Animal Nutrition 14 (2023) 111–120

Contents lists available at ScienceDirect

Animal Nutrition

journal homepage: http://www.keaipublishing.com/en/journals/aninu/

Original Research Article

High amylose to amylopectin ratios in nitrogen-free diets decrease the ileal endogenous amino acid losses of broiler chickens

Huajin Zhou [a], Tahir Mahmood [b], Wei Wu [a], Yanhong Chen [a], Yao Yu [a], Jianmin Yuan [a, *]

[a] State Key Laboratory of Animal Nutrition, College of Animal Science and Technology, China Agricultural University, Beijing 100193, China
[b] Adisseo Animal Nutrition, DMCC, Dubai 00000, United Arab Emirates

ARTICLE INFO

Article history:
Received 6 October 2022
Received in revised form
29 December 2022
Accepted 15 March 2023
Available online 19 May 2023

Keywords:
Broiler chicken
Endogenous amino acid losses
Nitrogen-free diet
Amylose
Amylopectin
Goblet cells

ABSTRACT

This study explored the variation of ileal endogenous amino acid (IEAA) losses and its influencing factors in chickens offered nitrogen-free diets (NFD) containing different ratios of amylose to amylopectin (AM/AP). A total of 252 broiler chickens at 28 d old were randomly allocated into 7 treatment groups for a 3-d trial. The dietary treatments included a basal diet (control), a NFD containing corn starch (CS), and 5 NFD with AM/AP ratios of 0.20, 0.40, 0.60, 0.80, and 1.00, respectively. As the AM/AP ratio increased, the IEAA losses of all AAs, starch digestibility and maltase activity linearly decreased ($P < 0.05$), but the DM digestibility linearly and quadratically decreased ($P < 0.05$). Compared with the control, the NFD increased the number of goblet cells and its regulatory genes mucin-2 and krüppel-like factor 4 (*KLF-4*) while decreasing serum glucagon and thyroxine concentrations, ileal villus height, and crypt depth ($P < 0.05$). Additionally, NFD with lower AM/AP ratios (0.20 and 0.40) decreased the ileal microbiota species richness ($P < 0.05$). In all NFD groups, the number of Proteobacteria increased whereas the abundance of Firmicutes dropped ($P < 0.05$). However, the broilers in the AM/AP 0.60 group were closer to the digestive physiological state of chickens fed the control diet, with no significant change in maltase activity and mucin-2 expression ($P < 0.05$). In conclusion, increasing AM/AP ratio in a NFD decreased the IEAA losses and the apparent ileal digestibility of starch but inevitably resulted in malnutrition and disruption of gut microbiota homeostasis. This study recommends AM/AP in NFD at 0.60 to measure IEAA of broiler chickens.

1. Introduction

The apparent ileal digestibility coefficients (AID) of amino acid (AA) are defined as the net disappearance of ingested dietary amino acid from the proximal digestive tract to the distal ileum, which has been widely used in diet formulations of broiler chickens (Ravindran et al., 2005; Stein et al., 2007). However, the AID ignores the contribution of AA from endogenous losses, thereby underestimating the actual digestibility of dietary AA in broilers (Adedokun et al., 2011).

Ileal endogenous amino acid (IEAA) losses are considered an inevitable loss in the gut, including pancreatic and bile secretions, salivary and gastric secretions, small intestinal secretions, sloughed epithelial cells, mucus, and microbial protein (Jansman et al., 2002). A poultry feed formula based on standardized ileal digestibility (SID) of AA could correct the AID by accounting for basal IEAA losses (Lemme et al., 2004). Therefore, AA digestibility in poultry is better explained based on SID value, and improving the detection of IEAA to determine the SID becomes very important for accurate diet formulation when evaluating low-protein ingredients.

In broiler chickens, several approaches have been used to determine IEAA losses (Golian et al., 2008; Hu et al., 2017), but the nitrogen-free diet (NFD) method is currently considered the best strategy among available techniques because of its simplicity, convenience, and cost-effectiveness (Ravindran, 2021). Mostly NFD consist of corn starch and dextrose or sucrose as the main

* Corresponding author.
E-mail address: yuanjm@cau.edu.cn (J. Yuan).
Peer review under responsibility of Chinese Association of Animal Science and Veterinary Medicine.

Production and Hosting by Elsevier on behalf of KeAi

https://doi.org/10.1016/j.aninu.2023.03.011

components. Kong and Adeola (2013) revealed that the ingredient composition of NFD has effects on IEAA estimation, and our previous study has also demonstrated that NFD with higher dextrose content could increase the IEAA losses of chickens (Zhou et al., 2022).

Additionally, there are numerous factors that can influence IEAA flow, such as mucin and sloughed epithelial cell turnover rate (Adedokun et al., 2011). Although corn starch is frequently used as a carbohydrate source in NFD, there are several concerns about the effect of starch type in NFD on IEAA measurements. Previous studies have demonstrated that the different chemical structures of starch (amylose or amylopectin) profoundly affect the net appearance of AA and glucose in the portal vein of animals due to their different digestive characteristics (van der Meulen et al., 1997; Li et al., 2008). A previous study in our lab also demonstrated that AA can be spared from catabolism in the gut mucosa by supplementation of amylose, thereby improving the post-enteral availability of AA in chickens (Yin et al., 2019). These studies suggested that the content of amylose or amylopectin in NFD may change the IEAA losses by affecting the AA availability in chickens.

Based on this evidence, we assumed that the IEAA losses evaluated under the normal digestive physiology state could be a true depiction of actual IEAA losses. The chickens fed the basal corn-soybean meal could be treated as control, and the major influencing factors of IEAA, for instance, digestive enzymes, intestinal microbiota, intestinal morphology, and goblet cell abundance could serve as a good basis for further evaluation. Therefore, this preliminary study aims to explore the effects of varying amylose to amylopectin ratios in the NFD on IEAA losses of broiler chickens, and try to find the optimum proportion of amylose and amylopectin in NFD for accurate estimation of IEAA losses by evaluating the digestive physiological state of broiler chickens.

2. Materials and methods

2.1. Animal ethics statement

All the experimental procedures involving animals in this study were conducted according to the Animal Welfare Committee guidelines and had the approval of the Ethics Committee of Animal Science and Technology College of China Agricultural University (No. AW60701202-2-3, Beijing, China).

2.2. Experimental design

A total of 252 male broiler chickens at 1 d old were fed the starter diet up to 27 d. At 28-d-old, chickens with similar body weight were allocated to 7 treatment groups (6 replicates with 6 chickens for each replicate) for a 3-day trial to estimate IEAA losses. The 7 treatment groups comprised 1 control group (corn-soybean meal), 1 NFD group containing corn starch (CS), and 5 NFD groups having different ratios of amylose to amylopectin (AM/AP), designated as AM/AP 0.20, AM/AP 0.40, AM/AP 0.60, AM/AP 0.80 and AM/AP 1.00, respectively.

2.3. Diet preparation and bird management

The ingredient composition and AA levels of NFD and the control diet are shown in Table 1. In this study, all NFD contained 40% total starch and 4% sodium carboxymethyl cellulose (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China). The dextrose, common corn starch, and amylopectin were purchased from Qinhuangdao Lihua Starch Co., Ltd. (Qinhuangdao, China). The purity of amylopectin was >98%. The amylose was purchased from Ingredion Incorporated (HI-MAIZE 260, Westchester, USA), with the amylose content accounting for 60%, and amylopectin accounting for 40%. The analysis of the amylose and amylopectin of each diet formulation was done by the Sanshubio Company (Shanghai, China), and the measured value was close to the calculated value, which met the experimental design requirements. The diet in the control group was corn-soybean meal-based with calculated metabolizable energy of 2,950 kcal/kg, crude protein 22.50%, Ca 1%, and non-phytate phosphorous 0.45% (as-fed basis). Each diet was evenly mixed, cold-pelleted, and contained 0.5% titanium dioxide (Sinopharm Chemical Reagent Co., Ltd., Shanghai, China) as an indigestible marker.

