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Research article

Exploring the Multifunctional Potential of Fructophilic *Enterococcus faecium* as a Probiotic and Extracellular Folate Producer Isolated from Wild *Apis dorsata* Honeybee Hives in East Nusa Tenggara

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Abstract

Folate or vitamin B_9 is an essential nutrient that supports a variety of biological functions. Folate deficiency can lead to a variety of health problems, including megaloblastic anemia, digestive problems, and impaired neurodevelopment in the fetus during pregnancy. Currently, folic acid used for fortification is generally synthesized chemically, which causes unwanted side effects. Bacteria are able to produce folate, but folate production from probiotics has not been widely reported. In fact, folate-producing probiotics can provide an additional source of folate for the body through its absorption in the intestine. This study aimed to select, isolate and identify fructophilic LAB strains from wild giant honeybee hives that had probiotic potential and the ability to produce folate. The results showed that the fructophilic strains *Enterococcus faecium* MD05, *Enterococcus* sp. MD23, and *E. faecium* MD29, identified based on the 16S rRNA gene, are lactic acid bacteria with Gram-positive and catalase-negative properties. All three have probiotic potential, indicated by in vitro tolerance tests to pH 2.5 and 0.3% bile salt, have antimicrobial activity, are able to form aggregates against pathogens, and are sensitive to antibiotics. *Enterococcus faecium* MD29 was successfully proven to produce extracellular folate up to 5 ng/mL. The multi-functional characteristics of fructophilic LAB have not been widely reported. The unique characteristics of fructophilic LABs, as demonstrated by this strain, remain underexplored. Genetically, *E. faecium* MD29 is closely related to commensal bacteria of the digestive tract, enhancing its potential as a functional food ingredient. These findings provide a strong basis for conducting in vivo studies to evaluate the strain's safety and validate its probiotic claims. Further research is also needed to determine the specific type of folate synthesized from fructose and its biofunctional properties.

Keywords: *Enterococcus*; folate; fructophilic; lactic acid bacteria; probiotic

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1. Introduction

Folate (vitamin B_9) is a crucial micronutrient essential for the metabolic processes of all living organisms. As a water-soluble B-group vitamin, folate plays a pivotal role in DNA replication, repair, and methylation, and the synthesis of nucleotides, vitamins, and amino acids. It is also involved in energy production and erythrocyte synthesis (de Giori & LeBlanc, 2018). Despite being present in many food sources, folate is highly susceptible to degradation during cooking and food processing, leading to widespread deficiencies. Folate deficiency has been linked to numerous health disorders, including Alzheimer's disease, coronary heart disease, osteoporosis, increased risk of breast and colorectal cancers, cognitive decline, hearing loss, and neural tube defects (Leblanc et al., 2011; Czeizel et al., 2013). Humans and animals cannot synthesize folate; however, many microorganisms and plants can produce folate derivatives de novo, starting with the formation of dihydrofolate. This inactive derivative must be reduced to tetrahydrofolate, the active form, via a dihydrofolate reductase enzyme using NADPH. Tetrahydrofolate and its derivatives are essential cofactors in one-carbon transfer reactions. Notably, folate differs from folic acid, a synthetic form of folate commonly used in food fortification. While folate refers to naturally occurring compounds, folic acid is the chemically stable version used in supplementation and fortification protocols (Saini et al., 2016).

One of the main advantages of folate production by microorganisms is its ability to provide a more affordable and sustainable source of folate. The fermentation process using lactic acid bacteria (LAB) can produce significant amounts of folate, which can be extracted and used as a dietary supplement. One of the main advantages of folate production by microorganisms is their ability to provide a more affordable and sustainable source of folate. Folate supplementation is particularly beneficial for individuals whose health is compromised by folate deficiency and is of general benefit for folate sufficiency in certain areas where access to natural folate sources is limited. Folate production by microorganisms can be achieved through environmentally friendly fermentation methods. Compared to conventional chemical synthesis, microbiological folate production using bacteria such as *Lactobacillus* spp. and *Bifidobacterium* spp. does not require hazardous chemicals or energy-intensive processes, making it more sustainable (D'Aimmo et al., 2023). Lactic acid bacteria function in the human digestive tract and can aid in the absorption of folate. Additionally, folate produced by these bacteria may be more bioavailable to the human body because the fermentation process can produce a form of folate that is more easily absorbed compared to some forms found in food. Certain types of lactic acid bacteria, such as *Lactobacillus* and *Bifidobacterium*, have the ability to colonize and proliferate in the human digestive tract. In addition to producing folate, they help maintain the balance of the gut microbiota, which supports digestive health and the immune system. This makes folate produced by microorganisms a beneficial addition to microbiota health (Liu et al., 2022). Folate production by lactic acid bacteria offers several advantages, making it a promising alternative to traditional folate supplements. Not only does it provide folate in larger and more affordable quantities, but it also enhances the balance of the gut microbiota, contributing to overall digestive health (Wang et al., 2021; Liu et al., 2022).

In East Nusa Tenggara, a tropical rainforest in Indonesia, giant honeybees (*Apis dorsata* L.) build their nests in the shady branches of large trees, often more than 10 m above the ground. These bees obtain nutrients from nectar and pollen from various tropical plants. Their nests harbor a wide variety of microorganisms, including lactic acid bacteria and yeasts, which are found in bee products such as bee bread, royal jelly, pollen,

honeydew, honey, and fermented honey (Leska et al., 2022). This microbial diversity is likely the result of the bees' foraging activities, transporting nectar and pollen from various plants to their nests. These nests can even support symbiotic cultures of bacteria and yeast (SCOBY). However, scientific research reporting the potential of microbes from honeybee nests is still limited.

