

# Determination of bacterial amino acid contribution to ileal digesta from pigs using <sup>35</sup>S and DAPA marker techniques

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## ABSTRACT

A study was conducted using two duodenal and ileal cannulated, 45 kg gilts, to determine the bacterial amino acid contribution to amino acids in ileal digesta by using <sup>35</sup>S and diaminopimelic acid (DAPA) as bacterial protein markers. Both the <sup>35</sup>S and DAPA marker techniques are based on the assumption that a representative purified bacterial pellet is collected from ileal digesta samples. The <sup>35</sup>S technique was modified from previous methods by isolating a specific methionine sulphone marker fraction. However, results from the <sup>35</sup>S-methionine sulphone marker technique exceeded sensible physiological estimates (mean of 190.6%) of the bacterial amino acid contribution to ileal digesta amino acids. Whereas, the mean bacterial amino acid contribution to ileal digesta was 29.4% when determined using the DAPA marker technique. The values determined by DAPA are within the 20 to 34% range of values that have been reported for pigs fed cereal based diets. The amino acid composition was different ( $P \leq 0.05$ ) between bacteria and undigested non-bacterial residues for isoleucine, aspartic acid, glycine, cysteine and tyrosine. The high concentration of glycine in non-bacterial residues relative to other amino acids measured and the corresponding low concentration in bacterial samples suggests that the bacterial pellet fraction was free of contamination. It was therefore concluded, that further research into determining the bacterial contribution to amino acids in ileal digesta is warranted.

KEY WORDS: pig, ileum, bacterial amino acid markers

## INTRODUCTION

Currently, the protein sources used in swine diets are often ranked on the basis of their supply of digestible amino acids, as determined using the ileal analysis method (Sauer and Ozimek, 1984; Sauer and de Lange, 1990). Until recently,

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bacteria content and activity proximal to the large intestine of the pig had been considered to be of little importance in nutritional terms (Hungate, 1984; Savage, 1986). However, the inclusion of subtherapeutic levels of antibiotics in diets has been shown to improve growth performance (Yen et al., 1987) and reduce intestinal bacterial ammonia production (Yen et al., 1990) in swine. Bacterial primary amine production and amino acid absorption efficiency (Dierick et al., 1986) and the level of lumenal adenosine triphosphate presumed to be of bacterial origin (Jensen, 1988) were also altered with the use of antibiotics.

The influence of intestinal bacteria on amino acid digestibilities is measured by determining the bacterial contribution to total protein found in ileal digesta. Of the ileal digesta crude protein (CP) and amino acids taken from pigs fed cereal based diets, 20 to 35% has been estimated to be of bacterial origin when diaminopimelic acid (DAPA) is used as a bacterial marker (Dierick et al., 1983; Poppe et al., 1983; Drochner, 1984). However, Drochner (1984) reported a large statistical variation in the bacterial amino acid contribution in ileal digesta. An initial study using  $^{35}\text{S}$  to label bacterial organic matter in pig ileal digesta and faeces was therefore undertaken to determine if the variability of bacterial amino acid estimations was due to the use of the DAPA technique (Dugan, 1992). Nevertheless, subsequent results using the  $^{35}\text{S}$  technique reported by Dugan (1992) overestimated (in some cases by up to 500%) the bacterial contribution of amino acids in ileal digesta and faeces.

The objective of this study is to determine the bacterial amino acid contribution to amino acids in ileal digesta taken from pigs using  $^{35}\text{S}$  and DAPA as bacterial markers.

## MATERIAL AND METHODS

### *Animals and Management*

Two gilts (Lacombe x Yorkshire), with an average initial body weight of 45 kg, were surgically fitted with sample T-cannulas in both proximal duodenum and distal ileum. The ileal cannula was designed according to Sauer et al. (1983) as modified according to de Lange et al. (1989). The duodenal cannula was similar to the re-entrant pancreatic cannula described by Hee et al. (1985). The surgical technique of Sauer et al. (1983) was adapted for the insertion of the ileal cannula, whereas the duodenal cannula was inserted using the technique described by Hee et al. (1985). The gilts were fasted for a 24 h period and then sedated one hour prior to surgery with a 1 ml intramuscular injection of Atravet (Ayerst Laboratories, Montreal, Quebec, Canada). Following surgery, the gilts were housed in individual stainless steel metabolic crates in a barn maintained at 20°C.

