Quality and Probiotic Lactic Acid Bacteria Diversity of Rabbit Meat Bekasam-Fermented Meat

Eka Wulandari¹*, Husmy Yurmiati², Toto Subroto³, and Kusmajadi Suradi¹

¹Departement of Livestock Product Technology, Faculty of Animal Husbandry, Universitas Padjadjaran, Jatinangor - Sumedang 45363, Indonesia
²Departement of Livestock Production, Faculty of Animal Husbandry, Universitas Padjadjaran, Jatinangor - Sumedang 45363, Indonesia
³Departemen of Chemistry, Faculty of Mathematics and Science, Universitas Padjadjaran, Jatinangor - Sumedang 45363, Indonesia

Abstract
Rabbit meat bekasam is a traditional fermentation product from Indonesia. This study aimed to determine the chemical and microbiological characteristics of rabbit meat bekasam during the fermentation process in order to isolate, characterize (in vitro and in vivo), and identify lactic acid bacteria (LAB) as the probiotic candidate. The chemical contents of bekasam on 7-day fermentation were investigated in explorative and experimental methods in a completely randomized design. A proximate analysis reported a decrease in the moisture content, fat and carbohydrate content, and an increase in protein content. Also, lactic acid content was increased from 0.48% to 1.12%, and pH was decreased from 5.3 to 4.3. Other properties indicated different values, such as bacteria (2.75×10⁶ to 4.45×10⁷ CFU/g), total LAB (3.82×10⁶ to 4.67×10⁸ CFU/g), total yeast (9.89×10⁶ to 3.82×10⁸ CFU/g) and total mould (4.34×10¹ to 4.86×10³ CFU/g). The experiment produced nine LAB isolates, including two probiotics subjected to further 16S rRNA gene analysis, which indicated that Lactobacillus buchneri was the potential probiotic isolate. After being tested on BALB/c mice, L. buchneri could improve the immune system by inhibiting the growth of Coliform and Salmonella.

Keywords  bekasam, rabbit meat, fermented meat, probiotic, microbiology

Introduction
Traditional fermented food products are prepared with biotechnological methods using indigenous microorganisms present in food (Utama et al., 2019). These methods are practical and economical to preserve food (Eze et al., 2014). Indonesia is home to traditional fermented food products such as bekasam, the naturally fermented fish from Central Java, South Sumatra, and Central Kalimantan. Today, bekasam is also made of rabbit meat.

Indonesians people generally avoid consuming rabbit meat because they consider rabbit as a pet. Therefore, rabbit meat is not widely distributed in Indonesian markets...
Quality and Lactic Acid Bacteria Diversity of Rabbit Meat Bekasam

(Priyanti and Raharjo, 2012). Interestingly, the nutritional quality of rabbit meat is superior to other species due to high proteins, high biological value, minerals, and vitamins despite the low saturated fatty acids, cholesterol, and sodium (El-Medany and El-Reffaei, 2015; Nistor et al., 2013). Accordingly, rabbit meat-based products are recently developed to implement food diversification and to increase the consumption of rabbit meat in Indonesia.

Three key steps in preparing rabbit meat bekasam include salting process, carbohydrate addition (rice), and fermentation process. The salting process aims to select microbes and prevent the growth of pathogen bacteria as spoilage microorganisms (Doyle et al., 2001). Then, rice as the source of carbohydrate is put to stimulate the growth of lactic acid bacteria (LAB) (Putri et al., 2015). On fermentation, LAB breaks down carbohydrates into lactic acid, propionic acid, acetic acid, and ethyl alcohol (Ahmed et al., 2013). These compounds are useful preservatives and impart sour taste to bekasam (Anihouvi et al., 2012).

LAB is the major component in bekasam fermentation. Previous study on eight types of fish bekasam from eight areas in Indonesia reported 62 LAB isolates including 19 which exhibited antimicrobial activity against Escherichia coli, Salmonella typhimurium ATCC 14028, Bacillus cereus, Staphylococcus aureus, and Listeria monocytogenes (Desniar et al., 2013). Therefore, LAB is the potential inhibitor of pathogen microbes and it may convert into probiotic bacteria (Kerry et al., 2018). Probiotics are beneficial bacteria; they alter the intestinal microflora balance and inhibit the growth of pathogen microbes.

To perform beneficial effects, probiotics must survive in the gastrointestinal tract, persist in the host and prove safety for consumers. To survive in the gut, the organism must be tolerant of low pH and bile toxicity prevalent in the upper digestive tract (Shokryazdan et al., 2016). A study had successfully isolated LAB from 144 kinds of plara (a fermented fish made in Thailand), namely A. viridans, E. avium, E. faecalis, E. faecium, E. hirae, E. thailandicus, L. plantarum, L. lactis, L. paracasei, P. pentosaceus, P. acidilactici, T. halophilus, W. cibaria, W. confusa, W. paramesenteroides, and W. viridescens (Miyashita et al., 2012).

Studies on rabbit meat bekasam are currently non-existent in Indonesia. Therefore, this study investigated the chemical and microbiological characteristics of rabbit meat bekasam (during the fermentation process) in order to isolate, characterize (in vitro and in vivo), and identify LAB as the candidate probiotic bacteria. The isolated bacteria can be used as the starter for rabbit meat bekasam.

Materials and Methods

Preparation of rabbit meat bekasam

Nine healthy New Zealand White crossbreed aged 3 months old weighing 1,800±53 g were purchased from Rajawali Farm, Sumedang, Indonesia. The rabbits were slaughtered used halal method according to Fuseini et al. (2017). Carcas was obtained by removing the blood, skin, distal portions of legs, distal part of the tail, organs located in the thorax and neck (lungs, oesophagus, trachea, thymus, and heart), genital organs, urinary bladder, gastrointestinal tract, liver, and kidneys. After deboning, the whole meat was chopped and rationed to seven batches for seven-day observation. Rabbit meat bekasam was prepared with a method by Sari et al. (2018) with a slight modification. Rabbit meat was marinated with 10% salt for 6 h and stored in a sterile container along with Setra Ramos® local rice (1:1 ratio). The container was tightly closed and incubated at room temperature (27.0±2.0°C) for 7 d.

