Clinical Research

The effect of *Bifidobacterium animalis lactis* HNO19 supplementation among pregnant and lactating women on interleukin-8 level in breast milk and infant's gut mucosal integrity

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ABSTRAK

Latar belakang: *Mukosa usus bayi baru lahir belum sepenuhnya berkembang sehingga bayi rentan mengalami diare. Pemberian probiotik diketahui dapat memicu maturasi saluran cerna. Penelitian ini bertujuan untuk mengidentifikasi apakah suplementasi probiotik sejak ibu hamil di trimester ketiga dapat meningkatkan integritas mukosa usus pada bayi baru lahir.*

Metode: *Uji klinis acak tersamar ganda dilakukan untuk mengetahui potensi efek pemberian probiotik terhadap kandungan probiotik dan interleukin-8 (IL-8) di ASI, kadar IFABP di urin ibu menyusui, dan kandungan α-1-antytripsin, serta kalprotektin pada feses bayi saat lahir (V_0) dan saat usia 3 bulan (V_3). Strain tunggal Bifidobacterium lactis animalis HNO19 (dikenal sebagai DR10) digunakan dalam studi ini karena bukan merupakan bakteri residen. Penelitian ini dilakukan di Rumah Sakit Budi Kemuliaan dan satelit kliniknya dari Desember 2014 hingga Desember 2015.*

Hasil: *Hasil penelitian menunjukkan bahwa sebanyak 14% (5/35) dan 20% (7/35) subjek memiliki DR10 pada kolostrum dan ASI saat bayi berusia tiga bulan. Nilai median IL-8 kelompok probiotik dibandingkan kelompok placebo pada V_0 dan V_3 berturut-turut adalah 2810,1 pg/mL vs. 1516,4 pg/mL (p=0,327) dan 173,2 pg/mL vs 132,7 pg/mL (p=0,211). IFABP 211,7 ng/mL vs 842,5 ng/mL (p=0,243) dan 25,3 ng/mL vs 25,1 ng/mL (p=0,466). AAT 136,2 mg/dL vs 148,1 mg/dL (p=0,466) dan 24 mg/mL vs 29,72 mg/mL (p=0,545). Kalprotektin 746,8 ng/mL vs 4645,2 ng/mL (p=0,233) dan 378,6 ng/mL vs 391,3 ng/mL (p=0,888).*

Kesimpulan: *Probiotik DR10 yang diberikan pada ibu hamil dan menyusui dapat ditemukan pada kolostrum dan ASI saat usia bayi 3 bulan, tetapi tidak memberikan efek terhadap kadar probiotik lain atau IL-8 dan integritas mukosa usus.*

ABSTRACT

Background: Newborn's gut mucosal is not fully developed, therefore infants are prone to diarrhea. Probiotic supplementation is known to induce the gut mucosal maturity. This study aimed to identify whether probiotics supplementation among pregnant women since the third trimester would increase the infant's gut mucosal integrity.

Methods: A double-blind, randomized clinical trial was conducted to understand the potential effect of probiotic supplementation on the level of probiotics and IL-8 in breastmilk, urine IFABP, faecal α-1-antytripsin (AAT) and calprotectin in infant's at birth (V_0) and three-months old (V_3). A single strain of *Bifidobacterium lactis animalis* HNO19 (known as DR10) was used since it was not the resident bacteria. The study was held at Budi Kemuliaan Hospital and its satellite clinics from December 2014 to December 2015.

Results: About 14% (5/35) and 20% (7/35) of the subjects had DR10 in the breastmilk's colostrum and at the age of 3-months. The median values of IL-8 in the probiotic group vs the placebo group at V_0 and V_3 were 2810,1 pg/mL vs 1516.4 pg/mL (p=0.327) and 173.2 pg/mL vs 132.7 pg/mL (p=0.211) respectively. IFABP level 211.7 ng/mL vs 842.5 ng/mL (p=0.243) and 25.3 ng/mL vs 25.1 ng/mL (p=0.466); AAT 136.2 mg/dL vs 148.1 mg/dL (p=0.466) and 24 mg/mL vs 29.72 mg/mL (p=0.545); Calprotectin 746.8 ng/mL vs 4645.2 ng/mL (p=0.233) and 378.6 ng/mL vs 391.3 ng/mL (p=0.888).

