ANTIMICROBIAL ACTIVITY OF BACTERIOCIN FROM INDIGENOUS *Lactobacillus plantarum* 2C12 AND ITS APPLICATION ON BEEF MEATBALL AS BIOPRESERVATIVE

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ABSTRAK

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Salah satu tujuan pengawetan pangan adalah untuk memperpanjang masa simpan. Bahan pengawet alami dapat digunakan sebagai senyawa antimikroba, salah satu contohnya adalah bakteriosin yang diproduksi oleh bakteri asam laktat. Tujuan penelitian ini adalah untuk mengevaluasi aktivitas antimikroba bakteriosin yang diproduksi oleh bakteri asam laktat indigenus *Lactobacillus plantarum* 2C12 yang diisolasi dari daging sapi lokal dan mempelajari kualitas bakso yang ditambah dengan pengawet bakteriosin 0,3%, nitrit 0,3% dan kontrol (tanpa pengawet) selama penyimpanan (0, 3 dan 6 hari) pada suhu dingin (4^{o}C). Hasil penelitian menunjukkan bahwa bakteriosin yang diproduksi oleh *L. plantarum* 2C12 mampu menghambat pertumbuhan bakteri patogen *Escherichia coli, Staphylococcus aureus* and *Salmonella* Typhimurium. Efektivitas bakteriosin sebagai pengawet alami pada produk bakso dicapai dengan menghambat pertumbuhan total mikroba dan *E.coli*, yang tidak berbeda dengan nitrit. Penambahan bakteriosin dari *L. plantarum* 2C12 juga tidak berpengaruh terhadap perubahan kualitas fisik dan kimia bakso, sesuai dengan Standar Nasional Indonesia (SNI 01-3818-1995) untuk bakso.

Kata kunci: bakso, bakteriosin, L. plantarum 2C12, pengawet alami

ABSTRACT

One purpose of food preservation is to extend the shelf life of foods. Biological preservations can be conducted by adding antimicrobial substances, such as bacteriocin produced by lactic acid bacteria and has been characterized as biopreservatives. The aims of this research were to evaluate antimicrobial activity of bacteriocin produced by indigenous lactic acid bacteria *Lactobacillus plantarum* 2C12 isolated from local beef and to study the quality of beef meatball with 0.3% bacteriocin as biopreservative at different storage times (0, 3, and 6 days) in cold temperature (4^{o}C), compared to 0.3% nitrite and control (without preservative). The results showed that bacteriocin from *L. plantarum* 2C12 could inhibit pathogenic bacteria such as *Escherichia coli, Staphylococcus aureus* and *Salmonella* Typhimurium. Bacteriocin was effective as well as nitrite as biopreservatives of meatballs by inhibiting the growth of total microbes and *E. coli*. The addition of bacteriocin did not lead the physical and nutritional changes in the meatballs. The quality of meatball with bacteriocin treatment conformed with Indonesia National Standard of meatball.

Keywords: bacteriocin, biopreservative,L. plantarum 2C12, meatballs

INTRODUCTION

Bacteriocins are ribosomally synthesized antimicrobial peptides or protein. They are ubiquitous in the microbial world and produced by *Lactobacillus* species, a Gram-positive lactic acid bacteria. Bacteriocins have cationic properties and kill target cells by causing disruption of the membrane-potential and/or leakage of cellular solutes that eventually leads to cell death (Diep *et al.*, 2009). The bacteriocins from lactic acid bacteria have attracted significant attention because of their potential use as non-toxic and safe additives for food preservation and

prevention of food spoilage by foodborne pathogenic bacteria (Hata *et al*., 2010; Savadogo *et al.,* 2006). Bacteriocins are considered to be safe biopreservative, since they are assumed to be degraded by protease in the gastrointestinal tract (Cleveland *et al.,* 2001).

