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Identification of Lactic Acid Bacteria (LAB) Consortium from Tempeh Jember Based on 16S rRNA Gene Sequences as Potential Probiotic Candidates

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Abstract

The development of pharmaceutical raw materials as local Indonesian probiotic concentrates requires attention to strain identity accuracy, safety, and health potential. This is due to the high potential of probiotics in preventing and addressing various health disorders from infancy to old age. Previous research successfully obtained lactic acid bacteria (LAB) isolates as probiotic candidates from tempeh produced in Jember, and preclinical testing showed that administering a consortium of five LAB isolates at a dose of 10^8 CFU/ml to BALB/c mice was an effective treatment for further application. This study aimed to perform molecular identification of the consortium of five LAB isolates from tempeh in Jember, East Java, based on 16S rRNA gene sequences. The consortium includes TA1, TB1, TK1, TK2, and TK4 isolates. This study used both qualitative descriptive and quantitative methods. Identification based on the 16S rRNA gene to determine LAB species strains involved bacterial genomic DNA isolation, amplification of the bacterial 16S rRNA gene, PCR product analysis, and phylogenetic identification and analysis. DNA amplification results showed that the consortium of five LAB isolates produced DNA fragments with bands of approximately 1300 bp. BLAST-N and phylogenetic analysis of the 16S rRNA gene showed that TB1 had 99.05% similarity with *Kosakonia cowanii* JCM 10956, TA1 had 99.46% similarity with *Lactiplantibacillus plantarum* JCM 1149, TK1 had 99.38% similarity with *Lactiplantibacillus pentosus* 124-2, TK2 had 96.86% similarity with *Pseudomonas fluvialis* ASS-1, and TK4 had 99.38% similarity with *Lactiplantibacillus plantarum* NBRC 15891. TA1, TB1, and TK4 were identified as non-pathogenic LAB strains, while TK1 and TK2 were not classified as LAB.

Keywords: Consortium; Lactic acid bacteria; Probiotics; Tempeh; 16S rRNA identification.

INTRODUCTION

Probiotics are beneficial living microorganisms for the host's health, when provided in adeaquate amount. Probiotics are commonly composed of *Lactobacillus* and *Bifidobacterium,* known as lactic acid bacteria (LAB). Moreover, most LAB, like *Lactobacillaceae, Pediococcus,* several *Streptococcus, Weissella*, and *Enterococcus* are safe for human as food-preserving agents (Martínez-Álvarez et al. 2017). LAB can be classified as probiotics, when they support human health, resist stomach acid and bile salt, adhere to mucose intestinal surface, and possess antimicrobial properties against pathogens (Ratna el al. 2021). The probiotics' potential are widely identified in human health. Probiotics reduce inflammation, induce mineral absorption, repair urogenital damage, attack colon cancer (Ma et al., 2023), remove pathogenic bacteria and increase good bacteria in colon (Kamil et al. 2022), decrease antibiotics associated diarrhea risk (Goodman et al. 2021), and cholesterol (Elisa and Lestari. 2021). Probiotic consortia have been shown to enhance memory power in Alzheimer's mice (Bonfili et al. 2020), improve cognitive performance during acute stress in healthy women (Bloemendaal et al. 2021), increase sperm quality (Sanchez-Rodriguez et al. 2024), and reduce the incidence of constipation, diarrhea, and acute respiratory infections in children (Mai et al. 2021).

Pramudito et al (2024) reported that LAB are present in tempeh, due to producing bioactives that reduce diarrhea by inhibiting the adhesion of *E. coli ETEC* to mammalian epithelial cells. Therefore, probiotics derived from Jember tempeh can be explored further for potential contribution to support human health in Indonesia. Previously, Azizah et al (2021) successfully isolated LAB from Jember tempeh as probiotic candidates and firstly demonstrated that LAB from Jember tempeh, East Java, Indonesia, can inhibit diarrhea-associated bacteria from both Gram-negative and Gram-positive groups, with strong inhibition activities. Additionally, LAB exhibit resilience to low pH and bile salts. Furthermore,

the LAB from Jember tempeh surpasses the capability of commercial *L. casei* strains.

