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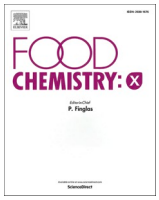

Study of volatile flavor derived from lipids degeneration in yak Milk based on Semiquantitative Lipidomics

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ABSTRACT

Milk lipids greatly affect the volatile flavor of milk, and the relationship between lipids and volatile flavor in yak milk was explored in this study. The volatile flavor compounds (VFCs), lipids profile, fatty acids in yak ordinary milk and colostrum were detected with HP/SPME-GC–MS, the semiquantitative lipidomics based on LC-MS/MS, GC–MS, respectively. The VFCs differences in yak milk were closely related to 1-((1 s,3ar,4r,7 s,7as)-4-hydroxy-7-isopropyl-4-methyloctahloctahydro-1h-inden-1-Yl)-ethanone,2,6,6-trimethyl-2,4-cycloheptadien-1-one, pentanal, 2-phenylethyl propionate, octanoic acid methyl ester, diphosphoric acid diisooctyl ester, (Z)-3,4,4-trimethyl-5-oxo-2-hexenoic acid and acetic acid. The volatile flavor in yak milk was well correlated with milk lipids, and TG(4:0_12:3_18:1), TG(6:0_8:0_18:1), TG(4:0_12:3_18:1), TG(12:0_18:2_18:3) and TG(16:0e_18:1_22:5) were the crucial lipid molecules affecting volatile flavor. The degeneration of above lipids by hydrolysis produced some fatty acids and alcohol, then these compounds were further derived into other VFCs especially above crucial 8 molecules. This study provided a theoretical basis for improving the volatile flavor by controlling lipids in yak milk.

1. Introduction

Milk and dairy products are very rich in nutrition contents and easy to be digested and absorbed, so are really loved by consumers (Aziz et al., 2022; Aziz et al., 2023). Many factors can affect the milk quality and the consumer choice to milk (Barbano, 2017), and mainly include sensory quality and nutritive value. Flavor is an index belonging to sensory quality, and is one of the most immediate factors for the consumer acceptance to milk (Bekele, Beuving, & Ruben, 2017). On the other hand, the quality of dairy products and modified milk heavily relies on the characteristic of raw milk. Diary processing companies tend to used the raw milk with high quality in practical production, and the flavor is a kind of crucial evaluation index for the raw milk in food industry too. The volatile flavor compounds (VFCs) play an important role in the formation of milk flavor, and include plenty of the substances with characteristic odor like alcohols, volatile fatty acids (VFAs), ethers, aldehydes, phenols, ketones and so on. The VFCs generation in milk is very complicated, and is mainly from the diet and the metabolism in animal. The degradation of lipids, proteins and other substances in vivo all can produce above VFCs in milk (Smith, Campbell, Jo, & Drake, 2016), and more and more researches show the effect of milk lipids to volatile flavor is more prominent by contrast with milk proteins and other substances. The milk lipids are the most complex lipids known at present (Mohan, O'Callaghan, Kelly, & Hogan, 2021), and possess the significant impact on the sensory quality of milk and milk products (Drake, Miracle, & McMahon, 2010). VFCs can be produced by the lipids decomposition and oxidation, and the specific lipids can form the unique volatile flavor of milk (Zhang et al., 2016). The effect of milk lipids to volatile flavor mainly depends on lipids content, composition and distribution (Saengsuk, Sangsawad, Paengkoum, & Pongsetkul, 2024); on the other

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hand, the physical characteristics of lipid globules and the interactions between lipid globule and other compounds also are the key factors affecting the volatile flavor of milk. The studies on milk lipids have been made great progress with the development of analytical technology in recent years, and more and more attention is payed to exploring the connection between milk lipids and volatile flavor (Omar, Gounga, Liu, Mlyuka, & Wang, 2016).

Yak (*Bos grunniens*) is a unique cattle in Tibetan Plateau (Wang et al., 2013; Wen et al., 2015). Yak milk an ideal high-end raw milk is a kind of pollution-free and green food, and possesses the advantages including the higher content of protein, functional fatty acids and essential amino acids (Lin et al., 2018; Wang et al., 2013). But yak milk itself possesses some relatively special flavor, which limits the consumer acceptance to it. The volatile flavor of yak milk is very unique, and the type and content of many flavor substances in yak milk are different from cow milk. The VFCs in yak milk are mainly composed of alcohols, aldehydes, lactones, phenols, ketones, acids, esters and terpenes (Hidalgo & Zamora, 2008), and the production of these VFCs depends on the complex flavor system. Due to the special growing environment, the content of yak milk lipids is very high, which obviously impacts the physicochemical properties and sensory quality of yak milk (Yang, Zheng, Wang, & Yang, 2018). In recent decades, plenty of researches on milk flavor were carried out, and the conclusion that the formation of unique milk flavor in milk is closely related to the higher content of milk lipid has been verified (Zhang et al., 2017). The lipids content of yak milk is higher than cow, water buffalo, camel and goat milk, which results in that the aroma flavor of yak milk is different from cow milk and other kinds of milk to some extent (Li, Zhang, & Zhao, 2022).

The volatile flavor of milk is influenced by many factors including breed, environment, diet (Li et al., 2022) and feeding system (Yayota, Tsukamoto, Yamada, & Ohtani, 2013). In addition, the lactation time also greatly impacts milk flavor (Park & Drake, 2014), and the volatile flavor of milk from the same individual in different lactation stages is different. The milk in early lactation stage possesses more abundant volatile flavor. On the other hand, many factors including breed, age, environment, feeding system, diet and gender also affect the content and type of milk lipids (Strekozov, Motova, & Fedorov, 2008). Beyond above factors, the lactation stage of cow is also an important factor affecting the characteristic of milk lipids. The nutritional need of calf at different growth stages is a dynamic process, so the milk secretion of cow is significantly changed to meet calf need. Generally speaking, the nutritive value of colostrum is better than ordinary milk, and the rate of milk lipid is different in the different lactation period too. Therefore, it is inferred both lipids and VFCs are different in the ordinary milk and colostrum of yak, and the two kinds of milk can be used as the model for exploring the relationship between the lipids and VFCs in yak milk.

The effect of lipids to the volatile flavor of yak milk was explored in this study, further the crucial lipids affecting the volatile flavor of yak milk were screened. First, the VFCs abundance in yak ordinary milk and colostrum was detected with the headspace solid phase microextraction gas chromatography-mass spectrum (HP/SPME-GC–MS); the lipids content in yak milk was detected by the semiquantitative lipidomics based on liquid chromatography-tandem mass spectrometry (LC-MS/MS); the fatty acids content in yak milk was detected with gas chromatography-mass spectrometer (GC–MS). Then, the characteristic of lipids and VFCs in two kinds of yak milk was revealed, and the different volatile flavor compounds (DVFCs), different lipids species (DLSs) and different fatty acids were screened out, respectively. Further, the Pearson correlation between DLSs, different fatty acids and DVFCs was analyzed, respectively. The crucial lipids and fatty acids affecting the volatile flavor of yak milk were explored, and their function in the formation of volatile flavor was expounded. This study establishes a theoretical basis for the improvement of the volatile flavor of yak milk, and can promote the development of yak milk industry. Based on the screened crucial lipids affecting the volatile flavor in yak milk, the bad volatile flavor to consumer in yak milk can be decreased. In future, yak milk lipids can be improved by diet intervention, then the volatile flavor of yak milk will be optimized; diary processing company also can produce the dairy products with better flavor by adjusting the lipids content and type in raw yak milk.

