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Identification and Antimicrobial Activity of Lactic Acid Bacteria Isolate from Fermented Cassava *(Growol)* to *Bacillus cereus* and *Morganella morganii*

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Abstract:- Most of the gastrointestinal infections (GI) are dominated by pathogenic bacteria, such as Bacillus cereus and Morganella morganii, which are spread through lack of hygiene in food. Increasing the intestinal microflora could prevent gastrointestinal infections. Intestinal microflora can be increased by consuming foods containing bacteria which have the potency to inhibit the growth of pathogenic microbes in digestive tract such as Lactic Acid Bacteria (LAB). LAB is usually found in fermented foods and growol, Indonesian local spontaneous fermented cassavas, is one of them. This study aims to identify LAB species in growol using molecular techniques and its antimicrobial activity. Identification of LAB isolates and the antimicrobial activity were done using 16S rRNA Gene Sequencing and Well Diffusion Method using Bacillus cereus and Morganella morganii as strain indicator. Results showed 5 LAB isolates can be isolated and had the characteristics of Gram positive, non-motile, and catalase negative. The 16S rRNA Gene Sequencing identified 4 Enterococcus faecium, and 1 Enterococcus durans. Identification of 5 isolates that been sequenced can be seen on accession number (MH793509 – MH793513). Antimicrobial activity of the Cell-Free Supernatants from 5 isolates inhibits Bacillus cereus (d = 2,48 mm) and Morganella morganii (d = 2,68 mm) and can be categorized as weak (0-3 mm).

Keywords:- *Lactic Acid Bacteria (LAB); Growol; Cell-Free Supernatants (CFS); Enterococcus; Bacillus cereus; Morganella morganii.*

I. INTRODUCTION

Gastrointestinal infections (GI) are a condition of diarrhea or vomiting with a total of 2 times or more in 24 hours period. Gastrointestinal infections symptoms include diarrhea, abdominal pain, nausea, and vomiting. Of 121 GI outbreaks cases, 51% were caused by bacteria and 45 % were transmitted through food (Lee and Greig, 2010; Ekkert, 2015). The spread pathogenic bacteria infection was mediated from food (Doménech, 2006).

Indigenous microflora that exists in human gastrointestinal tract such as Bacillus cereus and Morganella morganii can potentially causes GI and gastrointestinal diseases such as diarrhea (Müller, 1986; Doménech, 2006). Treatment by antibiotics, probiotics, prebiotics, or synbiotics can be used to prevent GI (Riddle et al., 2016). The balance of intestinal microflora is very important because the bacteria that live in the gastrointestinal tract play an important role in human systemic conditions, one of them is immunity system (Macpherson and Harris, 2004). Probiotic bacteria and bacteria can be used to maintain the balance of intestinal microflora. Lactic Acid Bacteria (LAB) are common bacteria that can be used to modulate the microflora balance. LAB produces antimicrobial compounds such as organic acids (lactic acid), H_2O_2, CO_2, diacetyl, D-amino acid and bacteriocin, that can inhibit pathogenic microbes (Piard and Desmazeaud, 1991; Cintas et al., 2001).

Lactic Acid Bacteria can be isolated from various sources. Traditional fermented foods are known to be one of the richest sources of LAB. Growol is one of the spontaneous fermented foods but unwell known to the public. Growol is a naturally fermented cassava (Manihot esculenta Crantz.) from Wates, Kulonprogo, Yogyakarta (Paramithiotis, 2017). Previous study has been successfully isolating LAB from Growol (Purwijantiningsih et al., 2017). Studies on the isolation of LAB from fermented foods have been conducted. Nevertheless, the isolation of antimicrobial producing LAB from Growol is still limited. Different isolation sources can isolate different LAB species and characteristics, moreover the antimicrobial activity. Therefore, the aim of this study is to isolate LAB from Growol and identify its antimicrobial activity against gastrointestinal infections (GI) causing bacteria, Bacillus cereus and Morganella morganii.

