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Metagenomic insights into bacterial communities in eco-enzymes and their microbial diversity and abundance

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Abstract. *Novianti T, Saraswati H, Hafsah H, Seprianto. 2025. Metagenomic insights into bacterial communities in eco-enzymes and their microbial diversity and abundance. Biodiversitas 26: 2157-2166.* The aim of microbial diversity analysis of eco-enzymes is to optimize their benefits in various fields, such as for use in disinfectants, household cleaning agents, liquid fertilizers, and more. Metagenomic profiling is an effective method of analyzing microbial diversity that involves determining the nucleotide sequences of microbial DNA and allows the identification and taxonomic classification of certain bacteria. In this study, metagenomic analysis was carried out on eco-enzymes derived from mangosteen peel, orange peel, and a mixture of 16S rRNA gene primers. We visualized the amplification results using electrophoresis to confirm the length of the DNA base band obtained. We then sequenced the Polymerase Chain Reaction (PCR) products using MinKNOW software (version 23.04.5; Oxford Nanopore Technologies, Oxford-UK). The bioinformatics metagenomic analysis included chronological visualization, Sankey phylogenetic analysis, heat map graphing, and Venn diagram visualization. The study's results showed that in the mangosteen peel eco-enzyme there were bacteria from 7 different phyla. The results of the alpha-diversity index analysis showed the highest value of 2,050 compared to other samples. However, the abundance of each species was lower than that in the other samples. In contrast, the orange peel eco-enzyme had only one bacterial phylum, and the mixed vegetable eco-enzyme had two bacterial phyla. This finding indicates that mangosteen peel eco-enzyme has the most abundant bacterial diversity from different phyla than orange peel eco-enzyme and mix-vegetables, which may cause diversity in the enzyme content produced.

Keywords: Bacteria, eco-enzyme, mangosteen peels, metagenomics, taxa

INTRODUCTION

Eco-enzymes are fermented liquids made from waste vegetables and fruit peel through the action of microorganisms. Fermentation leads to an eco-enzyme solution with a low acidity level below pH 4, which encourages the growth of beneficial bacteria while inhibiting harmful pathogenic strains (Hanifah et al. 2022). The environment this bacterial diversity fosters is particularly conducive to the growth of lactic acid. For example, eco-enzymes can be used in organic liquid fertilizers or to enhance water purification, aid in wound care, provide effective hand cleansing, clean household surfaces, and help treat dandruff. Eco-enzymes can also be used as air purifiers to remove odors and toxic particles (Permatananda et al. 2023).

During fermentation, microbes break down complex organic compounds into simpler compounds and beneficial chemicals, such as antioxidants and antibacterials. Eco-enzymes contain various secondary metabolite compounds produced by microbes (bacteria and fungi) during fermentation (Gutiérrez-Sarmiento et al. 2022; Kurniawan et al. 2024), including enzymes (amylase, trypsin, and lipase), phenol, alcohol, and organic acids. Polyphenols are known to be the primary natural antioxidants in food, and several metabolic processes that occur during fermentation contribute to the release or conversion of polyphenols into more active forms. It is also possible that several enzymes in eco-enzymes can function as antibiotics to inhibit the growth of pathogenic microorganisms (Low et al. 2020)

Lignocellulose, a key component of vegetables, is a renewable carbon source composed of cellulose, hemicellulose, and lignin. Its degradation relies on enzymes produced by microorganisms, which operate through complex mechanisms (Loe et al. 2020; Patel et al. 2021). Mangosteen (a fruit) has substantial nutritional benefits, and its peel contains abundant xanthone compounds, including bioflavonoids that are known for their antioxidant, antibacterial, antiallergic, antitumor, antihistamine, and anti-inflammatory properties (Indraloka et al. 2023). Xanthone acts as a powerful antioxidant that neutralizes free radicals, potentially prevents cell aging, heart disease, and cancer, and boosts immune function (Huang et al. 2021). Orange peel contains several chemical compounds, such as ascorbic acid, vitamin E, and polyphenols. Antioxidant polyphenols, especially flavonoids, can inhibit free radicals that play an important role in the pathogenesis of inflammation because they have anti-inflammatory, antioxidant, and antibacterial effects. Orange peel also contains citric, oxalic, malic, and succinic acids. The advantages of eco-enzymes stem from their rich bacterial compositions, which generate diverse beneficial enzymes (Hasanah et al. 2022; Muktiarni et al. 2023).

Analyzing the diversity of bacteria in eco-enzymes is critical for identifying potential enzymes for application in food, textile, and medical industries. The insights gained from identifying these bacteria can position eco-enzymes as a transformative force across various sectors (Mavani et al. 2020; Hanifah et al. 2022). Metagenomics is a powerful method of genomic analysis that facilitates the interpretation of DNA sequences from various microorganisms. DNA-based sequencing focused on the bacterial 16S ribosomal RNA (rRNA) gene can be employed to precisely identify specific bacterial species based on conserved genes (Berini et al. 2017; Wirajana et al. 2024). Metagenomic sequencing is crucial for determining species' abundance and understanding their taxonomies, genetic compositions, and biochemical capabilities. Analyzing eco-enzyme metagenomes from diverse biomass sources can reveal unique microbial communities, taxonomic diversity, functional gene abundance, metabolic potential and enzymatic profiles (Eck et al. 2019), potentially leading to the discovery of new enzymes linked to previously uncharacterized microorganisms. This approach considers microbial DNA across various taxonomic levels to provide insights into bacterial activity and to help identify enzymes and proteins in eco-enzymes (Meng et al. 2023).

