Probiotic Activity and Antibiotic Sensitivity of Lactic Acid Bacteria Isolated from Healthy Breastfed Newborn Baby Feces

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Abstract. Breast milk is a very nutritional food that helps with the production of good microbiota in the gut of an infant. Generally, two lactic acid bacteria (LAB), namely Lactobacillus and Bifidobacterium, are related to infant gut microbiota. LAB is often used as a probiotic agent, but some conditions required for every bacterium to be utilized for this purpose. This study aimed to investigate the acid and bile salt tolerance as a criterion of probiotic agent and antibiotic sensitivity of LAB isolated from the feces of a healthy breastfed newborn baby. The sample was obtained from the feces of the aforementioned subject (baby). The result showed that the total colony of LAB from the feces was 10.2 x 10^8 CFU/mL. In an acidic condition at pH 3, there was one isolate that had 100% viability in the first hour period, but all of them had 100% at bile salt tolerance. However, sensitivity test of the isolated LAB indicated resistance to 6 types of antibiotics namely Erythromycin 5 µg, Gentamicin 10 µg, Oxacillin 5 µg, Ofloxacin 5 µg, Amoxycillin 25 µg, and Cefotaxime 30 µg. The study showed that LAB isolated from healthy newborn baby feces have potency as a probiotic.

1. Introduction
Metabolite of carbohydrate fermentation is majorly produced by lactic acid which are a group of Lactic Acid Bacteria (LAB) gram-positive bacteria. They are cocc.i or rod shaped, non-sporing, but do not generate catalase [1]. During lactate fermentation, LAB produces many compounds such as diacetyl, organic acids, hydrogen peroxide, and bactericidal proteins [2].

There are several main genera of bacteria that are included in the LAB, including; Carnobacterium, Aerococcus, Enterococcus, Lactococcus, Lactobacillus, Leuconostoc, Weissella, Oenococcus, Pediococcus, Tetragnococcus, Streptococcus, and Vagococcus [3]. Furthermore, Lactobacillus has a beneficial effect on health when ingested by humans. Therefore, it is now widely isolated, and such strains are called probiotics [4], also, this type of bacteria usually grow (adhesion) and make colonies in the human intestine. Probiotics play a role in fighting against and preventing the colonization of pathogenic bacteria in the intestinal wall [5]. Some conditions required for bacteria to be a probiotic are safety, tolerant of acid and bile salt, capable of making colonies in the gastrointestinal tract, producing antimicrobial substances, and maintaining viability [6].

Many studies have shown that probiotics have a beneficial effect on health by preventing the growth of disease-causing bacteria, reducing the rate of colon cancer, increase the immune response, and decrease serum cholesterol [7]. Besides, they able to treat various diseases such as diarrhea, allergy, and hypertension [8].
In neonates, intestinal microbiota has the most important role in metabolism, nutrition, defense against pathogens and immune functions. *Bifidobacterium*, *Lactobacillus*, and *Enterococci* are anaerobic bacteria that are normally related to infant gut microbiota [7]. Breast milk is very nutritious and helps to produce good microbiota in an infant’s gut [9]. Exclusive breastfeeding of life for the first six months is highly recommended for newborns because it provides nutrients that are needed for healthy growth and development [9]. Oligosaccharides, carbohydrates, fatty acids, nucleotides, cytokines, immunoglobulins, immune cells, lactoferrin, lysozyme, and other immunomodulating are bioactive molecules found in breast milk [10]. All of these molecules fortify the breast milk with ability to develop infant immune systems and defense against gastrointestinal and respiratory infections, allergies and also reduces the risk of developing other complications, such as inflammatory bowel disease (IBD), diabetes, or obesity [11]. *Lactobacillus* and *Bifidobacterium* are intestinal microbiota that are transferred from mother-to-infant through breastfeeding [11].

