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The influence of substituting dietary peptide-bound with free amino acids on nitrogen metabolism and acid–base balance of broiler chickens depends on asparagine and glutamine supply

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Abstract

Reducing dietary crude protein (CP) concentration while maintaining adequate amino acid (AA) supply by free AA inclusion can contribute to attenuate the negative environmental effects of animal farming. This study investigated upper limits of dietary free AA inclusions without undesirable effects including the dependence on asparagine (Asn) and glutamine (Gln) supply. Ten broilers were allocated to sixty-three metabolism units each and offered nine experimental diets from day (d) 7–21 (n 7). One diet (167 g CP/kg) contained 80 g soya protein isolate (SPI)/kg. In the other diets, 25, 50, 75 and 100 % of the digestible AA from SPI were substituted with free AA. Digestible Asn+aspartic acid (Asp) and Gln+glutamic acid (Glu) were substituted with Asp/Glu or 50/50 mixes of Asp/Asn and Glu/Gln, respectively. Total excreta were collected from d 11–14 and from d 18–21. Growth and nitrogen accretion were unaffected by 25 and 50 % substitution without and with free Asn/Gln, respectively, but decreased at higher substitution ($P \leq 0{\cdot}024$). Circulating concentrations of Asp, Glu and Gln were unaffected by treatment, while Asn decreased at substitution higher than 50 % when Asn/Gln were not provided ($P \leq 0{\cdot}005$). Blood gas analysis on d 21 indicated a compensated metabolic acidosis at substitution higher than 50 and 75 % without and with free Asn/Gln, respectively ($P \leq 0{\cdot}017$). Results suggest that adding Asn/Gln increased an upper limit for proportion of dietary free AA from 10 to 19 % of dietary CP and enabled higher free AA inclusion without affecting the acid–base balance.

Keywords: **Free amino acids: Broiler chickens: Peptide-bound amino acids: Nitrogen metabolism: Acid–base balance**

The use of free amino acids (AA) in feed compounding supports adjustment of dietary AA concentrations in the feed to the requirement of animals while reducing dietary crude protein (CP). This leads to decreased intake of nitrogen (N) without affecting performance. This strategy reduces the environmental impact of animals and enables more efficient production of animal-based food because less N is excreted, and the N utilisation efficiency (NUE) is increased[1]. The need for free AA inclusion to adjust dietary AA concentrations in the feed increases as the dietary CP concentration is lowered.

Absorption processes in the intestine differ between peptide-bound and free AA. AA are mainly absorbed into the enterocytes as di- and tripeptides. In the enterocytes, peptides are mostly hydrolysed to free AA and then transferred to the systemic circulation[2]. When AA are provided as free AA, no peptides need to be hydrolysed by digestive processes and the AA are directly available for absorption in the small intestine[3]. Studies on broiler chickens[4], pigs[5–8], rats[9–13] and humans[10] found that free AA are absorbed more rapidly into the systemic circulation than peptide-bound AA. With different absorption rates, AA may be available in an unbalanced pattern for protein synthesis even though dietary AA supply is formulated as balanced. Excess AA are degraded and the contained N is excreted, thus limiting NUE. The different absorption processes have led to the suggestion that there is a certain threshold of dietary free AA upon which the utilisation of AA is limited[14]. The interpretation of a threshold of dietary free AA was also supported from studies where dietary CP was reduced while more free AA were included in diets[15]. However, only AA considered as relevant were supplemented in such studies. Hence, impaired growth may have been an effect of free AA or of an unrecognised AA deficiency.

The studies in which peptide-bound AA were substituted with free AA do not allow for a clear conclusion on whether an

Abbreviations: AA, amino acids; ADFI, average daily feed intake; ADG, average daily weight gain; Asn, asparagine; Asp, aspartic acid; Asp/Glu, aspartic acid and glutamic acid; Asx/Glx, aspartic acid + asparagine and glutamic acid + glutamine; BE, base excess; CP, crude protein; d, day; G:F, gain:feed ratio; Gln, glutamine; Glu, glutamic acid; HCO_3, bicarbonate; NH_3, ammonia; NUE, nitrogen utilisation efficiency; SPI, soya protein isolate; TCO_2, total carbon dioxide; UA, uric acid.

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upper limit of dietary free AA without impaired growth exists. In broiler chickens[16], approximately 41 % of dietary CP was provided by either soya protein isolate (SPI) or a mixture of 18 free AA, resulting in almost identical analysed dietary AA concentrations. The use of free AA reduced feed intake and growth, but the NUE was unaffected. In pigs, casein was substituted with a free AA mixture that resulted in similar analysed essential AA concentrations, but dietary non-essential AA concentrations differed[5,6]. In this series of experiments on pigs, substitution of peptide-bound with free AA did not affect NUE in one study[5] but reduced NUE in another[6] without known reasons for the different results. In another study on rats[9], substituting casein with a mixture of seventeen free AA resulted in lower weight gain and N retention in rats that received the free AA mixture.

Several possible reasons may have caused the different outcomes of the studies. Lower growth and NUE when peptide-bound AA were substituted with free AA may have been caused by a deficient supply of one or more non-essential AA if not considered in the respective free AA mix. The potential of Gly and Ser to reduce growth when not sufficiently provided was described for broiler chickens[1,17] and pigs[18]. A recent study on broiler chickens indicated that asparagine (Asn) and glutamine (Gln) can be growth-limiting in diets with very low CP concentrations[19]. None of the aforementioned studies on the effects of substituting peptide-bound with free AA considered aspartic acid (Asp), Asn, glutamic acid (Glu) and Gln individually. This may be explained by the common procedure of AA analysis where proteins are hydrolysed. Asn and Gln lose an amide residue during the hydrolysis and elute together with Asp and Glu, respectively[20].

Another explanation for effects of substituting peptide-bound with free AA is a difference in small intestine AA digestibility. Free AA are completely digestible[15] while the digestibility of peptide-bound AA is less than 100 %. This results in more AA being available for metabolic processes when supplied as free AA than as peptide-bound AA even if daily AA consumption is unchanged. The digestibility of AA has not been considered in any of the aforementioned studies where peptide-bound AA were substituted with free AA.

It is to be investigated whether substituting peptide-bound with free AA may alter the acid–base balance in a diagnostically detectable way. Overall, catabolism of AA has an acidifying impact on the acid–base balance, with effects of individual AA ranging from alkalising for dicarboxylic AA to strongly acidifying for sulphurous AA[21]. However, consequences of substituting peptide-bound with free AA on the acid–base balance of broiler chickens and whether high dietary proportions of free AA can overstrain regulatory mechanisms for acid–base homeostasis are unknown to our knowledge. The dietary supply with Asn and Gln may be relevant in this regard because Asn and Gln are involved in a mechanism to compensate acidifying impacts on the acid–base balance[22].

Therefore, the first objective of this study was to determine an upper limit of substitution of peptide-bound with free AA without impaired growth and N accretion in broiler chickens and whether such an upper limit depends on the Asn and Gln supply. The second objective was to investigate whether the substitution of peptide-bound with free AA affects the acid–base balance and whether this depends on the Asn and Gln supply. SPI as a source of peptide-bound AA was incrementally substituted, on a digestible AA basis, with free AA to achieve this. The relevance of Asn and Gln was investigated by substituting analysed Asp+Asn and Glu+Gln in SPI with Asp and Glu, or with 50/50 mixes of Asp/Asn and Glu/Gln, respectively. Nitrogenous compounds in excreta allowing for conclusions on the intermediary N metabolism and free AA in the blood plasma were analysed.

We hypothesised that there is an upper limit for proportion of free AA in diets for broiler chickens without impaired growth and that this proportion depends on the Asn and Gln supply. We further hypothesised that the acid–base balance of the animals is affected beyond the capacity of regulatory mechanisms to maintain acid–base homeostasis.

Materials and methods

Ethics approval

The animal trials were carried out at the Agricultural Experiment Station of the University of Hohenheim, Germany. All experimental protocols and procedures were conducted according to German animal welfare legislation and approved by the Regierungspräsidium Tübingen, Germany (permit number: HOH63/21_460a).

