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Original Research Article

Hulless barley and β-glucanase affect ileal digesta soluble beta-glucan molecular weight and digestive tract characteristics of coccidiosis-vaccinated broilers☆

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ABSTRACT

Exogenous β-glucanase (BGase) in barley-based feed has been shown to reduce digesta viscosity in chickens, and thereby improve performance. Less well studied is the potential for BGase to convert barley β-glucan into low molecular weight carbohydrates, which might influence digestive tract function and enteric disease. Coccidiosis-vaccinated broiler chickens were fed graded levels of hulless barley (HB) and BGase to determine their effects on β-glucan depolymerization and digestive tract characteristics. Broilers were fed high β-glucan HB (0%, 30% and 60% replacing wheat) and BGase (0%, 0.01% and 0.1%) in a 3 × 3 factorial arrangement. A total of 5,346 broilers were raised in litter floor pens and vaccinated for coccidiosis on d 5. Each treatment was assigned to 1 pen in each of 9 rooms. The significance level was set at $P \leq 0.05$. At both 11 and 33 d of broiler ages, peak molecular weight of β-glucan in ileal digesta decreased with increasing BGase for 30% and 60% HB. The maximum molecular weight for the smallest 10% β-glucan molecules (MW-10%) decreased with BGase at both ages for 30% and 60% HB; for birds fed 0% HB, only 0.1% BGase decreased MW-10%. The 0.1% BGase increased caecal short chain fatty acids (SCFA) compared to the 0.01% BGase at d 11 only for the 60% HB. Ileal pH increased with increasing HB and BGase at d 11 and 33. Caecal pH was lower for 0.1% BGase than 0% BGase for 60% HB at d 11. Relative mRNA expression of interleukin 6 (*IL-6*) and *IL-8* in the ileum increased with 0.1% BGase at d 11 and 33, respectively, whereas expression of ileal mucin 2 (*MUC2*) decreased with 0.1% BGase at d 33. In the caeca, interactions between HB and BGase were significant for monocarboxylate transporter 1 (*MCT1*) and mucin 5AC (*MUC5AC*) on d 11, but no treatment effects were found at d 33. In conclusion, BGase depolymerized high molecular weight β-glucan in HB in a dose-dependent manner. Hulless barley and BGase did not increase SCFA concentrations (except for 60% HB with 0.1% BGase at d 11) and caused minor effects on digestive tract histomorphological measurements and relative mRNA gene expression.

1. Introduction

Antibiotics have been commonly used in the feed at sub-therapeutic levels to control gastro-intestinal (GI) disease in broiler chickens for many decades, but their use has been reduced in many countries around the world with the growing awareness of antibiotic resistance issues (Garcia-Migura et al., 2014; Kaesbohrer et al., 2012). Alternative strategies to mitigate the increasing prevalence of enteric diseases with the reduction of prophylactic antibiotics include adherence to strict bio-security measures and

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vaccination protocols (Hoelzer et al., 2018; Mehdi et al., 2018). Further, the evaluation of feed additives as alternative products to antibiotics has also become a prominent area of research to control the increased susceptibility to infectious diseases that cause a substantial economic loss to the poultry industry (Diarra and Malouin, 2014; Suresh et al., 2018).

The use of prebiotics has been extensively studied as an alternative strategy to mitigate the adverse effects of reduced antibiotic use in the poultry industry. Recently the International Scientific Association of Probiotics and Prebiotics defined a prebiotic as "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017). Prebiotics may improve digestive tract characteristics and production performance in poultry through different mechanisms. These include promoting growth and metabolism of host microorganisms capable of competitive exclusion of pathogenic bacteria by competing for the sites of attachment in the host (Baurhoo et al., 2007; Corrigan et al., 2015; Ofek and Beachey, 1978), improving GI morphological structure (Ding et al., 2018; Shang et al., 2015), producing antimicrobial factors (Chen et al., 2007; Muñoz et al., 2012) and modulating the host immune system (Babu et al., 2012; Huang et al., 2015).

Commonly studied prebiotics in chickens are fructo-oligosaccharides, mannan-oligosaccharides, arabinoxylo-oligosaccharides, and xylo-oligosaccharides (Patterson and Burkholder, 2003). A common characteristic of prebiotics is that they are not digestible by chickens and, as a result, are potentially fermentable in the lower gastro-intestinal tract (GIT) (Gaggìa et al., 2010). Through their metabolism and subsequent production of fermentation products, they exert prebiotic properties by modifying the GI microbial population and epithelial integrity and stimulating the immune system through modulation of cytokine production (Pourabedin and Zhao, 2015). Most prebiotics tested in broiler diets are fed in the extracted form (often oligosaccharides) at specific doses (2 to 10 g/kg), but potential prebiotics also exists in common feed ingredients. For example, feeding arabinoxylo-oligosaccharides and xylo-oligosaccharides in wheat-based diets has resulted in modification of GIT microbiota (Courtin et al., 2008; Pourabedin et al., 2015), increased short chain fatty acids (SCFA) production including butyric, acetic and propionic acids (Keerqin et al., 2017; Yuan et al., 2018), improved intestinal epithelial morphology (De Maesschalck et al., 2015; Ding et al., 2018) and down-regulation of the pro-inflammatory response in chickens (Yuan et al., 2018). These oligosaccharides can also be formed by enzymatic depolymerization of arabinoxylan found in cereal grains and thereby produce similar effects to those found by adding purified oligosaccharides (Courtin et al., 2008; Lee et al., 2017; Masey-O'neill et al., 2014). The same principle can be used to project the prebiotic effects of other fiber types such as β-glucan, which is also found in cereal grains.

It is common practice to use exogenous β-glucanase (BGase) in barley-based poultry feed to reduce β-glucan-induced digesta viscosity (Fuente et al., 1995; Karunaratne et al., 2017a; Salih et al., 1991) and mitigate the adverse effects associated with viscosity. In addition to reducing these negative effects on digestibility and bird performance, dietary BGase also has been found to modify the microbial population in the GIT of chickens (Kaldhusdal and Hofshagen, 1992; Mathlouthi et al., 2002). Enzyme use has also been observed to affect bacterial fermentation (SCFA level), and GI pH, which are considered to be factors affecting digestive tract microbial populations (Józefiak et al., 2006, 2010). However, the effects of exogenous BGase on carbohydrate fermentation in broilers fed barley-based diets have not been consistent (Józefiak et al., 2005, 2006), possibly due to variation in grain β-glucan characteristics and BGase source (purity, dose). The research using exogenous BGase in poultry suggests that barley β-glucan may act as a prebiotic, but the evidence is not definitive.

A prebiotic effect of cereal β-glucan has been demonstrated in mammalian species (Metzler-Zebeli and Zebeli, 2013; Queenan et al., 2007; Shen et al., 2012), and almost complete digestion of cereal β-glucan at the end of the digestive tract has been shown in pigs (Bach Knudsen and Hansen, 1991; Fadel et al., 1988). However, extrapolation of these findings to chickens is ill-advised because of major differences in digestive tract anatomy and bacterial fermentation capacity. Further, information is lacking on the degree of β-glucan depolymerization in the digestive tract, which is accomplished by enzyme use in chickens and how this affects fermentation and other digestive tract characteristics. Moreover, most of the studies on enzyme use in barley diets have used mixed enzyme sources which contains both BGase and xylanase activities at a similarly high level, and there is minimal research using purified feed BGase to study the digestive tract characteristics in broiler chickens (Dos Santos et al., 2013; Karunaratne et al., 2017b).

The objective of the study was to evaluate the effects of diet hulless barley (HB) and BGase levels on ileal digesta soluble β-glucan molecular weight distribution and digestive tract characteristics of broiler chickens vaccinated for coccidiosis. It was hypothesized that the level of exogenous BGase would correlate with the degree of β-glucan depolymerization and the production of low molecular weight β-glucan in the digestive tract of broiler chickens. In turn, these changes will increase carbohydrate fermentation and affect other digestive tract characteristics.

2. Materials and methods

The experiment was approved by the Animal Research Ethics Board of the University of Saskatchewan and completed according to the Canadian Council on Animal Care guidelines for humane animal use (Canadian Council on Animal Care, 1993, 2009).

2.1. Experimental diets

The experiment was designed as a 3 × 3 factorial arrangement based on diet HB (cultivar - CDC Fibar; 0%, 30% and 60%) and BGase (Econase GT 200 P from ABVista, Wiltshire, UK; 0%, 0.01% and 0.1%) levels. The calculated BGase activities in diets were 0, 20,000 and 200,000 BU/kg for the 0%, 0.01% and 0.1% levels, respectively. Hulless barley, which contained 8.7% β-glucan, replaced wheat (Shaw; 93.8%; AC Domain 6.2%) in each experimental diet based on the assumption that the nutrient content of these grains was similar. Starter diets were fed from d 0 to 11, and grower diets were supplied from d 11 to the end of the study. The ingredients and calculated nutrient levels are shown in Table 1. Diet formulation was completed according to Ross 308 broiler nutrition specifications (Aviagen 2014). The starter diets were made in crumble form. The grower diets were given in a crumble form initially and then switched to a pellet form. The pelleting temperature was retained between 70 and 75 °C during feed processing to prevent BGase inactivation. Beta-glucanase (EC 3.2.1.6) and xylanase activities (EC 3.2.1.8) of the diets were analyzed using AB Vista methods of ESC Standard Analytical Method SAM042-01 and SAM038, respectively. Xylanase activity was not detected in the diets (<2,000 U/kg), and BGase activities approximated the calculated enzyme activity values (average values for the grower diets: 0% BGase − 16,267 BU/kg; 0.01% BGase − 46,333 BU/kg; 0.1% BGase − 296,033 BU/kg).

2.2. Birds and housing

A total of 5,346 newly hatched (Ross × Ross 308) broiler chickens were obtained from a commercial hatchery and randomly

Table 1
Ingredients and nutrient levels of starter and grower diets (as-is basis, %).

