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An *in vitro* model for caecal proteolytic fermentation potential of ingredients in broilers

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ABSTRACT

Fermentation of protein in the caeca of chickens may lead to the production of potentially detrimental metabolites, which can reduce gut health. A poor precaecal digestion is expected to increase protein fermentation (**PF**), as more proteins are likely to enter the caeca. It is unknown if the undigested protein that enters the caeca differs in fermentability depending on their ingredient source. In order to predict which feed ingredients increase the risk of PF, an *in vitro* procedure was developed, which simulates the gastric and enteric digestion, subsequent caecal fermentation. After digestion, amino acids and peptides smaller than 3.5 kD in the soluble fraction were removed by means of dialysis. These amino acids and peptides are assumed to be hydrolysed and absorbed in the small intestine of poultry and therefore not used in the fermentation assay. The remaining soluble and fine digesta fractions were inoculated with caecal microbes. In chicken, the soluble and fine fractions enter the caeca, to be fermented, while insoluble and coarse fractions bypass them. The inoculum was made N-free to ensure bacteria would require the N from the digesta fractions for their growth and activity. The gas production (**GP**) from the inoculum, therefore, reflected the ability of bacteria to use N from substrates and was an indirect measure for PF. The Maximum GP rate of ingredients averaged 21.3 ± 0.9 ml/h (mean ± SEM) and was in some cases more rapid than the positive control (urea, maximum GP rate = 16.5 ml/h). Only small differences in GP kinetics were found between protein ingredients. Branched-chain fatty acids and ammonia concentrations in the fermentation fluid after 24 hours showed no differences between ingredients. Results indicate that solubilised undigested proteins larger than 3.5 kD are rapidly fermented independent of its source when an equal amount of N is present.

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Implications

Feed-food competition for proteins could press poultry production towards feeding less-digestible protein ingredients to broilers, which could reduce their gut health as a result of protein fermentation. An *in vitro* method, as described in this paper, could help to predict the protein fermentability of an ingredient when fed to broilers in a cheap, non-animal invasive and fast manner. This information can aid researchers in selecting model ingredients to investigate protein fermentation. The model still requires validation. Results imply that solubilised undigested proteinaceous components entering the caeca are readily fermented, regardless of the ingredient from which it originated.

Introduction

Modern broilers are fed according to ileal digestible amino acid requirements, meaning that ingredients with high digestibility levels are preferred. However, with an increasing market feed-food competition for quality proteins, these highly digestible ingredients might not always be available. To reach ileal digestible amino acid requirements with less-digestible protein ingredients, the total amount of protein in the diet will increase. Growth performance in broilers reduces with increasing levels of undigestible dietary protein (De Lange et al., 2003), and this might be a result of protein fermentation (**PF**) in the caeca of broilers. The caeca, and not the colon, are the main sites for fermentation in the gastrointestinal tract of broilers (Elling-Staats et al., 2022a). Protein fermentation leads to the production of potentially toxic metabolites, such as ammonia, phenols, indoles, H_2S and biogenic amines, which might reduce enteric health (Qaisrani et al., 2015; Apajalahti and Vienola, 2016). As such, increased indigestible protein intake

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in broilers resulted in more necrotic lesions in the intestine (Liu et al., 2017). However, much is still unknown about the effects of PF in broilers, as well as which protein ingredients are more prone to fermentation.

With the current study, an *in vitro* model was developed, which simulates the precaecal digestion and subsequent fermentation of ingredients to determine their PF potential in broilers. This model could be useful for researchers to help them to select ingredients for the study of PF in broilers, but could also be helpful to feed manufacturers to select ingredients with a low PF potential. Such an *in vitro* model has been developed for pigs (Cone et al., 2005). In this model, protein ingredients were digested in two phases, simulating stomach and small intestine. Undigested residuals were freeze-dried and subsequently fermented using pig faeces in the inoculum. This inoculum was made nitrogen (N) free to ensure the bacteria would only use the N from the undigested residuals for their growth. Gas production (**GP**) measured from this N-limiting fermentation, therefore, reflected the availability of the protein sources for bacteria.

In the current study, we used a similar method to rank ingredients in their PF potential in broilers, with strong modification in how digesta is separated. Boisen and Fernández (1997) used a simple filtration to separate the 'digested' and 'undigested' fractions. However, this filter residue does not represent the digesta fraction that is exposed to fermentation in the caeca of broilers. Earlier research (De Vries et al., 2014) shows that soluble and very fine particles of digesta enter the caeca to be fermented, while the coarse and insoluble digesta fraction is excreted. Therefore, in order to predict the effect of protein source on PF in the caeca of broilers, we need to separate the fraction of digesta which is undigested and soluble or very fine. To enable this, multiple separation steps are required.

Heat-damaged proteins are of interest as they might be useful as model for poorly digestible protein in feed research (González-Vega et al., 2011; Hulshof et al., 2016). Heat damage reduces digestibility measured *in vivo* as well as measured via *in vitro* solubilisation with digestive enzymes (Salazar-Villanea et al., 2016). Moreover, in pig studies, heat-damaged proteins have been used to cause PF (Pieper et al., 2012; Richter et al., 2014).

We developed a whole intestinal tract *in vitro* method with multiple separation steps. The N concentrations in the different fractions were determined. Fermentation GP kinetics of broiler caeca bacteria were measured using soluble and fine digesta fractions at equal N concentrations as substrate in an N-limiting setting. Five protein ingredients were tested. Three of them were also evaluated at different levels of heat damage. The protein fermentability of soluble and fine digesta fractions of the different test ingredients are discussed.

Material and methods

Digestion

Two runs of *in vitro* digestion were performed in two phases, a gastric phase and enteric phase, using a slightly adjusted method of Boisen and Fernández (1997). The pH, temperature and durations were adjusted to better match physiological conditions of the broiler gastrointestinal tract. All test dry substrates were ground to pass through a 1-mm sieve.

For the gastric phase, 4 g of test substrate was incubated in 100 ml of phosphate buffer (0.89 g/L $Na_2HPO_4{\cdot}2H_2O$ and 13.245 g/L $NaH_2PO_4{\cdot}2H_2O$ in demineralised water) and 40 ml of 0.2 M HCl (pH adjusted to 3.5) with porcine pepsin (1.43 FIP-U/ml) for 1 hour at 40 °C with continuous gentle stirring. Then, for the enteric phase, the pH was increased to 6.8 by adding 40 ml of a second phosphate buffer (9.65 g/L $Na_2HPO_4{\cdot}2H_2O$ and 22.74 g/L $NaH_2PO_4{\cdot}2H_2O$) and 20 ml 0.6 M NaOH, and 4 ml porcine pancreatin solution (P7545, Sigma Aldrich, 0.1 g/ml) was added. Digestion continued for another 3 hours at 40 °C with continuous gentle magnetic stirring.

Digestion Run 1

In the first digestion run, the following ingredients were tested in duplicate: soybean meal (**SBM**), rapeseed meal (**RSM**), pea meal (**PM**) and corn-dried distiller's grain with solubles (**DDGSs**). Two blanks, where no ingredient was added to the mixture of enzymes and buffers, were also included. The DM and N concentrations of samples were determined using standard methods (for DM: ISO 6496; ISO, 153, 1999, for N: Dumas method, ISO 16634-1, ISO, 2008).

