



Original Research Article

Different starch sources and amino acid levels on growth performance, starch and amino acids digestion, absorption and metabolism of 0- to 3-week-old broilers fed low protein diet



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ARTICLE INFO

Article history:

Received 15 February 2024

Received in revised form

26 October 2024

Accepted 7 November 2024

Available online 28 November 2024

Keywords:

Starch

Intestine

Targeted metabolomics

Amino acid oxidation

Broiler

ABSTRACT

The synchronized absorption of amino acids and glucose in the gut is essential for amino acid utilization and protein synthesis in the body. The study aimed to investigate how the starch digestion rate and amino acid levels impact the growth and intestinal starch and amino acid digestion, transport, and metabolism in juvenile broilers. The experiment was conducted with 702 Arbor Acres Plus broilers at 1 d old, which were randomly divided into 9 treatments with 6 replicates of 13 chickens each. The treatments included 3 different starch sources (corn, waxy corn, and tapioca) with 3 different apparent ileal digestible lysine (AID Lys) levels (1.08%, 1.20%, and 1.32%). A notable interaction was noted for dietary starch sources and AID Lys levels in the feed-to-gain ratio (F/G) and distal ileal starch digestibility ($P < 0.01$). The tapioca starch and waxy corn starch diets with 1.32% of AID Lys significantly decreased F/G compared with corn starch ($P < 0.01$). There was no significant difference in F/G of broilers among waxy corn starch diet with 1.08% AID Lys level, tapioca starch diet with 1.20% AID Lys level, and corn starch diet with 1.32% AID Lys level ($P > 0.05$). The 1.32% AID Lys level and the waxy corn starch both improved the body weight (BW) of broilers from 0 to 3 weeks of age, intestinal starch digestibility, and intestinal villi height or the ratio of villi height to crypt depth ($P < 0.05$). Compared with the corn starch diet, waxy corn starch and tapioca starch diets significantly elevated the AID of Met, Glu, Lys, Arg, Asp, His, Ile, Tyr, Gly, and Val levels ($P < 0.05$). The carbon metabolomics results revealed that the waxy corn starch diet significantly reduced malic acid and cis-aconitic acid levels ($P < 0.05$) in the tricarboxylic acid cycle compared to the corn starch diet. It was concluded that a waxy corn starch diet improves the growth performance of broilers by improving intestinal morphology, increasing the absorption and transport of amino acids, reducing the amino acid oxidation for energy supply in the intestinal mucosa, and promoting protein synthesis in muscles, which not only reduces the need for dietary AID Lys but also saves on production costs.

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Peer review under the responsibility of Chinese Association of Animal Science and Veterinary Medicine.



Production and Hosting by Elsevier on behalf of KeAi

1. Introduction

Recently, poultry has overtaken pork as the most consumed meat in the world (OECD/FAO, 2023a). In poultry production, the diet is traditionally formulated using corn and soybean meal as the main ingredients. However, the cost of corn is increasing in China due to international imports, which affects the price of poultry feed prices (OECD/FAO, 2023b). Therefore, it is important to consider using other cereal grains, legume seeds, tubers, and roots as alternatives to corn.

Grains serve as the primary reservoirs of starch and energy. Starch is a complex dietary carbohydrate, which contains amylose and amylopectin. Starch utilization regulates the energy utilization rate (Patience et al., 2015). Moreover, starches exhibit variation in their rate and site of digestion in animals based on their source, amylose-to-amylopectin ratio, and structure influencing energy supply and production performance (Yin et al., 2010). Waxy corn starch and tapioca starch have abundant amylopectin, quickly releasing glucose in animals, triggering glucose and insulin response in the body (Luo et al., 2023a), thereby promoting animal growth (Petrilla et al., 2022). The intestinal uptake of glucose may antagonize amino acid transport, as both processes require coupled transport with Na^+ absorption, thereby impacting animal growth (Moss et al., 2018; van den Borne et al., 2007). Additionally, a reciprocal relationship exists between the digestion of starch and amino acids, whereby the digestion of starch influences the metabolic pathways of amino acids (Selle and Liu, 2019).

The well-timed provision of glucose could enhance amino acid utilization and body protein synthesis (Zhou et al., 2022), thereby diminishing the need for dietary crystal amino acids. This is because enterocytes utilize glucose for energy supply, consequently decreasing the amount of dietary crystal amino acid oxidized for energy supply and improving the likelihood and efficiency of converting dietary crystal amino acid into body proteins (Li et al., 2024). Moreover, Weurding et al. (2003) found that the effect of slowly digestible starch (SDS) on the feed-to-gain ratio (F/G) of broilers was pronounced at low dietary amino acid levels and in the early stage, suggesting that the rate of starch digestion affects the utilization of dietary amino acid and has a greater effect on young broilers. Recent studies indicate that the crystal amino acids are in monomeric form, and once they enter the intestinal tract they get absorbed into the bloodstream or oxidatively degraded by the intestinal cells, whereas digestible starch in the diet can only release glucose after a period of digestion in the intestine (Nolles et al., 2009). Hence, crystal amino acids in the diet can be released much more rapidly than starch-derived glucose, which may lead to a higher proportion of crystal amino acids in the diet being utilized directly for oxidative degradation, resulting in amino acid wastage. Previous studies have demonstrated that rapidly digestible starch (RDS) diets are quickly digested in the intestine, releasing glucose to provide energy for enterocytes, thereby enhancing the production performance and nitrogen efficiency of growing pigs (Zhou et al., 2021, 2022). Furthermore, our research team discovered that the RDS diet enhanced starch digestibility and the F/G of young broilers (Yin et al., 2019). Previous findings suggest that intestinal amino acid utilization can be improved, and the requirement of dietary crystal amino acids can be decreased, thereby potentially saving production costs by modulating the rate of dietary starch digestion without compromising the production performance of livestock and poultry. In summary, there is disagreement regarding the relationship between the rate of starch digestion and amino acid utilization. Furthermore, it remains unclear why increasing the dietary starch digestion rate would improve amino acid utilization. Therefore, further study is warranted.

The connection between the speed of starch digestion and the amino acid oxidation for energy supply is crucial for the energy metabolism of the intestinal mucosa. Energy metabolism is regulated by the tricarboxylic acid (TCA) cycle (Ewald et al., 2016), and this process decomposes amino acids and lipids to generate energy in cells (Zhu et al., 2022). The intestine, as an “energy-consuming organ”, digests and absorbs dietary nutrients, utilizing 20% to 35% of the animal's total energy (Van Der Schoor et al., 2002; Wang et al., 2018). The energy metabolism of the intestinal mucosa is more complex than that of other tissues, such as the liver, primarily because the energy source of intestinal mucosa consists of an

intricate blend of luminal and arterial matrix, and the oxidation pattern of intestinal matrix is altered by the nutrient composition of the diet (Li et al., 2019; van der Schoor et al., 2001; Zhou et al., 2022). Optimizing the rate of glucose release in the diet by changing the dietary starch pattern and promoting the simultaneous supply of amino acids and glucose may be a viable way to elevate the efficiency of amino acid utilization and protein synthesis. In addition, prior reports have noted that the slowly digestible dietary starch provides sufficient glucose to the animal's hindgut and reduces the intestinal mucosal amino acid oxidation for energy supply (Weurding et al., 2003; Yin et al., 2019).

In the present study, we hypothesized that young broilers have an immature digestive system. RDS helps release glucose quickly, making it easier for the animals to digest and absorb nutrients, thereby preventing amino acid oxidation. Therefore, we used different starch sources and different amino acid levels (based on apparent ileal digestible lysine [AID Lys]) to investigate the relationship between the rate of starch digestion and the amino acid oxidation for energy supply in juvenile broilers. Moreover, earlier studies lacked a substantial scientific basis for understanding the energy metabolism of the intestinal mucosa. Therefore, in the present study, intestinal mucosa-targeted metabolomics was focused on exploring the mechanism between the rate of starch digestion and the oxidation of intestinal amino acids for energy supply.

2. Materials and methods

2.1. Animal ethics statement

The animal procedures followed Beijing's Laboratory Animal Regulations and were authorized by the Laboratory Animal Welfare and Ethical Committee of China Agricultural University (approval number: AW40703202-1-4).

2.2. Trial design and diet formulation

The experiment was designed as a 3×3 factorial design and conducted with 702 one-day-old healthy male Arbor Acres Plus broilers (with an average body weight of 41.95 ± 0.024 g), randomly divided into 9 treatments. The treatments included 3 different starch sources (corn, waxy corn, and tapioca) with 3 different levels of AID Lys (1.08%, 1.20%, and 1.32%). The corn starch diet was used as the control, while waxy corn starch and tapioca starch were used as the RDS diets. Every group had 6 replicate cages with 13 male chickens per cage. The duration of the experiment was 21 d. In this study, the 1.20% AID Lys level was the recommended value for broilers aged 1 to 3 weeks (Aviagen, 2018), and other amino acids were adjusted according to amino acid balance. Apparent metabolizable energy (AME) and crude protein (CP) levels were set based on previous studies of our group. Near-infrared spectroscopy was employed to analyze the chemical components, AME, and digestible amino acid content in corn, soybean meal, and corn gluten meal. This study reduced CP diets that were formulated to contain 2% lower CP than the control (21% vs. 23%). According to the measured results of these ingredients, the experimental starch diets were then formulated (Table 1).

2.3. Bird husbandry

The broilers were managed following the guidelines for Arbor Acres Plus broilers (Aviagen, 2018). All broilers were provided unrestricted access to water and crumble-pellet feed via nipple drinkers and feed troughs.

Table 1
Ingredients and nutrient composition of experimental diet.