Lactobacillus plantarum was reported could produce bacteriocin, called plantaricin. Plantaricin EF and plantaricin JK were included into Class II bacteriocin (Diep *et al.,* 2009). Whereas plantaricin W from *Lactobacillus plantarum* LMG 2379 belongs to a new family of two-peptide lantibiotics (Holo *et al.,* 2001). *Lactobacillus plantarum* 2C12 is Indonesian indigenous lactic acid bacteria isolated from fresh beef, and has been identified by 16S rRNA sequencing. *L. plantarum* 2C12 has antimicrobial substances against pathogenic bacteria such as *Escherichia coli, Staphylococcus aureus* and *Salmonella Typhimurium* (Arief, 2011).

Meatball is one of highly consumed meat products in Indonesia and considered as economical foods in many Indonesian societies. Meatballs are produced from a mixture of finely ground meat with salt, tapioca starch and garlic. This batter is formed into balls ranging in size from a marble to a ping-pong ball and then cooked in boiling water (Purnomo and Rahardian, 2008). Nitrite is usually added in the meatball as preservative. Consuming 150 ppm nitrite for a long period of time is harmfull for people's health, because it could cause carcinogenic effect. The development of natural preservative is needed to reduce chemical preservatives level in processed foods. In this case, natural preservative refers to biological preservation, such as bacteriocin. The application of bacteriocins in meatball processing is expected to replace nitrite as preservative. The aims of this research were to evaluate the antimicrobial activity of bacteriocin from *L. plantarum* 2C12 and to study the quality of beef meatball with 0.3% bacteriocin as biopreservative during storage (0, 3, and 6 days) at low temperature (4^{o}C), compared with unpreservatived and 0.3% nitrite.

MATERIALS AND METHODS

Production of Bacteriocin

Culture of *Lactobacillus plantarum* 2C12, isolated from Indonesian local beef (Arief, 2011) at the concentration of 10% (v/v) was inoculated in to 1000 ml MRS broth supplemented with 3% (w/v) yeast extract and 1% (w/v) NaCl, and was incubated at 37 ^{o}C for 20 h. The culture was centrifuged (10000 rpm for 20 min, at 4 ^{o}C), and a cell-free supernatant was obtained. The cell-free supernatant was adjusted to pH 6.0 by adding 1 N NaOH, and then precipitated by ammonium sulphate. The mixture was stirred slowly for 2 h at 4^{o}C. The precipitates were dialyzed by using dialysis membrane and the buffer used for washing was potassium phosphate (pH 6.8) (Hata *et al.*, 2010). Bacteriocins were then collected and used in meatball processing.

Bacteriocin Activity Assay

This experiment was conducted according to Hata *et al*. (2010). The agar well diffusion method was used to examine the antibacterial spectrum of bacteriocin based on inhibition zone created toward the tested microorganisms. The inhibition was measured from the diameters of the clear zones. The pathogen bacteria used in this experiment were *Salmonella* Typhimurium ATCC 14028, *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923.

Preparation of Meatballs

Meatballs were formulated using beef, tapioca flour, ice block, salt, mixed spices, and preservative (bacteriocin and nitrite). The beef 400 g, 5% salt, and 15% ice block were grinded finely in food processor. Twenty percent of tapioca flour and spices (2% garlic and 2% pepper) were added into the meatball mix and then was grinded for the second time (percentage of additive materials were as % to the beef weight only). For bacteriocin treatment, 0.3% (v/v) bacteriocin was mixed with salt in to the first grinding, while 0.3% nitrite was added in the second grinding, then formed into meatballs in 60^{o}C water. The meatball samples were packed aerobically in polyethylene bag, stored at 4 ^{o}C, and taken for physical and microbiological analysis in every three days (0, 3, and 6 days).

Nutritional Quality Analysis of Meatballs

Moisture, protein, fat and ash contents of meatballs were determined by proximate analysis (AOAC, 2005). Crude protein content was determined by Micro Kjeldahl assay, and crude fat content was determined by Soxhlet method. Proximate analysis have been done using composite samples before storage. Composite

samples were expressed as mixture of three replication samples to be analyzed.

