BIODIVERSITAS
Volume 26, Number 6, June 2025
Pages: 2684-2695

ISSN: 1412-033X
E-ISSN: 2085-4722
DOI: 10.13057/biodiv/d260613

Metagenomic insights into microbial community dynamics of fermented *Indigofera zollingeriana* supplemented with probiotics and phytobiotics

IRWAN SUSANTO[1,♥], ANURAGA JAYANEGARA[2,♥♥], RONI RIDWAN[3], I KOMANG G. WIRYAWAN[2], ERIKA B. LACONI[2]

[1]Graduate Program of Nutrition and Feed Science, Faculty of Animal Science, Institut Pertanian Bogor. Jl. Agatis, Dramaga, Bogor 16680, West Java, Indonesia. Tel.: +62-251-8626213, ♥email: irwansusanto939@gmail.com

[2]Department of Nutrition and Feed Technology, Faculty of Animal Science, Institut Pertanian Bogor. Jl. Agatis, Dramaga, Bogor 16680, West Java, Indonesia. ♥♥email: anuraga.jayanegara@gmail.com

[3]Research Center for Applied Zoology, National Research and Innovation Agency. Jl. Raya Jakarta-Bogor Km 46, Cibinong, Bogor 16911, West Java, Indonesia

Manuscript received: 1 April 2025. Revision accepted: 20 May 2025.

Abstract. *Susanto I, Jayanegara A, Ridwan R, Wiryawan IKG, Laconi EB. 2025. Metagenomic insights into microbial community dynamics of fermented* Indigofera zollingeriana *supplemented with probiotics and phytobiotics. Biodiversitas 26: 2684-2695.* Silage fermentation is an effective method for preserving forage by utilizing microbial activity under anaerobic conditions to maintain its nutritional value and prolong storage stability. *Indigofera zollingeriana* is a high-protein and fibre-rich forage, making it a promising candidate for silage. However, fermentation success depends on microbial communities involved in organic matter degradation. This study employed Next-Generation Sequencing (NGS) with the Illumina platform to analyze the microbial composition of Indigofera silage, enabling precise genus-level identification via 16S rRNA sequencing. The study evaluated microbial diversity and population shifts in silage supplemented with phytobiotics, probiotics, and their combination. The results showed that Gammaproteobacteria dominated all treatments (>90%), with Bacilli and Alphaproteobacteria present in smaller proportions. The combination treatment exhibited the highest microbial diversity, with increased Betaproteobacteria and Actinomycetes, which aid fibre degradation. The low presence of *Clostridia* suggested well-controlled fermentation. At the family level, Enterobacteriaceae were dominant in single LAB and phytobiotic treatments, while the combination treatment reduced Enterobacteriaceae and increased Moraxellaceae and Enterococcaceae, which support fermentation stability. At the genus level, *Enterobacter* was prevalent, but the combination treatment increased *Pantoea* and *Leclercia*, indicating a more balanced fermentation ecosystem. Alpha diversity and Principal Coordinates Analysis (PCoA) confirmed that the combination treatment promoted microbial diversity and fermentation stability while suppressing *Enterobacter cloacae*. Treatment using acacia bark extract showed effectiveness in the smallest *Costridia* population (2%). These findings highlight the importance of combining probiotics and phytobiotics to enhance silage quality and microbial balance, emphasizing the need for further research on microbial interactions in fermentation.

Keywords: Fermentation, *Indigofera zollingeriana*, microbial diversity, phytobiotics, probiotics

Abbreviations: DNA: Deoxyribonucleic Acid; LAB: Lactic Acid Bacteria; MAE: Microwave-Assisted Extraction; NGS: Next-Generation Sequencing; PcoA: Principal coordinates Analysis; PCR: Polymerase Chain Reaction

INTRODUCTION

Silage fermentation is a widely practised technique for preserving forage, leveraging microbial activity under anaerobic conditions to maintain its nutritional integrity and prolong storage stability (McAllister et al. 2018). The process relies on the natural conversion of carbohydrates into organic acids, predominantly lactic acid, which lowers the pH and inhibits spoilage microorganisms. The success of silage fermentation largely hinges on the types and activities of microbial communities involved, which mediate organic matter breakdown and the production of key fermentation metabolites, such as lactic acid and acetic acid (Muck 2013). Enhancing microbial diversity and stability is essential to improve silage quality, especially for protein-rich forage crops like *Indigofera zollingeriana* Miq.

I. zollingeriana is an exceptional forage crop recognized for its rich protein content, balanced amino acid profile, and substantial fibre levels, making it a promising candidate for silage production (Harahap et al. 2023). However, high-protein silages are often prone to excessive proteolysis and ammonia accumulation, reducing protein availability and compromising silage quality. Functional additives, such as probiotics and phytobiotics, have been explored to enhance fermentation efficiency, suppress undesirable microbial activity, and improve the nutritive value of silage (Utama et al. 2021). Understanding the microbial dynamics in Indigofera silage supplemented with these bioactive compounds is crucial for optimizing outcomes and ensuring high-quality feed.

Next-Generation Sequencing (NGS) has revolutionized metagenomic research, enabling in-depth analysis of microbial populations in silage environments (Tennant et al. 2017). Unlike conventional microbiological techniques that require cultivation, NGS allows for the direct identification and functional profiling of microbial

communities, providing more comprehensive insights into microbial roles during fermentation (Satam et al. 2023). This study applies NGS to evaluate the effects of acacia bark extract as a phytobiotic, *Lactiplantibacillus plantarum* as a probiotic, and their combination on the microbiota composition of Indigofera silage, offering novel insights into microbial dynamics and fermentation performance.

Phytobiotics, which are plant-derived bioactive compounds, have been recognized for enhancing silage fermentation by promoting Lactic Acid Bacteria (LAB) growth while inhibiting pathogenic microorganisms (Utama et al. 2021). Acacia bark extract, rich in phenolic compounds and tannins, exhibits antimicrobial properties and has been reported to boost the production of organic acids, thereby contributing to improved fermentation stability (Lazzari et al. 2023). Recent studies suggest that including phytobiotics in silage can enhance fermentation efficiency, improve the nutritional profiles, and suppress spoilage microbial activity (Muck et al. 2018).

L. plantarum is one of the most frequently used probiotics in silage fermentation due to its ability to rapidly produce lactic acid, reduce pH, and inhibit the growth of undesirable bacteria and moulds (Okoye et al. 2023). Furthermore, this probiotic enhances fibre digestibility and increases nutrient bioavailability in silage (Arowolo and He 2018). Combining probiotics with phytobiotics may offer a synergistic effect, further improving fermentation stability and fostering beneficial microbial diversity (Stefańska et al. 2020).

Assessing silage microbiota is essential for evaluating the effects of LAB and phytobiotic supplementation on fermentation quality. DNA sequencing-based approaches such as NGS facilitate the identification of dominant microbial species in silage and provide insights into microbial interactions across different fermentation treatments (McAllister et al. 2018). This approach can elucidate the synergistic mechanisms of LAB and phytobiotics in creating an optimal fermentation environment. Analysis of alpha and beta microbial diversity and specific identification of bacteria involved in fermentation will provide deeper insight into the impact of natural additives on silage quality and safety (Tennant et al. 2017).

This study aimed to generate valuable insights into the microbial dynamics of Indigofera silage treated with acacia bark extract and *L. plantarum*, contributing to the advancement of natural, sustainable additives for silage production. The outcomes are expected to support the development of feeding strategies that minimize reliance on synthetic preservatives and antibiotics, thereby improving animal health and enhancing feed efficiency. Additionally, findings from this work bolster ongoing efforts to promote sustainable livestock systems and improve food security through bio-based innovations in feed preservation. By bridging microbial ecology with applied fermentation practices, this research provides a foundation for refining silage technology and developing eco-friendly alternatives tailored to tropical forage crops like *I. zollingeriana.*

MATERIALS AND METHODS

Sample preparation

This study was conducted at the Feed Science and Technology laboratory, Faculty of Animal Science, IPB University, and Research Center for Applied Zoology, National Research and Innovation Agency (BRIN), Cibinong. Samples of *Acacia mangium* Willd. bark were sourced from the forested area within the IPB University campus. These samples were then dried in an oven at 60°C for 48 hours to obtain the dried bark simplicia. Once dried, the bark was finely ground and passed through an 80-mesh sieve for uniform particle size. The extraction procedure was performed according to Makkiyah et al. (2024). The dried acacia bark powder was subjected to Microwave-Assisted Extraction (MAE). Specifically, 4 g of bark powder were mixed with 40 mL of pro-analysis methanol in a 1:10 (m/v) ratio within an Erlenmeyer flask. The prepared mixture was placed in a microwave oven (Sharp R-21D0(S)-IN) and exposed to 135 watts of power for three minutes. Following heating, the sample was left to cool at room temperature before undergoing vacuum filtration using filter paper to separate the clear filtrate. The final filtrate volume was standardised to 40 mL, yielding a 0.1 $g.mL^{-1}$ concentration.

Silage preparation

The forage material utilized in this study was *I. zollingeriana*, sourced from the Agrostology Grassland at the Faculty of Animal Science, IPB University. Only the edible portions of the plant, including leaves and tender stems, were selected for silage production.

Prior to ensiling, the forage was air-dried to reduce its moisture content before weighing. The plant material was then chopped into approximately 2-3 cm long pieces to facilitate the ensiling process. A total of 200 g of Indigofera was weighed and treated with 1% acacia extract, based on the silage weight. Silage making was carried out with five repetitions.

