Age-dependent response to fasting during assessment of metabolizable energy and total tract digestibility in chicken

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ABSTRACT Fasting is typically used to empty the gastrointestinal tract (**GIT**) and assess feed metabolizable energy (**ME**). However, the effects of fasting on energy and nutrient utilization are not well understood. This study aimed to explore the difference in GIT emptying, energy and nutrient utilization of broilers and adult roosters fed corn-soybean meal-based diet upon fasting. In experiment 1, 7 cages of broilers/adult roosters were selected and fasted for 72 h, and excreta were collected from 12 h of fasting and analyzed every 12 h to explore GIT emptying. Results indicated the GIT emptying time of free-feeding broilers or adult roosters is 12 or 24 h, respectively. In experiment 2, 4 treatments were used that consisted of 2 ages of birds (25 d broilers and 30 wk adult roosters) and 2 feeding forms (fed ad libitum or fasted for 36 h before formal feeding). Excreta was collected during refeeding, and the total collection method (**TCM**) and the index method (**IM**) were used for data analysis. Compared to non-fasted group, fasting increased the total tract digestibility of ME, gross energy (**GE**), and ether extract (**EE**) (by 1.80, 3.50 and 18.56%, respectively, all $P < 0.05$) in broilers, but decreased the total tract digestibility of nitrogen by 8.10% ($P < 0.05$). Conversely, fasting increased total tract digestibility of nitrogen in adult roosters (-0.37% vs. 11.65%, $P < 0.05$). The comparative analysis found that total tract digestibility of nitrogen obtained by TCM was greater than the result calculated by IM (17.76 % vs. -0.37). Similarly, total tract digestibility of GE calculated by TCM was significantly higher than the value observed by IM ($P < 0.05$). However, the results of total tract digestibility of GE and nitrogen in broilers calculated by TCM were consistent with those obtained by IM. Overall, fasting increases total tract digestibility in broilers and total tract digestibility of nitrogen in adult roosters, respectively. Additionally, total tract digestibility calculated by TCM may be overestimated.

Key words: fasting, age, gastrointestinal tract emptying, metabolizable energy, nutrient digestibility

INTRODUCTION

Fasting is routinely employed in classical total collection method (**TCM**) assays to ensure the emptying of the gastrointestinal tract (**GIT**) before measuring the feed metabolizable energy (**ME**). Besides, it is also used to account for endogenous losses of energy (**EEL**) and nitrogen (**ENL**, Sibbald and Price, 1978). However, GIT emptying has a direct connection with the age of the bird (Lilburn and Loeffler, 2015). In adult roosters, an earlier study found that 48 h of fasting is sufficient to empty the GIT by force-feeding (Sibbald, 1976). In recent years,

different fasting times such as 24 h (Kerr et al., 2014), 26 h (von Schaumburg et al., 2019; Matin et al., 2021), 30 h (Yadav et al., 2022), and 48 h (Liu et al., 2017) have been used to empty the GIT before the measurement of ME in adult roosters. Different fasting durations were also found in GIT emptying for broilers, such as 16 h (Peron et al., 2005) or 12 h (Yang et al., 2020). For the determination of ME, the ad libitum feeding method is becoming increasingly popular compared to the labor-intensive and time-consuming method of force-feeding. However, there is a lack of consensus on fasting duration (Lu et al., 2020; Yang et al., 2020), which necessitates investigating a new fasting procedure in ME assays.

Values of ME were influenced by fasting duration. Sibbald (1976) found that lengthening the fasting duration from 24 to 96 h, by 24 h intervals, had no significant effect on the true ME (**TME**) value in adult White Leghorn roosters. What's more, it has been reported that the values of TME increases with increased fasting

duration from 12 h to 48 h in adult roosters (Shires et al., 1979). However, it is worth noting that both Sibbald (1976) and Shires et al. (1979) did not compare the fasted birds with the non-fasted birds. More recently, researchers have found that fasting influences the development of the small intestine (Lu et al., 2020; Yang et al., 2020), thereof the absorptive functions. Development of digestive and absorptive functions in adult roosters is different from that of broilers (Lilburn and Loeffler, 2015; Fondevila et al., 2020), in terms of intestinal nutrient transporters expression, villus surface area (Li et al., 2020) and relative weight of digestive organs (Yang et al., 2020). Correspondingly, it has indicated that apparent metabolizable energy (**AME**), TME, and nutrient digestibility varied with age of birds (Dale and Fuller, 1980; Stefanello et al., 2016; Yang et al., 2020). As the main site for nutrient absorption, growing research finds that adult and young chickens respond differently to fasting. For example, fasted adult roosters showed atrophic intestinal villus height (Yamauchi et al., 1996) while fasted broilers showed increased or unchanged intestinal villus height (Thompson and Applegate, 2006; Wang et al., 2021). Similarly, fasting caused villus atrophy in aged mice much greater than in young mice (Song et al., 2009). Therefore, how fasting affects ME and total tract digestibility in birds of different ages is unclear.

In the present study, birds underwent long-time fasting and were used for determination GIT emptying time and EEL and ENL. The **TCM** and the index method (**IM**) were used to investigate the impact of fasting on the ME, total tract digestibility of gross energy (**GE**), ether extract (**EE**), nitrogen, starch of on refeeding d 1 (**RFD1**), and refeeding d 2 (**RFD2**) in broilers and adult roosters.

MATERIALS AND METHODS

All procedures have complied with the Beijing Regulations of Laboratory Animals, and the Laboratory Animal Ethical Committee of China Agricultural University (No. AW04110202-2-4).

Experiment 1 — GIT Emptying

Thirty-five broilers (Arbor Acres Plus, aged 25 d, 1.3 kg) and 7 adult roosters (White Leghorn, aged 30 wk, 1.9 kg) were divided into 7 cages, respectively. Each cage had 5 broilers or 1 adult rooster. Feed and water was available ad libitum. Feed formulation was present in Table 1. Feed was removed for 72 h. Then after 12 h of withdrawal, iron plates covered with plastic films were placed under each cage to begin collect excreta. Excreta were collected every 12 h and lyophilized and ground, then sieved through a 40 μm and then stored at $-20°C$ for nitrogen and energy analysis. Broilers were fed the same diet as adult roosters before fasting, and the feed formulation and nutrient content was listed in Table 1.

