Current Science
CS 5 (5), 4945-4961 (2025)
Received 23 July 2025 | **Revised 13 August** 2025 | **Accepted 10 September** 2025 | **Online Available 21 October** 2025
https://doi.org0/10.52845/CS/2025-5-5-47

Current Science

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OPEN ACCESS

ISSN(O)2795-8639

Original Article

Dynamics of Spoilage Bacteria Succession in *Oreochromis* Surimi Preserved Under Different Low-Temperature Conditions during Cold Chain Transportation

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Abstract:
This study investigates spoilage bacterial community changes in Tilapia (Oreochromis) surimi under different low-temperature conditions using 16S rRNA high-throughput sequencing. Samples were preserved at 4℃ and -20℃ for 20 days. Results showed that at 4℃, initial species richness and evenness were high but decreased over time. Dominant genera included Lactococcus_A, Pseudomonas_E, Psychrobacter and Vagococcus_B, which formed a “small group” with synergistic defense and complementary metabolism, but competed with Acinetobacter and Flavobacterium. At -20℃, only a few psychrotolerant genera like Acinetobacter and Lactococcus_A could survive, maintaining stable richness and evenness. Lactococcus_A was dominant under both temperatures, showing tolerance to low and ultra-low temperatures. Alpha and beta diversity analyses revealed that temperature significantly affected microbial composition and function, with more rapid succession at -20℃. This study provides a theoretical basis for optimizing Tilapia (Oreochromis) surimi’s cold chain transportation and extending shelf life.

Keywords: Tilapia(Oreochromis); Cold chain transportation; Spoilage bacteria; Temperature differences; 16S rRNA sequencing

Introduction

Tilapia(*Oreochromis*), originally from Africa and the Middle East, is a small to medium-sized warm-water fish. It has several advantages, including excellent flesh quality, absence of *intermuscular* bones, a wide range of environmental adaptability, extremely fast growth rate, and strong reproductive capacity. In 1956, Mozambique tilapia was first introduced to China. After years of development, tilapia has now become one of the important farmed fish species. At present, the tilapia industry has formed a four-dimensional pattern characterized by "concentrated production areas, full cold chain process, domestic sales as the main focus, and diversified exports"[1,2].

Tilapia is one of China's important exported aquatic products, and cold chain transportation can maintain its taste and freshness. Based on

temperature classification, there are three types of tilapia refrigeration methods: refrigeration (0–4℃), slight freezing (-3–-2℃), and freezing (≤-18℃). The commonly used transportation modes are chilled transport (0℃–4℃) and frozen cold chain transportation (≤-18℃).As the temperature decreases, the preservation time also increases. For example, domestic refrigeration can preserve tilapia for 1–2 days, while slight freezing can preserve it for 20–27 days.[3,4].Research has shown that there are significant differences in the growth dynamics of spoilage bacteria and the formation of their metabolic products at different temperatures, which in turn lead to changes in the shelf life and sensory quality of fish paste.[5].At low temperatures, the growth rate of spoilage bacteria is significantly reduced, and the lag phase is extended. This allows fish paste to maintain its freshness and better sensory quality for a longer period of time.[6].However, as the temperature rises, the growth rate of spoilage bacteria accelerates and the accumulation of metabolic products increases, leading to a shortened shelf life and a rapid decline in the sensory quality of fish paste.[7].Moreover, over time, the fish meat will inevitably undergo spoilage and deterioration[8].For example, the color of the fish meat will become dull and lose its luster, with spots or discoloration appearing on the surface; the mucus will increase, becoming viscous, and even pus-like substances may adhere to it, accompanied by a mild fishy odor and other characteristics. From the perspective of harmful microorganisms, spoilage bacteria are the main factors causing the deterioration of aquatic products.Under favorable conditions, spoilage bacteria can proliferate rapidly and metabolize to produce putrefactive compounds. For instance, they can break down fish meat proteins into ammonia and amines through protein decomposition and oxidize fats, thereby generating a pungent odor. This process not only degrades the sensory quality of the fish but also poses safety hazards.[9-11].Therefore, this experiment employs high-throughput sequencing of the 16S rRNA gene to investigate the changes in spoilage bacterial communities in fish paste at different time points under low-temperature transportation conditions of 4°C and -20°C, as well as the differences in spoilage bacteria produced under different temperature conditions at the same time point.

Materials and Methods

Experimental Design and Sampling

The tilapia samples used in this experiment were purchased from a fish market in Wuhan, Hubei. The tilapia had a body length of 22.56 cm (±2.87 cm) and a weight of 1145 g (±22.64 g). After purchase, the tilapia were first deboned to remove the obvious fish bones. The fish were then cut into pieces with scissors, and any small bones were removed. The fish pieces were subsequently placed into a blender and processed into fish paste. Finally, the fish paste was filtered through a fine sieve.The fish paste was then divided into Eppendorf (Ep) tubes and separated into two groups, which were stored in refrigerators set at 4°C and -20°C, respectively. This setup was designed to simulate the transportation and storage of fish paste under these two low-temperature conditions. On the day of purchase, one Ep tube was collected from each of the 4°C and -20°C refrigerators and labeled as the Day Zero group. Following this, one Ep tube was randomly collected from each refrigerator daily for a total of twenty days, resulting in twenty-one groups of tilapia fish paste samples.

