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Immunomodulatory effects of probiotics isolated from traditional fermented foods and beverages of Sumatra (Indonesia) and synbiotics in mice

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Abstract. *Harahap NIP, Munir E, Hutahaean S. 2023. Immunomodulatory effects of probiotics isolated from traditional fermented foods and beverages of Sumatra (Indonesia) and synbiotics in mice. Biodiversitas 24: 1157-1162.* Traditional Sumatran foods and beverages such as sugar palm sap, *dadih,* and *tempoyak* have a higher nutritional value than the original ingredients. Lactic Acid Bacteria (LAB), a group of gut bacteria, is the starter that commonly appeared abundantly during fermentation of these natural products. Probiotics are microorganisms that, when consumed in sufficient amounts, provide health benefits to the host, one of which is immunomodulation. When probiotics are coupled with prebiotics to create a unified product known as synbiotics, their function as immunomodulators improves. The goal of this study is to identify new probiotic candidate LAB strains from traditional Sumatran fermented products in combination with inulin as a prebiotic, that can act as immunomodulators in mice through phagocytosis activity. Eight bacterial isolates encoded as NI01, NI02, NI04, DA01, DA02, TE01, TE02 and TE03 were isolated as LAB strains. Four isolates i.e. NI02, NI04, DA01, and TE02 were identified molecularly as *Lactiplantibacillus plantarum*, *Leuconostoc mesenteroides*, *Lacticaseibacillus paracasei* and *Lactiplantibacillus plantarum*, respectively which were antagonists to human pathogenic bacteria, *Escherichia coli* and *Staphylococcus aureus*. The highest phagocytic activity of macrophages was observed through the administration of synbiotics from DA01 + Inulin at 98%, followed by TE02 (96%), NI04 (95%), and NI02 (92%). The findings of this study demonstrated the potential of LAB strains in traditional fermented Sumatran products as probiotic agents and immunomodulators for future application in the health sector.

Keywords: 16S-rRNA, fermented products, probiotics, Sumatra, synbiotics

INTRODUCTION

The island of Sumatra has a variety of natural and cultural resources, which result in the production of regional specialties like *Dadih*, a curd-like food prepared from coagulated buffalo milk from West Sumatra, *Nira* a sugar palm sap (*Arenga pinnata* Merr.) from North Sumatra, and *Tempoyak*, a fermented durian flesh cooked with spices from South Sumatra, which are all obtained through the fermentation of local foods and beverages (Surono 2016). The fermented products have a superior nutritional value than the original content because the bacteria present in the fermented product can disintegrate complex components into simple nutrients that are easier for humans to assimilate and metabolize (Sharma et al. 2020). The important function that bacteria play in the intestinal environment and thus have an impact on the health and well-being of the host has been extensively established over the years through animal research as well as findings obtained in human investigations (Koirala and Anal 2021). There have been reports of fermented foods such as *Calpis* (Japan), *Kaymak* (Turkey), *Kefir* (Russia), *Kimchi* (Korea), and *Tofu* (Southeast Asia) being consumed for its health-bearing effects by various communities (Bagchi et al. 2014). Lactic Acid Bacteria (LAB) are a class of starter that can reside in the digestive tract, restrict the growth of spoilage bacteria, and are resistant to food-borne pathogens either opportunistic or latent species (Cordeiro et al. 2019; Khaneghah et al. 2020).

LAB which plays a role in the fermentation process is a probiotic agent (de Simone 2019). The concept of probiotics therapy arose with the discovery of the gut microbiome, which is an inherent component of intestinal epithelial cells (Gallego and Salminen 2016). A probiotic is a dietary supplement containing living microorganisms that assist the human by enhancing the gut microbiome in the gastrointestinal tract (Fuller 1989). Probiotics are microorganisms which, when given in sufficient quantities, will provide health benefits to the host (Kechagia et al. 2013). One of the beneficial effects of probiotics is that they act as an immunomodulator in the host, influencing the expression of cytokines and enhancing the synthesis of immunoglobulin (IgA) antibodies and cytoprotective molecules (Azad et al. 2018). The role of LAB as probiotics can be strengthened by the addition of prebiotics, which are nondigestible food items that benefit the host by encouraging the growth and activity of certain

bacteria in the gut, hence boosting host health (Bodke and Jogdand 2022).