Table 1. Ingredient composition and nutrient content of the experimental diet (%, as-fed basis, unless otherwise indicated)[1].

Item	Composition
Corn	60.70
Soybean meal (44% CP)	22.20
Soybean oil	4.10
Wheat flour	2.00
Maize gluten meal	6.02
Lysine-HCl (98%)	0.60
Limestone	1.20
Calcium phosphate	1.70
Sodium chloride	0.30
Vitamins premix[2]	0.02
Mineral premix[3]	0.20
Phytase	0.02
Choline chloride (60%)	0.20
DL-Methionine (98%)	0.22
Antioxidant (30%)	0.02
Titanium dioxide	0.50
Total	100
Analyzed energy and nutrient content	
Gross energy (Mcal/kg)	4.11
Crude protein	19.50
Ether extract	6.00
Total starch	39.12
Calcium	0.93
Available phosphorus	0.46

[1]Diet was in pellet form (Φ, 5mm).
[2]The vitamin premix provided the following per kg of diets, Vitamin A 12,500 IU, Vitamin D3 2,500 IU, Vitamin E 18.75 mg, Vitamin K3 2.65 mg, Vitamin B1 2.00 mg, Vitamin B2 6.00 mg, Vitamin B6 6.00 mg, Vitamin B12 0.03 mg, biotin 0.03 mg, folic acid 1.25 mg, pantothenic acid 12.00 mg, nicotinic acid 50.00 mg.
[3]The trace mineral premix provided the following per kg of diets: Cu, 8.00 mg (as $CuSO_4 \cdot 5H_2O$); Zn, 75.00 mg (as $ZnSO_4$); Fe, 80.00 mg (as $FeSO_4 \cdot ;H_2O$); Mn, 100.00 mg (as $MnSO_4$); Se, 0.30 mg (as Na_2SeO_3); I, 0.35 mg (as $Ca(IO_3)_2$).

Experiment 2 — Metabolism Trial

Seventy broilers (Arbor Acres Plus, aged 23 d, average body weight was 1.2 kg) and 14 adult roosters (White Leghorn, aged 30 wk, 1.9 kg) were randomly assigned into fasting or non-fasting groups with 7 replicate cages (5 birds per cage for broilers, 1 bird per cage for adult roosters). Experimental treatments consisted of 2 factors: fasting or not (fed ad libitum, or fasted for 36 h) and 2 ages (d 25, or wk 30) in a 2 × 2 factorial design. Birds have received a light-to-dark ratio of 20L:4D (turn off at 22:00 p.m. and turn on at 02:00 a.m.). Broilers and roosters were fed the same basal diet (0.5% titanium dioxide [**TiO₂**] as a digestibility marker, Table 1). Water was available ad libitum.

As shown in Figure 1, after fasting, birds were refed for 2 d followed by another round of fasting. Excreta was collected every day until the end of the second round of fasting. Notably, for non-fasted broilers and adult roosters, feed containing chromic oxide (**Cr₂O₃**, 0.3%) was provided 6 h before formal feeding. After 4 h of feeding, birds fed diet with Cr_2O_3 were removed and vacated feeder for 2 h. Then birds were provided with the experimental diet. Similarly, at the end of the formal feeding, the diet with Cr_2O_3 was provided again. Diet with Cr_2O_3 used here was aimed to ensure that collected excreta samples were from the feed ingested during the formal feeding. Because excreta containing Cr_2O_3 is green, while excreta only containing TiO_2 is tawny.
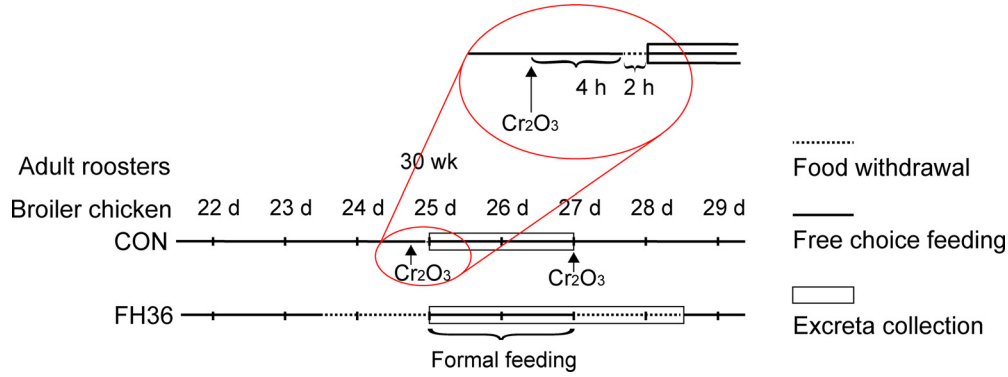
Figure 1. Fasting and excreta collecting procedure in broilers and adult roosters. Abbreviations: CON, birds fed ad libitum; FH36, birds fasted for 36 h.

Evacuate the feeder for 2 h to better distinguish between different colors of excreta. So, tawny excreta excreted from birds fed experimental diet were easily collected from non-fasted birds. All excreta samples were lyophilized and ground, then sieved through a 40-μm screen and then stored at −20°C for later analysis.

Chemical Analysis

The UV-spectroscopy assay was used to determine TiO_2 in the feed and excreta samples (Morgan et al., 2014). Feed and excreta samples were analyzed for GE using an oxygen bomb calorimeter (CALORIMETER, 6400, Parr [Moline, USA]) with benzoic acid as the calibration; Nitrogen by Kjeldahl method (Mountzouris et al., 2010); EE by Soxhlet apparatus while starch in feed and excreta was analyzed by a starch assay kit (KTSTA, Megazyme, Ireland).

