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Cell Surface and Adherence Properties of Indonesian Indigenous Lactic Acid Bacteria as Probiotic Candidate

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Abstract

Adherence to intestinal mucosa is one of the crucial probiotic traits. This ability varied among strains. This study aims to evaluate the cell surface properties and adherence potential of Indonesian Indigenous LAB. The adherence potential was evaluated using auto- aggregation, coaggregation against *Salmonella*, cell hydrophobicity, and adherence to stainless-steel surface. All strains classified as having medium high aggregation (>90%) after 24 h of incubation and can coaggregate with *Salmonella* (58-92%). Among all strains, *Levilactobacillus brevis* AB3 showed the highest hydrophobicity (36%) and adhesion to stainless steel (6.37 log CFU/mL). Current findings suggest that Indonesian Indigenous LAB, especially *L. brevis* AB3, possessed an adherence property and has a potential to be developed into probiotic bacteria.

Keywords: adherence, aggregation, hydrophobicity, intestinal mucosa, probiotic

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Introduction

Probiotics have gained significant attention in recent years due to their potential health benefits and therapeutic (Da Cruz Rodrigues et al., 2020; Harahap et al., 2021; Hossain et al., 2022). These living microorganisms, when consumed in adequate amounts, confer numerous advantages by modulating the gut microbiota, promoting immune responses, and improving overall gut health (Food and Agriculture Organization of the United Nations. & World Health Organization., 2006). However, one critical factor that influences their efficacy is their ability to adhere to the intestinal mucosa, as this determines their colonization potential and interaction with host cells (Monteagudo-Mera et al., 2019). Understanding the adhesion capability of probiotic candidates is crucial for optimizing their beneficial effects and developing more targeted and effective probiotic formulations.

The adhesion of probiotic strains to the intestinal epithelium is a multifaceted process involving various mechanisms, such as cell surface properties, specific molecular interactions, and host-related factors (Deepika & Charalampopoulos, 2010). Several studies have identified certain characteristics that contribute to the adhesive properties of probiotic bacteria, including the production of extracellular proteins in *Lactiplantibacillus plantarum subsp. plantarum* Dad-13 and Mut-7 (Darmastuti et al., 2021), expression of fimbriae or pili in *Lacticaseibacillus rhamnosus* GG (Krishnan et al., 2016), and the presence of mucus-binding proteins in *Lactobacillus sp.* (Muscariello et al., 2020). These attributes allow probiotics to adhere to the mucosal surfaces, form biofilms, and establish a close relationship with the host, which is essential for exerting their functional effects.

While numerous probiotic strains have been investigated for their potential health benefits, not all strains exhibit the same degree of adhesion capability (Krausova et al., 2019). Variations in adhesion abilities among different probiotic candidates can be attributed to inherent genetic variations, ecological niche

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adaptations, or specific host-microbe interactions (Deepika & Charalampopoulos, 2010). Consequently, the identification and characterization of probiotic strains with superior adhesion properties are pivotal for developing more targeted interventions to address specific health conditions.

Probiotic strains can be isolated from several sources. The most abundant probiotic sources are traditional fermented food. Indonesia is known for its rich traditional food, especially traditional fermented food. Several studies have isolated Indonesian Indigenous LAB have that possessed numerous health benefits (Amelia et al., 2021; Ramadhanti et al., 2021; Yusuf et al., 2020). Nevertheless, isolation of LAB that has superior adhesion properties is limited. Whereas adhesion properties is crucial to assessed the probiotic potential of microbes. Our laboratory successfully isolated several Lactic Acid Bacteria (LAB) strains from Indonesian traditional food. Isolated LAB has been proven to tolerate gastric juice and bile salts. Moreover, Indonesian Indigenous LAB possessed antimicrobial properties against several foodborne pathogen (Febriana et al., 2021); therefore, have a potential to be a probiotic candidate. Probiotic adherence properties and their mechanism differs among strains. This study aims to investigate the adherence properties, moreover the cell surface characteristics to determine the potential adherence mechanisms as probiotic candidates.

