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Metabolomic Approach to Understand the Effect of Lairage on the Quality of Pork Loin[a]

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Abstract: The objective of this study was to evaluate the effect of lairage on the metabolomic profiles and physicochemical quality of pork loin. The castrated commercial Landrace × Yorkshire × Duroc (LYD) pigs were assigned into 2 groups: slaughter without lairage (CON; $n = 20$) and slaughter after 24 h lairage (LRG; $n = 20$). The metabolomic profiles and physicochemical properties (pH, moisture content, water holding capacity, cooking loss, meat color, and tenderness) of pork loin from CON and LRG were evaluated. The effect of lairage was not found in the physicochemical quality of pork loin ($P > 0.05$). However, when considering their metabolites, CON and LRG were distinguished in orthogonal partial least squares-discriminant analysis majorly due to the 2 up-regulated (isoleucine and valine) and 5 down-regulated (glutamate, glycerol, glycine, lysine, and methionine) compounds in LRG ($P < 0.05$). Pathways such as amino acid metabolisms, glycerolipid metabolism, and glutathione metabolism were differentiated between CON and LRG. Despite the absence of observed quality changes, our findings contribute to the understanding of how lairage impacts muscle metabolism following slaughter.

Introduction

On-farm animals experience pre-slaughter stress from loading and unloading, transportation, rough handling, etc. (Zhen et al., 2013). These stressors can deteriorate meat quality considerably (Jung et al., 2022). Pigs in particular are exclusively sensitive to pre-slaughter stress due to the difficulty in releasing heat from the increased metabolic rate by stress responses (Flores-Peinado et al., 2020). Therefore, with the increase in public concern for animal welfare issues, the relief of the stress that pigs undergo has become crucial to maintaining high meat quality in the livestock industry (Velarde et al., 2015; Lee et al., 2022).

It was reported that the lairage period was a major factor that influenced pork quality among stress factors (Nanni Costa et al., 2002; Dokmanović et al., 2017). Lairage provides a resting period for animals, allowing them to relieve stress and fatigue from loading, unloading, and transportation and to recover from dehydration (Zhen et al., 2013; Díaz et al., 2014).

Major advantages of lairage in the livestock industry were reported as lowering blood cortisol concentration, reducing carcass loss by rehydration and glycogen recovery of the animal, and preventing the incidence of meat abnormalities (Rey-Salgueiro et al., 2018; Acevedo-Giraldo et al., 2020). Despite this, the influence of lairage on meat quality is still not defined because the results varied depending on the lairage condition such as lairage periods. In particular, the effect of overnight lairage has been controversial as some previous studies found improved meat quality as a result of overnight lairage such as higher pH, lower drip loss, and incidence of pale, soft, and exudative (PSE) meat (Warriss et al., 1998; Dokmanović et al., 2017), whereas other studies reported the detrimental effect of overnight lairage, including increased toughness and incidence of PSE meat (Zhen et al., 2013; Acevedo-Giraldo et al., 2020). Therefore, new approaches should be applied to elucidate the effect of lairage on meat quality attributes.

As metabolic changes in animals occur through the cascade reaction from the secretion of cortisol to the stimulated activity of enzymes such as catecholamines, glycogen phosphorylase, ubiquitin-proteasome, and lysosomes in response to a stressful condition (Gonzalez-Rivas et al., 2020), a metabolomic approach could be effective for the evaluation of the effect of lairage on pork quality (Xing et al., 2019; Gonzalez-Rivas et al., 2020). In particular, little is known about the change in pork muscle metabolites by lairage although previous studies reported the change in the concentrations of glucose or lactate in the pig blood plasma (Dokmanović et al., 2014; Acevedo-Giraldo et al., 2020). We previously compared the metabolomic profiles of chicken breast and thigh meat from conventional and animal-friendly farms through nuclear magnetic resonance (NMR) spectroscopy and found major differences in the metabolite contents related to glycolysis, purine metabolism, and amino acid metabolism, and these changes had a relationship with meat quality changes (Jung et al., 2022; Lee et al., 2022). Similarly, metabolomics combined with conventional analyses on the physicochemical quality of pork will provide new insights into understanding how lairage influences pork quality.

Therefore, we analyzed the metabolome in pork loin at 48 h postmortem from the unrested group after unloading and 24 h-rested groups, along with physicochemical analyses of pork loin, to understand comprehensively how lairage influences pork quality.

Materials and Methods

Animals

The experiment was approved by the Institutional Animal Care and Use Committee (IACUC) at Sunchon National University, Suncheon, Republic of Korea (SCNU IACUC-2020-12). The castrated commercial Landrace × Yorkshire × Duroc (LYD) pigs were used in this study. The animals were reared at two similar commercial animal-friendly farms where the essential environmental conditions were set according to the legislation of the Ministry of Agriculture, Food and Rural Affairs in Korea enforced from February 2, 2018. The pigs were raised in an animal-friendly farm, Cheil breeding stock farm (Icheon, Korea), until their live weight reached 30 kg (stocking density of >0.3 m^2 per animal) and then were transported to Yuunwoo animal-friendly farm (Hwasung, Korea) until their live weight reached 110 kg (stocking density of >1 m^2 per animal). Forty pigs from four rearing pens ($n = 10$ per pen) were used for the study, and the pigs from the same pen were randomly divided in half and classified into two groups: the pigs that were slaughtered immediately after unloading (CON; $n = 20$) and those that were provided with 24 h lairage (LRG; $n = 20$). Prior to transport, the live weight of the pigs was measured, and the average live weight of CON was 115.31 ± 0.91 kg and that of LRG was 118.11 ± 5.01 kg. The pigs were transported to an abattoir (Dodram, Anseong, Korea) for 2 h, which was about 90 km away from the farm. During transportation, the temperature was maintained at 15°C–18°C in an enclosed truck to minimize stress. The stocking density of the animals during the lairage period was >0.83 m^2, and the pigs could access water freely. The temperature and relative humidity were set to 15°C–18°C and 75%, respectively. After slaughter, the carcasses were cooled at 4°C for 24 h. Then, the carcass weight and backfat thickness of each carcass were measured, and loin cuts from the left half carcasses were obtained. The backfat thickness of the carcass was determined by measuring the average value of the backfat thickness between the last thoracic vertebra and the first lumbar vertebra and that between the 11th and 12th thoracic vertebrae of the left half carcass of the pig according to the Pig Carcass Grade System in Korea. The loin samples were vacuum-packaged and quickly transferred to the laboratory, and M. *longissimus thoracis* at 48 h postmortem from the loin samples was used for metabolomic profiling and physicochemical quality analyses.

Metabolomic analyses

Extraction of metabolites. The extraction process proceeded according to Kim et al. (2021). Five grams of loin muscle was added into 20 mL of 0.6 M perchloric acid and homogenized at 16,000 rpm for 1 min using a homogenizer (T25 digital ULTRA-TURRAX®, Ika Works, Staufen, Germany). The homogenates were centrifuged at $2{,}265 \times g$ for 20 min (Continent 512R, Hanil Co., Ltd., Daejeon, Korea). Then, the supernatant was neutralized and centrifuged at the same condition above. After filtration (No. 1, Whatman International Ltd., Kent, UK), the filtrates were lyophilized (Freezer dryer 18, Labco Corp., Kansas City, MO). The lyophilized extracts were reconstituted with the addition of 1 mL of 20 mM deuterium oxide-based phosphate buffer (pH 7.0) containing 1 mM of 3-(trimethylsilyl) propionic-2, 2, 3, 3-d_4 acid (TSP). The samples were centrifuged at $2{,}265 \times g$ for 20 min (Continent 512R, Hanil Co., Ltd.), and the supernatant was further centrifuged at $17{,}000 \times g$ for 10 min (HM-150IV, Hanil Co., Ltd.). The supernatant was transferred into NMR tubes and used for NMR analyses. The metabolite extraction process was conducted twice for each animal.

NMR analyses. NMR spectra were recorded using an 850 MHz cryo-NMR spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany). One-dimensional ^{1}H NMR for quantitative analysis of metabolites was performed using a zg30 pulse program with a spectral width of 17006.803 Hz with 128 scans, and acquisition time was 4.20 s. Additionally, two-dimensional NMR spectra were obtained for peak identification. [1]H-^{13}C heteronuclear single quantum coherence (HSQC) was performed with 11 ppm for the F2 and 233 ppm for the F1 axis, respectively, and 2 k data points in the t2 domain and 256 increments in the t1 domain with 16 scans. Correlation spectroscopy (COSY) was conducted with 11 ppm of spectral width, 2 k data points in the t2 domain and 128 increments in the t1 domain with 16 scans. The experimental condition of total correlation spectroscopy (TCOSY) was 2 k data points in the t2 domain and 256 increments in the t1 domain with 16 scans. Finally, heteronuclear multiple-bond correlation spectroscopy (HMBC) was recorded with 4 k data points in the t2 domain and 512 increments in the t1 domain with 16 scans. During the process, lock, tune, and shimming were performed automatically by ICON-NMR automation (Bruker Biospin GmbH).

