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Bioavailability of D-methionine relative to L-methionine for nursery pigs using the slope-ratio assay

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This experiment was conducted to determine the bioavailability of $_{D}$-methionine (Met) relative to $_{L}$-Met for nursery pigs using the slope-ratio assay. A total of 50 crossbred barrows with an initial BW of 13.5 kg (SD = 1.0) were used in an N balance study. A Met-deficient basal diet (BD) was formulated to contain an adequate amount of all amino acids for 10 to 20 kg pigs except for Met. The two reference diets were prepared by supplementing the BD with 0.4 or 0.8 g $_{L}$-Met/kg at the expense of corn starch, and an equivalent concentration of $_{D}$-Met was added to the BD for the two test diets. The pigs were adapted to the experimental diets for 5 d and then total but separated collection of feces and urine was conducted for 4 d according to the marker-to-marker procedure. Nitrogen intakes were similar across the treatments. Fecal N output was not affected by Met supplementation regardless of source and consequently apparent N digestibility did not change. Conversely, there was a negative linear response ($P < 0.01$) to Met supplementation with both Met isomers in urinary N output, which resulted in increased retained N (g/4 d) and N retention (% of intake). No quadratic response was observed in any of the N balance criteria. The estimated bioavailability of $_{D}$-Met relative to $_{L}$-Met from urinary N output (g/4 d) and N retention (% of intake) as dependent variables using supplemental Met intake (g/4 d) as an independent variable were 87.6 and 89.6%, respectively, but approximate 95% fiducial limits for the relative bioavailability estimates included 100%. In conclusion, with an absence of statistical significance, the present study indicated that the mean relative bioequivalence of $_{D}$- to $_{L}$-Met was 87.6% based on urinary N output or 89.6% based on N retention.

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Bioavailability of D-methionine relative to L-methionine for nursery pigs using the slope-ratio assay

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Short title: Bioavailability of methionine isomers

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Abstract

This experiment was conducted to determine the bioavailability of $_{D}$-methionine (Met) relative to $_{L}$-Met for nursery pigs using the slope-ratio assay. A total of 50 crossbred barrows with an initial BW of 13.5 kg (SD = 1.0) were used in an N balance study. A Met-deficient basal diet (BD) was formulated to contain an adequate amount of all amino acids for 10 to 20 kg pigs except for Met. The two reference diets were prepared by supplementing the BD with 0.4 or 0.8 g $_{L}$-Met/kg at the expense of corn starch, and an equivalent concentration of $_{D}$-Met was added to the BD for the two test diets. The pigs were adapted to the experimental diets for 5 d and then total but separated collection of feces and urine was conducted for 4 d according to the marker-to-marker procedure. Nitrogen intakes were similar across the treatments. Fecal N output was not affected by Met supplementation regardless of source and consequently apparent N digestibility did not change. Conversely, there was a negative linear response ($P < 0.01$) to Met supplementation with both Met isomers in urinary N output, which resulted in increased retained N (g/4 d) and N retention (% of intake). No quadratic response was observed in any of the N balance criteria. The estimated bioavailability of $_{D}$-Met relative to $_{L}$-Met from urinary N output (g/4 d) and N retention (% of intake) as dependent variables using supplemental Met intake (g/4 d) as an independent variable were 87.6 and 89.6%, respectively, but approximate 95% fiducial limits for the relative bioavailability estimates included 100%. In conclusion, with an absence of statistical significance, the present study indicated that the mean relative bioequivalence of $_{D}$- to $_{L}$-Met was 87.6% based on urinary N output or 89.6% based on N retention.

(Keywords: methionine isomers, nitrogen balance, pigs, relative bioavailability, slope-ratio assay)

