Intestinal permeability, microbiota composition, and expression of genes related to intestinal barrier function of broiler chickens fed different methionine sources supplemented at varying concentrations

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ABSTRACT Intestinal health of broiler chickens is influenced by the concentration of dietary amino acids but data are limited on the role of dietary methionine (**Met**). Two experiments were conducted to investigate the implications of different Met sources for performance, gut barrier function, and intestinal microbiota in broilers. In the first experiment, Ross 308 off-sex birds ($n = 900$) were assigned to 10 dietary treatments each replicated 9 times in a 35-day study. Three sources of Met included DL-Met, L-Met, or Met hydroxy analog free acid (**MHA-FA**), each supplemented at suboptimal (**SUB**) at 80%, adequate (**ADE**) at 100% and over-requirement (**OVR**) at 120% of the specifications against a deficient (**DEF**) diet with no added Met. The second experiment used 96 Ross 308 broilers in a 2 × 4 factorial arrangement. Four diets included 3 sources of Met supplemented at ADE level plus the DEF treatment. On d 17, 19, and 23, half of the birds in each dietary treatment were injected with dexamethasone (**DEX**) to induce leaky gut. In the first experiment, without an interaction, from d 0 to 35, birds fed DL-Met and L-Met performed similarly for BWG, feed intake, and FCR but birds fed MHA-FA had less feed intake and BWG ($P < 0.05$). At d 23, mRNA expression of selected tight junction proteins was not affected except for claudin 2. Ileal microbiota of DEF treatment was different from DL-MET or L-MET supplemented birds ($P < 0.05$). However, microbiota of MHA-FA treatments was only different at OVR from the DEF group. The abundance of Peptostreptococcus increased in DEF treatment whereas *Lactobacillus* decreased. In the second experiment, DEX independently increased ($P < 0.001$) intestinal permeability assayed by fluorescein isothiocyanate dextran, but diet had no effect. DL-Met and L-Met fed birds had a higher level of claudin 3 only in DEX-injected birds ($P < 0.05$). In conclusion, unlike the level of supplementation, DL-Met, L-Met, and MHA-FA were largely similar in their limited impacts on intestinal barrier function and gut microbiota in broilers.

Key words: methionine, tight junction protein, microbiota, dexamethasone, leaky gut

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INTRODUCTION

Methionine (**Met**) is the first limiting indispensable amino acid supplemented in broiler chicken diets mainly in 3 different synthetic sources, either DL-Met, L-Met or Met hydroxy analog free acid (**MHA-FA**) as an aqueous solution (Wickramasuriya et al., 2019). All these sources of Met can be utilized by poultry through a unique enzymatic pathway involving D-amino acid oxidase that can convert D isomer or analog to L-isomer. In poultry, the liver and kidney abundantly produce D-amino acid oxidase. The comparison between different sources of Met mainly DL-Met, L-Met, and MHA-FA for the growth performance of broiler chickens has been the subject of numerous studies over the last 3 decades. There is debate that there may be differences between D-isomer and L-isomer for intestinal functions and development as only L-isomer is utilized by enterocytes after being converted from D-isomer in the liver or kidney (Bauriedel, 1963). In this regard, data are limited on the specific impact of Met sources on intestinal health and integrity where previous studies mainly focused on some histological analysis, oxidative status, or microbiota composition.

The intestinal permeability as a key function of the intestinal barrier is controlled by tight junction (**TJ**) proteins, adherent junctions, and desmosomes (Gilani

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et al., 2021). Tight junction proteins in particular have been shown to be affected by dietary components and in particular amino acids (Gilani et al., 2021). In an in vitro assay, using Caco-2 cells, Martín-Venegas et al. (2013) found that hydroxy analog of Met and DL-Met both prevented an increase in intestinal permeability. However, they found that more of a positive response for intestinal barrier function was associated with MHA-FA owing to its ability to upregulate the trans-sulfuration pathway and produce metabolites with antioxidant properties.

High DL-Met concentration has recently been shown to downregulate gene expression of occludin and claudin-3 in a broiler study (Miao et al., 2021) but intestinal permeability was not measured. Nevertheless, there is no comprehensive study about Met supplementation and its different sources on intestinal barrier function, permeability, and gut integrity in broiler chickens. It is not clear from the literature whether the composition of intestinal microbiota can be differentially affected by the inclusion of different Met sources in broiler diets. It was hypothesized that different sources of Met supplementation may have different impact on intestinal barrier function and microbiota composition under normal and challenged conditions.

Thus, 2 experiments were conducted to address distinct objectives. The primary aim of the first experiment was to compare the effects of 3 main sources of Met and their dietary supplementation levels on performance, intestinal gene expression as a proxy for barrier function and intestinal microbiota of broiler chickens. The second study was conducted to test the effect of adequate supplementation of Met sources in birds subjected to a gut barrier dysfunction model using dexamethasone (**DEX**) to assess the intestinal permeability and barrier function in broiler chickens. Specifically, gene expression of selected TJ proteins including claudin 1, claudin 2, claudin 3, zonula occludens (***ZO***)-1 and -2, and occludin were investigated. Some mechanistically important genes influencing intestinal functions, inflammation and oxidative functions including interleukin 1-β (***IL1-β***), interleukin 10 (***IL-10***), nuclear factor kappa B (***NFκB***), Mucin 2 (***MUC-2***), intestinal fatty acid binding protein (***IFABP***), interferon-gamma (***IFN-γ***); glucagon-like peptide-2 (***GLP-2***); nuclear factor erythroid 2-related factor 2 (***Nrf2***), and glutathione peroxidase 1 (***GPx1***) were also studied.

MATERIALS AND METHODS

The experimental procedures of this study were approved by the Animal Ethics Committee of the Department of Primary Industries and Regions, South Australia.

Experiment 1

Experimental Design and Treatments The experimental treatments consisted of 3 different Met sources and 3 supplementation levels compared with a Met deficient diet (**DEF**, no Met supplemented). Sources of Met were, DL-Met (DL-2-amino-4-(methylthio)butanoic acid), MHA-FA (DL-2-hydroxy-4-(methylthio) butanoic acid), and L-Met (L-2-amino-4-(methylthio) butanoic acid). The DEF diet was formulated supplying only ~60% of recommended Met. The standardized ileal digestible amino acid specifications of AMINOChick software 2.0, (Evonik Industries AG, Essen, Germany) were followed for dietary formulation (Lemme et al., 2020). The supplementation levels were either adequate (**ADE**; 100% of AMINOChick specification), suboptimal (**SUB**; ~80% of the specification) or oversupplementation (**OVR**; ~120% of the specification). There was 1 basal diet for each phase of feeding to which Met sources were supplemented to construct the experimental treatments. The composition of the basal starter, grower, and finisher diets to which the Met sources were supplemented is shown in Table 1. The basal diets

Table 1. Composition of basal starter, grower and finisher diets with no supplemented methionine (% as fed unless specified).

Ingredients	Starter	Grower	Finisher
Wheat	26.550	33.514	34.020
Soybean meal	33.454	26.052	24.560
Corn	28.000	28.000	28.000
Peas	5.000	5.000	5.000
Canola oil	3.399	4.071	5.468
Dicalcium phosphate	1.259	1.026	0.786
Limestone	1.004	0.967	0.895
L-Lysine	0.303	0.284	0.215
Sodium bicarbonate	0.288	0.387	0.425
Vitamins and minerals premix[1]	0.200	0.200	0.200
Sodium chloride	0.190	0.190	0.213
L-Threonine	0.125	0.105	0.085
L-Valine	0.103	0.073	0.039
Choline	0.079	0.084	0.071
L-Isoleucine	0.036	0.037	0.012
Phytase	0.005	0.005	0.005
NSP enzyme	0.005	0.005	0.005
Calculated nutrients			
DM	88.8	88.8	88.9
CP	22.74	20.06	19.30
CF	3.00	2.85	2.80
Fat	5.84	6.49	7.86
AME (kcal/kg)	3000	3100	3200
Ca	0.96	0.87	0.78
P	0.73	0.66	0.61
Available P	0.48	0.44	0.39
Na	0.16	0.19	0.21
Cl	0.23	0.23	0.23
SID[2] Lys	1.28	1.10	1.01
SID Met	0.29	0.26	0.25
SID Cys	0.30	0.28	0.27
SID Met+Cys	0.59	0.53	0.52
SID Thr	0.81	0.70	0.66
SID Trp	0.24	0.21	0.20
SID Arg	1.37	1.17	1.13
SID Ile	0.87	0.76	0.71
SID Leu	1.60	1.41	1.37
SID Val	1.01	0.87	0.81
SID His	0.52	0.45	0.44
SID Phe	0.99	0.87	0.84
SID Tyr	0.70	0.61	0.58

[1]Supplied per kilogram 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]SID: standardized ileal digestible.

Table 2. Experimental treatments based on methionine (Met) sources and the levels of supplementation (%) to the basal starter, grower, and finisher diets.