The gilts were allowed a 10 d recovery period during which a 18% CP starter diet (Sauer et al., 1983) was fed twice daily at 6.00 and 18.00 h. The diet was introduced gradually the day after surgery from 50 g per feeding to to appetite intake by d 5. To reduce post-surgical pain, Torbugesic (Ayerst Laboratories, Montreal, Quebec, Canada) was added to the feed at 1.2 mg per kg of body weight for 2 d after surgery.

The experimental period lasted 13 d. During the experiment, the gilts were fed 16% CP grower diet (Dugan, 1992). Chromic oxide was included in the diet at a level of 4 g/kg as a digestibility marker. The diet was fed four times daily at 6.00, 12.00, 18.00 and 24.00 h; 550 g per feeding. Water was available at all times from a low pressure drinking nozzle. A 512 ml aqueous solution containing 4.35 g of NaCl and 1 g of  $\text{Na}_2\text{SO}_4$  was continuously infused into the duodenum of each pig per d throughout the experimental period. On d 12 and 13, 55.5 MBq of  $\text{Na}_2^{35}\text{SO}_4$  (ICN Biomedicals, Irvine, CA, U.S.A.) was added to the infusate for each gilt. During the last 12 h of infusion on d 13, two 6 h samples of ileal digesta were collected into flexible polyethylene tubing (1.5 m length, 4 cm width). The proximal end of the tubing was connected to the cannula and the distal end was submerged in ice water. Outflowing digesta was immediately shunted into tubing submerged in the ice water to reduce the rate of bacterial metabolism.

### *Laboratory Analyses*

The  $^{35}\text{S}$  technique was adapted from the method of Mathers and Miller (1980) which was used for determining the bacterial contribution to CP in sheep rumen digesta. To improve upon the methodology, the  $^{35}\text{S}$  technique was modified by increasing the  $^{35}\text{S}_4^{2-}$  activity and  $^{32}\text{SO}_4^{2-}$  concentration in the infusate solution, changing the site of infusion from the stomach to the duodenum, and by isolating a specific sulphur containing marker fraction (methionine sulphone) from both bacteria and digesta. Diaminopimelic acid (DAPA) was employed as a second marker for comparison with  $^{35}\text{S}$ .

After each collection period ice cooled digesta was immediately taken for bacterial separation. The samples were mixed and six 100 ml subsamples of digesta were taken and added to 800 ml of  $4^\circ\text{C}$  diluent containing 8.5 g/l NaCl, 6 g/l  $\text{MgSO}_4$  and 2 ml of Triton-x-100. The bacterial metabolic activity was reduced by keeping the digesta at  $4^\circ\text{C}$ . Triton-x-100 was added to aid in the separation of loosely bound bacteria from feed particles (Dugan, 1992). Magnesium sulphate was added to stabilize the gram-negative bacterial cell membranes (Gerhardt, 1981). The suspensions were mixed for 40 min using Nuova II stir plates (Thermolyne Corp., Dubuque, Iowa, U.S.A.) set at speed 7. Feed particles were removed from the suspension by centrifuging twice at 200 g for 5 min. Each centrifugation was followed by filtration through several

layers of glass wool. After low speed centrifugation, the supernatant was examined microscopically (with and without gram-staining) and found to be relatively free of feed particles. Bacteria in the supernatant were then pelleted by high speed centrifugation at 5100 g for 10 min at 4°C in a Beckman J-6B/P Centrifuge (Beckman, Palo Alto, CA, U.S.A.). The bacterial pellets were washed with 500 ml of 4 C diluent, centrifuged again at 5100 g for 10 min and then collected for subsequent analyses. The 5100 g centrifugation used in this study was reduced from 9100 g described previously (Dugan, 1992) to minimize bacterial lysis and to ensure that bacterial fragments, particularly cell walls, were not collected in the pellet (Schnaitman, 1981). A disproportionately high cell wall content in the pellet would result in an overestimation of the DAPA to amino acid ratio of ileal digesta due to a high concentration of DAPA in bacterial cell walls. Therefore, bacterial amino acid calculations represent the maximum bacterial amino acid contribution in ileal digesta.

### *Chemical Analysis*

Prior to analysis, samples of digesta, bacteria and feed were dried and ground through a 1 mm screen in a Wiley mill. Dry matter determinations were carried out on feed and digesta (Association of Official Analytical Chemists, 1984). Chromic oxide concentrations in feed and digesta were determined according to the procedure of Fenton and Fenton (1979).

