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Probiotic Potential of *Lactiplantibacillus plantarum* Isolates from Indonesian Kefir Grains Cultured in Goat Milk

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ABSTRACT

Background: The increasing interest in functional fermented dairy products has intensified research on lactic acid bacteria (LAB) as potential probiotics. Kefir, a symbiotic matrix of LAB and yeasts, represents a rich source of probiotic microorganisms, yet limited data exist on strains isolated from Indonesian goat milk kefir. Exploring the molecular identity and probiotic traits of these local isolates can contribute to the development of novel functional dairy cultures suited to regional production systems.

Methods: Sixteen LAB isolates were procured from Indonesian kefir grains sub-cultured in goat milk and evaluated for probiotic characteristics. Molecular identification was conducted *via* 16S rRNA sequencing and *in vitro* experiments assessed acid and bile salt tolerance, carbohydrate utilization with 4% inulin and antibacterial efficacy against *Staphylococcus aureus*, *Escherichia coli* and Salmonella Typhi. Statistical analysis was conducted to ascertain strain-level variations in probiotic characteristics.

Result: Twelve isolates were successfully identified, including eight *Lactiplantibacillus plantarum* and four *Lactobacillus plantarum* strains. Strains AN and QF showed high acid tolerance (>50% survival at pH 2.0 for 90 min) and strain NT exhibited strong bile salt resistance up to 1.5% (log 6.5 CFU/mL). All isolates metabolized inulin as the sole carbon source and inhibited pathogenic bacteria with inhibition zones of 3.7-6.0 mm, the strongest against *S. aureus*. *L. plantarum* strains obtained from goat milk kefir exhibited strong probiotic properties, validating their suitability as starting cultures for functional dairy fermentation and as possibilities for industrial probiotic formulations.

Key words: Food safety, Goat milk, Kefir grains, Lactic acid bacteria, Molecular identification, Probiotics.

INTRODUCTION

Probiotics are defined as live microorganisms that confer health benefits to the host when administered in adequate amounts and their global demand continues to grow due to their integration into functional foods and nutraceuticals (Biadała *et al.*, 2023; Bentahar *et al.*, 2024). Among these, lactic acid bacteria (LAB)-particularly Lactobacillus and Bifidobacterium-are widely recognized for their safety and functional attributes, including acid and bile tolerance, antagonism toward pathogens and the ability to metabolize prebiotic substrates (Kahraman-Ilikkan, 2024).

Fermented dairy products represent some of the most common vehicles for probiotic delivery (Szajnar *et al.*, 2025). This aligns with previous reports showing that fermentation by lactic acid bacteria significantly alters the biochemical and nutritional profile of milk, strengthening its value as a functional food matrix (Wulansari *et al.*, 2024). Kefir, produced through fermentation with kefir grains, contains a complex consortium of LAB and yeasts and has been associated with improved gut microbiota composition, immune regulation and enhanced nutritional value (Azizi *et al.*, 2021; Ströher *et al.*, 2025). However, the microbiota of kefir grains varies widely depending on geographic origin, milk source and fermentation practices, resulting in substantial differences in microbial composition and probiotic functionality (Sumarmono *et al.*, 2023; Abdi *et al.*, 2025). Notably, metagenomic analyses have shown that goat milk kefir harbors distinct LAB communities compared to cow milk kefir, highlighting the influence of substrate type on LAB diversity and performance (Sumarmono *et al.*, 2023; Ströher *et al.*, 2025).

Despite the recognition of kefir as a reservoir of beneficial microorganisms, systematic isolation and

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comprehensive genomic characterization of LAB from Indonesian kefir remain limited. Previous studies have primarily focused on phenotypic identification, while strain-level molecular characterization and functional probiotic assessment have not been fully explored. Such an integrated approach is critical for achieving precise taxonomic resolution and identifying strains with industrial relevance (Kahraman-Ilikkan, 2024).

Goat milk holds particular importance in Indonesia due to its increasing consumer demand and its role in smallholder dairy systems. Characterizing LAB from Indonesian goat milk kefir is therefore scientifically valuable and offers potential benefits for the development of locally sourced functional dairy products.