Antibiotic is the opposite of probiotic [6], but currently in many countries, antimicrobial resistance has caused high morbidity and mortality [12]. Actually, genetic mutation in bacteria is the primary cause of the resistance which is increased when antibiotics are underused, overused, or misused [13]. Patients’ incompliance with recommended treatment and demand, irrational use of antibiotics, and drug advertisement are factors that also influence the resistance response [13]. Probiotics are chromosomally encoded to ensure absence of antibiotic resistance genes that can be transferred from them to another organism [6]. Furthermore, they are able to inhibit the growth of pathogenic bacteria and that effect is expected to reduce the use and resistance cases of antibiotics. Therefore, this study aimed to investigate the probiotic activity and antibiotic sensitivity of LAB isolated from the feces of healthy breastfed newborn baby.

2. Materials and Methods

2.1. Sample Collection
The sample was obtained from feces of a healthy breastfed newborn baby. It was collected at the healthy infant room of Royal Prima Hospital Medan. Afterwards, it was stored in a sterile sample tube and then carried to the Biomolecular Laboratory of Universitas Prima Indonesia Medan for immediate processing.

2.2. Isolation of LAB from Healthy Breastfed Newborn Baby Feces
LAB was isolated using specific growth media namely MRS broth and agar. 1 mL of feces sample was added to 9 mL sterile MRS broth in a test tube and incubated for 24 hours at 37°C in anaerobic condition. Afterwards, serial dilution process was performed (10⁻¹ to 10⁻⁷) by adding 0.1 mL of the LAB culture in 0.9 mL sterile MRS broth contained in a 1.5 mL microtube and then homogenized. This is the first, but the process was continued until the seventh one using the same method as initially described by taking 0.1 mL of the previous dilution as the portion to be used for the next addition. Following the seventh dilution, the product obtained was inoculated in a petri dish containing MRS agar, then incubated for 48 hours at 37°C for bacterial growth, and the total colony of LAB was counted afterwards [5].

2.3. Acid Tolerance
Please LAB isolate was enriched on MRS broth for 24 hours at 37°C. Then, 1 mL of LAB culture was added into a sterile test tube that contained 9 mL MRS broth as a control. In the same tube, few drops of 5M HCl were added to reach pH 3. Furthermore, the mixture was homogenized and incubated for 4 hours and at every hour it was diluted to 10⁻³, followed by inoculation of the MRS agar. Then, incubated for 48 hours at 37°C, while the survival rate of LAB colonies was calculated in percentage [6].
2.4. Bile Salt Tolerance

1 mL of LAB culture was added into a sterile test tube that contained 9 mL MRS broth as a control. 0.03 gram bile salt was added in the same tube, after which it was homogenized and incubated for 4 hours and was diluted to $10^{-4}$, at every hour. Then, inoculated on MRS agar and incubated for another 48 hours at 37ºC, and the survival rate of LAB colonies was calculated in percentage [6].

2.5. Antibiotic Sensitivity

According to Kirby-Bauer, the antibiotic sensitivity test of LAB was conducted by disc diffusion method. LAB isolate was enriched on MRS broth for 24 hours at 37ºC. Afterwards, it was swabbed on the entire MRS agar surface using a sterile inoculation needle, and then, the antibiotic disc was positioned on the media. There are 6 types of antibiotics used in this test, namely Erythromycin 5 µg (E$_5$), Gentamicin 10 µg (CN$_{10}$), Oxacillin 5 µg (OX$_5$), Ofloxacin 5 µg (OFX$_5$), Amoxycillin 25 µg (AML$_{25}$), and Cefotaxime 30 µg (CTX$_{30}$). The petri dish was incubated at 37ºC for 48 hours, while after 48 hours, the diameter of the antibiotic disc and inhibition zone were measured using a calliper. Interpretation of the result of this test is sensitive when the diameter of inhibition zone ≥ 21 mm, intermediate when the diameter of inhibition zone 16 – 20 mm, and resistant when the diameter of inhibition zone ≤ 15 mm [6].

3. Results and Discussion

3.1. LAB Isolation from Healthy Breastfed Newborn Baby Feces

After incubating for 48 hours at 37ºC under anaerobic condition, the total colony of LAB from healthy breastfed newborn baby feces was $10.2 \times 10^8$ CFU/mL. The colonies were milky or bright cream colored, and large in size, that is >1.5 mm. The growth of LAB isolate on the MRS agar surface is shown in figure 1.