2. Materials and methods

2.1. Animals and sample collection

Six healthy female yaks with similar weight and age were chosen and used in this study, and all yaks were grazed in the natural pasture of Gannan region, Gansu province in China. After yaks calved, the yak colostrum and ordinary milk were collected at the first and 10th day before the calf sucking in the morning, respectively. Total 50 mL milk sample for each yak was put into a sterile sampling tubes, then the tubes were kept on an ice cooling box. The cooling box was sent back to the laboratory as soon as possible, then these yak milk samples were stored in an ultra-low temperature freezer at −80 °C (Haier-Biomedical, Qingdao, Shandong, China). Yak colostrum was the treated group, and yak ordinary milk was the control group.

2.2. Determination of volatile flavor compounds (VFCs) in yak colostrum and ordinary milk

The VFCs in yak milk were detected according the reference with some modification (Alemayehu, Hannon, McAuliffe, & Ross, 2014). The yak milk samples were taken out from the freezer, then was thawed at room temperature. Twenty μg/mL n-alkane solution (C_7 to C_{40}) was used as the internal standards. The solution from mixing all milk samples in equal volume was as the quality control (QC) sample. Two point five mL thawing yak milk was transferred into a headspace bottle, then the bottle was shaken for 10 min at 450 r/min. Next, the 50/30 μm DVB/CAR/PDMS extraction head (Supelco Corp., Bellefonte, Pennsylvania, USA) was aged for 2 h in the fiber conditioning station, and was heated for 10 min to desorbe the impurities. The extraction head was inserted into the headspace of yak milk samples, and the extraction process lasted for 30 min. Finally, the extracted VFCs were desorbed at 250 °C for 10 min.

The extracted VFCs were separated and analyzed with the 7890B/5977B/PAL-RSI 120 GC–MS (Agilent Corp., Santa Clara, CA, USA) coupled with DB-WAX capillary column (30 m × 0.25 mm × 0.25 μm) (Agilent Corp., Santa Clara, CA, USA). GC parameters were carrier gas high-purity helium (purity >99.999%), flow rate 1.0 mL/min, inlet temperature 250 °C and programmed temperature. The initial temperature was held at 30 °C for 1 min, then was increased to 250 °C at 5.5 °C/min. Mass spectrum (MS) parameters were EI source, ion source temperature 230 °C, quadrupole temperature 150 °C, ionization voltage 70 eV, full scan mode, and scanning range *m/z* 30–400. The obtained raw data in .D format was transferred into .abf format via software Analysis Base File Converter, then these data was imported into the software MS-DIAL. The qualitative analysis for the lipids of yak milk was based on the NIST database (https://webbook.nist. gov/chemistry/).

2.3. Determination of semiquantitative lipidomics in yak colostrum and ordinary milk

Lipomics in yak milk was detected according the reference with some modification (Chen et al., 2021). Twenty μL isotope internal standards of 14 lipids (SPLASH® Lipidomix Mass Sprcstandard, Avanti, 330,707-1EA) and 20 μL water were transferred into each milk sample, then the mixture was adequately vortexed. Eight hundred μL methyl tert-butyl ether (MTBE) was added into the mixture, then the solution was homogenised. Two hundred forty μL pre-cooled methanol was added into the mixture, then the solution was homogenised too. The mixture was extracted using ultrasound for 20 min, then the extracted solution was let stand for 30 min at room temperature and was centrifuged at

14,000 r/min for 15 min at 10 °C on the 5430R high speed centrifuge (Eppendorf, Hamburg, Germany). The upper organic solvent was transferred into a new tube, then was dried under nitrogen blow. The residue was redissolved in 200 μL the solution of isopropanol and acetonitrile (9:1, *v:v*), and the reconstitution solution was sufficiently vortexed. Ninety μL solution was transferred out, and centrifuged at 14,000 r/min for 15 min. The supernatant was transferred into a vial for analysis.

The extracted lipids were separated with the Nexera LC-30 A (SHIMADZU, Kyoto, Japan) UHPLC coupled with an ACQUITY UPLC CSH C_{18} column (1.7 μm × 2.1 mm × 100 mm) (Waters, Milford, Massachusetts, USA). The mobile phase was the solvent A acetonitrile–water (6:4, *v:v*) with 0.1% formic acid and 0.1 mmol ammonium formate and the solvent B acetonitrile–isopropanol (1:9, *v:v*) with 0.1% formic acid and 0.1 mmol ammonium formate. The elution program was as follows: 30% solvent B was held for 2 min, then was linearly increased to 100% in 23 min, followed by equilibrating at 5% solvent B for 10 min. The Q-Exactive Plus (Thermo Scientific, Massachusetts, USA) was used to collect MS data. MS parameters were Heater Temp 300 °C, Sheath Gas Flow rate 45 arb, Aux Gas Flow Rate 15 arb, Sweep Gas Flow Rate1arb, Spray Voltage 3.0 KV, Capillary Temp 350 °C, S-Lens RF Level 50%, and MS1 scan ranges 200–1800. LipidSearch was used to carry out peak identification and extraction, lipid identification, and the main parameters was precursor tolerance 5 ppm, product tolerance 5 ppm, product ion threshold 5%.

2.4. Determination of fatty acids in yak colostrum and ordinary milk

The organic horizon was transferred into a tube after milk sample was centrifuged, then the methanol containing potassium hydroxide was added into the tube. The mixture was shaken for 2 h and the lipids hydrolysis was completed. The pH value of solution was adjusted to 3 with hydrochloric acid. Ten mL n-hexane was transferred into the tube, and the mixture was let stand for 10 min after shaking. The supernatant was transferred into a new tube, then was dried under nitrogen. Two mL methanol containing 1% sulfuric acid was added into the tube, and the mixture was put on 80 °C water bath for half an hour, then the n-hexane was used to extract the solution. Two mL saturated salt solution was added into the solution, and the supernatant was transferred into a new tube after centrifuging. The solution was dried using anhydrous sodium sulfate, then was centrifugated. The supernatant was transferred out, and 25 μL methyl nonadecanoate (the internal standard) was added into the solution. The mixture was dried under nitrogen, and the residue was redissolved in n-hexane for analysis.

The 7890/5975 GC–MS (Agilent Corp., Santa Clara, CA, USA) coupled with DB-WAX capillary column (30 m × 0.25 mm ID × 0.25 μm) (Agilent Corp., Santa Clara, CA, USA) was used to separate the extracted lipids in yak milk. The initial temperature of column was 50 °C and maintained for 3 min, then was increased to 220 °C and was held for 5 min. The other GC parameters was injection volume 1 μL, the ratio of split/splitless 1:100, the flow rate 1.0 mL/min, and the inlet temperature 280 °C. The MS parameters were ion source temperature 230 °C, transmission line temperature 250 °C, EI source, signal ion monitoring (SIM) scanning mode, and electron energy 70 eV. At last, the external standard method was used to calculate the content of fatty acids in yak milk.

