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Original Article

Effect of probiotic on the fecal sIgA level in preterm infants (A randomized double-blind placebo control study)

Lucia P Retnaningtyas, Subijanto M Sudarmo, Ariyanto Harsono, Sylviati M Damanik

Abstract

Background Secretory immunoglobulin A (sIgA) plays an important role in the defense of gastrointestinal tract. Preterm infants that developed abnormal pattern of bowel colonization may benefit from strategy to support maturation of humoral immunity and endogenous production of sIgA by early colonization with probiotic.

Objective To evaluate the effect of probiotic on the fecal sIgA level in newborn preterm infants.

Methods A randomized control study of newborn preterm infants was conducted in NICU Dr Soetomo Hospital, Surabaya in November-December 2007. Probiotic group was given multistrain probiotic containing 107 cfu of Lactobacillus acidophilus, Bifidobacterium longum, and Streptococcus faecium once daily for 14 days from second day of life. Fecal sIgA was determined by ELISA before and after intervention. Subjects who got respiratory distress syndrome (RDS) or sepsis during the study was dropped out. Statistical analysis used in this study were chi-square, independent sample t-test, Mann-Whitney, Wilcoxon Signed-Ranks test, and multivariate analysis of variance (α=0.05).

Results Forty seven neonates were enrolled, seven of them were dropped out. Forty analyzed neonates were divided in probiotic (n=20) and placebo group (n=20). The basic characteristics of two groups were similar. At first examination, median of fecal sIgA level did not differ significantly between groups (P=0.512), 0.164 and 0.174mg/g feces in probiotic and placebo group respectively. There was higher increment of fecal sIgA level in probiotic than placebo group post treatment(1.735 versus 1.449 mg/g feces, P=0.003).

Conclusion Preterm infants may benefit from probiotic because of the clear tendency to increase fecal sIgA secretion **[Paediatr Indones 2008;48:225-30]**..

Keywords*: preterm infant, probiotic, fecal sIgA*

The mucosal immune system uses a number of mechanisms to protect the host against an aggressive immune response to luminal constituents. These include a strong physical barrier; the presence of luminal enzymes that alter the nature of the antigen itself; the presence of specific regulatory T cells in both the organized and disorganized lymphoid tissue of the gut; and the production of antibodies such as secretory immunoglobulin A (sIgA), which is highly suited for the hostile environment of the gut.[1] Immunoglobuline A is the predominant antibody class in many external secretions and has many functional attributes, both direct and indirect. IgA functions in mucosal membranes are often defined collectively as "immune exclusion". Whereas systemic defense aim at the ultimate destruction of intruding antigens, mucosal IgA prevent the attachment and penetration of microorganisms and molecular antigens, blocking their potential effects on the host. In secretions, sIgA

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From the Department of Child Health, Division of Gastroenterology, Medical School, Airlangga University, Soetomo Hospital, Surabaya.

Reprint request to: Lucia Pudyastuti, MD, Depatment of Child Health, Medical School, Airlangga University, Soetomo Hospital, Surabaya, Indonesia. Telp. 0315501693. Fax: 0315501748. E-mail: luciapudyastuti@yahoo.com

antibodies are well suited to these tasks because of their molecular characteristics. Their four or more antigen binding sites allow them to block adherence determinants, including microbial, neutralize microbial enzymes and toxins, and agglutinate microorganisms.[2,3]

The bacterial colonization of intestines of preterm infants may differ from that of term infants because methods of neonatal care, such as treatment with antibiotics or nursing in incubators, may delay or impair the colonization process.[4] The normal colonization of the mammalian intestine with commensal microbes is hypothesized to drive the development of the humoral and cellular mucosal immune systems during neonatal life.[5,6] Development of IgA producing plasmablast in intestinal mucosa and production of intestinal IgA are influenced by gut microfloras.[5] The level of fecal SIgA antibody is associated with increased neutralization and clearance of viruses. Breast-fed infants had the highest level in early life, and then gradually decline.[7] The sIgA level is lower in preterm infants who do not received breastmilk, because breastmilk contains more sIgA and bifidus factor.[1]

