Flavor properties of post-heated fermented milk revealed by a comprehensive analysis based on volatile and non-volatile metabolites and sensory evaluation

Xiaoxia Li [a,1], Zhi Zhao [a,1], Shaoqi Shi [a,g,1], Dongdong Li [a], Yue Sang [a], Pengjie Wang [a], Liang Zhao [a], Fuqing Wang [b], Bing Fang [a], Shuxing Chen [c], Yixuan Li [a], Zhengqiang Jiang [d], Jie Luo [e], Xiaoxu Zhang [f,**], Ran Wang [a,g,*]

[a] *Department of Nutrition and Health, Key Laboratory of Functional Dairy, Co-constructed by Ministry of Education and Beijing Government, China Agricultural University, Beijing, 100190, China*
[b] *Tibet Tianhong Science and Technology Co., Ltd, Tibet, 850000, China*
[c] *Food Laboratory of Zhongyuan, Luohe, Henan, 462001, China*
[d] *Key Laboratory of Food Bioengineering (China National Light Industry), College of Food Science and Nutritional Engineering, China Agricultural University, Beijing, 100083, China*
[e] *College of Food Science and Technology, Hunan Agricultural University, Changsha, 410114, China*
[f] *Research Center for Probiotics, China Agricultural University, Beijing, 100190, China*
[g] *National Center of Technology Innovation for Dairy, Hohhot, 010100, China*

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ABSTRACT

Existing research on the post-heating processing of fermented milk has primarily focused on single post-heating treatments and the texture, while research on how changes in metabolites during different post-heating treatments affect flavor and sensory properties is limited. This study investigates the changes in volatile metabolites in fermented milk treated at different post-heating temperatures to determine the characteristic aroma types and analyzes the changes in non-volatile metabolites associated with aroma-active compounds or their precursors to clarify the causes of the altered flavor and sensory properties. The results showed that in the 65 °C and 75 °C treatments, 63 volatile compounds were produced by Strecker degradation, lipid oxidation and esterification to produce ketones and aldehydes. Significantly higher odor activity values for 2,3-butanedione, hexanoic acid, and esters and significantly lower odor activity values for 2-heptanone enhanced the frankincense odors and creaminess of the post-heated fermented milk. With temperatures increasing to 95 °C, the increased ketones were primarily 2-heptanone, 2-nonanone, and 2-undecanone that originated from the oxidative decomposition of unsaturated phospholipids at high temperatures. The Maillard reaction of dipeptides produces nitrogenous heterocycles that trigger a caramelized flavor, while organic acids interact with proteins to form complexes that produce astringent flavors. These increase the oxidative off-flavors and reduce the overall palatability. These findings provide a scientific basis for optimizing the post-heating temperature process of fermented milk.

1. Introduction

Over the past few decades, yogurt has become one of the most popular dairy products worldwide. It is widely consumed as a daily health food due to its beneficial effects on regulating intestinal microbiota and modulating mucosal immunity (Gao et al., 2023). Yogurt typically requires at least 10^6 live bacteria per gram to maintain its probiotic benefits. To control acidity, ensure a consistent texture and taste, and keep the probiotics active, yogurt must be transported at low temperatures. The National Food Safety Standard-Fermented Milk (GB

19302-2010) in China mandates strict requirements for the transportation and storage of yogurt at temperatures between 2 °C and 6 °C to ensure food safety. The transportation process requires strict temperature control, which necessitates advanced technical support and monitoring systems. During its short shelf life, improper temperature control can cause yogurt to spoil, resulting in a loss of product quality.

Yogurt faces several challenges, including high transportation costs, stringent technical requirements, and market limitations. Consequently, post-heating yogurt (ambient yogurt) was developed, which is based on the original low-temperature yogurt process, with an added second heat treatment (Gao et al., 2023). The total market size of yogurt in China is expected to exceed RMB 210 billion by 2023, with post-heating yogurt accounting for a significant share and demonstrating strong market potential. Post-heating yogurt is not subject to refrigeration, reducing the dependence on refrigeration equipment and cold chain management. After heat treatment, the shelf life of the post-heated yogurt is significantly extended to 4–6 months, while maintaining stable taste and nutrient content. The post-heating treatment process does not alter the immune function of fermented milk, with post-heated fermented milk (PHT) and low-temperature yogurt exhibiting the same immune properties. PHT sample containing the heat-killed *Bifidobacterium longum* BBMN68, mitigates allergic airway inflammation by altering the structure and function of the gut microbiota to maintain systemic Th1/Th2 immune balance (Niu et al., 2023). This indicates no significant difference between post-heated and low-temperature yogurt regarding immunological parameters and gastrointestinal comfort. These advantages make post-heated yogurt an increasingly popular healthy food choice for modern consumers.

Current research on post-heating fermented milk primarily focuses on the effects of post-heating temperature on the content of flavor substances and texture. Studies have demonstrated that post-heating fermented milk changes textural properties and organic volatile compound content, contributing to the changes in texture and overall flavor of fermented milk. Subjecting fermented milk to heating between 55 °C and 85 °C for 25 s results in notable changes in its textural and rheological properties. Specifically, heating at temperatures below 65 °C enhances the gel strength and hardness due to moderate aggregation of microgels. Conversely, heating above 65 °C deteriorates these textural properties because of excessive aggregation, resulting in undesirable particulate properties (Gao et al., 2023). Combinations of post-heating processes can also induce changes in the odor of volatiles. Overall odor characteristics of pasteurized yogurt samples obtained using a smooth pump were superior to those obtained using a homogenizer during the same fermentation process. Compounds such as hexanal, (E)-2-octenal, 2-heptanone, and butyric acid may be important odor-active components in pasteurized yogurt (Zhao et al., 2023a). However, these studies have focused on the effects of single-temperature treatments on the volatile metabolic or textural profiles. However, a systematic assessment of the effects of different post-heating treatment temperatures on both the volatile and non-volatile metabolic profiles of yogurt is lacking. Therefore, it is important to understand how post-heating treatment temperatures affect changes in yogurt's intrinsic compounds and how these changes lead to alterations in the flavor profile and organoleptic attributes. This understanding is crucial for consumer acceptance and preference.

This study aims to identify and quantify volatiles in PHT samples using headspace solid-phase microextraction gas chromatography-mass spectrometry (HS-SPME-GC-MS) technology. It also seeks to identify aromatic active compounds in fermented milk (with odor activity values (OAVs) > 1) and determine the characteristic aroma types at each post-heating temperature. Additionally, non-volatile metabolites associated with aroma-active compounds, including creamy and frankincense odor precursors, are analyzed using an ultra-high performance liquid chromatography-quadrupole exactive high field X (UHPLC-Q Exactive HF-X) system. This study explores the potential differential flavor markers in PHT that will help optimize the post-heating temperature

process and improve the quality of fermented milk.

2. Materials and methods

2.1. Fermented milk manufacture

Milk (3.2% (*w/w*) protein, 4.0% (*w/w*) fat) (Inner Mongolia Mengniu Dairy (Group) Co., Ltd., Hohhot, China) was used as the substrate and heated to 50 °C in the fermentation tank (SY-PA-04-04, Shanghai Shunyi Technology Co., Ltd., Shanghai, China). 6.0% (*w/w*) granulated sugar was added and stirred with an electronic stirrer (FLUKO Technology, Shanghai, China) at 550 rpm until they were completely blended. Subsequently, the mixture was heated to 60 °C and then pressure-homogenized at 5 MPa/15 MPa (APV-2000, SPX Flow Technology Co., Ltd., Shanghai, China). It was maintained at 85 °C for 20 min and then inoculated with 1×10^7 CFU/g *Streptococcus salivarius* subsp. *thermophilus* (*S. thermophilus*) MN-ZLW-002 (CGMCC No. 3817) at 42 °C. Fermentation was terminated when the pH of the fermented milk reached 4.50 ± 0.05.

2.2. Post-heating temperature treatments

The fermented milk was divided into six portions. One portion underwent non-heated processing (NT), while five portions were heated in batches in the fermentation tank to center temperatures of 55 °C, 65 °C, 75 °C, 85 °C and 95 °C, each held for 25 s. After obtaining the PHT samples, they were stored at 4 °C overnight.

