Animal Feed Science and Technology 324 (2025) 116305

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci

A high dietary protein level or poorly digestible protein source may lower voluntary feed intake and limit pre-cecal digesta flow and cecal protein fermentation in broilers

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ARTICLE INFO

Keywords:
Broiler
Rapeseed
Protein digestibility
Pre-cecal digesta flow
Cecal fermentation

ABSTRACT

Cecal protein fermentation (PF) in broilers, as a result of increased pre-cecal protein flow, has been hypothesized to underly the negative effects on gut health when feeding high protein or poorly digestible protein diets. However, the effect of pre-cecal protein flow on cecal PF metabolites in broilers remains largely unknown. Also the effect of dietary fiber, which is hypothesized to increase saccharolytic fermentation and reduce PF, is still unclear in broilers. Male ROSS 308 broilers were fed either a low protein (LP, 19 %) or high protein (HP, 23 %) diet. Also, the digestibility level of the protein was varied, by using rapeseed meal (RSM; poorly digestible), rapeseed protein isolate (RPI; highly digestible), or a mixture of RSM and RPI. Finally, these 3 digestibility levels were fed with or without the addition of sunflower seed hulls (SFH), as a fiber source. HP vs. LP reduced feed intake of birds fed the RSM (106 vs. 121 g/d) or mixture (111 vs. 119 g/d) based diets. At the same time FCR was reduced when feeding HP vs. LP in RSM (1.35 vs. 1.42) and mixture (1.43 vs. 1.50) based diets. The apparent ileal N digestibility was higher in mixture and RPI diets than in RSM (78.7, 77.7, and 73.3 %, respectively). Pre-cecal digesta protein flows were not significantly increased by a higher protein level nor by reduced protein digestibility. The pre-cecal liquid AA flow, which was 3–8 % of the total AA flow, increased with HP vs. LP in the RSM-fed groups (415 vs. 167 mg/d). This increase corresponded with an increase in cecal histamine and tyramine contents (72 vs. 9 and 262 vs. 79 nmol/g freeze dried digesta). Addition of SFH in the diets did not reduce PF. A reduced feed intake of broilers fed HP and low digestible protein diets limited the total pre-cecal protein flow, which might explain the lack of a cecal PF metabolite response.

Abbreviations: PF, Protein fermentation; LP, Low protein; HP, high protein; RSM, rapeseed meal; RPI, rapeseed protein isolate; SFH, sunflower seed hulls; SBM, soybean meal; AA, amino acids; CP, crude protein; NDF, Neutral detergent fiber; SCFA, short-chain fatty acids; BCFA, branched-chain fatty acids; MRT, digesta mean retention time; GIT, gastrointestinal tract; AID, apparent ileal digestibility; ATTD, apparent total tract digestibility; FCR, feed conversion ratio; ADFI, average daily feed intake; ADG, average daily gain; CV, coefficient of variation; ADWI, average daily water intake; WFR, water to feed ratio.

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https://doi.org/10.1016/j.anifeedsci.2025.116305
Received 1 October 2023; Received in revised form 6 March 2025; Accepted 14 March 2025
Available online 22 March 2025

1. Introduction

Modern-day broilers require high levels of protein for their growth (Widyaratne and Drew, 2011; Ding et al., 2016; Liu et al., 2017). Protein sources, however, may become less available for broiler diets due to an increasing global market demand. Moreover, in some cases high protein levels can deteriorate gut health. For instance, necrotic enteritis lesions in the small intestine of broilers were increased when fed a 22.5 % crude protein (CP) diet compared to a group fed a 19.5 % CP diet, despite an improved growth and growth efficiency (Liu et al., 2017). Feeding broilers high levels of indigestible protein may even reduce performance (De Lange et al., 2003). Dietary protein level, but also protein digestibility and feed intake, determines the protein digesta flow towards the ceca, where subsequently, protein fermentation (PF) may occur. This PF could lead to the production of metabolites such as ammonia, indoles, phenols, sulfur-containing compounds, and biogenic amines. Some of these compounds may have damaging effects on the gut (Gilbert et al., 2018). However, the effects of PF-metabolites in chicken remains largely unknown.

Carbohydrates available to the cecal microbiota also influence PF, as beneficial microbes appear to favor carbohydrates as energy source (Smith and Macfarlane, 1998), while microbial proteolytic activity is associated with intestinal disease (Frees et al., 2013). Dietary fiber could, therefore, shift microbial activity towards more saccharolytic fermentation and reduce the production of PF metabolites. For instance, in piglets fed indigestible protein, dietary fiber reduced colon PF metabolite concentrations (Pieper et al., 2012). Moreover, replacing highly digestible soybean meal (SBM) with less digestible rapeseed meal (RSM) in broiler diets, did not increase concentrations of PF metabolites in the ceca (Qaisrani et al., 2014). The pre-cecal flow of protein and fiber was increased with the RSM diet, and PF was counteracted by these fibers.

Fiber fermentation is complex and differs depending to the structure and solubilization characteristics of the fiber source (de Vries, 2014). As fiber sources differ, it might not be enough to equalize the fiber level, when determining the effect of protein on fermentation

Table 1
Compositions of starter diets (fed to broilers from day 0–14).

Diet nr.	1 Low CP RSM +SFH	2 High CP RSM -SFH	3 Low CP Mixture +SFH	4 High CP Mixture -SFH	5 Low CP Isolate +SFH	6 Low CP RSM -SFH	7 Low CP Mixture -SFH	8 Low CP Isolate -SFH
Ingredients (g/kg)								
Rapeseed meal	87	240	45	120	0	94	47	0
Rapeseed protein isolate[1]	0	0	13.8	35.8	27.2	0	14.5	29
Sunflower seed hulls	56	0	27.4	0	35.2	0	0	0
Soybean meal	110	110	110	110	110	110	110	110
Soy protein[2]	60	60	60	60	60	60	60	60
Potato protein	40	40	40	40	40	40	40	40
Corn	300	300	300	300	300	300	300	300
Peas	60	60	60	60	60	60	60	60
Premix[3]	5	5	5	5	5	5	5	5
Corn starch	196.5	87.45	265.3	209.9	270.1	260.9	265.9	279.7
Soybean oil	41.5	65	15	18.5	15	15	15	15
DL-methionine	2.9	2.1	3	2.45	3.1	2.9	3	3.1
L-lysine HCl	2.15	0.95	2.35	1.65	2.6	2.05	2.3	2.6
L-threonine	0.6	0	0.6	0.05	0.6	0.55	0.55	0.55
L-tryptophan	0.15	0	0.15	0.1	0.15	0.15	0.15	0.2
L-valine	0.65	0	0.4	0	0.1	0.6	0.3	0
L-arginine	0.75	0	0.8	0.25	0.85	0.7	0.8	0.8
Salt	2.2	2.5	2.2	2.3	2.1	2.2	2.1	2.1
$NaHCO_3$	2.6	2	2.6	2.3	2.7	2.6	2.7	2.7
Potassium carbonate	2.5	0	4.1	2.8	5	3.5	4.6	5.7
Monocalcium phosphate	15.6	13	16.4	15	17.2	15.5	16.5	17.5
Magnesium oxide	0.5	0	0.9	0.5	1.2	0.6	0.9	1.3
Fine limestone	13.4	12	14	13.4	14.4	13.8	14.2	14.8
Diatomaceous earth[4]	0	0	11.0	0	27.5	10.0	34.5	50.0
Analyzed composition (g/kg, unless stated otherwise)								
Dry matter	905.1	905.0	901.9	900.6	917.6	900.4	906.1	913.7
Ash	57.1	61.5	65.5	58.7	78.6	65.6	85.1	97.3
Crude protein	198.1	241.2	199.8	244.6	206.5	199.7	201.7	200.6
Crude fat	66.5	92.0	34.8	40.6	31.5	38.3	33.9	23.2
Starch	386.2	299.2	435.2	396.3	450.4	443.7	437.5	457.7
NDF	108.7	105.9	79.8	79.4	71.4	73	65.9	46.7
ME (kcal/kg, calculated)	2859	2859	2860	2860	2860	2858	2858	2860

Abbreviations: CP = crude protein, RSM = rapeseed meal, SFH = sunflower seed hulls, +SFH = SFH is added, -SFH = SFH is not added, NDF= neutral detergent fiber. [1]CanolaPRO™. [2] Soycomil®. [3]Premix provided per kilogram of diet: 10,000 IU vitamin A, 2500 IU vitamin D_3, 50 mg vitamin E, 1.5 mg vitamin K_3, 2.0 mg vitamin B_1, 7.5 mg vitamin B_2, 35 mg vitamin B_3, 12 mg vitamin B_5, 3.5 mg vitamin B_6, 0.2 mg vitamin B_8, 1 mg vitamin B_9, 20 µg vitamin B_{12}, 460 mg choline chloride, 80 mg iron, 12 mg copper, 85 mg manganese, 60 mg zinc, 0.8 mg iodate and 0.15 mg selenium. Ground corn was used as a carrier. [4]Diamol®.

characteristics. The effect of the added fiber source should be included in the experimental design.

