Significance of combined nutritional and morphological precaecal parameters for feed evaluations in non-ruminants

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Chapter 1

General introduction

The small intestine can be described as a tube-shaped organ between stomach and large intestine serving the digestion by hydrolysis of contents derived from the stomach, and subsequently the absorption of the breakdown products, water and electrolytes. It comprises the digesta containing lumen and a wall with its mucosal epithelium. Digesta composition changes gradually from the proximal to the distal area as a consequence of the digestive processes and influx of endogenous components such as digestive enzymes, mucus and extruded epithelial cells. The amounts of nutrients and contents of hydrolytic enzymes in the digesta are relatively high in the proximal area and decrease towards the distal region. Therefore, the proximal region of the small intestine is the most important location for hydrolysis and absorption. Distally the digesta consist of indigestible components or components less readily digested by the enzymes of the animal.

Besides enzymatic digestion, microbial degradation of nutrients also occurs. Microbial activity in the upper part of the small intestine of the studied non-ruminants is limited and increases towards the distal region of the small intestine. Therefore, the whole digestive complex in the small intestine comprises activity of endogenous enzymes, mainly in the proximal area, and microbial activity, mainly in the distal area, whereas interactions between intestinal bacteria and the host's intestinal mucosa also occur (McCracken and Gaskins, 1999; Gaskins, 1999).

The most functional element of the small intestine is the mucosa, which can be characterised as a tissue, permeable to nutrients but with a barrier function against noxious compounds. Because of its selective properties the healthy small intestinal mucosa can cope with the apparent conflicting functions of permeability and resistance against passage of undesired substances (van Dijk, 1997). Both functions are essential for the elementary physiological processes of the animal. Nutrient digestion and absorption are essential to total body maintenance and production, whereas specific nutrients and energy sources are used for gut wall maintenance. Spreeuwenberg *et al*. (2001) observed in piglets fed iso-energetic diets, during the first days after weaning a preference for lactose compared to protein for gut wall maintenance, and glutamate has been shown to be beneficial to the mucosal function in models of bowel injury (Souba, 1993). The barrier function

of the mucosa as a resistance factor against micro-organisms is essential to the healthy animal and largely depends on dietary composition (Deitch, 1994).

Regarding its permeable characteristics, the mucosal membrane of the small intestine is specifically equipped for the absorption of amino acids, in contrast to the large intestine. Therefore, precaecal digestion of protein and amino acids is a specific nutritional-functional parameter of the small intestine. When free amino acids occur in the colonic lumen they will be degraded by microbes to ammonia and are no longer available to the animal (Zebrowska *et al*., 1978). This means that in contrast to whole tract digestion, precaecal digested feed proteins offer a more accurate estimate of the amount of protein available for body maintenance and production. Dierick *et al.* (1987) confirmed this hypothesis in performance experiments with pigs. The proportion of a nutrient that disappears from digesta in the gastro-intestinal (GI) tract proximal to the caecum, is termed (apparent) precaecal or ileal digestible. Data on (apparent) precaecal digestibility of proteins and amino acids determined in pigs have been compiled in tables (CVB, 2001). These digestibility values provide quantitative information for feed formulation and the use of precaecal protein and amino acid digestibility values can help to optimise diet composition in terms of feed protein conversion to body protein and minimise nitrogen waste in manure.

As mentioned, the precaecal digestibility of protein determined in the healthy animals is a functional-nutritional parameter, which differs between proteins of different feedstuffs (CVB, 2001) partly because of so-called antinutritional factors (Huisman, 1990). The amounts of proteolytic enzymes secreted by the pancreas are excessive (Makkink, 1993). However, unfamiliar feed may provoke inappropriate responses of the digestive system as shown after weaning in piglets (Van Beers-Schreurs, 1996), in starting calves (Reynolds, *et al.,* 1981) and in broilers fed high pectin diets (Langhout, 1998). A change in the function of the small intestinal mucosa in the upper part affects the amount of undigested proteins in the distal region causing bacterial proliferation. Bacterial activity in the small intestinal lumen may erase effects on precaecal digestibility of protein. Moreover, the excess of enzymes in digesta and absorptive capacity of the small intestinal mucosa may mask negative functional changes of the small intestinal mucosa. Changes of the mucosal function related to absorption and the barrier function are generally expressed by morphological parameters of the mucosa. Therefore, qualitative functional-morphological parameters of the small intestinal mucosa, such as villus and crypt dimensions and brush border enzyme activity, may provide additional information about the functional status of the small intestine. Other functional-morphological parameters, such as numbers of goblet cells

and type of mucin in the goblet cells, are parameters, which indicate the condition of the epithelium with respect to the mucosal function as a barrier between the (septic) intestinal lumen and body tissues.

In summary, it is assumed that both the hydrolytic and absorptive capacity of the small intestine have a large overcapacity. Secondly, in literature there is consensus that, despite this overcapacity there is still considerable variation in the quantitative digestion of crude protein between different feedstuffs. It is also generally accepted that a close and intensive relationship exists between microbial activity, the mucosal epithelium and the mucus composition. Other factors such as, differences in microbial status between herds (Van Beers-Schreurs, 1996), and the age-related changes of nutritional function (Pluske, 1993) may limit adaptation to diet composition and are at least partly responsible for the variation in gut morphology.

Therefore in this thesis the hypothesis is tested that evaluation of the digestive function requires both functional-nutritional and functional-morphological approaches. These approaches provide knowledge about both the quantitative result of the digestive processes and qualitative information about the interference of dietary components with the intestinal mucosa, respectively.

The thesis describes procedures for functional-nutritional and functional-morphological studies in several species (pigs, calves and poultry). Studies on the functional-nutritional parameters by the quantitative determination of the precaecal digestibility are presented in Part I. They comprise cannulation techniques (Chapter 2 and 4), procedures for digesta collection in pigs and roosters and present a comparison of results on apparent ileal crude protein digestibilities in both animal species (Chapter 3 and 4). In Part II of the thesis functional-morphological parameters of the small intestinal mucosa are described in relation to dietary protein sources, an antibiotic (virginiamycin), bioactive peptides (lactoperoxidase/ lactoferrin), and *Vicia faba* L. tannins as dietary constituents or additives (Chapters 5 to 8). The main results of these studies are discussed and evaluated in a general summarising discussion.

REFERENCES

CVB (2001) Veevoedertabel. Chemische samenstelling, verteerbaarheid en voederwaarde van voedermiddelen. Centraal Veevoederbureau, P.O. Box 2176, Lelystad, The Netherlands.

Deitch, E.A. (1994) Bacterial translocation; the influence of dietary variables. Gut, supplement 1: S23-S27.

Dierick, N.A., Vervaecke, I.J., Decuypere, J.A., van der Heyde, H. and Henderickx, H. K. (1987) Correlation of ileal and faecal digested protein and organic matter to production performance in growing pigs. In: Proceedings of the Symposium on Protein Metabolism and Nutrition, Rostock, DDR.

Gaskins, H.R. (1999) Nutritional modulation of mucosal defence In: Nutrition and gastrointestinal physiology –today and tomorrw-. A.J.M. Jansman and J. Huisman (editors). TNO Nutrition and Food Research Institute, Wageningen, The Netherlands, 23-33.

Huisman, J. (1990) Antinutritional effects of legume seeds in piglets, rats and chickens. (Thesis) Agricultural University Wageningen.

Langhout D.J. (1998) The role of the intestinal flora as affected by non-starch polysaccharides in broiler chicks. PhD Thesis, Agricultural University Wageningen, The Netherlands.

Makkink, C.A. (1993) Of piglets, dietary proteins, and pancreatic proteases. (Thesis) Agricultural University Wageningen, The Netherlands.

McCracken, V.J. and Gaskins, H.R. (1999) Intestinal microbes and the immune system. In Probiotics: A Critical Review, Tannock, G.W. Ed., Horizon Scientific Press, Norfolk, U.K., 85-112.

Pluske, J.R. (1993) Psychological and nutritional stress in pigs at weaning: production parameters, the stress response, and histology and biochemistry of the small intestine. PhD Thesis, University of Western Australia, Perth, Australia. SAS (1991).

Reynolds, D.J., Morgan, J.H., Chanter, H. (1981) Microbiology of calf diarrhoea in Southern Britain. Veterinary Records 119. 34-39

Souba, W. (1993) Review: Intestinal glutamine metabolism and nutrition. Journal of Nutritional Biochemistry 4: 2- 9.

Spreeuwenberg, M.A.M., Verdonk, J.M.A.J., Gaskins, H.R. and Verstegen M.W.A. (2001) Small intestine epithelial barrier function is compromised in pigs with low feed intake at weaning.

Journal of Nutrition 131:1520-1527.

van Dijk, J.E. (1997) Morphology of the gut barrier. The European Journal of Comparative Gastroenterology. 2: 2.

Van Beers-Schreurs, H.M.G. (1996) The changes in the function of the large intestine of weaned pigs. PhD Thesis. University of Utrecht, The Netherlands.

Zebrowska, T., Buraczewska, C., Horaczynski, H. (1978) Apparent digestibility of nitrogen and amino acids and utilization of protein given orally or introduced into the large intestine of pigs. Roczn. Nauk. Roln. Ser. 99B, 99-105.

Part I Functional-nutritional parameters

Chapter 2

The post valve T-caecum (PVTC) cannulation technique in pigs applied to determine the digestibility of crude protein and amino acids in maize, groundnut and sunflower meal

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SUMMARY

The post valve T-caecum (PVTC) technique has been described as a technique for cannulation of pigs in order to determine ileal digestibility. A digestibility experiment with PVTC cannulated pigs was conducted to apply this technique.

The PVTC cannula is placed directly opposite to the ileo-caecal valve. The cannulated pigs can be used over a long period of time without signs of discomfort. Chyme collection with PVTC cannulated pigs is nearly quantitative but a marker is needed.

The digestibility experiment was a pilot study comprising three rations with maize, groundnut meal and sunflower meal as main feedstuffs, respectively. The results of the ileal digestibility of crude protein and indispensable amino acids of the three feedstuffs were compared with data from literature, determined with different techniques. In groundnut meal the digestibilities varied between trials and, also in maize, the number of observations were limited. For sunflower meal there was a good agreement between the digestibility values of crude protein and of indispensable amino acids from this experiment and those of the referred studies.

INTRODUCTION

In the Netherlands a wide variety of raw materials are used in animal feed industry. Therefore it is important to have correct measurements for evaluating the nutritional value. The ileal digestibility of amino acids is such a measure (Zebrowska, 1973; Dierick *et al.,* 1987).

At the Institute for Animal Nutrition and Physiology (ILOB) extensive series of experiments have been performed with the aim to establish a table of ileal (precaecal) digestibility coefficients of crude protein (CP; N x 6.25) amino acids in pigs. In order to determine ileal digestibility, the ileal chyme has to be collected. Initial ILOB research on the field of amino acid digestion was based on experiments in animals fitted with ileo-caecal re-entrant cannulas according to Easter and Tanksley (1973). This cannulation method was not always suitable because of high incidence of blockages

in the cannulas, especially when feedstuffs with high crude fibre content were tested (Sauer and Ozimek, 1986). An infusion of physiological saline solution (0.9 % NaCl) into the cannula (van Leeuwen *et al*., 1987) has reduced the frequency of blockages, but this improved re-entrant method was not practical for large-scale studies.

Several techniques for chyme collection are used in various laboratories. Prerequisites for each method are that the animals are in a physiological state and samples of chyme are representative. Collection of digesta from a simple T-cannula, placed 5-10 cm anterior of the ileocaecal valve, is the method, which is mostly used. With this method there is doubt about obtaining representative samples of digesta (Sauer and Ozimek, 1986).

In the ileo-rectal anastomose technique of Laplace *et al.* (1985) and Souffrant *et al.* (1985) the colon has no function for the digestive activity. This bypass, however, may affect the physiological state of the animal and may cause compensatory adaptation of the small intestine (Köhler *et al.,* 1990). Also methods for ileo-colic post valve cannulation allowing total collection of digesta are described (Darcy *et al.,* 1980; Darcy and Laplace, 1980). The technique involves the transection of the intestine and collection of chyme after the ileo-caecal valve. Although, the re-entrant flow of digesta between the two cannulae was not always spontaneous.

An alternative collection method has been developed: the post valve T-caecum cannula (PVTC cannula) (van Leeuwen *et al.,* 1988). With this technique the anatomy of the transition from ileum into caecum is used. Normally, the ileo-caecal valve protrudes into the caecum. After PVTC cannulation the caecum is partially removed and replaced by a cannula, which is positioned opposite to the valve. When the cannula is opened, the valve protrudes into the cannula and digesta flow into the cannula.

First results of a comparison of techniques, where PVTC was included, are reported by Den Hartog *et al.* (1988). The surgical procedures for PVTC cannulation and the estimation of the ileal amino acid digestibility in a pilot experiment are described in this paper.

MATERIALS AND METHODS

PVTC cannula

The cannula (Figure 1) is composed of segments of silicon tubing (Medical grade; Medica, Den Bosch, The Netherlands) and medical Adhesive Type A (Raumedic® Adhesive SI 1511, Rehau, West Germany). The barrel consists of a 10 cm. tube (25 mm inside diameter (ID) and 30 mm outside diameter (OD)).

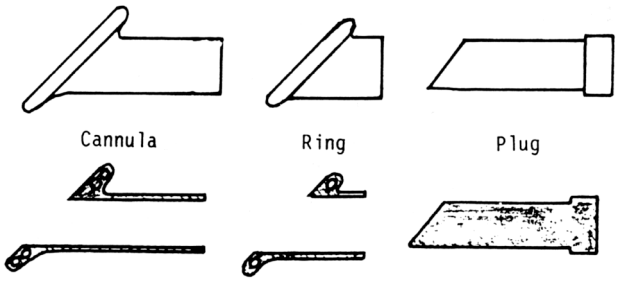

Figure 1. A design of the PVTC cannula, the ring and plug.

The circular flange consists of two rings of silicon tubing. Nylon straps are introduced in these rings to rigid the flange. The rings are filled with medical adhesive. The barrel is inserted at an angle of approximately 45 degrees into the described circular rings and fixed with medical adhesive (Figures 1 and 2). In the same way a ring is made to fix the cannula after surgery. Also a plug is prepared by filling a tube (24 mm. OD) with the adhesive.
The described cannula is used in pigs with a liveweight over 35 kg. For smaller pigs the dimensions must be adapted.

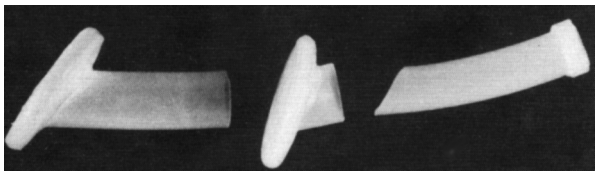

Figure 2. The PVTC cannula with ring and plug.

Surgery

Healthy crossbred barrows (Yorkshire x Dutch Landrace) with a live weight of approximately 50 kg were fasted overnight prior to surgery. Pigs were premedicated by an intramuscular injection of 4 mg Azaperon (Stresnil®) and 0.05 mg Atropinesulphate per kg bodyweight. After twenty minutes general anaesthesia was induced and maintained by inhalation of O_2/N_2O, Halothane, (Fluothane®).
The pigs were placed in the left lateral recumbency and the surgical area was shaved and scrubbed with a general disinfectant. Lidocaine with noradrenaline was administered in the area

of the incision subcutaneously and intramuscularly as local anaesthesia. A sterile plastic incision sheet was fixed to the skin with glue (Leukospray®).

Laparotomy was performed by an incision just dorsal to the inguinal fold. The caecum was mobilized by incision of the ileocaecal ligament through the avascular tissue. An intestinal clamp was used to isolate the apex and corpus of the caecum according to a projection perpendicular to the ileum (Figure 3A). After placing a purse string suture (Vicryl V 311 H) in the part of the caecum to be preserved (distance to the intestinal clamp was 0.5 cm) the caecum was transected between the clamp and the string (Figure 3 B). Now the flange of the cannula (Figure 3 C) was introduced and immediately fixed by tightening the pre-placed purse string suture. A second purse string suture (Vicryl V 311 H) was placed to reduce bleeding, to invert the intestinal wall and to secure proper positioning of the cannula as can be monitored by observation of the ileo-caecal valve (Figure 3 D). The cannula was then exteriorised through a stab incision in the body wall about 8 cm dorsal to the first incision.

After routine closure of the laparotomy incision the cannula was fixed externally by mounting the ring of silicon rubber. Finally the cannula was closed by the plug and the external ring was attached to the barrel with silicon adhesive. All parts were fixed with self-tightening nylon straps. Antimicrobial treatment of the animals and administration of an antiflogistic analgetic, 0.03 ml Trimethoprime/Sulfadiazine (Tribrissen®) and 2 mg/kg Flunixine (Finadyne®)/kg bodyweight/day, respectively, were applied during four days post surgery.

Digestibility experiment

The twelve crossbred PVTC cannulated barrows (Yorkshire x Dutch Landrace) were used to perform an experiment in order to determine ileal digestibility. Three diets were formulated, diet 1, with maize; diet 2, with groundnut meal; and diet 3 with sunflower meal, as main feedstuffs. The crude fibre content was for maize 1.8%, for groundnut meal 12.7% and for sunflower meal 23.6% (Table1 and 2). The three diets were added with chromiumoxide (Cr_2O_3).

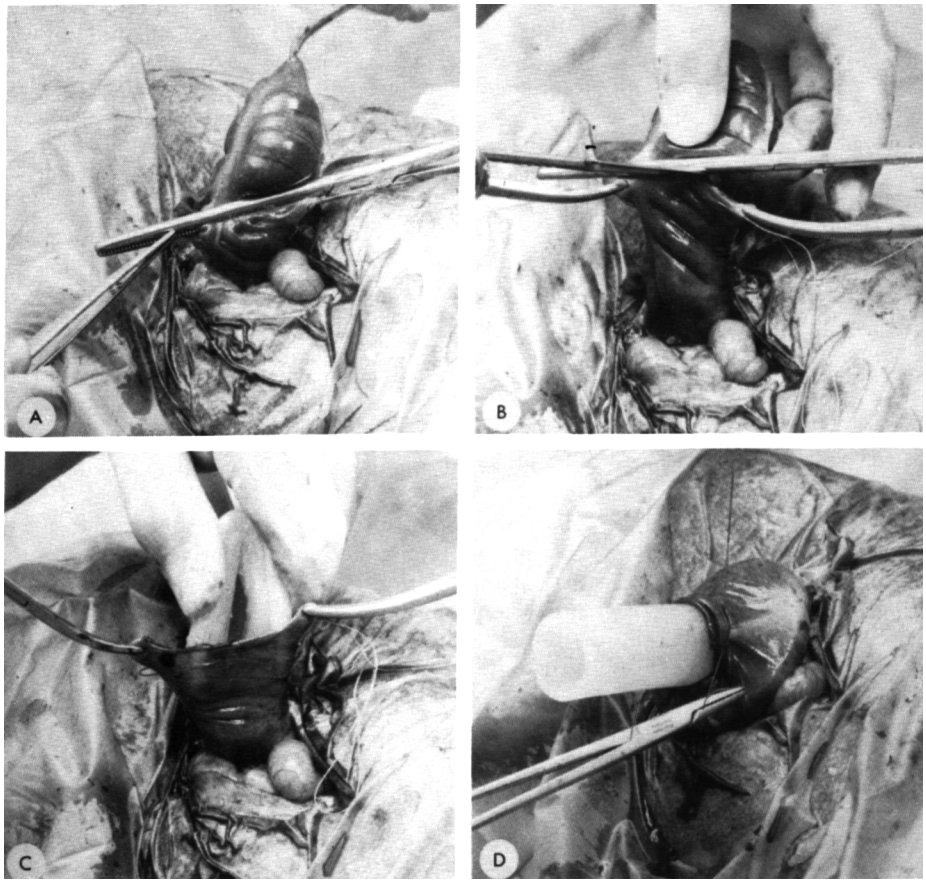

Figure 3. PVTC cannulation of a piglet. (A) An intestinal clamp is used to isolate the apex and corpus of the caecum according to a projection perpendicular to the ileum; (B) The caecum is transected between the clamp and the purse string; (C) The flange of to the cannula is introduced into the intestine; (D) The cannula is fixed by tightening the pre-placed and the second purse string suture.

After surgery the animals were allowed a recovery period of 14 days followed by an adaptation period of two days in which the ration gradually changed from a commercial diet to one of the three experimental diets. So, each of the diets were fed to four animals. The diets were ground through a 2.75 mm mesh screen and supplied at a level of 2.6 times the requirement of metabolizable energy for maintainance requirement. Maintenance level was assumed to be 420 kJ per kg metabolic body weight. The pigs were fed equal amounts of feed, twice daily, at 08.00 and 16.00 hours. During the collection period and two days in advance the animals received their feed at 8.00 and 20.00 hours during. The pigs were housed individually in adjustable metabolic cages in an environmentally controlled barn with continuous lightening and at an air temperature

in the range of 19-20^{0}C. Water was administered with the feed at a ratio of 2 : 1.Digestibility studies were carried out with pigs over the weight range from 55 to 65 kg.

Procedure for chyme collection

Three 12 h collection periods of digesta were carried out on alternate days from 8.00 to 20.00 hours from days 6, 7 and 8 after the first administration of the experimental rations. One hour in advance of the collection period the PVTC cannula was opened to adapt the animal and the digesta flow. During this hour the position of the valve changed from protruding into the intestinal lumen to protruding into the lumen of the cannula (Figure 4). At 8.00 hours the cannula was connected with a silicon tube, 1.5 m in length and 25 mm in diameter. Digesta flowed through the tubing into a container packed in crushed ice. Digesta were collected over periods of one hour and frozen (-20^{0}C) until analyses.

Analytical procedures

Chemical analyses (Table 1) were carried out in the three feedstuffs, in the three rations and in the samples of the ileal chyme. Prior to chemical analyses, the samples of feedstuffs and rations were ground through a 1 mm screen using a Retsch AM 1 grinder. The frozen chyme collected over the three days was thawed, pooled per animal and freeze-dried. The analyses of nitrogen and chromium were carried out in these samples. For amino acid analysis the freeze-dried chyme samples were pooled to one sample per ration.

Nitrogen was analysed using a Technicon auto-analyser after wet destruction in sulphuric acid, with a mixture of potassium sulphate and mercuric oxide as catalists. Nitrogen was bound by hypochlorite and phenol and this nitrogen complex was measured at 630 nm.

Chromium contents were determined by atomic absorption spectrometry after treatment with a digestion mixture containing perchloric and nitric acid. Most amino acids were determined after hydrolysis of the sample with 6 M hydrochloric acid under reflux for 22 h.

Table 1. Chemical analyses of the feedstuffs (%).

	Maize	Groundnut meal solv. extr	Sunflower meal Solv. extr.
Crude protein (N x 6.25)	8.9	51.8	29.9
Crude fibre	1.8	12.7	23.6
Indispensable amino acids			
Arginine	0.36	5.05	2.29
Histidine	0.23	1.06	0.69
Isoleucine	0.31	1.65	1.29
Leucine	1.06	3.10	1.93
Lysine	0.23	1.36	1.07
Methionine	0.17	0.51	0.68
Phenylalanine	0.40	2.31	1.32
Threonine	0.31	1.28	1.14
Tryptophan	0.06	0.45	0.34
Valine	0.43	2.03	1.55
Dispensable amino acids			
Alanine	0.62	1.75	1.25
Aspartic acid	0.55	5.35	2.67
Cysteine	0.19	0.54	0.55
Glutamic acid	1.59	9.05	5.80
Glycine	0.31	2.72	1.68
Proline	0.75	1.93	1.23
Serine	0.39	2.48	1.38
Tyrosine	0.33	1.57	0.67

Hydrolysates for amino acid analysis were chromatographed on an automatic amino acid analyser (Biotronic LC 6001), except tryptophan which was separated on a HPLC colomn (Lichrosorb 10 RP 18). Factors were used for correction of the destruction of threonine and serine (1.05 and 1.10, respectively) and of incomplete hydrolysis of isoleucine and valine (1.07 and 1.08, respectively) according to Slump (1969). The sulphur containing amino acids were analysed after oxidation with performic acid as described by Moore (1963). Tryptophan was

determined after hydrolysis with 2.7 N barium hydroxide during 8 h at 130^0C, according to Slump and Schreuder (1969.

Table 2. Formulation and chemical analyses of the diets (%).

	Diets		
	1	2	3
Maize	95.3	70.8	71.0
Groundnut meal solv. extr.	-	25.0	-
Sunflower meal solv. extr.	-	-	25.0
Chromiumoxide (Cr_2O_3)	0.1	0.1	0.1
Mineral mixture[1]	3.6	3.1	2.9
Vitamin-trace element mixture[2]	1.0	1.0	1.0
Analyses			
Organic mater	82.7	83.2	82.5
Crude protein (N x 6.25)	8.3	19.1	13.6

[1]Contributed were the following mineral sources per kg of diet: $Ca(H_2P0_4)_2.H_20$, 12.5 g; NaCl, 5.0 g; $NaHCO_3$, 3.0 g; $KHCO_3$, 7.0 g; $FeSO_4.7H_20$, 0.5 g; $CuSO_4.5H_20$, 0.5 g; MnO_2, the 0.05 g; $ZnSO_4.H_20$, 0.2 g. The diets were supplied with 20 ppm virginiamycin. Diet 1 was additional supplemented with 2.5 g $Ca(H_2P0_4)_2.H_20$ and 5 g $KHCO_3$, and diet 2, with 2.5 g $Ca(H_2P0_4)_2.H_20$ per kg of diet.

[2]Contributed were the following vitamin sources and trace elements per kg of diet: Vitamin E, 40 mg; riboflavin, 5 mg; niacin, 30 mg; D-pantothenic acid, 12 mg; choline chloride, 150 mg; vitamin B_{12}, 0.04 mg; vitamin K_3, 3 mg; vitamin A, 9000 IU; vitamin D_3, 1800 IU; KI, 0.5 mg; $CoSO_4.7H_20$, 2.5 mg. The remainder was made up of ground with maize.

Data analysis

The digestibilities of CP and amino acid of the three test rations were calculated with the formula described by Wünsche *et al.* (1984). Using diet 1, the amino acid digestibilities of maize were directly determined. The CP and amino acid digestibilities of groundnut meal and sunflower meal were calculated by difference from ration 1 and ration 2 and by difference from ration 1 and ration 3, respectively. The standard deviation (SD) was calculated for the CP digestibility derived from the results of the four pigs per ration. The SD of the CP digestibility of groundnut meal and sunflower meal were determined with respect to the SD of ration 1 and 2, or ration 1 and 3, respectively.

RESULTS AND DISCUSSION

Surgery

After the PVTC cannulation the recovery after surgery was rapid and was not associated with reduced appetite or other signs of discomfort. The PVTC cannula is located at the site of the caecum, therefore we assume that the PVTC cannula has minor consequences for the myo-electrical innervation of the small intestines. It is possible to check the motility of the intestine and the innervation of the ileo-caecal valve (Darcy *et al.,* 1980). Furthermore, the digestion in the colon appears to be normal after cecectomy (Gargallo and Zimmerman, 1981). So a comparison between faecal and ileal digestibility within the same animal is possible. At this moment we have experience with application of the PVTC cannula in animals ranging from 8 to 100 kg liveweight. Also in the piglets the post operative recovery was rapid and not associated with reduced appetite or signs of discomfort, and the use of the pigs was not limited by the cannulas.

Chyme collection

Digestibility of a diet or feedstuff is a quantative parameter for feed evaluation. For this reason an important prerequisite is that samples, for analysing the nutrients of the collected chyme, are representative for the total chyme passing at that location. When the chyme collection is completely quantitative, the mixed sample will necessarily be representative. By definition the re-entrant technique and the ileo-rectal anastomose give a quantitative collection and in this respect these methods are thought to be reliable.

The PVTC cannula is placed directly opposite to the ileo-ceacal valve. When the cannula is opened the valve protrudes into the cannula and chyme flows from the valve into the cannula. However, the PVTC technique can not guarantee a complete quantitative collection. It is possibile that during the collection periods some chyme flows into the colon. Therefore, a marker (Cr_2O_3) was added to the feed and the proportion of the collected chyme in relation to the total passage was measured. In the present experiment with PVTC cannulated animals the amount of collected marker over three times 12 h collection period was in average 99% of the marker given with the feed. The range of recoveries was between 80 and 112%. This range shows that chyme passage is not constant. The recovery proportions indicate that the collection of the chyme was semi quantitative.

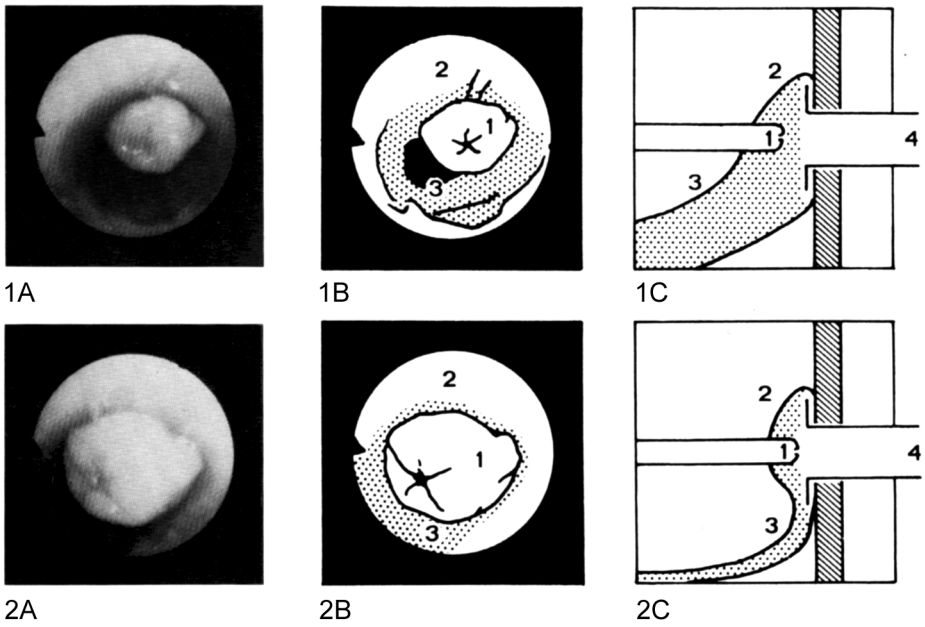

Figure 4. Photographic and schematic presentation of the position of the ileocaecal valve in a PVTC cannulated pig. After opening the cannula the position of the valve changes. Instead of protruding into the intestinal lumen after 15 minutes it protrudes into the cannula. Panels 1 (A-C) Directly after opening of the cannula. Panels 2 (A-C) About 15 minutes after opening of the cannula; A. Endoscopic views through the cannula; B. Schematic linedrawings of 1 A and 2 A; C. Schematic transection through cannula, abdominal wall and intestines (1. Ileocaecal valve, 2. Caecum, 3. Colon, 4. PVTC cannula).

Apparent precaecal, or ileal, protein and amino acid digestibility

The results of the digestibility experiment are given in table 3. The digestibility of protein from maize (65.1) was significantly lower than groundnut meal (80.3) and sunflower meal (75.6). Also the difference in digestibility between groundnut meal and sunflower meal was significant ($P < 0.05$).

The differences between the apparent digestibility of protein and the apparent digestibility of the indispensable amino acids differs between the three vegetable feedstuffs. Specially the digestibility of lysine, threonine and tryptophan from maize were, compared to the protein digestibility, lower (14, 15 and 21 percentage units, respectively). The negative deviation between the digestibility of the indispensable amino acids and the protein digestibility (DC amino acid minus DC protein) for groundnut meal were less than for maize. The digestibility of the indispensable amino acids from sunflower were almost equal or higher than protein.

In table 4 the apparent digestibility of protein and the apparent digestibility of the indispensable amino acids of the three feedstuffs are summarized and compared with data from literature. It needs to be recognized that there were differences between various trials i.a. in batches of feedstuffs and animals. Also the techniques were not identical; Green *et al.* (1987), Green *et al.* (1988) and Green and Kiener (1989) used the ileo-rectal anastomose; van Leeuwen *et al*.(1987) used ileo-caecal re-entrant cannulation; Jörgensen *et al.* (1984) and Knabe *et al.* (1989) simple T-cannulas. Protein digestibility of maize, compared with the mean of the results referred, was six percent units lower. Also the digestibilities of the indispensable amino acids were lower (4 to11 percent units).

The results from the groundnut meal show a considerable variation. Knabe *et al*. (1989) reported the results of three batches with a mean protein digestibility of 73 percentage units and Green *et al.*(1988) those one batch with a protein digestibility of 85 percent units. In the present experiment a protein digestibility of 80 percent units was measured. The digestibilities of the indispensable amino acids showed a similar variation between the different studies. The protein and indispensable amino acids digestibility in sunflower meal determined in present study were similar to the referred studies. The digestibility of this feedstuff is more constant.

Table 3. The apparent precaecal digestibility of crude protein and amino acids (% units).

	Maize	Groundnut meal solv. extr.	Sunflower meal solv. extr.
Crude protein (N x 6.25)	65.1 ± 2.1A[1]	80.3 ± 2.6B	75.6 ± 2.5C
Indispensable amino acids			
Arginine	72.2	93.8	89.3
Histidine	71.0	82.1	78.4
Isoleucine	67.0	85.7	78.3
Leucine	78.0	85.8	77.5
Lysine	50.8	75.6	73.8
Methionine	76.5	81.4	85.4
Phenylalanine	73.9	89.4	79.5
Threonine	50.2	75.4	73.0
Tryptophan	43.6	78.1	76.8
Valine	66.3	77.4	76.3
Dispensable amino acids			
Alanine	74.1	79.5	73.8
Aspartic acid	62.6	82.9	76.8
Cysteine	61.4	68.7	70.9
Glutamic acid	76.7	86.7	84.7
Glycine	38.3	66.6	68.5
Proline	71.4	82.8	78.8
Serine	65.9	80.3	75.4
Tyrosine	72.5	88.7	76.2

[1]Means ± Standard Deviation; A, B, C Means followed by a different letter in the same row differ significantly ($P < 0.05$).

Table 4. The apparent precaecal digestibility (% units) of crude protein (N x 6.25) and indispensable amino acids in maize, groundnut meal and sunflower meal; data of present experiment and from literature

	Maize			Groundnut meal solv. extr.			Sunflower meal solv. extr.		
Item[a]…	1	2, 3		1	4, 5		1	4, 6,7	
n ..	1	2	SD[b]	1	4	SD	1	5	SD
Crude protein	65	71	2	80	76	6	76	73	1
Arginine	72	79	3	94	91	3	89	89	1
Histidine	71	79	2	82	76	7	78	78	3
Isoleucine	67	75	1	86	79	7	78	77	2
Leucine	78	85	<0.5	86	80	6	78	76	1
Lysine	51	57	<1	76	70	8	74	74	3
Methionine	76	82	<1	81	84	nd	85	85	2
Phenylalanine	74	80	<1	89	86	4	80	78	5
Threonine	50	61	1	75	65	10	73	69	2
Tryptophan	44	48	nd[c]	78	71	5	77	76	1
Valine	66	75	<0.5	77	78	5	76	74	4

[a]1 = Present study; 2 = Green *et al*. (1987); 3 = van Leeuwen *et al.* (1987); 4 = Knabe *et al*. (1989); 5 = Green *et al.* (1988); 6 = Green and Kiener (1989); 7 = Jörgensen *et al.* (1984).[b]SD, = standard deviation. [c]nd = not determined.