After digestion, the whole mixture of sample + enzymes + buf fers of individual digestion assays was divided over 6-ml centrifuge tubes and centrifuged at 4 000*g* for 10 minutes at 4 °C. Supernatants (200 ml) were used in dialysis to separate the proteins and large peptides from the free amino acids and smaller peptides. The remaining supernatant was used to determine the N concentration, in the wet material. The sediment was rinsed back with demineralised water and filtered through nylon cloths with a pore size of 40 μm, using suction pressure. The residue resulting from this separation represented the solid coarse digesta fraction, while the filtrate represented the solid fine fraction. Nitrogen was determined in both fractions, after drying. A scheme of the different separation steps used in the first digestion run is shown in Fig. 1.

The dialysis was done using dialysis tubing of 40 cm (Medicell Membranes, London, UK) with a molecular weight cut-off (**MWCO**) of 3500 DaltonDM. Tubings were cleaned according to the manufacturer's instruction. Tubing was knotted on one side and filled with 200 ml of supernatant, air was gently removed and knotted on the other side. The filled tubing was immersed into a large beaker with 3000 ml demineralised water and kept at 4 °C for 2 h after which the water was replaced with fresh demineralised water and kept at 4 °C for another 2 h. The water was replaced one final time and then kept at 4 °C for another 15 hours. The fluid remaining in the tubing, the dialysate, was sub-sampled. One sub-sample was analysed for N, and the other sample was freeze-dried. These freeze-dried samples were subsequently used for the first *in vitro* fermentation run.

Digestion Run 2

In the second experiment, the effect of heat damage on the digestibility and fermentation of protein ingredients was investigated. SBM, RSM and sunflower meal (**SFM**) were used as is or additionally toasted in a pressurised steam toaster at 100 °C. Toasting durations were 15 and 30 minutes for SBM and RSM, and 30 minutes for SFM. Porcine mucin (M2378, Sigma Aldrich, Saint Louis, USA) was also included in the *in vitro* digestion, as representative of endogenous protein. All digestions, including those of the blanks, were run in duplicate.

The separation steps were simplified in this run, as shown in Fig. 2. The mixture of sample + enzymes + buffers was filtered through nylon (40 μm pore size), using suction, after which 200 ml of the filtrate was used for dialysis. The same method for dialysis was used as in run 1. The N concentration in the filtrate, the dialysate, and (after drying) the filter residue was measured. Dialysates were freeze-dried before being used as fermentation substrate in the gas production system.

Extreme values of N losses during dialysis (<25% or >95%) were excluded from the statistical analysis of GP kinetics, as these dialysates deviated significantly from their digestion replicate counterparts (samples are: replicate 1 of SBM t30, replicate 2 of RSM t15, replicate 1 of RSM t30 and replicate 2 of SFM t0).

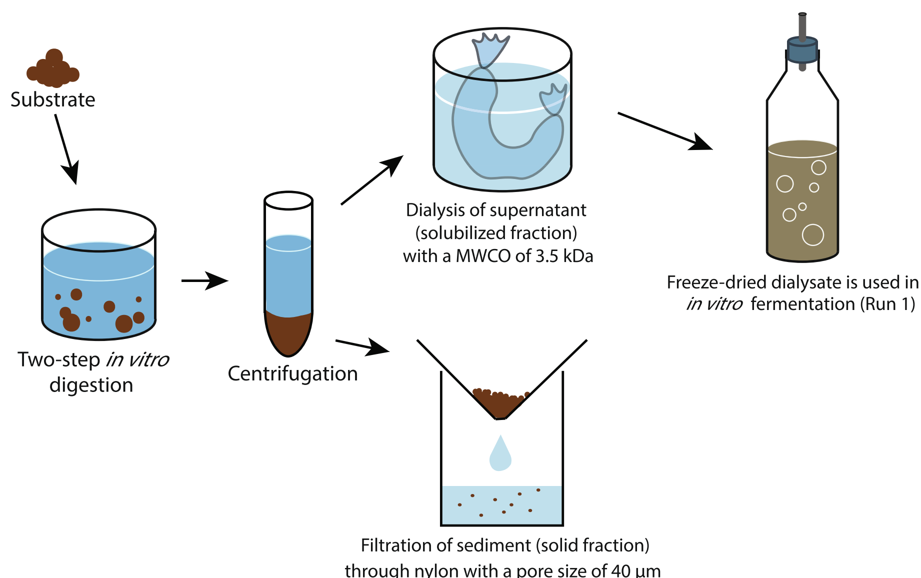

Fig. 1. Scheme of separations steps used in Digestion Run 1 simulating chicken digestion. Abbreviation: MWCO = molecular weight cut-off.

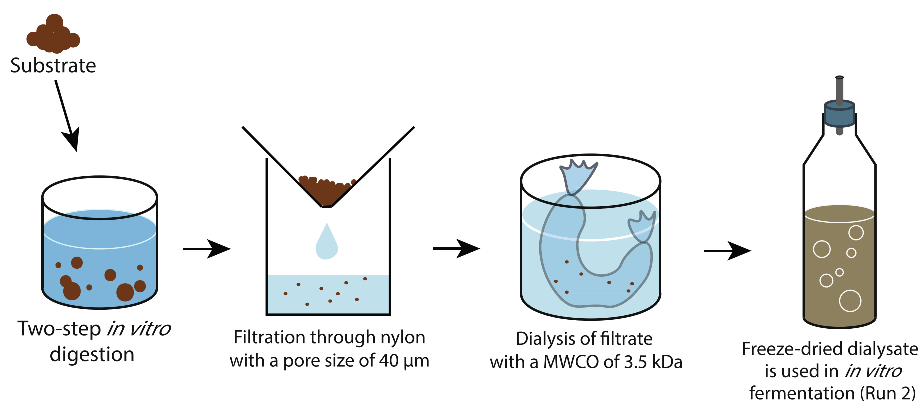

Fig. 2. Scheme of separations steps used in Digestion Run 2 simulating chicken digestion. Abbreviation: MWCO = molecular weight cut-off.

Fermentation

All fermentation kinetics were determined by using an automated GP system as described by Cone et al. (1996). The fermentation of test substrates took place in bottles placed in gently shaking water baths, maintained at 40 °C.

The buffer for both runs was made according to the method of Williams et al. (2005), using an N-free buffer. The caecal contents were retrieved from a commercial broiler slaughterhouse from birds that were destined for slaughter. Intact caeca were taken from 30 broilers within 15 minutes postmortem. Caeca were placed in flasks prefilled with CO_2 and transported on ice to the laboratory. All handling in the laboratory occurred under a continuous flow of CO_2. Caeca were cut open, and contents were removed by finger stripping. Contents were pooled, and then, 100 g was mixed with warm 500 ml saline. The caecal mixture was homogenised with a kitchen blender for 20 s, filtered through nylon gauze (0.04 mm pore size), inoculated at a ratio of 1:17 caecal fluid: buffer. Highly fermentable carbohydrates, maltose (4.3 g/l), soluble potato starch (2.16 g/l), xylose (2.16 g/l) and citric pectin (2.16 g/l) were added to the inoculum. From this mixture, 60 ml was added into two reference bottles and the bottles were connected to the GP measurement system. The inoculum was prefermented for 3.5 h at 40 °C with continuous gentle stirring to ensure that all available N was incorporated into microbial mass, making N limiting in this inoculum. The reference bottles were used to measure GP during the prefermentation.

