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Short Communication: Application of bacteriocin from *Lactobacillus plantarum* SRCM 1 004 34 strain isolated from okara as a natural preservative in beef sausage

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Abstract. *Aritonang SN, Roza E, Sandra A. 2020. Short Communication: Application of bacteriocin from* Lactobacillus plantarum *SRCM 1 004 34 strain isolated from okara as a natural preservative in beef sausage. Biodiversitas 21: 2240-2245.* Lactic Acid Bacteria (LAB) can be found in food and most of the LAB have beneficial effects for humans as potential probiotics. LAB isolated from okara (soy milk pulp) produces antimicrobial compounds called bacteriocins which can be used as natural preservatives in sausages. This study aims to determine the effectiveness of this bacteriocin from LAB as a natural preservative in sausages. The bacteriocin was isolated from *Lactobacillus plantarum* SRCM 1 004 34 strain isolated from Okara. Completely Randomized Design (CRD) was used in 4 X 4 factorial pattern by three replicates The first factor was the percentage addition of bacteriocin (B): 0% (B_0), 0.3% (B_1), 0.6% (B_2) and 0.9% v/b (B_3). The second factor was the length of sausage storage time at 4°C (L): 0 days (L0), 4 days (L_1), 8 days (L_2) and 12 days (L_3). The observed variables were moisture content, protein, fat, pH, total aerobic bacteria, and sausage fatty acid profile. The results showed that the content of protein, fat, and the bacterial count was significantly influenced by the interaction of the percentage addition of bacteriocin and storage time. pH was significantly affected by bacteriocin dose and storage time but was not affected by the interaction of other factors. The moisture content of the sausage was significantly affected by storage time. The results of this study showed that sausages treated with 0.9% bacteriocin (B_3) with a maximum storage time of 12 in the refrigerator was still safe to be consumed.

Keywords: Bacteriocin, lactic acid bacteria, preservative, storage time, sausage

INTRODUCTION

Lactic Acid Bacteria (LAB) are bacteria that produce lactic acid from the breakdown of carbohydrates and antibacterial substances including bacteriocins and hydrogen peroxide, which can inhibit the growth of microbes. Therefore it can be used as a preservative or natural antimicrobial (Alakomi et al. 2000). The effectiveness of LAB in inhibiting pathogenic and destroying bacteria is influenced by the density and composition of the media and the strain. Lactic acid bacteria such as *Lactobacillus lactis* and *Streptococcus thermophillus* are used to inhibit food spoilage and pathogenic bacteria and preserve the nutritive quality of food (Heller 2001). Lactic Acid Bacteria can be isolated from various natural sources and are produced especially during the fermentation process of some foods. LABs are probiotics that are Generally Recognized as Safe (GRAS) therefore they have no health risks health. Previous studies showed that LAB can reduce cholesterol (Liong and Shah 2005; Jeun et al. 2010), increased the nutritional value of food, control intestinal infections and improve digestion because LABs produce lactase in the digestive tract of humans and animals (LeBlanc et al. 2008).

One important characteristic of LAB is its ability to produce antimicrobial compounds bacteriocins that inhibit the growth of pathogenic microorganisms, therefore it can be used as bio-preservatives (Savadogo et al. 2006). There are several classes of bacteriocins, i.e. simple peptides or proteins and others contain lipid molecules (Salminen 2004). They act as bactericidal or bacteriostatic agents against other bacteria. They are easily degraded by proteolytic enzymes and they can inhibit the growth of microorganisms that phylogenetically close to bacteria that produced bacteriocins. (De-Vuyst and Leroy 2007). The bacteriocins produced by LAB can inhibit the growth of pathogenic microbes and those involved in decomposition such as *Bacillus cereus*, *Clostridium botulinum*, *Clostridium perfringens*, *Listeria monocytogenes*, and *Staphylococcus aureus* (Diop et al. 2007). The application of bacteriocins in food does not affect the taste and appearance of the product. Bacteriocins produced by LAB can be utilized in the form of supernatant, partially purified, or more completely purified products (Woraprayote et al. 2016). Bacteriocins are commonly used in the food industry especially in fermented foods to inhibit the growth of bacterial contaminants that cause food spoilage and food-borne diseases (Abdelbasset et al. 2008). The antimicrobial compounds may affect bacterial metabolism and toxin production (Rolfe 2000).