Item	Starter	Grower
Ingredients		
Cereal grain (wheat and hulless barley)	59.09	64.80
Soybean meal	32.97	26.93
Canola oil	3.29	4.03
Mono-dicalcium phosphate	1.40	1.20
Limestone	1.64	1.52
Sodium chloride	0.43	0.38
Vitamin-mineral broiler premix[1]	0.50	0.50
Choline chloride	0.10	0.10
DL-Methionine	0.30	0.27
L-Threonine	0.07	0.05
L-Lysine HCl	0.21	0.22
Nutrient level (calculated)		
AME, MJ/kg	12.56	12.97
Crude protein	23.46	21.24
Crude fat	4.74	5.57
Calcium	0.96	0.87
Chloride	0.38	0.36
Non-phytate phosphorous	0.48	0.44
Potassium	0.92	0.83
Sodium	0.20	0.18
Digestible arginine	1.50	1.35
Digestible isoleucine	0.90	0.81
Digestible leucine	1.61	1.47
Digestible lysine	1.28	1.15
Digestible methionine	0.60	0.54
Digestible methionine and cysteine	0.95	0.87
Digestible threonine	0.86	0.77
Digestible tryptophan	0.27	0.24
Digestible valine	0.96	0.87

[1] Vitamin-mineral premix provided the following per kilogram of complete diet: vitamin A, 11,000 IU; vitamin D, 2,200 IU; vitamin E, 30 IU; menadione, 2 mg; thiamine, 1.5 mg; riboflavin, 6 mg; pyridoxine, 4 mg; vitamin B_{12}, 0.02 mg; niacin, 60 mg; pantothenic acid, 10 mg; folic acid, 0.6 mg; biotin, 0.15 mg; copper, 10 mg; iron, 80 mg; manganese, 80 mg; iodine, 0.8 mg; zinc, 80 mg; selenium, 0.3 mg; calcium carbonate, 500 mg; ethoxyquin, 0.63 mg; wheat middlings, 3,773 mg.

placed (33 males and 33 females per pen) in 81 floor pens (2.3-m length and 2-m width) in 9 environmentally controlled rooms on d 0. Each of the 9 dietary treatments was randomly assigned to 1 pen per room, giving 9 replications per treatment. An equal amount of straw was placed in each pen at an approximate initial thickness of 7.5 to 10 cm. Room temperature was 33 °C on d 0, and then gradually decreased until it was 21 °C by d 25. Day length was 23 h at the trial start, and it was gradually reduced to 17 h by d 12. Light intensity was 20 lx at the beginning of the trial and gradually reduced to 10 lx by d 10. Each pen was supplied with a tube feeder having a pan diameter of 36 (0 to 25 d) or 43 cm (>25 d) to provide ad libitum feed. Each pen was provided with a height-adjustable nipple drinker, and each drinker contained 6 nipple drinkers (Lubing). Supplementary feed and water were provided to each pen using a cardboard egg tray and an ice cube tray from d 0 to 7 to assist chicks getting feed and water.

2.3. Coccidiosis vaccination

Coccidiosis vaccination was completed to evaluate the effects of HB and BGase levels on the digestive tract characteristics under a disease-challenge condition that might affect microbiota in the digestive tract in chickens. All the birds were vaccinated with the Coccivac B-52 live vaccine (Merck Animal Health, Madison, NJ). The vaccine comprises oocysts of *Eimeria acervulina, E. mivati, E. maxima, E. maxima* MFP and *E. tenella*. Vaccination was completed at 5 d of age to enable uniform intake of oocysts by spraying diluted vaccine (1,000 doses in 500-mL distilled water) onto 1 egg tray containing feed and 1 ice cube tray containing water in each pen (1.3 × recommended dose). Access to feed and water was removed from each pen before starting vaccination and returned when the vaccine containing supplementary feed and water were consumed. A Kraft brown paper strip (Model S-8511S, ULINE Canada, Milton, Ontario, Canada) of 30-cm width was put under the full length of the nipple drinker line before vaccination to facilitate coprophagy and thereby coccidian oocyst cycling (Blake and Tomley, 2014; Gilbert et al., 2011). Further, the humidity was raised to 60% in the rooms using humidifiers and spraying water on the litter to optimize the environmental conditions for oocyst sporulation and cycling.

2.4. Sample collection

At each sample collection (d 11 and 33), 2 birds from each pen were randomly sampled and individually weighed; extremes in body weight were replaced. Subsequently selected birds were euthanized by injecting T-61 containing embutramide, mebezonium iodide, and tetracaine hydrochloride (Merck Animal Health, Kirkland, Quebec, Canada) into the brachial vein. Samples for gene expression (6 rooms), SCFA analysis (6 rooms at d 11; 9 rooms at d 33) were collected, and pH measurements (9 rooms) were taken from both birds. The samples for histology were collected from 1 of the above 2 birds from each pen (6 rooms). Initially, in situ pH was measured in the content of the crop, gizzard, duodenum, jejunum, ileum, caeca and colon using a Beckman Coulter 34 pH meter (Model PHI 34, Beckman Instruments, Fullerton, CA). Samples (about 1 cm) of the mid ileum were removed and put into 10% neutral buffered formalin for histomorphology analysis; samples were stored at room temperature until examination. Samples (about 2 cm) were collected after removing contents from the mid ileum and caeca into sterile plastic bags and stored at −80 °C until analysis for gene expression. Total ileal and caecal contents were collected into plastic centrifuge tubes and stored at −20 °C for the analysis of SCFA. A portion of the pooled ileal contents was collected into plastic snap-cap vials and centrifuged at 17,013 × *g* at 40 °C for 5 min using a Beckman microfuge (Model E348720, Beckmann instruments, INC, Palo Alto, CA). The ileal supernatant was stored at −80 °C for the analysis of β-glucan molecular weight distribution (6 rooms).

2.5. Dietary analysis

Experimental diets and ingredients (wheat and HB) were ground using a Retsch laboratory mill (Retsch ZM 200, Germany) to 1-mm (for the analysis of insoluble and soluble dietary fiber, N, fat and ash) and 0.5-mm (for the analysis of total starch and β-glucan) screen-hole sizes. Insoluble and soluble dietary fibers (IDF and SDF) were analyzed using a Megazyme kit (Total dietary fiber assay procedure, Megazyme International Ireland Ltd., Bray Business Park, Bray, Co. Wicklow, Ireland) according to AOAC method 991.43 and AACC method 32-07.01, and total dietary fiber (TDF) was obtained by addition. Beta-glucan was analyzed (AOAC Method 995.16, 2006, AACC Method 32-23, 2010 and ICC Standard Method No. 168, 2011) using a Megazyme analysis kit (Mixed-linkage beta-glucan assay procedure/McCleary method, Megazyme International Ireland Ltd., Bray Business Park, Bray, Co. Wicklow, Ireland). The total starch analysis was completed based on AOAC method 996.11 and AACC method 76-13.01 using a Megazyme kit (Total starch assay procedure, Amyloglucosidase/α-amylase method, Megazyme International Ireland Ltd., Bray Business Park, Bray, Co. Wicklow, Ireland). A Leco protein analyzer (Model Leco-FP-528L, Leco Corporation, St. Joseph, MA, USA) was used to determine N, and 6.25 was used as the N to CP conversion factor. The fat analysis was completed by ethyl ether extraction using

Goldfish Extraction Apparatus (Labconco model 35001; Labconco, Kansas, MO, USA) following the AOAC method 920.39. Ash content was analyzed according to AOAC method 942.05 using a muffle oven (Model Lindberg/Blue BF51842C, Asheville, NC 28804, USA). Moisture was analyzed according to the AOAC method 930.15.

2.6. Beta-glucan molecular weight distribution

Ileal supernatant was boiled for 15 min to destroy BGase activity in the samples and centrifuged at 9,000 × *g* for 10 min using a Beckman microfuge (Model E348720, Beckmann instruments, INC, Palo Alto, CA). Ileal supernatant was analyzed for β-glucan molecular weight using size exclusion chromatography with calcofluor post-column detection for fluorescent recognition (Boyd et al., 2017). The HPLC used 2 columns (Shodex OHpak SB-806M column with OHpak SB-G guard column and a Waters Ultrahydrogel linear column). The mobile phase was 0.1 mol/L Tris buffer (pH = 8). Peak molecular weight (Mp) and weight average molecular weight (Mw) were obtained using a molar mass distribution curve. Peak molecular weight is the molecular weight of the highest β-glucan fraction. Weight average molecular weight is the average of all the molecular weights of β-glucan (based on the weight fraction of each type of molecule). In addition, the maximum molecular weight for the smallest 10% β-glucan molecules (MW-10%) was also assessed based on the molar mass distribution curve.

2.7. Short chain fatty acids analysis

Short chain fatty acids were analyzed using the method described by Zhao et al. (2006) with minor modifications. The internal standard for gas chromatography was prepared using 20 mL of 25% phosphoric acid, 300 μL of isocaproic acid, and deionized water. The standard solution was made up of pure and concentrated (100%) 300 μL of acetic acid, 200 μL of propionic acid, 100 μL of butyric acid and 50 μL of isobutyric, isovaleric, valeric, caproic and lactic acids and the amounts were brought up to 20 mL in 25% phosphoric acid. The digesta was thawed and mixed with 25% phosphoric acid at 1:1. It was kept at room temperature for 10 min with occasional shaking and centrifuged at 12,500 × *g* for 10 min. One milliliter of the supernatant was collected into a microcentrifuge tube and mixed with the internal standard at 1:1 and centrifuged at 12,500 × *g* for 10 min. Then the supernatant was filtered through a 0.45-micron nylon filter. The filtrate was added to a gas chromatographic autosampler vial. After that, it was injected into the Zebron Capillary Gas Chromatography column (Zebron ZB-FFAP, Phenomenex, Torrance, CA). The column length was 30 m and an internal diameter of 0.25 mm. The film thickness of the column was 0.25 μm. A Thermos Scientific Gas chromatography system (Model Trace 1310, Milan, Italy) equipped with a flame ionization detector was used for the analysis.

