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Deciphering Microbial Diversity and Functional Codes of Traditional Fermented Whole Grain Tianpei from Typical Regions of China

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Abstract: Whole grains are a crucial part of healthy and sustainable diets, attracting great attention. Tianpei is a popular traditional fermented whole grain food and beverage from China. It is suitable for all ages with lots of health benefits. However, its microflora and their functions, relations between microbial taxa and functions with Tianpei properties, were still little informed, limiting the fermentation optimization and quality improvement. In this study, the characteristics and distribution of the microbial flora taxa and their functions of the fermented whole grain Tianpei from typical regions of China were mainly deciphered through metagenomic methods. Phyla Mucoromycota, Firmicutes, Ascomycota, and Proteobacteria were the most abundant. *Rhizopus*, *Limosilactobacillus*, and *Lactobacillus* were the most abundant genera. Microbial COG functions carbohydrate transport and metabolism (mainly including fructose, galactose, glucose, L-arabinose, and mannose) and amino acid transport and metabolism (mainly including arginine, asparagine, glutamine, and glycine) kept a high abundance. PCA (Principal Component Analysis) illustrated that the microbial community and their functions of every Tianpei sample clustered individually based on the analysis, related with the factors of raw material and sources. The microbial taxa, microbial functions, and the Tianpei properties were significantly correlated. *Rhizopus*, *Limosilactobacillus*, and *Lactobacillus* contributed most COG functions in Tianpei samples. Analysis of quorum sensing, pfam, secretion protein, probio, and cytochromes P450 were also annotated and found among Tianpei microbial communities. A sum of 105 probiotics were classified, mainly belonging to *Lactobacillus*, *Leuconostoc*, *Acetobacter*, *Bacillus*, *Bifidobacterium*, *Pediococcus*, etc. Tianpei samples made in the library with the most abundant and functional microbial key taxa strains—*Rhizophus oryzae*, *Lactobacillus plantarum*, and *Limosilactobacillus fermentum*—showed rich nutrient chemicals. The results indicate that microbial taxa and their functions could determine Tianpei properties. Thus, the quality, nutrients, flavor, and industrial production of Tianpei could be further investigated, promoted, and improved in the future based on the characteristics of these microbial taxa and their functions, such as the regulations of the main carbohydrate and AA. The study will also lay a foundation for the fermentative characteristics and condition technology of fermented whole grain food.

Keywords: fermented whole grain; microbial diversity; Illumina sequencing; genomic functions; nutrient properties

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1. Introduction

Whole grains could be an essential part of healthy and sustainable diets and the direction of food development [1,2]. Whole grains contain rich dietary fiber and varieties of biological active ingredients, such as polyphenols, oryzanol, β-glucan, etc. [3–6]. The nutritional health benefits of whole grains are far superior to those of refined grains [3–6]. Long-term consumption of whole grains is beneficial to reduce blood lipids, enhance immunity, inhibit obesity, and other health effects, and growing researchers reported a reduction in stroke, heart disease, type II diabetes, colorectal cancer, a reduction of the risk of breast cancer and inflammation, and a slow aging of whole grains [7–9]. Its health promotion roles have reached international consensus, conforming to the people's higher pursuit of the diets "Natural, green, healthy" [3–6]. Whole grains could also greatly reduce environmental impacts [10]. More and more researchers and people are paying their attentions to the whole grains [3–9]. In 2021, the Whole Grain Initiative published the global definitions of whole grain and whole-grain foods. Whole grains should constitute the intact, ground, cracked, flaked, or otherwise processed kernel after removing the inedible parts, and whole-grain food should contain $\geq$50% whole-grain ingredients by dry weight [11]. Whole grain food has the problems of rough taste, poor palatability, poor quality stability, and difficult storage, as well as less variety of related deep-processed products and less public acceptance of whole grains, etc. [12]. It is urgent to strengthen the research and development of whole grains and related products and improve the utilization rate of whole grain resources.

Studies have shown that fermentation can affect food values by increasing nutrient content, improving biomass utilization, or reducing anti-nutrient content [13–16]. Fermentation could significantly increase the variety and content of bioactive substances of whole grain products, significantly enhance the functional activity, and enhance the taste and flavor of whole grain products [13–18], playing vital roles on improving protein solubility, texture, and the nutritional value of the products [14–20]. Fermentation technology has a good application prospect in whole grain processing. Fermentation can be used as an effective technical means to develop new types of whole grain foods and improve their nutritional and health properties, provide whole grain fermented foods to consumers with different nutritional needs, such as vegetarianism, lactose intolerant, high cholesterol, obese, etc. [15–20].

As the traditional fermented whole grain food and beverage from China, Tianpei is mainly made of oats, highland barley, and brown rice; it contains abundant microbial resources, which need to be studied and exploited urgently and deeply [3]. Tianpei is suitable for all ages with lots of health benefits, with fermented sauce like sugar-like water, gaining great popularity. The fermentation of Tianpei largely included natural fermentation and fermentation by the addition of a starter from a previous culture. At present, the production of sweet, fermented grains is mostly in the family-style workshop processing, the fermentation technology is unstable, the strain and product standards are not based, the shelf life is short, and it restricts the industrialized large-scale production [21,22]. The research on the microbial flora of the traditional fermented whole grains can not only protect the traditional characteristic food, but also promote the research level of the whole grain food. The research of Tianpei also integrates the whole grain food, fermentation technology, and microbial resources. It is a good model of research and the development of fermented whole grain food.

Thus, scientific questions that require attention and resolution in this study will be: 1. How about the characteristics and distribution of the microbial flora of the fermented whole grain Tianpei from typical regions of China? 2. Whether the microbes in the traditional fermented grains are significantly related to the characteristics of Tianpei? 3. What roles do the microbes play with the Tianpei and their functions for further investigation and use? The study will lay a foundation for the fermentative characteristics and condition technology of the fermented whole grain −Tianpei, and also provide theoretical and technical support for the comprehensive utilization of whole grains in the future. The quality and flavor promotion and industrial production of Tianpei could be investigated further.

