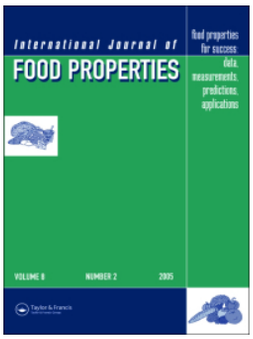

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Comparative characterization and correlation between lipids and volatile organic compounds in NingXiang and Berkshire-Ningxiang pork

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ABSTRACT

Lipids and volatile organic compounds (VOCs) are essential contributors to meat flavor. However, no studies to date have comprehensively explored the lipids and VOCs in raw pork from Ningxiang (NX) pigs and their hybrid breed Berkshire × Ningxiang (BN) pigs. This study aimed to identify the lipids and VOCs and reveal the crucial lipids for characteristic flavor formation. Samples were collected from the longissimus dorsi muscle of six NX and BN 8-month-old pigs each. The intramuscular fat (IMF) content of NX pork was 5.43%, almost twice that of BN pork ($p < .01$). Total 187 significantly different lipids were identified between NX and BN pork (variable importance in projection scores > 1, $p < .05$). Further analysis suggested 38 lipids were potential markers. Out of 66 identified VOCs, 16 key VOCs were screened both in NX and BN pork. Furtherly, hexanal-D, 2-methylbutanal, 3-methylbutanal, 1-penten-3-one, 1-octen-3-ol, and ethyl acetate-D were found to be key differential VOCs. Comparing with NX pork, BN pork significantly improved pungent odor. Correlation between the lipid markers and key VOCs demonstrated that phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylcholine (PC), and triglyceride (TG) were identified as key differentiating compounds for characteristic flavor. Our findings provided a novel understanding of pork identification and a basis for improving the flavor quality of NX and BN pork.

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Ningxiang pork; intramuscular fat; lipid; volatile organic compound; correlation

Introduction

With the development of animal husbandry and consumption level, the consumer requirements for meat products have changed from quantity to quality. Safe, high-quality, and flavorful pork is increasingly gaining favor. Flavor is an important component of meat quality and includes taste and smell. The material basis of taste includes nonvolatile organic compounds, such as fatty acids (FAs), amino acids, and inorganic salts. Volatile organic compounds (VOCs), such as aldehydes, alcohols, ketones, and esters form the material basis of odor. These non-VOCs and VOCs are collectively referred to as flavor substances.

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Intramuscular fat (IMF) has a critical effect on the eating quality of meat and is closely related to meat breed.[1–3] The IMF is a precursor of flavor substances that is mainly composed of phospholipids, triglycerides (TGs), and cholesterol.[4] Some studies in recent years indicated that the odor of raw meat is closely related to lipid oxidation, which determines its characteristic flavor.[5] The volatile derivatives produced by lipid degradation, whether directly as flavor compounds or reaction intermediates, play a crucial role in forming the ideal meat flavor. Most flavor substances are volatile, and VOCs largely contribute to pork flavor.[6] More than 1000 types of pork flavor substances have been studied both in China and abroad.[7] Most research on pork flavor has focused on its precursors, characteristic components, formation pathways, and influencing factors.[8,9]

The Ningxiang (NX) pig is a famous indigenous pig breed in China, and NX pork is the geographical indication of Chinese national agricultural products. The NX pig is a specialty of Ningxiang City in Hunan Province, with a breeding history of over 1000 years. It has a high farrowing rate, rough feeding tolerance, and fatness characteristics, and it exhibits tender succulent flavor.[10] Berkshire pig is native to England and is lean-type. Berkshire (♂) × Ningxiang (♀) (BN) pig shows excellent hybrid characteristics, and its meat is more suitable for market demands than that of crossbred Duroc (♂) × Ningxiang (♀) pig.[11] Recent studies have evaluated growth performance, meat quality, and gut microbes in NX pigs.[12–14] However, to date, few studies have comprehensively analyzed the lipids and VOCs in the raw meat of NX pigs and their hybrids. Furthermore, only little information is available on the correlation between lipids and VOCs in NX and BN pork. Lipidomics is the systematic study of all lipids in an organism. In the food quality and safety field, lipidomics has gradually attracted the attention of researchers, and many organizations and research institutions have been conducting relevant research.[15] The present study aimed to identify the differences in lipidomic and volatilomic profiles between NX and BN pork, characterize the potential lipid markers and key VOCs, and then investigate their correlation to reveal the crucial lipids for the formation of characteristic flavors.

Materials and methods

Sample collection and preparation

All the experiments in this study were reviewed and approved by the Institutional Animal Care and Use Committee of Hunan Institute of Animal & Veterinary Science (Approval number: 20211015). Six NX and six BN pigs were provided by Hunan Liushahe Ningxiang Pigs Ecological Husbandry Limited Company (Changsha City, China). These pigs were fed under the same conditions for eight months from birth. After slaughter, the average carcass weights of NX and BN were 53.58 ± 2.50 kg and 81.06 ± 4.46 kg, respectively. The approximate 200 g *longissimus dorsi* (LD) muscle was sampled from each pig. Six NX and six BN samples were stored at −80°C for analysis. Each test was repeated three times for each analysis item.

IMF analysis

The IMF content was quantified using the Soxhlet extraction method, and the samples (5 g) were extracted with anhydrous ether.[16]

Untargeted lipidomic analysis

Untargeted lipidomics was carried out by Shanghai Bioprofile Biotechnology (China). Each sample (20.00 g) was ground in liquid nitrogen and 0.06 g test ground sample was transferred to a centrifuge tube. Ice-cold methanol (75%, v/v, 400 µL) was added in the centrifuge tube and mixed with ultrasound for 15 min in an ice bath. Then, 1 mL of icy methyl-tert-butyl ether (MTBE) was added, vortexed fully, and mixed rotationally for 1 h in a refrigerator at 4°C. The

mixture was then ultrasonicated for 15 min in an ice bath. Water (250 μL) was added to the mixture, vortexed for 1 min, and incubated at 24–28°C for 10 min. After centrifugation at 14,000 ×g for 15 min at 4°C, the upper layer contained the lipids. The lipids were dried using a nitrogen-blowing instrument. The dried lipid components were redissolved in an appropriate amount of isopropanol/methanol (1/1, v/v) and transferred to the injection bottle. The lipid samples were stored at −20°C for analysis.

Lipid components were separated using an ultra-high-pressure liquid chromatography system (UHPLC, Ultimate 3000, Thermo Scientific, USA). The system parameters were as follows: column temperature, 60°C; flow speed, 300 μL/min; injection volume, 5 μL. Mobile phase A was acetonitrile/water (6:4, v/v) containing 0.77 g ammonium formate. Mobile phase B was acetonitrile/isopropyl alcohol (1:9, v/v). The gradient elution procedure was as follows: 0–10.5 min, 30%–100% B; 10.5–12.5 min, 100% B; 12.5–12.51 min, 100%–30% B; 10.5–16 min, 30% B. The automatic sampler was kept at 6°C all the time. After the UHPLC process, the next step was mass spectrometry (MS; Q-Exactive Plus, Thermo Scientific, USA). The MS parameters were as follows: heater temperature, 300°C; sheath gas flow rate, 45 arb; auxiliary gas flow rate, 15 arb; sweep gas flow rate, 1 arb; capillary temperature, 350°C; spray voltage, 3.0 KV for positive and 2.8 KV for negative mode. Lipids were determined using the software of LipidSearch (Version 4.1.30, Thermo Scientific, USA), which is based on the precursor ions and multistage MS data of each independent sample. The LipidSearch data were normalized to the total peak area.

