JProbiotic activity of lactic acid bacteria isolated from several commercial fermentation product in Medan, North Sumatera

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Abstract. Lactic Acid Bacteria (BAL) are bacteria that have been used to produce food, which is in the manufacture of fermented milk. In addition, LAB can also be used as a probiotic agent. This study was conducted to look at the resistance of antibiotics, acids and bile salts as a criterion as probiotics. Samples were taken from 6 commercial fermentation products containing BAL. The isolation results obtained all samples had a number of BAL $10^6$ colonies so that according to the probiotic criteria with the highest number of colonies were Y samples. Test results on 6 types of antibiotics (gentamicin, amoxicillin, ofloxacin, erythromycin, cefotaxime, oxacillin) U samples were resistant to all antibiotics and then product Y. In the test of acid and bile salts, only U samples were taken as the most resistant to antibiotic samples. The test results found that U samples were resistant to acid and bile salts with 100% viability.

Keywords: lactic acid bacteria; probiotics; antibiotics; fermented products.

1. Introduction

Lactic acid bacteria (LAB) is the type of beneficial bacterium that had been used more than thousands of years in producing food fermentation and alcohol. In fermenting lactic, LAB also producing some organic compound, diacetyl, hydrogen peroxide, and bacteriocin¹.

Along with the variation of the fermentation food product, then it would be more variations of lactic acid bacteria. The safest lactic acid bacteria and most widely used in fermentation dairy products are the genera of Lactobacillus. Lactobacillus believed to be probiotics with the ability to inhibit the growth of pathogenic bacteria that benefits for digestive system².

The LAB is the cocci or rods form bacteria³, gram-positive bacteria which nonspores forming, catalase negative, anaerobic facultative and able to survive in the acid. The majority of lactic acid bacteria have generally recognized as safe (GRAS)⁴, qualified presumptions of safety (QPS), status provided by US Food and Drug Administration (FDA) and European Food Safety Authority (EFSA)⁵. LAB have benefit in maintaining the health of our bodies.⁶ There is 12 genera of the LAB, that is Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Weissella, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus dan Vagococcus⁶.

LAB could maintain the balance of microorganisms in the gastrointestinal tract⁸. As probiotics, the LAB must not pathogenic, can survive in the bile salts and acid condition, can colonize in gastroduodenal, capable to inhibit the pathogenic bacterium and capable to produce in large quantities⁹.
LAB can inhibit the growth of pathogenic bacteria because LAB produce bacteriocins. Bacteriocin is a compound of peptide bioactive produced by the ribosome. Bacteriocins active in inhibiting the development of both gram-positive and gram-negative bacteria with intrusive stability of the outer membrane of bacteria.\textsuperscript{16}

LAB is needed to produce some dairy product as a starter for the fermentation process. There are three processes of making fermented milk including isolation, selection and confirmation. Every strain of LAB will produce different dairy products. LAB can change the milk so have a good taste and texture by breaking down milk proteins with proteolytic activity\textsuperscript{11}.

Yogurt is frequently contained some LAB and have a lot of beneficial effect for human, prevention of allergies and can improve gastrointestinal disorder. LAB can be isolated in yogurt easily. However, the isolated of BAL must be a culture with an adequate selective medium so can make their characteristics quite different from LAB in milk product\textsuperscript{12}.

The aim of the study is there are a lot of fermented milk products that are claimed to contain LAB which has good probiotic properties. Therefore it is necessary to test the ability of probiotics in commercial fermentation products that exist in the community in antibiotic sensitivity test, acid and the bile salt tolerance.

