ANTIBACTERIAL ACTIVITY OF SYNBIOTIC FERMENTED MILK (*Lactobacillus plantarum* DAD 13 - FRUCTOOLIGOSACCHARIDES) WITH IRON AND ZINC FORTIFICATION

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ABSTRACT
Indonesia suffers from high prevalence of stunting. Considering the benefits of iron (Fe) and zinc (Zn) for growth and development of the children, there is a chance to formulate fermented milk fortified with both micronutrients. The milk is expected to have good effects for health which indicate by the presence of antibacterial activity. Therefore, the researchers aimed to investigate the effect of $FeSO_4$ and NaFeEDTA fortification towards the antibacterial properties of the fermented milk.
Experimental design with in vitro method is used. The antibacterial activity will be tested against Escherichia coli and compared to ciprofloxacin. Fermented milk storage is done for 0 days, 7 days, 14 days, 21 days and 28 days. Data are grouped by test groups and analyzed using One Way ANOVA test using STATA 12.
No effect of fortification on the antibacterial properties are shown ($p > 0.05$) between the diameter of the clear zone on ciproloxacin (8 mm) compared to synbiotic fermented milk without fortification (10 mm), fermented milk with NaFeEDTA-Zinc fortification (9.33 ± 0.57 mm) and fermented milk with $FeSO_4$-Zinc fortification (10.33 ± 0.57 mm). There is no significant difference in the diameter of clear zone produced by NaFeEDTA milk and milk $FeSO_4$-Zinc ($p > 0.05$.
Fortification of either NaFeEDTA or $FeSO_4$ did not affect the antibacterial properties of the fermented milk in all storage categories. There was also no difference of the antibacterial activity between the fermented milk fortified with NaFeEDTA and $FeSO_4$.

Keywords: Synbiotic, Antibacterial activity, *Lactobacillus plantarum* Dad 13, Fructooligosaccharides, Iron Fortification, Zinc Fortification

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INTRODUCTION

Indonesian Basic Health Research 2013 showed that the national prevalence of stunted children (combination of short and very short) reached 37.2% (Ministry of Health of Republic Indonesia, 2013). Stunting is an important issue to be solved due to its negative effect on development of the children, physically and mentally. Moreover, stunting will also have an impact on cognitive development, school performance, productivity, and birth outcomes in adulthood (Dewey and Begum, 2011).
There are several factors associated with stunting include health status, poor maternal nutritional status, inadequate food intake and infectious diseases. Stunting is also determined by the health and nutritional status of the mother before, during and after pregnancy (WHO, 2012). Stunting in infants and young children are influenced by feeding and supplementary food given. World Health Organization reports that one of the most effective interventions to address stunting during the phase of complementary feeding is by improving the quality of the food (WHO, 2012). Foods provide essential nutrients which support growth of the children thus prevent and cope stunting in under-fives.
Iron (Fe) and zinc (Zn) considered as essential micronutrients for growth, development and maintenance of the immune system, especially for children. Fe plays roles in supporting psychomotor development, physical activity and defense against infection while Zn mainly

needed for growth and immune system. Therefore, Zn deficiency can cause alteration in the children growth, lower immune system, anorexia, and diarrhea (de Biasse-Fortin, 2003; Shao, et al., 2014; Cousins and Hempe, 1994). These two micronutrients can be obtained naturally from the food, or by fortification. Garrett et al. (2015) mentioned that food fortification provides a strong pillar in food security system which is help to solve the problem of micronutrient deficiencies.
Beside iron and zinc, probiotic is also has a good effect on child growth aged 2-5 years old (Saran et al., 2002). Moreover, the incidence of diarrhea and fever in children is also lower when compared with children who did not receive probiotics. In addition, probiotics promote the damaged intestine regeneration due to infection. The improvement of intestine health helps to prevent growth disorder.
Milk is the best choice as food vehicle for Fe, Zn, probiotics and prebiotics, in the form of fermented milk. Nutrients inside fermented milk are easier to digest and able to minimize the incidence of lactose intolerance. The addition of prebiotics increasingly provide many benefits since it can improve the vitality and activity of microflora in the gut (Gibson et al., 2004) and increase the absorption of minerals (Rastall, 2010).
Based on the above background, the researchers want to develop a formula that can help to overcome stunting in Indonesia. The addition of pre- and probiotics in the product are expected to maintain the balance of gut microflora. It is really important since the consumption of iron in high amount considered to increasing the number of pathogenic bacteria such as enterobacteria and lowering the number of lactobacilli. Gut microflora imbalance can lead to acute or persistent diarrhea (Zimmermann et al., 2010; Lee et al., 2008). On the other hand, zinc fortification helps to alleviate the severity of diarrhea and promote the child growth (Lazzerini, 2008; Wessells et al., 2012; Prasad, 2013). The quality and benefit of the milk can be expected by the presence of antibacterial activity against pathogenic bacteria (Helmyati et al., 2018).