		Deficient	Adequate (100% of supplementation)			Suboptimal (−50% of supplementation)			Over (150% of supplementation)		
Met source	Required dM+C (%)[1]	Basal dM+C (%)	DL-Met	MHA-FA[2,3]	L-Met	DL-Met	MHA-FA[4]	L-Met	DL-Met	MHA-FA[5]	L-Met
Treatment number		1	2	3	4	5	6	7	8	9	10
Starter (d 0−10)	0.93	0.59	0.34	0.52	0.34	0.17	0.26	0.17	0.51	0.78	0.51
Grower (d 11−24)	0.81	0.53	0.28	0.43	0.28	0.14	0.22	0.14	0.42	0.65	0.42
Finisher (d 25−35)	0.76	0.52	0.24	0.37	0.24	0.12	0.18	0.12	0.36	0.55	0.36

[1]Required digestible Met + Cys based on AMINOChick 2.0 (Ross 308 male broilers).
[2]DL-Met supplementation divided by 65%.
[3]Adequate supplementation in form of a product/carrier (Sipernat) mix containing 58.2% of MHA-FA (Sipernat supplemented at 0.89% of the starter diet, 0.74% of the grower diet, and 0.63% of the finisher diet).
[4]Suboptimal supplementation in form of a product/carrier (Sipernat) mix containing 58.2% of MHA-FA (Sipernat supplemented at 0.45% of the starter diet, 0.37% of the grower diet, and 0.32% of the finisher diet).
[5]Over supplementation in form of a product/carrier (Sipernat) mix containing 58.2% of MHA-FA (Sipernat supplemented at 1.34% of the starter diet, 1.10% of the grower diet, and 0.95% of the finisher diet).

without Met supplementation were used as DEF treatment for each feeding phase. The Met sources were supplemented to the basal diets in powder form at the expense of wheat. To allow better mixability of diets, the MHA-FA was provided as part of a product/carrier (Sipernat 2200, Evonik, Germany) mix containing 58.2% of the product (Zeitz et al., 2019). MHA-FA was provided at a higher concentration proportionate to an average 65% bioavailability of Met compared with DL-Met (Lemme et al., 2002; Lemme et al., 2020). DL-Met and L-Met were supplemented at a similar amount within each level or feeding phase. Full descriptions of the 10 experimental treatments according to the source of Met and associated supplementation levels are given in Table 2. The measured values for amino acids and protein of experimental treatments are provided in Supplementary materials (xlxs. File).

A total of 900 male off-sex Ross broiler chickens were obtained from Aviagen hatchery (Goulburn, NSW, Australia) and were transferred to SARDI poultry facilities located at the Roseworthy Campus of the University of Adelaide. Upon arrival, birds were weighed and assigned to 10 experimental treatments each replicated 9 times using a completely randomized design. A fully ventilated and temperature-controlled broiler shed was used for the experiment. Each pen accommodated 10 chicks. Birds were fed with starter diets from d 0 to 10, grower diets from d 11 to 24 and finisher diets from d 25 to 35 of age. Birds in each replicate were weighed at d 0, 10, 24, and 35 and feed consumption was recorded on a per pen bases. The feed conversion ratio was then calculated for each pen and corrected for mortality if occurred. Grower and finisher diets were fed as pellets and starter diets were fed as mash.

Birds had 24 h of light on the first day of age, then 22 h light and 2 h dark on d 2 followed by 18 h light and 6 h dark for the remainder of the study until d 35 of age. The shed temperature was around 33°C ± 1°C on the placement of birds and then gradually decreased to approximately 21°C at the end of the study.

Sampling On d 21, 1 bird from each pen was randomly selected and euthanized by cervical dislocation. For microbiota analysis, ileal and cecal contents were collected into screw-caped 5 mL tubes and placed on dry ice, and then stored in −80°C until used for analysis. Segments measuring approximately 2 cm were taken from mid jejunum, rinsed with PBS, immediately frozen in liquid nitrogen, and then stored at −80°C until used for RNA extraction and gene expression analysis.

Microbiota Analysis Microbial DNA of all the ileal and cecal contents was isolated using a QIAamp PowerFecal Pro DNA kit (Cat. No. 51804, Qiagen, Germany). The manufacturer's instructions were precisely followed including bead beating using a vortex adaptor (Cat. No. 1300-V1-24, Qiagen, Germany). The concentration of extracted DNA was measured using a NanoDrop One spectrophotometer (Thermo Fisher Scientific, Waltham, MA).

All steps regarding the amplification of the 16S gene, library preparation with sample pooling, and multiplexing and sequencing were performed at LGC Genomics laboratories in Berlin. As a rule of thumb, a sequencing depth of 60,000 to 80,000 reads per sample was aimed for. Amplicon PCR used the primer pair 341F and 785R, MyTaq DNA polymerase (Bioline, London, UK), and BioStab II PCR Optimiser (Cat. Nr. 53833-5ML-F, Sigma-Aldrich, Darmstadt, Germany). About 20 ng amplicon DNA of each sample was pooled for up to 48 samples carrying different barcodes. The amplicon pools were purified with one volume AMPure XP beads (Agencourt, Beverly, MA) to remove primer dimer and other small mispriming products, followed by an additional purification on MinElute columns (Qiagen, Germany). About 100 ng of each purified amplicon pool DNA was used to construct Illumina libraries using the Ovation Rapid DR Multiplex System 1-96 (NuGEN, Männedorf, Switzerland). Illumina libraries were pooled, and size selected by preparative gel electrophoresis. Sequencing was done on an Illumina MiSeq using V3 2 × 300 bp Chemistry according to Illumina's protocol for starting a sequencing run. For bioinformatics and statistics, in short, raw data were demultiplexed and primer clipped using Illumina bcl2fastq 1.8.4. and for merging of forward and reverse read BBMerge 34.48 was used. 16S preprocessing and OTU picking from amplicons was performed with Mothur 1.35.1 with an

alignment against the 16S Mothur-Silva SEED r119 reference. Uchime and FastTree were used for chimera removal and OUT binning. OTUs were annotated using BLAST+ 2.2.29.

Gene Expression Assays Similar to a previous study (Barekatain et al., 2021), prior to RNA extraction process, approximately 100 mg of each jejunal tissue sample was homogenized in Qiazol (Qiagen, Hilden, Germany) using an IKA T 25 digital ULTRA-TURRAX disperser (Staufen, Germany). Total RNA of the jejunal tissues was extracted using an RNeasy Plus Universal Mini Kit (Qiagen, Hilden, Germany). A NanoDrop One spectrophotometer (ThermoFisher Scientific, Waltham, MA) was used to measure the concentration of total RNA. The RNA integrity of 12 randomly selected samples was measured using a Tape Station (Agilent Technologies 2200, Waldbronn, Germany) with samples revealing RIN numbers above 8.5. Extracted RNA samples were then diluted to 200 ng/μL by an epMotion liquid handling robot (Eppendorf, Hamburg, Germany). The cDNA was synthesized following a high-capacity cDNA reverse transcription kit (Thermo Fisher Scientific, Waltham, MA). Diluted cDNA (1:20) in TE buffer was used in assaying the genes for quantitative PCR. The sequences of the primers and their references for all the assayed genes are given in Table 3. The standard curve method and Power SYBR Green PCR Master Mix (Life Technologies Ltd., Warrington, UK) were utilized for PCR assays. Standard reactions were constructed using 1:4 diluted pooled cDNA sample of the same tissues. Arbitrary numbers according to the dilutions were assigned to the standard reactions in each assay including reference genes. The PCR assays were performed using a QuantStudio 6 Flex (Thermo Fisher Scientific, Waltham, MA) having triple measurements for each sample in each assay. The PCR conditions were as follows: an initial 10 min denaturation at 95°C followed by 95°C for 15 s, 40 cycles of 60°C for 20 s, and 72°C for 40 s. A melting curve stage was also included. The values obtained for each assay were divided by the corresponding values obtained for TATA-Box binding protein (**TBP**) as the most stable reference gene. The resulting values expressed to the reference gene were used for statistical analysis.

Experiment 2

Experimental Design and Treatments The second experiment used 4 dietary treatments out of 10

Table 3. The primer sequences of the target genes used in PCR assays.

Gene name	Sequences (5′→3′)	References
Tight junction proteins		
Claudin 1	F: AAGGTGTACGACTCGCTGCT R: CAGCAACAAACACACCAACC	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)
ZO1	F: CCGCAGTCGTTCACGATCT R: GGAGAATGTCTGGAATGGTCTGA	Chen et al. (2015)
ZO2	F: GCCCAGCAGATGGATTACTT R: TGGCCACTTTTCCACTTTTC	Gilani et al. (2018)
Occludin	F: ACGGCAAAGCCAACATCTAC R: ATCCGCCACGTTCTTCAC	Gilani et al. (2018)
Immune function and inflammation		
IL1-β	F: CAGCCCGTGGGCATCA R: CTTAGCTTGTAGGTGGCGATGTT	Chen et al. (2015)
IL-10	F: ATGAACTTAACATCCAACTGCTC R: TGTTGCCCAGGTCGCCCAT	Xiao et al. (2018)
NFκB	F: GAAGGAATCGTACCGGGAACA R: CTCAGAGGGCCTTGTGACAGTAA	Lee et al. (2018)
MUC-2	F: ATTGAAGCCAGCAATGGTGT R: TTGTTGGCCTTGTCATCAAA	Gilani et al. (2018)
IFABP	F: AAAGATAATGGAAAAGTACTCACAGCAT R: CCTTCGTACACGTAGGTCTGTATGA	Chen et al. (2015)
IFN-y	F: ACACTGACAAGTCAAAGCCGC R: AGTCGTTCATCGGGAGCTTG	Brisbin et al. (2010)
GLP-2	F: CGTGCCACAGCCATTCTTA R: AGCGGCTCTGCAAATGATTA	Gilani et al. (2018)
Nrf2	F: GGAAGAAGGTGCTTTTCGGAGC R: GGGCAAGGCAGATCTCTTCCAA	Lee et al. (2018)
GPx1	F: TCCCCTGCAACCAATTCG R: AGCGCAGGATCTCCTCGTT	Greene et al. (2021)
Reference genes		
TBP	F: GTCCACGGTGAATCTTGGTT R: GCGCAGTAGTACGTGGTTCTC	Gilani et al. (2018)
GAPDH	F: CAACCCCCAATGTCTCTGTT R: TCAGCAGCAGCCTTCACTAC	Gilani et al. (2018)

ZO1: zonula occludens 1; ZO2: zonula occludens 2; *IL1-β*: interleukin 1-β; *IL-10*: interleukin 10; NFκB: nuclear factor kappa B; MUC-2: Mucin 2; *IFABP*: intestinal fatty acid binding protein; *IFN-y*: interferon-gamma; *GLP-2*: glucagon-like peptide-2; *Nrf2*: nuclear factor erythroid 2-related factor 2; GPx1: glutathione peroxidase 1; *TBP*: Tata-Box binding protein; *GAPDH*: glyceraldehyde 3-phosphate dehydrogenase.

described for Experiment 1. The experiment was conducted to compare different sources of Met supplemented at adequate levels (100% of AMINOChick specification) with a DEF diet, as a control diet, for intestinal permeability and selected gene expression under a leaky gut model using DEX. Using a 2 × 4 factorial arrangement of treatments, 4 diets included 1) DEF diet with no Met supplemented, 2) DL-Met supplemented, 3) MHA-FA supplemented, and 4) L-Met supplemented. The 4 diets were from the exact same mixing batch used in the first experiment (Treatments 1−4, Table 2) that had been kept in a cool room (4°C) for about 2 months.