An inoculum of *L. plantarum* was added at 0.2 mL per 200 g of forage material for the probiotic treatment. Each millilitre of *L. plantarum* inoculum contained 1×10^8 $CFU.mL^{-1}$. The treated forage was then packed into plastic bags with a capacity of approximately 300 mL, vacuum-sealed to establish anaerobic conditions, and stored for 30 days.

Following the incubation period, the solid and liquid fractions of the silage were separated. The silage was homogenized with distilled water in a 10 g: 90 mL ratio and filtered to obtain silage extract. These extracts were then analyzed to see the abundance of microbes by extracting DNA from samples at each replicate. Details of the experimental treatments are presented in Table 1.

Table 1. Details of the experimental treatments

Treatment	Code
Indigofera (Control)	A
Indigofera + LAB (*Lactiplantibacillus* plantarum)	B
Indigofera + Extract (*Acacia mangium* bark)	C
Indigofera + LAB + Extract	D

DNA extraction

DNA was extracted from silage fluid samples using the QIAamp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with modifications following Ridwan et al. (2015) and Adawiyah et al. (2025) which included the addition of RBC Lysis Buffer, Lysozyme, and RNAase with the Genomic DNA Mini Kit (Geneaid).

The extraction began with sample preparation, in which 3 mL of glycerol solution from the sample and a cotton swab were transferred to a 2 mL tube and added 2 mL of RBC Lysis Buffer. The sample was then centrifuged at 14,000 rpm for 10 minutes at 4°C. After centrifugation, the supernatant was discarded, and the resulting pellet was retained. Another 2 mL of RBC Lysis Buffer was added to the pellet, which was resuspended and vortexed to ensure uniformity. This centrifugation step was repeated two to three times for 5 minutes at the same speed and temperature until a sufficient pellet was formed.

The pellet was treated with 1 mL of InhibitEX Buffer for cell lysis and vortexed for 1 minute. Next, 20 µL of lysozyme was added, and the mixture was incubated at 70°C for 5 minutes, followed by vortexing for 15 seconds (Tube A). Meanwhile, 15 µL of proteinase K was added to a new tube (Tube B), and 200 µL of the suspension from Tube A was transferred into Tube B. To this, 200 µL of AL buffer was added, vortexed for 15 seconds, and incubated at 70°C for 10 minutes. After incubation, 5 µL of RNAse was introduced, and the tube was inverted every 3 minutes. Finally, 200 µL of cold ethanol (96-100%) was added to facilitate DNA binding (Ridwan et al. 2015; Adawiyah et al. 2025).

The DNA washing phase followed the method of Ridwan et al. (2015) and Du et al. (2024), specifically related to the fermentation process, where 600 µL of the suspension was transferred into a spin column tube and centrifuged at 14,000 rpm for 1 minute at 4°C. The spin column was then moved to a fresh collection tube, and the filtrate was discarded. 500 µL of buffer AW1 was added to further purify the DNA, followed by centrifugation for 1 minute at the same speed and temperature. The spin column was transferred to a new collection tube, and the filtrate was discarded. This procedure was repeated with 500 µL of buffer AW2, with centrifugation extended to 2 minutes. The spin column was then placed in a fresh tube and centrifuged for 3 minutes to remove residual contaminants (Ridwan et al. 2015).

The final elution step involved the addition of 25 µL of ATE Buffer, followed by a 5-minute incubation at room temperature. The sample was centrifuged at 14,000 rpm for 1 minute at 4°C. To maximize DNA yield and concentration, the elution step was repeated. DNA samples obtained from each extraction were pooled and submitted for further analysis through commercial services provided by PT Genetica Science (Indonesia).

Metagenomic analysis

The concentration of extracted DNA was measured using a NanoDrop™ Thermo Scientific Spectrophotometer (USA). Total DNA from each replicate within a treatment group was pooled for metagenomic analysis via Next Generation Sequencing (NGS). Qualitative genome detection was performed through agarose gel electrophoresis using 1% DNAsave. The genomic DNA obtained from PCR products was prepared at a final concentration of 100 ng. The V1-V9 (full-length) region of the 16S rRNA gene was targeted for PCR amplification using universal 16S primers: 27F (5'-AGAGTTTGATCCTGGTCCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') (Adawiyah et al. 2025).

DNA concentration was further assessed using both a NanoDrop spectrophotometer and a Qubit fluorometer. Library preparation was conducted using Oxford Nanopore Technology kits, and sequencing was carried out using the MinKNOW software version 24.02.16. Basecalling was performed with Dorado version 7.3.11, applying a high-accuracy model (Wick et al. 2019). The quality of FASTQ files was evaluated using NanoPlot, and quality filtering was conducted with NanoFilt (de Coster et al. 2018; Nygaard et al. 2020; Wening et al. 2024). Filtered reads were classified using the Centrifuge classifier (Kim et al. 2016). The bacterial and archaeal index was constructed using the NCBI 16S RefSeq database (https://ftp.ncbi.nlm.nih.gov/refseq/TargetedLoci/). Subsequent data analysis and visualizations were carried out using Pavian (https://github.com/fbreitwieser/pavian), Krona Tools (https://github.com/marbl/Krona), and RStudio with R version 4.2.3 (https://www.R-project.org/). The database from KEGG (Kyoto Encyclopedia of Genes and Genomes, https://www.genome.jp/kegg/) was used in determining the functional profile.

RESULTS AND DISCUSSION

Abundance by class

The microbial composition analysis based on class presented in Figure 1, in silage from treatments A, B, C, and D, revealed that the Gammaproteobacteria class was dominant in all treatments, showing a relative abundance exceeding 90%. This suggests that bacteria within this class play a crucial role in silage fermentation, potentially due to their capability to metabolize carbohydrates and generate metabolites that suppress pathogenic bacterial growth (Guan et al. 2020). Additionally, lower proportions of Bacilli and Alphaproteobacteria were identified, with treatment D showing an increased presence of microbial groups other than Gammaproteobacteria. Bacilli, commonly linked to lactic acid production, contribute to silage stabilization by reducing pH levels, creating an unfavourable environment for spoilage microbes (Keshri et al. 2019). In general, the microbiota that dominates each treatment is relatively the same, namely from the Gammaproteobacteria and Bacilli classes.

Variations in microbial abundance across treatments may be attributed to differences in fermentation conditions, such as moisture content, substrate availability, and potential environmental contaminants (McAllister et al. 2018). Treatment D exhibited greater microbial diversity than treatments A, B, and C, indicating more complex fermentation dynamics. Although Sphingobacteriia and Flavobacteriia were detected in relatively small proportions, their presence remains essential for maintaining microbial equilibrium in

the silage ecosystem. Compared to other treatments, the increased abundance of Betaproteobacteria and Actinomycetes in treatment D could be associated with variations in raw materials or distinct silage storage conditions. Actinomycetes, recognized for their lignocellulolytic activity, may contribute to silage fibre degradation, thereby enhancing livestock nutrient availability (Saini et al. 2015).

The results in Figure 1 highlight that *Clostridia* were detected in minimal quantities, particularly in treatment D, indicating successful silage fermentation. This bacterial class is generally undesirable in silage because it produces butyric acid, which deteriorates silage quality and promotes spoilage (Pahlow et al. 2023). *Clostridia* can also degrade lactic acid, increasing silage pH and creating conditions conducive to the proliferation of spoilage bacteria (Muck et al. 2018). The low presence of *Clostridia* suggests that the applied treatment effectively suppressed its growth, likely due to the dominance of lactic acid bacteria, which reduced pH and established an environment unfavourable for *Clostridia*. Additional factors contributing to *Clostridia* inhibition include maintaining anaerobic conditions during fermentation and competition from bacteria such as *Lactobacillus* spp., which are better adapted to such environments (Ridwan et al. 2015). Therefore, while the fermentation strategy employed in treatment D appears adequate, further attention to microbial diversity is necessary to ensure the long-term stability of silage.

The presence of this group of Gammaproteobacteria often includes facultative anaerobic and aerobic bacteria such as *Enterobacter*, *Pseudomonas* and *Klebsiella*. In the fermentation process, these bacteria do not play a direct role in the lactic acid fermentation process but instead utilize sugar compounds to produce acetic acid, ethanol and NH_3 (Durica-Mitic et al. 2018; Ridwan et al. 2023). This is because the substrate used is Indigofera which is a high-protein legume so that the acidic pH is more difficult to achieve and supports the environment of this type of bacteria to grow. This condition promotes deamination of amino acids into free nitrogen. Some members of Gammaproteobacteria, such as *Enterobacter* can deaminate amino acids, and produce free ammonia, which increases the pH of silage (Jayanegara et al. 2019; De Gouveia et al. 2024). Meanwhile, the presence of this taxonomic group of the Bacilli class indicates the success of fermentation during the ensilage process. The presence of these Bacilli is important for lowering the pH of silage and preventing spoilage bacteria. This is supported by Guan et al. (2020), who stated that Bacilli are an important genus, such as *Lactobacillus*, which plays a major role in lactic acid fermentation during the ensilage process. In treatment A (Control), without the addition of probiotics and phytobiotics, Gammaproteobacteria dominate compared to treatments with LAB *L. plantarum* inoculation and plant extract phytobiotics. This is following Xu et al. (2021) where the use of inoculants such as *L. plantarum* and *L. buchneri* is effective in suppressing the growth of Gammaproteobacteria and improving the quality of silage fermentation. Meanwhile, the role of plant extracts in this case is their ability as an antimicrobial, thus inhibiting the growth of unfavorable bacteria (Sinaga et al. 2021).