Blanks (no test substrate) were used as negative. Urea, which is a readily available N source for bacteria, was used as positive control.

Fermentation Run 1

Freeze-dried dialysate samples from digestion run 1 (5 mg N) were added to 60 ml prefermented inoculum using four replicates, leading to eight semi-replicates for each ingredient as these were digested in duplicate. Four blanks (no substrate) and four urea (5 mg N) samples were also included. Bottles were connected to the GP measurement system, and fermentation kinetics were measured for 52 h. After the test, 3 of the 40 incubations were omitted from the analysis due to gas leakage.

Fermentation Run 2

Freeze-dried dialysate samples from digestion run 2 (5 mg N) were inoculated with the prefermented inoculum. Four replicates of each digested sample were used (a total of eight semi-replicates per ingredient), as well as eight blank and eight urea (5 mg N) samples. Fermentation kinetics were measured for 24 h, after which bottles were sampled for analysis of

short-chain fatty acids (**SCFAs**), branched-chain fatty acids (**BCFAs**), and ammonia (NH_3).

Due to technical problems, 19 incubations had to be excluded from the results. Therefore, the number of replicated incubations used for data analysis varied between 3 and 7.

For NH_3 analysis, 0.75 ml of fermentation fluid was mixed vigorously with 0.75 ml trichloroacetic acid (10%) and stored at −20 °C until further analysis. Once thawed, samples were centrifuged for 10 minutes at 14 000g. The NH_3 was transformed by phenol and hypochlorite in an alkaline solution into a blue-coloured product by the Berthelot reaction, as described by Scheiner (1976). The blue colour was spectroscopically measured at 623 nm (Evaluation 201; Thermo Fisher Scientific, Waltham, USA).

For SCFA analysis, 0.6 ml of the fermentation fluid was mixed with 0.6 ml internal standard solution (85% ortho-phosphoric acid containing 19.7 mmol/l isocaproic acid) and stored at −20 °C until further analysis. Once thawed, samples were centrifuged at 21 000g for 5 min. The SCFAs in the supernatant were analysed by gas chromatography (Trace 1300; Rodano, Milan, Italy) with a flame ionisation detector and hydrogen as mobile phase. Quantification was based on a chemical standard solution after an internal standard correction, as described by Baert et al. (2016).

Calculations

The monophasic model of Groot et al. (1996) was fitted to the gas production data using SAS statistical software (version 9.4, SAS Intitute Inc., Cary, NC). The following variables were calculated: the maximum gas production rate (**Rmax**), the time after start of the incubation at which the Rmax is reached (**tRmax**) and the time after start of the incubation at which half of the asymptotic amount of gas has been formed (**halftime**).

Statistical analyses

All analyses were performed with SAS statistical software (version 9.4, SAS Institute Inc., Cary, NC). Differences were considered significant at $P < 0.05$ and a tendency towards significance at $0.05 < P < 0.1$. Data are presented as means ± SEM. Before all analyses, residuals were checked for normality and homogeneity of variance by inspection of histograms and Q-Q plots, using PROC UNIVARIATE.

Fermentation Run 1

The cumulative GP at specific timepoints, Rmax, tRmax and halftime were analysed for a substrate effect with the mixed linear model (PROC MIXED), according to the following formula:

$$Y_{ijk} = \mu + \alpha_i + \beta(\alpha)_{j(i)} + \varepsilon_{ijk},$$

in which Y is the measured response, α the substrate type (i = SBM, RSM, PM, DDGS or Urea), β the *in vitro* digestion replicate (j = 1 or 2) and ε the error term. Substrate types could originate from two replicates *in vitro* digestions. Therefore, these digestion replicates (β) were included as random factor, nested in substrate type, (with the variance components covariance structure) into the models. This model was run twice; once while including blanks in the dataset as another substrate type (i could be blank as well) and once without blanks in the dataset. Tukey-Kramer adjusted multiple comparisons were used to determine differences between substrate types and *in vitro* digestion replicates, in both models.

Fermentation Run 2

The following model in PROC MIXED was used:

$$Y_{ijt} = \mu + \alpha_i + \beta(\alpha)_{j(i)} + \gamma(\alpha\beta)_{k(ji)} + \varepsilon_{ijkt}.$$

in which Y is the measured response, α is the type of ingredient (i = SBM, RSM, SFM, mucin or urea), β is the type of processing (j = no toasting, toasted for 15 min, or toasted for 30 min) which is nested in ingredient type (α), γ is the *in vitro* digestion replicate (k = 1 or 2) which is nested in ingredient and type of processing ($\alpha\beta$), and ε is the error term. Tukey-Kramer adjusted multiple comparisons were made to compare substrate types and *in vitro* digestion replicates. A separate model was run including the blanks as another type of ingredient.

Results

Digestion Run 1

The DM and N concentrations of the ingredients used are shown in Table 1. The amounts of N in the substrates and in the different fractions after digestion are presented in Table 2. The relative amount of N in the solubilised fractions was 88% for SBM, 84% for PM, 73% for RSM and 66% DDGS. The solid fraction that was smaller than 40 μm was 10% for RSM and DDGS, and 7% and 6% for SBM and PM, respectively.

Between 56.1 and 69.5% of the N in the solubilised fractions passed through the membrane during dialysis.

Digestion Run 2

The DM and N concentrations of the ingredients used in the second run are presented in Table 3. The amounts of N in the substrates digested and in the fractions left after digestion and filtration are presented in Table 4. The percentage of N in the fine and solubilised fraction was, on average per ingredient, 78%, 93% and 90% in RSM, SBM and SFM, respectively. The additional toasting of ingredients prior to digestion did not affect the amount of N in the fine and solubilised fractions. The N losses during the dialysis of the filtrates were in most cases more than 40%.

Fermentation Run 1

Table 5 shows the cumulative GP of the incubations at different timepoints, as well as the kinetics parameters from the fitted models. No differences in GP were detected between predigested soluble ingredients and urea. Pea meal, however, tended to have a higher cumulative GP than urea and SBM after 12 and 24 h of incubation. Furthermore, Rmax and halftime did not differ between substrates. Yet, incubations of the predigested PM had a higher tRmax than incubations of the undigested SBM, predigested RSM and urea.

Blank incubations produced significantly less gas than all tested substrates at 3, 6 and 12 h of incubation ($P < 0.01$, at all three timepoints). After 24 h, cumulative gas production of SBM and urea no longer differed from the blank. At 52 hours, only DDGS showed a higher cumulative gas production than the blanks. The blanks

Table 1
DM and N concentration of ingredients used in Digestion Run 1 simulating chicken digestion.

Ingredient	DM (g/kg)	Nitrogen (g/kg DM)
Soybean meal	884	85.0
Rapeseed meal	887	61.7
Pea meal	864	39.1
Maize Dried Distiller's Grain with Solubles	899	50.9

Table 2
Nitrogen contents in the different fractions after *in vitro* digestion Run 1 simulating chicken digestion.

