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Milled and cryogenic micronised wheat bran inclusions stimulate fibre degradation and fermentation differently throughout the broiler's gastrointestinal tract

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ABSTRACT

Inclusion of fibre fractions in broiler feed can increase microbial short-chain fatty acid (**SCFA**) production upon fermentation, enhancing gut health and performance. The main site of fermentation is the caeca, and extensive particle size reduction of fibre fractions (< 100 μm) may increase their fermentability and influx into the caeca, extending the associated benefits. Coarser fibre fractions (> 400 μm) improve fermentation parameters as well, but are expected to act in the foregut (precaecal) as they seem too large to pass through the restricted caecal entrance. The mechanism behind the effect of different fibre particle sizes and their inclusion level on fibre degradation and fermentation is not yet fully understood. The role of fibre particle size along the gastrointestinal tract (**GIT**) and the possible increase of their effect with increasing inclusion level was studied by comparing 0.2% (**L02**) and 2% (**L2**) inclusion of coarse milled (452 μm, **WB452**) and cryogenically milled (27 μm, **WB27**) wheat bran in male Ross 308 broilers (570, d 1 to d 37) using a 2 × 2 factorial design. GIT parts, digesta and plasma samples were collected to measure GIT development, fibre degradation and fermentation, and gut health at d 10 and d 37. WB452 increased precaecal fibre degradation and fermentation through gizzard stimulation based on the decrease in gizzard pH and increase in ileal arabinoxylan digestion and ileal butyric acid content at d 37. Both bran sizes tended to increase the relative abundance of different SCFA-producing bacterial groups in the caeca at d 10, although this was not related to differences in caecal SCFA content. Inclusion level L02 had a stronger positive effect on caecal functioning and gut health through improving caecal lobe weight, rhamnose content and microbial composition and decreasing plasma D-lactate levels compared to L2. These results suggest that both fine and coarse fibre inclusions, particularly at a low inclusion level, can stimulate fibre fermentation through different mechanisms, highly depending on the GIT section and broiler age.

Introduction

A recent development in broiler nutrition is the inclusion of additional fibre fractions to improve broiler performance and gut health in a sustainable manner. A major part of these benefits is due to their fermentation by the gut microbiome, leading to the production of short-chain fatty acids (**SCFA**) (Jha and Mishra, 2021; Singh and Kim, 2021). SCFA can be used as an additional energy source by the host cells and the microbiome, and can inhibit pathogen invasion, colonisation and proliferation (Gomez-Osorio et al., 2021; Liu et al., 2021a). This beneficial fibre fermentation mostly happens in the caecal lobes, two blind sacs at the end of the small intestine containing the highest microbial load of the gastrointestinal tract (**GIT**) (Apajalahti, 2005; Clench and Mathias, 1995; Svihus *et al.*, 2013). The influx of fermentable fibre into the caeca, restricted to soluble and fine particles, mainly smaller than 100 μm, seems to be one of the main factors that limit fibre fermentation (Svihus et al., 2013; Vanderghinste et al., 2025a). This implies that fibre fermentation in broilers could be improved by the use of extensive

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particle-size-reduced fibre inclusions, although no studies have incorporated fibre fractions with a particle size below 100 μm to study their effect on fibre fermentation in in vivo broiler experiments. Aside from increasing caecal influx, reducing fibre particle size can facilitate more microbial and enzymatic interactions due to the increased surface area and the breakage of cell walls (De Bondt et al., 2020; Deroover et al., 2021; Stewart and Slavin, 2009; Vermeulen et al., 2016). Studies using fibre fractions of 280 to 500 μm in broiler feed have demonstrated these positive effects, for example by an increase in caecal SCFA content or a decrease in Salmonella colonisation compared to the inclusion of larger fibre fractions (Bautil et al., 2023; Vanderghinste et al., 2025b; Vermeulen et al., 2016).

Aside from fibre particle size, the fibre inclusion level in feed can also affect caecal fermentation, however, few studies have tested different inclusion levels of fine fibre particles in broilers. Boguslawska-Tryk et al. (2015) observed that 0.5% lignocellulose inclusion (200 to 300 μm) resulted in the highest caecal SCFA contents in 42-day-old broilers, compared to levels from 0.25 to 1%. Röhe et al. (2020) reported a decrease in caecal SCFA with increasing levels of lignocellulose (0.8 to 10%) in 70-day-old slow-growing broilers, although this was attributed to the limited fermentability of lignocellulose. It has been hypothesised that excessive inclusion levels (10%) of fine fibre particles (100 μm) may be detrimental for caecal fermentation in young broilers, possibly through overfilling of the immature caeca with these relatively slowly fermented substrates (Vanderghinste et al., 2025b). This evidence suggests an upper limit to the inclusion level of fine fibre particles for optimal caecal fermentation, but the interaction effect of fibre particle size, inclusion level and broiler age on caecal fermentation is not completely understood. A relatively low inclusion level ($\leq$ 0.5%) is often used for additives such as feed enzymes, prebiotics and more recently stimbiotics to stimulate microbial activity, while limiting their effect on other gastrointestinal processes (Bautil et al., 2021; Morgan, 2023; Ribeiro et al., 2018; Veluri et al., 2024). It can be hypothesised that increasing the inclusion level of fine, fermentable fibre added as a prebiotic to over 1% may increase its caecal influx and thus also fermentation, but the additional effects of this higher inclusion level still require investigation.

Despite the limited caecal influx of coarse fibre particles due to the filtering mechanism at the caecal entrance (Fenna and Boag, 1973; Svihus et al., 2013; Vanderghinste et al., 2025a), fibre inclusions with a particle size of 460 to 3000 μm can also positively alter the caecal microbiome composition (Jacobs et al., 2010; Kheravii et al., 2018, 2017; Pourazadi et al., 2020; Veluri *et al.*, 2024). As it is unlikely that these coarse fibre fractions enter the caeca in their initial particle size, they probably affect the caecal microbiome composition indirectly by stimulating beneficial processes in the precaecal GIT such as gizzard grinding and acidification. This can also enhance fibre degradation, defined as the combination of particle size reduction, acid hydrolysis and enzymatic solubilisation of the fibre fraction in the precaecal tract, improving later caecal fermentation (Amerah et al., 2008; Elling-Staats et al., 2021; Itani et al., 2024; Kheravii et al., 2018, 2017; Singh et al., 2014; Svihus, 2011; Veluri *et al.*, 2024). The extent of the benefits of coarse fibre inclusions on broiler performance will also depend on their inclusion level, as excessive inclusion may result in nutrient dilution or high energy consumption by the gizzard. This was observed in studies using coarse fibre fractions ($>$ 600 μm), where inclusion levels of 8 to 11% resulted in a poorer FCR compared to levels of 3 to 4% (Itani et al., 2024; Tejeda and Kim, 2021). Inclusion levels below 5% seem to avoid detrimental effects on FCR, but further research on coarse fibre inclusion levels in broiler feeds and the effect on caecal fermentation is still lacking.

To summarise, fine and coarse fibre inclusions can enhance caecal fermentation and its beneficial consequences for broiler performance and gut health in an inclusion level and particle size-dependent manner. The particle size of fibre inclusions studied in previous research remained relatively large for them to pass the restricted caecal entrance (Vanderghinste et al., 2025a). An extensive particle-size reduced fibre fraction ($<$ 100 μm) may further boost caecal fibre influx and consequently fibre fermentation and gut health, while coarser fibre inclusions can exert similar positive effects through gizzard stimulation. In addition, raising the fibre inclusion level may increase the amount of fermentable fibre available for the caecal microbiome. The effect of bran particle size and inclusion level on fermentation can further be expected to be age-dependent. This study hence investigated the potential of extensively particle-size-reduced wheat bran (27 μm) to enhance fibre fermentation and compared it to the inclusion of coarser conventionally milled wheat bran (452 μm). The working mechanisms behind the stimulating effects of these bran fractions on fibre degradation and fermentation in both the foregut and hindgut were studied at a low and higher inclusion level, respectively 0.2 and 2%, as a function of broiler age.

