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Sensitivity of broiler performance, organ weights and plasma constituents to amino acid supplementation and reused litter exposure using ideal protein-formulated rations

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ABSTRACT

Effects of amino acid supplementation to ideal protein (**IP**) formulated rations were investigated on growth performance, plasma metabolites and organ weights of broilers placed on 100% recycled (reused) litter. Day-old Ross308 male broilers were raised on either clean or reused litter and fed for three weeks on one of five isoenergetic diets, where an IP-based control diet (**C**) was compared with diets containing threonine (**T**) or arginine (**A**) at 25% above requirements, or with 1% supplemented glutamine (**G**), or with each amino acid added (**TAG**). Litter and diet treatments did not strongly interact on outcomes. Reused litter placement resulted in greater weight gain, smaller feed conversion ratio and heavier bursal weights ($P < 0.05$) compared to clean litter placement. Relative to C and T birds, TAG birds reduced weight gain and feed intake ($P < 0.05$). Plasma uric acid levels in G birds were greater than in C, T and A birds ($P < 0.001$). Collectively, since the outcomes of placement on reused litter increased performance and the control diet was IP formulated, the absence of increased growth performance in response to amino acid supplementation would be consistent with amino acids tested being excess to requirements.

Implications

This manuscript explores the sensitivity of using the ideal protein concept to reused litter exposure as a model to mimic litter-borne microbiome exposure in the poultry industry. Our findings confirm that the ideal protein concept remains an appropriate basis for an effective feeding strategy, with no extra benefit from selected amino acid supplementation as reused litter exposure increased performance. Thus, our study supports the view that multiple benefits can arise from reused litter usage, including increased feed and non-feed resource use efficiency, both contributing to reducing environmental footprint, which will be of global interest including Europe.

Introduction

Many studies have shown that supplementing broiler rations with selected essential amino acids (**AAs**) enhance performance, including for threonine (Min et al., 2017; Ji et al., 2019; Ahmed et al., 2020) and arginine (Ebrahimi et al., 2014; Pirsaraei et al., 2018; Omidi et al., 2020), but also for the non-essential AA glutamine (Nassiri Moghaddam and Alizadeh-Ghamsari, 2013; Ribeiro et al., 2015; Wu et al., 2021), suggesting that the availability of these AAs for growth performance was reduced from the control rations used. To minimise excess protein supply and excessive N excretion, the ideal protein (**IP**) concept has been introduced in diet formulations (Baker, 2003; Miles and Chapman, 2007; McGill et al., 2012). A diet is formulated to IP if it provides digestible AA in the ratios healthy birds require for maintenance and production (Deschepper and de Groote, 1995). Consequently, increased performance would not be expected from AA supplementation to an IP-formulated baseline. However, selected AA supplementation resulted in increased resilience and immune responses when birds were under a wide range of sub-clinical enteric health challenges, including sub-clinical coccidiosis, necrotic enteritis, and exposure to reused litter (Star et al., 2012; Keerqin et al., 2017; Bortoluzzi et al., 2020). These studies suggest that sub-clinical enteric microbial challenge results in different dietary AA ratios for optimal production and thus may influence the IP concept.

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Specific AAs are required for effective immune response, including lymphocyte proliferation and the synthesis of immunoglobulins, nitric oxide, and cytokines (Rubin et al., 2007). Among the essential AAs, threonine is required to synthesise mucin, which covers the intestinal mucosal surface (Ahmed et al., 2020), and threonine supplementation has resulted in increased antibody responses (Sigolo et al., 2017). During enteric disease challenges, reduced threonine availability may restrict mucin synthesis, impairing gut barrier function. It has been reported that threonine supplementation may enhance mucin synthesis and enhance mucosal integrity during enteric challenges (Faure et al., 2007; Mehdipour et al., 2020). Many studies have demonstrated that threonine supplementation can increase performance and gut health in broilers under enteric challenge (Kidd et al., 2003; Star et al., 2012; Mehdipour et al., 2020). However, others show no such effects (Chen et al., 2017), even in the presence of sub-clinical challenges (Corzo et al., 2007; Valizade et al., 2014).

Supplementing diets with arginine has increased broiler growth performance under commercial conditions, where sub-clinical challenge is assumed to be present to some degree (Montanhini Neto et al., 2013). In addition, supplementing diets with arginine may reduce intestinal permeability, indicating its protective role when intestinal inflammation and permeability are elevated (Barekatain et al., 2019; 2021). Moreover, arginine is essential for enterocyte growth and development (Rhoads et al., 2008) and is required to produce nitric oxide, a key component of innate immunity (Zhang et al., 2018). Whilst glutamine is known as non-essential AA, it has been considered a conditionally essential AA at times of stress and enteric challenges (Curi et al., 2007; Wang et al., 2009). Glutamine is the main energy source for immune and intestinal epithelial cells. It is also a precursor of N-acetyl-glucosamine and N-acetyl-galactosamine, major components of mucin (Coster et al., 2004). In addition, glutamine may enhance performance during sub-clinical challenges by acting on inflammatory processes and increasing the intestinal surface area and, thus, nutrient absorption (Xue et al., 2018; Oxford and Selvaraj, 2019).

Variability in response to AA supplementation likely has a multifactorial basis, including the level of sub-clinical challenge and deviation of baseline AA profile from IP. Here, we assessed the effects of individual or combined supplementation with threonine, arginine and glutamine and reused litter exposure on performance, blood biochemistry and organ weights in broilers where basal rations were formulated to IP. In a parallel paper, we have recently reported concurring responses in caecal microbiome parameters to link to the performance outcome for the combined AA supplementation and reused litter exposure (Hussein et al., 2023). Raising birds on reused litter may have variable outcomes on performance, as it has been associated with decreased (Khattak et al., 2019; González-Ortiz et al., 2021), similar (Yamak et al., 2016; Vieira and Moran, 1999) or increased (Garcés Gudiño et al., 2018) performance relative to birds raised on clean litter. Therefore, we hypothesised that the outcome of AA supplementation to IP-formulated basal rations is sensitive to the outcome of reused litter exposure, and that AA supplementation increases performance only when reused litter exposure reduces bird performance.

Material and methods

Housing and experimental design

A total of 800 one-day-old Ross 308 broiler male chickens were used in a 21-day experiment. Upon arrival, chicks were weighed and distributed to 80 experimental pens in a single animal room with 10 birds per pen and 8 pens per treatment. Stocking density, temperature and lighting schedule were maintained as described in Hussein et al. (2023). Birds were *ad libitum* fed and watered. Five dietary treatments and two litter treatments were combined in a 5×2 factorial arrangement. The resulting 10 treatment combinations were assigned within a complete randomised block design to one of 10 pens in 8 blocks to account for possible pen location effects within the single animal room used.

Diet treatments

The first diet treatment was the control (**C**), which was IP formulated and included crystalline lysine, methionine, threonine, tryptophan, arginine, valine, and isoleucine to meet all essential AA requirements on a digestible AA basis. The 2nd and the 3rd diet treatment consisted of additional threonine (**T**) and arginine (**A**), respectively, at 25% above requirements. The 4th diet treatment consisted of 1% glutamine supplementation (**G**). The 5th diet treatment combined additional threonine, arginine and glutamine at the aforementioned levels (**TAG**). The inclusion levels of T, A and G were informed by Corzo et al. (2007), Fasina et al. (2010) and Gottardo et al. (2016), respectively. The tested AAs were included in diet C at the expense of maize starch. Diets were calculated to be isoenergetic for apparent metabolisable energy and varying CP levels, as presented in Tables 1 and 2. The experimental diets were fed as mash during starter (days 0day –11) and grower (days 11day –21) phases and were formulated to Ross 308 recommendations (Aviagen, 2014).

