

Effect of Supplementation

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Zinc, Glutamine, Fiber and Prebiotic Supplementation

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Conflict of Interest

The authors have no financial conflicts of interest.

been more or less stable, with 13.5% in 2008, 13.9% in 2013, and 13.8% in 2018. Low intake and malabsorption caused by diseases, especially diarrhea, are the major causes of malnutrition [1]. Disturbance of the gut mucosal integrity is at the center of the pathogenesis of malnutrition in diarrheal disease inducing malabsorption, hormonal imbalance, bacterial overgrowth, secondary protein allergy, and disturbed villous regeneration. Gut mucosal integrity disturbance may cause inflammation [2], and nutritional factors that may modulate gut mucosal integrity include zinc, glutamine, fiber, and inulin [3].

Zinc is a component of more than 300 enzymes and is required for DNA synthesis, cell replication, and protein synthesis. Signs and symptoms of zinc deficiency are unclear, especially for mild deficiency. The prevalence of zinc deficiency in Indonesia is high (approximately 17%) [4]. According to a cohort study, the incidence of diarrhea is 47% higher in children with zinc deficiency [5]. However, studies in developing countries found that zinc supplementation might reduce the incidence of acute and persistent diarrhea [5,6]. Glutamine has a vital role in maintaining gut mucosal integrity and activating the immune system of the gastrointestinal tract [7,8].

The nutritional content of insoluble fiber may be low, but fiber has an important role that cannot be replaced by other compounds. Inulin is a prebiotic, an oligosaccharide, which is not digested but is fermented in the colon, resulting in the development of a bifidobacteria-dominated microbiota, enhancing the production of short chain fatty acids such as butyrate, which are very important nutritional substrates for the colonic mucosa [9,10].

To date, to the best of our knowledge, there have been no studies reporting the effect of zinc, glutamine, and fiber supplementation on gut mucosal wall integrity.

Fecal calprotectin (FC) and alpha-1-antitrypsin (α 1AT) are markers of mucosal integrity in toddlers [11,12]. Increased levels of FC may indicate inflammation and lead to decreased mucosal integrity. However, FC levels are age-dependent, and they decrease with age. In a Swedish population, the upper limits for normal values were 233, 615, 136, and 57 μ g/mg for infants aged 0, 6, 12 and 24 months, respectively [13]. The FC levels of infants aged 0–6 months reduced with age and were higher than the normal levels observed in healthy older infants [14]. This study aimed to measure the prevalence of intestinal mucosal integrity disturbance in presumed healthy children aged 1–3 years and assess the potential benefits of these supplements.

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MATERIALS AND METHODS

This study was conducted in Pasar Minggu district, South Jakarta, from August 2015 to June 2016.

A total of 200 subjects were recruited by consecutive sampling, and they were presumed healthy children aged 1–3 years. Nutritional status was determined based on weight/height using the World Health Organization's child growth standards. Children with a history of cows' milk allergy or children who took medication that could modify the results of the study such as anti-inflammatory drugs or pre-, pro- and synbiotics were excluded from this study.

This study consisted of two phases

The first phase was a cross-sectional analysis to evaluate the prevalence of abnormal intestinal mucosal integrity in presumed healthy children aged 1–3 years by measuring FC

and α 1AT. The cut-off point for FC was 214 mg/kg [11]. The cut-off for α 1AT was 26.8 mg/dL (Phical; Immundiagnostik, Bensheim, German).

The second phase was a double-blind randomized clinical trial. The 200 subjects were divided using block random sampling into two groups of 100 subjects each; the first group received formula supplemented with zinc, glutamine, fiber, and inulin (Test formula), whereas the second group (control) received the standard formula (Table 1: composition of formulas). All subjects were given 2×200 mL formula per day for 10 months, and they were not allowed to drink other formula.

Physical examination and anthropometric measurements were performed every month, whereas laboratory FC and α 1AT were measured every 2 months. Weekly and monthly forms were used to record fecal consistency and frequency for evaluation of constipation and diarrhea.

Per-protocol analysis was performed for all outcomes and for all eligible children who were randomly allocated and had consumed the intervention formula. Analyses were conducted according to a predefined data analysis protocol. The independent t -test and Mann-Whitney test were used for statistical analyses for the first phase of study. A p -value <0.05 was considered statistically significant. For the second phase, the Mann-Whitney U-test and

