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16S rDNA profiling of Loach (*Misgurnus anguillicus*) fed with soybean fermented powder intestinal flora in response to Lipopolysaccharide (LPS) infection

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ABSTRACT

Soybean fermentation has a balancing effect on the regulation of intestinal flora. Relative research between fermented soybeans and intestinal microbiota is limited. Our aim was to explore the effects of soybean fermented fowder on lipopolysaccharide (LPS) induced intestinal microflora and corresponding functions in loach. 16S rDNA high-throughout sequencing was applied to estimate differences in the intestinal microbiota and predict genes function. Analysis of the overall of sequencing data showed that the ratio of Effective Tags in both the control group and the treatment group was greater than 80 %. Based on six major classifications involved in the phylum, class, order, family, genus, and species, we acquired the changes in the composition of intestinal microorganisms after the supplement of soybean fermented powder. These results showed that the dominant bacteria in the two groups were basically distinct at different levels. Alpha diversity analysis indicated that the microbial richness and uniformity of soybean fermented powder decreased compared to the control group. PICRUSt and Taxfun tools analysis of intestinal flora illustrated the functional genes of the six groups were mainly involved in metabolism, genetic information processing, cellular processes, environmental information processing, and human diseases at the level 1. These data clearly demonstrated the effect of soybean fermented powder on the gut microbiome. Not only that, it provides new ideas and insights for achieving high-quality utilization of soybean fermented powder. The potential mechanisms of soybean fermented powder to alter gut flora and intestinal microbiome function can further be explored.

1. Introduction

The loach (*Misgurnus anguillicus*) is a rich source of high-quality protein [[1]]. Its meat is delicious and has a unique flavor. Moreover, it contains a variety of amino acids indispensable for humans [[2]]. It is widely distributed in China as a freshwater fish, mainly distributed in the Yangtze River basin, and is an endemic species in Asia [[3]]. In recent years, with the increase in domestic and foreign trade, the demand for loach has steadily increased, and it is one of the important aquatic products, generating significant economic effects [[4]]. The cultivation mode of loach has developed rapidly and the scale of cultivation continues to expand.

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However, lacking of scientific feeding can lead to an intestinal microbial imbalance in fish, leading to intestinal damage, while the application of fermented feed regulates the microecological balance in aquatic animals and meets the current development requirements of environmentally friendly fisheries [[5]].

The intestine is one of the important organs in our body. Hippocrates, the father of Western medicine, once said, 'All diseases begin in the gut'. Modern medical research has further verified that intestinal health plays a vital role in human health [[6,7]]. As a microbial community resident in the body's intestines, intestinal flora plays a decisive role in intestinal health and participates in regulating various physiological functions of the human body. Currently, it has been regarded as an important acquired "organ" of the human body [[8]], affecting our metabolic system [[9]], immune system [[10]], nervous system, etc [[11]]. Once the intestinal flora is out of balance, it will cause serious harm to the body and lead to various diseases, including obesity [[12]], diabetes [[13]], gastrointestinal cancer [[14]], and a series of other diseases.

Presently, research on the food functionality of fermented soybeans has been increasing both at home and abroad. Soybean fermented products at home and abroad include soy sauce, fermented bean curd, China, Cheonggukjang, Doenjiang, Korea, and natto, Japan [[15]]. Soybean is rich in dietary fiber and is one of the most important food crops in China. After fermentation, some proteins in soybean exhibit an increase in flavor amino acids, and functional polypeptides and small peptides increase [[16]]. Fermented soybeans have significant pharmacological effects, including antioxidants [[17]], blood pressure lowering [[18]], blood lipid lowering [[19]], improved digestion [[20]], and immune regulation [[21]]. In the final analysis, fermented soybean has more bioactive substances than unfermented soybean. As an example, fermented soybeans are rich in oligosaccharides (also known as bifidous factors), which are not directly allowed to digest and absorb by the body, while having a regulatory effect on the intestinal flora. Moreover, it can increase beneficial bacteria such as bifidobacteria in the intestine and degrade harmful bacteria [[22,23]]. And a large number of beneficial bacteria can even increase the number of white blood cells in the body, improving the immune ability of the body to pathogens. Similarly, it can also enhance the activity of macrophages in the body and strengthen the immune barrier [[24, 25]].