Ingredient	Replicate	Start ±4 g sample used		Residual Solid fraction > 40 µm		Filtrate Solid fraction < 40 µm		Supernatant Solubilised fraction		Dialysate[1] Solubilised fraction > 3.5 kD	
		mg	%	mg	%	mg	%	mg	%	mg	%
Soybean meal	1	383	100	19	04.9	22	05.8	342	89.3	150	39.2
	2	378	100	16	04.2	34	09.0	328	86.9	138	36.6
Rapeseed meal	1	272	100	49	17.9	27	09.8	197	72.3	071	26.0
	2	273	100	44	16.2	28	10.3	201	73.5	068	24.7
Pea meal	1	169	100	18	10.4	12	06.9	140	82.7	049	28.8
	2	172	100	18	10.5	9	05.3	145	84.2	050	29.0
DDGS[2]	1	230	100	53	23.0	22	09.4	156	67.6	051	22.3
	2	230	100	60	26.0	24	10.3	147	63.7	045	19.4

[1] Dialysate is a fraction of the supernatant. All N percentages are relative to the start sample.
[2] DDGS = Dried Distiller's Grain with Solubles from maize.

Table 3
DM and N concentration of ingredients used in Digestion Run 2 simulating chicken digestion.

Ingredient	Addition Processing	DM (g/kg)	Nitrogen (g/kg DM)
Soybean meal	None	884	085.5
	Toasted 15 min	956	087.2
	Toasted 30 min	956	087.5
Rapeseed meal	None	887	061.7
	Toasted 15 min	943	063.2
	Toasted 30 min	951	062.8
Sunflower meal	None	882	049.1
	Toasted 30 min	954	051.3
Mucin	None	-	120

had a lower Rmax and greater tRmax and halftime than all substrates ($P < 0.001$, $P < 0.001$, and $P < 0.01$, respectively).

Fermentation Run 2

Gas production parameters show that GP from urea incubations was generally slower than of that the predigested ingredient fractions (Table 6). No differences were found between the different processing levels of ingredients. No differences were found between digestion replicate pairs of the same ingredient and processing combination, despite a significant overall model effect of digestion replicate for cumulative GP at 3 and 6 h. All substrate types showed a greater GP than the blank incubations ($P < 0.01$ for all timepoints, Rmax, tRmax and halftime).

Concentrations of NH_3 and SCFA in the fermentation fluid after 24 h of incubation are shown in Table 7. The concentrations of NH_3 and SCFA were greater for all substrates than for the blanks ($P < 0.001$), with exception of valeric acid in urea and isovaleric acid in mucin incubations. Incubations with urea resulted in a higher concentration of NH_3 and butyric acid ($P < 0.001$), and a lower concentration of valeric acid, isobutyric acid and isovaleric acid ($P < 0.001$) than all other substrates. No differences in NH_3 or SCFA concentrations were found between digestion replicate pairs of the same ingredient and processing combination, despite significant overall model effects of digestion replicate.

Discussion

The objective of this study was to develop an *in vitro* model that can predict the potential of an ingredient to cause protein fermentation in the caeca of chickens. This was done using an *in vitro*

Table 4
Nitrogen contents in the different fractions after *in vitro* digestion Run 2 simulating chicken digestion.

Substrate[1]	Replicate	Start ±4 g sample used		Residual Solid fraction > 40 µm		Filtrate Solid and solubilised fraction < 40 µm		Dialysate[2] Solid and solubilised fraction < 40 µm and <3 500 Dalton	
		Mg	%	mg	%	mg	%	mg	%
Soybean meal t0	1	301	100.0	22	07.8	283	94.1	126	42.0
	2	305	100.0	20	06.6	281	92.2	730	23.9
Soybean meal t15	1	333	100.0	23	06.9	305	91.5	139	41.7
	2	334	100.0	27	08.1	313	93.8	141	42.4
Soybean meal t30	1	336	100.0	20	05.8	312	93.1	236	70.3
	2	342	100.0	21	06.2	319	93.5	149	43.7
Rapeseed meal t0	1	219	100.0	45	20.4	170	77.7	089	40.9
	2	220	100.0	49	22.4	175	79.6	091	41.6
Rapeseed meal t15	1	239	100.0	51	21.6	182	76.1	094	39.5
	2	240	100.0	57	23.9	172	71.5	135	56.0
Rapeseed meal t30	1	240	100.0	46	19.0	202	84.4	004	01.9
	2	239	100.0	47	19.4	185	77.2	093	39.0
Sunflower meal t0	1	177	100.0	22	12.6	161	91.3	089	50.2
	2	174	100.0	22	12.6	162	93.2	131	75.7
Sunflower meal t30	1	196	100.0	25	13.0	177	90.5	095	48.7
	2	196	100.0	24	12.3	169	86.0	084	43.0
Mucin	1	437	100.0	33	07.5	386	88.3	195	44.7
	2	433	100.0	03	00.6	421	97.2	135	31.0

[1] Ingredients (except for mucin) received heat treatment, additional to normal processing in the manufacturing of these products: t0 = no treatment, t15 = toasting for 15 min at 100 °C, t30 = toasting for 30 min at 100 °C.
[2] Dialysate is a fraction of the filtrate. All N percentages are relative to the start sample.

Table 5
Gas production measurements and kinetics of Fermentation Run 1 using chicken caecal inoculum.

	Substrate[1]					Pooled SEM	*P*-value	Blank	
Item	SBM	RSM	PM	DDGS	Urea			Mean	SEM
CGP[2] 3 h	65	70	68	71	68	2	0.44	20	1.4
CGP 6 h	89	94	99	99	88	2	0.09	38	2.2
CGP 12 h	109	114	122	118	107	2	0.05	69	2.9
CGP 24 h	119	122	129	126	116	2	0.07	105	3.6
CGP 52 h	129	133	136	140	130	2.3	0.14	121	3.9
Rmax[3] (ml/h)	26	28	27	28	27	1	0.7	7	0.4
tRmax[4] (h)	0.66[a]	0.72[a]	1.19[b]	0.96[ab]	0.56[a]	0.08	*0.023*	3.34b	0.36
Halftime[5] (h)	3.13	2.94	3.05	2.99	3	0.1	0.92	12.18	0.16
N[6]	7	8	8	7	3			4	

[a–b]Values within a row without a similar superscript differ significantly ($P < 0.05$).
[1] Substrate abbreviations: SBM = soybean meal, RSM = rapeseed meal, PM = pea meal, DDGS = Dried Distiller's Grain with Solubles from maize.
[2] CGP = Cumulative gas production (ml) at indicated time.
[3] Rmax = maximum rate of gas production.
[4] tRmax = time after the start of incubation at which Rmax is reached.
[5] Halftime = time after incubation at which half of the asymptotic amount of gas has been formed.
[6] N = the number of incubations.

Table 6
Gas production measurements and kinetics of Fermentation Run 2 using chicken caecal inoculum.

