

Nitrogen and *in vitro* Fermentation of Nitrogenous Substrates in Caecal Contents of the Pig

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Abstract

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Caecal contents of four 28-week-old pigs were analysed and used as inocula of cultures supplied with plant proteins (gluten, gliadin, zein), mucin, ribonucleic acid (RNA) and glucose. Pigs were fed *ad libitum* a 14.1% crude protein diet containing barley, wheat, wheat bran, extracted soybean meal and a feed supplement. The caecal digesta contained dry matter, total N, protein, trichloroacetic acid (TCA)-soluble N and NH₃-N at 120, 3.1, 14.9, 0.64 and 0.27 mg/g (on average), respectively. One g of the caecal content contained 70 μmol of glycine equivalents of amino acids in TCA-soluble fraction. Peptides rather than amino acids were the primary component of the TCA-soluble ninhydrin-reactive material in the caecum.

Fermentation of mucin yielded more volatile fatty acids (VFA) than fermentation of other nitrogenous substrates. Zein was the least fermentable substrate tested. Acetate was the principal fermentation end-product, followed by propionate and butyrate. Fermentation pattern differed in cultures grown on glucose and proteins: propionate molar proportions were higher and those of branched-chain VFA lower in former cultures than in cultures supplied with gluten, gliadin and zein. Low proportions of branched-chain VFA were also in VFA produced from mucin and RNA. It can be concluded, therefore, that caecal microorganisms convert nitrogenous substrates, which would be lost in faeces, into valuable products (VFA), utilizable by the host.

Pig, caecum, nitrogenous substances, protein, fermentation, in vitro, microbial metabolites

In pigs, most nutrients are released from the feed by action of endogenous enzymes, and absorbed in the small intestine. In the lower ileum, caecum and colon, an intensive microbial degradation of available nutrients takes place. A significant portion of the digestible energy thus could be ingested post-ileally, depending on the composition of pig the diet. In growing pigs, the hindgut fermentation contributed up to 15% to the total digestion of the organic matter, whereas this level can reach 50% in sows (reviewed by Drochner 1993). Microorganisms in the hindgut convert polysaccharides and nitrogenous substances into a mixture of metabolites and compounds of bacterial cells. Nitrogenous substances entering the hindgut are undegraded dietary proteins, i.e. plant proteins of low solubility (prolamins and glutelins), and proteins of endogenous origin, i.e. enzymes and mucin secretions, and cell desquamation products. Ileal solids contained 16.1 and 20.2 % of crude protein (N x 6.25) in pigs on a high-fibre diet with wheat bran, and on a low-fibre diet, respectively (Knudsen and Canibe 2000). Additional N sources in the hindgut are proteins and nucleic acids released from lysed bacterial cells. As shown by Dierick et al. (1990), the bacterial growth efficiency in pig hindgut is rather low: on average 13.6 g bacterial N was excreted in the faeces per kg of organic matter apparently digested in the hindgut. Corresponding values in the rumen

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vary around a mean of *ca* 30 g N per kg of organic matter apparently digested (Demeyer and Van Nevel 1986). A more extensive lysis of bacterial cells in the pig hindgut is a possible explanation of this difference.

As shown by Drochner (1984), the capability of pigs to digest dietary fibre is considerable if adequate N is supplied to the hindgut microflora. Whereas carbohydrate metabolism in the pig hindgut has been investigated in many studies, knowledge on fermentation of nitrogenous substrates in pigs is far from complete. The present study was carried out (i) to determine the amount of the total and trichloroacetic acid (TCA)-soluble nitrogen, protein, ammonia and amino acids in the caecal digesta of *ad libitum* fed pigs, and (ii) to examine fermentation pattern in cultures of the caecal contents supplied with plant proteins, mucin and nucleic acids. For comparison, the fermentation in caecal cultures with glucose was also investigated.

