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Asian Jr. of Microbiol. Biotech. Env. Sc. Vol. 20, No. (3) : 2018 : 718-724 © Global Science Publications ISSN-0972-3005 CHARACTERIZATION OF PROBIOTICS PROPERTIES OF LACTOBACILLUS FROM SOLID WASTE OF SOY MILK PRODUCTION EVY ROSSI12, ELLY ROZA3, YURNALIS SOFYAN3, SALAM N. ARITONANG3 AND ENDANG PURWATI3 1) Graduate Students of Animal Science Faculty, Universitas Andalas Padang, Indonesia 2) Department of Agricultural Technology, Faculty of Agriculture, Universitas Riau, Indonesia 3)Department of Animal Production, Animal Science Faculty, Universitas Andalas Padang, Indonesia (Received 19 November, 2017; accepted 25 January, 2018) Key words : Probiotics, Lactobacillus, Soy milk production Abstract– Lactobacillus isolated from solid waste of soy milk production was a potential source of isolates as a starter for the processing of functional foods made from soybeans. Therefore Sixteen Lactobacillus spp. which was isolated from this waste were evaluated further to see their probiotics properties. The results showed that all lactobacillus had the different ability (P<0.05) to inhibit the growth of pathogenic bacteria Escherichia coli 0157, Staphylococcus aureus ATCC-25293 and Listeria monocytogenes EP01. Isolates A21.1 and A21.3 displayed very high tolerance to acidic conditions (pH 2.5 and 3), and The viability LABs in 0.3 and 0.5% oxgall for 0 hr ranged from 21.43-188.89% and 76.97-162.96%, respectively. All LAB isolates showed resistance to all the antibiotic discs texted namely Penicillin (10 ˜g), Tetracycline (10˜g), Erythromycin (15 µg), Chloramphenicol (30 µg), Amoxicillin (25 µg), and Cefadroxil (15 µg), except for isolate A13.2 and A23.2 that were intermediate response to Amoxicillin. The conclusion of this study was all isolates were able to grow in acid condition (pH 2.5 and 3) and at 0.3 and 0.5% bile concentration. Based on probiotic characteristics and 16S-RNA identification, LAB isolated from solid waste of soybean milk production were all Lactobacillus, and most of them were the plantarum strain INTRODUCTION Lactic acid bacteria (LAB) are microbes that are widely found in nature, and these bacteria are commonly used as starter culture for fermenting food (Leroy and De Vuyst, 2004; Xu et al., 2007; Ho et al., 2015), processing of functional food (Gourbeyre, et al., 2011) ; and provide the aroma of food. The widespread use of LAB is due to the

bacteria being categorized as commonly recognized as safe (GRAS) organism (Holzapfel and Schillinger, 2002). These bacteria can be isolated from various sources in nature. Soy milk is one of the functional foods that are widely consumed, especially for patients with lactose intolerance. The process of making soy milk produced solid waste in the form of soybean dregs that were rarely consumed by humans so have a low economic value. Soybean or so-called okara still has a high nutritional value such as protein content, fat, dietary fiber, minerals, monosaccharides, and oligosaccharides. Soybean from soy milk contains 22% of isoflavones and is a potential source of antioxidants, prebiotic, lowers cholesterol and blood sugar (Marazza et al., 2013). The nutrient content it contains causes this waste to be an excellent medium for the growth of microorganisms, one of which was the LAB. Aritonang et al., (2017) had isolated and identified LAB from okara and found 16 LAB. Six of the LAB isolates have been identified using the 50 CHL KIT API and are known to have the Lactobacillus genus. In recent year, many works had been done to evaluate probiotics properties of the LAB. The LAB was isolated from various sources in nature, such as from Cameroon local milk product (Mbawala et al. 2011), Brazilian kefir grains (Leite et al., 2015), Corresponding author's email: rossi_brp@yahoo.com; evy.rossi@lecturer.unri.ac.id Iranian Koozeh traditional cheese (Tavakoli et al., 2017), soft cheese (Maia et al., 2017), and buffalo milk in