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PENGARUH SUSU SARI TEMPE FERMENTASI SINBIOTIK YANG DIFORTIFIKASI ZAT BESI TERHADAP ASAM PROPIONAT, ASAM BUTIRAT, DAN MIKROBIOMA NON PATOGEN PADA TIKUS WISTAR ANEMIA

Iron-Fortified Synbiotic Fermented Milk with Tempeh Extract to Enhances Propionic Acid, Butyric Acid, and Non-Pathogen Microbiome in Anemic Wistar Rat

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ABSTRACT

Background. *Anemia, as a global public health problem, needs to be solved. The previous study by the researcher found the potency of iron-fortified synbiotic fermented milk with tempeh extract as an alternative to overcome anemia.* ***Objective.*** *This study aims to determine the effects of iron-fortified synbiotic fermented milk with tempeh extract towards short-chain fecal fatty acids (SCFAs) and fecal microbiota.* ***Method.*** *The study was a randomized controlled trial with three groups, consisting of 8 anemic Wistar rats. The groups made into anemia within 17 days.The groups treated as follows: group NA: fermented milk with tempeh extract fortified by NaFeEDTA, group FE: fermented milk with tempeh extract fortified by* $FeSO_4$*, and group KO: fermented milk with tempeh extract without fortification. SCFAs and microbiota of the rat's feces determined using the Total Plate Count method. SCFAs were measured after the intervention, while fecal microbiota was measured before and after the intervention. One-way ANOVA was used to measure the difference between NA, FE, and KO groups with post hoc test Bonferroni.* ***Results.*** *There was a significant mean difference between propionic and butyric acid between NA and FE groups and the KO group. The highest Lactobacilli number was in the FE group, while Bifidobacteria and Enterobacteriaceae were highest in the KO group. Meanwhile, the NA group had the highest Escherichia coli number.* ***Conclusion.*** *Iron fortification has positive effects on increasing the production of SCFAs in the gut. Prebiotics and probiotics have positive effects on pathogenic bacteria. Further study is needed to determine the effects of iron-fortified synbiotic fermented milk with tempeh extract in human.*

Keywords: *iron, microbiota, SCFA, synbiotic, tempeh*

ABSTRAK

Latar belakang. Anemia sebagai masalah kesehatan masyarakat global perlu untuk diselesaikan. Penelitian sebelumnya menunjukkan potensi susu fermentasi sinbiotik dengan sari tempe yang difortifikasi oleh zat besi untuk mengatasi anemia. **Tujuan.** Penelitian ini bertujuan mengetahui efek susu sari tempe fermentasi sinbiotik yang difortifikasi oleh zat besi terhadap asam lemak rantai pendek (SCFA) dan mikroflora feses. **Metode.** Desain penelitian menggunakan *randomized* *controlled trial* yang terdiri dari tiga kelompok masing-masing sebanyak 8 tikus jenis Wistar. Semua sampel tikus tersebut dibuat menjadi anemia selama 17 hari. Ketiga kelompok diberikan intervensi sebagai berikut: 1) NA: susu fermentasi sinbiotik dengan sari tempe yang difortifikasi oleh NaFeEDTA, 2) FE: susu fermentasi sinbiotik dengan sari tempe yang difortifikasi oleh $FeSO_4$, dan 3) KO: susu fermentasi sinbiotik dengan sari tempe tanpa fortifikasi. Setelah intervensi, SCFA dan mikrobiota dalam feses sekum dari tikus diukur menggunakan metode *Total Plate Count*. Analisis data menggunakan uji statistik one way ANOVA yang dilakukan untuk mengukur perbedaan antara kelompok NA, FE, dan KO dengan *Post Hoc Test Benferroni*. **Hasil.** Terdapat perbedaan yang signifikan rerata kadar asam propionat dan butirat antara kelompok NA dan FE dengan kelompok KO. Jumlah *Lactobacilli* tertinggi terdapat pada kelompok FE, jumlah *Bifidobacteria* dan *Enterobacteriaceae* tertinggi terdapat pada kelompok KO. Adapun kelompok NA memiliki jumlah *Escherichia coli* tertinggi. **Kesimpulan.** Fortifikasi zat besi memilki efek positif untuk meningkatkan produksi asam lemak rantai pendek dalam saluran cerna. Prebiotik dan probiotik memiliki pengaruh positif dalam menghambat pertumbuhan bakteri patogen. Penelitian lanjutan dibutuhkan untuk menentukan dampak dari susu sari tempe fermentasi sinbiotik yang difortifikasi zat besi pada manusia.