Conclusion: Probiotic DR10 given to pregnant women since the 3rd trimester can be found in colostrum and 3-months breastmilk. However, it did not affect the level of other probiotics or IL-8 and the gut mucosal integrity.

Keywords: *Bifidobacterium lactis animalis* HNO19, breastmilk, gut mucosal integrity

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The infant's intestinal mucosal growth is not mature, yet this is called "leaky gut". The intestinal mucosal develops until the infants reach two years of age. Therefore, the highest incidence of diarrhea is found among children under two years old. The mucosal epithelial barrier and immunoregulatory network are poorly developed in newborns. The infant's abrupt introduction to life outside uterus and the exposure to antigens force the gastrointestinal (GI) tract to adapt quickly and commence its crucial duties.[1]

However, the neonate's adaptive immune system is still immature, leaving the newborn in a state of vulnerability and at increased risk for serious infection.[2] Human milk, the natural infant feeding, is a complex species-specific biological fluid adapted to perfectly satisfy the nutritional and immunological needs to neonate. The compounds presented in colostrum or mature milk may have anti-infective effect.[3]

Recently, extensive research has been conducted to understand the beneficial role of human milk oligosaccharides (HMO), introducing the new concept of the 'milk microbiome'. The mechanism of whether bacteria contamination or active migration helps bacteria to reach mammary gland is debatable[4] and further study is necessary. Numerous studies had identified probiotics in breastmilk.[3,5] Probiotic consumptions may help other bacteria to grow, for example supplementation of bifidobacteria affects lactobacillus growth.[6] Administration of *Enterococcus faecium* CRL 183 increases bifidobacteria and short chain fatty acid (SCFA) levels whereas *Lactobacillus acidophilus* CRL 104 increases *Bifidobacterium* and *Lactobacillus*, as well as acetate in an in vitro GI model.[7] Interleukin 8 (IL-8) presents in significant amount in human milk. Studies showed that a consistent increase in cell migration, proliferation, and differentiation occured when human fetal and adult intestinal cells were treated with rhIL-8 in vitro.[8] Other in vitro study found the relationship of *E. coli* LTH 634 and *Lactobacillus sakei* with the production of IL-8.[9]

This study aims to provide evidence whether probiotics are present in breastmilk and affect the growth of other bacterias, and also increase the IL-8 production and mucosal integrity of infants, compared to the placebo group. A single strain of *Bifidobacterium lactis animalis* HNO19, a non-resident bacterium, was used in this study.

METHODS

Study design

A double-blind, randomize controlled trial with two parallel groups was conducted to understand the effect of probiotic supplementation in pregnant women, assessing the IL-8 production in breast milk, urinary intestinal fatty acid binding protein (IFABP), α-1-antytripsin (AAT) and calprotectin in feses at birth and three-month old infant. This study was conducted from December 2014 to December 2015 at Budi Kemuliaan hospital and its satellite clinics. A total of 110 pregnant women in their third trimester, who came to the obstetrics and gynecology outpatient department in the selected hospitals were enrolled. The inclusion criteria were pregnant women at third trimester, normal pregnancy, plan to deliver spontaneously, no consumption of antibiotics, and plan to exclusively breastfeed the baby (at least until the baby is three months old). The newborns of the study were also included in the study. Exclusion criteria were pregnant women with pre-eclampsia, bleeding, infection, premature rupture of the membrane or other chronic disease. This study was approved by the Health Research Ethics Committee Faculty of Medicine University of Indonesia number 525/UNS.F1/ETIK/2014.