Calcium was determined by ethylene diamine tetra-acetic acid method. A total of 2.5 g dry excreta with three replicates were put into crucibles and carbonized on an electric stove. Then, the carbonized samples were placed into a muffle furnace and burned at 550°C for 3 h. And then took out for cooling. Then 10 mL HCL (V (HCL) / V (water) = 1: 3) and 4 drops of nitric acid were added into every cooled muffle furnace and boiled mildly. Boiled solution were then transfered to volumetric flask for calibration.

Next, 1 mL of decomposition liquor was popetted into a conical flask, 50 mL of water was added along wiht, 10 mL of starch solution, 2 mL of triethanolamine, 1 mL of ethylenediamine, 1 drop of malachite green, 10 mL of potassium hydroxide, 0.1 g of hydroxylamine hydrochloride, and a little calcein was titrated with EDTA standard titration immediately under the black background until the green fluorescence disappeared and turned purple as the titration end point. Then the concentration of Calcium was calculated according to the following formulation: Calcium (%) = T × (V2 − V1) × 100/m. Where T represents the concentration of EDTA, g/mL; V1 represents the volume of standard used in titration of blank, mL; V2 represents the volume of standard used in titration of the sample, mL; m, weight of the sample, g.

Take another 1 mL of decomposition liquor into a 50 mL volumetric flask, then add vanadium ammonium molybdate and water for use in calibration. After shaking, the absorbance was measured at a wavelength of 420 nm. Trichloroacetic acid was used to determinate the concentration of phytate phosphorus in diet. Available phosphorus was result from total phosphorus minus phytate phosphorus.

Calculations and Statistical Analysis

The AME, TME, and nitrogen corrected AME ($\mathbf{AME_n}$) and TME ($\mathbf{TME_n}$) were calculated by the following equations (Khalil et al., 2021; Lee et al., 2018):

$$AME = \left[\frac{GE\ intake\ -\ GE\ excretion}{Feed\ intake} \right].$$

$$TME = \left[\frac{GE\ intake\ -\ GE\ excretion\ +\ EEL}{Feed\ intake} \right].$$

$$AMEn = [AME - 8.22\ kcal/kg\ \times\ nitrogen\ retention]$$

$$TMEn = [TME - 8.22\ kcal/kg\ \times\ nitrogen\ retention]$$

Nitrogen retention ratio (%)

$$= \left[\frac{nitrogen\ intake\ -\ nitrogen\ excretion}{nitrogen\ intake} \right]$$
$$\times\ 100$$

8.22 kcal/kg is the energy required to metabolize 1 kg of urea (Hill and Anderson, 1958).

Total tract digestibility observed by TCM was calculated using the equation (Mustafa and Baurhoo, 2017):

Total tract digestibility (%) = $\left[\frac{Nutrient\ intake\ -\ Nutrinet\ excretion}{Nutrient\ intake} \times 100 \right]$.

Total tract digestibility observed by IM was calculated using the equation (Mustafa and Baurhoo, 2017):

Total tract digestibility (%) = $\left[\left(1 - \frac{nutrient\ excretion}{nutrient\ intake} \times \frac{TiO2\ in\ feed}{TiO2\ in\ excreta} \right) \times 100 \right]$

Data were analyzed by SPSS (Version 25.0, SPSS Inc., Chicago, IL). A 2 × 2 factorial arrangement of 2 fasting states and 2 ages (d 25 and wk 30) was used, and the main effect of age, fasting state, and interactions between age and fasting state were analyzed by general linear model. Items with the interaction effect were analyzed by ANOVA. Also, ANOVA was used for the analysis of the impact of the calculation method on the digestibility of GE and nitrogen and endogenous excretion. Significant means were separated by Duncan's Multiple Range test method. A probability level of less than 5% by a two-tailed test was considered to be statistically significant ($P \leq 0.05$).

RESULTS

GIT Emptying Time and Endogenous Excretion of Broilers and Adult Roosters

Energy excretion in broilers reached a relative plateau starting from 12 h post fasting (Figure 2A), whereas relative constant energy excretion in roosters was noted at 24 h post fasting (Figure 2B). The lowest energy excretions were found during 36 h to 48 h fasting in broilers. Nevertheless, this phenomenon was observed at 48 h to 60 h of fasting in adult roosters. We assumed that the means of values in relative plateau (post-feeding

between 12 h and 72 h in broilers, whereas 24 to 72 h in roosters) phases as the EEL, even though there was a sudden decrease in relative plateau stages. In this method, the EEL of broilers was 6.29 kcal/bird/12 h, while EEL of adult roosters was 5.33 kcal/bird/12 h.

In broilers, constant nitrogen output started from 12 h until 48 h fasting, then suddenly increased after 48 h of fasting (Figure 2C). In roosters, the constant ENL was noticeable from 24 h to 60 h of fasting, then increased after 60 h of fasting (Figure 2D). On the principle of constant ENL, it can be argued that 12 h of fasting is enough for GIT emptying in broilers, while 24 h of fasting is mandated for adult roosters. In this study, we regarded the mean of values in broilers post-feeding between 12 h and 48 h as ENL, while that of adult roosters was between 24 h and 60 h of fasting. In this method, we found the ENL of broilers was 0.19 g/bird/12 h, while that of adult roosters was 0.42 g/bird/12 h.