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II. MATERIALS AND METHODS

A. Microorganisms

The LAB used in the study was obtained form previous study (Purwijantiningsih et al., 2017) at the Laboratory of Teknobio-Pangan, Faculty of Biotechnology, Universitas Atma Jaya Yogyakarta, Indonesia. The LAB were cultured using De Mann Rogosa Sharpe (MRS) broth and agar (Merck, Germany). Meanwhile, the pathogen strains for the antimicrobial assay, *Bacillus Cereus* and *Morganella Morganii* were obtained from Food and Nutrition Culture Collection (FNCC), Center for Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia. The pathogen strains were cultured using Mueller-Hinton agar (Merck, Germany).

B. Lactic Acid Bacteria Identification

Five LAB isolates from *growol* (G1 – G5) were obtained from previous study (Purwijantiningsih et al., 2017). LAB isolates subculture in MRS agar medium with 0,5% $CaCO_3$ supplementation and stored in MRS broth (4 °C). LAB isolates were characterized by motility test, catalase test, and gram staining test (Aygan and Arikan, 2007; Nur et al., 2017).

Gene 16S rRNA from LAB isolates amplified by colony PCR method with primers LABF (5'-AGAGTTTGATYDTGGCTCAG-3') and LABR (5'-GGTTACCTTGTTACGACTT- 3'). Standard PCR protocol with a reaction volume of 50 µL consist of 25 µL MyTaqTM HS Red Mix 2x, 10 µM LABF and 0,5 µL LABR primer, a single colony LAB and 50 µL ddH2O. PCR begins with pre-denaturation for 60 seconds at 95 °C, followed by 35 cycles of three steps consisting of denaturation stage for 15 seconds at 95°C, annealing stage for 15 seconds at 55°C, and ending with final stage extension for 300 seconds at 72°C (Nanasombat et al., 2012 with modification). The PCR results were visualized using 1,5% agarose (EtBr as DNA stain) for 25 minutes at 100 V. The amplified sequence was used to identify the phylogenetic tree.

C. Determination of Antimicrobial Assay

The antimicrobial activity was determined using the well diffusion method. Antimicrobial activity of the LAB Cell-Free Supernatants (CFS) was tested using Mueller-Hinton agar (0,7% agar) (MHA). Pathogenic bacteria, *B. cereus* and *M. Morganii*, were used as strain indicators. The pathogen bacteria were inoculated using pour plate method (Papamanoli et al., 2003; Sujaya et al., 2008; Noordiana et al., 2013; Rahayu et al., 2015). The diameter of the clear zone formed was measured. The classification of antimicrobial activity was assessed according to Pan et al. (2009) i.e., diameter of 5 mm is equivalent to diameter of well, means no inhibiting ability, diameter 0 – 3 mm is equivalent to weak, diameter 3 – 6 mm is equivalent to medium, and diameter more than 6 mm is equivalent to strong.

D. Data Analysis

The experiment was performed using a completely randomized design, with 5 replications. Data was analyzed by ANOVA followed by Duncan's Multiple Range Test using SPSS ver. 15 with 95% confidence interval. The identification of the sequences was done using the Basic Local Alignment Search Tool (BLAST). Phylogenetic tree is created using MEGA ver. 6 with Neighbor Joining Tree method.

III. RESULTS AND DISCUSSION

➢ *LAB Identification*

The five LAB isolates (G1, G2, G3, G4, and G5) tested from *growol* have characteristics Gram positive, non-motile, and catalase negative. Characteristics of LAB are in accordance with Vos et al. (2009). Furthermore, the gene 16S rRNA from isolates was amplified using colony PCR and molecular analysis. Fig. 1 showed the DNA amplification of the five LAB isolates in the range of 1500 bp. Similar results were found by Nanasombat et al. (2012). The generated amplicon can be used for the next analysis phase.

