New statistical approach shows that hydroxy-methionine is noninferior to DL-Methionine in 35-day-old broiler chickens

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ABSTRACT The efficacy of a new molecule is assessed in the pharmaceutical industry through noninferiority tests to establish that it is not unacceptably less efficient than the reference. This method was proposed to compare DL-Methionine (**DL-Met**) as reference and DL-Hydroxy-Methionine (**OH-Met**) as alternative, in broiler chickens. The research hypothesized that OH-Met is inferior to DL-Met. Noninferiority margins were determined using 7 datasets comparing broiler growth response between a sulfur amino acid deficient and adequate diet from 0 to 35 d. The datasets were selected from the literature and internal records of the company. The noninferiority margins were then fixed as the largest loss of effect (inferiority) acceptable when OH-Met is compared to DL-Met. Three corn/soybean meal-based experimental treatments were offered to 4,200 chicks (35 replicates of 40 birds). Birds received from 0 to 35 d 1) a negative control diet deficient in Met and Cys; the negative control treatment supplemented on equimolar basis with 2) DL-Met or 3) OH-Met in amounts allowing to reach Aviagen Met+Cys recommendations. The three treatments were adequate in all other nutrients. Growth performance, which was analysed with one-way ANOVA, showed no significant difference between DL-Met and OH-Met. The supplemented treatments improved ($P < 0.0001$) the performance parameters compared to the negative control. The lower limits of the confidence intervals of the difference between means for the feed intake [-1.34; 1.41], body weight [-57.3; 9.8] and daily growth [-1.64; 0.28], did not exceed the noninferiority margins. This demonstrates that OH-Met was non-inferior to DL-Met.

Key words: methionine, hydroxy-methionine, broiler, noninferiority, efficacy

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INTRODUCTION

The pharmaceutical industry uses equivalence experiments to study the bioequivalence of 2 active compounds (Elie et al., 2008). Regulatory authorities in the pharmaceutical industry require that the final quality judgment of a test compound be based on its rate and extent of absorption compared to the reference product. It is agreed that if 2 formulations exhibit similar blood concentration-time profiles, they should exhibit similar therapeutic effects (Rani and Pargal, 2004; Elie et al., 2008). Thus, bioequivalence of 2 active compounds is demonstrated when the same biological effects that is, the same therapeutic effects, in terms of efficacy and tolerance are proven (Rani and Pargal, 2004). Bioequivalence studies are designed to show the equivalence between 2 galenic forms of the same molecule (for example, capsules vs. tablets) or 2 molecules of the same therapeutic category. According to Elie et al. (2008), however, these studies can face several challenges such as the fact that the use of plasma kinetics is irrelevant or the molecules have different routes of administration or as frequently observed, they are simply 2 different treatments. In addition, in many cases, the difference of efficacy between the new and the referent treatment is so small that it becomes difficult to prove statistical superiority to the reference treatment (Elie et al., 2008). Consequently, noninferiority studies have appeared in medical journals with increasing frequency (Suda et al., 2011). Noninferiority trials aim to assess whether the new molecule is 'not unacceptably worse' than the molecule used as the reference (Schumi and Wittes, 2011). Such trials aim to show that a new treatment is safer or easier to use without loss of efficacy, or both, rather than to demonstrate its superiority (Elie et al., 2008).

DL-Methionine (2-amino-4-(methylthio)butanoic acid, DL-Met) and DL-Hydroxy-Methionine (2-

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hydroxy,4-(methyl)-thio-butanoic acid, OH-Met) are used to meet animals' requirements in sulfur amino acids (methionine, Met and Cysteine, Cys). DL-Methionine is an amino acid with one basic amino group and one acidic carboxyl group. OH-Methionine is an organic acid until complete absorption and conversion by the animal, as the amino group on the α-carbon in Met has been replaced by a hydroxyl group (2-hydroxy-4-(methylthio)butanoic acid). Recently, the European Food Safety Authority (**EFSA**) issued new guidance on the assessment of the efficacy of feed additives (Rychen et al., 2018). This guidance states "*evidence of efficacy should be provided for amino acid analogues... Where evidence from literature is insufficient to reach a conclusion, a bioequivalence study is considered adequate to demonstrate efficacy for amino acid analogues...*". The EFSA also recommended that experiments which purpose is to assess the noninferiority between 2 groups should use tests for non-inferiority (e.g., bioequivalence). Despite the fact both DL-Met and OH-Met have been used for decades in both poultry and swine, studies have inconsistent findings concerning the efficacy of OH-Met relative to DL-Met (Hoehler et al., 2005a; Sauer et al., 2008; Agostini et al., 2016; Uddin et al., 2022). Therefore, we designed this study to compare the performance obtained with OH-Met to DL-Met using statistical methods appropriate to assess its noninferiority.