According to Azizah et al (2023), administering lactic acid bacteria (LAB) from Jember tempeh consortium at 10^8 CFU/ml could significantly affect small intestine histology in BALB/c mice. Administering single LAB strains and control group (*L. acidophilus*) at the same dose resulted in partial necrosis of intestinal tissue. In contrast, the LAB consortium and normal group showed no signs of intestinal necrosis. The LAB consortium was also more effective in reducing populations of *Salmonella* sp. and *E. coli* in feces. These findings highlight the importance of further probiotic potential investigation of LAB derived from Jember tempeh, due to meeting key criteria for probiotic candidates. However, the molecular identity of the five LAB strains, encoded as TA.1, TB.1, TKI, TK2, and TK4, remains unknown. As probiotic effects are strain-specific, T.S. Kemgang (2014) reported that potential probiotic strains must be accurately identified, like using 16S rRNA methods.

The 16S rRNA sequencing analysis offers higher accuracy, faster processing, more superior sensitivity and specificity, than biochemical methods (Adesulu-Dahunsi et al. 2017). For example, Muryany (2017) stated that biochemical identification kits classified *L8* and *S1* bacterial isolates as *B. megaterium* and *Pediococcus pentosaceus*, respectively. However, molecular techniques revealed that *L8* was *L. plantarum* and *S1* was *L. pentosus*. The ribosomal RNA (rRNA) gene is one of the most conserved genes, thus reliable for determining bacterial taxonomy, phylogeny, and species divergence estimation. Therefore, this study aimed to identify five lactic acid bacteria consortia derived from Jember tempeh, East Java using 16S rRNA molecular identification.

MATERIALS AND METHODS

Procedures

LAB Strains and Growth Conditions

Five isolates of lactic acid bacteria (LAB) consortium were obtained from the Laboratory of Microbiology, Politeknik Kesehatan, Jember. These isolates were previously isolated from tempeh (a traditional fermented soybean product), originated from Jember, East Java (Azizah et al. 2023). Five LAB isolates were rejuvenated on solid GYP medium (1% glucose, 1% yeast extract, 0.5% peptone, 0.2% beef extract, 0.01% sodium acetate, 0.04% $MgSO_4 \cdot 7H_2O$, 0.0002% $MnSO_4 \cdot 4H_2O$, 0.002% $FeSO_4 \cdot 7H_2O$, 0.002% NaCl, 1% Tween 80, 5% $CaCO_3$, and 1.2% bacto agar) using the quadrant streak method. After 48-h incubation at 37 °C, single colonies of each LAB isolate were identified by the formation of a clear zone around the colonies (Azizah et al. 2021; Azizah et al. 2023). These single colonies were subsequently subjected to molecular identification.

Genomic DNA Isolation of Bacterial Isolates

A 1.5 mL bacterial culture was transferred into a 1.5 mL Eppendorf tube and centrifuged at 8,000 rpm for 10 min. The pellet was washed three times with STE buffer (containing 0,3M sucrose, 25 mM Tris-HCl, and 25 mM EDTA 2Na, pH 8) to remove impurities and centrifuged at 8,000 rpm for 10 min. This washing step was repeated three times. The resulting pellet was resuspended in 200 µL STE buffer and supplemented with 45 µL lysozyme (20 mg/mL), gently mixed, and incubated at 55 °C for 1 h to form protoplasts. -Subsequently, 20 µL Proteinase K was added to degrade proteins, followed by incubation with 400 µL of 10% CTAB in 0.7 M NaCl to remove polysaccharides and other contaminants. The mixture was then extracted with an equal volume of phenol:chloroform (25:24) and centrifuged at 12,000 rpm for 10 min. The aqueous phase was transferred to a new tube, mixed with 0.6 volumes of isopropanol and 20 µL sodium acetate, and incubated overnight at (−20) °C. The sample was then centrifuged at 12,000 rpm for 10 min. The supernatant was discarded, and the pellet was washed with 1 mL of 70% ethanol. DNA pellets were air-dried for 1 h to remove residual ethanol and dissolved in 50 µL sterile ddH_2O. The extracted DNA was stored at 4 °C or −20 °C for further use (Sambrook and Russell. 2001).