2.3. A non-targeted metabolomics analysis revealed changes in volatile metabolites according to the HS-SPME-GC/MS

The volatile compounds were separated and identified using HS-SPME-GC/MS according to the method described by Wang et al. (2023a). Briefly, 0.5 mL of PHT sample and purified water, 1.5 g of NaCl, and 1.55 ppm of 2-methyl-3-heptanone were dispensed into headspace vials, solid-phase microextraction (SPME) was carried out after equilibrating for 15 min and then left in the headspace at 65 °C for 30 min, and the SPME fibers (50/30 μm divinylbenzene/Carboxen/polydimethylsiloxane. Bellefonte, Supelco®, Bellefonte, PA, USA) adsorb the sample after aging at 250 °C for 15 min to remove adsorbed impurities. Solid-phase microextraction fibers fitted with enriched components were placed in the chromatograph and maintained at a setting of 250 °C for 5 min for the detection of the desired substance components, with an Agilent 7200 Q-TOF (Agilent Technologies, Santa Clara, CA, USA) coupled with a Model 7890 chromatograph for the detection of volatile components and aroma substances, with helium gas Helium was used as the flow medium at a flow rate of 1.1 mL/min, and the injection chamber temperature was fixed at 250 °C. The oven temperature program was as follows: from 40 °C, heating was carried out at a rate of 8 °C per minute up to 120 °C, and then the heating was continued at a rate of 5 °C per minute up to 230 °C and maintained at this temperature for 10 min, with the detection equipment operating in the electron ionization (EI) mode. The specific parameters were as follows: ion source temperature: 230 °C; interface temperature: 200 °C; scanning range: 30–400 m/z. To identify specific compounds, their mass spectra were analyzed by comparison with mass spectral references in the National Standard Compound Spectroscopy Database (NIST)17. The minimum match mass value for the two-way match ratio was 75/100, and the aroma profile of each compound was derived from internal information to perform the identification Good Scents Company Information System (https://www.thegoodscentscompany.com).

2.4. Determination of the non-volatile components using UHPLC-Q Exactive HF-X

The extraction and identification of non-volatile metabolites from

the PHT samples were performed according to the method described by Zhao et al. (2023b). For a brief overview, 50 mg of lyophilized solid samples were accurately weighed and the extraction of metabolites was executed using 400 μL of methanol solution (80%, *v/v*) and 0.02 mg/mL of L-2-chlorophenylalanine as a reference, and the mixtures were allowed to stand at −10 °C and processed for 6 h at 50 Hz using a high throughput tissue pulverizer, the Wonbio-96c (Shanghai Wanbo Biotechnology Co., Ltd, Shanghai, China.) at 50 Hz for 6 min, immediately followed by half an hour of sonication at 40 kHz performed in a cryogenic environment. The sample material was stored - kept at a cold temperature for up to half an hour to solidify the proteins, and then centrifuged at 12,000 rpm for 15 min at 4 °C. The supernatant was carefully transferred into vials for liquid chromatography-mass spectrometry (LC-MS/MS) analysis.

Mass spectrometry data were collected with the aid of a UHPLC-Q Exactive HF-X system (Thermo Fisher Scientific, Waltham, MA, USA). Negative mode is −3500 V, positive mode is 3500 V, capillary temperature is 325 °C, heater temperature is 425 °C, MS resolution is 60,000, and MS/MS resolution is 75,000. mass range is set to 70–1050. For detailed information on mobile phase solvent configurations and gradient variations, please refer to our previous study. (Zhao et al., 2023b). Briefly, the mobile phase A was 0.1% formic acid (*v/v*) in acetonitrile. The mobile phase B was 47.5% acetonitrile (*v/v*), and 47.5% acetonitrile isopropanol (*v/v*) in ultrapure water. The solvent gradient changed according to the following conditions: from 0 to 3.5 min, 0% B to 24.5% B (0.4 mL/min); from 3.5 to 5 min, 24.5% B to 65% B (0.4 mL/min); from 5 to 5.5 min, 65% B to 100% B (0.4 mL/min); from 5.5 to 7.4 min, 100% B to 100% B (0.4 mL/min to 0.6 mL/min); from 7.4 to 7.6 min, 100% B to 51.5% B (0.6 mL/min); from 7.6 to 7.8 min, 51.5% B to 0% B (0.6 mL/min to 0.5 mL/min); from 7.8 to 9 min, 0% B to 0% B (0.5 mL/min to 0.4 mL/min); from 9 to 10 min, 0% B to 0% B (0.4 mL/min) for equilibrating the systems. The sample injection volume was 2 μL and the flow rate was 0.4 mL/min. The column temperature was maintained at 40 °C. During the analysis period, all the samples were stored at 4 °C.

2.5. Sensory evaluation

A panel of eight judges proficient in sensory evaluation was trained to evaluate the PHT samples using a quantitative descriptive analysis conducted using the generalized labeled magnitude scale (gLM). The gLM scale is divided into 10 points, with zero representing "no sensation," one representing "barely detectable," two representing "very weak," three representing "weak," four representing "somewhat weak," five representing "moderate", six representing "somewhat strong," seven means "strong," eight means "very strong," and nine means "strongest imaginable sensation of any kind." The evaluated attributes were as follows: frankincense odors, creaminess, sweetness, acidity, refreshing sensation, astringency, viscous (texture), and caramelized flavor. The PHT sample evaluations were performed at the sensory lab facility of the dairy product sensory tasting room in the Ambient Temperature R&D Center (Inner Mongolia, China) in individual booths under white light. The PHT samples (60 mL) were presented in plastic cups identified by a three-digit code. Between the sensory assessment of the samples, mouthwash, and mineral water were provided to rinse the mouths of the subjects to avoid possible cross-effects between the samples. Fifty consumers (29 women and 21 men, with a mean age of 42 ± 5 years) were randomly recruited, and they evaluated the PHT samples using a consumer preference analysis using the labeled affective magnitude (LAM) scale. The LAM scale involves subjects making unimodal estimates of phrases expressing different affective states on a nine-level harmonic scale. The study was approved by the China Agricultural University Ethics Committee (CAUHR-20220502) and written informed permission was acquired by each subject.

2.6. Multivariate statistical analysis

For detailed information on data screening, data normalization, and data analysis performed on the HS-SPME-GC/MS and LC-MS datasets, please refer to our previous studies (Zhao et al., 2023b). The HS-SPME-GC/MS datasets were processed for peak pick and deconvolution with the Unknowns Analysis tool of the MassHunter Quantitative Analysis software package (B.10.1, Agilent Technologies). The mass spectra of volatile substances were matched to the reference mass spectra in the NIST17 library, and those exhibiting a matching factor above 75 were chosen for further data analysis. The results are presented as microgram equivalents of 4-methyl-2-pentanol per 100 mL of sample. The pretreatment of LC/MS raw data was performed by Progenesis QI software, generating a three-dimensional data matrix in CSV format. The data matrix derived from the database search was uploaded to the Majorbio cloud platform (https://cloud.majorbio.com) for further data analysis. Firstly, the data matrix underwent pre-processing, following these steps: At least 80% of the metabolic features detected in any set of samples were retained. After filtering, for specific samples exhibiting metabolite levels below the lower limit of quantification, the minimum metabolite value was estimated, and each metabolic signature was normalized to the sum. To mitigate errors arising from sample preparation and instrument instability, the response intensities of the sample mass spectrometry peaks were normalized using the sum normalization method, yielding the normalized data matrix. Meanwhile, variables from quality control (QC) samples exhibiting a relative standard deviation (RSD) greater than 30% were excluded and log10-transformed to achieve the final data matrix for subsequent analysis.

During the data processing, the data were selected and prepared for processing using MetaboAnalyst's inbuilt statistical analyses involving the mean calculation and Pareto sizing of the dataset, and Partial Least Squares Discriminant Analyses (PLS-DA) were applied to present the data in MetaboAnalyst (https://www.metaboanalyst.ca) to demonstrate the discriminatory nature and differences in the control metabolome of the experimental data, and the parameters R^2Y and Q^2, which represent explanatory power and predictive power, respectively, were used to measure the accuracy and predictability of the PLS-DA model. Replacement tests were used to assess computational accuracy and overfitting, and one-tailed Student's t-tests ($p < 0.05$) were used to determine significant differences between groups and projected variable importance (VIP) scores, which were used as an indicator criterion for the importance of the variables presented in the PLS-DA model. Nonvolatile substances in the LC-MS data were identified based on retention time, m/z, and fragmentation information with the help of Progenesis QI software, an analytical tool that has been pre-programmed to automatically find, for example, selected databases such as Human Metabolome Data Bank (HMDB) (https://www.hmdb.ca), ChemSpider (which is available at http://www. chemspider.com) and the METLIN database (accessed at https://metlin.scripps.edu/) repositories. The path function component exhibits path parsing HTTP protocol text transfer protocol communication://www.metaboanalyst.ca/MetaboAnalyst/upload/PathUploadView.xhtml), with the mass accuracy set to 10.0 ppm and the p-value threshold set to 0.05 applied to the mummichog was performed when GraphPad Prism 9 was used to create graphs and fragmentation information was used to present the data difference significance ($p < 0.05$), all data were taken as mean ± standard deviation presentation.

