e-Journal
Peternakan Tropika
Journal of Tropical Animal Science
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Submitted Date: January 26, 2020 *Accepted Date: January 28, 2020*
Editor-Reviewer Article;: A.A.Pt. Putra Wibawa & I Wyn. Wirawan

THE LACTIC ACID BACTERIA OF *BEBONTOT* SPENT CHICKEN MEAT AND ANTIOXIDANT ACTIVITY OF THEIR ISOLATES

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Highlights* *Bebontot or buntilan of spent chicken a novel Balinese traditional meat fermented product * Species-specific PCR assays were used to confirm the identity of the LAB strains, discriminated 63 profiles out of 72 LAB isolates * Pediococcus pentosaceus was dominant, followed by Lactobaccillus plantarum were the main species of bebontot.* Lactobaccillus plantarum had strong radical scavenging activities of 1.1-Diphenyl-2-picrylhydrazyl, DPPH than Pediococcus pentosaceus*

Abstract* *The lactic acid bacteria (LAB) of bebontot or buntilan (Balinese traditional meat fermented product) of chicken breast meat of spent laying hen had been identified and these LAB were distributed as 58.33% Pediococcus acidilactici strain LMG 17680 and 41.67% Pediococcus acidilactici strain O-mls-1 at 0 day of the batters. The result observed at the end of fermentation (5 days) dried under the sun were isolate consist of 56.25% Pediococcus pentosaceus strain Ni 1386; 20.83% Lactobaccillus plantarum strain PA21 and 8.33% Lactobacillus plantarum strain Ni 1002. The average inhibition concentration (IC) of radical scavenging activity (RSA) of 1.1-diphenyl-2-picrylhydrazyl, DPPH after innoculation (0 day) was 66.83±3.55% and after 5days of fermentation was 83.37±4.06%. It is interesting to note that LAB isolates of bebontot spent-hen chicken breast meat of properness (nor isolated from human or infant feces) and could be benefit for antioxidant activity to process functional meat products for human consumption.*

Keywords: Identification, antioxidant activity, LAB isolates, *bebontot*

1. Introduction

Recent years most publications concerning incorporation of lactic acid bacteria (LAB) into foods have focused only on their survival during manufacture and storage, furthermore a few studies have considered the effect LAB as starter culture for adjuncts on the oxidative stability of raw fermented meat products [1]. A number of the LAB had studied or exerted an antioxidant action ([2]; [3]; [4]; [5]; [6]; [7]; [8]; [9]). [10] reported that since 1998 in Germany (a salami producer) and Japan (a meat-spread, *Breadton* producer) have developed meat products containing human intestinal LAB. Furthermore [11] reported that used of autochthonous LAB isolated from fermented sausages have been proposed to offer even better results as these bacteria are highly adapted to the food environment, hence they have a higher chance to outcompete against the growth of pathogenic bacteria. Traditionally meat fermentation is more an art depending on skill and experience of the meat manufacture rather than a process fully based on scientific and

technological means [12]. On the other hand [10] reported that the proteolyses system of LAB can contribute to release of health-enhancing bioactive peptides from food proteins, which generated to the fermentation and ripening process of meat by LAB, and they have bioactivities for functional foods. *Bebontot* or *buntilan* is a Balinese traditional meat fermented product, and this mostly prepared using air drying by traditional methods in small-scale enterprises. Generally it was made from mixed of lean meat and fat pork meat, chopped into 1.5-2.0 cm cubes, and mixed with fresh ground spices consist of turmeric, galangal, aromatic ginger, ginger, garlic, chilies, pepper and salt [13]; and in other region of Bali a difference mix spices used to produce *bebontot*, from pork with lard added, where it is also related to the environmental drying condition [14]. However there are no reports on identification of LAB isolate from *bebontot* spent chicken breast meat and hence the aims of this study were to find out the LAB strains involved in fermentation process from time of innoculation (0 day) and during at 5 days drying and to determine their antioxidant activity.

