has bactericidal effects (Surjawidjaja *et al*., 2004). However, the mechanism of zinc to prevent damage to intestinal function and structure induced by lipopolysaccharide (LPS) produced by *Escherichia coli* infection remains unclear. At present, many zinc supplements are sold as vitamins to aid in prevention; however, research supporting the effect of zinc supplementation on improving intestinal integrity is lacking. This study used LPS derived from *E. coli* serotype O55:B5 as a model of bacterial endotoxin, which was administered at a non-lethal dose that could still generate the expected response. The objectives of the present study were to investigate the effects of zinc on damage to intestinal integrity caused by administration of *E. coli*-derived LPS.

2. Materials and methods

2.1. Samples

This study received ethical approval from the Animal Care and Use Committee (ACUC) of the Veterinary Medicine School, Airlangga University (Indonesia). Male Wistar rats (n = 32, 5 weeks old, approximately 80 g) were housed in 16 cages (two per cage), and each cage was supplied with either a zinc-adequate (30 ppm) or a zinc-deficient (0.5 ppm) diet for 6 weeks. Rat were fed according to four dietary treatments, consisting of a normal diet (30 ppm of $ZnSO_4$), normal diet with zinc supplementation (60 ppm of $ZnSO_4$), zinc-deficient diet (0.5 ppm of $ZnSO_4$) or zinc-deficient diet with zinc supplementation (120 ppm of $ZnSO_4$), based on a preliminary study conducted by Soemyarso *et al*. (2019). Zinc supplementation was orally administered through a $ZnSO_4$ solution on day 15 in half of the animals that received the normal diet (n = 8) and half of the zinc-deficient rats (n = 8). Rats had *ad libitum* access to a feed diet containing 0.02–0.04 ppm zinc, and rats were routinely monitored for body weight (BW) and daily intake.

After 5 weeks of the dietary treatments, half of the animals receiving the normal diet (n = 8) and the deficiency diet (n = 8) were exposed to oral saline (control), and the other half receiving the normal diet (n = 8) and the deficiency diet (n = 8) were administered oral LPS (250 μg/kg BW in 0.2 ml) on day 36 to simulate a bacterial challenge. Administration of LPS would induce intestinal distress, similar to other diarrhoea models. One week after the LPS challenge, euthanasia by cervical dislocation was performed for all rats. This study examined the effects of zinc supplementation on intestinal morphology in LPS-challenged rats through the histological evaluation of intestinal tissue sections. Histological analysis of intestinal samples included evaluation of villi length and the number of enterocytes.

2.2. Small intestine tissue collection

Tissue sample collection was performed immediately after euthanasia, and blood was collected into heparinised tubes. The small intestine of each rat was dissected into sections of the ileum approximately 3 cm in length. Each intestinal sample was immediately washed with cold saline and then rinsed with 10% formalin. The sections were filled with phosphate-buffered formalin solution for 24 h and then moved to the morphohistology laboratory for histological preparation. Histological preparation of the ileal tissue was carried out through several stages beginning with dehydration, clearing, impregnation, embedding and finally fixation in paraffin. Sections were stained with haematoxylin and eosin and viewed through an Olympus CX 21 microscope under 200× magnification for villi length assessment and 1000× magnification to count the number of enterocytes in four areas. The villi length was measured from the tip of the villi to the villous crypt junction. The number of enterocytes was measured by counting the enterocytes per villi. Both villi length and enterocyte amount are expressed relative to the result of the control

group (normal diet and deficiency diet group). This observation was carried out in the Department of Anatomy and Pathology Dr Soetomo General Hospital and the Faculty of Math and Science at the Universitas Brawijaya Malang.

2.3. Statistical analysis

Intestinal morphology was analysed by analysis of variance (ANOVA). All data analyses were performed using SPSS software, version 17. Significance was assessed at $p<0.05$.

3. Results and discussions

Figures 1A-H present the variation in villi length and enterocyte number for each of the eight groups.

Figures 1A-H show the variation in villi length for each group, in addition to enterocytes (black arrows) and damaged cells (red arrows). The difference between the normal diet group (Figure 1A) and the normal diet group with zinc supplementation (Figure 1B) is that the zinc supplementation group had longer villi. Figure 1C represents the normal diet group treated with LPS, showing blunt villi and multiple damaged epithelial cells, which differs from Figure 1D, representing LPS-treated animals on a normal diet with zinc supplementation, as zinc supplementation ameliorated these negative effects, evidenced by longer villi, fewer damaged cells and more intact epithelium.

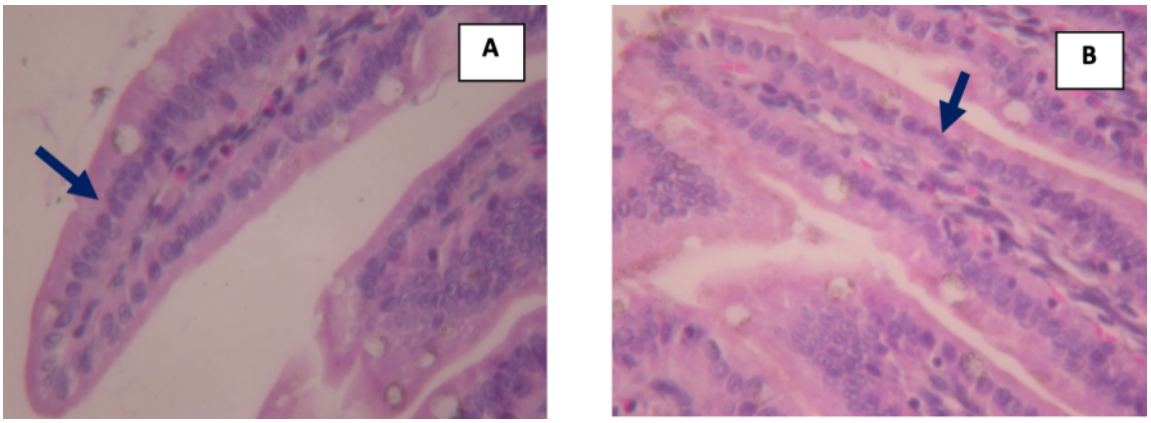

Figure 1A and B. Representative image of the small intestine of rats fed the (A) normal diet and (B) normal diet with zinc supplementation. Enterocytes are indicated by black arrows.

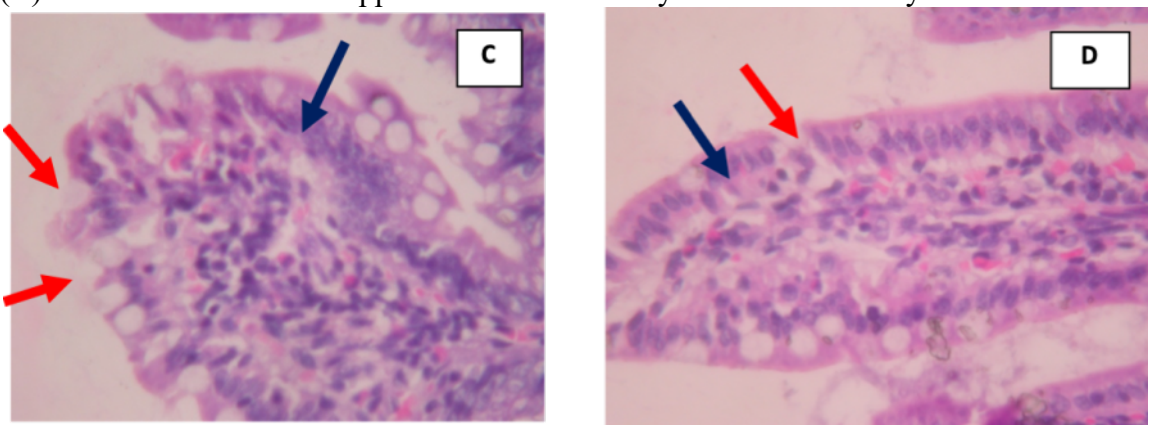

Figure 1C and D. Representative image of the small intestine or rats treated with *Escherichia coli*-derived lipopolysaccharide (LPS) fed the (C) normal diet and (D) normal diet with zinc supplementation. Enterocytes are indicated by black arrows and cell damage is shown by red arrows.

Figure 1E shows epithelial damage and blunt villi in the group fed the zinc-deficient diet. When zinc-deficient animals were supplemented with zinc (Figure 1F), animals showed longer villi and intact epithelium. Figure 1G represents the zinc-deficient group treated with LPS, showing blunt villi and damaged epithelial cells. In Figure 1H, animals in the zinc-deficient diet group treated with LPS which received zinc supplementation showed decreased epithelial cell damage. Zinc supplementation in the normal diet group had a protective effect on villi length ($p = 0.001$), while zinc supplementation in deficient animals protected the number of enterocytes ($p < 0.0001$) and villi length ($p < 0.0001$; Table 1). Administration of LPS to animals fed a normal diet led to significantly decreased villi length ($p = 0.001$) and number of enterocytes ($p < 0.0001$), and zinc-deficient animals showed a decrease in villi length ($p < 0.0001$) and enterocyte amount ($p < 0.0001$; Table 2). Zinc supplementation in both the normal and zinc-deficient groups protected the number of enterocytes ($p < 0.0001$) and villi length ($p < 0.0001$). Zinc supplementation in LPS-treated animals fed a normal diet and deficient diet resulted in improved villi length ($p < 0.0001$) and number of enterocytes ($p <0.0001$; Table 3). Administration of LPS to animals fed a normal diet and supplemented with zinc led to a decrease in the number of enterocytes, and LPS administration in zinc-deficient animals that had been given zinc supplementation resulted in decreased villi length (Table 4).

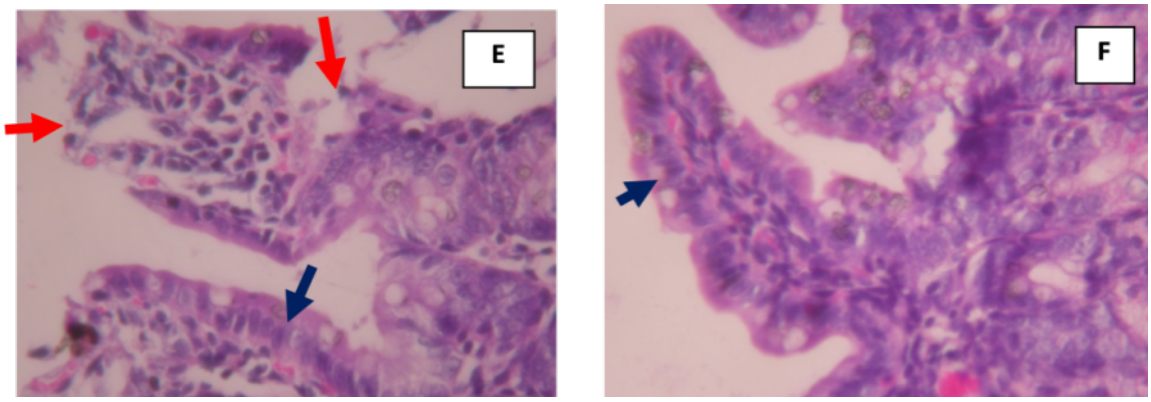

Figure 1E and F. Representative image of the small intestine of rats fed the (E) zinc-deficient diet and (F) zinc-deficient diet with zinc supplementation. Enterocytes are indicated by black arrows and cell damage is shown by red arrows.

The results of this study suggest that zinc supplementation in a normal diet could help to maintain villi length ($p = 0.001$) and protect against anatomical intestinal damage. In a zinc-sufficient condition (normal diet), zinc supplementation can improve the endogenous zinc reserve and induce metallothionein synthesis in intestinal cells. This metallothionein could provide anatomical protection against intestinal damage. Higher serum concentrations of zinc are usually associated with increased synthesis of metallothionein in the intestinal mucosa (Martínez *et al.*, 2004; Carlson *et al.*, 2007). Metallothionein is a binding protein that regulates the quantity of zinc absorbed by binding dietary zinc in mucosal cells, thereby controlling its transfer across the basolateral membrane into the circulation, and its subsequent deposition in the liver and intestine (Richards & Cousins, 1975).

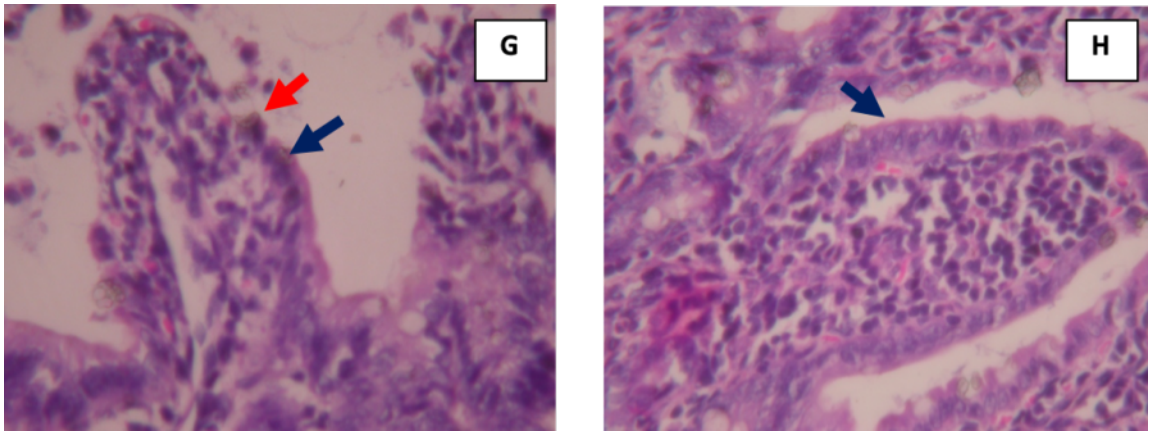

Figure 1G and H. Representative image of the small intestine or rats treated with *Escherichia coli*-derived lipopolysaccharide (LPS) fed the (G) zinc-deficient diet and (H) zinc-deficient diet with zinc supplementation. Enterocytes are indicated by black arrows and cell damage is shown by red arrows.

Table 1. Different effects of zinc supplementation in normal diet and zinc-deficient diet groups.

Group	Villi length (mm)	p	Enterocyte number	p
	Mean ± SD		Mean ± SD	
Normal diet	15.095 ± 0.843	***0.001****	22.187 ± 1.231	***0.871***
Normal diet + zinc	21.235 ± 2.597		22.312 ± 0.375	
Deficient diet	8.390 ± 0.388	***<0.0001****	12.250 ± 0.540	***<0.0001****
Deficient diet + zinc	19.640 ± 0.618		26.750 ± 2.131	

**Significance, $p \leq 0.05$.*

Zinc deficiency may enable oxidative damage to the structural integrity of cell membranes, which could alter the function of permeability channels and transport proteins in cell membranes (Hambidge *et al*., 1986). In the current study, we showed that zinc deficiencies can cause damage to intestinal anatomy and function, and zinc supplementation in deficient animals could protect villi length ($p < 0.0001$) and the number of enterocytes ($p < 0.0001$). Thus, in conditions of deficiency, zinc supplementation could protect against anatomical and functional intestine damage.

Table 2. Different effects of lipopolysaccharide (LPS) administration in normal diet and zinc-deficient diet groups.

Group	Villi length (mm)	p	Enterocyte number	p
	Mean ± SD		Mean ± SD	
Normal diet	15.095 ± 0.843	***0.001****	22.187 ± 1.231	***<0.0001****
Normal diet + LPS	9.095 ± 1.566		10.000 ± 0.204	
Deficient diet	8.390 ± 0.388	***<0.0001****	12.250 ± 0.540	***<0.0001****
Deficient diet + LPS	6.095 ± 0.495		5.375 ± 1.050	

**Significance, $p \leq 0.05$.*

Damage to the intestinal mucosal epithelium is a characteristic feature of numerous gastrointestinal diseases such as infectious diarrhoea or inflammatory bowel

diseases. The intestinal epithelium has enormous regenerative capabilities that allow rapid healing after damage. Firstly, cells beneath the damaged epithelial surface migrate into the wound within minutes to hours to seal the denuded area, a process that has been termed epithelial restitution. Secondly, cells proliferate to replenish the reduced cell pool. Finally, epithelial cells differentiate to mature enterocytes with diverse functional abilities within the intestinal epithelium (Goke & Podolsky, 1996).

The LPS challenge in rats fed a normal diet affected the villi length ($p = 0.001$) and enterocyte number ($p < 0.0001$), which was also observed in the zinc-deficient group, affecting villi length ($p < 0.0001$) and enterocyte number ($p < 0.0010$). Administration of LPS derived from *E. coli* in normal diet groups and deficient groups was found to destroy intestinal integrity, evidenced by decreases in villi length and enterocyte number. This experiment demonstrates that an oral LPS challenge is sufficient to induce intestinal inflammation. Excessive inflammation in the intestine may reduce the enterocyte barrier function, allowing additional bacterial and inflammatory challenges to occur. An adequate zinc status prevents barrier disruption in the enterocyte monolayer, as well as bacterial adhesion and internalization. Serum zinc status should be influenced to show positive developmental changes to intestinal integrity and immune function during the suckling period and after weaning. In this context, dietary supplementation with 3000 mg/kg zinc oxide was previously found to reduce the incidence of LPS-induced translocation of *E. coli* into mesenteric lymph nodes (Huang *et al*., 1999), and it also improved small intestinal mucosal epithelial morphology in recently weaned pigs (Li *et al*., 2001). Administration of LPS results in ischemia and destroys mature cells, and can also disrupt the growth and maturation of immature cells (Ruemmele, 2007). LPS derived from *E. coli* of an enteropathogenic serotype can be used as a pathogen because this endotoxin determines the innate immune response (Andonova *et al*., 2001) and increases bacterial translocation in the intestine (Huang *et al*., 1999).

Table 3. Different effects of lipopolysaccharide (LPS) administration and zinc supplementation in normal diet and zinc-deficient diet groups.

Group	Villi length (mm)	p	Enterocyte number	p
	Mean ± SD		Mean ± SD	
Normal diet + zinc + LPS	15.562 ± 2.016	***0.001****	18.375 ± 1.689	***<0.0001****
Normal diet + zinc	21.235 ± 2.597		22.312 ± 0.375	
Deficient diet + zinc + LPS	13.737 ± 0.739	***<0.0001****	12.750 ± 1.513	***<0.0001****
Deficient diet + zinc	19.640 ± 0.618		26.750 ± 2.131	

**Significance, $p \leq 0.05$.*

Zinc has been implicated in diarrhoea due to its effect on intestinal mucosal permeability (Rodriguez *et al*., 1996), and dietary zinc can help prevent or alleviate intestinal diseases. At pharmacological levels, this effect may be due to the antimicrobial properties of zinc (Owusu-Asiedu *et al*., 2003) and disruption of bacterial-enterocyte binding, as well as subsequent bacterial translocation (Huang *et al*., 1999).

We found that zinc supplementation in LPS-treated rats fed a normal diet or deficient diet helped to prevent damage to the intestine anatomy and function. Thus, zinc supplementation in the normal diet group and deficient diet group infected by LPS protected the intestinal integrity, showing improved villi length ($p<0.0001$) and number of enterocytes ($p<0.0001$). This study also demonstrated that zinc supplementation in rats in the group fed a normal diet with LPS treatment protected the

number of enterocytes ($p < 0.0001$), and zinc supplementation in the deficient diet group treatment with LPS protected villi length ($p < 0.0001$) and the number of enterocytes ($p < 0.0001$). LPS administration in rats fed a normal diet and provided zinc supplementation before only destroyed intestine function because anatomical structure had not yet been disrupted. There was likely a decrease in intestinal function due to the increased number of immature enterocytes, which were not yet functioning optimally. Immature enterocytes were increased due to the increased cell proliferation. Administration of LPS in deficient animals that had been given zinc supplementation showed a destroyed intestinal anatomical structure, but the function was not disrupted.

Table 4. Different effects of lipopolysaccharide (LPS) administration and zinc supplementation in normal diet and zinc-deficient diet groups.