Litter treatments

The litter within each pen was either unused wood shavings (clean litter) or with spent litter from a previous broiler study with no evidence of clinical disease (reused litter). The reused litter was stored in bags at room temperature for four weeks between collection and usage.

Chemical analysis of the diets

Representative samples of the experimental diets were analysed for DM, gross energy, CP, and all AA, as described elsewhere (Hussein et al., 2023). The analysed nutrient contents of the experimental diets were mostly consistent with the expected values (Tables 3 and 4), though analysed CP contents of T, A and G diets were, on average, ~1% smaller than calculated. However, the analysed individual AA contents generally accorded with the calculated figures.

Growth performance

Growth performance parameters, i.e., BW gain (**BWG**), feed intake (**FI**), and feed conversion ratio (**FCR**, calculated as FI over BWG), were calculated from mean BWs through bulk weighing and bird counting at pen level, and weights of feed offered on days 0 and 11, and feed refusals on days 11 and 21. The resulting BWG, FI and FCR were calculated for the periods of days 0–11 (starter phase), 11–21 (grower phase), and 0–21 (total trial). Dead or culled birds were weighed, and date recorded, to correct performance parameters for mortality.

Blood parameters and organ weights

Two birds per pen were selected randomly at trial end (day 21) in the morning, individually weighed, electrically stunned, and exsanguinated in the postmortem room (away from home cages) to collect 5 mL of blood in K-ethylenediaminetetraacetic acid-treated vacutainers. Plasma was separated through centrifugation at 2 000*g* for 15 min at room temperature and was stored at

Table 1
Feed ingredients and as fed calculated chemical compositions of the experimental starter rations fed to male broilers.

	Experimental starter rations				
	C[1]	T	A	G	TAG
Ingredients (%)					
Wheat	58.22	58.22	58.22	58.22	58.22
Maize starch	3.00	2.78	2.66	2.00	1.44
Soybean meal	31.59	31.59	31.59	31.59	31.59
Soya oil	2.20	2.20	2.20	2.20	2.20
Salt	0.05	0.05	0.05	0.05	0.05
Limestone	0.95	0.95	0.95	0.95	0.95
Dicalcium Phosphate	1.85	1.85	1.85	1.85	1.85
Sodium bicarbonate	0.50	0.50	0.50	0.50	0.50
L-Lysine HCl	0.39	0.39	0.39	0.39	0.39
DL-Methionine	0.24	0.24	0.24	0.24	0.24
L-Threonine	0.23	0.45	0.23	0.23	0.45
L-Valine	0.09	0.09	0.09	0.09	0.09
L-Tryptophan	0.14	0.14	0.14	0.14	0.14
L-Arginine	0.10	0.10	0.44	0.10	0.44
L-Isoleucine	0.05	0.05	0.05	0.05	0.05
L-Glutamine	0.00	0.00	0.00	1.00	1.00
Vitamin & Mineral premix[2]	0.40	0.40	0.40	0.40	0.40
Calculated chemical composition[3]					
DM (%)	88.80	88.80	88.80	88.80	88.80
CP (%)	22.47	22.63	23.16	23.67	24.51
Metabolisable energy (MJ/kg)	12.51	12.51	12.51	12.51	12.51
Ca (%)	0.96	0.96	0.96	0.96	0.96
P (%)	0.72	0.72	0.72	0.72	0.72
Available P %	0.48	0.48	0.48	0.48	0.48
Na (%)	0.19	0.19	0.19	0.19	0.19
Cl (%)	0.16	0.16	0.16	0.16	0.16
Digestible amino acids (%)					
Arginine	1.37	1.37	1.71	1.37	1.71
Histidine	0.48	0.48	0.48	0.48	0.48
Isoleucine	0.86	0.86	0.86	0.86	0.86
Leucine	1.4	1.4	1.4	1.4	1.4
Lysine	1.28	1.28	1.28	1.28	1.28
Methionine	0.51	0.51	0.51	0.51	0.51
Cysteine	0.31	0.31	0.31	0.31	0.31
Tryptophan	0.20	0.20	0.20	0.20	0.20
Threonine	0.86	1.08	1.08	0.86	1.08
Valine	0.96	0.96	0.96	0.96	0.96
Methionine + Cysteine	0.82	0.82	0.82	0.82	0.82
Phenylalanine + Tyrosine	1.58	1.58	1.58	1.58	1.58

[1] C, ideal protein-formulated control diet; T, threonine supplemented diet; A, arginine supplemented diet; G, glutamine supplemented diet; TAG, threonine, arginine, and glutamine supplemented diet.
[2] Vitamin and mineral premix provided (units kg^{-1} diets): vitamin A (retinyl acetate) 16 000 iu; vitamin D3 (cholecalciferol) 3 000 iu; vitamin E (dl-α-tocopherol acetate) 75 iu; vitamin B1 (thiamin) 3 mg; vitamin B2 (riboflavin) 10 mg; vitamin B6 (pyridoxine HCl) 3 mg; vitamin B12 (cyanocobalamin) 15 μg; phylloquinone 5 mg; nicotinic acid 60 mg; pantothenic acid 14.5 mg; folic acid 1.5 mg; biotin 275 μg; choline chloride 250 mg; iron 20 mg; copper 10 mg; manganese 100 mg; cobalt 1 mg; zinc 82 mg; iodine 1 mg; selenium 0.2 mg; molybdenum 0.5 mg.
[3] Derived from Premier Atlas data (2014).

−20 °C pending analysis. To underpin influences of reused litter exposure on performance and its possible modification through AA supplementation, plasma was analyzed for total protein, albumin, uric acid, triglycerides, total cholesterol, total bile acids, glucose, gamma-glutamyl transpeptidase, glutamate dehydrogenase, Na, K and Cl through an AU 5800 clinical chemistries analyzer (Beckman) at Synlab Vet GmbH (Geesthacht, Germany). Plasma globulin was calculated as the difference between the total protein and albumin. In addition, the gizzard with the intact yellow lining membrane, spleen and bursa were removed and weighed. The absolute organ weights were reported to give an indication of gut development (gizzard) and as associated with the immune system (spleen and bursa).

Litter analysis

Litter samples for both clean and reused litter treatments were collected at day 0 using sterilised gloves and plastic bags. The collected litter samples were mixed thoroughly in sterilised trays, packed inside sealed plastic bags, and kept at −80 °C pending pH, moisture content and microbiological analysis.

Litter pH and moisture

Litter samples were defrosted, spread onto a clean sheet of plastic, mixed thoroughly and sub-sampled in duplicate for pH and moisture content analysis, as described elsewhere (Hussein et al., 2023). Litter moisture was then calculated from the sample start and end weight difference. The resulting moisture content of the clean and reused litter at day 0 (±SE) was 10.8 ± 2.3% and 12.5 ± 2.3%, respectively, and had a pH of 5.8 ± 0.1 and 8.1 ± 0.1, respectively.