Strains isolated from unique ecological niches, such as wild bee hives, often show improved resistance and specialized and beneficial metabolic profiles. Previous studies have highlighted the probiotic potential of isolates obtained from wild beehives, especially in fructose-rich environments (Karyawati et al., 2018). Fructophilic lactic acid bacteria (FLAB), such as *Lactobacillus kunkeei*, prefer fructose as a substrate and thrive in fructose-rich niches such as flowers and fruits. These bacteria, which are commonly found in the digestive tract of bees and the hive environment, can contribute to the health of bee colonies (Endo & Salminen, 2013). Honeybee products are known to have nutraceutical value (Ajibola, 2012) associated with the vitamin B complex, especially folate. Probiotics have been reported to be able to produce folate through several common mechanisms and can be overproducers if the environment is supportive (Mahara, 2021). Zheng et al. (2021) reported that there are probiotics that have additional benefits such as producing health-beneficial metabolites. Therefore, there is a strong suspicion that the folate contained comes from the presence or activity of LAB with probiotic potential. This study aimed to select, isolate, and identify LAB strains from wild giant honeybee hives with probiotic potential and folate production ability, which were evaluated through in vitro assays. This study contributes to the growing interest in utilizing probiotics to address micronutrient deficiencies of compounds such as folate and to develop innovative probiotic applications.

2. Materials and Methods

2.1 Materials and chemicals

A total of 4 wild honeybee hives (*Apis dorsata*) from different trees were collected from the forest in the Amfoang area, East Nusa Tenggara Province, Indonesia (latitude: -9.5143746, longitude: 123.8634544). Hives were stored in a cooler at 4°C during transport and then transferred to a laboratory refrigerator for further analysis.

2.2 Screening and isolation of lactic acid bacteria using media containing bile salt

This method followed the protocol described by Yusuf et al. (2020). A 25-gram portion of the honeybee hive was homogenized in 225 mL of sterile phosphate-buffered saline (PBS, ML023, Himedia, Maharashtra, India). From this solution, 1 mL was taken and diluted into 9 mL of PBS to create a 10^{-2} dilution. Serial dilutions were continued until a concentration of 10^{-6} was achieved. *Lactobacillus* MRS agar (MRSA, M641I, Himedia, Maharashtra, India) plates were prepared and supplemented with 2% fructose (1040070250, Merck, Darmstadt, Germany), 0.15% bile salt (RM008, Himedia, Maharashtra, India), and 0.02% nystatin (N581, Phytotech, Kansas, USA) to inhibit fungal growth. Then, 1 mL of each solution from the 10^{-3} to 10^{-6} dilutions was spread evenly onto the surface of the prepared media. The plates were incubated in an inverted position at 37°C for 24-48 h. After incubation, the number of colonies that grew was enumerated to determine the total colony-forming units (CFU) of LAB. Single colonies were selected for isolation and purified using a four-quadrant streaking method on MRSA plates. Each single colony was examined for

cell morphology under a microscope, followed by Gram staining and catalase testing for preliminarily identifying as LAB (Ali et al., 2023).

2.3 Acid and bile tolerance

The method was modified from Yusuf et al. (2020) and Ali et al. (2023). LAB isolates, incubated for 24-48 h in *Lactobacillus* MRS broth (MRSB, (GM369, Himedia, Maharashtra, India), were centrifuged at 3000×g for 10 min at 4°C to separate pellet cells. The resulting pellet was washed twice with sterile PBS. The absorbance of the cell suspension was measured at 580 nm and adjusted to 0.50±0.05, ensuring a uniform cell concentration for further testing. Subsequently, 1 mL of the prepared cell suspension was inoculated into MRSB supplemented with 0.3% bile salts and acidified to pH 2.5 using 1M hydrochloric acid (7647-01-0, Merck, Darmstadt, Germany). The mixture was incubated at 37°C for 3 h. Samples of 1 mL were taken at 0 and 3 h to enumerate cell counts using the total plate count method on MRSA, followed by incubation at 37°C for 24-48 h. The difference in colony counts between 0 and 3 h represented the isolate's viability and resistance to acidic and bile conditions. The isolate exhibiting the highest viability was selected as a probiotic candidate.

2.4 Antimicrobial activity

The method was modified from Yusuf et al. (2020) and Ali et al. (2023). LAB isolates were cultured in MRSB at 37°C for 24 h. The cultures were centrifuged at 3000×g for 10 min at 4°C to obtain the supernatant. The supernatant was concentrated to half its volume using a vacuum evaporator and sterilized through a 0.22-μm filter membrane (SF127, Himedia, Maharashtra, India). The cell-free supernatant was tested for antimicrobial activity against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 13420 on Mueller-Hinton agar (MP173, Himedia, Maharashtra, India) using the disc diffusion method. Standard ampicillin discs (10 μg, WHA2017006, Merck, Darmstadt, Germany) served as positive controls, while sterile discs (74146, Sigma-Aldrich, Darmstadt, Germany) containing sterile water acted as negative controls. The diameter of the inhibition zones was measured in millimeters (mm) to determine antimicrobial activity.

2.5 Antibiotic resistance

The method was modified from Yusuf et al. (2020) and Ali et al. (2023). A 100 μL inoculum of the selected LAB isolate was mixed with 100 μL of antibiotic solutions containing chloramphenicol, ampicillin, or tetracycline and incubated at 37°C for 24 h. The minimum inhibitory concentration was determined by measuring the optical density at 600 nm (OD_{600}) using a microplate reader (Varioskan, Thermo Fisher Scientific, USA).

2.6 Auto-aggregation assay

The method was modified from Ali et al. (2023). The cell suspension of the three selected LAB was incubated at 37°C for 24 h. The OD_{600} were measured at 0, 2, 4, 6, and 24 h. The percentage of auto aggregation was determined using the equation (1).

$$\text{Auto aggregation (\%)} = 1 - \frac{OD_{time}}{OD_{initial}} \times 100 \quad (1)$$

OD_{time} = the absorbance at 2, 4, 6, or 24 h of incubation
$OD_{initial}$ = the absorbance at 0 h

2.7 Co-aggregation assay

The method was modified from Ali et al. (2023). Pathogenic strains *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 13420 were used. The inoculum preparation for both the selected LAB and pathogenic strains followed the procedure described earlier. Equal volumes of LAB and pathogen cell suspensions were mixed in a 1:1 ratio, vortexed for 30 s, and incubated at 37°C. The OD_{600} of the mixtures was measured at 0, 2, and 24 h of incubation. The percentage of co-aggregation was calculated using the following equation (2).

$$\text{Co-aggregation (\%)} = 1 - \frac{OD_{time}}{OD_{initial}} \times 100 \quad (2)$$

OD_{time} = the absorbance at 2 and 24 h of incubation
$OD_{initial}$ = the absorbance at 0 h

2.8 Scanning electron microscopy analysis

The formation of aggregates through auto- and co-aggregation was further confirmed using scanning electron microscopy (SEM) analysis. After removing the excess solution of the cell suspension, the precipitates formed were collected. Subsequently, the formation of aggregates within the precipitate was observed using low-vacuum SEM (JSM-5310LV; Jeol Ltd., Japan) at magnifications based on the morphology and dimensions of probiotics and pathogens.