Material and Methods

Microorganisms and Inoculum Preparation

The Indonesian Indigenous LAB used in the current study was shown at Table 1. The LAB strains were obtained from the Laboratory of Teknobio-Pangan, Faculty of Biotechnology, Universitas Atma Jaya Yogyakarta. The probiotic bacteria, *Lacticaseibacillus rhamnosus* GG was used as positive control and obtained from Paedicare Co. Ltd (United States). The pathogen bacteria *Salmonella typhimurium* IFO 12529 for the Coaggregation assay was obtained form the Food & Nutrition Culture Collection (FNCC), Center for Food and Nutrition Studies, Universitas Gadjah Mada, Yogyakarta, Indonesia.

The microorganisms were rejuvenated before used. Frozen stock culture of LAB and *Salmonella* was grown in the De Mann Rogosa Sharpe (MRS) Broth (Merck, Germany) and Nutrient Broth (NB) (Merck, Germany), respectively. Cultures were incubated (Memmert, Germany) at 37 °C for 24 hours twice. The initial viable cells were around 8 log CFU/mL.

Visual Aggregation Assay

The visual aggregation of LAB was determined by visual observation (Collado et al., 2008). Visual aggregation showed the LAB ability to form a colony which was indicated by aggregates formation. Culture of LAB was grown in the MRS Broth at 37 °C for 24 h. Visual appearance of the culture was visually observed. Positive results showed an aggregates formation, meanwhile negative results showed no aggregates formation. The viable cell was also determined using dilution and platting method using MRS Agar and optical density (OD600) using spectrophotometer (Thermo Scientific, United States).

Auto-aggregation Assay

The ability of LAB to interact with the same species was studied by the auto-aggregation assay (Grigoryan et al., 2018). The rejuvenated cultured were washed using phosphate buffer solution (PBS) (pH 7.2) twice. The culture was centrifuged (IKA, Germany) at 3500 rpm for 15 min. The supernatant was discarded, and PBS solution was added. The culture was vortexed and centrifuged again with the same condition. The washed culture was incubated at room temperature (27 ± 0,5 °C) for 24 h and the OD was measured at 600 nm. The auto-aggregation percentage was calculated using Eq. (1).

$$Autoaggregation\ (\%) = \left(A_0 - \frac{A_t}{A_0}\right) \times 100 \quad (1)$$

where A_t represents OD at 24 h and A_0 represents OD at 0 h.

Tabel 1. Indonesia Indigenous LAB Strains isolated from Indonesian Traditional Food Used

Isolate	Species	Origin	Origin Description
K1	*Leuconostoc mesenteroides*	Kepel	Fruit produced from *Stelechocarpus burahol*, annonaceous plant from the humid evergreen forests of Southeast Asia, grown in Central Java, Indonesia.
AB3	*Levilactobacillus brevis*	Asinan Bogor	Traditional pickled fruits from Bogor, consisted of papaya, unriped mango, jicama, snake fruit, pineapple, cucumber, guava, served with asinan sauce consist of peanut, vinegar, tamarind, and chilies.
G4	*Enterococcus faecium*	Gatot	Traditional snacks from Gunung Kidul, Indonesia, made from dried cassava.
R4	*Enterococcus durans*	Rusip	Traditional sambal from Bangka, made from anchovy. Salt and palm sugar was added and then spontaneously fermented.

Coaggregation Assay

The ability of LAB to interact with pathogen species, e.g., *Salmonella*, was studied by the coaggregation assay (Grigoryan et al., 2018). Two milliliters of LAB culture were washed by PBS and added with 2 mL of *Salmonella* culture (1:1, v/v). The suspension was vortexed and incubated at room temperature (27 ± 0,5 °C) for 5 and 24 h and the OD was measured at 600 nm. The coaggregation percentage was calculated using Eq. (2).

$$Coaggregation\ (\%) = \frac{\left(\frac{A_x + A_y}{2}\right) - A(x+y)}{\left(\frac{A_x + A_y}{2}\right)} \times \quad (2)$$

where x and y represent each of the two strains in the control tubes, and (x + y) the mixture.