2.8. Histomorphology of gastro-intestinal wall

The 10% formalin buffered saline preserved ileal tissue samples were cut into 2 longitudinal sections and embedded in paraffin. Two slides were made for each sample. One slide was stained with hematoxylin and eosin to obtain GIT morphology measurements, whereas the other slide was stained with Alcian Blue/Periodic Acid-Schiff for differentiation of goblet cells. Villi length and width, and crypt depth were measured in 8 to 10 well-oriented villi and crypts per section. Slides were observed, and images were captured using an Optika B-290TB digital microscope (Bergamo, Italy) with an HDCE-X3 digital camera. Optika vision lite software was used to capture the images. Ileal morphology measurements of captured images were obtained using Scope Image 9.0 professional imaging software (BP Integrated Technologies, Inc, Calamba City, Philippines). Villus length was considered as the length from the tip of a villus to the villus–crypt junction. Villus width was measured at half the height of a villus. Crypt depth was considered as the depth of the invagination between 2 adjacent villi. Goblet cells were categorized as acidic mucin-producing (appears in blue), neutral mucin-producing (appears in magenta), and mixed mucin-producing (appears in purple) (Osho et al., 2017). Goblet cells were counted around the perimeter of 8 to 10, well-oriented villi per section.

2.9. Gene expression

Ileal and caecal tissue samples, frozen in liquid nitrogen, were homogenized using a mortar and pestle and then stored at −80 °C until RNA extraction. RNA was extracted from the ground samples according to the user manual of the TRIzol (ThermoFisher Scientific) RNA extraction procedure. The RNA concentration was quantified, and RNA purity was assessed (based on absorbance values at 260 nm/280 nm and 260 nm/230 nm) using a spectrophotometer (NANODROP 2000 spectrophotometer, ThermoFisher Scientific, Mississauga, ON, Canada). Then each sample was diluted until the RNA concentration was ≤1,300 ng/μL using nuclease-free water. An RNA concentration of 1,000 ng was used to synthesize cDNA using a High-Capacity Reverse Transcription kit (Applied Biosystems; Thermo Fisher Scientific, Foster City, CA). The calculated amount of nuclease-free water and RNA was added into each tube. The master mix was prepared using 10 × RT buffer, 25 × dNTP mix (100 nm), 10 × RT random primers and MultiScribe reverse transcriptase, and then 5.8 μL of the master mix was added into each sample. The reaction was started at 25 °C and continued for 10 min. Then the temperature was raised to 37 °C for 2 h and followed by 85 °C for 5 min. The program was run using a C1000 Touch thermal cycler (Bio-Rad Laboratories, Hercules, CA). The synthesized cDNA was stored at −20 °C until use for qPCR reactions. Primers used for the gene expression are shown in Table 2; some were designed using primer 3 in primer BLAST (NCBI). Each PCR reaction included 0.8 μL of 10 μmol/L forward primer, 0.8 μL of 10 μmol/L reverse primer, 6.4 μL of nuclease-free water and 10 μL of SsoFast EvaGreen Supermix (Bio-Rad Laboratories, Inc., Hercules, CA). The PCR conditions for the primers were 1 × 95 °C for 30 s for initial denaturation, 40 cycles × (95 °C for 5 s for denaturation, annealing temperature for 5 s and 72 °C for 5 s for extension), followed by a melt curve analysis from 55 to 95 °C in 0.5 °C increments for 5 s each. The PCR of all the samples was run using a Bio-Rad CFX 96 Real-Time System (Bio-Rad Laboratories, Inc., Hercules, CA). The sequence and annealing temperature for each primer are mentioned in Table 2. The quantification of the products was completed using a Bio-Rad CFX Manager Software, version 3.1 (Bio-Rad Laboratories, Inc., Hercules, CA). The mRNA abundance was determined based on a serial 5-fold dilution curve of a pooled cDNA of all the samples. Then the calculated mRNA abundance of genes of interest was normalized to the level of housekeeping genes. Glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) and ribosomal protein L30 (*RPL30*) were used as house-keeping genes to normalize the genes of interest in the ileum for the d 11 and 33, respectively. The average of *GAPDH* and *RPL30* (d 11), and *RPL30* (d 33) was used to normalize the genes of interest in the caeca.

2.10. Statistical analysis

The experiment was a randomized complete block design with a room used as a block to account for potential environmental differences between rooms. Data were analyzed using a 2-way analysis of variance of SAS 9.4 Proc mixed model to determine the main

Table 2
Primer sequences used for quantitative real-time polymerase chain reaction.

Target	Function	Orientation	Sequence (5′-3′)	Size, bp	Annealing temperature, °C	Reference
GAPDH	Glycolysis	F	GTGAAAGTCGGAGTCAACGGA	101	60	Cheled-Shoval et al. (2011)
		R	AAGGGATCATTGATGGCCAC			
RPL30	Protein coding	F	GAGTCACCTGGGTCAATAA	160	57	Yang et al. (2013)
		R	CCAACAACTGTCCTGCTTT			
IL-6	Pro-inflammatory cytokine	F	GAAATCCCTCCTCGCCAATCTGA	281	63	Bhanja et al. (2015)
		R	TGAAACGGAACAACACTGCCATCT			
IL-8	Pro-inflammatory cytokine/chemotaxis	F	ATGAACGGCAAGCTTGGAGCT	312	62	Khatri and Sharma (2006)
		R	TCACAGTGGTGCATCAGAATTGA			
MUC2	Mucus secretion	F	GCCTGCCCAGGAAATCAAG	59	55	Chen et al., (2015)
		R	CGACAAGTTTGCTGGCACAT			
MUC5AC	Mucus secretion	F	TGTGGTTGCTATGAGAATGGA	244	60	Kitessa et al. (2014)
		R	TTGCCATGGTTTGTGCAT			
AvBD2	Anti-microbial peptide	F	GGCGGGACATGCTGTTCT	107	60	Designed with Primer 3 (NCBI)
		R	CCATTTGCAGCAGGAACG			
PCNA	DNA replication and repair	F	GGGTTCGGGCGGCATCAG	807	55	Withana Gamage (2015)
		R	TCTTCATTTCCAGCACACTTCAG			
MCT1	Mono-carboxylate transporter-1	F	CAAATCCATCACTGTGTTCTTCA	111	57	Designed with Primer 3 (NCBI)
		R	GGACCTCCTGCATACATAACA			

F = forward; R = reverse; *GAPDH* = glyceraldehyde 3-phosphate dehydrogenase; *RPL30* = ribosomal protein L30; *IL-6* = interleukin 6; *IL-8* = interleukin 8; *MUC2* = mucin 2; *MUC5AC* = mucin 5*AC*; *AvBD2* = avian β-defensin 2; *PCNA* = proliferating cell nuclear antigen; *MCT1* = monocarboxylate transporter 1.

effects of, and interaction between, HB and BGase (SAS 9.4, Carey, N.C. 2008). The significance level was $P \leq 0.05$, and trends were considered when $0.10 \geq P > 0.05$. Mean separation was completed using the Tukey–Kramer test. Data were tested for normality using the Shapiro–Wilk test and log-transformed when they were not normally distributed.

3. Results

3.1. Nutrient composition

The TDF, IDF and SDF in HB were analyzed as 26.7%, 18.9% and 7.8%, respectively. In wheat, 14.4% TDF, 12.4% IDF and 2.0% SDF were obtained. Total β-glucan was analyzed as 8.70% and 0.64% in HB and wheat, respectively. The content of total starch, CP, fat, and ash were determined as 53.7%, 16.2%, 2.8% and 2.4%, respectively, in HB, and 62.8%, 14.9%, 1.2% and 1.7% in wheat.

Beta-glucan Mp and Mw were measured as 762×10^3 and 648×10^3, respectively, in 60% HB-based diets without adding BGase. The diets containing 60% HB with 0.1% BGase consisted of β-glucan that having 758×10^3 and 624×10^3 for Mp and Mw, respectively.

3.2. Beta-glucan molecular weight distribution

Interactions between HB and BGase were observed for all β-glucan molecular weight parameters of the soluble ileal content of broiler chickens except for Mw at d 33 (Table 3). Overall, Mp, Mw

Table 3
Effects of hulless barley (HB) andβ-glucanase (BGase) on β-glucan molecular weight in the soluble ileal content of broiler chickens (g/mol).

