

Full-Length Article

Intestinal barrier function, caecal microbiota and growth performance of thermoneutral or heat stressed broiler chickens fed reduced crude protein diets supplemented with guanidinoacetic acid[☆]

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ABSTRACT

The effectiveness of guanidinoacetic acid (GAA) in reduced protein (RP) diets on performance and gut health of broilers under heat stress is largely unknown. A 35-d experiment was conducted using four dietary treatments: a standard protein diet (SP, 22.1 and 20.7% CP in grower and finisher), a RP diet (20.1 and 18.7% in grower and finisher), a RP diet with 0.092% GAA per kg diet substituting 50% of supplemented arginine (GAA50) at one-to-one ratio and a RP diet with the same amount of GAA added on top (GAAtop). Day-old male Ross 308 chicks were assigned to 64 pens (10 birds each) in two rooms. In each room, each diet was replicated 8 times. From d 25 to 35, birds in one room were subjected to a cyclic heat stress (32 ± 1 °C for 8 h). There was no interaction between diets and heat stress for any of the studied parameters. GAA50 followed by GAAtop significantly decreased the feed intake during the finisher phase ($P < 0.01$) and from d 10 to 35 ($P < 0.001$), compared with SP diet. Heat stress reduced ($P < 0.0001$) feed intake and body weight gain at all stages of the study but did not impact FCR. The GAA50 tended to reduce FCR from d 24 to 35 ($P = 0.086$) and d 10 to 35 ($P = 0.082$) compared with SP and RP. Heat stress increased ($P < 0.05$) intestinal permeability whereas diets had no effect. The gene expression of IL1 β was downregulated ($P < 0.01$) by GAA50 but diet had no effect on other selected genes. Heat stress upregulated the expression of several genes including Claudin 2, Claudin 3, GPX-1, HSP70, IL1 β , SOD-1 and AMPK- α 1. Caecal microbiota composition remained unaffected. The results indicate that replacing 50% of supplemented arginine with GAA tends to improve FCR by reducing the feed intake under both thermoneutral and heat stress conditions without any interaction. Supplementation of GAA or two percentage points reduction of dietary protein had no demonstrable effects on parameters of intestinal health.

Introduction

Supplementation of synthetic amino acids to compensate for the reduction of dietary crude protein in broiler chicken diets has been the subject of extensive recent research (Kidd et al., 2021). Arginine (Arg) is one such amino acid that is required to be supplemented, depending on the ingredient composition of the diet, to meet the breed's diet specification and support optimal growth and health. Arginine is an

indispensable amino acid in chickens due to an incomplete urea cycle and hence lack of sufficient de novo synthesis (Fouad et al., 2013; Uyanga et al., 2023). The benefits of additional Arg supplementation to reduced protein diets for growth performance and intestinal integrity of broilers have been documented (Barekattain et al., 2021; Barekattain et al., 2019a). However, additional supplementation of Arg may not always be the most economical approach and when supplemented at an adequate amount, more Arg may need to be used to support creatine

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synthesis in birds fed reduced protein diets that are naturally lower in precursors of creatine (i.e. glycine) compared with normal protein diets (Sharma et al., 2022). Creatine formation is fundamentally important to support energy metabolism, muscle growth, other organs and tissues and its supply is particularly vital for the mitigation of stress and its associated impacts on energy reserves (Bonilla et al., 2021).

The use of guanidinoacetic acid (GAA) as the only and direct precursor of creatine is shown to have additional implications beyond creatine loading including Arg-sparing effects. The relevance of GAA in poultry nutrition has been reviewed by Khajali et al. (2020) and the Arg-sparing effects of GAA range from 50% to 150% in broilers fed reduced protein diets have been shown in a recent study (Sharma et al., 2022). In addition, feeding GAA has been shown to reduce oxidative stress, increase glycogen stores, and enhance muscle energy metabolism in birds under cyclic heat stress (Majeduddin et al., 2023).

Beyond creatine deposition in the muscle, little is known about the association of GAA supplementation with creatine loading at the intestinal level and for the intestinal barrier function in poultry. From rodent models, there is evidence that creatine supplementation improves the intestinal barrier function (Mueller et al., 2018). In colitis, disruption of the creatine kinase pathway abrogates intestinal integrity by changing the dynamics of junction assembly and leads to mucosal inflammation (Glover et al., 2013). Creatine was able to attenuate the increased inflammation in the same study (Glover et al., 2013). Creatine may provide an energy buffer in the face of acutely increased energy requirements; it also facilitates the transfer of high-energy phosphate from the mitochondria to the cytosol via the phosphocreatine shuttle, which may be important during the regeneration of the mucosal barrier (Turer et al., 2017). It remains to be investigated whether GAA, by changing the intestinal level of creatine, can impact intestinal health in poultry.

Thus, as a precursor of creatine synthesis, it was hypothesized that supplementation of GAA to the diet of broilers can increase creatine concentration in intestinal tissues and hence may provide benefit by restoring the intestinal barrier function failure induced by heat stress in broilers. Thus, the current study was designed to investigate intestinal permeability, barrier function and creatine loading in broiler chickens fed reduced protein diets with and without heat stress.

Materials and methods

Animal ethics

The Animal Ethics Committee of the Primary Industries and Regions (SA, Australia) approved all the experimental procedures of this study (AEC 25/22).

Experimental design and dietary treatments

The experiment comprised of a 4 × 2 factorial arrangement of treatments to study dietary treatments with and without cyclic heat stress. Four dietary treatments included a standard crude protein diet (SP), a reduced crude protein diet (RP), a RP diet with GAA supplemented at 0.092% of the diet replacing 50% of supplemented Arg at a one-to-one ratio (GAA50) and a RP diet with the same amount of GAA added on top of the Arg of the diet (GAAtop). The required supplemented Arg in grower and finisher diets were 0.184% to achieve 1.26% and 1.16% of digestible Arg in grower and finisher diets, respectively. No uplift of energy was considered for GAA as part of diet formulation. The main ingredients including wheat, sorghum and soybean meal were analysed by a near-infrared (FT-NIR) spectrometer (Bruker MPA II Fourier transform) using Addissee PNE advanced calibration for amino acids and proximate analysis prior to feed formulation. Diets were formulated to meet or exceed the specifications of Ross 308 (Aviagen, 2022). All the experimental diets were mixed and pelleted using a cold-press pelleting machine at the SARDI feedmill facilities (Roseworthy, SA). Table 1 shows the composition of experimental grower and

Table 1

Composition of experimental grower and finisher diets (% unless specified otherwise).