The presence of secondary metabolites in mangosteen peel, orange peel, and various vegetables can significantly enrich fermentation processes, fostering bacterial growth and facilitating enzyme production (Acuña et al. 2023). In this study, we conducted a metagenomic analysis of eco-enzyme microorganisms derived from mangosteen peel, orange peel, and various vegetables to obtain data on the abundance of bacteria in each eco-enzyme sample and to reveal the potential benefits of enzymes and bacteria for various areas of life and diverse industrial sectors.

MATERIALS AND METHODS

We prepared eco-enzyme solutions from mangosteen peel, orange peel, and mixed vegetables fermented for three months using a 3:1:10 ratio of organic matter to brown sugar and water. The primer base sequences of the 16S rRNA gene used in the metagenomic assay were 5′-TTTCTGTTGGTGCTGATATTGC-3′ for the forward primer and 5′-ACTTGCCTGTCGCTCTATCTTC-3′ for the reverse primer. We extracted DNA using the Quick DNA Magbead Plus Kit (D4082; Zymo Research, California) and employed DNA primers for PCR quality control with an Illumina universal adapter. We used a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific, Waltham) to conduct initial quantification and purity assessment, then we visualized the PCR products using agarose gel electrophoresis, performed accurate quantification with Qubit dsDNA HS Assay Kits (Thermo Fisher Scientific, Waltham), and conducted sequencing using the Oxford Nanopore Technologies sequencing platform (Nanopore Technologies, Oxford).

DNA extraction and sequencing

We extracted DNA from each of three 50-mL eco-enzyme solution samples: mangosteen peel eco-enzyme (Sample 1), orange peel eco-enzyme (Sample 2), and vegetable eco-enzyme mix (Sample 3). After DNA extraction, we calculated the DNA concentrations using a NanoDrop spectrophotometer and a Qubit dsDNA high-sensitivity fluorometer to accurately measure concentrations of 0.1-120 ng/µL. Genomic DNA (gDNA) is the primary DNA extracted from the 16S rRNA gene and conserved in bacteria. We conducted gDNA amplification using PCR and visualized the amplification results using electrophoresis to confirm the presence of bacterial DNA, ensuring that the base length of the DNA primer is parallel to the base length of the bacterial DNA. PCR products of 20 ng in a volume of 10 µL were required for nanopore sequencing using a kit by ONT (Oxford Nanopore Technologies, Oxford) with MinKNOW software (version 23.04.5). We analyzed and interpreted the sequencing results using Pavian, Krona Tools, and the R version as bioinformatics tools.

Bioinformatics analysis

We initiated the base calling process after nanopore sequencing of the extracted DNA using MinKNOW (version 23.04.5) and Guppy (version 6.5.7) software with a high-accuracy model. We employed nano plotting to visualize the base results as FASTQ files, and we used NanoFit to filter the results and ensure their quality. We then applied a centrifuge classifier to classify the red filter results. We referred to the NCBI 16S RefSeq database (https://ftp.ncbi.nlm.nih.gov/refseq/TargetedLoci/) to identify bacteria and determine the Archaea index. We conducted downstream analysis and visualization using Pavian (https://github.com/fbreitwieser/pavian), Krona Tools (https://github.com/marbl/Krona), and the R version of R Studio.

RESULTS AND DISCUSSION

The results of DNA amplification using PCR with 16S rRNA gene primers from bacteria showed that the long DNA bands obtained from electrophoresis ranged from 1,300-1,500 bp. This indicates the presence of bacterial DNA in the eco-enzyme sample (Figure 1). The electrophoresis results showed a DNA band measuring 1,300 bp in Samples 1 and 2. In Sample 3, there was DNA with a length of 1,300-1,500 bp. Bacterial DNA genes generally have a DNA base length ranging from 1,000-2,000 bp (de la Fuente-Salcido et al. 2015; Brazda et al. 2020; Gutiérrez-Sarmiento et al. 2022).