3. Results and discussion

3.1. GC-MS analysis

Six fermented milk samples underwent analysis using HS-SPME-GC/MS, revealing a rich array of 63 volatile metabolites. These metabolites were systematically sorted into eight categories composed of 15 organic acids, 14 aromatic compounds, eight ketones, seven alcohols, six

aldehydes, five esters, three furans, and five others (Table S1). Some research has indicated that the post-heating method increases volatiles in fermented milk. Wen et al. identified 60 volatile compounds in goat's milk treated at 62.5 °C for 30 min, with olefins being the most abundant, followed by esters and ketones. In contrast, non-heat-treated goat milk exhibited the lowest concentration of flavor compounds (Wen et al., 2023). The post-heating temperature might be the primary cause of the changes in flavor (Geng et al., 2024). As the temperature rises, substances such as proteins and fats form richer flavor substances, such as ketones, aldehydes, and esters, through the Maillard, Strecker degradation, and lipid oxidation reactions (Chen et al., 2021; Yu et al., 2020).

A principle component analysis (PCA) was conducted on the relative content of 63 annotated volatiles to provide insight into the similarities within the volatile profiles at various post-heating temperatures. Principal components 1 (PC1) and 2 (PC2) encapsulated 45.3% and 17.4% of the total variance, respectively, collectively embodying the bulk of information on volatile organic compounds (VOCs) (Fig. 1A). In terms of PC1, three PHT samples (75 °C, 85 °C, and 95°C-PHT) exhibited significantly higher levels than the NT samples ($p < 0.01$, Fig. 1A). Regarding PC2, distinct differences were observed between all of the PHT and non-heated (NT) samples ($p < 0.05$, Fig. 1A). A total of 63 volatile metabolites were naturally separated into three discernible clusters in the PHT samples. Cluster A, which included the NT and 55°C-PHT samples, was characterized predominantly by organic acids. Cluster B, encompassing the 65 °C and 75°C-PHT samples, featured a predominance of aldehydes, esters, and aromatic compounds. Cluster C consisted of the 85 °C and 95°C-PHT samples and was marked by a dominance of ketones (Fig. 1B).

3.2. Analysis of volatile compounds

Across all of the PHT samples, acids, alcohols, and ketones were the most abundant volatile components. The proportion of volatile constituents (acids, alcohols, ketones, esters, aldehydes, furans, and aromatic compounds) in the 55°C-PHT samples was considerably decreased compared with that of volatile constituents in the NT samples. Conversely, there was a consistent increase in the levels of these volatile substances in direct correlation with the rise in 55–95 °C post-heating temperatures (Fig. 2A). During milk fermentation, volatile compounds in fermented milk are dominated by acids, ketones, and alcohols, such as lactic acid, hexanoic acid, 3-hydroxy-2-butanone, 2,3-pentanedione, and δ-decanolactone (Han et al., 2024). The formation of acids primarily results from microbial transformations, and this process involves the action of starter cultures such as *S. thermophilus* and *Lactobacillus lactis* (Xia et al., 2023). These microorganisms metabolize lactose, leading to the production of carboxylic acids by pyruvate metabolism and the tricarboxylic acid cycle. Although enzymatic or chemical transformations involving lactose, lipids, citric acid, and proteins may occur, changes in the acid, alcohol, and ketone content of the PHT samples are primarily attributable to the effects of temperature during heating. Tong et al. revealed that the concentrations of major flavor compounds (2-heptanone, 2-nonanone, decanal, and hexanoic acid) were significantly increased in the skim milk treated at 137−141 °C compared with that treated at 30 − 60 °C, and this enriched the creamy, frankincense odors, sweetness, and caramelized flavors in the skim milk (Tong et al., 2019). Furthermore, acids serve as precursors to several other compounds, including ketones, alcohols, aldehydes, and esters. Alcoholic compounds can also be synthesized from ketones, aldehydes, and/or amino acids, all of which may contribute to the milk flavor. Among the
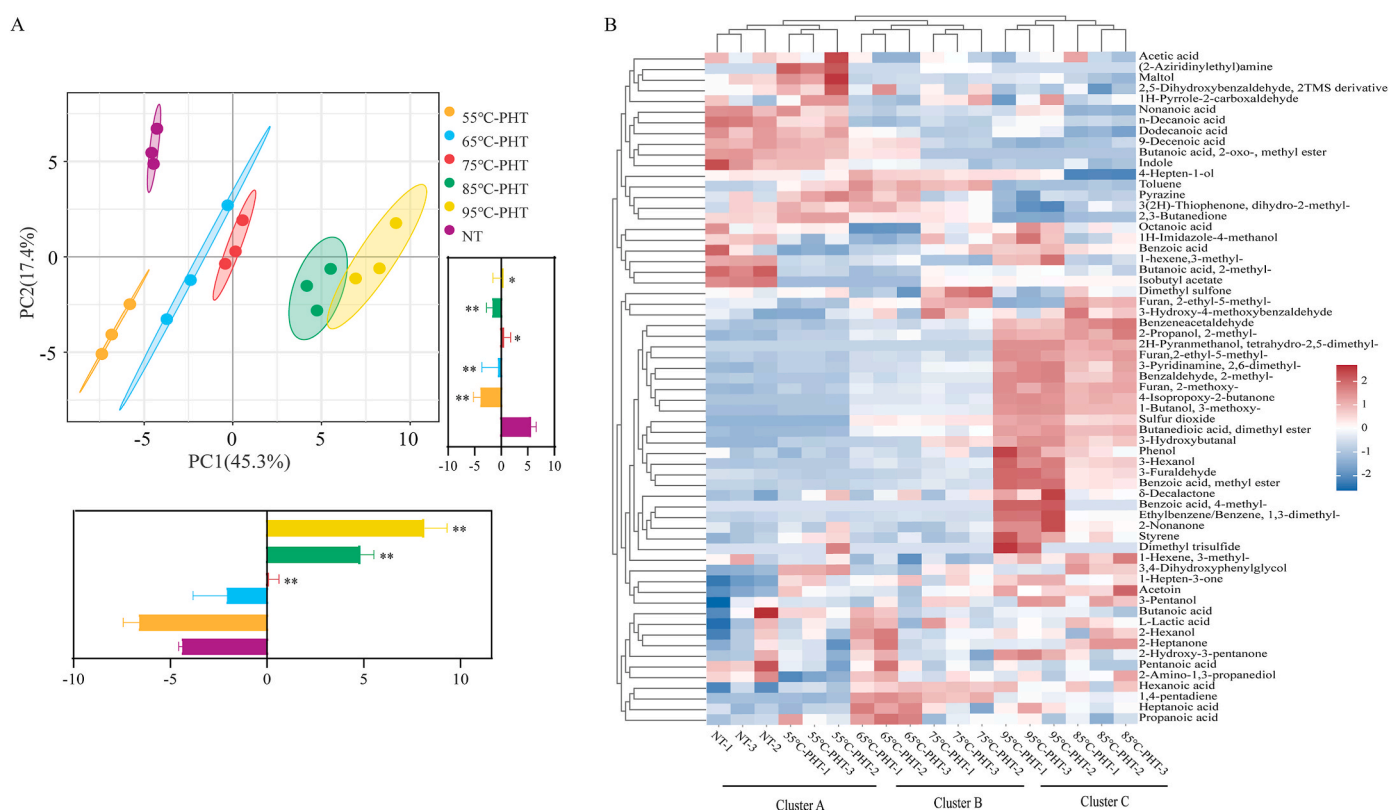