2. Materials and methods

2.1. Bebontot *chicken breast meat preparation.*

Skinless breast fillet (*Musculus Pectoralis Superficialis*, left and right breast muscles of each bird) of spent laying chicken (ISA-brown) after a period of laying of 76 weeks from an egg farm in Bali region were used in this study. The skinless breast fillet were packed in polyethylene pouches and stored at 4ºC before used for *bebontot* production. Meat were chopped into 1.5-2.0cm cubes and mixed with fresh ground spices consist of coriander (12.5-15%), white pepper (2%) (dry seed), fresh alpinia galangal (35-37.5%) and garlic (25%), then salt (8%), sugar (10%) and coconut oil (5%) were also added. The bebontot ingredients mixture were wrapped in dried *Areca catechu palm* sheaths and finally sun dried for 5 days.

2.2. *Isolation and identification of LAB.*

The wrappings *Areca catechu* sheaths were aseptically removed, and 10 g of the *bebontot* chicken meat were homogenized in 90 ml of sterile 0.85% sodium chloride (Merck, Darmstadt, Germany) diluents in a Stomacher (Lab-blender, Seward, London, UK) for 2 min. Serial decimal dilutions were prepared. The lactic acid bacteria isolation, count was determined after 48 h in an anaerobic-jar (*Gas-Pack, envelopes,* BBL, Baltimore, Maryland, USA) at 37^{o}C on MRS (De Man, Rogosa and Sharpe) agar (Pronadisa, cat.1043), after added with indicator of Brome Cresol Purple (BCP). Colonies were randomly selected from MRS plates containing less than 300 colonies and puried on MRS agar with added sterilizes 1% of calcium carbonate (Merck) and incubated in an anaerobic-jar at 37^{o}C for 24 h. After overnight growth, yellowish clear zone around the colonies. Typically all isolates of LAB were selected for morphological examination using Gram-staining under microscope, and examined for production of catalase test and oxidase/gas. Only Gram-positive, catalase-negative, oxidase/gas-negative isolates were considered and stored at -20^{o}C in MRS broth (Pronadisa, cat.1215) containing 30% glycerol (Panreac, Badalona, Spain) before being subjected to molecular identification. Species-specific PCR assays were used to confirm the identity of the strains belonging to the LAB group as previously reported [15], with slight modification. Briefly, DNA extraction was carried out from a single colony input in the PCR tubes were added 10 µl of sterile water distillate and then add 90 µl Insta Gene Matrix (Bio-Rad Laboratories, Hercules, CA) into the tube and incubated in a heat block/ thermal cycle with a temperature of 100^{o} C for 15 min, then after incubated vortex for 2 min vortex and centrifugation for 3 min at 12.000 rpm. Sample is ready for use as a template DNA for amplification and 25 micro-liters used for PCR amplifications. DNA was prepared using the 16S r-RNA was amplified by PCR using a 9F forward primer, GAGTTTGATYMTGGCTCAG; and a 1541R reverse primer, AAGGAGGTGWTCCARCC.

The reaction mixture (50 µl total volume) contained 30 µl ddH2O, 5 µl of MgCl2, 5 µl of 10X Buffer 4 µl of de-oxy-nucleoside-tri-phosphates, 1 µl of each primer, 0.25 µl of Takara Taq Polymerase (Takara Bio Inc, Japan), and 5 µl of cell lyses as the template. PCR conditions were as follows: denaturation at 98°C for 20 s, annealing at 52°C for 45 s, and elongation at 72°C for 2 min. A total of 30 cycles were performed, followed by a final elongation for 4 min at 72°C. PCR products were purified with a Gene-aid PCR Fragments Extraction Kit according to the manufacturer's instructions (Gene-aid, Taiwan). Amplicon was sequenced with an automatic sequence analyzer (Applied Bio-systems 3130 DNA Analyzer; Applied Bio-systems, CA, USA) using the Big-Dye_Terminator v3.1 Cycle Sequencing Kit (Applied Bio-systems). Related sequences were identified by performing sequence database searches using the Basic Local Alignment Search Tool (BLAST), the address: blast@ncbi.nlm.nih.gov. Sequence data for related species were retrieved from GenBank (retrieve@ncbi.nlm.nih.gov) ([16]; [17]).

