OPEN ACCESS
Advances in Animal and Veterinary Sciences

Research Article

Molecular Identification of Lactic Acid Bacteria from Black Soldier Fly Larvae Reared on Different Substrates

Aulia Nurul Saputri, I Komang Gede Wiryawan, Dilla Mareistia Fassah*, Dewi Apri Astuti

Department of Nutrition and Feed Technology, Faculty of Animal Science, IPB University, Bogor, 16680, Indonesia.

Abstract | Usage of antibiotic growth promoters (AGP) in livestock causes certain antibiotic resistance. Probiotic may be used as an alternative for AGP by providing microorganisms that have a health benefit for the host. This study was to identify the molecular characteristics of LAB isolated from the digestive tract of black soldier fly (BSF) larvae on different substrates as probiotic candidates. The LAB from the digestive tract of BSF larvae in chicken manure and palm kernel meal substrates was isolated and taxonomically identified using the 16S rRNA sequence homology and molecular identification. The LAB isolates were also tested for antimicrobial and hemolysis activities. Five isolates from BSF larvae reared on chicken manure substrate and three isolates from BSF larvae reared on palm kernel substrate showed a gram-positive bacteria characteristic. The LAB isolates from BSF larvae reared on chicken manure and palm kernel meal substrate showed no different in inhibition zones against pathogenic bacteria (*E. coli* and *S. typhimurium*). All isolates showed no hemolysis activity. Three isolates (A3, A4, and B1) are molecularly identified as *Enterococcus faecalis* which showed a potency as probiotic candidate. We conclude that *Enterococcus faecalis* is a potential probiotic that can be isolated from BSF larvae.

Keywords | 16S rRNA Gene; BSF Larvae; Chicken Manure; Lactic Acid Bacteria; Palm Kernel Meal

Received | August 18, 2022; **Accepted** | September 15, 2022; **Published** | October 10, 2022
***Correspondence** | Dilla Mareistia Fassah, Department of Nutrition and Feed Technology, Faculty of Animal Science, IPB University, Bogor, 16680, Indonesia;
Email: dilla_mareistia@apps.ipb.ac.id
Citation | Saputri AN, Wiryawan IKG, Fassah DM, Astuti DA (2022). Molecular identification of lactic acid bacteria from black soldier fly larvae reared on different substrates. Adv. Anim. Vet. Sci. 10(11): 2275-2284.
DOI | http://dx.doi.org/10.17582/journal.aavs/2022/10.11.2275.2284
ISSN (Online) | 2307-8316

INTRODUCTION

Currently, the use of antibiotics as a growth promoter is restricted or banned due to its residue effects in Indonesia since 1st January 2018 (Livestock and Health Law, 2014). Probiotics is defined as the living microorganisms when it is administered in adequate amount may have a health beneficial effect to the host. It can be an emerging alternative to increase livestock productivity (FAO/WHO, 2002). Some safety characteristics that must be owned by probiotics to be used in animal feed, e.g., non-toxic, non-pathogenic, have accurate taxonomic identification, survive in targeted sites, can survive, and colonize, and produce antimicrobial compounds (Ahasan et al., 2015). Lactic Acid Bacteria (LAB) is one of the most frequently used microorganisms as probiotics. Lactic acid bacteria capable to produce different inhibitory compounds such as bacteriocins, organic acids as lactic acid, hydrogen peroxide, diacetyl, and carbon dioxide (Saiz et al., 2019).

Black soldier fly (BSF) is reported to have antimicrobial peptides (AMP) which are bacteriocidal, as antibacterial activity (Park et al., 2014). In addition, BSF larvae are well known to have the high lauric acid and chitin functioned as a natural antimicrobial agent and immune response enhancer, respectively (Kim and Rhee, 2016; Bovera et al., 2016). It has been reported that BSF larvae can kill about 99.96% of the pathogenic bacteria *Staphylococcus aureus*

(gram-positive bacteria) and 99.94% of the pathogenic bacteria *E. coli* (Astuti and Komalasari, 2020). The LAB isolated from the digestive tract of BSF larvae can inhibit *E. coli* pathogenic bacteria (Astuti and Wiryawan, 2022). LAB is potentially used as probiotics in young ruminant, which could reduce the incidence of diarrhea, improve weight gain, and feed efficiency (Astuti and Wiryawan, 2022; Shehta et al., 2019).

The BSF larvae can convert chicken manure into a more useful and sustainable product. Chicken manure has a low C/N ration, high moisture content and pathogens, phytotoxic compounds, and an unpleasant odor (Bortolini et al., 2020). The use of palm kernel meal (PKM) as the main live substrate has been proven to have resulted in high BSF larvae production. Moreover, the PKM substrate may increase the protein content of BSF larvae due to the adequacy of the nutrient and water content needed for BSF life needs (Syahrizal et al., 2022).

This study aimed to identify, and molecularly characterized LAB isolated from the digestive tract of BSF larvae reared on chicken manure and palm kernel meal as substrates. The LAB isolates molecularly identified using the 16S rRNA gene sequencing. The phylogenetic relationships, prediction of active carbohydrate enzymes, and types of secondary metabolite were also studied.

MATERIALS AND METHODS

Isolation and morphological identification of LAB

Black soldier fly larvae were isolated from larvae reared on broiler chicken manure (14 days old) and palm kernel meal (16 days old). All the samples were obtained from PT Bio Cycle Indo, Riau, Indonesia. Isolation of LAB from BSF larvae was carried out by collecting 0.1 mg of the liquid sample by pressing the stomach of 11 BSF larvae. The serial dilutions were prepared in 9 mL of 0.85% NaCl solution and 0.1 mL of aliquots of 10^{-7} dilution was plated on de Man, Rogosa, and Sharpe Agar (MRS Agar) medium (Merck KGaA, Germany) into a petri dish. After anaerobic incubation at 41°C for 72 hours, the single colonies of LAB were picked and streaked on the surface of MRS Agar medium then incubated at 41°C for 48 hours to obtain pure cultures. The selected colonies were regrown and propagated on MRS Broth medium (Merck KGaA, Germany). After that, the pH of the medium was measured using pH meter (Mediatech, China) in the following way: digital pH meter was entered in the medium to obtain medium pH. Morphological identification including the shape, color, and diameter of the colony was determined according to Liu et al. (2012). The gram stain test was determined following Tripathi and Sapra (2021).