Influence of Fasting on Feed ME in Broilers and Adult Roosters

No interaction was found for feed intake ($P > 0.05$, Figure 3A). Broilers had greater feed intake than adult roosters (174.44 vs. 294.17, $P < 0.001$). Besides, fasting significantly increased feed intake in broilers (211.45 vs. 254.51, $P = 0.001$). Treatment interactions were
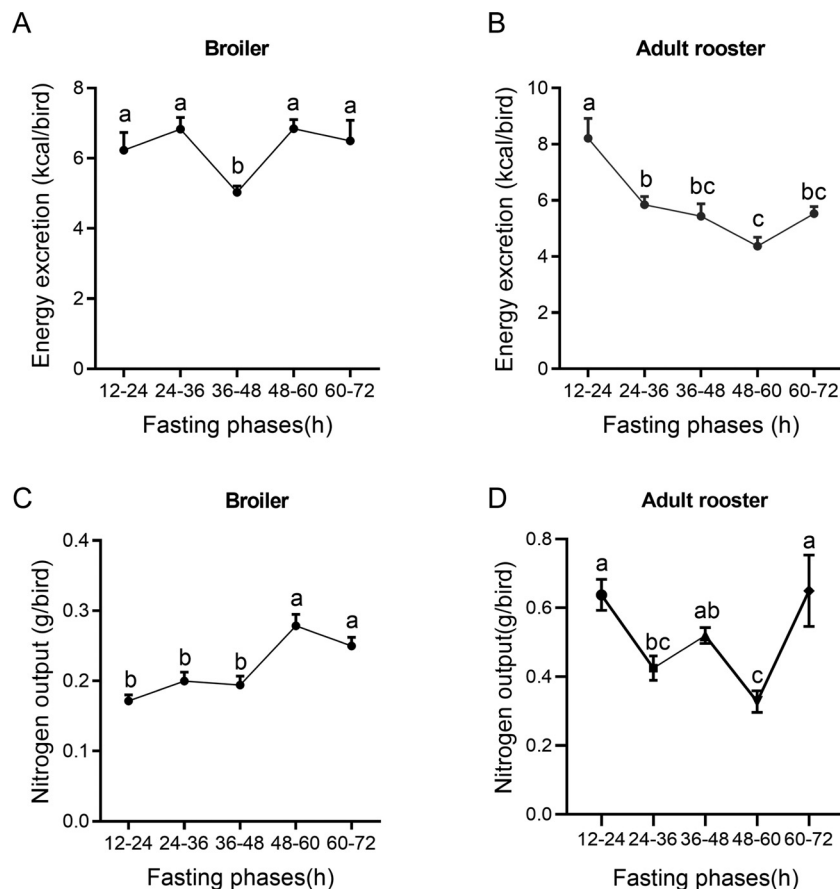

Figure 2. Changes of body weight and endogenous excretion at different fasting phases. (A) Changes of body weight for broilers. (B) Energy excretion during fasting in broilers. (C) Nitrogen output during fasting in broilers. (D) Changes of body weight for adult roosters. (E) Energy excretion during fasting in adult roosters. (F) Nitrogen output during fasting in adult roosters. Error bars represent the SEM, n = 7. Means without a common letter differ, $P \leq 0.05$.
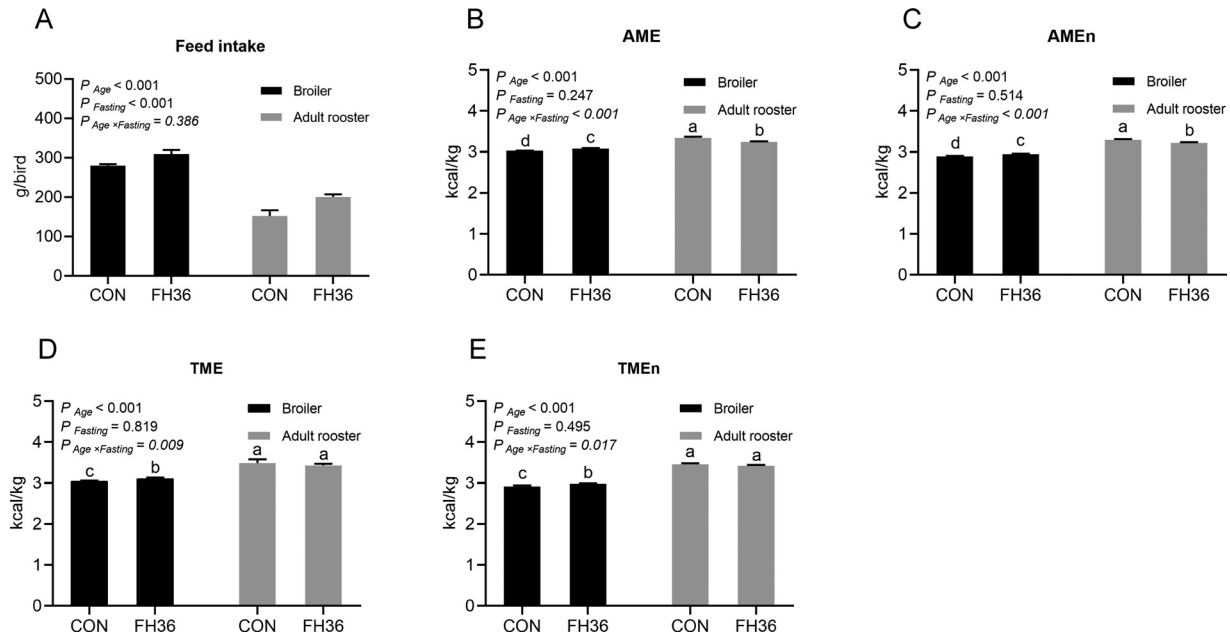
Figure 3. Effect of fasting on feed intake, ME in broilers and roosters. (A) Feed intake during refeeding. (B) AME in response to fasting. (C) AMEn in response to fasting. (D) TME in response to fasting. (E) TMEn in response to fasting. Error bars represent the SEM, n = 7. Means without a common letter differ, $P \leq 0.05$. Abbreviations: AME, apparent metabolizable energy; AMEn, nitrogen corrected AME; CON, birds fed ad libitum; FH36, birds fasted for 36 h; TME, true metabolizable energy; TMEn, nitrogen corrected TME.

observed for AME ($P < 0.001$, Figure 3B), TME ($P = 0.009$, Figure 3C), AMEn ($P < 0.001$, Figure 3D), and TMEn ($P = 0.017$, Figure 3E). The AME, TME, AMEn and TMEn of adult roosters were 8.20, 12.34, 11.64, and 16.61% more than broilers (all $P < 0.001$), respectively. For broilers, fasting increased AME, TME, AMEn, and TMEn by 1.59, 1.80, 1.90, and 2.05%, respectively (all $P < 0.05$). For adult roosters, fasting decreased AME and AMEn (both $P < 0.05$), but fasting had no influence on TME and TMEn (both $P > 0.05$).

