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Editor-in-Chief
Journal of Food Quality

February 18th , 2021

Dear Editor-in-Chief:

I am pleased to submit an original research article entitled "Antioxidant Activity, Microbiological Quality, and Acceptability of Spontaneous-Fermented Shrimp Sausage (Litopenaeus vannamei)" by Diana Nur Afifah, Uchida Titis Sari Dewi, Rizka Diana Anggraeni, Fahmy Arif Tsani, Nurmasari Widyastuti, Faizah Fulyani, and Gemala Anjani for consideration for publication in Journal of Food Quality.

In this manuscript, we show that the effect of varied different fermentation times production on variations in antioxidant activity, vitamin E content, total LAB, total pathogenic bacteria, pH, and acceptability of shrimp sausage. Significant differences were observed in the mean antioxidant activity, levels of vitamin E, total LAB, acidity value (pH), and acceptance level of shrimp sausages treated with different fermentation times (0, 1, 2, and 3 days) at $p \leq 0.05$. Based on the results, the day 1 products were selected as the optimal formulation, considering their bioactive content and the level of consumer acceptance. The product is assumed to be useful in neutralization of free radicals, hence showing an application tendency as a functional food for coronary heart disease.

We believe that this manuscript is appropriate for publication by the Journal of Food Quality very related to aims and scope this journal. Our manuscript creates a paradigm for future studies of the evolution of functional food from Indonesian traditional food.

This manuscript has not been published and is not under consideration for publication elsewhere.

Thank you for your consideration!

Sincerely,

Dr. Diana Nur Afifah
Doctor, Department of Nutrition Science
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Antioxidant Activity, Microbiological Quality, and Acceptability of Spontaneous-Fermented Shrimp Sausage (Litopenaeus vannamei)

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Abstract

Fermented shrimp sausages are prepared spontaneously with *Litopenaeus vannamei* as raw material; they contain chitosan as bioactive compounds, antioxidants, vitamin E, and probiotic lactic acid bacteria (LAB) produced by fermentation processes. The product is assumed to be useful in neutralization of free radicals, hence showing an application tendency as a functional food for coronary heart disease. This study aimed to analyze the variations in antioxidant activity, vitamin E content, total LAB, total pathogenic bacteria, pH, and acceptability of shrimp sausage produced at different fermentation times. A completely randomized experimental design study was performed using four levels of exposure time, including control (0 day), 1, 2, and 3 days. The treatment was conducted spontaneously with 1.2% salt concentration, a drying temperature of 50°C for 3 h, and fermentation at 35°C. The evaluated parameters included the antioxidant activity measured with radical 2,2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid method, vitamin E levels by high-performance liquid chromatography, total LAB with total plate count, and *Escherichia coli* bacteria by the most probable number method. *Salmonella* sp. and *Staphylococcus aureus* were estimated by the identification method. A pH meter was used to assess acidity, and hedonic organoleptic testing was performed for taste, aroma, color, and texture. The results show significant differences in antioxidant activity, vitamin E, and shrimp sausage acceptability at varied fermentation times ($p \leq 0.05$). However, the best formulation was obtained with the first-day fermented shrimp sausage, as shown by its bioactive content and level of acceptability.

Introduction

The Basic Health Research survey conducted in 2018 showed a 1.5% increase in coronary heart disease (CHD) prevalence in Indonesia. This estimate is expected to continuously rise to 23.3 million in 2030 [1]. High levels of cholesterol and low-density lipoproteins are linked as risk factors to CHD [2]. Consuming functional food products that are low in fat and rich in antioxidants can be an alternative choice for preventing CHD. Several marine organisms contain active compounds, such as antioxidants and anti-inflammatory compounds, that can be used as functional food for CHD prevention [3].

Shrimp is a marine product with rich bioactive antioxidant content in the form of chitosan, carotenoids, and monounsaturated fatty acids. The *astaxanthin* constituent has been identified as the most powerful antioxidant of *xanthophyll*. This condition is due to the carotenoid protein characteristics, which include the capability to reduce oxidative stress, protect from inflammation, and neutralize free radicals and oxidants [4]. Shrimps also contain vitamin E, which is assumed to confer protection on active compounds against the degradation process, with a possible value as an antioxidant [5]. The characteristics of bioactive contents indicate their potential application as raw material for fermented sausage for use as an alternative functional food. However, seafood products are susceptible to spoilage due to mechanical, physiological, and microbiological influences. The fermentation process is carried out to enhance shelf life, resulting a distinctive taste and increased product quality. The fermentation process can be possibly conducted spontaneously without the addition of any microorganisms [6]. This process involves protein and amino acid hydrolysis by enzymes of microbial origin, yielding bioactive peptides that are needed as antioxidants [7]. The lactic acid bacteria (LAB) produced demonstrate antioxidant effects that can inhibit free radicals and lower cholesterol levels [8], [9]. The microorganisms generated can reduce the number of harmful pathogens present in the digestive tract upon ingestion and inhibit the growth of spoilage bacteria. This activity results from the ability of LAB to produce several metabolites, including organic acids (lactic and acetic acid), hydrogen peroxide, diacetyl, and bacteriocin. Furthermore, LAB are assumed to possess probiotic effects, maintaining the existence of intestinal microflora and increasing endurance [10], [11]. The fermentation process increases the vitamin E content of shrimp sausages. This outcome results from the ability of LAB to produce primary and secondary metabolites following the hydrolysis of carbohydrates, fats, and proteins [12]. Moreover, the treatment reduces water content due to the capability of added salt to attract hydrogen ions, subsequently causing a decline in the half maximal inhibitory concentration (IC_{50}) [13]. This effect occurs simultaneously with a high antioxidant activity, which is estimated to ensure the capture of free radicals [14]. Referencing *Indonesian National Standard*, dry fermented sausages contain 30%–40% water content and have an aw value of 0.85–0.91 [15].

Sausages are processed meat products that are ground, seasoned, and wrapped in sleeve. The ingredients added affect the *bioactive* and *organoleptic* compound composition. Marination of shrimp with lime juice aims to reduce the fishy scent, therefore improving the product aroma. In addition, adding 2%–4% salt further improves the flavor and inhibits the activity of spoilage microbes, thus facilitating the optimal growth of beneficial LAB [6]. The addition of sugar as a source of carbohydrates for microorganisms to produce lactic acid affects the pH, flavor, and storability of products [16]. Moreover, pepper and ginger enhance the taste and aroma of sausages, with ginger specifically increasing the antioxidant content [17]. In addition, garlic adds to the aroma and is useful as a bacteriostatic and antioxidant agent due to its inherent phenolic compounds and allicin [18]. The incorporated egg whites and oils

serve as emulsifiers, whereas tapioca starch acts as filler to improve the texture [19], [20]. Furthermore, corn oil contains vitamin E (8%) and can confer antioxidant effects; hence, its addition increases bioactivity [21]. The smoking and roasting process affects product chemical composition and quality, and exposure to 50°C temperature for 3 h optimizes antioxidants and generates high protein levels [21]. Therefore, long duration of smoking process is implicated in high LAB generation [22]. The utilization of 5% liquid smoke improves the chemical, organoleptic, and microbiological quality and causes a simultaneous reduction in cholesterol [23]. Moreover, reducing the pH level of fermented sausages to a decent value of 4.5–5.0 inhibits the growth of pathogenic and spoilage bacteria. However, other studies suggest 4.8–5.4 as the proper range [22]. The duration of fermentation also influences the amount of pathogenic microorganisms present in food products, with *E. coli* (15%–20%), *Salmonella* sp. (1%–5%), and *Staphylococcus aureus* [23] being the most common in Indonesia. In addition, the existence of *E. coli* indicates the presence of other microorganisms capable of causing typhus, vomiting, and diarrhea. *Salmonella* sp. are usually found in foods with low environmental hygiene and closely related to the cleanliness of processing, storing, and serving. A poor setup facilitates the production of enterotoxins, which can initiate gastroenteritis and inflammation of the intestinal tract [24]. Based on this background, the study aimed to determine the differences in antioxidant activity, levels of vitamin E, total LAB, total pathogenic bacteria, acidity, and acceptance level of fermented shrimp sausage produced at varied fermentation durations.

Materials and Methods

This one-factor randomized complete design experimental study is classified into the field of Food Technology. The variables were tested at Undip Nutrition Science Laboratory, Integrated Services Unit of Undip Laboratory, Bogor Saraswanti Laboratory, and Health Laboratory of Central Java.

Preparatiom Process of Fermented Shrimp Sausage

The first step involved marinating 62.3% *Litopenaeus vannamei* with 2% lime juice before grinding. Then, 12% ice cubes, 9.3% egg whites, 7.5 % tapioca flour, 3.1% corn oil, 1.2% salt, 1.2% sugar, 0.6% garlic, 0.6% ginger, and 0.2% pepper were added; mixed sequentially; and poured into an edible sausage sleeve. The second step involved smoking and was conducted by soaking the shrimp sausage into a 5% liquid smoke solution for 30 min. The shrimp was subsequently placed in an oven set at 50°C for 3 h. The third stage involved fermenting the sausage from day 0 to day 3 [25]in an incubator with a set temperature of 35°C [11], [23]. The products obtained on day 0 were used as a control.