HB, %	BGase, %	Day 11			Day 33		
		Mp	Mw	MW-10%	Mp	Mw	MW-10%
0	0	37,056^{bc}	42,779^{de}	20,325^{b}	36,633^{c}	42,391	21,061^{b}
	0.01	45,834^{b}	47,864^{cd}	18,623^{b}	33,697^{cd}	37,326	17,855^{b}
	0.1	29,534^{bc}	28,659^{e}	10,691^{cd}	26,386^{cde}	29,852	10,039^{cd}
30	0	80,837^{a}	80,759^{ab}	37,329^{a}	53,072^{b}	57,846	20,471^{b}
	0.01	45,341^{b}	50,488^{cd}	24,771^{b}	32,500^{cd}	44,226	12,457^{c}
	0.1	27,570^{bc}	48,635^{cd}	8,251^{d}	23,664^{de}	37,806	8,719^{cd}
60	0	78,293^{a}	80,971^{a}	33,322^{a}	71,377^{a}	71,684	28,973^{a}
	0.01	42,727^{b}	50,008^{cd}	17,430^{bc}	33,677^{cd}	58,350	11,734^{c}
	0.1	23,611^{c}	62,930^{bc}	7,632^{d}	16,985^{e}	48,316	7,074^{d}
SEM[1]		3,050.8	2,535.9	1,490.8	2,298.7	1,926.3	987.1
Main effects							
HB, %							
0		37,475	39,767	16,546	32,238	36,523^{c}	16,319
30		51,249	59,961	23,450	36,412	46,626^{b}	13,822
60		48,210	64,636	19,461	40,680	59,450^{a}	15,927
BGase, %							
0		65,395	68,170	30,325	53,694	57,307^{a}	23,502
0.01		44,634	49,453	20,275	33,291	46,634^{b}	14,016
0.1		26,905	46,741	8,858	22,345	38,658^{c}	8,610
ANOVA *P*-value							
HB		0.0006	<0.0001	<0.0001	0.001	<0.0001	0.002
BGase		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
HB × BGase		<0.0001	<0.0001	<0.0001	<0.0001	0.39	<0.0001

Mp = peak molecular weight; Mw = weight average molecular weight; MW-10% = the maximum molecular weight for the smallest 10% β-glucan molecules.
[a-e] Means within a column not sharing a common superscript are significantly different ($P \leq 0.05$).
[1] SEM = pooled standard error of the mean ($n = 6$ birds per treatment).

and MW-10% were lower in 0% HB treatments compared to 30% and 60% HB treatments. Further, molecular weight parameters were higher for the 60% HB compared to 30% HB level with 0% BGase supplementation at d 33.

In the birds fed 0% HB diets, MW-10% was lower with the addition of 0.1% BGase than with 0% and 0.01% BGase levels, and Mw was lower with 0.1% BGase than with 0.01% BGase at d 11. When considering the birds fed 30% HB diets, both 0.01% and 0.1% BGase resulted in a lower Mp, Mw and MW-10% than with 0% BGase level at d 11. In addition, MW-10% of β-glucan molecules was lower in the birds aged 11 d with 0.1% BGase supplementation than with 0.01% BGase level. For the birds fed 60% HB diets, both 0.01% and 0.1% BGase resulted in lower values for Mp, Mw and MW-10% compared to 0% BGase at d 11. Further, 0.1% BGase supplementation compared to 0.01% BGase level showed lower Mp and MW-10%. As an example, the blue lines at the same point (1×10^4) of the horizontal axis in the graphs shown in Fig. 1A and B demonstrate the β-glucan curve had shifted to the left side (smaller β-glucan molecules) with the 0.1% BGase compared to the 0% BGase when the birds were fed 60% HB-based diets at d 11. Moreover, a bimodal distribution of molecules was noticed when the diets contained BGase (Fig. 1B). The movement of the curve with the use of 0.1% BGase compared to 0% BGase and the bimodal distribution curve were found in the broiler chickens fed both 30% and 60% HB levels at both ages.

At d 33, MW-10% was lower with the addition of 0.1% BGase than with 0% and 0.01% BGase levels in the birds fed 0% HB diets. For 30% HB treatments, both Mp and MW-10% were lower with 0.01% and 0.1% BGase levels in comparison to the 0% BGase. For the birds fed 60% HB diets, Mp and MW-10% decreased with the increasing level of BGase in the diets. Although the interaction was not found for Mw at d 33, Mw increased with increasing HB and decreased with increasing BGase in the diets.

Overall, molecular weight parameters for 0% HB treatments were numerically similar at d 11 and 33, although d 33 values were appeared to be lower than d 11 in 30% and 60% HB treatments.

3.3. Short chain fatty acids and gastro-intestinal pH

Interactions of main effects of HB and BGase or the main effects did not affect total SCFA or major individual SCFA concentrations (acetic acid, propionic acid, butyric acid and lactic acid) in the ileum at 11 d old broiler chickens (Table 4). However, interactions between HB and BGase were found for ileal valeric acid, isovaleric acid and caproic acid concentrations at d 11. Although these interactions were significant, no clear trends were identified in relationship to grain source or enzyme level.

Hulless barley did not affect the concentrations of total or major SCFA in the ileum of 33 d broilers (Table 4). However, BGase tended to increase total SCFA ($P = 0.06$), acetic acid ($P = 0.10$), propionic

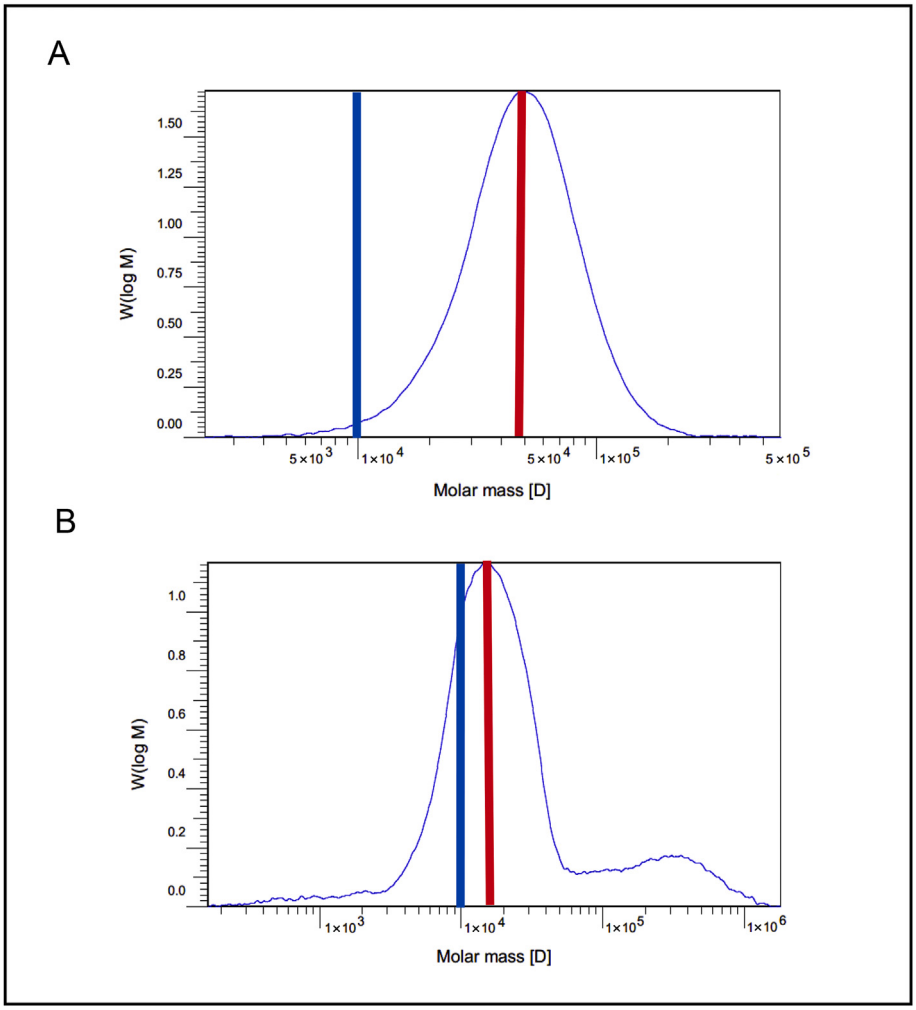

Fig. 1. Beta-glucan molecular weight distribution in soluble ileal digesta from 11-d-old broiler chickens fed 60% hulless barley diets. Blue lines denote point 1×10^4 on the x-axis and red lines indicate the Mp of the distribution curve. (A) 0% β-glucanase; (B) 0.1% β-glucanase. Mp = peak molecular weight.

acid ($P = 0.06$) and lactic acid ($P = 0.08$). Interactions between HB and BGase were significant for the ileal concentrations of isobutyric acid, valeric acid, isovaleric acid and caproic acid at d 33. Except for isobutyric acid, all the other minor SCFA concentrations in the ileum were higher with increasing BGase, when 33-d-old birds were fed 30% and 60% HB diets.

Interactions between the main effects were found for the concentrations of total SCFA and all individual SCFA concentrations in the caeca at 11 d (Table 5). Differences were small, and the most notable effect was the increase in SCFA values for the 60% HB combined with the 0.1% BGase treatment.

Interactions between HB and BGase were not found for caecal SCFA concentrations, but the use of BGase impacted caecal SCFA levels at 33 d of age (Table 5). Total, butyric acid, valeric acid and isovaleric acid values were lower for the 0.01% compared to the 0% BGase treatment and either numerically or statistically lower than 0.1% enzyme level. Acetic acid ($P = 0.06$), propionic acid ($P = 0.06$) and caproic acid ($P = 0.07$) levels also tended to be lowest for 0.01% BGase treatment.

Hulless barley and BGase affected the pH of GI contents at both d 11 and 33 (Table 6). There was an interaction between HB and BGase levels for crop pH at d 11. Statistical separation of interaction means demonstrated that the pH of birds fed the 0% HB diet with 0.01% BGase was higher than those fed the same diet without enzyme, and the 2 enzyme levels in the 60% HB diet; all other values were intermediate and not different than the extremes. Gizzard, duodenum, and jejunum pH values were not affected by dietary treatment. However, ileal pH increased with increasing levels of HB and BGase. The interaction between HB and BGase was significant for caecal pH. The highest level of BGase compared to 0% BGase significantly decreased caecal pH in the birds fed 60% HB diets.

At d 33, crop and gizzard pH values were not affected by treatment. Interaction between main effects was found for duodenal pH, where the highest level of BGase increased duodenal pH compared to 0% BGase when given a wheat-based diet. Jejunal pH increased with an increasing level of BGase, whereas both HB and BGase increased ileal pH. Caecal pH was higher at 30% HB compared to 0% and 60% HB in the diets.