![Figure 1. LAB growth from healthy breastfed newborn baby feces on MRS agar.](image)

The minimum number of colony that a probiotic should have is $10^6$ CFU/mL or gram, and to obtain its beneficial effect, $10^4$ to $10^9$ of these microorganisms must be consumed daily [6]. Park Y S et al carried out the same study, which reported that the total colony of LAB isolated from newborn
baby feces at Dongchimi was greater than $10^7$ CFU/mL [14]. Also, another study that was conducted by Medjaoui I et al, stated that the average number of LAB colonies isolated from breast milk and infant’s feces samples were $2.43 \times 10^{10}$ CFU/mL and $6.85 \times 10^{10}$ CFU/mL respectively [9].

3.2. Acid Tolerance of LAB Isolated from Healthy Breastfed Newborn Baby Feces

The growth of LAB isolated from healthy breastfed newborn baby feces on the MRS agar surface at pH 3 is shown in figure 2. Also, its tolerance test showed that only one isolate has 100% viability at pH 3 in the first hour period. The viability percentage of LAB from the feces in acidic conditions is shown in figure 3.

Figure 2. LAB growth on MRS agar in acidic conditions.

Figure 3. Viability percentage of Lab from healthy breastfed newborn baby feces at pH 3.
However, in order to assure viability, tolerance of acid conditions is one of the criteria that must be considered during the selection of potential probiotic isolates [15]. Same study by Park Y S et al also stated that LAB isolated from newborn baby feces at Dongchimi had the highest survival rate at pH 3 [14]. In addition, Adetoye A et al's study reported that LAB isolated from cattle feces were able to survive growth at pH 3 except four samples from *Lactobacillus* strains, namely *L.* *mucosae* C101, *L.* *ingluviei* C13, *L.* *ingluviei* C89, and *L.* *taiwanensis* C20 [16]. But, probiotic bacteria need to survive and stay in the stomach when in low pH conditions between 1.5 to 2.0 during the gastric emptying time of 4 hours or more [6].

3.3. Bile Salt Tolerance of LAB Isolated from Healthy Breastfed Newborn Baby Feces

Growth of the LAB on the MRS agar surface that contained 0.03 gram bile salt is shown in figure 4. All isolates from the feces have 100% viability of bile salt tolerance. Also, its viability percentage in the MRS agar is shown in figure 5.

![Figure 4. The growth of LAB on MRS agar that contained 0.03 gram bile salt.](image)

Probiotics must have tolerance for bile salts to ensure colonization and bacteria metabolic activity in the host's small intestine. This is to assist *Lactobacillus* spp. and *Lactococcus* spp. balance the intestinal microflora and reach the small intestine and colon [17]. Furthermore, Park Y S et al reported that LAB isolated from newborn baby feces at Dongchimi were able to survive at 1% high concentration of bile [14]. Adetoye A et al's report also stated that LAB isolated from cattle feces survived at 5% bile salt concentration, while six other samples failed to grow at 7% [16]. Dissociation of a lipid bilayer and integral protein of cell membranes occurred after bacterial exposure to bile salts, which caused content leakage and finally, cell death [18]. Detoxification of the bile salt by producing bile salt hydrolase (BSH) enzyme activity was an ability of the probiotic strains [6]. Therefore, bile salt tolerance is related to the activity of the BSH, which hydrolyzes conjugated bile, thus minimizing the strains’ bactericidal effect [19].
3.4. **Antibiotic Sensitivity**

The diameter of the antibiotic disc employed was 6.1 mm, and LAB was tested using 6 types of antibiotics (See Table 1). The LAB isolate was resistant with the diameter of inhibition zone ≤ 15 mm to all the antibiotics tested. The isolate’s antibiotic sensitivity test can be seen in figure 6.

| Isolate | The diameter of the inhibition zone (mm) |
|---------|-----------------------------------------|
|         | Erythromycin 5 µg (E<sub>5</sub>) | Gentamicin 10 µg (CN<sub>10</sub>) | Oxacillin 5 µg (OX<sub>5</sub>) | Ofloxacin 5 µg (OFX<sub>5</sub>) | Amoxycillin 25 µg (AML<sub>25</sub>) | Cefotaxime 30 µg (CTX<sub>30</sub>) |
| F1      | 6.1 | 0 | 0 | 0 | 0 | 0 |

Therefore, transferrable antibiotic resistance determinants need not be possessed by potential probiotic strains [16]. From Adetoye A et al's study, 88 LAB samples isolated from cattle feces were resistant to Clindamycin, Vancomycin, Gentamicin, and Kanamycin but sensitive to Chloramphenicol, Ampicillin, Amoxicillin-Clavulanic acid, Erythromycin, and Tetracycline [16].

