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Original Research Articles

Microbial-environmental interactions reveal the evaluation of fermentation time on the nutrient properties of soybean meal

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Abstract

Microbial fermentation techniques are often used to improve their quality, where the keys are fermentation strains and fermentation time. This study studied the interaction between microbiota and environmental (or nutritional) factors and microbiota at different fermentation times to determine the most appropriate time, using lactic acid bacteria as fermentation strains. It can be concluded that fermentation improved the nutritional value of soybean meals. In the early stages of fermentation, debris in soybean meal highly proliferated and destabilized the microbial community, while pH and nutritional conditions played an important role in helping its stabilization. In addition, we must pay attention to the interspecific interactions of microorganisms, which makes it easy to understand how the microbial community maintains community stability. A 4-day fermentation of soybean meal with *Lactobacillus* is recommended.

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Introduction

With the vast market demand, the scale of animal production is gradually rising alongside a marked increase in demand for feed, especially making protein sources increasingly available (Kim, et al., 2019). Raw materials are mainly divided into animal-derived, plant-derived, and single-cell proteins. Among these, fishmeal is a high-quality protein source commonly used in aquafeeds. However, due to limited resources and high prices, plant-derived or other low-cost proteins are widely employed (Montoya-Camacho, et al., 2019).

Soybean meal, a by-product obtained from soybean oil extraction from soybeans, has been developed as a very widely used plant protein source in the aquafeed industry (Hossain, et al., 2015). Compared to animal protein sources such as fishmeal, soybean meal is widely available, relatively low-cost, and less susceptible to contamination by pathogenic bacteria. The use of soybean meal as a partial replacement for fishmeal has become a research hotspot in aquatic animal nutrition and feed (Wirtz, et al., 2022). However, there are still shortcomings in the efficiency of the use of these protein sources by aquatic animals, mainly because some of the disadvantages of protein sources (including phytic acid, oligosaccharides, anti-nutritional factors, such as trypsin inhibitors and urease, poor palatability and low digestibility and absorption) have not been fully overcome in the actual feed production (Chen and Chen, 2021; Schedle, et al., 2013), which reduces the nutritional value of soybean meal and leads to wasting protein sources. Therefore, it has become a common problem for the aquafeed industry to comprehensively improve the efficiency of existing protein and improve the utilization of new low-cost protein to achieve high-value utilization of aquafeed. Wheat bran, a widely used feed ingredient, can also be added to concentrates appropriately (Renato Balandran-Quintana, et al., 2015). The content of dietary fiber in wheat bran is more than 50% of the total weight of wheat bran, of which more than 90% is insoluble dietary fiber (Hell, et al., 2014). Insoluble dietary fiber in wheat bran promotes intestinal motility and improves the composition of the intestinal flora of livestock.

Recently, microbial fermentation technology has been widely used in the high-value application of feed ingredients (Li, et al., 2015; Marcellin, et al., 2022; Vethathirri, et al., 2021). Microbial fermentation can promote the degradation of some polysaccharides, proteins, and fats in feed, reducing the level of anti-nutritional factors in feed, thus improving the immune function and maintaining the balance of the intestinal flora (Mukherjee, et al., 2016). In addition, feed fermentation can form a unique aroma, organic acids, soluble peptides, monosaccharides, and other small molecules easily digested and absorbed by animals (Zdunczyk, et al., 2015). Research into fermented soybean meal as a protein ingredient in aquaculture has gradually increased in recent years. Fermented baits have a variety of beneficial effects on fish, such as promoting the colonization of beneficial bacteria in the host gut, improving the morphological structure of the gut, promoting intestinal development, and improving production performance.

Fermentation time and strains are at the heart of microbial fermented feeds and play a decisive role in the application of fermented feeds (Li, et al., 2021). Long or too short a fermentation time is not conducive to highlighting the nutritional value of fermented feed. It may even undermine its existence, making animal production disadvantageous (Iram, et al., 2021). Some studies have shown that as the fermentation time changes, the crude protein content, as well as small peptides and free amino acids (Bratosin, et al., 2021). Too long a fermentation will lead to the accumulation of lactic acid and other substances, and the enzyme production of the bacterium will reach its maximum after entering the plateau period. The continued fermentation may affect the production and activity of protease (Emkani, et al., 2022). The fermentation strain used in this study is *Lactobacillus*, which are commonly used microbial fermentation strains and are relatively important probiotics (Uchida, Miyoshi, 2013; Widyastuti, et al., 2021). *Lactobacillus* can perform a regular metabolic activity in the body, breaking down proteins and sugars in food and synthesizing vitamins and fats (Adesulu-

Dahunsi, et al., 2022). With the action of *Lactobacillus* proteases, the large molecules of protein in food are partially degraded into small molecules of peptides and essential amino acids that can be used directly by the host, significantly increasing the digestibility and biomass value of feed and promoting gastrointestinal absorption. Lactose in food can be converted into glucose and galactose after fermentation by *Lactobacillus*, which can then be transformed into small molecule compounds such as lactic acid, which can be easily digested. In this experiment, Lactococcus lactis 17 was used as fermenting strain to ferment soybean meal. The microbial community diversity in the soybean solid-state fermentation system was investigated at six different fermentation times (0 d, 3 d, 4 d, 5 d, 6 d, and 7 d). The relationship between the quality of soybean fermented feedstuff and the microbial community structure was investigated, and the sensory evaluation and physicochemical properties of the fermented soybean meal were analyzed to provide a reference for the industrialization of functional fermented soybean meal bait for aquatic animals.

Materials and Methods

Experimental feedstock and strain

Soybean meal was purchased from Shandong Hoguan Food Co., Ltd., and wheat bran was purchased from e-commerce platforms. Among them, soybean meal was crushed using a grinder and passed through a 60-mesh sieve to take the sieve offsets for use.

The experimental strain was *Lactobacillus plantarum*, named *Lactobacillus plantarum* ATCC8014, collected and conserved at Shanghai Biotechnology Center for Conservation (SHBCC), accession number SHBCC D24318. Before being used, the experimental strain was cultured in MRS (Shanghai Maclean's Biotechnology Co., Ltd.) medium, beef paste peptone medium, and PDA medium, which were sterilized by moist heat at 120℃ for 20 min, and incubated at 30℃ for 23h, 12h, 16h and 22h at 150 rpm, respectively, when they were at the initial stage of stabilization and their CFU were kept greater than 10^8. (Baohu et al., 2021)

Fermentation and sampling

Solid-state fermentation was carried out in a fermentation breathing bag, and the volume used for fermentation was 1 kg. Soybean and wheat bran were mixed and bagged in a ratio of 8:2 by mass with 200 ml of tap water and 25 ml of culture solution each, sealed with a plastic sealer to expel air, and incubated in a constant temperature incubator at 30°C for 3 d, 4 d, 5 d, 6 d, and 7d.

