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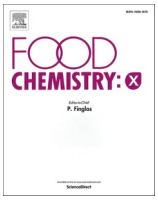

Machine learning analysis of pre-culture effects on rate-limiting steps in volatile compound dynamics of Mead

Xian Li [a,1], Tiantian Zhang [b,1], Ziwei Liu [a], Meng Jiao [c], Qian Li [a], Martin Gand [d], Kexin Zhu [a], Yibing Qiao [a], Wushuang Bai [a], Zisheng Guo [c,*], Bin Li [c], Yiran Wang [c], Jing Dong [c], Binglin Li [a,e,**]

[a] College of Food Science and Engineering, Northwest University, Xi'an 710069, China
[b] College of Petroleum and Chemical Engineering, Longdong University, Qingyang, Gansu 745000, China
[c] Key Laboratory of Resources Biology and Biotechnology in Western China, Ministry of Education, Provincial Key Laboratory of Biotechnology of Shaanxi Province, the College of Life Sciences, Northwest University, Xi'an 710069, China
[d] Institute of Food Chemistry and Food Biotechnology, Justus Liebig University Giessen, Giessen 35392, Germany
[e] Cangzhou Academy of Agriculture and Forestry Sciences, Cangzhou 061001, China

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Benzaldehyde (PubChem CID: 240)
2,3-Butanediol (PubChem CID: 262)
Nerolidol (PubChem CID: 5284507).
Benzeneacetaldehyde (PubChem CID: 9985)
3-Methylbutyl octanoate (PubChem CID: 5364423).

ABSTRACT

A novel two-step fermentation process was developed to enhance mead flavor quality. Headspace Solid-Phase Microextraction Gas Chromatography-Mass Spectrometry (HS-SPME-GC–MS) with three columns was used to analyze the volatile profiles of meads, along with sensory evaluation and machine learning. Compared to traditional mead (TM), our novel mead (NM) reduced off-flavor compounds by 37.6 %, with isoamyl alcohol decreasing 1.26-fold and ethyl laurate 2.09-fold. Meanwhile, aromatic compounds increased by 39.41 %, with isoamyl acetate rising 3.31-fold, ethyl caproate 2.79-fold, and phenylethyl alcohol 1.69-fold. Sensory evaluation revealed a significant reduction in bitterness (41.1 %) and irritation (42.5 %), while fruity, sweet, and pleasantly sour flavors increased by 27.4 %, 36.9 %, and 45.5 % for NM. Key aroma compounds (benzaldehyde, 2,3-butanediol, cedrol) were identified via recombination and omission experiments. Dynamic monitoring and machine learning identified key rate-limiting steps, including the oxidation of benzeneacetaldehyde (phenylethyl alcohol synthesis), isovaleraldehyde (isoamyl alcohol synthesis), and the conversion of octanoic acid to decanoic acid.

1. Introduction

Mead is considered one of the oldest known alcoholic drinks (Vidrih & Hribar, 2016). Studies suggested that moderate consumption of mead could offer health benefits due to its rich content of antioxidants, including phenolic acids and flavonoids, as well as essential nutrients like vitamins and minerals (Kružík et al., 2022). These bioactive compounds were shown to support immune function, enhance metabolic activity, and contribute to bone health (Gomes et al., 2013; Lopes et al., 2020; Socha, Pająk, Fortuna, & Buksa, 2015). Additionally, the rise of urban and backyard beekeeping, particularly in North America and Europe, further promoted the cultural and ecological relevance of honey-based products like mead (Matsuzawa & Kohsaka, 2021).

The one-step fermentation process is widely used for mead production. Yeast is first activated in warm water (~37 °C) for several minutes before being directly added into the honey solution for the fermentation.

* Corresponding author.
** Corresponding author at: College of Food Science and Engineering, Northwest University, Xi'an 710069, China.
E-mail addresses: zishengguo@nwu.edu.cn (Z. Guo), libinglin@nwu.edu.cn (B. Li).
[1] X. L. and T.Z. contributed equally to this work.

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Preliminary studies revealed that meads produced using this technique often exhibited a reduced fruity aroma and the presence of certain off-flavors (Silva et al., 2020). This issue is particularly evident in semi-sweet meads, where it negatively impacts consumer acceptance to varying degrees (Bednarek & Szwengiel, 2020).

The investigation of characteristic volatile compounds in alcoholic beverages is a significant topic in food science. For example, Pereira et al. found that when producing dark mead using Portuguese black honey, off-flavor substances such as caproic acid and caprylic acid were detected. These fatty acids produce unpleasant, rancid, and sweaty odors, which negatively affect the sensory quality of the mead (Pereira, Mendes-Ferreira, Oliveira, Estevinho, & Mendes-Faia, 2015). Wintersteen et al. reported that during the fermentation of mead made from soybean and buckwheat honey juice, which underwent two heat treatments, the formation of 4-methylphenol (p-cresol) was detected. This compound imparts "fecal, barnyard, burnt leather, and animal-like odors", which significantly detract from the sensory quality of the mead (Wintersteen, Andrae, & Engeseth, 2005). Langos et al. analyzed the key aromatic compounds in two Bavarian wheat beers and identified acetic acid (sour, irritating), butyric acid (sweaty), 2-methylbutyric acid, and 3-methylbutyric acid (both sweaty), as well as 3-hydroxy-4,5-dimethyl-2(5H)-furanone (seasoning-like, spicy) as the primary contributors to undesirable sensory attributes (Langos, Granvogl, & Schieberle, 2013). Similarly, Wang et al. investigated Australian rosé wines and found that 3-methyl-1-butanol (raw, nail polish, miscellaneous alcohols), ethyl lactate (chemical solvent-like), methyl decanoate (chemical, metallic, burnt), α-terpineol (oily, anise, spicy), decanoic acid (rancid, fatty), and dodecanoic acid (metallic) were the main volatile compounds responsible for sensory defects (Wang, Capone, Wilkinson, & Jeffery, 2016). In another study, Zheng et al. characterized the key odorants in Chinese sesame aroma-type liquor, identifying ethyl acrylate (plastic), hexanoic acid (sour, fatty, sweaty, cheesy), and butyric acid (sweaty) as the dominant contributors to undesirable sensory profiles (Zheng et al., 2016). Despite substantial progress in understanding characteristic volatile compounds in other alcoholic beverages, relatively little attention has been paid to their formation and impact in mead. To date, no targeted strategies have been proposed to reduce undesirable volatile compounds or enhance desirable ones during mead fermentation.

In this study, we identified the off-flavor substances and aroma components in mono-floral mead derived from rapeseed honey using Headspace Solid-Phase Microextraction Gas Chromatography-Mass Spectrometry (HS-SPME-GC–MS) with three different columns. Through principal component analysis (PCA), orthogonal partial least squares discriminant analysis (OPLS-DA), odor activity value (OAV) analysis, variable importance in projection (VIP) analysis, and sensory analysis, combined with aroma recombination and omission experiments, we identified the key compounds affecting the aroma of mead. Correlation analysis was performed to further investigate these characteristic compounds, and a machine learning approach (Networkx, Python 3.10 Toolkit) was employed to reveal the formation mechanisms of flavor and off-flavor compounds. The physicochemical properties, antioxidant activity, volatile compounds associated with the flavor profile, and sensory analysis all indicated that mead prepared by this method had superior flavor and higher quality compared to the traditional method.

2. Materials and methods

2.1. Materials

The rapeseed honey (purity >85 %, 107°03′-107°30′ E, 32°45′-33°40′ N) stored in a plastic barrel. The yeast strain *Saccharomyces cerevisiae* RV002 and yeast extract FN502 were sourced from Angie's Yeast Co. Ltd., located in Yichang City, Hubei Province, China. Internal standards, including methyl octanoate (≥99 %) and methyl isobutyl methanol (≥99 %), were obtained from Shanghai Titan Technology Co. Ltd., Shanghai, China. All other reagents utilized were of analytical grade.

2.2. Traditional fermentation for semi-sweet rapeseed mead

Traditional fermentation was conducted by diluting rapeseed honey to 20°Brix, adjusting pH to 3.5 with tartaric acid, and supplementing 0.08 g/L sodium metabisulfite and 2 g/L yeast extract FN502. After sterilization (80 °C, 20 min), *Saccharomyces cerevisiae* RV002 (0.3 g) was activated in 5 mL honey solution (38 °C, 20 min) and inoculated into 300 mL substrate. Fermentation proceeded at 25 °C for 15 days. After the fermentation, all meads should be separated by centrifugation by 4000 rpm for 30 min at 14 °C.

2.3. Two-step fermentation for mead semi-sweet rapeseed mead

Optimization of seed-culture medium. For the novel two-step process, a seed medium (2°Brix glucose, 0.48 g/L yeast extract) was sterilized (80 °C, 20 min), inoculated with 0.24 g/L RV002, and incubated (28 °C, 180 rpm, 12.2 h). Single-factor experiments evaluated yeast concentration (0.06–0.30 g/L), yeast extract (0.12–0.60 g/L), and incubation time (6–18 h). Response surface methodology (RSM) via a Box-Behnken design optimized three factors: yeast (0.21–0.27 g/L), yeast extract (0.42–0.54 g/L), and incubation time (10.5–13.5 h), with cell density (OD_{600}) as the response. Data were analyzed using Design-Expert 13, with OD_{600} conversions calculated per Supplementary Equations.

Mead was fermented in a fermentation tank (Fig. S1). Seed medium (20 L: 0.4 kg glucose, 9.6 g yeast extract) was sterilized, inoculated with 4.8 g RV002, and incubated (28 °C, 180 rpm, 12.2 h). Then, 20 L rapeseed honey (40°Brix, pH 3.5, 0.16 g/L sodium metabisulfite) was sterilized (80 °C, 20 min), mixed 1:1 with seed culture, and fermented (25 °C, 15 days). After the fermentation, all meads should be separated by centrifugation by 4000 rpm for 30 min at 14 °C.

2.4. HS-SPME-GC–MS analysis

A 5 mL mead sample was prepared for HS-SPME-GC–MS analysis. The sample was transferred into a 20 mL crimp-top vial with precise threading, to which 0.75 g sodium chloride, 20 μL methyl octanoate, and methyl isobutyl methanol were added. An HS-SPME fiber (DVB/C-WR/PDMS/10/80 μm) supplied by Agilent Technologies (California, USA) was introduced into the vial, reaching a depth of 40 mm for sample extraction. The extraction process was carried out at 45 °C for 30 min, utilizing a 5-s shaking cycle followed by a 2-s pause.

After extraction, the fiber was desorbed at 250 °C for 5 min and analyzed using an Agilent 7890B/5977B GC–MS system (Agilent Technologies, California, USA). The system featured dual columns, including an Agilent HP-5 ms Ultra Inert column (60 m × 0.25 mm × 0.25 μm), which facilitated initial separation. This dual-column configuration improved compound separation and identification, ensuring reliable analysis of the sample matrix. Helium was employed as the carrier gas at a flow rate of 1 mL/min, and the sample was injected in non-split mode with the inlet temperature maintained at 250 °C.

The oven temperature program began at 35 °C, held for 6 min, followed by a ramp to 70 °C at 2 °C/min, with an additional hold of 2 min. The temperature was then increased to 90 °C at a rate of 5 °C/min, subsequently ramped to 150 °C at 3 °C/min, and held for 10 min. Finally, the oven temperature was elevated to 230 °C at 8 °C/min. The mass spectrometer operated in electron ionization (EI) mode with an ionization energy of 70 eV. The ion source, quadrupole, and transfer line temperatures were set to 230 °C, 150 °C, and 200 °C, respectively. The mass range was scanned from 10 to 350 *m/z*.

To confirm compound identities, an Agilent DB-WAX122–7062 column (60 m × 0.25 mm × 0.25 μm) and a Shimadzu SH-I-5Sil MS column (60 m × 0.25 mm × 0.25 μm) were also utilized. The qualitative analysis

of major compounds was performed using external standard methods. Standard samples were categorized into three groups of mixed standards, and the TIC chromatograms were provided in Fig. S2.

N-alkanes ranging from C7 to C40 were employed to calculate the retention indices (RI) of the volatiles. The concentrations of the detected compounds were calculated using the internal standard method. To ensure accurate quantification of volatile compounds, we employed the internal standardization method and selected two internal standards: methyl octanoate and methyl isobutyl methanol. Prior to extraction, defined concentrations of the internal standards (1400 μg/L for methyl octanoate and 1296 μg/L for methyl isobutyl methanol) were added to all samples to compensate for matrix effects and instrument response variability. The use of two internal standards improves the accuracy of sample preparation and analysis corrections, enhancing the precision and reliability of quantification—a method widely adopted in numerous studies(Da Silva, Gauche, Gonzaga, Costa, & Fett, 2016; Jetti, Yang, Kurnianta, Finn, & Qian, 2007). Detailed formulae and calculation steps are provided in the supplementary material (Eq-C).

The identification of volatile compounds was achieved by matching the mass spectral fragmentation patterns of the compounds with those stored in the NIST Mass Spectral Library version 2.3, and by considering the characteristic odor descriptors. Simultaneously, the retention indices (RIs) and relative retention indices (RSIs) calculated using n-alkanes (C7-C40) on both polar and non-polar columns were compared with those of authentic standards. In the identification process, compounds with relative retention indices (RSIs) greater than 750 were considered to be correctly identified substances, and compounds with retention index (RI) errors less than 10 % between the sample and the authentic standard were deemed to be consistent with the standard. Additionally, the accuracy of the identification results was further verified by matching the mass spectra with those of authentic standards and by comparing the results with those reported in the literature.

2.5. Analysis of the physicochemical properties of mead

In this study, the physical properties of mead, including alcohol content, residual sugar, translucency, total acidity, and volatile acids, were evaluated according to the methods described by the International Organization of Vine and Wine (OIV, 2009), with minor modifications. The antioxidant activity was analyzed using 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and ferric reducing antioxidant power (FRAP) assays, as well as by determining total polyphenol content (TPC) and total flavonoid content (TFC), following the procedures outlined by Liang (Liang, Zhang, Ma, Zeng, & Fang, 2024), Fentie (Fentie et al., 2022), Zhou (Zhou, Hu, Yang, Li, & Zeng, 2023), Özdemir (Özdemir, Pashazadeh, Zannou, & Koca, 2022) and (Koguchi, Saigusa, & Teramoto, 2009).