Fig. 1. Volatile metabolomics characteristics of the post-heated fermented milk (PHT) samples. (A) The principal component analysis (PCA) score plot for the PHT samples was derived from the HS-SPME-GC/MS data. The asterisks indicate significant differences in PC1/PC2 among the PHT samples as determined via ANOVA and Duncan's test. "**" and "*" indicate highly significant ($p \leq 0.01$) and significant ($p \leq 0.05$) differences, respectively. (B) The heat map visualization and cluster analysis of the volatile metabolite data based on relative concentrations. Each cell within the heat map reflects the normalized volatile metabolite content concerning the color gradient range. A deeper red hue signifies a higher content of the respective compound. PHT = post-heated fermented milk; NT = non-heated processing.
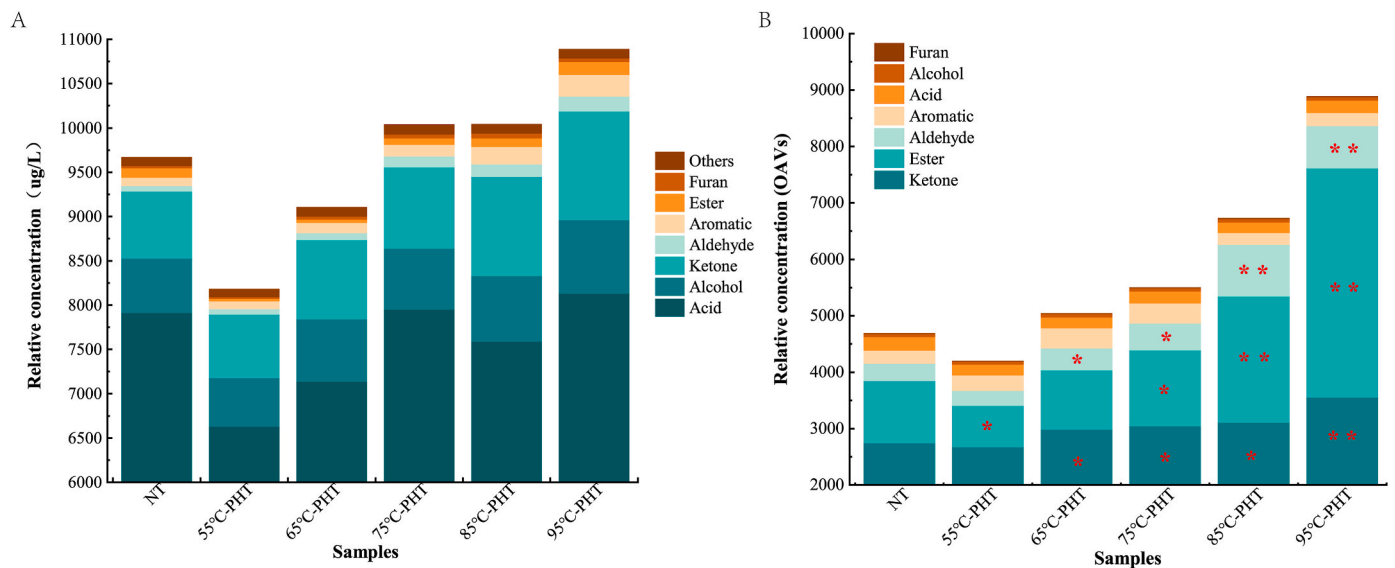
Fig. 2. Volatile compounds in the PHT and NT samples. (A) The relative concentration of volatile compounds. (B) The odor activity values (OAVs) of the volatile compounds. OAVs are a ratio of compound concentrations in the water to the odor threshold, and volatile organic compounds are considered key aromatic components when the OAVs of the flavor compounds are greater than 1.0. The asterisks denote the significance of the ANOVA and Duncan's test comparing the same class of substances after the post-heating treatment. "**" and "*" indicate highly significant ($p \leq 0.01$) and significant differences ($p \leq 0.05$), respectively. PHT = post-heated fermented milk; NT = non-heated processing.

identified volatile compounds, a total of 19 exhibited significant variations, with nine being carboxylic acids. Notably, the concentrations of hexanoic acid, 3-hydroxybutanal, 2H-pyranmethanol, tetrahydro-2,5-dimethyl-, furan, 2-methoxy-, 1-butanol, and 3-methoxy- escalated in tandem with the rise in pasteurization temperature. Conversely, the levels of n-decanoic acid and dodecanoic acid diminished as the thermal input intensified. The slight alterations in proteins and fats at different temperatures could affect the formation of volatile compounds, leading to significant differences in flavor. The impact of different pasteurization temperatures on yogurt flavor likely predominantly affects the unique volatile compounds.

3.3. Analysis of the characteristic OAVs

The effect of volatiles on the overall aroma profile of fermented milk depends not only on the concentration and relative abundance of VOCs but also on their respective odor thresholds (Ni et al., 2020). Consequently, the OAVs were investigated to delve deeper into the pivotal VOCs of the PHT samples. Referring to the odor thresholds delineated in the prior research (Wang et al., 2022; Yu et al., 2024), the OAVs of the 63 flavor compounds in the PHT samples were classified. Flavor compounds, such as ketones, esters, and aldehydes, comprised 80% of the total OAVs (Fig. 2B), and these encompassed characteristic fermented milk flavors that included acetaldehyde, diacetyl, ethyl butanoate, acetone, and 2-butanone that are frequently associated with yogurt (Table S2). The OAVs of esters, and aldehydes in the three PHT samples (65 °C, 75 °C, 85 °C, and 95°C-PHT) exhibited a significant increase compared with the NT samples (increases of 5.10–107.22%, and 56.11 − 147.19%, respectively). Conversely, the 55°C-PHT samples demonstrated a significant decrease in the OAVs of esters (−12.27%) ($p < 0.05$, Fig. 2B). The post-heating temperature (65 °C − 95 °C) was directly proportional to the OAVs of ketones, aldehydes, and esters. Ketones, aldehydes, and esters are very important differential odor compounds that can be used as odor markers for evaluating the overall flavor profile of PHT (Zhao et al., 2023a). Zhao et al. assessed the freshness of 80 pasteurized yogurts using off-flavor substances produced by lipid oxidation and revealed that ketones and aldehydes (2-heptanone, hexanal, and (E)-2-heptenal) could be used as potential indicators to determine the odor quality of heat-treated fermented milk (Zhao et al.,

2022).

Twenty-two important flavor-contributing substances (OAVs >1.0) were screened, contributing significantly to a comprehensive understanding of the PHT samples. These substances included acids (seven), aromas (four), ketones (three), esters (three), aldehydes (two), alcohols (two), and furans (one) (Table S3). Odor descriptions of these compounds were obtained from the Good Scents Company (https://thegoodscentscompany.com). Differential flavor substances were also analyzed in conjunction with the PHT samples separately from the NT samples (variable importance in the projection (VIP) > 1, $p < 0.05$, and fold change (FC) > 2 or FC < 0.5). Among the principal aromatic constituents, esters, aldehydes, and ketones registered the highest OAVs, with ranges of 73–82%, 7 − 14%, and 4 − 6%, respectively (Fig. 2B). The higher the post-heating temperature, the more pronounced the effect on the flavor of the fermented milk. The OAVs of methyl benzoate (from 861.45 to 3950.01), δ-decanolactone (from 2428.83 to 3260.80), ethylbenzene (from 7.89 to 38.13), and benzene acetaldehyde (from 301.06 to 737.53) exhibited a notable increase with rising post-heating temperatures ($p < 0.05$, Table S3). Conversely, the OAVs of indole (from 59.03 to 44.39), 2-methyl butanoic acid ethyl ester (from 18.75 to 9.49), and n-decanoic acid (from 6.41 to 2.35) decreased significantly as the post-heating temperature increased ($p < 0.05$, Table S3). Esters are formed by esterification of free fatty acids (FFA) with alcohols (Alewijn et al., 2005). The levels of 4-acetylbutyrate, Geranyl acetoacetate, 3-phenylpropyl acetate, and Glyceryl lactooleate were significantly higher in pasteurized fermented milks at 65 and 75 °C. Concentrations of these esters were found to be approximately 2–10 times higher in the 65 and 75 °C-PHT samples compared to the NT samples. Ethyl acetate and lactate butyrate were present in all fermented milk products because lactate and acetic acid are the predominant free fatty acids in fermented milk. Notably, δ-decalactone and methyl benzoate were the most significant odor-active compounds in PHT yogurt samples. The low odor thresholds values for δ-decalactone (threshold value = 0.037 mg/kg) and methyl benzoate (threshold value = 0.028 mg/kg)-mean that even slight variations can greatly affect odor intensity. The presence of δ-decanolactone in yogurt is closely linked to that of short-chain and medium-chain fatty acids, as δ-decalactone is produced through the lactonization of hydroxy fatty acid molecules. δ-decanolactone had a creamy or coconut flavor. (Qiu et al., 2023). The formation of lactones in

fermented milk most likely results from a non-enzymatic one-step transesterification reaction (Alewijn et al., 2007).

In NT samples, carboxylic acid compounds such as lactic acid, acetic acid, butyric acid, 2-methylbutanoic acid, pentanoic acid, nonanoic acid, and n-decanoic acid showed the highest OAVs. These compounds were used as volatile flavor-specific compounds, which significantly enhance the aroma and acidity of fermented milk (Table S3). The post-heating temperatures of 55 °C resulted in an increase in the OAVs of (2-aziridinylethyl) amine, while the OAVs of isobutyl acetate decreased (Fig. 3A, Table S3). Both (2-aziridinylethyl) amine and maltol were identified as volatile flavor-specific compounds that contribute to sweetness and fruity flavors in PHT samples (Wu et al., 2014). The post-heating temperatures of 65 °C and 75 °C markedly increased the OAVs of 2,3-butanedione (from 27.51 to 57.77), hexanoic acid (from 39.73 to 52.71), and methyl benzoate (from 861.45 to 1235.95) in fermented milk and also significantly decreased the OAVs of 2-heptanone (from 262.51 to 171.26) (Fig. 3B and C, Table S3). The 2,3-butanedione is the most important volatile compound in yogurt, which gives a buttery and creamy flavor. Esters are an important part of food aromas and generally bring floral and fruity notes to fresh dairy products, thus reducing the irritating odors caused by fatty acids. (Zhu et al., 2016). Hexanoic acid makes yogurt and other fermented dairy products more aromatic and palatable by imparting a floral and pungent flavor (Wang et al., 2019). However, with increasing post-heating temperatures (from 85 to 95 °C), the increased ketones were primarily 2-heptanone, 2-nonanone, and 2-undecanone, which produce oxidative off-flavors during fermented milk storage (Fig. 3D and E, Table S3). The emergence of benzoic acid, 4-methyl-(an acidic compound), and 2H-pyran-methanol, tetrahydro-2,5-dimethyl- (an aromatic compound) was observed at post-heating temperatures of 85 °C and 95 °C. These changes affect the overall flavor of fermented milk.

