JPSA
Japan Poultry Science Association

http://www.jstage.jst.go.jp/browse/jpsa
doi:10.2141/jpsa.2023032

Effects of Delaying Post-hatch Feeding on the Plasma Metabolites of Broiler Chickens Revealed by Targeted and Untargeted Metabolomics

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Exogenous nutrients are essential for body and skeletal muscle growth in newly hatched chicks, and delaying post-hatch feeding negatively affects body growth, meat yield, and meat quality. The aim of this study was to investigate the effects of delayed post-hatch feeding on the metabolic profiles of broiler chickens using a combination of targeted and untargeted metabolomics. Newly hatched chicks had either immediate free access to feed (freely fed chicks) or no access to feed from 0 to 2 days of age (delayed-fed chicks); both groups were subsequently provided feed *ad libitum* until 13 days of age. Untargeted metabolomic analysis was performed using gas chromatography-mass spectrometry, whereas targeted metabolomic analysis of amino acids was performed using high-performance liquid chromatography with *ortho*-phthalaldehyde derivatization. Delayed feeding increased the plasma levels of sucrose, maltose, serotonin, lactitol, gentiobiose, xylitol, threonic acid, and asparagine, and decreased the plasma levels of creatinine, aspartic acid, and glutamic acid. In addition, the digestibility of the nitrogen-free extract (starch and sugar) and the cecal butyric acid concentration increased in chicks subjected to delayed feeding. In contrast, delayed feeding did not affect muscle protein degradation or digestibility in chicks. Taken together, our results indicate that delaying feeding until 48 h post-hatch alters multiple metabolic pathways, which are accompanied by changes in intestinal carbohydrate digestion and cecal butyric acid content in broiler chickens.

Key words: broiler chickens, early nutrition, metabolites, metabolomics, neonatal chicks

J. Poult. Sci., 60: jpsa.2023032, 2023

Introduction

Exogenous nutrients are essential for body and skeletal muscle growth in newly hatched chicks[1–6]; however, in practice, hatching and transportation result in the delayed feeding of chicks by 10–60 h[2]. Such delays in post-hatch feeding negatively affect subsequent body growth, meat yields, and meat quality[3,5,7]. In a previous study, we demonstrated that delaying post-hatch feeding for the first 48 h increased lipid peroxidation levels and drip losses of the pectoralis major muscle of broiler chickens at 60 days old, thereby seriously impacting meat quality[7].

Mechanistically, delaying post-hatch feeding for the first 48 h depresses muscular satellite cell mitotic activity and expression of genes encoding growth hormone receptors[3]. Delayed feed-

Received: August 22, 2023, Accepted: October 30, 2023
Available online: December 22, 2023
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ing also alters the expression of genes associated with protein metabolism[8,9]; for example, *FBXO32* mRNA expression increased after 48 h of delayed feeding. In another previous study, we reported that 48 h of delayed feeding reduced the mRNA levels of *Cu/Zn-SOD* and *Mn-SOD* and consequently increased lipid peroxidation levels in the skeletal muscle and plasma of chicks at 13 days post hatch[10]. These results suggest that delaying post-hatch feeding may affect several metabolic pathways in chicks.

To evaluate this hypothesis, in the present study, we used gas chromatography-mass spectrometry (GC-MS)–based untargeted metabolomic analysis and high-performance liquid chromatography (HPLC) with *ortho*-phthalaldehyde derivatization and fluorescence detection for targeted metabolomic analysis of amino acids. These findings can help to clarify the effects of delaying post-hatch feeding for the first 48 h on the metabolic pathways of chicks 13 days post hatch when increased lipid peroxidation levels were observed[10].

Materials and Methods

Animal experiment

Experimental protocols and procedures were reviewed and approved by the Animal Care and Use Committee of Kagoshima University (Kagoshima, Japan; approval number: A16010). Twelve newly hatched male Ross308 broiler chicks (*Gallus gallus domesticus*) that had not been fed were supplied by a commercial hatchery (Kumiai Hina Center, Kagoshima, Japan) within 24 h of hatching. The chicks were randomly divided into two groups. One group was fed the experimental diet *ad libitum* (freely fed chicks), whereas the other group was fasted for an additional 48 h (delayed-fed chicks). After 48 h, both groups were provided *ad libitum* access to the experimental diet until 13 days of age. Six freely fed and delayed-fed chicks were housed in one cage until 3 days of age, and then two chicks of each group were housed in one cage until 8 days of age. Subsequently, the chicks were reared individually in stainless steel cages from 8 to 13 days of age. All chicks were kept in a thermo-regulated room (30°C, 60% humidity, and 24 h continuous light) with an electric heater as necessary.

