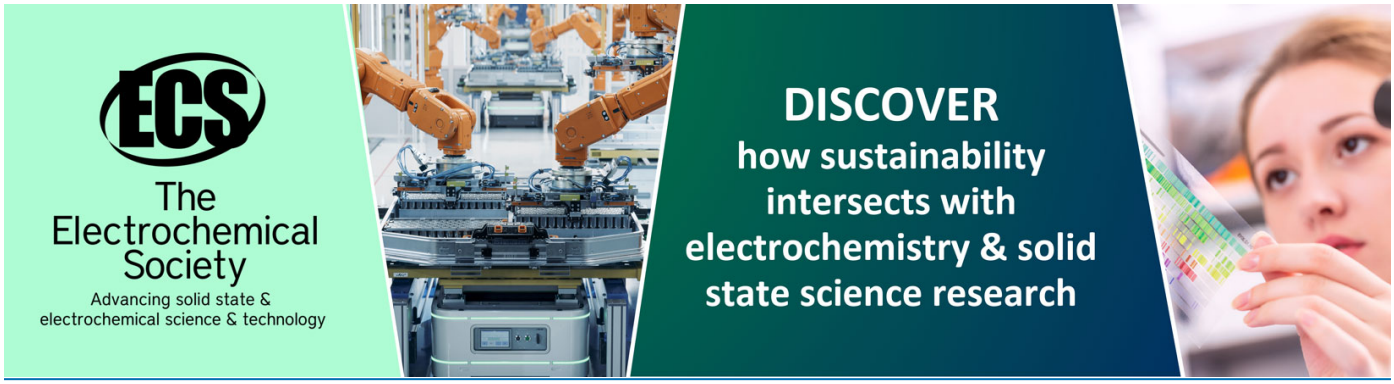
# Isolation, Characterization, Molecular Identification of Probiotic Bacteria from Meconium





# Isolation, Characterization, Molecular Identification of Probiotic Bacteria from Meconium

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**Abstract** Probiotic bacteria are a group of bacteria that have positive effects such as increasing the absorption ability of some nutrients, maintaining the intestinal pH so that it can be protected from pathogenic microorganisms, maintaining disturbances in water absorption, smoothing digestion by producing several digestive enzymes. Each bacterial species has different probiotic effects so selection and identification are needed to get a good strain of probiotics. The identification of probiotic bacteria in this study uses the molecular identification method with the 16S rRNA marker gene. 6 probiotic bacterial isolates (DA1, DA3, DA4, DA7, DA8, DA10) were identified as having the characteristics of a circular shape, the edge of the entire and flat elevation. negative. The antibacterial test results have a clear zone of 7.1 mm. The results of sequencing with the help of the Basic Local Alignment Search Tool (BLAST) program showed that probiotic bacteria from meconium had 99.78% similarity with Basillus coagulans NBRC.

## 1. Introduction

Infant Mortality Rate (IMR) is an indicator that determines the degree of health. Based on the results of the Indonesian Health Demographics and Survey (SKDI) (2012), it shows a significant decline from year to year. From 68 deaths per 1,000 live births in 1991, to 24 deaths per 1,000 live births in 2017.
The results of a study of 11,000 newborns (age <28 days) in the UK, 22% of babies could be saved if they received maximum colostrum. Babies who are given colostrum will receive greater protection from the threat of diarrhea infections and ARI than babies who do not receive colostrum (Pollard, 2015; Djaiman & Sihadi, 2015; Edmond et al., 2006).

The digestive tract is the most important organ that plays a role in the growth, development and health of children. The process of gastrointestinal maturation is stimulated by breast milk facilitated by colostrum. Colostrum, which is secreted by the breast glands on day 1 to day 4 after birth, contains bioactive components and beneficial microbiota which play a role in creating a balance of the neonatal gastrointestinal microbiota which affects the maturation and development of the gastrointestinal immune system of the newborn (Pediatrician Association) Indonesia, 2012).

Several studies have shown that breast milk, especially colostrum, is the largest source of probiotic bacteria for babies. Several bacterial genera that are included in the probiotic bacteria group include

Lactobacillus, Streptococcus and Bifidobacteria which are dominant in the feces of babies who are breastfed (Mcguire & Mcguire, 2015; Lee et al., 2015).