Samples were taken at the end of the initial mixing and used as samples for 0d of fermentation. After fermentation, samples were selected and placed in EP tubes at -80°C for freezing and storage. Each fermentation group was performed in 7 copies. The remaining samples were placed in a blast drying oven at 60℃ for drying treatment for 6h to constant weight, crushed and refrigerated.

Chemical analysis

The pH of the solution was measured using an electronic pH meter, and the pH was expressed as the adequate acidity of the sample. The sample was accurately weighed 5g (accurate to 0.001g), dissolved in 30ml of trichloroacetic acid for 5min, and filtered. Our previous studies measured the filtrate (Cai, et al., 2021; Cai, et al., 2022a). Analytical methods used for proximate analysis included urease activity (AOCS Official Method Ba 9-58), crude protein (AOAC 990.03), and ash (AOAC 942.05). Fructose, acid-soluble protein, energy, glucose, total phosphorus, and calcium were tested by Shandong New Hope Liuhe Testing Center concerning its internal method.

16S rRNA sequencing and microbial analysis

16S rDNA was extracted from soybean meal using the PowerFecal™ DNA Isolation Kit (MoBio Laboratories, Inc). After amplifying the 16S rRNA V3-V4 region with barcode fusion primers of 515F and 907R, the Illumina MiSeq platform was used for high-throughput sequencing. The raw sequences were sorted into different samples based on barcodes using the BIPES pipeline, and then chimeric sequences were filtered using UCHIME(Edgar et al., 2011). After removing low-quality scores with FASTX-Toolkit, all sequences were classified into operational taxonomic units (OTUs) with 100% similarity using QIIME2(Bolyen et al., 2019). Classification hierarchies were assigned to each OTU using uclust. The representative sequences obtained were subjected to multiple alignments using muscle5(Robert and Edgar, 2004) (-super mode), then trimmed afterward using trimAl. A phylogenetic tree was constructed based on the fasta file obtained above. The raw abundance data were later subsampled using the R package micro2eco to filter out the low-abundance OTUs and OTUs occurring within less than four samples are filtered out and combined for subsequent use described previously (Cai, et al., 2022b; Cai, et al., 2022c).

Alpha diversity (number of OTUs, Chao1 index (http://www.mothur.org/wiki/Chao), Shannon index (http://www.mothur.org/wiki/Shannon), and Simpson index (http://www.mothur.org/wiki/Simpson)) and beta diversity (Principal Coordinate Analysis (PCoA) based on Bray–Curtis dissimilarity and Jaccard similarity index distance) were also analyzed. Linear discriminant analysis Effect Size (LEfSe) analysis and linear models for microarray data (limma) analysis were then performed to draw leveled 16S amplicon data to construct S3 objects, obtained by performing Kruskal-Wallis tests (Threshold = 0.05) for each taxonomic stratum, and the LDA score was logged and set to 2.0.

Abundance and random matrix theory (RMT) based on OTUs abundance was used in constructing molecular ecological networks using micro2eco (Liu et al., 2020) to describe interspecific interactions within microbial communities. First, a similarity matrix was obtained using the correlation matrix based on Pearson's coefficient. This similarity matrix measures the degree of agreement in the abundance profiles of individual OTUs in different groups. Secondly, an RMT-based network approach was used to define an appropriate similarity threshold St for the network structure to obtain an adjacency matrix that encodes the strength of the connection between each pair of nodes. Thirdly, submodules within the co-occurrence network module can be detected by fast-greedy optimization (Thyagu, Mehta, 2011). Random networks are generated using the Maslov-Sneppen procedure, which randomly reprograms all connections of the original network by keeping the total nodes and total connections of each node constant (Maslov and Sneppen, 2002). Based on the Z-test, which examines the differences between empirical and random networks in terms of among-module connectivity (*Pi*) and within-module connectivity (*Zi*), the topological roles of different nodes can be divided into four categories: peripheral nodes ($Zi \leq 2.5$, $Pi \leq 0.62$), connected nodes ($Zi \leq 2.5$, $Pi > 0.62$), modular hubs ($Zi > 2.5$, $Pi \leq 0.62$) and network hubs ($Zi > 2.5$, $Pi > 0.62$). The visualization of the network was implemented by R package micro2eco, Circos (Zhang et al., 2013), and Cytoscape(Shannon et al., 2003).

Microbial source tracking analysis was performed via R package feast. Functional prediction of gut microbial communities was performed using Tax4Fun on 16S amplicon data based on individual OTUs compared with reference data from the Silva123 database (Quast, et al., 2013). Statistical differences in the relative abundance of microbial communities were analyzed by R package micro2eco based on a T-test, with $P < 0.05$ being considered significant.

All RNA-seq data from this study can be downloaded through the NCBI Sequence Read Archive (SRA) (http://www.ncbi.nlm.nih.gov/sra) under the BioProject number PRJNA846171.

Results

Microbial community composition

The soybean meal microbial community was investigated using 42 samples from six fermentation time treatments. A total of 1 721 622 high-quality sequences with an average read of 40991 were derived from bacterial profiles after subsampling. 221 bacterial OTUs (bOTUs) were detected in all samples, with over 90% sequencing depth coverage, of which 28 bOTUs were shared in all groups. The number of specific bOTUs originating from the individual group was 1 (M0), 51 (M3), 3 (M4), 16 (M5), 15 (M6), and 9 (M7), respectively (**Figure 1b**). A phylogenetic tree was constructed based on the trimming and alignment of sequences (**Figure 1a**), covering nineteen phyla annotated, with Cyanobacteria, Firmicutes, Proteobacteria, and Actinobacteriota dominating during the whole fermentation period (Figure 1c). *Lactobacillus* and an unidentified genus classified by Cyanobacteria contributed to the dominant classification at the genus level. Moreover, it could be found that the absolute abundance of Firmicutes and *Lactobacillus* was increased after fermentation but decreased with the fermentation time being, which shared a similar trend.