Ingredients	Grower		Finisher	
	Standard	Reduced protein	Standard	Reduced protein
Wheat	39.739	46.440	35.279	41.985
Sorghum	20.000	20.000	28.000	28.000
Soybean meal	33.254	26.350	29.955	23.047
Canola oil	3.538	2.718	3.754	2.933
Limestone	1.052	1.062	0.922	0.932
Dicalcium phosphate	0.817	0.880	0.577	0.639
Sodium chloride	0.214	0.136	0.217	0.140
Sodium bicarbonate	0.319	0.431	0.315	0.427
Choline Cl 70%	0.018	0.051	0.037	0.070
L-lysine HCl	0.279	0.475	0.254	0.450
DL-methionine	0.359	0.408	0.330	0.379
L-threonine	0.151	0.236	0.122	0.207
L-Valine	0.041	0.146	0.020	0.126
L-Isoleucine	0.000	0.105	0.000	0.105
L-Arginine	0.000	0.184	0.000	0.184
Glycine	0.000	0.160	0.000	0.159
Xylanase	0.010	0.010	0.010	0.010
Phytase	0.010	0.010	0.010	0.010
Vitamin-mineral premix ¹	0.200	0.200	0.200	0.200
Nutrient composition				
AME kcal/kg	3050.000	3050.000	3100.000	3100.000
Crude protein	22.085	20.086	20.717	18.717
Measured crude protein	22.73	20.16	20.12	18.43
Crude fat	5.294	4.496	5.603	4.804
Crude fiber	3.103	2.871	2.984	2.751
DEB ²	262.1	228.1	244.4	210.4
Dig ³ Gly equivalent	1.366	1.366	1.270	1.270
Dig Arg	1.260	1.260	1.162	1.162
Dig Lys	1.180	1.180	1.080	1.080
Dig Met	0.627	0.647	0.583	0.604
Dig Cys	0.291	0.271	0.275	0.255
Dig M+C	0.920	0.920	0.860	0.860
Dig Trp	0.248	0.217	0.231	0.201
Dig Leu	1.464	1.300	1.417	1.253
Dig Ile	0.800	0.800	0.750	0.750
Dig Thr	0.790	0.790	0.720	0.720
Dig Val	0.910	0.910	0.840	0.840
Dig Gly	0.726	0.799	0.672	0.745
Calcium	0.865	0.865	0.750	0.750
Available phosphorus	0.430	0.430	0.380	0.380
Sodium	0.180	0.180	0.180	0.180
Potassium	1.056	0.910	0.983	0.836
Chloride	0.230	0.230	0.230	0.230
Choline mg/kg	1700.0	1699.9	1700.0	1699.9

¹ Supplied per kg of diet: vitamin A, 12,000 IU; vitamin D3, 3,000 IU; vitamin E, 25 mg; vitamin K3, 3 mg; vitamin B1, 2 mg; vitamin B2, 6 mg; niacin, 45 mg; pantothenate, 15 mg; pyridoxine, 5 mg; folate, 1 mg; cyanocobalamin, 16 µg; biotin, 150 µg; Cu (sulfate), 10 mg; Fe (sulfate), 60 mg; I (iodide), 1 mg; Se, 0.3 mg; Mn (sulfate and oxide), 120 mg; Zn, 70 mg; antioxidant, 20 mg.

² DEB: Dietary electrolyte balance

³ Dig: Digestible

finisher diets. The analysed composition of dietary treatments for total amino acids and GAA concentration is given in Table 2. The GAA content of the diets was measured using methods described by Dobenecker and Braun (2015). Birds were fed the experimental grower and finisher diets from d 10 to 24 and d 24 to 35 of age, respectively. From d 0 to 10 of age, all birds received the same commercial diet with protein content of 23.4% and AME of 2950 kcal/kg.

A total of 640 day-old male Ross 308 (off-sex) broilers were sourced from an Aviagen hatchery (Goulburn, NSW) and were transferred to the poultry facilities at the Roseworthy Campus of the University of Adelaide. Upon arrival, birds were weighed (average weight, 39.9 g) and

Table 2

Analysed total amino acid (mg/g) and guanidinoacetic acid (mg/kg) contents in experiment diets.

Amino Acid	Grower SP	Grower-RP	Grower- GAA50	Grower - GAAtop	Finisher SP	Finisher-RP	Finisher- GAA50	Finisher - GAAtop
His	5.6	5.1	5.1	5.2	5.5	4.7	4.8	4.7
Ser	10.3	9.4	9.3	9.5	10.2	8.8	8.8	8.5
Arg	12.8	13.2	12.3	13.2	12.5	12.2	11.5	11.7
Gly	8.8	9.5	9.5	9.6	8.6	9.1	9.1	8.6
Aspartic acid	20.3	17.7	17.7	17.9	20.0	16.5	16.6	15.9
Glutamic acid	42.7	40.1	40.0	40.3	41.8	37.5	37.4	37.3
Thr	9.1	9.1	9.1	9.1	8.7	8.4	8.4	8.0
Ala	9.3	8.4	8.3	8.5	9.5	8.2	8.2	8.1
Pro	12.9	12.3	12.2	12.3	12.7	11.6	11.5	11.5
Lys	13.0	13.3	13.2	13.1	12.4	12.3	12.3	11.4
Tyr	5.6	4.8	4.8	5.0	5.4	4.7	4.8	4.4
Met	5.8	5.4	5.3	5.2	4.6	5.1	5.4	5.1
Val	10.4	10.4	10.4	10.5	10.2	9.8	9.7	9.3
Ile	9.2	9.1	9.2	9.2	9.1	8.6	8.6	8.2
Leu	16.7	15.1	15.0	15.3	17.0	14.6	14.6	14.2
Phe	10.4	9.4	9.4	9.5	10.3	8.8	8.8	8.6
Total	202.9	192.2	190.8	193.1	198.6	180.8	180.7	175.4
Guanidinoacetic acid	BD	BD	813	800	BD	BD	946	830

BD: Below detection limit (20 mg/kg)

assigned to 64 raised pens each accommodating 10 birds in two separate rooms that had identical heating, cooling, and husbandry management. In each room, each diet was replicated 8 times. All birds were weighed on d 0, 10, 24 and 35 of age and feed consumption and residues were recorded for each pen. Feed conversion ratio was calculated by dividing the feed intake by the body weight gain of each pen and was adjusted for any mortalities that occurred.

From d 25 to 35, birds in one room were subjected to cyclic heat stress by increasing the room temperature to 32 ± 1 °C for 8 hours from 9 am to 5 pm daily maintaining a 10 °C difference compared with birds in the thermoneutral room. The heat stress was applied only during the finisher phase, as its effects are more pronounced in birds older than 21 d (Andretta et al. 2021). Birds had access to feed and water on an ad libitum basis throughout the study. The lighting program of 6 h dark and 18 h light was assigned to birds after d 5 of age. In the first 2 days birds had 24 hr light which was then reduced to 23 hr light and 1 hour darkness until d 5 of age.

Intestinal permeability test

To assess intestinal permeability, on d 27, one bird per pen (8 birds per treatment) was given an oral gavage of 4.16 mg fluorescein isothiocyanate–dextran (FITC-d, average mol wt 4000, Sigma Aldrich) per kg BW followed by blood collection from jugular vein after precisely 150 min. Blood samples were allowed to clot at room temperature for 3 h before serum was separated by centrifuging the vacutainers for 15 min at 1500 g and 4 °C. Serum samples were stored at -20 °C until used for analysis. The determination of FITC-d concentration in blood was according to the procedures previously described by Barekatin et al. (2021). A CLARIOstar® Plus multimode plate reader was used to measure the sample absorbance at the excitation of 485 and emission of 530 nm.

Sampling

On d 29, one bird per replicate was randomly chosen, weighed, and euthanized by cervical dislocation and the required samples were collected. The entire breast tissue (left side) was collected and stored at -20 °C until further analysis. A section of ileum was also cut and rinsed with PBS solution and subsequently frozen in liquid nitrogen before storage at -80 °C and was later used for gene expression analysis. Additional sections of jejunum and ileum were collected for creatine analysis. The caecal contents of each sampled bird were also collected and kept on dry ice before being stored at -80 °C until used for microbial DNA extraction.

Creatine content of muscle and intestinal tissues

Breast samples and intestinal tissues were sent on dry ice to Alzchem (Germany) for creatine measurements. The creatine in all the muscle and intestinal tissues was measured using previously validated techniques (Dobenecker and Braun, 2015).

Gene expression assays

The ileal tissues were homogenized in QIAzol Lysis Reagent (Qiagen) using an IKA T25 Ultra Turrax disperser as per previously described procedures (Barekatin et al., 2021). The RNA contents of the homogenates were then extracted using a Qiagen RNA Universal Mini Kit following the manufacturer's instructions. The procedures for dilution of RNA samples, cDNA synthesis and setting up the gene expression assays were similar to Barekatin et al. (2021). The PCR conditions included an initial 10 min denaturation at 95 °C, then 95 °C for 15 s, 40 cycles of 60 °C for 20 s, and 72 °C for 40 s. The list of primers and their sequences used in the gene expression assays are shown in Table 3. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and TATA-Box Binding Protein (TBP) were chosen as reference genes (Gilani et al., 2018). The data of each assayed gene was divided by the values obtained for TBP as the most stable reference gene. Therefore, all the reported values are relative to the TBP.