2. Materials and Methods

2.1. Collections of Tianpei Samples

Based on our early sample sources and some primary screening and selections, considering sensory evaluations, flavor, nutrient chemicals, etc., three types of representative fermented whole grain Tianpei from typical regions of China were selected for this study. Tianpei HNTP is from the traditional family workshops of Hunai, south region of China, made from whole grain brown rice. Tianpei HZTP is from the traditional family workshops of Huizhou Gansu, north region of China, made from whole grain oats. While Tianpei LNTP is from the commercial goods of Lanzhou Hotel in Beijing, it is also made from whole grain oats. We speculate microbiota composition and their metagenomic functions might closely correlate with Tianpei properties. To study and decipher microbial diversity and their functions with the nutrient chemical properties, etc., of Tianpei, the experiments and analysis were carried out in this investigation. Each type of sample was prepared in triplicate for microbial metagenomic study.

2.2. Tianpei Genomic Extraction, Metagenomic Library Construction, and Sequencing

The metagenomic DNA of Tianpei samples was extracted with the DNA Kit (Omega Bio-tek, Norcross, GA, USA) following the manufacturer's instructions. The concentration and purity of genomic DNA was tested on TBS-380 and NanoDrop2000. The quality of genomic DNA was checked by 1.2% agarose gel electrophoresis. The metagenome was made into fragments with an average size of ca. 400 bp by Covaris M220 (Gene Company Limited, Shanghai, China) for the library. NEXTFLEX Rapid DNA-Seq from Bioo Scientific (USA) was used to construct the library and the adapters were added. Paired-end sequencing was carried out with Illumina Novaseq 6000 (Illumina Inc., San Diego, CA, USA) at Shanghai Majorbio Bio-Pharm Technology Co., Ltd. (China) by the NovaSeq Reagent Kits following the associated instructions. The metagenomic sequences of Tianpei were submitted to the NCBI (National Center for Biotechnology Information) under the project accession number PRJNA905197.

2.3. Sequence Processing and Megenomic Assembly

The sequencing data were mainly analyzed on the Majorbio Cloud Platform. Briefly, the raw reads were trimmed and the adaptors removed. The low-quality reads were removed with fastp 0.20.0 [23]. Sequencing reads were aligned to the oat and rice genome with BWA version 0.7.9a [24], and the hits related with the grain reads and their mated reads were all removed. MEGAHIT version 1.1.2 [25] was adopted to assemble the metagenomics data, making use of succinct de Bruijn graphs. Contigs with a length more than 300 bp were chosen as the final assembling results. These contigs were prepared and used for further microbial gene prediction and annotation.

2.4. Microbial Gene Prediction, Taxonomy, Functional Annotations, and Analysis

ORFs (open reading frames) of assembled contigs were predicted with Prodigal [26]/ MetaGene [27]. The predicted ORFs whose length was more than 100 bp were recovered and translated to amino acids by the NCBI translation table. Then, the non-redundant gene catalog construction was made by CD-HIT version 4.6.1 [28]. The identity and coverage of the sequence was 90%. High-quality reads were aligned to the non-redundant gene catalogs, calculating the gene abundance with 95% identity by SOAPaligner version 2.21 [29].

For taxonomic annotations, representative sequences from the non-redundant gene catalog were aligned to the NR database by Diamond version 0.8.35 [30]. The e-value cutoff was 1e-5. COG (cluster of orthologous groups of proteins) annotation of the representative sequences was also carried out by Diamond version 0.8.35 [30] with the EggNOG database. The KEGG annotation was performed by Diamond [30] with the Kyoto Encyclopedia of Genes and Genomes database.

Carbohydrate-active enzymes annotation was conducted by hmmscan [31] with the CAZy database [32].

PCA and RDA were adopted to analyze the microbial taxa and functions and correlation among microbial taxa, functions, and Tianpei properties. Kruskal-Wallis H test was used to test the taxa and functional differences (p-value < 0.05). Quorum sensing was analyzed with Diamond through BLASTP version 2.2.31+. Domain-related protein structures were analyzed with Pfam [33]. Secretion proteins were predicted by SignalP version 5.0 [34]. Probio database [35] was used to predict the probiotics. Cytochromes P450 was analyzed with Diamond through BLASTP version 2.2.3.

2.5. Tianpei Properties and Tianpei-Making in Laboratory

Oat Tianpei samples were fermented and made in laboratory with the representative microbial strains of the most abundant taxa and functions. After fermenting for 72h at 25 °C as terminal, the nutrient chemical properties—contents of starch (S), free fatty acid (FFA), cellulose (C), lactic acid (LA), flavonoid (F), protein (P), amino acid (AA), starch (S), and reducing sugar (RS)—were tested with the corresponding content assay kits from Suzhou Grace Biotechnology (China).

3. Results

3.1. Sequencing Data Overview of Tianpei Microbiome

After sequence quality evaluation and control, an average of more than 42,000,000 mean clean reads and 6,400,000,000 mean clean bases of every sample were conserved and the percentage in raw reads and bases (%) was higher than 98% (Table 1). When removing the host genomic sequences, an average of more than 22,000,000 mean clean reads and 3,400,000,000 mean clean bases of each sample were recovered and the percentage in raw reads and bases (%) was higher than 50% (Table 1).

Table 1. Sequences data overview of Tianpei samples.

Samples	**Mean Clean Reads**	**Mean Clean Bases (bp)**	**Percent in Raw Reads (%)**	**Percent in Raw Bases (%)**
HNTP	43,266,315.33	6,519,779,808	98.57	98.37
HZTP	47,782,730.67	7,197,564,757	98.63	98.39
LDTP	42,708,644.00	6,431,474,771	98.42	98.15
Removing the host genome				
	Mean Optimized reads	Mean optimized bases (bp)	Percent in raw reads (%)	Percent in raw bases (%)
HNTP	26,046,762	3,929,623,528	59.36%	59.31%
HZTP	24,257,476	3,659,932,373	50.05%	50.01%
LDTP	22,573,880	3,404,879,219	52.01%	51.95%

A non-redundant gene set was constructed. Before de-redundancy, there were 1,057,434 genes. The total length was 507,490,584 bp. The average length was 479.93 bp. After de-redundancy, the catalog genes were 284,511. The catalog total length was 152,936,139 bp. The catalog average length was 537.54 bp. It makes the following annotation and analysis of Tianpei microbial taxa and functions credible and reliable.

3.2. Microbial Community Composition among Tianpei Samples

Taxonomic annotations were analyzed by NR database. A total of microbial 87 phyla, 174 classes, 360 orders, 658 families, 1114 genera, and 2641 species were annotated. At the phylum level, the relative abundance of 5 phyla was >1%, including Mucoromycota (Fungi), Firmicutes (Bacteria), Ascomycota (Fungi), Proteobacteria (Bacteria), and uroviricota (virus) (Figure 1A). Mucoromycota dominated in Tianpei HNTP (84.6%) and LDTP (93.50%), while Firmicutes was richest in HZTP (53.61%).