Group	Villi length (mm)	p	Enterocyte number	p
	Mean ± SD		Mean ± SD	
Normal diet + zinc + LPS	15.562 ± 2.016	***<0.0001****	18.375 ± 1.689	***<0.0001****
Normal diet + LPS	9.095 ± 1.566		10.000 ± 0.204	
Deficient diet + zinc + LPS	13.737 ± 0.739	***<0.0001****	12.750 ± 1.513	***<0.0001****
Deficient diet + LPS	6.095 ± 0.495		5.375 ± 1.050	

**Significance, $p \leq 0.05$.*

In general, measurements of villus height and crypt depth provide an indication of the maturity and functional capacity of enterocytes (Roselli, 2003). Considering the role of zinc in RNA and DNA synthesis, these increases may be explained by an increase in cell proliferation and protein synthesis promoted by supplementary zinc (Hampson, 1986). Dietary zinc supplementation was found to exert a beneficial effect on intestinal morphology through increasing IGF-I expression in the small intestinal mucosa, which results in increased mucosal growth, brush border activity and nutrient absorption (Tako *et al*., 2005; Carlson *et al*., 2004). This is in agreement with other studies that reported no differences in the villus height and crypt depth of weaned pigs fed diets supplemented with zinc oxide (Li *et al*., 2006).

Enterocyte renewal in LPS-challenged rats might have been increased to maintain intestinal integrity in order to prevent bacteria from crossing the mucosal epithelial barrier. There is a relationship between intestinal architecture and the prevention of diarrhoea, as increased villus height and shorter crypt depth may have a higher absorption capacity and a lower rate of secretion from secretory cells, thereby helping to prevent diarrhoea (Namkung *et al*., 2006).

It is generally known that a lack of zinc may impair DNA synthesis and therefore decrease cell division and proliferation (Nabuur *et al*., 1993). Others have shown that zinc may promote the repair of wounded monolayers of cultured vascular endothelial cells through the lipo-oxygenase pathway via fibroblast growth factor signalling (Williams, 1989). Additionally, intracellular zinc may regulate various metalloproteins, transcription factors and other ligands in the intestinal epithelium. Thus, zinc may initiate several intracellular signal transduction pathways that directly or indirectly enhance cell migration. The effects of zinc on epithelial cell restitution *in vitro* are not as potent as the effects described previously for various cytokines and growth factors, for example TGF-alpha, epidermal growth factor, interferon-gamma and interleukin-1 beta, which were found to induce 2.3–5.5-fold enhancement of IEC-6 cell restitution *in vitro* (Kaji *et al*., 1994).

The protective role of zinc on resisting intestinal disease is well known (Brown *et al.*, 2002). Zinc plays an important role in maintaining membrane function and stability. Some researchers suggest that the mechanism of this activity involves the stabilisation of the membrane structure. The integrity of this intestinal barrier is fundamental to epithelial cells (Cario, 2000). Zinc plays a role in maintaining epithelial barrier integrity and function (Lu & Walker, 2001).

Zinc oxide has been shown to improve gastrointestinal tract function by increasing mucosal thickness, villi height and width of the small intestine (Li *et al*., 2001). Zinc primarily affects tissues with a high turnover rate, such as those of the gastrointestinal tract and immune system. Zinc is needed in these tissues for DNA and protein synthesis. Zinc stabilises the membrane structure and may modify membrane function, protect membranes from the effects of infectious agents, and may act at the tight junction to prevent the increased intestinal permeability associated with malnutrition or tumour necrosis factor. Zinc may also stimulate immune defences while minimising the adverse effect of immune cell activation by bacterial translocation on the epithelial layer (Huang *et al*., 1999).

4. Conclusions

Zinc supplementation led to improved villi length in animals fed a normal diet, and improved the villi length and enterocyte number in rats fed a zinc-deficient diet. In rats fed a normal diet, there were significant differences in villi length and enterocyte number in LPS-treated rats compared to those that were not exposed to LPS. In animals fed a zinc-deficient diet and treated with LPS, zinc supplementation improved villi length and enterocyte number.

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ZINC SUPPLEMENTATION ALTERED BRONCHUS MUCOSAL IMMUNE STATUS EXPRESSED BY IFN-γ, IL-6, DENDRITIC CELLS AND sIgA

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ABSTRACT

Zinc deficiency can cause suppression of the immune system and make it susceptible to infection, including infection of the respiratory tract. The benefits of zinc have not been widely studied and known to improve the bronchial mucosal immune status during respiratory tract infections. The aim of this study was to analyze the alteration of the bronchus mucosal immune status, expressed by IFN-γ, IL-6, dendritic cells and sIgA, that was caused by zinc deficiency. Twenty-four *Rattus norvegicus* strain Wistar species were divided into 4 experimental groups consisting of normal zinc + zinc supplementation (Z1), normal zinc (Z2), zinc deficiency + zinc supplementation (Z3) and zinc deficiency (Z4). The dose of zinc supplementation was 60 ppm in the normal diet group and 120 ppm in the zinc deficiency group. Dendritic cells, sIgA and the cytokines IFN-γ and IL-6 were examined using immunohistochemistry to assess bronchial immune status. The results of this study showed that dendritic cells and sIgA increased ($p<0.0001$) in the Z3 group but that cells producing cytokine IL-6 and IFN-γ ($p<0.0001$) decreased in the Z3 group. In the Z1 group, a statistically significant ($p<0.0001$) increase was seen in the number of dendritic cells and sIgA as well as an increase in cells producing cytokine IL-6 ($p<0.016$) compared with the Z2 group. According to the results, zinc is proven to alter the immune mucosa of the bronchi. Zinc supplementation can reverse the immune status by changing the dendritic cells, sIgA and cytokines IFN-γ and IL-6 in a zinc deficiency condition.

1. Introduction

In developing countries, respiratory tract infections are a major cause of morbidity and mortality in children under 5 years old (Rudan, 2004). In Indonesia, respiratory infections cause about 15.5% of mortalities in children aged 1-4 years old (Riskesdas, 2013). This is thought to be due to the immunological decline that occurs in malnourished children (De Francisco, 1993).

There is no complete study of the use of zinc with respiratory infections, but there is a relationship between zinc deficiency and respiratory diseases (Krebs, 2000). Zinc stimulates the immune response by activating dendritic cells and releasing cytokines to coordinate the immune response so that there is a balance of Th1 and Th2 (Haase, 2009).

Zinc is an essential substance in the cellular and humoral immune system components (Zalewski, 1996). Until now, there has been no research done on the effect of zinc that has been given orally on the immune status and immune response of dendritic cells, sIgA, IFN-γ and IL-6 in the bronchial mucosa of rats with normal zinc levels and rats with a zinc deficiency.

This study was conducted to prove that zinc alters the immune mucosa of the bronchi. This

mechanism is explained more thoroughly in experiments that were designed to determine the relationship between immune activation of dendritic cells, sIgA, IFN-γ and IL-6 in individuals with both normal zinc levels and zinc deficiency. This experiment used rats as subjects since the treatment and final inspection procedures have been proven to be fatal in human subjects.

2. Materials and methods

2.1. Animals

This research was ethically approved by a committee in the Faculty of Veterinary Medicine, Universitas Airlangga, Surabaya. Twenty-four white rats, *Rattus norvegicus* strain Wistar species, that fulfilled the inclusion criteria (healthy males, aged 5-6 weeks, weight ranging from 50 to 100 grams) were divided into 4 groups: normal zinc + zinc supplementation (Z1), normal zinc (Z2), zinc deficiency + zinc supplementation (Z3) and zinc deficiency (Z4). The dose of zinc supplementation was 60 ppm in the normal diet group and 120 ppm in the zinc deficiency group. The treatment took 42 days, and on day 42 a necropsy was performed.

2.2. Zinc Administration

The number of meals per day was determined by 10% of the body weight of each rat. The pellets without the zinc content included 25% protein, 5% fat and 50% carbohydrates. Drinking water was supplied ad libitum with the drop method using distilled water to avoid contamination with dirt. Zinc deficiency conditions were made based on the method used in a study by Soemyarso et al. (2019). Zinc was administered via an oral tube in 30 ppm (30 mg zinc/kg feed/day) with 0.2% zinc sulfate syrup in Groups Z1 and Z2, in 0.5 ppm (0.5 mg zinc/kg feed/day) with 0.002% zinc sulfate syrup in Groups Z3 and Z4, in 60 ppm (60 mg zinc/kg feed/day) with 0.4% zinc sulfate syrup in Group Z1 and in 120 ppm (120 mg zinc/kg feed/day) with 0.8% zinc sulfate syrup in Group Z3.

2.3. Bronchial Tissue Collection

On day 42, a necropsy was performed. The bronchial organs were cleaned and fixed in 10% buffered formalin solution, followed by dehydration, clearing, impregnation and embedding. Then, experts from the Pathology Anatomy Laboratory, Faculty of Medicine, Universitas Airlangga, Surabaya, fixed the organs by using the paraffin method. Expression of the cytokine-producing cells was detected with monoclonal antibodies (dendritic cells [Follicular DC Marker Antibody (Ki-M9R) sc-58529], IL-6 [IL-6 antibody (E-4)], sIgA [Sigma Receptor Antibody (B-5) sc-137075] and IFN-γ [IFN gamma Antibody Bioss Inc.]) before it was seen through a Nikon E100 microscope (Nikon Instruments Inc. [magnification 400x]) in the Biochemistry Laboratory, Faculty of Medicine, Universitas Brawijaya, Malang. The number of dendritic cells, sIgA and cytokines IFN-γ and IL-6 was determined by counting the number of cells per incision.

2.4. Statistical Analysis

Descriptive analysis was used to determine the results of the observations and profiles of the cells producing dendritic cells, sIgA and cytokines IFN-γ and IL-6 for each group. Expression data on the number of cells producing dendritic cells, sIgA and cytokines IFN-γ and IL-6 are expressed in mean ± standard deviation. Multivariate analysis of variance was used to analyze the effect of zinc on the changes in the number of cells producing dendritic cells, sIgA and cytokines IFN-γ and IL-6 in the bronchial mucosa in all treatment groups. To find the comparison between the treatment groups, a double comparison LSD test was performed. Data were analyzed using a 95% confidence level ($\alpha = 0.05$).

3. Results and discussions

This study used an immunohistochemical staining method. Then, a microscope was used to observe the dendritic cells, sIgA and cytokines IFN-γ and IL-6 on the bronchial tissue (Figure 1).

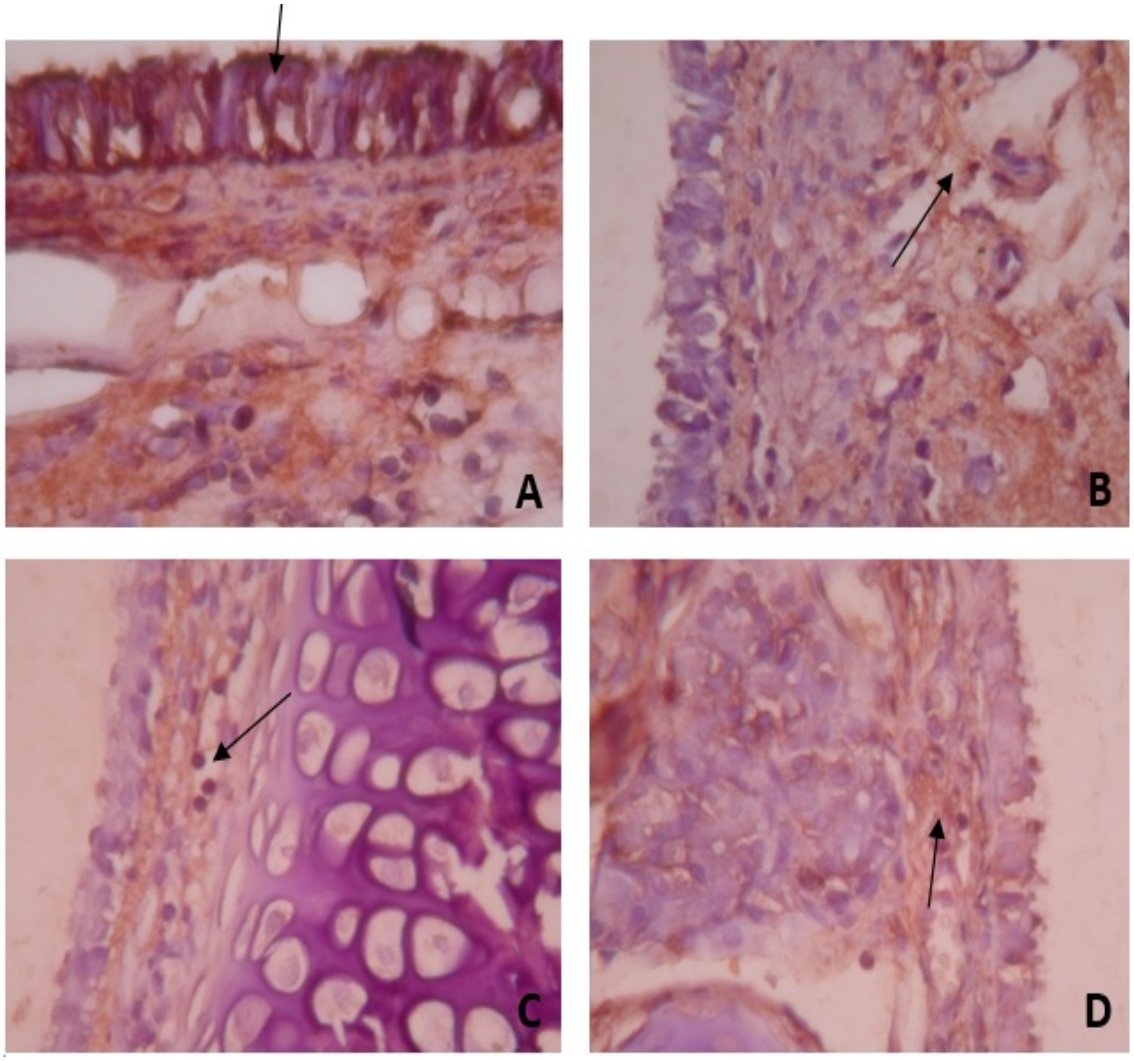

Figure 1. Rat's bronchus with immunohistochemical staining with monoclonal anti-mouse A) sIgA; B) IFN-γ; C) dendritic cell; D) IL-6 with 1000x magnification.

The effect of zinc supplementation on the immune status of the mucosal bronchus reflected by sIgA-producing cells, dendritic cells and cytokines IFN-γ and IL-6 in the zinc-deficient rats can be seen below (Figure 2).

When zinc-deficient rats are supplemented with zinc, changes in the immune status can be seen in the form of a statistically significant increase ($p<0.0001$) in bronchial mucosal cells producing secretory IgA and dendritic cells compared with the rats with a zinc deficiency. However, a statistically significant decrease ($p<0.0001$) is seen in cells producing cytokine IL-6 and IFN-γ compared with the rats with a zinc deficiency, so zinc can be said to be beneficial for the zinc-deficient rat in sIgA immune response and dendritic cells but not in the immune response of IL-6 and IFN-γ (Figure 2).

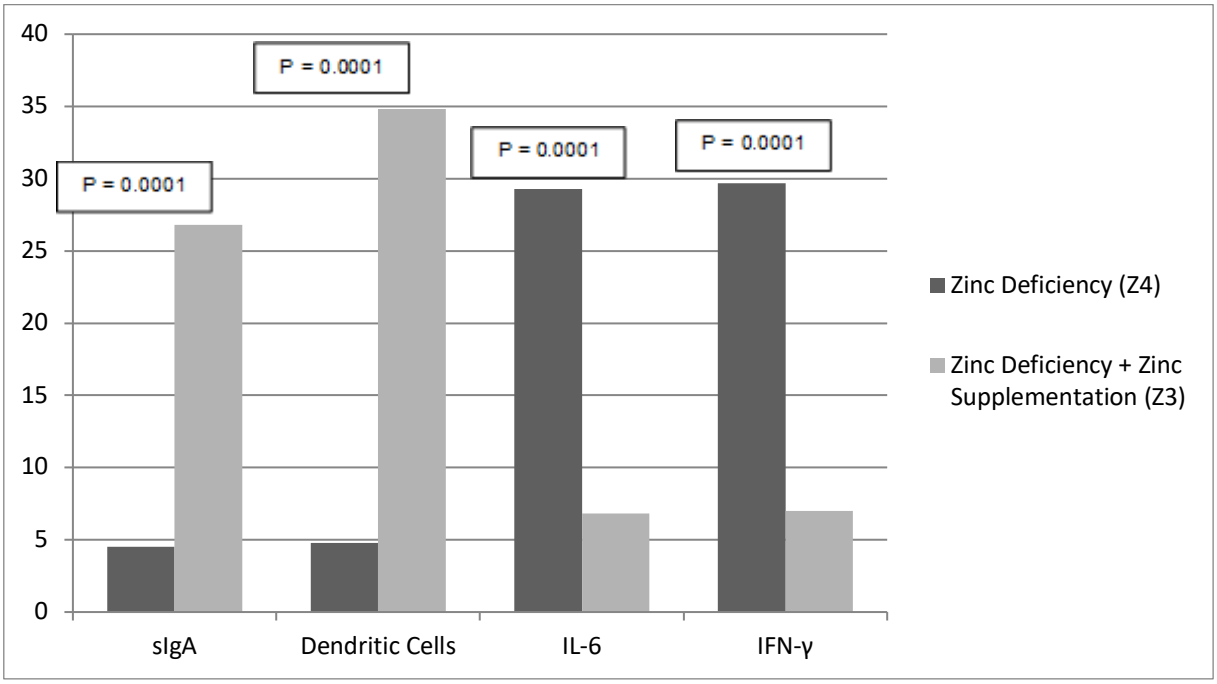

Figure 2. A profile comparing the cells producing sIgA, dendritic cells and the cytokines IFN-γ and IL-6 of the zinc-deficient group with the zinc deficiency + zinc supplementation group.

The effect of zinc supplementation on the immune status of the mucosal bronchus reflected by cells producing sIgA, dendritic cells and cytokines IFN-γ and IL-6 on a rat with normal zinc levels can be seen below (Figure 3). When rats with normal zinc levels are supplemented with zinc, changes in the immune status can be seen in the form of a statistically significant increase ($p<0.0001$) in bronchial mucosa cells producing secretory IgA and dendritic cells compared with the rats with normal zinc levels. There is also a statistically significant increase ($p<0.016$) in cells producing cytokine IL-6 compared with the rats with normal levels of zinc. The increase also occurs in cytokine IFN-γ but is not statistically significant ($p<1.000$) compared with rats with normal zinc levels, so zinc is determined to be beneficial in improving the immune status in rats with normal levels of zinc (Figure 3).

This study was conducted to prove that zinc deficiency alters the immune mucosa of the bronchi and to seek evidence regarding the effect of zinc administration on the bronchial immune system expressed by changes in dendritic cells, sIgA and cytokines IFN-γ and IL-6 of zinc-deficient rats and rats with normal zinc levels.

In the study conducted by Prasad, zinc deficiency also resulted in stress and activation of monocytes and macrophages, resulting in an increased generation of the inflammatory cytokine IL-6. In observational studies, individuals with normal post-intervention or serum zinc concentrations have a shorter bout of pneumonia. This is also evident in studies conducted by several student officers that show zinc may prevent upper respiratory tract infections (Prasad, 2009). In the same year, Prasad also reviewed the effects of zinc in patients that were infected with TB. It was found that zinc stimulated an increase in IFN-γ.

In 2004, Wieringa et al. conducted a study on 59 infants aged 3-10 months. They measured and compared levels of IFN-γ and IL-6 in infants with both normal zinc levels and zinc deficiency levels and on stimulation with LPS (Lipopolysaccharide). Results were obtained that showed a decline in the levels of the cytokines IFN-γ and IL-6 after stimulation with LPS in both normal zinc and zinc deficiency conditions.