The more frequently the baby breastfeeds, the more bioactive components and microbiota of breast milk are transferred from mother to baby. Kent et al., (2005) found that the average frequency or frequency of breastfeeding babies to their mothers in 24 hours was 11 times with a range of 6-18 times. Rahmagiarti et al., (2013) reported on the gastrointestinal tract of infants who were breastfed when they were 4 days old, showing the presence of bifidobacterium strains and will increase at 7 days of age. Bifidobacterium will more stable dominate the intestinal environment of infants at the age of 1 month. The factors that influence the colonization of the microbiota in the gastrointestinal tract of neonates in newborns are the way the baby is born, the type of baby's nutritional intake (breast milk or formula), gestational age, mother's diet, hospitalization (environment) and use of antibiotics (Penders et al. ., 2005). Babies are born in 2 ways, namely vaginal birth and birth by cesarean section, namely through an incision in the abdominal wall (uterus) and uterine wall (Cunningham et al., 2012). There are differences in the composition of the gastrointestinal microbiota in newborns between babies born by cesarean section and babies born vaginally (Biasucci et al., 2008). Babies born vaginally will be colonized from the start by the microbiota that comes from the mother's vagina (Kusumo, 2012). Research by Hansen et al., (2015) and Sihotang and Fachrial (2020) on feces for the first 24 hours after birth, found a low number of bacteria in the first meconium. One baby was dominated by Enterobacteriaceae, while the other sample was dominated by 2-5 genera of bacteria such as Bifidobacterium, Enterobacteriaceae, Enterococcaceae, Bacteroides and Prevotella.

## 2. Materials

*2.1 Sample preparation*

The sample used was the feces of newborns (meconium) who had not consumed breast milk or formula milk for the first 24 hours after birth. Taken from a hospital in the city of Medan. The research was conducted at the Biomolecular Laboratory of the Faculty of Medicine, Prima University, Medan from June to September 2019.

*2.2 Probiotic Bacteria Isolation*

Bacterial isolation was carried out based on the Syukur and Purwati method (2013). A total of 1 gram of meconium dissolved in 9 ml of MRS broth solution. This dilution is equal to $10^{-1}$. Furthermore, it was incubated for 24 hours in an incubator under anaerobic conditions with a temperature of 37ºC. After incubation, the dilution was carried out again to 10-7, then pipette as much as 0.1 ml and planted with the spread method on a petri dish containing 9 ml of MRS agar. The inoculum was incubated in an incubator for 48 hours at 37ºC. The growing probiotic bacteria colonies were examined using a colony counter to obtain a pure culture. Bacterial colonies growing on MRS media were observed both macroscopically and microscopically.

*2.3 Characterization of Probiotic Bacteria*

Characterization of bacteria refers to Yulfizar (2015). Briefly, characterization was carried out by observing the morphology, physiology, and biochemistry of the probiotic bacterial colonies that grew on MRS media.

*2.4 Probiotic bacteria identification using 16S rRNA*

The initial stage of 16S-rRNA analysis was carried out by isolating the genomic DNA from selected bacteria which was then amplified using PCR techniques. The primary combination used is 27F

AGAGTTTGATCCTGGCTGAG for forward direction with primary 1492 R GTTTACCTTACGACTT for reverse direction.
5 µL of isolated DNA samples were mixed with 12.5 µL of Mater Mix, 1 µL of Primer F, 1 µL of Primary R, 1 µL of dH2O. The mixture is then put in the PCR machine, the process begins with Pre-denaturation at 95oC for 2 minutes, Denaturation at 95oC for 45 seconds, Anneling at 56oC for 45 seconds, Extention at 72oC for 1 minute 40 seconds, Final extension at 72oC for 10 minutes and cooling down to 4oC. The amplification results were verified by agarose gel electrophoresis 1% with a voltage of 100 volts for 30 minutes. The DNA bands formed are then compared with markers.
The next stage is sequencing carried out at the Microbiology Laboratory of the Biology Research Center of the Indonesian Institute of Sciences. The sequencing results were used to determine the similarity of DNA sequences to other bacterial DNA sequences in GenBank using the Basic Local Alignment Search Tool (BLAST) program on the site http://www.ncbi.nml.nih.gov. Bacterial relationships are presented in the form of images created with the help of Mega7 software.