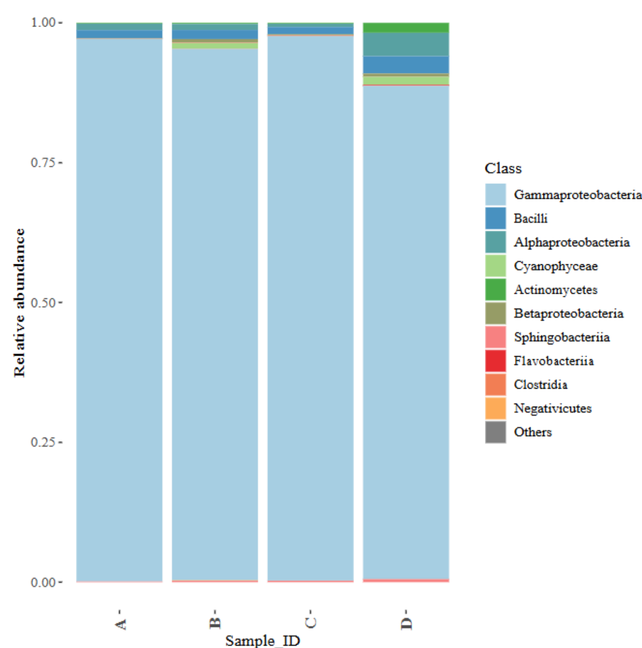

Figure 1. Top 10 distribution of bacterial abundance on the silage of the taxonomic class: A. Indigofera; B. Indigofera + LAB (*L. plantarum*); C. Indigofera + extract (*Acacia mangium* bark); D. Indigofera + LAB + extract

Relative abundance of family

Analysis of the microbial community in silage based on family is presented in Figure 2. The results show that the Enterobacteriaceae family is the dominant group in all treatments, with the highest proportion approaching 100% in treatments A, B, and C. However, in treatment D, a relative decline in Enterobacteriaceae was observed, accompanied by an increase in other bacterial families. The strong presence of Enterobacteriaceae in silage fermentation is generally associated with suboptimal fermentation conditions, as particular species within this family, such as *Escherichia* and *Klebsiella*, can generate undesirable metabolites, including ethanol and ammonia (Ni et al. 2015). A high abundance of Enterobacteriaceae may also suggest limited competition from Lactic Acid Bacteria (LAB), which play a crucial role in acidifying the environment and enhancing silage stability (He et al. 2018).

In addition to Enterobacteriaceae, other bacterial families such as Moraxellaceae, Xanthomonadaceae, and Erwiniaceae were detected in lower proportions across all treatments (Figure 2). Moraxellaceae, which includes aerobic bacteria like *Moraxella*, was more prevalent in treatment D than in the other treatments, potentially indicating shifts in fermentation conditions that fostered greater microbial diversity. Furthermore, an increase in Yersiniaceae, Enterococcaceae, and Rhizobiaceae was noted in treatment D, suggesting that this fermentation environment was more conducive to the proliferation of bacteria associated with stable silage fermentation. Notably, Enterococcaceae includes beneficial lactic acid bacteria such as *Enterococcus faecium*, which enhances lactic acid production and suppresses spoilage or pathogenic microbes (Guan et al. 2020).

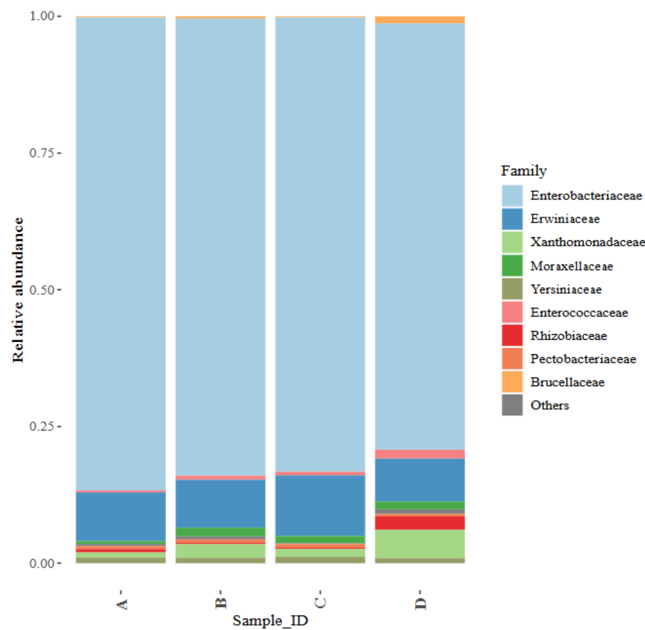

Figure 2. Top 10 distribution of bacterial abundance on the silage of the taxonomic-family: A. Indigofera; B. Indigofera + LAB (*L. plantarum*); C. Indigofera + extract (*Acacia mangium* bark); D. Indigofera + LAB + extract

Compared to the other treatments, treatment D exhibited higher microbial diversity, likely due to modifications in the fermentation process, such as the introduction of inoculants or variations in anaerobic conditions. The reduction of Enterobacteriaceae in treatment D indicates improved fermentation stability, aligning with previous findings that LAB inoculation can enhance fermentation quality by inhibiting spoilage and pathogenic bacteria (McGarvey et al. 2013). Increased microbial diversity in treatment D may also reflect a more balanced fermentation process, which positively impacts both the nutritional quality and storage longevity of silage. Therefore, treatment D supports a more optimal and stable fermentation process than treatments A, B, and C, where Enterobacteriaceae remained dominant.

In general, one of the dominant families seen in all samples was Enterobacteriaceae, which is a member of the Gammaproteobacteria class. In silage fermentation, the presence of Enterobacteriaceae is often associated with fermentation that is not running optimally, because they can consume sugars that should be used by lactic acid bacteria, and produce metabolites such as acetic acid, ethanol, and gases (CO_2 and H_2), which can cause nutrient loss and increase silage pH. If the Enterobacteriaceae population is too high, this is also related to the formation of ammonia from protein degradation, which reduces the quality of protein and the nutritional value of silage (Keshri et al. 2019; Xue et al. 2024). A study by Wang et al. (2024) also showed that Enterobacteriaceae have genes related to the production of ammonia-N and butyrate, which contribute to nutrient degradation during silage fermentation. In contrast, Lactobacillaceae are more related to the synthesis of serine from glycine, which can reduce ammonia-N production.

In addition to Enterobacteriaceae, other families such as Pectobacteriaceae, Erwiniaceae, and Rhizobiaceae, which are also included in Proteobacteria, are also present. Some members of this family, such as *Pectobacterium*, are known as plant-decomposing bacteria that can produce pectinase enzymes, which can cause decomposition of plant cell structures (Islamov et al. 2021) and contribute to silage damage, especially when anaerobic conditions are not perfectly achieved. The Yersiniaceae and Brucellaceae families, although present in small numbers, are still important because their presence can be an indicator of environmental contamination or further microbiological degradation, especially when the silage is exposed to air or is not fermented quickly. Thus, the microbial community profile shown in the image of treatment A (Control) has a higher Enterobacteriaceae population than B, C, and D, indicating that treatment A has a dominance of microbes from less favored groups. This shows that silage with the addition of phytobiotics and probiotics can suppress the population of unwanted bacteria and increase the prevalence of beneficial fermentative bacteria.

Relative abundance by genus

Based on genus analysis, the microbial community analysis in Figure 3 showed that *Enterobacter* predominated in all treatments, with treatments A, B, and C exhibiting the highest abundance. In contrast, the relative proportion of *Enterobacter* decreased slightly in treatment D compared to the other treatments, even though it was still the most prevalent genus. Significant amounts of the *Klebsiella* genus were also found in all treatments, in addition to *Enterobacter*. This suggests that the fermentation conditions continued to favour the persistence of facultative anaerobic bacteria, which could impede the best possible lactic acid fermentation (Ni et al. 2015). Because some species in this genus are known to produce unwanted metabolites like ethanol and ammonia, which lower fermentation efficiency, the presence of these bacteria can negatively affect silage quality (He et al. 2018).

Across all treatments, different amounts of several other genera (Figure 3) were found in addition to *Enterobacter* and *Klebsiella*. These genera included *Pantoea*, *Leclercia*, *Citrobacter*, and *Erwinia*. Treatment D had a higher relative abundance of *Pantoea* and *Leclercia* than the other treatments. This could mean that applying microbial inoculants or changes in anaerobic conditions have altered fermentation dynamics. *Pantoea*, often found on plant surfaces, has pathogenic potential and are tolerant to oxidative conditions. They can thrive when the ensilage process is slow or does not reach anaerobic conditions quickly (Duchateau et al. 2024). Members of the Enterobacteriaceae family, *Citrobacter* and *Erwinia*, are known to take part in plant fermentation; however, they can also compete with Lactic Acid Bacteria (LAB) for available sugars, which could have an impact on silage fermentation efficiency (Guan et al. 2020).