Kata kunci: zat besi, mikrobiota, SCFA, sinbiotik, tempe

INTRODUCTION

Anemia is a global health problem either in a high income or middle-income country. Anemia prevalence in Indonesia is still high, 21.7 percent.[1] Anemia considered having inter-generational impacts. It means that the effect of anemia can be passed on between generations. In some periods, it can lower the brain function and immune system, lower the productivity, and indirectly correlate with the level of public welfare.[2,3] It is essential to cut the chain of anemia, mainly through some innovation about food and nutrition.

Fortification is a cost-effective way to improve micronutrient deficiencies.[4] Several studies suggested that iron supplementation and fortification on humans may increase hemoglobin concentration.[5] NaFeEDTA is one type of iron fortificants which recommended to be added on high phytate meal to increase iron absorption in the gut.[6] Compared with $FeSO_4$, the absorption level of NaFeEDTA is 2-4 times higher.

Indonesian Ministry of Agriculture mentioned that the availability of soybeans were increasing from 7 million kg/capita/year in 2007 to 10 million kg/capita/year in 2011.[7] However, there are still many people who assume tempeh is a low social food.[8] Tempeh contains micronutrients which needed for hemoglobin syntheses such as vitamin B12, folic acid, protein, iron, Cu, and Zn. Every 100 g tempeh contains 20.8 g protein; 4 mg iron; 1.4 g fiber; and 155 mg calcium.[7,9] Tempeh is also known as a functional food to reduce the period of diarrhea.[10]

Iron supplementation and fortification do have adverse effects. It enhances pathogenic gut microbiota and lowers lactobacilli number.[11] In the study of pigs, iron supplementation increases the coliform number in the gut.[12] Iron supplementation also modulates free radicals, thus interfere with the body's immune system.[13] The change of gut microbiota composition is due to non-absorbed iron in the lower gastrointestinal tract. Considering the adverse effects of iron supplementation, a study by Yilmaz and Li[14] recommends investigation of the effects of iron supplementation on gut microflora.

Several types of research have been done to analyze the importance of gut microbiota balance. Sender *et al*,[15] mentioned there were approximately 10^{11}-10^{14} bacteria in the large intestine. These bacteria used undigested starch to produce short-chain fatty acids, which increase the energy modulation for the hosts. Short-chain fatty acids are essential metabolites that are estimated to correlate with a different condition.[16] Alteration of gut microbiota (or called dysbiosis) leads to different diseases such as obesity, asthma, metabolic syndrome, and diabetes.[17]

Prebiotic, probiotic, and its combination (or called synbiotic) can maintain lactic acid bacteria numbers such as Lactobacilli and Bifidobacteria in the gut.[12,18,19] Synbiotic can suppress the growth of pathogenic bacteria like *Salmonella enterica* serovar typhimurium[20] while prebiotic like fructooligosaccharides (FOS) increases iron absorption[21] and stimulates probiotic growth.[22] In this study, the researcher developed functional food using milk as a food vehicle for iron fortification. Milk would be fermented using probiotic and be added by prebiotic to increase its nutritional value.

Milk is an excellent vehicle for food fortification. The negativity of excessive iron intake will be lowered by the presence of synbiotic and tempeh extract in milk. In this study, the researcher developed synbiotic fermented milk with tempeh extract and iron fortification. Milk would be fermented by *Lactobacillus plantarum* and enriched by fructooligosaccharide (FOS). The experimental study aimed to determine the effect of the synbiotic milk towards gut microbiota and short-chain fatty acid (SCFA).

METHODS

Study Design

The study is randomized controlled trial with 3 groups consisting of 8 Wistar rats. The groups made into anemia within 17 days. The rats were divided into: (1) NA: the group with fermented milk with tempeh extract fortified by NaFeEDTA; (2) FE: the group with fermented milk with tempeh extract fortified by $FeSO_4$; (3) KO, as the control group: provided fermented milk with tempeh extract without fortification.