Sample selection

Pregnant women who fulfilled the inclusion criteria at Budi Kemuliaan Hospital and its satellite clinics would be recruited. They were asked to participate in the study and signed the informed consent. The subjects were divided into two groups by block permutted randomization with the size of ten. The subjects were restricted to consume food/drink with probiotic ingredients, such as yoghurt and yakult, during the study. Their diet was controlled by nutrition experts.

Intervention stage

The treatment group was given probiotic capsul of *Bifidobacterium lactis animalis* HNO 19 with the dose of 10^9 unit every day until their newborns were three-months old. While for the same period, the control group was given placebo daily. In case of bleeding during birth delivery or cesarean surgery or antibiotics treatments, the subjects would be excluded from the study (dropped out).

Initial breastmilk in the first days after birth (colostrum) was obtained and examined using real time (RT) polymerase chain reaction deoxyribose-nucleic acid (PCR DNA) to identify the total number of microbiota, genus *Bifidobacteria*, genus *Lactobacillus*, and strain *Bifidobacterium lactis* animalis HNO19 (DR10). A manual sampling was conducted; the nipples and mammary areole were cleaned with antiseptic. The researcher washed their hands and wore sterile gloves before doing the examination. Skin swab around the breast was also performed. Infants were examined for intestinal fatty acid binding protein (IFABP) in their urine and for calprotectin, alpha 1 antytripsin, in their stool. The gestational age, anthropometric status (birth weight, birth length, head circumference), mode of delivery, and Apgar score were recorded from the newborns. Infants with respiratory distress, asphyxia, seizures, congenital abnormalities, require NICU or were not able to drink orally are considered as drop-out (DO). During breastfeeding, subjects were monitored and counseled by a breastfeeding counselor in order to achieve exclusive breastfeeding. Same procedures were performed when the infants reached three-months old.

Specimen collection and examination of breastmilk

Colostrum were obtained after birth delivery (day 1–5). Trained persons collected the samples with aseptic and antiseptic action. After pumping, at least 0.1 mL of breastmilk was poured into a sterile tube containing 300 mg of zirconium beads (diameter 0.1 mm). Afterwards, the samples were centrifuged several times in the laboratoray, washed with ethanol, and centrifuged again before DNA separated. Real-time PCR was performed using the applied biosystems (ABI) 7500 Fast Sequence Detection System using software version 2.0 (Applied - Biosystems). Primers were designed based on 16S ribosa ribonucleic acid (rRNA) specific for *Bifidobacterium* sp., total bacteria, *Lactobacillus* sp. and *Bifidobacterium lactis animalis* HNO19 (Table 1). Each reaction run with duplication of the final volume 15 µl with a final concentration of 0.3 to 0.9 µM each primer, and 10 mL of appropriate dilution with the DNA sample. IL-8 in breastmilk was also examined using enzyme linked imunosorbent assay (ELISA) method.[10] Before collecting breastmilk, we did skin swab around the breast and examined for DR 10.

Examination of urine IFABP, stool AAT and calprotectine

In this study, I-FABP urine was measured by Elisa using human I- FABP ELISA kits HK406.[11] One mL urine was collected with aseptic procedure and removed to the poliprophylen tube, which then kept in -80°C storage. Before analysis, the specimens were removed to -20°C for one night, then they were removed again to room temperature (18–25°C), and mixed well. Centrifugation was conducted to remove the debris, then everything was done as in the kit procedure.

AAT and calprotectin was measured in stool.[12,13] Make sure reagen and specimen were mixed adequately. Put 100 mg stool to a plastic vial then add 5 mL Exbuff. Centrifugation and dilution for AAT and calprotectin were performed differently according to the kit procedure.

Data Analysis

All data were analysed using statistical product and service solutions (SPSS) version 20 and presented in text, tables, or graphs. We used 95% of confidence level 80% power. Data with normal distribution presented mean and standard deviations values while data with abnormal distribution used median values. Mann Whitney and independent t-test were applied in this study.