Accordingly, 96 male off-sex Ross broiler chickens (excluding any spare birds) were obtained from the same hatchery (Goulburn, NSW, Australia) as in the first study and was transferred to the research facility. All birds were kept on 4 floor pens with wood shavings as bedding material from d 0 to d 12 receiving a same starter diet. The procedure of this challenge experiment had some modifications compared with previous experiments conducted in the same lab (Barekatain et al., 2019a,b). Birds in this experiment were assigned to their experimental grower diets on d 16 of age to allow adequate growth of birds in particular Met-deficient diet being able to reach the drinker line and stand on wire-mesh cages. Accordingly, on d 16 from each treatment 24 broilers were transferred to individual metabolism cages in a total of 96 cages. On d 17, 19, and 23 of age, half of the birds in each dietary treatment were given a low dose of DEX (0.35 mg/kg) to induce gut barrier dysfunction. The low dosage was used to minimize the severe reduction in broiler growth previously observed (Barekatain et al., 2019a,b). The preparation of the DEX solution was already explained by Wideman and Pevzner (2012).

Fluorescein Isothiocyanate-Dextran Assay and Sampling On d 24, all 96 birds were orally gavaged with 4.16 mg/kg BW of FITC-d. The preparation of FITC-d solution dissolved in the water had been previously described in detail (Barekatain et al., 2021). After 150 min, blood sample was taken from the wing of the live bird into a vacutainer. Blood samples were left at room temperature for at least 3 h before being centrifuged at 1,500 × g for 15 min. The serum samples were then separated and stored at −20°C until used for analysis. The analysis of FITC-d was performed in duplicate including a blank sample from a previously stored serum sample from a bird without FITC-d. Sample absorbance was measured at the excitation of 485 and emission of 530 nm using a Synergy MX plate reader (Biotek Instruments, Bedfordshire, UK).

After completion of blood collection, each bird was euthanized by cervical dislocation to collect jejunal tissues. The tissues were collected and stored similar to Experiment 1. The gene expression assays, and related procedures were exactly similar to Experiment 1 and therefore it is not repeated herein.

Statistical Analysis

The data of Experiment 1 included 10 dietary treatments from which DEF treatment (no Met supplementation) were excluded for a 2-way ANOVA of General Linear Model of SAS 9.4 to assess the main effects of 3 sources of Met and 3 levels of supplementation and their interaction. One replicate or its corresponding collected sample was considered the experimental unit depending on the parameter analyzed. DEF treatment was included in linear regression analysis for the increasing levels (SUB, ADE, and OVR) of inclusion of each of the 3 Met sources against DEF diet using proc regression of SAS 9.4.

For microbiome, alpha and beta diversity analyses were based on QIIME1.9.0 and custom R scripts using R.4.0.2 with vegan and phyloseq packages. For alpha diversity measures and abundance estimates, Mann-Whitney U tests were run, and results were corrected with the Banjamini-Hochberg method for multiple testing. For beta diversity 1-way PERMANOVA of Bray-Curtis dissimilarity was run, with pairwise PERMANOVAs as post hoc tests. Pairwise comparisons were correct for their false discovery rate. To evaluate significance, we considered alpha < 0.05 as significant.

For Experiment 2, data were analyzed by 2-way ANOVA of General Linear Model of SAS 9.4 to assess the effect of 4 diets (DEF, Met, MHA-FA, and L-Met supplemented at ADE levels), DEX challenge, and their interaction. One bird or its representative samples was considered the experimental unit. For both experiments, if a significant effect was detected, the means were separated by Fisher's least square differences test. The level of significance was considered at $P < 0.05$ while $0.05 \leq P \leq 0.10$ was declared as a tendency.

RESULTS

Experiment 1

Growth Performance The BWG, feed intake, and FCR values are presented in Table 4. When the DEF treatment was included, linear regression analysis showed strong effects of all Met sources for BWG, feed intake, and FCR at all phases of the experiment ($P < 0.0001$). Excluding the DEF treatment, the 2-way ANOVA revealed no significant interaction between Met sources and the levels of supplementation for BWG, feed intake and FCR at any stage of the experiment except for FCR from d 0 to 35 ($P < 0.05$).

Regardless of the level of inclusion, birds fed DL-Met had similar BWG compared with birds fed L-Met at any stage of the experiment. However, birds fed MHA-FA had lower BWG compared with other sources of Met from d 0 to 10 ($P < 0.001$) and when assessed from d 0 to 35 ($P < 0.001$). From d 10 to 24, birds fed MHA-FA had less BWG only when compared with birds fed DL-Met. In terms of the source of Met, there was no significant difference for BWG during the finisher phase (d 24−35). There were no differences between the levels of Met

Table 4. Growth performance of broiler chickens fed different sources of methionine (Experiment 1).[1,2]

	Body weight gain (g/bird)				Feed intake (g/bird)				FCR (g feed intake: g weight gain)			
Items	D 0–10	D 10–24	D 24–35	D 0–35	D 0–10	D 10–24	D 24–35	D 0–35	D 0–10	D 10–24	D 24–35	D 0–35
Treatments												
Deficient	135	422	545	1102	195	770	1099	2064	1.455	1.815	2.007	1.863
DL-Met—SUB	239	1032	1229	2500	276	1345	1903	3524	1.162	1.304	1.549	1.410^{a}
MHA-FA—SUB	229	997	1221	2447	268	1289	1833	3390	1.175	1.293	1.502	1.385^{b}
L-Met—SUB	238	1015	1206	2458	273	1330	1861	3464	1.154	1.310	1.544	1.409^{a}
DL-Met—ADE	244	1018	1268	2530	275	1318	1865	3457	1.129	1.295	1.475	1.367^{c}
MHA-FA—ADE	222	1007	1239	2468	268	1279	1856	3403	1.216	1.270	1.498	1.379^{bc}
L-Met—ADE	253	1037	1272	2562	285	1345	1866	3496	1.126	1.297	1.464	1.365^{c}
DL-Met—OVR	250	1103	1280	2633	276	1376	1944	3595	1.105	1.248	1.518	1.366^{c}
MHA-FA—OVR	227	1026	1247	2499	268	1304	1855	3427	1.193	1.272	1.488	1.372^{bc}
L-Met—OVR	254	1040	1255	2550	290	1337	1888	3515	1.143	1.285	1.505	1.379^{bc}
SEM[3]	2.2	4.8	6.0	10.1	1.9	8.45	9.09	17.04	0.009	0.011	0.006	0.006
Source effect (3 × 3 factorial excluding deficient)												
DL-Met	244^{a}	1050^{a}	1259	2554^{a}	275^{ab}	1346^{a}	1903^{a}	3526^{a}	1.132^{b}	1.282^{ab}	1.514	1.380
MHA-FA	225^{b}	1010^{b}	1235	2471^{b}	268^{b}	1291^{b}	1848^{b}	3407^{b}	1.194^{a}	1.278^{b}	1.496	1.378
L-Met	248^{a}	1031^{ab}	1244	2523^{a}	282^{a}	1337^{a}	1872^{b}	3491^{a}	1.141^{b}	1.297^{a}	1.504	1.384
Level effect												
SUB	235	1015^{b}	1218^{b}	2468^{b}	272	1321	1865^{ab}	3512	1.163	1.302^{a}	1.532^{a}	1.401
ADE	239	1021^{b}	1259^{a}	2519^{a}	275	1314	1862^{b}	3452	1.156	1.287^{a}	1.479^{c}	1.370
OVR	244	1056^{a}	1260^{a}	2561^{a}	279	1339	1895^{a}	3512	1.146	1.268^{b}	1.503^{b}	1.372
P values												
Methionine	0.0004	0.0062	0.2914	0.0049	0.0121	0.0029	0.0044	0.0009	0.005	0.046	0.301	0.601
Level	0.331	0.0023	0.0082	0.0017	0.528	0.305	0.087	0.115	0.700	0.0002	0.0002	<0.0001
Met x level	0.553	0.092	0.735	0.282	0.563	0.611	0.332	0.489	0.408	0.110	0.071	0.014
Linear regression (level of methionine including deficient)	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

[1]Each value for each treatment represents the mean of 9 replicates.
[2]Values within a same column and the same main effect or treatment not sharing a superscript letter differ significantly at the *P* level shown.
[3]Pooled standard error of the mean.ADE: adequate supplementation; OVR: oversupplementation; SUB: suboptimal supplementation.

supplemented at SUB, ADE, and OVR for BWG during the first 10 d of age. However, during the grower phase, feeding Met at OVR independently increased BWG ($P < 0.01$) whereas there was no difference between the DL-Met and L-Met fed groups. For the finisher phase from d 24 to 35 and when assessed from d 0 to 35, feeding birds with SUB Met specification reduced ($P < 0.01$) BWG compared with ADE and OVR levels.

Independent of inclusion level, birds fed MHA-FA consumed less ($P < 0.05$) feed during the first 10 d of age compared with birds fed L-Met. In the grower phase and from d 0 to 35, birds fed MHA-FA had lower ($P < 0.001$) feed intake compared with birds fed DL-Met and L-Met but there was no difference between DL-Met and L-Met. In the finisher phase, birds fed DL-Met had the highest ($P < 0.01$) feed intake among all the sources of Met. There were no effects of inclusion rate on feed intake of the birds until d 24 of age or from d 0 to 35. Birds fed Met at OVR requirement, regardless of the source, consumed more feed during the finisher phase only compared with birds fed ADE.

