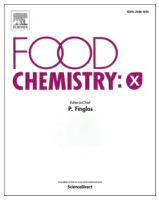
Food Chemistry: X 22 (2024) 101257

Contents lists available at ScienceDirect

Food Chemistry: X

journal homepage: www.sciencedirect.com/journal/food-chemistry-x



Characterization the microbial diversity and metabolites of four varieties of Dry-Cured ham in western Yunnan of China

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ARTICLE INFO

Keywords:
Dry-cured ham
Bacterium
Fungi
Metabolite
Metabolic pathways

ABSTRACT

In this study, high-throughput sequencing and metabolomics analysis were conducted to analyze the microbial and metabolites of dry-cured Sanchuan ham, Laowo ham, Nuodeng ham, and Heqing ham that have fermented for two years produced from western Yunnan China. Results showed that at the genus level, the dominant bacteria in the four types of ham were *Halomonas* and *Staphylococcus*, while the dominant fungi were *Aspergillus* and *Yamadazyma*. A total 422 different metabolites were identified in four types of ham, mainly amino acids, peptides, fatty acids, and their structural analogs, which were involved in pantothenate and coenzyme A biosynthesis, caffeine, and tyrosine metabolism. The dominant microorganisms of the four types of ham were mainly related to the metabolism of fatty acids and amino acids. This research enhances the identification degree of these four types of dry-cured ham and provides a theoretical basis for developing innovative and distinctive ham products.

Introduction

Dry-cured ham is a type of dry-cured meat product that is made from the front and back legs of pigs and processed through various processes such as dry curing, drying, dehydration, and fermentation with salt as the main pickling agent. It is a traditional specialty food with a long history in China (Xiao et al., 2010). Due to the unique climatic conditions, pig breeds, and processing techniques in different regions of Yunnan, there are many different varieties of dry-cured ham in Yunnan (Liu, Wang, Xiao, Pu, Ge, & Liao, 2019). There are many varieties of dry-cured ham in Yunnan, and their unique sensory characteristics are deeply loved by consumers both inside and outside Yunnan. Among them, Xuanwei ham and Norden ham are famous at home and abroad.

The production of dry-cured ham mainly involves trimming, squeezing out blood, curing with salt, fermenting, and air-drying. However, the production process of dry-cured ham varies in different production regions, and the processing characteristics of the four types of ham are shown in Table 1. Sanchuan ham (SC) is wrapped with white cotton paper during fermentation, then placed in a bamboo basket and buried with wood ash after air-drying (Y. H. Zhang, Shan, Gong, & Hu, 2022). Laowo ham (LW) is pressed to the salted ham on a grinding plate for 15 to 30 days, then smoked, fermented, and air-dried to obtain the finished ham. Heqing ham (PT) bends the fresh pig feet and inserts them into the skin's edge before curing them with salt. Nuodeng ham (ND) is cured with salt using the unique Nuodeng ancient well salt from the local area (Yang et al., 2022).

Due to different processes, environmental and geographical conditions of ham fermentation, there are differences in the richness and community compositions of microorganisms in dry-cured ham (Van Reckem, Charmpi, Van der Veken, Geeraerts, De Vuyst, & Leroy, 2019). Ge et al. found that the relative abundance of Staphylococcus was the highest in Jinhua ham produced by factories with a history of 5, 15, and 30 years of ham processing, while the abundance of Actinomycetes and Proteus increased with the age of the ham factory (Ge et al., 2017).

Previous research has analyzed the transformation mechanism of flavor substances in ham during fermentation by monitoring the changes in metabolites during the fermentation process of dry-cured ham, to enhance the flavor quality (Shi, Li, & Huang, 2019). Ham fermentation will produce numerous small molecular metabolites through different metabolic pathways, including amino acids and lipids. Conversely, these metabolites impact the quality of dry-cured ham (Zhang et al., 2018).

In recent years, studies have analyzed the differences in the quality of

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https://doi.org/10.1016/j.fochx.2024.101257
Received 14 November 2023; Received in revised form 6 February 2024; Accepted 25 February 2024
Available online 5 March 2024

Table 1
Characteristics of Four Ham Processing Techniques.

Types	Characteristics of processing technology
SC	SC is wrapped with white cotton paper during fermentation, then placed in a bamboo basket and buried with wood ash after air-drying.
LW	LW is pressed to the salted ham on a grinding plate for 15 to 30 days, then smoked, fermented, and air-dried to obtain the finished ham.
PT	PT bends the fresh pig feet and inserts them into the skin's edge before curing them with salt.
ND	ND is cured with salt using the unique Nuodeng ancient well salt from the local area.

Note: SC, Sanchuan Ham; ND, Norden ham; LW, Laowo Ham; PT, Heqing Ham.

dry-cured ham from the perspectives of microorganisms and metabolites. Understanding the differences in metabolites and microbial composition among different types of dry-cured ham during the fermentation process was key to comprehending the quality differences of ham in different regions, as these factors determine the appearance, flavor, nutrition, and safety of the varieties of dry-cured ham (Zhou et al., 2022). Nonanal and hexanal were the main flavor substances of Jinhua ham, and *Saccharomyces*, *Aspergillus,* and *Staphylococcus* were positively related to the characteristic flavor of Jinhua ham after ripening (Deng, Xu, Li, Wu, & Xu, 2022). The research on the relationship between metabolites and microorganisms in Panxian ham at different fermentation times showed that *Debaryomyces*, *Staphylococcus*, and *Aspergillus* were the dominant bacteria, thirty differential metabolites that showed significant correlation with flavor formation amino acids were identified in hams of different fermentation years, and which participated in eleven metabolic pathways relating to amino acid metabolism (Mu, Su, Mu, & Jiang, 2020). A study comparing the microorganisms and metabolites composition of Jinhua, Xuanwei, Rugao, Iberia, and Parma hams fermented for two years found that *Staphylococcus*, *Tetragenococcus,* and *Halomonas* were the main dominant microorganisms among these hams, which were significant correlations with many metabolites especially amino acids (Zhu, Guo, Yang, Zhou, Tang, & Zhou, 2021).

The purpose of this study is to explore the differences in microorganisms and metabolites of Sanchuan, Laowo, Nuodeng, and Heqing dry-cured ham fermented for two years in western Yunnan of China, as well as the correlation between microorganisms and metabolites. High-throughput sequencing was used to analyze the bacterial and fungal composition of hams from different origins, and metabolomics was used to analyze the diversity and expression patterns, and metabolic pathway enrichment analysis of four ham metabolites. This study provided new insights into the differences in microorganisms and metabolites, and the correlation between microorganisms and metabolites of four types of two-year dry-cured ham in western Yunnan.

Materials and methods

Experimental materials

The Sanchuan ham, Laowo ham, Norden ham, and Heqing ham used in the experiment have all undergone two years of fermentation. Sanchuan Ham (SC) was purchased from Sanchuan Town, Yongsheng County, Lijiang City, and is owned by Lijiang Sanchuan Ham Co., Ltd. Lao Wo Ham (LW) was purchased from Lao Wo Town, Lushui City, Nujiang Lisu Autonomous Prefecture, and is owned by Nujiang Lao Wo Ham Industry Development Co., Ltd. Norden Ham (ND) was purchased from Norden Ham Food Factory in Norden Village, Yunlong County, Dali Bai Autonomous Prefecture. Heqing Ham (PT) was purchased from Heqing Yixiang Ham Factory in Heqing County (Dali Bai Autonomous Prefecture). Microbial samples were collected from the surface of four types of dry-cured ham in the fermentation room. Five ham samples were used for each type of ham. Microbial samples were collected from the surface of each ham, stored in sterile sampling bags, mixed well, and brought back to the laboratory for microbiological analysis. When analyzing the differences in ham metabolites through metabolomics, 6 hams were used for each type of ham. The biceps femoris muscle was taken from each ham after removing subcutaneous fat and connective tissue, and vacuum packaged and stored at −80 °C for metabolomics analysis.

Experimental Methods

DNA extraction and sequencing of different varieties of dry-cured ham

The genomic DNA of ham samples was collected through the SDS method. The V3V4 region of the 16S rRNA gene was amplified through 341F and 806R, while TS5-1737F and ITS2-2443R were used to amplify the ITS1 region. The PCA reaction was carried out according to the method of Cui et al. (Cui et al., 2019).The amplified products were sequenced using NovaSeq 6000.

Metabolome processing

Sample preparation and UHPLC MS/MS analysis referred to Li et al. (Li, Al-Dalali, Zhou, & Xu, 2022), the sample (50 mg), grinding beads, and extraction solution (400 μL, water: methanol = 4: 1 (v: v)) were added to a 2 mL centrifuge tube together. The sample was then ground using a frozen tissue grinder at −10 °C and 50 Hz for 6 mins. Next, it was sonicated at 5 °C and 40 KHz for 30 mins, followed by standing at −20 °C for 30 mins. Finally, the sample was centrifuged at 4 °C and 13000g for 15 mins, and the supernatant was collected for UHPLC MS/MS analysis.

Liquid Chromatograph Mass Spectrometer (LC-MS) detection was performed using Thermo Fisher's ultra-high performance liquid chromatography-tandem Fourier transform mass spectrometry UHPLC-Q Exactive HF-X system. The chromatographic conditions were as follows, the ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm id, 1.8 μm; Waters, Milford, USA) was used; mobile phase A consisted of 95 % water and 5 % acetonitrile (containing 0.1 % formic acid), while mobile phase B consisted of 47.5 % acetonitrile, 47.5 % isopropanol, and 5 % water (containing 0.1 % formic acid); the injection volume was 3 μL, and the column temperature was maintained at 40 °C.