Hydrophobicity Assay

The hydrophobicity of the LAB cell surface was studied based on bacterial adhesion to hydrocarbons (BATH) (Rokana et al., 2018). The LAB culture was centrifuged at 3500 rpm for 15 min. The supernatant was discarded, and PBS solution was added to wash the culture. Three milliliters of culture suspension were added with 1 mL hydrocarbon (xylene). Xylene was chosen as an apolar solvent because it reflects cell surface hydrophobicity and hydrophilicity. The two-phase system was thoroughly mixed by vortexing. Cell suspension was then incubated at 37 °C for 5 min to let the suspension separate. The aqueous phase was removed and its absorbance at 600 nm was measured. Affinity to hydrocarbons (hydrophobicity) was reported as adhesion percentage according to Eq. (3).

$$Hydrophobicity\ (\%) = \left(OD_i - \frac{OD_t}{OD_i}\right) \times 100 \quad (3)$$

where OD_i and OD_t represent the OD before and after hydrocarbon addition, respectively.

Bacterial Adherence Assay

The LAB ability to adhere to the intestinal mucosa was simulated using the Bacterial Cell Adhesion to Stainless Steel Coupon Surface Method (Maciel et al., 2010). The Stainless-Steel Coupon (AISI 304, (2x25x27) mm) was sanitized and sterilized before used. The coupon was washed using acetone and soaked in alkaline detergent (1% NaOH) for 1 h to detach the natural biofilm microbe in the coupon. The coupon was rinsed with sterile water and dried for 2 h at 60 °C. The coupon was the sterilized using autoclave (Hirayama, Japan) at 121 °C for 15 minutes. The coupon was placed inside a sterile petri dish until used.

To determine the LAB ability to adhere to the stainless-steel coupon, the sterile coupon was immersed into the LAB suspension (8 log CFU/mL) or PBS (negative control) for 24 h. The coupon was incubated at 37 °C for 24 h. The coupon was aseptically removed and washed using 0.85% NaCl twice. One cm^2 part of the coupon was swabbed using sterile cotton bud. The swabs were transferred to test tubes containing 10 mL of saline solution and stirred in vortex for one minute. Serial dilutions of up to 10^{-6} were made in test tubes containing 9 mL of saline solution. Aliquots of 1000 µL of each dilution were inoculated in Petri dishes containing MRS Agar using the pour plate technique.

Data Analysis

Experiment was conducted in triplicate and three analysis replications. Data was shown in Mean±standard deviation. Data was analyzed using one way ANOVA with a significance level of $p<0.05$ followed by Duncan's Multiple Range Test. Analysis was performed using IBM SPSS Statistic 20 software (IBM, United States). P-values below 0.05 were considered statistically significant.

Results and Discussion

Aggregation Potential of Indonesian Indigenous LAB

The ability of Indonesian Indigenous LAB to interact with other microbe was observed from its visual aggregation, auto-aggregation, and coaggregation with *Salmonella*. Figure 1 shows the visual aggregation of the LAB isolates. All isolates demonstrated no visual aggregation (Fig 1.). No aggregate formation was found. On the other hand, the positive control, *L. rhamnosus* GG demonstrated distinct aggregate formation. Sumarni (2011) reported *L. plantarum* D-01, *L. lactis D-01*, *L. acidophilus* Y-01 dan *B. longum* Y-01 isolated from isolated from *dadiah* and yogurt have high aggregation proven by distinct aggregate formation. Nevertheless, results showed all LAB isolate viable cells reached 8 log CFU/mL and optical density (OD_{600}) ranging 0.8-1 (Table 2.). There is no significant difference between the OD_{600} even though the viable cell demonstrated a significant difference between isolates.