Amplification of 16S rRNA Gene from Bacterial Isolates

The 16S rRNA gene was amplified from genomic DNA using Polymerase Chain Reaction (PCR) with universal prokaryotic primers, specifically the forward primer 63f (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and the reverse primer 1387r (5′-GGG CGG WGT GTA CAA GGC-3′), as described by Marchesi et al (1998).The PCR reaction mixture consisted of GoTaq Green Master Mix (2×), 1.5 µL of each primer (10 pmol), 18.0 µL nuclease-free water (NFW), and 4.0 µL DNA template. The PCR cycling conditions included initial denaturation at 94°C for 4 minutes, 30 cycles of denaturation (94°C, 45seconds), annealing (55°C, 1minute), and extension (72°C, 1minute 10seconds), with a final extension at 72°C for 7 minutes.

PCR Product Analysis

PCR products were separated using 1% agarose gel electrophoresis in a mini-gel system at 75 V for 45 minutes. DNA bands were visualized under a UV transilluminator after staining with ethidium bromide (EtBr).

Id*entification and Phylogenetic Analysis of LAB Consortium Isolates*

The raw sequencing data were trimmed and assembled using *ChromasPro version 1.5*. The assembled sequences were compared with reference sequences in the *NCBI* database using the *BLAST* program

(http://www.ncbi.nlm.nih.gov/BLAST/). Closely related species and type strains identified from *GenBank* were selected for further analysis. Sequence alignment was performed using *MEGA version 5.0* (Tamura et al. 2011). A phylogenetic tree was constructed to determine the evolutionary relationship of the LAB isolates from tempe Jember with other lactic acid bacteria and non-lactic acid bacteria using the Neighbor-Joining method with 1,000 bootstrap replications (Felsenstein. 1985).

RESULTS AND DISCUSSION

Results

Amplification of the Five LAB Consortium Isolates

Genomic amplification of the five lactic acid bacteria (LAB) isolates from tempe in Jember, East Java, was carried out using PCR with primers 63F and 1387R. Visualization of the 16S rRNA gene amplification from isolates TA1, TB1, TK1, TK2, and TK4 produced DNA fragments with an approximate size of 1,300 bp (Figure 1). All five LAB isolates, which have potential as probiotic candidates, were molecularly identified based on the 16S rRNA gene and phylogenetic tree analysis.

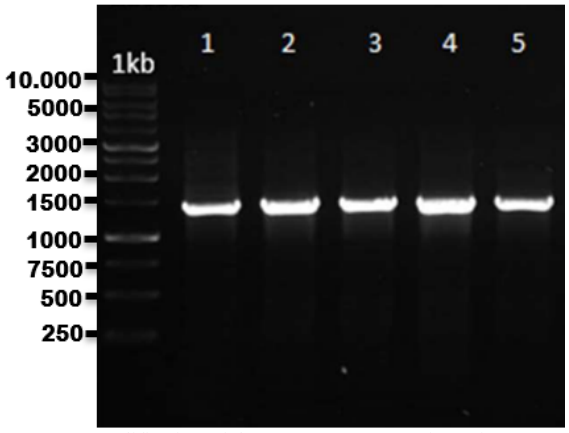

Figure 1. PCR amplification of the 16S rRNA gene using primers 63F and 1387R. M = 1 Kb DNA ladder (Fermentas); lanes 1–5 represent PCR products of bacterial isolates: 1 = TA1; 2 = TB1; 3 = TK1; 4 = TK2; 5 = TK4.