VOC analysis

VOCs were identified using a flavor analyzer (FlavourSpec®, G.A.S., German) and gas chromatography-ion mobility spectrometry (GC-IMS). Samples (2 g) were ground, transferred to a headspace vial (20 mL), and incubated at 60°C for 20 min (incubation speed: 500 rpm). Then, a headspace 0.5 mL was injected automatically with a heated syringe (85°C). The chromatographic column type was MXT-5 (15 m × 0.53 mm, 1 μm, RESTEK, USA). The samples were separated on a column maintained at 60°C using nitrogen carrier gas (99.99% purity). The carrier gas flow program was as follows: The starting velocity was 2 mL/min, which was maintained for 2 min and then increased to 100 mL/min for 20 min. The tested objects were ionized in the IMS detector at 45°C and transferred to a drift tube using nitrogen (99.99% purity) at a speed of 150 mL/min.

Each group of samples was named NXZ-01–NXZ-06 and BNZ-01–BNZ-06, and each sample number was consistent with the NX1–6 and BN1–6 samples, respectively. VOCs were identified qualitatively using VOCal software, which included two databases, the National Institute of Standards and Technology (NIST, version 20, USA) and Ion Mobility Spectrometry (IMS) Drift Time data library (G.A.S., German). Each VOC was identified according to its retention time and drift time using the corresponding NIST and IMS databases. VOCs were semi-quantified by comparing their peak volumes as relative abundance. The percentage relative content (%) was evaluated according to peak volumes normalization (the volume of a certain peak was expressed as a percentage of the total volumes of all peaks).

ROAV analysis

The relative odor activity values (ROAV) were used to determine the contribution of each VOC to the overall flavor profile. ROAV was calculated based on the following formula for characterizing the key VOCs. The higher the ROAV, the greater the contribution.

$$\mathrm{ROAV} = \mathrm{OAVi}/\mathrm{OAVmax} \times 100$$

Odor activity value (OAV) was measured by OAVi=Ci/Ti, where Ci was the relative content (%), and Ti presented the odor threshold of a given VOC in water. OAVmax was designated as the highest OAV

in all samples. The odor threshold was obtained from Odor & Flavor Detection Thresholds in Water (http://www.leffingwell.com) and other literature.[17–20]

Data processing

Data are expressed as means ± standard error. Student's t-test was performed using SPSS software (version 22.0; IBM Corp., Armonk, NY, USA). Statistical significance was set at $p < .05$, and extreme significance was set at $p < .01$ and $p < .001$. Principal component analysis (PCA), partial least squares discriminant analysis (PLS-DA), and orthogonal partial least squares discriminant analysis (OPLS-DA) were conducted using SIMCA-P 14.1 (Umetrics, Umea, Sweden). Clustering correlation heat map with signs was performed using the OmicStudio tools at https://www.omicstudio.cn.

Results

IMF contents of NX and BN pork

The IMF contents of NX and BN pork are listed in Table 1. The IMF contents of NX pork were almost twice as high ($p < .01$) as that of BN pork.

Lipid profiles of NX and BN pork

A total of 36 lipid classes were detected, including 1213 lipid molecular species of NX and BN pork (Table S1). The top five lipid molecular species were phosphatidylcholine (PC), phosphatidylethanolamine (PE), TG, sphingomyelin (SM), and phosphatidylserine (PS) (Figure 1). Total phospholipids and glycerolipids were the top two lipid classes, with 855 and 189 molecular species, respectively. Especially, TG with 153 molecular species occupied the vast majority of glycerolipids.

Multivariate statistics on the lipidomic data were used to distinguish the NX and BN pork. As shown in Figure 2a, the two groups of NX and BN samples were presented an obvious separation trend in t[1] and t[2]. To further investigate the difference between NX and BN groups, OPLS-DA model was established to analyze the lipids (Figure 2b). The NX group could be well separated from BN group, and the difference between groups was maximized at t[1]. Total 187 significantly different lipids were screened according to variable importance in projection (VIP) scores > 1 from OPLS-DA and $p < .05$ (Table S1). Hierarchical clustering was performed based on these 187 lipids (Figure 2c). Compared with the BN group, the NX group had 103 up-regulated lipids dominated by 32 TGs, 15 PEs, 15 DGs, and 11 LPEs ($p < .05$), and 84 down-regulated lipids primarily including 22 PCs, 14 PEs, 9 LPCs, and 6 PSs ($p < .05$). Furthermore, 38 lipids that met the criteria of VIP > 1, $p < .01$, and fold change (FC) >2 or < 0.5 were selected as potential markers (Figure 3). Among these lipid markers, the phospholipids accounted for the largest proportion, reaching 68.4%, followed by TG, reaching 15.8%.

VOCs of NX and BN pork

NX and BN pork were rich in VOCs. The GC-IMS spectra and VOCs peak volumes of the 12 samples were obtained (Figure S1, Table S2). As shown in Figure 4a, total of 66 identified VOCs and 7 unknown VOCs were detected in NX and BN pork. The color and area of the fingerprint dots indicate the contents of VOCs. The darker the color and the larger the area, the higher the contents. Obviously,

Table 1. IMF contents of NX and BN pork.

Item	NX	BN	*p* Value
IMF content (%)	5.43 ± 1.23^{A}	2.67 ± 1.00^{B}	.002

NX, Ningxiang; BN, Berkshire × Ningxiang; IMF, intramuscular fat.
^{A,B}Different capital letters within a row differ significantly ($p < .01$).

