Identification of key aroma compounds and core functional microorganisms associated with aroma formation for *Monascus*-fermented cheese

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Abstract

This study aimed to analyze the key aroma compounds and core functional microorganisms of *Monascus*-fermented cheese (MC). 36 key aroma compounds were identified according to gas chromatograph-mass spectrometer (GC-MS), aroma extract dilution analysis (AEDA), and odor activity values (OAV) analysis. And internal standard curves were used to clarify the changes in their concentration of them during cheese ripening. Furthermore, High-throughput sequencing was used to investigate the composition and dynamic changes of bacteria and fungi in MC, respectively. *Lactococcus lactis* was found to be the dominant bacterium while *Monascus* was confirmed to be the dominant fungus. In addition, Pearson correlation analysis showed that *Lactococcus lactis*, *Staphylococcus*, *Trichococcus*, and *Monascus* were strongly associated with the 36 key aroma compounds ($r>0.80$, $p<0.05$). Finally, a metabolic network containing biosynthetic pathways of the key aroma compounds was constructed. This study provides deeper insights into the unique aroma of MC and the contribution of cheese microbiota.

Keywords**:** *Monascus*-fermented cheese; Gas chromatograph-mass spectrometer (GC-MS); Aroma extraction dilution analysis (AEDA); Key aroma compounds; Core functional microorganism; Biosynthetic pathways

1. Introduction

The *Monascus*-fermented cheese (MC) is a new fermented dairy product that emerged in 2019 (Wu, et al., 2019). The introduction of molds during the cheese ripening stage, e.g. *Penicillium roqueforti* in Blue cheeses and *Pen. camemberti* (mainly) in Camembert cheeses, can bring stronger proteolytic and lipolytic activities that increase the contents of fatty acids, esters, aldehydes, and ketones, which makes cheeses richer in flavor (Kosikowski, 1988). So far, two main methods have been reported to make *Monascus*-fermented cheese (Xia, et al., 2020; S. Zhang, et al., 2022). One is to imitate the production process of Blue cheese, which makes MC with internal mold. The type of MC with internal mold requires a long ripening time to allow mold to grow throughout the channels of the cheese, which means that the proteolysis, lipolysis, and microbial growth more thoroughly, results in a strong and stimulating odor. The other is to simulate the preparation of Camembert cheese which makes MC with surface mold. It has a shorter ripening period, with soft sensory qualities such as fruity and milk aroma (S. Zhang, et al., 2022). Due to the mild flavor and short ripening period, we chose to make a surface-mold ripened MC.

The variety and contents of flavor compounds are important indicators of cheese quality and also key factors that affect consumer preference and consumption choices (Kilcawley, 2017). Due to the unique aroma of MC and the potential market value, several works have been conducted to investigate the aroma profile and sensory attribute of MC recently (Xia, et al., 2020; S. Zhang, et al., 2022). In their work, the content of methyl ketones, esters, and alcohols was high. In addition, sensory attribute analysis indicated that the aroma and sensory quality of MC were higher than those of commercial blue cheese. Despite previous efforts, there is currently only a preliminary

understanding of the flavor quality of MC, and there is a lack of research on the key (characteristic) aroma compounds of MC. Besides, the specific metabolic pathways that are responsible for the formation of those key aroma compounds are still not clear.

Cheese is a fermented dairy product, containing different microbial communities, which change with time and according to the type of cheese and their respective starter and adjunct cultures. These microorganisms are important factors that determine the flavor, quality, and safety of cheese products (Santiago-Lopez, et al., 2018; Suzuki-Iwashima, et al., 2020). To make clear the relationship between the formation of flavor compounds and microbial communities, previous studies have used gas chromatography-mass spectrometry (GC-MS) to resolve the volatile flavor compounds (Shi, et al., 2021; Wang, et al., 2021), and then used high-throughput sequencing to study microbial communities diversity of fermented foods (Z. Liu, et al., 2020). By analyzing the change in aroma compounds and microbial community structure, the function of the microbial community over the cheese maturation process and the formation mechanism of aroma compounds can be unveiled, which would help improve cheese quality, and lay the foundation for cheese standardization. However, the composition of microorganisms and the role of microorganisms in the formation of flavor compounds, and the shaping of the overall flavor profile of MC have not been reported.

In this study, GC-MS, aroma extract dilution analysis (AEDA), odor activity values (OAV) analysis, microbial correlation, and metabolic pathway analysis were conducted to obtain a quantitative and dynamic understanding of the key aroma compounds and associated functional core microbes for MC across a 90-day ripening period. The volatile compounds by GC-MS, the qualitative analysis of key aroma compounds by AEDA, and the quantitative determination of key

aroma compounds by OAV. The time-dependent changes in the composition and abundances of bacteria and fungi of MC were identified by 16S rDNA and ITS, respectively. The potential correlations between key aroma compounds and microorganisms were uncovered through Pearson coefficients, and the core functional microorganisms (i.e. microorganisms strongly associated with aroma formation) were determined. Finally, a metabolic network containing the biosynthetic pathways of key aroma compounds was assembled referring to the genome-scale metabolic networks of *Saccharomyces cerevisiae*, *Lactococcus lactis subsp. cremori*s and *Staphylococcus aureus subsp. Aureus* and metabolic databases like BiGG and MetaCyc. Overall, this work aims to boost the breadth and depth of the current knowledge base of *Monascus*-fermented cheese, which would contribute to the development of high-flavor-quality cheeses in the dairy industry.

2. Materials and methods

2.1 Cheese preparation

The bovine milk was collected from a local dairy farm. The preparation of MC was consistent with our previously published research (Zeng, et al., 2022). The modification was that the ripening time of MC was extended. 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 3×3×3 cm cubes. After draining off the whey, NaCl (2.00 g/100 g curd) was added to the curd. Then, the curd was placed into square molds and transferred to the incubator (KB450, Binder, Tuttlingen, Germany) at 26°C and 70% relative humidity for 5 days, and then at 8°C for

90 days. Samples were collected at seven different ripening stages (0, 5, 10, 25, 40, 60, and 90 days) for further analysis. The selection of sampling time was based on the rapid growth of microorganisms in the early stage and the significant changes in the cheese, therefore the sampling was relatively dense. In the later stage, cheese was in the condition of low-temperature maturation, and microbial growth was in a relatively stable stage, so the sampling interval was extended.

2.2 Gas chromatography-mass spectrometry (GC–MS) analysis of volatile compounds

The volatile extract of cheese is extracted in two methods, including solvent-assisted flavor evaporation (SAFE) and solid-phase microextraction (SPME) -Arrow. For SAFE, the isolation of volatiles was according to the previous literature studies with a little modification (Whetstine, et al., 2005). Volatile compounds from MC solvent extract with 10 μL internal standard solution containing 2-methyl-pentanoic acid (1266.8 μg/mL) and 2-methyl-3-heptanone (154.3 μg/mL) dissolved in dichloromethane were distilled. Connected the receiver tube and waste tube to the SAFE apparatus. The glassware was then connected to an Edwards nxds6i rotary vane pump as the vacuum source. Used a circulating water bath to keep the SAFE apparatus at a constant temperature of 40°C. A liquid nitrogen was poured into the cooling trap and the receiver flasks. Distillation was carried out for 2 h under vacuum (~10^{-4} Pa). After distillation, the distillate was dried over anhydrous sodium sulfate overnight and then concentrated to 1 mL under a gentle stream of high-purity nitrogen. The concentrated distillate was washed three times with 1 mL 0.5 M sodium bicarbonate and mixed thoroughly. After each washing, remove the bottom layer, namely the water phase of the distillate, and collect it into a separate test tube. For SPME-Arrow, the equilibrium and extraction conditions are as described above (Wang, et al., 2021; Zeng, et al., 2022).

The GC-MS (Agilent 7890B GC with Agilent 5977A Mass Selector Detector, Agilent Technologies, Santa Clara, California America) analyses parameters were consistent with previous studies (Zeng, et al., 2022). For SAFE, injected the liquid directly into the injection port. For SPME-Arrow, inserted the extracted fiber into the injector port and used the splitless mode. Samples were analyzed on both a DB-Wax column (60 m × 0.25 mm i.d. × 0.25 μm film, Agilent Technologies, Santa Clara, California America), and the injector port was held at 230°C. The column carrier gas was helium at a constant flow rate of 1.2 mL/min. The oven temperature was held at 40°C for 5 min, increased to 230°C at a rate of 3°C/min. The mass spectrometer was operated in the electron impact mode with 70 eV ionization energy, and the source temperature was set at 230°C. Full-scan acquisition was used in the 30 to 350 m/z range. High-purity helium (99.99%) was applied as the carrier gas for GC-MS analysis. Each analysis was performed in triplicate.