An intra-lab validation comparing the PVTC-, simple T-, and re-entrant cannulation technique was conducted by Den Hartog et al. (1988). Three diets with a different crude fibre content (3.6, 6.5 and 10.1 %) were involved. There was a good accordance between the results of the three methods in case of the 3.6 % and 6.5 % crude fibre diets. The protein digestibility of the crude fibre rich diet was, determined with the re-entrant cannula lower, than the protein digestibility determined with the simple T- and PVTC cannula.

Other aspects have been validated by Köhler *et al.* (1990). In a comparative study, the PVTC cannulation and the ileo-rectal anastomose techniques were used to measure the digestibility of various diets ranging from semi synthetic crude fibre free to crude fibre rich diets. CP digestibility determined with the PVTC cannulated animals was higher than determined with ileo-rectal anastomized animals. Furthermore they concluded that PVTC cannulated animals

were in a more physiological state than the ileorectal anastomized animals.

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REFERENCES

Darcy, B., Laplace, J.P., 1980: Ann. Zootech. 29, 137-145.

Darcy, B., Laplace, J.P., Villiers, P. A., 1980: Ann. Zootech. 29, 147-177

Den Hartog, L. A., van Leeuwen, P., Huisman,J., Zandstra, T., van Heugten, E., van Ommeren, E.J., van Kleef, D.J., 1988: Proc. IVth International Seminar on Digestive Physiology in the Pig, Jablonna, Poland.

Dierick, N.A., Vervaeke, I.J., Decuypere, J.A., van der Heyde, H., Henderickx, H.K., 1987: Proc. Vth Int. Symp. Protein Metabolism and Nutrition, Rostock, DDR.

Easter, R. A., Tanksley Jr., T.D., 1973: J. Anim. Sci. 36, 1099-1103.

Gargallo, J., Zimmerman, D. R., 1981: J. Anim. Sci. 53, 395-402.

Green, S., Bertrand, S.L., Duron, M.J.C., Maillard, R.A., 1987: J. Sci. Food Agric. 41, 29-43.

Green, S., Bertrand, S.L., Duron, M.J.C., Maillard, R.A., 1988: J. Sci. Food Agric. 42, 119-128.

Green, S., Kiener, T., 1989: Anim. Prod. *48,* 157-179.

Jørgensen, H., Sauer, W.C., Thacker, P.A., 1984: J. Anim. Sci. 58, 926-934.

Knabe, D.A., LaRue, D.C., Gregg, E.J., Martinez, G.M., Tanksley Jr.., T. D., 1989: J. Anim. Sci. 67, 441-458.

Köhler, T., Mosenthin, R., Verstegen, M.W.A., den Hartog, L.A., Huisman, J., Aherns, F., 1990: J. Anim. Physiol. a. Anim. Nutr. 64, 33-34.

Laplace, J.P., Darcy-Vrillon, B., Picard, M., 1985: Journees Rech. Porcine en France 17, 353-370.

Moore, S., 1963: J. Biol. Chem. *238,* 235-237.

Van Leeuwen, P., Huisman, J., Verstegen, M.W.A., van Baak, M.J., van Kleef, D.J., van Weerden, E.J., den Hartog, L.A., 1988: Proc. IVth International Seminar on Digestive Physiology

in the Pig, Jablonna, Poland.

Van Leeuwen, P., Sauer, W.C., Huisman, J., van Weerden, E.J., van Kleef, D.J., den Hartog, L.A., 1987: J. Anim. Physiol. a. Anim. Nutr. 58, 122-133.

Sauer, W.C., Ozimek, L., 1986: Livest. Prod. Sci. 15, 367-388.

Slump, P., 1969: Thesis. Free University of Amsterdam.

Slump, P., Schreuder, H.A.W., 1969: Analyt. Biochem. 27, 182-186.

Souffrant, W.B., Schumann, B., Matkowitz, R., Gebhardt, G., 1985: Arch. Tierernähr. 35, 781-784.

Wüsche, J., Borgmann, E., Hennig, U., Kreienbring, P., Bock, H.-D., 1984: Arch. Tierernahr. 34, 817-831.

Zebrowska, T., 1973: Roczn. Nauk. Roln. 95B, 85-90.

Chapter 3

Apparent ileal dry matter and crude protein digestibility of rations fed to pigs and determined with the use of chromic oxide (Cr_2O_3) and hydrochloric acid (HCl)-insoluble ash as digestive markers

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ABSTRACT

Two experiments were conducted to determine apparent ileal dry matter (DM) and crude protein (CP) digestibilities in rations fed to pigs. An evaluation was made of chromic oxide (Cr_2O_3) and hydrochloric acid (HCl)-insoluble ash as digestive markers. In addition, the effects of body weight (BW) on apparent ileal DM and CP (N x 6.25) digestibilities were studied.

In experiment 1, thirteen barrows averaging 35 kg BW were fitted with post valve T-caecum (PVTC) cannulas to determine the apparent ileal DM and CP digestibility of a wheat gluten-bran ration (B2) and a soybean-meal ration (E1). Immediately after morning feeding ileal digesta samples were collected on an hourly basis for a total of 12 h. Subsequently, N and marker contents were determined in the samples. The postprandial patterns of N and Cr were more similar than those of N and HCl-insoluble ash. Therefore Cr_2O_3 seems more suitable as marker than HCl-insoluble ash. The apparent ileal CP digestibility coefficient of ration B2 derived using Cr_2O_3 as a marker, was significantly ($P < 0.05$) higher by 0.018 compared with the value obtained using HCl-insoluble ash. The corresponding values for ration E1 obtained using Cr_2O_3 HCl-insoluble ash were both 0.825.

In experiment 2, apparent ileal DM and CP digestibilities were determined in eighteen rations using twelve barrows also fitted with PVTC cannulas (BW from 40 to 100 kg). The protein sources for these rations were from different groups of feedstuffs. In four and three of the rations apparent ileal DM and CP digestibilities respectively were significantly different ($P < 0.05$) when assessed using the two markers. The digestibility coefficients were not systematically higher or lower for either marker and the absolute differences were < 0.049. Small but significant effects ($P < 0.05$) of live weight on apparent ileal CP digestibilities were found.

INTRODUCTION

Several studies have reported comparisons of apparent faecal digestibilities, determined using the digestibility markers Cr_2O_3 and HCl-insoluble ash (Moughan *et al*., 1991; Bakker and Jongbloed, 1994). However, problems determining digestibilities using Cr_2O_3, because of interference from other minerals in the rations have been reported (Saha and Gilbreath, 1991). Moreover, mineral concentrations are much higher in undigested materials and, therefore, the authers proposed that analytical recovery factors should be considered. McCarthy *et al.* (1974) proposed HCl-insoluble ash as an alternative marker to Cr_2O_3. However, comprehensive information on the ileal digestibility of

DM and CP for many feedstuffs have not been reported in literature.

The objectives of this study were: (a) to determine postprandial changes in the contents of N, Cr and HCl-insoluble ash when feeding two rations different in crude fibre content (Experiment 1) and (b) to determine ileal digestibilities of DM and CP in eighteen different protein-source rations using Cr_2O_3 and HCl-insoluble ash as digestive markers (Experiment 2).

MATERIALS AND METHODS

Experimental protocol

Crossbred barrows ((Dutch Landrace x Yorkshire) x Finnish Landrace) were individually housed in smooth-walled metabolism cages (80 x 180 cm) with a plastisol surface (Tenderfoot®, 4530 Ibberbüren, Am Ring 1, West-Germany) without bedding. The animals could move freely in the cages. The cages were placed in an environmentally controlled barn with air temperature of 19-21°C. From 07.00 to 21.00 hours the experimental room was illuminated, during the night the lights were dimmed. Animals were surgically fitted with post valve T-caecum (PVTC) cannulas according to Leeuwen *et al.* (1991). The cannulas, of 25 mm internal diameter, were constructed from silicon rubber. Following the surgery the pigs were returned to the metabolism cages and allowed a recovery period of 10 days. Rations were fed at a level of 2.6 times the requirement of metabolizable energy for maintenance (420 kJ/ body weight $(BW)^{0.75}$). The pigs were given equal amounts of feed at 08.00 and 16.00 hours during the adaptation period and at 08.00 and 20.00 hours from 2 days before and during the collection periods. Water was mixed with the feed (2.5 : 1) just before feeding.

The following two experiments were approved by the TNO Committee for Animal Welfare.

Experiment 1.

Thirteen animals, with a mean BW of 35 kg, were divided into two groups (1 and 2). Seven animals of group 1 received ration B2 (Table 1), with wheat gluten and wheat bran as a protein source. The six animals of group 2 received ration E1, with soybean meal as a protein source. After 10 days of adaptation to the rations, digesta were collected on three successive days over a period from 08.00 to 20.00 hours for the determination of apparent ileal CP digestibility. Digesta samples were collected hourly and immediately frozen (-20°C). After the experiment the collected digesta over the three days were thawed, pooled on basis of animal, frozen again and freeze dried. On the fourth day digesta was collected hourly to study N and marker passage. Hourly samples were pooled per ration, immediately

frozen (-20°C) and freeze dried.

Experiment 2.

Twelve crossbred barrows were used to determine ileal digestibility of eighteen experimental rations (Table 1) in a split-plot design (Table 2). Digestibility determinations of these rations were conducted at three different body-weight ranges; 40 to 52, 57 to 70, and 87 to 100 kg. Each body-weight domain consisted of three separate test periods of one week duration each. Between the body-weight domains the adaptation period was at least 11 days. Within the three weeks three rations with feedstuffs from the same product group were given to the individual animals. The adaptation period within the domain was 4.5 days (9 feedings). After adaptation, on three successive days digesta were collected over a period from 08.00 to 20.00 hours for the determination of apparent ileal DM and CP digestibility. Digesta were collected hourly and immediately frozen (-20°C). After the experiment, the digesta of the three day collections were thawed, pooled per animal and freeze dried.

Rations.

Feedstuffs (Table 3) were divided into six product groups:

A, cereals; wheat, barley, maize.

B, by-products of cereals; wheat-gluten, wheat bran, maize-gluten feed.

C, legume seeds, group I; peas, faba beans *(Vicia faba)* with low tannin content, faba beans with high tannin content.

D, legume seeds, group II; lupins, toasted full-fat soybeans, toasted Phaseolus beans.

E, expellers; soybean meal, rapeseed meal, sunflower-seed meal.

F, products from animal origin; fish meal, casein, meat-and-bone meal.

Eighteen rations (Table 1) were formulated using the eighteen feedstuffs. In rations with a feedstuff containing a low protein percentage, additional wheat-gluten meal was included to a level of at least 14.6 CP g/kg. The feedstuffs (except wheat-gluten meal and casein which were manufactured as a powder) were ground through a 2.5 mm mesh screen in a hammer mill. Lupins were ground with a Urchul cutting mill to fineness similar to the other milled feedstuffs. As digestive markers both Cr_2O_3 (2.5 g/kg) (Merck, Darmstadt, Germany; cat. No. 1.02483) and HCl-insoluble ash (10 g/kg) (Diamol, purified diatomaceous shell; Biakon N.V., Parklaan 18, B2280 Grobbendonk, Belgium) were included in the rations.

Analytical procedures

Before chemical analysis, feedstuffs, rations and freeze-dried digesta were ground through a 1 mm screen using a Retsch AM 1 grinder. N was analysed by the Kjeldahl method in a semi-automatic Kjellfoss apparatus (Foss Electronic, Hilleerod, Denmark). DM contents were determined after drying at 80°C overnight.

Crude fibre was analysed according to NEN standard 5417 (Netherlands Normalization Institute, 1988). Briefly, samples were boiled 30 minutes in 0.13 M-H_2SO_4 and 30 min in 1.5 M-NaOH. After filtration, the samples were ashed and dried.

Crude fat was analyzed by treating the samples for 1 h with 3 N-HCl and drying for 3 h under vacuum at 100°C, followed by 9 h extraction with petroleum ether (European Commission, 1984).

Cr_2O_3 in the rations and digesta were analysed colorimetrically after destruction of the sample by ashing at 525 °C for 4 h followed by oxidation with Na_2O under strong heating with a gas flame. The ash was solubilized in water and the Cr concentration was measured at 372 nm as chromate.

HCl-insoluble ash was determined gravimetrically. Each ration and digesta sample was hydrolysed with 3 M-HCl at 100°C for 30 min. Subsequently samples were filtered through an ash-free filter and washed with boiling water until free of acid. Residues were ashed at 550°C.

Procedures used for the determination of the antinutritional factors in the feedstuffs were, for trypsin inhibitor activity (TIA), Van Oort *et al*. (1989); for condensed tannins (expressed as catechin equivalents), Kuhla and Ebmeier (1981); for alkaloids, European Commission (1971); for glucosinolates, European Commission (1990). Biogenic amines were analysed with an amino acid analyser (Biotronic LC6001, Biotronik, Hamburg, Germany) using ion-exchange column BTC2710 and u.v. detection.

Table 1. Composition and analysed contents of the maize-starch based diets with cereals, by-products of cereals, legume seeds, expellers and products of animal origin (g/kg as fed) (continued on next page).

Diet	A1	A2	A3	B1	B2	B3	C1	C2	C3
Substituted feedstuff	Wheat	Barley	Maize	Wheat-gluten meal	Wheat bran	Maize-gluten feed	Peas	Faba beans[1] (LT)	Faba beans[1] (HT)
Substituted feedstuff	848.0	840.0	828.2	179.0	300.0	600.0	750.0	510.0	582.0
Wheat-gluten meal	76.5	70.0	94.0	-	123.5	44.0	-	-	-
Maize starch	-	-	-	519.0	339.7	104.3	80.9	267.8	195.3
Glucose	-	-	-	150.0	150.0	150.0	100.0	150.0	150.0
Soyabean meal	5.0	20.0	-	15.0	10.0	40.0	10.0	10.0	10.0
Cellulose	-	-	-	50.0	-	-	-	-	-
$CaCO_3$	9.3	9.3	9.0	8.0	9.5	10.0	10.0	9.0	9.3
$CaHPO_4$. $2H_2O$	17.5	17.5	20.0	22.5	17.5	50.0	16.0	18.5	18.5
NaCl	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
MgO	-	-	-	2.0	-	-	0.5	-	-
$KHCO_3$	9.0	9.0	13.0	18.0	12.0	3.5	-	2.0	2.0
$NaHCO_3$	3.0	3.0	3.0	4.0	3.0	1.0	3.0	3.0	3.0
L-Lysine.HCl	4.2	3.7	5.0	5.0	6.0	4.3	-	-	-
L-Threonine	-	-	-	-	1.0	-	-	-	-
L-Tryptophan	-	-	0.3	-	0.3	0.4	0.4	0.2	0.2
DL-Methionine	-	-	-	-	-	-	1.7	2.0	2.5
Vitamin-trace element mixture*	22.5	22.5	22.5	22.5	22.5	22.5	22.5	22.5	22.5
CP (Nx 6.25)	165	158	166	159	158	152	167	171	169
NE[2] (MJ/kg)	9.20	8.87	9.48	9.83	9.04	8.87	9.20	9.33	9.12
Cr_2O_3	2.3	2.4	2.4	2.3	2.5	2.5	2.4	2.4	2.4
HCl-insoluble ash	11.5	14.5	11.1	9.8	10.3	29.4	12.4	9.9	9.9

[1]Faba beans, (*Vicia faba* L.), LT, low tannin; HT, high tannin;[2]NE, net energy.

*Contributed the following (/kg diet): DL-alfa-tocopheryl acetate, 37.5 mg; riboflavin, 6 mg; niacin, 30 mg; D-pantothenic acid, 15 mg; choline chloride, 120 mg; cyanocobalamin, 0.045 mg; menadione, 3 mg; renitol, 2.7 mg; cholecalciferol, 45 mg; KI, 0.81 mg; $CoSO_4.7H_2O$, 7mg; $FeSO_4.7H_20$, 0.4g; $CuSO_4.5H_20$, 0.1g; MnO_2, 0.07g; $ZnSO_4.H_20$, 0.3g. This mixture was supplied with 2.5 g Cr_2O_3 and 10 g Diamol per kg as digestibility markers and 20 ppm Tylosine.

D1	D2	D3	E1	E2	E3	F1	F2	F3	Category
Lupins	Soya Beans	Phaseolus beans	Soya-bean meal	Rape-seed meal	Sun-flower meal	Fish meal	Casein	Meat-a.bone meal	Substituted feedstuff
540.0	435.0	350.0	330.0	510.0	470.0	232.5	180.0	270.0	Substituted feedstuff
-	-	90.0	-	-	-	-	-	-	Wheat-gluten meal
249.8	328.0	301.0	433.0	262.5	282.5	519.0	533.5	480.5	Maize starch
150.0	150.0	150.0	150.0	150.0	150.0	150.0	150.0	150.0	Glucose
-	-	15.0	10.0	30.0	40.0	10.0	10.0	10.0	Soyabean meal
-	30.0	30.0	20.0	-	-	50.0	50.0	50.0	Cellulose
8.0	8.5	9.0	8.5	3.0	8.0	-	12.0	-	$CaCO_3$
16.0	17.5	18.5	17.5	14.0	15.0	-	16.0	-	$CaHPO_4.\ 2H_2O$
5.0	5.0	5.0	5.0	5.0	5.0	2.0	5.0	2.0	NaCl
-	-	1.0	-	-	-	1.0	2.0	1.0	MgO
4.0	-	2.0	-	-	-	13.0	15.0	12.5	$KHCO_3$
3.0	3.5	4.0	3.5	3.0	4.0	-	4.0	-	$NaHCO_3$
-	-	2.0	-	-	3.0	-	-	-	L-Lysine.HCl
-	-	-	-	-	-	-	-	-	L-Threonine
0.4	-	-	-	-	-	-	-	0.2	L-Tryptophan
1.3	-	-	-	-	-	-	-	1.3	DL-Methionine
22.5	22.5	22.5	22.5	22.5	22.5	22.5	22.5	22.5	Vitamin-trace element mixture*
161	148	164	171	166	149	162	160	146	CP (N x 6.25)
9.37	9.46	9.58	9.71	9.04	9.08	9.92	9.66	10.13	NE (MJ/kg)
2.4	2.5	2.8	2.6	2.6	2.5	2.5	2.2	2.5	Cr_2O_3
10.1	15.5	17.4	10.5	12.8	11.3	10.4	10.4	15.6	HCl-insoluble ash

Calculations

Apparent digestibilities of CP were corrected for N from synthetic amino acids included assuming 100 % digestibility. The digestibilities of DM and CP were calculated based on Cr_2O_3 and HCl-insoluble ash.

The formula used for the calculation of ileal digestibilities was:

$$DC = 1 - \frac{\text{N digesta (g/kg)}}{\text{M digesta (g/kg)}} \times \frac{\text{M feed (g/kg)}}{\text{N feed (g/kg)}},$$

Where DC is the digestibility coefficient of the nutrient; N feed (g/kg) is the content of the nutrient in feed (g/kg); N digesta (g/kg) is the content of the nutrient in digesta (g/kg); M feed (g/kg) is the content of the marker in feed (g/kg); M digesta (g/kg) is the content of the marker in digesta (g/kg).

Statistical analysis

In experiment 1, differences between digestibility values derived from the two markers were analysed using the paired sampling Student's *t*-test using Statistical Packages for the Social Sciences software (1992).

Table 2. Experimental design: split-plot design with twelve animals (1…12), three body-weight domains (P, Q, R), six product groups of rations (A…F) and three feedstuffs each product group ($A_{1,2,}$…$F_{1,2,3}$).

	Animals					
Body weight domains	1,4	2,5	3,6	7,10	8,11	9,12
P	$A_{1,2,3}$	$B_{1,2,3}$	$C_{1,2,3}$	$D_{1,2,3}$	$E_{1,2,3}$	$F_{1,2,3}$
Q	$B_{1,2,3}$	$C_{1,2,3}$	$A_{1,2,3}$	$E_{1,2,3}$	$F_{1,2,3}$	$D_{1,2,3}$
R	$C_{1,2,3}$	$A_{1,2,3}$	$B_{1,2,3}$	$F_{1,2,3}$	$D_{1,2,3}$	$E_{1,2,3}$

Experiment 2 was carried out according to a split-plot design with two blocking factors. The blocking factors were animal (twelve animals) and body weights (three domains). The whole plots are product groups (A,….,F) of rations with similar protein sources. Subplots were developed by variation of the feedstuffs within product groups (groups; A_1, A_2,…,F_2,F_3). The layout of the design is given in Table 2.

The model for data analysis was:

$y_{ijk} = \mu + BW_i + animal_j + \theta_{ij} + a\_ration_k + e_{ij(k)}$,

where: y_{ijk} is the analysed variable, μ is the overall mean, BW_i is the body-weight domain (i = 1.…3), $animal_j$ is the animal (j= 1….12), a_ration_k is the ration (A_1, A_2, A_3 …. F_2, F_3; k = 1.…18), θ_{ij} is the main plot error and $e_{ij(k)}$ is the subplot error. An analysis of variance was performed with the computer program GENSTAT 5, (Payne, 1994). GENSTAT instructions were: block (animal x BW/ subplot; treatment a_ration // group). The variables analysed were ileal digestibility of DM and CP using Cr_2O_3 and HCl-insoluble ash as digestive markers, respectively.

Digestibilities of three rations with feedstuffs of the same origin were determined in the same group of six animals. Using the same animals for the similar feedstuffs increased comparability within the groups. However, due to the layout of the design the groups and feedstuffs are partially confounded with animals. The degrees of freedom of the least significance difference (LSD) value for comparing feedstuffs of different product groups is calculated according to Satterthwaite's formula.

Correlation between recovery of the digestive markers and digestibility was calculated according to the Spearman rank correlation analysis also with the computer program GENSTAT 5. In addition, statistical analysis of DM and CP digestibility were performed with marker recovery as covariate. GENSTAT instructions were: treatment a_ration; covariate recovery.

Feed refusals of individual animals, due to palatability, occurred when feeding the maize-gluten-feed ration (B3, two animals), feeding the ration with the high-tannin faba-bean variety (C3, one animal) and feeding the lupin ration (D1, one animal). This resulted in four missing DM and CP digestibility values of the data set.

Table 3. Dry matter (DM), crude protein (CP; N x 6.25) and crude fibre (CFi) contents (g/kg as fed) of feedstuffs from six categories (A-F).

Feedstuff*		DM	CP	Cfi
A1	Wheat	873	110	24
A2	Barley	888	111	58
A3	Maize	885	95	26
B1	Wheat-gluten meal	921	857	ND
B2	Wheat bran	909	154	112
B3	Maize-gluten feed	889	177	74
C1	Peas	901	216	63
C2	Faba beans (*Vicia faba*) (LT)	893	333	84
C3	Faba beans (HT)	890	287	74
D1	Lupins	921	296	149
D2	Toasted full-fat soyabeans	917	339	67
D3	Toasted Phaseolus beans	905	240	54
E1	Soybean meal	902	519	44
E2	Rapeseed meal	913	317	116
E3	Sunflower meal	912	295	242
F1	Fish meal	924	687	ND
F2	Casein	912	889	ND
F3	Meat-and-bone meal	924	522	ND

ND = not determined; LT = low tannin; HT = high tannin.

* Contents of antinutritional factors: C1, 2.6 mg trypsin-inhibitor activity (TIA)/g; C2, < 5 mg tannins/g expressed as catechin equivalents, 1.9 mg TIA/g; C3, 5.5 mg tannins/g expressed as catechin equivalents, 1.9 mg TIA/g; D1, 4 mg alcaloids/g; D2, 1.5 mg TIA/g; D3, < 0.1 mg TIA/g; E1, 3.5 mg TIA/g; E2, 4 μmol glucosinolate/g; F1, 1.4 mg biogenic amines/g.

RESULTS

Contents of DM, CP, crude fibre and antinutritional factors in the feedstuffs are given in Table 3. Crude fat content in toasted full-fat soybeans, fish meal and meat-and-bone meal were 118, 60 and 92 g/kg, respectively. Content of CP in the rations ranged from 146 to 162 g/kg (Table 2). The analysed contents of the markers varied for Cr_2O_3 from 2.2 to 2.8 g/kg and HCl-insoluble ash from 9.9 to 29.4 g/kg.

Experiment 1. Postprandial change in content of nitrgen and markers and digestibility coefficients derived from Cr_2O_3 and HCl-insoluble ash

The N contents in freeze-dried digesta were, over the 12 h of collection, for both rations rather constant (Figure 1). Also, feeding ration E1 the content of Cr was rather constant (Figure 2). For ration B2, however, the content of Cr increased after the third hour of the collection period and decreased after the sixth hour of the collection period. The pattern of content of HCl-insoluble ash in the digesta varied for both rations more than for Cr.

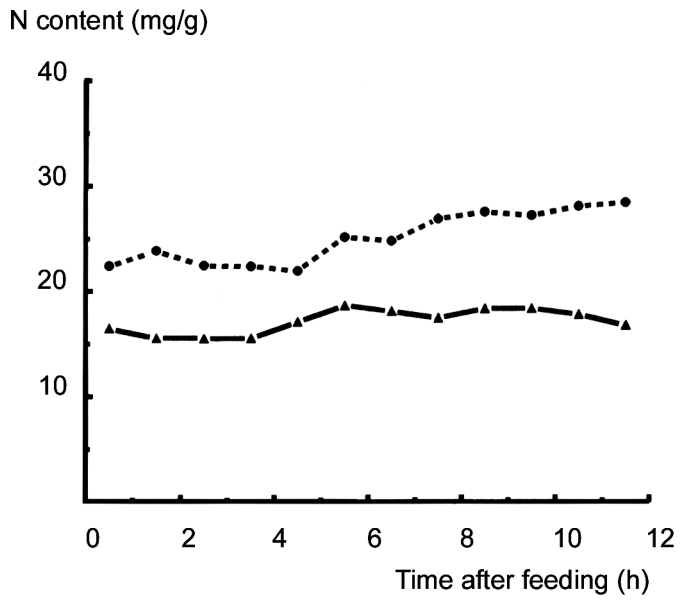

Figure 1. N content in hourly collected freeze dried digesta,
—▲— wheat bran/wheat gluten ration (B2),
- - ● - - - soybean meal ration (E1).

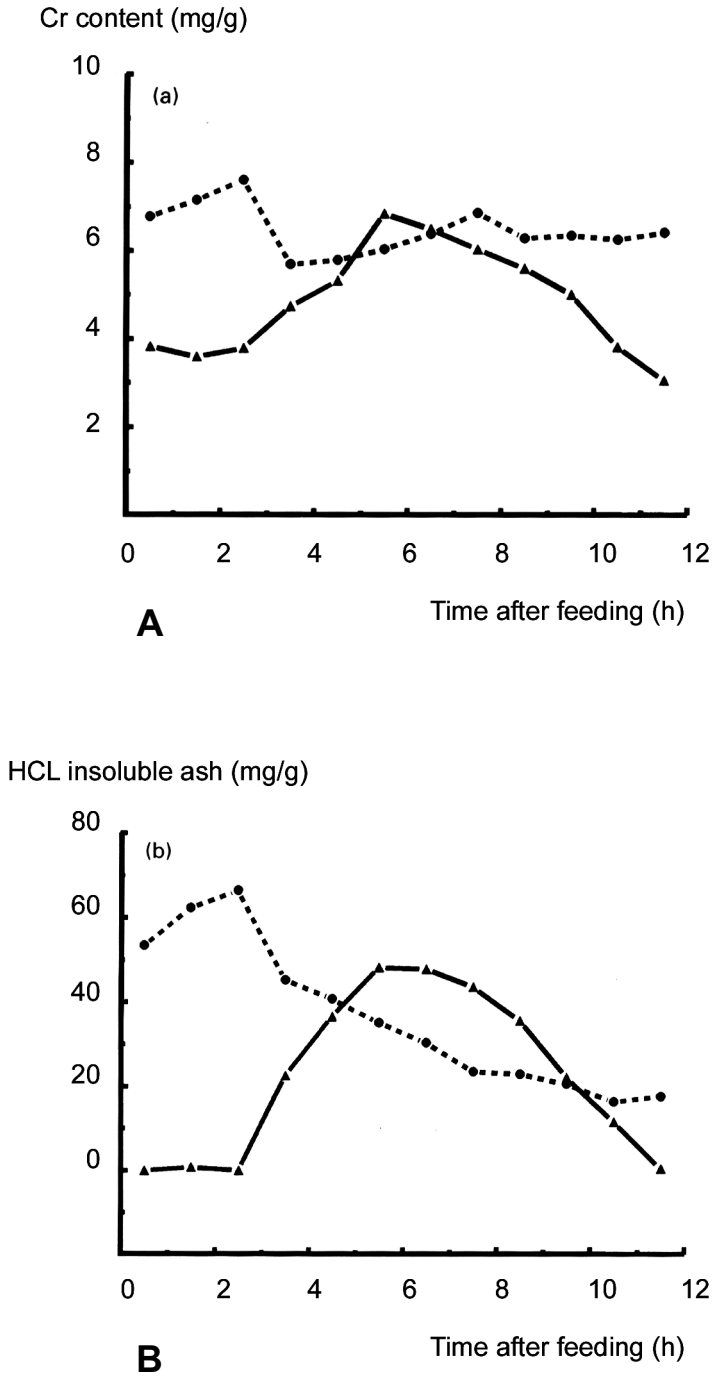

Figure 2. Content of Cr (A) and HCl-insoluble ash (B) in the hourly collected freeze dried digesta.,
—▲— wheat bran/wheat gluten ration (B2),
- - ● - - - soybean meal ration (E1).

The mean digestibility coefficients for CP, determined with Cr_2O_3 as a digestibility marker, were for ration B2 and E1, 0.834 and 0.825, respectively. Corresponding digestibility coefficients determined with HCl-insoluble ash as a marker were 0.816 and 0.825. The absolute difference in digestibility coefficients determined using Cr_2O_3 and HCl-insoluble ash were small (0.018 unit), but significantly different ($P < 0.05$) for ration B2.

Table 4. Mean apparent ileal digestibility of dry matter (DM) and crude protein (CP; N x 6.25) in pigs at different body weights (BW) determined with chromic oxide (Cr_2O_3) and hydrochloric acid (HCl)-insoluble ash as digestive markers.

	Cr_2O_3		Ash	
Mean BW (kg)	DM	CP	DM	CP
46	0.720	0.749	0.715	0.746
63 kg	0.718	0.749	0.720	0.752
94 kg	0.726	0.771	0.718	0.766
LSD (P = 0.05)	0.014	0.019	0.013	0.011

LSD = least significant difference.

Experiment 2. Evaluation of Cr_2O_3 and HCl-insoluble ash as marker in a digestibility experiment and the effect of body weight on digestibility.

CP digestibility coefficients increased significantly ($P < 0.05$) with BW (Table 4). This can be explained by the higher CP digestibility values in the highest body weight. Changes in DM digestibility were small. Between animals significant differences ($P < 0.01$) were found for both DM and CP digestibility.

In Table 5, the mean apparent ileal digestibilities of the eighteen individual rations are given with the LSD. The LSD of rations from different product groups were slightly higher than those within group of feedstuffs. For four out of the eighteen rations differences in DM digestibility between the two markers were significant ($P < 0.05$) and the CP digestibilities of three out of the eighteen rations were different ($P < 0.05$). The digestibility coefficients of DM values derived from Cr_2O_3 were significantly higher than those derived from HCl-insoluble ash for the DM of the wheat ration (A1), the maize-gluten-feed ration (B3), the lupin ration (D1) and the sunflower-seed-meal ration (E3). The CP digestibility coefficient of the maize-gluten feed ration (B3) was significantly ($P < 0.05$) higher and the CP digestibility coefficients of the soyabean-meal ration (E1) and fish-meal ration (F1) were

significantly lower when calculated with Cr_2O_3 as a marker rather than HCl-insoluble ash.

Table 5. Apparent ileal digestibility coefficients of dry matter (DM), crude protein (CP; N x 6.25) in pigs, for eighteen rations with feedstuffs from six categories (A-F) determined with chromic oxide (Cr_2O_3) and hydrochloric acid (HCl)-insoluble ash (Ash) as digestive markers.

		DM			CP		
Diet		Cr_2O_3	Ash	Difference[1]	Cr_2O_3	Ash	Difference[1]
A1	Wheat	0.797	0.776	*	0.871	0.859	Ns
A2	Barley	0.685	0.692	ns	0.788	0.792	Ns
A3	Maize	0.812	0.802	ns	0.838	0.830	Ns
B1	Wheat-gluten meal	0.854	0.863	ns	0.915	0.920	Ns
B2	Wheat bran	0.728	0.730	ns	0.826	0.827	Ns
B3	Maize-gluten feed	0.549	0.500	*	0.610	0.566	*
C1	Peas	0.715	0.713	ns	0.761	0.761	Ns
C2	Faba beans (LT[2])	0.701	0.698	ns	0.736	0.735	Ns
C3	Faba beans (HT)	0.688	0.684	ns	0.696	0.692	Ns
D1	Lupins	0.600	0.577	*	0.771	0.762	Ns
D2	Toasted full-fat soyabeans	0.694	0.699	ns	0.725	0.729	Ns
D3	Toasted Phaseolus beans	0.640	0.646	ns	0.655	0.659	Ns
E1	Soybean meal	0.802	0.817	ns	0.804	0.818	*
E2	Rapeseed meal	0.650	0.654	ns	0.582	0.585	Ns
E3	Sunflower meal	0.643	0.625	*	0.731	0.719	Ns
F1	Fish meal	0.821	0.833	ns	0.770	0.787	*
F2	Casein	0.869	0.867	ns	0.924	0.922	Ns
F3	Meat-and-bone meal	0.737	0.743	ns	0.612	0.619	Ns
LSD[3] within the same product group (P = 0.05)		0.023	0.025		0.030	0.032	
LSD from different product groups (P = 0.05)		0.025	0.025		0.033	0.036	

[1] Difference between digestibility values measured using Cr_2O_3 and HCl- insoluble ash were assessed using Student's paired *t* test is, * = significant ($P < 0.05$), ns = not significant ($P > 0.05$); [2] LT = low tannin, HT = high tannin; [3] LSD = least significant difference.

The recovery of the two markers collected in digesta ranged from 78 (C3) to 109 (F2) percentage

units of Cr_2O_3 dietary intake, and from 78 (C3) to 107 (F2) percentage units of the HCl-insoluble ash intake. The means of both markes recoveries of the rations were positively correlated with the means of the DM and CP digestibility of the rations ($P < 0.05$), however, the correlation coefficient (R^2) were < 0.29). Within diets no significant correlation between recoveries of the markers and digestibility were found.