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1. Introduction

Crystalline amino acids (**AA**) are commonly used to provide indispensable AA (also known as essential AA), which limit growth of pigs when protein sources are marginally used to reduce feed cost as well as N excretion. Methionine is an important indispensable AA for protein synthesis and is generally a limiting AA for the growth of nursery pigs fed diets containing dried blood products (plasma and cells) and dried whey (Cromwell, 2004). Methionine is often supplemented as a racemic mixture of D- and L-Met which is produced through chemical synthesis (Hoehler et al., 2005), but only L-Met can be directly incorporated into naturally-occurring proteins. Therefore, the conversion of D- to L-Met must be completed prior to protein synthesis in pigs, which requires a two-step enzymatic processes involving oxidative deamination followed by transamination (Dibner and Knight, 1984; Chung and Baker, 1992). Thus, it has been questioned whether the bioefficacy of D- and L-Met for pigs is equal or not. Little research has been conducted to compare the bioefficacy of both Met isomers for pigs and the results have been controversial when the growth performances of pigs were used as responses (Reifsnyder et al., 1984; Chung and Baker, 1992; Shen et al., 2014). Nitrogen balance has been used as the classical metabolic indicator of protein metabolism (Haymond, 1999) and is more sensitive and straightforward response than growth performance. However, to the authors' knowledge, there have been no published reports on the bioavailability of Met isomers for pigs using N balance as the response criterion. Therefore, it was hypothesized that supplementation of L-Met would have better effects on N balance of growing pigs compared with D-Met and the present study was conducted to determine the relative bioavailability of D- to L-Met in nursery pigs using the slope-ratio assay with N balance as the response criteria.

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2. Materials and methods

The Institutional Animal Care and Use Committee of Konkuk University reviewed and approved all protocols (KU13188) used in the present study.

2.1. Animals and experimental design

A total of 50 crossbred barrows with an initial BW of 13.5 kg (SD = 1.0) were used to estimate the relative bioavailability of $_D$- to $_L$-Met during 5 consecutive periods. In each 9-d period, 10 pigs were individually placed in metabolism cages, and allotted to 5 dietary treatments with 2 replicates per treatment in a randomized complete block design based on the initial BW.

2.2. Diets

A Met-deficient basal diet (**BD**) was formulated to meet or exceed the estimated requirements of all nutrients except for Met. The BD contained total Met at 18.1 g/kg which was about 66% of the Met requirement for 10 to 20 kg pigs (NRC, 1998; Table 1). Two reference diets were prepared by supplementing the BD with 0.4 or 0.8 g $_L$-Met/kg at the expense of corn starch, and an equivalent concentration of $_D$-Met was added to the BD for two test diets. To minimize orts, daily feed allowance was calculated as 3.5% of the BW of each animal at the beginning of each period. The feed was divided into 2 equal meals and fed to the pigs at 0900 h and 1700 h. The pigs were provided ad libitum access to water.

2.3. Sample collection

For the N-balance study, the pigs were adapted to the experimental diets for 5 d and then total but separated collection of feces and urine was conducted for 4 d according to the marker-to-marker procedure (Kong and Adeola, 2014). The collected feces and urine were immediately stored in a freezer at -20°C prior to further analyses.

2.4. Chemical analysis

At the completion of the study, the frozen fecal samples were dried in a forced-air oven at 55°C and finely ground prior to chemical analyses. The experimental diets, fecal and urine samples were determined for crude protein (CP) content (N × 6.25) by the Kjeldahl method (Kjeltec 1035; Foss, Hillerod, Denmark).

2.5. Calculations and statistical analysis

Apparent total tract N digestibility and retention were calculated using the following equations:

$$\text{Apparent total tract N digestibility (\%)} = (N_I - N_F) / N_I \times 100,$$

$$\text{N retention (\% of intake)} = (N_I - N_F - N_U) / N_I \times 100$$

Where: N_I is the amount of N ingested (g); N_F and N_U are the amount of N voided via the feces (g) and urine (g), respectively.

Experimental data were analyzed using the MIXED procedures of SAS (SAS Institute Inc., Cary, NC, USA). The independent variables in the model included diet as a fixed effect and period and block nested within period as random effects. The orthogonal polynomial contrast was used to examine the relationship between N balance response criteria and graded

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concentrations of Met isomers. The relative bioavailability of D-Met to L-Met was estimated using a multiple regression model and the slope-ratio analysis described by Littell et al. (1997). The statistical model used in the analysis as follows:

$$y = a + b_s x_s + b_t x_t + e,$$

in which y is response criterion; a is intercept; e is random error; b_s and b_t are the slopes for L- and D-Met, respectively; x_s and x_t are the concentrations of L- and D-Met intake, respectively. An individual pig served as the experimental unit and statistical significance was determined at $P < 0.05$.