*2.5 Antibacterial Test*

The antibacterial activity test used the agar diffusion method (Taheri et al., 2009, Heravi et al., 2011) against Escherria coli and Staphylacoccus aureus. The sterile disc paper was dipped in probiotic bacterial culture and placed on the surface of NA media which had been smeared with indicator bacteria culture. Petri dishes were stored in an incubator at 37ºC. Observe the clear zone forming after 24 hours.

## 3. Results and Discussion

*3.1 Probiotic Bacteria Isolation*

The results of probiotic bacteria isolation showed that the total probiotic bacteria colonies in the M sample were 15 x 107 CFU / g.
The results of culture on the meconium sample for 48 hours showed that the total probiotic bacteria colonies were 15 x 107 CFU / g. Macroscopic identification activities are by observing the size and shape of the bacterial colony, the surface / elevation, the color and the shape of the edges of the bacteria visually. Based on the identification of probiotic bacterial colonies, the appearance of probiotic bacteria colonies on MRS Agar media is round, milky white with smooth edges and convex elevations which can be seen in Figure 1 and the size of the bacterial colonies is medium, small and large. Macroscopic identification of probiotic meconium bacteria can be seen in Figure 1.

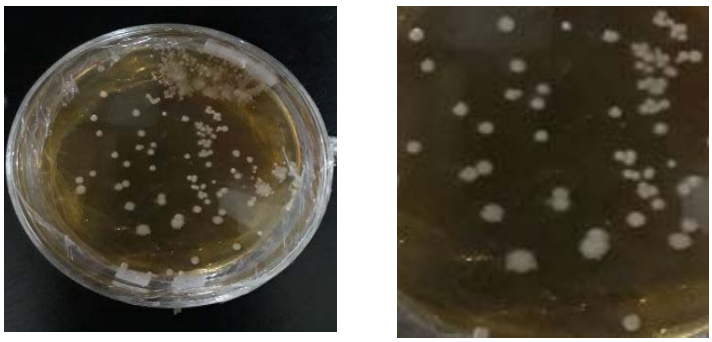


**Figure 1:** Macroscopic identification of probiotic bacteria from Meconium

Microscopic identification activity is to perform Gram stain on selected single colony which is based on the thickness or thinness of the peptidoglycan layer on the cell wall and the amount of fat layer on the bacterial cell membrane. Can be seen in Figure 2 below.

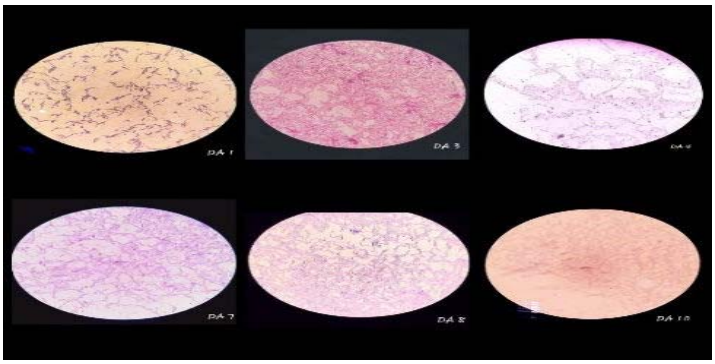


**Figure 2.** Gram stain of the meconium probiotic bacteria isolate

The isolates of probiotic bacteria from meconium were purified by 6 bacterial isolates. The staining results showed that the 6 isolates were gram-positive bacteria.

**Table 1.** Microscopic identification of meconium probiotic bacteria

| No | Sample code | Result |
|---|---|---|
| 1 | DA1 | Basil and purple |
| 2 | DA3 | Basil and purple |
| 3 | DA4 | Basil and purple |
| 4 | DA7 | Basil and purple |
| 5 | DA8 | Basil and purple |
| 6 | DA10 | Basil and purple |

From the 6 isolates from the above gram staining, it was obtained gram-positive bacteria in the form of bacilli. Bacteria classified as Gram positive and under a 1000x magnification microscope will display a purple color, which is caused by the bacteria absorbing the purple color from crystal violet.