Antimicrobial activity

The antimicrobial activity was tested towards pathogenic bacteria, *Escherichia coli* ATCC 8739 and *Salmonella typhimurium* b11 669 by following Klose et al. (2010). Briefly, the pathogenic bacteria were incubated for 14 hours and 1 mL (10^{-7} – 10^{-8} CFU mL^{-1}) was transferred into 20 mL of Nutrient Agar (Becton, Dickinson, and Company, United States). The agar wells were prepared using sterile pipette tips with a diameter of 7 mm. Each well was filled with 50 µL of LAB cultures and was incubated at 41°C for 24 hours. Antimicrobial activity was characterized by a clear zone indicating inhibition against pathogenic bacteria. Then, a clear zone diameter was measured using a ruler. According to Morales et al. (2003), inhibitory zone activity grouped 4 categories by weak (diameter < 5 mm), medium (diameter 5 – 10 mm), strong (diameter >10 – 20 mm), and very strong (diameter > 20 – 30 mm).

Hemolysis activity test

The hemolysis activity was assessed by LAB colonies streaked into a plate containing 15 mL of blood agar medium (Merck KGaA, Germany), then incubated at 37°C for 48 hours anaerobically. The *Staphylococcus aureus* ATCC 6538 was used as a positive control. Hemolysis activity was characterized from the growing colonies, if there was a clear zone around the colony, it showed a positive beta hemolysis reactio (Thakkar et al., 2015).

16S rRNA sequence homology and molecular phylogeny analysis

Total bacterial genomic DNA was extracted by the Presto™ mini gDNA Bacteria Kit (Geneaid Biotech, Ltd., Taiwan). The purification of DNA was performed if the DNA ratio was less than 1.8. All genome extracts had a value of genome comparison absorbance 260/280 about 2.01 – 2.09 (Supplementary file 1). The purification results are used in the process of DNA amplification using PCR. The 16S rRNA gene was amplified from the genomic DNA using DNA primers encoding 16S rRNA, namely 27F (5' AGA GTT TGA TCM TGG CTC AG 3') and 1492R (5' GGT TAC CTT GTT ACG ACT T 3') (Zhang et al., 2007) in Genetica Analyzer (ABI PRISM 3730xl Genetic Analyzer develop by Applied Biosystems, USA). PCR was performed by reacting with 25 µL My Taq HS Red Mix (Meridian Life Science Inc., USA), 2 µL primer (10 pmol), DNA template 100 ng, and sterile aquabidest in a final volume of 50 µL. Genomic DNA quality was tested using electrophoresis with 0.8% agarose gel and sequenced with an automated sequencer with a specific primer using the facility at PT Genetika Science Indonesia (Tangerang, Indonesia). The gene obtain a single band located at 1500 bp (Supplementary file 2).

The molecular phylogenetic constructed using software

MEGA version 11.0 (Penn State University) by comparing the DNA sequences with the sequences available at http://www.ncbi.nm.nih.gov/Genbank. BLAST result taken top 10 from these isolates. The 16S rRNA sequences were aligned using the clustal X alignment and saved as MEGA format using MEGA 11.0 The data were directly used to build the phylogenetic tree based on the MEGA 11.0 to describe the branch length between sequences isolates and blast sequences result. The genome of the closest phylogenetic relationship was mapped to the dbCAN database (https://cys.bios.niu.edu/dbCAN2) to identify the predicted active carbohydrate enzymes and identified the type of secondary metabolite through antiSMASH (https://antismash.secondarymetabolites.org/#!/start).

Growth curve of lactic acid bacteria

The growth curve of lactic acid bacteria was measured by optical density (OD) every 4 hours for 24 hours. The OD measurement started with the regrown LAB culture in MRS broth medium, then the OD was measured for each isolate using a spectrophotometer (LaMotte™ Smart Spectro™ 2000-02, Washington Ave, United States) at a wavelength of 620 nm. The OD value is obtained using the following formula.

$$OD = 2 - \log \%T$$

which represents the logarithm value (log) of percentage transmittance (T).

Statistical analysis

Parameter of medium pH and LAB isolates antimicrobial activity against pathogenic bacteria from BSF larvae reared on chicken manure and palm kernel meal substrates was analyzed by T-test. Data were presented as the means ± SD using IBM SPSS 25.0 (SPSS Inc., Chicago, IL, USA).

RESULTS

Morphological characterization of LAB isolates from BSF larvae

A total of 5 isolates of LAB from BSF larvae in chicken manure substrate and 3 isolates from BSF larvae in palm kernel meal substrate were selected. A comparison of LAB colonies from BSF larvae in chicken manure and palm kernel meal substrates can be seen in Figure 1. There are two colonies color of LAB isolates of BSF larvae in chicken manure and palm kernel meal substrates, i.e., yellowish white and milky white (Table 1). Both substrates, showed the same colonies color. Moreover, the diameter of LAB isolate colonies varied between 1.25 to 3.43 mm.

The isolates were incubated for 24 hours to measure of pH. After 24-hour incubation, the pH value of the inoculation medium decreased from 6.5 (initial pH) into a ranged of 4.34 to 4.6 (LAB isolates from BSF larvae reared on chicken manure substrate) and 4.1 to 4.4 (LAB isolates from BSF larvae reared on palm kernel meal substrate) (Table 1). The LAB isolates from BSF larvae reared on chicken manure and palm kernel meal substrates showed no different (*p value* 0.496) in pH of medium.