2.6. Calculation of odor and OAVs for volatile compounds

The OAVs of volatile compounds in mead was quantified. The contribution of each volatile compound to the characteristic aroma of mead was evaluated (Roldán, Van Muiswinkel, Lasanta, Palacios, & Caro, 2011). The formula was calculated as supplementary material Eq-D.

2.7. Sensory evaluation

A sensory evaluation of the mead samples was conducted by a trained panel consisting of 80 assessors (38 males and 42 females) from Northwest University (Xi'an, China) over a period of 5 to 8 academic terms. Sensory attributes were identified through frequency analysis, leading to the selection of 10 key descriptors: sweet, fruity, herbal, floral, sour, sweaty, waxy, bitter, pungent, and fatty. Each attribute was rated on a scale from 1 (not detectable) to 10 (extremely intense). Acidity, regarded as a separate parameter, was calculated according to the methodology outlined in supplementary material Eq-E.

2.8. Aroma recombination and omission experiments

To investigate whether volatile compounds with high OAVs significantly contributed to the aroma profiles of NM and TM meads, aroma recombination experiments were conducted. Recombination models were prepared by blending volatile compounds (OAVs $\geq$1) at their natural concentrations, as determined in the original mead samples, with ethanol solutions adjusted to match the alcohol content of the respective meads. These mixtures were allowed to equilibrate at room temperature for 3 h prior to sensory evaluation.

A trained sensory panel assessed the overall similarity between the aroma profiles of the original mead samples and their corresponding recombination models using a 100-point scale. Additionally, the panel rated the intensity of ten specific aroma attributes, such as floral, fruity, and woody notes, to evaluate the extent to which the recombination models replicated the sensory characteristics of the original meads.

To further analyze the role of individual or groups of volatile compounds in the overall aroma, omission models were developed. Separate omission models were constructed for NM and TM to assess the importance of compounds unique to each mead. A shared omission model was also created to evaluate the influence of compounds common to both meads (OAVs $\geq$1). In total, 38 omission models were tested. The three-alternative forced-choice (3-AFC) method was employed to evaluate these models. For each test, two complete recombination models and one omission model were presented in three 20 mL amber glass bottles, which were randomly labeled and arranged in random order. Panelists (8 males and 6 females) were tasked with identifying the omission model and describing the differences in its aroma compared to the complete models. All sensory evaluations were conducted following the standardized procedures outlined in Section 2.6.

2.9. Statistical analysis

All experiments were conducted in triplicate, and the results were expressed as mean ± standard deviation. Statistical analyses, including one-way ANOVA, were performed using SPSS 20.0 (SPSS Inc., Chicago, IL, USA). Response surface plots were generated using Design-Expert 13 (State-East, TN, USA), while heat maps were constructed with TBtools (v1.082, China). Data correlation analysis was carried out in Origin 2022 (OriginLab, Northampton, MA, USA). Principal component analysis (PCA) and orthogonal partial least squares discriminant analysis (OPLS-DA) were performed with SIMCA-P 14.1 (Umetrics, Sweden). Python 3.10 was employed for modeling rate-limiting steps, with Pandas used for data processing, Networkx for constructing reaction networks, and Matplotlib for generating visual representations.

3. Results and discussions

3.1. Fermentation of semi-sweet rapeseed mead

Fig. S3 presented the single-factor analysis of variables influencing the seed culture, including yeast concentration (0.06–0.30 g/L), yeast extract concentration (0.12–0.60 g/L), and incubation time (6–18 h). To further optimize the fermentation conditions, response surface methodology (RSM) was employed. As shown in Table S1–2, the model showed high significance, with an R^2 value of 0.991, an adjusted R^2 of 0.980, and a variation of 6.26 %. The lack-of-fit term was not significant ($P = 0.1444 > 0.05$), indicating a good fit of the model. Based on the F-values, the factors influencing fermentation were ranked in order of importance as: incubation time (C) > yeast concentration (A) > yeast extract concentration (B). Significant interactions between the factors were observed, as illustrated in Fig. S4, with the most notable interaction depicted in Fig. S4A. The optimal seed culture conditions were determined to be 12.20 h of incubation, 0.24 g/L yeast extract, and 0.12

g/L yeast, resulting in an OD_{600} of 0.85 ± 0.02, as verified through five independent tests.

3.2. Properties of two semi-sweet meads

As shown in Table S3, residual sugar levels were maintained at approximately 17 g/L, in accordance with the standards for semi-sweet wines. Compared to the traditional fermentation process, the proposed method significantly reduced both yeast and nitrogen inputs, while achieving a higher alcohol content (10.69 % vs. 9.93 %) and lower residual sugar levels (16.92 g/L vs. 18.41 g/L). Additionally, transparency improved by 36.40 %. Despite the reduced use of additives, no significant differences were observed in other properties. These findings indicate that the proposed method enhanced yeast activity and fermentation efficiency.

3.3. HS-SPME-GC–MS characterization of volatile compounds

HS-SPME-GC–MS was employed to analyze the volatile compounds in meads produced by different fermentation methods. TIC chromatograms of NM and TM were provided in Fig. S5. Volatile compounds in the honey solution (40 Brix), seed culture medium, yeast solution (2.00 g/L), and yeast extract solution (1.50 g/L) were also examined to elucidate the formation mechanisms of mead volatiles. The results are presented in Table 1. A total of 85 flavor compounds were identified in the meads, categorized as esters (41.18 %), alcohols (23.53 %), acids (12.94 %), aldehydes (7.06 %), olefins (7.06 %), phenols (2.35 %), alkanes (2.35 %), ketones (2.35 %), and ethers (1.18 %). Flavor descriptions and thresholds are detailed in Table S4.

Alcohols, as primary metabolic products of yeast, were the most abundant volatile compounds in mead. They were primarily synthesized through the degradation of glucose and amino acids (Liu & Sun, 2018). The total alcohol content in TM (42.90 mg/L) and NM (44.00 mg/L) showed negligible differences.

On the one hand, five alcohols were uniquely present in NM, including methyl benzyl alcohol, cis-linalool oxide, 1-dodecanol, γ-eudesmol, and 3-methyl-2-pentanol. Among these, 3-methyl-2-pentanol was found at a relatively high concentration of 112.72 μg/L, contributing to a pleasant cocoa aroma, slight citrus flavor, and sweetness. The other alcohols were present at lower concentrations (< 10.00 μg/L), potentially imparting nutty, carrot-like, and piney notes. On the other hand, two alcohols—2-ethylhexanol and 5-methyl-3-hexanol—along with eucalyptol, were uniquely found in TM. Despite their low concentrations, these compounds may contribute earthy and mushroom-like flavors.

Eight alcohols exhibited significant differences between NM and TM, namely phenylethyl alcohol, isoamyl alcohol, nerolidol, cedrol, trans-farnesol, trans-linalool oxide, 4-methylhexanol, and benzyl alcohol. Among these, higher concentrations of phenylethyl alcohol, nerolidol, trans-farnesol, trans-linalool oxide, cedrol, 4-methylhexanol, and benzyl alcohol were observed in NM. The concentration of phenylethyl alcohol in NM (16.22 mg/L) was approximately 1.69 times higher than that in TM (9.61 mg/L). The concentration of nerolidol increased from 26.68 μg/L to 61.83 μg/L, and that of trans-linalool oxide rose from 12.02 μg/L to 37.87 μg/L. Benzyl alcohol increased from 0.85 μg/L to 26.25 μg/L. These compounds contributed to more persistent woody, chicory, cool, and rose-like aromas in NM. Isoamyl alcohol, an undesirable compound, was found to contribute earthy, anesthetic, oily, and pungent characteristics (Fan et al., 2024). Its concentration was higher in TM (9.33 mg/L) but was obviously reduced in NM, decreasing to 7.36 mg/L. The differences in alcohol profiles between NM and TM were primarily attributed to the presence of higher alcohols, branched-chain alcohols, and certain terpene alcohols. These compounds were predominantly involved in the metabolism of amino acids. Initially, they were converted into α-keto acids through transamination reactions. The α-keto acids were then decarboxylated to form aldehydes, which were subsequently reduced to alcohols by the action of alcohol dehydrogenase (Kaluzna, Matsuda, Sewell, & Stewart, 2004).

Esters, as a significant class of flavor compounds, were responsible for imparting fruity, sweet, and floral notes (Sun et al., 2022). The total ester content in TM and NM was 7.64 mg/L and 8.1 mg/L, respectively. Seven esters were uniquely present in NM, including ethyl undecylenate, isobutyl phthalate, ethyl pentadecanoate, dibutyl phthalate, octyl acetate, phenylethyl caproate, and ethyl butyrate. Among these, ethyl undecylenate was present at a relatively high concentration of 41.56 μg/L, contributing to fruity and oily flavor notes. The other esters were found at lower concentrations, potentially imparting subtle sweet, rose, orange, and pineapple flavors, thus enhancing the freshness of the mead. Conversely, ethyl 3-hydroxytridecanoate, trimethylene acetate, ethyl propionate, ethyl isopentyl succinate, and ethyl isovalerate were detected only in TM. However, only ethyl propionate had a distinct flavor profile, providing a banana-like taste.

A total of 15 esters exhibited highly significant differences between the two meads, with ten being more prominent in NM. Notable compounds with higher concentrations in NM included phenethyl acetate, which increased from 335.63 μg/L to 822.74 μg/L; ethyl 9-decenoate, which rose from 5.29 μg/L to 515.50 μg/L; ethyl caproate, which increased from 100.31 μg/L to 379.78 μg/L; isoamyl acetate, which went from 50.60 μg/L to 218.28 μg/L. These compounds imparted a more intense aroma of banana, pear, apple, and pineapple, along with a subtle sweetness and a pleasant rose fragrance in NM. Other compounds present at lower concentrations included ethyl nonanoate (2.12 μg/L to 30.36 μg/L), ethyl myristate (6.84 μg/L to 63.16 μg/L), and ethyl phenylacetate (33.07 μg/L to 79.27 μg/L). These esters contributed additional sweetness, fruity, and herbal notes to NM.

Five esters were more prominent in TM, with ethyl 3-phenylpropionate increasing from 168.80 μg/L to 254.84 μg/L and diethyl succinate rising from 257.64 μg/L to 497.89 μg/L. These compounds contributed more fruity, astringent, and bitter flavors to TM. Compounds present at lower concentrations included gamma-butyrolactone, which rose from 13.47 μg/L to 36.89 μg/L; and 3-methylbutyl decanoate, which increased from 7.86 μg/L to 59.23 μg/L. These esters imparted a pleasantly warm taste, contributing herbal, rose, caramel, waxy, and soapy flavors to TM.

Fatty acids played an important role in the aromatic balance of honey mead (Peepall, Nickens, Vinciguerra, & Bochman, 2019). Low concentrations of acids could impart a mild sour taste to honey mead, but at higher concentrations, they would increase acidity and diminish the original flavor of the mead (Česlová, Pravcová, Juričová, & Fischer, 2022). Pentanoic acid and isovaleric acid were only detected in NM, imparting pear and apple aromas. Meanwhile, dl-3-methylvaleric acid was only detected in TM, contributing to a sweaty aroma.

Five fatty acids showed highly significant differences. Two of these were predominant in NM. Benzoic acid increased from 1.72 μg/L to 21.06 μg/L, contributing a sweet and sour taste; decanoic acid increased from 52.37 μg/L to 208.60 μg/L, which imparted a slightly rancid flavor. In TM, the predominant fatty acids were hexanoic acid, which increased from 1.08 μg/L to 53.93 μg/L; nonanoic acid, which increased from 81.18 μg/L to 454.25 μg/L; and octanoic acid, which increased from 245.71 μg/L to 358.25 μg/L. These acids contribute to more pronounced plastic, fatty, and acidic flavors. 2-Methylbutyric acid, with concentrations of 25.94 μg/L and 16.23 μg/L in NM and TM respectively, showed significant differences and was more concentrated in the latter. This acid can impart sweaty, spicy, and sour notes.

In addition, NM and TM contained aldehydes, alkenes, phenols, alkanes, ketones and ethers. The concentrations of these compounds were all below 100 μg/L. Seven compounds were uniquely present in NM, specifically 2-butyl-2-hexenal, 2,5-dimethylbenzaldehyde, 2-nonanone, 2-undecanone, 2,4-di-tert-butylpheno, α-curcumene and butylated hydroxytoluene. These compounds led to citrus, green grass, herbal and lava-like flavors. Three compounds were uniquely present in the TM group, namely styrene, γ-muurolene and decanal, contributing to the

Table 1
Qualitative and quantitative analysis of volatile components in two types of processed mead (TM, NM), honey solution (HS), seed medium (SCM), yeast solution (YS), and aqueous yeast extraction solution (YES). The following symbols indicate the associated odors: a denotes a fruity and sweet odor; b denotes an herbaceous odor; and c denotes an off-flavor. Significance analyses were conducted for the TM and NM samples, with results indicated in the p-columns: * represents $p > 0.05$; ** represents $0.05 < p < 0.01$; *** represents $p < 0.01$.
d. Sample: RSI values of compounds in the samples.
e. Standard: RSI values of standards.
f. Sample: Calculated RI values of compounds in the samples.
g. Standard: Calculated RI values of standards.
h. Reference: RI values in the NIST Mass Spectral Library v.2.3.

