Metagenomic and flavoromic profiling reveals the correlation between the microorganisms and volatile flavor compounds in *Monascus*-fermented cheese

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Abstract: The *Monascus*-fermented cheese (MC) is a unique cheese product that undergoes multi-strain fermentation, imparting it with distinct flavor qualities. To clarify the role of microorganisms in the formation of flavor in MC, this study employed SPME (arrow)-GC-MS, GC-O integrated with PLS-DA to investigate variations in cheese flavors represented by volatile flavor compounds across 90-day ripening periods. Metagenomic datasets were utilized to identify taxonomic and functional changes in the microorganisms. The results showed a total of 26 characteristic flavor compounds in MC at different ripening periods (VIP＞1, p<0.05), including butanoic acid, hexanoic acid, butanoic acid ethyl ester, hexanoic acid butyl ester, 2-heptanone and 2-octanone. According to NR database annotation, the genera *Monascus, Lactococcus, Aspergillus, Lactiplantibacillus, Staphylococcus, Flavobacterium, Bacillus, Clostridium, Meyerozyma*, and *Enterobacter* were closely associated with flavor formation in MC. Ester compounds were linked to *Monascus*, *Meyerozyma*, *Staphylococcus*, *Lactiplantibacillus*, and *Bacillus*. Acid compounds were linked to *Lactococcus, Lactobacillus, Staphylococcus*, and *Bacillus*. The production of methyl ketones was closely related to the genera *Monascus, Staphylococcus, Lactiplantibacillus, Lactococcus, Bacillus,* and *Flavobacterium*. This study offers insights into the microorganisms of MC and its contribution to flavor development, thereby enriching our understanding of this fascinating dairy product.

Keywords: flavoromic, metagenomic, microorganisms, *Monascus*, ripening periods, cheese

1. Introduction

Monascus-fermented cheese (MC) is a mold-ripened cheese, renowned for its unique flavor and quality. Boasting fruity notes and a fermented wine-like aroma, this cheese holds the potential for widespread consumption (Agboyibor, Kong, Chen, Zhang, & Niu, 2018; Chaudhary et al., 2021). The production of flavor compounds in MC, a type of fermented food, is inextricably linked to the type and growth of microorganisms. The secondary starter culture plays a facilitating role in the flavor formation of mold-ripened cheese (Y. Yang et al., 2024). Xia et al. improved the aroma quality of cheeses by adding *Monascus* fumeus x08 (Xia, Yuan, Weng, Wang, & Ai, 2020). Yang et al. enhanced the flavor profile and ripening characteristics of cheese by co-fermenting *Monascus* fumeus x08 with *Lactobacillus salivarius* AR809 (Y. Yang et al., 2024). However, the precise mechanism by which various strains influence the flavor of cheese remains elusive.

The metagenome represents the collective genetic material of all microorganisms present in a given environment (Mardanov, Kadnikov, & Ravin, 2018). In recent years, metagenomic sequencing has been a widely utilized tool in the exploration of cheese microbial genomes (Afshari et al., 2020; Erkus et al., 2016; Wolfe, Button, Santarelli, & Dutton, 2014). For example, A meta-analysis of cheese microorganisms and corresponding volatiles was conducted by Aaron M. Walsh et al. (Walsh, Macori, Kilcawley, & Cotter, 2020). A collection of 328 metagenomic assemblies was collected and differences in strain abundance were found to correspond to levels of volatiles. The cheese microorganisms have been provided with new and substantial technical and ecological insights through the overall findings. Using a metagenomic-based approach, N. Suárez and colleagues evaluated the taxonomic affiliation and functional potential of bacteriocin production by bacterial communities in artisanal cheeses made from milk in northwestern Argentina. Their

findings revealed that 92% of the coding sequences (CDSs) in the cheese metagenomics, which is associated with bacteriocin production, are encoding for class II bacteriocins. These bacteriocins are widely recognized as ribosomally synthesized antimicrobial peptides effective against food-borne pathogens. (Georgieva et al., 2010). Overall, metagenomic sequencing is a favorable tool for studying microbial communities, diversity, and function. Currently, there are only a few research on microbial species in MC (Y. Wang, Zeng, Qiu, Han, & Wang, 2024). However, there is a lack of metagenomic study of MC, which leads to current inadequate understanding of species and functional gene annotation mining of the microorganisms present in MC.

The formation of cheese flavor is a sophisticated and gradual process that primarily occurs during the ripening period. As the milk components undergo various chemical and biochemical reactions, three primary reactions stand out: lactose metabolism, protein hydrolysis, and lipolysis. These reactions collectively contribute to the development of the unique flavor profile in cheese (Anastasiou et al., 2022; Hassan & Gawad, 2012). Lactose in cheese is mainly produced by the action of fermenting agents to produce lactic acid (McSweeney, 2004). The proteins within cheese are broken down into smaller peptides and amino acids due to the action of rennet, protease, and enzymes secreted by microorganisms (Block, 1951). These peptides and amino acids serve as precursors for specific flavor substances or contribute directly to the flavor profile of cheese. The fat in cheese is enzymatically hydrolyzed by lipase into various compounds, including free fatty acids, aldehydes, and alcohols. At present, research on the flavor formation of MC is still in its infancy. Previous research results have shown that the main volatile flavor compounds of MC are acids, esters, and methyl ketones, such as butanoic acid, hexanoic acid, 2-heptanone, 2-nonanone, hexanoic acid butyl ester, and hexanoic acid hexyl ester (Y. Wang et al., 2024; Wu, Yu, Liu, & You,

2019; Zeng, Wang, Han, Cao, & Wang, 2022). These compounds collectively endow MC with unique aroma characteristics. Microorganisms associated with the flavor formation of MC include *Lactococcus lactis, Monascus*, and *Lactobacillus* (Y. Wang et al., 2024). However, how the microorganisms involved affect the formation of MC flavor compounds, i.e. which key enzymes play a role, is not clear.

In this study, volatile compound evolution in MC throughout various ripening stages was investigated using SPME arrow-GC-MS in conjunction with GC-O techniques. Additionally, PLS-DA was employed to distinguish characteristic flavor compounds among the samples. Furthermore, metagenomic analyses were conducted to analyze the microbiological profiles of MC at different ripening stages, encompassing species annotation and functional gene annotation. Finally, a relationship between the formation of flavor substances in MC and microorganisms was established using catalase as a bridge. This work provides insights into the study of flavor formation of MC, which contributes to the precision fermentation and control of cheese flavor and quality in future food industries.