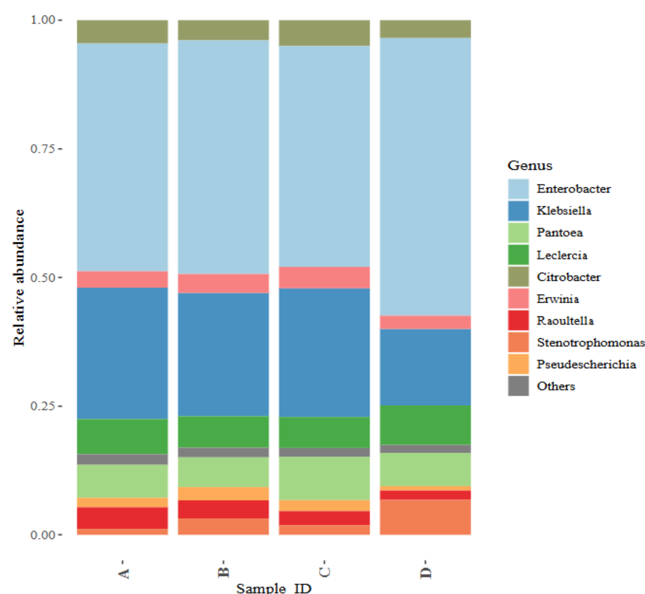

Figure 3. Top 10 distribution of bacterial abundance on the silage at the taxonomic-genus: A. Indigofera; B. Indigofera + LAB (*L. plantarum*); C. Indigofera + extract (*Acacia mangium* bark); D. Indigofera + LAB + extract

A more stable fermentation process, marked by a decrease in the dominance of potentially harmful bacteria like *Enterobacter* and *Klebsiella*, is suggested by the higher microbial diversity in treatment D compared to the other treatments. According to earlier research, LAB inoculation can improve fermentation quality by preventing the growth of unwanted enteric bacteria (McGarvey et al. 2013). Additionally, treatment D's higher percentage of bacteria like *Pantoea* and *Leclercia* might point to a more balanced microbial ecosystem, which over time might improve the stability of silage. Consequently, in contrast to the other treatments, which were still primarily controlled by *Enterobacter*. Furthermore, the presence of high numbers of *Enterobacter* is often associated with protein degradation, due to its ability to deaminate the amino acids into ammonia (NH_3), thus reducing the native protein content and increasing the proportion of Non-Protein Nitrogen (NPN) in silage (Wang et al. 2024).

The presence of the genus *Klebsiella* is often associated with suboptimal fermentation. A study by Liu et al. (2024) showed that genes related to butyrate production, such as butyryl-CoA: acetate CoA-transferase (but), were mainly associated with *Klebsiella oxytoca* and *Escherichia coli* in alfalfa silage without sucrose addition. In contrast, the lactate dehydrogenase gene related to lactate production was more dominant in the group with sucrose addition and was associated with *Lactobacillus*. *Klebsiella* was least detected in treatment D compared to other treatments. This supports the synergistic role between LAB and phytobiotic extracts in supporting the fermentation process of high-protein legume feed.

Relative abundance of species

According to Figure 4, species-level microbial community analysis, the *Enterobacter cloacae* complex predominated in all treatments. However, it was especially prevalent in treatments A, B, and C, comprising over half of the bacterial population. A facultative anaerobic bacterium frequently found in silage fermentation, *E. cloacae* is renowned for its ability to adapt to various environmental stressors, including less-than-ideal fermentation conditions (Ni et al. 2015). Given that these opportunistic bacteria can compete with Lactic Acid Bacteria (LAB) for fermentation substrates, the high prevalence of the *E. cloacae* complex in silage may be a sign of less than ideal fermentation (Guan et al. 2020).

Furthermore, as shown in Figure 4, in addition to the *E. cloacae* complex, several other species were found in considerable quantities in all treatments, including *Klebsiella pneumoniae*, *Klebsiella variicola*, and *E. cloacae*. Because *K. pneumoniae* is an opportunistic pathogen that can produce unwanted metabolites like ethanol and gases, which may compromise silage stability, its relatively high abundance is concerning (McGarvey et al. 2013). Smaller amounts of *Leclercia pneumoniae* were also found, indicating the presence of various fermentative bacteria in the silage. It is believed that *L. pneumoniae* contributes to secondary fermentation processes and is frequently found in anaerobic settings (He et al. 2018).

Along with a relative rise in other species like *Enterobacter quasimoraechei*, *Enterobacter chuandaensis*, and *Klebsiella* spp., treatment D showed a decrease in the *E. cloacae* complex. This change implies that treatment D may have established conditions favouring microbial diversification, resulting in more stable and higher-quality fermentation. Prior research has shown that using LAB inoculants can promote advantageous fermentative bacteria's dominance by inhibiting opportunistic bacteria growth like *Enterobacter* spp. and *Klebsiella* spp. (He et al. 2018). Treatment D's decrease in *E. cloacae* complex dominance is consistent with research showing that a balanced and diverse microbial community is generally linked to more stable fermentation (Ni et al. 2015).

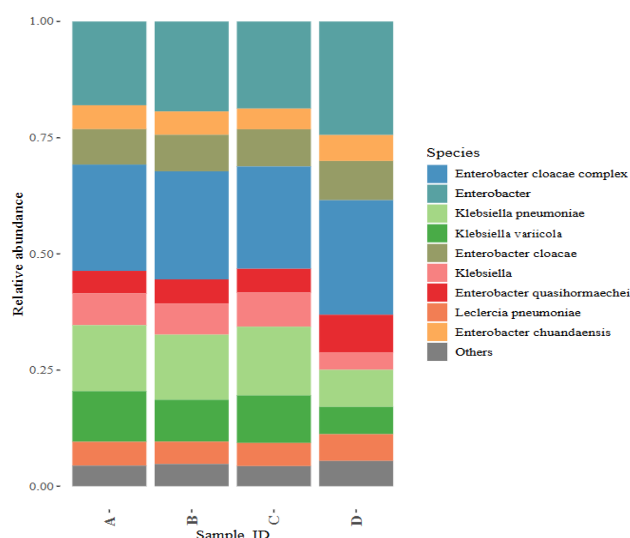

Figure 4. Top 10 distribution of bacterial abundance on the silage at the taxonomic-species: A. Indigofera; B. Indigofera + LAB (*L. plantarum*); C. Indigofera + extract (*Acacia mangium* bark); D. Indigofera + LAB + extract

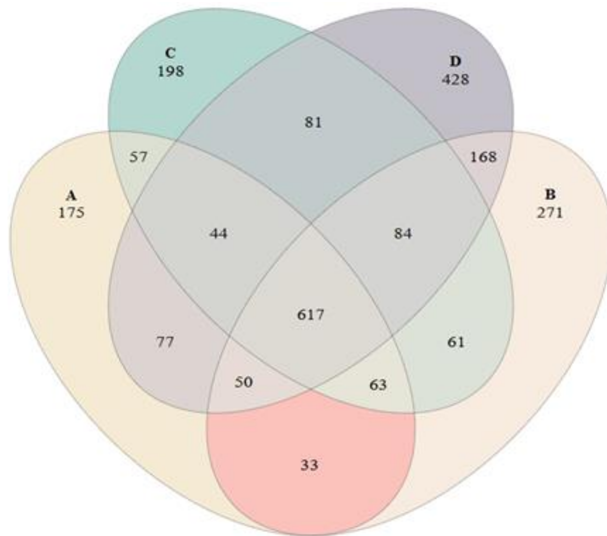

Figure 5. Venn diagram of the bacteria's abundant: A. Indigofera; B. Indigofera + LAB (*L. plantarum*); C. Indigofera + extract (*Acacia mangium* bark); D. Indigofera + LAB + extract

Overall, treatment D exhibited greater microbial diversity compared to the other treatments, with a reduction in *E. cloacae* complex dominance and an increase in other species that may contribute to a more stable silage fermentation process. The enhanced microbial diversity in treatment D suggests a more optimal fermentation process, likely due to a decline in the proportion of opportunistic bacteria that compete with LAB. This finding underscores the importance of effective silage fermentation management strategies, such as the use of LAB inoculants and strict anaerobic conditions, to enhance silage quality and inhibit the proliferation of spoilage-associated bacteria (Guan et al. 2020).

Venn diagram of microbial abundance

Microbial abundance in different treatment groups is impacted by the treatments applied to legume silage, according to the results of the bacterial abundance interactions analysis presented in a Venn diagram (Figure 5). The Venn diagram shows that 271 distinct microbial species were found in the treatment supplemented with Lactic Acid Bacteria (LAB) (B). In comparison, 175 microbial species were found exclusively in the control treatment (A). The combination of LAB and tannin extract (D) produced the most significant number of unique species, reaching 428, whereas the treatment containing tannin extract (C) contained 198 distinct species. All treatments shared a total of 617 microbial species, indicating the existence of a core microbial community that stayed constant despite treatment changes. Microbial diversity was higher in the LAB-containing treatments (B) and the LAB-tannin extract combination (D) than in the control. This pattern is consistent with other studies showing that LAB inoculation increases the abundance of advantageous fermentative microorganisms, like *Lactobacillus* species, which are essential for enhancing fermentation quality and preventing the growth of harmful bacteria (Muck et al. 2018).

However, the effects of adding tannin extract to treatments C and D variants, with treatment D showing the highest number of microbial species. Because of their selective antimicrobial qualities, tannins can inhibit the growth of some bacterial populations while encouraging the growth of other fermentative microorganisms (Ozogull et al. 2025). It is suggested that each treatment creates a unique microbial niche when exclusive microbial species are found in particular treatments, such as 168 species unique to treatment B and 198 species unique to treatment C. The high number of unique species in treatment C may be explained by tannin extract's ability to suppress proteolytic bacteria while fostering the dominance of bacteria more tolerant to phenolic compounds (Huang et al. 2023). In comparison to separate treatments, the synergistic effect of LAB and tannin extract in treatment D probably increased microbial diversity. The quality of the silage and fermentation stability may be improved by this greater diversity.

Therefore, the combination of LAB and tannin extract (treatment D) appears to be the most effective strategy for enhancing the abundance and diversity of beneficial microbes in legume silage. Additionally, Ridwan et al. (2019) examined how bacterial populations in silage influence the abundance of rumen bacteria, including methanogenic species. Further research is needed to clarify the specific interactions between fermentative microbes and the bioactive compounds present in tannin extracts, with the aim of optimizing inoculant formulations to enhance silage quality.