Class	Code	Compound	CAS	Reverse Similarity Index (DB-WAX122–7062)		Retention Index (HP-5 ms Ultra Inert)		Retention Index (SH-I-5Sil)		Concentration(μg/L)						Threshold (μg/L)	OAV		P
				Sample[d]	Standard[e]	Sample[f]	Standard[g] (Reference[h])	Sample[f]	Standard[g] (Reference[h])	NM	TM	HS	YS	SM	YES		NM	TM	
Acids	AC1	2-Methylbutyric acid[c]	116–53-0				861			25.93 ± 3.53	16.22 ± 1.46	13.86 ± 1.32	7.10 ± 0.12	67.19 ± 2.02		540	<1	<1	**
	AC2	Isovaleric acid[b]	503–74-2	777	792		863		811	36.48 ± 5.65						12	3.04		
	AC3	Pentanoic acid[b]	109–52-4	813	851		904		875	5.97 ± 0.28						500	<1		
	AC4	DL-3-Methylvaleric acid[c]	105–43-1	756	785		947		910		29.24 ± 1.44		4.42 ± 0.30	31.17 ± 0.23		280		<1	
	AC5	Hexanoic acid[c]	142–62-1	823	819	1000	990	949	974	1.08 ± 0.03	53.92 ± 2.94								***
	AC6	Benzoic acid[b]	65–85-0	840	854	1167	1170			21.05 ± 3.02	1.72 ± 0.21					10,000	<1		***
	AC7	Octanoic acid[c]	124–07-2	853	863	1194	1180	1184	1173	245.70 ± 11.12	358.24 ± 7.33	51.20 ± 1.84	3.91 ± 0.41	804.20 ± 62.82		500	<1	<1	***
	AC8	Nonanoic acid[c]	112–05-0	789	802	1285	1273	1220	1272	81.17 ± 9.03	454.24 ± 35.13			34.65 ± 3.75		71,100	<1	<1	***
	AC9	Decanoic acid[c]	334–48-5	792	803	1378	1373	1406	1372	208.59 ± 24.99	52.37 ± 8.43	33.39 ± 1.01		215.76 ± 8.97		1000	<1	<1	***
	AC10	Undecanoic acid[c]	112–37-8	758	789	1481	1475	1421	1471	9.57 ± 2.05	15.19 ± 6.06					10,000	<1	<1	*
	AC11	4-Methylvaleric acid	646–07-1	752	781	956	949	933	910			2.37 ± 0.20							
	AC12	Butanoic acid[c]	107–92-6				805		775					30.32 ± 1.95					
	AC13	3-Hydroxyisovaleric acid	625–08-1			978	980					0.31 ± 0.01							
Aldehydes	AD1	Benzaldehyde[a]	100–52-7			956	962	923	982	26.12 ± 2.69	21.41 ± 1.95	18.89 ± 1.84		14.77 ± 0.61	7.69 ± 1.10	3	8.7	7.13	*
	AD2	Benzeneacetaldehyde[b]	122–78-1	759	816	1033	1045	1137	1081	19.66 ± 2.31	12.82 ± 2.09			29.59 ± 1.47	6.04 ± 0.74	4	4.91	3.2	**
	AD3	Nonanal[b]	124–19-6	832	876	1093	1104	1182	1104	10.57 ± 1.69	7.44 ± 1.11	29.52 ± 2.52		1.78 ± 0.14	8.22 ± 0.54	40	<1	<1	*
	AD4	Decanal[a]	112–31–2	823	875	1197	1206	1128	1204		22.65 ± 2.27	8.27 ± 1.18			4.52 ± 0.69	1		22.65	
	AD5	2,5-Dimethylbenzaldehyde[a]	5779-94-2	889	907	1191	1208	1244	1208	28.12 ± 3.66									
	AD6	2-Butyl-2-hexenal[b]	13,019–16-4			1373	1378			50.87 ± 12.56						20	2.54		
	AD7	Isovaleraldehyde	590–86-3				(652)								5.62 ± 0.93				

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Table 1 (*continued*)

Class	Code	Compound	CAS	Reverse Similarity Index (DB-WAX122–7062)		Retention Index (HP-5 ms Ultra Inert)		Retention Index (SH-I-5Sil)		Concentration(μg/L)						Threshold (μg/L)	OAV		P
				Sample[d]	Standard[e]	Sample[f]	Standard[g] (Reference[h])	Sample[f]	Standard[g] (Reference[h])	NM	TM	HS	YS	SM	YES		NM	TM	
	AD8	Pentanal	110–62-3	756	784		699								5.90 ± 0.53				
	AD9	Heptanal	111–71-7			892	(901)	920	(905)						0.58 ± 0.10				
	AD10	Octanal	124–13-0			987	1003	1029	1005						2.64 ± 0.26				
	AD11	Hydroxycitronellal	107–75-5			1282	(1300)								0.94 ± 0.07				
	AD12	3,4-Dehydro-β-ionone	1203-08-3			1452	(1458)					0.05 ± 0.008							
	AD13	Cinnamaldehyde	104–55-2			1243	(1274)					2.49 ± 0.32							
	AD14	Camphor	76–22-2			1144	(1145)					1.01 ± 0.02							
	AD15	β-Cyclocitral	432–25-7			1220	(1220)					0.99 ± 0.04							
	AD16	Tridecanal	10,486–19-8			1495	1512					2.07 ± 0.32							
	AK1	Pentadecane[a]	629–62-9	885	830	1494	1500	1552	1512	45.41 ± 9.52	43.39 ± 5.32								*
	AK2	Heptadecane[c]	629–78-7	850	816	1715	1700	1761	1711	14.92 ± 4.76	8.85 ± 1.25			2.52 ± 0.38		10,000	<1	<1	*
	AK3	2-Methyl-octadecane	1560-88-9			1856	(1863)								0.53 ± 0.04				
	AK4	2,6,10,14-Tetramethyl-heptadecan	18,344–37-1			1859	(1872)	1802	(1852)						0.63 ± 0.06				
	AK5	2,6,10,15-Tetramethylheptadecane	54,833–48-6	804	823	1876	(1889)	1807	(1852)						0.80 ± 0.05				
	AK6	3-Methyleicosane	6418-46-8			2050	(2068)						1.23 ± 0.18						
	AK7	Pentacosane	629–99-2			2484	(2500)						0.68 ± 0.02		0.14 ± 0.03				
Alkanes	AK8	Pentylcyclopropane	2511-91-3			802	(813)						0.09 ± 0.01		0.27 ± 0.02				
	AK9	2,4,6-Trimethyldecane	62,108–27-4			1118	(1121)						0.93 ± 0.11		0.43 ± 0.06				
	AK10	4-Methyltridecane	26,730–12-1			1344	(1359)	1344	(1349)						0.26 ± 0.03				
	AK11	Farnesane	3891-98-3			1354	(1366)								1.94 ± 0.16				
	AK12	Octane	111–65-9				(800)		(816)			53.10 ± 5.22							
	AK13	p-Cymene	99–87-6			1021	(1025)					1.92 ± 0.30							
	AK14	Theaspirane	36,431–72-8			1296	(1302)					2.98 ± 0.13							
	AK15	Farnesane	3891-98-3			1349	(1366)					2.74 ± 0.39							

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Table 1 (*continued*)

Class	Code	Compound	CAS	Reverse Similarity Index (DB-WAX122–7062)		Retention Index (HP-5 ms Ultra Inert)		Retention Index (SH-I-5Sil)		Concentration(μg/L)						Threshold (μg/L)	OAV		P
				Sample[d]	Standard[e]	Sample[f]	Standard[g] (Reference[h])	Sample[f]	Standard[g] (Reference[h])	NM	TM	HS	YS	SM	YES		NM	TM	
Alcohols	AL1	Ethanol	64–17-5	953	976		427		463	19,811.90 ± 413.50	23,496.79 ± 570.79								*
	AL2	Isoamyl alcohol[c]	123–51-3	897	866		736		697	7362.81 ± 146.68	9311.61 ± 193.83	113.64 ± 8.98	147.17 ± 4.09	6814.10 ± 75.00		2500	2.95	3.73	***
	AL3	3-methyl-2-pentanol[b]	565–60-6				(778)		(716)	112.72 ± 6.08				25.98 ± 1.66					
	AL4	2,3-Butanediol[b]	513–85-9	767	789		788		743	369.47 ± 13.88	407.58 ± 58.08					150	2.46	2.71	*
	AL5	5-Methyl-3-hexanol[a]	623–55-2			829	(838)				2.70 ± 0.41	24.50 ± 0.95				500		<1	
	AL6	4-Methylhexanol[a]	818–49-5			937	(953)	932	(896)	5.64 ± 0.17	1.15 ± 0.11					2000			***
	AL7	2-Ethylhexanol	104–76-7			1028	1030	959	995		22.21 ± 0.82				1.24 ± 0.09				
	AL8	Eucalyptol	470–82-6			1015	(1032)				2.41 ± 0.40								
	AL9	Benzyl alcohol[a]	100–51-6	944	913	1020	1036	1003	1036	26.24 ± 1.52	0.84 ± 0.11	424.72 ± 12.66				5100	<1		***
	AL10	Methyl benzyl alcohol[a]	98–85-1			1055	1061			1.79 ± 0.14			6.59 ± 0.15	2.27 ± 0.14		1380	<1		
	AL11	cis-Linaloloxide[b]	1365-19-1			1046	(1066)			9.62 ± 0.54						320	<1		
	AL12	trans-Linalool oxide[a]	34,995–77-2	803	922	1066	(1086)	1136	(1164)	37.86 ± 0.57	12.02 ± 1.84	8.32 ± 0.33				3600	<1	<1	***
	AL13	Phenylethyl alcohol[a]	60–12-8	936	957	1113	(1116)			16,215.58 ± 1216.73	9612.23 ± 335.20	32.27 ± 0.71	6.46 ± 0.13	3087.50 ± 228.83		14,000	1.15	<1	***
	AL14	Citronellol[a]	106–22-9			1219	1228			7.02 ± 0.50	5.60 ± 0.59			10.06 ± 1.62		40	<1	<1	**
	AL15	1-Dodecanol[a]	112–53-8	845	911	1466	1473	1409	1457	5.00 ± 0.16						1000	<1		
	AL16	Nerolidol[a]	40,716–66-3	763	934	1545	1564			61.83 ± 5.33	26.68 ± 4.30			21.19 ± 0.80		10	6.18	2.66	***
	AL17	Cedrol[a]	77–53-2			1588	1598			2.66 ± 0.12	0.63 ± 0.27					0.5	5.33	1.27	***
	AL18	γ-Eudesmol[b]	1209-71-8			1613	(1631)			2.96 ± 0.11						10,000			
	AL19	2,3-Dihydrofarnesol[c]	51,411–24-6			1714	(1696)			3.98 ± 0.48	2.48 ± 0.31								**
	AL20	trans-Farnesol[a]	106–28-5			1706	1722			4.68 ± 1.08	1.56 ± 0.05					0.02	234.2	78.45	***
	AL21	2-Methyl-1-butanol	137–32-6				(739)		(697)			6.99 ± 0.46	11.78 ± 0.46						
	AL22	2-Methylcyclohexanol[b]	7443-70-1			929	(946)					0.15 ± 0.04			0.58 ± 0.101				
	AL23	1-Heptanol[a]	111–70-6			968	970	1043	960					79.72 ± 7.06		3			
	AL24	3-(hydroxymethyl)-2-nonanone	67,801–33-6	897	951	1079	(1093)								0.51 ± 0.03				

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Table 1 (*continued*)

Class	Code	Compound	CAS	Reverse Similarity Index (DB-WAX122–7062)		Retention Index (HP-5 ms Ultra Inert)		Retention Index (SH-I-5Sil)		Concentration(μg/L)						Threshold (μg/L)	OAV		P
				Sample[d]	Standard[e]	Sample[f]	Standard[g] (Reference[h])	Sample[f]	Standard[g] (Reference[h])	NM	TM	HS	YS	SM	YES		NM	TM	
	AL25	Dihydrocarveol	38,049–26-2			1179	(1192)							3.86 ± 0.14					
	AL26	isoGeraniol	5944-20-7			1226	(1240)							9.26 ± 0.89					
	AL27	Leaf alcohol	928–96-1			861	(857)					20.94 ± 0.85							
	AL28	1-Hexanol	111–27-3	768	886	857	868	813	860			16.49 ± 0.60							
	AL29	2-Heptanol	543–49-7			896	900	890	879			5.65 ± 1.25							
	AL30	Sulcatol	1569-60-4			945	(994)					7.49 ± 0.21							
	AL31	cis-5-Octenol	64,275–73-6			1080	(1074)					0.13 ± 0.01							
	AL32	Linalool	78–70-6			1103	1099					6.78 ± 0.43							
	AL33	1-Nonanol	143–08-8	862	894	1169	1173	1108	1159			28.13 ± 2.03							
	AL34	3-Phenyl-1-propanol	122–97-4	898	867	1241	1232	1286	1235			19.63 ± 1.95							
	AL35	Cubenol	21,284–22-0			1652	(1642)					0.64 ± 0.13							
	AL36	α-Santalol	115–71-9			1639	(1681)					0.07 ± 0.003							
	AL40	Cinnamyl alcohol	104–54-1			1299	(1312)					9.86 ± 0.84							
	ES1	Ethyl acetate[b]	141–78-6	928	912		612		586	6074.69 ± 307.40	5889.43 ± 227.13					17,000	<1	<1	
	ES2	Ethyl propionate[b]	105–37-3				710				8.75 ± 0.30					19,000			
	ES3	Ethyl butyrate[b]	105–54-4	889	923		802		785	9.37 ± 1.29						20	<1	<1	
	ES4	Ethyl lactate[c]	97–64-3	791	823		815		848	17.22 ± 0.96	26.10 ± 5.44					14,000	<1	<1	**
	ES5	Ethyl isovalerate	108–64-5	768	903	846	854	760	820		2.15 ± 0.10								
Esters	ES6	Isoamyl acetate[b]	123–92-2	774	904	870	876	849	820	218.28 ± 6.21	50.60 ± 2.66			66.13 ± 9.91		30	7.27	1.68	***
	ES7	Gamma-butyrolactone[b]	96–48-0			903	915			13.46 ± 1.075	36.88 ± 3.48					35	<1	1.05	***
	ES8	Ethyl 3-hydroxybutyrate[b]	5405-41-4				944			70.45 ± 7.24	85.33 ± 2.67					21,000	<1	<1	**
	ES9	Ethyl caproate[b]	123–66-0	892	878	998	1000			379.78 ± 24.54	100.31 ± 6.95	3.21 ± 0.25		553.01 ± 50.56		14	27.12	7.16	***
	ES10	Trimethylene acetate	628–66-0			1079	(1089)	947	(984)		3.74 ± 0.12			10.96 ± 0.66					
	ES11	Octyl acetate[a]	112–32-3			1096	1114			4.19 ± 0.53		3.49 ± 0.11				12	<1		

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Table 1 (*continued*)

