

Steering protein fermentation by adding fibres in pig diets

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ABSTRACT

Protein fermentation is associated with diarrhoea in pigs through the presence of potentially toxic metabolites, including ammonia, branched-chain fatty acids, biogenic amines, and indolic and phenolic compounds. One approach to reduce these metabolites is the dietary inclusion of fibres. In this experiment, 128 boars (mean BW \pm SEM; 24 \pm 1.5 kg) were assigned to diets without added fermentable fibres (**NFF**), with rapidly fermentable fibres (**RFF**), or with slowly fermentable fibres (**SFF**), using either bovine collagen (**BC**) or zein (**ZE**) as the only, low-digestible protein source. In addition, two control groups received easily digestible whey protein isolate (**WPI**) either with NFF or SFF. After 2 weeks, digesta contents from the ileum, cecum, proximal- and distal colon were analysed to assess protein digestibility, digesta mean retention time, and the concentrations and flows of protein-derived metabolites. Metabolite flow was used as a proxy for the extent of protein fermentation, reflecting the net appearance of metabolites, while the concentrations provided insight into the direct exposure of the intestinal epithelium to these metabolites. Metabolite flow was generally linked to the precursor amino acid profiles of the protein sources. Apparent ileal protein digestibility did not differ significantly between BC and ZE, except when RFF were added (Fibre \times Protein $<$ 0.05), where it was lower in ZE-fed pigs ($P <$ 0.05). Both fibre treatments, but particularly SFF, reduced the concentrations of most protein-derived metabolites. The effects of RFF and SFF on colonic metabolite flows varied depending on metabolite type and protein source. In the proximal colon of ZE-fed pigs, dietary fibres reduced the flows of branched-chain fatty acids (**BCFA**; RFF: 26%, SFF: 33%), indole (RFF: 70%, SFF: 59%), and p-cresol (SFF: 28%), while no effects were observed in BC-fed pigs (Fibre \times Protein $<$ 0.05). No fibre effects on the flows of ammonia, biogenic amines, valeric acid, phenol, and skatole were observed in either BC- or ZE-fed pigs. In WPI-fed pigs, SFF significantly increased the flows of BCFA (47%), p-cresol (75%), and biogenic amines (120%; all $P <$ 0.05) in the proximal colon. It can be concluded that protein fermentation primarily occurs in the proximal colon, starting in the ileum, with metabolite production generally linked to amino acid precursor composition. While dietary fibres reduced the concentrations of most protein-derived metabolites, the ability of different fibres to steer the extent of protein fermentation is strongly dependent on the source of proteins being fermented.

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Implications

Protein fermentation has been shown to be associated with the incidence of diarrhoea in pigs through the presence of potentially toxic metabolites. Feeding fibres is commonly proposed as a strategy to reduce the production of these protein-derived metabolites, but the effectiveness of various types of fibres and their interactions with different protein sources are unclear. This study shows that fibres reduce the concentrations of many protein-derived

metabolites, but that the ability of fibres to steer the extent of protein fermentation is strongly dependent on the source of proteins being fermented. These findings are important for optimising pig feed formulations and intestinal health.

Introduction

In pig feed, soybean meal is increasingly being replaced by alternative protein sources such as rapeseed meal and sunflower seed meal, to reduce costs and environmental impact of pork production (Florou-Paneri et al., 2014; Van Zanten et al., 2015). However, the relatively low digestibility of these alternative protein

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sources (CVB, 2023) increases the protein influx into the hindgut, where proteins are prone to fermentation by microbiota. Protein fermentation results in the production of protein-derived metabolites, such as ammonia, branched-chain fatty acids (BCFA), biogenic amines, and indolic and phenolic compounds. Some of these metabolites, including ammonia and phenolic compounds, may be harmful to intestinal health and increase the incidence of diarrhoea (Heo et al., 2008; Heo et al., 2009; Wellock et al., 2006).

One strategy to reduce the formation of these protein-derived metabolites is the inclusion of fermentable fibres in the diet (Bikker et al., 2006; Pieper et al., 2014). The addition of fermentable fibres has been shown to shift nitrogen excretion from urine to faeces (Bindelle et al., 2009; Canh et al., 1997; Mosenthin et al., 1992a), likely due to bacterial incorporation of amino acids for growth instead of being used as energy source or the incorporation of recycled urea (Bindelle et al., 2009; Krone et al., 2019; Mosenthin et al., 1992b).

Several studies have demonstrated the efficacy of adding fermentable fibres in reducing the faecal concentration of protein-derived metabolites. Uncertainty persists regarding the role of physical and chemical properties of fibres and whether their effect depends on the type of proteins in the digesta (Bikker et al., 2006; Jearend et al., 2008; Pieper et al., 2014). Assuming that protein fermentation primarily occurs in the distal colon, where carbohydrates become depleted (Macfarlane et al., 1992; Wen et al., 2018), the addition of slowly fermentable fibres has been hypothesised to be most effective. However, previous findings indicate that protein fermentation already occurs in the ileum, and that the highest concentrations of protein-derived metabolites may be found in either the proximal or distal colon, depending on the specific metabolite (Bikker et al., 2006; Pieper et al., 2014), suggesting that incorporating rapidly fermentable fibres or a mixture of rapidly and slowly fermentable fibres may yield greater effectiveness than incorporating slowly/poorly fermentable fibre sources only (Awati et al., 2006). The optimal fibre strategy could further hinge on the protein source used. Synchronising the availability of fibres and proteins at the site of fermentation may increase effectiveness. Furthermore, the amino acid profile of the protein sources may influence the interactions between fibre and protein fermentation by altering the microbial composition, and metabolism and decomposition of amino acids (Ma et al., 2022).

In this study, the effect of incorporating rapidly versus slowly fermentable fibres into the diet on the concentrations and flows of protein-derived metabolites is investigated along the gastrointestinal tract of pigs, fed with two low-digestible protein sources varying in amino acid composition, and one high-digestible protein source. The flows of protein-derived metabolites were used to reflect the extent of protein fermentation, while the concentrations may be important for direct exposure of these metabolites to the intestinal epithelium. It was hypothesised that rapidly fermentable fibres would most effectively reduce protein-derived metabolites in the proximal gastrointestinal tract, whereas slowly fermentable fibres would be more effective in reducing these metabolites further along the gastrointestinal tract. Additionally, it was hypothesised that the impact of fibres on the reduction of protein-derived metabolites depends on the protein source fed, with a greater effect when the location of fermentation of both fibres and proteins is synchronised. Insights gained from examining the interactions between dietary proteins, fibres and protein-derived metabolites throughout the gastrointestinal tract can be used to more effectively reduce the formation of protein-derived metabolites with future pig feed formulations, potentially improving intestinal health. This study is part of a larger research study, which not only explores the effects of dietary fibres on protein-derived metabolites but also the effects of protein fermentation on intestinal permeability and faecal consistency (data unpublished), and the

effects of dietary fibres and poorly digestible proteins on functional metabolic capacity of pig colonocytes (Bekebrede et al., 2022).

Material and methods

Experimental design and housing

The experiment was conducted as a randomised complete block design with the addition of two extra control treatments, resulting in eight dietary treatments, across two independent blocks (batches). The treatments were tested in a 2 × 3 factorial arrangement, with poorly digestible protein sources (bovine collagen; **BC** or zein; **ZE**) and dietary fibres (no addition of fermentable fibres; **NFF**, addition of rapidly fermentable fibres; **RFF**, or addition of slowly fermentable fibres; **SFF**) as factors. Bovine collagen and zein were selected as low-digestible protein sources due to their absence of intrinsic fibre. In addition, two control treatments were included, consisting of well-digestible whey protein isolate (**WPI**) without the addition of fermentable fibres (WPI-NFF) or with the addition of slowly fermentable fibres (WPI-SFF).

In total, 128 boars (TN70; Large White × Norwegian Landrace, TopigsNorsvin, Vught, The Netherlands) were selected from two commercial farms in the Netherlands. The pigs for the second batch were obtained from a specific-pathogen-free breeding farm, because of the presence of the commensal parasite *Balantidium coli* in the faeces of pigs from the first farm, which interfered with measurements on the metabolic function of isolated colonocytes (Bekebrede et al., 2022). The experimental period was 14 days. Pigs (mean BW ± SEM; batch 1: 25.4 ± 0.34 kg, batch 2: 22.6 ± 0.55 kg) were transported to the research facilities of Wageningen University & Research. Upon arrival, pigs were allocated to one of 16 pens, stratifying initial BW among pens. Four pigs were housed per pen, with the 16 pens divided between two housing rooms. Each experimental diet was assigned to one pen in each room. Temperature was maintained between 22 and 25 °C. Between 0700 and 1900 h lights and radio were switched on, and from 1900 till 0700 h, the lights were dimmed to 5% and the radio was off. In the first week of batch 1, two pigs (BC-NFF) were diagnosed with porcine reproductive and respiratory syndrome and were treated individually with meloxicam. They showed complete recovery before the start of frequent feeding. In the second batch, pigs were vaccinated against porcine reproductive and respiratory syndrome.

Diets and feeding

After arrival, pigs were gradually switched to one of the experimental diets outlined in Table 1 over a period of 3 days. The diets contained either thermally processed BC or ZE as the sole low-digestible protein source, or WPI as a high-digestible protein source (Noorman et al., 2024). In the RFF diets, maize starch was exchanged for a mixture of inulin, high-methylated apple and citrus-pectin (Herbstreith&Fox Classic CU 201, Germany), and native potato starch (Avebe 110 813 Potato Starch, the Netherlands; 60% assumed resistant; Sun et al., 2006), resulting in the addition of 200 g fermentable fibres/kg. In the SFF diets, maize starch was exchanged for a mixture of soybean hulls (estimated non-starch polysaccharides content of 648 g/kg; Feedipedia, 2022) and oat hulls (estimated non-starch polysaccharides content of 694 g/kg; Feedipedia, 2022), resulting in the addition of 192 g non-starch polysaccharides /kg. To compensate for the protein present in soybean hulls and oat hulls in the SFF diet, soy protein concentrate and wheat gluten meal were added to the NFF and RFF diets. All diets included 30 g/kg cellulose to promote gastrointestinal motility, 100 g/kg sugar to enhance palatability, and 2 g/kg titanium dioxide as an indigestible marker. Industrially produced

Table 1

Ingredients, and chemical composition of the experimental pig diets (NFF; no added fermentable fibres, RFF; rapidly fermentable fibres, SFF; slowly fermentable fibres).