Alpha diversity analysis

The alpha diversity analysis of the microbial communities in silage, as illustrated in Figure 6, revealed significant differences among the different diversity indices, including Observed OTUs, Chao1, ACE, Shannon, Simpson, Inverse Simpson, and Fisher. Sample D had the most species, while sample A had the fewest, based on the Observed OTUs index, which counts the species present in each sample. Similarly, the Chao1 and ACE indices, which determine total species richness, indicated that sample D had greater microbial abundance than the other samples.

The Shannon and Simpson indices, which gauge microbial diversity by taking into account species distribution within the community, further corroborated Sample D's higher diversity compared to the other samples. This suggests that the microbial ecosystem in this silage was more stable and varied. However, Sample A's diversity values were lower, indicating that a small number of dominant species predominated. The Inverse Simpson and Fisher indices displayed a similar pattern, supporting the conclusion that the microbial population in sample D was the most diverse of all treatments.

Variations in microbial diversity during silage production are likely caused by a number of factors, including nutrient composition, pH levels, inoculant application, and fermentation conditions (Guo et al. 2022). Increased microbial diversity is commonly associated with improved fermentation stability and silage quality because a balanced microbial population can help prevent the growth of pathogenic or spoiling organisms (Bai et al. 2020). Therefore, monitoring silage's microbial diversity is essential to ensuring a flawless fermentation process and producing high-quality animal feed.

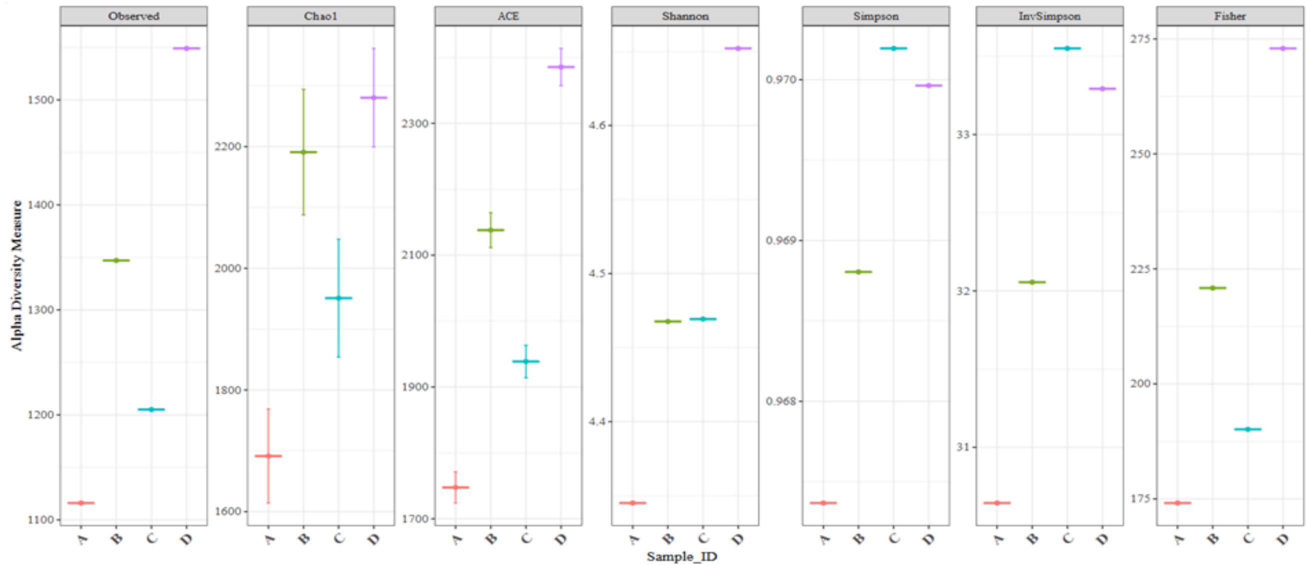

Figure 6. Alpha diversity analysis of silage microbial communities: A. Indigofera; B. Indigofera + LAB (*L. plantarum*); C. Indigofera + extract (*Acacia mangium* bark); D. Indigofera + LAB + extract

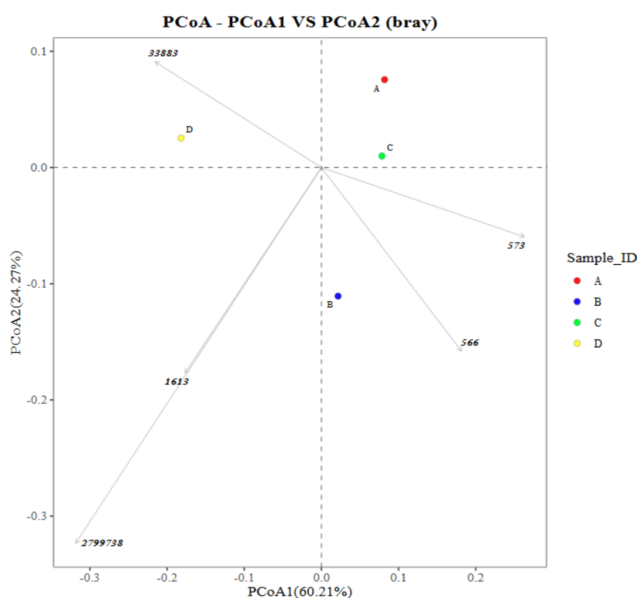

Figure 7. Principal coordinates analysis of silage microbial community: A. Indigofera; B. Indigofera + LAB (*L. plantarum*); C. Indigofera + extract (*Acacia mangium* bark); D. Indigofera + LAB + extract

Principal Coordinates Analysis (PCoA)

The results of Principal Coordinates Analysis (PCoA) based on the Bray-Curtis index, which are shown in Figure 7, showed that the silage samples' microbial community composition varied significantly. Together, they captured 84.48% of the variance, with the first principal coordinate (PCoA1) explaining 60.21% and the second (PCoA2) explaining 24.27%. This implies that these two dimensions can be used to effectively visualise the main variations in microbial structure between samples. The microbial profiles of samples A (red) and C (green) were similar, while samples B (blue) and D (yellow) showed more pronounced differences. The diagram's grey vectors stand for particular microbial taxa that are responsible for the observed variations, such as *Lactobacillus, Enterobacter*, and *Clostridium*, which are important for the fermentation and stability of silage (Guo et al. 2022).

Environmental variables like pH and moisture content, inoculant application, and fermentation conditions are probably some of the factors that affect the variation in microbial composition. *Lactobacillus* and other beneficial fermentative bacteria increase the production of lactic acid, which raises the quality of silage. Conversely, the presence of *Clostridium* and *Enterobacter* could indicate less than ideal fermentation, which could result in unwanted metabolic byproducts (Bai et al. 2020). Sample D's unique clustering indicates that its microbial community evolved differently, perhaps as a result of variations in raw materials or storage circumstances. Based on the results of a meta-analysis conducted by Ridwan et al. (2023), differences in raw materials as substrates also affect silage bacterial reports. These microbial differences are important because they can have a direct effect on the anaerobic stability, nutrient composition of silage, and overall livestock feed safety (Xu et al. 2021).

Sunburst Krona analysis

The population of *Clostridium* bacteria, as represented by four sunburst diagrams from Krona analysis, is one of the key parameters to be communicated from the study (Figure 8). Discussing this is crucial because silage from high-protein legume plants like *I. zollingeriana* may not ferment as well as it should if it is not managed, particularly if bacteria from the *Clostridia* class grow and produce toxic substances like butyrate and ammonia (Susanto et al. 2025). The taxonomic analysis of the microorganisms shown in the sunburst diagram indicates that the *Clostridia* population varies significantly across the four Indigofera silage treatments.