Lipopolysaccharide (LPS) is a major component of the cell wall of gram-negative bacteria and a stimulant of the intestinal immune system [[26]]. Infection with intestinal epithelial cells can lead to a series of reactions such as increased intestinal permeability, which can lead to intestinal inflammation and damage the normal barrier function of the intestine [[27]]. Given the above, we hypothesized that there may be an interaction between soybeans fermented powder and intestinal microbiota. Consequently, we investigated the impact of supplementation of soybean fermented products on LPS-induced intestinal microbiota imbalance. 16S rDNA sequencing has played a pivotal role in the accurate identification of bacterial isolates and the discovery of novel bacteria in microbiology [[28]].

Taken together, our present study applied 16S rDNA sequencing technology to explore the effects of soybeans fermented powder on the structure and composition of intestinal microflora of loach induced by LPS. These findings in this study will provide new clues for the treatment of LPS-mediated intestinal dysbacteriosis-related diseases with soybeans fermented powder, as well as provides ideas for regulating the microecological balance in aquatic animals, promoting healthy growth, and improving production performance.

2. Materials and methods

2.1. Experimental animal and LPS treatment

70 in total, 6 months of age, loach with an equal number of male and female loaches (mean weight of 8 ± 2 g) were purchased from a Loach feeding base (Wenzhou, China). After 7 days of feeding in basins, 70 loaches were randomly divided into two groups, namely control (A), and soybeans fermented powder treatment groups (B). Group A was fed fish feed and injected with 100 μl PBS. Group B was fed 100 g soybeans fermented powder, fed fish feed, and injected 5 mg/kg of LPS (Solarbio life science, China) in 100 μl PBS [[29]]. All animals were given normal water and kept in the same volume of water. After 24 h, loaches were euthanized to collect tissues such as midgut, spleen, blood, liver, etc. Eventually, the loaches' midgut were quickly frozen in dry ice to sequence under sterile and nuclease-free containers. Consequently, the other samples and tissues were stored at - 80 °C until analysis.

2.2. DNA isolation, library construction, and sequencing

Genomic DNA in total of collected midgut samples was isolated by the HiPure Stool DNA Kits methods (Magen, Guangzhou, China), and the agarose gel electrophoresis was employed to demonstrate DNA integrity and concentration. Moreover, we also used NanoDrop 2000 analyzer (Thermo Fisher Scientific, USA) to validate. The 16S rDNA target the V3–V4 regions of the ribosomal RNA gene were amplified by PCR (95 °C for 5 min, followed by 30 cycles at 95 °C for 1 min, 60 °C for 1 min, and 72 °C for 1 min and a final extension at 72 °C for 7 min) [[30]]. The PCR reaction was employed in a 50 μL reaction volume through TransGen High-Fidelity PCR SuperMix (TransGen Biotech, Beijing, China) according to the manufacturer's protocols. Later, diluted genomic DNA was acted as a template, PCR was executed, and the amplified DNA was demonstrated by 2 % agarose gel electrophoresis, and the PCR product was recycled using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.). The libraries were sequenced on an Illumina Novaseq 6000 sequencing platform. The library quality was constructed and then quantified by Qubit 3.0 Fluorometer (Thermo Fischer Scientific, USA).

2.3. Data analysis process and method

A total of the obtained sequencing raw data were filtered and truncated to create high-quality clean reads by using FASTP (version

0.18.0) [[31]]. Then, paired-end clean reads were combined with raw tags by employing FLASH (version 1.2.11) [[32]] with a minimum overlap of 10 bp and mismatch error rates of 2 %. After filtering and processing Raw tags, we acquired clean tags, which were clustered into operational taxonomic units (OTUs) with similarity using UPARSE (version 9.2.64) [[33]] pipeline. To remove chimeric tags, UCHIME algorithm [[34]] was applied and finally obtained effective tags with high abundance for further analysis.