Class	Code	Compound	CAS	Reverse Similarity Index (DB-WAX122–7062)		Retention Index (HP-5 ms Ultra Inert)		Retention Index (SH-I-5Sil)		Concentration(μg/L)						Threshold (μg/L)	OAV		P
				Sample[d]	Standard[e]	Sample[f]	Standard[g] (Reference[h])	Sample[f]	Standard[g] (Reference[h])	NM	TM	HS	YS	SM	YES		NM	TM	
	ES12	Diethyl succinate[c]	123–25-1	887	910	1193	1182	1102	1151	257.64 ± 19.51	497.89 ± 11.9					114,000	<1	<1	***
	ES13	Ethyl caprylate[b]	106–32-1	902	898	1179	1196	1273	1183	1715.35 ± 108.02	1082.98 ± 64.16	146.41 ± 21.09		2887.94 ± 648.00		1200	1.42	<1	***
	ES14	Ethyl phenylacetate[a]	101–97-3	897	844	1245	1246	1217	1259	79.26 ± 6.45	33.06 ± 1.14					407	<1	<1	***
	ES15	Phenethyl acetate[a]	103–45-7	892	910	1253	1258	1218	1259	822.73 ± 15.00	335.62 ± 4.26	7.89 ± 0.35		54.27 ± 4.44		250	3.29	1.34	***
	ES16	Ethyl nonanoate[b]	123–29-5	784	753	1276	1296	1349	1282	30.36 ± 0.19	2.11 ± 0.13	1.91 ± 0.15		11.33 ± 0.37		3150.61	<1	<1	***
	ES17	Ethyl 3-phenylpropionate[b]	2021–28-5	916	912	1347	1353	1372	1359	168.80 ± 4.33	254.84 ± 2.54								***
	ES18	Ethyl 9-decenoate[a]	67,233–91-4	898	835	1367	(1378)	1309	(1371)	515.50 ± 26.03	5.29 ± 0.67			320.72 ± 19.14		100,000	<1		***
	ES19	Ethyl decanoate[b]	110–38-3	754	898	1376	1396	1322	1381	2368.50 ± 104.76	3213.12 ± 362.61	1.41 ± 0.53		419.92 ± 20.67		510	4.64	6.3	**
	ES20	Ethyl isopentyl succinate	28,024–16-0	751	798	1425	(1436)	1369	(1385)		9.61 ± 1.00								
	ES21	3-Methylbutyl octanoate[a]	2035-99-6			1429	1446			13.58 ± 1.41	15.61 ± 2.35	0.33 ± 0.03		19.59 ± 0.50		0.15	90.56	104.08	
	ES22	Ethyl undecylenate[c]	692–86-4			1482	1469	1594	1471	41.56 ± 5.00									
	ES23	Ethyl 3-hydroxytridecanoate	107,141–15-1	803	812	1527	(1539)				1.31 ± 0.25								
	ES24	Isobutyl decanoate[b]	30,673–38-2	814	874	1539	1546			1.56 ± 0.34	2.94 ± 0.47								**
	ES25	2-Phenylethanol tiglate[a]	55,719–85-2			1583	(1589)			0.20 ± 0.01	0.56 ± 0.07								***
	ES26	Ethyl laurate[c]	106–33-2	757	846	1611	1595	1592	1580	811.35 ± 76.89	1687.15 ± 391.93			69.87 ± 9.84		500	1.62	3.37	**
	ES27	3-Methylbutyl decanoate[c]	2306-91-4			1652	1646			7.86 ± 2.72	59.23 ± 17.90								***
	ES28	Phenylethyl caproate[b]	6290-37-5			1632	1650			4.33 ± 0.07									
	ES29	Ethyl myristate[a]	124–06-1	750	879	1792	1794	1749	1779	63.16 ± 10.77	6.84 ± 0.69					2000	<1	<1	***
	ES30	Isobutyl phthalate	84–69-5			1868	1870			1.21 ± 0.31									
	ES31	Ethyl pentadecanoate[b]	41,114–00-5	797	822	1875	1894	1774	1878	2.72 ± 0.31									
	ES32	Dibutyl phthalate[b]	84–74-2			1945	1965			3.65 ± 0.72				3.82 ± 0.31		159	<1		
	ES33	13-Octadecenoic acid methyl ester	56,554–47-3			1984	(1993)			430.68 ± 3.05	119.23 ± 13.36								***
	ES34	Ethyl oleate	6114-18-7	752	791	2160	(2173)	2260	(2185)	17.95 ± 7.41	1.44 ± 0.52								**
	ES35	Ethyl stearate[c]	111–61-5			2201	2195	2102	2177	3.22 ± 0.59	2.97 ± 0.87					500	<1	<1	
	ES36	Pentyl acetate[b]	628–63-7	756	865	908	911	931	884			1.89 ± 0.29		18.78 ± 2.82		0.005			

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Table 1 (*continued*)

Class	Code	Compound	CAS	Reverse Similarity Index (DB-WAX122–7062)		Retention Index (HP-5 ms Ultra Inert)		Retention Index (SH-I-5Sil)		Concentration(μg/L)						Threshold (μg/L)	OAV		P
				Sample[d]	Standard[e]	Sample[f]	Standard[g] (Reference[h])	Sample[f]	Standard[g] (Reference[h])	NM	TM	HS	YS	SM	YES		NM	TM	
	ES37	Methyl hexoate[a]	106–70-7			907	(925)	795	(884)			4.38 ± 0.37	3.80 ± 0.08	6.27 ± 0.35	4.66 ± 0.30	100			
	ES38	Heptanoic acid, methyl ester[a]	106–73-0			1013	1023	926	984			0.62 ± 0.05	0.47 ± 0.04	20.79 ± 2.39	4.05 ± 0.66				
	ES39	Methyl octanoate	111–11-5			1116	(1126)	1086	(1083)				33.75 ± 0.16		1785.52 ± 86.17				
	ES41	Diisopentyl oxalate	2051-00-5			1424	(1443)								1.65 ± 0.26				
	ES42	Ethyl 2-methylbutyrate	7452–79-1			849	849	862	820			8.83 ± 0.86							
	ES43	Ethyl 3-methylvalerate	5870–68-8	893	931	945	960	962	920			4.94 ± 0.53							
	ES44	4-methyl-pentanoicaciethylester	25,415–67-2	797	890	987	969	960	920			4.34 ± 0.71							
	ES45	Benzyl acetate	140–11-4			1163	(1164)					3.89 ± 0.75							
	ES46	Hexyl isovalerate	10,032–13-0			1247	1244					0.80 ± 0.01							
	ES47	Tetradecane, 2,6,10-trimethyl-	14,905–56-7	769	822	1527	(1539)					0.52 ± 0.05	0.60 ± 0.01	1.82 ± 0.44					
	ES55	3-Phenylpropyl acetate	122–72-5	848	779	1348	(1373)	1394	(1359)			14.77 ± 2.35							
	ES63	Ethyl cinnamate	103–36-6	824	920	1436	(1464)					3.44 ± 0.33							
	ES74	Ethyl formate	109–94-4				468		584			1672.14 ± 102.28							
	ES75	Hexadecenoic acid ethyl ester	54,546–22-4	799	817	1986	1977	1935	1986	43.17 ± 10.76	4.14 ± 1.07								***
Ethers	ET1	(+)-Rose oxide	16,409–43-1			1109	1110			1.20 ± 0.18		1.10 ± 0.25							
	ET2	2H-pyran, 2-butoxytetrahydro-	1927-68-0			1102	(1110)								0.15 ± 0.02				
	ET3	2-Butyrylfuran	4208-57-5			1078	(1078)					1.43 ± 0.24							
Ketones	KE1	2-Nonanone[b]	821–55-6	827	908	1090	1092	990	1052	19.59 ± 0.63						100	<1		
	KE2	2-Undecanone[b]	112–12-9			1280	1294	1315	1251	17.69 ± 0.45						80	<1		
	KE3	4-Methyl-2-pentanone	108–10-1				(735)								10.49 ± 1.06				
	KE4	4-Methyl-2-hexanone [b]	105–42-0	816	832	846	(848)	728	(789)					2.94 ± 0.11					
	KE5	6-Methyl-5-hepten-2-one [b]	110–93-0			980	986							3.63 ± 0.15		68			
	KE6	β-Damascenone	23,726–93-4	871	897	1396	1386					12.00 ± 0.74							

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Table 1 (*continued*)

Class	Code	Compound	CAS	Reverse Similarity Index (DB-WAX122–7062)		Retention Index (HP-5 ms Ultra Inert)		Retention Index (SH-I-5Sil)		Concentration(μg/L)						Threshold (μg/L)	OAV		P
				Sample[d]	Standard[e]	Sample[f]	Standard[g] (Reference[h])	Sample[f]	Standard[g] (Reference[h])	NM	TM	HS	YS	SM	YES		NM	TM	
Terpenes	OL1	Styrene	100–42-5	932	955	875	893	852	883		1.19 ± 0.16								
	OL2	Cedrene[a]	11,028–42-5			1417	(1422)			4.35 ± 0.26	5.69 ± 0.36					0.03	145.06	189.9	**
	OL3	Humulene	6753-98-6			1442	(1454)			1.30 ± 0.20	1.13 ± 0.17			13.69 ± 0.73					*
	OL4	(*E*)-β-Famesene	18,794–84-8			1455	(1457)			9.16 ± 0.51	4.63 ± 0.44			5.39 ± 1.19					***
	OL5	γ-Muurolene	30,021–74-0			1466	(1477)				2.28 ± 0.25								
	OL6	α-Curcumene[a]	644–30-4			1482	(1483)			0.91 ± 0.19									
	OL7	5-Isopropylidene-1,3-cyclopentadiene	2175-91-9	814	850	842	(858)								0.92 ± 0.10				
	OL8	(e)-Limoneneoxide	4959-35-7			1119	(1138)						0.89 ± 0.02						
	OL10	α-Ylangene	14,912–44-8			1364	(1372)					0.66 ± 0.03							
	OL11	α-Terpinene	99–86-5			1000	(1017)					3.10 ± 0.16							
	OL12	α-Ionene	475–03-6	942	921	1254	(1258)					9.35 ± 0.35							
Phenols	PN1	Butylated hydroxytoluene	128–37-0			1504	1513			0.58 ± 0.20				0.78 ± 0.09					
	PN2	2,4-Di-tert-butylphenol[c]	96–76-4	871	790	1531	1519	1548	1555	8.06 ± 0.30						200	<1		
	PN4	Eugenol	97–53-0	913	918	1349	1357					16.77 ± 1.36							
	PN5	Methyleugenol	93–15-2	864	879	1405	1402					13.90 ± 0.35							

greenwood flavor.

As shown in Fig. 1A, a comprehensive sensory analysis revealed that off-flavors were detected less frequently in NM compared to TM. Specifically, the sensory scores for bitterness, irritation, and sweat were reduced by 41.1 %, 42.5 %, and 30.6 %, respectively, in NM. In contrast, fruit, sweet, and pleasant sour flavors increased by 27.4 %, 36.9 % and 45.5 %, respectively, in NM. An interesting observation was that male assessors were less inclined to favor sour meads (NM sourness score: 5.9 for males vs 7.3 for females), while both male and female panelists agreed that NM exhibited a softer, more pleasant acidity compared to TM. Regarding sweetness, an appropriate level of sourness was found to balance the taste, thereby enhancing the overall quality of the meads. Women tended to show greater sensitivity to off-flavors, as evidenced by the waxy flavor evaluation. Nearly all female panelists reported the presence of a waxy flavor in TM, but not in NM. This contrasted with male preferences, who may have a stronger inclination toward beverages with more intense flavors and higher alcohol content, such as dark beer, whiskey, or rum. In comparison, women often favored drinks with a lighter, more refreshing taste and rich fruity notes, such as wine, champagne, or cocktails with a more subtle profile (Spence & Wang, 2019). This could be one of the reasons, why sensory evaluations vary across genders. PCA was employed to visualize relationships between samples based on their sensory analysis (Fig. 1B) and PC1 along with PC2 accounted for 99.00 % of the total variance (73.80 % and 25.20 %, respectively). NM and TM were scattered separately. NM formed a cluster at the left side of the biplot, revealing that more flavor and less off-flavor aroma were observed in NM. The OPLS-DA model was employed to further analyze the sensory scores and its VIPs are shown in Fig. 1C. It was found that five sensory scores had VIPs >1. Among these, the sensory attributes contributing most to NM, ranked by VIPs, included sweetness, pleasant acidity and fruitiness. For the TM group mead, the most influential attributes were bitterness and astringency.

3.4. OAV of volatile compounds

As detailed in Table 1, aroma compounds were categorized into three main groups: fruity-sweet (30 types), herbal (29 types), and off-flavors (19 types). A total of 53 aroma compounds had documented odor thresholds, including 20 fruity-sweet types, 20 herbal types, and 13 off-flavors. Among these, 20 volatile compounds with OAV values greater than 1 were identified, comprising 9 fruity-sweet, 9 herbal, and 2 off-flavor compounds, which were the primary contributors to the mead's aroma, as illustrated in Fig. 1D.