MATERIALS AND METHODS

Understanding Noninferiority Trials

Noninferiority trials determine whether a new or alternate treatment is not unacceptably worse than a reference treatment. In other words, "not unacceptably worse" means that it is acceptable for the alternate treatment to have a smaller effect than the reference treatment, but within some threshold that must be defined before performing the experiment. Noninferiority and equivalence are 2 different tests. The expected outcome for equivalence trial is that the 2 products are the same (Schumi and Wittes, 2011). The methodology has been clearly described by Elie et al. (2008) and Schumi and Wittes (2011).

According to Schumi and Wittes (2011), one challenge of a noninferiority trial is defining 2 values: 1) a reliable estimate of the performance expected with the reference treatment (M_1), which could come from a meta-analysis or data synthesis, and 2) a definition of what it is meant by "unacceptably worse" (M_2). The "unacceptably worse" value (M_2) is often the most difficult of the 2 values to define because it involves clinical and regulatory judgment, and sometimes precedent from other compounds. One way to approach setting M_2 is to consider whether the alternate treatment would be worth pursuing if it preserved at least some percentage (e.g., 50, 80, 90, 95%) of the effect of the reference treatment. Often these design questions are a balance between statistical considerations and the practicalities of available resources.

Determining the Noninferiority Margins for the Comparison of Methionine Sources

The comparison of nutritional feed additives using noninferiority trials is quite recent. According to Althunian et al. (2017), the definition of noninferiority margin is not clear and there is no agreement on the ideal method allowing its accurate determination. Moreover, very little information is available on the definition of the noninferiority and equivalence margins in the feed industry. For the safety and efficacy of some feed additives, 80% is considered as a predefined noninferiority, considering up to 20% loss of efficacy compared to the control, as was applied for trials on 3-nitrooxypropanol in ruminants (Bampidis et al., 2021) and for ethyl ester of β-apo-8'-carotenoic acid in poultry (Bampidis et al., 2019). As for blood biochemistry, Bampidis et al. (2021) used the 2.5% and 97.5% percentiles, determined from the placebo.

In the case of comparison of Met sources, well-known nutritional feed additives ensuring broiler growth performance, a loss of efficacy of 20% for OH-Met in comparison to DL-Met would allow such a large difference in growth performance that the economic impact would be huge. Therefore, the 95-95 approach described by Schumi and Wittes (2011) was applied for the calculation of the noninferiority margins. The first step consisted of estimating with data from previous studies the difference between DL-Met (the reference treatment) and a basal diet (not supplemented in methionine). We considered 7 datasets from trials performed from 0 to 35 d. Four of these datasets were selected from the literature (Vazquez-Anon et al., 2006a; Zelenka et al., 2013; Drażbo et al., 2015; Zeitz et al., 2018) and three datasets were historical data of the company. Table 1 summarizes the difference between the control treatment (**DL-Met**) and the basal diet, calculated for the body weight (**BW**), the average daily feed intake (**ADFI**), the average daily gain (**ADG**), and the feed conversion ratio (**FCR**). The meta-mean is then calculated as average difference between control and basal diet from all the datasets selected. The 95% confidence interval around this difference (meta-mean) was generated from the database. M_1 was then determined as the lower limit of this confidence interval.

The second step determined the largest loss of effect (inferiority) referred to as M_2 acceptable when OH-Met is compared to DL-Met that is, from no preservation of effect (100% loss) to a preservation of all the effect (0% loss). When 100% loss is assumed with the alternate treatment (OH-Met), $M_2 = 0$ and when there is 0% loss (Full preservation), $M_2 = M_1$. Finally, we calculated the noninferiority margins ($-\delta_L$) by assuming no loss of efficacy for OH-Met compared to DL-Met (Table 4) as the difference between the mean and the confidence interval for each meta-mean presented Table 1.

Table 1. Determination of the meta-mean, difference between the reference treatment (DL-Methionine) and the basal diet for broiler's parameter of performance using historical and literature data.