Microbiota analysis

DNA of all caecal samples was extracted using a QIAamp PowerFecal Pro DNA Kit (Qiagen) and following the manufacturer's instruction. The V3-V4 region of 16S rRNA genes was PCR amplified using primers 5' ACTCCTACGGGAGGCAGCAG 3' and 5' GGACTACHVGGGTWTCTAAT 3' with Q5-HF polymerase (New England Biolabs), with the dual barcoding method as described by Fadrosch et al. (2014). The PCR-cycling conditions were as follows: 98 °C for 1 min; 35 cycles of 98 °C for 10 sec; 49 °C for 30 sec; and 72 °C for 30 sec; and a final 10 min extension at 72 °C. Sequencing was performed on an Illumina MiSeq system (2 × 300 bp).

Sequence data was analysed using Quantitative Insights into Microbial Ecology 2 (QIIME2 v220.6) (Bolyen et al., 2019), and using the Divisive Amplicon Denoising Algorithm (DADA2) plugin for quality filtering, denoising, chimera removal and identification of amplicon sequence variants (ASVs). ASVs were taxonomically classified using the SILVA database (v138.1) (Callahan et al., 2016). An ASV abundance table with taxonomic assignments was produced for further analysis and visualisation of alpha and beta diversity, and biomarker identification, in the online MicrobiomeAnalyst system (Lu et al., 2023).

Table 3

The primer sequences of the selected genes used in PCR assays.

Gene name	Sequences (5'→3')	Reference
Claudin 1	F: AAGGTGTACGACTCGTGCT R: CAGCAACAAACACCAACC	(Gilani et al., 2018)
Claudin 2	F: CCTACATTGGTTCAAGCATCGTGA R: GATGTCGGGAGGCAGGTTGA	(Gong et al., 2020)
Claudin 3	F: GCCAAGATCACCATCGTCTC R: R: CACCAGCGGGTTGTAGAAAT	(Gilani et al., 2018)
IL1-β	F: CAGCCCGTGGGCATCA R: CTTAGCTTGTAGGTGGCGATGTT	(Chen et al., 2015)
IL-6	F: AGGACGAGATGTGCAAGAAGTTC R: TTGGGCAGGTTGAGGTTGTT	(Lee et al., 2018)
IL-10	F: ATGAACCTTAACATCCAACGTCTC R: TGTTCGCCAGGTGCGCCAT	(Xiao et al., 2018)
NFκB	F: GAAGGAATCGTACCGGAACA R: CTCAGAGGGCCTTGTGACAGTAA	(Lee et al., 2018)
MUC-2	F: ATTGAAGCCAGCAATGGTGT R: TTGTTGGCCTTGTGTCATCAA	(Gilani et al., 2018)
Nrf2	F: GGAAGAAGGTGCTTTTCGGAGC R: GGGCAAGGCAGATCTCTCCAA	(Lee et al., 2018)
GPx1	F: TCCCTGCAACCAATTGC R: AGCGCAGGATCTCTCGTT	(Greene et al., 2021)
HSP-70	F: GGCTGGAGAGAAGAATGTGC R: CAGCTGTGGACTTCACCTCA	(Gilani et al., 2018)
SGLT-1	F: TGCCGGAGTATCTGAGGAAG R: CCCATGGCCAACTGTATAA	(Gilani et al., 2018)
SOD-1	F: TGGCTTCCATGTGCATGAAT R: ACGACCTGCGCTGGTACAC	(Greene et al., 2021)
AMPK-α1	F: AAGGTTGGCAAGCATGAGTT R: TTCTGGGCTGCATATAACC	(Proszkowiec-Weglarz et al., 2006)
TBP	F: GTCCACGGTGAATCTTGGTT R: GCGCAGTAGTACGTGGTTCTC	(Gilani et al., 2018)
GAPDH	F: CAACCCCAATGTCTCTGTT R: TCAGCAGCAGCCTTCACTAC	(Gilani et al., 2018)

IL1-β: Interleukin 1-β; IL-6: Interleukin 6; IL-10: Interleukin 10; NFκB: Nuclear factor kappa B; MUC-2: Mucin 2; Nrf2: Nuclear factor erythroid 2-related factor 2; GPx1: glutathione peroxidase 1; HSP-70: Heat shock protein 70; SGLT-1: Sodium/glucose cotransporter 1; SOD1: superoxide dismutase; AMPK- α1: AMP-activated protein kinase alpha-1; TBP: Tata-Box binding protein; GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

After quality control and removal of rare amplicons (those with fewer than 10 reads in the data set), a total of 1,236,181 sequences was produced from 63 samples, giving an average of 19,621 sequences per sample. A total of 3,458 ASVs were found. The raw sequencing data of all samples were submitted to Sequence Read Archive database (PRJNA1183917).

Statistical analysis

The General Linear Model of SAS statistical package (9.4) was used to analyse the data after testing for normal distribution. Two-way ANOVA was used to assess the main effects of diets, room temperature and their interactions. For any statistically significant results, Fishers' Least Square Differences test was used to separate the means. The level of significant was set at $P < 0.05$ while $0.05 \leq P \leq 0.10$ was declared as a tendency.

Results

Growth performance

Table 4 shows the data for growth performance of the birds. There was no interaction between the dietary treatments and room temperature for any of the performance related parameters. From d 10 to 24 of age, there were no differences between dietary treatments for feed

intake, body weight gain or FCR.

From d 24 to 35, birds fed RP diets had lower feed intake than birds fed SP diet with the lowest feed intake observed for birds fed GAA50 ($P < 0.001$). When assessed for the entire study period (d 10 to 35), birds fed the SP diet had the highest ($P < 0.01$) feed intake when compared with GAA50 and GAAtop. From d 24 to 35 and d 10 to 35, birds under heat stress consumed less ($P < 0.0001$) feed compared with birds in the thermoneutral group.

Body weight gain of the birds was not affected by dietary treatments at any stage of the experiment. Heat stress independently reduced ($P < 0.0001$) BWG during the finisher phase and when assessed from d 10 to 35 of age.

Birds fed GAA50 and GAAtop tended to have lower FCR compared with other birds from d 24 to 35 ($P = 0.086$) and the entire study ($P = 0.082$).

Creatine concentration in muscle, ileum and jejunum

As shown in Table 5, there was no interaction between diet and room temperature for creatine content in the breast, jejunum, and ileum of broilers. The supplementation of GAA on top of the Arg led to the highest level of creatine in breast muscle followed by GAA50 whereas the birds fed RP diet without GAA had the lowest creatine in breast muscle ($P < 0.0001$). Heat stress did not significantly alter creatine content in breast muscle, jejunal, or ileal tissues. However, while diets had no effect, birds under heat stress tended ($P = 0.078$) to have higher creatine in the jejunum. Either of the GAA supplemented diets markedly increased ($P < 0.0001$) creatine concentration in the ileal tissues.

Fluorescein isocyanate dextran-D concentration in serum (intestinal permeability test)

There was no interaction between the room temperature and diet for the serum FITC-d concentration (Fig. 1). Heat stress significantly elevated ($P < 0.01$) FITC-d in serum samples compared with birds in the thermoneutral group. There was no significant difference between dietary treatments for the serum concentration of FITC-d.

