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Molecular Phylogenetic Analysis of 16S rRNA Gene of Facultative Hetero-Fermentative Lactic Acid Bacteria

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Abstract

Facultative heterofermentative lactic acid bacteria are bacteria that ferment carbohydrates to produce lactic acid and by-products such as acetic acid, ethanol, and CO2. This group of bacteria is widely used in the food and health industries. However, research on BAL species, especially the facultative heterofermentative group, is still relatively limited, so further studies are needed with molecular phylogenetic analysis aimed at providing an overview of the kinship of BAL species in the facultative heterofermentative group using the 16S rRNA gene molecular marker for more accurate identification results. The research design was conducted as a molecular-based descriptive analysis using secondary data in the form of 16S rRNA gene nucleotide sequences taken from the National Center for Biotechnology Information (NCBI) GenBank. Data analysis was performed using bioinformatics tools, including sequence alignment using ClustalX, phylogenetic tree reconstruction using MEGA and PHYLIP, and similarity matrix analysis using PHYDIT. The results of the analysis showed that *Lactobacillus casei* and *Lactobacillus rhamnosus*, as well as *Lactobacillus pentosus* and *Lactobacillus plantarum*, were closely related with a bootstrap value of 100% and a similarity matrix of 98-99%. Meanwhile, *Lactobacillus alimentarius* showed a relatively close relationship with *Lactobacillus pentosus* and *Lactobacillus plantarum* with bootstrap values and similarity matrices of around 92%. Thus, molecular phylogenetic analysis is effective in analyzing, identifying, and proving the relationship between BALs of the facultative heterofermentative group.

Keywords: Lactic acid bacteria; Facultative Hetero-Fermentative; Phylogenetic, Molecular; 16S Rrna Gene.

INTRODUCTION

Approximately 30,000 species of bacteria have been officially identified, recognized, cultivated in pure form, and physiologically studied by scientists around the world (Dykhuizen, 2005), one of which is lactic acid bacteria, which are widely distributed in natural habitats such as water, soil, the digestive tracts of humans and animals, and various fermented food products (Ayivi *et al*., 2020; Karyawati *et al*., 2024). Lactic acid bacteria are characterized by their metabolic ability to produce lactic acid as the end product of carbohydrate fermentation (Axelsson, 2004; Finanda *et al*., 2021; Mokoena, 2017; Quinto *et al*., 2014) and beneficial non-pathogens (Surak *et al*., 2024). In addition, LAB has potential nutritional, probiotic, and antimicrobial properties, so it is widely used in various biological and biotechnological processes, especially in food fermentation, health, and the pharmaceutical and agricultural industries (Adamu *et al*., 2025; Ahmad *et al*., 2020; Ayivi *et al*., 2020). One important classification within lactic acid bacteria is based on the fermentation products, which are grouped into several types, one of which is the facultative heterofermentative bacteria.

Facultative heterofermentative bacteria are a group of lactic acid bacteria that, in addition to producing lactic acid as the end product of carbohydrate fermentation, also produce acetic acid, ethanol, and CO2 (Finanda *et al*., 2021; Kusnadie *et al*., 2025). Bintsis (2018), explains that there are several species of lactic acid bacteria classified as facultative heterofermentative, including *Lactobacillus alimentarius*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus pentosus*, and *Lactobacillus xylosus*. Much research has been conducted on lactic acid bacteria, but research on their phylogenetic relationships is still lacking.

Most studies have only conducted basic isolation and characterization of bacteria, without reviewing information on the relationship between lactic acid bacteria, including facultative hetero-fermentative groups, except for the species *Lactobacillus casei* and *Lactobacillus rhamnosus*. Some information shows that *Lactobacillus casei* and *Lactobacillus rhamnosus* are closely related phenotypically and genotypically and are

widely used in research around the world in fermented milk products, food supplements, and probiotics to improve host health (Hill *et al*., 2018; Huang *et al*., 2018). However, information regarding the relationship between other facultative heterofermentative bacteria is not yet known with certainty. Therefore, to complete and add to the information, a study is needed to prove and determine the relationship between facultative heterofermentative lactic acid bacteria, one of which is through molecular phylogenetic analysis.