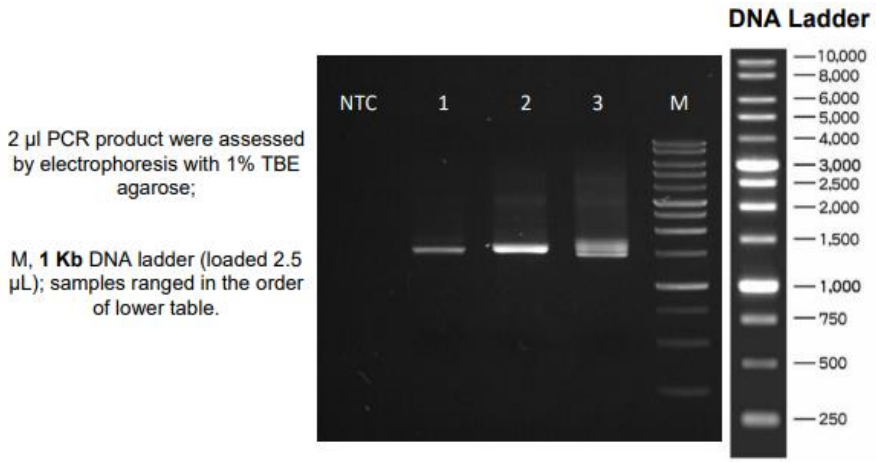

Figure 1. Visualization of PCR electrophoresis results following 16S rRNA gene amplification in Sample 1 (mangosteen peel), Sample 2 (orange peel), and Sample 3 (mixed vegetable), with a comparison of DNA markers (M)

The metagenomic analysis of eco-enzyme samples based on the bacterial 16S DNA primers effectively yielded DNA sequences, allowing us to successfully identify sequences of 1,300-1,500 bp. The results of metagenomic analysis showed that the highest frequency of DNA amplification occurred in sequences with a length of 1,300 bp. Brazda et al. (2020) stated that bacterial DNA is characterized by a base length exceeding 1,000 bp. The metagenomic results successfully used 16S rRNA DNA primer sequencing to successfully amplify bacteria from all eco-enzyme samples.

The bacterial diversity visualization at the species level demonstrated a notable presence of *Liquorilactobacillus nagelii* in Samples 1 and 2 (Figure 2.A), but the abundance of other species in Sample 1 was minimal. In contrast, Sample 2 showed that *L. nagelii* was the dominant species, accompanied by *Lacticaseibacillus* sp. playing a secondary role. The bar chart of Figure 2.A indicates that Samples 2 and 3 had an increased diversity of bacterial types. Interestingly, *L. nagelii* was absent in Sample 3, where *Acetobacter* sp. was identified as the dominant species. Research by Gutiérrez-Sarmiento suggests that *L. nagelii* is commonly linked with fermented fruit products due to its obligate homofermentative nature and its consumption of hexose sugars, including galactose, glucose, and fructose, for lactate production via glycolysis (Gutiérrez-Sarmiento et al. 2022; Abdulsalam et al. 2023).

When analyzing genus abundance across the three samples, we observed significant diversity, with *Liquorilactobacillus* predominating in Samples 1 and 2 but *Acetobacter* sp. predominating in Sample 3 (Figure 2). Regarding taxonomic orders, *Lactobacillales* were dominant in Samples 1 and 2, while *Alphaproteobacteria* sp. was dominant in Sample 3. Sample 1 showed more diversity across taxa from order to phylum levels compared to Samples 2 and 3. This indicates that the mangosteen peel eco-enzyme has greater bacterial diversity than the orange peel eco-enzyme and mix-vegetables eco-enzyme. The Bacillota phylum was the most common in all three samples; however, a more significant presence of the Pseudomonadota phylum was seen in Sample 3, highlighting its significance.

Studies on endophytic bacteria in vegetable plant seedlings have underscored the essential roles played by Bacillota and Pseudomonadota in bacterial communities in aiding in pathogen resistance and enhancing plant growth throughout the developmental stages (Acuña et al. 2023; Oviedo-León et al. 2024). The bacterial diversity analysis, represented with a bar chart and a corona diagram, revealed considerable species, genus, order, family, class, and phylum diversity in Sample 1 compared to Samples 2 and 3 (Figure 3.A). Sample 2, comprising waste orange peel, displayed variation mainly at the species level, with all representatives fitting within the same genus, order, family, class, and phylum (Figure 3.B). Conversely, the vegetable waste eco-enzyme (Sample 3), displayed more significant species variation than Sample 2, spanning two distinct phyla (Figure 3.C). Importantly, mangosteen peel is a valuable source of bioactive compounds that promote the growth of lactic acid bacteria (LAB), making it an ideal substrate for fermentation processes (Arundina et al. 2018; Indraloka et al. 2023).

The three eco-enzyme samples were dominated by the Bacillota phylum (i.e., by LAB). Patel et al. (2021) stated that during a fermentation process, acidity increased along with an increasing number and activity of microorganisms that converted lactose into lactic acid, thereby inhibiting the growth of pathogenic bacteria that grow at the normal pH level. LAB can inhibit and kill other bacteria by producing bacteriocin proteins, hydrogen peroxide, organic acids, diacetyl, acetoin, and reuterin (Patel et al. 2021; Permatananda et al. 2023). Antibacterial pathogens in eco-enzymes in gel form can clean pathogenic bacteria from wounds, thereby stimulating tissue regeneration in wound areas (Ramadona 2022).