3.4. Gastro-intestinal wall histomorphology and gene expression

There were only minor differences of GI histological measurements of broiler chickens at both d 11 and 33, and no interactions were found (Appendix). At d 11, villi width was lower for the birds fed 0.01% BGase in comparison to the broilers from the 0% BGase treatment. However, no differences were found for the birds fed the highest level of BGase. The number of goblet cells (neutral) per villi tended to increase with the level of HB ($P = 0.06$) at d 11. At d 33, villi height decreased with an increasing level of HB addition. The highest level of BGase increased the crypt depth compared to 0% BGase diets.

Both HB and BGase affected ileal gene expression in broiler chickens (Table 7), although statistical differences were minor. The mRNA expression of interleukin 6 (*IL-6*) in the ileum increased with the highest level of BGase compared to without enzyme treatment at d 11. Further, *IL-6* expression was higher at the 30% HB compared to 0% HB levels. There was no treatment effect on ileal *IL-8*, mucin 2 (*MUC2*) and Proliferating cell nuclear antigen (*PCNA*) expression at d 11. An interaction was found for Monocarboxylate transporter 1 (*MCT1*) expression at d 11. The 0% BGase resulted in the highest, whereas 0.01% BGase showed the lowest *MCT1* expression, and all the other treatment means were intermediate and equal according to mean separation. The interaction between HB and BGase was significant for ileal Avian β-defensin 2 (*AvBD2*) expression at d 11. Beta-glucanase dosages of 0.01% and 0.1% at 30% HB level had the highest, whereas 0% BGase level at the 30% HB had the lowest *AvBD2* expression, and all the other treatments showed intermediate and statistically similar means.

No interactions between HB and BGase were found for ileal gene expression at d 33. However, there were significant main effects

Table 4
Effects of hulless barley (HB) and β-glucanase (BGase) on ileal short chain fatty acids of broiler chickens (μmol/g of wet ileal content).

HB, %	BGase, %	Day 11								Day 33								
		Total	Ace	Pro	Buty	Val	Isov	Cap	Lac	Total	Ace	Pro	Buty	Isob	Val	Isov	Cap	Lac
0	0	126.1	48.0	18.5	8.2	2.4^{a}	2.7^{a}	1.1^{a}	44.9	121.4	46.6	17.6	7.9	0.14^{b}	2.6^{a}	2.4^{abc}	1.1^{a}	42.8
	0.01	117.0	45.6	17.2	7.7	0.8^{b}	2.5^{abc}	1.1^{a}	42.0	119.3	45.6	17.3	7.7	0.00^{b}	2.5^{a}	2.5^{ab}	1.1^{a}	42.2
	0.1	118.0	44.9	16.8	7.8	2.5^{a}	2.6^{ab}	1.1^{a}	42.0	124.5	47.7	18.1	8.1	0.00^{b}	2.6^{a}	2.5^{ab}	1.1^{a}	44.2
30	0	119.6	46.8	17.8	8.0	1.1^{b}	0.8^{c}	1.1^{a}	43.6	117.2	45.4	16.5	7.7	1.54^{a}	1.4^{b}	1.7^{bc}	0.7^{bc}	41.9
	0.01	122.4	46.7	18.4	8.3	0.9^{b}	0.9^{c}	0.4^{b}	46.4	125.6	47.8	18.1	8.1	0.00^{b}	2.6^{a}	2.7^{a}	1.1^{a}	44.8
	0.1	120.4	45.7	17.5	7.8	2.5^{a}	2.6^{ab}	1.1^{a}	42.9	120.7	46.3	17.4	7.8	0.14^{b}	2.5^{a}	2.5^{ab}	1.1^{a}	42.6
60	0	125.3	48.2	18.4	8.2	2.7^{a}	1.5^{abc}	1.1^{a}	44.9	115.2	44.6	17.0	7.6	0.00^{b}	1.5^{b}	1.6^{c}	1.0^{ab}	41.6
	0.01	122.4	45.3	17.8	8.1	2.7^{a}	2.7^{a}	1.2^{a}	43.7	123.7	48.0	18.2	8.2	0.17^{b}	1.6^{b}	1.9^{abc}	0.6^{c}	44.6
	0.1	122.5	47.6	18.3	8.1	1.5^{ab}	1.4^{bc}	0.7^{ab}	44.6	125.0	47.8	18.1	8.1	0.00^{b}	2.6^{a}	2.7^{a}	1.1^{a}	44.3
SEM[1]		1.40	0.49	0.20	0.10	0.11	0.12	0.04	0.60	1.01	0.37	0.16	0.06	0.05	0.07	0.07	0.02	0.35
Main effects																		
HB, %																		
0		120.4	46.2	17.5	7.9	1.9	2.6	1.1	43.0	121.7	46.6	17.6	7.9	0.04	2.6	2.5	1.1	43.1
30		120.8	46.4	17.9	8.0	1.5	1.5	0.9	44.3	121.2	46.5	17.4	7.9	0.56	2.2	2.3	1.0	43.1
60		123.2	47.0	18.1	8.1	2.3	1.9	1.0	44.4	121.3	46.8	17.8	8.0	0.05	1.9	2.1	0.9	43.5
BGase, %																		
0		123.7	47.7	18.2	8.1	2.1	1.7	1.1	44.5	117.9	45.5	17.0	7.7	0.56	1.8	1.9	0.9	42.1
0.01		120.4	45.9	17.8	8.0	1.5	2.0	0.9	44.0	122.9	47.2	17.9	8.0	0.05	2.3	2.4	0.9	43.9
0.1		120.3	46.0	17.5	7.9	2.2	2.2	1.0	43.2	123.4	47.3	17.9	8.0	0.04	2.6	2.6	1.1	43.7
ANOVA *P*-value																		
HB		0.69	0.77	0.53	0.68	0.003	0.0001	0.03	0.68	0.97	0.92	0.60	0.83	<0.001	0.001	0.02	0.009	0.87
BGase		0.53	0.24	0.31	0.72	0.02	0.10	0.01	0.65	0.06	0.10	0.06	0.18	<0.001	<0.001	0.001	0.003	0.08
HB × BGase		0.72	0.64	0.39	0.80	<0.001	<0.001	<0.001	0.55	0.24	0.26	0.39	0.25	<0.001	0.001	0.02	<0.001	0.25

Ace = acetic acid; Pro = propionic acid; Buty = butyric acid; Val = valeric acid; Isov = isovaleric acid; Isob = isobutyric acid; Cap = caproic acid; Lac = lactic acid.
[a-c] Means within a column not sharing a common superscript are significantly different ($P \leq 0.05$).
[1] SEM = pooled standard error of the mean (d 11; $n = 12$ birds per treatment, d 33; $n = 18$ birds per treatment).

Table 5
Effects of hulless barley (HB) and β-glucanase (BGase) on caecal short chain fatty acids of broiler chickens (μmol/g of wet caecal content).

HB, %	BGase, %	Day 11								Day 33							
		Total	Ace	Pro	Buty	Isob	Val	Isov	Cap	Total	Ace	Pro	Buty	Isob	Val	Isov	Cap
0	0	266.0^{ab}	157.1^{ab}	58.0^{ab}	26.4^{ab}	8.6^{ab}	3.4^{c}	8.61^{ab}	3.7^{ab}	229.4	132.5	49.1	22.9	7.3	7.1	7.2	3.0
	0.01	245.2^{ab}	143.1^{ab}	52.1^{ab}	24.1^{ab}	7.8^{ab}	7.7^{ab}	6.87^{ab}	3.3^{ab}	203.6	120.0	42.9	20.0	6.4	6.3	5.1	2.7
	0.1	217.9^{b}	126.8^{b}	46.2^{b}	21.3^{b}	6.9^{b}	6.7^{abc}	6.85^{b}	2.9^{b}	197.3	114.8	41.6	19.6	6.2	6.1	6.1	2.6
30	0	285.0^{ab}	166.6^{ab}	60.2^{ab}	27.4^{ab}	9.0^{ab}	8.8^{a}	8.97^{ab}	3.8^{ab}	225.6	131.6	47.3	22.5	7.0	6.9	7.0	3.0
	0.01	241.7^{ab}	140.0^{ab}	51.4^{ab}	24.0^{ab}	7.7^{ab}	7.6^{ab}	7.66^{ab}	3.2^{ab}	200.3	116.4	42.8	20.0	6.4	4.2	3.6	2.7
	0.1	223.4^{ab}	131.9^{ab}	49.2^{ab}	22.3^{ab}	7.4^{ab}	7.3^{abc}	7.34^{ab}	3.1^{ab}	241.2	141.4	50.4	24.2	7.0	7.4	7.4	3.2
60	0	224.6^{ab}	131.8^{ab}	48.9^{ab}	22.3^{ab}	7.3^{ab}	4.2^{bc}	7.31^{ab}	2.6^{b}	224.5	131.9	46.4	22.5	6.9	6.8	6.8	2.9
	0.01	208.2^{b}	121.0^{b}	44.2^{b}	20.3^{b}	6.6^{b}	6.4^{abc}	6.58^{b}	2.8^{b}	212.4	124.3	44.2	21.7	6.6	6.1	6.5	2.8
	0.1	309.4^{a}	178.1^{a}	66.9^{a}	30.2^{a}	10.0^{a}	9.9^{a}	9.92^{a}	4.2^{a}	231.1	135.2	48.3	23.0	7.2	7.1	7.1	3.0
SEM[1]		7.46	4.32	1.58	0.71	0.23	0.35	0.25	0.10	3.63	2.13	0.77	0.35	0.12	0.15	0.12	0.04
Main effects																	
HB, %																	
0		243.0	142.3	52.1	24.0	7.8	5.9	7.44	3.3	210.1	122.4	44.5	20.9	6.6	6.5	6.1	2.8
30		250.0	146.2	53.6	24.6	8.0	7.9	7.99	3.4	222.3	129.8	46.8	22.2	6.8	6.9	6.5	2.9
60		247.4	143.6	53.3	24.3	8.0	6.8	7.93	3.2	222.7	130.5	46.3	22.4	6.9	6.6	6.8	2.9
BGase, %																	
0		258.5	151.8	55.7	25.4	8.3	5.5	8.29	3.4	226.5^{a}	132.0	47.6	22.7^{a}	7.1	7.0^{a}	7.0^{a}	3.0
0.01		231.7	134.7	49.2	22.8	7.3	7.2	7.03	3.1	205.3^{b}	120.2	43.3	20.6^{b}	6.4	6.2^{b}	5.6^{b}	2.7
0.1		250.2	145.6	54.1	24.6	8.1	7.9	8.04	3.4	223.2^{ab}	130.5	46.7	22.3^{ab}	6.8	6.9^{ab}	6.9^{a}	2.9
ANOVA *P*-value																	
HB		0.91	0.92	0.90	0.93	0.87	0.03	0.58	0.81	0.25	0.21	0.43	0.13	0.63	0.48	0.15	0.35
BGase		0.28	0.23	0.18	0.29	0.19	0.003	0.08	0.39	0.04	0.06	0.06	0.04	0.13	0.03	0.0001	0.07
HB × BGase		0.001	0.002	0.001	0.002	0.001	0.001	0.01	0.001	0.12	0.11	0.11	0.08	0.38	0.17	0.07	0.13