2. Materials and methods

2.1 Manufacturing *Monascus*-fermented cheese

The preparation of MC followed the same protocol as outlined in our previously published research (Zeng et al., 2022). Specifically, the milk was pasteurized at 65°C for 30 min. Before renneting, the milk was inoculated with a commercial freeze-dried starter culture (CHR R-704, CHR Hansen, Hoersholm, Denmark) and *Monascus purpureus* M1 spore liquid (5.0×10^7 spores per milliliter) at 31°C and acidified for 60 min. Then, commercial rennet (CHR Hansen, Hoersholm, Denmark) was added (0.2 g/L milk) at 31°C for 45 min. The curd was cut into approximately 2×2×2

97 cm cubes. After draining off the whey, NaCl (2.00 g/100 g curd) was added to the curd. Then, the
98 prepared cheese was incubated in an incubator (KB450, Binder, Germany) at 26°C and 90% relative
99 humidity for 5 days, followed by storage at 8°C for 90 days. Samples were collected from the cheese
100 at seven distinct ripening stages (0, 5, 10, 25, 40, 60, and 90 days). Cluster analysis was conducted
101 based on the flavor information of cheese samples at seven distinct ripening stages, and three
102 ripening stages with significant flavor differences were selected for further analysis (Fig. S1).
103 Samples were taken in triplicate.

104 2.2 Gas chromatography-mass spectrometry analysis (GC-MS)

105 The volatile compounds from cheese were isolated using solid-phase microextraction (SPME)-
106 arrow with a specific fiber (a 120 μm DVB/CWR/PDMS fiber from Agilent Technologies, USA).
107 These compounds were then analyzed by gas chromatography-mass spectrometry (GC-MS),
108 employing an Agilent 7890B GC interfaced with an Agilent 5977A mass-selective detector. The
109 analytical parameters used for this analysis were aligned with those reported in previous scientific
110 studies (Zeng et al., 2022). Each sample (6.0 g) was prepared in headspace vials (40 mL) for
111 headspace solid-phase microextraction. 2-methyl-pentanoic acid (1266.8 μg/g) and 2-methyl-3-
112 heptanone (154.3 μg/g) (Aladdin, Shanghai, China) were used as an internal standard. The capillary
113 column was DB-WAX with a length of 60 m, an internal diameter of 250 μm, and a film thickness
114 of 0.25 μm (Agilent Technologies, USA). Extraction was then performed using a SPME-arrow fiber
115 at 50°C for an additional 30 min. Helium, flowing at a rate of 1 mL/min, served as the carrier gas.
116 The temperature program began at 40°C and held constant for 5 min, followed by a gradual increase
117 to 230°C at a rate of 3 °C/min.

118 2.3 Gas chromatography-olfactometry (GC-O)

An Agilent 7890B gas chromatograph (GC) integrated with a sniffing system (ODP 3 from Gerstel, Mülheim, Germany) was utilized to pinpoint odor-active compounds. The olfactory port and transfer line temperatures were maintained at 230°C and 250°C, respectively. During the GC-O analysis, three experienced panelists noted the aroma descriptors and the corresponding retention times of aroma-producing compounds. An odor-active compound was designated when two or more panelists concurred in detecting the aroma (Yin et al., 2023; Yu et al., 2023).

2.4 Qualitative and quantitative analysis

The flavor compounds were initially identified by comparing the mass spectra of each analyte with the mass spectra of library standards (MS). Actual retention indices (RI) were then calculated for each compound based on n-Alkanes (C_7~C_{30}). Furthermore, the compounds smelt were reconfirmed based on standard odor descriptions (O) and verified with the existing standards (S). In addition, all identified compounds were initially semi-quantified using the internal standard (2-methyl-pentanoic acid and 2-methyl-3-heptanone).

2.5 DNA extraction and sequencing analysis

1 g of the sample was ground in a sterile quartz mortar for 2 min and homogenized with 9mL of sterile NaCl solution (0.85% w/v) for 10 min. Subsequently, centrifuge the suspension at 5,000 × g for 10 minutes at 4℃. Use a sterile spoon to collect 1g of upper sediment aseptically into a sterile microcentrifuge tube. According to the manufacturer's plan, total DNA was extracted from sediment using the Qiagen DNA Mini kit (Qiagen, Hilden, Germany). Nanodrop ND 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, Germany) was used to determine the concentration of extracted DNA, and the purity of DNA was evaluated by 1.0% agarose gel electrophoresis. Samples were stored at a temperature of -20℃. DNA libraries were paired-end

sequenced using the Illumina HiSeq4000 sequencing platform (Novaseq, Beijing, China).

2.6 Bioinformatic analysis

The fastp software 0.20.0 (Haplos, Shenzhen, China) was used to remove the adapter sequences at the 3' and 5' ends of the original sequenced sequences and the reads that were less than 50 bp in length, had an average quality value of less than 20 and contained N bases. considering host gene contamination, the reads were compared to the host (cow) DNA sequences by the software BWA 0.7.17-r1188 (Heng Li, China), and the contaminated reads with high similarity were removed to obtain high-quality clean data for the next step of analysis. Megahit software 1.1.2 (https://github.com/voutcn/megahit, Shenzhen OSI Network Technology Co., Ltd., China) was used to assemble the data after quality control, and the succinct de Bruijn graph method was adopted, with the splicing parameters iteratively spliced from small k-mer to large k-mer, and the shortest length of 300 bp was retained. The shortest contigs of 300 bp were retained, and then MetaGene was used to predict the open reading frames (ORFs) of the assembled contigs to obtain gene sets. The predicted gene sequences were clustered using CD-HIT software 4.6.1 (http://weizhongli-lab.org/cd-hit/), and the redundant genes with identity and coverage greater than 90% were removed to construct a set of non-redundant catalog genes. Finally, the high-quality reads of each sample were compared with the non-redundant gene set using SOAP aligner soap2.21release software (https://github.com/ShujiaHuang/SOAPaligner, Shenzhen UW Genome Research Institute, China). Finally, the high-quality sequencing reads from each sample were aligned to the non-redundant gene set using the SOAP aligner software version soap2.21 (UW Genome Institute, Shenzhen, China). This comparison was conducted to determine the abundance of genes within the respective samples.

2.7 Statistical analysis

All statistical analyses were conducted with three biological replicates. The clustering heat map analysis by the OriginPro 2022 (OriginPro Lab Corp., Northampton, America). Additionally, Duncan's multiple tests were applied to verify significant differences in flavor compounds at a level of $p<0.05$ (the Statistical Program for the Social Sciences 23.0; SPSS Inc., Chicago, IL, USA). Partial least squares-discriminant analysis (PLS-DA) was accomplished by the SIMCA 14.1 (Umetrics, Malm¨o, Sweden). Metagenome annotation database information is shown below, NR species annotation (nr_202109, https://ftp.ncbi.nlm.nih.gov/blast/db/FASTA/), KEGG functional annotation (202109, https://www.genome.jp/kegg), COG functional annotation (2020, http://eggnog5.embl.de/#/app/downloads), carbohydrate-active enzyme analysis (v8, http://bcb.unl.edu/dbCAN2/download/Database). GC-O experiment was approved by the University Scientific Research Ethics Committee of BTBU (2024-64).