2.5. Statistical analysis

The independent-sample *t*-test in the software SPSS 16.0 (SPSS Inc., Chicago, IL, USA) was used to analyze the fatty acids content in yak milk. Principal component analysis (PCA) was used to observe the stability of the lipidomics and VFCs analyze; orthogonal partial least-squares-discriminant analysis (OPLS-DA) was utilized to distinguish DLSs and DVFCs, and 7-fold cross-validation and 200 Response Permutation Testing (RPT) were used to evaluate the model quality of OPLS-DA. Further, the two-tailed Student's t-test was used to verify whether the DLSs and DVFCs were significant. PCA and OPLS-DA were performed using R based. The DLSs and DVFCs with variable importance of projection (VIP) > 1.0 and $P < 0.05$ were selected out. Pearson correlation analysis was used to calculate the correlations between crucial DVFCs abundance, fatty acids content and crucial DLSs content, respectively. When P was <0.05, the differences were considered to be significant; when the correlation coefficient was >0.8, the correlation was considered to be high.

3. Results

3.1. Volatile flavor compounds (VFCs) in yak colostrum and ordinary milk

The base peak chromatograms (BPCs) for the VFCs in yak ordinary milk and colostrum were shown in **Fig. S1**. The score maps of the PCA and OPLS-DA for the VFCs in yak colostrum and ordinary milk were shown in Fig. 1**a** and **b**, respectively. It was found the PCA model for milk samples can completely distinguish the VFCs in yak colostrum and ordinary milk, which indicated the VFCs in two kinds of yak milk were significantly different. The values of R^2Y and the vertical intercept in the permutation test were 0.970 and − 0.133, respectively (Fig. 1**c**), and the OPLS-DA model for the VFCs in yak milk was not overfitting. The splot of OPLS-DA for VFCs was shown in Fig. 1**d**. Total 217 VFCs were detected in two kinds of yak milk, and the VFCs composition in yak milk at super class, class and sub class was shown in **Fig. 2a**, **b** and **c**, respectively. The lipids and lipid-like molecules, organic oxygen compounds, benzenoids and hydrocarbons were the predominant categories at superclass level, and were total 144 kinds; prenol lipids, organooxygen compounds, saturated hydrocarbons, benzene and substituted derivatives and fatty acyls were the predominant categories at class level, and were total 137 kinds; alkanes, carbonyl compounds, monoterpenoids and sesquiterpenoids were the predominant categories at subclass level, and were total 84 kinds.

Total 44 DVFCs were screened out in two kinds of yak milk (Fig. 3a). The abundance of 31 DVFCs was higher in yak colostrum, whereas the abundance of 13 DVFCs was down-regulated. The cluster heat map of the DVFCs was shown in Fig. 3b. The predominant DVFCs at class level in yak milk were prenol lipids (total 19 kinds), organooxygen compounds (total 6 kinds) and fatty acyls (total 2 kinds); the predominant DVFCs at subclass level in yak milk were sesquiterpenoids (total 9 kinds), monoterpenoids (total 9 kinds), carbonyl compounds (total 5 kinds) and fatty acid esters (total 2 kinds). Further, these DVFCs included 15 alcohols, 6 ketones, 4 esters, 11 aliphatic hydrocarbons, 3 aromatic hydrocarbons, 2 organic acids, 2 aldehydes and 1 nitrogen-containing organic compound. The lolipopmap and correlation for these DVFCs were shown in **Fig. 3c** and **d**, respectively. The DVFCs whose abundance was lower in yak colostrum mainly were aliphatic hydrocarbons and aromatic hydrocarbons (total 7 kinds), and the abundance of all ketones and acids in DVFCs was higher in yak colostrum. There were positive correlation among the most of DVFCs with the same variation trend of abundance in yak milk. It can be verified there were complicated interrelations among these VFCs in yak milk, and some VFCs were formed by the derivations of other VFCs.

The PCA for all DVFCs was carried out by the DVFCs abundance in all twelve yak milk samples to obtain the important VFCs affecting the volatile flavor of yak milk. The Loading-PAC of DVFCs in two kinds of yak milk (**Fig. S2**) showed the variance contribution rate of principal component 1 (PC1), PC2 and PC3 was 66.773%, 8.958% and 5.565%, respectively, and the aggregate-value was reached at 81.296%. Moreover, 1,6-dimethyl-4-(1-methylethyl)-naphthalene, 4-isopropyl-6-methyl-1-methylene-1,2,3,4-tetrahyd-ronaphthalene, 3,7,11-trimethyl-1-dodecanol, 2,4-dimethylphenethyl alcohol, 1-ethenyl-1-cyclohexanol, agarospirol, 1-((1 s,3ar,4r,7 s,7as)-4-hydroxy-7-isopropyisopropopropyl-4-methyloctahydro-1 h-inden-1-Yl)-ethanone,

