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Metagenomic Analysis for Indigenous Microbial Diversity in Soaking Process of Making Tempeh Jack Beans (*Canavalia Ensiformis*)

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Abstract

Jack Beans are a type of high-protein legume that can produce high nutritional value. One of the processed superfoods from *Jack* beans is tempeh. Soaking is essential in making tempeh as a pre-fermentation process utilizing microbial enzymes to increase product nutrition. The metagenomic analysis is a novel technique to know microbial communities based on culture-independent microorganisms. This study aims to determine the diversity of microbes in the soaking process at 12 hours and 24 hours. This analysis found ten OTUs genera, namely *Prevotella*, *Bacillus*, *Paenibacillus*, *Staphylococcus*, *Lactobacillus*, *Pediococcus*, *Saccharo fermentants*, *Klebsiella*, *Pantoea*, and *Acinetobacter*. Phylum Firmicutes is dominant in the soaking of *Jack* beans with a difference of 53.24% 12 h soaking time and 47.89% 24 h soaking time. This finding contributes to controlling the quality production of making tempeh.

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Microbial Diversity;
Microbial Enzymatic;
Soaking Process.

Introduction

Jack beans are one of the types of beans in Indonesia, especially in Java and Sumatra. *Jack* beans have nutritional contents, especially protein, which is relatively high at 24%.[1] However, the use of products from *Jack* beans is very minimal, with only 25% of products.[2] Dependence on the processing of legumes generally uses soybeans, so that the potential for *Jack* beans, especially protein, is not widely utilized. Products developed using *Jack* beans include raw materials for tempeh, milk, flour substitute for flour, cooking oil, and shredded.[3,4]

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Fermentation of *Jack* beans into tempeh produces high nutrition. The production of Tempeh starts from soaking, cooking, inoculation, storage, and packaging.[5] Each processing process has a different effect on legumes' nutritional and antinutritional components. *Jack* beans contain toxic substances, including choline, hydrozianine acid, trogonelin, glucocyanide, and phytic acid which are antinutritional compounds but can be neutralized and detoxified by process making tempeh.[6–8] Traditional processing or a combination of these reduces antinutritional factors and increases the bioavailability of nutrients. Tempeh made of *Jack* beans can affect the nutrient composition that is important for health, such as increasing protein content, producing several bioactive compounds like phenolic compounds, and developing vitamins, namely vitamin B_1 and vitamin B_2.[1,9] How an efficient process can affect the nutrient composition and the extent to which it will reduce antinutritional factors are still considered essential issues to be explored.[10,11]

Soaking the beans is an essential step in the process of making tempeh, among others, facilitating the release of seeds, the absorption of water for subsequent fungal fermentation, and acid fermentation. It inhibits the growth of spoilage fungi.[12] The soaking process is generally carried out using distilled water and using chlorine-free water overnight to lower the pH so that it is suitable for use mold growth and accelerated natural lactic fermentation.[11,13] The soaking process has been effective in removing anti nutritional factors, but some metabolic reactions affect the content of some compounds. The soaking process reduces some of the anti nutritional factors and increases *in vitro* protein digestibility, but this is dependent on legume cultivar and soaking process conditions. The soaking process depends on the type of solution, time soaking, and temperature.[6,14] *Canavalia ensiformis* with a ratio of water material is 1:3 in a soaking time of 72 hours to produce 36.06% crude protein.[15,16] The 24 h soaking time in *Canavalia ensiformis* with a 1:10 ratio of materials and water resulted in 31% crude protein.[17] Therefore, this is a strong assumption of the effect of the activity of microbes that can determine the nutritional value of the soaking process of *Jack* beans. Soaking the *Jack* beans is one of the pre-fermentation stages by utilizing the microorganisms that grow in them. These microorganisms have an important role in producing enzymatic hydrolytic (protease, lipase, and amylase). In the soaking process, it is found that there was the enzymatic activity of microbes produced, including *Bacillus licheniformis*, *Bacillus pumilus*, Enterococcus faecium, Pseudomonas luteola, *Aerococcus viridans*, *Bacillus mycoides*, and *Staphylococcus cohnii* are reported to have both protease and lipase activities.[18]

Microbes have produced an enzymatic process and their ability to produce enzymes can be elucidated further using metagenomic analysis. The metagenomic analysis is a technique using a next-generation sequencing based on culture-independent microorganisms.[19] Metagenomic is a precise way to determine the microbial diversity that is uncultured in an environment. Metagenomic based on DNA analysis from a community or ecosystem using the 16s rRNA gene.[20] 16S rRNA marker is used for amplification DNA and identification of various diversity microbial. Therefore, to determine the microbial diversity in the soaking process of *Jack* beans, metagenomic analysis with next-generation sequencing is required to elucidate the effects of nutritional factors of *Jack* beans tempeh and the method novelty applied on soaking process *Jack* beans.

