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To cite this article: S Melia *et al* 2020 *IOP Conf. Ser.: Earth Environ. Sci.* **454** 012095

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Physical and microbiological properties of sausage with addition of crude bacteriocin supernatant *Lactobacillus fermentum* L23 in cold storage

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Abstract. The purpose of this study was to determine the physical and microbiological properties of sausages with the addition of crude bacteriocin supernatant *Lactobacillus fermentum* L23 stored at cold temperatures. Research used a completely randomized design (CRD) 4x3 factorial pattern with 2 replications as a group. Factor A was application of bacteriocin concentration which consists of 4 levels, namely A1: 0%, A2: 3%, A3: 6%, A4: 9%. Factor B was the storage time at cold temperatures, namely B1: 0 days, B2: 6 days, B3: 12 days. The results showed that there was a significant interaction ($P < 0.05$) on differences in the addition of bacteriocin supernatant concentration and storage time in cold temperatures, against total aerobic bacterial colonies, but there was no interaction ($P > 0.05$) on moisture content and the pH value of sausages. Factor A can have a significant effect on the pH of the sausage, while factor B can have a significant effect on the moisture content of sausage. The conclusion of this study is that the best bacteriocin supernatant concentration for sausages is 6% and storage time in cold temperatures for 6 days with 62.52% moisture content, pH 5.75 and total plate count 4×10^3 CFU/g.

1. Introduction

Beef is very popular in Indonesia as a source of animal protein. Meat needs are increasing along with the increase in population, income, purchasing power, lifestyle and public awareness of nutrition. The nutritional content of beef consists of 75% water, 19% protein, 3.5% non-protein, soluble substances and 2.5% fat [1]. The meat is processed as meatballs, sausages, nuggets, and cornet, however, the favourite product are sausages.

Sausage is derived from the Latin word 'Salsus' meaning salted or preserved by salting. The term was probably originally applied to cured or salted meat. Sausage is a processed product of meat mixed with spices and inserted into the casing [2]. Fresh sausage, smoked sausage, and semi-dried sausage products have a shelf life in a sequence of 2 days at cold temperatures (4 °C). The sausage stored at -18 °C can still be stable up to 1-2 months [3]. Additionally, preservatives, such as vegetable and chemical preservatives, are usually added when processing food to extend the shelf life. Vegetable preservatives can be derived from spices and chemical preservatives using nitrite. However, the use of nitrites in food will have an impact on human health because it is carcinogenic. Therefore, bacteriocin can alternatively be used as a preservative to produce a food processing process that Safe, Healthy, Whole and Halal.

The use of Bacteriocins from LAB have several advantages: it is not a toxic, does not change the taste and texture of foodstuffs, is easily biodegradable, is easily digested by enzymes in the digestive tract and able to use as preservatives in food or as additives [4].



In this study, the bacteriocin is produced from Lactic Acid Bacteria (LAB) *Lactobacillus fermentum* L23, which was the isolation of lactic acid bacteria from buffalo milk from four districts in West Sumatra. The bacteriocin of *Lactobacillus fermentum* L23 has a good antimicrobial activity against *Lactobacillus monocytogenes* [5] and potential as probiotic [6].

Bacteriocins produced from *Lactobacillus fermentum* L23 are expected to be able to extend the shelf life of sausage products. The role of bacteriocins as a natural preservative in sausage products has been proven during the previous research implementation. The result showed that sausages without bacteriocin addition (0%) and sausages with addition bacteriocin 9% stored for 10 days' storage produced Total Plate Count (TPC) 1×10^6 CFU/g and TPC 1×10^4 CFU/g, respectively. This study, therefore, aimed to conducted on "physical and microbiology properties of sausage with addition of crude bacteriocin supernatant *Lactobacillus fermentum* L23 in cold storage".

2. Materials and methods

Materials 2400 g beef, 10% ice cubes, 10% oil, 15% tapioca flour, 10% skim milk, 0.4% garlic, 0.6% shallots, 0.15% pepper, 0.5% ginger, 1.5% sugar, 0.1% nutmeg, 2% salt and bacteriocin supernatant from *Lactobacillus fermentum* L23 (collection of Laboratory Animal Science and Technology, Andalas University). The design used was a Completely Randomized Design factorial 4x2 with 2 replications. Factor (A) was bacteriocin supernatant concentration (0%, 3%, 6% and 9%) and Factor (B) was storage period in refrigerator at 4°C (0 day, 6 days, 12 days). Data were analysed by ANOVA [7].