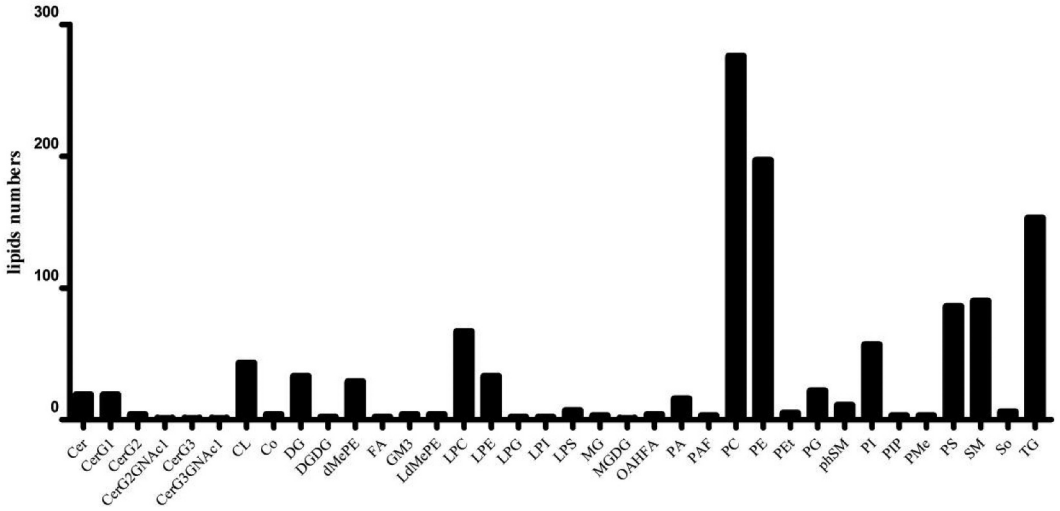


Figure 1. Numbers of detected lipid species of each lipid class. (Cer, ceramides; CerG1, monogylcosyceramide; CerG2, diglycosylceramide; CerG2GNAc1, simple glc series 1; CerG3, triglycosylceramide; CerG3GNAc1, simple glc series 2; CL, cardiolipin; Co, coenzyme; DG, diglyceride; DGDG, digalactosyldiacylglycerol; dMePE, dimethylphosphatidylethanolamine; FA, fatty acid; GM3, gangliosides; LdMePE, lysodimethylphosphatidylethanolamine; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; LPG, lysophosphatidylglycerol; LPI, lysophosphatidylinositol; LPS, lysophosphatidylserine; MG, monoglyceride; MGDG, monogalactosyldiacylglycerol; OAHFA, (O-acyl)-1-hydroxy fatty acid; PA, phosphatidic acid; PAF, platelet-activating factor; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEt, phosphatidylethanol; PG, phosphatidylglycerol; phSM, phytosphingosine; PI, phosphatidylinositol; PIP, phosphatidylinositol phosphate; PMe, phosphatidylmethanol; PS, phosphatidylserine; SM, sphingomyelin; So, sphingosine; TG, triglyceride.).

some VOCs showed great differences in contents between samples in the same column, such as 2-methylpropyl acetate, ethyl acetate-D, isobutyl butanoate, 3-methylbutanal, and propanol. In Figure 4b,c, the 66 VOCs were divided into nine categories: aldehydes (17), ketones (12), alcohols (11), esters (14), acids (5), furan (1), sulfur compounds (3), olefins (2), and amines (1). In NX pork, the percentage contents of alcohols occupied the most abundance, followed by ketones, aldehydes, esters, acids, and others. In BN pork, the highest contents were ketones, followed by alcohols, aldehydes, esters, sulfur compounds, and others in turn. The PCA score plot (Figure 4d) showed that PC1 and PC2 accounted for 41% and 19% of the variables' contribution, respectively. Samples from each group were clustered separately, and the two test groups could be well distinguished.

Based on Table 2, ROAV of decanal in NX and BN pork were both set to 100 and considered to be the highest. The VOCs with ROAV ≥ 1 were considered as key odor compounds, while VOCs with 0.1 < ROAV < 1 had modifying effects on flavor.[21] There were 16 key VOCs both in NX and BN pork, including 9 aldehydes, 4 esters, 1 ketone, 1 alcohol, and 1 sulfur compound. Among these key VOCs, hexanal-D, 2-methylbutanal, 3-methylbutanal, 1-penten-3-one, 1-octen-3-ol, and ethyl acetate-D were screened as key differential VOCs ($p < .05$). The VOCs with 0.1 < 1 mainly belonged to aldehydes, esters, sulfur compounds, and olefins. The relative contents of all acids were significantly higher in NX pork than that in BN pork ($p < .05$) except for hexanoic acid-D ($p > .05$), and the ROAV of acids were all smaller than 0.1.

Correlation between lipids and VOCs of NX and BN pork

As shown in Figure 5 and Table S3, correlation analysis was conducted between 38 potential lipid markers and 16 key VOCs. In terms of quantity, the top 5 lipid classes of PE, PI, PC, PS, and TG had 29, 21, 20, 20, and 16 high correlation coefficient (r) values with VOCs, respectively ($p < .05$). The top 5 lipid classes of PE, PI, PC, PS, and TG had 20, 13, 13, 13, and 12 high *r* values with the key differential