Materials and Methods

Sampling and Preparation

Soaked 500 grams of *Jack* beans from Central Java Indonesia, in sterile water with a ratio of 1:10. The soak is placed in the soaking box for 12 hours and 24 hours with 25°C and relative humidity (RH) of 60%. Ten ml of soaked water at different soaking times (12 h and 24 h) were used for the next analysis.

Metagenomic Analysis

Extraction of Bacterial Genome DNA and 16S rRNA gene amplification

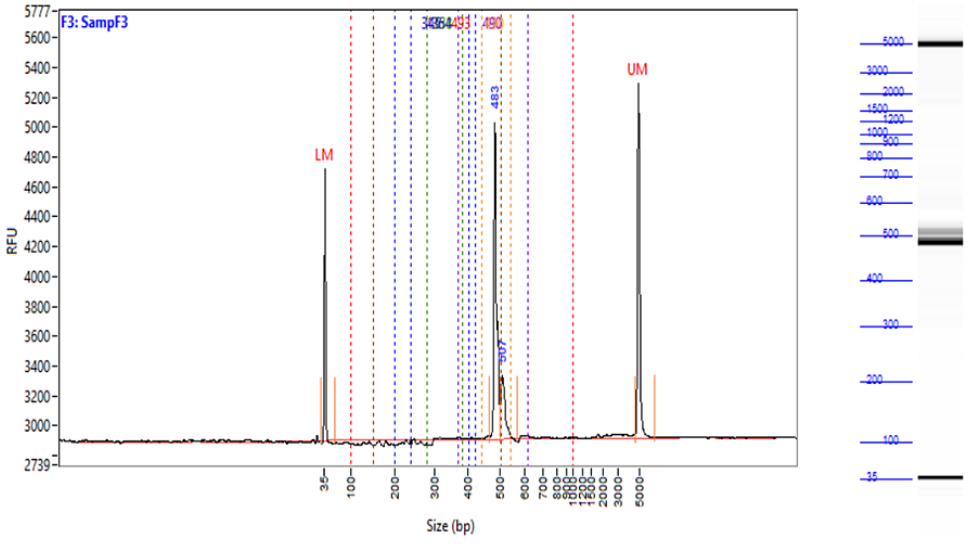
Extraction of bacterial genomic DNA was carried out in water baths for 12 h and 24 h. The soaking water is filtered first using a vacuum on a 0.45 m membrane filter (Thermo Fisher Scientific, United States). The filter was extracted using the DNA Isolation Kit with the CTAB/SDS method.[21,22] The extracted DNA samples were collected, centrifuged, and diluted to 1ng/µL using sterile water.

Identification of bacterial diversity 16S rRNA genes from different regions (16SV4/16SV3/16SV3-V4/16SV4-V5) were amplified using specific primers with barcodes.[20,22] The PCR reactions was by Phusion® High-Fidelity PCR Master Mix (New England Biolabs). The 30 cycles of PCR (Thermo Fischer Scientific, United States) were carried out under the following conditions: pre-denaturation 94ºC for 5 minutes, denaturation 92ºC for 30 seconds, annealing 62ºC for 30 seconds, elongation 72ºC for 30 seconds, and post-elongation 72ºC for 7 minutes.[23] The loading buffer was mixed once with the same volume as the PCR product and electrophoresis was operated on 2% agarose gel to detect results. DNA samples were selected with main strips between 400 bp-450 bp for further experiments. DNA bands from PCR products were cut by QIAquick Gel Extraction Kit and purified by Qiagen Gel Extraction Kit (Qiagen, Germany).