3. Results

The effects of supplemental L- or D-Met on N balance are shown in Table 2. Nitrogen intakes were similar across the treatments due to the restricted feeding based on the initial BW of pigs. Fecal N output was not affected by Met supplementation regardless of source and consequently apparent N digestibility did not change. In contrast, there was a linear response ($P < 0.05$) to Met supplementation from L-Met or D-Met in urinary N output, which resulted in increased ($P < 0.01$) retained N and N retention. No quadratic response was observed in any of the N-balance criteria. The estimated bioavailability of D-Met relative to L-Met from urinary N output (g/4 d) and N retention (% of intake) as dependent variables using supplemental Met intake (g/4 d) as an independent variable were 87.6 and 89.6%, respectively (Figures 1 and 2), but approximate 95% fiducial limits for the relative bioavailability estimates for both dependent variables included 100%.

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4. Discussion

Nutrient bioavailability assay provides relative information on the capacity of feed ingredients to supply a nutrient capable of being digested, absorbed and available for use or storage (Gabert et al., 2001; Adeola, 2009). Growth performance has generally been used as response criteria for AA bioavailability assay (Chung and Baker, 1992; Adeola, 2009; Shen et al., 2014). However, in the present study, growth responses to the supplemental Met were not significant among dietary treatments while N balance responses were affected by the supplemental Met. This may be attributed to the relatively greater sensitivity of N balance responses to AA adequacy compared to growth responses in a short-term experiment (Figueroa et al., 2001).

The results from the present study suggest that the relative bioavailability of D- to L-Met, using urinary N output (g/4 d) and N retention (% intake) as dependent variables, are 87.6 and 89.6%, respectively, and the 95% fiducial limits included 100%. To attain meaningful results from bioavailability assays, the validity tests of the assumptions for bioavailability assay should be performed (Littell et al., 1997). This tested for linearity of the slopes and lack of curvature, and for intersection of responses to reference and test diets at the response to basal diet. For urinary N output (g/4 d) and N retention (% intake) in the present study, validity tests were performed and all assumptions were valid.

Due to the lack of D-transaminase, pigs are not able to directly utilize D-Met for protein synthesis and S-adenosylmethionine formation. To become bioavailable, D-Met has to be converted to α-keto-γ-methiolbutyrate in a process catalyzed by D-Met oxidase, with subsequent transamination to L-Met (Lewis, 2003). However, the efficiency of these additional enzymatic

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processes has not been so clear and little information is available on the relative bioefficacy of D- to L-Met. Using phenylalanine as an indicator AA, the relative bioefficacy of D- to L-Met was only 50% when 10 to 14-day-old pigs were used in an indicator AA oxidation study (Kim and Bayley, 1983). Recently, Shen et al. (2014) conducted a relative bioavailability study for a period of 20 d using growth performance as response criteria and reported that the bioavailability of DL- to L-Met was calculated as 69.4% and 81.3% for the average daily gain and gain:feed of nursery pigs, respectively. However, several other studies showed no differences in bioefficacy between D- and L-Met. In the present study, the bioavailability of D-Met was comparable with L-Met in nursery pigs when urinary N output (g/4 d) was used as the response criterion. This was in agreement with Cho et al. (1980) who determined the urinary excretion of L- or D-Met in 6-week old miniature pigs infused with solutions containing DL-Met and observed little urinary excretion of D-Met, indicating no difference in utilization between Met isomers. Furthermore, no differences in BW gain or plasma urea levels were observed when 3-week-old pigs received low-protein-liquid diets containing either DL- or L-Met (0.51%) for 7 days (Reifsnyder et al., 1984) and Chung and Baker (1992) reported that the molar efficacy of D-Met was parallel to L-Met when the growth performance of pigs averaging 9.6 kg was used as response. It is difficult to explain the reason for discrepancy in the bioefficacy of Met isomers among studies but it may be attributed in part to the age of animals (Chung and Baker, 1992). The activity of D-Met oxidase, the key enzyme that converts D- to L-Met, was determined to be greater in older animals compared with younger animals (D'Aniello et al., 1993). In addition, the response criteria for bioavailability may also contribute to the equivocal results. Because N balance is a sensitive indicator of protein utilization (Kim et al., 2006), N retention was used as the response criterion for the bioavailability of Met isomers in the present study. In the study conducted by Shen et al.

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(2014), the bioavailability of $_{DL}$-Met was less than that of $_{L}$-Met when the average daily gain and gain:feed were used as the responses, whereas the relative bioavailability of 100.9% was observed for plasma urea N, indicating that the contrary results may be attributed to the use of different response criteria for the estimates of relative bioavailability.