Proximate composition

The proximate composition of rabbit meat bekasam was measured according to the methods of AOAC International
The moisture content of weight loss was calculated after 12 h oven-drying at 105°C (Digital drying oven DOD-150, Raypa, Barcelona, Spain). The protein content was measured using an automatic Kjeldahl nitrogen analyzer (AutoKjeldahl Unit K-370, Büchi Labortechnik, Flawil, Switzerland). Fat content was determined with the Soxhlet method using a solvent extraction system (Soxtec™ 2050 automated analyzer, FOSS Analytical, Hillerød, Denmark). A dry ashing method to determine ash content was conducted by incinerating the meat samples in a furnace (Thermolyne FD1410M, ThermoFisher Scientific, Waltham, MA, USA) at 550°C. Total carbohydrate was calculated (by difference) using the formula: Total CHO = 100 – (moisture% + fat% + protein% + ash%).

Lactic acid content and pH

The pH value of rabbit meat *bekasam* was measured using a pH meter (3510 Advanced Bench pH Meters, Jenway, Staffordshire, UK). Five gram rabbit meat *bekasam* was blended with 20 mL distilled water in a homogenizer for 60 s (Ultra-Turrax T25, IKA, Darmstadt, Germany). Lactic acid content was determined using the standard titration procedure for total titratable acidity.

Microbiological analysis

Twenty five grams *bekasam* sample from each treatment was transferred to 50 mL of sterile saline solution and homogenized for 90 s, and serial dilutions were prepared by mixing 1 mL of the homogenized sample with 9 mL of sterile saline solution. Total bacteria and total LAB were enumerated by plating samples on Nutrient Agar (NA, M001, HiMedia) and *Lactobacillus* MRS Agar (MRSA, M6411, HiMedia), respectively after aerobic incubation at 37°C for 24 h. Total yeast and moulds were counted by plating serial dilution on Malt Extract Agar (MEA, M137, HiMedia) and Potato Dextrose Agar (PDA, M137, HiMedia), respectively, after aerobic incubation at 27°C for 48 h. Total *Coliform* was counted on MacConkey Agar (MCA, MH081, HiMedia) media after incubation at 37°C for 48 h. The formed colonies were counted and expressed as colony forming units of the suspension (CFU/g).

Isolation of LAB

LAB was isolated by suspending 25 g sample in 225 mL of *Lactobacillus* MRS Broth (MRSB, M369, HiMedia) followed by anaerobic incubation at 37°C for 24 h for enrichment, and the MRSB was serially diluted with sterile saline solution. Appropriate dilutions were spread on MRSA plates containing 0.3% (w/v) CaCO₃ (Calcium Carbonate, GRM1044, HiMedia) for 48-hour incubation at 37°C. LAB produces lactic acid and reacts with CaCO₃ to produce soluble lactate calcium, characterized by a clear zone around the growing bacterial colonies. The possible identification of LAB colonies was tested for catalase activity, motility, and Gram staining. All catalase-negative, non-motil and positive Gram staining colonies were streaked on the MRSA and incubated at 37°C for 48 h to obtain pure colonies. They were stored in slanted semi-solid MRSA at 4°C.

Evaluation of probiotic potential (*in vitro*)

The viability in low pH (pH 2.0 and pH 3.0) were examined using 0.1 N HCl to manipulate the MRSB medium. One mL of 24-h-old LAB culture was pipetted into 9 mL of MRSB pH 2.0 and pH 3.0, incubated at 37°C for 3 h. Before and after a 3-hour incubation, total cells were enumerated in MRSA pouring method, followed by a 48-hour incubation at 37°C (Ngom,
To measure bacteria survival against bile salt, 1 mL of 24-h-old LAB culture was pipetted into 9 mL of MRSB that contained 0.5% and 1% bile salt (Bile salt, RM008, HiMedia), incubated at 37°C for 48 h, and count the cell using MRSA pouring method.

Antimicrobial activity was determined by agar well diffusion assay according to Tagg et al. (1976). The pathogens in this study were Gram-positive bacteria (*Staphylococcus aureus* ATCC 6538; *Listeria monocytogenes* ATCC 7644) and Gram-negative bacteria (*Escherichia coli* ATCC 11229; *Salmonella thypimurium* ATCC 14088) obtained from the Central Laboratory of Padjadjaran University, Jatinangor, Indonesia. Standard chloramphenicol 30 mg/mL (Chloramphenicol, TC204, HiMedia) were used as a positive control. The pathogens cultured in Nutrient Broth (NB, M002, HiMedia) were spread on the NA plate 5 mm diameter wells were made in the plate, poured with 40 μL of LAB isolate, and incubated at 37°C for 24 h. The diameter of the clear zone formed around the well after incubation was measured to confirm the antimicrobial activity.

Identification of probiotic LAB strains by genotypic characterization using 16S rRNA

LAB isolates were cultured in MRSB (pH 7.0) for 1d and bacterial cells were collected by centrifugation at 3,185×g for 10 min (Sigma 1-16K, Sigma-Aldrich, Osterode am Harz, Germany). The genomic DNA was extracted in a method by Zhu et al. (1993) with modification by Mustopa and Fatimah (2014). The pellet was resuspended with TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA), 40 μL of lysozyme (60 mg/mL; Lysozyme, PC0710, Vivantis), incubated at 37°C for 60 min and added with 200 μL 10% sodium dodecyl sulfate, 100 μL 5 M NaCl, and 80 μL 10% centrimide. Furthermore, the solution was warmed at 68°C for 30 min, added with equal amount of chloroform, and proceed with centrifugation at 14,953×g speed for 10 min. The supernatant was harvested and 1 mL of ethanol was added. The mixture was shaken again and then centrifuged at 14,953×g for 10 min. after being air-dried, the DNA was dissolved in TE buffer and the concentration was adjusted to 10 μg/mL DNA and stored at –20°C for further analysis.