At genus level, the relative abundance of 7 genera was more than 2%, including *Rhizopus*, *Limosilactobacillus*, *Lactobacillus*, *Weissella*, *Mucor*, *Pichia*. (Figure 1B). The variety and diversity of LZTP is lowest, with *Rhizopus* dominating at 92%. In Tianpei HZTP, *Rhizopus*, *Limosilactobacillus*, and *Lactobacillus* were most abundant. The Tianpei samples shared 594 species, which mainly belong to *Rhizopus*, *Lactobacillus*, *Mucor*, *Weissella*, *Pichia*, *Leuconostoc*, *Lactococcus*, *Bacillus*, *Aspergillus*, etc. (Figure 1C).

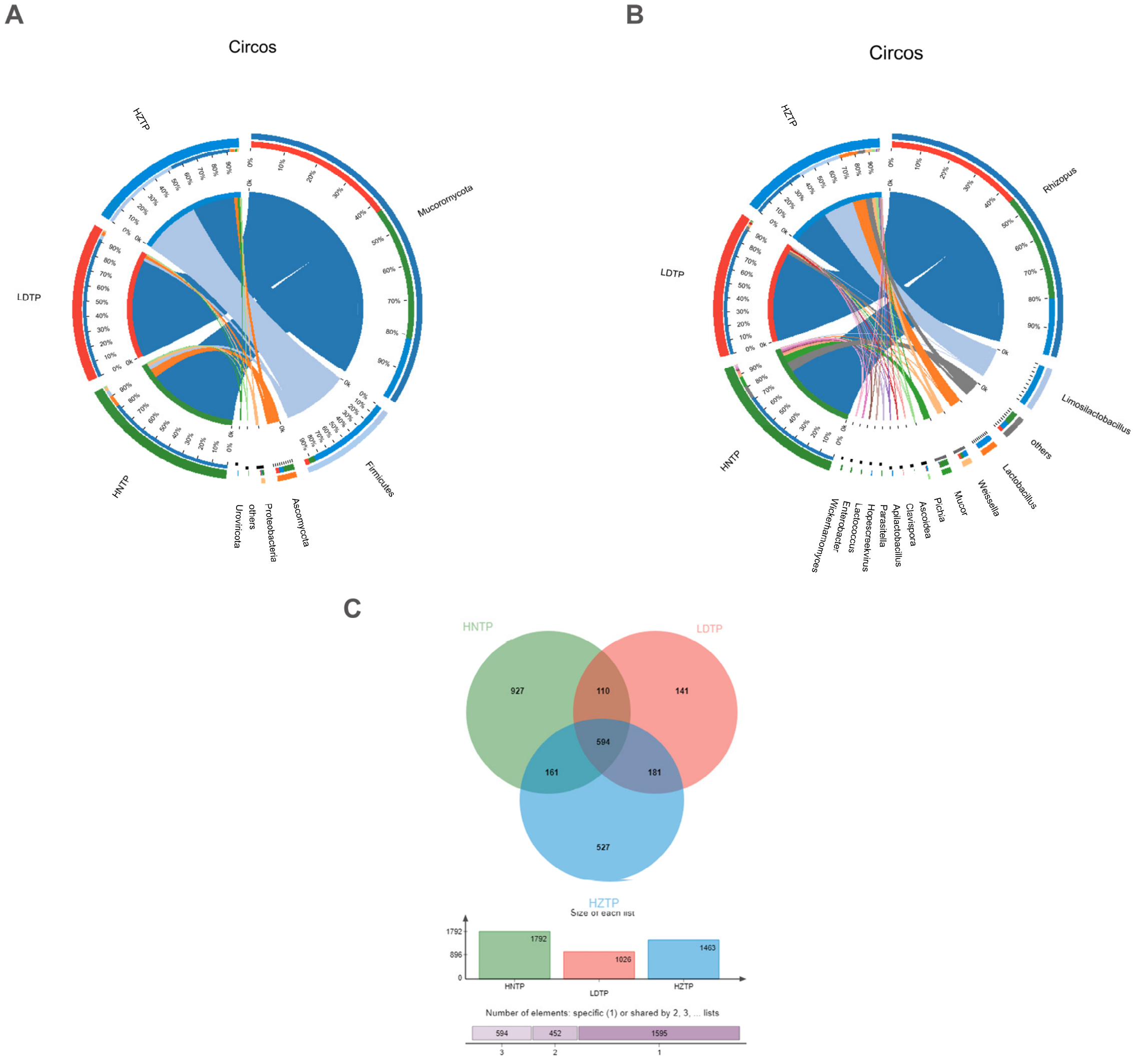

Figure 1. The Circos microbial composition of Tianpei samples with NR annotation. (**A**) At the phylum level and (**B**) at the genus level. (**C**) A Venn diagram illustrating shared and unique species among three Tianpei samples.

3.3. Microbial Functional Annotation and Compositions of Tianpei Samples

After being compared with the EggNOG database, a sum of 5949 functional genes were annotated. COG function C Energy production and conversion were richest in Tianpei HNTP (11.46%) and LDTP (10.66%), while its abundance value was only 4.77% in Tianpei HZTP. The relative abundance of the COG function was as follows: J—Translation, ribosomal structure and biogenesis, G—Carbohydrate transport and metabolism, E—Amino acid transport and metabolism. These kept a similar high abundance in three Tianpei samples (J-HNTP 8.92%, LDTP 9.01%, HZTP-9.72%, G-HNTP 9.29%, LDTP 9.11%, HZTP-7.65%, E-HNTP 8.57%, LDTP 8.49%, HZTP-8.14%) (Figure 2A). Carbohydrates mainly included fructose, galactose, glucose, L-arabinose, and mannose. AA metabolism incorporated most arginine, asparagine, glutamine, and glycine ones.