Item	Corn starch			Tapioca starch			Waxy corn starch		
Ingredients, % as-fed basis									
Corn	40.70	41.60	42.60	40.70	41.60	42.60	40.70	41.60	42.60
Corn starch (AM/AP = 0.29) ¹	15.00	15.00	15.00						
Tapioca starch (AM/AP = 0.11) ¹				15.00	15.00	15.00			
Waxy corn starch (AM/AP = 0.05) ¹							15.00	15.00	15.00
Corn gluten meal	4.57	5.00	5.00	4.57	5.00	5.00	4.57	5.00	5.00
Soybean meal	33.23	31.30	29.80	33.23	31.30	29.80	33.23	31.30	29.80
Soybean oil	1.60	1.40	1.10	1.60	1.40	1.10	1.60	1.40	1.10
Dicalcium phosphate	1.67	1.70	1.70	1.67	1.70	1.70	1.67	1.70	1.70
Limestone	1.15	1.15	1.15	1.15	1.15	1.15	1.15	1.15	1.15
Sodium chloride	0.20	0.20	0.15	0.20	0.20	0.15	0.20	0.20	0.15
Sodium bicarbonate	0.33	0.33	0.38	0.33	0.33	0.38	0.33	0.33	0.38
Vitamins premix ²	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03	0.03
Mineral premix ³	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Choline chloride (50%)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
Antioxidant	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
Phytase 10,000, U/g	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02	0.02
L-Lysine hydrochloride (98%)	0.27	0.47	0.67	0.27	0.47	0.67	0.27	0.47	0.67
DL-Methionine (98%)	0.22	0.33	0.42	0.22	0.33	0.42	0.22	0.33	0.42
L-Threonine (98%)	0.06	0.16	0.26	0.06	0.16	0.26	0.06	0.16	0.26
L-Arginine hydrochloride (98%)	0.07	0.24	0.40	0.07	0.24	0.40	0.07	0.24	0.40
L-Tryptophan (98%)	–	–	0.03	–	–	0.03	–	–	0.03
L-Valine (98%)	–	0.08	0.20	–	0.08	0.20	–	0.08	0.20
Titanium dioxide	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
L-Isoleucine (98%)	–	0.11	0.21	–	0.11	0.21	–	0.11	0.21
Total	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00	100.00
Nutritional level									
AME ⁴ , Mcal/kg	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00	3.00
AME ¹ , Mcal/kg	3.02	2.99	2.97	3.00	2.91	2.94	2.95	3.00	2.94
CP ⁴ , %	20.99	21.05	21.06	20.99	21.05	21.06	20.99	21.05	21.06
CP ¹ , %	21.03	21.01	21.08	20.96	21.07	21.03	21.02	21.08	21.07
Calcium ⁴ , %	0.95	0.95	0.94	0.95	0.95	0.94	0.95	0.95	0.94
NPP ⁴ , %	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37	0.37
Total Lys ¹ , %	1.32	1.42	1.56	1.25	1.39	1.51	1.26	1.46	1.57
Total Met ¹ , %	0.73	0.88	0.81	0.69	0.80	0.85	0.71	0.85	0.89
Total Met + Cys ¹ , %	1.12	1.26	1.22	1.09	1.17	1.14	1.14	1.30	1.28
Total Thr ¹ , %	1.09	1.07	1.06	0.97	0.97	1.02	0.99	1.04	1.14
Total Val ¹ , %	1.16	1.22	1.33	1.05	1.16	1.16	1.05	1.16	1.16
Total Arg ¹ , %	1.36	1.52	1.63	1.40	1.41	1.61	1.41	1.51	1.64
Total Ile ¹ , %	1.06	1.27	1.27	0.96	1.20	1.27	1.07	1.17	1.23
Total Leu ¹ , %	1.87	1.98	2.03	1.93	1.85	1.81	1.89	1.85	1.86
AID Lys ⁴ , %	1.08	1.20	1.32	1.08	1.20	1.32	1.08	1.20	1.32
AID Met ⁴ , %	0.50	0.60	0.69	0.50	0.60	0.69	0.50	0.60	0.69
AID Met + Cys ⁴ , %	0.78	0.89	0.96	0.78	0.89	0.96	0.78	0.89	0.96
AID Lys ¹ , %	1.10	1.19	1.37	1.09	1.22	1.33	1.05	1.20	1.33
AID Met ¹ , %	0.53	0.69	0.66	0.55	0.67	0.72	0.54	0.58	0.68
AID Met + Cys ¹ , %	0.81	0.93	0.94	0.83	0.92	0.91	0.86	0.91	0.98
AID Thr ¹ , %	0.81	0.80	0.85	0.78	0.76	0.84	0.77	0.75	0.85
AID Val ¹ , %	0.89	0.93	1.09	0.84	0.94	0.97	0.85	0.96	1.00
AID Arg ¹ , %	1.15	1.30	1.46	1.24	1.25	1.46	1.23	1.23	1.42
AID Ile ¹ , %	0.67	0.90	0.97	0.68	0.92	0.99	0.73	0.78	0.87
AID Leu ¹ , %	1.76	1.87	1.93	1.84	1.77	1.73	1.80	1.74	1.75
Total starch ¹ , %	31.97	32.67	33.30	31.15	34.26	34.99	32.15	32.42	33.70
AM/AP ¹	0.18	0.18	0.18	0.15	0.15	0.16	0.08	0.08	0.09

ME = apparent metabolizable energy; CP = crude protein; NPP = non-phytate phosphorus; AM = amylose; AP = amylopectin.

¹ Actually measured values of nutrient components.

² The vitamin premix provided (per kilogram of diets) the following: vitamin A, 15,000 IU; vitamin D₃, 3600 IU; vitamin E, 30 IU; vitamin K₃, 3.00 mg; vitamin B₂, 9.60 mg; vitamin B₁₂, 0.03 mg; biotin, 0.15 mg; folic acid, 1.50 mg; pantothenic acid, 13.80 mg; nicotinic acid, 45 mg.

³ The trace mineral premix provided (per kilogram of diets) the following: Cu, 16 mg; Zn, 110 mg; Fe, 80 mg; Mn, 120 mg; Se, 0.30 mg; I, 1.50 mg.

⁴ Design values of nutrient components.

2.4. Sample collection

On d 21, six birds of similar weight from each group (1 bird/replicate) were chosen for sampling. The birds were stunned using electronarcosis and then euthanized by exsanguination. Tissue samples about 2 to 3 cm from the mid-jejunum and mid-ileum were collected, rinsed off with saline solution, and then fixed in 4% paraformaldehyde for morphological detection. The digesta was collected from the entire proximal jejunum (PJ), distal jejunum (DJ), proximal ileum (PI), and distal ileum (DI). The four segments were

marked by locating the midpoints between the end of the duodenal loop, Meckel's diverticulum, and the ileocecal junction. One digesta sample was snap-frozen in liquid nitrogen to detect the activities of amylase, trypsin, chymotrypsin, and lipase. Another portion of the digesta samples was freeze-dried to determine the digestibility of amino acids and starch. The jejunum samples were collected into 1.5-mL RNase-free Cryo tubes and flash-frozen in liquid nitrogen for mRNA analysis of glucose and amino acid transporters. The ileal mucosa samples of the waxy corn starch and corn starch diets supplemented with 1.08% and 1.32% AID Lys were scraped with a

sterile glass microscope slide. These samples were then snap-frozen in liquid nitrogen for targeted energy metabolomics.

2.5. Growth performance

On d 21, following a 12-h fast, feed intake (FI) and body weight (BW) of broilers were measured for each cage, and body weight gain (BWG) and F/G were calculated. Mortality was recorded daily.

2.6. Jejunal digestive enzyme activity measurement

The digesta in the jejunum was centrifuged at 4 °C and $1500 \times g$ for 10 min, and the supernatant was used to determine the activities of lipase, chymotrypsin, trypsin, and amylase using commercially available kits from Nanjing Jianjian Bioengineering Institute Co., Ltd. (Nanjing, China).

2.7. Chemical analysis

The TiO_2 of the digesta and diet was determined based on the method of Short et al. (1996). The starch content assay was measured using an assay kit (code: #BC0705, Solarbio, Beijing, China) based on the use of thermostable α -amylase and amylo-glucosidase method (McCleary et al., 1997). Amylose and amylo-pectin content in starch was determined according to the instructions of Megazyme's commercially available kit by a colorimetric method (code: K-AMYL, Wicklow, Ireland). Dry matter was determined based on the China National Standard (GB/T 6435-2014). The CP of feed and feces was determined based on the Kjeldahl method (total nitrogen $\times 6.25$) using the technical shown in China National Standard (GB/T 6432-2018). The AME bioassay was adopted from those described by Bourdillon et al. (1990). The gross energy (GE) of feed and feces was determined by oxygen bomb calorimetry using a 6400 automatic isoperibol calorimeter (Parr Instrument Co., Moline, IL, USA) with benzoic acid as the standard.

$$\text{AME (kcal/kg)} = \frac{\text{GE}_{\text{diet}} \times \text{FI} - \text{GE}_{\text{feces}} \times \text{Fecal output}}{\text{FI}}$$

The freeze-dried samples of ileal digesta and diet were ground, sieved through a 40-mesh sieve, and then stored in a sealed bag at 4 °C for future testing. The method of Cohen (2000) was used to determine the amino acid contents of the digesta in the ileum and diet. The apparent ileal digestibility (AID) of starch and amino acid was determined using the following formula.

$$\text{AID}(\%) = \left[1 - \left(\frac{\% \text{Nutrient}_{\text{digesta}}}{\% \text{Nutrient}_{\text{diet}}} \right) \times \left(\frac{\% \text{Marker}_{\text{diet}}}{\% \text{Marker}_{\text{digesta}}} \right) \right] \times 100$$

2.8. Determination of intestinal morphology and structure

The jejunum and ileum tissue samples were dehydrated, embedded, fixed, and stained, then sliced for observation of intestinal morphology and structure. The specific procedure was referred to as the method described by Luo et al. (2023b).

2.9. Determination of gene expression related to intestinal glucose and amino acid transporters

Relevant steps such as RNA extraction, cDNA reverse transcription, and quantitative real-time PCR (qRT-PCR) of the jejunal

samples were based on the method of Luo et al. (2023b). The sequences of qRT-PCR primers for the intestinal glucose and amino acid transporters are shown in Table 2. All sample relative expression results were normalized to housekeeping gene (β -actin) expression using the $2^{-\Delta\Delta\text{Ct}}$ method.

