American Journal of Food Technology

ISSN 1557-4571

Academic Journals Inc.

www.academicjournals.com

OPEN ACCESS American Journal of Food Technology

ISSN 1557-4571
DOI: 10.3923/ajft.2018.32.41

Research Article
Synbiotic Fermented Milk with Tempeh Extract and Iron Fortification: Effect on Antibacterial Activity and Total Enterobacteriaceae

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Abstract

Background and Objective: Synbiotic fermented milk with tempeh extract and iron fortification is a diversification of Indonesian local food to overcome anemia. However, consuming excessive iron may increase the pathogen virulence in the gut. Antibacterial agents from the *Lactobacillus plantarum* Dad 13 and tempeh are assumed to kill the pathogens effectively. To determine the effect of synbiotic fermented milk with tempeh extracts and iron fortification on total Enterobacteriaceae and to measure the antibacterial activity of the milk against *Escherichia coli* and *Staphylococcus aureus*. **Materials and Methods:** There were three groups of synbiotic fermented milk with tempeh extract, with two groups fortified with: (1) NaFeEDTA (SN), (2) $FeSO_4$ (SF) and one group, (3) without fortification (SK). The milk groups were made and stored for 4 and 2 weeks and 1 day before analysis. Specifically, antibacterial activity was examined after the samples were stored f or 2 and 0 weeks while total Enterobacteriaceae was measured after stored for 0, 2 and 4 weeks. **Results:** The antibacterial activity of the study groups was almost the same against both *E. coli* or *S. aureus* ($p>0.05$). Total Enterobacteriaceae decreased in the longer shelf life (SN Group: week (0 2.26×10^8), week (2 1.45×10^6), week-4 estimated $<1\times10^5$, $p<0.05$). **Conclusion:** The addition of iron did not hamper the antibacterial activity of synbiotic fermented milk with tempeh extracts against *E. coli* and *S. aureus*. After confirming previous findings related to iron supplementation and anemia, this study further demonstrated the extract's effectiveness against Enterobacteriaceae.

Key words: Antibacterial activity, Enterobacteriaceae, iron, synbiotic, tempeh extract

Citation: Siti Helmyati, Lily Arsanti Lestari, Odilia Ratna Mayasari, Maria Wigati, Setyo Utami Wisnusanti, Endang Sutriswati Rahayu and Muhammad Juffrie, 2018. Synbiotic fermented milk with tempeh extract and iron fortification: Effect on antibacterial activity and total Enterobacteriaceae. Am. J. Food Technol., 13: 32-41.

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Anemia is one form of micronutrient deficiency, mainly caused by iron deficiency, which leads to low cognitive function, low birth weight, low hemoglobin concentration and increased risk of mortality and morbidity[1–4]. The World Health Organization (WHO) estimated around 800 million people in the world suffer from anemia, including children, pregnant women and women in reproductive age[5]. In Indonesia, anemia remains one of the significant health problems that cannot be solved yet, which is shown by the data of the Indonesian Basic Health Research that estimated around 21.7% of Indonesian people were anemic[6]. This high percentage was caused by the low compliance of iron supplement administration. The consumers reasoned they dislike the side effects of iron supplementations such as diarrhea, constipation and the black feces and refused taking them[7].

In order to address this problem, some programs of iron fortification with a food-based approach need to be pursued[2]. These programs are also important to increase the Indonesian local food utilization. Furthermore, iron fortification has been shown to be more effective to increase the compliance of iron consumption in a population[8]. However, excessive iron consumption has some negative impacts on pathogen virulence in the gut[9].

Escherichia coli and *Staphylococcus aureus* are common pathogenic bacteria found in the human body. As the extracellular Gram-positive bacteria, a high number of *S. aureus* have the ability to gain an access to the underlying tissues, which can cause severe diseases as well as *Escherichia coli* in the gut[10,11]. Excessive iron may be beneficial for the colonization of both pathogens[12,13]. They are able to bind to the iron, even in a low iron environment by forming a high affinity iron chelating agent called siderophore[14,15].

Tempeh is a local food widely consumed by Indonesian people. A survey in 2012 stated that the Indonesian people consumed more than 6 kg of tempeh per year on average[16]. Although Indonesian people are fond of tempeh, the raw tempeh is limited in preparation and shelf life. Conventionally, the tempeh is fried, boiled or stir fried only. Its shelf life is also very short, just 48 h, which restricts the utilization of tempeh[17]. It is unfortunate since the tempeh is a good source of vitamin B complexes, folic acid, protein, iron and zinc which are important for red blood cell formation[18]. Therefore, to increase the availability of tempeh for a longer period, a method is needed to increase the shelf life of tempeh and one of the examples is by extracting the tempeh. By making the extract, the nutritional value of tempeh can be maintained. Consuming iron-fortified tempeh could also reduce the risk of diarrhea, the side effect of iron consumption[19]. Thus, making new food formulations based on tempeh can be an alternative to treat and overcome anemia.

A functional food that can be made from tempeh is called synbiotic fermented milk with tempeh extract. The beverage is made from tempeh extract and fermented by *Lactobacillus plantarum* Dad 13. The product was first developed by Kandarina *et al.*[20], who conducted a randomized control trial study to investigate its effect on anemic rats. The previous study showed the positive effects of synbiotic tempeh extract with or without iron supplement on body weight and hemoglobin level of Wistar rats.

Addition of probiotics and prebiotics (synbiotics) in the milk will add to its nutritional value. The probiotic commonly used is *Lactobacillus plantarum* Dad 13 which is isolated from the Indonesian local food, *dadih*, while the prebiotic is using fructooligosaccharide (FOS) to support the growth of the probiotic[21,22]. Fermentation is beneficial to increase the shelf life and nutrients' bioavailability, to add favorable texture and flavor and to maintain gastrointestinal health through the action of probiotics and prebiotics[23]. Foods and beverages that contain probiotics are suggested to have antibacterial effects against pathogenic bacteria[24–27].