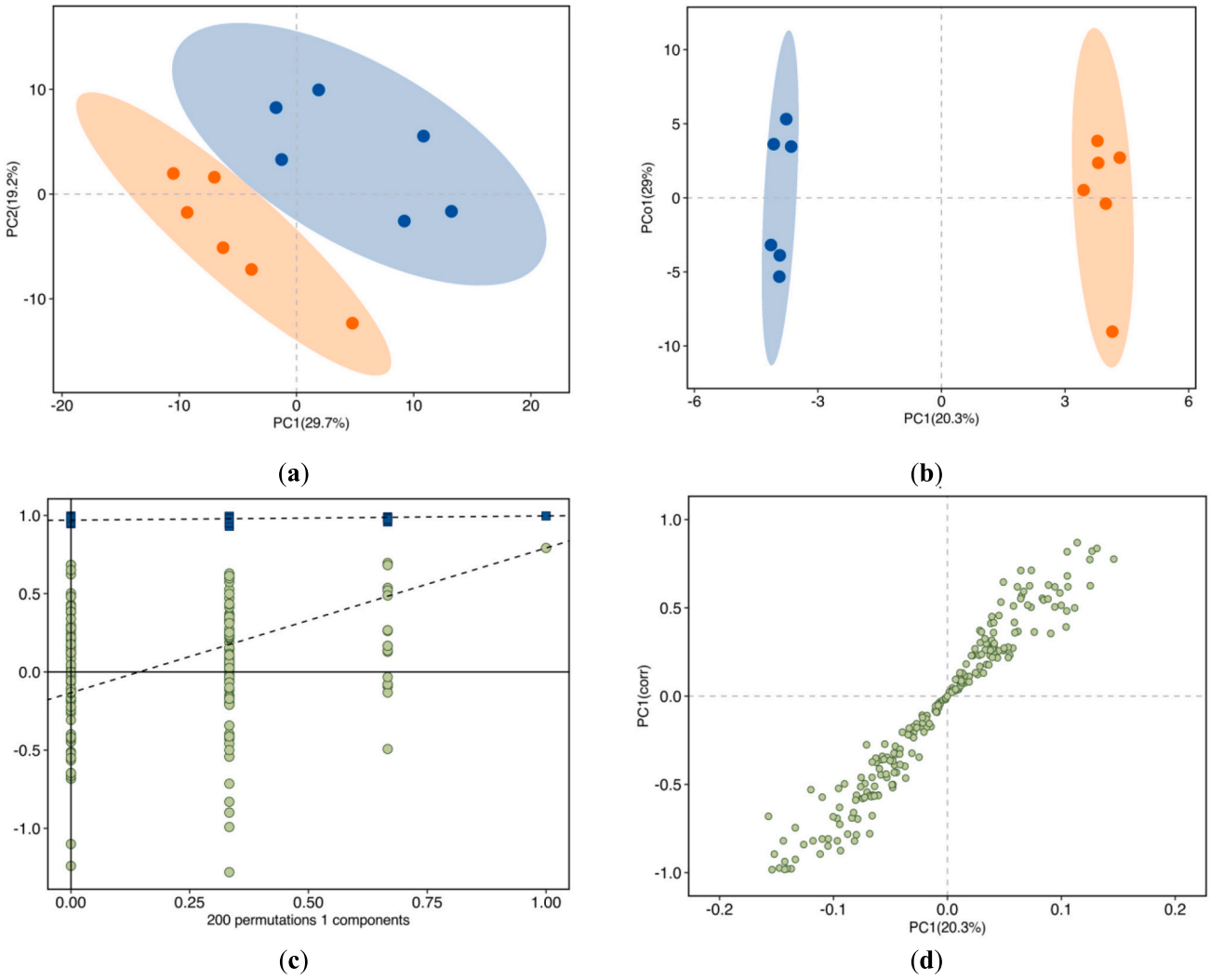

Fig. 1. (**a**) The results of principal component analysis (PCA) for the volatile flavor compounds (VFCs) in yak colostrum and ordinary milk. A circle represented a milk sample, and blue circles, red circles represented the yak colostrum, ordinary milk, respectively. PC1 represented principal component 1, and PC2 represented principal component 2; (**b**) The results of orthogonal partial least-squares-discriminant analysis (OPLS-DA) for the VFCs in yak colostrum and ordinary milk. PC1 represented principal component 1, and PCo1 represented principal component 2; (**c**) The results of Permutation test for the OPLS-DA model of the VFCs in yak milk; (**d**) The Splot of OPLS-DA for the VFCs in yak milk. The horizontal coordinate is the characteristic value of the effect of VFCs to the comparison group, and the vertical coordinate is the correlation between the sample score and the VFCs. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

alpha-cadinol, diphosphoric acid diisooctyl ester, gamma-elemene, octanoic acid methyl ester, 2,6,6-trimethyl-2,4-cyclohepeptadien-1-one, pentanal, decahydro-4a-methyl-1-methylene-7-(1-methylethyl)-[4ar(4aα,7α,8aβ)]-naphthalene, 2-phenylethyl propionate, m-cymen-8-ol, (Z)-3,4,4-trimethyl-5-oxo-2-hexe-noic acid, acetic acid, cis-calamenene, (−)-aristolene, p-Cymen-7-ol, thymol and (*R*)-4-methyl-1-(1-methylethyl)-3-cyclohexen-1-ol were highly correlated with PC1 in yak milk. Above VFCs can be as the candidate substances which played important roles in the volatile flavor of yak milk.

3.2. Lipids in yak colostrum and ordinary milk

The total ion chromatograms (TIC) for the lipids in yak ordinary milk and colostrum were shown in **Fig.S3**. Total 2268 lipid species in 41 lipid classes were detected in yak milk, and the absolute content of lipids was shown in **Table S1**. Nine hundred thirty five lipids species were found in negative ion mode, whereas 1333 lipids species were found in positive ion mode. The total lipids content in yak colostrum was higher than yak ordinary milk (62.75 μg/mL vs. 36.16 μg/mL, $P < 0.05$). The lipids in yak milk were mainly composed of 802 TGs, 233 DGs, 172 PCs, 153 PEs, 150 Cers, 132 Hex1Cers and 118 PSs (**Fig. S4**). The main lipids classes in yak colostrum included 96.78% TGs, 1.05% DGs, 0.63% Hex1Cers, 0.26% CerPs, 0.21% SPHs and 0.15% PEs (**Fig. S5**); the main lipids classes in yak ordinary milk included 95.84% TGs, 1.67% DGs, 0.54% Hex1Cers, 0.44% CerPs, 0.32% SPHs and 0.19% WEs (**Fig. S6**). The total content of AcCas, ChEs, GD2s, Hex1Cers, LPCs, LPEs, LPIs, LPSs, PGs, PhSMs, SiEs, SMs, StEs and ZyEs in yak colostrum was higher than yak ordinary milk ($P < 0.05$), whereas the total content of CLs, PCs, PEs and PIs in yak colostrum was lower than yak ordinary milk ($P < 0.05$).

The score plots of PCA and OPLS-DA for the lipids in two kinds of yak milk were shown in Fig. 4**a** and **b,** respectively. The lipids in yak colostrum and ordinary milk were distributed into two spaces in PCA, which indicated the analysis was reliable. The value of R^2Y and the vertical intercept in the permutation testing for OPLS-DA were 0.8489 and − 0.5617 (Fig. 4**c**), respectively, so there was no overfitting phenomenon in the OPLS-DA model. Further, the content of 66 lipid species was different in yak colostrum and ordinary milk (**Table S2**), and the DLSs included 58 TGs, 5 Hex1Cers, 1 WE, 1 PE and 1 PS (Fig. 4**d**). The