2.10. Ileal mucosa targeted metabolomics analysis

2.10.1. Sample preparation

Briefly, 10 mg of ileal mucosa sample was mixed with 20 μL of isotopic internal standard, 100 μL of 50% methanol in water, and 280 μL of acetonitrile in a 1.5-mL centrifuge tube. Then, two small steel beads were added, and the sample was ground in a freeze grinder for 6 min (-10 °C, 50 Hz). The tubes were centrifuged at $14,000 \times g$ for 20 min at 4 °C, and 100 μL of supernatant was taken; and added with 25 μL of 200 mmol/L 3-NPH·HCl solution and 25 μL of 120 mmol/L EDC·HCl (containing 6% pyridine) solution, vortexed for 30 s, and reacted at 60 °C for 40 min with a constant temperature oscillator. After the reaction was complete, the samples were mixed on a vortex mixer for 30 s, after which they were centrifuged at $14,000 \times g$ for 20 min at 4 °C, and the supernatant was transferred to the mass spectrometry vial for liquid chromatograph mass spectrometer (LC-MS) analysis.

2.10.2. LC-MS analysis

For the investigation at hand, the detection of the target substances in the samples was conducted using the liquid chromatography-electrospray ionization tandem mass spectrometry (LC-ESI-MS/MS) technique. Both qualitative and quantitative analyses were performed. The detailed parameters employed in the study are provided below.

Chromatographic conditions: The mobile phase A was 0.03% formic acid in water and the mobile phase B was 0.03% formic acid in methanol. ExionLC AD system, Waters HSS T3 (2.1 mm \times 150 mm, 1.8 μm) liquid chromatography column, column temperature was kept at 40 °C, and injection volume was 2 μL . The chromatographic separation was carried out using the gradient described in Table S1.

Mass spectrometry conditions: SCIEX QTRAP 6500+, mass spectrometric detection was carried out in positive and negative modes using an ESI source. The temperature was 550 °C, Ion Source Gas 2 was 55 psi, IonSpray Voltage was +4500/-4500 V, Curtain Gas was 35 psi, Ion Source Gas 1 was 55 psi, and collision Gas was medium. Central carbon metabolite ion pair parameter information is described in Table S2.

2.10.3. Metabolomics data analysis

The ileal mucosa targeted energy metabolomics data underwent processing using multivariate statistical analysis. Within this analysis, a comparison of metabolic profiles was conducted through the utilization of principal component analysis (PCA). To distinguish between various groups, orthogonal-projections-to-latent-structures discriminant analysis (OPLS-DA) was executed (Li and Song, 2019). To ensure the avoidance of overfitting, all models assessed underwent testing via permutation tests. Furthermore, differential metabolites could be further screened by merging *P*-values or fold changes in the univariate analysis. The R software (www.r-project.org/) enabled the performance of hierarchical cluster analysis on the accumulation patterns of metabolites among diverse samples. The findings' supporting data had already been deposited into the CNGB Sequence Archive of the China National Gene-Bank Database under the accession number METM0000139.

Table 2
Primer sequences of RT-PCR.

Gene	Forward sequences (5' to 3')	Reverse sequences (5' to 3')
β -Actin	GAGAAATGTGCGTGACATCA	CCTGAACCTCTCATTGCCA
<i>SGLT1</i>	AGATTTGGAGGGCAGAGGAT	GCCCAAAGAGATTTGGATGA
<i>GLUT2</i>	CCGCAGAAGGTGATAGAAGC	ATTGTCCCTGGAGGTGTT
<i>PepT1</i>	TACGCATACTGTACCATCA	TCCTGAGAACGGACTGTAAT
<i>B⁰AT</i>	TATCCTGGCTGGGTCTATGC	AGGCCTGTACGATCCCTTCT
<i>EAAT3</i>	TGATTGTTCTGAGCGCTGTC	TACCAAAGGCATCTCCAAG
<i>CAT1</i>	CACATGGATACGGTTTGACG	GTCCATGTTCTCTCCGTGT
<i>y⁺LAT1</i>	CACCAGTCCCTGCTCTTCTC	CTGCAATAGACAAGCCAC
<i>LAT1</i>	TACCTGCTGAAGCCATCTT	ACGGGTAGCAGCTTTCACAC
<i>b^{0,+}AT</i>	CAGTAGTAATTCTCTGAGTGAAGCT	GCAATGATTGCCAC AACTACCA

SGLT1 = sodium-glucose cotransporter 1; *GLUT2* = glucose transporter 2; *PepT1* = peptide-transporter 1; *B⁰AT* = Na⁺ dependent neutral amino acid transporter; *EAAT3* = excitatory amino acid transporter 3; *CAT1* = Na⁺ independent cationic amino acid transporter 1; *y⁺LAT1* = Na⁺ independent cationic and Na⁺ dependent neutral amino acid transporter 1; *LAT1* = Na⁺ independent neutral amino acid transporter 1; *b^{0,+}AT* = Na⁺ independent cationic and zwitterionic amino acid transporter.

2.11. Statistical analysis

To ensure the authenticity of the research, an examination of data homogeneity was performed prior to conducting the two-factor analysis of variance using the general linear model (GLM) from the widely used SPSS 20.0 statistical software (version 20.0, SPSS Inc., USA). Meanwhile, one-way ANOVA and Duncan's multiple comparisons were used when a significant interaction was observed. A significant difference was declared as having $P < 0.05$, and $0.05 \leq P \leq 0.10$ was defined as a statistical trend.

$$Y_{ijk} = \mu + \alpha_i + \beta_j + (\alpha \times \beta)_{ij} + \varepsilon_{ijk},$$

where Y_{ijk} = the k th observation of dependent variable recorded on i th and j th treatment, μ = overall mean, α_i = effect of i th treatment, β_j = effect of j th treatment, $(\alpha \times \beta)_{ij}$ = interaction effect of i th treatment \times j th treatment, ε_{ijk} = error associated with Y_{ijk} .

3. Results

3.1. Growth performance

A notable interaction was observed between the sources of dietary starch and AID Lys levels in relation to the F/G of broilers ($P = 0.001$) (Table 3). The tapioca starch and waxy corn starch with 1.32% AID Lys significantly decreased F/G compared with corn starch ($P = 0.001$). The waxy corn starch with 1.08% AID Lys significantly decreased F/G compared with corn starch with 1.08% AID Lys ($P = 0.001$), but the difference between tapioca starch and corn starch was not significant at 1.08% AID Lys ($P > 0.05$). However, no significant interaction was observed between dietary starch sources and AID Lys levels concerning the BW, ADG, and ADFI of broilers ($P > 0.05$). In addition, dietary starch sources had a significant effect on ADG ($P = 0.013$) and BW ($P = 0.008$), waxy corn starch increased BW and ADG of broilers significantly compared with corn starch and tapioca starch ($P < 0.05$). The AID Lys levels

Table 3
Effects of different starch sources and levels of AID Lys on growth performance of 0 to 21 d broilers.

Item	Initial BW, g	BW, kg	ADG, g	ADFI, g	F/G
Corn starch					
1.08% AID Lys	41.95	0.875	39.69	51.25	1.30 ^a
1.20% AID Lys	41.92	0.849	38.13	49.15	1.28 ^b
1.32% AID Lys	41.92	0.872	39.53	50.35	1.28 ^b
Tapioca starch					
1.08% AID Lys	42.05	0.860	38.97	52.05	1.30 ^a
1.20% AID Lys	41.97	0.845	38.24	48.83	1.28 ^b
1.32% AID Lys	41.93	0.865	39.20	49.22	1.25 ^c
Waxy corn starch					
1.08% AID Lys	41.97	0.884	40.08	50.83	1.27 ^{bc}
1.20% AID Lys	41.93	0.872	39.33	50.62	1.29 ^{ab}
1.32% AID Lys	41.92	0.915	41.59	52.02	1.25 ^c
SEM	0.024	0.048	0.237	0.302	0.003
Main effect					
Starch source					
Corn starch	41.93	0.866 ^b	39.12 ^b	50.25	1.29
Tapioca starch	41.98	0.857 ^b	38.80 ^b	50.03	1.28
Waxy corn starch	41.94	0.890 ^a	40.33 ^a	51.15	1.27
AID Lys level					
1.08%	41.99	0.873 ^{ab}	39.58 ^{ab}	51.37 ^a	1.29
1.20%	41.94	0.855 ^b	38.57 ^b	49.53 ^b	1.28
1.32%	41.92	0.884 ^a	40.10 ^a	50.53 ^{ab}	1.26
P-value					
Starch source	0.637	0.008	0.013	0.233	0.009
AID Lys level	0.532	0.031	0.016	0.035	<0.001
Starch source \times AID Lys level	0.988	0.703	0.674	0.197	0.001

BW = body weight gain; ADG = average daily gain; ADFI = average daily feed intake; F/G = feed to gain ratio; SEM = standard error of means; AID Lys = apparent ileal digestible lysine.

Means in the same column with different superscripts indicate significant differences ($P < 0.05$).

had a significant effect on BW, ADG, and ADFI of broilers ($P < 0.05$). The 1.32% AID Lys level markedly increased the BW and ADG of broilers as compared with 1.20% AID Lys ($P < 0.05$). The 1.20% AID Lys level significantly decreased the ADFI of broilers compared with the 1.08% AID Lys level ($P = 0.035$).

3.2. Starch digestibility

No significant interaction was observed between dietary starch sources and AID Lys levels regarding the starch digestibility of PJ, DJ, and PI of broilers ($P > 0.05$) (Table 4). A notable interaction was observed between dietary starch sources and AID Lys levels on the starch digestibility of DI of broilers ($P = 0.001$). The tapioca starch with 1.32% AID Lys noticeably increased the starch digestibility of DI compared with corn starch and waxy corn starch, or AID Lys level of 1.20% or 1.08% ($P = 0.001$). The starch digestibility of DI was lowest in the waxy corn starch with 1.08% AID Lys and in corn starch with 1.08% or 1.20% AID Lys.

Dietary starch sources significantly influenced the digestibility of starch in PJ, DJ, and PI ($P < 0.01$). In the PJ, the waxy corn starch exhibited the highest digestibility, followed by tapioca starch, while corn starch showed the lowest digestibility ($P < 0.001$). The waxy corn starch and tapioca starch displayed a significant enhancement in starch digestibility in the DJ compared to the corn starch ($P = 0.002$). The waxy corn starch notably improved the starch digestibility in PI ($P = 0.007$) compared to both corn starch and tapioca starch. Additionally, AID Lys levels significantly affected starch digestibility in PJ and DJ ($P < 0.001$). The 1.20% and 1.32% AID Lys levels significantly increased the starch digestibility of PJ and DJ compared to the 1.08% AID Lys level ($P < 0.001$).