The formulations and nutrient compositions of the experimental diets are listed in Table 1. The crude protein content of the experimental diet was determined using an NC analyzer (JM1000CN, J-Science Lab Co., Ltd., Kyoto, Japan), and its metabolized energy was calculated using the Standard Tables of Feed Composition in Japan[11]. Chromic oxide was added to the diets as an indigestible marker from 8 days of age to enable the determination of digestibility. Excreta samples were collected from each cage during the last 3 days of the experiment.

After measuring body weight, all chicks were sacrificed by cervical dislocation following carbon dioxide anesthesia. After dissection, the pectoralis major muscles were weighed, snap-frozen in liquid nitrogen, and stored at −80°C until analysis. The cecal content was collected to determine the organic acid contents, which was snap-frozen in liquid nitrogen and stored at −80°C until analysis. Blood samples were collected in heparinized test tubes, immediately centrifuged (5,900 ×*g*, 10 min, 4°C) to separate the plasma, and stored at −80°C until analysis.

Table 1. **Composition of the experimental diet.**

Ingredients	Amount (g/100 g
Corn meal	48.36
Soybean meal	41.87
Corn oil	5.71
$CaCO_3$	0.66
$CaHPO_4$	1.99
NaCl	0.50
DL-Methionine	0.11
Mineral and vitamin premix[a]	0.50
Chromium oxide	0.30
Crude protein (%)[b]	22.80
Metabolizable energy (MJ/kg)[c]	13.00

[a] Content per kilogram of the vitamin and mineral premix: vitamin A, 90 mg; vitamin D3, 1 mg; DL-alpha-tocopherol acetate, 2000 mg; vitamin K3, 229 mg; thiamin nitrate, 444 mg; riboflavin, 720 mg; calcium D-pantothenate, 2174 mg; nicotinamide, 7000 mg; pyridoxine hydrochloride, 700 mg; biotin, 30 mg; folic acid, 110 mg; cyanocobalamin, 2 mg; calcium iodinate, 108 mg; MgO, 198,991 mg; $MnSO_4$, 32,985 mg; $ZnSO_4$, 19,753 mg; $FeSO_4$, 43,523 mg; $CuSO_4$, 4,019 mg; and choline chloride, 299,608 mg.
[b] Actual measurement value determined using an NC analyzer.
[c] Calculated value using the Standard Tables of Feed Composition in Japan[11].

Sample preparation for GC-MS analysis

Metabolomic analysis was performed using GC-MS as previously described[12], with some modifications. Fifty microliters of plasma was suspended in 250 µL of methanol:chloroform:water (5:2:2) and 5 µL of 1 mg/mL 2-isopropylmalic acid as an internal standard. Samples were mixed in a shaker at 1,200 rpm at 37°C for 30 min and then centrifuged at 16,000 ×*g* at 4°C for 5 min. Next, 225 µL of the supernatant was vortex-mixed with 200 µL of distilled water, followed by centrifugation at 16,000 ×*g* at 4°C for 5 min. Subsequently, 250 µL of the supernatant was dried under a vacuum using a centrifugal evaporator (RD-400; Yamato Scientific, Tokyo, Japan) after cooling at –80 °C for 10 min. Methoxyamine hydrochloride in pyridine (20 mg/mL, 40 µL) was then added to the tubes, which were vortex-mixed and then shaken at 1,200 ×*g* at 30°C for 90 min in the dark for oximation. N-methyl-N-trimethylsilyltrifluoroacetamine (20 µL) was then added to each tube and the contents were vortex-mixed. To prepare trimethylsilyl derivatives, the tubes were shaken at 1,200 ×*g* at 37°C for 45 min in the dark.