The identification of peaks in the two-dimensional NMR spectra was accompanied using Topspin 4.0.8 (Bruker Biospin GmbH). The phase of each one-dimensional ^{1}H NMR spectra was first automatically corrected, and then manually corrected based on the signal of lactate (1.35 ppm), and the baseline of the spectra was corrected automatically. The concentration of identified peaks was quantified using Chenomx NMR suite 8.6 (Chenomx, Inc., Edmonton, Alberta, Canada) with the resonance of the TSP as a standard reference for both identification and quantification. An average value of metabolite concentration from two measurements in each animal was taken as one replicate.

Multivariate and pathway analysis. To identify the change in the metabolic process of pork loin by lairage, principal component analysis (PCA) coupled with orthogonal partial least squares-discriminant analysis (OPLS-DA) and pathway analysis were performed with the dataset of metabolite contents in pork samples using MetaboAnalyst 5.0 (www.metaboanalyst.ca). Prior to multivariate and pathway analysis, a log transformation and a Z-score normalization were applied to the metabolite data.

Physicochemical meat quality

pH. Two grams of loin muscle were homogenized with the addition of 18 mL of deionized distilled water at 11,000 rpm for 1 min (Polytron PT 10-35 GT, Kinematica AG, Luzern, Switzerland). The homogenates were filtered (No. 4, Whatman International Ltd.), and the pH of each filtrate was measured using a pH meter (Seven Excellence™, Mettler Toledo International Inc., Schwerzenbach, Switzerland) at room temperature.

Moisture content. The moisture content of pork samples was measured according to the AOAC official methods (2000) with minor modifications. Three grams of loin muscle was placed on the aluminum dish and dried in the dry oven at 104°C to a constant weight. The moisture content (%) was determined by the percentage of weight loss after drying.

Water holding capacity. Five grams of the loin muscle was put into the centrifuge tube and centrifuged at $1200 \times g$ for 10 min (Combi-514R, Hanil Co., Ltd.). The water holding capacity (WHC) of the sample was determined by the percentage of weight loss after centrifugation as follows:

$$WHC\,(\%) = \frac{\text{Weight before centrifugation (g)} - \text{Weight after centrifugation (g)}}{\text{Weight before centrifugation (g)}} \times 100$$

Cooking loss. The pork loin was cut into 30 mm × 50 mm × 10 mm and was cooked at 100°C using an electric grill with double heating surfaces at 1,400 W (Nova EMG-533, Evergreen Enterprise, Yongin, Korea) for 90 s until the internal temperature of the sample reached 75°C. Cooking loss (%) was calculated by the weight difference of the sample before and after cooking.

Meat color. The meat color of pork samples was measured using a colorimeter (CR-410, Minolta Co., Osaka, Japan). The colorimeter was pre-calibrated with a standard white plate (Y = 86.8; x = 0.3156; y = 0.3225). The instrumental color was expressed as Commission International d'Eclairage (CIE) $L*$ (lightness), $a*$ (redness), and $b*$ (yellowness) values using Spectra Magic Software (Minolta Co.). An average value of 3 measurements from each sample was taken as one replicate.

Warner-Bratzler shear force. The pork loin was cut into a meat block of 40 mm × 50 mm × 10 mm and heated until the core temperature of the meat block reached 75°C. Then, each sample of 10 mm × 20 mm × 10 mm was obtained from the cooked meat block, and used for shear force measurements using a texture analyzer (TA-XT2, Stable Micro Systems, Surrey, UK) equipped with a Warner-Bratzler blade. The muscle fiber direction was perpendicular to the blade, and the condition of the instrument was as follows: pre-test speed of 2.0 mm/s, test speed of 2.0 mm/s, and post-test speed of 5.0 mm/s.

Statistical analysis

The results were statistically analyzed by Student's t-test (SAS 9.4, SAS Institute Inc., Cary, NC). Prior to t-test, F-tests were employed for metabolite concentrations, carcass and physicochemical quality parameters to analyze the homoscedasticity of each variable. Welch-Satterthwaite t-test was applied for 4 metabolites which showed heterogenous variances including betaine, glycine, tyrosine, and β-alanine and 3 carcass characteristic parameters, while pooled t-test was applied for the other variables with homogenous variances (Tables S1 and S2). The significant differences between the mean value of the 2 groups were determined at $P < 0.05$. A volcano plot was performed using MetaboAnalyst 5.0, and metabolites with the fold change (FC) of >1.2 or <0.8 and $P < 0.05$ were considered as regulated by lairage.

Results and Discussion

Carcass characteristics

The live weight of LRG was significantly higher than that of CON; on the other hand, there was no significant difference in carcass weight between the two groups (Table 1). Previous studies reported that the weight loss of the carcass showed an increasing trend with lairage time from 0 h to 24 h or overnight lairage (Warriss et al., 1998; Díaz et al., 2014). However, LRG showed significantly higher backfat thickness compared to CON, which was the opposite phenomenon from the research conducted by Warriss et al. (1998) and Nanni Costa et al. (2002), wherein the pigs that were provided with an overnight lairage showed a reduction in the backfat thickness compared to unrested pigs or pigs with 2 h of lairage. The authors suggested that the loss of water from the fat tissue led to a decrease in the amount of fat. Nevertheless, it was reported that the water supply during lairage could rehydrate the animals (Díaz et al., 2014). On the other hand, Čobanović et al. (2020) observed that cortisol positively correlated with backfat thickness but negatively correlated with lean carcass content, and they supposed that cortisol might stimulate fat storage to compensate for the increased protein degradation in a stressful condition. The mechanism of the effect of lairage on the increase in backfat thickness of pigs remains unclear; however, the carcass characteristics of both CON and LRG correspond to 1+ carcass grade, which is the best carcass grade in the Korean pig carcass grade system with 83–92 kg of carcass weight and 17–24 mm of backfat thickness (Park et al., 2022).

Identification of metabolites and multivariate analysis

We identified a total of 32 metabolites in every sample through NMR analyses (Table 2). Those were classified as (1) free amino acids, dipeptides, and

Table 1. Carcass characteristics of pigs in relation to lairage

Treatment	CON	LRG	P value
Live weight (kg)	115.31 ± 0.91	118.11 ± 5.01	0.0186
Carcass weight (kg)	88.05 ± 0.69	89.50 ± 3.80	0.1017
Backfat thickness (mm)	22.20 ± 1.24	23.25 ± 1.41	0.0168

The results were expressed as mean ± standard deviation ($n = 20$ for each group). Pigs in the CON group were slaughtered immediately after unloading, whereas those in the LRG group were held in lairage for 24 h before slaughter.

Table 2. Assignment of metabolites in pork loin extracts using two-dimensional nuclear magnetic resonance analyses