West Sumatra Indonesia (Melia et al., 2017). Today, probiotic dairy products are one of the most highly developed functional foods industry. Meanwhile, functional food processed from soybeans are widely produced and preferred like soyghurt (Pinthong et al., 2007), or soy-kefir (Liu et al., 2006). Fermented products from soy milk have functional properties, not only containing LAB but also contain prebiotics and other compounds such as flavonoids and phytoestrogens contained by soybeans (Kim et al., 2011). Lactobacillus isolated from soybean or soybean processing waste is a potential source of isolates as a starter for the processing of functional foods made from soybeans. Therefore Lactobacillus spp. which was isolated by (Aritonang et al., 2017) should be evaluated further to see the probiotics properties, so that the future can be applied starter culture in the development of novel fermented soy milk, or other processing functional food made from soybean as raw material. The objectives of this investigation were to evaluate probiotics properties of Lactobacillus spp isolated from solid waste of soy milk production, MATERIALS AND METHODS Tested Microorganisms Lactobacilli isolated from solid waste of soy milk production (Aritonang et al., 2017) that had identified using morphological and physiological method (Lu et al. 2013). The LABs isolated from solid waste of soy milk production taken from three locations of soybean milk home industries in Taman Karya District, Pekanbaru, Indonesia. Antimicrobial Activity of LAB against Pathogen Bacteria Lactic acid bacteria isolates were preserved in MRS broth (Merck) with 20% (v/v) glycerol at -20 °C. To obtain the active culture of LAB, all LAB isolates were cultured in MRS broth and incubated for 24 h at 37 ° C. The pathogenic indicator strains, Escherichia coli 0157 and Staphylococcus aureus ATCC 25923 were inoculated into a test tube containing 5 ml nutrient broth (Merck) and incubated at 37 °C for 24 hours to obtain active cultures. An active culture was characterized by the occurrence of turbidity in the media. The pathogen bacteria used were obtained from the collection of livestock technology laboratory, Animal Science Department of Andalas University, Padang. Indonesia. Well diffusion method (Bromberg et al. 2004) was used to measure LAB antimicrobial activity. Indicator bacteria (0.1 ml) were inoculated on a nutrient agar (NA) and swabbed medium using hockey stick (Hawaz, 2014). To create 5 mm wells, the bottom of a sterile blue pipette tip, used to drill media agar and the bottom of the wellbore was filled with sterile agar. Then, 50 µL of each LAB isolate (an overnight culture) grown in MRS broth at 37 °C was added to the previously made wells and incubated at 37 °C for 24 hours.

The antimicrobial activities were evaluated by measuring a diameter of clear zone three times at different positions and averaged. Resistance Each trial was performed in four replications Resistance to Low pH and Bile Salt The resistance of LABs in acidic MRS broth at pH 2.5 and 3.0 were evaluated. The pH was adjusted with 1 N HCl. The resistance of bacteria was evaluated at 0 and 90 min of incubation. The survival LAB was monitored at OD 600nm using UV-Vis Spectrometer. The bile tolerance of LAB at bile salt was evaluated by inoculating 1 mL of LAB isolate into MRS broth containing 0.3 and 0.5 % bile salts (Sigma). After 0 and h of incubation at 37°C, an absorbance of the culture at 620 nm was determined. Experiments of acid and bile tolerance were repeated three times each with duplicate analysis. Antibiotics Sensitivity The antibiotic resistance test was performed by the method described by Babot et al. (2014) and modified by the Diffuse Agar method. LAB culture of 18 hours was poured 1 into 18 mL medium of gel jelly agar plate, then stirred until solidified. The well was made using a 7mm diameter sterile pipette tip. Wells were made of four wells in each gelatin cup. Each well was