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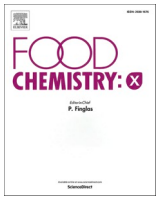

Characterization and discrimination of donkey milk lipids and volatiles across lactation stages

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ABSTRACT

The lipid and flavor in milk are key factors that affect its quality, which profiles during donkey lactation are not yet clear. In this study, the lipids and volatile compounds (VOCs) in donkey milk from stages of lactation were analyzed by using LC-MS and GC-IMS. A total of 1774 lipids were identified in donkey milk, spanning over 6 major categories and attributed to 30 subclasses. The 233 differentially expressed lipids were identified between donkey colostrum and mature milk, which participate in 20 metabolic pathways, including glycerophospholipid, linoleic acid, and sphingolipid. Additionally, 35 VOCs in donkey milk were identified, including 28.57% aldehydes, 28.57% ketones, 25.71% esters, and 8.57% alcohols. Of these VOCs, 15 were determined to be characteristic flavors in donkey milk, mainly including methyl 2-methylbutanoate, 2-pentanone, and butyl acetate. 11 significantly different VOCs were found between the groups. Acetone, 2-heptanone, and ethyl acetate-m were considered potential discriminatory markers.

1. Introduction

Milk, with its rich nutrients, diverse categories, and unique odors, is greatly beloved by consumers and has become an essential beverage today. Donkey milk is similar to breast milk in terms of composition, and has been widely considered an ideal substitute for breast milk. The lactose and protein contents in donkey milk are similar to those of breast milk, although the lipid content is much lower (Salimei & Fantuz, 2012). Donkey milk is used in infant formula, which is often supplemented with vegetable oil or fish oil. However, due to its low lipid content, donkey milk can also be consumed as part of a low-calorie diet by elderly individuals (Chiofalo et al., 2011). Therefore, it is necessary to conduct more comprehensive and in-depth research on the characteristics and profiles of donkey milk lipids, which would serve as a foundation for the development of donkey milk formula.

Previous studies have demonstrated that milk lipids serve important physiological functions, including energy storage, cell membrane formation and signal transmission (George et al., 2021; Ren et al., 2023). Milk lipids are mainly divided into glycerophospholipid (GP), glycerolipid (GL), sphingolipid (SP), and fatty acid (FA). These lipids consist of 98% non-polar lipids, mainly including triacylglycerols (TGs), located in fat globules and 0.5–1% polar lipids including GP and SP, found in the milk fat globule membrane (Zheng et al., 2014). It is speculated that milk lipids contain thousands of lipid molecules, making them the most complex substances in terms of lipid profile in nature. In bovine and donkey milk, a total of 335 lipids were identified across 13 subclasses including TG, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylglycerol (PG), sphingomyelin (SM), and phosphatidylinositol (PI) (Li, Li, Kang, et al., 2020; Li, Li, Wu, et al., 2020). In human milk and colostrum, previous studies detected 617 and 701 lipids, respectively (Garwolińska et al., 2017; Hewelt-Belka et al., 2020). In addition, flavor is a crucial factor in milk quality and significantly influences consumer repurchase. Volatile compounds (VOCs) play a critical role in milk flavor and can be categorized into aldehydes, ketones, esters, and alcohols. Although many types of VOCs are present in milk, only a small number of key flavor compounds significantly contribute to its overall flavor. Odor activity values (OAVs) are determined as characteristic flavor compounds in sensory flavor research by dividing the concentration by the threshold. VOCs with OAV ≥ 1 are generally considered to contribute to aroma components. According to a recent research report, the most important contributors to human milk flavor were 2,3-butanedione, (E)-2-decenal, nonanal, (E)-2-nonenal,

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octanal, 1-octen-3-one, hexanal, methional, and butanoic acid (Yu et al., 2024). In raw mare's milk, acetic acid, n-hexanoic acid, octanoic acid, decanoic acid, and lauric acid were identified as the characteristic flavor compounds (Xia et al., 2021). Through extensive an literature review, it has been found that only the flavor of donkey milk has been described as sweet and pleasant, with a milky white aroma and a sweet taste, without a lasting aftertaste (Malissiova et al., 2016). Fresh donkey milk has been found to contain 2-heptanone, 1,3-bis (1,1-dimethylethyl)-benzene, nonanal, D-limonene, octanoic acid, and 1-octanol (Vincenzetti et al., 2018). However, the characteristic VOCs profiles of donkey milk have not yet been reported.

Milk lipids, proteins, flavor and other nutrients are influenced by various factors such as animal species, dietary composition, season, environment, lactation periods, and production processes (Li, Li, Wu, et al., 2020). In bovine mature milk, the concentrations of total fat, TG, DG, PC, and PE were significantly lower compared to colostrum (Li, Li, Kang, et al., 2020). Similar results were also found in caprine milk lipid subclasses during lactation (Chilliard et al., 2003). However, lipids in human and donkey milk showed the opposite trend during lactation (Li, Li, Wu, et al., 2020; Zou et al., 2012). These findings confirm that lactation period is the most important factor affecting milk lipid profiles. The levels of esters, alcohols, and phenols in milk were significantly reduced for donkeys fed corn straw compared to wheat hull and wheat straw (Ren et al., 2023). The freeze-drying and spray-drying treatments significantly increased the levels of octanoic acid, 2-heptanone, and nonanal compared to fresh donkey milk (Vincenzetti et al., 2018). However, the variation pattern of VOCs in milk during lactation has not been explored, making it difficult to regulate the flavor of donkey milk.

Therefore, this study analyzed and screened the lipid profiles in donkey colostrum (DC) and mature milk (DM), as well as differentially lipids and key metabolism pathways using lipidomics technology based on LC-MS. Moreover, the VOCs profiles and characteristic flavor in donkey milk were comprehensively identified, and potential VOCs were identified to distinguish donkey milk from different lactation periods using gas chromatography–ion mobility spectrometry (GC-IMS) and multivariate analysis. The results provide novel insights into the lipid and VOC profiles underlying alterations in donkey milk during different lactation periods. The differential lipids and VOCs can serve as potential biomarkers, providing fundamental information for evaluating the quality of donkey milk and developing products.

2. Material and methods

2.1. Sample collection

Animal experiments were approved by the Animal Care and Use Committee of Liaocheng University (2023022706). Milk samples were collected from 12 Dezhou donkeys at Yucheng Huimin Agricultural Technology Co., Ltd. (Shandong, China), including 6 each for DC (3 day postpartum) and DM (3 month postpartum) samples. All donkeys were fed same diet and raised in a similar environment. They were healthy, aged between 3 and 5 years, and weighted 260–340 kg. The milk samples were immediately frozen in liquid nitrogen and stored at −80 °C in a refrigerator for further analysis.