Items	Diet							
	Bovine Collagen			Zein			WPI ¹	
	NFF	RFF	SFF	NFF	RFF	SFF	NFF	SFF
Ingredient (g/kg of feed, as fed)								
Bovine collagen	181.0	181.0	181.0					
Zein				200.0	200.0	200.0		
Whey protein isolate							210.0	210.0
Inulin		50.0			50.0			
Pectin ²		50.0			50.0			
Native potato starch ³		167.0			167.0			
Soybean hulls			155.0			155.0		155.0
Oat hulls			132.0			132.0		132.0
Maize starch	531.4	263.7	287.1	527.4	259.1	284.8	542.7	298.2
Soy protein concentrate	31.0	31.0	0.0	31.0	31.0	0.0	31.0	0.0
Wheat gluten meal	11.0	13.0	0.0	13.0	15.0	0.0	12.0	0.0
Cellulose	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Rapeseed oil	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Sugar	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Vitamin and mineral mix ⁴	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
L-Lysine HCl	6.4	6.3	5.8	11.5	11.5	11.0	0.5	0.0
DL-Methionine	4.4	4.4	4.8	1.6	1.6	2.2	0.0	0.4
L-threonine	3.6	3.6	4.0	1.8	1.8	2.3	0.0	0.4
L-tryptophan	1.5	1.5	1.7	1.3	1.3	1.6	0.0	0.2
L-isoleucine	2.7	2.7	3.5	0.0	0.0	0.0	0.0	0.7
L-histidine	2.8	2.8	3.3	1.6	1.6	2.2	0.0	0.6
L-arginine	0.0	0.0	1.2	1.2	1.2	2.5	0.0	1.2
L-phenylalanine	5.4	5.2	7.2	0.0	0.0	2.3	0.0	2.0
L-leucine	5.2	5.1	6.5	0.0	0.0	1.6	0.0	1.4
L-valine	2.7	2.6	3.4	0.0	0.0	0.9	0.0	0.7
Magnesium oxide	5.7	5.6	4.1	5.5	5.4	3.9	3.3	1.7
Calcium carbonate	14.4	14.2	12.0	14.0	13.9	11.2	12.6	10.3
Monocalcium phosphate	14.5	14.4	14.8	14.0	13.9	14.2	14.7	14.9
Potassium carbonate	3.7	3.4	0.0	4.0	3.7	0.3	0.0	0.0
Potassium chloride	0.5	0.5	0.6	0.0	0.0	0.0	2.2	0.0
Sodium hydrogen carbonate	5.1	5.0	5.0	5.1	5.0	5.0	2.5	0.0
Sodium chloride	0.0	0.0	0.0	0.0	0.0	0.0	1.5	3.3
Titanium dioxide	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Calculated chemical composition (g/kg) ⁵								
DM	924	924	925	924	924	925	920	921
CP	220	220	220	220	220	220	220	220
Digestible protein	177	178	162	164	166	150	194	179
Crude fat	34	32	38	34	32	38	36	40
Crude ash	42	42	49	40	41	48	40	49
Starch	455	344	272	451	338	267	457	272
Sugars	112	112	109	112	112	109	113	111
Dietary fibre ⁶	47	163	231	47	163	231	47	231
Net Energy (MJ/kg)	11.5	10.2	9.4	11.2	9.8	9.0	11.5	9.4
Analysed chemical composition (g/kg)								
DM	913	906	915	914	916	919	911	916
CP ⁷	253	244	250	233	235	241	208	208

¹ Whey protein isolate.² Herbstreith & Fox KG Classic CU 201, Germany.³ Avebe 110 813 Potato Starch, the Netherlands.⁴ Supplied per kilogram of feed: retinyl acetate, 10 000 IU; cholecalciferol, 2 000 IU; dl- α -tocopherol, 40 mg; menadione, 1.5 mg; thiamine 1.0 mg; riboflavin, 4 mg; pyridoxin-HCl, 1.5 mg; cyanocobalamin, 20 μ g; niacin, 30 mg; D-pantothenic acid, 15 mg; Choline chloride, 150 mg; Folic acid, 0.4 mg; Biotin, 0.05 mg; iron(II)sulphate monohydrate, 331 mg; copper(II)sulphate pentahydrate, 80 mg; manganese(II)oxide, 49 mg; zinc sulphate monohydrate, 194 mg; potassium iodate, 1 mg; sodium selenite, 0.56 mg.⁵ CVB, 2018, Feedipedia, 2022, FoodData Central, 2019, Mathai et al., 2017, Pedersen et al., 2016.⁶ Dietary fibre was calculated as: organic matter – CP – crude fat – starch – sugars.⁷ N conversion factor = 6.25.

amino acids were added to the diets to meet the minimal requirements for growth and to ensure consistent concentrations of these amino acids across diets with the same protein source (CVB, 2020).

The pigs were fed twice daily, at 0800 and 1600 h. From days 1 to 11 of batch 1, they received a daily allowance of 1.4 times the energy required for maintenance, calculated using the CVB formula (net energy for maintenance = 750 kJ \times BW^{0.60}; Everts, 2015), based on the diets with the highest energy contents. From day 11, the allowance was increased to 1.6 times maintenance. In batch 2, pigs were fed daily at 1.6 times the energy required for maintenance throughout the entire study period. To approach steady-state con-

ditions in the distal gastrointestinal tract, from 36 h prior to dissection, the pigs were fed every six hours with 25% of the daily portion (Martens et al., 2019; Schop et al., 2023). Water was available *ad libitum* throughout the entire study period.

Daily measurements and digesta and tissue sampling

Health and welfare were assessed visually twice a day during feeding. After 2 weeks, the pigs were sedated using an intramuscular injection of zolazepam/tiletamine (Zoletil® 100) and xylazine (5:2 ratio, 0.1 mL/kg BW), and euthanised by an injection with pen-

tobarbital (Euthasol® 20%; 24 mg/kg BW) into the ear vein and bled. After opening the abdominal cavity, the digestive tract was ligated at four positions to prevent digesta from flowing between gastrointestinal segments. Clamps were placed two metres proximal to the cecum (defined as the start ileum), at the ileal-cecal valve, between cecum and colon, and at the end of the colon. The small intestine was separated from the cecum and colon, and the length of the small and large intestine was recorded. Then, clamps were placed on 20 and 33% of the colon, based on length (segment defined as proximal colon), and 66 and 80% of the colon (segment defined as distal colon). The digesta of the ileum, cecum, proximal, and distal colon were collected and stored at -20°C .

Measurements and analyses

Digesta from the ileum, cecum, proximal- and distal colon were thawed, homogenised and subsampled for the different analyses. For the analysis of biogenic amines and indolic and phenolic compounds, digesta samples from one pen were pooled. For ammonia analysis, subsamples were acidified with trichloroacetic acid, and for volatile fatty acids with phosphoric acid (1:1 w/v). The subsamples were again stored at -20°C until further analysis. Analyses for nitrogen (N) and titanium were performed in duplicate. Other analyses were performed in singlicate.

Nitrogen was determined using the Kjeldahl method (ISO 5893, 2005). Ammonia concentration was measured colorimetrically at a wavelength of 623 nm using a UV spectrophotometer in the thawed sample, as described by Searle (1984). For the analysis of volatile fatty acids, the samples were shaken for 30 min in a mechanical shaker, and centrifuged for 10 min at $20\,817 \times g$. Then, the sample solutions were mixed with an internal standard (31 784 mM 2-methyl valeric acid), centrifuged ($20\,817 \times g$ for 5 min), and introduced into the gas chromatograph (HIP-FFAP, 30 m \times 0.32 mm \times 0.25 μm ; Agilent J&W, USA). After separation of the fatty acids, volatile fatty acids were detected by a flame ionisation detector.

Biogenic amines were extracted from the samples with dilute hydrochloric acid. Proteins were removed by adding 1 ml of 2% sulphosalicylic acid in 0.1 M hydrochloric acid to 0.5 g of sample at 4°C . After shaking for 15 min on a shaker with maximum speed, the samples were placed on an ice bath for 15 min, followed by centrifugation at 14 000 rpm for 10 min at 18°C . Subsequently, 0.5 ml of heptylchloride (215.8 $\mu\text{mol/L}$) was added to 0.5 mL of the supernatant. After derivatisation with Dansyl chloride, the biogenic amines were separated using high-performance liquid chromatography and fluorescence detection (S5200 analyser, fluorescence detector RF-20A). The analytical column used was a Agilent 5 TC-C18 (2) 250 \times 4.6 mm. Heptylchloride was used as an internal standard to correct the loss of components during preparation and injection. A chemical standard solution was used to identify and quantify the individual biogenic amines. Separation was achieved by mobile phase A (50 mL 0.2 M NH_4Ac ; pH 5, 300 mL Milli-Q water and 150 mL acetonitrile), and mobile phase B (50 mL 0.2 M NH_4Ac ; pH 5, 25 mL Milli-Q water and 425 mL acetonitrile). The flow was 0.62 mL/minute over the entire run, at 45°C and detection at an excitation wavelength of 250 nm and an emission of 540 nm.

In addition, samples of the digesta from ileum, cecum, proximal and distal colon were freeze-dried and analysed for titanium to determine mean retention time (MRT) based on titanium pool sizes, and assuming steady-state conditions (De Vries and Gerrits, 2018). DM was assessed using the freeze-dried samples without correction for the minor moisture content still present. Titanium was determined in the freeze-dried samples after hydrolysis with concentrated sulfuric acid in the presence of a copper catalyst at 420°C and subsequent addition of peroxide. The resulting

orange/yellow coloured complex was spectroscopically determined at 408 nm.

Indole, p-cresol, phenol, skatole, and 4-ethylphenol were also measured in the freeze-dried digesta samples of the proximal and distal colon, using HPLC, and fluorescence detection at an excitation wavelength of 275 nm and an emission of 310 nm, based on a modification of the method described by Gibis et al. (1991), as detailed in Supplementary Material S1. Briefly, separation was achieved using a gradient starting at 100% mobile phase A (80% water, 12% acetonitrile, 8% iso-propanol) and 0% mobile phase B (60% acetonitrile and 40% isopropanol) for 2 min, followed by a linear increase to 40% mobile phase B over 8 min. This ratio of mobile phase A and phase B was held for 5 min, and then the column was washed with 90% mobile phase B for 3 min and equilibrated with 0% mobile phase B for 4 min. The flow was 1 mL/minute over the entire run.

