

Assessment of Probiotic Characteristics of *Lactiplantibacillus plantarum* SU-KC1a Isolated from Human Breast Milk in Indonesia

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Abstract

Background and Objective: Lactic acid bacteria such as *Lactiplantibacillus* spp. and *Bifidobacterium* spp. are addressed for their beneficial characteristics. This study aimed to assess probiotic attributes of *Lactiplantibacillus plantarum* SU-KC1a isolated from human breast milk in Indonesia.

Material and Methods: *Lactiplantibacillus plantarum* SU-KC1a was anaerobically isolated from human breast milk and then morphologically and biochemically characterized. Probiotic characteristics, including tolerance to various pH levels and bile salt concentrations, antibiotic susceptibility, antimicrobial activity, mucosal adhesion ability and oxidative stress tolerance, were assessed.

Results and Conclusion: *Lactiplantibacillus plantarum* SU-KC1a exhibited rod-shaped, Gram-positive staining, negative catalase activity, and lacked acid-fastness and motility. Under stress conditions, this strain adopted a coccoid-like shape to protect its cell membrane and internal structures from external environments. Significantly, it demonstrated resistance to mupirocin, distinguishing it from other lactobacilli. The strain showed enhanced growth in the presence of prebiotics, particularly galactooligosaccharides. Additionally, it showed probiotic characteristics such as robust tolerance to pH variations and bile salts, potent antimicrobial activity against pathogens, strong adherence to mucosal surfaces and resistance to oxidative stress. These findings highlight *Lactiplantibacillus plantarum* SU-KC1a as a promising probiotic candidate appropriate for incorporation into functional foods.

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1. Introduction

Breast milk is renowned for its rich composition of essential nutrients, bioactive compounds and antibodies critical for infant growth and immune development. It contains a spectrum of elements, including carbohydrates, proteins, antibodies, vitamins, minerals and amino acids (notably L-arginine), making it a perfect natural nourishment for infants [1]. Studies have consistently highlighted advantages of breastfeeding, showing a lower susceptibility to infections and gastrointestinal disorders in breast-fed neonates, partly due to the presence of probiotics in breast milk [1, 2]. From these probiotics, lactic acid bacteria (LAB) such as *Lactiplantibacillus* spp. and *Bifidobacterium* spp. are particularly prominent for their

beneficial effects on the infants' immune system. Naturally, LAB play their significant roles in promoting human health by stimulating proliferation and differentiation of the host intestinal cells, regulating acidity in the gastrointestinal tract (GIT) and competing with harmful pathogens [3].

Breast milk contains prebiotics known as human milk oligosaccharides to increase growth and viability of LAB. Importance of prebiotics in breast milk is signified in industrial practices, where prebiotics are intentionally introduced to functional foods to support LAB growth and viability. This integration of prebiotics and probiotics in food production serves to nourish beneficial bacteria, enhancing their survival and functionality.

Oligosaccharides widely used as prebiotics in food industries include fructooligosaccharides (FOS) and galactooligosaccharides (GOS), lactose, isomaltooligosaccharides, xylooligosaccharides, maltodextrin and inulin [4]. In addition to its prebiotic characteristics, inulin is used to improve the quality of milk fermented by *Lactobacillus acidophilus* and *Bifidobacterium lactis* [5]. Although addition of prebiotics can stimulate growth of LAB, those used in food industries often facing challenging environments, resulting in decreased viabilities during processing and storage. As many LAB are facultative anaerobes or microaerophiles, they are highly susceptible to oxidative stress. Zhai et al. [6] reported that the growth of *L. plantarum* strain CAUH2 decreased when exposed to hydrogen peroxide. Another study by Dosan et al. [7] revealed that *B. animalis* subsp. *lactis* BR2-5 and *B. breve* BS2-PB3 cells were significantly depleted when exposed to oxygen, with no viable cells detected under aerobic conditions.

Categorized as probiotics, microorganisms must withstand stressful conditions, exhibit survival capability within GIT, show effective adherence to intestinal epithelia and be safe for animals and humans [8]. Adherence of probiotic bacteria to the intestinal lining includes particular significance due to its role in the bacterial survival within the dynamic digestive tract environment. Colonization, a critical aspect of this survival, is facilitated through bacterial adhesion to the intestinal wall [9]. Additionally, LAB, specifically *L. plantarum* strain WLPL04 isolated from human breast milk (HBM), has been reported for its capacity to prevent adhesion of pathogenic bacteria via competitive mechanisms. These inhibitory effects have been demonstrated *in vitro* against pathogens such as *Escherichia coli* O157:H7, *Salmonella* Typhimurium ATCC13311 and *Staphylococcus aureus* CMCC26003 [10].

Despite extensive global studies on probiotic potential of LAB, a significant gap remains in understanding their isolation and characterization from HBM samples across diverse geographical regions. Studies focusing on LAB from HBM in various regions of the world, including Indonesia, are significantly limited. This gap is critical as regional variations in microbial composition can affect beneficial characteristics of LAB, especially in promoting maternal-infant health. Addressing this gap is essential for gaining a comprehensive understanding of LAB diversity and their potential uses in nutrition and health worldwide. Therefore, this study aimed to isolate and characterize LAB strains from HBM samples in Indonesia. Through comprehensive biochemical profiling, molecular identification, assessment of growth capabilities on prebiotic substrates and assessment of the probiotic attributes of the identified strains, this study provided an overview of potential probiotic stains from HBM that could improve public health globally.

2. Materials and Methods

2.1. Isolation and identification

2.1.1 Subjects and ethical issue

All procedures were approved by the Ethical Committee of Rumah Sakit Anak dan Bunda Harapan Kita, Jakarta, Indonesia (922/2020 on 03/31/2020) and all the volunteers signed written informed consents. Overall, two healthy women with no underlying conditions or complications participated in this study. They provided breast milk samples 1–2 d after their caesarean deliveries.

2.1.2 Sample collection

Sampling method was adapted from Eglash and Simon [11] with some modifications. All the equipment for sampling were sterilized for 10 min in boiling water and dried at room temperature (RT) before use. Nipples and mammary areola were cleaned with sterile water and cotton soaked in chlorhexidine (0.1%). The first drops were discarded, then the milk samples were collected using electric breast pump (Real Bube, UK) and then transferred to sterile breast-milk storage bags. All samples were stored in a cool box with ice packs during transport to the laboratory.

