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Probiotic Community of Dage Banyumas: Next Generation Sequencing Approach

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Abstract. Tempeh is known as a traditional fermented food that has been popular in Indonesia for hundreds of years. One of the tempeh that is widely consumed in Banyumas regency is Dage, which is made from fermented coconut waste. Currently, there is no information regarding the presence of probiotics in Dage. Therefore, this study aims to detect the diversity of probiotic types in the Dage tempeh microbiome using the Next Generation Sequencing (NGS) approach. The research stages consisted of sampling, enrichment in MRSB and R2A media, DNA extraction, Polymerase Chain Reaction (PCR), Illumina NGS-based sequencing, and bioinformatics analysis using QIIME2 and MEGA XI software. The results showed that the Dage was dominated by bacteria from the genera *Lactobacillus, Weisella,* and *Acetobacter,* which are known as a group of probiotics. In addition, bacteria from the genus *Bifidobacterium and Enterococcus* were also detected. Diversity analysis showed that Dage enriched in R2A medium had higher species diversity than in MRSB medium. However, the presence of the probiotic bacteria group was more abundant in MRSB medium. The dominant amplicons by phylogenetic analysis were identified as *Lactobacillus spicheri, Lactobacillus zymae, Lactobacillus crustorum, Companilactobacillus farciminis, Weisella paramesenteroides*, and *Acetobacter indonesianensis,* which previously have several probiotic properties. These results provide novel information regarding the presence of probiotic bacteria in Dage, which can be explored further using a culturable approach.

Keywords: Dage; Probiotic; Lactobacillus; Next Generation Sequencing (NGS).

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INTRODUCTION

A group of non-pathogenic bacteria known as probiotics is crucial to preserving the host organism's health. In addition to bacteria from the genera Lactobacillus, Bacillus, or Bifidobacterium, the group of probiotic microorganisms also includes yeast microorganisms (fungi) from the species Saccharomyces (Dahiya & Nigam, 2022). By generating secondary metabolites like reuterin, biosurfactants, and bacteriocins, probiotic bacteria, such as those in the genus Lactobacillus, typically contribute to the inhibition of pathogen growth in the intestines. Furthermore, by improving T cell function, several probiotics from the Lactobacillus and Bifidobacterium genera can elicit an immunological response (Reid, 2016). Probiotic candidates produced from traditional foods are among the many sources of new probiotic candidates that researchers are still searching for (Parvez et al., 2006).

Dage tempeh is a fermented food product originating from Banyumas Regency, Central Java. Dage tempeh, which translates as food that tastes better when eaten immediately after frying, is an abbreviation of the Javanese terms “didag age-age”. Dage can be processed to make a variety of foods, including chips and fried tempeh (Handayani & Haryadi, 2001). The leftover material from the extraction of coconut oil is used to make Tempe Dage, which is then wrapped in banana leaves and inoculated with Rhizopus spp. starter (Romulo & Surya, 2021). Because of the black dots, Dage tempeh has a slightly distinct appearance from ordinary tempeh. However, there is still limited information available about studies on Dage, particularly with regard to the kinds of bacteria that live there.

At a far cheaper cost, next-generation sequencing (NGS) technology may provide sequencing data concurrently with precise results (Mardis, 2011). Furthermore, compared to earlier generation sequencing methods like Sanger and Maxam-Gilbert sequencing, the analysis is more efficient because gigabase-sized data can be produced in a matter of days or hours (Kchouk et al., 2017). Several sequencing techniques for identifying microbial diversity are available from Next Generation Sequencing (NGS) service providers, including Oxford Nanopore, Pacific Biosciences, ABI Life Technologies, Roche, and Illumina. Each sequencing approach has pros and cons specific to its platform (C. Y. Lee et al., 2013). The forward and reverse sequences generated by the Illumina MiSeq service provider, which will be employed in this investigation, have a length of roughly ±300 base pairs (Wen et al., 2017) (Bukin et al., 2019).

According to Next Generation Sequencing previous study, the most prevalent genera of probiotics in tempeh that have undergone prolonged fermentation are Lactobacillus, Lactococcus, and Klebsiella (Pangastuti et al., 2019). Furthermore, one of the most prevalent taxa during the tempeh production process was discovered to be Enterobacter (Yulandi et al., 2020). Additionally, prior studies have demonstrated that lactic acid bacteria (LAB) are frequently present during the tempeh production process (Efriwati et al., 2013). On the other hand, no information is currently available about the kinds of microbiomes that role as probiotics in Dage. Thus, the purpose of this research is to identify the different kinds of probiotics present in the Dage using an NGS approach.