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Data statistics and analysis

Based on valid data, OTUs (Operational taxonomic units) clustering and species classification analysis were conducted. Nonmetric Multidimensional Scaling (NMDS) and Principal Coordinate Analysis (PCoA) analysis of different varieties of ham microorganisms were conducted using R packages, and LEfSe software was used for Linear Discriminant Analysis Effect Size (LEfSe) analysis.

The metabolomics software Proggenesis QI (Waters Corporation,

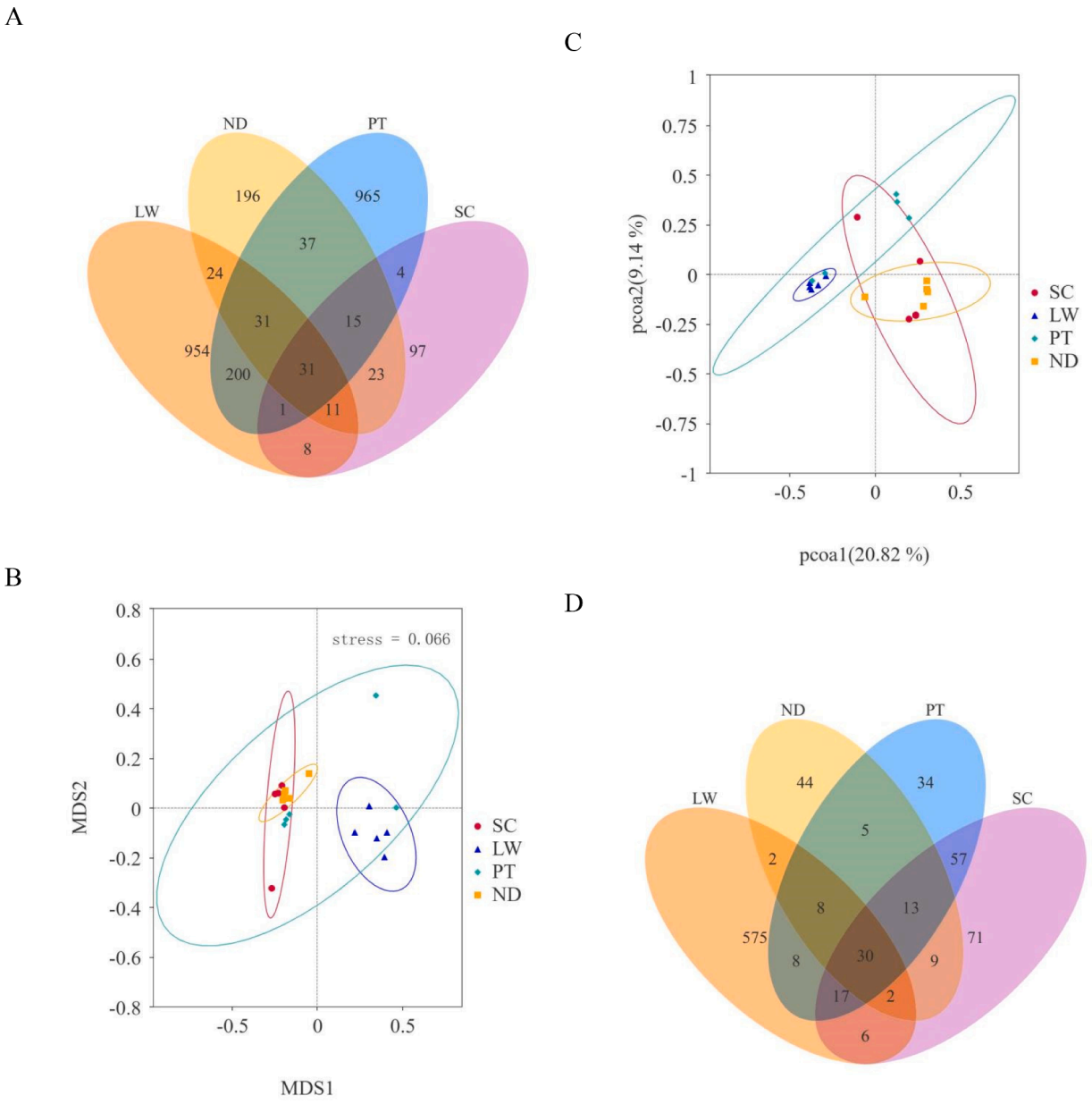

Fig. 1. The impact of microbial diversity and community structure on different varieties of ham. Veen plots of bacteria and fungi based on OTUS (A, D); NMDS scores of bacteria and fungi (B, E); PCoA scores of bacteria and fungi (C, F).

Milford, USA) was used to process the raw data, followed by feature peak search and identification. mass spectrometry information was compared with the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Human Metabolomics Database (HMDB) for matching. The MS quality error was set to less than 10 ppm, and metabolites were determined based on the MS/MS matching score.

The metabolite raw data underwent missing value filtering, simulation, data normalization, QC validation, and data transformation. PCA and PLS-DA analysis of metabolites using ropls (R packages), KEGG pathway enrichment, clustering analysis, and correlation analysis using scipy (Python).

Results and discussion

Relative abundance of bacteria and fungi in different varieties of dry-cured ham

As shown in Fig. 1A and B, at a similarity level of 97 %, 2599 OTUs were identified for bacteria in the four types of ham, of which 31 were common OTUs, and 881 OTUs were identified for fungi, of which 30 were common OTUs. The OTUs of bacteria and fungi in LW were the largest, indicating that their species richness was higher. Non-metric multidimensional scaling (NMDS) (Fig. 1C, Fig. 1D) and principal coordinates analysis (PCoA) (Fig. 1E, Fig. 1F) based on Binary-Jaccard distance were used to analyze the microbial composition to assess differences and similarities. The more similar the structure of species composition, the closer the distance between samples (Mu, Su, Mu, & Jiang, 2020). The stress values of different varieties of dry-cured ham (bacteria, 0.066; fungi, 0.061) were all less than 0.2, indicating that the

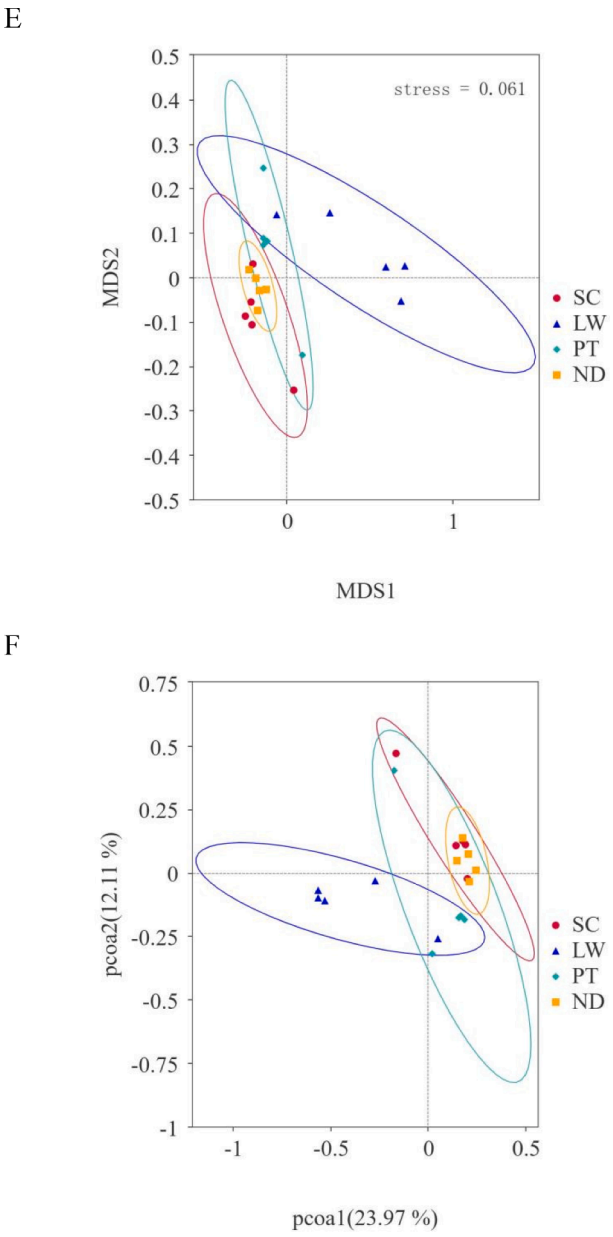

Fig. 1. (*continued*).

degree of difference between samples could be accurately reflected by NMDS. The results of PCoA and NMDS showed that the sample distances of SC and ND were closest, the fungal and bacterial communities of SC and ND were the least different, and the distance between LW and the other three types of ham was larger, indicating that the microbial composition of LW was quite different from that of other hams. This may be because LW is the only ham among the four types that have the smoking processing step.