		Difference between reference treatment and basal diet (DL-Methionine − Basal diet)[1]			
	Study identification	Body weight	Average daily feed intake	Average daily gain	Feed cconversion ratio
Historical company data	Study 1 (n = 35)[2]	888	23.48	25.38	−0.315
	Study 2 (n = 6)[2]	871	26.94	24.2	−0.241
	Study 3 (n = 8)[2]	652	9.84	18.11	−0.325
Literature data	Zelenka et al., 2013[2]	587	3.81	16.3	−0.509
	Zeitz et al., 2017[2]	793	16.0	22.7	−0.320
	Drazbo et al., 2015[2]	437	8.9	12.5	−0.190
	Vazquez-Anon et al., 2006a (n = 5)[3]	370	8.51	10.69	−0.238
Meta-mean (n = 7)[4]	Lower limit of 95% confidence interval (=M1)[5]	467	6.02	13.22	−0.401
	Mean	657	13.93	18.55	−0.305
	Upper limit of 95% confidence interval	847	21.83	23.89	−0.210

[1]Difference between the control treatment (DL-Methionine) and the basal diet (non-supplemented in methionine) for studies performed between 0 to 35 days, calculated as followed: DL-Methionine − Basal diet. For the trials presenting several supplementation levels, the reference DL-Methionine was chosen as the treatment presenting the most optimal data for body weight, weight gain, and feed conversion ratio.

[2]The number of replicates considered for the calculation of the difference between the control treatment, DL-Met and the basal diet is given in parentheses and was only given for the historical company data. Literature data considered treatment means.

[3]In (Vazquez-Anon et al., 2006a), only one study (Trial #2) was retained as it was performed on the desired period (from 0 to 35 d) with similar feed ingredients as the one to be used for the non-inferiority trial. Five replicates were used to calculate the difference between the control treatment, DL-Methionine and the basal diet.

[4]Seven studies were used in total to calculate the meta-mean. The meta-mean is the average percentage difference between the reference DL-Methionine and a basal diet (nonsupplemented in methionine) for studies performed between 0 to 35 days, calculated as followed: DL-Methionine − Basal diet.

[5]M1 is the lower limit of the confidence interval (95%) around the means for each of the performance criterion.

Animal Study

This experiment was conducted at SCEA de Kériotel Pluzerec, 22320 Plussulien (France). All experimental procedures were compliant with the European Standards relating to animal protection in broiler chickens and broiler breeding density.

Four Thousand Two Hundred male Ross 308 broiler chickens were reared in 120 floor pens from 0 to 35 d. During this period, a randomized complete block design was used. Three groups of broilers received 3 corn/soybean meal-based experimental treatments, consisting, respectively, of 1) a negative control diet deficient in Met and Cys (Table 2); the negative control treatment supplemented either with 2) DL-Met or 3) OH-Met on equimolar basis allowing them to reach Met+Cys requirements (Aviagen, 2019). The sample size was calculated prior to the start of the trial based on the following formula [Eq. 1] from (Elie and Touzé, 2012; Walker, 2019).

$$n = \frac{2\sigma^2}{\delta_L^2}\left(z_{1-\alpha} + z_{1-\beta}\right)^2 \quad (1)$$

Where n is the number of replicates per treatment, σ^2 is the variance of the criteria of response of the reference treatment (e.g., variance of the ADG obtained for DL-Met), δ_L the inverse of the noninferiority margin, α is Type I error rate ($P = 0.05$) and $1 - \beta$ is the power ($\beta = 0.20$). The variances used for DL-Met were of 15.38, 14.52, 0.0006, and 11,561, respectively for the ADFI, ADG, FCR, and final BW. The noninferiority margins are given Table 4.

The sample size calculated using [Eq. 1] was extremely small, giving 2 to 6 replicates, depending on the performance parameter considered. However, to avoid any lack of precision of the design as explained by Cohen (1988), we retained the sample size applied for one of the historical company studies, which data was used to calculate the noninferiority limits. Thus, each treatment consisted of 35 floor pens replicates of 40 birds. Each floor pen (1.90 m × 1.25 m × 0.80 m) consisted of a dedicated nipple line, one hung pan feeder and fresh wood shavings as bedding. All treatments were adequate in all nutrients accordingly to Ross 308 recommendations (Aviagen, 2019), except Met and Met + Cys which differed between the negative control and the supplemented treatments. Table 2 presented the feed ingredients and nutritional composition of the experimental diets used from d 0 to 10 (starter), d 11 to 24 (grower) and d 25 to 35 (finisher). The amino acids composition of the basal diet is presented Supplementary Table S1.