Research on the detection of probiotics in Dage’s microbiome also provides information on the diversity of microorganisms present in Dage tempeh. Furthermore, this study will offer novel insights into the probiotic content of Dage tempeh, which has not been investigated before. Previously, *Lactobacillus fermentum*, which is isolated from tempeh, has successfully improved cognitive function in older adults (Handajani et al., 2022). Therefore, this research is also useful to serve as the foundation for pursuiting novel probiotic candidates that may subsequently be utilized in the food and health sectors.

METHODS

Dage samples were obtained from several traditional markets in Sumbang, Banyumas regency, Central Java. The selected sample was the fresh sample which already undergone through fermentation process for three days.

Figure 1. The appearance of Dage from the traditional market in Banyumas

Sample Enrichment, DNA Isolation, and Quantification

Approximately 10 g of the composed samples were inoculated into 90 ml of De Man Rogosa and Sharpe Broth (MRSB) medium and Reasoner’s 2 (R2A) broth. The samples were incubated for three days at 30 ºC with a speed of 120 rpm. DNA isolation of the Dage sample was performed using the ZymoBIOMICS DNA/RNA Mini Kit (Zymo Research, California, USA) according to the manufacturer's protocol. The samples were quantified using the Nanodrop Spectrophotometer using 260/280 nm wavelength. DNA samples are stored at –20 °C until further analysis.

(a) (b)

Figure 2. The enrichment of Dage in (a) MRSB medium and (b) R2A medium

Polymerase Chain Reaction

The primers to ampify V3 and V4 of 16S rRNA region were 341F (5'-CCTAYGGGRBGCASCAG-3') and 806R (5'-GGACTACNNGGGTATCTAAT-3') (Hussain et al., 2021). The Polymerase Chain Reaction buffer consists of 25 µL containing 2.5 µL of DNA template, 5 µL of forward primer, 5 µL of reverse primer, and 12.5 µL of MyTaq DNA polymerase. The Polymerase Chain Reaction cycle consists of an initial denaturation at 95°C for 3 minutes; 30 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds, followed by a final extension at 72°C for 5 minutes (Illumina, 2025).

Electrophoresis

Electrophoresis of the amplification product was conducted based on previous research (Eshananda et al., 2020). Agarose gel with a concentration of 1X is then soaked in stain solution for 20 minutes. The agarose gel was then visualized using Gel Documentation. The targeted PCR products were compared with a 1 kb DNA ladder.

Next Generation Sequencing

The library preparation and Sequencing using the Next Generation Sequencing (NGS) method with the Illumina NovaSeq 6000 sequencing system of size 2X300 bp was conducted by PT. Genetika Science Indonesia (GSI). The output of the sequencing data was in .fastq format.

Bioinformatics Analysis

The analysis of 16S rRNA gene amplicon sequences was performed using the software Quantitative Insights Into Microbial Ecology (QIIME)2 (Bolyen et al., 2019) on the Linux operating system (Ubuntu version 20.02). The QIIME software version to analyze the sequence was 22.02. The analysis results files in QIIME2 have a QZA format. Thus, at each stage, it needs to be converted to the qzv format for visualization on the qiime2view.com website. Analysis using QIIME2 is performed using a sequence of commands in the Linux command line system. The first step was quality control and denoising sequences. This step also removed the barcodes and primers from targeted sequences. Chimeric sequences will be removed using the Divisive Amplicon Denoising Algorithm (DADA)2 subprogram found within QIIME2(Susanti et al., 2025) . The formation of Operational Taxonomic Units (OTUs) was also carried out using the DADA2 program (Callahan et al., 2016) based on the Amplicon Sequence Variants (ASVs) method without comparing the OTU data found in the database. Then, taxonomic analysis was conducted based on the Silva 138 database (https://www.arb-silva.de/) using the Silva138 Classifier program. It resulted in the identity of amplicon sequences at the genus level. Diversity analysis was conducted based on the Shannon-Wiener, Simpson, ACE, and Chao indices. Visualization of the Alpha diversity analysis results was performed using R Studio software by the ampvis2 package (Andersen et al., 2018). Amplicon datasets is available in NCBI-SRA database with accession number PRJNA1291423.