Sausages are meat products with the addition of fillers and binders such as vegetable flour or starch, seasonings, and other permitted food ingredients to be placed in sausage casings (Hui et al. 2001). In Indonesia, sausages must contain at least 75% of meat (SNI 1995). According to the USDA (2001), fresh sausages stored in the fridge (4°C) have a shelf life of 1-2 days.

The shelf life of fresh sausages can be extended by adding nitrite, but excessive consumption of nitrites can be harmful to health (Stringer and Pin 2005). Nitrites can bind to the amino and amides present in meat proteins to form carcinogenic nitrosamines (Zarringhalami et al.2009). The use of nitrite as a preservative in making sausages can be reduced by the addition of bacteriocin. The addition of 0.3% bacteriocin extracted from *Lactobacillus plantarum* 2C12 was able to inhibit the growth of *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella typhimurium* in meatballs without any changes of the taste (Arief et al. 2012).

Lactic Acid Bacteria in this study were isolated from Okara. Okara is the residue left from ground soybeans after filtration to produce soy milk, however, it still has a high nutritional content. Okara contains 28.36% crude protein, 5.52% fat, 7.6% crude fiber, lysine and methionine amino acids, and vitamin B (Hsieh and Yang 2003), therefore it can be used as growth media for bacteria including lactic acid bacteria. The aim of the research was to study the role of bacteriocin from *L. plantarum* SRCM 1 004 34 strain isolated from okara as a natural preservative in beef sausage.

MATERIALS AND METHODS

Materials

Bacteriocin used in this study was isolated from *L. plantarum* strain SRCM 1 004 34 from okara (Aritonang et al. 2017).

Research design

The study used a Completely Randomized Design (CRD) in 4x4 factorial pattern by three replicates The first factor was four levels of bacteriocin addition (B), i.e., 0% (B_0), 0.3% (B_1), 0.6% (B_2) and 0.9% (B_3). The second factor was the length of sausage storage time (L) at 4°C: 0 days (L_0), 4 days (L_1), 8 days (L_2) and 12 days (L_3).

Data analysis

The data were statistically analyzed using the Statistical Analysis System (SAS). Mean comparisons were carried out by Duncan's Multiple Range Test (DMRT) with significance at $P < 0.05$.

Bacteriocin extraction (Yang et al. 2012)

Ten ml of *L. plantarum* SRCM 1 004 34 strain culture was inoculated into 90 ml of MRS-B media, then incubated in a *shaker* incubator at a speed of 100 rpm at 37°C for 24 hours. After incubation, the growth media was centrifuged at 4,000 rpm at 4°C for 25 minutes and then filtered using a 0.22 µm membrane filter. The crude bacteriocin supernatant was used as sausages preservative.

The process of making sausages (Modified Erkkila 2001)

Beef ribs (meat and fat) were washed thoroughly, and then added 3% salt and 20% ice/ice water followed by grinding until smooth. Ground meat was added with 15% tapioca flour, 10% skim milk, and spices and a preservative (garlic 0.4%, onion 0.6%, ginger 0.5%, sugar 1.5%, salt 2%, nitrite 150 ppm, and pepper 0.15%) as a filler and mixed until well blended. Sausage mixture was cured at 4-7°C for 24 hours. The sausage mixture was divided into 48 parts and added with supernatant containing bacteriocin according to the treatment: 0% (B_0), 0.3% (B_1), 0.6% (B_2) and 0.9% (B_3). Stuff mixture into casings and steamed at 80°C for 40 minutes, and then cooled down to room temperature for 2 hours, followed by storing in a refrigerator according to the treatment of storage time: 0 days (L_0), 4 days (L_1), 8 days (L_2) or 12 days (L_3). Every treatment has three replicates

Determination of chemical composition

Chemical properties of sausages: The moisture, protein, and fat content were determined according to AOAC (2005).