3.3. Jejunal digestive enzyme activity

No notable interaction was observed between dietary starch sources and AID Lys levels on the digestive enzyme activities in the jejunum of the broiler ($P > 0.05$) (Table 5). The AID Lys levels

Table 5

Effects of different starch sources and levels of AID Lys on jejunal digestive enzymes activities of 21 d broilers.

Item	Jejunal digestive enzyme activity, U/mg prot			
	Amylase	Lipase	Trypsin	Chymotrypsin
Corn starch				
1.08% AID Lys	56.24	214.40	549.89	8.49
1.20% AID Lys	80.18	251.28	553.63	11.89
1.32% AID Lys	81.65	307.23	568.59	12.50
Tapioca starch				
1.08% AID Lys	76.26	271.50	536.80	10.83
1.20% AID Lys	85.60	298.44	534.93	13.27
1.32% AID Lys	93.78	297.93	566.72	12.01
Waxy corn starch				
1.08% AID Lys	77.73	294.98	617.22	13.22
1.20% AID Lys	87.64	339.05	654.63	13.06
1.32% AID Lys	96.39	362.82	649.02	15.78
SEM	3.337	10.842	19.245	0.439
Main effect				
Starch source				
Corn starch	72.69	257.63 ^b	557.37	10.96 ^b
Tapioca starch	85.21	289.29 ^{ab}	546.15	12.04 ^b
Waxy corn starch	87.26	332.28 ^a	640.29	14.02 ^a
AID Lys level				
1.08%	70.08 ^b	260.29 ^b	567.97	10.84 ^b
1.20%	84.47 ^{ab}	296.26 ^{ab}	581.06	12.74 ^{ab}
1.32%	90.60 ^a	322.66 ^a	594.78	13.43 ^a
P-value				
Starch source	0.147	0.014	0.118	0.008
AID Lys level	0.036	0.047	0.859	0.025
Starch source × AID Lys level	0.946	0.828	0.987	0.342

SEM = standard error of means; AID Lys = apparent ileal digestible lysine.

Means in the same column with different superscripts indicate significant differences ($P < 0.05$).

dramatically affected the jejunal lipase, chymotrypsin, and amylase activities ($P < 0.05$), and the 1.32% AID Lys level significantly increased jejunal lipase, chymotrypsin, and amylase activities of broilers compared with the 1.08% AID Lys level ($P < 0.05$). Dietary starch sources had remarkable effects on jejunal lipase ($P = 0.014$)

Table 4

Effects of different starch sources and levels of AID Lys on the starch digestibility (%) of 21 d broilers.

Item	Proximal jejunum	Distal jejunum	Proximal ileum	Distal ileum
Corn starch				
1.08% AID Lys	79.73	87.68	93.72	94.83 ^{cd}
1.20% AID Lys	85.57	90.23	93.57	94.43 ^d
1.32% AID Lys	83.60	90.57	94.37	95.57 ^{bc}
Tapioca starch				
1.08% AID Lys	82.32	89.08	93.73	95.52 ^{bc}
1.20% AID Lys	86.65	91.73	94.23	96.22 ^b
1.32% AID Lys	87.33	92.42	94.58	97.03 ^a
Waxy corn starch				
1.08% AID Lys	85.93	91.27	94.62	94.40 ^d
1.20% AID Lys	88.13	92.32	95.15	96.17 ^b
1.32% AID Lys	89.68	92.55	94.93	95.37 ^{bc}
SEM	0.595	0.336	0.153	0.142
Main effect				
Starch source				
Corn starch	82.97 ^c	89.49 ^b	93.88 ^b	94.94
Tapioca starch	85.43 ^b	91.08 ^a	94.18 ^b	96.26
Waxy corn starch	87.92 ^a	92.04 ^a	94.90 ^a	95.31
AID Lys level				
1.08%	82.66 ^b	89.34 ^b	94.02	94.92
1.20%	86.78 ^a	91.43 ^a	94.32	95.61
1.32%	86.87 ^a	91.84 ^a	94.63	95.99
P-value				
Starch source	<0.001	0.002	0.007	<0.001
AID Lys level	<0.001	<0.001	0.132	<0.001
Starch source × AID Lys level	0.677	0.751	0.847	0.001

SEM = standard error of the mean; AID Lys = apparent ileal digestible lysine.

Means in the same column with different superscripts indicate significant differences ($P < 0.05$).

and chymotrypsin ($P = 0.008$) activities of broilers. The waxy corn starch significantly increased the lipase and chymotrypsin activities in jejunum relative to the corn starch ($P < 0.05$).

3.4. Intestinal morphology analysis

No notable interaction was observed between dietary starch sources and AID Lys levels on the jejunal and ileal morphology of the broiler ($P > 0.05$) (Table 6). However, AID Lys levels dramatically affected the jejunal villi height (VH) and the ratio of villi height to crypt depth (VH/CD) ($P < 0.05$). The 1.32% AID Lys level markedly increased jejunal VH/CD and VH ($P < 0.05$) compared to the 1.08% AID Lys level. Dietary starch sources significantly affected the jejunal VH/CD ($P < 0.001$). As compared to corn starch, tapioca starch and waxy corn starch increased jejunal VH/CD significantly ($P < 0.001$). In addition, AID Lys levels had a notable effect on the ileal VH/CD and VH ($P < 0.01$). The 1.32% AID Lys level significantly increased ileal VH and VH/CD as compared with the 1.08% and 1.20% AID Lys levels ($P < 0.01$). Dietary starch sources had a notable effect on the ileal VH ($P = 0.003$) and CD ($P = 0.013$). Compared with corn starch, waxy corn starch increased ileal VH ($P = 0.003$), and tapioca starch decreased ileal CD significantly ($P = 0.013$).

3.5. Apparent ileal amino acid digestibility

A notable interaction was observed between dietary starch sources and AID Lys levels on the apparent ileal digestibility of Asp, Met, and Tyr ($P < 0.01$) (Table 7). At the 1.08% AID Lys level, the waxy corn starch significantly increased the apparent ileal digestibility of Asp, Met, and Tyr compared with corn starch ($P < 0.01$). At the 1.20% AID Lys level, the waxy corn starch significantly increased the apparent ileal digestibility of Asp and Tyr compared with corn starch ($P < 0.01$), however, the different starch sources had no notable effect on the apparent ileal digestibility of

Met ($P > 0.05$). At the 1.32% AID Lys level, different starch sources had no significant effect on the apparent ileal digestibility of Asp, Met, and Tyr ($P > 0.05$).

Dietary starch sources dramatically affected the apparent ileal digestibility of Asp, Arg, Tyr, Gly, Lys, Val, Cys, Ile, Glu, His, Met, and Thr ($P < 0.05$). Compared with corn starch, tapioca starch, and waxy corn starch significantly increased the apparent ileal digestibility of Asp, Ile, Gly, Lys, Val, Glu, Tyr, Arg, His, and Met ($P < 0.05$), waxy corn starch significantly increased the apparent ileal digestibility of Thr and Cys ($P < 0.05$).

AID Lys level dramatically affected the apparent ileal digestibility of Asp, Gly, Ala, Val, Met, Ile, and Lys ($P < 0.05$). Compared with the 1.08% AID Lys level, the 1.32% AID Lys level significantly increased the apparent ileal digestibility of Gly, Ala, Val, Met, Ile, and Lys ($P < 0.05$). The 1.32% AID Lys level significantly increased the apparent ileal digestibility of Asp and Val ($P < 0.05$) contrasted to the 1.20% AID Lys level.

3.6. Intestinal glucose and amino acid transporters

No notable interaction was observed between dietary starch sources and AID Lys levels on the mRNA expression of glucose and amino acid transporters in the jejunum of broilers ($P > 0.05$) (Table 8). However, dietary starch sources had a notable effect on the mRNA expressions of *GLUT-2*, *SGLT-1*, *CAT1*, and *y⁺LAT1* in the jejunum ($P < 0.05$). The tapioca starch and waxy corn starch significantly decreased the mRNA expression of *SGLT-1*, *GLUT-2*, *CAT1*, and *y⁺LAT1* in jejunum as compared with corn starch ($P < 0.05$). Dietary AID Lys levels did not affect glucose and amino acid transporters in the jejunum of broilers ($P > 0.05$) except for the *CAT1* gene. The 1.20% and 1.32% AID Lys levels significantly increased the mRNA expression of *CAT1* in jejunum as compared with the 1.08% AID Lys level ($P = 0.003$).

Table 6
Effects of different starch sources and levels of AID Lys on intestinal morphology of 21 d broilers.

Item	Jejunal morphology			Ileal morphology		
	VH, μm	CD, μm	VH/CD	VH, μm	CD, μm	VH/CD
Corn starch						
1.08% AID Lys	876.58	221.72	4.01	513.86	156.36	3.53
1.20% AID Lys	880.36	190.83	4.66	513.45	149.76	3.49
1.32% AID Lys	960.34	182.85	5.29	612.96	134.45	4.61
Tapioca starch						
1.08% AID Lys	895.74	201.32	4.45	490.31	130.53	3.78
1.20% AID Lys	920.47	185.83	4.98	523.21	138.24	3.68
1.32% AID Lys	1015.75	187.07	5.45	579.34	132.43	4.41
Waxy corn starch						
1.08% AID Lys	873.35	182.95	4.78	575.83	146.88	3.98
1.20% AID Lys	1083.40	207.40	5.25	602.90	159.09	3.71
1.32% AID Lys	1016.96	179.65	5.68	696.98	142.67	4.83
SEM	19.969	3.882	0.078	13.356	2.455	0.087
Main effect						
Starch source						
Corn starch	905.76	190.00	4.65 ^c	546.76 ^b	146.86 ^a	3.88
Tapioca starch	943.99	191.41	4.96 ^b	530.95 ^b	133.73 ^b	3.96
Waxy corn starch	991.24	198.47	5.24 ^a	625.23 ^a	149.55 ^a	4.17
AID Lys level						
1.08%	881.89 ^b	183.19	4.41 ^c	526.67 ^b	144.59	3.76 ^b
1.20%	961.41 ^{ab}	194.69	4.96 ^b	546.52 ^b	149.03	3.63 ^b
1.32%	997.68 ^a	201.99	5.47 ^a	629.76 ^a	136.52	4.62 ^a
P-value						
Starch source	0.190	0.613	<0.001	0.003	0.013	0.154
AID Lys level	0.046	0.127	<0.001	0.001	0.078	<0.001
Starch source \times AID Lys level	0.352	0.140	0.662	0.954	0.390	0.707

SEM = standard error of means; AID Lys = apparent ileal digestible lysine.