All of groups were given AIN-93 (*ad libitum*) with the composition following Reeves *et al*.[23] All examinations were in Microbiology Laboratory, Pusat Antar Universitas, Universitas Gadjah Mada, and Nutrition Laboratory, Faculty of Medicine, Universitas Gadjah Mada. The study secured ethical clearence from the Ethics Committee of Faculty of Medicine, Universitas Gadjah Mada, with the reference number KE/FK/655/EC/2015 on June 10th, 2015.

Production of Synbiotic Fermented Milk with Tempeh Extract and Iron Fortification

Tempeh was from a local market in Special Region Yogyakarta Province, Indonesia. To prepare the iron-fortified synbiotic fermented milk, the following ingredients used: skim milk, tempeh extract - which was obtained by blending tempeh and water in 1:1 ratio then squeezed to reduce water content, prebiotic fructooligosaccharides (FOS) (Beneo Orafti, Indonesia), sugar (Gulaku), probiotic *Lactobacillus plantarum*, and iron fortificant (Merck). The process of milk production was according to Helmyati *et al.*[24] It was categorized into three groups according to the type of fortificant added, 1) fermented milk fortified with NaFeEDTA (NA group), 2) fermented milk fortified with $FeSO_4$ (FE group), and 3) fermented milk without fortification (KO group). This product is in the process of patent registration with the number of P00201802758. Information about the nutritional value of the iron-fortified synbiotic fermented milk was in Table 1.

Table 1. Nutritional Value of Iron-Fortified Fermented Milk with Tempeh Extract

Nutrients	Value
Protein (%)	0.68
Fat (%)	0.83
Crude fiber (%)	0.17
Carbohydrate (%)	14.05
Energy (Kcal/100g)	64.57
Zinc (ppm)	3.18
Iron (ppm)	38.60
Folic acid (%)	3.21
Lactobacillus plantarum (CFU)	4.3×10^9

Hemoglobin and Body Weight Measurement

The photometric method (Diasys, Germany) used to measure the hemoglobin level of the rats. Hemoglobin was analyzed before and after 17 days of intervention using blood taken from sinus orbitalis.[25] The body weight of the rats examined using digital analytic scales and measured every three days.

Fecal Short-Chain Fatty Acids and Microflora Analysis

Short-chain fatty acids (SCFA) counted from the rats' feces. The measurement of SCFA post-intervention using High-Performance Liquid Chromatography (HPLC) method.[26,27] The number of gut microbiota were analyzed twice, pre- and post-intervention. There were four types of gut microbiota assessed, Lactobacilli, Bifidobacteria, Enterobacteria, and *Escherichia coli*. Gut microbiota was analyzed using Total Plate Count (TPC) following the method by Mailoa *et al*.[28] Lactobacilli were determined by MRS medium agar, Bifidobacteria by Bifidobacterium agar, Enterobacteria by MacConkey agar, and *Escherichia coli* by Tryptone Bile X-Glucuronide (TBX) agar.

Statistical Analysis

Data analysis used software SPSS to determine the difference between the three groups. One-way ANOVA was used to measure the difference between NA, FE, and KO groups with post hoc test Bonferroni.[29]

RESULTS

Body weight of the rats increased in all groups. It was measured before the intervention began and after the hemoglobin depletion and found not significantly different, $p>0.05$ (Table 2).

Table 2. Body Weight Differences before and after the Intervention

Body Weight (g)	NA	FE	KO
Before intervention	87±12	85±10	82±15
After intervention	120±10[a]	116±7[a]	114±13[a]

One-Way ANOVA

[a] different superscript in the same row means there was a significant difference

NA: NaFeEDTA fortified synbiotic fermented milk with tempeh extract, FE: $FeSO_4$ fortified synbiotic fermented milk with tempeh extract, KO: synbiotic fermented milk with tempeh extract without fortification

Table 3 shows hemoglobin concentration of the rats before and after given the intervention. From the results, the level of hemoglobin after the intervention was the same between the three groups.

Table 3. Hemoglobin Concentration

Hemoglobin (mg/dL)	NA	FE	KO
Before intervention	6.85±0.14[a]	6.41±0.14[b]	6.47±0.23[b]
After intervention	11.80±0.76[a]	11.48±0.31[a]	11.03±0.35[a]

One-Way ANOVA
[a,b] The same superscript letter in a row shows no significant difference
NA: NaFeEDTA fortified synbiotic fermented milk with tempeh extract, FE: $FeSO_4$ fortified synbiotic fermented milk with tempeh extract, KO: synbiotic fermented milk with tempeh extract without fortification

We also measured fecal short chain fatty acid (SCFA) concentration by the intervention. Three types of SCFA which were acetic acid, butyric acid, and propionic acid were analyzed. In the present study, it was found a significant mean difference of propionic and butyric acid concentration between the three groups. However, there was no significant mean difference of acetic acid concentration between the three groups. From the study, SCFA concentration was higher in the group given iron-fortified synbiotic fermented milk with tempeh extract compared to synbiotic fermented milk with tempeh extract without fortification (Table 4).