Fatty acid composition: was determined by extracting samples using chloroform: methanol (2: 1) solution. The extract was partitioned with methanol using the Morrison and Smith method (1964). Fatty acids were separated on a capillary column (30 m × 0.32 mm with 0.25 µm film thickness) that connected to a Gas Chromatography (GC, Model Star 3600, Palo Alto, USA). The GC was conditioned at 250°C for the injection port and 300°C for detectors. The free fatty acids in the samples were identified by comparing their retention time to those of standard fatty acids. The results were expressed as relative percentages based on the total peak area.

Total aerobic bacteria count (Fardiaz 1992): Five grams of the sausage was crushed until smooth, then put into a flask containing 45 ml of NaCl solution and homogenized. Take one ml of the solution and diluted to 10^{-7} and 10^{-8}. One hundred μl from each dilution was pipetted and placed on Plate Count Agar (PCA) media in a petri dish. Petri dishes were incubated at 37°C for 48 hours in an inverted position. The number of bacteria was determined by the plate count method and Standard Plate Count (SPC).

RESULTS AND DISCUSSION

Moisture content

Table 1. showed that the moisture content of beef sausages was not affected by the interaction between bacteriocin dose and sausage storage time ($P > 0.05$) but it was significantly affected by storage time ($P < 0.05$). The level of bacteriocin addition did not affect significantly moisture content ($P > 0.05$).

Storage time of 12 days (L_3) result in the highest level of moisture content in sausages but it was not significantly different from moisture content in 8 days (L_2) and 4 days (L_1) storage time. Naturally, food degraded during storage which can result in increased moisture content (Ray and

Bhuna 2008). The results of this study showed that 12 days of storage at 4ºC did not significantly change the chemical composition of sausages, but the moisture content was slightly increased. The increase of moisture content did not reduce sausage quality, because the moisture content still meets the Indonesian National Standard (INS 2015) requirements which is less than 67%. Arief et al. (2017) showed that the addition of bacteriocin derived from *L. plantarum* IIA-1A5 produces sausages with the moisture content of 57.57%. The addition of bacteriocin supernatant in this study did not significantly affect the moisture content of beef sausages (P> 0.05 because the primary content of bacteriocin is proteins.

Protein

The protein content of sausages was not significantly affected by the interaction between the bacteriocin dose and the storage time ($P < 0.05$) (Table 2). Increasing bacteriocin dose up to 0.9% (B3) result in increasing protein content due to the addition of protein from bacteriocin (Jack et al. 1995). This is because bacteriocins are a precursor protein that carries N-terminals in the main peptide that remains it is in the cytoplasm to play a role again in the synthesis of bacteriocins (Ray 2004).

Table 1. The effect of bacteriocin dose and storage time to the moisture content of sausages

Treatment	L_0	L_1	L_2	L_3	Average
B_0	52.74	52.12	53.17	54.04	53.02
B_1	52.60	52.32	53.59	54.20	53.18
B_2	52.85	52.37	53.45	54.10	53.19
B_3	52.75	52.79	52.87	53.69	53.11
Average	52.74^a	52.40^b	53.27^b	54.01^b	

Note: $^{a, b}$ Means in the same column with a different letter are significantly different (P<0.05). L: Length of storage time. B: Percentage of bacteriocin

Table 2. The effect of bacteriocin dose and storage time to the protein content of sausages (%)

Treatment	L_0	L_1	L_2	L_3
B_0	18.37^b	18.22^b	18.31^b	18.05^a
B_1	18.72^d	18.48^{ab}	18.46^{ab}	18.21^b
B_2	19.07^f	18.87^e	18.70^d	18.51^c
B_3	19.53^g	19.40^g	19.43^g	19.39^g

Note: $^{a-g}$: Means with a different letter are significantly different (P<0.05)

Table 3. The effect of bacteriocin dose and storage time to the fat content of sausages (%)

Treatment	L_0	L_1	L_2	L_3
B_0	6.11^c	6.06^{ab}	6.01^a	6.07^b
B_1	6.40^g	6.30^f	6.19^e	6.15^d
B_2	6.47^i	6.40^g	6.42^h	6.22^e
B_3	6.52^j	6.50^{ij}	6.48^{ij}	6.47^i

Note: $^{a-g}$: Means with a different letter are significantly different (P<0.05)

The protein content of sausages containing 0.9% bacteriocin was slightly decreased after being stored for 12 days at 4-10ºC, but it did not significantly different from sausages that were not stored (L_0B_3). The addition of bacteriocin to 0.9% did not change the nutrient content of sausages after being stored for 12 days. It might be caused by the ability of bacteriocins to inhibit the growth of microorganisms such as proteolytic bacteria that could potentially break down protein (Collins et al. 2010).