Microbiological analysis for bacterial detection in collected litter samples

Litter samples at day 0 were tested in duplicate for the presence of *Escherichia coli* and *Campylobacter jejuni* using the culturing method. Strains of *E. coli* and *C. jejuni* used as positive controls were retrieved from frozen beads and stored at −80 °C at SAC Veterinary Services, Auchincruive, UK.

Table 2
Feed ingredients and as fed calculated chemical compositions of the experimental grower rations fed to male broilers.

	Experimental grower rations				
	C[1]	T	A	G	TAG
Ingredients (%)					
Wheat	60.56	60.56	60.56	60.56	60.56
Maize starch	3.00	2.81	2.70	2.00	1.51
Soybean meal	28.50	28.50	28.50	28.50	28.50
Soya oil	3.50	3.50	3.50	3.50	3.50
Salt	0.05	0.05	0.05	0.05	0.05
Limestone	0.87	0.87	0.87	0.87	0.87
Dicalcium Phosphate	1.65	1.65	1.65	1.65	1.65
Sodium bicarbonate	0.50	0.50	0.50	0.50	0.50
L-Lysine HCl	0.32	0.32	0.32	0.32	0.32
DL-Methionine	0.21	0.21	0.21	0.21	0.21
L-Threonine	0.18	0.37	0.18	0.18	0.37
L-Valine	0.06	0.06	0.06	0.06	0.06
L-Tryptophan	0.12	0.12	0.12	0.12	0.12
L-Arginine	0.05	0.05	0.35	0.05	0.35
L-Isoleucine	0.03	0.03	0.03	0.03	0.03
L-Glutamine	0.00	0.00	0.00	1.00	1.00
Vitamin & Mineral premix[2]	0.40	0.40	0.40	0.40	0.40
Calculated chemical composition[3]					
DM (%)	88.85	88.85	88.85	88.85	88.85
CP (%)	21.01	21.15	21.61	22.21	22.95
Metabolisable energy (MJ/kg)	12.94	12.94	12.94	12.94	12.90
Ca (%)	0.87	0.87	0.87	0.87	0.87
P (%)	0.67	0.67	0.67	0.67	0.67
Available P (%)	0.44	0.44	0.44	0.44	0.44
Na (%)	0.19	0.19	0.19	0.19	0.19
Cl (%)	0.15	0.15	0.15	0.15	0.15
Digestible amino acids (%)					
Arginine	1.23	1.23	1.53	1.23	1.53
Histidine	0.45	0.45	0.45	0.45	0.45
Isoleucine	0.78	0.78	0.78	0.78	0.78
Leucine	1.31	1.31	1.31	1.31	1.31
Lysine	1.15	1.15	1.15	1.15	1.15
Methionine	0.47	0.47	0.47	0.47	0.47
Cysteine	0.30	0.30	0.30	0.30	0.30
Tryptophan	0.19	0.19	0.19	0.19	0.19
Threonine	0.77	0.96	0.77	0.77	0.96
Valine	0.87	0.87	0.87	0.87	0.87
Methionine + Cysteine	0.77	0.77	0.77	0.77	0.77
Phenylalanine + Tyrosine	1.48	1.48	1.48	1.48	1.48

[1] C, ideal protein-formulated control diet; T, threonine supplemented diet; A, arginine supplemented diet; G, glutamine supplemented diet; TAG, threonine, arginine, and glutamine supplemented diet.
[2] Vitamin and mineral premix provided (units kg^{-1} diets): vitamin A (retinyl acetate) 16 000 iu; vitamin D3 (cholecalciferol) 3 000 iu; vitamin E (dl-α-tocopherol acetate) 75 iu; vitamin B1 (thiamin) 3 mg; vitamin B2 (riboflavin) 10 mg; vitamin B6 (pyridoxine HCl) 3 mg; vitamin B12 (cyanocobalamin) 15 μg; phylloquinone 5 mg; nicotinic acid 60 mg; pantothenic acid 14.5 mg; folic acid 1.5 mg; biotin 275 μg; choline chloride 250 mg; iron 20 mg; copper 10 mg; manganese 100 mg; cobalt 1 mg; zinc 82 mg; iodine 1 mg; selenium 0.2 mg; molybdenum 0.5 mg.
[3] Derived from Premier Atlas data (2014).

A total of 10 g of litter samples were added to 50 mL sterile phosphate buffer saline in a sterilised stomacher bag and homogenised using a mechanical shaker for approx. 1 min. For *E. coli*, 10 μL of litter suspension were spot dropped onto MacConkey agar plates. The plates were aerobically incubated at 37 °C for 24 h. Isolated colonies were identified as *E. coli* by observing their cultural characterisation, gram's stain, oxidase test and biochemical reaction using indole test (Munoz et al., 2021), whereas, for *C. jejuni*, 100 μL of the litter mixture was applied onto charcoal cefoperazone deoxycholate agar plates (Thermo-Fisher Scientific) and spread evenly over the surface with a sterile spreader. Plates were incubated invertedly at 41.5 °C ± 2.0 °C in an atmosphere suitable for microaerophilic and capnophilic micro-organisms and examined after 48 hours of incubation. For confirmation, two colonies were picked up from each plate and sub-cultured onto blood agar plates (Oxoid PB0114) and incubated at 37 °C for 48 h, one plate aerobically and one plate microaerophilically. The presence of *C. jejuni* was indicated by a lack of growth aerobically and colonies with *C. jejuni* morphology that grew microaerophilically. Gram staining and oxidase strips (Oxoid Ltd., MB0266) were performed on all samples (Khattak et al., 2018). Both clean and reused litter were positive for oxidase-negative and indole-positive *E. coli* but negative for *C. jejuni*.

Statistical analysis

Data collected were analysed using the general ANOVA function of GenStat (16th Edition) and following the 5 × 2 factorial arrangement for diet treatments (C, T, A, G and TAG), litter treatments (clean vs reused) and their interaction, using pen location as a block and day 0 BW as a covariate for day 11 and day 21 BW. Prior to analysis, the data were checked for normality by examining residuals, histograms, and box plots. Data that were not normally distributed were log10 transformed prior to statistical analysis. Means presented without common superscript differed significantly as per Tukey's honest significance test at $P < 0.05$. Power analysis supported that at a 2% CV for BWG and FCR, typical for poultry trials at SRUC, Auchincruive, biologically meaningful differences of 3.5% in response to treatment were expected to be detected at $n = 8$.

Table 3
Analysed chemical composition, gross energy, and amino acids of the experimental starter diets fed to male broilers.