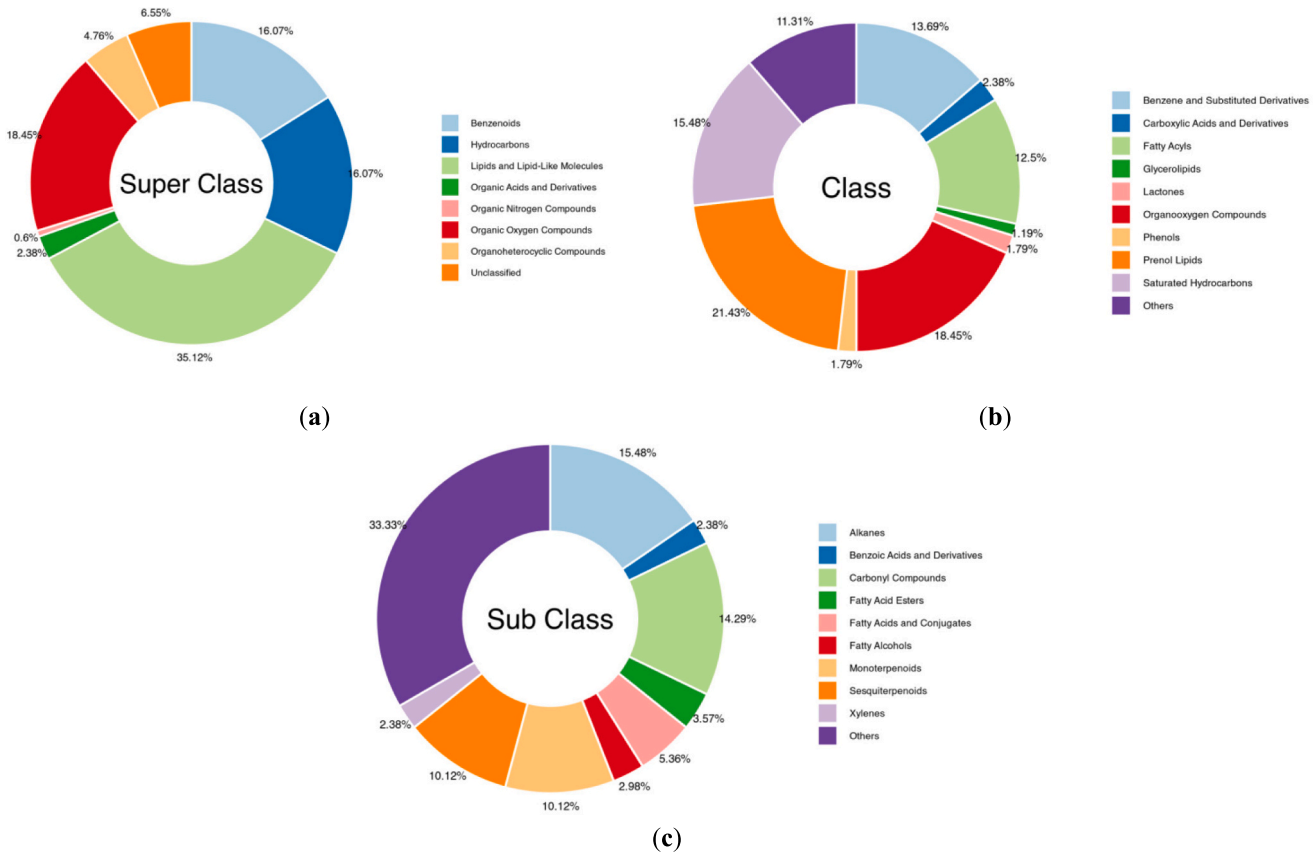

Fig. 2. (**a**) The pie chart for the composition of VFCs kinds in yak colostrum and ordinary milk at superclass level. If the numbers of VFCs categories in yak milk were >10, the 9 categories with the largest numbers of VFCs kinds were selected and shown in the pie chart. The different colour represented the different VFCs categories; (**b**) The pie chart for the composition of VFCs kinds in yak colostrum and ordinary milk at class level; (**c**) The pie chart for the composition of VFCs in yak colostrum and ordinary milk at subclass level.

content of Hex1Cer (d14:2_18:1) and TG(16:0_18:2_18:3) in yak colostrum was lower than yak ordinary milk ($P < 0.05$), whereas the content of other DLSs in yak colostrum was higher ($P < 0.05$). The PCA for all DLSs was carried out by the DLSs content in all twelve yak milk samples to obtain the important lipids affecting the volatile flavor of yak milk. The Loading of PAC for the DLSs (**Fig. S7**) showed the variance contribution rate of PC1, PC2 and PC3 was 83.523%, 5.139% and 4.358%, respectively, and the aggregate-value was reached at 93.020%. The most variables beside PE(18:1_18:1), PS(18,0_18:1), TG(4,0_16:0_16:0), TG(16,0_18:2_18:3) and Hex1Cer(d14:2_18:1) were all highly correlated with PC1. Therefore, the DLSs beside above 5 lipids can be preliminarily considered as the candidate substances in lipids greatly affecting the volatile flavor of yak milk.

3.3. Fatty acids content in yak colostrum and ordinary milk

The content of 40 fatty acids in yak milk was shown in **Table S3**. The content of C4:0, C10:0, C11:0, C12:0, C16:1n7, C17:0, C18:0, *t*-C18:1n9, C18:1n9, *t,t*-C18:2n6, C18:2n6, C18:3n6, C18:3n3, C20:3n6, C20:4n6, C22:2n6, C22:5n3 and C22:6n3 was different in yak colostrum and ordinary milk ($P < 0.05$). Of them, the content of C4:0, *t*-C18:1n9, C18:1n9, *t,t*-C18:2n6, C18:2n6, C18:3n6, C18:3n3, C22:5n3 and C22:6n3 in yak colostrum were higher than yak ordinary milk ($P < 0.05$), while the content of other fatty acids in yak colostrum was lower ($P < 0.05$).

3.4. Results of correlation analysis

The heat map of Pearson correlation for the important DVFCs and the fatty acids with different content in two kinds of yak milk was shown in **Fig. S8**. The abundance of decahydro-4a-methyl-1-ethylene-7-(1-methyle-(2-thenyl)-[4ar-(4aα,7α,8a)]-naphthalene, acetic acid and (*R*)-4-methyl-1-(1-methylethlethl)-3-cyclohexen-1-ol in yak milk was highly positively correlated with the most DLSs content; only cis-calamenene, (−)-aristolene, p-cymen-7-ol and thymol abundance was highly positively correlation with TG(4:0_12:3_18:1), TG(12:0_18:2_18:3), TG(16:2e_19:0_19:1), Hex1Cer(m17:0_16:0 + O) and Hex1Cer(m18:0_19:0) content; TG(4:0_12:3_18:1), TG(12:0_18:2_18:3), TG(16:2e_19:0_19:1), TG(16:0e_18:0_20:5), TG(16:0e_18:1_22:5), Hex1Cer(m17:0_16:0 + O) and Hex1Cer(m18:0_19:0) content was highly positively correlated with the most DVFCs abundance. The information of important VFCs, lipids affecting the volatile flavor in yak milk was shown in Tables 1, 2**,** respectively. Moreover, the *t*-C18:1n9, C18:1n9, *t, t*-C18:2n6, C18:2n6, C18:3n6 and C18:3n3 content was highly positively correlated with the most DVFCs abundance; the C22:2n6 content was highly positively correlated with the 3,7,11-trimethyl-1-dodecanol abundance; the C12:0 content was highly negatively correlated with the m-cymen-8-ol abundance.

4. Discussion

Milk lipids serve as the important precursor of the flavor substances in milk (Mao, Roos, Biliaderisc, & Miao, 2017). In recent years, a large number of researches on the flavor substances in bovine milk have shown the unique flavor in bovine milk is closely related to lipids content (Clarke et al., 2019). The higher the lipids content in bovine milk is, the stronger the aroma is. The total content of lipids in yak colostrum was higher than yak ordinary milk, and the abundance of DVFCs leading