Molecular phylogenetics is a method often used to determine the kinship between species by considering the evolutionary path of each species studied, either through phylogenetic tree reconstruction or similarity matrices (Yang & Rannala, 2012). Molecular phylogenetics aims to visualize evolutionary relationships between species, families, and genes, including microorganisms, and has become an important component in modern biological research (Zou *et al*., 2024), such as molecular biology, genetic populations, developmental biology, and evolutionary biology (Tindi *et al*., 2017).

As modern biology develops, the systematic approach, especially in molecular phylogenetic studies, has expanded, particularly through the use of DNA sequences as the main character in classification and phylogenetic analysis. DNA sequences are considered more informative because they provide more accurate genetic data, strengthen homology testing, and produce more natural phylogenetic tree reconstructions (Baldwin *et al*., 1995; Chase *et al*., 1993; Moritz, C. & Hillis, 1996). One important development that forms the basis of this research is the use of a molecular phylogenetic approach based on the 16S rRNA marker gene or ribosomal gene.

Gen 16S rRNA is a molecular marker in prokaryotes that is approximately 1550 bp long (Jumrah & Salnus, 2024) and contains variable and conserved regions that can be used to distinguish between species. (Akihary & Kolondam, 2020; Nurkanto & Agusta, 2015). The 16S rRNA gene has been widely used in various fields of research because it is considered highly effective, does not take too long to perform identification, and produces results with a high level of accuracy compared to other genes such as 5S and 23S, which are considered more difficult to apply in identification (Akihary & Kolondam, 2020; Noer, 2021). In addition, the 16S rRNA gene can identify organisms from the genus to species level (Jumrah & Salnus, 2024), including bacteria, because it is universal, evolves, and shows phylogenetic relationships (Woese *et al*., 1985). The reason for using ribosomes as target genes is that ribosomes are the most conserved elements among all organisms and function as ideal molecular chronometers (Prakash *et al*., 2013; Woese *et al*., 1985).

Based on the description of the background of the problem above, it is necessary to conduct molecular phylogenetic analysis of facultative heterofermentative lactic acid bacteria based on ribosomal genes or 16S rRNA, with the aim of analyzing, identifying, and proving kinship relationships through phylogenetic tree reconstruction and similarity matrix.

MATERIALS AND METHODS

Tools and Materials

The tools used in this study were the ClustalX software program for sequence alignment, MEGA and PHYLIP for phylogenetic tree reconstruction, and PHYDIT for similarity matrix creation. The materials used were nucleotide sequence data from the 16S rRNA gene or ribosomal gene taken from the *National Center for Biotechnology Information* (NCBI) (https://www.ncbi.nlm.nih.gov/). The sequences taken were data on lactic acid bacteria species from the Facultative Hetero-Fermentative group.

Research Design

This research is a molecular-based descriptive analysis study, namely molecular phylogenetics with a 16S rRNA gene nucleotide sequence analysis approach. The main objective of this analysis is to describe in detail the evolutionary relationships and kinship between strains of the Facultative Hetero-Fermentative BAL group based on comparisons of 16S rRNA gene nucleotide sequences through final output in the form of data analysis using bioinformatics tools, namely phylogenetic trees and similarity matrices.

Procedures and Data Collection

1. Sequens Data Collection

This study uses secondary data in the form of nucleotide sequence data from the 16S rRNA gene or ribosomal gene taken from the NCBI (National Center for Biotechnology Information) website (https://www.ncbi.nlm.nih.gov/). These sequences are data on lactic acid bacteria species from the facultative heterofermentative group, taken from published research journals to ensure data validity. Bintsis (2018)~~,~~ explains that there are several species of lactic acid bacteria classified as facultative heterofermentative, including *Lactobacillus alimentarius*, *Lactobacillus plantarum*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus pentosus*, and *Lactobacillus xylosus*.

However, in this study, not all identified bacterial species had 16S rRNA gene nucleotide sequence data available on NCBI. One bacterial species, Lactobacillus xylosus, could not be used because the 16S rRNA gene nucleotide sequence was not yet available on the NCBI website. Therefore, this study only used five species that had 16S rRNA gene nucleotide sequences, including *Lactobacillus alimentarius*, *Lactobacillus casei*, *Lactobacillus rhamnosus*, *Lactobacillus plantarum*, and *Lactobacillus pentosus*. One species from a different

genus was also used as an outgroup. The species used in the outgroup, *Lactococcus lactis,* was taken from the study by Muri *et al*. (2013).

Table 1. Sequence Data of Facultative Hetero-Fermentative Lactic Acid Bacteria from the NCBI Site.