2.3 Aroma extraction dilution analysis (AEDA)

For SAFE, the volatile extracts of MC were diluted stepwise with dichloromethane in serial dilutions of $1:3^1$, $1:3^2$, $1:3^3$, and so forth. For SPME-Arrow, Dilution analysis was performed by changing the GC inlet split ratio, which was set to $1:3^1$, $1:3^2$, $1:3^3$, and so forth. Three experienced panelists (two females and one male) used chromatography–olfactometry (GC–O), an Agilent 7890B series GC coupled with a sniffing system (ODP 3 Gerstel, Mülheim, North Rhine-Westphalia, Germany), for sensory evaluation of each dilution until no aroma was perceived, with corresponding dilution factors (FD) of 3, 9, 27, …, and 3^n, respectively (H. Zhang, et al., 2022).

2.4 Quantitative analyses of key aroma compounds

Internal standard curves were constructed using GC-MS for the quantitative analysis of the key aroma compounds. Selective ion monitoring MS was adopted. To get more accurate

quantitative results, a matrix to simulate MC was prepared with milk protein concentrate powder, water, and sunflower oil and used for matrix-matched recovery during analytical quantification (Wang, et al., 2021). The MPC/water/oil simulant matrix was prepared as follows: first, the milk protein concentrate powder (MPC 70, Saishang Dairy Company, Ningxia, China) and ultrapure water were mixed and pasteurized as the substrate. Then the substrate was incubated with rennet (CHY-MAX Powder Extra, Hensel, Germany) at 31°C until it coagulated. The fresh curd was prepared by the whey draining process, then freeze-dried and crushed in a grinder for later use. The crushed curd (protein phase), deodorized sunflower oil (fat phase), and ultrapure water (water phase) were mixed in the same proportion as in MC. 2-methyl-pentanoic acid (0.923 μg/μL in n-hexane for quantitation of carboxylic acids) and 2-methyl-3-heptanone (0.816 μg/μL in n-hexane for quantitation of other compounds) were added into the *Monascus*-fermented cheese matrix as the internal standards. The specific amount of addition to the internal standard depends on the type of compound to be quantified. To homogenize the internal standard in the sample, the MPC/water/oil simulant matrix was ground twice in a stirrer for 30 seconds each time. Then, according to the semiquantitative results of MC samples in the pre-experiment, the concentration gradient of each compound was set and added successively, and the standard curve of no less than 5 points was established. All standard curves were constructed by plotting the ratio of the peak area of the reference compound to the peak area of the internal standard against its concentration (Zhang, et al., 2018).

The calculation formula for the concentration of volatile compounds is

$$f = \frac{A_s / m_s}{A_r / m_r},$$

where A_s is the peak area of the internal standard compound, A_r is the peak area of the compound under test, m_s is the mass of the internal standard compound, m_r is the mass of the compound under test, and f is the average value of the calibration factor calculated for each concentration of the standard.

2.5 Odor activity values (OAV)

The olfactory intensity of each compound in the sample is highly correlated with its concentration and odor threshold. In this study, the odor activity values (OAV) were used to describe the intensity of odor compounds in the sample. The OAV is the ratio of the concentration of a single compound to the odor threshold of that compound. (Trabue, et al., 2006). When a compound has OAV > 1, it is considered to contribute to the overall aroma of the sample. The compound threshold used in this study is from references. (Gemert, 2013; Wang, et al., 2021)

2.6 Microbial analysis

Before extracting, 1 g of the sample was ground in a sterile quartz mortar for 2 min and homogenized with 9 mL of sterile NaCl solution (0.85%, wt/vol) for 10 min. To separate fat and coarse particles, the suspension was centrifuged at 5,000 × g for 10 min at 4°C, and 1 g of the upper sediment was collected into a sterile microcentrifuge tube using a sterile spoon. Total genomic DNA was extracted using the Qiagen DNA Mini kit (Qiagen, Dusseldorf, North Rhine-Westphalia, Germany) according to the manufacturer's protocol. The concentration and purity of the extracted DNA were determined using a Nanodrop ND 1000 spectrophotometer (Thermo Fisher Scientific, Wilmington, America) and 1.0% agarose gel electrophoresis, respectively. Samples were stored at -20°C.

For bacteria (Zhong, et al., 2018), the V3–V4 domains of 16S rDNA were amplified by PCR using primer pairs 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-

GGACTACHVGGGTWTCTAAT-3′) with specific barcode. For fungi (Zhong, et al., 2018), the ITS1 rDNA regions were amplified by PCR (95℃ for 2 min, followed by 30 cycles at 95℃ for 30 s, 61℃ for 30 s, and 72℃ for 45 s with a final extension at 72℃ for 10 min) with the primers ITS5F (5'-GGAAGTAAAAGTCGTAACAAGG-3') and ITS2R (5'-GCTGCGTTCTTCATCGATGC-3') with specific barcode. Further paired-end sequencing was performed using an Illumina MiSeq instrument (Illumina Inc., San Diego, California, America).

2.7 Determination of free fatty acids (FFAs)

The fatty acids (FFAs) extraction and methylation were performed according to Balthazar (Balthazar, et al., 2016). Collect the derived fatty acid (FAME) in hexane in dichloromethane and pass a 0.45 injection-μm Filter before injection for GC-MS analysis. The initial temperature was 150°C and held for 3 minutes, then ramped up to 160°C at 6°C/min and then to 250°C at 2°C/min for 1 minute. Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The temperature of the ion source was 200°C. The mass scan range was 30-500 m/z and the electron energy was 70 eV. The content of FFA is obtained from the peak area of the total ionic current.

2.8 Determination of organic acids

The extraction method of organic acids in MC refers to Bouzas with slightly modified (Bouzas, et al., 1993). 5 g of MC was weighed into 25 ml of 0.009N H_2SO_4 and stirred for 1 hour. Then centrifuge (5000 × g, 10 min) and filter the supernatant through a 0.2 mm membrane filter (Gelman). The sample volume of 10 μL. The mobile phases were 0.1% phosphoric acid and acetonitrile (8:2). The absorbance was 210 nm, the column temperature was 35℃, the flow rate was 1 mL/min, and the elution is 40 min. 14 organic acids (formic acid, oxalic acid, tartaric acid, malic acid, lactic acid, acetic acid, citric acid, succinic acid, maleic acid, fumaric acid, and pyruvic acid) were used as external standards.

2042.9 Determination of free amino acids (FAAs)

205 30 mg samples of cheese freeze-dried powder in different ripening periods were taken, and 206analyzed the content of free amino acids was by HPLC. Chromatographic analysis conditions: 207Zorbax Eclipse-AAA chromatographic column (4.6 mm × 150 mm, 5 μm); Mobile phase: A: 40 208mmol/L NaH_2PO4 (pH=7.8), B: Acetonitrile: methanol: water (45:45:10, V: V: V); Injection 209volume: 10 μL; Column temperature: 40℃. Detection wavelength: 338 nm. Qualitative and 210quantitative analysis with the mixed free amino acid standard.

2112.10 Data analysis

212 All statistical analyses were performed with three biological replicates. One-way ANOVA 213followed by Duncan's multiple tests was used to verify significant differences in key aroma 214compounds at a level of $p<0.05$ (the Statistical Program for the Social Sciences 23.0; SPSS Inc., 215Chicago, IL, America). Additionally, the microbial abundance, microbial diversity, and Pearson 216correlation analysis were performed using the OriginPro 2021 64-bit (OriginPro Lab Corp., 217Northampton, America). Linear discriminant analysis Effect Size (LEfSe) using Galaxy for 218analysis (http://huttenhower.sph.harvard.edu/galaxy/). The correlation network of microorganisms 219and environmental compounds was visualized using Gephi 0.9.

220**3. Results and discussion**

2213.1 Aroma profiles of *Monascus*-fermented cheese

2223.1.1 Characterization of volatile aroma compounds

223 To obtain an overview of the evolution of the aroma profile of *Monascus*-fermented cheese 224(MC) across the 90-day ripening period, the volatile aroma compounds of MC ripened for 0 days, 2255 days, 10 days, 25 days, 40 days, 60 days, and 90 days were analyzed by SPME-Arrow-GC-MS. 226Clustering analysis showed that the complete 90-day ripening period can be divided into four

227stages: 0 days (unripened/fresh cheese), 5-10 days, 25-60 days, and 90 days (Fig.1).
228Correspondingly, MC ripened for 0, 10, 40 and 90 days were selected as representations of each
229stage for further analysis (see below).

230 To obtain more comprehensive information on the volatile aroma compounds, two different
231extraction methods were used, i.e. solid-phase microextraction (SPME) -Arrow and solvent-
232assisted flavor evaporation (SAFE). SPME-Arrow has higher stability, longer service life, 6 times
233the adsorption area, and 10 times the sensitivity (Kremser, et al., 2016), which is suitable for
234extracting low boiling point compounds. SAFE is suitable for extracting high boiling point
235compounds. Combining the results of SPME-Arrow and SAFE, a total of 85 volatile aroma
236compounds were detected in MC across the 90-day ripening period (Fig.1), including 20 acid
237compounds, 27 ester compounds, 13 ketone compounds, and 42 other compounds.

2383.1.2 Determination of flavor dilution (FD) factors of key aroma compounds

239 Gas chromatography-olfactometry can be conducted to evaluate the relative contribution of
240odorants by AEDA, being based on threshold concentrations in air (Gemert, 2013; Wang, et al.,
2412021). The results of AEDA in MC with different ripening stages were shown in Table S1.