The birds were housed in an environmentally controlled facility and had ad libitum access to feed and water. They were fed with crumbs from d 0 to 10 and pellets from d 10 to 35. The lighting program comprised the following schedule: 24 h light from d 1 to 4 (60 lux), 100% light intensity; and 18 h light:6h dark (16L- 4D-2L- 2D), 30 to 40% light intensity from d 5 to 35 (25 lux). Collective body weight was measured at d 0, 10, and 24 and individual body weight was measured at d 35. The total feed intake was measured for each replicate (pen) at d 10, 24, and 35. The body weights of dead or culled broilers were measured to adjust the feed conversion ratio (FCR) of birds from 0 to 10 d, from 10 to 24 d, and from 24 to 35 d. The adjusted feed conversion ratio was calculated with the following formula (Total feed consumption/(Total weight of alive birds + Total weight of dead birds)). Temperature and humidity were recorded daily.

Table 2. Feed ingredients and nutrients composition of the experimental diets offered to broiler chickens in the starter, grower, and finisher phase.

	Starter phase (0−10 d)			Grower phase (10−24 d)			Finisher phase (24−35 d)		
	Basal diet	DL-Methionine	OH-Methionine	Basal diet	DL-Methionine	OH-Methionine	Basal diet	DL-Methionine	OH-Methionine
Ingredients, %									
Corn	43.64	44.02	43.91	46.69	46.97	46.89	53.62	53.91	53.83
Soybean meal 48	36.78	36.20	36.22	36.46	36.02	36.04	31.44	30.99	31.01
Wheat	10.00	10.00	10.00	7.00	7.00	7.00	5.00	5.00	5.00
Soybean oil	5.01	4.81	4.85	5.90	5.75	5.78	6.10	5.95	5.98
Dicalcium phosphate	2.87	2.87	2.87	2.53	2.53	2.54	2.40	2.40	2.40
Mineral and vitamin premix[1]	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50	0.50
Calcium carbonate	0.33	0.33	0.33	0.31	0.31	0.31	0.28	0.28	0.28
Sodium sulfate	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24	0.24
Salt	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19	0.19
L-Lysine HCl 98	0.26	0.28	0.28	0.11	0.12	0.12	0.15	0.16	0.16
L-Threonine	0.15	0.16	0.16	0.06	0.07	0.07	0.07	0.08	0.08
L-Valine	0.03	0.04	0.04						
DL-Methionine, 99%[2]		0.36			0.29			0.29	
OH-Methionine, 88%[2]			0.41			0.32			0.33
Calculated composition, %									
Crude protein	22.0	22.0	22.0	21.5	21.5	21.5	19.5	19.5	19.5
Fat	7.21	7.02	7.05	8.16	8.01	8.04	8.51	8.36	8.38
AMEn, kcal/kg	2,850	2,850	2,850	2,925	2,925	2,925	3,000	3,000	3,000
Total Lys	1.39	1.39	1.39	1.26	1.26	1.26	1.16	1.16	1.16
Total Met	0.32	0.67	0.67	0.32	0.60	0.60	0.29	0.58	0.58
Total Met+Cys	0.68	1.03	1.03	0.67	0.95	0.95	0.62	0.90	0.90
Digestible Lys	1.28	1.28	1.28	1.15	1.15	1.15	1.06	1.06	1.06
Digestible Met equivalent[3]	0.29	0.65	0.65	0.29	0.57	0.57	0.27	0.56	0.56
Digestible Met+Cys equivalent[3]	0.60	0.95	0.95	0.59	0.87	0.87	0.55	0.83	0.83
Digestible Thr	0.86	0.86	0.86	0.77	0.77	0.77	0.71	0.71	0.71
Digestible Trp	0.26	0.25	0.25	0.25	0.25	0.25	0.22	0.22	0.22
Digestible Arg	1.42	1.40	1.40	1.40	1.39	1.39	1.25	1.24	1.24
Digestible Val	0.96	0.96	0.96	0.92	0.91	0.91	0.83	0.83	0.83
Digestible Ile	0.91	0.90	0.90	0.90	0.89	0.89	0.80	0.79	0.79
Digestible Leu	1.66	1.64	1.64	1.65	1.64	1.64	1.53	1.52	1.52
Digestible His	0.51	0.50	0.50	0.50	0.50	0.50	0.46	0.45	0.45
Digestible Phe+Tyr	1.62	1.60	1.60	1.61	1.59	1.59	1.46	1.45	1.45
Ca	0.96	0.96	0.96	0.87	0.87	0.87	0.81	0.81	0.81
Av. P	0.48	0.48	0.48	0.43	0.43	0.43	0.40	0.40	0.40
Na	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
Cl	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16
K	0.95	0.93	0.93	0.94	0.93	0.93	0.85	0.84	0.84
Dietary electrolyte balance, mEq/kg	302	300	300	300	298	298	277	275	275
DL-Methionine		0.36			0.28			0.29	
OH-Methionine			0.36			0.28			0.29
Analyzed composition, %									
Crude protein	22.1	21.9	22	21.3	21.5	21.6	19.5	19.4	19.3
Crude fat	7.4	7.2	7.8	8.4	8.3	8.2	8.6	8.4	8.6
Ash	6.2	6.1	6.3	5.9	5.9	5.9	5.7	5.5	5.6
DL-Methionine		0.34			0.27			0.26	
OH-Methionine			0.35			0.26			0.26
Total Met equivalent[2]	0.33	0.67	0.68	0.31	0.58	0.57	0.29	0.55	0.55
Total Met+Cys equivalent[2]	0.68	1.02	1.03	0.66	0.93	0.92	0.61	0.87	0.87