2. Material

The material used in this study were LAB isolated from 6 commercially fermented products containing LAB which was purchased in one of the shopping centers in Medan shown in table 1, plastic wrap, de Man Rogosa and Sharpe Agar (MRSA), de Man Rogosa and Sharpe Broth (MRSB), Nutrients Agar, aluminum foil, cotton, tissue, label paper, aquadest and alcohol 70%.

| Sample | Preparation          | LAB                                      |
|--------|----------------------|------------------------------------------|
| C      | Yogurt drink         | \textit{Lactobacillus delbrueckii subsp bulgaricus} & \textit{Streptococcus thermophilus} |
| K      | Yogurt drink         | \textit{Lactobacillus bulgaricus}, \textit{Streptococcus thermophilus} |
| Byd    | Yogurt drink         | \textit{Streptococcus thermophilus}, \textit{Lactobacillus bulgaricus}, \textit{Lactobacillus acidophilus}, \textit{Bifidobacterium lactis} |
| Y      | Fermented skim milk  | \textit{Lactobacillus casei}              |
| Lb     | Powder sachet        | \textit{Lactobacillus acidophilus}, \textit{Lactobacillus casei}, \textit{Lactobacillus salivarius}, \textit{Bifidobacterium infantis}, \textit{Bifidobacterium lactis}, \textit{Bifidobacterium longum}, \textit{Lactococcus lactis} |
| U      | Powder sachet        | \textit{Lactobacillus rhamnosus LGG}, \textit{Bifidobacterium animalis BB-12} |

The tools used in this study are test tubes, Erlenmeyer flask, Bunsen, analytic balance, petri dish, measuring cup, stirring spoon, drip pipette, ose needle, magnetic stirrer, beaker glass, microtube, paper disc, autoclave, incubator, caliper and antibiotic discs (gentamycin, amoxicillin, ofloxacin, erythromycin, cefotaxime, oxacillin).
3. Method
3.1. Isolation and Enumeration
1 mL of fermented milk sample was put into a test tube containing 9 mL of MRS Broth. Incubated in anaerobic conditions at 37°C for 24 hours. Then lactic acid bacteria that have been grown are carried out for serial dilution \((10^{-1} - 10^{-7})\). 0.1 mL sample from test tube put into a microtube containing 0.9 mL MRS Broth and then homogenized \((10^{-1})\). Then take 0.1 sample from 1st microtube and put into other microtube containing 0.9 mL MRS broth and then homogenized \((10^{-2})\) make it until 7 times and each dilution incubated in a petri dish containing MRS agar for 48 hours at 37°C.3.2. Antibiotic Sensitivity
The incubated bacteria were then enriched and incubated for 24 hours at 37°C in the incubator. The bacteria tested for antibiotic resistance by Kirby-Bauer method. The enriched LAB is spread on the surface of the MRS using a sterileoose needle. The test is conducted using 6 types of antibiotics including Erythromycin 5 µg (E5), Gentamicin 10 µg (CN10), Oxacillin 5 µg (OX5), Ofloxacin 5 µg (OFX5), Amoxycillin 25 µg (AML25) and Cefotaxime 30 µg (CTX30), then incubated for 48 hours at 37°C in the incubator. Result interpretation as 3 category, sensitive (diameter of inhibition zone is ≥ 21 mm), intermediates (diameter of inhibition zone is 16-20 mm) and resistance (diameter of inhibition zone is 15 mm).3.3. Acid Tolerance
The LAB with the highest antibiotic resistance is used for acid tolerance test. 1 mL of enrichment that incubated for 24 hours were put into a test tube containing MRS Broth which had been dipped with 5M HCl to reach pH 3 and incubated for 4 hours. Every 1 hour the bacteria is inoculated in petri dish until the fourth hour incubation for 48 hours at 37°C. The percentage survival rate was calculated.
3.4. Bile Salt Tolerance
The sample used for bile salt tolerance test was LAB with the highest antibiotic resistance. 1 mL of enrichment that incubated for 24 hours were put into a test tube containing MRS Broth which had been supplemented with 0.03 gram bile salt which acts as bile salt. The mixture was incubated for 4 hours. Every 1 hour the bacteria is inoculated in petri until the fourth hour at 37°C. The percentage survival rate was calculated.