Table 4. Short Chain Fatty Acid Concentration based on the Intervention

Intervention	NA	FE	KO	*p*
Acetic acid	24.49±5.55	20.63±5.04	20.85±3.84	0.23
Propionic acid	11.13±1.91	9.71±1.83	8.66±1.14	0.02*
Butyric acid	6.37±1.30	5.64±1.16	4.4±0.50	0.004*

One-Way ANOVA
NA: NaFeEDTA fortified synbiotic fermented milk with tempeh extract, FE: $FeSO_4$ fortified synbiotic fermented milk with tempeh extract, KO: synbiotic fermented milk with tempeh extract without fortification

Different gut microbial was developed after the intervention. The highest Lactobacilli number was FE group, Bifidobacteria, and Enterobacteriaceae were highest in KO group. While NA group had the highest *Escherichia coli* number compared with the other groups (Table 5).

Table 5. Total Gut Microbiota Number before and after Intervention

Groups (CFU/g)		NA	FE	KO
Lactobacilli	Pre	4.3×10^6	4.3×10^6	4.3×10^6
	Post	4.0×10^8	6.7×10^7	3.3×10^8
Bifidobacteria	Pre	2.9×10^7	2.9×10^7	2.9×10^7
	Post	1.5×10^8	1.3×10^8	6.4×10^7
Escherichia coli	Pre	5.5×10^5	5.5×10^5	5.5×10^5
	Post	9.0×10^4	5.1×10^4	6.2×10^5
Enterobacteriaceae	Pre	3.5×10^5	3.5×10^5	3.5×10^5
	Post	3.7×10^5	5.6×10^5	9.2×10^6

NA: NaFeEDTA fortified synbiotic fermented milk with tempeh extract, FE: FeSO4 fortified synbiotic fermented milk with tempeh extract, KO: synbiotic fermented milk with tempeh extract without fortification

DISCUSSION

NaFeEDTA is a yellow, pale iron powder that has good solubility in the pH 7 solution. The stability of NaFeEDTA made it suitable to use in the long-stored foods or were made at high temperatures. The EDTA protects iron compounds from the formation of insoluble iron like iron-phytate. EDTA is influenced by pH to work.[30,31] Unlike the NaFeEDTA, $FeSO_4$ is more unstable, mainly when use in the phytate rich foods or stored in a long time. It is because $FeSO_4$ does not have chelating capacity, which could protect the fortificant from forming other compounds.[32]

Short-chain fatty acid (SCFA) is a fermented product of undigested fiber by gut microbiota in the colon. The main types of SCFAs are acetic, butyric, and propionic acid.[33] The present study suggests a significant difference of propionic and butyric acid between NaFeEDTA and $FeSO_4$ fortified synbiotic fermented milk with tempeh extract. SCFA concentration is higher in the group of iron-fortified compared with unfortified group.

A study by Dostal *et al,*[34] in rats suggested that availability of colonic iron modulated gut microbiota metabolites and the production of SCFA. It was supported by Louis and Flint[35], who stated that iron influenced butyrate production pathway and the gene of butyrate production in the gut. Yilmaz and Li[14] concluded that in iron-deficient conditions, the Bacteroides decreased while Lactobacilli and Enterobacteriaceae increased, which caused lower propionate and butyrate production. However, different results showed in the human study by Jaeggi *et al,*[36] who mentioned that iron did not have any effect on fecal SCFA concentration since 95-99 percent of SCFA produced by bacterial fermentation were absorbed in the colon.