Antioxidant Activity Test

Antioxidant activity was analyzed using the 2,2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) method [26]. This stock solution was created at a concentration of 7.4 mM. Potassium persulfate (2.6 mM) was also formulated. Both solutions were mixed at a ratio of 1:1 (v/v) and stored for 16–18 h, followed by dilution to an absorbance level of 1.1 ± 0.02 units at a wavelength of 750 nm. Subsequently, the samples were mixed with ABTS at a ratio of 1:2 (100 µL:200 µL) and stored at room temperature for 10 min. The antioxidant activity was expressed as the inhibition percentage and calculated using the following formula:

Inhibition (%) = (blank absorbance - sample absorbance) / (blank absorbance) x 100%

$$\text{Inhibition (\%)} = \frac{\text{absorbance control} - \text{absorbance sample}}{\text{absorbance blanko}}\ x\ 100\% \quad (1)$$

The IC_{50} value can be calculated by the following formula:

$$IC_{50}\ \text{value} = 50\%\ x\ \text{absorbance control} \quad (2)$$

Vitamin E Concentration Test

The vitamin E level was analyzed using high-performance liquid chromatography (HPLC) [27]. HPLC was performed on Shimadzu Prominence system (Shimadzu Corporation, Kyoto, Japan) equipped with solvent delivery system LC-20AD, with a SIL-20AC autosampler, DGU-AS on-line degasser, SPD-M20A DAD detector, CTO 20AC column oven, and CBM-20A communication module. Sample stock solutions were prepared by dissolving 100 g sample in 150 mL 70% ethanol, followed by shaking with 350 mL n-hexane solution. Then, the filtrate was separated using a separatory funnel. The oil part was collected and evaporated using a rotary evaporator vacuum regulated at a temperature of 50°C. Moreover, the HPLC system was set to a stable baseline, and about 25–100 µL sample was injected and analyzed using a mobile phase with a flow rate of 1.5 mL/min (normal phase).

LAB Test

The total LAB were calculated using total plate count with three repetitions. First, all the tools and materials to be used were sterilized in an autoclave at 121°C for 15 min at a pressure of 1 atm and used to create a liquid medium. Second, the samples were homogenized with distilled water. Third, a sterile test tube filled with 9 ml distilled water was combined with 1 ml suspension sample to achieve a homogenized dilution series. Fourth, the planting process was conducted by adding 1 ml distilled water to the control petri dish and 1 ml 10^{-1} dilution suspension sample to the 10^{-1} petri dish up to the last dilution. Then, the media were poured onto a petri dish and incubated at 35°C–37°C for 24 h after solidification. The grown white or yellowish LAB colonies were subsequently collected and counted.

Pathogenic Bacterial Test

Pathogenic bacterial tests were performed to evaluate the presence of *E. coli* using the most probable number method [28]. *S. aureus and Salmonella* sp. were detected using identification method with SNI-01-2332.2-2006. First, the liquid media were developed in an Erlenmeyer flask, followed by the sterilization of tools and materials by autoclaving at 121°C for 15–20 min. Then, 1 ml sample was mixed with 9 ml distilled water in the test tube up to a specific dilution value (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}). Therefore, 1 ml 10^{-1} dilution sample was collected and transferred to a petri dish containing the media before incubating at 35°C–37°C for 48 h. The samples were then evaluated for microbial growth. The affirmative test of *E. coli* was carried out by observing medium color changes to red-green metallic sheen [29] whereas *S. aureus* and *Salmonella* sp. were confirmed positive if the color of the medium agar changed to red and yellow, respectively [30].

Acidity Test (pH)

The pH indicator/electrode was examined and calibrated with a solution of pH = 7 before rinsing with distilled water and subsequently dried. A slice of fermented shrimp sausage sample was assessed using calibrated pH electrodes placed on a glass, pending the attainment of stable and readable values.

Organoleptic Test

Organoleptic properties were evaluated in 30 respondents using a hedonic test questionnaire. This tool examined the aspects of color, aroma, texture, and taste with four scales: 1 = worse, 2 = bad, 3 = good, and 4 = best (Lim, 2011). The best formulation was selected using a weighting technique considering the hedonic test attributes (color, aroma, texture, and taste) and the bioactive component.

Data Analysis

Data analysis was performed using a statistical software program with a p value of ≤ 0.05, whereas normality was evaluated using *Shapiro–Wilk* test for values <50. The antioxidant activity, vitamin E levels, and acidity (pH) were tested using *analysis of variance*. Meanwhile, *Kruskal–Wallis test* was adopted in the assessment of LAB, pathogenic bacteria, and the level of acceptance.

Results and Discussion

Antioxidant Activity

Table 1 presents the shrimp sausages produced at different fermentation times, demonstrating variation in the mean percentage of inhibition ($p < 0.05$). In addition, the highest value was obtained on the 3rd day at 42.09% per 0.1 g, whereas the least value was recorded on day 0 (control) at 23.93% per 0.1 g.

Table 1. Results of the inhibition percentage (%) of fermented shrimp sausages at different fermentation times

Duration	Inhibition (%)
Fresh Prawn	17.36 ± 2.746*
0 day	23.93 ± 2.927*
1 day	26.57 ± 3.935*
2 day	34.32 ± 4.956
3 day	42.09 ± 5.994*
	P = 0,005[a]

**Significance < 0.05; [a]ANOVA: analysis of variance*

Antioxidant activity is the capability for oxidants to capture free radicals, and a high level indicates the need for less food to ensure optimal acquisition [31]. The results of statistical tests showed differences in the average percent inhibition value (p = 0.005) and IC_{50} (p = 0.024). In addition, the highest antioxidant activity was measured in shrimp sausages treated with 3-day fermentation time. This sample demonstrated a percentage inhibition value of 42.09% per 0.1 g and an IC_{50} of 1.161 ppm and was hence classified in the very powerful category.

The increased activity was due to the protein and amino acid hydrolysis caused by LAB-produced enzymes into bioactive peptides with the capability to inhibit free radicals [7]. The fermentation process can produce probiotic LAB that have antihypertensive and cholesterol-lowering effects and inhibit the growth of pathogen bacteria [8], [9]. These activities are also influenced by the addition of ginger, garlic, and corn oils containing phenolic compounds, alongside the sausage preparation process before fermentation at 50°C. Furthermore, the smoking treatment using liquid smoke has been implicated due to the phenolic compounds, organic acids, and carbonyl content, which are assumed to serve as flavors, bacteriostatic, and antioxidant agents [32].

IC_{50} Value

Table 2 presents the average IC_{50} value of shrimp sausages produced at different fermentation durations varied significantly ($p < 0.05$). This finding is congruent with those of previous studies, including the report on kombucha fermentation for 0, 1, 2, and 3 days, in which IC_{50} increased from 120 ppm to 54.46 ppm [33]. Furthermore, the best outcome was recorded on the third day at 1.161 ppm, whereas the least (1.849 ppm) was observed on day 0 (control). Moreover, a food is considered to possess very strong antioxidant characteristics at $IC_{50} < 10$ ppm [34].

Table 2. Results of IC_{50} (ppm) of fermented shrimp sausages at different fermentation times

Duration	IC_{50} Value (ppm)
Fresh shrimp	2.99 ± 0.227*
0 day	1.849 ± 0.189*
1 day	1.655 ± 0.276
2 day	1.597 ± 0.256
3 day	1.161 ± 0.069*
	p=0.024[a]

**Significance <0.05; [a]ANOVA: analysis of variance*

Vitamin E Level

Table 3 presents the average levels of vitamin E, which differed between shrimp sausages produced at different fermentation durations *($p < 0.05$)*. In addition, the highest result at 4.985 mg/100 g was recorded on the third day, whereas the least (2.685 mg/100 g) was obtained on day 0 (control).

Table 3. Results of vitamin E level (mg/dl) of fermented shrimp sausages at different fermentation times

Duration	Vitamin E Levels (mg/100gr)
Fresh shrimp	1.515 ± 0.007*
0 day	2.685 ± 0.007*
1 day	3.355 ± 0.007*
2 day	4.090 ± 0.000*
3 day	4.985 ± 0.021*
	$P < 0{,}001$[a]

***Significance < 0.05; [a]ANOVA: analysis of variance*

Vitamin E is one of the compounds contained in shrimp; it is characterized by a phenol group on the 6-chromanol ring and believed to be capable of inhibiting free radicals and preventing fat oxidation. Processed foods are considered a source of vitamin E, possessing a minimum of 15% nutrition label reference in solid form or 7.5% nutrition label reference in liquid form. Meanwhile, the potential for high content is considered in cases where over twice the amount is present in the source [35].