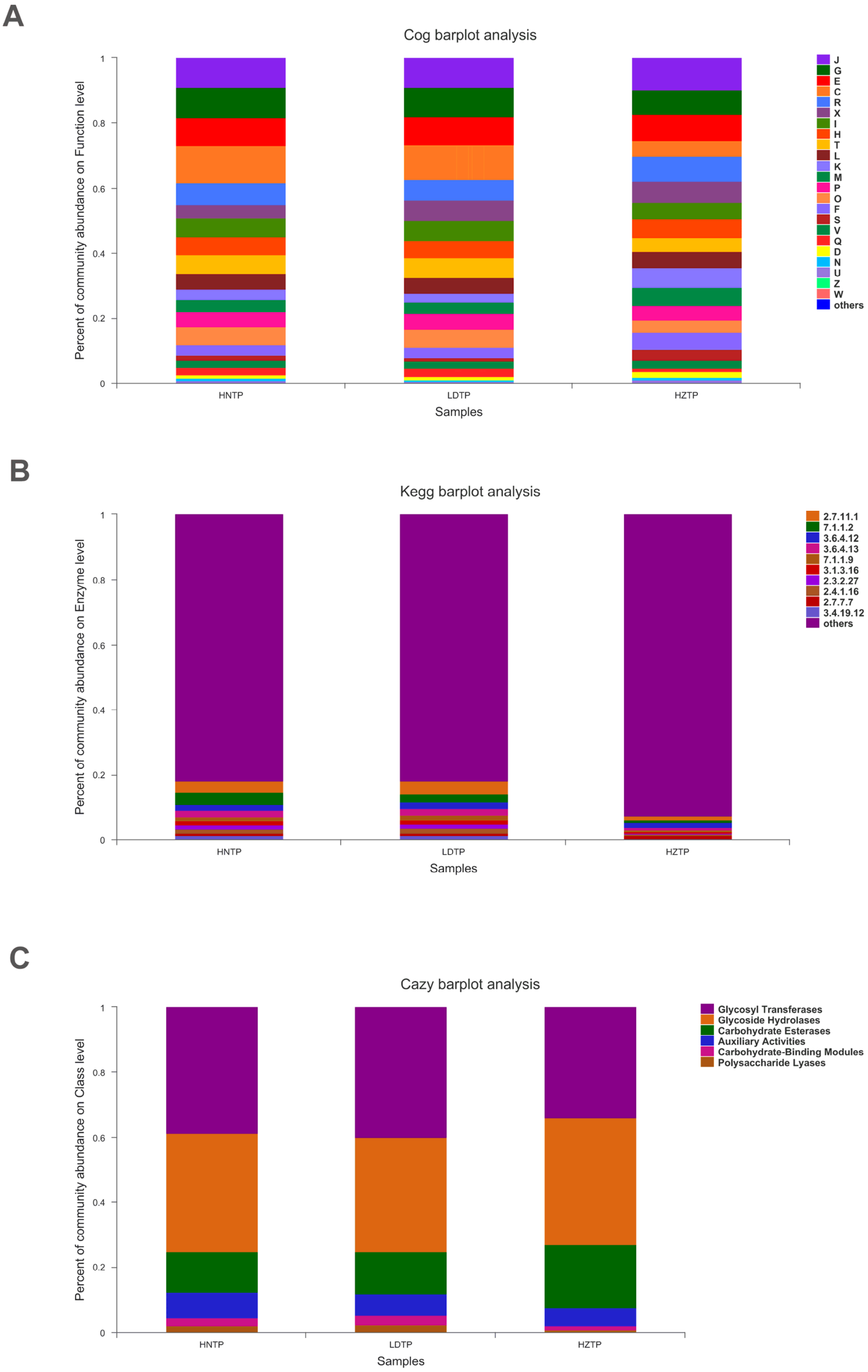

Figure 2. Microbial functional annotation and compositions of Tianpei samples. (**A**) COG analysis, (**B**) KEGG analysis-enzyme, and (**C**) CAZy analysis.

After aligning and comparing the KEGG database, we found that microbial pathways mainly included metabolism, cellular processes, genetic information processing, and environmental information processing at pathway level 1. The abundance of 10 enzymes' genes is over 1% (Figure 2B). Transferases (2.7.11.1) were most abundant (HNTP 3.55%, LDTP 4.16%, HZTP-1.06%). Translocases (7.1.1.2) and Hydrolases genes (3.6.4.12) followed. Translocases and Hydrolases genes in Tianpei HNTP and LDTP were higher than in Tianpei HZTP [Translocases: HNTP 3.80%, LDTP 2.54%, HZTP-0.85%; Hydrolases: HNTP 1.79%, LDTP 1.84%, HZTP-1.56%].

For CAZy annotation, glycoside hydrolases, glycoside transferases, carbohydrate esterases, auxiliary activities, carbohydrate-binding modules, and polysaccharide lyases were categorized and identified. Among them, glycoside transferases were most abundant in Tianpei HNTP (38.75%) and LDTP (40.01%), while glycoside hydrolases were richest in HZTP (39.06%).

3.4. Comparative Analysis of Microbial Taxa and Functions and Correlation among Microbial Taxa, Functions, and Tianpei Properties

PCA illustrated that every Tianpei sample microbial community and their functions clustered individually based on NR, COG, KEGG, and CAZy analysis (Figure 3A–D). ANOSIM verified the remarkable differences in microbial communities and their functions among the Tianpei samples (p = 0.002).