The aim of the current experiment was to study the effects of dietary protein level and protein digestibility on pre-cecal protein digesta flow, cecal protein fermentation metabolite concentrations and growth performance in broilers. The fiber level between corresponding diets was made similar by the addition of fiber in the low protein level diets and low protein-digestibility diets. An additional, secondary aim was to study the effect of this fiber addition, as the effects of fiber-sources on cecal fermentation might also differ. For this purpose, diets without fiber addition were also included in the design.

2. Material and methods

2.1. Ethics statement

A project license was granted by the Central Committee for Animal Experimentation (The Hague, the Netherlands) after approval by the Animal Care and Use Committee of Wageningen University and Research (AVD1040020171667, Wageningen, The Netherlands). The experiment was approved by the Animal Welfare Body of Wageningen University and Research (2017.W-0025.006).

Table 2
Compositions of grower diets (fed to broilers from day 14–31).

Diet nr.	1 Low CP RSM +SFH	2 High CP RSM -SFH	3 Low CP Mixture +SFH	4 High CP Mixture -SFH	5 Low CP Isolate +SFH	6 Low CP RSM -SFH	7 Low CP Mixture -SFH	8 Low CP Isolate -SFH
Ingredients (g/kg feed)								
Rapeseed meal	87	240	45	120	0	94	47	0
Rapeseed protein isolate[1]	0	0	13.8	35.8	27.2	0	14.5	29
Sunflower hulls	56	0	27.4	0	35.2	0	0	0
Soybean meal	80	80	80	80	80	80	80	80
Soy protein[2]	60	60	60	60	60	60	60	60
Potato protein	40	40	40	40	40	40	40	40
Corn	300	300	300	300	300	300	300	300
Peas	60	60	60	60	60	60	60	60
Premix[3]	5	5	5	5	5	5	5	5
Corn starch	228.4	119.5	297.3	241.7	302	292.6	298.2	312.3
Soybean oil	46.5	70	20	23.5	20	20	20	20
DL-methionine	2.9	2.1	3	2.45	3.1	2.9	3	3.05
L-lysine HCl	2.15	0.95	2.35	1.65	2.6	2.05	2.3	2.5
L-threonine	0.6	0	0.6	0.05	0.6	0.55	0.55	0.55
L-tryptophan	0.15	0	0.15	0.1	0.15	0.15	0.15	0.15
L-valine	0.65	0	0.4	0	0.1	0.6	0.3	0
L-arginine	0.75	0	0.8	0.25	0.85	0.7	0.8	0.8
Salt	2.2	2.5	2.2	2.3	2.1	2.2	2.1	2.1
$NaHCO_3$	2.6	2	2.6	2.3	2.7	2.6	2.7	2.7
Potassium carbonate	2.5	0	4	2.8	5	3.5	4.5	5.6
Monocalcium phosphate	10.5	8	11.3	10	12.2	10.5	11.5	12
Magnesium oxide	0.7	0	1.1	0.7	1.3	0.9	1.2	1.5
Fine limestone	11.4	10	12	11.4	12.4	11.8	12.2	12.8
Diatomaceous earth[4]	0	0	11	0	27.5	10	34	50
Titanium dioxide (marker)	1	1	1	1	1	1	1	1
Cobalt EDTA (marker)	1	1	1	1	1	1	1	1
Analyzed composition (g/kg, unless stated otherwise)								
Dry matter	901.8	903.5	899.7	899.7	907.9	905.8	906.7	907.0
Ash	50.9	56.1	59.7	52.9	73.0	61.0	79.0	91.0
Crude protein	184.6	237.4	190.3	230.4	187.9	187.9	188.4	182.1
Crude fat	70.4	96.3	35.6	44.2	28.5	40.0	29.3	24.1
Starch	424.1	320.8	471.9	437.6	470.9	455.6	461.8	483.4
NDF	108.6	110.0	81.3	86.4	79.2	71.6	56.9	45.6
Titanium	0.59	0.59	0.62	0.59	0.71	0.61	0.73	0.81
Cobalt (mg/kg)	120.3	119.9	111.8	119.9	116.8	111.1	116.8	119.7
ME (kcal/kg, calculated)	2950	2950	2951	2950	2950	2948	2950	2980

Abbreviations: CP = crude protein, RSM = rapeseed meal, SFH = sunflower seed hulls, +SFH = SFH is added, -SFH = SFH is not added, NDF= neutral detergent fiber. [1]CanolaPRO™. [2] Soycomil®. [3]Premix provided per kilogram of diet: 10,000 IU vitamin A, 2500 IU vitamin D_3, 50 mg vitamin E, 1.5 mg vitamin K_3, 2.0 mg vitamin B_1, 7.5 mg vitamin B_2, 35 mg vitamin B_3, 12 mg vitamin B_5, 3.5 mg vitamin B_6, 0.2 mg vitamin B_8, 1 mg vitamin B_9, 20 μg vitamin B_{12}, 460 mg choline chloride, 80 mg iron, 12 mg copper, 85 mg manganese, 60 mg zinc, 0.8 mg iodate and 0.15 mg selenium. Ground corn was used as carrier. [4]Diamol®.

2.2. Birds and housing

A total of 504 one-day old male broilers (ROSS 308) were obtained from a commercial hatchery (Kuikenbroederij Morren B.V., Lunteren, the Netherlands). Upon arrival, chicks received individual neck labels, were weighed and randomly assigned to one of 48 floor pens, with 10 or 11 birds per pen. Pens were divided over two climate-controlled rooms and organized into three blocks per room. To each block, eight experimental diets were assigned in random order. Room temperature was 32–34°C on the day of arrival and was gradually decreased to 20–22°C on d 30. Relative humidity in the rooms was maintained between 40 % and 70 %. The lights were on for 23 h on the day of arrival, after which the dark period was increased with one hour every day up to 8 h. Pens had a surface area of 2 m^2 and were bedded with pellets of finely ground lignocellulose (SoftCell®). Each pen contained one large round feeder, a drinking line with five drinking nipples and a perch. Drinking lines were attached via a hose to a water tank (one for each pen). Water intake was measured from the tanks.

2.3. Experimental diets

The experiment consisted of eight dietary treatments with contrasts in protein level, protein digestibility and fiber inclusion. Protein sources used were RSM and a highly digestible rapeseed protein isolate (RPI; CanolaPRO™, DSM Food Specialties, Delft, the Netherlands). The RPI consisted of cruciferin and napin, the main proteins of rapeseed, and contained 99 % CP in DM. A mixture of RSM and RPI was also used to create three protein digestibility levels. The first five diets formulated were: a low protein (LP) RSM diet, a high protein (HP) RSM diet, a LP mixture diet, a HP mixture diet and a LP RPI diet. Ground sunflower seed hulls (SFH) were added to the LP RSM and LP mixture diets to equalize the neutral detergent fiber (NDF) level with their corresponding HP diet. In the LP RPI diet SFH was added to equalize the NDF level to that of the LP RSM diet without SFH addition. Three more LP diets were included in the design: RSM, mixture and RPI without SFH addition, which allowed us to study the effect of this fiber addition (Tables 1 and 2).

Two feeding phases were applied in this experiment: the starter phase (1–14 d, Table 1) and the grower phase (14 d – dissections, between 24 and 32 d, Table 2). Grower diets contained 1 g/kg titanium dioxide and 1 g/kg cobalt-EDTA as inert markers for the solid and liquid digesta fractions, respectively. Diatomaceous earth was added to some of the diets, as a filler to obtain the desired contrasts in protein and NDF level. All diets were produced as pellets by Research Diet Services, Wijk bij Duurstede, The Netherlands. Feed and water were provided *ad libitum* per pen.

2.4. Measurements

Feed and water intake and individual body weights were recorded weekly. On day 24 and 25 four birds per pen were dissected for digesta mean retention time (MRT) measurements. On days 29, 30 and 31 the remaining birds were dissected for apparent ileal digestibility, digesta flow, organ weight and cecal metabolites measurements. Birds were culled by injection of sodium pentobarbital in the wing vein. Intact body weights were recorded. Then the body cavity was opened and the gastrointestinal tract (GIT) was carefully removed. On days 24 and 25, the GIT was separated into 8 segments; crop, stomach (proventriculus and gizzard combined), duodenum (from gizzard up to the hepatopancreatic duct), jejunum (from the hepatopancreatic duct to Meckel's diverticulum), proximal and distal half of the ileum (from Meckel's diverticulum to the ileocolic junction), ceca (combined) and colon. Each segment was closed using tie-raps and weighed, after which the contents were collected and the empty weight of the segment was also recorded. Segment digesta from the four birds per pen were pooled. On days 29, 30 and 31, the ileum was tied with tie-raps before separation, after which it was separated in half. The digesta of the distal half of the ileum was collected by gentle flushing with demineralized water. All GIT organs were emptied and weighed. The pH was measured in the proximal ileum and the ceca. The distal ileum and cecal digesta were pooled per pen and stored at −20°C pending analyses. Lights were on during the night prior dissections, to enable continuous feed intake.