Materials and methods

Preparation and analysis of bran material

Commercial wheat bran (*Triticum aestivum* L.) was obtained from Dossche Mills (Deinze, Belgium) and subjected to two different milling techniques to obtain two desired particle sizes. The first type of particle-size-reduced bran was obtained through Cyclotec milling (Cyclotec 1093 Sample mill, Foss, Höganäs, Sweden) using a sieve with a pore size of 500 μm. The particle size distribution of this Cyclotec milled bran was determined by dry sieving using a Retch Vibratory Sieve Shaker (Retch, Aartselaar, Belgium) with a sieve set with pore sizes of 2000, 1000, 710, 500, 400, 250, 125, 90 and 38 μm as described in Vanderghinste et al. (2025b). A 20 g of bran was shaken on the sieve set for 30 minutes at an amplitude of 1.5 mm and gently brushed in between to prevent clogging of the pores. The amount of bran left on each sieve was weighed afterwards to calculate the average particle size (d_{av}) using the following formula:

$$d_{av} = \Sigma d_i * m_i,$$

with d_i the average particle size of the particle on sieve i and m_i the mass percentage of the fraction of bran on sieve i. The Cyclotec milled bran had an average particle size of 452 μm and will be further referred to as **WB452** in this paper. The particle size distribution is shown in Figure S1 (Supplementary file).

The second type of particle-size-reduced bran was obtained by large-scale cryogenic milling as described in De Bondt et al. (2020) with a DemiNo® 2250 impact whirl mill (Noll, Bobingen, Germany) using an inlet temperature of −140°C and outlet temperature of −80°C. The particle size distribution and average volumetric particle size of this cryogenic milled bran was determined using the wet module of a Laser Diffraction Particle Size Analyzer LS 13 320 (Beckman Coulter Inc, Indianapolis, IN, USA) as described in Vanderghinste et al. (2025a), as the dry sieving method is not suitable for particles smaller than 50 μm. The Laser Diffraction Particle Size Analyser can measure particle sizes ranging from 0.375 μm to 2000 μm and assumes that all particles are spherical, making it an accurate technique for the cryogenic milled bran, but not for the Cyclotec milled bran due to its intact, non-spherical bran shape. The cryogenic milled bran had an average volumetric particle size of 27 μm and will be further referred to as **WB27**. The volumetric particle size distribution is shown in Figure S2 (Supplementary file).

Experimental design and diets

WB452 and WB27 were included in broiler feed by replacing 0.2% (**L02**) or 2% (**L2**) of the wheat fraction, resulting in a 2 × 2 factorial design, with the two main factors being wheat bran particle size and bran inclusion level, along with their interaction effect. A control group was included with no replacement by wheat bran. Two-phase diets consisting of a starter phase (d 1 to d 10) and a grower phase (d 11 to d 37) were used (Table 1). The diets were formulated to meet the nutritional requirements of Ross 308 broilers (Aviagen, 2022). Lipid and protein levels of the diets with a 2% bran replacement were slightly adapted to provide isocaloric diets for all treatments. No fibre-degrading enzymes were included, but phytase was included. The indigestible marker TiO_2 was included in all diets at 0.5% for digestibility measurements.

Broiler housing and sampling

This broiler trial was approved by the Ethical Committee for the experimental use of animals of the KU Leuven under accession number P140/2020.

570 one-day-old male broilers (Ross 308) were purchased from a commercial hatchery (Belgabroed NV, Merksplas, Belgium) and randomly divided over 30 floor pens containing fresh wood shavings. Each dietary treatment contained 6 replicate pens with 19 broilers per pen. Conventional heating, ventilation and lighting conditions for broiler housing were applied (Aviagen, 2018). The housing temperature was set to 34°C at d 1 and gradually decreased until 20°C at d 27, after which this temperature was kept constant until the end of the trial (d 37). All broilers received water and feed ad libitum. Inspection of feed and water supply and chick mortality was carried out daily. Pen weights and feed intake were measured on pen level at d 1, 10, 11, 15, 29 and 37 to calculate FCR, using 6 replication pens per treatment. At d 10 and d 37, 150 and 60 broilers, respectively, were stunned by electronarcosis followed by cervical dislocation. Digesta was pooled per 5 and 2 broilers from the same replicate pen on d 10 and d 37, respectively, to obtain 6 digesta replicates per treatment with sufficient material for further analysis at each sampling age. The chyme and digesta of the gizzard, duodenum-jejunum, ileum (the section between Meckel's diverticulum and ileocaecal intersect) and caeca were collected by gently stripping the segments. Digesta samples were stored at −20°C, with aliquots of ileal and caecal digesta at −80°C. The empty weight of the gizzard and

Table 1
Formulation of the experimental diets.

Ingredients (as-fed, g/kg)	Starter CTRL	Starter 0.2%	Starter 2%	Grower CTRL	Grower 0.2%	Grower 2%
Wheat	577.55	575.55	552.55	641.95	639.95	616.95
Wheat bran (452 µm or 27 µm)	**0.00**	**2.00**	**20.00**	**0.00**	**2.00**	**20.00**
Soybean meal (48.0% CP)	265.00	265.00	265.00	230.00	230.00	230.00
Full fat soyabeans	50.00	50.00	50.00	50.00	50.00	50.00
Soybean oil (37.5 MJ)	33.00	33.00	38.00	37.70	37.70	42.70
Rapeseedmeal (33.9% CP)	30.00	30.00	30.00	0.00	0.00	0.00
Premix[1]	5.00	5.00	5.00	5.00	5.00	5.00
Lime fine (38% Ca)	16.80	16.80	16.80	13.30	13.30	13.30
Monocalciumphosphate	6.50	6.50	6.50	4.30	4.30	4.30
Salt	1.90	1.90	1.90	2.00	2.00	2.00
Sodium carbonate	2.80	2.80	2.80	2.70	2.70	2.70
L-lysine HCl	2.30	2.30	2.30	2.60	2.60	2.60
DL-methionine	2.80	2.80	2.80	3.15	3.15	3.15
L-threonine	1.10	1.10	1.10	1.40	1.40	1.40
L-valine 96.5%	0.20	0.20	0.20	0.45	0.45	0.45
L-arginine	0.00	0.00	0.00	0.25	0.25	0.25
L-isoleucine 90%	0.00	0.00	0.00	0.15	0.15	0.15
Phytase (Axtra PHY, 500 FTU)	0.050	0.050	0.050	0.050	0.050	0.050
TiO_2	5.00	5.00	5.00	5.00	5.00	5.00
Calculated nutrient content (g/kg)						
Energy (kcal/kg)	2900	2900	2901	3000	3000	3000
DM	880.9	880.9	881.6	879.6	879.6	880.4
Ash	61.6	61.6	62.5	52.7	52.7	53.6
Crude protein	219.5	219.5	219.8	200.6	200.6	200.9
Crude fat	63.1	63.1	68.4	67.1	67.1	72.4
Crude fibre	29.1	29.1	31.1	25.6	25.6	27.5
Carbohydrates	499.9	499.9	492.5	526.0	526.0	518.6
Starch	349.2	349.2	337.0	385.4	385.4	373.1
Sugars	44.8	44.8	45.1	40.8	40.8	41.1
Calcium	9.0	9.0	9.0	7.0	7.0	7.0
Total phosphorus	5.5	5.5	5.7	4.7	4.7	4.8
Available phosphorus	4.4	4.4	4.4	3.9	3.9	3.9
Digestible phosphorus	4.2	4.2	4.2	3.6	3.6	3.6
Magnesium	1.7	1.7	1.7	1.5	1.5	1.5
Potassium	9.3	9.3	9.5	8.4	8.4	8.6
Sodium	1.6	1.6	1.6	1.6	1.6	1.6
Chloride	2.0	2.0	2.0	2.1	2.1	2.1
Digestible lysine (%)	1.15	1.15	1.15	1.05	1.05	1.05
Digestible leucine (%)	1.38	1.38	1.38	1.24	1.24	1.24
Digestible methionine + Cysteine (%)	0.85	0.85	0.85	0.83	0.83	0.83
Digestible arginine (%)	1.25	1.25	1.25	1.13	1.13	1.14
Digestible threonine (%)	0.75	0.75	0.75	0.71	0.71	0.71
Digestible tryptophan (%)	0.24	0.24	0.24	0.21	0.21	0.21

CP = crude protein; DM = dry matter.