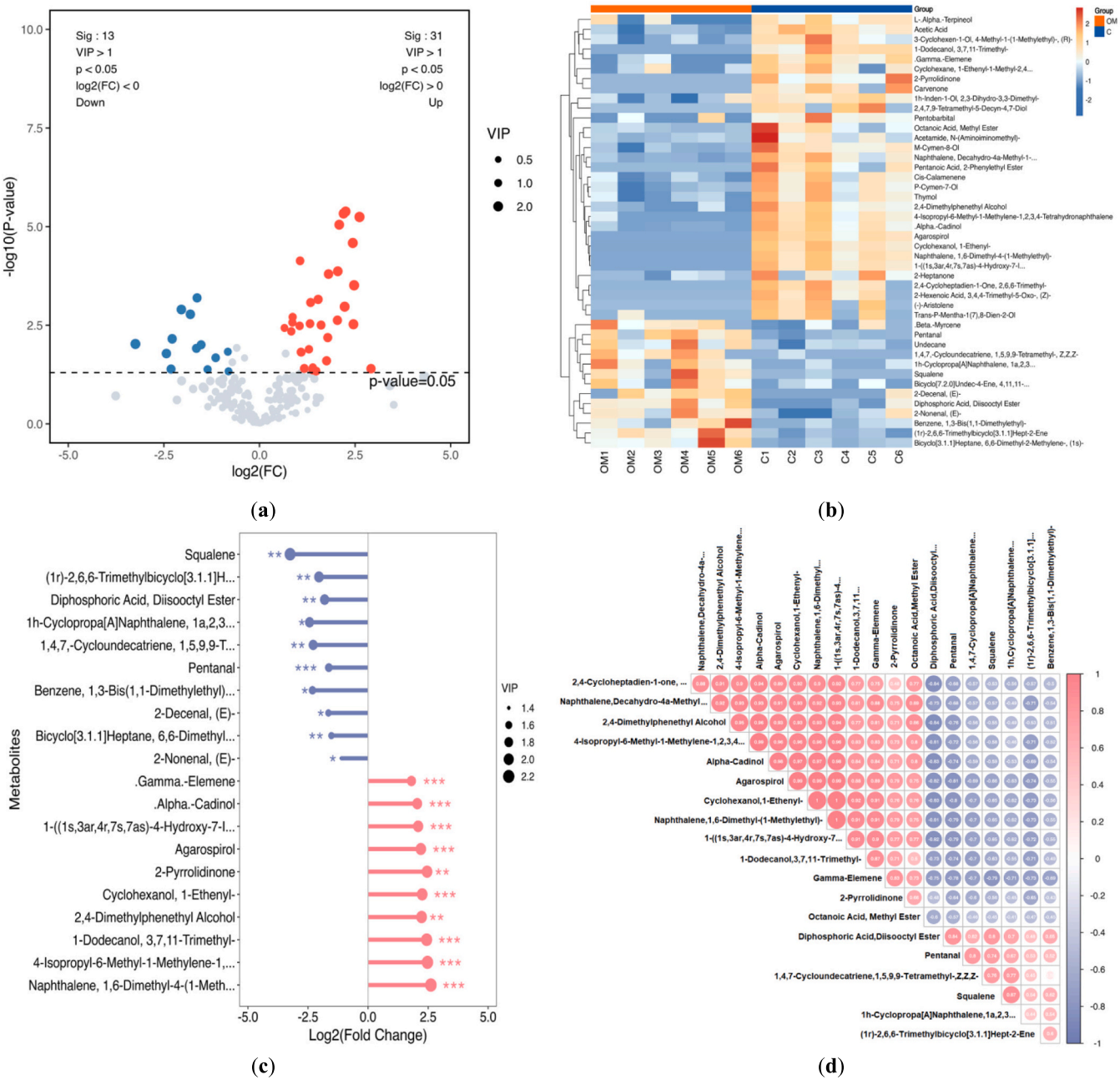

Fig. 3. (**a**) The volcano plot of VFCs abundance in yak colostrum by contrast with yak ordinary milk. The abscissa represented the value of $\log_2$ fold change (FC), and the ordinate represented the value of -log10 *P*. A point represented a kind of VFC. These VFCs with the values of FC > 1, variable importance of projection (VIP) > 1 and $P < 0.05$ were shown in red, whereas these VFCs with the value of FC < 1, VIP > 1 and $P < 0.05$ were shown in blue; non-significantly different volatile flavor substances (DVFCs) were shown in gray; (**b**) The cluster heat map of DVFCs in two kinds of yak milk. A row represented a kind of DVFC, and a column represented a yak milk sample. The C represented the yak colostrum milk; the OM represented the yak ordinary milk. The relative abundance of DVFCs was presented by the colour blocks at different positions. Red represented the higher abundance in yak colostrum, while blue represented the lower abundance in yak colostrum; (**c**) The lollipopmap for the DVFCs in two kinds of yak milk. The horizontal coordinate represented the $\log_2$ FC value, and a vertical coordinate represented a kind of DVFC. These 10 kinds of DVFCs with the largest VIP value in up-regulated, down-regulated DVFCs in yak colostrum were selected and shown, respectively. Red, blue represented the up-regulated, down-regulated DVFCs, respectively. The asterisk indicated the DVFCs significance, and the dot size was determined by VIP value; (**d**) The Pearson correlation of the DVFCs in two kinds of yak milk. Pearson correlation coefficient was used to measure the degree of linear correlation between two kinds of DVFCs. Red indicated the positive correlation, whereas blue indicated the negative correlation. The larger the dot was, the greater the correlation coefficient was. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to the change of violate flavor was almost up-regulated in yak colostrum too. Therefore, it also was inferred the higher the lipid content is, the stronger the volatile flavor in yak milk is too. Milk lipids can be broken down by some enzymes, then form methyl ketones, aldehydes and alcohols(Saerens, Descamps, & Dewettinck, 2008). Compared with cow milk, yak milk possesses more types and higher content of flavor compounds, and the ketones and acids from saturated fatty acids (SFAs) oxidation and triglyceride hydrolysis account for the relatively high proportion in all flavor substances. The aldehydes, esters, alkanes and aromatics production are also related to milk lipids oxidation or hydrolysis (Khan, Nadeem, Imran, & Khalique, 2020). The DVFCs in yak milk mainly included alcohols, ketones, aldehydes, esters, aliphatic