Litter quality was scored on d 28 by an accessor blinded to the experimental treatments according to the method of Van Harn et al. (2009), in which a score from 1 to 10 was given for wetness (1 = very wet to 10 = completely dry) and for friability (1 = bedding has become one hard agglomeration, to 10 = completely loose bedding).

Excreta were collected on the two consecutive days prior the dissections of the remaining birds. To enable excreta collection, a collection box (36 ×56 x 7 cm) with a plastic mesh lid, on which broilers could sit, was placed in each pen two days prior collections. Besides collection of excreta in the collection boxes, fresh droppings were collected once per day, by placing all the birds per pen in a plastic crate (with closed bottom and sides) outside the pen for approximately a minute. Fresh droppings in the crate were then collected. All excreta were stored at −20°C until further processing.

Immediately after removal of the last birds from a pen, a 0.4 m^2 surface area of litter was collected from 6 locations per pen (in each corner and on each side of the feeder in the center of the pen). The litter was weighed and oven-dried in at 100°C for 12 h in order to determine litter moisture content.

2.5. Analytical procedures

Digesta from the GIT segments for MRT measurements and pooled excreta samples were freeze dried. Flushed ileum digesta samples for digesta flow measurements were thawed at room temperature. A mixed subsample was taken and the rest was freeze dried (total fraction). The subsamples were centrifuged for 10 min at 3000 x g (Thermo Megafuge 40 R). The supernatant was poured off and the remaining solid fraction was also freeze dried.

Samples from the total and solid ileal digesta fractions, digesta from GIT segments, excreta and diets were ground to pass a 1 mm screen using a Retsch ZM200 mill. Titanium and cobalt were measured using inductively coupled plasma optical emission spectrometry, with an Avio 500 ICP-OES (Perkin Elmer, Waltham, USA). Samples were ashed at 550°C overnight, and digested, using the MARS 6 microwave digestion system (CEM, Matthews, USA). Yttrium was used as an internal standard. The total and solid ileal digesta fractions, the diets and the excreta were also analyzed for nitrogen content (N; Dumas method; ISO 16634–1; ISO, 2008) and for amino acid (AA) content including sulfur-containing AA measured after oxidation with performic acid (ISO 13903; ISO, 2005). Uric acid content was determined in the excreta samples by enzymatic colorimetry using a kit (Uric Acid liquicolor plus, 10694, Human GmbH, Wiesbaden, Germany). Diets were also analyzed for starch (ISO 15914; ISO, 2004), crude fat (after HCl hydrolysis; ISO 6492; ISO, 1999) and neutral detergent fiber (NDF) content. Neutral detergent fiber was determined as the remaining insoluble organic fraction after 60 min of hydrolysis with neutral detergent reagent and 30 min of incubation with amylase and alcalase at pH 7, according to the method of Van Soest et al. (1991).

Cecal contents of birds dissected on d 29, 30 and 31 were analyzed for ammonia, short-chain fatty acids (SCFA), branched-chain fatty acids (BCFA), and biogenic amines. For ammonia analysis, 2 g of homogenized cecal digesta were mixed vigorously with 2 mL trichloroacetic acid (10 %). Thereafter, samples were centrifuged for 10 min at 2500 x g. The NH_3 was transformed by phenol and hypochlorite in an alkaline solution into a blue colored product by the Berthelot reaction, as described by Scheiner (1976). The blue color was spectroscopically measured at 623 nm. For the SCFA and BCFA analysis, 2 g of the homogenized cecal digesta were mixed with 2 g of 0.1 M ortho-phosphoric acid. Thereafter, samples were centrifuged at 5000 x g for 10 min. The SCFAs in the supernatant were analyzed, after dilution with internal standard, by gas chromatography with a flame ionization 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). For biogenic amines analysis, 50 mg of freeze-dried cecal sample was mixed with 20 mg sulfosalicyl acid and 1 mL 0.1 M hydrochloric acid, after which tubes were placed in an ice-bath for 15 min. Samples were subsequently centrifuged at 20,000 x g for 10 min and filtered through a syringe filter of 0.2 µm. Biogenic amines were detected by ion chromatography in combination with fluorescence detection. A chemical standard solution was used to identify and quantify the individual biogenic amines.

2.6. Calculations

Digesta flows of the solid and liquid fraction where calculated according to the method described by Armentano and Russell (1985), using the following equation in matrix notation:

$$\begin{bmatrix} F_s \\ F_l \end{bmatrix} = \begin{bmatrix} Ti_s & Ti_l \\ Co_s & Co_l \end{bmatrix}^{-1} \times \begin{bmatrix} I_{Ti} \\ I_{Co} \end{bmatrix}$$

in which, F_s and F_l represent the digesta flow of the solid and liquid phase, Ti and Co represent the concentrations of markers (mg/kg) in the solid (s) or liquid (l) fractions, and I_{Ti} and I_{Co} represent the intake rate of the markers Ti and Co (mg/day). The marker concentrations in the liquid fraction were calculated from concentrations analyzed in the total (before centrifugation) and solid fraction (after centrifugation). Ileal flows of a specific nutrient were then calculated as

$$Nu_{Fs} = F_s \times Nu_s$$

and

$$Nu_{Fl} = F_l \times Nu_l$$

in which Nu_{Fs} and Nu_{Fl} represent the solid and liquid digesta flow of the nutrient (mg/day), and Nu_s and Nu_l represent the concentration of the nutrient in the solid and liquid phase (mg/kg). The total flow of a nutrient was determined by summing the solid and liquid nutrient flow.

The apparent ileal digestibility (AID) was calculated as:

$$AID = \frac{I_{Nu} - Nu_{Ft}}{I_{Nu}} \times 100$$

in which I_{Nu} represents the intake rate of the nutrient of interest and Nu_{Ft} the total digesta flow of the nutrient.

The apparent total tract digestibility (ATTD) was calculated as:

$$ATTD = \frac{(Nu/Ti)_{feed} - (Nu/Ti)_{excreta}}{(Nu/Ti)_{feed}} \times 100$$

in which $(Nu/Ti)_{feed}$ represents the ratio of the nutrient of interest (DM, N or an AA) and Ti in the feed and $(Nu/Ti)_{excreta}$ represents this ratio of in the excreta. For the ATTD of nitrogen, the nitrogen concentration in excreta excluded uric acid nitrogen.

Mean retention time (MRT, min) per GIT segment was calculated separately using Ti or Co, using the following equation:

$$MRT = \frac{T \times M_D \times D}{FI \times M_F}$$

in which T represents the length of the feed intake-period prior to dissection (ranging from 1440 to 1860 min), M_D represents the concentration of marker in the digesta (g/kg) of the GIT segment, D represents the weight of digesta (g) in that segment, FI represents the feed intake (g) in the measurement period T, and M_F represents the concentration of marker in the feed (g/kg).

2.7. Statistical analyses

All statistical analyses were performed using the MIXED procedure in SAS (version 9.4, SAS Institute Inc., Cary, NC) with diet as fixed effect. Effects of protein level, protein source and fiber level were evaluated using pre-set contrast statements. The effect of protein level was analyzed separately for RSM-fed (diets 1 and 2) and for mixture-fed birds (diets 3 and 4: see Table 1 for diet numbers). The effect of protein source was analyzed by comparing diets 6, 3 and 5. The effect of SFH addition was evaluated separately for RSM-fed birds (diet 1 and 6), mixture-fed birds (diets 3 and 7), and RPI-fed birds (diets 5 and 8). Block was included as a random factor. Pens were the experimental units. Effects were considered to be significant when $P < 0.05$ and a trend when $P < 0.1$. For the analysis of litter quality score and litter moisture percentage, the sum of BWs of birds in the pen (on day 28) was included as a co-variable in the model. Model residuals were checked for homogeneity of variance by inspection of histograms and Q-Q plots. The ileal flow of histidine in the liquid fraction and the cecal concentration of propionic acid, butyric acid, isocaproic acid and the biogenic amines were analyzed in the GLIMMIX procedure with a lognormal distribution, using the same contrast statements, to obtain homogeneity of variance of the model residuals. For the same reason the ATTD of a number of AA was reflected and analyzed using the GLIMMIX procedure with a lognormal distribution. The MRT of cobalt in the stomach of one pen deviated more than five times the standard deviation of that variable and was therefore considered a measuring error and removed from analysis. All data are presented as means and standard errors of the mean, except for litter quality score and litter moisture percentage, which are shown as least square means.

3. Results

3.1. Performance

A high protein level reduced average daily feed intake (ADFI) of birds fed RSM or the mixture of RSM and RPI in both the starter (Table 3) and grower phase (Table 4). Average daily gain (ADG) was lower in HP RSM vs. LP RSM-fed broilers, in the grower phase, and did not differ between HP and LP mixture-fed birds. The feed conversion ratio (FCR) was lower in HP vs. LP-fed birds in both RSM and mixture diets in both phases. The RPI diet increased ADG in both phases compared with the RSM and mixture diets, without altering FCR significantly. Adding fiber via SFH had no effect on growth performance in the starter phase. In the grower phase SFH addition

Table 3
Growth performance of broilers in the starter phase (0–14 d of age).