4. Result and Discussion
4.1. LAB Isolation From Commercially Fermented Products
After incubation for 48 hours at 37°C, all products produce a round lactic acid bacteria colony with shiny white color. The number of colonies found in each product showed in table 2.

| Sample | Total Colony |
|--------|--------------|
| C      | 0.69 x 10^7  |
| K      | 1 x 10^7     |
| Byd    | 4.6 x 10^7   |
| Y      | 9.6 x 10^7   |
| Lb     | 3.5 x 10^7   |
| U      | 2.3 x 10^7   |

From table 2, it can be seen that the highest number of colonies is found in sample Y with the amount of 9.6 x 10^7 then samples of Byd and Lb each of 4.6 x 10^7 and 3.5 x 10^7. The same study was reported by Purwijantiningisih (2014), in testing 12 commercial fermentation products, 2 products did not contain a LAB. Long-term and improper environments storage can reduce the viability of lactic
acid bacteria contained in the product. The best storage of lactic acid bacteria is in cold conditions. Sykur et al (2015) reported LAB isolated from dadih to have 80 x 10^8 CFU/mL colonies. The minimum amount must be owned by fermentation product is 10^6 CFU/mL LAB and to get the benefits of the LAB must reach 10^8 to 10^9 by every day consuming.

![Figure 1. The growth of LAB in petri dish contains MRS agar.](image)

### 4.2. Antibiotic sensitivity

Antibiotic sensitivity test with the Kirby-Bauer method using antibiotic discs measuring 6.1 mm. Antibiotic resistance test results can be seen in table 3.

| Sample | E5  | CN10 | OX5  | OFX5 | AML25 | CTX30 |
|--------|-----|------|------|------|-------|-------|
| C      | 32.5| 20.9 | 27.3 | 24.5 | 8.4   | 6.1   |
| Byd    | 38.7| 21.2 | 39.8 | 15.8 | 14.2  | 6.1   |
| Y      | 7.2 | 6.1  | 7.2  | 6.1  | 7.2   | 6.1   |
| K      | 33.1| 22.5 | 16.1 | 35.9 | 6.3   | 6.1   |
| Lb     | 36.1| 11   | 15.2 | 25.2 | 9.8   | 6.1   |
| U      | 6.1 | 6.1  | 6.1  | 6.1  | 6.1   | 6.1   |

LAB in U sample has resistance to 6 antibiotics. Six sample have resistance to a cephalosporin (cefotaxime) and other samples are most sensitive to macrolide (erythromycin). The same results were reported by Emmawati (2015), LAB isolated from mandai showed resistance to cephalosporin group antibiotics and were still sensitive to the aminoglycoside group. Different results can be seen in the tests on quinolone groups (Ofloxacin), LAB isolated from mandai being able to have resistance to quinolone groups. Antibiotic resistance can be intrinsic and acquired. Intrinsic antibiotic resistance is nature, encoded in chromosome, and inherited when miosis and cannot be inherited to other species. There is some composition in the cell membrane that makes it resistance. Acquired resistance encode in plasmid and can be transferred to other species. LAB isolated from the commercial fermented product cannot be ascertained intrinsic or acquired antibiotic resistance. The different results obtained in studies reported by Fachrial (2018) of LAB isolated from palm sap resistance to aminoglycoside class of antibiotics. The LAB is uncontrollably formed and have a risk factor to spread of antibiotic resistance gen. Gad M (2014) showing LAB isolated from food products was still sensitive to gentamicin and erythromycin. Most LAB susceptibility to a beta-lactam antibiotic like ampicillin and amoxicillin. Lactobacillus strains are the highest prevalence of penicillin resistance (20.3%). Lactobacillus strains have the highest resistance to vancomycin (40.6%) and streptomycin (17.4%) but Lactococcus and Streptococcus strains are susceptible to vancomycin. Lactococci have
higher percentage resistance to erythromycin (22.2%). High resistance to tetracycline showed in Lactococcus (29.6%), followed by Streptococcus (12.3%) and Lactobacillus (11.6%). Many factors can affect the result such as the incubation time and temperature, size of inoculum, composite of the atmosphere and the growth medium. Elevated antibiotic minimum inhibitory concentration (MIC) for some species caused by the increase of inoculum size and extended incubation time.