Table 1. Composition of the test formula and standard formula

Nutrient	Unit	Test formula	Standard formula
		Per 100 mL	Per 100 mL
Energy	kcal	74	74
Protein	g	2.6	2.6
Fat	g	2.8	2.8
Multi fiber*	mg	950.5	0.0
Inulin*		480	
Cellulose*		203	
Arabic GUM*		122	
Resistant starch*		115	
FOS*		30.5	
Mineral			
Calcium (Ca)	mg	91.0	91.0
Phosphor (P)	mg	61.8	61.8
Magnesium (Mg)	mg	11.1	11.1
Sodium (Na)	mg	55.3	55.3
Potassium (K)	mg	161.7	161.7
Chloride (Cl)	mg	92.6	92.6
Iron (Fe)	mg	1.4	1.4
Zinc (Zn)*	mg	1.5	0.2
Iodine (I)	mcg	24.4	24.4
Manganese (Mn)	mcg	108.1	108.1
Selenium (Se)	mcg	3.2	3.2
Amino acids			
Isoleucine	g	0.16	0.16
Leucine	g	0.26	0.26
Lysine	g	0.23	0.23
Methionine	g	0.07	0.07
Phenylalanine	g	0.11	0.11
Treonine	g	0.16	0.16
Tryptophane	g	0.05	0.05
Valine	g	0.18	0.18
L-glutamine*	G	0.175	0.0
Total amino acids	G	1.39	1.22

Test formula: SGM Digestive, Sari Husada, FOS: fructo-oligosaccharide.

*The difference between both formulas.

Wilcoxon test³² applied for data that were not distributed normally. SPSS PASW Statistic for Windows, Version 17.0.3 (SPSS Inc., Chicago, IL, USA) was used for all analyses.

This study was approved²¹ the ethical review from the Permanent Committee on Medical/Health Research Ethics, Faculty of Medicine, University of Indonesia (number 117/UN2.F1/ETIK/2015) and informed consent was obtained.

RESULTS

The characteristics of the 200 included children are listed in Table 2; none of the characteristics showed a significant difference suggesting adequate randomization. After 10 months, 144 subjects completed the study, with 72 in each group.

FC ranged at baseline in the 200 children from 10.62 to 805.83 mg/kg feces with a median of 199.02 mg/kg; 91 (45.5%) had an FC above the cut-off limit of 214 mg/kg. Fecal α 1AT ranged from 3.44 to 237.14 mg/dL, with a median of 30.22 mg/dL; in 116 (58.0%) subjects, fecal α 1AT was above the cut-off of 26.27 mg/dL. After 10 months, 72 out of the 100 subjects from each group completed the study. There were no significant differences between the test and placebo groups in relation to FC and α 1AT during the 10 months of study as shown in Table 3.

Among 144 subjects who completed the study, there were 66 (45.8%) with FC levels above the cut-off value at baseline. The data were³⁴ a posteriori re-analyzed excluding those subjects with FC baseline >214 mg/kg. Data from³³ subjects in the test formula group and 37 subjects in the control group were re-analyzed. A statistically significant difference in the decrease in calprotectin levels between the test and control group at baseline in 6 months was observed, which disappeared at 10 months. Data were also a posteriori re-analyzed for children with FC levels that are above the cut-off limit at baseline. In this subgroup, there was no statistical significant difference at any measuring time point. However, there was a trend for a larger decrease in the control than in the test group (Table 4).

Table 2. Subject's characteristics

Variable	Test formula (n=100)	Standard formula (n=100)	p-value
Sex			0.777**
Male	48 (51.1)	46 (48.9)	
Female	52 (49.1)	54 (50.9)	
Age (mo)	23.6 (6.18)	24.2 (6.40)	0.748***
Birth weight (g)	3,141.2 (477.71)	3,112.8 (467.72)	0.974***
Birth length (cm)	48.9 (2.03)	48.7 (2.15)	0.546***
Lactation history			0.098**
Exclusive breastmilk	68 (68.0)	67 (67.0)	
Breastmilk and formula	27 (27.0)	27 (27.0)	
Never got breastmilk	5 (5.0)	6 (6.0)	
Weight (kg)	11.1 (2.0)	10.9 (1.9)	0.330*
Height (cm)	83.7 (6.5)	83.6 (6.0)	0.470*
Head circumference (cm)	46.5 (1.4)	46.5 (1.7)	0.051*
Nutrition status			0.129**
Undernutrition (wasted)	6 (6.0)	1 (1.0)	
Normal	92 (92.0)	98 (98.0)	
Overweight	2 (2.0)	1 (1.0)	

Values are presented as number (%) or mean (SD).

*Independent t-test, ** χ^2 test, ***Mann-Whitney test.