3. Results and discussion

3.1 Volatile flavor compound characteristics of *Monascus*-fermented cheese at different ripening periods

The volatile flavor compounds of MC across 90-day ripening periods were determined by SPME (arrow)-GC-MS and a total of 75 compounds were identified. Cluster analysis (Fig. S1) revealed three distinct ripening periods, namely 10 days, 40 days, and 90 days with significant flavor profile differences, which were also visualized in the heat map (Fig. 1A). Subsequently, we identified 43 compounds that could be detected by olfaction, i.e., compounds contributing to the odor profile of MC, using GC-O analysis (Table S1). Finally, PLS-DA identified 26 characteristic flavor compounds (VIP > 1, $p < 0.05$).

The 26 different compounds in MC are mainly acid compounds, ester compounds, and methyl

ketone compounds, among others. The acid compounds were butanoic acid, hexanoic acid, octanoic acid, decanoic acid, undecanoic acid, benzoic acid, and dodecanoic acid. These acid compounds of medium and short carbon chains have a low threshold value and produce special milky, rancid, and other irritating flavors at higher levels. The ester compounds in MC were butanoic acid ethyl ester, hexanoic acid butyl ester, hexanoic acid hexyl ester, heptanoic acid ethyl ester, octanoic acid ethyl ester, nonanoic acid ethyl ester, and dodecanoic acid ethyl ester. The ester compounds in MC are mostly ethyl esters. Ethyl esters can commonly impart milky, fruity, and creamy aromas to cheeses (Romain, Marie-Bernadette, Jean-Rencb, Sylvie, & Anne, 2008; Senoussi et al., 2022). Butanoic acid ethyl ester, heptanoic acid ethyl ester, octanoic acid ethyl ester, and nonanoic acid ethyl ester are common medium and short carbon chain fatty acid ethyl esters that have a low threshold, and mixtures of these esters are known as butter esters, which usually produce a pleasant odor. The methyl ketone compounds in MC were 2-heptanone, 2-octanone, 2-nonanone, 2-decanone, and 2-undecanone. The methyl ketone compounds are the typical volatile flavor compounds of blue cheese, giving the cheese a fruity and creamy aroma (Cao, Fonseca, Schoenfuss, & Rankin, 2014; Qian, Nelson, & Bloomer, 2002). Other compounds also include δ-decanolactone, acetophenone, 1-dodecanol, benzaldehyde, limonene, styrene, and dimethyl sulfone. δ-decanolactone has a creamy, fruity aroma of coconut and peach (Peterson & Reineccius, 2003). Acetophenone exudes a fruity aroma (Paschke, Hutzler, Henkler, & Luch, 2015), 1-dodecanol possesses a pungent odor, benzaldehyde imparts a bitter almond scent (Q. Wang, Jiang, Qin, Liu, & Wu, 2020), limonene emanates an orange aroma (Norman, Craft, & Davis, 2010), styrene has a slightly sweet odor, and dimethyl sulfone carries a slightly foul scent.

PLS-DA is a supervised classification method that integrates the benefits of principal

component analysis (PCA), canonical correlation analysis (CCA), and multiple linear regression analysis (MLRA), enabling it to effectively extract predictive information from high-dimensional data (Feng et al., 2023). PLS-DA has been widely applied to analyze the interrelation of different volatile compounds (P. Yang et al., 2022; Zhang et al., 2022). The flavor compounds of MC under different ripening periods were compared by PLS-DA ($R^2X = 0.894$, $R^2Y = 0.993$, $Q2 = 0.976$, $p < 0.05$). The VIP diagram and the Permutation test of PLS-DA were shown in Fig. S2 and Fig. S3. As depicted in Fig. 1B, the respective contribution rates of the first and second principal components, t1 and t2, were 45.4% and 37.2%. Together, these two principal components accounted for a cumulative contribution rate of 82.6%.

In the PLS-DA model, MC samples ripened for 10 days, and 90 days were on the positive end of t1. MC ripened for 40 days and was on the negative side of t1 and t2. This suggests that MC from different ripening periods can be clearly distinguished based on the type and content of flavor compounds. Further analysis as shown in Fig. 1C, compounds such as benzaldehyde (69), propanoic acid (2), 2-heptanol (57), and 2-heptanone (46) were enriched in cheeses ripened for 10 days, with 2-heptanone (46) among the compounds with VIP > 1. Compounds such as decanoic acid (12), undecanoic acid (14), dodecanoic acid (16), 2-methyl-propanoic acid (3), benzaldehyde (68) and were enriched in cheeses ripened for 40 days, among which compounds with VIP > 1 were decanoic acid (12), undecanoic acid (14) and dodecanoic acid (16). This suggests that differences in the content of methyl ketones and acids can be responsible for the differences in volatile flavor profiles of MC at different ripening times. The differential compounds with VIP > 1 are important contributions to the identification of MC from different ripening periods using volatile flavor compounds. These compounds provide basic data for subsequent studies on the formation pathways

of volatile flavor compounds in MC.

3.2 Metagenome-based analysis of the composition and function of Monascus-fermented cheese microorganisms across 90-day ripening periods

As described previously, there were significant differences in flavor compounds among samples, which were ripened for 10, 40, and 90 days. To better understand the role that microorganisms played in altering the flavor profiles, we used metagenomics to obtain genetic information on MC (Table 1). Species and functional annotations were carried out to analyze the microbial community composition and to compare the differences in species composition of MC in different ripening periods.

3.2.1 The microbial community of *Monascus*-fermented cheese across 90-day ripening periods

A total of 42 phyla, 81 classes, 172 orders, 323 families, 620 genera, and 1733 species were annotated from the samples of MC at different ripening periods. The top 30 genera in terms of abundance were shown in Fig. 2A, including *Lactococcus, Monascus, Staphylococcus, Aspergillus, Lactiplantibacillus, Meyerozyma, Streptococcus, Penicillium, Bacillus, Mycobacteroides, Kaistella, Flavobacterium, Enterobacter, Talaromyces, Rasamsonia, Enterococcus, Paecilomyces, Histoplasma, Streptomyces, Fusarium, Penicilliopsis, Schinkia, Oenococcus, Citrobacter, Virgibacillus, Anaerosalibacter, Coccidioides, Blastomyces, Fictibacillus, Blautia*. Changes in the abundance of 30 genera in MC at different ripening periods can also be observed in Fig. 2B. The most abundant bacterium was *Lactococcus*, which gradually increased during MC ripening. This is consistent with the previous 16S sequencing results obtained in our study (Y. Wang et al., 2024). *Lactococcus* is a lactic acid bacterium that has been used for centuries in the production of a variety of cheeses, as these bacteria rapidly acidify milk and greatly contribute to the flavor of the

fermentation end-products (Bert, Bron, Sijtsma, De Vos, & Hugenholtz, 2014). The fungus with the highest abundance was *Monascus*, which initially rises and subsequently declines throughout the ripening process of MC. This observation aligns with the previous ITS sequencing results reported in our study (Y. Wang et al., 2024). During the ripening process, *Monascus* produces several enzymes involved in the ripening of the cheese, such as proteases and esterases (Chung, Suh, Choi, Noh, & Bae, 1999). In addition, *Monascus* produces reddish pigments that give MC its distinctive reddish appearance (Liu, Zhao, Huang, Xin, & Wang, 2018). During fermentation, *Monascus* also produces a variety of secondary metabolites, such as γ-aminobutyric acid and Monacolin K (Chen, He, Zhou, Shao, & Chen, 2015), which may provide potential functionality to MC. The decrease in the abundance of *Monascus* in the later periods of cheese ripening may be due to the absolute dominance of *Lactococcus*.