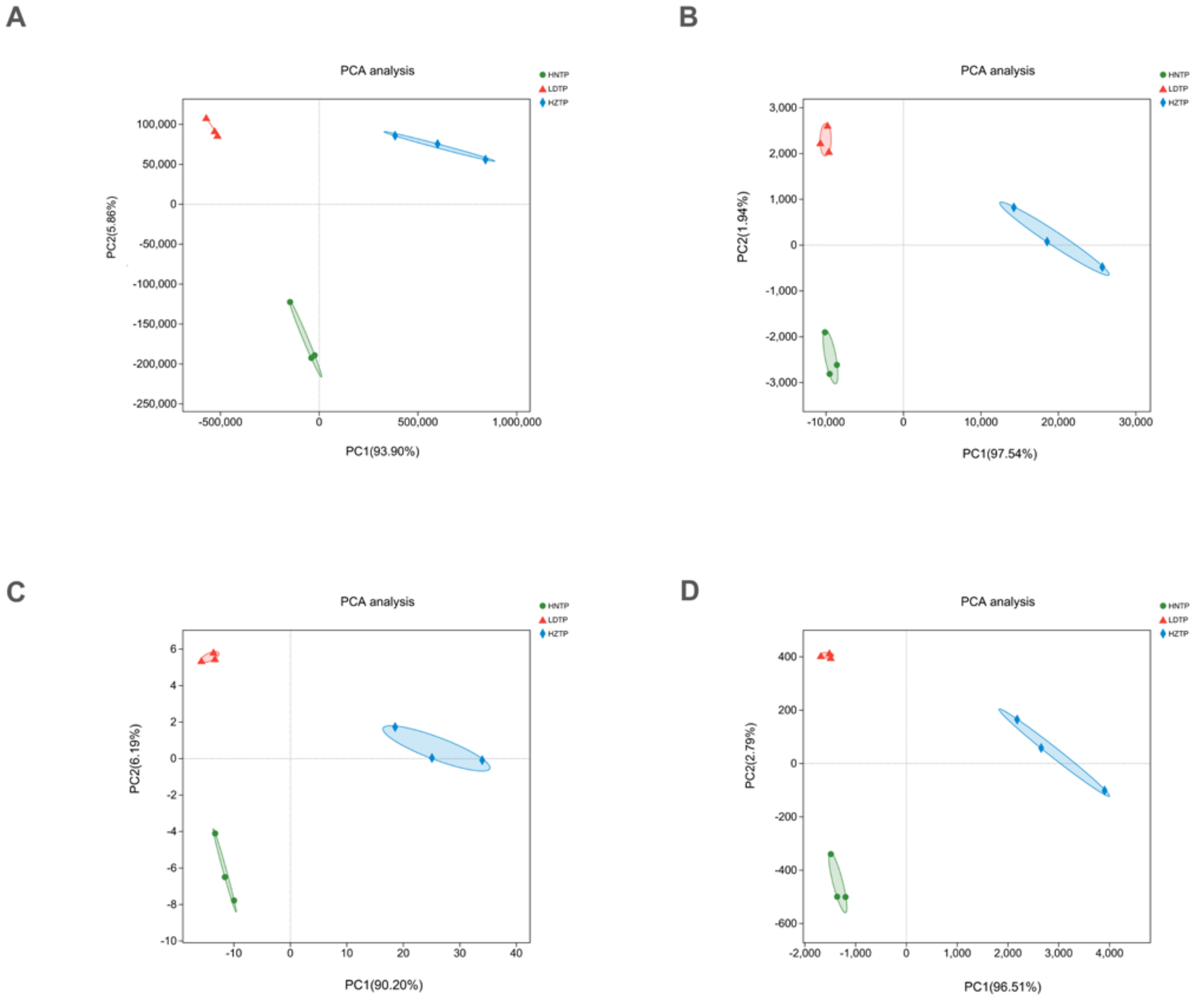

Figure 3. PCA of Tianpei samples: (**A**) PCA of NR; (**B**) PCA of COG; (**C**) PCA of KEGG; (**D**) PCA of CAZy.

RDA illustrated that the microbial taxa, microbial functions, and the Tianpei properties [content of lactic acid (LA), flavonoid (F), amino acid (AA), soluble protein (P), starch

(S), cellulose (C), free fatty acid (FA), reducing sugar (RS)] were significantly correlated (Supplementary Figure S1). Genera Rhizopus and *Mucor*, and genera *Lactobacillus* and *Limosibacillus* showed a converse effect trend (Supplementary Figure S1). The Tianpei properties were detailed in Supplementary Table S1.

3.5. Analysis of Taxa and Functional Differences, Taxa and Function Contribution Analysis of Tianpei Samples

According to the Kruskal-Wallis H test, 15 genera exhibited remarkable differences, including *Rhizopus*, *Limosilactobacillus*, *Lactobacillus*, *Mucor*, *Pichia*, etc. (Supplementary Figure S2A). Among COG categories, the cellular processes and signaling information storage and processing differed significantly (Supplementary Figure S2B). As for KEGG, pathways related to organismal system, environmental information processing, genetic information processing, and cellular processes showed great differences (Supplementary Figure S2C). When subdivided to pathway level 3, it included amino sugar and nucleotide sugar metabolism, cell cycle-yeast, RNA degradation, glycerophospholipid metabolism, protein processing in endoplasmic reticulum, meiosis-yeast, glycerolipid metabolism, etc. Five CAZy classes, including glycosyl transferases, glycoside hydrolases, carbohydrate esterases, etc., illustrated remarkable differences (Supplementary Figure S2D).

Based on the relative abundance of taxa and the function of the samples, the association analysis between taxa abundance and function abundance was carried out to discover the detailed function contributions of specific taxa and the taxa contribution of specific functions. This analysis can either investigate which taxa TOP/specific functional or metabolic pathways are predominantly present, or it can analyze which major functional or metabolic pathways TOP/specific taxa have. *Rhizopus* contributed most COG functions in Tianpei HNTP and LDTP. *Limosilactobacillus* contributed most COG functions in Tianpei HZTP, followed by *Lactobacillus* in Function E, G, J, C, R (E—amino acid transport and metabolism; G—carbohydrate transport and metabolism; J—translation, ribosomal structure, and biogenesis; C—energy production and conversion; R—general function prediction) (Figure 4A). *Rhizopus* contributed most KEGG functions in Tianpei HNTP and LDTP. *Limosilactobacillus* contributed most CAZy functions in Tianpei HZTP, including global and overview maps, carbohydrate metabolism, signal transduction, amino acid metabolism, and energy metabolism (Figure 4B). *Rhizopus* contributed most CAZy functions in Tianpei HNTP and LDTP, followed by *Lactobacillus*. *Limosilactobacillus* contributed most CAZy functions in Tianpei HZTP, including GT, GH, CE, and AA (Figure 4C).

3.6. Analysis of Quorum Sensing, Pfam, Secretion Protein, Probio, and Cytochromes P450

Based on quorum sensing analysis, ten types of gene types were annotated—quorum sensing activator, autoinducer, receptor, producer, decomposer, effector, regulator, repressor, transporter, and unclear (Supplementary Table S2). Besides the unclear type, autoinducer, activator, decomposer, regulator, and transporter were the most abundant (Supplementary Table S2). With Pfam analysis, 7456 protein structures of domain, family, repeat, motifs, and coiled coil were classified, including many descriptions of glycosyl hydrolase, DNA polymerase, RNA polymerase, amino acid permease, secretion system, ubiquitin, peptidase, oxidoreductase, methyltransferase, and lactococcin. A lot of secretion proteins were also annotated among Tianpei samples (Supplementary Table S3). A total of 105 probiotics were classified, mainly belonging to *Lactobacillus*, *Leuconostoc*, *Acetobacter*, *Bacillus*, *Bifidobacterium*, *Pediococcus*, etc. Three hundred and eighty-two cytochromes P450 related genes and taxa were annotated. The taxa mainly included *Aspergillus*, *Bacillus*, *Mucor*, and *Rhizopus.* The homologous family concluded CYP116B, CYP620-like, CYP102A, etc., and superfamily CYP116, CYP51, CYP102, etc.