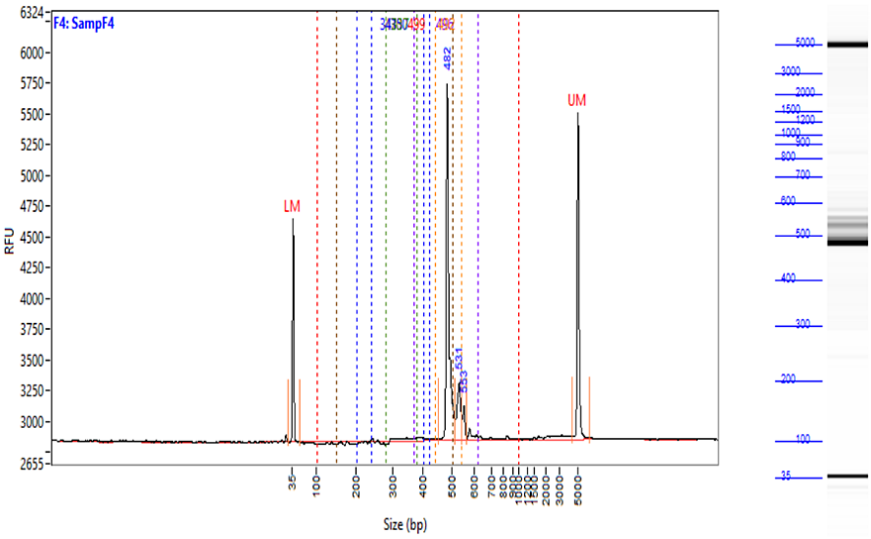
Data and Information analysis (Operational Taxonomic Unit Cluster and Alpha Diversity)

Analysis of PCR products for data and information sequences was with FLASH. FLASH is the method to combine overlapping read-paired of DNA fragments, similar DNA fragments, and a sequence of splicing. Raw tag DNA fragments from FLASH were purified and filtered to obtain high-quality clean tags by Qiime.[24,25] Clean tags were compared with reference databases using the UCHIME algorithm and detected by chimera sequences. The chimera sequence can obtain an effective tag database by deleting the sequence.[26] All effective tags classified with 97% similarity are considered OTU (Operational Taxonomy Units) and all sequence clones were submitted to BLASTN-NCBI database (http://www.ncbi.nlm.nih.gov/BLAST/).[27,28] The OTUs cluster was analyzed using UPARSE software.[26] The annotation of bacteria by the taxonomic tree, such as kingdom, phylum, class, order, family, genera, and species. Taxonomic trees were analyzed using Mothur software with a database from the SILVA.[29,30]

The phylogenetic tree representative OTU sequences were obtained by MUSCLE software. OTU abundance information was normalized using the sequence number standard corresponding to the sample with the least sequence.[31] Alpha diversity was applied in analyzing the complexity of biodiversity for the sample. The alpha diversity can indicated Observed-species, Chao1, Shannon Wiener Index, Simpson index, ACE, Good-coverage.[11,32] All of these indices in our sample were calculated by QIIME and displayed with R software.[24,33] All measured parameters of this study used Statistical Package for the Social Sciences Statistical software (IBM-SPSS) version 23 (NC, USA).[34,35]



(a)



(b)

Fig. 1: 16S rRNA gene amplification and PCR Product (a) 12 h of soaking time (b) 24 h of soaking time