Influence of Fasting on Total Tract Digestibility of GE in Broilers and Adult Roosters

The IM was used for the total tract digestibility on RFD1 and RFD2, and TCM was used for total energy utilization assessment. There was no interaction between age and fasting on total tract digestibility of GE on RFD1 and RFD2 ($P = 0.902$ and $P = 0.625$, respectively, Figures 4A and 4B). Food deprivation significantly increased total tract digestibility of GE on RFD1 (72.25% vs. 74.27%, $P < 0.001$, Figure 4A), but did not influence total tract digestibility of GE on RFD2 (72.31% vs. 72.46%, $P = 0.282$, Figure 4B). No age effect was found on total tract digestibility of GE on RFD1 and 2 ($P = 0.080$ and $P = 0.504$, respectively). An interaction effect was observed on the total tract digestibility of GE obtained from the TCM ($P = 0.001$, Figure 4C). Fasting decreased total tract digestibility of GE that obtained by TCM in roosters (79.38 vs. 81.65%, $P < 0.05$, Figure 4C), which is similar to the decreased AME upon fasting. However, fasting had no influence on broilers (75.07 vs. 74.13%, $P > 0.05$, Figure 4C), and

digestibility of GE was increased with age (80.51 vs. 74.60%, $P < 0.001$, Figure 4C).

Influence of Fasting on Total Tract Digestibility of Nitrogen in Broilers and Adult Roosters

Interaction effects were observed for the total tract digestibility of nitrogen both on RFD1 and RFD2 ($P < 0.001$, $P = 0.002$, respectively, Figures 5A and 5B), but there was no interaction between age and fasting on total tract digestibility of nitrogen calculated by TCM and nitrogen retention ratio ($P = 0.862$ and $P = 0.967$, respectively). By the IM, on RFD1, fasting decreased total tract digestibility of nitrogen were found in broilers (57.22 vs. 52.58%, $P < 0.05$), whereas increased total tract digestibility of nitrogen was found in roosters (0.37 vs. 11.74%, $P < 0.05$). However, on RFD2, fasting showed no influence on total tract digestibility of nitrogen in broilers ($P > 0.05$), but increased total tract digestibility of nitrogen of adult roosters (0.37 vs. 11.57%, $P < 0.05$).

By TCM, we found that fasting decreased the nitrogen retention ratio (37.10 vs. 29.17%, $P = 0.019$, Figure 5D) and total tract digestibility of nitrogen (45.21 vs. 35.73%, $P < 0.001$, Figure 5C). Similarly, both nitrogen retention ratio and total tract digestibility of nitrogen calculated by TCM were decreased with age (51.80 vs. 14.47%, $P = 0.002$ and 51.99% vs. 14.24%, $P = 0.001$, respectively). Notably, adult roosters fed ad libitum showed a low total tract digestibility of nitrogen at RFD1 and RFD2 (both −0.37%), which is different from total tract digestibility of nitrogen calculated by TCM.
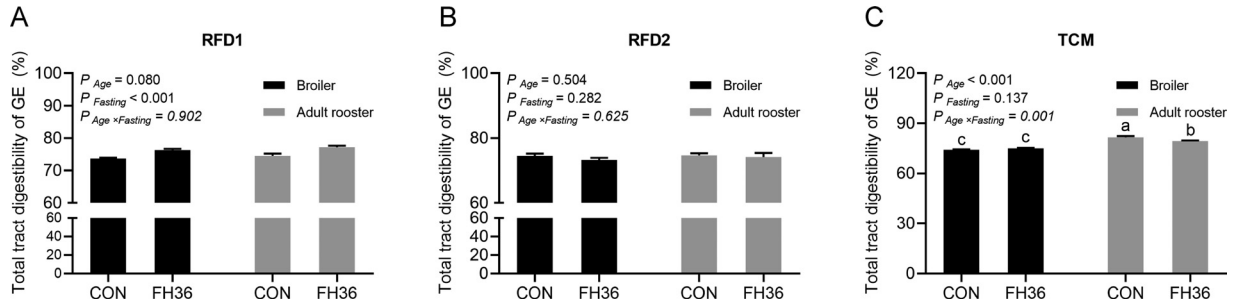
Figure 4. Effect of fasting on total tract digestibility of GE in broilers and roosters. (A) Total tract digestibility of GE observed by IM on RFD1. (B) Total tract digestibility of GE observed by IM on RFD2. (C) Total tract digestibility of GE observed by TCM. Error bars represent the SEM, n = 7. Abbreviations: CON, birds fed ad libitum; FH36, birds fasted for 36 h; GE, gross energy; RFD1, refeeding d 1; RFD2, refeeding d 2; TCM, the total collection method. Means without a common letter differ, $P \leq 0.05$.

Influence of Fasting on Total Tract Digestibility of EE and Starch in Broilers and Adult Roosters

The interaction effect was found for the total tract digestibility of EE on RFD1 ($P < 0.001$, Figure 6A), but no interaction effect was found on RFD2 ($P = 0.421$, Figure 6B). Total tract digestibility of EE was increased with age on RFD1 (77.84 vs. 95.12%) and RFD2 (73.78 vs. 96.75%) regardless of fasting or not (both $P < 0.001$, Figures 6A and 6B). On RFD1, fasting increased the total tract digestibility of EE of broilers (71.23 vs. 84.44%, $P < 0.05$, Figure 6A) but no difference was found in adult roosters (94.55 vs. 95.68%, $P > 0.05$, Figure 6A).