Microbial community composition of different varieties of dry-cured ham

The results of bacteria and fungi of four types of hams were classified at the phylum and genus levels, and the top ten species with the relative abundance of bacteria at the phylum and genus levels for four types of ham samples were shown in Fig. 2A and Fig. 2B. Proteobacteria and Firmicutes were the dominant bacteria among the four types of hams, and the relative abundance of Firmicutes in the other three hams were greater than that of Proteobacteria except for LW (Fig. 2A). Firmicutes, the mostly beneficial bacteria, produce many substances that are beneficial to human health, and produce spores to help them resist dehydration and extreme environments (Zhou et al., 2023). The dominant bacterial genera of LW were *Halomonas*, *Chromohalobacter,* and *Lentibacillus*, the dominant bacterial genera of SC were *Staphylococcus* and *Cobetia*, the dominant bacterial genera of PT were *Staphylococcus* and *Solitalea*, and the dominant bacterial genera of ND were *Staphylococcus* and *Psychrobacter* (Fig. 2B). *Halomonas* can promote the degradation of flavor substances by producing lipolytic enzymes (Jeong, Heo, & Lee, 2017). Similarly, Lin et al. (Lin, Cai, Luo, Gu, Ahmed, & Long, 2020) found that *Halomonas* was the dominant bacterial genera in LW fermented for one year, and *Staphylococcus* tended to become the dominant genus in ham as the fermentation time prolonged. Moreover, ham is a high-salt product, so salt-tolerant bacteria are better able to adapt to the high-salt and complex nutritional environment of the ham fermentation system, further affecting the metabolic product changes during ham fermentation (Zhu, Guo, Yang, Zhou, Tang, & Zhou, 2021). Most *Staphylococci* are salt-tolerant. Wang et al. (Wang, Zhang, Ren, & Zhan, 2018) found that *Staphylococcus* was the dominant bacteria in the meat fermented process which can degrade fat and protein, promote the

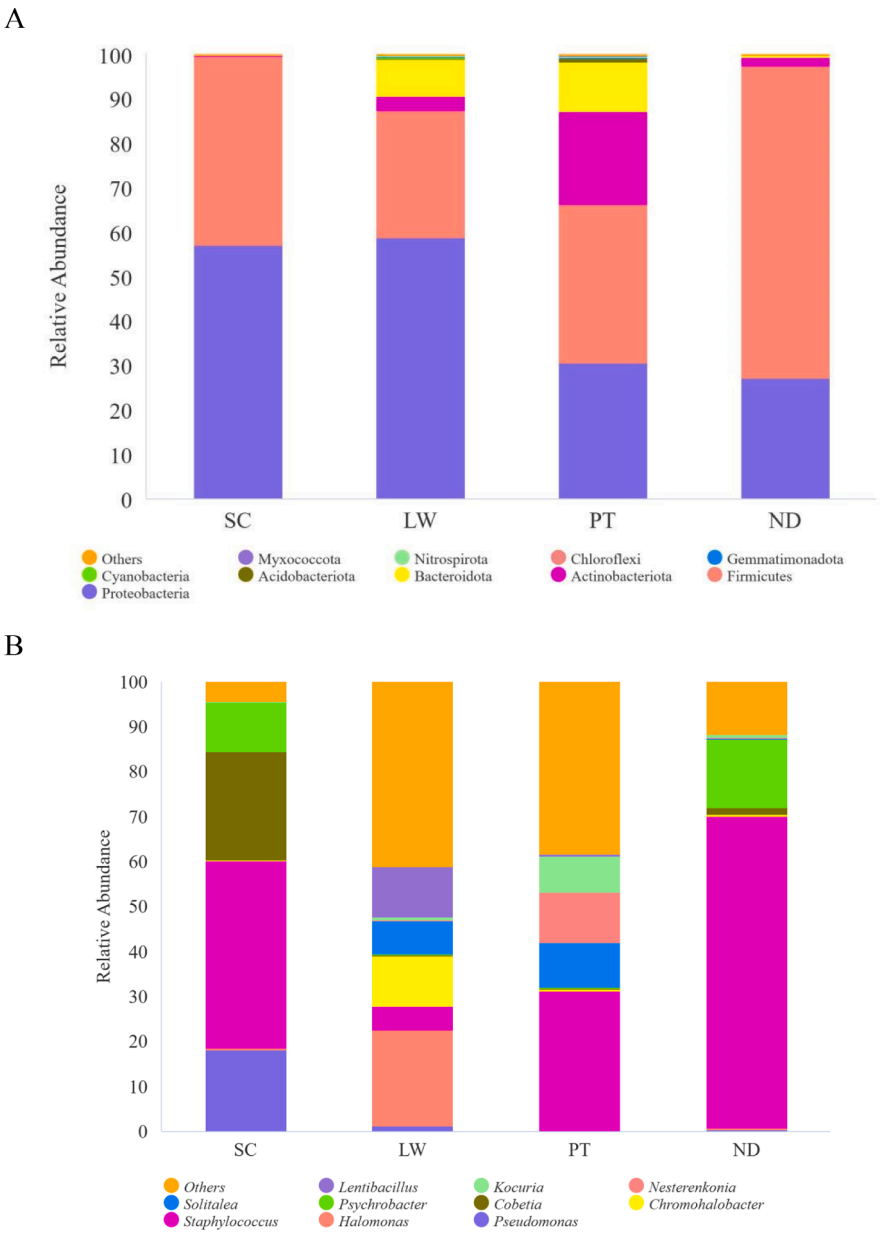

Fig. 2. Relative species abundance of bacteria (A) and fungi (C) in different varieties of ham at the phylum level; The relative abundance of bacteria (B) and fungi (D) at the genus level in different varieties of ham.

production of fatty acids and flavor amino acids, reduce the content of biogenic amines, and promote the improvement of flavor and nutritional quality of fermented meat. Most *Staphylococci* also play an important role in the color of ham because they can reduce nitrate to nitrite, which improves the color of meat by reacting with myoglobin and producing rose nitroso myoglobin (Langille et al., 2013). Research has shown that *Staphylococcus* is also the dominant bacteria in Panxian ham (Mu, Su, Mu, & Jiang, 2020).

According to Fig. 2C, the dominant fungal phyla among four varieties of dry-cured ham were *Ascomycota* and *Basidiomycota*. *Aspergillus* had the highest relative abundance among the four ham fungal genera. The second and third highest relative abundance in SC were *Knufia* (4.86 %) and *Yamadazyma* (2.41 %), while *Yamadazyma* and *Wallemia* had the second and third highest relative abundance in fungal genera among ND, LW, and PT (Fig. 2D). *Aspergillus* is one of the most abundant molds on the surface of ham (Mu, Su, Mu, & Jiang, 2020), which can avoid direct exposure to oxygen and sunlight, prevent rancidity, and improve quality. High mold content not only improves the safety of ham but also promotes the formation of a unique flavor of ham (Wen, Sun, Li, Chen, & Kong, 2021). Yeasts show good salt tolerance and are usually in relatively high abundance in high-salt foods (Mi et al., 2021). Proteus is closely related to lipid and amino acid metabolism, Firmicute is a major member of the ham microbiota that is closely related to carbohydrate metabolism (Bhutia, Thapa, Shangpliang, & Tamang, 2021), Yeast and *Staphylococcus* increase the production of flavor substances by decomposing fats and proteins (Mi et al., 2021)).

Microbial LEfSe analysis of different varieties of dry-cured ham

The differences in microbial species among different varieties of ham could be revealed through LEfSe (LDA threshold ≥4, Fig. 3). The bacterial (Fig. 3A) and fungi (Fig. 3B) LEfSe analysis showed that Biomaker had the most significant differences at various levels in the LW ham samples, and had a significant impact, which is consistent with the

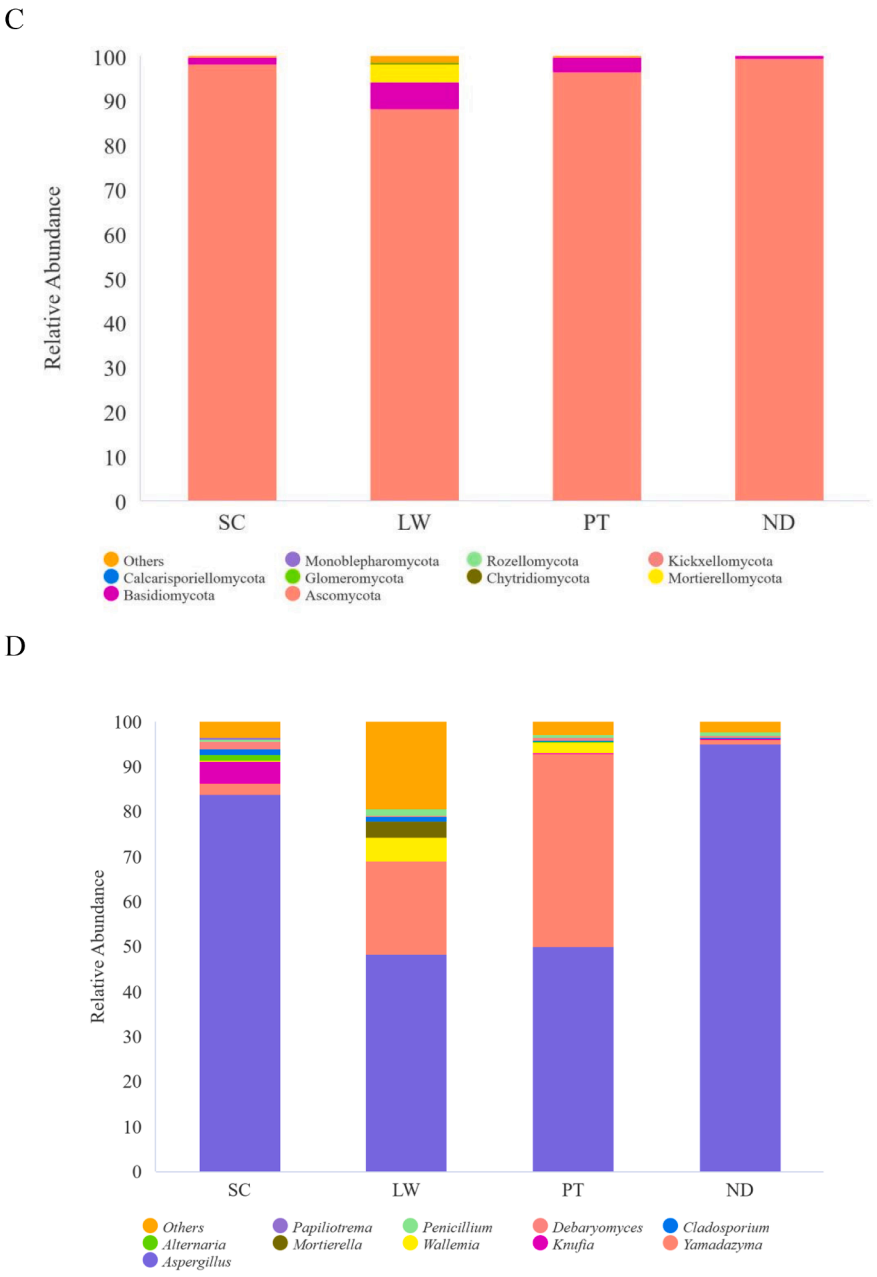

Fig. 2. (*continued*).

relative abundance results of species at the bacterial and fungal genus levels in the LW samples.