Upon arrival at d 1, birds were placed immediately in conventional cages (0.7 m^2/per cage), with 6 chickens per cage. On d 28, chickens with similar body weight were allocated to 1 of 7 treatment groups, each group had 6 replicate cages, and each cage had 6 chickens. The stocking density was about 0.12 m^2/per bird from 1 to 31 d. The room temperature was kept at 33 to 35 °C for the first 2 d, and then the temperature was gradually decreased by one degree every 2 d until 21 °C. For the first 2 d, 24 h of light was provided, and then it was reduced by 1 h every day until 6 d and maintained at 20 h until the end of the trial. The relative humidity was maintained at 65% to 70% for the first 7 d, and 50% to 65% from 8 to 31 d. The feed and water were provided ad libitum throughout the trial. No animal died during the experimental period.

2.4. Sample collection

On d 31, all the broilers were weighed, and 1 bird near average body weight from each replicate was selected to collect blood from the wing vein. The blood samples were centrifuged at 4 °C, 1,500 × *g* for 10 min to separate the serum, which was stored at −20 °C until further analyses. Later, these chickens were euthanized via injecting pentobarbital sodium (50 mg/kg body weight). The ileum starting from Meckel's diverticulum to the ileocecal junction was carefully excised. A 1 cm piece of intestinal tissue was excised from the middle of the ileum, fixed in Carnoy fixative (G2312, Solarbio, Japan) for Alcian blue-periodic acid-Schiff (AB-PAS) stain.

The ileal digesta sample was collected in a bacteria-free tube by gently squeezing from the ileocecal junction, and then quickly snap-freezing in liquid nitrogen and stored at −80 °C for microbiota analysis. The remaining ileal segments were opened longitudinally, digesta was flushed out with ice-cold PBS (HyClone, Logan, USA), and the ileal mucosa was scraped off using sterilized microscope slides, collected in tubes, snap-frozen in liquid nitrogen, and stored at −80 °C for the further analysis of disaccharidase activity and gene expression. The remaining 5 chickens in each replicate cage were euthanized with pentobarbital sodium (50 mg/kg BW), the intestine removed, and the digesta of terminal ileum (lower half of the ileum) was collected and pooled within a replicate cage, immediately stored in −80 °C overnight and then freeze-dried using a vacuum freeze dryer (FD-2, Biocool Co., Ltd., Beijing, China).

2.5. IEAA losses and AID of nutrients

The diet and ileal digesta samples were finely ground and sifted through a 40-mesh sieve after being freeze-dried. The diet and ileal digesta samples were analyzed for dry matter (DM), starch, and AA. Briefly, the contents of DM were determined according to Association of Official Analytical Chemists method 930.15 (AOAC, 2007). The content of starch was measured according to the instruction of a commercial kit for starch content detection (A148-1-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Before AA analysis, samples were hydrolyzed with 6 mol/L hydrochloric acid for 24 h at 110 °C (method 930.15; AOAC, 2007), and then the content of AA was analyzed by an amino acid analyzer (A-300, Membrapure GmbH, Frankfurt, Germany). The concentration of

Table 1
Diet formulations and nutrient content.

Item	AM/AP 0.20	AM/AP 0.40	AM/AP 0.60	AM/AP 0.80	AM/AP 1.00	CS	Control[1]
Ingredients, g/kg as-fed basis							
Dextrose	488.80	488.80	488.80	488.80	488.80	488.80	0.00
Corn starch	0.00	0.00	0.00	0.00	0.00	400.00	0.00
Amylose (60%)	111.20	190.70	250.00	296.30	333.30	0.00	0.00
Amylopectin (>98%)	288.80	209.30	150.00	103.70	66.70	0.00	0.00
Cellulose	40.00	40.00	40.00	40.00	40.00	40.00	0.00
Soybean oil	30.00	30.00	30.00	30.00	30.00	30.00	30.00
Dicalcium phosphate	19.00	19.00	19.00	19.00	19.00	19.00	19.00
Limestone	10.00	10.00	10.00	10.00	10.00	10.00	10.00
Sodium chloride	3.00	3.00	3.00	3.00	3.00	3.00	3.00
Choline chloride	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Vitamin premix[2]	0.20	0.20	0.20	0.20	0.20	0.20	0.20
Trace mineral premix[3]	2.00	2.00	2.00	2.00	2.00	2.00	2.00
Titanium dioxide	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Corn (7.5% CP)	0.00	0.00	0.00	0.00	0.00	0.00	544.30
Soybean meal (46% CP)	0.00	0.00	0.00	0.00	0.00	0.00	380.50
DL-Methionine (98%)	0.00	0.00	0.00	0.00	0.00	0.00	2.00
L-Lysine HCL	0.00	0.00	0.00	0.00	0.00	0.00	2.00
Total	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00	1000.00
Analyzed nutrients contents, g/kg DM basis							
Amylose	67.04	107.21	138.70	167.96	178.25	72.71	76.06
Amylopectin	310.59	276.04	238.18	200.11	175.76	305.53	294.08
AM/AP ratios	0.22	0.38	0.58	0.84	1.01	0.24	0.26
Asp	0.02	0.02	0.03	0.02	0.03	0.03	20.37
Thr	0.00	0.00	0.00	0.01	0.00	0.00	7.03
Ser	0.00	0.00	0.01	0.01	0.03	0.04	10.62
Glu	0.02	0.00	0.00	0.02	0.00	0.03	34.28
Gly	0.00	0.01	0.00	0.10	0.00	0.00	8.13
Ala	0.08	0.05	0.05	0.06	0.08	0.08	9.73
Val	0.02	0.01	0.00	0.00	0.05	0.08	7.92
Ile	0.02	0.01	0.00	0.00	0.02	0.04	7.79
Leu	0.08	0.07	0.10	0.09	0.10	0.12	15.43
Tyr	0.00	0.01	0.00	0.00	0.03	0.04	6.74
Phe	0.04	0.07	0.02	0.03	0.05	0.06	10.63
His	0.00	0.00	0.00	0.01	0.01	0.00	4.92
Lys	0.00	0.00	0.01	0.00	0.00	0.00	11.92
Arg	0.02	0.00	0.02	0.03	0.01	0.04	13.12
Pro	0.00	0.05	0.06	0.05	0.07	0.07	11.47

AM/AP = the ratio of amylose to amylopectin; CS = the nitrogen-free diet containing corn starch; CP = crude protein; DM = dry matter.
[1] Nutrient level of the control diet: metabolizable energy, 2,950 kcal/kg; crude protein, 22.50%; calcium, 1%; non-phytate phosphorous, 0.45%.
[2] The vitamin premix provided the following per kilogram of diets: vitamin A, 10,000 IU; vitamin D_3, 2,400 IU; vitamin E, 20 mg; vitamin K_3, 2 mg; vitamin B_1, 2 mg; vitamin B_2, 6.4 mg; vitamin B_6, 3 mg; vitamin B_{12}, 0.02 mg; biotin, 0.1 mg; folic acid, 1 mg; pantothenic acid, 10 mg; nicotinamide, 30 mg.
[3] The trace mineral premix provided the following per kilogram of diets: Zn, 110 mg (as $ZnSO_4 \cdot H_2O$); Fe, 80 mg (as $FeSO_4 \cdot H_2O$); Mn, 120 mg (as $MnSO_4 \cdot H_2O$); Se, 0.3 mg (as Na_2SeO_3); I, 1.5 mg (as $Ca(IO_3)_2$); Co, 0.5 mg ($CoCl_2 \cdot H_2O$).

TiO_2 was determined according to the method described by Myers et al. (2004). Briefly, the diet and ileal digesta samples were ashed and then digested using sulphuric acid (7.4 mol/L) and subjected to reaction with hydrogen peroxide, and the absorbance was measured at 410 nm using a spectrophotometer (Ultrospec 2100 pro, Amersham Biosciences Inc., New Jersey, American).

The IEAA were calculated as milligrams of amino acid flow per 1 kg of DM intake using Eq. (1) by Moughan and Marlies Leenaars (1992). The apparent digestibility of DM and starch was calculated using Eq. (2). All the data were expressed on DM basis for calculations:

$$\text{IEAA flow} = \left(\frac{TiO_{2\,\text{diet},\%}}{TiO_{2\,\text{ileal digesta},\%}}\right) \times AA_{\text{ileal digesta}}, \text{mg/kg of DM} \quad (1)$$

$$\text{AID of Nutrients}, \% = \left[1 - \left(\frac{TiO_{2\,\text{diet},\%}}{TiO_{2\,\text{ileal digesta},\%}}\right) \times \left(\frac{\text{Nutrient}_{\text{ileal digesta},\%}}{\text{Nutrient}_{\text{diet},\%}}\right)\right] \times 100 \quad (2)$$

where $AA_{\text{ileal digesta}}$ represented the AA concentration in terminal ileal digesta (mg/kg of DM); $TiO_{2\,\text{diet}}$ and $TiO_{2\,\text{ileal digesta}}$ represented the TiO_2 concentrations (%) in the diets and terminal ileal digesta, respectively; $\text{Nutrient}_{\text{diet}}$ and $\text{Nutrient}_{\text{ileal digesta}}$ represented the DM or starch concentrations (%) in the diets and terminal ileal digesta, respectively.