	Experimental starter rations				
	C[1]	T	A	G	TAG
Chemical composition (as fed)					
DM (%)	88.00	88.00	88.00	88.10	89.00
CP (%)	22.82	22.32	22.06	23.83	24.12
Gross energy (MJ/kg)	16.45	16.39	16.53	16.53	16.63
Amino acids (%)					
Methionine	0.55	0.52	0.50	0.51	0.50
Cysteine	0.36	0.37	0.34	0.37	0.36
Methionine + Cysteine	0.90	0.89	0.84	0.88	0.86
Lysine	1.45	1.43	1.26	1.39	1.31
Threonine	0.98	1.12	0.91	0.97	1.16
Arginine	1.48	1.56	1.64	1.50	1.76
Isoleucine	0.95	0.99	0.85	0.96	0.90
Leucine	1.55	1.63	1.38	1.57	1.49
Valine	1.10	1.13	0.98	1.10	1.02
Histidine	0.52	0.55	0.47	0.53	0.50
Phenylalanine	1.02	1.07	0.90	1.03	0.98
Glycine	0.87	0.93	0.78	0.89	0.85
Serine	1.04	1.09	0.92	1.06	1.02
Proline	1.33	1.38	1.22	1.37	1.33
Alanine	0.88	0.93	0.78	0.90	0.85
Aspartic acid	2.04	2.17	1.76	2.08	1.95
Glutamic acid	4.31	4.49	3.98	5.19	5.19

[1] C, ideal protein-formulated control diet; T, threonine supplemented diet; A, arginine supplemented diet; G, glutamine supplemented diet; TAG, threonine, arginine, and glutamine supplemented diet.

Table 4
Analysed chemical composition, gross energy, and amino acids of the experimental starter diets fed to male broilers.

	Experimental grower rations				
	C[1]	T	A	G	TAG
Chemical composition (as fed)					
DM (%)	88.30	88.60	88.40	88.20	88.30
CP (%)	21.14	20.29	20.56	20.51	23.21
Gross energy (MJ/kg)	16.77	16.75	16.68	16.49	16.77
Amino acids (%)					
Methionine	0.46	0.46	0.45	0.41	0.50
Cysteine	0.35	0.33	0.36	0.34	0.35
Methionine + Cysteine	0.81	0.79	0.81	0.74	0.85
Lysine	1.26	1.16	1.25	1.07	1.35
Threonine	0.87	1.03	0.87	0.79	1.10
Arginine	1.36	1.25	1.62	1.20	1.68
Isoleucine	0.88	0.81	0.89	0.78	0.90
Leucine	1.47	1.35	1.49	1.32	1.50
Valine	0.99	0.91	1.02	0.90	1.02
Histidine	0.50	0.46	0.51	0.45	0.51
Phenylalanine	0.97	0.89	0.99	0.87	1.00
Glycine	0.84	0.76	0.85	0.76	0.84
Serine	0.99	0.92	1.00	0.90	1.01
Proline	1.27	1.21	1.32	1.25	1.33
Alanine	0.84	0.76	0.85	0.75	0.86
Aspartic acid	1.91	1.72	1.95	1.65	1.97
Glutamic acid	4.17	3.91	4.21	4.67	5.16

[1] C, ideal protein-formulated control diet; T, threonine supplemented diet; A, arginine supplemented diet; G, glutamine supplemented diet; TAG, threonine, arginine, and glutamine supplemented diet.

Results

Growth performance and organ weights

Tables 5a and 5b show the effect of litter and diet treatments on FI. During the starter phase, litter and diet treatments did not interact. Birds placed on reused litter ate less than birds placed on clean litter, whilst TAG birds ate less than C and T birds, with A and G birds being intermediate. During grower phase and total trial, litter and diet treatments interacted; TAG birds ate less than C and A birds placed on clean litter, whilst diet did not affect FI of birds placed on reused litter.

Table 6 shows the effect of litter and diet treatments on BWG and FCR. Initial BW (day 0) did not differ significantly between treatments (P = 0.265) and averaged (±SD) 39.3 ± 0.81 g. Litter and diet treatments did not interact on BWG and FCR. However, birds placed on reused litter had greater BWG and smaller FCR than birds placed on clean litter, whilst TAG birds had smaller BWG and greater FCR than C and T birds, with A and G birds being intermediate and the effect on FCR limited to the starter phase. Mortality was low at 0.5% (4 out of 800 birds placed) and was not treatment associated.

Table 6 also shows the effect of litter and diet treatments on empty gizzard, spleen, and bursa weights. There were no main

Table 5a
Main effects for average feed intake (g) during starter phase (days 0–11), grower phase (days 11–21) or total trial (days 0–21) of male broilers placed as day-old on either clean or reused litter and fed five different diets.[1,2]

	Litter			Diet[3]						*P*-values		
	Control	Reused	SED	C	T	A	G	TAG	SED	Litter	Diet	Litter × Diet
Feed intake (g)												
Starter phase	231[a]	225[b]	2.4	238[a]	230[ab]	226[bc]	228[ab]	217[c]	3.9	0.030	<0.001	0.528
Grower phase	752	762	9.7	793	754	763	756	719	15.3	0.286	<0.001	0.035
Total trial	711[b]	744[a]	11.1	1 032	985	989	984	936	17.8	0.657	<0.001	0.048

[1] Each litter-diet combination was allocated to 8 pens of 10 birds per pen.
[2] Means within the same row for each model main effect outcome differ at $P < 0.05$ when presented with different superscripts; see Table 5b for such differences between simple means in the presence of significant litter × diet interactions.
[3] C, ideal protein-formulated control diet; T, threonine supplemented diet; A, arginine supplemented diet; G, glutamine supplemented diet; TAG, threonine, arginine, and glutamine supplemented diet.

Table 5b
Simple means for average feed intake (g) during starter phase (days 0–11), grower phase (days 11–21) or total trial (days 0–21) of male broilers placed as day-old on either clean or reused litter and fed five different diets.[1,2]

	Clean litter					Reused litter					
	C[3]	T	A	G	TAG	C	T	A	G	TAG	
Feed intake (g)											
Starter phase[4]											
Grower phase	802[a]	745[abc]	781[ab]	731[bc]	702[c]	785[ab]	764[abc]	746[abc]	781[ab]	736[abc]	21.6
Total trial	1 047[a]	977[abc]	1 011[ab]	960[bc]	919[c]	1 017[ab]	993[abc]	967[abc]	1 007[ab]	952[bc]	25.0

[1] Each litter-diet combination was allocated to 8 pens of 10 birds per pen.
[2] Means within the same row for each model outcome presented with different superscripts differ at $P < 0.05$.
[3] C, ideal protein-formulated control diet; T, threonine supplemented diet; A, arginine supplemented diet; G, glutamine supplemented diet; TAG, threonine, arginine, and glutamine supplemented diet.
[4] Simple means during starter phase are not shown as litter × diet interaction was not significant (Table 5a).

effects of diet treatment or interaction between diet and litter treatments on organ weights. However, litter treatments independently affected organ weights, as birds placed on reused litter had a lighter empty gizzard and a heavier bursa than birds placed on clean litter, with no effect on spleen weight.

Plasma parameters

Most read-outs on plasma biochemistry were not affected by the experimental treatments, which averaged (±SD) 29.5 ± 2.7 5 g/L for total protein, 12.2 ± 1.20 g/L for albumin, 17.3 ± 1.73 g/L for globulin, 19.0 ± 3.70 U/L for gamma-glutamyl transpeptidase, 18.9 ± 7.58 μmol/L for bile acids, 3.6 ± 0.52 mmol/L for cholesterol, 1.6 ± 0.46 mmol/L for triglycerides, 16.1 ± 1.03 mm ol/L for glucose and 95 ± 7.9 mmol/L for Cl. However, diet treatments influenced plasma uric acid, which averaged 444[ab], 436[ab], 369[a], 553[c] and 520[bc] μmol/L for C, T, A, G and TAG birds, respectively (SED 38.7 mmol/L; $P < 0.001$). In addition, litter treatments influenced plasma Na and K concentrations. Plasma Na averaged 143.7 and 138.2 mmol/L for birds placed on clean and reused litter, respectively (SED 2.63 mmol/L; $P < 0.05$), whilst their plasma K averaged 27.1 and 24.5 mmol/L, respectively (SED 0.86 mmol/L; $P < 0.05$). Gamma-glutamyl transpeptidase averaged 4.3 ± 1.23 U/L, and although diet and litter treatment interacted ($P = 0.046$), Tukey's honest significance test at $P < 0.05$ revealed no diet treatment effect within litter treatment, or vice versa.