2.4. Community composition and indicator species analysis

The abundance data of classifications containing phylum, class, order, family, genus and species was screened with Krona (version 2.6) [[35]]. The stacked bar plot of the community constitution was described in R project ggplot2 package (version 2.2.1) [[36]]. Between groups A and B, Venn analysis was drawn in R project VennDiagram package (version 1.6.16) [[37]] to validate specially appointed and general species or OTUs or ASVs.

2.5. Alpha diversity analysis

Alpha-diversity index was involved in Chao, Shannon, Simpson, Pielou's evenness, and Good's coverage, which was calculated in QIIME (version 1.9.1) [[38]] and applied to examine the intestinal microbial community diversity [[39]]. While PD-whole tree index was calculated in picante (version 1.8.2) [[40]]. Rarefaction curve and rank abundance curves were performed to evaluate the differences in OTU/ASV richness and evenness of intestinal microbial communities with R project ggplot2 package (version 2.2.1) [[33]] in groups A and B. Concrete speaking, rarefaction curve was evaluated whether the sequencing amount was sufficient to cover all groups, and indirectly reflect the richness of species in the sample. The Rank Abundance curve can intuitively reflect the classification abundance and uniformity in the groups, the wider the horizontal curve, the higher the classification abundance, and the smoother the vertical curve, the more uniform the species distribution.

2.6. Functional prediction based on PICRUSt and Tax4Fun tools

The functional potential prediction, namely, the KEGG pathway analysis of the OTUs/ASV was inferred using Tax4Fun (version 1.0) [[41]] and PICRUSt (version 2.1.4) [[42]]. The Functional group (guild) of the Fungi was inferred using FUNGuild (version 1.0).

PICRUSt functional prediction software was annotated the function of the KEGG Pathway for bacteria/archaea or predicted the MetaCycle Pathway for fungi (ITS) on the ground of the OTU's species annotation and abundance statistics, and was counted the abundance of information on each Pathway.

Tax4Fun functional prediction was first achieved by combining the 16S rRNA sequence of prokaryotes in the KEGG database with the 16S rRNA sequence in the SILVA database. Finally, Sequence interruption of the existing prokaryotic species genomes in the KEGG database was performed, and the KO sequences of all genomes were statistically analyzed to implement KEGG prediction and KO abundance statistics using UproC [[43]]. All data were presented as mean ± standard deviation. $p < 0.05$ was counted as statistically significant.

3. Results

3.1. Quality control and overview of sequencing data

In the present study, over 120,000 Clean Reads, 110,000 Clean Tags, 11,000 Chimera, and 100,000 Effective Tags were developed from sequencing data in groups A and B (Table 1). The effective ratios of the two groups were greater than 80 %. These information demonstrated the high-quality sequencing data, which can be applied to delve into subsequent experiments. Moreover, we further excavated the effective tags in the two groups and represented that the maximum and minimum sequence lengths were 476 and 223 in group A, respectively, while 469 and 227 in group B, respectively (Table 2). Not only that, we further demanded to conduct abundance statistics on the OTU based on the effective Tags (Table 3). The number of Taxon Tags in groups A and B were 75,036 and 86,028, respectively. Then, unclassified tags were 0. These results had proven that the tags obtained by sequencing have excellent quality and wide coverage.

Table 1
Summary of the sequence analyses.

Sample	Raw Reads	Clean Reads	Raw Tags	Clean Tags	Chimera	Effective Tags	Effective Ratio (%)
A	124,819	124,687	122,848	122,514	18,747	103,767	83.13
B	120,803	120,669	118,693	118,323	11,524	106,799	88.41

Raw reads: The number of original reads obtained without filtering low-quality sequences. Clean reads: The number of reads after removing low-quality sequences. Raw tags: The number of original Tags obtained from the Clean Reads after overlap assembly. Clean Tags: high-quality tag data obtained from raw tags spliced through quality and length filtering processing. Chimera: The number of chimeric Tags detected during clustering. Effective Tags: The number of high-quality tags after removal of chimeras. The subsequent analysis is based on effective tags. Effective Ratio (%): Effective Tags as a percentage of Raw Reads.