Experimental setup

Two trials were conducted; a digestibility trial and a substitution trial. The results of the AA digestibility trial (online Supplementary Table 1) were used to determine the precaecal digestible AA amount from SPI based on differences in the digested AA amounts between a basal diet plus SPI and the basal diet plus maize starch. A mixture of free AA was then formulated with the same AA pattern as the digestible AA pattern determined for SPI. This mixture of free AA was used in the substitution trial so that the digestible AA concentration in all experimental diets of the substitution trial was the same.

Experimental diets

The diets were prepared at the certified feed mill of the University of Hohenheim. Concentrations of essential AA in the diets were calculated at 105 % of the recommendations of the Gesellschaft für Ernährungsphysiologie[23]. Concentrations of non-essential AA including glycine equivalents were calculated to avoid limitations due to non-essential AA deficiency[19,24]. The basal mixture for all diets represented 92 % of the final diets and mainly consisted of maize, maize starch and casein (Table 1). SPI (Euroduna Feed Ingredients GmbH, Barmstedt, Germany) was used as a source of peptide-bound AA because SPI has a low hydrolysation degree of up to 3 % according to the supplier and almost completely consisted of AA (Table 1). Hence, concentrations of nutrients other than AA hardly varied when SPI was substituted with free AA. Mass differences between the diets after addition of treatment-specific

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Table 1. Ingredient composition of the experimental diets (g/kg)

	Digestibility trial		Substitution trial								
Diet*	BM + SPI	BM + maize starch	0FAA	25FAA-Asp/Glu	25FAA-Asx/Glx	50FAA-Asp/Glu	50FAA-Asx/Glx	75FAA-Asp/Glu	75FAA-Asx/Glx	100FAA-Asp/Glu	100FAA-Asx/Glx
Maize						583					
Casein†						40·3					
Soybean oil						30					
L-Lysine·HCl						3·08					
DL-Methionine						2·50					
L-Cysteine						1·06					
L-Tryptophan						0·19					
L-Threonine						1·65					
L-Arginine						3·40					
L-Isoleucine						1·09					
L-Valine						3·29					
Glycine						3·07					
L-Alanine						0·35					
L-Aspartic acid						0·16					
Trace element premix‡						1					
Vitamin premix§						2					
Monocalcium phosphate						19·7					
Limestone						10·49					
Sodium bicarbonate						5					
Choline chloride						2					
Sodium chloride						1					
SPI‖	80	0	80	60	60	40	40	20	20	0	0
Free AA mix¶	0	0	0	11·62	11·62	23·24	23·24	34·86	34·86	46·48	46·48
L-Aspartic acid	0	0	0	1·92	0·96	3·84	1·92	5·75	2·88	7·67	3·84
L-Glutamic acid	0	0	0	3·32	1·66	6·64	3·32	9·95	4·98	13·27	6·64
L-Asparagine·H_2O	0	0	0	0	1·07	0	2·15	0	3·22	0	4·29
L-Glutamine	0	0	0	0	1·66	0	3·32	0	4·98	0	6·64
Titanium dioxide	5	5	0	0	0	0	0	0	0	0	0
Maize starch	200·67	280·67	205·67	208·81	208·7	211·95	211·72	215·11	214·75	218·25	217·78

BM, basal mix; SPI, soya protein isolate.

* 0FAA (no free amino acid substitution): basal mix + 80 g/kg SPI; digestible amino acids in 80 g SPI/kg contained in 0FAA were substituted by 25 (25FAA), 50 (50FAA), 75 (75FAA), or 100 (100FAA) percent with free amino acids using a mixture of all twenty proteinogenic amino acids except for asparagine, aspartic acid, glutamine and glutamic acid; digestible asparagine+aspartic acid and glutamine+glutamic acid in SPI of the 0FAA diet were substituted by the same levels as the other amino acids, either with aspartic acid and glutamic acid (Asp/Glu) or with 50/50 mixes of asparagine/aspartic acid and glutamine/glutamic acid (Asx/Glx).

† Food grade casein 110 mesh, Meggle GmbH & Co.KG, Wasserburg am Inn, Germany; 966 g crude protein/kg dry matter.

‡ Trace element premix (Gelamin Gesellschaft für Tierernährung mbH) provided per kg of diet: 160 mg manganese from manganese-(II)-oxide, 120 mg zinc from zinc-sulphate, 50 mg iron from iron-(II)-sulphate monohydrate, 15 copper from cupric-(II)-sulphate pentahydrate, 1·2 mg iodine from calcium iodate anhydrous and 0·4 mg selenium from sodium selenite.

§ Vitamin premix (MIAVIT Stefan Niemeyer GmbH, Essen, Germany) provided per kg of diet: 10 000 IE vitamin A, 3000 IE vitamin D3, 30 mg vitamin E, 2·4 mg vitamin K3, 100 μg biotin, 1 mg folic acid, 3 mg vitamin B_1, 6 mg vitamin B_2, 6 mg vitamin B_6, 30 μg vitamin B_{12}, 50 mg niacinamide and 14 mg calcium-D-pantothenate.

‖ Analysed concentrations per kg dry matter: 146 g nitrogen, 994·4 g sum of analysed amino acids, 42·0 g alanine, 116·5 g aspartic acid, 73·6 g arginine, 11·6 g cysteine, 196·4 g glutamic acid, 39·7 g glycine, 27·3 g histidine, 46·3 g isoleucine, 80·1 g leucine, 62·6 g lysine, 13·2 g methionine, 52·4 g phenylalanine, 51·1 g proline, 53·3 g serine, 37·2 g threonine, 11·2 g tryptophan, 33·5 g tyrosine and 46·4 g valine.

¶ Free AA mix with 5·9 % L-alanine, 10·9 % L-arginine, 1·4 % L-cysteine, 5·4 % glycine, 4·0 % L-histidine, 7·0 % L-isoleucine, 11·3 % L-leucine, 11·9 % L-lysine·HCl, 1·9 % DL-methionine, 7·6 % L-phenylalanine, 7·2 % L-proline, 7·8 % L-serine, 5·0 % L-threonine, 1·4 % L-tryptophan, 4·7 % L-tyrosine and 6·6 % L-valine.

ingredients were compensated by maize starch. All diets were pelleted without using steam through a 3 mm die to prevent feed selection or de-mixing.

Nine diets were investigated in the substitution trial (Table 1) with one diet containing 80 g SPI/kg, which accounted for ~38 % of dietary CP. In the other diets, 25, 50, 75 or 100 % of the digestible AA provided by SPI were substituted with free AA using a mixture of all proteinogenic AA except for Asp, Asn, Glu and Gln and assuming complete digestibility of free AA[15]. Digestible Asp+Asn and Glu+Gln in SPI was substituted with Asp and Glu ('Asp/Glu'), or with 50/50 mixes of Asp/Asn and Glu/Gln ('Asx/Glx'). All diets were formulated to have the same concentration of digestible AA. Two diets containing either 80 g SPI/kg or maize starch were prepared for the digestibility trial. Titanium dioxide was included as an indigestible marker at a level of 5 g/kg. Concentrations of all nutrients reported herein are based on a standardised dry matter (DM) of 88 %, unless otherwise stated. Differences in AA concentrations between the diets of the digestibility trial resulted from the SPI only. Results of AA analysis of the feed confirmed the formulated values (online Supplementary Table 2).

Birds and housing

In both trials, male Ross 308 broiler hatchlings were obtained from a commercial hatchery (Brüterei Süd ZN der BWE-Brüterei Weser-Ems GmbH & Co. KG). Birds were raised in floor pens (3 m × 4 m) on dedusted wood shavings and received a commercial starter diet containing 215 g CP/kg and 12·5 MJ ME/kg (315042025 Club Mastkükenstarter, Deutsche Tierernährung Cremer GmbH & Co. KG) until experimental diets were introduced. Feed and water were provided for *ad libitum* consumption throughout the trials. Lighting was continuous during the first 3 d after placement, followed by 18-h light and

6-h dark cycle. The temperature was set at 34°C for the first 3 d of the trials and was gradually decreased to 22°C on d 21. Birds were allocated on a mesh-wired floor in metabolism units on d 18 and d 6 in the digestibility and substitution trial, respectively, to achieve an equal mean bird weight in every unit within each trial. In the digestibility trial, 240 birds were distributed to 16 units (2 m × 1 m × 1 m) of fifteen birds each. The experimental diets were provided in eight units each from d 18 to 21. In the substitution trial, 630 broiler chickens were distributed to 63 units (1 m × 1 m × 1 m) of ten birds each. Each diet in the substitution trial was fed from d 7 to 21 and tested in seven units in a randomised complete block design optimised using the OPTEX procedure of SAS (Version 9·4, SAS Institute, Cary, USA).