DISCUSSION

Content of both markers varied in the diets (Table 1). For Cr_2O_3 the content was, with the exception of Phaseolus beans ration (D3), close to or lower than the intended dosage (2.5 g/kg). Analytical difficulties, such as interference with phosphorus (Saha and Gilbreath, 1991), possibly explain part of the variation of the Cr_2O_3 content in the rations. Variation of HCl-insoluble ash content of the rations can be explained by the differences in the HCl-insoluble ash content in the feedstuffs (Wünsche *et al*., 1984) and by the different amounts of minerals added to the rations.

Comparing digestibility coefficients derived from Cr_2O_3 versus total collections, Bakker and Jongbloed (1994) showed the validity of Cr_2O_3 as a digestive marker. However, the use of chromium, which is a heavy metal, is limited for routine experiments because of national and international environmental legislations (Besluit Gevaarlijke Afvalstoffen, 1993; European Commission, 1976). An alternative to Cr_2O_3 is HCl-insoluble ash. Bakker and Jongbloed (1994) concluded that HCl-insoluble ash is not suitable for the determination of faecal digestibility. However, in their experiment no extra HCl-insoluble ash was added to the rations making accurate qualitative analysis more critical (McCarthy *et al.,* 1974). On the other hand, Wünsche *et al*. (1984) using barley-soybean-meal rations found apparent ileal DM and CP digestibility values, assessed with HCl-insoluble ash from the feedstuffs, similar to those obtained by quantitative collection of digesta. Moughan *et al.* (1991) support the use of natural dietary HCl-insoluble ash as a marker and have suggested the addition of diatomaceous earth when the natural level of insoluble ash is low. Also, Jongbloed *et al.*(1991) have suggested, based on results of an experiment where the overall digestibility was measured, addition of milled diatomaceous shells to decrease the variation of digestibility values. In the present experiment diatomaceous shells (Diamol) were added to all rations to guarantee that the HCl-insoluble ash level was high enough for accurate analysis.

A prerequisite for the use of a marker is that the nutrient : marker content ratio in the collected digesta is representative of the total digesta that passes the terminal ileum. However, the ratios in the

undigested material can change postprandially (Moore, 1957). Only quantitative collection, semi-quantitative collection or frequent collection provides representative samples. The results of the first experiment showed rather constant N content in the freeze-dried digesta during collection. Variation in Cr_2O_3 content in the digesta during collection, however, differed between the rations. Cr_2O_3 content was rather constant after feeding the soybean ration (calculated crude fibre content: 14 g/kg) but varied after feeding the wheat bran-gluten (calculated crude fibre content: 34 g/kg). However, variation of HCl-insoluble ash contens were found when feeding both the soybean and wheat gluten-bran rations. The observed variation implies differences in the nutrient : marker content ratio in digesta during collection and means that the method used to collect digesta is critical for accurate calculation of digestibilities. In the present experiments, digesta was collected semi-quantitatively. In experiment 2, apparent DM digestibilities derived from the two markers were similar for fourteen out of the eighteen rations. For four rations the difference in DM digestibility between the two markers was significant ($P < 0.05$). On these occasions the values derived from Cr_2O_3 were higher than those derived from HCl-insoluble ash. The CP digestibility of three out of the eighteen rations were different ($P < 0.05$); one values for Cr_2O_3 was higher compared to HCl-insoluble ash and two were lower. The absolute differences in appartent DM and CP digestibility were, with the exception of the relatively poorly digestible ration B3, < 0.023. Further, results showed that digestibilities derived from Cr_2O_3 were not systematically higher or lower than those derived from HCl-insoluble ash and that the LSD were similar. However, validity of markers in general, can be improved when variation in the nutrient and marker contents in the undigested material are reduced. This could possibly be achieved by feeding the animals more frequently. In the present study, animals were fed every 12 h and digesta was collected, also, over 12 h. The recovered amount of marker should be 100 %, or less when some digesta passed the collection cannula. However, feeding the casein ration (F2) the collected amount of the markers was over 100 % (for Cr_2O_3,109 % and for HCl-insoluble ash,107 %). The recovery of the markers were higher in rations with a high CP digestibility which have, in general, a low crude fibre content. In high-fiber rations relatively more digesta flows through to the colon thereby passing the collection cannula. The differences in recovery were also observed within rations and between animals. Within rations, no correlations were found between recovery of the marker and DM or CP digestibility. However, the observations indicate an increased passage rate during digesta collection. The explanation for this phenomenon may be the effect of a change of the abdominal pressure after opening the cannula and a difference in activity of the animals during the night (without collection) and during the day (collection period). Furthermore, during digesta collection, no colo-ileal reflux is

possible. Malbert *et al.* (1994) concluded that this reflux alters the gastro-duodenal motility. The observation that no correlations were found within rations between recovery and digestibility suggests that the possible effect on motility does not alter digestibility. However, frequent feeding and shortening of the collection period would be more in accordance with the physiology of the animal because the period of the interruption of the colo-ileal reflux is shorter. Moreover, frequent feeding may be more comparable to conventional pig feed management systems in Europe.

The apparent ileal CP digestibility values of the rations with a single feedstuff as protein source in the present study are compared with data from the literature (van Leeuwen *et al.*, 1993). The apparent ileal CP digestibility values from present experiment (exp.) are in good agreement with values from literature (lit.) for, peas (0.76 (exp.) *v.* 0.74 (lit.)), faba beans (0.71 (exp.) *v.* 0.73 (lit.)), soybean meal (0.81 (exp.) *v.* 0.79 (lit.)), sunflower-seed meal (0.72 (exp.) *v.* 0.74 (lit.)), fish meal (0.78 (exp.) *v.* 0.78 (lit.)) and casein (0.92 (exp.) *v.* 0.91 (lit.)). The CP digestibility values of three feedstuffs of present experiment were lower compared to literature values (lupins, 0.77 (exp.) *v.* and 0.82 (lit.), rapeseed meal, 0.58 (exp.) *v.* 0.69 (lit.), meat-and-bone meal, 62 (exp.) *v.* 0.70 (lit.)). The latter observations illustrate the possible differences between the digestibility value of different individual batches of the same type feedstuff. Also, apparent digestibility DM and CP was significantly ($P < 0.05$) different between individual animals. The variation between individual crossbred animals may alter factors such as, enzyme activity of the intestinal mucosa (van Leeuwen *et al.*, 1995) and possibly contribute to the differences between digestibility values determined in the present experiment and the literature values. A slight increase in the apparent digestibility coefficient CP (0.021) was observed over the BW range of 46-94 kg. For DM, no BW effect was found. These observations indicate that the digestion capacity in this BW range changes to a minor extent.

In summary, apparent ileal digestibility coefficients of DM and CP when using Cr_2O_3 and HCl-insoluble ash as digestive markers were similar when 10 g/kg diatomaceous shells (Diamol) was added to the rations and digesta was collected semi-quantitatively. Shortening of the collection periods in combination with frequent feeding might improve the measurements for ileal digestibility experiments and needs further investigation.

REFERENCES

Besluit Aanwijzing Gevaarlijke Afvalstoffen. (1993). Staatsblad, 617, 25/11/1993. Staatsdrukkerij, PO Box 20014, 2500 EA Den Haag, The Netherlands.

Bakker, G.C.M. and Jongbloed, A.W. (1994). The effect of housing system on apparent digestibility in pigs using classical and marker (Chromic oxide, acid HCl-insoluble ash) techniques, in relation to dietary composition. *Journal of Science of Food and Agriculture* 64, 107-115.

European Commission (1971). *Determination of Alkaloid Content in Lupin Seed*, p. L155/35. Luxembourg: Publication Office of European Communities.

European Commission (1976). *Environment, Nuclear Safety and Civil Protection. Commission of the European Communities* no. DC XI, p. L129/23. Brussels, European Commission.

European Commission (1984). *Determination of Crude Fat Content*, p. L15/29. Luxembourg: Publication Office of European Communities.

European Commission (1990) *Determination of Glucosinolates,* p. L170/28. Luxembourg: Publication Office of European Communities

Jongbloed, A.W., Bakker, J.G.M., Goedhart, P.W. and Krol-Kramer, F. (1991). Evaluation of chromic oxide and of HCl-insoluble ash as markers for measuring overall apparent digestibility of some dietary nutrients for pigs. In: *Proceedings of the Vth International Symposium on Digestive physiology in pigs,* pp. 325-329 [M.W.A. Verstegen, J. Huisman, L.A. den Hartog, editors]. Wageningen: PUDOC, Wageningen, The Netherlands.

Kuhla, S. and Ebmeier, C. (1981). Untersuchungen zum Tanningehalt in Ackerbohnen. *Archives of Animal Nutrition* 31, 573-588.

Malbert, C.H., Montfort, I., Mathis, C., Guérin, S. and Laplace, J.P. (1994). Remote effects of ileo-colic SCFA's levels on gastric motility and emptying In: *Proceedings of the VIth International Symposium on Digestive Physiology in pigs. EAAP Publication* no. 80, pp 283-286 [W.B. Souffrant and H. Hagemeister, editors]. Bad Doberan, Germany.

McCarthy, J.F. Aherne, F.X. and Okai, D.B. (1974). Use of HCl-insoluble ash as an index for deterning apparent digestibility with pigs. *Canadian Journal of Animal Science* 54: 107-109.

Moore, J.H. (1957). Diurnal variations in the composition of the faeces of pigs on diets containing chromium oxide. *The British Journal of Nutrition* 11, 273-288.

Moughan, P.J., Smith, W.C., Schrama, J. and Smits, C. (1991). Chromic oxide and insoluble ash as faecal markers in digestibility studies with young pigs. *New-Zealand Journal of Agricultural Research* 34, 85-88.

Netherlands Normalization Institute (1988). *Standards of the Netherlands Normalization Institute* no. 5417. Determination of Crude Fibre Contents in Feedstuffs. Delft: Netherlands Normalization Institute.

Payne, R.W. (1994). *Users manual* GENSTAT 5, Oxfort: Clarendon Press.

Saha, D.C. and Gilbreath, R.L. (1991). Analytical recovery of chromium from diet and faeces determined by colorimetry and atomic absorption spectrophotometry. *Journal of Science of Food and Agriculture* 55, 433-446.

Statistical Package for Social Sciences (1992). *System Users' Guide. SPSS/PC+ Version 5.0 Base* Chicago. IL: Marija J. Norusis.

Van Leeuwen, P., van Kleef, D.J., van Kempen, G.J.M., Huisman, J. and Verstegen, M.W.A. (1991). The post valve T-caecum cannulation technique in pigs applicated to determine the digestibility of amino acid in maize, groundnut and sunflower meal. *Journal of Animal Physiology and Animal Nutrition* 65, 183-193.

Van Leeuwen, P., Jansman, A.J.M., van Kempen, G.J.M., Verstegen, M.W.A. and Huisman, J. (1993). Mathematical analysis of apparent ileal digestibility of amino acids in feedstuffs for pigs. *Livestock Production Science* 36, 255-272.

Van Leeuwen, P., Jansman A.J.M. and Wiebenga, J. (1995). Dietary effects of faba-bean (*Vicia faba* L.) tannins on the morphology and function of the small intestinal mucosa of weaned pigs. *British Journal of Nutrition* 73, 31-39.

Van Oort, M.G., Hamer, R.J. and Slager, E.A. (1989). The trypsin inhibitor assay: improvement of an existing method. In: *Recent Advances of Research in Antinutritional Factors in Legume Seeds* pp. 110-113 [J. Huisman, A.F.B. van der Poel and I.E. Liener, editors]. Wageningen: PUDOC.

Wünsche, J., Borgmann, E., Hennig, U., Kreienbring, K. and Bock, H.-D. (1984). Anwendung der HCl-unlöslichen Asche als Indicator zur bestimmung der Nährstoff- eischließlich Aminosäurenverdaulichkeit am Ende des Dünndarms und des Gesamtverdauungstraktes beim Schwein (Application of the HCl-insoluble ash as an indicator for the determination of nutrient, including amino acid, digestibility at the end of the small intestine and the whole digestive tract of pigs). *Archives of Animal Nutrition* 12, 817-831.

Chapter 4

A procedure for ileostomy in adult roosters to determine apparent precaecal digestibility of protein and amino acids of diets: a comparison of six diets in roosters and growing pigs

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ABSTRACT

A procedure for ileostomy in adult roosters has been described with the use of flexible silicon cannulas. Apparent ileal digestibility coefficients for dry matter (aDC DM), crude protein (aDC CP) and amino acids (aDC AA) of six diets, formulated with maize/wheat gluten meal, wheat gluten meal, faba beans, lupins, soybean meal and casein as the main protein sources were determined in the ileostomized roosters fitted with silicon cannulas. In addition, aDC data determined using roosters (present study) were correlated with previously published aDC data of the same diets determined with pigs (van Leeuwen *et al.,* 1996a, 1996b).

The ileal aDC CP in roosters ranged from 0.81 to 0.92. Significant ($P < 0.05$) differences in aDC CP and aDC AA were observed between diets. Between aDC in roosters and in pigs linear relations were found. The linear models explained 85 % of the variation in ileal aDC CP between the six diets determined in roosters and pigs. For ileal aDC AA, the explained part of the variation between roosters and pigs, ranged from 62 to 90 %, depending on the particular amino acids, with the exception of aDC of Arg. The standard errors (SE) of the models for the prediction of the aDC AA in roosters from aDC AA of the pigs was <0.04 units.

INTRODUCTION

Karasawa and Meada (1994) investigated the nitrogen (N) metabolism of chickens with regard to the role of the caeca and the effects of the retroperistaltic movement of digesta from the cloaca to the caeca. The amino acids (AA) synthesized in the caeca by microbial activity can not be used in birds (Mortensen and Tindall, 1981). The undigested AA which reach the caeca can be deaminated by the microflora but the endproducts have no nutritional value (McNab, 1989). Moreover, Parsons (1986) observed a higher agreement between amino acid availability measured in chick growth assays, and digestibility determined in caecectomised rather than in intact birds. This means that, in poultry, digestion in the distal part of the intestines, more specifically the caeca, is mainly fermentative and that the AA synthesized or disappearing in the caeca are not available for protein synthesis in the animal. As in poultry, it has been shown that in pigs the amino acids from proteins digested in the large intestine do not contribute to protein synthesis in the animal (Zebrowska *et al*. 1978). The ratio of the proteins digested in the small and large intestine of pigs differs between ingredients. So, ileal digestibility estimates amino acid

availability in pigs better than faecal digestibility (Dierick *et al*., 1987). Several studies have been reported on the apparent ileal digestibility of amino acids of feedstuffs in pigs and data for pig diet formulation are commonly used (van Leeuwen *et al*., 1993; CVB, 1998; Degussa, 1993; Eurolysin, 1995; Rhône Poulenc, 1993).

Also in birds, the relative contribution of the distal part of the gastro-intestinal (GI) tract to protein and AA digestion of different feedstuffs is not constant (Raharjo and Farrell, 1984; Green and Kiener, 1989; Angkanaporn *et al.,* 1997) and technological treatments on feedstuffs may have effects on the proportion of protein digested in the different sections of the GI tract (Johns *et al.* 1986). This implies that for poultry, as in pigs, ileal digestibility values of protein and amino acids in feedstuffs may give a more accurate estimate of the amino acids potentially available for protein synthesis than faecal digestibility data (Raharjo and Farrell, 1984).

Apparent ileal digestibility values for poultry have been determined using caecectomised roosters (Green and Kiener, 1989). In caecectomised birds, digesta flows from the ileum into the rectum and cloaca where it is stored and diluted with the urine until excretion. It is usually assumed that the AA in the excreta originate only from the digesta. The amount of AA in urine has been generally assumed to be low and can be ignored (McNab, 1989). However, digesta in the cloaca is not sterile and microbiota may convert some urine N to micribial protein. Moreover, post-ileum changes in amino acid composition of the digesta may occur because of microbial activity in the cloaca of caecectomised birds.

An alternative method for the collection of ileal digesta uses surgically cannulated roosters at the terminal ileum (Fussel, 1969; Okumura, 1976; Raharjo and Farrell, 1984). In these ileostomized birds, the digesta can be directly collected from the cannulas without contamination with feathers or urine. The described procedures for intestinal cannulation use glass cannulas. More recently, flexible medical silicon tubing was demonstrated to be very effective for cannulations in pigs (van Leeuwen *et al*., 1988 and 1991).

Slaughter methods are frequently used to determine ileal digestibility of diets (Ravindran *et al*., 1999). With this method the small amounts of digesta are derived from small intestine over 40 mm proximal ileal-caecal junction, on a certain moment of the day. This method needs many animals (40-60 per treatment) to collect enough digesta for analysis and to have a representative sample of the digesta over a longer period. Also the way of sampling is critical, because, from the dead intestine easily mucosa can be scraped off.

Green and Kiener (1989) have studied the relation between ileal digestibility values determined in

precise-fed (force-fed) caecectomised roosters and the same diets determined in ileo-rectal anastomized pigs. Even though many differences exist in the GI tract and in the digestive processes between poultry and pigs (Moran Jr., 1982), they found similarities in ileal digestibility of CP and AA of diets between both species.

The objectives of the present study were, (a) to describe a surgical procedure for ileostomy in roosters with the use of cannulas made of flexible medical silicon tubing, (b) to determine the ileal digestibility of diets with different protein sources in roosters, (c) to relate the ileal digestibility data of six diets determined in ileal cannulated roosters with values previously determined for the same diets in cannulated pigs (van Leeuwen, 1996b).

The experiment was approved by the TNO Committee for Animal Welfare.

MATERIALS AND METHODS

Experimental protocol

Ten adult roosters (Lohmann brown) with an average body weight (BW) of 2.8 kg were individually housed in metabolic (galvanized wire mesh) cages (40 x 75 x 60 cm, width x height x depth), with a feed and water bowl. The cages were placed in an environmentally controlled room with an air temperature of 19-21^{o}C. Birds were maintained under a 16 h light : 8 h twilight cycle throughout.

Surgical procedure for ileostomy

The principle of the surgical procedure used in the present experiment was briefly described, previously, by Schutte *et al*. (1991). From 3 weeks prior to the surgeries and for 3 weeks post-surgery the roosters were fed a highly digestible diet with soyflour meal (410 g/kg), maize starch (180 g/kg), glucose (200 g/kg), cellulose (Arbocel, 100 g/kg) and a premix of vitamins and minerals mixed with maize (110 g/kg). The cannulas consisted of a barrel with a flange (Figure 1), both segments of the same medical grade silicon tubing, Type SR 16 (Maxxim B.V., s'Hertogenbosch, The Netherlands), with a 8 mm inner diameter (ID) and an 11 mm outside diameter (OD). The barrel and the flange were glued together with a silicon adhesive, Elastosil E41 (Wacker- Chemie GmbH, München, Germany).

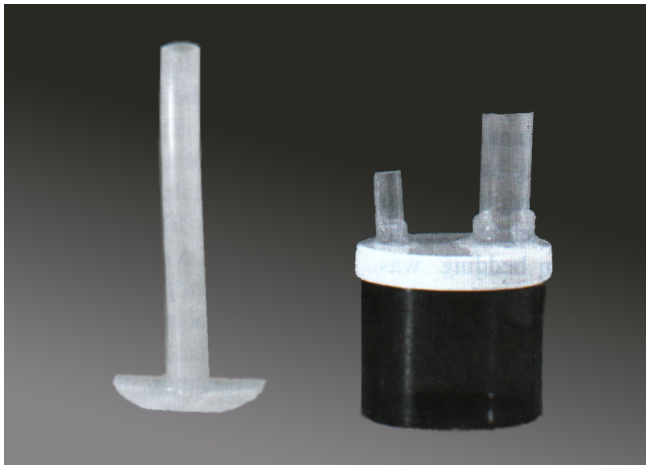

Figure 1. T-shaped silastic cannula and a plastic bottle for digesta collection.

Feed was removed 24 h prior to surgery, but water was always freely available. Each rooster was premedicated with an intramuscular injection of 1 mg Ketamin (Nimatec®, Eurovet, Bladel, The Netherlands; given as a sedative), 10 mg flunixine-meglumine (Finadyne®, Schering Plough Animal Health, USA; as an analgestic), 0.3 ml Depomycine® 20/20 (Mycofarm, De Bilt, The Netherlands; as a wide spectrum antibiotic) and 0.05 mg atropinsulphate (Eurovet, Bladel, The Netherlands; as a cholinergic blocker). Oxygen (O_2) with isoflurane as anaesthesia were given with a mask. After sedation, the rooster was intubated (OD, 3 mm) and the rooster was placed on its back on the surgery table. The area ventral of the pubis was cleaned with a general disinfectant and the anaesthetics were given by the tube. Laparotomy was performed by a 4 cm straight incision at the ventral side of the right pubis. The ileo-ceacal junction was positioned at the incision. The intestine was closed with two absorbable sutures (Polysorb GL-181/CV-25) around the terminal ileum, with a distance of 3 mm between the sutures. The ileum was transected between the sutures. A purse string suture (Polysorb GL-181/CV-25) was placed at the antimesenteric side of the proximal part of the transected intestine. An incision was made in the intestine between the purse string suture, the flange of the cannula was inserted and fixed immediately with the ligature. A second ligature was placed around the cannula. The cannula was then exteriorized through a stab incision in the body wall about 2 cm ventral of the first incision (Figure 2). After a routine closure of the laparotomy, the cannula was fixed externally with tape with a slight pressure between the intestine and the abdominal wall. Too much pressure would increase local necrosis. After a period of three weeks, the digestibility trial started. Time needed

for surgery was, included the introduction of the anaesthesia, about one hour. Number of animals alive after two months was over 90%.

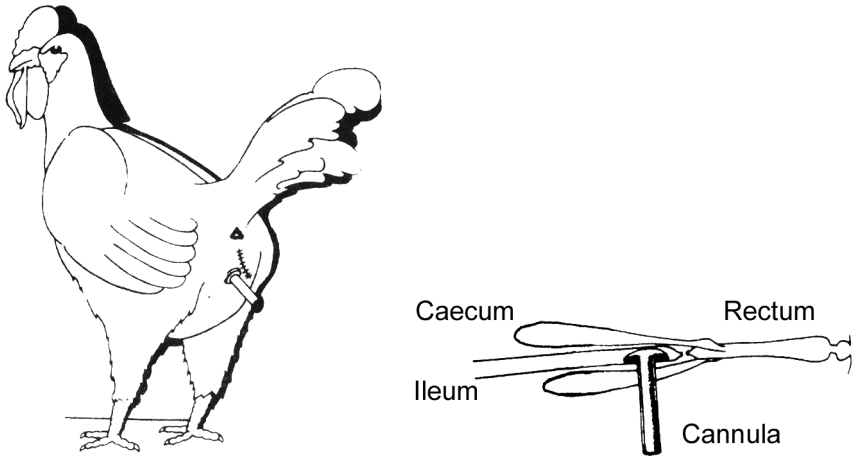

Figure 2. Left: cannulated rooster with an indication of the incision.
Right: the ileo-caecal junction and the position of the cannula.

Experimental diets and determination of ileal digestibility

After a pre-test period of three weeks, the roosters were fed maize starch based diets with maize/wheat gluten (diet 1), wheat gluten (diet 2), faba beans with a low tannin content (LT) (diet 3), lupins, angustifolius, white (diet 4), soybean meal (diet 5) and casein (diet 6) as main feedstuffs (Tables 1 and 2). The incorporation rates of the feedstuffs were calculated in order to obtain diets containing 150 g/kg CP. In diet 1, 94 g/kg wheat gluten meal was added to elevate the CP content. The same batch of the diets were used in previously conducted digestibility experiments with pigs (van Leeuwen *et al*., 1996a, 1996b). Diets were stored at -20^{0}C for two years until the use in the present experiment and the meal was ground over a 1 mm screen and not pelleted. The feed intake was restricted to 80 g/day, equivalent to a semi *ad libitum* level.

The ileal digestibility experiment consisted of four periods (P1, P2, P3 and P4) of 14 days each. Diets 1- 6 were assigned randomly with no rooster receiving the same diet twice. The digestibility coefficients of each diet were determined in at least five different roosters.

Table 1. Dry matter (DM), crude protein (CP; N x 6.25) and crude fibre (CFi) of the feedstuffs (%).

Feedstuffs	DM	CP	Cfi
Maize	88.5	9.5	2.6
Wheatgluten	92.1	82.2	nd[1]
Faba beans (LT[2])	89.3	32.0	8.4
Lupins[3]	92.1	28.4	14.9
Soybean meal[4]	90.2	48.9	4.4
Casein	91.2	86.4	n.d.

[1]nd = not determined; [2]*Vicia faba* L.; Low tannins, < 0.5 % tannins expressed as catechin equivalents (Kuhla and Ebmeier, 1981), 0.19% trypsin inhibitor activity (TIA)(van Oort *et al*., 1989); [3] 0.4% alkaloids (European Commision, 1971); [4] 0.35% TIA.

After 11 days of adaptation to the rations, without fasting periods, digesta were collected over of three successive days (72 h) in a plastic bottle connected to the cannula (Figure 1). Digesta bottles were removed hourly over the day (8.00 - 20.00 hours) and each 6 h during the night. The collected digesta were and immediately frozen (-20^{o}C).

Digesta collection was more frequently during the day rather than in the night. This was done: (1) to have the possibility to compare digesta composition for the two different periods separate, and (2) to keep the roosters quiet during the night, maintaining the normal bio-rhythm of the roosters as much as possible. Unpublished results showed no systematically differences between the contents in the digesta collected over the day and collected during the night. After each experimental period, digesta of the three day collections were thawed and pooled per bird.

Analytical procedures and calculations

Feedstuffs, diets and freeze-dried digesta were ground through a 1 mm screen using a Retsch AM 1 grinder prior to chemical analysis. Nitrogen (N) was analyzed by the Kjeldahl method and crude protein (CP) was calculated as N x 6.25. Dry matter (DM) contents were determined after drying at 80^{o}C overnight. Amino acids (AA) were determined according to Bech-Anderson *et al.* (1990). The apparent digestibility for DM, CP and AA of the diets were calculated from nutrient intake and the total collected amounts of digesta over periods of 3 days (72 h). A correction was made for the included crystaline amino acids, assuming that they are completely absorbed.

The data set for the calculation of the correlations between ileal aDC CP and AA were derived from the cannulated roosters in the present experiment and from cannulated pigs from the previous experiment (van Leeuwen *et al.*, 1996b).

Table 2. Dietary compositions (%).

Diet	1	2	3	4	5	6
Substituted feedstuff	Maize	Wheat-gluten meal	Faba beans (LT)	Lupins	Soya-bean meal	Casein
Substituted feedstuff	82.82	17.90	51.00	54.00	33.00	18.00
Wheat-gluten meal	9.40	-	-	-	-	-
Maize starch	-	51.90	26.78	24.98	43.30	53.35
Glucose	-	15.00	15.00	15.00	15.00	15.00
Soyabean meal	-	1.50	1.00	-	1.00	1.00
Cellulose	-	5.00	-	-	2.00	5.00
$CaCO_3$	0.90	0.80	0.90	0.80	0.85	1.20
$CaHPO_4.\ 2H_2O$	2.00	2.25	1.85	1.60	1.75	1.60
NaCl	0.50	0.50	0.50	0.50	0.50	0.50
MgO	-	0.20	-	-	-	0.20
$KHCO_3$	1.30	1.80	0.20	0.40	-	1.50
$NaHCO_3$	0.30	0.40	0.30	0.30	0.35	0.40
L-Lysine.HCl	0.50	0.50	-	-	-	-
L-Threonine	-	-	-	-	-	-
L-Tryptophan	0.03	-	0.02	0.04	-	-
DL-Methionine	-	-	0.20	0.13	-	-
Vitamin-trac element mixture*	2.25	2.25	2.25	2.25	2.25	2.25
CP (Nx 6.25)	16.6	15.9	17.1	16.1	17.1	16.0
NE (MJ/kg)	9.48	9.83	9.33	9.37	9.71	9.66
Cr_2O_3	0.24	0.23	0.24	0.24	0.26	0.22
HCl-insoluble ash	1.11	0.98	0.99	1.01	1.05	1.04

[1]Contributed the following (/kg diet): DL-alfa-tocopheryl acetate, 37.5 mg; riboflavin, 6 mg; niacin, 30 mg; D-pantothenic acid, 15 mg; choline chloride, 120 mg; cyanocobalamin, 0.045 mg; menadione, 3 mg; renitol, 2.7 mg; cholecalciferol, 45 mg; KI, 0.81 mg; $CoSO_4.7H_2O$, 7mg; $FeSO_4.7H_20$, 0.4g; $CuSO_4.5H_20$, 0.1g; MnO_2, 0.07g; $ZnSO_4.H_20$, 0.3g. This mixture was supplied 20 ppm Tylosine.

Statistical analysis

Data were subjected to analysis of variance using the SPSS/PC+V5.0 software (Norusis, 1992). The diet type was treatment factor using the following model:

$y_{ij} = \mu + D_i + e_{ij}$,

where: y_{ij} = response measurements, μ = mean value, D_i = diet type, e_{ij} = residual error. Preliminary analysis showed no significant ($P>0.1$) effect of periods and therefore period was not included in the model as a factor. Treatment means were tested for difference by use of the Least Significant Difference test (Snedecor and Cochran, 1980). All statements of significance are based on a probability of $P < 0.05$.

Correlations were calculated between ileal aDC determined in the present experiment using roosters and from previously determined aDC data using pigs (van Leeuwen *et al.,* 1996b). The model used to correlate the aDC CP and aDC AA determined in roosters and pigs was:

$y = a \cdot x + c$,

where y = aDC CP determined in roosters, x = aDC CP determined in pigs.

The calculations were conducted using SPSS/PC+V5.0 software (Norusis, 1992).

RESULTS

Feed intake of the roosters two weeks post-surgery was similar to the feed intake prior to the surgeries and the weight of roosters decreased in average of approximately 100 g following surgery. The roosters were kept for more than one year and were used for additional experiments (not presented). During this period, weights of the animals were slightly higher or unchanged compared to the weight prior to the surgeries.

Results of the amino acid analysis of the diets have been previously reported (van Leeuwen *et al.,* 1996b) and the ileal aDC CP and aDC AA measured in roosters are presented in Table 3. The aDC CP of the maize-wheat gluten meal based diet (diet 1), the wheat gluten diet (diet 2), and the casein diet (diet 6) were significantly ($P < 0.05$) different from those of the faba bean diet (diet 3), lupin diet (diet 4) and soybean meal diet (diet 5). Also differences between diets were found for the apparent digestibility of the amino acids. The standard error of the mean (S.E.M.) was, in general, less than 0.02 of the mean digestibility values.

Table 3. Apparent ileal digestibility coefficients of dry matter (DM), crude protein (CP= N x 6.25) and amino acids of six diets determined in roosters.

	Diet						
	1	2	3	4	5	6	
	Maize/ wheat gluten	Wheat-gluten meal	Faba beans *(Vicia faba)* (LT)	Lupins	Soya-bean meal	Casein	SEM
Dry matter	0.83^{b,c}	0.86^{a,b}	0.82^{c}	0.66^{d}	0.82^{c}	0.87^{a}	0.011
Crude protein	0.88^{b}	0.92^{a}	0.81^{c}	0.84^{c}	0.81^{c}	0.90^{a,b}	0.012
Indispensable amino acids							
Arginine	0.85^{a,b}	0.91^{d}	0.85^{a,b}	0.87^{b,c}	0.84^{a}	0.89^{c,d}	0.010
Histidine	0.87^{a}	0.85^{a}	0.76^{b}	0.80^{b}	0.78^{b}	0.88^{a}	0.015
Isoleucine	0.86^{b}	0.92^{a}	0.77^{d}	0.82^{b,c}	0.78^{c,d}	0.86^{b}	0.014
Leucine	0.91^{a}	0.94^{a}	0.81^{c}	0.85^{b}	0.81^{c}	0.93^{a}	0.012
Lysine	0.73 ^{a}	0.78^{a}	0.80^{b,c}	0.84^{c}	0.83^{c}	0.93^{d}	0.015
Methionine	0.86^{b}	0.93^{a}	0.72^{d}	0.81^{c}	0.84^{b,c}	0.92^{a}	0.016
Phenylalanine	0.88^{b}	0.93^{a}	0.80^{c}	0.82^{c}	0.81^{c}	0.90^{a,b}	0.012
Threonine	0.81^{a}	0.84^{a}	0.73^{b}	0.74^{b}	0.74^{b}	0.86^{a}	0.021
Valine	0.85^{b}	0.92^{a}	0.77^{d}	0.82^{b,c}	0.79^{c,d}	0.90^{a}	0.012
Dispensable amino acids							
Alanine	0.85^{a}	0.89^{a}	0.73^{b}	0.76^{b}	0.75^{b}	0.85^{a}	0.017
Aspartic acid	0.81^{a}	0.82^{a}	0.81^{a}	0.83^{a}	0.80^{a}	0.88^{b}	0.015
Glutamic acid	0.94^{b}	0.97^{a}	0.85^{d}	0.90^{c}	0.86^{d}	0.90^{c}	0.010
Glycine	0.84^{b}	0.89^{a}	0.77^{d}	0.82^{b,c}	0.79^{c,d}	0.85^{a,b}	0.014
Proline	0.91^{b}	0.96^{a}	0.82^{d}	0.85^{c}	0.83^{c,d}	0.92^{b}	0.011
Serine	0.88^{b}	0.92^{a}	0.80^{c}	0.84^{b,c}	0.81^{c}	0.84^{b,c}	0.015
Tyrosine	0.85^{b}	0.91 ^{a}	0.77^{d}	0.82^{b,c}	0.79^{c,d}	0.91^{a}	0.014
Sum analysed AA	0.88^{b}	0.92^{a}	0.80^{d}	0.84^{c}	0.81^{c,d}	0.89^{a,b}	0.012

SEM = Standard error of means,^{a,b} Different letters in the same row indicates a significant difference ($P < 0.05$).

Correlations between ileal aDC CP and AA determined in roosters and pigs are presented in table 4. Figure 3 shows the aDC of CP, Ile, Lys, Met, Thr and Ser determined in the pigs (x-axis) and roosters (y-axis). The correlation coefficients (R^2) of aDC CP and aDC AA determined in roosters and pigs ranged from 0.62 to 0.90, with the exception of Arg ($R^2 = 0.49$).

DISCUSSION

Previous experiments (van Leeuwen, not published) and the present experiment have shown that ileal cannulated animals maintained for more than one year at an almost constant body weight and the flexible silicon cannulas did not give any tissue reaction. However, due to the caecal-colonic bypass, sodium absorption in the distal part of the GI tract, as observed by van der Klis *et al.* (1993), was not possible. But, the observation that the body weight of the ileostomised adult roosters did not change indicates an adequate absorption of the minerals.