2.6. Serum metabolites

The concentration of glucose, total protein (TP), albumin, and uric acid (UA) were determined by an automated biochemical analyzer (TBA-120FR, Toshiba Corporation, Tokyo, Japan). The content of glucagon (GLUN), insulin (INS), triiodothyronine (T_3), and thyroxine (T_4) was determined by an automatic radioimmunocounter (XH-6080, Xi'an Nuclear Instrument Factory, Xi'an, China).

2.7. Intestinal morphology and digestive enzymes

The tissue sections and AB-PAS stain of the ileum was prepared by Servicebio Co., Ltd. (Beijing, China). The intestinal morphology was measured based on 8 representative complete villi in the same AB-PAS-stained slide. Mucosal villus height was defined as the length from the villus tip to the villus-crypt junction, and the associated crypt depth was measured from the crypt mouth to the

crypt base. The number of goblet cells was manually counted in 8 villi and presented as the average number of stained goblet cells per 100 μm length of the villus.

Approximately 0.2 g ileal mucous was homogenized (55 Hz, 60 s) by an Ultra-Turrax homogenizer (JIUPIN-92, Jiupin Instrument Co., Ltd., WuXi, China) in 6 fold (vol:wt) of saline, and then centrifuged at 4 °C, 362 × *g* for 10 min to collect the homogenate supernatant. The activities of sucrase and maltase were measured ($n = 6$) according to the commercial kit instructions (A082-2-1 and A082-3-1, Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The data were collected by optical density (all 505 nm) measurement on a microplate reader (SpectraMax i3x, Molecular Devices LLC, California, American).

2.8. Microflora analysis of ileal digesta

The DNA in ileal digesta was extracted using a QIAamp Fast DNA Stool Mini Kit (51604, Qiagene N.V., Hilden, Germany). Amplicon sequencing of the 16S V3–V4 region was performed on Illumina NovaSeq PE250 platform (Novogene Biotech Co., Ltd., Beijing, China). A total of 2,079 operational taxonomic units (OTU) were clustered using USEARCH drive5 at a 97% similarity level. The OTU sequences were annotated with the Silva132 database, and the calculations of alpha diversity, beta diversity, and the relative abundance taxonomic summaries were performed using QIIME 1.8.

2.9. Gene expression

Total RNA of ileal mucosa was extracted using the RNA Easy Fast Tissue/Cell Kit (DP451, Tiangen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol. The concentration and purity of each RNA sample were assessed using the NanoDrop Lite Spectrophotometer (Thermo Fisher Scientific Inc., Massachusetts, America). After that, first-strand cDNA was synthesized from 1 μg of total RNA using a kit (RR047A, Takara Bio Inc., Kyoto, Japan). The gene expressions were quantitated by real-time PCR using the ABI 7500 Fluorescent Quantitative PCR system (Applied Biosystems LLC., Massachusetts, America) with SYBR Premix ExTaq system (RR420A, Takara Bio Inc., Kyoto, Japan). The program of the primer–dissociation curve was set to 95 °C for 30 s; 40 cycles of 95 °C for 5 s and 60 °C for 34 s. The commercially manufactured specific primers (Sangon Biotech Co., Ltd., Shanghai, China) are shown in Table 2. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), TATA-box binding protein 1 (*TBP-1*), and β-actin were chosen as the house-keeping gene. The relative gene expression levels were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

2.10. Statistical analysis

The data were analyzed by SPSS, version 20.0 (SPSS, IBM, Chicago, IL, USA). Data distribution was checked by Shapiro–Wilk test. Normally distributed data were analyzed by one-way ANOVA for comparisons among groups and then followed by Duncan's post hoc test. In addition, polynomial regression analysis was also applied to test the linear and quadratic response to increasing AM/AP ratios in NFD. Data were displayed as means and the standard error of the mean (SEM) was represented for all pooled data. *P*-values less than 0.05 were considered statistically significant.

3. Results

3.1. Growth performance

The growth performance of broiler chickens is shown in Table 3. Compared with the control group, the chickens in all NFD groups experienced significant weight loss and reduced feed intake after the 3-d trial ($P < 0.001$). There was a linear trend of increased average feed intake with increasing AM/AP ratios in NFD ($P = 0.086$). In addition, increasing AM/AP ratios in NFD linearly reduced the body weight loss of broilers ($P = 0.020$).

3.2. Serum biochemical indices

Table 4 demonstrates the serum biochemical changes among groups. The serum insulin level was linearly decreased with increasing AM/AP ratios ($P = 0.029$), which significantly decreased in groups AM/AP 0.60, 0.80 and 1.00 when compared with control ($P = 0.008$). In addition, the levels of glucagon and T_4 were linearly increased with increasing AM/AP ratios ($P < 0.05$), and both levels were significantly decreased in all NFD groups relative to the control ($P < 0.001$). Additionally, a linear and quadratic increase in albumin and UA levels were observed in 5 NFD groups ($P < 0.05$), but all significantly decreased when compared with the control ($P < 0.001$). However, NFD did not substantially affected the level of glucose and T_3 in the serum.

Table 2
Nucleotide sequence of primers for gene expression analysis.

Target gene	F:forward R: reverse	Primer sequence (5′→3′)	Accession no.	Size, bp
GAPDH	F	TGCTGCCCAGAACATCATCC	NM_204305.1	142
	R	ACGGCAGGTCAGGTCAACAA		
TBP-1	F	TAGCCCGATGATGCCGTAT	NM_001396190.1	147
	R	GTTCCCTGTGTCGCTTGC		
β-actin	F	TGTTACCAACACCCACACCC	NM_205518	110
	R	TCCTGAGTCAAGCGCCAAAA		
SGLT-1	F	CATCTTCCGAGATGCTGTCA	XM_015275173	169
	R	CAGGTATCCGCACATCACAC		
GLUT-2	F	CCGCAGAAGGTGATAGAAGC	NM_207178	87
	R	ATTGTCCCTGGAGGTGTT		
Mucin-2	F	TCACCCTGCATGGATACTTGCTCA	NM_001318434.1	228
	R	TGTCCATCTGCCTGAATCACAGGT		
KLF-4	F	TCAAGGCACACCTGAGAACC	XM_004949369	119
	R	GCCCGTGTGTTTTCGGTAAT		

GAPDH = glyceraldehyde-3-phosphate dehydrogenase; *TBP-1* = TATA-box binding protein 1; *SGLT-1* = Na(+)-glucose cotransporter 1; *GLUT-2* = glucose transporter type 2; *KLF-4* = Krüppel like factor 4.

Table 3
The effects of different nitrogen-free diets on growth performance of broiler chickens.[1]

Item	AM/AP 0.20	AM/AP 0.40	AM/AP 0.60	AM/AP 0.80	AM/AP 1.00	CS	Control	SEM	*P*-value		
									Treatment	Linear[2]	Quadratic[2]
28 d BW, g	1447.83	1452.47	1446.30	1448.90	1449.37	1454.43	1453.83	3.530	0.994	0.987	0.990
31 d BW, g	1397.20^{b}	1403.63^{b}	1399.27^{b}	1410.97^{b}	1410.67^{b}	1421.67^{b}	1733.13^{a}	3.296	<0.001	0.225	0.937
AFI, g/chick	203.17^{b}	200.47^{b}	204.33^{b}	221.00^{b}	222.90^{b}	218.07^{b}	364.40^{a}	3.574	<0.001	0.086	0.589
ABWG, g/chick	−50.63^{b}	−48.83^{b}	−47.03^{b}	−37.93^{b}	−38.70^{b}	−32.77^{b}	279.3^{a}	2.158	<0.001	0.020	0.897

AM/AP = the ratio of amylose to amylopectin; CS = the nitrogen-free diet made with common corn starch; SEM = standard error of the mean; BW = body weight; AFI = average feed intake; ABWG = average body weight gain.
[1] The values are based on 6 birds for each treatment ($n = 6$). Labeled means without a common letter are significantly different, $P < 0.05$.
[2] Linear and quadratic regression of 5 groups: AM/AP 0.20; AM/AP 0.40; AM/AP 0.60; AM/AP 0.80; AM/AP 1.00.