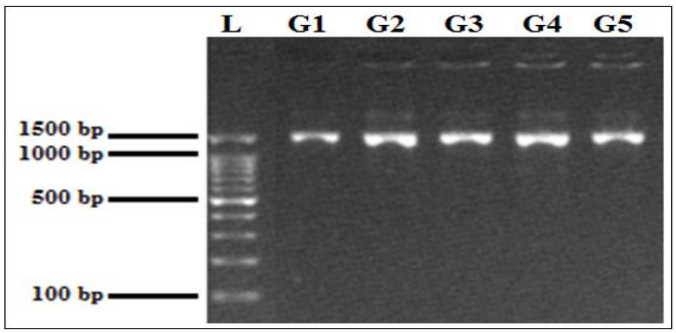

Fig 1 DNA Visualization of LAB Isolated from *Growol* (G1-G5) and Smobio 100 Bp DNA Ladder (L)

The 16S rRNA gene was chosen because it has parts that are conserve (not mutated) or variable parts (different from each species), so the amplification results have a unique part for each species (Rinanda, 2011). The sequences of 16S rRNA gene have been widely used for bacterial phylogeny and taxonomy studies. It contains common housekeeping gene markers (Janda and Abbott, 2007). The DNA sequence that has been processed will be used for LAB identification. The identification of LAB isolates from *growol* was shown in Table 1.

Table 1 Identification of Lab Isolates from Growol

Strain	Homology	Reference species	Accession number
G1	98%	Enterococcusfaeciumsf69	KM978216.1
G2	99%	Enterococcus durans 075	JN560931.1
G3	98%	Enterococcus faecium CAU:242	MF369891.1
G4	98%	Enterococcus faecium sf69	KM978216.1
G5	100%	Enterococcus faecium ZDHHM1	KC222512.1

Four isolates were identified as Enterococcus faecium and one isolate as Enterococcus durans. The homology of 16S rRNA gene (if aligned) by >97%, indicates two sequences are still in the same species. The 16S rRNA gene has about 3% variable parts between species (Stackebrandt and Goebel, 1994). The DNA sequence of each LAB isolated from growol (G1-G5) was uploaded to Genebank and named "strain BALG1-BALG5" which can be accessed through accession number MH793509-MH793513.

Genus Enterococcus is a genus which is commonly found in the gastrointestinal tract as well as plant-based fermented foods (Gomes et al., 2010), rice (Yonzan and Tamang, 2010), and corn (Abriouel et al., 2006). Enterococcus faecium and E. durans can be used as a food starter culture since they hold the role of flavor formation. Some strains can be potential probiotics, inhibit pathogenic bacteria and food spoilage bacteria (Gomes et al., 2010; Franz et al., 2011).

The result of bacterial sequences can be used to build phylogenetic trees. Phylogenetic trees are not only used to known kinship but also the basis and branching of the evolution of organism (Woese, 2000). Fig. 2 demonstrated the phylogenetic tree from each LAB isolate. Bacillus cereus was chosen as the outgroup of the phylogenetic tree because its kinship with LAB was not close; therefore, suitable as a comparison. Kinship and similarity can be seen through branches formed by phylogenetic trees. The LAB isolates from growol (G1 – G5) were still in 1 clade with E. faecium and E. durans. Both LAB still had similarity in near 16S rRNA gene (Franz et al., 2011) and not closely related to comparison species (L. plantarum and L. mesenteroides).