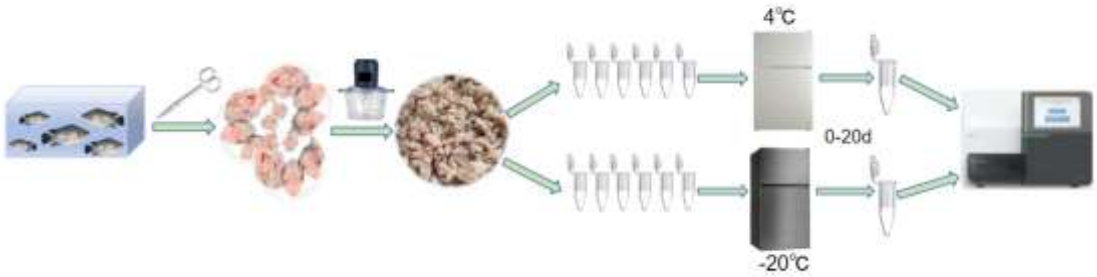

Figure 1. Experimental Flowchart

Gut Microbiota Detection

Bacterial Genomic DNA Extraction

The fish paste samples taken from the refrigerators at 4°C and -20°C were quickly placed into the laminar flow hood. The E.Z.N.A.® Stool DNA Kit (D4015, Omega Bio-tek, USA) was used to extract DNA from different samples according to the manufacturer's instructions, with RNase-free water as the negative control. The purified bacterial genomic DNA was eluted with 50 μL elution buffer and stored at -80°C. The concentration and purity (A260/A280 ratio) of the DNA were measured using a NanoDrop spectrophotometer, and the integrity of the DNA was checked by 1.2% agarose gel electrophoresis to ensure that the extracted DNA met the requirements for subsequent PCR amplification.

16S rRNA gene PCR Amplification

The PCR amplification was performed using TransGen Pfu high-fidelity DNA polymerase, which is known for its high fidelity that helps in accurately copying the template and reducing amplification errors. The number of cycles was strictly controlled (minimized and kept consistent within the same batch of samples) to enhance sequencing accuracy. A negative control was set up to detect contamination, and any sample group showing amplification bands in the negative control was discarded. The primers had sample-specific barcodes and universal sequencing primers at the 5' end. The 25 μL reaction mixture consisted of: 25 ng of bacterial genomic DNA template, 2.5 μL of forward primer, 2.5 μL of reverse primer, 12.5 μL of PCR premix, and the remaining volume was filled with PCR water. The amplification program was set as follows: initial denaturation at 98°C for 30 seconds; followed by 32 cycles, each including denaturation at 98°C for 10 seconds, annealing at 54°C for 30 seconds, and extension at 72°C for 45 seconds; and a final extension at 72°C for 10 minutes. The PCR products were confirmed by 2% agarose gel electrophoresis. A volume of 25 μL of the PCR product was purified using approximately 0.8 times the volume of Vazyme VAHTSTM DNA Clean Beads. After confirmation by 2% agarose gel electrophoresis, 25 μL of the PCR product was purified with 0.8 times the volume of Vazyme beads and quantified using a Microplate reader (BioTek FLx800) in combination with Quant-iT PicoGreen dsDNA reagent. The library was prepared using the Illumina TruSeq Nano kit and sequenced on the NovaSeq PE250 platform.

16S rRNA Data Analysis

For the raw paired-end sequencing data (PE250) generated by the Illumina NovaSeq platform, the first step is to conduct quality assessment using FastQC or FastP. This involves checking key metrics such as base quality (with a Phred score ≥20 being acceptable), sequence length (theoretically 250bp), and GC content. Subsequently, the Trimmomatic software is employed to remove adapters by setting the ILLUMINACLIP parameter, trim low-quality bases at both ends using the LEADING and TRAILING parameters, and perform sliding wind

ow quality filtering with the SLIDINGWINDOW parameter. Sequences with a length of ≥150bp are retained to obtain high-quality clean data. Then, b ased on sample-specific barcode and index inform ation, the QIIME2 demux plugin or Cutadapt tool is used to assign sequences to corresponding sam ples and remove barcode sequences. The dada2 pi peline in QIIME2 can be utilized to denoise the q uality-controlled sequences and generate high-pre cision amplicon sequence variants (ASVs). Altern atively, the Vsearch software can be used to clust er sequences into operational taxonomic units (O TUs) at a 97% similarity threshold. Finally, the SI LVA database (or Greengenes database) is emplo yed to annotate the taxonomy of the obtained AS Vs/OTUs, thereby completing the entire analysis process from raw data to species information.

The following analyses were conducted based on the annotation results:(1) Alpha Diversity Analysi s: The Chao1 richness index and Shannon diversit y index were calculated for each sample, and raref action curves were plotted to assess sequencing d epth.(2) Beta Diversity Analysis: A distance matri x based on Bray-Curtis dissimilarity was calculate d, and principal coordinates analysis (PCoA) and UPGMA clustering were performed. The significa nce of differences between groups was tested usin g PERMANOVA.(3) Taxonomic Composition an d Differential Analysis: The relative abundance of taxa at different taxonomic levels (from phylum t o genus) was calculated. The LEfSe method was used to identify differentially abundant taxa betwe en different transport time groups.(4) Statistical A nalysis of Microbial Abundance: The relative abu ndance of dominant bacterial taxa in fish paste sa mples under different temperatures and storage ti mes was analyzed to identify patterns of microbia l abundance and composition changes. One-way ANOVA was used to compare microbial abundan ce and diversity indices among different treatment groups, with $P<0.05$ indicating statistical significa nce.(5) Microbial Co-occurrence Network Analys is: Spearman correlation coefficients between tax a at the genus level were calculated to construct c o-occurrence networks and identify key nodes.(6) Functional Potential Prediction: The PICRUSt2 to ol was used to predict the KEGG metabolic pathw ay functions of microbial communities. All statisti cal analyses and visualizations were performed in the R programming environment[12].

Results

The sequence length distribution plot can detect data outliers and assess sequencing depth and integrity [13],Under both 4°C and -20°C conditions, the sequence lengths were concentrated in the range of 400 to 450 bp, indicating good data integrity. This provides a crucial basis for quality control and hypothesis generation for subsequent analyses (Figure 2A-B). From the taxonomic annotation results, the x-axis is arranged according to days, and the y-axis represents the number of ASVs/OTUs that can only be classified at each taxonomic level (phylum, class, order, family, genus, species) in each sample. The figure shows that under both 4°C and -20°C conditions, the bars representing the genus and species levels account for the majority of the total bar length, while the phylum level bars account for a smaller portion, indicating high-resolution annotation (Figure 2C-D).