This study is developed from the previous research[20]. After the good findings were confirmed in animal trials, further research is needed to investigate its effect on humans. The novelty of the present study is the replication and extension of the trial formulations. The research replicates the previous study by using the same sample, synbiotic tempeh extract. It also extends the findings by modifying the formula (adding iron fortification) and changing the parameters. This continuation is very important for analyzing both the safety and quality of the product before conducting a clinical study on humans.

Although the synbiotic fermented milk with tempeh extract has the beneficial nutrients for hemoglobin improvement, there is a possibility of food contamination during the production phase that hampers the quality of synbiotic fermented milk. One of the bacteria that could be an indicator of food contamination is Enterobacteriaceae[28]. Large numbers of Enterobacteriaceae in the product increase the risk of food-borne pathogens[29].

The present study aimed to determine the antibacterial activity of iron-fortified synbiotic fermented milk with tempeh extract and to know the possibility of food contamination by Enterobacteriaceae. The antibacterial activity was measured against *E. coli* and *S. aureus* as representatives of Gram-positive and Gram-negative bacteria in humans.

Measuring the antibacterial activity and total Enterobacteriaceae is expected to guarantee the health effects and safety of the synbiotic fermented tempeh product for human consumption.

MATERIALS AND METHODS

Complete Random Design with the variation of shelf life and groups of synbiotic fermented milk with tempeh extract as factor variables were used in this study. There were 2 categories of shelf life (0 and 2 weeks) for antibacterial activity examination and 3 categories of shelf life (0, 2 and 4 weeks) for total Enterobacteriaceae. The study was conducted in the Microbiology Laboratory of Pusat Antar Universitas, Universitas Gadjah Mada, Yogyakarta, Indonesia from September 1st to October 30th 2016 as a part of undergraduate thesis by Mayasari[30] and Wigati[31].

Materials: This research uses tempeh called "ATTEMPE" which is made from a variety of Anjasmoro soybeans, skim milk "Lactona" and sugar "Gulaku". They were purchased from a local market in Yogyakarta, Indonesia. Fructooligosaccharide (FOS) Orafti P95 was procured from the Beneo Institute of Indonesia. The strain of *Lactobacillus plantarum* Dad 13 used in this study was obtained from the Food and Nutrition Culture Collection (FNCC), Centre for Food and Nutrition Studies, Universitas Gadjah Mada, Indonesia.

Chemicals: This study uses Gallic acid (Sigma Aldric), phenolphthalein (Merck), sodium hydroxide (Merck) and ethanol 96%. All chemicals and selective agar media are analytical grade approved.

Preparation of synbiotic fermented milk with tempeh extract and iron fortification: The making process of tempeh extract and fermented tempeh extract followed the procedures as published by Kandarina *et al.*[20]. For tempeh extract preparation, the fresh tempeh was crushed with the following procedures: (1) Adding hot water to tempeh in 2:1 ratio, (2) Blending, (3) Heating, (4) Straining, (5) Pouring into sterile bottles and (6) pasteurizing at 80°C. After the extract was obtained, it was mixed with skim milk, FOS and sugar. Then it was pasteurized and fermented using *L. plantarum* Dad 13. The synbiotic tempeh extracts were formulated and categorized according to the type of iron compound used. The first group was given $FeSO_4$ 50 ppm (SF), the second was given NaFeEDTA 50 ppm (SN) and the third was not given the fortification (SK or control). The samples were made and stored for 4 weeks, 2 weeks and 1 day before analysis. The product made 1 day before analysis was considered as 0 weeks of shelf life. All analyses were done on the same day to determine the difference of antibacterial activity and total Enterobacteriaceae of the synbiotic tempeh extract with and without iron fortification after the extracts were kept in 4°C for the several categories of storage period. The formula of synbiotic milk with tempeh extract and iron fortification is now in the process of Patent Registration number P00201802758.

Characterization: Sample characterization was conducted to understand the basic features of the sample, especially the acid and phenol content. It was done in both indicators to identify the concentration of the sources of antibacterial agents in the sample. Analysis of total phenol was done using the methods by Slinkard and Singleton[32] while the total titrated acids was quantified using phenolphthalein 1% which then was converted with the formula developed by the Food Safety and Standard Authority of India[33].

Antibacterial activity measurements: Antibacterial activity was investigated using the Well Diffusion Method with the procedure as described by Balouiri *et al.*[34]. It is applied to measure the antibacterial activity of all groups of extracts with 0 and 2 weeks of shelf life. In this method, the agar plate was inoculated by microbial inoculums over the entire surface. Then, an approximately 6-8 mm diameter hole was punched aseptically and a volume of the extract solution was introduced into the well. After 18-24 h of incubation, the antimicrobial agent diffuses and inhibits the growth of bacteria in the plate. The antibacterial activity of the synbiotic tempeh extract was examined for *Escherichia coli* as representative of Gram-negative and *Staphylococcus aureus* as representative of Gram-positive bacteria commonly found in the human body.

The three study groups were compared with a standard antibiotic, ciprofloxacin. Ciprofloxacin is a broad spectrum antimicrobial agent for oral consumption, meaning it is sensitive towards Gram-positive and Gram-negative bacteria[35]. The comparison was important to know the difference between the antibacterial activity of synbiotic fermented milk with tempeh extract and the standard antibiotic.

Total Enterobacteriaceae enumeration: The Enterobacteriaceae were counted using the Total Plate Count Method with the Pour Plate Technique which followed the procedure developed by the Food Safety and Standard Authority of India[33]. The selective agar medium, MacConkey Agar (Oxoid) was used specifically for Gram-negative and lactose-fermenting bacteria. Its isolation action works after

being incubated in 35-37°C for 18-24 h and is marked by pink to red color for lactose-fermenting colonies and colorless for non-lactose-fermenting colonies[27,36]. Since the agar medium was used in isolation, the method may have some limitations. The cells often showed as clumps, clusters, chains and were not distributed very well which made it hard to count the microbial cells. To avoid miscalculation of the cells, the Quebec Colony Counter was used. In spite of the limitations, the procedure was considerably cheap and simple. The samples of the milk were stored for 0, 2 and 4 weeks before analysis.