Table 6
Average BW gain and feed conversion ratio during starter phase (days 0–11), grower phase (days 11–21) and total trial (days 0–21), and day 21 organ weight (g) of male broilers placed as day-old on either clean or reused litter and fed five different diets.[1,2]

	Litter			Diet[3]						*P*-values		
	Control	Reused	SED	C	T	A	G	TAG	SED	Litter	Diet	Litter × Diet
BW gain (g)												
Starter phase	196[b]	203[a]	3.0	212[a]	205[ab]	198[bc]	197[bc]	186[c]	4.7	0.028	<0.001	0.628
Grower phase	515[b]	541[a]	8.7	542	535	532	524	505	13.8	0.004	0.108	0.914
Total trial	711[b]	744[a]	11.1	754[a]	741[a]	730[ab]	721[ab]	691[b]	17.7	0.004	0.013	0.913
Feed conversion ratio												
Starter phase	1.18[a]	1.11[b]	0.009	1.13[b]	1.13[b]	1.15[ab]	1.16[ab]	1.17[b]	0.015	<0.001	0.010	0.396
Grower phase	1.47[a]	1.41[b]	0.026	1.47	1.41	1.44	1.44	1.44	0.041	0.029	0.798	0.504
Total trial	1.42[a]	1.36[b]	0.021	1.40	1.36	1.39	1.39	1.39	0.032	0.004	0.786	0.497
Organ weight (g)												
Empty gizzard	20.93[a]	20.04[b]	0.404	20.11	20.23	20.52	20.22	21.34	0.639	0.028	0.295	0.317
Spleen	0.774	0.789	0.040	0.825	0.841	0.772	0.697	0.772	0.064	0.710	0.186	0.501
Bursa of Fabricius	1.800[b]	1.995[a]	0.068	1.988	1.888	1.975	1.875	1.763	0.107	0.005	0.225	0.502

[1] Each litter-diet combination was allocated to 8 pens of 10 birds per pen.
[2] Means within the same row for each model outcome presented with different superscripts differ at $P < 0.05$.
[3] C, ideal protein-formulated control diet; T, threonine supplemented diet; A, arginine supplemented diet; G, glutamine supplemented diet; TAG, threonine, arginine, and glutamine supplemented diet.

Discussion

Here, we assessed the effect of specific AA supplementation to IP-formulated rations on growth performance, blood biochemistry, and organ weights of birds placed on clean or reused litter. Since the control diet was formulated to IP, it was hypothesised that the effect of AA supplementation is sensitive to the outcomes of reused litter exposure. Our data support the hypothesis, as having observed that reused litter increased bird performance, AA supplementation reduced the growth performance of birds in both litter treatments.

The results support the view that birds fed C diets had greater BWG, FI and smaller FCR than those fed TAG diets. The C diets were formulated on digestible AA requirements (IP-basis) to maximise growth performance and reduce N excretion into the environment (Adedokun et al., 2016; Maharjan et al., 2020). Our results showed that the individual T, A, and G diets tended to reduce bird performance. It can be proposed that these relatively small individual negative effects accumulated in the larger penalty on performance when these AAs were combined in the TAG diet. The latter could be attributed to multiple causes. Firstly, diets were formulated to be isoenergetic, whilst threonine, arginine, and glutamine supplementation in TAG diets increased the CP content. This greater CP level above IP needs to be metabolised, requiring additional energy use for the process of excess AA deamination and N elimination. It has been estimated that six molecules of ATP are used to excrete each excess N molecule, which contrasts with the four molecules of ATP required per N molecule for protein synthesis (Aletor et al., 2000). This accords with observations that a reduced apparent metabolisable energy to CP ratio reduces energy available for growth to benefit uric acid synthesis (Dessimoni et al., 2019) and agrees with elevated levels of uric acid observed here. The latter may also indicate an enhanced caecal protein and/or AA fermentation (Elling-Staats et al., 2022), a view that is supported by elevated levels of caecal branched-chain fatty acids in TAG birds (Hussein et al., 2023). Secondly, an increased N excretion from excess AA supplementation might result in elevated ammonia production in the litter, e.g. through uric acid degradation, which may detriment broiler health, environment and performance (Aletor et al., 2000; Corzo et al., 2005). It should also be recognised that the final BW was 20% below Ross 308 target (Aviagen, 2014). This is likely due to mash feeding rather than crumbs (starter) and pellets (grower). Compared to mash, pelleted feeds usually result in enhanced bird performance arising from amongst others decreased feed wastage, increased feed intake, avoidance of selective feeding, pathogen reduction, improved palatability, and increased nutrient digestibility (Lv et al., 2015).

Raising birds on reused litter has shown variable outcomes on performance, as it has been associated with decreased (Torok et al., 2009; Khattak et al., 2019; González-Ortiz et al., 2021), similar (Yamak et al., 2016; Vieira and Moran, 1999) or increased (Garcés Gudiño et al., 2018) performance relative to birds raised on clean litter. It might be speculated that such variability in response to reused litter exposure could result from variations in pathogen load, moisture, pH, and recycled nutrients from the previous flock. In our study, birds on reused litter had 4.5% greater final BW than those on clean litter by day 21. Gut-derived microbiome in the reused litter may have acted as a probiotic or a direct-fed microbial supplementation that can alter the gastrointestinal tract microbiota of newly hatched chicks, leading to enhanced performance (Torok et al., 2009; Shanmugasundaram et al., 2012). In addition, the more alkaline pH of reused litter used here may indicate greater ammonia production, which may influence pathogen load, as indicated from earlier studies (Kennard et al., 1959). Furthermore, reused litter microbiome has been shown to reduce the caecal abundance of *Salmonella* spp. and delay *Clostridium perfringens* colonisation with a potential increase in broiler performance (Cressman et al., 2010; Wei et al., 2013). This view is supported by a shift towards more beneficial caecal microbiome composition for the birds placed on reused litter (Hussein et al., 2023). Finally, although the rations used were supplemented with vitamin premix, the reused litter might have been a source of vitamins with greater bioavailability, which could also have increased broiler performance. Earlier studies showed that the synthesis of vitamin B12 and riboflavin by microorganisms found in chicken droppings increased the bird's growth and feed efficiency when raised on reused litter (Kennard et al., 1959; Roll et al., 2011). The reduced FI of birds on reused litter during the starter phase concurred with a smaller FCR, which suggests that ingesting reused litter competes with the feed offered. This supports previous evidence by Malone (1992), who showed that broiler chickens may consume up to 4% of the daily intake as litter. Although there was no significant effect of reused litter on FI in the grower phase or the whole growth period, by day 21, birds on reused litter had a significantly greater BW, BWG, and smaller FCR compared to birds on clean litter. Since reused litter exposure increased performance, AA supplementation would have resulted in excess AA and as such did not increase bird's performance despite the two contrasting litter treatments used.