RESULTS

About 110 subjects were recruited and enrolled to this study, but 40 of them were dropped out, leaving only 70 subjects (Figure 1). Primer quantitative PCR for microbiota in breastmilk showed in Table 1. The majority were housewives, aged 20–29 years, giving birth once, graduated from high school, and received income of 2–9 million IDR (Table 2). About 14% (5/35) subjects in probiotic group had DR10 positive in their breast milk during birth delivery (V_0) and 20% (7/35) at three-months postpartum (V_3). However, none were positive in the placebo group, both during birth delivery and at three-month postpartum. Skin swab indicated negative result in all groups (Table 3). Composition of microbiota and IL-8 in breastmilk showed in Table 4. Result of urine IFABP, stool AAT and calprotectin showed in Table 5.

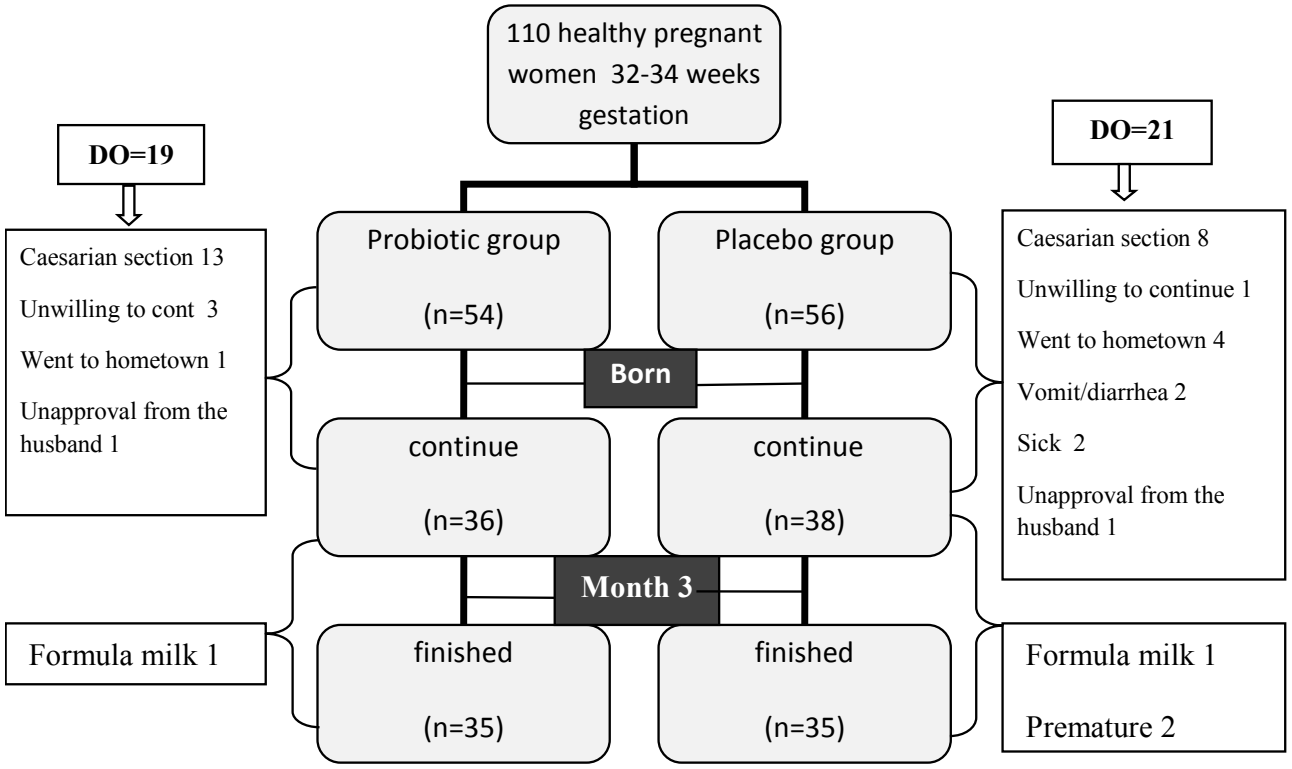

Figure 1. Subject recruitment. DO= drop out

Table 1. Primer quantitative PCR for microbiota in breastmilk.[14]