Means in the same column with different superscripts indicate significant differences ($P < 0.05$).

Table 7
Effects of different starch sources and levels of AID Lys on apparent ileal amino acid digestibility (%) of 21 d broilers.

Item	Asp	Thr	Glu	Pro	Gly	Ala	Cys	Val	Met	Ile	Tyr	Phe	His	Lys	Arg
Corn starch															
1.08% AID Lys	82.42 ^b	78.79	85.94	82.63	68.21	82.12	70.84	76.23	72.81 ^d	63.62	68.74 ^d	96.06	84.88	83.38	84.41
1.20% AID Lys	75.68 ^c	76.69	84.33	81.42	78.62	86.03	63.61	75.83	78.41 ^c	70.50	65.87 ^d	94.17	84.65	83.93	85.91
1.32% AID Lys	83.69 ^{ab}	81.16	87.94	82.15	78.00	88.13	66.76	82.33	82.10 ^{abc}	76.54	76.96 ^c	94.88	89.00	87.94	89.50
Tapioca starch															
1.08% AID Lys	81.66 ^b	79.85	88.02	84.42	78.72	88.23	69.43	79.49	80.75 ^{abc}	81.29	82.85 ^{abc}	94.68	88.56	86.68	89.54
1.20% AID Lys	85.10 ^{ab}	80.77	89.44	85.42	80.95	87.67	68.01	81.00	83.72 ^{abc}	76.35	84.90 ^{ab}	94.96	91.17	87.77	89.22
1.32% AID Lys	86.50 ^a	82.02	89.01	83.20	81.48	86.46	64.88	83.12	85.01 ^{ab}	78.04	77.60 ^c	93.83	89.72	88.27	90.44
Waxy corn starch															
1.08% AID Lys	86.61 ^a	83.53	89.70	86.05	78.23	84.39	74.07	80.86	85.80 ^a	79.97	87.05 ^a	94.78	87.17	86.92	90.44
1.20% AID Lys	84.29 ^{ab}	82.64	87.99	82.61	82.27	87.61	75.61	82.12	79.20 ^{bc}	76.22	76.34 ^c	93.87	89.48	87.30	87.46
1.32% AID Lys	86.59 ^a	81.80	88.53	83.40	80.85	88.45	75.25	86.30	83.32 ^{abc}	80.80	79.27 ^{bc}	92.35	87.78	88.61	89.67
SEM	0.591	0.488	0.386	0.511	0.801	0.511	1.002	0.713	0.773	1.061	1.160	0.488	0.455	0.467	0.482
Main effect															
Starch source															
Corn starch	80.59 ^b	78.88 ^b	86.07 ^b	82.07	74.94 ^b	85.42	67.07 ^b	78.13 ^b	77.77 ^b	70.22 ^b	70.52 ^b	95.50	86.18 ^b	85.08 ^b	86.61 ^b
Tapioca starch	84.42 ^a	80.88 ^{ab}	88.82 ^a	84.34	80.38 ^a	87.45	67.44 ^b	81.20 ^a	83.16 ^a	75.23 ^a	81.78 ^a	94.49	89.82 ^a	87.57 ^a	89.63 ^a
Waxy corn starch	85.83 ^a	82.66 ^a	88.74 ^a	84.02	80.45 ^a	86.81	74.97 ^a	83.09 ^a	82.77 ^a	78.99 ^a	80.88 ^a	93.66	88.14 ^a	87.61 ^a	89.19 ^a
AID Lys level															
1.08%	83.56 ^{ab}	80.72	87.88	84.36	75.06 ^b	84.91 ^b	71.77	78.86 ^b	79.79 ^b	71.62 ^b	79.54	95.17	86.87	85.66 ^b	88.03
1.20%	81.69 ^b	80.03	87.25	83.15	80.61 ^a	87.10 ^{ab}	69.08	79.65 ^b	80.44 ^{ab}	74.36 ^{ab}	75.70	94.33	88.43	86.33 ^{ab}	87.53
1.32%	85.60 ^a	81.66	88.49	82.92	80.11 ^a	87.68 ^a	68.96	83.92 ^a	83.48 ^a	78.46 ^a	77.94	93.68	88.83	88.27 ^a	89.87
P-value															
Starch source	<0.001	0.004	0.002	0.156	0.001	0.206	0.001	0.008	0.001	0.001	<0.001	0.547	0.002	0.032	0.013
AID Lys level	0.002	0.325	0.341	0.469	0.001	0.048	0.445	0.004	0.043	0.009	0.146	0.493	0.102	0.048	0.077
Starch source × AID Lys level	0.002	0.242	0.184	0.634	0.180	0.094	0.454	0.835	0.010	0.137	0.001	0.917	0.084	0.645	0.206

SEM = standard error of means; AID Lys = apparent ileal digestible lysine.
Means in the same column with different superscripts indicate significant differences ($P < 0.05$).

Table 8
Effects of different starch sources and levels of AID Lys on jejunal glucose and amino acid transporters (copy/ μ g total RNA) of 21 d broilers.

Item	<i>SGLT-1</i>	<i>GLUT-2</i>	<i>CAT1</i>	<i>b⁰⁺AT</i>	<i>y⁺LAT1</i>	<i>LAT1</i>	<i>EAAT3</i>	<i>B⁰AT</i>	<i>PepT1</i>
Corn starch									
1.08% AID Lys	1.45	4.07	1.80	1.10	1.20	1.02	0.95	1.12	0.49
1.20% AID Lys	0.87	2.70	2.53	1.37	1.04	0.92	0.97	0.93	0.47
1.32% AID Lys	1.36	2.89	2.36	1.49	1.29	1.08	1.02	1.14	0.44
Tapioca starch									
1.08% AID Lys	0.84	1.60	1.22	1.16	0.77	0.73	0.86	1.13	0.46
1.20% AID Lys	0.90	2.27	2.05	1.17	1.16	1.13	0.81	0.89	0.34
1.32% AID Lys	0.79	2.18	2.49	1.45	0.91	0.92	1.11	1.13	0.95
Waxy corn starch									
1.08% AID Lys	0.86	1.84	1.23	1.48	0.84	1.08	1.08	0.86	0.51
1.20% AID Lys	1.08	1.49	1.52	1.57	0.78	1.00	1.16	1.31	0.55
1.32% AID Lys	0.98	1.69	1.96	1.59	1.11	0.96	1.16	0.98	0.56
SEM	0.054	0.173	0.111	0.070	0.047	0.038	0.038	0.054	0.059
Main effect									
Starch source									
Corn starch	1.22 ^a	3.22 ^a	2.23 ^a	1.32	1.18 ^a	1.00	0.98	1.06	0.47
Tapioca starch	0.84 ^b	2.02 ^b	1.92 ^{ab}	1.26	0.95 ^b	0.92	0.93	1.05	0.58
Waxy corn starch	0.97 ^b	1.67 ^b	1.57 ^b	1.55	0.91 ^b	1.01	1.13	1.05	0.54
AID Lys level									
1.08%	1.05	2.50	1.42 ^b	1.24	0.94	0.94	0.96	1.03	0.49
1.20%	0.95	2.15	2.03 ^a	1.37	0.99	0.98	0.98	1.04	0.45
1.32%	1.04	2.25	2.24 ^a	1.51	1.10	1.01	1.10	1.08	0.65
P-value									
Starch source	0.008	<0.001	0.029	0.227	0.034	0.599	0.066	0.992	0.716
AID Lys level	0.653	0.611	0.003	0.321	0.300	0.767	0.269	0.921	0.349
Starch source × AID Lys level	0.057	0.196	0.612	0.931	0.120	0.166	0.699	0.178	0.332

SEM = standard error of means; AID Lys = apparent ileal digestible lysine; *SGLT1* = sodium-glucose cotransporter 1; *GLUT2* = glucose transporter 2; *PepT1* = peptide-transporter 1; *B⁰AT* = Na⁺ dependent neutral amino acid transporter; *EAAT3* = excitatory amino acid transporter 3; *CAT1* = Na⁺ independent cationic amino acid transporter 1; *y⁺LAT1* = Na⁺ independent cationic and Na⁺ dependent neutral amino acid transporter 1; *LAT1* = Na⁺ independent neutral amino acid transporter 1; *b⁰⁺AT* = Na⁺ independent cationic and zwitterionic amino acid transporter.
Means in the same column with different superscripts indicate significant differences ($P < 0.05$).

3.7. Central carbon metabolomic characteristics of ileal mucosa

The waxy corn starch (WC) group vs corn starch (C) group (Fig. 1): The PCA and OPLS-DA plot demonstrated the differences in metabolites between the WC group and the C group (Fig. 1A and B).