Statistical analysis: All data-sets went through coding, editing and cleaning phases to ensure the data quality. Statistical analyses were performed in SPSS 19. For multivariate analyses, the antibacterial activity and total Enterobacteriaceae between the study groups were compared using the One-Way ANOVA test, while to compare the differences between two or three categories of shelf life in one group of the milk, the Paired t-test or the Repeated ANOVA tests were performed. The confidence interval was set at 95% with the significant value if $p<0.05$.

RESULTS

Characterization: The characteristics of the sample were determined using total phenol and total titrated acid measurements as shown in Table 1. Total titrated acid of the samples was almost the same in all groups ($p>0.05$) while the total phenol was significantly different between the SN and SF groups ($p<0.05$) but not different with the control. This result suggested that the addition of iron does not alter the concentration of acid and phenol of the synbiotic fermented milk with tempeh extracts.

Table 1: Characterization of synbiotic fermented milk with tempeh extract

Samples	Total titrated acid (%)	Total phenol (ppm)
SF	3.8±0.3[a]	151.7±2.1[a]
SN	4.1±0.5[a]	167.6±1.1[b]
SK	3.6±0.0[a]	162.3±3.2[a,b]
p	0.60	0.01

One-way ANOVA, $p<0.05$ data shown as mean±standard deviation (SD), [a,b]same superscript letter in the same column means no significant difference SF: Synbiotic fermented milk with tempeh extract and $FeSO_4$ fortification, SN: Synbiotic fermented milk with tempeh extract and NaFeEDTA fortification, SK: Synbiotic fermented milk with tempeh extract without fortification/control

Table 2: Antibacterial activity against *Staphylococcus aureus* (mm)

	Shelf life		
Samples	Week-0	Week-2	P_2
SF	12.3±1.5[a]	17.9±2.5[a]	0.01
SN	13.8±2.0[a]	16.7±3.4[a]	0.13
SK	15.2±2.0[a]	15.5±2.4[a]	0.85
SC	27.3±2.7[b]	25.3±1.8[c]	0.19
P_1	<0.001	<0.001	

P_2: Paired t-test, $p<0.05$, data shown as Mean±standard deviation (SD), [a,b]same superscript letter in the same column means no significant difference, SF: Synbiotic fermented milk with tempeh extract and $FeSO_4$ fortification, SN: Synbiotic fermented milk with tempeh extract and NaFeEDTA fortification, SK: Synbiotic fermented milk with tempeh extract without fortification/control, SC: Ciprofloxacin antibiotic

Table 3: Antibacterial activity against *Escherichia coli* (mm)

	Shelf life		
Samples	Week-0	Week-2	P_2
SF	15.7±2.1[a]	12.0±2.4[a]	0.05
SN	15.8±1.2[a]	15.8±1.5[b,c]	1.00
SK	15.5±1.4[a]	13.2±0.7[a,b]	0.02
SC	24.2±1.5[b]	17.7±1.9[c]	<0.001
P_1	<0.001	<0.001	

P_1: One-Way ANOVA, P_2: Paired t-test, $p<0.05$, data shown as mean±standard deviation (SD), [a,b]same superscript letter in the same column means no significant difference, SF: Synbiotic fermented milk with tempeh extract and $FeSO_4$ fortification, SN: Synbiotic fermented milk with tempeh extract and NaFeEDTA fortification, SK: Synbiotic fermented milk with tempeh extract without fortification/control

Antibacterial activity: As seen in Fig. 1, the zone of inhibition was measured by the diameter of the clear zone around the well. The sharpness of the inhibition diameter differed between antibacterial activity against *E. coli* and *S. aureus*. The inhibition diameter of antibacterial activity against *S. aureus* was sharper and clearer than that of antibacterial activity against *E. coli*. It could be assumed that the antibacterial activity of synbiotic fermented milk with tempeh extract is stronger against Gram-positive bacteria.

As presented in Table 2 and 3, there was no difference between the antibacterial activity of SN, SF and control groups. The significant difference only happened between the samples either with or without fortification and the ciprofloxacin group, with the antibacterial activity of ciprofloxacin group results higher ($p<0.001$). Although the antibacterial activity of synbiotic fermented milk with tempeh extract was lower, the result suggested that the addition of iron fortification does not affect the antibacterial activity significantly.

Total number of Enterobacteriaceae: The present study suggested that there was a declining trend of total Enterobacteriaceae when the shelf life was longer as shown in Table 4. The significant difference appeared in the total Enterobacteriaceae of the SN group between the extracts stored for 0 and 4 weeks and between the extracts stored for