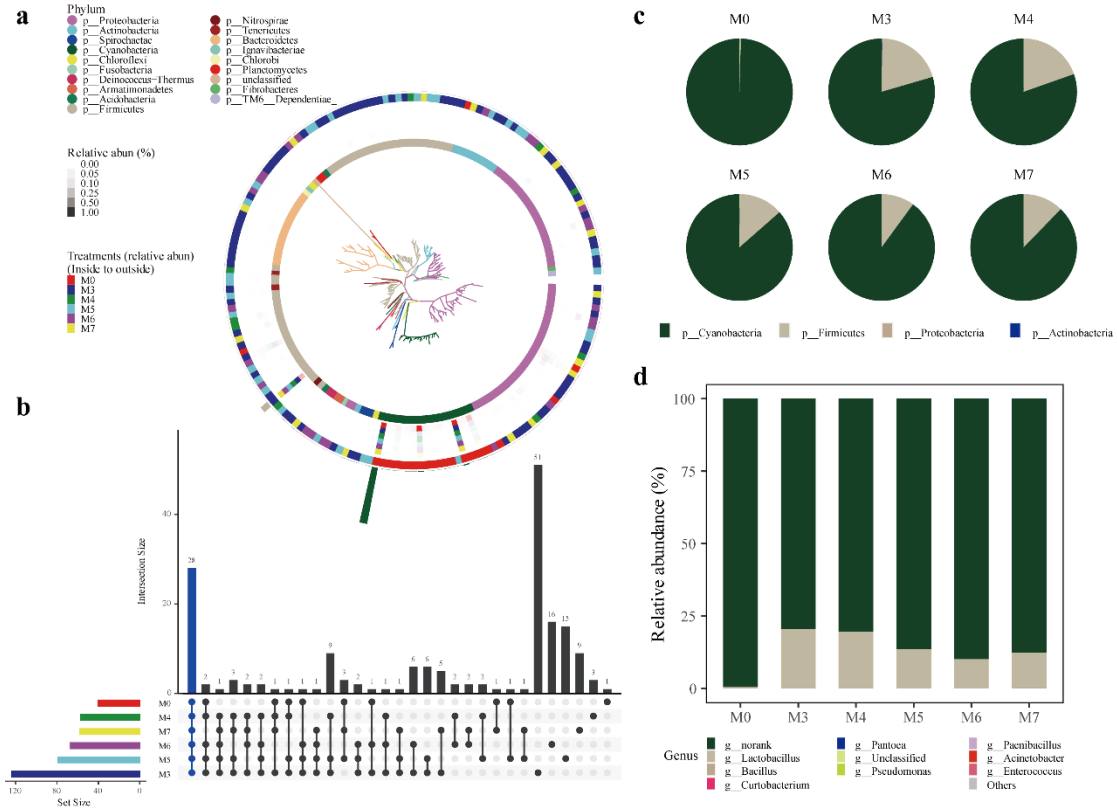

Figure 1 Microbial composition

No significant differences could be found in observed bOTUs and Chao1 index among all groups, based on the Kruskal-Wallis test ($P > 0.05$), with the Shannon and Simpson index holding opposite results ($P < 0.05$) (**Figure 2a**). The value of the Shannon and Simpson index showed a significant increase after a 3-day fermentation when compared to the M0 group, which decreased with time being then. Based on Bray–Curtis dissimilarity and Jaccard similarity index distance, the transposed distance matrix for microbial abundance and microbial community structure got calculated. Principal coordinate analysis (PCoA) revealed that the 2-dimensional planes could adequately explain the variations in microbial abundance and community structure contributed by fermentation time (Bray–Curtis dissimilarity: PCoA1 = 96.3%, PCoA2 = 3.05%; Jaccard similarity index: PCoA1 = 80.6%, PCoA2 = 10.48%) (**Figure 2b**). Furthermore, the M0 group samples were grouped in separate clusters, isolated from the other experimental groups. In contrast, the experimental group samples were interwoven using permutational multivariate analysis of variance (Permanova, Bray–Curtis dissimilarity: $P = 0.037$, $R^2 = 0.275$; Jaccard similarity index: $p = 0.003$, $R^2 = 0.172$).

To uncover the possible causes of the above variation from a microbiological perspective, a joint analysis of linear discriminant analysis effect size (LEfSe, LDA score = 4.0) and linear models for microarray data (limma) was put into use for testing microbial data. LEfSe analysis of microbiota revealed that differential OTUs were few of the biomarkers with high scores at the corresponding taxonomic level (**Supplementary File Figure 2**), in which the differential OTUs were mainly distributed among the groups in the dominant phylum, such as Cyanobacteria, Firmicutes, Proteobacteria, and Actinobacteriota. Together with the distribution cladogram findings, differential indicator species ranked top in M0 were coincidentally classified into Cyanobacteria. Fermentation contributed to the significant increase of Bacilli in experimental groups and Clostridia in M3 and M5 groups. Combined with limma analysis, the biomarkers in this study were OTU57 (classified into *Lactobacillus*), OTU97, and OTU166 (classified into Cyanobacteria), of which OTU57 was up-regulated in the fermentation groups, and another two bOTUs were down-regulated.