Early gut colonization with probiotic may benefit for preterm infants. Probiotics are alive microorganisms if administered in adequate amounts confer a health benefit on the host.[8] Several studies shown that probiotic feeding could increase intestinal sIgA level in mice,[9] and children;[10] and serum sIgA level in preterm infants.[11] Study about fecal sIgA level have been done to adult[12] and children.[13] There was also positive effect of pre and probiotic feeding in term newborn infant,[7] but no study had done to preterm infant. Determination intestinal sIgA level from intestinal secretion is too invasive for newborn infants. A study had shown that fecal sIgA level give good representation to sIgA level in colon. [9] This study will prove the influence of probiotic feeding to increase intestinal mucosa immune response in preterm infant by examine fecal sIgA level.

Methods

This was a randomized placebo controlled clinical trial. Preterm newborns admitted to Neonatal Intensive Care Unit at Dr Soetomo Hospital – Surabaya, Indonesia, from November to December 2007 were enrolled. The inclusion criteria were preterm infant with gestational age range from 28 to 36 weeks according to Ballard Score, with physiologic delivery (per vaginam without intervention), with no asphyxia (the fifth minute Apgar Score $\geq$ 7), no oropharyngeal anomaly causing difficulty of oral feeding, no major congenital anomaly, and less than 24 hours of age.

Written informed consent was obtained from the parents of all the patients before enrollment. Patients were excluded from the study if they had been suspected for respiratory distress syndrome. Ethical approval had been released from Ethic Clearance Committee Department of Child Health - Soetomo Hospital Surabaya. The patients were enrolled in two groups (placebo and probiotic group) using random number table. Patients will be dropped out if the data are not complete.

Treatment protocol

Both groups were managed with standard management for preterm newborn infants in NICU at Dr Soetomo Hospital – Surabaya. If there was suspicion of bacterial infection, they were given antibiotic (Ampicillin-Sulbactam 100 mg per kilogram body weight per day divided in two doses intravenously, and Gentamycin 5 mg per kilogram body weight divided in two doses intramuscularly if the amniotic fluid was not clear). These drugs were given for minimal three days with observation of sign and symptom of sepsis. When there were signs and symptoms of sepsis, the antibiotics were continued; but if there were not, the drugs were stopped. Criteria of FIRS (fetal inflammatory response syndrome) and sepsis in this study were based on Criteria of 1st International Sepsis Forum on Sepsis in Infant and Children, 2005. Observation was done everyday until the patient sent to home or for minimal 2 weeks. Side effect was observed by clinical sign and symptoms of sepsis and blood culture.

One group was given multistrain of probiotic (*Lactobacillus acidophillus, Bifidobacterium longum, dan Streptococcus fecalis*) 1 x 10^7cfu/day for 14 days orally started at second day of age; another was given placebo in a same package. Feeding of probiotic or placebo was stopped if there were signs and symptoms of sepsis. Probiotic or placebo was saluted by 5 mL Dextrose 5%, given to the infants by spoon or gastric tube.

The probiotic used in this study was Lacto-B® produced by Novell Pharmaceutical. The placebo was packaged by Production Department of Pharmacy Installation Soetomo Hospital, Surabaya. The department gave the code of probiotic or placebo, and open them at the end of the study.

Laboratory examination

Fecal specimen

Fecal specimen was taken at postnatal day 0, and 15 or 16 - 17. The specimen was taken from the diaper made from plastic as soon as possible after defecation, collected in feces containers (*cryotube*), and stored immediately in freezer. Before 24 hours, fecal specimen was transported in a portable *freezer* (minimal temperature -15^{o}C) to the laboratory (Microbiology and Virology Laboratory - Veterinary Faculty of Airlangga University)

Fecal homogenates

For the determination of the SIgA concentration by an enzyme-linked immunosorbent assay (ELISA), 10% (w/v) fecal homogenates were prepared according to standard procedures. Briefly, the frozen (-20^{o}C) fecal specimens were defrosted on ice. Suspensions were made by adding 0.1 g feces to 0.9 ml of phosphate-buffered saline (PBS) and homogenizing by 1500 rpm for 5 min at 4^{o}C using a centrifuge. The homogenates were stored at -80^{o}C until further processing.

