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Original Article

Gut wall integrity in exclusively breastfed *vs.* formula-fed infants

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Abstract

Background Breast milk has bioactive substances that modulate gastrointestinal maturation and maintain mucosal integrity of the gut in infants. Markers that are both non-invasive and reliable, such as fecal alpha-1 antitrypsin (AAT), calprotectin, and secretory immunoglobulin A (sIgA) have been used to assess gut integrity in adults. Higher AAT levels may imply greater enteric protein loss due to increase intestinal permeability of immaturity gut.

Objective To assess and compare gut integrity of exclusively breastfed (BF) and exclusively formula fed (FF) infants aged 4-6 months.

Methods Subjects were 80 healthy infants (BF=40; FF=40), aged 4-6 months who visited the Pediatric Polyclinic at St. Carolus Hospital, and lived in Pasar Minggu or Cempaka Putih Districts, Jakarta. The fecal AAT was analyzed by an ELISA method. Mann-Whitney and unpaired T-test were used to analyze possible correlations between feeding type and gut integrity.

Results The BF group had significantly higher mean fecal AAT than the FF group (P=0.02). Median sIgA levels were not significantly different between groups (P=0.104). The FF group had a higher mean fecal calprotectin level but this difference was also not significant (P=0.443). There was a significant correlation between breastfeeding and mean fecal AAT level (P=0.02), but no significant correlation with calprotectin (P=0.65) or sIgA (P=0.26).

Conclusion The breastfed group shows better mucosal integrity compared to the formula fed group. Higher mean fecal AAT level in the BF group is related to the AAT content of breast milk. Therefore AAT content of BF group is actually lower than formula fed group which shows greater mucosal integrity in BF group. **[Paediatr Indones. 2016;56:199-204. doi: 10.14238/pi56.4.2016.199-204]**.

Keywords: *infants; breastfeeding; markers; gut wall integrity; fecal; alpha-1 antitrypsin; calprotectin; secretory IgA*

Adequate levels of nutritional intake and utilization are critical to optimize children's ongoing growth, development, and health.[1-2] Breast milk is the ideal nutrition for infants and has been recommended to be given exclusively for 6 months of life.[3-5] Although breastfeeding is optimal for infants, there are few conditions under which breastfeeding may not be in the best interest of the infant. As such, formula is an alternative type of feeding. The notion that human milk supplies only nutrients to the infant has been rectified by the discoveries of a wide spectrum of bioactive agents that have potential targets in the gastrointestinal tract, modifying its function and having profound effects on its integrity in experimental animals.[6-8] Infant formula provides the nutrients needed for adequate growth, but not all components of human milk can be duplicated in formula.[8]

Maturation of the gastrointestinal tract is a dynamic process that continues from birth to 1 to 2 years of life. Intestinal maturation may be assessed by its permeability, secretory immunoglobulin,

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and antimicrobial peptides.[9] Gut wall integrity is affected by gastrointestinal maturity, as defined by permeability changes and intestinal inflammation.[7,10] Assessment of gut wall integrity in clinical practice is a challenge. Indirect examinations that are non-invasive, rapid, easy, and accurate are needed to assess gut wall integrity. Markers of gut wall integrity that fulfill these criteria are fecal alpha-1 antitrypsin (AAT), calprotectin, and secretory IgA (sIgA). Studies with human subjects on nutritional effects on the growth and development of the post-natal gastrointestinal tract have been rare because of ethical reasons related to nutritional intervention and the need for gut specimens from healthy subjects. Studies on differences in gut wall integrity in breastfed (BF) and formula fed (FF) infants using fecal AAT, calprotectin, and sIgA levels have had varying results.[12-17] We aimed to assess gut maturity of infants in 4-6 months age interval using fecal AAT, calprotectin, and sIgA levels on exclusively BF and FF babies.

Methods

An analytic, cross-sectional study of 80 healthy babies was undertaken in the Pediatric Clinic at St. Carolus Hospital, as well as Pasar Minggu and Cempaka Putih Districts, Jakarta from June to August 2013. Infants recruited were 4–6 months of age and were divided into groups according to their diet. Infants were eligible for inclusion as breastfed or formula fed if the mother had either exclusively breast fed or exclusively formula fed her baby (i.e., the mothers did not combine the two or add complementary food) for the entire 4-6 months before enrolling in the study. All infants were healthy, with histories of full term pregnancy (gestational age 37–42 weeks) and birth weights of 2.5-4.75 kg.