Table 2
General overview of the Tag sequence analyses.

Sample	Tags Number	Total length	Max length	Min length	N50	N90
A	103,767	48,188,674	476	223	466	461
B	106,799	49,335,550	469	227	461	461

Tags Number: The number of Effective Tags obtained from two groups of samples, respectively. Total length: Total length of all Tags. Max length: Sequence length of the maximum Tag. Min length: Sequence length of the minimum Tag. N50 (bp): Arrange all sequences from long to short, and add the sequence lengths in this order. When the added length reaches 50 % of the total length of the Tags sequence, that is, the length of the last sequence. N90 (bp): Arrange all sequences from long to short, and add the sequence lengths in this order. When the added length reaches 90 % of the total length of the Tags sequence.

Table 3
Distribution overview in groups A and B of OTU.

Sample	Total Tags	Taxon Tags	Unclassified Tags	Singleton Tags	OTUs
A	103,767	75,036	0	28,731	922
B	106,799	86,028	0	20,771	972

Tags Number: The number of Effective Tags were acquired from groups A and B of samples. Taxon Tags: The number of Tags with species annotations. Unclassified Tags: The number of tags without species annotations. Singleton Tags: The number of Tags corresponding to OTUs with a total abundance of 1 (OTUs filtered out). OTUs: The number of OTUs ultimately obtained after preprocessing.

3.2. Effects of supplying soybeans fermented powder on intestinal microbial composition

Dominant species largely determined the ecological and functional structure of microbial communities. Understanding the species composition of communities at various levels can effectively interpret the formation, variation, and ecological impact of community structures. We calculated the species composition of each sample at each level of classification, and then visually displayed the changes in species abundance of different samples at each level of classification using a stack diagram.

As shown in Fig. 1, the composition of Taxa was reflected at different levels of classification, including phylum (Fig. 1A), class (Fig. 1B), order (Fig. 1C), family (Fig. 1D), genus (Fig. 1E), and species (Fig. 1F). We selected species with an average abundance of the top 10 from all samples to demonstrate. At the phylum level, the main phyla in group A were Proteobacteria (84.25 %) and Bacteroidota (7.87 %). On the contrary, the dominant phyla in group B were Bacteroidota (61.92 %), followed by Proteobacteria (32.01 %). As shown at the class level, the staple classes were Gammaproteobacteria (84.12 %), following Bacteroidota (7.87 %), and Verrucomicrobiae (3.05 %) in group A. The major classes in group B were Bacteroidota (61.91 %), followed by Gammaproteobacteria (31.82 %). At the order level, Pseudomonas (42.15 %) and Altermonas (35.58 %) were the essential orders in Group A, while Flavobacteria (59.77 %) and Pseudomonas (26.87 %) were the major orders in group B. At the family level, the dominating families in group A were Shewanellaceae (35.58 %), followed by Pseudomonas (21.70 %) and Moraxellaceae (20.38 %). In Group B, the dominant families were Moraxellaceae (59.70 %), Moraxellaceae (19.05 %), and Pseudomonas (7.80 %). At the genus level, the primary genera