Fructooligosaccharides, the prebiotics added to fermented milk with tempeh extract, function to transform ferric ion into a ferrous form in the colon and the probiotics.[21] The ferrous form is more readily to absorb than ferric. Beside of it, prebiotic helps the probiotic against pathogen bacteria. The result proved that the hemoglobin level increased in all groups. Although before the intervention, the hemoglobin level is different between NA and FE and KO group, after the intervention, the hemoglobin level was the same. It suggested that the consumption of synbiotic fermented milk with tempeh extracts with or without iron fortification was able to increase the rat's hemoglobin concentration. Perez-Conesa *et al,*[37] explained that iron absorption mainly happened in the colon. Administration of prebiotic and synbiotic diet could help increase iron bioavailability through balancing gut microbiota composition. As written in Reeves *et al,*[23] AIN-93 also contained iron in a small portion.

The present study suggested the positive effects of probiotics and prebiotics to prevent the adverse effects of the pathogen. This study showed that the highest Lactobacilli number was in the FE group. It was supported by the research of Lin *et al*,[38] about the supplementation of prebiotic/probiotic to tackle the adverse effect of excessive iron intake. Lactobacilli is a probiotic which added to the iron-fortified fermented milk with tempeh extract. It is possible that some parts of probiotic pass the upper gastrointestinal and colonize in the colon, reflects in feces.

Administration of excessive iron, either due to supplementation or fortification, can lead to gut microbiota imbalance. A clinical study about iron fortification for the children revealed an increasing number of Enterobacteria.[11] Naikare *et al,*[39] suggested that gram-negative bacteria, such as Enterobacteria, had feoB as a modulation gene to modulate both commensal and infectious bacteria colonization in the host gut. This transporter could be used to obtain an iron compound for bacteria virulence.[40] Bifidobacteria and Enterobacteriaceae were highest in KO group. The result is following the study of Dostal *et al*.[41] It stated that Enterobacter and Bifidobacteria were excellent iron scavengers and could grow in the limited iron environment.

The present study gave new evidence on how synergistic effects of pre- and probiotic may affect gut microbiota balance and its production of short-chain fatty acids. It also contributed to the research of local-based functional foods, especially tempeh development and utility of *L. plantarum*.

CONCLUSION

Iron fortification has positive effects on increasing the production of SCFAs. Moreover, the prebiotics and probiotics have positive effects against pathogenic bacteria growth.

RECOMMENDATION

Further study is needed to determine the effects of iron-fortified synbiotic fermented milk with tempeh extract in human.

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REFERENCES

1. Kementerian Kesehatan RI. *Riset Kesehatan Dasar (RISKESDAS) 2013*. Jakarta: Kementerian Kesehatan R1; 2013. Available from: *http://www.depkes.go.id/resources/download/general/Hasil Riskesdas 2013.pdf*
2. Barkley JS, Kendrick KL, Codling K, Muslimatun S, Pachón H. Anaemia Prevalence Over Time in Indonesia: Estimates from the 1997, 2000, and 2008 Indonesia Family Life Surveys. *Asia Pac J Clin Nutr*. 2015;24(3):452–5.
3. World Health Organization. *The Global Prevalence of Anaemia in 2011*. Geneva: World Health Organization; 2011.
4. Le HT, Brouwer ID, Burema J, Nguyen KC, Kok FJ. Efficacy of Iron Fortification Compared to Iron Supplementation Among Vietnamese Schoolchildren. *Nutr J*. 2006;5(1):1–8.
5. Huo J, Sun J, Miao H, Yu B, Yang T, Liu Z, et al. Therapeutic Effects of NaFeEDTA-Fortified Soy Sauce in Anaemic Children in

China. *Asia Pac J Clin Nutr*. 2002;11(2):123-7.