Table 4
The effects of different nitrogen-free diets on serum biochemical parameters of broiler chickens.[1]

Item	AM/AP 0.20	AM/AP 0.40	AM/AP 0.60	AM/AP 0.80	AM/AP 1.00	CS	Control	SEM	*P*-value		
									Treatment	Linear[2]	Quadratic[2]
Glucose, mmol/L	10.45	10.63	10.32	10.96	10.46	11.50	12.10	0.224	0.310	0.860	0.864
Insulin, μIU/mL	7.52^{abcd}	7.99^{ab}	6.61^{d}	6.70^{cd}	6.81^{bcd}	7.84^{abc}	8.44^{a}	0.144	0.008	0.029	0.597
Glucagon, pg/mL	98.71^{c}	119.41^{c}	115.38^{c}	120.84^{c}	155.15^{b}	106.49^{c}	184.80^{a}	3.774	<0.001	0.001	0.342
T_3, ng/mL	1.71	1.88	1.88	1.83	1.89	2.23	1.73	0.075	0.605	0.551	0.665
T_4, ng/mL	22.09^{d}	26.65^{cd}	24.91^{cd}	25.16^{cd}	36.69^{b}	34.74^{bc}	49.07^{a}	1.204	<0.001	0.014	0.211
TP, g/L	25.30^{ab}	25.17^{ab}	24.28^{bc}	27.87^{a}	27.53^{ab}	21.53^{c}	26.83^{ab}	0.397	0.002	0.038	0.303
Albumin, g/L	10.17^{cd}	10.28^{cd}	9.68^{d}	11.57^{bc}	12.43^{ab}	8.15^{e}	13.10^{a}	0.187	<0.001	<0.001	0.026
UA, μmol/L	105.83^{b}	103.17^{d}	131.00^{c}	107.33^{d}	254.30^{b}	124.67^{c}	267.20^{a}	1.479	<0.001	<0.001	<0.001

AM/AP = the ratio of amylose to amylopectin; CS = the nitrogen-free diet made with common corn starch; SEM = standard error of the mean; T_3 = triiodothyronine; T_4 = thyroxine; TP = total protein; UA = uric acid.
[1] The values are given as the means based on 6 birds for each treatment ($n = 6$). Labeled means without a common letter are significantly different, $P < 0.05$.
[2] Linear and quadratic regression of 5 groups: AM/AP 0.20; AM/AP 0.40; AM/AP 0.60; AM/AP 0.80; AM/AP 1.00.

3.3. Basal IEAA losses

As shown in Table 5, the group AM/AP 0.20 had the highest IEAA flow for all AA. As the AM/AP ratio further increased, a linear decrease in IEAA losses of all AAs and a quadratic decrease in the IEAA losses of Gly, Ala, Val, Lys, and Pro were observed in this study ($P < 0.05$). Group AM/AP 0.60 had a moderate level of IEAA losses, with no significant difference in the IEAA losses of Asp, Thr, Ser and His when compared with group AM/AP 0.40, whereas it was significantly higher than that in the AM/AP 0.80 and 1.00 groups ($P < 0.05$).

3.4. Ileal morphology

To assess the impact of NFD on ileal morphology in broiler chickens, the villus height, crypt depth, and the number of goblet cells was detected. As shown in Table 6, we observed that feeding chickens with NFD was accompanied by a sharp increase in the number of goblet cells in the intestinal villi ($P < 0.001$), with the arranged purple AB-PAS-stained goblet cells more tightly packed in 6 NFD treatments than the control group (Fig. 1). Moreover, the villus height and crypt depth in all of the NFD groups were significantly lower than that in the control group ($P < 0.01$; Table 6).

Table 5
The losses of basic IEAA in the ileum of broiler chickens (mg/kg DM intake).[1]

Item	AM/AP 0.20	AM/AP 0.40	AM/AP 0.60	AM/AP 0.80	AM/AP 1.00	CS	SEM	*P*-value		
								Treatment	Linear[2]	Quadratic[2]
Asp	1153.23^{a}	1010.60^{b}	929.48^{bc}	806.40^{d}	836.04^{cd}	1018.07^{b}	15.790	<0.001	<0.001	0.054
Thr	1037.84^{a}	816.05^{b}	758.85^{b}	558.50^{c}	554.80^{c}	850.54^{b}	16.490	<0.001	<0.001	0.079
Ser	852.92^{a}	752.94^{b}	743.88^{b}	599.44^{c}	629.17^{c}	785.43^{ab}	13.217	<0.001	<0.001	0.318
Glu	1338.15^{a}	1170.24^{b}	1049.97^{bc}	890.16^{d}	928.37^{cd}	1068.94^{bc}	20.189	<0.001	<0.001	0.058
Gly	646.50^{a}	578.44^{b}	496.92^{c}	433.20^{d}	459.91^{cd}	592.12^{ab}	7.848	<0.001	<0.001	0.010
Ala	602.78^{a}	494.53^{b}	425.82^{c}	327.56^{d}	352.20^{d}	532.44^{b}	8.631	<0.001	<0.001	0.010
Val	621.41^{a}	515.32^{bc}	505.15^{c}	416.01^{d}	467.60^{cd}	575.17^{ab}	8.485	<0.001	<0.001	0.008
Ile	439.36^{a}	387.61^{abc}	361.46^{bc}	297.26^{d}	334.25^{cd}	399.50^{ab}	7.883	<0.001	<0.001	0.077
Leu	725.34^{a}	660.86^{ab}	639.86^{abc}	560.95^{c}	615.49^{cd}	694.54^{ab}	11.671	0.005	0.002	0.120
Tyr	458.71^{a}	406.17^{ab}	378.68^{bc}	316.10^{d}	331.68^{cd}	429.34^{ab}	7.151	<0.001	<0.001	0.148
Phe	529.67^{a}	494.53^{ab}	366.70^{cd}	271.06^{e}	325.70^{de}	430.70^{bc}	12.340	<0.001	<0.001	0.078
His	1075.10^{a}	814.57^{bc}	877.84^{b}	719.00^{cd}	646.27^{d}	1072.33^{a}	17.969	<0.001	<0.001	0.379
Lys	533.32^{a}	470.03^{bc}	432.56^{b}	379.97^{b}	406.91^{b}	480.89^{ab}	9.126	<0.001	<0.001	0.028
Arg	527.52^{a}	430.67^{ab}	403.37^{bcd}	329.20^{d}	335.96^{cd}	426.63^{b}	10.425	<0.001	<0.001	0.123
Pro	723.19^{a}	579.18^{b}	505.15^{bc}	443.85^{c}	463.33^{c}	672.84^{a}	10.509	<0.001	<0.001	0.001

IEAA = ileal endogenous amino acid; DM = dry matter; AM/AP = the ratio of amylose to amylopectin; CS = the nitrogen-free diet made with common corn starch; SEM = standard error of the mean.
[1] The values are given as the means based on 6 birds for each treatment ($n = 6$). Labeled means without a common letter are significantly different, $P < 0.05$.
[2] Linear and quadratic regression of 5 groups: AM/AP 0.20; AM/AP 0.40; AM/AP 0.60; AM/AP 0.80; AM/AP 1.00.