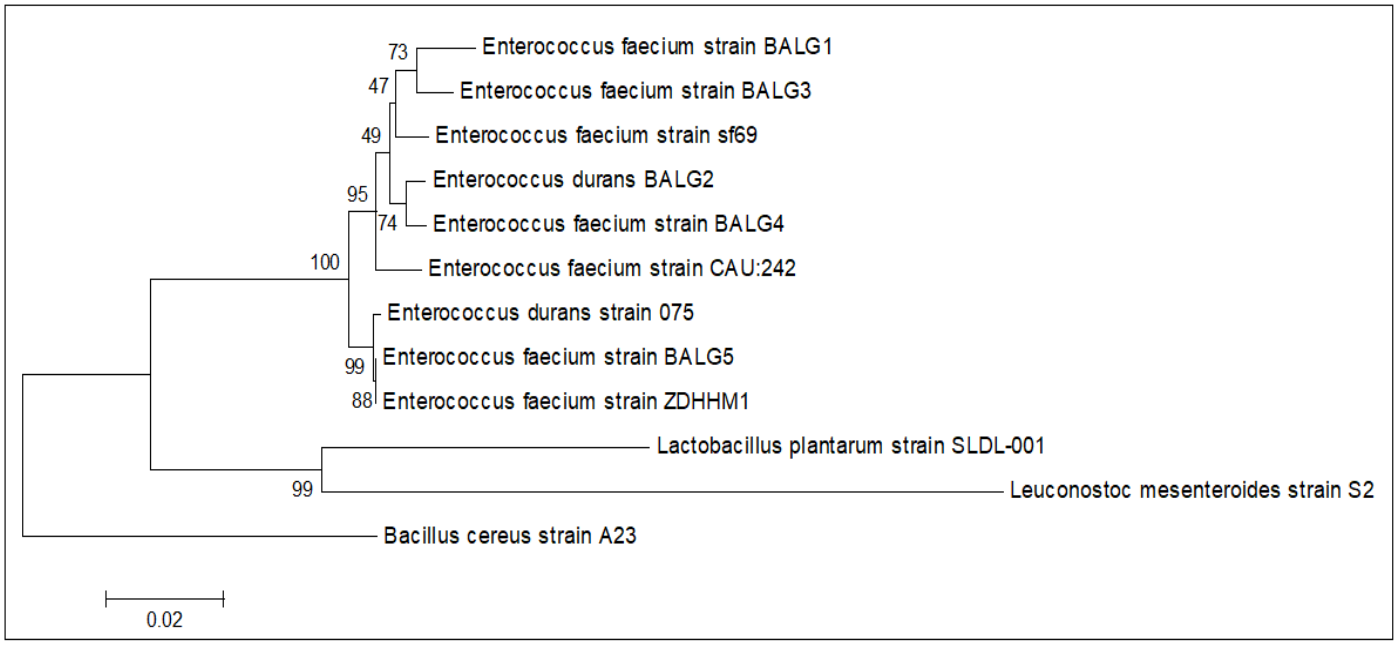

Fig 2 Phylogenetic tree of E. Facieum and E. Durans from Growol with other LAB Species and Bacillus Cereus as Outgroup.

➢ *Antimicrobial Properties of LAB*

Antimicrobial compounds produced by LAB from *growol* have been tested and can inhibit the growth of several pathogenic bacteria such as *Salmonella typhi, Shigella dysenteriae, E. coli, S. aureus, S. typhimurium, B. cereus*, and *M. morganii* (Rahayu et al., 2015; Djaafar et al., 1996; and Rahayu et al., 1996). Fig. 3 showed the antimicrobial activity from CFS. Clear zone formed around the well indicated the inhibition properties of the CFS (blue arrow). The clear zone formed indicated the production of antimicrobial compounds and inhibited the growth of B. cereus and M. morganii. Table 2 shows the diameter of the clear zone formed during incubation. Organic acid such as lactic acid is one of the antimicrobial compounds that are produced by LAB. The production of lactic acid resulting in a pH drop. The pH of the CFS can be seen in Fig. 4.

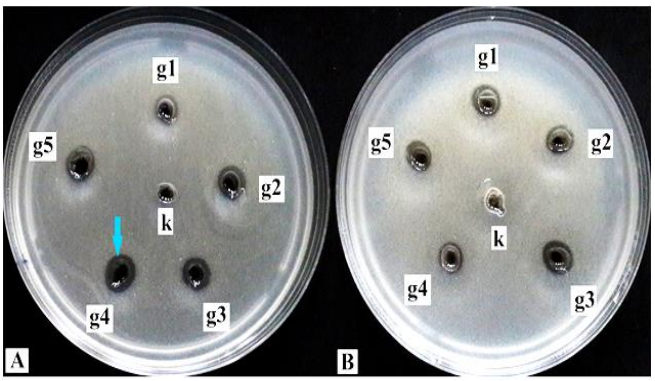

Fig 3 Antimicrobial activity of LAB isolates from growol againts B. cereus (A) and M. morganii (B)