Accordingly, this study aimed to characterize LAB isolates from Indonesian kefir grains using 16S rRNA-based genotypic identification and to evaluate their *in vitro* probiotic properties, including acid and bile tolerance, inulin utilization and antimicrobial activity. These findings provide new insights into the probiotic potential of locally derived kefir LAB and support their prospective application as starter cultures in functional fermented dairy products.

MATERIALS AND METHODS

Sample collection

Sixteen lactic acid bacteria (LAB) isolates derived from kefir grains sub-cultured in goat milk, as reported in the previous study by Wulansari *et al.* (2023), were used as samples. All experimental procedures were conducted between 2024 and 2025 at the Microbiology Laboratory, Faculty of Agriculture, Universitas Perjuangan Tasikmalaya; the Animal Science Learning Center (ASLC), Faculty of Animal Science, Universitas Gadjah Mada; and the Integrated Genome Factory, Faculty of Biology, Universitas Gadjah Mada. The overall experimental design is presented in Fig 1.

Reculture of isolate

Isolates were recultured in de Man, Rogosa and Sharpe broth (MRSB; Merck, Germany) supplemented with 0.15% (w/v) bile salts (Oxoid, UK) and incubated at 37°C for 24 hours under anaerobic conditions. The recultured isolates were subsequently used for DNA extraction and probiotic assessment.

Extraction of genomic DNA

Genomic DNA was isolated from 1.5 mL of overnight cell culture using 400 µL of SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris-HCl, pH 7.5) supplemented with 30 mg/mL lysozyme. The mixture was incubated at 37°C for 1 hour, after which 50 µL of 10% SDS was added and the sample was incubated at 65°C for an additional hour. Subsequently, 167 µL of 5 M NaCl was added and incubation was continued for 1 hour. DNA was extracted with 400 µL of chloroform, incubated at room temperature for 10 minutes and centrifuged at 13,000 rpm for 10 minutes. The aqueous phase was precipitated with isopropanol at -20°C overnight, centrifuged at 13,000 rpm for 10 minutes, washed with 500 µL of 70% ethanol and finally resuspended in TE buffer. DNA quality was assessed by electrophoresis on a 0.8% agarose gel in TAE buffer at 70 V for 45 minutes, using a 1 Kb DNA ladder (Promega, USA).

Polymerase Chain Reaction amplification of 16S rRNA genes

The 16S rRNA gene (~1,500 bp) was amplified using the universal primers F21 (5′ -AGAGTTTGATCMTGGCTCAG-3′) and R1492 (5′ -TACGGYTACCTTGTTACGACTT-3′) [7]. PCR reactions were performed on a BOECO TC-SQ thermal cycler under the following conditions: Initial denaturation at 96°C for 30 seconds, annealing at 55°C for 30 seconds and extension at 72°C for 45 seconds for 30 cycles, followed by a final extension at 72°C for 4 minutes. Amplification products were visualized on a 1% agarose gel stained with ethidium bromide.

DNA sequencing and phylogenetic assessment

Purified PCR products were sequenced using the Applied Biosystems 3730XL Analyzer (1st Base Sequencing, Singapore). The resulting sequences were compared with those in the NCBI database using BLAST and species identities were assigned based on a similarity threshold of >98%. The 16S rRNA gene is widely recognized as a reliable molecular marker for bacterial identification because its conserved and variable regions allow differentiation among closely related species (Ehmud *et al.*, 2025). Phylogenetic trees were constructed using MEGA version 5.05 with the UPGMA method and 1,000 bootstrap replications, following the approach described by Kumar *et al.* (2018).

In vitro evaluation of probiotic characteristics

Tolerance to acids

Isolates were cultured in MRS broth adjusted to pH 2.0 using HCl. One microliter of overnight culture was inoculated into 9 µL of pH-adjusted MRS broth and incubated at 37°C. Viable cell counts were determined at 0, 45 and 90 minutes by plating on MRS agar supplemented with L-cysteine. Results were expressed as log CFU/mL, following the method described by Nawaz *et al.* (2024).