α-Diversity defines the variety and abundance of species in a microbial community. *β*-Diversity indexes are calculated for each pair of groups and represent either a similarity or difference between the 2 groups (Ianni et al., 2020). The *α*-diversity was the highest overall in cheese samples in ripened 10 days, and lowest in the cheese samples on day 90 (Fig. 2C), according to the Simpson index. The reduction of taxa during ripening followed the increment (relative abundance) of the starter culture *Lactococcus* and *Monascus*, which together dominated the microbial community. Furthermore, utilizing the weighted UniFrac distance metric, *β*-diversity analysis revealed a statistically significant distinction between the 90-day cohort and the remaining groups, with a significance level of $p<0.05$ (Fig. 2D). This divergence can be primarily attributed to the substantial decrease in *Monascus* abundance observed for 90 days of MC ripening.

3.2.2 Functional gene category by blasting to EggNOG, KEGG, CAZy databases

Functional annotation using Evolutionary genealogy of genes Non-supervised Orthologous Groups (EggNOG), Kyoto Encyclopedia of Genes and Genomes (KEGG), and Carbohydrate-active enzymes (CAZy) databases were used to compare functional gene metabolism information of MC at different ripening periods and to differentially compare them at the level of gene function. As shown in Fig. 3, the Circos plot demonstrates the distribution of functions present in samples of MC at different ripening periods. The bar graph illustrates the differences in the average relative abundance of the same function between the different groups.

The sequences of the non-redundant gene sets were aligned with the EggNOG database to obtain the clusters of orthologous groups of proteins (COG) corresponding to the genes and calculate the corresponding gene abundance. The statistical results of the overall microbial functional annotation of MC were shown in Fig. 3A. A total of 24 COG functional genes were annotated in MC. The genes with high relative abundance were 8% amino acid transport and metabolism(E), 8.0% carbohydrate transport and metabolism (G), 7.0% translation, ribosomal structure and biogenesis (J), and 7% transcription (K). The primary components of cheese are proteins, with a minor amount of lactose. Microbial amino acid metabolism genes, energy metabolism genes, and carbohydrate metabolism genes account for a relatively high proportion, ensuring the normal ripening of cheese to some extent. Additionally, during the ripening process, cheese microorganisms undergo enrichment and expansion, leading to a high density and activity of the flora. The growth, proliferation, and genetic stability of these microorganisms are determined by the replication, recombination, repair, and transcription of DNA, ultimately leading to the presence of numerous related functional genes. The differences in functional genes of MC at different ripening periods were further compared. A total of 9 COG functional genes were

295 significantly different (p<0.05), and the abundance of 5 COG functional genes in 90-day ripened
296 cheese was significantly higher than that in other ripened cheeses, including E, J, K, P (Inorganic
297 ion transport and metabolism), F (Nucleotide transport and metabolism). This may indicate that
298 these functional genes may be derived from *Lactococcus*. The highest abundance of *Lactococcus*
299 was found in MC ripened for 90 days.

300 The statistical results of the overall functional annotation of the microorganisms of MC were
301 compared with the KEGG database and were presented in Fig. 3C. A total of 30 subclasses of
302 metabolic pathways were annotated at level 2 level metabolic pathways. The abundance of related
303 genes in MC were all significantly different. Among them, the highest abundance of genes related
304 to global and overview maps metabolic pathways, followed by carbohydrate metabolism, amino
305 acid metabolism, and metabolism of cofactors and vitamins. Furthermore, there were significant
306 differences in 15 categories of metabolic pathways in MC at different ripening periods. Among them,
307 global and summary maps, carbohydrate metabolism, amino acid metabolism, nucleotide generation,
308 lipid metabolism, cofactor and vitamin metabolism, and metabolism of terpenoids and polyketides
309 terpenoids and polyketides had a higher abundance of the relevant genes in cheeses ripened for 90
310 days than cheeses at other ripening periods. The biosynthesis of the most important fatty acids,
311 esters, and methyl ketones in MC involves carbohydrate metabolism, amino acid metabolism, and
312 lipid metabolism. Pyruvic acid from carbohydrate metabolism enters the tricarboxylic acid cycle to
313 synthesize citric, succinic, and malic acids, which are common organic acids in cheese. Meanwhile,
314 acetyl CoA, which is generated by oxidative decarboxylation, is the hub for the interconversion of
315 sugars, fats, and amino acids, leading to differences in the content of fatty acid compounds, esters,
316 and methyl ketones, and affecting the metabolic production of cheese flavor substances.

317 Carbohydrate metabolism, amino acid metabolism, and lipid metabolism were shown to be
318 important metabolic pathways during cheese ripening.

319 Carbohydrate metabolism was an important metabolic pathway during cheese ripening that
320 affects the production of cheese flavor substances, as shown by the results of the annotation of the
321 egg NOG database and the KEGG database. The CAZy annotation results were shown in Fig. 3E.
322 The highest percentage of the cheese samples were glycosyl transferase genes with an average
323 relative abundance of 38.11%. Glycosyl transferases were mainly involved in the synthesis of
324 polysaccharides, oligosaccharides, and disaccharides (Zhao, Ma, Yin, Shi, & Ding, 2021), and the
325 related genes had the highest abundance in cheeses ripened for 10 days, suggesting that more
326 polysaccharide-producing microorganisms were present in cheeses ripened for 10 days. The
327 glycoside hydrolase gene (37.56%) was the second most abundant gene among cheese
328 microorganisms and had a higher percentage of genes in cheeses ripened for 10 days than in cheeses
329 ripened for other ripening times. Glycoside hydrolases hydrolyze or rearrange glycosidic bonds and
330 function in the breakdown of carbohydrates (Wardman, Bains, Rahfeld, & Withers, 2022), e.g.
331 hydrolysis of lactose. The higher the abundance of glycoside hydrolase genes, the faster the
332 breakdown of carbohydrates, i.e. the faster the rate of saccharification during the early stages of
333 cheese ripening, favoring the metabolism of lactic acid bacteria to produce acetic acid and ethanol.
334 The third highest gene abundance was the carbohydrate esterase gene (15.89%), which had a higher
335 percentage of genes in cheeses ripened for 90 days and 10 days than in cheeses ripened for 40 days.
336 Esters are important flavor compounds in cheese and carbohydrate esterases have the function of
337 synthesizing or hydrolyzing esters (Nakamura, Nascimento, & Polikarpov, 2017).