Table 6
The ileum morphology, disaccharidase activity, nutrient digestibility and relative mRNA expression of broiler chickens.[1]

Item	AM/AP 0.20	AM/AP 0.40	AM/AP 0.60	AM/AP 0.80	AM/AP 1.00	CS	Control	SEM	*P*-value		
									Treatment	Linear[2]	Quadratic[2]
Distal ileum morphology											
Villus height	665.82^{b}	685.02^{b}	663.41^{b}	682.10^{b}	667.21^{b}	732.89^{b}	971.89^{a}	11.807	<0.001	0.999	0.772
Crypts depth	121.47^{b}	132.30^{b}	119.21^{b}	134.45^{b}	124.49^{b}	123.02^{b}	155.80^{a}	2.423	0.004	0.685	0.579
V/C	5.39	5.22	5.65	5.25	5.43	6.06	5.72	0.114	0.450	0.876	0.902
Goblet cells[3]	13.94^{ab}	12.40^{b}	13.40^{ab}	12.58^{b}	12.48^{b}	15.06^{a}	9.73^{c}	0.220	<0.001	0.059	0.506
Disaccharidase activity, U/mg prot											
Sucrase	31.58^{b}	30.06^{ab}	26.99^{c}	21.98^{d}	39.19^{a}	30.93^{ab}	20.52^{d}	0.500	<0.001	0.106	<0.001
Maltase	166.67^{a}	166.36^{a}	130.71^{c}	173.30^{a}	146.02^{b}	163.56^{a}	119.07^{c}	1.852	<0.001	0.024	0.164
Digestibility of starch and DM, %											
Starch	93.34^{a}	86.15^{c}	83.61^{c}	83.68^{c}	75.37^{d}	95.12^{a}	89.82^{b}	0.415	<0.001	<0.001	0.924
DM	82.66^{a}	74.85^{b}	73.02^{c}	69.73^{d}	67.70^{e}	74.63^{b}	62.28^{f}	0.104	<0.001	<0.001	0.005
Relative mRNA expression											
GLUT-2	8.33^{a}	7.66^{ab}	8.89^{a}	7.56^{ab}	7.23^{ab}	6.45^{b}	1.00^{c}	0.220	<0.001	0.274	0.455
SGLT-1	2.65^{a}	3.50^{a}	3.43^{a}	3.35^{a}	2.93^{a}	3.23^{a}	1.00^{b}	0.119	<0.001	0.693	0.049
Mucin-2	1.50^{ab}	1.42^{abc}	1.33^{abc}	1.61^{a}	1.77^{a}	1.12^{bc}	1.00^{c}	0.057	0.012	0.147	0.145
KLF-4	2.45^{a}	1.86^{ab}	1.53^{bc}	2.21^{ab}	2.58^{a}	1.67^{bc}	1.00^{c}	0.094	0.001	0.511	0.012

AM/AP = the ratio of amylose to amylopectin; CS = the nitrogen-free diet made with common corn starch; SEM = standard error of the mean; V/C = the ratio of villus height to crypt depth; DM = dry matter; *GLUT-2* = glucose transporter type 2; *SGLT-1* = Na(+)-glucose cotransporter 1; *KLF-4* = krüppel-like factor 4.
[1] The values are given as the means based on 6 birds for each treatment ($n = 6$). Labeled means without a common letter are significantly different, $P < 0.05$.
[2] Linear and quadratic regression of 5 groups: AM/AP 0.20; AM/AP 0.40; AM/AP 0.60; AM/AP 0.80; AM/AP 1.00.
[3] The number of goblet cells were quantified by counting the number of stained goblet cells per 100 μm length of villus, and presented as the average number of goblet cells per 8 intestinal villi.

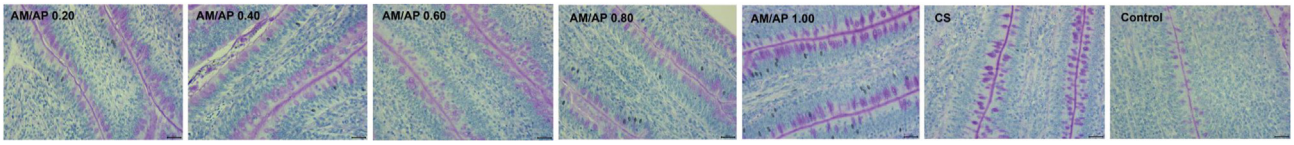

Fig. 1. The AB-PAS stained transverse section of the chicken ileal epithelium. AM/AP = the ratio of amylose to amylopectin; CS = the nitrogen-free diet made with common corn starch. Goblet cells appear in an intense purple color with Alcian blue-periodic acid-Schiff stain. The number of goblet cells was manually counted in 8 villi and presented as the average number of stained goblet cells per 100 μm length of the villus. The scale bar is 20 μm.

However, the different groups did not show significant differences in the ratio of ileal villus height to the crypt depth, and there was no linear or quadratic change in ileal morphology index.

3.5. Disaccharidase activity and the digestibility of starch and dry matter

As shown in Table 6, a quadratic change in sucrase and a linear change in maltase were found with the increasing ratio of AM/AP ($P < 0.05$). Higher sucrase and maltase activity were found in all NFD groups, AM/AP 0.20, 0.40, 1.00, and the CS group compared with the control ($P < 0.001$). The starch digestibility of NFD was linearly decreased with increasing ratio of AM/AP ($P < 0.001$). The starch digestibility in the CS group was similar to the AM/AP 0.20 group, which significantly higher than that in the control ($P < 0.001$). The DM digestibility of all NFD groups was significantly higher than that of the control ($P < 0.001$). Group AM/AP 0.20 had the highest DM digestibility ($P < 0.001$), and a linear decrease in DM digestibility was detected in NFD groups as AM/AP increased ($P < 0.001$).

3.6. Gene expression

As for ileal gene expression, there was a quadratic change in *KLF-4* ($P = 0.012$) and *SGLT-1* ($P = 0.049$) expression in 5 NFD groups (Table 6), however, no linear or quadratic effects were detected for other genes. Overexpression of the glucose transporter genes *GLUT-2* and *SGLT-1* was observed in the NFD treatments ($P < 0.001$; Table 6). In addition, the expression of goblet cell marker mucin-2 and *KLF-4*, a known up-regulator of goblet cell development, were significantly increased in AM/AP 0.20, 0.80, and 1.00 compared with the control ($P < 0.05$).

3.7. Ileal microbial community composition

To explore the microbial community diversity of the different treatments, we conducted alpha and beta diversity analyses (Fig. 2). As shown in Fig. 2A, NFD changed the ileal microbiota species richness of broiler chickens. Compared with the control, the NFD treatment with less amylose resulted in a significant decrease in the total number of ileal microorganisms, with a lower number of species observed in the group AM/AP 0.20 and group AM/AP 0.40 ($P < 0.001$). Fig. 2B shows that the Shannon diversity index of group AM/AP 0.20, AM/AP 0.40, and AM/AP 0.60 were significantly different relative to the control ($P = 0.0074$, $P = 0.0005$, and $P = 0.0185$, respectively). That is, the NFD with a lower AM/AP ratio had lower species richness.

Beta diversity analyses were applied to indicate the similarity between microbial communities. Principal-coordinate analysis (PCoA) based on weighted UniFrac distance matrices showed a clear separation among groups (Fig. 2C). Besides, a significant difference was also observed between group AM/AP 0.40 and AM/AP 0.80, indicating that the different content of amylose in NFD results in obviously distinct in ileal microbiota community structure ($P < 0.05$).

The relative abundances of the top 10 phyla are shown in Fig. 2D. A wide variation in the microbial community composition was observed between the NFD groups and the control. The