Diet nr.	Protein level	Protein source	SFH addition	ADFI g/d	ADG g/d	FCR g/g	CV of ADG %	ADWI mL/d	WFR mL/g
1	**Low**	RSM		34.5	32.1	1.07	9.7	78.2	2.27
2	**High**	RSM		30.3	31.0	0.98	9.7	82.6	2.72
			SEM	0.62	0.49	0.009	1.18	2.13	0.046
			P-value	**< 0.001**	0.13	**< 0.001**	0.99	0.13	**< 0.001**
3	**Low**	Mixture		34.0	31.3	1.09	9.2	81.1	2.39
4	**High**	Mixture		31.2	31.7	0.98	12.2	75.7	2.43
			SEM	0.64	0.60	0.007	1.68	2.07	0.053
			P-value	**0.001**	0.51	**< 0.001**	0.16	*0.06*	0.59
6		**RSM**		34.0	31.2[b]	1.09	11.2	84.8	2.50
3		**Mixture**		34.0	31.3[b]	1.09	9.2	81.1	2.39
5		**RPI**		35.5	33.0[a]	1.08	9.2	83.8	2.36
			SEM	0.54	0.52	0.006	1.99	2.11	0.057
			P-value	*0.09*	**0.02**	0.23	0.56	0.41	0.15
1		RSM	**Yes**	34.5	32.1	1.07	9.7	78.2	2.27
6		RSM	**No**	34.0	31.2	1.09	11.2	84.8	2.50
			SEM	0.52	0.48	0.004	2.03	1.42	0.051
			P-value	0.52	0.20	*0.09*	0.47	**0.03**	**0.01**
3		Mixture	**Yes**	34.0	31.3	1.09	9.2	81.1	2.39
7		Mixture	**No**	33.8	31.2	1.08	10.5	80.5	2.38
			SEM	0.72	0.64	0.007	1.19	2.26	0.052
			P-value	0.81	0.95	0.57	0.55	0.83	0.96
5		RPI	**Yes**	35.5	33.0	1.08	9.2	83.8	2.36
8		RPI	**No**	34.4	32.0	1.08	7.8	82.6	2.39
			SEM	0.39	0.31	0.004	1.09	2.03	0.063
			P-value	0.15	0.27	0.91	0.50	0.67	0.68

[a-b]In case of a protein source effect: means within a column lacking a common superscript differed significantly ($P < 0.05$).
Abbreviations: RSM: Rapeseed meal, RPI: Rapeseed protein isolate, Mixture: RSM and RPI mixed, SFH: sunflower seed hulls, ADFI: average daily feed intake, ADG: average daily gain, FCR: feed conversion ratio, CV: coefficient of variation in individual ADG, ADWI: average daily water intake, WFR: water to feed ratio.

Table 4
Growth performance and litter quality of broilers in the grower phase (14–31 d of age).

Diet nr.	Protein level	Protein source	SFH addition	Growth performance						Litter quality[1]	
				ADFI g/d	ADG g/d	FCR g/g	CV of ADG%	ADWI mL/d	WFR mL/g	Moisture %	Quality Score
1	**Low**	RSM		120.5	84.8	1.42	13.0	229.2	1.90	19.9	8.4
2	**High**	RSM		106.1	78.6	1.35	15.7	233.1	2.19	21.4	8.1
			SEM	2.45	2.47	0.016	2.26	6.05	0.024	0.77	0.23
			P-value	**< 0.001**	**0.01**	**0.01**	0.42	0.56	**< 0.001**	**0.05**	0.36
3	**Low**	Mixture		119.2	79.3	1.50	11.8	221.5	1.86	20.1	8.4
4	**High**	Mixture		111.2	78.2	1.43	22.1	212.9	1.91	19.4	8.0
			SEM	2.31	2.13	0.030	3.31	7.03	0.047	0.76	0.23
			P-value	**< 0.01**	0.63	**< 0.01**	**< 0.01**	0.20	0.23	0.32	0.29
6		**RSM**		115.9^{b}	78.5^{b}	1.48	10.0	223.8	1.93	19.4	8.1
3		**Mixture**		119.2^{ab}	79.3^{b}	1.50	11.8	221.5	1.86	20.1	8.4
5		**RPI**		123.1^{a}	84.2^{a}	1.46	9.5	227.2	1.85	18.9	8.2
			SEM	1.93	1.25	0.015	1.45	3.35	0.033	0.18	0.05
			P-value	**0.02**	**0.03**	0.28	0.79	0.68	0.13	0.26	0.64
1		RSM	**Yes**	120.5	84.8	1.42	13.0	229.2	1.90	19.9	8.4
6		RSM	**No**	115.9	78.5	1.48	10.0	223.8	1.93	19.4	8.1
			SEM	1.85	1.63	0.020	1.34	3.57	0.033	0.76	0.23
			P-value	*0.06*	**0.01**	**0.04**	0.39	0.41	0.51	0.56	0.26
3		Mixture	**Yes**	119.2	79.3	1.50	11.8	221.5	1.86	20.1	8.4
7		Mixture	**No**	118.8	80.8	1.47	13.5	223.0	1.88	20.6	8.0
			SEM	1.61	0.79	0.011	2.77	3.14	0.030	0.76	0.23
			P-value	0.88	0.50	0.20	0.61	0.82	0.68	0.52	0.26
5		RPI	**Yes**	123.1	84.2	1.46	9.5	227.2	1.85	18.9	8.2
8		RPI	**No**	118.4	79.5	1.49	10.8	227.7	1.92	20.1	8.3
			SEM	1.83	1.47	0.008	1.18	3.49	0.032	0.77	0.23
			P-value	*0.06*	**0.05**	0.30	0.72	0.94	*0.09*	0.12	0.64

[a-b] In case of a protein source effect: means within a column lacking a common superscript letter differed significantly ($P < 0.05$).

Abbreviations: RSM: Rapeseed meal, RPI: Rapeseed protein isolate, Mixture: RSM and RPI mixed, SFH: sunflower seed hulls, ADFI: average daily feed intake, ADG: average daily gain, FCR: feed conversion ratio, CV: coefficient of variation in individual ADG, ADWI: average daily water intake, WFR: water to feed ratio. [1] Litter quality scores were measured on day 23, ranging from 1 to 10 in which 1 = ‘very wet and agglomerated litter’ and 10 = ‘perfectly dry and loose litter’. The sum of BW of all birds in a pen was included as co-variable in the statistical model for litter quality score and litter moisture percentage.

increased ADG of RSM and RPI-fed birds and reduced FCR of RSM-fed birds. Litter quality scores (Table 4) were unaffected by dietary treatments. However, litter moisture content was higher for HP RSM compared to LP RSM-fed birds.

3.2. Digestibility

The high protein level had no effect on the AID of N and the AID of DM was lower in both the RSM and mixture diets (Table 5), compared with the low protein counterparts. A high protein level in the RSM diets increased AID of cysteine, histidine, glycine and proline (supplementary document, Table S1). Replacing half of the RSM protein with RPI increased the AID of N, most of the AA and DM. The AID of most AA was numerically higher in for RPI than in for RSM, which was significant for cysteine, glutamate and serine. For the mixture diet, AID of AA was even higher, significantly for histidine, methionine, valine, alanine, aspartate and glycine, as compared with RPI. Adding SFH increased the AID of N in the RSM and protein isolate diets, but not in the mixture diets. In line with the effects on AID of N, the AID of most AAs was numerically higher with SFH addition (Table S2).

The ATTD of a few AA was lower in the high protein diets in both RSM and mixture-fed birds (Table S3). Replacing RSM protein with RPI had no effect on ATTD. Furthermore, SFH addition did not affect ATTD of N, AA or DM (Table S4).

3.3. Pre-cecal digesta flows

The high protein level in the RSM diets increased pre-cecal flow of the sum AA (Table 6) and most AA in the liquid fraction, but not in the solid fraction or total digesta (Tables S5 and S6). In the mixture diets, high protein tended to increase N and AA flows, with a significant increase of histidine in the solid fraction (Table S5). Protein source had no effect on pre-cecal digesta flows (Table 6, S5 and S6). Adding SFH had no effect on N and most of the AA flows but did increase DM, histidine and taurine solid digesta flow of birds fed RSM (Table S7). Adding SFH reduced histidine liquid digesta flow of RSM-fed birds and cysteine and lysine liquid digesta flows of RPI-fed birds (Table S8).

3.4. Mean gastrointestinal retention times and empty organ weights

Mean retention time from crop to distal ileum averaged 186 ± 69 min for the solid marker and 174 ± 110 min for the liquid marker. Both were little affected by protein level or source in any of the GIT segments. The MRT of both the solid and liquid marker in the stomach (proventriculus and gizzard combined) increased with SFH addition in the RSM diets, but not in the mixture or RPI diets (Table 7). The relative empty weight of the gizzard and the gizzard to proventriculus ratio was higher with SFH addition in the birds fed the mixture and RPI diets (Table S9). Adding SFH tended to increase the gizzard weight of RSM-fed birds. High protein increased the gizzard to proventriculus ratio in both RSM as well as mixture-fed birds, without significantly affecting the relative empty weights of

Table 5
Apparent ileal digestibility coefficients in broilers at 29–31 days of age.