Duplicate samples of 60 mg of bacteria, 200 mg of digesta or 100 mg of feed were hydrolyzed for 20 h at 100°C in 6N HCl and then taken for analyses of amino acids, DAPA and sulphur containing amino acids using three separate techniques, respectively. The amino acid content in the bacterial pellet, digesta and feed were analyzed using high performance liquid chromatography (HPLC) according to the procedure described by Jones and Gilligan (1983). The

TABLE I  
Solvent gradient for High Performance Liquid Chromatography  
analysis of diaminopimelic acid

Time (min) <sup>1</sup>	% Acetate Solvent
0	100
0.1	69
38.3	59
38.4	0
41.4	0
41.5	100

<sup>1</sup>Total run time of 42 min

DL-DAPA concentration in samples was analyzed by HPLC according to the method of Dugan et al. (1989) based on an external standard instead of the internal standard ( $\beta$ -amino-butyric acid), due to the presence of unknown compound which co-eluted with the internal standard. Each hydrolysate was analysed in quadruplicate for DL-DAPA content. The solvent gradient used for the HPLC analysis of DAPA (Table 1) was altered from the procedure of Dugan et al. (1989). Under these conditions DL-DAPA was found to elute in 27.4 min.

The sulphur containing amino acids were quantified and prepared for scintillation counting using a modified combination of the methods described by Beever et al. (1974), Mathers and Miller (1980) and Jones and Gilligan (1983). Specifically, test samples were weighted, in duplicate, into closed 16 x 150 mm screw capped test tubes and oxidized in 4 ml of performic acid for 16 h at 40°C. The performic acid was prepared as described by Mathers and Miller (1980). Oxidation was halted with the addition of 0.6 ml of 48% hydrogen bromide. After the hydrogen bromide addition, the samples were dried using the dryer described by Dugan et al. (1992) then hydrolyzed and re-dried using a Speed Vac Concentrator (Savant Instruments, Farmingdale, NY, U.S.A.) and subsequently resuspended in 6 ml of water. Aliquots of 0.5 ml were taken in duplicate for methionine sulphone and cysteic acid analysis by modification of the method described by Jones and Gilligan (1983). The analysis was modified by changing the solvent gradient (Table 2), using the internal standard L -  $\alpha$ -amino- $\beta$ -guanidino-propionic acid (Sigma Chemical Co., St. Louis, MO, U.S.A.) and changing the acetate solvent composition from 180 ml to 120 ml methanol and adjusting the pH from 7.2 to 6.75.

The remaining 5 ml of the oxidized hydrolysed samples were subjected to cation exchange clean-up to separate methionine sulphone from other <sup>35</sup>S containing compounds such as sulphate and cysteic acid. The remaining 5 ml was

TABLE 2  
Solvent gradient for High Liquid Chromatography analysis of  
the sulphur containing amino acids

Time (min <sup>1</sup> )	% Acetate Solvent
0	100
3.0	95
3.1	86
29.5	83
29.6	0
31.6	0
32.6	100

<sup>1</sup>Total run time of 33 min

loaded onto a 6 x 180 mm glass column a glass wool plug containing Dowax-50 hydrogen form resin (8 x cross linked, 100 to 200 dry mesh; Sigma Chemical Co., St. Louis, MO, U.S.A.). Sulphur containing compounds other than methionine sulphone were eluted with 11 ml of water followed by 5 ml of 2.5M  $\text{NH}_4\text{OH}$ . The methionine sulphone fraction was then eluted with 8 ml of 2.5M  $\text{NH}_4\text{OH}$ . The eluants were dried in a Speed Vac concentrator and resuspended in 5 ml of water. From each resuspension, duplicate 0.5 ml samples were taken and analyzed to determine the methionine sulphone recovery to check for complete removal of cysteic acid. To ensure  $^{35}\text{SO}_4^{2-}$  removal from the remaining 4 ml of sample, 0.4 ml 0.5M  $\text{Na}_2\text{SO}_4$  was added, mixed and left to equilibrate with the sample for 15 min. A 0.25 ml of 1M  $\text{BaCl}_2$  was then added to each sample, mixed and left to stand for 15 min. Soluble  $\text{BaSO}_{4(s)}$  was then removed from the suspension by centrifugation at 2300 g for 15 min. Supernatants were prepared for scintillation counting by adding and mixing, duplicate 2 ml of sample with 12 ml of Ecolite™ Scintillation Counting Fluid (ICN Biomedicals Inc., Irvine, CA, U.S.A.). Scintillation counting was carried out using a Searle Mark III 6880 Liquid Scintillation System (Searle Analytical Inc., Des Plaines, ILL, U.S.A.). The  $^{35}\text{S}$  activity in the samples were determined after corrections for background and counting efficiency.