Peak	Metabolite	Moiety	δ^1H (ppm) and multiplicity[1]	Assignment
1	Isoleucine	δ-CH$_3$	0.95 (t)	HSQC (13.8), COSY (1.27, 1.48), HMBC (16.7, 27.2, 62.4)
2	Leucine	δ-CH$_3$	0.97, 0.98 (d)	HSQC (23.8), COSY (1.72), TCOSY (1.74, 3.76), HMBC (26.9, 42.6)
3	Valine	γ-CH$_3$	1.01 (d)	HSQC (19.5), COSY (2.30), TCOSY (3.64), HMBC (20.8, 32.1, 63.1)
4	Isoleucine	γ-CH$_3$	1.03 (d)	HSQC (17.5), COSY (2.00), HMBC (38.8, 62.4)
5	Valine	γ-CH$_3$	1.06 (d)	HSQC (20.8), COSY (2.30), TCOSY (3.64), HMBC (19.5, 32.1, 63.1)
6	Isoleucine	γ'-CH$_2$	1.27 (m)	HSQC (27.2)
7	Lactate	β-CH$_3$	1.35 (d)	HSQC (23.0), COSY (4.15), HMBC (71.2, 185.3)
8	Lysine	γ-CH$_2$	1.46 (m)	HSQC (24.9), TCOSY (1.72, 3.02)
9	Isoleucine	γ'-CH$_2$	1.48 (m)	HSQC (27.2)
10	Alanine	β-CH$_3$	1.50 (d)	HSQC (19.0), COSY (3.81), HMBC (53.3, 178.7)
11	Lysine	δ-CH$_2$	1.72 (m)	HSQC (29.1), TCOSY (1.46, 3.02)
12	Leucine	β-CH$_2$	1.74 (m)	HSQC (42.6), COSY (0.98), TCOSY (3.76)
13	Leucine	β-CH$_2$	1.74 (m)	HSQC (26.9), COSY (0.98), TCOSY (3.76), HMBC (23.8, 24.4)
14	Lysine	β-CH$_2$	1.93 (m)	HSQC (32.6)
15	Acetate	α-CH$_3$	1.94 (s)	HSQC (26.2), HMBC (184.3)
16	Isoleucine	β-CH	2.00 (m)	HSQC (38.8), COSY (1.03), TCOSY (3.70)
17	Glutamate	β-CH$_2$	2.15 (m)	HSQC (29.9)
18	Methionine	S-CH$_3$	2.15 (s)	HSQC (16.8), HMBC (31.7)
19	Glutamine	β-CH$_2$	2.18 (m)	HSQC (29.0), COSY (3.80), HMBC (57.0)
20	Valine	β-CH	2.30 (m)	HSQC (32.1)
21	Glutamate	γ-CH$_2$	2.37 (m)	HSQC (36.2), TCOSY (2.08), HMBC (29.8, 57.6)
22	Succinate	α-CH$_2$	2.43 (s)	HSQC (36.8), HMBC (185.2)
23	Glutamine	γ-CH$_2$	2.47 (m)	HSQC (33.7), TCOSY (3.79)
24	β-Alanine	α-CH$_2$	2.58 (t)	HSQC (36.3), COSY (3.21), HMBC (39.3, 181.2)
25	Methionine	γ-CH$_2$	2.65 (t)	HSQC (31.7), COSY (2.15), HMBC (16.8, 32.6, 56.7)
26	Anserine	NH$_2$-CH$_2$	2.72 (m)	HSQC (34.8), COSY (3.25), HMBC (174.5)
27	Carnosine	NH$_2$-CH$_2$	2.72 (m)	HSQC (34.8)
28	Aspartate	β-CH$_2$	2.75 (dd)	HSQC (39.6)
29	Aspartate	β-CH$_2$	2.83 (d)	HSQC (39.6), COSY (3.94), HMBC (55.2)
30	Lysine	e-CH$_2$	3.02 (t)	HSQC (41.8), COSY (1.72), TCOSY (1.46), HMBC (57.5)
31	Creatine	N-CH$_3$	3.05 (s)	HSQC (39.7), COSY (3.94), HMBC (56.6, 159.9)
32	Anserine	β-CH$_2$	3.08 (dd)	HSQC (28.9), COSY (3.25, 4.49, 7.17)
33	Carnosine	β-CH$_2$	3.08 (dd)	HSQC (30.5), COSY (4.50)
34	Carnosine	β-CH$_2$	3.19 (dd)	HSQC (30.7), COSY (4.50)
35	β-Alanine	N-CH$_2$	3.21 (t)	HSQC (39.3), COSY (2.58), HMBC (36.3)
36	Anserine	β-CH$_2$/NH$_2$-CH$_2$-CH$_2$	3.25 (m)	HSQC (28.3), COSY (2.72, 3.08), HMBC (35.4, 174.6)
37	Carnitine	N(CH$_3$)$_3$	3.25 (s)	HSQC (56.8), COSY (3.44)
38	Carnosine	NH$_2$-CH$_2$-CH$_2$	3.25 (m)	HSQC (38.6), COSY (2.72, 3.08, 3.25), HMBC (30.7, 34.8)
39	Glucose	CH-2 (ring)	3.25 (m)	HSQC (56.9), HMBC (98.8)
40	Betaine	N(CH$_3$)$_3$	3.28 (s)	HSQC (56.2), HMBC (69.1)
41	Taurine	N-CH$_2$	3.30 (t)	HSQC (50.4), COSY (3.43), HMBC (38.1)
42	Taurine	S-CH$_2$	3.43 (t)	HSQC (38.1), COSY (3.30), HMBC (50.4)
43	Carnitine	γ-CH$_2$	3.44 (m)	HSQC (72.4), COSY (4.58)
44	Glucose	CH-4 (ring)	3.44 (m)	HSQC (72.5), TCOSY (3.75)
45	Glucose	CH-3 (ring)	3.49 (m)	HSQC (72.4), HMBC (63.5)
46	Glucose	CH-2 (ring)	3.57 (m)	HSQC (74.0)
47	Glycerol	CH$_2$-1,3	3.58 (dd)	HSQC (65.2), HMBC (75.0)
48	Glycine	α-CH$_2$	3.59 (s)	HSQC (44.2), HMBC (175.4)
49	Glycerol	CH$_2$-1,3	3.67 (dd)	HSQC (65.3), HMBC (75.0)
50	Isoleucine	α-CH	3.70 (d)	HSQC (62.4), TCOSY (1.03, 1.27, 2.00)

Table 2. (*Continued*)