The intestine houses up to 80% of the human immune system because it is the first organ to be exposed to the outside world through feeding (Wiertsema et al. 2021). The intestine is not only responsible for food absorption and digestion, but it is also part of the body's greatest immune system, which deals with incoming antigens and hazardous substances. The host-microbe interaction is crucial for the development of gastrointestinal immunity in the first few weeks after birth (Zheng et al. 2020). The multiplication and expansion of gut bacteria continues until the age of two, when the intestinal immune system is said to be mature (Zhang et al. 2017). Intestinal habitat is largely stable for gut microbiota, especially at the species and genus levels. Aside from that, irrational antibiotic usage, pathogenic parasites, malnutrition, and cold and heat stress all affect the structural diversity of gut microbiota (Jandhyala et al. 2015). As a result, gut health also promotes endurance to host. The availability of prebiotics, which when combined into a single product are called synbiotics, can help improve the function of probiotics as immunomodulators (Yahfoufi et al. 2018). Probiotic research has exploded in recent years, with nearly all studies aiming to identify new strains of lactic acid bacteria, or candidate probiotics, as natural immunomodulators with less adverse effects than synthetic immunomodulators. As a result, a study on screening probiotic candidate LAB isolates from traditional Sumatran fermented products as immunomodulatory agents is being investigated.

MATERIALS AND METHODS

Isolation and characterization of LAB isolates from fermented products

The isolation technique of LAB strains followed the standard dilution and spread plate method. *Nira* (*Arenga pinnata* Merr.) was randomly collected from trees in Padang Lawas Utara Regency, North Sumatra, Indonesia. One mL of liquid sample was diluted ten-fold into sterile physiological saline solution until 10^5 dilution. Aliquot of each dilution was spread on solid deMann, Rogosa, and Sharpe (MRS) medium supplemented with 0.5% (w/v) of $CaCO_3$. The plates were incubated at 37°C for 48 h. *Dadih* was collected from Bukit Tinggi City, West Sumatra, Indonesia. *Tempoyak* from durian fruits was collected from Palembang City, South Sumatra, Indonesia. One g of each sample was diluted into sterile physiological saline until 10^8 dilution. Aliquot from each sample dilution was inoculated and incubated in the same manner as previously described. Colonies showing a clear zone indicate the presence of LAB and purified into a new solid MRS medium. Each isolate was characterized based on their colony morphology, gram staining, formation of endospore, catalase activity using 3% (v/v) of H_2O_2, and motility using sulfide indole motility (SIM) medium.

Tolerance assay under simulated gastric and intestinal environment

Approximately 100 µL of each LAB isolate pre-grown 24 hr in MRS broth was inserted into a 1.5-mL tube containing 900 µL of liquid MRS medium pre-adjusted for its pH to simulate the gastric (pH = 2.0) and intestinal (pH = 8.0) environment. The tubes were incubated at 37°C for 3 h. The samples were centrifuged at 3,000 rpm for 5 min to obtain pellets. The pellets were washed with 300 µL of sterile physiological saline solution. Fifty µL of samples were then inoculated into 5 mL of liquid MRS medium (neutral pH) and incubated at 37°C for 48 h under anaerobic condition. The optical density (OD_{600}, OD_{660}) of each suspension was measured using a spectrophotometer to evaluate the growth tolerance of each LAB isolate when OD_{600} or $OD_{660} \geq 1.0$ (Febrianti et al. 2016). In addition, the growth tolerance assay of LAB was later monitored in bile salts or oxgall solutions (0.1%, 0.2%, 0.3%, w/v) following the similar protocol as described before.