2.9 Semi-qualitative analysis for folate production

The semi-qualitative analysis for folate production was conducted following the method described by Mahara et al. (2021). Approximately 2% of the cell pellet was added to a folate-free medium, and a folic acid casei medium (FACM, M543, Himedia, Maharashtra, India), with MRSB used as a control. The cultures were incubated at 37°C for 24 h. To ensure adequate growth in the folate-free medium, the cultures were subcultured twice in fresh media under identical growth conditions. Culture growth was then evaluated by measuring OD_{600} using a UV-visible spectrophotometer (BioSpec-1601, Shimadzu Corp.). Isolates exhibiting high absorbance were selected for further extracellular folate production measurement.

2.10 Extracellular folate production and quantification

The method was modified from Mahara et al. (2021). The selected isolates were cultured twice in both FACM and MRSB using the same procedure outlined above. Following the final subculture, extracellular folate was extracted and quantified. The extraction process involved centrifugation at 8,000×g for 10 min, followed by filtration of the supernatant through a 0.2-µm nylon filter membrane. The filtered supernatant was then analyzed for extracellular folate using the Vitafast folic acid test kit (P1001, R-Biopharm, Darmstadt,

Germany), following the manufacturer's instructions. Briefly, the supernatant was added to the wells of a microtiter plate pre-coated with folate-consuming bacteria, which served as the indicator bacteria (their growth was dependent on the folate present in the sample). The plate was incubated in darkness at 37°C for 48 h. Culture growth was then evaluated by measuring OD_{600} using a UV-visible spectrophotometer. Folate concentration was determined by referencing a standard curve constructed from samples with known folate concentrations, which were measured in the same manner as the test samples.

2.11 Molecular identification of probiotic candidates

Each pure culture of the selected LAB isolates was grown in MRSB at 37°C for 18 h. DNA was extracted from bacterial cell pellets using the GF-1 Bacterial DNA Extraction kit (GF-1, Vivantis, Selangor, Malaysia), following the manufacturer's instructions. DNA purity and concentration were assessed using a Nanodrop ND-1000 spectrophotometer (Thermo-Fisher Scientific Inc., USA). For PCR amplification, each reaction was performed in a 20 µL reaction mixture. This mixture consisted of 10 µL of 2x PCR mix (MB208, GeneDireX, Taiwan), 1 µL of 0.5 µM of each forward primer (27F: 5'–AGAGTTTGATCCTGGCTCAG–3') and reverse primer (1495R: 5'–CTACGGCTACCTTGTTACGA–3'), 1 µL of DNA template (≥150 ng), and 7 µL of nuclease-free water (BUF-1180, 1st Base, Selangor, Malaysia). PCR amplification was carried out in an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, USA) using the following program: an initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 2 min, and extension at 72°C for 2 min. A final extension step was performed at 72°C for 10 min. Negative control, without DNA template, was included in the amplification process. The PCR products were analyzed by gel electrophoresis (100 V, 30 min) using a 2% agarose (MB080, Himedia, Maharashtra, India) gel stained with GelRed in 1x Tris-acetate-EDTA buffer.

The PCR products were sequenced using the Sanger method by 1st Base Laboratories. The resulting sequences were analyzed using MEGA software, and homology searches were performed using the NCBI BLAST (Basic Local Alignment Search Tool) algorithm. Phylogenetic trees based on the 16S rDNA gene sequences were constructed. The 16S rDNA sequences of the bacterial isolates, along with sequences from the NCBI Nucleotide database (Nuccore), were aligned using MAFFT version 7.526 (Katoh & Standley, 2013). The aligned sequences were then trimmed to remove uninformative gaps using trimAL version 1.4.rev 22 (Capella-Gutiérrez et al., 2009). Phylogenetic trees were constructed using a Python script with the DendroPy 5 library version 5.0.1 (Moreno et al., 2024), interoperating with PAUP* (version 4.0a build 169). The final phylogenetic tree was visualized using MEGA7 (Saini et al., 2016).

2.12 Statistical analysis

Statistical analysis was performed using SPSS version 20.0 (IBM Corp., USA), with statistical significance set at $P<0.05$. One-way analysis of variance (ANOVA) was used to compare differences among strains in each test (acid and bile tolerance, antibacterial activity, auto- and co-aggregation ability). When significant differences were detected, Duncan's post hoc test was applied. Differences in LAB growth (absorbance) between FACM and MRSB media were assessed using paired t-tests. Data were presented as the mean ± standard deviation. All experiments were done in triplicate.

3. Results and Discussion

3.1 Screening of potential probiotic lactic acid bacteria

A total of 40 bacterial colonies were isolated from a wild giant honeybee hive, grown on MRSB media containing 0.15% bile salts. Morphological analysis revealed 16 Bacillus-like and 14 Coccus-like isolates. Gram staining and catalase assays showed that all isolates were catalase-negative, with 16 being Gram-negative. Based on these results, 34 isolates were identified as potential LAB and were subsequently purified for further analysis. Detailed data are provided in Table 1.