Enzyme-linked immunosorbent assay

ELISA plates were coated overnight at 4^{o}C with 100 μl *polyclonal goat anti-human IgA antibody* (2.0 μg/ml), diluted 1:50 in *sodium bicarbonate buffer* (SCB; 0,1 mol/l, pH 9.6). After thoroughly washing the plates six times with buffer washing (SCB-Tween 0,05%), the plates were incubated for 1 hour at room temperature (37^{o}C) with 200 μl *buffer blocking* (PBS Tween 20 containing 1% (w/v) of bovine serum albumin(BSA), pH 7.4) to block non-specific protein binding sites. After blocking, the plates were again washed thoroughly. The supernatants of the fecal homogenates was used as specimens, and *purified human IgA* diluted twice (1.25–80 ng/ml) with SCB-BSA was used as a positive standard; 100 μl of the specimen and the standards were added to each well in *duplo*, and were incubated for 2 hours at room temperature. Plates were then washed five times and *horsedish peroxidase-conjugated goat anti-human IgA antibody* diluted 1:1000 in PBS Tween 20 containing 0.5% BSA was added to the plates for 1 hour at room temperature. The wells were then washed and incubated for 15 min with 100 μL of a *ortho phenylene diamine* (OPD) substrate diluted in 1:10 citrat phosphate. Enzymatic colour development was stopped by adding 100 μL 3 mol/L NaOH. The absorbance was measured at 450 nm by using an ELISA reader (BIORAD). Concentrations of sIgA were calculated from the standard curve.

Complete blood count (CBC), C-reactive protein (CRP), blood culture were taken at the day when signs and symptoms of sepsis suspected. CBC was examined by Pediatric Hematology Laboratory Soetomo Hospital; CRP and culture of blood were performed by Regional Health Laboratory – East Java.

Statistical analysis

Values were expressed as means (SD) unless otherwise noted. Differences between the groups were tested for significance with the two-tailed independent t-test. Because the concentration of sIgA was not normally distributed, results between the groups are analyzed with Mann-Whitney test. Wilcoxon Signed Ranks test was used to analyze sIgA level before and after trial. Factors influencing the outcome were tested by multivariate analysis of variance. A P value of less than 0.05 was considered to statistical significance.

Results

There were 79 newborn preterm infants (15.4%) of 513 newborn infants admitted to our institution during the study period. Forty seven neonates were enrolled, 7 of them were drop out (two with RDS, one from each group; five with sepsis, four neonates from placebo group). Forty analyzed neonates were divided in probiotic (n=20) and placebo group (n=20). Probiotic group was given multistrain

probiotic containing 10^7 cfu of *Lactobacillus acidophilus, Bifidobacterium longum,* and *Streptococcus faecium* once daily for 14 days since second day of life. The control group was given placebo. Fecal sIgA was determined by an ELISA before and after intervention. The repeated examination was done at 15 -18th day of age.

Table 1. Baseline characteristics of infants from both groups

	Probiotik Group (n= 20)	Placebo (n= 20)
Sex		
Male	6	11
Female	14	9
Gestational Age (weeks)	32.6 (1.88)	33.3 (1.37)
30-33 weeks	11	10
34-36 weeks	9	10
Apgar Score 1st min	6 (3-8)	7 (5-8)
Apgar Score 5th min	8 (5-9)	8 (7-9)
Birth weight (g)	1572.50 (263.32)	1742.50 (354.39)
≤ 1500 g	12	6
> 1500 g	8	14
PRoM	5	4
Turbid amniotic fluid	2	2

PRoM: Premature Rupture of the Membrane

Table 2. Distribution of subjects with breastfeeding and antibiotics

	Probiotic Group (n= 20)	Placebo Group (n= 20)
Received antibiotic/s	14	14
Duration received antibiotic		
3 days	8	10
7 days	6	4
Median of starting breastfeeding (days)	3 (1-4)	2 (1-5)

There was no significant difference in sex, gestational age, Apgar Score, birth weight, mode of delivery, history of premature rupture of the membrane and turbid amniotic fluid between the groups (**Table 1**). There was no infant with gestational age less than 30 weeks.