Ramachandra et al (2017) reported the antibiotic resistance of LAB isolated from “dahi” traditional fermented milk product from North Bengaluru. The result showed that all strain of L. rhamnosus strain were susceptible against 7 class of antibiotics as with diameter of the inhibitory zone was more than 10 mm. The probiotics normally do not carry transferable antibiotic resistance gene because they are chromosomally encoded, but in certain foods, the LAB was carried the transferable antibiotic resistance genes, which may be related to chromosomal, transposon or plasmid located genes. Associated with certain specific antibiotics, lactobacillus is usually sensitive to antibiotics that target the cell wall including penicillin and β-lactamase, but more resistant to cephalosporin. Many Lactobacillus species show a high level of resistance of vancomycin. On the other hand, lactobacillus is generally susceptible to protein synthesis inhibitors even at low concentration including chloramphenicol, macrolides, lincosamide, and tetracycline.

S. thermophilus is susceptible to penicillin G and roxithromycin, whereas 23.5% and 64.7% But L. bulgaricus isolated were resistant. Moderate levels of resistance to ampicillin and chlorotetacycline were observed among Lb. Bulgaricus (29.4 dan 47.1%) and S. thermophilus (44.4% and 27.8%). Resistance to tetraacycline varied significantly between the 2 species, as 88.2% for Lb. Bulgaricus and 44.4% for S. thermophilus. High level resistance to chloramphenicol and lincomycin was observed for L.bulgaricus (88.2% and 94.1%) and for S.thermophilus (88.9% and 88.9%). Both L.bulgaricus and S.thermophilus strains tend to be strongly resistant to aminoglycosides (kanamycin, streptomycin, neomycin, and gentamycin). In fact, only 5.9% of L.bulgaricus isolates showed susceptibility to streptomycin, neomycin, or gentamycin and 33.3% and 11.1% of S.thermophilus isolated were susceptible to streptomycin and gentamycin, respectively.

LAB strain have acquired the generally regarded as safe status and are used as fermenting lactic starter culture in the food industry. LAB when used as probiotic and enter to our intestines in large number, may occur interaction with the natural intestinal microbiota and gen transfer. Lab can be prone to gene exchange to enhance survival in antibiotic-containing environments. LAB might be horizontally transferred their antibiotic resistant genes to other species. In the meantime, a series of measures inspired to a principle of precaution should be undertaken, the most important of which is to systematically test strains for the presence of transferable antibiotic resistance genes before they are used as commercial starters or probiotic culture in food product. Also, this measure should be complemented by a more prudent use of antibiotic in all fields, including agriculture, veterinary, and human medicine.

Lactobacillus has been reported to be intrinsically resistant to aminoglycosides, fluoroquinolones, and glycopeptides. Nevertheless, 30% of Lactobacillus isolates in this study were resistant to amikacin and gentamycin. However, none of them was resistant to high level of streptomycin and no more than 50% of the isolates were resistant to ciprofloxacin, not supporting the intrinsic resistance of Lactobacilli to these antibiotics. Enterococci are naturally resistant to all cephalosporins, but susceptible to vancomycin and erythromycin in the clinical environment. Vancomycin-resistant Enterococci are commonly associated with nosocomial infection in hospitals and the resistance of Enterococci to vancomycin-resistant is transferable that indicating Enterococcus is a controversial species that should not be used as probiotic because its potential pathogenicity and its notable resistance to some of the widely used antibiotics.
Figure 2. Antibiotic sensitivity test for lactic acid bacteria from commercial fermented product.
(a) Isolated sample Y (b) Isolated sample Byd (c) Isolated sample U (d) Isolated sample C (e) Isolated sample K (f) Isolated sample Lb

Table 4. Antibiotic susceptibility and resistance of LAB (modified from Moracanin, 2017).