Gene expression in the ileum

The relative mRNA expression of selected genes in the ileum of broilers are shown in Tables 6 and 7. Diet and room temperature did not interact for any of the quantified genes. Considering the main effects, diet only affected the expression of IL1β, where GAA50 downregulated ($P < 0.01$) the mRNA expression compared with SP and RP treatments. Heat stress markedly upregulated the gene expression of claudin 2 ($P < 0.05$), claudin 3 ($P < 0.0001$), GPX-1 ($P < 0.05$), HSP70 ($P < 0.01$), IL1β ($P < 0.01$), IL10 ($P < 0.05$), NFκB ($P < 0.001$), SOD-1 ($P < 0.001$) and AMPK-α1 ($P < 0.001$).

The expression of claudin 1, IL6, MUC2, Nrf2 and SGLT1 remained unaffected by either dietary treatments or room temperature.

Caecal microbiota composition

The caecal microbiota of broilers at the phylum level was predominantly comprised of Firmicutes, Actinobacteriota, Campilobacteriota, Bacteroidota, Proteobacteria and Cyanobacteria, whereby Firmicutes comprised on average over 80% of the bacteria in all groups (Fig. 2). Microbiota characterization at the family level is presented in Fig. 3 and demonstrates the animal-to-animal variation in microbiota composition within treatments. All alpha (Shannon diversity, $P = 0.396$) and beta diversity (PERMANOVA; $P = 0.888$, $R^2 = 0.032$) analysis revealed no significant differences between treatment groups.

Table 4

– Growth performance of broiler chickens fed experimental diets with and without cyclic heat stress from day 25 to 35 of age.

		BWG (g/bird)			Feed intake (g/bird)			FCR (feed/gain)		
		d10-24	d24-35	d10-35	d10-24	d24-35	d10-35	d10-24	d24-35	d10-35
Temperature										
SP ¹	TN ⁵	1127	1445	2582	1398	2290	3693	1.242	1.587	1.431
RP ²	TN	1123	1389	2524	1404	2234	3644	1.251	1.609	1.444
RP-GAA50 ³	TN	1111	1422	2532	1380	2189	3574	1.243	1.541	1.412
RP-GAAtop ⁴	TN	1124	1423	2544	1385	2222	3603	1.232	1.562	1.417
SP	HS ⁶	-	1246	2362	-	2013	3406	-	1.617	1.443
RP	HS	-	1232	2342	-	1969	3366	-	1.600	1.438
RP-GAA50	HS	-	1208	2318	-	1898	3273	-	1.571	1.412
RP-GAAtop	HS	-	1229	2356	-	1970	3358	-	1.603	1.425
SEM ⁷		3.8	5.2	7.1	4.5	7.8	10.4	0.0026	0.0069	0.0042
Main effects										
Diet										
SP ¹		1127	1346	2472	1398	2151 ^a	3549 ^a	1.242	1.602	1.437
RP ²		1123	1310	2433	1404	2101 ^b	3505 ^{ab}	1.251	1.604	1.441
RP-GAA50 ³		1111	1315	2425	1380	2043 ^c	3423 ^c	1.243	1.556	1.412
RP-GAAtop ⁴		1124	1326	2450	1385	2096 ^b	3481 ^{bc}	1.232	1.583	1.421
Temperature										
TN		-	1419 ^a	2546 ^a	-	2233 ^a	3628 ^a	-	1.575	1.426
HS		-	1229 ^b	2344 ^b	-	1962 ^b	3351 ^b	-	1.597	1.429
P values										
Diet		0.504	0.12	0.15	0.230	0.0004	0.002	0.139	0.086	0.082
Room temperature		-	<.0001	<.0001	-	<.0001	<.0001	-	0.13	0.66
Diet x Room temperature			0.31	0.77		0.87	0.81		0.65	0.90

^{a-c} Means that do not share a same superscript letter are significantly different at the P-level shown for the main effects. Values for d10 to 24 are from both rooms.¹ SP: Standard protein diet² RP: Reduced protein diet³ RP-GAA50: Reduced protein diet supplemented with GAA replacing 50% of supplemented Arg⁴ RP-GAAtop: Reduced protein diet supplemented with GAA on top of the supplemented Arg⁵ TN: Thermoneutral⁶ HS: Heat stress⁷ SEM: Pooled standard error of the mean**Table 5**

– Creatine concentration (mg/kg) in muscle, jejunum and ileum of broilers on d 29 of age.

Main effects	Breast	Jejunum	Ileum
Diet			
SP ¹	4300 ^{bc}	250	232 ^b
RP ²	4159 ^c	243	229 ^b
RP-GAA50 ³	4425 ^b	250	290 ^a
RP-GAAtop ⁴	5035 ^a	263	311 ^a
Temperature			
Thermoneutral	4488	258	243
Heat stress	4471	273	260
SEM ⁵	31.1	4.7	4.9
P values			
Diet	<.0001	0.48	<.0001
Room temperature	0.79	0.078	0.13
Diet x Room temperature	0.74	0.29	0.59

^{a-c} Means that do not share a same superscript letter are significantly different at the P-level shown for the main effects.¹ SP: Standard protein diet² RP: Reduced protein diet³ RP-GAA50: Reduced protein diet supplemented with GAA replacing 50% of supplemented Arg⁴ RP-GAAtop: Reduced protein diet supplemented with GAA on top of the supplemented Arg⁵ SEM: Pooled standard error of the mean

Discussion

Birds in this study outperformed the breed standards under thermoneutral conditions (Aviagen, 2022) even with a 2-percentage point reduction of dietary crude protein. The reduced dietary protein provided an opportunity to test the GAA supplementation both as a partial replacement of Arg as well as its independent inclusion. The lack of

interaction between diets and room temperature for all the parameters in this study highlights that the effect of GAA supplementation and a moderate reduction of dietary crude protein can be considered independent in both thermoneutral, and heat stressed birds. Hence, the discussion is devoted to the main effects. The muscle creatine loading and Arg-spring effect resulting from GAA supplementation have been shown to be particularly effective in alleviating the detrimental effect of heat stress in broilers (Majdeldin et al., 2020). Albeit only by tendency, the overall FCR of the birds that received GAA was lower compared with other treatments. This result corroborates with the findings of several other studies as reviewed by Khajali et al. (2020). In the current study, GAA used in both diets increased the creatine deposition in breast muscle. Despite not being significantly different, birds fed GAA50 tended to have lower FCR than GAAtop in the finisher phase. Two plausible explanations can explain this tendency. First, the Arg requirement of birds fed GAAtop may have already been satisfied by the supplemented Arg under the circumstances of this study. Second, the analysis of the treatment GAAtop at the finisher phase revealed a slightly lower concentration of GAA compared with the treatment GAA50 which may have contributed to this observation.

In this study, thermal stress was used more in the context of gut health and barrier function rather than performance or other routine heat stress related parameters (i.e. panting, rectal temperature) as it was already known that the GAA would be effective under high environmental temperature. Nonetheless, the reduced feed intake and BWG of the birds under heat stress were expected responses to restore homeostasis and redirecting energy to avoid elevation of body temperature which limits the available energy and nutrients for body growth (Apalowo et al., 2024).