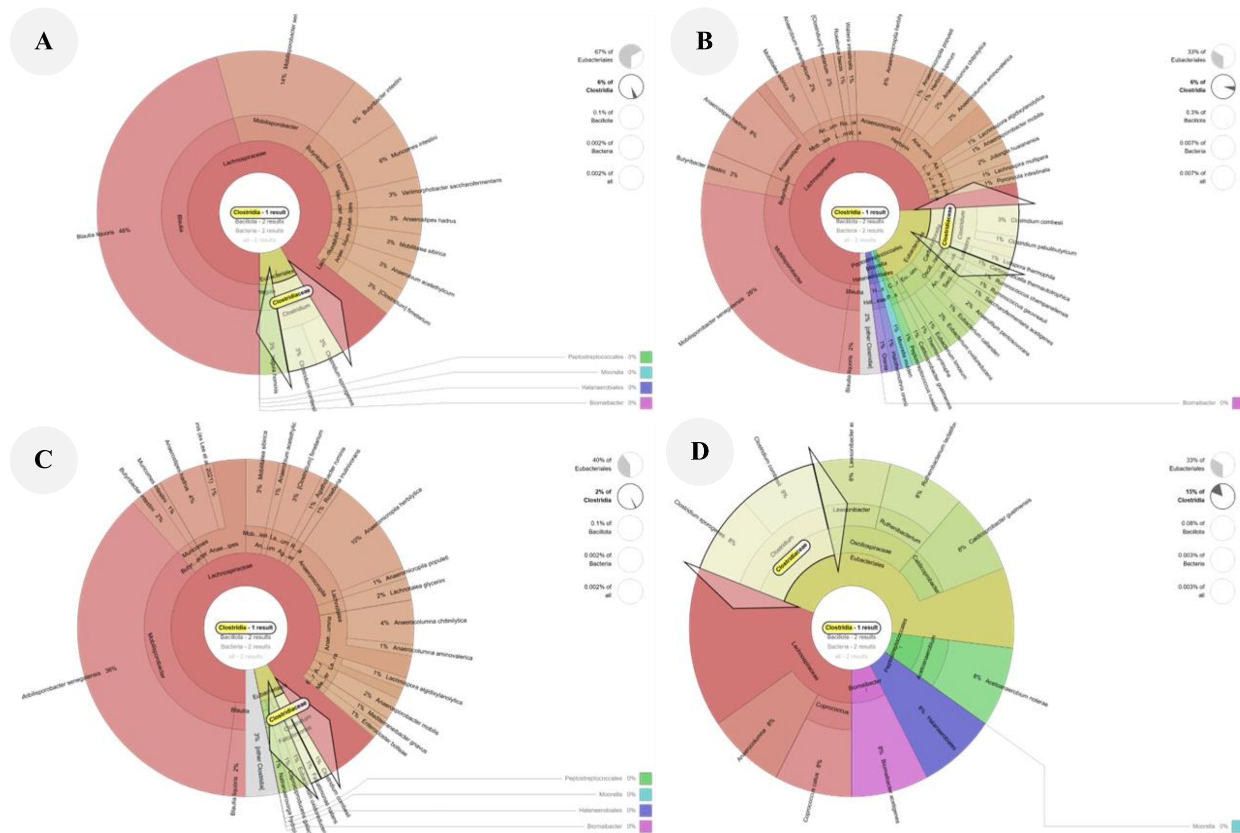

Figure 8. Sunburst Krona analysis of *Clostridia* abundance on silage microbial community: A. Indigofera; B. Indigofera + LAB (*L. plantarum*); C. Indigofera + extract (*Acacia mangium* bark); D. Indigofera + LAB + extract

Clostridium makes up about 6% of the entire microbial population, according to Figure 8.A, suggesting a minor but noticeable presence. This is consistent with findings that *Clostridium* growth is generally supported by anaerobic conditions with high protein levels (Kung et al. 2018). Since *Clostridia* are obligate anaerobes that may harm the ensilage process, it is necessary to monitor this population. Because *Clostridium* ferments amino acids into ammonia, butyrate, and other odorous compounds, even trace amounts of this species in silage can degrade its quality. Butyrate and other *Clostridium* metabolic products can decrease feed palatability, increase energy loss, and raise the possibility of toxin contamination of silage (Susanto et al. 2025). Consequently, the presence of *Clostridium* is still a significant indicator in determining the quality of silage, despite the small proportion.

Even though *Clostridium* is still found in the Clostridiaceae family (Clostridia class), Figure 8.B with *L. plantarum* supplementation demonstrates that the proportion is only about 6% or even lower at the genus level, where the genera *Clostridium*, *Lachnoclostridium*, and *Ruminiclostridium* are present. This suggests that probiotic supplementation has caused a change in the microbial composition. By producing lactic acid, *L. plantarum* supplementation is known to hasten the pH drop during ensilage, thereby reducing the growth of facultative anaerobic microbes like *Clostridium* spp. (Muck et al. 2018). In the early phases of the ensilage process, these probiotics dominate fermentative activity, compete for substrate consumption, and produce antimicrobial compounds like bacteriocins that can stop the growth of harmful bacteria like *Clostridium* (Dong et al. 2022).

Compared to probiotics, adding acacia extract (Figure 8.C), which contains antimicrobial tannins and phenolic compounds, effectively suppresses *Clostridia*, although the population is only about 2%. This demonstrates that supplementing with acacia phytobiotics effectively inhibits *Clostridium* growth during the ensilage process. It is well known that acacia extract contains a high concentration of secondary metabolite compounds, particularly condensed tannins (proanthocyanidins), which have potent antibacterial effects against Gram-positive bacteria like *Clostridium* spp. According to Jayanegara et al. (2018), tannins precipitate proteins, break down cell walls, and block vital enzymes that microbes require to proliferate. This antimicrobial effect aids in preventing proteolytic fermentation and producing substances such as butyric acid and ammonia which impair silage quality during the fermentation process. Because *Clostridium* spores are so resilient to harsh environmental factors, such as low pH, small amounts of *Clostridium* can still be found. However, these findings suggest that acacia extract can inhibit *Clostridium* germination and metabolic activity, particularly during the early stages of silage when the pH of the silage is still relatively high (Sinaga et al. 2021).

Unique results were found in Figure 8.D, where the population results of *Clostridia* were higher than other treatments, which were 15%. The high presence of *Clostridia* reflects the failure of the combination strategy in suppressing microorganisms that play a role in proteolytic fermentation and the formation of harmful butyric acid. This is believed to happen because the extract compound present prevents the probiotic *L. plantarum*, which should be able to control lactic acid fermentation in the early stages of this ensilage, from functioning at its best. The growth of *Clostridium* will use a process that does not result in a sharp drop in pH. However, phytobiotics derived from acacia extract, abundant in secondary metabolite compounds like tannins, prevent bacterial cell membrane integrity, prevent enzymatic activity, and precipitate microbial proteins (Sinaga et al. 2021). As a result, probiotics and phytobiotics do not work very well together.

An anaerobic environment that prevents *Clostridium* from growing further is produced by the combination of tannin activity and the pH drop brought on by the predominance of lactic acid bacteria. In addition, using acacia-based phytobiotics can also increase the dominance of beneficial fermentative microbes such as *Lactobacillus* or members of Lachnospiraceae. This change suggests that, depending on the dosage and kind of tannin, tannins can also modulate the microbial communities in rumen or silage and act as antimicrobials (Lazzari et al. 2023). Strategies to enhance fermentation quality and reduce nutrient losses during silage storage should consider this. Overall, this figure confirms that adding acacia extract to Indigofera silage effectively inhibits the growth of *Clostridium*, preserves microbiological stability, and enhances fermentation quality. This method offers a bio-based, sustainable feed fermentation management solution.

Metabolite pathway overview

Phenylpropanoid biosynthesis (KEGG ID: MAP01061) is a secondary metabolic process in plants that yields a variety of phenol derivative compounds, including flavonoids, tannins, and several other derivative compounds that are abundant in acacia extract. Figure 9 provides an overview of the pathway metabolite analysis of this biosynthesis. Based on molecular-level data, particularly large-scale molecular data sets produced by genome sequencing, KEGG is database resource for comprehending the purpose and usefulness of biological systems, including cells, organisms, and ecosystems (Kanehisa and Goto 2000). Metabolites from this pathway are crucial to the fermentation of silage feed because they contribute to the stability and preservation of nutrients, particularly those involved in protein deamination during the ensilage process (the anaerobic storage of green feed).

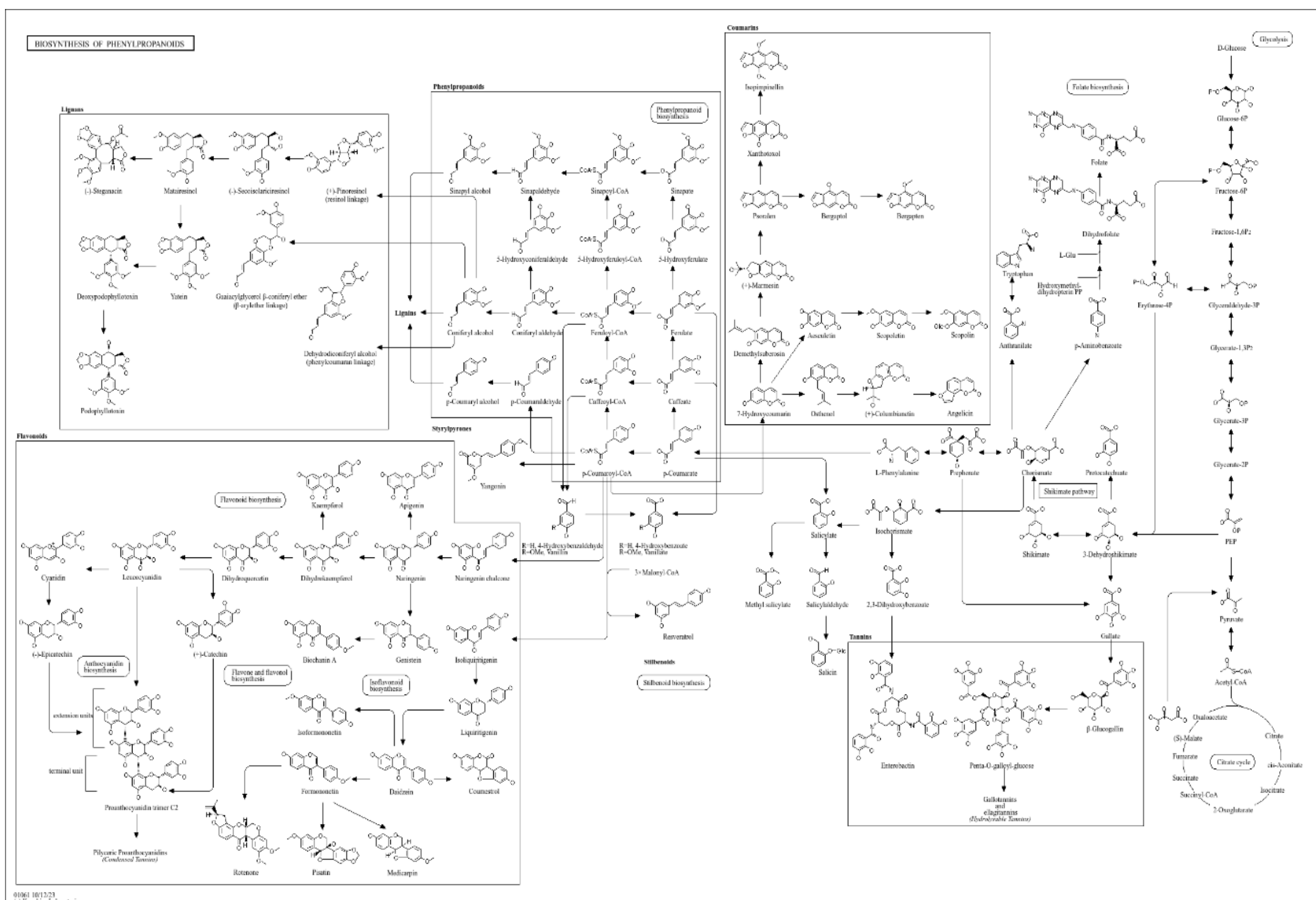

Figure 9. Metabolite pathway of bioactive compound specific biosynthesis of phenylpropanoids (KEGG ID: MAP01061)