The permutation test diagram of the OPLS-DA model showed that the intercept of Q2 was -0.1491 (200-time response permutation testing, Fig. 1B), it could be seen that the OPLS-DA model was effective and not over-fitting ($Q2 < 0.05$), which further demonstrates that the material is sufficiently reproducible and suitable for

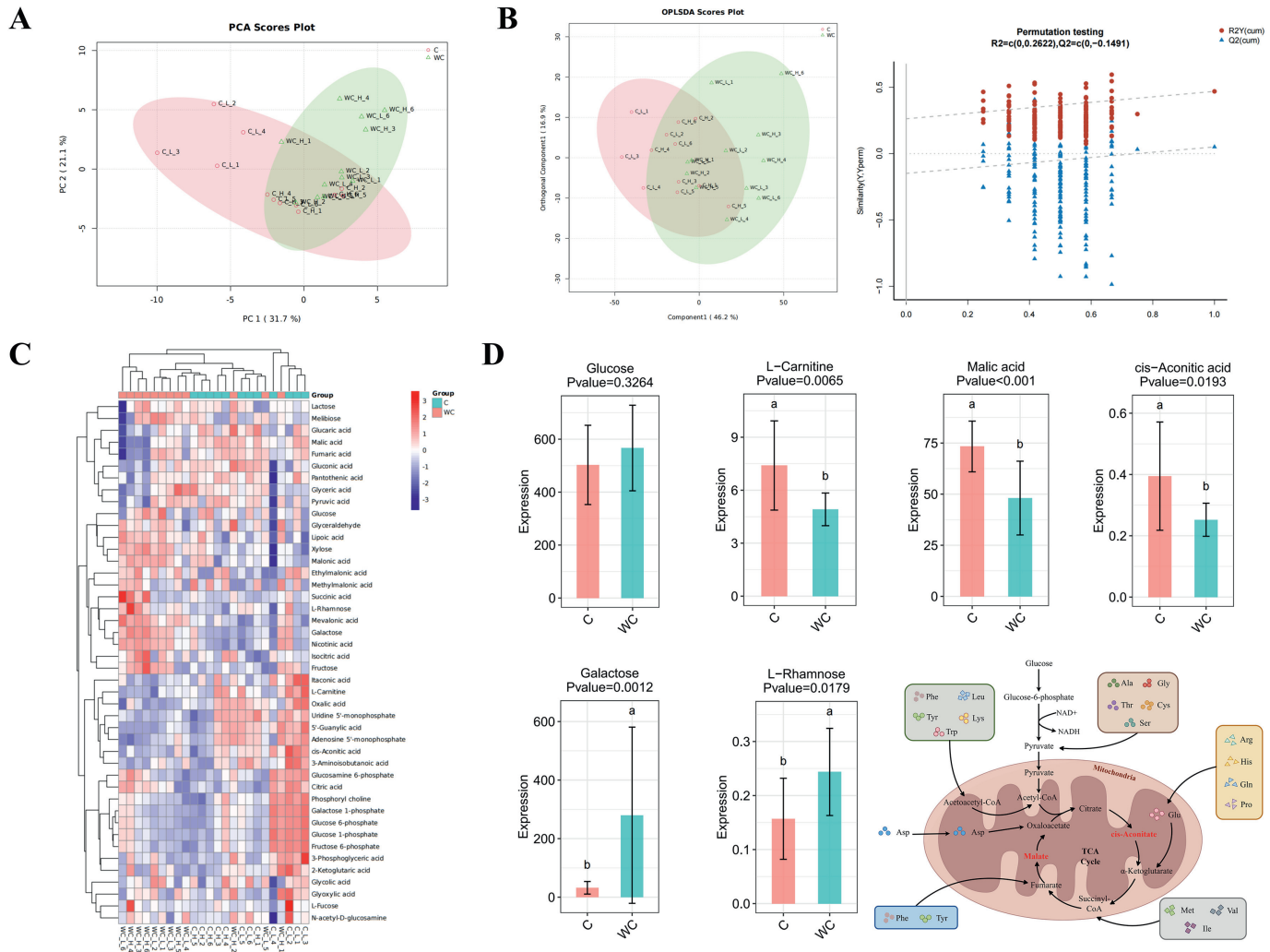


Fig. 1. Targeted central carbon metabolomic analysis between group WC and group C ($n = 12$ for each group). (A) PCA score plot. (B) OPLS-DA score plot and the corresponding permutation test plot. (C) Hierarchical clustering heat map of 44 differential metabolites between group WC and group C. (D) Content analysis of key expressed metabolites between group WC and group C. WC = waxy corn starch; C = corn starch; TCA = tricarboxylic acid cycle; PCA = principal component analysis; OPLS-DA = orthogonal-projections-to-latent-structures discriminant analysis. Bars with different superscripts indicate significant differences ($P < 0.05$).

subsequent validation of qualitative and quantitative analyses. The cluster analysis results showed (Fig. 1C) that 18 metabolites were up-regulated in the WC group and 26 metabolites were up-regulated in the C group. Among them, the WC group significantly decreased the contents of L-carnitine, malic acid, and cis-aconitic acid, and significantly increased the contents of galactose ($P = 0.001$) and L-Rhamnose ($P = 0.002$) as compared with the C group (Fig. 1D).

The 1.32% AID Lys (H) group vs 1.08% AID Lys (L) group (Fig. 2): the PCA and OPLS-DA plot demonstrated the similarity of metabolites between the H group and the L group (Fig. 2A and B). The cluster analysis results showed (Fig. 2C) that 22 metabolites were up-regulated in the H group and 22 were up-regulated in the L group. Combined with the results of the quantitative analysis, it can be seen that some of the differential metabolites, which we are concerned about (Glucose, xylose, L-Rhamnose, L-carnitine, malic acid, and cis-aconitic acid) had no significant differences between the H and L groups ($P > 0.05$) (Fig. 2D).

The corn starch + 1.08% AID Lys (C_L) group vs (waxy corn starch + 1.08% AID Lys) WC_L group (Fig. 3): The PCA and OPLS-DA plot demonstrated the differences in metabolites between the C_L

group and the WC_L group (Fig. 3A and B). The cluster analysis results showed (Fig. 3C) that 16 metabolites were up-regulated in the WC_L group and 28 were up-regulated in the C_L group. Among them, compared with the C_L group, the WC_L group tended to increase the galactose content ($P = 0.055$), and significantly decreased the contents of glucosamine 6-phosphate, glucose 1-phosphate, fructose 6-phosphate, galactose 1-phosphate, and glucose 6-phosphate ($P < 0.05$) (Fig. 3D).

The corn starch + 1.32% AID Lys (C_H) group vs waxy corn starch + 1.08% AID Lys (WC_L) group (Fig. 4): The PCA and OPLS-DA plot demonstrated the differences in metabolites between the C_H group and the WC_L group (Fig. 4A and B). The cluster analysis results showed (Fig. 4C) that 19 metabolites were up-regulated in the WC_L group and 25 were up-regulated in the C_H group. Among them, compared with the C_H group, the WC_L group significantly increased the contents of galactose ($P = 0.010$) and L-Rhamnose ($P = 0.037$), and significantly decreased the content of malic acid ($P = 0.025$) (Fig. 4D). However, no significant difference was found in the contents of glucosamine 6-phosphate, glucose 1-phosphate, fructose 6-phosphate, galactose 1-phosphate, and glucose 6-phosphate between the C_H group and the WC_L group ($P > 0.05$).

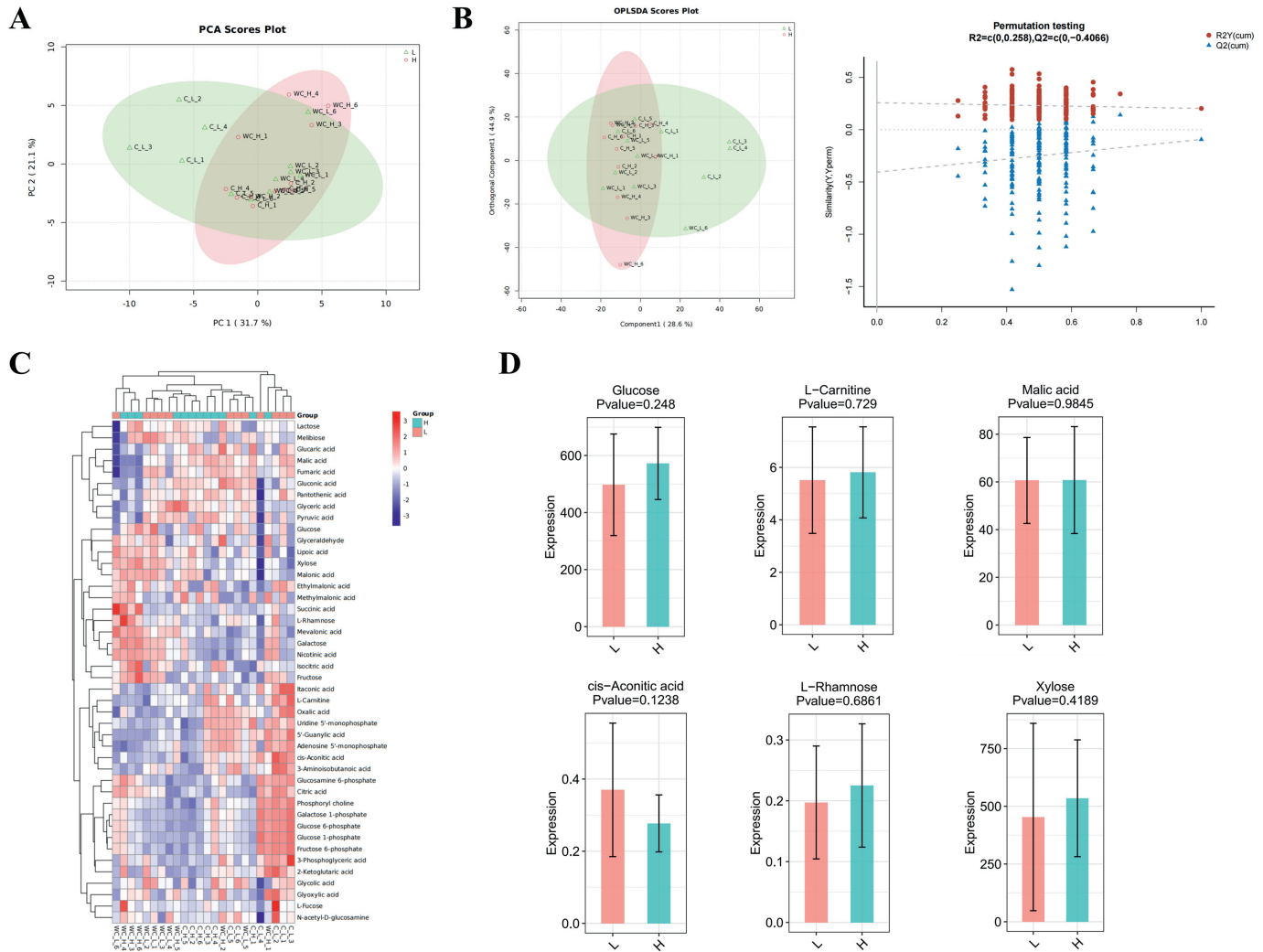


Fig. 2. Targeted central carbon metabolomic analysis between group H and group L ($n = 12$ for each group). (A) PCA score plot. (B) OPLS-DA score plot and the corresponding permutation test plot. (C) Hierarchical clustering heat map of 44 differential metabolites between group H and group L. (D) Content analysis of key expressed metabolites between group H and group L. H = 1.32% AID Lys; L = 1.08% AID Lys; PCA = principal component analysis; OPLS-DA = orthogonal-projections-to-latent-structures discriminant analysis.