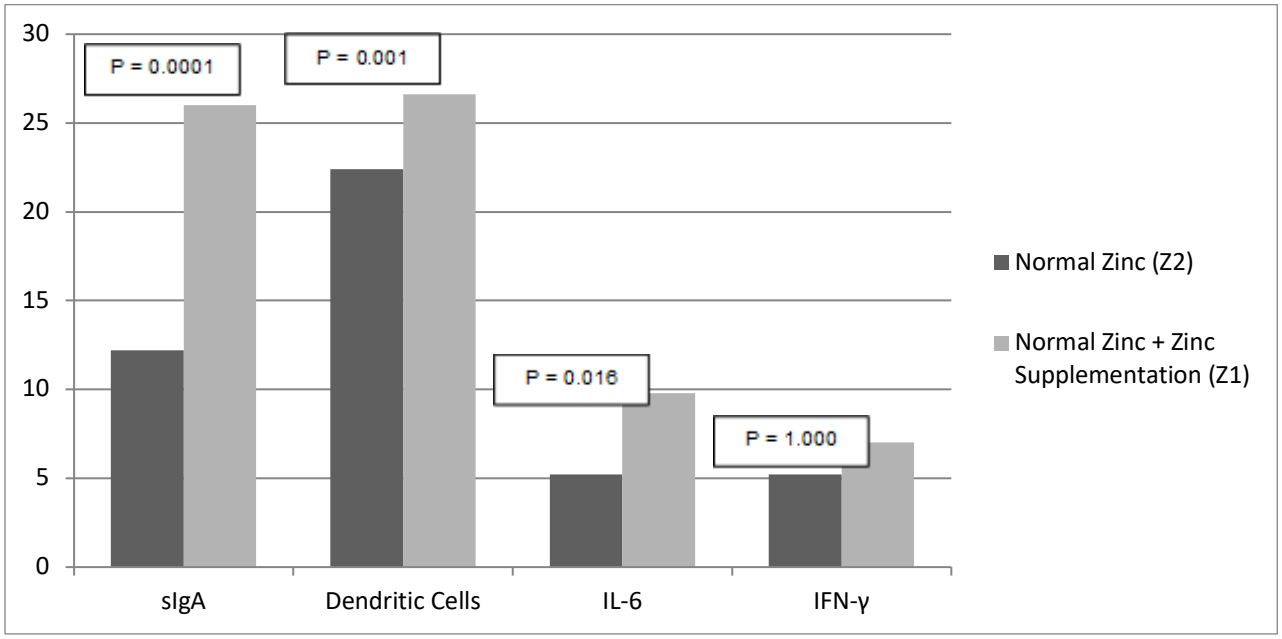

Figure 3. A profile comparing the cells producing sIgA, dendritic cells and the cytokines IFN-γ and IL-6 of the normal zinc group with the normal zinc + zinc supplementation group.

In a study, Brandtzaeg (2007) suggested that the effector site is where sIgA is secreted through the mucosal epithelium. Antigen presentation and activation of B cells in one area of the mucosa can result in sIgA appearing in the mucosa of different organs in accordance with the principle of the "common mucosal immune system" (Brandtzaeg, 2007).

In the same study, he also explained that the inductive sites for mucosal immunity are initiated by regional MALT, in which exogenous antigens will be streamed actively to achieve the antigen presenting cell (APC), including the dendritic cell (DC) and macrophage and B cells. An intra- or subepithelial dendritic cell that is not moving will capture antigens on effector sites and will migrate through the lymphatic flow toward the local lymph nodes and then become an active APC that stimulates T cells that will be productive or suppress the immune response. Once recognized as B cells and effector T cells, they will be streamed from MALT and the lymph nodes into the bloodstream to extravasation at mucosal effector sites (Brandtzaeg, 2007).

Holmgren and Czerkinsky (2005) proved the presence of kinetic-producing cells with specific IgA antibodies in peripheral blood circulation after administration of oral immunization in humans. They also showed that, after being given oral bacterial antigens, the cells that will secrete specific IgA antibodies appear in the peripheral blood circulation and are followed by an increase of the sIgA antibodies in saliva and tears. These studies also support/justify the concept of the common mucosal immune system in humans, and they also showed that oral immunization can be effectively used to induce the common sIgA antibodies in several external secretions (Holmgren and Czerkinsky, 2005).

Some evidence has suggested that mucosal surfaces, including the gastrointestinal and respiratory tract, are part of the common mucosal immune system and that both B and T lymphoblasts from one area of the mucosa in particular will return to the mucosal areas that are as far away as the original area. In research conducted on respiratory tract secretions in sheep, IgA is the major Ig in the upper respiratory tract (nasopharynx, trachea and main bronchi), and the large of numbers of IgA plasma cells reflected on the respiratory mucosa suggest that some of these cells may be derived from distant mucosa areas or elsewhere (Scicchitano, 1984).

Studies concerning the effects of zinc supplementation in pregnant rats conducted by

Raqib et al. (2007) found that administration of zinc actually decreases IgA levels. This finding contrasts with the results of our study where bronchial sIgA levels increased in rats with normal zinc levels that were given zinc supplementation.

New Research Findings

This study has provided new findings with evidence that zinc administration can improve the immune system in rats with normal zinc levels and rats with a zinc deficiency. The immune response in bronchial mucosa increases dendritic cell-producing cells, sIgA, IFN-γ and IL-6. Several studies on the effect of zinc were limited to the clinical improvement of respiratory tract infections, but no studies have been done in terms of changes and improvements in the immune system.

4. Conclusions

Zinc is proven to alter the immune mucosa of the bronchi. Zinc supplementation can reverse the immune status by changing dendritic cells, sIgA and the cytokines IFN-γ and IL-6. Further research is needed both immunologically and clinically on the use of zinc as a supplementation for the prevention of possible exposure to oral pathogens that may affect the bronchus.

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ANALYSIS OF IMMUNOGLOBULIN LEVELS AFTER EXPOSURE OF COW'S MILK PROTEIN IN MICE

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ABSTRACT

This research aimed to analize immunoglobulin levels after exposure of cow's milk protein in mice. We conducted a true experiment with a posttest-only control group method. This experiment used female Balb/C mice and neonate mice (pregnant mice 10 weeks old n=15, neonatal mice 2 weeks old). The sample was divided into low dose group (1 mg CMP per gram weight of mice), high dose group (10 mg CMP per gram weight of mice) and control group that consisted 5 pregnant mice respectively. The cow's milk protein allergens were induced in pregnant mice. We took blood from the pregnant mice, and their fetuses were taken for subclass immunoglobulin examination (Ig A, IgM, IgE, Ig G) by using an ELISA (Enzyme-Linked Immunosorbent Assay) kit. In low dose group we found a significant increase ($p<0,05$) in IgG-1, IgG-2a, and IgG-2b levels in pregnant mice and IgG-1, IgG-2a, IgG-2b, and IgG-3 in neonate mice which described in table 1. While for the high dose group described in table 2, similar results occurred in IgG-1, IgG-2a, and IgG-3 levels ($p<0.05$) in pregnant mice and IgG-1, IgG-2a, IgG-2b, and IgG-3 in neonate mice. In high-dose group we also found an increase in IgA, IgM, and IgE ($p<0.05$) in either the neonate or pregnant mice. However, in low dose group there was only a significant increase in IgM ($p=0.004$) and IgE ($p=0,000$) in neonate mice and only IgE ($P=0,000$) in pregnant mice. In conclusion, in low dose group we found a significant increase in IgG-1, IgG-2a, IgG-2b in both pregnant and neonate mice, IgG-3 and IgE in pregnant mice, IgM and IgE in neonate mice. While for the high dose group similar results occurred in IgG-1, IgG-2a, IgG-3, IgA, IgM, and IgE levels in both pregnant and neonate mice and IgG-2b in neonate mice.

1.Introduction

Allergy is a health problem with a high prevalence. In the human population, the prevalence of allergy has reached 20%, with 5%–15% occurring in children. In Southeast Asia, the prevalence of allergy reached 3.3% in children (Platts-Mills et al., 2003). While the prevalence is getting higher, efforts to prevent allergy is yet to be optimized, and the immunology process of *in utero* prevention has not been understood (Jones, Holloway, and Warner, 2000; Boyle, Robins-Browne, and Tang, 2006; Fusaro et al., 2009). Today, suspected allergic events are associated with the fetus's exposure to allergens, but this has yet to be explained. Some studies suggest that the child of a pregnant mother whose family has a history of dust mite allergy may become allergic to dust mites while exposure to cat hair

may induce tolerance to cat hair within the child (Prescott et al., 2003). It is predicted that different doses and types of allergen will induce different immune responses from the fetus. A study shows that fetal dendritic cells do not express the Th2 allergen, but they express the Th1 allergen (Platts-Mills et al., 2003). This leads to a discussion on how to treat allergy and whether mothers should expose allergens to their child to prevent allergies from developing or avoid them. Allergy is a chronic disease that can alter the growth and development of the child. Therefore, an experiment on pregnant Balb/C mice is conducted exposing them to low-dose and high-dose cow's milk protein allergen to analize immunoglobulin levels after exposure of cow's milk protein in mice (Kunert and Lavitska, 2001; Prescott et al., 2003; Lara-Villoslada, Olivares, and Xaus, 2005).

2. Materials and methods

We conducted a true experiment with a posttest-only control group method to analize immunoglobulin levels after exposure of cow's milk protein in mice.

2.1. Animals

The study was conducted in the Virology and Immunology Laboratory, Department of Microbiology, Airlangga University, Surabaya, East Java.

This experiment used female Balb/C mice and neonates (pregnant mice 10 weeks old n=15, neonatal mice 2 weeks old) acquired from the Farma Center of Veterinary, Surabaya. All the animals were inspected by a veterinary consultant to ensure pregnancy and the neonates' health condition. Animals were excluded if they were pregnant with fetuses with congenital disorders, have differences in meal behavior, or show signs of sickness such as decreased weight, breathing patterns, and diarrhea. Cow's milk protein allergens were induced in pregnant mice. After the pregnant mice gave birth, the neonates were kept for two weeks before having their blood taken, and the serum of both the female mice and their fetuses were examined. The samples were homogeneous in gender, age, and weight. The sample was divided into low dose group (1 mg CMP per gram weight of mice), high dose group (10 mg CMP per gram weight of mice) and control group. Then the data were analyzed using inferential statistics to achieve the research objectives.

2.2. Cows Milk Protein Allergen

Materials used were cow's milk protein allergen (Indoor Biotechnologies, Natural Bos d5 (NA-BD5-1). The cow's milk protein allergens were administered intraperitoneal in pregnant mice 5mg/5ml: low dose 1 mg CMP per gram weight of mice (Jones, Holloway, and Warner, 2000; Kunert and Lavitska, 2001; Prescott et al., 2003; Lara-Villoslada, Olivares, and Xaus, 2005). The high dose used the same reagent with 10 times the low dose.

2.3.ELISA

Immunoglobulin was analyzed using an IgG, IgM, and IgA ELISA kit with the Sandwich method. The ELISA kit (Ig isotyping Mouse Instant: Thermo Fisher Scientific company, catalog number 88-50660-22) was used to examine the optical density (OD) of IgG1, IgG2a, IgG2b, IgG3, IgM, IgA and IgE.

The 80 ml serums were collected into a microplate tube and processed by duplo. A standard 20 µl solution of IgE with a titer of 0, 50, 100, 200, 500, and 1000 was added into each tube, commencing to the A 1 and 2 until the G 1 and 2 columns. The microplate was incubated for an hour in room temperature and then washed three times with buffer washing. Then 100 µl of HRP enzyme was added to each tube. The microplate was then incubated for an hour and washed with buffer washing. Next, 100 µl of TMB was added and incubated for 30 minutes.

Absorbant value was analyzed using the 450 nm wavelength of the ELISA reader and then interpreted by linear regression analysis.

2.4. Statistics Analysis

The experiment was analyzed using ANOVA homogeneity test and Kolmogorov-

Smirnoff and normal probability plot for normality test for normal distribution. For testing differences between each group, we used ANOVA for normal distributed data and Kruskal Wallis A, Brown-Forsythe, and Mann-Whitney for abnormal distributed data.

3.Results and discussions

3.1. Results

In low dose group we found a significant increase in IgG-1, IgG-2a, and IgG-2b levels in pregnant mice ($p<0.05$) and IgG-1, IgG-2a, IgG-2b, and IgG-3 ($p<0,05$) in neonate mice which described in table 1. While for the high dose group described in table 2, similar results occurred in IgG-1, IgG-2a, and IgG-3 levels ($p<0.05$) in pregnant mice and IgG-1, IgG-2a, IgG-2b, and IgG-3 in neonate mice ($p<0.05$). Between high-dose and low-dose group, we found significant differences in IgG-1, IgG-2a, IgG-2b, and IgG-3 in neonate mice ($p(r-t)<0.05$) and IgG-1, IgG-2a, and IgG-2b in pregnant mice ($p(r-t)<0.05$). In high-dose group we found an increase in IgA, IgM, and IgE ($p<0.05$) in either the neonate or pregnant mice. However, in low dose group there was only a significant increase in IgM ($p=0.004$) and IgE ($p=0,000$) in neonate mice and only IgE ($P=0,000$) in pregnant mice. In the high-dose group and the low-dose group, there was a significant increase in IgA, IgM, and IgE in either the neonate or pregnant mice ($p(r-t)<0.05$).

Table 1. Immunoglobulin level after being exposed to low dose cow's milk allergen

	Low Dose Group (p)		Control Group	
	Pregnant mice	Neonate mice	Pregnant mice	Neonate mice
IgG-1	1.85± 0.07 (p = 0.000)	1.90± 0.08 (p = 0.004)	1.26± 0.07	0.18± 0.03
IgG-2a	2.66 ± 0.04 (p = 0.000)	2.88 ± 0.23 (p = 0.000)	1.41 ± 0.05	1.48 ± 0.18
IgG-2b	3.38± 0.08 (p = 0.000)	2.87± 0.42 (p = 0.001)	2.58± 0.06	1.95± 0.18
IgG-3	0.47± 0.14 (p = 0.078)	2.09± 0.47 (p = 0.000)	0.36± 0.04	0.09± 0.01
IgA	0.16 ± 0.05 (p = 0.749)	0.12 ± 0.03 (p = 0.192)	0.15 ± 0.09	0.10 ± 0.04
IgM	1.01 ± 0.05 (p = 0.184)	0.73 ± 0.10 (p = 0.004)	1.16 ± 0.22	0.24 ± 0.08
IgE	183.21± 5.91 (p = 0.000)	109.04±10.61 (p = 0.000)	21.89 ± 0.84	22.37 ± 3.01

Table 2. Immunoglobulin level after being exposed to high dose cow's milk allergen

	High Dose Group (p)		Control Group	
	Pregnant mice	Neonate mice	Pregnant mice	Neonate mice
IgG-1	1.49± 0.04 (p = 0.000)	1.31± 0.09 (p = 0.004)	1.26± 0.07	0.18± 0.03
IgG-2a	1.33 ± 0.46 (p = 0.688)	0.76 ± 0.17 (p = 0.000)	1.41 ± 0.05	1.48 ± 0.18
IgG-2b	2.68± 0.05 (p = 0.009)	2.39± 0.12 (p = 0.001)	2.58± 0.06	1.95± 0.18
IgG-3	0.45± 0.05 (p = 0.007)	0.20± 0.05 (p = 0.001)	0.36± 0.04	0.09± 0.01
IgA	0.32 ± 0.05 (p = 0.002)	0.04 ± 0.03 (p = 0.020)	0.15 ± 0.09	0.10 ± 0.04
IgM	1.55 ± 0.05 (p = 0.007)	0.52 ± 0.11 (p = 0.000)	1.16 ± 0.22	0.24 ± 0.08
IgE	192.96± 7.32 (p = 0.000)	124.26± 9.31 (p = 0.000)	21.89 ± 0.84	22.37 ± 3.01

3.2. Discussion

After exposure to cow's milk allergen, there were different responses from IgG2b both in pregnant mice and neonate mice and with high doses and low doses whereas for IgG3, significant response was only in pregnant and neonate mice with high-dose exposure and neonate mice with low-dose exposure. It can be explained in general that exposure to high doses and low doses of both pregnant and neonate mice gives the same response, meaning that the cow's milk antigen can be transferred through the placenta to the neonate and responded to by neonate plasma cells even with a lower response ability in neonate mice. Neonatal B cells express low levels of coreceptors including CD28 and CD40 ligand on Th2 or follicular T helper cells with their corresponding binding partners HLA-peptide, CD80/86, and CD40 on antigen-specific B cells. This limits their capacity to respond to B cells from neonates and infants aged less than two months. Hence, the immune system is immature and markedly impaired in neonates and more so in fetuses (Mcmichael, Simon, and Hollander, 2015).

Response to the exposure to high-dose cow's milk allergen occurred in IgA and IgM whereas for low-dose cow's milk, there was no significant increase from the control. It can be explained that the immune system remains inferior to exposure whereas the IgE response shows a potent allergy parameter both in pregnant mice and neonates with allergy. In food allergy, it is believed that food-specific IgE antibodies bind to high-affinity FcεRI receptors on mast cells, basophils, macrophages, and dendritic cells, as well as to low-affinity FcεRII receptors on macrophages, monocytes, lymphocytes, eosinophils, and platelets. When food allergens penetrate mucosal barriers and contact IgE antibodies bound to mast cells or basophils, histamine and other mediators that induce symptoms of immediate hypersensitivity are released (Li et al., 2005)

We found significant differences in IgE levels between pregnant mice and neonate mice exposed to low-dose and high-dose allergens. Efforts to induce allergy tolerance in mice exposed to high-dose allergens were unsuccessful. This is contrary to a study conducted by Adel-Patient (2005), where the dose of allergens exposed did not affect IgE regulation. There was an increase in IgE levels both after the exposure to low-dose or high-dose allergens (Adel-Patient, Ah-Leung, Creminon, Nouaille, Chatel, Langella, and Wal, 2005; Wavrin, 2015).

4. Conclusions

In low dose group we found a significant increase in IgG-1, IgG-2a, IgG-2b in both pregnant and neonate mice, IgG-3 and IgE in pregnant mice, IgM and IgE in neonate mice. While for the high dose group similar results occurred in IgG-1, IgG-2a, IgG-3, IgA, IgM, and IgE levels in both pregnant and neonate mice and IgG-2b in neonate mice.

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EFFECTS OF POMEGRANATE EXTRACTS ON MMP-1, TIMP-1, MMP-1/TIMP-1 RATIO, AND TYPE I COLLAGEN TO INHIBIT RIGHT VENTRICULAR FIBROSIS IN ANIMAL MODELS

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ABSTRACT

Pulmonary arterial hypertension (PAH) may cause myocardial fibrosis. The process involves collagenase, the metalloproteinase matrix (MMPs), and tissue inhibitors of metalloproteinase (TIMPs). The accumulation of type I collagen in the right ventricular heart muscle may result in right ventricular fibrosis. *Pomegranate* extracts contain active ellagic acid ingredients with anti-inflammatory, antiproliferative, antioxidant, and ACE inhibitory effects on tissue fibrosis with no side effects. The aim of this study to evaluate the effectiveness of pomegranate extracts on the fibrosis process due to PAH especially on right ventricular myocardium by observing the number of cells expressing MMP-1, TIMP-1, MMP-1/TIMP-1 ratio and type 1 collagen on the right ventricular heart muscle of pulmonary arterial hypertension (PAH) animal models. This study is an experimental laboratory research conducted on Sprague-Dawley white mice (*Rattus norvegicus*). Subjects were grouped as control and treatment groups that received CMC for two and four weeks, respectively. Monocrotaline was injected to mice to induce pulmonary arterial hypertension. MMP-1 expression of the right ventricular heart muscle in the control group was lower ($p < 0.001$ and $p < 0.001$). The number of cells expressed in TIMP-1 was higher in the control group than the observed pomegranate extracts for two weeks and four weeks ($p < 0.001$). The ratio of cell numbers expressing MMP-1/TIMP-1 was higher in the pomegranate extract group than in the control group ($p < 0.001$). The number of cells expressing type I collagen in the pomegranate extract group was lower than that of the control group ($p < 0.001$). According to the result, pomegranate extracts may inhibit the initial process of fibrogenesis.

1. Introduction

Pulmonary arterial hypertension (PAH) is defined as the mean pulmonary artery pressure (pPA) at rest exceeding 25 mmHg (Wardle and Tulloh, 2012). PAH may cause myocardial fibrosis due to an imbalance of metalloproteinase-tissue inhibitors of metalloproteinases resulting in an accumulation of extracellular matrix (Jeffery et al., 2002; Mandal, 2014). The process involves collagenase, that is, the metalloproteinase matrix (MMPs) and tissue inhibitors of metalloproteinase (TIMPs) that affect the balance of type I collagen synthesis and degradation. The accumulation of type I collagen in the right ventricular heart muscle can cause right ventricular fibrosis. This results in a low cardiac output and activates the RAA system, triggering fibroblast proliferation and differentiation into collagen-secreting myofibroblasts.