Table 1 indicated that the types of off-flavor compounds did not differ substantially between TM (15 types) and NM (16 types). However, the total concentration of off-flavor compounds was significantly reduced by approximately 37.60 %, from 12.58 mg/L in TM to 9.10 mg/L in NM. Significant concentration changes were observed for several off-flavor compounds, including isoamyl alcohol (decreasing from 9.31 to 7.36 mg/L), ethyl laurate (from 1.69 to 0.81 mg/L), diethyl succinate (from 497.89 to 257.64 μg/L), nonanoic acid (from 454.25 to 81.18 μg/L), and octanoic acid (from 358.25 to 245.71 μg/L). Additionally,

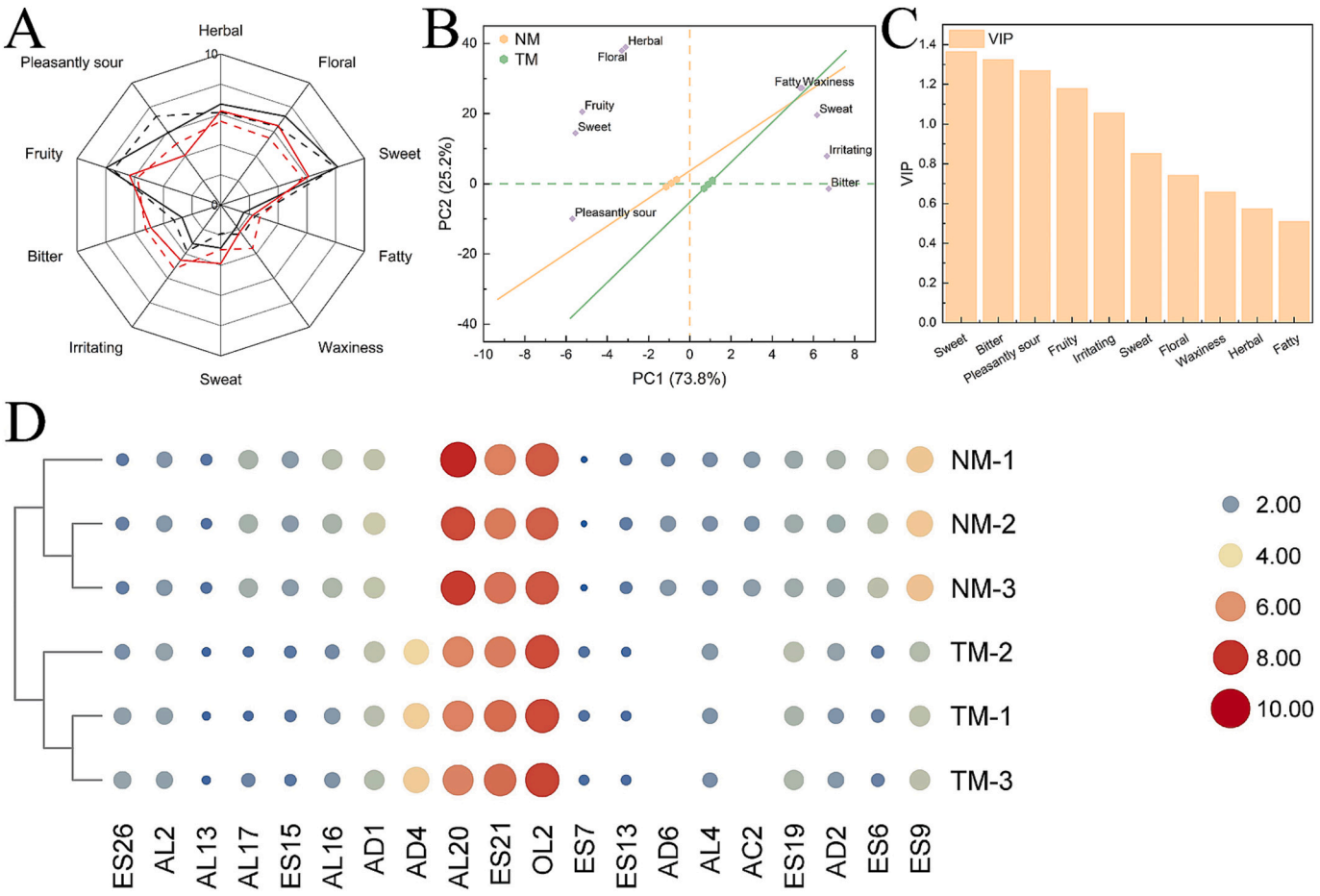

Fig. 1. (A). Radar plots of sensory attributes, NMB (black straight line) represents sensory attributes to NM for male panelists; TMB (red straight line) represents sensory attributes to TM for male panelists; NMG (black dotted line) represents sensory attributes to NM for female panelists; TMG (red dotted line) represents sensory attributes to TM for female panelists; (B). PCA plots of sensory scores for mead in NM and TM groups; (C). VIP plots of sensory scores for mead in NM and TM groups; (D). OAV of compounds NM and TM. Only compound with OAV > 1 was shown. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

although compounds such as 3-methylbutyl decanoate, hexanoic acid, and ethyl lactate were detected at low concentrations (~50 μg/L), their levels were further reduced, reaching concentrations below 10 μg/L. This reduction notably diminished the plastic, pungent, and sweaty characteristics of NM.

Overall, among the off-flavor compounds, only isoamyl alcohol and ethyl laurate exhibited OAVs greater than 1. The OAVs of isoamyl alcohol and ethyl laurate in TM (3.72 and 3.37, respectively) were approximately 1.3 and 2.1 times higher than those in NM (2.95 and 1.62), contributing to more pronounced anesthetic, oily, pungent, sweaty, and astringent notes in TM. While the OAVs of other compounds were lower, this did not imply they had no effect on the meads. Upon analyzing off-flavor compounds with OAVs greater than 0.05, common compounds such as 2-methylbutyric acid, decanoic acid, and octanoic acid were found, contributing noticeable oily, bitter, and astringent characteristics. These compounds have also been reported in studies of Chinese Baijiu (Mu et al., 2023), but the higher alcohol content masked these off-flavors.

The total concentrations of fruity and sweet compounds in TM and NM were 11.16 mg/L and 11.74 mg/L, respectively. The number of fruity and sweet compounds was 15 in TM and 26 in NM. While the total concentration did not exhibit significant changes after the process improvement, the diversity of compounds increased. This was likely due to the enhanced yeast metabolism, leading to the production of new fruity and sweet compounds, such as ethyl pentadecanoate, γ-eudesmol, dibutyl phthalate, phenylethyl caproate, pentanoic acid, ethyl butyrate, cis-linalool oxide, 2-undecanone, 2-nonanone, isovaleric acid, 2-butyl-2-hexenal, and 3-methyl-2-pentanol. Among these, the concentrations of 3-methyl-2-pentanol, 2-butyl-2-hexenal, and isovaleric acid notably increased to 112.72 μg/L, 50.88 μg/L, and 36.49 μg/L, respectively, contributing pineapple, pear, and peach flavors. Common compounds identified in both meads included benzoic acid, isobutyl decanoate, nonanal, gamma-butyrolactone, benzeneacetaldehyde, ethyl nonanoate, ethyl 3-hydroxybutyrate, ethyl 3-phenylpropionate, isoamyl acetate, 2,3-butanediol, ethyl caproate, ethyl caprylate, and ethyl acetate. Notably, the concentrations of isoamyl acetate, ethyl caproate, and ethyl caprylate significantly increased in NM compared to TM. Isoamyl acetate rose from 50.60 μg/L to 218.28 μg/L, a 3.31-fold increase; ethyl caproate increased from 100.31 μg/L to 379.78 μg/L, a 2.79-fold increase; and ethyl caprylate increased from 1.08 mg/L to 1.72 mg/L, a 58.39 % increase. These compounds enhanced the pineapple, pear, and apple-like aromas in NM, contributing to its sweet, refreshing taste. In contrast, ethyl 3-phenylpropionate increased from 168.80 μg/L (NM) to 254.84 μg/L (TM), a 51.19 % increase. These compounds likely contributed to more pronounced fruity and sweet flavors in TM. For compounds with OAV values greater than 1, common substances included 2,3-butanediol, ethyl caproate, benzeneacetaldehyde, isoamyl acetate, and ethyl caproate. Isoamyl acetate and ethyl caproate exhibited notable increases in their OAVs in NM (7.27 and, 27.12 respectively) compared to TM (1.68 and 7.17), enhancing the pineapple-like fruity aroma in NM. In TM, compounds such as gamma-butyrolactone (OAV = 1.05) and ethyl decanoate (OAV = 6.30) showed increased OAVs compared to NM (0.39 and 4.64), contributing to a subtle caramel aroma. Most of the newly identified compounds in NM had OAVs below 1, except for 2-butyl-2-hexenal (2.54) and isovaleric acid (3.04), which imparted citrus, green grass, and mild sweetness. Analysis of OAVs demonstrated that NM possessed a greater variety of fruity and sweet compounds with higher corresponding OAVs, indicating a more complex and pronounced flavor profile.

The number of herbal compounds increased from 19 in TM to 22 in NM, with the total concentration rising approximately 1.8 times, from 10.15 mg/L to 17.97 mg/L. Unique aromatic compounds identified in NM included α-curcumene, methyl benzyl alcohol, octyl acetate, 1-dodecanol, and 2,5-dimethylbenzaldehyde, which contributed herbal, fresh grass, and rose aromas. In contrast, TM contained unique compounds such as 5-methyl-3-hexanol and decanal, which imparted woody and mushroom-like aromas. Significant changes were observed in NM, with compounds such as ethyl 9-decenoate, which increased from 5.29 μg/L to 515.50 μg/L; phenethyl acetate, which rose from 335.63 μg/L to 822.74 μg/L; and phenylethyl alcohol, which increased from 9.61 mg/L to 16.22 mg/L. These substances contributed more pronounced floral, sweet, and long-lasting rose-like aromas. Additionally, compounds including benzyl alcohol (26.25 μg/L in NM, 0.85 μg/L in TM), trans-linalool oxide (37.87 μg/L in NM, 12.02 μg/L in TM), benzyl alcohol (26.25 μg/L in NM, 0.85 μg/L in TM), nerolidol (61.83 μg/L in NM, 26.68 μg/L in TM), and ethyl phenylacetate (79.27 μg/L in NM, 33.07 μg/L in TM) were detected at significantly higher concentrations in NM. These compounds imparted oak, rose, vanilla, and cocoa-like aromas. The analysis of OAVs revealed that most compounds with OAVs greater than 1 were common to both meads. Some low-concentration substances also exhibited higher OAVs due to their lower odor thresholds. In comparison with TM, NM showed significantly higher OAVs for compounds such as phenylethyl alcohol (0.69 in TM, 1.16 in NM), phenethyl acetate (1.34 in TM, 3.29 in NM), cedrol (1.27 in TM, 5.34 in NM), nerolidol (2.67 in TM, 6.18 in NM), benzaldehyde (7.14 in TM, 8.71 in NM), and trans-farnesol (78.45 in TM, 234.20 in NM), which contributed more pronounced rose, almond, floral, chicory, and woody aromas, enhancing the overall sensory experience with a cooler sensation. In TM, two compounds exhibited higher OAVs than in NM, including 3-methylbutyl octanoate (104.08 in TM, 90.56 in NM) and cedrene (189.90 in TM, 145.07 in NM), both of which imparted woody aromas. Overall, the concentration and OAVs of herbal compounds were significantly higher in NM, which enhanced its floral complexity. Although the OAVs of some compounds also increased in TM, their contributions were predominantly woody, leading to a more monotone flavor profile compared to NM's more diverse and aromatic herbal characteristics.

3.5. Principal component and orthogonal partial least squares discriminant analysis

PCA was employed to visualize relationships between samples based on their aroma compounds (Fig. 2A) and PC1 along with PC2 accounted for 91.90 % of the total variance (86.50 % and 5.40 %, respectively). NM and TM were scattered separately. As shown in the figure, the two groups of samples exhibit a clear separation trend on the two-dimensional plot, with no outliers and good clustering within the same sample type. The samples are mainly separated along PC1, primarily due to the significant differences in the types and concentrations of aromatic compounds produced during fermentation under different yeast activity levels.

The overlap existed between the aroma of TM and NM. Therefore, these aroma compounds with fruity-sweet (30 types), herbal (29 types), and off-flavors (19 types), were respectively analyzed by PCA between two meads.

As shown in Fig. 2C-D, 14 kinds of off-flavor compounds with VIPs scores greater than 1 were found. Among them, the substances that contributed most to NM in order of VIPs are as follows: 2-methylbutyric acid (AC1), decanoic acid (AC9), 2,3-dihydrofarnesol (AL19), ethyl undecylenate (ES22), butylated hydroxytoluene (PN1) and 2,4-di-tert-butylphenol (PN2). The total concentration of these compounds led to the plastic, clay, lava, and fatty smell. The substances that contributed most to TM in order of VIPs were found as dl-3-methylvaleric acid (AC4), hexanoic acid (AC5), octanoic acid (AC7), nonanoic acid (AC8), isoamyl alcohol (AL2), diethyl succinate (ES12) and 3-methylbutyl decanoate (ES27). The total concentration of these compounds led to bitter, irritating, sweaty and fatty flavors.

As shown in Fig. 2E-F, there were 22 kinds of fruity-sweet compounds with VIPs greater than 1.Among them, the substances that contributed most to NM in order of VIPs are as follows: isovaleric acid (AC2), pentanoic acid (AC3), benzoic acid (AC6), 2-butyl-2-hexenal (AD6), cis-linaloloxide (AL11), γ-eudesmol (AL18), 3-methyl-2-pentanol (AL3), ethyl caprylate (ES13), ethyl propionate (ES2), phenylethyl