GC-MS analysis

GC-MS was performed as previously described[13] using a QP2010Ultra system (Shimadzu Corporation, Kyoto, Japan) with a DF:0.25-mm InertCap 5MS/NP column (30 m × 0.25 mm internal diameter; GL-Science, Tokyo, Japan). The inlet temperature was 230°C and the column flow rate was 1.12 mL/min. Helium was used as the carrier gas. The column temperature was main-

tained at 80°C for 2 min, increased to 320°C at a rate of 15°C/min, and held for 6 min. The transfer line and source temperatures were 250°C and 200°C, respectively. Electron ionization was performed at 70 V. Twenty scans per second were recorded over a mass range of 85–500 m/z. In addition, a standard alkane mixture (C9-C40) was injected through the column before sampling. The retention time data for each peak in the mixture were used as a reference for tentative identification.

Data processing

The GC-MS data were exported in net CDF format and converted to ABF format, and peak detection and alignment were performed using MS-DIAL software version 5.05[14]. Raw peak extraction, baseline filtering, calibration of the baseline data, peak alignment, deconvolution analysis, peak identification, and integration of peak heights were performed. To minimize the number of missing values, peaks with similarity of >70% and a retention index within ±10% were accepted via comparison with the compound database (GL-Science DB InertCap 5MS-NP, Kovats RI, 494 records) available from PRIMe (http://prime.psc.riken.jp/). Metabolite levels were semi-quantified using the peak area of each metabolite relative to that of the internal standard (2-isopropylmalic acid). The level of each metabolite in control cells was set to 100. The levels of each metabolite are shown as ratios derived from comparisons with the control group.

Determination of free amino acid concentrations

Analysis of free amino acid concentrations in plasma samples from chickens was performed using a pre-column technique with HPLC according to a previously reported method[15]. The analysis was performed with the NexeraX2 HPLC system (Shimadzu Co., Ltd.) and a Kinetex 2.6 µm column (EVO C18; 100 × 3.0 mm) according to the manufacturer instructions. A gradient elution program was used with two mobile phases. Mobile phase A comprised 17 mM potassium dihydrogen phosphate and 3 mM dipotassium hydrogen phosphate, whereas mobile phase B was prepared using a mixed solution of water:acetonitrile:methanol (15:45:40). Pre-column derivatization was performed by the HPLC system with 45 µL, 22 µL, and 7.5 µL of mercaptopropionic acid, *ortho*-phthalaldehyde, and samples, respectively. The mixture was incubated for 2 min, mixed with 5 µL of fluorenylmethyl chloroformate, and left to react for 2 min. Finally, 1 µL of the mixture was injected into the column. The flow rate and column temperature were set 0.85 mL/min and 35°C, respectively. The RF-20Axs high-sensitivity fluorescence detector was set to excitation at 350 nm/emission at 450 nm in channel 1 and excitation at 266 nm/emission at 305 nm in channel 2.

A total of 20 compounds were measured in the analysis, including the following basic amino acids and associated molecules: arginine, asparagine, aspartic acid, cystine, glutamic acid, glutamine, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, serine, threonine, tryptophan, tyrosine, valine, and N^{τ}-methylhistidine. The concentrations of the amino acids in the plasma are expressed in units of µmol/L.

Digestibility

The dietary and fecal amounts of moisture, crude protein, ether extract, crude fiber, crude ash, nitrogen-free extract, neutral detergent fiber, acid detergent fiber, and chromic oxide were measured according to Association of Official Analytical Chemists procedures[16].

Determination of organic acids in the cecal content

The amount of specific organic acids (lactic, acetic, propionic, butyric, isobutyric, isovaleric, and valeric organic acids) present in the cecal content was measured using an HPLC system (Jasco, Tokyo, Japan) according to the method described by Miwa et al.[17]. The ultraviolet-visible detector (UV-2075 PLUS, Jasco) was set to 400 nm and a YMC-Pack FA column (6 × 250 mm; YMC Co., Ltd., Kyoto, Japan) was used with the column oven (CO-2065 PLUS) heated to 50°C. The mobile phase consisted of acetonitrile:methanol:water (30:16:54, v/v; pH 4.5) at a flow rate of 1.2 mL/min. Organic acids were labeled using a Short and Long-Chain Fatty Acid Analysis Kit (YMC) according to the manufacturer's instructions.

Statistical analysis

Data are presented as the mean ± standard error of the mean. Student's t-test was used to compare the means of two groups using R software[18]. Statistical significance was set at $P < 0.05$ for all comparisons.