Peak	Metabolite	Moiety	δ^1H (ppm) and multiplicity[1]	Assignment
51	Glucose	CH$_2$-6 (ring)	3.75 (m)	HSQC (63.6), TCOSY (3.44, 3.57, 3.91), HMBC (72.4, 74.0)
52	Glucose	CH-3 (ring)	3.75 (m)	HSQC (75.2), COSY (3.91)
53	Leucine	α-CH	3.76 (m)	HSQC (56.4), COSY (1.72), TCOSY (0.98, 1.74)
54	Glutamine	α-CH	3.80 (t)	HSQC (57.0), COSY (2.18), TCOSY (2.47), HMBC (29.0, 33.7)
55	Glycerol	CH-2	3.80 (m)	HSQC (75.0)
56	Alanine	α-CH	3.81 (q)	HSQC (53.3), COSY (1.50), HMBC (19.0)
57	Anserine	N-CH$_3$	3.82 (s)	HSQC (35.4), HMBC (139.0)
58	Glucose	CH-5 (ring)	3.86 (m)	HSQC (63.5), COSY (3.44), HMBC (72.4)
59	Inosine	CH$_2$-5 (ribose)	3.86 (dd)	HSQC (63.6)
60	Methionine	α-CH	3.88 (m)	HSQC (56.7)
61	Glucose	CH$_2$-6 (ring)	3.91 (m)	HSQC (63.7)
62	Inosine	CH$_2$-5 (ribose)	3.91 (dd)	HSQC (63.6), HMBC (88.3)
63	Betaine	α-CH$_2$	3.92 (s)	HSQC (69.1), HMBC (56.2, 172.1)
64	Aspartate	α-CH	3.94 (dd)	HSQC (55.1)
65	Creatine	α-CH$_2$	3.94 (s)	HSQC (56.6), COSY (3.05), HMBC (39.7, 159.9, 177.4)
66	Tyrosine	α-CH	3.94 (q)	HSQC (59.0), HMBC (118.6, 176.9)
67	UMP	CH$_2$-5 (ribose)	3.98 (m)	HSQC (65.8)
68	Phenylalanine	α-CH	4.02 (q)	HSQC (58.8), TCOSY (7.41), HMBC (131.9)
69	UMP	CH$_2$-5 (ribose)	4.03 (m)	HSQC (65.8), HMBC (65.8, 76.5)
70	IMP	CH$_2$-5 (ribose)	4.04 (m)	HSQC (65.9)
71	Lactate	α-CH	4.15 (q)	HSQC (71.2), COSY (1.35), HMBC (23.0)
72	Inosine	CH-4 (ribose)	4.29 (m)	HSQC (88.3), TCOSY (3.86, 3.91, 4.79), HMBC (73.1)
73	UMP	CH-3 (ribose)	4.33 (m)	HSQC (72.9), TCOSY (5.97)
74	IMP	CH-4 (ribose)	4.39 (m)	HSQC (73.0), COSY (4.53), TCOSY (4.79, 6.14), HMBC (73.4)
75	UMP	CH-2 (ribose)	4.40 (m)	HSQC (76.5), COSY (6.00)
76	Inosine	CH-3 (ribose)	4.45 (t)	HSQC (73.1), COSY (4.79), TCOSY (3.91, 4.29, 6.10), HMBC (88.3, 91.0, 143.0)
77	Anserine	CH-COOH	4.49 (m)	HSQC (56.3), COSY (3.08, 8.24), TCOSY (4.49, 7.17), HMBC
78	Carnosine	CH-COOH	4.50 (m)	HSQC (57.4), COSY (3.08), HMBC (30.5)
79	IMP	CH-3 (ribose)	4.53 (dd)	HSQC (73.4), COSY (4.79), HMBC (73.0, 90.2)
80	Carnitine	β-CH	4.58 (m)	HSQC (72.9), COSY (3.44)
81	Glucose	CH-1 (ring)	4.67 (d)	HSQC (98.8), TCOSY (3.75, 3.91), HMBC (75.2)
82	IMP	CH-2 (ribose)	4.79 (t)	HSQC (77.5), COSY (6.14, 8.56), TCOSY (4.39, 4.53, 6.14)
83	Inosine	CH-2 (ribose)	4.79 (t)	HSQC (77.4), COSY (3.91, 8.24), HMBC (63.6)
84	Glucose	CH-1 (ring)	5.26 (d)	HSQC (95.0), COSY (3.75, 3.86), TCOSY (3.44, 3.57), HMBC (74.0, 75.2)
85	UMP	CH-5 (uracil)	5.97 (d)	HSQC (105.4), TCOSY (8.09)
86	UMP	CH-1 (ribose)	6.00 (d)	HSQC (91.4), COSY (4.03, 4.40)
87	NAD	N9-CH (adenine)	6.04 (d)	HSQC (89.8), COSY (8.21, 9.36)
88	Inosine	CH-1 (ribose)	6.10 (d)	HSQC (91.1), COSY (3.86, 4.79), TCOSY (3.91, 4.45), HMBC (63.6, 143.0)
89	IMP	CH-1 (ribose)	6.14 (d)	HSQC (90.2), COSY (4.79), TCOSY (4.39, 4.53), HMBC (73.4, 77.5, 142.8)
90	Fumarate	CH = CH	6.54 (s)	HSQC (138.1), HMBC (177.4)
91	Tyrosine	CH-3,5 (Ring)	6.87 (d)	HSQC (118.6), COSY (7.18), TCOSY (3.94)
92	Anserine	δ-CH (His)	7.17 (s)	HSQC (122.3), COSY (3.08, 3.25, 8.24), TCOSY (2.72, 3.82, 4.50), HMBC (56.3)
93	Carnosine	δ-CH (His)	7.18 (s)	HSQC (119.8), COSY (3.08, 3.24, 8.32), HMBC (30.5, 57.4, 136.9)
94	Tyrosine	CH-2,6 (Ring)	7.18 (d)	HSQC (133.6), TCOSY (3.07, 6.87)
95	Phenylalanine	CH-2,6 (Ring)	7.33 (d)	HSQC (132.1)
96	Phenylalanine	CH-4 (Ring)	7.36 (t)	HSQC (130.6), TCOSY (7.33)
97	Phenylalanine	CH-3,5 (Ring)	7.41 (t)	HSQC (131.9), TCOSY (7.33)
98	Nicotinate	CH-5 (ring)	7.60 (m)	HSQC (127.0), COSY (8.71), TCOSY (8.93), HMBC (150.5)
99	Xanthine	CH-8 (purine)	7.96 (s)	HSQC (140.4)
100	UMP	CH-6 (uracil)	8.09 (d)	HSQC (145.0), COSY (5.97)

Table 2. (*Continued*)

Peak	Metabolite	Moiety	δ^1H (ppm) and multiplicity[1]	Assignment
101	Hypoxanthine	CH-2 (purine)	8.19 (s)	HSQC (148.4)
102	Hypoxanthine	CH-8 (purine)	8.21 (s)	HSQC (149.1)
103	NAD	CH-2 (adenine)	8.21 (s)	HSQC (131.3), TCOSY (8.86, 9.16), HMBC (89.8)
104	Anserine	ϵ-CH (His)	8.24 (s)	HSQC (139.0), TCOSY (3.08, 3.25, 4.49), HMBC (17.3)
105	IMP	CH-8 (purine)	8.24 (s)	HSQC (149.2), TCOSY (6.14, 8.56)
106	Inosine	CH-2 (purine)	8.24 (s)	HSQC (149.1), COSY (4.29), HMBC (143.0)
107	Carnosine	ϵ-CH (His)	8.32 (s)	HSQC (136.9), COSY (2.72, 3.08, 3.25, 7.18), HMBC (119.8)
108	Inosine	CH-8 (purine)	8.35 (s)	HSQC (143.0)
109	NAD	CH-8 (adenine)	8.42 (s)	HSQC (142.6), HMBC (131.3)
110	IMP	CH-2 (purine)	8.56 (s)	HSQC (142.8), COSY (8.24)
111	Nicotinate	CH-6 (ring)	8.71 (m)	HSQC (154.7), COSY (7.60), TCOSY (8.93), HMBC (150.5)
112	NAD	CH-4 (nicotinamide)	8.86 (d)	HSQC (148.3), COSY (8.21, 9.36), TCOSY (9.16), HMBC (131.3, 145.1)
113	Nicotinate	CH-2 (ring)	8.93 (m)	HSQC (150.5), COSY (7.60), TCOSY (8.71)
114	NAD	CH-6 (nicotinamide)	9.16 (d)	HSQC (145.1), COSY (8.21), TCOSY (8.86, 9.36)
115	NAD	CH-2 (nicotinamide)	9.36 (s)	HSQC (142.8), COSY (8.21, 8.86), TCOSY (9.16)

[1]Represents the type of peak splitting: s, singlet; d, doublet; t, triplet; q, quartet; dd, double of doublet; m, multiplet.

COSY, correlation spectroscopy; HMBC, heteronuclear multiple-bond correlation spectroscopy; HSQC, heteronuclear single quantum coherence; IMP, inosine monophosphate; NAD, nicotinamide adenine dinucleotide; TCOSY, total correlation spectroscopy; UMP, uridine monophosphate.

derivatives; (2) nucleotide-related products; (3) organic acids; and (4) others (Table S1). Free amino acids, dipeptides, and derivatives included 19 metabolites (alanine, anserine, aspartate, betaine, carnitine, carnosine, creatine, glutamate, glutamine, glycine, isoleucine, leucine, lysine, methionine, phenylalanine, taurine, tyrosine, valine, and β-alanine); nucleotide-related products included 6 metabolites (hypoxanthine, inosine monophosphate [IMP], inosine, nicotinamide adenine dinucleotide [NAD], uridine monophosphate [UMP], and xanthine); organic acids included 5 metabolites (acetate, fumarate, lactate, nicotinate, and succinate); and others included glucose and glycerol, respectively. Free amino acids, dipeptides, and derivatives comprised a large proportion of the metabolic profiles identified in the present study due to (1) that the extraction process conducted here mainly focused on the polar metabolites and (2) a large content of amino acids present in the skeletal muscle (Gondret et al., 2021; Kim et al., 2021).

Based on the metabolomic profiles of pork loins, PCA and OPLS-based multivariate analyses were performed. PCA is an effective analysis for the identification of the overall difference between two groups (Kim et al., 2021). On the other hand, OPLS-DA is a supervised methodology that can maximize the between-class difference by using both the covariate matrix and response matrix compared to unsupervised PCA (Debik et al., 2021). In Figure 1, the red and green dots indicate the metabolomic profiles of CON and LRG, respectively. The red and green circled area indicate the 95% confidence regions for CON and LRG, respectively. It was clearly observed that CON and LRG were hardly separated by PCA as shown in Figure 1a, implying that both CON and LRG had similar metabolomics profiles in common. On the other hand, with OPLS-DA, a better distinction was found between CON and LRG compared with a PCA-based result as shown in Figure 1b. It suggests that the overall metabolomic profiles between the two classes were similar as shown in the PCA result, yet certain metabolite contents were different by the effect of lairage. Similarly, Gao et al. (2020) utilized multivariate analysis to elucidate the effect of heat stress on pork metabolomic profiles and found that the classes were clearly distinguished under the OPLS-DA method but overlapped under the PCA method.

Metabolic pathway analysis

Among the metabolites in the pork loin, the amount of lactate was the highest, followed by creatine and carnosine. However, we did not observe changes in lactate or carnosine contents after lairage (Figure 2a; $P > 0.05$). Instead, isoleucine and valine were up-regulated, whereas glutamate, glycerol, glycine, lysine, and methionine were down-regulated in pork loin by lairage (Figure 2b; $P < 0.05$). Creatine, although its FC was below 1.2, increased in LRG compared to CON (Figure 2a; $P = 0.027$).