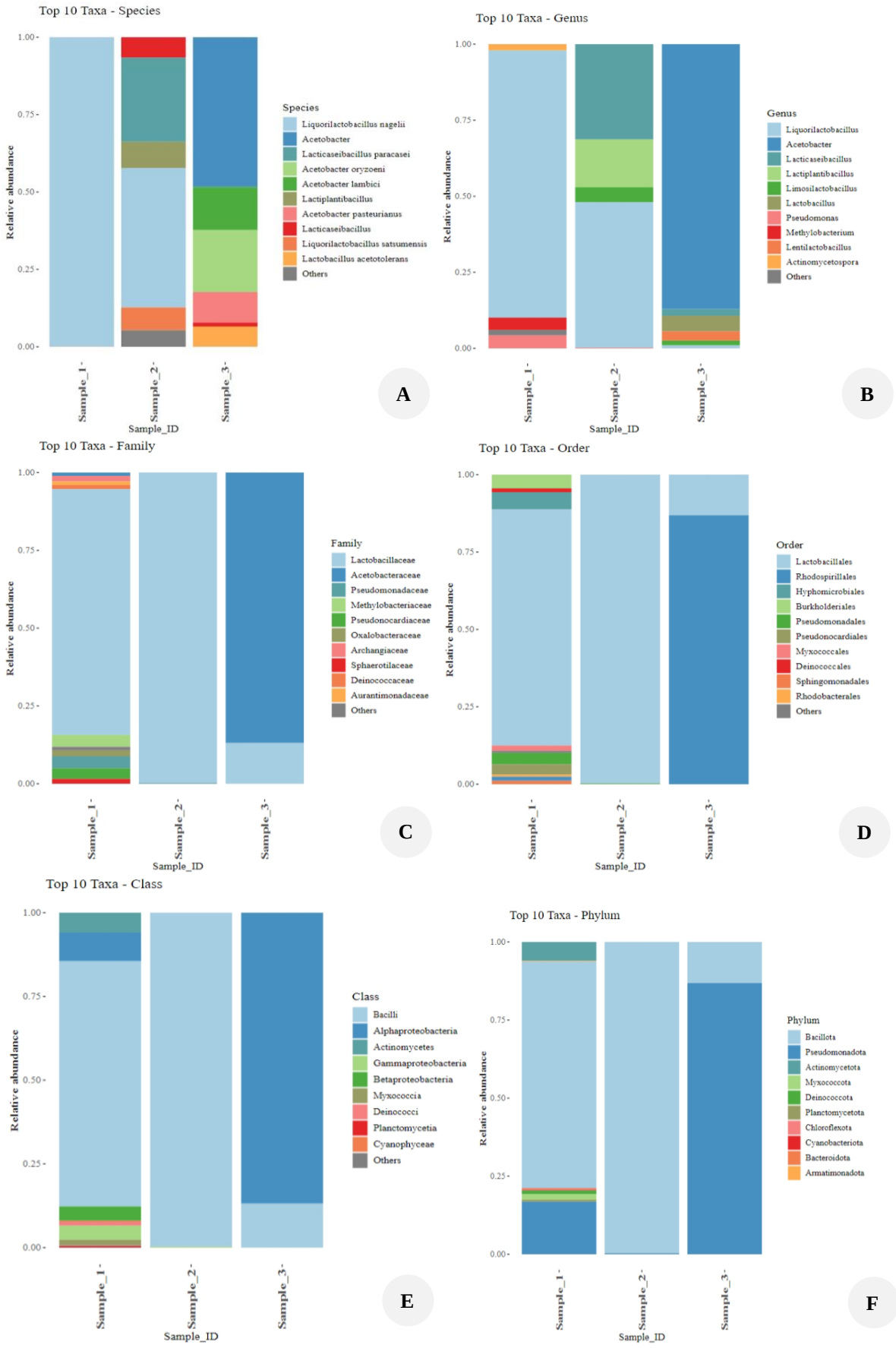

Figure 2. Bar chart of bacterial abundance categorized by taxa: A. Species, B. Genus, C. Family, D. Order, E. Class, and F. Phylum across the three samples