2.1.3 Media

Two different media were used in this study, including de Man, Rogosa and Sharpe (MRS) (Liofilchem, Italy) and trypticase phytone yeast extract (TPY). One liter of TPY broth was prepared by mixing 17.6 g of tryptone soya broth (TSB) (Oxoid, UK), 5 g of peptone (Merck, USA), 5 g of glucose (Merck, USA), 2.5 g of yeast extract (Merck, USA), 1 ml of Tween-80 (Merck, USA) and 2 g of dipotassium phosphate (Merck, USA) in distilled water (DW). The two media were modified by the addition of L-cysteine (2.5 g/l) (Nowfood, USA) and mupirocin (0.05 mg/ml) (Genero, UK). Modified MRS and TPY were referred to as mMRS and mTPY, respectively. To prepare solid media, 1.5% bacteriological agar was added to the media.

2.1.4 Isolation of lactic acid bacteria

Milk samples were diluted using sterile peptone water (0.1%) (Merck, Germany) and spread-plated (50 µl) on media. To support growth of LAB at the isolation stage, mMRS agar supplemented with 30% pasteurized cow milk (Greenfields, Indonesia) was used. All plates were incubated at 37 °C for 96 h in an anaerobic jar (Oxoid, UK) with anaerobic atmosphere generator sachets (Oxoid, UK). After incubation, a colony was selected and purified using four-way streak method with the purified isolate referred to as SU-KC1a.

2.1.5 Molecular identification and phylogenetic analysis

For molecular identification, isolate was processed by PT Genetika Science Indonesia for Species Barcoding (DNA extraction, 16S rRNA gene amplification and molecular



identification based on 16S rRNA sequence). The PCR reactions were prepared using universal primers 27F (5'-AGAGTTTGAATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). The 16S rRNA sequence was analyzed using Basic Local Alignment Search Tool (BLASTN 2.12.0+). Then, a phylogenetic tree was constructed using MEGA 11. MUSCLE alignment was carried out and a maximum likelihood phylogenetic tree was constructed. Bootstrap values were set at 1000 replicates. Sequences of other microorganisms used in the phylogenetic tree were retrieved from GenBank.

2.2 Characterization

2.2.1 Morphological study and biochemical activity test

The isolate was characterized based on morphology (Gram and acid-fast staining), motility and biochemical activity (catalase test). To assess effects of oxygen on cell morphology, the isolate was cultured on mMRS agar plates and incubated at 37 °C for 72 h under microaerophilic and anaerobic conditions. Furthermore, its capacity to thrive under various oxygen levels, encompassing microaerophilic and anaerobic conditions, was assessed.

2.2.2 Mupirocin resistance test

Since the isolation procedure involved a medium supplemented with mupirocin, resistance of the isolate to mupirocin was assessed. For comparison, *Lactiplactibacillus plantarum* F75 from the Biology Department culture collection at Universitas Pelita Harapan was used. This strain was originally isolated from the chicken crop. The two bacterial strains were cultured on mMRS agar at 37 °C, under anaerobic and microaerophilic conditions. Following a 72-h incubation time, growth of the strains on agar plates was recorded.

2.2.3 Carbohydrate fermentation test

The isolate ability to ferment various carbohydrates was analyzed using a method described by Dosan et al. [7]. For this test, MRS broth without carbon source was prepared manually by mixing 2.5 g of L-cysteine, 0.05 g of mupirocin, 10 g of peptone, 4 g of yeast extract, 8 g of beef extract, 5 g of NaCl, 2 g of di-ammonium hydrogen citrate, 0.2 g of MgSO₄·7H₂O, 2 g of FeSO₄·7H₂O, 0.04 g of MnSO₄·7H₂O and 1 g of Tween 80 in 1 l of sterile water and mixed. Then, 2% carbohydrate was added to the mixture; this medium was then referred to as MRSP broth. The MRS broth without carbon sources served as the negative control. Carbohydrates included glucose (Merck, USA), fructose (Merck, USA), lactose (Merck, USA), fructo-oligosaccharide (FOS; Xi'an Virgin Biotechnology, China), GOS (Xi'an Virgin Biotechnology, China), inulin (Beneo, Germany) and maltodextrin (Qinhuangdao Lihua Starch, China). To monitor acid production, pH indicator of bromocresol purple (30 mg/L) (Merck, Germany) was added to all media. Isolate was inoculated into MRSP broth

and incubated anaerobically at 37 °C for 24 h. The isolate ability to ferment carbohydrates was indicated by a drop in pH, resulting in color change of the medium to yellow. Additionally, the total viable cell count was carried out by serially diluting the liquid culture using 0.1% bacteriological peptone. Then, a 50-μl aliquot of the diluted liquid culture was spread onto MRSP agar plates and incubated at 37 °C for 72 h. Colony forming unit (CFU) was used to assess the bacterial growth in presence of various prebiotics. Results were present as relative growth percentage, with bacterial growth in MRS without carbohydrate serving as the reference point for comparison. Relative growth percentage (%) included ratio of bacterial growth in presence of prebiotics to its growth in absence of prebiotics.

2.3 Assessment of probiotic characteristics

2.3.1 Tolerance to various pH levels and various concentrations of bile salts

Assessment of the isolate tolerance to various pH levels and bile salt concentrations was carried out using a modified method from Khushboo et al. [12]. The isolate was cultured overnight in mTPY broth. Then, a 10% inoculum of the liquid culture was added to mTPY broth adjusted to various pH levels (2, 4, 7 and 9) or various bile salt concentrations (0, 0.5, 1 and 2%). All tubes were incubated anaerobically at 37 °C for 3 h. To assess total viable cell, an aliquot of 50 μl was withdrawn before and after incubation and then spread onto mTPY agar plates. All plates were incubated anaerobically at 37 °C for 72 h.

2.3.2 Antibiotic susceptibility

To assess isolate susceptibility to antibiotics, disk diffusion method was used according to Jorgensen and Turnidge [13]. Isolate was grown anaerobically in mTPY broth at 37 °C overnight. Then, a liquid culture (50 μl) was spread onto mTPY agar plate. A total of 12 commonly used antibiotics from various classes were used in this study (μg per disc): ampicillin, 10; erythromycin, 15; chloramphenicol, 30; bacitracin, 10; rifampin, 5; vancomycin, 30; neomycin, 30; streptomycin, 10; kanamycin, 30; gentamicin, 10; nalidixic acid, 30; and tetracycline, 30 (Liofilchem, Italy). Each disc was transferred onto the surface of the agar plates, which were then incubated anaerobically at 37 °C for 72 h. After incubation, diameter of inhibition zones was recorded. Antibiotic susceptibility of the isolate was categorized based on the zone of inhibition. This categorization was carried out by comparing susceptibility patterns of *Lactobacillus* spp. and *Bifidobacterium* spp., as the isolate was initially suspected as *Bifidobacterium* sp.