Experimental procedures and sample collection

In the digestibility trial, birds were stunned with a gas mixture of 35 % CO_2, 35 % N_2 and 30 % O_2 and euthanised with CO_2 exposure, except for one animal per unit being bled after anaesthesia by concussion to obtain blood samples. Digesta was collected from the distal two-thirds of the section between Meckel's diverticulum and 2 cm anterior to the ileo-caeca-colonic junction[25] by flushing with cold, double-distilled water. Digesta of the birds in each metabolism unit was pooled and immediately frozen at −20°C.

In the substitution trial, birds and feed were weighed on d 7, 11, 14, 18 and 21 on a unit basis to determine the average daily weight gain (ADG), average daily feed intake (ADFI) and the gain:feed ratio (G:F). Feed DM was also determined on these days. The birds were inspected at least twice daily. Dead birds were removed and weighed, and the feed intake of the remaining birds in the respective unit was recorded. Spilled feed pellets were collected daily from trays located under each unit, dried and weighed to correct ADFI data. Total excreta were collected in 12 h intervals on d 11–14 and d 18–21. Excreta was immediately frozen at −20°C after each collection. Blood samples were collected on d 21 from the brachial vein of the bird with the body weight that was closest to the group average. Four ml of total blood was placed in lithium heparin tubes (Sarstedt AG & Co, Nürnbrecht, Germany). Within 10 min after collection, about 0·1 ml of blood was used for blood gas analysis. Preliminary measurements performed in the course of the digestibility trial showed constant results within this time period after bleeding (online Supplementary Table 3). The remaining blood was centrifuged at 4°C for 10 min at 1500 *g*, and the obtained plasma was stored at −80°C until analysis.

Sample preparation and chemical analyses

Diets were ground through a 0·5 mm sieve in a centrifugal mill (ZM 200; Retsch GmbH) for analyses of crude ash, crude fat, crude fibre, starch, Na, K and Cl. A vibrating disk mill (Pulverisette 9, Fritsch GmbH) was used for all other analyses. The official methods for nutrient analysis in Germany[26] were used for the determination of DM, N, crude fat, crude fibre, crude ash, Na, K and Cl. CP was calculated as total N multiplied by 6·25[27]. The AA in diets and digesta were analysed after oxidation and acid hydrolysis[28], where Asn and Gln eluted together with Asp and Glu, respectively, because of a lost amide residue of Asn and Gln during acid hydrolysis[20]. Tryptophan was analysed using an HPLC[29]. Analysis of free AA in the blood plasma was carried out without preceding hydrolysis, which allowed for separate quantification of Asp and Asn as well as Glu and Gln.

Excreta samples were thawed at +3°C, weighed and homogenised. Excreta DM was determined in triplicate. Excreta N concentration was analysed in duplicate by using Kjeldahl digestion. Excreta ammonia (NH_3; herein, ammonia includes both NH_3 and NH_4^+) and uric acid (UA) concentration were analysed according to methods described previously[24].

Blood gas analysis was carried out with the i-STAT®Alinity system (Abaxis Inc., Union City, USA) fitted with EC8+ cartridges. This included Na, K, Cl, glucose, blood urea nitrogen, pH, carbon dioxide partial pressure, total carbon dioxide (TCO_2), bicarbonate (HCO_3), the anion gap, the base excess (BE), haematocrit and haemoglobin. Blood urea N was not within the measurement range in any observation.

Calculations and statistical analysis

The ADFI on a standardised DM of 88 %, ADG, G:F, N accretion and NUE were calculated on a metabolism unit basis. The N accretion, NUE and precaecal AA digestibility were calculated as

$$\text{N accretion (g/d)} = \text{N intake (g/d)} - \text{N excretion (g/d)} \quad (1)$$

$$\text{NUE (\%)} = (\text{N accretion (g/d)}/\text{N intake (g/d)}) \times 100 \quad (2)$$

$$\text{Digestibility (\%)} = 100 - ((TiO_{2\text{Diet}} \times AA_{\text{Digesta}})/(TiO_{2\text{Digesta}} \times AA_{\text{Diet}})) \times 100 \quad (3)$$

where AA_{Diet} and $AA_{Digesta}$ are the concentrations of the individual AA (g/kg DM) and TiO_{2Diet} and $TiO_{2Digesta}$ are the TiO_2 concentrations in the diet and the digesta (g/kg DM), respectively.

The sample size was calculated prior to the trial using the POWER procedure of SAS. Seven replicates per diet in the substitution trial were determined to be necessary to determine a difference in NUE between two diets of 0·01 g/g with a standard deviation of 0·006 g/g as significant in a two-sided *t* test ($\alpha = 0{\cdot}05$, $\beta = 0{\cdot}2$). The number of 10 birds per replicate reduced individual variation between individual birds.

All traits were statistically analysed by one-way ANOVA using the MIXED procedure of SAS. The metabolism unit was considered the experimental unit for all traits except for blood data, where individual birds were the experimental unit. No data were excluded from statistical evaluation. The statistical model was

$$y_{ij} = \alpha + trt_i + block_j + e_{ij} \quad (4)$$

where y_{ij} is the dependent trait, α is the overall mean, trt_i is the fixed effect of diet i and e_{ij} is the residual error. A random block j effect was included if model accuracy was improved, as indicated by Akaike information criterion. Statistical significance was set at $P < 0{\cdot}050$.

Results

Performance of birds

The mean bird weight on d 7 of the substitution trial ranged between 179 and 184 g/bird and was not significantly different among treatments ($P = 0{\cdot}88$). The survival rate during the experimental period was 99·4 % and was not related to any treatment (4 out of 630 birds in 4 different treatments). The ADFI from d 7–21 decreased at AA substitution higher than 25 % and 50 % with addition of Asp/Glu and Asx/Glx, respectively (Fig. 1) and decreased continuously at higher AA substitution levels ($P \leq 0{\cdot}040$). The ADG and G:F from d 7–21 was unaffected by 25 % AA substitution and by 50 % AA substitution with addition of Asp/Glu and Asx/Glx, respectively (Fig. 1) and decreased continuously at higher AA substitution levels ($P \leq 0{\cdot}011$).

Nitrogen metabolism

The N accretion from d 11–14 and d 18–21 was unaffected by 25 % AA substitution and by 50 % AA substitution with addition of Asp/Glu and Asx/Glx, respectively (Fig. 2) and decreased continuously at higher AA substitution levels ($P \leq 0{\cdot}024$). The NUE on d 11–14 and d 18–21 were on a high level in diet without AA substitution with values of 76 % and 75 %, respectively (Fig. 2). The NUE on d 11–14 decreased up to 50 % AA substitution ($P = 0{\cdot}002$) and remained at this level at higher AA substitution when Asx/Glx was added. When Asp/Glu was added, NUE was unaffected up to 75 % AA substitution and then increased at 100 % AA substitution ($P = 0{\cdot}049$). On d 18–21, NUE decreased up to 50 % AA substitution ($P \leq 0{\cdot}029$) and increased to the initial level at higher AA substitution with no difference between the addition of Asp/Glu and Asx/Glx.

The NH_3-N excretion on d 11–14 and d 18–21 increased with increasing AA substitution (Fig. 3; $P < 0{\cdot}001$). The UA-N excretion on d 11–14 and d 18–21 increased at 50 % AA substitution (Fig. 3; $P \leq 0{\cdot}001$) and then decreased to the basal level at 100 % AA substitution when Asx/Glx was added. When Asp/Glu was added, UA-N excretion was unaffected up to 50 % AA substitution and decreased at higher AA substitution levels ($P < 0{\cdot}020$). The NH_3-N/(NH_3-N + UA-N) ratio on d 11–14 and d 18–21 increased with increasing AA substitution (Fig. 3; $P < 0{\cdot}001$). This increase was significant for the substitution higher than 50 % on d 11–14 and d 18–21 ($P \leq 0{\cdot}014$). Addition of Asx/Glx decreased the NH_3-N/(NH_3-N + UA-N) ratio compared with Asp/Glu at 75 % and 100 % substitution ($P \leq 0{\cdot}002$).