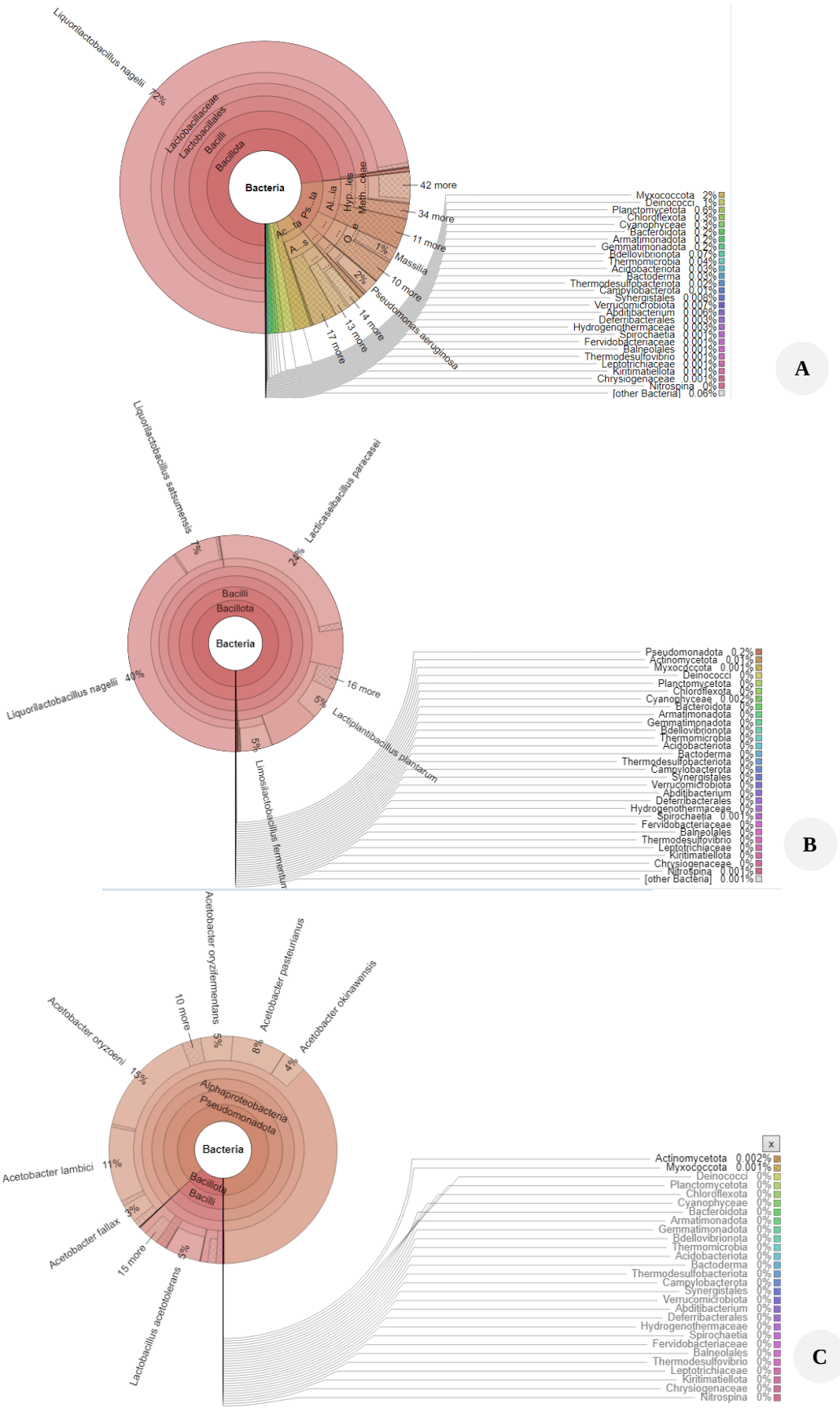

Figure 3. Corona visualization of bacterial diversity data across taxa from species to phylum: A. Bacterial diversity in the mangosteen peel eco-enzyme sample; B. Bacterial diversity in the orange peel eco-enzyme sample; C. Bacterial diversity in the vegetable eco-enzyme sample

The visualization in Figure 4 presents an in-depth comparative analysis of the abundance of various bacterial types across different taxa, providing valuable insights into microbial composition. The brightness of the colors used in the heat map shows the relative abundance of each bacterial type in the samples analyzed. Specifically, Samples 1 and 2 revealed that *L. nigelii* had the highest abundance and, therefore, the greatest prominence in these samples. However, in Sample 3, the presence of *L. nigelii* was markedly reduced, with only minimal quantities detected. For Sample 1, the heat map displays (Figure 4) a very bright color for a single species, signifying that one species dominated the overall composition of that sample. This finding is consistent with the results shown in the bar chart in Figure 4.A for Sample 2, where six distinct species exhibit similarly bright colors, reflecting a diverse array of bacterial species. Conversely, Sample 3 demonstrated broader variation in the dominant species compared to the more homogenous profiles in Samples 1 and 2 (Figure 4.A).

Remarkably, the dominant species abundance index in Sample 3 exceeded the threshold value of 4,096, indicating high microbial activity. The genera abundance in each sample emphasized the clear dominance of the genus *Liquorilactobacillus* in Samples 1 and 2. The abundance index value of *Liquorilactobacillus* in these samples exceeded 65,536, indicating its significant role in the microbial community (Figure 4). Sample 1 showed more variation in genus representation than Sample 2, with index values exceeding 4,096. Furthermore, in particular, several genera in Sample 2 showed abundance index values below 16, indicating lower microbial diversity compared to Sample 1. Although Samples 2 and 3 shared some genera with low or negligible abundance, Sample 1 included genera with notably high abundance of bacteria, such as *Pseudomonas*, *Pseudorhodoferax*, and *Cystobacter*. The genera *Lacticaseibacillus* and *Limosilactobacillus* were present in Samples 2 and 3, but they were absent from Sample 1. Furthermore, the genus *Lactiplantibacillus* was identified exclusively in Sample 2, while *Lentilactobacillus*, *Acetobacter*, and *Lactobacillus* were found solely within Sample 3. Oviedo-León et al.'s (2024) research provided significant insights into the genomic features of *L. nagelii*, revealing that its genome harbors the dextransucrase gene GH70 (EC 2.4.1.5). This gene plays a crucial role in producing dextran from sucrose, an exopolysaccharide known for its diverse applications in the food industry and biomedical fields. These findings emphasize the potential utility of *L. nagelii* for various biotechnological applications, highlighting its value for the research and commercial sectors (Hasanah et al. 2020; Oviedo-León et al. 2024).