To explore the metabolic pathways affected by delayed feeding, quantitative pathway analysis was performed using the pathway-associated metabolite sets of MetaboAnalyst 5.0[14], an established form of metabolite set pathway analysis, using plasma metabolite concentrations obtained via non-targeted analysis. Differences were considered statistically significant at $P < 0.05$.

Results

The freely fed chicks gained 17.65 ± 1.80 g in body weight from 0 to 2 days of age, whereas the body weight of the delayed-fed chicks slightly decreased during this period. The body weight gain in the delayed chicks was significantly lower than that in the freely fed chicks during this period (Table 2). From 2 days onward, although the delayed-fed chicks were fed the experimental diet *ad libitum* and gained weight, their final body weight was lower than that of the age-matched freely fed chicks at 13 days of age. However, the net body weight gain of the delayed-fed chicks did not differ from that of the freely fed chicks from 2 to 13 days of age. The feed intake of the delayed-fed chicks tended to be lower ($P = 0.08$) than that of the freely fed chicks from 8 to 13 days of age. The skeletal muscle weights (pectoralis major muscle and thigh muscles) of the delayed-fed chicks were also lower than those of the freely fed chicks at 13 days of age (Table 2). However, the heart and liver weights did not differ between the two groups.

Untargeted GC-MS–based metabolomic analysis identified 99 metabolites in the plasma of chicks (Table 3 and Table S1). Of these metabolites, eight were significantly affected by delayed feeding: sucrose, maltose, serotonin, lactitol, gentiobiose, xylitol, and threonic acid increased, whereas creatinine decreased in the plasma of chicks subjected to delayed feeding. Table 4 shows the plasma concentrations of free amino acids in chicks. The plasma

Table 2. **Effects of a delayed feeding on growth performance and tissue weights of broiler chickens.**

Metric	Control	Delayed feeding
Growth performance		
Initial body weight (g)	44.50 ± 0.71	45.78 ± 1.05
Final body weight (g)	333.52 ± 7.85	305.43 ± 4.68*
Body weight gain (g/day 0 to day 2)	17.65 ± 1.80	-2.65 ± 0.21*
Body weight gain (g/day 2 to day 13)	271.37 ± 8.10	262.30 ± 4.34
Feed intake (g/day 0 to day 2)	12.23	–
Feed intake (g/day 2 to day 8)	134.99	104.64
Feed intake (g/day 8 to day 13)	91.00 ± 4.55	80.17 ± 3.05
Tissue weights		
Pectoralis major muscle (g)	37.33 ± 1.52	28.65 ± 1.45*
Thigh muscles (g)	28.91 ± 0.67	26.01 ± 0.94*
Heart (g)	1.94 ± 0.12	1.88 ± 0.09
Liver (g)	8.20 ± 0.37	7.83 ± 0.26

Results are expressed as mean ± standard error of the mean (SEM) (n = 6)
* $P < 0.05$ (vs. control).

Table 3. **Effects of delayed feeding on plasma metabolites of broiler chickens (% of control).**

Metabolite	Control	Delayed feeding
Sucrose	100.00 ± 43.33	9.19 ± 5.55*
Maltose	100.00 ± 41.74	10.38 ± 3.22*
Serotonin	100.00 ± 23.13	17.52 ± 4.59*
Lactitol	100.00 ± 29.64	36.73 ± 5.87*
Gentiobiose	100.00 ± 27.78	40.00 ± 7.36*
Xylitol	100.00 ± 9.52	71.01 ± 4.13*
Threonic acid	100.00 ± 3.85	86.58 ± 4.52*
Creatinine	100.00 ± 26.67	168.52 ± 9.64*

Results are expressed as mean ± standard error of the mean (SEM) (n = 6).
* $P < 0.05$ (vs. control)

concentrations of aspartic acid and glutamic acid in the delayed-fed chicks were significantly lower than those in the freely fed chicks, whereas the plasma concentration of asparagine was higher in the delayed-fed chicks. Other amino acids measured in this study did not differ between the two groups of chicks.