242 During the ripening process, a total of 36 volatile aroma compounds had FD factor ≥ 81 (the
243FD of the compound in one or more stages is greater than or equal to 81), which were identified as
244the key aroma compounds of MC. Specifically, 12 aroma compounds with FD factor ≥ 81 were
245found in unripened MC (day 0). Among them, butanoic acid (pungent, FD = 243) and decanoic
246acid (rancid, FD = 243) had the highest FD factors, indicating their key role in the overall aroma
247at this stage. In addition, pentanoic acid (sour, FD = 81), hexanoic acid (sour and cheesy, FD =
24881), octanoic acid (rancid, FD = 81), benzoic acid (sour, sulfur, and musty, FD = 81) mainly
249contributed to the sour and cheesy flavors while 2-Heptanone (fruity and creamy, FD = 81) and 2-

tridecanone (fruity, creamy, FD = 81) contributed to the fruity and creamy flavors in unripened MC. *δ*-Decanolactone (fruit and wine aroma, FD = 81), limonene (lemon, FD = 81), and benzene ethanol (fruity, alcohol, FD = 81) contributed to the fruity, alcoholic flavors in unripened MC (Wang, et al., 2021).

Moving on to the second stage of the whole ripening period (represented by MC sampled on day 10), 26 aroma compounds with FD factor ≥ 81 were detected. Among them, butanoic acid (pungent, FD = 729), octanoic acid (rancid, FD = 279), decanoic acid (rancid, FD = 729), and dodecanoic acid (lemon and sour, FD = 729) had the highest FD factors, which showed that acid compounds are still the most important aroma compounds of MC at the initial stage of the ripening process. Following, acetic acid (sour and vinegar, FD = 243), 3-methyl-butanoic acid (sour, FD = 243), heptanoic acid (sour and cheesy, FD = 243), nonanoic acid (sour and cheesy, FD = 243) contributed to the sour and cheesy flavors. Decanoic acid ethyl ester (wine and apple, FD = 243), 2-tridecanone (fruity, creamy, FD = 243), 2-pentanone (fruit, creamy, FD = 81), 2-heptanone (fruit and wine aroma, FD = 81), 2-decanone (fruit and wine aroma, FD = 81), and hexanoic acid hexyl ester (fresh vegetable, FD = 81) contributed to fruity and creamy flavors in MC. *δ*-Decanolactone (fruit and wine aroma, FD = 243), *δ*-dodecalactone (fruit and wine aroma, FD = 243), octanoic acid ethyl ester (fruit and wine aroma, FD = 81), contributed to fruity and wine flavors. Limonene (lemon, FD = 81) contributed to lemon flavors. 2,3-butanediol (fruity, buttery, FD = 243), and benzene ethanol (fruity, alcohol, FD = 243) contributed to the fruity, alcoholic flavors.

For the third stage of ripening (represented by MC sampled on day 40)), there were 29 aroma compounds with FD factor ≥ 81 found in MC. Among them, decanoic acid (rancid, FD = 729),

butanoic acid (pungent, FD = 729), octanoic acid (rancid, FD = 729), decanoic acid, ethyl ester (wine and apple, FD = 729), 2-tridecanone (fruity, creamy, FD = 729) and *δ*-decanolactone (fruit and wine aroma, FD = 729) had the highest FD factor. During this stage, esters and ketones have become important aroma compounds of MC. The FD factor of acid compounds in MC decreased slightly, while the FD factor of ester compounds and ketone compounds increased. The decrease in acid may be due to the activation of the esterification reaction, which converts the accumulated short-chain fatty acids into esters (Xu, et al., 2020). As for the ketones, recalling the biochemical reactions that occurred in Blue cheese, acid compounds can undergo *β*-oxidation and be converted to methyl ketone by lipoxygenase (Kranz, et al., 1992). Correspondingly, in this work, we saw that the FD factors of ketone compounds in MC increased as the FD factors of acids decreased.

Finally, for the last stage of ripening (represented by MC sampled on day 90), there were 25 aroma compounds with FD factor ≥ 81 in MC. Among them, butanoic acid (pungent, FD = 729), 2-tridecanone (fruity, creamy, FD = 729), and 3-hydroxy-2-butanone (fruity, creamy, FD = 729) had the highest FD factors. During this stage, the FD factor of most acid compounds in MC continued to decrease. Ketones were still the important aroma compounds in MC.

3.1.3 Quantification of key aroma compounds and their OAV

To better assess the contribution of key aroma compounds, further calculations of odor activity values (OAV) were carried out for 36 key aroma compounds with FD ≥ 81. OAV is an important indicator for the quantitative evaluation of the contribution of volatile compounds to food aroma.

The results of the OAVs of 36 key aroma compounds in MC were listed in Table 1, which showed a similar trend to the AEDA results. 14 compounds were found with OAV ≥ 1 for unripened MC (sampled on day 0), including hexanoic acid, octanoic acid, decanoic acid, 2-

heptanone, 2-nonanone, 2-undecanone, 3-hydroxy-2-butanone, δ-decanolactone, δ-dodecalactone, limonene, benzaldehyde, benzene ethanol, and 1-dodecanol. Notably, no ester compounds were presented at the beginning of the ripening process. As ripening proceeded, more and more ester compounds developed, such as hexanoic acid ethyl ester, hexanoic acid butyl ester, and octanoic acid ethyl ester.

The number of aroma compounds with OAV ≥ 1 increases to 33, 36, and 33 for MC ripened for 10 days, 40 days, and 90 days, respectively. Most of them are acids, esters, and methyl ketones. Among these compounds, hexanoic acid ethyl ester has the highest OAV (4843.76) on day 40, followed by butanoic acid (OAV = 1112.93, day 40) and 2-heptanone (OAV = 1094.68, day 10). It has been reported that these compounds are important flavor contributors of other mold-ripened cheese, such as Blue cheese, Brie cheese, and Camembert cheese (Suzuki-Iwashima, et al., 2020). But the difference is that the content of these compounds in MC is about multiple higher than that of corresponding compounds in Blue cheese, Brie cheese, and Camembert cheese (Karahadian, et al., 1985; S. Zhang, et al., 2022). For example, the content of the ester compounds in MC is about 4 times as much as that in Blue cheese(Lawlor, et al., 2003; S. Zhang, et al., 2022). Therefore, esters, acids, and methyl ketones are the key aroma compounds in MC, which have important contributions to cheese senses. The implication is that the aroma quality of MC may be richer and softer compared to cheeses, such as Blue cheese and aged Cheddar cheese, dominated by acid compounds.

Considering the AEDA and OAV analysis in combination, except for unripened MC, the key aroma compounds of MC with different ripening stages were similar, including acids compounds (acetic acid, butanoic acid, pentanoic acid, hexanoic acid, heptanoic acid, octanoic acid, nonanoic

317acid, decanoic acid, undecanoic acid, benzoic acid, and dodecanoic acid), ester compounds (
318hexanoic acid ethyl ester, hexanoic acid butyl ester, octanoic acid ethyl ester, nonanoic acid ethyl
319ester, hexanoic acid hexyl ester, decanoic acid ethyl ester, and dodecanoic acid ethyl ester), ketone
320compounds (2-pentanone, 2-heptanone, 2-octanone, 2-decanone, 2-undecanone, 2-tridecanone,
321and 3-hydroxy-2-butanone), other compounds (*γ*-octalactone, *δ*-decanolactone, *δ*-dodecalactone,
322limonene, styrene, 2,3-butanediol. benzaldehyde, benzene ethanol, and 1-dodecanol). However,
323the amount of these compounds continuously changed with the MC ripening process. Specifically,
324acid compounds, such as hexanoic acid, octanoic acid, and decanoic acid, contribute more to the
325volatile flavor of cheese on 0 and 10 days of ripening. As ripening proceeded, more and more
326ester compounds and ketone compounds developed, such as hexanoic acid ethyl ester, hexanoic
327acid butyl ester, 2-pentanone, and 2-octanone.

3283.2 Microbiological analysis of *Monascus*-fermented cheese

329 Microorganisms play an important role in the formation of cheese aroma. This study
330characterized the composition and dynamic changes of microbial abundances and investigated the
331different stages of ripening (0 days, 10 days, 40 days, 90 days) of MC.

3323.2.1 Analysis of bacterial diversity during the ripening process

333 63 genera were detected at the level of bacterial community genera in MC. As shown in
334Fig.2A, the top 10 species in the genus level were *Lactococcus*, *Staphylococcus*, *Lactobacillus*,
335*Streptococcus*, *Bifidobacterium*, *Alloprevotella*, *Bacteroides*, *GpI*, and *Barnesiellah*. Among them,
336*Lactococcus* and *Staphylococcus* were the main dominant species during ripening. *Lactococcus*
337was the dominant group with the largest proportion. Its relative abundance ranged from 90.33% to
33898.50%. The relative abundance of *Lactococcus* first decreased, then increased, and reached the
339highest on day 90. *Staphylococcus* was the second dominant bacteria in MC, but its relative

abundance was far from that of *Lactococcus*. It only accounted for 0.090% on day 0 and reached the maximum value of 6.60% on day 10. Then, gradually decreased, accounting for 2.96% and 0.21% on day 40 and day 90, respectively. The changing trend of the relative abundance of *Staphylococcus* was opposite to that of *Lactococcus*.