During the starter phase, birds fed MHA-FA had the highest FCR ($P < 0.01$). In the grower phase, however, the highest FCR was observed in birds fed L-Met which was different only when compared with birds fed MHA-FA. FCR was not impacted by the source of Met in the finisher and the entire study (d 0–35). The level of Met inclusion did not influence FCR in the first 10 d of age. Increasing the supplementation level to OVR decreased FCR in the grower phase ($P < 0.001$) whereas there was no difference between SUB and ADE levels. During the finisher phase, supplementation of Met at ADE regardless of the source, resulted in the lowest FCR followed by birds fed OVR and SUB Met levels ($P < 0.001$). When assessed from d 0 to 35, there was an interaction ($P < 0.05$) between Met sources and levels of supplementation when birds fed SUB levels of L-Met and DL-Met had the highest FCR values.

Expression of Genes Related to Gut Integrity, Inflammation, and Oxidative Stress Table 5 shows the results of selected genes encoding TJ proteins and selective genes related to intestinal integrity, inflammation, and oxidative stress in Experiment 1. Inclusion of Met regardless of source and supplementation levels linearly increased ($P < 0.001$) the gene expression of claudin 2 in the jejunum of broilers at d 21. Supplementation of Met, its sources, and levels had no impact on mRNA expression of other measured TJ, *IL1-β, NFκB, MUC2, IFABP, GLP-2*, and *GPx1*.

Ileal and Cecal Microbiota Composition At d 21, the ileal microbiota consisted mainly of *Lactobacillus* and a genus of the Peptostreptococcaceae family (Supplementary materials, Figure S1). The alpha diversity as measured in Shannon diversity was not affected by treatment (Kruskal-Wallis, $P > 0.05$). The beta-diversity from ileum microbiota of DEF-fed birds was different from DL-Met or L-Met supplemented birds at any level of supplementation (PERMANOVA, $P < 0.05$; Supplementary materials, Table S1). However, for MHA-FA supplemented birds the composition of ileal microbiota was different only at OVR supplementation compared with DEF-fed birds. Additionally, at the OVR level, microbiota of MHA-fed birds differed from DL-Met and L-Met groups, whereas DL-Met and L-Met

Table 5. Relative mRNA expression of selected tight junction proteins and mechanistic genes in jejunum of broilers fed different methionine sources (Experiment 1).[1]

Items	Claudin 1	Claudin 2	Claudin 3	*ZO-1*	*ZO-2*	Occludin	*IL1-β*	*NFκB*	*MUC-2*	*IFABP*	*GLP-2*	*GPx1*
Treatments												
Deficient	0.921	0.497	0.950	1.804	0.858	1.902	0.776	0.626	0.441	1.121	1.319	1.157
DL-Met—SUB	0.873	0.753	0.862	1.591	0.878	1.587	0.831	0.690	0.483	1.022	1.118	1.313
MHA-FA—SUB	0.771	0.716	0.860	1.699	0.887	1.668	0.906	0.646	0.443	0.816	1.117	1.109
L-Met—SUB	0.850	0.713	0.831	1.648	0.936	1.718	0.896	0.672	0.448	1.027	1.284	1.348
DL-Met—ADE	0.892	0.738	0.764	1.644	0.832	1.738	0.894	0.628	0.414	1.016	1.256	1.256
MHA-FA—ADE	0.929	0.824	0.840	1.743	0.893	1.787	0.997	0.682	0.442	1.031	1.281	1.240
L-Met—ADE	0.938	0.789	0.866	1.799	0.928	1.864	0.951	0.693	0.440	0.993	1.372	1.340
DL-Met—OVR	0.853	0.827	0.861	1.677	0.872	1.802	0.787	0.648	0.460	1.090	1.361	1.287
MHA-FA—OVR	0.819	0.850	0.850	1.724	0.911	1.751	0.849	0.636	0.420	0.873	1.244	1.187
L-Met—OVR	0.967	0.842	0.814	1.522	0.858	1.649	0.788	0.671	0.387	0.881	1.174	1.283
SEM[2]	0.020	0.0196	0.0195	0.0290	0.0168	0.0376	0.0390	0.0112	0.0138	0.0324	0.0259	0.0266
P values (3 × 3 factorial analysis excluding deficient diet)												
Met source	0.33	0.89	0.91	0.46	0.52	0.93	0.74	0.62	0.74	0.25	0.65	0.94
Level	0.25	0.093	0.85	0.39	0.89	0.35	0.41	0.79	0.59	0.68	0.16	0.92
Met x level	0.68	0.89	0.76	0.45	0.78	0.73	0.99	0.66	0.82	0.52	0.25	0.79
Regression analysis of levels including deficient diet												
Linear												
DL-Met	0.49	0.0004	0.24	0.51	0.99	0.83	0.86	0.97	0.95	0.84	0.53	0.49
MHA-FA	0.62	0.0002	0.22	0.62	0.48	0.49	0.55	0.69	0.68	0.25	0.87	0.50
L-Met	0.39	0.001	0.19	0.10	0.97	0.18	0.86	0.38	0.39	0.10	0.28	0.24

[1]Each value for each treatment represents the mean of 9 replicates.
[2]Pooled standard error of the mean.ADE: adequate supplementation; OVR: oversupplementation; SUB: suboptimal supplementation.

groups did not differ from each other (Figure 1A). Particularly, the abundance of an unclassified *Lachnospiraceae* genus and OTU11 of the *Peptostreptococcus* genus increased in DEF treatment whereas OTU3 of the *Lactobacillus* genus decreased. OTU2 of the *Lactobacillus* genus reacted differentially to MHA-FA treatment (Kruskal-Wallis, $P < 0.05$, Supplementary materials, Figure S2).

The cecal microbiota consisted mainly of Faecalibacterium and 2 genera of the Lachnospiraceae family but also Blautia, *Lactobacillus*, and others were present (Supplementary materials, Figure S3). Cecal microbiota of DEF birds did not show a change in OTU richness but had an increased Shannon diversity (Kruskal-Wallis, $P < 0.05$, Figure 1B). A subsequent test indicated that this could likely be explained by as increase in evenness in the cecal microbiota of DEF birds (Pielou's index of evenness, Kruskal-Wallis, $P < 0.05$, Figure 1C). Furthermore, also the cecal microbiota of DEF birds was significantly different in composition from ADE or OVR-supplemented birds, except for ADE treatment of DL-Met (PERMANOVA $P < 0.05$, Figure 1D, Supplementary materials, Table S1). Particularly, an unclassified *Lachnospiraceae* genus consisting of several OTUs decreased with an increase in Met supplementation. Additionally, also

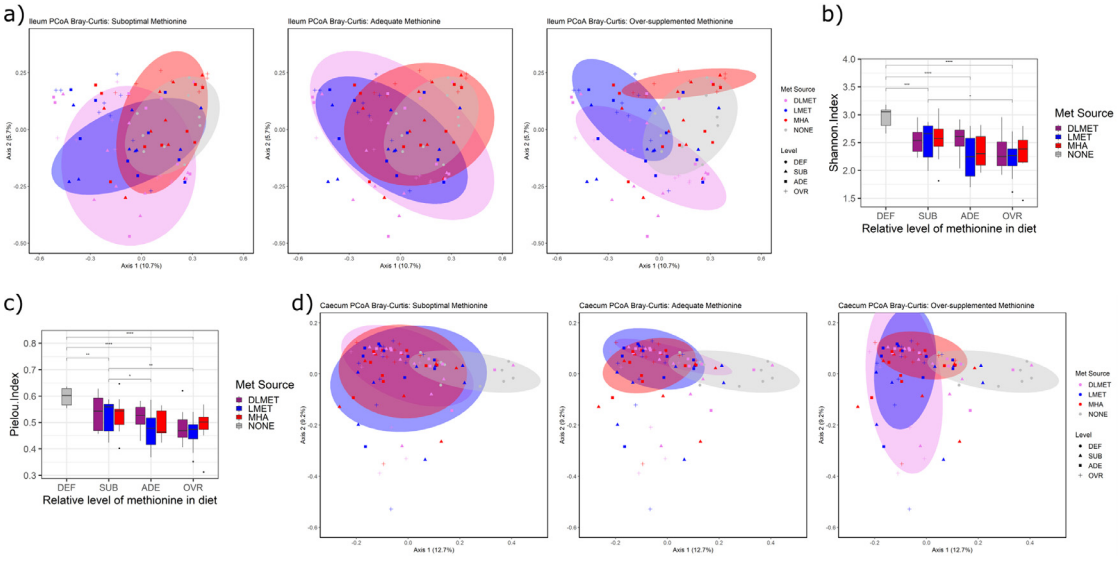

Figure 1. Microbiota diversity based on OTU level. (A, D) Principle coordinate analysis of Bray-Curtis dissimilarity of ileum (A) and cecum (D) microbiota. Ellipses represent multivariate *t* distribution at level 0.8 (stat_ellipse function from ggplot2). All 3 plots represent the same analysis, ellipses are focusing on different treatments for clarity. (B, C) Alpha diversity analysis of cecal microbiota. Boxplots represent Shannon index (B) and Pielou's index of evenness (C). ADE: adequate supplementation; OVR: Oversupplementation; SUB: suboptimal supplementation.

the *Anaerotruncus* genus decreased with increasing supplementation of Met. Like in ileum, also in cecum OTU11 of the *Peptostreptococcus* genus was higher in the DEF treatment group than in Met supplemented groups (Kruskal-Wallis, $q < 0.05$, Supplementary materials, Figure S4).

Experiment 2

Growth Performance Indications of Individually Housed Birds Growth performance data in Experiment 2 are shown in Table 6. Diet and DEX interacted significantly for feed intake ($P < 0.01$) and BWG ($P < 0.0001$). Unchallenged birds fed all 3 sources of Met consumed equal amounts of feed and more than the control group fed a Met-deficient diet. In challenged birds, DEX reduced the feed intake of the birds fed MHA-FA to a level similar to the DEF control diet. For BWG, the lowest value was observed for DEX-injected birds fed the DEF diet whereas unchallenged birds fed either of the 3 Met sources gained similar BW. There was no interaction between diet and DEX for FCR. Independently, DEX increased FCR ($P < 0.0001$). The DEF diet increased ($P < 0.0001$) FCR compared with supplemented diets, but no significant difference observed between the sources of Met.