Antagonistic test of LAB isolates against human pathogenic bacteria

Approximately 25 µL of bacterial suspension (*Escherichia coli*, *Staphylococcus aureus*) in sterile physiological saline (OD_{600} = 0.1 ≈ 10^8 CFU/mL) was inoculated on top of Mueller Hinton Agar (MHA) medium and spread thoroughly to generate a bacterial lawn. Suspension of each LAB isolate was prepared in a sterile physiological saline at OD_{600} = 0.1 and immersed with 6-mm blank discs for 30 s. The discs were removed, dried in a chamber for 1 min and placed on top of indicator lawn. Ciprofloxacin discs were used as a standard antibiotic or a positive control. The plates were incubated at 37°C for 48 h. The presence of clear zone around discs indicate the antagonistic activity of LAB isolates against human pathogenic bacteria and measured using a digital caliper in mm.

In vivo assay of immunomodulatory effects by probiotics and synbiotics in experimental mice

Eighty male-specific pathogen-free mice with a body weight of 20 g were obtained and maintained in Animal House, Universitas Sumatera Utara, Medan, Indonesia. Animals were acclimatized to laboratory conditions for 1 wk before experimentation. Animals and experimental procedures used in this study were approved by the Animal Care Review Committee, Universitas Sumatera Utara. All mice were randomly divided into 18 groups and five replications each: a negative control group (0.5%, w/v of Na-CMC), a positive control group (w/o LAB isolates), eight treatment groups each with different LAB isolates (NI01, NI02, NI04, DA01, DA02, TE01, TE02, TE03), and eight treatment groups using synbiotics (each LAB+inulin). All bacterial suspensions were prepared within 48-h of incubation prior experimentation. The concentrations of LAB isolates were adjusted to 10^8 CFU/mL and diluted to produce suspensions of safe doses for oral administration. The LAB treatments were conducted with 10 mL/kg BW by oral administration once daily for seven days. On the eighth day, all mice were injected with 0.5 mL/kg BW of *S.*

aureus suspension 10^8 CFU/mL through intraperitoneal region and maintained for 1 hr. The mice were anesthetized and sacrificed then the peritoneal fluids were harvested and fixed with MeOH, stained with 3% (w/v) of Giemsa and observed under light microscope at 1,000×. The immunomodulatory activity is expressed by the percentage of active macrophages (out of 100 cells) during observation (Ilyas et al. 2020).

Molecular identification of potential probiotics as immunomodulators

A series of molecular identification i.e. DNA extraction, PCR amplification and DNA sequencing of 16S-rRNA was conducted commercially by Macrogen, Inc., Singapore to four potential LAB isolates. The sequencing was performed at site specific region using 785F and 907R primers to obtain the nucleotide composition. The sequence of each isolate was checked for its similarity to GenBank database using BLAST, followed by a construction of phylogenetic tree to validate their species identities using MEGA-11 software (Tamura et al. 2021).

RESULTS AND DISCUSSION

Isolation of suspected LAB from traditional fermented products of Sumatra resulted into 10 isolates with four isolates from sugar palm sap, three isolates from both *dadih* and *tempoyak*. The bacterial colonies with clear zones were picked up and purified into a pure culture/ isolate. Lactic acid bacteria generate numerous organic acids, specifically lactic acid (up to 90%) during their growth. As a result, calcium carbonate ($CaCO_3$) is supplemented into solid MRS medium as an indication for acid-producing strains since it dissolves with acid, resulting in a clear zone around bacterial colonies (Onda et al. 2002). The colony morphology and biochemical characteristics of each LAB isolate is presented in Table 1. All isolates were Gam-positive bacteria with only one isolate (DA02) producing catalase and forming endospore. Motility was only observed from isolate NI03. Members of LAB are generally non-motile, rods or cocci in cellular shape, and classified into Gram-positive bacteria. The majority of Gram-positive bacteria known as probiotics to date are non-spore forming species from *Lactobacillus* and *Bifidobacterium* with only few exceptions to certain Gram-negative species (Behnsen et al. 2013). Catalase is an enzyme that converts hydrogen peroxide (H_2O_2) to water (H_2O) and oxygen (O_2) which is not a functional trait to LAB species that are anaerobes or facultative anaerobes (Ismail et al. 2018). We then excluded two LAB isolates, NI03 and DA02, from further testing based on the characterization results.