2.3.3 Antimicrobial activity

Isolate ability to inhibit growth of pathogenic bacteria was assessed using well diffusion assay [14]. In this study, *E. coli* and *S. aureus* from Biotechnology Culture Collection



(BTCC) were used. Isolate was cultured anaerobically in TPY broth, while pathogenic bacteria were cultured aerobically in nutrient broth (NB) (Merck, Germany). All tubes were incubated overnight at 37 °C. After incubation, the isolate liquid cultures were centrifuged at 5000× g for 20 min at 4 °C and the resulting cell-free supernatant (CFS) was collected. Liquid cultures of pathogenic bacteria (1%) were mixed with liquefied nutrient agar (NA) (Merck, Germany) in Petri dishes. Upon solidification, wells with a diameter of 6 mm were prepared using sterile pipettes. Then, cell-free supernatant (100 µl) was added into each well and plates were incubated at 37 °C for 24 h. Ampicillin (1 mg/ml) was used as positive control, while TPY broth was used as negative control. After incubation, diameter of inhibition zones was recorded. In well diffusion assay, inhibition zones might be achieved by subtracting diameter of the well from that of the clear area.

2.3.4 Mucosal adhesion ability

To assess mucosal adhesion ability of the isolate, three steps were carried out. First, porcine mucus was purified using modified method from Garcia-Gonzalez et al. [15]. Small intestine of a pig was opened, rinsed with cold PBS and the mucosal layer on the inner lining of the intestine was gently scraped off using spatula. Collected mucus was transferred into 50 ml of cold PBS. The mucosal suspension was centrifuged twice, first at 11,000× g for 10 min and then at 20380× g for 20 min. Supernatant was collected and filtered using cellulose acetate (CA) membrane syringe filters (0.2 µm). Protein concentration in the filtered solution, considered mucin, was estimated using Biuret method. First, bovine serum albumin (BSA) solution was prepared to match the estimated mucin protein concentration. The BSA was filtered using CA membrane syringe filters (0.2 µm). Second, mucin and BSA were immobilized on microplates based on a modified method from Tallon et al. [16]. Mucin and BSA solutions (100 µl per well) were added to various wells of 96-well microplates. Microplates were incubated overnight at 4 °C, then each well was washed twice with 200 µl of PBS. Third, adhesion assay was carried out using the immobilized mucus and BSA on the microplates. The liquid bacterial culture incubated at 37 °C for 16 h was diluted with PBS and three various dilution factors (100 µl each) were selected (N-initial). Diluted liquid cultures were added to each well coated with mucin or BSA and then microplates were incubated at 37 °C for 1, 2 and 3 h. Microplates were washed twice with sterile PBS (200 µl per well) and 200 µl of 0.05% (v/v) Triton X-100 solution were added to the microplates, followed by incubation at 25 °C with agitation at 50 rpm using shaker incubator to allow desorption of adherent bacteria (N-adhere). After 2 h of incubation, N-initial and N-adhere were spread-plated on MRS agar and

incubated anaerobically at 37 °C for 48 h. Bacterial colonies on the agar plates were enumerated and the percentage of adhesion cells was calculated using the Eq. 1:

Eq.1

$$\% \text{ Adhesion} = [\log (\text{N-adhere}) / \log (\text{N-initial})] \times 100\%$$

2.3.5 Oxidative stress tolerance

Isolate tolerance to oxidative stress was assessed based on a method from Watanabe et al. [17] with modifications. For this assay, MRS broth without manganese was prepared manually. The composition was as follows: 10 g/l bacteriological peptone, 8 g/l beef extract, 4 g/l yeast extract, 20 g/l D (+) glucose monohydrate, 1 ml of tween 80, 2 g/l diammonium citrate, 5 g of sodium acetate and 0.2 g of magnesium sulfate heptahydrate. MRS broth with manganese was prepared by adding 0.04 g/l manganese (II) sulfate. Isolate was cultured in MRS broth and incubated at 37 °C for 24 h under anaerobic conditions. Then, 1% culture (v/v) was inoculated into two various test tubes, each contained MRS broth with or without manganese. The two test tubes were incubated anaerobically at 37 °C and aliquots of the cultures were sampled upon reaching the exponential and stationary phase. Oxidative stress tolerance was assessed by adding 0.5% (v/v) H₂O₂ to the cultures. Sampling was carried out within 15–20 min after addition of H₂O₂. Collected samples were then serially diluted and spread-plated onto MRS agar and then CFUs were counted. The relative abundance (%) [18] included ratio of the cell number after incubation to that before incubation at 100%

3. Results and Discussion

3.1 Isolation and identification

A pure isolate from the isolation and purification processes was named SU-KC1a. Morphological observation and biochemical activity tests suggested that SU-KC1a was a rod-shaped, Gram positive, catalase negative, non-acid-fast and non-motile bacterium (Figure 1). All these characteristics were similar to those of *Bifidobacterium* and *Lactiplantibacillus* species [19].

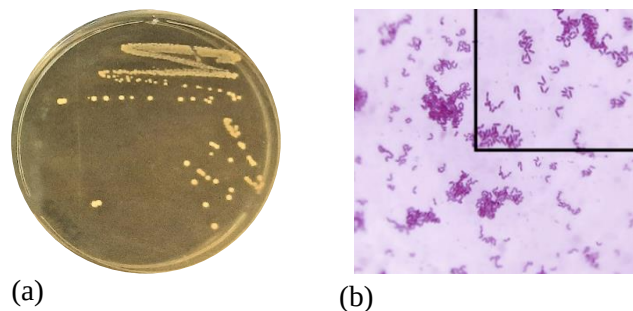


Figure 1. *Lactiplantibacillus plantarum* SU-KC1a, (a) colony morphology on mTPY agar, (b) cell morphology after Gram staining



Isolation and purification processes were carried out anaerobically to achieve anaerobic lactic acid bacteria such as *Bifidobacterium* spp. However, molecular identification based on 16S rRNA showed that SU-KC1a was a species of *L. plantarum*. Since taxonomic nomenclature of *L. plantarum* species was revised to *L. plantarum* in April 2020, isolate was named *L. plantarum* SU-KC1a [20].