338 3.3 Predicted roles that microbes played in the formation of characteristic flavor compounds

Taking the characteristic flavor compounds of MC as a starting point, the catalase enzymes in the metabolic pathways related to the production of volatile flavor compounds of the main body of MC were sorted out by comparing them with the known metabolic pathways in the KEGG database (Fig. S5). Using the coding genes of catalase as a bridge, the metabolic relationship between the formation of flavor compounds in MC and microorganisms was established. Metabolic networks were predicted for the catabolism of three substrates (fat, protein, and lactose) and the formation of 17 main flavors in the MC microbial community (Fig. 4). The enzymes involved in the different metabolic pathways are shown in Table 2.

As shown in Fig. 4, glucose, phosphoenolpyruvate, pyruvate, and acetyl CoA produced by two metabolic pathways, glycolysis (ko00010) and pyruvate metabolism (ko00620), were the backbone of the metabolic network of volatile flavor compounds in MC. Glucose can produce histidine through the pentose phosphate pathway (ko00030) and histidine metabolic pathway (ko00340). Glucose can also produce phosphoenolpyruvate through the ko00010 pathway. Phosphoenolpyruvic acid is a precursor of phenylalanine and tyrosine and can be produced via the ko00400 pathway. Phenylalanine and tyrosine are produced via the ko00360 and ko00350 pathways, respectively, to produce β-phenylethanol and p-hydroxyphenylethanol, which are the main higher alcohols in MC, contributing to floral and fruity flavors. Phosphoenolpyruvic acid is produced by continuing the ko00010 pathway to pyruvate. Pyruvic acid is the precursor of many flavor substances, through the ko00620 pathway to generate the main substance of MC ethanol, and the main organic acids, lactic acid and acetic acid. The acetyl CoA can enter the TCA cycle to produce the flavor organic acid citric acid, but also through the ko00061, ko00650 pathway to generate hexanoic acid, decanoic acid, and other compounds. Acid compounds generated by esterification and higher alcohols, such

as octanoic acid ethyl ester, and nonanoic acid ethyl ester, are the main ester compounds in MC and contribute significantly to its unique aromatic qualities. β-Keto acids are oxidized to generate methyl ketones, such as 2-heptanone, 2-nonanone, etc., which contribute fruity and creamy aromas. β-Keto acids are used to generate aspartate acid and glutamate, which are intermediates of the TCA cycle, through the ko00250 pathway to produce aspartate and glutamate. Aspartate is an important nodal substance in amino acid metabolism, which can generate lysine and arginine via ko00300, and ko00220, respectively. The interconversion of serine, glycine, threonine, methionine, and cysteine via ko00260 and ko00270 produces the main amino acids in MC.

In addition, the genes coding for catalase were used as a bridge to predict the relationship between microorganisms and flavor compounds. As shown in Fig. 4, the genera *Monascus, Lactococcus, Aspergillus, Lactiplantibacillus, Staphylococcus, Flavobacterium, Bacillus, Clostridium, Meyerozyma*, and *Enterobacter* were considered to be closely associated with flavor production in MC. The ester compounds in MC were mainly ethyl esters, which were produced by the esterification reaction of alcohol and acid compounds. Based on the annotation results, it was presumed that the genera of *Monascus, Meyerozyma, Staphylococcus, Lactiplantibacillus*, and *Bacillus* played an important role in the synthesis of acetate esters. *Lactococcus, Lactobacillus, Staphylococcus*, and *Bacillus* played an important role in the synthesis of acids, which were produced by carbon chain extension reactions using acetyl CoA as a precursor. Methyl ketone compounds are mainly derived from the β-oxidation pathway of fatty acids, and the genera of *Monascus, Staphylococcus, Lactiplantibacillus, Lactococcus, Bacillus,* and *Flavobacterium* played an important role in the synthesis of methyl ketone compounds.

4. Conclusion

In this work, we performed flavoromic and metagenomic analyses to investigate hidden liaisons between volatile flavor compounds and microorganisms of *Monascus*-fermented cheese (MC) across 90-day ripening periods. Firstly, a total of 26 characteristic flavor compounds were identified in cheeses ripened for 10, 40, and 90 days. Secondly, metagenomic annotation revealed that the genera *Monascus, Lactococcus, Aspergillus, Lactiplantibacillus, Staphylococcus, Flavobacterium, Bacillus, Clostridium, Meyerozyma*, and *Enterobacter* were closely associated with flavor formation in MC. Furthermore, using catalase as a bridge, multi-omic analysis divulged that ester compounds formed in MC were most likely derived from the genera *Monascus*, *Meyerozyma*, *Staphylococcus*, *Lactiplantibacillus*, and *Bacillus*; acid compounds were closely linked to *Lactococcus, Lactobacillus, Staphylococcus*, and *Bacillus;* methyl ketone compounds was strongly related to the genera *Monascus, Staphylococcus, Lactiplantibacillus, Lactococcus, Bacillus,* and *Flavobacterium*. Finally, given the above, we predicted a map of the metabolic pathways of characteristic flavor compounds of MC. Nevertheless, given the complexity of intertwined chemical, biochemical, and physical changes that occurred during the cheese maturation process, it is essential to validate these findings through for instance controlled-variable fermentation of specific combinations of starter cultures, coupling with flavoromics, meta-transcriptomics, and proteomics in future studies.

Abbreviations

MC, *Monascus*-fermented cheese; GC-MS, Gas chromatograph-mass spectrometer; SPME-Arrow, Solid-phase microextraction Arrow; GC-O, Gas chromatography-olfactometry; EggNOG, Evolutionary genealogy of genes Non-supervised Orthologous Groups; KEGG, Kyoto Encyclopedia of Genes and Genomes; CAZy, Carbohydrate-active enzymes.

CRediT authorship contribution statement

Yadong Wang: Formal analysis, Investigation, Methodology, Writing—original draft and editing. Ying Wang: Writing—original draft and editing. Sizhe Qiu: Writing—review and editing. Bei Wang: Supervision, Validation, Methodology, Writing—review and editing, Funding acquisition, Project administration. Hong Zeng: Writing—review and editing, 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 the work reported in this paper.

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Tables and figures

Table 1 Overview of metagenomic data

Basic information	Samples		
	10d	40d	90d
Raw reads	94148656	89040678	92018048
Clean reads	92860312	87893800	91070000
Raw base(bp)	14216447056	13445142378	13894725248
Clean base(bp)	13992447834	13181209084	13713397417
Contigs	17857	17761	27254
Contigs(bp)	50371629	43737903	50002159
Number of ORFs	58194	51779	57222
Average length of ORFs(bp)	689.23	577.57	618.98

Table 2 Key enzymes in substrate decomposition and flavor metabolism of *Monascus*-fermented cheese microbiota

