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Evaluation of the Apparent Ileal Digestibility (AID) of Protein and Amino Acids in Nursery Diets by *In vitro* and *In vivo* Methods

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ABSTRACT : The objective was to evaluate *in vitro* prediction of ileal digestibility of protein and amino acids (AA) for current nursery pig diets (n = 10) by using pepsin and pancreatin incubations. To compare *in vivo* ileal digestibility, forty nursery pigs (4 pigs per diet) with an initial BW of 12.2±2.7 kg were surgically equipped with T-cannula in the distal ileum. In all cases, the values of *in vitro* digestibility were higher than those of *in vivo* digestibility ($p<0.05$). With regard to the relationships of essential and non essential AA (CP), the r^2 value was 0.76. With regard to AA, high relationships were observed in Ile, Thr, and Gly (0.85, 0.83, and 0.89, respectively). Also, there was a lower relationship for Arg, Met, Ala, Asp, Glu, Pro, Ser, and Tyr with r^2 values of 0.56, 0.54, 0.40, 0.54, 0.45, 0.24, 0.49, and 0.35, respectively between *in vitro* and *in vivo* digestibility. The EAA relationship ($r^2 = 0.71$) was generally higher than that of NEAA ($r^2 = 0.50$) numerically. In conclusion, there were strong linear relationships between *in vivo* and *in vitro* ileal digestibility (CP, Ile, Thr, and Gly). *In vitro* prediction of ileal digestibility (CP, Ile, Thr, and Gly) seems to have significant potential for practical application. (**Key Words :** *In vivo*, *In vitro*, Ileal Digestibility, Nursery Pigs)

INTRODUCTION

Ileal and total tract digestibility should be measured by *in vivo* trial. The ileal digestibility of amino acid and nitrogen is one of the most valuable measurements for the evaluation of nursery pig diets. However, *in vivo* methods to evaluate of digestibility require complicated surgery and are time-consuming and costly. In 1991, a model for feed evaluation based on *in vitro* digestible dry matter and protein was developed by Boisen (1991). Since *in vitro* digestibility was not influenced by endogenous losses, Boisen and Fernandez (1995) reported that values of *in vitro* digestibility for protein were higher than those of apparent ileal digestibility and equation for prediction of apparent ileal digestibility of protein and amino acid for pigs diets. Recently, Huang et al. (2000) demonstrated that dialysis tubing and phosphate buffered saline (PBS) solution might be ideal materials for imitating the digestion environment of the intestinal tract. There were significant linear relationships ($0.96<r\text{-values}<0.99$) between *in vivo* and *in vitro* digestibility for amino acids. Most *in vitro* researches have predicted the equations for relationship and reported the digestibility of feedstuffs compared with *in vivo*. However, few have examined the relationship between *in vitro* and *in vivo* digestibility in modern pigs. Therefore, the objective of this study was to evaluate *in vitro* prediction of ileal digestibility of protein and amino acids for current nursery pig diets by using pepsin and pancreatin incubations.

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MATERIALS AND METHODS

The experimental protocols used were approved by the Animal Care and Use Committee of Dankook University.

Experimental diets, animals and *in vivo* samples

10 nursery pig diets (Table 1) from commercial feed company were sampled for 2 month (5 diets per month). Diets were formulated to meet or exceed the nutrient requirements recommended by the NRC (1998). 40 nursery pigs (4 pigs per diet; 20 pigs per month) with an initial BW of 12.2±2.7 kg were surgically equipped with T-cannula in the distal ileum (Stein et al., 1998). Pigs were provided a daily quantity of feed that supplied 2.4 times the estimated maintenance requirement of energy (i.e., 106 kcal ME/kg$^{0.75}$; NRC, 1998) during the experiments. Daily feed allotments were offered as two meals fed in 12 h intervals (0600 and 1800). Water was available to the pigs *ad libitum*.