In conclusion, the relative bioavailability of $_{D}$- to $_{L}$-Met in nursery pigs averaging 13.5 kg with the slope-ratio comparison of urinary N output (g/4 d) and N retention (% of intake) using supplemental Met intake (g/4 d) as the independent variable were 87.6 and 89.6%, respectively, but 95% fiducial limits for the relative bioavailability estimates included 100%.

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Table 1. Ingredient and chemical composition of experimental diets fed to pigs (as-fed basis)

Item[†]	Basal diet	Supplemental L-Met, %		Supplemental D-Met, %	
		0.04	0.08	0.04	0.08
Ingredient composition, %					
Ground corn	55.00	55.00	55.00	55.00	55.00
Dried whey	10.00	10.00	10.00	10.00	10.00
Spray dried animal plasma	10.00	10.00	10.00	10.00	10.00
Corn starch	19.92	19.88	19.84	19.88	19.84
Soybean oil	2.00	2.00	2.00	2.00	2.00
L-Met	-	0.04	0.08	-	-
D-Met	-	-	-	0.04	0.08
L-Lys·HCl	0.32	0.32	0.32	0.32	0.32
L-Thr	0.04	0.04	0.04	0.04	0.04
L-Trp	0.03	0.03	0.03	0.03	0.03
L-Ile	0.14	0.14	0.14	0.14	0.14
Dicalcium phosphate	0.67	0.67	0.67	0.67	0.67
Ground limestone	1.18	1.18	1.18	1.18	1.18
Salt	0.20	0.20	0.20	0.20	0.20
Vitamin-mineral premix[‡]	0.50	0.50	0.50	0.50	0.50
Calculated composition					
Metabolizable energy, kcal/kg	3,552	3,551	3,549	3,551	3,549
CP, %	14.08	14.10	14.13	14.10	14.13
Ether extract, %	4.48	4.48	4.48	4.48	4.48
Methionie, %	0.18	0.22	0.26	0.22	0.26
Cystein, %	0.41	0.41	0.41	0.41	0.41
Choline, %	0.32	0.32	0.32	0.32	0.32
Ca, %	0.72	0.72	0.72	0.72	0.72
Available P, %	0.34	0.34	0.34	0.34	0.34

[†]Met = methionine; Lys = lysine; Thr = threonine; Trp = tryptophan; Ile = isoleucine

[‡]Provided the following quantities per kg of complete diet: vitamin A, 25,000 IU; vitamin D_3, 4,000 IU; vitamin E, 50 IU; vitamin K, 5.0 mg; thiamin, 4.9 mg; riboflavin, 10.0 mg; pyridoxine, 4.9 mg; vitamin B_{12}, 0.06 mg; pantothenic acid, 37.5 mg; folic acid, 1.10 mg; niacin, 62 mg; biotin, 0.06 mg; Cu, 25 mg as copper sulfate; Fe, 268 mg as iron sulfate; I, 5.0 mg as potassium

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iodate; Mn, 125 mg as manganese sulfate; Se, 0.38 mg as sodium selenite; Zn, 313 mg as zinc oxide; butylated hydroxytoluene, 50 mg.

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Table 2. Effects of dietary L-methionine (L-Met) and D-Met on nitrogen (N) balance of weaning pigs†

Item	Basal diet	Supplemental L-Met, %		Supplemental D-Met, %		SEM	*P*-values for contrast			
							Linear		Quadratic	
		0.04	0.08	0.04	0.08		L-Met	D-Met	L-Met	D-Met
BW, kg										
Initial	13.9	14.0	14.4	14.0	14.3	0.3	0.116	0.243	0.443	0.733
Final	15.1	15.1	15.6	15.4	15.3	0.4	0.142	0.537	0.277	0.548
Collection period (4 d)										
Feed intake, g	1973	1973	1973	1955	1973	40	0.974	0.974	0.985	0.081
N intake, g	44.5	44.5	44.6	44.1	44.6	0.9	0.607	0.607	0.983	0.080
Fecal N output, g	8.33	7.85	8.07	8.24	7.70	0.43	0.630	0.243	0.450	0.612
N digestibility, %	81.3	82.4	82.0	81.4	82.7	0.8	0.537	0.234	0.480	0.555
Urinary N output, g	14.6	14.3	11.1	13.5	12.1	0.6	< 0.001	0.014	0.086	0.862
Retained N, g	21.4	22.4	25.4	22.4	24.8	0.7	< 0.001	< 0.001	0.170	0.337
N retention, % of intake	48.5	50.4	57.0	50.8	55.4	1.2	< 0.001	< 0.001	0.132	0.465

†Each least squares mean represents 10 observations except the basal diet (9 observations).