Assessing the evolutionary distance of *L. plantarum* SU-KC1a to other *Lactiplantibacillus* strains and *Bifidobacterium* involved constructing a phylogenetic tree using 16S rRNA sequences of *L. plantarum* SU-KC1a, three *L. plantarum* isolated from HBM, three *L. plantarum* isolated from various fermented foods and two *Bifidobacterium* spp. isolated from HBM as an outgroup (Figure 2). Interestingly, lack of clear separations between *L. plantarum* isolated from HBM and those from various fermented foods suggested that niche alone might not be an assessing factor in the evolution of *Lactiplantibacillus* spp. This finding challenged assumption that various niches led to distinct evolutionary paths within the *Lactiplantibacillus* genus. Variability in microbiota within breast milk, affected by factors such as maternal diet and antibiotic use during

pregnancy and lactation, indicates a complex interplay of environmental and genetic factors shaping microbial evolution. This suggests separate evolution of multiple *Lactiplantibacillus* strains within HBM environments.

As many as 590 various genera in breast milk have been detected through 16S rRNA sequencing, ten most frequently detected genera include *Staphylococcus*, *Streptococcus*, *Lactobacillus*, *Pseudomonas*, *Bifidobacterium*, *Corynebacterium*, *Enterococcus*, *Acinetobacter*, *Rothia* and *Cutibacterium* [21,22]. Taghizadeh et al. [23] isolated *Lactobacillus* spp. from breast milk, including *L. gasseri*, *L. rhamnosus*, *L. acidophilus*, *L. plantarum*, *L. reuteri*, *L. fermentum*, *L. animalis*, *L. brevis*, *L. helveticus*, *L. oris*, *L. casei*, *L. gastricus*, *L. vaginalis*, *L. crispatus*, and *L. salivarius*. To further explain relationships between the niche and evolution of *Lactiplantibacillus*, additional analyses such as whole-genome comparisons and metabolic profile assessments are recommended. These analyses can provide deeper insights into the genetic and functional differences between *Lactiplantibacillus* strains originating from various niches, shedding light on their adaptive strategies and evolutionary trajectories.

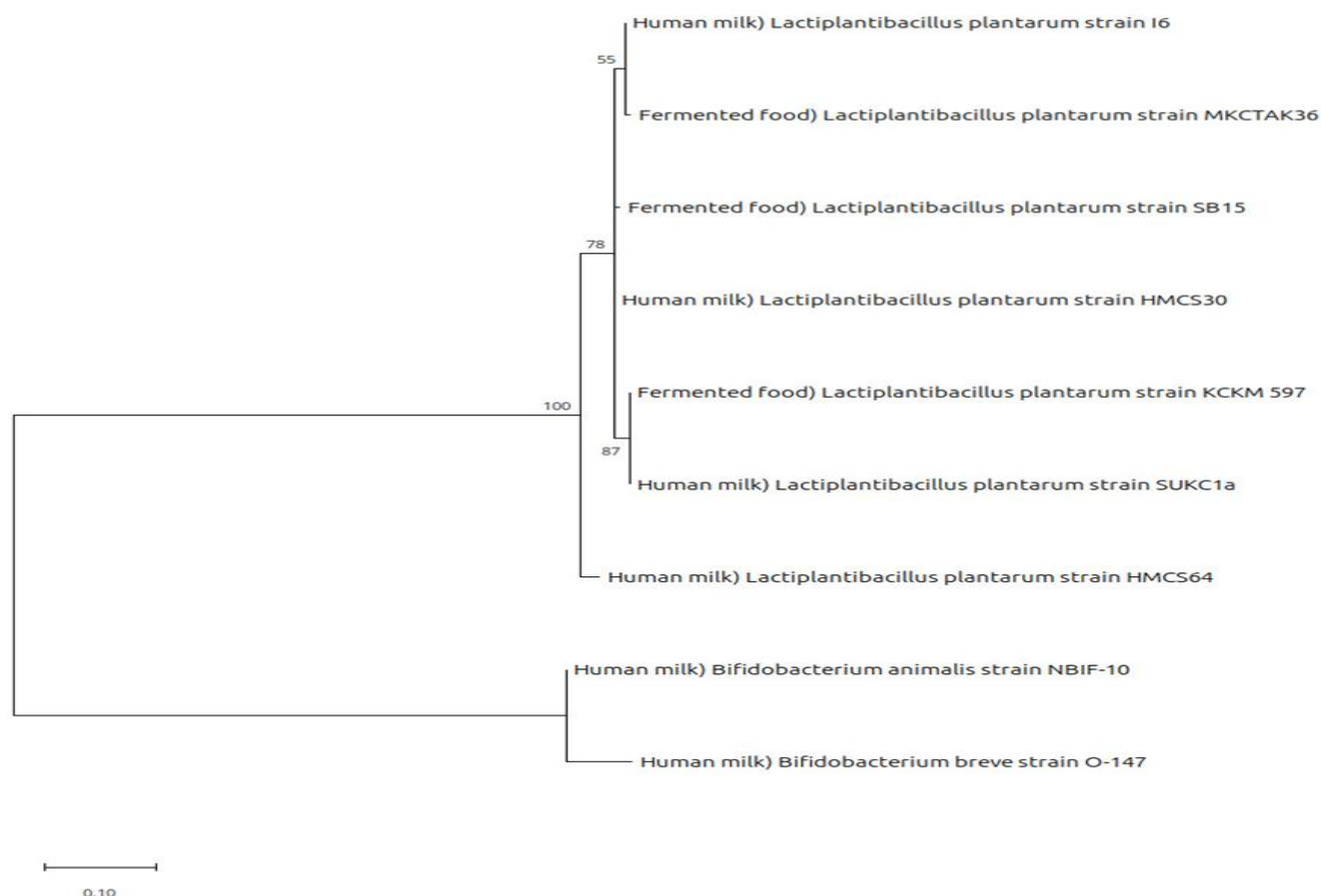


Figure 2. Evolutionary analysis using maximum likelihood method. Evolutionary history was studied using maximum likelihood and Tamura-Nei model [24]. A tree with the highest log likelihood (-4631.69) is shown. Percentage of trees; in which, associated taxa clustered together is shown next to the branches. Initial trees for the heuristic search were generated automatically using neighbor-join and BioNJ algorithms to a matrix of pairwise distances estimated using Tamura-Nei model and then selecting the topology with superior log likelihood value. This analysis involved nine nucleotide sequences. Codon positions included 1st + 2nd + 3rd + noncoding. Totally, 2087 positions were reported in the final dataset. Evolutionary analyses were carried out using MEGAX [25].