[1]Supplies per kilogram of diet: Vitamin A: 10,000 IU; Vitamin D3: 4,800 IU; Vitamin E: 45 mg; Vitamin K3: 3 mg; Vitamin B1: 3 mg; Vitamin B2: 9 mg; Vitamin B6: 4.5 mg: Vitamin B12: 40 μg; Folic acid: 1.8 mg; Biotin: 150 μg; Calcium panthotenate: 16.5 mg; Niacin: 51 mg; Mn (as MnSO4.H2O): 90 mg; Zn (as ZnO): 66 mg; I (as KI): 1.2 mg; Fe (as FeSO4.H2O): 54 mg; Cu (as CuSO4.5H20): 12 mg; Se (as NaSeO3): 0.18 mg; BHT: 25 mg; Calcium formate, 5 mg; Silicic acid, dry and precipitated, 25 mg; Calcium stearate, 25 mg; Calcium carbonate, 4 g.

[2]DL-Methionine and OH-Methionine are supplemented on equimolar basis.

[3]Met and Met+Cys equivalent are calculated for OH-Methionine-based treatments as the sum between the Met supply from feed ingredients and the OH-Methionine level.

Feed samples were collected during the experiment and analyzed in the experimental diets using methods of the International Organization for Standardization (ISO methods, http://www.iso.org). Samples were analyzed for Dry Matter (ISO 6496:1999), ash (NEN-ISO 5984:2003), crude protein (NEN-EN-ISO 16634-1:2008), Crude fibre (ISO 6865:2000) and crude fat (6492:1999). Dietary Met, Cys and OH-Met were analyzed using the methods previoulsy described by Agostini et al. (2016). Briefly, feed samples were grounded at 0.5 mm for added methionine sources extraction. OH-Met was extracted using water-methanol solution under stirring. The solution was treated under alkaline solution to hydrolyze oligomers and then neutralized before H PLC injection using a reverse phase column. The OH-Met peak was detected using UV detection at 214 nm. For DL-Met, extraction was done with 0.1N HCl solution containing thiodiglycol and adjusted to pH 2.2 by dilution in a citric/citrate buffer. DL-Met was separated using ion-exchange chromatography and determined after post column ninhydrin derivatization with colorimetric detection at 570 nm. Dietary total amino acids contents were obtained using ion-exchange chromatography on an autoanalyzer (ISO 13903-2005). The calculated and analyzed amino acids profile of the basal diet are presented in supplemented material S1.

Statistical Analyses

All data were analyzed using JMP software (JMP version 17.0.0, JMP Statistical Discovery LLC 2022). Growth performance data obtained on each period of age was analyzed using a one-way ANOVA to determine the effect of the experimental treatments. Treatment means were compared with a Tukey's test, with significant differences being declared at $P < 0.05$. The added Met equivalent intake was calculated as the product between the analyzed supplemental Met level (DL-Met or OH-Met) and the daily feed intake, and statistically analyzed using a t test.

Data obtained on the overall period (0−35 d) was submitted to the noninferiority tests in JMP. In the Two one-sided pooled-variance t tests (**TOST**) method (Schuirmann, 1987), only the one-sided t tests are used for noninferiority. The null hypothesis assumes that the true difference exceeds the threshold value. The difference in the means does not statistically exceed the threshold value if the null hypothesis is rejected ($P < 0.05$). Therefore, the groups are considered noninferior. (JMP 17.0.0, JMP Statistical Discovery LLC 2022).