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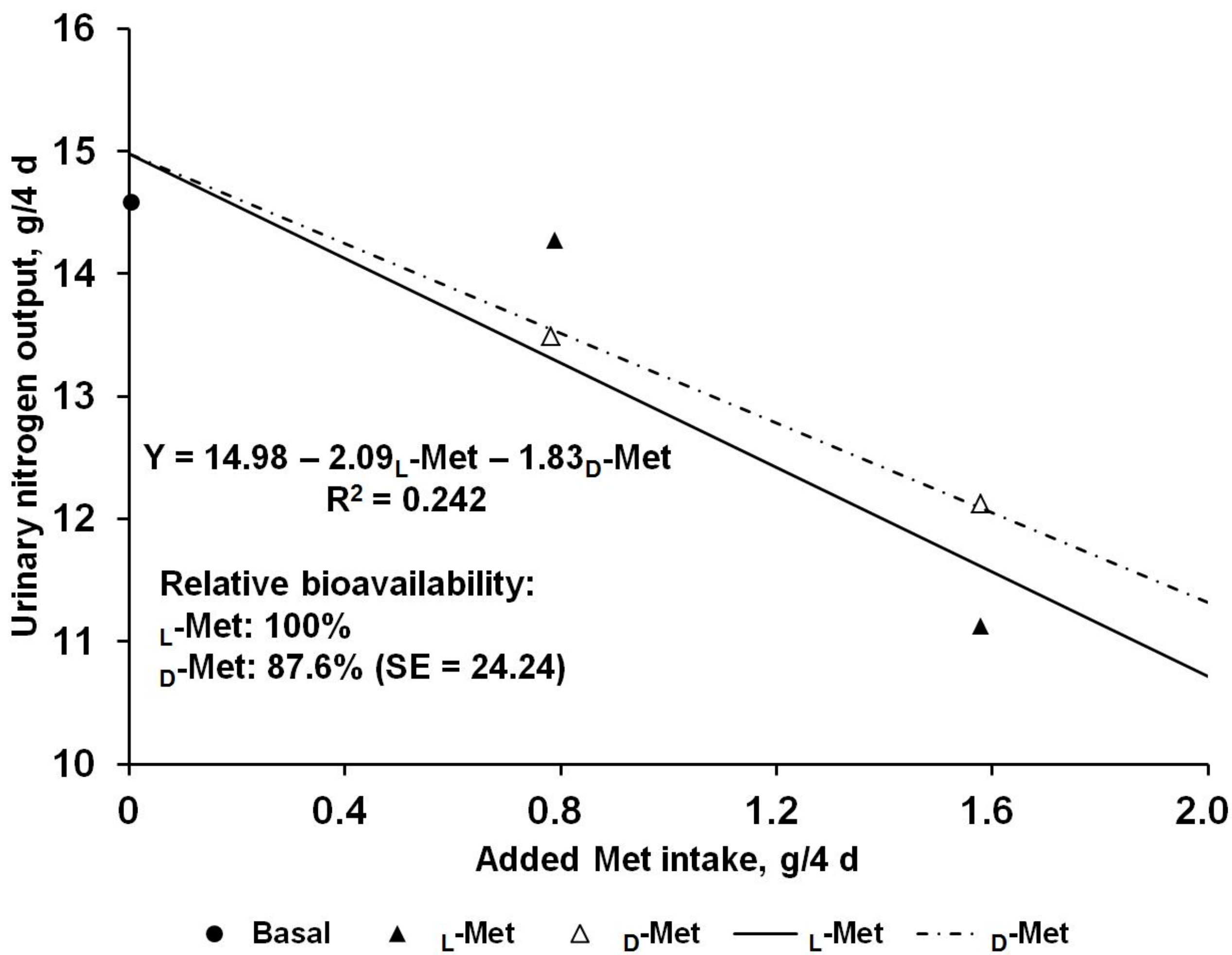

253

254 **Figure 1.** Slope-ratio comparison based on the urinary nitrogen output (g/4 d) of nursery pigs fed diets with graded levels of $_{D}$-

255 methionine ($_{D}$-Met) or $_{L}$-Met. Each data point represents least squares mean of 10 observations except the basal diet (9 observations).