No	Target	Primer	Sequence	Reference
1.	*Bifidobacterium* sp.	F_Bifid	CGGGTGAGTAATGCGTGACC	14
		R_Bifid	TGATAGGACGCGACCCCA	
2.	Total microbiota	8f_All	GRGTTYGATYMTGGCTCAG	14
		340R	ACTGCTGCCTCCCGTAGGAGT	
3.	*Lactobacillus* sp.	F_Lacto	AGCAGTAGGGAATCTTCCA	14
		R_Lacto	CGCCACTGGTGTTCYTCCATATA	
4.	*Bifidobacterium animalis* subsp. *lactis* strain HNO19 (DR10)	F_DR10	CCCTTTCCACGGGTCCC	14
		R_DR10	AAGGGAAACCGTGTCTCCAC	

DISCUSSION

This is the first study explaining the correlation between probiotic diet and breastmilk microbiota among women after birth delivery in Indonesia. Many factors influence the viabillity and survival of probiotic in breastmilk, such as genetic, culture, environment, diet, mode of delivery, and antibiotic treatment during pregnancy and lactation.[15] Breastmilk of women who received antibiotics during pregnancy or lactation had lower content of *Lactobacilli* or *Bifidobacteria* compared to women who received no antibiotics.[16] In this study, both the intervention and the placebo group had *Lactobacillus* and *Bifidobacterium* in their breastmilk. Subjects who received antibiotics during pregnancy had been excluded from this study. One subject with premature rupture of membrane was excluded due to caesarean section. There were few subjects who received antibiotics during delivery or maximum five days postpartum via oral or three days via parenteral. However, their test were still positive. These

Table 2. Subject charasteristic

	Group					
	Probiotic		Placebo		Total	
	n	(%)	n	(%)	n	(%)
Age (year)						
<20	3	(5.6)	3	(5.4)	6	(5.5)
20–29	35	(64.8)	31	(55.4)	66	(60.0)
30–39	14	(25.9)	20	(35.7)	34	(30.9)
>40	2	(3.7)	2	(3.6)	4	(36)
Education						
Illiterate	0	(0)	0	(0)	0	(0)
Primary school	11	(20.4)	10	(17.9)	21	(19.1)
High school	39	(72.2)	39	(69.6)	78	(70.9)
Bachelor	2	(3.7)	4	(7.1)	6	(5.5)
Diploma	2	(3.7)	3	(5.4)	5	(4.5)
Parity						
1	29	(53.7)	26	(46.4)	55	(50.0)
2	14	(25.9)	14	(25.0)	28	(25.5)
3	5	(9.3)	11	(19.6)	16	(14.5)
4	6	(11.1)	4	(7.1)	10	(9.1)
5	0	(0.0)	1	(1.8)	1	(0.9)
Income (IDR)						
<2	6	(11.1)	10	(17.9)	16	(14.5)
2–9	48	(88.9)	42	(75.0)	90	(81.8)
>9	0	(0)	4	(7.1)	4	(3.6)
Job						
Housewife	28	(51.9)	31	(55.4)	59	(53.6)
Enterpreneur	2	(3.7)	4	(7.1)	6	(5.5)
Employee	24	(44.4)	21	(37.5)	45	(40.9)
Total	54	(100.0)	56	(100.0)	110	(100.0)