3.4. Non-targeted analysis of the non-volatile metabolites

The non-volatile metabolic profiles were determined using UHPLC-Q Exactive HF-X in conjunction with ESI (±) scanning modes. A comprehensive assessment of the non-volatile metabolic profiles spanning eight categories and totaling 2820 and 3238 compounds was identified in the six fermented milk samples. Among these, 452 and 619 substances were successfully matched with the library of authentic chemical standards. We focused solely on analyzing these identified metabolites, amounting to a total of 869. The eight categories were composed of esters (41), aldehydes (49), ketones (61), amino acids, peptides, and derivatives (73), alcohols (107), sugars and derivatives (108), lipids and lipid-like molecules (129), acids and derivatives (251), and other metabolites (50) (Fig. 4A). The prevalence of alcohols, sugars and derivatives, lipids and lipid-like molecules, and acids and derivatives was observed among the non-volatile metabolites in the PHT samples. The identification of non-volatile metabolites relies heavily on the sample extraction process and the instrumental analysis. Various factors, including substance separation, sensitivity, and the detection threshold, collectively determine the number of detectable non-volatile substances. Peng et al. employed the UPLC-Q-TOF-MS method using acetonitrile containing 0.1% (v/v) formic acid as the mobile phase and an elution time of 22 min, resulting in the detection of 222 substances (Peng et al., 2021). In this study, optimization of the mobile phase (where phase A consisted of 0.1% formic acid (v/v) in acetonitrile, and phase B consisted of 47.5% acetonitrile (v/v) and 47.5% acetonitrile isopropanol (v/v) in ultrapure water) and an elution time to 10 min, among other parameters, facilitated the use of the UHPLC-Q Exactive HF-X method. This resulted in the detection of 869 substances. This method could separate and identify a wide range of metabolites with enhanced sensitivity and specificity, particularly at low concentrations.

An investigation of the non-volatile metabolic profile of fermented milk showed that the PCA plot unveiled a significant disparity between the PHT and NT samples. The combined contribution of the first two

principal components comprised 71.4% of the total variance, and this is visually depicted in a two-dimensional scatter plot (Fig. 4B). Notably, all samples exhibited segregation along the PC1 and PC2 axes, except for the 55°C-PHT samples that displayed some overlap with the NT samples along the PC2 axis. Among the various post-heating temperatures assessed, those ranging from 65 °C to 95 °C notably influenced the non-volatile metabolic profile. The non-volatile metabolomic profiles of the PHT and NT samples were depicted using a heat map plot complemented by a hierarchical clustering analysis. The non-volatile constituents were distinctly segregated into three clusters. Cluster A exhibited a higher relative abundance of non-volatile components in the NT samples, as well as in the 55 °C and 75°C-PHT samples. Cluster B showcased an increased relative abundance of non-volatile constituents in the 75 °C and 85°C-PHT samples. Finally, cluster C illustrated a decline in the relative abundance of non-volatile components solely in the 75°C-PHT samples, while the abundance in the other samples remained relatively consistent (Fig. 4C). These results highlight the significant impact of temperature on yogurt's non-volatile metabolites, emphasizing the crucial importance of temperature control during the heat treatment process.

3.5. Characterization of the potential differential markers

Based on the VIP from the orthogonal partial least squares discriminant analysis (OPLS-DA) model, further screening was performed using a *t*-test and fold change (FC) analysis with criteria of $p < 0.05$ and FC > 2 or FC < 0.5. This process led to the identification of 136 metabolites as potential differential markers for distinguishing the PHT and NT samples (Table S4). These differential compounds were crucial for the characteristics of PHT samples and primarily fall into categories such as lipids and lipid-like molecules, organic acids and derivatives, organic oxygen compounds, organoheterocyclic compounds, benzenoids, nucleosides and nucleotides, phenylpropanoids and polyketide metabolites. Specifically, 111 differential markers were identified by comparing the 75°C-PHT and NT samples, with 46 markers significantly increased and 65 significantly decreased. Additionally, 98 markers were observed for the 85°C-PHT samples, 92 for the 95°C-PHT samples, and 64 for the 65°C-PHT samples. The lowest number of differential markers, totaling 26, was observed in the 55°C-PHT samples (Fig. 5A). By combining the results of univariate and multivariate analyses, we obtained a smaller set of variables that effectively indicated the true differences between the two groups. The results indicated that the 75 °C post-heating treatment had the most substantial effect on the non-volatile metabolites of fermented milk.

An analysis of the differential marker species showed that only a limited number of species, primarily acids, and their derivatives, as well as lipids and lipid-like molecules, changed their relative content in the 55°C-PHT samples, similar to the non-volatile metabolites in the NT samples (Fig. 5B). In contrast, the relative content of acids and their derivatives, lipids and lipid-like molecules, sugars and their derivatives, and alcohols exhibited a significant decrease as the temperature increased from 65 °C to 95 °C (Fig. 5C–F). This reduction in the four substance types contributed to an enhancement of the flavor compounds. Furthermore, the relative contents of amino acids, peptides, and their derivatives, ketones, aldehydes, and esters showed significant increases in the 65 °C and 75°C-PHT samples (Fig. 5C and D).

3.6. Sensory analysis of the fermented milk samples

The sensory attributes, such as aroma and taste, of fermented milk underwent dynamic changes during the post-heating treatment process. In the PHT samples, the aroma was dominated by creamy and frankincense odors that contributed to a pleasant sensation and influenced the characteristic flavor of yogurt (Fig. 6A, Table S5). The intensities of the creamy and frankincense odors increased as the temperature treatment rose from 55 °C to 75 °C, reaching their highest ratings of 3.61 and 3.60,
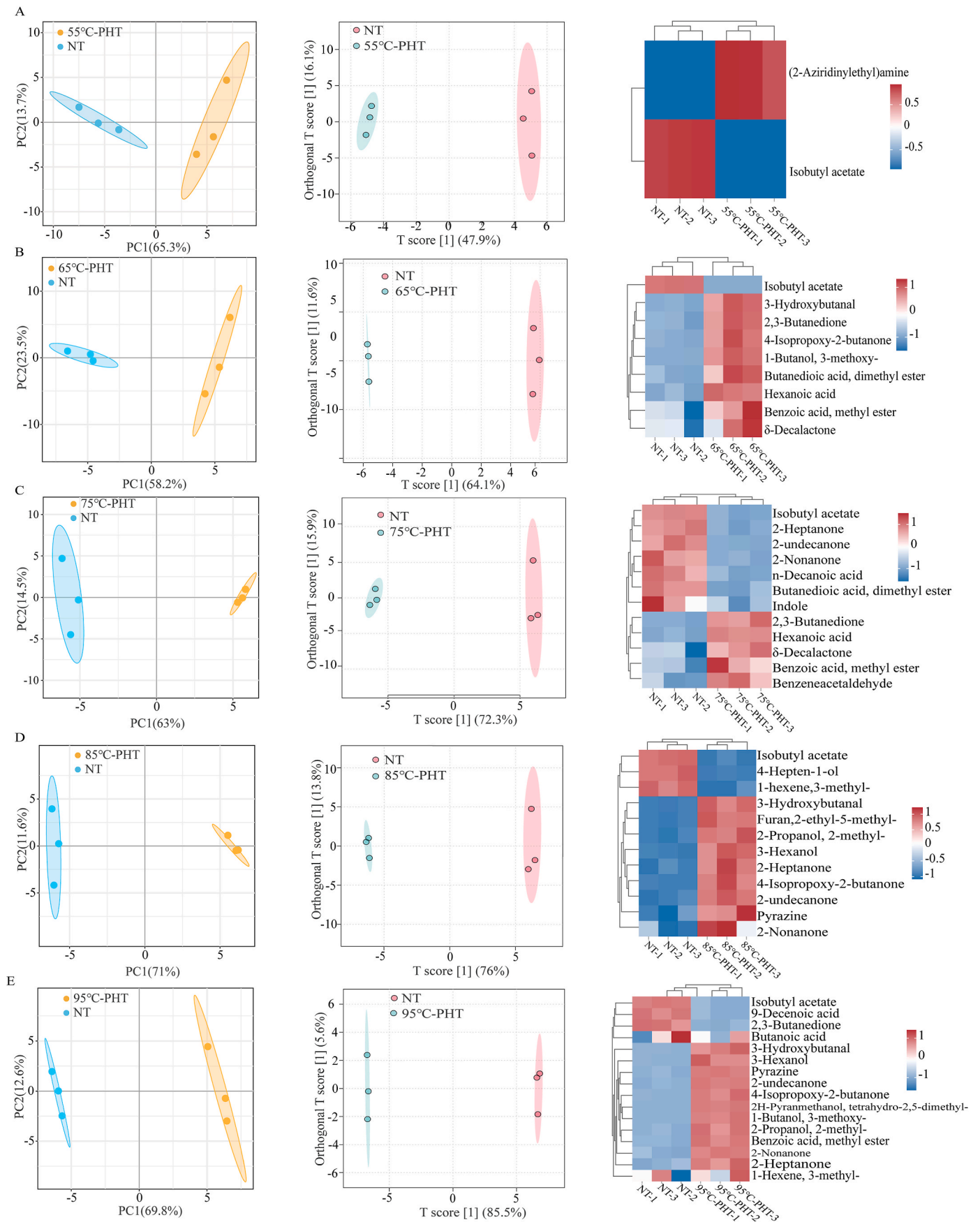
(caption on next page)