Free amino acids in blood plasma

Concentrations of free AA in blood plasma on d 21 did not differ significantly among treatments ($P \geq 0{\cdot}085$), except for Asn and lysine ($P < 0{\cdot}001$; Fig. 4; Table 2). The Asn concentration was unaffected by AA substitution when Asx/Glx were added. When Asp/Glu was added, free Asn in the blood plasma was unaffected up to 50 % AA substitution and decreased continuously at higher AA substitution ($P \leq 0{\cdot}005$). Lysine concentration was significantly increased at AA substitution higher than 50 % irrespective of whether Asp/Glu or Asx/Glx was added ($P < 0{\cdot}013$). Other measured free AA in blood plasma on d 21 did not differ significantly among treatments (Table 2).

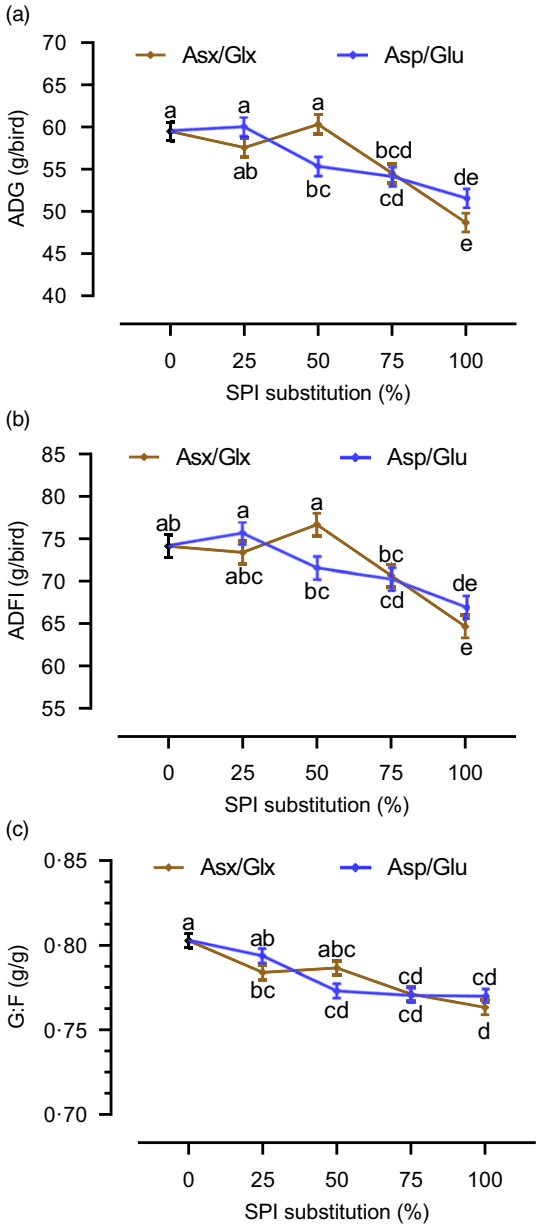

Fig. 1. Effects of incremental substitution of digestible amino acids from 80 g soya protein isolate (SPI)/kg of diet with free amino acids on average daily weight gain (ADG; panel a), average daily feed intake (ADFI; panel b), and the gain:feed ratio (G:F; panel c) of broiler chickens from day 7–21 post-hatch (n 7 units, 10 birds each). Digestible asparagine+aspartic acid and glutamine+glutamic acid in SPI were substituted with free aspartic acid and glutamic acid ('Asp/Glu'), or with 50/50 mixes of asparagine/aspartic acid and glutamine/glutamic acid ('Asx/Glx'), respectively. Error bars indicate the pooled standard error. Treatments not sharing the same letter were significantly different ($P \leq 0{\cdot}050$).

Blood traits related to the acid–base balance

Blood pH on d 21 was not significantly influenced by treatment (Fig. 5). Blood HCO_3, BE and TCO_2 decreased and Cl increased with the increasing AA substitution ($P \leq 0{\cdot}006$). When Asp/Glu was added, HCO_3, BE, TCO_2 and Cl were not significantly affected up to 50 % AA substitution, but HCO_3, BE and TCO_2 decreased and Cl increased at higher AA substitution (Fig. 5;

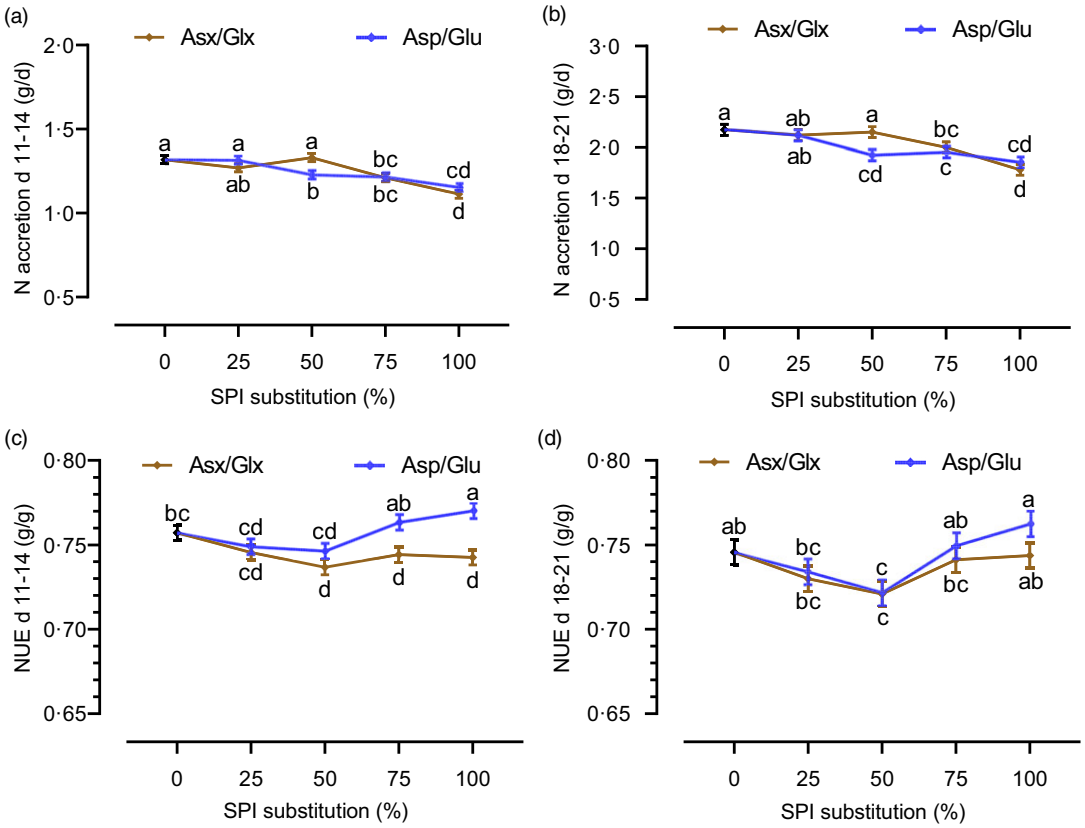

Fig. 2. Effects of incremental substitution of digestible amino acids from 80 g soya protein isolate (SPI)/kg of diet with free amino acids on nitrogen (N) accretion (panels a and b) and N utilisation efficiency (NUE; panels c and d) determined from days (d) 11–14 and d 18–21 of broiler chickens (*n* 7 units, 10 birds each). Digestible asparagine+aspartic acid and glutamine+glutamic acid in SPI were substituted with free aspartic acid and glutamic acid ('Asp/Glu'), or with 50/50 mixes of asparagine/aspartic acid and glutamine/glutamic acid ('Asx/Glx'), respectively. Error bars indicate the pooled standard error. Treatments not sharing the same letter were significantly different ($P \leq 0{\cdot}050$).