Table 1. Physicochemical characterization of isolates suspected to be lactic acid bacteria

Isolate Code	Shape	Gram Stain	Catalase	Isolate Code	Shape	Gram Stain	Catalase
*MD01	Coccus	-	-	MD21	Coccus	+	-
MD02	Bacillus	+	-	MD22	Coccus	+	-
MD03	Bacillus	+	-	MD23	Coccus	+	-
MD04	Bacillus	+	-	MD24	Bacillus	+	-
MD05	Coccus	+	-	MD25	Coccus	+	-
MD06	Coccus	+	-	MD26	Coccus	+	-
MD07	Coccus	+	-	MD27	Bacillus	+	-
MD08	Bacillus	+	-	MD28	Coccus	+	-
*MD09	Coccus	-	-	MD29	Coccus	+	-
MD10	Bacillus	+	-	MD30	Coccus	+	-
MD11	Coccus	+	-	MD31	Bacillus	+	-
MD12	Bacillus	+	-	MD32	Bacillus	+	-
MD13	Coccus	+	-	MD33	Bacillus	+	-
MD14	Coccus	+	-	*MD34	Bacillus	-	-
MD15	Coccus	+	-	MD35	Bacillus	+	-
*MD16	Bacillus	-	-	*MD36	Coccus	-	-
MD17	Coccus	+	-	MD37	Coccus	+	-
MD18	Coccus	+	-	MD38	Coccus	+	-
MD19	Bacillus	+	-	MD39	Bacillus	+	-
*MD20	Coccus	-	-	MD40	Coccus	+	-

*Not lactic acid bacteria

3.2 Tolerance towards low pH and bile salts

The 34 purified isolates were tested for their tolerance to low pH (2.5) and bile salts (0.3%). The initial colony count was around 8.0–8.6 log CFU/mL. The results showed that most isolates showed a significant decrease in viability upon exposure to low pH and bile salts. The number of live colonies decreased by 4–5 log CFU/mL. There were three isolates that showed a small decrease in the number of colonies, which was around 3 log CFU/mL. The three isolates indicated better viability or had resistance to the artificial digestive tract environment. The codes of the three isolates were MD05, MD23, and MD29. Based on these results, the three isolates were selected for further probiotic potential testing. Complete data are provided in Table 2.

Table 2. Tolerance of selected lactic acid bacteria isolates towards low pH and bile salt

Isolate Code	Decrease of Viable Cells (log CFU/mL)		Total Decrease of Viable Cells (log CFU/mL)
	after 3 h at pH 2.5	after 3 h at 0.3% bile salt	
MD05	2.07±1.55	1.47±0.81	3.54
MD23	2.09±0.90	1.20±0.67	3.29
MD29	2.03±0.40	1.90±1.18	3.93

CFU means colony forming units. Values are presented as mean±standard deviation (SD) from three repetitions.

Probiotics must survive the harsh conditions of the digestive tract, including exposure to hydrochloric acid at a pH of 2.5 and bile salts around 0.3%, in order to exert beneficial effects in the colon. These extreme acidic conditions can significantly reduce the viability of microorganisms, especially bacteria (Sensoy, 2021). Most bacteria are intolerant to environments with a pH below 3.0, with few exceptions (Corcoran et al., 2005). Acid-tolerant bacteria often possess defense mechanisms, such as H^+-ATPase pumps on the cell membrane, which expel protons (H^+) to maintain cytoplasmic pH balance. Acid-tolerant strains typically exhibit higher H^+-ATPase activity than acid-sensitive ones (Schwarz et al., 2022). In addition to pH homeostasis, some bacteria have systems to repair damage caused by low pH (Schwarz et al., 2022). Similarly, bile salts secreted in the digestive tract act as antimicrobial agents, limiting microbial penetration into the intestines. At a concentration of approximately 0.3%, bile salts pose a challenge for microbes, and strains tolerant to this concentration are considered promising probiotic candidates (Gangadharan et al., 2010). Upon exposure to bile salts, bacteria produce proteins that alter the cell wall structure to minimize damage (Ruiz et al., 2013). Moreover, bacteria capable of producing bile salt hydrolase (BSH) can neutralize bile salts by hydrolyzing them into less toxic free bile acids, further enhancing survival (Ruiz et al., 2013). Tolerance to both low pH and bile salts is a fundamental characteristic for microorganisms proposed as probiotics. The findings of this study support previous reports (Yusuf et al., 2020), which highlight that the survival of LAB in the digestive environment is strain-dependent. However, further testing is required to confirm their status as probiotics based on their tolerance to pH and bile salts.

3.3 Antimicrobial activity

Isolates MD05, MD23, and MD29, which exhibited the highest resistance to low pH and bile salts, were evaluated for antimicrobial activity. All three isolates demonstrated antimicrobial activity against *E. coli* and *S. aureus*, as indicated by the inhibition zone diameters. Inhibition zones were comparable to the positive control, 10 µg chloramphenicol, which had wider inhibition zones against *E. coli* and *S. aureus*. Complete data are presented in Table 3.

Probiotics should also possess antimicrobial properties to inhibit the growth of pathogens in the colon. In this study, we evaluated the antimicrobial activity of LAB against two common foodborne pathogens: *E. coli* (Gram-negative) and *S. aureus* (Gram-positive). These pathogens are not only implicated in foodborne illness outbreaks but are also part of the normal human gut microbiota. LAB are known to produce antimicrobial metabolites, including lactic acid, bacteriocins, and hydrogen peroxide. Lactic acid reduces the pH of the environment, creating conditions unfavorable for pathogen growth (Ibrahim et al., 2021). It also exerts direct antimicrobial effects by diffusing into pathogenic cells in its undissociated form, dissociating, and lowering the intracellular pH, which inhibits growth (Alakomi et al., 2000). Bacteriocins, which inhibit cell wall synthesis, and hydrogen peroxide, which causes oxidative stress to microbial macromolecules, also contribute to pathogen inhibition (Monika et al., 2021; Darbandi et al., 2022; Iorizzo et al., 2022).

Table 3. Mean inhibition zones of isolates against pathogenic bacteria

Isolates	Average of Inhibition Zone* (mm)	
	Escherichia coli	***Staphylococcus aureus***
MD05	9.91±0.12[a]	10.41±0.31[a]
MD23	10.00±0.35[a]	10.08±0.77[a]
MD29	10.33±0.31[a]	9.91±0.96[a]
Chloramphenicol (10 µg)	11.00±0.82[a]	11.67±0.47[b]

*From outward edge of isolates to outward edge of clear zone. Values are presented as mean±standard deviation (SD) from three repetitions.