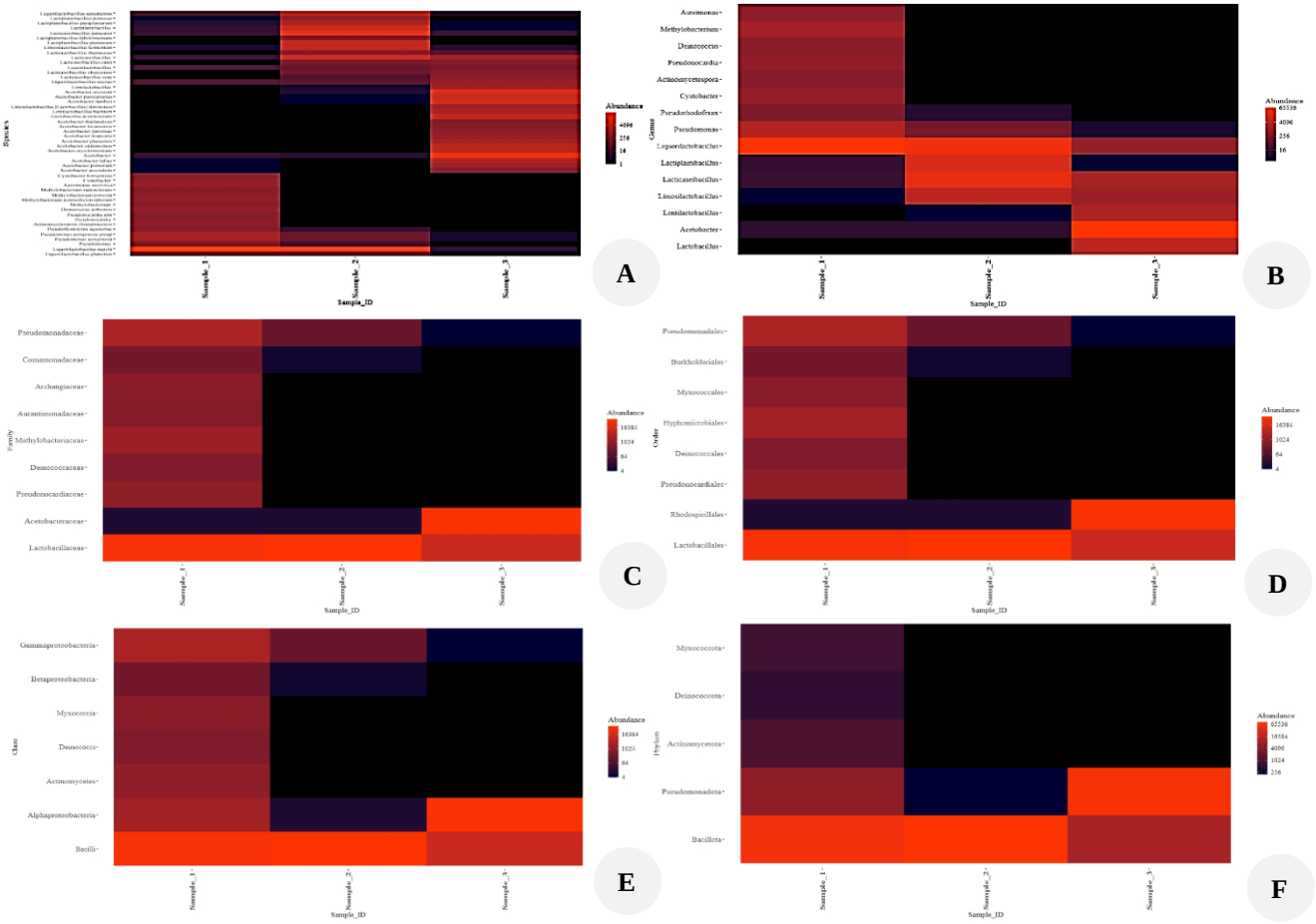

Figure 4. Visualization of bacterial abundance comparison using heat maps for Samples 1 (mangosteen peels), Sample 2 (orange peels), and Sample 3 (mixed vegetables) accross taxa: A. Species; B. Genus; C. Order; D. Family; E. Class; F. Phylum

Regarding the overall taxa abundance, Sample 1 displayed the most extensive family variation, encompassing nearly all family types within the eco-enzyme except for the Acetobacteraceae family. In contrast, sample 2 exhibited complete dominance by the Lactobacilaceae family. Sample 3 was characterized by the co-dominance of the Acetobacteraceae and Lactobacilaceae families. Regarding the other taxa, *Lactobacillus* was identified across all three samples, whereas Rhodospirillales was predominantly present in Sample 3. Sample 1 included a comprehensive array of all orders, except for Rhodospirillales, highlighting its diverse microbial composition. Moreover, all classes of bacteria were detected in Sample 1, in contrast to Sample 2, which contained only the Bacilli class. Sample 3 presented a dual dominance of classes featuring both Bacilli and Alphaproteobacteria. The predominant phylum in Sample 1 was identified as Bacillota, followed by Pseudomonadota, Actinomycetota, Myxococcota, and Deinococcota. Conversely, the dominant phylum in Sample 2 was primarily Bacillota, while Sample 3 predominantly featured Pseudomonadota, marking a significant divergence from the dominance of Bacillota observed in Sample 1 (Figure 4.F). This detailed examination provided a deeper understanding of the microbial dynamics within the samples, highlighting the complexity and variation present in bacterial communities across different conditions.