Phylogenetic Construction

The selected amplicon sequence variants (ASVs) with high abundance from the NGS data were compared with National Center for Biotechnology Information (NCBI) data (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The phylogenetic tree constructed using MEGA XI (Tamura et al., 2021) software by the Neighbor-Joining method with 1000 replications with Kimura two parameters' criteria.

RESULTS AND DISCUSSION

DNA Isolation and Amplification

The two samples of Dage enriched in MRSB and R2A show adequate DNA purity and concentration (Table 1), according to (Lucena-Aguilar et al., 2016) the outcomes of DNA extraction using the ZymoBIOMICS DNA Miniprep Kit. Based on DNA with a purity of 1.8–2.0 at absorbance of 260/280 nm is considered

"pure DNA". The lower number indicated the presence of phenol or proteins, while the increasing number exceeded the range as observed in these samples, which might lead to RNA presence. However, even though spectral profiles and purity ratios are significant markers of sample quality, functionality in the intended downstream application is the most reliable predictor of DNA quality (Matlock, 2015) . In addition, the DNA concentration of both samples in this research is also sufficient for the PCR process, as it ranged from 5- 50 ng/µL (Thermofischer, 2025). Thus, the two samples were reliable to proceed to the 16S rRNA gene amplification stages.

Table 1. DNA extraction results

Sample	Volume (ul)	Concentration	A260/280
MRSB	50	26.3	2.06
R2A	50	28.6	2.03

The V3–V4 region of the 16S rRNA gene is the focus of amplification when employing the pooling approach. Gel electrophoresis on 1% Agarose was then used to visualize the amplification results (Figure 3). Agarose gel electrophoresis, which separates DNA products according to size and charge, is the most popular technique for evaluating the PCR products. It is also the simplest technique for viewing and evaluating the PCR results (Garibyan & Avashia, 2013). Thus, it makes it possible to determine the presence and length of PCR products. The 16S rRNA in this research gene was successfully amplified with a length of roughly 350 base pairs, according to the comparison with a 1 kb DNA marker. This is in line with earlier studies by Bukin et al., 2019, which stated that the 16S rRNA gene's V3-V4 region is between 250 and 400 base pairs long.

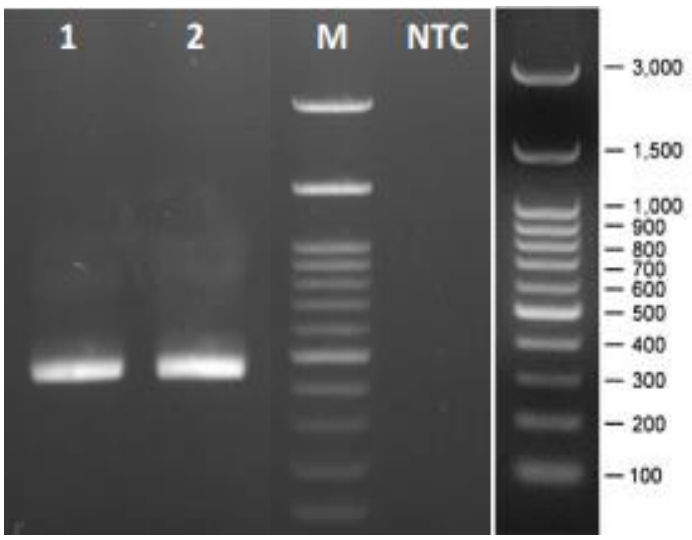
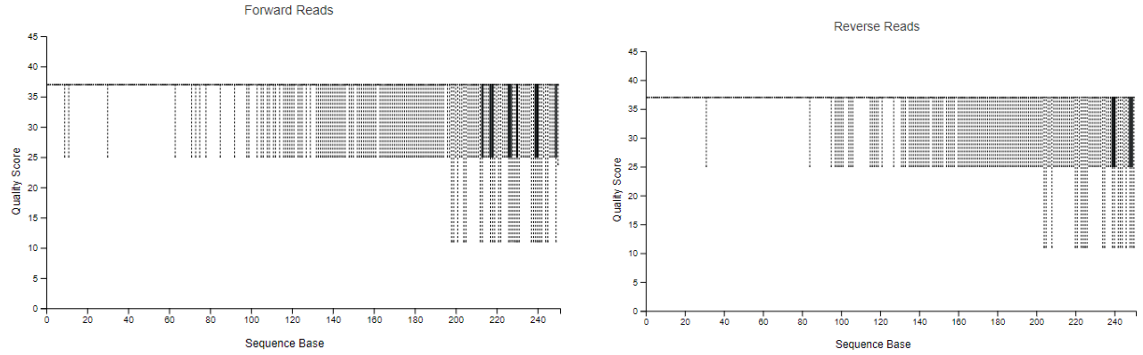