Bacterial Name	Abbreviated Name	Accesion Number	Long	Researcher
Lactobacillus pentosus (strain MH92)	LacpenMH92	FJ542304.1	1446 bp	Lee *et al.* (2008)
Lactobacillus alimentarius (strain HBUAS59653)	Lacal5963	ON125463.1	1496 bp	Guo *et al.* (2022)
Lactobacillus casei (strain LI3)	LacaseiLI3	KJ806307.1	1450 bp	Yang (2014)
Lactococcus lactis (strain NCDO604)	Laclac064	NR040955.1	1499 bp	Muri *et al.* (2013)
Lactobacillus rhamnosus (strain LOCK 0997)	Lacrha0997	KP773477.1	1453 bp	Nowak & Outlewska (2015
Lactobacillus plantarum (strain LP6)	LacplaLP6	PQ345794.1	1486 bp	Indrajeet *et al.* (2024)

2. Sequence Alignment with the Program "ClustalX"

Sequence alignment was performed using ClustalX version 2.0 (Larkin *et al*., 2007). The purpose of this alignment process is to arrange or align the 16S rRNA gene sequences so that they are placed according to their homology positions between sequences, enabling accurate comparison of the 16S rRNA genes between microbial strains. Alignment with ClustalX was performed after all sequence data sets were collected in a Notepad file in FASTA format, with each sequence prefixed with a ">" symbol and the abbreviation name of each strain sequence, with a maximum of 10 letters, is placed in front of the ">" sign. The data set is then saved in a folder and loaded into the ClustalX program. The program will display a list of sequence identities on the left and the nitrogen base sequence on the right. A total alignment (do complete alignment) is performed to obtain compatible file formats, including CLUSTAL (.aln) for accessing the MEGA program, PHYLIP (.phy) for accessing the PHYLIP program, and GDE (.gde) for accessing the PHYDIT program.

Molecular Phylogenetic Data Analysis

1. Reconstruction of Phylogenetic Tree with the Program "MEGA"

Reconstruction of the phylogenetic tree in the MEGA 5.1 program (Tamura *et al*., 2011) was performed using a sequence data set aligned using the ClustalX 2.1 program (Larkin *et al*., 2007). Because the ClustalX file format is different from MEGA, the sequence data file needs to be converted first so that it can be read and processed compatibly, namely by using the CLUSTAL (.aln) data file format generated from ClustalX to produce a new file format, namely the MEGA alignment file (.mas) and MEGA sequence data (.meg), in order to process the phylogenetic tree reconstruction analysis in the MEGA program. The algorithm used in phylogenetic tree reconstruction using the MEGA program is Neighbor and reconstructed using the Test of Phylogeny bootstrap method with 1000 replications. The evolutionary model used is Maximum Composite Likelihood. The evolutionary rate (Rate among lineages) uses "Uniform rates". The lineage pattern (Pattern among lineages) uses "same (homogenous)". Treatment of missing data uses the "complete deletion" model.

2. Reconstruction of Phylogenetic Tree with the Program "PHYLIP"

Phylogenetic tree reconstruction in the PHYLIP version 3.69 program (Felsenstein, 2005) was performed using a sequence data set resulting from alignment with the ClustalX 2.1 program (Larkin et al., 2007) in the form of a PHYLIP data file (.phy). The PHYLIP program contains many executable applications that can support sequence data analysis using various algorithms. The applications used in the PHYLIP program are "dnadist" to create a distance matrix, namely the Felsenstein 84 (F84) substitution model, and "Neighbor" for phylogenetic tree reconstruction using the Neighbor Joining distance matrix algorithm. The phylogenetic tree generated by the PHYLIP program can be accessed using the "Treeview" application.

3. Reconstruction of Phylogenetic Tree with the Program "PHYDIT"

The nucleotide similarity matrix between sequences in the PHYDIT program (Chun, 1995) was created using a sequence data set aligned by the ClustalX 2.1 program (Larkin *et al*., 2007) in GDE (gde.) file format. The nucleotide similarity matrix was generated using the SimTable: Generating Similarity Table analysis mode. The similarity matrix output was then copied into Microsoft Excel for better display and detail. The nucleotide similarity matrix display was presented in two parts, consisting of an upper-right triangle containing the number of nucleotides and a lower-left triangle containing the nucleotide similarity data (NT Similarity.