Finally, Tianpei samples were fermented and made in the library with microbial strains *Rhizophus oryzae*, *Lactobacillus plantarum*, and *Limosilactobacillus (Lactobacillus) fermentum*. The lab made Tianpei obtained a rich abundance of nutrient chemicals (Supplementary Table S4).

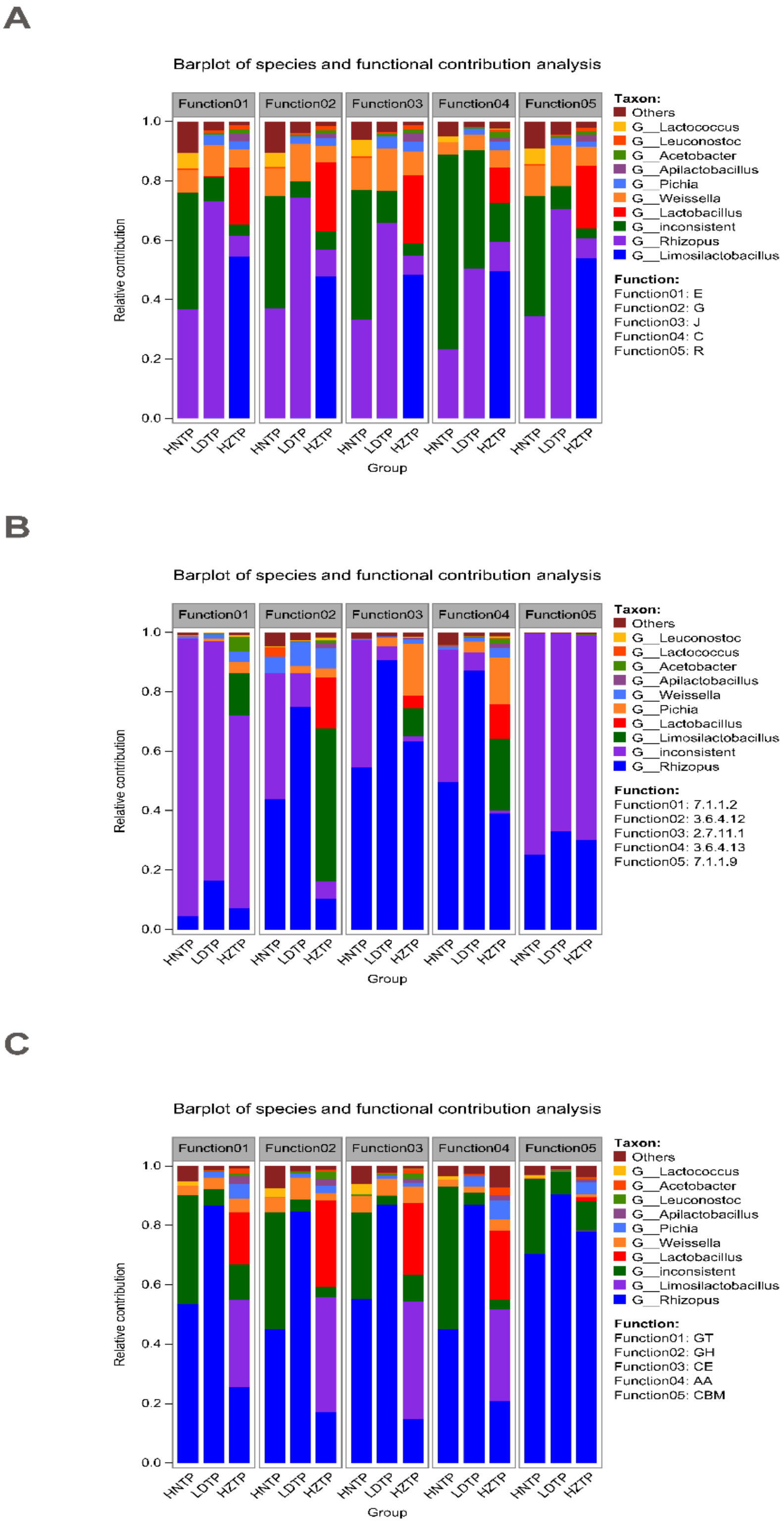

Figure 4. Taxa and function contribution analysis of Tianpei samples: (**A**) taxa and COG contribution, (**B**) taxa and KEGG contribution, (**C**) taxa and CAZy contribution.

4. Discussion

Whole grains held abundant dietary fiber and varieties of biological active ingredients. The nutritional health benefits of whole grains are far superior to those of refined grains [3,6]. Tianpei is a popular traditional fermented whole grain food and beverage from China. However, its microflora and their functions, relations among microbial taxa, and functions with Tianpei properties are little reported, restricting the industrialization process, which need to be studied and exploited deeply. With an increasing understanding of microbial importance and the development of molecular biotechnology, technologies represented by high-throughput sequencing and meta-omics give us the opportunity to re-examine and study the traditional fermented food [36,37] from a new view. In this study, the characteristics and distribution of the microbial flora taxa and functions of the fermented whole grain Tianpei, from typical regions of China, were mainly deciphered through metagenomic methods. This study provided precise references for further research on the microbiome of Tianpei and other fermented whole grain food as well.

Fungal phyla Mucoromycota and Ascomycota, and bacteria phyla Firmicutes and Proteobacteria (bacteria) were most abundant in Tianpei samples. These phyla were reported as widespread in many microbial communities of fermented food [36,37]. They were also dominant groups in a lot of other environmental systems since they are strong and widely adaptable [38–40]. Genera *Rhizopus*, *Lactobacillus*, *Limosilactobacillus*, *Weissella*, *Mucor*, *Pichia*, et al., were most abundant. *Rhizopus* can be used for immobilized fermentation to produce fumaric acid [41]. *Rhizopus oryzae* can be used for solid-state fermentation to improve the physicochemicals, nutrients, and antioxidant properties of whole grains [42]. *Lactobacillus* members are a common and important group taxa of lactic acid bacteria (LAB) and play vital roles in food production [42,43]. They are also one of most widely used microorganisms in cereal fermentations to enhance nutrient and improve flavor and texture. *L. plantarum* has been applied to several oat-based probiotic beverages [20]. *Limosilactobacillus* belongs to *Lactobacillus* formerly, including the most famous *L fermentum* and *L reuteri*, with a good probiotic and anti-inflammatory effect [44,45]. *Weissella* could be exopolysaccharide-forming, helping to improve the flavor of fermented food [36,46]. *Mucor* is widely used in fermentation, making ethanol production, and the extracting of crustacean and fungal chitosan, etc. [47,48]. *Pichia* is commonly found in fermented foods and can be used to produce wine, ethanol, acetic acid, and ethyl acetate [49,50].