Calculations

Apparent ileal, cecal, and total tract N digestibility were calculated using the following equation:

$$\text{Digestibility coefficient N} = \left(1 - \frac{[\text{Ti diet}] \times [\text{N digesta}]}{[\text{Ti digesta}] \times [\text{N diet}]} \right) \times 100\%$$

where [Ti diet] is the titanium concentration in the diet (g/kg DM), [N digesta] is the N concentration in the digesta (g/kg DM), [Ti digesta] is the titanium concentration in the digesta (g/kg DM), and [N diet] is the N concentration in the diet (g/kg DM).

Apparent DM digestibility in ileum, cecum, proximal and distal colon was calculated using the following equation:

$$\text{Digestibility coefficient DM} = \left(1 - \frac{[\text{Ti diet}]}{[\text{Ti digesta}]} \right) \times 100\%$$

where [Ti diet] is the titanium concentration in the diet (g/kg DM), and [Ti digesta] is the titanium concentration in the digesta (g/kg DM).

The MRT of titanium in ileum, cecum, proximal and distal colon was calculated using the following equation, assuming steady-state conditions (De Vries and Gerrits, 2018):

$$\text{MRT (h)} = 24 \times \frac{[\text{Ti digesta}] \times W}{I}$$

where MRT is the mean retention time in hours in the compartment of the gastrointestinal tract, [Ti digesta] is the marker concentration in the digesta (g/kg DM), W is the weight of digesta in the corresponding segment (kg DM) and I is the marker intake in 24 h. Mean retention time of the sampled part of the colon (13% based on length for both the proximal and distal colon) was recalculated for the entire proximal or distal colon (assuming each represents 50% of the total colon length) using the formula: $\text{MRT} / 0.13 \times 0.5$.

The flow of the protein-derived metabolites in the ileum, cecum, proximal and distal colon was calculated using the following equation:

Metabolite flow in intestinal segment (mmol/hour)

$$= \frac{[\text{metabolite}] \times W}{\text{MRT}}$$

where [metabolite] is the concentration of the metabolite of interest in the ileum, cecum, proximal or distal colon (mmol/g), W the weight of digesta in the ileum, cecum, proximal or distal colon (g), and MRT the digesta mean retention time in the ileum, cecum, proximal or distal colon (h).

Statistical analyses

For all statistical analyses, R for Windows 3.6.0 was used, packages: dplyr (Wickham et al., 2023), emmeans (Lenth et al., 2018), FactoMineR (Lê et al., 2008), ggplot2 (Wickham, 2016), moments (Komsta and Novomestky, 2022), and tidyr (Wickham et al., 2024). Pen (consisting of four pigs) was considered as the experimental unit. The homogeneity and normality of model residuals were verified graphically and with the Shapiro-Wilk test. If the residuals were not normally distributed, statistical analyses were performed on transformed data, as indicated below. Data are reported as least-square means \pm SEM, and differences were considered significant if $P < 0.05$ and a trend at $P < 0.10$.

With the use of principal component analysis (PCA), we identified protein-derived metabolites in the colon that shared common variance within the data set. Principal component analysis was done on both metabolite flows and metabolite concentrations in the colon. The principal components were calculated using a Varimax rotation in the FactoMineR package. Subsequently, treatment effects on the calculated values for each PC explaining variance exceeding 10% were analysed as described below.

To investigate the effects of protein source and fibre addition on digestibility, digesta MRT, metabolite flows and concentrations, and the principal components (PC1 and PC2) based on metabolite flows and concentrations, different datasets were used. This distinction was based on the fact that pigs were fed BC and ZE in combination with NFF, RFF and SFF, while the pigs receiving WPI were fed NFF and SFF, without RFF. The analyses were performed using two-way ANOVA: (1) comparing NFF, RFF and SFF in pigs fed BC and ZE, (2) comparing NFF and SFF in pigs fed WPI, (3) comparing BC, ZE and WPI in pigs fed NFF.

$$Y_{ijkl} = \mu + P_i + F_j + B_k + (P \times F)_{ij} + (P \times B)_{ik} + (F \times B)_{jk} + (P \times F \times D)_{ijk} + e_{ijkl} \quad (1)$$

where Y_{ijkl} = dependent variable, μ = overall mean, P_i = protein source (BC or ZE), F_j = fibre source (NFF, RFF, or SFF), B_k = batch effect (1 or 2), $(P \times F)_{ij}$ = interaction effect between P and F, $(P \times B)_{ik}$ = interaction effect between P and B, $(F \times B)_{jk}$ = interaction effect between F and B, $(P \times F \times B)_{ijk}$ = 3-way interaction effect between P, F and B, and e_{ijkl} = residual error. Non-significant interactions were omitted from the model.

In case of an interaction between $F \times P$, differences among all means were compared using type III least squares statistics, using Tukey adjustments for multiple comparisons. In case of a significant fibre effect in the absence of $F \times P$ interaction, differences among means were tested using type III least squares statistics.

$$Y_{ijk} = \mu + F_i + B_j + (F \times B)_{ij} + e_{ijk} \quad (2)$$

where Y_{ijk} = dependent variable, μ = overall mean, F_i = fibre source (NFF or SFF), B_j = batch effect (1 or 2), $(F \times B)_{ij}$ = interaction effect between F and B, and e_{ijk} = residual error. Non-significant interactions were omitted from the model. Mean retention time in the cecum was transformed using $1/x$.

$$Y_{ijk} = \mu + P_i + B_j + (P \times B)_{ij} + e_{ijk} \quad (3)$$

where Y_{ijk} = dependent variable, μ = overall mean, P_i = protein source (WPI, BC or ZE), B_j = batch effect (1 or 2), $(P \times B)_{ij}$ = interaction effect between P and B, and e_{ijk} = residual error. Non-significant interactions were omitted from the model. The net N disappearance between ileum and distal colon, the flow of cadaverine, histamine, tyramine, BCFA, isovaleric acid, and 4-ethylphenol, and the concentration of cadaverine, p-cresol, and phenol in the proximal colon, and the flow of biogenic amines, cadaverine, tryptamine, BCFA, isobutyric acid, isovaleric acid, isocaproic acid, and p-cresol, and the concentration of cadaverine, tryptamine, BCFA, acetic acid, iso-

valeric acid, indole, and p-cresol in the distal colon were transformed using \sqrt{x} . In case of a significant protein effect, differences among means were tested using type III least squares statistics.

Results

Lower feed intake in pigs fed zein + no fermentable fibres and zein + rapidly fermentable fibres

The feed intake of ZE-fed pigs, particularly when combined with NFF and RFF, was lower as compared with BC and WPI, due to unintended feed refusals (Table 2). During frequent feeding, the feed intake of ZE-fed pigs came close to BC, resulting in an 11 g/pig per day lower feed intake for ZE-NFF in batch 1 as compared with the other diets. In batch 2, the feed intake during frequent feeding was 208 g/pig per day lower for ZE-NFF as compared with BC, WPI and ZE-SFF, and 50 g/pig per day for ZE-RFF. In batch 2, we started the experiment with a higher feeding level as compared with batch 1. This resulted in a higher feed intake in batch 2 until the start of frequent feeding at 36 h prior to dissection.

Protein \times fibre interactions for nitrogen and DM digestibility, not for digesta mean retention time

As anticipated, apparent ileal protein digestibility was higher in pigs fed the highly digestible WPI diets as compared with ZE and numerically higher as compared with BC (Table 3). Apparent ileal protein digestibility did not differ significantly for BC and ZE, except when RFF were added (Fibre \times Protein < 0.05), where it was lower in ZE-fed pigs ($P < 0.05$). The effect of dietary fibres on total tract protein digestibility, measured in the distal colon, differed between protein sources. In BC-fed pigs, it was reduced by the addition of SFF, but not by RFF, but contrary in ZE-fed pigs, where it was lowered by the addition of RFF, but not SFF (Fibre \times Protein < 0.001). Also in WPI-fed pigs, the addition of SFF reduced total tract protein digestibility ($P < 0.001$). Net N disappearance between ileum and distal colon did not differ among protein sources. Feeding RFF resulted in a lower net N disappearance as compared with NFF in BC- and ZE-fed pigs. The net N disappearance between cecum and distal colon was lower in pigs fed WPI-NFF as compared with ZE-NFF ($P < 0.01$), while no differences were observed between fibre sources.

Feeding RFF and SFF reduced ileal DM digestibility in both BC- and ZE-fed pigs (Table 3). In the proximal and distal colon, DM digestibility was reduced by feeding SFF as compared with NFF ($P < 0.001$), and in the proximal colon of ZE-fed pigs, also when fed RFF (Fibre \times Protein < 0.05). Ileal digesta MRT tended to be 33 min prolonged in ZE-fed pigs as compared with BC ($P = 0.05$, Table 3). No differences in digesta MRT between ZE, BC, or WPI were observed in the other intestinal segments. In the distal colon of BC- and ZE-fed pigs, the digesta MRT was approximately five hours longer in RFF-fed pigs as compared with SFF-fed pigs ($P < 0.05$).

Digesta metabolite profiles varied among protein sources

The flow of protein-derived metabolites was higher in the proximal colon as compared with the distal colon, regardless of protein source ($P < 0.05$). The metabolite profiles of digesta in the proximal and distal colon varied among protein sources (Tables 4 and 5). Across all protein sources, the flow of biogenic amines was by far the greatest of all metabolites. In both the proximal and distal colon, the flow of the sum of biogenic amines was not significantly different between BC- and ZE-fed pigs, or between pigs fed BC-NFF,

Table 2

Average feed intake per day of pigs fed with different protein sources (bovine collagen, zein, whey protein isolate; WPI) and different fibre sources (no fibre source; NFF, rapidly fermentable fibres; RFF, slowly fermentable fibres; SFF)¹.

Items (gram/day)	Experimental diets									SEM
	Bovine Collagen			Zein			WPI			
	NFF	RFF	SFF	NFF	RFF	SFF	NFF	SFF		
Batch 1										
No. of pens ²	2	2	2	2	2	2	2	2	2	
Day 5 until 36 h prior to dissection	652	652	652	487	526	628	652	652	652	35.8
36 h before dissection until dissection ³	710	710	710	699	710	710	710	710	710	2.5
Batch 2										
No. of pens ²	2	2	2	2	2	2	2	2	2	
Day 5 until 36 h prior to dissection	701	707	709	363	505	597	710	710	710	66.5
36 h before dissection until dissection ³	710	710	710	502	660	710	710	710	710	37.6

¹ Data are presented as least square means ± pooled SEM.

² Number of replicate pens with four pigs per pen.

³ Frequent feeding.

Table 3

Apparent digestibility of nitrogen and DM, net N disappearance between the ileum/cecum and distal colon, and digesta mean retention time in pigs fed bovine collagen, zein, or whey protein isolate (WPI) as protein source in the absence (NFF) or presence of rapidly fermentable fibres (RFF), or slowly fermentable fibres (SFF)¹.