2.2. Lipid analysis

Lipids from donkey milk samples were extracted using the chloroform/methanol method, as described in a previous report (Ren et al., 2023). Briefly, 1.0 mL of milk and 750 μL of chloroform/methanol (2:1, *v/v*) were mixed and then extracted at 4 °C for 2 h. The mixture was centrifuged at 12,000 rpm for 10 min at 4 °C. The supernatant was dried using a water bath nitrogen blowing instrument (WT-12, Hangzhou Miou Instrument Co., Ltd., China). Finally, the sample was dissolved in 200 μL of isopropanol, and the supernatant was filtered through a 0.22-μm PTFE membrane, and stored at −80 °C for lipid analysis.

Lipid profiles of the milk samples were analyzed using a UPLC-Q-Exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, MA, USA) with a C18 column (1.7 μm, 100 × 2.1 mm) for lipidomics. The column was maintained at a temperature of 50 °C. The mobile phase comprised (A) 60% acetonitrile and 40% water (*v:v*), and (B) 90% isopropanol and 10% acetonitrile (*v/v*), both containing 0.1% formic acid and 10 mM ammonium formate. The injection volume was 2 μL, and the gradient elution with a flow rate of 0.25 mL/min was as follows: 0 min, 30% B; 5 min, 43% B; 5.1 min, 50% B; 14.1 min, 70% B; 21–24 min, 99% B; 24.1–28 min, 30% B. An electrospray ion source (ESI) was used for MS, with spray voltages of 3.5 kV and − 2.5 kV in positive and negative modes, respectively. The capillary temperature and normalized collision energy were set at 325 °C and 30 eV, respectively. The Orbitrap analyzer scanned a mass range of *m/z* 150–2000 for full scan at a mass resolution of 35,000. Data dependent acquisition (DDA) MS/MS experiments were conducted using high-energy collision dissociation (HCD) scan. Dynamic exclusion was applied to remove unnecessary information in the MS/MS spectra. Lipids in the milk were analyzed and identified using LipidSearch v3.2 (Thermo Fisher Scientific, MA, USA) by matching the mass-to-charge ratio (*m/z*) and intensity, and were relatively quantified by calculating the relative peak areas.

2.3. VOC analysis

The VOC profiles of milk samples were analyzed using GC–IMS equipped with an automatic headspace sampling unit (G.A.S., Dortmund, Germany) and a 15 m × 0.53 mm × 1.0 μm capillary column (MXT-5, CTC-PAL, CTC Analytics AG, Zwingen, Switzerland). 3 mL of milk sample was injected into a headspace bottle (20 mL), which was incubated at 50 °C for 15 min with spinning at 500 rpm. Then, 0.5 mL of the headspace gas was injected into GC–IMS. The injector and GC column temperatures were 85 °C and 65 °C, respectively. The carrier gas and drift gas used were purity N2 (99.999%). The carrier gas flow rate was as follows: 2 mL/min at 0–2 min; 2–20 mL/min at 2–10 min; 20–100 mL/min at 10–20 min. The IMS instrument temperature, drift tube voltage, and drift gas flow rate were 45 °C, 5 kV, and 150 mL/min, respectively. The retention indices (RIs) of the VOCs were calculated using C4-C9 *n*-ketones (Sinopharm Chemical Reagent Beijing Co., Ltd., China) as external references under the same detection conditions. The VOCs were identified using RI and the drift time (DT) of standards in the GC-IMS database (G.A.S., Dortmund, Germany) and NIST library. 2-methyl-3-heptanone was used as an internal standard to quantify VOCs in donkey milk. The OAVs were obtained by dividing the concentration of VOCs by the threshold.

2.4. Statistical analysis

The data were analyzed with Tukey's test using SPSS 24 software (SPSS Inc., Chicago, IL, USA), which were presented as mean ± standard error of mean, and were considered to have statistical significance when $P < 0.05$. The principal component analysis (PCA), orthogonal partial least squares discriminant analysis (OPLS-DA), volcano plot, variable importance in projection (VIP) value, lipid metabolism pathway analysis, and receiver operating characteristic (ROC) analysis were performed by using MetaboAnalyst 5.0 online software. Gallery Plot, and Reporter of GC-IMS instrumental analysis software were using to analysis the spectra and fingerprints, respectively. Differential lipids were determined using VIP > 1 and $P < 0.05$. The screening criteria of potential marker was area under the ROC curve (AUC) > 0.9.

3. Results

3.1. Lipid profiles

As shown in Table 1, a total of 1774 lipid molecules were identified in donkey milk. These lipids were categorized into six major lipid

Table 1
Lipid profiles of donkey milk.

No.	Name	Subclass	Category	Number	Percentages of number (%)	Percentages of category number (%)
1	Triglyceride	TG		1099	61.95	
2	Diglyceride	DG	Glycerolipids [GL]	136	7.67	70.18
3	Monoglyceride	MG		10	0.56	
4	Phosphatidylcholine	PC		108	6.09	
5	Phosphatidylethanolamine	PE		92	5.19	
6	Phosphatidylserine	PS		24	1.35	
7	Phosphatidylinositol	PI		12	0.68	
8	Cardiolipin	CL		8	0.45	
9	Lysophosphatidylcholine	LPC	Glycerophospholipids [GP]	8	0.45	14.83
10	Lysophosphatidylethanolamine	LPE		4	0.23	
11	Phosphatidylglycerol	PG		3	0.17	
12	Phosphatidic acid	PA		3	0.17	
13	Glycerophosphoethanolamine-n-(biotinyl)	BiotinylPE		1	0.06	
14	Sphingomyelin	SM		82	4.62	
15	Ceramide	Cer	Sphingolipids [SP]	45	2.54	9.24
16	Simple glc series	HexCer		32	1.80	
17	Sphingosine	SPH		5	0.28	
18	Methyl phosphatidylcholine	MePC		40	2.25	
19	Bis-methyl phosphatidic acid	BisMePA	Derivatized lipids [DL]	23	1.30	3.89
20	Dimethylphosphatidylethanolamine	dMePE		5	0.28	
21	Bis-methyl phosphatidylethanolamine	BisMePE		1	0.06	
22	Campesterol ester	CmE		4	0.23	
23	Stigmasterol ester	StE	Sterol Lipids [ST]	3	0.17	0.51
24	Cholesterol ester	ChE		2	0.11	
25	Zymosterol ester	ZyE		17	0.96	
26	Acylglcsitosterol ester	AcHexSiE		2	0.11	
27	Wax esters	WE	Other lipids	2	0.11	1.35
28	N-acylethanolamine	AEA		1	0.06	
29	Acyl carnitine	AcCa		1	0.06	
30	Acylglccampesterol ester	AcHexChE		1	0.06	
Total	–	–	–	1774	100	100