During the fermentation process, LAB produce metabolites from the hydrolysis of carbohydrates, fats, and proteins, including the primary forms from small compounds, e.g., vitamins and minerals, and secondary forms, e.g., antibiotics, enzyme inhibitors, and growth boosters [12]. Previous studies on gude seed *tempe* have attributed the increase in content of dissolved proteins, vitamins, and available minerals to the presence of molds. These microorganisms grow on the sample and hydrolyze intrinsic complex compounds into simpler forms [36], [37]. The increase in levels of vitamin E present in shrimp sausages is also caused by the addition of vitamin E (8%)-containing corn oil, which acts as antioxidants by inhibiting free radicals.

Total LAB

Table 4 shows the average difference in the total LAB of shrimp sausages treated with varied fermentation durations ($p < 0.05$). The highest value (13.99 x 10^9 CFU/ml) was recorded on day 3, where the least (0.429 x 10^9 CFU/ml) was observed on day 0 (control).

Table 4. Result of total LAB of fermented shrimp sausages at different fermentation times

Duration	Total of LAB (10^9CFU/ml)
0 day	0.429 ± 0.422
1 day	5.055 ± 4.879
2 day	8.437 ± 8.676
3 day	13.88 ± 9.988
	p = 0.048*[b]

*****Significance < 0.05; [b]Kruskal–Wallis Test*

The total LAB were positively influenced by the fermentation duration. This finding is an indicator of the ability for growth in the initial phase, prompting adjustments to the inhabiting substrate. Furthermore, this condition causes cell multiplication and active transport processes, which are expected to facilitate the production of protons as nutrients for survival. In addition, a high concentration of LAB was observed alongside the extended fermentation duration, with a tendency to overhaul the nutrients contained in the substrate. This phenomenon possibly allows the accumulation of organic acids in high amounts [38].

The initial phase of fermentation features the growth pattern adaptation of LAB. The first-day fermented shrimp sausage formulation showed an increase in total bacteria, resulting from the dominant activity of the microorganism to convert carbohydrates into lactic acid. This phase is classified as logarithmic, at which the bacteria quickly and constantly experienced a rapid growth rate. The speed of development was greatly influenced by the growth media, pH of nutrient content, and environmental conditions, including temperature, light, and air humidity. In addition, the media chemical composition changed because of the product synthesis following substrate utility. The concentration of alcohol and CO_2 produced increased, possibly up to levels toxic for the survival of microbes [39]. In addition, an

increase in the LAB population caused a decline in pH and an increase in acidity. This condition is unsuitable for certain LAB, e.g., *Lactobacillus bulgaricus, Streptococcus thermophilus, and Lactobacillus acidophilus*, thus leading to bacterial death.

Pathogenic Bacteria

The identification test results from days 0, 1, 2, and 3 showed negative/25 gr for *Salmonella* sp., <3/gr in the case of *Escherichia coli*, and 1 x 10^2 CFU/gr for *Staphylococcus aureus*. These findings are congruent with the Indonesian National Standard for food and beverages. The cumulation of large amount of LAB in the product can inhibit pathogenic bacteria. This result is due to the high bactericidal effect at low pH [40]. In addition, different types of LAB produced varied inhibition and activities due to the influence of metabolite components produced. Acetic acid, formic acid, succinic acid, ethanol, hydrogen peroxide, and diacetyl were also generated by LAB action. These products are antagonistic and can inhibit the growth of other bacteria [41].

Acidity Level (pH)

Table 5 presents the average pH level of shrimp sausages with differences in fermentation durations ($p < 0.05$). The highest value was observed on day 0 (6.6), whereas the least was recorded on day 3 (5.71). The fermentation duration affected the extent of pH decline, resulting from the conversion of glucose to lactic acid. Fermented catfish sausage by smoking for 30 min at temperature of 30°C can increase LAB levels and lower pH [25]. Therefore, low values alongside elevated concentrations of lactic acid further inhibit pathogenic bacterial growth [42]. The recommended pH for fermented sausage is between 4.8–5.4[43].

Table 5. Results of acidity value (pH) of fermented shrimp sausages at different fermentation times

Duration	pH Value
0 day	6.600 ± 0.327
1 day	6.290 ± 0.209
2 day	6.027 ± 0.347
3 day	5.710 ± 0.471*
	*p = 0.022**

**Significance < 0.05; [a]ANOVA: analysis of variance*

Organoleptic Test

Table 6. Results of organoleptic analysis of fermented shrimp sausages at different fermentation times

Duration	Categories (Mean ± SD)				Mean
	Color	Aroma	Texture	Taste	
0 day	3.20 ± 0.76[a]	2.97 ± 0.99[a]	2.30 ± 0.87[a]	2.47 ± 0.86[a]	2.73
1 day	2.73 ± 0.82[b]	2.73 ± 0.94[b]	2.57 ± 0.77[a]	1.97 ± 0.76[a]	2.5
2 day	2.63 ± 0.66[b]	1.60 ± 0.81[c]	1.83 ± 0.69[b]	1.57 ± 0.72[b]	1.9
3 day	2.00 ± 0.94[c]	1.90 ± 0.88[d]	1.50 ± 0.73[c]	2.17 ± 1.05[a]	1.89
	p < 0.001*	*p* < 0.001*	p < 0.001*	*p* = 0.001*	

*Different superscript letters (a, b, c, and d) show significant differences between groups; *Kruskal–Wallis test*

Differences were observed in shrimp sausage color, following the variations in fermentation duration ($p < 0.05$) presented in Table 6. In addition, the highest intensity was observed on day 0 and the least on day 3. Color changes were also observed during storage. This finding is attributed to the naturally existing pigments in food, caramelization, and Maillard reactions, interaction between organic compounds and air [44]. Moreover, prolonged fermentation duration increased the dark color concentration of fermented shrimp sausage. This phenomenon was due to the presence of H_2O_2 produced by microorganisms through *aerobic metabolism*. Furthermore, the reactive compound formed is assumed to cause a decline in the red pigmentation, following the formation of brown *metmyoglobin* [45]. The smoking process has also been implicated in color changes, resulting from the smoke absorption capacity of product surface, as evidenced by a reaction between carbonyl groups. This condition further leads to a brownish pigmentation in sausages [46].

A significant difference was observed between the aroma of shrimp sausages prepared at varied fermentation durations ($p < 0.05$). The highest level of aroma was observed on day 0 and the lowest on day 2. Moreover, Maillard reactions occurred following an interaction between amino groups and reducing sugars. This phenomenon was assumed to produce volatile compounds during the fermentation process [45]–[47]. Therefore, a long treatment duration causes a decline in the pH, alongside an increase in the amount of lactic acid produced from LAB metabolism. This condition further stimulates the aroma of fermented shrimp sausages to be more acidic [48].

The texture of shrimp sausages differed across the treatments with varied durations of fermentation ($p < 0.05$). In addition, samples obtained on day 1 demonstrated the best texture, whereas the poorest result was recorded on day 3. A chewy texture was caused by the presence of *amylose* and *amylopectin* in tapioca flour [35]. This shrinking is affiliated with the fermentation duration and assumed to play a role in hardening the texture. In addition, this parameter is influenced by product pH, given that low values are implicated in the high tendency for meat shrinkage [35].

The flavor of shrimp sausages varied at different fermentation durations ($p < 0.05$). Furthermore, the highest flavor acceptance was observed on day 0 and the lowest on day 2. Flavor is a stimulus of food, especially by the sense of taste and odor [35]. The fermentation process is characterized by the degradation of compounds, including the conversion of proteins into amino acids and peptides. These activities cause specific flavors [35]. In addition, long processes and the decline in pH instigate a rise in acid levels. The increased amount of lactic acid generated leads to high acidity in the taste of sausages.

Selected Formula Weigthing

Tables 7 and 8 present the weighting results of hedonic test attributed to the indicators of color, aroma, texture, and taste. A 25% weighting on each indicator, including color, aroma, texture, and taste, was provided on the fermented shrimp sausages. The first rank was obtained by treatment samples for day 0, followed by F1, F2, and F3. Therefore, F1 was selected because F0 was not fermented.

Table 7. Results of weighting hedonic test

Indicator	Day 0 (F0/Control)	Day 1 (F1)	Day 2 (F2)	Day 3 (F3)
Color (25%)	0.8	0.68	0.65	0.5
Aroma (25%)	0.74	0.68	0.4	0.47
Texture (25%)	0.57	0.64	0.45	0.37
Flavor (25%)	0.61	0.49	0.39	0.54
Total	2.72	2.49	1.89	1.88

Table 8. Determination of selected formulation score

Treatment	Hedonic Test (25%)	Score	Antioxidant Test (25%)	Score	Vitamin E Test (25%)	Score	Total
F0	1	0.25	1	0.25	1	0.25	0.75
F1	4	1	2	0.5	2	0.5	2
F2	3	0.75	3	0.75	3	0.75	2.25
F3	2	0.5	4	1	4	1	2.5

Based on the weighting results, the third-day fermented shrimp sausage formulation (F3) was selected because of its bioactive content. However, its hedonic test score was the lowest and was not favored by panelists. Therefore, F1 was selected considering the bioactive constituents and the level of consumer acceptance. The hedonic quality assessment showed the panelists an acceptance level of 1.97, which indicates a low preference, whereas the acceptance values of color, aroma, and texture were 2.73, 2.73, and 2.57, respectively. Therefore, the first-day fermented shrimp sausage (F1) was selected, considering the bioactive content and the level of consumer acceptance.