Polymerase chain reaction (PCR) amplification for 16S rRNA

For 16S rRNA sequencing, primers 8F (5'-AGA GTT TGA TCA TGG CTC AG-3'; positions 8 to 27 bp) and 15R (5'-AAGGAG GTG ATC CAA CCG CA-3'; positions 1,541 to 1,522 bp) (Invitrogen, ThermoFisher Scientifc) were used to amplify the full length of bacterial 16S rRNA fragment (Chao et al., 2008). Each 25 μL polymerase chain reaction (PCR) mixture (Invitrogen, 10572014, ThermoFisher Scientifc) contained 10 mM Tris-L-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 200 μM of each dNTP, 400 nM of each primer, 1 U of Taq polymerase, and 10 ng of the DNA template. The PCR was operated at 96°C for 5 min, and performed 35 cycles consisting of 96°C for 1 min, 58°C for 3 min, 72°C for 1 min; and 72°C for 7 min. The PCR products were subjected to gel electrophoresis in 1% agarose gel, followed by ethidium bromide staining.

DNA sequencing and phylogenetic analysis

The DNA sequencing was performed in Macrogen, Korea. An online BLAST analysis performed similarity searches with sequences were performed by an online BLAST analysis in the National Centre for Biotechnology Information (NCBI). For the phylogenetic analysis, sequences were aligned by using the CLUSTAL X software (Thompson et al., 1997), while the phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei, 1987).
**Evaluation of *Lactobacillus buchneri* (in vivo)**

*Lactobacillus buchneri* was cultured in MRSB, incubated at 37°C for 24 h, and centrifuged 8,848×g for 10 min at 4°C. Supernatants were discarded, and cell pellets were washed three times with deionized water. For cell suspension preparation, sterile saline was used as a diluent.

The study was performed on 20 male BALB/c mice aged 6–8 weeks old, weighing about 22–32 g, purchased from PT Biofarma (Bandung, Indonesia). Experimental animal handling was following the Polish law on the protection of animals. The Ethics Committee of Universitas Padjadjaran approved all experiments with Ethical Committee No. 690/UN6.KEP/EC/2019. The mice were housed (three per cage) and maintained under conventional conditions (room temperature 22±2°C, 12 h day/night cycle). The standard diet contained 13% maximum water content, 10%–12% protein, 5% fat, 8% fibre, 14% ash, 3% calcium, and 0.7% phosphor.

The mice were distributed into five dietary groups (four replicates each). After seven days of acclimatization and fasting for 16 h, each group was administered (by oral gavage) a single dose of one of five treatments. The treatments included P1: 0.2 mL sterile saline (negative control); P2: 0.2 mL 1×10⁸ CFU of *Bifidobacterium bifidum* in 1 mL sterile saline (positive control); P3: 0.2 mL 1×10⁷ CFU/mL of *L. buchneri* in 1 mL sterile saline; P4: 0.2 mL 1×10⁸ CFU/mL of *L. buchneri* in 1 mL sterile saline; and P5: 0.2 mL 1×10⁹ CFU/mL of *L. buchneri* in 1 mL sterile saline. The mice were fed for seven consecutive days. On the eighth day, each animal was inoculated with a single 0.2 mL dose of 1×10⁷ CFU/mL of *Salmonella typhimurium* in sterile saline. After three days, the mice were sacrificed by cervical dislocation and were anesthetized by 100 mg/kg (w/w) intraperitoneal injection of ketamine (Ketamil, Illium, New South Wales, Australia) and 20 mg/kg (w/w) xylazine (Xyla, Interchemie, AC Castenray, Netherlands).

**Microbiological analysis of fecal contents**

Fresh mice feces were collected and pooled daily from each group from day 0 to day 4 to enumerate the LAB, *Coliform*, and *Salmonella* using the spread plate method. 1 g fecal was suspended briefly in 9 mL phosphate-buffered saline (PBS) and vortexed for 1 min. The samples were serially diluted in sterile diluents, and 100 μL 10⁻⁴ to 10⁻⁶ dilutions were streaked on different media for bacterial count, MRSA for total LAB, MacConkey agar for coliform, and Xylose Lysine Deoxycholate agar (XLDA, M031, HiMedia) for *Salmonella*. After 24 h incubation at 37°C, the colonies on the plates were counted and the microbial population was expressed as CFU/g.

**Statistical analysis**

The proximate analysis was conducted in a randomized design with three replicates for each experiment to obtain the mean values with p<0.05 significance level. The data were analyzed using SPSS version 20.

**Results and Discussion**

**Physicochemical properties of rabbit meat *bekasam***

The proximate composition of rabbit meat *bekasam* during fermentation is presented in Table 1. The moisture content of *bekasam* was significantly decreased (p<0.05) from 66.50% to 55.05%. Salt which was added on *bekasam* have the ability to decrease the moisture content. According to Fennema (1996), salt can decrease moisture content is due to the ability of sodium and chloride ions to associate with water molecules. Similarly, Majumdar and Basu (2010) reported that the water...
content of fermented fish product (*Lona ilish*) from India was decreased during fermentation. The fat content of *bekasam* was significantly decreased (p<0.05) during fermentation process. The bacteria and yeast performed lipolytic activity which hydrolyzed fat molecules into free fatty acids, hence, reducing the fat content. It was in line with Desniar et al. (2009) on the fermentation process of chub mackerel (*Rastrelliger* sp.).