Table 3. Effect of supplementation of zinc, glutamine, fiber, and prebiotics on fecal calprotectin, α 1AT

Marker	Test formula (n=72)	Standard formula (n=72)	p-value
Calprotectin			
V ₀ (mg/kg)	197.9 (38.1-798.5)	248.8 (31.0-805.8)	p=0.897 [†]
V ₆ (mg/kg)	99.2 (20.1-479.4)	125.6 (15.9-358.0)	4 0.102 [†]
V ₁₀ (mg/kg)	148.3 (26.9-412.8)	173.2 (31.4-808.5)	p=0.169 [†]
p (0-6)*	p<0.001	p<0.001	p (0-6) [‡] =0.29
p (0-10) [†]	p=0.002	p=0.12	p (0-10) [§] =0.33
α1AT mg/dL			
V ₀ (mg/dL)	28.3 (6.48-190.13)	32.6 (3.4-237.1)	p=0.441 [†]
V ₆ (mg/dL)	13.3 (1.13-114.14)	14.4 (1.0-59.5)	4 0.790 [†]
V ₁₀ (mg/dL)	21.1 (2.46-63.32)	25.0 (3.1-51.6)	p=0.085 [†]
p (0-6)*	p<0.001	p<0.001	p (0-6)=0.39 [†]
p (0-10) [†]	p<0.001	p<0.001	p (0-10)=0.97 [†]

Values are presented as median (range).

α 1AT: alfa-1-antitrypsin, V₀: month-0, V₆: month-6, V₁₀: month-10.

Results in median with confidence of 95%; p-value shows interaction among groups and the difference of laboratory results between month-0 and month-10.

*Significance between month-0 and month-6 in each group. [†]Significance between month-0 and month-10 in each group. [‡]Significance between the changes of month-0 and month-6 between trial group and placebo group.

[§]Significance between the changes of month-0 and month-10 between trial group and placebo group. [¶]Wilcoxon test, ^{††}Mann-Whitney test.

Table 4A. Analysis in subgroups with fecal calprotectin cut-off of 214 mg/kg

Calprotectin <214 mg/kg	Test formula (n=41)	Standard formula (n=37)	p-value*
Diff calprotectin 6-0 (mg/kg)			
Median	-34.9200	27.0335	0.023
Diff calprotectin 10-0 (mg/kg)			
Median	16.2800	41.7350	0.127 (NS)

Diff calprotectin 6-0: Difference calprotectin month-6 and baseline, Diff calprotectin 10-0: Difference calprotectin month 10 and baseline; NS: not significant.

*Mann-Whitney test.

Table 4B. Analysis in subgroups with fecal calprotectin cut-off of 214 mg/kg

Calprotectin >214 mg/kg	Trial formula (n=31)	Standard formula (n=35)	p-value*
Diff calprotectin 6-0 (mg/kg)			
Median	-225.47	-175.14	0.500 (NS)
Diff calprotectin 10-0 (mg/kg)			
Median	-197.89	-132.33	0.386 (NS)

Diff calprotectin 6-0: Difference calprotectin month-6 and baseline, Diff calprotectin 10-0: Difference calprotectin month 10 and baseline; NS: not significant.

*Mann-Whitney test.

At baseline, 16.7% of the children in the test group and 4.2% in the control were constipated. According to the collected information, no child was constipated after 6 and 10 months. Regarding diarrhea, an incidence of 6.9% in the test and 13.9% in the control group at baseline was reported. After 29 months, this was 4.2% in both groups, and at 10 months, the incidence of diarrhea was 0 in the test group and 2.8% in the control group. None of the differences were statistically significant (Table 5).

There was no significant difference of z-score mean of anthropometry indicators between trial formula group and placebo group both after 6 months and 10 months (Table 6). As for increment of weight, there is a similar number of median weights raise count for both group, which is 1.65 kg for 10 months.

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Table 5. Effect of supplementation of zinc, glutamine, fiber and prebiotics to intestinal morbidity (diarrhea and constipation)

Morbidity	Test formula (n=72)	Standard formula (n=72)	χ^2 test
Constipation			
V_0			
Yes	12 (16.7)	3 (4.2)	
No	60 (83.3)	69 (95.8)	
V_6			
Yes	0 (0.0)	0 (0.0)	
No	72 (100.0)	72 (100.0)	
V_{10}			
Yes	0 (0.0)	0 (0.0)	
No	72 (100.0)	72 (100.0)	
p (0-6)*			$p=0.029$
Cured	12 (16.7)	3 (4.2)	
Stable	60 (83.3)	69 (95.8)	
Not cured	0 (0.0)	0 (0.0)	
Worsened	0 (0.0)	0 (0.0)	
p (0-10)†			$p=0.029$
Cured	12 (16.7)	3 (4.2)	
Stable	60 (83.3)	69 (95.8)	
Not cured	0 (0.0)	0 (0.0)	
Worsened	0 (0.0)	0 (0.0)	
Diarrhea			
V_0			
Yes	5 (6.9)	10 (13.9)	
No	67 (93.1)	62 (86.1)	
V_6			
Yes	3 (4.2)	3 (4.2)	
No	69 (95.8)	69 (95.8)	
V_{10}			
Yes	0 (0.0)	2 (2.8)	
No	72 (100.0)	70 (97.2)	
p (0-6)			$p=0.48$ (NS)
Cured	5 (6.9)	9 (12.5)	
Stable	64 (88.9)	60 (83.3)	
Not cured	0 (0.0)	1 (1.4)	
Worsened	3 (4.2)	2 (2.8)	
p (0-10)			$p=0.13$ (NS)
Cured	5 (6.9)	10 (13.9)	
Stable	67 (93.1)	60 (83.3)	
Not cured	0 (0.0)	0 (0.0)	
Worsened	0 (0.0)	2 (2.8)	

Values are presented as number (%).