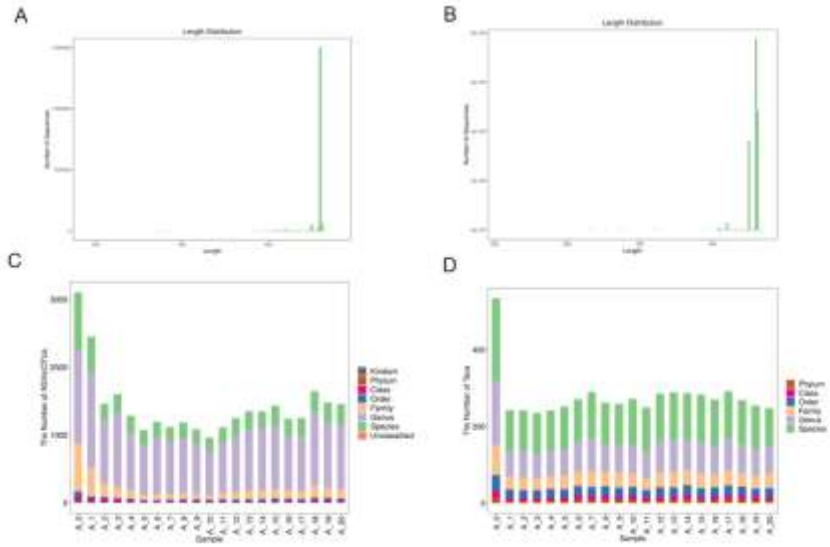

Figure 2. Statistics of Sequence Length Distribution and Taxonomic Annotation
A. Sequence length distribution at 4°C
B. Sequence length distribution at -20°C
C. Taxonomic annotation results at 4°C
D. Taxonomic annotation results at -20°C

At the phylum level, under both 4°C and -20°C conditions, the top three most abundant phyla were *Firmicutes*_D, *Proteobacteria*, and *Bacteroidota*. At 4°C, the relative abundance of *Firmicutes*_D rapidly increased from Day 0 to Day 5, while that of *Proteobacteria* and *Bacteroidota* quickly decreased during the same period and remained stable from Day 6 to Day 20. At -20°C, the relative abundance of *Firmicutes*_D slowly increased from Day 0 to Day 4, *Bacteroidota* gradually decreased during this period, and *Proteobacteria* maintained a stable relative abundance from Day 0 to Day 9 (Figure 3A-B).

At the genus level, under 4°C conditions, the top three most abundant genera were *Lactococcus*_A, *Psychrobacter*, and *Pseudomonas*_E. Their relative abundances rapidly increased in the first three days and then remained relatively stable from Day 4 to Day 20 (Figure 3C). Under -20°C conditions, the top three most abundant genera were *Acinetobacter*, *Lactococcus*_A, and *Flavobacterium*. The relative abundance of *Acinetobacter* and *Lactococcus*_A quickly increased on Day 2, but while *Acinetobacter*'s relative abundance gradually decreased from Day 1 to Day 5, *Lactococcus*_A continued to rise during this period and remained stable until Day 20. *Flavobacterium* maintained a stable relative abundance throughout the entire 20-day period (Figure 3D).

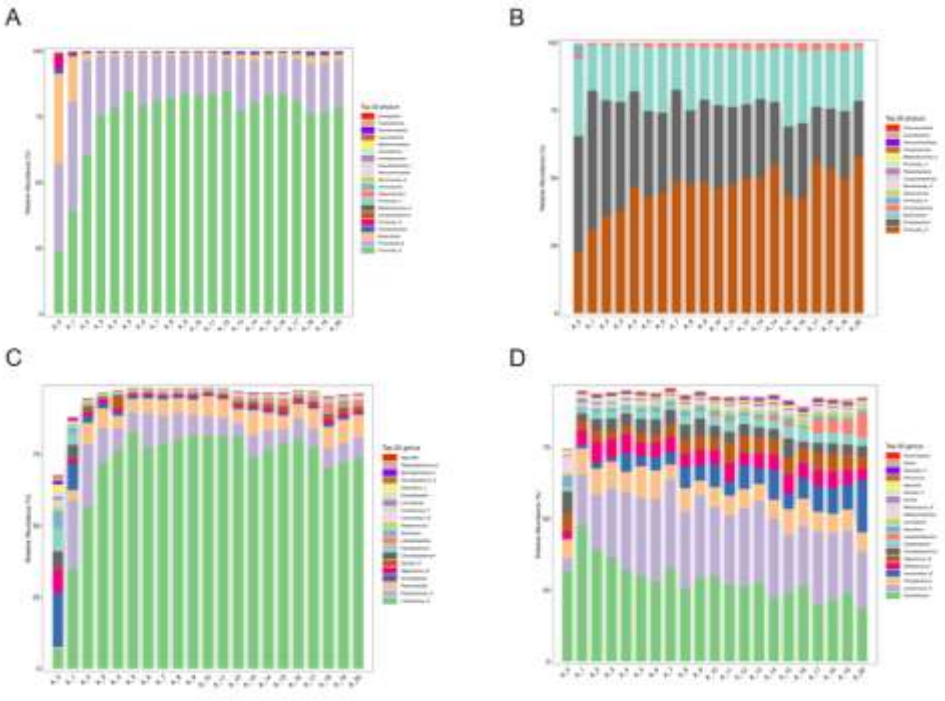

Figure 3. Analysis of the Species Composition of Tilapia Fish Paste Microbiota
A. Analysis of the phylum-level composition of fish paste microbiota at 4°C
B. Analysis of the phylum-level composition of fish paste microbiota at -20°C
C. Analysis of the genus-level composition of fish paste microbiota at 4°C
D. Analysis of the genus-level composition of fish paste microbiota at -20°C

To fully reveal the structural characteristics and ecological functions of microbial communities, we conducted alpha diversity analysis. In the abundance rank curve plot, the degree of flatness of the line reflects the evenness of the community composition. The flatter the line, the smaller the abundance differences among ASVs/OTUs, indicating higher community evenness. At 4°C, the lines for Day 0 and Day 1 were steeper compared to other days. At -20°C, the line for Day 0 was steeper than other days. This indicates that the relative abundance of species changed significantly on these days. From Day 2 to Day 20, the species curves were flatter, suggesting that the community composition became more stable (Figure 4A-B).