Twenty eight infants got antibiotics since first day of life, 14 infants from each group (P=1.000) (**Table 2**). The duration of receiving antibiotics between groups was not significant differ.

There was no significant difference of sIgA level before study between groups ({P=0.512). But, after study, the probiotic group level was 19.7% higher than placebo with Prevalence Rate of 9.45. (**Table 3**).

Table 4 revealed fecal sIgA level in each group before and after study. And there was significant difference in each group (both P=0.0001), the repeated results were higher than the first.

Four infants suffered from diarrhea (all from placebo group), but there was no bacteria found in their feces culture. Gastric retention was found lesser in probiotic group (five versus nine infants).

Signs and symptoms of sepsis were found in five infants, so the study was stopped. One was from probiotic group and got signs and symptoms of sepsis at seven days of age. The infant was getting better and discharged from hospital at 18 days of age. The four infants were from placebo group and got sepsis at third, forth, fifth and sixth days of age. One of them died at tenth days of age with positive blood culture (*Enterobacter aeruginosa*), the others (one with blood culture result *Pseudomonas aeruginosa*) were getting better and discharged from hospital at 18th, 19th and 20th days of age.

Table 3. Fecal sIgA level before and after study between groups

Fecal sIgA (mg/g feces)	Probiotic Group (n= 20)	Placebo Group (n= 20)	P
Before study	0.164 (0.018; 0.965)	0.174 (0.018; 1.424)	0.512
After study	1.735 (1.637; 3.602)	1.449 (0.799; 2.085)	0.003*
Increment	1.704 (0.5492)	1.336 (0.4543)	0.027*

Table 4. Fecal sIgA level before and after study in each group.

Goup	Fecal sIgA level (mg/g feces)		P
	Before	After	
Probiotic	0.164 (0.018; 0.965)	1.735 (1.637; 3.602)	0.0001*
Placebo	0.174 (0.018; 1.424)	1.449 (0.799; 2.085)	0.0001*

Discussion

All of the first fecal specimens in this study were taken at zero to first day of life. We noted that the median fecal sIgA level between groups was similar. Other study found that on the first day of age the median of fecal sIgA level in breastfed fullterm infants was 10.918 mg/gram feces; and in nonbreastfed infants was zero.[7] The positive fecal sIgA in our study might be caused by breastmilk consumed by all of the infants. The low level that compared than the study before revealed that preterm infants had very low level of fecal sIgA in first day of life. This supported theory that preterm newborn infants still had weak mucosal immune response. This condition for one; was caused by a delay of intestinal colonization, whereas the normal intestinal colonization was an intestinal mucosa immune response stimulant.[5]

Seventy percent of infants in this study received antibiotics with different duration, but there was no significant difference in number of infants received antibiotics or duration of receiving antibiotics between groups. Duration of receiving antibiotic was needed in this study because antibiotic had strong effect to mucosal immune response by influence intestinal microflora survival. The length duration of receiving antibiotic could kill the flora and reduce intestinal mucosa immune response stimulation. A study reveal that seven days of antibiotic reduced the number of lymphocytes phenotype in Peyer's patch.[14]

After study, there was increment of fecal sIgA level in both groups, but in probiotic group had higher increment than placebo group. Naturally, intestinal sIgA production will increase gradually by age.[1] Other factors that influence sIgA synthesis are breasfeeding, formula milk feeding, normal intestinal colonization, intraluminal infection, and antibiotics.[3] In this study, several factors had been controlled by randomization such brestfeeding and antibiotics.

This study used multistrain of probiotic containing Lactobacillus acidophillus, Bifidobacterium longum, dan Streptococcus fecalis. This regimen was chosen because each strain had been tested in many studies as a good mucosa immunomodulator,[15-16] and this regimen was available in Indonesia. Multistrain probiotic was expected to have synergy immunomodulation effect, because probiotic had strain specific effect. In this study, which strain that had the best effect to increase IgA synthesis was not studied. Ideally, the study used several single strain probiotic to prove this effect, but when this study was done, there was no single strain of probiotic in Indonesia market.