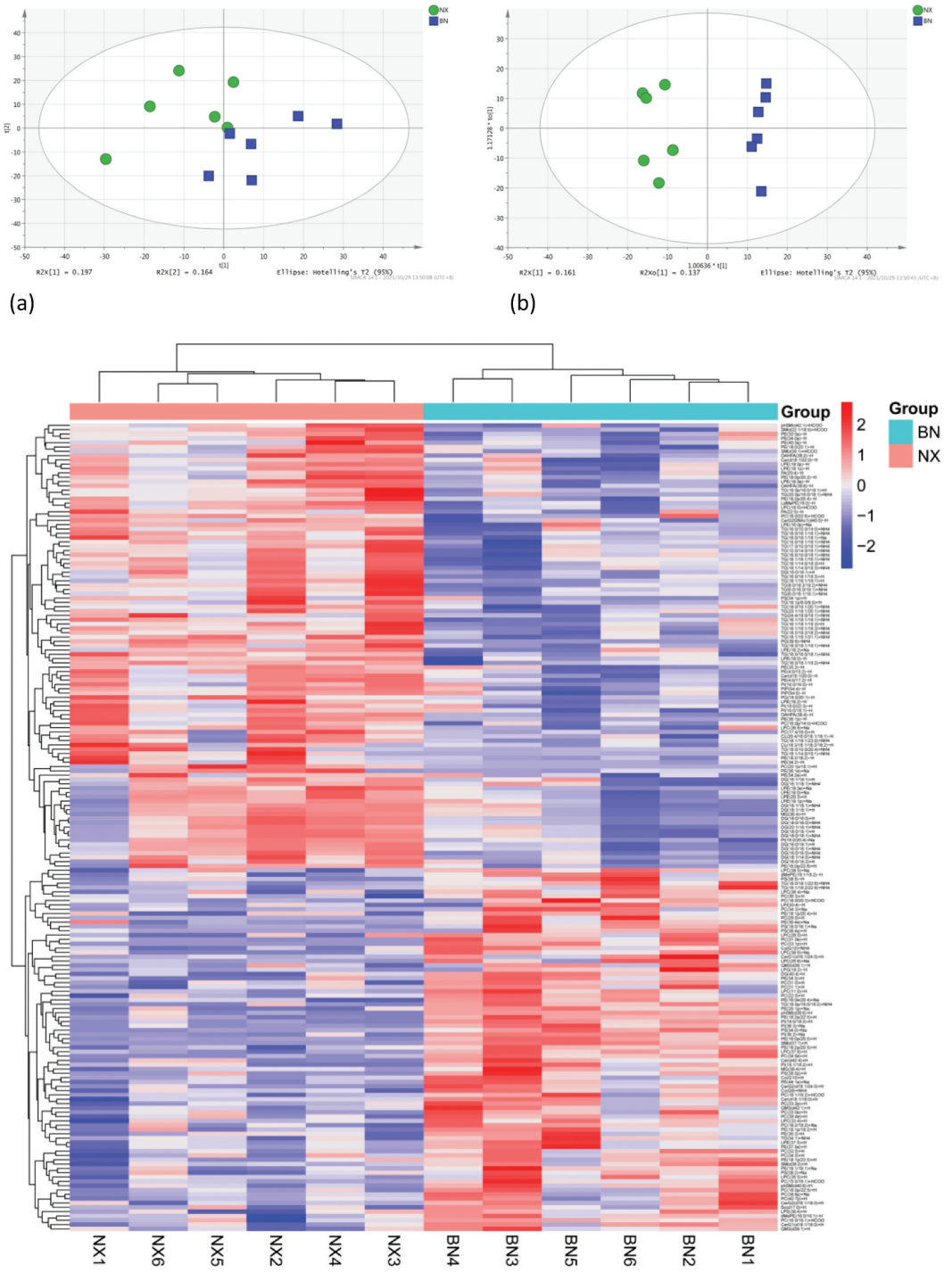
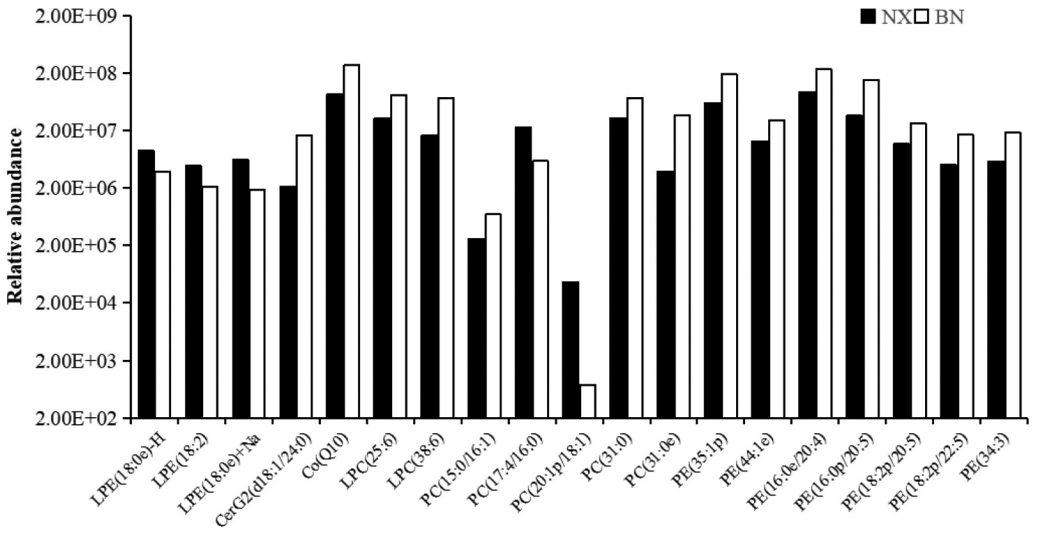
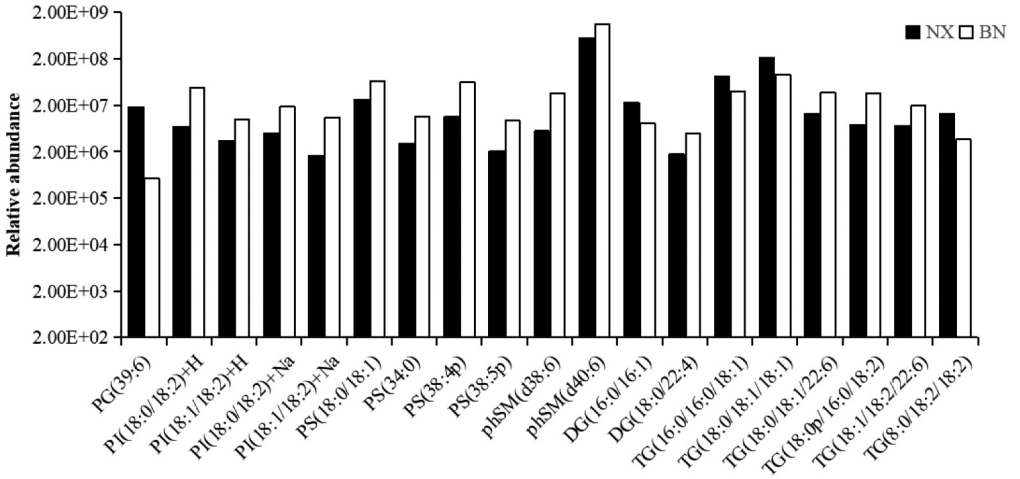


Figure 2. Multivariate statistics based on the lipidomic data between Ningxiang (NX) and Berkshire × Ningxiang (BN) pork. (a) Principal component analysis score plot, (b) orthogonal partial least squares discriminant analysis score plot, and (c) heat map of hierarchical cluster analysis.



(a)



(b)

Figure 3. (a & b) Potential lipid markers between Ningxiang (NX) and Berkshire × Ningxiang (BN) pork (variable importance in projection > 1, $p < .01$, fold change > 2 or < 0.5).

VOCs except for ethyl acetate-D, respectively ($p < .05$). Among the top 5 classes, all lipids contained unsaturated FAs except for PC (31:0), PC (31:0e), and PS (34:0). There were almost no significant correlation between lipids and esters ($p > .05$), except for 4 lipids being well correlated with ethyl acetate-M ($p < .05$).

Discussion

IMF contents of NX and BN pork

It's well known that the local purebred pigs in China are almost obese breeds. In general, the IMF contents of purebred pork are remarkably higher than that of hybrid pork. The IMF content in the