Fig. 3. Multivariate statistical analysis of the characteristic volatile compounds: (A) 55°C-PHT vs. NT samples, (B) 65°C-PHT vs. NT samples, (C) 75°C-PHT vs. NT samples, (D) 85°C-PHT vs. NT samples, and (E) 95°C-PHT vs. NT samples. A principal component analysis (PCA) was used to discern discernible trends in differentiation between the PHT and NT samples. Increased separation among samples signified a more pronounced influence of treatment on the metabolites. The partial least squares discriminant analysis (PLS-DA) was used to identify compounds influenced by varying post-heating temperatures, resulting in distinct metabolomic patterns. The parameters R^2Y and Q^2 were used to validate the model's accuracy and predictability. The results are considered more precise as the values of R^2 and Q^2 approach one. Following 200 permutation tests, the intersection of the Q^2 regression line with the vertical axis occurred at a point below zero, indicating that the model was not overfitted and effective model validation. With $R^2Y > 0.99$ and $Q^2 > 0.96$ for all five models, it was evident that each model closely fit the data. Visualization via the heat map and cluster analysis was conducted on the data of differential metabolites, and we considered the relative metabolite concentrations. Substances were categorized as differential metabolites when meeting the criteria of variable importance in projection (VIP) > 1, $p < 0.05$, and fold change (FC) > 2 or FC < 0.5. PHT = post-heated fermented milk; NT = non-heated processing.
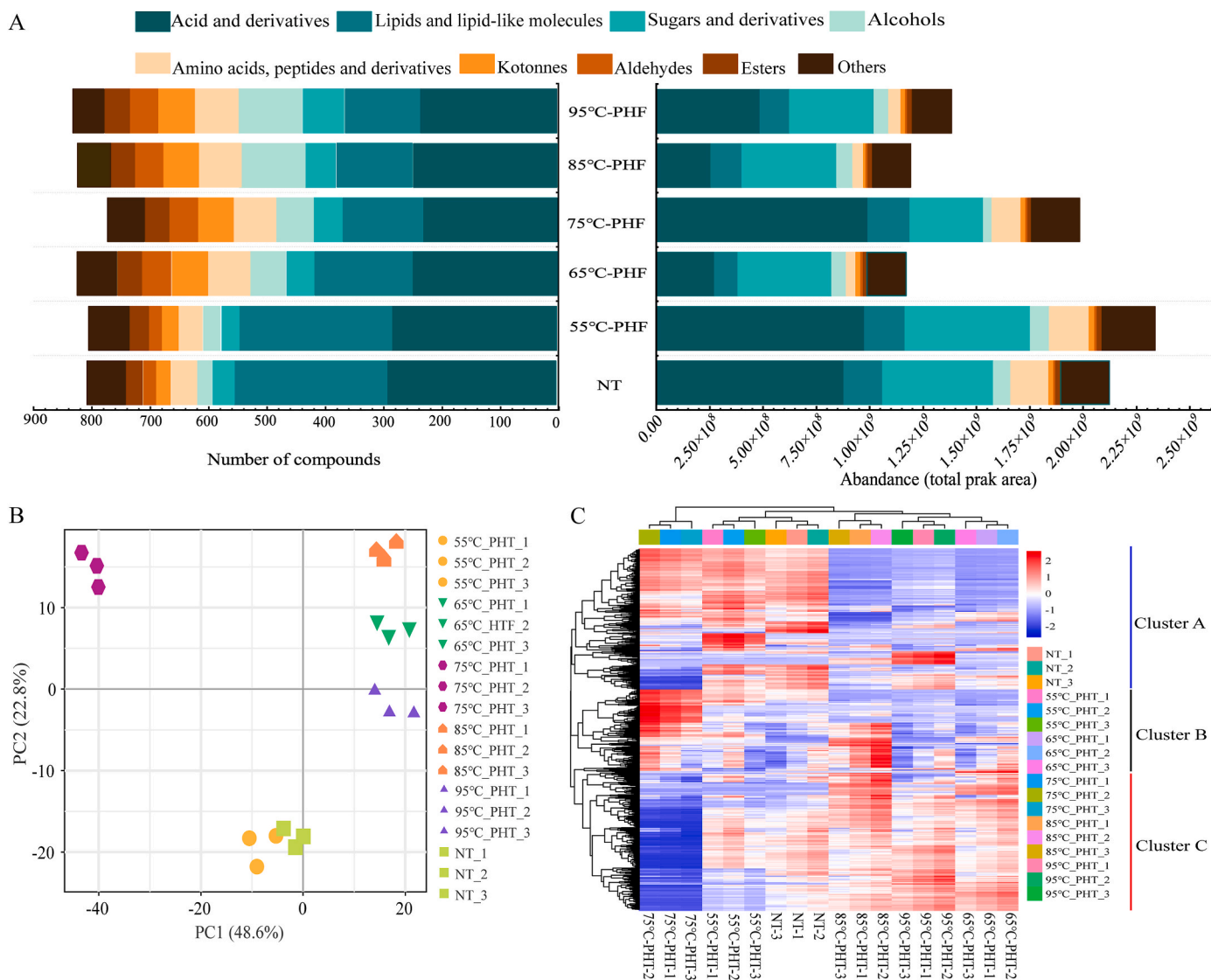

Fig. 4. Non-volatile metabolomics characteristics of the six fermented milk samples. (A) Bar chart illustrating the quantity and concentration of non-volatile classifications in both the PHT and NT samples. The analysis is based on the 869 identified non-volatile compounds successfully matched with a library of authentic chemical standards. (B) The PCA score plot is presented for the fermented milk samples that was generated from the UHPLC-Q Exactive HF-X data. (C) Heat map visualization and cluster analysis of the non-volatile metabolite data based on relative concentrations. PHT = post-heated fermented milk; NT = non-heated processing.

respectively, in the 75°C-PHT samples. These ratings were significantly higher than those observed at other post-heating temperatures ($p < 0.05$) (Fig. 6A–Table S5). However, both of these flavors significantly decreased due to the temperature treatment rose from 75 °C to 95 °C ($p < 0.05$). As the temperature treatment increased to 85 °C and 95 °C, a caramelized flavor emerged in the samples, with scoring values rising correspondingly (Fig. 6A). This caramelized flavor originated from substances produced by the Maillard reaction at high temperatures, such

as nitrogen-containing heterocycles including pyrazine and pyrrole, which are characteristic products of this process (Zhao et al., 2022).