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Table 2 Diameter of Inhibition Zone of LAB Isolates from Growol againts B. Cereus and M. Morganii

Strain	Strain Indicator	
	B. Cereus	M. morganii
Enterococcus faecium G1	2,8[a]	2,6[a]
Enterococcus durans G2	2,6[a]	2,2[a]
Enterococcus faecium G3	2,6[a]	2,4[a]
Enterococcus faecium G4	2,6[a]	2,2[a]
Enterococcus faecium G5	2,8[a]	3,0[a]

- Values are expressed as mean±SD (n=5). Values of each strain with different superscripts are significantly different ($p < 0.05$) by Duncan's multiple range test.

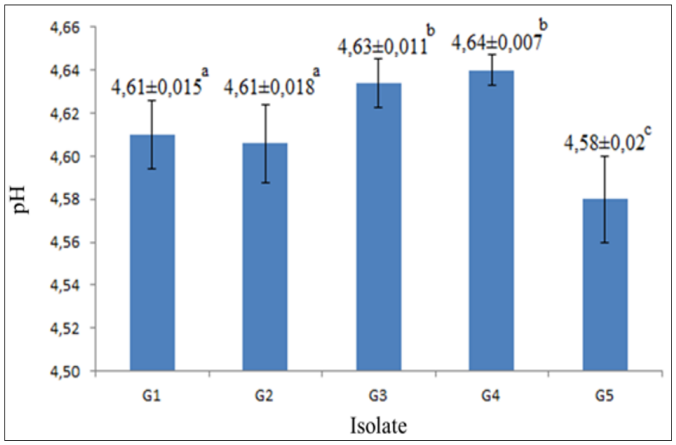

Fig 4 Ph of the CFS from LAB Isolates from Growol

Antimicrobial activity of all LAB isolates from growol (Table 2) are classified as weak (0-3 mm) (Pan et al., 2009) due to CFS' pH value (Fig. 4) produced by each LAB isolate from *growol* (4,58-4,64) are still in the growth range of *B. cereus* (pH 4,5-9,3) (Doyle, 1989). The acidity of CFS still higher than the maximum limit of acid tolerance of *M. morganii* (pH 3,5) (Zarei et al., 2013) although it is lower than the optimum growth range in the medium (5,8-6,8) (Chen et al., 1989). The acidity is not strong enough to inhibit the growth of indicator microbes. There may be other antibacterial substances that work to inhibit the growth, such as bacteriocin or other organic acids (Cintas et al., 2001).

Bacteriocin can inhibit cell performance by forming pores on cell membranes, which have an impact in transmembrane potential changes and or pH gradients resulting in leakage and death in target cells (Abee et al., 1995; Güllüce et al, 2013). Bacteriocin interacts easily with peptidoglycan wall of Gram-positive bacteria compared to peptidoglycan wall of Gram-negative bacteria, because gram-negative bacteria have an outer membrane consisting of phospholipid, protein, and lipopolysaccharide which makes it harder to penetrate and requires compounds that can disrupt the permeability of the outer membrane (van der Wal et al., 1995; Miller, 2016). Lactic acid is an organic acid that has the ability to interfere and increase the outer membrane permeability of Gram-negative bacteria (Alakomi, 2007). The presence of lactic acid and bacteriocin resulting in the ability of bacteriocins to inhibit bacteria and have inhibitory activity that was not significantly different between inhibition of Gram-positive bacteria as well as Gram-negative bacteria.

IV. CONCLUSION

Isolates of LAB which identified in *growol* belong to genus Enterococcus (4 isolates *E. facieum* and 1 isolate *E. durans*). LAB isolates from *growol* can inhibit *B. cereus* and *M. morganii* showed by the inhibition zone formed even though it is classified as weak. Further study on the specific antimicrobial substances and their mechanisms might be needed. Furthermore, other indicator strains might also be studied since the antimicrobial properties differ among strains.

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