Tolerance to bile salts

The ability of the isolates to tolerate bile salts was assessed in MRS broth supplemented with 0.3, 0.5, 1.0 and 1.5% (w/v)

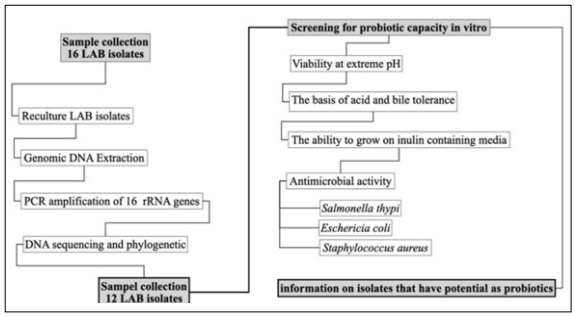


Fig 1: Schematic overview of the experimental design used in this study.

bile salts (Sigma-Aldrich, USA). One milliliter of overnight culture was inoculated into 9 mL of bile salt-enriched MRS broth and incubated at 37°C for 2 hours. Viable cell counts were determined by plating on MRS agar and incubating at 37°C for 48 hours. Results were expressed as log CFU/mL, following established protocols for probiotic LAB screening (Meena *et al.*, 2022).

Degradation of inulin

The ability of the LAB isolates to metabolize inulin (Orafti, Belgium) was evaluated in MRS broth supplemented with 4% (w/v) inulin. The 4% concentration was selected because it is widely used in probiotic carbohydrate utilization assays, providing an adequate substrate level to distinguish strain-specific differences in prebiotic fermentation efficiency while avoiding the osmotic inhibition associated with higher concentrations (Roos and Jonsson, 2002; Sanchez *et al.*, 2010). Growth was assessed by determining viable cell counts using modified methods for probiotic carbohydrate utilization.

Antimicrobial efficacy

Antagonistic activity was evaluated against *Salmonella Typhi*, *Escherichia coli* and *Staphylococcus aureus*. Cell-free supernatants from overnight LAB cultures were obtained by centrifugation and assessed using the agar well diffusion method. Inhibition zones were measured after incubation at 37°C, following the procedure described by Nawaz *et al.* (2024). Ampicillin (10 µg/mL) served as the positive control, while uninoculated MRS broth was used as the negative control. Controls were included in all antimicrobial assays to ensure the validity of the results. Each assay was performed in duplicate with three independent biological replicates.

Statistical examination

The data were analyzed using SPSS version 16.0 (IBM Corp., USA). Differences among treatments were evaluated using one-way ANOVA followed by Duncan's multiple range test at a significance level of $\alpha = 0.05$. Paired sample t-tests were used to compare pre- and post-incubation results in the bile salt tolerance assays.

RESULTS AND DISCUSSION

Molecular characterization of LAB isolates

PCR amplification of the 16S rRNA gene yielded approximately 1,500 bp fragments for all 16 isolates (Fig 2). Twelve amplicons were successfully sequenced, while four isolates were excluded due to poor quality. BLAST analysis revealed that eight isolates showed 96-100% similarity with *Lactiplantibacillus plantarum* and four with *Lactobacillus plantarum*. The phylogenetic tree clustered all isolates within the *L. plantarum* group, clearly separated from *L. casei* and *L. acidophilus* (Fig 3; Table 1).

Acid tolerance at pH 2.0

All isolates survived at pH 2.0 for 90 min, although viability declined significantly ($p<0.05$). Survival rates ranged from 32.4% to 57.6% (Table 2). *L. plantarum* strains AN and QF exhibited the highest survival (>50%), indicating superior tolerance to acidic conditions (Fig 4).

Bile salt tolerance

Isolates exhibited varying tolerance to bile salts at concentrations ranging from 0.3% to 1.5% following 2 hours of incubation. Most strains showed decreased viability with increasing bile salt concentration ($p<0.05$). Notably, *L. plantarum* strain NT maintained stable counts across all concentrations and even increased viability at 1.5% bile salts (Fig 5).

Inulin utilization

All isolates were capable of growing in medium containing 4% inulin as the sole carbon source, although growth was lower than in glucose-supplemented control medium. Growth curves demonstrated consistent inulin metabolism across strains (Fig 6).

Antimicrobial activity

Cell-free supernatants of all isolates inhibited the growth of *Salmonella Typhi*, *Escherichia coli* and *Staphylococcus*

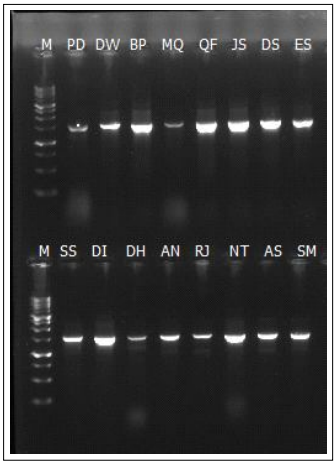


Fig 2: Visual of 16S rRNA gene amplification in agarose (1%).