2.1. Crude bacteriocin preparation

The amount of 10 ml *Lactobacillus fermentum* culture was incubated 24 hours at 37°C. Then, the culture was centrifuged for 8 minutes at 4 °C, and the supernatant was filtered. The supernatant was set at pH 6.5 with 1 N NaOH to eliminate the effect of resistance due to the presence of organic acids.

2.2. Sausage preparation

Milled beef mixed with ingredients (15% tapioca flour, 10% skim milk, 0.4% garlic, 0.6% shallots, 0.15% pepper, 0.5% ginger, 1.5% sugar, 0.1% nutmeg, 2% salt) and included crude bacteriocin supernatant (0%, 3%, 6% and 9%). The mixture was homogenized for 2 minutes, and added 10% oil, then the sausages were filled up into synthetic cellulose casings. The sausages, were then steamed at 80°C for 30 minutes. Once cooked, the sausage were cooled at room temperature for \pm 2 hours, and stored in refrigerator at 4°C (0 day, 6 days, 12 days).

3. Result and discussions

3.1. Moisture content

The result of the statistical analysis showed that there was no interaction ($P > 0.05$) between the bacteriocin supernatant concentrations (Factor A) and the storage period at cold temperature (Factor B). The mean of the moisture content of a sausage in each treatment is presented in Table 1.

Table 1. The Moisture Content (%) of sausages at various concentrations of bacteriocin supernatant (Factor A) and the storage time of cold temperature (Factor B)

(Factor A)	Factor B (days)			Mean
	B1 (0)	B2 (6)	B3 (12)	
A1 (0%)	64.20	61.82	61.54	62.52
A2 (3%)	63.15	62.76	61.80	62.57
A3 (6%)	63.20	62.52	61.57	62.43
A4 (9%)	63.86	62.02	61.98	62.62
Mean	63.60 ^a	62.28 ^b	61.72 ^b	

Note: The mean with superscript written in small letter showed a significant difference ($P < 0.05$)

The non significant interaction could happen because the mechanism of bacteriocin supernatant (Factor A) was directly proportional to the storage time at a cold temperature (Factor B). The bacteriocin was suppressing the spoilage caused by pathogenic bacteria, so that it acts as preservatives. Meanwhile, the storage at cold temperature also support the decrease of spoilage bacteria. However, at the cold temperature, there was still a type of psychrophilic bacteria that was able to survive. Hadiwiyo [8] also stated that the species of a bacterium that contaminates the food product stored at low temperature is included in psychrophilic bacteria i.e. the bacteria that can grow well at a temperature of 15°C to 20°C. In this case, such bacteria can also survive at a temperature between -10°C to 40°C.

Table 1 shows that a sausage with bacteriocin supernatant at different concentrations (Factor A) contributes a non significant effect ($P>0.05$) on the moisture content of sausage. It was found that the concentration of bacteriocin supernatant had no impact on the moisture content of sausage. Bacteriocin is a peptide synthesized in ribosome by the bacteria to inhibit other bacteria. Although bacteriocin also acts as antibiotics, there are some characteristics that are different from antibiotic, i.e. the bacteriocin is synthesized in the ribosome and the host cell is resistant to the bacteriocin [9].

Moisture content of the sausages, amounting 61.72% - 63.60%, stored for various time at cold temperature (Factor B) can be seen in Table 1. The result of the analysis of variance showed that the sausage with storage duration at cold temperature (Factor B) contributed to a significant impact ($P<0.01$) on the moisture content of sausage. The highest moisture content found in sausage with 0 day (B1) storage time (63.60%), while sausage stored for 6 day (B2) dan 12 days (B3) had similar moisture content. The result of Duncan's Multiple Range Test showed that the moisture content of fresh sausage (B1) was significantly different from ($P<0.05$) the treatment with storage duration at cold temperature for 6 days (B2) and 12 days (B3). In other words, storage time of 6 days (B2) and 12 days (B3) gave similar effect ($P>0.05$) on the moisture content of sausages.

It was found that extended storage time at cold temperature could result in a decrease in the moisture content of sausage. The decrease in moisture content was caused by the evaporation during storage at cold temperature. The longer the storage duration is, the more the decrease in moisture content. The decrease in moisture content during the storage was caused by the evaporation at low temperature (4°C) [8].

The change in humidity around the storage room would result in water evaporation or water absorption in packaging [10]. If the humidity around the storage room were lower than the humidity of a product, it would result in a decrease in the moisture content (dehydration) of a product and vice versa. That the decrease in moisture content during the storage duration could be caused by the packaging method and the storing method that was done [11]. In this study, the moisture content of sausage during storage was around 61.72% - 63.60%.