$P \leq 0{\cdot}012$). The HCO_3, BE, TCO_2 and Cl were not affected up to 75 % AA substitution when Asx/Glx was added, while HCO_3, BE and TCO_2 was decreased and Cl was increased at 100 % AA substitution ($P \leq 0{\cdot}001$). Other measured blood traits related to the acid–base balance did not differ significantly among treatments (Table 2).

Discussion

Effects on growth and nitrogen metabolism

The first objective of this study was to determine whether there is an upper limit of substituting dietary peptide-bound AA with free AA in feed for broiler chickens without impaired growth and N accretion and if this upper limit depends on whether Asn and Gln are supplied. The highest AA substitution without impaired growth and N accretion was found at 25% when only Asp/Glu were added, while 50% AA substitution was possible when Asx/Glx were included. Hence, the first hypothesis was confirmed. Given that the substitution was investigated in 25 %-unit increments, the highest substitution without impaired growth and N accretion was between 25 and 50% and between 50 and 75% when Asp/Glu and Asx/Glx was provided, respectively. This substitution was equivalent to ~10–19% of dietary CP when Asp/Glu was provided and ~19–29 % of dietary CP when Asx/Glx was provided. Considering that the basal diet also contained free AA, the dietary concentration of free AA was at a level of ~37 g/kg, ~54 g/kg, ~71 g/kg in diets with 25%, 50% and 75% AA substitution, respectively. The performance of broilers in the treatments with no or low substitution was higher than the performance objectives of the breeding company[30], suggesting that growth was not limited in those treatments by dietary AA supply.

The reduced ADFI may have caused the decreased growth and N accretion at high AA substitution because the pattern of ADG and ADFI responses was almost identical. However, the reasons for reduced ADFI are difficult to identify. Different taste of peptide-bound AA and free AA[14] may have contributed to reduced ADFI, but taste perception in birds is largely unknown and difficult to measure[31]. Possibly, reduced ADFI at high AA substitution was caused by increased insulin release. Feeding peptide-bound AA resulted in lower plasma insulin concentrations than feeding free AA in pigs,[8] and intracerebroventricular insulin administration had anorexic effects in broiler chickens[32]. However, insulin was not determined in the present study.

The dietary supply of Asn from the basal mixture and endogenous Asn formation apparently was not sufficient at an AA substitution of more than 50 % when only Asp/Glu were added. This is indicated by decreasing Asn concentrations in

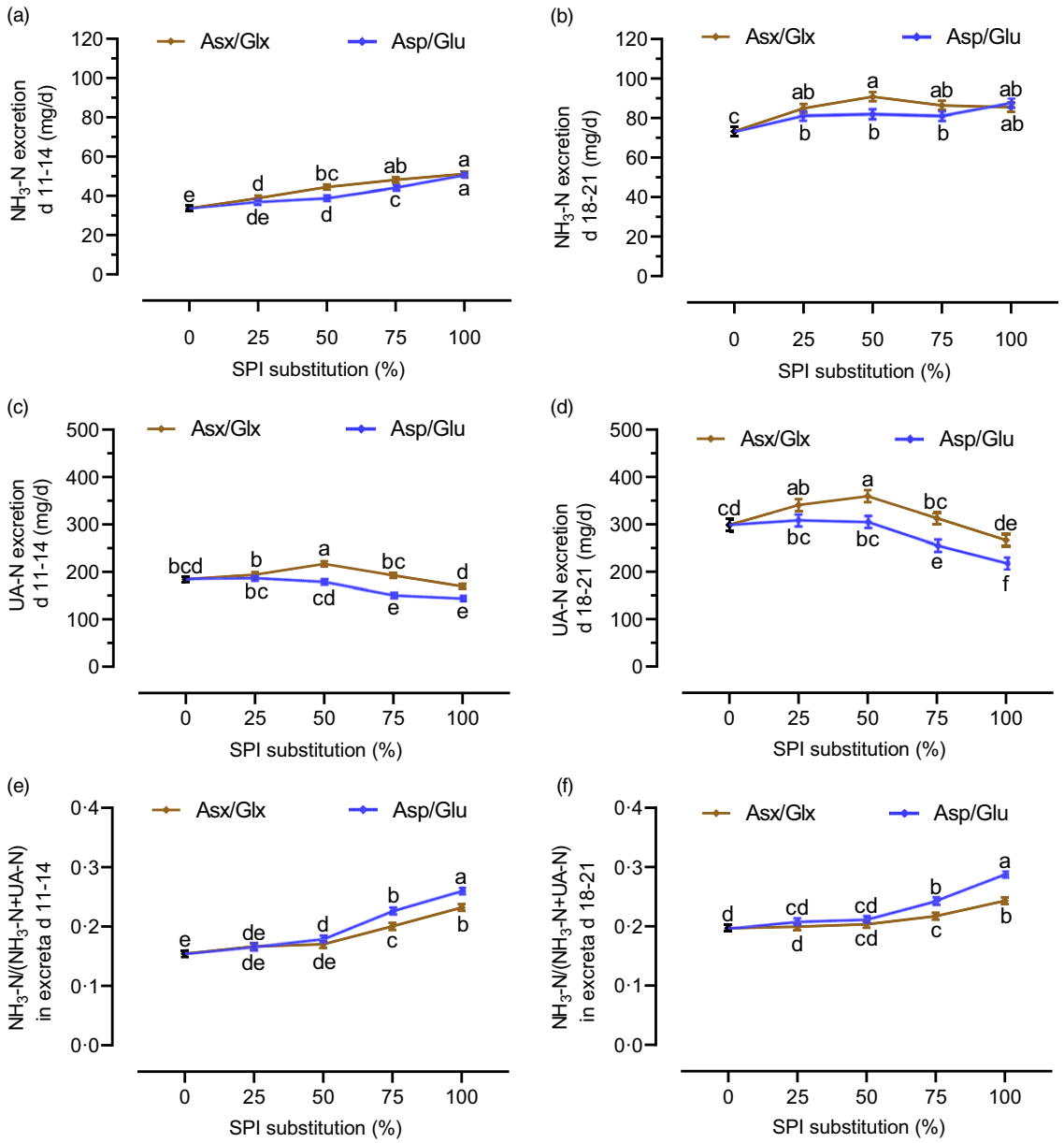

Fig. 3. Effects of incremental substitution of digestible amino acids from 80 g soya protein isolate (SPI)/kg of diet with free amino acids on excretion of ammonia-nitrogen (NH_3-N; panels a and b) and uric acid-nitrogen (UA-N; panels c and d) as well as the NH_3-N/(NH_3-N + UA-N) ratio (panels e and f) of broiler chickens from days (d) 11–14 and d 18–21 (n 7 units, 10 birds each). Digestible asparagine+aspartic acid and glutamine+glutamic acid in SPI were substituted with free aspartic acid and glutamic acid ('Asp/Glu'), or with 50/50 mixes of asparagine/aspartic acid and glutamine/glutamic acid ('Asx/Glx'), respectively. Error bars indicate the pooled standard error. Treatments not sharing the same letter were significantly different ($P \leq 0{\cdot}050$).

blood plasma, while Asn concentrations were unaffected when Asx/Glx was added to the diets. The increasing Asp provision with increasing AA substitution when Asp/Glu was added did not affect Asp concentrations in the blood plasma. Hence, surplus Asp may have been converted to Asn or other AA. This may have contributed to numerically higher alanine concentrations in the blood plasma when Asp/Glu was added because Asp can be converted to alanine in a one-step reaction[33]. Alternatively, surplus Asp may have been converted to nitrogenous compounds other than AA, including NH_3. In contrast to Asn, there are no indications that provision of Gln was relevant because blood plasma concentrations of Glu and Gln were unaffected by treatment. This is consistent with previous findings, where growth of broiler chickens fed very low CP diets was higher when supplemented with an Asn/Asp mixture than with the same amount of Asp, while there was no difference between supplementation of a Gln/Glu mixture and the same amount of Glu[19]. Nonetheless, provision of Gln may have been relevant for other metabolic purposes than those measured herein, such as energy metabolism. Parts of dietary Glu and Gln are known to be used as substrates by enterocytes to provide energy to intestinal tissues[34]. The amount of Glu used for energy provision can be remarkably high because