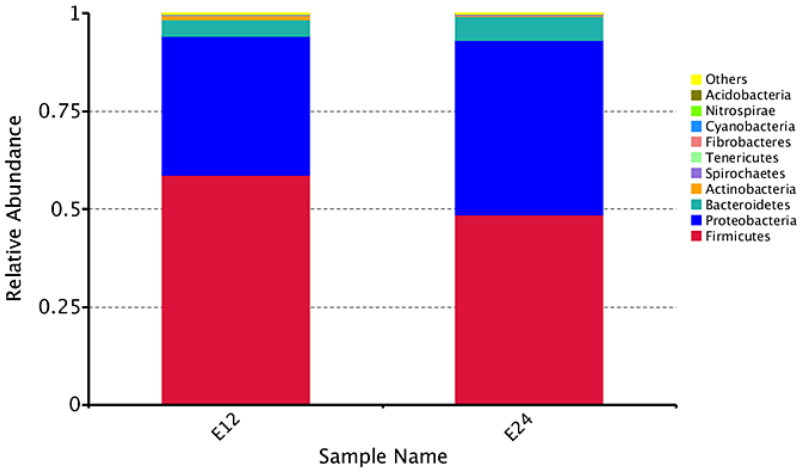
Results

Amplification of Gen 16S rRNA and PCR Product

The product DNA produced in each soaking was amplified using the 16S rRNA gene. The 16S rRNA amplicon is a technique microbiome analysis where different samples are analyzed simultaneously using multiplexing. The results can be used to perform microbial annotation diversity by genera, family, order, class, and phylum levels. The amplified region used is 16SV34/16S rRNA amplicon sequencing has been widely applied to the microbial community from various natural or endozoic environments such as soil, water, and host intestine.[19] The 16S rRNA gene sequence was amplified again for attachment of unique regions (multiplex or index) as a differentiator between samples, and primers for sequencing.[20]. The amplicons entered the cluster generation stage by Mi Seq, which aimed to attach the amplicons to the flow cell and multiply the amplicons using the bridge amplification principle to form clusters. After that, the amplicons would undergo a sequencing process based on sequencing-by-synthesis (SBS).[36] The high-sequence conservation of 16S genes can separate bacteria diversity in the phylogenetic tree and identify of new taxa. Most bacterial phyla are known only from 16S surveys and have no cultured representatives.[20] The electrogram amplification of Gen 16S rRNA and PCR product can be seen in Figure 1.

Operational Taxonomy Unit (OTUs) cluster

The number of amplicon sequences is quite large and varies because the soaking process is a treatment allowing bacteria to grow from the environment and triggers fermentation in the soaking process. The known DNA amplicons were then continued with the analysis of bacterial diversity with OTUs. A taxonomy tree was obtained could be compared with the variety of bacteria in soaking the *Jack* beans. OTUs analysis was done by the USEARCH algorithm and taxonomically through nucleotide blast (BLASTn) were identified.[37] OTUs were created as taxonomic annotations for representative sequences of each OTUs to obtain appropriate taxa information and taxa-based abundance distributions.[32,38] OTUs can analyze species annotation, species distribution, ternary plot, and phylogenetic tree. Identification and annotation of species on microbial commodities are made in clusters with 97% identity.[32] Relative abundance and Heatmap OTUs of the soaking process can be seen in Figure 2.



(a)

(b)

Fig. 2: (a) Relative abundanceand (b) Heatmap OTUs of soaking tempeh Jack Beans in different soaking times of 12 h and 24 h

Alpha Diversity

Two samples were analyzed, namely soaking the *Jack* beans for 12 h and 24 h. The total sequences produced by soaking the *Jack* beans for 12 h produced 178762 more sequences than the soaking of the *Jack* beans for 24 h. A total of 3.64% of the sequences were removed after filtering the length of the sequence, the presence of ambiguous sequences, and unassigned sequences to obtain clean sequences with 163193 sequences at 12 h of soaking time and 126066 sequences at 24 h of soaking time, respectively. The soaking of *Jack* beans produced many sequences because the soaking process came from an environment influenced by temperature and humidity, allowing a large variety of microbes to grow.[11] Total reads and clean reads sequence can be shown in Table 1.

Table 1: Alpha diversity index in different of soaking time Jack beans

Sample	Total reads	Clean reads	Total OTU	Shannon Wiener Index (H')	Simpson Index (D)	Goods Coverage (%)
12 h of soaking time	178762	139141	423	4.231	0.892	1.000
24 h of soaking time	163193	126066	410	4.262	0.886	1.000

Taxonomic Annotation

The taxonomic annotation on the *Jack* beans soaking can be seen in the phylogenetic tree in Figure 3, indicating a difference in the percentages produced in the bacterial population of 12 h soaking time compared to 24 h. The taxonomic annotation will be represented by using a phylogenetic tree. Phylogenetic analysis is useful in analyzing and illustrating gene/protein/species evolution. Besides that, phylogenetic analysis is known for an increasing number of species on DNA/genome sequenced. The phylogenetic tree will annotate the kingdom, phylum, class, order, family, genera, and species from product sample DNA extraction.[32] This study suggested that the bacterial community on the soaking process was diversified, in which 423 OTUs in 12 h of soaking time and 410 OTUs in 24 h of soaking time. The major phylum of the soaking bacteria was Firmicutes and Proteobacteria, but minor phylum Bacteroidetes. A phylogenetic tree can represent the taxonomy tree between soaking time. The results show by differentiating the number of microbial populations at 12 h of soaking time in red color and microbial populations at 24 h soaking time in blue color.