Conclusions

Significant differences were observed in the mean antioxidant activity, levels of vitamin E, total LAB, acidity value (pH), and acceptance level of shrimp sausages treated with different fermentation times (0, 1, 2, and 3 days) at $p \leq 0.05$. Based on the results, the day 1 products were selected as the optimal formulation, considering their bioactive content and the level of consumer acceptance.

Data Availability

The fermented shrimp sausage's ingredients, inhibition percentage (%), IC_{50} (ppm), vitamin E level (mg/dl), total LAB (10^9CFU/ml), acidity value (pH), organoleptic analysis, and weighting hedonic test of fermented shrimp sausages at different fermentation times used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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Mail FK UNDIP

diana nurafifah <d.nurafifah.dna@fk.undip.ac.id>

5553432: Revision requested

Zotta Teresa <support@hindawi.com> 17 August 2021 at 15:36
Reply-To: Alaiza Alaiza <alaiza.montuano@hindawi.com>
To: Afifah Diana Nur <d.nurafifah.dna@fk.undip.ac.id>

Hindawi

Dear Afifah Diana Nur,

In order for your submission "Antioxidant Activity, Microbiological Quality, and Acceptability of Spontaneous-Fermented Shrimp Sausage (Litopenaeus vannamei)" to Journal of Food Quality to proceed to the review process, there needs to be a revision.

Reason & Details:

“

Dear Authors, I am regret to inform you that the manuscript cannot be published in this form. Major revisions are required. Please, find the reviewers' comments below: Reviewer 1 The work was prepared to analyse effects of the spontaneous fermentation on the parameters of the shrimp sausage. Introduction: The Basic Health Research survey conducted in 2018 showed a 1.5% increase in coronary heart disease (CHD) prevalence in Indonesia. This estimate is expected to continuously rise to 23.3 million in 2030 Answer: First Authors mention percentage, then millions. It is not clear how many millions is meant in the first sentence. Consuming functional food products that are low in fat and rich in antioxidants can be an alternative choice for preventing CHD A: Alternative to what? The smoking and roasting process affects product chemical composition and quality, and exposure to 50°C temperature for 3 h optimizes antioxidants and generates high protein levels [21] A: Firstly - smoking and roasting cannot generate protein. It can, however, denature protein and make it easier to digest. Furthermore, reference 21 is about mayonnaise and not smoking. Therefore, long duration of smoking process is implicated in high LAB generation A:I do not see the reason for that. High concentration of protein is not needed for high LAB generation, main goal is high sugar concentration. Moreover, reducing the pH level of fermented sausages to a decent value of 4.5–5.0 inhibits the growth of pathogenic and spoilage bacteria A: What is ‘decent’ value? Wrong word. Materials and methods: The first step involved marinating 62.3% Litopenaeus

Materials and methods: The first step involved marinating 62.5% Litopenaeus vannamei with 2% lime juice before grinding. Then, 12% ice cubes, 9.3% egg whites, 7.5 % tapioca flour, 3.1% corn oil, 1.2% salt, 1.2% sugar, 0.6% garlic, 0.6% ginger, and 0.2% pepper were added A: What kind of percentage is this? Please clarify. Also, how were the shrimp prepared before? Were they frozen or not? At what temperature were they frozen? Were some of the ingredients pasteurised or not? What kind of garlic, ginger and pepper was used? What was the sugar used (was it saccharose or not?). In how many repetitions were the samples prepared? This section needs clarifying. Also, what kind of 'liquid smoke' was used? Organoleptic Test A: What was the temperature of the samples tested? What were the lightning conditions? How were the samples stored before testing? Were all the samples tested at the same day, or not? If on the same day, then how were they stored before testing? Results and discussion This sample demonstrated a percentage inhibition value of 42.09% per 0.1 g and an IC50 of 1.161 ppm and was hence classified in the very powerful category. A: By what criteria it was classified? The increased activity was due to the protein and amino acid hydrolysis caused by LAB-produced enzymes into bioactive peptides with the capability to inhibit free radicals A: How is it known? These parameters were not tested, this is just the hypothesis based on the results from other studies. It cannot be said that this was precisely the reason preparation process before fermentation at 50°C A: Fermentation was performed at 35 degrees, smoking was performed at 50. Vitamin E is one of the compounds contained in shrimp; it is characterized by a phenol group on the 6-chromanol ring and believed to be capable of inhibiting free radicals and preventing fat oxidation A: It is not only believed, but it is a proven fact. Also, add reference. Furthermore, this condition causes cell multiplication and active transport processes, which are expected to facilitate the production of protons as nutrients for survival. A: Describing metabolic process with the H+ transfer as "production of protons as nutrients for survival" is a little bit far-fetched. Describe it as it truly is. Furthermore, a reference. The speed of development was greatly influenced by the growth media, pH of nutrient content, and environmental conditions, including temperature, light, and air humidity. In addition, the media chemical composition changed because of the product synthesis following substrate utility. A: Once again - how is it known? There is no data about these conditions in the experiments. All the sausages were fermented in the same condition, so Authors cannot say whether light or humidity had an impact on the microbial metabolism. Furthermore - speed of development was not precisely monitored during the experiment. Acetic acid, formic acid, succinic acid, ethanol, hydrogen peroxide, and diacetyl were also generated by LAB action. These products are antagonistic and can inhibit the growth of other bacteria A: How is this known, that these compounds were produced? They were not analysed there is no data. the dark color concentration A: What is concentration of dark color? This phenomenon was due to the presence of H_2O_2 produced by microorganisms through aerobic metabolism A: Was the process of sausage production an fermentation (anaerobic metabolism) or not? A chewy texture was caused by the presence of amylose and amylopectin in tapioca flour [35] A: Amylose and amylopectin are enzymes, which can affect the starch in the flour. They do not create a 'chewy texture'. These details should be corrected. Also, manuscript needs expanded discussion and comparison with work of other authors. Reviewer 3 The authors investigated the effect of varied different

authors. Reviewer 3 The authors investigated the effect of varied different fermentation times on variations in antioxidant activity, vitamin E content, total LAB, total pathogenic bacteria, pH, and acceptability of shrimp sausage. This topic is interesting and worthwhile in practice. Overall, the manuscript was well written and it can be considered for publication in Journal of Food Quality, yet the authors should make the following revisions. 1. Lines 14-18, abstract should be concise, even though a short background sentence can be allowed in Abstract section. 2. Lines 58, the two references [8],[9] should be merged into [8, 9]. Please check the whole manuscript with the same citation format. 3. Line 116, please provide the incubator brand and manufacturer. 4. line 144, please add a reference 5. line 172, please add a reference for hedonic test questionnaire. 6. line 177, please be specific for statistical software program used. 7.lines 245-246, The highest value (13.99 x 109 CFU/ml) , (0.429 x 109 CFU/ml), please use the correct mark for “x” 8. References, please be unified for journal name, I noticed that some use journal abbreviation, others use full journal name. Some references are with a doi , while others are not. Sincerely, Teresa Zotta

For more information about what is required, please click the link below.

MANUSCRIPT DETAILS

Kind regards,
Zotta Teresa

Journal of Food Quality

Antioxidant Activity, Microbiological Quality, and Acceptability of Spontaneous-Fermented Shrimp Sausage (Litopenaeus vannamei)

Diana N. Afifah,[1,2] Uchida T. S. Dewi,[1] Rizka D. Anggraeni,[1] Fahmy A. Tsani,[1,2] Nurmasari Widyastuti,[1,2] Faizah Fulyani,[3] and Gemala Anjani[1,2]

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Correspondence should be addressed to Diana N. Afifah; d.nurafifah.dna@fk.undip.ac.id

Abstract

Fermented shrimp sausages are prepared spontaneously with *Litopenaeus vannamei* as raw material. ~~; they~~ Shrimp is one of marine source whith rich ~~contain~~ chitosan as bioactive compounds, antioxidants, vitamin E, and probiotic lactic acid bacteria (LAB) produced by fermentation processes. ~~The product is assumed to be useful in neutralization of free radicals, hence showing an application tendency as a functional food for coronary heart disease~~. This study aimed to analyze the variations in antioxidant activity, vitamin E content, total LAB, total pathogenic bacteria, pH, and acceptability of shrimp sausage produced at different fermentation times. A completely randomized experimental design study was performed using four levels of exposure time, including control (0 day), 1, 2, and 3 days. The treatment was conducted spontaneously with 1.2% salt concentration, a drying temperature of 50°C for 3 h, and fermentation at 35°C. The evaluated parameters included the antioxidant activity measured with radical 2,2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid method, vitamin E levels by high-performance liquid chromatography, total LAB with total plate count, and *Escherichia coli* bacteria by the most probable number method. *Salmonella* sp. and *Staphylococcus aureus* were estimated by the identification method. A pH meter was used to assess acidity, and hedonic organoleptic testing was performed for taste, aroma, color, and texture. The results show significant differences in antioxidant activity, vitamin E, and shrimp sausage acceptability at varied fermentation times ($p \leq 0.05$). However, the best formulation was obtained with the first-day fermented shrimp sausage, as shown by its bioactive content and level of acceptability.