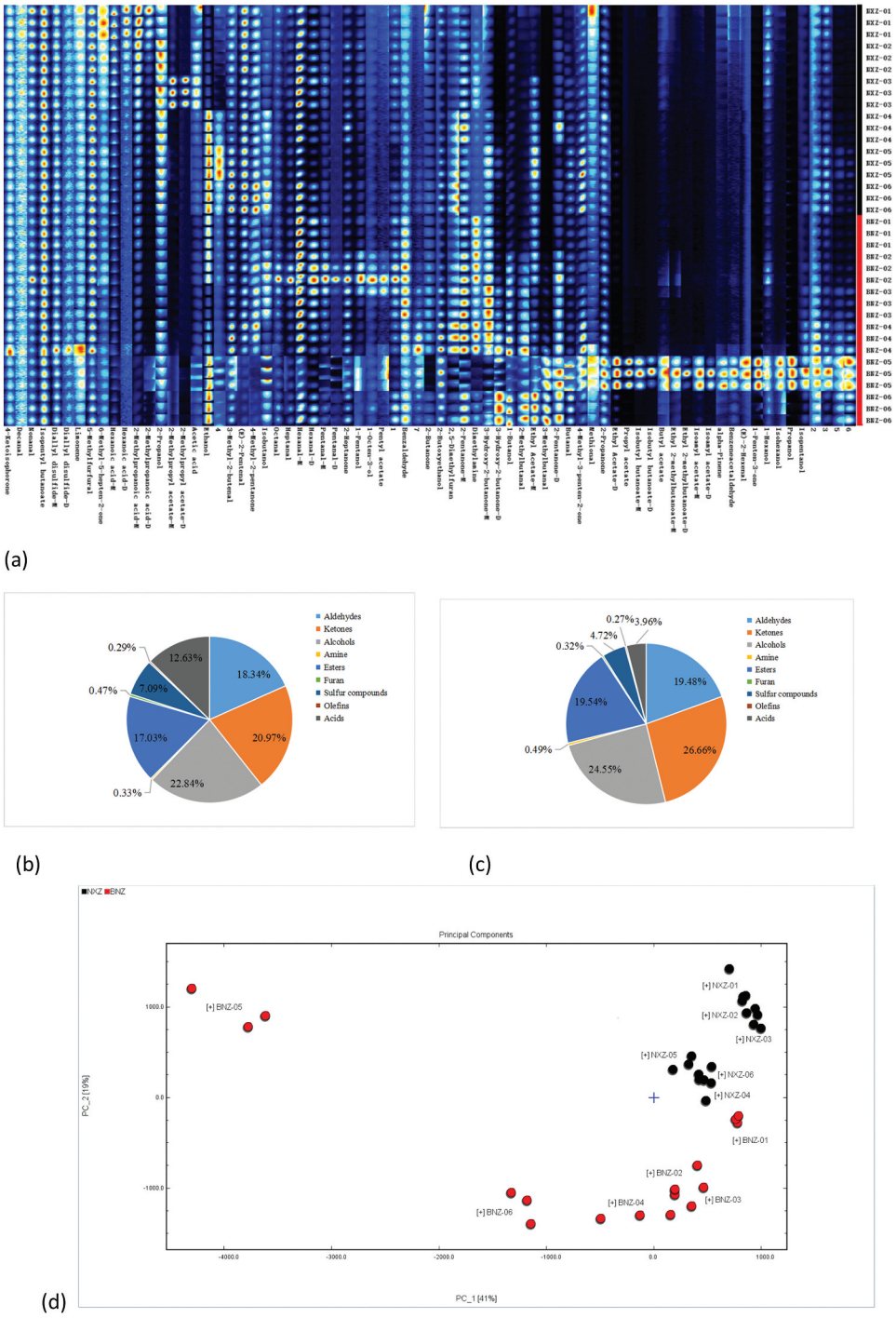


Figure 4. The volatile organic compounds (VOCs) of Ningxiang (NX) and Berkshire × Ningxiang (BN) pork. (a) Fingerprints plot in the gas chromatography-ion mobility spectrometry spectrum, (b) the VOCs composition in NX pork, (c) the VOCs composition in BN pork, and (d) PCA score plot. In (a), each line showed all signal peaks selected from a sample and each column showed the signal peaks of the same VOC in different samples. M: monomer; D: dimer.

Table 2. Identified volatile organic compounds (VOCs) in NX and BN pork.

Count	Compound	RI	Rt [sec]	threshold (ppb)	Relative content(%)		ROAV	
					NX	BN	NX	BN
				Aldehydes				
1	Decanal	1262.7	727.16	0.1	0.97 ± 0.12	0.69 ± 0.21	100.00	100.00
2	Nonanal	1107.8	504.68	1	2.13 ± 0.39	1.53 ± 0.49	21.959	22.174
3	Octanal	1003.7	355.29	0.7	0.63 ± 0.17	0.58 ± 0.29	2.165	2.802
4	Heptanal	898.1	261.69	3	0.60 ± 0.06	0.54 ± 0.20	8.837	11.180
5	5-Methylfurfural	962.6	316.29	20	2.91 ± 0.34	2.02 ± 0.72	1.500	1.464
7	Hexanal-M	788.3	202.70	5	3.58 ± 0.82	3.41 ± 1.47	7.381	9.884
8	Hexanal-D	787.3	202.15	5	2.64 ± 1.12^{b}	4.16 ± 2.43^{a}	5.443	12.058
9	3-Methyl-2-butenal	767.1	193.57	—	0.87 ± 0.14	0.57 ± 0.16	—	—
10	(E)-2-Pentenal	739.0	182.13	1500	1.38 ± 0.24	0.62 ± 0.35	0.009	0.006
11	Butanal	587.5	134.92	9	0.61 ± 0.19	0.62 ± 0.16	0.699	0.998
12	2-Methylbutanal	653.4	152.34	1	0.16 ± 0.03^{B}	0.93 ± 0.53^{A}	1.649	13.478
13	3-Methylbutanal	644.2	149.90	1.1	0.17 ± 0.02^{B}	2.80 ± 2.69^{A}	1.593	36.891
14	Pentanal-M	689.2	161.85	12	0.84 ± 0.30	0.99 ± 0.55	0.722	1.196
15	Pentanal-D	690.9	162.57	12	0.14 ± 0.03	0.23 ± 0.12	0.120	0.278
16	(E)-2-Hexenal	833.4	226.14	17	0.09 ± 0.01	0.10 ± 0.03	0.055	0.085
17	Benzaldehyde	955.7	310.40	750.9	0.21 ± 0.03^{b}	0.28 ± 0.11^{a}	0.003	0.005
18	Benzeneacetaldehyde	1037.3	403.58	6.3	0.20 ± 0.07	0.20 ± 0.09	0.327	0.460
	Ketones							
19	6-Methyl-5-hepten-2-one	988.6	338.23	50	0.24 ± 0.06	0.14 ± 0.05	0.049	0.041
20	2-Butanone-M	567.6	129.670	50000	2.96 ± 1.17	2.19 ± 1.11	0.001	0.001
21	4-Ketoisophorone	1156.6	574.83	1250	3.35 ± 0.35	2.53 ± 0.83	0.028	0.029
22	2-Heptanone	889.6	255.35	140	0.81 ± 0.25	1.00 ± 0.43	0.060	0.104
23	2-Pentanone-M	675.4	158.15	70000	1.75 ± 0.35	1.78 ± 0.77	0.000	0.000
24	2-Pentanone-D	679.6	159.25	70000	0.79 ± 0.43	1.35 ± 0.37	0.000	0.000
25	2-Propanone	492.2	109.76	500000	4.07 ± 0.71	3.21 ± 0.43	0.000	0.000
26	3-Hydroxy-2-butanone-M	706.5	168.93	800	3.16 ± 0.77	4.26 ± 2.45	0.041	0.077
27	3-Hydroxy-2-butanone-D	707.2	169.21	800	0.96 ± 1.07^{b}	6.81 ± 5.58^{a}	0.012	0.123
28	4-Methyl-3-penten-2-one	789.9	203.49	—	0.90 ± 0.20	0.68 ± 0.27	—	—
29	1-Penten-3-one	692.3	163.14	1	0.36 ± 0.10^{b}	1.91 ± 2.60^{a}	3.711	27.681
30	4-Methyl-2-pentanone	729.1	178.10	170	0.94 ± 0.14^{a}	0.54 ± 0.34^{b}	0.057	0.046
	Alcohols							
31	Ethanol	449.3	98.43	100000	15.74 ± 10.52^{A}	13.35 ± 5.32^{B}	0.002	0.002
32	Propanol	535.0	121.06	9000	0.59 ± 0.09^{b}	2.74 ± 4.82^{a}	0.001	0.004
33	2-Propanol	495.3	110.59	9787.9	0.68 ± 0.13	0.33 ± 0.08	0.001	0.000
34	1-Butanol	647.1	150.68	500	0.72 ± 0.08^{B}	1.95 ± 0.95^{A}	0.015	0.057
35	Isobutanol	607.4	140.18	7000	0.46 ± 0.20	0.43 ± 0.19	0.001	0.001
36	1-Pentanol	756.8	189.41	4000	0.69 ± 0.27	0.94 ± 0.67	0.002	0.003
37	Isopentanol	726.2	176.93	250	0.44 ± 0.08^{B}	0.58 ± 0.31^{A}	0.018	0.034
38	1-Hexanol	867.1	243.65	2500	0.70 ± 0.39^{A}	0.52 ± 0.20^{B}	0.003	0.003
39	Isohexanol	827.3	222.93	—	0.13 ± 0.03^{B}	0.24 ± 0.17^{A}	—	—
40	2-Butoxyethanol	914.3	275.34	—	0.82 ± 0.10	0.85 ± 0.24	—	—
41	1-Octen-3-ol	981.8	332.53	1	0.23 ± 0.04^{b}	0.31 ± 0.17^{a}	2.371	1.483
	Esters							
42	2-Methylpropyl acetate-M	744.6	184.42	66	0.90 ± 1.03	0.29 ± 0.13	0.141	0.021
43	2-Methylpropyl acetate-D	743.2	183.86	66	0.38 ± 0.56	0.09 ± 0.02	0.059	0.007
44	Isobutyl butanoate-M	963.7	317.18	—	0.18 ± 0.02^{b}	0.43 ± 0.62^{a}	—	—
45	Isobutyl butanoate-D	964.1	317.54	—	0.15 ± 0.02	0.22 ± 0.21	—	—
46	Isopentyl butanoate	1085.4	472.61	—	11.92 ± 1.27	8.82 ± 2.78	—	—
47	Ethyl Acetate-M	595.8	137.13	5	1.30 ± 0.70	1.28 ± 0.50	2.680	1.224
48	Ethyl Acetate-D	598.0	137.69	5	0.91 ± 0.32^{b}	3.78 ± 6.62^{a}	1.876	3.616
49	Ethyl 2-methylbutanoate-M	838.2	228.64	0.1	0.09 ± 0.04	0.19 ± 0.17	9.278	9.087
50	Ethyl 2-methylbutanoate-D	839.6	229.36	0.1	0.09 ± 0.01	0.11 ± 0.07	9.278	5.261
51	Isoamyl acetate-M	870.5	245.42	2	0.07 ± 0.01	0.13 ± 0.18	0.361	0.311
52	Isoamyl acetate-D	871.2	245.78	2	0.08 ± 0.01	0.11 ± 0.09	0.412	0.263
53	Propyl acetate	705.3	168.44	—	0.43 ± 0.10	0.74 ± 1.03	—	—
54	Butyl acetate	801.8	209.72	66	0.10 ± 0.05	0.13 ± 0.09	0.016	0.009
55	Pentyl acetate	910.1	271.79	5	0.09 ± 0.02^{b}	0.11 ± 0.05^{a}	0.186	0.105
	Acids							
56	Acetic acid	579.1	132.71	22000	0.85 ± 0.44^{A}	0.33 ± 0.10^{B}	0.000	0.000
57	2-Methylpropanoic acid-M	769.0	194.35	8100	5.09 ± 2.45^{a}	1.36 ± 0.79^{b}	0.006	0.001
58	2-Methylpropanoic acid-D	770.3	194.87	8100	1.22 ± 1.28^{a}	0.28 ± 0.14^{b}	0.002	0.000