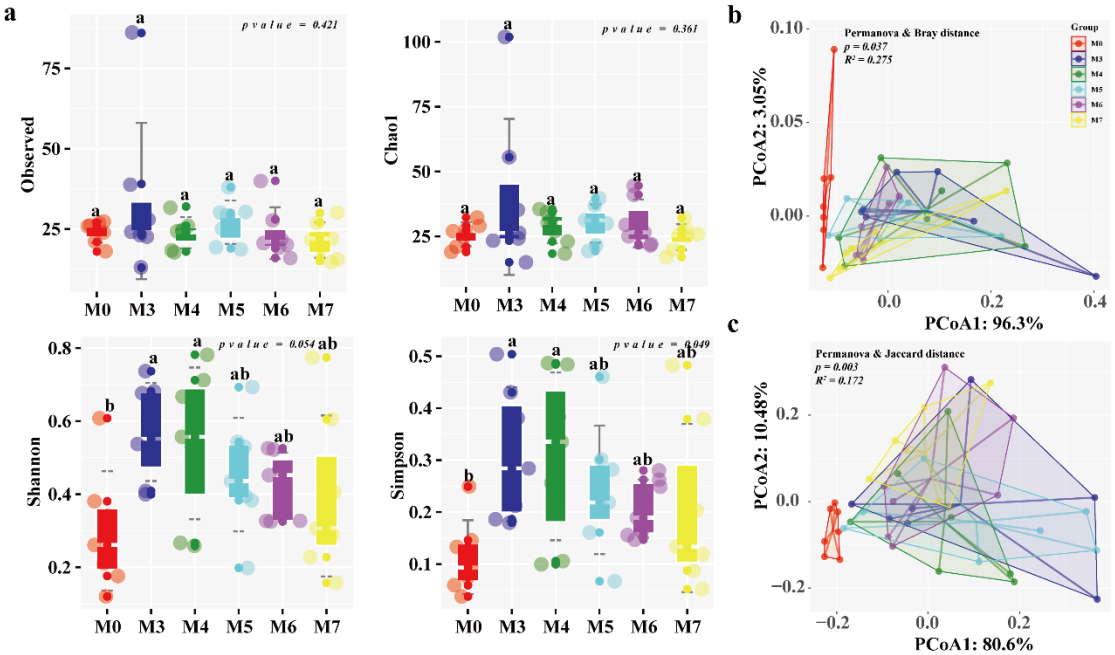

Figure 2 Microbial diversity

Microbial co-occurrence network

Microbial networks were constructed based on a uniform similarity threshold (0.75) using the RMT method (**Figure 3-4, Supplementary File Tab. 1**). The scale-free property (power-law r^2=0.330 - 0.617) suggested a non-random co-occurrence pattern in this microbial network, which was consistent with the result of the comparison between the empirical network and random network (**Supplementary File Tab. 1, Figure 1**). The microbial composition and abundance profiles of co-occurrence networks shared similar patterns to their microbial community via Circos plots (**Figure 3a-b**). The dominant phyla remained Cyanobacteria and Firmicutes, and the number of all taxa appeared to be increased and then decreased

Six representative networks were presented to identify combinations of potential interactions in the water microbial community. Outright, all networks inclined to co-exist rather than co-exclude, with positive correlations accounting for over 90% of the potential interactions were scanned, and the proportion of negative edges increased with the fermentation time increasing. Notably, an enhanced interaction of the microbial network can be found in the M3 group, evidenced by a significant decrease in the proportion of negative edges (1.89%), which consisted of the interactions between Firmicutes and other phyla. Besides, the M3 group showed the most significant network size (node number), average node degree (avgK), and average clustering coefficient (avgCC), coupled with the lowest module number and modularity (M). As the fermentation time increased, node number, avgK, and avgCC appeared to be decreased after 3-day fermentation. Moreover, 4-day fermentation contributed to a connector (OTU223) classified into Firmicutes based on the Zi-Pi plot (**Figure 3c**), considered keystone species in the molecular ecological network.

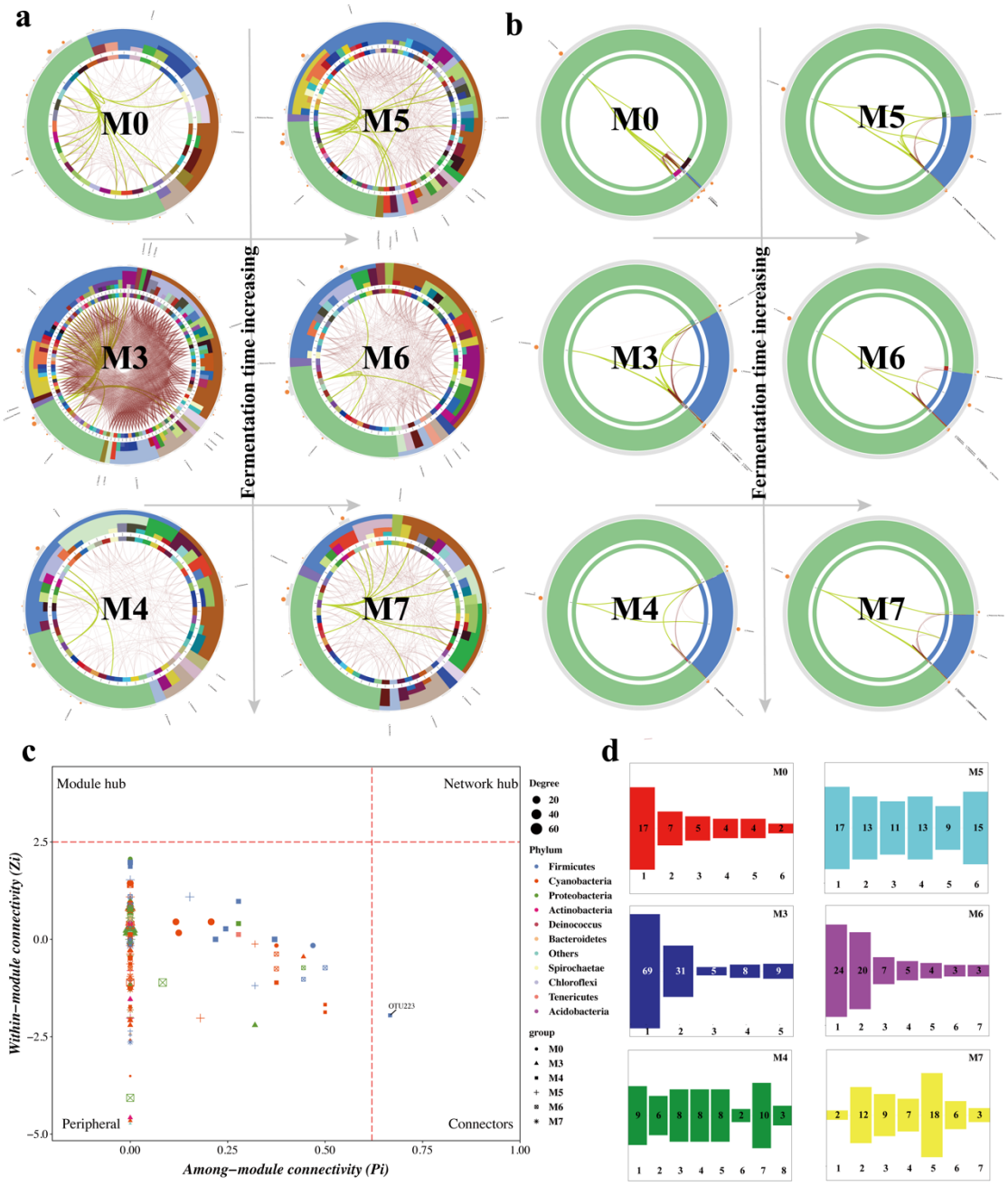

Figure 3 Microbial co-occurrence network analysis

Upon examining the individual modules of all networks, it was found that the M3 group had only a small number of modules but that the nodes were concentrated mainly within the first two modules. In contrast, the other group's networks were high in module number, and the nodes were evenly distributed (**Figure 4a**). Fermentation promoted interaction at all taxonomic levels, and the strength and amount of this interaction decreased with increasing fermentation time, initiating changes at the family level when fermentation reached 4 days (**Figure 4c-d**).