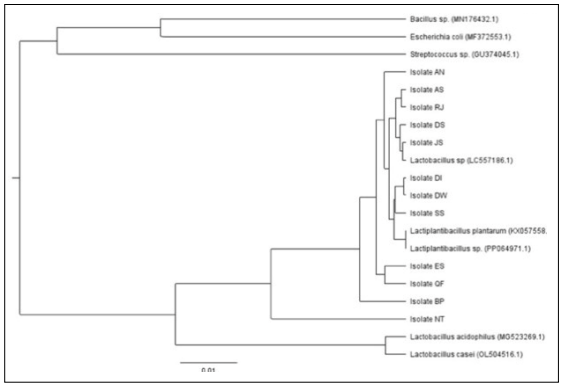


Fig 3: Phylogenetic tree of lactic acid bacteria isolates based on 16S rRNA sequences (UPGMA method, 1,000 bootstrap replications).

aureus (Fig 7). Inhibition zones varied significantly among strains for *S. Typhi* and *S. aureus* ($p<0.05$), while activity against *E. coli* was comparable across isolates. *L. plantarum* strain DS showed the strongest inhibition against *S. aureus*, whereas strain AS had the weakest effect.

This study identified and characterized lactic acid bacteria (LAB) isolates derived from Indonesian kefir grains and demonstrated that these isolates possess several desirable probiotic traits. Molecular analysis revealed that the majority of isolates belonged to *Lactiplantibacillus plantarum* and *Lactobacillus plantarum*, a finding consistent with global reports highlighting the predominance of these species in fermented dairy ecosystems (Aziz *et al.*, 2023; Lappa *et al.*, 2024). The presence of similar species in kefir from Tibet, Greece and Brazil suggests that *L. plantarum* exhibits strong ecological adaptability to fermentation environments, though differences in strain distribution across regions likely arise from variations in milk source, fermentation temperature and microbiota composition (Ströher *et al.*, 2025). The detection of these strains in Indonesian goat milk kefir-an underexplored substrate-indicates that local fermentation practices may select for unique *L. plantarum* variants with potential functional value.

The isolates demonstrated clear evidence of gastrointestinal resilience based on their acid and bile tolerance. Two strains (AN and QF) exhibited superior survival at pH 2.0, indicating strong adaptation to acidic environments similar to those reported in LAB isolates from Thai fermented foods (Suwannasom *et al.*, 2025). Acid tolerance in *L. plantarum* is generally associated with proton pump regulation, membrane-stabilizing proteins and the activity of F1F0-ATPase, which help maintain intracellular pH homeostasis (Aziz *et al.*, 2023; Liu *et al.*, 2024). Likewise, strain NT displayed exceptional tolerance to bile concentrations up to 1.5%, surpassing levels physiologically encountered in the small intestine. Comparable findings from *L. plantarum* F42 isolated from Chinese sauerkraut support the notion that certain strains possess strong bile stress–response systems involving bile salt hydrolase (BSH) and membrane restructuring (Fidanza *et al.*, 2021; Han *et al.*, 2025). These results confirm that the isolates studied here possess several of the key physiological characteristics required for persistence and activity within the gastrointestinal tract.

Table 1: Molecular identification of lactic acid bacteria isolates based on 16S rRNA sequencing.

Isolates	Accession No. of isolate	Matches to 16S rRNA sequences from genbank database	Identity similarity (%) with genbank database
DW	PP499271.1	*Lactiplantibacillus plantarum* strain UK1	99.85%
QF	MH333182.1	*Lactiplantibacillus plantarum* strain HBUAS51032	99.60%
JS	KX057558.1	*Lactiplantibacillus plantarum* strain a78	99.61%
DS	ON117110.1	*Lactiplantibacillus plantarum* strain HBUAS59669	99.51%
ES	OM108219.1	*Lactiplantibacillus plantarum* strain TMPC 20737	96.77%
AN	MF369875.1	*Lactiplantibacillus plantarum* strain CAU:222	99.79%
NT	OQ202181.1	*Lactiplantibacillus plantarum* strain MUT HDS27	99.88%
AS	ONO75517.1	*Lactiplantibacillus plantarum* strain B101	100%
BP	OR502188.1	*Lactobacillus plantarum* strain HBUR51130	98.70%
SS	MG590106.1	*Lactobacillus plantarum* strain SX-3	99.62%
DI	MT613642.1	*Lactobacillus plantarum* strain 3359	100%
RJ	MZ749634.1	*Lactobacillus plantarum* strain 240	100%