RESULTS AND DISUCUSSION

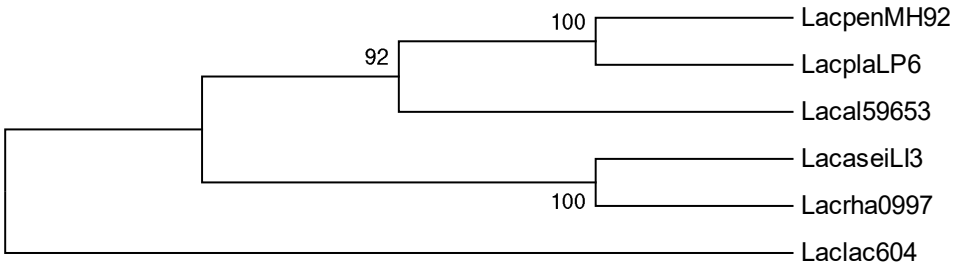

Figure 1. Phylogenetic Tree in the MEGA 5.1 Program using the Neighbor Joining Algorithm, Model Maximum Composite Likelihood 1000 Bootstrap.

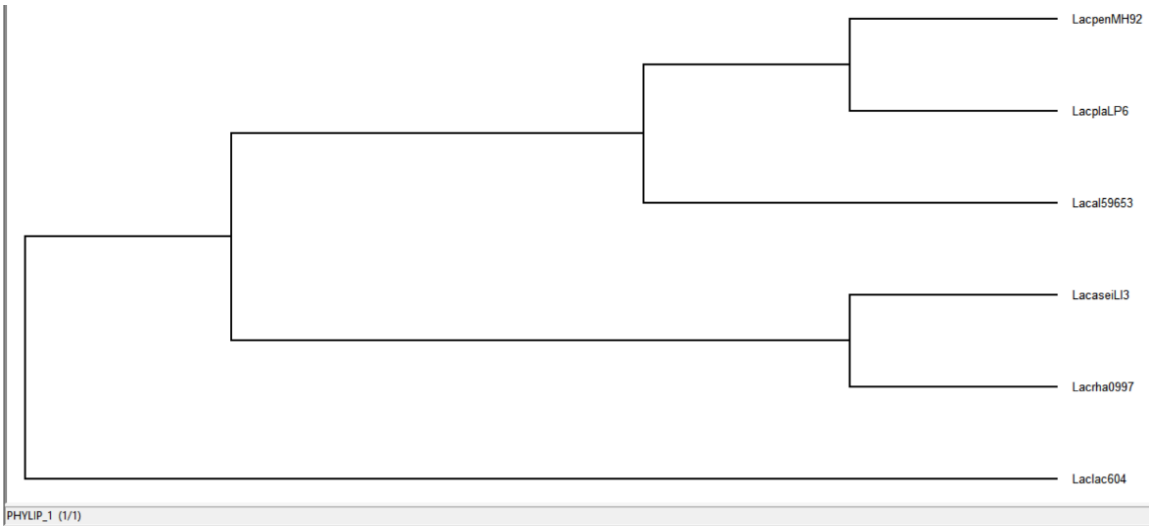

Figure 2. *Phylogenetic Tree* in the PHYLIP 3.69 Program Using the *Neighbor Joining* Algorithm.

Based on molecular phylogenetic analysis through phylogenetic tree reconstruction using the MEGA 5.1 software program (Tamura *et al*., 2011) shown in **Figure 1** and the PHYLIP 3.69 program (Felsenstein, 2005) shown in **Figure 2** using the Neighbor Joining algorithm, a cladogram visualization with the exact same shape and position was obtained. However, the difference between PHYLIP and MEGA 5.10 is that in PHYLIP, the phylogenetic tree reconstruction must first be opened using the Tree View application, and the phylogenetic tree chart does not display information such as numbers that index bootstrap values. However, in the Mega 5.10 software, phylogenetic tree reconstruction can be done directly in the MEGA application without opening the Tree View application, and the phylogenetic tree chart also displays numbers that index bootstrap values.

Phylogenetic tree analysis is used to examine the degree of kinship between different Lactate Acid Bacteria sequences that are interrelated, namely the Facultative Hetero-Fermentative group. The resulting phylogenetic tree consists of three clusters, including **Cluster I**, which consists of the Lacpen MH92 (*Lactobacillus Pentosus*), LacplaPL6 (*Lactobacillus plantarum*), and Lacal59653 (*Lactobacillus alimentarius*) strains. **Cluster II** consists of the LacaseiL13 (*Lactobacillus casei*) and Lacrha0997 (*Lactobacillus rhamnosus*) strains. Finally, **Cluster III** consists of one strain, Laclac604 (*Lactococcus lactis*).