To understand the effect of lairage on the metabolic profiles of pork loin, pathway analysis was conducted. Pathway analysis could provide valuable information
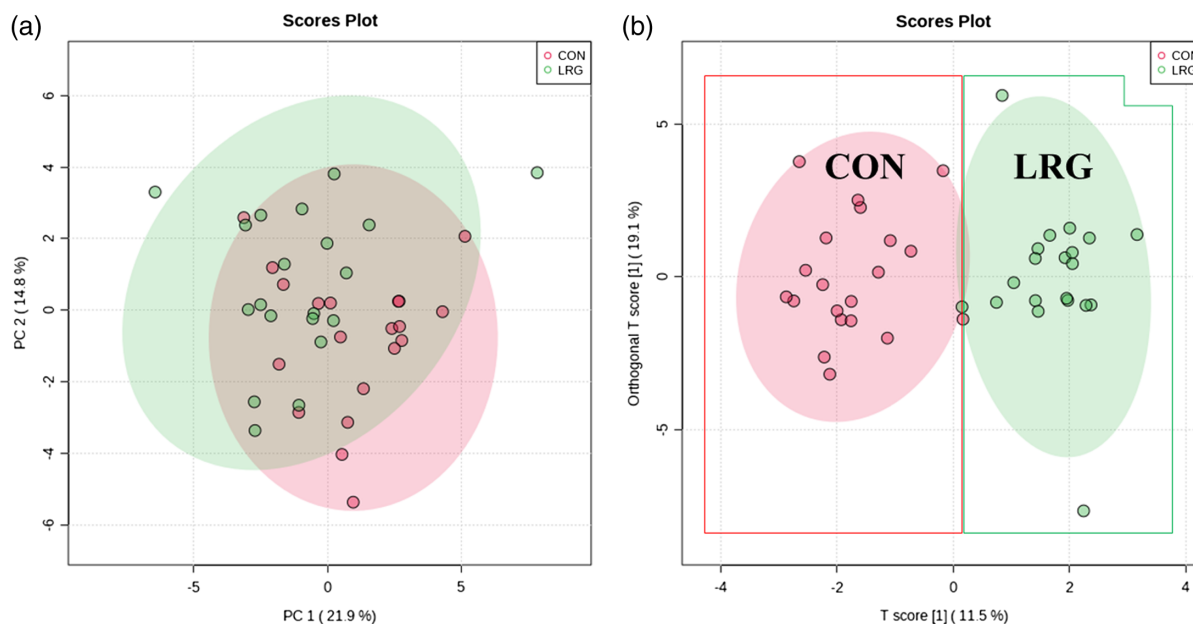
Figure 1. Principal component analysis (a) and orthogonal partial least squares-discriminant analysis (b) plot for the discrimination of metabolomic profiles of pork loin in relation to lairage. Pigs in the CON group were slaughtered immediately after unloading, whereas those in the LRG group were held in lairage for 24 h before slaughter.

about the role of each metabolite in biological reactions, and therefore the data of differentiated metabolomic profiles become interpretable in terms of biological context. Among 33 pathways identified, 13 pathways with $P < 0.05$ and pathway impact value > 0 were considered significantly differentiated between the two groups (Table 3). These included amino acid metabolisms (alanine, aspartate and glutamate metabolism, arginine and proline metabolism, arginine biosynthesis, cysteine and methionine metabolism, D-amino acid metabolism, glycine, serine and threonine metabolism, and histidine metabolism), citrate cycle, glutathione metabolism, glyoxylate and dicarboxylate metabolism, pantothenate and CoA biosynthesis, and primary bile acid biosynthesis. Most differentiated pathways by lairage belonged to amino acid metabolisms, which may explain the difference in free amino acids (glutamate, glycine, isoleucine, lysine, methionine, and valine) in pork loin by lairage (Figure 2b).

The results imply the beneficial effect of lairage on pigs in terms of the stability of (1) energy supply through amino acid and glycerolipid metabolisms and (2) antioxidant system via glutathione metabolism. Previous studies reported the relationship between stress and the metabolism of animals and suggested that the metabolic changes occurred to compensate for the energy needs induced by the physiological responses to stressful conditions such as increased heart rate and body temperature, impaired metabolism and hormonal

regulation, and behavioral abnormalities (Zou et al., 2020; Lee et al., 2022). In this study, the up-regulation of isoleucine and valine after lairage may be related to energy metabolism as these branched-chain amino acids were reported to be utilized as nitrogen donors to produce alanine, which contributes to glycolysis (Jung et al., 2022). On the other hand, the increase of free amino acids and glycerol contents in CON may derived from the stimulated proteolysis and lipid metabolism by the action of corticosterone and norepinephrine to produce free amino acids with the action of proteases and the degradation products of lipids including triacylglycerols with glycerol and esterified three fatty acids, respectively (Zhang et al., 2021). These aforementioned metabolites can also be utilized as energy sources via glycolysis (Figure 3). Furthermore, the increase in creatine after lairage supports the idea that lairage could alleviate energy usage in response to environmental changes (Antonelo et al., 2020). Creatine is an important energy source and is involved in ATP synthesis and metabolism, so it might be possibly assumed from the result of the lower content of creatine in CON compared to LRG that creatine was utilized as an energy source (Yu et al., 2021). Other differentiated pathways, such as the citrate cycle and pantothenate and CoA biosynthesis, further support the hypothesis that the muscle required additional energy when the pigs were immediately slaughtered.
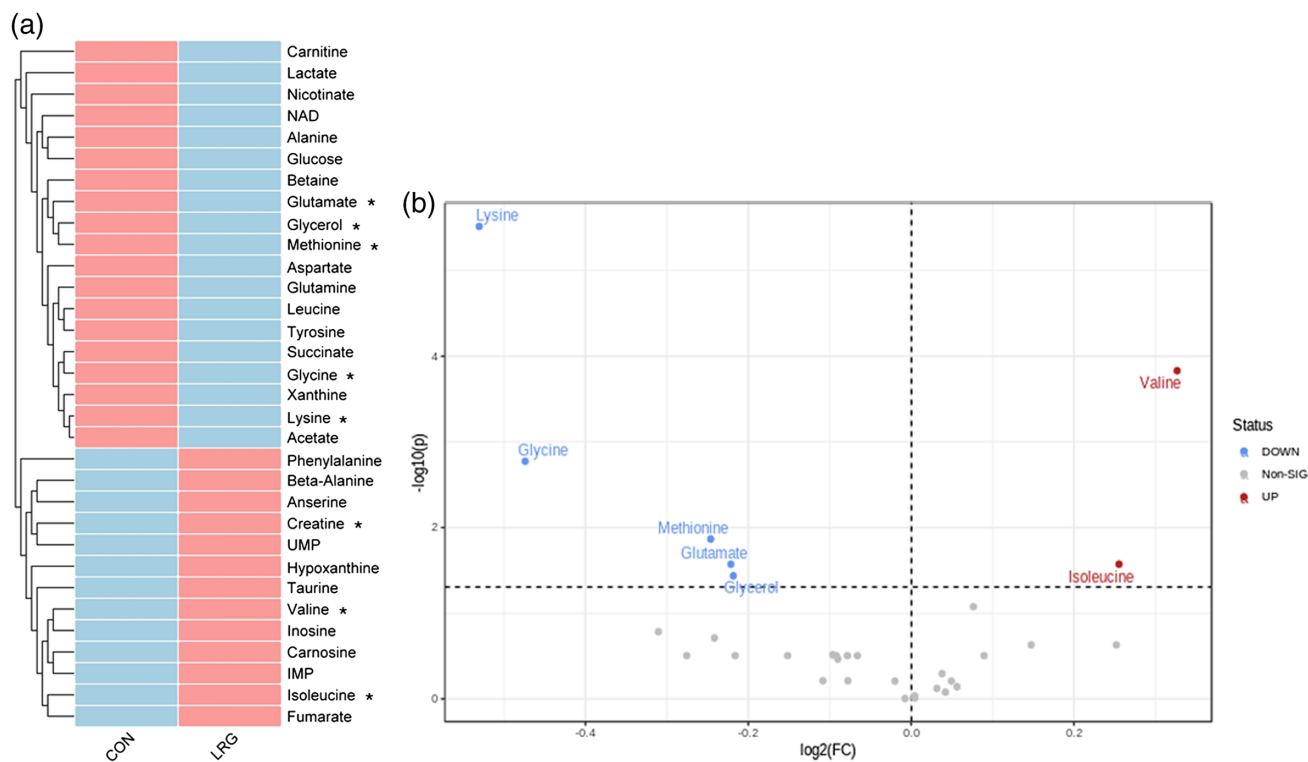
Figure 2. Heatmap analysis (a) and volcano plot (b) of metabolites from pork loin of CON and LRG. Pigs in the CON group were slaughtered immediately after unloading, whereas those in the LRG group were held in lairage for 24 h before slaughter. The red-blue color system was used in heatmap analysis to represent the relative abundance of each metabolite in the pork loin, and the * mark indicates significant differences in metabolite content between the two groups. The scattering of metabolites in the volcano plot includes up-regulated metabolites (red circle) on the right side and down-regulated metabolites (blue circle) on the left side in relation to 24 h of lairage. Metabolites with a fold change of >1.2 or <0.8 and $P < 0.05$ were considered as regulated by lairage.