Birth weights, lengths, head circumferences, age at recruitment, gender, and mode of delivery were recorded for all subjects. Fecal specimens were obtained from infants' diapers, put in sterile plastic tubes, then stored at -80^{o}C until analysis. Calprotectin, AAT, and sIgA levels were measured in duplicate fecal aliquots using an enzyme-linked immunoassay (*Phical*®, Immundiagnostik, Germany). The normal levels of fecal AAT, calprotectin, and sIgA were defined (<26.8 mg/dL, 85-988 mg/kg, and 510-2040 μg/mL, respectively) based on the manual guide of the test kits.

Data was analyzed using *SPPS version 20* and expressed as medians, means and standard deviations (SD) with 95%CI. Statistical analyses on correlations between two qualitative variables were performed using Mann-Whitney and unpaired T-test. Cut off point levels for fecal AAT, calprotectin, and sIgA of this study subject population was defined using ROC curves. The correlation of qualitative and quantitative variables was analyzed using student's T-test. A P value of < 0.05 was considered to be statistically significant. Informed consent was obtained from parents at the time of recruitment. Protocols were approved by the Medical Research Ethics Committee of the University of Indonesia Medical Schhool/Dr. Cipto Mangunkusumo Hospital, Jakarta, Indonesia.

Results

The characteristics of the subjects in both groups were similar (**Table 1**).

Table 1. Characteristics of subjects

Characteristics	BF group	FF group	P value
Mean age, days	153.43 95% CI 147.21 to 159.64	147.85 95% CI 140.83 to 154.87	0.233[b]
Median birth weight (range), g	3,275 (2,700-3,700)	3,232.75 (2,600-4,500)	0.605[a]
Median birth length (range), cm	49 (47-52)	49 (47-56)	0.957[a]
Mean body weight, kg	7.25 95% CI 6.93 to 7.58	7 95% CI 6.68 to 7.3	0.249[b]
Mean body length, cm	64.79 95% CI 64.03 to 65.56	63.58 95% CI 62.41-64.74	0.083[b]
Mean head circumference, cm	41.93 95% CI 41.55 to 42.3	41.41 95% CI 40.96 to 41.86	0.078[b]

[a]Mann-Whitney test, [b]unpaired T-test

Mean fecal AAT in the BF group was significantly higher than in the FF group, but there were no significant differences in calprotectin and sIgA levels between groups (Table 2).

The marker cut-off points of the fecal level of the three markers based on this study subject population were AAT 133.875 mg/dL, calprotectin 205.5 mg/kg, and sIgA 777.78 μg/mL. There was a significant correlation between feeding type and increased mean AAT level, but no significant correlation between feeding type and mean calprotectin or median sIgA levels **(Table 3, Table 4, Table 5)**

subjects less than 6 weeks of age (BF 97.66 mg/dL; 95%CI 60.56 to 134.77 mg/dL, SF 38.4 mg/dL; 95%CI 0.32 to 76.39 mg/dL). In a second sampling 3 months later, they found the following mean levels: (BF 195.19 mg/dL; 95%CI 157.35 to 233.03 mg/dL; SF 61.7 mg/dL; 95%CI 22.25 to 101.15 mg/dL).[12]

A prospective, longitudinal study on 820 healthy babies also had significantly higher AAT levels in BF babies at all age intervals. That study divided the subjects into six age groups, with 2 groups of feeding types in subjects less than 6 months of age (BF and FF), and 3 groups of

Table 2. Fecal AAT, calprotectin, and sIgA levels in the BF and FF groups

Variables	BF group (n=40)	FF group (n=40)	P value
Mean AAT,mg/dL	147.43 95% CI 135.86 to 158.99)	121.84 95% CI 103.51 to 140.17	0.02[a]
Mean calprotectin, mg/kg	199.08 95% CI 174.48 to 223.69	213.79 95% CI 184.08 to 243.51	0.443[a]
Median sIgA (range), μg/mL	928.81(75.1-1676.95)	530.87 (50.82-1376.95)	0.104[b]

[a]unpaired T-test, [b]Mann-Whitney test

Table 3. Analysis of fecal AAT, calprotectin, sIgA levels and feeding types

Variables	Feeding groups	Levels		P value	Odds ratio	95%CI
		Higher*	Lower*			
AAT, n(%)	BF (n=40)	25 (62.5)	15 (37.5)	0.02	2.78	1.12 to 6.87
	FF (n=40)	15 (37.5)	25 (62.5)			
Calprotectin, n(%)	BF (n=40)	20 (47.6)	20 (52.6)	0.65	1.22	0.51 to 2.94
	FF (n=40)	22 (52.4)	18 (47.4)			
sIgA, n(%)	BF (n=40)	17 (43.6)	23 (56.1)	0.26	1.65	0.68 to 4.00
	FF (n=40)	22 (56.4)	18 (43.9)			