Analysis of the microbiota *a*-diversity (indicated by Shannon index) indices suggests that the microbial diversity of MC sampled on day 10 was significantly higher than that at other periods ($p<0.05$) (Fig.2B). After day 10, it was observed that the Shannon index decreased significantly, and the species diversity was the lowest on day 90, which may be resulted from the high relative abundance of *Lactococcus* at that time. In addition, based on the weighted uniFrac distance metric, *β*-diversity analysis showed a significant difference between the 10-day group and other groups ($p<0.05$) (Fig.2C).

LEfSe could analyze the differences between groups of bacteria and find out the types of microorganisms with differences between groups (Fig.2D) (Zhao, et al., 2022). 20 biomarkers with LDA scores > 2 were obtained after LEfSe analysis of MC ripened for 0, 10, 40, and 90 days. *Lactococcus*, *Streptococcaceae*, and *Lactobacillales* were identified as biomarkers at the genus level in the 90-day group (LDA scores greater than 4). Finally, the cluster thermogram analysis was carried out to show the differences in the species composition of MC at different ripening periods. As shown in Fig.2E, 0-day and 10-day groups can be clustered together, while the rest two can be grouped into one, indicating that the bacterial species and content in MC changed significantly within cluster 1 (0-10 day) to cluster 2 (40-90 day).

3.2.2 Analysis of fungi diversity during the ripening process

A total of 103 genera were detected at the level of the fungi community genera in MC across the 90-day ripening period. As shown in Fig.3A, the top 10 species in the genus level were

Monascus, Meyerozyma, Fusarium, Mortierella, Funneliformis, Aspergillus, Gibellulopsis, Cladosporium and *Plectosphaerella*. *Monascus* and *Meyerozyma* are the main dominant fungi, accounting for more than 99% of the overall abundance. The relative abundance of *Monascus* first increased and then decreased from day 0 to day 90, being 46.54%, 81.41%, 53.42%, and 54.47% respectively. On the other hand, the relative abundance of *Meyerozyma* was 52.70% on day 0, decreased to 18.16% on day 10, and increased significantly and became stable on day 40 and day 90, with the proportion of 46.21% and 45.19%, respectively.

The analysis of the fungi *α*-diversity index of MC at different ripening periods showed that the Shannon index on day 10 was significantly lower than that on day 0, day 40, and day 90 ($p < 0.05$), indicating that the fungi diversity at this time points decreased (Fig.3B). This may be due to the high proportion of *Monascus* and *Meyerozyma* at this time. The *β*-diversity analysis based on the weighted uniFrac distance metric showed that the *β*-diversity of the 90-day group was significantly higher than that of the others ($p < 0.05$) (Fig.3C). Fig.3D showed that 0-day, 10-day, and 40-day were clustered into one group, and the 90-day was significantly different from other groups in species composition. The LEfSe analysis results of fungi were not shown as the LDA scores of fungi were less than 2.

3.3 Metabolomics analysis of *Monascus*-fermented cheese

Free fatty acids (FFAs) were a major group of metabolites that related to flavor formation during cheese ripening. Long-chain unsaturated fatty acids can be further oxidized and decomposed to produce aromatic compounds such as aldehydes, alcohols, and ketones, which lead to flavors in fermented products. Apart from FFAs, organic acids are another major group of compounds that are closely related to flavor formation in cheeses. The organic acids themselves

have a fruit acid aroma at low concentrations and pungent sour at high concentrations. In addition, the organic acids produced during cheese ripening can be esterified with alcohols to form esters, which not only provide an enhanced aroma but also reduces the irritating taste of organic acids (Li, et al., 2020). In addition, free amino acids (FAAs) were metabolic precursors of volatile sulfur compounds, aldehydes, ketones, and fatty acids, which also play an important role in the formation of cheese aroma (Izco, et al., 2000).

In this work, the dynamic changes in the concentrations of FFAs in MC were determined via GC-MS, and the dynamic changes in the concentrations of organic acids and FAAs in MC were determined via high-performance liquid chromatography (HPLC). The production and accumulation of high concentrations of FFAs and organic acids were observed in MC across the 90-day ripening period. 23 kinds of FFAs were identified (Table S2), among which oleic acid (C18:1) was the most abundant one. It has been suggested that oleic acid can undergo autoxidation and convert into nonanal, octanal, and other aldehydes (Zhao, et al., 2022). In addition, high levels of linoleic acid (C18:2), myristic acid (C14:0), palmitic acid (C16:0), and stearic acid (C18:0) were also found. As for organic acids, lactic acid, pyruvic acid, citric acid, oxalic acid, maleic acid, and tartaric acid were detected (Table S2). The content of lactic acid is 30121.84 mg/kg on day 40, which is mainly produced by glycolysis of halophilic microorganisms, such as *Staphylococcus* (S. Liu, et al., 2019). In addition, the content of tartaric acid in MC was also found to be at a high level (6152.24 mg/kg) on day 40. As shown in Table S3, During the ripening of MC, a total of 15 FAAs was detected, and the total content increased first, then on day 40 decreased. Among them, glutamic acid (Glu), phenylalanine (Phe), and leucine (Leu) were higher, accounting for 45.99% of the total FAAs in MC. Glutamic acid is a common flavor enhancer in

foods, which can cooperate with GMP and IMP to enhance the umami taste characteristics of cheese (Phat, et al., 2016). Phenylalanine and leucine are bitter amino acids that may bring bitterness to the cheese. But as important precursors of related aroma compounds, they also have a positive effect on cheese flavor development.

3.4 The correlation between flavor metabolites and microorganisms

During the fermentation process of MC, the flavor metabolites and microorganisms changed dynamically with the ripening process. Pairwise correlation analysis was performed for flavor metabolites and microorganisms to analyze the potential interaction (Qian, et al., 2023). Pearson correlation coefficients revealed that the populations of several microorganisms were significantly correlated with the abundance of flavor metabolites ($|r|>0.60$, $p<0.05$) (Fig.4A). *Lactococcus*, *Staphylococcus*, *Trichococcus*, *Monascus* showed significant correlations with *δ*-dodecalactone, pentanoic acid (C5:0), hexanoic acid (C6:0), tridecanoic acid (C13:0), pentadecanoic acid (C15:0), hexadecanoic acid (C16:0), nonadecanoic acid (C19:0), heneicosanoic acid (C21:0), 10-methyl nonadecanoic acid (C19:1), maleic acid, citric acid, tartaric acid, lactic acid, and leucine (Leu). In addition, *Muribaculaceae*, *Lactobacillus*, *Bacteroides*, *Alistipes*, *Limnohabitans*, *Enterococcus*, *Faecalibacterium*, *Meyerozyma*, *Candida*, *Aspergillus*, *Russula*, *Fusarium*, *Mycochlamys*, *Mortierella*, *Chaetomium*, and *Preussia* showed significant negative correlations with oxalic acid, lactic acid, hexanoic acid butyl ester (Butyl ester- C6:0), 2-tridecanone, eicosanoic acid (C20:0), valine (Val), proline (Pro), asparagine (Asp), phenylalanine (Phe), leucine (Leu), and arginine (Arg). Among these paired connections, *Lactococcus*, *Staphylococcus*, *Trichococcus*, and *Monascus* had strong significant positive correlations with flavor metabolites ($r>0.80$, $p<0.05$), which may be used as the core functional microorganism in MC.

Detailed visualization of significant correlations was presented as an undirected network graph (Fig.4B). In bacteria, *Lactococcus*, the dominant genus, had significant positive correlations with *δ*-dodecalactone and C6:0. Previous works have also indicated that *Lactococcus* is an important source of *δ*-lactones in fermentation (Zia, et al., 2022). *Staphylococcus* had significant positive correlations with several fatty acids, such as maleic acid and citric acid. Research on mixed culture in fermented meat indicated that *Staphylococcus* has high lipase activity to generate free fatty acids, which contributes to flavor formation (Lawlor, et al., 2003). In addition, *Trichococcus* positively correlated with tartaric acid, C5:0, and Leu. The high positive correlation between *Trichococcus* and tartaric acid was also observed in other correlation studies for flavor metabolites and microbial communities (Sacks, et al., 2018). The positive correlations between *Trichococcus* and C5:0 and Leu were not reported by previous studies as found. There were fewer significant positive correlations between fungi and metabolites: only *Monascus* was positively correlated with lactic acid and C5:0. Though lactic acid bacteria usually would be major lactic acid-producing organisms in fermented food, *Monascus* could also act as a "cell factory for lactic acid production" (Santiago-Lopez, et al., 2018).