Table 3. List of differentiated metabolic pathways in pork loin during lairage

Pathway	P value	Pathway impact
Alanine, aspartate, and glutamate metabolism	0.0063	0.5369
Arginine and proline metabolism	0.0007	0.0981
Arginine biosynthesis	0.0085	0.1168
Citrate cycle	0.0368	0.0625
Cysteine and methionine metabolism	0.0017	0.1045
D-amino acid metabolism	0.0133	0.5000
Glutathione metabolism	0.0001	0.1084
Glycerolipid metabolism	0.0080	0.2368
Glycine, serine, and threonine metabolism	0.0004	0.2961
Glyoxylate and dicarboxylate metabolism	0.0004	0.1058
Histidine metabolism	0.0289	0.1393
Pantothenate and CoA biosynthesis	0.0001	0.0214
Primary bile acid biosynthesis	0.0001	0.0152

Pathway impact value indicates the cumulative percentage of the importance of matched metabolites in each metabolic pathway.

The changes in glycine, glutamate, lysine, and methionine contents might be further related to the antioxidant system. Glutathione metabolism was one of the differentiated pathways between CON and LRG, and glutathione can act as an antioxidant compound against reactive oxygen species and free radicals (Yu et al., 2021). Interestingly, glutamate, glycine, and methionine are constitutive amino acids of glutathione, and lysine plays an important role in the biosynthesis of glutathione; therefore, these 4 free amino acids participated in the glutathione metabolism pathway (Gondret et al., 2021; Zhang et al., 2022). Therefore, the increased glycine, glutamate, lysine, and methionine content in CON compared to LRG may result from the necessity of additional glutathione to protect the cellular components from reactive substances that were formed during stress-induced lipid and protein oxidation (Xing et al., 2019).

In brief, lairage led to the up-regulation of isoleucine and valine and the down-regulation of glutamate, glycerol, glycine, lysine, and methionine in pork loin, which suggested that lairage might help the animals to recover by lowering the energy need and reduce the stress-induced oxidative damage by stimulating glutathione metabolism. On the contrary, without lairage, the muscle proteins and lipids in pork loin would be degraded into energy substrates to fulfill the energy
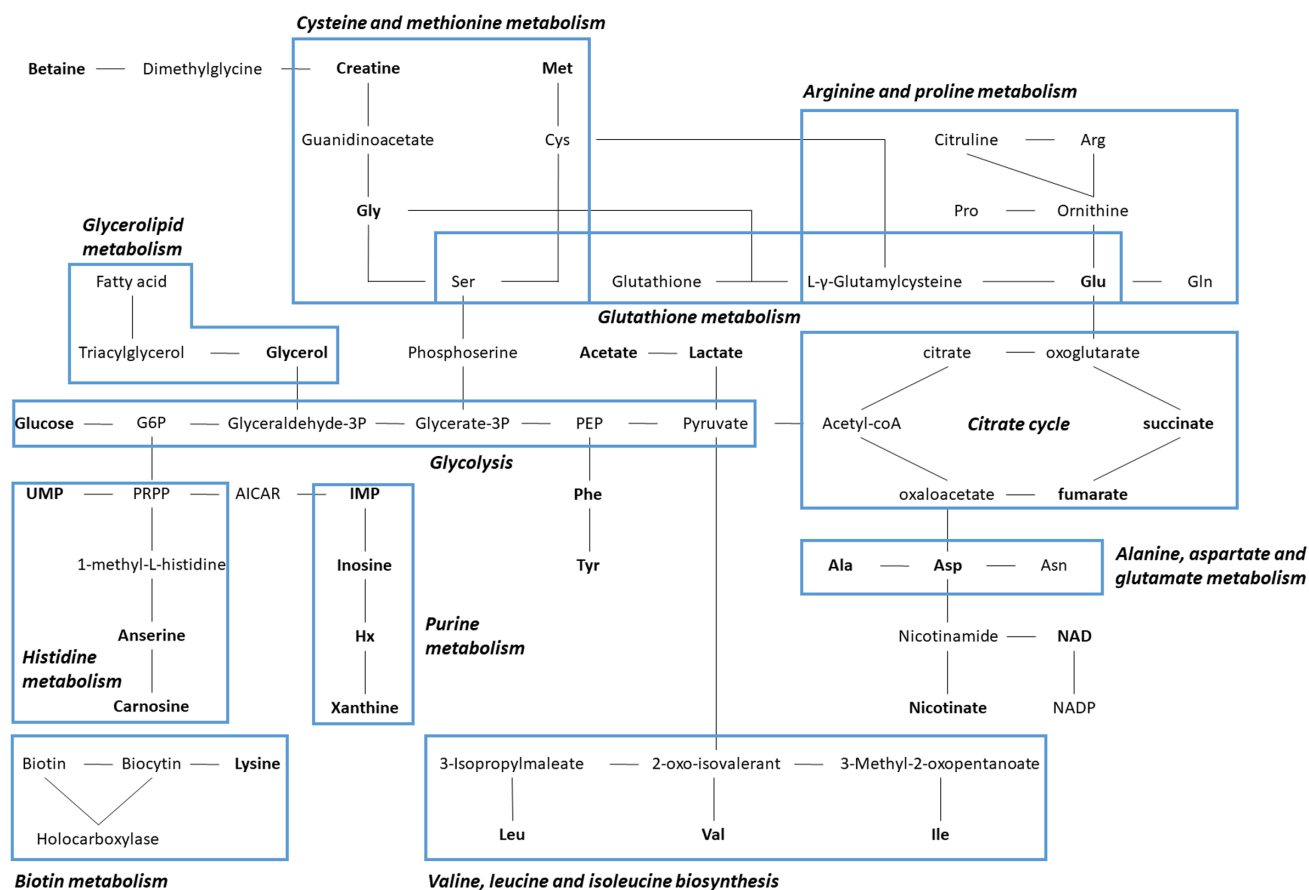
Figure 3. A schematic figure of the metabolic pathways of the metabolites. The name of the identified metabolites in this study was expressed in bold. AICAR, 5-aminoimidazole-4-carboxamide ribonucleotide; AMP, adenosine monophosphate; G6P, glucose 6-phosphate; Hx, hypoxanthine; IMP, inosine monophosphate; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; PEP, phosphoenolpyruvate.

requirements as a result of physiological responses to stressful conditions.

Physicochemical meat quality

pH. The pH of CON and LRG did not differ significantly (5.71 ± 0.10 vs. 5.74 ± 0.13; $P = 0.337$), and both treatments showed a normal pH range (Table 4). On the other hand, previous studies in which the ultimate pH of pork with a long-term lairage for 8 h or more was higher than that with or without a short period of lairage for about 1 to 3 h (Warriss, 2003; Díaz et al., 2014; Dokmanović et al., 2014). The degree of pH decline is mainly affected by the muscle glycogen reserves, and the rate of pH change is dependent on postmortem muscle metabolism (Dalla Costa et al., 2019; Acevedo-Giraldo et al., 2020). Specifically, the glycogen after slaughter is converted into glucose which is used for glycolysis, and the accumulated lactate by glycolysis decreases the muscle pH (Lee et al., 2022). Further, the depletion of creatine phosphate and the accumulation of hydrogen ions from the hydrolysis of ATP also contribute to the decrease of meat pH

Table 4. Physicochemical properties of pork loin in relation to lairage

Traits	CON	LRG	P value
pH	5.71 ± 0.10	5.74 ± 0.13	0.3367
Moisture (%)	74.11 ± 0.55	73.61 ± 1.08	0.0730
WHC (%)	69.20 ± 3.63	69.32 ± 2.73	0.9045
Cooking loss (%)	20.74 ± 0.93	21.06 ± 1.35	0.3903
CIE L^*	55.73 ± 2.05	54.85 ± 1.64	0.1445
CIE a^*	15.89 ± 1.03	15.80 ± 0.81	0.7564
CIE b^*	5.40 ± 0.58	5.56 ± 0.61	0.3980
WBSF (kgf)	5.70 ± 1.20	6.01 ± 1.11	0.4132

The results were expressed as mean $\pm$ standard deviation ($n = 20$ for each group). Pigs in the CON group were slaughtered immediately after unloading, whereas those in the LRG group were held in lairage for 24 h before slaughter.