(*Continued*)

Table 2. (Continued).

Count	Compound	RI	Rt [sec]	threshold (ppb）	Relative content(%)		ROAV	
					NX	BN	NX	BN
59	Hexanoic acid-M	987.3	337.16	3000	5.27 ± 1.91[a]	2.19 ± 0.76[b]	0.018	0.003
60	Hexanoic acid-D	987.3	337.16	3000	0.34 ± 0.10	0.18 ± 0.05	0.001	0.000
	Furan							
61	2,5-Dimethylfuran	701.9	167.05	—	0.44 ± 0.18	0.36 ± 0.18	—	—
	Sulfur compounds							
	Methional	905.0	267.54	0.2	0.12 ± 0.03	0.07 ± 0.02	6.186	1.674
62	Diallyl disulfide-M	1060.8	437.23	30	6.27 ± 0.66	4.60 ± 1.41	2.155	0.733
63	Diallyl disulfide-D	1061.2	437.83	30	0.57 ± 0.06[a]	0.45 ± 0.14[b]	0.196	0.072
	Olefins							
64	Limonene	1028.5	390.88	10	0.13 ± 0.02	0.10 ± 0.03	0.134	0.048
65	alpha-Pinene	943.9	300.40	6	0.16 ± 0.07	0.14 ± 0.08	0.275	0.112
	Amine							
66	Dimethylamine	586.4	134.65	33	0.33 ± 0.12[b]	0.58 ± 0.29[a]	0.103	0.084

NX, Ningxiang; BN, Berkshire × Ningxiang; RI, retention index; Rt, retention time; ppb, parts per billion; M, Monomer; D, Dimer; ROAV, relative odor activity value; —, threshold not found.
[a,b]Different lowercase letters within a row differ significantly ($p < .05$); [A,B]different uppercase letters within a row differ significantly ($p < .01$).

longissimus thoracis muscle of Guangdong small-ear spotted pigs was significantly higher than that of their crossbred pigs.[22] In addition, the IMF content in the *longissimus thoracis* muscle of local Jiaxing pigs was greatly higher than that of hybrid Qinglian Black pigs.[23] In this study, the IMF content of NX pork was 5.43%, and it was about two times that of BN pork. These results were consistent with the report that NX pigs had over 5% IMF, while the IMF content of crossbred pigs was approximately 2%.[24] This indicates that NX pork has more flavor precursors than BN pork, which could provide a better flavor material basis.

Comparative characterization of lipidomics between NX and BN pork

Pork flavor is largely determined by the metabolic reaction involved in lipid production and deposition. Therefore, the lipid composition of pork must be explored to improve its flavor traits. Lipids play a key role in the production of cooked and raw meat flavors through thermal oxidation or autoxidation.[5,25] In this study, 1213 lipid molecules were detected, including 855 phospholipid molecules (70.49%) and 153 TG molecules (12.61%). A large number of phospholipids and TGs can significantly contribute to pork flavor.[26] Phospholipids and TGs are the main components of the IMF. Fernandez et al. reported that the IMF content is closely related to TG, and the increase in TG content almost completely reflects the increase in the IMF.[27] However, this study found no linear relationship between the IMF and TG content, for TG content had no significant difference between NX and BN pork. In this study, the number of phospholipid molecules was the highest, and TGs were the second among the potential lipid markers. Zhou et al. demonstrated that phospholipids were the most significantly differential lipids, followed by TGs in the LD muscle of Xidu black pigs between IMF-H group and IMF-L group,[28] which was consistent with our results. In a recent study, phospholipids and TGs were found to be the main lipids that differentiated Jianhe White Xiang and Large White pork.[29] Perhaps, the distinct lipids between NX and BN pork are related to their lipogenic potential. The expression levels of acyl-CoA synthetase long-chain family member 1 (*ACSL1*), pyruvate dehydrogenase kinase 4 (*PDK4*), uncoupling protein 3 (*UCP3*), and fatty acid-binding protein (*FABP3*) in lipogenic metabolism in the LD muscle of Chinese purebred Wei pigs were higher than those of Yorkshire pigs, and these differentially expressed genes were mainly involved in phospholipid catabolism, TG anabolism, and lipid storage regulation.[30] In porcine adipose tissue, TGs were hydrolyzed by adipose triglyceride lipase (*ATGL*) at the first ester bond.[31] The potential genes that affect lipids in NX and BN pork need further researches.