Proteolytic enzymes from microorganisms and plants cause protein deamination of feed during silage fermentation, resulting in ammonia and free nitrogen compounds that can lower feed quality. The degradation of crude proteins during silage storage can be lessened by phenolic metabolites, such as p-coumaric acid, and flavonoid and tannin derivatives, which are known to inhibit the activity of protease and deaminase enzymes. According to Makkar (2003), phenylpropanoid compounds are antimicrobial against spoilage bacteria and can inhibit proteolytic enzymes, which could help lower the formation of ammonia.

Additionally, the astringent tannin compounds (such as enthebactin, gallotanin, and pentagalloylglucose) in the lower right portion of the image are capable of binding proteins to form tannin-protein complexes that are impervious to microbial degradation (Ozogull et al. 2025). This complex increases the efficiency of protein use by decreasing the amount of protein available for deamination during fermentation (Jayanegara 2018; Susanto et al. 2025). Tannins can therefore aid in shielding proteins from excessive deamination during the fermentation process of silage. Phenolic compounds and lignin from the phenylpropanoid pathway are crucial for preserving protein quality during silage storage, according to studies by Makkar (2003). To increase the quality and stability of silage, they recommended using feed that contains a lot of phenolic secondary metabolites or adding additives like tannins.

According to Dias et al. (2021), flavonoids and compounds like vanillin and coumarin, which are the transformation products of phenolic acid, are also known to have antimicrobial activity. They may inhibit the growth of *Clostridium* bacteria and other microorganisms that cause excessive protein degradation. In addition to promoting the dominance of more advantageous fermentative microorganisms like *L. plantarum*, this mechanism is crucial for preserving nitrogen stability during silage storage. Furthermore, lignin and its derivatives, which are also derived from the phenylpropanoid pathway, physically fortify plant cell walls, increasing the protection of proteins from microbial degradation, particularly in high-fibre forages like legumes and elephant grass. This is supported by the results of a meta-analysis conducted by Irawan et al. (2021), where LAB inoculation containing fibrolytic enzymes was able to improve silage quality.

In conclusion, microbial community alterations can significantly affect fermentation results and the general quality of silage. Animal health may be at risk due to the production of toxic compounds and the alteration of organic acid profiles caused by the dominance of specific microorganisms in a sample. To maximise fermentation processes and lower the possibility of pathogenic bacterial contamination, genomic-based monitoring of microbial populations is essential. Fermentation conditions, the kind of bacterial inoculants used, the source of the extract, substrate characteristics, and storage conditions are the primary factors affecting these microbial variations. Furthermore, this pathway should be used in silage quality improvement strategies because phenylpropanoid metabolites are crucial in controlling protein stability during silage fermentation, either by complexing with proteins, inhibiting deaminase enzymes, or controlling spoilage and *Clostridium* microbes.

ACKNOWLEDGEMENTS

This research was funded by the Directorate General of Higher Education, Ministry of Education, Culture, Research, and Technology under the PMDSU program, by grant number 001/E5/PG.02.00/PL.PMDSU/2024, year 2024. The authors acknowledge to Research Center for Applied Zoology, BRIN's facilities, scientific, and technical support through E-Layanan Sains Genomic Cibinong-Indonesia, National Research and Innovation Agency.

REFERENCES

Adawiyah A, Meryandini A, Ridwan R, Fidriyanto R, Sarwono KA, Wiryawan KG. 2025. The rumen microbiome and metabolome profile of Ongole crossbreed cattle fed probiotics and protected amino acids. Trop Anim Health Prod 57: 148. DOI: 10.1007/s11250-025-04400-z.

Arowolo MA, He J. 2018. Use of probiotics and botanical extracts to improve ruminant production in the tropics: A review. Anim Nutr 4 (3): 241-249. DOI: 10.1016/j.aninu.2018.04.010.

Bai J, Xu D, Xie D, Wang M, Li Z, Guo X. 2020. Effects of antibacterial peptide-producing *Bacillus subtilis* and *Lactobacillus buchneri* on fermentation, aerobic stability, and microbial community of alfalfa silage. Bioresour Technol 315: 123881. DOI: 10.1016/j.biortech.2020.123881.

De Coster W, D'Hert S, Schultz DT, Cruts M, van Broeckhoven C. 2018. NanoPack: Visualizing and processing long-read sequencing data. Bioinformatics 34: 2666-2669. DOI: 10.1093/bioinformatics/bty149.

De Gouveia MIM, Bernalier-Donadille A, Jubelin G. 2024. Enterobacteriaceae in the human gut: Dynamics and ecological roles in health and disease. Biology 13 (3): 142. DOI: 10.3390/biology13030142.

Dias MC, Pinto DCGA, Silva AMS. 2021. Plant flavonoids: Chemical characteristics and biological activity. Molecules 26 (17): 5377. DOI: 10.3390/molecules26175377.

Dong J, Li S, Chen X, Sun Z, Sun Y, Zhen Y, Qin G, Wang T, Demelash N, Zhang X. 2022. Effects of *Lactiplantibacillus plantarum* inoculation on the quality and bacterial community of whole-crop corn silage at different harvest stages. Chem Biol Technol Agric 9: 57. DOI: 10.1186/s40538-022-00326-y.

Du Z, Nakagawa A, Fang J, Ridwan R, Astuti WD, Sarwono KA, Sofyan A, Widyastuti Y, Cai Y. 2024. Cleaner anaerobic fermentation and greenhouse gas reduction of crop straw. Microbiol Spectr 12 (7): e0052024. DOI: 10.1128/spectrum.00520-24.

Duchateau S, Crouzet J, Dorey S, Aziz A. 2024. The plant-associated *Pantoea* spp. as biocontrol agents: Mechanisms and diversity of bacteria-produced metabolites as a prospective tool for plant protection. Biol Control 188: 105441. DOI: 10.1016/j.biocontrol.2024.105441.

Durica-Mitic S, Göpel Y, Görke B. 2018. Carbohydrate utilization in bacteria: Making the most out of sugars with the help of small regulatory RNAs. Microbiol Spectr 6 (2): 10.1128/microbiolspec.rwr-0013-2017.

Guan H, Shuai Y, Yan Y, Ran Q, Wang X, Li D, Cai Y, Zhang X. 2020. Microbial community and fermentation dynamics of corn silage prepared with heat-resistant lactic acid bacteria in a hot environment. Microorganisms 8 (5): 719. DOI: 10.3390/microorganisms8050719.

Guo X, Xu D, Li F, Bai J, Su R. 2022. Current approaches on the roles of lactic acid bacteria in crop silage. Microb Biotechnol 16 (1): 67-87. DOI: 10.1111/1751-7915.14184.

Harahap RP, Nahrowi, Suharti S, Jayanegara A, Fakhri S. 2023. Enhancing silage quality of Elephant grass and Indigofera legume using shrimp shell-derived chitin and chitosan additives. Livest Res Rural Dev 35 (11): 100.

He L, Zhou W, Wang Y, Wang C, Chen X, Zhang Q. 2018. Effect of applying lactic acid bacteria and cellulase on the fermentation quality, nutritive value, tannins profile and in vitro digestibility of

Neolamarckia cadamba leaves silage. J Anim Physiol Anim Nutr 102 (6): 1429-1436. DOI: 10.1111/jpn.12965.

Huang R, Ma C, Zhang F, Wang X. 2023. Effects of condensed tannins on bacterial and fungal communities during aerobic exposure of sainfoin silage. Plants 12 (16): 2967. DOI: 10.3390/plants12162967.

Irawan A, Sofyan A, Ridwan R, Hassim HA, Respati AN, Wardani WW, Sadarman, Astuti WD, Jayanegara A. 2021. Effects of different lactic acid bacteria groups and fibrolytic enzymes as additives on silage quality: A meta-analysis. Bioresour Technol Rep 14: 100654. DOI: 10.1016/j.biteb.2021.100654.

Islamov B, Petrova O, Mikshina P, Kadyirov A, Vorob'ev V, Gogolev Y, Gorshkov V. 2021. The Role of *Pectobacterium atrosepticum* exopolysaccharides in plant-pathogen interactions. Intl J Mol Sci 22 (23): 12781. DOI: 10.3390/ijms222312781.

Jayanegara A, Sujarnoko TUP, Ridla M, Kondo M, Kreuzer M. 2018. Silage quality as influenced by concentration and type of tannins present in the material ensiled: A meta-analysis. J Anim Physiol Anim Nutr 103 (2): 456-465. DOI: 10.1111/jpn.13050.