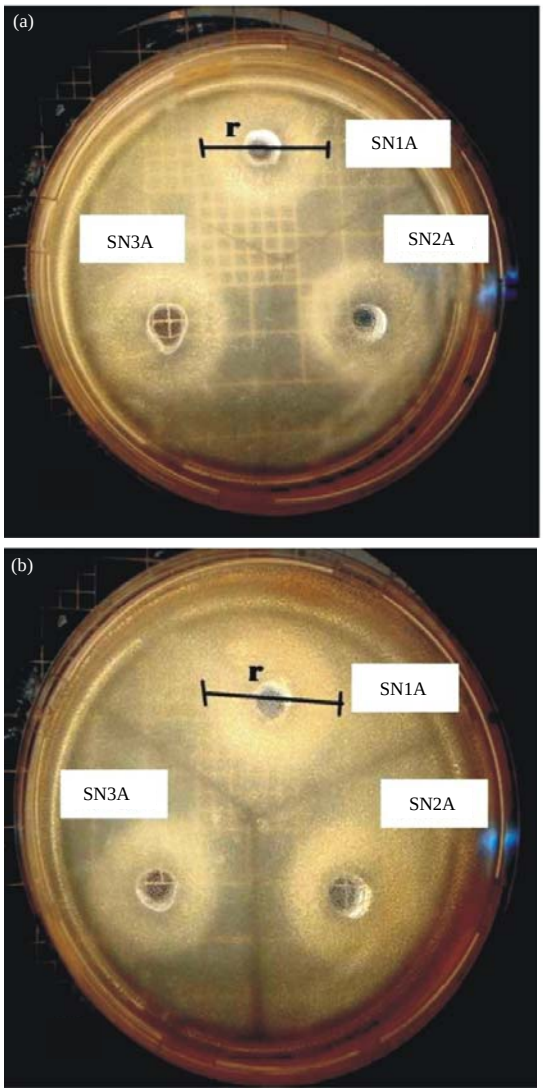

Fig. 1(a-b): Antibacterial activity measurements of synbiotic fermented milk with tempeh extract and NaFeEDTA fortification

A: Antibacterial activity towards *Staphylococcus aureus*, B: Antibacterial activity towards *Escherichia coli*, r: Diameter of inhibition zone

2 weeks and 4 weeks ($p<0.05$). Meanwhile, when the study groups were compared, the significant difference only occurred in samples with week 0 of shelf life ($p<0.05$). Although there was a statistical difference in total Enterobacteriaceae in samples with week 0 of shelf life and in SN group, the number of Enterobacteriaceae remained high. In week-0 of shelf life, the lowest total number of Enterobacteriaceae was in the SN group (2.26×10^8 CFU mL^{-1}) while after 4 weeks of storage the number of Enterobacteriaceae in the SF group reached 2.6×10^6 CFU mL^{-1}, much higher than the other groups which were estimated lower than 1×10^3 CFU mL^{-1}.

Table 4: Total Enterobacteriaceae of synbiotic fermented milk with tempeh extract and Iron fortification (CFU mL^{-1})

	Shelf life			
Samples	Week-0	Week-2	Week-4	P_2
SF	3.04×10^{8b}	$3.06\times10^{7a,b}$	2.6×10^{6a}	0.263
SN	2.26×10^{8b}	$1.45\times10^{6b*}$	Est. $<1\times10^{3a*}$	0.020
SK	1.065×10^{9a}	7.55×10^{7a}	Est. $<1\times10^{3a*}$	0.067
P_1	0.012	0.034	0.130	

P_1: One-Way ANOVA, P_2: Repeated ANOVA, $p<0.05$, data shown as mean±standard deviation (SD), ^{a,b}same superscript letter in the same column means no significant difference, SF: Synbiotic fermented milk with tempeh extract and $FeSO_4$ fortification, SN: Synbiotic fermented milk with tempeh extract and NaFeEDTA fortification, SK: Synbiotic fermented milk with tempeh extract without fortification/control

DISCUSSION

The present study is a part of a larger research about the utilization of tempeh, an Indonesian functional food to treat and overcome anemia. The first stage of the study was conducted by Kandarina *et al.*[20] by developing the synbiotic tempeh extract and investigating its effect on anemic Wistar rats. The second stage of the study was done to investigate the quality and safety of the product which is important before the 3rd stage research is conducted. The third stage of study is planned to analyze the effect of synbiotic tempeh extract with iron fortification on anemia through a human trial. This research, the second stage of the extended study, revealed that phenol and acids exist in the synbiotic fermented milk with tempeh extracts with a similar amount. Although the difference between total phenol of the SN and SF groups was statistically significant, it was not different with that of the SK group. This result suggested that the addition of iron did not affect the quantity of the phenol and acid compounds of the milk.

Phenols and acids are types of antibacterial agents contained in some foods or beverages. In this research, the formation of phenolic and acidic compounds could be due to the activity of *Lactobacillus plantarum* Dad 13. A study by Fitrotin *et al.*[37] stated that *L. plantarum* Dad 13 has the ability to produce antibacterial agents after the incubation for 24 h. The lactic acid bacteria will produce ferulic acid reductase and vinyl phenol reductase and then degrade the hydroxycinnamic acid which leads to higher quantity of total phenol in the sample[38,39].

The clear zone that formed around the well diffusion confirmed the inhibition effect of the antibacterial agents of

the sample against *Escherichia coli* and *Staphylococcus aureus*. The bactericidal effect of lactic acid or phenol has been proved by several studies[23,38,40]. It is possible that the lactic acid will lower the pH environment, while the phenol disrupts the cell membrane formation, breaking down the permeability of the membrane itself causing the envelope of the cells to lysis[41].

The results of the present study showed that the addition of iron fortification does not affect the antibacterial activity of synbiotic fermented milk with tempeh extract towards *E. coli* and *S. aureus*. Moreover, in the longer shelf life, the antibacterial activity of the sample also increased.

Based on the categorization by Davis and Stout[42], the length of the inhibition zone can be a determinant of the antibacterial activity towards certain bacteria. The SN, SF and SK groups are included in the strong category against *E. coli* and *S. aureus* since the length of inhibition diameter is longer than 10 mm. Milk is known for having several antibacterial agents that inhibit the activity of pathogens[43]. The studies about isolated products from goat and camel's milk also suggested strong antibacterial activity in the samples, which were proved by the inhibition diameter that ranged[24,25] between 13-30 mm.