In the bacterial flora of SC, *Pseufomonadales* was significantly enriched. *Bacteroidota*, *Actinobacteriota*, *Enterobacteria*, and *Micrococcales* were PT core bacterial taxa. The bacterial communities were significantly enriched in ND including *Alteromonadales*, Staphylococcales, *Pseudoalteromonas*, *Psychrobacter*, and *Moraxellaceae*. The LW bacterial groups were significantly enriched in the phylum mainly including Halomonadaceae, Oceanosipirillales, Proteobacteria, and Bacillales.

Trichomeriaceae and *Dothideomycetes* were significantly enriched in SC. Debaryomycetaceae and Saccharomycetales were the main fungi in PT. *Aspergillaceae* and *Eurotialets* were significantly enriched in ND. While *Wallemiaceae* and *Mortierellaceae* were significantly enriched in LW. Among them, *Debaryomycetaceae* can improve the aroma and flavor of dry-cured meat products and has been widely used as a leavening agent (Long, Nie, & Liu, 2016).

Metabolites of different varieties of dry-cured ham

As shown in Fig. 4A, there are 2320 metabolic products shared by four varieties of ham. The types of metabolites identified in LW were the most, with 3120 metabolites and 162 unique metabolites, while the least metabolites (2596 metabolites identified and 6 unique metabolites) were identified in ND. PCA analysis (Fig. 4B) and PLS-DA analysis (Fig. 4C) demonstrated that the distance between the four varieties of ham is far, indicating significant differences in metabolite composition.

According to the screening conditions $P < 0.05$, VIP pred OPLS-DA > 1, and upper and lower differential multiples of 1.5. A total of 422 differential metabolites were selected, and a metabolic set was established to compare and identify the differential metabolites with HMDB and KEGG (Wishart et al., 2022). Compared with the HMDB, 367 differential metabolites were identified in the four types of ham, mainly including 71 amino acids, peptides, and analogs (19.35 %), 15 fatty acid esters with a relative content of 4.09 %, followed by 12 fatty acids and

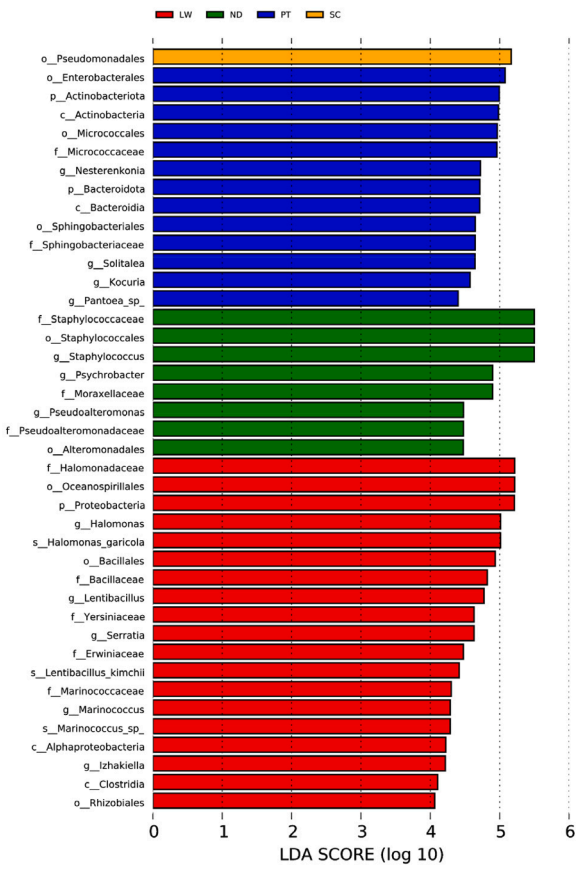

Fig. 3. LDA discrimination histogram of bacteria (A) and fungi (B) in different varieties of ham; LEfSe multi-level classification tree of bacteria (C) and fungi (D) in different varieties of ham.

conjugates (3.27 %) (Fig. 5A). Because the contents of protein and fat in pork are relatively high, amino acids and fatty acids are usually the most important metabolites in ham (Zhang, et al., 2018). Amino acids are precursors of various volatile flavor compounds (Liu, Wang, Xiao, Pu, Ge, & Liao, 2019). Glutamic acid, aspartic acid, and pyroglutamic acid are related to the umami taste of ham, while serine, valine, and alanine are related to the sweetness of ham, lysine and tyrosine are related to the ripe flavor (Zheng, Tao, Gong, Gu, & Xu, 2015).

The KEGG database can provide comprehensive annotations of the enzymes that catalyze each step of the reaction, as well as the gene products and metabolites involved in the integrated metabolic pathway (Zhang, Chen, Zhang, & Jia, 2023). Fig. 5B indicated that a total of 11 compounds with biological effects were identified in the four types of ham by comparing with the KEGG database, and the substances with relatively high content were phospholipids (3 types), eicosanoids (3 types), and steroid hormones (2 types). Eicosanoids and n-6 fatty acids such as arachidonic acid can be decomposed into flavor substances such as hexanal (Marusic, Vidacek, Janci, Petrak, & Medic, 2014).

Differential metabolites and metabolic pathway of different varieties of dry-cured ham

According to the expression abundance and expression patterns of metabolites in different samples, cluster analysis was performed on the metabolites of the four varieties of ham, as shown in the clustering heat map (Fig. 6A), with significant differences in metabolites between different varieties of ham. The clustering heat map showed 20 significantly different metabolites. Similar to the results of Zhu et al. (Zhu, Guo, Yang, Zhou, Tang, & Zhou, 2021). The differential metabolites of different varieties of ham were lipid and peptide compounds. Through ANOVA, the differences in the top 20 relative abundance metabolites among four varieties of ham were compared. As shown in Fig. 6B, the relative abundance of the top 20 metabolites in the four varieties of ham were significant differences, including creatine, p-chlorophenylalanine, N, *N*-dimethylbutylamine, cis linoleic acid, 2-hydroxycinnamic acid, cinnamic acid, hypoxanthine, and dioctyl succinate ($P \leq 0.001$). Hypoxanthine can enhance the flavor of ham (Zhang, et al., 2018). The odor threshold of lipid oxidation products, such as esters, ketones, alcohols, and aldehydes is lower than that of flavor substances such as fatty hydrocarbons, which are the main contributors to ham flavor (Wang et al., 2016).

Based on the comparison of metabolites and KEGG compound IDs, using the KEGG database can classify metabolites and obtain information on the metabolic pathways involved in metabolites to evaluate their impact on biological metabolic processes (Cai et al., 2023). The metabolites of four varieties of ham were mainly involved in five primary metabolic pathways including amino acid metabolism (14 metabolites involved), lipid metabolism (12 metabolites involved), and biodegradation and metabolism of foreign organisms (8 metabolites involved) (Fig. S1A).

The metabolites of the four types of ham were enriched and analyzed, and the pathways with significant enrichment of the four