How probiotic mechanism increase intestinal sIgA was not done in this study. Several references noted that peptidoglycan in the cell wall of probiotic worked as antigen in intestinal lumen then stimulate the intestinal mucosa immune response.[5] This response could also be stimulate by pathogen bacteria, but it would lead to infection. Peptidoglycan in the cell wall of gram positive bacteria was a pathogen-associated molecular patterns (PAMPs) than would be caught specifically by surface receptor of dendritic cell at intestinal mucosa or pattern recognition receptors (PRR) or Toll-like receptor (TLRs) then presented to T cell. There were 11 TLRs found in human. TLR and four were TLR that recognize PAMPs from gram positive bacteria such as probiotics.[6]

Secretory IgA is the predominant antibody class product of mucosal immune system, so it could be stated that determining intestinal or fecal sIgA reflect intestinal mucosa immune response competency.[1,2] It is caused by the relative stabilty of intestinal sIgA in intestinal lumen because it is protected from luminal proteases by an epithelial cell produced glycoprotein, secretory component (SC). This molecule envelops the Fc portion of the dimeric antibody and hides potential proteolytic cleavage sites.[1,3] It caused intestinal sIgA found completely in feces, so examination of sIgA from feces could be used to reflect mucosal immune response in intestines.[9] This was why we used fecal sIgA determination to avoid invasive procedure to the preterm newborn infants.

This study only found that there was increment of fecal sIgA level in preterm newborn infants after probiotic feeding for 14 days. This result supported the hypothesis that normal colonization of the mammalian intestine with commensal microbes is needed to drive the development of the humoral and cellular mucosal immune systems during neonatal life,[5,6] so the abnormal colonization of microflora in preterm infants need to be stimulated by early colonization with probiotic. This similar results (increment of intestinal sIgA level) were found in mice,[9] and children.[10]

There was still controversial about administering probiotic to preterm infant.[17,18] One of the reasons is the possibility of bacterial translocation of this probiotic

to bloodstream and result in sepsis in preterm infant that immunocompromized one. But many data showed that probiotic can decrease intestinal permeability so that bacterial translocation could be prevented.[19] Many studies had been done in preterm infants, and there was no side effect found.[4,16,20-22] This study also did not find any side effects. Signs and symptoms of sepsis happened in one infant from probiotic group was found at seven days of age, but then the infant was getting better and the blood culture was negative. In placebo group, there were four infants with signs and symptoms of sepsis, two of them had the signs and sympstoms since three days of age (one infant with positive blood culture, and died at 10th days of age). Two other infants started getting sepsis at fifth and sixth days of age, one of them had positive blood culture (*Pseudomonas aeruginosa*). It might be hypothezised that early probiotic feeding since born had protective effect to early infection in newborn infants that usually happen in mucosa (gastrointestinal or respiratory).

Small number of samples is one of the limitations of our study. Serial examinations day by day of fecal sIgA level can reveal better data, and examination of specific fecal sIgA level can minimize the false effect of probiotic. Similar study that compare several single strain probiotic was needed to know which strain of probiotic that has the strongest effect to increase fecal sIgA level.

Despite the limitations, the consistently higher fecal sIgA levels in the probiotic group allow the conclusion that it is possible to stimulate the development of the mucosal immune response with a multistrain probiotic of *Lactobacillus acidophilus*, *Bifidobacterium longum*, and *Streptococcus faecium*.

In conclusion, it is evident that that probiotic administration to preterm infants results in a higher levels of fecal sIgA. There is increment of fecal sIgA level in preterm infants who both receive probiotic and who do not, but the increment in probiotic group is higher than placebo group.