When GAA was used to replace 50% of supplemented Arg, the feed intake was reduced, independent of the heat stress. The exact mechanism of such reduction is not known but it is consistent with some other studies (Majdeldin et al., 2023). In the literature, there were some

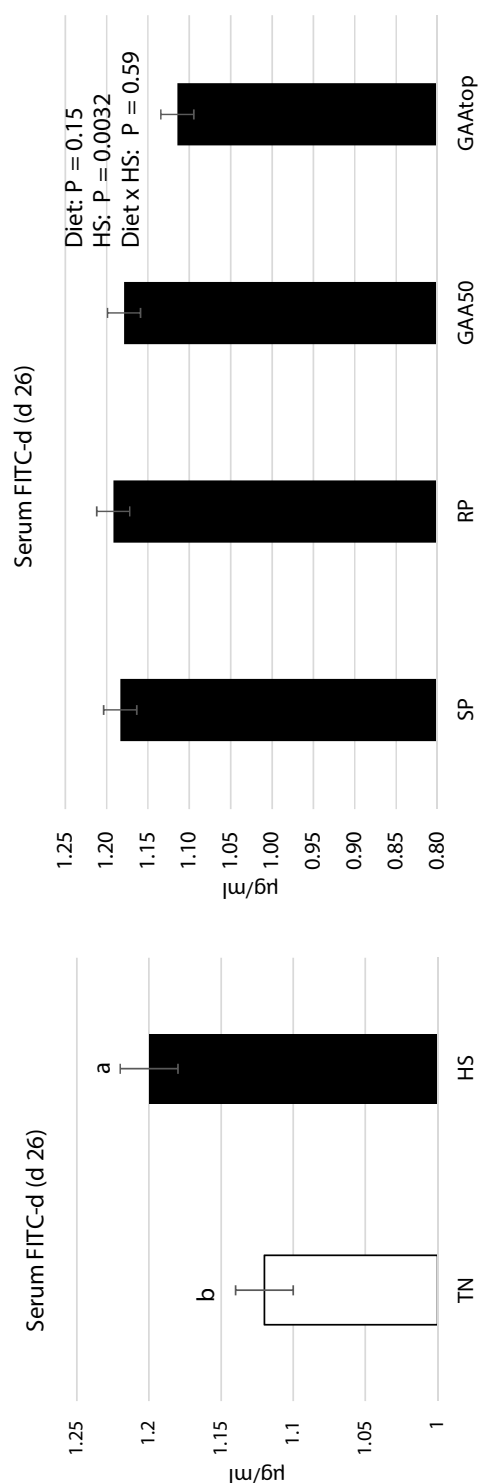


Fig. 1. Serum concentration of FITC-d ($\mu\text{g}/\text{ml}$) at d 26 of age. TN: Thermoneutral; HS: Heat stress; SP: Standard protein diet; RP: Reduced protein diet; GAA50: Reduced protein diet supplemented with GAA replacing 50% of supplemented Arg. GAAtop: Reduced protein diet supplemented with GAA on top of the supplemented Arg.

speculations that GAA may affect cellular energy or reduce the stimulation of AMP-activated protein kinase (AMPK) as the main sensor of cellular energy status, which could in turn affect the energy metabolism and feed intake (Majdoddin et al., 2023). In the present study, the expression of AMPK- $\alpha 1$ (catalytic subunit alpha) was quantified in the ileum but no difference was observed between the dietary treatments and as such there was no correlation of this gene expression to any effect observed in birds fed GAA. Quantification of this gene in the liver is likely to be more informative in future studies. However, the over-expression of AMPK- $\alpha 1$ in birds under heat stress is related to the activation of AMPK in response to a decline in intracellular ATP levels as seen in heat stressed birds (Liang et al., 2020). AMPK promotes catabolic pathways to produce more ATP and impedes anabolic pathways.

In the present study, cyclic heat stress during the finisher phase of feeding was used as a relevant model to induce dysfunction of the intestinal barrier in broilers. Heat stress has consequences for intestinal barrier function by causing hyperthermia and hypoxia resulting from repartitioning the blood. Specifically, the peripheral blood flow increases whereas the splanchnic blood flow decreases leading to intestinal hypoxia and the breakdown of intestinal integrity (Lian et al., 2020). In the current study, the increased passage of FITC-d into blood confirmed the elevated intestinal permeability in birds under hyperthermia. The unaltered intestinal permeability between different dietary treatments indicated that the diets likely provided sufficient nutrients to maintain the intestinal integrity. A moderate reduction of dietary protein is not expected to adversely impact the intestinal integrity of broiler chickens compared with an excessive protein reduction (Barekatain et al., 2023; Barekatain et al., 2019b). There had previously been an indication of improved intestinal barrier function when broilers were fed with additional Arg above the breed specification (Barekatain et al., 2021). It is plausible to assume that the mechanisms and the dietary levels by which Arg impacts intestinal integrity may be different to what could be expected as an indirect sparing effect of GAA supplemented on the top or when partially replacing Arg.

Tight junction proteins control intestinal permeability to avoid the passage of undesirable toxins, pathogens, microorganism and regulate the diffusion of ions and solutes across the epithelium (Anderson and Van Itallie, 2009). The mRNA of two barrier-forming tight junction proteins, claudin 1 and 3, as well as a pore-forming claudin 2, were quantified. Reduction of dietary protein or addition GAA had no effect on the expression of these tight junction genes, which is consistent with the unaltered intestinal permeability reported in the study. A recent study by Peng et al. (2023) also showed no effect of GAA on gene expression of selected tight junction protein encoding genes in the jejunum of broilers. To our knowledge, there are no other studies on the effect of GAA on the expression of tight junction genes and permeability in poultry. Research in other species supports that creatine alongside creatine transporter 1 and creatine kinase and phosphocreatine affect tight junction integrity in the intestinal epithelial cell (Wallimann et al., 2021). The data of the present study does not support the notion that GAA, as the precursor of creatine, could influence the assembly of tight junctions or permeability. Future studies with highly deficient Arg or gene knockout studies may provide insight into a similar role for GAA as for creatine.

Intestinal tight junction proteins are constantly remodelled and respond to thermal stress in a differential and time dependent manner (Uerlings et al., 2018). Increase in the mRNA expression of claudin 2 and 3 genes in response to hyperthermia can be explained by elevated intestinal permeability and possible physiological defense mechanisms to restore intestinal function in those birds. Higher expression of claudin 2 is specifically associated with a leaky epithelium (Oami et al., 2024). In the present study, the unaffected claudin 1 expression after 4 days of heat exposure is consistent with Uerlings et al. (2018).

The production of reactive oxygen species (ROS) induced by heat stress overwhelms the cellular redox defense system including glutathione, glutathione peroxidase, superoxide dismutase (SOD), and haem

Table 6

– Relative mRNA expression of selected genes in the ileum of broiler chickens on d 29 of age.

Main effects	<i>Claudin 1</i>	<i>Claudin 2</i>	<i>Claudin 3</i>	<i>GPX-1</i>	<i>HSP70</i>	<i>IL1β</i>	<i>IL6</i>	<i>IL10</i>
<i>Diet</i>								
SP ¹	1.10	0.86	0.45	1.05	1.47	1.30 ^a	0.54	0.60
RP ²	1.08	0.76	0.46	1.07	1.48	1.22 ^a	0.53	0.91
RP-GAA50 ³	1.29	0.73	0.46	1.08	1.62	0.95 ^b	0.47	0.72
RP-GAAtop ⁴	1.07	0.77	0.43	0.98	1.38	1.15 ^{ab}	0.55	0.73
<i>Temperature</i>								
Thermoneutral	1.10	0.70 ^b	0.41 ^b	0.98 ^b	1.17 ^b	1.05 ^b	0.49	0.57 ^b
Heat stress	1.16	0.85 ^a	0.49 ^a	1.11 ^a	1.80 ^a	1.25 ^a	0.55	0.91 ^a
SEM ⁵	0.054	0.028	0.010	0.028	0.099	0.036	0.060	0.065
<i>P values</i>								
Diet	0.42	0.43	0.63	0.56	0.87	0.0092	0.97	0.42
Room temperature	0.59	0.012	0.0001	0.030	0.0022	0.0072	0.589	0.014
Diet x Room temperature	0.38	0.45	0.93	0.18	0.92	0.51	0.17	0.39

^{a-b} Means that do not share a same superscript letter are significantly different at the P-level shown for the main effects.¹ SP: Standard protein diet² RP: Reduced protein diet³ RP-GAA50: Reduced protein diet supplemented with GAA replacing 50% of supplemented Arg⁴ RP-GAAtop: Reduced protein diet supplemented with GAA on top of the supplemented Arg⁵ SEM: Pooled standard error of the mean**Table 7**

– Relative mRNA expression of selected genes in the ileum of broiler chickens on d 29 of age.