Alpha diversity reflects the abundance and diversity of microbial communities. The Chao1 index was higher at 4°C than at -20°C on most days, such as from Day 0 to Day 9 and from Day 11 to Day 20. This indicates that the community abundance was higher at 4°C on most days (Figure 4C). The Shannon index is influenced by both species richness and relative abundance (evenness). The more species there are, the higher the Shannon index. The Simpson index primarily measures "relative abundance" and is not sensitive to "how many species" there are. Even if there are many species, if a few dominate, the index will still be high. The figure shows that the Shannon index at -20°C was initially lower than at 4°C, but the Shannon index at 4°C continuously decreased, while the Shannon index at -20°C remained unchanged. As a result, it surpassed the 4°C index on Day 2 to Day 3. This indicates that there were more species in the fish paste at 4°C in the first two days, but the number of species decreased subsequently, and the dominant species became more dominant. In contrast, the number of species in the fish paste at -20°C remained almost unchanged, with only a

few species dominating (Figure 4D-E).

The Pielou_e index is higher (closer to 1) when the community evenness is higher. The Pielou_e index was higher at -20°C than at 4°C, indicating higher community evenness at -20°C (Figure 4F). The Observed_species index is higher when the number of detected actual species (or ASVs/OTUs) is greater, indicating higher alpha diversity of the community. The Observed_species index at -20°C and 4°C fluctuated over the 20 days, but their means were similar, indicating that the number of detected actual species was almost the same (Figure 4G). The Good's coverage index represents the coverage. The index values for both -20°C and 4°C were close to 1, indicating that the sequencing depth was sufficiently high, and almost all species were detected. The number of species that appeared only once was very low, indicating good data coverage (Figure 4H).

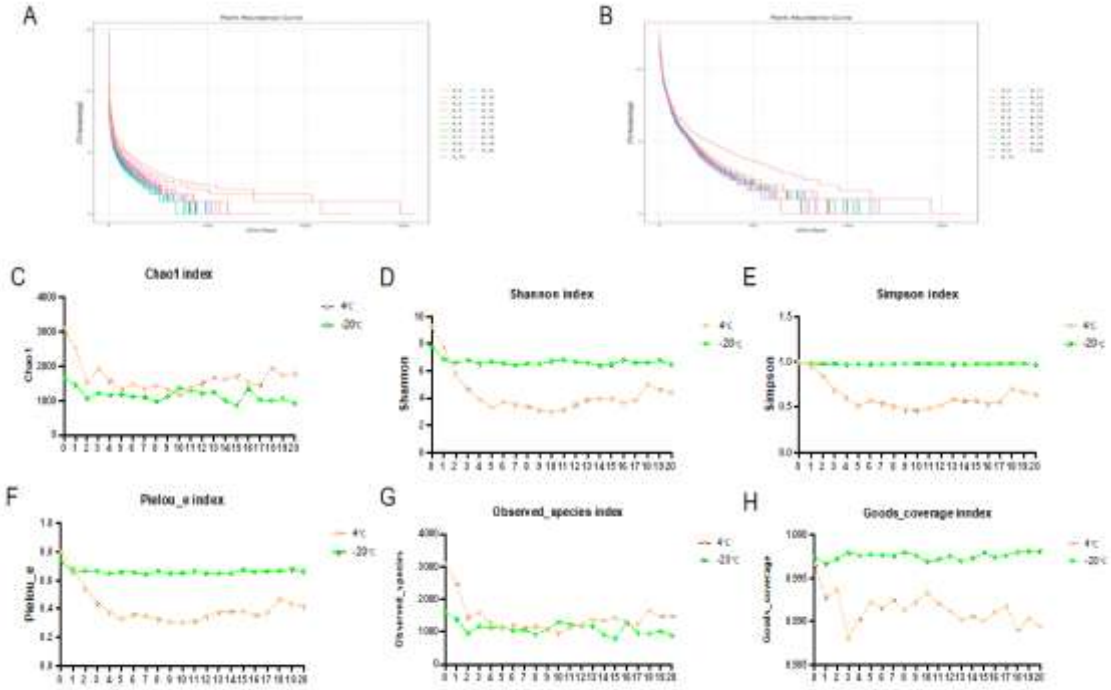

Figure 4. Alpha Diversity Analysis of Tilapia Fish Paste Microbiota
A. Abundance rank curve at 4°C
B. Abundance rank curve at -20°C
C. Chao1 index D. Shannon index E. Simpson index F. Pielou_e index G. Observed_species index H. Good's coverage index

The results of beta diversity analysis indicate that under 4°C conditions, the projection distance of the Day 0 group on the coordinate axis is the farthest from the other groups, with the Day 1 group being the second farthest. This suggests that the microbial community composition on these two days is quite different and that the microbial species are undergoing rapid succession. Starting from Day 5, the projection distances of the subsequent groups become more concentrated, indicating that the community composition becomes more stable (Figure 5A). In the UPGMA clustering analysis, Day 1 and Day 0 form one cluster, Day 7 and Day 5 form another, and Day 12 and Day 15 form another, which also indicates that the microbial community composition tends to become more consistent over time (Figure 5C).

Under -20°C conditions, the projection distance of the Day 0 group on the coordinate axis is again the farthest from the other groups, with the Day 1 group being the second farthest. However, the projection distances of Day 2 and Day 3 begin to become more concentrated, indicating that microbial succession occurs more rapidly at this temperature compared to 4°C conditions. The projection distances of Day 5 and Day 7, as well as Day 8, Day 11, and Day 13, are very close, indicating that the microbial community composition also stabilizes (Figure 5B). Similarly, in the UPGMA clustering analysis, Day 6 and Day 8 form one cluster, and Day 17 and Day 18 form another, indicating that the microbial community composition tends to become more consistent over time (Figure 5D).