Metabolic pathway analysis indicated that delayed feeding affected the following 14 metabolic pathways in the plasma: tryptophan metabolism; starch and sucrose metabolism; D-glutamine and D-glutamate metabolism; glutathione metabolism; butanoate metabolism; porphyrin and chlorophyll metabolism; nitrogen metabolism; histidine metabolism; galactose metabolism; aminoacyl-tRNA biosynthesis; β-alanine metabolism; nicotinate and nicotinamide metabolism; pantothenate and coenzyme A biosynthesis; and alanine, aspartate, and glutamate metabolism (Table 5).

Delayed feeding had no significant effect on the digestibility of crude protein, ether extract, or crude ash, but tended to reduce the digestibility of crude fiber ($P = 0.07$), neutral detergent fiber ($P = 0.06$), and acid detergent fiber ($P = 0.13$) (Table 6). In contrast, delayed feeding significantly increased the digestibility of the nitrogen-free extract.

Table 7 shows the organic acid concentrations in the cecal contents of chicks. Delayed feeding significantly increased acetic and butyric acid concentrations. Although delayed feeding did not affect the concentrations of lactic acid, propionic acid, isobutyric acid, isovaleric acid, or valeric acid, the total organic acid concentration in the cecal contents increased.

Discussion

We confirmed that delaying post-hatch feeding for 48 h negatively affected body and skeletal muscle growth in chicks (Table 2). This result agreed with previous studies showing that a 48 h delay in feeding decreased body weight compared with that of age-matched, freely fed control chicks at 14 days of age[3,7]. Moreover, in this study, we evaluated the plasma metabolites after delaying post-hatch feeding in broiler chickens for the first

Table 4. **Effects of delayed feeding on plasma amino acids concentrations of broiler chickens (μmol/L).**

Amino acids	Control	Delayed feeding
Arginine	24.52 ± 1.52	26.11 ± 1.09
Asparagine	2.93 ± 0.49	4.53 ± 0.43*
Aspartic acid	25.21 ± 2.59	17.64 ± 1.17*
Cystine	6.09 ± 0.76	7.96 ± 1.23
Glutamic acid	5.22 ± 0.44	3.90 ± 0.13*
Glutamine	330.26 ± 18.16	338.71 ± 35.49
Glycine	43.97 ± 1.68	38.71 ± 3.02
Histidine	2.95 ± 0.29	3.56 ± 0.58
Isoleucine	7.84 ± 0.86	7.58 ± 0.42
Leucine	13.43 ± 1.12	12.80 ± 0.52
Lysine	24.53 ± 3.70	21.44 ± 3.08
Methionine	3.78 ± 0.34	4.59 ± 0.50
Phenylalanine	8.28 ± 0.40	8.16 ± 0.37
Proline	1.93 ± 0.38	3.15 ± 0.61
Serine	39.83 ± 1.70	37.02 ± 3.81
Threonine	35.96 ± 2.80	35.72 ± 3.84
Tryptophan	3.41 ± 0.24	3.20 ± 0.14
Tyrosine	8.81 ± 0.47	9.92 ± 0.37
Valine	15.97 ± 1.51	15.54 ± 1.23
N^{τ}-methylhisitidine	24.30 ± 1.67	23.07 ± 0.89

Results are expressed as mean ± standard error of the mean (SEM) (n = 6)
*P < 0.05 (vs. control).

Table 5. **Effects of delayed feeding on metabolisms of broiler chickens.**

Metabolism pathway	P-value
Tryptophan metabolism	<0.01
D-Glutamine and D-glutamate metabolism	<0.05
Glutathione metabolism	<0.05
Butanoate metabolism	<0.05
Porphyrin and chlorophyll metabolism	<0.05
Nitrogen metabolism	<0.05
Alanine, aspartate and glutamate metabolism	<0.05
Aminoacyl-tRNA biosynthesis	<0.05
Histidine metabolism	<0.05
β-Alanine metabolism	<0.05
Nicotinate and nicotinamide metabolism	<0.05
Pantothenate and CoA biosynthesis	<0.05
Starch and sucrose metabolism	<0.05
Galactose metabolism	<0.05

time. We identified 99 metabolites in the plasma of chicks (Tables 3 and S1). The number of identified metabolites was comparable to that reported in a previous study, in which Tomonaga *et al.*[19] identified 92 metabolites in the plasma of chickens using GC-MS–based non-targeted metabolomic analysis. Therefore, the results obtained in this study are reliable.