Figure 3. Electrophoresis results of the Dage samples (1=MRSB, 2=R2A, M=Marker, NTC=Negative Control). The marker used is the 100 kb marker.



(a) (b)

Figure 4. The Illumina sequencing results of forward (a) and reverse (b) reads by QIIME2 software analysis

Sequence quality

The quality of Next Generation Sequencing (NGS) by Illumina-Miseq sequencing platform in two Dage samples was explained in Figure 4 by using QIIME2 software created by Bolyen et al. (2019). Q-scores, or phred-like quality scores, are used to quantify the accuracy of nucleotide identity after the sequencing process. An individual base's likelihood of being named wrongly decreases with a greater quality score based on equity of $Q = -10\log10$ (e). According to Q20, there is a 1 in 100 chance of making a base call incorrectly; Q30, 1 in 1000; and Q40, 1 in 10,000. Bases detected during the run with a high-quality score are represented by a graph value greater than 30 (Q30) (Eastman & Yuan, 2014) . The forward and reverse sequences in Figure 4 have an average quality (Q) score ranging from 10 to 37. However, most bases in both reads have a Q score of more than 30 and are considered high-quality. Based on Mishra et al. (2022)Phred quality value is still the most widely used technique for base-call error estimation and has established itself as the industry standard since its launch in 1998.

The sequencing depth of the Dage sample was analyzed using a rarefaction curve. The curve shows the depth of sequencing results using Next Generation Sequencing (NGS) in estimating species diversity. The curve that shows a continuously rising line indicates that the species diversity has not yet been found. The curve that has plateaued indicates a sequencing depth that is representative of or can depict the diversity in the sample (Shamsuri & Ab Majid, 2023). The analysis results show that the sequencing depth in the Dage sample is sufficiently representative in estimating the bacterial species diversity in Dage.