Items (%)	Experimental diets							SEM	P-values ^{2,3}				Reference diets			
	Bovine Collagen			Zein			Fibre		Protein	Batch	F × P ⁵	WPI		SEM	P-value ^{2,4}	
	NFF	RFF	SFF	NFF	RFF	SFF						NFF	SFF			NFF-SFF
No. of pens ⁶	4	4	4	4	4	4						4	4			
CP (%)																
Ileum	62 ^{ab#*}	76 ^b	57 ^{ab}	52 ^{ab#}	48 ^a	65 ^{ab}	7.0	0.68	0.062	0.78	0.032	84 [*]	71	4.1	0.012	
Cecum	86 ^{d#}	80 ^{cd}	78 ^{cd}	69 ^{b#}	59 ^a	75 ^{bc}	4.7	<0.001	<0.001	0.84	<0.001	93 [×]	84	2.3	<0.001	
Distal colon	91 ^{d#}	88 ^{cd}	85 ^c	76 ^{b#}	66 ^a	79 ^b	4.4	<0.001	<0.001	0.65	<0.001	94 [×]	87	2.1	<0.001	
DM (%)																
Ileum	70 [#]	65	55	72 [#]	60	63	4.1	<0.001 ⁷	0.51	0.006	0.074	84 [*]	63	6.2	0.004	
Cecum	85 [#]	78	62	81 [#]	74	62	4.7	<0.001 ⁸	0.027	0.44	0.44	91 [*]	65	6.9	<0.001	
Proximal colon	89 [#]	84 ^c	73 ^a	86 ^{c#}	79 ^b	74 ^{ab}	3.2	<0.001	0.031	0.86	0.032	92 [*]	76	4.4	<0.001	
Distal colon	91 [#]	89 ^c	73 ^a	87 ^{bc*}	84 ^b	75 ^a	3.5	<0.001	<0.001	0.063	<0.001	93 [×]	77	4.5	<0.001	
Net N disappearance (%) ⁹																
Ileum – Distal Colon	36	13	26	27	22	12	7.5	0.045 ¹⁰	0.30	0.061	0.11	30	15	12.3	0.44	
Cecum – Distal Colon	5 ^{#*}	8	7	6 [#]	7	4	1.4	0.11	0.39	0.12	0.21	2 [*]	3	0.4	0.39	
Mean retention time (h:min)																
Ileum	1:00	1:08	0:51	0:59	1:30	1:34	0:15	0.32	0.052	0.26	0.24	1:31	1:02	0:17	0.27	
Cecum	15:19	15:58	9:48	7:34	11:31	10:17	5:17	0.49	0.13	<0.001	0.42	20:54	10:18	7:48	0.052	
Proximal colon	13:30	18:06	12:48	15:36	18:12	16:24	3:09	0.27	0.34	<0.001	0.77	22:54	15:42	3:53	0.21	
Distal colon	11:04	14:38	7:46	8:56	12:43	9:36	2:11	0.035 ¹¹	0.62	0.016	0.48	22:06	13:42	6:10	0.30	

¹ Data are presented as least square means ± pooled SEM.

² Model established P-values for the fixed effects of protein source, fibre source, and blocking factor batch, and their interactions. In case of a significant fibre × protein interaction ($P < 0.05$), letters (a,b,c,d) indicate differences among treatments. In case of a significant effect of protein source within NFF diets ($P < 0.05$), symbols (#, *, ×) indicate differences among protein sources within NFF.

³ For apparent digestibility of DM in the distal colon, interactions were observed between protein × batch ($P = 0.041$), and fibre × protein × batch ($P = 0.028$). For net N disappearance between ileum and distal colon, an interaction was observed between fibre × batch ($P = 0.019$). For mean retention time in the ileum, an interaction was observed between fibre × batch ($P = 0.029$).

⁴ For mean retention time in the cecum, a batch effect was observed ($P < 0.001$).

⁵ Interaction between fibre × protein.

⁶ Number of replicate pens with four pigs per pen.

⁷ Difference between NFF-RFF and NFF-SFF.

⁸ Difference between NFF-RFF, NFF-SFF, and RFF-SFF.

⁹ Difference N digestibility between the ileum/cecum and distal colon.

¹⁰ Difference between NFF-RFF.

¹¹ Difference between RFF-SFF.

ZE-NFF, and WPI-NFF. Also, for most individual biogenic amines, no significant differences were found between protein sources, except for agmatine, spermidine and tryptamine in the proximal colon and agmatine, spermidine and spermine in the distal colon. These biogenic amines had generally lower flows in pigs fed WPI-NFF as compared with BC-NFF (proximal colon: agmatine, spermidine, tryptamine, distal colon: spermidine, spermine; $P < 0.05$) and/or ZE-NFF (proximal colon: agmatine, distal colon: agmatine, spermidine; $P < 0.05$). In the distal colon, the flow of biogenic amines

was about halved as compared with the proximal colon, mainly caused by a reduced flow of agmatine.

In both the proximal and distal colon, the flows of BCFA (isobutyric-, isovaleric- + isocaproic acid), indole, p-cresol, phenol and skatole were higher in ZE- than in BC-fed pigs ($P < 0.05$). Moreover, the flows of BCFA, indole, and p-cresol were higher in pigs fed ZE-NFF as compared with BC- and WPI-NFF ($P < 0.01$). Similarly, the flows of phenol and skatole were higher in pigs fed ZE-NFF than in WPI-NFF in both the proximal and distal colon, and the flow of

Table 4

Flow of metabolites in the proximal colon of pigs fed bovine collagen, zein, or whey protein isolate (WPI) as protein source in the absence (NFF) or presence of rapidly fermentable fibres (RFF), or slowly fermentable fibres (SFF)¹.

Items (mmol/h)	Experimental diets						SEM	P-Values ^{2,3}				Reference diets			
	Bovine Collagen			Zein								WPI	SEM	P-value ^{2,4}	
	NFF	RFF	SFF	NFF	RFF	SFF		NFF	SFF	NFF	SFF	NFF-SFF			
No. of pens ⁶	4	4	4	4	4	4						4	4		
Biogenic amines	146	198	151	146	141	101	28.2	0.38	0.18	0.89	0.61	58.3	128.1	21.02	0.005
Agmatine	77 [#]	97	78	99 [#]	87	70	10.4	0.20	0.90	0.46	0.24	45.4*	70.2	8.36	0.033
Putrescine	8.4	17.2	7.1	4.4	11.0	5.4	3.58	0.014 ⁷	0.079	0.99	0.69	2.6	7.2	1.46	0.011
Cadaverine	14.2	23.8	10.8	26.4	10.5	5.4	9.71	0.47	0.80	0.31	0.45	3.1	27.8	8.95	<0.001
Histamine	3.6	1.9	1.6	3.8	1.9	1.3	1.05	0.12	0.96	0.30	0.95	0.8	3.3	1.15	0.16
Tyramine	0.6	4.7	0.7	1.4	5.9	0.5	1.97	0.030 ⁷	0.73	0.076	0.92	0.2	3.3	1.70	0.24
Spermidine	9.1 [#]	13.5	9.8	6.4 ^{#*}	15.0	7.4	1.88	<0.001 ⁸	0.23	0.67	0.17	3.4*	8.0	1.28	<0.001
Spermine	0.7	0.8	0.6	0.4	0.5	0.4	0.13	0.40	0.008	0.99	0.99	0.3	0.7	0.12	0.009
Tryptamine	9.3 [#]	32.2 ^a	34.3 ^a	1.1 ^{a*}	2.0 ^a	1.3 ^a	10.0	0.032	<0.001	0.035	0.041	0.6*	2.2	0.48	0.002
Pyrrolidine	3.8 ^{ab}	7.3 ^b	7.3 ^b	3.1 ^a	6.7 ^{ab}	4.6 ^{ab}	1.31	<0.001	0.002	<0.001	0.045	1.9	5.4	0.99	<0.001
BCFA	0.078 ^{a#}	0.082 ^a	0.096 ^{ab}	0.197 ^{c*}	0.145 ^b	0.132 ^b	0.0024	0.067	<0.001	0.075	0.004	0.058 [#]	0.084	0.0086	0.026
Isobutyric acid	0.036 ^{a#}	0.038 ^{ab}	0.046 ^{abc}	0.059 ^{c*}	0.055 ^c	0.050 ^{bc}	0.0053	0.85	<0.001	0.015	0.014	0.029 [#]	0.042	0.0043	0.017
Isovaleric acid	0.041 ^{a#}	0.044 ^{ab}	0.050 ^{ab}	0.137 ^{d*}	0.089 ^c	0.081 ^{bc}	0.0190	0.024	<0.001	0.16	0.004	0.029 [#]	0.041	0.0042	0.033
Isocaproic acid	0.0012	0.0005	0.0006	0.00012	0.0007	0.0004	0.0003	0.11	0.97	0.71	0.89	0.0007	0.0007	0.00028	0.97
SCFA, excl. BCFA	3.59	4.95	5.62	2.50	4.60	3.47	0.486	0.008 ⁹	0.012	0.008	0.25	1.73	4.00	0.690	0.016
Acetic acid	2.34	3.29	4.19	1.68	2.95	2.49	0.568	0.006 ⁹	0.010	0.011	0.20	1.17	2.83	0.482	0.002
Propionic acid	0.78	0.80	0.90	0.56	0.85	0.66	0.126	0.38	0.15	0.027	0.36	0.35	0.81	0.158	0.025
Butyric acid	0.28	0.68	0.30	0.16	0.66	0.24	0.116	<0.001 ⁸	0.13	0.046	0.67	0.16	0.29	0.060	0.14
Caproic acid	0.003 ^a	0.019 ^b	0.003 ^a	0.003 ^a	0.029 ^c	0.004 ^a	0.0054	<0.001	<0.001	0.10	<0.001	0.003	0.003	0.0008	0.98
Valeric acid	0.18 ^{cb#}	0.16 ^{bcd}	0.23 ^b	0.10 ^{ad*}	0.11 ^{ac}	0.08 ^a	0.035	0.44	<0.001	<0.001	0.004	0.05*	0.06	0.014	0.75
4-ethylphenol	0.0003 ^a	0.0000 ^a	0.0156 ^a	0.0018 ^a	0.0130 ^a	0.0590 ^b	0.01163	<0.001	<0.001	0.21	0.004	0.0000	0.0306	0.00887	0.003
Indole	0.53 ^{ab#}	0.21 ^a	0.41 ^{ab}	2.40 ^{c*}	0.71 ^{ab}	0.98 ^b	0.393	<0.001	<0.001	0.13	<0.001	0.79 [#]	0.57	0.099	0.15
p-Cresol	3.18 ^{a#}	3.62 ^a	4.04 ^a	13.07 ^{c*}	10.97 ^{bc}	9.37 ^b	2.116	0.23	<0.001	0.99	0.035	2.20 ^x	3.84	0.545	0.023
Phenol	0.18 [#]	0.14	0.29	1.49 [*]	0.80	0.34	0.345	0.15	0.005	0.081	0.078	0.13 [#]	0.11	0.028	0.72
Skatole	0.59 [#]	0.46	0.71	1.07 [#]	1.06	1.24	0.216	0.52	0.003	0.84	0.94	0.25*	1.13	0.382	0.10

Abbreviations: BCFA = branched-chain fatty acids; SCFA = short-chain fatty acids.