[1] Mineral-vitamin premix provided per kg diet: Vitamin A 10,000 IU; Vitamin D_3 2,500 IU; Vitamin E 50 mg; Vitamin K_3 2.3 mg; Vitamin B_1 2.0 mg; Vitamin B_2 7.5 mg; Vitamin B_6 3.5 mg; Vitamin B_{12} 20 µg; Niacinamide 35 mg; D-pantothenic acid 12 mg; Choline chloride 460 mg; Folic acid 1.0 mg; Biotin 0.2 mg; Iron 80 mg; Copper 12 mg; Manganese 85 mg; Zinc 60 mg; Iodine 1.2 mg; Selenium 0.25 mg; milled maize used as carrier.

the full weight of the caecal lobes were also measured during sampling. At d 37, blood samples (2 mL) of 6 broilers per treatment were collected via the wing vein before culling and stored in EDTA-coated tubes on ice during sampling. Blood samples were centrifuged at 3,000 g for 15 min at 4°C, after which the plasma fraction was collected for storage at −20°C.

Determination of gizzard digesta pH

The pH of the gizzard digesta was measured on extracts of stored digesta (−20°C) collected during sampling ($n = 6$ per treatment and per age group). Digesta was thawed for 45 min at room temperature, then 10 mL of demineralised water was added to 1 g digesta. The suspension was vortexed and put in a water bath at 27°C, based on the actual temperature of the freshly sampled gizzard digesta during pH measurement in a previous trial. After 15 minutes, the pH of the suspension was measured at 27°C. This method was validated prior to this analysis by using gizzard digesta samples from a previous trial during which the pH and the temperature were also measured on fresh digesta immediately after sampling.

Determination of ileal and caecal volumetric particle size distribution

The volumetric particle size distribution of the ileal and caecal digesta was measured on 6 digesta replicate pools per treatment at d 10 and d 37 with a Laser Diffraction Particle Size Analyzer as described in Section 2.1. The 50th percentile or average particle size (**D50**) was also calculated for each measurement.

Determination of ileal and caecal carbohydrate content

The total arabinoxylan (**TOT-AX**), water-extractable arabinoxylan (**WE-AX**) and rhamnose content of feed ($n = 3$ per phase) and digesta samples ($n = 6$ per treatment and per age group) were determined by gas chromatography (**GC**) using the procedure based on Cummings and Englyst (1987) and adapted by Gebruers et al. (2009). Prior to the determination of the WE-AX content, wheat endogenous enzymes in the feed samples and microbial enzymes in the digesta sample were inactivated following the procedure described by Bautil et al. (2019). Lyophilised sample (100 to 125 mg) was heated in 10 mL ethanol (80%) in a boiling water bath until all ethanol was evaporated. Next, aqueous extracts were made by dissolving the dried pellets in 10 mL HCl/KCl buffer (20 mM, pH 3) and shaking them for 30 min at 150 rpm at 7°C. The extracts were centrifuged (4,000 g, 10 min, 7°C) and filtered (Whatman MN 615, Macherey-Nagel, Düren, Germany). Subsequently, all polysaccharides in the extract (WE-AX measurement) or lyophilised sample (TOT-AX and rhamnose measurement) were hydrolysed to monosaccharides in trifluoroacetic acid (TFA, 2 N) during 1 h at 110°C. The resulting aldoses were reduced to alditols in alkaline conditions using $NaBH_4$ (2 M), after which the alditols were derivatised to alditol acetates using $(CH_3CO)_2O$ and catalyst $CH_3C_3H_3N_2$. The concentration of the volatile alditol acetates was measured by an Agilent 6890 Series A GC (Agilent Technologies, Wilmington, DE, USA) using a Supelco SP-2380 polar column (30 m x 0.32 mm, film thickness 0.2 μm) with helium as carrier gas (flow rate 20 mL/min). TOT-AX and WE-AX were calculated as follows:

$$AX\ \% = ([arabinose\ \%] + [xylose\ \%])\ x\ 0.88$$

The water-unextractable arabinoxylan (**WU-AX**) content was calculated as the difference between the TOT-AX and WE-AX content.

Quantification of TiO_2 and determination of ileal WU-AX digestibility

The TiO_2 content of the feed ($n = 3$ per phase) and ileal digesta samples ($n = 6$ per treatment and per age group) was measured based on the method of Short et al. (1996) with adaptations proposed by Myers et al. (2004). Lyophilised digesta (100 mg) was mixed with 12 mL sulfuric acid (H_2SO_4, 95%) and a reaction catalyst containing 3.5 g K_2SO_4 and 0.4 g $CuSO_4$ (CK, Thompson & Capper Ltd, Cheshire, UK) in a 250 mL macro-Kjeldahl tube (Kjeldahltherm block and digestion unit, Gerhardt, Königswinter, Germany) for incubation at 420°C during 60 min. After cooling for at least 30 min, 10 mL hydrogen peroxide (H_2O_2, 30% wt/wt) was added. After another 30 min of cooling, demineralised water was added until a total liquid weight of 50.0 g was obtained. The liquid was filtered (Whatman MN 615) and the extinction was measured at 410 nm, using air as a reference (Ultraspec II UV/vis spectrophotometer, Pharmacia Biotech, Uppsala, Sweden).

Ileal WU-AX digestibility was calculated using the following formula:

$$WU-AX\ digestibility\ (\%) = 1 - \left[\left(TiO_{2\ feed} \times WU-AX_{digesta}\right) \div \left(TiO_{2\ digesta} \times WU-AX_{feed}\right)\right] \times 100$$

with $TiO_{2\ feed}$ and $TiO_{2\ digesta}$ the measured TiO_2 contents (g/kg), and $WU\text{-}AX_{feed}$ and $WU\text{-}AX_{digesta}$ the measured WU-AX contents (g/kg) in the feed and ileal digesta, respectively.

Determination of ileal and caecal SCFA content

Acetic, propionic, butyric and valeric acid in the ileal and caecal digesta ($n = 6$ per treatment and per age group) were measured by GC, based on the procedure described by Van Craeyveld *et al.* (2008) with slight adaptations described in Vanderghinste et al. (2025b). In short, 20 μL of 2-ethyl butyric acid was added as an internal standard to 500 mg wet digesta (stored at −80°C), after which 400 μL of sodium-chloride solution (25 g/100 mL) and 200 μL of 1:1 sulfuric acid solution was added. The samples were vortexed and after 2 min, 800 μL of diethyl ether was added. Next, the samples were horizontally shaken at 60 rpm for 2 min at 4°C, after which they were centrifuged at 3,000 g for 5 min at 6°C. The ether phase was transferred into vials to measure the SCFA content using an Agilent 6890 Series A GC (Agilent Technologies) equipped with a capillary J&W DB-FFA packed column (30 m x 0.53 mm, film thickness 1.0 μm, Bellefonte, PA, USA) using helium (flow rate 20 mL/min) as carrier gas.

Determination of ileal and caecal microbial endoxylanase activity

Ileal and caecal microbial endoxylanase activity was measured on the digesta extracts using the Xylazyme AX method (Megazyme, Bray, Ireland). Digesta samples ($n = 6$ per treatment and per age group) stored at −80°C were thawed on ice, and 400 mg or 200 mg was mixed with 7 mL or 10 mL phosphate buffer (100 mM, pH 6.5, including 0.02% sodium azide) for the ileum or caeca, respectively. Samples were shaken at 150 rpm for 30 min, centrifuged at 40,000 g for 30 min at 7°C, and the supernatant collected through filtering (Whatman MN 615). Next, duplicates of 1 mL extract were pre-incubated for 10 min at 40°C, then one Xylazyme azurine-cross-linked AX tablet was added to each extract for further incubation at 40°C for 16 h (ileum) or 3 h (caeca). One blank per digesta sample was created by incubating the extract without an AX tablet. A control sample was created by incubating only the phosphate buffer with and without (blank control) an AX tablet to correct for spontaneous colour release. Next, 10 mL Tris-(hydroxymethyl)aminomethane solution (1.0% wt/vol) was added to terminate the reaction and each extract was vortexed, filtered (Whatman MN 615) and transferred to a cuvette. The extinction was measured at 590 nm using air as a

reference (Ultraspec II UV/vis spectrophotometer). Xylanase activity was expressed as activity per h incubation per g digesta, and calculated using the following formula:

$$Xylanase\ activity = \frac{[E590\ sample - E590\ blank\ sample - (E590\ control - E590\ blank\ control)]\ x\ V}{weight\ sample\ (g)\ x\ hours\ of\ incubation}$$

with E590 the absorbance measured at 590 nm, and V the volume of phosphate buffer added in mL.