3.4 Antibiotic resistance

The antibiotic susceptibility of isolates MD05, MD23, and MD29 was tested against chloramphenicol, ampicillin, and tetracycline. All three isolates were found to be sensitive to these antibiotics, with concentrations of 16 µg/mL for chloramphenicol, 2 µg/mL for ampicillin, and 4 µg/mL for tetracycline, as per European Food Safety Authority (EFSA) guidelines. Complete data are provided in Table 4. Another essential consideration for probiotic safety is antibiotic resistance. Probiotic strains should not harbor transferable antibiotic resistance genes that could potentially spread to pathogenic or commensal bacteria. Different antibiotics target specific bacterial processes: for instance, β-lactam antibiotics (e.g., ampicillin) inhibit cell wall synthesis, while broad-spectrum antibiotics like tetracycline and chloramphenicol block protein synthesis (Sharma et al., 2014).

Table 4. Antibiotic resistance patterns of isolates against three antibiotics

Isolate	Chloramphenicol	Ampicillin	Tetracycline
	16 µg/mL*	2 µg/mL*	4 µg/mL*
MD05	S	S	S
MD23	S	S	S
MD29	S	S	S

*Maximum concentration recommended by European Food Safety Authority. S means sensitive.

3.5 Auto-aggregation and co-aggregation ability

The results of the autoaggregation test showed that isolates MD05, MD23, and MD29 gradually formed autoaggregation over time. After 6 h of incubation, autoaggregation was still minimal, around <23%, but began to increase after 24 h, up to 67% in isolate MD23, 55% for MD05, and 34% for MD29. Complete data are presented in Figure 1. SEM analysis also confirmed the presence of autoaggregation (Figure 2). MD05, MD23, and MD29 cells can be seen sticking to each other and forming aggregates. Further testing, namely co-aggregation, was carried out by exposing the isolates to pathogenic bacteria *E. coli* and *S. aureus*. After 2 h of incubation, coaggregation occurred between the isolates and pathogenic bacteria of around 6-17%, and there was no significant difference between all isolates. Meanwhile, after 24 h of incubation, coaggregation increased to 39-54%, which also had no significant difference between all isolates (Table 5). These results indicate that the three isolates tested had moderate and not too significant auto- and co-aggregation abilities. The auto-aggregation and co-aggregation properties of probiotics are very important for the formation of a stable probiotic ecosystem and are able to compete or suppress the development of pathogenic microbes in the host intestine.

Table 5. Co-aggregation percentage of LAB with different pathogenic bacteria as measured after 2 and 24 h of incubation at 37°C.

Pathogenic Bacteria	Co-aggregation (%)					
	Isolate MD05		Isolate MD23		Isolate MD29	
	2 h	24 h	2 h	24 h	2 h	24 h
E. coli ATCC 25922	11.36±6.1[a]	49.56±6.86[b]	7.92±2.7[a]	54.29±1.03[b]	11.05±4.9[a]	52.8±1.68[b]
S. aureus ATCC 13420	17.96±1.29[a]	41.06±1.13[b]	6.58±2.9[a]	39.45±2.29[b]	6.54±3.1[a]	41.76±1.21[b]

Superscript letters within the same row indicate that the treatment results were significantly different according to Duncan tests ($p<0.05$). Values are presented as mean±standard deviation (SD) from three repetitions.

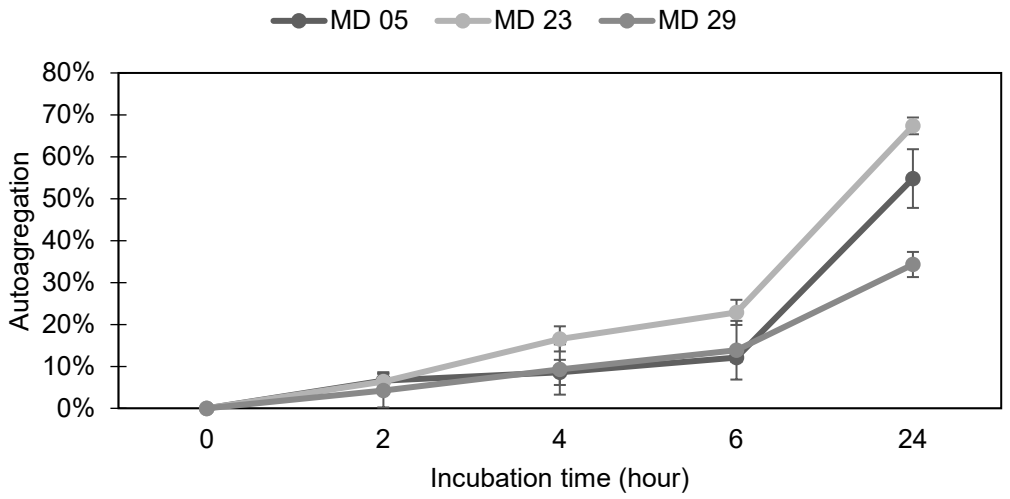

Figure 1. Auto-aggregation percentage. Values are presented as mean±standard deviation (SD) from three repetitions.

The ability of probiotic bacteria to auto-aggregate and co-aggregate is crucial for their role in pathogen suppression and colonization. Auto-aggregation reflects a strain's capacity to clump together, facilitating high cell density and promoting adhesion to intestinal epithelial cells. Co-aggregation evaluates a strain's ability to aggregate with pathogens, forming a protective barrier to prevent pathogen colonization and facilitating pathogen elimination through feces (de Melo Pereira et al., 2018). Strains that exhibit strong auto-aggregation (≥80%) are considered highly aggregative, while those with weaker aggregation (≤10%) are not. This phenotype is often linked to surface proteins like S-layer proteins that aid in bacterial adhesion and protection (Kos et al., 2003). In this study, all three LAB isolates showed similar co-aggregation profiles with foodborne pathogens, suggesting their potential to release antimicrobial substances in close proximity to these pathogens.