Diet nr.	Protein level	Protein source	SFH addition	Nitrogen	Sum of AA[1]	Dry matter
1	**Low**	RSM		76.9	85.2	88.1
2	**High**	RSM		77.0	87.6	86.2
			SEM	0.86	0.59	0.29
			P-values	0.94	*0.07*	**0.01**
3	**Low**	Mixture		78.7	87.2	90.2
4	**High**	Mixture		78.8	87.5	87.9
			SEM	0.81	0.66	0.36
			P-values	0.97	0.84	**< 0.01**
6		**RSM**		73.3[a]	83.5[a]	88.4[a]
3		**Mixture**		78.7[b]	87.2[b]	90.2[b]
5		**RPI**		77.7[b]	85.0[ab]	89.5[ab]
			SEM	1.47	1.25	0.61
			P-values	**< 0.01**	**0.03**	**0.04**
1		RSM	**Yes**	76.9	85.2	88.1
6		RSM	**No**	73.3	83.5	88.4
			SEM	1.5	1.17	0.40
			P-values	**0.04**	0.20	0.70
3		Mixture	**Yes**	78.7	87.2	90.2
7		Mixture	**No**	76.0	86.4	89.7
			SEM	0.7	0.45	0.25
			P-values	0.11	0.56	0.48
5		RPI	**Yes**	77.7	85.0	89.5
8		RPI	**No**	72.6	84.1	89.0
			SEM	1.4	1.20	0.73
			P-values	**< 0.01**	0.53	0.52

[a-b]In case of a protein source effect: means within a column lacking a common superscript letter differed significantly ($P < 0.05$).
Abbreviations: RSM: Rapeseed meal, RPI: Rapeseed protein isolate, Mixture: RSM and RPI mixed, SFH: sunflower seed hulls.
[1]The sum of the amino acids measured in the digesta includes all proteogenic amino acids except tryptophan.

Table 6
Pre-cecal nutrient flows in the solid, liquid and total digesta fractions of broilers at 29, 30 and 31 days of age.

Diet nr.	Protein level	Protein source	SFH addition	**Solid digesta flow**			**Liquid digesta flow**			**Total digesta flow**		
				N (mg/d)	**AA**[1](mg/d)	**DM** (g/d)	**N** (mg/d)	**AA**[1](mg/d)	**DM** (g/d)	**N** (mg/d)	**AA**[1](mg/d)	**DM** (g/d)
1	**Low**	RSM		907	5240	15.2	519	167	7.2	1426	5407	22.4
2	**High**	RSM		1031	4694	15.5	622	415	8.0	1653	5109	23.4
			SEM	73.4	349.4	0.87	65.9	57.8	0.96	127.4	354.4	1.43
			P-value	0.15	0.32	0.84	*0.07*	**< 0.01**	0.45	*0.07*	0.57	0.49
3	**Low**	Mixture		827	4142	10.9	474	321	6.7	1301	4463	17.6
4	**High**	Mixture		963	5109	13.1	586	278	8.4	1549	5373	21.5
			SEM	43.7	435.6	0.99	51.3	33.6	0.88	78.1	423.5	0.85
			P-value	0.11	*0.08*	*0.06*	*0.05*	0.562	0.13	*0.05*	*0.09*	**0.01**
6		**RSM**		881	4906	11.5	492	223	6.7	1374	5129	18.2
3		**Mixture**		827	4142	10.9	474	321	6.7	1301	4463	17.6
5		**RPI**		920	4709	13.0	469	326	6.2	1388	5035	19.1
			SEM	63.1	358.3	0.94	35.1	54.5	0.60	81.4	357.8	1.02
			P-value	0.55	0.35	0.19	0.91	0.294	0.85	0.75	0.40	0.60
1		RSM	**Yes**	907	5240	15.2	519	167	7.2	1426	5407	22.4
6		RSM	**No**	881	4906	11.5	492	223	6.7	1373	5129	18.2
			SEM	68.9	445.7	0.99	36.1	38.6	0.53	96.2	437.0	1.61
			P-value	0.76	0.54	**< 0.01**	0.63	0.45	0.64	0.67	0.60	**< 0.01**
3		Mixture	**Yes**	827	4142	10.9	474	321	6.7	1301	4463	17.6
7		Mixture	**No**	967	4664	12.1	535	365	7.2	1502	5029	19.3
			SEM	48.1	256.5	0.92	40.3	35.1	0.70	77.8	266.5	0.75
			P-value	0.10	0.34	0.31	0.28	0.554	0.67	0.11	0.29	0.28
5		RPI	**Yes**	920	4709	13.0	469	326	6.2	1388	5035	19.1
8		RPI	**No**	1015	4920	11.1	562	446	8.4	1577	5366	19.5
			SEM	62.3	340.3	0.67	41.8	69.7	0.66	84.4	340.6	0.74
			P-value	0.27	0.69	0.12	*0.10*	0.11	**0.05**	0.13	0.53	0.79

Abbreviations: RSM: Rapeseed meal, RPI: Rapeseed protein isolate, Mixture: RSM and RPI mixed, SFH: sunflower seed hulls.
[1]The sum of the amino acids measured in the digesta includes all proteogenic amino acids except tryptophan.

Table 7
Mean retention times[1] of the solid and liquid marker in gastrointestinal tract segments of broilers at 29, 30 and 31 days of ages.

Diet nr.	Protein level	Protein source	SFH addition	**Solid marker (titanium)**						**Liquid marker (cobalt)**					
				Crop	**Stomach**	**Duodenum**	**Jejunum**	**Proximal Ileum**	**Distal Ileum**	**Crop**	**Stomach**	**Duodenum**	**Jejunum**	**Proximal Ileum**	**Distal Ileum**
1	**Low**	RSM		24.7	35.3	5.9	52.9	39.7	32.7	25.9	25.1	7.3	50.6	35.5	32.4
2	**High**	RSM		18.9	34.3	5.0	53.7	39.8	43.1	18.0	23.8	6.5	52.0	35.1	38.1
			SEM	4.5	8.0	1.1	5.3	3.4	4.1	5.6	4.9	1.5	7.9	3.4	3.9
			P-value	0.40	0.90	0.44	0.89	0.99	*0.07*	0.32	0.77	0.64	0.87	0.95	0.49
3	**Low**	Mixture		20.0	20.2	4.0	56.5	46.6	35.1	21.7	17.1	5.6	52.9	43.2	33.4
4	**High**	Mixture		9.1	20.6	4.1	49.3	35.4	42.8	12.2	15.8	5.9	45.7	31.4	46.3
			SEM	6.3	5.8	0.8	4.8	3.8	4.9	6.3	3.3	1.2	6.4	5.2	7.8
			P-value	0.12	0.96	0.96	0.23	**0.05**	0.17	0.23	0.75	0.88	0.41	*0.08*	0.12
6		**RSM**		11.1	15.8	4.5	58.1	39.4	42.9	13.0	14.3	5.8	51.2	36.3	40.9
3		**Mixture**		20.0	20.2	4.0	56.5	46.6	35.1	21.7	17.1	5.6	52.9	43.2	33.4
5		**RPI**		22.5	27.2	3.1	48.9	46.9	41.6	22.1	17.8	4.4	49.8	43.7	42.8
			SEM	5.5	3.9	0.8	3.5	3.6	3.9	5.5	2.4	0.9	5.6	4.9	5.4
			P-value	0.22	0.35	0.48	0.26	0.29	0.33	0.42	0.69	0.67	0.94	0.46	0.49
6		RSM	**No**	11.1	15.8	4.5	58.1	39.4	42.9	13.0	14.3	5.8	51.2	36.3	40.9
1		RSM	**Yes**	24.7	35.3	5.9	52.9	39.7	32.7	25.9	25.1	7.3	50.6	35.5	32.4
			SEM	4.0	5.1	1.2	4.4	4.7	4.4	5.5	2.3	1.5	7.4	5.6	6.0
			P-value	*0.05*	**0.02**	0.22	0.38	0.95	*0.07*	0.10	**0.02**	0.36	0.95	0.91	0.31
7		Mixture	**No**	25.6	23.0	4.7	62.2	44.7	42.4	24.3	16.9	5.2	55.7	38.1	37.2
3		Mixture	**Yes**	20.0	20.2	4.0	56.5	46.6	35.1	21.7	17.1	5.6	52.9	43.2	33.4
			SEM	7.5	4.9	0.7	3.2	4.1	4.4	6.7	2.6	0.9	4.2	4.6	4.8
			P-value	0.41	0.72	0.57	0.34	0.72	0.19	0.74	0.95	0.82	0.76	0.44	0.64
8		RPI	**No**	32.2	16.0	4.4	54.7	46.8	39.6	35.5	13.4	0.4	48.3	40.1	34.2
5		RPI	**Yes**	22.5	27.2	3.1	48.9	46.9	41.6	22.1	17.8	4.4	49.8	43.7	42.8
			SEM	2.9	2.0	0.4	2.7	2.7	3.3	5.4	1.7	0.7	4.1	2.9	4.1
			P-value	0.16	0.16	0.27	0.33	0.99	0.72	*0.09*	0.31	0.39	0.87	0.59	0.30

Abbreviations: RSM: Rapeseed meal, RPI: Rapeseed protein isolate, Mix: RSM and RPI mixed, SFH: Sunflower seed hulls.
[1]Mean retention time (min) $= (Time \times M_{Digesta} \times Digesta)/(FI \times M_{Feed})$

either the proventriculus or gizzard. High protein decreased relative jejunum weight in the mixture-fed birds. High protein increased relative colon weights in both RSM and mixture-fed birds. The RSM-fed birds had higher relative colon weights than mixture or RPI-fed birds. Adding SFH increased relative jejunum weight in RSM and RPI-fed birds, but not in the mixture-fed birds.