Table 1. Analysed contents of ME, CP, and amino acids of 10 nursery pig diets[1]

Items	1	2	3	4	5	6	7	8	9	10	Mean	Min.	Max.	SD
ME (kcal/kg)	3,500	3,510	3,510	3,500	3,420	3,450	3,520	3,500	3,520	3,450	3,488	3,420	3,520	35
CP (%)	21.30	21.40	21.60	21.60	21.70	21.60	21.20	22.40	22.30	22.10	21.72	21.20	22.40	0.41
Lys (%)	1.43	1.45	1.50	1.43	1.38	1.45	1.39	1.43	1.51	1.55	1.45	1.38	1.55	0.05
Met (%)	0.54	0.55	0.56	0.54	0.51	0.56	0.50	0.50	0.49	0.54	0.53	0.49	0.56	0.03
Thr (%)	0.94	0.93	0.92	0.94	0.89	0.92	0.94	0.95	0.94	0.99	0.94	0.89	0.99	0.03
Trp (%)	0.24	0.24	0.25	0.24	0.28	0.28	0.28	0.24	0.27	0.31	0.26	0.24	0.31	0.02
Val (%)	1.10	1.11	1.18	1.09	1.08	1.11	1.20	1.10	1.17	1.12	1.13	1.08	1.20	0.04
Ile (%)	0.92	0.94	0.93	0.97	0.99	0.96	0.94	0.91	0.95	0.90	0.94	0.90	0.99	0.03

[1] 10 samples for 2 months (5 diets per month) and based on corn-soy diets.

In each period, the 7 d was a period of adaptation to the experimental diets and ileal digesta were collected on d 8 and 9 (12 h/d) for each pig. The ileal digesta were collected between 0600 and 1800 for 2 days by attaching a transparent 100-ml latex collection bag to the cannulas. During the 12 h collection period, digesta were collected every 30 min and immediately frozen at -20°C. The samples were then freeze-dried and finely grounded prior to analysis for chromium (Kimura and Miller, 1957).

In vitro method

Step 1 : About 0.5 g of feed was weighed within an accuracy of ±0.1 mg into 100-ml conical flasks with a blank was included in each series. Each diet has 20 replicates because 0.5 g feed is not enough for analysis. A small magnetic rod and 25 ml of phosphate buffer (0.1 M, pH 6.0) were added to each flask, the sample and buffer then were mixed carefully by gentle magnetic stirring. 10 ml a 0.2 M HCl was added to the slurry, and pH was then adjusted to pH 2 using a 1 M HCl or a 1 M NaOH solution. 1 ml of a freshly prepared pepsin solution containing 10 mg pepsin (porcine, 2000 FIP U/g, Merck art no. 7190) was then added to the mixture. In order to prevent bacteria growth, 0.1 ml of a chloramphenicol solution (0.5 g chloramphenicol (ICN no. 190321) per 100 ml ethanol) was also added to the mixture. The flasks were then closed with a rubber stopper and the flasks were incubated in a heating chamber at 40°C for 75 minutes with constant magnetic stirring.

Step 2 : After incubation, 10 ml of a phosphate buffer (0.2 M, pH 6.8) plus 5 ml of a 0.6 M NaOH solution were added. The slurry was adjusted to a pH of 6.8 with a 1 M HCl or a 1 M NaOH solution, then mixed with 1 ml freshly prepared pancreatin solution containing 50 mg pancreatin (porcine, grade IV, Sigma no p 1750). After closing with a rubber stopper, the sample was incubated under constant magnetic stirring in a heating chamber at 40°C for three hours and thirty minutes.

A minimum of 0.5 g Celite (545, Tecator) was added to glass filter crucibles and rinsed. Then, samples were dried at 100°C for at least 4 h and crucibles were weighed after cooling in a dessicator. The undigested residues were then collected in a filtration unit (Fibertee System M, Tecator, Sweden) by using dried and pre-weighed glass filter crucibles (d: 3 cm; pore size: 40-90 pm) containing about 0.5 g celite (545, Tecator) as a filter aid. All material was then transferred with 1% sulphosalicylic acid to the crucible. After consecutive washings with 2×10 ml of ethanol and acetone, respectively, the crucible was suctioned (with the water pump) to be as dry as possible. The undigested residues were then dried at 100°C overnight. The crucible was placed in an ashing oven and the content was ashed at 525°C for about 4 hours. After ashing, the crucibles were cooled in a dessicator and subsequently weighed.