Determination of plasma D-lactate concentration

The D-lactate concentration in the plasma samples collected at d 37 ($n = 6$ per treatment) was measured using the 'D-lactic acid (D-lactate) rapid assay' of Megazyme. In a cuvette, 750 μL distilled water was mixed with 100 μL plasma sample, 250 μL buffer solution (pH 10), 50 μL dissolved NAD^+ and 10 μL D-Glutamate-pyruvate transaminase suspension. After 3 minutes, the absorbance (A_1) was measured at 340 nm (Ultraspec II UV/vis spectrophotometer). Next, 10 μL of D-Lactate dehydrogenase suspension was added to initiate the reaction. The absorbance of this mixture after the reaction (A_2) was measured after 5 minutes at 340 nm (Ultraspec II UV/vis spectrophotometer). The D-lactate concentration was calculated using the following formula:

$$c = \frac{V \times MW}{\varepsilon \times d \times v} \times \Delta A$$

with c the concentration of D-lactate (g/L), V the final volume (mL), MW the molecular weight of D-lactate (g/mol), ε the extinction coefficient of NADH at 340 nm (mol^{-1} x cm^{-1}), d the light path (cm), v the plasma sample volume (mL) and ΔA the absorbance difference before and after the reaction ($A_2 - A_1$).

Caecal microbiome analysis

DNA for microbiome analysis was extracted from caecal samples stored at −80°C using the QIAmp Powerfecal Pro DNA Kit (Qiagen, Venlo, The Netherlands) ($n = 6$ per treatment and per age group). 250 mg of frozen digesta sample was mixed with 800 μL of CD1 lysis solution in a bead-beating tube and heated in a water bath at 70°C for 5 min, then shaken at 5000 rpm for 3 min. After centrifugation (1 min, 14,000 rpm), the supernatant was mixed with 200 μL CD2 solution and centrifuged (1 min, 14,000 rpm). The remaining supernatant was mixed with 600 μL CD3 solution and filtered through a spin-column with silica membrane via centrifugation (1 min, 14,000 rpm). Next, the membrane was washed consecutively with 500 μL and 200 μL C5 solution, followed by centrifugation (1 and 4 min, 14,000 rpm). Finally, the extracted DNA was collected by adding 50 μL of C6 solution on the membrane and centrifuging for 1 min at 14,000 rpm. The concentration and the quality of the extracted DNA were assessed through spectrophotometry (Nanodrop 2000, Thermo Fisher Scientific, Merelbeke, Belgium). The universal primers 27F: AGAGTTTGATCCTGGCTCAG and 1514R: GNTACCTTGTTACGACTT were used for the amplification of the full-length (V1-V9) 16S rRNA gene through PCR, followed by PacBio sequencing, carried out by Novogene (Munich, Germany). The resulting data was cleaned by splitting according to barcode and filtering, after which amplicon sequence variance analysis using the DADA2 method, and species annotation using QIIME2's classify-sklearn algorithm, were performed. Alpha and beta diversity were studied, and linear discriminant analysis (**LDA**) as well as the relative abundancy on genus and species level were used to investigate differences in microbial composition between treatments.

Statistical analysis

Statistical analysis was performed using JMP Pro 16 Software. Zootechnical performance was measured on pen level with 6 pen replicates per treatment. All chemical analyses were carried out on 6 digesta replicate pools, one pool from each replicate pen, per treatment and per age group. The normality of the data was assessed through a normal quantile plot, and its homoscedasticity through a residuals by predicted plot. Performance, intestinal, digesta and plasma parameters were analysed using a two-way ANOVA with bran particle size, bran inclusion level and their interaction as model effects on the four treatments with a wheat bran inclusion, excluding the control group (no bran inclusion). Significantly different means between bran size and bran level groups were further identified by performing a posthoc Student t-test. In addition, a one-way ANOVA was performed on all the treatments (including the control) followed by a posthoc Dunnett's test to identify significant differences between the treatment means and the mean of the control group. Due to the non-normality of the relative abundance data of the caecal microbiome analysis, significant differences between means of each bran particle size and inclusion level group were identified with a Van der Waerden test per bacterial group, as well as for the difference between each treatment and the control group. Effects and

Table 2
Effect of bran size and inclusion level on BWG (body weight gain), FI (feed intake) and FCR (feed conversion ratio) during the starter (d 1 – 10) and grower phase (d 11 – 37).

Parameter	BWG (g)	FI (g)	FCR[1]
d 1 – 10[2]			
Control	177.80 ± 17.79	187.50 ± 17.64	1.06 ± 0.03
WB452 L2	171.12 ± 20.48	178.77 ± 19.23	1.05 ± 0.04
WB452 L02	172.94 ± 14.82	178.95 ± 12.78	1.04 ± 0.02
WB27 L2	168.63 ± 10.93	176.03 ± 12.28	1.04 ± 0.04
WB27 L02	160.94 ± 9.12	167.81 ± 10.00	1.04 ± 0.02
***P* values**			
Size	0.235	0.239	0.863
Level	0.626	0.490	0.642
Size x Level	0.431	0.471	0.721
d 11 - 37			
Control	2326.99 ± 90.71	3397.95 ± 168.03	1.46 ± 0.03
WB452 L2	2267.27 ± 99.98	3288.80 ± 159.82	1.45 ± 0.03
WB452 L02	2338.09 ± 89.07	3360.68 ± 133.05	1.44 ± 0.03
WB27 L2	2288.75 ± 87.54	3260.60 ± 114.94	1.42 ± 0.02
WB27 L02	2233.42 ± 124.27	3220.23 ± 123.21	1.44 ± 0.03
***P* values**			
Size	0.327	0.138	0.379
Level	0.853	0.776	0.813
Size x Level	0.143	0.317	0.178

$n = 6$ per treatment group per age.
Significant differences with the control group are indicated by a plus sign (Dunnett test, $0.05 \leq P \leq 0.10$).
[1] FCR calculated as the feed intake divided by the body weight gain over a certain period per pen, corrected for mortality.
[2] Day of hatching considered as d 1.

Table 3
Effect of bran size and inclusion level on gizzard weight and pH, average ileal particle size (D50), full caecal lobe weight and caecal D50 on d 10 and d 37.

Parameter	Gizzard		Ileal D50 (μm)	Caeca	
	Empty weight (g)	pH		Weight full lobes (g)	D50 (μm)
d 10					
Control	7.19 ± 0.46	3.42 ± 0.15	529.97 ± 27.40	2.05 ± 0.25	6.64 ± 2.44
WB452 L2	7.15 ± 0.66	3.42 ± 0.08	575.99 ± 53.76	2.13 ± 0.16	**4.62 ± 0.72**$^{+}$
WB452 L02	7.42 ± 0.53	3.29 ± 0.30	564.23 ± 67.98	2.14 ± 0.43	5.22 ± 1.47
WB27 L2	6.86 ± 0.37	3.35 ± 0.14	557.36 ± 75.03	2.17 ± 0.30	4.86 ± 0.55
WB27 L02	7.01 ± 0.40	3.41 ± 0.17	535.65 ± 90.56	2.24 ± 0.31	5.19 ± 1.66
***P* values**					
Size	0.110	0.768	0.438	0.576	0.829
Level	0.313	0.698	0.581	0.748	0.348
Size x Level	0.779	0.234	0.869	0.805	0.785
d 37					
Control	20.31 ± 0.81	3.71 ± 0.36	643.71 ± 64.31	14.27 ± 4.87	11.96 ± 2.61
WB452 L2	22.18 ± 2.90	3.69 ± 0.27	704.98 ± 54.51	14.52 ± 2.49	10.51 ± 2.25
WB452 L02	20.47 ± 2.17	3.82 ± 0.27	661.92 ± 87.31	15.97 ± 3.99	9.07 ± 1.96
WB27 L2	20.74 ± 2.27	4.01 ± 0.61	713.93 ± 32.02	14.37 ± 1.91	10.08 ± 3.69
WB27 L02	22.15 ± 2.82	4.24 ± 0.44	675.35 ± 79.60	18.68 ± 3.50	**8.09 ± 1.82**$^{++}$
***P* values**					
Size	0.912	**0.059**	0.689	0.368	0.502
Level	0.883	0.322	0.154	**0.062**	0.114
Size x Level	0.150	0.780	0.936	0.399	0.797

$n = 6$ per treatment group per age.
P values of significant main and interaction effects are indicated in bold ($P < 0.10$, 2-way ANOVA), significant differences with the control group are indicated by a single ($0.05 \leq P \leq 0.1$) or a double ($P < 0.05$) plus sign (Dunnett test).

differences were considered significant at $P \leq 0.05$ and interpreted as a trend at $0.05 < P \leq 0.10$.