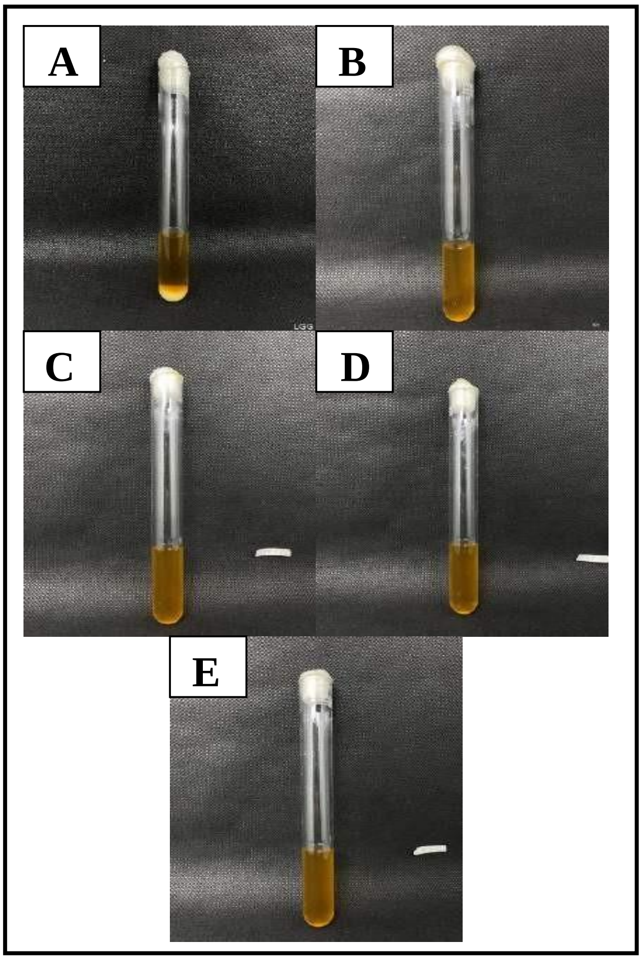

Figure 1. Visual Aggregation of (**A**) *L. rhamnosus* GG; (**B**) *L. mesenteroides* K1; (**C**) *L. brevis* AB3; (**D**) *E. faecium* G4; and (**E**) *E. durans* R4.

Tabel 2. Viability of Indonesia Indigenous LAB Strains

Isolate Cell	Viable (log CFU/mL)	OD_{600}
L. mesenteroides K1	8.48 ± 0.08^a	0.87 ± 0.10^a
L. brevis AB3	8.84 ± 0.06^c	0.94 ± 0.01^a
E. faecium G4	8.67 ± 0.07^b	1.04 ± 0.17^a
E. durans R4	8.91 ± 0.04^c	0.86 ± 0.01^a

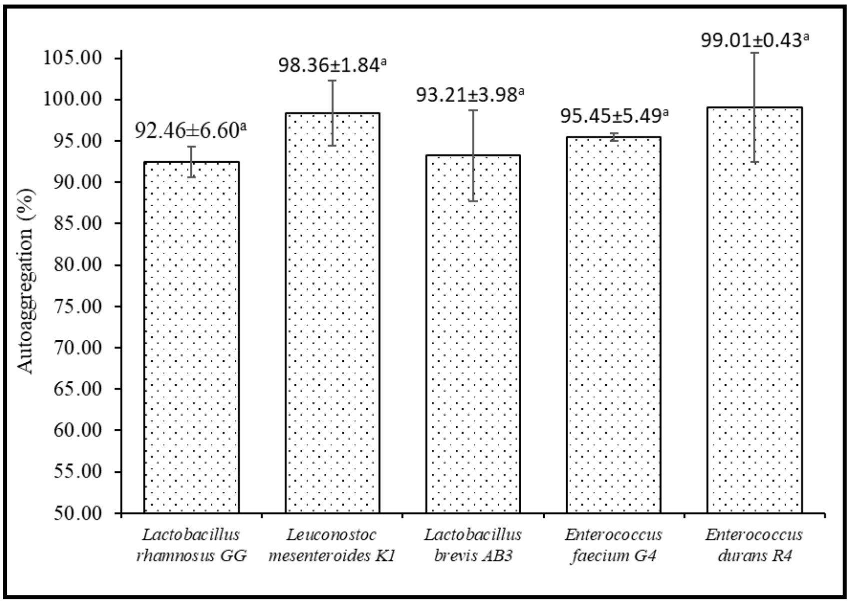

Figure 2. Auto-aggregation Properties of Indonesian Indigenous LAB. Values are expressed as mean±SD. Values with different superscripts are significantly different ($p < 0.05$) by Duncan's multiple range test.

Even though the visual aggregation did not show a distinct aggregate formation, the auto- aggregation results demonstrated a high aggregation (Figure 2). The auto-aggregation percentage of all LAB isolates were between 92.46-99.01% after 24 h incubation. All LAB isolates have a higher auto- aggregation percentage compared to probiotic bacteria *L. rhamnosus* GG. However, statistical analysis revealed no significant difference between probiotic and Indonesian Indigenous LAB. This finding points out that even though there is no distinct visual aggregate formation, LAB can still interact with the same species.

