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Research Article
Application of Purified Bacteriocin from *Lactobacillus plantarum* IIA-1A5 as a Bio-preservative of Beef Sausage

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Abstract

Background: Beef sausage is a meat product that is potentially susceptible to microbial growth. Most chemical preservatives in current use may cause unhealthy effects in humans if consumed in large doses; therefore, the use of bio-preservatives, such as bacteriocin from *Lactobacillus plantarum* IIA-1A5 is needed. **Objective:** This study aimed to assess the characteristics of bacteriocins produced by *Lactobacillus plantarum* and their effects on sausage quality. **Methodology:** In this experimental study an attempt was made to investigate the microbiological, physicochemical and sensory characteristics of beef sausages supplemented with various preservatives (control, 0.3% nitrate and 0.3% bacteriocin) for various periods (0, 3, 6 and 9 days) in a cold temperature (4-6°C). Parameters measured included: Acidity, water binding activity, water activity and total acid number. **Results:** The results showed that the presence of bacteriocin in the sausages inhibited the growth of pathogenic *Staphylococcus aureus* and *Escherichia coli* bacteria until day 6, which was better than the inhibition observed in the presence of nitrite. **Conclusion:** The study concluded that bacteriocin from *Lactobacillus plantarum* IIA-1A5 could replace nitrite as a preservative for 6 days of storage.

Key words: Bacteriocin, *Lactobacillus plantarum* IIA-1A5, bio-preservatives, beef sausage, meat

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

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

INTRODUCTION

Meat is edible animal tissues, including muscle tissue, organs (liver, spleen, kidneys and brain), used as a food ingredient[1]. Current technology has produced various products made from meat, such as meatballs, sausages and salami. These variations encourage the manufacturing of tools to support the production process[2].

Sausage is processed from meat. The meat is minced, mashed, mixed with selected seasonings and transferred to an artificial container. The sausage can be cooked and smoked[3]. According to the Indonesian Standardization Body (Indonesian: Badan Nasional Standardisasi, BSN), the International Organization for Standardization (ISO) member body for Indonesia, sausage is a mixture of minced meat (not less than 75%) and flour or starch with or without the addition of seasonings and other permitted food additives placed to sausage casings. To prolong its shelf life, chemical preservatives, such as nitrite are used by many industries. The preservatives used should be safe for health; thus, natural compounds, such as bacteriocin are good candidates for preserving sausages.

Lactic Acid Bacteria (LAB) can produce bacteriocin as a bio-preservative and have been widely used. Bacteriocin can inhibit the development of pathogens that have a close relationship with bacteriocin-producing bacteria. Cotter *et al.*[4] explained that bacteriocin is a ribosomal peptide produced by bacterial strains that has antagonistic properties against other bacterial strains. However, the bacteriocin-producing bacteria are protected against bacteriocin activity. Hata *et al.*[5] stated that this antimicrobial compound is not toxic to humans, is easily degraded by proteolytic enzymes and is not harmful to intestinal microflora because it is easily digested by digestive enzymes. Bacteriocin is also stable against changes in pH and temperature.

Lactobacillus plantarum is naturally found in beef and produces a bacteriocin known as plantaricin. Arief *et al.*[6] found that the *Lactobacillus plantarum* isolates IIA-2C12, IIA-1A5, IIA-1B1 and IIA-2B2 from local beef in Indonesia produced bacteriocin as an antimicrobial compound. Bacteriocin from different strains of *Lactobacillus plantarum* has different characteristics and inhibitory spectrums. The effectiveness of these bacteriocins are expected to equal that of nisin, which is also a natural preservative.

This study aimed to assess the characteristics of bacteriocins produced by *Lactobacillus plantarum* and their effects on sausage quality.

MATERIALS AND METHODS

Bacteriocin production: MRS Broth media (1 L) with 3% of yeast extract was inoculated with a 10% (v/v) culture of *L. plantarum* IIA-1A5, incubated at 37°C for 20 h and then stored at 4°C for 2 h. The suspension was centrifuged at 5000 rpm for 20 min at 4°C and filtered through a sartorius membrane filter; then, the cell-free supernatant pH was neutralized to pH 6 using 0.1 N NaOH.