Ingredient	Soybean meal			Rapeseed meal			Sunflower meal		Mucin	Urea	Pooled SEM	*P*-values[1]			Blank	
Processing[2]	t0	t15	t30	t0	t15	t30	t0	t30				Ingr.	Pro.	Rep.	Mean	SEM
CGP[3] 3 h	44[abc]	47[ab]	46[ab]	47[ab]	48[a]	47[ab]	44[abc]	46[ab]	42[bc]	37[c]	1.4	*<0.001*	0.47	*0.04*	14	0.7
CGP 6 h	59[ab]	60[ab]	59[ab]	60[a]	62[a]	60[ab]	58[ab]	59[ab]	56[ab]	53[b]	1.3	*<0.001*	0.67	*0.03*	28	0.7
CGP 12 h	65[ab]	66[ab]	65[ab]	66[ab]	69[a]	66[ab]	64[ab]	66[ab]	65[ab]	60[b]	1.4	*0.009*	0.75	0.12	48	1.0
CGP 24 h	68[ab]	68[ab]	67[ab]	69[a]	72[a]	69[ab]	67[ab]	69[a]	69[a]	60[b]	1.6	*0.002*	0.82	0.22	56	1.1
Rmax[4] (ml/h)	21[ab]	22[a]	22[a]	22[a]	22[a]	21[a]	20[ab]	21[a]	20[ab]	16[b]	0.87	*<0.001*	0.66	0.09	6	0.1
tRmax[5] (h)	0.81[a]	0.86[a]	0.82[a]	0.97[a]	0.97[a]	0.94[a]	0.78[ab]	0.84[a]	0.49[b]	1.51[c]	0.05	*<0.001*	0.99	0.76	2.7	0.2
Halftime[6] (h)	2.09[ab]	1.93[a]	1.95[a]	2.01[ab]	2.07[ab]	2.01[ab]	2.07[ab]	2.01[a]	2.40[ab]	2.49[b]	0.09	*<0.001*	0.73	0.54	6.71	0.12
N[7]	6	3	3	7	4	3	3	7	7	5					4	

[a–c]Values within a row without a similar superscript differ significantly ($P < 0.05$).
[1] Sources of variation were: Ingr. = ingredient type, Pro. = extra processing level, Rep. = *In vitro* digestion replicates.
[2] Extra processing: t0 = no heat treatment, t15 = toasting for 15 min at 100 °C, t30 = toasting for 30 min at 100 °C. Mucin and urea received no extra processing.
[3] CGP = Cumulative gas production (ml) at indicated time.
[4] Rmax = maximum rate of gas production.
[5] tRmax = time after incubation at which Rmax is reached.
[6] Halftime = time after incubation at which half of the asymptotic amount of gas has been formed.
[7] N = the number of incubations.

Table 7
Ammonia and short-chain fatty acid concentrations (mmol/l) in fermentation fluid after 24 h of fermentation in Run 2 using chicken caecal inoculum.

Ingredient	Soybean meal			Rapeseed meal			Sunflower meal		Mucin	Urea	Pooled SEM	*P*-values[1]			Blank	
Processing[2]	t0	t15	t30	t0	t15	t30	t0	t30				Ingr.	Pro.	Rep.	Mean	SEM
Ammonia	0.53[a]	0.63[a]	0.72[a]	0.64[a]	0.49[a]	0.61[a]	0.43[a]	0.70[a]	0.78[a]	2.44[b]	0.08	*<0.001*	*0.01*	*<0.001*	0.00	0.00
Acetic acid	35.24[a]	36.21[ab]	35.96[ab]	36.42[ab]	36.23[ab]	37.56[b]	35.25[ab]	36.55[ab]	36.46[ab]	35.35[ab]	0.46	*0.01*	*0.02*	*<0.001*	27.43	0.06
Propionic acid	6.41[a]	6.60[ab]	6.53[ab]	6.63[ab]	6.66[ab]	6.80[ab]	6.45[ab]	6.65[ab]	6.84[b]	6.73[ab]	0.1	*0.01*	0.18	*<0.001*	5.25	0.08
Butyric acid	7.79[a]	7.83[a]	7.74[a]	7.83[a]	7.88[a]	7.99[a]	7.93[a]	7.77[a]	7.74[a]	6.17[b]	0.07	*<0.001*	0.30	0.94	7.07	0.08
Valeric acid	0.38[ac]	0.41[abc]	0.40[abc]	0.43[b]	0.42[ab]	0.41[abc]	0.38[ac]	0.38[c]	0.46[d]	0.22[e]	0.01	*<0.001*	0.30	*0.001*	0.18	0.01
Isobutyric acid	0.35[a]	0.34[a]	0.34[a]	0.33[a]	0.33[a]	0.34[a]	0.35[a]	0.33[a]	0.33[a]	0.13[b]	0.01	*<0.001*	0.08	*<0.001*	0.18	0.00
Isovaleric acid	0.34[a]	0.34[a]	0.33[a]	0.33[ab]	0.33[a]	0.33[ab]	0.34[a]	0.33[ab]	0.29[b]	0.14[c]	0.01	*<0.001*	0.76	0.24	0.28	0.01
N[3]	6	3	3	7	4	3	3	7	7	5					4	

[a–e]Superscripts indicate differences between Ingredients and processing combinations determined by pairwise comparisons of least square means. Values within a row without a similar superscript differ significantly ($P < 0.05$).
[1] Sources of variation were: Ingr. = ingredient type, Pro. = extra processing level, Rep. = *In vitro* digestion replicates.
[2] Processing level. Ingredients (except for mucin) received heat treatment, additional to normal processing in the manufacturing of these products: t0 = no treatment, t15 = toasting for 15 min at 100 °C, t30 = toasting for 30 min at 100 °C.
[3] N = the number of incubations.

model with a precaecal digestion step and a fermentation step. An important aspect of this model was the separation of the undigested fine and soluble particles, as in chicken, these are the particles that enter the caeca after leaving the small intestine (De Vries et al. 2014). The undigested soluble and fine fraction left from the *in vitro* digestion were used as substrate for an *in vitro* batch culture fermentation, while measuring the gas produced by bacteria in time as a measure for their activity.

We used dialysis to separate the solubilised fraction, from the digestion step, as free amino acids and small peptides are assumed

to be hydrolysed and absorbed in the small intestine of the chicken and will therefore not be fermented in the caeca. While the first run focussed on the soluble particles alone (≤3.5 kD), in the second run, the fraction used for fermentation also included very fine (<40 μm), but still solid, particles. This mix mimics the digesta fraction that enters the caeca in the chicken better.

In vitro digestion

The protein digestibility coefficients of the tested ingredients based on *in vivo* data are 90% for SBM, 84% for SFM, 76% for PM, 76% for RSM (Lemme et al., 2004) and 76% for DDGS (Adedokun et al., 2015). In comparison to these values, the fractions considered undigested in the current *in vitro* study (solid fraction + solu ble > 3.5 kD) are 20–40% larger. Dialysis, as a method to simulate the disappearance of peptides, may not be an accurate predictor of the *in vivo* situation in the small intestines. In the small intestine free amino acids, di- and tripeptides are absorbed directly (<500 Da), while larger peptides are broken down by brush border peptidases before absorption (Peters, 1970; Miner-Williams et al., 2014). Therefore, presumably, it takes longer for larger peptides to be absorbed (Adibi and Morse, 1977). The current *in vitro* model does not simulate brush border peptidases activity, and for this reason, we assumed that an MWCO of just 500 D would provide us with a poorer approximation of *in vivo* disappearance than 3.5 kD. Others also used dialysis with an MWCO of 3.5 kD to separate nutrients likely to be absorbed (Van den Abbeele et al., 2018; Verstrepen et al., 2021). Even larger MWCOs (10–20 kD) are used in more complex *in vitro* simulations which allow for simultaneous digestion and absorption (Zyla et al., 1995; Minekus, 2015; González et al., 2020). To determine what the most appropriate MWCO would be for separating the soluble undigested fraction via dialysis, study of peptide chain lengths in the distal ileum and caeca of chicken is required.