Ace = acetic acid; Pro = propionic acid; Buty = butyric acid; Isob = isobutyric acid; Val = valeric acid; Isov = isovaleric acid; Cap = caproic acid.
[a-c] Means within a column not sharing a common superscript are significantly different ($P \leq 0.05$).
[1] SEM = pooled standard error of the mean (d 11; $n = 12$ birds per treatment, d 33; $n = 18$ birds per treatment).

(Table 7). The expression of *IL-8* was higher with increasing levels of BGase in the diets, whereas *MUC2* expression was lower with increasing BGase supplementation. In addition, *MCT1* expression was lower when HB was included in the diet.

At d 11, no interaction was noted for caecal *MUC2* expression. However, it was higher at 60% compared to 30% HB. Further, *MUC2* expression was higher for the 0.1% than 0% BGase level. Interactions were observed for caecal *MUC5AC* and *MCT1* expression. The expression of *MUC5AC* was higher at 0% compared to 0.01% BGase level when the birds were fed 0% HB diets; however, no significant differences were noted due to enzyme level at 30% and 60% HB levels. There were minor differences in *MCT1* expression, even though an interaction was found (Table 8). No treatment effects were found for caecal *PCNA* expression.

There were no treatment effects on caecal gene expression at 33-d-old broiler chickens.

4. Discussion

The β-glucan molecular weight of soluble ileal digesta was affected by both cereal grain and the use of exogenous enzymes. Both Mp and Mw of β-glucan in the ileal digesta were higher when the birds were fed HB-based diets compared to wheat-based diets without the addition of BGase, which was not unexpected because HB contains higher molecular weight β-glucan compared to wheat (Cui et al., 2000; Storsley et al., 2003). The β-glucan molecular weight of barley and wheat ranges from 31 to 2,700 × 10^3 g/mol and 209 to 416 × 10^3 g/mol, respectively (Biliaderis and Izydorczyk, 2006).

Analysis of the diet β-glucan molecular weight in the current study demonstrated a similar molecular weight for HB with and without exogenous BGase (60% HB and 0% BGase: Mp 762 × 10^3 g/mol, Mw 648 × 10^3 g/mol; 60% HB and 0.1% BGase: Mp 758 × 10^3 g/mol, Mw 624 × 10^3 g/mol) suggesting little or no enzyme activity before feed consumption. Further, these values demonstrate β-glucan molecular weight is reduced to a large degree in the ileal digesta, even without the addition of BGase (Mp 78 × 10^3, Mw 80 × 10^3 with 60% HB and 0% BGase). There are several potential explanations for the molecular weight reduction between diet and digesta in the current study. The digestive process, including moistening, may lead to the activation of endogenous BGase from HB in the upper GIT of chickens (Ribeiro et al., 2011). Alternately, it may be due to the action of BGase derived from the microbes colonizing the upper chicken GIT (Cardoso et al., 2014; Józefiak et al., 2006). Grain cell walls have a complex structure, and β-glucan is associated with other non-starch carbohydrates, including heteroxylans, as well as protein and phenolic acids (Burton and Fincher, 2014), and these compounds might hold β-glucan molecules together and contribute to the high molecular weight determined for the diet β-glucan. However, these β-glucan molecules may separate with the digestion of the cell wall components other than β-glucan, and therefore reduce molecular weight even without the addition of exogenous BGase. Furthermore, β-glucan molecular weight might be reduced due to the exposure to gastric acidity because it has been found that cereal β-glucan is hydrolyzed at an extremely low pH (Johansson et al., 2006).

Beta-glucan Mp, Mw and MW-10% in the soluble ileal digesta decreased with the addition of BGase when the birds were fed an HB-based diet. These data confirm the ability of exogenous BGase to depolymerize high molecular weight β-glucan in the GIT of chickens and demonstrate the degree and nature of the depolymerization associated with exogenous enzyme use. The reduction in molecular weight with an increasing level of BGase indicates the positive response of a very high dosage of enzyme compared to

Table 6
Effects of hulless barley (HB) and β-glucanase (BGase) on gastro-intestinal pH of broiler chickens.

HB, %	BGase, %	Day 11						Day 33					
		Crop	Giz	Duo	Jej	Ileum	Caeca	Crop	Giz	Duo	Jej	Ileum	Caeca
0	0	4.58^{b}	2.68	6.00	5.87	6.26	6.03^{abc}	4.93	3.35	5.78^{b}	5.92	6.51	6.38
	0.01	5.19^{a}	2.63	6.00	5.92	6.37	6.24^{ab}	4.96	3.72	5.99^{ab}	5.95	6.55	6.14
	0.1	4.80^{ab}	2.69	6.00	5.96	6.23	5.90^{bc}	5.03	3.38	6.07^{a}	6.05	6.67	6.22
30	0	4.76^{ab}	2.45	5.96	5.86	6.17	6.37^{a}	4.85	3.56	6.09^{a}	5.99	6.44	6.61
	0.01	4.82^{ab}	2.73	6.08	5.98	6.25	5.91^{bc}	4.81	3.47	6.12^{a}	5.97	6.57	6.46
	0.1	4.78^{ab}	2.58	6.02	5.91	6.56	6.06^{abc}	4.99	3.43	6.24^{a}	6.04	6.79	6.45
60	0	4.78^{ab}	2.81	5.88	5.91	6.29	6.36^{a}	4.94	3.67	6.15^{a}	5.93	6.50	6.22
	0.01	4.69^{b}	2.48	5.93	5.88	6.54	6.17^{abc}	5.03	3.44	6.21^{a}	6.00	7.01	6.43
	0.1	4.62^{b}	2.41	5.99	5.92	6.61	5.78^{c}	4.83	3.44	6.01^{ab}	5.99	6.94	6.03
SEM[1]		0.034	0.034	0.018	0.014	0.035	0.035	0.033	0.036	0.023	0.012	0.043	0.036
Main effects													
HB, %													
0		4.86	2.67	6.00	5.92	6.29^{b}	6.06	4.97	3.48	5.95	5.97	6.58^{b}	6.25^{b}
30		4.78	2.59	6.02	5.91	6.33^{ab}	6.11	4.88	3.48	6.15	5.99	6.60^{ab}	6.51^{a}
60		4.70	2.57	5.93	5.90	6.48^{a}	6.10	4.93	3.51	6.12	5.97	6.82^{a}	6.23^{b}
BGase, %													
0		4.71	2.64	5.95	5.88	6.24^{b}	6.25	4.91	3.53	6.01	5.95^{b}	6.48^{b}	6.41
0.01		4.90	2.61	6.00	5.93	6.39^{ab}	6.11	4.93	3.54	6.10	5.97^{ab}	6.71^{a}	6.34
0.1		4.73	2.56	6.01	5.93	6.47^{a}	5.91	4.95	3.41	6.10	6.03^{a}	6.80^{a}	6.23
ANOVA *P*-value													
HB		0.13	0.42	0.13	0.90	0.03	0.75	0.51	0.92	<0.0001	0.53	0.02	0.001
BGase		0.04	0.61	0.32	0.27	0.01	0.0001	0.85	0.28	0.09	0.02	0.004	0.10
HB × BGase		0.01	0.20	0.66	0.28	0.08	0.002	0.40	0.07	0.004	0.43	0.30	0.10

Giz = gizzard; Duo = duodenum; Jej = jejunum.
[a-c] Means within column not sharing a common superscript are significantly different ($P \leq 0.05$).
[1] SEM = pooled standard error of the mean ($n = 18$ birds per treatment).

Table 7
Effects of hulless barley (HB) and β-glucanase (BGase) on relative mRNA levels in the ileum of broiler chickens.