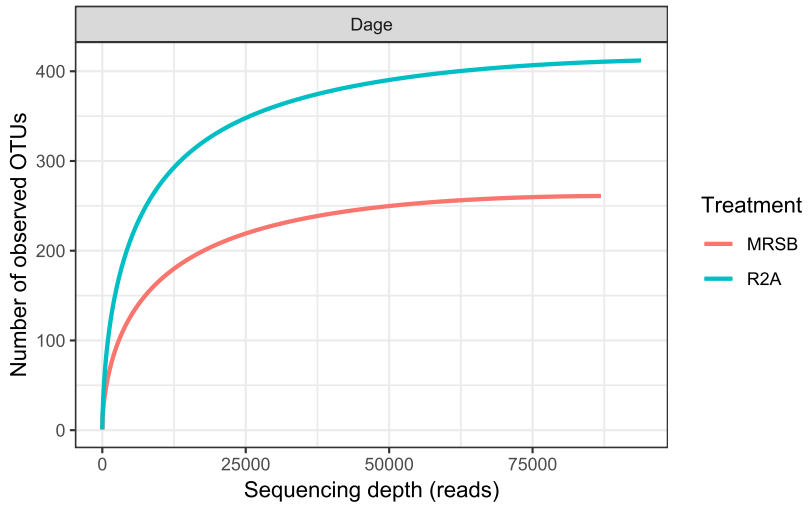


Figure 5. Rarefaction curve of samples by the Ampvis2 package

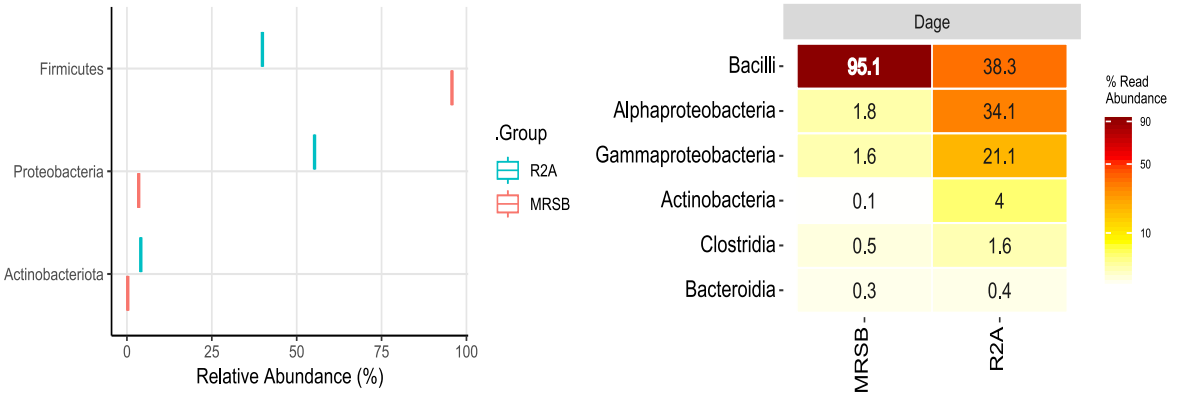


Figure 6. Taxonomic diversity at the Phylum (A) and Class level (B) in the Dage sample

Bacterial Diversity

Overall, there were only three phylum that were present in the sample. The results of the taxonomic analysis based on the SILVA 138 database on MRSB medium and R2A medium show different compositions. The Firmicutes phylum was most frequently detected in the MRSB medium (89%), while the Proteobacteria phylum was the most abundant in the R2A medium (55%). The phylum Actinobacteriota, or known as Actinobacteria, was detected in very small amounts in each sample (<5%). In addition, the Bacilli class is the most dominantly detected class in both types of media, which accounted for 95,1 % (MRSB) and 38,3% (R2A). Based on Figure 6. (b), members of that group might be more suitable for growth in MRSB medium compared to R2A. However, the R2A medium shows a higher number of bacteria from other taxonomic classes, such as Alphaproteobacteria, Gammaproteobacteria, and Actinobacteria. This is probably because of the difference in nutrients between MRSB and R2A media. Also, obtaining a particular bacterium in this kind of medium is challenging. In fact, all of the bacteria that were obtained from the sample, which were enriched in the culture broth, were mixed. On the other hand, liquid culture media make it easier for bacteria to obtain nutrients. As the culture medium is incubated under agitation, these nutrients become even more available, enabling bacterial nutritional regeneration (Bonnet et al., 2020).

A total of 15 and 40 ASVs were present across MRSB and R2A medium. The results of the analysis using the Venn diagram on the MRSB and R2A media showed quite different outcomes. The diagram shows the core OTUs or ASVs, which are OTUs with a quantity of at least 50% in the samples and has 1% of minimum relative abundance (Jory Brinkerhoff et al., 2020). As many as 18 OTUs, or 76.5% are core OTUs that are shared between the R2A and MRSB media.