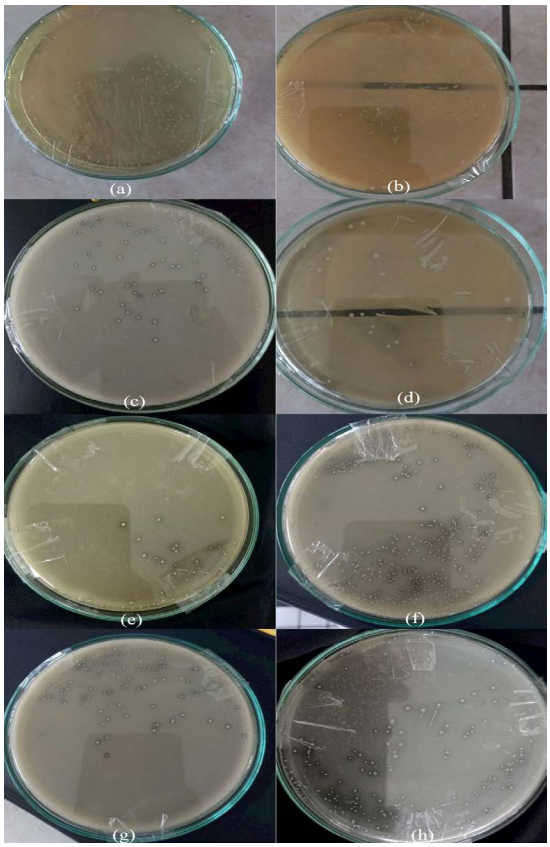

Figure 1: Morphology identification of LAB isolates. a) Isolate A2. b) Isolate A3. c) Isolate A4. d) Isolate A5. e) Isolate A6. f) Isolate B1. g) Isolate B2. h) Isolate B3.

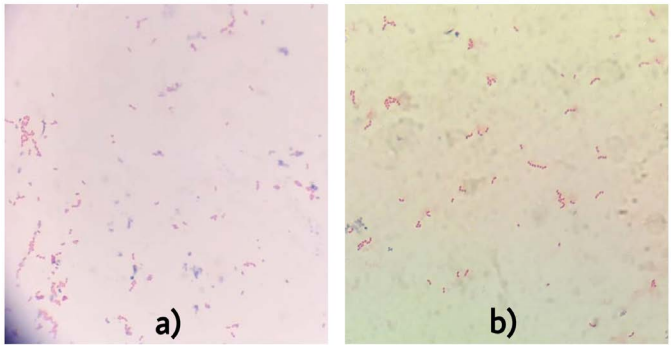

Figure 2: Gram staining of LAB isolates. a) Isolate A4. b) Isolate B1

The gram-stained results showed purple color and cocci form (Figure 2) indicates the gram-positive bacteria characteristics. The gram-positive bacteria were characterized by cocci (round) or bacillus (rod) shaped (Tripathi and Sapra, 2021).

Table 1: Comparison of phenotype characteristics of LAB isolated from BSF in chicken manure and palm kernel cake substrates

Substrate	Chicken manure substrate						Palm kernel meal substrate				
Characteristics	A2	A3	A4	A5	A6	Average	B1	B2	B3	Average	*p*
Color	Yellowish white	Yellowish white	Milky white	Yellowish white	Yellowish white		Yellowish white	Yellowish white	Milky white		
Diameter (mm)	1.94	1.62	3.43	2,24	1.41		1.25	2.32	2.93		
pH	4.60	4.34	4.34	4.50	4.50	4.43 ± 0.15	4.10	4.34	4.40	4.28 ± 0.16	0.496

p were presented by T-test (P > 0.05)

Table 2: Comparison of the diameter of inhibitory zone against pathogenic bacteria and hemolysis activity of LAB isolated from BSF in chicken manure and palm kernel cake substrates

Substrate	Chicken manure						Palm kernel meal				*p*
Parameter	A2	A3	A4	A5	A6	Average	B1	B2	B3	Average	
Inhibitory zone											
E. coli	9.57	14.83	10.13	10.20	9.33	10.81 ± 2.35	15.33	9.67	9.73	11.58 ± 3.03	0.968
S. typhimurium	9.07	9.98	9.10	9.60	7.90	9.13 ± 1.04	9.23	9.66	8.26	9.06 ± 0.91	0.433
Hemolysis activity	No	No	No	No	No		No	No	No		

p were presented by T-test (P > 0.05)

Table 3: Top 10 of BLAST by sequence which are analyzed for molecular identification

No isolate	Description	Per. Ident (%)	Accession
A3	*Enterococcus faecalis* strain S1-4-5 16S ribosomal RNA gene, partial sequence	99.90	MT509943.1
	Enterococcus faecalis strain G1-6-15 16S ribosomal RNA gene, partial sequence	99.90	MT509898.1
	Enterococcus faecalis strain PBA_120 16S ribosomal RNA gene, partial sequence	99.90	MT484114.1
	Bacterium strain J44FV 16S ribosomal RNA gene, partial sequence	99.90	MT425056.1
	Bacterium strain J34MR 16S ribosomal RNA gene, partial sequence	99.90	MT424970.1
	Bacterium strain J29FV 16S ribosomal RNA gene, partial sequence	99.90	MT424956.1
	Enterococcus faecalis strain SM30 16S ribosomal RNA gene, partial sequence	99.90	MT356185.1
	Enterococcus faecalis strain SM29 16S ribosomal RNA gene, partial sequence	99.90	MT356184.1
	Enterococcus faecalis strain DUT1805 16S ribosomal RNA gene, partial sequence	99.90	MN880256.1
	Enterococcus faecalis strain JM102 16S ribosomal RNA gene, partial sequence	99.90	MN758862.1
A4	*Enterococcus faecalis* strain SM30 16S ribosomal RNA gene, partial sequence	99.79	MT356185.1
	Enterococcus faecalis strain SM29 16S ribosomal RNA gene, partial sequence	99.79	MT356184.1
	Enterococcus faecalis strain DUT1805 16S ribosomal RNA gene, partial sequence	99.79	MN880256.1
	Enterococcus faecalis strain FDAARGOS_528 chromosome, complete genome	99.79	CP033787.1
	Enterococcus faecalis strain YN771 16S ribosomal RNA gene, partial sequence	99.79	MH919370.1
	Enterococcus faecalis strain DL1505 16S ribosomal RNA gene, partial sequence	99.79	MG736061.1
	Enterococcus faecalis strain SI 16S ribosomal RNA gene, partial sequence	99.79	KX527572.1
	Enterococcus faecalis strain NS2 chromosome, complete genome	99.79	CP078162.1
	Enterococcus faecalis isolate 27688_1#342 genome assembly_chromosome: 1	99.79	LR962850.1
	Enterococcus faecalis isolate 28157_4#42 genome assembly_chromosome: 1	99.79	LR962843.1