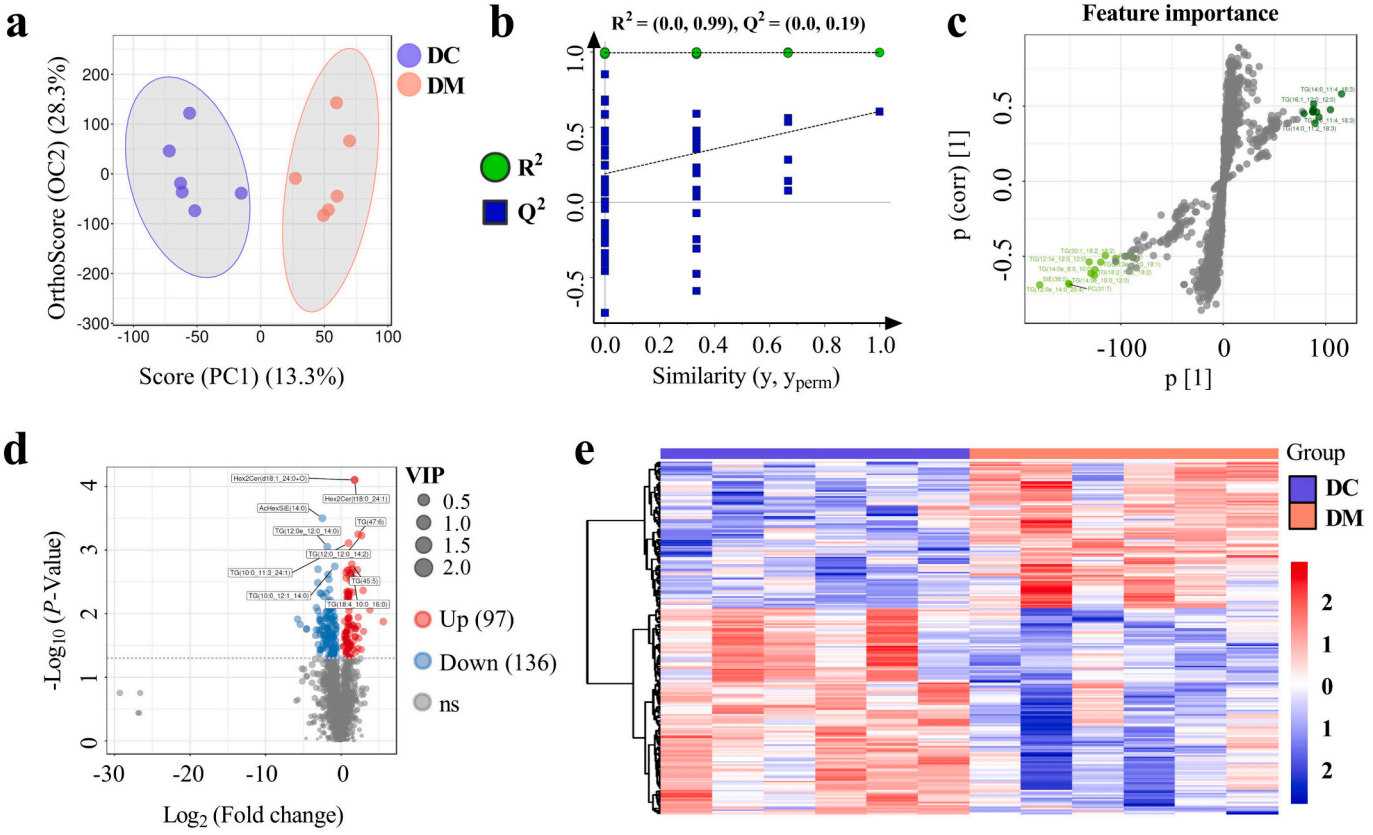

Fig. 1. Differential lipids in milk from donkey different lactation stages. Orthogonal partial least squares discriminant analysis (OPLS-DA) score plot (a), corresponding validation plots (b), and S-plots (c) of the lipidomic data for donkey milk. Differential lipids between DC and DM (d). Heatmap for the differential lipids (e). Donkey mature milk (DM), donkey colostrum milk (DC).

categories; glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), derivatized lipids (DL), sterol lipids (ST), and other lipids. Furthermore, these lipid molecules were classified into 30 subclasses. The most abundant subclasses were; 61.95% triglyceride (TG), 7.67% diglyceride (DG), 6.09% phosphatidylcholine (PC), 5.19% phosphatidylethanolamine (PE), 4.62% sphingomyelin (SM), 2.54% ceramides (Cer), and 2.25% methyl phosphatidylcholine (MePC).

3.2. Differential lipids and metabolism pathways

The qualitative lipid analysis achieved excellent separation for DC and DM by OPLS-DA (Fig. 1a). The corresponding OPLS-DA validation plots were R^2 and Q^2 intercept parameters of (0.0, 0.99) and (0.0, 0.16), respectively (Fig. 1b), which indicated that OPLS-DA was not overly fitted, OPLS-DA was reliable. The TG, including TG (14:0_11:4_18:3), TG (16:1_12:0_12:0), TG(14:0e_8:0_10:0), and TG(12:0e_14:0_20:4) was very important in donkey milk (Fig. 1c). A total of 233 differentially lipids were identified between DM and DC ($P < 0.05$, VIP > 1.0; Fig. 1d). Out of these, 136 lipids were downregulated in DM compared to DC. This group mainly consisted of 67 TGs, 15 PEs, 13 DGs, 9 SMs, and 8 PCs. On the other hand, 97 lipids were upregulated, including 82 TGs, 7 Hex2Cers, 3 BisMePAs, 2 MePCs, 2 SMs, and 1 DG (Fig. 1e; Table S1). These significantly different lipids were mapped to KEGG databases and were found to participate in 20 metabolic pathways (Fig. 2a). The five most relevant metabolic pathways were obtained at a significance level of $P < 0.05$, included GP metabolism, linoleic acid metabolism, sphingolipid metabolism, α-linolenic acid metabolism, and glycerolipid metabolism (Fig. 2b).

3.3. VOC profiles

As shown in Fig. 3 and Table 1, the 35 VOCs were identified in donkey milk (Fig. 3a), including 28.57% aldehydes, 28.57% ketones, 25.71% esters, 8.57% alcohols, and 8.57% unidentified (Fig. 3b). The ketones were the most abundant VOCs in donkey milk, followed by esters and aldehydes (Fig. 3c). The levels of ketone and ester were significantly higher in DM than in DC ($P < 0.05$; $P < 0.01$; Fig. 3d). A total of 15 characteristic VOCs with OAVs >1 were identified (Fig. 3e). These VOCs include methyl 2-methylbutanoate, 2-pentanone, butyl acetate, octanal, heptanal, hexanal, ethyl acetate, acetone, nonanal, pentanal, 2-butanone, 2,3-butanedione, ethyl hexanoate, 2-methylbutanal, and 2-heptanone (Fig. 3e). It is worth noting that the OAVs of ethyl acetate, octanal, and hexanal significantly increased in DM.