Materials and Methods

Pigs

Castrated male pigs (Czech White) were fed *ad libitum* a standard diet containing barley (39%), wheat (29%), wheat bran (20%), extracted soybean meal (9%) and a feed supplement MastSan (SANO). The diet contained 14.1% of crude protein, 4.5% of fibre and metabolizable energy at 12.3 MJ/kg. Four pigs were slaughtered at the age of 28 weeks (average live weight of 106 kg), their caeca emptied, pooled, mixed and used for incubation of *in vitro* cultures. Individual samples of digesta were taken for analyses.

Analyses of caecal contents

The pH of the digesta was measured immediately after sampling. Dry matter (DM) of the caecal contents was determined by drying at 105 °C overnight. Total nitrogen (N) was determined using the Kjeltac Auto 1030 Analyser (Tecator AB, Sweden). To determine TCA-soluble N, ammonia and amino acids in TCA-supernatant, caecal contents (20 g) were mixed with 7% (w/v) trichloroacetic acid (60 ml). The mixture was refrigerated overnight, then centrifuged at 8 000 *g* for 20 min. TCA-soluble N was determined in the supernatant by the Kjeldahl procedure, and ammonia colorimetrically with Nessler reagent, after prior separation from interfering compounds by microdiffusion in Conway units. TCA-precipitate was washed with 7% trichloroacetic acid and protein extracted with 1 M NaOH at 100 °C for 1 h. Extracted protein was determined by the Lowry method (Herbert et al. 1971). Free amino acids in the TCA-supernatant were detected by the ninhydrin reagent containing ninhydrin (1 g), hydrindantin (50 mg), methylcellulose (38 ml) and acetate buffer (12.6 ml). The method was calibrated with glycine. A portion of the TCA-supernatant was mixed 1 : 1 with 12 M HCl and heated at 110 °C for 22 h. The hydrolysate was neutralized and free amino acids assayed using the same procedure. Volatile fatty acids were determined in samples of caecal contents diluted with physiological saline 1 : 3 by titration, after steam distillation. Lactic acid in these samples was determined by a microdiffusion method (Conway 1957).

Incubation

Incubations were carried out in triplicate on a shaking water bath at 39 °C, in 300 ml bottles. The caecal contents (25 g) were added to 75 ml of the McDougall buffer (McDougall 1949) containing microelements and vitamins. Substrates were added at 1 g per incubation. Gluten from wheat and zein from maize were purchased from Sigma. Lipids from gluten were removed by diethylether extraction. Gliadin from wheat and mucin from pig stomach were bought from Fluka. Sodium ribonucleate (RNA) from yeast was supplied by ICN. The sixth substrate was glucose (a positive control). Sodium sulphide was added to the incubation fluid at 0.5 g/l as a reducing agent. Bottles were thoroughly flushed with CO₂, closed with rubber stoppers and incubated for 19 h. Blank incubations were carried out by incubating diluted caecal contents without substrate.

Analyses of incubation fluid and gas

The VFA concentrations were determined by titration, after steam distillation in the Markham apparatus. Molar percentages of acetate (C2), propionate (C3), isobutyrate (iC4) butyrate (C4), isovalerate (iC5) and valerate (C5) were estimated on a gas chromatograph with a FI detector, equipped with a column of the Chromosorb WAW with 15% SP 1200 and 1% H₃PO₄ (Supelco). The separation was carried out at 140 °C. Lactate was assayed as described above. Samples of the headspace gas were taken at the end of the incubation and analysed with the gas chromatograph, using the same column operated at room temperature. Production of metabolites from substrates tested was calculated by correcting findings for amounts present in incubated blanks.

The analysis of variance and Bonferroni test were used to evaluate differences in VFA production. Hydrogen recovery in cultures supplied with glucose was calculated according to Marty and Demeyer (1973).

Results and Discussion

Concentrations of DM, VFA, lactic acid and pH values of the caecal contents were more or less stable, only limited animal variation was observed. Data presented in Table 1 do not differ much from our previous findings in pigs fasted for 16 h before slaughter (Marounek et al. 1997). Crude protein (calculated using the factor 6.25) and true protein (determined by the Lowry method) represented 16.1 and 12.4% of digesta DM, respectively (Table 2).