6. Hurrell RF. Fortification: Overcoming Technical and Practical Barriers. *J Nutr*. 2002;132(4):806S–812S.
7. Pusat Data dan Sistem Informasi Pertanian. *Statistik Konsumsi Pangan Tahun 2012*. Jakarta: Kementerian Pertanian; 2012.
8. Astuti M, Meliala A, Dalais FS, Wahlqvist ML. Tempe, a Nutritious and Healthy Food from Indonesia. Asia Pac J Clin Nutr. 2000;9(4):322–5. Available from: *https://pdfs.semanticscholar.org/e6f5/10b088d5ae8cf3dbdeb48acdd471fe7c2095.pdf*, cited July 9th 2019.
9. Mahmud MK, Zulfianto NA. *Tabel Komposisi Pangan Indonesia*. Jakarta: Elex Media Komputindo; 2009.
10. Sari DK, Nurrohmah A. Bubur Tempe Membantu Penanganan Diare pada Balita. *GEMASSIKA Jurnal Pengabdian Kepada Masyarakat*. 2019;3(1):10-8.
11. Zimmermann MB, Chassard C, Rohner F, N'Goran EK, Nindjin C, Dostal A, et al. The Effects of Iron Fortification on the Gut Microbiota in African Children: A Randomized Controlled Trial in Côte d'Ivoire. *Am J Clin Nutr*. 2010;92(6):1406-15.
12. Bomba A, Nemcová R, Gancarciková S, Herich R, Guba P, Mudronová D. Improvement of the Probiotic Effect of Micro-Organisms by Their Combination with Maltodextrins, Fructo-Oligosaccharides and Polyunsaturated Fatty Acids. *Br J Nutr*. 2002;88(1):S95–S99.
13. Gera T, Sachdev HP. Effect of Iron Supplementation on Incidence of Infectious Illness in Children: Systematic Review. *Br Med J*. 2002;325(7373):1142.
14. Yilmaz B, Li H. Gut Microbiota and Iron: The Crucial Actors in Health and Disease. Pharmaceuticals (Basel). 2018; 11(98). Available from: *https://www.mdpi.com/1424-8247/11/4/98/pdf*, cited July 3rd 2019.
15. Sender R, Fuchs S, Milo R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biol*. 2016;14(8):e1002533. Available from: *http://www.ncbi.nlm.nih.gov/pubmed/27541692%0Ahttp://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=PMC4991899*
16. Heiss CN, Olofsson LE. Gut Microbiota-Dependent Modulation of Energy Metabolism. *J Innate Immun*. 2018;10(3):163–71. Available from: *https://www.karger.com/Article/FullText/481519*, cited July 1st 2019.
17. Carding S, Verbeke K, Vipond DT, Corfe BM, Owen LJ. Dysbiosis of the Gut Microbiota in Disease. *Microb Ecol Heal Dis*. 2015;26(0):1–9. Available from: *http://www.microbecolhealthdis.net/index.php/mehd/article/view/26191.*
18. Fooks LJ, Gibson GR. In Vitro Investigations of the Effect of Probiotics and Prebiotics on Selected Human Intestinal Pathogens. *FEMS Microbiol Ecol*. 2002;39(1):67–75.
19. Bartosch S, Woodmansey EJ, Paterson JCM, McMurdo MET, Macfarlane GT. Microbiological Effects of Consuming a Synbiotic Containing Bifidobacterium bifidum, Bifidobacterium lactis, and Oligofructose in Elderly Persons, Determined by Real-Time Polymerase Chain Reaction and Counting of Viable Bacteria. *Clin Infect Dis*. 2005;40(1):28–37.
20. Asahara T, Nomoto K, Shimizu K, Watanuki M, Tanaka R. Increased Resistance of Mice to Salmonella enterica Serovar Typhimurium Infection by Synbiotic Administration of Bifidobacteria and Transgalactosylated Oligosaccharides. *J Appl Microbiol*. 2001;91(6):985–96.
21. Yeung CK, Glahn RE, Welch RM, Miller DD. Prebiotics and Iron Bioavailability - Is there

a connection?. *J Food Sci*. 2005;70(5):R88–92.