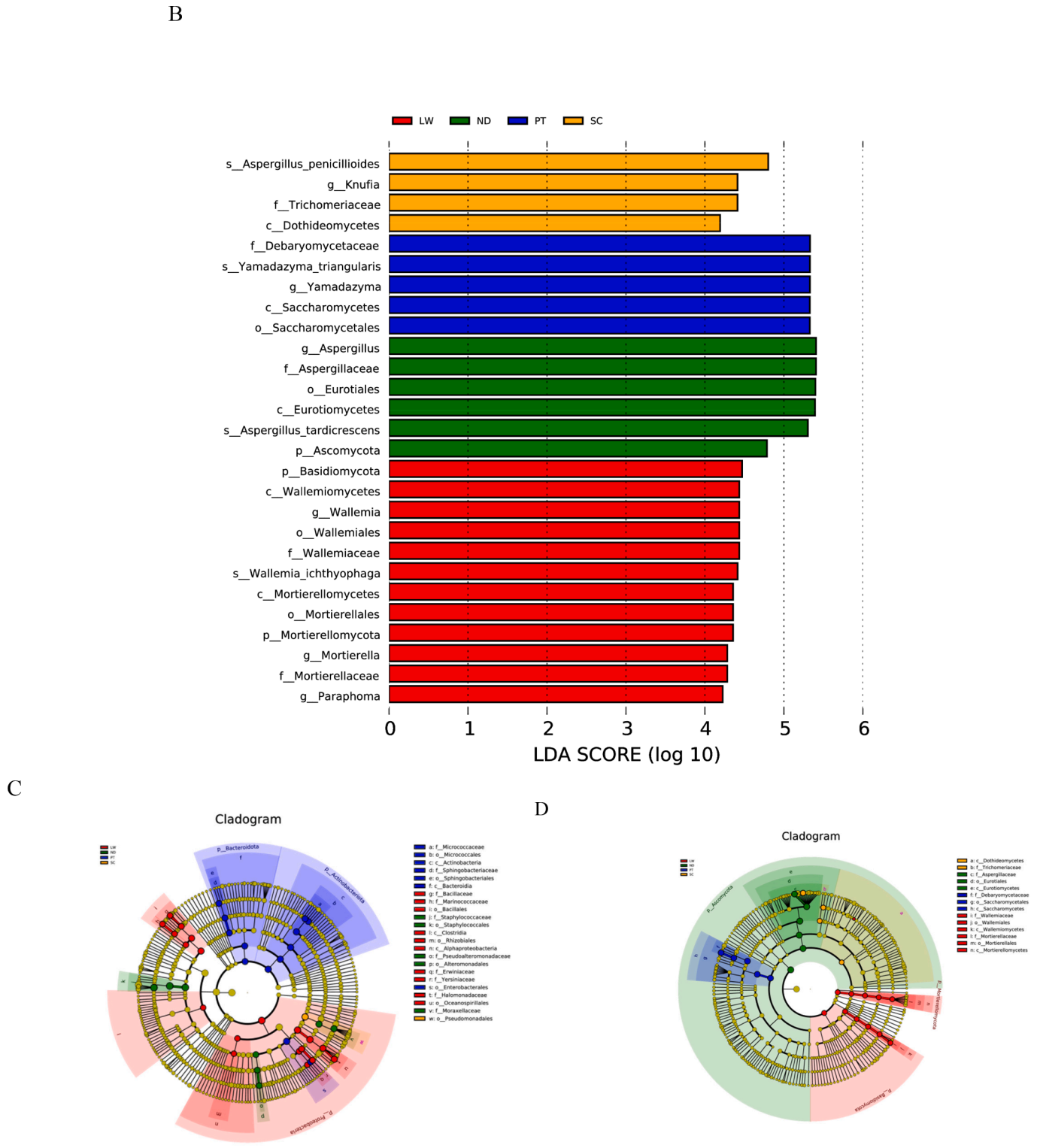

Fig. 3. (*continued*).

metabolites were obtained using the hypergeometric distribution algorithm. As shown in Fig. S1B, the pantothenate and coenzyme A (CoA) biosynthesis metabolic pathways showed the most significant difference among the four hams, and the pathway importance score was the highest. Pantothenic acid is vitamin B5 and a key precursor for CoA biosynthesis (Ku, Chen, & Lan, 2020). CoA is a cofactor in multiple metabolic pathways that can participate in various metabolic pathways during ham fermentation, including phospholipid synthesis, fatty acid and amino acid biosynthesis, as well as tricarboxylic acid cycling, which leads to the degradation of protein, fat, and glycogen in ham into fatty acids, amino acids, and pyruvate, these compounds further undergo various chemical reactions such as Strecker degradation, deamination, Maillard reaction, and β-oxidation to produce the characteristic flavor compounds of dry-cured ham (Flores, 2018; Martinez-Onandi, Rivas-Canedo, Avila, Garde, Nunez, & Picon, 2017).

In addition, a large number of metabolites of hams were involved in

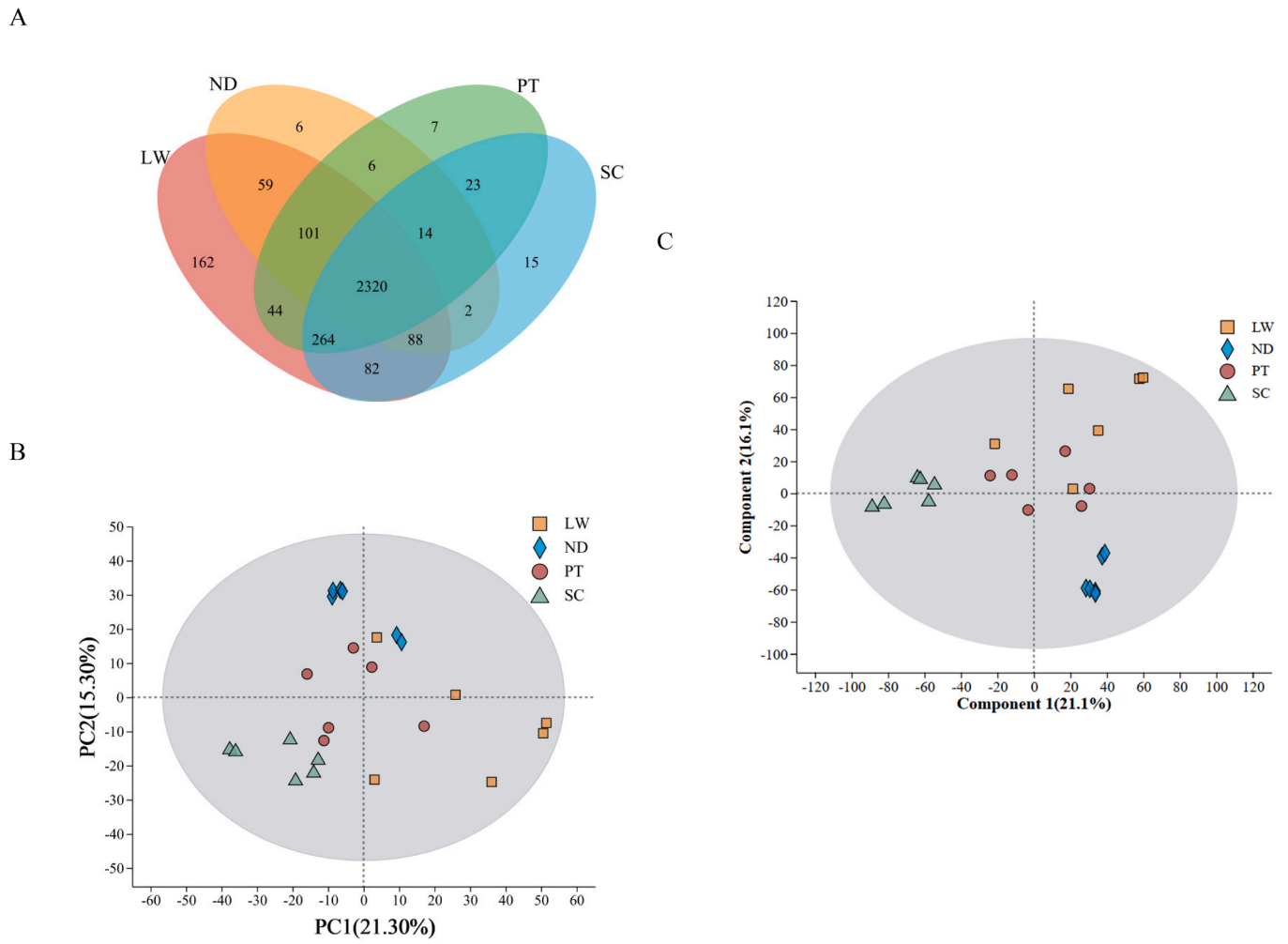

Fig. 4. Metabolite analysis of different varieties of ham. Venn diagram (A); PCA analysis (B); PLS-DA analysis (C).

caffeine metabolism, tyrosine metabolism, glycerophospholipid metabolism, and histidine metabolism. Since caffeine is mainly metabolized into hypoxanthine (Benowitz, Jacob, Mayan, & Denaro, 1995; Kim et al., 2019), and pig legs contain a large amount of adenosine triphosphate, during the fermentation process of ham, adenosine triphosphate is ultimately metabolized into hypoxanthine by the action of enzymes, leading to an increase in the content of hypoxanthine in the ham (Hernández-Cázares, Aristoy, & Toldrá, 2011; Tikk et al., 2006). In addition, the presence of caffeine in feed and the abundance of metabolites related to the caffeine pathway in ham may also be related to the intake of feed.

Microorganisms actively participate in metabolic pathways related to flavor development, including protein, lipid, and carbohydrate metabolism, to improve the unique flavor characteristics of ham. Among them, microorganisms (such as Lactobacillus, Staphylococcus, Cobeta, Saccharomycetes, Penicillium, and Aspergillus) can secrete extracellular enzymes and decompose carbohydrates in ham through intracellular enzymes and secondary metabolism, thereby producing precursor substances for flavor compounds while meeting their own nutritional needs (Li, Bao, Wang, Su, Zhou, & Xu, 2023; Petrova, Aasen, Rustad, & Eikevik, 2015).

Storrustlokken et al. (Storrustlokken et al., 2015) investigated the changes in metabolites during ham fermentation and found that lipid degradation and oxidation were important factors in producing unique flavors of ham. The hydrolysis and oxidation of phospholipids and triglycerides are the basic reactions that form ham-flavor substances (Petrova, Aasen, Rustad, & Eikevik, 2015). During the fermentation process of dry-cured ham, phospholipids and triglycerides are hydrolyzed by phospholipase and lipase to produce free fatty acids, which are more easily oxidized than triglycerides and are conducive to the formation of volatile substances (Guo, Lu, Wang, Dong, Ji, & Wang, 2019). In addition, during the fermentation of Jinhua ham, histidine and lysine could undergo a Maillard reaction with reducing sugars in the ham to produce alcohols, aldehydes, ketones, esters, and other flavor compounds (Zhu, Zhao, Tian, Liu, Li, & Zhao, 2018). The degradation of amino acids can form branched aldehydes, branched ketones, toluene, and phenylacetaldehyde. The degradation of isoleucine and leucine can produce benzaldehyde, which can participate in the degradation of aromatic amino acids in the Strecker reaction and the Maillard reaction, providing meat and floral aromas, respectively (Ruiz, Ventanas, Cava, Andres, & Garcia, 1999; Zhu et al., 2022).