The texture of the PHT samples was dominated by sweetness, acidity, a refreshing sensation, astringency, and viscosity. Sweetness increased as the temperature treatment rose from 55 °C to 65 °C, reaching the highest score of 4.06 in the 65°C-PHT samples, which was significantly higher than those of the other temperatures ($p < 0.05$). However, sweetness significantly decreased to 3.12 as the temperature treatment
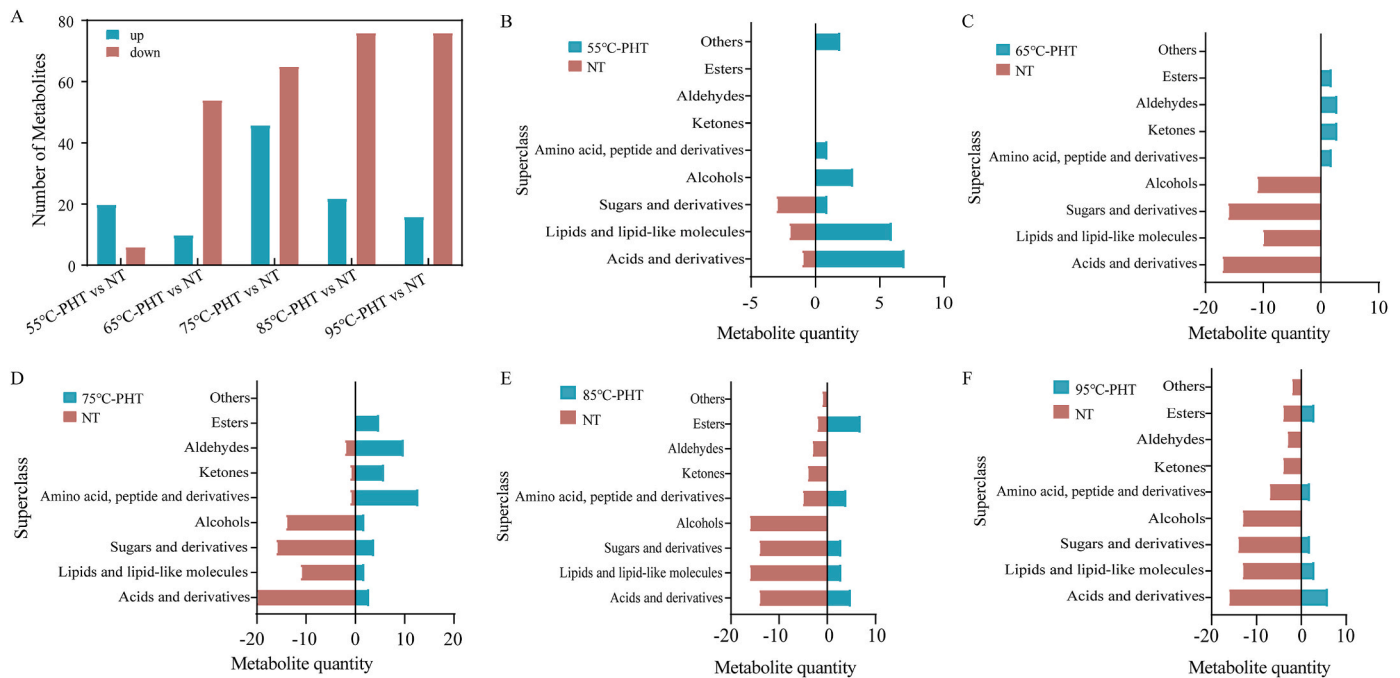
Fig. 5. Potential differential markers for the PHT and NT samples. (A) Comparison of the differential markers. (B) Statistics on the types of differential markers. Species counting of substances in 136 differential markers based on the Human Metabolome Database (HMDB). PHT = post-heated fermented milk; NT = non-heated processing.
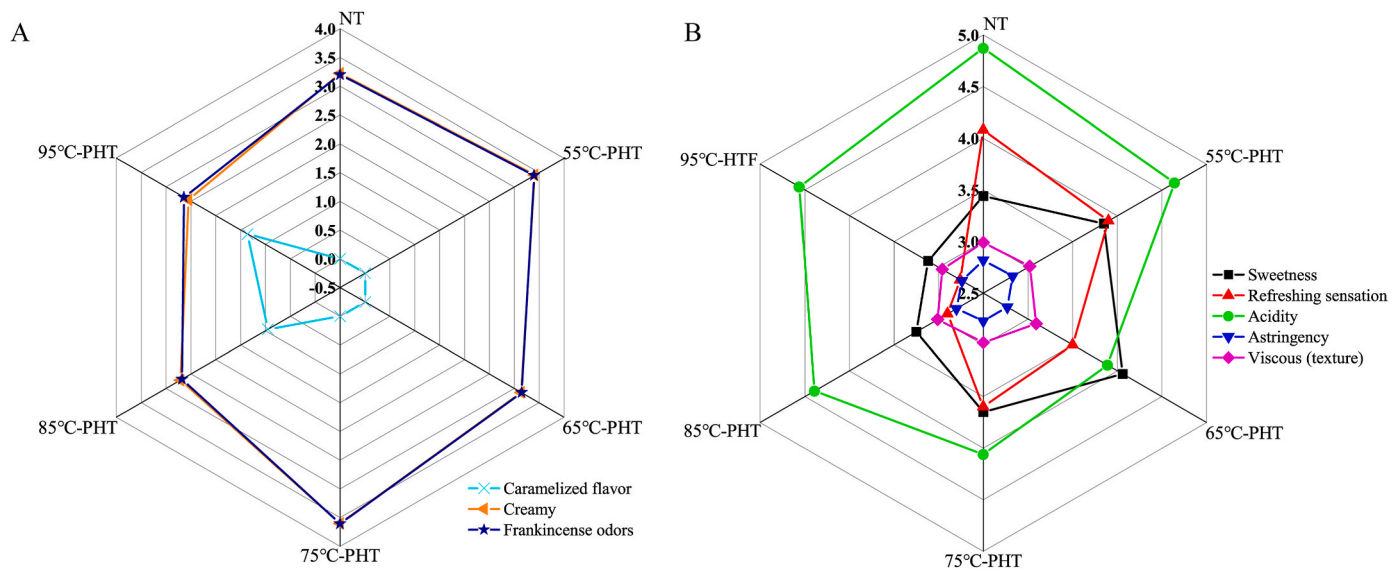

Fig. 6. Sensory analysis of the PHT and NT samples. (A) The aroma sensory attributes. (B) The taste sensory attributes. PHT = post-heated fermented milk; NT = non-heated processing.

rose from 75 °C to 95 °C ($p < 0.05$) (Fig. 6B–Table S5). Both acidity and astringency decreased as the temperature treatment rose from 55 °C to 65 °C, with the lowest ratings of 3.89 and 2.07, respectively, in the 65°C-PHT samples, which were significantly lower than those of the other post-heating processes ($p < 0.05$) (Fig. 6B–Table S5). Then, the ratings of acidity and astringency significantly increased to 4.56 and 3.74, respectively, as temperature treatment rose from 65 °C to 95 °C ($p < 0.05$) (Fig. 6B–Table S5). Despite its significant benefits during fermentation, *S. thermophilus* fermented milk often exhibits undesirable sensory characteristics, particularly in terms of acidity and astringency. Acidity is a crucial sensory attribute of fermented dairy products, and when it surpasses a certain threshold, the resulting taste can become

overwhelmingly sharp, overshadowing other flavor notes and rendering the product's taste profile harsh and unbalanced. Astringency arises primarily from certain metabolic byproducts produced during fermentation, such as specific organic acids and polyphenolic compounds (Wang et al., 2023b). These substances can interact with milk proteins to form complexes that elicit a dry, puckering sensation in the mouth, detracting from the overall palatability and consumer preference. As a result of the aforementioned changes in sweetness, acidity, and astringency, there was a direct effect on the refreshing sensation, which consistently decreased in the 55–95 °C post-heating temperature.

In terms of aroma and taste, the 65 °C and 75°C-PHT samples showed typical creamy and frankincense odors. These two samples effectively

reflected the characteristic flavors of yogurt without being too acidic, which could otherwise mask the yogurt's flavor. The evaluation of flavor and mouthfeel showed a significant positive correlation with the consumer preference scores. The consumer preference scores for the 65 °C and 75°C-PHT samples were 5.94 and 5.92, respectively, indicating a significant preference compared to the other samples. Additionally, the PHT samples exhibited viscoelasticity attributed to moderate aggregation and enhanced microgel linkage after heating (Gao et al., 2023).

3.7. Changes in substances of the 65 °C and 75°C-PHT samples

Proteins can undergo hydrolysis, yielding free amino acids and peptides. These compounds further transform, such as Strecker degradation and the Maillard reaction, resulting in the formation of multiple flavor compounds, including ammonia, amines, aldehydes, phenols, indoles, and alcohols, that contribute to the characteristic flavor of fermented milk. In the 65 °C and 75°C-PHT samples, a total of 22 significantly different amino acids and peptides were found, including four sweet amino acids (O-Succinyl-L-homoserine, L-phenylalanine, L-Proline, and Threonic acid), nine bitter amino acids (leucylleucine, (+)-γ-Hydroxy-L-homoarginine, N-Stearoyl-valine, 4-Chloro-L-phenylalanine, N-Acetyl-L-tyrosine, L-valine, L-Tyrosine, DL-Norleucine, and Pre-tyrosine), and four dipeptides (Fig. 7A). Notably, sweet amino acids, such as l-proline and threonic acid, were significantly increased by 4- to 10-fold following heat treatment at 65 − 75 °C. Conversely, bitter amino acids, such as isoleucine, l-valine, and l-tyrosine, were significantly reduced by 5- to 10-fold after heat treatment at 65 − 75 °C. Through Strecker degradation and the Maillard reaction, isoleucine and l-valine can generate ketones and aldehydes, such as 2-hydroxy-3-pentanone and 3-hydroxy butyraldehyde, consequently enhancing the frankincense odors and creaminess of PHT (Teng et al., 2023). The dipeptides, glycyl-arginine (G-X type) and valylproline (V-X type), exhibited a two-fold decrease in the 75°C-PHT samples and a notable 15-fold decrease when the post-heating temperature was further increased to 95 °C. These dipeptides, the G-X and V-X types, actively engage in the Maillard reaction, leading to the formation of nitrogen-containing heterocyclic compounds, such as pyrazines, that contribute to a caramelized flavor profile (Li et al., 2021).