*3.2 Characterization of Probiotic Bacteria*

The biochemical test of probiotic bacteria was carried out by using the catalase test. The catalase test was carried out on DA1, DA3, DA4, DA7, DA8 and DA10 isolates in order to determine the ability of bacteria to produce catalase enzymes and isolate tolerance to oxygen. This biochemical test is carried out by dropping hydrogen peroxide (H2O2) on the bacterial review on a glass preparation. The catalase test for probiotic bacteria isolates can be seen in Figure 3.

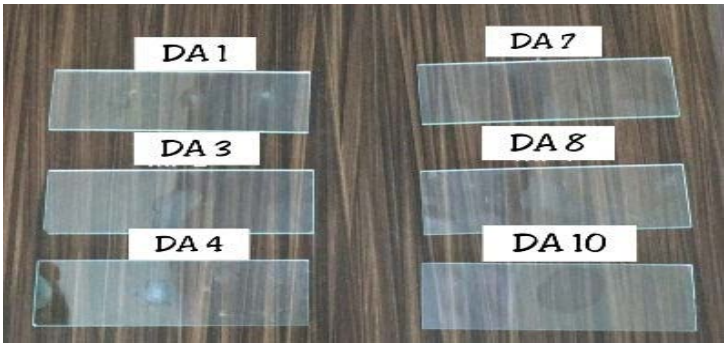



**Figure 3.** Catalase test for meconium probiotic bacteria isolates

Catalase test is one indicator of probiotic bacteria determination. Negative catalase is characterized by the absence of gas bubbles. If the results of the study do not find gas, it means that the bacteria are probiotic bacteria. Figure 3 shows the absence of gas bubbles in the bacterial review. If gas bubbles appear, this indicates that the bacteria is catalase positive, while no gas is catalase negative. As the characteristics of probiotic bacteria in general are having a positive Gram stain, react negatively to catalase and do not form spores.

Apart from the catalase test, another biochemical test is the fermentation type test. The purpose of this test is to classify probiotic bacteria into homofermentative groups or heterofermentative groups. Observations were made by looking at the presence of air bubbles that appeared on the durham tube. The results of fermentation type testing on meconium probiotic bacterial isolates are as shown in Table 2.

**Table 2**.Test of the type of meconium probiotic bacteria fermentation

| No | Code isolate | Type of Fermentation |
|---|---|---|
| 1 | DA1 | Homofermentation |
| 2 | DA3 | Homofermentation |
| 3 | DA4 | Homofermentation |
| 4 | DA7 | Homofermentation |
| 5 | DA8 | Homofermentation |
| 6 | DA10 | Homofermentation |

The results of the fermentation type test on 6 bacterial isolates found that the type of fermentation was homofermentatife. The homofermentative type is characterized by the absence of gas bubbles in the durham tube. Table 2 shows that the probiotic bacterial isolates from meconium are homofermentative type bacteria, namely bacteria whose main product is lactic acid. This is indicated by the absence of gas bubbles on the durham tube placed in the MRS Broth MERCK medium.

Characterization of potential probiotic bacterial isolates from meconium included: 1. Morphological tests including shape, edge and elevation; 2. Biochemistry which includes starch, citrate, motility and gelatin. The results of testing the characterization of potential probiotic meconium bacterial isolates can be seen in Table 3 below.

**Table 3.** Characterization of potential probiotic meconium bacterial isolates

| **Morfology** | 1 | 3 | 4 | 7 | 8 | 10 |
|---|---|---|---|---|---|---|
| Shape | Circular | Circular | Circular | Circular | Circular | Circular |
| Edge | Entire | Entire | Entire | Entire | Entire | Entire |
| Elevation | Flat | Flat | Flat | Flat | Flat | Flat |
| **Biochemistry** | | | | | | |
| Starch | - | - | - | - | - | - |
| Citric | - | - | - | - | - | - |
| Motility | + | + | + | + | + | - |
| Gelatin | | | | | | - |

**Table 3** shows that the test results on all bacterial isolates from meconium have the same results. In the morphological test, which includes a circular shape, entire edge and flat elevation. Furthermore, for biochemical tests, namely starch, citrate and gelatin showed negative results and mortality showed positive results.