3.2 Effects of oxygen on cell morphology

Lactobacillus spp. are addressed for their ability to grow in anaerobic and microaerophilic conditions [26]. Based on the findings of this study, *L. plantarum* SU-KC1a was able to grow without oxygen (anaerobic) and with low oxygen concentrations (microaerophilic). Watanabe et al. [17] reported that *L. plantarum* could capture oxygen in the presence of manganese metal ions and thus provide aerotolerance characteristics. Moreover, MRS media typically contain manganese metal ions in the form of MnSO_4 (0.04 g/l) as a source of nutrition. Figure 3 shows morphology of *L. plantarum* SU-KC1a cells after anaerobic and microaerophilic incubations. Although cells were rod-shaped, anaerobic incubation resulted in shorter rod shape and smaller cell size. According to Parlindungan et al. [27], stress conditions could cause morphological changes in *Lactobacillus* spp. as an adaptive response of bacteria to survive. For microaerophilic *Lactobacillus* spp., anaerobic incubation might cause stress for the cells. To improve survival under stress conditions, rod-shaped LAB formed coccoid-like shapes, which were expected to preserve cell membrane and safeguard internal structures from the external environment, ensuring genetic material intact and preserving low rates of metabolic activities [28]. Morphological changes in *L. plantarum* SU-KC1a under anaerobic and microaerophilic conditions further emphasized adaptive responses of *Lactobacillus* spp. to various oxygen environments. However, further studies are needed to assess the effects of oxygen concentration on the growth and stress responses of *L. plantarum*.

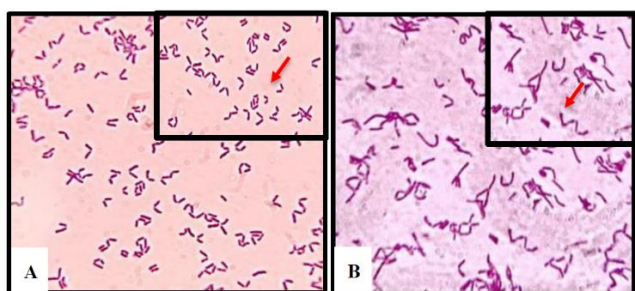


Figure 3. Morphology of *Lactiplantibacillus plantarum* SU-KC1a cells under various incubation conditions, (A) anaerobic and (B) micro-aerophilic. Magnification 1000 $\times$.

3.3 Mupirocin resistance test

In this study, *L. plantarum* SU-KC1a could grow on mupirocin selective media (mMRS) anaerobically and microaerophilically. Furthermore, *L. plantarum* F75 showed no growth on mMRS media (data not shown). This finding suggested that *L. plantarum* SU-KC1a might possess inherent resistance to mupirocin, indicating potential trait variations within strains of similar species. Pechar et al. [29] reported that mupirocin was often added

to the media for isolating bifidobacterial cultures because of the intrinsic resistance characteristics of *Bifidobacterium* spp. However, it is reported that *Lactobacillus* spp. are susceptible to mupirocin. Similar to Sunardi et al. [18], *L. plantarum* SU-KC1a demonstrated high-level resistance to mupirocin that might be acquired by several *L. plantarum* strains. However, *mupA* gene, causing high-level mupirocin resistance, was not detected in the genome of *L. plantarum* SU-KC1a. Therefore, the specific gene responsible for the isolate high-level resistance to mupirocin is still unidentified.

3.4 Carbohydrate fermentation test

L. plantarum SU-KC1a demonstrated ability to ferment glucose, fructose, lactose, FOS, GOS, inulin and maltodextrin, as evidenced by the color changes of the media from purplish brown to yellow-brown. Media without carbon sources, serving as a negative control, showed no color changes overall. This alteration in color within the media indicated shifts in pH (Figure 4). Markowiak-Kopec and Slizewska reported that carbohydrate fermentation by *Lactobacillus* spp. produced organic acids such as lactic acid, acetic acid and short chain fatty acids (SCFA), leading to decreases in pH in the large intestine [30]. Since pH of the media supplemented with various carbohydrates decreased after incubation, these results indicated that *L. plantarum* SU-KC1a was capable to ferment FOS, GOS, inulin and maltodextrin. Analysis of various responsible enzymes for carbohydrate fermentation has been carried out (data are published soon).

The highest growth of *L. plantarum* SU-KC1a was observed in media supplemented with GOS, followed by FOS, inulin and maltodextrin. Ability of bacteria to ferment carbon sources can be affected by the degree of polymerization (DP). GOS, as a prebiotic, includes a DP range of 2–7, making it easier to ferment by bacteria. FOS, inulin and maltodextrin include DPs of > 10, 2–60 and 3–11, respectively. Higher DP values might prolong the fermentation duration of FOS, inulin and maltodextrin, compared to GOS fermentation. Bacterial ability to ferment oligosaccharides with a DP > 10 includes half of the speed; at which, oligosaccharides ferment with a DP < 10 [31]. Cao et al. [32] reported that three oligosaccharides (GOS, FOS and MOS/mannooligosaccharides) were able to enhance growth of *L. plantarum*. In this study, bacterial growth rate was slower when cultured on oligosaccharides rather than glucose. Mandadzhieva et al. [33] reported that most of *Lactobacillus* strains could ferment mono, di and trisaccharides; however, each strain demonstrated a unique fermentation pattern for various carbohydrate sources. Generally, carbohydrates with lower DP are easier to utilize.

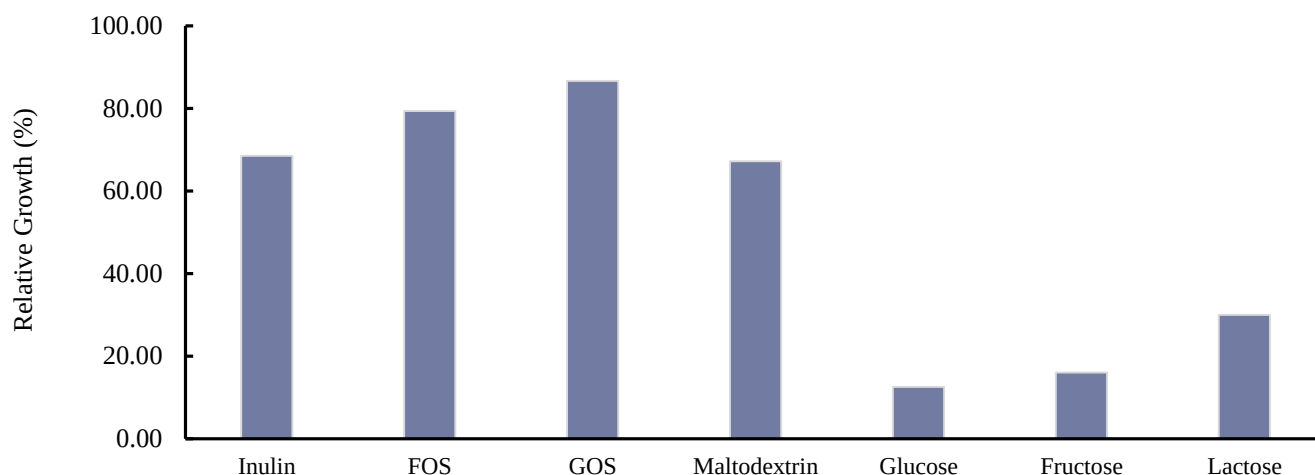


Figure 4. Relative growth of *Lactiplantibacillus plantarum* SU-KC1a cells after 24 h of incubation in MRS broth supplemented with various prebiotics (2%). Growth in MRS broth without prebiotics served as the negative control for comparison.