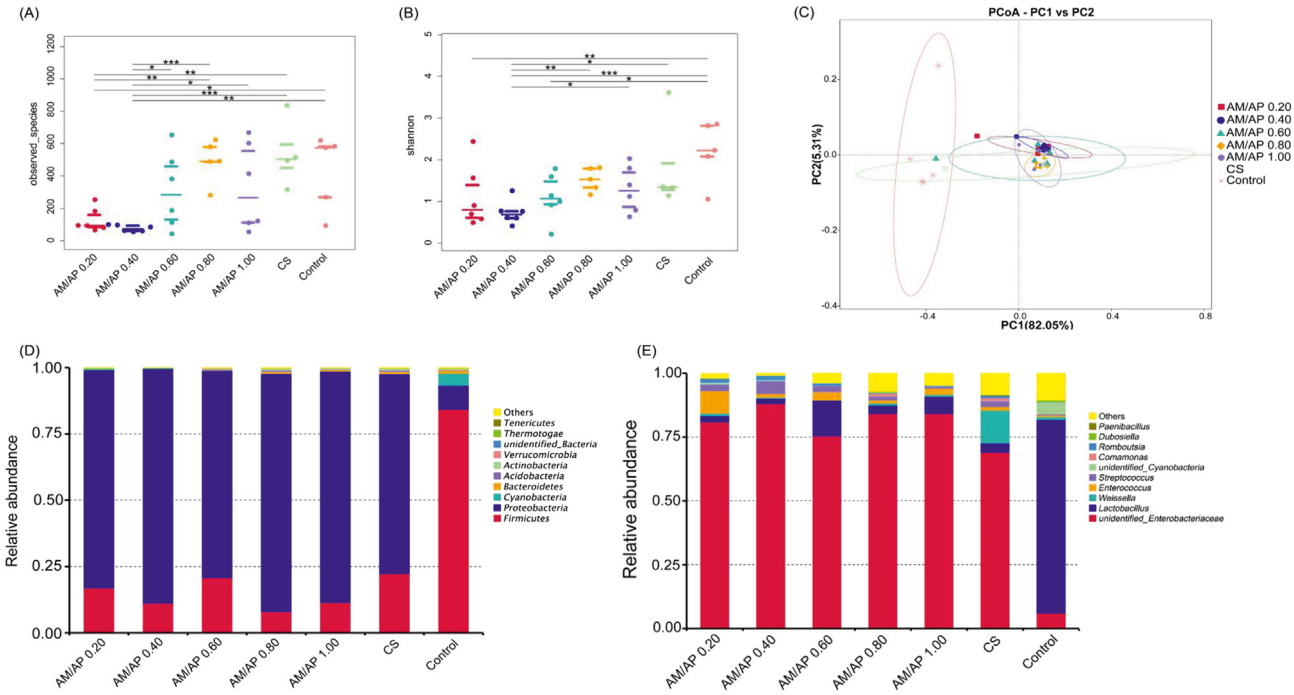

Fig. 2. The analysis of alpha and beta diversity of microbial community composition in the ileum of broiler chickens. (A) Observed species in the ileal digesta among groups, representing the scatter distribution of the total number of species among different groups. (B) Shannon index, reflecting the differences of species diversity and evenness among different groups. (C) Principal coordinates analysis (PCoA) of the microbial community. The analysis is generally based on the UniFrac distance, each point in the graph represents a sample, the distance between points represents the degree of difference, and the samples of the same group are represented by the same color. (D) The relative abundance of the top 10 species at phylum level. (E) The relative abundance of the top 10 species at genus level. Group CS was fed nitrogen-free diet (NFD) with common corn starch. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

dominant microbial flora were Firmicutes (84.43%), Proteobacteria (8.90%), and Cyanobacteria (4.57%) in the control group. In contrast, Proteobacteria was the most dominant microbial flora in every NFD group, with the proportions of 82.10%, 88.40%, 78.10%, 89.66%, 87.09%, and 75.16% in NFD groups with AM/AP ratio from 0.20 to 1.00 and group CS, respectively. At the genus level (Fig. 2E), *Lactobacillus* (75.90%) and *unidentified_Enterobacteriaceae* (6.01%) were the first and second most dominant genera in the control group. However, *unidentified_Enterobacteriaceae* consistently represented the vast majority of genera in every NFD group, with the proportions of 80.93%, 88.18%, 75.43%, 84.21%, 84.18%, and 69.07% in NFD groups with AM/AP ratio from 0.20 to 1.00 and group CS, respectively. Notably, group AM/AP 0.60 had the highest proportion of *Lactobacillus* (14.00%) among all groups, suggesting that the ratio of AM/AP in NFD at 0.60 contributes to gut homeostasis.

4. Discussion

Consistent with previous studies, the NFD-fed chickens showed a significant drop in growth performance due to severe deficiency of essential amino acid (Adedokun and Applegate, 2014). Despite the inherent flaw, the NFD method is generally considered to be an effective method for the detection of IEAA losses in broiler chickens. In this study, we assumed that the IEAA losses evaluated under normal digestive physiology status could represent the actual IEAA loss. Therefore, some serum biochemical parameters related to blood glucose homeostasis and nutritional status were determined in this study to indicate the metabolic functions and basic physiological status of chickens.

It was noted that a high-amylose starch diet led to slower release and absorption of glucose, and a higher insulin sensitivity index than a high-amylopectin starch diet (Breyton et al., 2021). Ma et al. (2020) found that the serum insulin concentration was significantly reduced with the increase in dietary AM/AP ratio, but the blood glucose was not changed in chickens. Consistently, the present study also observed reduced serum insulin levels in NFD groups with higher AM/AP ratios (0.60, 0.80, and 1.00) compared with the AM/AP ratio in NFD at 0.40, indicating that high-amylose NFD led to higher insulin sensitivity in chickens. It is interesting to note that the glucagon level was significantly decreased in all NFD treatments compared to the control, as insulin and glucagon are normally perceived to operate in an opposite manner in mammals. This could be related to the nutritional composition of NFD, since there are positive relationships between dietary protein levels and plasma glucagon in chickens (Chendrimada et al., 2006), and exposure to the exogenous glucose has been found to suppresses glucagon secretion (Ma et al., 2005).

Previous research has shown that serum thyroid hormone is correlated with dietary protein level in chickens, with thyroxine concentrations increasing as crude protein level increased from 150 to 200 g/kg diet (Rosebrough and McMurtry, 1993). The results of the current study provide further support for the relationship between thyroxine and dietary protein, with serum thyroxine in NFD groups significantly lower than the control group. Besides, thyroxine to triiodothyronine conversion is a regulated step, which is mainly catalyzed by deiodinases (Ortiga-Carvalho et al., 2016). The starch-fed animals had higher rates of hepatic thyroxine monodeiodinase activity than animals fed the other dietary carbohydrates (Smith and Lukaski, 1992). This suggests that the chickens fed with starch-enriched NFD may have a strong capacity for thyroxine to triiodothyronine conversion, which might be the reason for triiodothyronine keeping stable in the face of decreased thyroxine in this study.

Uric acid is the major end product of nitrogen metabolism in chickens (Machín et al., 2004; Wang et al., 2022). Decreased plasma uric acid levels induced by dietary protein restriction have been previously noted in chickens (Darsi et al., 2012), which is consistent with the findings in the NFD treatments in this study. Moreover, blood albumin acts as a conventional marker of protein status, and a decreased albumin level could be an indicator of protein and amino acid deficiency in chickens (Jariyahatthakij et al., 2018). We observed that NFD decreased body weight as well as serum uric acid and albumin levels in chickens due to protein malnutrition, which suggests that the IEAA could also be affected under this abnormal physiological state.

In the current study, the NFD with higher AM/AP ratios (0.80 and 1.00) significantly decreased the IEAA losses compared with lower AM/AP ratios (0.20 and 0.40). Since there was no significant difference in feed intake among different NFD treatments, therefore, these differences in IEAA losses could partly be explained by the differences in starch digestion rate, the extent of starch digestion, or both. There are negative relationships between amylose content and starch digestion rate (Zhong et al., 2021), and it has been observed that slowly digestible starch continuously supplies glucose, resulting in a gradual insulin release and leading to more efficient utilization of amino acid (Weurding et al., 2001). Moreover, diets with a high AM/AP ratio have been found to reduce the consumption of AA by the intestinal epithelial cells, promoting more AA entry into the bloodstream to maintain protein synthesis, which has been reported in goats (Lv et al., 2022), pigs (van der Meulen et al., 1997), and chickens (Selle and Liu, 2019; Yin et al., 2019). Normally, endogenous protein mixed with dietary protein is digested, and the resulting AA are absorbed, thus, IEAA represent the net balance between ingested protein and endogenous protein secretions minus absorption of dietary protein and reabsorption of the endogenous protein (Moughan, 2003; Ravindran, 2021). The NFD as an extreme protein constraint may induce the intestine to respond with a net breakdown of endogenous proteins to secure AA availability for the body (Ten et al., 2012), and the reabsorption of endogenous protein may play a more prominent role in chickens fed with NFD. Therefore, the NFD with high amylose (AM/AP 0.80 and 1.00), which increased the reabsorption efficiency of endogenous AA by reducing the consumption of AA by the intestinal epithelial cells could be one of the reasons for decreased IEAA losses. In addition, the starch digestibility also decreased significantly with increasing dietary AM/AP ratios which is also consistent with previous research in chickens (Ma et al., 2020).