RESULTS AND DISCUSSION

Growth Performance of Broilers Fed With DL-Methionine and Hydroxy-methionine

The supplementation of Met source significantly improved ($P < 0.05$) broiler performance in comparison to the basal diet in all age periods (Table 3). DL-Met and OH-Met based diets resulted in higher ADFI ($P < 0.0001$), ADG ($P < 0.0001$), and BW (P < 0.0001), thus a lower FCR ($P < 0.0001$) in comparison to the basal

Table 3. Performance of broiler chickens fed either a deficient basal diet in sulfur amino acids or the basal diet supplemented with DL-Methionine (DL-Met) or OH-Methionine (OH-Met) to reach sulfur amino acids requirements from 0 to 35 d[1].

	Basal diet (n = 35)[2]	DL-Methionine (n = 35)[2]	OH-Methionine (n = 35)[2]	SEM	*P*-value
Starter phase (0−10 days)					
Body weight, g	240.4^{b}	315.7^{A}	314.7^{A}	1.60	< 0.0001
Average daily feed intake, g/d	23.99^{C}	29.86^{A}	29.27^{B}	0.17	< 0.0001
Added Met equivalent intake, g/d[3]	-	0.102	0.102	0.0005	0.242
Average daily gain, g	20.01^{B}	27.54^{A}	27.43^{A}	0.16	< 0.0001
Feed conversion ratio	1.001^{A}	0.948^{B}	0.932^{C}	0.002	< 0.0001
Grower phase (11-24 days)					
Body weight, g	1,033^{B}	1,427^{A}	1,407^{A}	7.23	< 0.0001
Average daily feed intake	80.02^{B}	100.0^{A}	99.55^{A}	0.51	< 0.0001
Added Met equivalent intake, g/d3	-	0.270^{A}	0.259^{B}	0.001	< 0.0001
Average daily gain, g	56.62^{B}	79.35^{A}	78.04^{A}	0.46	< 0.0001
Feed conversion ratio	1.416^{A}	1.266^{B}	1.279^{B}	0.004	< 0.0001
Finisher phase (25−35 days)					
Body weight, g	1,958^{B}	2,741^{A}	2,718^{A}	11.73	< 0.0001
Average daily feed intake	155.8^{B}	188.6^{A}	189.0^{A}	0.94	< 0.0001
Added Met equivalent intake, g/d[3]	-	0.490	0.491	0.002	0.745
Average daily gain, g	84.11^{B}	119.5^{A}	119.2^{A}	0.67	< 0.0001
Feed conversion ratio	1.874^{A}	1.597^{B}	1.609^{B}	0.009	< 0.0001
Overall (0−35 days)					
Average daily feed intake, g/d	87.50^{B}	107.2^{A}	107.1^{A}	0.46	< 0.0001
Added Met equivalent intake, g/d[3]	-	0.287^{a}	0.284^{b}	0.0009	0.039
Average daily gain, g	54.80^{B}	77.17^{A}	76.51^{A}	0.34	< 0.0001
Feed conversion ratio	1.578^{A}	1.385^{B}	1.395^{B}	0.004	< 0.0001

[1]Basal diet: negative control diet without supplementation of Methionine sources, deficient in Met+Cys. DL-Methionine and OH-Methionine were supplemented on equimolar basis.

[2]Values are means of 35 replicates of 40 birds each per treatment. Values within a row without a common superscript letter (a,b,c) indicate differences among treatments by Tukey's test. Uppercase letters (A, B) indicate $P \leq 0.01$ or less.

[3]Added Met equivalent intake, g/d was calculated as the product between the analyzed supplemental Met level (DL-Methionine or OH-Methionine) and the daily feed intake. It was statistically analyzed using a t test.

diet. These results agree with the results of Conde-Aguilera et al. (2016) who showed that a dietary deficiency in Met+Cys altered performance of broiler chickens. Overall, the effects found in the grower and finisher phase were also observed in the overall period (0−35 d), that is, the 2 groups supplemented with Met source performed better ($P < 0.0001$) than those on the basal diet and were not different from each other. Similar results have been observed by several authors (Agostini et al., 2016; Zhao et al., 2018; Uddin et al., 2022) who concluded that both DL-Met and OH-Met maintained similar broiler growth performance at Met+Cys requirement level when supplemented on equimolar basis. These results are also in contradiction with the findings of Hoehler et al. (2005a) on turkeys and Lemme et al. (2020) on broilers chickens. In the latter study, Lemme et al. (2020) compared OH-Met to DL-Met and DL-Met diluted to 65% purity and found a lower efficacy for OH-Met. In the meta-analysis of Uddin et al. (2022), the use of a bayesian approach allowed to cope with the difference of experimental methods used in studies comparing Met sources. These authors concluded that the comparison of DL-Met and OH-Met based on the Met intake or Met+Cys intake resulted in similar performance in broiler chickens when supplemented at the requirements.