3.6. Correlation analysis of metabolites and microbial diversity

Correlations between the top 20 microorganisms in relative abundance and the top 20 metabolites in relative expression at the genus level of the four types of ham were analyzed using Pearson to identify key microbial genera. At the bacterial genus level, *Lentibacillus* was significantly positively correlated with 3,4-Dimethoxy-benzaldehyde, Prolyl-Histidine and Gibberellin A36 ($P < 0.001$), while it was significantly negatively correlated with *Serratia*, *Izhakiella* and 4-Hydroxytrideca-7, 10-dienoylcarnitine ($P < 0.001$) (Fig. S2A). *Acinetobacter* and *Marinococcus* were significantly negatively correlated with 2,4-Dihydroxybenzophenone ($P < 0.001$). The relative content of Toremifene was

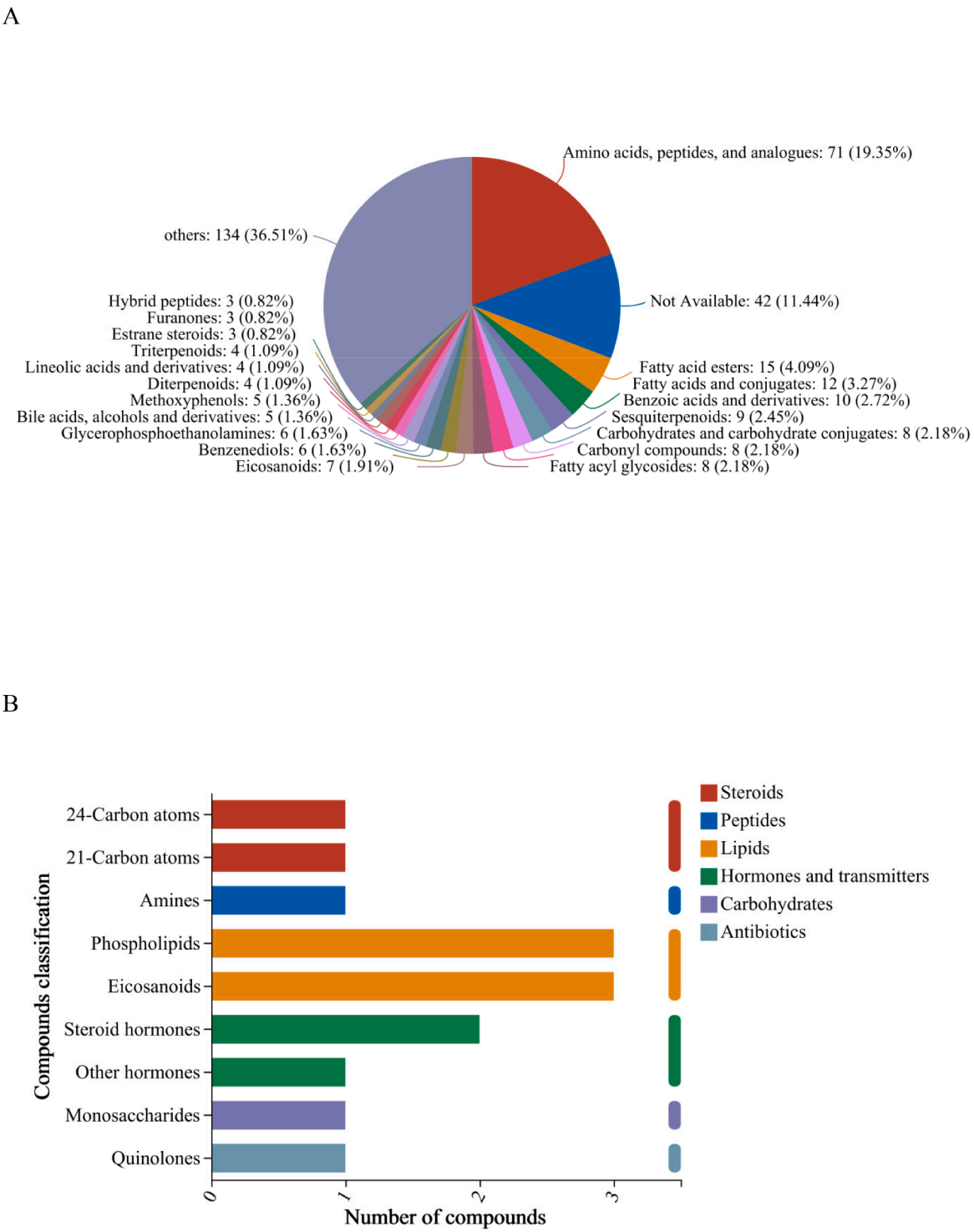

Fig. 5. Classification of metabolites in different varieties of ham. HMDB compound classification(A); KEGG compound classification (B).

significantly negatively correlated with *Mitochondria* ($P < 0.05$), and *Kocuria* ($P < 0.001$). The relative content of *N*-Desmethyltramadol was significantly negatively correlated with *Pseudoalteromonas* ($P < 0.001$). At the fungal genus level, P-coumaroyltriacetic acid lactone was significantly positively correlated with *Yamadazym* ($P < 0.001$), and a variety of fungi were positively correlated with Gibberellin A36 (Fig. S2B). *Aspergillus* was significantly negatively correlated with P-coumaroyltriacetic acid lactone, Gibberellin A36, and 3,4-Dimethoxybenzaldehyde ($P < 0.001$). At the genus level, the four types of ham microorganisms mainly had a high correlation with peptides and amino acid analogs. The three types of metabolites with the highest relative content among the four types of ham were amino acids, peptides and assays, fat acid esters, fat acids and aggregates, and the four types of ham metabolites mainly involved lipid and amino acid metabolism pathways. Corresponding to the relative content of metabolites and metabolic pathway analysis results in four types of ham, the dominant bacteria mainly had a high correlation with peptides, amino acids, and their analogs, as well as fat and aggregates. *Yamadazyma,* a genus in the *Saccharomyceae* subphylum of the *Ascomycota* phylum, produces extracellular enzymes with high proteolytic and lipolytic activity, which can

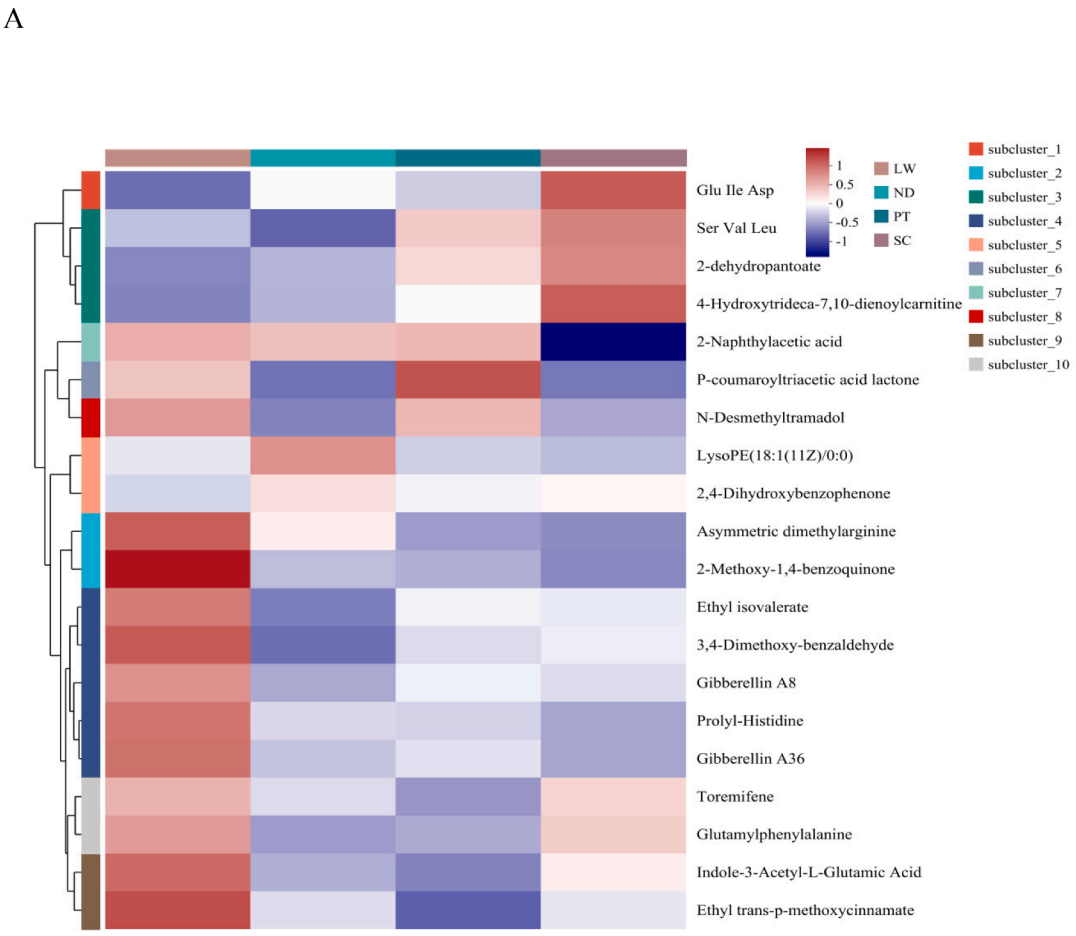

Fig. 6. Analysis of different metabolites in different varieties of ham. Heat map of metabolite cluster analysis (A); multi group comparison column chart (B).

enhance the sensory characteristics of ham for the production of free amino acids, fatty acids, and flavor substances (Wang, Ma, Jiang, Peng, & Yang, 2006). *Aspergillus* can improve the appearance of the hams, promote the breakdown of fat and protein by protease and lipase to generate flavor substances, and thus will be beneficial to format the unique flavor of ham (Takenaka et al., 2021). Amino acids and lipid metabolism produce many volatile flavor compounds, such as cysteine and cysteine, phenylalanine, methionine, and other amino acids.