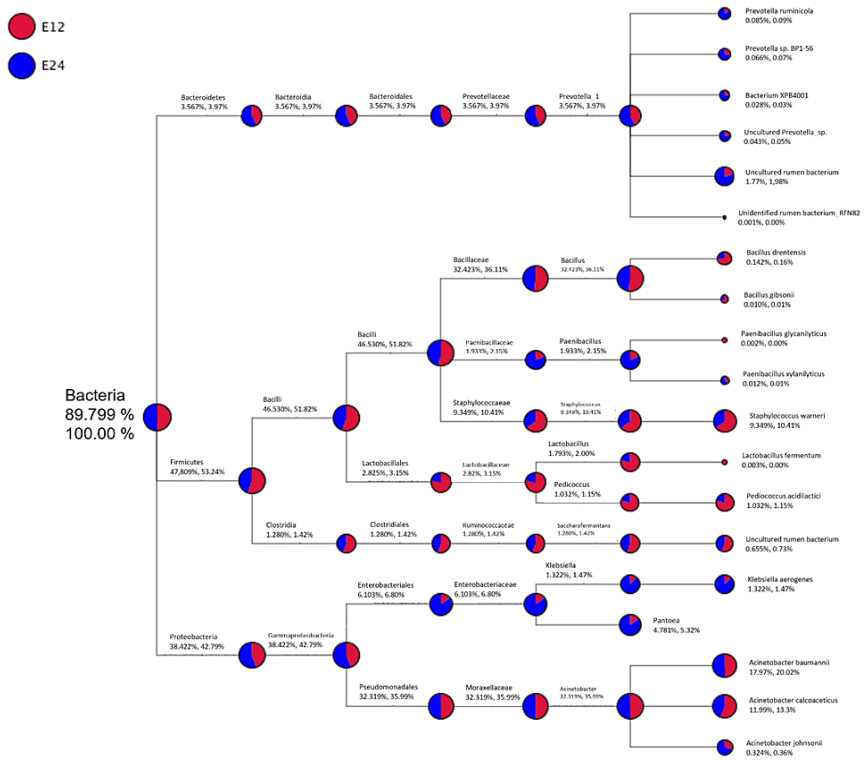


Fig. 3: The phylogenetic tree in different 12 h and 24 h of soaking times process of Jack beans

Discussion

Amplification of Gen 16S rRNA and PCR Product

In the soaking process, microorganisms can grow because the appropriate temperature and humidity support them for these microorganisms.[39] The foam on the surface indicated the presence of microbial growth. The soaking water was extracted using the Microbial DNA Isolation Kit to obtain DNA and amplified using the 16S rRNA gene amplification.[11] Most current work with 16S rRNA produce single-end reads only up to 350 bp and paired-end reads up to 2 × 300–350 bp. The experimental diversity microbial used NGS (Next-Generation Sequencing) to estimate the taxonomic diversity of the products 19,38. The primers amplified the V1 to V9 regions of the 16S rRNA gene, resulting in an amplicon length of ~1500 bp. Species-level identification uses the first 500 bp or ~1500 bp 16S rRNA gene sequences so that they can be identified for species level in metagenomics.[40,41]

The electrogram in Figure 1a shows that the 12 h of soaking time contained several colonies that were successfully amplified using the 16S rRNA sequence. Variations in amplicon length of 35 bp, 500 bp, and 5000 bp (base pair) indicated that the DNA fragment band was detected, indicating amplified bacterial fragments presence.[42] Several amplicons were successfully amplified at 24 h soaking time using 16S rRNA sequences, with variations of 35 bp, 480-500bp, and 5000 bp of the amplicon, as shown in Figure 1b. At 24 h of soaking time produced different amplicons compared to 12 h of soaking time, at 480 bp-500 bp DNA amplicon produced varying thicknesses compared to 12 h of soaking time. DNA amplification in the soaking process indicated the diversity of bacteria. The amplicon of DNA at 24 h of soaking time was greater than the 12 h soaking, which would be further analyzed to determine the diversity of bacteria and taxa obtained.[36]