2.3. *DPPH radical scavenging activity of LAB isolate*

The radical-scavenging activity (RSA) was estimated with the aqueous supernatant obtained from raw breast meat according to the method of Blois as described by [18]; [3], with slight modifications. 0.1 ml quantity of aqueous supernatant of isolate diluted to 1 ml of water and vortex, then filtered using Millipore 0.45 μm filter membrane, then take into test tube 0.1 ml and was added 1.0 ml fresh of methanolic DPPH solution (0.2 mM). The mixture was vortexed and left to stand at room temperature for 30 min. A tube containing 1.0 mL of methanol and 1.0 mL of methanolic DPPH solution (0.2 mM) served as the control. The absorbance of the solution was measured at 517 nm (GENESYS 10S UV-VIS Spectrophotometer, Thermo Scientific). Each strains of isolated at 0 day and 5 days were prepared in three replicates and absorbance measured in two replicate readings. The percentage of DPPH radical scavenging activity was obtained from the following equation:

Radical-scavenging activity (%) =

$$\left(1 - \frac{\text{absorbance value of testing solution}}{\text{absorbance value of control solution}}\right) x\ 100$$

3. Results

3.1. *Identification of LAB isolate from bebontot spent chicken meat.*

Results of the 16S rDNA sequence analysis in these research (Table 1) on microbial diversity in spontaneously fermented of LAB *bebontot* chicken meat products were distributed two strains of *Pediococcus acidilactici* at 0 day of the batters (before wrapped) as 58.33% *Pediococcus acidilactici strain LMG 17680* (530 base pair, 100% maximum identity, accession number AJ249891.1; GenBank ID number 7672203) and 41.67% of *Pediococcus acidilactici strain O-mls-1* (649 base pair, 99% maximum identity, accession number JN836485.1; GenBank ID number 374722731) were found in this study. *Pediococcus acidilactici* strain LMG 17680, it was similar with the reference strains, and it was

corroborated with other researchers as well as [19], and *Pediococcus acidilactici* strain –O mls-1 similarity with isolate from yak milk cheese [20].

One strain of the isolate was 56.25% of *Pediococcus pentosaceus* strain Ni 1386 (1538 base pair, 100% maximum identity, accession number AB598987.1; GenBank ID number

Table 1. Distribution of lactic acid bacteria of *bebontot* from spent chicken meat at day 0 to the end (at day 5) fermentation.

Accession number	Description spesies	Length of sequences (basepair)	GenBank ID number	Maximum Identifications (%)	Total of Isolate (%)	DPPH RSA (%)
before wrapping/ the batters						
AJ249891.1	Pediococcus acidilactici strain LMG 17680	530	7672203	100	7 (58.33)	68.76%
JN836485.1	Pediococcus acidilactici strain O-mls-1	649	374722731	99	5 (41.67)	66.34%
the end (at day 5) ripening/fermentation						
AB598987.1	Pediococcus pentosaceus strain Ni 1386	1538	385541184	100	27(56.25)	85.58%
JX244277.1	Lactobaccillus plantarum strain PA21	1561	396576520	100	10(20.83)	84.69%
AB598950.1	Lactobaccillus plantarum strain Ni 1002	1529	385541147	99	4 (8.33)	82.93%
at day 4 ripening/fermentation						
AB362734.1	Lactobaccillus plantarum strain NRIC 1725	1554	157907466	99	2 (4.17)	NE
AB261004.1	Lactobaccillus plantarum strain G51104J1	503	106365513	99	2(4.17)	NE
AB598983.1	Pediococcus pentosaceus strain Ni 1382	1538	385541180	100	1 (2.08)	NE
AB598980.1	Pediococcus pentosaceus strain Ni 1379	1537	385541177	100	1 (2.08)	NE
AB481102.1	Pediococcus pentosaceus strain KT3CE27	1539	245002877	99	1 (2.08)	NE