3.2. pH

The result of the statistical analysis showed that there was no interaction ($P>0.05$) between the bacteriocin supernatant at different concentrations (Factor A) and the storage duration at cold temperature (Factor B) toward pH level. The mean value of the pH level in a sausage in each treatment is presented in Table 2.

Table 2. The pH of sausages at various concentrations of bacteriocin supernatant (Factor A) and the storage time at cold temperature (Factor B)

(Factor A)	Factor B (days)			Mean
	B1 (0)	B2 (6)	B3 (12)	
A1 (0%)	5.90	5.90	6.00	5.93 ^a
A2 (3%)	5.90	5.80	6.10	5.93 ^a
A3 (6%)	5.95	5.75	5.90	5.87 ^a
A4 (9%)	5.75	5.70	5.90	5.78 ^b
Mean	5.88 ^a	5.79 ^a	5.98 ^b	

Note: The mean with superscript written in small letters showed a significant difference ($P<0.05$)

The result of the statistical analysis showed that there was no interaction effect ($P > 0.05$) between the concentration of bacteriocin supernatant (Factor A) and storage duration at cold temperature (Factor B) toward pH value. Table 2 shows that the sausage with bacteriocin supernatant at different concentrations (Factor A) contributed to a significant impact ($P < 0.05$) on the pH value of sausage. The mean pH value in sausage with bacteriocin supernatant at different concentrations (Factor A) was around 5.78 – 5.93. In that, the highest activity in the treatment with 0% bacteriocin supernatant (A1) and the average pH value of sausage of 5.93 and the lowest pH value in 9% bacteriocin supernatant (A4) with an average pH value of sausage of 5.78.

The result of Duncan's Multiple Range Test showed that the pH value of sausage added at 0% bacteriocin supernatant (A1) was not significantly different ($P > 0.05$) from the addition of 3% bacteriocin supernatant (A2) and 6% bacteriocin supernatant (A3). The addition of 6% bacteriocin supernatant (A3) was significantly different from ($P < 0.05$) the addition of 9% bacteriocin supernatant (A4).

It showed that the addition of bacteriocin supernatant resulted in a decrease of pH of the sausages. The decrease in pH value as seen in the research finding (Table 2) by adding the highest concentration of 9% (Factor A) resulted in the lowest pH value of 5.78. It happened due to organic acid that had been formed, and the acid dissociated in the form of hydrogen (H^+) ions. The result was in line with the previous statement by Kusnadi [12]. Therefore, the measurement of pH value using an electrode in a pH meter showed a gradual decrease of the pH value.

Storage time at cold temperature (Factor B) gave pH value of around 5.79 – 5.98. The result of the analysis of variance showed that the storage time at a cold temperature (Factor B) contributed to a significant impact ($P < 0.05$) on the pH value of sausage. Sausage pH stored for 12 days (5.98) was higher than the pH of sausages (5.79) stored for 12 days (B3). It showed that there was a correlation between the storage duration at cold temperature and the pH value in a sausage.

The result of Duncan's Multiple Range Test showed that the pH value of fresh sausage (the storage duration was 0 days) (B1) was not significantly different ($P > 0.05$) from the treatment with storage time at a cold temperature for 6 days (B2). Meanwhile, sausages stored for 6 and 12 days gave a significant differences on the pH sausages. It was found that the storage duration at cold temperature could give an impact on the increase in the pH value of a sausage. The storage for up to 12 days could result in the growth of psychrophilic bacteria, resulting in the secondary metabolite leading to the increase in the pH of the food material.

Previous study stated that the increase of pH in the sausage was due to the chemical base compound such as ammonia, H_2S , and amine during the storage [13]. Moreover, the increase in pH was also caused by the reaction between protein and acid that resulted in alkaline ammonia [14]. The accumulation of lactic acid would reduce the protein content in sausage, and the degradation produced ammonia. The result in this study is supported by Rabban et al. [15] who stated that the storage duration would result in the increase of pH value in sausage.

According to Reddy's research [16], the increase of pH value was caused by the increase of the basic substance of volatile i.e. ammonia due to the bacterial activity. The pH value extremely influenced the shelf life of processed meat products. The changes in pH value were strongly correlated with the color and the texture of meat. Additionally, pH could influence the quality of meat since it was related to the color, tenderness, taste, Water Holding Capacity, and shelf life [17].

3.3. Total colony of aerob bacteria

The result of the statistical analysis showed that there was an interaction between ($P < 0.05$) the bacteriocin supernatant at different concentrations (Factor A) and the storage duration at cold temperature (Factor B) toward the total value of aerobic colony. The mean of the total value of aerobic bacteria in sausage for each treatment is presented in Table 3 below.