Identification and Phylogenetic Construction of the Five LAB Consortium Isolates Using the 16S rRNA Gene

DNA sequence analysis was performed using the *Basic Local Alignment Search Tool* (*BLAST*) against the National Center for Biotechnology Information (NCBI) database. Table 1 shows that the 16S rRNA gene sequences of the five LAB isolates exhibited the highest similarity with the 16S rRNA gene sequences of several bacterial species. Table 1 also presents the alignment of LAB isolate sequences with available data in *NCBI (BLAST-N)* along with their accession numbers.

Table 1. Alignment of 16S rRNA gene sequences of the five LAB consortium isolates with available data in NCBI (BLAST-N).

Isolate Name	Description	Max score	Total score	Query cover	E value	Per. Identity	Accession number
TA1	*Lactiplantibacillus plantarum* strain JCM 1149	2359	2359	100 %	0.00	99.46%	NR_115065.1
	Lactiplantibacillus pentosus strain 124-2	2353	2353	100 %	0.00	99.38%	NR_029133.1
	Lactiplantibacillus paraplantarum strain DSM 10667	2342	2342	100 %	0.00	99.23%	NR_025447.1
TB1	*Kosakonia cowanii JCM 10956 = DSM 18146* strain 888-76	2278	2278	100 %	0.00	99.05%	NR_025566.1
	Atlantibacter hermannii strain CIP 103176	2259	2259	100 %	0.00	98.82%	NR_104940.1
	Enterobacter timonensis strain mt20	2230	2230	100 %	0.00	98.42%	NR_179439.1
TK1	*Atlantibacter hermannii* strain CIP 103176	2254	2254	100 %	0.00	98.81%	NR_104940.1
	Enterobacter timonensis strain mt20	2213	2213	100 %	0.00	98.26%	NR_179439.1
	Salmonella enterica subsp. *enterica* serovar Typhimurium strain LT2	2209	2209	100 %	0.00	98.18%	NR_074910.1
TK2	*Pseudomonas fluvialis* strain ASS-1	2074	2074	100 %	0.00	96.86%	NR_159318.1
	Pseudomonas pharmacofabricae strain ZYSR67-Z	2058	2058	100 %	0.00	96.62%	NR_165768.1
	Pseudomonas tohonis strain TUM18999	2047	2047	100 %	0.00	96.47%	NR_179382.1
TK4	*Lactiplantibacillus plantarum* strain NBRC 15891	2346	2346	99 %	0.00	99.38%	NR_113338.1
	Lactiplantibacillus pentosus strain 124-2	2353	2353	99 %	0.00	99.38%	NR_029133.1
	Lactiplantibacillus paraplantarum strain DSM 10667	2342	2342	99 %	0.00	99.23%	NR_025447.1

BLAST-N analysis of the 16S rRNA gene sequences revealed that three isolates (TB1, TK1, and TK2) were identified as different species. Isolate TB1 was identified as *Kosakonia cowanii* and showed similarity with *Atlantibacter hermannii* and *Enterobacter timonensis*. Isolate TK1 was identified as *Atlantibacter hermannii* and exhibited similarity with *Enterobacter timonensis* and *Salmonella enterica subsp. enterica serovar typhimurium*. Isolate TK2 was identified as *Pseudomonas fluvialis* and showed similarity with

Pseudomonas pharmacofabricae and *Pseudomonas tohonis*. Isolates TA1 and TK4 were both identified *as Lactiplantibacillus plantarum*, albeit of different strains. Furthermore, isolates TA1 and TK4 consistently clustered closely with *Lactiplantibacillus pentosus* and *Lactiplantibacillus paraplantarum* based on BLAST-N sequence analysis and phylogenetic reconstruction.

Table 2. Identification results of the five LAB consortium isolates based on 16S rRNA sequences.