**Antibacterial Activity Test**

Probiotic bacterial isolate screening is carried out with the aim of selecting and obtaining the most potential bacterial isolates that will be used as probiotic candidates and to determine the ability to suppress the growth of pathogenic bacteria, namely Staphylococcus aureus and Escherichia coli. This test bacteria is a Gram-positive pathogenic bacterium that can cause food poisoning and can cause diarrhea, and Staphylococcus aureus is a type of Gram-positive pathogenic bacteria, one of its natural habitats, namely the surface of the human body skin and mucous membranes that can contaminate food through physical contact with the skin surface. The screening process uses the diffusion well method. The diameter of the clear zone formed in the antimicrobial resistance test can be seen in Table 4.

**Table 4.**Diameter of the clear zone of the antimicrobial resistance test (mm)

| No | Isolat | *Staphylococcus aureus* | *Escherichia coli.* |
|---|---|---|---|
| 1 | DA1 | 7,2 | 0,0 |
| 2 | DA3 | 6,6 | 0,0 |
| 3 | DA4 | 7,1 | 0,0 |
| 4 | DA7 | 6,3 | 0,0 |
| 5 | DA8 | 6,5 | 0,0 |
| 6 | DA10 | 7,0 | 0,0 |

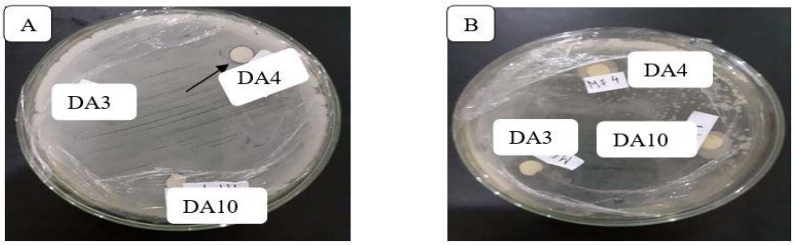



**Figure 4.** Clear zones of probiotic antibacterial test results: A. S. aureus; B. E. coli

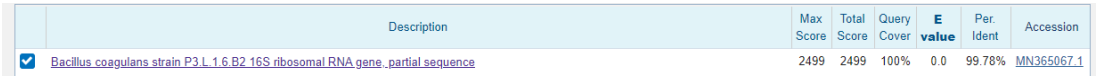
Table 3 and Figure 4 show the results of the antimicrobial inhibition zone in pathogenic bacteria Staphylococcus aureus and Escherichia coli. Isolates DA1, DA4 and DA10 were the isolates with the greatest inhibition zone in suppressing the pathogenic bacteria Staphylococcus aureus, namely 7.2 mm, 7.1 mm and 7.0 mm compared to other isolates. Meanwhile, DA1, DA3, DA4, DA7, DA8 and DA10 isolates were not capable of Escherichia coli bacteria. This is because the Escherichia coli bacteria have a trilayer cell wall structure so that they are not easily damaged by probiotic candidate bacterial isolates from meconium.

Identification of Probiotic Bacteria with 16S rRNA

The identification of probiotic bacteria from meconium showed a PCR product fragment with a size of 1300 bp which is the expected size if using a 27F AGAGTTGATCCTGGCTGAG primer combination for the forward direction with 1492 R GTTTACCTTACGACTT primer for the reverse direction. This indicates that the PCR product fragments indicate that the bacterial isolate from meconium is a probiotic bacterial isolate.

The results of sequencing of meconium isolates were compared with GeneBank data using the BLAST program conducted online on the NCBI website (http://www.ncbi.nlm.nih.gov).

The BLAST results show that the identity value between DA4 and Bacillus coagulans NBCR 12583 is 99.78% with a cover query of 100%.



| Description | Max Score | Total Score | Query Cover | E value | Per. Ident | Accession |
|---|---|---|---|---|---|---|
| Bacillus coagulans strain P3.L.1.6.B2 16S ribosomal RNA gene, partial sequence | 2499 | 2499 | 100% | 0.0 | 99.78% | MN365067.1 |