In vitro and *in vivo* DM, CP and AA digestibility

In vitro digestibility of dry matter was calculated from the difference between the dry matter in the sample and the

Table 2. Comparison of *in vitro* and *in vivo* digestibility of CP and AA in 10 nursery pig diets[1]

Items (%)	*In vitro*	*In vivo*	SE[2]
CP*	91.25	84.61	2.38
EAA (essential AA)*			
Arg	90.25	80.97	3.25
His	87.60	78.82	2.14
Ile	93.82	84.75	2.52
Leu	91.71	83.10	4.05
Lys	94.56	82.44	3.02
Met	90.88	81.53	2.09
Phe	87.21	78.47	3.44
Thr	89.20	76.43	3.80
Val	92.77	80.83	2.99
NEAA (non-essential AA)*			
Ala	91.68	81.00	2.95
Asp	90.56	82.96	3.02
Cys	92.38	79.53	2.44
Glu	90.81	80.20	3.56
Gly	89.54	77.74	4.01
Pro	89.76	74.48	2.10
Ser	91.79	77.21	2.74
Tyr	92.76	80.82	2.56

[1] Each mean represents 40 observations for *in vivo* and *in vitro*, respectively.
[2] Standard error. * $p<0.05$.

undigested residue, correction was made for dry matter in the blank.

Undigested materials together with the celite were wrapped into a piece of nitrogen-free paper, and undigested nitrogen was measured by using the Kjeldahl method in a semi-automatic Kjellfoss apparatus (Foss Electric, Denmark). The *in vitro* digestibility of protein was calculated from the difference between nitrogen found in the sample and the undigested residue after correction for nitrogen in the blank.

For the *in vivo* measure, the chromium concentration was determined via UV absorption spectrophotometry (Shimadzu, UV-1201, Japan) and the ileal apparent digestibilitiy was calculated via indirect method. N content was determined by using a Kjeltec 2300 Analyzer (Foss Tecator AB, Hoeganaes, Sweden). Amino acids (excluding tryptophan) were analyzed by dansylation (Beckman Intruments Inc., Fullerton, CA) and HPLC after acid hydrolysis for 24 h in 6 M HCl. Sulfur-containing amino acids were analyzed after overnight cold performic acid oxidation and subsequent hydrolysis.

Statistical analyses

Pig means served as the experimental unit. The means of the treatments were also compared by using Duncan's multiple range test (Duncan, 1955) with an alpha level of $p<0.05$. Variability in the data was expressed as the SEM. All 40 pigs were fed the experimental diets at the 2 different times (20 pigs per month). However, there was no significant difference about time factor. The relationship between *in vitro* digestibility and *in vivo* digestibility measured in nursery pigs was determined by regression analyses using a general linear model (GLM) in a standard SAS package (SAS Institute Inc., Cary, NC).

RESULTS AND DISCUSSION

***In vitro* and *in vivo* Digestibility of CP and AA**

The *in vitro* digestibility of CP and AA of 10 nursery diets are presented in Table 3. In all cases, the values of *in vitro* digestibility were higher than those of *in vivo* digestibility ($p<0.05$). Boisen and Fernandez (1995) compared the *in vitro* ileal digestibility of protein of 17 feedstuffs with the *in vivo* digestibility by using the *in vitro* enzyme digestion method, and reported that ileal protein digestibility of the *in vitro* was higher than those of *in vivo*. Endogenous losses of protein at the ileal level might have a great influence on *in vivo* digestibility (Boisen and Eggum, 1991). Thus the reduced values of *in vivo* digestibility, compared with values of *in vitro* digestibility, can be explained by the presence in the feces of endogenous loss. Boisen and Fernandez (1995) reported that the *in vivo* digestibility of CP and AA could be predicted from *in vitro* digestibility, since *in vitro* digestibility is not influenced by endogenous losses.