But, fasting didn't influence the total tract digestibility of EE on RFD2, irrespective of age ($P = 0.362$, Figure 6B).

Treatment interaction was observed for total tract digestibility of total starch on RFD1 ($P = 0.040$, Figure 6C). Roosters had greater total tract digestibility of total starch than broilers (increased by 1.76%, $P < 0.001$), irrespective of fasting. However, fasting decreased total tract digestibility of total starch on RFD1 in broilers (decreased by 0.6%, $P < 0.05$), but showed no influence on adult roosters ($P > 0.05$). Given that birds fed ad libitum have a great total tract digestibility of starch on RFD1 (close to 100%), and a negligible influence was observed for fasting and age on RFD1, total tract digestibility of starch on RFD2 was not completed.
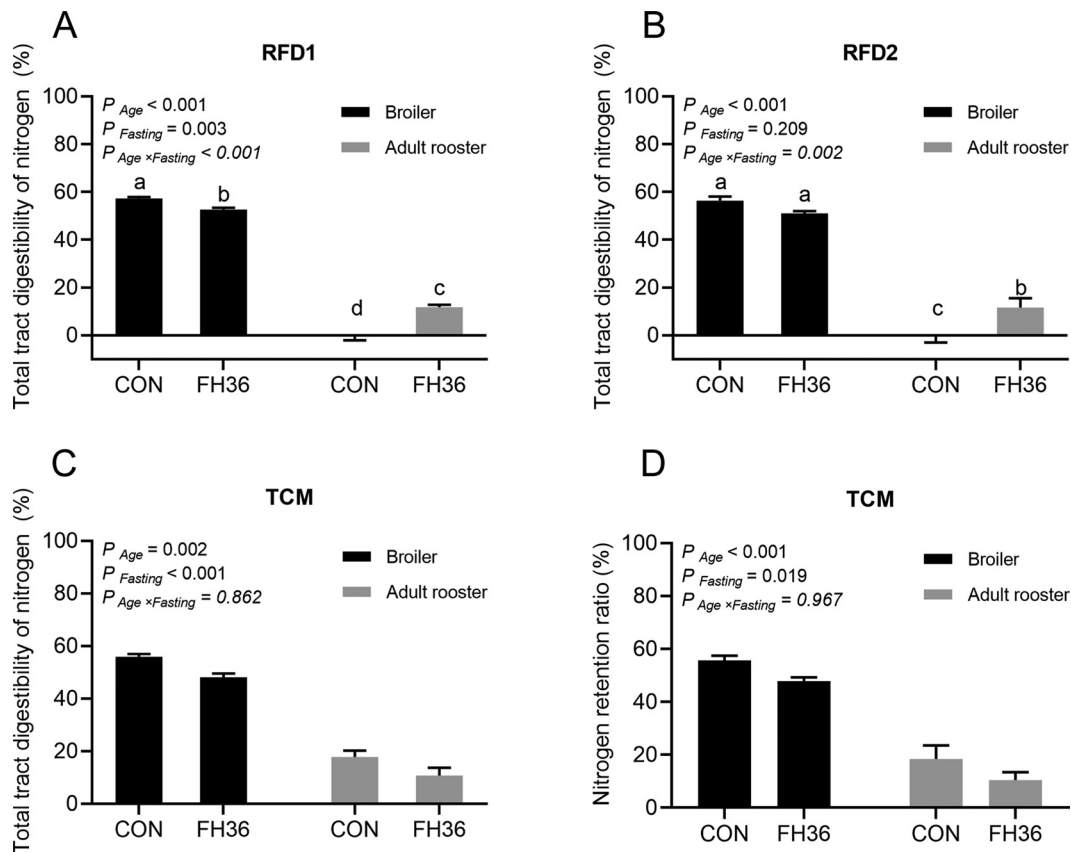

Figure 5. Effect of fasting on total tract digestibility of nitrogen in broilers and roosters. (A) Total tract digestibility of nitrogen observed by IM on RFD1. (B) Total tract digestibility of nitrogen observed by IM on RFD2. (C) Digestibility of nitrogen observed by TCM. (D) Nitrogen retention ratio observed by TCM. Error bars represent the SEM, n = 7. Abbreviations: CON, birds fed ad libitum; FH36, birds fasted for 36 h. RFD1, refeeding d 1; RFD2, refeeding d 2; TCM, the total collection method. Means without a common letter differ, $P \leq 0.05$.
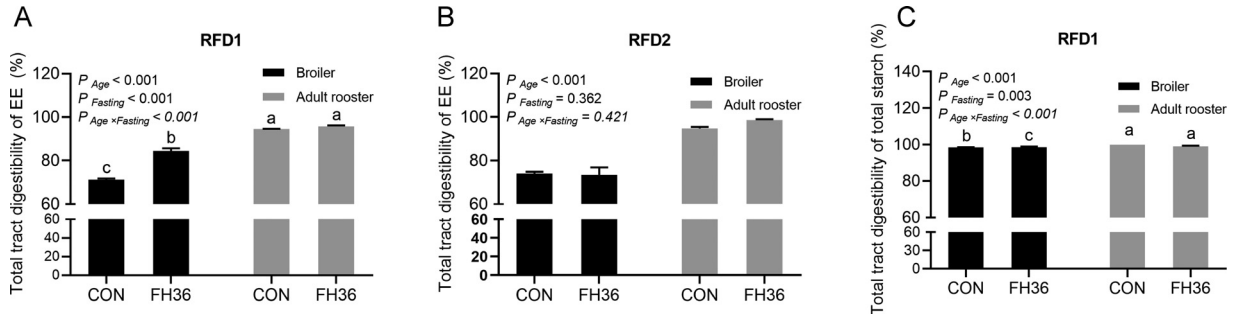
Figure 6. Effect of fasting on total tract digestibility of EE and total starch in broilers and roosters. (A) Total tract digestibility of EE observed by IM on RFD1. (B) Total tract digestibility of EE observed by IM on RFD2. (C) Total tract digestibility of total starch observed by IM on RFD1. Error bars represent the SEM, n = 7. Abbreviations: CON, birds fed ad libitum; EE, ether extract; FH36, birds fasted for 36 h; RFD1, refeeding d 1; RFD2, refeeding d 2. Means without a common letter differ, $P \leq 0.05$.