Medication to prevent PAH progression while waiting for a definitive therapy is

necessary to reduce morbidity and mortality (Rahman, 2016). Compared with calcium channel blockers, prostanoids, endothelin antagonists, and phosphodiesterase inhibitors (PDEs), each of which has different therapeutic points, pomegranate extracts have several advantages. Pomegranate extracts contain the active ingredient ellagic acid, which is rich in anti-inflammatory, antiproliferative, antioxidant, and ACE inhibitors that are beneficial for cardiovascular diseases and for the liver as an antifibrotic in mice models (Yuniarti et al., 2013; Haber et al., 2011). In addition to previous studies, pomegranates are said to have no side effects, so they are safe to be used as an alternative therapy (Vidal et al., 2003; Jurenka, 2008). However, there has been no research on pomegranates' effects on the right ventricular myocardial fibrosis process due to PAH caused by CHD with left-to-right shunting. The objective of this study is to examine the effectiveness of pomegranate extracts on fibrosis due to PAH especially on the right ventricular heart muscle by observing the number of cells expressing MMP-1, MMP-1/TIMP-1 ratio, and type-1 collagen in the right ventricular hearts of PAH mice models.

2. Materials and methods

This study is an experimental research conducted in the Biochemistry Laboratory of the Faculty of Medicine, Universitas Airlangga. Ethical clearance was issued by the Animal Care and Use Committee, Veterinary Faculty, Universitas Airlangga, no: 758-KE, issued on August 10, 2017

2.1. Sample

This study used three-month-old Sprague-Dawley white mice (*Rattus norvegicus*) weighing 300–500 grams. Model mice were grouped as control and treatment groups. The control group was divided into two, which received CMC 0.3% treatment for two weeks (K1) and four weeks (E1), respectively. Treatment groups were divided into a group that received pomegranate extracts with CMC 0.3% for two weeks (K2) and another that received pomegranate extracts with CMC for 0.3% four weeks (E2).

2.2. Monocoraltine and pomegranate extracts

Monocrotaline (Chengdu Biopurify Phytochemicals, Chengdu, Sichuan, China) was subcutaneously injected to mice to induce PAH at 60 mg/kg.

The materials in this research used extracts from all parts of pomegranate (*Punica granatum L*) in powder form and have been standardized, containing 40% ellagic acid, and produced by Xi'an Biof Bio-Technology Co., Ltd. (Room 1-1111, High-tech Venture Park, No. 69 Jinye Road, Gaoxin District of Xi'an, People's Republic of China).

2.3. Blood analysis

We performed a immunohistochemical analysis of monoclonal antibodies for MMP-1, TIMP-1, type 1 collagen, and MMP-1/TIMP-1 ratio, which were measured after two and four weeks of treatment.

2.4. Data analysis

We used SPSS 20 for Windows. Statistical analysis used ANOVA and least significant difference test with a level of significance of < 0.05.

3. Results and discussions

There were 24 subjects that met the inclusion criteria of this study. Two subjects died from the K1 and K2 group. The mean initial body weight of the K1 group was 303.83 ± 37.46 grams, with a final body weight of 294.00 ± 39.81 grams. The mean initial body weight of the E1 group was 312.50 ± 62.40 grams, with a final body weight of 318.50 ± 66.46 grams. The mean initial body weight of the K2 group was 319.00 ± 26.57 grams, with a final body weight of 294.40 ± 53.23 grams. The mean initial body weight of the E2 group was 300.33 ± 18.55 grams, with a final body weight of 275.33 ± 33.57 grams.

The results of this study showed that the mean systolic and diastolic pressure of the pulmonary artery in the group of pomegranate extract–treated mice was observed to be lower

than that of the control group after two weeks or four weeks ($p = 0.002$). The mean pulmonary artery pressure of the group treated with pomegranate extracts was lower than that of control model mice, significantly different ($p = 0.001$) in the observation after two weeks or four weeks. This suggests that the administration of pomegranate extracts may prevent pulmonary artery pressure increase and inhibit PAH progress.

The mean systolic and diastolic pressure of Sprague-Dawley mice with similar age and weight in the preliminary study was 45 mmHg and 19 mmHg, respectively. The mean systolic pressure of PAH model mice in a previous study was 80 mmHg, and the mean systolic pulmonary artery pressure of normal control mice was 30 mmHg (Bogart et al., 2009). A lower mean pulmonary artery pressure in the pomegranate extract group proved that these extracts may decrease pulmonary artery pressure in monocrotaline-induced PAH model mice. The pulmonary arterial pressure of monocrotaline PAH model mice treated with pomegranate extracts significantly decreased compared to that of the control group (Hanif, 2010).

Figure 1 described about the comparison the number of cells expressing MMP-1 between treatment and control groups after two and four weeks. The number of cells expressing MMP-1 in the right ventricular heart muscle of the control mice was less than that of the group given pomegranate extracts after two weeks or four weeks ($p < 0.001$ and $p < 0.001$, respectively).

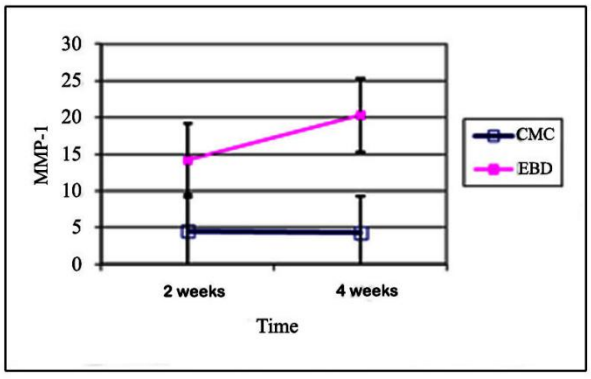

Figure 1. MMP-1 expression

The average number of cells that expressed MMP-1 in PAH model mice that received pomegranate extracts increased compared with PAH model mice not given pomegranate extracts at two weeks and four weeks of observation. Transgenic expression of human MMP-1 decreases smooth muscle cell proliferation and inhibits the excessive accumulation of collagen in the pulmonary artery. Upregulation of MMP-1 may decrease severe PAH and open the possibility of therapeutic intervention.

Administration of pomegranate extracts in the liver fibrosis model mice led to an increase in MMP-1 expression (Yuniarti, 2012).

While the number of cells expressing TIMP-1 was explained in figure 2. On the contrary with MMP-1, the number of cells expressing TIMP-1 actually was more apparent in the control group mice than in the treatment group, which was given pomegranate extracts for two weeks and four weeks of observation ($p < 0.001$).

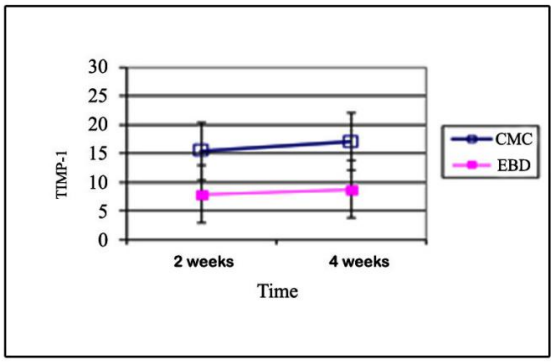

Figure 2. TIMP-1 expression

In figure 3, the ratio of cell numbers expressing MMP-1/TIMP-1 was higher in the group of mice treated with pomegranate extracts than in the control group mice and significantly different in observations after two weeks or four weeks ($p < 0.001$). The TIMP-1 expression rate of the treatment group mice decreased and is significantly different from that of the control group. Expression of TIMP-1 increased again in the control group mice as well as the treatment group mice after four weeks but did not differ significantly between two weeks and four weeks. This proves that there are obstacles to

MMP-1 activity that can cause the accumulation of MES protein and right ventricular heart muscle fibrosis. This is in line with other studies that considered the use of pomegranate extracts as an adjunctive therapy to the existing standard therapy to decrease PAH-related morbidity and mortality.

The increased MMP-1/TIMP-1 ratio after pomegranate extract admission can mean that there is an increase in MMP-1 activity and reduced resistance to MMP-1 activity. MMP-1 more actively degrades the MES protein so that right ventricular heart muscle fibrosis can be inhibited or reduced. The ratio remained significantly increased after two weeks and four weeks of pomegranate extracts. The increased MMP-1/TIMP-1 ratio in this study is due to increased MMP-1 expression and decreased TIMP-1 expression. Another study also found an increase in the ratio of MMP-1/TIMP-1 in the tunica adventitia pulmonary vein model of PAH experimental animal (Rahman, 2016). The MMP-1/TIMP-1 ratio under normal circumstances is 1:1. Increased ratios led to increased MMP-1 activity and increased MES protein degradation including collagen degradation.

There was a significant decrease in the number of TIMP-1 expression cells in the tunica adventitia of the pulmonary vein models of the PAH-induced treatment group mice compared with control group mice (Rahman, 2016).

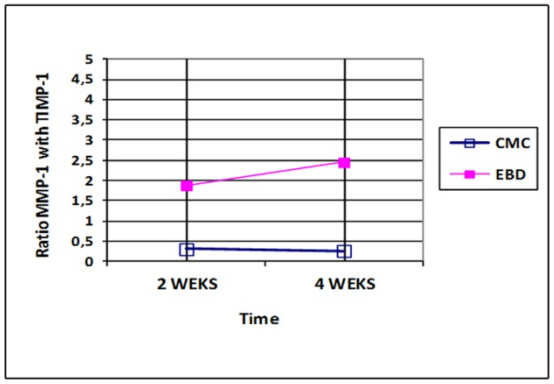

Figure 3. The ratio of the number of cells expressing MMP-1 / TIMP-1

Figure 4 showed that the number of cells expressing type 1 collagen in the treatment group mice was no different than in the control group mice after two weeks of observation (p = 0.193).

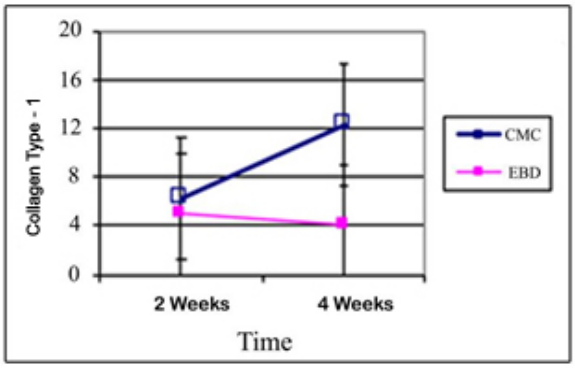

Figure 4. Expression of collagen type-1

In contrast, the number of cells expressing type I collagen in the group of mice treated with pomegranate extracts was much lower than in the control group mice after four weeks of observation and was significantly different ($p < 0.001$). Pomegranate extracts have the ability to inhibit the increase of the number of cells expressing type I collagen of the right ventricular heart muscle. The number of cells expressing type 1 collagen in the four-week control group was more statistically different than in the two-week control group. These results are consistent with other studies showing a decrease in the number of cell expression of type I collagen in the pulmonary vein of the monocrotaline-induced PAH in treatment group mice (Rahman, 2016). The effect of type I collagen decrease is related to the active ingredient ellagic acid and polyphenols in pomegranate extracts. These results suggest that pomegranate extract administration may inhibit PAH progression, but more research is needed to evaluate the effectiveness of the long-term use of PAH progression.

4. Conclusions

The effectiveness of pomegranate extracts in increasing MMP-1 levels, lowering TIMP-1 levels, increasing MMP-1/TIMP-1 ratio, and decreasing type I collagen in two weeks and four weeks of observation showed that pomegranate extracts may inhibit the initial fibrogenesis process while increasing fibrosis resolution.

Pomegranate extracts are expected to prevent fibrosis and reduce the degree of fibrosis that has already occurred. The results of this study are expected to be a consideration for the use of pomegranate extracts as an adjunctive therapy to existing standard therapy to decrease PAH-related morbidity and mortality.

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DIETARY INTAKES AND HIGH SENSITIVITY CRP (hsCRP) IN ADOLESCENTS WITH OBESITY

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ABSTRACT

Obesity is related to the over-expression of pro-inflammatory cytokines and causes chronic systemic inflammation. The identification of dietary intake at risk of inflammation led to optimal interventions. The aim of this study was to determine the correlation between dietary intake and the inflammatory biomarker in adolescents with obesity.

A cross-sectional study was performed on adolescents with obesity at the Pediatric Nutrition Clinic of Dr Soetomo General Hospital, Surabaya from July to October 2018. Dietary intake was obtained from food recall. The assessment of high sensitivity CRP (hsCRP) was performed using ELISA. Statistical analysis was performed using a correlation test with $p<0.05$ indicating significance.

A total of 59 adolescents were included, 32 (54.2%) of which were male and 27 (45.8%) were female. The age range was 13–16 years. The mean calorie intake was 1955.9 ± 778 calories, and the mean hsCRP level was 2308.83 ± 470.95 ng/ml. There was no correlation between total calories, carbohydrate, protein, and fat level intake and hsCRP ($p>0.05$). No substantial effect of dietary intake was found for hsCRP.

1.Introduction

In developing countries, the prevalence of obesity has increased in all age groups (Rachmi et al., 2017). The cause of obesity is energy imbalance between energy intake and energy expenditure. Diet is important in the development and progression of obesity.

Obesity is related to chronic low-grade inflammation (Lee et al., 2013) and oxidative stress (Paltoglou et al., 2017). Chronic inflammation causes metabolic disease (Castro et al., 2017). Abdominal obesity is a predictive factor of cardiometabolic risk (Amato et al., 2013). Even in obese individuals with a healthy metabolic state, they are at an increased risk of long-term mortality (Kramer et al., 2013).

Over-nutrition is associated with immune-activation and inflammatory conditions (Lee et al., 2013). Dietary excess produces an accumulation of lipids in adipocytes (Gómez-Hernández et al., 2016). Excess weight has an inflammatory effect (García-Hermoso et al., 2016). However, exercise can reduce the systemic inflammation associated with obesity (Sirico et al., 2018). The complications of obesity are due to metabolic disorder induced by an excessive accumulation of fat, which leads to cardiovascular disease (CVD) and type 2 diabetes mellitus (Morandi and Maffeis, 2014). Diet and nutrition are modifiable variables in obesity (Hale et al., 2015). The aim of this study was to determine the correlation between dietary

intake and inflammatory biomarkers (hsCRP) in adolescents with obesity.

2. Materials and methods

This is a cross-sectional study conducted at The Pediatric Nutrition Clinic of Dr Soetomo General Hospital, Surabaya from July to October 2018.

2.1. Samples

Adolescents (age 13–16 years old) with obesity were enrolled. The following exclusion criteria were applied: (a) steroid or medical therapy, (b) hormonal therapy, (c) alcohol consumption, (d) smoking, (e) infection, (f) a medical history including an endocrinology disorder, immunological disorder, cardiovascular disease, or other known chronic pathology, and (h) secondary obesity (having a medical condition that causes gain weight). Health status was obtained through self-report to exclude acute and chronic diseases. The study procedures and objectives were explained to the parents and informed written consent was obtained from all participants before they were enrolled. This study was approved by the Ethics Committee in Health Research of the Dr Soetomo General Hospital, Surabaya.

2.2. Anthropometric measurements

Body weight was measured with the patients barefoot and lightly clothed to the nearest 0.1 kg using calibrated digital scales (Seca, Germany ref. 224 1714009). The barefoot standing height was measured using a fixed stadiometer to the nearest 0.1 cm (Seca, Germany ref. 224 1714009). Following inclusion, the body mass index (BMI) of all participants was calculated using the following formula: body weight (Kg)/height (m^2); obesity was defined as a BMI greater than the 95th percentile for sex and age based on The United States Centers for Disease Control and Prevention (CDC 2000).

2.3. Data Collection

The nutritional profiles of participants were established through food recall using a food model. The total daily intake of calories, carbohydrates, proteins, and fat were calculated.

2.4. Blood Analyses

Blood samples were collected in 5 cc aliquots from subjects using tubes containing EDTA. They were centrifuged and shipped to the Laboratory of the Institute of Tropical Disease, Universitas Airlangga for the analysis of inflammatory biomarkers, including hsCRP. Serum levels of hsCRP (ref. CAN-CRP-4360) were measured by specific ELISA kits (*Bioassay Technology Laboratory, China*) following the manufacturer's instructions.

2.5. Statistical Analysis

Data were analyzed using the SPSS software. The value of $p<0.05$ was taken as statistically significant. The Pearson correlation analysis was performed to examine the relationship between calories, protein, carbohydrate, fat, and hsCRP.

3. Results and Discussion

A total of 59 adolescents were included in this study, consisting of 32 (54.2%) males and 27 (45.8%) females. The age range was 13–16 years. Anthropometric measurements, dietary intake, and laboratory characteristics are shown in Table 1

Table 1. Characteristics of subjects

Variable	Mean (SD)
Body weight (kg)	80.77 ± 13.35
Body height (cm)	158.76 ± 7.12
Body Mass Index (kg/m^2)	31.99 ± 3.67

Calories (Kcal/day)	1955.96 ± 778.01
Carbohydrate (Kcal/day)	1005.95 ± 421.67
Protein (Kcal/day)	361.66 ± 221.43
Fat (Kcal/day)	589.03 ± 313.25
hsCRP (ng/ml)	2308.83 (285.79–2941.37)

The mean hsCRP level was 2308.83 ± 470.95 ng/ml. The mean calorie intake was 1955.96 ± 778 calories. The correlation between dietary intake and hsCRP is given in Table 2.

Table 2. Correlation between dietary intake and inflammatory biomarkers

Variable	Total Calories	Carbohydrate	Protein	Fat
hsCRP				
r	-0.167	-0.178	-0.015	-0.164
p	0.207	0.177	0.910	0.215

In this study, there were no significant correlations between total calories, carbohydrate, protein, and fat level intake and hsCRP ($p>0.05$) (Table 2.).

Obesity is associated with oxidative stress and aseptic inflammation (Paltoglou et al., 2017). This inflammation is associated with the development of many comorbidities, including metabolic syndrome, cardiovascular disease (CVD) and type 2 diabetes (DeBoer, 2013). Fat cell size and fat cell number are increased in obesity or with a combination of the two (Coelho et al., 2013). Adipose tissue is an endocrine organ secreting adipocytokines, which exert endocrine, paracrine, and autocrine actions, both locally and systemically (Kelishadi et al., 2017). There is an increase in the adipocyte number (hyperplasia) and adipocyte size (hypertrophy), which releases various cytokines such as leptin, adiponectin, tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1, IL-4, IL-6, IL-10, and CRP (Castro et al., 2017). These cytokines induce oxidative stress and inflammation (Ellulu et al., 2017). Elevated inflammatory biomarkers were higher in abdominal adiposity (Steckhan et al., 2016).

HsCRP and TNF-α were significantly higher in obese compare to non-obese individuals (Ayoub et al., 2015). HsCRP is an acute phase protein produced by the liver. HsCRP is negatively associated with anti-oxidation in pre-pubertal obese boys (Paltoglou et al., 2017). In inflammatory conditions, hsCRP plasma levels are increased and associated with metabolic syndrome, insulin resistance, and cardiovascular disease (DeBoer, 2013). TNF-α is a pro-inflammatory cytokine produced by macrophages and T lymphocytes (Kelishadi et al., 2017). Elevated TNF-α is associated with insulin resistance and cardiovascular risk (Khosravi et al., 2013). Higher BMI is associated with a higher TG/HDL ratio and insulin resistance in pre- and post-pubertal children (Olson et al., 2012).

Dietary imbalances characterized by an excessive intake of calories, fat, and carbohydrate are observed in obese subjects (Ayoub et al., 2015). High fat and energy-dense foods are a dietary habit in obesity (Manna and Jain, 2015). The excessive fat and carbohydrate is associated with inflammation (Ayoub et al., 2015). Polyunsaturated fatty acids, an appropriate glycemic index and glycemic load in carbohydrates are associated with improving inflammation in obesity (Lee et al., 2013). Dietary fatty acids influence the expression of the TNF-α cytokine gene and alter TNF-α production (Joffe et al., 2013). The excessive

intake of carbohydrate and fat is associated with increased TNF-α (Ayoub et al., 2015).