Gizzard weight was recorded as a marker for gut development, including in response to variation in intake, whilst spleen (primary lymphoid organ) and bursa (secondary lymphoid organ) weights were recorded to infer possible influence on immune system development (Madej et al., 2015). Diet treatments did not influence gizzard weight, though gizzards of reused litter birds were lighter than those on clean litter, which concurred with reduced FI. Although mash diets were used, which may require less gizzard action than pelleted diets (Svihus, 2011), using the latter would likely have resulted in similar effects on gizzard weight due to its sensitivity to variation in feed intake per se.

The heavier bursa of Fabricius arising from raising birds on reused litter observed may be an indicator of B-lymphocyte development (Rajput et al., 2013). This would be consistent with previous studies where early exposure to the reused litter microbiome increased antigens' subsequent uptake through cloacal reflex, stimulated B-cell development, and resulted in an effective antibody response (Sorvari et al., 1975). On the other hand, there was no significant effect of AA supplementation on bursa weight. Whilst our outcome agrees with Xue et al. (2018), a heavier bursa was reported in birds following supplementation with arginine (Toghyani et al., 2018) and glutamine (Ribeiro et al., 2015), where production responses were also observed. This would further support the view that AA availability in the current study was unlikely to be scarce in the absence of AA supplementation. Spleen weight was not affected in our study, which accords with other studies on threonine supplementation (Toghyani et al., 2018) or arginine and glutamine supplementation (Szabó et al., 2014).

Blood biochemical can assist in evaluating bird health and metabolic status, and how these respond to internal and external factors (Abdul Basit et al., 2020). Most of the measured plasma parameters in the current study were within the reference range of broilers, supporting the observation that placement on reused litter did not detriment the birds and that homeostatic mechanisms were operating within normal physiological range in response to the AA supplementation. Indeed, whilst plasma uric acid levels are also within a normal range (Clinical Diagnostic Division, 1990), they seem to be greater with G supplementation, both in G and TAG birds, which agrees with the excess nature of the AA supplemented. Only plasma K was elevated beyond normal values, which may have resulted from using K-

ethylenediaminetetraacetic acid-treated tubes (Cornes et al., 2008).

Conclusion

Placement on reused litter increased BWG and reduced FCR compared to placement on clean litter. Because of this outcome, and since the control diet was based on the ideal AA profile, the absence of increased growth response to specific AA supplementation would be consistent with AA being excess.

Ethics approval

All the experimental animal procedures in the current study were carried out under the Animals [Scientific Procedures] Act (1986) and approved by SRUC's Animal Welfare and Ethical Review Body (AU AE 33-2018) and carried out under Home Office authorisation (PPL P32D394C9). All methods were carried out in accordance with the Code of Recommendations for the Welfare of Livestock: Meat Chickens and Breeding Chickens (Department for Environment and Food & Rural Affairs, 2018).

Data and model availability statement

The datasets generated during and/or analysed during the current study and the models used are available from the corresponding author upon reasonable request.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors did not use any artificial intelligence-assisted technologies in the writing process.

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Declaration of interests

None.

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References

Abdul Basit, M., Abdul Kadir, A., Loh, T.C., Abdul Aziz, S., Salleh, A., Kaka, U., Banke Idris, S., 2020. Effects of inclusion of different doses of *Persicaria odorata* leaf meal (POLM) in broiler chicken feed on biochemical and haematological blood indicators and liver histomorphological changes. Animals 10, 1209.
Adedokun, S.A., Helmbrecht, A., Applegate, T.J., 2016. Investigation of the effect of coccidial vaccine challenge on apparent and standardized ileal amino acid digestibility in grower and finisher broilers and its evaluation in 21-day-old broilers. Poultry Science 95, 1825–1835.
Ahmed, I., Qaisrani, S.N., Azam, F., Pasha, T.N., Bibi, F., Naveed, S., Murtaza, S., 2020. Interactive effects of threonine levels and protein source on growth performance and carcass traits, gut morphology, ileal digestibility of protein and amino acids, and immunity in broilers. Poultry Science 99, 280–289.
Aletor, V.A., Hamid, I.I., Niess, E., Pfeffer, E., 2000. Low-protein amino acid-supplemented diets in broiler chickens: effects on performance, carcass characteristics, whole-body composition and efficiencies of nutrient utilisation. Journal of the Science of Food and Agriculture 80, 547–554.
Aviagen, 2014. Ross 308 Nutrition Specifications – All plant-protein based feeds. Retrieved on 17 July 2018 from http://eu.aviagen.com/assets/Tech_Center/Ross_Broiler/Ross-308-Broiler-Nutrition-Specs-plant-2014-EN.pdf.
Baker, D.H., 2003. Ideal amino acid patterns for broiler chicks. In: D'Mello, J.P.F. (Ed.), Amino acids in animal nutrition. 2nd edition. CAB International, Wallingford, UK, pp. 223–235.
Barekatain, R., Chrystal, P.V., Howarth, G.S., McLaughlan, C.J., Gilani, S., Nattrass, G. S., 2019. Performance, intestinal permeability, and gene expression of selected tight junction proteins in broiler chickens fed reduced protein diets supplemented with arginine, glutamine, and glycine subjected to a leaky gut model. Poultry Science 98, 6761–6771.
Barekatain, R., Chrystal, P.V., Gilani, S., McLaughlan, C.J., 2021. Expression of selected genes encoding mechanistic pathways, nutrient and amino acid transporters in jejunum and ileum of broiler chickens fed a reduced protein diet supplemented with arginine, glutamine and glycine under stress stimulated by dexamethasone. Journal of Animal Physiology and Animal Nutrition 105, 90–98.
Bortoluzzi, C., Fernandes, J.I.M., Doranalli, K., Applegate, T.J., 2020. Effects of dietary amino acids in ameliorating intestinal function during enteric challenges in broiler chickens. Animal Feed Science and Technology 262, 114383.
Chen, Y.P., Cheng, Y.F., Li, X.H., Yang, W.L., Wen, C., Zhuang, S., Zhou, Y.M., 2017. Effects of threonine supplementation on the growth performance, immunity, oxidative status, intestinal integrity, and barrier function of broilers at the early age. Poultry Science 96, 405–413.
Clinical Diagnostic Division, 1990. Veterinary Reference Guide. Eastman Kodak Company, Rochester, New York, NY, USA.
Cornes, M.P., Ford, C., Gama, R., 2008. Spurious hyperkalaemia due to EDTA contamination: common and not always easy to identify. Annals of Clinical Biochemistry 45, 601–603.
Corzo, A., Fritts, C.A., Kidd, M.T., Kerr, B.J., 2005. Response of broiler chicks to essential and non-essential amino acid supplementation of low crude protein diets. Animal Feed Science and Technology 118, 319–327.
Corzo, A., Kidd, M.T., Dozier III, W.A., Pharr, G.T., Koutsos, E.A., 2007. Dietary threonine needs for growth and immunity of broilers raised under different litter conditions. Journal of Applied Poultry Research 16, 574–582.
Coster, J., McCauley, R., Hall, J., 2004. Glutamine: metabolism and application in nutrition support. Asia Pacific Journal of Clinical Nutrition 13, 25–31.
Cressman, M.D., Yu, Z., Nelson, M.C., Moeller, S.J., Lilburn, M.S., Zerby, H.N., 2010. Interrelations between the microbiotas in the litter and in the intestines of commercial broiler chickens. Applied and Environmental Microbiology 76, 6572–6582.
Curi, R., Newsholme, P., Procopio, J., Lagranha, C., Gorjão, R., Pithon-Curi, T.C., 2007. Glutamine, gene expression, and cell function. Frontiers in Bioscience 12, 344–357.
Department for Environment, Food & Rural Affairs, 2018. Code of Recommendations for the Welfare of Livestock. Retrieved on 2 February 2017 from https://www.gov.uk/government/uploads/system/ uploads/attachment_data/file/69372/pb7275meat-chickens-020717.pdf.
Deschepper, K., de Groote, G., 1995. Effect of dietary protein, essential and non-essential amino acids on the performance and carcase composition of male broiler chickens. British Poultry Science 36, 229–245.
Dessimoni, G.V., Dalólio, F.S., Moreira, J., Teixeira, L.V., Bertechini, A.G., Hermes, R.G., 2019. Protease supplementation under amino acid reduction in diets