A variety of databases were used to aid in microbial functional annotations. The EggNOG database is a widely recognized professional annotation database for orthologous groups [51]. The KEGG database is a large repository for the systematic analysis of gene function, linking genomes, and functional information [52]. The CAZy is a specialized database for enzymes involved in the synthesis or decomposition of complex carbohydrates and sugar complexes [53]. Carbohydrate transport and metabolism, and amino acid transport and metabolism, kept a high abundance in three Tianpei samples. Transferases were most abundant, followed by Translocases and Hydrolases. Transferases can transfer the groups containing phosphorus, protein-serine/threonine kinases, and ATP protein phosphotransferase [54]. Translocases catalyze the translocation of hydrons, linking to oxidoreductase reactions, NADH ubiquinone oxidoreductase [55]. Hydrolases act on acid anhydrides to facilitate cellular and subcellular movement and ATP phosphohydrolase (DNA helix unwinding) [56]. Carbohydrate-active enzymes are a large group of important enzymes. They are classified into glycosidase, glycosyltransferases, polysaccharide lyases, and sugar esterase, etc., having the functions of degrading, modifying, and forming glycosidic bonds [57]. Glycoside transferases were most abundant in Tianpei HNTP, while glycoside hydrolases were richest in HZTP. Further research on CAZy enzymes is very important so as to reveal the metabolic mechanism of these microbial carbohydrates. Carbohydrates are a crucial component in cereals, not only in our Tianpei materials oats and brown rice, but other kinds of grains [3]. Amino acids and protein metabolism is also very essential, with a high abundance in microbial functions, meriting further investigation. In future research, microbial functions could be studied on the specific regulations by the main carbohydrate (fructose, galactose, glucose, L-arabinose, and mannose) and AA (arginine, asparagine, glutamine, and glycine) found in this study.

PCA illustrated that every Tianpei sample's microbial community and their functions formed an individual cluster based on NR, COG, KEGG, and CAZy analysis. Tianpei samples in this study had different raw material oats and brown rice, and different geographic sources. Samples also held differences for traditional family workshops and commercial goods. All these factors have an effect on the microbial community and their functions, and vice versa. Different fermented food samples host different microbial communities [36,37]. Traditional fermented food is a valuable microbial storehouse. It is very important to constantly research and exploit them with modern technical methods.

Microbial community taxa, microbial functions, and the Tianpei properties were significantly correlated (content of lactic acid, flavonoid, amino acid, soluble protein, starch, cellulose, free fatty acid, reducing sugar). Fungal genera *Rhizopus* and *Mucor*, and bacterial genera *Lactobacillus* and *Limosibacillus*, showed a converse effect trend. It gave a clue that fungi and bacteria might not play the same roles for the Tianpei properties, and correlation among fungi and bacteria in Tianpei samples is worth paying more attention to. Thus, in the next step, microbial community taxa and microbial functions exhibiting remarkable differences were found. Microbial taxa included *Rhizopus*, *Limosilactobacillus*, *Lactobacillus*, etc. The taxa contribution of specific taxa and specific functions were also found. *Rhizopus* contributed most COG functions in Tianpei HNTP and LDTP. *Limosilactobacillus* contributed most COG functions in Tianpei HZTP. Both were then followed by *Lactobacillus*. According to the series of analyses, the microbial taxa and functions were connected. Putting all the information together, *Rhizopus*, *Limosilactobacillus*, and *Lactobacillus* should be key taxa and functional contributors for these Tianpei samples, mainly related with carbohydrate transport and metabolism, amino acid transport and metabolism, signal transduction, and energy metabolism.

Quorum sensing (QS) is one of the mechanisms that regulate microbial population behavior. Many bacteria can synthesize and release autoinducers, as signal molecules for information exchange, through sensing signal molecules; bacteria can judge the colony density and the change of the surrounding environment and start the expressions of associated genes to regulate the colony behavior of bacteria. This regulation mechanism has become a hot research topic in medicine, pathogenic bacteria control, and many other fields [58–60]. The QS genes were classified into ten categories: activator, autoinducer, decomposer, regulator, repressor, transporter, etc. Besides unclear type, autoinducer, activator, decomposer, regulator, and transporter were most abundant. It is shown that quorum sensing exists in the microbial community of Tianpei. It will help us understand the interactions among microbial communities.

Generally, proteins consist of one or more functional regions, which are often referred to as domains. Different domains could determine the protein functions, so the identification of protein domains is particularly essential to analyze the function of proteins. Structures of Tianpei microbial protein involved in glycosyl hydrolase, DNA polymerase, RNA polymerase, amino acid permease, secretion system, ubiquitin, peptidase, oxidoreductase, methyltransferase, and lactococcin indicate microbial active roles in carbohydrate, nucleic acids, AA, and protein metabolisms. Lactococcin is a kind of bacteriocin from *Lactococcus*, which was first isolated and characterized in 1991 [61,62]. It is natural, green, and safe for food preservation. In the later analysis, only a few antibiotic genes were found in the microbes of Tianpei samples involved in bacitracin, aminoglycoside, glycylcycline, macrolide, etc., showing few potentials to produce antibiotic resistance. Currently, under the conditions of the abuse of antibiotics and the spread of resistance genes, it is worthy of attention, especially in these traditional fermented foods, to find green and healthy preservatives, such as lactococcin, and their natural producing microbial strains for the future. Correspondingly, many secretion proteins were also found among Tianpei samples. Secretory proteins participated in cell signal transduction, cell proliferation, differentiation, and apoptosis regulation; the development of organisms and other important life processes, the most important feature being a DNA-encoded hydrophobic amino acid peptide at the 5′ end, play a vital role in protein secretion pathway, and is called signal peptide. It is significant to understand the expressions of secretory proteins in different developmental and growth stages.