Results

Zootechnical parameters and GIT development

Bran particle size

Bran particle size did not significantly affect body weight gain, feed intake or FCR during the entire trial (Table 2). The effect of bran particle size on GIT development is shown in Table 3. Bran size had a limited effect on these parameters at d 10. Gizzard pH at d 37 tended to decrease for WB452 ($P = 0.059$). No effect of bran size on ileal digesta D50 was observed at d 37.

Bran inclusion level

No significant effect of bran inclusion level was observed on body weight gain, feed intake or FCR during the entire trial (Table 2). Gizzard weight, pH and ileal digesta D50 were also not affected by inclusion level at either age (Table 3). Full caecal lobe weight tended to increase for L02 compared to L2 ($P = 0.062$) at d 37.

Interaction effects of bran particle size and level

No significant interaction effects of bran size and level on performance parameters (Table 2) or GIT development (Table 3) were observed. In comparison to the control, the combination of L2 with WB452 tended to decrease the caecal digesta D50 at d 10 ($P = 0.100$), while the combination of L02 with WB27 decreased this D50 at d 37 ($P = 0.047$).

AX degradation and fermentation

Bran particle size

Bran particle size had a limited effect on the measured parameters of AX degradation and fermentation at d 10 in the ileum (Table 4) and caeca (Table 5). At d 37, WB452 affected AX degradation in the hindgut by increasing the ileal WU-AX digestibility compared to WB27 ($P = 0.018$) and the control group ($P < 0.05$). Although the control group showed a biological unfeasible negative digestibility, the relative comparison of these digestibility values is still informative. WB452 further tended to increase ileal butyric acid contents ($P = 0.073$), especially when included at the highest level compared to the control ($P = 0.031$). Few effects of bran size on caecal fermentation parameters were detected at d 37.

Bran inclusion level

Across both ages, the higher L2 level improved measures of precaecal fibre fermentation compared to L02, such as increased ileal endoxylanase activity at d 10 ($P = 0.075$) and increased ileal butyric acid content at d 37 ($P = 0.010$) (Table 4). However, the lower L02 levels also showed positive effects in a different area, as is tended to improve gut health indicators by increasing caecal rhamnose content ($P = 0.057$) and decreasing plasma D-lactate ($P = 0.053$) compared to L2 at d 37 (Table 5). Caecal microbial endoxylanase activity was not affected by bran inclusion level (Table S1, Supplementary file).

Interaction effects of bran particle size and level

Ileal WU-AX digestibility at d 10 was subject to an interaction effect between bran size and level ($P = 0.050$), with the lowest value observed for WB27 L2 (Table 4). At d 37, the combination of WB27 and L02 tended to decrease ileal propionic acid content ($P = 0.077$). In the caeca, L02 combined with WB27 tended to lower propionic acid contents compared to the control at this age ($P = 0.091$).

Caecal microbiome analysis

Bran particle size and inclusion level did not affect the alpha diversity, beta diversity or LDA scores of the caecal microbiome. Still, the dietary treatments altered the relative abundance of specific genera and species, and the affected bacterial groups are shown for d 10 and d 37 in Table 6 and Table 7, respectively. The complete relative abundance data set on genus and species level can be consulted in the KU Leuven RDR data repository [https://doi.org/10.48804/0WP30M].

Bran particle size

Both bran particle sizes increased the relative abundance of potential beneficial bacterial groups at d 10 (Table 6). For WB27, the abundance of the genera *Oscillibacter* ($P = 0.003$), *Subdoligranulum* ($P = 0.042$) and *Eisenbergiella* ($P = 0.082$) was increased compared to WB452. Meanwhile, WB452 increased the abundance of the *Ruminococcus gauvreauii* group ($P = 0.035$) and *Bacterium ic1296* ($P = 0.035$) compared to WB27 at this age. At d 37, less effect of bran particle size on the caecal microbiome composition was observed.

Bran inclusion level

At d 10, the higher inclusion level L2 tended to increase the relative abundance of the potential beneficial bacteria of the genera *Subdoligranulum* ($P = 0.067$) and *Butyricicoccus* ($P = 0.032$), together with the related species *Butyricicoccus pullicaecorum* ($P = 0.056$) (Table 6), compared to L02. The lower level L02 had a different positive effect by

Table 4
Effect of bran size and inclusion level on WU-AX digestibility (dig), total short-chain fatty acid (SCFA), acetic (AA), propionic (PA) and butyric acid (BA) content, and microbial endoxylanase activity in the ileum on d 10 and d 37.

Parameter	WU-AX dig (%)	Total SCFA (μmol/g)	AA (μmol/g)	PA (μmol/g)	BA (μmol/g)	Endoxylanase activity (E590/g*h)
d 10						
Control	6.52 ± 7.32	1.31 ± 0.79	1.31 ± 0.79	0.00 ± 0.00	ND	0.301 ± 0.018
WB452 L2	12.67 ± 10.10	1.61 ± 0.25	1.61 ± 0.25	0.01 ± 0.03	ND	0.335 ± 0.046
WB452 L02	8.96 ± 7.78	1.52 ± 0.32	1.52 ± 0.32	0.00 ± 0.00	ND	0.290 ± 0.042
WB27 L2	2.68 ± 7.71	1.42 ± 0.74	1.42 ± 0.74	0.00 ± 0.00	ND	0.337 ± 0.061
WB27 L02	12.71 ± 6.27	1.66 ± 0.17	1.65 ± 0.17	0.00 ± 0.00	ND	0.308 ± 0.043
***P* values**						
Size	0.355	0.865	0.865	0.329	/	0.607
Level	0.350	0.673	0.673	0.329	/	**0.075**
Size x Level	**0.050**	0.375	0.357	0.329	/	0.679
d 37						
Control	−3.79 ± 6.95	5.74 ± 1.42	5.61 ± 1.37	0.10 ± 0.05	0.03 ± 0.01	0.329 ± 0.086
WB452 L2	**6.32 ± 4.96**$^{++}$	6.82 ± 2.59	6.61 ± 2.47	0.10 ± 0.06	**0.11 ± 0.09**$^{++}$	0.321 ± 0.052
WB452 L02	**6.60 ± 4.76**$^{++}$	6.68 ± 1.77	6.55 ± 1.67	0.13 ± 0.10	0.02 ± 0.02	0.293 ± 0.075
WB27 L2	0.54 ± 4.21	7.35 ± 2.73	7.16 ± 2.72	0.14 ± 0.05	0.04 ± 0.05	0.287 ± 0.041
WB27 L02	1.77 ± 6.15	6.00 ± 1.09	5.93 ± 1.07	0.06 ± 0.03	0.01 ± 0.01	0.348 ± 0.158
***P* values**						
Size	**0.018**	0.933	0.971	0.620	**0.073**	0.790
Level	0.718	0.407	0.459	0.373	**0.010**	0.668
Size x Level	0.819	0.494	0.503	**0.077**	0.162	0.253

Total SCFA = sum of acetic, propionic and butyric acid.
$n = 6$ per treatment group per age.
P values of significant main and interaction effects are indicated in bold ($P < 0.10$, 2-way ANOVA), significant differences with the control group are indicated by a single ($0.05 \leq P \leq 0.1$) or a double ($P < 0.05$) plus sign (Dunnett test).
ND = not detected.