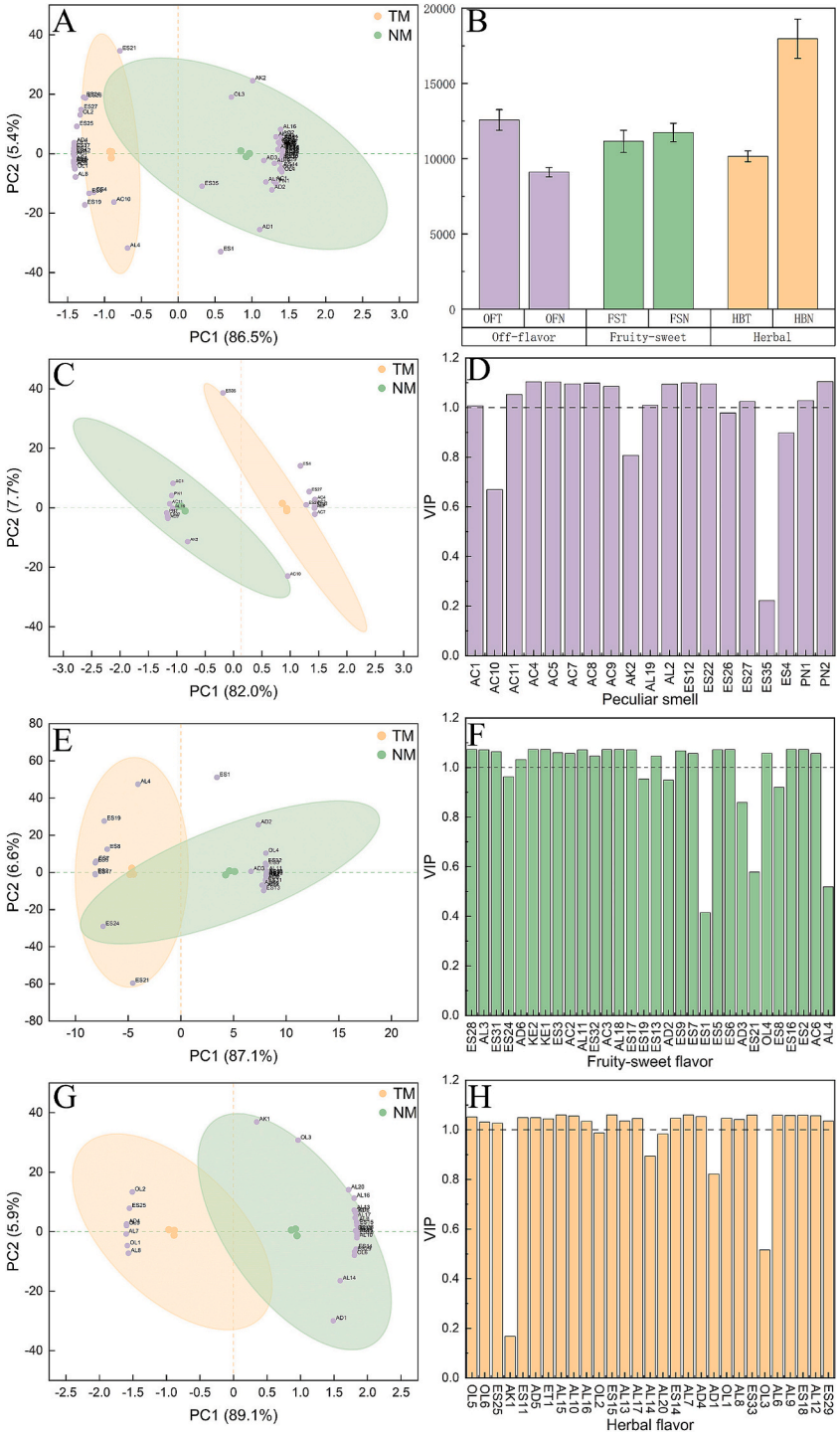

Fig. 2. (A). PCA score plots for all substances; (B). Total concentration of volatile compounds in different flavors of NM and TM, OFT represents off-flavor substances in traditional mead, OFN represents off-flavor substances in novel mead, FST represents fruity and sweet substances in traditional mead, FSN represents fruity and sweet substances in novel mead, HBT represents herbaceous substances in traditional mead, and HBN represents herbaceous substances in novel mead; (C). PCA score plots for off-flavors; (D). VIP plots of OPLS-DA for off-flavors; (E). PCA score plots for fruity and sweet substances; (F). VIP plots of OPLS-DA for fruity and sweet substances; (G). PCA score plots for herbaceous substances; (H). VIP plots of OPLS-DA for herbaceous substances. Orange dots represent TM samples, green dots represent NM samples, and purple dots represent volatile compounds. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

caproate (ES28), ethyl butyrate (ES3), ethyl pentadecanoate (ES31), dibutyl phthalate (ES32), isoamyl acetate (ES6), ethyl caproate (ES9),2-nonanone (KE1) and 2-undecanone (KE2). The total concentration of these compounds led to the refreshing apple, citrus, pineapple flavors. The substances that contributed most to TM in order of VIPs as followed: ethyl isovalerate (ES5), (*E*)-β-famesene (OL4), gamma-butyrolactone (ES7), ethyl 3-phenylpropionate (ES17) and ethyl propionate (ES2). The total concentration of these compounds led to the caramel and banana flavors.

As shown in Fig. 2G-H, there were 23 kinds of herbal compounds

with VIPs greater than 1. Among them, the substances that contributed most to NM in order of VIPs as followed: 2,5-dimethylbenzaldehyde (AD5), methyl benzyl alcohol (AL10), trans-linalool oxide (AL12), phenylethyl alcohol (AL13), 1-dodecanol (AL15), nerolidol (AL16), cedrol (AL17), 4-methylhexanol (AL6), benzyl alcohol (AL9), octyl acetate (ES11), ethyl phenylacetate (ES14), phenethyl acetate (ES15), ethyl 9-decenoate (ES18), ethyl myristate (ES29) and α-curcumene (OL6). The total concentration of these compounds led to rich flavors of the almond, pine, cocoa floral, woody, rose and mushroom. The substances that contributed most to TM in order of VIPs as followed: γ-muurolene (OL5), 2-phenylethanol tiglate (ES25), 2-ethylhexanol (AL7), eucalyptol (AL8), (+)-rose oxide (ET1), 13-octadecenoic acid methyl ester (ES33), decanal (AD4) and styrene (OL1). The total concentration of these compounds led to the rose, chicory and floral flavors.

3.6. Mechanism of flavor compound formation

To analyze the potential mechanism of the formation of aroma compounds, volatile compounds of the honey solution (HS), yeast solution (YS), yeast extraction solution (YES) and seed culture medium (SCM) were analyzed, as shown Table 1 and Fig. 3A. NM and TM were different with unfermented groups. YES is a kind of fermented products, which showed the more similarity with NM. However, their contents were completely different. The composition of volatile compounds of SCM was between NM and TM. After the full fermentation, the types and contents of volatile compounds were generally increased. Many new volatile compounds were found, not only flavor but also off-flavor.

The total concentration of herbal compounds was increased in NM than that in TM (Fig. 3B). For example, ethyl 9-decenoate was obviously accumulated in SCM and further increased in NM significantly. Unfortunately, it was barely detected in TM. Three compounds were only found in NM including 2,5-dimethylbenzaldehyde (AD5), 1-dodecanol (AL15) and α-curcumene (OL6). These phenomena indicated yeast showed high activities in NM than TM during the fermentation. Pentadecane (AK1), cedrol (AL17), trans-farnesol (AL20), 4-methylhexanol (OL6), ethyl phenylacetate (ES14), 2-phenylethanol tiglate (ES25), ethyl myristate (ES29), and cedrene (OL2) were only detected in meads. Phenylethyl alcohol (AL13) and phenethyl acetate (ES15) were barely detected in SCM or HS, but a huge accumulation was found in TM and NM. These compounds might be unique metabolites of the anaerobic respiration from yeasts in honey solutions. Benzaldehyde (AD1), methyl benzyl alcohol (AL10), citronellol (AL14), benzyl alcohol (AL9), and 3-methylbutyl octanoate (ES21) existed in the raw materials (HS and YES) and were also metabolites of the aerobic respiration from yeasts. After the anaerobic respiration, their concentrations were all reduced compared with HS. Therefore, they might be the important intermediates during the fermentation considering less accumulation in the final products. For example, benzyl alcohol (AL9) would be converted into phenylethyl alcohol (AL13) by alcohol dehydrogenase and ketoreductase.

More significant differences were observed in compounds with fruit-sweet flavors and higher total concentration was observed in NM than that in TM (Fig. 3C). Characterized volatile compounds in NM consisted of isovaleric acid (AC2), pentanoic acid (AC3), 2-butyl-2-hexenal (AD6), cis-linaloloxide (AL11), γ-eudesmol (AL18), phenylethyl caproate (ES28), ethyl butyrate (ES3), ethyl pentadecanoate (ES31), 2-nonanone (KE1) and 2-undecanon (KE2). TM only had one characterized compound, ethyl propionate (ES2). Many compounds were only detected in mead samples, such as benzoic acid (AC6), 2,3-butanediol (AL4) and etc. These compounds might be unique metabolites of the anaerobic respiration from yeasts in honey solutions. Benzeneacetaldehyde (AD2), ethyl caprylate (ES13), pentyl acetate (ES36), ethyl caproate (ES9), 4-methyl-2-hexanone (KE4), and 6-methyl-5-hepten-2-one (KE5) were all detected in SM and mead samples. However, their concentrations were sharply decreased in the later, implying that they were mainly accumulated in the aerobic respiration and, then, converted into other aroma compounds in the anaerobic respiration. Although ethyl decanoate (ES19) was obviously increased from 1.50 to 418.00 μg/L during the yeast growth, it sharply accumulated into about 2300.00–3200.00 μg/L during the anaerobic respiration and became one of the main aroma compounds in meads.

The main differences in concentrations of off-flavor compounds between TM and NM were caused by isoamyl alcohol (AL2), ethyl laurat (ES26), octanoic acid (AC7), nonanoic acid (AC8), and decanoic acid (AC9). The total concentration was decreased about 38.19 % in NM than that in TM (Fig. 3D). The reduction in irritation might be due to the decrease in isoamyl alcohol (AL2) content, while the reduction in fatty and oily flavors would be caused by the decrease in ethyl laurate (ES26). Additionally, the sweaty odor and acidity of NM were improved because the content of undecanoic acid (AC10), ethyl lactate (ES4), hexanoic acid (AC5), 3-methylbutyl decanoate (ES27) and diethyl succinate (ES12) in the NM group is reduced.

Some off-flavor compounds were detected in both SM and TM, but significantly reduced or even disappeared in NM group, such as dl-3-methylvaleric acid (AC4), octanoic acid (AC7), nonanoic acid (AC8), isoamyl alcohol (AL2), ethyl laurat (ES26). This phenomenon implying that yeasts with higher activities would be beneficial to metabolize/convert some off-flavor compounds during the anaerobic respiration and improve the mead quality. Compared with TM, less amount of nitrogen sources and yeasts were used NM. Meanwhile, these nitrogen sources and yeasts would not introduce a large amount of flavor compounds basing on the cluster analysis. Therefore, the yeast activity should be the crucial factor on the formation of the aroma and off-flavor compounds during the mead fermentation. Yeasts with higher activities should be beneficial to synthesize more flavor compounds and minimize the off-flavor compounds, as shown in NM group.

3.7. Metabolomics of volatile compound

Dynamic monitoring was employed to investigate the relationships among volatile compounds during fermentation (Table S5 and S6). The correlations between the changes in volatile compound concentrations during dynamic fermentation in the NM and TM groups are presented in Fig. 4A-B, while the *P*-values for the concentration differences of volatile compounds between the NM and TM groups are shown in Fig. 4C-D. The special attention was paid on the key volatile compounds based on their values of VIPs (>1) or OAVs (>1) or concentrations (100 μg/L). Among the compounds contributing to the fruity and sweet taste differences, five were identified as isovaleric acid (AC2), 2-butyl-2-hexenal (AD6), ethyl caprylate (ES13), isoamyl acetate (ES6) and ethyl caproate (ES9), all of which were found in higher concentrations in NM. Eight compounds were identified as contributing to the herbal taste differences between the two types of mead. Among these, phenylethyl alcohol (AL13), nerolidol (AL16), cedrol (AL17), trans-farnesol (AL20) and phenethyl acetate (ES15) were found in higher concentrations in NM. In contrast, concentrations of decanal (AD4), 3-methylbutyl octanoate (ES21) and cedrene (OL2) were higher in TM. Six compounds would lead to off-flavors. In TM, they were dl-3-methylvaleric acid (AC4), octanoic acid (AC7), nonanoic acid (AC8), isoamyl alcohol (AL2) and ethyl laurate (ES26). Only decanoic acid (AC9) was higher in NM.

Fig. 5A and Fig. 6A-B mainly demonstrated the synthesis pathways of phenylethyl alcohol (AL13), which involved the metabolic pathways of the Ehrlich pathway, Shikimic acid /3-Phenylpyruvic acid pathway, Phenylethylamine pathway and “styrene-derived” pathway (Warhurst & Fewson, 1994; Y. Wang et al., 2019). The enzymatic conversion from benzeneacetaldehyde (AD2) into phenylethyl alcohol (AL13) catalyzed by alcohol dehydrogenases enzyme was identified as the rate-limiting step. Compared with TM, the reaction speed of this rate-limiting step was faster in NM. There are two synthetic pathways for the precursor substance benzeneacetaldehyde (AD2) of phenylethyl alcohol (AL13), but there is no obvious metabolic difference. Meanwhile, no significant difference was observed in the consumption of phenylethyl alcohol

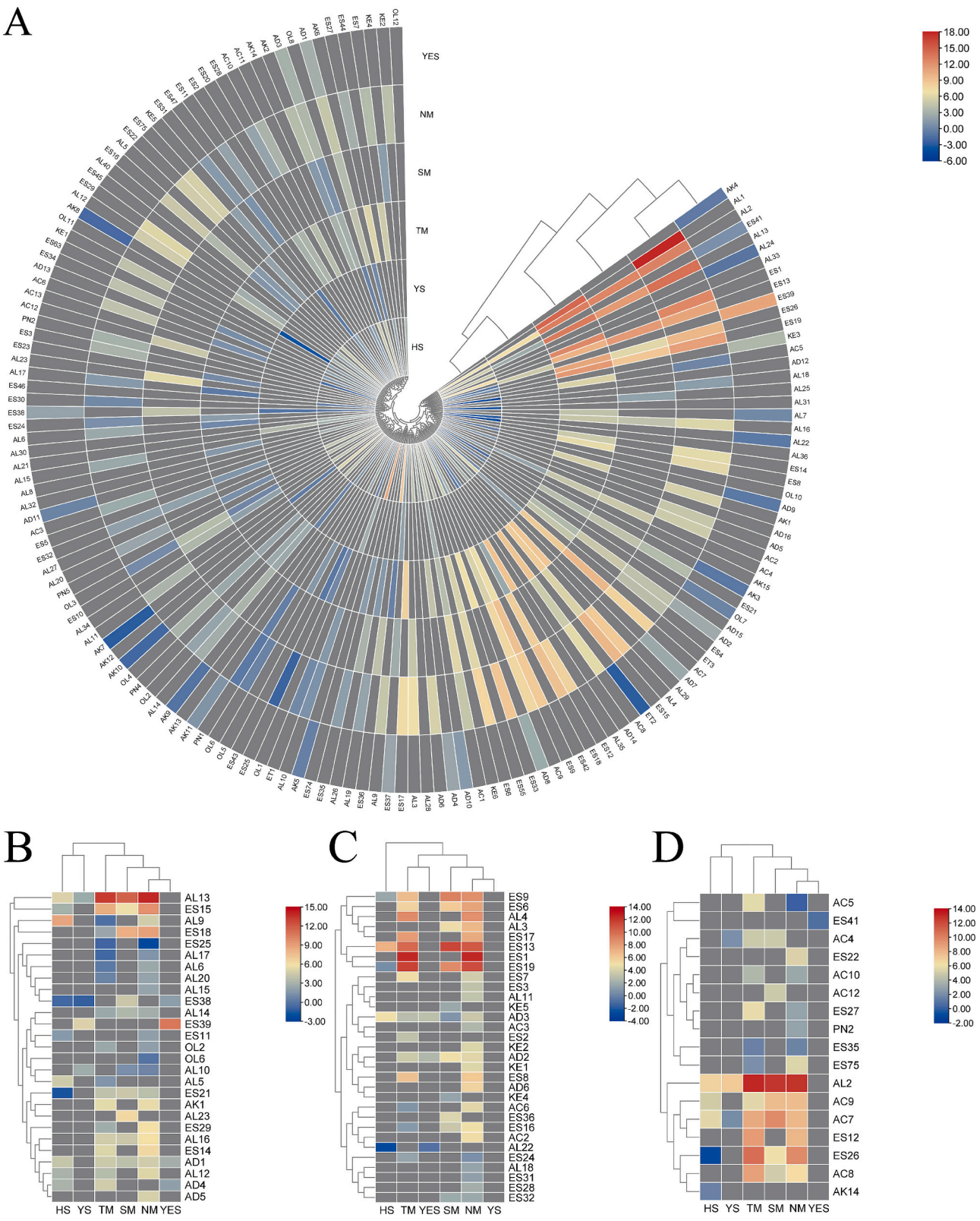

Fig. 3. (A). Heat map analysis of aroma components of mead (TM, NM), honey solution (HS), yeast solution (YS), yeast extraction solution (YES) and seed culture medium (SCM); (B-D). Heat map analysis of herbal flavor, fruity-sweet flavor and off-flavor aroma components in the samples. Red colour indicates high concentration; blue colour indicates low concentration. The samples were clustered using hierarchical analysis and the similarity measure was calculated using pearson distance. Concentrations were log2-transformed. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