385541184), two strains lactobacilli as 8.33% of *Lactobacillus plantarum* strain Ni 1002 (1529 base pair, 99% maximum identity, accession number AB598950.1; GenBank ID number 385541147) and 20.83% of *Lactobaccillus plantarum* strain PA21 (1561 base pair, 100% maximum identity, accession number JX244277.1; GenBank ID number 396576520) were found during of fermentation (5 days dried under the sun) of *bebontot*. In a comparison of ribosomal proteins *Pediococcus pentosaceus* are related to *Lactobacillus brevis* and *Lactobacillus plantarum*, these were similarity with the reference strains, and it was corroborated with other researchers as well as [21] isolate from mixed pasture of timothy and orchardgrass silage, this bacteria is also similar with *Pediococcus pentosaceus* strain Ni1386 and *Lactobacillus plantarum* strain Ni1002. Then for *Lactobaccillus plantarum* strain PA21 was similarity with isolate from *Pandanus amaryllifolius* [22].

3.2. *Antioxidant activity of LAB isolate from bebontot spent chicken meat.*

The radical scavenging activity (RSA) of DPPH as inhibition concentration (IC) of *Pediococcus acidilactici* strain LMG 17680 (68.76%) and *Pediococcus. acidilactici* strain O-mls-1 (range between 52.00%-73.05%) or the average IC of RSA was 66.83±3.55% at 0 day of the batters.

The result observed at the end of ripening/fermentation (5 days) dried under the sun were isolate consist of *Pediococcus pentosaceus* strain Ni 1386 (85.58% IC of RSA); *Lactobaccillus plantarum* strain PA21 (the mean IC of RSA 79.89%) and *Lactobacillus plantarum* strain Ni 1002 (the mean IC of RSA 82.93%). The average IC of RCA after 5days of fermentation was 83.37±4.06%.

4. Discussion

4.1. *Identification of LAB isolate*

[23] also reported that *Pediococcus acidilactici* had been described as one of the predominant LAB isolated from Iberian dry fermented sausages (10 *chorizos* and 12 *salchichones*), traditional meat products produced in the central-west of Spain (Extremadura), and this species according to [24] was commonly used for commercial starter culture. While [25] noted as commercial probiotic cultures for human use, and [26] stated that it was used for fermented meat product in pilot processing of Iberian sausages.

It is interesting to note that in this study *Pediococcus acidilactici* and *Lactobacillus plantarum* were similar to the one reported by [27] by using SDS-PAGE-sequencing of 16S-rRNA gene; and for *Pediococcus acidilactici, Pediococcus pentosaceus* and *Lactobacillus plantarum* were similar with the one who founded by [28], where they use phenotypic identification approach. *Lactobacillus plantarum* found in *bebontot* chicken breast meat was similar to the one reported by [29], and it was also found dominate the LAB flora in a Greek sausage [30]. Furthermore [31] reported that *Lactobacillus plantarum, Pediococcus acidilactici* and *Pediococcus pentosaceus* BT520 have been used for the production of Som-fug, a Thai fermented fish at 30^{o}C.

The dominant species identified in this study are different than the one reported by [15] where in their study on identification of dominant species in other indigenous Balinese traditional meat fermented poduct known as *urutan* pork meat they found *Lactobacillus plantarum, Pediococcus acidilactici, Lactobacillus. farciminis, Lactobacillus fermentum* and *Lactobacillus hilgardii. Urutan* pork meat was prepared using pork meat with 30% fat added with spices/herb and packed in natural casing then sun dried for 5 days. While [14] prepared bebontot from lean meat and pork fat and wrapped in *Areca cathecu palm* sheaths (*upih*, Balinese word) and found LAB isolate were dominated by genus *Lactobacillus sp*., and *Streptococcus sp.* However *Leuconostoc* sp was found in *bebontot* from Tabanan region besides both of LAB, and it is possibly due to different mixed spices, type of meat and fat used and difference in temperature and relative humidity at one region to other regions in Bali island. Furthermore [32] reported that the several lactobacilli dominate the endogenous LAB had been isolated from infant feces, and identify by partial 16S rRNA sequencing, they confirming as potential starter cultures for fermented sausage. [33] noted that difference in identified microorganisms involved during traditional meat fermentation (depend on the type of meat products) were related to the diversity in formulation (the ingredients and raw materials used), and to the technology applied (fermentation and ripening time) such as different temperature, duration and relative humidity.