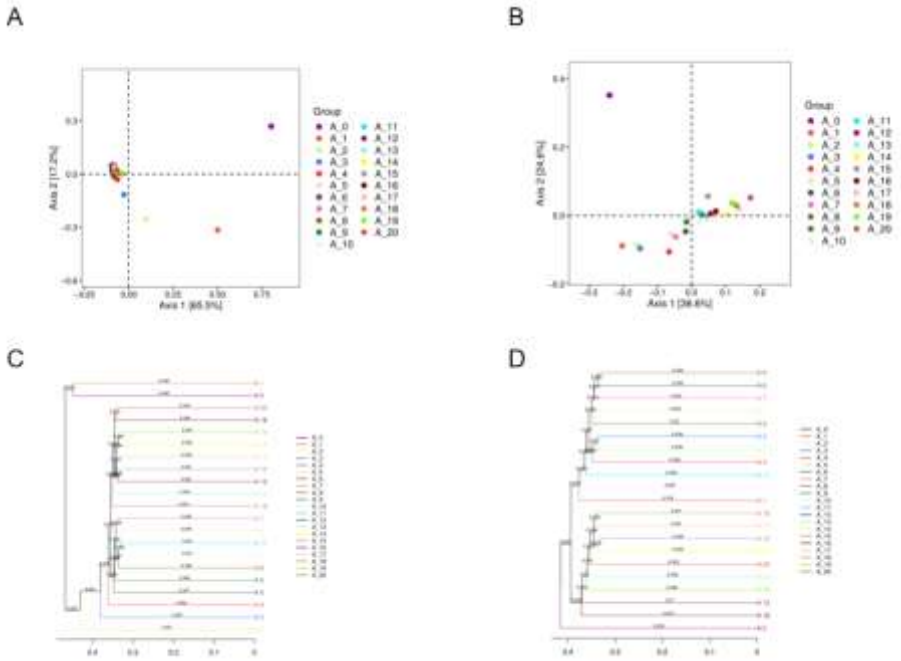

Figure 5. Beta Diversity Analysis of Tilapia Fish Paste Microbiota
A. PCoA plot at 4°C
B. PCoA plot at -20°C
C. UPGMA clustering at 4°C
D. UPGMA clustering at -20°C

To further explore the key factors causing differences in microbial community composition, we conducted differential species analysis. The Venn diagram revealed that under 4°C conditions, the number of shared ASVs/OTUs among all samples (groups) was 77, while under -20°C conditions, it was 179. Excluding the initial days with significant species changes at 4°C, most samples (groups) had fewer than 300 ASVs/OTUs, whereas under -20°C conditions, all samples (groups) had more than 300 ASVs/OTUs (Figure 6A/C). This indicates that -20°C exerts stronger selective pressure on microbial communities. In the genus-level heatmap of species composition, under 4°C conditions, genera such as *Soonwooa*, *Vagococcus*_B, and *Acinetobacter* had lower abundance in the early stages of transport compared to later stages, while *Leuconostoc*_B, *Pantoea*_A, and *Psychrobacter* had higher abundance in the early stages (Figure 6B). Under -20°C conditions, genera such as

Soonwooa, *kaistella*, and *Deinococcus*_D had lower abundance in the early stages, whereas *Leuconostoc*_B, *Lactiplantibacillus*, and *Levilactobacillus* had higher abundance in the early stages (Figure 6D). The shift in the ranking of genus abundance indicates the occurrence of microbial succession.

In the differential genus plot, we selected the top six most abundant genera and one genus, *Flavobacterium*, which showed significant differences due to temperature. These selected genera primarily represent the main composition of microbial communities under the respective temperature conditions and include genera that appear under both low-temperature conditions. Ranked by abundance, under 4°C conditions, the genera were *Lactococcus*_A_343306, *Pseudomonas*_E_647464, *Psychrobacter*, *Acinetobacter*, *Vagococcus*_B, *Serratia*_D_727245, and *Flavobacterium*. The genus *Lactococcus*_A_343306 maintained a dominant position from Day 1 to Day 20, with a significant lead over others. Its growth rate was similar to that of *Pseudomonas*_E_647464 and *Psychrobacter*, while the remaining genera showed little change (Figure 7A-B). Under -20°C conditions, the genera were *Acinetobacter*, *Lactococcus*_A, *Flavobacterium*, *Leuconostoc*_B, *Streptococcus*, *Vagococcus*_B, and *Chryseobacterium*. *Acinetobacter* and *Lactococcus*_A had the highest abundance, with a significant difference from other genera. *Leuconostoc*_B showed an increasing trend over the 20 days, with a sharp rise in relative abundance on the last day, aligning with the abundance of *Acinetobacter* and *Lactococcus*_A. Other genera exhibited only minor fluctuations in abundance (Figure 7C-D).

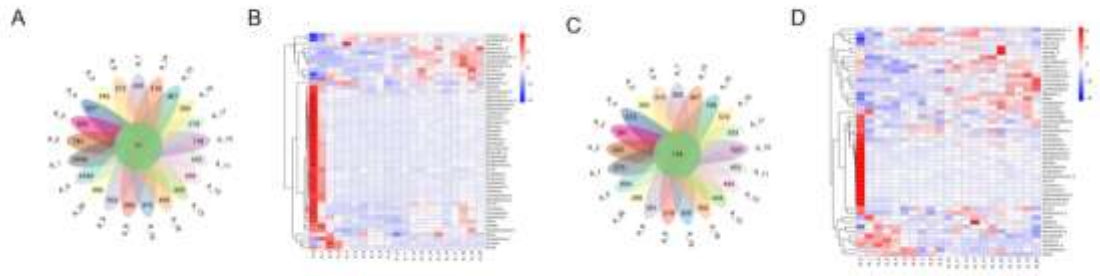

Figure 6. Differential Species Analysis of Tilapia Fish Paste Microbiota
A.ASV/OTU Venn diagram at 4°C
B. Genus-level species composition heatmap at 4°C
C ASV/OTU Venn diagram at -20°C
D. Genus-level species composition heatmap at -20°C