3.5. Cecal metabolite concentrations and digesta pH

Cecal ammonia concentrations were unaffected by dietary treatments (Table 8). High protein level in the RSM-fed birds increased cecal concentrations of histamine and tyramine. Replacing RSM protein with RPI increased cadaverine and reduced tyramine concentration. Adding SFH increased putrescine concentration in birds fed the RPI diets.

The high protein level reduced cecal pH in birds fed RSM and mixture diets. However, SCFA or BCFA concentrations were not affected by protein level or source (Table 9). Ileal pH increased when half of the RSM protein was replaced with protein isolate, but did not increase further when it was fully replaced. Adding SFH reduced the cecal concentration of acetic acid, propionic acid and isocaproic acid in the mixture-fed birds and caproic acid in the RPI-fed birds, but had no effect in RSM-fed birds.

4. Discussion

The current study investigated the effects of dietary protein level and protein digestibility on pre-cecal protein digesta flows in broilers, and the subsequent effects of these digesta flows on cecal PF. A high dietary protein level as well as a low protein digestibility were expected to increase pre-cecal flow of N and AA. This increased flow of N and AA towards the ceca could then increase PF (assessed by cecal concentrations of NH_3, BCFA and biogenic amines) and affect gut health. Pre-cecal digesta flows of protein are a better predictor of PF than ileal protein digestibility (Heo et al., 2010; Elling-Staats et al., 2022), as it shows in absolute terms what may become available in the ceca. Also the possible effects of fiber, included as SFH, on protein fermentation were measured.

4.1. Protein level and digestibility

A protein digestibility contrast was created by replacing half of the RSM protein with RPI in the mixture diet. The AID of N, DM and most of the AA increased. This effect was however non-linear, as fully replacing RSM protein with RPI showed no further increase. Surprisingly, the AID of histidine, methionine, valine, alanine, aspartate and glycine was even lower for the RPI compared to the mixture diet. A lack of structural components in the RPI diet might have reduced amino acid digestibility (Amerah et al., 2007; Jiménez-Moreno et al., 2013).

Despite differences in AID, the dietary treatments had little effect on pre-cecal flows in the current study. The higher AID of the mixture diet did not result in a lower pre-cecal N or AA flow in birds fed this diet compared to the RSM-fed birds, due to a lower voluntary feed intake of the RSM-fed birds. Both digestibility and feed intake determine the nutrient flow. Feeding high protein within the RSM and mixture diets only tended to increase total pre-cecal N flow. This is also the result of a lower feed intake in birds fed the high protein diets. However, high protein in the RSM diets did increase AA flow in the liquid fraction. Although this fraction was just 3–8 % of the total AA flow, it is considered important as mostly fine and soluble particles from the ileum enter the ceca (de Vries et al., 2014).

Some discrepancy was found between N and AA flows, as the total pre-cecal flow of N tended to increase in the high vs. low protein RSM-fed birds, while this tendency was not seen in the total AA flow. The pre-cecal flow of total AA was numerically even slightly lower. Not all the N in the ileal digesta is in the form AA; also non-protein N such as ammonia and other metabolites are present (Rehman et al., 2008; Goodarzi Boroojeni et al., 2014). Therefore, this tendency of increased total pre-cecal N flow in the high protein RSM-fed birds, might indicate increased PF in the distal ileum.

Dietary protein level or ileal digestibility level (created by protein source differences) had no effect on the cecal concentration of isobutyric acid, isovaleric acid and isocaproic acid. These BCFAs are exclusively formed by bacterial fermentation of valine, leucine and isoleucine (Smith and Macfarlane, 1997) and, therefore, considered PF indicators. This lack of response is in line with the limited effects of dietary treatment on pre-cecal flows. A high protein level did not affect the solid flows of valine, leucine and isoleucine, but increased the liquid flow of these AA in RSM-fed birds. High protein in RSM-fed birds also increased the liquid flow of histidine and tended to increase the liquid flow of tyrosine. The only indication that the increased AA flow in the liquid fraction led to more PF is the higher cecal histamine and tyramine concentrations of the high protein RSM-fed birds. Biogenic amines, such as histamine and tyramine, are produced via the decarboxylation of amino acids (Erdag et al., 2018).

Protein source had contradicting effects on cecal biogenic amine concentrations as cadaverine increased and tyramine decreased with increasing inclusion of RPI in the diet. These effects do not correspond with flows of lysine and tyrosine as these were not significantly affected by protein source, suggesting that other factors than pre-cecal AA flow affect cecal metabolite concentrations.

High protein in the mixture diets had no significant effect on flows and cecal PF metabolites. Apparently, the smaller fraction of undigested protein in this group compensates the effect of protein level.

In the high protein RSM-fed group the litter moisture content was higher, which is in line with the increased liquid flow of AAs and the higher cecal histamine and tyramine concentrations. Low protein digestibility can increase excreta moisture content and reduce growth performance (Hossain et al., 2013) and may be a sign of poor gut health (Collett, 2012). No effect was found on litter quality score, despite the negative correlation between litter moisture and litter quality score ($r = -0.44$, $P = 0.002$). Despite this higher litter moisture content, growth performance in the current study was not hampered. In both RSM and mixture diets, high protein reduced feed intake and FCR, in both the starter in grower phases. This was similar in an earlier study in which broilers fed dehulled RSM ate

Table 8
Freeze dry matter, ammonia and biogenic amine concentrations in cecal digesta of broilers at 29, 30 and 31 days of age.

Diet nr.	**Protein level**	**Protein source**	**SFH addition**	FDM mg/g	NH_3 μg/g FDM	Cadaverine nmol/g FDM	Histamine nmol/g FDM	Putrescine nmol/g FDM	Spermidine nmol/g FDM	Spermine nmol/g FDM	Tyramine nmol/g FDM
1	**Low**	RSM		89.2	4471	561	8.9	239	1849	82.0	79.3
2	**High**	RSM		77.9	5647	774	72.0	267	1963	74.2	262.0
			SEM	9.6	847	254	14.4	18.1	215	10.3	67.5
			P-value	0.43	0.20	0.19	**< 0.01**	0.64	0.87	0.57	**0.001**
3	**Low**	Mixture		91.6	4011	1433	7.7	360	1793	85.0	97.0
4	**High**	Mixture		81.4	5054	2134	33.0	249	1817	86.0	125.2
			SEM	11.9	701	948	17.0	88.5	276	14.9	15.5
			P-value	0.48	0.26	0.64	0.40	0.41	0.99	0.92	0.33
6		**RSM**		91.1	4536	383^{a}	12.3^{a}	239	1774	81.8	151.7^{a}
3		**Mixture**		91.6	4011	1433^{ab}	7.7^{ab}	360	1793	85.0	97.0^{ab}
5		**RPI**		87.0	4500	7060^{b}	3.4^{b}	449	1418	63.8	70.0^{b}
			SEM	10.0	502.9	2548	4.8	137	268	14.3	34.6
			P-value	0.94	0.81	**0.04**	0.13	0.39	0.29	0.24	*0.08*
1		RSM	**Yes**	89.2	4471	561	8.9	239	1849	82.0	79.3
6		RSM	**No**	91.1	4536	383	12.3	239	1774	81.8	151.7
			SEM	7.8	611.2	241	2.7	14.7	221	10.6	39.7
			P-value	0.89	0.94	0.88	0.43	0.99	0.62	0.87	0.15
3		Mixture	**Yes**	91.6	4011	1433	7.7	360	1793	85.0	97.0
7		Mixture	**No**	66.4	5296	252	12.9	207	1228	53.3	58.9
			SEM	9.4	371.9	506	6.7	88.0	170	11.6	11.6
			P-value	*0.09*	0.17	0.14	0.73	0.11	*0.10*	*0.06*	*0.09*
5		RPI	**Yes**	87.0	4500	7059	3.4	449	1418	63.8	70.0
8		RPI	**No**	81.4	4391	1564	7.6	169	1128	63.8	48.1
			SEM	10.4	471.6	3130	2.5	142.7	222	14.6	11.0
			P-value	0.70	0.91	0.11	0.38	**< 0.01**	0.52	0.80	0.31

Abbreviations: RSM: Rapeseed meal, RPI: Rapeseed protein isolate, Mixture: RSM and RPI mixed, SFH: sunflower seed hulls.

Table 9
Ileal and cecal pH and short-chain fatty acid concentrations[1] in cecal digesta of broilers at 29, 30 and 31 days of age.