Main effects	<i>MUC2</i>	<i>NFκB</i>	<i>Nrf2</i>	<i>SGLT1</i>	<i>SOD-1</i>	<i>AMPK-α1</i>
<i>Diet</i>						
SP ¹	1.05	0.43	0.85	0.85	1.07	1.60
RP ²	1.07	0.45	0.78	0.81	1.01	1.55
RP-GAA50 ³	1.19	0.43	0.90	0.80	1.04	1.59
RP-GAAtop ⁴	1.10	0.44	0.87	0.74	0.97	1.58
<i>Temperature</i>						
Thermoneutral	1.06	0.42 ^b	0.83	0.80	0.92 ^b	1.39 ^b
Heat stress	1.14	0.46 ^a	0.86	0.79	1.11 ^a	1.77 ^a
SEM ⁵	0.028	0.006	0.031	0.020	0.019	0.027
<i>P values</i>						
Diet	0.26	0.77	0.57	0.29	0.25	0.89
Room temperature	0.15	0.0008	0.63	0.93	<.0001	<.0001
Diet x Room temperature	0.19	0.68	0.90	0.68	0.90	0.38

^{a-c} Means that do not share a same superscript letter are significantly different at the P-level shown for the main effects.¹ SP: Standard protein diet² RP: Reduced protein diet³ RP-GAA50: Reduced protein diet supplemented with GAA replacing 50% of the supplemented Arg⁴ RP-GAAtop: Reduced protein diet supplemented with GAA on top of the supplemented Arg⁵ SEM: Pooled standard error of the mean

oxygenase 1 (Lian et al., 2020). The overexpression of mRNA associated with antioxidant enzymes, SOD-1 and GPX-1 in heat stressed birds can be regarded as protective responses against surplus superoxide formation in those birds (Akbarian et al., 2016). The observed 54% increase in the mRNA abundance of heat shock protein 70 (HSP70) may also be a physiological response in heat stressed birds to prevent the aggregation of actin fibers in the intestinal epithelial cells (Dokladny et al., 2016). HSP70 also possesses anti-inflammatory properties and can impact tight junction assembly and intestinal permeability (Bidmon-Fliegenschnee et al., 2015). The supplemented GAA or the type of diet had no impact on these genes. The lack of GAA effect on HSP70 is, however, consistent with another study (Li et al., 2023).

Through the action of the phosphocreatine shuttle, creatine helps transfer high-energy phosphate from the mitochondria to the cytosol which may impact the intestinal barrier function. Under inflammation, creatine facilitates the process of cell survival and maintains pathways involved in cell proliferation (Turer et al., 2017). Although most creatine is deposited in the muscle, it can find its way to other tissues including the intestine. Our data demonstrated that GAA supplementation as a precursor of creatine synthesis increased the creatine concentration in the ileum of the birds irrespective of whether it replaced Arg or was added on its own. The differences between dietary treatments for

intestinal creatine content were only seen in the ileum, not the jejunum. This observation may be related to different creatine uptake at jejunum and ileum as shown in rats (Peral et al., 2005).

Among all the immune related genes only IL-1 β responded to the dietary treatment where GAA downregulated the expression. IL-1 β is a pro-inflammatory cytokine and its expression is increased under HS conditions. This result is consistent with another recent study (Peng et al., 2023) which may indicate some anti-inflammatory effects of GAA. It should, however, be noted that change in the expression of only one gene, in isolation, is insufficient to discern any conclusive effect associated with GAA for intestinal health. In future it would be informative to undertake complete RNA-Seq analysis to characterize the total transcriptome under these feed and HS conditions.

Data is sparse on the effect of dietary GAA on the intestinal microbiota in poultry. It was hypothesized that GAA may indirectly influence microbiota through an Arg-sparing effect (Zhang et al., 2018) or impact the synthesis of GAA by the gut microbiota (Ostojic, 2020). The lack of the effect of GAA supplementation on cecal microbiota is consistent with a recent study that used limited enumeration culture techniques (Hossain et al., 2024), although the present study is the first to consider it in the context of reduced protein diets. The lack of differences between heat stress and thermoneutral birds for caecal microbiota composition

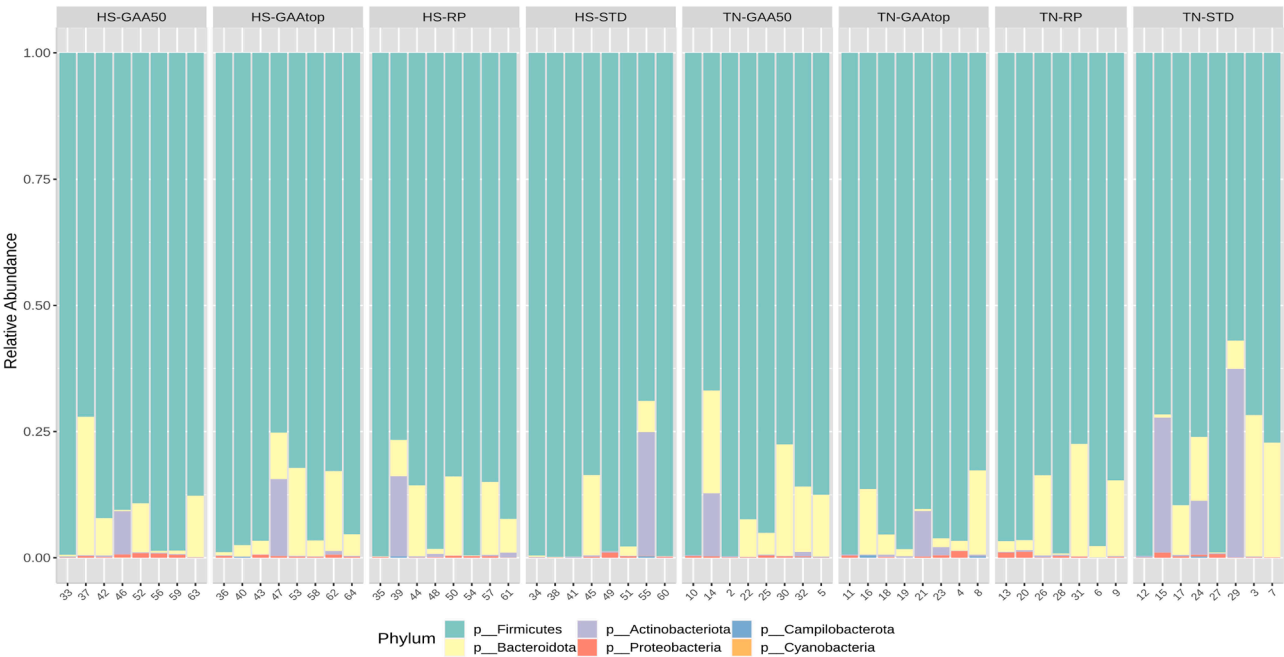


Fig. 2. Bacterial phylum in the caeca of broiler chickens at d 26 of age. TN: Thermoneutral; HS: Heat stress; SP: Standard protein diet; RP: Reduced protein diet. GAA50: Reduced protein diet supplemented with guanidinoacetic acid replacing 50% of supplemented Arg, GAAtop: Reduced protein diet supplemented with guanidinoacetic acid on top of the supplemented Arg.