**Figure 5.** Blast results of DA4 sequences and Bacillus coagulans NBRC

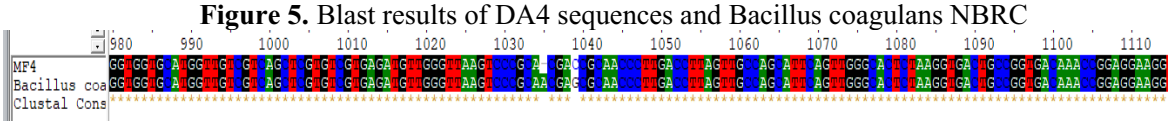



**Figure 6.** Alignment results of DA4 sequences and Bacillus coagulans NBRC

## 4. Discussion

The isolation of probiotic bacteria from meconium resulted in six isolates (Table 2). The bacterial isolates were stored in the biomolecular laboratory, Universitas Prima Medan. The macroscopic and microscopic appearance of some of these endophytic fungi are as shown in Figures 1 and 2. Of the six bacterial isolates tested, they showed antimicrobial activity which was characterized by an inhibition zone against S. aureus but did not show antimicrobial activity on E. coli.

Probiotic bacteria are bacteria that help and provide protection and maintenance for the health of the digestive system from various diseases, especially in the stomach and intestines. These bacteria belong to normal flora that are beneficial to health because they maintain the balance of the intestinal microflora.

According to FAO / WHO (2001), the total colonies of probiotic candidate bacteria are around 106-108 CFU / g. This is consistent with the results of research which showed that the total bacterial colonies of meconium were around 106-108 CFU / g. This result is in accordance with the opinion of Suryono (2003), which states that probiotic isolate bacteria are said to be of good quality if they contain bacterial colonies of 106-108 CFU / g. The presence of probiotic candidate bacteria in meconium is caused by complex microbial colonization due to the influence of prenatal non-pathogenic bacteria from mother to fetus.

The results of the study by Widodo et al., 2012 stated that baby feces is one of the best sources of bacteria for probiotic isolation. Furthermore, the research results of Jimenez et al., (2008) showed that the types of bacteria found in meconium were dominated by probiotic candidic acid bacteria from the Lactobacillusm Bacillus strain while some were dominated by enteric bacteria similar to E. coli.

The results of macroscopic observations on six probiotic bacteria isolates showed that the colony was microscopic round, milky white with wild edges and convex elevation (Figure 1). Furthermore, the microcopic identification of six ptobiotic bacteria isolates showed the results of the isolate with a stem (bacillus) and purple color. Fachrial and Jamsari (2014) probiotic bacterial colonies are characterized by milky white color with wild edges and convex elevation, gram-positive, and include facultative anaerobic bacteria. The same thing was stated by Salminen et al., (2007) that probiotic bacteria are facultative anaerobic bacteria, gram positive, rod-shaped (bacillus), do not have spores and produce lactic acid as the main product of carbohydrate fermentation.

According to Abun (2008), probiotic bacteria are unable to produce the catalase enzyme which is used to break down hydrogen peroxide dihydrioxy into oxides. According to Suryani, Santoso, and Jufrie (2010) there are two types of probiotic bacterial fermentation, namely homofermentative and heterofermentative. Homofermentative probiotic bacteria only produce lactic acid as the main product of fermentation, while heterofermentative probiotic bacteria besides lactic acid also produce ethanol, other acids such as acetic acid and CO2 gas, so that if the tested probiotic bacteria produce gas stored in the durham tube, the probiotic bacteria are declared as heterofermentative, while the isolates that do not produce or produce gas are called homofermentative. These results are in accordance with the opinion of Syukur and Purwati (2013) which states that homofermentative probiotic bacteria involve the Embden Meyernof-Parnas pathway, namely glycolysis which produces lactic acid, 2 moles of ATP from 1 glucose

/ hexose molecule under normal conditions, does not produce CO2 and produces cell biomass. twice as many as heterofermentative probiotic bacteria.

According to Samosir (2016), probiotic potential bacteria have a circular, entire and flat morphology. The same thing was stated by Lasari (2019) that probiotic bacteria have a morphological form, which includes circular, entire edges and flat elevations, and biochemical tests, namely starch, citrate and gelatin, show negative results and mortality shows positive results.

According to Rattanachaikunsopon and Phumkachorn (2010), during growth, most of the sugar is converted by probiotic bacteria into lactic acid which causes the pH to become acidic and provides inhibition against other microorganisms. When lactic acid is produced, there is a decrease in pH, as a result of which the organic acids do not decompose or do not dissociate.