WBSF, Warner-Bratzler shear force; WHC, water holding capacity.

(Yu et al., 2021). Among the metabolites identified in this study, the contents of glucose, lactate, and acetate (which participates in the glycolysis pathway) were not significantly different, which could explain our unexpected result (Figure 2a).

It is known that stress can affect meat pH. High levels of stress can induce the secretion of cortisol as a stress response of the animal, and cortisol stimulates postmortem glycogen degradation to produce energy (Dokmanović et al., 2014; Gonzalez-Rivas et al., 2020). In this regard, many studies reported that lairage can decrease the extent of glycolysis early postmortem because the animals can recover from pre-slaughter stress (Zhen et al., 2013). However, it was also known that under a prolonged lairage period, pigs face other stressors such as food deprivation, mixing, and fighting with unfamiliar pigs (Rey-Salgueiro et al., 2018). The increased muscular activity as a result of stressors during lairage leads to the consumption of muscle glycogen (Zhen et al., 2013). Therefore, the result of the present study might result from both the positive and negative effects of lairage. The lairage period was set to 24 h in this experimental design with the consideration of the commercial abattoir system in Korea; however, the pH of pork after a shorter lairage may exhibit a different result compared with that of the pork that was slaughtered immediately.

Moisture content, water holding capacity, and cooking loss. There were no significant differences between CON and LRG in the moisture content (74.11 ± 0.55 vs. 73.61 ± 1.08), WHC (69.20 ± 3.63 vs. 69.32 ± 2.73), or cooking loss (20.74 ± 0.93 vs. 21.06 ± 1.35; Table 4). In general, it is known that pigs can have a chance to rehydrate during the lairage period, which may affect moisture content and reduce carcass weight loss (Díaz et al., 2014). Previous studies reported that overnight lairage led to the increase of WHC with a pH rise in pork loin compared to short-term lairage due to the high ultimate pH or delayed muscle metabolism (Nanni Costa et al., 2002; Warriss, 2003; Dokmanović et al., 2017). Furthermore, Zhen et al. (2013) reported a decrease in the cooking loss of pork after 24 h lairage compared to pork without lairage. Our result was not in accordance with the results from the previous literature, possibly because these attributes can be influenced by pH, structural changes of myofibril, or postmortem storage conditions. Here, pH and the amounts of pH-associated metabolites such as lactate and acetate did not vary significantly by lairage, which might explain the present results.

Meat color. There was no significant difference in pork color between CON and LRG including lightness (55.73 ± 2.05 vs. 54.85 ± 1.65), redness (15.89 ± 1.03 vs. 15.80 ± 0.81), and yellowness (5.40 ± 0.58 vs. 5.56 ± 0.61) as shown in Table 4 ($P > 0.05$). It is well known that the appearance of color is important in assessing meat quality. It was reported that the lightness of pork loin showed a positive correlation with consumer preference in South Korea (Lee et al., 2021). The lightness of meat is highly attributed to the meat pH or the structure of muscles, such as myofibril diameter, sarcomere length, and distribution of sarcoplasmic proteins, which all affect light scattering (Purslow et al., 2020; Ali et al., 2021). Higher L^* values were observed in pork loin that was assigned to lairage groups compared to groups without rest (Zhen et al., 2013). However, compared to a long-term lairage of 8 h or longer, a small lairage period of within 3 h was more effective in the increase in the lightness of pork meat (Dokmanović et al., 2014; Acevedo-Giraldo et al., 2020).

Similarly, the a^* and b^* values of pork loin were higher after a short lairage instead of a long lairage in previous studies (Dokmanović et al., 2017), whereas other studies reported an insignificant change in the b^* values during the lairage period (Warriss, 2003). The myoglobin redox form can affect a^* and b^* values, and the relative composition of myoglobin changes depending on the mitochondrial oxygen consumption rate, metmyoglobin reducing activity, the content of NAD(H) and pH (Aroeira et al., 2017). In the present study, we could not find any significant differences in meat pH or NAD content between CON and LRG, which might be one reason why lairage did not lead to the change in meat color.

Warner-Bratzler shear force. Similar to other physicochemical traits, the shear force of pork loin was not significantly different between CON (5.70 ± 1.20) and LRG (6.01 ± 1.11) groups (Table 4; $P = 0.413$). In accordance with the results of physicochemical analyses, the trained sensory panels did not find any significant differences in the eating quality between CON and LRG pork (data not shown). On the other hand, Zhen et al. (2013) reported the increase in the shear force of pork loin was in accordance with the increase in the lairage time. Meat tenderization is attributed to muscle proteolytic activity, and this activity is regulated by pH, temperature, oxidation or nitrosylation of proteins, etc. (Xing et al., 2019; Ali et al., 2021). In pork and chicken meat, the degradation of muscle protein could occur by the cascade effect as a consequence of the increased corticosterone under stressful conditions (Lee et al., 2022). However, although the lairage had an effect on the relief of pre-slaughter stress that animals suffer, including transportation, loading, unloading, and dehydration, whether these kinds of stress induce change in meat tenderness or not, and related mechanisms, have been little known. Therefore, the relationship

between lairage and shear force remains unclear and requires further study.

Overall, there was no considerable change in physicochemical quality parameters between CON and LRG in this study. As stated earlier, these attributes could be influenced much by pH; however, the content of organic acids including lactate between CON and LRG were not significantly different, which may lead to similar pH values between the two groups (Figure 2a). While previous studies reported the effect of overnight lairage on livestock such as the increase in pH and WHC as well as the decrease in drip loss and incidence of PSE meat (Warriss, 2003; Dokmanović et al., 2017), some studies stated that the influence of other stressors like fighting and mixing with unfamiliar animals could possibly be increased as the period of lairage increased (Rey-Salgueiro et al., 2018; Dalla Costa et al., 2019). Possibly, the effects of the changes in metabolite contents and metabolic pathways such as energy metabolism and antioxidant system on meat quality become more evident during storage as enzymatic and non-enzymatic degradation of taste compounds and the protein and lipid oxidation process also influence the meat quality.

Conclusions

In this study, lairage had no significant effect on the physicochemical quality of pork loin. However, lairage led to the up-regulation of isoleucine and valine and the down-regulation of glutamate, glycerol, glycine, lysine, and methionine. Differentially activated pathways between CON and LRG suggested that the stressful pre-slaughter environmental factors stimulate the production of additional energy substrates and the activation of the antioxidant system, and these metabolic changes can be alleviated by providing a period of lairage to animals. Our findings explained how lairage drives metabolic change in pork loin through a metabolomic approach, and suggested the need for further studies on how these changes will affect meat quality in a specific condition, e.g., prolonged storage condition.