3.5 Tolerance to various pH levels and concentrations of bile salts

To qualify as a probiotic candidate, resistance of cells to various pH levels and bile salt concentrations is critical for their survival in the digestive tract and during the processing of functional foods. Sunardi et al. [18] reported that *L. plantarum* F75 and *L. plantarum* SU-KC1a were susceptible to simulated gastric fluid (pH 2) but able to tolerate the fluid after 30 and 60 min of exposure. After 120 min of exposure, *L. plantarum* SU-KC1a was still detected but at low numbers [18]. Compared to Sunardi et al. [18] who used simulated gastric fluid with pepsin supplementation, this study used MRS broth adjusted to various pH levels. Figure 5A demonstrates that *L. plantarum* SU-KC1a could preserve its high survival after 3 h of exposure to pH 2. Similar to Huang et al. [34], several *L. plantarum* strains

demonstrated minimal or no decreases in cell viability even after 6 h of exposure to low pH and digestive enzymes (pepsin and trypsin).

A previous study by de Melo Pereira et al. [35] included that bile salt resistance within a concentration range of 0.3–2% was a prerequisite for surviving passage through the human digestive tract. As depicted in Figure 5B, number of *L. plantarum* SU-KC1a cells decreased with increasing bile salt concentration; however, cells demonstrated robust survival within all concentrations of bile salts. Another study by Sunardi et al. [18] reported that *L. plantarum* F75 and SU-KC1a were able to survive exposure to 0.3% of bile salts for 3 h. This phenotype was attributed to the capacity of various *L. plantarum* strains to produce bile acid hydrolases, which catalyzed conversion of conjugated bile salts into free bile salts [36].

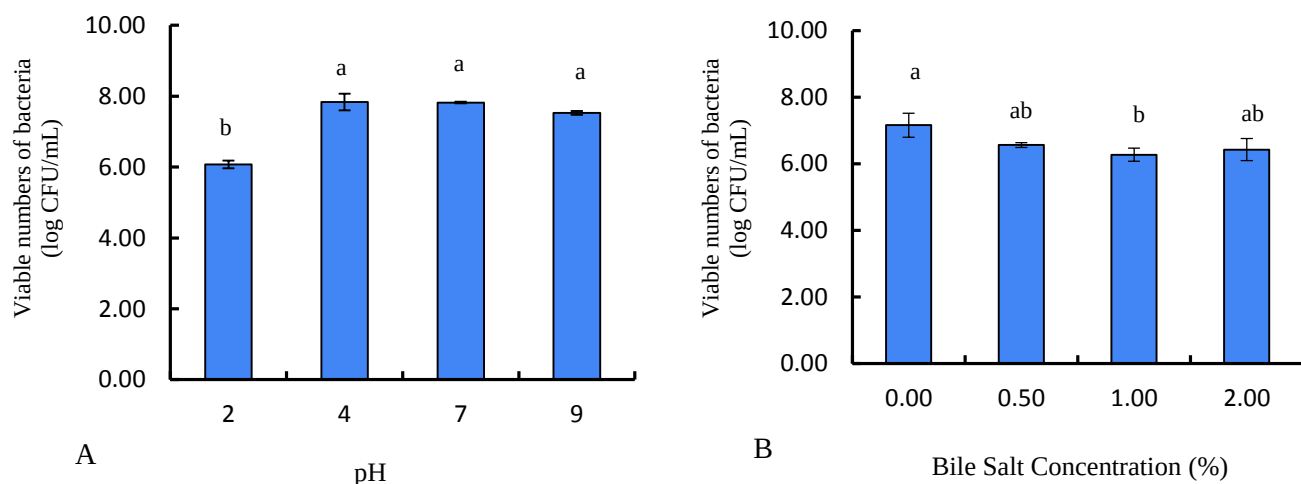


Figure 5. Survival of *Lactiplantibacillus plantarum* SU-KC1a for 3 h at 37 °C under various conditions. (A) Effects of various pH levels, and (B) effects of various bile salt concentrations. Initial cell concentrations for each test were similar.



3.6 Antibiotic susceptibility

Table 1 shows that *L. plantarum* SU-KC1a demonstrated resistance to three antibiotics, including vancomycin, streptomycin and nalidixic acid from various classes. These findings were partially similar to those of Sunardi et al. [18] who reported that *L. plantarum* SU-KC1a was resistant to antibiotics such as vancomycin and nalidixic acid. Antibiotic susceptibility tests in the two studies were carried out using different media: mTPY agar in this study and Mueller-Hinton agar in Sunardi et al. [18]. It is noteworthy that resistance profile of *L. plantarum* SU-KC1a was not unique, as antibiotic resistances in *Lactobacillus* spp. to β -lactam, glycopeptides, tetracycline, lincosamides and quinolones (nalidixic acid) have previously been documented. According to Mancino et al. [37], *L. plantarum* was typically resistant to aminoglycosides (gentamycin, neomycin, streptomycin and kanamycin), bacitracin, quinolone (nalidixic acid) and vancomycin. One of these resistances is intrinsic resistance to *Lactobacillus* spp., meaning that this resistance type cannot be transferred to other bacteria, suggesting that *L. plantarum* SU-KC1a may be safe for use as a probiotic. If resistance genes are located on plasmids, there is a risk of horizontal gene transfer to other bacteria [38].

3.7 Antimicrobial activity

Since the beginning of this century, numerous serious bacterial infections have evolved resistance to commonly prescribed antibiotics, creating major healthcare issues worldwide. Hence, there are urgent needs to investigate novel natural sources for antibacterial compounds. Antimicrobial activity of *L. plantarum* SU-KC1a against *E. coli* and *S. aureus* was assessed based on the inhibition zone. Based on the diameter of inhibition zones (Figure 6), *L. plantarum* SU-KC1a demonstrated greater inhibitions to *E. coli* (5.0 ± 0.7 mm), compared to *S. aureus* (2.0 ± 0.7

mm). Malanovic and Lohner [41] reported that antimicrobial activity of *Lactiplantibacillus* spp. was more visible in Gram-negative bacteria than Gram-positive bacteria. Gram-negative bacteria include outer membranes that are selective for antimicrobials. In this study, concentration of antimicrobial compounds in the cell-free supernatant (CFS) could not be assessed, potentially leading to various results. Further investigations regarding antimicrobial activity can be carried out using broth dilution and time-kill assays [42].