### *Calculations*

The bacterial percentage of amino acids in ileal digesta were determined using their individual concentrations, instead of crude protein, with reference to both  $^{35}\text{S}$ -methionine sulfone and DAPA as markers (Dugan, 1992).

### *Statistical Analysis*

The amino acid composition of bacteria and digesta (corrected for its bacterial content) were analyzed according to General Linear Model procedures for a repeated measures ANOVA using the Statistical Analysis System Institute, Inc. (1988) statistical software. The repeated measures were the percentage of amino acids in bacteria and digesta in two pigs over two time periods according to analysis procedures as described by Steel and Torrie (1980).

## RESULTS AND DISCUSSION

After the experiment, the pigs were sacrificed and *post-mortem* examinations conducted to inspect the cannulation sites for abnormalities. There were no intestinal interconnections or irregular intestinal adhesions observed.

TABLE 3

Bacterial amino acid contribution as a percent of amino acids in ileal digesta as determined using  $^{35}\text{S}$ -methionine sulphone and diaminopimelic acid (DAPA) techniques

Pig	Period	$^{35}\text{S}$ -methionine sulphone	DAPA
1	1	288.0	32.6
1	2	95.5	26.7
2	1	255.0	33.1
2	2	107.5	25.1
Mean <sup>1</sup>		190.6 $\pm$ 50.0	29.4 $\pm$ 2.0

<sup>1</sup>Mean standard error

The bacterial percentage of amino acid in ileal digesta amino acids, estimated using the  $^{35}\text{S}$ -methionine sulphone marker, exceeded the maximum possible limit of 100%, while estimates based on DAPA fell within a physiologically sensible range (Table 3).

The results determined using  $^{35}\text{S}$ -methionine sulphone were likely confounded by either bacterial or endogenous sources. Either bacterial or endogenous compounds could have elevated the  $^{35}\text{S}$  activity in digesta resulting in a 190.6% overestimation of the bacterial contribution to amino acids in ileal digesta. Future  $^{35}\text{S}$  experiments to provide specific explanations for these results should concentrate on the isolation of a purified marker fraction instead of scintillation counts on whole aliquots as described by Beever et al. (1974) and Mathers and Miller (1980).

The bacterial contribution to amino acids in ileal digesta was 29.4  $\pm$  2.0% when measured using the DAPA marker technique (Table 3). This is within the range of 20 to 35% reported in previous studies in pigs fed cereal based diets (Dierick et al., 1983; Poppe et al., 1983; Drochner, 1984). Although a limited number of animals were sampled in this study, there appears to be a similarity of bacterial amino acids as a percent of ileal digesta amino acids within pigs over time. However, a large 7 percentage unit difference was noted between the two pigs. Large variations between pigs fed corn-soya bean meal diets were also reported by Drochner (1984). The large variation in the bacterial amino acid content between pigs may be due to relatively small changes in the apparent digestibility of amino acids. Partition of amino acids in ileal digesta (Table 4) indicate that as the apparent ileal digestibility of amino acids changed, there were corresponding increases or decreases in bacterial, undigested endogenous and dietary residual amino acid levels. This would indicate that small changes in apparent ileal digestibility of amino acids are not totally explained by changes in bacterial amino acids levels but are also partly due to changes in digesta levels of non-bacterial amino acids.

TABLE 4

Partitioning of the total dietary amino acids, %

Period	Pig 1			Pig 2		
	D <sup>1</sup>	B	R	D	B	R
1	83.7	5.3	11.0	82.8	4.6	12.6
2	81.8	6.0	12.2	84.7	3.9	11.5
Mean <sup>2</sup>	82.8±0.7	5.7±0.4	11.6±0.5	83.8±0.7	4.3±0.4	12.1±0.5

<sup>1</sup>D=digested amino acids; B=bacterial amino acids; R=undigested endogenous and dietary residual amino acids

<sup>2</sup>Mean ± standard error

TABLE 5

Amino acid composition of bacteria (B) and undigested endogenous and dietary residual amino acids (R); expressed as a percentage of total amino acids