Serum FITC-d as Intestinal Permeability Test As shown in Figure 2, there was no interaction between DEX and diet for the concentration of FITC-d in Experiment 2. Diet had no effect on serum FITC-d concentration. DEX, however, increased ($P < 0.001$) the passage of FITC-d into blood compared with sham-injected birds.

Jejunal Gene Expression As presented in Table 7, without interaction, claudin 1 was only affected by DEX injection ($P < 0.01$) where challenged birds had a reduced gene expression compared with unchallenged birds. Independent of DEX, supplementation of Met from any of the 3 sources tended ($P = 0.069$) to upregulate mRNA expression of claudin 2 compared with an unsupplemented diet.

Table 6. Growth performance parameters broilers fed different methionine sources under dexamethasone (DEX) challenge or sham injections (Experiment 2).

Items		Feed intake (g/bird)	Weight gain (g/bird)	FCR
Treatments	DEX			
Deficient	−	657[d]	377[b]	1.76
DL-Met	−	827[a]	620[a]	1.34
MHA-FA	−	819[a]	607[a]	1.35
L-Met	−	834[a]	617[a]	1.35
Deficient	+	703[cd]	266[d]	2.7
DL-Met	+	762[b]	324[c]	2.38
MHA-FA	+	735[bc]	297[bc]	2.49
L-Met	+	760[b]	304[cd]	2.56
SEM		6.8	5.0	0.024
Main effect				
Diets				
Deficient		680	321	2.23[a]
DL-Met		794	472	1.85[b]
MHA-FA		776	452	1.92[b]
L-Met		797	460	1.95[b]
DEX				
Sham		784	555	1.45[b]
Injected		740	297	2.53[a]
P values				
DEX		<0.0001	<0.0001	<0.0001
Diet		0.002	<0.0001	<0.0001
Diet × DEX		0.003	<0.0001	0.24

[1]Each value for each treatment represents the mean of 12 replicates.
[2]Values within a same column, the same main effect or treatment not sharing a superscript letter differ significantly at the *P* level shown.
[3]Pooled standard error of the mean.

There was a significant interaction ($P < 0.05$) between DEX and diet for the expression of claudin 3. A higher expression of claudin 3 was observed in DEX injected birds fed DL-Met and L-Met than birds fed MHA-FA and DEF diet. The genes of *ZO1, ZO2, MUC2, NFκB*, and *IL-10* remained unaffected. No interaction was observed between DEX and diet for *IFNγ* and *IL1-β* and these genes were also not influenced by dietary treatments. Both *IFNγ* ($P < 0.01$) and *IL1-β* ($P < 0.01$) were independently downregulated by DEX injections.

Diet and DEX interacted for mRNA expression of *Nrf2* ($P < 0.01$) and *GLP2* ($P < 0.01$). For *Nrf2*, while

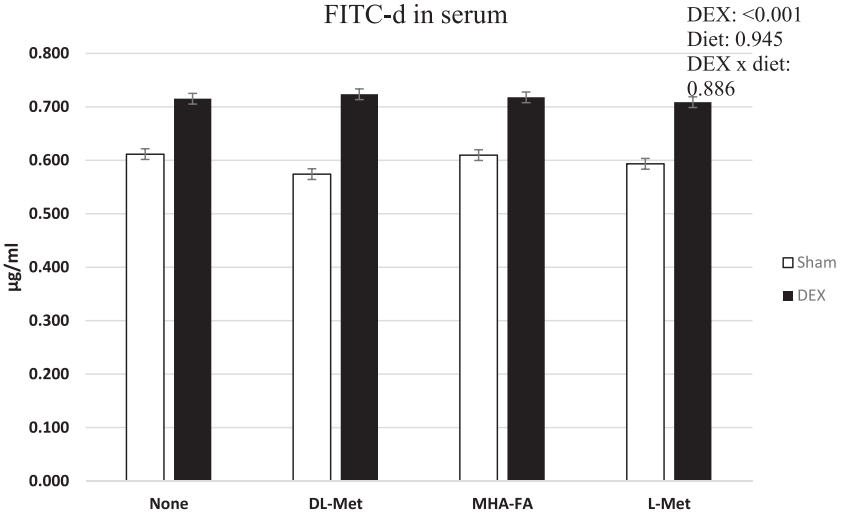

Figure 2. FITC-d concentration in serum of broilers on d 24 of age (Experiment 2).

Table 7. Relative mRNA expression of selected genes in broilers fed different methionine sources under dexamethasone (DEX) challenge or sham injections (Experiment 2).[1,2]

Items		Claudin-1	Claudin 2	Claudin 3	*ZO-1*	*ZO-2*	*MUC-2*	*IFNγ*	*IL1β*	*NFκB*	*Nrf2*	*GLP-2*	*IL-10*
Treatments	DEX												
Deficient	−	1.124	0.936	0.268^{c}	1.063	1.291	0.546	1.027	0.454	1.09	1.300^{ab}	1.726^{a}	0.671
DL-Met	−	0.994	1.354	0.236^{c}	1.017	0.987	0.451	0.919	0.481	0.956	1.047^{c}	1.490^{b}	0.909
MHA-FA	−	0.997	1.298	0.203^{c}	0.986	1.04	0.431	1.27	0.462	0.934	1.059^{bc}	1.243^{cd}	0.825
L-Met	−	0.834	1.286	0.208^{c}	0.951	1.088	0.485	0.954	0.418	0.905	0.976^{c}	1.296^{bcd}	0.797
Deficient	+	0.911	0.651	0.350^{b}	0.91	1.121	0.402	0.515	0.268	0.881	0.997^{c}	1.168^{d}	0.498
DL-Met	+	0.895	0.793	0.432^{a}	1.178	1.059	0.486	0.834	0.315	0.898	1.349^{a}	1.421^{bc}	0.722
MHA-FA	+	0.85	0.836	0.349^{b}	1.059	1.137	0.446	0.747	0.339	1.071	1.201^{abc}	1.288^{bcd}	0.755
L-Met	+	0.738	0.705	0.440^{a}	0.982	1.081	0.407	0.827	0.363	0.931	1.222^{abc}	1.375^{cbd}	0.889
SEM[3]		0.0421	0.0409	0.0092	0.0281	0.0382	0.0138	0.0525	0.0205	0.0318	0.0306	0.0276	0.0637
Main effect													
Diets													
Deficient		0.935	0.794	0.309	0.986	1.205	0.474	0.771	0.361	0.985	1.148	1.447	0.585
DL-Met		0.944	1.073	0.334	1.097	1.023	0.468	0.876	0.398	0.927	1.198	1.455	0.817
MHA-FA		0.923	1.067	0.276	1.022	1.088	0.438	1.001	0.401	1.002	1.129	1.265	0.789
L-Met		0.786	0.995	0.324	0.966	1.084	0.446	0.890	0.390	0.918	1.098	1.335	0.843
DEX													
Sham		0.987a	1.218a	0.229	1.004	1.101	0.478	1.042^{a}	0.454a	0.971	1.095	1.438	0.800
Injected		0.807b	0.746b	0.392	1.032	1.099	0.435	0.731^{b}	0.321b	0.945	1.192	1.313	0.716
P values													
DEX		0.009	<0.0001	<0.0001	0.62	0.98	0.12	0.005	0.002	0.689	0.12	0.028	0.51
Diet		0.29	0.069	0.15	0.38	0.41	0.76	0.47	0.89	0.73	0.71	0.05	0.47
Diet × DEX		0.39	0.56	0.036	0.26	0.60	0.095	0.29	0.68	0.28	0.005	0.001	0.86

[1]Each value for each treatment represents the mean of 6 replicates.
[2]Values within a same column, the same main effect or treatment not sharing a superscript letter differ significantly at the *P* level shown.
[3]Pooled standard error of the mean.

DEX decreased the expression for birds fed the DEF diet, increased it for birds fed DL-Met but not for the other 2 sources of Met. Among challenged birds, birds fed DL-Met had the highest mRNA expression of *GLP-2* whereas, in sham-injected birds, the highest expression was observed in Met-deficient fed birds.

DISCUSSION

In general, the growth performance of broilers excluding the DEF diet exceeded the Ross 308 performance objectives (Aviagen, 2019). This study was designed with the main objective of investigating the effect of Met sources and supplementation levels on gut barrier function-related factors and intestinal microbiota and not the relative bioavailability of Met sources. However, growth performance was studied to correlate any possible differences in intestinal measurements with animal performance.

In the present study, the effects of Met sources and level of supplementation remained independent for almost all the performance-related parameters except for the overall FCR of the suboptimally fed birds. As expected, Met+Cys at DEF and SUB levels markedly depressed the growth performance and ADE-formulated treatments exceeded the expected BWG of Ross 308 performance objectives (2,519 g vs. 2,333 g). However, feeding Met at approximately 20% over the requirement improved BWG and FCR of broilers only from d 10 to 24 but not during other phases, which may highlight that broilers at the grower phase may have had a slightly higher requirement of Met+Cys.