4. Discussion

Balancing glucose and amino acid uptakes in the intestine is important to obtain optimum growth performance in broilers. Sydenham et al. (2017) found a quadratic relationship between the rate at which starch and protein disappear in the jejunum of broilers and their weight gain ($y = -28.874x^2 + 207.49x + 892.42$, $R^2 = 0.722$) and F/G ($y = 0.0219x^2 - 0.1699x + 1.6165$, $R^2 = 0.702$). In the present study, we investigated the interactive effect of different sources of starch and amino acid levels (based on AID Lys) in young broilers. The findings indicate that the addition of waxy corn starch at different AID Lys levels significantly improved the F/G of broilers in comparison to the corn starch diet. The negative effect of the corn starch diet can be linked to the underdeveloped digestive capabilities of young chickens. However, the rapid digestion rate of the RDS diet is more suitable for enhancing intestinal morphology and the absorption of nutrients (Luo et al., 2023a, Tables 4 and 6), and the positive effects contribute to enhanced feed efficiency and production performance in young broilers (Herwig et al., 2019; Yin et al., 2019). Furthermore, the results of the present study revealed no significant differences in the BW, ADG, and F/G between broilers fed waxy corn starch with 1.08% AID Lys level and those fed corn

starch with 1.32% AID Lys level, which suggested that the use of waxy corn starch reduces the dietary requirement for AID Lys while maintaining consistent growth performance.

The digestibility of starch correlates with its amylose-to-amylopectin ratio and the molecular weights of amylose and amylopectin (Ma et al., 2020). The current study observed improved starch digestibility in the jejunum when broilers were fed tapioca starch and waxy corn starch diets. The tapioca starch and waxy corn starch diets were rapidly digested in the proximal small intestine, resulting in the release of glucose and triggering an insulin response (Luo et al., 2023a). The results of this study are consistent with the findings of Zhu et al. (2011), who observed a negative correlation between the quantity of starch hydrolyzed by amylase in the intestine and the dietary content of amylose, which occurred because the hydrogen bonds linking the glucose residues in amylose rendered it less vulnerable to amylase action, leading to a reduction in starch digestibility (Svihus et al., 2005).

The digestive enzyme activities in the gastrointestinal tract are crucial for nutrient absorption and digestion and are linked to the bird's performance. Alterations in the diet affect the endogenous enzyme activity in the gut (Yang et al., 2018). However, Ma et al. (2020) found that different dietary amylose/amylopectin ratios

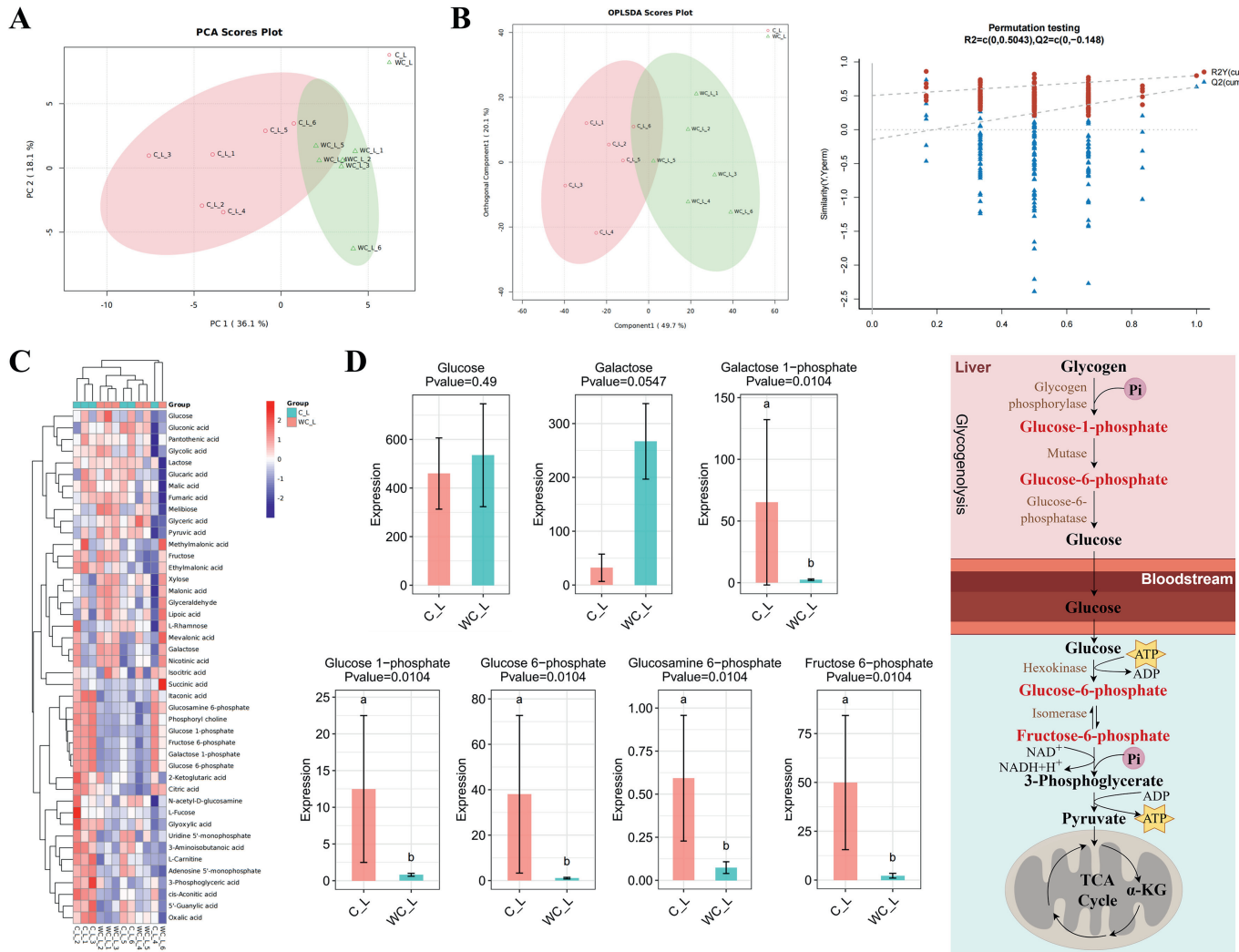


Fig. 3. Targeted central carbon metabolomic analysis between group C_L and group WC_L ($n = 6$ for each group). (A) PCA score plot. (B) OPLS-DA score plot and the corresponding permutation test plot. (C) Hierarchical clustering heat map of 44 differential metabolites between group C_L and group WC_L. C_L = corn starch + 1.08% AID Lys; WC_L = waxy corn starch + 1.08% AID Lys; WC_L = waxy corn starch + 1.08% AID Lys; ATP = adenosine triphosphate; ADP = adenosine diphosphate; TCA = tricarboxylic acid cycle; α -KG = α -ketoglutaric acid; PCA = principal component analysis; OPLS-DA = orthogonal-projections-to-latent-structures discriminant analysis. Bars with different superscripts indicate significant differences ($P < 0.05$).

did not affect enzyme activities like α -amylase, trypsin, and lipase. In contrast, in the present study, the waxy corn starch diet increased jejunal lipase and chymotrypsin activities in broilers. The addition of waxy corn starch may have contributed to regulating the secretion of endogenous enzymes in response to changes in the dietary amylose/amylopectin ratio as reported by Liu et al. (2019). In addition, our research indicates that increasing the AID Lys levels in the diet resulted in elevated activities of amylase, lipase, and chymotrypsin in the jejunum, which suggests that Lys may have the potential to enhance intestinal health and improve small intestinal digestion and absorption function.

The small intestine facilitates the absorption and digestion of nutrients by broilers, including the decomposition of feed in the duodenum, and absorption of nutrients in the jejunum, followed by continued absorption and fermentation of products in the ileum (Moreno-Mendoza et al., 2021). Hence, young broilers need to have normal intestinal morphology development to effectively absorb and digest nutrients. The current study revealed that high AID Lys levels in the diet significantly increased the VH/CD and VH in the jejunum and ileum of broilers. This finding aligns with the results

reported by Vaezi et al. (2011) and Nunes et al. (2015), suggesting that the increased intestinal absorptive surface resulting from higher Lys metabolism could be the contributing factor. The increases in VH were associated with increased digestion and absorption of nutrients as well as brush border enzymes and nutrient transport systems (Maqsood et al., 2022). The higher the VH/CD, the stronger the intestinal absorptive capacity (Du and Guo, 2021). In addition, the current study demonstrated that the waxy corn starch diet improved the jejunal VH/CD, ileal VH, and CD of broilers. Similarly, Wang et al. (2022b) showed that the waxy corn starch diet supported the early intestinal development of broilers, leading to improved nutrient digestion, absorption, and overall growth performance.