Commented [i1]:
Reviewer 3:
(2) Line 15-17, Please rewrite the sentence “Shrimp is one of marine source with rich chitosan as bioactive compounds, antioxidants, vitamin E, and probiotic lactic acid bacteria (LAB) produced by fermentation processes”.

Answer:
Done

Commented [i2]:
Reviewer 3:
(1) Abstract: Lines 14-18. Please be concise for the background introduction

Answer:
Done

Commented [i3]:
Reviewer 3:
Lines 14-18, abstract should be concise, even though a short background sentence can be allowed in Abstract section.

Answer: The correction have been done

Introduction

Coronary heart disease (CHD) is one of the major causes of death in Indonesia and ~~The Basic Health Research survey conducted in 2018~~ its prevalence showed an increase of 1.5% ~~increase in coronary heart disease (CHD) prevalence~~ (15.259 people) in 2018 [1]. ~~in Indonesia~~. This estimate is expected to continuously rise to 23.3 million in 2030 [1]. High levels of cholesterol and low-density lipoproteins are linked as risk factors to CHD [2]. Consuming functional food products that are low in fat and rich in antioxidants can be an alternative diet choice for preventing CHD. Several marine organisms contain active compounds, such as antioxidants and anti-inflammatory compounds, that can be used as functional food for CHD prevention [3].

Shrimp is a marine product with rich bioactive antioxidant content in the form of chitosan, carotenoids, and monounsaturated fatty acids. The *astaxanthin* constituent has been identified as the most powerful antioxidant of *xanthophyll*. This condition is due to the carotenoid protein characteristics, which include the capability to reduce oxidative stress, protect from inflammation, and neutralize free radicals and oxidants [4]. Shrimps also contain vitamin E, which is assumed to confer protection on active compounds against the degradation process, with a possible value as an antioxidant [5]. The characteristics of bioactive contents indicate their potential application as raw material for fermented sausage for use as an alternative functional food. However, seafood products are susceptible to spoilage due to mechanical, physiological, and microbiological influences. The fermentation process is carried out to enhance shelf life, resulting a distinctive taste and increased product quality. The fermentation process can be possibly conducted spontaneously without the addition of any microorganisms [6]. This process involves protein and amino acid hydrolysis by enzymes of microbial origin, yielding bioactive peptides that are needed as antioxidants [7]. The lactic acid bacteria (LAB) produced demonstrate antioxidant effects that can inhibit free radicals and lower cholesterol levels [8,9]. The microorganisms generated can reduce the number of harmful pathogens present in the digestive tract upon ingestion and inhibit the growth of spoilage bacteria. This activity results from the ability of LAB to produce several metabolites, including organic acids (lactic and acetic acid), hydrogen peroxide, diacetyl, and bacteriocin. Furthermore, LAB are assumed to possess probiotic effects, maintaining the existence of intestinal microflora and increasing endurance [10,11]. The fermentation process increases the vitamin E content of shrimp sausages. This outcome results from the ability of LAB to produce primary and secondary metabolites following the hydrolysis of carbohydrates, fats, and proteins [12]. Moreover, the treatment reduces water content due to the capability of added salt to attract hydrogen ions, subsequently causing a decline in the half maximal inhibitory concentration (IC_{50}) [13]. This effect occurs simultaneously with a high antioxidant activity, which is estimated to ensure the capture of free radicals [14]. Referencing *Indonesian National Standard*, dry fermented sausages contain 30%–40% water content and have an aw value of 0.85–0.91 [15].

Sausages are processed meat products that are ground, seasoned, and wrapped in sleeve. The ingredients added affect the *bioactive* and *organoleptic* compound composition. Marination of shrimp with lime juice aims to reduce the fishy scent, therefore improving the product aroma. In addition, adding 2%–4% salt further improves the flavor and inhibits the activity of spoilage microbes, thus facilitating the optimal growth of beneficial LAB [6]. The addition of sugar as a source of carbohydrates for microorganisms to produce lactic acid affects the pH, flavor, and storability of products [16]. Moreover, pepper and ginger enhance the taste and aroma of sausages, with ginger specifically increasing the antioxidant content [17]. In

Commented [i4]:
Reviewer 1:
First Authors mention percentage, then millions. It is not clear how many millions is meant in the first sentence.

Answer:
The correction have been done in this sentences according recommendation from reviewer.

Commented [i5]:
Reviewer 1:
Alternative to what?

Answer:
The correction have been done in this sentences.
Consumig functional food products that are low in fat and rich in ntioxidants can be an alternative diet choice for preventing CHD

Commented [i6]:
Reviewer 3:
Lines 58, the two references [8],[9] should be merged into [8, 9]. Please check the whole manuscript with the same citation format.

Answer:
Done

addition, garlic adds to the aroma and is useful as a bacteriostatic and antioxidant agent due to its inherent phenolic compounds and allicin [18]. The incorporated egg whites and oils serve as emulsifiers, whereas tapioca starch acts as filler to improve the texture [19,20]. Furthermore, corn oil contains vitamin E (8%) and can confer antioxidant effects; hence, its addition increases bioactivity [21]. The smoking and roasting processes affects ~~product~~ the texture, chemical composition and quality of the product, such as the colour, flavour and juiciness [22]. Another study indicated that the interaction of pH 4 and ~~exposure to~~ 50°C temperature for 10 min~~3 h~~ optimizes antioxidant~~s~~ activity and increases the concentration of solubility ~~generates high~~ protein ~~levels~~ [23]. Therefore~~, long duration of smoking process~~ the LAB generation during fermentation process is implicated to reduce the ph matrix through production of lactic acid from the fermentation of sugar~~in high LAB generation~~ [24] . The utilization of 5% liquid smoke improves the chemical, organoleptic, and microbiological quality and causes a simultaneous reduction in cholesterol [25]. Moreover, reducing the pH level of fermented sausages ~~to a decent value of~~ (4.5–5.0) inhibits the growth of pathogenic and spoilage bacteria. However, other studies suggest 4.8–5.4 as the proper range [26]. The duration of fermentation also influences the amount of pathogenic microorganisms present in food products, with *E. coli* (15%–20%), *Salmonella* sp. (1%–5%), and *Staphylococcus aureus* [25] being the most common in Indonesia. In addition, the existence of *E. coli* indicates the presence of other microorganisms capable of causing typhus, vomiting, and diarrhea. *Salmonella* sp. are usually found in foods with low environmental hygiene and closely related to the cleanliness of processing, storing, and serving. A poor setup facilitates the production of enterotoxins, which can initiate gastroenteritis and inflammation of the intestinal tract [27]. Based on this background, the study aimed to determine the differences in antioxidant activity, levels of vitamin E, total LAB, total pathogenic bacteria, acidity, and acceptance level of fermented shrimp sausage produced at varied fermentation durations.

Materials and Methods

This one-factor randomized complete design experimental study is classified into the field of Food Technology. The variables were tested at Undip Nutrition Science Laboratory, Integrated Services Unit of Undip Laboratory, Bogor Saraswanti Laboratory, and Health Laboratory of Central Java.

Preparatiom Process of Fermented Shrimp Sausage

The first step involved marinating 62.3% *Litopenaeus vannamei* with 2% lime juice before grinding. Then, 12% ice cubes, 9.3% egg whites, 7.5 % tapioca flour, 3.1% corn oil, 1.2% salt, 1.2% sugar, 0.6% garlic, 0.6% ginger, and 0.2% pepper were added; mixed sequentially; and poured into an edible sausage sleeve. The second step involved smoking and was conducted by soaking the shrimp sausage into a 5% liquid smoke solution for 30 min. The shrimp was subsequently placed in an oven set at 50°C for 3 h. The third stage involved fermenting the sausage from day 0 to day 3 [28]in an incubator (Esco,® model IFA-54-8 Esco Micro Pte. Ltd.)with a set temperature of 35°C [11,25]. The products obtained on day 0 were used as a control.

Antioxidant Activity Test

Antioxidant activity was analyzed using the 2,2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) method [29]. This stock solution was created at a concentration of 7.4 mM. Potassium persulfate (2.6 mM) was also formulated. Both solutions were mixed at a ratio of

Commented [i7]:
Reviewer 1:
Firstly - smoking and roasting cannot generate protein. It can, however, denature protein and make it easier to digest. Furthermore, reference 21 is about mayonnaise and not smoking.

Answer: The correction have been done in this sentences. Author added some reference to complete this part.

Commented [i8]:
Reviewer 1:
I do not see the reason for that. High concentration of protein is not needed for high LAB generation, main goal is high sugar concentration.