Operational Taxonomy Unit (OTUs) cluster

The soaking process of *Jack* beans affects the abundance of bacteria diversity. The abundance of relative microbes can be seen in Figure 2a. The Heatmap is an interactive web with a presentation of the taxonomic annotation corresponding to OTUs in Figure 2b. This figure indicates that color coding was used to represent the percentage of each OTU to the total OTU count. The blue color contributed a low percentage of OTUs to the sample, and the red color contributed a high percentage of OTUs. In the taxa histograms, the soaking of *Jack* beans at 12 hours and 24 hours showed that the dominant bacteria were taxa Firmicutes, Proteobacteria and Bacteroidetes were the most important groups and accounted for 0.95, meanwhile Actinobacteria, Spirochaetes, Tenericutes, Fibrobacteres, Cyanobacteria, Nitrospirae, and Acidobacteria were also found with minor abundance. Relative species abundance refers to the biodiversity on how common one taxa is relative to other species in a particular community, especially in the process of soaking beans.[43]

At 12 h soaking time of *Jack* beans, phylum Firmicutes, Proteobacteria Bacteroidetes value relative abundances were 0.58, 0.35, and 0.04, respectively. In 24 h of *Jack* bean soaking, phylum Firmicutes, Proteobacteria, and Bacteroidetes have relative abundances were 0.48, 0.44, and 0.06, respectively. The relative abundance of species and species richness in a community describes the biodiversity associated with the population of microorganisms.[43] The abundance of bacteria found 89,31% of the total microorganisms in the soaking process. The abundance of the bacterial kingdom is known by analyzing the taxonomy level, such as phylum, class, order, family, genera, and species.[19] Three dominant phylum's that dominate in both processes of soaking the *Jack* beans are Firmicutes (52.36%), Proteobacteria (33.925%), and Bacteroidetes (3.03%).

Alpha Diversity

Alpha diversity is an index of bacterial diversity that can calculate the value of Shannon-Wiener, Simpson, and good coverage index.[32] Alpha diversity can determine the diversity, richness, and probability of bacteria. The alpha diversity of the soaking time index can be seen in Table 1. The soaking of *Jack* beans for 12 h resulted in more OTUs values than soaking at 24 h, indicating that the microbes are influenced by ideal temperature (28°C) and humidity (60%) during the 12 h soaking time. Meanwhile, at 24 h of soaking time, several dominant microbes grown can potentially have enzymatic bacteriocins, which allows these bacteria to have antimicrobial compounds. For example, *Klebsiella* and Lactic Acid Bacteria could act as bacteriocins.[44]

The Shannon-Wiener index (H') is a suitable index to calculate the level of species diversity.[36] If H' value is less than 1.0, the sample has low diversity and very low productivity, which indicates heavy pressure and an unstable ecosystem. If H's value ranges from 1 < H' > 3.5, the sample has moderate species diversity, sufficient productivity, and balanced ecosystem conditions.[45] The analysis results of the two samples, namely soaking in beans for 12 h and 24 h, had a Shannon-wiener (H') value with high species diversity, and the condition of the ecosystem species in the soaking *Jack* beans was quite balanced. Simpson's index (D) is an index that calculates dominance in bacteria. If Simpson's value is close to 0, then the value of species diversity is low 45. In the analysis results of soaking *Jack* beans for 12 h and 24 h, the Simpson value is more than 0, namely 0.892 and 0.882, respectively. The result showed a high species diversity, coinciding with the high value of OTUs generated in the process.

Good coverage is an estimator of the completeness of the sampling process, which is characterized by the probability that an amplicon is drawn from a random sample that has been successfully sequenced.[32] Good coverage above 95% stated that the number of samples taken per sampling was sufficient to evaluate the diversity of bacteria in soaking legumes. Table 1 shows that at 12 h and 24 h beans soaking samples were successfully amplified and comprised of a range of bacterial diversity. The composition of the bacterial community in the soaking of legumes is influenced by several things, including the raw materials used, water sources, environmental conditions, and the tools used.[12,45] The bacterial diversity results in the sample using NGS (Next Generation Sequencing) succeeded in identifying bacterial diversity and identified taxonomy, namely phylum, class, order, family, the genera to species.[19]

Taxonomic Annotation

Soaking legumes showed that the major microbial populations comprised Lactic acid bacteria, Enterobacteriaceae, and yeast. The dominant bacteria during the soaking process are caused by ambient temperature and uncontrolled conditions soaking water that make it enriched in Lactic acid bacteria.[12,45] The accession number of soaking water 12 h was SAMN30222989 (https://www.ncbi.nlm.nih.gov/biosample/30222989) and soaking water 24 h was SAMN30222990 (https://www.ncbi.nlm.nih.gov/biosample/30222990).