Microbiological Analysis of Meatballs

Total of microbe and *E.coli*

A five grams of meatball sample was suspended in 45 ml sterile of 0.85% (w/v) NaCl. Microbiological analyses were performed by pour plate method using plate count agar (PCA, Merck) for total plate count (TPC), and eosyn methylen blue agar (EMBA, Merck) for *E.coli* test. For total plate count (TPC), 10^{-3} to 10^{-5} number of dilutions were used, and for *E. coli* test as much as 10^{-1} to 10^{-3} number of dilutions were used. Samples were incubated for 24 h, at 37^{o}C (AOAC, 2005).

Qualitative Analysis of *Salmonella sp.*

A five gram of meatball sample was aseptically transferred into sterile plastic containing 45 ml lactose broth, homogenized for 1-2 minutes, and then incubated at 37°C for 24h. One ml of the solution was transferred into 9 ml Rappaport-Vassiliadis (RV, Merck) media and tetrathionate broth (TTB, Difco) media, followed by incubation at 37°C for 24h. Then one colony from each RV and TTB was streaked (1 Ose) onto bismuth sulfite agar, xilose lysine desoxycholate agar (Difco), and hektoen enteric agar (Difco) plate, incubated at 37°C for 24h. The colonies suspected of being *Salmonella* sp. were selected and streaked onto triple sugar iron agar (Difco), lysine iron agar (Difco), and incubated at 37°C for 24h. The specific *Salmonella* sp. colonies observed were confirmed using standard biochemical result (BAM, 2001).

Physical Quality Analysis of Meatballs

Analysis of pH and Water Activity

The meatball sample (5 g) was homogenized with 45 ml of distilled water. The pH of the sample was determined with pH-meter (Hanna) by dipping the pH electrode into sample for a few seconds and then the pH value will be obtained (AOAC, 2006). Water activity was measured by a_w – meters Novasina SAL-T & Sensor-Check SC number 75.

Analysis of Water Absorption Capacity

One gram of meatball sample was mashed and transferred into the reaction tube, homogenized with 10 ml of distilled water. Sample was stored at 27^{o}C for 30 minutes and then sample in reaction tube was centrifuged at 3500 rpm for 30 minutes. Water absorption capacity was determined by measuring the volume of the supernatant obtained (Fardiaz, 1992).

Statistical Analysis

Completely randomized design was used in this experiments. After verification of the normal distribution of data, ANOVA was used. Tukey's test was used to test for differences between the paramaters (Steel and Torrie, 1995).

RESULTS AND DISCUSSION

Antimicrobial Activity of Bacteriocin from *Lactobacillus plantarum* 2C12

The antimicrobial activities of bacteriocin against three pathogenic bacteria as indicator strains were detected as inhibition zones diameters. Bacteriocin from *L. plantarum* 2C12 showed strong antimicrobial activity against *E. coli*, *S. aureus* and S.Typhimurium (Table 1). Pan *et al.* (2009) stated that high level of antimicrobial activities were detected if diameters of inhibition zones were over than 6 mm. The broad spectrum of antimicrobial activity of crude bacteriocin of *L. plantarum* 2C12 was shown by it's capability to inhibit the growth of Gram positive bacteria (*S. aureus*) and Gram negative bacteria (*E.coli* and *S.*Typhimurium). Analysis of the protein content of bacteriocin from *L.plantarum* 2C12 showed the concentration was 6.97 mg/ml. Some strains of *L. plantarum* have been reorted produce bacteriocin, called plantaricin, wich have antimicrobial activities against pathogenic bacteria, such as *L. plantarum* LR/14 (Tiwari and Srivasta, 2008) and *L. plantarum* A-1 isolated from tortilla (Hata *et al.*, 2010). All of these strains have broad spectrum antimicrobial activities.

Nutritional Quality of Meatball

Table 2 shows the nutritional quality of all the meatball samples before storage. Application of bacteriocin (0.3% v/w) into the meatball formula increased the water content (WC), ash, crude protein (CP), and crude fat (CF) content of meatball compared with control, but the carbohydrate (C) content decreased. Bacteriocin produced by *L. plantarum* consisted of peptides or peptide complexes (Jeevaratnam *et al.*, 2005), so the addition of bacteriocin in meatball was capable to increase the crude protein content of