Concerning significant negative correlations, oxalic acid negatively correlated with most bacteria and fungi, C20:0 negatively correlated with almost all bacteria except *Lactococcus*, *Staphylococcus*, and *Trichococcus*, and proline showed significant negative correlation with all fungi except *Monascus* and bacteria including *Enterococcus*, *Limnohabitans*, and *Faecalibacterium*. In previous research on antimicrobial compounds from fungi extracts, oxalic acid was detected to exhibit antibacterial activity against phytopathogenic bacteria, such as *Streptococcus faecalis* (minimal inhibitory concentrations = 10 μg/mL – ≥ 128 mg/L) (Alves, et

al., 2012). C20:0 (30 μM/10 mL of Nutrient Broth) also showed antimicrobial activity against pathogenic infection in existing studies (Bravo-Santano, et al., 2019). In addition, proline-rich peptides (2 to 8 mg/L) have been found to have an antibiotic effect against bacteria like Enterobacteriaceae in a few previous research works (Luzia, et al., 2018). Therefore, oxalic acid, C20:0, and proline-rich peptides might act as anti-microbial agents for unwanted microorganisms in cheese. In the future, to accurately evaluate the association between flavor metabolites and microorganisms during the ripening of MC, the core functional microbiota responsible for the production of volatile aroma should be further explored.

3.5 Formation of flavor metabolites in *Monascus*-fermented cheese based on microbial metabolic network

The metabolic network containing biosynthetic pathways of flavor metabolites, including short-chain carboxylic acids (lactic acid, citric acid, oxalic acid, maleic acid, and tartaric acid), saturated and unsaturated fatty acids, fatty acid derivatives (hexanoic acid butyl ester, 2-tridecanone, and *δ*-dodecalactone) and amino acids (Pro, Phe, Asp, Arg, Leu, and Val), was assembled referring to genome-scale metabolic networks of *Saccharomyces cerevisiae*, and gram-positive bacteria, such as *Lactococcus lactis subsp. cremoris* and *Staphylococcus aureus subsp. aureus*, BiGG database, MetaCyc database, and previous works on biosynthesis of flavor metabolites.

Short-chain carboxylic acids, e.g., lactic acid, mostly derive from intermediates of glycolysis, for example, the biosynthesis of oxalic acid starts from phosphoenolpyruvate and D-Erythrose 4-phosphate (Fig.5A). Maleic acid and tartaric acid can both be synthesized from fumarate, an intermediate of the TCA cycle. Maleate cis-trans isomerase (MALEI) catalyzes the conversion of

fumarate to maleic acid. Another biosynthetic pathway of maleic acid presented in the genome-scale metabolic network of *Staphylococcus aureus subsp. aureus* suggests that maleic acid can be formed by the oxidation of nicotinate through nicotinic acid 6 hydroxylase (NACHY), (6HNACMO), 2,5-dihydroxypyridine dioxygenase (PYRDOX), N-Formyl maleate deformylase (NFMLDF) and pyrazinamidase (PYRZAM) (Becker, et al., 2005). For tartaric acid, the epoxidation of fumarate (FUMEPO) forms epoxy succinic acid, and then cis-epoxy succinate hydrolase (CESH) catalyzes the dehydration of epoxy succinic acid (Xuan, et al., 2019).

The biosynthesis of fatty acids and their derivatives are all initiated by acetyl-CoA (coenzyme A), which formed malonyl-ACP with bicarbonate by acetyl-CoA carboxylase (ACCOA) and malonyl-CoA-ACP trans acylase (MCOATA) (Fig. 5B). Malonyl-ACP is the building block of both even and odd-numbered fatty acids: the elongation of even-numbered ones starts from C4:0 (butanoic acid) and malonyl-ACP, and that of odd-numbered ones starts from C3:0 (propionic acid) and malonyl-ACP. Fatty acid derivatives (i.e., esters and ketones) were found to correlate significantly with microorganisms in MC were hexanoic acid butyl ester, 2-tridecanone, and *δ*-dodecalactone. Hexanoic acid butyl ester derives from C6:0 (hexanoic acid) by butyl esterification (BTES60), which is common in microorganisms. For 2-tridecanone, the dehydration of 3-oxotetradecanoyl-CoA by thioester hydrolase (THH) forms its precursor, 3-oxo-myristate, and then, the decarboxylation catalyzed by 3-monolaurate decarboxylase (OXGDC3, EC 4.1.1.56) forms 2-tridecanone. Previous studies have indicated that 2-tridecanone was synthesized by bacteria instead of fungi (Mohanan, et al., 2020). No significant positive correlation of 2-tridecanone or hexanoic acid butyl ester (butyl ester C6:0) was found in section 3.4 to show its possible source. *δ*-dodecalactone is a *δ*-lactone formed by the lactonization of 5-

hydroxy alkanoic acid (5HDCALAC) (Zia, et al., 2022), which is 5-hydroxydodecanoic acid in this case.

The biosynthesis of amino acids is mainly mediated by aminotransferases, such as aromatic amino acid aminotransferase phenylalanine (araphe), transaminase, and ornithine transaminase (ORNTA) (Fig. 5C). Proline can be synthesized from L-Glutamate 5-semialdehyde (glu5sa) through L-glutamate 5-semialdehyde dehydratase (G5SADs) and pyrroline-5-carboxylate (P5CR), and glu5sa can be formed either from glutamate through glutamate 5-kinase (GLU5K) and glutamate-5-semialdehyde dehydrogenase (G5SD), or from 2-oxoglutarate and ornithine through ornithine transaminase (ORNTA). The removal of an amino group by L-asparaginase (ASNN) from asparagine forms aspartate, and then aspartate and citrulline can form argininosuccinate, the precursor of arginine, by arginine-succinate synthase (ARGSS). And argininosuccinate lyase (ARGSL) will split argininosuccinate to produce arginine. With phenylpyruvate (phpyr), also involved in the synthesis of oxalic acid, as the precursor, phenylalanine can be formed by the transfer of the amino group from other amino acids such as tyrosine. Similarly, Leu and Val can be synthesized from 4-Methyl-2-oxopentanoate and 3-Methyl-2-oxobutanoate respectively. Only the significant positive correlation between *Trichococcus* and Leu suggests the potential source of Leu, other amino acids' sources cannot be deduced from the correlation analysis in section 3.4.

4. Conclusion

A total of 36 key aroma compounds of *Monascus*-fermented cheese (MC) were identified during ripening. Among them, acid compounds, such as hexanoic acid octanoic acid, and decanoic acid contribute more to the volatile flavor of cheese on 0 and 10 days of cheese ripening. As ripening proceeded, more and more ester compounds and ketone compounds developed, such as

hexanoic acid ethyl ester, hexanoic acid butyl ester, 2-pentanone, and 2-octanone. In addition, *Lactococcus lactis* and *Monascus* were the dominant bacterium and fungus in MC, respectively. Furthermore, Pearson correlation analysis suggested that *Lactococcus lactis*, *Monascus*, *Staphylococcus*, and *Trichococcus*, were strongly associated with *δ*-dodecalactone, hexanoic acid, and pentanoic acid (members of the 36 key aroma compounds, $r>0.80$, $p<0.05$), and was identified as the core functional microorganisms. Finally, a metabolic network containing biosynthetic pathways of key aroma compounds concluded that short-chain carboxylic acids mostly derive from intermediates of glycolysis, and the biosynthesis of fatty acids and their derivatives (i.e., esters and ketones) are all initiated by acetyl-CoA. The biosynthesis of amino acids is mainly mediated by aminotransferase. This study constitutes a more comprehensive atlas of *Monascus*-fermented cheeses via quantifying the key aroma compounds, core functional microorganisms, and the metabolic pathways towards the formation of key aroma compounds, which can contribute to the development of potential strategies to improve the flavor quality of mold-ripened cheeses.

Acknowledgements

This work was supported by National Natural Science Foundation of China (project No. 32072345; Beijing) and the Cultivation Project of Double First-Class Disciplines of Food Science and Engineering, Beijing Technology & Business University (BTBUYXTD202205).

Abbreviations:

MC, *Monascus*-fermented cheese; GC-MS, Gas chromatograph-mass spectrometer; AEDA, Aroma extract dilution analysis; OAV, Odor activity values; SPME-Arrow, Solid-phase microextraction Arrow; SAFE, Solvent-assisted flavor evaporation.

Author contributions

Yadong Wang: Formal analysis, Methodology, Writing—original draft and editing. Hong Zeng: Methodology, Writing—review and editing, Supervision. Sizhe Qiu: Writing—original draft and editing. Haoying Han: Writing—original draft and editing. Bei Wang: Supervision, Validation, Methodology, Writing—review and editing, Funding acquisition, Project administration.

Conflicts of Interest

The authors declared that they have no conflicts of interest to this work.

Data Availability

The raw data supporting the conclusions included in the article can be obtained from the corresponding author upon reasonable request.