¹ Data are presented as least square means ± pooled SEM.

² Model established P-values for the fixed effects of protein source, fibre source, and blocking factor batch, and their interactions. In case of a significant fibre × protein interaction ($P < 0.05$), letters (a,b,c,d) indicate differences among treatments. In case of a significant effect of protein source within NFF diets ($P < 0.05$), symbols (#, *, ×) indicate differences among protein sources within NFF.

³ For putrescine and spermine, interactions were observed between fibre × protein × batch ($P = 0.035$ and $P = 0.029$, respectively). For tryptamine and pyrrolidine, interactions were observed between fibre × batch ($P = 0.011$ and $P = 0.014$, respectively), protein × batch ($P = 0.026$ and $P < 0.001$, respectively), and fibre × protein × batch ($P = 0.013$ and $P = 0.042$, respectively). For spermine and caproic acid, interactions were observed between fibre × batch ($P = 0.046$ and $P = 0.006$). For valeric acid, an interaction was observed between protein × batch ($P = 0.002$).

⁴ For cadaverine and pyrrolidine, a batch effect ($P < 0.01$) was observed. For SCFA, excl. BCFA, and cadaverine, interactions were observed between fibre × batch ($P < 0.05$).

⁵ Interaction between fibre × protein.

phenol in the proximal colon was also higher in pigs fed ZE-NFF as compared with BC-NFF ($P < 0.05$).

Conversely, the flow of the short-chain fatty acids excl. BCFA (acetic-, propionic-, butyric-, caproic- + valeric acid) in the proximal colon was higher in BC- than in ZE-fed pigs ($P < 0.05$), with no significant differences observed between BC and ZE in the distal colon. Furthermore, their flow was lower in pigs fed WPI-NFF as compared with BC-NFF and ZE-NFF in both the proximal and distal colon ($P < 0.05$). The flow of ammonia in both the proximal and distal colon was similar between pigs fed BC and ZE (Table 6). In the proximal colon, ammonia flow was also not significantly different between BC-, ZE-, and WPI-NFF. However, in the distal colon, the flow of ammonia was higher in pigs fed ZE-NFF than in WPI-NFF ($P < 0.05$).

Effects of dietary fibres on protein-derived metabolite flows varied depending on the protein sources, metabolites, and intestinal segments

In the ileum, both RFF and SFF tended to reduce ammonia flows as compared with NFF, in BC- and ZE-fed pigs ($P < 0.1$; Table 6). No such trend was observed in pigs fed WPI-SFF as compared with WPI-NFF and no significant fibre effects were observed in the proximal colon. In contrast, in the cecum and distal colon, feeding SFF increased ammonia flows as compared with NFF in BC-, ZE-, and WPI-fed pigs ($P < 0.05$). In the proximal colon of ZE-fed pigs, both RFF and SFF reduced the flows of BCFA, indole, and, for SFF, p-cresol, whereas no significant effects of dietary fibres were observed in BC-fed pigs (Fibre × Protein < 0.05 ; Table 4). Both fibre treatments numerically increased the flows of BCFA and p-cresol in

Table 5

Flow of metabolites in the distal colon of pigs fed bovine collagen, zein, or whey protein isolate (WPI) as protein source in the absence (NFF) or presence of rapidly fermentable fibres (RFF), or slowly fermentable fibres (SFF)¹.

Items (mmol/h)	Experimental diets						SEM	P-Values ^{2,3}				Reference diets			
	Bovine Collagen			Zein								WPI	SEM	P-Value ^{2,4}	
	NFF	RFF	SFF	NFF	RFF	SFF		Fibre	Protein	Batch	FxP ⁵	NFF	SFF	NFF-SFF	
No. of pens ⁶	4	4	4	4	4	4									
Biogenic amines	66	120	87	88	76	50	19.5	0.35	0.36	0.26	0.17	38.6	57.4	6.16	0.030
Agmatine	40 ^{a#}	61 ^a	50 ^a	57 ^{a#}	40 ^a	42 ^a	7.6	0.83	0.49	0.99	0.046	27.6*	31.6	2.72	0.37
Putrescine	1.6	4.1	2.2	1.4	3.3	1.0	1.20	0.13	0.46	0.21	0.92	0.5	4.5	1.10	<0.001
Cadaverine	11.3	12.8	3.6	20.5	4.9	1.8	7.82	0.24	0.98	0.071	0.53	0.7	10.7	3.53	0.002
Histamine	0.9	1.5	0.7	1.1	0.9	0.4	0.43	0.47	0.62	0.68	0.71	0.3	1.2	0.84	0.52
Tyramine	0.5	4.5	0.9	0.7	5.6	0.5	2.26	0.13	0.99	0.18	0.97	0.2	0.8	0.34	0.20
Spermidine	5.3 [#]	8.9	7.5	4.3 ^{#*}	11.6	4.8	2.39	0.074	0.86	0.55	0.50	2.4*	4.6	0.63	<0.001
Spermine	0.3 [#]	0.3	0.7	0.2*	0.5	0.3	0.16	0.31	0.58	0.84	0.31	0.2*	0.5	0.08	<0.001
Tryptamine	5.3	14.6	15.7	0.8	1.0	0.7	4.73	0.42	0.006	0.55	0.42	0.3	0.8	0.16	0.007
Pyrrolidine	1.7	3.5	4.7	1.8	2.9	2.7	0.79	0.045 ⁷	0.26	0.34	0.41	0.8	2.6	0.56	0.006
BCFA	0.061 ^{a#}	0.092 ^{ab}	0.137 ^{bc}	0.167 ^{c*}	0.151 ^{bc}	0.150 ^{bc}	0.0145	0.13	<0.001	0.28	0.016	0.042 [×]	0.095	0.0153	0.001
Isobutyric acid	0.029 ^{a#}	0.041 ^{ab}	0.062 ^c	0.049 ^{c*}	0.057 ^{bc}	0.055 ^{bc}	0.0067	<0.001	0.009	0.17	0.005	0.020 [×]	0.043	0.0066	0.001
Isovaleric acid	0.032 ^{a#}	0.050 ^{ab}	0.074 ^{abc}	0.117 ^{cd*}	0.094 ^{bd}	0.094 ^{bd}	0.0174	0.51	<0.001	0.35	0.025	0.022 [#]	0.052	0.0085	0.001
Isocaproic acid	0.0002 [#]	0.0008	0.0014	0.0009 [*]	0.0010	0.0010	0.00034	0.20	0.54	0.52	0.29	0.0001 [#]	0.0007	0.00017	<0.001
SCFA, excl. BCFA	1.58 [#]	2.38	3.80	1.56 [#]	2.18	2.44	0.322	0.003 ⁷	0.11	0.66	0.20	0.66 [*]	2.67	0.546	<0.001
Acetic acid	1.11 [#]	1.64	2.72	1.04 [#]	1.47	1.67	0.369	0.003 ⁷	0.074	0.54	0.17	0.48 [*]	1.72	0.337	<0.001
Propionic acid	0.28 [#]	0.37	0.67	0.32 [#]	0.36	0.48	0.090	0.003 ⁸	0.36	0.97	0.28	0.11 [*]	0.62	0.138	<0.001
Butyric acid	0.11	0.28	0.27	0.12	0.26	0.21	0.048	0.004 ⁹	0.51	0.85	0.71	0.05	0.28	0.064	<0.001
Caproic acid	0.002	0.012	0.004	0.003	0.018	0.007	0.0033	<0.001 ¹⁰	0.015	0.43	0.17	0.001	0.004	0.0012	0.21
Valeric acid	0.07 [#]	0.08	0.14	0.07 [#]	0.06	0.07	0.020	0.15	0.038	0.84	0.099	0.02 [*]	0.05	0.008	0.001
4-ethylphenol	0.0002 [#]	0.0003	0.0409	0.0193 [*]	0.0039	0.0508	0.01122	<0.001 ⁸	0.002	0.002	0.12	0.0008 ^{#*}	0.0508	0.01369	<0.001
Indole	0.49 [#]	0.57	0.84	2.13 [*]	1.55	1.33	0.362	0.53	<0.001	0.86	0.091	0.55 [#]	0.32	0.107	0.11
p-Cresol	2.39 ^{a#}	4.55 ^{ab}	10.00 ^{bc}	14.91 ^{cd*}	10.44 ^{bc}	11.32 ^{bcd}	2.607	0.20	<0.001	0.86	0.015	1.68 [×]	6.90	1.481	<0.001
Phenol	0.17 ^{**}	0.05	0.17	0.88 [*]	0.57	0.22	0.264	0.39	0.042	0.13	0.38	0.05 [#]	0.10	0.027	0.15
Skatole	0.60 ^{**}	0.79	1.43	1.37 [*]	1.36	1.34	0.263	0.19	0.031	0.10	0.16	0.36 [#]	1.19	0.283	0.024

Abbreviations: BCFA = branched-chain fatty acids; SCFA = short-chain fatty acids.

¹ Data are presented as least square means ± pooled SEM.

² Model established P-values for the fixed effects of protein source, fibre source, and blocking factor batch, and their interactions. In case of a significant fibre × protein interaction (P < 0.05), letters (a,b,c,d) indicate differences among treatments. In case of a significant effect of protein source within NFF diets (P < 0.05), symbols (#, *, ×) indicate differences among protein sources within NFF.

³ For putrescine and spermine, interactions were observed between fibre × protein × batch (P = 0.035 and P = 0.029, respectively). For 4-ethylphenol, an interaction was observed between fibre × batch (P = 0.035).

⁴ For phenol, cadaverine, and spermine, a batch effect (P < 0.05) was observed. For isocaproic acid, butyric acid, and caproic acid, interactions were observed between fibre × batch (P < 0.05).