The ability of the LAB isolates to interact with other species, especially pathogens like *Salmonella* was shown in Figure 3. Results showed that the coaggregation was lower compared to the auto-aggregation. Coaggregation at 24 h was higher than to 5 h. At 5 h incubation, *L. mesenteroides* K1 demonstrated the highest coaggregation percentage. On the contrary, the coaggregation after 24 h was found to be the lowest. This finding indicates that *L. mesenteroides* K1 can interact with *Salmonella* faster but weaker compared to other strains. Two Indonesian Indigenous LAB, *L. brevis* AB3 and *E. faecium* G4, showed a significantly higher coaggregation with *Salmonella* compared to probiotic bacteria *L. rhamnosus* GG after 24 h of incubation. Current finding indicate that the Indonesian indigenous LAB strains have similar aggregation properties with probiotic bacteria L. rhamnosus GG even though did not show visual aggregation.

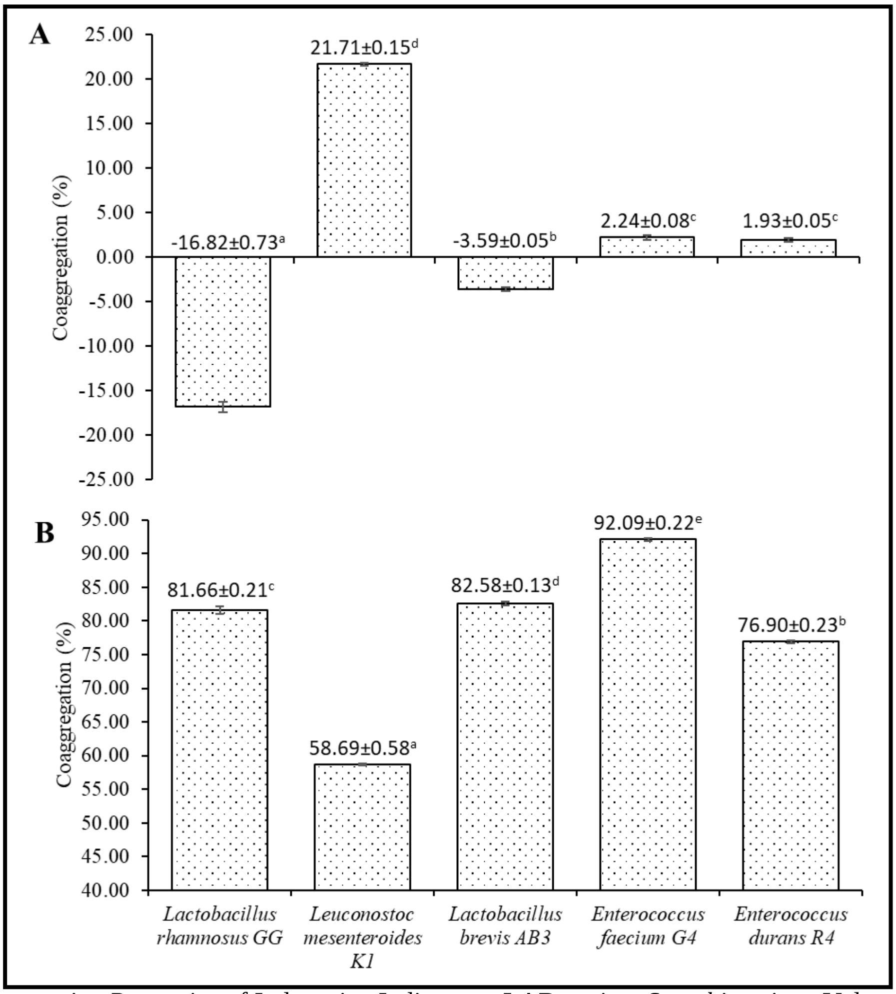

Figure 3. Coaggregation Properties of Indonesian Indigenous LAB against *S. typhimurium*. Values are expressed as mean±SD. Values with different superscripts are significantly different ($p < 0.05$) by Duncan's multiple range test.