The measurements made in this experiment have a low variation (Table 3) and compare well with measurements in other reports (Green and Kiener, 1989; Fuller *et al.,* 1994;). In the present experiment, diets which were already prepared for an experiment with pigs (van Leeuwen *et al.*, 1996b), were given to the roosters. However, the diets were reground to avoid blockages in the cannulas. Regrinding may affect the digestibility for coarse diets, as demonstrated in pigs by Fuller *et al.* (1994). Even though the differences between the studies in grinding procedures, feeding level, and methods of digesta collection, the values of aDC CP and aDC AA for the soybean meal diets were similar. The mean aDC CP for the soybean meal diet with restricted-fed cannulated roosters in the present experiment was 0.81. Green and Kiener (1989) reported aDC CP of 0.83 for a similar diet in caecectomised forced-fed adult roosters. The aDC AA of soybean meal determined in the present study and determined by Green and Kiener (1989) were for Lys, 0.83 and 0.84, for Met, 0.84 and 0.85, and for Thr, 0.74 and 0.72, respectively. Angkanaporn *et al.* 1997, reported higher values (aDC Lys, 0.88, and aDC Thr, 0.85). However, they used a batch of soybean meal with a 0,8 % higher CP, and 0.3 % lower CFi content compared with the soybean meal used in the present study.

Amino acids, which are in low concentrations in the diet, will give a lower apparent digestibility than expected on basis of apparent digestible protein. From studies in pigs (Dammers, 1964; Fan *et al.,* 1994) and birds (Angkanaporn *et al.,* 1997), it is known that a low intake of CP, or a

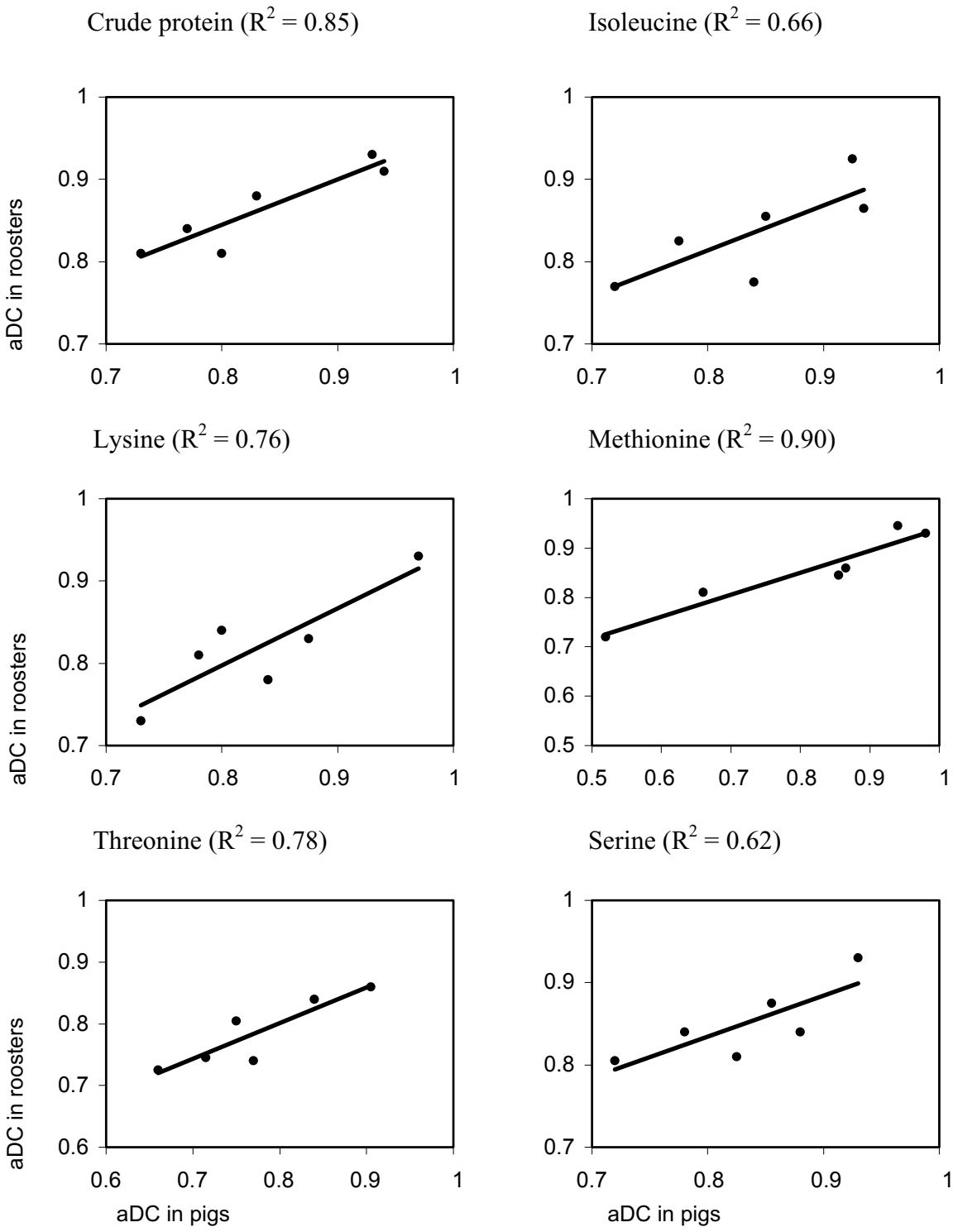

Figure 3. Apparent ileal digestibility coefficients (aDC) of crude protein (CP), Ileu, Lys, Met, Thr and Ser determined in the pigs (x - axis) and roosters (y - axis).

particular AA, results in a relatively low aDC, due to the higher amounts of endogenous protein or AA in the digesta relative to the intake. The aDC Lys from the diets with proteins from cereals (diet 1 and 2) were low compared with the CP digestibility of the corresponding diets, and compared with the digestibility of the other diets. This finding is related with the relatively low Lys content in the protein of cereals compared to the diets with legume seeds and casein. The low content of Met in the faba beans diet also resulted in a relatively low aDC of Met.

For each of the diets, the aDC of the sum of the individual AA, determined in roosters, was similar to the aDC CP. Green and Kiener (1986) eliminated the uric acid N in the excreta by the lead acetate method (Terpstra and de Hart, 1974) in order to determine the aDC CP in caecectomised roosters. They also found good correspondence between aDC CP and for the aDC of the sum of the analysed AA for diets with vegetable protein sources.

In spite of the large differences in anatomy and physiology between roosters and pigs (Moran Jr., 1982), the aDC CP showed a high correlation. The explained part of the variation of aDC of CP between roosters and pigs using a linear model was 85%. For the individual amino acids, the R^2 was 0.62 to 0.90, with the exception of aDC Arg ($R^2 = 0.47$). The latter can be explained because of the small range of the values of aDC for Arg in roosters and pigs (In roosters the range was 0.84- 0.91 and in pigs 0.87- 0.96). In the present experiment, the aDC of highly digestible diets (aDC > 0.85) were in roosters similar or lower than in pigs. The aDC values in roosters tended to be higher than the values determined in pigs when the aDC in pigs was <0.8.

In summary,

- after the presented procedure for ileostomy, roosters can be used over a long period of time.
- significant differences in aDC CP and AA were observed between diets.
- aDC of CP and AA of soybean meal determined in the present experiment were comparable with data from the literature in which adult caecectomised roosters were used.
- significant correlations were observed between aDC CP of various feedstuffs determined in roosters and in pigs ($R^2 = 0.85$). Although, more work is needed to validate these correlations, it is likely that this approach can be used for the prediction of aDC values for roosters from values determined in pigs and reverse.

Table 4. Correlations between apparent digestibility coefficients in roosters as dependent variable (y) with apparent digestibility coefficients in pigs as independent variable (x) in a linear model (y = ax + c; n = 6).

	a	c	R^2	SE*	P
Dry matter	0.8782	0.2857	0.83	0.035	0.01
Crude protein	0.5512	0.4008	0.85	0.022	0.01
Indispensable amino acids					
Arginine	0.6691	0.2556	0.47	0.021	0.13
Histidine	0.6237	0.2801	0.76	0.027	0.02
Isoleucine	0.5002	0.4139	0.66	0.036	0.05
Leucine	0.6111	0.3446	0.82	0.028	0.01
Lysine	0.6874	0.2354	0.76	0.036	0.02
Methionine	0.4168	0.5139	0.90	0.027	0.01
Phenylalanine	0.5034	0.4419	0.86	0.022	0.01
Threonine	0.6105	0.3140	0.78	0.030	0.02
Valine	0.5679	0.3716	0.77	0.032	0.02
Dispensable amino acids					
Alanine	0.5884	0.3382	0.74	0.038	0.03
Aspartic acid	0.3732	0.5201	0.69	0.017	0.04
Glutamic acid	0.6332	0.3351	0.76	0.025	0.02
Glycine	0.5087	0.4404	0.85	0.019	0.01
Proline	0.5048	0.4493	0.85	0.024	0.01
Serine	0.4861	0.4433	0.62	0.030	0.06
Tyrosine	0.5794	0.3448	0.82	0.028	0.01

*SE = Standard error of the model.

REFERENCES

Angkanaporn, K., Ravindran., V. and Bryden, W.L. (1997) Influence of caecectomy and dietary protein concentration on apparent excreta amino acid digestibility in adult cockerels. Br. Poultr. Sci. 38, 270-276.

Bech-Anderson, S., Mason, V.C. and Dhana, M.S. (1990) Hydrolysate preparation for amino acid determinations in feed constituents. 9. Modification to oxidation and hydrolysis conditions for steamlined procedures. J. Anim. Physiol. a. Anim. Nutr. 63, 188-197.

CVB (1998) Veevoedertabel Chemische samenstelling, verteerbaarheid en voederwaarde van voedermiddelen. Centraal Veevoederbureau, P.O. Box 2176, Lelystad, The Netherlands.

Dammers, J. (1964) Etudes de la digestibilité chez le porc les facteurs, qui influencent la digestibilité des aliments digestibilité des amino acides. *Thesis*. Leuven, Belgium.

Degussa (1993) Scheinbare verdauliche Aminosäuren Für Schweine. Degussa AG, Geschaftsgebiet Futtermittel Additive, Frankfurt am Main, Germany.

Dierick, N., Vervaeke, I., Decuypere, J., van der Heyde, H. and Henderickx, H. (1987) Correlation of ileal and fecal digested protein and organic matter to production performances in growing pigs. Proc. Vth Int. Symp. Protein Metabolism and Nutrition, Rostock (DDR).

Eurolysin (1995). Ileal digestibility of amino acids in feedstuffs for pigs. Eurolysin, 16 Rue Ballu, 75009 Paris, France.

European Commission (1971) Determination of Alkaloid Content in Lupin Seed, p. L155/35. Luxembourg: Publication Office of European Communities.

Fan, M.Z., Sauer, W.C., Hardin, R.T. and Lien, K.A. (1994) Determination of apparent ileal amino acid digestibility in pigs: Effects of dietary amino acid level. J. Anim. Sci. 72, 2851-2859.

Fussel, M.H. (1969) A method for the separation and collection of urine and faeces in the fowl *Gallus domesticus.* Res. Vet. Sci. 10, 332-337.

Fuller, M.F., Darcy-Vrillon, B., Laplace, J.P., Picard, M., Cadenhead, A., Jung, J., Brown, D., Franklin, M.F. (1994) The measurement of dietary amino acid digestibility in pigs, rats and chickens: A comparison of methodologies. Anim. Feed Sci. Technol. 48, 305-324.

Green, S. and Kiener, T. (1989) Digestibilities of nitrogen and amino acids in soya-bean, sunflower, meat and rapeseed meals measured with pigs and poultry. Anim. Prod. 48, 157-

179.

Johns, D.C., Low, C.K., Sedcole, J.R., James, K.A. (1986) Determination of amino acid digestibility using caecectomised and intact adult cocerels. Br. Poult. Sci. 27, 451-461.

Karasawa, Y. and Maeda, M. (1994) Role of caeca in the nitrogen nutrition of the chicken fed on a moderate protein diet or a low protein diet plus urea. Br. Poult. Sci. 35, 383-391.

Kuhla, S. and Ebmeier, C. (1981) Untersuchungen zum Tanningehalt in Ackerbohnen. Arch. Tierernähr. 33, 47-56.

McNab, J.M. (1989) Digestibility of amino acids by poultry. Amino Acids in Animal Production Conference. Forum Feeds. London.

Moran Jr. E.T. (1982) Comparative Nutrition of Fowl and Swine. The Gastro Intestinal Systems. E.T. Moran Jr. Office for Educational Practise, University of Guelph, Guelph, Ontario N1G 2W1, Canada.

Mortensen, A. and Tindall, A.R. (1981) On cecal synthesis and absorption of amino acids and their importance for nitrogen recycling in willow ptarmigan *(Lagopus lagopus lagopus).* Acta Physiol. Scand. 113, 465-469.

Norusis, M.J. (1992) ystem Users' Guide SPSS/PC Version 5.0 Base.Statistical Package for Social Sciences Chicago. IL.

Okumura, J. (1976) Method of colostomy and cannulation of the chicken. Br. Poult. Sci. 17, 547-551.

Parsons, C.M. (1986) Determination of digestible and available amino acids in meat meal using conventional and caecectonized cockerels or chick growth assays. Br. J. Nutr. 56, 227-240.

Ravindran, V., Hew, L.I., Ravindran, G., Bryden, W.L. (1999) A comparison of ileal digesta and excreta analysis for the determination of amino acid digestibility in food ingredients for poultry. Br. Poult. Sci. 40, 266-275.

Raharjo, Y. and Farrell, D.J. (1984) A new method for determining amino acid digestibility in poultry feedstuffs using a simple cannula, and the influence of dietary fibre on endogenous amino acid output. Anim. Feed Sci. Technol. 12, 29-45.

Rhône Poulenc (1993) Rhodimet Feed Formulation Guide, 6Th Edition, Rhône Poulenc Animal Nutrition 42, Avenue Aristide Briand, BP 100, 92164 Antony Cedex, France.

Schutte, J.B., van Leeuwen, P., and Lichtendonk, W.J. (1991) Ileal digestibility and urinary excretion of xylose and arabinose in ileostomized adult roosters. Poult. Sci. 70, 884-891.

Snedecor, G.W. and Cochran, W.G. (1980) Statistical methods. 7th edition. The Iowa State

University Press. Ames, 1A.

Terpstra, K. and de Hart, N. (1974) The estimation of urinary nitrogen and faecal nitrogen in poultry excreta. Z. Tierphysiol. Tierernähr. Futtermittelkd. 32, 306-320.

Van Leeuwen, P., Jansman, A.J.M., van Kempen, G.J.M. and Verstegen M.W.A. (1993) Mathematical analysis of apparent ileal digestibility values of amino acids in feedstuffs for pigs. Livest. Prod. Sci. 36, 255-272.

Van Leeuwen, P., Sauer, W.C., Huisman, J., van Kleef, D.J. and den Hartog, L.A. (1988) Ileo-cecal re-entrant cannulation in baby pigs. J. Anim. Physiol. Anim. Nutr. 59, 59-63.

Van Leeuwen, P., van Kleef, D.J., van Kempen, G.J.M., Huisman, J. and Verstegen, M.W.A. (1991) The post valve T-caecum cannulation technique in pigs applied to determine the digestibility of amino acid in maize, groundnut and sunflower meal. J. Anim. Physiol. Anim. Nutr. 65, 183-193.

Van Leeuwen, P., Veldman, A., Boisen, S., Deuring, K., van Kempen, G.J.M., Derksen, G.B., Verstegen, M.W.A. and Schaafsma, G. (1996a) Apparent dry matter and crude protein digestibility of rations fed to pigs and determined with the use of chromic oxide (Cr2O3) and insoluble ash as digestive markers. Br. J. Nutr. 76, 551-562.

Van Leeuwen, P., Gdala, J., Boisen, S., Buraczewski, S., van Kempen, G.J.M., Verstegen, M.W.A. and Schaafsma, G. (1996b) Validation of a mathematical model to explain variation in apparent ileal amino acid digestibility of diets fed to pigs. J. Anim. Feed Sci. 5, 303-315.

Van der Klis, J.D., Verstegen, M.W.A., van Voorst, A. (1993) Effect of a soluble polysaccharide (carboxy methyl cellulose) on the absorption of minerals from the gastrointestinal tract of broilers. Br. Poult. Sci. 34, 377-389.

Van Oort, M.G., Hamer, R.J. and Slager, E.A. (1989) The trypsin inhibitor assay: Improvement of an existing method. In: Recent Advances of Research in Antinutritional Factors in Legume Seeds (J. Huisman, A.F.B. van der Poel and I.E. Liener, editors). PUDOC, Wageningen, The Netherlands, pp. 110-113.

Zebrowska, T., Buraczewska, C., Horaczynski, H. (1978) Apparent digestibility of nitrogen and amino acids and utilization of protein given orally or introduced into the large intestine of pigs. Roczn. Nauk. Roln. Ser. 99B, 99-105.

Part II Functional-morphological parameters

Chapter 5

Morphology of the small intestinal mucosal surface of broilers in relation to age, diet formulation, small intestinal microflora and performance

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SUMMARY

Three experiments were performed with broilers in an attempt to relate morphological characteristics of the small intestinal mucosal surface to the age, dietary factors, small intestinal microflora and performance. Characterisation of the small intestinal mucosal surface using a dissecting microscope was based on the orientation of the villi, villus shape and the presence of convoluted villi.

In trial 1, the morphological changes of the mucosal surface were studied weekly in the period from 7 to 28 days of age. At day 7 mainly tongue- and leaf-shaped villi together with some ridge-shaped ones were observed in the middle section of the small intestine, displaying a regular zigzag pattern for 53% of the mucosal surface. During the period from day 7 to 14, the area with ridge-shaped villi increased significantly ($P < 0.05$) from 7 to 63% and did not change significantly over the next two weeks.

In trial 2, three protein sources and L-Glutamine (Gln) were studied with respect to their impact as dietary components on villus morphology in the mid-small intestine and performance. Diets were fed with (0 - 14 days) and without pectin (14 - 21 days). Feed conversion ratio on the hydrolysed wheat gluten diet improved significantly ($P < 0.05$) in comparison to the native wheat gluten diet. During the period of 0 to 14 days of age the mucosal area with zigzag orientated villi increased significantly ($P < 0.008$) when the pectin diet was supplemented with Gln. Moreover, weight gain of birds fed the Gln diet increased significantly ($P < 0.05$) in the period of 14 - 21 days.

In trial 3, a study was made of the morphological response of the villi to a stimulation of microbial activity in the digesta after addition of highly methylated pectin to the diet. This was performed with and without inoculation of a non-virulent *Salmonella typhimurium*. The animals fed the pectin diet showed impaired weight gain and a significantly ($P < 0.01$) higher feed conversion. The pectin affected the mucosal surface by decreasing the area with the zigzag pattern ($P = 0.02$) and increasing the area with convoluted, mainly ridge shaped villi ($P < 0.01$). *Salmonella typhimurium* infection increased the effects of pectin on performance and mucosal morphology.

INTRODUCTION

Both form and function of the gastro-intestinal tract in birds have been reviewed by King and McLelland (1979). During the development of the embryonic small intestine a regular zigzag pattern of so-called pre-villus ridges are formed after 14 to17 days of incubation. On the seventeenth day of incubation crest cells appeared on the top of the ridges where two rows of villi developed (Lim and Low, 1977), which are broad finger-shaped or more narrow plate-like over the whole length of the small intestine (Bayer *et al.,* 1975). In a previous study mainly finger-shaped villi were observed with a few tongue- and leaf-shaped ones in the small intestine of one-day old chickens (van Leeuwen, unpublished). King and McLelland (1979) postulated that the surface area of the small intestine of birds is increased by being thrown into a series of projections, showing a large variation in arrangements and forms.

The first objective of the present study was to describe the morphological characteristics of the small intestinal villi in 7 to 28 day-old broilers in relation to the age. Secondly, a study was made of the effects of some dietary factors and a *Salmonella typhimurium* infection on the morphology of the small intestinal villi and on the performance.

MATERIALS AND METHODS

Morphological description of the small intestinal villus shape and villus orientation

The morphological description of the small intestinal mucosa concerns the orientation and shape of the villi. Differences in villi orientation and villus shape are demonstrated in figures 1 to 6. Figure 1 shows the mucosa taken from one-day-old chicken (van Leeuwen, unpublished), whereas the mucosa in figures 2 to 6 are derived from broilers at day 21 from trial 1. The pictures have been made using an Olympus dissecting microscope connected to a video system.

In one-day old chickens the villi are mainly cylindrical, with a diameter of ~ 0.2 to 0.3 mm, and have a coned top (Figure 1). These villi, standing close to each other, are called finger-shaped. From day 7 onwards more variation in villus orientation and shape occurs.

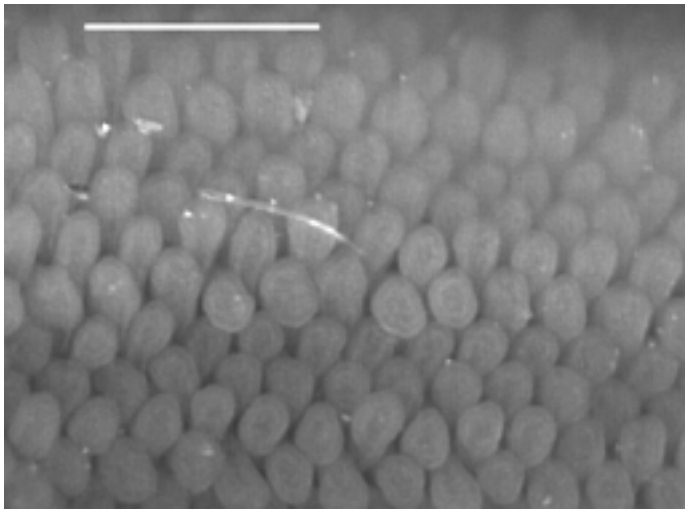

Figure 1. One-day old broilers with mainly finger shaped villi.

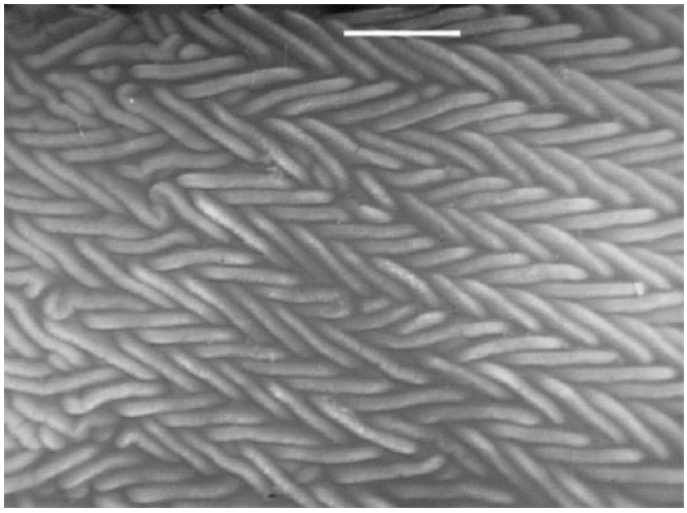

Figure 2. 21-days old broilers with mainly zigzag oriented ridges.

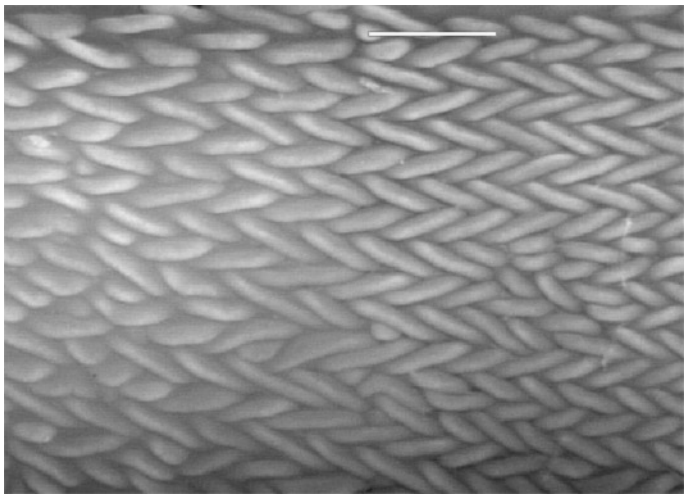

Figure 3. 21-days old broilers with different villus types and ridges partly positioned in a regular zigzag pattern.

Figures 1-6. Dissection microscopically observations of the small intestinal mucosa of broilers (The bar in the picture represents 1 mm).

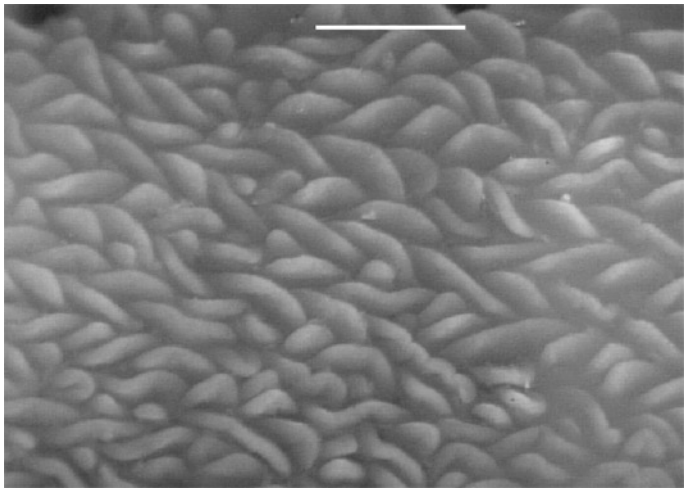

Figure 4. 21- days old broilers with tongue shaped villi (coned top) and ridges and with few ridges in a zigzag position.

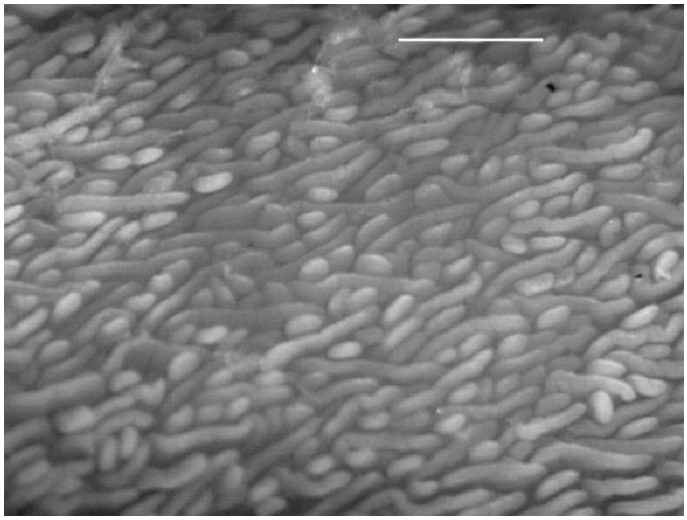

Figure 5. 21-days old broilers with tongue shaped villi (coned top), leaf shaped villi (straight top) and ridges, without zigzag oriented ridges.

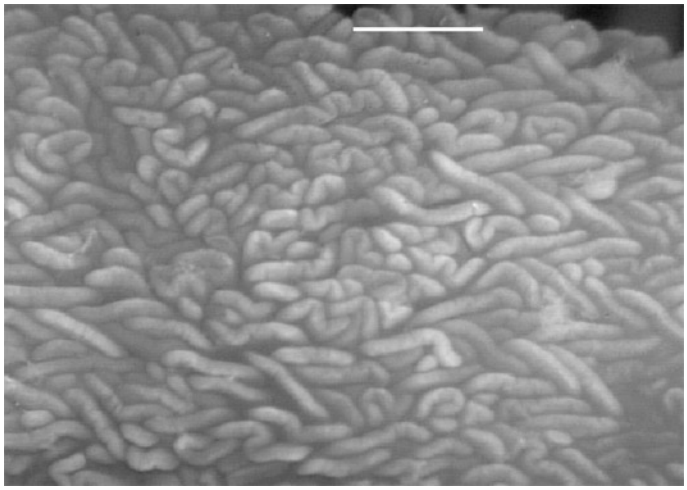

Figure 6. 21-days old broilers with mainly convoluted ridges and a few convoluted leaf shaped villi, without zigzag oriented ridges or villi.

Table 1. Composition of the diets (g/kg).

Ingredients	Trial 1 and 2, SI	Trial 2, WG	Trial 2, HWG	Trial 2, SI+Gln	Trial 3, SBM
Maize	629.8	624.8	624.8	629.6	451.5
Soya isolate (SI)	50.0	-	-	39.0	-
Wheat gluten (WG)	-	50.0	-	-	-
Hydrolysed wheat gluten (HWG)	-	-	50.0	-	-
L-Glutamine (Gln)	-	-	-	15.0	-
Soya bean meal (SBM)	140.0	140.0	140.0	140.0	221.0
Toasted soya beans	100.0	100.0	100.0	100.0	25.0
Casein	-	-	-	-	63.0
Fish meal	15.0	15.0	15.0	15.0	20.4
Tapioca	-	-	-	-	130.9
Soya oil	-	-	-	-	5.0
Animal fat	30.0	30.0	30.0	25.0	40.0
Limestone	12.0	12.0	12.0	12.0	12.5
$Ca(H_2PO_4)_2$	8.5	9.0	9.0	8.5	13.4
NaCl	1.5	1.5	1.5	1.5	2.2
$NaHCO_3$	-	2.0	2.0	-	1.9
$KHCO_3$	-	-	-	-	1.7
L-lysine HCl	1.1	3.2	3.2	1.7	-
DL-methionine	1.8	1.4	1.4	2.0	1.5
L-threonine	0.1	0.7	0.7	0.4	-
L-tryptophan	0.2	0.4	0.4	0.3	-
Vitamin + mineral mix[1]	10.0	10.0	10.0	10.0	10.0
Calculated contents (g, MJ/kg)[2]					
Dry matter	879	880	880	880	880
Crude protein (N x 6.25)	210	210	210	210	217
Crude fat	77	79	79	72	71
Crude ash	50	49	49	49	63
Ca	7.1	7.2	7.2	7.1	8.8
P	5.9	5.6	5.6	5.8	6.9
Digestible lysine	10.2	10.2	10.2	10.2	11.6
Digestible methionine+cystine	7.4	7.4	7.4	7.4	7.9
Metabolizable energy	12.6	12.6	12.6	12.6	12.1

[1] Contributed were the following vitamins and trace elements per kg of diet: vitamin A (retinol acetate), 10,000 IU; cholecholciterol, 2,000 IU; vitamin E (DL- alfa- tocopheryl acetate), 20 IU; menadione, 5 mg; riboflavin, 4 mg; D-pathothenic acid, 12 mg; nicotinamide, 40 mg; cobalamin, 0.015 mg; choline choride, 500 mg; biotin, 0.1 mg; folic acid, 1 mg; Fe, 60 mg; Mn, 60 mg; Cu, 25 mg; Zn, 100 mg; Se, 0.05 mg; I, 3.5 mg; anti-oxidant , 100mg.

[2] Calculated from the data provided by the Dutch Livestock Committee.

The figures 2 and 3 show a regular zigzag villus orientation, whereas the villi in figures 4, 5 and 6 display irregular patterns. The mesenteric ligament lies transversely behind the area viewed, which implies that the zigzag villi forms a spiral pattern.

Differences in villus-shape are related to the dimensions of the top of the villi compared to the base. Besides finger-shaped villi with a coned top, there are also villi with a coned top and a base of ~0.2 ≤ 0.5-mm length (Figure 4). These triangular villi are called tongue-shaped villi. Villi with similar dimensions and a straight top instead of a coned one are termed leaf-shaped (Figure 5). When the length of the villus base is > 0.5 mm the villus is called ridge-shaped (Figure 2). The ridge-, leaf- and tongue-shaped villi are generally flat and straight (Figure 1 and 2), but may also be curved or convoluted (Figure 6).

Judgement of the small intestine mucosal surface comprised estimates of the percentage area occupied with different types of villi according to their descriptions. All judgements were conducted, without knowledge of the treatment factor during classification (blind classification).

Animals, housing, diets and treatments

In all trials conventional one-day-old male Ross hybrid broiler chickens were housed in electrically heated battery cages in two tiers with wire floor space of 2.34 m^2. The cages were continuously (24 h/day) illuminated and located in an insulated room with controlled temperature and humidity. The temperature decreased from 33^0C in week 1 to 23^0C in week 4, whereas humidity remained above 60% and on average 65%. The study comprised three trials and five diets (Table 1). Each diet was formulated to meet the requirements of broiler chicks (National Research Council, 1994) and pelleted without steam addition. Feed and water were supplied for *ad libitum* intake. Antimicrobial growth promoters and coccidistatics were not included in the diets, as they may interfere with the treatment factors. Day-old broiler chickens were vaccinated against NCD.

In trial 1, a study was made of the changes in shape and orientation of the villi during the period

7 to 28 days of age. Twenty-four chickens are fed a diet with soy isolate (SI), soya bean meal, toasted soya beans and fish meal as main protein sources and kept in one cage. At the age of 7, 14, 21 and 28 days, six broilers are taken at random for dissection and sampling of the small intestine. In trial 2, the effects of protein source on villus characteristics are studied. Soy isolate (SI) was exchanged for wheat gluten (WG; Amytex 100, Amylum group, Belgium) and hydrolysed wheat gluten (HWG, Solpro 500, Amylum group, Belgium) or the SI diet was supplemented with Glutamine (Gln). The SI, WG, HWG and SI + Gln diets were equalized on the basis of crude protein and metabolizable energy. From day 1 to 14, 30 g pectin (HMC; type CU 301, methoxylation > 65%, Contined B.V., Bennekom, The Netherlands)/kg diet was added to all diets as a fermentable non-starch polysaccharide (NSP) to stimulate microbial activity, as previously described by Langhout (1998). The study comprised of 6 cages each with 15 chickens per treatment group. Chicks were individually weighed at days 14 and 21 and feed consumption for each cage was recorded over the periods 0 - 14 and 14 - 21 days. At the age of 14 days and at the end of the trial, two broilers were taken at random from four cages of each group, for dissection.

Trial 3 consisted of four treatment groups (1 - 4) to study the effects of microbial activity on villus characteristics and on production parameters. Each experimental group comprised of 6 cages each with 24 chickens. Groups 1 and 2 were fed a soya bean meal (SBM) diet, the birds of groups 3 and 4 also received the SBM diet, however, supplemented with 30g of HMC/ kg. The pectin product was supplied as anhydrous polysaccharide and has been substituted in the diet by tapioca on the basis of similar metabolizable energy (ME) contents. At an age of 7 days, an infection has been induced in each of the birds in group 2 and 4 with an oral dosage of 10^9 of a non-virulent *Salmonella typhimurium* strain (SL3261 AroA). The trial was terminated on day 21. Each chick was weighed at day 7 and 21 and feed consumption was recorded for each cage from day 7 to day 21. Upon completion of the trial, four broilers were taken at random from 3 cages of each group from each experiment and dissected.

All treatments and procedures were performed with approval of the Ethical Committee on Animal Welfare, ID Lelystad, The Netherlands.