All LAB isolates were subjected to growth tolerance assay under different pH (2.0, 8.0) to mimick the human gastric and intestinal environment and exposure to different bile salts concentrations (0.1%, 0.2%, 0.3%) using oxgall (Table 2). All LAB isolates showed obvious growth through $OD_{600,660}$ reading in different pH, however some of them, i.e. NI01, DA03, TE01, and TE03 showed no growth in 0.3% of oxgall. This tolerance assay is the most important factor in the development of probiotics from the LAB group. Probiotics that are administered orally, will confront a variety of barriers as they pass through the mouth, stomach, intestine, and colon. The health benefits of probiotics are lost mostly due to a significant decline in viable population of probiotics in the gastrointestinal tract and colonization hindered by commensal bacteria (Han et al. 2021). The probiotics will enter the small intestine after passing via the pylorus (stomach), where there is an abundance of pancreatic juice and bile. The pH of the small intestine is around 6.0-7.0 due to the neutralizing action of intestinal fluid, which is substantially lower than the pH of stomach fluid. Bile acids, on the other hand, can have an effect on probiotic viability by disrupting cell membranes and causing DNA damage (Yao et al. 2020). Aritonang et al. (2022) reported that the viability of LAB isolates from *dadiah*, a buffalo dairy product was below 60% under 0.3% of oxgall. Roza et al. (2022) reported the probiotics potential of LAB isolates from *dadiah kapau*, a fermented foods from different region, and reported the viability below 87% after exposure to 0.3% of oxgall. In this study, we did not calculate the total density of viable LAB isolates but only observed it through the OD or turbidometry approach. Based on the reported studies, it can be seen that the growth tolerance of each LAB strain and isolate is different depending on the origin of the isolate and its species.

Table 1. Morphological and biochemical characteristics of LAB isolates from traditional fermented products of Sumatra

Isolates	Cellular shape	Colony color	Gram stain (+/–)	Endospores (+/–)	Catalase (+/–)	Motility (+/–)
NI01	Cocci	Whitish yellow	+	–	–	–
NI02	Cocci	Whitish yellow	+	–	–	–
NI03	Rod	Whitish yellow	+	–	–	+
NI04	Cocci	Whitish yellow	+	–	–	–
DA01	Cocci	Whitish yellow	+	–	–	–
DA02	Cocci	Whitish yellow	+	+	+	–
DA03	Rod	Beige/Cream	+	–	–	–
TE01	Cocci	Whitish yellow	+	–	–	–
TE02	Cocci	Whitish yellow	+	–	–	–
TE03	Cocci	Whitish yellow	+	–	–	–

Note: NI: sugar palm sap, DA: *dadih*, TE: *tempoyak*.

Table 2. Growth tolerance of LAB isolates under different pH and bile salts concentrations

Isolates	Gastric (pH 2.0)	Intestine (pH 8.0)	Oxgall (0.1%)	Oxgall (0.2%)	Oxgall (0.3%)
NI01	+	+	+	+	–
NI02	+	+	+	+	+
NI04	+	+	+	+	+
DA01	+	+	+	+	+
DA03	+	+	+	+	–
TE01	+	+	+	+	–
TE02	+	+	+	+	+
TE03	+	+	+	+	–

Note: NI: sugar palm sap, DA: *dadih*, TE: *tempoyak*. Growth: (+): tolerant, (–): intolerant.