References

1. Mayer L. Mucosal Immunity. Pediatrics 2005;111 Suppl 6:1595-1600.
2. Mestecky J, Russel MW, Elson CO. Intestinal IgA: novel views on its function in the defence of the largest mucosal surface. Gut 1999;44:2-5.
3. Woof JM, Mestecky J. Mucosal immunoglobulins. Immunol Rev 2005;206:64-82.
4. Millar MR, Bacon C, Smith SL,Walker V, Hall MA. Enteral feeding of premature infants with Lactobacillus GG. Arch Dis Child 1993;69:483-7.
5. Cebra JJ. Influences of microbiota on intestinal immune system development. Am J Clin Nutr 1999;69 Suppl 5:1046S-51S.
6. Stagg AJ, Hart AL, Knight SC, Kamm MA. The dendritic cell: its role in intestinal inflammation and relationship with gut bacteria. Gut 2003;52:1522-9.
7. Zierikzee AM, van Tol EAF, Kroes H. Faecal SIgA secretion in infants fed on pre- or probiotic infant formula. Pediatr Allergy Immunol 2006;17:134-40.
8. FAO/WHO. Health and Nutritional Properties of Probiotics in Food including Powder Milk with Live Lactic Acid Bacteria. Report of a Joint FAO/WHO Expert Consultation on Evaluation of Health and Nutritional Properties of Probiotics in Food Including Powder Milk with Live Lactic Acid Bacteria, 2001.
9. Grewal HM, Karlsen TH, Vetvik H. Measurement of specific IgA in faecal extracts and intestinal lavage fluid for monitoring of mucosal immune responses. J Immunol Methods 2000;239:53-62.
10. Fukushima Y, Kawata Y, Hara H, Terada A, Mitsuoka T. Effect of a probiotic formula on intestinal immunoglobulin A production in healthy children. Int J Food Microbiol 1998;42 Suppl 1:39-44.
11. Cukrowska B, Lodi RÂ, Enders C, Sonnenborn U, Schulze J, Tlaskalova-Hogenova H. Specific Proliferative and Antibody Responses of Premature Infants to Intestinal Colonization with Nonpathogenic Probiotic E. coli Strain Nissle 1917. Scand J Immunol 2002;55:204-9.
12. Watanabe S, Narisawa Y, Arase S, Okamatsu H, Ikenaga T, Tajiri, Y, et al. Differences in fecal microflora between patients with atopic dermatitis and healthy control subjects. J Allergy Clin Immunol 2003;111 Suppl 3:112-9
13. Dion C, Montagne P, Bene MC, Faure G. Measurement of faecal immunoglobulin A levels in young children. J Clin Lab Anal 2004;18:195-9.
14. Yaguchi Y, Fukatsu K, Moriya T, Maeshima Y, Ikezawa F, Omata J. *et al*. Influences of Long-Term Antibiotic Administration on Peyer's Patch Lymphocytes and Mucosal Immunoglobulin A Levels in a Mouse Model. J Parenter Enteral Nutr 2006;30:395-399
15. Hoyos AB. Reduced incidence of necrotizing enterocolitis associated with enteral administration of Lactobacillus acidophilus

and Bifidobacterium infantis to neonates in an intensive care unit. Int J Infect Dis 1999;3 Suppl 4:197–202.

16. Lin HC, Su BH, Chen AC, Lin Tw, Tsai CH, Yeh TH, *et al*. Oral probiotics reduce the incidence and severity of necrotizing enterocolitis in very low birth weight infants. Pediatrics 2005;115:1-4.
17. Millar M, Wilks M, Costeloe K. Probiotics for preterm infants? Arch Dis Child Fetal Neonatal 2003;88:354-8.
18. Zhang L, Li N, Neu J. Probiotics for preterm infants. NeoReviews 2005;Suppl 5:227-32.
19. Ishibashi N, Yamazaki S. Probiotics and safety. Am J Clin Nutr 2001;73 Suppl:465S–70S.
20. Kitajima H, Sumida Y, Tanaka R, Yuki N, Takayama H, Fujimira M. Early administration of Bifidobacterium breve to preterm infants: randomised controlled trial Arch Dis Child Fetal Neonatal Ed 1997;76 Suppl 2:101-7.
21. Uhlemann M, Heine W, Mohr C, Plath C, Pap S. Effects of oral administration of bifidobacteria on intestinal microflora in premature and newborn infants. Z Geburtshilfe Neonatol 1999;203 Suppl 5:213-7.
22. Costalos C, Skouteri V, Gounaris A, Sevastiadou S, Triandafilidou A, Ekonomidou C, *et al*. Enteral feeding of premature infants with Saccharomyces boulardii. Early Human Dev 2003;74:89-96.