The carbohydrate content of *bekasam* was reduced significantly (p<0.05). *bekasam* as a rice-based fermentation product was degraded by yeast and mould that produced an extracellular amylolytic enzymes (*α*-amylase and *glucoamylase*). Kumar et al. (2013) stated that starch was hydrolyzed into maltose and glucose by amylolytic enzyme which then converted into lactid acid and other organic acid, decreasing the carbohydrate content during fermentation. It was confirmed by the decreasing pH and the increasing lactid acid content in *bekasam* during fermentation in this research. On the other hand, protein content was increased significantly (p<0.05). Protease was hydrolyzing protein into polypeptide and amino acid which was used by microbes to increase microbial cell. Microbial cells are mostly build on protein; hence, microbes are known as Single Cell Protein (SCP) or natural protein concentrates (Kurbanoğlu, 2001). It was reflected from the increasing LAB and yeast in this study (Table 2).

There was no significant difference in the ash content (p>0.05). It indicated that during the fermentation process, the microbe used and produced mineral in a small amount. The minerals of the rabbit meat included potassium, phosphor, sodium, magnesium, calcium, zinc, iron, copper, and manganese (Hermida et al., 2006).

### Table 1. The proximate composition of rabbit meat *bekasam* during fermentation

| Fermentation days | Moisture (%) | Protein (%) | Fat (%) | Ash (%) | Carbohydrate (%) |
|------------------|--------------|------------|---------|---------|------------------|
| 1                | 66.50±0.02a  | 9.59±0.01c | 8.58±0.02a | 3.18±0.03a | 12.15±0.10a      |
| 2                | 63.37±0.22b  | 13.39±0.04d | 8.09±0.03a | 3.15±0.06a | 12.00±0.12a      |
| 3                | 61.53±0.04b  | 16.16±0.01d | 7.67±0.02b | 3.08±0.04a | 11.56±0.03a      |
| 4                | 58.42±0.03c  | 19.13±0.08d | 7.33±0.07b | 2.87±0.18a | 12.25±0.10a      |
| 5                | 57.79±0.10c  | 21.41±0.01d | 6.86±0.07c | 2.97±0.15a | 10.97±0.02b      |
| 6                | 56.35±0.03c  | 24.61±0.02b | 5.93±0.07c | 2.57±0.04a | 10.54±0.05b      |
| 7                | 55.05±0.10d  | 26.86±0.01a | 5.22±0.10a | 2.55±0.08a | 10.32±0.10b      |

*a–e* Different superscripts in the same row represent significant differences (p<0.05).

### Table 2. Microbiological properties of rabbit meat *bekasam* during fermentation

| Fermentation days | Total viable counts (CFU/g) | Total LAB counts (CFU/g) | Total yeast counts (CFU/g) | Total mould counts (CFU/g) | Total coliform counts (CFU/g) |
|-------------------|-----------------------------|--------------------------|----------------------------|---------------------------|-----------------------------|
| 1                 | 2.75×10⁶                    | 3.82×10⁶                 | 9.89×10⁶                   | 4.86×10⁴                  | 2.3×10                      |
| 2                 | 6.20×10⁷                    | 4.64×10⁶                 | 3.54×10⁶                   | 4.48×10³                  | 1.5×10                      |
| 3                 | 6.54×10⁷                    | 4.68×10⁷                 | 4.21×10⁷                   | 7.20×10²                  | -                           |
| 4                 | 4.77×10⁸                    | 5.72×10⁷                 | 5.51×10⁷                   | 5.47×10²                  | -                           |
| 5                 | 6.80×10⁸                    | 5.85×10⁷                 | 3.82×10⁸                   | 8.48×10²                  | -                           |
| 6                 | 6.86×10⁷                    | 2.75×10⁸                 | 4.62×10⁸                   | 6.20×10¹                  | -                           |
| 7                 | 4.45×10⁷                    | 4.67×10⁸                 | 3.42×10⁸                   | 4.34×10¹                  | -                           |

LAB, lactic acid bacteria.
Alteration in lactic acid content and pH level during the fermentation process

Titratable acidity and pH are illustrated in Fig. 1. The pH was reduced from 5.8 to 4.3, whereas the titratable acidity was increased from 0.428% to 1.12%. It was due to the accumulation of lactic acid and organic acid generated during the fermentation process which promoted the decrease in pH value. The growth of LAB converted carbohydrates to lactic, acetic, formic, caproic, propionic, butyric, and valeric acids (Zalán et al., 2010). Our findings are in agreement to previous studies on lower pH values of fermented fish during fermentation (Desniar et al., 2012; Paludan-Muller et al., 2002).

Microbiological characteristics of rabbit meat bekasam

Microbiological characteristics of bekasam during fermentation are shown in Table 2. The total bacteria was improved across 6 days of fermentation and decreased on the 7th day because low pH (4.4) was not suitable for the growth of several bacteria. Total LAB and yeast was increased from $3.82 \times 10^6$ to $4.67 \times 10^8$ CFU/g, and $9.89 \times 10^6$ to $3.42 \times 10^8$ CFU/g, respectively. In this study, total yeast was linear to total LAB during fermentation. LAB generates organic acids that may reduce pH and potentially stimulates the growth of yeast. Meanwhile, yeast produces vitamin and amino acid to support LAB growth (Fleet, 1990). Paludan-Muller et al. (2002) reported that isolated LAB and yeast were the dominant fermenting microorganisms for plaa-som, the fermented fish produced in Thailand.