Isolate Code	Sample Origin	Identification Based on 16S rRNA Sequence
TA1	Antirogo, Jember, East Java	*Lactiplantibacillus plantarum* strain JCM 1149, 16S ribosomal RNA gene, partial sequence (99.46% similarity)
TB1	Baratan, Jember, East Java	*Kosakonia cowanii* JCM 10956 = DSM 18146 strain 888-76, 16S ribosomal RNA gene, partial sequence (99.05% similarity)
TK1	Kaliwates, Jember, East Java	*Atlantibacter hermannii strain CIP 103176 16S ribosomal RNA gene, partial sequence (98.81% similarity)*
TK2	Kaliwates, Jember, East Java	*Pseudomonas fluvialis* strain ASS-1_16S ribosomal RNA gene, partial sequence (96.86% similarity)
TK4	Kaliwates, Jember, East Java	*Lactiplantibacillus plantarum* strain NBRC 15891_16S ribosomal RNA gene, partial sequence (99.38% similarity).

Based on the nucleotide sequence identification of the 16S rRNA gene using BLAST-N, isolate TB1 showed 99.05% similarity with *Kosakonia cowanii* JCM 10956. Isolate TA1 exhibited 99.46% similarity with *Lactiplantibacillus plantarum* strain JCM 1149. Isolate TK1 demonstrated 99.38% similarity with *Lactiplantibacillus pentosus* strain 124-2. Isolate TK2 showed 96.86% similarity with *Pseudomonas fluvialis* strain ASS-1. Isolate TK4 displayed 99.38% similarity with *Lactiplantibacillus plantarum* strain NBRC 15891. Among these, isolates TA1, TB1, TK1, and TK4 had sequence similarity values above 97% compared to the nucleotide sequences of 16S rRNA genes available in GenBank, indicating that these isolates belong to the same species. In contrast, isolate TK2 exhibited a similarity value below 97%, suggesting that it may represent a different species or potentially a novel bacterium. According to Rosahdi et al (2018), a similarity value of less than 97% indicates that the identified bacterium is distinct and could be classified as a new species.

The phylogenetic tree reconstruction, which illustrates the evolutionary relationships, was performed using *MEGA version 5* and is presented in Figure 2.

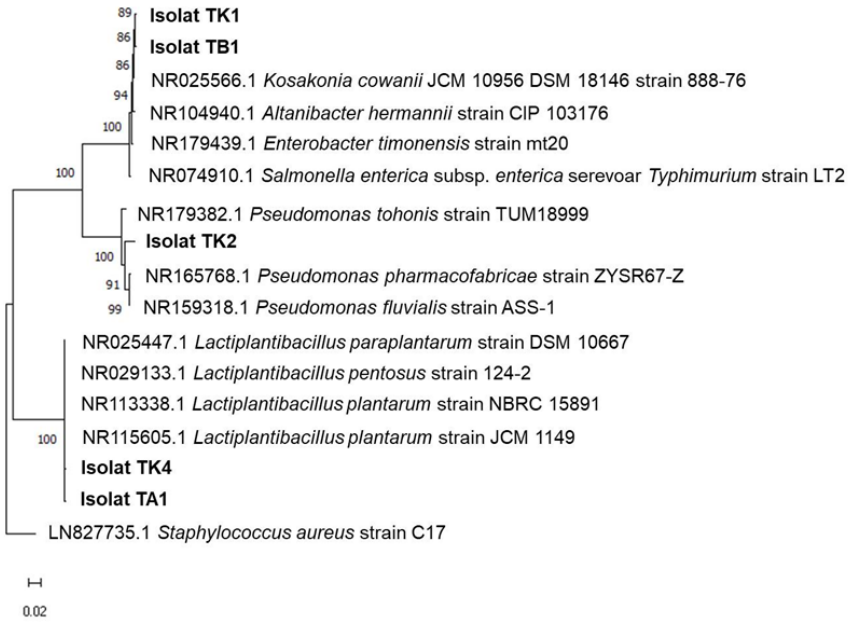

Figure 2. Phylogenetic tree of the five LAB isolates from tempeh Jember, East Java, Indonesia, compared with bacterial strains available in *GenBank*.