The Probio database provided functional and integrative information on 448 probiotic strains that have been marketed: 167 have undergone clinical/field trials, 382 have been reported, 329 are human probiotics, 89 are animal probiotics, and 52 are plant probiotics. It can also be used in human, animal, and plant research, and some literature has reported potential applications of probiotic data in research, which can be used to explore the function and mechanism of probiotics and help find new probiotics. One hundred and five probiotics were found in Tianpei, mainly belonging to *Lactobacillus*, *Leuconostoc*, *Acetobacter*, *Bacillus*, *Bifidobacterium*, *Pediococcus*, etc., confirming Tianpei is a treasure trove for probiotics. Cytochromes P450 are a large group of proteins with heme as a cofactor playing a role in foreign chemicals, drug metabolism, arachidonic acid and eicosanoids, cholesterol metabolism and bile-acid biosynthesis, steroid synthesis and metabolism, vitamin D metabolism, etc. [63,64]. They could catalyze the oxidation of many kinds of substrates [65]. These proteins are also named P450 because of their maximum absorption wavelength at 450nm when they bind to CO in a reduced state. P450 participated in the metabolism of both endogenous and exogenous substances in drugs and environmental compounds. CYP has many families and subfamilies, including CYP1, CYP2, CYP3A4, CYP2D6, CYP2C19, and so on.

Based on the metagenomic analysis, with the microbial taxa of the most abundant functions, Tianpei samples were fermented and preliminarily made in the library with the representative microbial strains *Rhizophus oryzae*, *Lactobacillus plantarum*, *and Limosilactobacillus fermentum*. The lab made Tianpei obtained a rich abundance of nutrient chemicals. Considering the safety aspects of traditional fermented food, we also tested the *Escherich coli*, *Staphylococcus aureus*, and mounds in the Tianpei samples in a later study, and they were all within the standard range. The study tried to precisely find and combine the microbial strains to make fermented whole food, to some degree, according to the metagenomic analysis. With the further analysis of metatranscriptome, proteome, metabolome, etc., characteristics of microbial taxa and their functions could be better achieved. The microbial strain combinations and precise fermentation, and the quality and flavor promotion of Tianpei, could be further investigated and improved. The research ideas, process, main results, and future study is summarized in Figure 5.

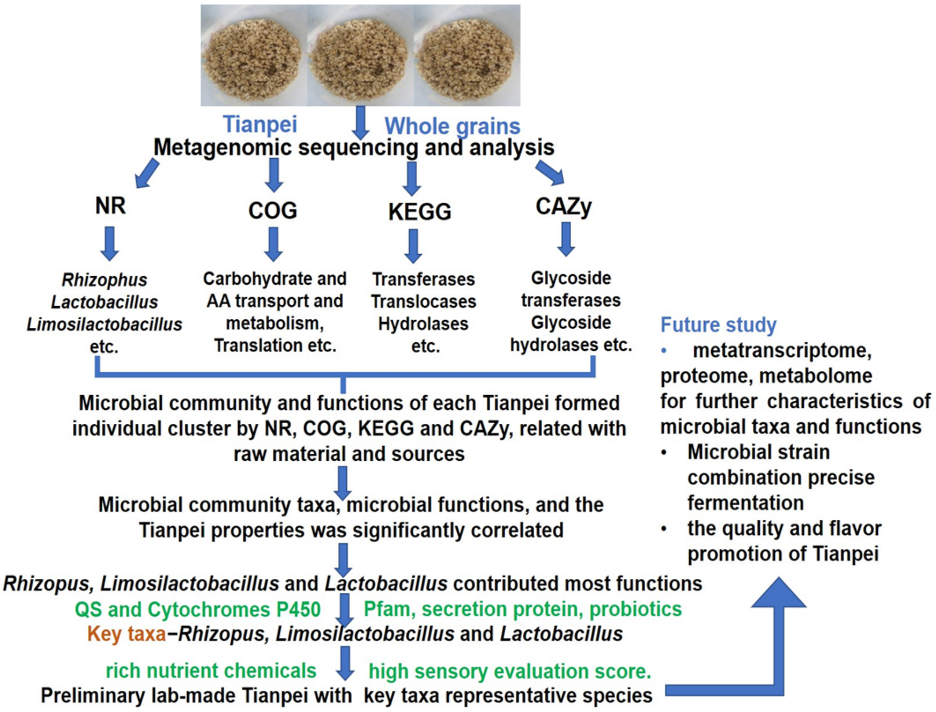

Figure 5. The summary of the research ideas, process, main results, and future study.

5. Conclusions

Phyla Mucoromycota, Firmicutes, Ascomycota, and Proteobacteria dominated among Tianpei samples. *Rhizopus*, *Limosilactobacillus*, and *Lactobacillus* were the most abundant genera. Each Tianpei sample's microbial community and their functions formed an individual cluster based on NR, COG, KEGG, and CAZy analysis. The microbial taxa, microbial functions, and the Tianpei properties were significantly correlated. *Rhizopus*, *Limosilactobacillus*, and *Lactobacillus* contributed most COG functions in the Tianpei samples. Quorum sensing and cytochromes P450 existed in microbial communities. Protein domains and secretion protein were found among Tianpei microbial communities. A sum of 105 probiotics were classified, mainly belonging to *Lactobacillus*, *Leuconostoc*, *Acetobacter*, *Bacillus*, *Bifidobacterium*, *Pediococcus*, etc. Tianpei samples were made in the library with microbial strains *Rhizophus oryzae*, *Lactobacillus plantarum*, and *Limosilactobacillus fermentum*, showing rich nutrient chemicals and receiving a high sensory evaluation score. The results indicate that microbial taxa and functions are closely related with Tianpei properties. Microbial functions could be further regulated by the main carbohydrate and AA. The characteristics of microbial taxa and functions, microbial strain combination and precise fermentation, the analysis of metatranscriptome, mataproteome, and metabolome, quality and flavor promotion, and the industrial production of Tianpei could be further investigated and improved.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/fermentation9010053/s1, Figure S1: Correlation among microbial taxa, functions, and Tianpei properties: (A) RDA of NR; (B) RDA of COG; (C) RDA of KEGG; (D) RDA of KEGG. Figure S2: Analysis of taxa and functional differences: (A) NR; (B) COG; (C) KEGG; (D) KEGG. Table S1: Chemical properties of Tianpei samples. Table S2: Analysis of QS abundance among Tianpei samples. Table S3: Secretion proteins of microbial communities among Tianpei samples. Table S4: Chemical properties of lab made Tianpei samples.

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