3.6 Extracellular folate production ability

Isolates MD05, MD23, and MD29 showed high growth in FACM, with absorbance at 600 nm ranging from 1.51 to 2.20 after 24 h of incubation. MD29 exhibited the highest growth compared to MD05 and MD23. However, growth in the FACM medium did not exceed the growth observed in MRSB or media containing folate (Figure 3A), suggesting the potential for natural folate production. To confirm this, isolate MD29 was tested for extracellular folate production, resulting in the production of 5 ng/mL of folate (Figure 3B).

The MD29 isolate, which exhibited the highest growth, was selected for further investigation of its extracellular folate production potential. This isolate was tested under different conditions, including folate-free (FACM) and folate-containing (MRSB) media. The results indicated that MD29 could produce extracellular folate in FACM, but not in folate-containing media, where it consumed available folate. This suggests that MD29 is a folate-efficient strain, capable of producing folate when it is absent from the medium, but not when it is available. Folate-efficient bacteria synthesize folate only as needed, while folate-

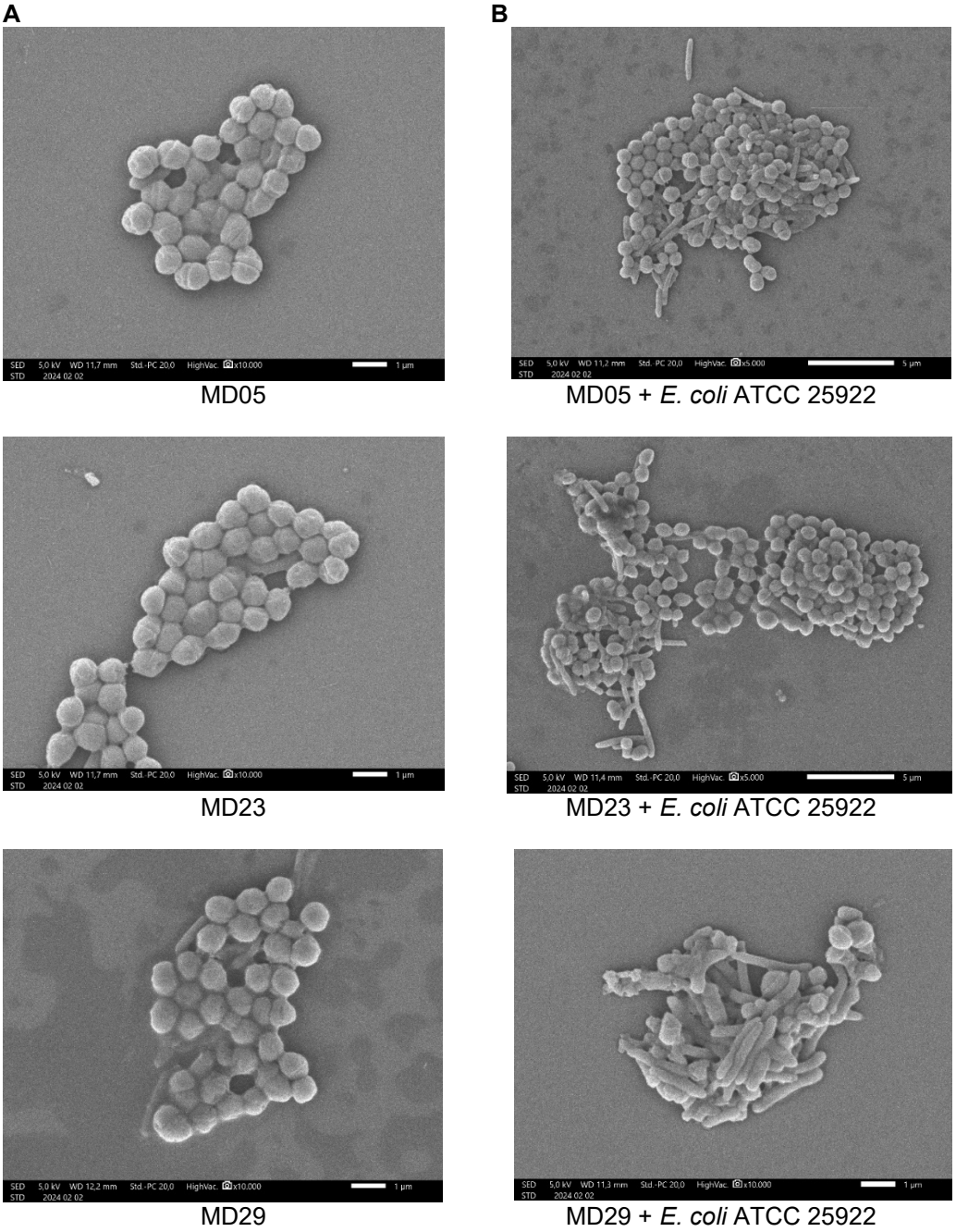

Figure 2. SEM images of (A) auto-aggregation of MD05 and MD23 (magnification 10,000x), and (B) co-aggregation between MD05 or MD23 with *E. coli* ATCC 25922 (magnification 5,000x) after 24 h incubation in PBS solution