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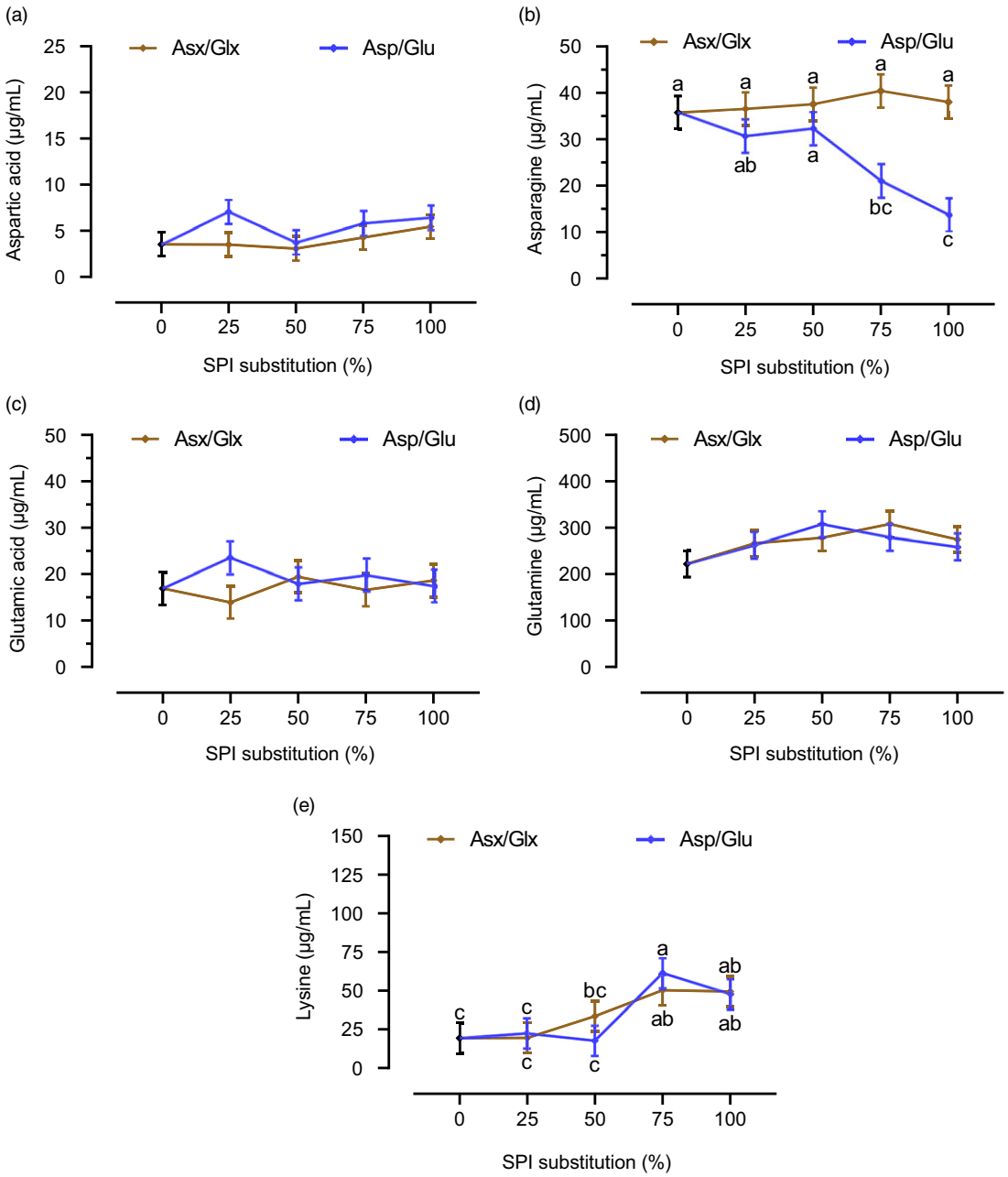

Fig. 4. Effects of incremental substitution of digestible amino acids from 80 g soya protein isolate (SPI)/kg of diet with free amino acids on concentrations of selected amino acids in blood plasma of broiler chickens on day 21 of the trial (n 7 birds/treatment). Digestible asparagine+aspartic acid and glutamine+glutamic acid in SPI were substituted with free aspartic acid and glutamic acid ('Asp/Glu'), or with 50/50 mixes of asparagine/aspartic acid and glutamine/glutamic acid ('Asx/Glx'), respectively. Error bars indicate the pooled standard error. Treatments not sharing the same letter were significantly different ($P \leq 0{\cdot}050$). Results of other analysed AA are presented in Table 2.

Glu and glucose were the major energy sources used by the enterocytes of post-hatching broiler chickens[35].

Gln and Asp apparently were not limiting factors for UA formation, and the limited UA formation did not seem to be the cause for reduced growth and N accretion at high AA substitution. The daily UA-N excretion decreased at more than 50 % AA substitution irrespective of whether Asx/Glx or Asp/Glu was added, but this effect was minor for UA-N excretion relative to body weight (Fig. 6). Hence, the decreasing UA-N excretion at AA substitution higher than 50 % in part was a consequence of animals being lighter. The possibility that Asn and Gln, or both may limit UA-N formation was discussed in a previous study[16], where substituting AA contained in SPI with all proteinogenic AA except for Asn and Gln led to increasing NH_3-N/(NH_3-N + UA-N) ratios in excreta of broiler chickens. The sum of NH_3-N and UA-N can be used as an indicator for urine-N because those compounds characterise most of the urinary N in birds[36]. Increasing NH_3-N/(NH_3-N + UA-N) ratios indicate that less urine-N was excreted as UA-N at more than 50 % AA substitution. Two molecules of Gln and one molecule of

Table 2. Effects of incremental substitution of digestible amino acids from 80 g soya protein isolate/kg of diet with free amino acids on blood traits of broiler chickens in the substitution trial on day 21 (*n* 7 individual birds/treatment)

Diet*	0FAA	25FAA-Asp/Glu	25FAA-Asx/Glx	50FAA-Asp/Glu	50FAA-Asx/Glx	75FAA-Asp/Glu	75FAA-Asx/Glx	100FAA-Asp/Glu	100FAA-Asx/Glx	PooledSEM	*P*-value
Blood gas analysis in blood											
Sodium (mmol/l)	143	142	143	143	143	143	143	142	143	0·5	0·272
Potassium (mmol/l)	3·6	3·9	3·7	3·8	3·7	3·9	3·8	3·5	3·4	0·2	0·321
Anion gap (mmol/l)	15·6	15·2	16·7	15·1	17·3	15·7	15·6	15·1	15·0	0·9	0·562
Glucose (mmol/l)	15·1	17·1	14·4	16·9	15·2	14·6	13·8	13·5	13·5	0·8	0·058
Haematocrit (% PCV)	17·7	18·7	19·0	16·9	17·1	18·9	18·0	16·7	18·0	0·6	0·110
Haemoglobin (g/l)	60·1	63·4	64·6	57·4	58·4	64·0	61·1	56·7	61·4	2·2	0·129
Free amino acids in blood plasma (μg/ml)											
Alanine	78·4	104·4	79·0	108·9	81·5	114·6	82·2	106·4	84·5	78·4	0·085
Arginine	37·8	26·1	23·4	24·0	26·6	30·8	21·0	43·6	35·3	7·2	0·197
Cystine	14·1	24·6	13·4	18·6	15·7	20·8	19·8	17·9	17·8	3·9	0·171
Cysteic acid	1·4	1·5	1·2	1·6	1·6	1·1	1·2	1·5	1·0	0·6	0·984
Glycine	55·9	71·7	51·1	72·6	64·7	72·4	69·2	60·7	53·7	12·4	0·472
Histidine	6·0	11·9	5·1	6·0	6·2	7·8	7·2	7·9	7·9	1·8	0·108
Isoleucine	10·2	13·0	8·6	10·7	9·7	9·0	8·9	8·8	9·0	2·0	0·376
Leucine	22·4	27·7	19·7	23·6	21·1	22·6	21·9	22·3	22·9	4·3	0·748
Methionine	18·0	19·2	17·1	19·8	17·8	20·9	19·0	18·9	18·8	3·4	0·922
Phenylalanine	19·2	25·0	16·7	20·9	18·4	19·1	20·3	20·8	19·4	3·6	0·439
Proline	48·7	61·1	48·7	57·4	50·8	61·3	57·7	57·4	56·7	9·9	0·730
Serine	59·9	74·8	58·8	82·5	78·7	82·8	81·9	76·3	65·3	14·6	0·526
Threonine	60·4	72·7	57·6	100·7	86·5	64·7	74·5	75·6	74·4	15·0	0·222
Tyrosine	41·7	50·5	28·8	44·0	39·1	37·2	37·3	39·4	35·5	7·7	0·477
Valine	42·2	48·4	35·0	42·8	40·2	40·4	37·3	39·6	39·4	7·4	0·759

SEM, standard error of the mean; PCV, packed cell volume.