A high protein diet did not improve anthropometric measurements and cardiovascular risk factors (LDL, HDL, insulin, and blood pressure) in obese children (Izadi et al., 2018). However, the energy-restricted high protein diet is associated with a reduction of waist circumference and hsCRP in women (Azadbakht et al., 2013). A low fat diet can reduce CRP (Steckhan et al., 2016).

In this study, no correlation was found between calories, carbohydrate, protein, fat level intake and hsCRP (Figure 1-4.). There was ethnicity difference with another study that showed correlation between dietary intake and inflammatory biomarkers.

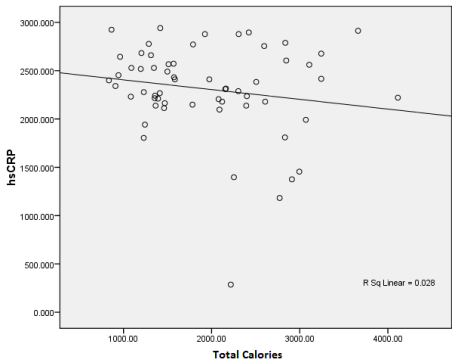

Figure 1. Correlation between Total Calories and hsCRP.

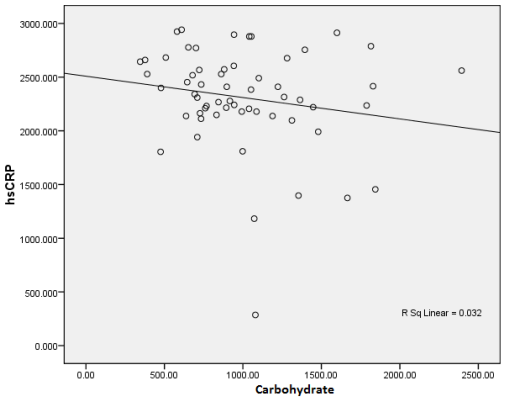

Figure 2. Correlation between Carbohydrate and hsCRP.

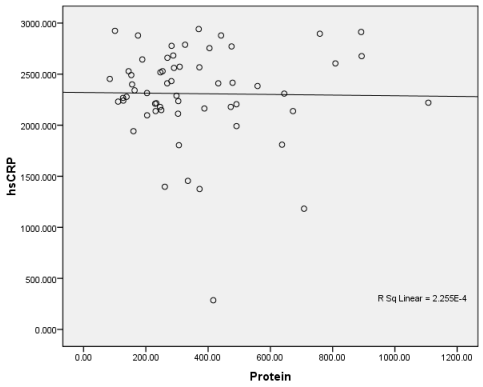

Figure 3. Correlation between Protein and hsCRP.

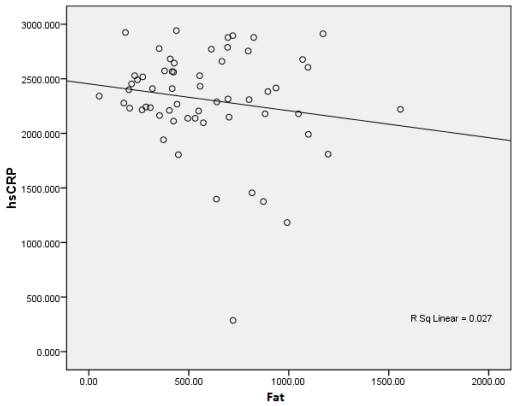

Figure 4. Correlation between Fat and hsCRP.

Dairy product consumption does not influence the biomarkers of inflammation (Labonté et al., 2013). Recent studies in the literature have reported that dietary intake has a positive effect on inflammatory biomarkers in patients with metabolic syndrome (Steckhan et al., 2016). The metabolic syndrome was not examined in this study.

There are limitations on this study. First, the sample size was small. Second, the study only obtained 24 hour recalls once. Third, there was a factor affecting of hsCRP, such as physical activity.

4. Conclusions

No effect of dietary intake of calories, carbohydrate, fat, and protein was found for hsCRP. Further studies are needed with larger sample sizes to investigate dietary food composition and its influence on inflammatory biomarkers in adolescents with obesity.

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ANTHROPOMETRIC MEASUREMENTS AND INFLAMMATORY BIOMARKERS IN OBESE ADOLESCENTS

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ABSTRACT

Obesity is related to chronic inflammation. Various anthropometric measurements have been shown to be associated with complications of obesity. Identification of the most accurate anthropometric measurement correlated with inflammation could lead to early interventions. The aim of this study was to determine the correlation between anthropometric measurements and inflammatory biomarkers in obese adolescents.

A cross-sectional study was performed on obese adolescents at the Pediatric Nutrition Clinic of Dr Soetomo Hospital, Surabaya. The inflammatory markers High Sensitivity C-Reactive Protein (hsCRP) and Tumor Necrosis Factor Alpha (TNF-α) were measured using ELISA. Anthropometric measurements including BMI (kg/m^2), waist circumference (cm), and waist to hip ratio (WHR) were performed. Statistical analysis was performed using a correlation test with significance set at $p<0.05$.

In total, 59 adolescents aged 13-16 years were included. The mean BMI was 31.99 (26.6–41.13) kg/m^2 and the mean waist circumference was 100.18 (75–122) cm. There was no correlation between TNF-α and BMI (r=-0.094; p=0.479), waist circumference (r=-0.041; p=0.757), or WHR (r=0.041; p=0.759). There was also no correlation between hsCRP and BMI (r=0.184; p=0.162) or WHR (r=0.146; p=0.274). However, hsCRP had a weak positive correlation with waist circumference (r=0.315; p=0.015). Waist circumference could serve as an indicator of a systemic inflammatory state in adolescents with obesity.

1. Introduction

Obesity is a global issue that is related to morbidity and mortality. Obesity causes chronic inflammation of the adipose tissue which is involved in the production of adipocytokines, such as IL-1, IL-6, IL-8, TNF-α, resistin, and leptin (Castro et al., 2017). Adipocytokine production by adipose tissue results in a pro-inflammatory condition and oxidative stress (Ellulu et al., 2017). This condition impacts cell function, thus causing diseases such as metabolic syndrome (Castro et al., 2017). Metabolic syndrome is a cluster of cardio metabolic risk factors which includes obesity (Roberts et al., 2013).

Anthropometric measurements of waist circumference and the WHO method of defining obesity in adults have been studied. A waist circumference of 76.8 cm (men) and 71.7 cm (women) is associated with obesity and a waist to hip ratio (WHR) of 0.86 (men) and 0.77 (women) is the cut-off used to define obesity (Hastuti et al., 2017). A study in adults showed that WHR is a poor predictor of obesity, especially in women, compared to other

anthropometric parameters (Sinaga et al., 2018). On the other hand, another study in adults revealed that waist circumference has an impact on inflammatory conditions and a further study mentioned that waist circumference is a weak indicator of elevated hsCRP and decreased adiponectin (Schlecht et al., 2016). A study in children stated that a WHR of greater than or equal to 0.51 is associated with a higher risk of inflammation (Mendes et al., 2017). However, the results of the above-mentioned studies remain controversial. The aim of this study was to determine the association between anthropometric measurements (BMI, waist circumference, and WHR) with inflammatory biomarkers in obese adolescents.

2. Materials and methods

This was a cross-sectional study in adolescents aged 13-16 years with obesity. The exclusion criteria for this study were corticosteroid therapy in the 6 months immediately prior to the study, antibiotic use, hormonal therapy, alcohol consumption or smoking, the presence of an infection, immunity or endocrine disorder.

2.1. Anthropometric measurements

Anthropometric measurements are a series of measurements performed by trained medical practitioners which include weight, height, waist circumference, and thigh circumference. Weight was measured with the individuals not wearing shoes, and wearing clothes with a weight of less than 0.1 kg, without any other accessories, using a digital scale (Seca, Germany). Height was measured in an upright position, without the presence of clothes or head covers, and using a Seca stadiometer. Body mass index (BMI) was defined as weight (in kilograms) divided by the square of height (in metes). Waist and thigh circumference were measured using metlin. Waist circumference was measured from the midpoint between the iliac crest and the lowest rib.

2.2. TNF alpha test

TNF alpha is a pro-inflammatory cytokine synthesised in macrophages in adipose tissue (Lee et al., 2013). The TNF-α test was performed using the ELISA method from *Bioassay Technology Laboratory* (China), and measured in ng/L. The concentration was determined using a standardised curve established by the manufacturer.

2.3 CRP test

CRP is a systemic inflammation marker and a nonspecific acute phase reactant produced in the liver. CRP is commonly used to detect inflammation and infections which cause injury to the liver. The hsCRP test was performed using an ELISA method from *Diagnostic Biochem Canada Inc.* (Canada), and measured in ng/mL. A study in adults showed that an increased CRP level of >10 mg/L in obese women is more likely to be caused by chronic inflammation than acute inflammation; thus, a CRP level of >10 mg/L could distinguish between acute and chronic inflammation in obese women (Ishii et al., 2012).

2.4. Statistics method

Average values, minimum values, and maximum values were analysed using quantitative parameters. The correlation between TNF-α, hsCRP, BMI, waist circumference, and WHR was analysed using bivariate analysis with a significant p value of <0.05. If the data showed normal distribution, analysis was conducted using Pearson correlation; otherwise, Spearman's rho was used. Analysis was performed using the SPSS software package.

3. Results and discussions

In this study, there were 59 adolescents with obesity, consisting of 32 (54.2%) male adolescents and 27 (45.8%) females, as shown in Table 1.

There was no correlation between TNF-α and BMI (r=-0.094; p=0.479), waist circumference (r=-0.041; p=0.757), or WHR (r=0.041; p=0.759). hsCRP did not show any correlation with BMI (r=0.184; p=0.162) or WHR (r=0.146; p=0.274). However, hsCRP showed a low

positive correlation with waist circumference (r=0.315; p=0.015), as shown in Table 2

Table 1. Characteristics of study subjects

Variable	**Number (percentage)**
Sex	
Male	32 (54.2)
Female	27 (45.8)
Maternal Education	
Uneducated	2 (3.4)
Elementary School	4 (6.8)
Middle School	5 (8.5)
High School	25 (44.1)
Diploma	5 (8.5)
Bachelor	17 (28.8)
Paternal Education	
Uneducated	1 (1.7)
Elementary School	5 (8.5)
Middle School	3 (5.1)
High School	31 (52.5)
Diploma	4 (6.8)
Bachelor	15 (25.4)
Maternal Occupation	
Employed	36 (61)
Homemaker	23 (39)
Mean (Average)	
Weight (kg)	80.77 (53.5–112)
Height (cm)	158.76 (140.8–175.5)
Body Mass Index (kg/m^2)	31.99 (26.6–41.13)
Waist Circumference (cm)	100.18 (75–122)
Waist to hip ratio	0.9477 (0.79–1.04)
TNF-α (ng/l)	147.17 (20.63–337.11)
hsCRP (ng/ml)	2308.83 (285.79–2941.37)

Table 2. Correlation among variables

Variable	**BMI**	**Waist circumference**	**WHR**
TNF-α			
r	-0.094	-0.041	0.041
p	0.479	0.757	0.759
hsCRP			
R	0.184	0.315	0.146
P	0.162	0.015	0.274

Obesity is associated with subclinical inflammation because there is an imbalance in inflammation mediators (Todendi et al., 2016). Subclinical inflammation takes place when BMI rises (del Mar Bibiloni et al., 2013). Adipose tissue produces adipokines (such as resistin, adiponectin, leptin, and visfatin), pro-inflammatory cytokines, and anti-inflammatory markers (Lee et al., 2013).

Inflammation is associated with a risk of cardiovascular diseases in children (Caminiti et al., 2016). Levels of the inflammatory marker hsCRP rise when there is an increase in body fat levels (Singer et al., 2014). Body fat and adipose tissue reserves are associated with cardio metabolic diseases in children and adults (Staiano and Katzmarzyk, 2012). As fat percentage increases, there is a greater risk of cardiovascular disease in children, especially with a body fat percentage of greater than 20% in both sexes (Going et al., 2011).

Anthropometric measurements are appropriate for detecting overweight/obesity in children; these include BMI, waist circumference, and arm circumference (Shafiee et al., 2018). An increase in abdominal fat level is associated with an increase in inflammatory markers (Toemen et al., 2015). Inflammatory conditions in obesity are marked by an increase in TNF- α and hsCRP (Ayoub et al., 2015).

A study in children with an average age of 10.03±0.74 years showed that waist circumference is positively correlated with TNF-α levels (Guedes et al., 2016). This is in accordance with another study in adults which mentioned that waist circumference is positively correlated with TNF-α levels (Marques-Vidal et al., 2012). In this study there was no correlation between anthropometric measurements with TNF-α level (Table 2.)

HsCRP could be utilised as an early inflammatory marker in obesity. The prevalence of hsCRP levels higher than 3 mg/L in obese children is 4.15, falling to 1.91 in overweight children (Todendi et al., 2016). In this study, there was a positive correlation between hsCRP and waist circumference (Figure 1.). Waist circumference has a strong correlation with inflammation (Arbel et al., 2012). Another study showed that CRP is not correlated with waist circumference (Bea et al., 2018). A study in obese pre-adolescent girls showed that waist circumference could be a better indicator of cardio metabolic risk than BMI (Hetherington-Rauth et al., 2017). Waist circumference is a predictor of insulin resistance in obese boys and girls (Reyes et al., 2011). A study in non-obese girls also revealed that waist circumference is an independent predictor of insulin resistance compared to BMI (Wolfgram et al., 2015); this is due to visceral fat in the abdomen being pro-inflammatory. Visceral fat in the abdomen is exposed to macrophage infiltration, which causes cell dysfunction and metabolic syndrome (Weber et al., 2014).

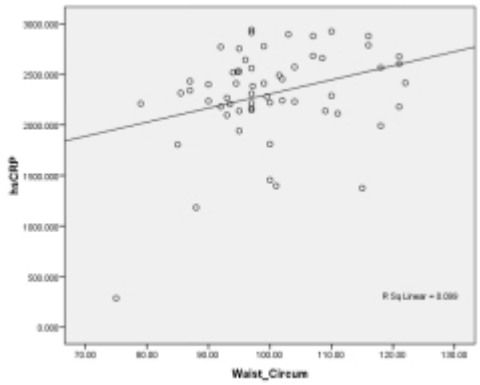

Figure 1. Correlation between hsCRP and waist circumference.

In this study, there were no correlation between hsCRP with BMI and WHR (Figure 2. and 3.). BMI has a strong correlation with inflammation (Arbel et al., 2012), but another study showed that BMI is not correlated with inflammation (Bea et al., 2018).

The combination of BMI and waist circumference increase inflammation, showed in increased hsCRP levels (Todendi et al., 2016). Another study showed that BMI with WHR are more accurate at predicting inflammation in obese children (Samouda et al., 2015).

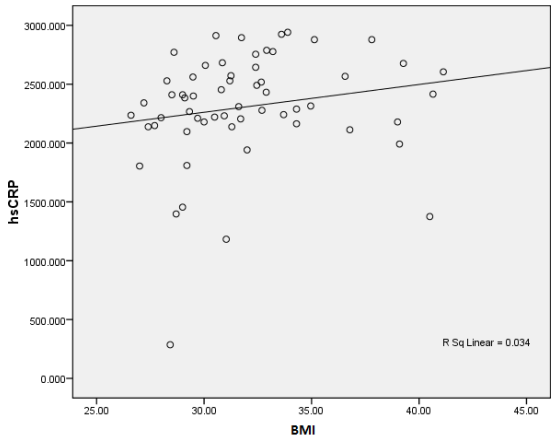

Figure 2. Correlation between hsCRP and BMI.

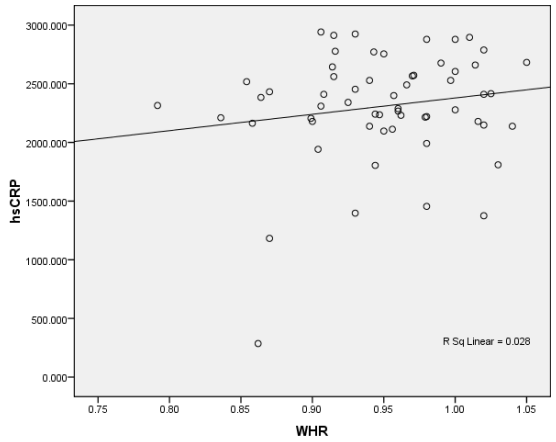

Figure 3. Correlation between hsCRP and WHR.

Waist circumference and WHR have stronger effects on increasing the risk of cardiovascular diseases than BMI (Goh et al., 2014). A study in obese children showed that insulin resistance and triglyceride levels are predicted more accurately by the combination of BMI and WHR or waist circumference (Samouda et al., 2015).

This study has some limitations, include physical activity and diet can influence the inflammatory response, and small sample size. Further study with large sample size and controlling of diet and physical activity are needed to examine the correlation between anthropometric measurements and inflammatory biomarker in obese adolescents in Indonesia.

4. Conclusions

Waist circumference could serve as an indicator of inflammatory states in obese adolescents.

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COMPARISON BETWEEN BABY LED WEANING AND TRADITIONAL SPOON-FEEDING ON IRON STATUS AND GROWTH IN BREASTFED INFANTS

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ABSTRACT

Background: The current guideline recommends infants are initially offered smoothly pureed foods, known as the traditional method of spoon feeding. Currently, an alternative method known as 'baby-led weaning' (BLW) has been really popular. With BLW, infants are allowed to self-feed family foods in their whole form instead special-prepared foods. Infants following BLW may be at increased risk of faltering growth.

Objective: To compare traditional and BLW methods in the risk of iron deficiency anemia (IDA) and growth faltering of breastfed infants.

Methods: A cross-sectional study was undertaken in 9 – 15 months breastfed infants admitted to Bedah Hospital Surabaya from August – October 2017. Exclusion criteria were infants suffering from chronic conditions. Biochemical assessment of Haemoglobin (Hb), Serum Transferrin (ST) and Serum Ferritin (SF) was measured. Diagnosis of IDA and growth chart interpretation was made based on WHO criteria.

Results: Out of all, 12/30 boys and 15/30 following BLW. Mean age was 12.6±2.14 months old. Mean Hb level, ST level and SF in BLW group was 10.9±0.55 g/dl, 11.6±7.13%, 19.1±18.40ug/dl prospectively, while in the traditional group were 12.5±0.75 g/dl, 24.6±7.92%, 57.6±18.78 ug/dl prospectively. IDA was higher in BLW group than in the traditional group. (13/15 vs 3/15, OR 26.000 95%CI 3.686-183.418, p <0.001). Underweight was higher in BLW. (13/15 vs 3/15, OR 26.000 95%CI 3.686-183.418, p <0.001). Stunted were higher in BLW (2/15 vs none, OR 1.667 95%CI 1.103-2.519. p=0.017).

Conclusion: In breastfed infants, those who were following BLW are in higher risk of IDA, underweight and stunted than traditional spoon feeding.

1. Introduction

The current guideline of the World Health Organization recommends that infants are initially offered smoothly pureed foods, and gradually increased food consistency and variety as the infant gets older, adapting to the infant's requirements and abilities. The guideline also recommends practicing responsive feeding, specifically feed infants directly and assist older children when they feed themselves. This is known as the traditional method of spoon-feeding (WHO, 2001). Over the last 10 years, an alternative method known as 'baby-led weaning' (BLW) has been really popular in Indonesia.

Baby-led weaning is a method for introducing complementary foods in which

the infant feeds themselves hand-held foods or finger foods instead of being spoon-fed by an adult. Infants following BLW share family food and are offered breast milk on demand until they wean. (Cameron *et al.*, 2012; Brown *et a*l., 2017)

Baby-led weaning was introduced with the publication of the book of *Baby-Led Weaning* by Gill Rapley and Tracey Murkett. It has been suggested that BLW may encourage greater acceptance of foods with a variety of textures and flavors and that this may result in higher intakes of "healthier" foods such as vegetables and unprocessed foods as the child grows, so it could lower the risk of obesity. In BLW, infants share family food and meal times. The food is offered as ''graspable" pieces that they can pick up and they learn to feed themselves. The infant is in control of exactly what and how much they eat, They never hurried or forced to eat food. Combined method of BLW and traditional spoon-feeding is not defined as BLW (Rapley, 2008).