Reference

Tables and figures

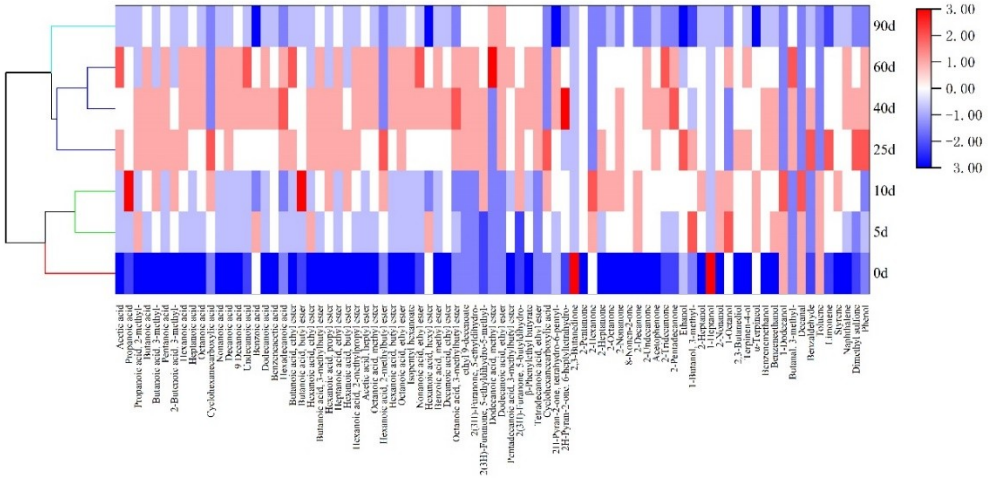
Table 1 Quantitation and odor activity values (OAV) of key aroma compounds in *Monascus*-fermented cheese. Abbreviation: 0d, 10d, 40d, 90d refers to cheese ripened for 0 day, 10 days, 40 days, and 90 days, respectively.

Compounds	Concentration (±SD) (μg/kg)				Threshold[1]	OAV			
	0d	10d	40d	90d	(μg/kg)	0d	10d	40d	90d
Acids									
Acetic acid	523.87± 18.63	4348.1± 1207.36	3029.76± 305.97	2188.79± 409.52	700	0.75	6.21	4.33	3.13
Butanoic acid	-	2513276.38 ±279129.31	3338781.1± 191432.83	1187692.05 ±235689	3000	23.58	837.76	1112.93	395.9
3-Methyl-butanoic acid	-	286114.93± 202689.54	315179.64± 100063.98	93072.73± 28051.73	1000	-	286.11	315.18	93.07
Pentanoic acid	-	11955.97± 46282.7	19311.89± 143761.66	10320.86± 278841.12	9000	-	1.33	2.15	1.15
Hexanoic acid	11199.08± 1481.82	626329.85± 73102.74	688624.63± 46940.46	155018.19± 55584	4300	2.6	145.66	160.15	36.05
Heptanoic acid	254.96± 30.34	9152.04± 3994.86	20097.31± 3325.32	7136.13± 1725.43	640	0.4	14.3	31.4	11.15
Octanoic acid	7048.69± 792.13	125731.29± 45366.71	131813.01± 33641.82	44499.06± 7237.25	3000	2.35	41.91	43.94	14.83
Nonanoic acid	957.36± 222.89	10157.12± 4680.78	31860.03± 7001.97	7838.69± 2275.58	8800	0.11	1.15	3.62	0.89
Decanoic acid	29676± 6300.09	438920.88± 183055.72	917612.43± 124206.46	182675.37± 57984.68	2300	12.9	190.84	398.96	79.42
Undecanoic acid	93.4± 11.22	494.9± 182.24	895.07± 102.21	236.94± 47.82	100	0.93	4.95	8.95	2.37
Benzoic acid	769.62± 229.68	778.1± 23.87	7648.22± 3825.43	239.43± 105.81	1000	0.77	0.78	7.65	0.24
Dodecanoic acid	4011.13± 1242.34	75144.96± 8851.45	2459.37± 910.7	36659.72± 8256.78	10000	0.4	7.51	4.21	3.67
Esters									
Hexanoic acid, ethyl ester	-	4998.95± 3519.56	14531.27± 3151.7	5798.92± 1607.14	3	-	1666.32	4843.76	1932.97
Hexanoic acid, butyl ester	-	257.66± 446.27	598.46± 49.26	429.57± 3.28	70	-	3.68	8.55	6.14
Octanoic acid, ethyl ester	-	2212.39± 997.13	13130.27± 1125.17	5097.73± 1007.44	960	-	2.3	13.68	5.31
Nonanoic acid, ethyl ester	-	-	287.36± 60.21	283.97± 13.76	50	-	5.92	2.59	1.7
Hexanoic acid, hexyl ester	-	295.89± 36.29	129.61± 5.55	85.1± 3.62	1	-	295.89	129.61	85.1

Decanoic acid, ethyl ester	-	242.47± 225.59	4218.54± 869.78	1614.47± 428.51	530	-	0.46	7.96	3.05
Dodecanoic acid, ethyl ester	-	-	652.03± 131.46	358.64± 261.06	2	-	-	326.02	179.32
Ketones									
2-Pentanone	-	13512.56± 256.46	6731.38± 118.45	2905.68± 472.04	1380	-	9.79	4.88	2.11
2-Heptanone	1258.28± 41.76	25177.56± 6984.67	6210.18± 1600.4	1718.47± 162	23	54.71	1094. 68	270.01	74.72
2-Octanone	-	2172.09± 659.31	899.16± 97.73	566.64± 17.67	50	-	43.44	17.98	11.33
2-Nonanone	740.19± 39.86	24159.58± 12521.58	10988.25± 1978.68	2962.06± 337.93	200	3.7	120.8	54.94	14.81
2-Decanone	-	1859.47± 832.32	804.02± 171.93	196.68± 18.72	110	-	16.9	7.31	1.79
2-Undecanone	438.25± 33.56	3491.68± 990.37	1821.6± 243.41	620.63± 32.33	10	43.83	349.17	182.16	62.06
2-Tridecanone	-	189.33± 5.43	157.49± 4.39	102.94± 2.23	1	-	189.33	157.49	102.94
2-Butanone, 3-hydroxy-	1087.13± 340.43	4168.13± 2475.63	2642.49± 473.92	1003.42± 884.98	14	77.65	297.72	188.75	71.67
Others									
γ-Octalactone	-	251.27± 300.49	199.02± 58.93	23.74± 4.15	20	-	12.56	9.95	1.19
δ-Decanolactone	62.61± 55.47	297.4± 28.32	222.68± 90.21	36.68± 3.93	50	1.25	5.95	4.45	0.73
δ-Dodecalactone	4710.42± 1296.31	16908.59± 5045.8	18359.09± 6727.92	5682.26± 1431.58	100	47.1	169.09	183.59	56.82
Limonene	111.54± 96.62	458.09± 96.91	279.48± 35.66	163.84± 2.67	70	1.59	6.54	3.99	2.34
Styrene	-	18604.51± 2377.14	4839.02± 755.08	1023.72± 35.18	70	-	265.78	69.13	14.62
2,3-Butanediol	-	87.68± 151.86	2058.66± 433.62	283.02± 16.47	3	-	29.23	686.22	94.34
Benzaldehyde	489.31± 31.12	2159.59± 314.94	3709.81± 390.72	1151.38± 232	60	8.16	35.99	61.83	19.19
Benzene ethanol	10010.55± 11594.39	629432.06± 284276.66	489171.04± 193940.69	98774.66± 55268.44	390	25.67	1613. 93	1254.28	253.27
1-Dodecanol	270.65± 6.04	316.24± 21.53	3068.17± 58.83	213.35± 184.8	16	16.92	19.76	191.76	13.33

[1]Threshold from the literature (Gemert, 2013; Wang, et al., 2021).

"-" means not detected.



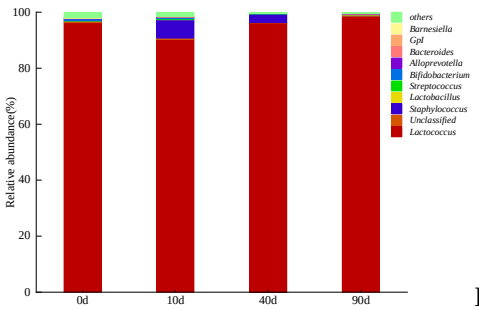
556

557Fig.1 Heat map of the relative contents of volatile aroma compounds in *Monascus*-fermented

558cheese (MC) across the 90-day ripening. Data presented in the heat map are in the log10 scale. 0d:

559MC ripened for 0 day.