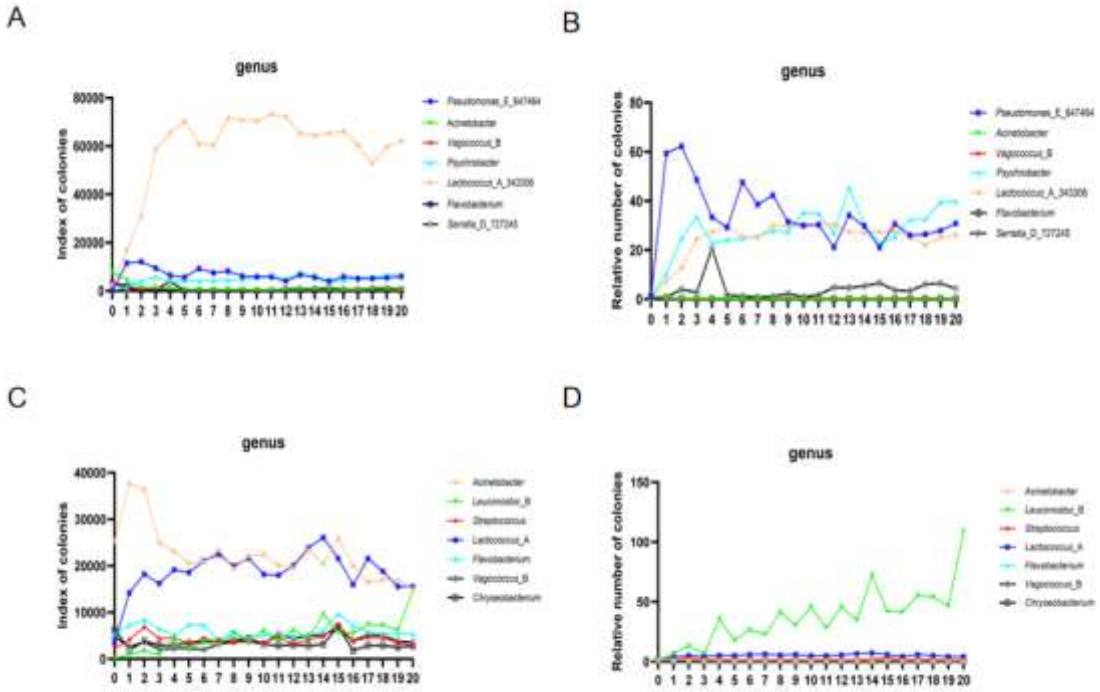

Figure 7. Differential Genus Analysis of Tilapia Fish Paste Microbiota
A. Absolute abundance of differential genera at 4°C
B. Relative abundance of differential genera at 4°C
C. Absolute abundance of differential genera at -20°C
D. Relative abundance of differential genera at -20°C

Correlation analysis was performed on the selected genera. Under 4°C conditions, the genera *Lactococcus*_A_343306, *Vagococcus*_B, *Pseudomonas*_E_647464, and *Psychrobacter* showed positive correlations with each other. *Acinetobacter* was positively correlated with *Flavobacterium*, while *Vagococcus*_B was negatively correlated with both *Acinetobacter* and *Flavobacterium* (Figure 8A). Under -20°C conditions, the strongest positive correlation was between *Chryseobacterium* and Streptococcus, while the strongest negative correlation was between *Lactococcus*_A and Streptococcus (Figure 8B).

In the KEGG level 2 functional pathway abundance plot, functional prediction analysis indicated that under both 4°C and -20°C conditions, the metabolic pathways with higher abundance in the "Metabolism" category included Amino acid metabolism, Carbohydrate metabolism, and Xenobiotics biodegradation and metabolism. In the "Human Diseases" category, the Infectious diseases pathway had a higher abundance (Figure 8C-D).

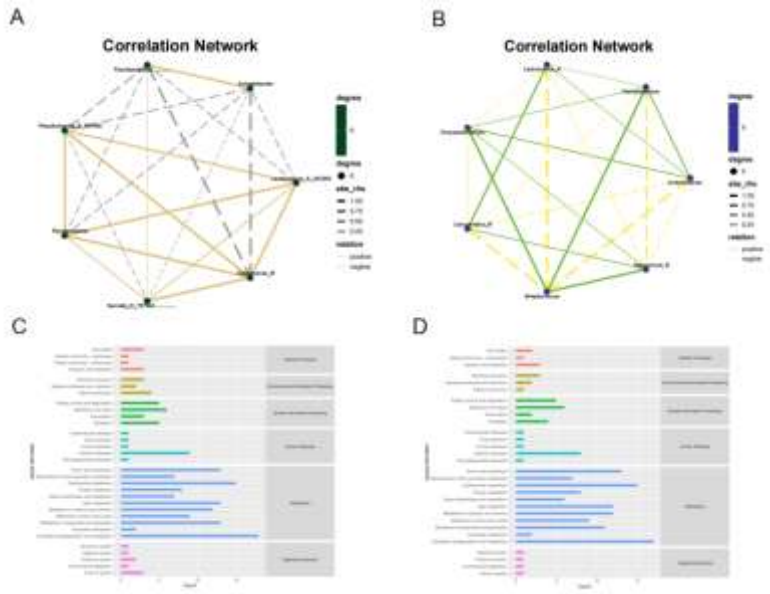

Figure 8. Differential Genus Correlation Network Analysis and Functional Potential Prediction of Tilapia Fish Paste Microbiota
A. Correlation network analysis of differential genera at 4°C
B. Correlation network analysis of differential genera at -20°C
C. KEGG metabolic pathway analysis at 4°C
D. KEGG metabolic pathway analysis at -20°C