Pathway analysis of the metabolomic data revealed that delayed feeding induced changes in 14 plasma metabolic pathways (Table 5). Among these, 11 pathways were related to amino acid or nitrogen metabolism, suggesting that protein metabolism might be influenced in delayed-fed chicks. However, it has previously been reported that protein synthesis does not differ between delayed-fed chicks and age-matched control chicks when they are fed freely[5]. In addition, the plasma N^{τ}-methylhisitidine

Table 6. **Effects of delayed feeding on the feed digestibility of broiler chickens (%).**

Component	Control	Delayed feeding
Crude protein	85.62 ± 0.36	85.46 ± 0.31
Ether extract	92.93 ± 1.05	93.92 ± 0.51
Crude fiber	66.18 ± 2.09	53.41 ± 6.38
Crude ash	72.25 ± 0.33	72.24 ± 0.63
Nitrogen-free extract	94.05 ± 0.49	95.02 ± 0.57*
Neutral detergent fiber	81.68 ± 2.19	80.27 ± 1.68
Acid detergent fiber	69.35 ± 2.13	61.49 ± 4.66

Results are expressed as mean ± standard error of the mean (SEM) (n = 6).
* $P < 0.05$ (vs. control).

Table 7. **Effects of delayed feeding on cecal organic acid contents of chicks (nmol/g cecal content).**

Organic acids	Control	Delayed feeding
Lactic acid	12.14 ± 2.07	19.44 ± 3.91
Acetic acid	166.48 ± 22.44	234.41 ± 5.16*
Propionic acid	16.45 ± 1.17	18.33 ± 2.68
Isobutyric acid	1.79 ± 0.55	3.28 ± 0.09
Butyric acid	80.54 ± 16.90	162.40 ± 8.55*
Isovaleric acid	2.48 ± 0.13	4.19 ± 0.30
Valeric acid	2.35 ± 0.31	4.19 ± 1.72

Values are means ± standard error of the mean (n = 6).
*$P < 0.05$ (vs. control).

concentration, which is an index of muscle protein degradation[20,21], did not differ between these two groups of chicks. Therefore, delayed feeding did not affect protein synthesis or degradation levels. Body weight gain from 2 to 13 days of age, during which both groups of chicks were given access to food *ad libitum*, did not differ between the two groups. The protein digestibility of delayed-fed chicks did not differ from that of freely fed chicks (Table 6). Furthermore, in our preliminary experiment, we confirmed that the delayed-fed chicks at 15 days of age did not differ in body weight from freely fed chicks at 13 days of age (unpublished data). Taken together, these results suggest that differences in plasma amino acid concentrations do not contribute to changes in protein turnover in chicks.

Metabolic pathway analysis revealed that delayed feeding resulted in changes in starch, sucrose, and galactose metabolism, which are all pathways related to carbohydrate metabolism. Specifically, the GC-MS–based metabolomic analysis indicated that the plasma levels of sucrose, maltose, lactitol, gentiobiose, xylitol, and threonic acid (a derivative of threose) were lower in delayed-fed chicks than in freely fed chicks. As chickens lack the ability to synthesize these metabolites, these metabolic pathways may not be altered in chicks *in vivo*. Alternatively, delaying feeding for 48 h was reported to affect the intestinal flora of chicks[22,23], suggesting that the production of these metabolites by intestinal bacteria might change. Furthermore, this may indicate that intestinal absorption changed because delayed feeding significantly increased the digestibility of the nitrogen-free extract. This suggests that delaying post-hatch feeding improves intestinal carbohydrate digestion. However, Corless and Sell[24] reported that delayed feeding resulted in decreased pancreatic amylase activity in chicks. Furthermore, the effects of delayed feeding on chick intestinal morphology remain controversial. It has been reported that delaying post-hatch feeding did not affect villus height and crypt depth in the duodenum and ileum at 7 days old[25]. In contrast, Li *et al.*[22] showed that delayed feeding for 48 h significantly decreased villus height and the villus height-to-crypt depth ratio and increased the crypt depth in the duodenum at 50 days of age. Therefore, the relationship between delayed post-hatch feeding and intestinal digestion remains unclear. Although the biological implications of sucrose and maltose in chick plasma are unclear, the partial absorption of maltose and sucrose in the small intestine has been reported, resulting in detectable plasma levels[26]. Additionally, the mRNA expression and catalytic activity of maltase and sucrase are predominantly found in enterocytes; however, they are also found in other non-intestinal tissues (i.e., blood leukocytes and kidneys) in mice[27]. Therefore, the results of these studies, coupled with those of the present study, suggest that these starches or sugars are metabolized in cells that are not of gastrointestinal origin in broiler chickens.