* 0FAA (no free amino acid substitution): basal mix + 80 g soya protein isolate/kg; digestible amino acids in 80 g soya protein isolate/kg contained in 0FAA were substituted by 25 (25FAA), 50 (50FAA), 75 (75FAA), or 100 (100FAA) % with free amino acids using a mixture of all twenty proteinogenic amino acids except for asparagine, aspartic acid, glutamine and glutamic acid; digestible asparagine+aspartic acid and glutamine+glutamic acid in soya protein isolate of the 0FAA-mix diet were substituted by the same levels as the other amino acids, either with aspartic acid and glutamic acid (Asp/Glu) or with 50/50 mixes of asparagine/aspartic acid and glutamine/glutamic acid (Asx/Glx).

Asp are needed to form the purine ring of UA[37]. The unaffected free Asp, Glu and Gln concentrations in blood plasma give no indication of Gln and Asp being deficient when Gln was not added to the diets. Therefore, Gln and Asp most likely were not limiting factors for UA formation.

The relevance of a potentially deficient supply of constituents of SPI other than AA, including non-AA-N, at high AA substitution was probably minor. The concentration of constituents in SPI other than AA cannot be determined precisely because AA analysis only gives information on Asp+Asn and Glu+Gln and the molar mass of Asn and Gln is higher than that of Asp and Glu, respectively. Analysed AA explained 994·4 and 996·6 g/kg DM of SPI assuming that analysed Asp+Asn and Glu+Gln in SPI consisted only of Asp and Glu or only of Asn and Gln, respectively. Therefore, constituents of SPI other than AA differed in the range of 0·27–0·45 g/kg DM among diets.

The overall high level and the small differences in NUE give no indication that increasing proportions of free AA lead to a marked decrease in AA utilisation, which is an often-suggested consequence of the different absorption rates of peptide-bound and free AA. This may partly be attributed to the *ad libitum* access to feed in the present study, which leads to a more continuous feed intake in broiler chickens than in species fed in meals[38,39]. Further, crop and gizzard portion the amount of feed to the posterior digestive tract so that feed enters the small intestine more continuously[40]. These factors may mute peaks of AA entering the systemic circulation[16]. Further, the availability of AA may act as an anabolic signal leading to protein synthesis[8]. Such an effect would support a high NUE despite temporarily high amounts of AA in the systemic circulation. Another possible process leading to high NUE is that UA in the urine can be transported to the caeca via reverse peristalsis. In the caeca, NH_3 formed from UA degradation can then be absorbed and used for the synthesis of non-essential AA. The relevance of non-essential AA synthesis from NH_3 was shown to be of increased relevance when availability of non-essential AA was insufficient[41,42], e.g. in consequence of increased AA catabolism. In addition, a decreased protein turnover as a consequence of limited AA availability[43] may have contributed to high NUE.

The similar response patterns of N accretion, NUE and forms of urine-N excretion determined on d 11–14 and on d 18–21 provide no evidence for an adaption to the presence of high proportions of free AA in diets after more than 7 d of feeding the experimental diets. Nonetheless, adaptations may have been relevant within the first days of feeding the experimental diets because feeding free AA is known to influence the abundance of peptide and AA transporters in the small intestine[44,45]. Therefore, adaptations to the presence of high proportions of free AA in diets within few d of feeding experimental diets warrant further investigation.

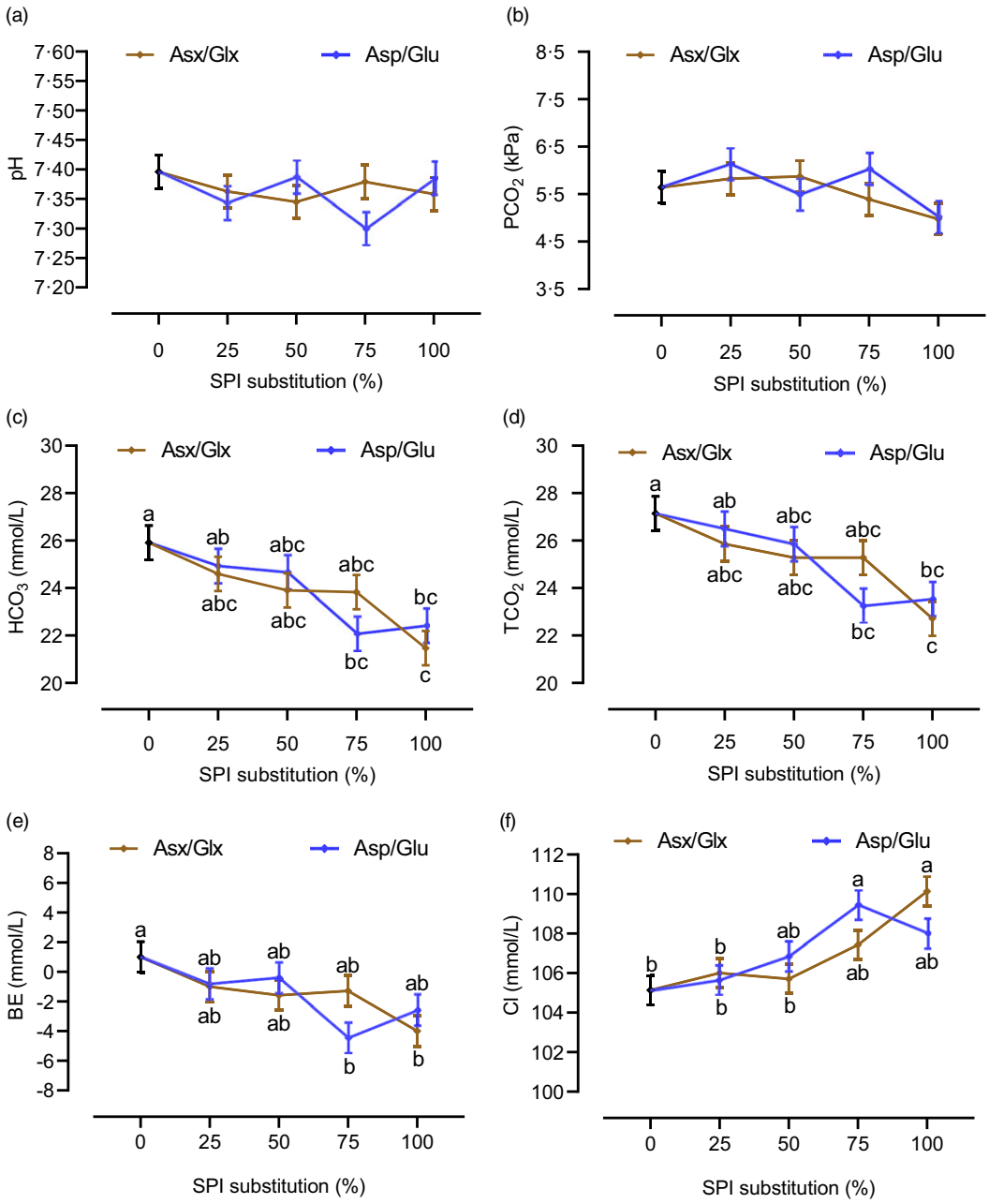

Fig. 5. Effects of incremental substitution of digestible amino acids from 80 g soya protein isolate (SPI)/kg of diet with free amino acids on selected blood traits related to the acid–base balance (pH, panel a; PCO_2, carbon dioxide partial pressure, panel b; HCO_3, bicarbonate panel c; TCO_2, total carbon dioxide, panel d; BE, base excess, panel e; Cl, chloride, panel f) of broiler chickens on day 21 of the trial (n 7 birds/treatment). Digestible asparagine+aspartic acid and glutamine+glutamic acid in SPI were substituted with free aspartic acid and glutamic acid ('Asp/Glu'), or with 50/50 mixes of asparagine/aspartic acid and glutamine/glutamic acid ('Asx/Glx'), respectively. Error bars indicate the pooled standard error. Treatments not sharing the same letter were significantly different ($P \leq 0{\cdot}050$). Results of other analysed blood traits related to the acid–base balance are presented in Table 2.