Haynes and Playne[44] mentioned that the antibacterial activity of a product depended on the temperature, pH, storage period, total number of probiotic, oxygen and bacteriocin concentration. The present study showed higher concentration of total titrated acids and total phenol in samples with a longer shelf life. It was supported by Cabrita *et al.*[39] and Ray[45] which stated that fermentation products contain antibacterial compounds such as plantaricin, phenol and lactic acid to combat pathogenic bacteria in the gut. A total of 15 lactic acid bacteria from *Lactobacillus* genus which were isolated from goat milk are proved to inhibit the growth of *B. cereus*, *B. subtilis*, *E. faecalis*, *E. coli* O157:H7, *E. coli* V517 and *S. typhi*[24]. *Lactobacillus* sp. which is commonly used in food fermentation is able to produce bacteriocins, hydrogen peroxide (H_2O_2), carbon dioxide and diacetyl[46]. The components will penetrate into the pathogen's cytoplasm and destroy the intracellular activity. In the samples with longer shelf life, the diameter inhibition will spread since the amounts of lactic acid, phenolic compounds, acetic acid, hydrogen peroxide, diacetyl and bacteriocins increase.

According to some studies, iron is an important nutrient to support a pathogen's vitality which leads to lower antibacterial activity of the product[12,13,47]. However, the present study showed the opposite results. Antibacterial activity of the SF group against *S. aureus* was higher than that in the SN and SK groups although the antibacterial activity of the SF group against *E. coli* was lower. Type and characteristic of the iron bond used in fortification affects the pathogen ability to use the iron. In NaFeEDTA forms, the iron is strongly bound to the EDTA compound in low pH environment thus the pathogens cannot use the iron to aid in their proliferation[48]. It is predicted to be the reason why the antibacterial activity of the SN and SK groups was almost the same. A study by Mirdalisa *et al.*[49] suggested that fermented milk produced several antibacterial agents such as lactic acids, acetic acids, bacteriocins, hydrogen peroxide which inhibit the growth of *E. coli* and *S. aureus.* On the other hand, the iron compound in $FeSO_4$ has weak bonds which can easily be undone[48]. This characteristic causes Gram-negative bacteria such as *E. coli* to more easily obtain the iron for their virulence and reduces the antibacterial activity of the SF group. However, other studies stated that iron exhibits weak antibacterial activity. The zero-valent iron nanoparticles were observed to have antimicrobial activity against *E. coli* and *S. aureus*[50]. The research on antibacterial activity of iron oxide (Fe_3O_4) nanoparticles against several species of bacteria showed longer zone of inhibition against *Staphylococcus epidermidis* (14 ± 0.44) than *Escherichia coli* (11.0 ± 0.44)[51].

The result of total Enterobacteriaceae enumeration in the SN, SF and SK groups showed high numbers of the bacteria on average ($>1\times10^5$ CFU mL^{-1}). Despite the high numbers of Enterobacteriaceae, the result also showed that the Enterobacteriaceae would be reduced in samples with longer storage periods, supporting the finding of the stronger antibacterial activity of the samples with the longer period of shelf life.

Total Enterobacteriaceae examination in foods and beverages is important to know the possibility of product contamination[30]. Enterobacteriaceae refers to a large group of bacteria related genetically and biochemically. *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella* and others are genera included in Enterobacteriaceae[27,39]. The presence of Enterobacteriaceae indicated there might be failures in the food manufacturing processes such as in the preparation, making process and post-processing[52]. Recently the food industries have moved to identifying the presence of Enterobacteriaceae beside the Coliform. Different with the specific regulations of Coliform, Enterobacteriaceae comprises various types of bacteria, so that further investigation is needed to detect certain specific bacteria[27].

The growth and survival of the Enterobacteriaceae are depending on the characteristics of the genus or species. The optimum temperature for most Enterobacteriaceae species range between 15-40°C, while some strains are categorized as psychrotrophic bacteria which still grow in 0°C. Several

thermotolerant strains including *E. coli* and Klebsiella are capable to grow[51] up to 44°C and the extreme ones can grow in the temperature[27] below -8°C. Heating the products for a period of time above 60°C or freezing should be enough to destroy the bacteria[27,42]. However, the present study showed the opposite results.

All of the study groups went through several heating methods and sterilization. In the preparation phase, the tempeh extract was boiled until above 60°C, the bottle and lactic acid bacteria culture also were sterilized in the temperature above 100°C and in the making process, the samples were heated again to above 70°C. All the heating methods seem to fail to prevent the growth of Enterobacteriaceae in the synbiotic fermented milk with tempeh extract, which suggested post-processing contamination.

Despite the contamination which is most likely to happen in post-processing, several studies suggested that microbial contaminants often grow in fermented products manufactured from milk or soy. Melkamsew *et al*.[53] revealed that the presence of *Escherichia coli*, *Klebsiella pneumonia*, *Enterobacter aerogenes* and *Enterobacter cloacae* in overall content of 28.7% are found in Ethiopian Traditional Cheese. Another study about yogurt sold by street vendors in Nigeria detected the contamination of *E. coli* in the value[54] of 4.4×10^5 CFU mL^{-1}. Westling *et al*.[55] also identified several microbial contaminants such as Staphylococci, *Bacillus cereus*, *Hafnia alvei*, *Escherichia coli* and *Klebsiella pneumonia* growing in soft cheese made from raw milk in different values. All the results and previous studies proposed that Enterobacteriaceae is a good indicator for food contamination.

The present study demonstrates that the antibacterial activity of the synbiotic fermented milk with tempeh extract actively reduced the presence of Enterobacteriaceae in the samples with longer shelf life. The notable difference can be seen between the SN and SF groups. The sample fortified with NaFeEDTA had lower number of Enterobacteriaceae than that fortified with $FeSO_4$. It is possible because the ferric compound in NaFeEDTA is strongly binding with the EDTA so that the bacteria could not utilize the iron for their growth[56]. It was different with the sample fortified with $FeSO_4$, where the ferrous compounds have a weak binding complex with its chelating agent and thus made it easier to undo the bonds[57].