560A



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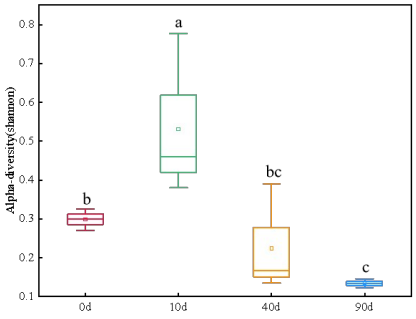

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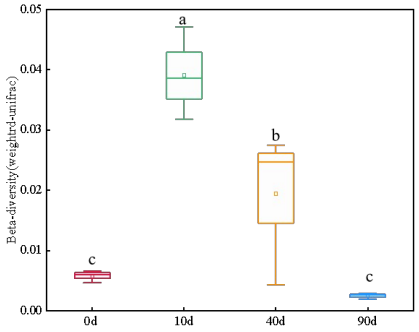

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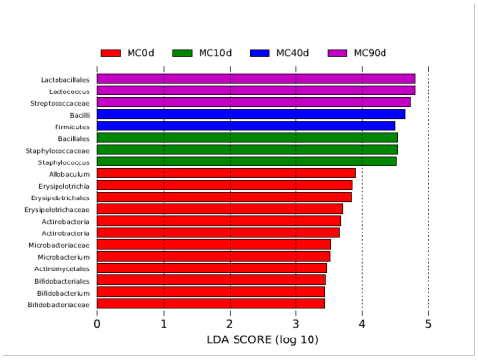

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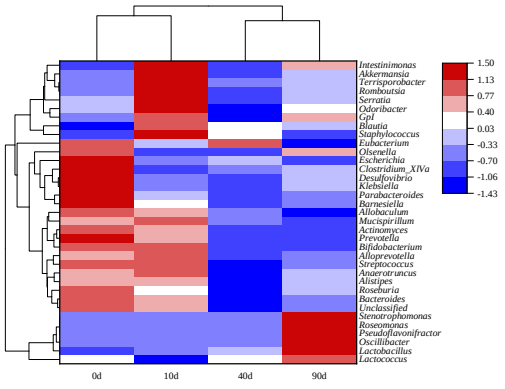


Fig.2 Diversity and composition of the bacterial community of *Monascus*-fermented cheese (MC) at different ripening periods. (A) Changes in the relative abundance of bacterial communities at the genus level during ripening of MC. (B) Changes of bacteria community diversity in samples of MC (within community). Based on Shannon index of Turkey test *α*-Diversity. (C) Changes of bacteria community diversity among samples of MC. Based on weighted uniFrac distance metric *β*-Diversity (D) Species differences of MC in different ripening stages. Based on LEfSe analysis. (E) Cluster heat maps of the first 35 genera of bacteria annotated according to species and abundant information. Abbreviation: 0d, 10d, 40d, 90d refers to cheese ripened for 0 day, 10 days, 40 days, and 90 days, respectively.

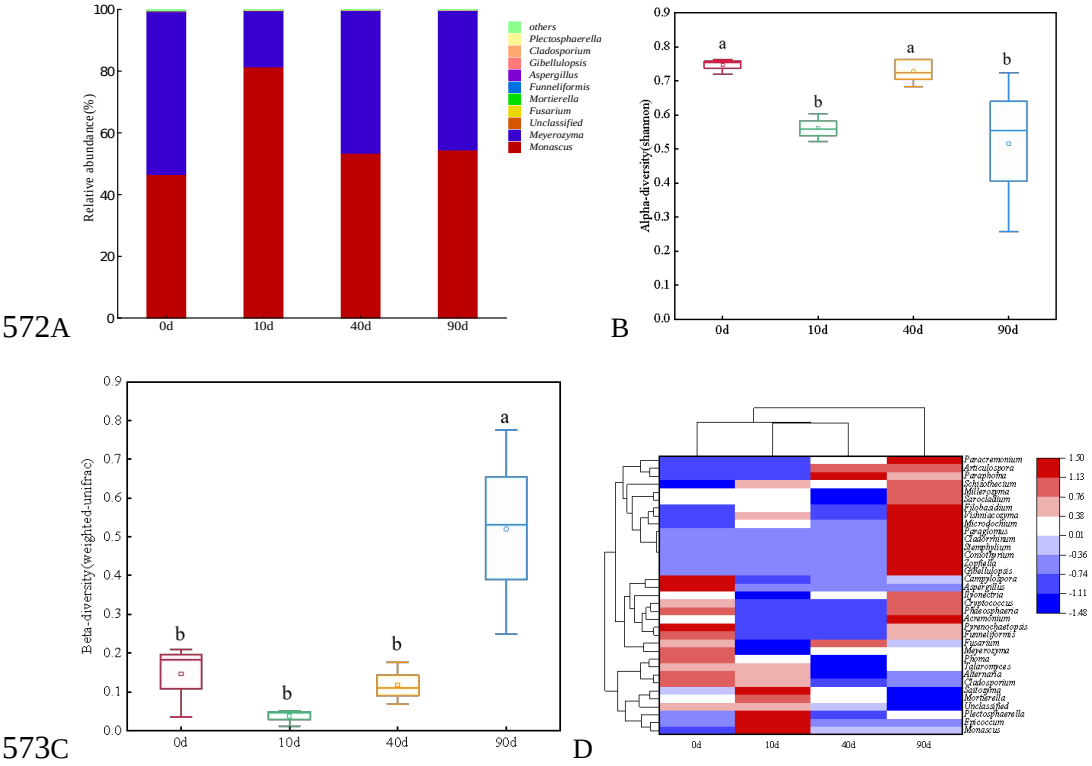
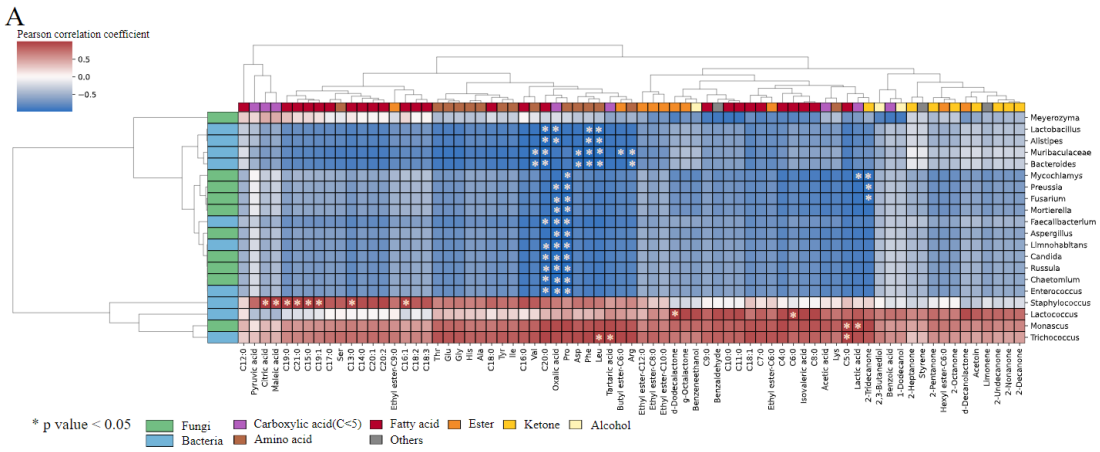


Fig.3 Diversity and composition of fungi community at different ripening periods of *Monascus*-fermented cheese (MC) (A) Changes in the relative abundance of fungi communities at the genus level during the ripening of MC. (B) Changes of Fungi community diversity in samples of MC (within community). Based on Shannon index of Turkey test *α*-Diversity. (C) Changes of Fungi community diversity among samples of MC. Based on weighted uniFrac distance metric *β*-Diversity. (D) Cluster heat maps of the first 35 genera of fungi annotated according to species and abundant information. Abbreviation: 0d, 10d, 40d, 90d refers to cheese ripened for 0 day, 10 days, 40 days, and 90 days, respectively.



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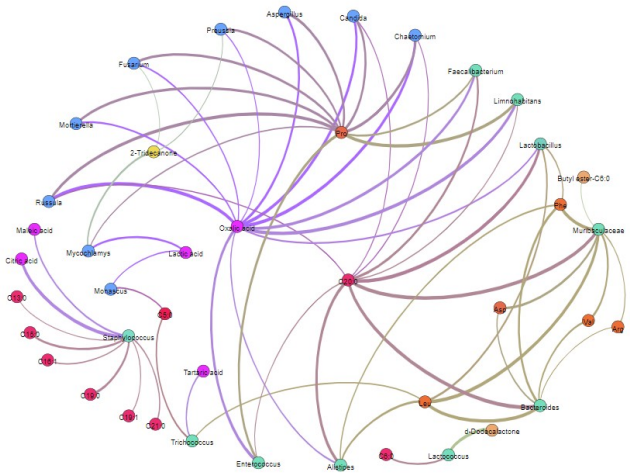
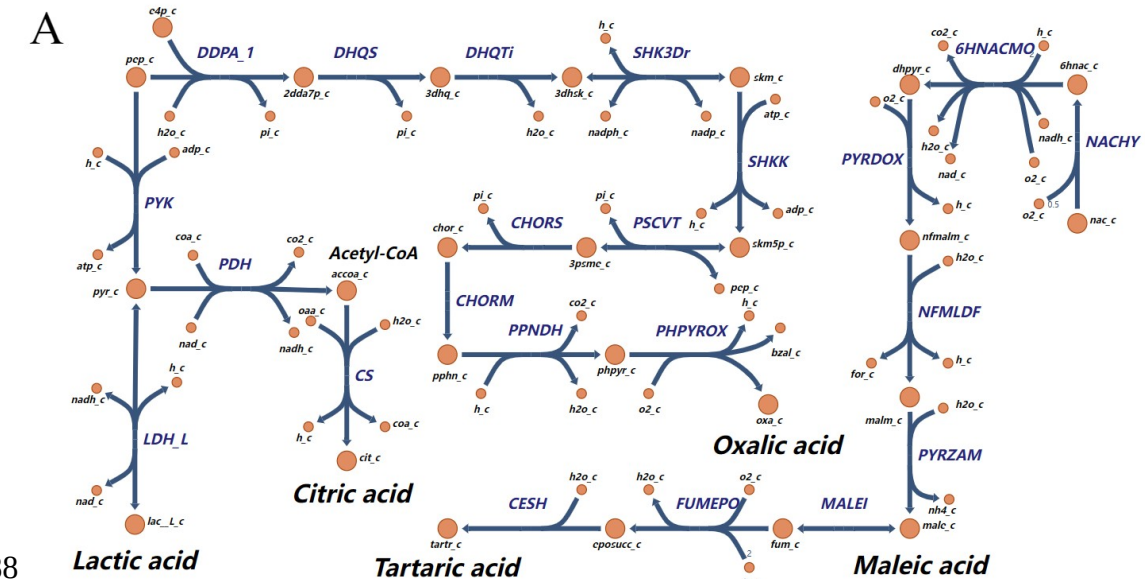