The specific transport proteins mediate glucose and amino acid absorption in the intestine (Khwatenge et al., 2020; Zhou et al., 2021). In chickens, glucose absorption is facilitated by SGLT1 at the GLUT2. The SGLT1 transports glucose by utilizing the downhill gradient of Na^+ maintained by Na^+/K^+ -ATPase (Li et al., 2019). Intestinal GLUT2 helps the entry of glucose, galactose, and fructose into the bloodstream, and maintains glucose balance in the body

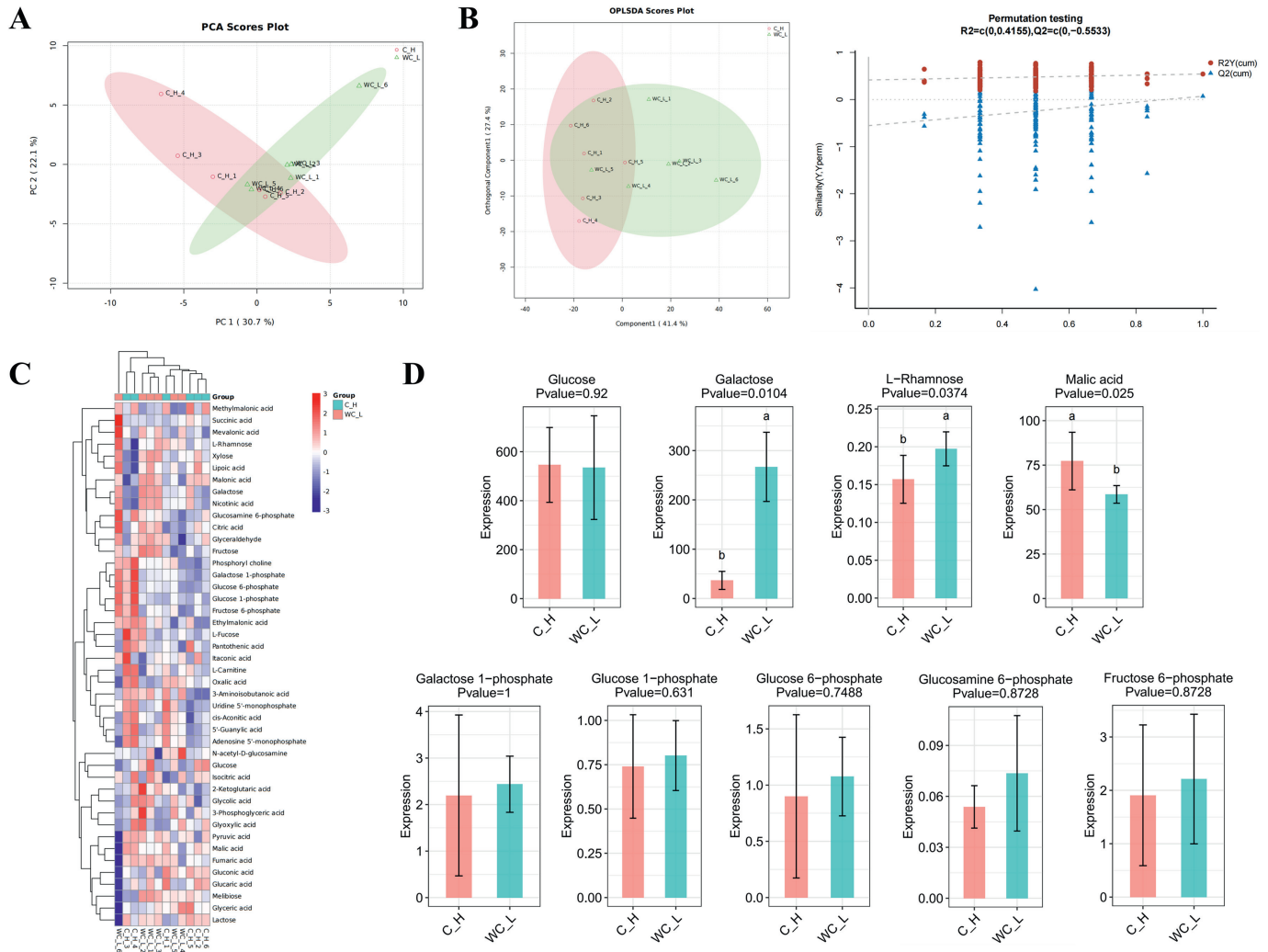


Fig. 4. Targeted central carbon metabolomic analysis between group C_H and group WC_L ($n = 6$ for each group). (A) PCA score plot. (B) OPLS-DA score plot and the corresponding permutation test plot. (C) Hierarchical clustering heat map of 44 differential metabolites between group C_H and group WC_L. (D) Content analysis of key expressed metabolites between group C_H and group WC_L. C_H = corn starch + 1.32% AID Lys; WC_L = waxy corn starch + 1.08% AID Lys; PCA = principal component analysis; OPLS-DA = orthogonal-projections-to-latent-structures discriminant analysis. Bars with different superscripts indicate significant differences ($P < 0.05$).

(Schmitt et al., 2017). Proteins are first degraded to small oligopeptides and amino acids in the stomach and small intestinal tract before being absorbed. Amino acid is transported intracellularly in the free form via a variety of transporters with different specificities (Palacín and Kanai, 2004) or in the form of dipeptides and tripeptides via peptide transporters (Leibach and Ganapathy, 1996). Cationic amino acids are transported by CAT family proteins (Bröer, 2008). The *CAT1* has a high affinity for Lys, ornithine, and Arg (Hatzoglou et al., 2004). In the current study, dietary AID Lys levels increased the mRNA expression of *CAT1*, which was also reported by Morales et al. (2013). The current study suggests that increasing levels of dietary AID Lys promote the up-regulation of the expression of its related transporters, which in turn promotes the absorption and utilization of amino acids in the intestinal tract. Moreover, in the current study, a high amylose diet upregulated the jejunal mRNA expression of glucose and amino acid transporters. Contrary to previous studies, it has been shown that akin to glucose transport, the absorption of amino acids also requires co-transport with sodium ions (Na^+), potentially leading to competition between amino acids and glucose for intestinal absorption (Moss et al., 2018; van den Borne et al., 2007). However, we found from

the serum glucose and insulin responses in broilers after 2 h of feeding (Luo et al., 2023a) and the results of the above test that the rate of starch digestion and glucose release had a negative correlation with the expression of glucose transporters, but an interaction was noted for glucose and amino acid transport in the intestine. Hence, the mechanism of glucose and amino acid transport needs further exploration in the future.

The state of glucose and amino acids in the intestinal tract also affects the energy metabolic balance of the intestinal mucosa. Intestinal homeostasis and renewal of intestinal epithelial cells depend on adenosine 5'-triphosphate (ATP) supplied by the energy metabolism of the intestinal mucosal cells (Cao et al., 2021; Wang et al., 2022a). The energy metabolism of the intestinal mucosa is more complex than that of other tissues such as the liver, because the energy source of intestinal mucosa consists of an intricate blend of luminal and arterial matrix, and the oxidation pattern of intestinal matrix is altered by nutrients composition of the diet (Li et al., 2019; van der Schoor et al., 2001; Zhou et al., 2022). In recent years, more and more studies have found that changing the digestion rate of dietary starch could improve the efficiency of amino acid utilization and protein synthesis (Yin et al., 2019; Zhou et al., 2021,

2022). In the present study, targeted carbon metabolomics showed that compared with the corn starch diet, the waxy starch diet significantly decreased the contents of malic acid and cis-aconitic acid in the ileal mucosa. Malic acid and cis-aconitic acid are key metabolites in the TCA cycle, a key pathway for energy metabolism (Sweetlove et al., 2010). Amino acids like Asp, Glu, and glutamine are metabolized into TCA cycle intermediates and supply energy (Katragkou et al., 2017). Specifically, Glu serves as a major metabolic fuel in the enterocytes of chickens (He et al., 2022). In addition, the contents of TCA cycle intermediates affect the digestibility of amino acids in the intestine (Amaral et al., 2016). In the present study, it was found that compared with the corn starch diet, the apparent ileal amino acid digestibility (such as Lys, Asp, Glu, etc.) of the waxy corn starch diet was significantly improved. The current study further demonstrated that the rapid digestion rate of waxy corn starch diet could save the amounts of amino acids oxidized for energy in the intestinal tract, improve intestinal amino acids digestibility, and ultimately improve the growth performance of broilers in the early stages. However, this study found that some of the differential metabolites we focused on (malic acid and cis-aconitic acid) had no significant differences in the 1.08% and 1.32% AID Lys groups, which indicates that the energy metabolism of the intestinal mucosa is not influenced by the levels of AID Lys. An unanticipated result was that the waxy corn starch diet with 1.08% AID Lys group significantly decreased ileal mucosal fructose 6-phosphate, glucose 6-phosphate, and glucose 1-phosphate compared with the corn starch diet with 1.08% AID Lys group, but these differential metabolites were not significantly different between the waxy corn starch diet with 1.08% AID Lys and corn starch diet with 1.32% AID Lys groups. Glycogenolysis is strictly controlled through the activities of rate-limiting enzymes such as glucose 6-phosphate and glucose 1-phosphate (Li et al., 2020). Glycogen in the liver is converted into glucose by glucose 1-phosphate and glucose 6-phosphate, and glucose is further converted into pyruvate by fructose 6-phosphate and glucose 6-phosphate to enter the TCA cycle to provide the energy needs for the animal's body and intestinal tract (Ma et al., 2021; van Leeuwen et al., 2013). Therefore, the results of this study indicated that feeding SDS at low AID Lys levels led to glycogenolysis in broilers to supplement insufficient intestinal energy supply.

5. Conclusion

Our study showed that a waxy corn starch diet supplemented with 1.32% AID Lys effectively improved intestinal starch and amino acid digestibility, digestive enzyme activities, and intestinal morphology, which ultimately improved the growth performance of young broilers. In addition, based on the results of the targeted center carbon metabolome, we hypothesized that the rapid glucose release rate of waxy corn starch can meet the energy needs of the intestinal tract of young broilers, reduce the amino acids oxidized for energy supply, and improve the utilization of amino acid, thus decreasing the requirement level of dietary AID Lys and saving the production cost of broilers. However, the determination of the amount of glucose and amino acids entering the TCA cycle for energy supply was lacking in the present study, and further validation through gut organoid techniques combined with isotope labeling is needed subsequently.

CRedit authorship contribution statement

Caiwei Luo: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Visualization, Writing – original draft. **Jinping Wang:** Formal analysis, Investigation, Visualization, Writing – review & editing. **Wei Jiang:** Investigation, Resources.

Dafei Yin: Methodology, Visualization, Writing – review & editing. **Gang Meng:** Investigation, Resources. **Jiwei Wang:** Investigation, Resources. **Jing Xu:** Investigation, Resources. **Jianmin Yuan:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Writing – review & editing.

Data availability

The data presented in this study are available on request from the corresponding author.

Declaration of competing interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

Acknowledgments

This research was supported by the National Key R&D Program of China (2021YFD1300404). Thanks to Dr Muhammad Suhaib Shahid for the revision and polishing of this article.

Appendix Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.aninu.2024.11.004>.

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