HB, %	BGase, %	Day 11						Day 33					
		IL-6	*IL-8*	*MUC2*	*PCNA*	*MCT1*	*AvBD2*	*IL-6*	*IL-8*	*MUC2*	*PCNA*	*MCT1*	*AvBD2*
0	0	0.53	0.75	0.81	1.22	1.19^{a}	0.80^{ab}	0.59	0.37	1.09	0.98	0.71	0.17
	0.01	0.44	0.60	0.88	1.00	0.77^{b}	0.60^{ab}	0.59	0.62	0.94	1.04	0.70	0.91
	0.1	0.61	0.67	1.03	1.28	1.03^{ab}	0.80^{ab}	0.95	0.50	0.63	0.78	0.68	0.49
30	0	0.38	0.58	0.88	0.94	0.79^{ab}	0.22^{b}	0.45	0.28	0.99	0.81	0.59	0.76
	0.01	1.73	0.58	0.88	1.18	1.04^{ab}	2.21^{a}	0.49	0.39	0.68	0.75	0.50	0.38
	0.1	2.61	0.89	1.00	1.26	0.98^{ab}	3.21^{a}	0.33	0.58	0.73	1.01	0.47	0.46
60	0	0.71	0.96	1.07	1.05	0.99^{ab}	0.87^{ab}	0.43	0.23	0.75	0.69	0.52	0.44
	0.01	0.50	1.09	1.19	1.08	0.94^{ab}	1.03^{ab}	0.61	0.40	1.00	0.81	0.54	0.54
	0.1	1.88	0.47	0.92	1.17	0.87^{ab}	1.39^{ab}	0.33	0.51	0.65	0.86	0.46	0.32
SEM[1]		0.182	0.060	0.045	0.032	0.032	0.215	0.052	0.036	0.045	0.034	0.029	0.067
Main effects													
HB, %													
0		0.52^{b}	0.67	0.91	1.17	1.00	0.73	0.71	0.50	0.88	0.93	0.70^{a}	0.63
30		1.57^{a}	0.68	0.92	1.13	0.94	1.88	0.42	0.42	0.80	0.86	0.52^{b}	0.53
60		1.03^{ab}	0.84	1.06	1.10	0.93	1.10	0.45	0.38	0.80	0.79	0.50^{b}	0.43
BGase, %													
0		0.54^{b}	0.76	0.92	1.07	0.99	0.63	0.49	0.29^{b}	0.94^{a}	0.82	0.60	0.57
0.01		0.89^{ab}	0.75	0.98	1.09	0.92	1.28	0.56	0.47^{ab}	0.87^{ab}	0.87	0.58	0.61
0.1		1.70^{a}	0.68	0.98	1.23	0.96	1.80	0.54	0.53^{a}	0.67^{b}	0.88	0.54	0.42
ANOVA *P*-value													
HB		0.02	0.60	0.36	0.64	0.63	0.08	0.06	0.39	0.60	0.19	0.01	0.47
BGase		0.01	0.86	0.80	0.09	0.61	0.03	0.85	0.01	0.02	0.75	0.60	0.51
HB × BGase		0.09	0.07	0.52	0.15	0.01	0.03	0.46	0.74	0.14	0.11	0.96	0.36

IL-6 = interleukin-6; *IL-8* = interleukin 8; *MUC2* = mucin 2; *PCNA* = proliferating cell nuclear antigen; *MCT1* = monocarboxylate transporter 1; *AvBD2* = avian β-defensin 2.
[a-b] Means within a column not sharing a common superscript are significantly different ($P \leq 0.05$).
[1] SEM = pooled standard error of the mean ($n = 12$ birds per treatment).

0.01% BGase on β-glucan depolymerization at both broiler ages. The higher response of 0.1% BGase level compared to the 0.01% BGase in terms of ileal soluble β-glucan depolymerization might be associated with the relatively short transit time of digesta in chicken GIT and therefore less time for enzyme and substrate interaction (Hughes, 2008; Rougière and Carré, 2010). Further, the optimum pH for BGase is 4.5, although it has activity over a broader range of pH (Econase GT 200 P, 2019). Therefore, more efficient enzyme action

Table 8
Effects of hulless barley (HB) and β-glucanase (BGase) on relative mRNA levels in the caeca of broiler chickens.

HB, %	BGase, %	Day 11				Day 33			
		MUC2	*MUC5AC*	*PCNA*	*MCT1*	*MUC2*	*MUC5AC*	*PCNA*	*MCT1*
0	0	0.23	0.43^{a}	0.27	0.19^{ab}	2.43	2.13	1.78	1.76
	0.01	0.12	0.13^{b}	0.13	0.18^{ab}	1.48	1.03	1.59	1.21
	0.1	0.14	0.19^{ab}	0.20	0.24^{ab}	1.36	1.04	2.79	3.24
30	0	0.10	0.13^{b}	0.17	0.13^{b}	1.88	1.45	1.80	2.47
	0.01	0.22	0.32^{ab}	0.28	0.21^{ab}	1.51	1.09	2.34	1.87
	0.1	0.06	0.10^{b}	0.08	0.08^{b}	2.00	1.35	1.92	2.20
60	0	0.32	0.32^{ab}	0.33	0.45^{a}	1.99	1.47	2.08	2.18
	0.01	0.22	0.27^{ab}	0.25	0.23^{ab}	1.59	1.58	1.80	2.20
	0.1	0.16	0.20^{ab}	0.22	0.18^{ab}	2.11	1.57	2.08	2.17
SEM[1]		0.015	0.021	0.017	0.019	0.159	0.128	0.158	0.182
Main effects									
HB, %									
0		0.16^{ab}	0.24	0.20	0.20	1.76	1.40	2.05	2.07
30		0.13^{b}	0.18	0.18	0.14	1.80	1.30	2.02	2.10
60		0.23^{a}	0.26	0.27	0.29	1.90	1.54	1.99	2.18
BGase, %									
0		0.22^{a}	0.29	0.26	0.26	2.10	1.68	1.88	2.14
0.01		0.19^{ab}	0.24	0.22	0.21	1.53	1.23	1.91	1.76
0.1		0.12^{b}	0.16	0.17	0.16	1.82	1.32	2.26	2.45
ANOVA *P*-value									
HB		0.01	0.18	0.12	0.01	0.90	0.85	0.96	0.93
BGase		0.02	0.03	0.12	0.16	0.24	0.11	0.34	0.10
HB × BGase		0.06	0.002	0.06	0.04	0.88	0.47	0.34	0.13

MUC2 = mucin 2; *MUC5AC* = mucin 5AC; *PCNA* = proliferating cell nuclear antigen; *MCT1* = monocarboxylate transporter 1.
[a-b] Means within a column not sharing a common superscript are significantly different ($P \leq 0.05$).
[1] SEM = pooled standard error of the mean ($n = 12$ birds per treatment).

is restricted to specific GIT locations, which further reduces the availability of time for the enzyme to act on β-glucan. The reduction of MW-10% also supports the depolymerization of β-glucan, because it indicates the molecular weight distribution curve has shifted towards the direction of smaller β-glucan molecules, and BGase addition has resulted in a higher quantity of small molecular weight β-glucan.

The bimodal size distribution shows two distinct peaks, which indicates two distinct populations of β-glucan molecules when 0.1% BGase is added to 30% and 60% HB diets; major peak associated with Mp and larger molecular weight peak, which was not found for the 0% BGase treatment. The reason for the larger peak is not obvious but might relate to aggregation of smaller β-glucan molecules, which has been previously shown to occur, particularly with increasing pH found in the distal small intestine (Gaborieau and Castignolles, 2011; Holtekjølen et al., 2014). It might also be associated with the release of insoluble β-glucan, which had not yet been depolymerized. However, 0.1% BG did not increase ileal viscosity in the birds given the same barley diets (Karunaratne et al., 2017a), although the use of enzyme produced a novel population of larger molecular weight β-glucan. Therefore, current research suggests that all high molecular weight β-glucans are not viscous despite the assumption of high molecular weight β-glucan increasing digesta viscosity. Nevertheless, the concentration of the second population is much lower than the main population of β-glucan according to the area under the curve, and this lower concentration of β-glucan might not be sufficient to increase ileal viscosity despite the comparatively high molecular weight. The larger molecular weight peak affects Mw of β-glucan, although it does not disturb Mp because the major peak is distinctive from the larger molecular weight peak, which is originated with the use of 0.1% BGase. The effect of the larger peak on Mw is exemplified by the minimum BGase effect on Mw at d 11 (no clear trends at 60% HB level).

There was a minimum or no BGase effect on molecular weight parameters of the birds given wheat-based diets (0% HB) at both d 11 and 33. Wheat might be less susceptible to exogenous BGase in comparison to HB, which in turn might be associated with the structural differences in wheat and barley β-glucan. The ratio of trisaccharides to tetrasaccharides units (DP3-to-DP4 ratio) in wheat and barley β-glucan are 3.0 to 4.5 and 2.3 to 3.4, respectively. The trisaccharides and tetrasaccharides in wheat and barley are 67% to 72% for DP3, 21% to 24% for DP4 and 52% to 69% for DP3, 25% to 33% for DP4, respectively (Biliaderis and Izydorczyk, 2006). Therefore, wheat β-glucan has a more regular structure in comparison to barley and is thus possibly less susceptible to endo-β-1,3-1,4-glucanase attack. Furthermore, β-glucan with a high predominant molar proportion is more uniform, causing increased aggregation and reduced β-glucan solubility (Burton and Fincher, 2014), which possibly results in reduced susceptibility to exogenous BGase.

Overall, molecular weight parameters in this study were lower in broilers aged 33 d compared to 11 d (on average - Mp; 19.2%, Mw; 11.1%, Mw-10%; 16.5% reductions at 33 d in comparison to 11 d). This reduction may be associated with the adaptation of the digestive tract microbial population with age (Bautil et al., 2019) and the ability of the more complex and diversified gut microbiota of the older birds to secrete more non-starch polysaccharidases including BGase. In addition, the diseased state induced by coccidiosis vaccination at d 5 might also influence the gut microbial composition at d 11, which affects β-glucan depolymerization in the digestive tract. This type of gut microbial adaptation to the diets with age may also be related to the lower ileal viscosity, which was found at d 33 compared to d 11 in the broilers fed HB-based diets (Karunaratne, 2020).