| Strains of LAB     | Susceptibility                                                                 | Resistance                                                   |
|--------------------|-------------------------------------------------------------------------------|--------------------------------------------------------------|
| Lactobacillus lactis | Amikacin, ampicillin, 1st generation cephalosporine, chloramphenicol, erythromycin, gentamicin, penicillin, imipenem, oxacillin, sulfonamide, tetracycline, vancomycin | Colistin, fosfomycin, pipemidic acid, rifamycin              |
| Bifidobacterium    | Ampicillin, penicillin G, Bacitracin, cephalosporin, chloramphenicol, erythromycin, clindamycin, nitrofurantoin, tetracycline | Vancomycin, gentamycin, fusidic acid, streptomycin, polymyxin B, trimethoprim, aminoglycosides, colistin, metronidazole |
| Lactobacillus spp. | Chloramphenicol, streptomycin, gentamycin, penicillin G, tetracycline, erythromycin | Aminoglycosides, fluoroquinolones, glycopeptides, vancomycin |

4.3. Acid Tolerance of LAB Isolation from Commercially Fermented Products

The sample tested was sample U as the most antibiotic-resistant samples. Samples were tested with the acidity of pH 3. 8 samples were tested with the acid, only one sample has viability 100%. As a probiotic agent, the LAB must be able to pass through the stomach acid in order to colonize in colon\(^9\). Bawole (2018) LAB isolated from fermented red cabbage. The number of LAB colonies had lower growth ability than control colonies. LAB relative tolerance to low pH. As the probiotic, LAB must have to survive in gaster at pH 2.0-4.0, low pH condition can cause cell damage and decrease of LAB viability so the amount of LAB colonies will decrease too. LAB can survive in low pH condition because LAB has a mechanism to keep their cell in neutral pH\(^{21}\). Fachrial (2018) reported that LAB isolated from palm oil showed a viability of 72% of the resistance to acid. pH and variations of strain can be affected the viability of LAB in low pH condition. As the probiotic LAB must survive from stomach acid persist during the gastric emptying time almost 4 hour\(^8\). Study reported by Wang C (2014), LAB resistance in low pH condition are mainly focused on neutralization process, biofilm and cell density, proton pump, protection of macromolecules, pre-adaptation, cross-protection and effect of soluted\(^{22}\). Menconi (2014) report that Lactobacillus strains are very resistant to low pH, with a high viability rate at pH 3.0 for 1 hour. Bifidobacterium strains are high sensitive to pH 2.0 and pH 3.0.
LAB are acidophilic, that mean they are low pH tolerance, but they need to be differentiated from a low pH conditions because free acid may inhibited LAB growth\textsuperscript{23}.

\textbf{Figure 3.} The growth of LAB on MRS contains HCl 5M (pH 3.0).

\textbf{Figure 4.} Acid tolerance of lactic acid bacteria from commercial fermented product

4.4. Bile Salt of LAB Isolation from Commercially Fermented Product

Sample taken are a sample that has the strongest antibiotic resistance, LAB was isolated from sample U has the best tolerance to the bile salts viability of 100%. From the results of this study, LAB isolated from the U product have criteria as probiotics\textsuperscript{9}. Adawiyah (2015) reported the isolation of LAB from Dangke, also resistance to bile salt though does not show a significant figure\textsuperscript{24}. Fachrial E (2018) reported that LAB isolation from palm oil showed a viability of 70% of the resistance to bile salts. The result shows that LAB can produce Bile Salt Hydrolase (BHS) Enzyme\textsuperscript{8}.

\textbf{Figure 5.} The growth of LAB on MRS contains 0.03 gram bile salt.
Tolerance to bile salt is not related to specific mechanisms but rather to the complex regulation of gene expression. The protonated form of bile salt leads to dissociation of the lipid bilayer and integral protein of cell membrane, causing leakage to the cell wall and resulting cell death. Tolerance to bile salt is related to the activity of bile salt hydrolase (BSH) which hydrolizes conjugated bile salt to minimize the bactericidal effect of the LAB strain.

Figure 6. Bile salt tolerance of lactic acid bacteria from commercial fermented product.

5. Conclusion
The result showed that LAB from commercial fermentation products has the criteria as probiotics, LAB colonies exceed 10^6 CFU/mL, from the results of antibiotic tests, sample U has the best antibiotic resistance and second best is Y. Sample U have good tolerance with acid and bile salt and its viability reaches 100% of the 6 products, U products have a good probiotics criterion.

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