Table 5
Effect of bran size and inclusion level on rhamnose content, total short-chain fatty acid (SCFA), acetic (AA), propionic (PA), butyric (BA), valeric acid (VA) content in the caeca and plasma D-lactate levels on d 10 and d 37.

Parameter	Rhamnose (%)	Total SCFA (μmol/g)	AA (μmol/g)	PA (μmol/g)	BA (μmol/g)	VA (μmol/g)	Plasma D-lactate (g/L)
d 10							
Control	0.894 ± 0.181	83.96 ± 25.39	61.57 ± 18.32	6.29 ± 2.51	14.91 ± 6.97	1.18 ± 0.25	NM
WB452 L2	0.908 ± 0.078	83.85 ± 16.08	59.32 ± 12.48	5.64 ± 1.64	17.55 ± 4.85	1.35 ± 0.18	NM
WB452 L02	0.995 ± 0.213	79.68 ± 14.99	57.25 ± 11.11	5.28 ± 3.15	16.09 ± 4.60	1.07 ± 0.40	NM
WB27 L2	0.935 ± 0.160	77.23 ± 7.54	58.28 ± 5.99	4.00 ± 1.03	13.94 ± 4.54	1.02 ± 0.30	NM
WB27 L02	0.982 ± 0.171	72.54 ± 12.04	51.47 ± 9.00	5.76 ± 2.74	14.31 ± 2.35	1.00 ± 0.27	NM
***P* values**							
Size	0.721	0.212	0.412	0.543	0.133	0.114	/
Level	0.531	0.416	0.288	0.466	0.756	0.228	/
Size x Level	0.864	0.961	0.566	0.271	0.599	0.312	/
d 37							
Control	1.002 ± 0.305	95.45 ± 23.98	67.29 ± 19.73	12.29 ± 4.19	14.34 ± 5.57	1.53 ± 0.33	0.033 ± 0.021
WB452 L2	0.994 ± 0.158	96.52 ± 20.98	68.74 ± 13.53	11.17 ± 4.73	15.19 ± 5.63	1.41 ± 0.21	0.023 ± 0.007
WB452 L02	1.192 ± 0.425	99.63 ± 8.04	69.37 ± 6.32	8.61 ± 1.93	20.15 ± 2.31	1.50 ± 0.17	0.020 ± 0.008
WB27 L2	0.929 ± 0.054	94.51 ± 16.10	65.63 ± 10.69	8.22 ± 1.62	19.25 ± 5.02	1.42 ± 0.16	0.036 ± 0.018
WB27 L02	1.242 ± 0.419	95.11 ± 21.56	67.80 ± 15.46	**7.83 ± 3.07**$^{+}$	17.93 ± 5.06	1.55 ± 0.41	0.021 ± 0.010
***P* values**							
Size	0.951	0.654	0.638	0.154	0.637	0.779	0.171
Level	**0.057**	0.798	0.779	0.255	0.350	0.318	**0.053**
Size x Level	0.654	0.862	0.877	0.340	0.117	0.817	0.251

Total SCFA = sum of acetic, propionic, butyric and valeric acid.
$n = 6$ per treatment group per age.
P values of significant main and interaction effects are indicated in bold ($P < 0.10$, 2-way ANOVA), significant differences with the control group are indicated by a single ($0.05 \leq P \leq 0.1$) or a double ($P < 0.05$) plus sign (Dunnett test).
NM = not measured.

decreasing the abundance of the pathogenic *Escherichia-Shigella* compared to L2 ($P = 0.041$). At d 37, little effect of inclusion level on the microbiome composition was observed.

Interaction effects of bran particle size and level

The interaction of WB27 with the different inclusion levels positively altered the abundance of several bacterial groups in the caeca at d 10. Combined with L2, it led to the highest abundance of *Subdoligranulum* ($P = 0.052$) and tended to increase the abundance of *Eisenbergiella* compared to the control group ($P = 0.050$). Its combination with L02 resulted in the highest abundance of *Oscillibacter* ($P = 0.029$) and the lowest abundance of *Escherichia-Shigella* ($P = 0.077$). The interaction of WB452 with these inclusion levels tended to affect the abundance *Sellimonas* ($P = 0.097$), which was the highest when combined with L02 and the lowest for L2. At d 37, WB27 combined with L2 increased the relative abundance of the genus *Defluviitaleaceae UCG-011* ($P = 0.022$), while WB452 L02 resulted in the lowest abundance of the species *Lactobacillus reuteri* ($P = 0.097$) (Table 7).

Discussion

The different impact of coarse milled and cryogenic milled wheat

Table 6
Relative abundance of genera and species affected by dietary treatment in the caecal microbiome at d 10.

d10	Relative abundance on genus and species level (%)								
	Butyricicoccus	*Oscillibacter*	*Subdoligranulum*	*Ruminococcus gauvreauii* group	*Sellimonas*	*Eisenbergiella*	*Escherichia-Shigella*	*Bacterium ic1296*	*Butyricicoccoccus pullicaecorum*
Control	0.0248 ± 0.0270	0.0019 ± 0.0032	0.0097 ± 0.0128	0.0004 ± 0.0009	0.0035± 0.0047	0.0005± 0.0009	0.0004 ± 0.0009	0.0004 ± 0.0009	0.0074 ± 0.0165
WB452 L2	0.0170 ± 0.0160	0.0002 ± 0.0004	0.0065 ± 0.0059	0.0005 ± 0.0009	0.0002 ± 0.0004	0.0019 ± 0.0017	0.0060 ± 0.0146	0.0005 ± 0.0009	0.0083 ± 0.0108
WB452 L02	0.0100± 0.0119	0.0002 ± 0.0004	0.0037 ± 0.0040	0.0005 ± 0.0009	0.0063 ± 0.0084	0.0012 ± 0.0022	0.0002 ± 0.0004	0.0005 ± 0.0009	0.0012 ± 0.0014
WB27 L2	0.0272± 0.0275	0.0023 ± 0.0020	0.0165 ± 0.0117	0.0000 ± 0.0000	0.0026 ± 0.0025	**0.0056 ± 0.0049**$^{+}$	0.0081 ± 0.0088	0.0000 ± 0.0000	0.0081 ± 0.0119
WB27 L02	0.0070± 0.0053	0.0025 ± 0.0035	0.0070 ± 0.0055	0.0000 ± 0.0000	0.0011 ± 0.0018	0.0042 ± 0.0058	0.0000 ± 0.0000	0.0000 ± 0.0000	0.0016 ± 0.0025
***P* values**									
Size	0.689	**0.003**	**0.042**	**0.035**	0.994	**0.082**	0.427	**0.035**	0.803
Level	**0.032**	0.986	0.067	1.000	0.411	0.340	**0.041**	1.000	**0.095**
Size x Level	0.179	**0.029**	**0.052**	0.215	**0.097**	0.265	**0.077**	0.215	0.413

$n = 6$ per treatment group per age.
P values of significant tendencies in the main and interaction effects are indicated in bold ($P < 0.10$, Van der Waerden test), significant differences with the control group are indicated by a plus sign ($P < 0.10$, Van der Waerden test).