Diet nr.	Protein level	Protein source	SFH addition	Ileal pH	Cecal pH	Acetic acid	Propionic acid	Butyric acid	Valeric acid	Caproic acid	Isobutyric acid	Isovaleric acid	Isocaproic acid
1	**Low**	RSM		7.23	7.17	665.3	86.0	71.0	10.9	0.30	9.0	7.9	0.17
2	**High**	RSM		7.24	6.88	790.0	88.9	77.3	12.1	0.38	8.3	7.1	0.11
			SEM	0.09	0.07	93.2	9.1	20.5	2.1	0.08	0.8	0.9	0.05
			P-values	0.96	**0.001**	0.49	0.98	0.97	0.61	0.48	0.77	0.63	0.28
3	**Low**	Mixture		7.36	7.19	634.3	102.4	53.9	10.3	0.32	10.3	7.9	0.16
4	**High**	Mixture		7.25	7.02	850.9	130.3	66.2	12.9	0.32	13.0	10.1	0.17
			SEM	0.09	0.10	135.7	23.9	9.2	1.4	0.03	1.8	1.3	0.03
			P-values	0.44	**< 0.01**	0.24	0.53	0.28	0.26	0.97	0.25	0.21	0.99
6		**RSM**		6.96^{a}	7.20	575.4	95.2	47.3	9.6	0.37	9.6	7.5	0.16
3		**Mixture**		7.36^{b}	7.19	634.3	102.4	53.9	10.3	0.32	10.3	7.9	0.16
5		**RPI**		7.17^{ab}	7.28	760.7	149.6	67.2	12.8	0.44	11.8	9.5	0.28
			SEM	0.13	0.06	105.4	22.4	10.6	1.4	0.08	1.2	0.9	0.04
			P-values	**0.02**	0.48	0.58	0.32	0.44	0.36	0.53	0.64	0.49	0.22
1		RSM	**Yes**	7.23	7.17	665.3	86.0	71.0	10.9	0.30	9.0	7.9	0.17
6		RSM	**No**	6.96	7.20	575.4	95.2	47.3	9.6	0.37	9.6	7.5	0.16
			SEM	0.12	0.07	61.4	12.9	15.0	1.5	0.10	0.8	0.9	0.05
			P-values	**0.04**	0.67	0.62	0.89	0.20	0.57	0.53	0.80	0.81	0.64
3		Mixture	**Yes**	7.36	7.19	634.3	102.4	53.9	10.3	0.32	10.3	7.9	0.16
7		Mixture	**No**	7.16	7.19	1001.7	198.0	62.4	13.4	0.47	12.6	9.6	0.34
			SEM	0.08	0.08	168.4	39.7	8.3	1.6	0.06	2.2	1.6	0.07
			P-values	0.15	0.91	**0.05**	**0.04**	0.37	0.19	0.19	0.33	0.33	**0.03**
5		RPI	**Yes**	7.17	7.28	760.7	149.6	67.2	12.8	0.44	11.8	9.5	0.28
8		RPI	**No**	7.27	7.28	633.9	140.1	52.3	11.1	0.96	10.0	7.5	0.24
			SEM	0.11	0.07	132.6	28.5	14.4	2.1	0.08	1.3	1.1	0.05
			P-values	0.46	0.98	0.49	0.91	0.19	0.46	**< 0.001**	0.46	0.25	0.99

Abbreviations: RSM: Rapeseed meal, RPI: Rapeseed protein isolate, Mixture: RSM and RPI mixed, SFH: sunflower seed hulls.
[1]Concentrations of short- and branched chain fatty acids are in μmol/g FDM.

less but were also more efficient in their growth (Elling-Staats et al., 2022). Overall, the reduction in voluntary feed intake prevented high levels of PF and potential subsequent deteriorating effects on gut health.

4.2. Effects of fiber

In the current study, SFH were added to diets to equalize the level of NDF in the low protein diets to their high protein counterparts and in the mixture and RPI diet to that of RSM, in order to evaluate the effect of protein level and protein digestibility at equal NDF level. Including diets without SFH addition allowed the evaluation of the effect of SFH addition on digestion and fermentation as well. Sunflower seed hulls are likely less fermentable than the fiber in RSM, because of the higher concentration of cellulose and the lower concentration pectic polysaccharides and hemicellulose in SFH (Lannuzel et al., 2022). Moreover, SFH addition increased the level of insoluble fiber in the diet, which could bypass the ceca (de Vries et al., 2014) and, therefore, does not contribute to fermentation.

The SFH addition increased DM flow in RSM diets but had no effect on N and AA flows. Ileal N digestibility and growth performance in RSM and RPI diets improved with SFH addition, which agrees with findings of Mateos et al. (2012). This improved digestion might have been the result of a slower passage through the proximal part of the gastro-intestinal tract. In the RSM-fed birds, SFH addition tended to increase the MRT of the solid phase in the crop with 4 min and it increased the MRT in the stomach with 20 min for the solid phase and 11 min for the liquid phase. An increased MRT in the stomach is associated with improved protein digestion (Rougière and Carré, 2010), as it permits more time for the mixing of digesta with pepsin and hydrochloric acid (Svihus, 2011). Moreover, for the RPI and mixture-fed birds, the empty weights of the gizzard and the gizzard to proventriculus ratio were increased with SFH addition. A heavier gizzard indicates a better grinding of feed and hence an improved digestibility (Svihus, 2011). Particularly, in the RPI diets this effect was expected to be strong as it had the lowest fiber level. Our current results suggest that SFH may improve digestion via increased gastric retention.

A higher level of soybean oil was included in the RSM diets to create iso-caloric diets, increasing the crude fat level of these two diets and its subsequent ingestion (from 4.4 to 8.0 g/d). Such differences were small in other diet comparisons (< 2 g/d). In ruminants, unsaturated fat may reduce rumen fiber fermentation (Pantoja et al., 1994). Whether such an effect occurs in ceca is presently not clear. As soybean oil is highly digestible (apparent ileal digestibility of 97 %; Kierończyk et al., 2018), little of this fat will enter the ceca. Therefore, we speculate that cecal fermentation was little affected by this difference in dietary oil levels.

Adding SFH reduced cecal acetate and propionate concentrations in mixture-fed birds and reduced cecal caproic acid in RPI-fed birds, indicating less fermentation took place. These SCFA may be produced both during carbohydrate and protein fermentation (Macfarlane et al., 1992). Moreover, high protein RSM-fed birds had a lower cecal pH, indicating more fermentation. Therefore, it appears that fiber from RSM ferments to a larger extent than SFH or RSM was degraded to smaller particles, which were able to enter the ceca to a higher extend than fiber originating from SFH. Furthermore, we found no reducing effect of fiber from SFH on PF as cecal ammonia and BCFAs were unaffected.

On the contrary, adding SFH tended to increase cecal spermidine, spermine and tyramine in mixture-fed birds and significantly increased cecal putrescine in RPI-fed birds. As these biogenic amines are products of amino acid decarboxylation an increase of these products might indicate increased PF. However, carbohydrate fermentation could coincide with increased biogenic amines. Rehman et al. (2008) found that inulin supplementation increased cecal butyrate and reduced ammonia concentration, but at the same time increased the concentration of biogenic amine putrescine. Hence, without other indications (such as ammonia and BCFAs), these higher concentrations of biogenic amines may not directly reflect increased PF.

4.3. Conclusions

The low voluntary feed intake of broilers fed diets containing a high protein level or a low digestible protein source limited pre-cecal protein flow. Consequently, no effects were observed on cecal concentration of ammonia, BCFAs and most biogenic amines. The pre-cecal AA flow in the liquid fraction was increased with a high protein level in birds fed the RSM diet. This corresponded with an increase in cecal histamine and tyramine concentrations and increased litter moisture content. Despite these indications of a slightly increased PF, growth performance was not hampered. High protein fed birds had a reduced feed intake, but FCR was improved. The addition of SFH increased growth performance and N digestibility. It is, however, unlikely that the variations in PF among the ingredients are caused by their intrinsically different fiber contents.

Funding source

This publication is part of the project ‘Dynamics of proteolytic fermentation and the effects on intestinal health of broilers and piglets,’ with project number 14943, of the research program ‘DSM: Animal Nutrition and Health’ which is financed by the Dutch Research Council (NWO) domain Applied Engineering Sciences, and the Dutch Ministry of Economic Affairs and Climate Policy. The research program is co-financed by DSM Nutritional Products.

CRediT authorship contribution statement

Miranda Elling-Staats: Writing – review & editing, Writing – original draft, Investigation, Formal analysis. **Arie Kies:** Writing – review & editing, Validation, Supervision. **Myrthe Gilbert:** Writing – review & editing, Validation, Supervision. **René Kwakkel:** Writing – review & editing, Validation, Supervision.

Declaration of Competing Interest

There is no conflict of interest.