There were many causes leading to the decline of Enterobacteriaceae in the samples with longer shelf life. Firstly, the synbiotic tempeh extract contains antibacterial agents in origin. The plantaricin, phenol, acetic acid and lactic acid are the common forms of antibacterial agents in the sample[26]. The concentration of the acids further increased during the longer shelf life as the pH of the extracts reduced. The Enterobacteriaceae also has the possibility of acid stress response failure and thus could not survive in low pH environment[58]. The acidic environment also leads to lower lactose content in the extracts. In the samples with longer shelf life, it is more difficult for the bacteria to obtain lactose as their nutrient source, for both *Lactobacillus plantarum* Dad 13 and Enterobacteriaceae.

Categorization of the shelf life helps to determine whether there was an alteration of the antibacterial activity and total Enterobacteriaceae either due to the iron addition or the storage itself. Fermented tempeh extract with iron fortification had been analyzed for its efficacy in hemoglobin improvement of anemic Wistar rats and the result suggested a significant improvement compared to the control group[31]. Another study conducted by Sudargo *et al*.[59] about NaFeEDTA fortification on tempeh showed better bioavailability of the iron and it did not change the sensory properties significantly.

The studies about formulation of novel foods containing antibacterial agents have been developing rapidly. This type of study is important since there is an increase of microbial infection in humans which causes higher risk of morbidity and mortality[60]. The present study proposed the broad spectrum of inhibitory effect of the synbiotic fermented milk with tempeh extract and iron fortification towards gram-positive and gram-negative bacteria. It was proved by the ability of the sample to inhibit the growth of *E. coli* and *S. aureus* even though the bacteria species used in this study was limited.

The well diffusion method conducted in this study proposes a relatively quick and simple method to measure the antibacterial activity. An *in vitro* study is powered for its ease of culture compared to an *in vivo* study and is more economical. This method also prevents the actual human contamination and confounding factors that can alter the results although the conclusions cannot be generated as if the intervention was conducted in human trial[34].

The enumeration of Enterobacteriaceae in the product also supports the result of antibacterial activity measurements. It proves that the iron-fortified synbiotic fermented milk with tempeh extract contains antibacterial agents which are beneficial to prevent the growth of pathogens. However, it is the present limitation of the study to not investigate and determine the source of contamination yet. The hazards that may occur were anticipated by not giving the high dose of sample with short shelf life to humans.

More research is needed to improve the effectiveness of iron-fortified synbiotic fermented milk with tempeh extract in order to address anemia in the future. The identification of the source contamination is urgently needed to prevent the

hazards of Enterobacteriaceae or specific bacteria which are included in the Enterobacteriaceae family. The measurements of antibacterial activity against other pathogenic bacteria and in longer categories of shelf life are also needed.

CONCLUSION

Synbiotic fermented milk with tempeh extract contains antibacterial agents that have the potential to combat either gram-negative or Gram-positive bacteria. The addition of iron fortification does not change the antibacterial activity of the fermented extract but may support the growth of Enterobacteriaceae, mainly if the fortification is in $FeSO_4$ forms. Due to the presence of total Enterobacteriaceae, consuming excessive amounts of synbiotic fermented milk with tempeh extract with short storage period is discouraged.

SIGNIFICANCE STATEMENT

The study discovered the ability of antibacterial activity of tempeh in fermented extract form against gram-positive and gram-negative bacteria. The research showed that the extracts were able to lower the number of Enterobacteriaceae. These findings support the previous study and encourage further research to investigate the effect of synbiotic tempeh extract with iron fortification in human trials. Synbiotic tempeh extract with iron fortification has the potential to be a functional food to treat patients with anemia in the future.

ACKNOWLEDGMENT

The study was funded by *Hibah Riset Unggulan Desentralisasi* Universitas Gadjah Mada 2016. The ethics approval was given by the Institutional Review Board of Faculty of Medicine, Public Health and Nursing of Universitas Gadjah Mada, Indonesia no. KE/FK/257/EC/2016.

REFERENCES

1. Weise, A., 2014. WHA global nutrition targets 2025: Low birth weight policy brief. World Health Organization, Geneva.
2. WHO., 2016. Strategies to prevent anaemia: Recommendations from an expert group consultation. World Health Organization, New Delhi, India.
3. Allen, L., B. de Benoist, O. Dary and R. Hurrell, 2006. Guidelines on Food Fortification with Micronutrients. World Health Organization, Rome, ISBN: 9789241594011, Pages: 370.
4. Emmanuel-Ikpeme, C.A., O.I. Godwin and B.I.A. Mgbeje, 2013. Effect of iron-fortified 'Gari', cassava meal on serum iron, hemoglobin concentration and total iron-binding capacity in Albino Wistar rats. Am. J. Food Technol., 8: 124-132.
5. WHO., 2015. The global prevalence of anaemia in 2011. World Health Organisation, Geneva.
6. Ministry of Health, 2013. Basic health research. Agency for Healthcare Research and Development, Ministry of Health, Jakarta.
7. Lestari, L., 2012. Relation between the knowledge and iron tablet consumption during menstruation phase of the adolescents in state senior high school 2 banguntapan bantul. Universitas Sebelas Maret, Indonesia.
8. Le, H.T., I.D. Brouwer, J. Burema, K.C. Nguyen and F.J. Kok, 2006. Efficacy of iron fortification compared to iron supplementation among Vietnamese schoolchildren. Nutr. J., Vol. 5. 10.1186/1475-2891-5-32.
9. WHO., 2002. World health report 2002. World Health Organization, Geneva.
10. Hammer, N.D. and E.P. Skaar, 2011. Molecular mechanisms of *Staphylococcus aureus* iron acquisition. Annu. Rev. Microbiol., 65: 129-147.
11. Kortman, G.A.M., M. Raffatellu, D.W. Swinkels and H. Tjalsma, 2014. Nutritional iron turned inside out: Intestinal stress from a gut microbial perspective. FEMS Microbiol. Rev., 38: 1202-1234.
12. Andrews, S.C., A.K. Robinson and F. Rodriguez-Quinones, 2003. Bacterial iron homeostasis. FEMS Microbiol. Rev., 27: 215-237.
13. Paganini, D., M.A. Uyoga and M.B. Zimmermann, 2016. Iron fortification of foods for infants and children in low-income countries: Effects on the gut microbiome, gut inflammation and diarrhea. Nutrients, Vol. 8. 10.3390/nu8080494.
14. Silva-Stenico, M.E., F.T.H. Pacheco, J.L.M. Rodrigues, E. Carrilho and S.M. Tsai, 2005. Growth and siderophore production of *Xylella fastidiosa* under iron-limited conditions. Microbiol. Res., 160: 429-436.
15. Naikare, H., K. Palyada, R. Panciera, D. Marlow and A. Stintzi, 2006. Major role for FeoB in *Campylobacter jejuni* ferrous iron acquisition, gut colonization and intracellular survival. Infect. Immunity, 74: 5433-5444.
16. National Standardization Agency, 2006. Guidelines for organoleptic and sensory testing. National Standardization Agency of Republic Indonesia, Jakarta, Indonesia.
17. Shurtleff, W. and A. Aoyagi, 2011. History of Tempeh and Tempeh Products (1815-2011): Extensively Annotated Bibliography and Sourcebook. Soyinfo Center, Lafayette, Louisiana, ISBN: 9781928914396, Pages: 990.
18. Babu, P.D., R. Bhakyaraj and R. Vidhyalakshmi, 2009. A low cost nutritious food Tempeh: A review. World J. Dairy Food Sci., 4: 22-27.
19. Kiers, J.L., J.C. Meijer, M.J.R. Nout, F.M. Rombouts, M.J.A. Nabuurs and J. van der Meulen, 2003. Effect of fermented soya beans on diarrhoea and feed efficiency in weaned piglets. J. Applied Microbiol., 95: 545-552.