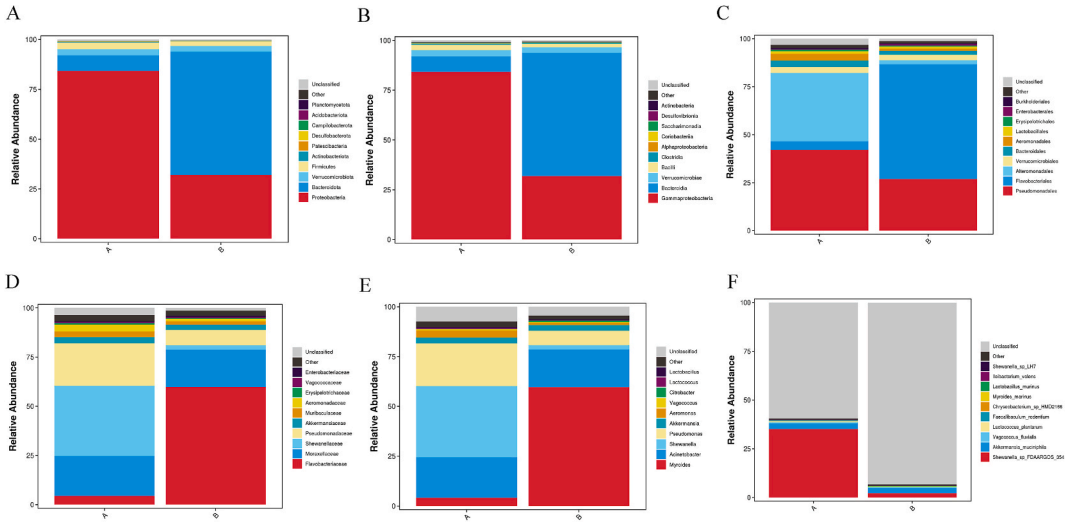

Fig. 1. Effect of dietary Soybean fermented powder supplementation on the relative abundance of gut microbiota in different taxa levels. (A) phylum, (B) class, (C) order, (D) family, (E) genus, (F) species.

in Group A were Shewanella (35.58 %), Pseudomonas (21.61 %), and Acinetobacter (20.39 %), while Myroutes (59.58 %) and Acinetobacter (19.03 %), in group B. At the species level, the largest species in groups A and B were Unclassified, which were 59.56 % and 93.30 %, respectively. In addition, Shewanella_sp_FDAARGOS_354 (35.07 %) , Akkermansia_Muciniphila (2.70 %), and Shewanella_sp_FDAARGOS 354 (3.19 %) were the principal species in group A and Group B, respectively.

To explore distinct biomarkers in loach microbiota induced by LPS, we executed the different regions of the Venn diagram based on ASV/OUT abundance to acquire the difference or similarities between groups A and B. Results demonstrated that a total of 1558 OTUs were shown in groups A and B. Concretely speaking, 336 OTUs were shared in groups A and B, while the unique 586 OTUs and 636 OTUs in group A (Fig. 2A) and B (Fig. 2B), respectively (Fig. 2).

3.3. Effects of adding soybean fermented powder on the alpha diversity

The alpha-diversity index of Sob, Shannon, Simpson, Chao1, ACE, Good's Coverage, Pielou's evenness, and PD-whole tree was shown in Table 4. Of note, the Good's Coverage values were significantly variable in groups A and B, indicating that the amount of sequencing data was relatively adequate. Shannon index explained that the species richness and evenness of Group A were greater than those in group B. This indicated that LPS mediated diminution in the diversity of intestinal flora to a certain extent. Soybean fermented powder may improve this impairment to some extent. As shown in Fig. 3A, the two groups of samples reached the plateau stage in rarefaction curve in the Simpson index, reflecting that the sequencing depth well covered all species in the sample. The Rank Abundance curve (Fig. 3B) in the vertical direction had flat solid lines in groups A and B, revealing the uniformity of the OTU composition of the sample.

3.4. Prediction of community function by PICRUSt and Tax4Fun

To dig into the function annotation related to variation in intestinal microflora of loaches supplied for soybean fermented powder, we adopted PICRUSt and Tax4Fun functions tools to predict. Of note, we generated cumulative histograms of the relative abundance of the function, and visually scanned the functions and corresponding proportions of the relevance in groups A and B's abundance at level 2 using the two methods, then we employed Tax4Fun to mark the clustering heat map at levels 2 and 3.