Jayanegara A, Yaman A, Khotijah L. 2019. Reduction of proteolysis of high protein silage from moringa and indigofera leaves by addition of tannin extract. Vet World 12 (2): 211-217. DOI: 10.14202/vetworld.2019.211-217.

Kanehisa M, Goto S. 2000. KEGG: Kyoto Encyclopedia of Genes and Genomes. Nucleic Acids Res 28: 27-30. DOI: 10.1093/nar/28.1.27.

Keshri J, Chen Y, Pinto R, Kroupitski Y, Weinberg ZG, Saldinger SS. 2019. Bacterial dynamics of wheat silage. Front Microbiol 10: 1532. DOI: 10.3389/fmicb.2019.01532.

Kim D, Song L, Breitwieser FP, Salzberg SL. 2016. Centrifuge: Rapid and sensitive classification of metagenomic sequences. Genome Res 26 (12): 1721-1729. DOI: 10.1101/gr.210641.116.

Kung Jr L, Shaver RD, Grant RJ, Schmidt RJ. 2018. Silage review: Interpretation of chemical, microbial, and organoleptic components of silages. J Dairy Sci 101 (5): 4020-4033. DOI: 10.3168/jds.2017-13909.

Lazzari G, Münger A, Eggerschwiler L, Borda-Molina D, Seifert J, Camarinha-Silva A, Schrade S, Zähner M, Zeyer K, Kreuzer M, Dohme-Meier F. 2023. Effects of *Acacia mearnsii* added to silages differing in nutrient composition and condensed tannins on ruminal and manure-derived methane emissions of dairy cows. J Dairy Sci 106 (10): 6816-6833. DOI: 10.3168/jds.2022-22901.

Liu Y, Du S, Sun L, Li Y, Liu M, Sun P, Bai B, Ge G, Jia Y, Wang Z. 2024. Volatile metabolomics and metagenomics reveal the effects of lactic acid bacteria on alfalfa silage quality, microbial communities, and volatile organic compounds. Commun Biol 7: 1565. DOI: 10.1038/s42003-024-07083-8.

Makkar HPS. 2003. Effects and fate of tannins in ruminant animals, adaptation to tannins, and strategies to overcome detrimental effects of feeding tannin-rich feeds. Small Rumin Res 49 (3): 241-256. DOI: 10.1016/S0921-4488(03)00142-1.

Makkiyah FA, Rahmi EP, Mahendra FR, Maulana F, Arista RA, Nurcholis W. 2024. Polyphenol content and antioxidant capacities of *Graptophyllum pictum* (L.) extracts using in vitro methods combined with the untargeted metabolomic study. J Appl Pharm Sci 14 (3): 55-63. DOI: 10.7324/japs.2024.153548.

McAllister TA, Dunière L, Drouin P, Xu S, Wang Y, Munns K, Zaheer R. 2018. Silage review: Using molecular approaches to define the microbial ecology of silage. J Dairy Sci 101 (5): 4060-4074. DOI: 10.3168/jds.2017-13704.

McGarvey JA, Franco RB, Palumbo JD, Hnasko R, Stanker L, Mitloehner FM. 2013. Bacterial population dynamics during the ensiling of *Medicago sativa* (alfalfa) and subsequent exposure to air. J Appl Microbiol 114 (6): 1661-1670. DOI: 10.1111/jam.12179

Muck RE, Nadeau EMG, McAllister TA, Contreras-Govea FE, Santos MC, Kung Jr L. 2018. Silage review: Recent advances and future uses of silage additives. J Dairy Sci 101 (5): 3980-4000. DOI: 10.3168/jds.2017-13839.

Muck RE. 2013. Recent advances in silage microbiology. Agric Food Sci 22 (1): 3-15. DOI: 10.23986/afsci.6718.

Ni K, Wang Y, Cai Y, Pang H. 2015. Natural lactic acid bacteria population and silage fermentation of whole-crop wheat. Asian-Australas J Anim Sci 28 (8): 1123-1132. DOI: 10.5713/ajas.14.0955.

Nygaard AB, Tunsjø HS, Meisal R, Charnock C. 2020. A preliminary study on the potential of Nanopore MinION and Illumina MiSeq 16S rRNA gene sequencing to characterize building-dust microbiomes. Sci Rep 10: 3209. DOI: 10.1038/s41598-020-59771-0.

Okoye CO, Wang Y, Gao L, Wu Y, Li X, Sun J, Jiang J. 2023. The performance of lactic acid bacteria in silage production: A review of modern biotechnology for silage improvement. Microbiol Res 266: 127212. DOI: 10.1016/j.micres.2022.127212.

Ozogull Y, Ucar Y, Tadesse EE, Rathod N, Kulawik P, Trif M, Esatbeyoglu T, Ozogul F. 2025. Tannins for food preservation and human health: A review of current knowledge. Appl Food Res 5 (1): 100738. DOI: 10.1016/j.afres.2025.100738.

Pahlow G, Muck RE, Driehuis F, Elferink SJWHO, Spoelstra SF. 2003. Microbiology of ensiling. In: Buxton DR, Muck RE, Harrison JH (eds). Silage Science and Technology. American Society of Agronomy, Madison, Wisconsin. DOI: 10.2134/agronmonogr42.c2.

Ridwan R, Abdelbagi M, Sofyan A, Fidriyanto R, Astuti WD, Fitri A, Sholikin MM, Rohmatussolihat, Sarwono KA, Jayanegara A, Widyastuti Y. 2023. A meta-analysis to observe silage microbiome differentiated by the use of inoculant and type of raw material. Front Microbiol 14: 1063333. DOI: 10.3389/fmicb.2023.1063333.

Ridwan R, Rusmana I, Widyastuti Y, Wiryawan KG, Prasetya B, Sakamoto M, Ohkuma M. 2019. Bacteria and methanogen community in the rumen fed different levels of grass-legume silages. Biodiversitas 20 (4): 1055-1062. DOI: 10.13057/biodiv/d200417.

Ridwan R, Rusmana I, Widyastuti Y, Wiryawan KG, Prasetya B, Sakamoto M, Ohkuma M. 2015. Fermentation characteristics and microbial diversity of tropical grass-legumes silages. Asian-Australas J Anim Sci 28 (4): 511-518. DOI: 10.5713/ajas.14.0622.

Saini A, Anggarwal NK, Sharma A, Yadav A. 2015. Actinomycetes: A Source of Lignocellulolytic Enzymes. Enzyme Res 2015: 279381. DOI: 10.1155/2015/279381.

Satam H, Joshi K, Mangrolia U, Waghoo S, Zaidi G, Rawool S, Thakare RP, Banday S, Mishra AK, Das G, Malonia SK. 2023. Next-generation sequencing technology: Current trends and advancements. Biology 12 (7): 997. DOI: 10.3390/biology12070997.

Sinaga DM, Laconi EB, Jayanegara A. 2021. Inhibitory effect of tannin extract from Acacia bark (*Acacia mangium* Willd.) against gastro-intestinal pathogenic bacteria. J Phys: Conf Ser 1869: 012010. DOI: 10.1088/1742-6596/1869/1/012010.

Stefańska B, Sroka J, Katzer F, Goliński P, Nowak W. 2020. The effect of probiotics, phytobiotics and their combination as feed additives in the diet of dairy calves on performance, rumen fermentation and blood metabolites during the preweaning period. Anim Feed Sci Technol 272: 114738. DOI: 10.1016/j.anifeedsci.2020.114738.

Susanto I, Mahendra FR, Rahmadani M, Ridwan R, Jayanegara A. 2025. Potential of cumin essential oil as inhibitor of deamination during ensiling process: A meta analysis and in-silico approach. Bio Web Conf 155: 10008. DOI: 10.1051/bioconf/202515510008.

Tennant RK, Sambles CM, Diffey GE, Moore KA, Love J. 2017. Metagenomic analysis of silage. J Vis Exp 2017 (119): 54936. DOI: 10.3791/54936.

Utama C, Sulistiyanto B, Sumarsih S. 2021. Chemical and microbiological quality of phytobiotics from blends plant with different fermentation ripening. Online J Anim Feed Res 11 (5): 171-179. DOI: 10.51227/ojafr.2021.26.

Wang Y, Sun Y, Huang K, Gao Y, Lin Y, Yuan B, Wang X, Xu G, Nussio LG, Yang F, Ni K. 2024. Multi-omics analysis reveals the core microbiome and biomarker for nutrition degradation in alfalfa silage fermentation. mSystems 9 (11): e00682-24. DOI: 10.1128/msystems.00682-24.

Wening S, Setiowati RD, Pratiwi DR, Pangaribuan IF, Ginting EN. 2024. Bacterial diversity indifferent growing mediums of oil palm seedlings associated with potassium use efficiency. Biodiversitas 25 (8): 3386-3393. DOI: 10.13057/biodiv/d250809.

Wick RR, Judd LM, Holt KE. 2019. Performance of neural network basecalling tools for Oxford Nanopore sequencing. Genome Biol 20 (1): 129. DOI: 10.1186/s13059-019-1727-y.

Xu D, Wang N, Rinne M, Ke W, Weinberg ZG, Da M, Bai J, Zhang Y, Li F, Guo X. 2021. The bacterial community and metabolome dynamics and their interactions modulate fermentation process of whole crop corn silage prepared with or without inoculants. Microb Biotechnol 14 (2): 561-576. DOI: 10.1111/1751-7915.13623.

Xue Y, Wu N, Na N, Sun J, Sun L, Qili M, Li D, Li E, Yang B. 2024. Dynamics of gas and greenhouse gases production during fermentation of barley silage with lactic acid bacteria. Chem Biol Technol Agric 11: 82. DOI: 10.1186/s40538-024-00606-9.