Phylogenetic tree analysis is performed by looking at bootstrap values. Muth'iah & Jannah (2023) explain that bootstrap values are indices used to test how well the sequence model data set is used. If the bootstrap values in the phylogenetic tree reconstruction are low, then the sequences from the analysis to obtain a phylogenetic tree become unreliable. A good and reliable phylogenetic tree reconstruction is one with bootstrap values above 70% (>70%) (Subari et al., 2021). According to Hillis & Bull (1993), if the bootstrap value is above 70%, it can be confirmed with ≥ 95% certainty that the organisms have significant similarities and are reliable. Of the six bacterial strains, those with the highest bootstrap values were *Lactobacillus casei* (strain LacaseiL13) and *Lactobacillus rhamnosus* (strain Lacrha0997), as well as *Lactobacillus pentosus* (strain Lacpen MH92) and *Lactobacillus plantarum* (strain LacplaPL6) at 100%. followed by *Lactobacillus alimentarius* (Lacal5963 strain) with *Lactobacillus Pentosus* (Lacpen MH92 strain) and *Lactobacillus plantarum* (LacplaPL6 strain) at 92%. meaning that the bootstrap values of these bacterial strains are consistent with the theoretical explanations provided by Subari et al (2021) and Hillis & Bull (1993). Ubaidillah and Sutrisno (2009) also explain

that high bootstrap values result in high topology confidence values from the phylogenetic tree results they constructed. A bootstrap value of 100% indicates that the species have a very high and close relationship, such as *Lactobacillus casei* (strain LacaseiL13) and *Lactobacillus rhamnosus* (strain Lacrha0997) as well as *Lactobacillus Pentosus* (strain Lacpen MH92) and *Lactobacillus plantarum* (strain LacplaPL6) (Bramasta et al., 2021; Sahadeva & Pertiwi, 2024).

Analysis of the *Lactococcus lactis* (Laclac604) strain shows that it is the most distantly related bacterial strain compared to the others. When observed carefully on the phylogenetic tree, the Laclac604 strain has no known bootstrap value and its position on the cladogram is in the outgroup because it is separated from the other strains in quotation marks "has no pair and close kinship relationship". However, the existence of an outgroup is very much needed in phylogenetic tree reconstruction. This is based on the statement made by Hidayat & Adi (2008) that the existence of an outgroup in phylogenetic tree analysis is very necessary to form character polarization, namely apomorphic characters (characters that change and are inherited in the in-group) and plesiomorphic characters (primitive characters found in the outgroup).

PHYDIT - Similarity analysis
1531 nucleotides analysed
Lower-left triangle contains [NT] Similarity.
Uppper-right triangle contains [NT] Different/Total nucleotides.

The following table contains tab-delimited numbers. Copy and paste to MS Excel or Word.

	LacaseiLI3	Lacrha0997	LacpenMH92	LacplaLP6	Lacal59653	Laclac604
LacaseiLI3	---	18/1439	131/1446	133/1446	149/1447	205/1425
Lacrha0997	98.75	---	117/1435	121/1449	141/1450	210/1428
LacpenMH92	90.94	91.85	---	8/1445	103/1445	194/1422
LacplaLP6	90.80	91.65	99.45	---	107/1476	204/1461
Lacal59653	89.70	90.28	92.87	92.75	---	214/1466
Laclac604	85.61	85.29	86.36	86.04	85.40	---

Figure 3. Matrix of Similarities in the PHYDIT Program.