In the current study, 55–70% of N passed through the dialysis membrane in the first run and 45–74% in the second run. In the second run, different levels of additional toasting were applied to ingredients prior to the *in vitro* digestion. This was done in order to examine the effect toasting could have on digestion, solubilisation and subsequently fermentation. Heat damage can reduce the digestibility of ingredients, as shown in chickens and pigs (Zhang and Parsons, 1994; Hulshof et al., 2016; Salazar-Villanea et al., 2018a; 2018b; Elling-Staats et al., 2022b). In the current study, however, toasting for 15 or 30 minutes at 100 °C caused no changes in the *in vitro* digestion or solubilisation. Toasting SBM, RSM or SFM at a higher temperature (>125 °C) or for a longer duration (>120 minutes) reduced protein *in vivo* or *in vitro* digestibility (González-Vega et al., 2011; Almeida et al., 2014; Salazar-Villanea et al., 2016, 2018a, b; Elling-Staats et al., 2022b). The toasting applied in the current study may have been too mild to have altered *in vitro* digestion.

In vitro fermentation

The kinetics of gas production during *in vitro* fermentation reflect bacterial activity. In the current study, bacterial activity depends on the availability of the tested proteinaceous substrates, as N was limiting for the bacteria. Nitrogen was indeed limiting in both runs, as incubation with urea, a source of non-protein N for bacteria, showed a significantly higher and faster GP than the blank incubations.

Current GP kinetics show that all tested substrates are readily used by bacteria as a source of N, as the kinetics are quite similar to the incubations with urea. The GP of incubations with urea were less than that of the predigested ingredients in the second run. This coincided with a higher concentration of NH_3 in the fermentation fluid after 24 h incubations of urea, as compared to the ingredients. Ammonia interferes with the equilibrium of the buffer and reduces gas production as it binds H+ ions (Cone et al., 2005). As a result, Cone and van Gelder (1999) calculated that 1 mmol of NH_3 produced prevents the release of 20.9 ml of gas.

Blank incubations, although slower, do continue to produce gas throughout both runs, which might be the result of dead bacteria becoming an available N source. This microbial-protein turnover normally occurs in the last phase seen in GP kinetics, in which GP no longer is the result of substrate fermentation (Cone et al., 1997). It is likely that the phase of microbial-protein turnover is reached shortly in the current study, as substrates are soluble and quickly depleted.

Kinetics did not differ between tested ingredients (or toasting levels), except in the first run where the Rmax of the PM substrate was reached later than of other substrates. Mucin was included in the second run, as representative of endogenous losses, a source of N from the host itself. Mucin was readily available for bacteria, time needed to reach the Rmax was shorter than ingredient fractions, but this had no effect on the Rmax itself or halftime. Based on the GP kinetics in both runs, it is concluded that, at equal amounts of N, the feed ingredient or toasting level of these ingredients had little effect on the level of PF of the solubilised proteinaceous fraction.

Short-chain fatty acids and ammonia in fermentation fluid

Short-chain fatty acids, acetic, propionic and butyric acid, are the main end-products of caecal fermentation in chickens (Annison et al., 1968). Fermentation of mostly carbohydrates, but also protein, contributes to the production of SCFA (Macfarlane et al., 1992; Cone and van Gelder, 1999). SCFA concentrations were expected to be high, as excess carbohydrates were added to the mixtures for prefermentation. Concentrations of acetic, propionic and butyric acid in the current study were higher than in the incubations of Santos et al. (2012), whom used a similar carbohydrate prefermentation with equine caecal contents. The SCFA concentrations in the blank incubations were lower than with urea and all substrates, as the limited N reduced overall fermentation.

Branched-chain fatty acids, isobutyric acid and isovaleric acid, are derived from specific branched-chain AA (Smith and Macfarlane, 1997). As the blank and urea incubations do not provide these AAs, the BCFA production in these incubations are likely the result of microbial-protein turnover. Our results are in agreement: isobutyric acid and isovaleric acid were lower for blanks and urea than for the predigested ingredients. These results indicate that, indeed, little microbial-protein turnover occurred. The higher level of isovaleric acid for the blanks than for urea is unexpected and cannot be easily explained.

NH_3 concentrations of the substrate incubations were lower than those measured by Santos et al. (2012) who employed an identical fermentation procedure to our trial but using equine caecal content as inocula. Low levels were expected as N was limiting for fermentation, meaning that the available N was incorporated in the microbial mass and little NH_3 cumulation occurred. NH_3 concentrations in urea incubations were higher than the other substrate incubations and agrees with Santos et al. (2012 and 2013), whom found higher NH_3 concentrations in equine caecal digesta incubations with urea and fermentable carbohydrates than with casein (milk protein) and fermentable carbohydrates. It remains unclear why urea has this effect. Bacteria can synthesise the AA they require from urea and carbohydrates alone, as has been shown in ruminal and caecal microbiota of different species (Bryant and Robinson, 1963; Forsythe and Parker, 1985; Maczulak et al., 1985). To do so, bacteria first hydrolyse urea into NH_3 through urease activity (Patra and Aschenbach, 2018), after

which the NH_3 is used to synthesise AAs. This appears to be no different for chicken caecal bacteria (Karasawa, 1999). The process of utilising N from urea for microbial-protein synthesis might be less efficient than from protein sources in the medium (Argyle and Baldwin, 1989; Santos et al., 2012). Possibly, hydrolysis of urea into NH_3 exceeded the rate of incorporation of NH_3 into microbial protein in the current study, as is also speculated by Santos et al. (2012). Indeed ruminal microbiota research suggests that urea hydrolysis is faster than NH_3 incorporation into amino acids (Salter et al., 1979). Moreover, experiments with ruminal microbiota fed increasing levels of urea demonstrated that a concentration in the rumen of 8.5 mg NH_3-N/100 ml (Kang-Meznarich and Broderick, 1980) or 5 mg NH_3-N/100 ml (Satter and Slyter, 1974) was the optimum for bacterial protein formation. This optimum might have been reached in the current study as 5 mg urea-N was added to 60 ml caecal inoculum. Future study in which different levels of urea or NH_3 salts are inoculated should help to determine this optimum.

The high SCFA concentrations in combination with low NH_3 concentrations indicate that N was indeed limiting during the fermentation. The lack of differences in fermentation metabolites between test ingredient fractions hampers the ranking of ingredients for their PF potential.

Caecal protein fermentation potential of tested sources

The fine and solubilised undigested fractions of the different ingredients in the current study were rapidly available for microbiota. Little difference in protein fermentability was found between ingredients, which is in contrast to Cone et al. (2005). In the latter *in vitro* study, the solid and not the solubilised fractions were fermented, making this a less relevant model for the chicken caeca. *In vivo* studies show that high intake of (poorly digestible) protein increases PF (De Lange et al., 2003; Heo et al., 2010; Nery et al., 2012; Villodre Tudela et al., 2015; Qaisrani et al., 2020). The amount of N available in the caeca is, therefore, expected to be of more importance for PF than the origin. If so, an accurate prediction of the undigested soluble fraction likely to enter the caeca should be enough to predict the PF potential of the ingredients.