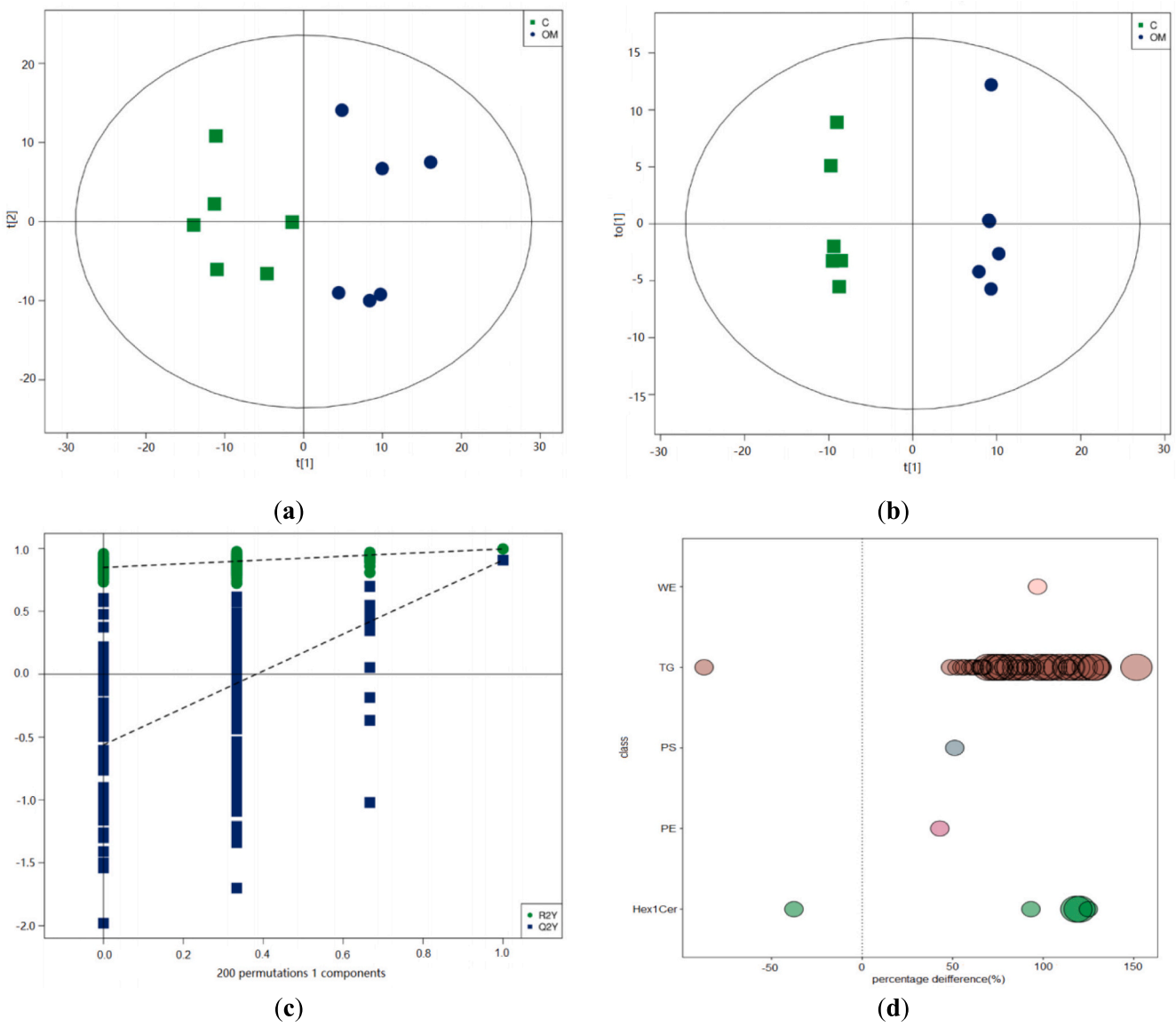

Fig. 4. (**a**) The score plots for the PCA of lipids in yak colostrum and ordinary milk. t[1] represented principle component 1, and t[2] represented principle component 2. Green, blue circles represented the yak colostrum, ordinary milk samples, respectively; (**b**) The score plots for the OPLS-DA of lipids in yak colostrum and ordinary milk. t[1] represented principal component 1, and to[1] represented principle component 2; (**c**) The results of permutation test for the OPLS-DA model of lipids in yak colostrum and ordinary milk; (**d**) The bubble diagram for the different lipids species (DLSs) in yak colostrum and ordinary milk. The bubbles represented the DLSs; the ordinate represented the lipid subclass; the bubble size represented the difference significance of DLSs. There were two kinds of bubbles in terms of area. These smaller bubbles represented the significant difference ($P < 0.05$), while these larger bubbles represented the very significant difference ($P < 0.01$).

Table 1
The information of important volatile flavor compounds (VFCs) in yak milk.

Volatile flavor compound	m/z	CAS	FC	VIP
1-((1 s,3ar,4r,7 s,7as)-4-hydroxy-7-isopropyl-4-methyloctahlo-ctahydro-1 h-inden-1-Yl)-ethanone	153.0514	1911-78-0	4.24	2.02
acetic acid	44.99714	64–19-7	2.09	1.38
pentanal	44.00625	110–62-3	0.32	1.68
2,6,6-trimethyl-2,4-cycloheptadien-1-one	107.0456	503–93-5	2.09	1.38
2-phenylethyl propionate	104.0325	7460-74-4	3.45	1.62
octanoic acid methyl ester	74.02834	111–11-5	7.56	1.76
diphosphoric acid diisooctyl ester	83.00667	72,101–07-6	0.29	1.87
(Z)-3,4,4-trimethyl-5-oxo-2-hexenoic acid	110.0371	14,919–56-3	3.05	1.54

FC: Foldchange; VIP: variable importance of projection.

hydrocarbons, aromatic hydrocarbons and organic acids, so it was inferred the change of volatile flavor in yak milk was also closely related to ketones, esters, aliphatic hydrocarbons, aromatic hydrocarbons, organic acids and aldehydes. On the other hand, the fatty acids from lipids degradation also can be transferred into the VFCs. Eighteen fatty acids content was different in yak colostrum and ordinary milk, and C4:0, *t*-C18:1n9, C18:1n9, *t,t*-C18:2n6, C18:2n6, C18:3n6, C18:3n3, C22:5n3 and C22:6n3 cotent in yak colostrum were higher than ordinary milk. Therefore, the change of violate flavor in yak milk was also closely related to the lipids derived from above fatty acids.

Aldehydes and ketones enhancing milk volatile flavor are the main products in lipids degradation (Licón et al., 2012). Ketones like methyl ketones are the important characteristic aroma components in milk (Allen & Parks, 1969). Aldehydes flavor is a kind of light and fruity aroma in a general way, and the aldehydes contribution to milk flavor is moderate. The DVFCs in yak colostrum and ordinary milk included 6

Table 2
The information of important lipid molecules affecting the volatile flavor in yak milk.

Lipid ion	Class	Fatty acid	Ion formula	FC	VIP
TG(4:0_12:3_18:1) + H	TG	C4:0, C12:3, C18:1	$C_{37}H_{63}O_6$	4.61	1.62
TG(12:0_18:2_18:3) + NH_4	TG	C12:0, C18:2, C18:3	$C_{51}H_{92}O_6N_1$	3.08	1.63
TG(16:2e_19:0_19:1) + Na	TG	C16:2e, C19:0, C19:1	$C_{57}H_{106}O_5Na_1$	2.65	1.50
Hex1Cer(m17:0_16:0 + O) + H-H_2O	Hex1Cer	Cm17:0, C16:0 + O	$C_{39}H_{76}O_7N_1$	3.90	1.32
Hex1Cer(m18:0_19:0) + H	Hex1Cer	Cm18:0, C19:0	$C_{43}H_{86}O_7N_1$	4.01	1.08
TG(16:0e_18:0_20:5) + H	TG	C16:0e, C18:0, C20:5	$C_{57}H_{103}O_5$	3.18	1.18
TG(16:0e_18:1_22:5) + H	TG	C16:0e, C18:1, C22:5	$C_{59}H_{105}O_5$	2.97	1.84