The protein content of sausages in this study ranged from 18-19%, which was above the minimum protein content of 13%, according to the Indonesian National Standard (SNI 2015). these results were in line with the results of Melia (2018) that the addition of bacteriocin from *Lactobacillus fermentum* L23 produced sausages with protein content range 17-18%.

Fat

The fat content of the sausages was significantly affected by the interaction between bacteriocin dose and sausage storage time ($P < 0.05$) (Table 3). The addition of bacteriocin increased the fat content of sausages regardless of storage time. Increasing the dose of bacteriocin results in increasing the fat content of sausages.

The increase of fat content in sausages containing bacteriocin due to lipid content in bacteriocins (Ouattara et al. 2011). Sausages containing 0.9% bacteriocin (B3) stored for 12 days at cold temperatures (4-10ºC) have the highest fat content but are not significantly different from sausages that are stored for 4 days and 8 days.

These results indicated that the addition of bacteriocin can maintain the fat content of beef sausages at cold storage for 12 days. Bacteriocins contain antimicrobial compounds that inhibit lipid decomposition thereby reducing fat degradation. When bacteriocin peptides attach to target bacterial cell membranes, the positive end of the peptide binds to the fatty acids present in the membrane phospholipid, and then was separated by formation of pores which finally the bacterial death was in own cell (Song and Zheng 2015). The fat content of sausages in this study was around 6% which was below the maximum level (20%) according to the Indonesian National Standard (INS 2015). This result was in line with the result of Melia (2018) that the addition of bacteriocin from *L. fermentum* L23 produced sausage with the fat content of 6.85%.

pH

Table 4. showed that the pH of beef sausages was affected significantly by the bacteriocin dose and storage period ($P < 0.05$) but was not affected by the interaction between these two factors ($P > 0.05$).

Table 4. The effect of bacteriocin dose and storage time to the pH of sausages

Treatment	L_0	L_1	L_2	L_3	Average
B_0	5.40	5.40	5.50	5.50	5.45^a
B_1	5.40	5.40	5.40	5.50	5.40^a
B_2	5.30	5.40	5.40	5.40	5.40^a
B_3	5.20	5.30	5.30	5.30	5.30^b
Average	5.32^a	5.39^b	5.40^b	5.43^b	

Note: $^{a, b}$ Means in the same column/row with a different letter are significantly different (P<0.05)

Table 5. The effect of bacteriocin dose and storage time to the Total Aerobic Bacteria (x 10^3 Cfu/mL)

Treatment	L_0	L_1	L_2	L_3
B_0	14.37^a	14.86^{bc}	16.57^e	19.33^f
B_1	14.30^a	14.60^b	15.57^c	16.05^d
B_2	14.27^a	14.65^b	15.35^c	16.06^d
B_3	14.26^a	14.37^a	14.98^{bc}	15.70^c

Note: $^{a-f}$: Means with a different letter are significantly different ($P<0.05$)

The highest addition of bacteriocin (0.9%, B_3) produced the lowest sausage pH (5.2). The decrease in pH of beef sausages due to the amino acids in bacteriocin which contain hydrogen atoms in the carboxyl group dissociates to produce H^+ ions thereby increasing acidity and reducing pH.

The length of storage time from 4 to 12 days (L_1, L_2, L_3) did not significantly affect the pH of sausages but slightly higher than control (L_0). An increase of sausage pH accord along with an increase of storage duration in low temperature due to enzyme activity and chemical decomposition of compounds such as proteins which results in the production of alkaline compounds such as indole, scatole, and cadaverine (Suradi 2012).