At level 1, the functional genes of the six groups mainly included metabolism, genetic information processing, cellular processes, environmental information processing, and human diseases. Through the PICRUST community function prediction at level 2 (Fig. 4A), we found that the top 10 significantly different metabolic pathways were 'Amino acid metabolism', 'Metabolism of cofactors and vitamins', 'Carbohydrate metabolism', 'Xenobiotics biodegradation and metabolism', 'Metabolism of insects and polymers', 'Lipid metabolism', 'Metabolism of other amino acids', 'Energy metabolism', 'Replication and repair', and 'Folding, sorting, and upgrading'. In the top 10 pathways, 'Xenobiotics biodegradation and metabolism', 'Metabolism of insects and polymers', and 'Lipid metabolism' were significantly different than the other pathways.

At level 2, we detected the top 10 differential pathways, namely: 'Carbohydrate Metabolism', 'Membrane Transport', 'Amino Acid Metabolism', 'Signal Transmission', 'Metabolism of Cofactors and Vitamins', 'Energy Metabolism', 'Nucleotide Metabolism', 'Translation', 'Replication and Repair' and 'Lipid Metabolism' through the prediction of Tax4Fun tool (Fig. 4B). At level 2, we found that using these two genes function of prediction program, which were shared six pathways for significant differences involved in 'Carbohydrate Metabolism', 'Amino Acid Metabolism', 'Metabolism of Cofactors and Vitamins', 'Energy Metabolism', 'Replication and Repair', and 'Lipid Metabolism' through stacking diagram. Further analysis of the clustering heat map at the level 2 (Fig. 5A) revealed that 20 pathways were significantly enriched in groups A and B, with a significant increase in the abundance of 'Carbohydrate Metabolism', 'Membrane Transport', 'Lipid Metabolism', 'Infectious Diseases', 'Xenobiotics Biodegradation and metabolism', 'Cell

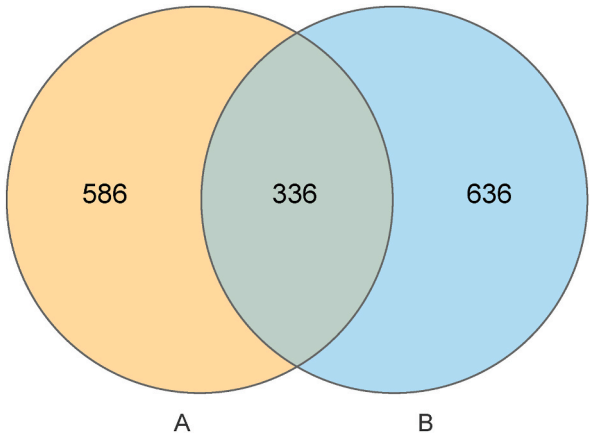

Fig. 2. Different species and marker species analysis through Venn diagram in loach microbiota induced by LPS. Groups A and B in the figure represent the control and soybean fermented powder treatment, respectively.

Table 4
Statistical analysis of index in alpha diversity.

Index	Sob	Shannon	Simpson	Chao1	ACE	Goods_Coverage	Pielou	PD
A	922	3.813720	0.818855	939.414	967.624	0.998654	0.387234	116.31911
B	972	3.462349	0.785793	990.167	1018.681	0.998721	0.348858	125.95995

Sob, Chao1, ACE, Shannon, Simpson, Good's Coverage, Pielou and PD, which is mainstream alpha diversity index and their correlation analysis results.