22. Rowland IR, Rumney CJ, Coutts JT, Lievense LC. Effect of Bifidobacterium Longum and Inulin on Gut Bacterial Metabolism and Carcinogen-Induced Aberrant Crypt Foci in Rats. *Carcinogenesis*. 1998;19(2):281–5.
23. Reeves PG, Nielsen FH, Fahey GC. AIN-93 Purified Diets for Laboratory Rodents: Final Report of the American Institute of Nutrition Ad Hoc Writing Committee on the Reformulation of the AIN-76A Rodent Diet. *J Nutr*. 1993;123(11):1939–51.
24. Helmyati S, Sudargo T, Kandarina I, Yuliati E, Wisnusanti SU, Puspitaningrum VAD, et al. Tempeh Extract Fortified with Iron and Synbiotic as a Strategy Against Anemia. *Int Food Res J*. 2016;23(5):2296–9.
25. Van Herck H, Baumans V, Boere HA, Hesp AP, Van Lith HA, Beynen AC. Orbital Sinus Blood Sampling in Rats: Effects Upon Selected Behavioural Variables. *Lab Anim*. 2000;34(1):10–9.
26. Reuhs BL, Rounds MA. High-Performance Liquid Chromatography. In: Nielsen SS, editor. *Food Analysis*, 4th Edition. New York: Springer Science+Business Media, LLC; 2010. p. 499–512.
27. Kotani A, Miyaguchi Y, Kohama M, Ohtsuka T, Shiratori T, Kusu F. Determination of Short-Chain Fatty Acids in Rat and Human Feces by High-Performance Liquid Chromatography with Electrochemical Detection. *Anal Sci*. 2009;25(8):1007–11.
28. Mailoa MN, Tapotubun AM, Matrutty TEAA. Analysis Total Plate Counte (TPC) on Fresh Steak Tuna Applications Edible Coating Caulerpa sp During Stored at Chilling Temperature. *IOP Conference Series: Earth and Environmental Science*. 2017;89(1):012014.
29. Sirait AM. Analisis Varians (ANOVA) dalam Penelitian Kesehatan. *Media Penelitian dan Pengembangan Kesehatan*. 2001; 11(2):39-43. Available from: https://media.neliti.com/media/publications/156905-ID-analisa-varians-anova-dalam-penelitian-k.pdf, cited March 22nd 2020.
30. World Health Organization. *Guidelines on Food Fortification with Micronutrients*. Geneva: World Health Organization; 2006.
31. Wreesmann CTJ. Reasons for Raising The Maximum Acceptable Daily Intake of EDTA and The Benefits for Iron Fortification of Foods for Children 6-24 Months of Age. *Matern Child Nutr*. 2014;10(4):481–95.
32. Husna NN. Effectiveness of FeSO4 and FeSO4 + Na2EDTA as Iron Fortificant in Soy Milk and Tempe. *Skripsi*. Depok: Fakultas Matematika dan Ilmu Pengetahuan Alam Universitas Indonesia, 2011.
33. Topping DL. Short-chain Fatty Acids Produced by Intestinal Bacteria. *Asia Pac J Clin Nutr*. 1996;5(1):15-9.
34. Dostal A, Chassard C, Hilty FM, Zimmermann MB, Jaeggi T, Rossi S, et al. Iron Depletion and Repletion with Ferrous Sulfate or Electrolytic Iron Modifies the Composition and Metabolic Activity of the Gut Microbiota in Rats. *J Nutr*. 2012;142(2):271–7.
35. Louis P, Flint HJ. Diversity, Metabolism and Microbial Ecology of Butyrate-Producing Bacteria from the Human Large Intestine. *FEMS Microbiol Lett*. 2009;294(1):1– 8.
36. Jaeggi T, Kortman GAM, Moretti D, Chassard C, Holding P, Dostal A, et al. Iron Fortification Adversely Affects the Gut Microbiome, Increases Pathogen Abundance and Induces Intestinal Inflammation in Kenyan Infants. *Gut*. 2015;64:731-42.
37. Pérez-Conesa D, López G, Ros G. Effect of Probiotic, Prebiotic, and Synbiotic Follow-

up Infant Formulas on Iron Bioavailability in Rats. *Food Sci Tech Int.* 2007;13(1):69-77.

38. Lin F, Wu H, Zeng M, Yu G, Dong S, Yang H. Probiotic/Prebiotic Correction for Adverse Effects of Iron Fortification on Intestinal Resistance to Salmonella Infection in Weaning Mice. *Food Funct*. 2018;9(2):1070–8. Available from: *http://www.ncbi.nlm.nih.gov/pubmed/29355277*, cited March 22nd 2020.
39. Naikare H, Palyada K, Panciera R, Marlow D, Stintzi A. Major Role for FeoB in Campylobacter jejuni Ferrous Iron Acquisition, Gut Colonization, and Intracellular Survival. *Infect Immun*. 2006;74(10):5433–44.
40. Boyer E, Bergevin I, Malo D, Gros P, Cellier MFM. Acquisition of Mn(II) in Addition to Fe(II) is Required for Full Virulence of Salmonella enterica Serovar Typhimurium. *Infect Immun*. 2002;70(11):6032–6042.
41. Dostal A, Lacroix C, Bircher L, Pham VT, Follador R, Zimmermann MB, et al. Iron Modulates Butyrate Production by a Child Gut Microbiota In Vitro. *mBio*. 2015;6(6):e01453-15.