ketones and 2 aldehydes, and 1-((1 s,3ar,4r,7 s,7as)-4-hydroxy-7-isopropyl-4-methyloctahydro-1 h-inden-1-Yl)ethanone, 2,6,6-Trimethyl-2,4-cycloheptadien-1-one and pentanal abundance was highly correlated with the volatile flavor in yak milk. Esters are another important VFCs in milk, and their contribution to milk flavor is very great due to low threshold value. Two-phenylethyl propionate, octanoic acid methyl ester and diphosphoric acid diisooctyl ester abundance was highly correlated with the volatile flavor in yak milk. Fatty acids are mainly produced by triglycerides hydrolysis in milk (Iranmanesh, Ezzatpanah, Akbari-Adergani, & Torshizi, 2018). Fatty acids threshold is medium, and especially short chain fatty acids threshold is relatively low (Toelstede & Hofmann, 2008), so the fatty acids contribution to milk flavor is very great too. Further, fatty acids themselves are the precursors of ketones, aldehydes and ester (Pan, Tong, & Chi, 2019). The DVFCs (Z)-3,4,4-trimethyl-5-oxo-2-hexenoic acid and acetic acid content was highly correlated with the volatile flavor in yak milk. The odor threshold of ethanols is relatively high (Lirong, Shihao, & Ailing, 2023), and their contribution to the volatile flavor of milk is relatively small (Wu, Zhan, Tang, Li, & Duan, 2022). The 2,4-dimethylphenethyl alcohol, 3,7,11-trimethyl-1-dodecanol, 1-ethenyl-1-cyclohexanol, agarospirol, alpha-cadinol, m-cymen-8-ol, p-cymen-7-ol, thymol and (*R*)-4-methyl-1-(1-methylethyl)-3-cyclohexen-1-ol abundance was highly correlated with the volatile flavor in yak milk. Hydrocarbons have a relatively small impact on milk flavor (Hu, Zheng, Liu, & Deng, 2017) due to high threshold value, so the effect of hydrocarbons to the volatile flavor of yak milk was not discussed. In a conclusion, 1-((1 s,3ar,4r,7 s,7as)-4-hydroxy-7-isopropyl-4-methyloctahydro-1 h-inden-1-Yl)ethanone,2,6,6-trimethyl-2,4-cycloheptadien-1-one, pentanal, 2-phenylethyl propionate, octanoic acid methyl ester, diphosphoric acid diisooctyl ester, (Z)-3,4,4-trimethyl-5-oxo-2-hexenoic acid and acetic acid played crucial roles in the formation of the volatile flavor in yak milk.

The ketones in milk is mainly from lipids degradation (Jo, Benoist, & Barbano, 2018) and free fatty acids oxidation (Vagenas & Roussis, 2012). Aldehydes are mainly from lipids oxidation in milk too, and this process is realized by Maillard reaction (Oh et al., 2013) or fatty acid oxidation (Contador, Delgado, & Garcia-Parra, 2015). The lipids hydrolysis in milk system can produce free fatty acids and alcohols, and these compounds further derive into some esters by esterification under the action of endogenous esterase (Aziz et al., 2021). Alcohols mainly come from lipids oxidation and the reduction synthesis of carbonyl groups (Xu et al., 2022). Hydrocarbons can be formed by automatic lipids oxidation too. TG(4:0_10:0_15:0), TG(4:0_12:3_18:1), TG (6:0_8:0_18:1), TG(4:0_10:0_15:0), TG(4:0_12:3_18:1), TG(12:0_18:2_18:3), TG(16:2e_19:0_19:1), TG(16:0e_18:0_20:5), TG(16:0e_18:1_22:5), Hex1Cer(m17:0_16:0 + O) and Hex1Cer(m18:0_19:0) content was highly positive correlation with the most crucial DVFCs abundance in yak milk. Moreover, lipids can generate free fatty acids by hydrolytic action, then these fatty acids were further transferred into other VFCs (Aziz et al., 2021). Polyunsaturated fatty acids (PUFAs) oxidation can produce aldehydes, alcohols, ketones, esters and heterocyclic compounds (Ba, Ryu, Lan, & Hwang, 2013). The higher the unsaturation degree of UFAs is, the easier UFAs oxidation is. Meawhile, the higher the UFAs content is, and the stronger the volatile flavor is (Madruga, Elmore, Oruna-concha, Balagiannis, & Mottram, 2010). Therefore, the effect of UFAs to the volatile flavor of yak milk was greater than saturated fatty acid (SFAs). The content of *t*-C18:1n9, C18:1n9, *t,t*-C18:2n6, C18:2n6, C18:3n6 and C18:3n3 from the lipids hydrolysis in yak milk was highly positive correlation with the abundance of the most DVFCs including above 8 crucial VFCs. All above 6 fatty acids belong to UFAs, so these UFAs greatly affected the violate flavor of yak milk. Combing the correlation analysis for the crucial VFCs and lipids in yak milk, it can be inferred TG(4:0_12:3_18:1), TG(6:0_8:0_18:1), TG(4:0_12:3_18:1), TG (12:0_18:2_18:3) and TG(16:0e_18:1_22:5) greatly affected the volatile flavor of yak milk. The 5 lipids in yak milk can produce some UFAs, monounsaturated fatty acid (MUFAs), SFAs and alcohol by the hydrolysis, then these compounds can be further transferred into other VFCs by the oxidation, saturation, esterification and dehydrogenation. In future, the volatile flavor of yak milk may be improved by the diet intervention based on this research results. The concentration of the crucial 5 lipids in feed for yak can be adjusted during feeding yak, then the volatile flavor in yak milk will be changed correspondingly.

5. Conclusions

There were significant differences in lipids, fatty acids, volatile flavor between yak colostrum and ordinary milk. Total 217 VFCs were detected in yak milk, and prenol lipids, organooxygen compounds, saturated hydrocarbons, benzene and substituted derivatives and fatty acyls were predominant components at class level. Of them, 44 DVFCs included 15 alcohols, 6 ketones, 4 esters, 11 aliphatic hydrocarbons, 3 aromatic hydrocarbons, 2 organic acids, 2 aldehydes and 1 nitrogen-containing organic compound. The differences of the volatile flavor in yak milk were mainly caused by 1-((1 s,3ar,4r,7 s,7as)-4-hydroxy-7-isopr-opyl-4-methyloctahdro-1 h-inden-1-Yl)ethan-one, 2,6,6-trimethyl-2,4-cycloheptadien-1-one, pentanal, 2-phenylethyl propionate, octanoic acid methyl ester, diphosphoric acid diisooctyl ester, (Z)-3,4,4-trimethyl-5-oxo-2-hexenoic acid and acetic acid; total 66 DLSs were detected, and 64 DLSs content were higher in yak colostrum; 18 fatty acids content was different in two kinds of yak milk, and 2 MUFAs and 9 PUFAs were contained. Further, there were positive correlation between crucial DVFCs and DLSs in yak milk, and the lipids contained PUFAs played important role in the the formation of volatile flavors in yak milk. The effect of lipids to the violate flavor in yak milk was mainly realized by the degradation of TG(4:0_12:3_18:1), TG(6:0_8:0_18:1), TG (4:0_12:3_18:1), TG(12:0_18:2_18:3) and TG(16:0e_18:1_22:5). These crucial milk lipids can produce some fatty acids and alcohol by hydrolysis, then these compounds further derived into others VFCs especially above crucial 8 VFCs by oxidation, saturation and esterification. The optimization of volatile flavor in yak milk and products may be realized by the control of the crucial 5 lipids in yak diet and dairy processing.

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Institutional review board statement

The animal study protocol was approved by the Ethics Committee of the Lanzhou Institute of Husbandry and Pharmaceutical Sciences, Chinese Academy of Agricultural Sciences.

CRediT authorship contribution statement

Lin Xiong: Writing – review & editing, Writing – original draft, Resources, Methodology, Investigation, Data curation. **Jie Pei:** Writing – review & editing, Validation. **Xingdong Wang:** Writing – original draft. **Shaoke Guo:** Data curation. **Mengli Cao:** Resources. **Zhiqiang Ding:** Validation. **Yandong Kang:** Resources. **Xiaoyun Wu:** Writing – original draft. **Min Chu:** Writing – review & editing. **Pengjia Bao:** Resources. **Qianyun Ge:** Resources. **Xian Guo:** Supervision, Project administration, Funding acquisition, Conceptualization.

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.

Data availability

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

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Appendix A. Supplementary data

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