filled with one test antibiotic each of 50 µL (10 µg Penicillin, 10µg Tetracycline, 15 µg Erythromycin, 30 µg Chloramphenicol, 25 µg Amoxicillin, and 15 µg Cefadroxil). The MRS agar plate was incubated for 24 hours at 37 ° C. The clear zone diameter was measured by caliper (mm). Each isolate was tested for three replications. The sensitivity of LAB isolates was interpreted as resistant, intermediate, and sensitive according to the standards described by (Rubin, 2013) and (Swenson et al. 1990). Identification of Microorganism Using 16S rRNA The species of a selected LAB was identified based on 16S rRNA gene sequence analysis. Genomic DNA from isolate was extracted using kit Presto™ Mini gDNA Bacteria. The DNA gene was amplified by PCR using the universal primers 27 F (5'-GAGTTTGATCCTGGCTAG-3'), 1525 R (5'- AGAAAGGAGGTGATCCAGCC-3'). The conditions of PCR amplification were programmed as follows: Initial denaturation for 5 min at 95°C, 40 denaturation cycles at 94°C for 45 sec/each, 1 min annealing time at 56°C, 1 min and 30 sec for the extension at 72°C, and 7 min for final extension at 72°C. The products of amplification were evaluated by electrophoresis using 1% (w/v) agarose gel and visualized. The electrophoresis gel was stained with 5 µg / ml ethidium bromide solution by immersion, then visualized over UV light (WiseUV WUV-M20) and photographed with a digital camera (Olympus SP-500 UZ). The 16S rRNA sequence was compared with the sequences available in the nucleotide database using the BLAST (Basic Local Alignment Search Tool) at the NCBI server. RESULTS AND DISCUSSION Antimicrobial Activity of LAB against Pathogen Bacteria Lactic acid bacteria isolated from solid waste soybean milk production had the different ability ($P<0.05$) to inhibit the growth of pathogenic bacteria Escherichia coli 0157, Staphylococcus aureus ATCC- 25293 and Listeria monocytogenes EP01. The cell masses of LAB isolates were tested for antimicrobial activities against three pathogenic bacteria (Escherichia coli 0157, Staphylococcus aureus ATCC- 25293 and Listeria monocytogenes EP01). Figure 1 showed that all LAB isolates having different antimicrobial activities by developing a clear zone around the wells which were this LAB isolate inside. The difference in the diameter of the clear zone around the wells was due to structural differences in the cell wall of pathogenic bacteria determining penetration, bonding, and microbial activity. Antimicrobial activities of LAB isolates were accumulation reaction of several substances that were organics acid, diacetyl, hydrogen peroxide, and bacteriocin. The antimicrobial effects were directly caused by organic acids, namely lactic acid, acetate and propionate, this organic acid activity in the bacterial cytoplasmic membrane which can affect the maintenance of membrane potential and inhibit active transport. The higher active isolate against Listeria monocytogenes EP01 was A.22.4, A.23.2, and A22.1 with inhibitory activity zone of 14,49 mm. 13.56 and 12.85 mm respectively. The lowest antimicrobial activities of LAB isolates against

Staphylococcus aureus ATCC-25293 was A11.4 with inhibitory activity zone of 2.13 mm. For Escherichia coli 0157, the most active isolate was A22.3 with 9.90 mm of diameter clear zone. The results of this study indicate that Listeria monocytogenes EP01 was more sensitive to antimicrobial LAB activities compared with another pathogen. The results indicated that the antimicrobial produced by this LAB isolate has a relatively high activity to inhibit the growth of L.monocytogenes. This implies this LAB isolate can be an alternative to protect humans against diseases caused by L. monocytogenes. The disease that caused by this bacteria was listeriosis in animals and humans characterized by fever, diarrhea and other indigestion (Gandhi and Chikindas, 2007). The gastrointestinal tract is the main entry point of infection of L. monocytogenes (Gahan and Hill, 2014). Su et