Discussion

This study employed full-length 16S rRNA sequencing to analyze the microbial communities in fish paste samples stored for 20 days under 4°C and -20°C conditions. The findings revealed that in the initial stages of storage, the abrupt temperature drop from room temperature to low temperatures significantly altered microbial α-diversity (Chao1, Shannon, Simpson, Observed_species, Pielou_e, and Good's coverage indices) and species abundance, and also led to marked differences in community structure (β-diversity) (Figures 4-5). The vast majority of microbial communities that thrive at room temperature were eliminated. For instance, *Bacteroides*_E and *Clostridium*, which are typical room-temperature dominant genera with an optimal growth temperature range of 30°C to 37°C [14].experienced a dramatic decline in relative abundance. At 4°C, the relative abundance of *Bacteroides*_E decreased nearly 170-fold from Day 0 to Day 8, while that of *Clostridium* decreased 150-fold over the same period. At -20°C, the relative abundance of *Bacteroides*_E dropped from 1.7% on Day 0 to 0% by Day 12, and that of *Clostridium* fell from 1.6% on Day 0 to 0% by Day 3. Cold-tolerant species quickly took over the dominant positions. Compared to the 4°C condition, microbial changes and adaptation occurred more rapidly under -20°C conditions (Figures 3-4). Meanwhile, the same genera were still present at different temperatures, but their abundances varied significantly. For example, *Acinetobacter*, which had the highest abundance under -20°C conditions, had its abundance reduced by several hundred folds under 4°C conditions. Similarly, the abundance of *Flavobacterium* and *Vagococcus*_B was tens of times higher at -20°C than at 4°C. However, under 4°C conditions, the genera *Psychrobacter*, *Lactococcus*_A_343306, *Vagococcus*_B, and *Pseudomonas*_E_647464 exhibited positive correlations with each other. Studies have shown that *Lactococcus*_A_343306 produces lactic acid, which lowers the

environmental pH. This lactic acid can also be utilized by other genera. For instance, *Psychrobacter*, *Pseudomonas*_E_647464, and *Vagococcus*_B can use lactic acid as a carbon source for their metabolic activities[15,16].Moreover, *Psychrobacter* and *Pseudomonas*_E_647464 produce antimicrobial substances that inhibit the growth of other spoilage bacteria[17].*Psychrobacter* can break down organic compounds that are difficult for other genera to utilize at low temperatures, while *Pseudomonas*_E_647464 can decompose complex organic compounds to produce small-molecule metabolites that can be utilized by *Lactococcus*_A_343306 and *Vagococcus_B*[18].This forms a "small group" with synergistic defense and complementary metabolism. Meanwhile, *Acinetobacter* and *Flavobacterium* also showed a positive correlation, but these two "small groups" had a negative correlation with each other (Figure 7). In the differential genus plot (Figure 7), from Day 0 to Day 3, the abundance of *Lactococcus*_A_343306, *Psychrobacter*, and *Pseudomonas*_E_647464 increased, while that of *Flavobacterium* and *Acinetobacter* decreased, suggesting a competitive or inhibitory relationship between them.

Under 4°C conditions, the dominant genus of spoilage bacteria on Day 0 was *Acinetobacter*. *Acinetobacter* is a Gram-negative bacterium with an optimal growth temperature range of 28°C to 30°C. This strain can still grow at low temperatures (such as 4°C), albeit at a slower rate. It is widely found in refrigerated foods, including mackerel, and is an important spoilage bacterium in refrigerated aquatic products [19,20]. From Day 1 onwards, the abundance of *Lactococcus*_A_343306 increased sharply while the abundance of Acinetobacter decreased rapidly (Figure 3C). This is because the temperature drop caused a dramatic change in the abundance of Acinetobacter [21], and the metabolic products of *Lactococcus*_A_343306 (such as lactic acid) can inhibit the growth of *Acinetobacter* [22]. Subsequently, *Lactococcus*_A_343306 remained the dominant species. *Lactococcus*_A_343306 is a Gram-positive cocci, belonging to lactic acid bacteria. These bacteria typically play an important role in fermentation, producing lactic acid through the fermentation of sugars, which inhibits the growth of other harmful microorganisms [23]. Generally, *Lactococcus*_A_343306 is considered a beneficial bacterium. However, in this experiment, due to its overgrowth, it produced a large amount of organic acids and other metabolic products, leading to an increase in the acidity of the fish paste, thereby acquiring spoilage capabilities[24]. *Pseudomonas*_E_647464 is a Gram-negative bacillus that can grow well in neutral to slightly acidic low-temperature environments. It can decompose proteins, fats, and carbohydrates, producing off-flavors and bad odors that cause food spoilage and is one of the main spoilage bacteria in aquatic products[25]. *Psychrobacter* is a Gram-negative coccobacillus widely distributed in cold environments. It can influence lipid spoilage processes and hydrolyze amino acids to produce off-flavors [26]. *Vagococcus*_B is a Gram-positive cocci, usually arranged in pairs or short chains, with a certain degree of acid tolerance, and grows well in neutral to slightly acidic environments [27]. These genera form a "small group." These genera mutually defend against each other and share common adaptive traits, such as acid tolerance and cold tolerance, allowing them to coexist. *Flavobacterium* is a Gram-negative bacterium with cold-resistant characteristics and can grow under refrigerated conditions[28]. *Flavobacterium* and *Acinetobacter* also form a "small group." Fish paste contains abundant nutrients such as proteins, carbohydrates, lipids, and minerals. These resources and living spaces are limited, and microbial communities must compete to obtain sufficient nutrients and necessary living space to

support their growth and metabolism[29]. Meanwhile, *Pseudomonas*_E_647464 can produce antibiotics and siderophores, which can inhibit the growth of *Acinetobacter* and *Flavobacterium* but do not negatively affect *Lactococcus*_A_343306, *Vagococcus*_B, and *Psychrobacter*, and may even provide some protection[30]. This further intensifies the competition. However, *Acinetobacter* and *Flavobacterium* have relatively weak competitive abilities in the spoilage bacterial community and are in a disadvantaged position, but they together maintain the ecological balance of the microbial community.