⁵ Interaction between fibre × protein.

⁶ Number of replicate pens with four pigs per pen.

⁷ Difference between NFF-SFF.

⁸ Difference between NFF-SFF and RFF-SFF.

⁹ Difference between NFF-RFF and NFF-SFF.

¹⁰ Difference between NFF-RFF, NFF-SFF, and RFF-SFF.

BC-fed pigs, and SFF significantly increased their flows in WPI-fed pigs (P < 0.05). In the distal colon, RFF and SFF numerically reduced the flows of indole, p-cresol, and BCFA in ZE-fed pigs, while they numerically increased their flows in BC-fed pigs, with SFF significantly increasing BCFA flow (Table 5). Similarly, in WPI-fed pigs, feeding SFF significantly increased the flows of BCFA and p-cresol in the distal colon (P < 0.01).

In the proximal colon, feeding SFF increased the flow of 4-ethylphenol in ZE-fed pigs (F × P < 0.01) and WPI-fed pigs (P < 0.01). In the distal colon, feeding SFF increased the flow of 4-ethylphenol in ZE-fed, BC-fed, and WPI-fed pigs (P < 0.001). In

BC- and ZE-fed pigs, the flows of biogenic amines, valeric acid, phenol and skatole were not affected by RFF or SFF in either the proximal or distal colon, while in WPI-fed pigs, the flow of biogenic amines was increased by feeding SFF in the proximal colon (P < 0.01), and the flows of biogenic amines, valeric acid, and skatole in the distal colon (P < 0.05). In the proximal colon of BC- and ZE-fed pigs, both fibre treatments increased the flow of the short-chain fatty acids, excl. BCFA (P < 0.01). In the distal colon, only SFF increased their flow (P < 0.01). Similarly, in the proximal and distal colon of WPI-fed pigs, SFF increased the flow of the short-chain fatty acids, excl. BCFA (P < 0.05).

Table 6

Ammonia flow in different intestinal segments of pigs fed bovine collagen, zein, or whey protein isolate (WPI) as protein source in the absence (NFF) or presence of rapidly fermentable fibres (RFF), or slowly fermentable fibres (SFF)¹.

Items (mmol/h)	Experimental diets						SEM	P-values ^{2,3}				Reference diets		SEM	P-value ^{2,4}	
	Bovine Collagen			Zein				Fibre	Protein	Batch	F × P ⁵	WPI			NFF-SFF	
	NFF	RFF	SFF	NFF	RFF	SFF						NFF	SFF			
No. of pens ⁶	4	4	4	4	4	4						4	4			
Ileum	1.07	0.65	0.98	0.95	0.78	0.84	0.179	0.088	0.67	<0.001	0.49	1.09	0.52	0.460	0.40	
Cecum	0.45 [#]	0.33	0.64	0.38 [#]	0.22	0.46	0.142	<0.001 ⁷	<0.001	<0.001	0.18	0.24 [*]	0.40	0.097	0.003	
Proximal colon	0.64	0.76	0.92	0.71	0.69	0.71	0.145	0.23	0.31	<0.001	0.22	0.41	0.60	0.171	0.36	
Distal colon	0.35 ^{#*}	0.52	0.70	0.47 [*]	0.52	0.65	0.138	0.008 ⁸	0.74	<0.001	0.53	0.19 [#]	0.41	0.096	0.014	

¹ Data are presented as least square means ± pooled SEM.

² Model established P-values for the fixed effects of protein source, fibre source, and blocking factor batch, and their interactions. In case of a significant effect of protein source within NFF diets (P < 0.05), symbols (#, *) indicate differences among protein sources within NFF.

³ For apparent digestibility of DM in the distal colon, interactions were observed between protein × batch (P = 0.041). For ammonia flow in the cecum, interactions were observed between fibre × batch (P = 0.002), and protein × batch (P < 0.001).

⁴ For the cecum and proximal and distal colon, a batch effect (P < 0.05) was observed.

⁵ Interaction between fibre × protein.

⁶ Number of replicate pens with four pigs per pen.

⁷ Difference between NFF-RFF, NFF-SFF, and RFF-SFF.

⁸ Difference between NFF-SFF.

Dietary fibres generally reduced protein-derived metabolite concentrations

In the proximal colon of ZE-fed pigs, the effects of RFF and SFF on the flows and concentrations of many metabolites were similar. Conversely, in BC- and WPI-fed pigs, feeding RFF and/or SFF led to a numerical increase in the flow of these metabolites, while reducing

their concentrations (Fig. 1, Tables 4, 6, and Supplementary Table S1). In BC- and ZE-fed pigs, feeding RFF and SFF did not affect the flows of ammonia and biogenic amines, while RFF and SFF reduced ammonia concentrations and SFF reduced the concentrations of biogenic amines. In WPI-fed pigs, SFF tended to reduce the concentrations of ammonia and biogenic amines. In the distal colon, less significant effects of dietary fibres on the concentrations

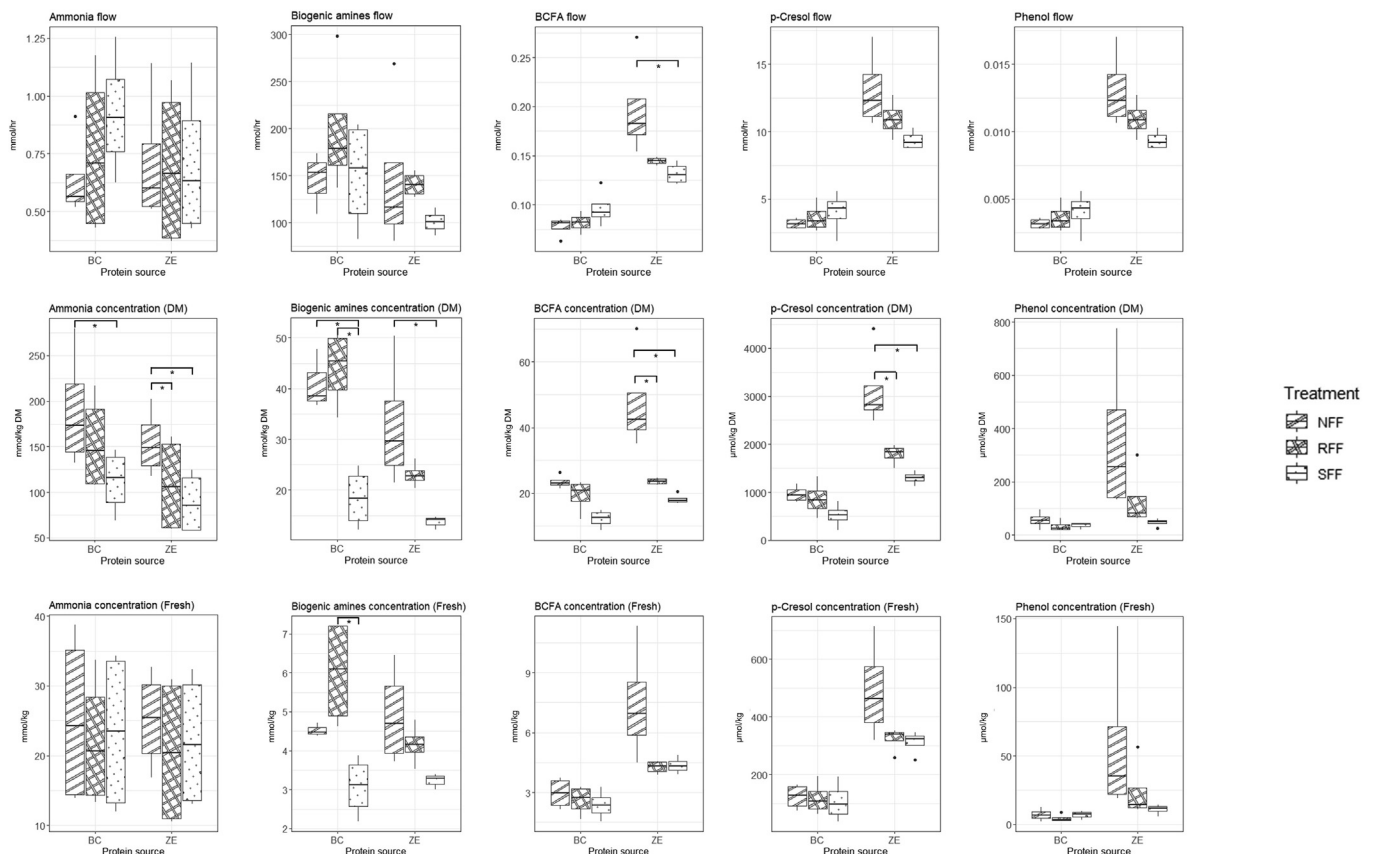


Fig. 1. Flows and concentrations of ammonia, biogenic amines, branched-chain fatty acids (BCFA), p-cresol, and phenol in the proximal colon of growing pigs fed bovine collagen or zein as protein source in the absence (NFF) or presence of rapidly fermentable fibres (RFF), or slowly fermentable fibres (SFF). * indicate differences P < 0.05. The data points outside the whiskers of the box plot show the outliers.

of protein-derived metabolites were observed and feeding RFF did not show any significant effect on the concentrations of protein-derived metabolites in the distal colon for either ZE- or BC-fed pigs (Supplementary Table S2).

Similar principal component patterns for metabolite flows and concentrations

Principal component analysis was performed on metabolite flows and concentrations in the colon. For each treatment, the average values of the proximal and distal colon were calculated to represent overall colonic flows and concentrations. The results showed 23 PC's, of which the first two of both analyses explained >57% of the variance (Fig. 2, Supplementary Figure S1); the other PC's explained <10% of the variance each. For the PCA on colonic metabolite flows, PC1 explained 33.0% of the variation and included high, positive loadings of most biogenic amines –except cadaverine and histamine–, and acetic acid, propionic acid, butyric acid, and valeric acid. PC2 explained 23.7% of the variation and included high, positive loadings of BCFA, indole, p-cresol, phenol and skatole. Similar patterns were observed for the PCA performed on the colonic metabolite concentrations. PC1 explained 36.6% of the variation and included high, positive loadings of the same metabolites as for the flow, except for tyramine, which had a lower loading. PC2 explained 20.5% of the variation and also included high loadings of the same metabolites as for the flow, except for isocaproic acid, which had a lower loading.