DL-Met and MHA-FA must be converted to L-Met for absorption and used in protein synthesis in an enzymatic conversion process taking place in the liver and kidney. The complete bioefficiency of such conversion is not 100% (Marrett and Sunde, 1965; Ribeiro et al., 2005) and as such the performance of birds fed different sources of Met may differ, a topic that has been subject of debate. In the present study, DL-Met and L-Met supplementation regardless of their levels of supplementation produced similar BWG and FCR highlighting a comparable efficacy of the 2 products for growth performance. Our results agree with several other studies reporting equal efficacy of DL-Met and L-Met for broiler performance (Garlich, 1985; Pacheco et al., 2018; Çenesiz et al., 2022), although there are some studies reporting superiority of L-Met over DL-Met (Esteve-Garcia and Khan, 2018; Wang et al., 2019). The birds fed MHA-FA regardless of the dietary Met concentration consumed less feed compared with other sources of Met which translated to lower BWG when assessed for the entire period of study. The diets had been formulated to contain similar Met bioavailability from all 3 sources of Met considering the bioavailability of MHA-FA as 65% of the DL-Met. The exact reason for the lower feed intake of these birds cannot be immediately explained by the data of the current study. While in some cases, bioavailability of less than 65% has been reported for MHA-FA (Payne et al., 2006), it needs to be acknowledged that in the present study, the MHA-FA was used in form of a mixed product with the carrier (Sipernat 2200, Evonik, Germany) for the ease of mixability and transportation. It was assumed that the inclusion of that carrier had no effect on the growth performance of broilers as shown in other studies (Zeitz et al., 2019). However, any possible negative effect of this silicon-based carrier on feed consumption and therefore performance parameters cannot be ruled out

completely, and the interpretation of the results for MHA-FA should be considered in this context.

Available data on the effect of Met supplementation on TJ expression in broilers are limited and contradictory (Del Vesco et al., 2020; Miao et al., 2021). In mice, however, Met restriction was shown to improve intestinal barrier function and reduce inflammation (Ren et al., 2021) but such responses may not be applicable to poultry. The present study, to our knowledge, is the most comprehensive analysis of TJ expression in response to different sources of Met and their concentrations in the diet of broilers. In Experiment 1, among all tested TJ, only claudin-2 expression was linearly upregulated by the inclusion of Met in the diet regardless of the source and level of supplementations against the DEF group. Higher expression of claudin 2 has been shown to be associated with leaky epithelia and can be upregulated by certain cytokines (Venugopal et al., 2019). However, there is also evidence that overexpression of claudin-2 offered resistance to epithelial damage in mice and that claudin-2 by itself did not cause inflammation (Ahmad et al., 2014). In addition, claudin 2 is permeable to cations, Na+, K+, and Li+ as well as water molecules. We are not aware of any previous research on the role of Met sources on claudin 2 expression. However, as there was no effect on other TJ expression at least at mRNA level, changes in claudin 2 in our study may be more attributed to its physiological roles in shuttling cations in particular Na+ which is critical in the transport of Met in enterocytes (Romanet et al., 2020). It should also be noted that changes in individual claudins may be less critical than an overall alteration in the relative expression of claudins compared to each other because claudin extracellular loops interdigitate in homotypic and heterotypic interactions (Mullin et al., 2016).

In Experiment 2, DEX injections successfully caused leaky gut in broilers evidenced by changes in expressions of claudin 1, 2, and interactively influencing claudin 3. Indicative and significant retardation of body weight and higher FCR of DEX challenged birds were consistent with previous studies (Barekatain et al., 2019a,b). The DEX challenge successfully increased intestinal permeability as shown by a higher level of FITC-d compared with sham-injected birds. These results are largely supported by previous research using the same model (Barekatain et al., 2019a,b, 2021). In general, stress-induced changes and increased gut permeability is mediated by corticotrophin-releasing factor. The specific mechanisms of DEX effect as stress-inducing factor on gut disturbance are triggered with preferential binding to type II receptors causing stress-like physiological responses including immune suppression and selective uptake, and use of glucose (Turner et al., 2012). Such mechanism inflicts a wide range of changes including changes in gut motility, permeability, mast cell, and mucin production (Ünsal and Balkaya, 2012).

As for the effect of Met sources, under a DEX-induced leaky gut challenge, gene expression of the same TJ again remained largely unaffected with exception of claudin 3. The interaction between DEX and Met source highlighted that only DL-Met and L-Met upregulated claudin 3 in challenged birds. Upregulation of claudin 3 may be regarded as a sign of strengthening the intestinal barrier function (Patel et al., 2012). However, given that there were no other significant changes in other TJ and in particular intestinal permeability in Experiment 2, the change observed for Claudin-3 per se cannot be sufficient to associate any gut integrity-promoting effect to the tested Met sources. Importantly, changes in the expression of TJ are structural changes that may underpin the alteration of intestinal barrier function and are not necessarily molecular mechanisms affecting intestinal barrier function.

In our study, the permeability assay of FITC-d was not affected by the inclusion of DL-Met, MHA-FA, or L-Met against DEF treatment in both sham and DEX-injected birds. This study is the first report on the effect of Met on intestinal permeability in broiler chickens. Two plausible explanations can be given for the unaffected intestinal permeability. First, Met alone may not be involved in the regulation of intestinal permeability given that TJ were only partially affected in this study. Second, the birds fed DEF and SUB diets may have still had sufficient Met from the dietary ingredients required to maintain intestinal integrity but evidently not for optimal growth and productivity of the birds. The lack of effect of Met and their level of supplementation indicates that Met may have limited implications for the expression of TJ and intestinal permeability in broiler chickens. Although intestinal permeability as the main function of the intestinal barrier was not affected in this study, Met impacts other cellular and cytoskeletal changes and pathways in the GIT or other organs that were not specifically studied here (Lauinger and Kaiser, 2021).

Glucagon-like peptide-2 (**GLP-2**) is a hormone secreted by enteroendocrine L cells of the intestinal epithelium (Markovic and Brubaker, 2019). GLP2 enhances gut barrier function, motility, cell proliferation, and control nutrient absorption, digestion, and amino acid transport (Lee et al., 2017; Markovic and Brubaker, 2019). In the present study, in Experiment 1, GLP-2 expression remained unaltered, in Experiment 2 however, challenged birds fed DEF treatment had a lower expression of GLP-2 than birds fed ADE treatments. The mechanistic nature of such interactive effect of Met deficiency and DEX administration is unknown, but it may show that birds fed DEF diet are more susceptible to enteric issues possibly due to lower expression of GLP-2. Nevertheless, no differences were detected for GLP-2 expression between different sources of Met in both experiments.

The Nrf2 is a transcription factor with a cytoprotective role involved in the integration of cellular stress signals and resistance to oxidative stress (Sporn and Liby, 2012). It also reduces inflammation by regulating inflammatory factors such as various interleukins, and TNFα (Ahmed et al., 2017). In the present study, the downregulation of Nrf2 in DEX-injected birds fed DEF diet

may be associated with either an underlying acute inflammation or higher oxidative stress.

Methionine is turned to S-adenosylmethionine (**SAM**) facilitated by methyltransferases in the process of Met metabolism. The SAM serves as a precursor for glutathione and is shown to modulate *IL-10* and *IL-6*, 2 major anti-inflammatory cytokines (Song et al., 2005). In the challenge study, DL-Met, L-Met, or MHA-FA had no effect on *IL-10, IL1-β, NFκB, IFNy*, and *MUC-2* mRNA expression compared with DEF treatment. Recently Adhikari et al. (2022) found no effect of different levels of Met supplementation to a basal diet deficient in total sulfur amino acids on ileal gene expression of *IL-10, IL1-β, IFNy* of broiler under normal conditions. On the other hand, some positive effects of higher inclusion of DL-Met and MHA-FA on IL-10 expression in the liver of heat-stressed birds have been shown (Liu et al., 2019). Possible tissue effect or involvement of other immune pathways and regulatory functions not specifically studied here might explain the lack of differences observed in our study. In addition, in this study, the background Met+Cys may have been sufficient to sustain selected genes in the gut at least at the mRNA level.

A comprehensive study of intestinal microbiota was undertaken to ascertain whether sources of Met and their levels of supplementation have effects on the composition of ileal and cecal microbiota. Besides, intestinal microbiota plays a pivotal role in the intestinal health of poultry with specific bacteria known to promote or hinder performance (Diaz Carrasco et al., 2019). Alpha diversity measures describe the diversity within a single sample, whereas beta diversity measures describe the diversity between samples. The alpha diversity measured as Shannon index represents the species richness and the community evenness whereas the Pielou index reflects only the evenness of the bacterial community (Wang et al., 2018). Shannon diversity and Pielou index did not differ between treatment groups for the ileum. However, in the cecum, birds fed DEF or SUB supplemental Met had a higher Shannon index, probably caused by a more even community distribution as evidenced by a higher Pielou index. No differences in cecal alpha diversity were observed between ADE and OVR supplemented birds and between Met sources. For beta diversity, from the PCoA plots and the PERMANOVA analysis, we found an effect of Met level on the microbiota composition in ileum and cecum, particularly in ileal microbiota where the SUB level was already significantly different from DEF. From this, we conclude that Met level in the diet overall does not change which bacteria are present in the respective digesta but Met deficiency can have a strong effect on the distribution of bacteria within the ileal and cecal microbiota.

Similar to our results, the dominance of *Lactobacillus* in the ileum of broilers is widely reported (Bjerrum et al., 2006). The ileum is the place of water and mineral resorption but also contributes to starch and lipid digestion (Richards-Rios et al., 2020). Richards-Rios et al. (2020) suggest that the ileal microbiota dominated by *Lactobacillus* and other fast-growing facultative anaerobes like Enterococcus can function as an amino acid sink preventing a high nitrogen influx into the ceca where anaerobic fermentation could lead to toxic by-products (Apajalahti and Vienola, 2016). As the microbiota composition changes along with Met supplementation level and FCR of the birds, we cannot distinguish direct effects of Met deficiency from effects caused by a higher nutrient availability for the bacteria.

Cecal microbiota often reflects more fermentation activity resulting mainly from protein and carbohydrates (Apajalahti and Vienola, 2016). As Met-deficient birds had a much higher FCR, more undigested feed and/or unabsorbed nutrients may likely have entered the ceca and affected the composition of microbiota and diversity. Two other studies conducted addressing the same question have shown no significant changes in cecal alpha diversity in response to Met deficiency in poultry (Kumar et al., 2019; Liu et al., 2022), but 1 found an effect on beta diversity as well (Kumar et al., 2019). Liu et al. (2022) investigated a regional Chinese breed of laying hens at 6 wk of age and found no effect of Met deficiency on the cecal microbiota, potentially because the birds were much older. Kumar et al. (2019) investigated Cobb 500 broiler at 21 and 42 d of age, respectively, and found an effect of Met level on cecal beta diversity. However, the cecal microbiota composition consisting mostly of Clostridia Custer XI and *Lactobacillus* in that trial was highly different from our trial, which prevents a comparison of changes of individual genus.