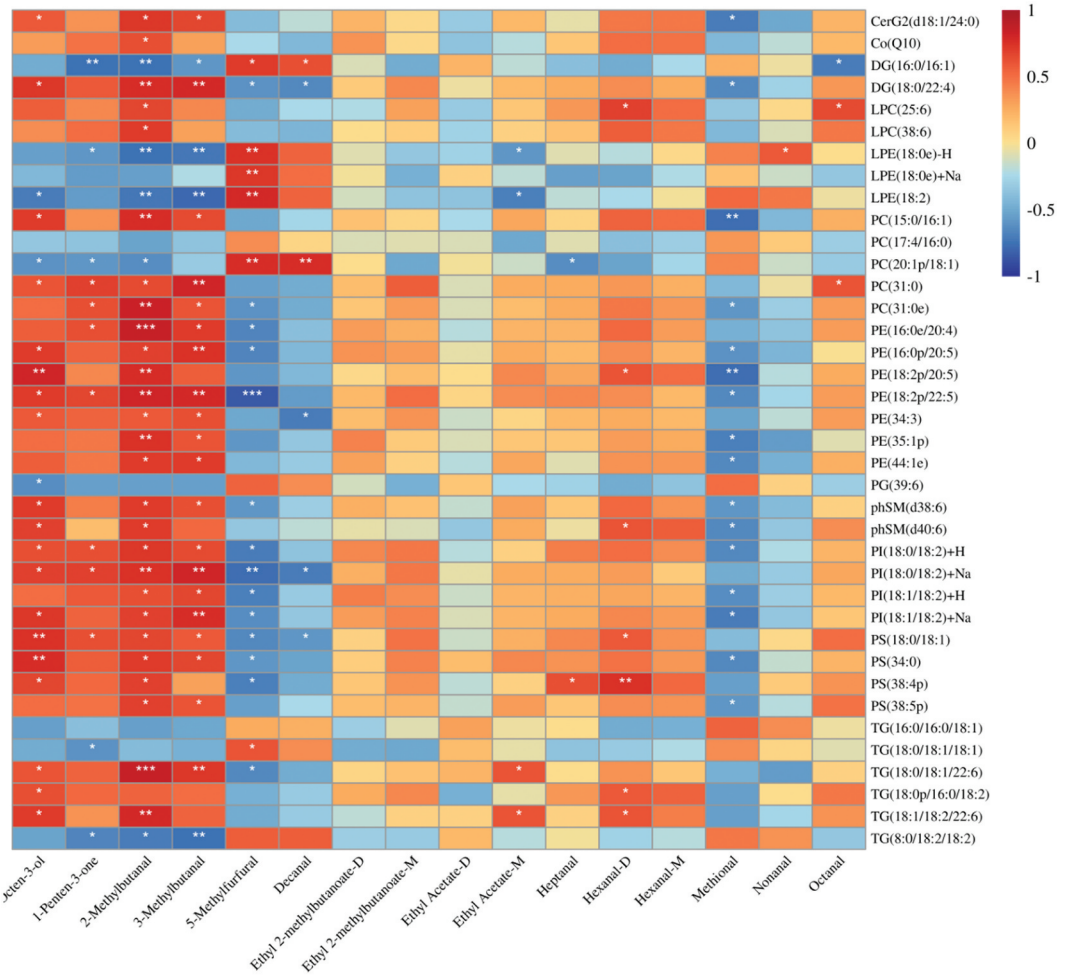


Figure 5. Correlation analysis of the lipid markers and key VOCs in NX and BN pork. The red and blue units indicate positive and negative correlation, respectively. The darker the color, the stronger the correlation (*$p < .05$, **$p < .01$, ***$p < .001$).

The lipid composition in the cluster heat map was consistent with the composition of overall lipids, both of them indicating that phospholipids and glycerolipids were the dominant lipids. What's more, the heat map illustrated visually that NX group has more up-regulated lipids than BN group. It further confirmed that IMF content in NX pork was significantly higher than that in BN pork. This was similar to previous researches about pork lipids. Zhang et al. proved that IMF content of Luchuan pigs was significantly higher than that of Duroc pigs by comparing the regulating lipids in cluster heat map.[32] Wang et al. investigated the local pure pigs and their hybrid progeny using lipid heat map, showing that the significant differences in IMF content corresponded to differences in lipid composition.[22]

Comparative characterization of VOCs between NX and BN pork

Some studies have compared flavor substances in indigenous Chinese pork and hybrid pork.[33,34] Flavor compounds have a close relationship with the type of pork. In this study, the number with 17 of aldehydes was the highest among VOCs, but their relative abundances were lower than those of alcohols and ketones. Aldehydes are the main flavor substances in pork, with a low threshold and great

contribution to flavor.[35,36] For flavor contribution of VOCs, it not only depended on the content, but also on the odor threshold, and ROAV was an important indicator for determining the contribution to the entire flavor.[37] In this study, decanal with a sweet and flower aroma had the highest ROAV of 100 due to its super low threshold, explaining its vast contribution to flavor in NX and BN pork. Nonanal and hexanal were the main aldehydes with high contents and high ROAV. Nonanal contains the fragrance of rose, citrus, and fat, and it was reported that its content was very high in five pig breeds.[36] Hexanal is produced by the oxidation of arachidonic acid or linoleic acid, which imparts the flavor of raw oil, fragrant grass, and pungent smell. Hexanal is regarded as a predictor of the oxidation flavor of meat, especially it had a good correlation with the oxidation flavor after a week of storage.[38] In our study, hexanal was the main VOCs in NX pork, while the previous study was reported that hexanal was not the main flavor component in NX raw pork.[39] Perhaps, it was due to the different sample suppliers and VOC test methods in spite of the similar carcass weight of about 50 kg and the same sampling location. Octanal has strong orange and fatty odor, providing the characteristic flavor for samples, and its ROAV was greater than 8 despite their low relative contents. 2-Methylbutanal and 3-methylbutanal are both fruit flavored compounds, especially 3-methylbutanal giving sharp apple-like aroma with so high ROAV in BN pork. These two methyl-branched aldehydes are the metabolites from fat and carbohydrate or generated by degradation of meat protein.[40] What's more, there were a few aldehydes played a modifying role for flavor both in NX and BN pork, such as butanal (pungent, fruity), pentanal-D (fermented bread, fruity), and benzeneacetaldehyde (hyacinth).

Ketones are generated by the oxidation of fatty acids and degradation of amino acids.[41] 1-Penten-3-one contributed greatly to entire aroma in NX and BN pork, and it held a strongly pungent odor of pepper, garlic, and onion. 2-Heptanone (fruity, milky) and 3-hydroxy-2-butanone-D (cream) modified the flavor only in BN pork. Nevertheless, the other ketones almost were odorless on entire flavor, which was largely attributed to their high odor threshold.