Table 2: Viability of lactic acid bacteria isolates at pH 2.0 for 90 min (%).

Lactic acid bacteria strain	Viability percentage after 90 mins (%)
Lactiplantibacillus plantarum strain DW	41.48 ± 0.29^{cd}
Lactiplantibacillus plantarum strain QF	50.85 ± 1.58^{b}
Lactiplantibacillus plantarum strain JS	45.87 ± 3.88^{bc}
Lactiplantibacillus plantarum strain DS	44.87 ± 3.93^{c}
Lactiplantibacillus plantarum strain ES	35.06 ± 1.18^{ef}
Lactiplantibacillus plantarum strain AN	57.62 ± 8.08^{a}
Lactiplantibacillus plantarum strain NT	36.99 ± 0^{def}
Lactiplantibacillus plantarum strain AS	42.40 ± 1.62^{cd}
Lactobacillus plantarum strain BP	41.94 ± 0.26^{cd}
Lactobacillus plantarum strain SS	38.09 ± 1.53^{de}
Lactobacillus plantarum strain DI	35.95 ± 1.19^{ef}
Lactobacillus plantarum strain RJ	32.43 ± 0.61^{f}

^{a,b,c,d,e,f} Different superscript in each strain shows significant difference $P<0.05$.

All isolates exhibited the ability to utilize inulin, reinforcing their potential for application in synbiotic formulations. This observation is consistent with studies showing that *L. plantarum* strains efficiently ferment inulin and fructooligosaccharides, enhancing growth stability and increasing tolerance to gastrointestinal stress (Parhi *et al.*, 2021). The capacity to metabolize inulin is noteworthy because prebiotic supplementation has been associated with elevated production of short-chain fatty acids (SCFAs) such as acetate and butyrate-metabolites that support intestinal barrier integrity and exert anti-inflammatory effects (Cui *et al.*, 2022; Xu *et al.*, 2025). The ability of Indonesian kefir-derived isolates to metabolize inulin therefore indicates functional equivalence to internationally studied strains and suggests that these isolates may contribute to beneficial metabolic outputs in synbiotic applications. Future studies should quantify SCFA profiles and evaluate these strains *in vivo*.

The antimicrobial properties observed across isolates further support their probiotic potential. All strains inhibited *Salmonella Typhi*, *Escherichia coli* and *Staphylococcus aureus* and these findings are consistent with previous reports showing that fermented milk containing *Lactobacillus* species exhibits strong antibacterial effects, particularly