Fig. 3. Bacterial families in the caeca of broiler chickens at d 26 of age. TN: Thermoneutral; HS: Heat stress; SP: Standard protein diet; RP: Reduced protein diet. GAA50: Reduced protein diet supplemented with guanidinoacetic acid replacing 50% of supplemented Arg, GAAtop: Reduced protein diet supplemented with guanidinoacetic acid on top of the supplemented Arg.

was unexpected. While the exact reason for the lack of differences cannot be ascertained by the data of the present study, the time of sampling, only 4 days after the commencement of the heat stress regime as well as the cyclic nature of the applied HS, may have contributed to the lack of difference in caecal microbiota diversity and composition.

Using a chronic heat stress model throughout the study, [Schreier et al. \(2022\)](#) found limited effects of hyperthermia on caecal microbiota and could not associate any main changes in microbiota solely due to heat stress in a combined and separate *Enterococcus cecorum* infection and heat stress model. In contrast, using 16S rRNA sequencing, a wide range

of differences in abundance and species diversity, particularly at the phylum level, caused by heat stress were observed in another study (Shi et al., 2019). Other studies have also observed changes in microbiota composition associated with heat stress in poultry, but the extent of the temperature increase, duration, and experimental conditions differ from the present study (Hatipoglu et al., 2024; Liu et al., 2020). It should be acknowledged that in the current study, there was considerable animal to animal variation in microbiota composition within the groups. Because of this variation, it was not possible to discern any statistically significant differences between groups in either alpha or beta diversity. The observed high sample variation within treatment groups has been previously noted in other studies (Stanley et al., 2013).

The results of the present study indicate that depending on the required Arg supplementation, GAA can partially replace Arg with a consistent tendency to improve the FCR of broilers under thermoneutral and HS conditions, demonstrated by the lack of interaction between diets and heat stress. Despite an increase of creatine concentration in the ileum of the birds, under the conditions of the present study, there was no conclusive evidence that the GAA supplementation *per se* could directly impact intestinal permeability, barrier function or caecal microbiota composition. More comprehensive analysis including transcriptomics in future studies can be suggested to provide more insights into any immune system regulation or changes in metabolic pathways not specifically studied here. A cyclic heat stress during the finisher phase was shown to have a profound impact on intestinal permeability, expression of genes related to tight junction protein, oxidative stress, inflammation, and AMPK, making it an attractive model of intestinal barrier function in broiler chickens.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: The co-author is employed by Alzchem GmbH as the funder of the project. VI. The co-author is employed by Feedworks Ltd that sells guanidinoacetic acid. DC.

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References

- Akbadian, A., Michiels, J., Degroote, J., Majdeddin, M., Golian, A., De Smet, S., 2016. Association between heat stress and oxidative stress in poultry; mitochondrial dysfunction and dietary interventions with phytochemicals. *J. Anim. Sci. Biotechnol.* 7, 1–14.
- Anderson, J.M., Van Itallie, C.M., 2009. Physiology and function of the tight junction. *Cold Spring Harb. Perspect. Biol.* 1, a002584.
- Andretta, I., Kipper, M., Schirrmann, G.D., Franceschina, C.S., Ribeiro, A.M.L., 2021. Modeling the performance of broilers under heat stress. *Poult. Sci.* 100, 101338.
- Apalowo, O.O., Ekunseitan, D.A., Fasina, Y.O., 2024. Impact of heat stress on broiler chicken production. *Poultry* 3, 107–128.
- Aviagen, 2022. Ross 308 Broiler: Performance Objectives, 2022. Ross Breeders Limited, Newbridge, Midlothian, Scotland, UK.
- Barekatain, R., Chalvon-Demersay, T., McLaughlan, C., Lambert, W., 2021. Intestinal barrier function and performance of broiler chickens fed additional arginine, combination of arginine and glutamine or an amino acid-based solution. *Animals* 11, 2416.
- Barekatain, R., Chrystal, P., Howarth, G., McLaughlan, C., Gilani, S., Nattrass, G., 2019a. 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. *Poult. Sci.* 98, 6761–6771.
- Barekatain, R., Chrystal, P.V., Nowland, T., Moss, A.F., Howarth, G.S., Van, T.T.H., Moore, R.J., 2023. Negative consequences of reduced protein diets supplemented with synthetic amino acids for performance, intestinal barrier function, and caecal microbiota composition of broiler chickens. *Anim. Nutr.* 13, 216–228.
- Barekatain, R., Nattrass, G., Tilbrook, A., Chousalkar, K., Gilani, S., 2019b. Reduced protein diet and amino acid concentration alter intestinal barrier function and performance of broiler chickens with or without synthetic glucocorticoid. *Poult. Sci.* 98, 3662–3675.
- Bidmon-Fliegenschnee, B., Lederhuber, H.C., Csacsich, D., Pichler, J., Herzog, R., Memaran-Dadgar, N., Huber, W.-D., Aufrecht, C., Kratochwill, K., 2015. Overexpression of Hsp70 confers cytoprotection during gliadin exposure in Caco-2 cells. *Pediatr. Res.* 78, 358–364.
- Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghathil, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat. Biotechnol.* 37, 852–857.
- Bonilla, D.A., Kreider, R.B., Stout, J.R., Forero, D.A., Kerksick, C.M., Roberts, M.D., Rawson, E.S., 2021. Metabolic basis of creatine in health and disease: a bioinformatics-assisted review. *Nutrients* 13, 1238.
- Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583.
- Chen, J., Tellez, G., Richards, J.D., Escobar, J., 2015. Identification of potential biomarkers for gut barrier failure in broiler chickens. *Front. Vet. Sci.* 2. <https://doi.org/10.3389/fvets.2015.00014>. <https://pmc.ncbi.nlm.nih.gov/articles/PMC4672187/pdf/fvets-02-00014.pdf>.
- Dobenecker, B., Braun, U., 2015. Creatine and creatinine contents in different diet types for dogs—effects of source and processing. *J. Anim. Physiol. Anim. Nutr. (Berl.)* 99, 1017–1024.
- Dokladny, K., Zuhl, M.N., Moseley, P.L., 2016. Intestinal epithelial barrier function and tight junction proteins with heat and exercise. *J. Appl. Physiol.* 120, 692–701.
- Fadrosh, D.W., Ma, B., Gajer, P., Sengamalai, N., Ott, S., Brotman, R.M., Ravel, J., 2014. An improved dual-indexing approach for multiplexed 16S rRNA gene sequencing on the Illumina MiSeq platform. *Microbiome* 2, 1–7.
- Fouad, A., El-Senousey, H., Yang, X., Yao, J., 2013. Dietary L-arginine supplementation reduces abdominal fat content by modulating lipid metabolism in broiler chickens. *Animal* 7, 1239–1245.
- Gilani, S., Howarth, G., Nattrass, G., Kitesa, S., Barekatain, R., Forder, R., Tran, C., Hughes, R., 2018. Gene expression and morphological changes in the intestinal mucosa associated with increased permeability induced by short-term fasting in chickens. *J. Anim. Physiol. Anim. Nutr. (Berl.)* 102, e653–e661.
- Glover, L.E., Bowers, B.E., Saeedi, B., Ehrentraut, S.F., Campbell, E.L., Bayless, A.J., Dobrinskikh, E., Kendrick, A.A., Kelly, C.J., Burgess, A., 2013. Control of creatine metabolism by HIF is an endogenous mechanism of barrier regulation in colitis. *Proc. Natl. Acad. Sci.* 110, 19820–19825.
- Gong, Y., Xia, W., Wen, X., Lyu, W., Xiao, Y., Yang, H., Zou, X., 2020. Early inoculation with caecal fermentation broth alters small intestine morphology, gene expression of tight junction proteins in the ileum, and the caecal metabolomic profiling of broilers. *J. Anim. Sci. Biotechnol.* 11, 1–13.
- Greene, E.S., Emami, N.K., Dridi, S., 2021. Research note: phytochemicals modulate the expression profile of circulating inflammasome and cyto (chemo) kine in whole blood of broilers exposed to cyclic heat stress. *Poult. Sci.* 100, 100801.
- Hatipoglu, D., Senturk, G., Aydin, S.S., Kirar, N., Top, S., Demircioglu, I., 2024. Ryegrass-derived probiotics alleviate heat stress effects on broiler growth, health, and gut microbiota. *J. Therm. Biol.* 119, 103771.
- Hossain, M.M., Cho, S.B., Kang, D.-K., Nguyen, Q.T., Kim, I.H., 2024. Comparative effects of dietary herbal mixture or guanidinoacetic acid supplementation on growth performance, cecal microbiota, blood profile, excreta gas emission, and meat quality in Hanhyup-3-ho chicken. *Poult. Sci.* 103, 103553.
- Khajali, F., Lemme, A., Rademacher-Heilshorn, M., 2020. Guanidinoacetic acid as a feed supplement for poultry. *J. World's Poult. Sci.* 76, 270–291.
- Kidd, M., Maynard, C., Mullenix, G., 2021. Progress of amino acid nutrition for diet protein reduction in poultry. *J. Anim. Sci. Biotechnol.* 12, 1–9.
- Lee, M., Lin, W., Wang, S., Lin, L., Yu, B., Lee, T., 2018. Evaluation of potential antioxidant and anti-inflammatory effects of Antrodia cinnamomea powder and the underlying molecular mechanisms via Nrf2- and NF- κ B-dominated pathways in broiler chickens. *Poult. Sci.* 97, 2419–2434.
- Li, X., Bian, J., Xing, T., Zhao, L., Li, J., Zhang, L., Gao, F., 2023. Effects of guanidinoacetic acid supplementation on growth performance, hypothalamus-pituitary-adrenal axis, and immunity of broilers challenged with chronic heat stress. *Poult. Sci.* 102, 103114.
- Lian, P., Braber, S., Garssen, J., Wichers, H.J., Folkerts, G., Fink-Gremmels, J., Varasteh, S., 2020. Beyond heat stress: intestinal integrity disruption and mechanism-based intervention strategies. *Nutrients* 12, 734.
- Liang, Y., Tabler, G.T., Dridi, S., 2020. Sprinkler technology improves broiler production sustainability: from stress alleviation to water usage conservation: a mini review. *Front. Vet. Sci.* 7, 544814.
- Liu, G., Zhu, H., Ma, T., Yan, Z., Zhang, Y., Geng, Y., Zhu, Y., Shi, Y., 2020. Effect of chronic cyclic heat stress on the intestinal morphology, oxidative status and cecal bacterial communities in broilers. *J. Therm. Biol.* 91, 102619.
- Lu, Y., Zhou, G., Ewald, J., Pang, Z., Shiri, T., Xia, J., 2023. MicrobiomeAnalyst 2.0: comprehensive statistical, functional and integrative analysis of microbiome data. *Nucleic Acids Res.* 51, W310–W318.
- Majdeddin, M., Braun, U., Lemme, A., Golian, A., Kermanshahi, H., De Smet, S., Michiels, J., 2020. Guanidinoacetic acid supplementation improves feed conversion in broilers subjected to heat stress associated with muscle creatine loading and arginine sparing. *Poult. Sci.* 99, 4442–4453.
- Majdeddin, M., Braun, U., Lemme, A., Golian, A., Kermanshahi, H., De Smet, S., Michiels, J., 2023. Effects of feeding guanidinoacetic acid on oxidative status and creatine metabolism in broilers subjected to chronic cyclic heat stress in the finisher phase. *Poult. Sci.* 102, 102653.