Alcohols production is related to fat oxidation and ketone reduction.[42] The relative contents of ethanol in this experiment were the highest among VOCs in NX and BN pork, reaching 15.74% and 13.35%, respectively, but its ROAV was less than 0.1 for high odor threshold. The low contents of almost other alcohols as well as their high threshold led to little contribution to flavor in NX and BN pork except for 1-octen-3-ol, which was consistent with the result that 1-octen-3-ol was the only alcohol in the key odor-active compounds.[34] Here, 1-octen-3-ol with ROAV > 1 conferring a characteristic mushroom smell had an important effect on aroma, which was in agreement with the previous reports.[43,44]

The VOCs with sample individual differences in the fingerprint most were esters. Various types of pork contain different esters, and there are no ubiquitous esters that can reflect the characteristic pork flavor.[45] This may be related to the ester formation pathway, which involves a reaction between acids and alcohols. Different pig breeds, acid and alcohol reaction substrates, and reaction conditions could be the factors influencing the individual differences in NX and BN esters in this experiment. Generally, esters played an important role in meat flavor, although their effect was not as strong as that of aldehydes in this study. Many esters have a pleasant fruity aroma, such as ethyl 2-methylbutanoate with ROAV > 1 possessing a strong apple and pineapple aroma, and isoamyl acetate with 0.1 < ROAV < 1 having an odor of banana and pear. However, ethyl acetate with ROAV > 1 had not only slight fruit aroma but also especially pungent odor-like ether.

Sulfur compounds usually exist in pork, and most of them are associated with meaty aroma. Methional was the key VOC in spite of its very low contents, which possessed a characteristic flavor of onion, meat, and cooked potatoes. It's generally produced from the interaction between L-methionine and reducing sugar in Strecker's degradation.[46] Diallyl disulfide-M with a strong garlic odor contributed significantly to NX flavor. Compared to NX pork, the contents of three sulfur compounds in BN pork were lower, and it showed that hybrid pork could inhibit the generation of sulfur compounds to some extent.

In this experiment, all acids were contributed poorly to meat flavor because of their high threshold. Acids mainly come from fatty acids of small molecules produced by hydrolysis and oxidation of fats.

The acid contents in BN pork were significantly lower than that in NX pork, showing that hybrid breed greatly decreased acids formation. Methylamine has a strong unpleasant ammonia odor, and BN pork significantly enhanced its relative content. Probably the cross-bred pork would provide more malodorous substrates. Additionally, the relative contents of 6 key differential VOCs were significantly higher in BN pork than that in NX pork, and most of them conferring pungent odor. It inferred that the hybrid pork enhanced the pungent odor. Overall, the above results showed that these differences were attributed to breed dependant.

Correlation between differential lipids and key volatiles in NX and BN pork

Lipids play a key role in VOC formation and lipids form volatile substances through two major processes: lipid oxidative degradation and the Maillard reaction.[7,47] In this study, besides PS and PI, PE, PC, and TG were the top key classes of high *r* values with VOCs, consistent with the results which revealed that PE, PC, and TG might be the crucial lipids for volatiles formation.[48] In the top 5 classes, more than half of lipids contained palmitoleic acid (C16:1), oleic acid (C18:1), and linoleic acid (C18:2), in accord with the results of Zhang et al. who found that C16:1, C18:1, and C18:2 might be the key FAs for VOC formation.[36] For example, PC (18:0/18:1) was oxidized to generate nonanal, octanal, heptanal, and 1-hexanol, while PC (18:0/18:2) was oxidized to produce pentanal, (E)-2-hexenal, 1-octen-3-ol, and 2-heptanone.[49] Previous study reported that C18:2 and C20:4 could be degraded to generate 1-octene-3-ol.[48,50] In this study, 1-octen-3-ol was positively correlated with PE (16:0e/20:4) and had a strong correlation with many lipids containing C18:2, such as LPE (18:2), PE (18:2p/22:5), PI (18:1/18:2)+Na, and TG (18:1/18:2/22:6). Based on the above results, it indicated that a kind of FA could be involved in formation of several VOCs, and a VOC could be affected by different FAs. It was reported that heptanal and decanal were derived from C18:1,[51] but in this study these two VOCs were not highly correlated with those lipids having C18:1 except for PC (20:1p/18:1). Hexanal was found to be produced from C18:1, C18:2, and C20:4,[51] while in this experiment hexanal-D and hexanal-M were neither strongly correlated with the lipids containing these FAs except for PE (18:2p/20:5) and TG (18:1/18:2/22:6). Reportedly, the study showed that FA composition played a crucial role in the formation of carbonyl compounds, such as aldehydes and ketones.[52] So, the correlation results in the study might be attributed to the comprehensive effects of different FAs.

In this study, PE, PI, PC, PS, and TG are strongly correlated with 5 key differential VOCs, respectively. It indicated that the 5 lipid classes were the great contributors to the formation of key differential VOCs in NX and BN pork. Yet, the lipids had a weak correlation with ethyl esters. This might be because ethyl esters were not generated directly by lipids degradation, but formed from reactions between ethanol and carboxylic acids.[53] Collectively, the network of VOCs generated by lipids is quite complex, and the basis of correlation between lipids profiles and volatiles in NX and BN pork requires further research.

Conclusion

The present study identified lipids and VOCs in NX and BN pork through IMF, lipidomics, and volatile profiles analysis. It was found that IMF contents in NX pork were much higher than that in BN pork, with NX pork reaching (5.43 ± 1.23)% and BN pork reaching (2.67 ± 1.00)%. A total of 1213 lipids out of 36 classes were detected in NX and BN pork. The top 5 lipid classes with species numbers were PC, PE, TG, SM, and PS. And the NX group could be well separated from the BN group by PCA and OPLS-DA. Total 187 significantly different lipids were identified to discriminate between NX and BN pork, and 38 potential markers were further screened, such as LPE (18:2), LPC (25:6), PC (20:1p/18:1), PE (16:0e/20:4), PI (18:0/18:2)+H, PS (18:0/18:1), DG (16:0/16:1), and TG (18:1/18:2/22:6). Briefly, 66 VOCs were identified in NX and BN pork besides of 7 unknown VOCs. The VOCs were divided into 9 categories, and in terms of abundance, alcohols were the most in NX pork, while ketones in BN pork. The two test groups could be well differed. Also, 16 key VOCs were selected both

in NX and BN pork. Among these, hexanal-D, 2-methylbutanal, 3-methylbutanal, 1-penten-3-one, 1-octen-3-ol, and ethyl acetate-D were considered as key differential VOCs. Comparing with purebred pork, the hybrid pork significantly decreased acid contents and strengthened pungent flavor, which both due to breed dependant. The correlation between the lipids markers and key VOCs showed that PE, PS, PI, PC, and TG were found to be the crucial differentiating compounds for characteristic flavor. In addition, lipids had no significant correlation with ethyl esters. The composition of fatty acids in lipids might comprehensively influence the formation of VOCs. In a word, lipids were potentially responsible for flavor in pork. Our findings provide novel insights into the lipidomics, volatile profiles, and correlations between lipids and VOCs in NX and BN pork.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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Author contributions

Conceptualization, H.L.L., Y.Y.L., and C.C.; methodology, H.L.L., Y.L.P., and S.L.Y.; software, D.Y. and X.G.H.; validation, H.B.R. and J.Z.; formal analysis, H.L.L. and Y.Y.L.; data curation, Q.M.C., S.Y.Z., and J.B.Z; writing-original draft preparation, H.L.L. and Y.Y.L.; writing-review and editing, C.C. and L.H.C. All authors have read and agreed to the published version of the manuscript.

Data availability statement

The data in this article are available from the corresponding author on reasonable request.

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