When assessing the effects of dietary fibres and proteins on PC estimates for each observational unit, no differences between fibres and proteins were observed for PC1 derived from PCA on the metabolite flows, while PC1 derived from PCA on metabolite concentrations was lower in WPI-NFF as compared with WPI-SFF ($P < 0.05$, Table 7). Principal components 2, derived from both PCA on metabolite flows and metabolite concentrations, were

higher in ZE-fed pigs as compared with BC ($P < 0.05$). No significant effects of dietary fibre were observed on PC2, except in WPI-fed pigs. In these pigs, similarly to PC1, feeding SFF increased PC2 derived from PCA on metabolite concentrations ($P < 0.05$).

Discussion

The main objective of this experiment was to investigate the effect of RFF versus SFF on protein fermentation end-products when added to diets containing poorly versus easily digestible protein sources. To achieve this, digesta from boars fed with different combinations of protein and fibre sources were analysed to determine protein digestibility, digesta mean retention time, and the concentrations and flows of protein-derived metabolites.

Protein fermentation primarily occurred in the proximal colon

In contrast to the general assumption that protein fermentation primarily takes place in the distal colon (Macfarlane et al., 1992), the flow of protein-derived metabolites was the highest in the proximal colon, regardless of protein source. This indicates that the main site of protein fermentation may be dependent on diet composition. The concentrations of protein-derived metabolites reported by Pieper et al. (2014) were highest either in the proximal- or distal colon, depending on the specific metabolite. However, it is important to consider that these concentrations were expressed in mmol/L, meaning that a reduced moisture content in the distal colon may lead to higher concentrations. Based on the ammonia flow, which was also analysed in the ileum and cecum, we postulate that protein fermentation starts in the ileum. However, it is worth noting that urea recycling may also contribute to the reported ammonia flows in ileum, cecum, proximal- and distal colon (Krone et al., 2019; Mosenthin et al., 1992b).

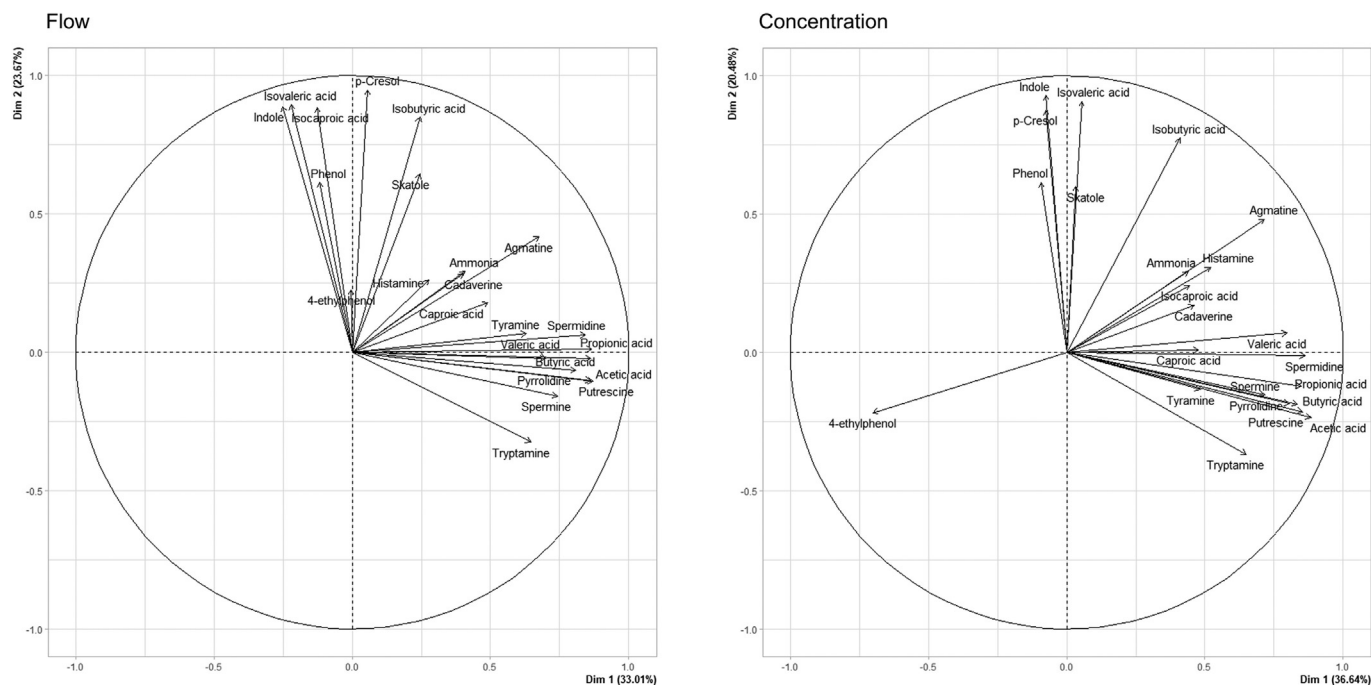


Fig. 2. Principal components derived from principal component analysis done on metabolite flow (left) and metabolite concentration (right) in colonic digesta (for each treatment, the values of the proximal and distal colon were averaged to represent overall colonic flows) of pigs fed bovine collagen, zein, or whey protein isolate in the absence or presence of rapidly or slowly fermentable fibres.

Table 7

Effects of dietary fibres and proteins on principal components derived from principal component analysis done on metabolite flows and metabolite concentrations in pigs fed bovine collagen, zein, or whey protein isolate (WPI) as protein source in the absence (NFF) or presence of rapidly fermentable fibres (RFF) or slowly fermentable fibres (SFF)¹.

Items	Experimental diets						SEM	P-values ^{2,3}				Reference diets			
	Bovine Collagen			Zein				Fibre	Protein	Batch	F × P ⁴	WPI		SEM	P-value ²
	NFF	RFF	SFF	NFF	RFF	SFF						NFF	SFF		
No. of pens ⁵	4	4	4	4	4	4						4	4		
Metabolite flow (mmol/h)															
PC1	-4	49	-4	7	12	0.4	21.5	0.30	0.68	0.31	0.50	12	4	11.4	0.69
PC2	-6	-14	-13	37	4	1	14.7	0.23	0.037	0.80	0.52	-9	6	5.2	0.052
Metabolite concentration (mmol/kg DM)															
PC1	5	84	17	1	6	41	25.7	0.70	0.100	0.68	0.13	9	29	6.8	0.035
PC2	-5	-20	17	48	1	11	15.3	0.051	0.039	0.54	0.082	0.03	24	7.4	0.008

¹ Data are presented as least square means ± pooled SEM.

² Model established P-values for the fixed effects of protein source, fibre source, and blocking factor batch, and their interactions.

³ For PC2 based on metabolite concentration, an interaction was observed between fibre × batch ($P < 0.05$).

⁴ Interaction between fibre × protein.

⁵ Number of replicate pens with four pigs per pen.

Lower ileal digestibility and increased protein fermentation in pigs fed bovine collagen and zein

The apparent ileal protein digestibility for BC (57–76%), ZE (48–65%), and WPI (71–84%) was relatively low as compared with findings published by Noorman et al. (2024; BC: 77%, ZE: 70%, WPI: 91%). The younger age of the pigs used in the current study (mean BW of 24 kg vs 27.6 at the start and 80 kg at the end of the experiment of Noorman et al., 2024) may have contributed to the lower digestibility observed (Ouweltjes et al., 2018). Furthermore, in the current study, nutrients were analysed in pooled digesta from the last two metres of the small intestine, which may have reduced the observed ileal N disappearance (Katsura and Hira, 2024; Tretola et al., 2024) as compared with the ileal cannulas used in the study of Noorman et al. (2024).

Apparent ileal protein digestibility did not differ significantly between BC and ZE, except when RFF were added (Fibre × Protein < 0.05), where it was lower in ZE-fed pigs ($P < 0.05$). Diets containing ZE combined with NFF and RFF formed relatively stiff mixtures, which likely contributed to a reduced feed intake and may have influenced the kinetics of digestibility and feed passage (Selling et al., 2005). In contrast, when zein was mixed with SFF, a more liquid mixture was formed. This may explain the numerically lower ileal digestibility in pigs fed zein combined with NFF or RFF as compared with SFF.

In the NFF and RFF diets, soy protein concentrate and wheat gluten meal were included to compensate for the protein present in soybean hulls and oat hulls in the SFF diets. It should be noted that soy protein concentrate and wheat gluten meal may be digested more proximally as compared with the proteins in soybean hulls and oat hulls. However, these protein sources account for only 15% of the total CP content.

To our knowledge, data on protein-derived metabolite flows have not been previously published in pigs, but the concentrations generally fall within the range of data reported by Bikker et al. (2006) and Pieper et al. (2014). Remarkably, the concentrations of 4-ethylphenol in our study (proximal colon: 0–2.04 µmol/kg fresh digesta, distal colon: 0–2.58 µmol/kg fresh digesta) were much lower as compared with the data reported by Pieper et al. (2014; proximal colon: 22–88 µmol/L, distal colon: 35–127 µmol/L). The reason for these lower concentrations is unknown.

It was anticipated that the protein influx into the hindgut would be higher in pigs fed the low digestible protein sources BC and ZE as compared with the highly digestible protein source WPI. This hypothesis was supported by the high ileal protein digestibility

for WPI (71–84%), as compared with BC (57–76%) and ZE (48–65%). The numerically lower flow of most protein-derived metabolites in pigs fed WPI-NFF, as compared with BC-NFF and ZE-NFF, further supports the anticipated contrast in protein fermentation. The most pronounced differences in the flow of protein-derived metabolites were observed between WPI-NFF and ZE-NFF, highlighting the greatest contrast in protein fermentation between these two protein sources.

Relatively long hindgut digesta mean retention time in pigs fed bovine collagen and zein + rapidly fermentable fibres, and whey protein isolate + no fermentable fibres

In most pigs, except for pigs fed WPI-NFF, BC-RFF, and ZE-RFF, digesta MRT was in line with previously reported total tract MRT (Hecker and Grovum, 1975; Le Goff et al., 2002; Owusu-Asiedu et al., 2006). In those pigs, the digesta solids remained on average 7–15 h in the cecum, and 20–29 h in the colon. However, it is important to consider that under steady-state conditions, gastric emptying and intestinal peristalsis may differ from those in practical settings. In pigs fed WPI-NFF and in pigs fed BC or ZE combined with RFF, the digesta MRT in the hindgut was relatively long. In pigs fed WPI-NFF, the digesta solids remained on average 21 h in the cecum, and 45 h in the colon, and in pigs fed BC or ZE combined with RFF digesta solids remained 12–16 h in the cecum, and 31–33 h in the colon. The extended retention in pigs fed WPI-NFF may possibly be attributed to the low amount of indigestible nutrients, which could have resulted in a slower intestinal passage rate (Cummings et al., 1992; Slavin et al., 1981). For pigs fed BC or ZE combined with RFF, possibly, the volatile fatty acids produced during fibre fermentation delayed gastric emptying via the ileal and colonic break (Chambers et al., 2015; Nightingale et al., 1996; Ratanpaul et al., 2019). Concomitantly, RFF-pigs had an increased colonic length (mean ± SEM; 282 ± 41 cm), as compared with 259 ± 31 cm in NFF- and 254 ± 29 cm in SFF-fed pigs, which may partially explain the longer digesta MRT. However, also, transit rates were found to be lowest for RFF-fed pigs (0.25 cm/min) as compared with NFF (0.31 cm/min) and SFF (0.32 cm/min), indicating that apart from the higher colonic length, also digesta transit rate was slower.