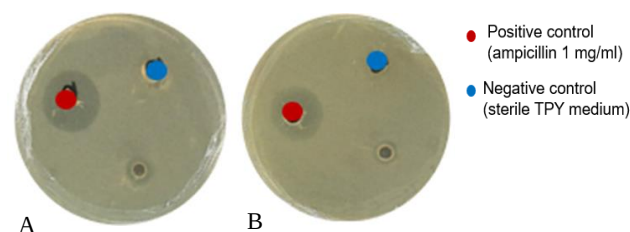


Figure 6. Antibacterial activity of *Lactiplantibacillus plantarum* SU-KC1a against pathogens of (A) *Escherichia coli*, and (B) *Staphylococcus aureus*

Lactiplantibacillus spp. possess numerous mechanisms for eliminating pathogenic bacteria, including production of inhibitory components such as bacteriocins and lactic acid. Bacteriocins produced by *L. plantarum*, commonly known as plantaricins, encompass various peptide structures and are generally classified into two main classes. Currently, various types of plantaricin have been identified, including plantaricins A, C, D, E, F, J, K, S, T, Y, 423, 163, 149, 35D, BN, SA6, LC74, KW30, ZJ008, LD1, UG1, NC8, C11 and NA [43]. Plantaricin EF, targets the bacterial membrane, leading to the leakage of intracellular compounds and eventual cell death [44].

Table 1. Susceptibility of *Lactiplantibacillus plantarum* SU-KC1a to various antibiotics

Class	Antibiotic	Zone of Inhibition (mm)	Category	
			<i>Bifidobacterium breve</i> BS2-PB3 ¹	<i>Lactobacillus</i> species
Aminopenicillins	Ampicillin	34.5	Susceptible	Susceptible ²
Macrolides	Erythromycin	35.0	Susceptible	Variable ²
Phenicol	Chloramphenicol	37.0	Susceptible	Variable ²
Polypeptide	Bacitracin	13.5	Susceptible	N/A
Rifampin	Rifampicin	25.0	Susceptible	Variable ²
Glycopeptide	Vancomycin	6.0	Susceptible	Resistance ²
Aminoglycoside	Neomycin	16.0	N/A	N/A
	Streptomycin	10.0	Susceptible	Resistance ²
	Kanamycin	12.0	Susceptible	Variable ³
	Gentamicin	19.0	N/A	Variable ²
Quinolones	Nalidixic acid	6.0	N/A	Resistance ²
Tetracycline	Tetracycline	15.0	Susceptible	Variable ²

Note: ¹Marbun et al. [39]; ²Parlindungan et al. [27]; ³Campedelli et al. [40]. Antibiotic susceptibility test was carried out using mTPY broth. All measurements include a 6-mm disc diameter. Numbers enclosed in grey boxes indicate the resistance phenotype.



3.8 Mucosal adhesion ability

In this study, modified adherence assay was carried out to assess the bacterial adhesive capability on purified mucin from pig intestines. Figure 7 shows that *L. plantarum* SU-KC1a could adhere to purified mucin from pig intestines as well as BSA. However, adhesion percentage to mucin was consistently higher than that to BSA. Bacterial adhesion does not only depend on protein-protein interactions, but depends on hydrophilic interactions between non-protein components. These non-protein components include polysaccharides, which are present in mucin [45].

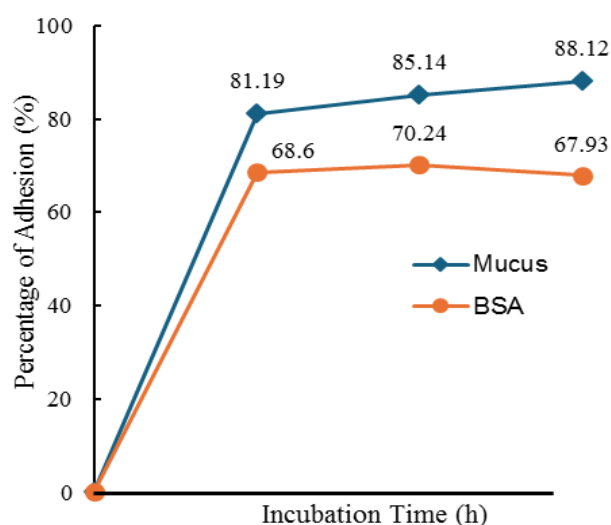


Figure 7. Proportions of adhesion of *Lactiplantibacillus plantarum* SU-KC1a to mucin and BSA.

Adhesion of *L. plantarum* SU-KC1a to mucin reached 81.2% after 1 h of incubation, indicating high adhesive capabilities of the bacteria to mucin. Adhesion ability was nearly twice as high as the adhesion percentage of *L. plantarum* 299V, a *Lactiplantibacillus* strain with an adhesion percentage of $48.49\% \pm 1.36$ [46]. However, the modified procedures in this study might contribute to the results. Uraipan et al. [46] carried out rinsing 12 times, while this study only carried out two rinses. Decision to rinse twice was made based on the preliminary experiments, which showed no significant differences in the bacterial adhesion proportions after two and five rinses (data not shown). Additionally, effects of incubation time on the adherence of *L. plantarum* SU-KC1a to immobilized mucin on microplates were assessed. In this study, incubation times for adhesion were set to 1, 2 and 3 h to cover time needed by the food to be emptied from the human small intestine, approximately 2–7.5 h [47]. Adhesion percentage of *L. plantarum* SU-KC1a to mucin increased with longer incubation times, suggesting that the contact time between the cells and mucin material might affect adhesion ability *in vitro*. A similar trend was observed in the adhesion of *L. paracasei* SP5 to HT-29 colon cancer cells (*in vivo*), which

increased from 40 to 60% after 4 h of incubation [48]. A higher percentage of adhesion during longer incubation times might be attributed to sedimentation or settling of bacteria. According to Mays et al. [49], further bacterial cells settled on the mucin surface as the incubation time increased, leading to increased bindings between the bacterial cells and mucin. However, this condition was unique to *in-vitro* adherence assays and might not necessarily occur *in vivo*.