In the starter period of the current study (Table 3), OH-Met-fed birds had a significantly lower ADFI (29.27 ± 0.17 g/d) in comparison to DL-Met-fed birds (29.86 ± 0.15 g/d) whereas the ADG was similar between the 2 Met sources, thus resulting in a lower FCR for OH-Met (0.932 ± 0.002) than for DL-Met (0.948 ± 0.002). These significant differences in ADFI and FCR between the 2 Met sources are not in line with results in the literature that showed no difference between the 2 Met sources (Zhao et al., 2018). This effect was not observed in the other rearing periods. Notably, we used 35 replicates per treatment in this study. As explained by Cohen (1988), the larger the sample size, assuming equality of other factors, the smaller the error and the better the precision of the results. Therefore, it can be expected that with this powerful design, any difference between DL-Met and OH-Met would become evident due to the high statistical power and a smaller least significant difference.

Despite the large sample size and low variability observed in this study, OH-Met was not different, or even slightly better than DL-Met in the starter period, which contradicts some results from the literature (Hoehler et al., 2005a; Lemme et al., 2002). Therefore, the data shown herein support that OH-Met and DL-Met have similar effects in broiler chickens fed from 0 to 35 d.

Noninferiority and Equivalence of Hydroxy-methionine to DL-Methionine

The results of the noninferiority assessment of OH-Met in comparison of DL-Met are presented Table 4. The differences between OH-Met and DL-Met and the 95% confidence intervals for the differences are presented for the average daily feed intake, daily weight gain, and final body weight. The differences as well as their 95% confidence intervals are strictly above the noninferiority limits determined using literature and historical data (Table 1). The lower the FCR, the better; thus, OH-Met was found noninferior to DL-Met because the difference between the 2 and its confidence interval was below the noninferiority margin (0.096). These results are in agreement with previous results which used rather ANOVA (Willemsen et al., 2011; Zhao et al., 2018), multiregression (Vazquez-Anon et al., 2006b) or a Bayesian analysis (Uddin et al., 2022) to demonstrate that the 2 Met sources similarly sustained growth performance.

Noninferiority Tests: What Margins to Use as Reference for Nutritional Feed Additives?

The use of noninferiority trials for comparison of animal feed additives and analogues is recent. A search performed on PubMed in May 2022 with the keyword

Table 4. Evaluation of the noninferiority of OH-Methionine compared to the reference treatment, DL-Methionine in broilers chickens fed from 0 to 35 d, for body weight, average daily feed intake, average daily gain, and feed conversion ratio.

	Body weight, g	Average daily feed intake, g/d	Average daily gain, g	Feed conversion ratio
Basal diet + DL-Methionine[1]	2,741	107.2	77.17	1.385
Basal diet + OH-Methionine[1]	2,718	107.1	76.51	1.395
Difference (OH-Methionine - DL-Methionine)	−23.2	−0.09	−0.66	0.010
95% Confidence Interval of the difference between OH-Methionine and DL-Methionine	[-50.8, 4.46]	[-1.03, 0.85]	[-1.45, 0.13]	[0.0014, 0.0194]
Non-inferiority limit (-δL)[2]	−190.1	−7.906	−5.334	0.096
P-value noninferiority[3]	< 0.0001	< 0.0001	< 0.0001	< 0.0001
Conclusion	Noninferior	Noninferior	Noninferior	Noninferior

[1]DL-Methionine and OH-Methionine were supplemented on equimolar basis to a basal diet to reach total sulfur amino acids requirements.

[2]The noninferiority margins have been determined using literature (Vazquez-Anon et al., 2006a; Zelenka et al., 2013; Drażbo et al., 2015; Zeitz et al., 2018) and three historical datasets from the company. The margins are calculated as the difference between the mean and the confidence interval for each meta-mean presented Table 1. The meta-mean is the average difference between the reference DL-Methionine and a basal diet (nonsupplemented in methionine) for studies performed between 0 to 35 days, calculated as followed: DL-Methionine − Basal diet.

[3]The null hypothesis assumes that the true difference exceeds the threshold value. If the null hypothesis is rejected ($P < 0.05$), the difference in the means does not statistically exceed the threshold value, thereby demonstrating the noninferiority.

"noninferiority" indicated more than 6,500 publications from 2011 to 2021, mostly on humans; only a few of these articles were published on animal species. Suda et al. (2011) conducted a literature search covering a period of 2 decades (from 1989 to 2009) and found 583 clinical studies evaluating drug therapies, which applied a noninferiority test. A yearly upward trend in number of publications was observed with zero study in 1989 and 133 studies in 2009 (Suda et al., 2011).