Acknowledgements

We gratefully acknowledge the help of Nisola Ayenfe and Ahmad Alsalim (master students) and Bert Beukers and Sabine van Woudenberg for their skilled assistance in the broiler study, and Niels Wever, Saskia van Laar, Erika Beukers-van Laar and Michel Breuer for their skilled assistance with the laboratory procedures. The authors also thank DSM Food Specialties (Delft, the Netherlands) for providing us with the rapeseed protein isolate and Gerard Beelen (RDS, Wijk bij Duurstede, the Netherlands) for producing the feeds.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.anifeedsci.2025.116305.

References

Amerah, A., Ravindran, V., Lentle, R., Thomas, D., 2007. Feed particle size: implications on the digestion and performance of poultry. World's Poult. Sci. J. 63, 439–455. https://doi.org/10.1017/S0043933907001560.

Armentano, L., Russell, R., 1985. Method for calculating digesta flow and apparent absorption of nutrients from nonrepresentative samples of digesta. J. Dairy Sci. 68, 3067–3070. https://doi.org/10.3168/jds.S0022-0302(85)81204-2.

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. J. Dairy Sci. 99, 8041–8052. https://doi.org/10.3168/jds.2016-11069.

Collett, S.R., 2012. Nutrition and wet litter problems in poultry. Anim. Feed Sci. Technol. 173, 65–75. https://doi.org/10.1016/j.anifeedsci.2011.12.013.

De Lange, L., Rombouts, C., Elferink, G.O., 2003. Practical application and advantages of using total digestible amino acids and undigestible crude protein to formulate broiler diets. World'. Poult. Sci. J. 59, 447–457.

de Vries, S., Kwakkel, R., Pustjens, A., Kabel, M., Hendriks, W., Gerrits, W., 2014. Separation of digesta fractions complicates estimation of ileal digestibility using marker methods with Cr2O3 and cobalt-ethylenediamine tetraacetic acid in broiler chickens. Poult. Sci. 93, 2010–2017. https://doi.org/10.3382/ps.2013-03845.

Ding, X., Li, D., Li, Z., Wang, J., Zeng, Q., Bai, S., Su, Z., Zhang, K., 2016. Effects of dietary crude protein levels and exogenous protease on performance, nutrient digestibility, trypsin activity and intestinal morphology in broilers. Livest. Sci. 193, 26–31. https://doi.org/10.1016/j.livsci.2016.09.002.

Elling-Staats, M.L., Kies, A.K., Gilbert, M.S., Kwakkel, R.P., 2022. Over-toasting dehulled rapeseed meal and soybean meal, but not sunflower seed meal, increases prececal nitrogen and amino acid digesta flows in broilers. Poult. Sci. 101, 101910. https://doi.org/10.1016/j.psj.2022.101910.

Erdag, D., Merhan, O., Yildiz, B., 2018. Biochemical and pharmacological properties of biogenic amines. Biog. Amines 8, 1–14.

Frees, D., Brøndsted, L., Ingmer, H., 2013. Bacterial proteases and virulence. In: Dougan, D. (Ed.), Regulated Proteolysis in Microorganisms. Subcellular Biochemistry, 66. Springer, Dordrecht. https://doi.org/10.1007/978-94-007-5940-4_7.

Gilbert, M.S., Ijssennagger, N., Kies, A.K., van Mil, S.W., 2018. Protein fermentation in the gut; implications for intestinal dysfunction in humans, pigs and poultry. Am. J. Physiol. Gastrointest. Liver Physiol. 315 (2), 159–170. https://doi.org/10.1152/ajpgi.00319.2017.

Goodarzi Boroojeni, F., Vahjen, W., Mader, A., Knorr, F., Ruhnke, I., Röhe, I., Hafeez, A., Villodre, C., Männer, K., Zentek, J., 2014. The effects of different thermal treatments and organic acid levels in feed on microbial composition and activity in gastrointestinal tract of broilers. Poult. Sci. 93, 1440–1452. https://doi.org/10.3382/ps.2013-03763.

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. Anim. Feed Sci. Technol. 160, 148–159. https://doi.org/10.1016/j.anifeedsci.2010.07.005.

Hossain, M.A., Islam, A.F., Iji, P., 2013. Growth responses, excreta quality, nutrient digestibility, bone development and meat yield traits of broiler chickens fed vegetable or animal protein diets. South Afr. J. Anim. Sci. 43, 208–218. https://doi.org/10.4314/sajas.v43i2.11.

ISO). 1999. ISO 6492:1999. Animal Feeding Stuff - Determination of Fat Content. ISO, Geneve, Switzerland.

ISO). 2004. ISO 15914:2004. Animal Feeding Stuff - Enzymatic Determination of Total Starch Content. ISO, Geneve, Switzerland.

ISO). 2005. ISO 13903:2005. Animal Feeding Stuff - Determination of Amino Acids Content. ISO, Geneve, Switzerland.

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.

Jiménez-Moreno, E., Frikha, M., de Coca-Sinova, A., García, J., Mateos, G., 2013. Oat hulls and sugar beet pulp in diets for broilers 1. Effects on growth performance and nutrient digestibility. Anim. Feed Sci. Technol. 182, 33–43. https://doi.org/10.1016/j.anifeedsci.2013.03.011.

Kierończyk, B., Rawski, M., Józefiak, A., Mazurkiewicz, J., Świątkiewicz, S., Siwek, M., Marek Bednarczyke, M., Szumacher-Strabela, M., Cieślaka, A., Benzertihaf, A., Józefiak, D., 2018. Effects of replacing soybean oil with selected insect fats on broilers. Anim. Feed Sci. Technol. 240, 170–183. https://doi.org/10.1016/j.anifeedsci.2018.04.002.

Lannuzel, C., Smith, A., Mary, A., Della Pia, E., Kabel, M., de Vries, S., 2022. Improving fiber utilization from rapeseed and sunflower seed meals to substitute soybean meal in pig and chicken diets: a review. Anim. Feed Sci. Technol., 115213 https://doi.org/10.1016/j.anifeedsci.2022.115213.

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. Anim. Feed Sci. Technol. 234, 110–117. https://doi.org/10.1016/j.anifeedsci.2017.09.013.

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 Microbiol. Ecol. 10, 81–88. https://doi.org/10.1111/j.1574-6941.1992.tb00002.x.

Mateos, G., Jiménez-Moreno, E., Serrano, M., Lázaro, R., 2012. Poultry response to high levels of dietary fiber sources varying in physical and chemical characteristics. J. Appl. Poult. Res. 21, 156–174. https://doi.org/10.3382/japr.2011-00477.

Pantoja, J., Firkins, J.L., Eastridge, M.L., Hull, B.L., 1994. Effects of fat saturation and source of fiber on site of nutrient digestion and milk production by lactating dairy cows. J. Dairy Sci. 77 (8), 2341–2356. https://doi.org/10.3168/jds.S0022-0302(94)77177-0.

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. J. Nutr. 142, 661–667. https://doi.org/10.3945/jn.111.156190.

Qaisrani, S., Moquet, P., Van Krimpen, M., Kwakkel, R., Verstegen, M., Hendriks, W., 2014. Protein source and dietary structure influence growth performance, gut morphology, and hindgut fermentation characteristics in broilers. Poult. Sci. 93, 3053–3064. https://doi.org/10.3382/ps.2014-04091.

Rehman, H., Böhm, J., Zentek, J., 2008. Effects of differentially fermentable carbohydrates on the microbial fermentation profile of the gastrointestinal tract of broilers. J. Anim. Physiol. Anim. Nutr. 92, 471–480. https://doi.org/10.1111/j.1439-0396.2007.00736.x.

Rougière, N., Carré, B., 2010. Comparison of gastrointestinal transit times between chickens from D+ and D- genetic lines selected for divergent digestion efficiency. Animal 4, 1861–1872. https://doi.org/10.1017/S1751731110001266.

Scheiner, D., 1976. Determination of ammonia and Kjeldahl nitrogen by indophenol method. Water Res. 10, 31–36. https://doi.org/10.1016/0043-1354(76)90154-8.

Smith, E. a, Macfarlane, G.T., 1997. Dissimilatory amino acid metabolism in human colonic bacteria. Anaerobe 3, 327–337. https://doi.org/10.1006/anae.1997.0121.

Smith, E.A., Macfarlane, G.T., 1998. Enumeration of amino acid fermenting bacteria in the human large intestine: effects of pH and starch on peptide metabolism and dissimilation of amino acids. FEMS Microbiol. Ecol. 25, 355–368. https://doi.org/10.1111/j.1574-6941.1998.tb00487.x.

Svihus, B., 2011. The gizzard: function, influence of diet structure and effects on nutrient availability. World's Poult. Sci. J. 67, 207–224. https://doi.org/10.1017/S0043933911000249.

Van Harn, J., I.C. De Jong, T. Veldkamp. 2009. Effect strooiselmateriaal, strooiselhoeveelheid, opvangschoteltjes en waterdruk op resultaten vleeskuikens. Rapport 220. Wageningen UR Livestock Research, Lelystad. ISSN 1570-8616.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583–3597. https://doi.org/10.3168/jds.S0022-0302(91)78551-2.

Widyaratne, G., Drew, M., 2011. Effects of protein level and digestibility on the growth and carcass characteristics of broiler chickens. Poult. Sci. 90, 595–603. https://doi.org/10.3382/ps.2010-01098.