The total mould declined from $4.86 \times 10^3$ to $4.34 \times 10^1$ CFU/g which may due to high salinity in fermentation. Coliform was present until the second day of fermentation because it could not survive in high salinity. Salt render microbial cells to undergo osmotic shock, resulting in the loss of water from the cell, and subsequently, cell death or retarded growth (Anihouvi et al., 2012). Total Coliform in this study was similar to <10 CFU/g in fermented fish (Hout-Kasef) produced in Saudi Arabia (Gassem, 2019).

Isolation and characterisation of LAB from rabbit meat bekasam

LAB isolation using MRSA with calcium carbonate produced 9 isolates. The colony morphology of isolated bacteria illustrated in Table 3 includes Gram-positive, catalase-negative, and non-motile. It indicated that indicating all bekasam isolates were corresponded to the LAB criteria proposed by Kerry et al. (2018).

Fig. 1. The changes of pH and lactic acid content during fermentation time; where ▲: pH and ■: lactic acid content.
Viability in low pH and bile salt

Resistance to low pH and bile salts are the key factors to predict the survival and growth of potential probiotic strains in gastrointestinal conditions. According to Sahadeva et al. (2011), low pH incubation was viable at pH 2.0 and pH 3.0 for 3 h as it stimulates bacterial residency. The viability of LAB in low pH and bile salt are as presented in Table 4 and Table 5 respectively. In general, total LAB counts of the nine isolates declined after exposure to low pH, and they were tolerant in pH 3.0 compared to pH 2.0. The acidity tolerance of LAB is attributed to a constant gradient between extracellular and cytoplasmic pH. When internal pH reached the threshold, cellular functions were inhibited and the cells died (Kashket, 1987).

Total LAB declined after exposure to 0.5% bile salts, and four LAB isolates (3.1, 5.1, 5.2, and 6.3) could not grow after exposure to 1.5% bile salts. Exposure to bile salts triggered the disruptions of cellular homeostasis which dissociated lipid bilayer and integral protein from their cell membranes, causing leakage in bacterial content and finally, cell death. (Tokatli et al., 2015). Isolate 6.1 and 7.1 in this study were highly tolerant to low pH and bile salt, producing $10^6$ CFU/mL total LAB which was the minimum total probiotic microbes (Sahadeva et al., 2011).

### Table 4. Effect of pH on the survival of LAB cells

| Isolate code | LAB counts (CFU/mL) pH 3.0 | LAB counts (CFU/mL) pH 2.0 |
|--------------|----------------------------|----------------------------|
|              | 0 hour                     | 3 hours                    | 0 hour                     | 3 hours                    |
| 3.1          | $3.58 \times 10^8$         | $3.23 \times 10^3$         | $2.42 \times 10^8$         | $3.46 \times 10^4$         |
| 3.2          | $2.58 \times 10^8$         | $3.52 \times 10^6$         | $1.65 \times 10^8$         | $2.55 \times 10^5$         |
| 4.1          | $3.76 \times 10^7$         | $4.26 \times 10^3$         | $2.78 \times 10^7$         | $5.62 \times 10^4$         |
| 5.1          | $4.71 \times 10^8$         | $2.52 \times 10^6$         | $3.52 \times 10^8$         | $4.68 \times 10^5$         |
| 5.2          | $5.52 \times 10^7$         | $6.31 \times 10^4$         | $4.76 \times 10^7$         | $3.59 \times 10^3$         |
| 6.1          | $3.58 \times 10^8$         | $4.72 \times 10^7$         | $4.56 \times 10^8$         | $3.75 \times 10^6$         |
| 6.2          | $7.33 \times 10^8$         | $4.87 \times 10^6$         | $6.42 \times 10^8$         | $5.21 \times 10^4$         |
| 6.3          | $2.44 \times 10^8$         | $3.97 \times 10^5$         | $1.78 \times 10^8$         | $6.43 \times 10^3$         |
| 7.1          | $5.77 \times 10^8$         | $4.62 \times 10^7$         | $4.64 \times 10^8$         | $4.32 \times 10^6$         |

LAB, lactic acid bacteria.
Antimicrobial activity of LAB isolates

The antimicrobial activity of all LAB isolates is presented in Table 6. All isolates exhibited a broad spectrum antimicrobial active against Gram-positive and Gram-negative pathogenic bacteria. It was in line with Sari et al. (2018) who examined the antimicrobial activities of fermented fish and discovered that LAB isolates might inhibit Gram-positive (S. aureus ATCC 25923 with 12.7 mm inhibition zone) and Gram-negative (Salmonella sp. with 7.3 mm) pathogenic bacteria. The activity of isolate 6.1 was highest against S. aureus with a 16.4 mm inhibition zone, and isolate 3.1 was the least for E. coli (6.2 mm).

LAB isolates can inhibit the growth of pathogenic microbes because LAB produces antimicrobial compounds during the fermentation process, such as organic acid (lactic acid and acetic acid), diacetyl, ethanol, hydrogen peroxide, reuterin, acetaldehyde, acetoin, carbon dioxide, and bacteriocins (García-Cano et al., 2014). This study indicated that isolate 6.1 exhibited the highest antibacterial activity against all pathogens tested except L.monocytogenes.

### Table 5. Effect of bile salt on the survival of LAB cells

| Isolate code | LAB counts (CFU/mL) bile salt 0.5% | LAB counts (CFU/mL) bile salt 1.5% |
|--------------|---------------------------------|---------------------------------|
|              | 0 hour                          | 24 hours                        |
|              | 0 hour                          | 24 hours                        |
| 3.1          | 3.58×10^8                      | 4.33×10^3                      | 3.26×10^8                      |
| 3.2          | 2.58×10^8                      | 6.25×10^7                      | 2.75×10^8                      | 3.68×10^5                      |
| 4.1          | 3.76×10^7                      | 7.43×10^5                      | 4.89×10^7                      | 4.24×10^4                      |
| 5.1          | 4.71×10^8                      | 2.66×10^5                      | 5.43×10^8                      | -                             |
| 5.2          | 5.52×10^7                      | 4.27×10^2                      | 4.70×10^7                      | -                             |
| 6.1          | 3.58×10^8                      | 7.65×10^7                      | 4.56×10^8                      | 2.79×10^6                      |
| 6.2          | 7.33×10^8                      | 5.32×10^5                      | 4.53×10^8                      | 4.54×10^4                      |
| 6.3          | 2.44×10^8                      | 4.21×10^4                      | 3.25×10^8                      | -                             |
| 7.1          | 5.77×10^8                      | 5.77×10^7                      | 6.72×10^8                      | 6.56×10^6                      |

LAB, lactic acid bacteria.