B1	*Enterococcus faecalis* strain SM30 16S ribosomal RNA gene, partial sequence	100	MT356185.1
	Enterococcus faecalis strain SM29 16S ribosomal RNA gene, partial sequence	100	MT356184.1
	Enterococcus faecalis strain FDAARGOS_528 chromosome, complete genome	100	CP033787.1
	Enterococcus faecalis strain Y533 16S ribosomal RNA gene, partial sequence	100	MZ323922.1
	Enterococcus faecalis strain NS2 chromosome, complete genome	100	CP078162.1
	Enterococcus faecalis strain DUT1805 16S ribosomal RNA gene, partial sequence	100	MN880256.1
	Enterococcus faecalis strain YN771 16S ribosomal RNA gene, partial sequence	100	MH919370.1
	Bacterium strain Glm16 16S ribosomal RNA gene, partial sequence	100	MH569446.1
	Enterococcus faecalis strain DL1505 16S ribosomal RNA gene, partial sequence	100	MG736061.1
	Bacterium JNMFL L5 16S ribosomal RNA gene, partial sequence	100	KM401459.1

Antimicrobial activity

Our study revealed that all isolates showed antimicrobial activity against *E. coli* and *S. typhimurium* (Table 2). This study showed no different (*p value* > 0.05) in inhibition zone of LAB isolates from BSF larvae reared on chicken manure and palm kernel meal substrates against *E. coli* and *S. typhimurium*. The zone of inhibition against *E. coli* formed from LAB isolates from BSF larvae reared on chicken manure substrate ranged from 9.33 to 14.83 mm, while LAB isolates from BSF larvae reared on palm kernel meal substrate ranged from 9.67 to 15.33 mm. Thus, all LAB isolates in inhibiting *E. coli* are categorized as medium to strong. The zone of inhibition LAB isolates against *S. typhimurium* from BSF larvae reared on chicken manure substrate ranged from 7.90 to 9.98 mm, while LAB isolates from BSF larvae reared on palm kernel meal substrate ranged from 8.26 to 9.66 mm. Thus, all LAB isolates in inhibiting *S. typhimurium* are categorized as medium.

Figure 3: Hemolysis activity of all isolates. c; Positive control (*Staphylococcus aureus*). A3; isolate A3. A4; isolate A4. A5; isolate A5. B1; isolate B1. B2; isolate B2.

Hemolysis Activity

The evaluation of hemolysis activity is a primary safety assessment which also one of the prerequisite criteria for probiotics to be used in animal feed (Saiz et al., 2019). In our study, there was no zone formed around the LAB colony (Figure 3).

Molecular identification using 16S rRNA sequence

Among all the isolates, we selected candidates with wide inhibition zone against pathogenic bacteria (*E. coli*) for molecular identification. Two isolates (A3 and A4) from BSF larvae reared on chicken manure substrate and one isolate (B1) from BSF larvae reared on palm kernel meal substrate were selected. The BLAST results showed that three isolates from chicken manure and palm kernel meal exhibit a similarity index to 99.90% (A3), 99.79% (A4), and 100% (B1) to *Enterococcus faecalis* (Table 3).

Based on the sequence analysis, we constructed the phylogenetic tree analysis using the MEGA 11.0. The phylogenetic tree may construct the strains relationship and estimate the differences that occur from one ancestor to the descendants (Lin et al., 2016). The phylogenetic tree showed that the A3 and B1 isolates have a close relationship (Figure 4). In addition, the A3 was affiliated with *Enterococcus faecalis* strain SM29. Isolate B1 was close to *Enterococcus faecalis* isolate 28157 and *Enterococcus faecalis* isolate 27688 with bootstrap value of 62. However, A4 isolate was close to outgroup species, which is Bacterium JNMFL L5.

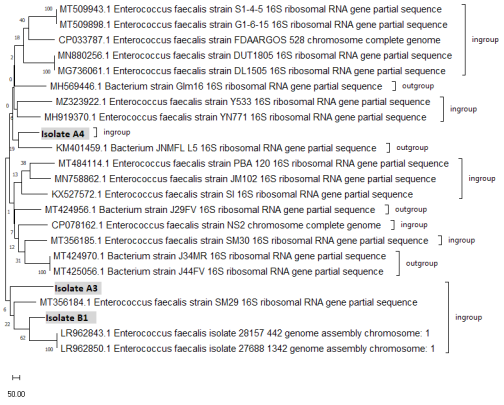

Figure 4: Phylogenetic tree of three isolates (A3, A4, and B1).

Identification of predicted carbohydrate active enzymes

The genome was mapped to the dbCAN database via CA

Table 4: List of predicted carbohydrate-active enzyme BLAST complete genome of of *Enterococcus faecalis* FDAARGOS 528

ID Gen	HMMER	Activities	Total
CP033787.1_1065	Glycoside Hydrolase Family 1	β-glucosidase; β-galactosidase; β-mannosidase; β-glucuronidase; β-xylosidase; β-D-fucosidase; phlorizin hydrolase; exo-β-1,4-glucanase; 6-phospho-β-galactosidase; 6-phospho-β-glucosidase; strictosidine β-glucosidase; lactase; amygdalin β-glucosidase; prunasin β-glucosidase; vicianin hydrolase; raucaffricine β-glucosidase; thioglucosidase; β-primeverosidase; isoflavonoid 7-O-β-apiosyl-β-glucosidase; ABA-specific β-glucosidase; DIMBOA β-glucosidase; β-glycosidase; hydroxyisourate hydrolase; β-rutinosidase /α-L-rhamnose-(1,6)-β-D-glucosidase; protodioscin 26-O-Î²-D-glucosidase	24
CP033787.1_1076	Glycoside Hydrolase Family 65	α,α-trehalase; maltose phosphorylase; trehalose phosphorylase; kojibiose phosphorylase; trehalose-6-phosphate phosphorylase; nigerose phosphorylase; 3-O-α-glucopyranosyl-L-rhamnose phosphorylase; 1,2-α-glucosylglycerol phosphorylase; α-glucosyl-1,2-β-galactosyl-L-hydroxylysine α-glucosidase; 1,3-α-oligoglucan phosphorylase; α-1,2-glucosidase; α-glucan phosphorylase	12
CP033787.1_1183	Glycoside Hydrolase Family 31	α-glucosidase; α-galactosidase; α-mannosidase; α-1,3-glucosidase; sucrase-isomaltase; α-xylosidase; α-glucan lyase; isomaltosyltransferase; oligosaccharide α-1,4-glucosyltransferase; α-N-acetylgalactosaminidase; sulfoquinovosidase; α-6-glucosyltransferase	12
CP033787.1_1187	Glycoside Hydrolase Family 31	α-glucosidase; α-galactosidase; α-mannosidase; α-1,3-glucosidase; sucrase-isomaltase; α-xylosidase; α-glucan lyase; isomaltosyltransferase; oligosaccharide α-1,4-glucosyltransferase; α-N-acetylgalactosaminidase; sulfoquinovosidase; α-6-glucosyltransferase	12
CP033787.1_1188	Glycoside Hydrolase Family 13/Subf 31	Hexosyltransferases; oligosaccharide a-4-glucosyltransferase; palatinase; [retaining] a-amylase; [retaining] oligo-a-1,6-glucosidase; a-glucosidase; glucodextranase; [retaining] isomaltulose synthase / sucrose isomerase / sucrose glucosylmutase	8