The blast results of the meconium isolates were Bacillus coagulans. Based on the PCR (Polymerase Chain Reaction) results that have been carried out and after being analyzed using BLAST as shown in Figures 9 and 10, it is found that the meconium isolate bacteria has 99.78% similarities with Bacillus coagulans, complete genome. This indicates that the type of bacterial isolate found in meconium is Bacillus coagulans strain NBRC.

Bacillus coagulans (Lactobacillus sporogenes) has heat-resistant and aerobic coagulants so that it is easily made in powder form. Bacillus coagulans is capable of forming spores, producing lactic acid, resistant to high temperatures, resistant to acidic conditions, resistant to high concentrations of salt solutions (> 10%), grows well in the small intestine, is able to maintain the balance of intestinal flora, is antagonistic to pathogenic bacteria, and produces some vitamins. Bacillus coagulans is quite economical because it has a coagulant that is resistant to heat so that it can be formed into a powder and is not damaged at feed-making temperatures (Wizna et al. 2013).

Bacillus bacteria are capable of producing various types of enzymes that are able to remodel food substances such as carbohydrates, fats and proteins so that they are easier for chickens to digest, while B. coagulans is able to produce enzymes such as alpha-amylase, alpha-ecetolactate decarboxylase, beta-glucanase, hemicellulase, maltogenic amylase. , protease and xylanase.

The results of the study by Kurniasih et al., (2013) succeeded in isolating potential probiotic bacteria from scars and testing the inhibitory ability of probiotic bacteria against several types of pathogenic bacteria, namely E. coli, S. typimurium, B. cereus, S. aureus, and L. monocytogenes. The antibacterial compounds possessed by probiotic candidate bacterial isolates are generally the bacteriocin group that can inhibit peptidoglycan synthesis because of the ability of these compounds to inhibit enzymes that play a role in the formation of peptidoglycan carboxypeptidase, endopeptid and transpeptidase. If the activity of these enzymes is disturbed, the autolytic enzyme as a regulator is lost and the enzyme is unable to control its activity so that the cell wall will experience degradation (Murray et al., 1998).

Antimicrobial mechanisms of bacteria include the production of organic acids, hydrogen peroxide, diacetyl and broad-spectrum antimicrobial compounds such as reuterin and bacterioisin. The anti-microbial effect of hydrogen peroxide causes denaturation of a number of enzymes that can increase the permeability of cell membranes so that antimicrobial substances enter the cells younger. The bactericidal effect of hydrogen is influenced by a strong oxidation effect on bacterial cells and the destruction of the basic molecular structure of protein cells (Pelzcar and Chan, 2005).

Probiotics are based on efforts to improve the efficiency of growth and development as well as health. Sumardi et al., (2010) reported that giving Bacillus coagulans can suppress the growth of Salmonella sp, whereas the effect of Escherichia coli is not seen. Ledezma-Torres et al., (2015) reported that the use of probiotics can increase intestinal villi height, crypt depth and surface area. The greater the surface area of the villi and the denser the villi, the more absorption area will be and the absorption of nutrients will increase (Lenherdt and Mozes 2003). Food substances that can be digested and absorbed through the

intestine are precursors for the formation of blood cells. Duka et al., (2015) reported that giving probiotics to broilers had no effect on hemoglobin, hematocrit, erythrocyte, and leukocytes.

## 5. Conclusion

Based on the research results it can be concluded that: 1. Probiotic bacterial isolate from meconium with 16S rRNA gene was obtained, namely Bacillus coagulans strain NBCR 12583 with 99.78% similarity. 2. The antimicrobial test results found that Bacillus coagulans strain NBCR 12583 has 7.1 mm. 3. The results of morphological characterization showed that Bacillus coagulans strain NBCR 12583 has a circular shape, entire edge and flat elevation, then the Biochemistry test showed negative catalase, homofermentative type, starch negative, negative citrate, positive motility and negative gelatin. Suggestion Based on the conclusions obtained, it is advisable to carry out further research on Bacillus coagulans strain NBCR 12583 in an antimicrobial test on other types of pathogenic bacteria.

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