Metabolic pathway analysis also revealed a change in the butanoate metabolism pathway in chicks subjected to delayed

post-hatch feeding. In addition, we found that delayed feeding increased the concentrations of acetic and butyric acids in the cecal contents of chicks (Table 7). It has been shown that delaying feeding by 48 h affects the intestinal flora of chicks[22,23]. These results suggest that delayed feeding may affect the intestinal flora of chicks and consequently increase cecal organic acids. Although delayed feeding increased nitrogen-free extract digestibility, it appeared to decrease the fiber digestibility of the chicks. Therefore, the source of increased organic acid content remains unclear. Because delayed feeding decreases the pancreatic amylase activity of chicks[24], some starch may escape digestion by the host and would consequently become available for utilization by intestinal bacteria.

In our previous studies, delayed feeding for 48 h resulted in the reduced expression of *Cu/Zn-SOD* and *Mn-SOD* mRNA levels, which also resulted in increased plasma and muscle lipid peroxidation[7,10]. In this study, the metabolic pathway analysis of the metabolomic data indicated that delayed feeding resulted in changes in glutathione metabolism, a pathway that plays an important role in antioxidant defense[28]. Taken together, these results suggest that delayed feeding enhances oxidative stress levels in chicks during the subsequent growth period. Furthermore, GC-MS–based metabolomic analysis indicated that delayed feeding affected tryptophan metabolism in chicks. The metabolism of tryptophan and its derivatives is important for the prevention of oxidative stress. For example, 5-hydroxytryptophan, a derivative of tryptophan, improves the antioxidant capacity of chickens[29]. In this study, although the GC-MS–based metabolomic analysis did not detect plasma 5-hydroxytryptophan, the plasma level of serotonin, a derivative of 5-hydroxytryptophan, was lower in delayed-fed chicks than in free-fed chicks. In agreement with this result, chickens exhibit decreased serum serotonin concentrations under oxidative stress[30]. Furthermore, under oxidative stress conditions, chickens have increased serum creatinine concentrations, which is a marker of impaired renal function or oxidative stress[31,32]. In the present study, we found that plasma creatinine levels increased in delayed-fed chicks, further indicating the possibility of oxidative stress. Moreover, in a non-obese diabetic mouse model[33], higher oxidative stress levels were accompanied by lower plasma levels of threonic acid, a breakdown product of ascorbic acid[34]. Our GC-MS–based metabolomic analysis indicated that the delayed-fed chicks had lower plasma levels of threonic acid. These results suggest that delayed feeding affects multiple metabolic pathways and metabolites involved in the regulation of oxidative stress in chickens.

In conclusion, delaying feeding for 48 h in post-hatch chicks resulted in changes in multiple metabolites related to amino acid, nitrogen, carbohydrate, and butanoate metabolism, accompanied by changes in intestinal carbohydrate digestion and cecal acetic acid and butyric acid content in broiler chickens.

Acknowledgments

We would like to thank Kagoshima Chicken Foods Company, Ltd. (Kagoshima, Japan) for supplying the chicks used in this study. We thank Wendy Hempstock, Ph.D., from Edanz (https://jp.edanz.com/ac) for editing the draft of this manuscript. This study was supported by a Grant-in-Aid for Scientific Research (C) (No. 19K06357) from The Japan Society for the Promotion of Science (JSPS) to D. Ijiri.

Author Contributions

Conceptualization, S. S., K. N., A. O., and D. I.; methodology, D. I. and S. T.; software, S. T. and D. I.; validation, A. K., S. S., and S. T.; formal analysis, A. K., S. S., and M. K.; investigation, A. K., S. S., and M. K.; statistical analysis, A. K. and S. I.; original draft preparation, A. K. and D. I.; manuscript review and editing, S. S., S. T., K. N., S. I., A. O., and D. I.; supervision, A. O.; project administration, D. I. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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