Table 7
Relative abundance of genera and species affected by dietary treatment in the caecal microbiome at d 37.

d 37	Relative abundance on genus and species level (%)	
	Defluviitalaeaceae UCG	*Lactobacillus reuteri*
Control	0.0000 ± 0.0000	0.0030 ± 0.0027
WB452 L2	0.0000 ± 0.0000	0.0042 ± 0.0054
WB452 L02	0.0000 ± 0.0000	0.0005 ± 0.0013
WB27 L2	0.0007 ± 0.0009	0.0014 ± 0.0013
WB27 L02	0.0000 ± 0.0000	0.0023 ± 0.0018
***P* values**		
Size	**0.073**	0.591
Level	**0.073**	0.331
Size x Level	**0.022**	**0.097**

$n = 6$ per treatment group per age.
P values of significant tendencies in the main and interaction effects are indicated in bold ($P < 0.10$, Van der Waerden test).

bran and its inclusion level on performance, gut health, precaecal fibre degradation and caecal fermentation was studied as a function of broiler age. Bran size and inclusion level had a limited effect on performance parameters, possibly related to the relatively low inclusion levels used in this trial, as no negative consequences on performance are expected from fibre inclusions below 4% (Kheravii et al., 2018). Compared to the Ross 308 reference (2022), the FCR in this experiment was similar during the starter phase and improved during the grower phase. This illustrates that further enhancement in Ross 308 broiler performance through low inclusions of feed additives may be difficult to obtain when reared under optimal environmental conditions using appropriate diet formulations (Aviagen, 2022; Martínez and Valdivié, 2021). The effect of fibre inclusions on performance and gut health may be more pronounced when using a dietary or infection challenge, although this is outside of the scope of the current study (Sadeghi *et al.*, 2020; Vermeulen et al., 2016; Woyengo et al., 2023). However, bran size and level did affect GIT development, AX digestibility and caecal microbial composition.

Coarser bran stimulates precaecal fibre degradation

The increase of ileal WU-AX digestibility by the coarser WB452 demonstrates its positive effect on precaecal fibre degradation and fermentation at d 37. The obtained negative digestibility for the control group (Table 4) is biologically unfeasible and can be assigned to differences in separation and accumulation of the TiO_2 marker and digesta fractions (Bautil et al., 2019; De Vries et al., 2014). More specifically, if this marker is not completely inert as previously assumed, the decrease in marker concentration in the digesta caused by in vivo losses results in an increase of the right-hand term of the equation discussed in Section 2.7, leading to a final negative result if this term exceeds the value of 1. The potential non-inertness of this marker is supported by previous reports of negative digestibility values using this method, and observations that addition of TiO_2 alters the caecal SCFA and microbial composition in vitro (Bautil et al., 2019; Kiani, 2025). Alternative methods to measure (fibre) digestibility include the use of Cr_2O_3, ash or acid-insoluble ash as a marker, although similar concerns have been raised on their use in broilers (De Vries et al., 2014; Sales and Janssens, 2003). Despite these concerns about the absolute values of the obtained digestibility results, the relative comparison of these values remains valid. The increased AX digestibility for WB452 can be explained by the stimulation of the gizzard, demonstrated by the decrease in gizzard pH for this bran size, leading to a stronger acid dissolution of the fibre fraction in the foregut. This solubilising effect of low pH environments on fibre-rich substrates has also been observed in vitro, where the WE-AX% increased for wheat bran incubated at pH 2 compared to pH 4 to 8, without any enzyme additions (unpublished data). The described positive relationship between coarser fibre inclusions, gizzard functioning and diet digestibility aligns with previous research (Amerah et al., 2008; Kheravii et al., 2017, 2018; Xu et al., 2015). It can be hypothesised that an increase in gizzard retention time caused by coarse inclusions in this low pH environment can further have contributed to fibre dissolution, although retention time was not measured in the current study (Amerah et al., 2008; Kheravii et al., 2018; Svihus, 2011). Coarse particles can also enhance digesta particle size reduction through stimulating gizzard grinding, but this seemed of minor importance compared to the pH decrease, as no effect of bran size on ileal digesta D50 was observed. Detecting differences in ileal digesta D50 caused by coarse inclusions can be impeded by their varying effect in the gizzard, as they can directly increase the ileal digesta particle size by passing through the gizzard without being extensively ground, but also indirectly decrease digesta particle size by stimulating gizzard development and the extent of its grinding (Amerah et al., 2007; Ghimire et al., 2024; Hetland et al., 2002; Svihus et al., 1997; Vanderghinste et al., 2025b; Xu et al., 2015). Finally, the increase in ileal butyric acid for WB452 at d 37 confirms an early start of beneficial fibre fermentation in the hindgut due to the improved feed degradation in the foregut. The possible reflux of caecal material into the ileum could also explain this increase, but

consensus on the impact of this in broilers has yet to be reached (Svihus et al., 2013). These effects of bran size show that the gizzard stimulation by coarser bran, rather than the increased accessibility of fine bran, can improve fibre degradation and fermentation in the precaecal tract.

A low bran inclusion level benefits caecal functioning and gut health

L02 induced several desired effects in the caeca at d 37, such as a decreased caecal D50 in combination with WB27, increased full caecal lobe weights and caecal rhamnose content, and decreased plasma D-lactate levels. Rhamnose can indicate the presence of *Lactobacillus* species, and is important in host-microbiome interactions and resulting immune responses (Mistou *et al.*, 2016; Vinogradov et al., 2016), while lower plasma D-lactate levels imply improved gut integrity (Ducatelle et al., 2018). The decrease in pathogenic *Escherichia-Shigella* abundance for L02 at d 10, and the higher abundance of the beneficial genera *Oscillibacter* and *Sellimonas* when combined with WB27 and WB452, respectively, further confirms its beneficial effect on gut health. On the other hand, L2 also stimulated several SCFA-producing genera, such as *Subdoligranulum* and *Butyricicoccus,* compared to L02 at this age, and W452 L02 showed the lowest abundance of probiotic *Lactobacillus reuteri* at d 37 (Burrows et al., 2025; Eeckhaut et al., 2011; Gonmei et al., 2019; Liu et al., 2021a; Mu et al., 2018). The overall positive effects of L02 compared to L2 in the caeca oppose the hypothesis that increasing the inclusion level of fermentable fibre would lead to an increase in caecal fibre fermentation and the following health benefits. These positive effects of L02 seem limited to the caeca, as this level did not stimulate other fibre degradation parameters and even decreased ileal endoxylanase activity at d 10 compared to L2. The slightly lower presence of particle-size-reduced wheat bran in the ileum for L02 compared to L2 may explain this, decreasing the expected stimulating effect of partially fermentable fibre in young broilers in the precaecal tract. As the caecal lobes have a more complex filling and emptying mechanism, the positive effects of the lower compared to the higher inclusion level on caecal functioning can be linked to the previously described phenomenon of overfilling of the caeca in young broilers. Here, a high inclusion level (10%) of 100 μm wheat bran induced the undesired increase in caecal D50 and decrease in SCFA content, attributed to the influx of too many relatively large fibre particles with respect to the microbial fermentative capacity (Vanderghinste et al., 2025a, 2025b). Even the 27 μm bran used in the current study is still larger than the D50 found in the caeca at both studied ages (5 to 12 μm), which may explain why a lower bran inclusion seems more beneficial on caecal level. This further aligns with previous positive effects of lower fibre inclusion levels (< 1 to 5%) on caecal fermentation compared to higher levels (Boguslawska-Tryk et al., 2015; Fenna and Boag, 1973; Itani et al., 2024; Röhe et al., 2020; Tejeda and Kim, 2021). The uncertainty on how much of the initially included fibre fractions enter the caecal lobes still impedes a complete understanding of the effect of inclusion level on caecal fermentation (Vanderghinste et al., 2025a). The observed benefits of L02 are also remarkable when comparing the amount of included bran at this low level to the fraction of wheat inherently present in the diet (58 to 64%, Table 1), of which 10 to 14% is also bran (Khalid *et al.*, 2023). A first hypothetical explanation for the effects of a low inclusion of additional bran in this wheat matrix can be the induced physical differences between the milled bran inclusions and the bran originating from the dietary wheat fraction. Milling techniques can impact the shape, particle size distribution and extent of physical breakdown of the end product differently, as described for wheat bran, whole wheat and teff flour (Barretto *et al.*, 2022; De Bondt et al., 2020; Navarro *et al.*, 2020). As the bran inclusions were subjected to a greater and different type of particle size reduction by the cyclonic or cryogenic milling compared to the hammer milling used on the dietary wheat fraction, this can have impacted the bran's susceptibility to fermentation. A second hypothesis is the effect of the structural difference between bran inclusions and the bran originating from the dietary wheat fraction on their fermentability. The latter is typically still adhered to other wheat segments, such as the endosperm and the germ, while the included bran already provides a purer fibre fraction at the start of digestion, possibly stimulating early fibre degradation. The more pure occurrence of bran inclusions compared to wheat-intrinsic bran was also utilised in a previous study of Vanderghinste et al., 2025b) to identify the origin of exceptionally large particles (> 100 μm) present in caecal digesta, which seemed to be isolated pericarp and aleurone layers belonging to the included wheat bran fraction. The hypothesised stimulation of fermentation through the more pure bran of the inclusions is supported by in vitro studies showing differences in fermentation outcomes depending on the bran fraction, but requires more research through the comparison of the fermentability of wheat and bran fractions, possibly in vitro (De Paepe et al., 2019; Stewart and Slavin, 2009). So, it is hypothesised that the included bran may provide a better substrate for fermentation than the wheat bran inherently present in the diet due to differences in physical structure, shape and breakdown through milling. Together with the low inclusion levels required by the complex caecal influx mechanism, this can explain the observed effects of low bran inclusion levels in wheat-based diets.