No consistent or distinguishable difference between DL-Met, L-Met, or MHA-FA could be substantiated for the beta-diversity microbiota composition of ileal or cecal contents at levels up to ADE. The changes in the composition of the ileal microbiota at the OVR supplementation level in response to MHA-FA could be explained by a possible interaction of MHA-FA with intestinal microbiota in chickens as has been previously claimed (Drew et al., 2003). We also found that *Lactobacillus* sequence variants react differently to high levels of MHA-FA supplementation than to high levels of L- or DL-Met supplementation. Others found similar varying responses in the *Lactobacillus* genus toward different treatments (Van der Hoeven-Hangoor et al., 2013) indicating that variety in function between different *Lactobacillus* species might contribute to its consistent dominance in the chicken ileum. However, the mechanism or the nature of such possible interaction between MHA-FA and the intestinal microbiota was beyond the scope of this study and requires further research.

Additionally, in the present study, DEF treatment with no Met supplementation reduced the abundance of one of the *Lactobacillus* genera with no distinguishable differences between sources of Met. *Lactobacillus* species are often associated with improved performance of broiler chickens, but their abundance can also vary over time (Richards-Rios et al., 2020). Therefore, deficiency of Met and significantly reduced performance is consistent with a reduction in *Lactobacillus* in the ileum of the same birds.

Besides *Lactobacillus* species, Met level affected *Peptostreptococcus* abundance. Particularly in the ileum but to a lower extent also in the ceca, *Peptostreptococcus* was more abundant in Met deficient birds. While Peptostreptococcus is part of the normal microbiota in the ileum (Kollarcikova et al., 2019), in humans it can be an opportunistic pathogen (Murphy and Frick, 2013). We found only 1 publication where its abundance changed in poultry. Yan et al. (2022) found that coarsely ground corn increased *Peptostreptococcus* in the ileum, indicating that potentially *Peptostreptococcus* thrive in nutrient-rich conditions. A low FCR in Met deficient birds means many nutrients are not absorbed from the digesta and can potentially be used by the microbiota.

Also in the ceca, the level of Met concentration is more influential than the source of Met as Lachnospiraceae were found to be higher in cecal content of birds fed DEF and SUB level of L- and DL-Met and there was no difference between sources of Met at ADE or OVR supplementation levels. Also in the ileum, several low abundant OTUs of the Lachnospiraceae family were higher in birds fed DEF diet compared to all other levels. In general, an increase in the Lachnospiraceae family has been linked with improved growth performance parameters in chickens (Diaz Carrasco et al., 2019). However, performance in our trial (Experiment 1) was limited by a missing nutrient and not by a gut health challenge which could have been ameliorated by change in the cecal microbiota. Therefore, we saw an increase in performance with Met supplementation despite a decrease in Lachnospiraceae indicating that the cause and consequence of microbial changes need to be carefully considered when the challenge is less clearly defined.

Lachnospiraceae can degrade some sugars including nonstarch polysaccharides and starch (Biddle et al., 2013) and in high abundance can be associated with some metabolic disorders in humans by affecting glucose and/or lipid metabolism (Vacca et al., 2020). Considering that Met deficiency led to a high FCR in our study and can impact digestive dynamics including amino acid and protein digestibility (Macelline et al., 2022), changes in Lachnospiraceae may be indirectly explained. However, the exact mechanisms by which Met deficiency promoted Lachnospiraceae in our study remain elusive.

The cecal Anaerotruncus taxa at family level was more abundant in birds fed DEF and L-Met and MHA-FA at the SUB level and not at other levels. Cecal Anaerotruncus abundance has been shown to be correlated with low productivity in chickens including low BW and BWG, high residual feed intake, and poor feed efficiency (Diaz Carrasco et al., 2019; Metzler-Zebeli et al., 2019). As such, the poor performance of birds fed SUB and DEF treatments are consistent with a higher cecal abundance of Anaerotruncus. As in our study performance was limited by nutrient deficiency, it indicates that an increase of Anaerotruncus is a consequence of low growth or feed efficiency rather than a cause. Further research is warranted on underlying mechanisms of changing the abundance of Anaerotruncus by different Met sources and nutrients in general.

As expected, dietary Met supplementation had profound effects on the growth performance of broiler chickens regardless of the tested sources. Despite a reduced growth performance of birds observed in this study in response to MHA-FA, no difference was found between DL-Met and L-Met sources. In summary, there were no consistent differences between 3 sources of Met at any levels of supplementation for gut health parameters assessed by expression of selected TJ, immune function, and inflammation-related genes. In the second experiment, largely similar results were observed in birds submitted to a leaky gut challenge induced by repeated injections of DEX exhibiting no effect of Met supplementation on intestinal permeability as the main function of the intestinal barrier. The 3 sources of Met also had a minor impact on ileal microbiota and no impact on cecal microbiota composition compared with the level of Met supplementation. Therefore, it can be concluded that, at comparable levels, the 3 tested sources of Met may largely be regarded as similar in terms of their implications for intestinal microbiota and gut barrier function in broiler chickens.

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DISCLOSURES

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SUPPLEMENTARY MATERIALS

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REFERENCES

Adhikari, P. A., F. L.d. S. Castro, G. Liu, and W. K. Kim. 2022. Effects of total sulfur amino acids on growth performance, immunity, and meat yield in broilers fed diets with and without antibiotics. Front. Vet. Sci. 9:903901.

Ahmad, R., R. Chaturvedi, D. Olivares-Villagómez, T. Habib, M. Asim, P. Shivesh, D. Polk, K. T. Wilson, M. K. Washington, and L. Van Kaer. 2014. Targeted colonic claudin-2 expression renders resistance to epithelial injury, induces immune suppression, and protects from colitis. Mucosal Immunol. 7:1340–1353.

Ahmed, S. M. U., L. Luo, A. Namani, X. J. Wang, and X. Tang. 2017. Nrf2 signaling pathway: pivotal roles in inflammation. Biochim. Biophys. Acta - Mol. Basis Dis. 1863:585–597.

Apajalahti, J., and K. Vienola. 2016. Interaction between chicken intestinal microbiota and protein digestion. Anim. Feed Sci. Technol. 221:323–330.

Aviagen. 2019. Ross 308 Broiler: Performance Objectives, 2019. Ross Breeders Limited, Newbridge, Midlothian, Scotland, UK.