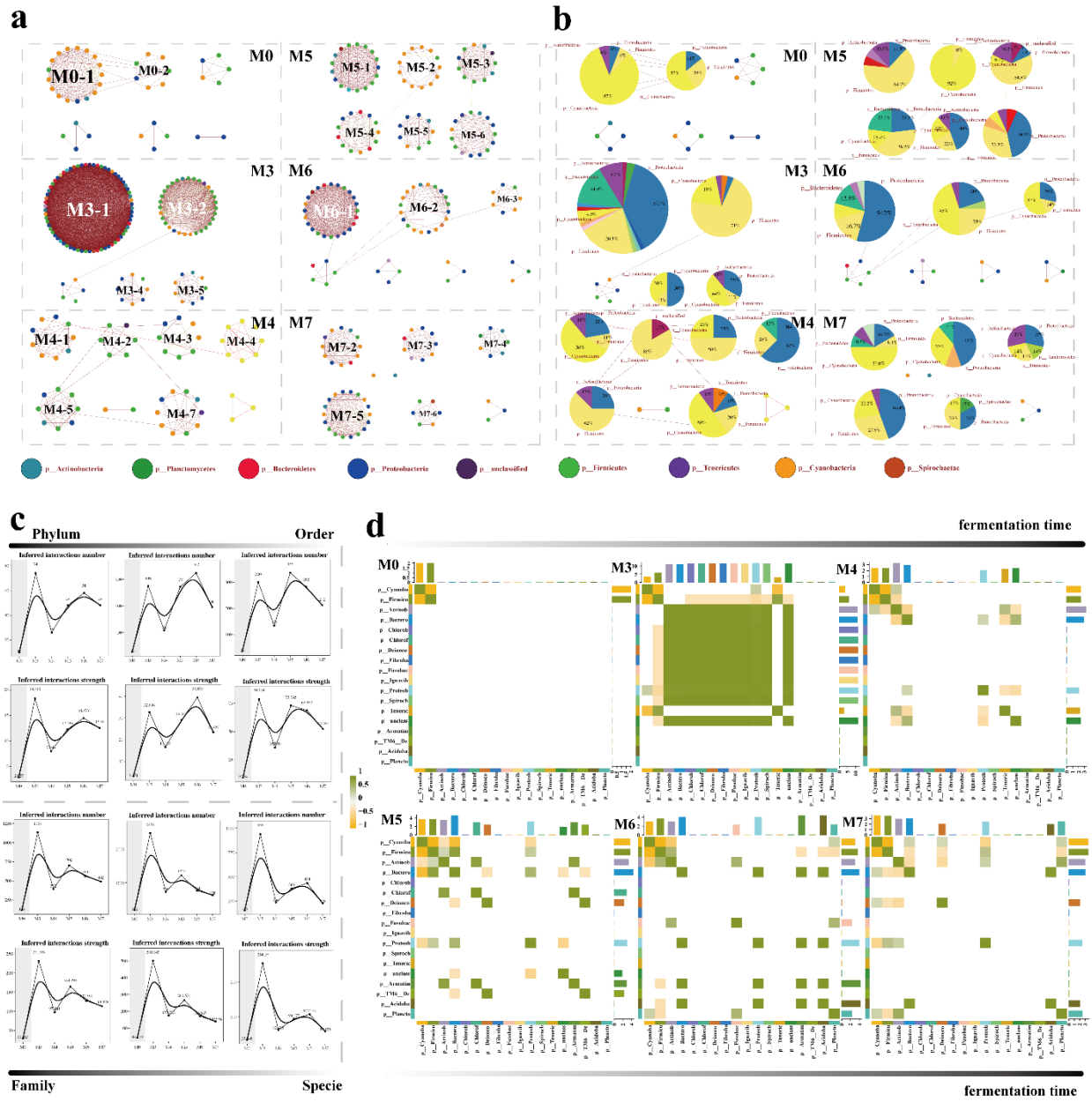

Figure 4 Microbial co-occurrence network topological roles

Microbial source-tracking analysis

Based on the above analysis steps, we can find that the M3 group of microbial communities exhibits diversity differences and functional redundancy. To investigate the sources of diversity differences, a dynamic Bayesian inference neural information flow network was used for retrospective analysis. Microbes fermented for 0, 3, 4, 5, and 6 days were used as sources,

and those fermented for 3, 4, 5, 6, and 7 days were used as the sink microbe, respectively. The overall data was analyzed, and it was found that the weight of each fermentation time as a source decreased as the fermentation time increased, whose proportions were 33.4% (M0), 23.99% (M3), 21.69% (M4), 11.49% (M5), 5.28% (M6) and 4.15% (unknown source), mainly manifested by the fact that the previous time point was the primary source for the next time point and that microbiota of unknown origin contributed mainly to the M3 group (**Figure 5a-b**).