Weaning period is a peak period for faltering in a child's growth. (Abeshu *et al*., 2016) Infants following BLW may be at increased risk of growth faltering, based on the assumption that not all infants will have the motor skills to feed themselves the amount of food they require, and that many of the first foods offered will be low in energy and iron.

2.Materials and methods

2.1.Materials

All breastfed infants between 9 months old to 15 months old were prospectively enrolled. Exclusion criteria included chronic conditions such as cerebral palsy, congenital deformities and chromosomal disorders. Infants with urinary tract infection, hypothyroid, and tuberculosis were also excluded.

2.2.Methods

This study was approved by the institutional review board of the Bedah Hospital Surabaya. The present study was carried out in the Outpatient Clinic Bedah Hospital Surabaya, during the period from August 2017 until October 2017. Based on the anamnesis, we classified all the subjects into 2 groups based on their feeding methods, the BLW group and the traditional group. Assessment of Haemoglobin (Hb), Serum Transferrin (ST) and Serum Ferritin (SF) was measured, as well body weight and body length. Diagnosis of IDA and growth anthropometry was interpreted based on WHO criteria. SPSS for Mac was used for analyses. To compare continuous variables, we used a two-sample *t* test or Mann- Whitney rank sum test, and to compare categorical variables we used the Chi-square or Fisher's exact test, as indicated.

3.Results and discussions

There were 30 infants included in the study. Of these, 12 infants were boys. All the infants started the complementary feeding at 6 months old. There was 15 infants who were following BLW, and there was 15 infants who were following traditional spoon-feeding method. Mean age was 12.6±2.14 months old. There was no significant difference between two groups with respect to age or sex (see Table 1). There was significant difference between two groups on Hb level, Serum Transferrin and Serum Ferritin (P<0.001). Iron Deficiency Anemia was higher in BLW group than in the traditional group. (13/15 vs 3/15, OR 26.00 95%CI 3.686-183.418, p <0.001) (see Table 2).

3.1.Comparison on Iron Status

Iron Deficiency Anemia was higher in BLW group than in the traditional group. (13/15 vs 3/15, OR 26.00 95%CI 3.686-183.418, p <0.001).

3.2.Comparison on Growth Status

Underweight was higher in BLW group then in the traditional group. (13/15 vs 3/15, OR 26.00 95%CI 3.686-183.418, p <0.001). Severe underweight was higher in BLW group then in the traditional group. (2/15 vs 0/15). Stunted were higher in BLW group (2/15 vs none, OR 1.67 95%CI 1.103-2.519. p=0.017) than in the traditional group, meanwhile wasted

was higher in BLW group then in the traditional group (11/15 vs 2/15).

To date, there are limited researches about BLW. Our study showed there was no difference noted with both groups with respect to gender and age. This was in line with some previous study (Morison et al., 2016; Cameron et al., 2013).

A significant difference was noted in iron status between the two groups (P<0.001).There was no consideration about iron status before study. Until now, there were no studies examining iron status in infants following BLW. But, there was a study revealed that infants following BLW appeared to have significantly lower intakes of iron, zinc and vitamin B12 (Morison *et al*., 2016). This was consistent with another study revealed that infants following BLW ate more fruits and vegetables which were low-iron foods while the infants following traditional spoon-feeding eat more baby rice cereal (Cameron *et al*., 2013).

Food presentation significantly influences food preferences (Blossfeld *et al*., 2007), so it is possible that differences in the presentation of foods between the two groups impacted on preferences. Meat may be easier to eat when pureed and spoon-fed (Morison *et al*., 2016). This can be explained by the fact that infants' early chewing is a primitive up-down munching pattern (Morris et al., 2000; Rudolph et al., 2002). For small pieces of soft foods, this pattern may be adequate. But for hard foods such as meat or strips, it is inadequate (Morris *et al*., 2000). BLW may not be suitable for age and infant's feeding skill development.

Our study noted a significant difference in growth status between both groups. Infants following BLW were at increased risk to have underweight, stunted and wasted. Consistent with this, some previous studies also showed underweight was higher in infants following BLW compared to those who were following traditional spoon- feeding (Townsend *et al*., 2012; Brown *et a*l.,2015). According to one study in the United Kingdom which compared body weight for age in infants following BLW and infants following traditional spoon-feeding. For the BLW group, 86.5% were of normal weight, 8.1% overweight and 5.4% underweight. In comparison, 78.3% of those in the traditional spoon-feeding group were normal weight, 19.2% overweight and 2.5% underweight (Brown *et al*., 2015).

Mothers following BLW method estimated that their babies ate more milk feeds and less solid food compare to those following a traditional spoon-feeding. This may provide inadequate nutrient intake such as energy and iron requirements for infants from 6 months of age onwards (Brown *et a*l., 2015).

Since in BLW approach, the infants are in full control to eat, there are some risks of inadequate intake. When the infants do not have interest in eating, when foods of inappropriate consistency are offered, and infants with poor self-feeding skills will nou able to have enough foods to meet their needs for rapid growth. This risk of inadequate energy and nutrient intake could lead to failure to thrive.

Table 1. Characteristic of samples

Variable	**BLW (n=15)**	**Traditional (n=15)**	**P**
Age (mo) 12.6±2.14	13±1.78	12.2±2.30	0.396
Sex Boys Girl	 12 18	 18 12	0.143

Iron Status			
Hb (g/dl)	10.9±0.55	12.5±0.75	<0.001*
ST (%)	11.6±7.13	24.6±7.92	<0.001*
SF (ng/ml)	19.1±18.40	57.6±18.78	<0.001*

Table 2. Logistic Regression Analysis

	Odds Ratio	**P**	**95% CI**
Iron Deficiency Anemia	26.00	<0.001	3.686-183.418
Underweight	26.00	<0.001	3.686-183.418
Stunted	1.67	0.017	1.103 -2.519

* Chi-square test

Table 3. Comparison of growth parameter between two groups

Growth Parameter	**BLW**	**Traditional**
Weight for age		
Normal	2	12
Underweight	11	3
Severe underweight	2	0
Length for age		
Normal	9	15
Stunted	6	0
Weight for length		
Normal	4	13
Wasted	11	2

4.Conclusions

Those who were following BLW are in higher risk of IDA, underweight and stunted than those who were fed traditionally. There are still some controversies of BLW that need further research.

5.References

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THE EFFECT OF EARLY PARENTERAL NUTRITION ON RETURN TO BIRTH WEIGHT AND GAIN WEIGHT VELOCITY OF PREMATURE INFANTS WITH LOW BIRTH WEIGHT

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Preterm infant.

ABSTRACT

Early aggressive parenteral nutrition has shown its benefits in preventing extra-uterine growth restriction. However, in daily practice, clinicians still in doubts to implement it in newborns. This study aims to analyze the effect of early parenteral nutrition on gaining weight pattern. This analytical study uses randomized-unblinded-controlled trial design. The study carried out on 44 preterm infants with gestational age less than 33 6/7 weeks, birth weight between 1000-2500 grams and unable to receive daily nutritional needs through oral and enteral. A control group (n=23) received early parenteral nutrition since day 3 and a treatment group (n=21) received parenteral nutrition since day 1. Return to Birth Weight (RTBW) and Gaining Weight Velocity (GWV) are measured to represent gaining weight pattern. The results of this study, RTBW mean have no significant difference ($p \geq 0.05$) in both groups. The treatment group has a higher weight loss on day 1 and day 3 ($p<0.001$; p0.02) and did not have a weight loss difference on day 7, day 10, and day 14 (all $p \geq 0,05$). Both groups has faster GWV on day 1 and 3 but similar on day 14 (18 gram/kg/day and 16 gram/kg/day respectively). Early parenteral nutrition has no significant effect on RTBW and GWV.

1.Introduction

Preterm infants are likely to develop extrauterine growth restriction. The incidence of preterm birth in Indonesia is estimated to be more than 15%, while the incidence of low birth weight infants is at 9% per year (UNICEF, 2004; WHO, 2012). Earlier studies found that 68.4% of underweight infants who were born in Dr. Soetomo General Hospital have an abnormal General Movement and were at high risk for neurodevelopmental disorders in the future (Rochmah *et al.,* 2012). Extrauterine growth restriction increases the incidence of behavioural disorders, mental retardation and persistent learning impairment later in life (Moyses *et al.*, 2013). Consequently, early life nutrition is important. Inadequate nutrition intake, protein and energy deficits in the first few days of life, bacterial infections and organ system disorders due to prematurity are the risk factors of extrauterine growth restriction (Moyses *et al.*, 2013).

Once the umbilical cord clamped, maternal nutrition support is stopped immediately. Early parenteral nutrition seems mimicking previous nutrition support from the mother. Early parenteral nutrition effects on preterm infants have various results (Deidre and Diane, 2004; Hay, 2013; Kotiya and Zhu, 2015; Moyses *et al.*,

2013). American Academy of Pediatric Committee on Nutrition (AAP-CON) and the European Society for Paediatrics Gastroenterology, Hepatology, and Nutrition Committee on Nutrition (ESPGHAN-CON) suggest that early parenteral and enteral nutrition can prevent the incidence of extrauterine growth restriction in preterm infants and improve long-term outcomes, especially in neurocognitive function. Protein and energy deficits can be reduced, RTBW can be faster, and the incidence of necrotizing enterocolitis and late onset of sepsis will decrease. Anthropometric parameters showed improvement at 37 weeks of corrected age with a shorter duration of care in NICU (Neonatal Intensive Care Unit) (Deidre and Diane, 2004; Hay, 2013; Moyses *et al.*, 2013). Meta-analysis and systematic review studies suggest that early parenteral nutrition in preterm infants has no significant effect on mortality, NEC incidence, retinopathy of prematurity (ROP), intraventricular hemorrhage and cholestasis (Kotiya and Zhu, 2015; Moyses *et al.*, 2013).

Despite of existing evidences, it is still difficult to motivate and make sure all hospital staff members to implement early parenteral nutrition in daily practice. There is a need to conduct a study which analyses the effect of early parenteral nutrition on simple anthropometric-related parameters such as Return to Birth Weight (RTBW) and Gain Weight Velocity (GWV). RTBW and GWV are seen as parameters that reflect the short-term growth outcome. Therefore, this study aims to analyse the effect of early parenteral nutrition on RTBW and GWV.

2. Materials and methods

2.1. Materials

The design of this study is an interventional analytic study with a randomized unblended controlled trial design. This study was conducted in the intensive neonatology observation room at the Department of Pediatrics, Dr.Soetomo General Hospital, since August 2016. The subjects of the study were preterm infants born in NICU Dr. Soetomo General Hospital and in accordance with the inclusion criteria of the study. The ethical clearance of this research has been obtained from Dr. Soetomo General Hospital Ethical Commission Board. The parents signed the informed consent form before their children were included in the study. Information for consent was given before the parents sign the informed consent forms. A total of 44 infants were divided into two groups. The first group, the control group, consisted of 23 preterm infants who received parenteral nutrition since day 3. The second group, the intervention group, consisted of 21 premature infants who received parenteral nutrition since day 1. The inclusion criteria of the study are (1) preterm infants with gestational age of less than 33 6/7 weeks with the birth weight between 1000 and 1500 grams, (2) preterm infants with gestational age more than 33 6/7 weeks up to 36 6/7 weeks and birth weight more than 1500 up to 2500 gram that are not able to be given nutrition by oral or enteral (full enteral feeding) due to neonatal asphyxia and/or respiratory distress syndrome. The exclusion criterion is preterm infants who have multiple congenital anomaly and congenital heart disease and need fluid restriction. The weight of the new-borns were measured on daily basis. Then, GWV and RTBW were calculated from the daily weight measurement. GWV is the velocity of weight gain (gram/kg/ day) which is calculated by reducing the weight of the 14th day with the lowest body weight of preterm infant, then it is divided by the number of days since the preterm infant starts to gain weight. RTBW is defined as the time needed (in days) for preterm infants to regain weight at the same level as birth weight.

2.2. Statistical Analysis

The data were analyzed using Microsoft Excel 2016, Windows 10 and IBM SPSS Statistics 21. The data were presented descriptively for the basic characteristics of the two study groups. The Chi Square test and independent sample t-test were used to analyze homogeneity. The Chi Square test, Fisher test, Mann-Whitney U test and unpaired T-test were

used to analyse the difference between GWV and RTBW from both groups. A 95% confidence interval and p value of <0.05 were used to determine the significance in this study.

3.Results and discussions

From 44 subjects who had met the inclusion criteria, 6 preterm infants were dropped out during the study period, 2 subjects from the treatment group and 4 subjects from the control group. The dropped-out subjects were all diagnosed septicemia. At the end of the study period, a final analysis of all the research subjects including the dropped-out subjects were performed. In this study, average gestational age when the baby were born was 31.52 weeks and the average birth weight was 1598.86 grams.

The average gestational age of the treatment group (31 weeks) is lower than the control group (33 weeks). The average birth weight of the treatment group was lower than that of control group ($p < 0.05$) (Table 1).

Table 1 Growth Patterns in Both Groups

Growth Patterns	Treatment Group (n=21)	Control Group (n=23)	p
TBW, median (min-max) (day)	8 (0-20)	7 (0-21)	0.85[1]
RTBW >14 days, n(%)	2 (9.50)	4 (17.40)	0.67[2]
GWV, median (min-max) (gram/kg/day)	18 (10-34.30)	16.33 (5-33)	0.14[1]
Post natal weight loss(%), median (min-max)	11.20 (0-20.20)	5.22 (0-23.66)	0.04[1]*

*Significant for $p < 0.05$, [1]Mann-Whitney U Test, [2]Fisher Test

Table 2. Parenteral Nutrition Composition in Both Groups

Parenteral Nutrition Composition	Treatment Group (n=21)	Control Group (n=23)	p
Carbohydrate (kcal/kg/day), mean (SD)			
a. Total	81.23 (±17.01)	69.18 (±13.70)	<0.001[2]*
b. Day-0	40.15 (±4.49)	33.64 (±6.86)	0.01[2]*
c. Day-3	72.34 (±9.77)	62.25 (±13.71)	<0.001[1]*
d. Day-7	88.93 (±16.06)	81.70 (±9.45)	0.09[2]
Protein (gram/kg/day), mean (SD)			
a. Total	3.25 (±0.25)	3.07 (±0.14)	0.02[1]*
b. Day-0	3	0	<0.001[1]*
c. Day-3	3.43 (±0.46)	3	<0.001[2]*
d. Day-7	3.25 (±0.58)	2.81 (±0.62)	0.06[2]
Lipid (gram/kg/day), mean (SD)			
a. Total	1.97 (±0.53)	1.55 (±0.13)	0.01[1]*
b. Day-0	1.5	0	<0.001[2]*
c. Day-3	2.13 (±0.64)	1.5	<0.001[2]*
d. Day-7	2.25 (±0.80)	1.70 (±0.27)	0.11[2]
GIR (mg/kg/minute), mean (SD)	7.06 (±1.70)	7.62 (±1.22)	0.21[2]

*Significant for $p < 0.05$, [1]Mann-Whitney U Test, [2]Independent T-Test

3.1. Mean Differences of RTBW and GWV between Treatment and Control Groups

Both groups did not differ significantly (p ≥0,05) in the mean of RTBW, number of subjects with RTBW more than 14 days, and GWV. The statistical analysis of the postnatal weight loss parameter showed that the treatment group had a greater weight loss than the control group (p = 0.04) (Table 1).

The treatment group had a Δ weight loss of -1.26% from the birth weight at day 1, while the control group had a Δ weight loss of -0.55%. On day 3, Δ weight loss in the treatment group was -7.07% from the birth weight and in the control group was -3.29%. On day 7, Δ weight loss in the treatment group was-3.11% and in the control group was -0.12%. Using the statistical analysis, the treatment group had a larger Δ weight loss compared to the control group on day 1 and day 3 (p = <0.001; 0.02, respectively). The treatment groups did not have a Δ weight loss difference with control group on day 7, 10, and 14 (all p > 0.05).

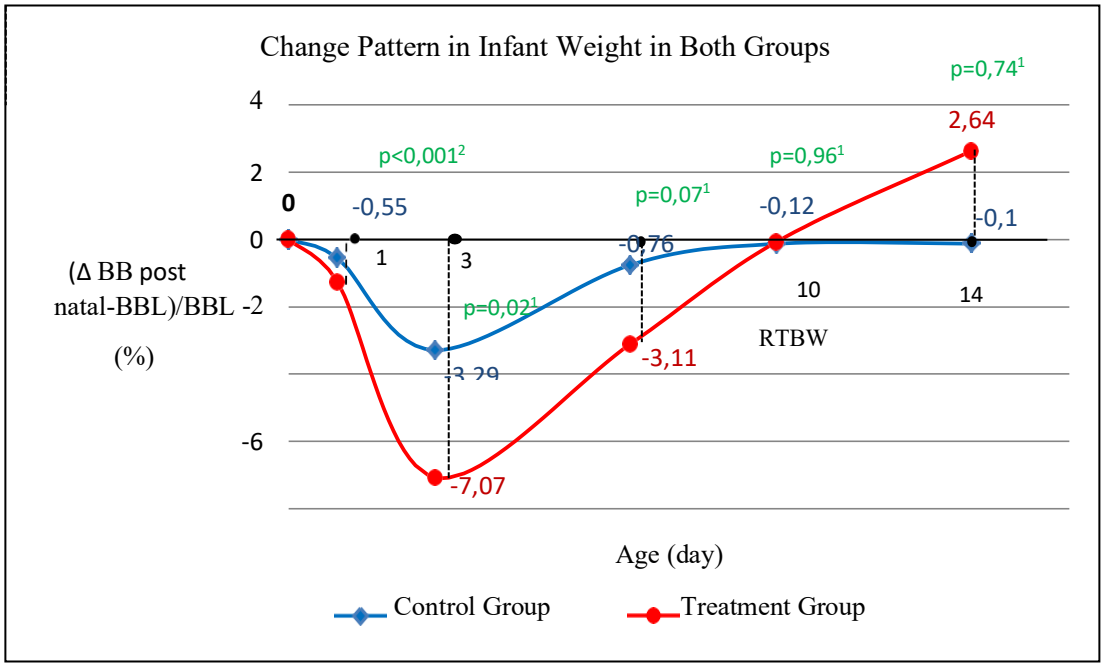

Figure 1. Change Pattern in Infant Weight in Both Groups

In this study, we analyzed the difference of parenteral nutrition mean composition between the two groups. The total and daily means of carbohydrates, proteins, lipids were counted since the subject received parenteral nutrition for the first time. The daily average of the parenteral nutrition components of both groups was calculated on day 0 (when parenteral nutrition was firstly initiated to the treatment group), day 3 (when parenteral nutrition was firstly initiated to the control group), and day 7 (mean of duration of parenteral nutrition given to both groups). The treatment group had a higher mean in total calories (p <0.001), calories on day-0 (p = 0.01), calories on day-3 (p <0.001), total protein (p = 0.02), protein on day-0 (p <0.001), protein on day-3 (p <0.001), total lipids (p = 0.01), lipids on day-0 (p <0.001) and lipids on day-3 (p <0.001). The mean calories on day-7, protein on day-7, lipids on day-7, and GIR did not differ between the two groups (Table 2). The treatment group had a lower median birth weight and lower first minute Apgar score, and faster GWV compared to the control group on observation day 1 (p <0.001), day 3 (p = 0.02), and had similar GWV on day 7, day 10, and day 14. Therefore, the RTBW between both groups did not differ at the end of the observation period (Figure 1).

3.2. Biochemical Monitoring of Treatment and Control Groups

On biochemical side effects monitoring, both groups had no difference in the random glucose test level, occurrences of hypoglycemia, serum triglyceride level, hypertriglyceridemia, direct bilirubin levels, total bilirubin levels, direct hyperbilirubinemia, mean BUN level, mean creatinine serum level, mean sodium level, mean potassium level, median chloride serum level and mean calcium serum level (all p ≥0.05) within the 24 hours of life. Both groups also had no difference in fluid balance and urine production on day 1, 3, and 7 (p ≥0,05)

3.3. Discussion

This study showed that aggressive parenteral nutrition did not seem to make difference in GWV and RTBW within the first week of life, although the treatment group had a higher weight loss than the control group. This could be explained because the mean birth weight was lower in the treatment group. Earlier studies showed that low birth weight infants are more at risk of extrauterine growth restriction than normal birth weight infants (Bolisetty *et al.,* 2014; Namiiro *et al.,* 2012). The treatment group in this study had one-minute Apgar score lower than the control group, but the five-minute Apgar score of both groups did not differ significantly.