The 100% of Kingdom Bacteria have three phyla found in soaking process 12 h and 24h. There are Firmicutes, Proteobacteria, and Bacteroidetes. The dominant phylum were Firmicutes with a relative abundance value of 53.24% (12 h of soaking time) and 47.809% (24 h of soaking time). Phylum Proteobacteria was the second population in the soaking process with 42.79% on 12 of soaking times; 38.42% on 24 h of soaking time, and the lowest relative abundance was phylum Bacteroidetes with the relative abundance of 3.97% on 12 h of soaking time; 3.567% on 24 h of soaking time.

The top 10 genera found in the soaking process of *Jack* beans for 12 h and 24 h of time soaking were Prevotella, *Bacillus*, *Paenibacillus*, *Staphylococcus*, *Lactobacillus*, *Pediococcus*, *Saccharofermentants*, *Klebsiella*, *Pantoea*, and *Acinetobacter*. The bacterial *Lactobacillus* casei, Streptococcus daecium, *Staphylococcus* epidermis, and Streptococcus dysgalactiae dominated the soaking fermentation process of tempeh12. Some other species such as *Klebsiella* pneumoniae, *Klebsiella ozaenae*, *Enterobacter cloacae*, *Enterobacter agglomerans*, Citrobacter diversus and *Bacillus* brevis, and *Bacillus pumilus* found in soaking process of tempeh[46]. Phylum Firmicutes were dominant in the soaking process of *Jack* beans. It consists of 2 major classes which were ±50% for Bacilli and ±1.3% for Clostridia. The class of Bacilli found three families were *Bacillaceae*, *Paeni bacillaceae*, and *Staphylococcaceae*. Two OTUs of the genera *Bacillus* were detected in this soaking process for 12 h and 24 h; *Bacillus* drentensis and *Bacillus* gibsonii. The soaking process of peanut seeds found *Bacillus* spp, which significantly affected crop yields.[47] *Bacillus* spp. is a soil bacterium that is often found in the rhizosphere of plants. Some species produce extracellular enzymes that can hydrolyze complex proteins and polysaccharides. As the most significant bacteria, *Bacillus* produces an enzyme in large quantities.[48] *Bacillus* drentensis is one bacterium can produce an enzyme, such as amylase[49]. These bacteria are known for their ability to secrete large amounts of alkaline proteases with significant proteolytic activity and it is stable at considerably

high pH, temperatures, and saline environments.[50] *Bacillus* is a Gram-positive bacterium, aerobic-facultative endospore-forming motile bacteria that belongs to the family *Bacillaceae*. *Bacillus* gibsonii is one bacterium from the soil that significantly enhanced beans and wheat growth. *Bacillus* gibsonii is a novel protease producer, which is more closely related to the high-alkaline proteases belonging to the Subtilisin family.[51]

In the soaking of *Jack* beans, *Paenibacillus* and *Lactobacillus* species were the most dominant group in the soaking process. Two major species of *Paenibacillus* glycanilyticus and Paena*bacillus* xylanilyticus were identified. *Paenibacillus* is one of the endophytic bacterium found in peanut seeds with several special properties, such as the formation of endospores, cell motility, tolerance to high osmotic pressure, and amylase activity.[52] *Paenibacillus* glycanilyticus is a novel species to degrade heteropolysaccharides and produce catalase.[53] *Paeni*bacilli can metabolize aliphatic and aromatic organic pollutants by applying oxygenase's, dehydrogenases, and ligninolytic enzymes.[54] Based on Figure 3, *Staphylococcus* sp is one of the genera identified in soaking *Jack* beans for 12 h and 24 h where *Staphylococcus* warneri was identified. In the soaking process of various types of beans, the growth of *Staphylococcus* was found when the pH was increased, but its growth was inhibited due to the presence of *Lactobacillus* plantarum bacteria.[55] S. warneri is a new source of lipase, extracellular protease, and the proteolytic cascade. In addition, S. warneri is a bacterium that can be an antimicrobial agent, namely bacteriocin.[56]