Phylogenetic analysis of the 16S rRNA gene sequences from the five LAB isolates and 11 bacterial strains obtained from GenBank was conducted using *MEGA version 5*. The phylogenetic tree was constructed

to determine the evolutionary relationships between the LAB consortium isolates and the 11 bacterial reference strains in GenBank. *Staphylococcus aureus* C17 was used as an outgroup. The phylogenetic tree is presented in Figure 2. The analysis revealed that isolates TK1 and TB1 clustered on the same branch, distinct from the other three LAB isolates. This clustering is explained by their high similarity (98%) with *Atlantibacter hermannii* strain CIP 103176 and *Enterobacter timonensis* strain mt20 (accession numbers: NR_104940.1 and NR_179439.1). Isolate TK2 was positioned on a separate branch from the other LAB isolates, grouping instead with *Atlantibacter hermannii* strain CIP 103176. Meanwhile, isolates TA1 and TK4 clustered together with *Lactiplantibacillus plantarum* strains, as well as *Lactiplantibacillus pentosus* and *Lactiplantibacillus paraplantarum* from GenBank (accession numbers: NR_029133.1 and NR_025447.1).

Discussion

The molecular identification results based on the 16S rRNA gene sequences of the TA1 and TK4 isolates indicate that both were identified as *Lactiplantibacillus plantarum*, showing close genetic relatedness to *Lactiplantibacillus pentosus* and *Lactiplantibacillus paraplantarum*. These isolates originated from tempehh produced in two different subdistricts: TA1 from Antirogo and TK4 from Kaliwates. Despite the geographic difference, *Lactiplantibacillus plantarum* was consistently found in tempeh originating from Jember. According to Fallo and Sine (2022), *Lpb. plantarum* was also successfully isolated and identified via 16S rRNA gene sequencing from tempeh produced in East Nusa Tenggara Province. Ilyanie et al (2023) further reported that seven LAB isolates from the fermented foods belacan and bosou were also identified as *Lpb. plantarum*, suggesting that *Lpb. plantarum* is capable of ecological and metabolic adaptation, allowing it to inhabit a wide range of ecological niches, including fermented foods, meat, plants, and the mammalian digestive tract. According to Azizah et al (2023), the TA1 and TK4 isolates are Gram-positive bacteria with rod-shaped cells, non-spore-forming, and occur either singly or in short chains, making the molecular identification results consistent with characterizations reported in previous studies.

Lactiplantibacillus plantarum has demonstrated broad-spectrum antibacterial potential against a wide range of Gram-positive and Gram-negative bacteria. Pathogenic food spoilage organisms and enteropathogens that can be inhibited by *Lpb. plantarum* include *E. coli*, *H.pylori*, *P.aeruginosa*, *Y.enterocolitica*, *C.r jejuni*, *L. monocytogenes*, *Klebsiella, Bacillus*, *Clostridium*, *Salmonella*, *S. aureus*, *Shigella*, and *Enterococcus*. In addition to its antibacterial properties, *Lpb. plantarum* also exhibits antifungal activity against several molds and yeasts, including *Candida* spp., *Aspergillus*, *Mucor*, and *Fusarium* (Dinev et al. 2018). This species has been utilized as a starter culture in fermented food products (Arena et al. 2016) and in livestock feed applications (Tian et al. 2023). Notably, *Lpb. plantarum* subsp. *plantarum* Kita-3, isolated from cheese produced by Mazaraat Artisan Cheese in Yogyakarta, Indonesia, has been recognized as a probiotic candidate that passed preclinical safety testing in animal models (A'inurrofiqin et al. 2022). Therefore, the *Lpb. plantarum* strain is acknowledged as a promising probiotic candidate in both the food and pharmaceutical industries, with potential applications as a biopreservative and alternative biotherapeutic agent (Gonzalez et al. 2021).