Barekatain, R., T. Chalvon-Demersay, C. McLaughlan, and W. Lambert. 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., P. Chrystal, G. Howarth, C. McLaughlan, S. Gilani, and G. Nattrass. 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., G. Nattrass, A. Tilbrook, K. Chousalkar, and S. Gilani. 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.
Bauriedel, W. 1963. The effect of feeding D-methionine on the D-amino acid oxidase activity of chick tissues. Poult. Sci. 42:214–217.
Biddle, A., L. Stewart, J. Blanchard, and S. Leschine. 2013. Untangling the genetic basis of fibrolytic specialization by Lachnospiraceae and Ruminococcaceae in diverse gut communities. Diversity 5:627–640.
Bjerrum, L., R. Engberg, T. Leser, B. Jensen, K. Finster, and K. Pedersen. 2006. Microbial community composition of the ileum and cecum of broiler chickens as revealed by molecular and culture-based techniques. Poult. Sci. 85:1151–1164.
Brisbin, J. T., J. Gong, P. Parvizi, and S. Sharif. 2010. Effects of lactobacilli on cytokine expression by chicken spleen and cecal tonsil cells. Clin. Vaccine Immunol. 17:1337–1343.
Çenesiz, A., İ. Çiftci, and N. Ceylan. 2022. Effects of DL-and L-methionine supplementation on growth performance, carcass quality and relative bioavailability of methionine in broilers fed maize-soybean-based diets. J. Anim. Feed Sci. 31:142–151.
Chen, J., G. Tellez, J. D. Richards, and J. Escobar. 2015. Identification of potential biomarkers for gut barrier failure in broiler chickens. Front. Vet. Sci. 2:14.
Del Vesco, A. P., A. de Souza Khatlab, T. P. Santana, P. C. Pozza, M. A. M. Soares, C. O. Brito, L. T. Barbosa, and E. Gasparino. 2020. Heat stress effect on the intestinal epithelial function of broilers fed methionine supplementation. Livest. Sci. 240:104152.
Diaz Carrasco, J. M., N. A. Casanova, and M. E. Fernández Miyakawa. 2019. Microbiota, gut health and chicken productivity: what is the connection? Microorganisms 7:374.
Drew, M., A. Van Kessel, and D. Maenz. 2003. Absorption of methionine and 2-hydroxy-4-methylthiobutoanic acid in conventional and germ-free chickens. Poult. Sci. 82:1149–1153.
Esteve-Garcia, E., and D. R. Khan. 2018. Relative bioavailability of DL and L-methionine in broilers. Open J. Anim. Sci. 8:151–162.
Garlich, J. 1985. Response of broilers to DL-methionine hydroxy analog free acid, DL-methionine, and L-methionine. Poult. Sci. 64:1541–1548.
Gilani, S., P. V. Chrystal, and R. Barekatain. 2021. Current experimental models, assessment and dietary modulations of intestinal permeability in broiler chickens. Anim. Nutr. 7:801–811.
Gilani, S., G. Howarth, G. Nattrass, S. Kitessa, R. Barekatain, R. Forder, C. Tran, and R. Hughes. 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.
Gong, Y., W. Xia, X. Wen, W. Lyu, Y. Xiao, H. Yang, and X. Zou. 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., N. K. Emami, and S. Dridi. 2021. Research Note: Phytobiotics 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.
Kollarcikova, M., T. Kubasova, D. Karasova, M. Crhanova, D. Cejkova, F. Sisak, and I. Rychlik. 2019. Use of 16S rRNA gene sequencing for prediction of new opportunistic pathogens in chicken ileal and cecal microbiota. Poult. Sci. 98:2347–2353.
Kumar, S., P. Adhikari, B. Oakley, and W. Kim. 2019. Changes in cecum microbial community in response to total sulfur amino acid (TSAA: DL-methionine) in antibiotic-free and supplemented poultry birds. Poult. Sci. 98:5809–5819.
Lauinger, L., and P. Kaiser. 2021. Sensing and signaling of methionine metabolism. Metabolites 11:83.
Lee, J., J. Koehler, B. Yusta, J. Bahrami, D. Matthews, M. Rafii, P. B. Pencharz, and D. J. Drucker. 2017. Enteroendocrine-derived glucagon-like peptide-2 controls intestinal amino acid transport. Mol. Metab. 6:245–255.
Lee, M., W. Lin, S. Wang, L. Lin, B. Yu, and T. Lee. 2018. Evaluation of potential antioxidant and anti-inflammatory effects of Antrodia cinnamomea powder and the underlying molecular mechanisms via Nrf2-and NF-κB-dominated pathways in broiler chickens. Poult. Sci. 97:2419–2434.
Lemme, A., D. Hoehler, J. Brennan, and P. Mannion. 2002. Relative effectiveness of methionine hydroxy analog compared to DL-methionine in broiler chickens. Poult. Sci. 81:838–845.
Lemme, A., V. Naranjo, and J. C. de Paula Dorigam. 2020. Utilization of methionine sources for growth and Met+ Cys deposition in broilers. Animals 10:2240.
Liu, G., A. D. Magnuson, T. Sun, S. A. Tolba, C. Starkey, R. Whelan, and X. G. Lei. 2019. Supplemental methionine exerted chemical form-dependent effects on antioxidant status, inflammation-related gene expression, and fatty acid profiles of broiler chicks raised at high ambient temperature. J. Anim. Sci. 97:4883–4894.
Liu, Y., D. Wang, L. Zhao, J. Zhang, S. Huang, and Q. Ma. 2022. Effect of methionine deficiency on the growth performance, serum amino acids concentrations, gut microbiota and subsequent laying performance of layer chicks. Front. Vet. Sci. 9:878107.
Macelline, S. P., P. V. Chrystal, L. R. McQuade, B. V. Mclnerney, Y. Kim, Y. Bao, P. H. Selle, and S. Y. Liu. 2022. Graded methionine dietary inclusions influence growth performance and apparent ileal amino acid digestibility coefficients and disappearance rates in broiler chickens. Anim. Nutr. 8:160–168.
Markovic, M. A., and P. L. Brubaker. 2019. The roles of glucagon-like peptide-2 and the intestinal epithelial insulin-like growth factor-1 receptor in regulating microvillus length. Sci. Rep. 9:1–13.
Marrett, L., and M. Sunde. 1965. The effect of other D amino acids on the utilization of the isomers of methionine and its hydroxy analogue. Poult. Sci. 44:957–964.
Martín-Venegas, R., M. T. Brufau, A. M. Guerrero-Zamora, Y. Mercier, P.-A. Geraert, and R. Ferrer. 2013. The methionine precursor DL-2-hydroxy-(4-methylthio) butanoic acid protects intestinal epithelial barrier function. Food Chem. 141:1702–1709.
Metzler-Zebeli, B. U., S.-C. Siegerstetter, E. Magowan, P. G. Lawlor, R. M. Petri, N. E. O′Connell, and Q. Zebeli. 2019. Feed restriction modifies intestinal microbiota-host mucosal networking in chickens divergent in residual feed intake. mSystems 4 e00261-00218.
Miao, Z., Y. Dong, X. Qin, J. Yuan, M. Han, K. Zhang, S. Shi, X. Song, J. Zhang, and J. Li. 2021. Dietary supplementation of methionine mitigates oxidative stress in broilers under high stocking density. Poult. Sci. 100:101231.
Mullin, J. M., S. M. Skrovanek, A. Ramalingam, K. M. DiGuilio, and M. C. Valenzano. 2016. Methionine restriction fundamentally supports health by tightening epithelial barriers. Ann. N. Y. Acad. Sci. 1363:59–67.
Murphy, E. C., and I.-M. Frick. 2013. Gram-positive anaerobic cocci −commensals and opportunistic pathogens. FEMS Microbiol. Rev. 37:520–553.
Pacheco, L. G., N. K. Sakomura, R. M. Suzuki, J. C. Dorigam, G. S. Viana, J. Van Milgen, and J. C. Denadai. 2018. Methionine to cystine ratio in the total sulfur amino acid requirements and sulfur amino acid metabolism using labelled amino acid approach for broilers. BMC Vet. Res. 14:1–11.
Patel, R. M., L. S. Myers, A. R. Kurundkar, A. Maheshwari, A. Nusrat, and P. W. Lin. 2012. Probiotic bacteria induce maturation of intestinal claudin 3 expression and barrier function. Am. J. Clin. Pathol. 180:626–635.
Payne, R. L., A. Lemme, H. Seko, Y. Hashimoto, H. Fujisaki, J. Koreleski, S. Swiatkiewicz, W. Szczurek, and H. Rostagno. 2006. Bioavailability of methionine hydroxy analog-free acid relative to DL-methionine in broilers. Anim. Sci. J. 77:427–439.
Ren, B., L. Wang, A. Mulati, Y. Liu, Z. Liu, and X. Liu. 2021. Methionine restriction improves gut barrier function by reshaping diurnal rhythms of inflammation-related microbes in aged mice. Front. Nutr. 8:746592.

Ribeiro, A. M. L., F. Dahlke, and A.d. M. Kessler. 2005. Methionine sources do not affect performance and carcass yield of broilers fed vegetable diets and aubmitted to cyclic heat stress. Braz. J. Poult. Sci. 7:159–164.

Richards-Rios, P., J. Fothergill, M. Bernardeau, and P. Wigley. 2020. Development of the ileal microbiota in three broiler breeds. Front. Vet. Sci. 7:17.

Romanet, S., J. R. Aschenbach, R. Pieper, J. Zentek, J. K. Htoo, R. A. Whelan, and L. Mastrototaro. 2020. Dietary supplementation of DL-methionine potently induces sodium-dependent l-methionine absorption in porcine jejunum ex vivo. J. Nutr. 150:1782–1789.

Song, Z., S. Uriarte, R. Sahoo, T. Chen, S. Barve, D. Hill, and C. McClain. 2005. S-adenosylmethionine (SAMe) modulates interleukin-10 and interleukin-6, but not TNF, production via the adenosine (A2) receptor. Biochim. Biophys. Acta - Mol. Cell Res. 1743:205–213.

Sporn, M. B., and K. T. Liby. 2012. NRF2 and cancer: the good, the bad and the importance of context. Nat. Rev. Cancer 12:564–571.

Turner A.I., Keating C.L. and Tilbrook A.J., Sex differences and the role of sex steroids in sympatho-adrenal medullary system and hypothalamo-pituitary adrenal axis responses to stress, *Pages 115 −136 in Sex Steroids*, 2012, Chapter 6. Accessed Apr. 2023. https://www.intechopen.com/chapters/27068.

Ünsal H.m. and Balkaya M., Glucocorticoids and the intestinal environment, *Glucocorticoids*, 2012. Accessed Apr. 2023. https://www.intechopen.com/chapters/41151.

Vacca, M., G. Celano, F. M. Calabrese, P. Portincasa, M. Gobbetti, and M. De Angelis. 2020. The controversial role of human gut lachnospiraceae. Microorganisms 8:573.

Van der Hoeven-Hangoor, E., J. Van Der Vossen, F. Schuren, M. Verstegen, J. De Oliveira, R. Montijn, and W. Hendriks. 2013. Ileal microbiota composition of broilers fed various commercial diet compositions. Poult. Sci. 92:2713–2723.

Venugopal, S., S. Anwer, and K. Szászi. 2019. Claudin-2: roles beyond permeability functions. Int. J. Mol. Sci. 20:5655.

Wang, X., J. Feng, M. Zhang, X. Li, D. Ma, and S. Chang. 2018. Effects of high ambient temperature on the community structure and composition of ileal microbiome of broilers. Poult. Sci. 97:2153–2158.

Wang, W., J. Wang, S. Wu, X. Dong, C. Guo, H. Zhang, and G. Qi. 2019. Bioavailability of l-methionine relative to dl-methionine in Broiler chickens. Ital. J. Anim. Sci. 18:1231–1238.

Wickramasuriya, S. S., E. Kim, H.-M. Cho, T.-K. Shin, B. Kim, M. Lee, S. Seo, J.-M. Heo, and H. Choi. 2019. Differential effects of dietary methionine isomers on broilers challenged with acute heat stress. J. Poult. Sci. 56:195–203.

Wideman, R., and I. Pevzner. 2012. Dexamethasone triggers lameness associated with necrosis of the proximal tibial head and proximal femoral head in broilers. Poult. Sci. 91:2464–2474.

Xiao, M., Y. Mi, L. Liu, C. Lv, W. Zeng, C. Zhang, and J. Li. 2018. Taurine regulates mucosal barrier function to alleviate lipopolysaccharide-induced duodenal inflammation in chicken. Amino Acids 50:1637–1646.

Yan, L., S. An, Z. Lv, M. Choct, G. Zhou, Y. Li, J. Zhuo, Z. Wang, J. Lai, and M. Lv. 2022. Effects of corn particle size on growth performance, gastrointestinal development, carcass indices and intestinal microbiota of broilers. Poult. Sci. 101:102205.

Zeitz, J. O., S. Mohrmann, S. C. Käding, M. Devlikamov, I. Niewalda, R. Whelan, A. Helmbrecht, and K. Eder. 2019. Effects of methionine on muscle protein synthesis and degradation pathways in broilers. J. Anim. Physiol. Anim. Nutr. (Berl.) 103:191–203.