formulated with different nutritional requirements for broilers. Brazilian Journal of Poultry Science 21, 1–8.
Ebrahimi, M., Zare Shahneh, A., Shivazad, M., Ansari Pirsaraei, Z., Tebianian, M., Ruiz-Feria, C.A., Adibmoradi, M., Nourijelyani, K., Mohamadnejad, F., 2014. The effect of feeding excess arginine on lipogenic gene expression and growth performance in broilers. British Poultry Science 55, 81–88.
Elling-Staats, M.L., Gilbert, M.S., Smidt, H., Kwakkel, R.P., 2022. Caecal protein fermentation in broilers: a review. World's Poultry Science Journal 78, 103–123.
Fasina, Y.O., Bowers, J.B., Hess, J.B., McKee, S.R., 2010. Effect of dietary glutamine supplementation on *Salmonella* colonization in the ceca of young broiler chicks. Poultry Science 89, 1042–1048.
Faure, M., Choné, F., Mettraux, C., Godin, J.P., Béchereau, F., Vuichoud, J., Papet, I., Breuillé, D., Obled, C., 2007. Threonine utilization for synthesis of acute phase proteins, intestinal proteins, and mucins is increased during sepsis in rats. The Journal of Nutrition 137, 1802–1807.
Garcés Gudiño, J.A., Merino Guzmán, R., Cevallos Gordón, A.L., 2018. Litter reuse reduces *Eimeria spp* oocyst counts and improves the performance in broiler chickens reared in a tropical zone in Ecuador. European Poultry Science 82, 1–9.
González-Ortiz, G., Dos Santos, T.T., Bedford, M.R., 2021. Evaluation of xylanase and a fermentable xylo-oligosaccharide on performance and ileal digestibility of broiler chickens fed energy and amino acid deficient diets. Animal Nutrition 7, 488–495.
Gottardo, E.T., Prokoski, K., Horn, D., Viott, A.D., Santos, T.C., Fernandes, J.I., 2016. Regeneration of the intestinal mucosa in *Eimeria* and *E. coli* challenged broilers supplemented with amino acids. Poultry Science 95, 1056–1065.
Hussein, M.A., Khattak, F., Vervelde, L., Athanasiadou, S., Houdijk, J.G.M., 2023. Growth performance, caecal microbiome profile, short-chain fatty acids, and litter characteristics in response to placement on reused litter and combined threonine, arginine and glutamine supplementation to juvenile male broiler chickens. Animal Microbiome 5, 18.
Ji, S., Qi, X., Ma, S., Liu, X., Liu, S., Min, Y., 2019. A deficient or an excess of dietary threonine level affects intestinal mucosal integrity and barrier function in broiler chickens. Journal of Animal Physiology and Animal Nutrition 103, 1792–1799.
Keerqin, C., Wu, S.B., Svihus, B., Swick, R., Morgan, N., Choct, M., 2017. An early feeding regime and a high-density amino acid diet on growth performance of broilers under subclinical necrotic enteritis challenge. Animal Nutrition 3, 25–32.
Kennard, D.C., Chamberlin, V.D., Moore, E.N., Winter, A.R., 1959. The use of compost (built-up) litter in chicken houses. Retrieved on 14 August 2020 from https://kb.osu.edu/bitstream/handle/1811/62632/1/OARDC_research_bulletin_n0843.pdf.
Khattak, F., Paschalis, V., Green, M., Houdijk, J.G.M., Soultanas, P., Mahdavi, J., 2018. TYPLEX® Chelate, a novel feed additive, inhibits *Campylobacter jejuni* biofilm formation and cecal colonization in broiler chickens. Poultry Science 97, 1391–1399.
Khattak, F., Kanbur, G., Houdijk, J.G.M., 2019. Re-used litter impacts on post-hatch broiler performance, litter quality and caecal morphology. British Poultry Abstracts 15, 40–41.
Kidd, M.T., Barber, S.J., Virden, W.S., Dozier III, W.A., Chamblee, D.W., Wiernusz, C., 2003. Threonine responses of Cobb male finishing broilers in differing environmental conditions. Journal of Applied Poultry Research 12, 115–123.
Lv, M., Yan, L., Wang, Z., An, S., Wu, M., Lv, Z., 2015. Effects of feed form and feed particle size on growth performance, carcass characteristics and digestive tract development of broilers. Animal Nutrition 1, 252–256.
Madej, J.P., Stefaniak, T., Bednarczyk, M., 2015. Effect of in ovo-delivered prebiotics and synbiotics on lymphoid-organs' morphology in chickens. Poultry Science 94, 1209–1219.
Maharjan, P., Mullenix, G., Hilton, K., Caldas, J., Beitia, A., Weil, J., Suesuttajit, N., Kalinowski, A., Yacoubi, N., Naranjo, V., England, J., 2020. Effect of digestible amino acids to energy ratios on performance and yield of two broiler lines housed in different grow-out environmental temperatures. Poultry Science 99, 6884–6898.
Malone, G.W., 1992. Nutrient enrichment in integrated broiler production systems. Poultry Science 71, 1117–1122.
McGill, E., Kamyab, A., Firman, J.D., 2012. Low crude protein corn and soybean meal diets with amino acid supplementation for broilers in the starter period. 1. Effects of feeding 15% crude protein. International Journal of Poultry Science 11, 161–165.
Mehdipour, Z., Golian, A., Nassiri-Moghadam, H., Javadmanesh, A., 2020. Effect of threonine supplementation on growth performance, metabolizable energy, morphological changes and immune response in broiler chickens challenged with coccidia. Poultry Science Journal 8, 95–107.
Miles, R.D., Chapman, F.A., 2007. The concept of ideal protein in formulation of aquaculture feeds. EDIS 2007, FA144.
Min, Y.N., Liu, S.G., Qu, Z.X., Meng, G.H., Gao, Y.P., 2017. Effects of dietary threonine levels on growth performance, serum biochemical indexes, antioxidant capacities, and gut morphology in broiler chickens. Poultry Science 96, 1290–1297.
Montanhini Neto, R., Ceccantini, M.L., Fernandes, J.I.M., 2013. Effects of methionine source, arginine: lysine ratio and sodium chloride level in the diets of grower broilers reared under high-temperature conditions. Brazilian Journal of Poultry Science 15, 151–160.
Munoz, L.R., Pacheco, W.J., Hauck, R., Macklin, K.S., 2021. Evaluation of commercially manufactured animal feeds to determine presence of *Salmonella*, *Escherichia coli*, and *Clostridium perfringens*. Journal of Applied Poultry Research 30, 100142.