4.2. *Antioxidant activity of LAB isolate*

The results in this study, it was higher than reported by [3], these probably due to mix between meat (spent laying hen meat, it abundant heme) and of spices/herbs contained of micronutrients on the batters as substrates for grow of LAB *bebontot*, may active and harbors systems for protection against reactive oxygen species in fermentation process. [34] reported that presence of micronutrients, especially manganese of spices and herbs could increase of the antioxidant activity.

The results of the radical scavenging ability of DPPH (0.2mM) on the intact cells and intracellular extract of intestinal bacteria *Bifidobacterium longum* ATCC 15708 (41.6% - 52.1%) and *Lactobacillus acidophilus* ATCC 4356 (20.8% - 43.2%) contributes to the antioxidant effect [3]. It was probably due to caused by using substrates for grew both (*Bifidobacterium longum* and *Lactobacillus acidophilus*) isolated from infant and human intestinal. It could also probably due to the influence on metabolism of nutrients for growing LAB, and therefore it was not properness for used as cultured meat products to human foods.

Furthermore [8] reported that for the DPPH (0.2 mM) radical of *Lactobacillus. fermentum* isolated from gastrointestinal mucosa of healthy weaning piglets showed 64.26% scavenging activity at 10^6 *cfu*/ml and 87.89% scavenging activity at 10^9 *cfu*/ml. It was used as feed supplement on basal diet of crossbred pigs. These result showed that antioxidant capacity of LAB was analyses as a function of LAB cell concentration as described by [4]. This result was later contradicted with the results of LAB isolate of *bebontot* chicken meat in this study, because it appears antioxidant activity of LAB isolate not only oriented towards cell concentration but also towards related with material nutrition original source for grow bacteria autochthonous or where of LAB isolated from meat product or based to other materials. Although all of LAB can grow in optimal medium but first growing influence physiologies condition of LAB when isolated. It seems that the RSA of LAB isolated from *bebontot* samples were possibly related to the length of the sequence of each native bacteria species which was identified from enzymatic activity of raw material at high drying temperature. This tight regulation of the intracellular Mn concentration would explain why antioxidant activity in LAB isolates of *bebontot* could be increased by the addition of spices /herbs to the growth medium during drying or fermentation .

Although LAB have long been considered as catalase-negative microorganisms, two groups of LAB with of catalase activity (heme-dependent catalase and nonheme Mn-containing catalase) have been reported in the last decade in genera *Lactobacillus*, *Pediococcus*, and *Leuconostoc* [35]. It is probably due to that in this study also contained of two group of catalase activity of LAB isolate *bebontot* chicken meat, can eliminate oxygen in a reaction that produces H_2O_2, there by preventing the formation of extremely damaging ROS like O_2^- (superoxide) and $OH^{\cdot}$ (hydroxyl radical). Furthermore [36] reported that *MnKat* is the only manganese-dependent catalase isolated from LAB where higher Mn concentrations intracellular (varies greatly among LAB) have been implicated ini oxidative stress resistance, acting as an O_2^- scavenger that could replace superoxide dismutase.

The several author ([10]; [1]) reported that microorganisms can producing antioxidant factors have been considered to play an important role in ameliorating the aging process, cardiovascular disease and diabetes, because meat proteins are hydrolyzed during the fermentation (ripening and drying) of meat products by muscle and microbial protease exerts a combination action, where they can contribute to release health-enhancing bioactive peptides. Our results should open the way to improve these products by introducing antioxidative LAB strains.

5. Conclusion

The bacteria of *Pediococcus pentosaceus* strain Ni 1386 and *Lactobaccillus plantarum* strain PA21 as autochthonous LAB, which was isolated from *bebontot* spent chicken meat has been proposed to offer even better results with good to the end (5days) drying or fermentation as antioxidant activity sources. These strains therefore constitute promising candidates for functional meat cultures and properness to the improvement of quality and safety to the choice of a selective starter culture.

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