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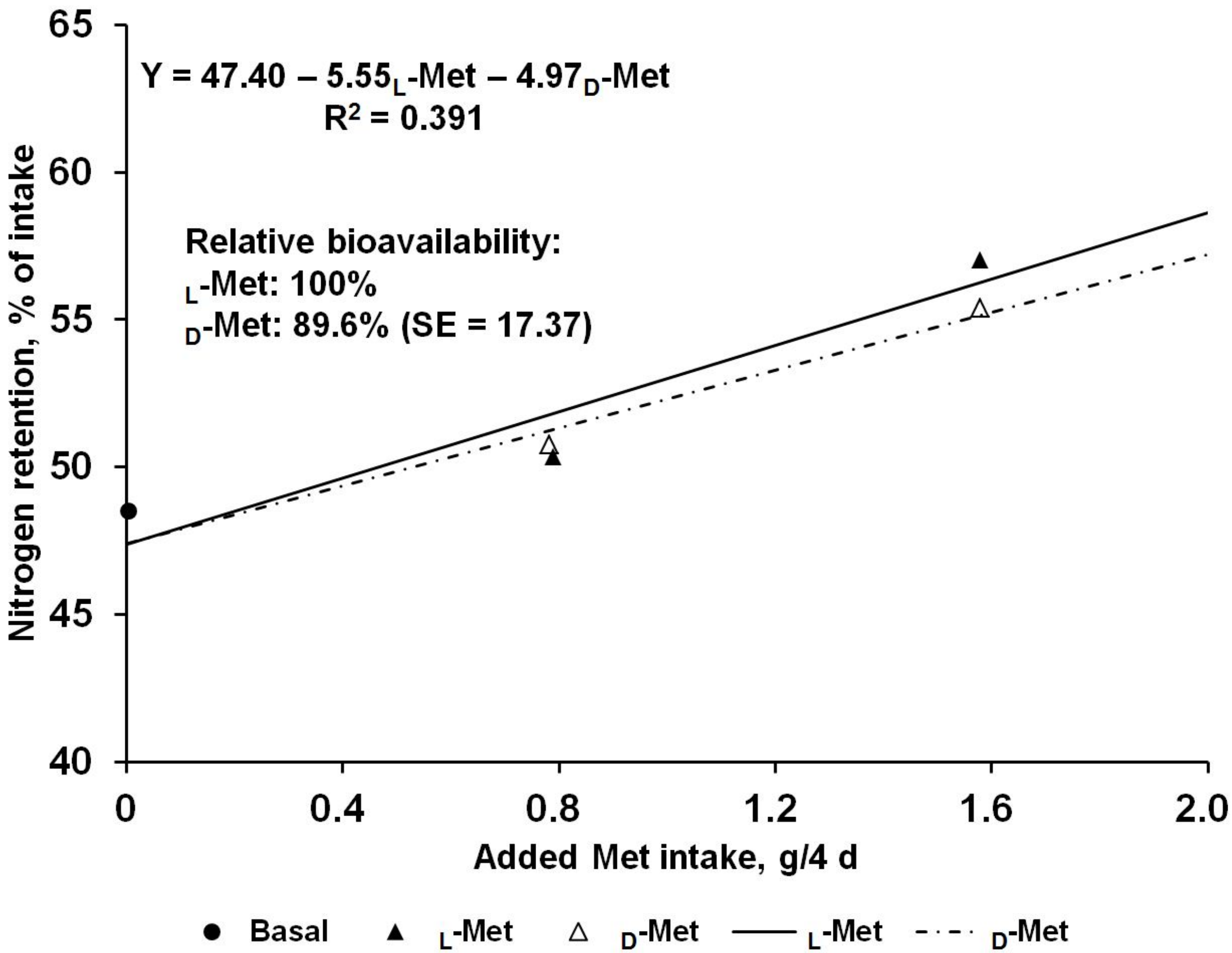

Figure 2. Slope-ratio comparison based on the nitrogen retention (%) of nursery pigs fed diets with graded levels of D-methionine (D-Met) or L-Met. Each data point represents least squares mean of 10 observations except the basal diet (9 observations).

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