Table 5: List of predicted carbohydrate-active enzyme BLAST complete genome of *Enterococcus faecalis* 27688

ID Gen	HMMER	Activities	Total
LR962850.1_104	Glycoside Hydrolase Family 20	β-hexosaminidase; Lacto-N-biosidase; β-1,6-N-acetylglucosaminidase; β-6-SO3_N-acetylglucosaminidase	4
LR962850.1_1095	GlycosylTransferase Family 51	Murein polymerase	1
LR962850.1_1185	Carbohydrate Esterase Family 7	Acetyl xylan esterase; Cephalosporin-C deacetylase	2
LR962850.1_1187	Glycoside Hydrolase Family 3	β-glucosidase; Xylan 1,4- β-xylosidase; β-glucosylceramidase; β-N-acetylhexosaminidase; α-L-arabinofuranosidase ; Glucan 1,4- β--glucosidase; Isoprimeverose-producing oligoxyloglucan hydrolase; Coniferin β-glucosidase; Exo-1,3-1,4-glucanase; β-N-acetylglucosaminide phosphorylase; β-1,2-glucosidase; β-1,3-glucosidase; Xyloglucan-specific exo- β-1,4-glucanase; Stevioside- β-1,2-glucosidase; Lichenase; Protodioscin 26-O-β-D-glucosidase	15
LR962850.1_1188	Glycoside Hydrolase Family 94	Cellobiose phosphorylase; Laminaribiose phosphorylase; Cellodextrin phosphorylase; Chitobiose phosphorylase; Cellobionic acid phosphorylase; β-1,2-oligoglucan phosphorylase; 4-O- β-D-glucosyl-D-galactose phosphorylase	7

Zyme annotation to identify predicted carbohydrate-active enzymes. In this study, the identification used the complete genome sequence in FASTA format from the bacteria with the closest phylogenetic relationship. The closest relationship of A4 and B1 were *Enterococcus faecalis* FDAARGOS 528 and *Enterococcus faecalis* 27688, respectively. The A3 was not further identified because there are no bacteria with complete genome sequence on the top

10 BLAST result. The results of five predicted carbohydrate-active enzymes of *Enterococcus faecalis* FDAARGOS 528 are Glycoside Hydrolase Family 1, Glycoside Hydrolase Family 65, Glycoside Hydrolase Family 31, Glycoside Hydrolase Family 31, and Glycoside Hydrolase Family 31/Subf 31 (Table 4). Whereas, the results of five predicted carbohydrate-active enzymes of *Enterococcus faecalis* 27688 are Glycoside Hydrolase Family 20, Glycosyltransferase Family 51, Carbohydrate Esterase Family 7, Glycoside Hydrolase Family 3, and Glycoside Hydrolase Family 94 (Table 5).

Identification of secondary metabolite types

In this study, the prediction of secondary metabolite types of isolates was using the complete genome sequence of bacteria with the closest phylogenetic relationship, i.e. *Enterococcus faecalis* FDAARGOS 528 and *Enterococcus faecalis* 27688. This study predicts that the A4 have secondary metabolite type is Lanthipeptide-class II, while the B1 is cyclic-lactone-autoinducer (Table 6).

Table 6: Identification of secondary metabolite types

Spesies	Accesion ID	Secondary Metabolite Type	Similarity
Enterococcus faecalis strain FDAARGOS_528	CP033787.1	Lanthipeptide-class-II	100%
Enterococcus faecalis 27688	LR962850.1	Cyclic-lactone-autoinducer	100%

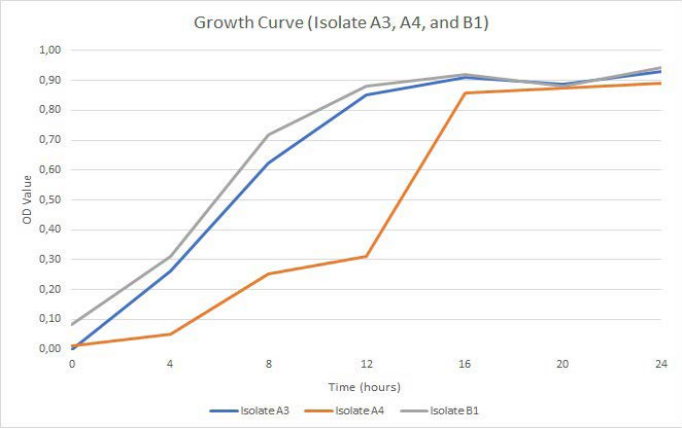

Figure 5: Growth curves of three isolates were determined by OD value for 24 hours every 4 hours. (a) Isolate A3. (b) Isolate A4. (c) Isolate B1.

Growth of lactic acid bacteria

The growth of LAB was determined by checking the optical density of the LAB cultures for 24 hours every 4 hours visualized by the LAB growth curve. The adaptation phase of three isolates (A3, A4, and B1) were between 0 – 4 hours (Figure 5). The logarithmic phase of these isolates started after 4 hours to 16 hours, which showed the significant growth of cells, whereas the isolate B1 was at 12 – 16 hours. The stationary phase occurred at 16 hours (Figure 5).