Answer: The correction have been done in this sentences according recommendation from reviewer. Author replaced another reference to complete this part.

Commented [i9]:
Reviewer 1:
What is 'decent' value? Wrong word.

Answer: The correction have been done in this sentences.

Commented [i10]:
Reviewer 1:
1.What kind of percentage is this? Please clarify.
2.Also, how were the shrimp prepared before?
3.Were they frozen or not?
4.At what temperature were they frozen?
5.Were some of the ingredients pasteurised or not?
6.What kind of garlic, ginger and pepper was used?
7.What was the sugar used (was it saccharose or not?).
8.In how many repetitions were the samples prepared? This section needs clarifying.
9.Also, what kind of 'liquid smoke' was used?

Answer:
1.This percentage is a ratio composition of each ingredients from the whole raw material (100%)
2.The fresh raw shrimp were sorted, rinsed and soaked in lime juice to avoid fishy smell then rinsed again before grinding
3. This study using fresh shrimp (not frozen)
4. Not frozen, but during the grinding process added with 12% ice cubes
5.No, they were not pasteurised
6.Softneck garlic, white ginger and white pepper play role as aromatic condiment
7.Yes, it was saccharose
8.Each sample was prepered and analysed with three repetition
9.The La Fronthea® liquid smoke used in this study manufactured by PT. ACM Indonesia

Commented [i11]:
Reviewer 3:
Line 116, please provide the incubator brand and manufacturer.

Answer: Done

Formatted: Superscript

1:1 (v/v) and stored for 16–18 h, followed by dilution to an absorbance level of 1.1 ± 0.02 units at a wavelength of 750 nm. Subsequently, the samples were mixed with ABTS at a ratio of 1:2 (100 µL:200 µL) and stored at room temperature for 10 min. The antioxidant activity was expressed as the inhibition percentage and calculated using the following formula:

Inhibition (%) = (blank absorbance - sample absorbance) / (blank absorbance) x 100%

$$\text{Inhibition (\%)} = \frac{\text{absorbance control} - \text{absorbance sample}}{\text{absorbance blanko}}\ x\ 100\% \quad (1)$$

The IC_{50} value can be calculated by the following formula:

$$IC_{50}\ \text{value} = 50\%\ x\ \text{absorbance control} \quad (2)$$

Vitamin E Concentration Test

The vitamin E level was analyzed using high-performance liquid chromatography (HPLC) [30]. HPLC was performed on Shimadzu Prominence system (Shimadzu Corporation, Kyoto, Japan) equipped with solvent delivery system LC-20AD, with a SIL-20AC autosampler, DGU-AS on-line degasser, SPD-M20A DAD detector, CTO 20AC column oven, and CBM-20A communication module. Sample stock solutions were prepared by dissolving 100 g sample in 150 mL 70% ethanol, followed by shaking with 350 mL n-hexane solution. Then, the filtrate was separated using a separatory funnel. The oil part was collected and evaporated using a rotary evaporator vacuum regulated at a temperature of 50°C. Moreover, the HPLC system was set to a stable baseline, and about 25–100 µL sample was injected and analyzed using a mobile phase with a flow rate of 1.5 mL/min (normal phase).

LAB Test

The total LAB were calculated using total plate count with three repetitions[24]. First, all the tools and materials to be used were sterilized in an autoclave at 121°C for 15 min at a pressure of 1 atm and used to create a liquid medium. Second, the samples were homogenized with distilled water. Third, a sterile test tube filled with 9 ml distilled water was combined with 1 ml suspension sample to achieve a homogenized dilution series. Fourth, the planting process was conducted by adding 1 ml distilled water to the control petri dish and 1 ml 10^{-1} dilution suspension sample to the 10^{-1} petri dish up to the last dilution. Then, the media were poured onto a petri dish and incubated at 35°C–37°C for 24 h after solidification. The grown white or yellowish LAB colonies were subsequently collected and counted.

Commented [i12]:
Reviewer 3:
line 144, please add a reference

Answer: Done

Y. Liu et al., "Functional Characteristics of Lactobacillus and Yeast Single Starter Cultures in the Ripening Process of Dry Fermented Sausage," Front. Microbiol., vol. 11, no. January, pp. 1–15, 2021, doi: 10.3389/fmicb.2020.611260

Pathogenic Bacterial Test

Pathogenic bacterial tests were performed to evaluate the presence of *E. coli* using the most probable number method [31]. *S. aureus and Salmonella* sp. were detected using identification method with SNI-01-2332.2-2006. First, the liquid media were developed in an Erlenmeyer flask, followed by the sterilization of tools and materials by autoclaving at 121°C for 15–20 min. Then, 1 ml sample was mixed with 9 ml distilled water in the test tube up to a specific dilution value (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}). Therefore, 1 ml 10^{-1} dilution sample was collected and transferred to a petri dish containing the media before incubating at 35°C–37°C for 48 h. The samples were then evaluated for microbial growth. The affirmative test of *E. coli* was carried out by observing medium color changes to red-green metallic sheen

[32] whereas *S. aureus* and *Salmonella* sp. were confirmed positive if the color of the medium agar changed to red and yellow, respectively [33].

Acidity Test (pH)

The pH indicator/electrode was examined and calibrated with a solution of pH = 7 before rinsing with distilled water and subsequently dried. A slice of fermented shrimp sausage sample was assessed using calibrated pH electrodes placed on a glass, pending the attainment of stable and readable values.

Organoleptic Test

Organoleptic properties were evaluated in 30 respondents using a hedonic test questionnaire. This tool examined the aspects of color, aroma, texture, and taste with four scales: 1 = worse, 2 = bad, 3 = good, and 4 = best [34]. The best formulation was selected using a weighting technique considering the hedonic test attributes (color, aroma, texture, and taste) and the bioactive component.

Data Analysis

Data analysis was performed using ~~a statistical software program~~ the SPSS 25.0 sofware for Windows with a p value of ≤ 0.05, whereas normality was evaluated using *Shapiro–Wilk* test for values <50. The antioxidant activity, vitamin E levels, and acidity (pH) were tested using *analysis of variance*. Meanwhile, *Kruskal–Wallis test* was adopted in the assessment of LAB, pathogenic bacteria, and the level of acceptance.

Results and Discussion

Antioxidant Activity

Table 1 presents the shrimp sausages produced at different fermentation times, demonstrating variation in the mean percentage of inhibition ($p < 0.05$). In addition, the highest value was obtained on the 3rd day at 42.09% per 0.1 g, whereas the least value was recorded on day 0 (control) at 23.93% per 0.1 g.

Table 1. Results of the inhibition percentage (%) of fermented shrimp sausages at different fermentation times

Duration	Inhibition (%)
Fresh Prawn	17.36 ± 2.746*
0 day	23.93 ± 2.927*
1 day	26.57 ± 3.935*
2 day	34.32 ± 4.956
3 day	42.09 ± 5.994*
	P = 0,005[a]

**Significance < 0.05; [a]ANOVA: analysis of variance*

Antioxidant activity is the capability for oxidants to capture free radicals, and a high level indicates the need for less food to ensure optimal acquisition [35]. The results of statistical tests showed differences in the average percent inhibition value (p = 0.005) and IC_{50} (p = 0.024). In addition, the highest antioxidant activity was measured in shrimp sausages treated

Commented [i13]:
Reviewer 1:
1.What was the temperature of the samples tested?
2.What were the lightning conditions?
3.How were the samples stored before testing?
4.Were all the samples tested at the same day, or not? If on the same day, then how were they stored before testing?

Answer:
1.Respondents assessed the samples after the different fermentation times including control (0 day), 1, 2, and 3 days which were served in pieces with room temperature
2.Respondents assessed the samples in the quiet room with sufficient lightning
3.The incubator with a set temperature of 35°C used for fermenting the sausage from day 0 to day 3 before served to respondents in pieces with room temperature
4.No. The samples tested in different time accoding fermentation times (control (0 day), 1, 2, and 3 days)

Commented [i14]:
Reviewer 3:
line 172, please add a reference for hedonic test questionnaire.

Answer: Done

Mervina, M. Clara, and S. Marliyati, "Formulasi biskuit dengan substitusi tepung ikan lele dumbo (Clarias gariepinus) dan isolat protein kedelai (Glycine max) sebagai makanan potensial untuk anak balita gizi kurang," J. Teknol. dan Ind. Pangan, vol. 23, no. 1, pp. 9–16, 2012

Commented [i15]:
Reviewer 3:
line 177, please be specific for statistical software program used.

Answer: Done

Commented [i16]:
Reviewer 1:
These details should be corrected. Also, manuscript needs expanded discussion and comparison with work of other authors.

Answer: The correction have been done in this part. Author added some reference to complete this part.

with 3-day fermentation time. This sample demonstrated a percentage inhibition value of 42.09% per 0.1 g and an IC_{50} of 1.161 ppm and was hence classified in the very powerful category [36].