The general characteristics of both groups in this study were same in terms of mean maternal age during delivery, mothers' perception towards pregnancy, history of premature pregnancy, and female sex's infant. The mother age during delivery which is more than 30 years [OR 0.41 (95% CI 0.20-0.82)], history of premature pregnancy [OR 2.4 (95% CI 1, 0-5,6)], female sex's infant [OR 1.23 (95% CI 0.84 to 1.81)], and unwanted pregnancy [OR 0.56 (95% CI 0.34-0, 93)] are the risk factors for intrauterine growth restriction in preterm infants (Viengsakhone, 2010; Zambonato, *et al.,* 2004). Preterm infants with intrauterine growth restriction had a double-risk for delayed GWV [OR 2.36 (95% CI 1.34-4.14)] and gain length velocity [OR 2.13 (95% CI 1.30-3, 50)] in the first month of life compared to low birth weight in term infants. In the third months of life, the risk of failure to grow increased [OR 5.89 (95% CI 3.07-11.30)] (Kiy *et al.,* 2015).

The daily measurement of infants' weight will not reflect gaining weight pattern because it only reflects the body water balance (Anchieta *et al.,*2004). Fenton and Kim (2013) recommend weekly weight measurement to observe the growth pattern of infants.

The effect of prenatal, perinatal, and post natal risk factors in the incidence of GWV and RTBW delay between both groups do not differ. Preeclampsia was not a risk factor for GWV delay [OR 0.47 (95% CI -0.10 s / d 1.05)] and height velocity gain delay [OR 0.2 (95% CI -0.29 s / d 0.69)] in the first 3 months of life (Kiy *et al.,* 2015). In this study, the number of preeclamptic mothers in the treatment group was higher than that of the control group. An earlier study showed that the difference in proportion of mothers with pre-eclampsia in both groups might explain why the median infants' birth weight and mean Apgar score in the treatment group is lower than the control group (Chen *et al.,* 2014).

In this study, the treatment group showed a lower median birth weight and one-minute Apgar score, but their GWV was faster than the control group on day 1 and day 3, and had the same GWV with the control group on day 7, 10, and 14. Despite the faster rate of GWV in infants with early parenteral nutrition, GWV with or without early parenteral nutrition was still within the normal range of 14.8-20 grams/kg/day (Bertino *et al.,* 2008).

The treatment group received a higher daily intake of parenteral nutrition since day 0 because of the early initiation. On day 3, the control group has just started the parenteral nutrition intake, while the treatment group received a higher dose because of the increased titration dose. On day 7, the parenteral nutrition dose in both groups are the same. A higher dose of carbohydrate in the treatment group is followed by protein nutrition intake of 3gram/kg/day on the same initiation day, so that the mean GIR in both groups are the same.

The absence of abnormal laboratorial results of both groups showed that aggressive parenteral nutrition is considered safe to be implemented in infants (Liu *et al.,* 2015). Hypoglycemia was found to be asymptomatic in three infants in the treatment group and two infants in the control group. Infants have a higher risk of developing hypoglycemia within the first 24 hours after initiation of parenteral nutrition (Lee, 2015). There was no difference in incidence of hypertriglyceridemia in both groups. Elevated triglyceride levels are not a risk factor for cardiovascular disease or obesity in adulthood (Cosmi *et al.,* 2011; Skilton *et al.,* 2011).

3.4. Limitation of study

This study was limited on the evaluation of GWV and RTBW for the growth parameter. The results do not reflect low birth weight growth pattern comprehensively. This study also only observes a short period of post natal age, so it is less likely to represent overall gaining weight patterns. Further studies are needed to expand the birth weight categories and include more preterm infants.

4. Conclusions

Early initiation of parenteral nutrition in preterm infants with low birth weight had no effect in the duration of Return to Birth Weight and Gain Weight Velocity. However, the acceleration of Gain Weight Velocity observed in the first 3-days of life reflects body fluids balance instead of growth.

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GAIN VELOCITY AND IGA SECRETORY FECAL BETWEEN PRETERM BABY RECEIVED HUMAN MILK AND HUMAN MILK FORTIFIED

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ABSTRACT

Preterm baby suffered from metabolic stress and hypogammaglobulinemia after birth. Extrauterine growth restriction (EUGR) is a common problem and related to neurodevelopmental outcome. The Independent risk factor of EUGR is necrotizing enterocolitis (NEC). Concentration secretory IgA (sIgA) as main immunity system decreased by age. Human milk fortification may resolve EUGR and organ immaturity of preterm baby.

Objective : Analyzed difference gain velocity and sIgA fecal between preterm baby received human milk and human milk fortified.

Methods : Prospective analytic observational study between December 2015-July 2016 at Soetomo Hospital Surabaya. Inclusion criteria consisted of gestational age ≤ 34 weeks and birth weight 1000 till less than 2000 g. Multiple congenital anomaly and enteral nutrition avoidance as exclusion criteria. Indication human milk fortification were stable period, no suckling reflex and gain weight velocity (GWV) <10 g/kg/d. Preterm baby was recruited and followed in 14 days. Chi-square, Mann-whitney and t-test independent sample used to analyzed discrepancies GWV, gain length velocity (GLV), gain head circumference velocity (GHC) and sIgA fecal.

Results : Human milk fortification (22(12,86-51,76) g/kg/day) showed significance difference to GWV than human milk (14,28(-12,86-(+32,86)) g/kg/day) group (p=0,020). GLV(p=0,257), GHC (p=0,215) and sIgA fecal (p=0,418) revealed no difference. Side effects (feeding intolerance and NEC) not found during observation.

Conclusions : Human milk fortification showed higher GWV than human milk group. Follow up still needed to evaluate anthropometric parameter.

1.Introduction

Prematurity infants have growth problems at 36 weeks postmenstrual age (91%) and mature (30%) (Fanaroff *et al*., 2007; Leppänen *et al*., 2014). Growth disorders cause neurodevelopmental disorders, cognitive function, and quality of life of prematurity infants, one of the disturbances in gastrointestinal organs (Cooke *et al.*, 2003). In prematurity infants, there is Immunoglobulin A (the main body's immune system in the gastrointestinal tract) and breast milk is the main source of IgA but its levels decline with the age of prematurity infants (Araújo and Gonçalves, 2005). Growth rates of weight gain were lower in 171 underweight infants who received breast milk > 75% (Colaizy, 2012). Therefore, it needs optimal nutritional support in prematurity infants to fit the intrauterine growth rate based on postconceptional age guidelines (American Academy of Nutrition Committee on Nutrition, 1977).

The technique of giving fortification to proper infant breastmilk is still continuously studied, as it found in the Gross study that the standard fortification for infants less than 24 kcal/oz was not able to meet the growth rate (Gross, 1987). Meanwhile, standard fortification according to Schutzman of 22 kcal/oz is recommended in prematurity infants with a birth weight of 1000-2000 grams. In 2 cases of underweight infants at Dr. Soetomo General Hospital Surabaya with fortification standard 22 kcal / oz (gestational age 31-33 weeks and birth weight 1400-1500 gram) showed that short-term growth rate (body weight 21.21 g /kg/day, 1 cm/week body length and head circumference 1.75 cm/week) whereas in 4 underweight babies with standard fortification 24 kcal/oz often showed sepsis with feeding intolerance. Adjustable fortification is an invasive technique that shows the growth rate of body length is not significantly different, and the constraint of its implementation is the availability of protein supplementation. (Arslanoglu *et al*., 2006). Tailored fortification is believed to be an appropriate fortification technique but it is expensive and the procedure is complicated (Reali *et al*., 2010).

Accordance with the Republic of Indonesia Government Regulation No. 33 the Year 2012 on exclusive breastfeeding and Regulation of the Republic of Indonesia Health Minister No.39 Year 2013 on infant formula and other infant products, strict evaluation and monitoring of infant formula usage, both from government and related institutions. In this study, observation and analysis of short-term growth rate and immune system of underweight infants receiving breast milk and breast milk were fortified with HMF by anthropometric method and examination of secretory IgA, IgE levels. This study aims to find a proper milking fortification technique and safe to overcome extrauterine growth restriction events and reduce the incidence of infection in infants less months. Thus, researchers observed and analyzed prematurity infants as indicated by breastfeeding fortification with HMF.

2. Materials and methods

This design of this study is a prospective analytic observational design. The study was conducted in the nursery Dr. Soetomo General Hospital Surabaya, which it begins December 2015 - July 2016. Samples were taken by consecutive sampling with a sample of 17 babies. The population of the samples was breastfed infants and breastmilk fortified with HMF (ASI + HMF) with inclusion criteria: Pregnancy age ≤ 34 weeks of gestational, birth weight 1000-2000 grams, subjects whose parents had signed an informed consent at the start of the study.

3.Results and discussions

The collection of research subjects was conducted from December 2015 to July 2016 and obtained 17 infants underwent indication of breastfeeding fortification (gestational age ≤34 weeks, birth weight 1000-2000 grams) and with the approval of the Neonatology Division staff Dr. Soetomo General Hospital Surabaya. Other considerations include poor feeding ability, no history of feeding intolerance, not being treated with oxygen supplementation, not in sepsis. Infants less than matched according to the above criteria will receive HMF fortified milk for 14 days and evaluated anthropometric and secretory IgA parameters before and after the study and monitored adverse effects of feeding intolerance and necrotizing enterocolitis (NEC).

The characteristics of mothers at the research subjects showed no significant differences in age, nutritional status, history of parity, history of preeclampsia / eclampsia, risk of delivery and history of specific illness during pregnancy in Table 1 ($p>0.05$). Characteristics in socio-economic status of both groups showed no significant difference ($p>0.05$) in the education of the father and mother, father's work and mother, income and status home ownership in Table 2. Characteristics of study subjects in terms of sex, type of labor, Apgar score 1 min, Apgar score 5 min gestational age, birth weight (z-score), z-score, premature rupture of membranes, amniotic fluid, history of corticosteroid administration before delivery,

neonatal jaundice, seizures, sepsis, oxygen source and type of breastmilk did not get significant difference (p>0.05) in Table 3. Characteristics of the subjects before breastfeeding fortification did not show significant differences (p>0.05) between breastfed and breast-fed groups were fortified with HMF in terms of body weight, z-score, body length (cm), z-score, head circumference (cm), z-score head, fecal concentration of secretory IgA, chronological age, average enteral volume (ml / day) and (ml / kg / day) in Table 4.

Table 1. Characteristics of research subject mothers

Characteristics	Breastfed (n=17)	Breastfed Fortification HMF (n=17)	p
Age (years)	28,82±5,90	28,82±5,71	1,000[3]
Nutritional Status (kg/m²)			
Malnutrition	2	2	0,714[2]
Normal	13	14	
Overweight	2	1	
History of parity			
Gravida 1	9	7	0,571[2]
Gravida 2	5	6	
Gravida 3	2	4	
>Gravida 3	1	0	
pre-eclampsia/eclampsia history	6	5	1,000[1]
Risk of Labor			
High	10	12	0,721[1]
Low	7	6	
history of specific illness during pregnancy	1	1	1,000[1]

Description: The value of p is significant when the value <0,05. Chi-square test[1], Mann-Whitney[2] test and independent sample[3] test

Table 2. Characteristics of socioeconomic status of parents

Characteristic	Breastfed (n=17)	Breastfed Fortification HMF (n=17)	p
Father's education			
Didn't School	0	1	0,736[2]
Elementary School	1	0	
Junior High School	3	3	
Senior High School	10	10	
Bachelor (S1/S2/S3)	3	3	
Mother's Education			
Elementary School	3	1	0,740[2]
Junior High School	2	3	

Senior High School	9	10	
Bachelor (S1/S2/S3)	3	3	
Father's Job			
Working	17	16	1,000[1]
Not Working	0	1	
Mother's Job			
Working	4	13	1,000[1]
Not Working	13	12	
Income			
Insufficient	10	7	0,494[2]
Sufficient	7	10	
More than enough			
Home Ownership Status	5	5	1,000[2]
One's Own	9	9	
Family Owner	3	3	
Someone else			

Description: The value of p is significant when the value <0,05. Chi-square[1] Test and Mann-Whitney[2] Test

Table 3. Characteristic of Research Subject

Characteristic	Breastfed (n=17)	Breastfed Fortification HMF (n=17)	p
Sex (n)			
Male	8	8	1,000[1]
Female	9	9	
Type of Parity			
Normal	7	7	1,000[2]
Cesarean section	10	10	
Apgar score 1 minute	6(1-8)	6(1-8)	0,722[2]
Apgar score 5 minute	8(3-9)	8(3-9)	0,750[2]
Gestational Age (week)	32(30-34)	31(30-34)	0,110[2]
Aterm	17	17	-
Birth Weight (g)	1700 (1000-1900)	1650 (1000-1950)	0,986[2]
Birth Weight to Age (z-score)	-0,73±0,82	-0,26±0,80	0,101[3]
Birth Length (cm)	41,58±2,92	41,58±3,04	1,000[3]
Birth Length (z-score)	-0,72±1,25	-0,32±1,07	0,329[3]
Birth Head Circumference(cm)	29(25-32)	29(22-31)	0,169[2]
Birth Head Circumference (z-score)	-0,36(-2.02-(+0,59))	-0,59(-1,90-(+1,25))	0,581[2]
Prematurity of Rupture Membrane	4	10	0,080[1]
Amniotic fluid			
Clear	16	15	1,000[1]
Murky	1	2	

History of antenatal corticosteroid administration	6	9	0,491[1]
Icterus Neonatal	16	16	1,000[1]
Seizure	0	1	1,000[1]
Sepsis	6	6	1,000[1]
Oxygen			
Room	2	0	0,220[1]
CPAP	15	15	
Ventilator	0	1	
Type of breastfed			
Week 1	3	2	0,949[2]
Week 2	11	13	
Week 3/4	3	2	

Description: P value means when value <0,05. Chi-square[1] test, Mann-whitney[2] test and t-test independent sample[3]

Table 4. Characteristic of Subject before Breastfeeding Fortification

Characteristic	Breastfed (n=17)	Breastfed Fortification HMF (n=17)	p
Weight (g)	1590 (1070-1950)	1620 (1160-1770)	0,629[1]
Weight to Age (z-score)	-1,52±0,65	-1,32±0,56	0,379[2]
Length (cm)	42,53±2,70	43,09±2,50	0,322[2]
Length to Age (z-score)	-1,20±1,29	-0,51±1,02	0,930[2]
Head Circumference (cm)	29,26±2,05	29,32±1,86	0,931[2]
Head Circumference to Age (z-score)	-1,17±0,87	-0,86±1,12	0,381[2]
IgA secretory fecal (µg/ml)	1312,90(194,43-2304,60)	1299,65(63,18-1373,62)	0,082[1]
Age of Chronology (day)	11,41±3,43	11,41±3,54	1,000[2]
Enteral Volume (ml/day)	192(180-300)	216(180-300)	0,133[1]
Enteral Volume (ml/kg/day)	130±21,50	140,94±22.7	0,151[2]

Description: The value of p is significant when the value <0,05.Mann-Whitney[1] test and independent t-test sample [2]

Weight (gram) and age-weighted (z-score) parameters showed significantly different changes (p<0.05) and in the breastmilk group 192.65±170.78 grams and the breastfeeding group was fortified with HMF 355. 88±162.30 grams. In the parameter of body length (cm), head circumference (cm) and the secretory IgA did not show significantly different changes (p>0.05) in Table 5. The breastfed group of fortified HMF had a faster growth rate of body weight of 22 (12.86-51.76) g / kg / day than the breastfeeding group 14.28 (-12.86 - (+ 32.86) g / kg / day (p = 0,020). While growth rate of body length and head circumference did not show significant difference (p>0.05) presented in Table 6. The mean age-to-weight (z-score) score was higher in the HMF-fortified breastfeeding group than in the breast milk group. Mean age-weighted values did not show significant differences at birth, before fortification and day 7 (p>0.05). However, the mean value of body

weight according to age at day 14 showed significant difference ($p<0.05$) in Figure 1.

The mean length-for-age (z-score) rate was higher in the HMF-fortified breastfeeding group than in the breastmilk group. The mean value of body length according to age did not show significant difference at birth ($p>0.05$), before fortification and day 7. The mean value of body length according to age at day 14 showed a significant difference ($p<0.05$) in Figure 2. The mean age of z-score head circumference was higher in the breastfed fortified HMF group than in the breastmilk group. The mean head circumference value according to age did not show significant difference at birth ($p>0.05$), before fortification, day 7 and day 14 in Figure 3.

The mean of enteral volume in breastmilk group was 178,42±17,85 ml / kg / day and breastfed group was fortified HMF 175,04±13,91 ml / kg / day were given in Table 7 indicating no significant difference. While caloric mean showed significant difference that was in breastfed group of HMF 144,47±10,03 kkal / kg / day and milk group 119,17±14,17 kcal / kg / day ($p<0.05$) in Table 8. The mean protein values were significantly different in the breastfed group of HMF 5.44±0.49 g / kg / day and breastfed group 3,43±0,42 g / kg / day ($p<0.05$) presented in Table 9. During the study, side effects of feeding intolerance with systemic symptoms and NEC were not found.

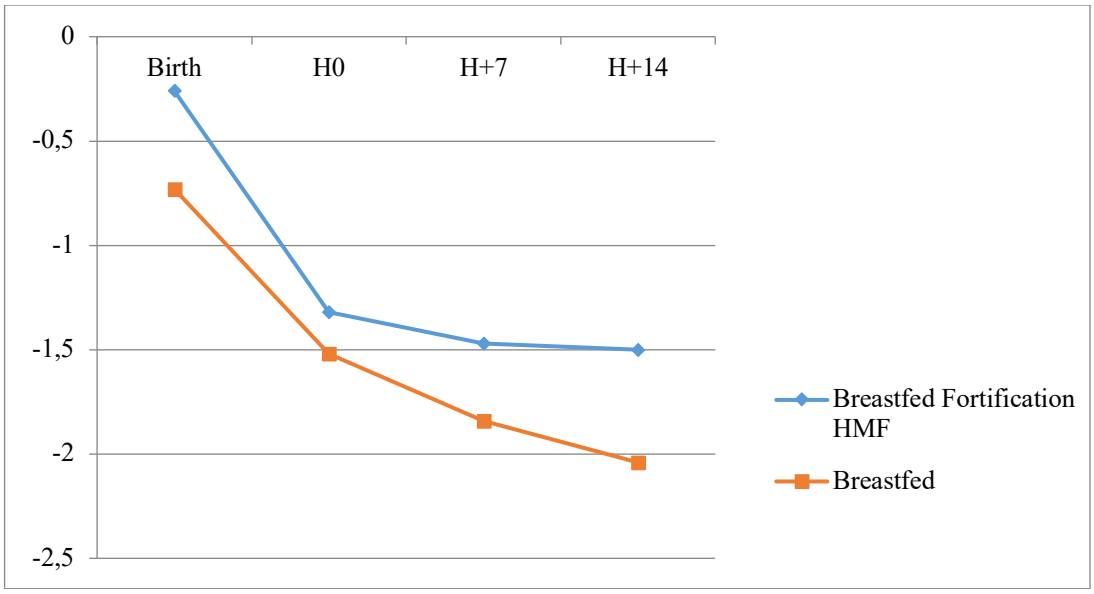

Figure 1. Comparison of mean age-weight (z-score) between breastfeeding and breast milk group was fortified by HMF. Description: blue line is a group of breastfed fortified HMF and red line is a breastmilk group. The p value is significant when the value is <0,05. Mann-Whitney[1] test and independent t-test sample[2].