DISCUSSION

Morphological identification includes color and diameter of LAB colonies. The diameter of LAB colonies is indicated by the presence of a clear zone around the colony. The clear zone indicated calcium lactate formation which is produced form organic acids (e.g. lactic acid and acetic acid) as the main products of LAB with $CaCO_3$ (Calcium carbonate) (Putri et al., 2020). The pH plays an important role in acid production in LAB. The pH decrease after LAB is inoculated resulted from the accumulation of lactic acid from LAB metabolism during the fermentation process (Liptakova et al., 2017).

Gram staining test can represent one of the characteristics of LAB. This study showed the LAB is gram-positive bacteria. Lactic acid bacteria are Gram-positive, non-sporulating, non-pigmented and non-motile rods and cocci, most of which are non-respiring but aerotolerant anaerobes (Liptakova et al., 2017). Gram positive bacteria commonly used as a probiotic. According to Cross et al. (2004), the vast majority of immunomodulatory probiotic are gram positive bacteria. Probiotics have been shown to play a protective role by directly competing with intestinal pathogens through the release antibacterial substances (Behnsen et al., 2013). Thus, all isolates were morphologically identified as having a clear zone around the colony, produce acid, and are gram-positive bacteria.

Antimicrobial activity of probiotics is of prime importance to control pathogenic microorganisms. The main component produced by LAB, organic acids, can reduce the pH value and change the environment into a growth medium that is not suitable for the development of some pathogenic bacteria and food contamination (Reis et al., 2012). Probiotics from *Enterococcus faecalis* adhere to intestinal cells and produce antimicrobial substances (Kim et al., 2017). It can be concluded that all isolates have antimicrobial activity which is the primary criteria of probiotics.

Hemolysis activity is considered a bacterial safety test for probiotic candidates. The clear zone formed is a sign of hemolysin. It is proteins that can be identified based on their lysis ability to break down red blood cells in vitro. Some hemolysins are toxins in pore forms, which can break cells membranes and kill the host cells (Haenen, 2017). All isolates in our study showed no hemolysis activity, thus characterized them as harmless bacteria.

The 16S rRNA gene results of selected isolates (A3, A4, and B1) were *Enterococcus faecalis*. *Enterococcus faecalis* is a species of *Enterococcus* genus that have been studied as probiotics (Adnan et al., 2017). Such probiotics are administered to treat diarrhea, antibiotic-associated diarrhea or irritable bowel syndrome, to lower cholesterol levels to improve host immunity (Franz et al., 2011). *Enterococcus* strains can survive, compete, and adhere to host cells in the GIT, an important feature for the successful use a probiotic, as a normal inhabitant of the gut (Laukova et al., 2017). Furthermore, the killing ability of bacteriocins produced by *Enterococcus* strains in considered a successful strategy for maintaining the population and reducing the numbers of competitors (Yang et al., 2014).

Identification of predicted carbohydrate active enzymes can be used to predict the enzyme activity of carbohydrate enzyme groups; conserved sequence motifs are functionally important (Busk and Lange, 2013). In the lactic acid fermentation process, LAB uses glucose as a carbon source to produce pyruvate through glycolysis and then produces lactic acid under the action of lactate dehydrogenase (Wang et al., 2021). Glycoside hydrolases are a group of enzymes that hydrolyse glycosidic bonds between two or more carbohydrates or between carbohydrates and non-carbohydrate. Generally, the hydrolysis of glycosidic bonds is catalysed by two amino acid residues of the enzyme, i.e., a common acid (donor/proton) and a nucleophile/base (Davies and Henrissat, 1995). Glycosyltransferases are involved in the biosynthesis of disaccharides oligosaccharides, and polysaccharides. Glycosyltransferase is an enzyme that catalyzes the transfer of a sugar moiety from an activated donor molecule to a specific acceptor molecule by forming a glycosidic bond (Sinnot, 1990). Carbohydrate esterase catalyze the de-O or de-N-acylation of substituted saccharides. There are two classes of carbohydrate esterase substrates, namely sugars acting as acids, such as pectin methyl esters, and sugars acting as alcohols such as xylan acetate. Therefore, our study suggests isolates A4 and B1 can produce Glycoside Hydrolase, Glycosyl Transferase, and Carbohydrate Esterase enzymes.

Secondary metabolites produced by bacteria and fungi are important source of antimicrobials and other bioactive compounds (Blin et al., 2019). According to Repka et al. (2017), Lanthipeptides are synthesized in ribosome and post-translationally modified peptides (RiPPs) that display a wide variety of biological activities, from antimicrobial to antiallodynic. Lanthipeptides is one group of bacteriocins that display antimicrobial activity called lantibiotics (lanthionine-containing lantibiotic group) (Chen et al., 1999). This is revealed by Almeida-Santos et al. (2021), that *Enterococcus faecalis* produces enterocins, one of the enterocins from lantibiotics, or can be called lanthipeptide as antimicrobial. It is known that the enterocin of this lantibiotic is very highly in killing gram-positive pathogenic bacteria (Cotter et al., 2013).

Cyclic lactone autoinducer, is autoinducing peptides that involved in intercellular communication in Gram positive bacteria. Autoinducing peptides involved in the production of class II antimicrobials proteins (also known as bacteriocins) and genetic competence are linear peptides. A specific type of modification is found in the autoinducing peptides that are involved in *Enterococcus faecalis*, where the peptides contain, respectively, a cyclic thiolactone or cyclic lactone (Sturme et al., 2002). Our study concludes that isolate A4 and B1 may produce lanthipeptide class II and cyclic lactone autoinducer are needed as antimicrobial activity.

The growth curve of LAB is important to determine the optimum production time of LAB. Growth curve composed of four distinct phases of growth: the lag or adaption phase, the exponential or logarithmic phase, the stationary phase, and the death or decline phase. The lag phase is an adaption period, where the bacteria are adjusting to their new conditions. Typically, cells in the lag period are adjusting to environmental changes such as changes in temperature, pH, or oxygen availability. Logarithmic phase is marked by predictable doublings of the population, where 1 cell become 2 cells, becomes 4, etc. Cells in optimum condition will result in very rapid growth. Stationary phase is a point the number of new cells being produced is equal to the number of cells dying off or growth has entirely ceased, resulting in a flattening out of growth on the growth curve (Bruslind, 2021). The LAB population of three isolates: A3, A4, and B1 in stationary phase (16 hours incubation) are 7.82 Log CFU mL^{-1}, 7.35 Log CFU mL^{-1}, and 7.55 Log CFU mL^{-1}, respectively. The LAB population of isolate A3 is higher than other isolates. Thus, it may affect to the higher inhibition zone in reduction pathogenic bacteria.