- Mueller, K., Kokotilo, M.S., Carter, J.M., Thiesen, A., Madsen, K.L., Studzinski, J., Khadaroo, R.G., Churchill, T.A., 2018. Creatine-loading preserves intestinal barrier function during organ preservation. *Cryobiology* 84, 69–76.
- Oami, T., Abtahi, S., Shimazui, T., Chen, C.-W., Sweat, Y.Y., Liang, Z., Burd, E.M., Farris, A.B., Roland, J.T., Tsukita, S., 2024. Claudin-2 upregulation enhances intestinal permeability, immune activation, dysbiosis, and mortality in sepsis. *Proc. Natl. Acad. Sci.* 121, e2217877121.
- Ostojic, S.M., 2020. Human gut microbiota as a source of guanidinoacetic acid. *Med. Hypotheses* 142, 109745.
- Peng, X.Y., Xing, T., Li, J.L., Zhang, L., Jiang, Y., Gao, F., 2023. Guanidinoacetic acid supplementation improves intestinal morphology, mucosal barrier function of broilers subjected to chronic heat stress. *J. Anim. Sci.* 101, skac355.
- Peral, M., Galvez, M., Soria, M., Ilundain, A., 2005. Developmental decrease in rat small intestinal creatine uptake. *Mech. Ageing Dev.* 126, 523–530.
- Proszkowiec-Weglarz, M., Richards, M.P., Ramachandran, R., McMurtry, J.P., 2006. Characterization of the AMP-activated protein kinase pathway in chickens. *Comp. Biochem. Physiol. B. Biochem. Mol. Biol.* 143, 92–106.
- Schreier, J., Rychlik, I., Karasova, D., Crhanova, M., Breves, G., Rautenschlein, S., Jung, A., 2022. Influence of heat stress on intestinal integrity and the caecal microbiota during *Enterococcus cecorum* infection in broilers. *Vet. Res.* 53, 110.
- Sharma, N.K., Cadogan, D.J., Chrystal, P.V., McGilchrist, P., Wilkinson, S.J., Inhuber, V., Moss, A.F., 2022. Guanidinoacetic acid as a partial replacement to arginine with or without betaine in broilers offered moderately low crude protein diets. *Poult. Sci.* 101, 101692.
- Shi, D., Bai, L., Qu, Q., Zhou, S., Yang, M., Guo, S., Li, Q., Liu, C., 2019. Impact of gut microbiota structure in heat-stressed broilers. *Poult. Sci.* 98, 2405–2413.
- Stanley, D., Geier, M.S., Hughes, R.J., Denman, S.E., Moore, R.J., 2013. Highly variable microbiota development in the chicken gastrointestinal tract. *PloS one* 8, e84290.
- Turer, E., McAlpine, W., Wang, K.-w., Lu, T., Li, X., Tang, M., Zhan, X., Wang, T., Zhan, X., Bu, C.-H., 2017. Creatine maintains intestinal homeostasis and protects against colitis. *Proc. Natl. Acad. Sci.* 114, E1273–E1281.
- Uerlings, J., Song, Z., Hu, X., Wang, S., Lin, H., Buyse, J., Everaert, N., 2018. Heat exposure affects jejunal tight junction remodeling independently of adenosine monophosphate-activated protein kinase in 9-day-old broiler chicks. *Poult. Sci.* 97, 3681–3690.
- Uyanga, V.A., Sun, L., Liu, Y., Zhang, M., Zhao, J., Wang, X., Jiao, H., Onagbesan, O.M., Lin, H., 2023. Effects of arginine replacement with L-citrulline on the arginine/nitric oxide metabolism in chickens: an animal model without urea cycle. *J. Anim. Sci. Biotechnol.* 14, 9.
- Wallimann, T., Hall, C.H., Colgan, S.P., Glover, L.E., 2021. Creatine supplementation for patients with inflammatory bowel diseases: a scientific rationale for a clinical trial. *Nutrients* 13, 1429.
- Xiao, M., Mi, Y., Liu, L., Lv, C., Zeng, W., Zhang, C., Li, J., 2018. Taurine regulates mucosal barrier function to alleviate lipopolysaccharide-induced duodenal inflammation in chicken. *Amino acids* 50, 1637–1646.
- 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*. *Front. Microbiol.* 9, 1716.