al. (2016) concluded that animal-derived foods might serve as the primary vehicle in transmitting human listeriosis in Shanghai. LAB isolate Fig. 1. Lactic acid bacteria inhibition zone diameter (mm) against pathogenic bacteria Resistance to Low pH and Bile Salt The successful probiotics, they must be able to tolerate gut conditions (Messaoudi et al., 2013). To survive on reaching the intestine, LAB should be able to live at low pH conditions found in the stomach (low pH 2-3). The time for food through the stomach is estimated to be 90 minutes. These conditions can result in LAB viability due to low pH. Figure 2. showed that not all isolated LAB could survive at pH 2.5 and 3 for 90 min. Isolates A21.1 and A21.3 displayed very high tolerance to acidic conditions (pH 2.5 and 3), and two isolates (A13.2 and A23. 4) displayed relatively low resistance at pH 2.5 and 3, with a survival rate of less than 50%. Food is known to stay in the stomach for an average of 3 hours (Mansour et al., 2014). Fig. 2. Resistant rate of lactic acid bacteria isolates at low pH conditions Bile salt tolerance was also required for probiotics to survive in the small intestine (Novak and Katz, 2006). The survival rate of LAB isolates on 0. 3 and 0.5% oxgall as shown in Figure 3. The viability LABs in 0.3 and 0.5% oxgall for 0 hr ranged from 21.43- 188.89% and 76.97-162.96%, respectively. The After incubation for 5 hours, LAB isolates A22.4 demonstrated the highest survival rate (195.54%) compared with other isolate survival rates. These results showed that Lactobacilli was mostly capable of surviving in the gastrointestinal tract environment which had the high concentration of bile salt (Oluwajoba et al., 2013). This decrease in the survival rate of some LAB at 0 hr was likely caused by changes in the cell membrane permeability of gram-positive bacteria, resulting in leakage of intracellular material due to cell lysis, resulting in cell death. Antibiotics Sensitivity Lactic acid bacteria resistance test against antibiotics was one of the stages in the selection of LAB as probiotics. Lactic acid bacteria as probiotic candidates should be resistant to antibiotics so that Fig. 3. Resistant rate of lactic acid bacteria isolates at bile salt (oxgall) Table 1. Antibiotic susceptibility profiles of the Lactobacillus isolates as obtained by well diffusion method Isolate Antibiotic susceptibility profile of Lactobacillus isolates Penicillin Tetracycline Erytromycin Chloramphenicol Amoxicillin Cefadroxil A.13.2 R R R R I R A.21.1 R R R R R R A.21.3 R R R R R R A.22.4 R R R R R R A.23.2 R R R R I R A.23.4 R R R R R R Note: R: resistant, I: intermediate, S: sensitive. Antibiotic resistance based on NCCL (2005): ≤ 10 mm resistant, 11-19 mm intermediates, ≥ 20 sensitive. Penicillin (10 µg), tetracycline (30 µg), erythromycin (15 µg), chloramphenicol (30 µg), amoxicillin (25 µg), cefadroxil (15 µg) Table 2. Lactic acid bacteria strain from solid waste of soymilk production identified by 16S rRNA No Isolate Code 16S rRNA Sequence 1 A.13.2 Lactobacillus pentosus strain lac 18. 2 A.21.1 Lactobacillus plantarum strain SRCM 1 004 34 3 A.21.3 Lactobacillus plantarum strain idyl20 4 A.22.4 Lactobacillus plantarum strain SRCMC 5 A.23.2 Lactobacillus plantarum strain MF 1298 6 A.23.4 Lactobacillus plantarum strain VP1.1 probiotic LAB can perform its therapeutic function. The LAB isolate from solid waste of soybean milk processing in this study showed different sensitivity to different antibiotics (Table 1). All LAB isolates were found to be resistant to