Most metabolites in digesta originated from identifiable dietary amino acids

The metabolite profiles in the digesta of the proximal colon differed among protein sources, generally consistent with their pre-

cursor amino acid composition. When comparing the flows of p-cresol and phenol relative to all metabolites analysed (relative flows), their contributions were higher in ZE-fed pigs as compared with BC. This was in line with the higher levels of tyrosine, which is the precursor for phenols, found in ZE (5 vs 0.9%; Portune et al., 2016; Supplementary Figure S2). None of the added fibre sources contained precursors for these phenolic metabolites. In pigs fed the highly digestible WPI, we expected that the substrate for protein fermentation would primarily consist of endogenous protein, including sloughed off intestinal epithelial cells, digestive secretions, mucoproteins, immunoglobulins, and bacteria. Considering these endogenous protein losses, tyrosine levels of the WPI-fed pigs were expected to fall between those of ZE and BC (endogenous protein consists of 3.2% tyrosine; Miner-Williams et al., 2009). Concomitantly, the relative flows of p-cresol and phenol were also in between those for ZE and BC. Moreover, ZE-fed pigs had higher PC2 estimates as compared with BC-fed pigs (Table 7), and PC2 had high positive loadings of p-cresol, phenol, and BCFA, for both the PCA on metabolite flows and metabolite concentrations (Fig. 2). This may be related to the high tyrosine content in ZE as precursor for phenols, and the high leucine content in ZE as precursor for BCFA. Remarkably, the relative flows of indole and skatole, that both have tryptophan as precursor, were found not to be the highest in WPI-fed pigs, but in ZE-fed pigs, despite endogenous proteins having the highest tryptophan levels (1.5 vs 0.1% in ZE and 0.01% in BC). In ZE-fed pigs, the flows of the sum of biogenic amines relative to all metabolites measured were the lowest, which may have partly influenced these findings, by increasing the relative contributions of the other metabolites.

Since BC contained more arginine (9% of all amino acids) as compared with ZE (1.6% of all amino acids) and endogenous protein (0%), the relative flow of arginine was expected to be the highest in BC-fed pigs. Conversely, the relative was the highest in WPI-fed pigs. However, the relative flows of putrescine, spermidine, and spermine, metabolites for which agmatine is a precursor (Alcázar et al., 2010), were highest in BC-fed pigs. Cadaverine showed the highest relative flows in ZE-fed pigs, while levels of lysine, its precursor, were higher in endogenous protein and BC as compared with ZE. This is remarkable, as zein contains hardly any lysine. This indicates that endogenous protein may have contributed, or bacterial production of lysine. Tryptamine was most abundant in ZE-fed pigs, aligning with the highest tyrosine content in ZE as compared with BC and WPI. Overall, it is important to consider that endogenous protein likely also contributes to the substrate for protein fermentation in BC- and ZE-fed pigs, but to a much lesser extent as compared with WPI (Noorman et al., 2024).

The impact of fibres on the extent of protein fermentation depended on protein source

Feeding RFF decreased the net N disappearance between the ileum and distal colon, while no effect was observed between the cecum and colon. It is important to note that the net N disappearance represents the difference in N digestibility between the ileum/cecum and distal colon. Given that it cannot be excluded that some digesta may bypass the cecum, the difference between cecum and distal colon may not accurately reflect N disappearance (Stevens and Hume, 1998; Wilson and Leibholz, 1981). Therefore, drawing conclusions about the net N disappearance between cecum and colon is challenging. Furthermore, N disappearance, which primarily reflects N fermentation, may also be influenced by variations in endogenous N losses, including microbial mass, which can either increase or decrease the N difference between these intestinal segments.

The flow of ammonia and biogenic amines in the proximal colon of BC- and ZE-fed pigs remained unaffected by dietary fibres, indicating that the extent of protein fermentation in the proximal colon was

not affected by the inclusion of either RFF or SFF in the diet. Remarkably, in the distal colon, the flow of ammonia and 4-ethylphenol, and BCFA for BC-fed pigs, was increased in SFF-fed pigs as compared with NFF, suggesting an increased extent of protein fermentation, despite a numerically lower net N disappearance between ileum and distal colon. It is likely that both the ammonia flow and N disappearance were affected by urea influx (Krone et al., 2019; Mosenthin et al., 1992b); however, urea influx does not affect the flow of BCFA and 4-ethylphenol. The observed increase in the flow of ammonia and BCFA in SFF-fed pigs aligns with the findings of Lammers-Jannink et al. (2024), who reported higher faecal excretion of ammonia and BCFA in pigs fed 12.5% soybean- and 12.5% oat hulls as compared with those fed 2.5% of each. These results cast doubt on the strategy of feeding fibres to reduce the extent of protein fermentation, as it appears that such additions can sometimes even exacerbate protein fermentation in the distal colon.

Results showed that the effects of dietary fibres on the flow of protein-derived metabolites varied depending on the protein sources. It is likely that the bacterial preference for utilising carbohydrates or proteins as an energy source is not solely determined by the presence of carbohydrates at the site of fermentation, but also by factors such as the microbiome, protein structure and amino acid profile, and the fermentability of all available substrates (Bindelle et al., 2007; Ma et al., 2022; Yang et al., 2013; Zhang et al., 2024). These factors may affect the suitability of the proteins present at the fermentation site to be incorporated by the bacteria for their growth.

Including pro- and prebiotics to reduce the impact of protein fermentation on intestinal health may hold potential for the future. By promoting the growth of saccharolytic bacteria, pre- and/or probiotics could reduce the abundance of proteolytic bacteria such as *Bacteroides*, *Clostridium*, *Fusobacterium*, *Prevotella*, and *Veillonella* (Aguirre et al., 2016; Lin et al., 2017). Moreover, microbiome modulation may allow protein fermentation to be steered towards the degradation of specific amino acids over others (Dai et al., 2010; Dai et al., 2012; Pugin et al., 2017; Zhu et al., 2020). However, more information is needed on the efficacy of specific probiotic strains, as their effectiveness is influenced by host-related factors, diet, environment, and the specific pattern of protein fermentation. These factors vary across individual pigs and farms (Dai et al., 2012; Martinen et al., 2023).

Dietary fibres generally reduced the concentrations of protein-derived metabolites

In contrast to the observed treatment effects on the flow of metabolites, feeding fermentable fibres led to a decrease in the concentrations (g/kg DM) of most protein-derived metabolites, particularly in the proximal colon, consistent with the results reported by Pieper et al. (2014). This reduction in metabolite concentrations may be partly due to the dilution effect caused by the presence of undigested fibres in the digesta (Birkett et al., 1996), which is only accounted for when metabolite concentrations are expressed relative to an inert marker.

In studies where protein-derived metabolite concentrations were expressed in fresh digesta, not adjusted for DM content, dilution due to moisture could also influence the results (Pieper et al., 2012; Pieper et al., 2014). Metabolite flows are more reflective of metabolite production than concentrations, even though these are also affected by their disappearance through absorption, microbial metabolism, and passage. Luminal concentrations of protein-derived metabolites may, however, provide valuable insights into exposure of the intestinal wall to these potential toxic metabolites. In our study, luminal concentrations not adjusted for DM content (g/kg of wet digesta) for all protein-derived metabolites except 4-ethylphenol and total biogenic amines in the proximal colon were numerically reduced by feeding RFF and SFF. For ammonia,

BCFA, short-chain fatty acids excl. BCFA, and indole, these reductions were significant ($P < 0.05$, data not shown). In the distal colon, no effects of feeding RFF and SFF on metabolite concentrations (g/kg of wet digesta) were observed.

Biogenic amines, acetic, propionic, butyric, and valeric acids were clustered in principal component 1

The production of short-chain fatty acids, although also produced during protein fermentation, is primarily associated with fibre fermentation (Davila et al., 2013; Jha et al., 2010), while the production of biogenic amines is linked to protein fermentation (Alcázar et al., 2010; Portune et al., 2016). Biogenic amines are produced through the decarboxylation of amino acids (Rodríguez-Romero et al., 2022). The cluster of most biogenic amines, acetic-, propionic-, butyric-, and valeric acid in PC1 indicates that the production of these metabolites partially overlaps. In line, Lammers-Jannink et al. (2023) found associations between acetic-, butyric-, propionic acid and acetylspermidine, ethylamine, and spermidine. The absence of a dietary fibre effect for PC1, determined for metabolite flows (Table 7), indicates that the production of the metabolites in PC1 is not primarily driven by fibre fermentation. However, it is important to consider that PC1, determined for metabolite flow, accounts for only 33% of the variation. When examining individual metabolites, it was observed that short-chain fatty acid flows were increased with dietary fibre intake.

Conclusions

As anticipated, dietary inclusion of low-digestible protein sources leads to an increase in protein fermentation end products, as compared with a high-digestible protein source. Our findings indicate that protein fermentation primarily occurs in the proximal colon, starting in the ileum, with the metabolites produced being generally linked to the composition of amino acid precursors in the digesta. While dietary fibres reduced the concentrations of most protein-derived metabolites, the ability to steer the extent of protein fermentation is strongly dependent on the source of proteins being fermented.

Supplementary material

Supplementary Material for this article (<https://doi.org/10.1016/j.animal.2025.101615>) can be found at the foot of the online page, in the Appendix section.

Ethics approval

A project licence was granted by the Central Committee for Animal Experimentation (AVD1040020209705, The Hague, the Netherlands), and the experiment was approved by the Animal Welfare Body of Wageningen University (2017.W-0025.004).

Data and model availability statement

The data/models were not deposited in an official repository. The data/models that support the study findings are available from the authors upon request.

Declaration of Generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) did not use any AI and AI-assisted technologies.

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Declaration of interest

At the beginning of the project, Arie K. Kies was employed at DSM Nutritional Products.

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