Conclusion and recommendations

Gas production kinetics and concentrations of fermentation metabolites did not differ between protein ingredient types and toasting levels, suggesting that these factors do not play a major role in the protein fermentation potential, when solubilised fractions are provided at a similar N level. In our *in vitro* model, a dialysis step was added to obtain a more representative fraction of proteins that is likely to enter the caeca *in vivo*. Our data suggest that this dialysis step might not fully reflect intestinal absorption *in vivo*. Therefore, more knowledge on the protein fraction likely to enter the caeca is needed. An *in vitro* model that can accurately predict this protein fraction might be valuable to rank feed ingredients on their protein fermentation potential.

Ethics approval

Not applicable.

Data and model availability statement

The data and SAS syntaxes are available from the authors upon request. The data/models were not deposited in an official repository.

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M.L. Elling-Staats performed the research and drafted manuscript; **M.L. Elling-Staats, A.K. Kies, J.W. Cone, W.F. Pellikaan** and **R.P. Kwakkel** designed the research and revised the manuscript; **M.L. Elling-Staats, A.K. Kies, J.W. Cone, W.F. Pellikaan** and **R.P. Kwakkel** approved the final version of manuscript.

Declaration of interest

The authors declare that they have no conflicts of interest.

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References

Adedokun, S., Jaynes, P., Payne, R., Applegate, T., 2015. Standardized ileal amino acid digestibility of corn, corn distillers' dried grains with solubles, wheat middlings, and bakery by-products in broilers and laying hens. Poultry Science 94, 2480–2487.
Adibi, S.A., Morse, E.L., 1977. The number of glycine residues which limits intact absorption of glycine oligopeptides in human jejunum. The Journal of Clinical Investigation 60, 1008–1016.
Almeida, F., Htoo, J., Thomson, J., Stein, H., 2014. Digestibility by growing pigs of amino acids in heat-damaged sunflower meal and cottonseed meal. Journal of Animal Science 92, 585–593.
Annison, E., Hill, K., Kenworthy, R., 1968. Volatile fatty acids in the digestive tract of the fowl. British Journal of Nutrition 22, 207–216.
Apajalahti, J., Vienola, K., 2016. Interaction between chicken intestinal microbiota and protein digestion. Animal Feed Science and Technology 221, 323–330.
Argyle, J.L., Baldwin, R.L., 1989. Effects of amino acids and peptides on rumen microbial growth yields. Journal of Dairy Science 72, 2017–2027.
Baert, N., Pellikaan, W.F., Karonen, M., Salminen, J.P., 2016. A study of the structure-activity relationship of oligomeric ellagitannins on ruminal fermentation in vitro. Journal of Dairy Science 99, 8041–8052.
Boisen, S., Fernández, J.A., 1997. Prediction of the total tract digestibility of energy in feedstuffs and pig diets by in vitro analyses. Animal Feed Science and Technology 68, 277–286.
Bryant, M., Robinson, I., 1963. Apparent incorporation of ammonia and amino acid carbon during growth of selected species of ruminal bacteria. Journal of Dairy Science 46, 150–154.
Cone, J.W., Van Gelder, A.H., 1999. Influence of protein fermentation on gas production profiles. Animal Feed Science and Technology 76, 251–264.

Cone, J.W., Van Gelder, A.H., Visscher, G.J., Oudshoorn, L., 1996. Influence of rumen fluid and substrate concentration on fermentation kinetics measured with a fully automated time related gas production apparatus. Animal Feed Science and Technology 61, 113–128.

Cone, J.W., Van Gelder, A.H., Driehuis, F., 1997. Description of gas production profiles with a three-phasic model. Animal Feed Science and Technology 66, 31–45.

Cone, J.W., Jongbloed, A.W., Van Gelder, A.H., De Lange, L., 2005. Estimation of protein fermentation in the large intestine of pigs using a gas production technique. Animal Feed Science and Technology 123, 463–472.

De Lange, L., Rombouts, C., Oude Elferink, G., 2003. Practical application and advantages of using total digestible amino acids and undigestible crude protein to formulate broiler diets. World's Poultry Science Journal 59, 447–457.

De Vries, S., Kwakkel, R.P., Pustjens, A.M., Kabel, M.A., Hendriks, W.H., Gerrits, W.J.J., 2014. Separation of digesta fractions complicates estimation of ileal digestibility using marker methods with Cr2O3 and cobalt-ethylenediamine tetraacetic acid in broiler chickens. Poultry Science 93, 2010–2017.

Elling-Staats, M.L., Gilbert, M.S., Smidt, H., Kwakkel, R.P., 2022a. Caecal protein fermentation in broilers: a review. World's Poultry Science Journal 78, 103–123.

Elling-Staats, M.L., Kies, A.K., Gilbert, M.S., Kwakkel, R.P., 2022b. Over-toasting dehulled rapeseed meal and soybean meal, but not sunflower seed meal, increases prececal nitrogen and amino acid digesta flows in broilers. Poultry Science 101, 101910.

Forsythe, S.J., Parker, D.S., 1985. Nitrogen metabolism by the microbial flora of the rabbit caecum. Journal of Applied Bacteriology 58, 363–369.

González, C., González, D., Zúñiga, R.N., Estay, H., Troncoso, E., 2020. Simulation of Human Small Intestinal Digestion of Starch Using an In Vitro System Based on a Dialysis Membrane Process. Foods 9, 913.

González-Vega, J.C., Kim, B.G., Htoo, J.K., Lemme, A., Stein, H.H., 2011. Amino acid digestibility in heated soybean meal fed to growing pigs. Journal of Animal Science 89, 3617–3625.

Groot, J.C., Cone, J.W., Williams, B.A., Debersaques, F.M., Lantinga, E.A., 1996. Multiphasic analysis of gas production kinetics for in vitro fermentation of ruminant feeds. Animal Feed Science and Technology 64, 77–89.

Heo, J., Kim, J., Hansen, C.F., Mullan, B., Hampson, D., Pluske, J., 2010. Feeding a diet with a decreased protein content reduces both nitrogen content in the gastrointestinal tract and post-weaning diarrhoea, but does not affect apparent nitrogen digestibility in weaner pigs challenged with an enterotoxigenic strain of Escherichia coli. Animal Feed Science and Technology 160, 148–159.

Hulshof, T.G., Bikker, P., Van der Poel, A.F.B., Hendriks, W.H., 2016. Assessment of protein quality of soybean meal and 00-rapeseed meal toasted in the presence of lignosulfonate by amino acid digestibility in growing pigs and Maillard reaction products. Journal of Animal Science 94, 1020–1030.

International organization for standardization (ISO). 1999a. ISO 6496:1999. Animal feeding stuffs – Determination of moisture and other volatile matter content. ISO, Geneva, Switzerland.

International organization for standardization (ISO), 2008. ISO 16634-1:2008. Food products—Determination of the total nitrogen content by combustion according to the Dumas principle and calculation of the crude protein content—Part 1: Oilseeds and animal feeding stuffs. ISO, Geneva, Switzerland.

Kang-Meznarich, J., Broderick, G., 1980. Effects of incremental urea supplementation on ruminal ammonia concentration and bacterial protein formation. Journal of Animal Science 51, 422–431.

Karasawa, Y., 1999. Significant role of the nitrogen recycling system through the ceca occurs in protein-depleted chickens. Journal of Experimental Zoology Part A: Ecological Genetics and Physiology 283, 418–425.

Lemme, A., Ravindran, V., Bryden, W.L., 2004. Ileal digestibility of amino acids in feed ingredients for broilers. World's Poultry Science Journal 60, 423–438.