The purpose of this study was to investigate the effect of fortification towards antibacterial properties of fermented milk fortified with iron and zinc. The study also aimed to compare the effect difference of the antibacterial activity between the fermented milk which has been fortified using either NaFeEDTAor$FeSO_4$and fermented milk without fortification.

MATERIAL AND METHODS

Study Design

The study was conducted using experimental with *in vitro* research design. There were 2 types of fortifican used in the study, zinc ($ZnSO_4$) and iron (NaFeEDTA and $FeSO_4$). The ratio of zinc and iron fortification in the milk is used 1:2 which is means 50 ppm of $ZnSO_4$ and 100 ppm of either NaFeEDTA or $FeSO_4$ will be added to the fermented milk. The milk will be fermented using synbiotic *Lactobacillus plantarum* Dad 13 and fructooligosaccharides then stored for 0 days, 7 days, 14 days, 21 days and 28 days. Antibacterial activity will be measured against *Escherichiacoli*. Synbiotic fermented milk without iron fortification is considered as the control. Each treatment will be repeated three times. If the results indicate the presence of antibacterial properties, the antibacterial strength will be compared with the antibiotic ciprofloxacin.

Preparation of Fermented Milk with Iron and Zinc Fortification

The tools used for the manufacture of fermented milk and antibacterial activity measurement are incubators, laminar air flow, Petri dishes, test tubes, driglasky, Erlenmeyer 100 ml, ose, aluminum foil, centrifuges, Bunsen lamp, water bath, cool room, vortex, and analytical balance, The ingredients used in the testing of antibacterial activity is the culture of Lactobacillus obtained from the Food and Nutrition Culture Collection Universitas Gadjah Mada, MRS, distilled water, skim milk powder, iron fortifican (NaFeEDTA and $FeSO_4$), fructooligosaccharides (FOS), sugar, distilled water, nutrient broth, nutrient agar, the antibiotic ciprofloxacin, *E. coli*.

The making process of fermented milk consists of (1) blending the ingredients such as skim milk, sugar, FOS, fortifican, and water, (2) heating, (3) pouring into sterile bottle, (4) pasteurizing, (5) adding the inoculum, and (6) incubating. There were 4 groups of samples we use: 1) fermented milk fortified using NaFeEDTA and $ZnSO_4$; 2) fermented milk fortified using $FeSO_4$ and $ZnSO_4$; 3) ciprofloxacin antibiotic; 4) fermented milk without fortification (control).

Antibacterial activity measurements
Antibacterial activity test was conducted by pitting. A volume of samples is added into the hole. After about 18- 24 hours of incubation, there will be formed a clear zone around the hole. The diameter of the clear zone indicates the degrees of inhibition. It was due to antibacterial compounds in the fermented milk and ciprofloxacin which is diffused into the surrounding media thus inhibit microbial growth (Davidson and Parish, 1989). The diameter was measured and expressed in millimeters.

Statistical Analysis
The clear zone data as the result of antibacterial activity will be expressed in mean. Data are grouped by test groups and analyzed using STATA 12 with One Way ANOVA Test. If there are significant differences, the test will be followed by Bonferroni Post Hoc Test.

RESULTS AND DISCUSSION

Table 1 shows a comparison between the antibacterial strength of the three types of milk with ciprofloxacin on each shelf life. On day-0 storage, fermented milk fortified with zinc NaFeEDTA and FeSO4 does not differ significantly from the strength of the resulting antibacterial ciprofloxacin. As with the fermented milk without fortification, at the time of storage days 0, the strength of the resulting lower antibacterial/differ significantly compared with ciprofloxacin. Storage of milk on the 7th day, just fermented milk fortified FeSO4-Zn which differs significantly from the antibacterial strength generated by ciprofloxacin. Fortified milk fermented with FeSO4-Zn decreased strength of antibacterial activity at the store 7th.
Storage of milk on day 14 and 21, all three have the power fermented milk antibacterial activity similar to those produced by ciprofloxacin. On the 28th day of storage, three dairy strength greater antibacterial activity compared with those produced by ciprofloxacin significantly.
Based on table 2, fermented milk without fortification trend has increased the storage ranging from day 0 until the 28th, but decreased in the storage period to 21 days. Fermented milk fortified with zinc and $FeSO_4$ NaFeEDTA also increased at day 14 and day 28. However, the antibacterial activity declined in the storage day 7 and day 21.