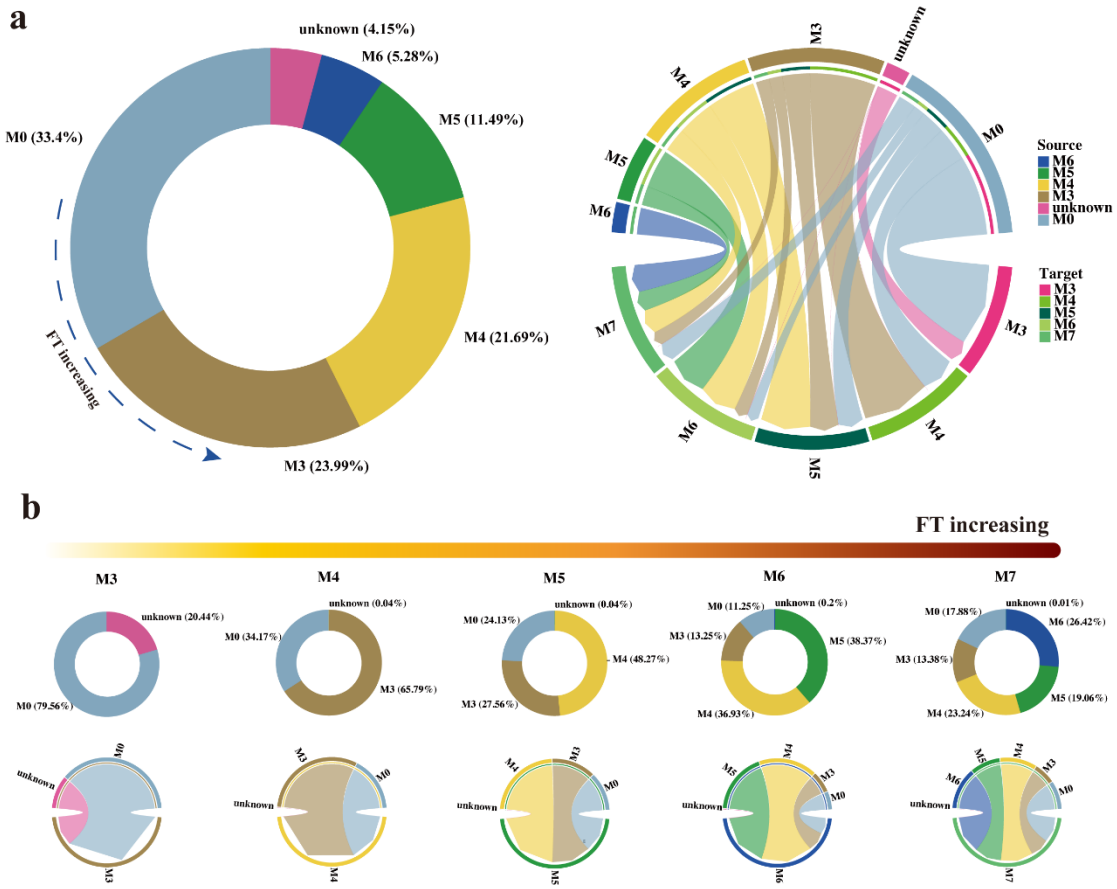

Figure 5 Microbial source tracking analysis

Functional prediction

LEfSe analysis (LDA score = 1) revealed that all functional prediction biomarkers were enriched in the M0 group (**Figure 6a**). Regarding the metabolism pathway at KEGG level 2, we could find the Amino acid metabolism, Energy metabolism, Metabolism of terpenoids and polyketides, Xenobiotics biodegradation and metabolism, Metabolism of cofactors and vitamins, and Metabolism of other amino acids. Subsequently, the t-test was used to compare two groups for metabolism-related pathways (**Figure 6b-f**). It could be found that all experimental groups shared similar patterns of functional predicted pathways when compared to the M0 group. Notably, upon fermentation, the pathways associated with restrictive amino acids in soybean meal and Carbohydrate metabolism got activated ($P < 0.05$), such as fructose and mannose metabolism, Cysteine and methionine metabolism, and Lysine biosynthesis, their associated functional genes were activated. Accompanied by the increase in fermentation time, the pathway related to Lysine degradation and Lipopolysaccharide biosynthesis got down-regulated ($P < 0.05$).

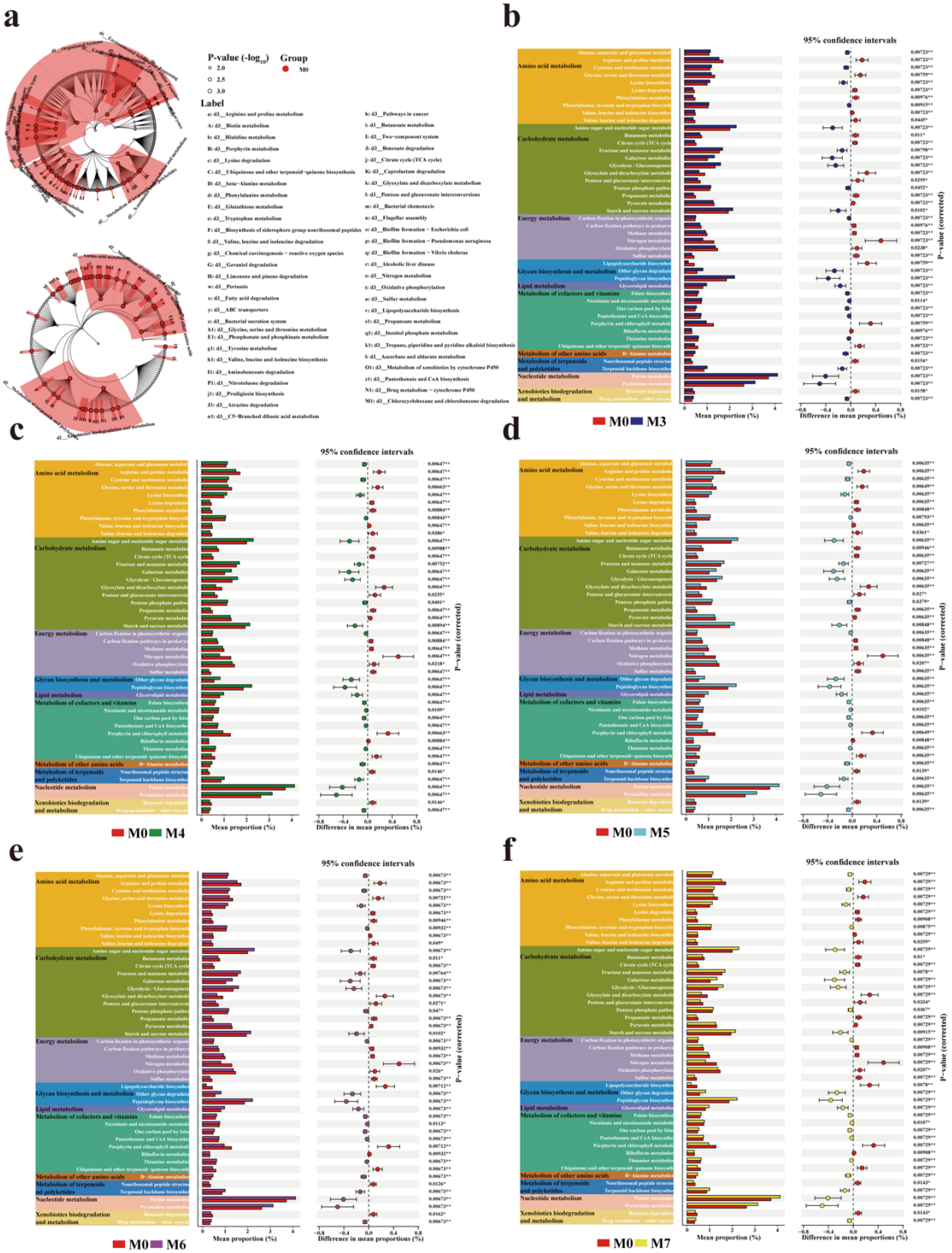

Figure 6 Microbial functional prediction

Soybean meal nutrition assessment

As can be seen from **Figure 7**, crude protein, acid-soluble protein, ash, glucose, total phosphorus, and energy were significantly higher than the M0 group with increasing fermentation time. At the same time, urease and pH showed a significant decrease. Other parameters, such as fructose and total calcium content, were not significantly different.