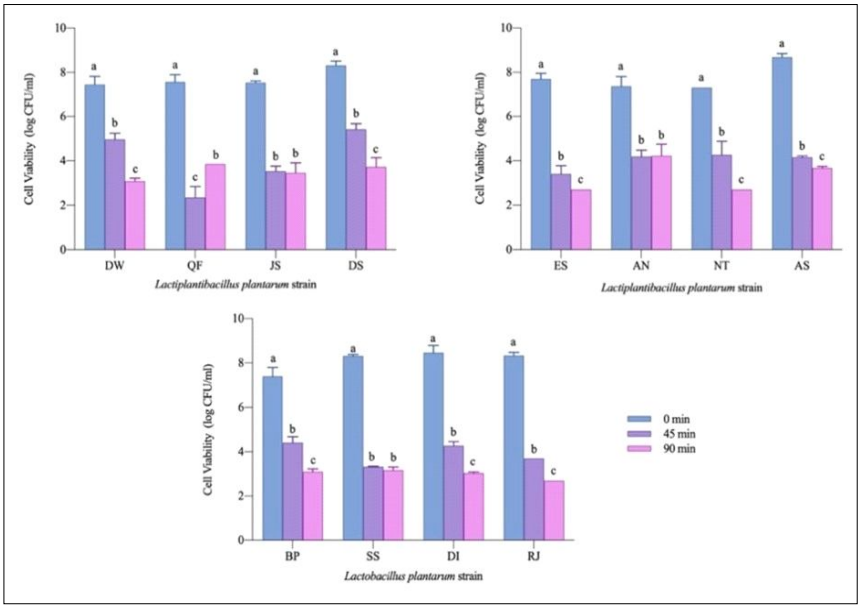


Fig 4: Viability of lactic acid bacteria isolates at pH 2.0 after 90 min incubation. Different superscripts indicate significant differences ($p<0.05$).

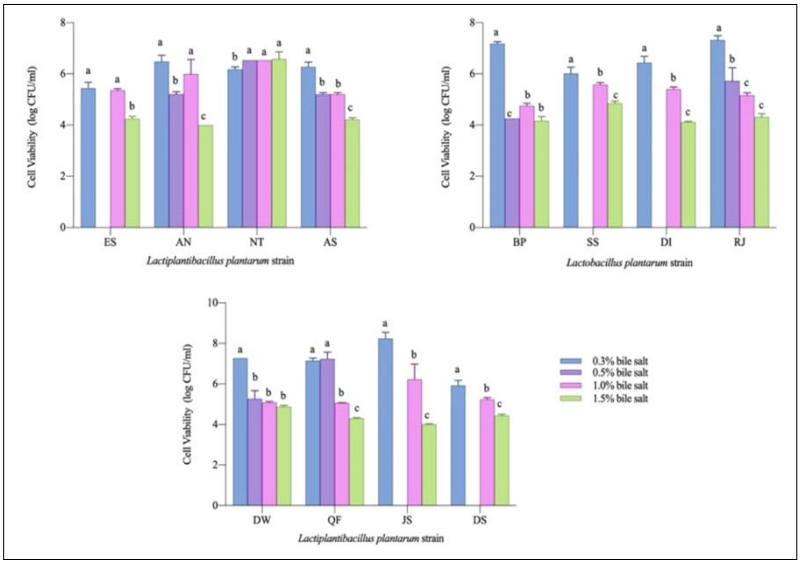


Fig 5: Survival of lactic acid bacteria isolates in bile salt concentrations (0.3-1.5%, w/v) after 2 h incubation. Different superscripts indicate significant differences ($p<0.05$).

against *S. aureus* (Hamad *et al.*, 2025). This aligns with recent studies reporting that *L. plantarum* produces organic acids, hydrogen peroxide and bacteriocin-like substances that suppress pathogenic organisms (Huang *et al.*, 2024; Bui *et al.*, 2025). The strong inhibition shown by certain isolates suggests the possible presence of bacteriocin gene clusters or other antimicrobial metabolites, mechanisms that have been identified in *L. plantarum* strains from diverse fermented foods. Considering the rising global concern regarding antimicrobial resistance, these findings highlight the relevance of Indonesian kefir-derived LAB as promising natural biopreservatives. Nonetheless, the precise compounds responsible for pathogen inhibition remain undetermined, emphasizing the need for metabolomic profiling and purification of antimicrobial agents in future work (Obafemi *et al.*, 2025). Overall, this study provides clear evidence that Indonesian kefir grains harbor LAB strains with strong probiotic potential, including robust gastrointestinal tolerance, prebiotic utilization and broad-spectrum antimicrobial