Ammonium sulfate powder was gradually added to 80% (20, 40, 60 and 80%) to the supernatant to obtain the protein deposits. The antimicrobial supernatant solution was slowly homogenized and stirred at 4°C for 2 h. The next supernatant was discarded and the precipitate obtained was collected in 50 mL Falcon tubes. The bacteriocin precipitate was dialyzed using a membrane (Ø 2 cm) and potassium phosphate buffer with a pH of 6.8 for 12 h. Then, the buffer was replaced twice at 2 and 4 h to obtained the crude extract of bacteriocin. The bacteriocin extract obtained was free of ammonium sulfate. The buffer replacement was performed by dialysis using distilled water (pH 7.0) to obtain bacteriocin for sausage treatment.

Sausage preparation: Sausage was produced according to the methods proposed by Arief *et al.*[7]. Meat and other ingredients (salt, STPP and ice) and preservatives (without natural preservatives; nitrite 0.3% and 0.3% bacteriocins) were ground in a food processor for 5 min. Fats, oils, spices, skim milk and ice were added and mixed for 3 min. Tapioca was then incorporated into the dough and mixed for 2 min. The dough was transferred into a sausage casing and boiled for 45 min at 60-70°C. The boiled sausage was packaged and stored in a refrigerator until analysis.

Parameters

Acidity: To observe acidity, the pH was measured using a pH meter (Hanna) calibrated at pH 4 and 7. The pH was measured by inserting the electrode into the center of the sausage.

Water binding activity[8]: The sample (1 g) was added in a centrifuge tube to 10 mL of distilled water and centrifuged at 6000 rpm for 30 min. The supernatant volume was measured with a 10 mL measuring cup. Water-binding was calculated as the difference between the starting water volume (10 mL) and the volume of the supernatant and expressed in g g^{-1} assuming the specific gravity of water is 1 g mL^{-1}.

Water activity (a_w): Water activity was measured using an a_w meter (Novasina) based on the device manual.

Total acid number[9]: The sample (5 g) was ground, added to 45 mL of distilled water and 3 drops of 1% phenolphthalein and stirred. The solution was titrated with 0.1 N NaOH to obtain a pH of 7. The titrated acid total was measured using the following formula:

$$\text{Lactic acid (\%)} = \text{Volume NaOH (mL)} \times \text{N NaOH} \times \text{BM} \times \text{FK} \times 100\% \text{ sample (g)} \quad (1)$$

where, N is normality, BM is molecular weight of lactic acid (90), 1 mL NaOH 0.1 N is exactly equal to 0.009 g lactic acid, FK is factor of reagent.

Chemical analysis: The analysis of the chemical composition included moisture content, protein content, fat content and ash content based on AOAC[9] and carbohydrate content using the difference method. Nitrite residue was also determined using AOAC[9].

Organoleptic test[10]: The organoleptic test included the hedonic quality test and hedonic test (Test A) on the sausage using 1-5 scales. The experiment involved 50 untrained panelists (hedonic test) and 40 semi-trained panelists (hedonic quality).

Microbiological evaluation: Total plate count was measured by Bacterial Analytical Methods (BAM)[11]. The sample (25 g) was mixed with Buffer Peptone Water (BPW), Oxoid (225 mL) to aquire a 10^{-1}-10^{-6} dilution. The growth of microbes was assessed in each of the desired dilutions as 1 mL of dilution was pipetted into a petri dish with Plate Count Agar (PCA) media added. The next petri dishes were incubated at 37°C in an inverted position. The colonies were counted after 24-48 h of incubation. The number of bacteria was determined by the plate count method and Standard Plate Count (SPC) was used to report the results.

For the quantitative analysis of *Escherichia coli*[11], the growth of *E. coli* was counted in dilutions of 10^{-1}-10^{-3} of BPW. The specific media used was eosin methylene blue agar (EMBA, Oxoid). Samples were evenly spread using a sterile hockey stick and incubated at 37°C for 48 h. *Escherichia coli* colonies were presented as purple-blue parts.