No.	Group name	Pathway in KEGG	EC number of enzymes	Examples (reads > 500)
1	Glucose utiliser	ko00010, ko00020, ko00030, ko00270	3.1.3.10,2.7.1.1,5.3.1.9,2.7.1.2,5.1.3.1,2.7.1.11, 3.1.3.11	*Monascus, Meyerozyma, Clostridium, Lactococcus, Lactiplantibacillus, Anaerosalibacter, Bacillus, Vibrio, Flavobacterium, Streptomyces, Kaistella, Flavobacterium, Staphylococcus*
2	Ethanol utiliser	ko00010, ko00061	1.1.1.1,1.1.1.2	*Lactiplantibacillus, Lactococcus, Staphylococcus, Meyerozyma*
3	Lactate utiliser	ko00010, ko00620, ko00640	1.1.1.27,1.2.4.1,4.1.1.1	*Staphylococcus, Kaistella, Lactococcus, Lactiplantibacillus, Flavobacterium, Bacillus, Meyerozyma*
4	Acetate producer	ko00010, ko00620	1.2.1.3,1.1.1.1,1.1.1.2,6.2.1.1	*Aspergillus, Capronia, Flavobacterium, Kaistella, Meyerozyma, Monascus, Staphylococcus, Lactiplantibacillus, Lactococcus, Bacillus*
5	Acetate utiliser	ko00010, ko00620	6.2.1.1	*Meyerozyma, Staphylococcus, Kaistella, Monascus, Bacillus, Flavobacterium*
6	Lactate producer	ko00010, ko00620, ko00640	1.1.1.27,5.1.2.1,3.1.2.6,1.1.2.3	*Lactococcus, Staphylococcus*
7	Lactose utiliser	ko00010, ko00030, ko00040, ko00520, ko00051, ko00052,	3.2.1.108,2.7.1.207	*Lactococcus, Staphylococcus*
8	Pyruvate utilization	ko00010, ko00020, ko00260, ko00250, ko00061, ko00290, ko00300, ko00071, ko00620	1.2.4.1,4.1.1.1,6.4.1.1,4.1.2.14,2.3.3.21,2.2.1.6	*Staphylococcus, Kaistella, Lactococcus, Lactiplantibacillus, Flavobacterium, Bacillus, Aspergillus, Bacillus, Clostridium, Lactococcus, Meyerozyma, Monascus*
9	Succinate producer	ko00020	6.2.1.4,6.2.1.5,2.8.3.18,1.3.5.4,1.3.5.1	*Enterobacter, Lactococcus, Staphylococcus*
10	Glycine, serine, and threonine group	ko00260	2.7.1.39,1.1.1.1,1.1.1.29,1.1.1.381,1.1.1.399,1.1.1.95,1.1.1.79,1.1.1.215,1.1.1.81,1.1.99.1,1.2.1.8,1.4.4.2,1.8.1.4,2.1.2.10,2.3.1.29,2.3.1.37,2.	*Enterobacter, Flavobacterium, Kaistella, Lactiplantibacillus, Lactococcus, Meyerozyma, Monascus, Staphylococcus, Clostridium, Anaerosalibacter, Ralstonia, Clostridium*

			6.1.76,2.7.1.31,2.7.1.39,2.7.8.8,3.1.3.3,4.2.1.20 ,4.2.1.22,4.2.3.1,4.3.1.19,4.4.1.1	
11	Valine, leucine, and isoleucine group	ko00290	4.2.1.33,1.1.1.85,1.1.1.86,1.4.1.9,2.2.1.6,2.3.1. 182,2.3.3.13,2.6.1.42,2.6.1.66,2.6.1.2,4.2.1.33, 4.2.1.35,4.2.1.9,4.3.1.17,4.3.1.19	*Aspergillus, Bacillus, Clostridium, Flavobacterium, Kaistella, Lactiplantibacillus, Lactococcus, Meyerozyma, Monascus, Staphylococcus*
12	Alanine, aspartate, and glutamate group	ko00250	2.1.3.2,2.6.1.2, 6.3.4.4,6.3.4.5, 4.3.2.2,4.3.2.1, 6.3.5.4, 6.3.5.5,6.3.1.2	*Flavobacterium, Anaerosalibacter, Bacillus, Chryseobacterium, Clostridium, Enterobacter, Flavobacterium, Janthinobacterium, Kaistella, Lactiplantibacillus, Lactococcus, Meyerozyma, Monascus, Pseudomonas, Raoultella, Staphylococcus*
13	Lysine biosynthesis	ko00300	2.7.2.4,1.2.1.11,1.1.1.3,4.3.3.7,1.17.1.8,2.6.1.1 7,3.5.1.18,3.5.1.47,2.6.1.83,6.3.2.13,6.3.2.10	*Bacillus, Anaerosalibacter, Clostridium, Coccidioides, Enterobacter, Flavobacterium, Janthinobacterium, Kaistella, Lactiplantibacillus, Lactococcus, Meyerozyma, Monascus, Pseudomonas, Staphylococcus,*
14	Cysteine and methionine group	ko00270	4.3.1.17,4.4.1.13,2.5.1.47,2.6.1.52,1.1.1.95,1.1. 1.27,2.6.1.42,2.7.2.4,2.3.1.46,2.3.1.31,2.1.1.14, 2.1.1.37	*Flavobacterium, Bacillus, Clostridium, Enterobacter, Flavobacterium, Kaistella, Lactiplantibacillus, Lactococcus, Meyerozyma, Monascus, Staphylococcus*
15	Arginine and proline group	ko00220	6.3.1.2,1.4.1.2,2.3.1.1,2.7.2.8,1.2.1.38,2.6.1.11, 3.5.1.16,2.3.1.35,3.5.3.1,4.3.2.1,6.3.4.5,2.1.3.3, 3.5.1.16,2.7.2.2	*Meyerozyma, Anaerosalibacter, Bacillus, Clostridium, Enterobacter, Flavobacterium, Kaistella, Lactiplantibacillus, Lactococcus, Meyerozyma, Monascus, Raoultella, Staphylococcus, Streptococcus*
16	Phenylalanine and tyrosine group	ko00400	2.5.1.54,4.2.3.4,4.2.1.10,1.1.1.25,2.7.1.71,2.5.1 .19,4.2.3.5,4.1.3.27,4.2.1.20,4.1.1.48,5.3.1.24,2 .6.1.57,4.2.1.512.6.1.9,2.6.1.5,1.3.1.12	*Anaerosalibacter, Bacillus, Chryseobacterium, Clostridium, Enterobacter, Flavobacterium, Kaistella, Lactiplantibacillus, Lactococcus, Meyerozyma, Monascus, Staphylococcus,*
17	Histidine biosynthesis	ko00340	2.4.2.17,3.6.1.31,3.5.4.19,5.3.1.16,4.3.2.10,4.2. 1.19,2.6.1.9,3.1.3.15,1.1.1.23,1.2.1.3,1.4.3.4	*Lactococcus, Anaerosalibacter, Bacillus, Chryseobacterium, Clostridium, Enterobacter, Flavobacterium, Janthinobacterium, Kaistella, Lactiplantibacillus, Lactococcus, Meyerozyma, Monascus, Staphylococcus*