~~The increased activity was due to the protein and amino acid hydrolysis caused by LAB-produced enzymes into bioactive peptides with the capability to inhibit free radicals [7].~~ The fermentation process can produce probiotic LAB that have antihypertensive and cholesterol-lowering effects and inhibit the growth of pathogen bacteria [8], [9]. These activities are also influenced by the addition of ginger, garlic, and corn oils containing phenolic compounds, alongside the sausage preparation process before fermentation at 50°C. Furthermore, the smoking treatment using liquid smoke has been implicated due to the phenolic compounds, organic acids, and carbonyl content, which are assumed to serve as flavors, bacteriostatic, and antioxidant agents [37].

Commented [i17]:
Reviewer 1:
By what criteria it was classified?

Answer: Author added reference to complete this part. Based on the reference, antioxidant activity is classified as "very powerful" if the IC_{50} value is < 50 ppm, "strong" if the IC_{50} value is 50-100 ppm, "medium" if the IC_{50} value is 101-150 ppm, and "weak" if the IC_{50} value is 150-200 ppm.

Molyneux Philip, "The Use Of The Stable Free Radical Diphenylpicryl-hydrazyl (DPPH) For Estimating Anti-oxidant Activity," Songklanakarin J. Sci. Technol., vol. 26, no. May, pp. 1–10, 2004

Commented [i18]:
Reviewer 1:
How is it known? This parameters were not tested, this is just the hypothesis based on the results from other studies. It cannot be said that this was precisely the reason

Answer: Authors decided to delete this sentences

Commented [i19]:
Reviewer 1:
Fermentation was performed at 35 degrees, smoking was performed at 50.

Answer: There were two temperature that used in the process, shrimp sausages was placed in an oven set at 50°C for 3 h and fermenting in an incubator with a set temperature of 35°C

IC_{50} Value

Table 2 presents the average IC_{50} value of shrimp sausages produced at different fermentation durations varied significantly ($p < 0.05$). This finding is congruent with those of previous studies, including the report on kombucha fermentation for 0, 1, 2, and 3 days, in which IC_{50} increased from 120 ppm to 54.46 ppm [38]. Furthermore, the best outcome was recorded on the third day at 1.161 ppm, whereas the least (1.849 ppm) was observed on day 0 (control). Moreover, a food is considered to possess very strong antioxidant characteristics at $IC_{50} < 10$ ppm [39].

Table 2. Results of IC_{50} (ppm) of fermented shrimp sausages at different fermentation times

Duration	IC_{50} Value (ppm)
Fresh shrimp	2.99 ± 0.227*
0 day	1.849 ± 0.189*
1 day	1.655 ± 0.276
2 day	1.597 ± 0.256
3 day	1.161 ± 0.069*
	p=0.024[a]

**Significance <0.05; [a]ANOVA: analysis of variance*

Vitamin E Level

Table 3 presents the average levels of vitamin E, which differed between shrimp sausages produced at different fermentation durations *($p < 0.05$)*. In addition, the highest result at 4.985 mg/100 g was recorded on the third day, whereas the least (2.685 mg/100 g) was obtained on day 0 (control).

Table 3. Results of vitamin E level (mg/dl) of fermented shrimp sausages at different fermentation times

Duration	Vitamin E Levels (mg/100gr)
Fresh shrimp	1.515 ± 0.007*
0 day	2.685 ± 0.007*
1 day	3.355 ± 0.007*
2 day	4.090 ± 0.000*
3 day	4.985 ± 0.021*
	P < 0,001[a]

Significance < 0.05; [a]ANOVA: analysis of variance

Vitamin E is one of the compounds contained in shrimp; it is characterized by a phenol group on the 6-chromanol ring and ~~believed to be capable of~~ has useful function of protecting in~~hibiting~~attacking free radicals [40]~~ and preventing fat oxidation~~. Processed foods are considered a source of vitamin E, possessing a minimum of 15% nutrition label reference in solid form or 7.5% nutrition label reference in liquid form. Meanwhile, the potential for high content is considered in cases where over twice the amount is present in the source [41]. During the fermentation process, LAB produce metabolites from the hydrolysis of carbohydrates, fats, and proteins, including the primary forms from small compounds, e.g., vitamins and minerals, and secondary forms, e.g., antibiotics, enzyme inhibitors, and growth boosters [12]. Previous studies on gude seed *tempe* have attributed the increase in content of dissolved proteins, vitamins, and available minerals to the presence of molds. These microorganisms grow on the sample and hydrolyze intrinsic complex compounds into simpler forms [42], [43]. The increase in levels of vitamin E present in shrimp sausages is also caused by the addition of vitamin E (8%)-containing corn oil, which acts as antioxidants by inhibiting free radicals.

Commented [i20]:
Reviewer 1:
It is not only believed, but it is a proven fact. Also, add reference.

Answer: The correction have been done in this sentences. Author added reference to complete this part.

Total LAB

Table 4 shows the average difference in the total LAB of shrimp sausages treated with varied fermentation durations ($p < 0.05$). The highest value (13.88~~99~~ x 10^9 CFU/ml) was recorded on day 3, where the least (0.429 x 10^9 CFU/ml) was observed on day 0 (control).

Commented [i21]:
Reviewer 3:
lines 245-246, The highest value (13.99 x 109 CFU/ml) , (0.429 x 109 CFU/ml), please use the correct mark for "x"

Answer: The correction have been done in this sentences.

Table 4. Result of total LAB of fermented shrimp sausages at different fermentation times

Duration	Total of LAB (x 10^9CFU/ml)
0 day	0.429 ± 0.422
1 day	5.055 ± 4.879
2 day	8.437 ± 8.676
3 day	13.88 ± 9.988
	p = 0.048*[b]

Significance < 0.05; [b]Kruskal–Wallis Test

The total LAB were positively influenced by the fermentation duration. This finding is an indicator of the ability for growth in the initial phase, prompting adjustments to the inhabiting substrate. Furthermore, this condition causes cell multiplication and active transport processes, which microorganism maintan their pH in cytoplasm by pH homeostasis mechanism based on the transport ~~are expected to facilitate the production of~~ protons across the cell membrane [44]. The anerobic bacteria during fermentation process perform H^+ transport through molecules of H^+ -ATPase with the use of energy, in this way the main product of lactic acid contribute to maintain acid-base balance [44]. ~~as nutrients for survival.~~ In addition, a high concentration of LAB was observed alongside the extended fermentation duration, with a tendency to overhaul the nutrients contained in the substrate. This phenomenon possibly allows the accumulation of organic acids in high amounts [45].

Commented [i22]:
Reviewer 1:
Describing metabolic process with the H^+ transfer as "production of protons as nutrients for survival" is a little bit far-fetched. Describe it as it truly is. Furthermore, a reference

Answer: The correction have been done in this sentences. Author added reference further explanation of that mechanism.

The initial phase of fermentation features the growth pattern adaptation of LAB. The first-day fermented shrimp sausage formulation showed an increase in total bacteria, resulting from the dominant activity of the microorganism to convert carbohydrates into lactic acid. This phase is classified as logarithmic, at which the bacteria quickly and constantly experienced a rapid

growth rate. Certain spesies of bacteria extreamly multiply due to the content of nutrients, water activity (a_w), pH value, storage temperature, composition atmosphere, physical/chemical treatment during processing and the specific growth rate of the bacteria themselves [44]. ~~The speed of development was greatly influenced by the growth media, pH of nutrient content, and environmental conditions, including temperature, light, and air humidity. In addition, the media chemical composition changed because of the product synthesis following substrate utility.~~ The concentration of alcohol and CO_2 produced increased, possibly up to levels toxic for the survival of microbes [46]. In addition, an increase in the LAB population caused a decline in pH and an increase in acidity. This condition is unsuitable for certain LAB, e.g., *Lactobacillus bulgaricus, Streptococcus thermophilus, and Lactobacillus acidophilus*, thus leading to bacterial death.

Commented [i23]:
Reviewer 1:
Once again - how is it known? There is no data about these conditions in the experiments. All the sausages were fermented in the same condition, so Authors cannot say whether light or humidity had an impact on the microbial metabolism. Furthermore - speed of development was not precisely monitored during the experiment.

Answer: Based on recommendation, author decided delete this sentencess.

Pathogenic Bacteria

The identification test results from days 0, 1, 2, and 3 showed negative/25 gr for *Salmonella* sp., <3/gr in the case of *Escherichia coli*, and 1 x 10^2 CFU/gr for *Staphylococcus aureus*. These findings are congruent with the Indonesian National Standard for food and beverages. The cumulation of large amount of LAB in the product can inhibit pathogenic bacteria. This result is due to the high bactericidal effect at low pH [47]. In addition, different types of LAB produced varied inhibition and activities due to the influence of metabolite components produced. Acetic acid, formic acid, succinic acid, ethanol, hydrogen peroxide, and diacetyl were also generated by LAB action. These products are antagonistic and can inhibit the growth of other bacteria [48].

Commented [i24]:
Reviewer 1:
How is this known, that these compounds were produced? They were not analysed there is no data.

Answer: True. All theses compounds were not analyzed in this study. This claim is the result based on the results from other studies.