20. Kandarina, I., T. Sudargo, S.U. Wisnusanti, V.A.D. Puspitaningrum, S. Helmyati, M. Juffrie and E. Yuliati, 2016. Tempeh extract fortified with iron and synbiotic as a strategy against anemia. Int. Food Res. J., 23: 2296-2299.
21. Yeung, C.K., R.E. Glahn, R.M. Welch and D.D. Miller, 2005. Prebiotics and iron bioavailability-is there a connection? J. Food Sci., 70: R88-R92.
22. Utami, T., Kasmiati, E. Harmayani and E.S. Rahayu, 2016. Survival of *Lactobacillus plantarum* Dad 13 during spray drying and its application for yoghurt fermentation. Int. Res. J. Biol. Sci., 5: 16-22.
23. Widyastuti, Y., Rohmatussolihat and A. Febrisiantosa, 2014. The role of lactic acid bacteria in milk fermentation. Food Nutr. Sci., 5: 435-442.
24. Kaboosi, H., 2011. Antibacterial effects of probiotics isolated from yoghurts against some common bacterial pathogens. Afr. J. Microbiol. Res., 5: 4363-4367.
25. Rozila, A., I.E. Suryani, M.N. Lani, M.D. Sharina, M.S. Hasmah, H. Asma and M.D. Sharida, 2012. Antibacterial activity of lactic acid bacteria isolated from goats' milk. Proceedings of the UMT 11th International Annual Symposium on Sustainability Science and Management, July 9-11, 2012, Terengganu, Malaysia, pp: 540-543.
26. Lafta, H., E.M. Jarallah and A. Darwash, 2014. Antibacterial activity of fermented camel milk using two lactic acid bacteria. J. Babylon Univ./Pure Applied Sci., 22: 2377-2382.
27. El-Sayed, E.M., H.M. El-Zeini, S.A. Hafez and F.A. Saleh, 2014. Antibacterial activity of probiotic yoghurt and soy-yoghurt against *Escherichia coli* and *Staphylococcus aureus*. J. Nutr. Food Sci., Vol. 4. 10.4172/2155-9600.1000303.
28. Baylis, C., M. Uyttendaele, H. Joosten and A. Davies, 2011. The Enterobacteriaceae and their significance to the food industry. International Life Sciences Institute (ILSI), Europe, Belgium.
29. Nagarjun, P.A. and P.N. Rao, 2015. Identification of novel food borne pathogen, enterobacteriaceae bacterium from fresh vegetables and egg products. Int. J. Curr. Microbiol. Applied Sci., 4: 54-64.
30. Mayasari, O.R., 2016. Antibacterial activities of tempeh extract milk from synbiotic fermentation under $FeSO_4$ and NaFeEDTA iron fortifications toward *Escherichia coli* and *Staphylococcus aureus* bacterium. Undergraduate Thesis, Universitas Gadjah Mada, Indonesia.
31. Wigati, M., 2016. Effects of iron fortified synbiotic tempeh milk's shelf life on total Enterobacter, physical characteristic and organoleptic properties. Undergraduate Thesis, Universitas Gadjah Mada, Indonesia.
32. Slinkard, K. and V.L. Singleton, 1977. Total phenol analysis: Automation and comparison with manual methods. Am. J. Enol. Vitic, 28: 49-55.
33. FSSAI., 2012. Manual of methods of analysis of foods. Food Safety and Standards Authority of India (FSSAI), Ministry of Health and Family Welfare, New Delhi, pp: 20-35.
34. Balouiri, M., M. Sadiki and S.K. Ibnsouda, 2016. Methods for *in vitro* evaluating antimicrobial activity: A review. J. Pharm. Anal., 6: 71-79.
35. Masadeh, M.M., K.H. Alzoubi, S.I. Al-Azzam, O.F. Khabour and A.M. Al-Buhairan, 2016. Ciprofloxacin-induced antibacterial activity is atteneuated by pretreatment with antioxidant agents. Pathogens, Vol. 5, No. 1. 10.3390/pathogens5010028.
36. Microbiology Services of Public Health England, 2013. UK standards for microbiology investigations identification of enterobacteriaceae. Bacteriology, 4: 1-34.
37. Fitrotin, U., T. Utami, P. Hastuti and U. Santoso, 2015. Antioxidant properties of fermented sesame milk using *Lactobacillus plantarum* Dad 13. Int. Res. J. Biol. Sci., 4: 56-61.
38. Kunaepah, U., 2008. Effect of fermentation period and glucose concentration on antibacterial activity, total polyphenol and chemical quality of the red bean milk kefir. Ph.D. Thesis, Diponegoro University, Indonesia.
39. Cabrita, M.J., V. Palma, R. Patao and A.M.C. Freitas, 2012. Conversion of hydroxycinnamic acids into volatile phenols in a synthetic medium and in red wine by *Dekkera bruxellensis*. Food Sci. Technol., 32: 106-112.
40. Musikasang, H., N. Sohsomboon, A. Tani and S. Maneerat, 2012. Bacteriocin-producing lactic acid bacteria as a probiotic potential from Thai indigenous chickens. Czech J. Anim. Sci., 57: 137-149.
41. Wu, Y., J. Bai, K. Zhong, Y. Huang, H. Qi, Y. Jiang and H. Gao, 2016. Antibacterial activity and membrane-disruptive mechanism of 3-*p*-trans-coumaroyl-2-hydroxyquinic acid, a novel phenolic compound from pine needles of *Cedrus deodara*, against *Staphylococcus aureus*. Molecules, Vol. 21. 10.3390/molecules21081084.
42. Davis, W.W. and T.R. Stout, 1971. Disc plate method of microbiological antibiotic assay. I. Factors influencing variability and error. Applied Microbiol., 22: 659-665.
43. Hancock, J.T., V. Salisbury, M.C. Ovejero-Boglione, R. Cherry, C. Hoare, R. Eisenthal and R. Harrison, 2002. Antimicrobial properties of milk: Dependence on presence of xanthine oxidase and nitrite. Antimicrob. Agents Chemother., 46: 3308-3310.
44. Haynes, I.N. and M.J. Playne, 2002. Survival of probiotic cultures in low-fat ice-cream. Aust. J. Dairy Technol., 57: 10-14.
45. Ray, B., 2004. Fundamental Food Microbiology. 3rd Edn., CRC Press, Boca Raton, London, pp: 1-529.
46. Barefoot, S.F. and C.G. Nettles, 1993. Antibiosis revisited: Bacteriocins produced by dairy starter cultures. J. Dairy Sci., 76: 2366-2379.
47. Ovali, F., I.H. Ciftci, Z. Cetinkaya and A. Bukulmez, 2006. Effects of human milk fortifier on the antimicrobial properties of human milk. J. Perinatol., 26: 761-763.
48. Hurrell, R.F., M.B. Reddy, J. Burri and J.D. Cook, 2000. An evaluation of EDTA compounds for iron fortification of cereal-based foods. Br. J. Nutr., 84: 903-910.