CONCLUSIONS

In conclusion, eight isolates potential LAB were successfully isolated from BSF larvae reared on chicken manure (5 isolates) and palm kernel meal (3 isolates) substrates. Isolates have a pH ranging from 4.34 to 4.6 (from BSF larvae reared on chicken manure substrate) and 4.1 to 4.4 (from BSF larvae reared on palm kernel meal substrate). All isolates had inhibition zones against pathogenic bacteria (*E. coli* and *S. typhimurium*) and showed no hemolysis activity. Isolates A3, A4, and B1 are potential candidates for probiotic which molecularly identified as *Enterococcus faecalis.*

ACKNOWLEDGEMENTS

This study was supported by Matching Fund Program 2021 grant (Contract No. 2914/E3/PKS.08/KL/2021) funded by Directorate General of Higher Education of Republic Indonesia.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

NOVELTY STATEMENT

Previous research has never been done on molecular identification of Lactic Acid Bacteria isolated from digestive tract of Black Soldier Fly Larvae. This study succeeded in identifying *Enterococcus faecalis*, a species of Lactic Acid Bacteria isolates from digestive tract of BSF larvae reared on chicken manure and palm kernel meal as substrates. In addition, this study was successfully constructed a phylogeny tree and predicted the carbohydrate active enzymes and secondary metabolites of *Enterococcus faecalis*.

AUTHOR'S CONTRIBUTION

Wiryawan IKG, Fassah DM, Astuti DA: Conceptualization, Funding acquisition, Project administration, Supervision, Validation, Writing – review and editing. Saputri AN and Fassah DM: Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft.

REFERENCES

Adnan M, Patel M, Hadi S (2017). Functional and health promoting inherent attributes of *Enterococcus hirae* F2 as a novel probiotic isolated from the digestive tract of the fresh water fish Catla catla. Peer. J., 5:3085. https://doi.org/10.7717/peerj.3085

Ahasan ASML, Agazzi A, Invernizzi G, Bontempo V, Savoini G (2015). The beneficial role of probiotics in monogastric animal nutrition and health. J. Dairy. Vet. Anim. Res., 2:116-132. https://doi.org/10.15406/jdvar.2015.02.00041

Almeida-Santos AC, Novais C, Peixe L, Freitas AR (2021). *Enterococcus spp*. as a producer and target of bacteriocins: A double-edged sword in the antimicrobial resistance crisis context. Antibiotics., 10:1-23. https://doi.org/10.3390/antibiotics10101215

Astuti DA, Komalasari K (2020): Feed and animal nutrition: insect as animal feed. In: Proceedings of the 3rd International Conference Tropical Animal Production for Food Security. IOP Conf Ser Earth and Environ Sci., 465:012002. https://doi.org/10.1088/1755-1315/465/1/0120025

Astuti DA, Wiryawan KG (2022). Black Soldier Fly as feed ingredient for ruminants. Anim. Biosci., 35:356-363. https://doi.org/10.5713/ab.21.0460

Behnsen J, Deriu E, Sassone-Corsi M, Raffatellu M (2013). Probiotics: properties, examples, and specific applications. California, US: Cold Spring Harb Perspect Med.

Blin K, Shaw S, Steinke K, Villebro R, Ziemert N, Lee SY, Medema MH, Weber T (2019). antiSMASH 5.0: updates to the secondary metabolite genome mining pipeline. Nucleic Acid Res., 47:81-87. https:doi.org/10.1093/nar/gkz310

Bortolini S, Macavei LI, Saadoun JH, Foca G, Ulrici A, Bernini F, Malferrari D, Setti L, Ronga D, Maistrello L (2020). *Hermetia illucens* (L.) larvae as chicken manure management tool for circular economy. J. Clean. Prod., 262:1-10.

Bovera F, Loponte R, Marono S, Piccolo G, Parisi G, Laconisi V, Gasco L, Nizza A (2016). Use of Tenebrio molitor larvae meal as protein source in broiler diet: Effect on growth performance, nutrient digestibility, and carcass and meat traits. J. Anim. Sci., 94:639-647. https://doi.org/10.2527/jas2015-9201

Bruslind L (2021): Microbial Growth Available at: https://bio.libretexts.org/Bookshelves/Microbiology/Book%3A_Microbiology_(Bruslind)/09%3A_Microbial_Growth. (accessed Jun 2022).

Busk PK, Lange L (2013). Function-based classification of carbohydrate-active enzymes by recognition of short, conserved peptide motifs. Appl. Environ. Microbiol., 79:3380-3391. https://doi.org/10.1128/AEM.03803-12

Chen P, Qi F, Novak J, Caufield PW (1999). The specific genes for lantibiotic mitacin II biosynthesis in Streptococcus mutans T8 are clutered and can be transferred en bloc. AEM., 65:1356-1360. https://doi.org/10.1128/aem.65.3.1356-1360.1999

Cotter PD, Ross RP, Hill C (2013). Bacteriocins-A viable alternative to antibiotics?. Nat. Rev. Microbiol., 11:95-105. https://doi.org/10.1038/nrmicro2937

Cross ML, Ganner A, Teilab D, Fray LM (2004). Patterns of cytokine induction by gram positive and gram negative probiotic bacteria. FEMS Microbiol. Immunol., 42:173-180. https://doi.org/10.1016/j.femsim.2004.04.001

Davies G, Henrissat B (1995). Structures and mechanisms of glycosyl hydrolases. Structure., 3:853-859

FAO/WHO (2002): Joint FAO/WHO (Food and Agriculture Organization/World Health Organization) working group report on drafting guidelines for the evaluation of probiotics in food. Joint working group report on drafting. London, UK.

Franz CMAP, Huch M, Abriouel H, Holzapfel W, Galvez A (2011). Enterococci as probiotics and their implications in food safety. Int. J. Food Microbiol., 151:125-140. https://doi.org/10.1016/j.ijfoodmicro.2011.08.014

Haenen O (2017): Major bacterial disease affecting aquaculture. Mangalore, India: FAO Aquatic AMR Workshop.