Dissection of the broilers and storage of mucosal samples

Broilers were dissected after euthanasia with an intravenous injection of T61 (containing a combination of embutramide, mebezoniumiodide and tetracainhydrochloride in solution, Hoechst

Holland N.V., 1100 AZ Amsterdam, The Netherlands). Laparatomy was performed and in trial 1, 1.5-cm wide samples were dissected for microscopic determinations taken from the mid-duodenum (proximal small intestine), 10 cm proximal to the Meckels diverticulum (mid-small intestine), and 5 cm proximal to the ileo-caecal ligament (distal small intestine). In trials 2 and 3 sampling was limited to the mid-small intestine representing the major part of the small intestine. The intestinal samples are cut open longitudinally on the anti-mesenteric side, affixed on dental wax with the villi on the upper side, fixed in 0.1 M phosphate-buffered formaldehyde solution (40 g/l).

Statistical analysis

Trial 1, was an experiment using bird age as an experimental factor. Data were analysed statistically according to the following model:

$$y_{ij} = \mu + A_i + e_{ij},$$

where y= response parameter, μ = the mean value, A_i = bird age in weeks, and e_{ij} = residual error. As variations within age were not equal Student *t*-test's were conducted to determine statistical differences between age groups.

Trial 2, consisted of four diets, using pen as experimental unit for performance and bird for morphology. Data were analysed according to the following model:

$$y_{ijk} = \mu + T_i + D_j + e_{ijk},$$

where y = response parameter, μ = the mean value, T_i = tier level, D_j = diet, and e_{ijk} = residual error.

Trial 3, comprised two dietary treatments and a microbial challenge. Pen was used as experimental unit for performance and bird for morphology. The following model was used for statistical analyses:

$$y_{ijkl} = \mu + T_i + D_j + C_k + D \,.\, C_{ik;i} + e_{ijkl},$$

where y= response parameter, μ = the mean value, T_i = tier level, D_j = diet,
C = challenge, and e_{ijkl} = residual error.

Treatment means were tested for difference by using of the Least Significant Difference test (Snedecor and Cochran, 1980). All statements of significance are based on a probability of $P < 0.05$. Data were subjected to analysis of variance using the SPSS/PC+V5.0 software (Norusis, 1992).

Table 2. Villus scores of the small intestine of 7, 14, 21 and 28 days old broilers at three different sites (Trial 1).

Section of the small intestine	Age (days)	Area with zigzag villus orientation (%)	Area with tongue-, leaf-, and ridge-shaped villi (%)			Area with convoluted villi (%)
			Tongue	Leaf	Ridge	
Proximal	7	0^{a}	77^{a}	23^{a}	0^{a}	2^{a}
	14	3^{a}	58^{a}	42^{a}	0^{a}	8^{a}
	21	3^{a}	57^{a}	40^{a}	3^{a}	18^{a}
	28	16^{a}	50^{a}	22^{a}	28^{a}	13^{a}
	P =	>0.05	>0.05	>0.05	>0.05	>0.05
Middle	7	53^{a}	71^{a}	22^{a}	7^{a}	1^{a}
	14	77^{a}	27^{b}	10^{ab}	63^{b}	1^{a}
	21	63^{a}	18^{b}	2^{b}	80^{b}	21^{a}
	28	60^{a}	15^{b}	0^{b}	85^{b}	24^{a}
	P =	>0.05	<0.01	0.04	<0.01	>0.05
Distal	7	2^{a}	98^{a}	2^{a}	0^{a}	0^{a}
	14	7^{a}	49^{b}	23^{b}	28^{b}	2^{a}
	21	33^{a}	45^{b}	11^{ab}	44^{b}	7^{a}
	28	35^{a}	23^{c}	3^{ab}	74^{c}	12^{a}
	P =	>0.05	<0.01	=0.05	<0.01	>0.05
Average of the three sections	7	18^{a}	82^{a}	16^{ab}	2^{a}	1^{a}
	14	29^{ab}	45^{b}	25^{a}	30^{b}	3^{a}
	21	33^{ab}	40^{b}	18^{ab}	42^{b}	15^{a}
	28	37^{b}	29^{b}	8^{b}	63^{c}	16^{a}
	P =	0.19	<0.01	0.07	<0.01	>0.05

[a,b] Mean values within column and within location with different superscript differ significantly (P≤ 0.05).

RESULTS

The results from trial 1, concerning the morphological characteristics of the mucosal surface at the different locations in the small intestine at 7, 14, 21 and 28 days of age, are presented in table 2. In the middle section of the small intestine a substantial area of the mucosa (53 – 70%) was occupied with zigzag-orientated villi and the villus shape changed with age. The average percentage of the three locations with tongue-shaped villi decreased significantly ($P < 0.05$) from 82 % on day 7 to 29 % on day 28, whereas the area with ridge-shaped villi increased significantly ($P < 0.05$) from 2 % on day 7 to 62 % on day 28.

Table 3. Performance of broilers fed diets with soya isolate (SI), wheat gluten (WG), hydrolysed wheat gluten (HWG), and the soya isolate diet with Glutamine (SI+Gln), with pectin from day 0 to 14 and without pectin from day 14 to 21 (Trial 2).

Treatment group	Weight gain (g)	Feed intake (g/day)	Feed conversion (g/g)
Performance from day 0 to 14; diets with 30g pectin/kg from day 0 to day 14			
SI	417	41	1.38
WG	420	42	1.41
HWG	421	41	1.35
SI+Gln	426	41	1.37
P	>0.1	>0.1	>0.1
LSD	30	2	0.07
Performance from day 14 to 21; diets without pectin from day 14 to day 21			
SI	851[a]	58[ab]	1.43[ab]
WG	837[a]	59[ab]	1.48[b]
HWG	846[a]	57[a]	1.42[a]
SI+Gln	897[b]	61[b]	1.43[ab]
P	<0.05	0.06	<0.05
LSD	44	3	0.05

[a,b] Mean values within column and within period with different superscript differ significantly ($P \leq 0.05$); 1 LSD = least significant difference.

Table 4. Villus scores of the middle section of the small intestine of 14 and 21 days old broilers fed diets with soya isolate (SI), wheat gluten (WG), hydrolysed wheat gluten (HWG), and the soya isolate with Glutamine (SI+Gln), with pectin from day 0 to day 14 and without pectin from day 14 to 21 (Trial 2).

Treatment group	Area with zigzag villus orientation (%)	Area with tongue-, leaf-, and ridge-shaped villi (%)			Area with convoluted villi (%)
		Tongue	Leaf	Ridge	
Dissection at day 14; diets with 30g pectin/kg from day 0 to day 14					
SI	21[a]	73	17	10	4
WG	27[a]	46	25	29	6
HWG	43[a]	69	9	22	1
SI+Gln	76[b]	58	26	16	4
P	0.008	>0.1	>0.1	>0.1	>0.1
LSD	33	29	17	24	6
Dissection at day 21; diets without pectin from day 14 to day 21					
SI	48	41	17	42	8
WG	59	31	12	57	4
HWG	64	25	4	71	14
SI+Gln	74	31	7	62	3
P	>0.1	>0.1	>0.1	>0.1	>0.1
LSD[1]	31	21	17	25	14

[a,b] Mean values within column and within age group with different superscript differ significantly (P≤ 0.05). [1] LSD = least significant difference.

The results from trial 2, concerning the morphology of the small intestine mucosal surface and broiler performance, are presented in tables 3 and 4, respectively. No significant (P > 0.1) between treatment differences were observed in broiler performance during the period between 0 to 14 days of age when the SI, WG, HWG and SI+ Gln diets were supplemented with pectin. In the period from 14 to 21 days no pectin were added and daily weight gain of the birds on the SI+Gln diet was significantly (P < 0.05) higher to that of birds on the SI diet. Feed conversion was significantly (P < 0.05) higher in birds fed the non hydrolysed wheat gluten (WG) compared to those fed hydrolysed wheat gluten (HWG).

Table 5. Performance of broilers from day 7 to 21 fed a soya bean meal diet (SBM), with or without pectin and with or without a *Salmonella typhimurium* challenge at day 7 (Trial 3).

Treatment group	Weight gain (g)	Feed intake (g/day)	Feed conversion (g/g)
Diets without pectin			
1, SBM	798[a]	51[ab]	1.35[a]
2, SBM + challenge	795[a]	50[a]	1.32[a]
Diets with 30g pectin/kg			
3, SBM	598[b]	51[ab]	1.78[b]
4, SBM + challenge	599[b]	53[b]	1.84[c]
P pectin	<0.01	>0.10	<0.01
P challenge	>0.10	>0.10	>0.10
P pectin x challenge	>0.10	0.07	<0.01
LSD[1] (P=0.05)	20	2	0.03

[a,b,c] Mean values within column with different superscript differ significantly (P≤ 0.05). [1] LSD = least significant difference.

Regarding the mucosal morphology, the percentage of the area with zigzag orientation of the villi was significantly (P < 0.008) higher when the SI+Gln diet was fed during the period that pectin was supplemented. No significant changes occurred in relation to the villus shape.

The results from trial 3, concerning the performance and the morphology of the small intestine mucosal surface of the broilers, are presented in tables 5 and 6, respectively. Two factors, the supplementation of pectin and the challenge with the non-virulent *Salmonella typhimurium* have been studied. Supplementation of pectin resulted in a significant (P < 0.01) decreased weight gain and an increase of feed conversion ratio. Feed intake remained almost unchanged. The challenge with the non-virulent *Salmonella typhimurium* increased feed conversion significantly (P < 0.05). The statistical interaction between pectin and the microbial challenge displayed a tendency to be significant (P = 0.07) and was significant for feed conversion (P < 0.01).

Regarding the morphology of small intestine mucosal surface, pectin significantly decreased the area with zigzag orientated villi (P = 0.02), and increased the area with convoluted villi (P < 0.01). The challenge did not have significant (P > 0.1) effects on the villus shape, but in combination with pectin the effects were more pronounced.

Table 6. Villus scores of the middle section of the small intestine of 21 days old broilers fed a soya bean meal diet (SBM) with or without pectin and with or without a *Salmonella typhimurium* challenge at day 7 (Trial 3).

Treatment group	Area with zigzag villus orientation (%)	Area with tongue-, leaf-, and ridge-shaped villi (%)			Area with convoluted villi (%)
		Tongue	Leaf	Ridge	
Without pectin					
1, SBM	40[a]	52[a]	22[a]	26[a]	1[a]
2, SBM + challenge	28[ab]	37[a]	31[a]	32[a]	2[ab]
With 30g/kg pectin					
3, SBM	18[ab]	39[a]	26[a]	35[a]	20[bc]
4, SBM + challenge	4[b]	34[a]	33[a]	33[a]	27[c]
P pectin	0.02	>0.10	>0.10	>0.10	<0.01
P challenge	>0.10	>0.10	>0.10	>0.10	>0.10
P pectin x challenge	>0.10	>0.10	>0.10	>0.10	>0.10
LSD[1] (P=0.05)	28	29	22	25	19

[a,b] Mean values within column with different superscript differ significantly (P≤ 0.05). [1] LSD = least significant difference.

DISCUSSION

The development of the villus shape in chickens of 7 to 28 days can be characterised by broadening of the villi from tongue- and leaf- shaped villi to mainly ridge shaped villi. These villus types were also present in 18 weeks old laying hens (Van Leeuwen, unpublished results). Also according to Vodovar (1964) the villus shape in pigs is characterised by broadening with increasing age. The origin of tongue-, leaf- and ridge- shaped villi is unknown. Possibly they result from villus fusion. In addition, ridge-shaped villi may represent the previllus ridges after loss of villi (Mouwen, 1972). A second aspect of the development is the characteristic zigzag villus orientation, present mainly in the middle part of the small intestine (Figures 1 and 2). Such a villus pattern was also present in 18 weeks old laying hens (Van Leeuwen, unpublished results). In birds many types of mucosal patterns have been described. Regular longitudinal

zigzag folds occur in the mid- and distal jejunum of the adult *Serinus canaria* (Bormans, 1973), whereas in different families of passerines, so-called zigzag folds, displaced lamellae and fold networks were observed (Ziswiler, 1967). As illustrated in figures 2 and 3, the zigzag ridges in the broilers are positioned transverse to the length of the intestine and seems to be characteristic for poultry *(Gallus domesticus).* The transverse position of the villi may slow down the passage of the outer layer of the digesta and may improve its contact with the epithelium. In trial 1 the most significant age related morphological changes were observed in the middle and distal part of the small intestine.

Substitution of SI with WG had minor effects on performance and morphology, whereas the feed conversion ratio of the HWG diet was significantly improved compared to the WG diet. The HWG diet also tended to increase the percentage with zigzag oriented villi at day 14 (27 % and 43% in the WG and HWG group, respectively). The improved feed conversion ratio and increased zigzag oriented villi suggest an advantage of the HWG related to the absorption of the amino acids due to hydrolyzation. The Gln addition had a beneficial effect on growth in the period of 14 - 21 days and a significantly ($P < 0.05$) increased percentage of villi placed in a regular zigzag orientation was observed in the previous period. The percentage of zigzag orientated villi in the SI + Gln group at day 14 was 76%, which was comparable to tissue from the middle part the small intestine of broilers of the same age from trial 1 (= 77 %). After the release of the of pectin administration, the percentage of zigzag-orientated villi of the other groups also increased. The positive effects of the Gln feeding the pectin diet may be related to the higher glutamate need of the gut, to maintain normal function, under pathological rather than physiological conditions (Souba, 1993; Gardiner *et al.,* 1995). The beneficial effects of Gln are demonstrated by the prevention of jejunal atrophy in weaned piglets (Wu *et al*., 1996). Pectin decreased daily weight gain while feed intake remained at the same level resulting in a negative effect on feed conversion ratio. The lower performance on pectin diets was possibly related to a reduction in nutrient digestibility. In particular, fat digestibility decreased (Langhout, 1998) when microbial activity increased. In pectin fed chickens he found increased numbers of *Enterococci, Bacteroidaceae, Clostridia* and *E. coli* and an increased deconjugation of bile salts. Conjugated bile salts are essential components for fat absorption. The nutritional implications of the microbiota mentioned are reviewed by Anderson *et al.* (2000). They concluded that the competition between microbes and host for nutrients may also be a factor, and the formation of growth depressing metabolites in the intestine may have negative effects on the small intestine

mucosa. In the present study a decrease in the percentage of the zigzag villus orientation and an increase in convoluted ridge-shaped villi were observed as a morphological response to the pectin. The decrease in zigzag orientation is in agreement with the effects described by Langhout (1998) using the same scoring system. An infection with a non-virulent *Salmonella typhimurium* had a significant negative effect ($P < 0.05$) on feed conversion in the pectin-fed chickens in addition to the effect of the dietary pectin. Increased feed conversion together with equal growth of the challenged pectin group indicates an increased requirement and/or a reduced nutrient absorption. Fourteen days after the *Salmonella typhimurium* infection the area with zigzag relief was significantly ($P < 0.05$) reduced and the area with convoluted villi significantly increased ($P < 0.05$) when challenged chicken were fed pectin compared to the control diet. The effects of the *Salmonella typhimurium* infection on the chickens fed control diet showed the same direction, but were not significant.

Conclusions:

- In clinically healthy broilers the shape and orientation of small intestine villi is related to age and intestinal location.
- Fermentable pectin reduced performance and reduced the area with zigzag villus-orientation.
- Gln addition to the diet limited the reduction in the zigzag villi-orientation caused by pectin.
- A non-virulent *Salmonella typhimurium* increased the effects of dietary pectin on performance and small intestine morphology.

REFERENCES

Anderson D.B., McCracken V.J., Aminov R.I., Simpson J.M., Mackie R.I., Verstegen M.W.A. and Gaskins H.R. (2000) Gut microbiology and growth-promoting antibiotics in swine. *Nutrition Abstracts and Reviews* 70: 101 - 108.

Bayer R.C., Chawan C.B., Bird F.H. and Musgrave S.D. (1975) Characteristics of the absorptive surface of the small intestine of the chicken from 1 day to 14 weeks of age. *Poultry Science* 54: 155 - 69.

Bormans, J.C.P. (1973) De microscopische anatomie van de dunne darm van vogels, in het bijzonder de kanarie *(Serius Canara).* Referaat, Ziektekunde der Bijzondere Dieren, Universiteit van Utrecht, Nederland.

Gardiner K.R., Kirk S.T.J. and Rowlands B.J. (1995) Novel substances to maintain gut integrity.

Nutrition Research Reviews 8: 43 - 66.

King, A.S. and McLelland, J. (1979) Form and Function in Birds. Volume 1. Academic Press New York (USA).

Langhout D.J. (1998) The role of the intestinal flora as affected by non-starch polysaccharides in broiler chicks. PhD Thesis, Agricultural University Wageningen, The Netherlands.

Lim S. and Low F.N. (1977) Scanning electron microscopy of the developing alimentary canal in the chick. *American Journal of Anatomy* 150: 149-174.

Mouwen, J.M.V.M. (1972) White scours in piglets at three weeks of age. PhD Thesis, University of Utrecht, The Netherlands.

National Research Council (1994) Nutrient requirements of poultry, 9th revised edn. (Washington DC, National Academy of Sciences).

Norusis, M.J. (1992) SPSS/PC+Base User's Guide Version 5.0 (SPPS inc. Chicago, Illinois, USA).

Snedecor and Cochran. (1980) Statistical Methods. 7th Edition. The Iowa State University Press, Ames, USA.

Souba, W. (1993) Review: Intestinal glutamine metabolism and nutrition. *Journal of Nutritional Biochemistry 4: 2- 9.*

Vodovar, N. (1964) Intestin grêle du porc. *Annales de Biologie animale, Biochimie, Biophysique* 4: 113- 139.

Wu., (1996) Dietary glutamine supplementation prevents jejunal atrophy in weaned pigs. *Journal of Nutrition* 126: 2578- 2584.

Ziswiler, V. (1967) Vergleichend morphologische Untersuchungen am Verdauungstract körnerfressender Singvögel zur Abklärung ihrer systematischen Stellung. *Zoologische Jahrbücher, Abteilung für Systematik, Ökologie und Geographie der Tiere* 94: 427- 520.

Chapter 6

Effects of virginiamycin, as a feed additive, on small intestinal mucosal morphology and performance in piglets

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SUMMARY

The objective of the present study was to determine the effects of virginiamycin (VM), given as a feed additive, on the morphology of the small intestinal mucosa, and on the performance in weaned piglets. The study comprised three trials (1, 2 and 3), each with a control group (C) and a group fed a diet with 40 mg/kg VM.

In experiment 1, five piglets received the C diet and five the VM diet from day 7 post-weaning until the end of the experiment at day 38 post-weaning. At day 31 post weaning, the piglets were challenged by an oral dose of K88 positive enterotoxigenic *Escherichia (E.) coli* (ETEC). Experiment 2 comprised the period from day 14 - 28 post-weaning and the C and VM group consisted of 16 piglets each. Samples of the small intestinal wall were taken by biopsy (Experiment 1) and at dissection (Experiment 1 and 2). These samples were studied dissecting microscopically and histologically. Experiment 3, was a growth experiment over the period from day 14 - 28 post- weaning with 32 piglets in each group.

In experiment 1, seven days after the *E. coli* challenge , the mean crypt depth of the VM group was significantly ($P = 0.05$) decreased, the number of crypt goblet cells (n/µm) increased ($P = 0.01$) and the villi in the VM group were more finger- shaped, compared to those of the C group. In experiment 2, the mean villus length in the VM group was increased ($P < 0.06$), while the mean crypt depth was similar to that of the C group. Significant ($P < 0.05$) improvements in feed conversion of the VM group compared to the C group were observed in experiment 3. Statistical evaluations of the determined parameters indicated litter effects on the morphological parameters in experiment 1 and 2 and a significant positive correlation between weight gain and height ($R = 0.71$; $P = 0.022$) in experiment 1.

In conclusion, the addition of VM to the diet had probiotic and protective effects on the morphology of the small intestinal mucosa and the performance of piglets.

INTRODUCTION

Kies *et al.* (1991) and Crovetto *et al.* (1993) have demonstrated the probiotic effects of virginiamycin (VM) and other antibiotics, as additive, in diets for pigs and piglets. Body weight gain and feed conversion ratio were improved when VM was administered compared to control diets without antibiotics. The mechanisms by which antibiotics enhanced animal growth and feed efficiency were poorly understood (Anderson *et al.*, 2000; Commission on Antimicrobial Feed Additives, 1997). Most of the studies showed little or no changes in the composition of gut flora during antimicrobial growth promoter supplementation (Corpet, 1999), although Jensen (1988) found a significantly reduced microbial activity in the precaecal part of the gastro-intestinal (GI) tract. It has been suggested that inhibition of the microbial activity by antibiotics reduced the competition with the host for nutrients (Hedde and Lindsey, 1986). Beside this direct functional-nutritional factor, also a reduced production of growth depressing metabolites occurred by microbial activity (Visek, 1978; Greypens *et al.*, 1997), decreasing turnover of muscle protein (Hathaway *et al.*, 1990 a, b) and gut epithelium (Corpet, 1999). These effects of additives might be related to the morphology of the small intestinal mucosa.

The present study with piglets intended to answer two questions: 1) does VM improve the morphology of the small intestinal mucosa and the performance of piglets kept under practical conditions, and 2) does virginiamycin (VM) protect the morphology of the small intestinal mucosa of piglets after an *E. coli* infection.

MATERIALS AND METHODS

Experiments, animals and rations

Three experiments (1, 2 and 3) were conducted with piglets weaned at 21 days of age and each experiment comprised a control (C) group, fed a diet without antibiotics, and a VM group, fed a diet with 40 ppm VM. In the experiment 1 and 2 the morphology of the small intestinal mucosa was studied, whereas in experiment 3 feed intake and weight gain were examined. The composition of the diets is presented in table 1.

The experiments were approved by the Committees for Animal Welfare of TNO (Experiment 1 and 2) and of the Centre de Mas Bové (Experiment 3).

Experiment 1 was conducted with ten piglets (Dutch Landrace x Yorkshire), derived from five

sows each delivering two male piglets. The sows were vaccinated against K88 positive ETEC and on the second day after weaning, blood samples were collected to determine the serum IgG titres against *E. coli* K88 antigen.

From weaning until allocation at day 7, the piglets received a diet without antibiotics. One week after weaning, the piglets were divided into the C and VM group. The two piglets originating from the same sow were distributed over the C and VM groups. Further criteria for allocation to the groups were body weight and serum IgG titres against K88. The piglets were individually housed in metabolic cages, feed intake was restricted to 2.9 times the requirements of metabolizable energy for maintenance and water was freely available.

One week after allocation the piglets were provided surgically with a cannula in the proximal jejunum, 90 cm distal to the ligament of Treitz, according to the procedure described by Kik *et al.* (1988). A challenge with ETEC K88 (O149K91 K88, 1.5 ml x 10^9 bacteria) was given orally at day 31 post weaning according to Meijer *et al.* (1997). Via the cannula, mucosal biopsies for dissecting microscopically examination were taken 50 cm proximal to the cannula, using a biopsy capsule (Kik *et al.*, 1988) twice prior to the challenge and at days 1, 3, 4, 5, and 6 after the challenge. The experiment ended with dissection at day 38 after weaning. At dissection, samples were taken from the small intestinal wall for dissecting microscopically and histological evaluations, from the peripheral blood to determine the antibody titres against K88 in sera, and from the small intestinal digesta to test the occurrence of ETEC (O149K91 K88). The timetable of experiment 1 has been summarized in figure 1.

Weaning	Allocation	Surgery	ETEC challenge	Dissection
Day 0	Day 7	Day 14	Day 31	Day 38

Days post weaning

Figure 1. Timetable of experiment 1 with the days post-weaning.

Table 1. Composition of the diets (g/kg).

Ingredients	Experiment 1	Experiment 2 and 3
Barley	500.0	200.0
Soybean meal	75.0	308.0
Wheat	-	150.0
Maize	-	228.0
Peas	50.0	-
Sunflower meal	76.0	-
Tapioca	102.0	-
Fish meal	53.0	30.0
Milk whey, sweet	46.0	30.0
Soybean oil	20.0	-
Lard	-	29.0
Corn starch	53.2	-
Synthetic amino acids[1]	4.4	1.6
Minerals[1]	16.4	18.9
Vitamins and trace elements[1]	4.0	4.0
Nutrients (Calculated)		
Crude protein (N x 6.25)	177	210
Crude fat	30	52
Crude fibre	40	40
Net energy (=ME) (MJ/kg)	9.8	13.4
Cu (mg/kg)	120	120

[1] Contributed were per kg of diet for experiment 1: L lysine, 2.3g; DL methionine, 0.7g; L threonine, 1.1g; L tryptophan, 0.3g, limestone, 10.4g; monocalciumphosphate, 5.5g; NaCl, 0.5g; for experiment 2 and 3: L lysine, 0.9g; DL methionine, 0.7g; calcium carbonate, 8.3; dicalcium phosphate, 8.3; NaCl, 2.3g; and for each experiment:Vitamin A, 10 000 IU; Vitamin D_3, 2 000 IU; Vitamin E, 15 mg; Vitamin B_1, 1.3 mg; Vitamin B_2, 3.5 mg; Vitamin B_{12}, 0.025 mg; Vitamin B_6, 1.5 mg; Ca- panthothenate, 10 mg; Nicotinic acid, 15 mg; Biotin, 0.1 mg; Folic acid, 0.6 mg; Vitamin K_3, 2 mg; Fe, 80 mg; Cu, 100 mg; Co, 0.75 mg; Zn, 185 mg; Mn, 60 mg; I, 0.75 mg; Se, 0.10 mg and Ethoxyquin, 0.15 mg.

The experiments 2 and 3 were conducted with piglets (Hybrid: Large White x Landrace) weaned at 21 days of age. The animals received during the first 14 days post-weaning a commercial diet with carbadox (Mecadox®) as a feed additive.

Experiment 2 comprised 32 piglets originating from 16 litters, each litter delivered two piglets, and one piglet of each litter was allocated to the C and one to the VM group. The piglets were kept in pens of 4 animals, according to practical standards with freely available feed and water. The experiment started with allocation at day 14 post weaning and ended at day 28 post weaning with dissection. Samples of the small intestinal wall were taken for dissecting microscopical and histological evaluations, and the peripheral blood to determine sera antibody titres against K88.

Experiment 3 comprised a C and VM group each with 32 piglets allocated to 8 pens of 4 piglets, which were equalised on basis of live weight, sex and litters. Duos of the same sex pigs were selected by descending weight order. Pigs within one duo were allocated at random across the two treatments. This resulted in one pen per treatment of heavier pigs in the batch with equal sex ratio for all treatments and one pen per treatment with lighter pigs with equal sex ratio's for all treatments etc. A maximum of 2 piglets from the same litter was allowed in the same pen. Feed intake and daily weight gain were determined from the start of the experiment, at day 14 post weaning, until the end of the experiment at day 28, and feed conversion (feed intake/weight gain) of the groups were calculated.

Procedure of dissection and storage of intestinal samples

The piglets of experiment 1 and 2 were dissected under general anaesthesia. Laparotomy was performed and 1.5 cm wide samples were taken from the jejunal wall, 5.5 m distal to the ligament of Treitz. The intestinal samples, taken at dissection, were cut open longitudinally at the antimesenteric side, affixed on dental wax with the villi on the upper side and fixed in 0.1 M phosphate-buffered formaldehyde solution (40 g/l). After sampling, the animals were euthanized with an intravenous injection of T61 (a watery solution containing a combination of embutramide, mebezoniumiodide and tetracainhydrochloride, Hoechst Holland N.V., 1100 AZ Amsterdam, The Netherlands). The mucosal samples of experiment 1, taken with the biopsy capsule, were fixed in the same buffered formaldehyde solution.

Morphological examination of the small intestinal mucosa and immunological determinations in blood sera

The villus shape was examined with a dissecting microscope and characterized according to a previously described procedure (Mouwen, 1972) using the criteria as shown in table 2. For histology, 3 mm wide longitudinal zones from the mesenteric side of the intestinal wall were cut at right angles to the surface of the mucosa and embedded in paraffin wax. Sections were cut (5 µm) and stained with the periodic acid-Schiff method (PAS). The procedure for the determinations of villus height, crypt depth and the number of goblet cells (n/100 µm crypt) was as described by Kik *et al.* (1990). The serum titres against *E.coli* K88 were determined using a K88 enzyme-linked immunosorbent assay (ELISA). All determinations were conducted with animal number and treatment being unknown during the observations (blind determinations).

Table 2. Description of villus gradation (score) determined by dissecting microscopy according to Mouwen (1972).

Score	Description
0	All finger-shaped villi or mostly finger-shaped villi with a few tongue-shaped villi
0.5	Mixed finger- and tongue-shaped villi
1.0	Predominantly long to short tongue-shaped villi with few finger- and leaf- shaped ones
1.5	Predominantly short tongue- and leaf-shaped with few long tongue- and ridge-shaped villi
2.0	Mixture of short tongue-, leaf- and ridge-shaped and convoluted villi
2.5	Similar to grade 2, with flat areas
3.0	Flat mucosa

Statistical analysis

The following three statistical evaluations were carried out:

Data of trials 1 and 2 were subjected to analysis of variance according to the following model:

$y_{ijk} = \mu + D_i + L_j + e_{ijk}$,

where: y = response to the measurements; µ = mean value; D_i= diet; L_{ij}= litter and e_{ijk} = residual error.

Experiment 3 was analysed as a Randomised Complete Block Design with 2 dietary treatments and 4 blocks corresponding to initial weight and location within the house according to the following model:

$y_{ijk} = \mu + \beta_i + D_j + e_{ijk}$,

where: y = response to the measurements; μ = mean value; β_i = block effect; D_j = diet effect and e_{ijk} = residual error.

The serum antibody titres were statistically evaluated according to dilution models described by McCullagh and Nelder (1989).

The morphological characteristics of the small intestinal mucosa and the titres against *E. coli* K88 determined in the sera were correlated with the daily weight gain from challenge until dissection.

Data were analysed using the SPSS/PC+V5.0 software (Norusis, 1992).). For experiment 3 data were analysed using the SAS® V6.03 package (SAS, 1991).

RESULTS

Experiment 1

In the pre-challenge period incidentally a lower faecal consistency and feed refusals occurred. After the *E. coli* challenge from the first day until the fifth day, one piglet of the VM group had pasty to thin faeces and this piglet refused about 20% of the offered feed. From the third day until the end of the experiment one C piglet refused about 30% of the daily offered feed. At dissection, the jejunal digesta of the C piglet were positive for *E. coli* K88, whereas the digesta of the other piglets were negative.

The serum titres against *E. coli* K88 differed between litters significantly in the beginning ($P < 0.001$) of the experiment as well as at dissection ($P < 0.01$) (Table 3). During the experiment the titres in both groups declined and at dissection the serum titres in both groups did not differ significantly ($P > 0.1$).

Before the ETEC challenge the villus shape scores of the C and VM groups were similar (Table 3). However, after the challenge the mean of villus shape scores of the VM group, of the biopsies taken at day 1, 3, 4, 5 and 6 after the challenge, were significantly ($P= 0.02$) lower compared to those of the C group and the litter effect was significant ($P = 0.03$). The villus shape scores of

Table 3. Experiment 1, serum titres against *E. coli* K88 and mean villus shape scores of biopsies from the small intestinal mucosa taken pre- and post- challenge piglets fed a diet without (C) or with 40 ppm virginiamycin (VM).

	Diets		P value between:	
	C	VM	Diets	Litters
IgG titres against E. coli K88 (ln)				
Two days after weaning	0.72	0.72	ns[1]	0.001
At dissection	0.44	0.56	ns	0.01
Villus shape score of the biopsies[2]				
Pre-challenge[3]	1.25	1.22	ns	ns
Post-challenge[4]	1.40	1.13	0.02	0.03

[1]ns= not significant (P > 0.1); [2]villus shape score according to Mouwen (1972) (Table 2); [3]mean value of two biopsies per piglet taken 7 and 2 days pre-challenge; [4]mean value of five biopsies per piglet taken at 1, 3, 4, 5 and 6 days post-challenge.

Table 4. Experiment 1, morphological characteristics of the small intestinal mucosa of piglets at dissection (7 days post challenge) fed a diet without (C) or with 40 ppm virginiamycin (VM).

	Diets			P value between:	
	C	VM	SEM[1]	Diets	Litters
Villus shape score[2]	1.13	0.93	0.20	ns[3]	ns
Villus height (µm)	490	473	43	ns	ns
Crypt depth (µm)	250	218	12	0.05	0.1
Villus/ crypt ratio (µm/µm)	2.0	2.2	0.3	ns	ns
Goblet cells (n/100 µm crypt)	6.4	8.7	0.5	0.01	0.08

[1] Standard error of means; [2] villus shape score according to Mouwen (1972) (Table 2); [3] ns= not significant (P > 0.1).

these biopsies did not change essentially from day to day and therefore were not presented. The results of the dissecting microscopical and histological investigations of the small intestinal mucosa at dissection have been presented in table 4. The villus shape score of the VM group, tended to be lower compared to that of the C group. The villus heights of both groups were similar, whereas the crypt depths were significantly (P = 0.05) smaller in the VM group. The

villus/crypt (V/C) ratio was increased in the VM group compared to that of the C group. Significantly ($P < 0.01$) more goblet cells occurred in the crypts of the VM group compared to those of the C group. Correlation analysis showed a significant positive correlation for both groups between villus length and body weight gain. The correlation between villus height and growth did not significantly differ between both groups and therefore were pooled and presented in figure 2. No significant ($P > 0.1$) correlations were found between weight gain, the other morphological parameters or the serum titres against *E. coli* K88.

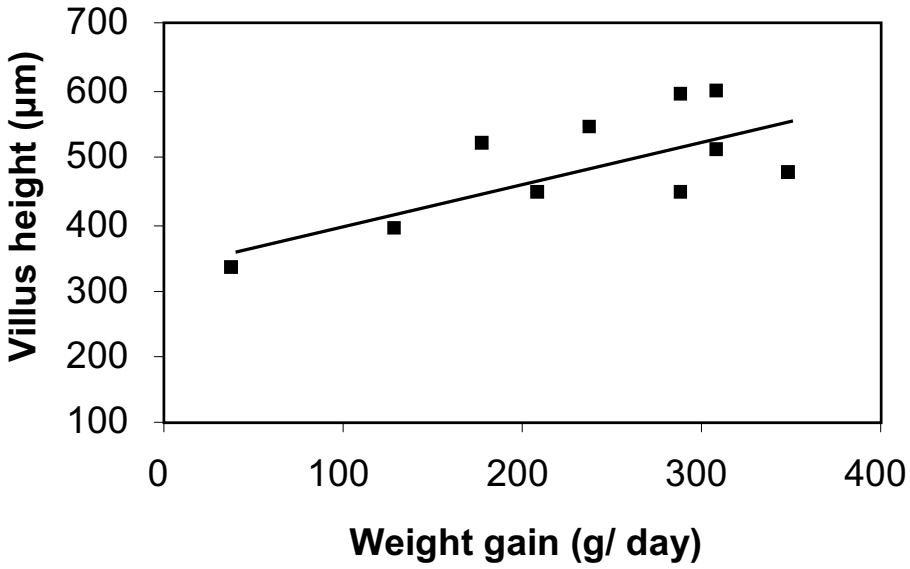

Figure 2. Correlation between average daily weight gain and villus height in the mid-jejunum of piglets ($R = 0.71$; $P = 0.022$)

Experiments 2 and 3

Neither clinical nor adverse reactions were observed in both experiments and the serum titres against E. coli K88 were low. The results of the morphological studies of experiment 2 are presented in table 5. The villus shape score of the VM group was slightly lower than that of the C group, indicating more slender, finger-shaped villi. The villus heights of the VM group were increased ($P = 0.06$) and crypt depths of the VM group were slightly smaller compared to those of the C group. As in experiment 1, differences ($P < 0.1$) between litters were observed regarding the crypt depths. No significant ($P > 0.1$) correlations were observed between the morphological

characteristics of the small intestinal mucosa and the average daily weight gain.