Table 3. *Bifidobacterium lactis* HNO19 in breast milk and skin swab

Bifidobacterium lactis HNO19	Group	
	Probiotic n=35	Placebo n=35
Breast milk n (%)		
V_0	5(14)	0
V_3	7(20)	0
Copy number/m Median (min – max)		
V_0	53.6 (25.3–74.4)	0
V_3	80.4 (6.7–85.7)	0
Skin swab		
V_0	0	0
V_3	0	0

V_0= at delivery; V_3= at 3 months

might happen because the microbiota depression period ranges usually between 3–8 weeks while the time period between V_0 and V_3 examination was three months. Our findings also support that *Bifidobacterium animalis lactis* HNO19 (DR 10) is transient microbiota that can be transferred from mother to fetus through mammary lymph nodes.[17,18] After consumption, ingested bacteria enter a hostile environment where subsequent passage through stomach and duodenum exposes them to highly stressful physiochemical and biological conditions such as gastric acid and bile salt.[15] DR 10 that reach gastric lumen are taken up by dendritic cell (DC) to the mesenteric lymph nodes, respiratory tract, genitourinary tract, salivary and lacrimal glands, and eventually mammary gland. DCs and macrofags are able to discriminate between pathogenic and non-

Table 4. Composition of breast milk microbiota and level of IL-8 in breast milk

	Group		p-value*
	Probiotic n=35	Placebo n=35	
Total microbiota Median (min-max) copy number/mL			
V_0	963.8 (184.9–94106.7)	2523.2 (123.6–26821.6)	0.242
V_3	1803.6 (143.1–551256)	2201.1 (273.1–870017.1)	0.819
Lactobacillus Median (min-max) copy number/mL			
V_0	3.3 (0–16.8)	2.7 (0.06–1201.1)	0.819
V_3	3.7 (0.11–1389.9)	6.62 (0.42–165.5)	0.073
% *Lactobacillus* from total microbiota Median (min-max)			
V_0	0.185 (0–6)	0.105 (0.001–38.0)	0.435
V_3	0.226 (0.001–30)	0.360 (0.002–34)	0.190
Bifidobacterium Median (min-max) copy number/mL			
V_0	122.3 (11.7–348.4)	120.6 (37.4–236.5)	0.911
V_3	61.3 (3.8–603.3)	61.9 (12.2–191.8)	0.577
% *Bifidobacterium* from total microbiota Median (min-max)			
V_0	8.8 (0.060–83)	6.3 (0.534–98)	0.442
V_3	3.4 (0.075–43)	1.9 (0.022–45)	0.930
IBreast milk IL8 Median (min-max) pg/mL			
V_0	2810.1 (94.5–66246.9)	1516.4 (28.8–514157)	0.327
V_3	173.2 (24.8–8118.2)	132.7 (35.4–3150.7)	0.211

*= Mann Whitney; V_0= at delivery; V_3= at the baby 3 months of age

Table 5. Urine IFABP, stool AAT and calprotectin

	Group		p-value*
	Probiotic n=35	Placebo n=35	
Urine IFABP median (min-max) ng/mL			
V_0	211.7 (23.8–6652.2)	842.5 (14.9–9619.8)	0.243
V_3	25.3 (18.1–952.6)	26.1 (17.2–255.3)	0.466
Stool AAT median (min-max) mg/dL			
V_0	136.2 (6.8–576.6)	148.1 (18.6–670.4)	0.466
V_3	24.0 (7.4–168.5)	29.72 (3.1–130.7)	0.545
Stool calprotectin median (min-max) ng/mL			
V_0	746.8 (39.4–1950.8)	465.2 (72.8–2622.6)	0.233
V_3	378.6 (169.69–1416.7)	391.3 (83.1–2132.8)	0.888

*Mann Whitney; V_0= at delivery; V_3= at the baby 3 months of age

pathogenic compounds through the expression of various pattern-recognition receptors (PRRs).[17,18] Bacterial signaling on the mucosal surfaces is dependent on the network between bacteria, epithelial cells, and the immune system. Therefore, not all subjects in the probiotic group had positive DR 10 in their breastmilk.