Total aerobic bacteria

Total aerobic bacteria were significantly affected by the interaction between the bacteriocin dose and the storage time of sausage ($P<0.05$) (Table 5). The addition of bacteriocin significantly inhibits the growth of aerobic bacteria during storage. Bacteriocin addition to 0.9% (B3) suppressed the increase of total aerobic bacteria. Total aerobic bacteria in the B_3L_3 treatment (0.9% bacteriocin, 12 days of storage) was 15.70 x 10^3 CFU/mL which was not significantly different from B2L2 treatment (0.6% bacteriocin, 8 days of storage) which was 15.35 x 10^3Cfu/mL.

Total aerobic bacteria in sausages with B_3L_3 treatment were higher compared to the results of Wibowo et al. (2017) showed that the addition of the bacteriocin supernatant from *L. plantarum* IBL-2 to fresh ground beef and stored for 12 days had total aerobic bacteria of 0.96 x 10^4 CFU / ml. Because *L. plantarum* IBL-2 of Wibowo research added with nisin amide. While the addition of nisin influenced decreased bacterial growth. So that why total aerobic bacterial was lower than the result of this study.

Result of this research is also higher than Arief et al. (2017) study which is Total aerobic bacteria in sausages with the addition of bacteriocin from *L. plantarum* IIA-1A5 is 3.66 x 10^3 CFU/mL. It caused the storage time of sausage at Arief's et al studied up to 9th days. Total aerobic bacteria are still under the maximum number (1 x $10^{5)}$ permitted by SNI (1995). The results suggest that 0.9% bacteriocin addition to sausages that sored for 12 in the refrigerator are still safe for consumption. SNI (1995). Therefore sausages with the addition of 0.9% bacteriocin stored for 12 days in the refrigerator are still safe for consumption.

Fatty acid profile

The percentage of bacteriocin addition and storage time affected the lipolysis process in sausages, results in changing the profile of fatty acids. Fatty acid profile of sausages was presented in Table 6. showed there were 13 types of fatty acids were detected in all samples. Percentage of stearic acid (C18:0), linolenic acid (C18:3n3), erucic acid (C20:1n9), and arachidonic acid (C20:4n6) were significantly different among treatments ($P<0.05$). The highest level of C18:0 (13.85%) was in sausages treated with 0.9% bacteriocin without storage (B_3L_0), while the lowest was sausages without the addition of bacteriocin and stored 12 days (B0L3).
The results suggest that the highest dose of bacteriocin (0.9%) inhibited fat decomposition by lipolytic bacteria and result in a high level of stearic acid. Bacteriocins are known to inhibit lipolytic bacteria (Abdelbasset et al. 2008). Levels of highly unsaturated fatty acids such as C18:3n3 and C20:4n6 were significantly higher in sausages which were treated with 0.9% bacteriocin and stored for 4 days (B_3L_2) were 0.67% and 0.49% respectively.

Unsaturated Fatty Acids (UFA) levels and n6/n3 ratio also differed among treatments. UFA levels were higher in bacteriocin-treated sausages than controls and being the highest was in sausages treated with 0.9 % bacteriocin on 0 days of storage (B_3L_0). The ratio of n6/n3 was lower in sausages treated with high bacteriocin than control, and being the lowest ratio was sausages with 0.9% bacteriocin with a storage period of 12 days. UFA especially PUFAs such as C18:3n3 have a highly significant influence on human health (Jump 2002), and a high ratio of n6/n3 is good for health (Krauss et al. 1998). Differences in fatty acid content between treatments due to differences in the lipase activity of microbes (Chen et al. 2017). Fatty acid levels may be influenced by the antimicrobial activity of bacteriocin that capable of inhibiting the growth of microorganisms (Collins et al. 2010).

In conclusion, the protein and fat content, pH, total aerobic bacteria, and fatty acid profile of the beef sausages were significantly influenced by bacteriocin dose and storage time. The moisture content of sausages was significantly influenced by storage time. The addition of 0.9% bacteriocin produces sausages that are still suitable for consumption after 12 days of storage at the refrigerator based on the chemical composition and total aerobic bacteria.