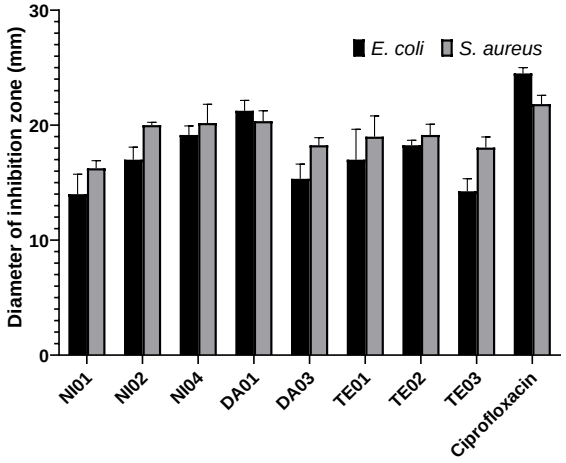

Figure 1. Antagonistic activity of LAB isolates as expressed in the diameter of inhibition zone (mm) through disk diffusion assay against *E. coli* and *S. aureus*

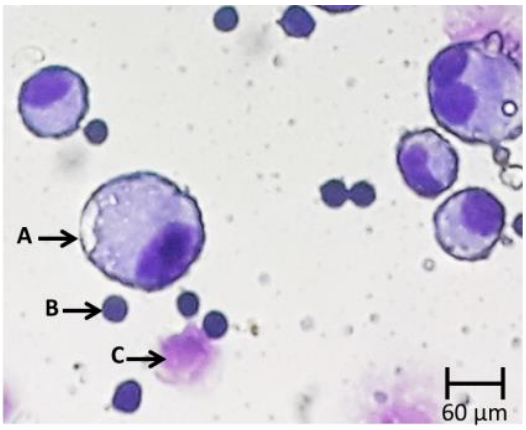

Figure 2. Phagocytosis activity of macrophage cells harvested from the peritoneal fluid of experimental mice infected with *S. aureus*. A: Active macrophage cells. B: Inactive macrophage cells. C: Artifact

All LAB isolates showed different degree in inhibiting the growth of indicator bacteria (*E. coli*, *S. aureus*). The strongest inhibition was observed from DA01 with the diameter of inhibition zone (DIZ) of 21.25 and 20.35 mm against *E. coli* and *S. aureus*, respectively. The weakest inhibition was observed from NI01 with DIZ of 14.00 and 16.25 mm against *E. coli* and *S. aureus*, respectively.

The pathogenic bacterium, *S. aureus* was considered as the most sensitive indicator in this study. Meanwhile, ciprofloxacin as a positive control or antibiotics produced a DIZ of 25.00 mm and 22.00 mm against *E. coli* and *S. aureus*, respectively which was still potent than our LAB isolates. Resistance and suppression of probiotic bacteria can be accomplished by releasing organic acids, which are typically produced by LAB. Organic acids will diffuse into the bacterial cell by disrupting the ions on the target cell surface; once the target cell surface has been damaged, LAB will diffuse into the bacterial cell. Organic acids will hinder substrate transport, energy production, macromolecule synthesis, and cytoplasmic fluid balance in the target bacterium (Desniar et al. 2020).

Eight LAB isolates as probiotic candidates were then tested for the immunomodulatory activity on increased macrophage phagocytosis activity in male mice infected with *S. aureus* with the positive control using a prebiotic, inulin. Inulin was used in this study as a form of soluble fiber that is fermented by the gut microbiota rather than processed by human or mouse digestive enzymes (Desai et al. 2016). Observation of mouse macrophage phagocytosis activity was observed through thin smear preparations of peritoneal fluid which had been stained with Giemsa. The results of staining using Giemsa dye showed staining of the nucleus of macrophage cells which was marked by a change in color to bluish purple. Observations were made on macrophage cells that were actively carrying out the process of phagocytosis from the 100 observed macrophage cells (Figure 2). All probiotic candidate LAB isolates were added with a prebiotic in the form of inulin. The tests showed that there was an increase in macrophage phagocytosis activity (Figure 3). Inulin enhances the growth and activity of these LAB bacteria in the colon, increasing the number of LAB bacteria as probiotic candidates while suppressing the number of *S. aureus* bacteria and their immunomodulatory potential.