For the quantitative estimation of *Salmonella*[11], the growth of *Salmonella* spp., was calculated in dilutions of 10^{-1}-10^{-3} of BPW. The sample (1 mL) was poured into the petri dish, covered with Xylose Lysine Deoxycholate Agar (XLDA) media (Oxoid) and incubated at 37°C for 48 h. The murky-yellow colonies with black spots of *Salmonella* spp., were calculated.

For the quantitative analysis of *Staphylococcus aureus*[11], the growth of *S. aureus* was calculated in dilutions of 10^{-1}-10^{-3} of BPW. The samples were inoculated in Baird Parker Agar (BPA) media with egg yolk, potassium tellurite and NaCl. *Staphylococcus aureus* colonies were presented as black spots surrounded by yellow.

RESULTS AND DISCUSSION

Prior to the sausage processing, the physical and chemical qualities of the beef were measured. Table 1 shows the physical quality of the beef. The results showed that the pH value was in an acceptable range of 5.4-5.8. Furthermore, the water activity (a_w) obtained was 0.87, suggesting that the beef was susceptible to microbial deterioration. This a_w value was lower than that of fresh meat (0.99). The total titrated acid of the fresh meat was 0.39%, which was categorized as low. This acid was produced by the degradation of lactic acid.

Table 2 presents the chemical profiles of the meat. The moisture content was 69.56%, which was low. The low water content indicated increased durability of the food[12]. In addition, the protein content of the fresh meat was 16.15%, which was also lower than a previous report (19%)[12]. The fat and carbohydrate contents were 2.22 and 0.11%, respectively.

The effects of the preservative addition on the sausage are presented in Table 3. The water content of the sausages was lower than that of the fresh meat since the heating induced water evaporation. The water content of the sausages treated with bacteriocin 0.3% was lower than that of the other treatments. The level of water content in the products may be affected by factors, such as raw material, additive ingredients, processing, packaging and storage. However, the ash content of the sausages treated with bacteriocin 0.3% was higher than that of the other treatments. Combustion or incineration processes may contribute to the degradation of bacteriocin, which results in a higher ash content[12]. Andarwulan *et al.*[13] stated that the ash content was influenced by the presence of mineral content in the materials.

Table 1: Results of physical analysis on fresh meat

Variables	Values
pH	5.48
a_w	0.87
Total acid number (%)	0.39

a_w: Water activity

Table 2: Chemical composition of fresh meat

Variables (wb%)	Fresh meat	*Fresh meat
Water content	69.46	75.0
Protein content	16.15	19.0
Fat content	2.22	2.5
Ash content	1.23	-
Carbohydrates	0.11	1.2

Source: *Lawrie[26], wb%: Wet basis%

Table 3: Chemical composition of sausage

Variables	Treatments		
	Control	Nitrite 0.3%	Bacteriocin 0.3%
Water content (wb%)	58.52±0.81	59.19±1.95	57.57±1.42
Ash content (wb%)	2.67±0.10	2.83±0.03	3.15±0.14
Protein content (wb%)	13.47±0.85	12.87±0.45	14.92±0.68
Fat content (wb%)	11.51±0.76^{a}	9.21±0.83^{b}	8.65±0.49^{b}
Carbohydrates (wb%)	13.83±0.36^{a}	15.90±0.63^{b}	15.71±0.76^{b}
Nitrite residue (ppm)	6.87±0.20^{a}	10.00±0.46^{b}	1.37±0.55^{c}

wb%: Wet basis%, Different superscripts following values in the same rows indicate significant difference ($p<0.05$)

Table 4: Appearance of sausage evaluated by hedonic test

Variables	Treatments		
	Control	Nitrite 0.3%	Bacteriocin 0.3%
Color	3.43±0.23^{a}	3.41±0.25^{a}	2.98±0.14^{b}
Taste	3.45±0.43^{a}	3.41±0.23^{a}	3.00±0.24^{b}
Aroma	3.53±0.28^{a}	3.38±0.07^{a}	3.11±0.11^{b}
Texture	3.31±0.25^{b}	3.58±0.23^{a}	3.11±0.24^{b}
Chewiness	3.34±0.20^{b}	3.56±0.24^{a}	3.23±0.18^{b}