Generally, *Lactobacilli* strains were susceptible to the antibiotics such as clindamycin, chloramphenicol, erythromycin, and tetracycline, which all have mechanism of action that inhibits protein synthesis. But, the strains were more resistant to aminoglycosides such as streptomycin, neomycin, kanamycin, and gentamicin. *Bifidobacteria* strains were resistant to some nalidixic acid, namely metronidazole, polymixin B, vancomycin, gentamicin, kanamycin, streptomycin, colistin, trimethoprin, and fusidic acid but susceptible to gram-positive spectrum (macrolides, novobiocin, teicoplanin, bacitracin, erythromycin, vancomycin, and lincomycin), broad-spectrum (rifampicin, chloramphenicol, and spectinomycin) and Beta-lactams antibiotics (piperacillin, amoxicillin, ampicillin, penicillin). Fundamentally, *Pediococci* strains had resistance to streptomycin, kanamycin, tetracycline, sulphamethoxazole, ciprofloxacin, doxycycline, trimethoprin-sulphamethoxazole, and glycopeptides antibiotics such as vancomycin and teicoplanin but susceptible to penicillin G,
imipenem, netilmicin, clindamycin, gentamicin, chloramphenicol, daptomycin, rifampicin, and ramoplanin. Also, Leuconostoc species were mostly susceptible to rifampicin, erythromycin, clindamycin, tetracycline, and chloramphenicol. Furthermore, these strains had resistance to glycopeptides antibiotics such as vancomycin, cefoxitin, and metronidazole and partially to gentamicin, kanamycin, streptomycin, nitrofurantoin, nalidixic acid, trimethopine, and sulphadiazine. Lactococcus lactis strains were susceptible to 1st generations of cephalosporin, erythromycin, chloramphenicol, amikacin, ampicillin, gentamicin, oxacillin, imipenem, vancomycin, penicillin, trimethoprim, and sulfamethoxazole. They also showed resistance to chloramphenicol, streptomycin, erythromycin, tetracycline, and clindamycin [20].

Enterococci indicated resistance to cephalosporins and low levels of aminoglycoside and clindamycin. Naturally, Lactobacilli, Pediococci, and Leuconostoc spp. all had high resistance to vancomycin and some of the Lactobacilli also had high resistance to bacitracin, cefoxitin, fusidic acid, ciprofloxacin, gentamicin, kanamycin, nitrofurantoin, metronidazole, norfloxacin, sulphadiazine, streptomycin, trimethoprim/sulphamethoxazole, teicoplanin, and vancomycin. In addition, Lactobacilli had a very high frequency of spontaneous mutation to nitrofurazone, kanamycin, and streptomycin [21].

Normally, probiotics are chromosomally encoded in order to prevent transferable antibiotic resistance genes [7]. Nevertheless, some reports showed that probiotics used in certain foods carry the transferable antibiotic resistance genes. Chromosomal, transposon, or plasmid located genes are possibly related to the occurrence of this resistance. The LAB has shown intermediate resistance, and susceptibility to various antibiotics. Therefore, international organizations such as European Food Safety Authority (EFSA) recommended microbiological breakpoint for the antibiotic to regulate the use of probiotic bacteria in food usage. In fact, after antibiotic treatment, the microbiota in infant’s gut are restorable by some probiotic strains with antibiotic resistance [22].

4. Conclusion
In conclusion, LAB isolated from healthy breastfed newborn baby feces have potential as a probiotic and the total number of their colony exceeded 10^6 CFU/mL. Also, they have good tolerance to acid and bile salt, which the viability reached 100%. And from the results of the antibiotic sensitivity test,
LAB were resistant (the diameter of inhibition zone ≤ 15 mm) to 6 types of antibiotics which include 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) tested.

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