3.4. Comparison of VOCs in DC and DM

As shown in Fig. 4a, the VOCs of DC and DM show good repeatability according to planar graph. The VOC profiles of donkey lactation stages were significant difference by further comparing the fingerprints (Fig. 4b). The ethyl acetate-d, ethyl acetate-m, acetone, 2-pentanone-d, 2-pentanone-m, 2-heptanone-d, and 2-heptanone-m present different signals in DC and DM according to fingerprint (Fig. 4c and d). Thus, represent differential components in the fingerprint regions of the donkey milk from different lactation. In total, 11 significantly different VOCs were identified between the groups ($P < 0.05$), including 3 esters, 6 ketones, 1 alcohol, and 1 unidentified (Table 2). The levels of the ethyl hexanoate, ethyl acetate-M, ethyl acetate-D, and acetone were significantly higher in DM than in CM ($P < 0.05$), whereas the opposite was true for 2-heptanone-M, 2-heptanone-D, 2-pentanone-M, 2-pentanone-D, 2-butanone-M, unidentified 2, and 3-methyl-3-buten-1-ol ($P < 0.05$). The PCA based on VOCs data showed a clear separation between DC and DM (Fig. 5a), which the contribution rate of the first principal component is 84.1%, and the contribution rate of the second principal component is 8.1%. The ROC curves and parameters for the top 3 VOCs were show in Figs. 5b-d, with an area under the ROCs curve (AUC) are >0.9. the specificity and sensitivity are >80% for acetone, 2-heptanone, and ethyl acetate-D. The normalized intensity of acetone and ethyl acetate-D were significantly higher in DM than in DC ($P < 0.05$), whereas 2-heptanone show the opposite trend ($P < 0.05$).

4. Discussion

Lipid structures are complex and diverse, usually were divided into 6 major categories and further divided into 30–50 subclasses, with over 40,000 lipid molecules, making their analysis and identification difficult. Lipidomics technology, specifically LC-MS, has emerged as an approach for lipid detection in milk. While it is speculated that milk contains thousands of lipids, but only about 700 lipid molecules have been identified so far, leaving many lipids still unknown (Ren et al., 2023). In a study by Li, Li, Wu, et al. (2020), it was discovered that donkey milk contains 335 lipids that belong to 13 different subclasses. However, the study identified a total of 1774 lipids categorized into 6

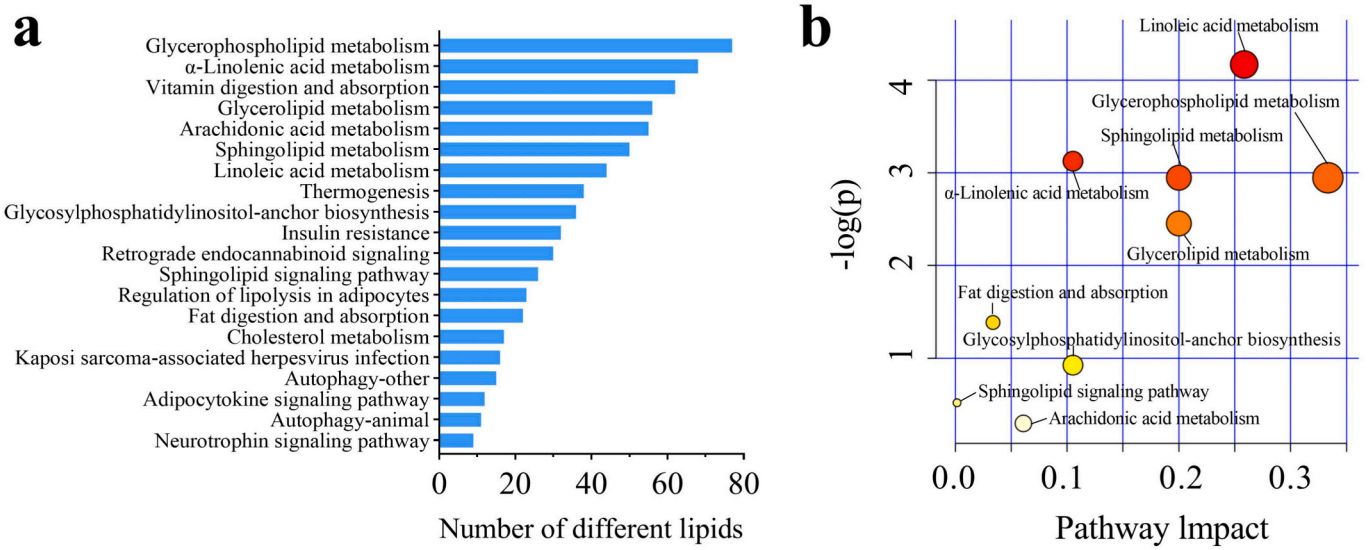

Fig. 2. Metabolic pathways involved in different lipid species in milk from donkey different lactation stages. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment pathways of significantly different lipids between donkey mature milk (DM) and donkey colostrum milk (DC) (a). Map of significant metabolic pathways related to lipids in DC compared with DM.