The results of the alpha diversity analysis, evaluated based on the observed Chao1, abundace-based coverage estimator (ACE), Shannon, and Simpson parameters, revealed significant differences among the eco-enzyme samples. Sample 1 displayed the highest observed value of variation, indicating more significant bacterial variation than in Samples 2 and 3 (Table 1). The Chao1 metric corroborated that Sample 1 had the highest number of species, which was also reflected in the abundance measure (ACE). The Shannon index, representing the relative abundance of species, was highest for Sample 1, followed by Sample 3, while Sample 2 had the lowest value. Conversely, Sample 1 exhibited the lowest Simpson index value, suggesting that it contained the fewest dominant species compared to Samples 2 and 3. The Shannon index further indicated that Sample 1 had a high level of species diversity and a low proximity index between species, pointing to a rich bacterial community comprising various phyla. This implies that Sample 1 (mangosteen peel) supported a high abundance of microorganisms with notable species variation. Although Sample 1 had the highest average abundance of each species, it featured the fewest dominant species, reflecting a greater evenness in bacterial numbers relative to Samples 2 and 3.

The results of the Sankey phylogenetic analysis illustrated the grouping of bacteria in each taxon from phylum to species (Figure 5). Sankey phylogenetics helps analyze the variants of each taxon of microorganism (Semumu et al. 2024). Sample 1 exhibited more phyla and branches, indicating greater species diversity across different taxa orders, families, classes, and phyla.

We visualized the similarities and differences in the abundance of data on organisms according to the metagenomic analysis using a Venn diagram (Prakash and Taylor 2012). The results indicated that Sample 1 had highly varied bacteria, with 1,921 types not found in Samples 2 and 3. Sample 2 contained approximately 94 unique types of bacteria not found in Samples 1 or 3. In contrast, Sample 3 contained 103 species not found in Samples 1 or 2 (Figure 6). The highest bacterial similarity existed between Samples 1 and 2, possibly due to their shared fruit origins. Eco-enzymes derived from similar organic materials are thought to harbor similar types of bacteria (Mursyid et al. 2022; Oviedo-León et al. 2024).

The high bacterial diversity observed in the mangosteen peel eco-enzyme suggests that it was the most effective medium for fostering bacterial growth among the three eco-enzyme samples. Mangosteen peel is rich in the active chemical compound xanthone—a bioflavonoid known for its antioxidant, antibacterial, antiallergic, antitumor, antihistamine, and anti-inflammatory properties (Indraloka et al. 2023). As a naturally occurring compound, xanthone is widely present in higher plants, fungi, lichens, and bacteria. Thus, the presence of xanthone in mangosteen peel promotes the growth of a diverse array of bacterial types across various phyla (Huang et al. 2021). The results of the phytochemical assessment of the orange peel eco-enzyme showed that it contained secondary metabolites in the form of alkaloids, flavonoids, phenols, saponins, and triterpenoids (Kurniawan et al. 2024). The results of Tigrero-Vaca et al.'s (2022) study on cocoa bean fermentation showed high microbial diversity during fermentation, indicating a low content of organic acids that inhibited microbial growth (Tigrero-Vaca et al. 2022). Likewise, the results of eco-enzyme fermentation in the mangosteen peel sample with the highest abundance value suggest that bacterial growth in the eco-enzyme was not inhibited by the organic compounds it contained.

Table 1. Alpha diversity measures of the samples

Sample	Alpha diversity measure				
	Observed	**Chao1**	**ACE**	**Shannon**	**Simpson**
1	2,050	3,200	3,000	2.95	0.40
2	240	400	400	1.80	0.76
3	230	250	300	2.80	0.82