Table 3. The total value of the colony of aerobic bacteria of sausages at various concentrations of bacteriocin supernatant (Factor A) and the storage time at cold temperature (Factor B)

Factor A	Factor B (days) ($\times 10^3$ CFU/g)			Mean
	B1 (0)	B2 (3)	B3 (9)	
A1 (0%)	16 ^{bc}	15 ^c	36 ^a	22
A2 (3%)	11 ^e	13 ^d	17 ^b	14
A3 (6%)	10 ^c	4 ^g	4 ^g	6
A4 (9%)	8 ^f	7 ^f	4 ^g	6
Mean	11	10	15	

Note: The mean with superscript written in small letters showed a significant difference ($P < 0.01$)

It can be seen in Table 3 that there is a significant and different interaction ($P < 0.01$) between the concentration of bacteriocin supernatant (Factor A) and the storage duration at cold temperature (Factor B) toward the total colony of aerobic bacteria. The result of Duncan's Multiple Range Test showed that the total colony of aerobic bacteria in the A3B2, A3B3 and A4B3 were similar, i.e. 4×10^3 CFU/g. Yet, the A3B2 4×10^3 CFU/g treatment was significantly different from A4B2 7×10^3 CFU/g, A3B1 8×10^3 CFU/g, A3B1 10×10^3 CFU/g, A2B1 11×10^3 CFU/g, A2B2 13×10^3 CFU/g, A2B2 15×10^3 CFU/g, A1B1 16×10^3 CFU/g, A2B3 17×10^3 CFU/g, and A1B3 36×10^3 CFU/g. The interaction between bacteriocin supernatant concentration (Factor A) and the storage duration at cold temperature (Factor B) decreased the total colony of aerobic bacteria.

The Table above shows the mean of the total aerobic bacteria by adding of 0%, 3%, 6%, and 9% bacteriocin and storage duration of the sausages (0, 6, 12 days) at a cold temperature were ranging from 4×10^3 CFU/g to 36×10^3 CFU/g. The highest number of total colony of aerobic bacteria in the A1B3 treatment (0% bacteriocin supernatant stored for 12 days at a cold temperature) was due to the absence of bacteriocin supernatant that had a function as an antimicrobial agent for decreasing the growth of bacteria.

The substance of bacteriocin, a peptide compound, is an antimicrobial resulted from the secondary metabolite of lactic acid bacteria, so that it could inhibit the growth of pathogenic bacteria. Other researcher stated that bacteriocin supernatant isolated from *Lactobacillus fermentum* L23 can inhibit the growth of pathogenic microorganisms. e. *Listeria Monocytogene* [5]. It is in line with others [18], which reported that the inhibitory activity of bacteriocin resulted by *Lactobacillus sp.* can work at room temperature (27°C) and at cold temperature (4°C). Therefore, it can reduce the spoilage microorganism in processed meat products.

According to Gonzalez et al. [19], there were some ways of an antimicrobial agent in its action to fight off microorganism i.e. by giving either bacteriostatic effect, bactericidal or bacteriolysis. In addition, the mechanism of inhibiting the growth of microbes by antimicrobial compound can be done by breaking down the cell wall until it became less or by changing and inhibiting the formation of cell wall in a growing cell, changing the permeability of cytoplasmic membrane that resulted in nutrient leaks in a cell, denaturation of cellular protein, and the destruction of metabolic system in a cell by inhibiting the activity of intracellular enzyme [20].

Low-temperature storage is usually between 1°C to 4°C [21]. Therefore, refrigeration prevents the growth of thermophilic microbes (the microbes that grow at 35°C - 55°C) and some mesophilic microbes (the microbes that grow at 10°C-40°C). The rate of an enzymatic reaction will be retarded at lower the temperature, resulting in the slower chemical change in a food product leading to the longer shelf life. The influence of refrigeration towards microbes i.e. prolonging the lag phase and inhibiting or decreasing the growth rate (Estiasih and Ahmadi, 2011). The total value of the aerobic colony of bacteri in this research is appropriate to the Indonesian national standard number 3820-2015, in which the permitted maximum limit of total microbes in a sausage product is 1×10^5 CFU/g.

4. Conclusion

There was no significant interaction between the addition of different bacteriocin supernatant concentrations and the storage time of cold temperatures on the moisture content and pH value, but there were significant interactions with the total aerobic colonies sausage. The best results of this research using 6% bacteriocin concentration, 6 days storage time (62.52% moisture content, pH value 5.75 and total value of 4×10^3 CFU/mg colonies aerobic bacteria).

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Acknowledgments

This research was supported by Publication Research Cluster Towards Professors (Project No. T/66/UN.16.17/PP-KP-KRP2GB/LPPM/2019) LPPM Andalas University.