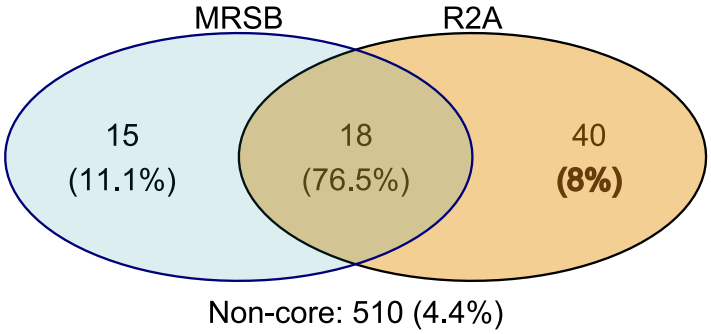


Figure 7. ASVs Venn diagram for MRSB and R2A by the Ampvis2 package

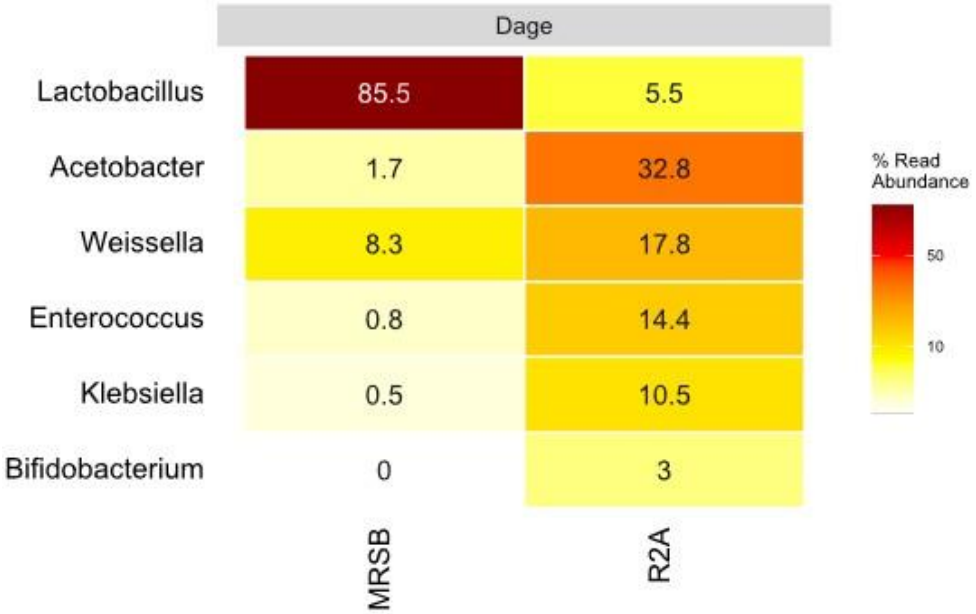


Figure 8. Taxonomic diversity up to the Genus level in the Dage sample

Probiotic Analysis

The results of the taxonomic analysis based on Figure 8 using the SILVA 138 database on the MRSB medium show that members of the genus Lactobacillus have the highest number of amplicons (85.5%) compared to the Acetobacter and Weisella groups. However, the taxonomic analysis on the R2A medium showed different results, with Acetobacter (32.8%) being the most dominant taxon. Members of the genus Lactobacillus on the R2A medium were only detected at around 5.5%. However, on the R2A medium, members of the genus Bifidobacterium were detected, which were not found on the MRSB medium. All the mentioned groups, except Bifidobacterium, have been detected in earlier research about other kinds of tempeh (Pangastuti et al., 2019; Yulandi et al., 2020).

The Lactobacillus genus is characterized by being rod-shaped, Gram-positive, non-spore-forming, and facultatively anaerobic. The genus degrades carbohydrates to produce lactic acid, which makes these bacteria the largest members of lactic acid bacteria (LAB). In the human digestive system, Lactobacillus plays a role in helping to break down certain types of substrates and protecting against pathogenic bacterial attacks. In addition, members of this genus are also widely used in the fermentation process of meat, vegetables, and bread (Dempsey & Corr, 2022). Based on the research findings, the Lactobacillus is suspected to be involved in the fermentation process of Dage, although there is no information regarding this matter yet.

Lactobacillus is also widely used as a probiotic, which is are beneficial living microorganisms that enhance the health aspects of the host. This group of microorganisms produces hydrogen peroxide, diacetyl, bacteriocins, and antifungal compounds during the lactic acid fermentation process. Bacteriocins play an important role in inhibiting pathogenic bacteria by disrupting their metabolic processes and inhibiting the toxins produced by pathogenic bacteria (Karami et al., 2017). Besides Lactobacillus, the genus Weisella is also known as a probiotic agent that can inhibit several pathogenic bacteria such as *Staphylococcus aureus, Listeria monocytogenes, Salmonella typhi,* and *Salmonella enterica* (Ahmed et al., 2022).

Acetobacter is a group of acetic acid bacteria that has been extensively developed industrially for commercial purposes. Some members of the genus, such as *Acetobacter aceti* and *Acetobacter syzygii,* can be categorized as probiotics (Haghshenas et al., 2015). Based on the research that has been conducted, the genus in the R2A medium has the highest number of ASVs or OTUs compared to other probiotic groups such as Enterococcus and Bifidobacterium. Previous research shows that members of the Enterococcus genus can be used in the treatment of urinary tract inflammation, bronchitis, or sinusitis (Krawczyk et al., 2021). Whereas Bifidobacterium is a probiotic that possesses antimicrobial, anticancer, anti-inflammatory, and antiviral capabilities, which can function as either a single strain or a multi-strain. The multistrain probiotic formulation of Bifidobacterium has better efficacy against infections compared to the single-strain formulation (Chen et al., 2021).