Table 1. Antimicrobial Activity of Bacteriocin from *L.plantarum* 2C12

Pathogenic Bacteria	Diameter of Inhibition Zone (mm)
Esherichia coli ATCC 25922	11.83 ± 0.83
Staphylococcus aureus ATCC 25923	10.95 ± 0.09
Salmonella Thypimurium ATCC 14028	11.28 ± 0.24

Table 2. Nutritional Quality of Beef Meatball using Different Type of Preservative

Treatment	Water Content	Ash	Crude Protein	Crude Fat	Carbohydrates
	----------% wb----------				
Control	76.67	1.29	11.76	0.23	10.05
Nitrite 0.3%	72.49	1.68	14.13	0.13	11.57
Bacteriocin 0.3%	79.09	1.66	12.34	0.52	6.39

meatball. However in this experiment (Table 2) addition of nitrite resulted in higher protein content than those of bacteriocin. Although by definition all bacteriocins are made of proteins, some have been reported to consist of combinations of different proteins or are composites of proteins together with lipid or carbohydrate moieties (Jack *et al.*, 1995). The significant increment of water content was caused by liquid form of bacteriocin. The Indonesia National Standard (SNI) for meatball required the composition as follows : moisture content appoximately 70%, ash content maximum 3%, protein content minimum 9%, and fat content minimum 2% (DSN, 1995). Nutritional quality of meatball samples in this experiment showed good conformance with SNI.

Microbiological Quality of Meatball during Preservation

The microbial analyses of all treated meatballs are shown in Table 3. As shown in Table 3, the microbial total plate count (TPC) of meatballs increased during 6 days of storage. Total plate count is intented to indicate the level of microorganism in a product. At day 0, there were a number of bacteria presented in all of treated meatballs. The total microbes were 4.20 log cfu/g (control), 4.65 log cfu/g (0.3% nitrite), and 3.65 log cfu/g (0.3% bacteriocin). The microbial quality of the meatballs depends on the microbiological contaminants of ingredients such as ground beef, herbs-spices and personal hygiene (Elmali and Hilmi, 2005). Addition of 0.3% bacteriocin into meatballs significantly reduced the TPC about 1 log cfu/g lower than control and nitrite treated samples at day 0, 3 and 6. After 6 days of storage, the TPC of both of nitrite and bacteriocin treated meatballs were still in accordance with the SNI 01-3818-1995 for total plate count, that is required maximum at level of $1x10^5$ cfu/g or 5.0 log cfu/g (DSN, 1995). This results had proven that bacteriocin from *L. plantarum* 2C12 was effective to control the microbial growth of meatballs

Analysis of *E. coli* population are used as sanitation indicators in food preparation. The results of *E. coli* assay in meatballs are shown in Table 3. A small numbers were observed in control meatballs after six days of storage period. The same number (0.46 log cfu/g) of *E.coli* population were also observed in nitrite and bacteriocin treated meatballs at day 0, but none observed at day 3 and 6. The results showed that bacteriocin from *L. plantarum* could reduce *E.coli* as well as nitrite. Bacteriocin can disrupt the cell wall of bacteria, causing dead of bacteria (Hata *et al*., 2010).

The result of *Salmonella* sp. detection in all of meatball samples and at all time of storage were negative. *Salmonella* is a genus of bacteria known as a major cause of foodborne illness

Table 3. Microbiological Quality of Beef Meatball using Different Type of Preservative and Different Storage Time at 4 ^{o}C

	Treatments	Storage Time (Day)		
		0	3	6
TPC	Control	4.20±0.80	5.00±0.49	5.15±0.99
(log cfu/g)	Nitrite 0.3%	4.65±0.44	5.01±0.63	4.73±0.58
	Bacteriocin 0.3%	3.65±0.25	4.40±0.00	4.39±0.00
E.coli	Control	0.47±0.81	0.60±0.02	0.59±0.02
(log cfu/g)	Nitrite 0.3%*	0.46±0.81^{a}	0.00±0.00^{b}	0.00±0.00^{b}
	Bacteriocin 0.3%*	0.46±0.81^{a}	0.00±0.00^{b}	0.00±0.00^{b}
Salmonella	Control	Negative	Negative	Negative
	Nitrite 0.3%	Negative	Negative	Negative
	Bacteriocin 0.3%	Negative	Negative	Negative