The choice of the margin for noninferiority tests is important. Among the papers included in their systematic review, Rehal et al. (2016) indicated that 98% of the authors specified the noninferiority margins but less than half of articles (45%) justified the choice of these margins. Most often, clinical basis (17%) was used as explanation in the choice of margins with little clarity; 8% of articles fetched results from previous studies or statistical reviews (Rehal et al., 2016). Schumi and Wittes (2011) suggested several methods to help choose the inferiority margin. One such method could be to propose to subject matter experts that how much loss of efficacy they could forego in return for the putative benefits of a new treatment. Other approaches may include a potential placebo, also known as synthesis method and the 95-95 approach (applied here).

The latter requires performing a meta-analysis of the previous placebo trials, as done in the current study. However, a meta-analysis is very time-consuming, and data are not always available. Moreover, it is often assumed that a loss of 20% of efficacy would be acceptable for a new treatment. Thus, the use of the margins [80%, 120%] of the performance of the reference treatment is in common practice, as performed by Bampidis et al. (2019) and Bampidis et al. (2021). We also compared the 2 Met sources assuming 20% loss of efficacy of OH-Met in comparison to DL-Met. The new noninferiority margins are obviously larger than the ones calculated previously, for example, for ADFI (-21.4), ADG (-15.4), final BW (-548.2), and FCR (0.277). Again, we concluded that OH-Met was noninferior to DL-Met in broiler chickens. Nevertheless, a 20% loss of efficacy for amino acids is unacceptable on a practical point of view. Amino acids are essential components used for protein synthesis, immune function, etc. (Baker, 2006). Their deficiency would be detrimental for animal production and health. For instance, beyond its role in protein accretion, Met also acts as a methyl donor and is precursor of Cysteine, glutathione which is an important intracellular antioxidant of the body (Brosnan and Brosnan, 2006). A deficiency in Met altered the performance, reduced the protein gain and tissue composition while increasing the lipid gain (Conde-Aguilera et al., 2013, 2016). Therefore, the choice of a smaller limit could be advised for general use in animal nutrition, when data or ressources, or both, are not available for a meta-analysis. Should an arbitrary range become more convenient, one could advise to target 3 to 5% improvement or loss of efficacy as noninferiority margins for nutritional feed additives authorization, as this would make more sense from the nutritionist point of view.

Perspectives of Utilization of Noninferiority Tests in Animal Studies

The evaluation of the efficacy of OH-Met relative to DL-Met has generated extensive number of studies in poultry species using different statistical approaches. Some authors compared treatments means after applying ANOVA (Agostini et al., 2016) and others applied regression models in dose-response studies (Lemme et al., 2002; Hoehler et al., 2005b) whereas others have used nonparametric binomial test that is, sign test (Kratzer and Littell, 2006) or meta-analyses (Sauer et al., 2008; Uddin et al., 2022). The regression models usually intended to quantify the equivalence among the 2 sources, with slope-ratio in linear models or steepness-coefficient ratio in nonlinear models. In addition to the performance comparison debate, the use of a common-plateau vs. separate plateaus in nonlinear regression models has only added unnecessary controversy (Kratzer and Littell, 2006). As mentioned by Kratzer and Littell (2006), OH-Met must be a dilution of DL-Met with the same form of growth response curve and same asymptote to allow the application of common plateau regression models. Finney (1978) described it further by stating that even a small discrepancy between the 2 response curves would invalidate the assumption that the 2 responses have the same asymptote.

The work presented herein is not meant to replace any other statistical method but rather to present a different approach, with a fresh view on this old debate. Because, it requires setting the noninferiority limits prior to the start of an experiment, the noninferiority approach, mostly used in medical field to determine whether a new molecule is not unacceptably worse than an old one (Suda et al., 2011), might be difficult to apply on a regular basis in animal nutrition. However, despite the complexity to determine the limits, one could dare to conclude that the noninferiority approach would give a qualitative evaluation of whether OH-Met is inferior to DL-Met whereas slope-ratio or steepness-coefficient ratio could be viewed as a quantitative way to express one Met source as percentage of the other. Once the limits determined or defined with subject matter experts, the noninferiority approach could be perceived as a "Yes or No" question allowing to decide whether the animal can efficiently use the product tested to sustain performance. The decision to which approach is the best to apply should be balanced with the most important concern of the final user which may be to know whether the product is efficacious or present any adverse effects on animal's performance.

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DISCLOSURES

DBA, FR, and YM are employees of Adisseo.

SUPPLEMENTARY MATERIALS

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