In experiment 3, daily growth of the VM piglets was higher than that of the C piglets (566 g/d and 543 g/d, respectively) and the daily feed intake was, not significantly ($P > 0.05$), lower (Table 6).

Table 5. Experiment 2, morphological characteristics of the small intestinal mucosa of piglets fed a diet without (C) or with 40 ppm virginiamycin (VM) at day 28 post weaning.

	Diets			P value between:	
	C	VM	SEM[1]	Diets	Litters
Villus shape score[2]	1.45	1.30	0.20	ns[3]	ns
Villus height (µm)	395	436	30	0.06	ns
Crypt depth (µm)	329	315	18	ns	0.1
Villus/ crypt ratio (µm/µm)	1.3	1.4	0.1	ns	ns
Goblet cells (n/100 µm crypt)	6.9	7.0	0.4	ns	ns

[1]Standard error of means, [2]Villus shape score according to Mouwen (1972) (Table 2), [3]ns= not significant ($P > 0.1$)

Table 6. Experiment 3, zootechnical results of piglets fed a diet without (C) or with 40 ppm virginiamycin (VM) from day 14 till day 28 post weaning.

	Diets			
	C	VM	SEM[1]	P value
Initial body weight (kg)	13.5	13.4	0.25	ns[2]
Daily weight gain (g)	543	566	15.3	ns
Daily feed intake (g)	915	871	30.7	ns
Feed conversion (kg/kg)	1.671	1.535	0.0384	<0.05

[1]Standard error of means, [2]ns= not significant ($P > 0.05$)

The increased growth and the lower feed intake the VM group resulted in a significantly lower ($P < 0.05$) feed conversion of the VM group (1.535 kg/kg) compared to the feed conversion of the C group (1.671 kg/kg).

DISCUSSION

In a review Anderson *et al.* (2000) concluded that in young pigs antibiotics, fed as additives, improved feed efficiency and growth. The observed significantly ($P < 0.05$) improved feed efficiency in the VM piglets in experiment 3 corresponded with this conclusion but the effects on growth were not significant. The animals of experiment 3 were kept according to practical standards, however, sanitary conditions at the Institute were possibly unintentional better than in average practice facilities which might have decreased the effects (Hays, 1991).

Both the decreased crypt depht of the VM group in experiment 1 and the increased villus height of the VM group in experiment 2 indicated a reduced turnover of the gut epithelium. Possibly these morphological effects after addition of antibiotics to the diet were related to a suppressed bacterial activity and decomposition of bile salts as suggested by Corpet (1999) and Anderson et *al.* (2000). Increased villus heights indicate an increased mucosal surface and absorption capacity, which agreed with the improved precaecal nutrient digestibility of diets with VM observed by Decuypere *et al*. (1991).

In experiment 1 the piglets were individually kept and an oral challenge with K88 positive ETEC was given. The clinical reactions after the infection were limited. However, a mean villus shape score of the biopsies taken after challenge from the VM group was significant ($P = 0.02$) lower compared to those of the C group, indicating more finger shaped villi. At dissection, seven days post challenge, a significantly ($P < 0.05$) smaller mean crypt depth was present in the VM group compared to the C group, while the mean villus height was similar. Deepening of the crypts and shortening of villi were observed in the first days post-weaning (Kenworthy, 1976; Hampson and Kidder, 1986; Nabuurs *et al.,* 1993). Also van Beers-Schreurs (1996) and Verdonk *et al.* (2000) found post-weaning, increased crypt depths and decreased villus heights in the small intestinal mucosa. However, the effects on villus height appeared to alter over a short period, while the effect on crypt depth remained over a longer period of time. The smaller crypts observed seven days after the challenge in the VM group compared to the C group might suggest that VM inhibited the effect of the infection with K88 positive ETEC on the small intestinal mucosa as proposed by Corpet (1999) and Anderson *et al.* (2000).

The numbers of goblet cells per 100 μm crypt depth of the VM group, seven days post challenge, were increased compared to those in the C group. Also when the smaller crypt depth in the VM group was taken into account, the number of goblet cells per crypt was higher. This indicated a

probiotic effect of VM (Mouwen, 1996).

Comparing the morphological parameters measured in experiment 1 and 2, a difference in V/C ratio was observed. In average V/C ratio was in experiment 1 and 2, 2.1 and 1.4, respectively. This difference illustrated the variation in the morphological characteristics between clinically healthy piglets. Also differences in villus/crypt ratios were observed by van Beers-Schreurs (1996) between piglets derived from sows with a history of post-weaning diarrhoea and piglets from SPF sows, free of *E. coli* associated with diarrhoea.

The correlation between daily weight gain and villus length was significant in experiment 1, as also described by Pluske (1993) in post- weaning piglets, whereas this correlation was not significant in experiment 2. Indications that genetic variation is a factor with regard to intestinal morphology were also found in experiment 1 by the observed litter differences in the numbers of goblet cells. The differences in the dimensions of villi and crypts, and the explaining value of these parameters seem to depend on factors as genetics and experimental conditions.

In conclusion, the addition of virginiamycin to the diet had probiotic and protective effects on the small intestinal mucosal morphology and on the performance of piglets.

REFERENCES

Anderson, D.B., V.J. McCracken, R.I. Aminov, J.M. Simpson, R.I. Mackie, M.W.A. Verstegen,. Gaskins H.R (2000) Gut microbiology and growth-promoting antibiotics in swine. Nutrition Abstracts and Reviews 70: 2, 101 - 108.

Commission on Antimicrobial Feed Additives (1997) Antimicrobial Feed Additives. Government Official reports, SOU 1997: 132, Ministry of Agriculture, Stockholm, Sweden.

Corpet, D.E. (1999) Mechanism of antimicrobial growth promoters used in animal feed. Comptes Rendus de l'Academie d'Agriculture de France 85: 7, 197-205.

Crovetto, G.M., Tamburini, A., Rapetti, L., Boselli, E (1993) Effect of including growth promoters in diets with different nutritive value for pigs. Atti del XX Meeting Annuale della Societa Italiana di Patologia ed Allevamento dei Suini, Parma. Italy. 135- 140.

Decuypere, J.A., Dierick, N.A., Vervaeke, I.J., Henderickx, H.K. (1991) Influence of virginiamycin on the digestive physiology in precaecal re-entrant cannulated pigs. Archieves of Animal Nutrition 41: 4, 373-393.

Greypens, B., Claus, D., Evenepoel, P., Hiele, M., Maes, B., Peeters, M., Rutgeerts P., Ghoos, Y. (1997) Influence of dietary protein supplements on the formation of bacterial metabolites in the colon. Gut 41, 70-76.

Hampson, D.J. and and Kidder, D.E. (1986) Alterations in piglet small intestinal structure at weaning. Res. Vet. Sci. 40, 126-133. Suppl.

Hathaway, M,R., Kretchmar, D.H., Allen, C.E. Cornelius, S.G., Dayton, W.R. (1990a) In vitro muscle cell proliferation and protein turnover as affected by serum from pigs fed antimicrobials. Journal of Animal Science. 68, 3190-3197.

Hathaway, M,R., Pampusch, M.S., Cornelius, S.G., Allen, C.E., Dayton, W.R. (1990b) Antimicrobial supplementation of growing pigs: The effect of porcine sera fractions on in vitro muscle cell proliferation. Journal of Animal Science. 68, 3642-3148.

Hays, V.W. (1991) Effects of antibiotics. In: Growth Regulation in Farm Animals. Advances in Meat Research. Volume 7. Eds: A.M. Pearson; T.R. Dutson. Elsevier Applied Science, New York, 299-320.

Hedde, R.D., and Lindsey, T.O. (1986) Virginiamycin: A nutritional tool for swine production. Agri-Practise. 7, (3) (4).

Jensen, B.B. (1988) Effect of diet composition and virginiamycin on microbial activity in the digestive tract of pigs. Proc. of the 4th International Symposium on Digestive Physiology in the Pig. Edited by Buraczewska, L., Buraczewski, S., Pastuszewska, B., Zebrowska, T. Jablonna, Poland.

Kenworthy, R. (1976) Observations on the effect of weaning in the young pig. Clinical and histopathological studies of the intestinal function and morphology. Res. Vet. Sci. 21, 69-75.

Kies, A., Kiener, T., Maillard, R., Venuat, M., Richard, A., Herin, J.F. (1991) Spiramycin : comparison with other growth promoters and influence on the true ileal digestibility. Journees de la Recherche Porcine en France. 23 : 135-140.

Kik, M.J.L., Huisman., J., van der Poel, A.F.B, and Mouwen J.M.V.M. (1990) Pathological changes of the small intestinal mucosa of pigs after feeding Phaseolus vulgaris beans. Veterinary Pathology 27. 329-334.

Kik, M.J.L., P. van Leeuwen, J.E. van Dijk, and Mouwen, J.M.V.M (1988) A small intestinal biopsy technique in cannulated piglets. Journal of Animal Physiology and Animal Nutrition. 60, 123-127

McCullagh, A. and Nelder, J.A. (1989) Generalized Linear Models. 2nd ed. Chapman and Hall (Editors), 2- 6 Boundary Row, London SE7 8 HN.

Meijer, J.C., L. van Gils, B. Blokland, B. de Geus, M. Harmsen, M. Nabuurs, F. van Zijderveld (1997) Weaned piglets orally challenged with E. coli following stress as a model of post weaning diarrhoea. In: Gastrointestinal disorders, 15 September 1997, ID- DLO, Lelystad, The Netherlands.

Mouwen, J.M.V.M. (1972) White scours in piglets at three weeks of age. PhD Thesis. University of Utrecht, The Netherlands.

Mouwen, J.M.V.M. (1996) Enteric condition in relation to nutrition and animal health. 5th International Symposium on Animal Nutrition, Kaposvár, Hungary, p. 2-10.

Nabuurs, M.J.A., Hoogendoorn, A., van der Molen, E.J., van Ostra A.L.M. (1993) Villus height and crypt depth in weaned pigs, reared under various circumstances in the Netherlands. Res. Vet. Sci. 55, 78-84.

Norusis, M.J. (1992). System Users' Guide SPSS/PC Version 5.0 Base. Statistical Package for Social Sciences Chicago. IL.

Pluske, J.R. 1993 Psychological and nutritional stress in pigs at weaning: production parameters, the stress response, and histology and biochemistry of the small intestine. PhD Thesis, University of Western Australia, Perth, Australia. SAS (1991).

SAS® User's Guide: Statistics. Version 6.03 Edition, SAS Institute, Inc. Cary, NC.

Van Beers-Schreurs, H.M.G. (1996) The changes in the function of the large intestine of weaned pigs. PhD Thesis. University of Utrecht, The Netherlands.

Verdonk„ J.M.A.J., Spreeuwenberg, M.A.M., Bakker, G.C.M. and Verstegen M.W.A. (2000) Nutrient intake level affects histology and permeability of the small intestine in newly weaned piglets. Proc. of the 8th Symposium on Digestive Physiology in Pigs. Edited by J.E. Lindberg and B. Ogle, Uppsala, Sweden.

Visek, W.J. (1978) The mode of growth promoting by antibiotics. Journal of Animal Science 46, 1447-1469.

Chapter 7

Effects of a lactoperoxidase system and lactoferrin, added to a milk replacer diet, on severity of diarrhoea, intestinal morphology and microbiology of digesta and faeces in young calves

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ABSTRACT

The objective of the present study was to determine the effects of the combination of a lactoperoxidase system (LP-s) and lactoferrin (LF) added to a milk replacer diet on severity of diarrhoea, morphology of the small intestinal mucosa, and microbiology of digesta and faeces in young calves compared to a control diet. The experiment was conducted with 15 young calves per treatment, during the period of 7 to 21 days of age. During this period, calves are sensitive to gastrointestinal disturbances that can cause diarrhoea.

The results showed in the LP-s/LF group a significantly ($P < 0.05$) reduced severity of diarrhoea compared to the control group as assessed by faecal consistency scores. Numbers of CFU (colony forming units) of *Escherichia coli* in jejunal and colonic digesta and in faeces were lower in the LP-s/LF group compared to the control group. The differences were significant in both colonic digesta ($P < 0.1$) and in faeces ($P < 0.05$). Dissecting microscopy of the small intestinal mucosa indicated more finger shaped villi in the distal jejunum of LP-s/LF treated calves compared to the control group ($P < 0.05$). Histometrical measurements showed that these villi were significantly ($P < 0.05$) longer.

INTRODUCTION

In dairy herds in the Netherlands, calves, which are destinated for fattening, are weaned before three weeks of age, moved to a calf-fattening unit and changed from cow's milk to a milk replacer. Following weaning, the incidence of diarrhoea and mortality is usually higher than that for unweaned calves (Reynolds *et al*., 1981). In conventional veal calf production, antibiotics are added to the milk replacer to reduce gastrointestinal (GI) disorders caused by pathogenic bacteria in the gut. However, recent legislation restricts the addition of antibiotics in diets for calves (EC, 1998) because of possible repercussions on human health (Van den Boogaard and Stobberingh, 1996).

In the present study a combination of two bioactive proteins, lactoperoxidase system (LP-s) and lactoferrin (LF), which occur naturally in raw milk and in other biological fluids such as saliva and tears, were added to the milk replacer. The chemical and biological properties of the LP-s have been reviewed by De Wit and Van Hoyden (1996) and Reiter and Perraudin (1991). The LP-s consists of three components, the LP enzyme (EC 1.11.1.7), hydrogenperoxide (H_2O_2) and

thiocyanate (SCN^-). The lactoperoxidase catalyses the redox reaction: $H_2O_2 + SCN^- \rightarrow OSCN^- + H_2O$. The hypothiocyanous acid ($OSCN^-$) is in equilibrium with hydrogen hypothiocyanite (HOSCN) at pH 5.3. Both compounds, particularly the uncharged HOSCN, have antibacterial properties (de Wit and van Hooydonk, 1996).

The structure and functions of LF were reviewed by Lönnerdal and Iyer (1995). The LF, which is a glycoprotein present in raw milk, is able to bind two ferric ions like transferrin in blood plasma. Due to this iron binding capacity, LF has effects on the microbial composition of the intestinal contents (Bullen *et al.,* 1972). Several studies have shown that LF acts directly against bacterial cells (Dalmastri *et al.,*1988; Ellison *et al.,*1988; Ellison *et al.,* 1991; Erdei *et al.,* 1994). The effect of LF on iron absorption possibly depends on its ability to bind to the species specific lactoferrin receptors on the surface of the intestine (Gislason *et al.,* 1993; Lönnerdal, 1996; Kume and Tanabe, 1996). When the LF is unable to bind with the epithelium, the protein may be digested by proteases in the intestinal lumen and the ferrous iron is absorbed. The LF also has antioxidative effects (Cohen *et al.*, 1992).

The present study was carried out to investigate the effect of adding both LP-s and LF to milk replacer on the incidence and severity of diarrhoea, the intestinal microbiology, and morphology of the small intestinal mucosa.

MATERIALS AND METHODS

Animals and rations

Thirty Frisian-Dutch calves approximately 7 days of age were purchased from Dutch dairy farms. After arrival at the Research Institute (day 0), each calf was assigned to one of two treatment groups (n = 15), equalised on body weight. The calves were housed in individual wooden stalls and were fed a commercial milk replacer diet (Table 1) without antibiotics.

Prior to feeding, the milk replacer powder was mixed with water at an approximate temperature of 70˚C. After 4 min of mixing, delactosed whey powder concentrate with the appropriate amount of bovine LP-s/bovine LF (DMV International, Veghel, The Netherlands) was added to the milk for the LP-s/LF group. For the control group, the same amount of delactosed whey powder was added to the milk as placebo. During a further 2 min of mixing, additional water with a temperature of 20˚C was added to reduce the temperature of the milk mixture. The final concentration of milk

replacer was 110 g milk replacer powder in 890 g water, and the milk had a temperature of approximate 40˚C.

Table 1. Composition of calf milk replacer diet.

Ingredients	Concentration (g/kg)
Cheese whey powder	345
Delactosed whey powder	339
Whey permeate	101
Hydrolysed wheat protein	55
Fat (90 % lard, 10 % cocos)	138
Minerals and vitamins	2
Calcium formiate and citric acid	15
Synthetic amino acids (methionine, lysine)	4
Flavours and odours	1
Nutrients (Calculated)	
Moisture	37
Crude protein	191
Crude fat	150
Crude fibre	0
Ash (Fe 100 ppm)	100
Lactose	467
Starch	3
Lysine	14.4
Tryptophan	2.4
Methionine	4.5
Cystine	3.8

For the LP-s/LF group, the milk was added with 11.5 g delactosed whey powder containing 200 mg LP, 120 mg potassium thiocyanate (KSCN), 225 mg sodium carbonate peroxyhydrate ($2Na_2CO_3.3H_2O_2$) and 1000 mg of bovine LF (20 % Fe saturated)/kg milk replacer powder, which means a final concentration of 22 mg LP and 110 mg LF/kg liquid milk. Both treatment groups were fed twice daily (08.00 and 15.00 hours) according to a restricted feeding schedule, as shown in table 2. Each day, calves were exposed to 9 h of light starting from one hour before the first feeding in the morning until one hour after the second feeding in the afternoon. The temperature in the experimental unit was approximately 22°C.

Table 2. Feeding schedule during the experimental period.

Day on the test	Amounts fed
Day 0	Water only
Day 1	1.5 l electrolyte solution* and 1.5 l milk replacer
Day 2 and 3	3.0 l milk replacer
Day 4	1.5 l electrolyte solution and 1.5 l milk replacer
Day 5 and 6	3.5 l milk replacer
Day 7	1.75 l electrolyte solution, 1.75 l milk replacer
Day 8, 9 and 10	4.0 l milk replacer
Day 11 and 12	4.5 l milk replacer
Day 13	5.0 l milk replacer

*34g sodium carbonate, 44g sodium chloride, 5g potassium chloride and 167g dextrose/l water.

The experiment was concluded on day 13 after arrival. Feed intake was determined daily, on day 0 and day 13, body weights (BW) were determined and over the period day 0 to day 13 body weight gains (BWG) were calculated. From day 1 to day 11, a record was made on visual observations of the faecal consistency with: 0, for normal pasty faeces; 1, for thin faeces and 2, for water-thin faeces and 3, for diarrhoea. Faecal samples were taken daily from the rectum of each animal from day 6 to day 10 for determination of bacterial counts. On day 14, animals were placed under general anaesthesia for sampling the intestinal tissues. At 30 min after feeding animals, ring shaped intestinal segments, 1 cm wide, were taken at the following sites: sample 1, 0.75 m distal from the ligament of Treitz; sample 2, 3 m distal from the ligament of Treitz; and sample 3, 0.5 m proximal to the ileocaecal ligament. Samples 1, 2 and 3 were evaluated using a dissecting

microscope. In addition, sample 3 was examined for histometrical parameters.
All procedures were approved by the TNO Committee for Animal Welfare.

Morphological and microbiological parameters

Samples of the small intestine were cut open longitudinally at the antimesenteric side, affixed on dental wax with the villi on the upper side and fixed in 0.1 M phosphate-buffered 4% formaldehyde solution. The shape of the villi was studied with a dissecting microscope and characterized according to a previously described procedure (Mouwen, 1972) using the criteria as shown in table 3.

Table 3. Description of villi gradation determined by dissection microscopic examination (Mouwen, 1972).

Score	Description
0	All finger-shaped villi or mostly finger-shaped villi with a few tongue shaped villi
1	Mixed finger- and tongue-shaped villi
0.5	Predominantly long to short tongue-shaped villi with few finger shaped and leaf shaped ones
1.5	Predominantly short tongue- and leaf-shaped villi with few long tongue and ridge shaped villi
2	Mixture of short tongue-, leaf- and ridge-shaped and convoluted villi
2.5	Similar to grade 2, with flat areas
3	Flat mucosa

In this evaluation the mesenteric part of the mucosa, outside of the Peyer's patches, was taken into consideration. After the dissecting microscopic characterization of the samples from the distal jejunum, 3 mm wide longitudinal zones from the mesenteric site were cut at right angles to the surface of the mucosa and embedded in paraffin wax. Sections were cut (5 μm) and stained with haematoxylin and eosin (HE) and the periodic acid-Schiff method (PAS). The procedures for staining and determinations of villus height, crypt depth and the numbers of goblet cells (n/100 μm crypt) were described by Kik *et al.* (1990).

The number of colony forming units (CFU) of *Escherichia coli (E. coli)* in faeces was determined according to the IDF standard (1985), *E. coli* in digesta according to the IDF standard (1997),

lactic acid producing bacteria (LAB) in digesta according to the FNZ standard (1986), and salmonellae according to ISO (1993) and the NEN standards (1994).

Statistical analysis

Data were subjected to analysis of variance using the software SPSS/PC+V5.0 software (Norusis, 1992). The diet type was treatment factor according to the following model:

$y = \mu + D_i + e_{ij}$,

where: y = response to the measurements, μ= mean value, D_i= diet type, e_{ij} = residual error.

RESULTS

Feed intake, daily weight gain and faecal consistency

Animals arrived at the Institute in good health. During the first few days after the arrival some calves refused feed and developed diarrhoea. Diarrhoea was treatment by feeding the individual animal an electrolyte solution instead of milk replacer.

Table 4. Mean feed intake, body weights, growth and faecal scores.

	Control (n = 14)	LP-s/LF (n = 15)	Significance[1]
Feed intake (g/animal per day)	322 (12.0)[2]	347 (11.6)	ns
BW[3] at day 0 (kg)	43.1 (0.39)	43.0 (0.38)	ns
BW at day 13 (kg)	42.7 (0.55)	43.6 (0.53)	ns
BWG[4] (g/animal per day)	-32 (42.8)	42 (41.4)	ns
Faecal score[5]	0.80 (0.064)	0.61 (0.062)	*

[1] ns = difference was not significant (P>0.1); * = difference was significant (P < 0.05); [2] standard errors of means
[3]BW = body weight, [4]BWG= body weight gain;[5] faecal score: 0, pasty faeces; 1, thin faeces; 2, water thin faeces; 3, diarrhoea.

At day 6, one animal from the control group was euthanized because intramuscular injections with an antibiotic (Exenel®, Pharmacia and Upjohn, Woerden, The Netherlands) failed to alleviate the severe diarrhoea, which it suffered. This was the only calf treated in this way; none of the others received antibiotics.

Data concerning feed intake, BW, BWG and faecal scores are presented in table 4. Feed intake was similar between groups. The BWG of the control group was negative (-32 g/animal per day) while the LP-s/LF group had a positive (42 g/animal per day) growth, which was not significantly different ($P > 0.1$). The faecal consistency scores over the 13 days experimental period are given in table 2 and figure 1. The mean faecal consistency score for the control was 0.80 and that for the LP-s/LF group 0.61 ($P < 0.05$). For the major part of the experimental period the LP-s/LF group had a lower mean faecal consistency score than the control group. The differences were significant ($P < 0.05$) at days 4, 8 and 9.

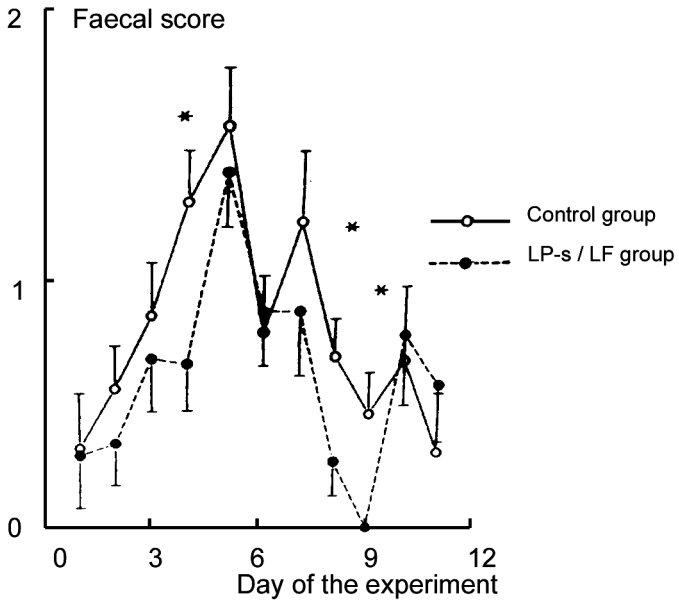

Figure 1. Mean faecal scores with: 0, pasty faeces; 1, thin faeces; 2, water thin faeces; 3, diarrhoea (y-axis) and days of the experimental period (x-axis). Bars represent standard errors of means, *significant difference ($P < 0.05$).

Small intestinal morphology

Under the dissecting microscope, no differences in morphology of the villus shape were found in the proximal and mid jejunum (Table 5). However, in the distal jejunum, the villus shape scores were higher ($P < 0.05$), indicating more slender villi, in the control calves compared to those of the LP-s/LF group. Histometrically, the mean villus height in the distal jejunum of the LP-s/LF group was significantly higher ($P < 0.05$) than that of the control group. The crypt depth, and goblet cell density were not significantly different ($P > 0.1$) between the groups.

Table 5. Morphological and histometrical characteristics at the proximal jejunum (0.75 m distal from the ligament of Treitz), mid jejunum (3 m distal from the ligament of Treitz) and distal jejunum (0.5 m proximal of the ileo caecal ligament).

Site, parameter	Control (n = 14)	LP-s/LF (n = 15)	Significance[1]
Proximal jejunum			
Villus shape score[2]	0.4 (0.01[3])	0.4 (0.01)	ns
Mid jejunum			
Villus shape score	0.3 (0.07)	0.2 (0.07)	ns
Distal jejunum			
Villus shape score	1.3 (0.15)	0.7 (0.15)	*
Villus height (µm)	229 (13.8)	295 (13.3)	*
Crypt depth (µm)	260 (14.6)	263 (14.1)	ns
Villus height/crypt depth	0.91 (0.62)	1.15 (0.60)	*
Goblet cells (n/100µm crypt)	6.1 (0.65)	6.9 (0.62)	ns

[1]ns = difference was not significant (P > 0.05); * = difference was significant (P < 0.05); [2]scale: 0 (ideal) to 3 (highly affected; table 3); [3] standard errors of means.

Microbiological parameters

The CFU of *E. coli* in the faeces of the control calves was significantly (P < 0.05) higher than in the LP-s/LF group (Table 6). Also the CFU of *E. coli* in the colonic digesta of the control calves was significantly (P < 0.1) higher than in the LP-s/LF group. There were no significant differences (P > 0.1) for the CFU of *E. coli* in the digesta from the jejunum. Counts of lactic acid producing bacteria (LAB) in the jejunum and colon were not significantly different (P > 0.1) between the groups. The colonic contents of one animal of the control group were positive for *Salmonella* whereas no *Salmonella* positive animals were found in the LP-s/LF group.

Table 6. Microbiological counts different sites of the gastro-intestinal tract.

Site	Control (n = 14)	LP-s/LF (n = 15)	Significance[1]
Faeces (day 1 to 11)			
E.coli (log CFU[2])	7.5 (0.11[3])	7.0 (0.10)	*
Digesta from jejunum (samples taken at dissection, day 14)			
E.coli (log CFU)	2.8 (0.03)	2.7 (0.02)	ns
LAB[4] (log CFU)	4.8 (0.35)	4.5 (0.34)	ns
Salmonella	negative	negative	
Digesta from colon (samples taken at dissection, day 14)			
E.coli (log CFU)	5.3 (0.44)	4.2 (0.48)	+
LAB (log CFU)	7.9 (0.21)	7.6 (0.22)	ns
Salmonella	one animal positive	negative	

[1] ns = difference was not significant (P>0.1); * = difference was significant (P < 0.05); + = difference was significant (P < 0.1); [2] CFU = colony forming units; [3] standard errors of means [4] LAB = lactic acid producing bacteria.

DISCUSSION

The concentration of lactoperoxidase (LP) in colostrum is initially low, and increases to a maximum concentration at 4 to 5 days postpartum and thereafter, the concentration of LP in bovine milk is on average 10 - 30 mg/l (de Wit and van Hooydonk, 1996; Reiter and Perraudin, 1991). The concentration of lactoferrin (LF) in cow's milk is approximately 10 mg/l, however, the concentration of LF in the early lactation is higher than in later lactation (Lönnerdal, 1996). The relative high concentrations of LP and LF in the colostrum indicate their significance for the new-born calf. The naturally occurring proteins LP and LF are at least partly inactivated in industrial processes such as pasteurisation and evaporation (de Wit and van Hooydonk, 1996). Because of their inactivation at temperatures above 65 - 70˚C (Kussendrager, 1993), dairy milk replacers, which have whey proteins as the main protein source, therefore lack LP and LF.

In the present experiment 22 mg LP and 110 mg LF/kg liquid milk was added and the effects of LP-s/LF was added to a whey powder-based milk replacer. To study the bio-activity of the LP-s/LF concentrate, the added activity of LP was similar and concentration of LF was higher than the naturally occurring levels in milk.

In the present experiment faecal consistency score of the LP-s/LF group was significantly (P <

0.05) improved compared to the control group. Possibly due to the short experimental period (13 days) or to low level of diarrhoea in this experiment, the average of feed intake, BW and BWG of the LP-s/LF group were only slightly, not significant ($P > 0.1$), improved compared to the control group. Reiter and Perraudin (1991) showed positive effects of LP-s on live weight change in field trials whereas they found more pronounced effects in animals with increased frequency of scouring. Still *et al.* (1989) studied the effects of a combination of LP-s and LF on the severity of diarrhoea in calves for the period 0 to 6 days after an experimental *E. coli* infection. They concluded that LP-s/LF had preventing and curing effects in the *E. coli*-infected calves. The significantly lower CFU of the colonic contents and faeces of the LPs/LF group ($P < 0.10$ and $P < 0.05$, respectively), compared to the control group confirm the results of Still *et al.* (1989).

Antibacterial activity of LP-s towards enterotoxigenic stains of *E. coli* were proven *in vitro* by Grieve *et al.* (1992). Hampson *et al*. (1985) suggested that in weaned piglets the hemolytic *E. coli*, which causes malaborption, colonizes the anterior small intestine from the distal part of the GI tract. Assuming similarities between the development of the GI disorders in weaned calves and in piglets, then in calves also the pathogenic *E. coli* appear to colonize the anterior small intestine from the colon. This explains why the effects of LP-s/LF on the *E. coli* in calves were more pronounced in the distal part of the GI tract and less in the proximal part and that no significant difference ($P > 0.1$) for the CFU of *E. coli* was observed in the jejunal digesta.

In the present experiment colonic contents from one animal of the control group were positive on *Salmonella* whereas no *Salmonella* positive animals were found in the LP-s/LF group. Reiter and Perraudin (1991) also observed inhibiting effects of LP-s on Gram-negative bacteria such as *Salmonella*.

The CFU of lactic acid producing bacteria (LAB) in the jejunum and colon were similar for both groups, which indicate no major implications of the bacteriostatic effects of LP-s/LF on the LAB. Saito *et al.* (1996) investigated the effect of LF on the growth of *Bifidobacterium* strains *in vitro* and they found a slight increase of bifidobacteria proliferation.

In the distal jejunum, significantly more ($P < 0.05$) finger-shaped villi and the higher ($P < 0.05$) villi were observed in the LP-s/LF group compared to the control group. This indicates less noxious stress, possibly *E. coli*, from the intestinal lumen (Mouwen *et al.,* 1983). The cell growth-stimulating activity of LF in an intestinal cell line of rats has also been observed (Hagiwara *et al.,* 1995).

In summary, the addition of LP-s/LF addition to milk replacer decreased severity of diarrhoea and

had a beneficial effect on the shape and height of the villi in the distal jejunum of young calves in comparison with a control group without supplementation. Furthermore, the numbers of *E. coli* in faeces and colonic digesta were decreased in young calves fed a milk replacer diet supplemented with LP-s/LF. The results of present study confirm the results of Still *et al.* (1989).

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REFERENCES

Bullen J.J., Rogers, H.J. and Leigh L. (1972) Iron-binding proteins in milk and resistance to Escherichia coli infections in infants. *British Medical Journal* 1 (792) 69-75.

Cohen, M.S., Mao, J., Rasmussen, G. T., Serody, J.S., Britigan, B.E. (1992) Interaction of lactoferrin and lipopolysaccharide (LPS): Effects on the antioxidant property of lactoferrin and the ability of LPS to prime human neutrophils for enhanced superoxide formation. *Journal of Infectious Diseases* 166. 1372-1378.

Dalmastri C., Valenti P., Visca P., Vittorioso P. and Orsi N. (1988) Enhanced antimicrobial activity of lactoferrin by binding to the bacterial surface. *Microbiologica* 11. 225-230.

De Wit J.N. and van Hooydonk A.C.M. (1996) Structure, functions and applications of lactoperoxidase in natural antimicrobial systems. *Netherlands Milk and Dairy Journal* 50. 227-224.

EC (European Community) (1998) Regulation 2821/ 98/ EC, 17-12-1998. Publication Office of EC, 11 Rue Christofophe Plantin L- 2339, Luxembourg.

Ellison R.T., Giehl T.J. and Laforce F.M. (1988) Damage of the outer membrane of enteric gram-negative bacteria by lactoferrin and transferrin. *Infection and Immunity* 56. 2774-2781.

Ellison R.T., Giehl T.J. and Laforce F.M. (1991) Killing of gram-negative bacteria by lactoferrin and lysozyme. *Journal of Clinical Investigation* 88. 1080-1091.

Erdei J., Forsgren A. and Naidu A.S. (1994) Lactoferrin binds to porcins Omp F and Omp C in Escherichia coli. *Infection and Immunnity* 62. 1236-1240.

FNZ (Federatie Nederlandse Zuivelindustrie) (1986) 53.29. Present name: NZO (Nederlands Zuivel Organizatie), P.O.Box 165, 2700AD Zoetermeer, The Netherlands.

Gislason, J., Suhasini, I., Hutchens, W.T. and Lönnerdal, B. (1993) Lactoferrin receptors in piglet small intestine: Lactoferrin binding properties, ontogeny, and regional distribution in the gastrointestinal tract. *Journal of Nutrition Biochemistry* 4. 528- 533.

Grieve, P.A., Dionysius D.A. and Vos, A.C. (1992) In vitro antibacterial activity of the lactoperoxidase system towards enterotoxigenic strains of Escherichia coli. *Journal of Veterinary Medicine* B39. 537-545.