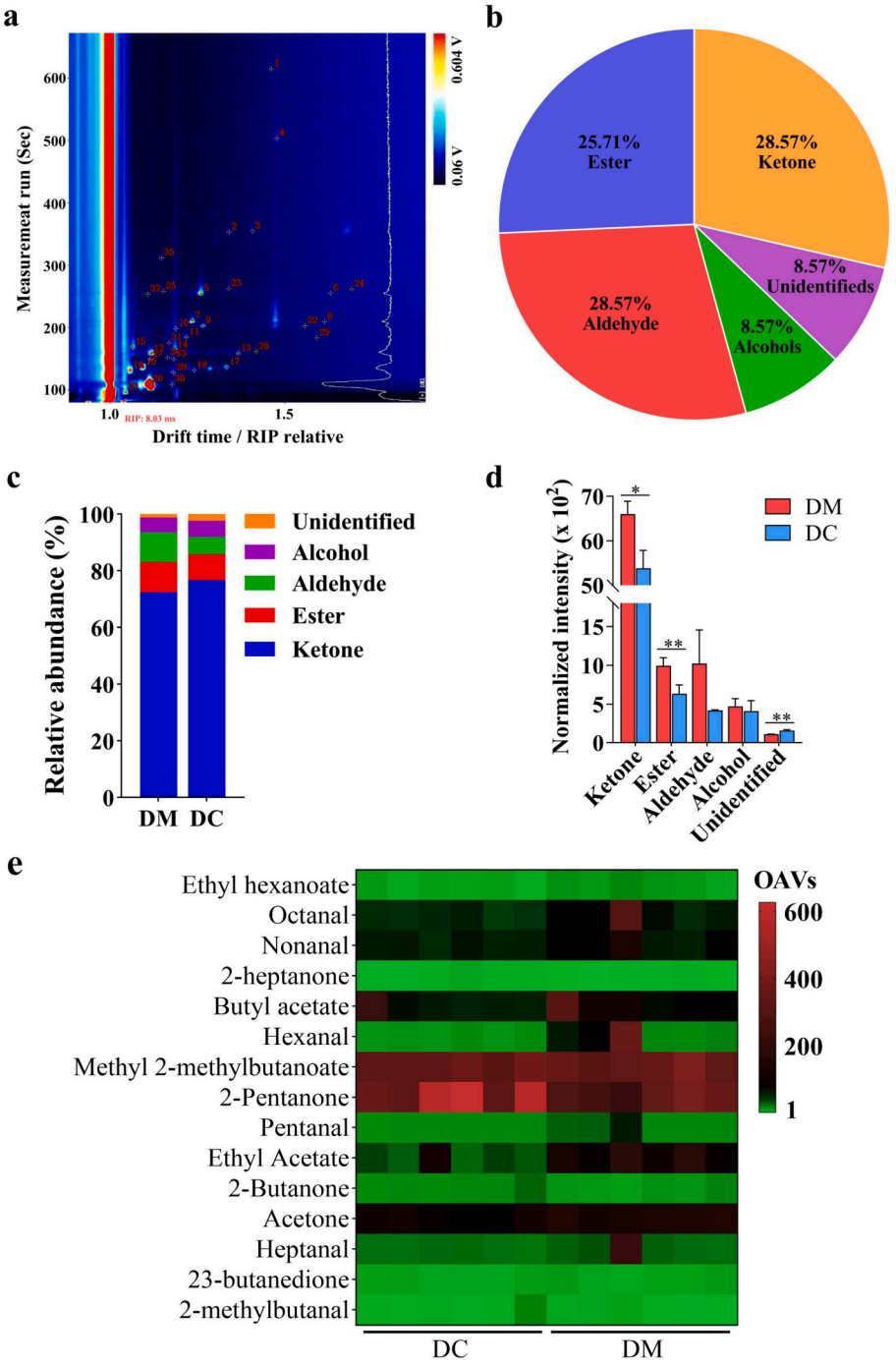

Fig. 3. Volatile compound (VOC) profiles of donkey milk. Number of VOCs (a). Number percentage (b) of VOC classes. Relative abundance (c) and concentrations (d) of VOC classes. The odor activity values (OAVs) were greater than or equal to 1 (e). The valus are presented as mean ± SEM ($n = 6$), $^{*}P < 0.05$, $^{**}P < 0.01$. Donkey mature milk (DM), donkey colostrum milk (DC).

major categories and further divided into 30 subclasses in donkey milk. A greater number and subclass of lipids have been found in donkey milk, enhancing our understanding of its composition and providing a foundation for utilizing milk lipids. The subclasses of lipids identified in donkey milk include TG, PC, PE, SM, PI, PS, Cer, and HexCer (Chiofalo et al., 2011). In our current study, GLs were found to be the most abundant, followed by GPs and SPs, mainly including subclasses TG, DG, PC, PE, SM, and Cer. Among them, TGs are the most abundant, aligning with findings in bovine milk (Li, Li, Kang, et al., 2020), and are an important source of energy and essential fatty acids for offspring. DG is a crucial lipid subclass in living organisms, functioning as a second messenger in various cellular activities and regulating gene expression related to lipid metabolism. Furthermore, this study also discovered two additional subclasses, namely MGs and sphingosine (SPH). MGs act as precursors for synthesizing functional lipids including TG, glycolipids, and phospholipids. The MGs containing PUFAs have physiological activities similar to PUFAs themselves, such as the antioxidant and anti-atherosclerotic effects of oleic acid 2-MGs (Cho et al., 2010). SPH, on the other hand, is a hydrolysis product of milk phospholipids. It reduces LPS levels in the blood and systemic inflammation by inhibiting the expression of pro-inflammatory genes in macrophages (Norris et al., 2017). The discovery of these previously unidentified lipids in donkey