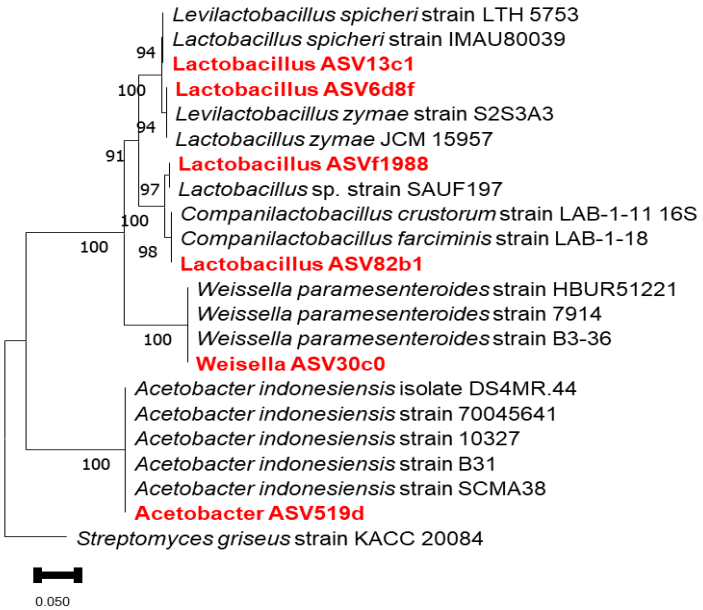


Figure 9. Phylogenetic analysis of the most abundant amplicon sequence variants (ASV)

To identify the probiotic species, we analyze the amplicon sequence from the representative genus as mentioned in Figure 9. Lactobacillus ASV13c1 and Lactobacillus ASV6d8f were closely related to *Lactobacillus spicheri* and *Lactobacillus zymae,* respectively. Previous research showed that both bacteria possess several characteristics of probiotic bacteria, such as bile salt tolerance and bacteriocin release (Gautam & Sharma, 2015; S. J. Lee et al., 2021). Lactobacillus ASV82b1 depicted the close relationship with two lactid acid bacteria comprises including *Companilactobacillus crustorum* and *Companilactobacillus farciminis*. Previously *C. crustosum* as probiotic can modulate microenvironment of intestinal organ through suppressing specific pathway and decreasing pathogenic bacteria (T. Wang et al., 2021). *C. farciminis* also has immunomodulatory actions that impact hormone synthesis (Kingkaew et al., 2023). Meanwhile, Lactobacillus ASVf1988 does not show a close relationship to any species. Moreover, Weisella ASV30c0 was identified as *Weisella paramesenteriodes,* which was previously known as a probiotic with therapeutic properties (Yadav et al., 2022). Lastly, Acetobacter ASV529d formed a monophyletic group with the indigenous species of *Acetobacter indonesiensis,* which obtained from Indonesian fruits and flowers (Lisdiyanti et al., 2000) and has anticancer properties (Haghshenas et al., 2015). All amplicons form a monophyletic group with high bootstrap values (more than 90). Thus, these findings showed that Dage, as a traditional food from Banyumas, has various kinds of probiotics with a broad range of abilities and might be able to enhance the human health level.

Bacterial diversity is shown with several indices consisting of Shannon, Simpson, Chao1, and FaithsPD. Based on the Shannon diversity index, the bacteria in Dage grown on R2A medium have a higher abundance (2.80) compared to the bacterial diversity on MRSB medium (2.55). However, the bacterial community grown on the MRSB medium had a higher evenness number compared to the R2A medium. Diversity index value improves both when the number of species increases and when evenness increases. Shannon index is a popular diversity metric used in microecological research. Community variety increases with a higher Shannon index value. Another metric that may be used to gauge microbial diversity is the Simpson index. The variety of the community decreases as the Simpson index value increases. There are fewer bacterial species and a more straightforward classification of fecal microbial communities in the low-Shannon index group (Yin et al., 2019). Based on the Faith's PD and Chao1 indices, the abundance of bacterial species on the R2A medium was also higher compared to the MRSB medium. Faith's PD value (Faith's phylogenetic diversity) is a good measure of phylogenetic diversity (J. Wang et al., 2022). The Chao estimator is utilized to determine the abundance of OTUs in a sample. There are more OTUs or ASVs in a sample with a higher Chao index, indicating a comparatively higher species diversity (He et al., 2013). Based on these indices, R2A medium has higher species richness, even though MRSB medium showed higher species evenness.