Kim JY, Awji EG, Park NH, Park JY, Kim JC, Lee SP, Suh JW, Park SC (2017). Probiotic properties and adsorption of *Enterococcus faecalis* PSCT3-7 to vermiculite. J. Vet. Sci., 18:95-99.

Kim SA, Rhee MS (2016). Highly enhanced bactericidal effects of medium chain fatty acids (caprylic, capric, and lauric acid) combined with edible plant essential, oils (carvacrol, eugenol, β-resorcylic acid, trans-cinnamaldehyde, thymol, and vanillin) against *Escherichia coli* O157:H7. Food Control., 60:447-454. https://doi.org/10.1016/j.foodcont.2015.08.022

Klose V, Bayer K, Bruckbeck R, Schatzmayr G, Loibner AP

(2010). In vitro antagonistic activities of animal intestinal strains against swine-associated pathogens. Vet. Microbiol., 144:515-21. https://doi.org/10.1016/j.vetmic.2010.02.025

Laukova A, Kandricakova A, Bunkova L, Pleva P, Scerbova J (2017). Sensitivity to enterocins of biogenic amine-producing faecal enterocci from ostriches and pheasants. Probiotics & Antimicro. Prot., 9:483-491. https://doi.org/10.1007/s12602-017-9272-z

Lin Y, Chen Y, Hsieh H, Chen M (2016). Effect of *Lactobacillus mali* APS1 and *L. kefiranofaciens* M1 on obesity and glucose homeostasis in diet-induced obese mice. J. Funct. Foods., 23:580-589. https://doi.org/10.1016/j.jff.2016.03.015

Liptakova D, Matejcekova Z, Valik L (2017). Lactic acid bacteria and fermentation of cereals and pseudocereals. Fermentation Processes., 10:223-254. https://doi.org/10.5772/65459

Liu W, Bao Q, Jirimutu, Qing M, Siriguleng, Chen X, Sun T, Li M, Zhang J, Yu J, Bilige M, Sun T, Zhang H (2012). Isolation and identification of lactic acid bacteria from Tarag in Eastern Inner Mongolia of China by 16S rRNA sequences and DGGE analysis. Microbiol. Res., 167:110-115. https://doi.org/10.1016/j.micres.2011.05.001

Livestock and Health Law (2014): Husbandry and Animal Health No 41. Indonesia, ID.

Morales G, Sierra P, Mancilla, Paredes A, Loyola LA, Gallardo O, Borquez J (2003). Secondary metabolites from four medicinal plants from Nurthern Chile, antimicrobial activity, and biotoxicity against Artemia salina. J. Chile. Chem., 48:44-49. https://doi.org/10.4067/S0717-97072003000200002

Park SI, Chang BS, Yoe SM (2014). Detection of antimicrobial substances from larvae of the black soldier fly, *Hermetia illucens* (Diptera: Stratiomyidae). Entomol. Res., 44:58-64. https://doi.org/10.1111/1748-5967.12050

Putri I, Jannah SN, Purwantisari S (2020). Isolation and characterization of lactic acid bacteria *Apis mellifera* and their potential as antibacterial using in vitro test against growth of *Listeria monocytogenes* and *Escherichia coli*. J. Trop. Biol., 3:26-34. https://doi.org/10.14710/niche.3.1.26-34

Reis JA, Paula AT, Casarotti S, Penna ALB (2012): Lactic acid bacteria antimicrobial compounds: characteristics and applications. New York, US: Springer. https://doi.org/10.1007/s12393-012-9051-2

Repka LM, Chekan JR, Nair SK, van der Donk WA (2017). Mechanistic understanding of lanthipeptide biosynthetic enzymes. Chem. Rev., 117:5457-5520. https://doi.org/10.1021/acs.chemrev.6b00591

Saiz NV, Belguesmia Y, Raspoet R, Auclair E, Gancel F, Kempf I, Drider D (2019). Benefits and inputs from lactic acid bacteria and their bacteriocins as alternatives to antibiotic growth promoters during food-animal production. Front. Microbiol., 10:1-17. https://doi.org/10.3389/fmicb.2019.00057

Shehta A, Omran H, Kiroloss F, Azmi M (2019). Effect of probiotic on growth performance and frequency of diarrhea in neonatal buffalo calves. Adv. Anim. Vet., 7: 876-881. https://dx.doi.org/10.17582/journal.aavs/2019/7.10.876.881

Sinnott ML (1990). Catalytic mechanisms of enzymatic glycosyl transfer. Chem. Rev., 90: 1171-1202

Sturme MHJ, Kleerebezem M, Nakayam J, Antoon DL, Akkermans, Vaughan EE, de Vos WM (2002). Cell to cell communication by autoinducing peptides in gram positive bacteria. Antonie Van leeuwenhoek., 81:233-243. https://doi.org/10.1023/A:1020522919555

Syahrizal, Ediwarman, Safratilofa, Ridwan M (2022). Analysis of the use of media resulting from bioconversion of organic waste in the production of maggots BSF (black soldier fly). JAI., 21:1-10.

Thakkar P, Modi HA, Prajapati JB (2015). Isolation, characterization, and safety assessment of lactic acid bacterial isolates from fermented food products. Int. J. Curr. Microbiol. App. Sci., 4:713-725

Tripathi N, Sapra A (2021): Gram staining Available at: https://pubmed.ncbi.nlm.nih.gov/32965827/. (accessed Jan 2022).

Wang Y, Wu J, Lv M, Shao Z, Hungwe M, Wang J, Bai X, Xie J, Wang Y, Geng W (2021). Metabolism characteristics of lactic acid bacteria and the expanding applications in food industry. Front Bioeng. Biotechnol., 9:1-9.

Yang SC, Lin CH, Sung C, Fang JY (2014). Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. Front Microbiol., 5:241. https://doi.org/10.3389/fmicb.2014.00241

Zhang M, Liu B, Zhang Y, Wei H, Lei Y, Zhao L (2007). Structural shifts of mucosa-associated *Lactobacili* and *Clostridium leptum* subgroup in patients with ulcerative colitis. J. Clin. Microbiol., 45:496-500. https://doi.org/10.1128/JCM.01720-06