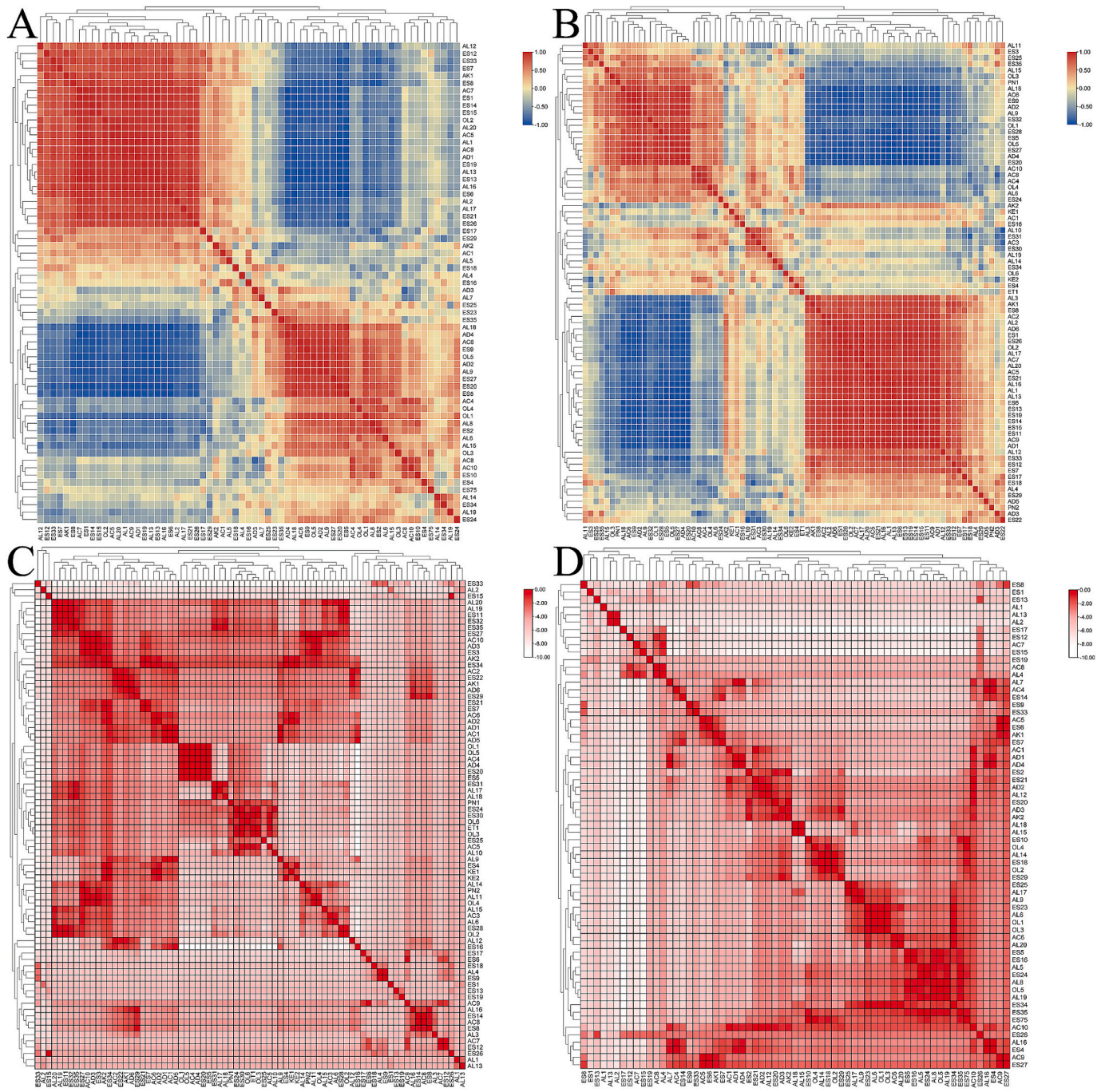

Fig. 4. (A - B). Heat maps represent the correlation between the concentration changes of volatile compounds during the fermentation of NM and TM, respectively. The correlation analysis was performed using Spearman's correlation analysis ($P < 0.05$). The R-values are shown in the legend on the right side.
(C - D). The heatmaps show log10-transformed P-values between the concentrations of volatile compounds during the fermentation of NM and TM, respectively.

(AL13) between NM and TM. This phenomenon explained the accumulation of phenylethyl alcohol (AL13) in NM.

Through the mevalonate and methylerythritol phosphate pathways, terpenoids could be synthesized, including nerolidol (AL16), cedrol (AL17), trans-farnesol (AL20) and cedrene (OL2), as shown in Fig. 5B and Fig. 6C -D (Frank & Groll, 2017; Zhao, Chang, Xiao, Liu, & Liu, 2013). However, the concentration of all terpenoids were not high. Metabolic calculations indicated that each step in these pathways was rate-determining. Nerolidol (AL16), cedrol (AL17) and trans-farnesol (AL20) showed slight increases in NM, while TM favored the synthesis of cedrene (OL2). They were the dominant herbal flavor compounds in NM and TM, respectively.

Fig. 5C and Fig. 6E-F illustrated the Ehrlich and Harris pathway of amino acids, in which the crucial off-flavor compound isoamyl alcohol (AL2) was involved (Plata, Mill, Mauricio, & Ortega, 2003; Yoshimoto, Fukushige, Yonezawa, & Sone, 2002). The rate-limiting step was confirmed as the oxidation of isovaleraldehyde (3-MEBU) to isoamyl alcohol (AL2) catalyzed by alcohol dehydrogenase. Meanwhile, this rate-limiting step proceeded faster in TM than that in NM. Isoamyl alcohol (AL2) as a precursor substance participated in the synthesis of four types of esters. Most of them were faster in NM. These phenomena explained the serious accumulation of isoamyl alcohol (AL2) in TM. As the precursor of isoamyl alcohol (AL2), isovaleraldehyde (3-MEBU) could also beconverted into isovaleric acid (AC2) by aldehyde

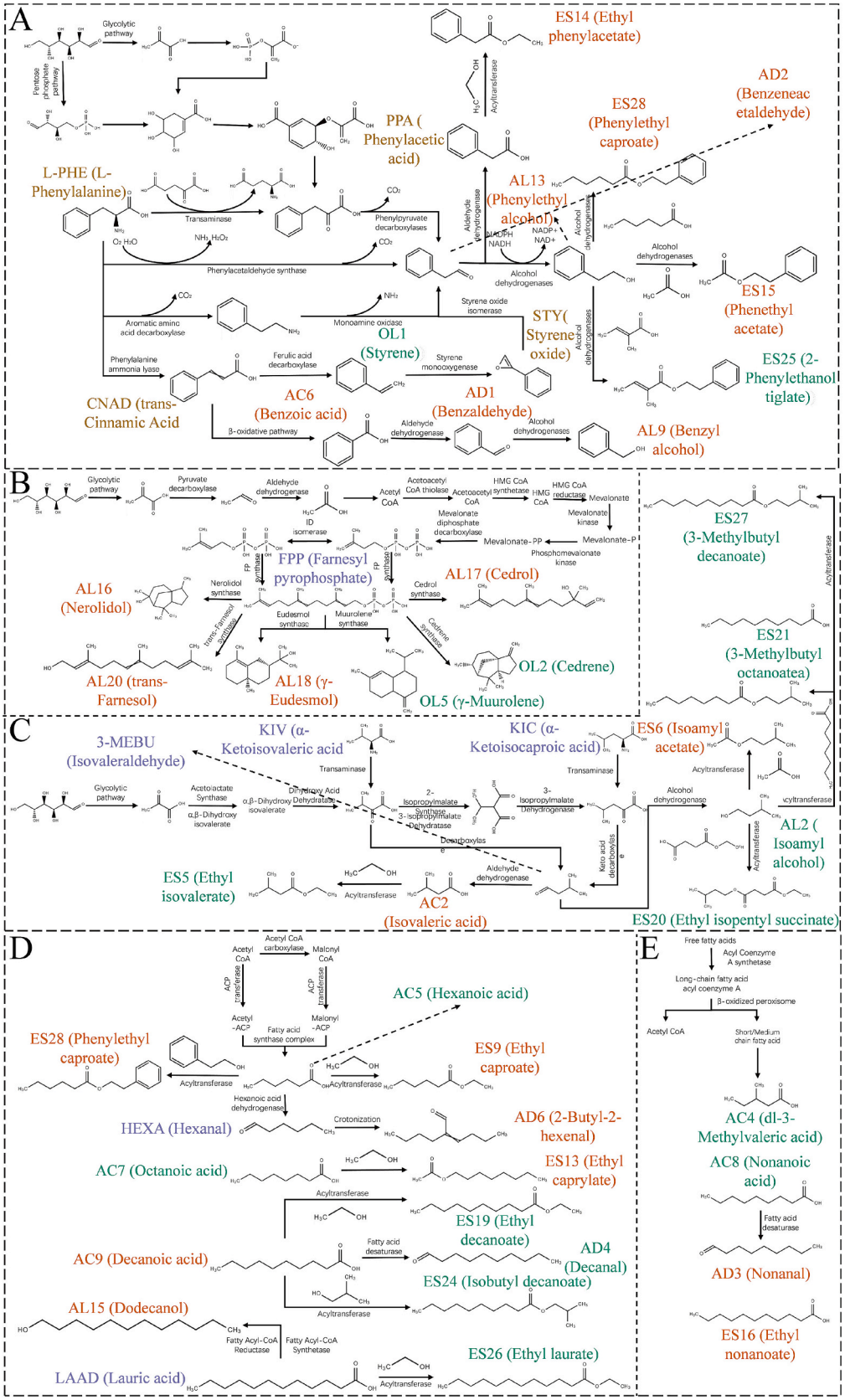

Fig. 5. (A). Metabolic pathways of phenylethanol production in *Saccharomyces cerevisiae*; (B). Metabolic pathways of terpenoid pathways in *S. cerevisiae*; (C). Metabolic pathways of isoamyl alcohol in *S. cerevisiae*; (D). Metabolic pathways of even-numbered fatty acids in *S. cerevisiae*; (E). Metabolic pathways of other acids in *S. cerevisiae*. The orange substances represent the dominant compounds in NM, while the green substances represent the dominant compounds in TM. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

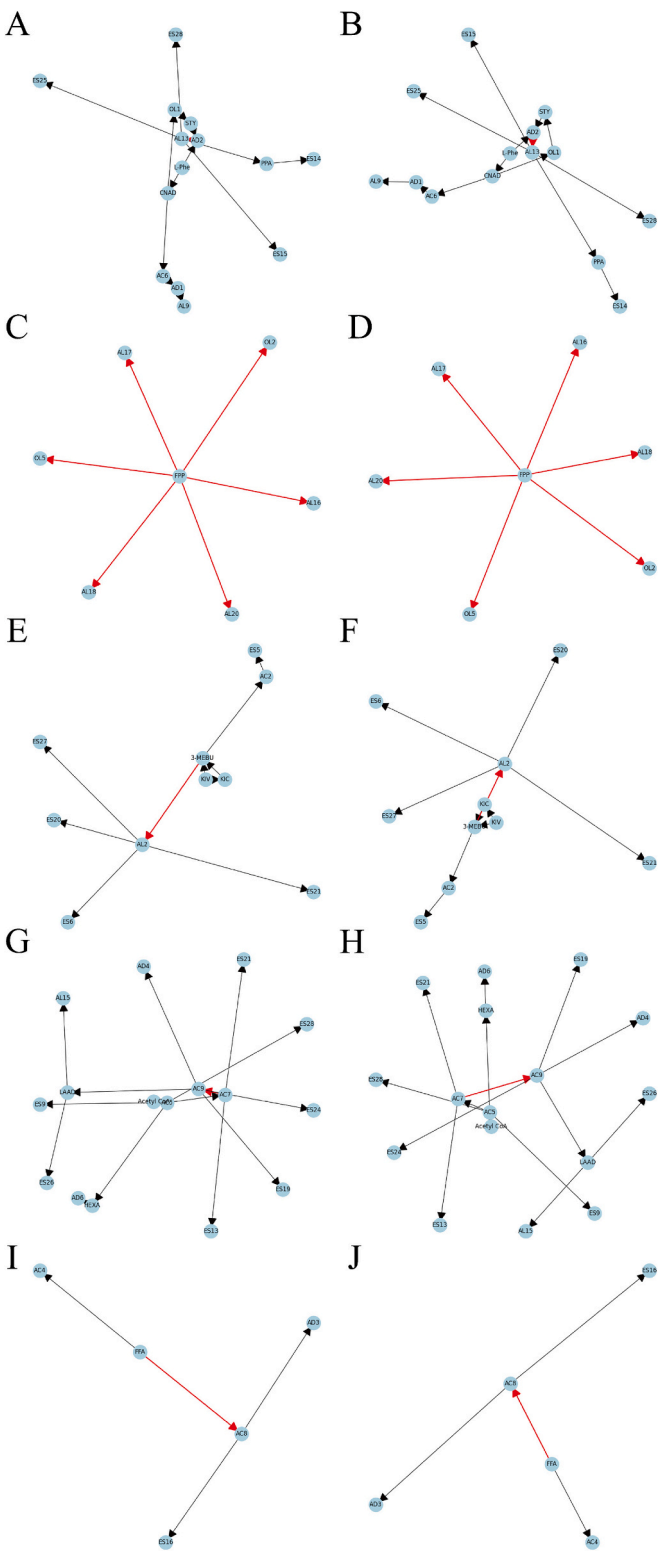

Fig. 6. (A - B). Reaction rate diagram of phenylethanol production in NM and TM; (C - D). Reaction rate diagram of terpenoid in NM and TM; (E - F). Reaction rate diagram of isoamyl alcohol in NM and TM; (G - H). Reaction rate diagram of even-numbered fatty acids in NM and TM; (I - J). Reaction rate diagram of other acids in NM and TM. Red arrows represent the rate-limiting steps, with the length of the segments indicating the difficulty of the reaction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

dehydrogenase. More isoamyl alcohol (AL2) was detected in TM and, thus, more isovaleric acid (AC2) was detected in NM. Isovaleric acid (AC2) was a fruity flavor compound leading to a better flavor for NM. Isoamyl acetate (ES6) and 3-methylbutyl octanoate (ES21) were two main products of isoamyl alcohol (AL2) catalyzed by acyltransferase. The former as a fruity flavor compound was detected with a higher generation speed in NM, the later as an herbal flavor compound was more accumulated in TM, which fitted with our sensory analysis (Fig. 1A).