Aggregation properties of LAB is the ability of microbe to interact with other microbe. Auto-aggregation is the ability to interact with the same species; on the other hand, coaggregation is the ability to interact with different species. Several auto-aggregation properties of probiotic isolated from traditional foods have been reported. *Leuconostoc mesenteroides* J.27 isolated from kimchi, *Enterococcus faecium* R2 isolated from Intestinal microbiota of carp, *Lactiplantibacillus plantarum subsp. plantarum* Mut-3 isolated from Gatot, had varied auto-aggregation percentage, ranging from 66.677 ± 0.95% (Hossain et al., 2022); 93.726 ± 3.87% (Manvelyan et al., 2023); and 57.5 ± 5.5 (Darmastuti et al., 2021), respectively. Current strains possessed a higher auto-aggregation percentage compared to previous study. Nevertheless, Hojjati et al. (2020) reported a faster auto-aggregation formation of *Levilactobacillus brevis* AB3 isolated from "Khikki", Iranian Traditional Cheese, which was 40% after 30 minutes of incubation. Moreover, Grigoryan et al. (2018) found a faster auto-aggregation in *E. durans*, which was 20-40% after 1 hour of incubation. Auto-aggregation of probiotic candidates isolated from other sources has also been reported. Several *Lactobacillus* species from animal origin and human milk possessed auto-aggregation ability ranging from 6.1-76.0% and 40-80%, respectively (Krausova et al., 2019; Rokana et al., 2018).

Coaggregation against pathogen was a crucial factor in determining the probiotic potential of LAB strains. Inhibitory or bacteriocin producing LAB that co-aggregate with pathogens may play an important role in host defense mechanisms against infection (Rickard et al., 2003). *Salmonella* is a pathogen that can cause foodborne diseases from bacterial infections. Previous studies have reported the coaggregation potential of probiotic candidates against *Salmonella*. *Lactobacillus plantarum* isolated from homemade cow and sheep cheese possessed coaggregation properties against *S. thyphimurium* between 30.5-40.5% (Janković et al., 2012), lower compared to current strains. Coaggregation against other pathogens has also been studied. Aziz et al., (2019) reported that several *Lactobacillus* species isolated from chicken gut can co-aggregate with *Citrobacter* and *L. monocytogenes*, ranging from 44-61% and 21-79%, respectively. Kumar et al. (2020) demonstrated coaggregation of *Lactobacillus casei ssp. casei* and *Lactobacillus acidophilus* against *Escherichia coli* which were 7.8% and 19.73%, respectively.

Several mechanisms of bacterial aggregation properties have been reported. (Fukao et al., 2019) mentioned that exopolysaccharide production mediated by the plasmid-encoded glycosyltransferase is responsible for the auto-aggregation. It was predicted that exopolysaccharide mediates cell to cell attachment. Secretion of other components of the cells also might play an important role in cell interaction. Adhesin, one of the proteins found on the surface of LAB cells, responsible for the colonization process of the bacterial cell surfaces (Re et al., 2000). It was also reported that this ability varied among strains (Rokana et al., 2018). Apart from secretion of specific cell components, cell surface properties also play a role in the cell interaction with other cells and gastrointestinal surface through hydrophobic group interaction (Collado et al., 2008).

Cell Surface Hydrophobicity

The four Indonesian Indigenous LAB isolates along with probiotic reference strains of *L. rhamnosus* GG strains were investigated for the cell surface hydrophobicity using BATH assay (Figure 4). All strains show a positive hydrophobicity. Moreover, three isolates; *L. rhamnosus* GG, *L. brevis* AB3, and *E. durans* R4, demonstrated a higher hydrophobicity cell surface compared to other strains. *Levilactobacillus brevis* AB3 has the highest percent hydrophobic value, 39.97%. It was significantly higher compared to the probiotic bacteria, *L. rhamnosus* GG. *Enterococcus durans* R4 also demonstrated a significantly higher percent hydrophobic value than *L. rhamnosus* GG even though did not surpass *L. brevis* AB3.