Based on the phylogenetic analysis, four probiotic candidates were identified as potent immunomodulators including the genus *Lactiplantibacillus* namely *Lactiplantibacillus plantarum* for isolates NI02 and TE02, the genus *Leuconostoc* namely *Leuconostoc mesenteroides* for isolate NI04 and the genus *Lacticaseibacillus* namely *Lacticaseibacillus paracasei* for isolate DA02 as presented in Figure 4. *Lactiplantibacillus plantarum* is a Gram-positive LAB that can live in a variety of environments and is one of numerous probiotics that can colonize the human gastrointestinal mucosa. *Lactobacillus plantarum* ST-III was able to develop resistance to stomach acid and survive in the intestines of mice by performing specific functional activities (Zhang et al. 2022). *Leuconostoc mesenteroides* LVBH107, by its auto- and co-aggregation activities, can produce a barrier that protects the host epithelium by preventing prospective pathogens from binding to host cell receptors, possesses good biosafety properties (no antibiotic resistance plasmid), as evidenced by its sensitivity to erythromycin, doxycycline, minocycline,

ampicillin, and chloramphenicol. In addition, *L. mesenteroides* LVBH107 has been studied as a probiotic that modulates immune function by regulating the production of pro- and anti-inflammatory cytokines (Luan et al. 2022). *Lacticaseibacillus paracasei* secreted effector molecules in the form of exopolysaccharides into the gut system that was assumed to be linked to its health-promoting properties (Oleksy and Klewicka 2018). Kim et al. (2020) found that *L. paracasei* strain KBL382 modified the structure of gut microbiota in oral-treated mice, resulting in a protective role against infection.

Screening of ten bacterial isolates based on their colony growth on deMann Rogosa Sharpe Agar (MRSA) supplemented with 0.5% $CaCO_3$ solution as Lactic Acid Bacteria (LAB) revealed eight potential isolates (NI01, NI02, NI04, DA01, DA02, TE01, TE02 and TE03) are probiotics candidate. Four isolates with the code NI02, NI04, DA01 and TE02 were identified molecularly as *Lactiplantibacillus plantarum, Leuconostoc mesenteroides, Lactocaseibacillus paracasei* and *Lactiplantibacillus plantarum*. The bacterium, *Lactocaseibacillus paracasei* DA01 displayed the best probiotics traits as an immunomodulator than the three LAB isolates in terms of antagonistic properties with *E. coli* and *S. aureus* bacteria, as well as macrophage phagocytosis activity as a probiotics and synbiotics when combined with inulin administration to experimental mice.

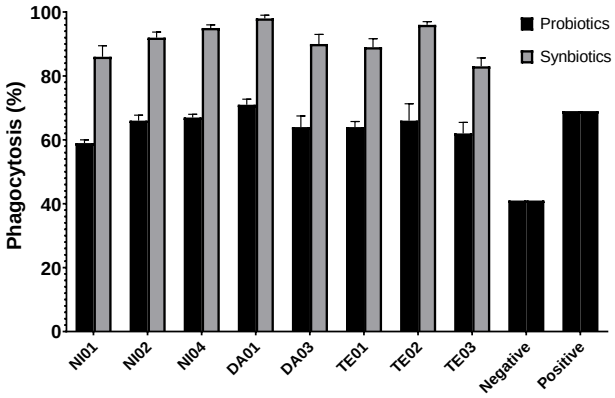

Figure 3. Phagocytosis activity (%) of macrophages in the peritoneal fluid infected with *S. aureus* and treated with probiotics (LAB isolates) and synbiotics (probiotics+inulin)