Mucus rich in Thr, Pro, and Ser (Bansil and Turner, 2018) is secreted from goblet cells and is considered one of the major contributors to IEAA (Ravindran, 2021). In this study, the NFD increased the number of AB-PAS-stained goblet cells that may potentially increase the mucin secretion capacity of the mucosa (Sharma and Schumacher, 2001), thereby influencing the IEAA losses of Thr and Pro. Previous reports indicated that dietary changes are influential in modifying epithelial mucin predominantly in the small intestine (Sharma et al., 1997; Sharma and Schumacher, 2001). Goblet cells depend heavily on absorbed glutamine and glucose to synthesize mucins, which can be readily provided by prolamines and starch in grain (Moran Jr, 2016). It has been shown that feeding carbohydrates to the late-term embryo of broiler chickens can be seen to enhance mucin-2 expression and goblet cell development (Sharma et al., 1997; Smirnov et al., 2006). In our study, the chickens fed NFD had higher sucrase activity, maltase activity, and upregulation of glucose transporter *GLUT-2* and *SGLT-1*. This demonstrates stronger glucose absorption from the gut in NFD-fed groups. It is noteworthy that the AID of DM in the control group was substantially decreased compared with all NFD groups which highlights that the NFD-fed groups could obtain more glucose from the gut than the control when consuming the same amount of feed. In addition, the transcription factor *KLF-4* is involved in the renewal of the intestinal epithelium, and its increased expression toward the terminal ileum has been shown to increase goblet cells (Iwasaki et al., 2011). The present study showed that NFD-treated chickens had higher *KLF-4* levels, which has the potential to promote the goblet cell marker gene mucin-2 expression, but the result should be taken with caution and a more detailed investigation is warranted. Collectively, the NFD might positively influence goblet cells thus increasing mucin synthesis in the ileum. Since no significant difference in maltase activity, as well as the mucin-2 and *KLF-4* expression between AM/AP 0.60 and the control was observed, it can be postulated that the chickens fed with NFD with AM/AP 0.60 might obtain a better digestive physiology status compared with other the AM/AP fed groups. The exact mechanism of goblet cell differentiation through cell experiments could further elucidate the underlying mechanism.

In addition to the change in goblet cells, great variability in the composition and diversity of ileal microbiota also indicated that the IEAA losses induced by NFD are different from that in chickens under a normal physiologic state. Dietary protein deficiency reduced intestinal bacterial diversity in hens (Dong et al., 2017) and pigs (Pollock et al., 2019), which is in accordance with our study. According to nutrient niche theory, the ecological niches in the gut are defined by the available nutrients, and a species can only colonize if it can most efficiently utilize a particular limiting nutrient (Freter et al., 1983). From a nutritional point of view, glucose and ammonia are the best carbon and nitrogen sources for *Escherichia_coli*, respectively (Reitzer, 2003). However, *Lactobacillus salivarius* is a nutritionally fastidious microorganism, since inorganic nitrogen sources are ineffective in stimulating its growth (Dong et al., 2014), and it needs 2 to 3 fold higher organic nitrogen than carbon sources to reach the maximum biomass value (Tomás et al., 2010). Studies have already demonstrated that a high monosaccharide diet significantly changed gut microbiota, characterized by a markedly increased proportion of Proteobacteria (Do et al., 2018). Therefore, the limited organic nitrogen sources from digested NFD might be the primary cause of decreased levels of *Lactobacillus*, while *unidentified_Enterobacteriaceae* increased their fitness under high monosaccharide conditions induced by NFD. Proteobacteria with adherent and invasive properties are a rich source of lipopolysaccharides (LPS) (Mukhopadhya et al., 2012). Therefore, the uncontrolled expansion of Proteobacteria in NFD-fed chickens represents an active feature of metabolic disturbance, which can further facilitate inflammation or invasion by exogenous pathogens (Shin et al., 2015). A previous study noted that the inflammation caused by LPS infection could stimulate the production and secretion of gel-forming mucins (Smirnova et al., 2003). This could be another reason for the increased number of ileal goblet cells in NFD-fed chickens but further mechanistic studies are needed to completely verify this idea. It is noteworthy that the NFD with AM/AP 0.60 showed the highest *Lactobacillus* abundance which is beneficial for gut health.

However, this study was limited to quantitative measurement techniques of mucin and bacterial protein, and thus lacking of firm evidence to prove the most appropriate AM/AP ratio. In addition, we noted that broilers fed the NFD with different AM/AP ratios could not recover from malnutrition induced by the absence of dietary N. Therefore, the enzyme-hydrolyzed casein method could be a better option to maintain normal physiologic levels of endogenous N flow throughout the intestinal tract.

5. Conclusion

In conclusion, the increased ratio of AM/AP in NFD decreased the IEAA losses and the AID of starch in broilers. In this study, all NFD invariably resulted in malnutrition and increased ileal *unidentified_Enterobacteriaceae* concentrations in broiler chickens regardless of the change in AM/AP ratios. However, the broilers in the AM/AP 0.60 group were closer to the digestive physiological state of birds fed the control diet, with no significant change in maltase activity, and mucin-2 expression. Therefore, this study recommends AM/AP in NFD at 0.60 to measure the IEAA losses of broiler chickens.

Author contributions

Huajin Zhou: Investigation, Data curation, Writing - Original draft preparation. **Yanhong Chen**: Supervision. **Wei Wu**: Resources. **Yao Yu**: Validation. **Tahir Mahmood**: Writing - Reviewing and Editing. **Jianmin Yuan**: Conceptualization, Methodology, Funding acquisition. All authors have read and agreed to the published version of the manuscript.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://submit.ncbi.nlm.nih.gov/subs/, PRJNA880570.

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 Natural Science Foundation of China (No. 31772620).