*compared to cut off level in this study

Discussion

The fecal AAT examination is an easy and accurate method to assess intestinal permeability and protein enteric loss in children and adults. Higher fecal AAT level shows greater gut wall permeability which cause greater protein enteric loss.[12,18] However, using AAT measurements to assess gut wall integrity in infants has had varied results. We found a significantly higher mean AAT level in the BF group compared to the FF group (P=0.02). Similar results were found in another study that involved 160 healthy, exclusively BF babies and infants who received probiotic/prebiotic-supplemented formula (SF). The BF group had a significantly higher mean AAT level (P=0.019) in

feeding types in subjects more than 6 months of age (BF, FF, and cows' milk). Fecal AAT levels tended to be higher in the first 6 months of life and tended to decrease and stabilize 6 months later.[14] In contrast, a study on 232 subjects (aged 7 days-18 months) with 5 different feeding types (exclusively BF, non-exclusively BF, exclusively FF, cows' milk, and soy formula) reportedly had higher fecal AAT level in the exclusively BF group at < 90 days of age, but significantly lower at 90-179 days of age (3-6 months). Their results showed differences in age and feeding in the concentration of alpha 1-antitrypsin which then support the theory that human milk can modulate the maturation process

of gastrointestinal tract and decrease enteric protein loss because the differences may reflect subtle changes in gastrointestinal tract function in infancy.[13]

In our study, elevated fecal AAT concentrations were found in both the BF and FF groups. The comparison of higher and lower value was assessed by both the cut-off in the test kit module (<26.8 mg/dL) and the cut off point from this study's subject population (133.875 mg/dL). The higher fecal AAT level in exclusively BF babies is derived from human milk.[15] The role of AAT is to protect human milk protein content (such as immunoglobulin) escape intestinal degradation. The AAT only works in an intact form.[15] One study demonstrated high AAT concentrations in human milk (0.3-0.6 mg/mL) in the early weeks of lactation which persisted for the first few months of life. The AAT consumed by exclusively BF babies is excreted and significant quantities of intact AAT (25-60%) can be found in feces.[15] In our study, eventhough we found a significant correlation between mean fecal AAT level in both group, anyhow those values were obtained without taking intact AAT from breastmilk in feces into account. Based on literature, 25-60% of the fecal AAT levels in the BF group in our study implied that 36.85-88.46 mg/dL AAT came from the breast milk. Accordingly, the AAT level that solely reflect protein entering the intestine from the intra-vascular space was 58.97-110.88 mg/dL and that value was still lower than FF group's fecal AAT level (121.84 mg/dL). Since the FF group did not get AAT from formula milk so it could be concluded that the high fecal AAT level of this group is caused purely by the protein enteric loss. Similar to a previous study on 820 healthy babies, we found a significant correlation between fecal AAT level and exclusive breastfeeding. In that study, the exclusively BF babies had higher AAT levels at all age intervals (2-52 weeks).[13]

Calprotectin has been used as an accurate fecal and gut inflammation marker for the last 10 years. Calprotectin is resistant to enzyme degradation, stabile at room temperature, and easy to measure in fecal specimens. High calprotectin levels are indicative of increased mucosal leukocyte and granulocyte migration to the intestinal lumen.[11] Gut inflammation is caused by several factors such as mucosal deterioration, or intestinal leakage related to pathological processes or immaturity, as is the focus of this study. Fecal calprotectin levels are relatively high in the first few weeks in life of term or premature infants compared to healthy children and adults. However, we found no significant difference in fecal calprotectin levels between the BF and FF groups (P=0.443). In comparison, a study of 69 four-day-old infants who were healthy and exclusively BF or FF, also found no significant difference in fecal calprotectin levels between groups. The study consisted of 3 groups: 18 subjects on standard formula, 19 subjects on prebiotic-supplemented formula, and 32 subjects were exclusively BF. The FF group showed a higher level of fecal calprotectin, but it was not significantly different compared to the other 2 groups. All groups showed a higher calprotectin level compared to the reference value in healthy adults (50 μg/g). That study demonstrated no correlation between feeding type and fecal calprotectin level.[17] Another report on 70 healthy 3-month-old babies also revealed no difference in fecal calprotectin levels between exclusively BF and FF babies (P=0.09).[18] In addition, another study on 160 healthy babies who were either exclusively BF or received prebiotic/probiotic supplemented formula revealed no significant difference in fecal calprotectin level between the first sampling (< 6 weeks of age) and at second (3 months later) (P=0.393).[12] Our study could not be compared to previous studies due to differences in age intervals of the subjects, thus the result consistently showed a higher level of fecal calprotectin in FF babies.