Different superscripts following values in the same rows indicate significant difference ($p<0.05$). Hedonic scale: 1: Dislike extremely, 2: Dislike, 3: Neutral, 4: Like, 5: Like extremely

Table 5: Appearance of sausage evaluated by hedonic quality test

Variables	Treatments		
	Control	Nitrite 0.3%	Bacteriocin 0.3%
Color	3.79±0.05	4.09±0.27	3.99±0.22
Taste	2.39±0.30	2.71±0.15	2.80±0.30
Aroma	2.20±0.35^{b}	2.59±0.12^{a}	2.59±0.34^{a}
Texture	3.31±0.25^{b}	3.58±0.23^{a}	3.11±0.24^{b}
Chewiness	3.34±0.20^{b}	3.56±0.24^{a}	3.23±0.18^{b}

Different superscripts following values in the same rows indicate significant difference ($p<0.05$). Color: Red extremely, Red, Brownish-red, Brown, Brown extremely; Taste: Highly meaty, Meaty, Neutral, Not meaty, Not highly meaty; Texture: Highly soft, Soft, Neutral, Coarse, Highly coarse; Chewiness: Highly chewy, Chewy, Neutral, Not chewy, Not highly chewy

The protein content in the sausage with added bacteriocin was also higher than that of the other treatments. Bacteriocin is ribosomally synthesized antimicrobial peptides or proteins[14]. Savadogo *et al.*[15] reported that bacteriocin may be a combination of proteins, carbohydrates and fats. Masuda *et al.*[14] stated that most bacteriocin was produced as protein precursors, in which N-terminal leader peptides were removed during secretion, yielding mature bacteriocin peptides. Therefore, the bacteriocin-treated sausage had a higher protein content. The sausage treated with bacteriocin and control sausages have acceptable protein content based on Badan Standarisasi Nasional[16]. The fat content of the sausages with or without preservatives meets the standards set by Badan Standarisasi Nasional[16], while the carbohydrate levels exceed the standard maximum of 8%. The fat and carbohydrate contents in the sausage are influenced by additive materials, such as emulsifier and stabilizer[17].

Sausages supplemented with nitrite still include residues, while the nitrite residue in the bacteriocin sausage is low. Nitrite residues may produce nitrosamine compounds when consumed in excessive amounts or continuously[18]. The compound is carcinogenic, which promotes health problems, such as cancer. Use of bacteriocin as a nitrite replacement could produce healthier products because of the reduction in nitrite residue. Savadogo *et al.*[15] reported that lactic acid bacteria produce bacteriocin secreted enzymes that produced glucuronidase, azoreductase and nitroreductase, which contribute to nitrite reduction.

Table 4 and 5 present the organoleptic evaluation of the sausages. The results indicated that the flavor, aroma, texture and elasticity were acceptable to the panelists. The color of the nitrate and control sausages was rated as "like", while the bacteriocin sausage was rated as "neutral". However, hedonic tests showed that the sausages were brown in color in all sausage treatments. Arief *et al.*[19] described that the brown color in the nitrite sausage was due to the oxidation of myoglobin color pigments in meat to metmyoglobin. The protein binds to Fe^{2+} (ferrous) and porphyrin, resulting in a brown color or myoglobin denatured into heme (protein