The composition of breastmilk microbiota is complex. From the hundreds of operational taxonomic units (OTUs) detected in the milk of every woman, only nine were present. Surprisingly, these nine "core" OTUs represented about half of the microbial community observed. Infant's immune system was greatly influenced by their maternal immunity which transferred via placenta and breast milk. IL-8 in breastmilk indicated the leukocyte movement from mothers to infants. The levels of cytokine were high in colostrum and transient milk; then it would reduce in the first 21 days and 60 days of breastmilk production.[19] This study confirmed that IL-8 level in colostrum was found higher in the probiotic group than in the placebo group after birth (V_0), but their level of IL-8 was equal in the probiotic as well as the placebo group at three-month postpartum. Probiotics did not affect the chemokine content from colostrum to mature milk.

Intestinal fatty acid binding protein (IFABP) is an indicator of enterocyte damage.[20] In this study, the median IFABP level at delivery (V_0) was lower in the probiotic group than in the placebo group (p=0.243). This means less enterocyte damage happened in the probiotic group. The low level on the probiotic group at V_0 seemed to correlate with the high level of IL-8, which improved gut maturity, then decreased enterocyte damage although it was not significant. During breastfeeding, the guts had microbiota to protect enterocyte and therefore, no differences were found in terms of IFABP level in both the probiotic and the placenta group 3-month after birth. The supplementation of DR10 had no effect to make any difference on IFABP level.

AAT is a serum protein that is resistant to enzymatic proteolysis in the gastrointestinal tract. This protein does not exist in the diet. Since this protein is excreted, testing the content of AAT in faeces could reflect protein entering the intestine from the intravascular space. Faecal AAT has been considered as a reliable and inexpensive method for the estimation of enteric protein loss.[21] The AAT level in both group at birth was similar, meaning that DR10 supplementation showed no effect in reducing enteropathy. While in the age of 3 months, the AAT level was decreased in both groups since the breastmilk already contained cytokine, secretory Ig A, and fatty acids.

Calprotectin, calcium and zinc binding protein, presents in monocytes, macrophages, and epithelial cells. Its function is to regulate inflammatory processes.[22] High faecal calprotectin levels correlate with an increased turnover of leukocytes in the intestinal mucosa and granulocyte migration to intestinal lumen. Faecal calprotectin levels have been reported to be much higher during the first few weeks of life, both in healthy full-term and pre-term infants. The gut mucosa in newborn infants tends to have higher risk of inflammation.[23] In gut inflammation, calprotectin can be detected in stool and plasma. Therefore, stool calprotectin could be used as a good marker for necrotizing enterocolitis (NEC). The calprotectin level in this study were both decreasing and found to have similar level at V_3. This might happened because breastmilk naturally had the ability to reduce inflammation in gut mucose. Longer follow-up after delivery is necessary to explore and analyse the effect of probiotics to the gut mucosal integrity and its role to improve gut mucosal integrity by comparing subjects with and without probiotics.

A high number of dropped-out subjects was found in this study because of unexpected cesarean section and low compliance. The socio-economic condition of the subjects may influence their compliance. Dropped-out due to formula milk usage was relatively low. In the first three months after birth, the rate of exclusive breastfeeding was still high. The same condition was found by Collado et al[24] babies who got breast milk at delivery, 66.1% of them continued to give exclusive breastfeeding until 6 months postpartum. However, the average exclusive breastfeeding duration is three months. The data from Basic National Research (Riskesdas) in 2012, 42% of 0–6 months exclusive breastfeeding.[25]

In conclusion, probiotic *Bifidobacterium animalis lactis* HNO19 (DR10) given to pregnant women since the 3rd trimester can be found in colostrum and breastmilk (three-months postpartum).

However, it did not affect the level of other probiotics or IL-8 and the gut mucosal integrity.

Conflict of Interest

The funding and source of probiotic strain were supported by Friesland Campina Innovation and Fontera.

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