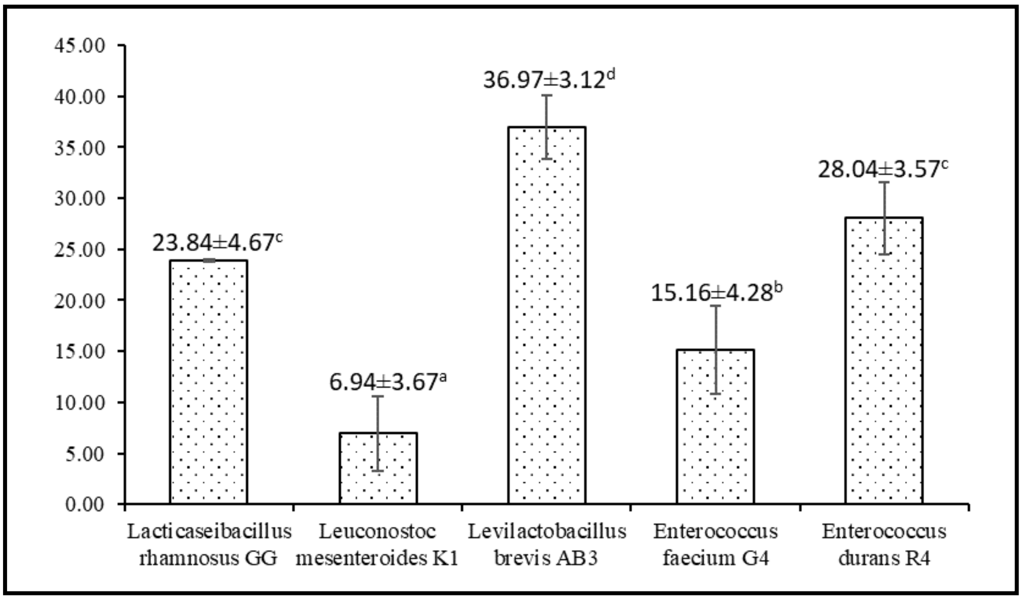

Figure 4. Hydrophobicity Properties of Indonesian Indigenous LAB. Values are expressed as mean±SD. Values with different superscripts are significantly different ($p < 0.05$) by Duncan's multiple range test.

Generally, probiotic bacteria have a hydrophobic cell surface. Several studies on probiotic LAB hydrophobicity have been conducted using xylene as the solvent. Dlamini et al. (2019) reported the hydrophobicity of *L. reuteri* and *L. salivarius* was up to 70%. Qureshi et al. (2020) found a high hydrophobic cell surface of *L. paracasei* ZMF54 reaching 84%. Juntarachot et al. (2023) and Harnentis et al. (2020) also found similar results in several *Lactobacillus* species and LAB isolated from dadih, respectively. Current findings did not correspond to previous study. The hydrophobicity of *L. rhamnosus* GG was found lower compared to other study, which was 64%.

The diversity of hydrophobicity percentage monitored by the BATH method was caused by the influence of the strains, cultivation time duration, medium used, the presence of acids, and the type of solvent used (Krausova et al., 2019). Hydrophobicity properties is corresponded to the presence of cell components, such as phospholipids, polysaccharides, and other external components on the bacterial cell surface (Fadda et al., 2017). Cell surface properties such as hydrophobicity correlate with the cell coaggregation properties since there is a hydrophobic interaction between cell membrane resulting in aggregation properties (Collado et al., 2008). Even though several bacteria are reported to have low hydrophobicity or even hydrophilic properties, those bacterial strains still possessed adhesion ability. Different cell components showed different mechanisms. Proteins and lipoteichoic acids on cell surfaces provide cells with hydrophobic properties, whereas polysaccharides play role in cell hydrophilic properties (Romaniuk & Cegelski, 2018). Since polysaccharides also contributed to cell adhesion it is possible that hydrophilic cell properties also showed adhesion ability, monitored using various methods, one of which is adherence to stainless steel surface.

Adherence Properties to Stainless Steel Surface

The adherence potential of the current strains was studied using a stainless-steel coupon. Figure 5 shows the viable cell count of the LAB isolates that are attached to the stainless-steel coupon. All strains exhibit an ability to attach to stainless steel. Moreover, three Indonesian Indigenous LAB; *L. brevis* AB3, *E. faecium* G4, and *E. durans* R4, have significantly higher viable cell count compared to *L. rhamnosus* GG. The viable cell counts of the Indonesian Indigenous LAB was around 6 log CFU/cm^2, 1 log cycle higher than *L. rhamnosus* GG. *Levilactobacillus brevis* AB3 has the highest viable cell count (6.37 ± 0.48 log CFU/cm^2) but not significantly different compared to *E. faecium* G4 and *E. durans* R4. Results revealed that the current LAB strains have similar adherence properties compared to probiotic bacteria.