3.9 Oxidative stress tolerance

In this study, oxidative stress tolerance of *L. plantarum* SU-KC1a to hydrogen peroxide was assessed at exponential and stationary phases. Cells were incubated anaerobically to induce oxidative stress alone through H_2O_2 exposure. Two different media were used, including MRS broths with and without manganese. Manganese plays an important role in response to oxidative stresses; therefore, addition of manganese to media was expected to enhance viability of the culture when assessed with H_2O_2 [17]. Figure 8 highlights the significance of manganese for bacterial growth in the presence of H_2O_2 . In *L. plantarum*, the role of manganese in combating oxidative stress is critical, particularly because the microorganism lacks the superoxide dismutase (SOD) enzyme and relies solely on pseudocatalase alone (catalase with a manganese metal core). Naturally, manganese complexes with polyphosphate in the absence of SOD and serves as a cofactor for the enzymes such as peroxidases [50]. This may explain why H_2O_2 did not delay the growth of *L. plantarum* SU-KC1a. Furthermore, the effects of manganese supplementation varied between cultures in the exponential and stationary phases. Cultures supplemented with manganese showed higher resistance rates to H_2O_2 exposure, while those lacking manganese demonstrated greater susceptibilities. This observation was similar to that of Watanabe et al. [16], who reported similar divergent effects of manganese supplementation in *L. plantarum* strain WCFS1 cultures within various growth phases in response to H_2O_2 stress.

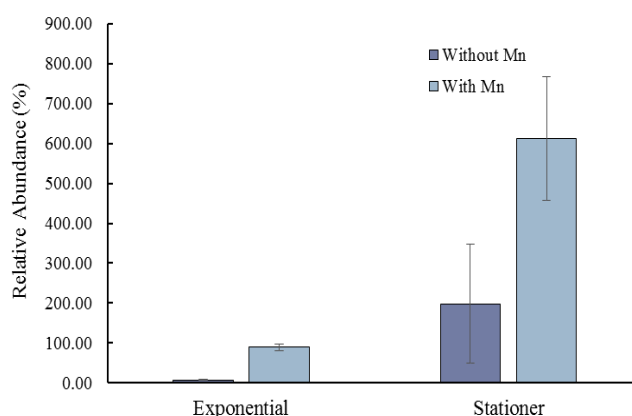


Figure 8. Relative abundance of *Lactiplantibacillus plantarum* SU-KC1a after exposure to hydrogen peroxide



They detected that exponential-phase cultures were more susceptible to H₂O₂, compared to that stationary-phase cultures were. According to Sousa-Lopes et al. [51], cell membrane permeability to H₂O₂ increased in exponential phase, compared to stationary phase.

4. Conclusion

In conclusion, *L. plantarum* SU-KC1a, originally isolated from HBM in Indonesia, demonstrated significant characteristics as a promising candidate for probiotic uses. This strain demonstrated robust oxidative stress tolerance and antibiotic resistance, particularly to mupirocin, as well as showing ability to thrive with various prebiotic supplementations. Its strong tolerance to acidic conditions and bile salts as well as its significant antimicrobial characteristics and adhesive capabilities to mucin underscore the microbial suitability for diverse environments. These attributes not only highlight its relevance in local contexts, but also suggest its potential as a valuable candidate for probiotics development on a global scale, offering possible benefits for human health and well-being. Further studies are essential to fully realize the microbial therapeutic and commercial potentials.

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6. Conflict of Interest

The authors report no conflict of interest.

7. Authors Contributions

Conceptualization, T.T.J.; methodology, M.S. and H.V.; data collection, Y.C., A.F.R, J.O., E.T.P., A.C.J.; writing—original draft preparation, M.S.; writing—review and editing, J.J. and T.T.J. All authors have read and approved final version of the manuscript.

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ارزیابی ویژگی‌های زیست‌یاری لاکتی پلانتهی باسیلوس پلانتروم SU-KC1a جدا شده از شیر مادر در اندونزی

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۶۲-۲۱-۵۴۶۰۹۱۰+

چکیده

سابقه و هدف: باکتری‌های لاکتیک اسید مانند گونه‌های لاکتی پلانتهی باسیلوس و بیفیدوباکتریوم به دلیل دارا بودن ویژگی‌های مفید مورد توجه قرار می‌گیرند. این مطالعه با هدف ارزیابی ویژگی‌های زیست‌یاری^۱ لاکتی پلانتهی باسیلوس پلانتروم SU-KC1a جدا شده از شیر مادر در اندونزی انجام شد.

مواد و روش‌ها: لاکتی پلانتهی باسیلوس پلانتروم SU-KC1a به صورت بی‌هوازی از شیر مادر جدا و سپس از نظر ریخت‌شناسی^۲ و بیوشیمیایی بررسی شد. ویژگی‌های زیست‌یاری، از جمله تحمل سطوح گوناگون pH و غلظت‌های نمک صفراوی، حساسیت آنتی‌بیوتیکی، فعالیت ضد میکروبی، توانایی چسبندگی به مخاط و تحمل استرس اکسیداتیو مورد ارزیابی قرار گرفت.

یافته‌ها و نتیجه‌گیری: لاکتی پلانتهی باسیلوس پلانتروم SU-KC1a میله‌ای شکل، فعالیت کاتالاز منفی، رنگ‌آمیزی گرم مثبت و فاقد ثبات اسیدی و تحرک بود. در شرایط استرس، این سویه شکلی شبیه کوکوئید به خود گرفت تا از غشای سلولی و ساختارهای داخلی خود در برابر محیط‌های خارجی محافظت کند. به طور قابل توجهی، به موپیروسین مقاومت نشان داد که آن را از سایر لاکتوباسیل‌ها متمایز کرد. این سویه در حضور پری‌بیوتیک‌ها، به ویژه گالاتولیگوساکاریدها، رشد بیشتری داشت. علاوه بر این، ویژگی‌های زیست‌یاری مانند تحمل بالا نسبت به تغییرات pH و نمک‌های صفراوی، فعالیت بالای ضد میکروبی در برابر عوامل بیماری‌زا، چسبندگی قوی به سطوح مخاطی و مقاومت در برابر استرس اکسیداتیو مشاهده شد. این یافته‌ها لاکتی پلانتهی باسیلوس پلانتروم SU-KC1a را به عنوان زیست‌یاری امیدوارکننده مناسبی برای ترکیب در غذاهای فراسودمند^۳ برجسته می‌کند.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافع مرتبط با انتشار این مقاله ندارند.

^۱ Probiotic attributes

^۲ Morphologically

^۳ Functional foods