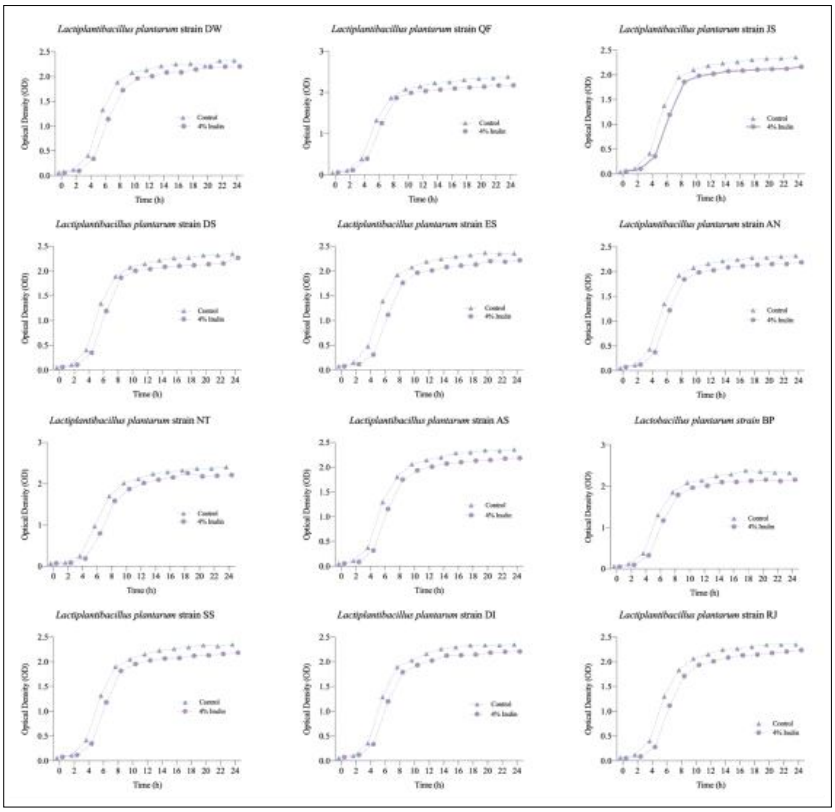


Fig 6: Growth of lactic acid bacteria isolates in MRS medium supplemented with 4% inulin compared to control medium.

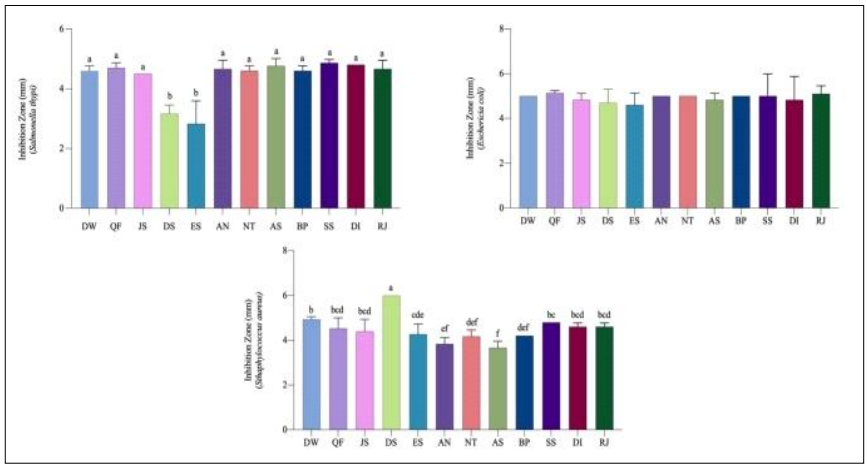


Fig 7: Antimicrobial activity of lactic acid bacteria isolates against *Salmonella Typhi*, *Escherichia coli* and *Staphylococcus aureus*. Different superscripts indicate significant differences in inhibition zones ($p<0.05$).

activity. These findings not only expand the scientific understanding of LAB diversity in Southeast Asian fermented foods but also underscore the potential of these strains for application in functional dairy fermentation and probiotic product development. Further genomic and metabolomic studies are needed to elucidate the molecular basis of these probiotic traits and to validate their functionality in real food systems and *in vivo* settings.

CONCLUSION

This study revealed that lactic acid bacteria extracted from Indonesian goat milk kefir were primarily classified as *Lactiplantibacillus plantarum* and displayed functional probiotic characteristics, such as acid and bile resistance, inulin metabolism and antimicrobial efficacy against prevalent pathogens. Strains AN, QF and NT showed superior resilience, underscoring their potential as promising probiotic candidates. These findings fill a regional knowledge gap by characterizing kefir-derived LAB from Indonesia, which has been less studied compared to kefir from other regions and highlight their industrial relevance as starter cultures for functional fermented dairy products and natural biopreservatives. Future research should focus on whole-genome sequencing to identify probiotic-related genes, metabolomic profiling of bioactive metabolites and *in vivo* validation to confirm health benefits and support application in food and nutraceutical industries.

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Disclaimers

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Conflict of interest

All authors declare that they have no conflicts of interest.

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