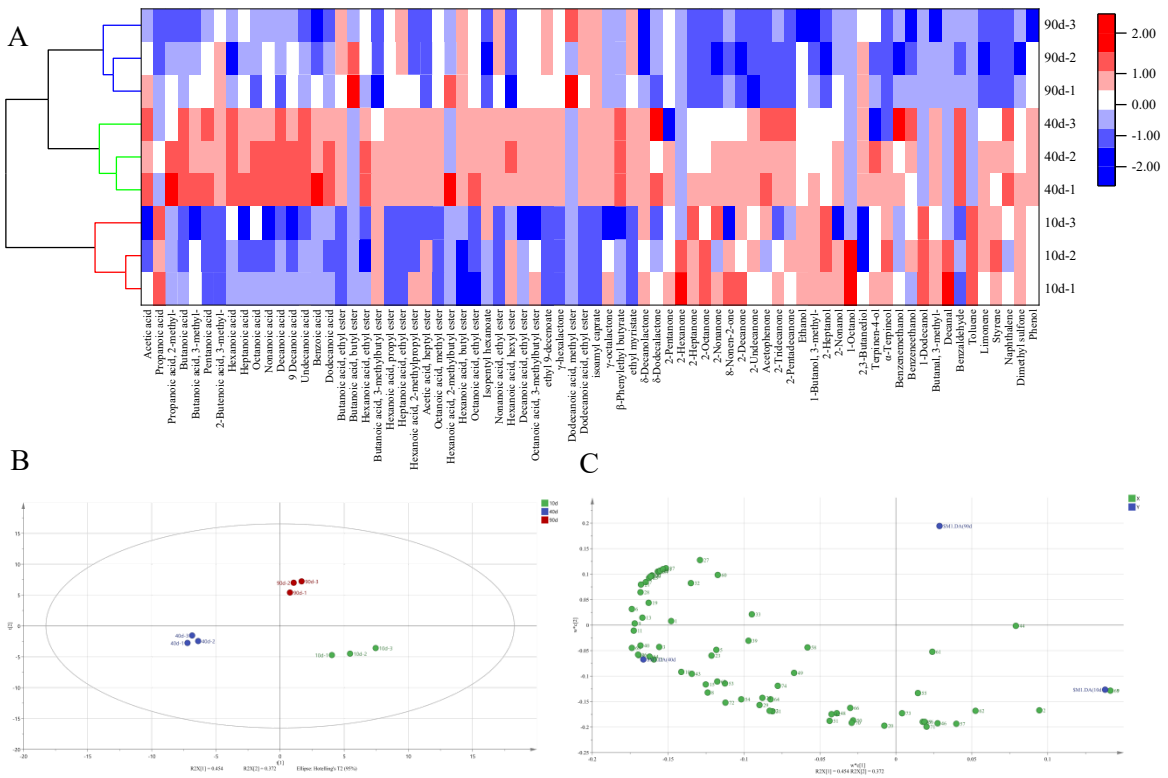

Fig. 1. The volatile flavor profile of Monascus-fermented cheese across 90-day ripening periods. (A), Heat map of volatile compounds. Ellipse: Hotelling's T2 (95%); (B), the PLS-DA sample diagram; (C), the PLS-DA loading diagram, X is the volatile compound. Y is the Monascus-fermented cheese sample across 90-day ripening periods. The corresponding relationship between compound and numbering information is shown in Table S1. Abbreviations: 10d, 40d, and 90d refer to cheese ripened for 10 days, 40 days, and 90 days, respectively.

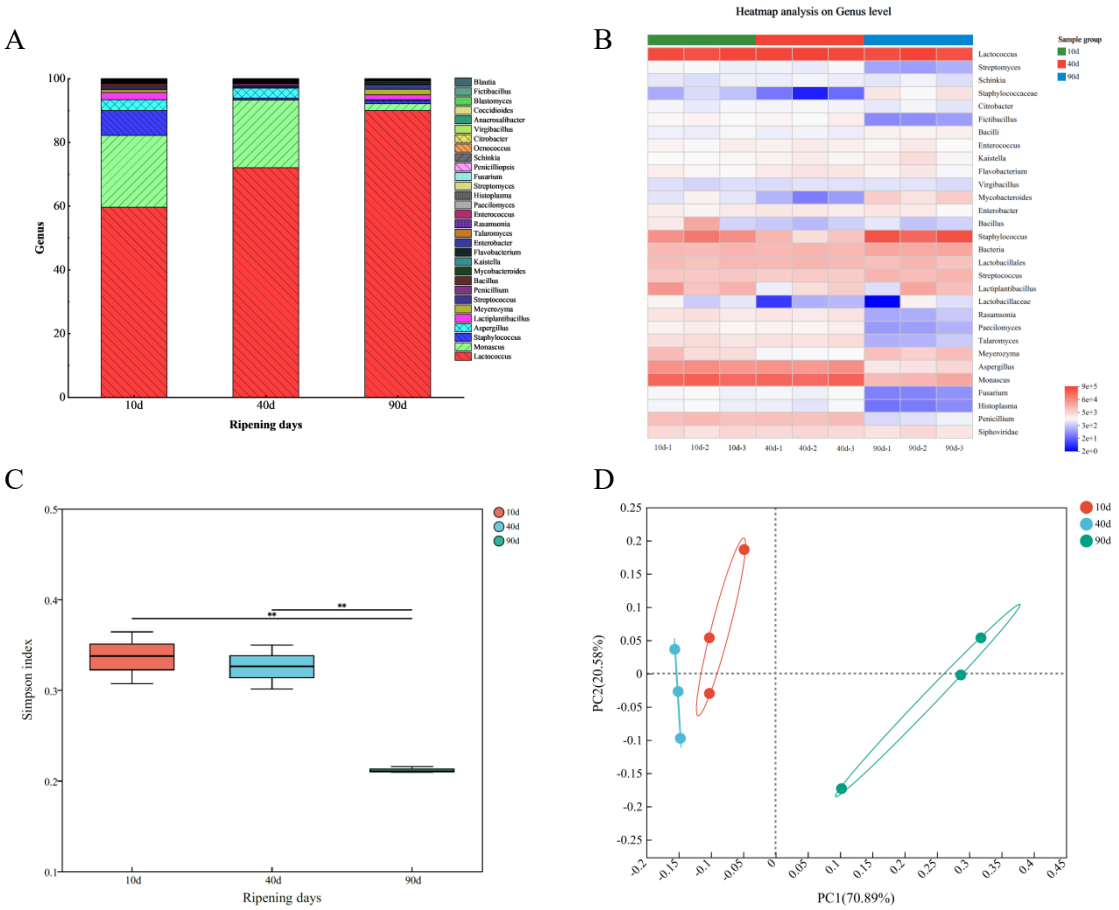

Fig. 2. Annotation of microbial of Monascus-fermented cheese (MC) across 90-day ripening periods. (A), Genus-level species abundance maps (top 30). (B), The Heat map of species at the genus level (top 30). (C), Changes in microbial community diversity in MC. Based on Shannon index of Turkey test α-Diversity. (D), Changes in microbial community diversity in MC. Based on weighted uniFrac distance metric β-Diversity. Abbreviations: 10d, 40d, and 90d refer to cheese ripened for 10 days, 40 days, and 90 days, respectively.