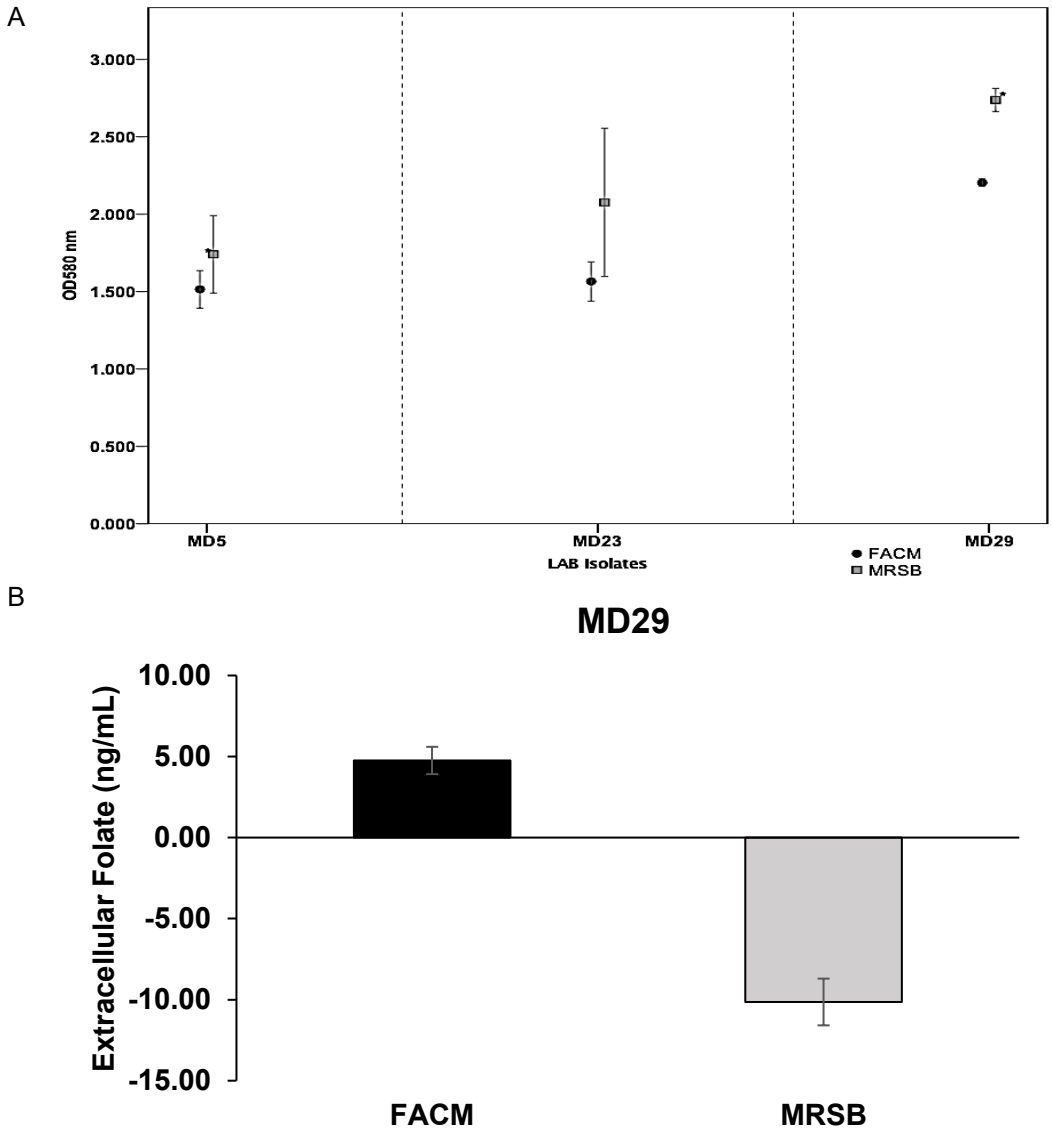

Figure 3. (A) Growth of three LAB potential probiotics in folate-free medium (FACM) and folate-containing medium (MRSB); (B) Extracellular folate production by isolate MD29 in FACM and MRSB

overproducing strains synthesize it in excess, regardless of external folate availability (Mahara et al., 2023). The MRSB medium used in this study contained folate and its precursors (para-aminobenzoic acid and glutamic acid), while FACM lacked folate and glutamic acid but included p-ABA. The findings suggest that MD29 is a folate-efficient LAB, as it produced folate only under folate-limiting conditions, consistent with the feedback inhibition mechanism regulating folate biosynthesis (Mahara et al., 2023). This property is important for the potential application of folate-producing bacteria in foods with low folate

content but may limit their use in folate-rich foods to avoid depletion of available folate. In this context, the MD29 isolate, which was obtained from honeybee hives, an environment characterized by low folate availability, is suspected to harbor folate biosynthesis genes, likely as an adaptation to meet its folate requirements for growth. Extracellular folate was reported to be produced by the probiotic candidate *Latilactobacillus sakei* LZ217 (Liu et al., 2022). *Latilactobacillus sakei* LZ217 showed the highest folate production in folic acid assay broth, which was 239.70 ± 0.03 ng/μL. The concentration of extracellular folate produced was still smaller than that of strain MD29 in this study. It is still necessary to test the production capacity in a biological environment and efforts need to be made to significantly increase extracellular folate production.

The key indicators that determine folate-producing bacteria have not been confirmed and are still being discussed by experts. Nevertheless, as a probiotic candidate, the capacity of MD29 for extracellular folate production, which we confirmed in this study, may be beneficial when the strain colonizes the colon, where it may contribute to the folate supply to the host (Greppi et al., 2017; Mahara et al., 2023). This trait is critical for the potential application of folate-producing bacteria in foods with low folate content but may limit its use in folate-rich foods to avoid depleting available folate. Nevertheless, as a probiotic candidate, MD29's capacity for extracellular folate production could be beneficial when the strain colonizes the large intestine, where it could contribute to folate supply for the host (Greppi et al., 2017; Mahara et al., 2023).

3.7 The 16S rRNA-based bacterial identification

Identification of isolates MD05, MD23, and MD29 was conducted using 16S rRNA gene sequencing. DNA concentrations of 114.45 ng/μL for MD05, 243.8 ng/μL for MD23, and 113.35 ng/μL for MD29 were obtained, with DNA purity values of 1.94, 1.99, and 1.85, respectively. PCR amplification yielded bands between 1000-1500 bp, confirming successful amplification (Figure 4). Sequence analysis revealed that MD05 was *Enterococcus faecium* strain BP106 (99.58% identity), MD23 was *Enterococcus sp.* strain 43-2 (94.35% identity), and MD29 was *Enterococcus faecium* strain AH2 (97.11% identity). These sequences were deposited in GenBank under accession numbers PQ571726 for MD05, PQ571750 for MD23, and PQ571749 for MD29 (Table 6). A phylogenetic tree (Figure 5) was constructed, showing that MD23 and MD29 were closely related, while MD05 was more distantly related. *Streptococcus* sp. was used as the control for the tree construction. The phylogenetic tree confirmed the position of this strain, which is closely related to other LAB commensals.