Molecular phylogenetic analysis was reinforced by using the PHYDIT program (Chun, 1995) to determine the nucleotide similarity matrix containing the percentage of nucleotide similarity between sequence pairs shown in **Figure 3**. Based on the similarity matrix table generated by the PHYDIT program, it can be seen that the strain with the highest similarity matrix value is *Lactobacillus pentosus* (Lacpen MH92 strain) with *Lactobacillus plantarum* (LacplaPL6 strain) at 99.45%. It can also be seen that these two strains have a value of 8/1445, which means that out of a total of 1445 nucleotide bases in the 16S rRNA gene, there are only 8 differences. This is followed by *Lactobacillus casei* (strain LacaseiL13) with *Lactobacillus rhamnosus* (strain Lacrha0997) at 98.75%. The two strains also had a value of 18/1439, meaning that out of a total of 1439 nucleotide base sequences of the 16S rRNA gene, there were only 18 differences. Thus, this is consistent with the cladogram in the PHYLIP and MEGA programs, in which the *Lactobacillus pentosus* strain (Lacpen MH92 strain) is grouped with *Lactobacillus plantarum* (LacplaPL6 strain) and *Lactobacillus casei* (LacaseiL13 strain) is grouped with *Lactobacillus rhamnosus* (Lacrha0997 strain), indicating that these strains are closely related. Furthermore, for *Lactobacillus alimentarius* (Lacal59653) has the closest matrix similarity percentage with *Lactobacillus plantarum* (LacplaPL6) at 92.75% and is known to have a value of 107/1476, indicating that out of a total of 1476 nucleotide base sequences of the 16S rRNA gene, there are only 107 differences. Meanwhile, the species strain with the lowest matrix similarity value is *Lactococcus lactis* (Laclac640) with *Lactobacillus rhamnosus* (strain Lacrha0997) at 85.29%.

Based on the above description and analysis, the 16S rRNA gene can provide informative visualization of the molecular phylogenetics of facultative hetero-fermentative BAL. Molecular phylogenetics using the 16S rRNA gene marker has proven the close relationship between *Lactobacillus casei* and *Lactobacillus rhamnosus* based on information obtained from Hill *et al*. (2018) and Huang *et al*. (2018). Molecular phylogenetics using the 16S rRNA gene marker has also provided other information regarding the kinship of other species of facultative hetero-fermentative Lactic Acid Bacteria that were previously unknown. *Lactobacillus pentosus* and *Lactobacillus plantarum* are very closely related, as are *Lactobacillus casei* and *Lactobacillus rhamnosus*, so this information can be used for further research and studies to confirm its validity. Meanwhile, *Lactobacillus alimentarius* has a close kinship relationship with *Lactobacillus pentosus* and *Lactobacillus plantarum.*

Meanwhile, in the outgroup species Lactococcus lactis, based on the PHYDIT similarity matrix, it has 85–86% similarity with other members of the BAL group of facultative heterofermentative bacteria. This percentage is still high enough to determine kinship. This is based on the taxospecies concept that a group of strains is classified as one species if it has a similarity index value of ≥ 70% (Meurant, 2011). This is because the PHYDIT program ignores any evolutionary model, so that when viewed on the *Lactococcus lactis* phylogenetic tree, it has no similarity at all because both the MEGA and PHYLIP programs consider the evolutionary path in terms of genus similarity. Then, based on other information obtained, the *Lactococcus lactis* species was formerly the *Lactobacillus xylosus* species but has now been classified as *Lactococcus lactis* (Bernardeau et al., 2006). When searching for the *Lactobacillus xylosus* species on the NCBI website, it was never found, but the *Lactococcus lactis* species always appeared. From this statement, *Lactococcus lactis* may still have inherent and similar properties to lactic acid bacteria even though they belong to different groups, resulting in a high similarity value with other members of the facultative heterofermentative group (85-86%).

CONCLUSIONS

The conclusion of this study is that molecular phylogenetic analysis using the 16S rRNA gene marker helps in analyzing, identifying, and proving the phylogenetic relationships of facultative hetero-fermentative lactic acid bacteria species. Based on branch position, bootstrap values, and similarity matrices, *Lactobacillus casei* and *Lactobacillus rhamnosus*, as well as *Lactobacillus pentosus* and *Lactobacillus plantarum*, have very close phylogenetic relationships with bootstrap values of 100% and similarity matrices of 98-99%. Meanwhile, *Lactobacillus alimentarius* shows a relatively close relationship with *Lactobacillus pentosus* and *Lactobacillus plantarum* with bootstrap values and similarity matrices of around 92%. Meanwhile, *Lactococcus lactis* has the furthest relationship because it is an outgroup.

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Authors' Contributions: Habib Ramadhan was responsible for designing the research, collecting data, analyzing data, and writing the research draft, including the abstract, introduction, results and discussion, and conclusion. Erna Wulandari served as the research supervisor and editor, correcting the research draft from the abstract to the conclusion to ensure that it was in line with the research objectives, appropriate methodology, scientific principles, and applicable systematic procedures. All authors have read and approved the final version of this research manuscript.

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