49. Mirdalisa, C.A., Y. Zakaria and Nurliana, 2016. Effect of temperature and the shelf life on antimicrobial activity of fermented milk with *Lactobacillus casei*. Agripet, 16: 46-55.
50. Mahdy, S.A., Q.J. Raheed and P.T. Kalaichelvan, 2012. Antimicrobial activity of zero-valent iron nanoparticles. Int. J. Mod. Eng. Res., 2: 578-581.
51. Behera, S.S., J.K. Patra, K. Pramanik, N. Panda and H. Thatoi, 2012. Characterization and evaluation of antibacterial activities of chemically synthesized iron oxide nanoparticles. World J. Nano Sci. Eng., 2: 196-200.
52. Centre for Food Safety, 2014. Microbiological guidelines for food: For ready-to-eat food in general and specific food items. Food and Environmental Hygiene Development, Hong Kong, pp: 3-21.
53. Melkamsew, A.T., M. Pal and A.H. Beda, 2012. Bacteriological study on Coliform organisms from Ethiopian traditional cheese West Showa zone, Ethiopia. Int. J. Microbiol. Res., 3: 188-191.
54. Ifeanyi, V.O., E.O. Ihesiaba, O.M. Muomaife and C. Ikenga, 2013. Assessment of microbiological quality of yogurt sold by street vendors in onitsha metropolis, Anambra State, Nigeria. Br. Microbiol. Res. J., 3: 198-205.
55. Westling, M., M.L. Danielsson-Tham, J. Jass, A. Nilsen, A. Ostrom and W. Tham, 2016. Contribution of *Enterobacteriaceae* to sensory characteristics in soft cheeses made from raw milk. Procedia Food Sci., 7: 17-20.
56. Viteri, F.E., R. Garcia-Ibanez and B. Torun, 1978. Sodium iron NaFeEDTA as an iron fortification compound in Central America. Absorption studies. Am. J. Clin. Nutr., 31: 961-971.
57. Messenger, A.J.M. and R. Barclay, 1983. Bacteria, iron and pathogenicity. Biochem. Educ., 11: 54-63.
58. Bearson, S., B. Bearson and J.W. Foster, 1997. Acid stress responses in enterobacteria. FEMS Microbiol. Lett., 147: 173-180.
59. Sudargo, T., F.Z. Nisa, S. Helmiyati, R.J. Kusuma, T. Arjuna and R.D. Septiana, 2013. Tempeh with iron fortification to overcome iron deficiency anemia. Pak. J. Nutr., 12: 815-820.
60. Gagliotti, C., A. Balode, F. Baquero, J. Degener and H. Grundmann *et al*., 2011. *Escherichia coli* and *Staphylococcus aureus*: Bad news and good news from the European antimicrobial resistance surveillance network (EARS-Net, formerly EARSS), 2002 to 2009. Euro Surveill., Vol. 16.