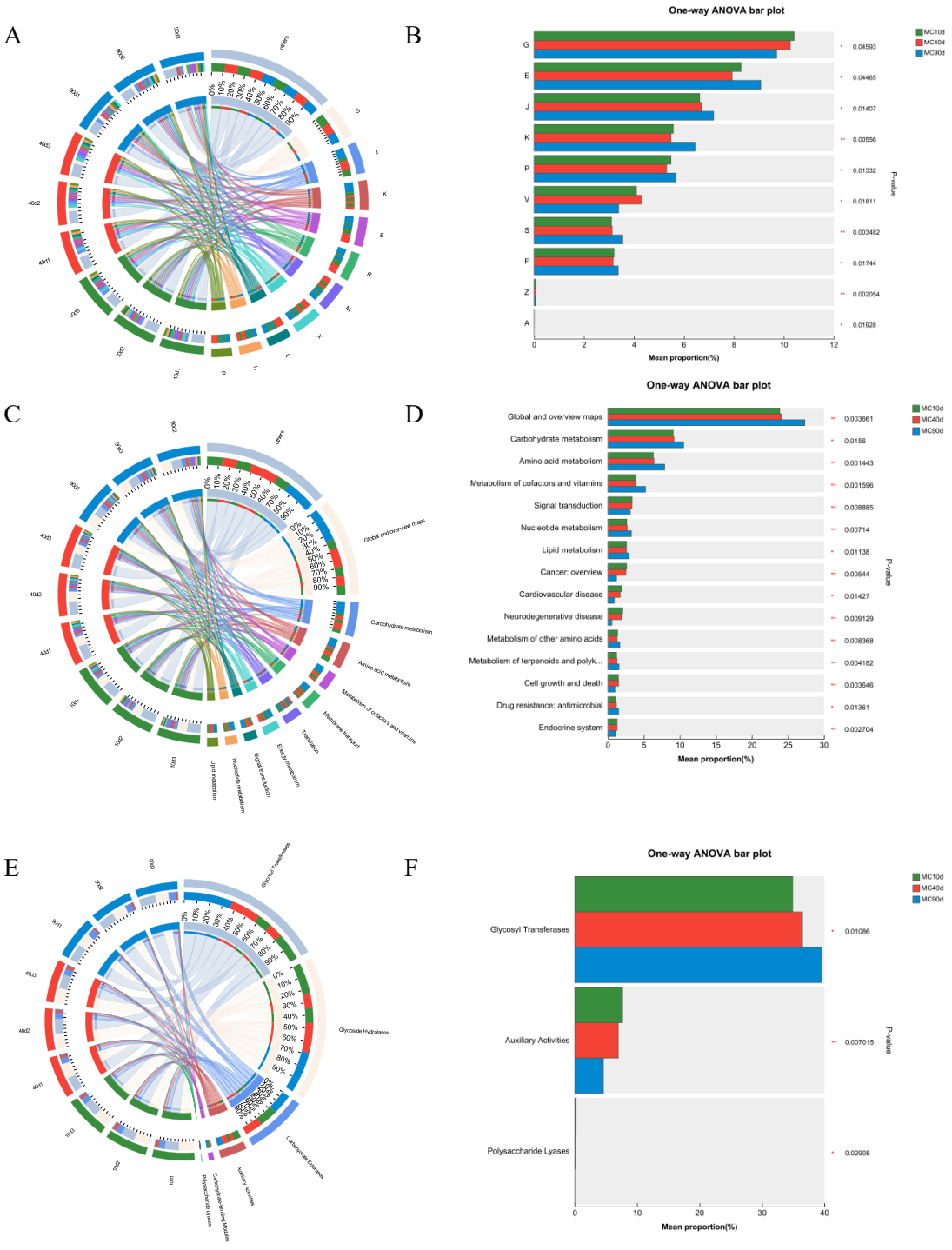

Fig. 3. Functional gene annotation plots. Circos sample-function relationship plots are commonly used to show the distribution of functions present in different microbial samples. The bar graphs show the differences in the mean relative abundance of the same function between different groups and are annotated as to whether the differences are significant (p-value values and asterisks indicate significant differences). (A), COG annotated Circos plot. (B), COG annotated bar graph plot. (C), KEGG annotated Circos plot. (D), KEGG annotated bar graph plot. (E), CAZy annotated Circos

plot F, CAZy annotated bar graph. 0.01<p<=0.05 *, 0.001<p<=0.01 **, p<=0.001 ***. Abbreviations: MC10d, MC40d, and MC90d refer to cheese ripened for 10 days, 40 days, and 90 days, respectively.

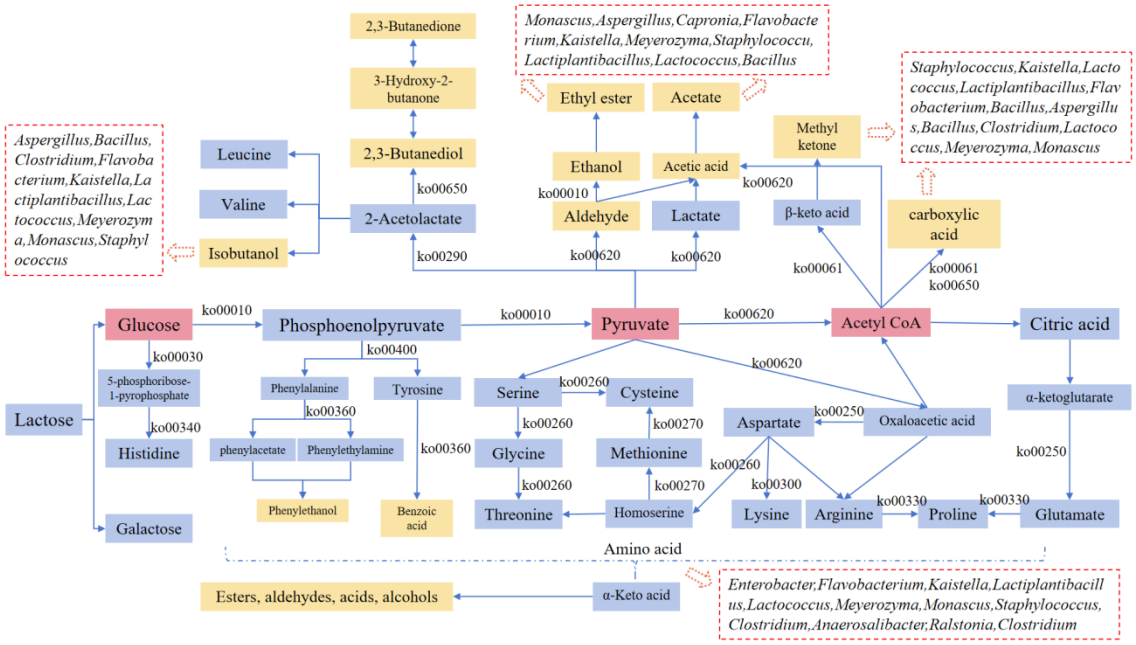

Fig. 4. Microbial and flavor metabolic pathways (KEGG pathway information, flavor compounds, and related microorganisms)