The molecular identification based on the 16S rRNA gene sequence revealed that the TB1 isolate was identified as *Kosakonia cowanii*, showing 98% genetic similarity to *Atlantibacter hermannii* and *Enterobacter timonensis*. This result aligns with the characterization of the TB1 isolate reported by Azizah et al (2023), which described TB1 as a Gram-negative bacterium with rod-shaped cells. This morphology is consistent with *Kosakonia cowanii*. According to Espinosa et al (2023), *Kosakonia cowanii* isolated from *Capsicum annuum* L. has potential as a biocontrol agent in suppressing the growth of pathogenic fungi. This ability is attributed to its capacity to produce volatile compounds with antifungal activity. Furthermore, phylogenetic analysis of *Kosakonia cowanii* indicates a close relationship with plant pathogenic strains, although several virulence genes were not detected.

El-Sheshtawy et al (2022) reported that the *K. cowanii* (B2) isolate was capable of producing a maximum yield of lactic acid (LA) using agro-industrial waste (cotton and coffee), reaching 28.14 g/L in a fermentor after 72 hours, as determined by HPLC analysis. Kacaribu and Darwin (2024) noted that *K. cowanii* is one of the LAB strains capable of producing LA from renewable sources for applications in petroleum-based products. Salatein et al (2025) also demonstrated that under optimal conditions (40 °C and pH 8.0), *K. cowanii* converted 87% of sugars from sugarcane and beet into LA, achieving a maximum yield of 14.2 g/L. Under optimal conditions, this strain achieved 85% efficiency, supporting the development of environmentally friendly production, bioeconomy, and enhanced lactic acid output. *Kosakonia* communities have also been found in silage feed fermented with *L. plantarum* SXC48 for livestock (Tian et al., 2023). However, isolation and screening of *K. cowanii* from fermented foods or beverages have not been previously reported, nor has *K. cowanii* been identified as a human pathogen. Therefore, this study represents the first report of *Kosakonia cowanii* being found in a fermented food, namely tempeh.

Based on 16S rRNA gene sequence analysis, the TK1 isolate was identified as *Atlantibacter hermannii*, showing 98% genetic similarity to *Enterobacter timonensis* and *Salmonella enterica*. According to Mohammad and Alyousif (2023), *A. hermannii* is a Gram-negative bacterium with rod-shaped cells. The

molecular identification of TK1 is consistent with its phenotypic characterization reported by Azizah et al (2023). Although *A. hermannii* is generally considered non-pathogenic, it has been isolated from human wound and eye infections (Girlich et al., 2021). Additionally, *A. hermannii* was detected as a contaminant in both local and imported frozen chicken meat in Basrah, Iraq, based on 16S rDNA analysis (Mohammad and Alyousif. 2023). *Atlantibacter hermannii* is not classified as a lactic acid bacterium (LAB) and is regarded as a rare opportunistic pathogen occasionally associated with human infections. Taxonomically, it belongs to the family Enterobacteriaceae.

Enterobacteriaceae is not classified as part of the LAB group, as LAB are defined as bacteria that convert carbohydrates into lactic acid (LA). Enterobacteriaceae belongs to a different bacterial family, although some of its members, such as *Enterococcus*, are capable of producing lactic acid in certain amounts. However, their primary function is not as clearly defined as that of true LAB. According to Wang et al. (2021), microorganisms capable of producing LA from various substrates through fermentation are highly diverse. These include bacteria, yeasts, algae, and fungi. Among these, only the bacterial group, specifically LAB, represents the genera most commonly utilized (Wang et al., 2021). LAB falls under the family *Lactobacillales*, which includes *Lactobacillus*, *Lactococcus Pediococcus*, *Aerococcus*, *Carnobacterium*, *Tetragenococcus*, *Vagococcus*, *Leuconostoc*, *Oenococcus*, *Streptococcus*, and *Weissella* (Sedó et al. 2022).

In the study conducted by Azizah et a. (2023), the TK1 isolate produced a clear zone measuring 5.31 ± 1.18 mm around the colony, indicating its ability to secrete lactic acid (LA) into GYP agar medium supplemented with $CaCO_3$. Calcium carboanate ($CaCO_3$) neutralizes the acid produced by TK1 and leads to the formation of a clear zone, which serves as an established indicator of LAB activity. This approach serves as an initial selection method in the isolation and purification of LAB. Based on molecular identification and supporting literature, TK1 can be classified as LAB. However, further confirmation through molecular re-identification is necessary to verify its classification and ensure that TK1 belongs to a non-pathogenic species. Such verification is essential for evaluating the safety of candidate probiotic strains, and requires precise species-level identification using 16S rRNA gene sequencing to support future applications in food and health biotechnology.