Hagiwara, T., Shinoda, I., Fukuwatari, Y., Shimamura, S. (1995) Effects of lactoferrin and its peptides on proliferation of rat intestinal epithelial cell line, IE-18 in the presence of epidermal growth factor. *Bioscience, Biotechnology and Biochemistry* 59, 10. 1875-1881.

Hampson, D.I., Hinton, M., Kidder, D.E. (1986) *Journal of Comparative Pathology*. 95, 353-362.

IDF (International Dairy Federation) (1985) Standard 73A. IDF, 41, Square Vergrote, B-1030 Brussels, Belgium.

IDF (International Dairy Federation) (1997) Standard 170, part 3. IDF, Square Vergrote 41, B-1030 Brussels, Belgium.

ISO (International Organization for Standardization) (1993) Publication 6579 . ISO, P.O.Box 5059, 2600 GB Delft, The Netherlands.

Kik M.J.L., Huisman J., van der Poel A.F.B. and Mouwen J.M.V.M. (1990) Pathologic changes of the small intestinal mucosa of pigs after feeding Phaseolus vulgaris beans. *Veterinary Pathology* 27. 329-334.

Kume S.I. and Tanabe S. (1996) Effect of supplemental lactoferrin with ferrous iron on iron status of newborn calves. *Journal of Dairy Science* 79. 459-464.

Kussendrager, K.D. (1993) Effects of heat treatment on structure and iron-binding capacity of bovine lactoferrin. In *Proceedings of the IDF Seminar Indigenous Antimicrobial Agents of Milk* pp.133-146. Uppsala, Sweden: IDF, Brussels, Belgium.

Lönnerdal, B. and Iyer, S. (1995) Lactoferrin molecular structure and biological function. *Annual Review Nutrition* 15. 93-110.

Lönnerdal B. (1996) Lactoferrin in milk. *Annales Nestlé* 54. 79-87.

Mouwen J.M.V.M. (1972) White scours in piglets at three weeks of age. (PhD Thesis), University of Utrecht, The Netherlands.

Mouwen J.M.V.M., Egberts H.J.A. and Koninkx J.F.J.G. (1983) The outermost mucosal barrier of the mammalian small intestine. *Deutsche Tierärztliche Wochenschrift* 90. 477-482.

NEN (Nederland Normalisatie Instituut) (1994) Publication 6871. NEN, P.O. Box 5059, 2600 GB

Delft, The Netherlands.

Norusis, M.J. (1992). System Users' Guide SPSS/PC Version 5.0 Base.Statistical Package for Social Sciences Chicago. IL.

Reiter, B. and Perraudin J-P. (1991) Lactoperoxidase. Biological Functions. In *Peroxidases in Chemistry and Biology* Vol. 1, pp. 143- 180 [J. Everse, K.E. Everse, M.B. Gruisham, editors] Boca Raton, FL, U.S.A.: C.R.C. Press Inc.

Reynolds, D.J., Morgan, J.H., Chanter, H. (1981) Microbiology of calf diarrheoa in Southern Britain. *Veterinary Records* 119. 34-39.

Saito, H., Miyakawa, H., Ishibashi, N., Tamura. Y., Hayasawa., H. and Shimamura S. (1996) Effect of Iron-free and metal bound forms of lactoferrin on the growth of bifidobacteria, E. coli and S. aureus. *Bioscience Microflora* 15 (1). 1-7.

Still, J., Delahaut, P., Coppe, P., Kaeckenbeeck, A. and Perraudin J.-P. (1990) Treatment of induced enterotoxigenic colibacillosis (scours) in calves by the lactoperoxidase system and lactoferrin. *Annales de Recherche Vétérinaires* 21. 143-152.

Van den Boogaard, A.E. and Stobberingh, E.E. (1996) Time to ban all antibiotics as animal growth- promoting agents? *The Lancet* 348. 619.

Chapter 8

Dietary effects of faba bean (*Vicia faba* L.) tannins on the morphology and function of the small intestinal mucosa of weaned pigs

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ABSTRACT

The objective of present study was to evaluate effects of condensed tannins in faba beans (*Vicia faba* L.) on morphological and functional parameters of the small intestinal mucosa of piglets. In an experiment with young piglets (8 to 17 kg body weight), fed either a control diet or a diet containing 200 g/kg of low- or high-tannin faba bean hulls (with < 0.10 % and 3.3% catechin equivalents of condensed tannins, respectively), morphological and functional characteristics of the jejunal mucosa were determined.

Results of present showed that the morphological variables of the mucosa of the three groups of piglets were similar. Also, no changes due to dietary tannins in sucrase (EC 3.2.1.48) -isomaltase (EC 3.2.1.10) activity in homogenates of mucosa plus submucosa were observed. However, aminopeptidase (EC 3.4.11.2) activity in these homogenates in the proximal part of the small intestine of the animals of the group fed the high-tannin diet was significantly lower than that in the animals fed on control diet or the diet with low-tannin hulls ($P < 0.05$).

INTRODUCTION

Faba beans (*Vicia faba* L.) are an important protein source in rations for livestock. However, their nutritive value is limited by the presence of condensed tannins (Marquardt *et al.*, 1977; Martin-Tanguy *et al.*, 1977; Jansman, 1993). Condensed tannins are water-soluble polyphenolic compounds with the ability to precipitate proteins from aqueous solutions (Bate-Smith and Swain, 1962) and are found particularly in the hull fraction of coloured-flowering faba bean varieties (Bos and Jetten, 1989). Tannins have been shown to reduce apparent protein digestibility in diets for non-ruminant animal species (Salunkhe *et al.*, 1990). The white-flowering varieties have a much lower tannin content than the coloured varieties and are generally more digestible. An explanation for the antinutritional effect of tannins is that these compounds have an affinity for proteins which results in poorly digestible tannin-protein complexes. Experiments with rats proved that the protein of these complexes originates partly from feed and partly from salivatory protein (Jansman, 1993). Also, an increased endogenous N excretion was observed in piglets after feeding tannin-containing diets (Jansman *et al.*, 1993). This finding indicates an enhanced turnover of mucus and/or glycocalyx. The mucus layer and the glycocalyx are important factors in the protection of epithelial integrity of the small intestine (Mouwen *et al.*, 1983; Egberts *et al.*, 1984). In addition, the glycocalyx contains

various digestive enzymes (Egberts *et al.*, 1984). Binding of tannins to mucins or the glycocalyx may change their biochemical and physical properties. Effects of tannins on morphological variables of the intestinal mucosa have been observed in rats (Mitjavila *et al.*, 1977). Moreover, this binding could affect the functional capacity of the mucosa, such as the activity of brush-border enzymes.
The nutritional implications of tannin-rich hulls of faba beans have been studied in a digestibility experiment with piglets (Jansman *et al.*, 1993). The present study evaluated the effects of condensed tannins in hulls of faba beans on the mucosal structure and the activities of digestive enzymes of the small intestinal mucosa using tissue from the piglets used in the digestibility experiment.

MATERIALS AND METHODS

Animals and rations

Three groups of eight, seven and eight conventional piglets, with a mean body weight of 12 kg, were fed either a control ration (ration 1, treatment group 1) or any of the two rations containing 200 g/kg of hulls of a white- (ration 2; treatment group 2) and a coloured-flowering variety (ration 3; treatment group 3) of faba beans (*Vicia faba* L.). Each diet contained barley and maize as major ingredients. In ration 1 autoclaved hulls from peas (*Pisum sativum*) were added to ensure that the fibre content of the rations were the same. After a period of adaptation the animals were fed on the rations over an experimental period of 23 d. Throughout the experiment the animals were fed twice daily, at 08.00 and 17.00 hours. Water was freely available via drinking nipples. Details of the preparation of the hulls, feed formulation and on the zootechnical protocol have been described elsewhere (Jansman *et al.*, 1993).
On day 24, three samples of small intestine from each animal were taken under general anaesthesia. Sites were: sample a, 0.5 m distal from the ligament of Treitz; sample b, 5.5 m distal from the ligament of Treitz; sample c, 0.5 m proximal to the ileal-caecal ligament. The samples were evaluated on morphological and functional variables.
The experiment was approved by the TNO Committee for Animal Welfare.

Assays on morphological characteristics

Dissecting microscopy Each sample of intestinal tube was cut open longitudinally at the antimesenteric attachment, affixed on dental wax with the villi on the upper side and fixed in 0.1 M-phosphate-buffered formaldehyde solution (40 g/l). The shape of the villi was studied with a dissecting microscope and characterized according to a previously described procedure (Mouwen, 1972) using the following gradation system: grade 0, a normal villus pattern with almost all finger-shaped villi; grade 0.5, mixed finger- and tongue-shaped villi; grade 1, predominantly long to short tongue-shaped villi with few finger-shaped and leaf-shaped ones; grade 1.5, predominantly short tongue- and leaf-shaped villi with few long tongue- and ridge-shaped villi; grade 2, a mixture of short tongue-, leaf- and ridge-shaped and convoluted villi; grade 2.5, similar to grade 2, but with flat areas; grade 3, flat mucosa. For this evaluation the mesenteric part of the mucosa, outside of the Peyer's patches, was taken into consideration.

Morphometry and histochemistry After the dissecting microscopical study a 3 mm wide zone from the mesenteric site was cut at right angles to the surface of the mucosa and embedded in paraffin wax. As the Peyer's patches are on the antimesenteric site of the intestine the regions with Peyer's patches were not included in the microscopic slides studied. Sections were cut (5 μm) and stained with haematoxylin and eosin (HE staining) and the periodic acid-Schiff method (PAS staining). From these stained sections villus height, crypt depth, villus/crypt ratio, index of mitosis (meta- and anaphases) per 100 crypt cells, goblet cells (number per 100 μm crypt and number per crypt) were determined according to previously described procedures (Kik *et al.*, 1990).

Transmission electron microscopy (TEM) From each sample a piece of luminal tissue was taken from the site of the mesenteric attachment for TEM to determine the length of the microvilli. Tissue processing consisted of a first fixation in 0.1 M cacodylate-buffered glutaraldehyde (25ml/l; pH 7.35; 440 mosm) for 5 h. After this period the samples were rinsed with 0.1 M cacodylate buffer (pH 7.35) and stored in 0.1 M cacodylate buffer (pH 7.35) with 70 g sucrose/l at 4°C until final processing. Final processing consisted of post-fixation with osmium tetroxide in 0.1 M cacodylate buffer (20g/l; pH 7.35) for 16 h at 4°C followed by dehydration in graded water-acetone mixtures and embedding in Epon Araldite mixture. From the embedded samples ultrathin (600 - 800 Ä) sections were cut and stained with uranylmagnesium acetate and lead citrate. The stained sections were examined with a Philips CM 10 electron microscope at 80 kV.

Enzyme activity in homogenates of mucosa and submucosa

The functional variables investigated were sucrase-isomaltase and aminopeptidase activity of homogenates of jejunal mucosa plus submucosa. Samples of the intestinal tissue for these determinations were frozen in liquid nitrogen and stored at -70°C until analysis. Homogenates of mucosa plus submucosa were made and analysed for sucrase-isomaltase and aminopeptidase activity. The enzyme activities were expressed in units (U) per g of protein in the homogenates. Protein contents of the homogenates were determined as described (Lowry *et al.*, 1951).

Sucrase-isomaltase enzyme activity. The principle of the determination of sucrase (EC 3.2.1.48) - isomaltase (EC 3.2.1.10) activity is based on the degradation of sucrose (substrate) in glucose and fructose by sucrase-isomaltase in the homogenate. The determination of sucrase-isomaltase activity has been described previously (Messer and Dahlqvist, 1966). Briefly, the reaction time was 60 min at 37˚C and the glucose is determined after glucose-oxidase (EC 1.1.3.4)/peroxidase (EC 1.11.1.7) treatment, staining and measuring the intensity of the colour at 405 nm spectrophotometrically. The analyses were carried out as a micro method on microtitre plates. On the same plate standard dilutions of glucose and dilutions of the samples were made and measured after incubation with a microplate reader (BioRad model 3550; BioRad, Veenendaal, The Netherlands). Sucrase-isomaltase activity is expressed in units (U) per g of protein. One unit is equal to the production of 1 μmol glucose/min from the sucrose substrate

Aminopeptidase enzyme activity The determination of the aminopeptidase (EC 3.4.11.2) activity assay is based on the degradation of L-alanine-p-nitroanilide (substrate) in p-nitroaniline en L-alanine by the aminopeptidase in the homogenate. The method for the determination of aminopeptidase activity has been described previously (Maroux *et al.*, 1973). Briefly, the reaction time was 20 min at 37°C and the p-nitroaniline is determined by staining and measuring the intensity of the colour at 405 nm spectrophotometrically. The analyses were carried out as a micro method on microtitre plates. On the same plate standard dilutions of p-nitroaniline and dilutions of the samples were made and measured after incubation with a microplate reader (Biorad model 3550; BioRad, Veenendaal, The Netherlands). Aminopeptidase activity is expressed in units (U) per gram of protein. One unit is equal to the production of 1 μmol p-nitroaniline/min from the L-alanine-p-nitroanilide substrate.

Statistical analysis

One-way analysis of variance was carried out with software package SPSS/PC+ V5.0 (SPSS, 1992)

on the experimental data using treatment as a factor. If the treatment effect was significant, the differences between means were tested using the least significance difference (LSD) test (Snedecor and Cochran, 1980). The correlation between protein digestibility and aminopeptidase activity was analysed with software package SPSS/PC+ V5.0 (SPSS, 1992).

RESULTS

Morphological characteristics of the small intestinal mucosa

Villus height and crypt depth No significant differences ($P < 0.05$) in the villus height and crypt depth between the three groups of animals were observed (Table 1). The results show rather large differences in morphological parameters between animals (standard errors of means were 5 - 10 % of the absolute values). There was a general tendency for villus height and crypt depth to decrease from the proximal to the distal part of the small intestine.

Index of mitosis and number of goblet cells The results with respect to these variables were similar for the different groups and no significant changes due to the presence of tannins in the ration were observed (Table 2). The index of mitosis tended to be higher in the distal part of the small intestine.

Length of the microvilli No significant differences in length between the three groups were observed (Table 3). There was a tendency for the microvilli in samples b and c of group 2 to be longer than those of groups 1 and 3. This difference was not related with the tannin content in the rations.

Enzyme activity in homogenates of mucosa and submucosa

Sucrase-isomaltase activity The data of the biochemical analyses showed large standard errors of means (Table 4). The sucrase-isomaltase activities in sample b of the animals fed the rations with the faba bean hulls (groups 2 and 3) tended to be higher than that of the control group (1). This tendency was also found in sample c. However, these differences were not significant.

Aminopeptidase activity Aminopeptidase activity in samples a and b of animals on the high-tannin ration (3) was lower than in the groups fed the control ration (1) or the diet with low-tannin faba bean hulls (group 2, Table 4). No differences in aminopeptidase activity were found between three groups in sample c. The differences in aminopeptidase activity between group 3 (high-tannin) and groups 1 (control) and group 2 (low-tannin) were significant for sample 1 and for the mean values of samples a and b ($P < 0.05$).

Table 1. Morphological characteristics of the villi and crypts in different parts of the small intestinal mucosa in animals of the control (1) and experimental groups (2 and 3); samples a and b, respectively 0.5 and 5.5 m distal from the ligament of Treitz; sample c, 0.5 m proximal to the ileal-caecal ligament.

	Groups			
	1 (n=8)	2 (n=7)	3 (n=8)	SEM[1]
Dissecting microscopical gradation (0-3)				
Sample a	1.3	1.1	1.3	0.1
Sample b	1.2	1.3	1.2	0.2
Sample c	1.1	0.7	0.7	0.2
Mean a,b,c	1.2	1.0	1.1	0.1
Villus height (μm)				
Sample a	590	600	642	55
Sample b	540	559	599	38
Sample c	415	448	454	40
Mean a,b,c	515	536	565	27
Crypt depth (μm)				
Sample a	313	350	339	25
Sample b	309	304	318	20
Sample c	251	243	222	10
Mean a,b,c	291	299	293	14
Villus/crypt ratio (μm/μm)				
Sample a	1.9	1.8	2.0	0.2
Sample b	1.8	1.9	1.9	0.1
Sample c	1.7	1.9	2.1	0.2
Mean a,b,c	1.8	1.8	2.0	0.1

[1] Standard errors of means.

Table 2. Index of mitosis and number of goblet cells in different parts of the small intestinal mucosa in animals of the control (1) and experimental groups (2 and 3); samples a and b, respectively 0.5 and 5.5 m distal from the ligament of Treitz; sample c, 0.5 m proximal to the ileal-caecal ligament.

	Groups			
	1 (n=8)	2 (n=7)	3 (n=8)	SEM[1]
Index of mitosis (n/ 100 crypt cells)				
Sample a	1.8	1.9	1.6	0.3
Sample b	2.7	3.0	2.9	0.4
Sample c	4.3	3.1	4.1	0.5
Mean a,b,c	2.9	2.7	2.9	0.2
Goblet cells (number per crypt)				
Sample a	23.7	24.6	23.2	3.2
Sample b	20.8	20.2	21.3	1.9
Sample c	24.4	25.4	22.1	1.5
Mean a,b,c	22.9	23.4	22.2	1.6
Goblet cells (number per 100 μm crypt)				
Sample a	7.4	6.9	6.8	0.6
Sample b	6.8	6.8	6.6	0.5
Sample c	9.7	10.5	9.9	0.5
Mean a,b,c	8.0	8.1	7.8	0.4

[1] Standard errors of means.

Table 3. Length of microvilli in different parts of the small intestinal mucosa in animals of the control (1) and experimental groups (2 and 3); samples a and b, respectively 0.5 and 5.5 meter distal from the ligament of Treitz; sample c, 0.5 meter proximal to the ileal-caecal ligament.

	Groups			
	1 (n=8)	2 (n=7)	3 (n=8)	SEM[1]
Length of microvilli (µm)				
Sample a	1.5	1.6	1.6	0.1
Sample b	1.9	2.0	1.8	0.2
Sample c	1.9	2.1	1.6	0.2
Mean a,b,c	1.8	1.9	1.7	0.1

[1] Standard errors of means.

Table 4. Functional characteristics of the small intestinal tissue in animals of the control (1) and experimental groups (2 and 3); samples a and b, respectively 0.5 and 5.5 meter distal from the ligament of Treitz; sample c, 0.5 meter proximal to the ileal-caecal ligament.

	Groups			
	1 (n=8)	2 (n=7)	3 (n=8)	SEM[1]
*Sucrase-isomaltase activity (*Units/gram protein)				
Sample a	45	49	40	10
Sample b	61	78	86	14
Sample c	61	66	64	13
Mean a,b	53	64	63	9
Mean a,b,c	56	64	63	8
Aminopeptidase activity (Units/gram protein)				
Sample a	118^{a}	$111^{a,b}$	66^{b}	16
Sample b	109^{a}	108^{a}	65^{a}	19
Sample c	109^{a}	140^{a}	145^{a}	24
Mean a,b	113^{a}	110^{a}	65^{b}	13
Mean a,b,c	112^{a}	120^{a}	92^{a}	15

[1] Standard errors of means; [a,b]Different letters in the same row indicate significant differences ($P < 0.05$).

Correlation between apparent faecal digestibility of protein and aminopeptidase activity (Figure 1)
The aminopeptidase activity was depressed in the mucosa of animals fed on the ration with the high-tannin faba bean hulls (group 3). From the previous described nutritional evaluation (Jansman *et al.,* 1993), with the same rations, it was clear that a high tannin content in the ration decreases protein digestibility. Combining both observations the correlation between protein digestibility and aminopeptidase activity was calculated for the animals of group 3. Figure 1 shows the relationship in faecal protein digestibility (y-axis) and mean aminopeptidase activity of the samples a, b and c (x-axis). A significant positive correlation was found between protein digestibility and aminopeptidase activity in the mucosa ($R = 0.91$; $P < 0.002$; $y = 0.0745x + 67.8$). For groups 1 and 2 no significant correlations were found.

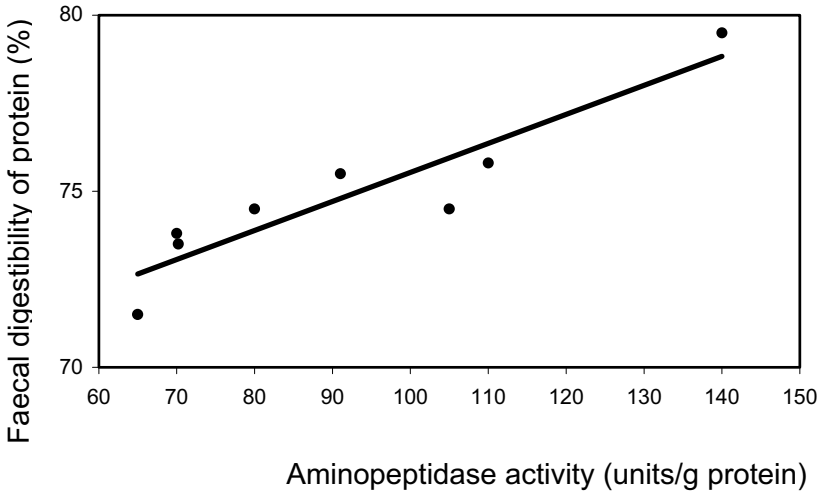


Figure 1. Correlation between aminopeptidase activity of the small intestinal tissue (x-axis) and the apparent faecal digestibility of protein (y-axis) determined in animals of the high-tannin group (3) ($y = 0.0745 x + 67.8$; $R = 0.91$; $P < 0.002$).

DISCUSSION

One of the properties of condensed tannins is their affinity to proteins. Tannins in the intestinal tract bind proteins from feed as well as endogenous proteins. The hypothesis underlaying the study was that, due to binding of tannins with proteins of the glycocalyx, the biochemical and physical properties of this layer change and induce morphological and/or functional changes of the small intestinal mucosa.

Samples of the intestinal tissue were derived from a nutritional experiment with weaned pigs

(Jansman *et al.*, 1993). The results of that study showed that protein digestion decreases due to dietary tannins from faba beans. The increase of faecal N losses was partly explained by the increase of excretion of low-digestible tannin-feed protein complexes. Also the excretion of endogenous N was increased (Jansman *et al.*, 1993).

The results of present study showed that morphological characteristics of the three groups of animals were similar and that dietary tannins did not induce significant changes. This means that tannins did not induce morphological changes of the small intestinal mucosa. However, morphological parameters of the small intestinal mucosa of the conventional piglets showed rather large differences between animals. These differences are related with differences in the local morphological structure of the mucosa, the conventional environment and/or genetic variation among the animals.

The biochemical activity in homogenates of mucosa and submucosa was also related with rather large variation. In man, distinct differences in intestinal brush border enzyme activity due to genetic variation are observed (Junqueira and Carneiro, 1983). Sucrase-isomaltase activities in the three experimental groups were similar. However, aminopeptidase activity in homogenates of the proximal jejunum was depressed in the high-tannin group (3) compared to the control (1) and the low-tannin group (2).

The effect of the tannins could be explained by a binding of dietary tannins with the protein site of the aminopeptidase-active enzymes. The difference in effect of tannins on sucrase-isomaltase and aminopeptidase activity is probably due to a difference in physical properties of the two enzymes. Sucrase has an extremely hydrophobic amino acid sequence at the N-terminal end (Brunner *et al.*, 1979). Aminopeptidase have a hydrophilic "head" that emerges entirely from the external microvillus membrane and two short domains inserted in the membrane and penetrating into the cytoplasm respectively (Maroux *et al.*, 1979; Svenssen, 1979).

Low activities of aminopeptidase and sucrase-isomaltase were found in piglets fed on *Phaseolus vulgaris* beans (Kik *et al.*, 1990). The decrease of the functional capacity of the mucosa in this case was associated with morphological abnormalities.

In the present study the enzyme activity analyses were performed in homogenates of the mucosa and submucosa. This means that the total activity of the brush-border and cytoplasmatic enzymes were measured. Aminopeptidase is essential for both hydrolysis of small peptides and for active transport of amino acids over the brush-border membrane of the enterocytes. To study this aspect the correlation between protein digestibility and aminopeptidase activity was analysed. Results showed a significant ($P < 0.002$) positive correlation for the data from the group with the high-tannin ration

(Group 3; Figure 1). This observation indicates that aminopeptidase activity was a limiting factor for the rate of protein digestion of the ration with a high level of tannins.

REFERENCES

Bate-Smith E.C. and Swain T. (1962). Flavonoid compounds. In *Comparative Biochemistry,* Vol III. pp 755-809 [M. Florkin and H.S. Mason, editors]. Academic Press, New York.

Bos K.D. and Jetten J. (1989). Determination of tannins in faba beans. In *Recent Advances of Research in Antinutritional Factors in Legume Seeds*. pp 285-296 [J. Huisman, A.F.B. van der Poel and I.E. Liener, editors]. PUDOC, Wageningen, The Netherlands.

Brunner J.H., Hauser H., Braun H., Wilson K.J., Wacker H. and O'Neil B., Semenza G. (1979). The mode of association of the enzyme complex sucrase-isomaltase with the intestinal brush border membrane. *Journal of Biological Chemistry* 254: 1821-1828.

Egberts H.J.A., Koninkx J.F.J.G., van Dijk J.E. and Mouwen J.M.V.M. (1984). Biological and pathobiological aspects of the glycocalyx of the small intestinal epithelium. *Veterinary Quarterly* 6: 186-199.

Jansman A.J.M. (1993). Tannins in faba beans (*Vicia faba* L.) -antinutritional properties in monogastric animals- (PhD Thesis). Agricultural University Wageningen, The Netherlands.

Jansman A.J.M., Verstegen M.W.A. and Huisman J. (1993). Effects of inclusion hulls of faba beans (*Vicia faba* L.) with low and high content of condensed tannins on digestibility and some physiological parameters in piglets. *Animal Feed Science and Technology* 43: 239-257.

Junqueira L.C. and Carneiro J. (1983). Basic Histology. Chapter 16. 4th. Edition. Lange Medical Publications.

Kik M.J.L., Huisman J., van der Poel A.F.B. and Mouwen J.M.V.M. (1990). Pathologic changes of the small intestinal mucosa of pigs after feeding Phaseolus vulgaris beans. *Veterinary Pathology* 27: 329-334.

Lowry O.H., Rosebrough N.J., Farr A.L. and Randell R.J. (1951). Protein measurement with the folin-phenol reagent. *Journal of Biological Chemistry* 193: 265-275.

Maroux S., Louvard D. and Baratti J. (1973). The aminopeptidase from hog intestinal brush border. *Biochimica et Biophysica Acta* 321: 282-295.

Maroux S., Louvard D., Semeriva M. and Desnuelle P. (1979). Hydrolyses bound to the brush border: an example of transmembrane proteins. *Annales de Biologie Animale, Biochimie,*

Biophysique 19: 787.

Marquardt R.R., Ward A.T., Campbell L.D. and Cansfield P.E. (1977). Purification, identification and characterization of a growth inhibitor in faba beans (*Vicia faba* L. var. minor). *Journal of Nutrition.* 107: 1313-1324.

Martin-Tanguy J., Guillaume J. and Kossa A. (1977). Condensed tannins in horse bean seeds: Chemical structure and apparent effects on poultry. *Journal of the Science of Food and Agriculture* 28: 757-765.

Messer M. and Dahlqvist A. (1966). A one step ultramicromethod for the assay of intestinal disaccharidases. *Analytical Biochemistry* 14: 376-392.

Mitjavila S., Lacombe C., Carrera G. and Derache R. (1977). Tannic acid and oxidized tannic acid on the functional state of rat intestinal epithelium. *Journal of Nutrition* 107: 2113-2121.

Mouwen J.M.V.M. (1972). White scours in piglets at three weeks of age. (PhD Thesis), University of Utrecht, The Netherlands.

Mouwen J.M.V.M., Egberts H.J.A. and Koninkx J.F.J.G. (1983) The outermost mucosal barrier of the mammalian small intestine. *Deutsche Tierärztliche Wochenschrift* 90: 477-482.

Salunkhe D.K., Chavan J.K. and Kadam S.S. (1990). Nutritional consequences of dietary tannins. In *Dietary Tannins: Consequences and Remedies.* pp. 113-146 [D.K. Salunkhe, J.K. Chavan and S.S. Kadam, editors]. CRC Press, Boca Raton, USA.

Snedecor and Cochran. (1980). Statistical Methods. 7th Edition. The Iowa State University Press, Ames, USA.

SPSS. (1992).*System User's Guide* Marija J. Norusis/SPSS Inc., SPSS/PC+ Version 5.0 Base. System User's Guide.

Svenssen B. (1979). Covalent cross-linking of porcine small-intestine microvilli aminopeptidase. Subunit structure of the membrane-bound and the solubilized enzyme. *Carlsberg Research Communications* 44: 417-427.

Chapter 9

General summarising discussion

In this thesis the hypothesis is tested that the nutritional evaluation of dietary formulations in non-ruminants requires both functional-nutritional and functional-morphological parameters. The functional-nutritional parameters provide data on the outcome of the digestive process. Additionally, the functional-morphological parameters provide information about the effects of feed components on the small intestinal mucosa.
Part I (chapters 2 - 4) considers the apparent digestibility as a functional-nutritional parameter for feed evaluation in pigs and roosters, whereas Part II (chapters 5 - 8) presents studies with functional-morphological parameters of the small intestinal mucosa of chickens, calves and piglets in relation to feed composition and additives.

FUNCTIONAL-NUTRITIONAL PARAMETERS (PART I)

The amount of protein and amino acids, which disappears in the large intestine of pigs, is not available for animal body maintenance and production (Zebrowska, *et al*., 1978). Degradation of protein in the large intestines is mainly fermentative resulting in non-amino acid N end products, which are not available to the animal. This finding implies that precaecal digestion rather than whole tract digestion provides a more accurate parameter for the estimation of protein availability (Dierick *et al.,* 1987). The *in vivo* determination of precaecal protein digestion relies on quantifying the ratio between the amount of the ingested protein to that which disappears proximal to the caecum. In digestibility experiments the diets and digesta, collected immediately after the ileum, are analysed on their protein contents. But digesta also contain undigested dietary protein of endogenous origin. Therefore, this ratio is determined as the apparent digestibility. Apparent digestibility is a quantitative parameter providing information on the digestive progress measured by nutrient disappearance at a defined site.
Quantitative studies concerning the digestive processes in the small intestine require reproducible collection of digesta from the small intestine. Present procedures can be divided into techniques

by which digesta are collected after sacrifying the animals and techniques based on a surgical intervention. Collection of digesta from animals after euthanasia is often used in experiments with broilers (Ravindran *et al.,* 1999). This method, however, requires a large number of animals and for this reason is not commonly used in pigs. There are different surgical techniques described in literature for precaecal digesta collection. It is generally concluded that flexible (silicone) rubber is preferable to rigid materials. Regarding surgical techniques for intestinal studies in pigs, there is a consensus that simple T-shaped cannulae in the ileum and ileo-rectal anastomose (IRA) may not provide representative samples of digesta and/or may interfere with the animal's physiology (Köhler, 1992), whereas collection of digesta from re-entrant cannulae is considered to be hampered by technical difficulties (van Leeuwen *et al*., 1987).

In part I of the thesis surgical techniques and procedures for digesta collection in pigs and roosters are described and results of digestibility determinations are given.

Chapter 1 describes a surgical procedure, which is called the Post Valve T-Caecum (PVTC) cannulation and is considered to be an alternative to the existing digesta collection methods. The prerequisites of this technique are that there is minimal hinder of the animal's physiology. Moreover, digesta samples should be representative, and the surgical technique acceptable in terms of animal welfare. The PVTC technique relies on partial caecectomy followed by placement of a wide flexible silicone T-cannula in the caecum. A considerable advantage of this technique is that the region of the intestine to be studied is not surgically treated. Gargallo and Zimmerman (1981) studied the possible effects of caecectomy on digestion in pigs. They observed small effects on overall digestibility of cellulose and nitrogen. Their final conclusion was that the absence of the caecum in pigs did not significantly alter digestive function. Darragh and Hodgkinson (2000) commented that the PVTC cannulation procedure appears to be the preferred method for the collection of ileal digesta.

Chapter 2 describes digesta collection procedures and implications when using PVTC cannulated pigs. Collection of digesta after PVTC cannulation necessitates the use of an inert marker in the diets, to quantify the amounts of nutrients present in ileal digesta for determination of diet digestibility. Two experiments were conducted to evaluate chromic oxide (Cr_2O_3) and HCl-insoluble ash as digestive markers by determining the apparent digestibility of dry matter (DM) and crude protein (CP). In addition, studies were performed of the effects of age (i.e. three different body weight (BW) classes) on apparent ileal DM and CP digestibilities. In experiment 1, barrows were fitted with PVTC cannulae to determine apparent ileal DM and CP digestibility of a wheat gluten/wheat bran ration and

a soybean meal ration. Immediately after the morning feeding ileal digesta were collected on an hourly basis for a period of 12 hours. Subsequently, nitrogen (N) and marker contents were determined in these samples. The postprandial Cr/N ratio was more constant than the HCl-insoluble ash/N ratio. Therefore, chromic oxide is considered more suitable as a marker than HCl-insoluble ash when apparent digestibility of protein is the parameter to be studied. In experiment 2, apparent ileal DM and CP digestibilities were determined in 18 rations using twelve barrows fitted with PVTC cannulas (BW from 40 - 100 kg). The protein sources for these rations were derived from feedstuffs of different origin. Apparent precaecal digestibility differed significantly ($P < 0.05$) on the marker in four rations for DM and in three rations for CP. Digestibility coefficients were not systematically higher or lower for either marker. Besides these methodological aspects, a slight increase in apparent ileal CP digestibility was observed with an increase in body weight.

Chapter 3 examines precaecal digestion of protein and amino acids (AA) in roosters. Similar to pigs, undigested AA which reach the caeca are deaminated by the microflora and the end-products have no nutritional value (McNab, 1989). Moreover, Parsons (1986) observed a closer relationship between amino acid availability measured in chick growth assays, and digestibility determined in caecectomised rather than in intact birds. This means that, in poultry, digestion in the distal region of the intestines, more specifically the caeca, is mainly fermentative and that the AA synthesized in, or disappearing from the caeca, are not available for protein synthesis by the animal. Therefore, a procedure for ileostomy in adult roosters has been described with the use of flexible silicon cannulae. Apparent ileal digestibility coefficients for dry matter (aDC DM), crude protein (aDC CP) and amino acids (aDC AA) were determined in diets formulated with maize/wheat gluten meal, wheat gluten meal, faba beans, lupins, soybean meal and casein as the main protein sources. These determinations were performed in ileostomised roosters fitted with silicon cannulae. In addition, aDC data determined using roosters (present study) were correlated with previously published aDC data of the same diets determined with pigs (van Leeuwen *et al.,* 1996a, 1996b).