To identify the main factors shaping microbial abundance and community structure, we quantified the relative importance of several environmental and nutritional factors on the composition of soybean meal microbial communities using redundancy analysis. Variance decomposition revealed that the unique effect of total phosphorus explained the most significant variation in soybean meal microbial communities, followed by fructose. Acid-soluble protein, ash, fructose, total phosphorus, and glucose contributed to the highest common effect (**Figure 7c-f**). Mantel's test revealed that Several factors mentioned above working in synergy were highly correlated to the changes of Firmicutes (**Figure 7b**).

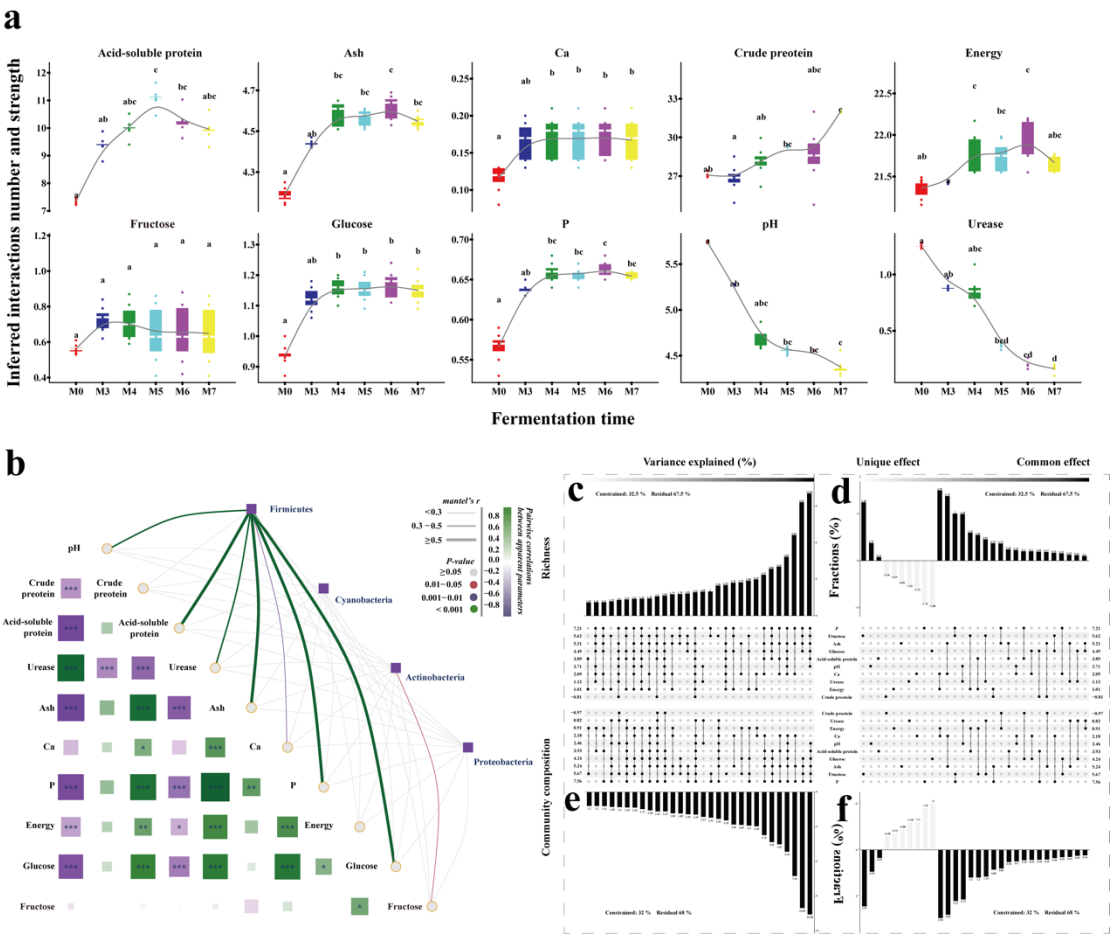

Figure 7 Nutritional assessment of soybean meal and its microenvironmental and microbial interactions

Discussion

Microbial composition analysis showed that microbial community diversity changed dynamically during solid-state fermentation. In the early stages of fermentation, the Shannon and Simpson microbial diversity indices increased and then decreased, indicating no statistical differences among the newly produced species. In contrast, the homogeneity between species

at different fermentation times clearly showed differences, suggesting that the abundance of some species may have changed significantly. We can identify significant species composition and abundance differences between the fermented and unfermented groups combined with beta diversity. These bacteria were necessary for assessing soymeal fermented feeds and can be used as indicator species to understand the fermentation stage. To understand the potential causes of this difference, we considered the following two aspects: (1) interactions between microbiota and (2) interactions between microbiota and the environment (or nutrition).

Recently, plant-based fermented foods have gained attention for their potential benefits. Studies have reported that fermentation reduces anti-nutritional factors and makes the original substrate more palatable and nutritious without leaving out the role of strains and fermentation time (Mukherjee, et al., 2016; Uchida and Miyoshi, 2013; Widyastuti, et al., 2021). The appropriate microbial fermentation time could improve the nutritional value of soybean meal, promoting animal digestion and absorption of protein and sugar and improving the feed utilization rate. Firstly, microbial fermentation reduced urease activity, a major anti-nutritional factor in soybean meal (Schedle, et al., 2013), and showed a significant reduction in urease activity as the fermentation time increased. This reduction in antinutritional factors indicates that adding fermented soybean meal to animal feeds facilitates the digestion and absorption of the feed and promotes the high-value application of the food or feed (Mukherjee, et al., 2016).