all the antibiotic discs texted, except for isolate A13.2 and A23.2 that were intermediate response to Amoxicillin. These results were similar to antibiotics test for lactobacilli by (Srinu et al., 2013). Bacterial resistance to antibiotics can be seen from the large diameter of the resulting clear zone. The small clear diameter of the clear zone indicates the resistance and the greater width of the clear zone indicate the an intermediate or sensitive properties of the antibiotic. BAL isolate resistance shows that BAL isolates can survive in the gastrointestinal tract of antibiotics and can still function as probiotics. The various resistance of lactic acid bacteria to antibiotics has been widely reported (Jerome et al., 2016) Lactic acid bacteria were resistant to the antibiotic substances due to bacteria capable of producing enzyme inactivation of antibiotic compounds. Also, the LAB cell wall may also be the second defense when inactivating enzymes that secrete bacteria are unable to inhibit the antibiotic compound in high concentrations. The cell wall structures of the LAB have the greater thickness than bacteria susceptible to antibiotics. The thickness of the peptidoglycan determines the resistance of bacteria to antibiotics whereby the greater the membrane permeability system becomes tighter in preventing the insertion of antibiotic components across the cell membrane so that LAB will tend to be antiseptic. Other factors that can cause bacteria to be resistant to antibiotics may also be due to the continued use of antibiotics. Antibiotics are substances produced by microorganisms that serve as bactericidal and can kill the growth of other microorganisms. This allows LAB probiotics to have antibiotic-resistant properties. Identification Microorganism Using 16S rRNA The LAB isolates had been molecularly identified by amplifying and sequencing 16S rRNA gen and comparing the results to the database of well-known 16S rRNA, The BLAST results of this study showed at Tabel 2. The most dominant microorganisms in solid waste of soy milk production that had antimicrobial activities was Lactobacillus plantarum. The results of this study were similar to those of (Piayura et al., 2017) who found L. plantarum as much as 78% of the lab from traditional Thai fermented minced fish (som-fak) products and L plantarum has antimicrobial activity (Chen et al., 2014) also found L. plantarum 510, isolated from a koshu vineyard in Japan and produces antimicrobial substances and has been purified and characterized as bacteriocin. CONCLUSION The probiotic properties of LAB isolated from solid waste of soy milk production varied among individual isolates. Results showed that all LAB isolate had antibacterial activity and the highest of their antimicrobial activity was against Listeria monocytogenes. All isolates were able to grow in acid condition (pH 2.5 and 3) and at 0.3 and 0.5% bile concentration. Based on probiotic characteristics and 16S-RNA identification, LAB isolated from solid waste of soybean milk production were all Lactobacillus and most of them were the plantarum strain. ACKNOWLEDGMENTS This research was supported by BOPTN Research Funding from Universitas Andalas, Ministry of Research Technology and Higher Education Republic of Indonesia through contract No. 71/ UN.16.17/PP.HGB/LPPM/2017. We are very grateful to the Rector of Universitas Andalas and the Minister of Research Technology and Higher Education for funding this work. REFERENCES Aritonang, S. N., Roza, E., Rossi, E., Purwati, E. and Husmaini. 2017. Isolation and identification of lactic acid bacteria from okara and evaluation of their potential as candidate probiotics. Pakistan Journal of Nutrition. 16(8) : doi: 10.3923/pjn.2017.618.628. Babot, J. D., Argañaraz-Martínez, E., and Saavedra, L. 2014. Selection of indigenous lactic acid bacteria to reinforce. Research in Veterinary Science. 97(1) : 8-17. Bromberg, R., Moreno, I. and Zaganini, C. 2004. Isolation of bacteriocin-producing lactic acid bacteria from meat and meat products and its spectrum of inhibitory activity. Brazilian Journal of Microbiology. 35 : 135-144. Chen, Y. Wang, Y., Chow, Y. Yanagida, F., Liao, C., and Chiu, C. 2014. Purification and characterization of plantaricin Y, a novel bacteriocin produced by Lactobacillus plantarum 510. Archives of Microbiology.

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