The ileal aDC CP in roosters significantly ($P < 0.05$) differed in aDC CP and aDC AA between diets. Over diets significant linear relationships were found for the digestibility data determined with roosters and pigs and inturn explained 85 % of the variation in ileal aDC CP between the six diets evaluated in roosters and pigs. Variation between roosters and pigs in ileal aDC AA could be explained for 62-90%, for the individual amino acids, with the exception of aDC of arginine. The standard errors of prediction of the models for aDC AA in roosters using aDC AA in pigs were <

0.04 percentage units. Although, more work is needed to validate these correlations, it is likely that this approach can be used for the prediction of aDC values for roosters from values determined in pigs. The results showed a similarity in the level of digestibility coefficients for protein and amino acids in both species. This means that, despite the differences in anatomy between pigs and poultry (Moran Jr., 1982) the differences in apparent precaecal digestibility of CP and AA were limited. The two animal species with their differences in intestinal structures, differences in amounts and activity of the endogenous components were both capable of digesting protein to a similar extent suggesting a similar precaecal digestive capacity.

Regarding methodological aspects the study showed comparable aDC CP and AA for soybean meal determined in the present experiment with the cannulated roosters and data from literature using adult caecectomised roosters. Secondly, the roosters provided with cannulae introduced after ileostomy can be used for periods up to a year after surgery.

FUNCTIONAL-MORPHOLOGICAL PARAMETERS (PART II)

The qualitative functional-morphological parameters of the small intestinal mucosa are examined in the chapters 5 - 8.

Chapter 5 considers the morphology of the mucosal surface of the small intestine of broilers and the relationship with age, diet formulation, small intestinal microflora and growth performance. The villi of the small intestine were examined with a dissecting microscope and the surface was described using a morphological scoring scale. As illustrated by pictures, zigzag oriented ridges were observed in the broilers, which seem to be characteristic for poultry.

The results showed that in clinically healthy broilers the shape and orientation of the small intestine villi were related to the age of the animal and the intestinal location. Effects of dietary composition and microflora are also demonstrated. Fermentable pectin as dietary component decreased the zigzag villus orientation and reduced performance. Addition of glutamin to a soybean diet limited the decrease of the zigzag villus-orientation caused by pectin and had a beneficial effect on performance. An oral challenge with a non-virulent *Salmonella typhimurium* increased the effects of dietary pectin on the small intestine morphology and performance.

Chapter 6, contains a study of the functional-morphological effects of virginiamycin (VM), used as feed additive in piglets. The objective of this study was to determine the effects of VM on morphological parameters of the small intestinal mucosa, animal growth and feed conversion ratio

(feed intake/weight gain) in piglets. The study comprised three trials: two experiments to study the morphological effects of VM on the small intestinal mucosa, whereas the third experiment was a performance study. Each experiment comprised a control group fed a diet without VM, and a VM group fed a diet containing 40 mg/kg VM. In the first experiment, the piglets were individually kept and an oral dose of K88 positive enterotoxigenic *Escherichia (E.) coli* (ETEC) was given as a sub-clinical challenge. The housing conditions in experiments 2 and 3 were according to practical standards. The results showed that the VM decreased feed conversion ratio and increased villus heights in conventionally kept piglets. Crypt depths were decreased in the individually kept piglets seven days after the ETEC challenge. Corpet (1999) and Anderson *et al.* (2000) reviewed the mode of action of antibiotics as feed additives and suggested that the antibiotics suppress bacterial activity and decomposition of bile salts resulting in a more slender villus structure. Increased villus heights indicated an increased mucosal surface and absorption capacity, which is in agreement with the improved precaecal nutrient digestibility of diets with VM, as observed by Decuypere *et al*. (1991). The difference in morphological response to the VM illustrated variation in the morphological characteristics between clinically healthy piglets.

In chapter 7 the effect of the use of the combination of two bioactive proteins, lactoperoxidase-system (LP-s) and lactoferrin (LF), on a milk replacer diet were investigated. This study examined the severity of diarrhoea, morphology of the small intestinal mucosa and the microbiology of digesta and faeces in young weaned calves.

Following weaning, the incidence of diarrhoea and mortality of calves is usually higher than that for unweaned calves (Reynolds *et al*., 1981). In conventional calf production, antibiotics are added to the milk replacer to reduce gastrointestinal disorders caused by pathogenic bacteria in the gut. Recent legislation restricts the addition of antibiotics in diets for calves (EC, 1998) because of possible repercussions on human health (Van den Boogaard and Stobberingh, 1996).

LP and LF are both specific protein constituents of colostrum. These naturally occurring proteins are probably at least partly inactivated during the processing of milk because of their thermo-instability, and the remaining levels are not constant. Moreover, in dairy milk replacers a significant part of the protein is of vegetable origin and therefore lacks LP and LF.

The experiment with calves comprised the first two weeks post weaning. One group received a control diet and a second group a diet with LP-s/LF. Results showed that faecal consistency of the LP-s/LF group, as assessed by faecal consistency scores, was significantly improved compared to the control group. The numbers of *E.* coli in faeces were significantly lower and the villi in the

distal jejunum more finger shaped and longer in those of the LP-s/LF group compared to the control group. These findings showed that the effects of LP-s/LF are mainly located in the distal region of the gastrointestinal tract. Reiter and Perraudin (1991) also showed positive effects of LP-s on live weight change in field trials. Still *et al*. (1989) studied the effects of a combination of LP-s and LF on the severity of diarrhoea in calves for a period 0 to 6 days after an experimental *E. coli* infection. They concluded that LP-s/LF had preventive and curing effects after the *E. coli* challenged infection. The results of the present experiment were in agreement with their observations.

Chapter 8 considers the functional-morphological implications of condensed tannins in faba beans *(Vicia faba* L.). The nutritional value of faba beans is limited by the presence of these tannins (Marquardt *et al.,* 1977). Jansman *et al.* (1993) studied the effects of tannins on the apparent faecal digestibility of a control diet, a diet containing hulls of white flowering, low-tannin faba beans, and a diet with hulls of coloured flowering, high-tannin faba beans. They concluded that whole tract crude protein digestibility of the high-tannin diet was significantly ($P < 0.05$) lower than the control and low-tannin diets. This effect was partly explained by an increase of the endogenous fraction in the faeces and by an increase of the undigested tannin-feed complexes. In addition, the present study investigated samples of the proximal-, mid- and distal jejunum were investigated histologically and biochemically. The histological differences between the diets were not significant. However, differences in aminopeptidase activity were observed in the proximal small intestine. The amino-peptidase activity of the high tannin group was significantly ($P < 0.05$) depressed compared to the control and low-tannin groups. Furthermore, a correlation was calculated within the three groups between amino peptidase activity, as a functional parameter of the brush border, and the apparent faecal digestibility of CP, as a quantitative nutritional characteristic. No significant correlations were found between apparent CP digestibility and the aminopeptidase activity in the animals fed the control or low-tannin diet. But when the high tannin diet was fed, the correlation was significantly positive ($P < 0.002$; $R = 0.91$). This correlation indicated that a decreased aminopeptidase activity of the small intestine mucosa explained, at least in part, the effects of tannins on CP digestibility.

CONCLUSION

Precaecal protein digestibility is a functional-nutritional parameter, which describes the digestive function quantitatively. Besides this quantitative parameter, qualitative functional-morphological parameters, demonstrate effects of the interaction between dietary constituents and the small intestine mucosa. Therefore, in animal nutrition, the use of morphological-functional parameters is complementary to the more conventional functional-nutritional parameters.

REFERENCES

Anderson, D.B., V.J. McCracken, R.I. Aminov, J.M. Simpson, R.I. Mackie, M.W.A. Verstegen, H.R. Gaskins (2000) Gut microbiology and growth-promoting antibiotics in swine. *Nutrition Abstracts and Reviews* 70: 2, 101 - 108.

Corpet, D.E. (1999) Mechanism of antimicrobial growth promoters used in animal feed. Comptes Rendus de l'Academie d'Agriculture de France 85: 7, 197-205.

Darragh, A.J., Hodgkinson, S.M. (2000) Quantifying the digestibility of dietary protein. *Journal of Nutrition* 130: 1850S-1856S.

Decuypere, J.A., Dierick, N.A., Vervaeke, I.J., Henderickx, H.K. (1991) Influence of virginiamycin on the digestive physiology in precaecal re-entrant cannulated pigs. *Archieves of Animal Nutrition* 41: 4, 373-393.

Dierick, N. A.; Vervaeke, I. J.; Decuypere, J. A.; van der Heyde, H.; Henderickx, H. K. (1987) Proceedings of the Vth Symposium on Protein Metabolism and Nutrition, Rostock, DDR.

EC (European Community) (1998) Regulation 2821/ 98/ EC, 17-12-1998. Publication Office of EC, 11 Rue Christofophe Plantin L- 2339, Luxembourg.

Gargallo, J., Zimmerman, D. R. (1981) Effects of dietary cellulose levels on intact and cecectomized pigs. *Journal of Animal Science*. 53, 395-402.

Jansman A.J.M., Verstegen M.W.A. and Huisman J. (1993). Effects of inclusion hulls of faba beans *(Vicia faba* L.) with low and high content of condensed tannins on digestibility and some physiological parameters in piglets. *Animal Feed Science and Technology* 43: 239-257.

Köhler, T. (1992) Evaluation of techniques to collect ileal digesta in pigs (Thesis) Agricultural University Wageningen, The Netherlands.

Marquardt R.R., Ward A.T., Campbell L.D. and Cansfield P.E. (1977) Purification, identification and characterization of a growth inhibitor in faba beans (*Vicia faba* L. var. minor). *Journal of*

Nutrition. 107: 1313-1324.

McNab, J.M. (1989) Digestibility of amino acids by poultry. Amino Acids in Animal Production Conference. Forum Feeds. London.

Moran Jr. E.T. (1982) Comparative Nutrition of Fowl and Swine. The Gastro Intestinal Systems. E.T. Moran Jr. Office for Educational Practise, University of Guelph, Guelph, Ontario N1G 2W1, Canada.

Parsons, C.M. (1986) Determination of digestible and available amino acids in meat meal using conventional and caecectonized cockerels or chick growth assays. *British Journal of Nutrition.* 56, 227-240.

Ravindran, V., Hew, L.I., Ravindran, G., Bryden, W.L. (1999) A comparison of ileal digesta and excreta analysis for the determination of amino acid digestibility in food ingredients for poultry. *British Poultry Science* 40, 266-27.

Reiter, B. and Perraudin J-P. (1991) Lactoperoxidase. Biological Functions. In *Peroxidases in Chemistry and Biology* Vol. 1, pp. 143- 180 [J. Everse, K.E. Everse, M.B. Gruisham, editors] Boca Raton, FL, U.S.A.: C.R.C. Press Inc.

Reynolds, D.J., Morgan, J.H., Chanter, H. (1981) Microbiology of calf diarrheoa in Southern Britain. *Veterinary Records* 119. 34-39.

Still, J., Delahaut, P., Coppe, P., Kaeckenbeeck, A. and Perraudin J.-P. (1990) Treatment of induced enterotoxigenic colibacillosis (scours) in calves by the lactoperoxidase system and lactoferrin. *Annales de Recherche Vétérinaires* 21. 143-152.

Van den Boogaard, A.E. and Stobberingh, E.E. (1996) Time to ban all antibiotics as animal growth- promoting agents? *The Lancet* 348. 619.

Van Leeuwen, P., Veldman, A., Boisen, S., Deuring, K., van Kempen, G.J.M., Derksen, G.B., Verstegen, M.W.A. and Schaafsma, G. (1996a) Apparent dry matter and crude protein digestibility of rations fed to pigs and determined with the use of chromic oxide (Cr2O3) and insoluble ash as digestive markers. *British Journal of Nutrition*. 76, 551-562.

Van Leeuwen, P., Gdala, J., Boisen, S., Buraczewski, S., van Kempen, G.J.M., Verstegen, M.W.A. and Schaafsma, G. (1996b). Validation of a mathematical model to explain variation in apparent ileal amino acid digestibility of diets fed to pigs. *Journal Animal Feed Science* 5, 303-315.

van Leeuwen, P., W.C. Sauer, W.C., Huisman, J., van Weerden, E.J., van Kleef, D.J., and den Hartog, L.A. (1987) Methodological aspects for the determination of amino acid

digestibilities in pigs fitted with ileocecal re-entrant cannulas. *Journal of Animal Physiology and Animal Nutrition* 58: 122-133.

Zebrowska, T., Buraczewska, C., Horaczynski, H. (1978) Apparent digestibility of nitrogen and amino acids and utilization of protein given orally or introduced into the large intestine of pigs. *Roczn. Nauk. Roln.* Ser. 99B, 99-105.

SAMENVATTING

De betekenis van nutritionele naast morfologische precaecale parameters voor voedingsonderzoek bij niet-herkauwers

De hypothese van dit proefschrift is gebaseerd op het idee dat in voedingsonderzoek bij landbouwhuisdieren zowel voedings-fysiologische als functioneel-morfologische benaderingen van belang zijn. Zij geven respectievelijk, kwantitatieve kennis van het verteringsproces op een bepaalde plaats in het maagdarmkanaal en kwalitatieve kennis over het functioneren van het darmslijmvlies. In dit proefschrift zijn studies bij verschillende diersoorten beschreven. Deel I handelt over de meting van de schijnbare pre-caecale verteerbaarheid van eiwit en aminozuren bij varkens en hanen als voedings-fysiologische parameter. In deel II zijn studies opgenomen waarin functioneel-morfologische parameters van de dunne darm centraal staan.

Inleiding

De maag en dunne darm omvat het gedeelte van het maagdarmkanaal dat voor de dikke darm ligt en wordt het pre-caecale deel van het maagdarmkanaal genoemd. Voor de vertering is de dunne darm hiervan het belangrijkste onderdeel. Het verteringsproces in de dunne darm omvat verkleining van voerdeeltjes, hydrolyse van nutriënten en de daaropvolgende absorptie van voedingstoffen door het slijmvlies van de dunne darm. Naast voedingsstoffen worden in de dunne darm ook vitaminen, mineralen en water opgenomen. Kleine uitstulpingen aan de binnenzijde van de darmwand, de zogeheten darmvlokken, vergroten het absorptieoppervlak van de dunne darm.

De verteringsprocessen hebben tot gevolg dat de samenstelling van de darminhoud in de dunne darm verandert. De dunne darm bevat in het gedeelte direct na de maag veel voedingsstoffen en toevoegingen als maagsap en gal. In de richting van de dikke darm bestaat de darminhoud vooral uit materiaal dat resistent is tegen hydrolyse en niet verteerbaar is.

De hydrolyse vindt plaats onder invloed van enzymen die in de maag en door de pancreas worden uitgescheiden. Verder bevinden zich enzymen in het slijmvlies van de dunne darm. De vertering die onder invloed van deze enzymen plaatsvindt wordt de enzymatische vertering genoemd. Naast enzymatische vertering van voedingsstoffen vindt in de darminhoud fermentatie plaats, onder invloed van de microflora. In het gebied juist na de maag is de bacteriële activiteit

nog gering. Die neemt toe in de richting van het laatste deel van de dunne darm. De vertering in de dunne darm omvat dus een complex van processen waarbij in het eerste deel de nadruk ligt op de activiteit van endogene enzymen die door het dier worden aangemaakt, terwijl de fermentatieve activiteit in de richting van de dikke darm belangrijker wordt. Dat wil echter niet zeggen dat er in de maag en het begin van de dunne darm geen microflora actief is. Deze bacteriële activiteit kan een effect hebben op het functioneren van het slijmvlies van de dunne darm.

Het slijmvlies van de dunne darm is doorlaatbaar voor voedingsstoffen maar vormt daarnaast een barrière tegen bacteriën en schadelijke stoffen als toxinen. Door de specifieke selectieve eigenschappen van het slijmvlies kan het voldoen aan deze schijnbaar tegenstrijdige functies van doorlaatbaarheid en weerstand. Beide functies zijn essentieel voor de elementaire fysiologische processen voor het dier. De vertering en absorptie van nutriënten zijn nodig voor het onderhoud van het lichaam en voor de aanmaak van bijvoorbeeld spiereiwit. Daarnaast zijn specifieke voedingsstoffen nodig voor het onderhoud van het darmwand zelf. Zo lijkt uit recent onderzoek naar voren te komen dat bij jonge biggen de wand van de dunne darm bij verstrekking van een dieet met een hoog gehalte aan lactose in een betere conditie verkeerd dan wanneer veel eiwit wordt versterkt. Verder blijkt glutamine een gunstig effect te hebben op het functioneren van het darmslijmvlies bij beschadigingen van het slijmvlies. Deze voorbeelden geven aan dat de voeding een effect kan hebben op de integriteit en functie van het slijmvlies.

Het slijmvlies van de dunne darm is toegerust voor de absorptie van aminozuren. Vrije aminozuren die vanuit de dunne darm doorstromen naar de dikke darm, of in de dikke darm bij hydrolyse van eiwit ontstaan, worden door bacteriën afgebroken tot ammoniak en zijn niet beschikbaar voor het dier. Om die reden is de mate van vertering van eiwit over het darmgedeelte voor de overgang van de dunne darm naar de dikke darm (pre-caecaal) een belangrijke voedings-fysiologische parameter. De hoeveelheid eiwit die pre-caecaal wordt verteerd is dan ook een betere maat voor beschikbaarheid van eiwit voor het dier dan de faecale verteerbaarheid. Gegevens over de precaecale verteerbaarheid eiwit en aminozuren van veevoedergrondstoffen zijn samengebracht in tabellen en deze kennis wordt gebruikt voor het samenstellen van rantsoenen voor varkens. Het toepassen van de pre-caecale verteerbaarheid bij het samenstellen van rantsoenen heeft geleid tot een verbetering van de conversie van voereiwit naar dierlijk eiwit en vermindering van N-verliezen.

De precaecale verteerbaarheid van eiwit van verschillende veevoedergrondstoffen kan bij een

gezond dier variëren o.a. door variatie in voersamenstellingen en het al dan niet voorkomen van zogeheten anti-nutritionele factoren in het voeder. Ook andere oorzaken kunnen de precaecale verteerbaarheid verlagen, zoals een verstoring van de activiteit van de pancreasfunctie. Die verstoring kan optreden wanneer biggen en kalveren worden gespeend. Ook een voercomponent als pectine en de daarmee gepaard gaande fermentatieve afbraak van nutriënten kan een verstoring van de enzymatische vertering gedeeltelijk tenietdoen. Verder kan de overmaat aan enzymen en absorptiecapaciteit in de dunne darm het uiteindelijke effect van de verstoring op de precaecale verteerbaarheid verkleinen.

Veranderingen in de functie van het slijmvlies ten aanzien van de absorptie en de barrière-functie zijn veelal gerelateerd aan veranderingen van morfologische parameters. Zo worden vloklengte, crypte-diepte en de activiteit van borstelzoomenzymen informatie gezien als parameters die gerelateerd zijn aan de absorptie van het slijmvlies. Andere functioneel-morfologische parameters, als de aantallen darmslijm-producerende cellen in het slijmvlies en het type mucinen in deze cellen geven kennis over de conditie van het slijmvlies als barrière tegen de passage van ongewenste stoffen en bacteriën.

Samengevat: de capaciteit van de dunne darm bij het gezonde dier is, zowel ten aanzien van hydrolyse als absorptie, bijzonder hoog. Ondanks deze overcapaciteit blijkt uit literatuurgegevens dat er toch sprake is van duidelijke verschillen in eiwit-verteerbaarheid tussen veevoedergrondstoffen. Verder is er een relatie tussen microbiële activiteit in de dunne darm en het epitheel waarbij veranderingen in de morfologische kenmerken van de dunne darm kunnen optreden.

Deel I, voedings-fysiologische parameters

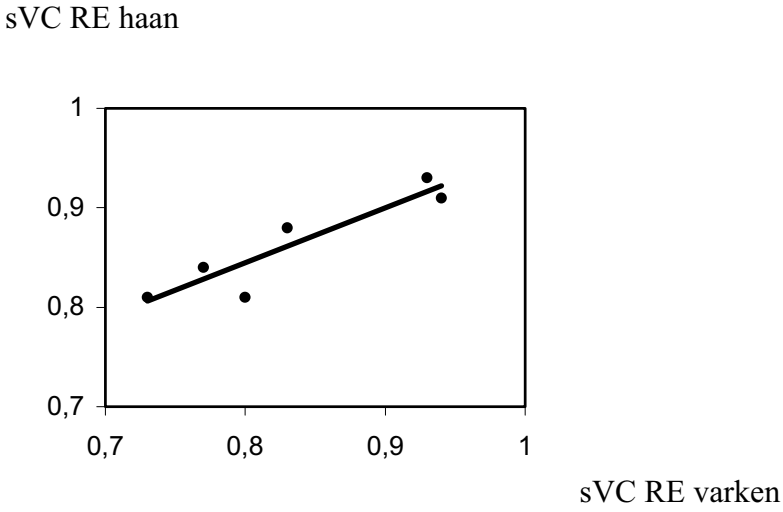
De vertering in de dikke darm berust op fermentatieve afbraak. Eiwit en aminozuren die in dit darmgedeelte uit de darminhoud verdwijnen, worden omgezet in ammoniak dat niet aan het dier ten goede komt (Zebrowska e.a., 1978). In het gedeelte van het maagdarmkanaal dat voor de dikke darm ligt (het pre-caecale gedeelte) worden deze nutriënten overwegend enzymatisch afgebroken en door het slijmvlies geabsorbeerd. Om die reden is de pre-caecale vertering van eiwit en aminozuren voor de diervoeding een belangrijke kwantitatieve parameter. In dit onderdeel van het proefschrift zijn methoden beschreven waarmee de pre-caecale vertering van

eiwit kan worden bepaald. Deze methoden zijn gebaseerd op het vaststellen van de hoeveelheid van een nutriënt die over een bepaalde tijdsperiode door het dier wordt opgenomen en de hoeveelheid van dezelfde voedingsstof die over dezelfde tijdsperiode op een bepaalde plaats in de darm wordt aangetroffen, en dus niet verteerd is. Door de opname van de nutriënt te verminderen met hoeveelheid die niet is verteerd, wordt vervolgens de verteerde hoeveelheid bepaald. Tenslotte wordt het verteerde deel uitgedrukt als het quotiënt van de opgenomen hoeveelheid van de onderzochte nutriënt. Het aldus bepaalde quotiënt wordt de verteringscoëfficiënt genoemd.

Voor het vaststellen van de hoeveelheid onverteerd materiaal dient de darminhoud verzameld te worden. Hiertoe worden proefdieren operatief voorzien van een opening in het betreffende gedeelte van het darmkanaal.

In de hoofdstukken 2 en 4 zijn operatiemethoden beschreven die voor het verzamelen van de darminhoud uit de dunne darm bij varkens en hanen. Hiertoe wordt een opening in de darm gemaakt en een buisje (canule) in deze opening geplaatst. Voor varkens is de zogeheten post-valve T-caecum (PVTC) canule ontwikkeld. De canule maakt het mogelijk om darminhoud die de dunne darm verlaat, te verzamelen. Deze methode wordt inmiddels bij diverse instituten voor diervoedingsonderzoek toegepast (Darragh en Hodgkinson, 2000). Voor het verzamelen van darminhoud bij hanen is een canule in het laatste gedeelte van de dunne darm geplaatst (ileostomie). Met behulp van deze methoden kan over een lange periode darminhoud worden verzameld.

In de hoofdstukken 3 en 4 wordt verder ingegaan op aspecten die van belang zijn voor het meten van de pre-caecale verteerbaarheid. Vastgesteld is dat de mate waarin het eiwit in dit darmgedeelte verteerbaar is, samenhangt met de veevoedergrondstoffen die in de rantsoenen waren opgenomen. Verder is een zekere mate van overeenstemming waargenomen tussen de verteringscoëfficiënten van eiwit en aminozuren van verschillende voeders die bij varkens en vervolgens bij hanen zijn gemeten (Figuur 1).

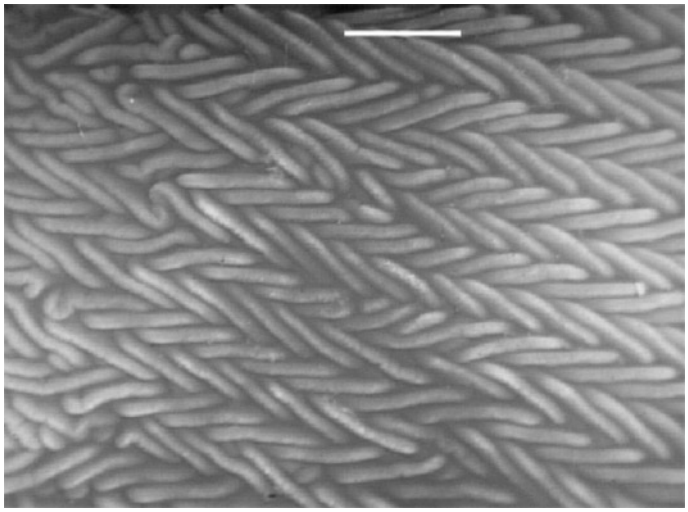


Figuur 1. Schijnbare verteerbaarheid van ruw eiwit van 6 rantsoenen gemeten bij varkens (x-as) en bij volwassen hanen (y-as); $R^2 = 0.85$.

Deel II, functioneel-morfologische parameters

Aan de binnenzijde van de dunne darm bevinden zich grote aantallen uitstulpingen, de zogeheten darmvlokken. Afhankelijk van de vorm en lengte van deze uitstulpingen wordt het darmoppervlak hierdoor vergroot. Het membraan van de laag cellen waarmee de darmvlokken zijn bedekt wordt gepasseerd bij absorptie van nutriënten, vitaminen, mineralen en water. De capaciteit van de absorptie hangt samen met de grootte van het darmoppervlak en dus met vorm van deze vlokken. Morfologische parameters van het darmoppervlak worden dan ook gezien als functionele parameters die op een bepaalde plaats in de darm een kwalitatief beeld geven over de capaciteit van de absorptie.

In de hoofdstukken 5 - 8 zijn kwalitatieve functioneel-morfologische parameters van het slijmvlies van de dunne darm onderzocht in proeven met vleeskuikens, varkens en kalveren.



Figuur 2 Darmvlokken van kuikens die een zigzag relief vormen (balk = 1 mm).

In hoofdstuk 5 is het effect onderzocht van voersamenstelling op morfologische karakteristieken van de dunne darm bij kuikens. Hiertoe zijn criteria opgesteld voor het beschrijven van de darmvlokken. Vervolgens zijn verschillende voervarianten verstrekt en zijn monsters van het dunne darm onderzocht. Uit dit onderzoek bleek dat het slijmvlies gedurende de eerste levensweken van het kuiken sterk verandert. Opvallend was dat een belangrijk deel van de vlokken regelmatig zijn gerangschikt en daarbij een zigzag reliëf vormen (Figuur 2). Bij kuikens vanaf zeven dagen leeftijd was in het midden gedeelte van de dunne darm gemiddeld 50% van de darmwand of meer bezet met dit karakteristieke zigzag reliëf (Tabel 1). Verder viel op dat na een leeftijd van 7 dagen de vlokken breder werden en hierdoor het percentage richelvormige vlokken sterk toenam en dat bij verstrekking van pectine dit percentage lager was. Na beëindiging van de pectine verstrekking nam zowel het aandeel zigzag reliëf als richelvormige vlokken toe. Pectine, en de daarmee gepaard gaande verhoging van de microbiële activiteit (Langhout, 1998), hebben dus bij kuikens een effect op de vorm van de vlokken in de dunne darm.

Tabel 1. Het percentage van de dunne darmvlokken dat een zigzag relief vormt en het percentage richelvormige dunne darmvlokken, bij kuikens op een leeftijd van 7, 14 en 21; met en zonder pectine verstrekking

Leeftijd in dagen	Zigzag reliëf (%)	Richelvormige vlokken (%)
Bij verstrekking van een standaardvoeder		
7	53	7
14	77	63
21	63	80
Bij verstrekking van 30 g/kg pectine van dag 0 - 14		
14	42	19
Na beëindiging van de pectine verstrekking, dag 14 – 21		
21	61	58

In hoofdstuk 6 zijn de effecten van een geringe dosering antibioticum (virginiamycine) in het voer onderzocht op morfologische kenmerken van de vlokken van de dunne darm. Daarnaast is een groeiproef uitgevoerd waarin het effect van het antibioticum is onderzocht. Uit dit onderzoek kwam naar voren dat de groei van de biggen per kilogram voeder met antibioticum hoger was dan bij een controlegroep zonder antibioticum. Het voerbesparende effect komt tot uiting in een lagere voederconversie (voer (kg)/groei (kg)). Verder is vastgesteld dat bij antibioticum-verstrekking de vlokken in de dunne darm langer waren (Tabel 2).

Tabel 2. Effecten van een antibioticum (virginiamycine) in het voer op groei, voederconversie en vlokhoogte in de dunne darm van biggen.

	Controlegroep, zonder antibioticum	Proefgroep, met antibioticum
Groei (g/dag)	543	566
Voederconversie (kg/kg)	1,671	1,535
Vlokhoogte (μm)	395	436

In hoofdstuk 7 is de combinatie bestudeerd van twee bioactieve componenten, lactoperoxidase-lactoferrine (LPs-LF), als een alternatief voor antibiotica bij jonge kalveren. Het ging om het vaststellen van de mogelijke beschermende effecten van LPs-LF, dat van nature in koemelk voorkomt. De proef omvatte de eerste twee weken nadat de kalveren waren overgezet van koemelk op een koemelkvervangend rantsoen. In deze periode zijn de kalveren gevoelig voor maagdarminfecties en treedt vaak diarree op. De proef toont een positief effect aan van LPs-LF verstrekking. De frequentie en mate waarin afwijkende faeces voorkwam was minder bij verstrekking van LPs-LF dan bij de controlegroep (Tabel 3). Dit positieve effect werd bevestigd door lagere aantallen *E. coli* in de faeces en dikke darm, en langere darmvlokken in het laatste deel van de dunne darm bij de kalveren van de LPs-LF groep.

Tabel 3. Effect van lactoperoxidase-lactoferrine (LPs-LF) verstrekking aan jonge kalveren.

	Controlegroep, zonder(LPs-LF)	Proefgroep, met (LPs-LF)
Gemiddelden van dag 0 -14		
Faeces score[1]	0,8	0,6
E. coli in faeces (log KVE[2])	7,5	7,0
Waarnemingen op dag 14		
E. coli in dikke darminhoud (log KVE)	5,3	4,2
Vlokhoogte, dunne darm (μm)	229	295

[1] Faeces score: 0 = normale faeces, 1 = dunne faeces; [2] KVE, kolonie vormende eenheden.

In hoofdstuk 8 zijn de effecten van tanninen uit veldbonen bij biggen onderzocht op morfologische kenmerken en op de enzymactiviteit van het dunne darmoppervlak. De tanninen hebben bij varkens een antinutritionele werking wat resulteert in een verlaging van de eiwit verteerbaarheid. Er is geen effect van de tanninen op de morfologische kenmerken gevonden. Wel bleek dat activiteit van aminopeptidase in het slijmvlies van de dunne darm bij biggen die tanninen verstrekt kregen gemiddeld lager was dan bij de controledieren. Verder is een verband tussen aminopeptidase activiteit en eiwitverteerbaaarheid vastgesteld.

Conclusie

De pre-caecale verteerbaarheid, als voedings-fysiologische parameter, geeft een kwantitatieve beschrijving van de verteringsfunctie van de dunne darm. Afhankelijk van de voersamenstelling kan de pre-caecale verteerbaarheid verschillen. Uit dit onderzoek bleek verder dat de darmvlokken in de dunne darm kunnen veranderen gevolg van bestanddelen in de voeding en de daarmee gepaard gaande bacteriële activiteit in de dunne darm. Ook stoffen met een antimicrobiële activiteit bleken bijvoorbeeld de lengte van de vlokken te beïnvloeden. Morfologische karakteristieken van de dunne darm zijn mede bepalend voor het functioneren van het darmslijmvlies en dienen voor het diervoedingsonderzoek dan ook als aanvullend op andere voedings-fysiologische parameters te worden gezien.

Referenties

Darragh, A.J., Hodgkinson, S.M. (2000) Quantifying the digestibility of dietary protein. *Journal of Nutrition* 130: 1850S-1856S.

Langhout D.J. (1998) The role of the intestinal flora as affected by non-starch polysaccharides in broiler chicks. PhD Thesis, Agricultural University Wageningen, The Netherlands.

Zebrowska, T., Buraczewska, C., Horaczynski, H. (1978) Apparent digestibility of nitrogen and amino acids and utilization of protein given orally or introduced into the large intestine of pigs. *Roczn. Nauk. Roln. Ser.* 99B, 99-105.

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De introductie van morfologische technieken bood nieuwe mogelijkheden voor het onderzoek. Het idee was dat de voeding effecten kan oproepen in het vlokepitheel van de dunne darm van gezonde landbouwhuisdieren. Bij het onderzoek naar deze effecten is op een plezierige wijze samengewerkt met de vakgroep Veterinaire Pathologie. De resultaten zijn gepubliceerd dankzij de steun die ik ondervond van Jaap van Dijk en mijn tweede promotor Johan Mouwen.

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Piet van Leeuwen

Curriculum vitae

Piet van Leeuwen is geboren in 1945 op Texel, volgde daar de Lagere Landbouwschool en ging vervolgens naar de Middelbare Landbouwschool in Schagen. Daarna werkte hij 1 jaar bij de Coöperatieve Aan- en Verkoopvereniging "De Ster" in Barsingerhorn en vervulde zijn militaire dienstplicht. Van 1966 tot 1970 was hij in dienst van de Nederlandse Organisatie voor toegepast-natuurwetenschappelijk onderzoek (TNO), en werkte als Technisch assistent bij de uitvoering van dierproeven op het Instituut voor Onderzoek Biochemische Producten (ILOB) in Wageningen. Dit instituut was destijds onderdeel van de Koninklijke Gist en Spiritus Fabrieken. In deze periode werden met succes de cursussen MULO A en B gevolgd. Hierna werkte hij drie jaar bij de B.V. Chemische Pharmaceutische Industrie "LUXAN", Elst (OB) als Chemisch analist op de afdeling ontwikkeling en controle van gewasbeschermingsmiddelen. Via een avondstudie verwierf hij het diploma HBO chemisch analist. In 1973 volgde een tweede periode bij het ILOB, aanvankelijk als medewerker van de Stichting ILOB en later opnieuw in dienst van TNO. Vanaf 1973 lag de nadruk op het ontwikkelen van proeftechnieken voor voedingsonderzoek bij landbouwhuisdieren. De bij het ILOB ontwikkelde dierproeftechnieken hadden betrekking op het aanbrengen van darmcanules ten behoeve van verteringsfysiologisch en histologisch onderzoek en katheters in bloedvaten voor onderzoek naar de kinetiek van geabsorbeerde nutriënten. In de avonduren is het diploma Atheneum en het Propedeuse diploma Biologie, van de deeltijdopleiding van de Universiteit Utrecht, behaald.

In 2000 is het ILOB opgegaan in het ID TNO Diervoeding in Lelystad waar Van Leeuwen op dit moment werkzaam is.