Table 3. Linear regression analysis between *in vivo* (y) and *in vitro* digestibility (x) of CP and AA in 10 nursery pig diets[1]

Items	Equation	r^2	RSD
CP	y = 0.91x+1.42	0.76	5.72
EAA (essential AA)			
Arg	y = 1.15x-23.24	0.56	7.67
His	y = 0.82x+6.94	0.75	7.46
Ile	y = 0.83x+7.12	0.85	7.18
Leu	y = 0.61x+27.29	0.75	7.10
Lys	y = 0.84x+2.83	0.68	9.68
Met	y = 0.69x+19.27	0.54	7.67
Phe	y = 0.78x+10.35	0.77	7.47
Thr	y = 1.04x-16.32	0.83	10.91
Val	y = 1.41x-49.81	0.69	9.73
NEAA (non-essential AA)			
Ala	y = 0.36x+48.24	0.40	8.74
Asp	y = 0.68x+21.64	0.54	6.19
Cys	y = 0.93x-11.69	0.60	15.13
Glu	y = 0.77x+10.24	0.45	8.78
Gly	y = 1.06x-17.16	0.89	9.98
Pro	y = 0.44x+35.21	0.24	13.16
Ser	y = 0.85x-0.60	0.49	10.31
Tyr	y = 0.82x+4.42	0.35	9.73

[1] Each mean represents 10 observations (each observation is the average for 4 samples per diet) for *in vivo* and *in vitro* respectively.

The relationships between *in vitro* and *in vivo* digestibility of CP and AA

The statistical relationships between *in vitro* and *in vivo* digestibility of CP and AA as linear regression equations are shown in Table 4. With regard to relationships concerning CP, the r^2 value was 0.76. This relationship was stronger than those of EAA and NEAA numerically. With regard to AA, Ile, Thr, and Gly had strong relationships (0.85, 0.83, and 0.89, respectively). Also, several equations had relatively weak relationships (Arg, Met, Ala, Asp, Glu, Pro, Ser, and Tyr were 0.56, 0.54, 0.40, 0.54, 0.45, 0.24, 0.49, and 0.35, respectively) between *in vitro* and *in vivo* digestibility. The EAA relationship ($r^2 = 0.71$) was generally stronger than that of NEAA ($r^2 = 0.50$) numerically. *In vitro* digestibility techniques using enzymes and length of incubations that mimic *in vivo* digestion can be used to predict the AID of protein and AA among feedstuffs and compound feeds in swine. And these techniques are valid, less expensensive and with reasonable accuracy (Boisen and Fernández, 1997; Noblet and Jaguelin-Peyraud, 2007). The accuracy of the equation of the *in vitro* to predict the AA or CP availability may be affected by many factors, such as the feedstuffs characteristics, the enzymatic method

used during the fermentation step (Regmi et al., 2008), and the endogenous losses of the animals. Boisen and Fernandez (1995) found that the relationship between predicted and determined apparent ileal digestibility of protein in the diets was substantially lower than that found in single feedstuffs (r^2 = 0.57 vs. r^2 = 0.92). Similarly, Huang et al. (2000) demonstrated that the statistical equations between *in vitro* and *in vivo* digestibility of CP and AA for single protein stuffs (fish meals, rapeseed meal, and cottonseed meal) had higher linear relationships (r^2 = 0.96 to 0.99). Jezierny et al. (2010) demonstrated that there were strong linear relationships ($p<0.05$) between *in vivo* and *in vitro* predicted values of standardized ileal CP and AA digestibility in grain legumes. However, Cone and Van der Poel (1993) reported that equations for digestibility of diets had a low relationship (r^2 = 0.23). In the present study, the r^2 values of NEAA have a lower tendency than those of EAA numerically. Wünsche et al. (1987) reported that Pro has high endogenous losses, because of significant variation of the mucine losses. Thus, low r^2 values of each AA are generally assumed to be caused by variations of endogenous losses at *in vivo* status. Based on the results that have been obtained so far, it is assumed that the low relationship between digestibility value of protein from vivo and vitro method is also caused by the heat damage (Maillard reaction) during the process (Pujol and Torrallardona, 2007). Results from the present study indicated that some improvement must be made in the *in vitro* digestion technique or its execution so that predictions of the AID of protein are more accurate.

In conclusion, there were strong linear relationships between *in vivo* and vitro ileal digestibility (CP, Ile, Thr, and Gly). *In vitro* prediction of ileal digestibility (CP, Ile, Thr, and Gly) seems to have significant potential for practical application.

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