Comparison of Total Tract Digestibility Observed From the IM and TCM in Broilers and Adult Roosters

Given that total tract digestibility of energy and nitrogen calculated by IM showed some difference from that calculated by TCM, we decided to make a comparison in nitrogen and energy utilization between IM and TCM (Figure 7). Notably, a comparison of evaluation methods of total tract digestibility of nitrogen and GE was carried on the non-fasted birds. The result showed no difference in total tract digestibility of GE and nitrogen between the RFD1 and RFD2 (both $P > 0.05$, Figures 7A–7D). We found that total tract digestibility of GE and nitrogen calculated by IM were comparable to the result from TCM in broilers ($P = 0.443$, $P = 0.739$, respectively, Figures 7A and 7C). However, in roosters, total tract digestibility of GE and nitrogen
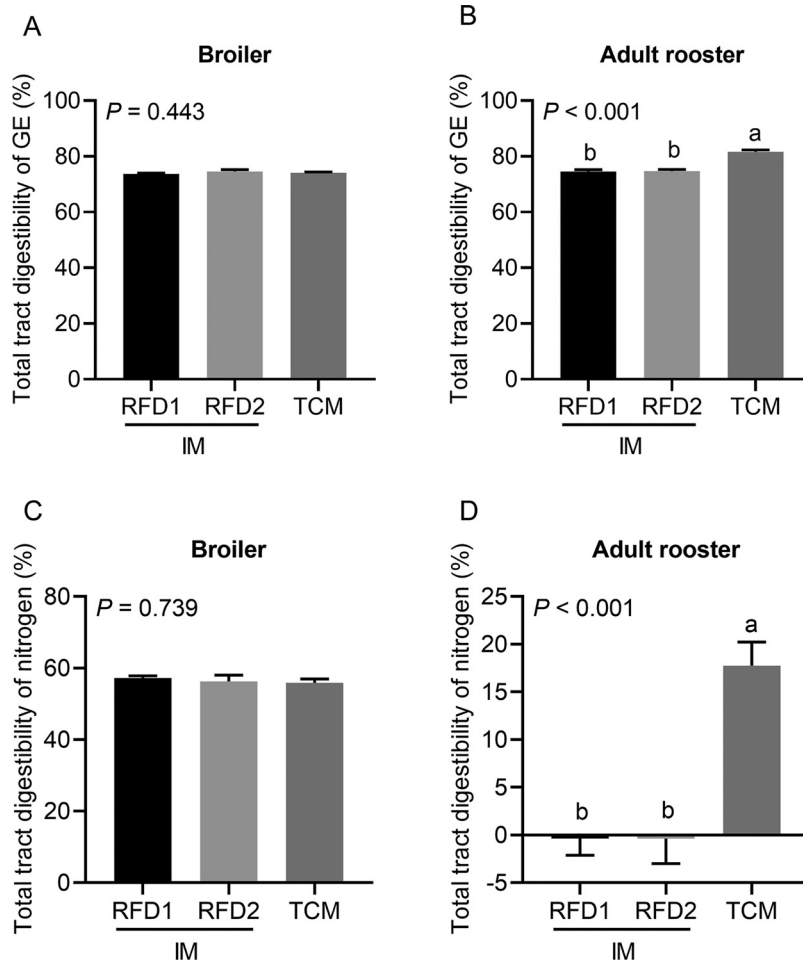

Figure 7. Effect of evaluation method on total tract digestibility of GE and nitrogen in birds fed ad libitum. (A) Total tract digestibility of GE observed by IM and TCM. (B) Total tract digestibility of GE observed by IM and TCM in adult roosters fed ad libitum. (C) Total tract digestibility of nitrogen observed by IM and TCM in broilers fed ad libitum. (D) Total tract digestibility of nitrogen observed by IM and TCM in adult roosters fed ad libitum. Error bars represent the SEM, n = 7. Abbreviations: IM, the index method; RFD1, refeeding d 1; RFD2, refeeding d 2; TCM, the total collection method. Means without a common letter differ, $P \leq 0.05$.

calculated by IM were significantly lower than that calculated by TCM (both $P < 0.001$, Figures 7B and 7D).

DISCUSSION

Traditionally, adult White Leghorn roosters are usually used to estimate the bioavailable energy of feedstuffs then form the nutrient database. These adult roosters are fasted to ensure the emptying of the GIT before force-feeding the experimental diet. This study compared the differences in GIT emptying, ME, and total tract digestibility of broiler chicken and adult rooster in detail.

Constant excreta output during fasting is taken as an indicator for an accurate GIT emptying time. A study on White Leghorn roosters aged between wk 25 and 52 showed that birds fasted for 24 h had 0.83 g and 2.50 g of residues in the GIT (Mcnab and Blair, 1988). But, the weight of residue is susceptible to sand and other sundries. Considering the practical difficulty of force-feeding and collecting the residues in the GIT, the time required to reach a constant excreta energy or nitrogen output during continuous fasting in birds initially fed ad libitum is taken as GIT emptying time. In the present study, constant EEL and ENL output suggest that 12 h of fasting is enough for GIT emptying in broilers initially fed ad libitum. However, we found that a constant EEL started at 24 h post fasting in adult roosters, which showed that 24 h fasting is required for GIT emptying in adult roosters initially fed ad libitum. Inconsistently, it has been reported that constant EEL was observed after 24 h of fasting in force-fed broilers (Murakami et al., 1994) and 48 h of fasting inforce-fed adult White Leghorn roosters (Sibbald, 1976). The feeding method that is, force-feeding in Murakami et al. (1994) and Sibbald's (1976) studies and ad libitum method used in the present study could be the putative reason for the difference in GIT emptying time. We also observed that constant nitrogen excretion occurred at 12 h after fasting in broilers and 24 h in adult roosters. However, a sudden increase in nitrogen excretion after 48 h and 60 h of fasting was observed in broilers and adult roosters, respectively. This may be attributed to the strengthened tissue protein catabolism for energy supply (Saneyasu et al., 2015). What's more, this also suggests that ENL doesn't apply to GIT emptying determination because it's instability. In summary, in order not to cause the breakdown of body protein in the GIT emptying assessment, the fasting duration of broilers should not exceed 48 h, and the fasting duration of adult roosters should not exceed 60 h.