Molecular identification based on 16S rRNA gene sequencing revealed that the TK2 isolate was identified as *Pseudomonas fluvialis*, showing 96% genetic similarity to *Pseudomonas pharmacofabricae* and *Pseudomonas tohonis*. This result is consistent with the phenotypic characterization reported by Azizah et al (2023), which described TK2 as a Gram-negative, rod-shaped bacterium. While certain species within the genus *Pseudomonas* are beneficial, others are known to be pathogenic. For instance, *P. aeruginosa* is associated with various human infections, *P. syringae* acts as a plant pathogen and *P. fluorescens* is a free-living soil bacterium with beneficial properties. Additionally, several *Pseudomonas* species have been isolated from contaminated environments and show potential as effective bioremediation agents (Sudan et al. 2018).

Previous studies have not reported *Pseudomonas fluvialis* as a member of the lactic acid bacteria (LAB) group. This is because *P. fluvialis* belongs to the family Pseudomonadaceae, which is not classified as LAB, as LAB are defined by their ability to convert carbohydrates into lactic acid (LA). However, in the study by Azizah et al (2023), the TK2 isolate produced a clear zone measuring 5.62 ± 1.50 mm around the colony, an indicator of LA secretion into GYP agar medium containing $CaCO_3$, suggesting LAB-like activity. The alkaline $CaCO_3$ neutralizes the acid produced, resulting in the clear zone formation. Despite this functional trait, the molecular identification of TK2 in this study does not align with existing literature. Therefore, re-identification using 16S rRNA gene sequencing is necessary to confirm whether TK2 belongs to a non-pathogenic LAB species.

Based on molecular identification using 16S rRNA gene sequencing and supporting literature review, the consortium of five lactic acid bacteria (LAB) isolates revealed that TA1, TB1, and TK4 belong to the LAB group and are non-pathogenic strains. In contrast, TK1 and TK2 require further molecular re-identification using 16S rRNA gene sequencing, as they were identified as non-LAB strains and exhibit characteristics that differ from previous findings. TA1 and TK4 were identified as *Lactiplantibacillus plantarum*, while TB1 was identified as *Kosakonia cowanii*. TK1 was identified as *Atlantibacter hermannii*, and TK2 as *Pseudomonas fluvialis*.

CONCLUSIONS

Based on the results of the study, it can be concluded that the 16S rRNA coding DNA from a consortium of five lactic acid bacteria (LAB) isolates derived from tempeh produced in Jember, East Java, was successfully isolated and sequenced. The identification of the five LAB isolates, collected from three different subdistricts in Jember, revealed the presence of bacterial species with distinct strain identites. The TA1 isolate, obtained from tempeh in Antirogo, Jember, was identified as *Lactiplantibacillus plantarum* strain JCM 1149. The TB1 isolate, also from tempeh in Antirogo, was identified as *Kosakonia cowanii* strain JCM 10956. Meanwhile, the TK1, TK2, and TK4 isolates, which were collected from tempeh in Kaliwates, Jember, were identified respectively as *Lactiplantibacillus pentosus* strain 124-2,

Pseudomonas fluvialis strain ASS-1, and *Lactiplantibacillus plantarum* strain NBRC 15891.

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Authors' Contributions: Siti Nur Azizah prepared the LAB isolates, designed the study, and drafted the manuscript. Rosida and Rizka Yolanda Febiaocti contributed to manuscript writing and performed proofreading. Dewi Riska Nurmalasari conducted genomic DNA isolation in the laboratory. Sipriyadi carried out bacterial amplification and identification. All authors were involved in drafting and refining the manuscript and approved the final submitted version

Competing Interests: Competing Interests: The authors declare that there are no competing interests.

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