Both bran sizes can stimulate the caecal fibre-fermenting microbiome

The absence of an effect of bran size on caecal SCFA levels at both studied ages contrasts with previous in vitro and in vivo studies indicating that increasing fine material in the caeca could boost fibre fermentation (Stewart and Slavin, 2009; Tuncil et al., 2018; Vanderghinste et al., 2025b; Vermeulen et al., 2016). These studies also report an increase in propionic acid for fine fibre inclusions, in contrast to the observed decreases in both the ileum and caeca for WB27 L02 at d 37 compared to the control group here. This treatment also resulted in the smallest caecal D50 at d 37. Studies on human fecal and soil samples have shown that particle size and the composition of the associated microbial community are closely related, which implies that this can further lead to differences in microbial fermentation products (Bach et al., 2018; Fan et al., 2025; Letourneau *et al.*, 2024). Together with the assumed positive effect of a low inclusion level on caecal influx as discussed above, WB27 L02 may have provided a sufficiently different substrate at an optimal level to stimulate bacterial species in the caeca that diverge from those in the other treatments, and especially from those in the control group receiving no additional wheat bran. However, this hypothesis could not be confirmed by corresponding changes in the caecal microbiome composition at d 37. This specific combination of WB27 and L02 did result in the highest abundance of *Oscillibacter* at d 10, and not d 37, which has been previously described in a negative correlation with propionic acid in humans, possibly through its conversion into other SCFA by this genus (Boets et al., 2017; Martínez-Cuesta et al., 2021). Future studies focusing on the effect of particle size and shape on microbial fermentation, possibly in vitro to reduce the variability coming from SCFA uptake by the host, may aid in a more direct interpretation of these results. Microbial analysis can also provide extra depth for caecal fibre fermentation and gut health measurements to overcome the difficulties in measuring consistent changes in SCFA content, partly caused by these cross-feeding dynamics and uptake by the host (Boets et al., 2017; González-Ortiz et al., 2019; Liu et al., 2021a). In the current study, the abundance of multiple different SCFA-producing bacterial groups was increased upon inclusion of both WB27 and WB452 at d 10, such as the genera *Subdoligranulum, Oscillibacter* and *Eisenbergiella* for the former (Burrows et al., 2025; Hou et al., 2016; Jia et al., 2024; Polansky et al., 2016), and *Ruminococcus* and *Bacterium ic1296* species for the latter (Liu et al., 2021b; Xiao et al., 2017). The combination of WB452 and L02 further increased the abundance of *Sellimonas* genus, a biomarker for gut homeostasis (Liu et al., 2025; Muñoz et al., 2020). The increase in the abundance of caecal SCFA-producing bacteria indicates that both bran fractions can stimulate different types of fibre fermentation depending on their particle size, despite the lack of response in caecal SCFA profiles and microbial

diversity measurements. Further research should clarify whether this particle size effect is induced by precaecal stimulation of the gizzard, the physical limitations of caecal influx, or the preference of certain bacterial groups for a specific fibre size inside the caecal lobes (De Paepe et al., 2019; Kheravii et al., 2018; Vanderghinste et al., 2025a; Vermeulen et al., 2016). At d 37, the enrichment of bacterial groups by the included bran sizes was limited and inconclusive, as WB27 L2 increased the abundance of *Defluviitaleaceae UCG-011*, a beneficial genus regarding host metabolism, while the same inclusion level of WB452 stimulated *Lactobacillus reuteri*, a species known for its probiotic potential (Gonmei et al., 2019; Kang et al., 2023; Mu et al., 2018; Zhang et al., 2025). The stimulation of SCFA-producing bacteria by both bran inclusions in the caeca at d 10 can indicate that boosting the fibre-fermenting microbiome in the caeca through fibre inclusions is especially important early in the broiler's life, as reported before for prebiotic fibre-derived oligosaccharide (Bautil et al., 2020). The caecal microbiome density and composition are further expected to stabilise after the first two weeks of life, creating a bigger opportunity to steer these early in life and explaining the observed age effect in the current study (Apajalahti *et al.*, 2004; Van Der Wielen et al., 2002). To conclude, both the fine and coarser bran inclusions positively stimulated the caecal microbiome at d 10, showing the potential to improve fibre fermentation and possibly gut health through different mechanisms.

The inconsistency of changes in relative microbial abundance and in caecal SCFA contents, together with the variable effects of inclusion level and bran particle size per age and GIT section, may imply that the prebiotic potential of the wheat bran inclusions was not fully reached within the settings of this study. The variability of the inclusion level effect further emphasises the importance of adjusting fibre inclusion levels to the targeted GIT section and broiler age. Fibre inclusions with a higher fermentability, such as AXOS, or the combination with fibre-degrading enzymes, may further improve fibre fermentation responses, although these strategies are often more expensive than the inclusion of milled wheat bran (Bautil et al., 2020, 2021; Bedford and Apajalahti, 2022; Singh et al., 2021)

Conclusion

This study compared the effect of coarse milled (452 μm) and cryogenically milled (27 μm) wheat bran at varying feed inclusion levels on the degradation and fermentation of fibre in growing broilers. Finer bran was expected to increase fibre degradation and fermentation, but this was improved by coarser bran on the precaecal level instead, through the stimulation of the gizzard. In addition, both bran sizes increased the abundance of different SCFA-producing bacteria in the caeca, which demonstrated their positive effects on the fibre-fermenting potential of the microbiome through different physiological mechanisms. The hypothesised positive effects of particle-size-reduced bran on fermentation were, however, not reflected in caecal SCFA content. The effect of inclusion level was most clear on caecal level, where the lower level (0.2%) stimulated caecal functioning and gut health indicators compared to the control and the higher inclusion level (2%), contrasting the hypothesis that increased fibre levels lead to increased caecal influx and fermentation. The results from this study highlight the additional importance of stimulating the gizzard and consequently the precaecal fibre degradation and the resulting fibre fermentation with coarser bran, aside from the expected positive effects of particle-size-reduced bran on fibre fermentation. For ideal stimulation of fibre degradation and fermentation, the inclusion of wheat bran with a particle size and level adapted to the targeted GIT part and the broiler's age seems to be the most advantageous to study in further experiments.

List of abbreviations

D50, volumetric average particle size; FCR, feed conversion ratio; GC, gas chromatography; GIT, gastrointestinal tract; L02, 0.2% inclusion level; L2, 2% inclusion level; LDA, linear discriminant analysis; SCFA, short-chain fatty acids; TOT-AX, total arabinoxylan; WB27, 27 μm wheat bran; WB452, 452 μm wheat bran; WE-AX, water-extractable arabinoxylan; WU-AX, water-unextractable arabinoxylan

Data availability

The dataset supporting the conclusions of this article is available in the KU Leuven RDR repository [https://doi.org/10.48804/0WP30M].

CRediT authorship contribution statement

Paulien Vanderghinste: Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Conceptualization. **An Bautil:** Writing – review & editing, Validation, Supervision, Conceptualization. **Steven J. Simmonds:** Writing – review & editing, Supervision. **Michael R. Bedford:** Writing – review & editing, Conceptualization. **Gemma González-Ortiz:** Writing – review & editing, Conceptualization. **Christophe M. Courtin:** Writing – review & editing, Validation, Supervision, Conceptualization.

Disclosures

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Supplementary materials

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