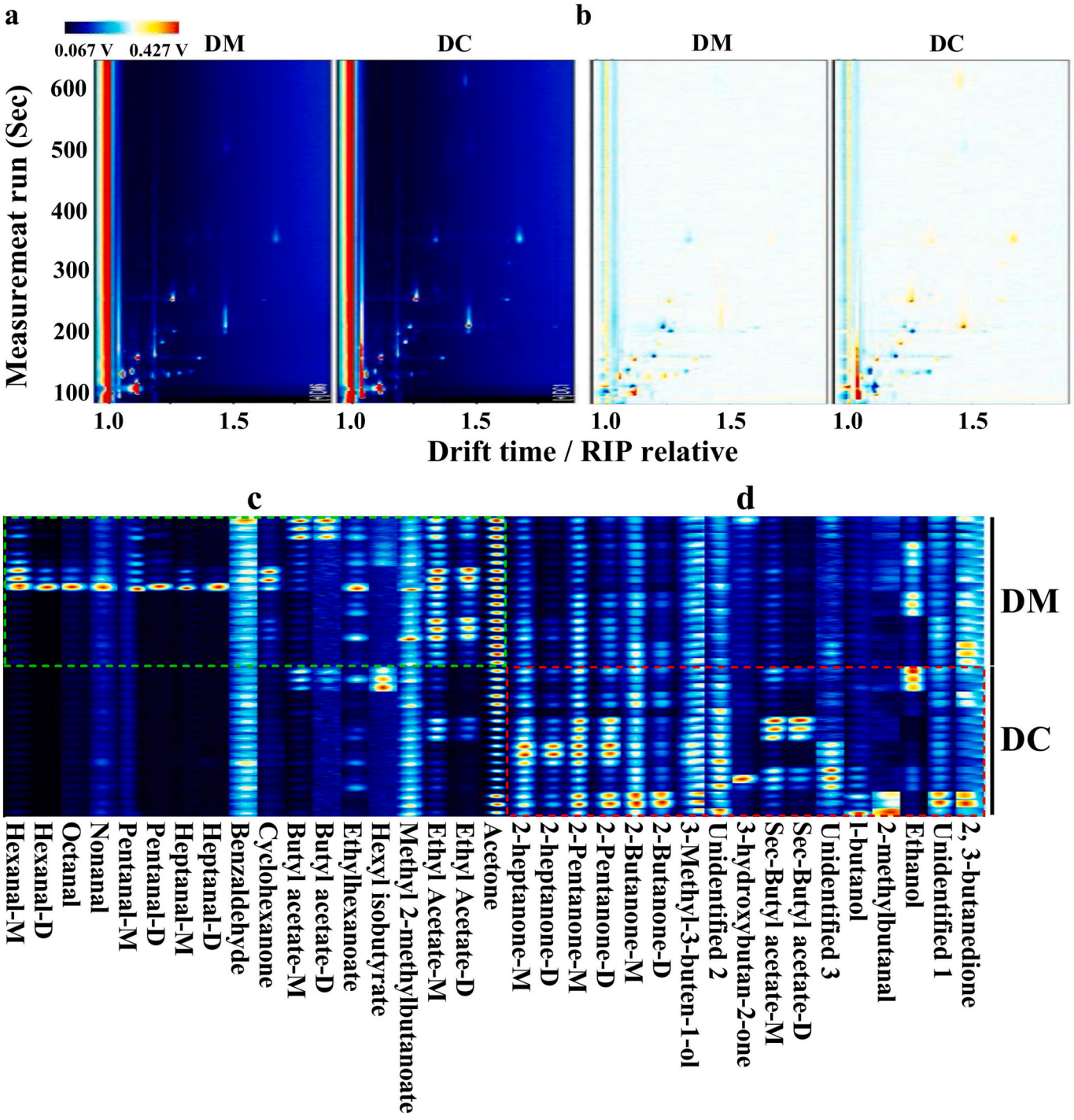

Fig. 4. Topographic plots of VOCs fingerprints for in lactation stage of donkey. The component (a) and differential spectrogram (b) of VOCs in donkey colostrum (DC) and mature milk (DM). The fingerprint gallery plots (c and d) for VOCs identified in DM and DC.

milk will enhance our in-depth understanding of its lipid composition and provide a foundation for the development of functional applications for donkey milk.

The lipid profiles in donkey milk were found to vary during different lactation periods. For instance, the levels of fatty acids, such as C18:0, C18:1, and C18:2n-6, showed significant differences at various stages of lactation (Li, Liu, Li, et al., 2020). Furthermore, a total of 60 different lipid molecules were identified between DC and DM (Li, Li, Wu, et al., 2020). In this study, we identified 233 lipids that exhibited significant differences across lactation periods, including 149 TGs, 15 PEs, and 14 DGs. It is evident that TGs were the most abundant different lipids, and their higher content in DM may be closely related to the higher energy requirements for growth and development in mammals (Silva et al., 2018). Furthermore, PEs were the second most common different lipid, with higher content in DC compared to DM. A previous study indicated hat PEs contribute to improved memory and brain function development (Modica-Napolitano & Renshaw, 2004). The higher levels of PEs in early lactation may play an important role in the development of the nervous system in donkey foals. It has been reported that the milk lipid profiles vary in relation to season and lactation (Martini et al., 2015). The different lipids were mapped to the KEGG database, and 20 metabolic pathways were identified. Among these, five were found to be most

Table 2
The detailed information on the volatile compounds in donkey milk.

No.	Compound	Class	CAS#	Formula	MW	RI	Rt [s]	Dt [ms]	DM	DC	*P* value
1	Hexyl isobutyrate	ester	C2349077	C10H20O2	172.3	1162.9	614.297	1.4639	55.94 ± 5.44	60.28 ± 14.27	0.7820
2	Ethyl hexanoate	ester	C123660	C8H16O2	144.2	1004.4	353.087	1.34321	60.11 ± 6.44[b]	37.54 ± 5.05[a]	0.0201
3	Octanal	aldehyde	C124130	C8H16O	128.2	1005.3	354.245	1.40904	57.09 ± 24.87	26.19 ± 0.81	0.2430
4	Nonanal	aldehyde	C124196	C9H18O	142.2	1105.7	503.094	1.47899	77.25 ± 13.11	54.94 ± 1.3	0.1210
5	2-heptanone-M	ketone	C110430	C7H14O	114.2	886.1	254.123	1.26261	260.07 ± 27.02[a]	447.09 ± 54.59[b]	0.0118
6	2-heptanone-D	ketone	C110430	C7H14O	114.2	887.2	254.728	1.63269	28.56 ± 4.87[a]	83.04 ± 23.16[b]	0.0441
7	Butyl acetate-M	ester	C123864	C6H12O2	116.2	805.7	210.839	1.23832	80.8 ± 28.53	51.16 ± 20.5	0.4180
8	Butyl acetate-D	ester	C123864	C6H12O2	116.2	802	209.023	1.61554	10.51 ± 2.44	8.51 ± 1.07	0.4700
9	Hexanal-M	aldehyde	C66251	C6H12O	100.2	788.1	202.363	1.26832	246.77 ± 129.03	47.66 ± 2.75	0.1540
10	Methyl 2-methylbutanoate	ester	C868575	C6H12O2	116.2	778.3	197.823	1.19117	87.88 ± 3.39	85.71 ± 2.83	0.6330
11	Sec-Butyl acetate-M	ester	C105464	C6H12O2	116.2	747.7	184.202	1.22117	80.37 ± 4.2	148.26 ± 44.98	0.1640
12	2-Pentanone-M	ketone	C107879	C5H10O	86.1	681.9	158.474	1.11972	365.43 ± 30.41[a]	515.49 ± 54.27[b]	0.0366
13	2-Pentanone-D	ketone	C107879	C5H10O	86.1	681	158.222	1.36911	62.93 ± 8.34[a]	125.78 ± 25.03[b]	0.0385
14	Pentanal-M	aldehyde	C110623	C5H10O	86.1	696.4	163.468	1.18852	292.17 ± 53.26	182.28 ± 1.62	0.0661
15	3-hydroxybutan-2-one	ketone	C513860	C4H8O2	88.1	709.4	168.496	1.06786	44.34 ± 5.02	38.4 ± 10.67	0.6260
16	Ethyl Acetate-M	ester	C141786	C4H8O2	88.1	600.8	135.68	1.09751	490.98 ± 60.51[b]	193.21 ± 51.25[a]	0.0038
17	Ethyl Acetate-D	ester	C141786	C4H8O2	88.1	602.3	136.078	1.33565	120.14 ± 28.71[b]	29.31 ± 9.97[a]	0.0136
18	2-Butanone-D	ketone	C78933	C4H8O	72.1	582.1	130.907	1.24288	71.25 ± 12.05	121.33 ± 25.26	0.1040
19	2-Butanone-M	ketone	C78933	C4H8O	72.1	581.3	130.708	1.06021	370.6 ± 30.48[a]	503.34 ± 41.51[b]	0.0275
20	Acetone	ketone	C67641	C3H6O	58.1	483.7	108.434	1.11664	5292.26 ± 228.18[b]	3479.42 ± 387.05[a]	0.0024
21	Ethanol	alcohol	C64175	C2H6O	46.1	419.6	95.904	1.04778	382.53 ± 99.18	294.5 ± 134.34	0.6100
22	Hexanal-D	aldehyde	C66251	C6H12O	100.2	788.4	202.504	1.55945	140.99 ± 117.21	12.65 ± 0.29	0.2990
23	Heptanal-M	aldehyde	C111717	C7H14O	114.2	898	261.879	1.34193	145.37 ± 77.35	58.03 ± 1.49	0.2850
24	Heptanal-D	aldehyde	C111717	C7H14O	114.2	897.3	261.408	1.69288	18.5 ± 10.27	8.52 ± 0.26	0.3540
25	Cyclohexanone	ketone	C108941	C6H10O	98.1	892	257.636	1.15459	56.79 ± 18.9	27.05 ± 1.04	0.1470
26	Pentanal-D	aldehyde	C110623	C5H10O	86.1	689.8	160.962	1.42036	26.18 ± 12.98	8.1 ± 0.47	0.1940
27	Unidentified 1	unidentified	–	–	–	648.7	148.712	1.11752	30.62 ± 2.85	36.3 ± 5.8	0.3990
28	2,3-butanedione	ketone	C431038	C4H6O2	86.1	567.2	127.224	1.18387	47.93 ± 4.85	43.61 ± 3.92	0.5040
29	Sec-Butyl acetate-D	ester	C105464	C6H12O2	116.2	745.5	183.253	1.59265	7.02 ± 0.38	18.08 ± 7.71	0.1830
30	Unidentified 2	unidentified	–	–	–	486.5	109.008	1.17962	67.77 ± 0.92[a]	103.49 ± 6.83[b]	0.0004
31	3-Methyl-3-buten-1-ol	alcohol	C763326	C5H10O	86.1	726.2	175.213	1.17047	27.91 ± 1.26[a]	32.18 ± 1.27[b]	0.0380
32	Unidentified 3	unidentified	–	–	–	885	253.453	1.11002	12.2 ± 1.56	17.86 ± 3.1	0.1340
33	1-butanol	alcohol	C71363	C4H10O	74.1	650.7	149.304	1.18445	60.2 ± 1.7	82.58 ± 14.43	0.1550
34	2-methylbutanal	aldehyde	C96173	C5H10O	86.1	658.4	151.512	1.16711	4.99 ± 0.7	6.69 ± 2.33	0.5000
35	Benzaldehyde	aldehyde	C100527	C7H6O	106.1	960.6	311.135	1.15046	13.33 ± 0.44	13.85 ± 0.38	0.3900