Table 1
Contents of dry matter (DM), volatile fatty acids (VFA), lactic acid and pH values of the caecal digesta of pigs.

	Per g of digesta	Per g of digesta DM
Dry matter (g)	0.120 ± 0.008	1.000
VFA (µmol)	156.3 ± 14.4	1 302 ± 120
Lactic acid (µmol)	2.19 ± 0.25	18.3 ± 2.1
pH	6.03 ± 0.22	

Mean values and standard deviations. Caecal contents of 4 pigs were analyzed.

Table 2
Contents of total nitrogen (N), protein, trichloroacetic acid (TCA)-soluble N, ammonia and amino acids in the caecal digesta of pigs

	Per g of digesta	Per g of digesta DM
Total N (mg)	3.10 ± 0.28	25.83 ± 1.84
Protein(mg)	14.91 ± 1.87	124.25 ± 21.6
<i>TCA-soluble N</i>		
Total (mg)	0.64 ± 0.04	5.33 ± 0.04
NH ₃ -N (mg)	0.27 ± 0.02	2.25 ± 0.02
<i>Amino acids in TCA-supernatant*</i>		
Before hydrolysis (mmol/l)	0.015 ± 0.001	0.125 ± 0.009
After hydrolysis (mmol/l)	0.070 ± 0.006	0.583 ± 0.021

Mean values and standard deviations. Caecal contents of 4 pigs were analyzed.

* Ninhydrin-reactive compounds expressed as glycine equivalents

Corresponding values found in the rabbit caecal contents were 27.6 and 18.2% DM (Marounek et al. 2000). The main fraction of crude protein in the pig caecum, thus, was the true protein. True protein accounted for three-quarters, and TCA-soluble nitrogenous substances for 20.6% of the total N in the caecal digesta. Ammonia N represented 42.2% of TCA-soluble N. Caecal contents contained 15 µmol of glycine equivalents of TCA-soluble amino acids per 1 g. Acid hydrolysis increased this amount to 70 µmol/g. This indicates that low-molecular-weight peptides were the primary component of the TCA-soluble ninhydrin-reactive material in the caecum. Similarly, also in the rumen (Chen et al. 1987), and in the rabbit caecum (Marounek et al. 2000), peptides rather than amino acids were the principal source of non-protein non-ammonia N.

Proteins tested and RNA differed in their fermentability. Fermentation of mucin yielded significantly more VFA than fermentation of other nitrogenous substrates (Table 3). Mucin is a glycoprotein, present in saliva, gastric juice and intestinal juice. Pig gastric mucin contains 77% of carbohydrates and 21% of protein (Hill 1986). Holmes et al. (1974) were probably the first authors who reported that in pigs large amounts of mucin were fermented in the large intestine. Zein was the poorest substrate out of the five nitrogen compounds tested. Zein is a very hydrophobic protein, the principal protein of maize. Acetate was the principal fermentation end-product in all incubations, followed by propionate and butyrate.

Table 3
Effect of substrate on production* and molar proportions of volatile fatty acids (VFA), lactate and methane in cultures of pig caecal contents supplied with proteins, ribonucleic acid (RNA) and glucose

Substrate	Total VFA (mmol/l)	Molar percentages						Lactate (mmol/l)	Methane (mmol/l)
		C2	C3	iC4	C4	iC5	C5		
Gluten	46.1a (5.8)	41.2 (1.9)	29.6 (4.7)	2.3 (0.9)	10.9 (2.2)	5.4 (0.8)	10.6 (0.6)	0.05 (0.05)	1.11 (0.15)
Gliadin	24.5b (3.1)	46.5 (0.4)	21.5 (0.1)	3.0 (0.2)	11.1 (1.8)	6.5 (0.3)	11.4 (0.7)	0	0.89 (0.12)
Zein	12.5b (3.3)	50.9 (19.8)	18.0 (10.6)	2.3 (0.7)	14.0 (6.0)	9.3 (2.5)	5.5 (1.5)	0	0.83 (0.48)
Mucin	114.2c (3.7)	55.7 (0.8)	28.5 (1.0)	1.0 (0.1)	9.3 (0.2)	0.8 (0.2)	4.7 (1.6)	0	1.19 (0.10)
RNA	48.6a (4.8)	58.0 (0.9)	32.4 (1.4)	0.5 (0.1)	8.7 (0.4)	0	0.4 (0.1)	0	0.76 (0.42)
Glucose	96.6d (3.1)	48.9 (0.7)	41.7 (0.4)	0.1 (0.1)	8.8 (0.4)	0.1 (0.0)	0.4 (0.1)	0.10 (0.05)	1.12 (0.15)

Means of 3 cultures. Standard deviations are given in parentheses.