Effects on the acid–base balance

The second objective of the study was to investigate whether the substitution of peptide-bound with free AA affects the acid–base balance and whether this depends on the Asn and Gln supply. The results point to an increasingly challenged acid–base homoeostasis with increasing AA substitution, as indicated by decreasing HCO_3, TCO_2 and BE as well as increased Cl concentrations in the blood[46,47]. However, such a challenge apparently was compensated because blood pH was unaffected, suggesting that the animals were in the state of a compensated metabolic acidosis[47]. Increasing urinary NH_3 excretion with increasing AA substitution supports the interpretation of a compensated metabolic acidosis because increased urinary NH_3 excretion represents an adaptive response to excrete acid[21]. Accompanying the increased NH_3 excretion, an increased renal HCO_3 excretion might explain the observed decrease of plasma HCO_3 concentration. Hence, the hypothesis of the acid–base balance being challenged beyond the capacity of regulatory

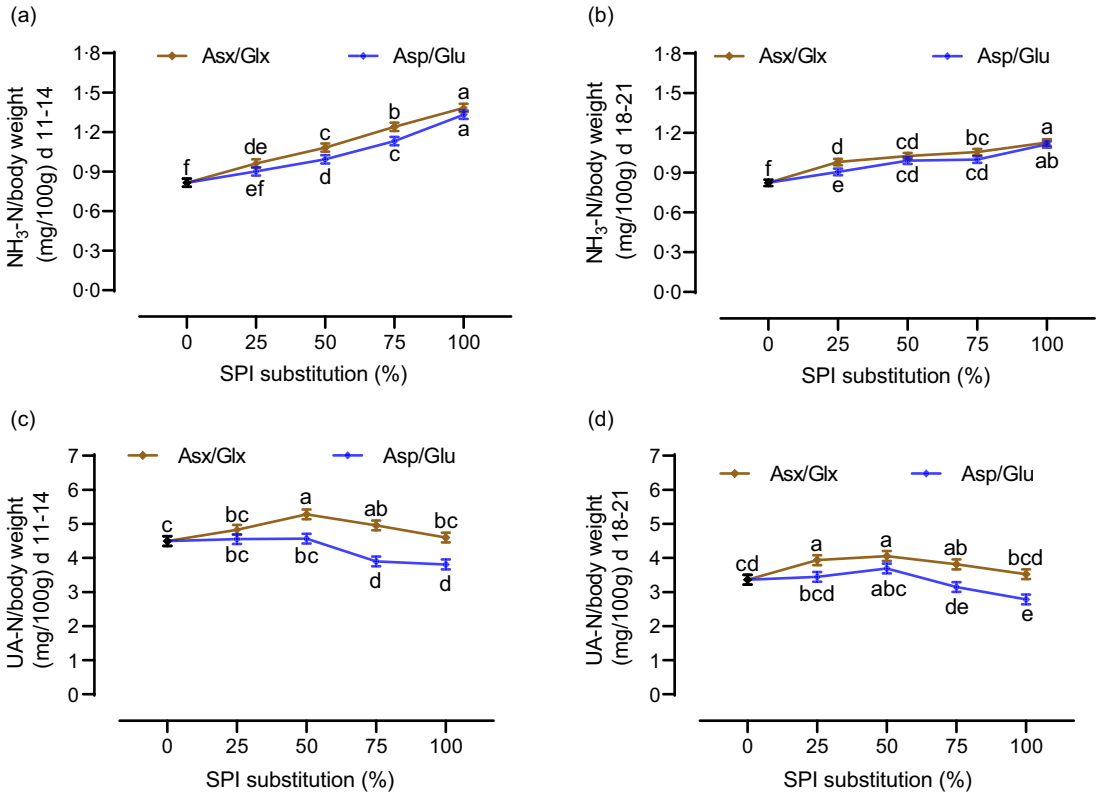

Fig. 6. Effects of incremental substitution of digestible amino acids from 80 g soya protein isolate (SPI)/kg of diet with free AA on excreta characteristics of broiler chickens relative to average body weight determined from days 11–14 and days 18–21 of the experiment (n 7 units, 10 birds each). Digestible asparagine+aspartic acid and glutamine+glutamic acid in SPI were substituted with free aspartic acid and glutamic acid ('Asp/Glu'), or with 50/50 mixes of asparagine/aspartic acid and glutamine/glutamic acid ('Asx/Glx'), respectively. Error bars indicate the pooled standard error. Treatments not sharing the same letter were significantly different ($P \leq 0{\cdot}050$).

mechanisms was rejected. Nonetheless, adaptive mechanisms to maintain the acid–base balance may have contributed to the reduced growth and N accretion at high AA substitution.

The addition of Asn and Gln seemed to have attenuated the challenge on the acid–base balance because higher AA substitution was possible until indications of a metabolic acidosis were measured. The addition of Asn and Gln may have allowed for more acid excretion enabled by NH_3 excretion because formation of Asp from Asn and of Glu from Gln are major sources of NH_3[22]. Decreased Asn in blood plasma of the birds provided with Asp/Glu at high AA substitution may be explained by Asn used for NH_3 formation to a degree exceeding the capacity for endogenous Asn synthesis. In the literature, UA excretion was shown to be decreased in severe metabolic acidosis, possibly because NH_3 excretion is a more efficient mechanism to excrete acid than UA excretion[48]. Hence, the lower UA excretion of the birds when provided with Asp/Glu than with Asx/Glx is in line with the interpretation of a challenge on the acid–base balance.

The HCl provided by inclusion of free lysine probably contributed to a shift in the acid–base balance by providing anions when peptide-bound AA were substituted with free AA. The HCl contained in each gram of dietary L-lysine·HCl would contribute 7 mEq/kg DM of acid[21]. In consequence, the acid contribution from HCl would then account for 60 mEq/kg DM in the diets at 100 % of substitution, which contained approximately 1·6 g/kg DM more HCl than the diet with no AA substitution.

It is difficult to estimate whether the affected acid–base balance at high AA substitution contributed to reduced growth. In rats, metabolic acidosis stimulated tissue protein degradation and led to reduced N retention[49], but the increasing NUE at high AA substitution gives no evidence for such a phenomenon in the present study. Alternatively, metabolic acidosis may have been the cause for low ADFI. Increased NH_3 excretion was correlated with NH_3 concentrations in the blood in a study on broiler chickens[50], and high NH_3 concentrations in the blood are associated with anorexia[51,52]. However, reduced ADFI was observed at one AA substitution increment lower than the indications of a metabolic acidosis. Acid–base balance was determined after 14 d of feeding the experimental diets. It appears possible that a challenge on the acid–base homeostasis was more pronounced at an earlier stage after the transition to diets containing high levels of free AA, resulting in the observed low impaired growth.

Summary

The present study indicated that an upper limit of free AA inclusion without effects on growth and N accretion depended on whether free Asn and Gln was supplied. This suggested that Asn, Gln or both were limiting growth in the diets with Asp and

Glu and more than 50 % AA substitution. Supplying free Asn and Gln increased the upper limit of AA substitution without reduced growth and N accretion from 10 % to 19 % of dietary CP and the proportion of dietary free AA from ~37 to ~54 g/kg. Blood measurements after 14 d of feeding the experimental diets indicated a compensated metabolic acidosis. Supply of Gln and Asn seemed to attenuate the challenge on the acid–base balance.

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A. I., M. R. and W. S. designed the research and performed the trials; A. I. and W. S. were responsible for data collection, statistical analyses and carrying out chemical analyses. A. I., M. R., Á. K. and W. S. contributed to the drafting of the manuscript. All authors have read and approved the final version of the manuscript.

The authors declare no conflict of interest.

Supplementary material

For supplementary material/s referred to in this article, please visit https://doi.org/10.1017/S0007114523001617

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