MW, molecular weight; RI, retention index; Rt, retention time; Dt, drift time; DM, donkey mature milk; DC, donkey colostrum milk.

related to lipid metabolism, including GP metabolism, linoleic acid metabolism, sphingolipid metabolism, α-linolenic acid metabolism, and glycerolipid metabolism. The lactation process is strongly linked to animal energy balance, and the results of this study suggest that different metabolic pathways during lactation contribute to these differences. These findings are consistent with previous studies on bovine milk (Li, Li, Kang, et al., 2020). In fact, the concentrations of lipids in milk are closely related to lactation, especially GP metabolism (Mesilati-Stahy & Argov-Argaman, 2014). PEs are one of the important components of milk GP and are part of mammalian cell membranes, serving essential physiological functions such as cell apoptosis and signaling processes (Li, Li, Kang, et al., 2020; Li, Li, Wu, et al., 2020; Li, Liu, Li, et al., 2020; Vance, 2008). Therefore, the changes in GP metabolism during lactation may have a significant impact on the development of donkey cubs. This relationship indicates that the lactation period influences lipid metabolism pathways, subsequently affecting specific lipids, such as TGs and PEs. Metabolic pathway analysis has identified key pathways involved in milk lipid metabolism, emphasizing the significant influence of lactation on these biochemical processes.

The characteristic VOCs in milk play a decisive role in the overall flavor, endowing it with unique aroma and taste. These sensory characteristics are essential for distinguishing different types of milk and dairy products. The primary VOCs contributing to these unique flavors include aldehydes, ketones, esters, nitrogen-containing compounds, sulfur-containing compounds, and terpene compounds (Chen et al., 2024). In this study, 35 VOCs were identified in donkey milk, which is consistent with the reported 45 VOCs in donkey milk identified using GC–MS (Ren et al., 2023). The VOCs are mainly classified into aldehydes, ketones, esters, and alcohols in donkey milk, of which ketones are the most abundant VOCs, followed by esters and aldehydes. These findings are consistent with those reported in a study on camel milk (Zhao et al., 2023). The predominance of ketones aligns with their known presence as common flavor compounds in milk and its products (Vagenas & Roussis, 2012). Additionally, the levels of ketones and esters in DM were significantly higher than those in DC. The VOCs in milk are influenced by the lactation period, likely due to variations in the level and profile of free fatty acids in milk.

The importance of flavor compounds in samples depended not only on their contents but also on their OAVs. OAVs $\geq$1 are generally considered to have a significant contribution to flavor (Liu et al., 2022; Sohail et al., 2022). We identified 15 VOCs with OAVs $\geq$1 in donkey milk, including methyl 2-methylbutanoate, 2-pentanone, butyl acetate, octanal, heptanal, and hexanal. These findings align with previous research on human milk (Yu et al., 2024), further underscoring the similarities between donkey milk and human milk (Aspri et al., 2017). Ester compounds, known for their low odor thresholds, are crucial flavor contributors in milk and dairy products. They are primarily formed through the esterification of free fatty acids and alcohols, resulting in buttery, fruity, and floral aromas (Gou et al., 2023; Zhang et al., 2016). Consistent with these findings, our study identified methyl 2-methylbutanoate, butyl acetate, ethyl acetate, and ethyl hexanoate as key flavor compounds of donkey milk. Aldehydes also play a significant role in the flavor profile of milk and its derivatives, originating mainly from Maillard reaction and fatty acid oxidation (Contador et al., 2015). At low concentrations, aldehydes impart herbal aromas, contributing to the fresh taste of milk, while at higher concentrations, they can produce unpleasant odors. Specifically, hexanal, with its grassy flavor, is a common flavor compound in milk (Francis et al., 2005; Gioacchini et al.,