583

584Fig.4 Correlations between key aroma compounds and flavor metabolites and microorganisms.
585The cluster map (A) and undirected network graph (B) demonstrate the Pearson correlation
586coefficient of fungi or bacteria and flavor metabolite, * indicates a significant correlation (p <
5870.05). Corresponding Table S4 for compound abbreviations.


A
c4p_c
DDPA_1
pep_c
2dda7p_c
DHQS
3dhq_c
DHQTi
3dhsk_c
SHK3Dr
skm_c
SHKK
CHORS
chor_c
3psme_c
PSCVT
skm5p_c
CHORM
pphn_c
PPNDH
phpyr_c
PHPYROX
bzal_c
oxa_c
Oxalic acid
PYK
pyr_c
PDH
Acetyl-CoA
accoa_c
CS
cit_c
Citric acid
LDH_L
lac__L_c
Lactic acid
CESH
tartr_c
Tartaric acid
FUMEPO
cposucc_c
MALEI
fum_c
malc_c
Maleic acid
6HNACMO
6hnac_c
NACHY
nac_c
PYRDOX
nfmalm_c
NFMLDF
for_c
malm_c
PYRZAM
nh4_c
B
Acetyl-CoA
Initiation
ACOATA
KASI or KASII
KAS14
KASIII
KAS15
3OAR40
3HAD40
EAR40x
C4:0
KASI or KASII
ACCOAC
MCOATA
3OAS60
3OAR60
3HAD60
EAR60x
C6:0
Hexanoic acid, butyl ester
BTES60
FA60ACPHi
Even numbered saturated FA elongation
C8:0
3OAS100
3OAR100
3HAD100
EAR100x
FA100ACPHi
C12:0
C12:0
ACACT6p
THH
OXGDC3
2-Tridecanone
5-hydroxydecanoate
FADBA
SHDCALAC
Delta-Dodecalactone
Citric acid
ACONTa
ACONTb
ICDHyr
ARGDH
MMM
MMCD
C3:0
PPCOATA
3HAD50
3OAR50
EAR50x
3OAS50
C5:0
Odd numbered saturated FA elongation
C14:1
3OAS161
3OAR161
3HAD161
EAR161x
3OAS181
ACPPAT161
C16:1
EAR181x
3HAD181
3OAR181
ACPPAT181
APH181
C18:1
Unsaturated FA elongation

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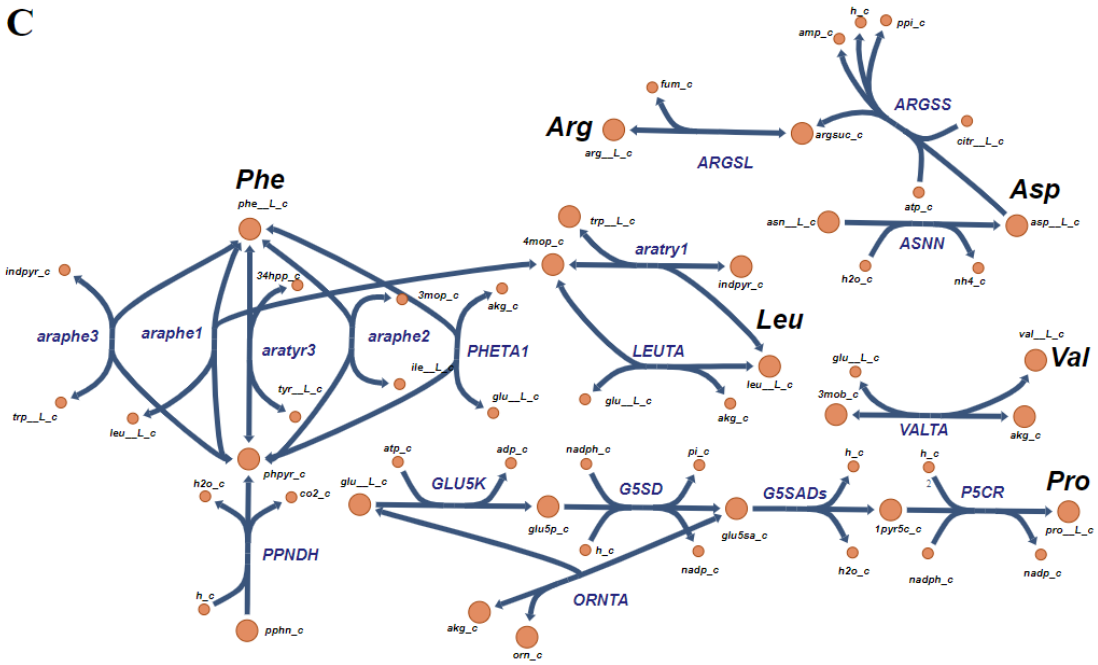

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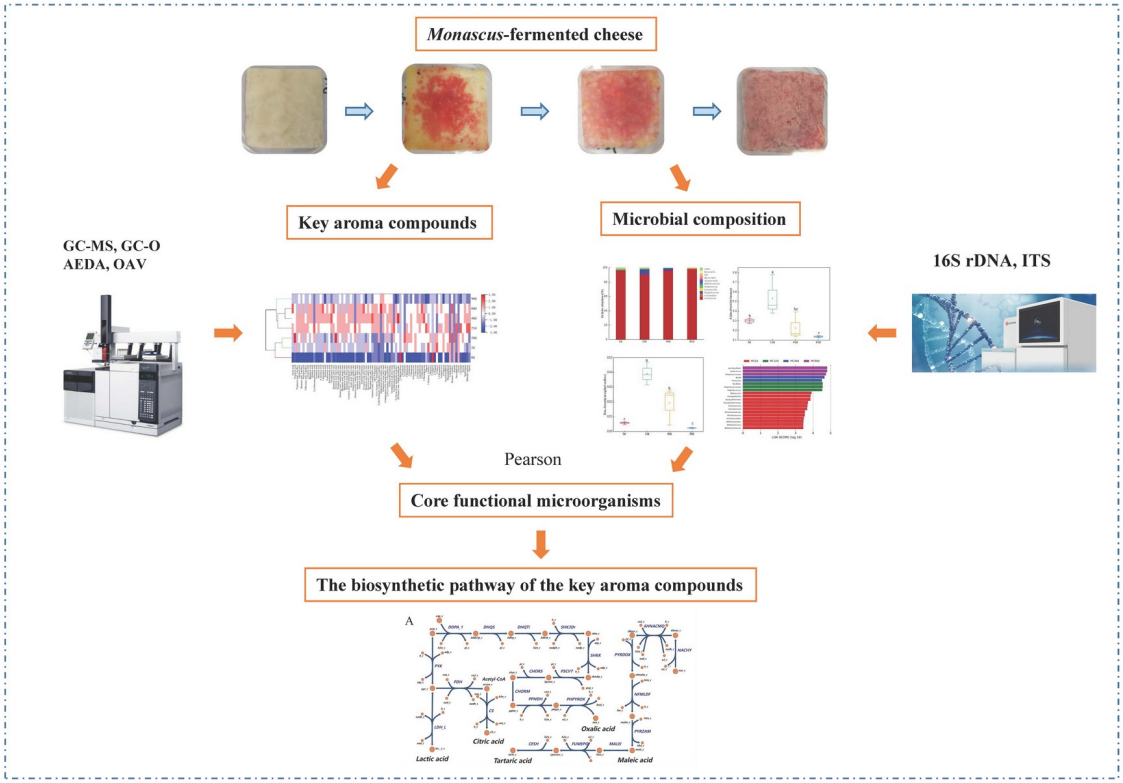
591Fig.5. Biosynthetic pathways of short-chain carboxylic acids (A), medium-chain fatty acids,
592esters, ketones (B) and amino acids (C) that showed significant correlations with microorganisms
593in this study. Corresponding Table S4 for compound abbreviations. The blue letters in the figure
594represent enzymes, which were sourced from the BiGG database and MetaCyc database.

595**Graphical abstract**



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