On *Lactobacillus* genera, *Lactobacillus* fermentum species dominated at 12 h of soaking time with a percentage of 0.003%; however, the bacterium was not found at 24 h of soaking time. *Pediococcus* genera was dominated at 12 h of soaking time with a percentage of 1.15%, while in 24 h of soaking time with a percentage of 1.032%. In the species, *Pediococcus acidilactici* was found to be dominant at 12 h of soaking time. On family *Lactobacillaceae*, two genera were found in the soaking process. There are *Lactobacillus* and *Pediococcus*, with each species were *Lactobacillus* fermentum and *Pediococcus acidilactici*. L. fermentum is one of the Lactic acid bacteria capable of exhibiting amylolytic activity, generally isolated from soil.[57] L. fermentum is a starter culture that could optimize fermented African kale's nutrient components. L. fermentum can hydrolyze proteins, so it can form unique fermented flavours and an important proteolytic enzyme, with protease activity of 20 U/ml.[58] P*. acidilactici* is a probiotic bacterium with high antioxidant, cryoprotective activity against alloxan, and produced extracellular proteolytic.[59]

The Class *Clostridia* found in process soaking in 12 h and 24 h with lower relative abundance was 1.42% dan 1.280%, respectively. One OTUs genera found in the soaking process was *Saccharofermentans*. *Saccharofermentans* is Gram-positive bacterium, non-motile, and aerobic growth. *Saccharofermentans* is predominant cellular fatty acids, and several hexose, polysaccharides, and alcohols are fermented with fermentation products from glucose.[60] The Bacteroidetes had a minor abundance phylum in the soaking process, approximately 3% of total taxa. In this study, the only genera found was Prevotella with six OTUs species. The Six OTUs species include Protovella ruminicola, Protovella sp, bacterium Protovella XPB, uncultured Protovella sp, uncultured bacterium Protovella, and unidentified bacterium Protovella. Protovella are the important bacteria that play important role in the metabolism of protein with proteolytic and characteristic of dipeptidyl peptidase and produce degradative enzymes, such as amylase and xylanase.[61–63]

Phylum Proteobacteria is the second dominant population of bacteria in the soaking process. One class bacterium found in the process was Gammaproteobacteria with two Orders such as *Enterobacteriales* and *Pseudomonadales*. At 12 h soaking time, the relative abundance value of phylum proteobacteria was lower than 24 h of soaking time. Five OTUs genera found in the process soaking includes *Klebsiella aerogenes*, *Pantoea*, *Acinetobacter baumannii*, *Acinetobacter calcoaceticus*, and *Acinetobacter* johnsonii. Proteobacteria are classified as pathogenic bacteria, but these bacteria can produce health benefits in the tempeh process. *Klebsiella* is one type of bacteria that can synthesize vitamin B_{12}, a resource for vegetarian's. *Klebsiella* has a role bacterium that can produce acidification (decrease until pH 4) during the soaking process.[64] Meanwhile, *Klebsiella* has the potential to produce bacteriocins and hydrolytic

enzymes.[44] *Acinetobacter* is a group bacterium commonly found in the environment, mostly water. *Acinetobacter baumannii* is a Gram-negative and *amylolytic bacterium*, and a potential proteolytic activity.[65]

Conclusion

Soaking is an essential step in making tempeh a pre-fermentation process utilizing microbial enzymes to increase product nutrition. Soaking has uncultured microbial diversity analyzed using metagenomic data with next-generation sequencing. The different times of soaking for 12 h and 24 h gave a different percentage of microbial population, indicate that the uniqueness of fermentation of *Jack* beans tempeh. Firmicutes phylum was the predominant phylum in both times soaking processes. Ten OTUs genera were found in both soaking times; Prevotella, *Bacillus*, *Paenibacillus*, *Staphylococcus*, *Lactobacillus*, *Pediococcus*, *Saccharofermentants*, *Klebsiella*, *Pantoea*, and *Acinetobacter*. Six OTUs genera bacteria were dominant in soaking for 12 h, and four OTUs genera were dominant in soaking for 24 h. The microbial diversity indicated different enzymes were presented and contributed nutrients *Jack* beans Tempeh.

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Conflict of Interest

All authors have no conflict of interest.

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