Amino acid	B	R	SE <sup>1</sup>
Indispensible			
Arginine	7.34	5.48	0.51
Histidine	2.62	2.78	0.56
Isoleucine	6.46 <sup>a</sup>	5.37 <sup>b</sup>	0.32
Leucine	9.76	9.37	0.38
Lysine	7.95	5.76	0.72
Methionine	1.16	1.69	0.20
Phenylalanine	6.62	6.12	0.40
Threonine	4.84	6.44	0.43
Valine	6.49	6.75	0.24
Dispensible			
Alanine	6.42	6.13	0.65
Aspartic acid	14.49 <sup>a</sup>	13.10 <sup>a</sup>	0.50
Glutamic acid	9.51	11.28	0.57
Glycine	4.83 <sup>b</sup>	7.67 <sup>a</sup>	0.06
Cysteine	1.85 <sup>b</sup>	4.56 <sup>a</sup>	0.06
Serine	5.07	4.69	0.05
Tyrosine	4.58 <sup>a</sup>	2.72 <sup>b</sup>	0.17

<sup>1</sup>Pooled standard error of mean (n=4)

a, b - Means in the same row with different superscripts are different ( $P \leq 0.05$ )

Differences between the amino acid composition of bacteria and undigested non-bacterial residuals were determined (Table 5). Percentages of five of the 16 amino acids analyzed (isoleucine, aspartic acid, glycine, cysteine and tyrosine) were significantly ( $P \leq 0.05$ ) different. This would indicate that the amino acid composition of protein in ileal digesta may be influenced by the corresponding bacterial amino acid content. However, the influence of bacteria on apparent

ileal digestibility of amino acids will likely be limited due to the level (approximately 5%) of bacterial amino acids in the digesta when expressed as a percentage of dietary amino acids.

The significantly lower ( $P \leq 0.05$ ) glycine content in bacteria compared to the undigested endogenous and dietary residue is of particular interest. The glycine content of endogenous protein in digesta collected from the distal ileum was found to be quite high relative to the other amino acids (de Lange et al., 1989). The lower glycine of bacteria would therefore suggest that the bacterial fraction was relatively free of contamination from endogenous sources.

In conclusion, with the use of the DAPA marker technique, the bacterial contribution to ileal amino acids is within the 20 to 34% range of values which have been previously reported for pigs fed cereal based diets. On the other hand, the  $^{35}\text{S}$ -methionine sulphone technique overestimated the bacterial contribution to amino acids in ileal digesta by 190.6%. Therefore, further investigation into using the  $^{35}\text{S}$ -methionine sulphone technique is warranted.

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## STRESZCZENIE

**Oznaczanie udziału aminokwasów pochodzenia bakteryjnego w treści jelita biodrowego świni przy zastosowaniu wskaźników  $^{35}\text{S}$  i DAPA**

Oznaczano zawartość aminokwasów pochodzenia bakteryjnego w treści jelita biodrowego dwóch 45 kg loszek z przetokami do dwunastnicy i jelita biodrowego, stosując  $^{35}\text{S}$  i kwas dwuaminopimelinowy (DAPA) jako wskaźniki białka bakteryjnego. Obydwie metody oparto na założeniu, że próba masy bakteryjnej wyizolowanej z treści jest reprezentatywna dla całej treści przepływającej przez jelito biodrowe. Udział aminokwasów pochodzenia bakteryjnego w ogólnej puli aminokwasów w treści jelita biodrowego oznaczony metodą z zastosowaniem  $^{35}\text{S}$ -sulfometioniny przewyższał niemal dwukrotnie (190,6%) całkowitą zawartość aminokwasów w treści jelita biodrowego. Przy użyciu DAPA wykazano, że aminokwasy pochodzenia bakteryjnego stanowią 20 do 34% (średnio 29,4%) ogólnej puli aminokwasów w treści jelita biodrowego świń żywionych dawkami z dużym udziałem śruty zbożowej.

Zawartość izoleucyny, kwasu asparaginowego, glicyny, cysteiny i tyrozyny w białku bakteryjnym różniła się istotnie ( $P \leq 0,05$ ) od zawartości tych aminokwasów w niebakteryjnej frakcji białka treści jelita biodrowego. Wysoki udział glicyny we frakcji niebakteryjnej w stosunku do innych aminokwasów i odpowiednio niski udział we frakcji bakteryjnej sugerują, że wydzielona z treści frakcja bakteryjna była wolna od zanieczyszczeń. Uzyskane wyniki wskazują na potrzebę dalszych badań nad oznaczaniem zawartości białka bakteryjnego w treści jelita biodrowego.