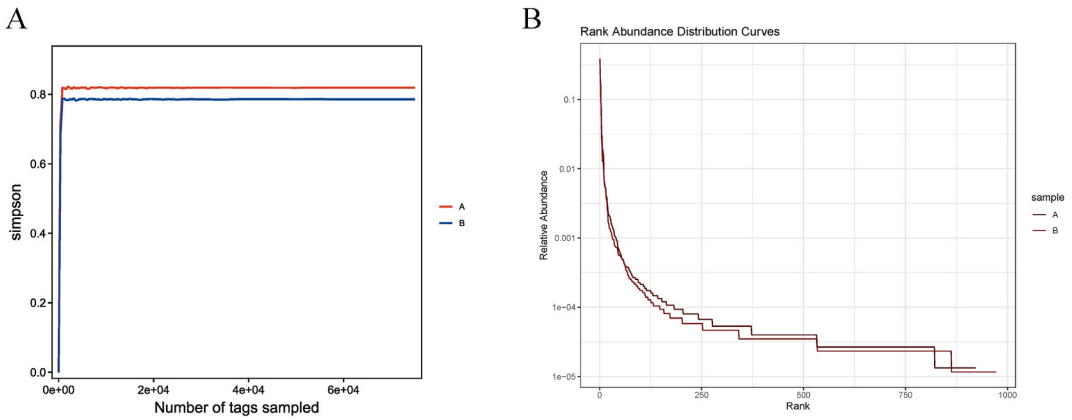

Fig. 3. Alpha-diversity index analysis. (A) Rarefaction curve, (B) RankAbundance. Groups A and B in the figure represent the control and soybean fermented powder treatment, respectively.

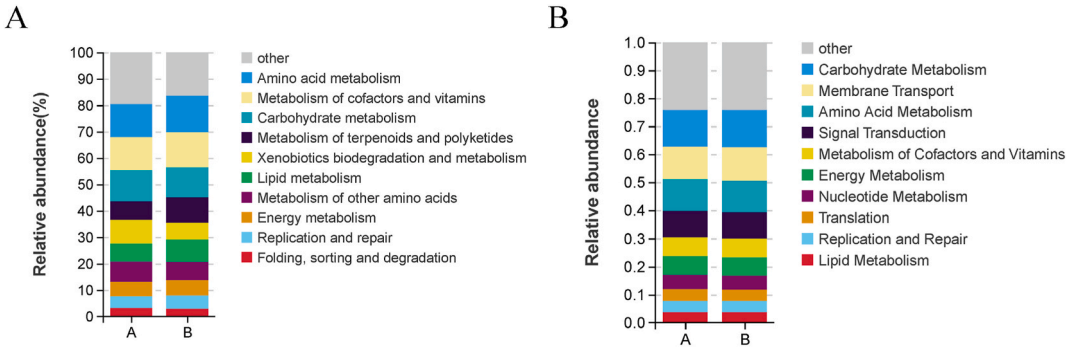

Fig. 4. Histogram of functional annotation relative abundance between the two groups using PICRUSt2 (A) and Tax4Fun (B) on level 2. The color represents the type of function, and the vertical axis represents the percentage of each function in the group. Groups A and B in the figure represent the control and soybean fermented powder treatment, respectively.

Motility', 'Metabolism of Other Amino Acids', 'Metabolism of Terpenoids and Polyketides', 'Biosynthesis of Other Secondary and Metabolites' and 'Signal Transduction' in group B significantly increased, while an apparent decrease in group A. At level 3 (Fig. 5B), the significantly different pathways between different treatment groups may indicate unique targets for different drugs at level 3. 20 pathways were significantly enriched in groups A and B. Compared with group A, such as 'Two-component system', 'ABC transporters', 'Bacterial secretion system', 'Arginine and proline metabolism', 'Starch and sucrose metabolism', 'Glycine, serine and threonine metabolism', 'Bacterial chemotaxis', and 'Flagellar assembly' in group B were greatly enhanced.

4. Discussion and conclution

In our present study, 16S rDNA sequencing was performed on the midgut from loaches in the control group, and soybean fermented powder treatment group, eventually high-quality Tags were obtained. To investigate the composition of two groups of intestinal flora, we detected the classification levels of phylum, class, order, family, genus, and species were significantly different. For example, at the genus level, the primary genera in Group A were Shewanella (35.58 %), Pseudomonas (21.61 %), and Acinetobacter (20.39 %), while Myroutes (59.58 %) and Acinetobacter (19.03 %), in group B.