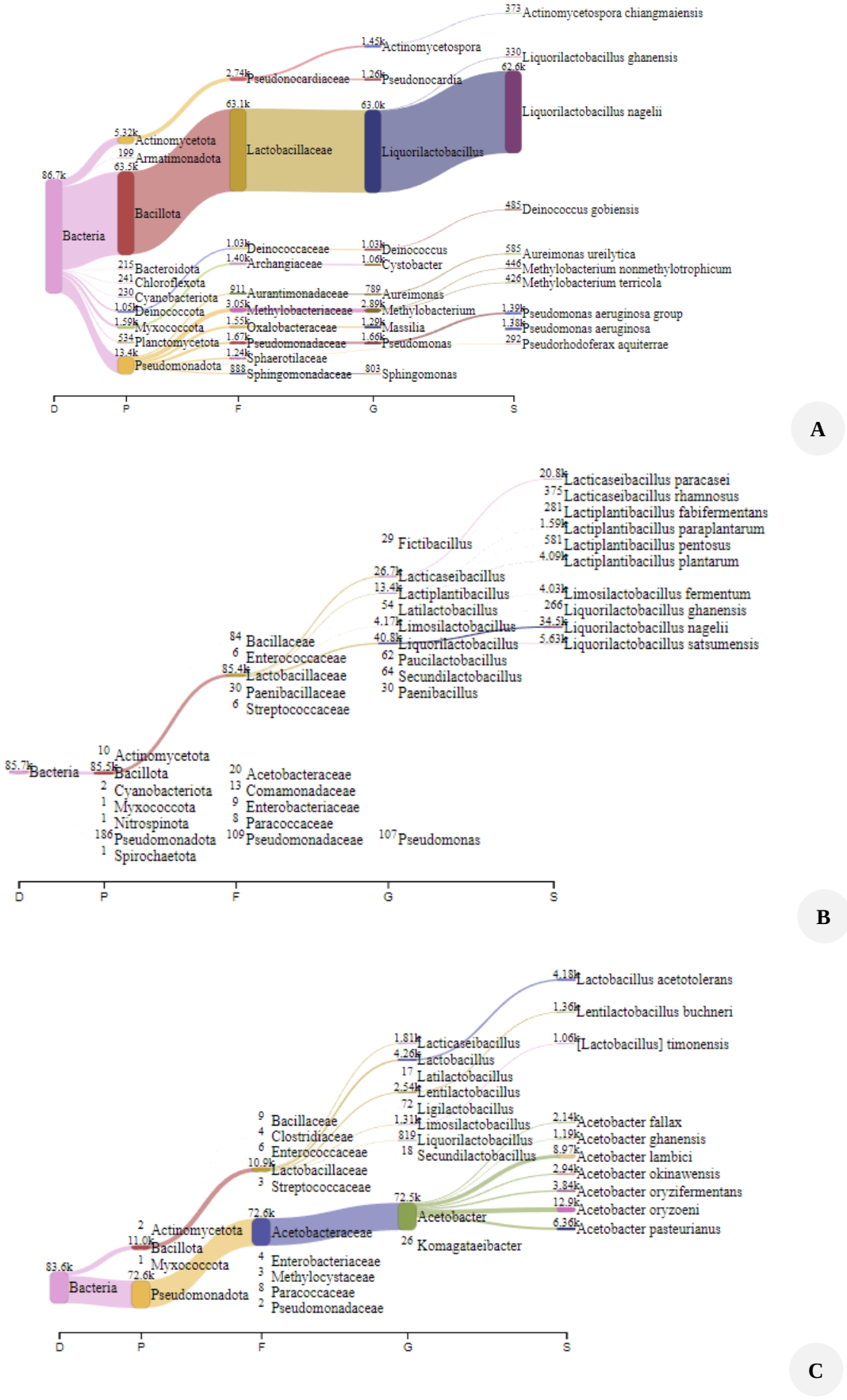

Figure 5. Phylogenic comparison of the abundance of bacterial species in each taxon for: A. Sample 1 (mangosteen peels); B. Sample 2 (orange peels); C. Sample 3 (mixed vegetables)

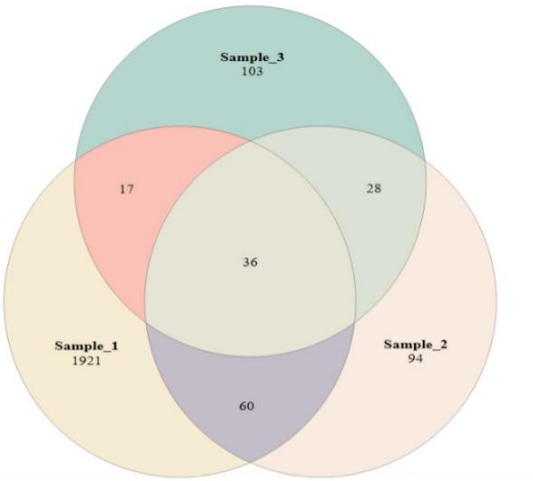

Figure 6. The similarity among bacterial abundance data for samples 1 (mangosteen peels), 2 (orange peels), and 3 (mixed vegetables)

In conclusion, the results of this study showed that the eco-enzyme solution derived from mangosteen peel had greater bacterial diversity than eco-enzymes derived from orange peel and various vegetables with different phyla. The mangosteen peel eco-enzyme had a highly dominant phylum with the greatest diversity of bacterial species, and it was dominated by *Liquorilactobacillus nagelii*. This high diversity of bacteria is thought to be due to the xanthone compound content in mangosteen peel, which can increase bacterial growth. We suspect that the enzymes found in the three eco-enzyme solutions were amylase, protease, and lipase, which can inhibit the growth of pathogenic bacteria. Further research is needed to identify the potential of other organic compounds derived from fermentation that can be used across all areas of life.

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