*****Significantly different ($p<0.05$) at same row

Table 4. Physical Quality of Beef Meatball using Different Type of Preservative and Different Storage Time at 4 ^{o}C

	Treatments	Storage time (Day)		
		0	3	6
Water	Control	0.90± 0.03	0.90± 0.02	0.91± 0.01
Activity	Nitrite 0.3%	0.91± 0.01	0.89± 0.01	0.89± 0.01
	Bacteriocin 0.3%	0.91± 0.02	0.89± 0.01	0.89± 0.01
pH	Control	6.24±0.07	6.31± 0.07	6.31± 0.10
	Nitrite 0.3%	6.23± 0.12	6.28± 0.14	6.27± 0.13
	Bacteriocin 0.3%	6.18± 0.05	6.30± 0.07	6.23± 0.08
WAC	Control	0.90± 0.20	1.33± 0.12	0.98± 0.18
	Nitrite 0.3%	1.03± 0.32	1.32± 0.06	1.03± 0.06
	Bacteriocin 0.3%	0.97± 0.06	1.12± 0.06	1.27± 0.12

throughout the world. Based on SNI 01-3818-1995 for meatball, the maximum total population of *E.coli* allowed are maximum 3 APM/g and for *Salmonella* sp. is negative (DSN, 1995). The microbiological quality of meatball treated by 0.3% nitrite and 0.3% bacteriocin from *L. plantarum* 2C12 were in accordance with SNI (DSN, 1995).

According to microbiological analysis in this research, 0.3% bacteriocin treatment was effective to inhibit the growth of bacteria in meatball. Bacteriocin produced by *L. plantarum* 2C12 tended to be active against a wide range of Gram positive and Gram negative bacteria, in accordance with the activity of bacterocin examined by in vitro analysis (Table 1). Bacteriocin (0.3%) produced by *L. plantarum* 2C12 was effective to inhibit *E. coli* and

Salmonella sp. (Gram negative) as well as 0.3% nitrite in meatball. The result suggested that bacteriocin from *L. plantarum* 2C12 can be used as biopreservatives for meatballs.

Physical Quality of Meatball during Storage

Water activity relates to water presents in food in free form for biological function and necessary for microbial growth. Water activity of meatballs are shown in Table 4. In this research, water activity decreased at day 3 and increased at day 6. The use of preservative in meatball processing tended to decline the water activity in the meatballs. Bacteriocin treated meatball had the lowest water activity after sixth day of storage. In fact, the declining of water activity is appropriate condition to inhibit the growth of bacteria in food. It can be achieved by adding solids, ions, hydrophilic colloids, freezing and drying (Ray, 2000).

Table 4 showed the pH of beef meatball using different type of preservatives during storage time at 4^{o}C. During sixth day of storage time, pH value of all treated meatballs were stable and relatively similar, with the pH values were ranging from 6.18 to 6.31.

Water absorption capacity (WAC) of beef meatball using different type of preservative during storage time at 4^{o}C is shown in Table 4. Bacteriocin treated meatball had the highest water absorption capacity than other treatments at day 3 and 6. Water absorption capacity influences the texture quality of the product. The high water absorption capacity will create better product texture. Water absorption capacity of meatballs are influenced by pH value of product and myofibrilar protein in meat (Intarapichet, 2006).

CONCLUSSION

Bacteriocin produced by indigenous *L. plantarum* 2C12 had antimicrobial activity that could inhibit the growth of pathogenic bacteria such as *E. coli, S. aureus* and *Salmonella* Typhimurium. Bacteriocin from *L. plantarum* 2C12 was effective as well as nitrite as biopreservatives for meatball. Bacteriocin (0.3% v/w) was able to extend the self life of meatballs kept at 4^{o}C.

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REFERENCES

AOAC. 2005. Official Methods of Analysis of AOAC International. 18th ed. Assoc. Off. Anal. Chem., Arlington.