Conclusion

This study revealed the significant differences in microbial diversity and metabolites of four varieties of ham in Western Yunnan China. At the phylum level, Proteobacteria and Firmicutes are the dominant bacteria in LW. Except for the relative abundance of Proteobacteria in LW which is higher than that in Firmicutes, the relative abundance of Firmicutes in the other three types of ham is higher than that in Proteobacteria. Basidiomycota and Ascomycota were the dominant fungal phyla in the four varieties of ham. *Staphylococcus* was the dominant genus of bacteria among the four types of ham, except for LW, while the dominant bacterium for LW was *Halomonas*. *Aspergillus* was the dominant fungal genus of four varieties of ham. These advantageous microorganisms are of great significance in shaping the quality characteristics of different varieties of ham. A total of 422 differential metabolites were identified in four types of ham which were mainly involved in amino acid metabolism, caffeine metabolism, tyrosine metabolism, glycerophospholipid metabolism, and histidine metabolism, and the highest proportion of differential metabolites (19.35 %, 71 metabolites) were amino acids, peptides and analogs. Eight of the top 20 metabolites in the relative content of the four hams were extremely significantly different, mainly fatty acids ($P < 0.001$). Compared to other metabolic pathways, the microorganisms of the four varieties of ham had a higher correlation with metabolites related to amino acid and lipid metabolism pathways. Amino acids and fatty acids play an important role in the formation of ham flavor. The differences in the microbial communities of the four types of ham can affect metabolic pathways in the ham, leading to differences in metabolites and resulting in differences in the quality and flavor of the four types of ham. The research results are of great significance for understanding the quality differences between the four types of dry-cured ham. However, due to the lack of comparison with fresh pig leg samples, the study has certain limitations.

CRediT authorship contribution statement

Yu Qin: Data curation, Visualization, Writing – original draft, Writing – review & editing. **Wenwen Li:** Data curation, Formal analysis, Methodology. **Wenwen Zhang:** Data curation, Formal analysis, Methodology. **Beibei Zhang:** Conceptualization, Resources. **Dengjie Yao:** Software, Validation. **Chunyin Zeng:** Methodology, Software. **Jianxin Cao:** Resources. **Lirong Li:** Project administration, Supervision, Writing – review & editing. **Rui Huang:** Project administration, Supervision.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

B

Fig. 6. (*continued*).

the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This study was financially supported by Yunnan Scientific and Technological Projects Translation Yunnan Modern Food Processing Key Technology Research and Development and New Product Creation (Grant No. 202202AG050009); Department of Science and Technology of Shaanxi Province Department of City Linkage Weinan Modern Agricultural Key Projects (Grant No. 2022GD-TSLD-58-1); Major Scientific and Technological Project of Yunnan Provincial Department of Science and Technology (Grant No. 202102AE090025); Special Project for High-level Scientific and Technological Talents and Innovation Teams of Yunnan Province (Grant No. 202405AS350005); Yunnan Province Talent Training Project (Grant No. KKRD202023026).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101257.

References

Benowitz, N. L., Jacob, P., 3rd, Mayan, H., & Denaro, C. (1995). Sympathomimetic effects of paraxanthine and caffeine in humans. *Clinical Pharmacology and Therapeutics, 58*(6), 684–691.

Bhutia, M. O., Thapa, N., Shangpliang, H. N. J., & Tamang, J. P. (2021). Metataxonomic profiling of bacterial communities and their predictive functional profiles in traditionally preserved meat products of Sikkim state in India. *Food Research International, 140*. https://doi.org/10.1016/j.foodres.2020.110002

Cai, X., Liao, R. Y., Pan, D. D., Xia, Q., Wang, Y., Geng, F., Zhou, C. Y., & Cao, J. X. (2023). H-1 NMR reveals the mechanism of potassium lactate on proteolysis and taste metabolites of Rugao ham. *Foods, 12*(7). https://doi.org/10.3390/foods12071453

Cui, M., Xiao, H. W., Li, Y., Zhang, S. Q., Dong, J. L., Wang, B., Zhu, C. C., Jiang, M., Zhu, T., He, J. B., Wang, H. C., & Fan, S. J. (2019). Sexual dimorphism of gut microbiota dictates therapeutics efficacy of radiation injuries. *Advanced Science, 6*(21). https://doi.org/10.1002/advs.201901048

Deng, J., Xu, H., Li, X., Wu, Y., & Xu, B. (2022). Correlation of characteristic flavor and microbial community in Jinhua ham during the post-ripening stage. *Lwt, 171*. https://doi.org/10.1016/j.lwt.2022.114067

Flores, M. (2018). Understanding the implications of current health trends on the aroma of wet and dry cured meat products. *Meat Science, 144*, 53–61. https://doi.org/10.1016/j.meatsci.2018.04.016

Ge, Q. F., Gu, Y. B., Zhang, W. G., Yin, Y. Q., Yu, H., Wu, M. G., Wang, Z. J., & Zhou, G. H. (2017). Comparison of microbial communities from different Jinhua ham factories. *Amb Express, 7*. https://doi.org/10.1186/s13568-017-0334-0

Guo, X., Lu, S. L., Wang, Y. Q., Dong, J., Ji, H., & Wang, Q. L. (2019). Correlations among flavor compounds, lipid oxidation indices, and endogenous enzyme activity during the processing of Xinjiang dry-cured mutton ham. *Journal of Food Processing and Preservation, 43*(11). https://doi.org/10.1111/jfpp.14199