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Table S1. T-test for the equality of the metabolite profiles (mg/100 g) of pork loin in relation to lairage

Item	Method	CON	LRG	Df	t	Pr > |t|	F	Df	Pr > F
							t-test for equality of variances		
Free amino acid, dipeptides, and derivatives									
Alanine	Pooled	12.57 ± 1.46	11.76 ± 2.22	38	1.37	0.1798	2.33	19	0.0734
	Satterthwaite			32.79	1.37	0.1810			
Anserine	Pooled	8.95 ± 0.99	9.19 ± 0.76	38	−0.85	0.3995	1.69	19	0.2613
	Satterthwaite			35.65	−0.85	0.3998			
Aspartate	Pooled	0.49 ± 0.21	0.41 ± 0.14	38	1.52	0.1367	2.36	19	0.0683
	Satterthwaite			32.64	1.52	0.1380			
Betaine	Pooled	5.43 ± 1.88	4.67 ± 1.12	38	1.54	0.1317	2.84	19	0.0279
	Satterthwaite			30.90	1.54	0.1335			
Carnitine	Pooled	4.96 ± 1.15	4.93 ± 1.14	38	0.07	0.9442	1.02	19	0.9740
	Satterthwaite			38.00	0.07	0.9442			
Carnosine	Pooled	154.24 ± 14.87	157.66 ± 21.32	38	−0.59	0.5594	2.06	19	0.1250
	Satterthwaite			33.95	−0.59	0.5598			
Creatine	Pooled	195.25 ± 12.27	205.88 ± 16.58	38	−2.30	0.0267	1.83	19	0.1986
	Satterthwaite			35.01	−2.30	0.0272			
Glutamate	Pooled	3.30 ± 0.59	2.83 ± 0.39	38	2.96	0.0053	2.31	19	0.0764
	Satterthwaite			32.87	2.96	0.0057			
Glutamine	Pooled	4.68 ± 1.42	4.34 ± 1.23	38	0.81	0.4245	1.33	19	0.5361
	Satterthwaite			37.24	0.81	0.4246			
Glycine	Pooled	10.78 ± 3.00	7.76 ± 1.75	38	3.88	0.0004	2.93	19	0.0237
	Satterthwaite			30.61	3.88	0.0005			
Isoleucine	Pooled	1.05 ± 0.17	1.25 ± 0.20	38	−3.44	0.0014	1.53	19	0.3653
	Satterthwaite			36.42	−3.44	0.0015			
Leucine	Pooled	2.52 ± 0.38	2.37 ± 0.27	38	1.48	0.1476	2.02	19	0.1344
	Satterthwaite			34.11	1.48	0.1485			
Lysine	Pooled	4.53 ± 0.68	3.13 ± 0.77	38	6.07	<.0001	1.31	19	0.5674
	Satterthwaite			37.35	6.07	<.0001			
Methionine	Pooled	1.31 ± 0.23	1.10 ± 0.16	38	3.31	0.0020	2.14	19	0.1062
	Satterthwaite			33.59	3.31	0.0022			
Phenylalanine	Pooled	1.12 ± 0.14	1.12 ± 0.11	38	−0.09	0.9322	1.60	19	0.3111
	Satterthwaite			36.06	−0.09	0.9322			
Taurine	Pooled	14.82 ± 2.62	16.41 ± 3.00	38	−1.79	0.0812	1.31	19	0.5616
	Satterthwaite			37.33	−1.79	0.0813			
Tyrosine	Pooled	1.57 ± 0.20	1.50 ± 0.09	38	1.43	0.1601	4.38	19	0.0023
	Satterthwaite			27.25	1.43	0.1633			
Valine	Pooled	1.65 ± 0.24	2.07 ± 0.28	38	−5.16	<.0001	1.34	19	0.5282
	Satterthwaite			37.21	−5.16	<.0001			
β-alanine	Pooled	8.28 ± 1.65	8.53 ± 3.69	38	−0.27	0.7878	5.01	19	0.0010
	Satterthwaite			26.30	−0.27	0.7885			
Nucleotide-related products									
Hypoxanthine	Pooled	2.89 ± 0.62	3.00 ± 0.75	38	−0.53	0.6002	1.46	19	0.4148
	Satterthwaite			36.71	−0.53	0.6003			
IMP	Pooled	37.76 ± 2.33	37.87 ± 3.27	38	−0.13	0.9010	1.97	19	0.1484
	Satterthwaite			34.34	−0.13	0.9011			
Inosine	Pooled	8.70 ± 1.20	9.26 ± 1.25	38	−1.44	0.1585	1.09	19	0.8588
	Satterthwaite			37.94	−1.44	0.1586			
NAD	Pooled	0.71 ± 0.41	0.67 ± 0.11	38	0.90	0.3726	1.56	19	0.3412
	Satterthwaite			36.27	0.90	0.3728			

Table S1. (*Continued*)

Item	Method	CON	LRG	Df	t	Pr > |t|	F	Df	Pr > F
							t-test for equality of variances		
UMP	Pooled	0.66 ± 0.06	0.68 ± 0.10	38	−0.86	0.3973	2.48	19	0.0544
	Satterthwaite			32.17	−0.86	0.3983			
Xanthine	Pooled	0.42 ± 0.16	0.34 ± 0.12	38	1.85	0.0714	1.80	19	0.2090
	Satterthwaite			35.13	1.85	0.0721			
			Organic acid						
Acetate	Pooled	5.49 ± 0.83	5.16 ± 1.13	38	1.06	0.2972	1.88	19	0.1785
	Satterthwaite			34.76	1.06	0.2978			
Fumarate	Pooled	0.38 ± 0.13	0.46 ± 0.15	38	−1.65	0.1078	1.38	19	0.4897
	Satterthwaite			37.06	−1.65	0.1080			
Lactate	Pooled	572.48 ± 34.22	564.61 ± 51.65	38	0.57	0.5730	2.28	19	0.0806
	Satterthwaite			32.99	0.57	0.5735			
Nicotinate	Pooled	5.07 ± 0.62	4.80 ± 0.64	38	1.35	0.1846	1.05	19	0.9121
	Satterthwaite			37.98	1.35	0.1846			
Succinate	Pooled	6.14 ± 1.60	5.19 ± 1.99	38	1.66	0.1058	1.56	19	0.3407
	Satterthwaite			36.26	1.66	0.1062			
			Others						
Glucose	Pooled	37.39 ± 8.16	33.66 ± 5.18	38	1.73	0.0924	2.48	19	0.0544
	Satterthwaite			32.17	1.73	0.0938			
Glycerol	Pooled	20.89 ± 3.42	17.96 ± 4.25	38	2.41	0.0211	1.55	19	0.3511
	Satterthwaite			36.33	2.41	0.0213			

Df, degree of freedom.

Pigs in the CON group were slaughtered immediately after unloading, whereas those in the LRG group were held in lairage for 24 h before slaughter.

IMP, inosine monophosphate; NAD, nicotinamide adenine dinucleotide; UMP, uridine monophosphate.

Table S2. T-test for the equality of the carcass and physicochemical quality parameters of pork loin in relation to lairage

Item	Method	CON	LRG	Df	t	Pr > |t|	t-test for equality of variances F	Df	Pr > F
Carcass characteristics									
Live weight (kg)	Pooled	115.31 ± 0.91	118.11 ± 5.01	38	−2.46	0.0186	30.46	19	<.0001
	Satterthwaite			20.25	−2.46	0.0230			
Carcass weight (kg)	Pooled	88.05 ± 0.69	89.50 ± 3.80	38	−1.68	0.1017	30.73	19	<.0001
	Satterthwaite			20.24	−1.68	0.1088			
Backfat thickness (mm)	Pooled	22.20 ± 1.24	23.25 ± 1.41	38	−2.50	0.0168	1.29	19	0.5812
	Satterthwaite			37.39	−2.50	0.0169			
Physicochemical quality parameters									
pH	Pooled	5.71 ± 0.10	5.74 ± 0.13	38	−0.97	0.3367	1.79	19	0.2138
	Satterthwaite			35.18	−0.97	0.3372			
Moisture (%)	Pooled	74.11 ± 0.55	73.61 ± 1.08	38	1.84	0.0730	3.90	19	0.0047
	Satterthwaite			28.15	1.84	0.0757			
WHC (%)	Pooled	69.20 ± 3.63	69.32 ± 2.73	38	−0.12	0.9045	1.77	19	0.2233
	Satterthwaite			35.28	−0.12	0.9046			
Cooking loss (%)	Pooled	20.74 ± 0.93	21.06 ± 1.35	38	−0.87	0.3903	2.11	19	0.1119
	Satterthwaite			33.70	−0.87	0.3910			
CIE L^*	Pooled	55.73 ± 2.05	54.85 ± 1.64	38	1.49	0.1445	1.55	19	0.3482
	Satterthwaite			36.31	1.49	0.1449			
CIE a^*	Pooled	15.89 ± 1.03	15.80 ± 0.81	38	0.31	0.7564	1.60	19	0.3119
	Satterthwaite			36.06	0.31	0.7565			
CIE b^*	Pooled	5.40 ± 0.58	5.56 ± 0.61	38	−0.85	0.3980	1.09	19	0.8487
	Satterthwaite			37.93	−0.85	0.3980			
WBSF (kgf)	Pooled	5.70 ± 1.20	6.01 ± 1.11	38	−0.83	0.4132	1.17	19	0.7390
	Satterthwaite			37.77	−0.83	0.4133			

Df, degree of freedom.

Pigs in the CON group were slaughtered immediately after unloading, whereas those in the LRG group were held in lairage for 24 h before slaughter.

WHC, water holding capacity; WBSF, Warner-Bratzler shear force.