* Data were corrected for amounts present in incubated blanks.

a-d Values with different superscripts differ significantly ($P < 0.05$).

Fermentation pattern differed in cultures supplied with glucose and proteins. Propionate molar proportion was higher and proportions of branched-chain VFA lower in former cultures. Molar proportions of branched-chain VFA were also lower in cultures supplied with mucin and RNA, i.e. supplied with substrates containing a carbohydrate moiety. Both lactate and methane productions were low. Low value of metabolic hydrogen recovery (61.0% in glucose-grown cultures, on average) indicated the presence of reductive acetogenesis, which is an alternative electron sink to methanogenesis.

In pigs, the disappearance of total N between ileum and rectum represents about 10% of the total N intake. Holmes et al. (1974) found that in growing pigs fed diets containing soyabean meal and rapeseed meal 8.0 and 12.4% of the total N intake, respectively, was digested in the hindgut. The nutritional significance of the hindgut nitrogen metabolism is inevitably reduced by the fact that neither bacterial protein nor non-volatile products of catabolism of nitrogenous substrates were utilized by the host. Thus, as it has been emphasized by Dierick et al. (1990), a low bacterial growth efficiency combined with a large VFA production is preferred in this digestive segment. Hindgut microbes convert macromolecular nitrogenous substances, which would be lost in faeces, into VFA, available to the host. Branched-chain VFA are growth factors for cellulolytic bacteria (Dehority et al. 1967). In addition, ammonia released from N-containing compounds during this process is the main source of nitrogen for fibrolytic microflora.

Frakce dusíku a fermentace dusíkatých látek *in vitro* v obsahu slepého střeva prasat

Analyzovali jsme obsahy slepých střev čtyř prasat poražených ve věku 28 týdnů. Tyto obsahy jsme současně použili jako inokula kultur v nichž substrátem byly rostlinné proteiny (gluten, gliadin, zein), mucin, ribonukleová kyselina (RNA) a glukosa. Prasata byla před porážkou krmena *ad libitum* dávkou s obsahem 14,1% N-látek, složenou z ječmene, pšenice, otrub, extrahovaného sojového šrotu, s přídatkem krmného doplňku. Zažitina slepého střeva v 1 g obsahovala v průměru 120 mg sušiny, 3,1 mg celkového N, 14,9 mg proteinu,

0,64 mg N rozpustného v 5,25% kyselině trichloroctové (TCA) a 0,27 mg NH₃-N. Dále bylo v 1 g zažitény 70 μmolů aminokyselin rozpustných v TCA (v přepočtu na glycin). Hlavní složkou této frakce nebyly volné aminokyseliny, ale nízkomolekulární peptidy.

V kulturách obsahu slepého střeva vznikalo nejvíce těkavých mastných kyselin (TMK) fermentací mucinu a nejméně fermentací zeinu. Hlavním fermentačním produktem byl acetát, následovaný propionátem a butyrátem. Stechiometrie fermentace v kulturách s glukosou a proteiny byla odlišná, neboť při fermentaci glukosy vznikalo více propionátu a méně TMK s rozvětveným řetězcem než při fermentaci glutenu, gliadinu a zeinu. Nízký podíl TMK s rozvětveným řetězcem byl nalezen také v kulturách s mucinem a RNA. Lze shrnout, že fermentací sloučenin dusíku v slepém střevu prasat vznikají těkavé produkty využitelné hostitelem, z makromolekulárních látek, které by jinak byly ztraceny ve výkalech.

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