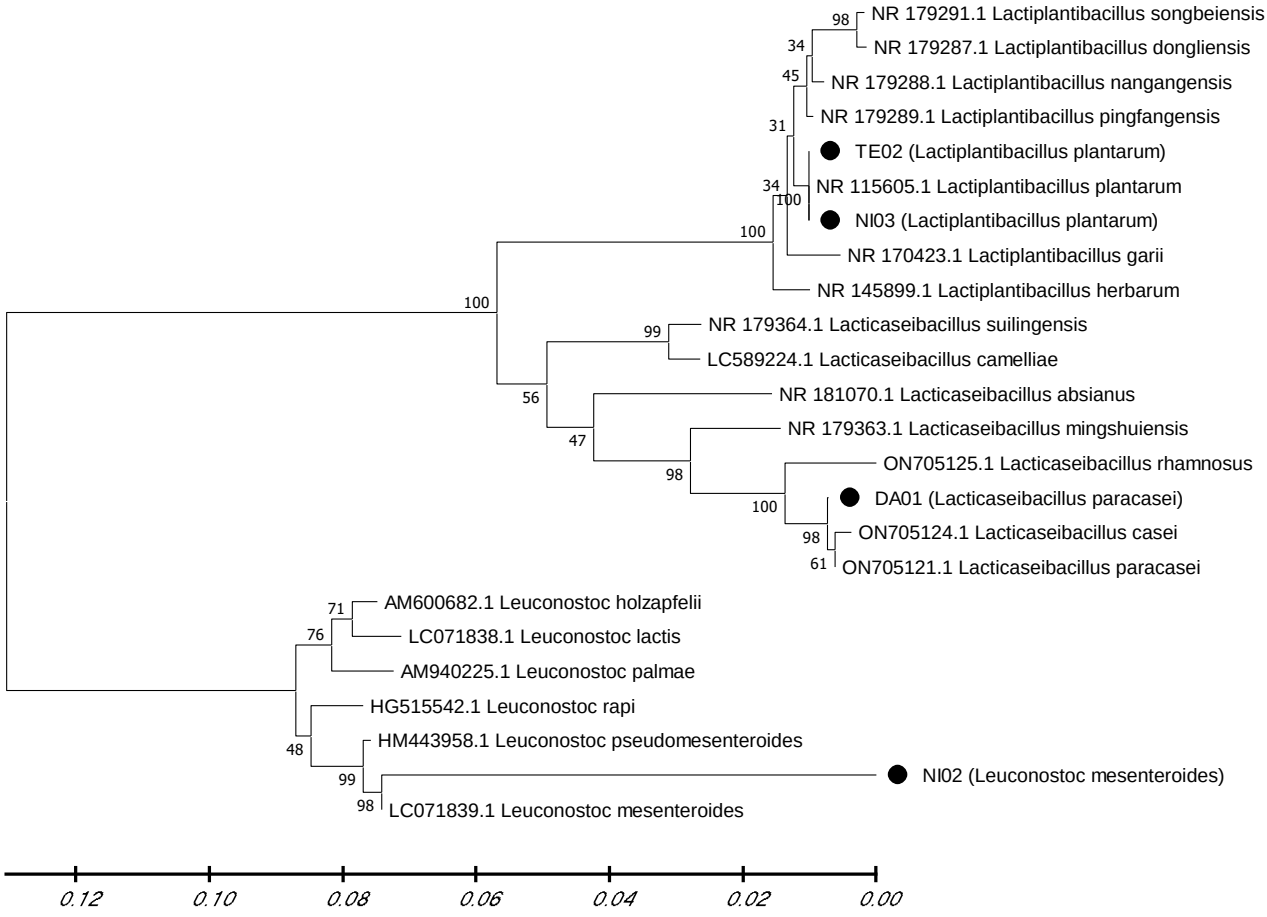

Figure 4. Phylogenetic position of LAB isolates (•) from fermented products of Sumatra among other LAB species retrieved from GenBank as inferred by neighbor-joining method and Kimura-2 parameter statistics. Bootstrapping at 1000×

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