Carbohydrates are fermented by the starter, yielding primary fermented milk flavor compounds that include lactic acid, citric acid, methanol, acetaldehyde, diacetyl, and ethylene glycol (Yu et al., 2021). During post-heating treatment at 65 − 75 °C, esters, such as isobutyl acetate, methyl benzoate, and dimethyl succinate, are synthesized through the esterification of organic acids (such as lactic, succinic, and acetic acids) and alcohol compounds present in fermented milk. These esters impart fruity and sweet flavors to the fermented milk that are generally favored by consumers. Additionally, citric acid, being the primary organic acid, plays a pivotal role in promoting the production of fermented milk with a refreshing flavor profile (da Costa et al., 2016).

The post-heating temperature has a strong correlation with lipid oxidation, such as phospholipids in fermented milk. At temperatures below 75 °C, lipid oxidation primarily results in the production of beneficial flavor substances, while concurrently reducing the relative content of volatile off-flavor substances. Lipids are broken down through oxidation into aldehydes, alcohols, ketones, esters, and other volatile compounds, which play an essential role in shaping the milk flavor. A total of 25 lipids and lipid-like molecules were found in the 65 °C and 75°C-PHT samples, comprising 11 fatty acids, eight phospholipids, five steroids and steroid derivatives, and one triglyceride (Fig. 7B). Fatty acids in fermented milk form hexanal or unsaturated aldehydes and ketones through lipolysis or oxidation, which is one of the major pathways for the production of flavor components in fermented milk. Following heating at 65 − 75 °C, compounds such as 5-hydroxyvaleric acid, myristoleic acid, azelaic acid, and 12-hydroxy octadecanoic acid exhibited significant reductions, ranging from 3- to 10-fold. Conversely, there was a corresponding increase in the content of 2,3-butanedione, 2-hydroxy-3-pentanone, 3-furaldehyde, and phenyl acetaldehyde among the volatiles, imparting frankincense odors and fruity flavors to the fermented milk. These changes might contribute to the enhanced consumer preference for PHT yogurt. However, as the temperature treatment rose to 95 °C, there was a significant 50-fold reduction in the content of unsaturated phospholipids. This reduction in the unsaturated phospholipid contents may lead to their oxidation and decomposition into compounds such as 2-heptanone, 2-nonanone, and 2-undecanone,
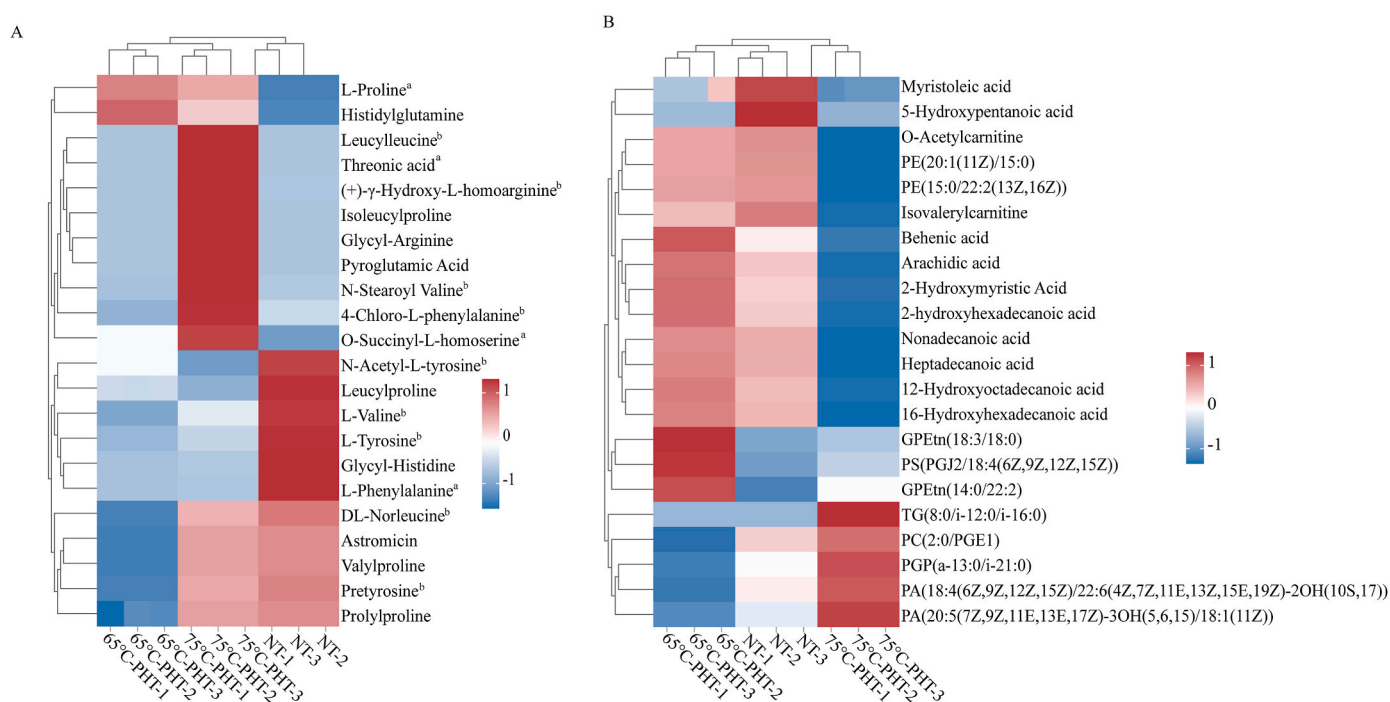

Fig. 7. Heat map visualization and cluster analysis of the non-volatile differential metabolite data of the 65 °C and 75°C-PHT samples. (A) Amino acids, peptides, and their derivatives. (B) Lipids and lipid-like molecules. [a] Sweet amino acid; [b] bitter amino acids. PHT = post-heated fermented milk; NT = non-heated processing.

the primary contributors to off-flavors in stored milk. Off-flavors resulting from lipid oxidation triggered by high temperatures diminish consumer acceptance of a product. Consequently, the post-heating treatment of fermented milk at lower temperatures (65 − 75 °C) enhances its frankincense odors and fruity flavors while concurrently mitigating the risk of off-flavors.

4. Conclusions

This study examined the impact of five post-heating temperatures on the characterization of volatile and non-volatile metabolites in fermented milk. After post-heating, changes in the fermented milk aroma and texture were further analyzed using a sensory analysis that triggered changes in consumer preference for the samples. Among the five post-heating temperatures, 65 °C and 75 °C were ideal for improving the flavor of fermented milk. These temperatures markedly increased the OAVs of 2,3-butanedione, hexanoic acid, and esters while significantly decreasing the OAVs of 2-heptanone. As the temperature increased from 85 °C to 95 °C, the increased ketones were primarily 2-heptanone, 2-nonanone, and 2-undecanone, which increase oxidative off-flavors during fermented milk storage. The texture of these higher-temperature samples was dominated by acidity, astringency, and caramelized flavors. Consequently, the 65 °C and 75 °C post-heating treatments of fermented milk enhanced the typical creamy, frankincense odors, and fruity and sweet flavors. The 65 °C and 75 °C post-heating treatments effectively reflected the characteristic flavors of fermented milk and were generally favored by consumers while concurrently mitigating the risk of oxidative off-flavors produced by 2-heptanone, 2-nonanone, and 2-undecanone. This study provides useful information for performing the post-heating temperature process.

Ethical statement

This study complied with all applicable international and domestic ethical regulations and was conducted in accordance with the World Medical Association Declaration of Helsinki. For research involving human sensory evaluation, we have been approved by the China Agricultural University Ethics Committee (CAUHR-20220502) and have ensured that all participants have provided written informed consent and have the right to withdraw from the study at any time. The collection and processing of research data follow privacy protection principles, and all personally identifiable information has been deleted or encrypted. We promise that the results of this study will be reported in an honest, objective, and responsible manner, and that no modifications will be made that may mislead readers or compromise the integrity of the study.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.crfs.2024.100892.

Data availability

Data will be made available on request.

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