Under -20°C conditions, the dominant spoilage bacterial communities commonly found in the transportation of ultra-low-temperature freshwater products mainly include genera such as *Acinetobacter*, *Lactococcus*_A, *Flavobacterium*, and *Psychrobacter*[31], which are consistent with the dominant genera observed in this experiment. *Flavobacterium* is a Gram-negative coccobacillus that can decompose proteins and fats, producing off-flavors and bad odors that lead to food spoilage [32]. In the early stages of spoilage (Days 0-4), the abundance of most spoilage bacteria initially increased and then decreased. This was due to the temperature change, which caused the death of many mesophilic bacteria, such as *Soonwooa* and *kaistella*. This provided some living space for the growth of psychrotolerant spoilage bacteria, such as *Acinetobacter*, which then dominated in the new environment[33]. As the abundance of spoilage bacteria increased, the acidic substances (such as lactic acid) produced by the metabolism of spoilage bacteria (such as *Lactococcus*_A) led to a decrease in the pH value of fish paste. This acidic environment was unfavorable for the growth of some spoilage bacteria, such as *Acinetobacter* and *Flavobacterium*, resulting in a decline in their numbers[34]. In the middle stages of spoilage (Days 5-11), all spoilage bacteria fluctuated within a certain range, reaching a stable growth phase. At this time, the dominant species were *Acinetobacter* and *Lactococcus*_A. In the later stages of spoilage (Days 12-20), the abundance of *Leuconostoc*_B slowly increased and became one of the dominant spoilage bacteria in the later period (Figure 6). *Leuconostoc*_B, a type of lactic acid bacteria, can produce acid, which inhibits the growth of other genera, ultimately leading to a rapid decline in other genera[35]. From Figure 7, it can be seen that under 4°C conditions, *Lactococcus*_A_343306 was the absolute dominant species over the 20 days, while under -20°C conditions, *Acinetobacter* and *Lactococcus*_A were the absolute dominant species over the 20 days. The difference in temperature led to differences in dominant species [36]. The fact that the same genera remained dominant under different temperatures indicates that *Lactococcus*_A has the ability to tolerate low and even ultra-low temperatures. According to the correlation network analysis (Figure 8), under 4°C conditions, *Flavobacterium* was negatively correlated with the main genera such as *Lactococcus*_A_343306, *Pseudomonas*_E_647464, and *Psychrobacte*r. Under -20°C conditions, *Flavobacterium* was positively correlated with the dominant species *Acinetobacter* and *Lactococcus*_A. Since *Pseudomonas*_E_647464 and *Psychrobacter* were dominant bacterial communities under 4°C conditions, their abundance levels would affect the abundance of *Flavobacterium* to some extent[37]. In contrast, under -20°C conditions, they promoted the growth of *Flavobacterium*, resulting in a completely different abundance of *Flavobacterium* under the two temperatures (Figure 6). Under 4°C conditions, the species richness and evenness of the microbial communities were higher in the early stages of the experiment but gradually decreased, indicating that low temperature has a significant impact on the dynamic changes of microbial communities.

Under -20°C conditions, the species richness and evenness of the microbial communities were relatively stable, indicating that extreme low temperature exerts stronger selective pressure on microbial communities, with only a few genera able to maintain higher relative abundance in such an environment.

Conclusions

This study comprehensively analyzed the microbial community structure and dynamics of tilapia fish paste under 4°C and -20°C conditions using high-throughput sequencing technology. The results demonstrated that temperature significantly affects the dynamics of microbial communities, with different selective pressures on microbial communities under varying temperature conditions. Only a few genera can maintain higher relative abundance in specific environments. Under 4°C conditions, *Lactococcus*_A_343306 was one of the dominant genera, and its metabolic products significantly inhibited the growth of other genera such as *Acinetobacter*. Due to its high abundance, *Lactococcus*_A_343306 exhibited spoilage capabilities. A "group" formed by *Psychrobacter*, *Lactococcus*_A_343306, *Vagococcus*_B, and *Pseudomonas*_E_647464 competed with another "group" formed by *Acinetobacter* and *Flavobacterium* for resources. The "group" formed by *Acinetobacter* and *Flavobacterium* had weaker competitive ability, lower abundance, and was in a disadvantaged position. Under -20°C conditions, *Acinetobacter* and *Lactococcus*_A were the main dominant genera, but in the later stages of spoilage, the abundance of *Leuconostoc*_B continuously increased, becoming the dominant spoilage genus in the later period. The abundance of *Flavobacterium* under different temperatures was affected by other dominant genera. This study provides valuable references for targeting dominant spoilage bacterial species in the low-temperature transportation of fish paste.

Author Contributions: Data Collation: Qibiao Xiao, Zhuoqi Ying. Official Analysis: Qibiao Xiao, Juzhe Wu, Shengao Xiao. Resources: Zhuoqi Ying,Zhang Chi. Software: Qibiao Xiao, Junzhe Wu,Zhuoqi Ying.Supervision: Zhuoqi Ying. Verification: Zhang Chi, Zhuoqi Ying. exceptional. Draft Writing: Qibiao Xiao, Junzhe Wu, Shengao Xiao. Review and Editing: Zhang Chi, Zhuoqi Ying.

Declaration of Conflict of Interest: The authors declare that they have no conflict of interest in this work.

Acknowledgements: This work was supported by project of Hubei Key Laboratory of Animal Nutrition and Feed Science, Wuhan Polytechnic University (202405); The Research and Innovation Initiatives of WHPU (2023); Science and Technology Research Program of Chongqing Municipal Education Commission (KJZD-K 202403501) Chongqing Wanzhou District Science and Technology Plan Project wzstc20230212.

Funding: This work was supported by National Key Research and Development Program (2022YFF1100902)

Availability of Data and Materials: The data associated with this paper are publicly available to readers.

Competing interests: The authors declare no conflicts of interest.

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