Fig. 5E-F and Fig. 6G-J showed the metabolic pathways of acids, esters, aldehydes, and alcohols. Acids were produced through the cleavage and metabolism pathways (Ferreira & Mendes-Faia, 2020; Yoshida & Yokoyama, 2012). Alcohols were formed catalyzed by acid dehydrogenase and alcohol dehydrogenase. Esters were synthesized by acyltransferase. Aldehyde compounds were obtained through a series of redox reactions.

Under the cleavage pathway, some medium- and long-chain fatty acids were released to produce dl-3-methylvaleric acid (AC4) and nonanoic acid (AC8). The latter was the rate-determining step. Both of them could proceed faster in TM. Nonanoic acid (AC8) could be respectively catalyzed by fatty acid desaturase and acyltransferase to generate nonanal (AD3) and ethyl nonanoate (ES16). However, the consumption speeds of both reactions were slower in TM. Thus, the more serious accumulations of dl-3-methylvaleric acid (AC4) and nonanoic acid (AC8) were found in TM.

During the fermentation, hexanoic acid (AC5) was synthesized from acetyl-CoA, which was further converted to octanoic acid (AC7) and then to decanoic acid (AC9). The conversion from octanoic acid (AC7) to decanoic acid (AC9) was a rate-limiting step and occurred faster in NM. This explained why more decanoic acid (AC9) accumulated in NM, while more octanoic acid (AC7) accumulated in TM. Hexanoic acid (AC5) could be converted into octanoic acid (AC7), hexanal (HEXA), ethyl caproate (ES9) and phenylethyl caproate (ES28). Although there was no difference in ethyl caproate (ES9) in NM and TM, other steps were faster in TM. Thus, the accumulation of ethyl caproate (ES9) in TM was lower. Decanoic acid (AC9) could be further converted into lauric acid (LAAD), which was further converted to ethyl laurate (ES26) and 1-dodecanol (AL15). The generation speed of lauric acid (LAAD) was obviously higher in TM leading to the accumulation of ethyl laurate (ES26) in TM.

Aldehydes were synthesized involving acids. In NM, hexanal (HEXA) was readily catalyzed by fatty acid desaturase to produce 2-butyl-2-hexenal (AD6), imparting a fruity flavor. Decanal (AD4) as an herbal flavor compound, was synthesized by fatty acid desaturase from decanoic acid (AC9). Calculations indicated that this process was more efficient in TM, explaining why decanal (AD4) is the dominant herbal flavor compound in TM.

3.8. Aroma recombination and omission experiments

Aroma recombination experiments were conducted using quantitative data from NM and TM. For NM, 17 flavor compounds with odor activity values (OAVs) ≥ 1 were selected, while 15 were chosen for TM. These compounds were incorporated into simulation systems at concentrations identical to those in the original samples. Fig. 7A and B illustrate the sensory analysis results for the recombination models of NM (RNM) and TM (RTM), respectively. The results demonstrated a close match between the aroma profiles of the recombination models and the original NM and TM samples.

A total of 38 omission models were developed and evaluated to determine the contributions of aromatic compounds with high odor activity values (OAV ≥ 1) to the overall aroma profiles of the meads (Fig. 7C-D; Table S7). When all alcohols, aldehydes, and esters were omitted, all 13 panelists accurately identified the omission models, showing significant differences ($p < 0.01$). For NM, the omission of isoamyl alcohol, phenylethyl alcohol, trans-farnesol, cedrene, ethyl

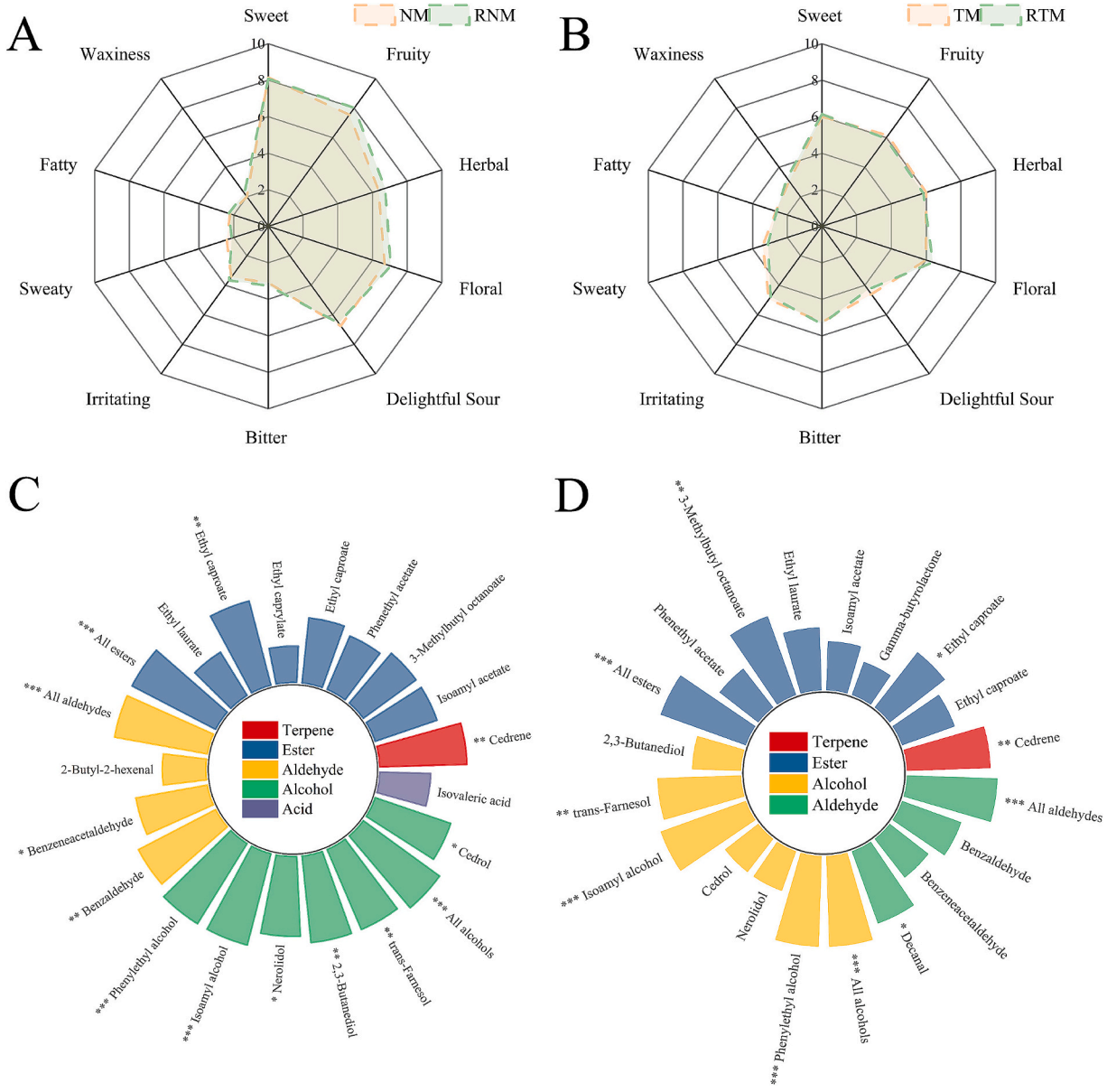

Fig. 7. (A - B). Comparison of the aroma characteristics of the RNM and RTM aroma model (18 compounds, OAV ≥ 1;17 compounds, OAV ≥ 1) with the original NM and TM aroma profile. Note: RNM and RTM represents the recombination model. (C—D). Omission tests for NM and TM recombination models. The levels of statistical significance and the corresponding correct answer counts are as follows: For very highly significant results ($p < 0.001$), the number of correct answers (k) needs to be greater than or equal to 12 ($k \geq 12$); for highly significant results ($p < 0.01$), k needs to be greater than or equal to 10 ($k \geq 10$); and for significant results ($p < 0.05$), k needs to be greater than or equal to 9 ($k \geq 9$).

caproate, benzaldehyde, and 2,3-butanediol was correctly identified by more than 10 panelists, emphasizing their critical roles in shaping NM's aroma. Additionally, significant differences ($p < 0.05$) were observed when cedrol, nerolidol, and benzeneacetaldehyde were omitted. For TM, the omission of isoamyl alcohol, phenylethyl alcohol, 3-methylbutyl octanoate, cedrene, and trans-farnesol was correctly identified by more than 10 panelists, highlighting the essential role of these compounds in TM's aroma. Additionally, significant differences ($p < 0.05$) were observed when Decanal and Ethyl caproate were omitted.

A comparison of the omission results for NM and TM revealed both similarities and differences in the key aroma contributors of the two meads. Isoamyl alcohol, phenylethyl alcohol, trans-farnesol, cedrene, and ethyl caproate were identified as critical to the aroma profiles of both meads. However, NM's distinctive aroma was more influenced by benzaldehyde, 2,3-butanediol, and cedrol, whereas TM's profile was shaped more significantly by 3-methylbutyl octanoate and decanal. Furthermore, 3-methylbutyl octanoate, cedrol, and isoamyl acetate emerged as important compounds for distinguishing the flavor profiles of NM and TM. These findings highlight that the unique flavors of NM and TM result from specific combinations of volatile compounds, providing a scientific basis for optimizing and customizing mead flavor profiles.

4. Conclusion

This study developed a novel two-step fermentation process to enhance the flavor quality of mead by optimizing pre-culture conditions. To comprehensively analyze the volatile compounds and flavor profiles of rapeseed mono-floral mead, we employed HS-SPME-GC–MS with three columns. Compared to TM, NM significantly reduced the concentrations of off-flavor compounds, and increased the concentrations of aromatic compounds. Sensory evaluation revealed that NM's flavor

profile was superior to TM's, with bitterness and irritation decreasing by 41.1 % and 42.5 %, respectively, while fruity, sweet, and pleasantly sour flavors increased by 27.4 %, 36.9 %, and 45.5 % ($P < 0.05$). Key aroma compounds, including benzaldehyde, 2,3-butanediol, and cedrol, were identified through recombination and omission experiments. Dynamic fermentation monitoring and machine learning identified key rate-limiting steps in the production of volatile compounds, such as the oxidation of benzeneacetaldehyde to phenylethyl alcohol, isovaleraldehyde to isoamyl alcohol, and the conversion of octanoic acid to decanoic acid. These steps play a crucial role in regulating the generation of volatile compounds. This research provides a scientific basis for improving mead brewing through data-driven fermentation optimization strategies, demonstrating the significant advantages of the novel two-step fermentation process in enhancing the flavor quality of mead.

Ethical Statement

The authors certify that this study was conducted in strict accordance with the ethical principles outlined in the World Medical Association's Declaration of Helsinki for research involving human participants.

In the context of sensory evaluation, national regulations do not require formal ethical approval, and no established ethics committee or formal documentation process is available for such studies.

Despite this, the authors implemented rigorous protocols to safeguard the rights and privacy of all participants. These protocols included ensuring voluntary participation without coercion, providing comprehensive information about the study's aims, procedures, and potential risks, obtaining verbal informed consent from each participant, ensuring that no participant data was disclosed without prior consent, and allowing participants the freedom to withdraw from the study at any point without prejudice.

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CRediT authorship contribution statement

Xian Li: Writing – original draft, Visualization, Software, Conceptualization. **Tiantian Zhang:** Formal analysis, Data curation. **Ziwei Liu:** Methodology, Investigation, Funding acquisition, Conceptualization. **Meng Jiao:** Software, Resources. **Qian Li:** Validation, Supervision. **Martin Gand:** Writing – review & editing, Conceptualization. **Kexin Zhu:** Software, Resources. **Yibing Qiao:** Software. **Wushuang Bai:** Project administration. **Zisheng Guo:** Supervision, Software, Funding acquisition. **Bin Li:** Conceptualization. **Yiran Wang:** Project administration, Methodology. **Jing Dong:** Investigation, Formal analysis. **Binglin Li:** Writing – review & editing, Writing – original draft.

Declaration of competing interest

The authors declare that there are no conflicts of interest regarding the publication of this paper. The research was conducted independently and was not influenced by any external financial support, commercial interests, or affiliations.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2025.102313.

Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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