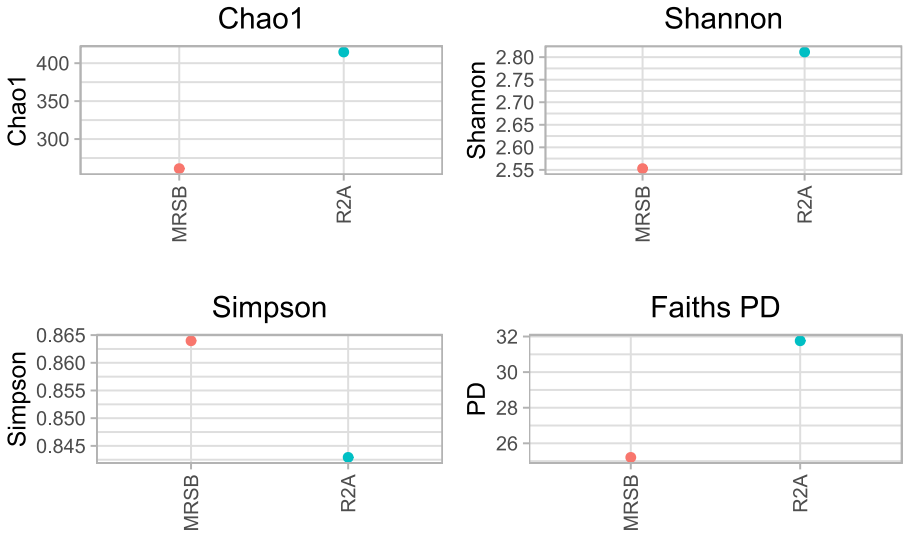


Figure 10. The diversity index of bacteria in Dage samples

Based on our findings, the different enrichment media result in differences in probiotic composition in Dage. However, the probiotic taxa of both R2A and MRSB media still showed the same genera. The main reason is probably the discrete nutrient compositions between these media. The R2A culture medium is classified as a low-nutrition culture medium, characterized by a comparatively broad spectrum of nutritional constituents (Cao et al., 2024) while MRS medium is frequently employed for the isolation and cultivation of Lactic Acid Bacteria (LAB). Nevertheless, due to the inadequacy of this medium in fulfilling the diverse nutritional needs of all LAB strains, it has undergone modifications to distinct objectives in various research studies (Ko et al., 2024). Therefore, it confirmed our results that Acetobacter is the most dominant genus in R2A medium categorized as Acetic acid bacteria (AAC) (Mitina, 2025). On the other hand, MRSB medium is dominated by the Lactobacillus genus, which mostly has LAB traits.

The finding of this research is the first report regarding the presence and diversity of the probiotic community in Dage, a traditional food originally from Banyumas regency, Indonesia. Such bacteria might be beneficial for people's health simply when they consume them. Also, the microbial community present in Dage could be explored further by bioprospection assay through its pure culture to obtain possibly novel secondary metabolites.

CONCLUSION

Next Generation Sequencing approach revealed the abundance of probiotic communities and several probiotic candidates in Dage that belong to the genera Lactobacillus, Weisella, and Acetobacter. In addition, it also detected bacteria from the genera Bifidobacterium and Enterococcus. This finding could be further developed in the related field, such as health and industry. The isolation using a specific medium to obtain culturable probiotic bacteria is needed for further bioassay exploration. Amplicon datasets in this study is available in NCBI-SRA database with accession number: PRJNA1291423

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AUTHOR CONTRIBUTION STATEMENT

Y.E. designed the study, performed the experiments, data analysis, and writing the manuscript. S.H.F.: sampling and data collection. N.F.(1): diversity analysis and project administration. N.F.(2): taxonomy analysis. H.P.K.: supervision and proofreading.

CONFLICT OF INTEREST

The authors declare no conflict of interest related to the research and content of the article.

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