In the present study, we found that fasting increased the ME in broilers, which may be explained by feed restriction-induced compensatory effect in growing animal (Le Floc'H et al., 2014), such as fasting increases villus height to increase nutrient absorption (Wang et al., 2021). But, fasting decreased the AME and AMEn in adult roosters. This means that EEL plays an important role in this difference. Most work has shown that fasting destroys intestinal villi and shorten villi height in adult

animals (Yamauchi et al., 1996; Song et al., 2009), but fasting promotes the growth of intestinal villi in growing animals (Thompson and Applegate, 2006; Gilani et al., 2018). The destroyed villi partly influenced the EEL and thus the AME and AMEn. However, no statistical difference in TME and TMEn was observed in adult roosters. Likewise, previous findings on adult White Leghorn roosters starved for 24 h or 48 h recorded no effect on TME (Shires et al., 1979). ME in all forms were increased with age, which aligns well with previous works performed by Yang et al. (2020) and Dale et al. (1980). This can be explained by progressive improvement in the digestive system in birds with age (Yang et al., 2020). In summary, ME in broilers is more susceptible to fasting than in adult roosters.

Total tract digestibility was studied to explore the underlying reason which could influence energy utilization. In this study, decreased total tract digestibility of nitrogen was noted on RFD1 and RFD2 of refed broilers, which is further evidenced by the values of total tract digestibility of nitrogen and nitrogen retention ratio calculated by TCM. Consistently, previous work also found fasting decreased the protein digestibility in broilers (Peron et al., 2005). The decreased total tract digestibility of nitrogen upon fasting may be attributed to the fasting-induced upregulated oxidation of absorbed amino acid and increased uric acid output into the excreta (Wang et al., 2021). What's more, increased feed intake also promoted nitrogen excretion (Wang et al., 2021), which partly explained the decreased digestibility of nitrogen. Intriguingly, fasting increased nitrogen digestibility on RFD1 and RFD2 in roosters. Adult roosters have zero nitrogen balance, while fasted adult roosters showed increased total tract digestibility of nitrogen. This increased total tract digestibility of nitrogen is possibly used to replenish the consumed protein during fasting. We observed that broilers had higher total tract digestibility of nitrogen than adult roosters, which can be explained by the fact that broilers grow faster and use more nitrogen for protein accretion, whereas adult roosters utilize nitrogen primarily for maintenance requirements.

In this study, roosters had a greater total tract digestibility of EE than broilers. Given that fat deposition increases with age (Sato et al., 2009), it could be possible that roosters have greater capability to absorb fatty acids than broilers. Besides, fasting increased the total tract digestibility of EE on RFD1 in broilers, which is consistent with the previous work that fasting increased the expression of the extracellular fatty acid-binding protein and transporters (Wang et al., 2021). However, no difference was found in roosters, which is possibly attributable to the already high total tract digestibility of EE that is, 95%. The high total tract digestibility of EE and the low total tract digestibility of nitrogen suggest that a high-fat diet could be an economical and environmentally friendly feed for adult roosters.

It can be further noted that fasting slightly decreased the total tract digestibility of total starch in broilers. The lower total tract digestibility of total starch in

broilers than adult roosters in this study may be explained by the lower monosaccharide transporter expression in broilers compared to adult roosters (Li et al., 2020). Similarly, decreased total tract digestibility of total starch was found in 20-day-old broilers that fasted for 16 h (Peron et al., 2005). Although fasting significantly reduces starch total tract digestibility in broilers, it was numerically negligible. In summary, total starch is almost digested thoroughly in both broilers and adult roosters.

Though it has been previously shown that the digestibility marker was controversial in recovery rate (Schaafstra et al., 2019), it was found that the values of GE and nitrogen obtained by IM in non-fasted broilers were very close to the results observed by TCM in this study. However, we found that the total tract digestibility of energy and nitrogen calculated by TCM was greater than results obtained by the IM in non-fasted adult roosters. As we know, adult rooster has a nearly zero nitrogen balance. Consistently, in this study, total tract digestibility of nitrogen obtained by IM was close to zero, which indicates that result obtained by IM is accurate for adult roosters. However, total tract digestibility of nitrogen observed by TCM was more than 15%, which implies that the TCM overestimate nitrogen utilization of adult roosters. Accordingly, GE observed by IM was reliable and total tract digestibility of GE observed by TCM was overvalued. These findings explained why the TME and total tract digestibility of GE obtained by TCM in adult roosters upon fasting were inconsistent with significant increased total tract digestibility of nitrogen observed by IM. Due to this, we speculate that the ME obtained from adult roosters calculated by TCM overestimate the nutritional value.

In conclusion, the GIT emptying time of free-feeding broilers and adult roosters is 12 and 24 h, respectively. It's better to collect excreta between 12−48 h and 24−60 h for EEL and ENL determination of broilers and adult roosters, respectively. Fasting increases TME of broilers mainly through improved EE absorption, even though it reduced total tract digestibility of nitrogen and total starch. Fasting increased total tract digestibility of nitrogen in adult roosters. Additionally, total tract digestibility obtained by IM is more accurate than TCM and the TCM may overestimate feed nutritional utilization.

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DISCLOSURES

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