### Table 6. Inhibition test of isolates towards several pathogenic bacteria

| No | Isolate code | S. aureus (mm) | L. monocytogenes (mm) | S. thypimurium (mm) | E. coli (mm) |
|----|--------------|----------------|----------------------|---------------------|-------------|
| 1  | Chloramphenicol | 27.2          | 28.0                  | 26.6                | 27.0        |
| 2  | 3.1          | 7.3            | 7.8                   | 6.4                 | 6.2         |
| 3  | 3.2          | 8.5            | 8.1                   | 7.2                 | 7.8         |
| 4  | 4.1          | 8.2            | 7.3                   | 6.3                 | 6.4         |
| 5  | 5.1          | 8.1            | 7.4                   | 7.2                 | 7.0         |
| 6  | 5.2          | 9.2            | 10.5                  | 8.3                 | 7.2         |
| 7  | 6.1          | 16.4           | 12.5                  | 15.2                | 13.1        |
| 8  | 6.2          | 11.5           | 7.2                   | 11.3                | 12.2        |
| 9  | 6.3          | 13.2           | 13.1                  | 10.2                | 7.5         |
| 10 | 7.1          | 15.2           | 15.3                  | 11.9                | 10.2        |

This data has been published in Scientific Papers-Animal Science Series: Lucrări Ştiinţifice - Seria Zootehnie.
Identification of probiotic LAB strains by genotypic characterization using 16S rRNA

Probiotic candidate test on the viability of pH, bile salt, and antimicrobial activity concluded that isolates 6.1 and 7.1 were the best probiotic candidate isolates. Both isolates were subject to molecular identification using 16S rRNA gene analysis. The result of the 16S rRNA gene amplification could be identified from any fragment of PCR product with the size of 1,500 base pairs (bp) as the desired measurement (Fig. 2).

The homology analysis based on BLAST and SIM revealed that isolate 6.1 had a genetic relationship with *Lactobacillus buchneri*, and isolate 7.1 had the closest genetic relationship with *Weisella paramesentoroides* with 99% homology level. According to Clarridge (2004), the similarity level of a species is 94%. Phylogenetic tree of isolates 6.1 and 7.1 based on gene sequence of 16S rRNA is shown in Fig. 3.

One of the identified isolates (*L. buchneri*) was selected as a single starter for *bekasam* future production because it...
acquired the Generally Recognized As Safe (GRAS) status (FAO/WHO, 2002). According to Fessard and Remize (2017), *Weisella* spp. was not a GRAS starter.

**In vivo probiotic activities**

*L. buchneri* probiotic isolates were studied *in vivo* using BALB/c strain mice to identify the safety use in fermented food. Mice body weight was recorded (Table 7) to indicate the adverse effect of substrate in animal study. Table 7 showed that mice body weight increased during *L. buchneri* treatment, and maintained after infection with *S. typhimurium*. It indicated that *L. buchneri* positively affected mice’s immune system. It was in line with El-Jakee et al. (2010) and Shokryazdan et al. (2016) that mice receiving probiotics did not undergo body weight loss.

The results of microbiological test in mice feces are shown in Fig. 4. Results revealed that total fecal LAB population in the negative control (P1) was lower throughout the observation days. Total LAB declined after the third day of *S. typhimurium* infection in mice which was administered with probiotic treatment (P2, P3, and P4). *L. buchneri* cells treatment

### Table 7. Body weight of mice

| Treatment | Day 1 (g) | Day 7 (g) | Day 12 (g) |
|-----------|-----------|-----------|------------|
| P1        | 22.6±0.9  | 24.5±0.5  | 25.1±1.3   |
| P2        | 22.5±0.7  | 24.8±0.8  | 26.2±1.0   |
| P3        | 20.5±1.4  | 22.7±1.2  | 24.5±0.7   |
| P4        | 21.5±1.0  | 23.1±1.3  | 24.6±0.9   |
| P5        | 22.8±1.2  | 24.5±1.2  | 25.6±1.2   |

Values are mean±SD of 4 replications. P1, 0.2 mL of sterile saline (negative control); P2, 0.2 mL dose of $1\times10^8$ CFU of *Bifidobacterium bifidum* in 1 mL sterile saline (positive control); P3, 0.2 mL dose of $1\times10^7$ CFU/mL of *Lactobacillus buchneri* in 1 mL sterile saline; P4, 0.2 mL dose of $1\times10^8$ CFU/mL of *L. buchneri* in 1 mL sterile saline; P5, 0.2 mL dose of $1\times10^9$ CFU/mL of *L. buchneri* in 1 mL sterile saline.