Alpha diversity can indicate the richness and diversity of species in a specific habitat or ecosystem, and reflect the degree of species

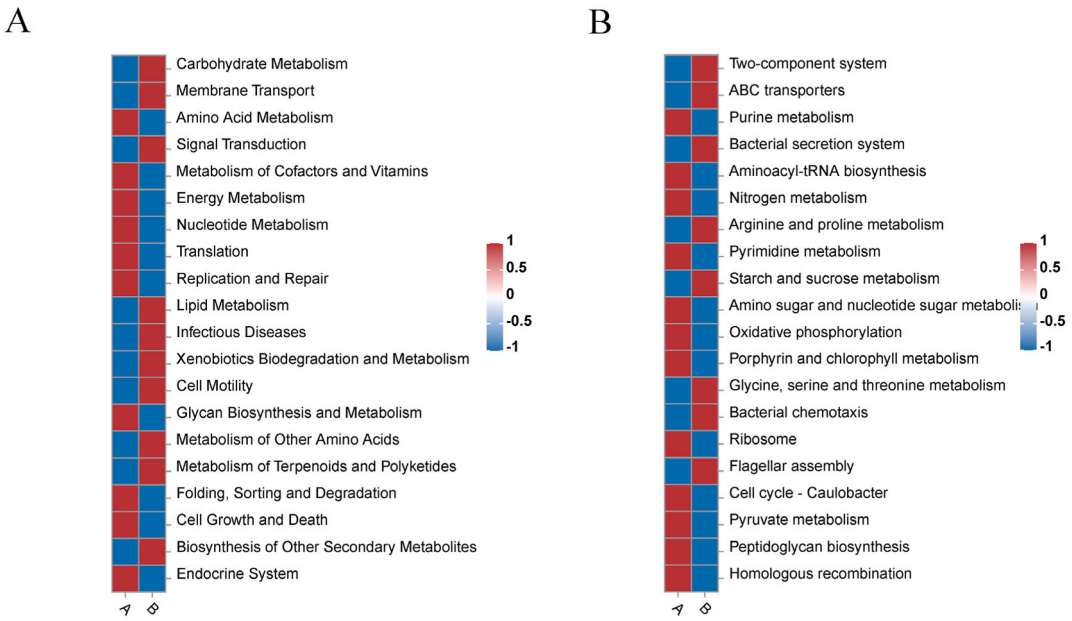

Fig. 5. Functional annotation clustering heat map using Tax4Fun on level 2 (A) and level 3 (B). The color scale reveals indicates the level of abundance in two groups. Groups A and B in the figure represent the control and soybean fermented powder treatment, respectively.

separation. Species richness and species evenness were valuable signs, which are used to reveal species classification and distribution in relative research. In this study, the species richness and evenness of group A were higher than group B. This data showed that LPS mediated reduction in the diversity of intestinal flora to a certain extent.

To predict genes functions, we employed PICRUSt software at level 2 and Tax4Fun tool at levels 2 and 3. At level 2, we used the two methods and found that the KEGG function of intestinal flora was mainly enriched in 'Carbohydrate Metabolism', 'Amino Acid Metabolism', 'Metabolism of Cofactors and Vitamins', 'Energy Metabolism' and 'Replication and Repair, and Lipid Metabolism' pathways in groups A and B. Further analysis of clustering heat map on levels 2 and 3 through Tax4Fun tool, we found that there were significant differences in the abundance of enrichment pathways between groups A and B.

In conclusion, this present study demonstrates that soybean fermented powder alters the LPS-induced intestinal microbial function of loaches. Studying the variance in the structure and distribution of intestinal flora and seeking the possible role of fermented powder of soybeans in intestinal inflammation will help us further realize value-added of fermented powder to prevent resource waste and clearly explore the pathophysiology of intestinal inflammation. Our research lays the foundation for achieving high-quality utilization of soybean fermented powder and improving their added-value, as well as for further analyzing diseases caused by intestinal flora imbalance in freshwater fish.

Data availability

Data associated with this study hasn't been deposited into a publicly available repository. The authors do not have permission to share data.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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