Table 1. Comparisons between groups milk antibacterial activity on each shelf life

Groups	Diameter of clear zone on the antibacterial activity of each of the shelf life **(mm)**				
	D-0	**D-7**	**D-14**	**D-21**	**D-28**
Control	6,67 ± 0,57 [ac]	7,33 ± 1,53	9 ± 0	8,33 ± 0,57	10 ± 0[a]
NaFeEDTA-Zinc Acetat	7 ± 0 [b]	5,67 ± 1,15	9 ± 0	7,33 ± 0 ,57	9,33 ± 0,57[a]
FeSO4- Zinc Acetat	8,67 ± 0,57 [bc]	5,33 ± 0,57 [a]	9 ± 1	8,33 ± 1,15	10,33 ± 0,57[a]
Ciprofloxacin	8 ± 0 [a]	8 ± 0 [a]	8 ± 0	8 ± 0	8 ± 0[a]
p value	0,0012*	0,0310*	0,0951	0,3300	0,0005*

Control : Fermented milk without fortification
NaFeEDTA-Zinc Acetat : Fermented milk with NaFeEDTA-Zinc Acetat fortification
FeSO4- Zinc Acetat : Fermented milk with $FeSO_4$-Zinc Acetat fortification
Ciprofloxacin : Quinolone class of antibiotics
Data were presented as mean± Standard Deviation, D = day
*Significant, p value <0.05
Test used One Way ANOVA test

Table 2. Comparison of antibacterial activity between the shelf life of each group of fermented milk

∆ Diameter of clear zone (mm)	Control	NaFeEDTA-Zn Acetat	$FeSO_4$-Zn Acetat
The shelf life between day 0 to day 7	0,67 ± 1,52 (0,5286)	-1,33 ± 1,15 (0,1835)	-3,33 ± 0,57 (0,0099)*
The shelf life between day 7 to day 14	1,67 ± 1,52 (0,1994)	3,33 ± 1,15 (0,0377)*	3,67 ± 1,15 (0,0315)*
The shelf life between day 14 to day 21	-0,67 ± 0,57 (0,1835)	-1,67 ± 0,57 (0,0377)*	-0,67 ± 0,57 (0,1835)
The shelf life between day 21 to day 28	1,67 ± 0,57 (0,0377)*	2 ± 1 (0,0742)	2 ± 1 (0,0742)

Control : Fermented milk without fortification
NaFeEDTA-Zinc Acetat : Fermented milk with NaFeEDTA-Zinc Acetat fortification
FeSO4- Zinc Acetat : Fermented milk with $FeSO_4$-Zinc Acetat fortification
Data presented as mean± Standard Deviation (*p* value), D = day
*Significant, *p* value<0.05
Test used One Way ANOVA test

Fermented milk with iron fortification NaFeEDTA did not differ significantly with fermented milk with iron fortification $FeSO_4$ at various times during storage. Both also tend to decrease or increase the incidence of the same on some shelf life. This can be because the content *L. plantarum* in fermented milk is successful in inhibiting the activity of *E. coli* bacteria. This is also supported by the results of research that says that the addition of fortification does not affect the growth of the yoghurt starter culture. In the absence of interference *L.plantarum* culture growth in the presence of fortification, the antibacterial strength of the lactobacillus bacteria remains optimal (Simova et al., 2008).

Antibacterial strength on the third optimal fermented milk on storage day 14 and day 28. In both these storage period there is an increased force on the third antibacterial milk fermentation and the result is greater than the force generated by the antibacterial ciprofloxacin. Antibacterial strength has several criteria. The clear zone considered as "very strong" when the diameter is 20 mm or higher. 10-20 mm of the diameter is called strong while if the diameter is 5-10 mm considered as medium. If the clear zone that produced less than 5 mm, it is called weak. Fermented milk in this study, both the control and fortified milk have the power antibacterial moderate. The resulting antibacterial strength is not inferior to the power generated by the antibacterial ciprofloxacin.

Based on research conducted by Selvamohan (2010), *Lactobacillus plantarum* produce bacteriocins that can serve sebakan antimicrobial and has a large power resistor against *E.coli*. *Escherichia coli* is a gram-negative bacteria that are sensitive to low levels of acidity (Ray, 2003). Acidity in fermented milk synbiotic (*L. plantarum* and fructoolygosacharida) derived from lactic acid or an organic acid contained in milk (Branen and Davidson, 2003). Synbiotic lactic acid in milk can damage the outer membrane of gram-negative bacteria. The mechanism of damaging the membrane of gram negative bacteria in the presence of antimicrobial substrates such as diacetyl, bacteriocins, hydrogen peroxide and lacto-peroxide that goes into the cytoplasmic membrane. With the existence of this mechanism will weaken the permeability of gram-negative bacteria (Alokomi et al, 2000).

CONCLUSION

Either NaFeEDTA of $FeSO_4$ does not affect the antibacterial properties of fermented milk with iron and zinc fortification. There is also no difference between antibacterial activity of fermented milk fortified with zinc-$FeSO_4$ and fortified with zinc-NaFeEDTA. Fermented milk with iron and zinc fortification has good strength antibacterial activity.

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