The lack of a significant difference in fecal calprotectin levels in our study may be explained by several factors. The first factor is that human milk contributed to the increasing calprotectin level in BF babies. Human milk contains monocytes/macrophages (59-63%) and those are known to contain calprotectin.[2,6] The second factor is that most FF subjects received prebiotic/probiotic-supplemented formula. The acid environment that created by the fermentation process may increase the *Bifidobacteria* and *Lactobacillus* populations, precipitating sIgA production. Prebiotics could also prevent pathogen attachment. Those two conditions would decrease the inflammation process in the intestinal lumen.[2,19-21] The first factor made the fecal calprotectin level of BF babies tend to increase and the second made the calprotectin level tend to decrease because of the decreasing inflammation process in the intestinal lumen.

The normal median fecal calprotectin level for infants aged 3 to 6 months is 278 mg/kg (range 85-988 mg/kg).[22] The mean level of both groups in our study were in the normal range and not significantly different. However, the FF group had a higher mean level than that of the BF group, based on the cut off point of this study subject population. The fact was revealed a higher inflammation process in FF babies compared to BF babies.

Fecal sIgA has been known to have a strong correlation with neutralizing ability and clearing of pathogenic microbes.[2,6,23] Another study on infants < 2 months of age revealed that the level of fecal sIgA in the BF group was significantly higher at weeks 2, 4, and 8 compared to that of the FF group. In another report on 34 healthy term babies, it was demonstrated that BF infants had significantly higher levels of fecal sIgA than FF infants at 1, 2, and 5 months of age. Fecal sIgA peaked at the age of 1 month, then gradually decreased until the age of 5 months in the BF group. This could be due the mean sIgA concentration in breast milk decreasing from 1 to 0.5 mg/mL between 1 month and 6 months of age. Thus, fecal sIgA levels were markedly influenced by the intake of breast milk.[24]

Another study on the immunological development of the gastrointestinal system in the first 8 months of life found that fecal sIgA was detected in all infants from birth, with comparable levels in preterm and term infants. Levels in BF infants were highest at birth, then decreased. The BF group had higher levels compared to FF group throughout the study. We thus inferred that fecal IgA levels reflect breast-milk IgA content, because along with the decrease in breast milk IgA level, the fecal IgA level decreased. Since fecal IgA levels were 3-9 times higher than levels in BF infants, either breast milk may have a stimulatory effect, or breast milk IgA may be concentrated in the colon, or both.[25]

The two groups in our study had normal fecal sIgA levels based on the literature (510-2,040 μg/mL). However, the BF group had higher median fecal sIgA level of based on the cut off point of this study subject population compared to that of the FF group. The high fecal sIgA level in the BF group may be explained by bioactive substances in human milk such as TNF-alpha, IL-1, IL-6, and IL-10 that activate both B- and T-lymphocytes, increasing sIgA production.[2,23,27]

We found no significant difference between groups that could be caused by the use of prebiotic/probiotic-supplemented formula in most of the FF group subjects. Nine subjects (22.5%) had standard formula, 26 subjects (65%) had prebiotic-supplemented formula, and 5 subjects (12.5%) had probiotic-supplemented formula. While comparing standard formula to prebiotic-supplemented formula, a previous study demonstrated a significantly higher fecal sIgA level in the prebiotic-supplemented group until 4 months of age.[21] Prebiotics or oligosaccharides in the intestinal lumen undergo fermentation that can increase the growth and activity of *Bifidobacteria* and *Lactobacillus*. The acid environment caused by fermentation acts as a signal to the gut defense mechanism to produce sIgA. Probiotics have a role in the fermentation process.[2,21]

There was no significant correlation between feeding type and increased fecal sIgA in this study. A previous study demonstrated decreased sIgA in human milk in the first 6 months along with increased fecal sIgA at 1-5 months of age.[24] Thus, the increased fecal sIgA is not only caused by human milk consumption, but also due to the maturity of GIT immunity that increases with age.

In conclusion, BF infants have better mucosal integrity compared to FF infants as shown by the significantly higher fecal AAT level in BF babies related to the AAT from breastmilk. The FF infants have a higher level of calprotectin, although they do not get calprotectin from formula as the BF infants get from breastmilk. The BF babies have a higher fecal sIgA level, although most of the FF subjects used prebiotic-supplemented formula.

Conflict of interest

None declared.

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