Gene identification revealed that the probiotic candidates *Enterococcus faecium* MD05, *Enterococcus* sp. MD23, and *Enterococcus faecium* MD29 were potential folate producers. Enterococcus species offer numerous benefits as commensals, including immune modulation, bacteriocin production, and cholesterol reduction (Viaud et al., 2013; Krawczyk et al., 2021). However, due to their opportunistic pathogenic potential (e.g., urinary tract infections, endocarditis, food poisoning), *Enterococcus* spp. lack the safety status required for probiotic use in the Europe and USA (Cheng et al., 2020). Therefore, further genotypic investigations, including whole genome sequencing and susceptibility assays, are needed to assess virulence factors and antibiotic resistance. Additionally, evaluating features like hydrophobicity, auto-aggregation, co-aggregation, adhesion to intestinal cells, and antimicrobial activity will be essential for determining the safety and efficacy of *Enterococcus* spp. in medical and food applications.

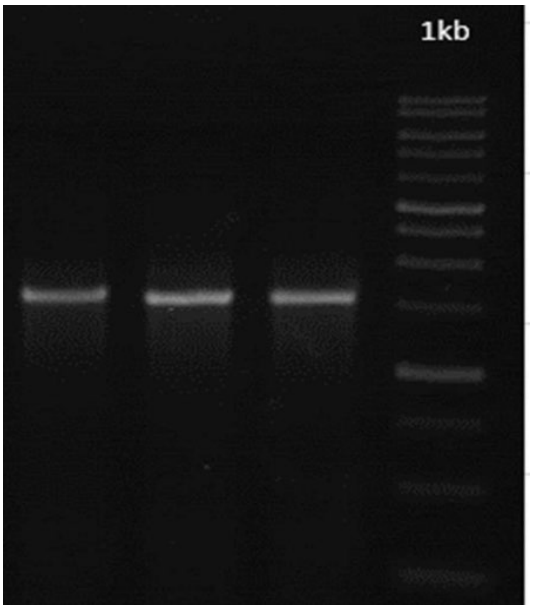

Figure 4. PCR products of 16S rRNA gene on agarose gel

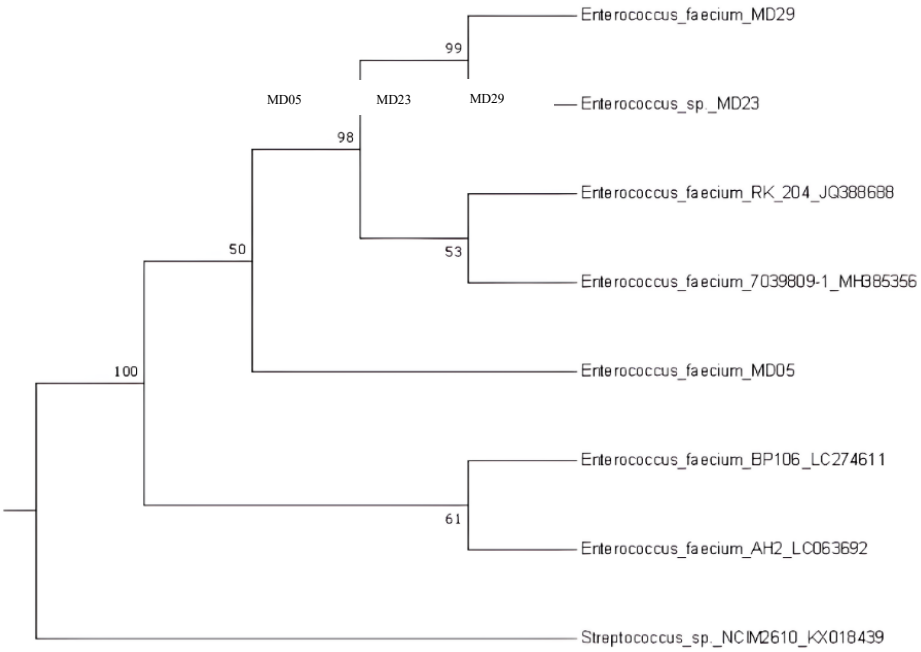

Figure 5. Phylogenetic tree based on the homology of 16S rRNA gene sequences of MD05, MD23, and MD29 isolated from East Nusa Tenggara Wild Giant Honeybee Hive (*Apis dorsata*)

Table 6. Identification of 16S rRNA gene sequence

Isolate	DNA Concentrate (ng/µL)	DNA Purity (A_{260}/A_{280})	bp	BLAST	% ID	NCBI No.
MD05	144.45	1.94	1490	*Enterococcus faecium* strain BP106	99.58	PQ571726
MD23	243.8	1.99	1347	*Enterococcus* sp. strain 43-2	94.35	PQ571750
MD29	113.35	1.85	1512	*Enterococcus faecium* strain AH2	97.11	PQ571749

4. Conclusions

This study successfully obtained *Enterococcus faecium* MD29 which was selected in vitro as fructophilic, a probiotic candidate, and an extracellular folate producer from Wild *Apis dorsata* Honeybee Hives in East Nusa Tenggara. The strain identity in the form of the 16S rRNA gene was successfully obtained and deposited in NCBI. Genetically, *E. faecium* MD29 is closely related to commensal bacteria in the human digestive tract. These findings provide a strong basis for conducting further studies to validate the probiotic claim and evaluate its capacity as a folate producer.

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6. Authors' Contributions

Concept and design: all authors. Analysis and interpretation: all authors. Data collection: Dandy Yusuf. Writing the article: Dandy Yusuf. Critical revision of the article: all authors. Final approval of the article: all authors. Statistical analysis: Dandy Yusuf. Obtained funding: Dandy Yusuf. Overall responsibility: all authors.

7. Conflicts of Interest

Authors have declared that no competing interests exist.

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