**Fig. 4.** Microbiological analysis of mice feces. P1, 0.2 mL of sterile saline (negative control); P2, 0.2 mL dose of $1\times10^8$ CFU of *Bifidobacterium bifidum* in 1 mL sterile saline (positive control); P3, 0.2 mL dose of $1\times10^7$ CFU/mL of *Lactobacillus buchneri* in 1 mL sterile saline; P4, 0.2 mL dose of $1\times10^8$ CFU/mL of *L. buchneri* in 1 mL sterile saline; P5, 0.2 mL dose of $1\times10^9$ CFU/mL of *L. buchneri* in 1 mL sterile saline.
increased total LAB in mice feces on the seventh day of *L. buchneri* administration, and decreased after the *L. buchneri* administration stopped and *S. typhimurium* infection started. Accordingly, *L. buchneri* could inhibit *S. typhimurium* infection by competing for essential nutrition; therefore, total LAB decreased after the *L. buchneri* administration stopped.

Total *Coliform* and *Salmonella* of mice feces in probiotic treatment were decreased. *Salmonella* was non-existent in mice feces on the day before infection. It indicated that the mice were healthy and not infected by *Salmonella*. The effect of probiotic administration on total coliform may increase due to competition between bacteria to adhere to the epithelial cells of the intestine Sherman et al. (2005). Previous studies reported the declining total intestinal *Salmonella* due to probiotics effect (El-Jakee et al., 2010; Vasilica and Balotescu, 2006).

**Conclusion**

Rabbit meat *bekasam* is considered a nutritional and health-promoting food. This study isolated *Lactobacillus buchneri* from rabbit meat *bekasam* and further analysis showed that it was resistant to acid pH and bile salts *in vitro*, and exhibited *in vitro* antimicrobial activity against Gram-positive and Gram-negative pathogen bacteria. The *in vivo* analysis demonstrated that *L. buchneri* was safe up to $10^9$ CFU/g in a 7-day treatment period. *L. buchneri* could reduce the populations of harmful intestinal bacteria and pathogenic bacteria while increasing beneficial bacterial populations. No adverse effects were observed on the growth of the experimental animals.

**Conflicts of Interest**

The authors declare no potential conflicts of interest.

**Acknowledgements**

This work was funded by Academic Leadership Grant (ALG) Universitas Padjadjaran 2018, so the authors thank to Rector Universitas Padjadjaran and Directorate Research and Community Services Unpad for supporting funding of research.

**Author Contributions**

Conceptualization: Wulandari E. Data curation: Suradi K. Formal analysis: Wulandari E, Yurmiati H. Methodology: Subroto T. Software: Subroto T. Validation: Suradi K. Investigation: Wulandari E. Writing - original draft: Wulandari E. Writing - review & editing: Wulandari E, Subroto T, Yurmiati H, Suradi K.

**Ethics Approval**

All experiments were approved by the Ethics Committee of Universitas Padjadjaran with Ethical Committee No. 690/UN6. KEP/EC/2019.

**References**

Ahmed S, Dora KC, Sarkar S, Chowdhury S, Ganguly S. 2013. Quality analysis of shidal: A traditional fermented fish
product of Assam, North-East India. Indian J Fish 60:117-123.
Anihouvi VB, Kindossu JM, Hounhouigan JD. 2012. Processing and quality characteristic of some major fermented fish products from Africa: A critical review. Int Res J Biol Sci 1:72-84.
AOAC. 2012. Official methods of analysis. 19th ed. Association of Official Analytical Chemist, Washington, DC, USA. p 931.
Chao SH, Tomii Y, Watanabe K, Tsai YC. 2008. Diversity of lactic acid bacteria in fermented brines used to make stinky tofu. Int J Food Microbiol 123:134-141.
Clarridge JE. 2004. Impact of 16S rRNA gene sequence analysis for identification of bacteria on clinical microbiology and infectious diseases. Clin Microbiol Rev 17:840-862.
Desniar, Poernomo D, Wijatur W. 2009. The influence of salt concentration on peda chub mackerel (*Rastrelliger* sp.) with spontaneous fermentation. Indones Fish Process J 12:73-87.
Desniar, Rusmana I, Suwanto A, Mubarik NR. 2013. Characterization of lactic acid bacteria isolated from an Indonesian fermented fish (*bekasam*) and their antimicrobial activity against pathogenic bacteria. Emir J Food Agric 25:489-494.
Desniar, Setyaningsih I, Sumardi RS. 2012. Chemical and microbiological parameter changes and isolation of acid-producing bacteria during fermentation process of common carp (*Cyprinus carpio* *bekasam*). Indones Fish Process J 15:232-239.
Doyle MP, Beuchat LR, Montville TJ. 2001. Food microbiology: Fundamentals and frontiers. 2nd ed. ASM Press, Washington, DC, USA. pp 687-688.
El-Jakee J, Moussa IM, Nada SA, Mohamed KF, Ashgan MH, Mohamed ML. 2010. Influence of probiotics mixture on *Salmonella typhimurium* in mice. Int J Microbiol Res 1:50-61.
El-Medany SA, El-Reffaei WHM. 2015. Evaluation canola meal on growing rabbits; nutritionally and on their nutritional meat quality. J Food Nutr Res 3:220-234.
Eze VC, Onwuakor CE, Ukeka E. 2014. Proximate composition, biochemical and microbiological changes associated with fermenting african oil bean (*Pentaclethra macrophylla* Benth) seeds. Am J Microbiol Res 2:138-142.
FAO, WHO. 2002. Guidelines for the evaluation of probiotics in food. Available from: http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf. Accessed at Sep 9, 2019.
Fennema OR. 1996. Food chemistry. 3rd ed. Marcel Dekker, New York, NY, USA. pp 32-33.
Fessard A, Remize F. 2017. Why are *Weissella* spp. not used as commercial starter cultures for food fermentation. Fermentation 3:38.
Fleet GH. 1990. Yeasts in dairy products: A review. J Appl Bacteriol 68:199-211.
Fuseini A, Wotton SB, Hadley PJ, Knowles TG. 2017. The compatibility of modern slaughter techniques with Halal slaughter: A review of the aspects of “modern” slaughter methods that divide scholarly opinion within the Muslim community. Anim Welf 26:301-310.
García-Cano I, Serrano-Maldonado CE, Olvera-García M, Delgado-Arciniega E, Peña-Montes C, Mendoza-Hernández G, Quirasco M. 2014. Antibacterial activity produced by *Enterococcus* spp. isolated from an artisanal Mexican dairy product, Cotija cheese. LWT-Food Sci Technol 59:26-34.
Gassem MA. 2019. Microbiological and chemical quality of a traditional salted-fermented fish (Hout-Kasef) product of Jazan region, Saudi Arabia. Saudi J Biol Sci 26:137-140.
Hermida M, Gonzalez M, Miranda M, Rodriguez-Otero JL. 2006. Mineral analysis in rabbit meat from Galicia (NW Spain). Meat Sci 73:635-639.
Kashket ER. 1987. Bioenergetics of lactic acid bacteria: Cytoplasmic pH and osmotolerance. FEMS Microbiol Rev 3:233-244.
Kerry RG, Patra JK, Gouda S, Park Y, Shin H, Das G. 2018. Benefaction of probiotics for human health: A review. J Food Drug Anal 26:927-939.