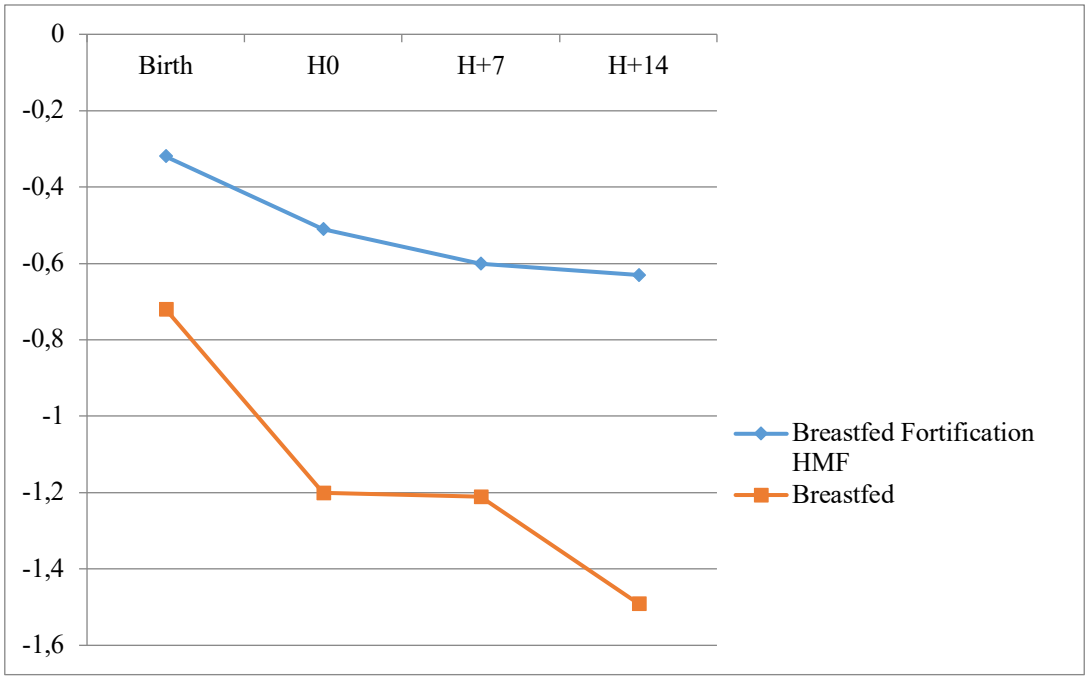

Figure 2. Comparison of mean age-for-age (z-score) scores between Breastfed and breast milk groups was fortified by HMF. Description: blue line is a group of Breastfed fortified HMF and red line is a breastmilk group. The p value is significant when the value is <0,05. Independent t-test test sample.

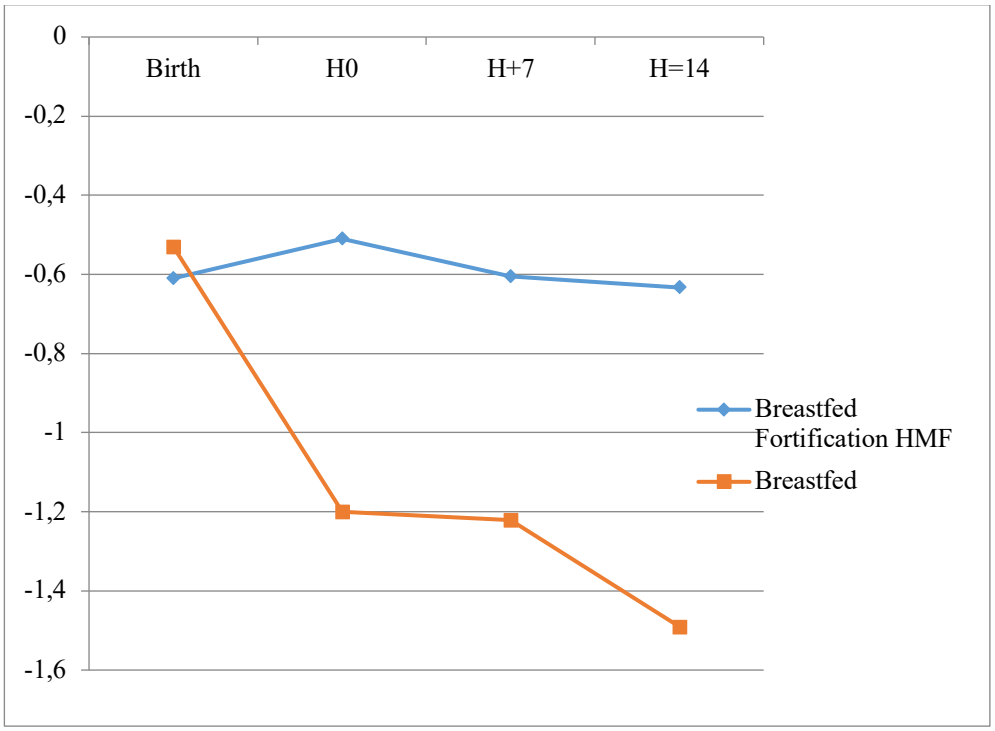

Figure 3. Comparison of mean z-score head circumference between breastmilk and breast milk group was fortified by HMF. Description: blue line is a group of breastmilk fortified HMF and red line is a breastmilk group. The p value is significant when the value is <0,05. Mann-Whitney[1] test and independent t-test sample[2].

The median birth weight of breastfed milk group HMF in this research was 1650 (1000-1950) gram, according to Mukhopadhyay et al., the average birth weight of 1202±202 grams (Mukhopadhyay *et al*., 2007). The Cochrane Review also mentions the indication of breastfeeding fortification as a term infant with a mean birth weight of 900-1850 grams (Kuschel and Hardling, 2004). The mean of body length was born in breastmilk group 41,58±2,92 cm and breastfeeding group was fortified HMF 41,58±3,04 cm. Both groups had normal mean birth rates and no significant differences. Research by Arslanoglu showed prematurity infants has a mean length of body born 38,9±2,2 cm (Arslanoglu *et al*., 2006). In this study, head circumference was born in the ASI group of 29 (25-32) cm and the breastfed group was fortified HMF 29 (22-31) cm. Both groups had normal head circumference and no significant differences. In contrast to previous studies, the mean birth circumference of underweight infants who received breast milk fortification was 27.7±2.2 cm (Arslanoglu *et al*., 2006).

Both groups showed no significant differences in body weight, body length (cm) and head circumference (cm). Body weight in breastfed group 1590 (1070-1950) grams and breastfed groups fortified HMF 1620 (1160-1770) grams. The mean body length in the breastmilk group was 42.53±2,70 cm and the breastfed group was fortified HMF 43,09±2,50 cm. The breastmilk group had a head circumference of 29 (25-32) cm and the breastmilk group fortified HMF 29 (22-31) cm (Table 4). In previous RCT studies showed the initial weight of the study 1189±209 grams (Mukhopadhyay *et al*., 2007). The study by Morlacchi *et al*. also showed less-than-matured infants with an initial body weight of 1412±231 gram (Morlacchi *et al*., 2016). The mean weight-to-age (z-score) and length-for-age (z-score), body weight before breast milk fortification showed a decline compared to birth weight and length of birth. However, both groups showed no meaningful differences (Figure 1).

Prematurity Infants get breastfeeding fortified HMF at chronological age of 11.41 ± 3.54 days. Both groups showed no significant difference (Table 4). Breastfeeding fortification begins at the age of chronologically 11 days (Adamkin, 2009) or at chronological age 4-15 days (Kuschel and Hardling, 2004). RCT study in 85 under-term infants stated that breastfeeding fortification was performed at the age of 11.8 ± 5.7 days (Mukhopadhyay *et al*., 2012). Other studies initiated breastfeeding fortification in underweight infants at chronological age of 13 (10-16) days (Miller *et al*., 2012).

Breastmilk fortification was given when the study subjects had enteral nutrition ability of 140.94±22.7 ml / kg / day. Both groups showed no significant difference to the mean enteral volume at baseline (Table 4). Previous studies have found that breastfeeding fortification begins when the prematurity infant had an enteral ability on 150 ml / kg / day (Arslanoglu *et al*. 2006, Adamkin, 2009), 45-170 ml / kg / day (Kuschel and Hardling, 2004). An RCT study of 85 underweight babies mentioned fortification when achieving enteral ability of 168±14.4 ml / kgbb / day (Mukhopadhyay *et al.*, 2012). Miller *et al*. did fortification of breastfeeding when prematurity infant had an enteral ability of 120 (94-140) ml / kg / day (Miller *et al*., 2012).

In this research, 4 underweight infants (gestational age 28-33 weeks and birth weight 750-1500 grams) who received HMF of 24 kcal / oz showed sepsis incidence with feeding intolerance more often than 2 infants less months (gestational age 31-33 week and birth weight 1400-1500 gram) that get HMF of 22 kcal / oz. Schutzman et al. mentions the fortification technique of ASK 22 kkal / oz given to prematurity infants with birth weight 1000-1500 gram since chronological age 10 days for 6 days continued fortification of ASK 24 kkal / oz. While in prematurity infants with birth weight 1500-2000 gram get fortification 22 kcal / oz since chronological age 9 days for 4 days (Schutzman *et al*., 2012). Radmacher and Adamkin recommend giving fortification of

breastmilk for 2-8 weeks with protein intake of 3.5-4.4 g / kg / day and calories of 24 kcal / oz (Radmacher and Adamkin, 2016).

In this study, the growth rate of body weight showed significant differences between the two groups. The growth rate of breastfeeding fortification HMF group weight was found on 22 (12,86-51,76) g / kg / day to be greater than breastfeeding group 14,28 (-12,86 - (+ 32,86)) g / kg / day (Table 6). Changes in weight (grams) and age-related weight (z-score) also showed significant differences (Table 5). Other studies with fortified breastfeeding techniques of 24 kcal / oz had a lower body weight growth rate of 18.2 ± 0.7 g / kg / day (Barrus *et al*., 2012) and 15.1 ± 4 g / kg / day (Mukhopadhay *et al*., 2007). The differences in the rate of weight gain can be influenced by the characteristics of the study subjects (low birth weight, gestational age, history of steroid delivery before delivery, gender and APGAR score) and HMF composition (Kartal *et al*., 2016). In this study, only a small proportion of preterm infants had prematurely ruptured membranes and no significant difference was found (Table 3).

The researchers used the fortification technique of ASK 22 kkal / oz of 2 sachets of HMF + 100 ml of breast milk. HMF product in this study contains protein 0.6 g / 100 ml of milk, fat 0.18 g / 100 ml of milk and carbohydrate 0.9 g / 100 ml of breastmilk. The fortified milk fortification technique 24 kcal / oz in the Porcelli *et al*. study contained 1g / 100 ml of milk protein, 0.05 g / 100 ml of breast milk and 2 g / 100 ml of breast milk and the study showed 27% feeding intolerance, 30% respiratory distress and 36% cardiovascular disorders (Porcelli *et al*., 2000).

The caloric value used in the study was 144.47 ± 10.03 kcal / kg / day (HMF fortified breastfeeding group) and 119.17±141.7 kcal / kg / day (breastfeeding group) (Table 8). Caloric mean of both study groups was in accordance with the recommendation of prematurity infants' caloric needs on 105-135 kcal / kg / day (Canadian pediatric society nutrition committee, 1995). Caloric mean showed significant differences (Table 8).

The mean of enteral volume in this study did not show significant difference (Table 7). The mean of enteral volume in breastmilk group was fortified HMF 175.04±13,91 ml/ kg/day and breastfeeding group 178,42±17,85 ml / kg / day. The mean of breast milk protein content of prematurity infants with chronological age 15,3±1,5 day is 2g/100 ml of breastmilk (Porcelli *et al*., 2000). The HMF product used contained 0.6 g /100 ml of breastmilk in this study. Thus, the mean protein was 5.44±0.49 g /kg/day (HMF fortified breastfeeding group) and 3.43±0.42 (breastfeeding group). The breastfed group of fortified HMF had a higher mean protein and was significantly different for the breastmilk group (Table 9). Levels of protein intake in this study have been in accordance with the recommendation of protein needs for prematurity infants 3-3.6 g/kg/day (Canadian pediatric society nutrition committee, 1995). Systematic reviews indicate that administration of high-dose protein (3-4 g/kg/day) may increase body weight by 23.6 g/kg/day (Miller *et al*. 2008). Other studies have suggested that giving 2-4 g/kg /day protein can increase BB, linear growth, nitrogen retention and albumin levels (Kuschel and Hardling, 2004). The HMF product in this study contains MCT of 9.6%. Meta-analysis showed no significant difference between the number of doses of MCT on weight gain, body length (Klenoff-Blumberg and Genen, 2003).

Breastfed fortified HMF groups had a higher mean age-to-weight (z-score). The breastfed fortified HMF group had a positive effect on the mean age weight (z-score) (Figure 1). In this study, mean age weight (z-score) showed a decrease in both groups. The decrease in body length by age (z-score) is greater than the z-score (Figure 1 and 2). Previous research has shown that age-related z-score is greater than z-score (Ramel *et al*., 2012; Olsen *et al*., 2014). Z-score weight loss was lower in the group receiving high-dose calories and protein (5 grams FM 85 + 100 ml of breast milk and 1-2.5 grams of Protifar + 100 ml of breast milk). The administration of high-dose protein had a significant difference to age-z-score (Roggero *et*

al., 2012). Larger weight growth rates (36 g/kg/day and levels of 6.3 g/kg/day) are expected to match the intrauterine growth rate (Olsen *et al*., 2010).

Table 5. Changes in anthropometric and immunoglobulin A values during the study

Changes	Breastfed (n=17)	Breastfed Fortification HMF (n=17)	p
Anthropometry			
Weight (g)	192,65±170,78	355,88±162,30	**0,008**[2]
Weight to Age (z-score)	-0,52±0,46	-0,18±0,36	**0,022**[2]
Length (cm)	1(0-5)	1,5(0-4)	0,146[1]
Length to Age (z-score)	-0,51(-1,01-(+1,28))	-0,39(-0,86-(+0,80))	0,185[1]
Head Circumference (cm)	1(0-3,5)	1,5(1-3)	0,154[1]
Head Circumference to Age (z-score)	-0,14±0,55	0,18±0,43	0,068[2]
Immunoglobulin A			
IgA secretory fecal (μg/ml)	32,92(5,13-1172,77)	46,18(0,43-1269,39)	0,796[1]

Description: The value of p is significant when the value <0,05. Mann-Whitney[1] test and t-test independent sample[2] test.

Table 6. Rate of short-term growth

Characteristic	Breastfed (n=17)	Breastfed Fortification HMF (n=17)	p
Weight (g/kg/day)	14,28(-12,86-(+32,86))	22(12,86-51,76)	**0,020**
Length(cm/week)	0,50(0-2,50)	0,75(0-2)	0,257
Head Circumference (cm/week)	0,50(0-1,75)	0,75(0,50-1,50)	0,215

Description: The value of p is significant when the value <0,05Mann-Whitney Test.

Table 7. The average of enteral volume

Volume	Breastfed (n=17)	Breastfed Fortification HMF (n=17)	p
Enteral Volume (ml/kg)	298,43(232,29-338,57)	304,29(219,43-334,39)	0,809[1]
Enteral Volume (ml/kg/day)	178,42±17,85	175,04±13,91	0,151[2]

Description: The value of p is significant when the value <0,05. Mann-Whitney[1] test and independent t-test sample[2].

Table 8. Caloric average

Caloric	Breastfed (n=17)	Breastfed Fortification HMF (n=17)	p
Caloric (kkal/day)	190,72±27,69	227,53±21,81	**<0,0001**
Caloric (kkal/kg/day)	119,17±14,17	144,47±10,03	**<0,0001**

Description: The value of p is significant when the value <0,05. Test t-test independent sample.

Table 9. The mean protein

Protein	Breastfed (n=17)	Breastfed Fortification HMF (n=17)	p
Protein (gram/day)	5,82(4,65-6,77)	9,26(6,78-10,17)	**<0,0001**[1]
Protein (gram/kg/day)	3,43±0,42	5,44±0,49	**<0,0001**[2]

Description: The value of p is significant when the value <0,05. Mann-Whitney[1] test and independent t-test sample[2].

The growth rate of body length showed no significant difference. The breastfed milk group HMF has a growth rate of body length of 0.75 (0-2) cm / week (Table 6). The growth rate of length breastfeeding fortified HMF group showed that varied results on recommendation of ideal infant growth less than 0.9 cm / week (Bertino *et al*., 2008). Breast fortification 24 kcal / oz for 2 weeks showed growth rate of body length 0,9±0,1 cm / week and did not show significant difference. The protein content in this study was 1 g / 100 ml of breast milk (Porcelli *et al*., 2000). Breastfed fortification 24kcal / oz other shows the growth rate of body length is 0.86±0,2 cm / week while breastfeeding group 1.04±0,3 cm. HMF is given in prematurity infants until it reaches 2000 grams (Mukhopadhyay *et al*., 2007). Research by Reis *et al* showed that a growth rate is 1.09±29 cm / week (Reis *et al*. 2000). HMF with high-dose protein has a growth rate of 1.15 (1.10-1.19) cm / week (Miller *et al*., 2012). Administration of adjustable fortification (1,3±0,5 cm / week) did not show significantly different body length growth rate against standard fortification (1,1,0,4 cm / week) (Arslanoglu *et al*., 2006). High doses of protein did not show any significant difference to the increase in body length (Miller *et al*., 2012; Roggero *et al*., 2012). This is caused by the level of milk protein is dynamic (Gidrewic *et al*., 2014). Thus, the calorie and protein levels given in this study, Miller et al and Roggero *et al*. were lower than those of Arslanoglu *et al*.

The breastfed fortified HMF group had a mean length of body-age (z-score) higher than the breastmilk group and showed a significant difference. The breastfeeding fortified HMF group was able to maintain a z-score average in the normal range (Figure 2). A z-score reduction in mean age was greater than the mean z-score (Figure 1 and Figure 2). In line with previous studies, breastfeeding fortified HMF showed a decline in mean age-for-age (z-score) (Miller *et al*., 2012; Ramel *et al*., 2012; Olsen *et al.,* 2014).

The HMF product in this study contained 58.4 mg calcium and 33.6 mg phosphorus per 100 ml of breast milk. The use of HMF products with calcium 87 mg and phosphorus 50 mg to body weight 2000 gram showed growth rate of body length is 0.86 ± 0.08 cm. The rate of body length growth in the Gross et al study, have not met the recommended recommendation of 0.9 cm / week (Bertino *et al*., 2008). Although the mineral content in this study was lower but the growth rate of body length was faster than that of Gross *et al*. This is due to the composition of breastmilk and the characteristics of research subjects.

In this study, head circumference growth rate showed no significant difference (p = 0,215)

(Table 6). The breastfed group of fortified HMF had a larger head circumference growth rate of 0.75 (0.50 to 1.50) cm / week than the breastmilk group of 0.50 (0-1.75) cm / week (Table 6). Fortification techniques of 24 kcal / oz milk and protein 0.9 g / 100 ml of breast milk showed a change in head circumference of 1.04□0.23 cm / week (p = 0.743) (Reis et al., 2000). A study by Porcelli et al. gave HMF to less than 2 months of gestational weight to a body weight of 2000 grams but this did not show any significant difference (Porcelli et al. 2000). High doses of protein did not show significantly different head circumference growth rates (p = 0.330). The rate of head circumference growth in less-than-term infants who received high-dose protein was 0.94 (0.9-0.98) cm / week (Miller et al., 2012). In contrast to the milk fortification technique of 24 kcal / oz with protein of 0.9 g / 100 ml of breast milk and 0.8 g / 100 ml showed a larger head circumference growth rate of 1±0.1 cm / week and 0.8 ± , 1 cm / week (Porcelli *et al*., 2000). The mean age-zero head circumference (z-score) of breastmilk group was fortified with HMF higher than in breastmilk group. Breastfeeding fortification maintains an average z-score head circumference in the normal range (Figure 4). Higher calorie and protein intake showed significant differences in mean age-added z-score (Miller *et al*., 2012).

4. Conclusions

Short-term growth rate based on body weight showed significant differences while the parameters of body length and head circumference did not show significant differences between prematurity infants of breastfeeding and breastfeeding were fortified with HMF, and the secretory IgA fecal content showed no significant difference between breastfed infants who were breastfed and breast milk fortified with HMF.

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