 V_0 : month-0, V_6 : month-6, V_{10} : month-10, NS: not significant.Results in mean, p -value shows difference morbidity of constipation and diarrhea between groups.

*Significance between the changes of month-0 and month-6 between trial group and placebo group.

†Significance between the changes of month-0 and month-10 between trial group and placebo group. Independent t -test.

DISCUSSION

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FC is a protein used as a diagnostic marker for inflammatory bowel diseases and is thus a fecal marker in gastrointestinal inflammation [13]. Calprotectin is a calcium- and zinc-binding protein of the S100 family expressed mainly by neutrophils with important extracellular activity. Increasing evidence suggests the implication of FC in the diagnosis, follow-up, assessment of relapses, and response to treatment in pediatric pathological conditions, such as inflammatory bowel disease, necrotizing enterocolitis, celiac disease, and many other inflammatory gastrointestinal conditions. In a Chinese cohort, the following median levels of FC in healthy

Table 6. Anthropometric status of subjects in each group

Mean z-score	Parameter	Test formula (n=72)	Standard formula (n=72)	p-value*
Baseline	W	-0.48	-0.70	0.319
	H	-0.57	-0.71	0.442
	W/H	-0.29	-0.49	0.304
Month-6	W	-0.28	-0.33	0.660
	H	0.11	-0.146	0.962
	W/H	-0.34	-0.39	0.745
Month-10	W	-0.52	-0.56	0.69
	H	-0.66	-0.55	0.54
	W/H	-0.41	-0.46	0.57

W: weight, H: height.

Results in mean z-score.

*p-value shows difference mean z-score between groups, on baseline, month-6 and month-10. Independent t-test.

children were reported, according to [13]: 12–24 months, 96.14 µg/g; 24–36 months, 81.48 µg/g; and 36–48 months, 65.36 µg/g [12]. This non-invasive test is useful for screening children with gastrointestinal symptoms to avoid unnecessary invasive procedures [15]. Our study shows that the prevalence of intestinal mucosal integrity disturbance in this cohort of presumed healthy children aged 1–3 years was 45.5% according to FC levels. The baseline median FC in this study was relatively higher (199 mg/kg feces) than that reported in two Chinese studies with a median of 83.19 and 104 mg/kg in children aged 1–4 years and 12–18 months, respectively [12,16]. Given that all the subjects in our study were presumed healthy children, the high level of FC at baseline raises the possibility that there may be intestinal inflammation in apparently healthy Indonesian children. After 6 months intervention with the test formula, FC decreased significantly, but only in those children with a FC below the cut-off. Furthermore, no change was observed in the group with high FC. If a true beneficial effect would be induced by the addition of the different supplements, a decrease would have been expected in those with elevated FC levels. The natural evolution of FC is a decrease over time in infants and toddlers. The younger the infant, the higher and the wider the range of FC [11,12,17,18].

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Prebiotics are known to stimulate the development of bifidobacteria in the gastrointestinal microbiome, which may strengthen the mucosal resistance toward gastrointestinal infection [19,20]. Prebiotics aside, zinc supplementation is known to increase the immunocompetence that affects the clearance of diarrhea-causing pathogens [21]. Guerrant et al. [22] showed that glutamine is a key component of cell replication and that glutamine improves and stimulates gut crypt cell proliferation. Accordingly, the supplementation of formula with prebiotics, zinc, and glutamine might reasonably be considered significant in reducing the intestinal inflammation in subjects of intervention group. An important limitation of this study is that overall daily nutrition was not registered.

We could not confirm the results from a meta-analysis by Mayo-Wilson et al. [23] showing that zinc supplementation to healthy children may reduce the incidence of diarrhea by 13% (95% confidence interval 0.85–0.89). This meta-analysis also measured zinc supplementation dosage; low dose zinc supplementation (0–5 mg/day) [24] reduced the incidence of diarrhea by 5% [23]. In our trial, zinc supplementation was given at 6 mg/day in the intervention group and 0.8 mg/day in the control group.

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In conclusion, there appeared to be a high prevalence of mucosal integrity disturbance in apparently healthy Indonesian children aged 1–3 years. Supplementation of infant formula with zinc, glutamine, fiber, and prebiotics failed to show a clinical benefit.

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