In this study, the pathways associated with restrictive amino acids in soybean meal and Carbohydrate metabolism got activated, such as Cysteine and methionine metabolism and Lysine biosynthesis, which served as the markers of soybean meal quality improvement. Secondly, the increase in crude protein and acid-soluble protein content indicates that nitrogenous substances such as peptides and free amino acids, which are more suitable for absorption and utilization by the organism, have also been increased. This may be because, during the fermentation of soya bean meal by bacteria, the microbial metabolism causes the organic matter to be completely oxidized to CO_2 and H_2O and generates bacteriophage proteins (Uchida, Miyoshi, 2013). The loss of carbon, hydrogen and oxygen in the dried fermented soybean meal resulted in a relative concentration of the nitrogen it contains, thus increasing the crude protein content of the fermented soybean meal. The total energy of the soybean meal is increased after fermentation. Regardless of protein, we found a significant change in the content of another energy source, with the glucose content in fermented soybean meal increasing significantly with fermentation time. This may be caused by the fact that the fermentation harbored some degradable bacteria and broke them down into usable monosaccharides, significantly increasing the efficiency of sugar utilization by the animals (Montoya-Camacho, et al., 2019). As it turns out, we found the presence of Clostridium in the indicator species in the pre-fermentation phase using LEfSe analysis, and the primary function of Clostridium is to utilize the cellulose in the raw material and to promote the degradation of the difficult-to-utilize cellulose to monosaccharides.

To some extent, changes in clostridia can reflect the effects of fermentation. Furthermore, along with the lower pH, fermentation brings a unique flavor to the soybean meal, improving palatability and enhancing animal feeding, thus promoting animal production. And a lower pH level inhibits the growth of harmful bacteria thanks to the high production of lactic acid (Emkani, et al., 2022). In addition, the fermenting strains *Lactobacillus* used in this study, as common probiotics, could produce lactic acid, improve the animal's intestinal micro-ecological environment, and inhibit the growth of harmful bacteria after their fermented feed is ingested by animals, which acted as an indicator species in the fermentation process itself. And the ash content in the soybean meal was also increased after microbial fermentation, which may result from the increased content of total phosphorus, which effectively optimized the calcium-to-phosphorus ratio of the fermented soybean meal.

Source tracking analysis allows the origin of microbiota to be tracked throughout the fermentation process, which can be used to determine the conditions of the initial fermentation

- the amount of inoculum of the fermenting strain in this study. To further investigate the sources of diversity differences, a retrospective analysis using a dynamic Bayesian inference neural information flow network revealed that the proportion of each fermentation time as a source decreased with increasing fermentation time. The unknown origin could be found to contribute mainly to the M3 group. This may reflect that functional redundancy existed in the M3 group, promoting the emergence of new species rather than from the M0 microbial community. In contrast, after 4 days of fermentation, the proportion of microbiota of unknown origin was close to zero, meaning that no new species were produced; combined with the species composition, foreign disturbances were reduced, and the microbial community stabilized.

The stability of a complex microbial ecosystem is maintained by a complex ecological network formed by the interactions between different species (OTUs) within the microbial community (Coyte, et al., 2015). Most OTUs are peripheral nodes in the network and a few OTUs act as connecting nodes or modular hubs. From an ecological perspective, peripheral nodes may represent specialists, while connecting nodes and modular hubs may act as generalists. According to Olesen (M, et al., 2007), functional key OTUs performed by generalists play a crucial role in maintaining the stability of the entire network. In the search for OTUs sensitive to fermentation time, the experimental results revealed that the central microbial communities showed good delineation across the different treatments. This is also consistent with the PCoA results mentioned above. Modularity is one of the critical structural features of networks, presenting the extent to which the network is divided into well-defined submodules (Deng, et al., 2012). Co-occurrence networks showed a decrease in modularity after 3 days of fermentation, suggesting that the M3 group microbial community performs fewer functions. This may be related to an increase in microbial species in the M3 group, with an increase in species with the same ecological function and the emergence of functional redundancy. It is assumed that soybean meal is still in a relatively good nutritional and environmental state at 3-day fermentation. In complex networks, the identification of submodules will not only reveal the pattern in which these networks are built into tightly connected communities. Perhaps more important is the link between this construction pattern and its function (McWilliams, et al., 2019). In this study, there were three main ecological network models (pre-fermentation, 3 days of fermentation, and 3 days post-fermentation) with characteristic sub-modules for 3 days of fermentation and the main component of these modules, was the dominant microbial community, implying that the dominant microbial community plays a vital role in the M3 network. According to Yan (Yan, et al., 2014), OTUs from the same category are most likely to have the same function as they share the same genes, confirming the existence of functional redundancy in M3 again. Furthermore, after performing network modularity analysis for each treatment separately, the fermentation time can significantly influence the ecological network structure of the soybean meal microbial community by changing the mean degree (avgK), mean clustering coefficient (avgCC), mean path distance (GD) and modularity coefficient. There was the lowest GD and the highest avgCC in the M3 group, which stated that 3-day fermentation contributed to the decrease of the presumed interspecies distances, suggesting the proximity of species in the spatial structure of soybean meal, the stability of the microbial community is more vulnerable to environmental perturbations.

Previous studies suggest ecological competition can promote microbial community stability (Coyte, et al., 2015). Moreover, interspecies interactions were strengthened in the M3 group, whereas they were substantially weakened at 4-day fermentation and beyond, of which the negative correlations were strengthened. On the one hand, the number of species decreases in the later stages of fermentation. On the other hand, the community stabilizes, interspecific cooperation weakens, and negative feedback is strengthened. Thus, enhanced interspecific interactions within microbial communities may be due to these species performing similar or

complementary functions or sharing ecological niches. The nutrients provided in the soybean meal were still adequate for microbial growth at three days of fermentation.
In contrast, after four days, the nutritional conditions and pH changed, no longer suitable for most microorganisms' growth. Increased species cannot synthesize many essential nutrients due to the lack of necessary pathways or key genes (Mee, et al., 2014; Zengler, Zaramela, 2018). Therefore, the survival of this increased auxotroph depends heavily on the community to ensure the exchange of carbon flows and by-products. Species in a cooperative relationship are prone to environmental dependence because of the coupling of species and positive feedback loops caused by the cooperative relationship. When species decline, there may be a rapid decline in other species, reducing the stability of the community. Therefore, inhibiting positive feedback loops and weakening interactions can improve the stability of the network.

Conclusion

Taken above, our results stated that fermentation improved the nutritional value of soybean meals. In the early stages of fermentation, the soybean meal was highly overgrown with stray bacteria that destabilized the microbial community, with pH and nutrient conditions playing an essential role in helping to make it stable. Moreover, we must attach importance to microbial interspecific interactions, which made it easy to understand how microbiota keeps the community stable. 4-day fermentation with *Lactobacillus* was recommended for soybean meal.

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