Liu, N., Wang, J., Gu, K., Deng, Q., Wang, J., 2017. Effects of dietary protein levels and multienzyme supplementation on growth performance and markers of gut health of broilers fed a miscellaneous meal based diet. Animal Feed Science and Technology 234, 110–117.

Macfarlane, G., Gibson, G., Beatty, E., Cummings, J.H., 1992. Estimation of short-chain fatty acid production from protein by human intestinal bacteria based on branched-chain fatty acid measurements. FEMS Microbiology Ecology 10, 81–88.

Maczulak, A.E., Dawson, K.A., Baker, J.P., 1985. Nitrogen utilization in bacterial isolates from the equine cecum. Applied and Environmental Microbiology 50, 1439–1443.

Minekus, M., 2015. The TNO gastro-intestinal model (TIM). In The Impact of Food Bio-Actives on Gut Health, (ed. Verhoeckx, K., Cotter, P., López-Expósito, I, Kleiveland, C., Lea, T., Mackie, A., Requena, T., Swiatecka, D., Wichers, H.). Springer, Cham Switzerland, Heidelberg Germany, New York NY USA, Dordrecht The Netherlands, London UK, pp. 37–46. DOI: 10.1007/978-3-319-16104-4_5.

Miner-Williams, W.M., Stevens, B.R., Moughan, P.J., 2014. Are intact peptides absorbed from the healthy gut in the adult human? Nutrition Research Reviews 27, 308–329.

Nery, J., Goudez, R., Biourge, V., Tournier, C., Leray, V., Martin, L., Thorin, C., Nguyen, P., Dumon, H., 2012. Influence of dietary protein content and source on colonic fermentative activity in dogs differing in body size and digestive tolerance. Journal of Animal Science 90, 2570–2580.

Patra, A.K., Aschenbach, J.R., 2018. Ureases in the gastrointestinal tracts of ruminant and monogastric animals and their implication in urea-N/ammonia metabolism: a review. Journal of Advanced Research 13, 39–50.

Peters, T., 1970. Intestinal peptidases. Gut 11, 720–725.

Pieper, R., Kröger, S., Richter, J.F., Wang, J., Martin, L., Bindelle, J., Htoo, J.K., Von Smolinski, D., Vahjen, W., Zentek, J., Van Kessel, A.G., 2012. Fermentable Fiber Ameliorates Fermentable Protein-Induced Changes in Microbial Ecology, but Not the Mucosal Response, in the Colon of Piglets. The Journal of Nutrition 142, 661–667.

Qaisrani, S.N., Van Krimpen, M.M., Kwakkel, R.P., Verstegen, M.W.A., Hendriks, W.H., 2015. Dietary factors affecting hindgut protein fermentation in broilers: a review. World's Poultry Science Journal 71, 139–160.

Qaisrani, S.N., Van Krimpen, M.M., Verstegen, M.W.A., Hendriks, W.H., Kwakkel, R.P., 2020. Effects of three major protein sources on performance, gut morphology and fermentation characteristics in broilers. British Poultry Science 61, 43–50.

Richter, J.F., Pieper, R., Zakrzewski, S.S., Günzel, D., Schulzke, J.D., Van Kessel, A.G., 2014. Diets high in fermentable protein and fibre alter tight junction protein composition with minor effects on barrier function in piglet colon. British Journal of Nutrition 111, 1040–1049.

Salazar-Villanea, S., Hulshof, T., van der Poel, A., Bruininx, E., Bikker, P., 2016. Predicting the standardized ileal protein digestibility of processed soybean meal and rapeseed meal in growing pigs using two in vitro methods. Journal of Animal Science 94, 202–206.

Salazar-Villanea, S., Bruininx, E.M.A.M., Gruppen, H., Hendriks, W.H., Carré, P., Quinsac, A., Van der Poel, A.F.B., 2018a. Pelleting and extrusion can ameliorate negative effects of toasting of rapeseed meal on protein digestibility in growing pigs. Animal 12, 950–958.

Salazar-Villanea, S., Butré, C.I., Wierenga, P.A., Bruininx, E.M.A.M., Gruppen, H., Hendriks, W.H., Van der Poel, A.F.B., 2018b. Apparent ileal digestibility of Maillard reaction products in growing pigs. PloS One 13, e0199499.

Salter, D.N., Daneshvar, K., Smith, R.H., 1979. The origin of nitrogen incorporated into compounds in the rumen bacteria of steers given protein- and urea-containing diets. The British Journal of Nutrition 41, 197–209.

Santos, A.S., Ferreira, L.M.M., Martin-Rosset, W., Cotovio, M., Silva, F., Bennett, R.N., Cone, J.W., Bessa, R.J.B., Rodrigues, M.A.M., 2012. The influence of casein and urea as nitrogen sources on in vitro equine caecal fermentation. Animal 6, 1096–1102.

Santos, A.S., Ferreira, L.M.M., Martin-Rosset, W., Cone, J.W., Bessa, R.J.B., Rodrigues, M.A.M., 2013. Effect of nitrogen sources on in vitro fermentation profiles and microbial yield using equine caecal contents. Animal Feed Science and Technology 182, 93–99.

Satter, L.D., Slyter, L.L., 1974. Effect of ammonia concentration of rumen microbial protein production in vitro. The British Journal of Nutrition 32, 199–208.

Scheiner, D., 1976. Determination of ammonia and Kjeldahl nitrogen by indophenol method. Water Research 10, 31–36.

Smith, E.A., Macfarlane, G.T., 1997. Dissimilatory Amino Acid metabolism in human colonic bacteria. Anaerobe 3, 327–337.

Van den Abbeele, P., Kamil, A., Fleige, L., Chung, Y., De Chavez, P., Marzorati, M., 2018. Different oat ingredients stimulate specific microbial metabolites in the gut microbiome of three human individuals in vitro. ACS Omega 3, 12446–12456.

Verstrepen, L., Van den Abbeele, P., Pignataro, G., Ribecco, C., Gramenzi, A., Hesta, M., Marzorati, M., 2021. Inclusion of small intestinal absorption and simulated mucosal surfaces further improve the Mucosal Simulator of the Canine Intestinal Microbial Ecosystem (M-SCIMETM). Research in Veterinary Science 140, 100–108.

Villodre Tudela, C., Boudry, C., Stumpff, F., Aschenbach, J.R., Vahjen, W., Zentek, J., Pieper, R., 2015. Down-regulation of monocarboxylate transporter 1 (MCT1) gene expression in the colon of piglets is linked to bacterial protein fermentation and pro-inflammatory cytokine-mediated signalling. British Journal of Nutrition 113, 610–617.

Williams, B.A., Bosch, M.W., Boer, H., Verstegen, M.W., Tamminga, S., 2005. An in vitro batch culture method to assess potential fermentability of feed ingredients for monogastric diets. Animal Feed Science and Technology 123, 445–462.

Zhang, Y., Parsons, C., 1994. Effects of overprocessing on the nutritional quality of sunflower meal. Poultry Science 73, 436–442.

Zyla, K., Ledoux, D.R., Garcia, A., Veum, T., 1995. An in vitro procedure for studying enzymic dephosphorylation of phytate in maize-soyabean feeds for turkey poults. British Journal of Nutrition 74, 3–17.