Acidity Level (pH)

Table 5 presents the average pH level of shrimp sausages with differences in fermentation durations ($p < 0.05$). The highest value was observed on day 0 (6.6), whereas the least was recorded on day 3 (5.71). The fermentation duration affected the extent of pH decline, resulting from the conversion of glucose to lactic acid. Fermented catfish sausage by smoking for 30 min at temperature of 30°C can increase LAB levels and lower pH [28]. Therefore, low values alongside elevated concentrations of lactic acid further inhibit pathogenic bacterial growth [49]. The recommended pH for fermented sausage is between 4.8–5.4[50].

Table 5. Results of acidity value (pH) of fermented shrimp sausages at different fermentation times

Duration	pH Value
0 day	6.600 ± 0.327
1 day	6.290 ± 0.209
2 day	6.027 ± 0.347
3 day	5.710 ± 0.471*
	*p = 0.022**

**Significance < 0.05; [a]ANOVA: analysis of variance*

Organoleptic Test

Table 6. Results of organoleptic analysis of fermented shrimp sausages at different fermentation times

Duration	Categories (Mean ± SD)				Mean
	Color	Aroma	Texture	Taste	
0 day	3.20 ± 0.76[a]	2.97 ± 0.99[a]	2.30 ± 0.87[a]	2.47 ± 0.86[a]	2.73

1 day	2.73 ± 0.82^{b}	2.73 ± 0.94^{b}	2.57 ± 0.77^{a}	1.97 ± 0.76^{a}	2.5
2 day	2.63 ± 0.66^{b}	1.60 ± 0.81^{c}	1.83 ± 0.69^{b}	1.57 ± 0.72^{b}	1.9
3 day	2.00 ± 0.94^{c}	1.90 ± 0.88^{d}	1.50 ± 0.73^{c}	2.17 ± 1.05^{a}	1.89
	$p < 0.001$*	$p < 0.001$*	$p < 0.001$*	$p = 0.001$*	

*Different superscript letters (a, b, c, and d) show significant differences between groups; *Kruskal–Wallis test*

Differences were observed in shrimp sausage color, following the variations in fermentation duration ($p < 0.05$) presented in Table 6. In addition, the highest intensity was observed on day 0 and the least on day 3. Color changes were also observed during storage. This finding is attributed to the naturally existing pigments in food, caramelization, and Maillard reactions, interaction between organic compounds and air [51]. Moreover, prolonged fermentation duration increased the darker ~~color concentration~~ of fermented shrimp sausage. This phenomenon was due to the presence of H_2O_2 produced by microorganisms through aerobic metabolism. Furthermore, the reactive compound formed is assumed to cause a decline in the red pigmentation, following the formation of brown *metmyoglobin* [52]. The smoking process has also been implicated in color changes, resulting from the smoke absorption capacity of product surface, as evidenced by a reaction between carbonyl groups. This condition further leads to a brownish pigmentation in sausages [53].

A significant difference was observed between the aroma of shrimp sausages prepared at varied fermentation durations ($p < 0.05$). The highest level of aroma was observed on day 0 and the lowest on day 2. Moreover, Maillard reactions occurred following an interaction between amino groups and reducing sugars. This phenomenon was assumed to produce volatile compounds during the fermentation process [52]–[54]. Therefore, a long treatment duration causes a decline in the pH, alongside an increase in the amount of lactic acid produced from LAB metabolism. This condition further stimulates the aroma of fermented shrimp sausages to be more acidic [55].

The texture of shrimp sausages differed across the treatments with varied durations of fermentation ($p < 0.05$). In addition, samples obtained on day 1 demonstrated the best texture, whereas the poorest result was recorded on day 3. A chewy texture was caused by the presence of amylose and amylopectin in tapioca flour [41]. This shrinking is affiliated with the fermentation duration and assumed to play a role in hardening the texture. In addition, this parameter is influenced by product pH, given that low values are implicated in the high tendency for meat shrinkage [41]. Furthermore, acid conditions during fermentation process also aid in texture formation due to meat protein coagulation and in color formation through the reactions of nitrite and nitrogen monoxide with myoglobin [44].

The flavor of shrimp sausages varied at different fermentation durations ($p < 0.05$). Furthermore, the highest flavor acceptance was observed on day 0 and the lowest on day 2. Flavor is a stimulus of food, especially by the sense of taste and odor [41]. The fermentation process is characterized by the degradation of compounds, including the conversion of proteins into amino acids and peptides. These activities cause specific flavors [41]. In addition, long processes and the decline in pH instigate a rise in acid levels. The increased amount of lactic acid generated leads to high acidity in the taste of sausages.

Commented [i25]:
Reviewer 1:
What is concentration of dark color?

Answer: This means the color is darker during the fermentation time.

Commented [i26]:
Reviewer 1:
Was the process of sausage production an fermentation (anaerobic metabolism) or not?

Answer: It was anaerobic environment. The fermentation process in this study conducted spontaneously without the addition of any microorganisms. This process involves protein and amino acid hydrolysis by enzymes of microbial origin, yielding bioactive peptides.

Commented [i27]:
Reviewer 1:
Amylose and amylopectin are enzymes, which can affect the starch in the flour. They do not create a 'chewy texture'.

Answer: Amylose and amylopectin in this sentences are two types of polysaccharides that can be found in strach garnules. The fermented shrimp sausages in this study using tapioca starch that caused the chewy texture.

Selected Formula Weigthing

Tables 7 and 8 present the weighting results of hedonic test attributed to the indicators of color, aroma, texture, and taste. A 25% weighting on each indicator, including color, aroma, texture, and taste, was provided on the fermented shrimp sausages. The first rank was obtained by treatment samples for day 0, followed by F1, F2, and F3. Therefore, F1 was selected because F0 was not fermented.

Table 7. Results of weighting hedonic test

Indicator	Day 0 (F0/Control)	Day 1 (F1)	Day 2 (F2)	Day 3 (F3)
Color (25%)	0.8	0.68	0.65	0.5
Aroma (25%)	0.74	0.68	0.4	0.47
Texture (25%)	0.57	0.64	0.45	0.37
Flavor (25%)	0.61	0.49	0.39	0.54
Total	2.72	2.49	1.89	1.88

Table 8. Determination of selected formulation score

Treatment	Hedonic Test (25%)	Score	Antioxidant Test (25%)	Score	Vitamin E Test (25%)	Score	Total
F0	1	0.25	1	0.25	1	0.25	0.75
F1	4	1	2	0.5	2	0.5	2
F2	3	0.75	3	0.75	3	0.75	2.25
F3	2	0.5	4	1	4	1	2.5

Based on the weighting results, the third-day fermented shrimp sausage formulation (F3) was selected because of its bioactive content. However, its hedonic test score was the lowest and was not favored by panelists. Therefore, F1 was selected considering the bioactive constituents and the level of consumer acceptance. The hedonic quality assessment showed the panelists an acceptance level of 1.97, which indicates a low preference, whereas the acceptance values of color, aroma, and texture were 2.73, 2.73, and 2.57, respectively. Therefore, the first-day fermented shrimp sausage (F1) was selected, considering the bioactive content and the level of consumer acceptance.

Conclusions

Significant differences were observed in the mean antioxidant activity, levels of vitamin E, total LAB, acidity value (pH), and acceptance level of shrimp sausages treated with different fermentation times (0, 1, 2, and 3 days) at $p \leq 0.05$. Overall, different fermentation times played role changes in bioactive content and the quality characteristics of fermented sausages. A long treatment duration causes a decline in the pH, alongside an increase in bioactive content. However, the best formulation was obtained with the first-day fermented shrimp sausage, as shown by its bioactive content and level of acceptability. It scored of the total weigthing hedonic test at 2.49 with 26.57% antioxidant activity, 1.655 ppm IC_{50}, 3.355 mg/100 gr Vitamin E, 5.055 x 10^9 CFU/ml total LAB and 6.29 pH. ~~Based on the results, the day 1 products were selected as the optimal formulation, considering their bioactive content and the level of consumer acceptance.~~

Commented [i28]:
Reviewer 3:
(3)Conclusion: please conclude the main finding from the research work.

Answer:
The correction have been made in this section based on reviewer's recommendation.

Data Availability

The fermented shrimp sausage's ingredients, inhibition percentage (%), IC_{50} (ppm), vitamin E level (mg/dl), total LAB (10^9CFU/ml), acidity value (pH), organoleptic analysis, and weighting hedonic test of fermented shrimp sausages at different fermentation times used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

Funding Statement

The authors are grateful to the Directorate of Research and Community Service of the Directorate General of Research and Development Strengthening, Ministry of Research, Technology and Higher Education for funding this study through *Higher Education Leading Basic Research Scheme with No. Contract: 257-39/UN7.P4.3/PP/2019.*

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Commented [i29]:
Reviewer 3:
References, please be unified for journal name, I noticed that some use journal abbreviation, others use full journal name. Some references are with a doi , while others are not

Answer: This paper were using reference including textbook, report and journal article/review. References in this paper are automacally formatted using Mendeley.

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