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Table 6. Percentage of fatty acid in sausage (%)

	B0.0	B0.4	B0.8	B0.12	B1.0	B1.4	B1.8	B1.12	B2.0	B2.4	B2.8	B2.12	B3.0	B3.4	B3.8	B3.12
C14:0	1.40	1.41	1.48	1.46	1.51	1.51	1.45	1.57	1.52	1.52	1.52	1.55	1.56	1.52	1.55	1.58
C16:0	22.45	22.70	22.85	22.15	22.87	22.72	22.15	22.18	23.25	23.05	22.85	22.35	23.60	23.35	23.20	23.15
C16:1n7	1.25	1.25	1.23	1.15	1.18	1.38	1.25	1.23	1.57	1.40	1.48	1.35	1.85	1.60	1.67	1.45
C18:0	12.47^{b}	12.40^{b}	12.20^{b}	11.25^{a}	12.85^{bc}	12.25^{b}	12.37^{b}	11.87^{a}	13.57^{c}	13.05^{c}	13.23^{c}	12.15^{b}	13.85^{d}	13.50^{cd}	13.25^{c}	12.87^{bc}
C18:1n9	40.90	40.60	40.53	39.95	40.85	40.63	40.67	40.45	40.95	40.70	40.25	40.00	40.73	40.75	40.78	40.55
C18:2n7	0.15	0.14	0.13	0.10	0.16	0.14	0.13	0.12	0.17	0.15	0.15	0.14	0.18	0.15	0.15	0.10
C18:2n6	15.51	15.50	15.48	15.27	15.78	15.73	15.65	15.35	16.41	16.47	16.35	16.05	16.75	16.53	16.45	16.25
C18:3n6	0.06	0.05	0.05	0.04	0.06	0.06	0.05	0.04	0.07	0.07	0.07	0.06	0.05	0.07	0.06	0.04
C18:3n3	0.52^{a}	0.52^{a}	0.50^{a}	0.49^{a}	0.52^{a}	0.51^{a}	0.50^{a}	0.48^{a}	0.57^{b}	0.53^{ab}	0.51^{a}	0.47^{a}	0.62b^{c}	0.67^{c}	0.57^{b}	0.54^{ab}
C20:1n9	0.62^{a}	0.83^{bc}	0.75^{b}	0.85^{c}	0.87^{c}	0.83^{b}	0.81^{b}	0.76^{b}	0.95^{d}	0.87^{c}	0.85^{c}	0.79^{a}	0.98^{d}	0.95^{d}	0.87^{c}	0.84^{bc}
C20:4n6	0.32^{ab}	0.30^{a}	0.26^{a}	0.24^{a}	0.37b	0.33^{ab}	0.30	0.27^{a}	0.39^{c}	0.37^{b}	0.37^{b}	0.31^{a}	0.45^{cd}	0.49^{d}	0.39^{c}	0.35^{ab}
C20:5n3	0.01	0.01	0.009	0.008	0.013	0.013	0.011	0.009	0.014	0.013	0.011	0.009	0.015	0.015	0.012	0.009
C22:4n6	0.13	0.13	0.12	0.10	0.16	0.15	0.12	0.11	0.16	0.16	0.13	0.11	0.18	0.16	0.14	0.12
SFA	38.70	38.70	38.20	38.00	39.70	39.67	38.50	38.10	39.87	39.65	39.10	38.80	39.92	39.85	39.50	38.80
UFA	55.40^{b}	55.25^{ab}	55.03^{a}	54,92^{a}	57.90^{de}	57.50^{d}	56.47^{c}	56.40^{c}	59.20^{f}	58.12^{e}	57.50^{d}	57.01^{cd}	62.20^{g}	61.81^{h}	61.35^{h}	60.95^{g}
MUFA	42.42	42.40	42.35	42.05	42.92	42.75	42.45	41.95	42.81	42.80	42.68	42.49	42.98	42.87	42.62	42.43
PUFA	17.43	17.37	17.15	17.05	18.00	17.87	17.63	17.57	18.02	18.01	17.85	17.55	18.23	18.05	17.86	17.51
n6/n3	29.60^{cd}	29.15^{bc}	28.87^{b}	28.85^{b}	29.89^{d}	29.57^{cd}	29.03^{bc}	28.67^{b}	29.87^{e}	29.76^{de}	29.15^{bc}	28.90^{b}	29.30^{c}	28.45^{a}	28.37^{a}	28.23^{a}

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