References

Adedokun SA, Adeola O, Parsons CM, Lilburn MS, Applegate TJ. Factors affecting endogenous amino acid flow in chickens and the need for consistency in methodology. Poult Sci 2011;90(8):1737–48.
Adedokun SA, Applegate TJ. Dietary electrolyte balance influences ileal endogenous amino acid losses in broiler chickens. Poult Sci 2014;93(4):935–42.
AOAC International. Official Methods of Analysis. rev. 2. 18th ed. Gaithersburg, MD: AOAC International; 2007.
Bansil R, Turner BS. The biology of mucus: composition, synthesis and organization. Adv Drug Deliv Rev 2018;124:3–15.
Breyton AE, Goux A, Lambert-Porcheron S, Meynier A, Sothier M, VanDenBerghe L, Brack O, Disse E, Laville M, Vinoy S, Nazare JA. Starch digestibility modulation significantly improves glycemic variability in type 2 diabetic subjects: a pilot study. Nutr Metab Cardiovasc Dis 2021;31(1):237–46.
Chendrimada T, Adams K, Freeman M, Davis AJ. The role of glucagon in regulating chicken hepatic malic enzyme and histidase messenger ribonucleic acid expression in response to an increase in dietary protein intake. Poult Sci 2006;85(4):753–60.
Darsi E, Shivazad M, Zaghari M, Namroud NF, Mohammadi R. Effect of reduced dietary crude protein levels on growth performance, plasma uric acid and electrolyte concentration of male broiler chicks. J Agr Sci Tech-Iran 2012;14(4):789–97.
Do MH, Lee E, Oh MJ, Kim Y, Park HY. High-glucose or -fructose diet cause changes of the gut microbiota and metabolic disorders in mice without body weight change. Nutrients 2018;10(6).
Dong XY, Azzam M, Zou XT. Effects of dietary threonine supplementation on intestinal barrier function and gut microbiota of laying hens. Poult Sci 2017;96(10):3654–63.
Dong ZX, Gu L, Zhang J, Wang M, Du GC, Chen J, Li HZ. Optimisation for high cell density cultivation of Lactobacillus salivarius BBE 09-18 with response surface methodology. Int Dairy J 2014;34(2):230–6.
Freter R, Brickner H, Botney M, Cleven D, Aranki A. Mechanisms that control bacterial populations in continuous-flow culture models of mouse large intestinal flora. Infect Immun 1983;39(2):676–85.
Golian A, Guenter W, Hoehler D, Jahanian H, Nyachoti CM. Comparison of various methods for endogenous ileal amino acid flow determination in broiler chickens. Poult Sci 2008;87(4):706–12.
Hu R, Li J, Soomro RN, Wang F, Feng Y, Yang X, Yao J. The 15N-leucine single-injection method allows for determining endogenous losses and true digestibility of amino acid in cecectomized roosters. PLoS One 2017;12(11):e188525.
Iwasaki M, Tsuchiya K, Okamoto R, Zheng X, Kano Y, Okamoto E, Okada E, Araki A, Suzuki S, Sakamoto N, Kitagaki K, Akashi T, Eishi Y, Nakamura T, Watanabe M. Longitudinal cell formation in the entire human small intestine is correlated with the localization of Hath1 and Klf4. J Gastroenterol 2011;46(2):191–202.
Jansman AJM, Smink W, van Leeuwen P, Rademacher M. Evaluation through literature data of the amount and amino acid composition of basal endogenous crude protein at the terminal ileum of pigs. Anim Feed Sci Technol 2002;98(1):49–60.
Jariyahatthakij P, Chomtee B, Poeikhampha T, Loongyai W, Bunchasak C. Effects of adding methionine in low-protein diet and subsequently fed low-energy diet on productive performance, blood chemical profile, and lipid metabolism-related gene expression of broiler chickens. Poult Sci 2018;97(6):2021–33.
Kong C, Adeola O. Ileal endogenous amino acid flow response to nitrogen-free diets with differing ratios of corn starch to dextrose in broiler chickens. Poult Sci 2013;92(5):1276–82.
Lemme A, Ravindran V, Bryden WL. Heal digestibility of amino acid in feed ingredients for broilers. World Poultry Sci J 2004;60(4):423–37.
Li TJ, Dai QZ, Yin YL, Zhang J, Huang RL, Ruan Z, Deng Z, Xie M. Dietary starch sources affect net portal appearance of amino acid and glucose in growing pigs. Animal 2008;2(5):723–9.
Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25(4):402–8.
Lv X, Zhou C, Ran T, Jiao J, Liu Y, Tan Z, Tang S, Kang J, Xie J, Chen L, Ren A, Xv Q, Kong Z. Dietary amylose:amylopectin ratio influences the expression of amino acid transporters and enzyme activities for amino acid metabolism in the gastrointestinal tract of goats. Br J Nutr 2022;127(8):1121–31.
Ma J, Yang T, Yang M, Yan Z, Zhao L, Yao L, Chen J, Chen Q, Tan B, Li T, Yin J, Yin Y. Effects of dietary amylose/amylopectin ratio and amylase on growth performance, energy and starch digestibility, and digestive enzymes in broilers. J Anim Physiol Anim Nutr 2020;104(3):928–35.
Ma X, Zhang Y, Gromada J, Sewing S, Berggren PO, Buschard K, Salehi A, Vikman J, Rorsman P, Eliasson L. Glucagon stimulates exocytosis in mouse and rat pancreatic alpha-cells by binding to glucagon receptors. Mol Endocrinol 2005;19(1):198–212.
Machín M, Simoyi MF, Blemings KP, Klandorf H. Increased dietary protein elevates plasma uric acid and is associated with decreased oxidative stress in rapidly-growing broilers. Comp Biochem Physiol B Biochem Mol Biol 2004;137(3):383–90.
Moran Jr ET. Gastric digestion of protein through pancreozyme action optimizes intestinal forms 552 for absorption, mucin formation and villus integrity. Anim Feed Sci Technol 2016;221:284–303.
Moughan PJ. Amino acid digestibility and availability in foods and feedstuffs. In: Proceedings of the 9th international symposium on digestive physiology in pigs; 2003. p. 199–221.
Moughan PJ, Marlies Leenaars GS. Endogenous amino acid flow in the stomach and small intestine of the young growing pig. J Sci Food Agric 1992;60(4):437–42.
Mukhopadhya I, Hansen R, El-Omar EM, Hold GL. IBD-what role do Proteobacteria play? Nat Rev Gastroenterol Hepatol 2012;9(4):219–30.
Myers WD, Ludden PA, Nayigihugu V, Hess BW. Technical note: a procedure for the preparation and quantitative analysis of samples for titanium dioxide. J Anim Sci 2004;82(1):179–83.
Ortiga-Carvalho TM, Chiamolera MI, Pazos-Moura CC, Wondisford FE. Hypothalamus-pituitary-thyroid axis. Compr Physiol 2016;6(3):1387–428.
Pollock J, Hutchings MR, Hutchings K, Gally DL, Houdijk J. Changes in the ileal, but not fecal, microbiome in response to increased dietary protein level and enterotoxigenic Escherichia coli exposure in pigs. Appl Environ Microbiol 2019;85(19).
Ravindran V. Progress in ileal endogenous amino acid flow research in poultry. J Anim Sci Biotechnol 2021;12(1):5.
Ravindran V, Hew LI, Ravindran G, Bryden WL. Apparent ileal digestibility of amino acid in dietary ingredients for broiler chickens. Anim Sci 2005;81(1):85–97.
Reitzer L. Nitrogen assimilation and global regulation in Escherichia coli. Annu Rev Microbiol 2003;57:155–76.
Rosebrough RW, McMurtry JP. Protein and energy relationships in the broiler chicken. Br J Nutr 1993;70(3):667–78.
Selle P, Liu SY. The relevance of starch and protein digestive dynamics in poultry. J Appl Poultry Res 2019;28(3):531–45.
Sharma R, Fernandez F, Hinton M, Schumacher U. The influence of diet on the mucin carbohydrates in the chick intestinal tract. Cell Mol Life Sci 1997;53(11):935–42.
Sharma R, Schumacher U. Carbohydrate expression in the intestinal mucosa. Adv Anat Embryol Cell Biol 2001;160:1–91.

Shin N, Whon TW, Bae J. Proteobacteria: microbial signature of dysbiosis in gut microbiota. Trends Biotechnol 2015;33(9):496–503.
Smirnov A, Tako E, Ferket PR, Uni Z. Mucin gene expression and mucin content in the chicken intestinal goblet cells are affected by in ovo feeding of carbohydrates. Poult Sci 2006;85(4):669–73.
Smirnova MG, Guo L, Birchall JP, Pearson JP. LPS up-regulates mucin and cytokine mRNA expression and stimulates mucin and cytokine secretion in goblet cells. Cell Immunol 2003;221(1):42–9.
Smith SM, Lukaski HC. Type of dietary carbohydrate affects thyroid hormone deiodination in iron-deficient rats. J Nutr 1992;122(5):1174–81.
Stein HH, Sève B, Fuller MF, Moughan PJ, de Lange CF. Invited review: amino acid bioavailability and digestibility in pig feed ingredients: terminology and application. J Anim Sci 2007;85(1):172–80.
Ten HG, Engelen MP, Soeters PB, Deutz NE. Absence of post-prandial gut anabolism after intake of a low quality protein meal. Clin Nutr 2012;31(2):273–82.
Tomás MS, Bru E, Nader-Macías ME. Estimation of combined effects of carbon and nitrogen sources on the growth and bacteriocin production of Lactobacillus salivarius from human source. J Basic Microbiol 2010;50(2):190–9.
Van der Meulen J, Bakker JGM, Smits B, De Visser H. Effects of source of starch on net portal flux of glucose, lactate, volatile fatty acids and amino acid in the pig. Br J Nutr 1997;78(4):533–44.
Wang W, Chen Y, Wang J, Lv Z, Li E, Zhao J, Liu L, Wang F, Liu H. Effects of reduced dietary protein at high temperature in summer on growth performance and carcass quality of finishing pigs. Animals (Basel) 2022;12(5):599.
Weurding RE, Veldman A, Veen WA, van der Aar PJ, Verstegen MW. Starch digestion rate in the small intestine of broiler chickens differs among feedstuffs. J Nutr 2001;131(9):2329–35.
Yin D, Selle PH, Moss AF, Wang Y, Dong X, Xiao Z, Guo Y, Yuan J. Influence of starch sources and dietary protein levels on intestinal functionality and intestinal mucosal amino acid catabolism in broiler chickens. J Anim Sci Biotechnol 2019;10:26.
Zhong Q, Li M, Atiba EM, Yin Y, Yang Q, Zhang L, Sun Z. Effects of physicochemical and structural properties of single and double feedstuffs derived from different botanic sources on in vitro starch digestion and glucose release kinetics. J Anim Physiol Anim Nutr 2021;105(3):493–506.
Zhou H, Wu W, Mahmood T, Chen Y, Xu Y, Wang Y, Yuan J. Comparison of endogenous amino acid losses in broilers when offered nitrogen-free diets with differing ratios of dextrose to corn starch. Sci Rep 2022;12(1):5689.