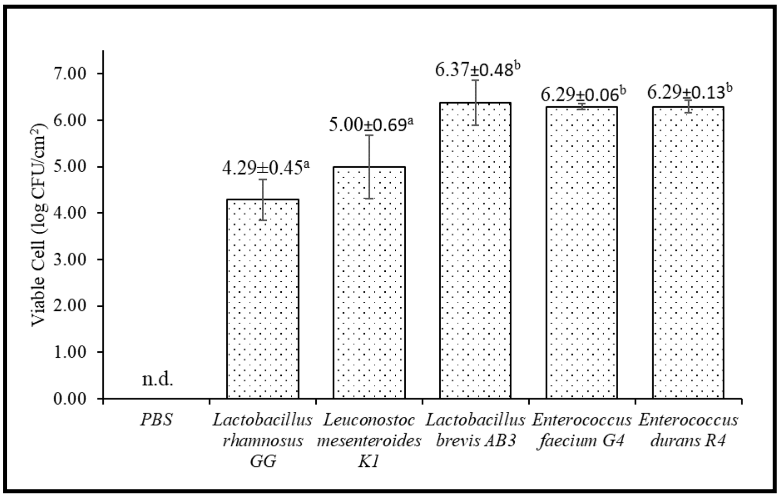

Figure 5. Adherence to Stainless Steel Coupon Properties of Indonesian Indigenous LAB. Values is expressed as mean±SD. Values with different superscripts are significantly different ($p < 0.05$) by Duncan's multiple range test.

Previous studies have been using stainless steel coupons to observe the LAB potential to adhere to epithelium. *Lactiplantibacillus plantarum subsp. plantarum* and *Lacticaseibacillus paracasei* isolated from Ethiopian traditional fermented foods showed a promising ability to adhere in stainless steel, ranging from 33.17% to 36.30% (Mulaw et al., 2019). Moreover, *L. mesenteroides* M2-8 isolated from kimchi showed ability to adhere in stainless steel based on crystal violet assay (Kim et al., 2022). On the other hand, *Lactobacillus johnsonii* isolated from chicken gut showed low adherence properties, around 4% adherence (Dertli et al., 2015). Other media also has been reported to study the adherence properties of LAB such as polystyrene, aluminum, and glass (Dutta et al., 2018; Reda, 2019).

Adherence properties of LAB is one of the important characteristics of probiotics. Several adherence properties mechanism have been reported. Generally, there are two main mechanisms, which were non-specific and specific interactions. The non-specific mechanisms were facilitated by the secretion of cell components such as exopolysaccharide (Fukao et al., 2019) and the cell surface physicochemical interaction (Deepika & Charalampopoulos, 2010). Meanwhile, the specific mechanism was facilitated by the production of specific proteins, adhesions. Darmastuti et al. (2021) reported that *L. plantarum* Dad-13 and *L. plantarum* Mut-7 possessed genes encoding fibronectin- binding proteins (ptrF/polymerase I and transcript release factor) and chaperonin (hsp33/heat shock protein 33) which regulate the adhesion of bacteria. Current findings point out that even though Indonesian Indigenous LAB showed low hydrophobic cell surface properties and no visual aggregation, those strains still have adherence properties to stainless steel surfaces. This might be due to the specific adhesion mechanism mediated by the release of adhesin properties. Future studies are required to confirm this hypothesis.

Conclusion and Suggestions

Indonesian Indigenous LAB possessed a promising adherence property even though all strains have a low hydrophobic cell surface. Current study points out the potential of the Indonesian indigenous LAB as a promising candidate. Based on adherence properties to stainless steel surface, *L. brevis* AB3 showed higher results, indicating *L. brevis* AB3 has the most potential to adhere in the intestinal mucosa. Future study on the specific mechanism of the LAB to adhere in intestinal mucosa might be needed. Evaluation of the adherence to intestinal mucosa using other media also needed to further confirm the current strains can adhere in the human intestinal mucosa.

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