Kumar RS, Kanmani P, Yuvaraj N, Paari KA, Pattukumar V, Arul V. 2013. Traditional Indian fermented foods: A rich source of lactic acid bacteria. Int J Food Sci Nutr 64:415-428.

Kurbanoğlu EB. 2001. Production of single-cell protein from ram horn hydrolysate. Turk J Biol 25:371-377.

Majumdar RK, Basu S. 2010. Characterization of the traditional fermented fish product Lona ilish of Northeast India. Indian JTraditKnowl 9:453-458.

Miyashita M, Yukphan P, Chaipitakchonlatarn W, Malimas T, Sugimoto M, Yoshino M, Potacharoen W, Tanasupawat S, Nakagawa Y, Kirtikara K, Tanticharoen M, Suzuki KI. 2012. 16S rRNA gene sequence analysis of lactic acid bacteria isolated from fermented foods in Thailand. Microbiol Cult Collect 28:1-9.

Mustopa AZ, Fatimah. 2014. Diversity of lactic acid bacteria isolated from Indonesian traditional fermented foods. Microbiol Indones 8:48-57.

Ngom MO. 2000. Induction and production of specific extracellular lipases from selected microorganisms. M.S. thesis. McGill University, Montreal, Canada.

Nistor E, Bampidis VA, Păcală N, Pentea M, Tozer J, Prundeanau H. 2013. Nutrient content of rabbit meat as compared to chicken, beef and pork meat. J Anim Prod Adv 3:172-176.

Paludan-Muller C, Madsen M, Sophanodora P, Gram L, Møller PL. 2002. Fermentation and microflora of plaa-som, a Thai fermented fish product prepared with different salt concentrations. Int J Food Microbiol 73:61-70.

Priyanti A, Raharjo YC. 2012. Market driving to develop rabbit meat products in Indonesia. Wartazoa 22:99-106.

Putri F, Indah H, Utama GL. 2015. Preliminary identification of potential halophilic bacteria isolated from ‘Asam Sunti’ - Indonesian traditional herbs, in inhibiting the growth of E. coli and Salmonella spp. Int J Adv Sci Eng Info Technol 5:152-154.

Sahadeva RPK, Leong SF, Chua KH, Tan CH, Chan HY, Tong EV, Wong SYW, Chan, HK. 2011. Survival of commercial probiotic strains to pH and bile. Int Food Res J 18:1515-1522.

Saitou N, Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4:406-425.

Sari M, Suryanto D, Yurnaliza. 2018. Antimicrobial activity of lactic acid bacteria isolated from bekasam against Staphylococcus aureus ATCC 25923, Escherichia coli ATCC 25922 and Salmonella sp. IOP Conf Ser Earth Environ Sci 130:012011.

Sherman PM, Johnson-Henry KC, Yeung HP, Ngo PSC, Goulet J, Tompkins TA. 2005. Probiotics reduce enterohemorrhagic Escherichia coli O157:H7- and enteropathogenic E. coli O127:H6-induced changes in polarized T84 epithelial cell monolayers by reducing bacterial adhesion and cytoskeletal rearrangements. Infect Immun 73:5183-5188.

Shokryazdan P, Jahromi MF, Liang JB, Kalavathy R, Sieo CC, Ho YW. 2016. Safety assessment of two new Lactobacillus strains as probiotic for human using a rat model. PLOS ONE 11:e0159851.

Tagg JR, Dajani AS, Wannamaker LW. 1976. Bacteriocins of gram-positive bacteria. Bacteriol Rev 40:722-756.

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG. 1997. The CLUSTAL_X windows interface: Flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 25:4876-4882.

Tokatli M, Gülgör G, Elmacı SB, İşleyen NA, Özçelik F. 2015. In vitro properties of potential probiotic indigenous lactic acid bacteria originating from traditional pickles. Biomed Res Int 2015:315819.
Utama GL, Meliana S, Djali M, Yuliana T, Balia RL. 2019. Probiotic candidates yeast isolated from dangke-Indonesian traditional fermented buffalo milk. Acta Univ Agric Silvic Mendelianae Brun 67:179-187.

Vasilica B, Balotescu C. 2006. Adherence pattern of Lactobacillus brevis 16GAL to HeLa cells and competition for sites against pathogens. Bulletin USAMV-CN 62:425.

Zalán Z, Hudáček J, Štětina J, Chumchalová J, Halász A. 2010. Production of organic acids by Lactobacillus strains in three different media. Eur Food Res Technol 230:395-404.

Zhu H, Qu F, Zhu LH. 1993. Isolation of genomic DNAs from plants, fungi and bacteria using benzyl chloride. Nucleic Acids Res 21:5279-5280.