Arief, I.I. 2011. Karakterisasi Bakteri Asam Laktat Indigenus asal Daging Sapi sebagai Probiotik dan Identifikasinya dengan Analisis Urutan Basa Gen 16S rRNA. Disertasi. Institut Pertanian Bogor.

BAM. 2001. Bacteriological Analytical Manual, Chapter 3: The Aerobic Plate Count (APC). [Online]. http://www.fda.gov/food/scienceresearch/laboratorymethods/BAM/ucm063346.htm

Cleveland, J., T.J Montville, I.F. Ness, and M.L. Chikindas. 2001. Bacteriocin: Safe, Natural Antimicrobial for Food Preservation. Int. J. Food Microbiol 71: 1-20.

Dewan Standardisasi Nasional. 1995. Standar Nasional Indonesia 01-3818-1995. Bakso Daging. Jakarta.

Dıaz, J.R., J.L. Ruız-Barba, D.P. Cathcart, H. Holo, I.F. Nes, K.H. Sletten, and P.J. Warner. 1995. Purification and partial amino acid sequence of plantaricin S, a bacteriocin produced by *Lactobacillus plantarum* LPCO10, the activity of which depends on the complementary action of two peptides. Appl. Environ. Microbiol. 61: 4459-4463.

Emali M, and Y. Hilmi. 2005. Microbiological Quality of Raw Meatballs: Produced and Sold In The Eastern of Turkey. Pakistan J. Nutrition 4 : 197-201.

Fardiaz, D., N. Andarwulan., H. Wijaya and N.I. Puspitasari. 1992. Teknik Analisis Sifat Kimia dan fungsional komponen pangan. Pusat Antar Universitas Pangan dan Gizi, Institut Pertanian Bogor. Bogor

Hata, T., R. Tanaka, and S. Ohmono. 2010. Isolation and Characterization of Plantaricin ASM1 : A new bacteriocin produced by *Lactobacillus plantarum* A-1. J.Food Microbiol.. 137:94-99.

Holo, H., Z. Jeknic, M. Daeschel, S. Stevanovic, and I. F. Nes. 2001. Plantaricin W from *Lactobacillus plantarum* belongs to a new family of two–peptide lantibiotics.

Microbiol. 147: 643-651.

Intarapichet, K. 2006. The Use of Crude Bacteriocin from *Lactobacillus lactis* TISTR 1401 as Biopreservative to Extend Shelf Life of Aerobically Packed Pork Meatballs, *Master Thesis.* Suranaree University of Technology, Thailand.

Jack, R.W., J.R. Tagg, and B. Ray. 1995. Bacteriocin of Gram-Positive Bacteria, Microbiological Reviews, American Society for Microbiology, pp 171-200.

Jeevaratnam, K., M. Jamuna and A.S. Bawa. 2005. Biological Preservation for Foods– Bacteriocins of Lactic Acid Bacteria. Indian J.Biotechnol.4:446-454.

Pan, X., F. Chen, T. Wu, H. Tang, and Z. Zhao. 2009. The acid, bile tolerance and antimicrobial property of *Lactobacillus acidophilus* NIT. Food Control 20 : 598-602.

Purnomo H, and D. Rahardian. 2008. Indonesian Traditional Meatball, Review Article. Int. Food Research J.15:101-108.

Soeparno. 2005. Ilmu dan Teknologi Daging. Gadjah Mada University Press. Yogyakarta.

Ray, B. 2000. Fundamental Food Microbiology 3rd Edition. CRC Press, Boca Raton, London, New York, Washington D.C, 2000.

Savadogo, A., C. A. T. Outtara, I. H. N. Bassole, and A. S. Traore. 2006. Bacteriocins and lactic acid bacteria – a mini review. Afr J. Biotechnol 5 (9): 678-683.

Steel, R. G. D. and J. H. Torrie. 1995. Principles and Procedurs of Statistic: a Biomedical Approach. 3rd Edition, Mc. Graw Hill, Inc. Singapore.

Tiwari, S.K. and S. Srivastava. 2008. Purification and Characterization of plantaricin LR14 : a novel bacteriocin produced by *Lactobacillus plantarum* LR/14. Appl. Microbiol. Biotechnol. 79:759-767.