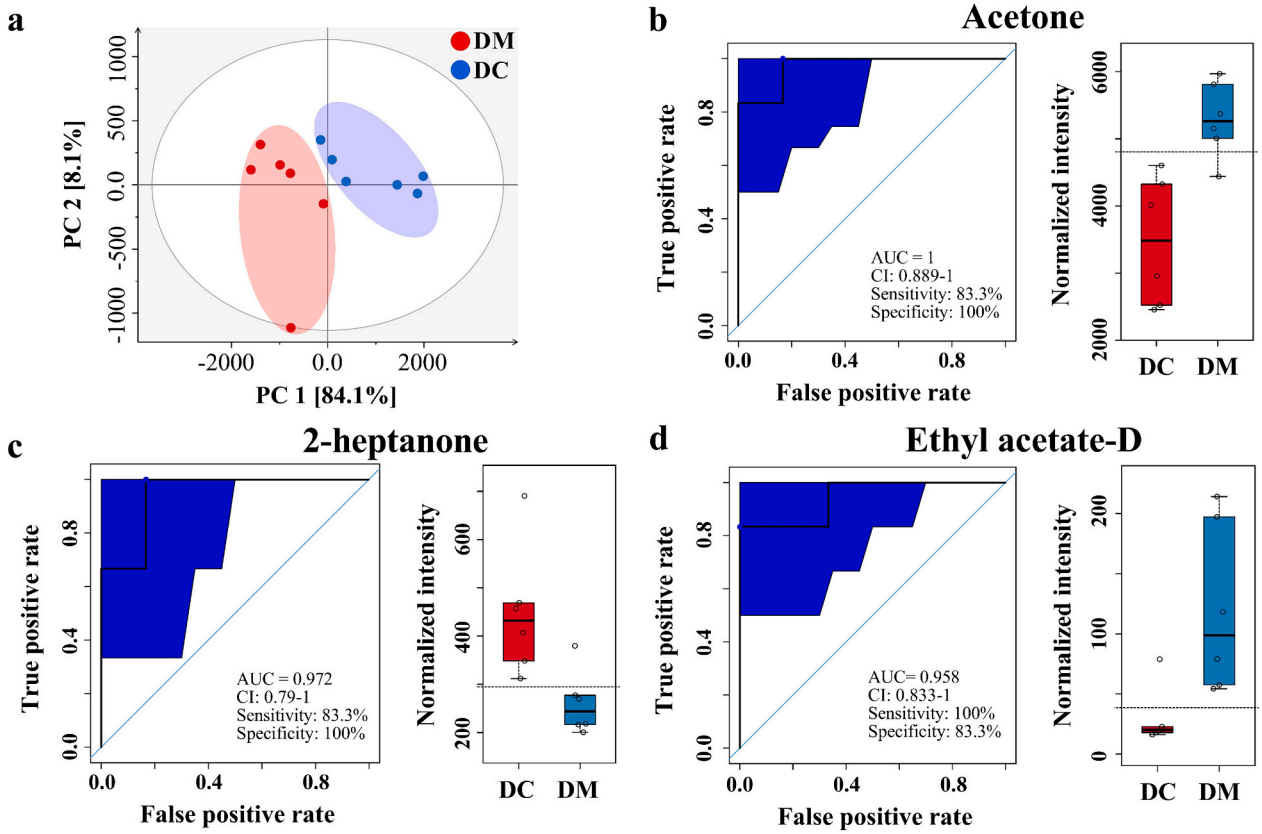

Fig. 5. Potential volatile marker compounds in milk from donkey different lactation stages. Principal component analysis (PCA) volatile components in milk (a). Receiver operating characteristic curve (ROC) and normalized intensity of volatiles in DM and DC (b-d). Area under the ROC curve (AUC) is the area under the ROC curve; CI 1–1 is the lower and upper limit of the AUC confidence interval. Donkey mature milk (DM), donkey colostrum milk (DC).

2010). In this study, we detected aldehydes such as octanal, heptanal, hexanal, nonanal, pentanal, and 2-methylbutanal as characteristic flavor compounds in donkey milk. Previous studies have reported that ethyl acetate, octanal, and hexanal typically exhibit sweet, green, green, and grassy aromas in milk, respectively (Clarke et al., 2022; Spitzer & Buettner, 2010). Our current findings show that the OAVs of ethyl acetate, octanal, and hexanal were significantly higher in DM than in DC, suggesting that mature milk has a stronger sweetness and grassy taste.

In this study, the PCA analysis was conducted without grouping samples, which does not account for random errors within the groups (He et al., 2021). To comprehensively compare the VOCs in donkey milk, we employed various methods, including fingerprint analysis and PCA, to characterize the overall differences in components between different samples. Our findings indicate that the VOCs of DC and DM exhibit good repeatability according to fingerprint and PCA analysis. This suggests that significant changes in VOCs may occur during the lactation period. These results align with previous studies which reported that fingerprint and PCA analysis could effectively differentiate between various milk and dairy products (Gou et al., 2023; Tan et al., 2024). Furthermore, previous research has demonstrated that ROC analysis can be utilized to screen biomarkers for identifying different animal species' meat, various animal tissues, and distinguishing between raw and cooked meat (Li et al., 2021; Li et al., 2022). The acetone, 2-heptanone, and ethyl acetate-D were identified as potential biomarkers for differentiating between DM and DC using ROC curves. These findings underscore that PCA and ROC analysis are the promising approaches for distinguishing between colostrum and mature milk.

5. Conclusion

In this study, we comprehensively analyzed and compared the lipid and VOC profiles in donkey milk from different lactation periods. A total of 233 significantly differential lipids were observed from a pool of 1774 lipids belonging to 30 subclasses in both DM and DC. These lipids were found to participate in 20 metabolic pathways, with particular emphasis on the glycerophospholipid, linoleic acid, sphingolipid, α-linolenic acid, and glycerolipid metabolism pathways. Among the 35 VOCs were identified in donkey milk, 15 compounds were determined to be characteristic flavors, including methyl 2-methylbutanoate, 2-pentanone, and butyl acetate. The VOCs profiles were found to significantly differ across different lactation periods, and we identified 3 VOC biomarkers that can discriminate between DM and DC. These findings enhance our understanding of the lipid and VOC characteristics in donkey milk and provide essential information for the development of high quality donkey milk production.

CRediT authorship contribution statement

Mengmeng Li: Writing – original draft, Resources, Methodology, Data curation, Conceptualization. **Lingyun Sun:** Investigation, Formal analysis, Data curation. **Xinyi Du:** Investigation, Formal analysis, Data curation. **Yan Zhao:** Resources, Investigation, Data curation. **Wei Ren:** Resources, Investigation, Data curation. **Limin Man:** Resources, Investigation, Data curation. **Mingxia Zhu:** Investigation, Data curation. **Guiqin Liu:** Investigation, Data curation. **Muhammad Zahoor Khan:** Investigation, Data curation. **Changfa Wang:** Writing – review & editing, Supervision, Project administration.

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

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fochx.2024.101740.

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