Table 6: Physicochemical quality of sausage during storage

Parameters	Treatments	Storage time (day) 0	3	6	9
pH	Control	5.56±0.24	5.69±0.11	5.92±0.14	5.79±0.10
	Nitrite 0.3%	5.92±0.07	5.73±0.07	5.73±0.19	5.91±0.10
	Bacteriocin 0.3%	5.74±0.22	5.65±0.04	5.56±0.25	5.83±0.09
a_w	Control	0.84±0.02	0.83±0.01^{a}	0.90±0.01	0.92±0.02
	Nitrite 0.3%	0.85±0.01	0.89±0.01^{b}	0.89±0.00	0.93±0.03
	Bacteriocin 0.3%	0.83±0.01	0.83±0.01^{a}	0.89±0.00	0.91±0.01
WBC (%)	Control	1.93±0.15^{a}	0.85±0.13^{a}	0.77±0.10^{a}	0.87±0.10^{a}
	Nitrite 0.3%	1.02±0.54^{b}	1.00±0.22^{b}	0.68±0.19^{a}	0.69±0.02^{b}
	Bacteriocin 0.3%	1.65±0.30^{ab}	0.97±0.19^{ab}	0.58±0.14^{b}	0.62±0.18^{b}
Total acid number (%)	Control	0.30±0.05	0.62±0.10^{a}	0.27±0.05^{b}	0.31±0.06^{a}
	Nitrite 0.3%	0.29±0.03	0.71±0.10^{b}	0.25±0.03^{a}	0.35±0.01^{b}
	Bacteriocin 0.3%	0.28±0.01	0.71±0.25^{b}	0.24±0.05^{a}	0.34±0.04^{ab}

a_w: Water activity, Different superscripts following values in the same columns indicate for each parameter significant difference ($p<0.05$)

and Fe^{2+} binds to porphyrins) and then oxidized to hemin (Fe^{3+} (ferry) that binds to the porphyrin.

Furthermore, based on the tests of hedonic quality, the taste of the sausage was not affected by the treatments. All sausages had a meaty taste. This result is due to the insignificant pH differences among the preservatives. Arief *et al.*[20] explained that the aroma of cooked meat was determined by the fat and water-soluble precursors and the release of volatile substances in the meat. The preservative treatment had no effects on the sausage texture. This is because of the similar pH levels[21]. The sausage textures and chewiness ranged from fine to coarse and chewy to not chewy, respectively. The sausage elasticity was also the same in all treatments.

The physicochemical properties of the sausages treated with various preservatives and length of cold storage are presented in Table 6. The results indicated that the pH value ranged from 5.56-5.92. This pH value was relatively constant, indicating that the amount of lactic acid produced by glycolysis tends to be constant. This pH range was also desirable since it is an undesirable condition for pathogenic growth. Soeparno[21] reported that the optimum conditions for their growth was at a pH of 7.0.

The addition of various preservatives showed no significant difference in a_w values and moisture content. However, storage periods positively correlated with a_w values. Different preservatives caused no significant differences in water absorption. It was found that a longer storage time increased the water absorption of the sausages.

Various preservatives were added to the sausages and resulted in an almost similar titrated acid value, meaning that the micro-organisms produced the same amount of lactic acid in both preserved sausages and control sausages. The acid content increased in day 3 of storage since the microorganisms contained in the sausage were at the log phase. In this stage, the micro-organisms were able to adapt to the environment, proliferate and increase acid production[22]. However, decreased acid content was observed at day 6 of storage. The reduction of the acid level in this phase was associated with a decreased availability of the nutrition that is required by micro-organisms for their growth[22]. Furthermore, there was an increase in the acid content on day 9, which was caused by the metabolism of bacterial activity in the sausages stored at cold temperatures. Soeparno[21] explained that the psychrophilic micro-organisms were able to grow at refrigeration temperatures but the optimal temperature was from 20-30°C.

Table 7 exhibits the microbiological evaluation of the sausages treated with different preservatives and cold storage periods. The total number of microbes is necessary to ensure the viability of food. The total bacteria still meets the standard of SNI 01-3818-1995, the threshold of microbial contamination is less than 10^5 colonies g^{-1}. According to the total plate count, the value of the bacteriocin-treated sausage was lower than that of the nitrite-treated sausage and the control sausage. Microbial growth in food is closely related to the amount of water content. The need for water is usually expressed as water activity (a_w). Sausage with a high a_w (0.83-0.91) is a good medium for micro-organisms. Fardiaz[8] reported that the minimum a_w value required for bacterial growth was 0.91.