Hernández-Cázares, A. S., Aristoy, M. C., & Toldrá, F. (2011). Nucleotides and their degradation products during processing of dry-cured ham, measured by HPLC and an enzyme sensor. *Meat Science, 87*(2), 125–129.
Jeong, D. W., Heo, S., & Lee, J. H. (2017). Safety assessment of Tetragenococcus halophilus isolates from doenjang, a Korean high-salt-fermented soybean paste. *Food Microbiology, 62*, 92–98.
Kim, H. J., Choi, M. S., Rehman, S. U., Ji, Y. S., Yu, J. S., Nakamura, K., & Yoo, H. H. (2019). Determination of urinary caffeine metabolites as biomarkers for drug metabolic enzyme activities. *Nutrients, 11*(8).
Ku, J. S. T., Chen, A. V. Y., & Lan, E. I. (2020). Metabolic engineering design dtrategies for increasing Acetyl-CoA Flux. *Metabolites, 10*(4). https://doi.org/10.3390/metabo10040166
Langille, M. G. I., Zaneveld, J., Caporaso, J. G., McDonald, D., Knights, D., Reyes, J. A., Clemente, J. C., Burkepile, D. E., Thurber, R. L. V., Knight, R., Beiko, R. G., & Huttenhower, C. (2013). Predictive functional profiling of microbial communities using 16S rRNA marker gene sequences. *Nature Biotechnology, 31*(9), 814-+. https://doi.org/10.1038/nbt.2676
Li, C., Al-Dalali, S., Zhou, H., & Xu, B. C. (2022). Influence of curing on the metabolite profile of water-boiled salted duck. *Food Chemistry, 397*. https://doi.org/10.1016/j.foodchem.2022.133752
Li, P., Bao, Z. J., Wang, Y., Su, X. L., Zhou, H., & Xu, B. C. (2023). Role of microbiota and its ecological succession on flavor formation in traditional dry-cured ham: A review. *Critical Reviews in Food Science and Nutrition*.
Lin, F. K., Cai, F., Luo, B. S., Gu, R. H., Ahmed, S., & Long, C. L. (2020). Variation of microbiological and biochemical profiles of Laowo dry-cured ham, an indigenous fermented food, during ripening by GC-TOF-MS and UPLC-QTOF-MS. *Journal of Agricultural and Food Chemistry, 68*(33), 8925–8935. https://doi.org/10.1021/acs.jafc.0c03254
Liu, S. Y., Wang, G. Y., Xiao, Z. C., Pu, Y. H., Ge, C. R., & Liao, G. Z. (2019). 1H-NMR-based water-soluble low molecular weight compound characterization and free fatty acid composition of five kinds of Yunnan dry-cured hams (vol 108, pg 174, 2019). *Lwt-Food Science and Technology, 112*. https://doi.org/10.1016/j.lwt.2019.04.080
Liu, S. Y., Wang, G. Y., Xiao, Z. C., Pu, Y. H., Ge, C. R., & Liao, G. Z. (2019). H-1-NMR-based water-soluble low molecular weight compound characterization and free fatty acid composition of five kinds of Yunnan dry-cured hams. *Lwt-Food Science and Technology, 108*, 174–182. https://doi.org/10.1016/j.lwt.2019.03.043
Long, Q., Nie, Q., & Liu, C. (2016). State of the art of functional starters for fermented meat products. *Food Science, 37*(17), 263–269.
Martinez-Onandi, N., Rivas-Canedo, A., Avila, M., Garde, S., Nunez, M., & Picon, A. (2017). Influence of physicochemical characteristics and high pressure processing on the volatile fraction of Iberian dry-cured ham. *Meat Science, 131*, 40–47. https://doi.org/10.1016/j.meatsci.2017.04.233
Marusic, N., Vidacek, S., Janci, T., Petrak, T., & Medic, H. (2014). Determination of volatile compounds and quality parameters of traditional Istrian dry-cured ham. *Meat Science, 96*(4), 1409–1416. https://doi.org/10.1016/j.meatsci.2013.12.003
Mi, R. F., Chen, X., Xiong, S. Y., Qi, B., Li, J. P., Qiao, X. L., Chen, W. H., Qu, C., & Wang, S. W. (2021). Predominant yeasts in Chinese Dong fermented pork (Nanx Wudl) and their aroma-producing properties in fermented sausage condition. *Food Science and Human Wellness, 10*(2), 231–240. https://doi.org/10.1016/j.fshw.2021.02.013
Mu, Y., Su, W., Mu, Y. C., & Jiang, L. (2020). Combined application of high-throughput sequencing and metabolomics reveals metabolically active microorganisms during Panxian Ham processing. *Frontiers in Microbiology, 10*. https://doi.org/10.3389/fmicb.2019.03012
Petrova, I., Aasen, I. M., Rustad, T., & Eikevik, T. M. (2015). Manufacture of dry-cured ham: A review. Part 1. Biochemical changes during the technological process. *European Food Research and Technology, 241*(5), 587–599. https://doi.org/10.1007/s00217-015-2490-2
Ruiz, J., Ventanas, J., Cava, R., Andres, A., & Garcia, C. (1999). Volatile compounds of dry-cured Iberian ham as affected by the length of the curing process. *Meat Science, 52*(1), 19–27.
Shi, Y. N., Li, X., & Huang, A. X. (2019). A metabolomics-based approach investigates volatile flavor formation and characteristic compounds of the Dahe black pig dry-cured ham. *Meat Science, 158*. https://doi.org/10.1016/j.meatsci.2019.107904
Storrustlokken, L., Devle, H. M., Haseth, T. T., Egelandsdal, B., Naess-Andresen, C. F., Hollung, K., Berg, P., Ekeberg, D., & Alvseike, O. (2015). Lipid degradation and sensory characteristics of M. biceps femoris in dry-cured hams from Duroc using three different processing methods. *International Journal of Food Science and Technology, 50*(2), 522–531. https://doi.org/10.1111/ijfs.12699
Takenaka, S., Ogawa, C., Uemura, M., Umeki, T., Kimura, Y., Yokota, S., & Doi, M. (2021). Identification and characterization of extracellular enzymes secreted by Aspergillus spp. involved in lipolysis and lipid-antioxidation during katsuobushi fermentation and ripening. *International Journal of Food Microbiology, 353*. https://doi.org/10.1016/j.ijfoodmicro.2021.109299
Tikk, M., Tikk, K., Torngren, M. A., Meinert, L., Aaslyng, M. D., Karlsson, A. H., & Andersen, H. J. (2006). Development of inosine monophosphate and its degradation products during aging of pork of different qualities in relation to basic taste and retronasal flavor perception of the meat. *Journal of Agricultural and Food Chemistry, 54*(20), 7769–7777.
Van Reckem, E., Charmpi, C., Van der Veken, D., Geeraerts, W., De Vuyst, L., & Leroy, F. (2019). Exploring the link between the geographical origin of European fermented foods and the diversity of their bacterial communities: The case of fermented meats. *Frontiers in Microbiology, 10*. https://doi.org/10.3389/fmicb.2019.02302
Wang, X. H., Ma, P., Jiang, D. F., Peng, Q., & Yang, H. Y. (2006). The natural microflora of Xuanwei ham and the no-mouldy ham production. *Journal of Food Engineering, 77*(1), 103–111. https://doi.org/10.1016/j.jfoodeng.2005.06.047
Wang, X. H., Zhang, Y. L., Ren, H. Y., & Zhan, Y. (2018). Comparison of bacterial diversity profiles and microbial safety assessment of salami, Chinese dry-cured sausage and Chinese smoked-cured sausage by high-throughput sequencing. *Lwt-Food Science and Technology, 90*, 108–115. https://doi.org/10.1016/j.lwt.2017.12.011
Wang, Y., Jiang, Y. T., Cao, J. X., Chen, Y. J., Sun, Y. Y., Zeng, X. Q., Pan, D. D., Ou, C. R., & Gan, N. (2016). Study on lipolysis-oxidation and volatile flavour compounds of dry-cured goose with different curing salt content during production. *Food Chemistry, 190*, 33–40. https://doi.org/10.1016/j.foodchem.2015.05.048
Wen, R. X., Sun, F. D., Li, X. A., Chen, Q., & Kong, B. H. (2021). The potential correlations between the fungal communities and volatile compounds of traditional dry sausages from Northeast China. *Food Microbiology, 98*. https://doi.org/10.1016/j.fm.2021.103787
Wishart, D. S., Guo, A. C., Oler, E., Wang, F., Anjum, A., Peters, H., Dizon, R., Sayeeda, Z., Tian, S. Y., Lee, B. L., Berjanskii, M., Mah, R., Yamamoto, M., Jovel, J., Torres-Calzada, C., Hiebert-Giesbrecht, M., Lui, V. W., Varshavi, D., Varshavi, D., Allen, D., Arndt, D., Khetarpal, N., Sivakumaran, A., Harford, K., Sanford, S., Yee, K., Cao, X., Budinski, Z., Liigand, J., Zhang, L., Zheng, J. M., Mandal, R., Karu, N., Dambrova, M., Schioth, H. B., Greiner, R., & Gautam, V. (2022). HMDB 5.0: The human metabolome database for 2022. *Nucleic Acids Research, 50*(D1), D622–D631. https://doi.org/10.1093/nar/gkab1062
Xiao, S., Zhang, W. G., Yang, Y., Ma, C. W., Ahn, D. U., Li, X., Lei, J. K., & Du, M. (2010). Changes of hormone-sensitive lipase (HSL), adipose tissue triglyceride lipase (ATGL) and free fatty acids in subcutaneous adipose tissues throughout the ripening process of dry-cured ham. *Food Chemistry, 121*(1), 191–195. https://doi.org/10.1016/j.foodchem.2009.12.029
Yang, Z. J., Liao, G. Z., Wan, D. Q., Kong, W. C., Li, C., Gu, D. H., Pu, Y. H., Ge, C. R., & Wang, G. Y. (2022). Combined application of high-throughput sequencing and LC-MS/MS-based metabolomics to evaluate the formation of Zn-protoporphyrin in Nuodeng ham. *Food Research International, 162*. https://doi.org/10.1016/j.foodres.2022.112209
Zhang, C., Chen, Z. W., Zhang, M. M., & Jia, S. L. (2023). KEGG_extractor: An effective extraction tool for KEGG orthologs. *Genes, 14*(2). https://doi.org/10.3390/genes14020386
Zhang, J., Ye, Y. F., Sun, Y. Y., Pan, D. D., Ou, C. R., Dang, Y. L., Wang, Y., Cao, J. X., & Wang, D. Y. (2018). H-1 NMR and multivariate data analysis of the differences of metabolites in five types of dry-cured hams. *Food Research International, 113*, 140–148. https://doi.org/10.1016/j.foodres.2018.07.009
Zhang, Y. H., Shan, B., Gong, J. S., & Hu, Y. J. (2022). Mechanism of biogenic amine synthesis of Enterococcus faecium isolated from Sanchun ham. *Food Science & Nutrition, 10*(6), 2036–2049. https://doi.org/10.1002/fsn3.2820
Zheng, J. Y., Tao, N. P., Gong, J., Gu, S. Q., & Xu, C. H. (2015). Comparison of non-volatile taste-active compounds between the cooked meats of pre- and post-spawning Yangtze Coilia ectenes. *Fisheries Science, 81*(3), 559–568. https://doi.org/10.1007/s12562-015-0858-7
Zhou, C. Y., Xia, Q., Du, L. H., He, J., Sun, Y. Y., Dang, Y. L., Geng, F., Pan, D. D., Cao, J. X., & Zhou, G. H. (2022). Recent developments in off-odor formation mechanism and the potential regulation by starter cultures in dry-cured ham. *Critical Reviews in Food Science and Nutrition*. https://doi.org/10.1080/10408398.2022.2057418
Zhou, Y. L., Zhou, Y., Wan, J., Zhu, Q. J., Liu, L. G., Gu, S., & Li, H. Y. (2023). Effects of sorbitol-mediated curing on the physicochemical properties and bacterial community composition of loin ham during fermentation and ripening stages. *Food Chemistry-X, 17*. https://doi.org/10.1016/j.fochx.2022.100543
Zhu, C. Z., Zhao, J. L., Tian, W., Liu, Y. X., Li, M. Y., & Zhao, G. M. (2018). Contribution of histidine and lysine to the generation of volatile compounds in Jinhua ham exposed to ripening conditions via maillard reaction. *Journal of Food Science, 83*(1), 46–52. https://doi.org/10.1111/1750-3841.13996
Zhu, L., He, S. Y., Lu, Y., Gan, J. H., Tao, N. P., Wang, X. C., Jiang, Z. L., Hong, Y. X., & Xu, C. H. (2022). Metabolomics mechanism of traditional soy sauce associated with fermentation time. *Food Science and Human Wellness, 11*(2), 297–304.
Zhu, Y. Y., Guo, Y., Yang, F. H., Zhou, C. Y., Tang, C. B., & Zhou, G. H. (2021). Combined application of high-throughput sequencing and UHPLC-Q/TOF-MS-based metabolomics in the evaluation of microorganisms and metabolites of dry-cured ham of different origins. *International Journal of Food Microbiology, 359*. https://doi.org/10.3389/fmicb.2019.03012