Nassiri Moghaddam, H., Alizadeh-Ghamsari, A.H., 2013. Improved performance and small intestinal development of broiler chickens by dietary L-glutamine supplementation. Journal of Applied Poultry Research 41, 1–7.
Omidi, S., Ebrahimi, M., Janmohammadi, H., Moghaddam, G., Rajabi, Z., Hosseintabar-Ghasemabad, B., 2020. The impact of in ovo injection of l-arginine on hatchability, immune system and caecum microflora of broiler chickens. Journal of Animal Physiology and Animal Nutrition 104, 178–185.
Oxford, J.H., Selvaraj, R.K., 2019. Effects of glutamine supplementation on broiler performance and intestinal immune parameters during an experimental coccidiosis infection. Journal of Applied Poultry Research 28, 1279–1287.
Pirsaraei, Z.A., Rahimi, A., Deldar, H., Sayyadi, A.J., Ebrahimi, M., Shahneh, A.Z., Shivazad, M., Tebianian, M., 2018. Effect of feeding arginine on the growth performance, carcass traits, relative expression of lipogenic genes, and blood parameters of Arian broilers. Brazilian Journal of Poultry Science 20, 363–370.
Premier Atlas, 2014. Ingredients Matrix. Premier Nutrition, a division of AB Agri Limited, Rugely, UK.
Rajput, N., Naeem, M., Ali, S., Zhang, J.F., Zhang, L., Wang, T., 2013. The effect of dietary supplementation with the natural carotenoids curcumin and lutein on broiler pigmentation and immunity. Poultry Science 92, 1177–1185.
Rhoads, J.M., Liu, Y., Niu, X., Surendran, S., Wu, G., 2008. Arginine stimulates cdx2-transformed intestinal epithelial cell migration via a mechanism requiring both nitric oxide and phosphorylation of p70 S6 kinase. The Journal of Nutrition 138, 1652–1657.
Ribeiro Jr, V., Albino, L.F.T., Rostagno, H.S., Hannas, M.I., Ribeiro, C.L.N., Vieira, R.A., de Araújo, W.A.G., Pessoa, G.B.S., Messias, R.K.G., da Silva, D.L., 2015. Effects of dietary L-glutamine or L-glutamine plus L-glutamic acid supplementation programs on the performance and breast meat yield uniformity of 42-d-old broilers. Brazilian Journal of Poultry Science 17, 93–98.
Roll, V.F.B., Dai Prá, M.A., Roll, A.P., 2011. Research on *Salmonella* in broiler litter reused for up to 14 consecutive flocks. Poultry Science 90, 2257–2262.
Rubin, L.L., Ribeiro, A.M.L., Canal, C.W., Silva, I.C., Trevizan, L., Vogt, L.K., Pereira, R.A., Lacerda, L., 2007. Influence of sulfur amino acid levels in diets of broiler chickens submitted to immune stress. Brazilian Journal of Poultry Science 9, 53–59.
Shanmugasundaram, R., Lilburn, M.S., Selvaraj, R.K., 2012. Effect of recycled litter on immune cells in the cecal tonsils of chickens. Poultry Science 91, 95–100.
Sigolo, S., Zohrabi, Z., Gallo, A., Seidavi, A., Prandini, A., 2017. Effect of a low crude protein diet supplemented with different levels of threonine on growth performance, carcass traits, blood parameters, and immune responses of growing broilers. Poultry Science 96, 2751–2760.
Sorvari, T., Sorvari, R., Ruotsalainen, P., Toivanen, A., Toivanen, P., 1975. Uptake of environmental antigens by the bursa of Fabricius. Nature 253, 217–219.
Star, L., Rovers, M., Corrent, E., Van der Klis, J.D., 2012. Threonine requirement of broiler chickens during subclinical intestinal *Clostridium* infection. Poultry Science 91, 643–652.
Svihus, B., 2011. The gizzard: function, influence of diet structure and effects on nutrient availability. World's Poultry Science Journal 67, 207–224.
Szabó, J., Andrásofszky, E., Tuboly, T., Bersényi, A., Weisz, A., Heténvi, N., Hullár, I., 2014. Effect of arginine or glutamine supplementation on production, organ weights, interferon gamma, interleukin 6 and antibody titre of broilers. Acta Veterinaria Hungarica 62, 348–361.
Toghyani, M., Tahmasebi, S., Modaresi, M., Fosoul, S.S.A.S., 2018. Effect of arginine and threonine in ovo supplementation on immune responses and some serum biochemical attributes in broiler chickens. Italian Journal of Animal Science 18, 342–349.
Torok, V.A., Hughes, R.J., Ophel-Keller, K., Ali, M., MacAlpine, R., 2009. Influence of different litter materials on cecal microbiota colonization in broiler chickens. Poultry Science 88, 2474–2481.
Valizade, M.R., Sadeghi, A.A., Chamani, M., Shawrang, P., Feizi, F., 2014. The effect of increasing dietary threonine to lysine ratio on carcass characteristics, mucin gene expression and morphological analysis of ileum of male broiler chickens challenged with *Salmonella*. International Journal of Biosciences 5, 138–146.
Vieira, S.L., Moran Jr, E.T., 1999. Effects of delayed placement and used litter on broiler yields. Journal of Applied Poultry Research 8, 75–81.
Wang, W.W., Qiao, S.Y., Li, D.F., 2009. Amino acids and gut function. Amino Acids 37, 105–110.
Wei, S., Gutek, A., Lilburn, M., Yu, Z., 2013. Abundance of pathogens in the gut and litter of broiler chickens as affected by bacitracin and litter management. Veterinary Microbiology 166, 595–601.
Wu, Q.J., Jiao, C., Liu, Z.H., Cheng, B.Y., Liao, J.H., Zhu, D.D., Ma, Y., Li, Y.X., Li, W., 2021. Effect of glutamine on the growth performance, digestive enzyme activity, absorption function, and mRNA expression of intestinal transporters in heat-stressed chickens. Research in Veterinary Science 134, 51–57.
Xue, G.D., Barekatain, R., Wu, S.B., Choct, M., Swick, R.A., 2018. Dietary L-glutamine supplementation improves growth performance, gut morphology, and serum biochemical indices of broiler chickens during necrotic enteritis challenge. Poultry Science 97, 1334–1341.
Yamak, U., Sarica, M., Boz, M., Ucar, A., 2016. Effect of reusing litter on broiler performance, foot-pad dermatitis and litter quality in chickens with different growth rates. Kafkas Universitesi Veteriner Fakultesi Dergisi 22, 85–91.
Zhang, B., Lv, Z., Li, Z., Wang, W., Li, G., Guo, Y., 2018. Dietary L-arginine supplementation alleviates the intestinal injury and modulates the gut microbiota in broiler chickens challenged by *Clostridium perfringens*. Frontiers in Microbiology 9, 1716.