Escherichia coli is not only the dominant anaerobic Gram-negative in the digestive system but it is also a potential pathogen capable of causing a variety of diseases with complicated mechanisms. *Escherichia coli* can cause no less than six overlapping clinical syndromes including diarrhea but with different signs and epidemiology; *E. coli* can cause urinary tract infections ranging from asymptomatic bacteriuria to urosepsis and neonatal meningitis, pneumonia, cholecystitis and wound infection. *Escherichia coli* is a bacterium used as an indicator of sanitation. The presence

Table 7: Microbiological quality of sausage during storage

Parameters (log CFU g^{-1})	Treatments	Storage time (day) 0	3	6	9
TPC	Control	3.18±0.25	2.15±0.36	3.26±0.84[a]	4.75±2.05[a]
	Nitrite 0.3%	3.00±0.26	2.23±0.27	3.60±0.32[a]	3.65±0.17[b]
	Bacteriocin 0.3%	3.25±0.28	2.24±0.47	2.30±1.21[b]	3.66±0.22[b]
Escherichia coli	Control	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Nitrite 0.3%	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Bacteriocin 0.3%	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Salmonella	Control	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Nitrite 0.3%	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	Bacteriocin 0.3%	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Staphylococcus aureus	Control	2.46±0.14[a]	2.86±0.34[a]	3.43±0.36[a]	3.77±0.13[a]
	Nitrite 0.3%	2.09±1.33[a]	2.29±0.26[a]	2.91±0.25[b]	3.36±0.45[a]
	Bacteriocin 0.3%	1.48±1.32[b]	1.03±0.90[b]	1.54±1.42[c]	2.41±0.22[b]

TPC: Total plate count, Different superscripts following values in the same columns indicate for each parameter significant difference ($p<0.05$)

of *E. coli* is one indicator of a poor sanitary application. The results showed that the average population of *E. coli* bacteria in the control, nitrite and bacteriocin sausages was still under the threshold as determined by SNI 01-3818-1995 at 10^3 colonies g^{-1}. The absence of *E. coli* in the sausage indicated that it is safe for consumption. *Escherichia coli* bacteria was not found on any sausage. This is due to the lack of *E. coli* bacteria in the raw meat material used.

The sausages were produced in accordance with the quality requirements established by SNI 01-3818-1995 requiring that *Salmonella* contamination must be negative. It was found that the produced sausages were not contaminated by *Salmonella* spp. Additionally, *S. aureus* produces an enterotoxin that is also as an indicator of pathogens. Therefore, it is important to consider the microbiological safety of the product. *Salmonella aureus* originally already exceeded the maximum contamination according to ISO-3820-1995 in the amount of 10^2 colonies g^{-1} for the control, the addition of nitrite and the addition of 0.3% bacteriocin stored for 9 days. Thus, the bacteriocin-treated sausage was safe for 6 days of storage based on the microbiological aspect. Bacteriocin produced by Lactic Acid Bacteria (LAB) was applicable for food preservation and has a potential as a substitute for antibiotics[23]. Arief *et al.*[24] found that bacteriocin from *L. plantarum* showed antimicrobial activity that inhibits the growth of *S. aureus*; thus, it had bacteriostatic properties. Kia *et al.*[25] reported a decrease of *S. aureus* bacterial contamination in meatballs, which were acceptable for consumption after 20 h at room temperature.

CONCLUSION

It can be concluded that bacteriocin purified from *Lactobacillus plantarum* IIA-1A5 was effective as a bio-preservative. It could inhibit the growth of *Staphylococcus aureus* and *Escherichia coli* pathogenic bacteria on beef sausage until after 6 days of storage time, which is better than sausages treated with nitrite. This result showed that bacteriocin could replace nitrite as a preservative for up to 6 days of storage time.

SIGNIFICANCE STATEMENTS

This study discover the application of plantaricin IIA-1A5 as novel bacteriocin that can be beneficial for biopreservative for animal food products, especially in sausage. In this study, highlighted the effect of the introduction of plantaricin IIA-1A5 in beef sausages upon storage for up to 9 days at refrigerated temperature. This result showed that plantaricin IIA-1A5 could replace nitrite as a preservative for up to 6 days of storage time. This study will help the researcher to uncover the critical areas of food biopreservatives that many researchers were not able to explore. Thus a new theory on application of novel bacteriocin as biopreservatives in sausages may be arrived at.

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