Performance data from the current research has previously been reported (Karunaratne et al., 2017a), and it may provide evidence of the relevance of molecular weight changes caused by BGase. The production data were within a normal range according to Ross 308

Broiler Performance Objectives (Aviagen, 2014). The data show that birds fed HB-based diets gained less and had poor feed efficiency compared to the birds given wheat-based diets. The reduced performance with HB compared to wheat might be attributed to the comparatively higher fiber and lower starch content in HB as well as lower nutrient digestibility caused by the increase in large molecular weight soluble β-glucan in HB diets. Interactions between HB and BGase for most production criteria demonstrate that the effect of BGase was larger, with increasing levels of HB, which is to be expected based on the level of β-glucan in the diets and the purity of the BGase source. Of particular interest is the response to BGase in birds fed 60% HB, which varied with bird age. In young birds (<11 d), 0.01% BGase improved growth rate and feed efficiency compared to the un-supplemented control treatment, whereas 0.1% BGase did not affect growth rate and significantly reduced feed efficiency. In contrast, both body weight gain and feed efficiency improved with increasing enzyme dose at older ages. At both ages, the effect of enzyme dose on β-glucan molecular weight was similar (see above paragraph), with decreasing values with increasing enzyme levels with the only exception of Mw at d 11, probably due to the presence of the larger molecular weight peak. Therefore, it might be speculated that larger amounts of low molecular weight β-glucan caused a negative effect in young birds despite the marked increase in SCFA levels and a decrease in pH in the caeca, and a positive effect in older birds where SCFA and pH levels were unaffected. A potential reason for the difference may relate to the status of the gut microbiota at the two ages. In young birds, the microbiota would still be evolving, and in this study may also have been affected by the coccidiosis vaccination. In older birds, the gut microbiota would have stabilized and adapted to the diets, and birds would have developed immunity to coccidiosis. If this is the case, it would suggest that high levels of soluble low molecular weight fiber should be avoided in young birds in antibiotic-free production, whereas the same levels of fiber would be beneficial in older and diet adapted broilers.

Microbial fermentation products (SCFA levels) in the lower GIT were assessed to determine the effect of exogenous BGase on providing low molecular weight, soluble HB β-glucan as substrates to increase carbohydrate fermentation in broilers, and GI pH was determined as an indication of the changes in microbial fermentation. Dietary treatments had only minor effects on ileal SCFA levels in this study, and levels did not relate to ileal pH. Major SCFA concentrations did not change with treatment, whereas both increasing levels of HB and BGase resulted in increased ileal pH. In contrast, caecal SCFA concentrations (total and major SCFA levels) at d 11 increased with the highest level of BGase, but only at the 60% HB level, which is related to caecal pH because caecal pH decreased with BGase at the highest HB level. Carbohydrate metabolizing microbes are abundant in chicken caeca compared to other categories of microbiota (Danzeisen et al., 2011; Qu et al., 2008), and they might be associated with more significant treatment effects in the caeca than the ileum in broilers. However, it is difficult to conclude the BGase effect on increasing carbohydrate fermentation in the caeca based on the treatment effect only for one treatment (60% HB, 0.1% BGase) in broiler chickens. There is little previous research that examined BGase effect on SCFA levels in broilers fed barley, and the results were inconsistent and demonstrated a minimum enzyme effect (Józefiak et al., 2005, 2006). Nevertheless, the results may not demonstrate precise SCFA production because the digesta samples of broilers were collected only at a point in each collection, and the digesta levels relate to the balance between production and utilization by bacteria or the host, as well as frequency of ileum and caecal evacuation. A portion of SCFA may be absorbed into the portal circulation before the digesta samples were collected in the study, and it might be expected that the gene expression of SCFA transporters would increase with increasing SCFA levels. However, *MCT1* expression in the ileum decreased with increasing HB, which appears to agree with ileal pH increasing with HB level. There are several proposed mechanisms involve in SCFA transport in ruminants, including passive diffusion, electro-neutral facilitated transport, and nitrate-sensitive pathway, although 50% of trans-epithelial SCFA transportation has been characterized as active and proton-mediated transport that occurs via MCT1 (Halestrap and Meredith, 2004; Schurmann et al., 2014). Therefore, transportation of SCFA across the intestinal epithelium might be associated with several mechanisms, including passive diffusion in chickens, and relative expression of *MCT1* might not indicate total SCFA transportation across the intestinal epithelium.

Caecal pH decreased with the addition of BGase to the 60% HB-based diet, which is an indication of increased carbohydrate fermentation because of the high availability of low molecular weight β-glucan originating from high molecular weight β-glucan depolymerization. In contrast, ileal pH increased with HB and BGase, but these treatments did not affect SCFA at both ages. Further, there was a trend ($P = 0.08$) for the interaction of HB and BGase on ileal pH at d 11, showing increased pH with increasing HB and BGase levels. According to β-glucan molecular weight distribution data, BGase resulted in an increased amount of low molecular weight soluble β-glucan in the ileum, which might be fermentable. However, feed passage rate may have increased in the ileum with the reduction of soluble β-glucan molecular weight, and therefore less time is available for the bacterial fermentation in the ileum. Consequently, low molecular weight material may enter the caeca and increase bacterial fermentation. It is supported by the reduction of mean retention time of the stomach in the growing pigs with increasing nutrient solubility in the diets (Schop et al., 2019). However, many other factors contribute to intestinal pH, including protein and minerals in the diet. Increased protein fermentation in the lower GIT of chickens increases intestinal pH due to protein fermentation metabolites, including ammonia, phenol, indole, and biogenic amines (Apajalahti, 2005). Minerals in the diet also enhance in buffering the acidity in GIT that results in increased pH.

Gastro-intestinal wall histomorphology is an indication of GI health in chickens, and increased epithelial integrity of the GIT wall is associated with improved nutrient digestion and absorption, and GI health in chickens (Choct, 2009; Onrust et al., 2015). However, there were few treatment effects on the histomorphological parameters in the current study. Short chain fatty acids, and in particular butyrate in chickens positively affect GI epithelial integrity as shown by measurements including villi height and width, crypt depth, and goblet cell distribution according to the previous research (Liu et al., 2017; Wu et al., 2018). In the current study, the highest level of HB decreased the villi height compared to wheat, and it might be attributed to the higher digesta viscosity that damage epithelial villi in the ileum. Previous research has also found that feeding high levels of soluble non-starch carbohydrates to chickens causes a reduction of villi height (Rakowska et al., 1993). Crypt depth in the ileum increased with the highest level of BGase, which is an indication of epithelial growth, which might be due to the beneficial effect of butyrate. The addition of dietary sodium butyrate increased intestinal villi height and goblet cell numbers (Wu et al., 2018), crypt depth (Antongiovanni et al., 2007; Panda et al., 2009), and villi height to

crypt depth ratio (Hu and Guo, 2007) in broilers. However, butyrate concentration in the ileum did not increase with dietary BGase in the current study, but this may relate to the inaccuracies mentioned above of estimating SCFA production. The number and distribution of goblet cells in the ileum were not affected by the treatment; however, ileal *MUC2* expression was lower with the highest level of BGase compared to the control. This finding may relate to BGase-mediated improvement of broiler immune defense mechanisms, which results in less requirement of mucin to combat against pathogens (Kufe, 2009). In addition, HB increased the expression of ileal *MUC2*, and this might relate to an increase in this front-line epithelial defense mechanism because HB mediated high ileal viscosity can increase the colonization of pathogenic microbes in the digestive tract of chickens (Hansson and Johansson, 2010). Of note, the expression of IL-6 and IL-8 in the ileum were higher for the highest level of BG compared to no enzyme at d 11 and 33 of age, respectively. This may reflect the bird's inflammatory response to changes in GI microflora due to disease status and age (Chow et al., 2010; Lu et al., 2003).

In conclusion, exogenous BGase causes the depolymerization of high molecular weight β-glucan in HB in a dose-dependent manner in the digestive tract of broiler chickens. However, there were minor effects of HB and BGase on the GI physiological and histomorphological measurements. Except for an increase of caecal SCFA concentrations with 0.1% BGase at 60% HB-fed 11-d-old broilers, HB and BGase did not increase SCFA levels, despite the exogenous BGase dependent depolymerization of high molecular weight β-glucan of HB at both ages.

Author contributions

Namalika D. Karunaratne: Conceptualization, Methodology, Formal analysis, Investigation, Data Curation, Writing - Original Draft, Visualization. **Rex W. Newkirk**: Conceptualization, Methodology, Investigation, Data Curation, Writing - Review & Editing, Supervision. **Nancy P. Ames**: Methodology, Resources, Writing - Review & Editing. **Andrew G. Van Kessel**: Methodology, Writing - Review & Editing. **Michael R. Bedford**: Methodology, Resources, Writing - Review & Editing. **Henry L. Classen**: Conceptualization, Methodology, Investigation, Resources, Data Curation, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Conflict of interest

We declare that we have no financial and personal relationships with other people or organizations that might inappropriately influence our work, and there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the content of this paper.

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Appendix

Effects of hulless barley (HB) and β-glucanase (BGase) on histomorphology parameters in the ileum of broiler chickens.

HB, %	BGase, %	Day 11							Day 33						
		Villi height, μm	Villi width, μm	Number of goblet cells per villus			Crypt depth, μm	Villi height: Crypt depth	Villi height, μm	Villi width, μm	Number of goblet cells per villus			Crypt depth, μm	Villi height: Crypt depth
				Acidic	Neutral	Mixed					Acidic	Neutral	Mixed		
0	0	479	102	41	10	5	130	3.7	709	113	78	19	8	138	5.2
	0.01	490	101	45	14	8	128	4.2	710	130	79	23	12	140	5.2
	0.1	461	103	39	10	4	115	4.2	725	113	67	15	6	160	4.6
30	0	402	106	43	13	6	124	3.2	625	113	75	22	10	144	4.8
	0.01	441	89	39	11	5	120	3.7	703	119	75	19	8	159	4.4
	0.1	465	100	39	13	7	136	3.6	776	117	72	21	9	161	4.9
60	0	403	102	30	13	5	136	3.1	662	116	78	20	8	134	5.0
	0.01	440	91	41	18	10	126	3.6	608	121	74	24	11	132	4.6
	0.1	446	93	35	17	6	139	3.2	652	117	64	21	10	161	4.1
SEM[1]		17.0	1.5	2.0	0.8	0.6	3.9	0.2	13.6	1.7	2.0	1.3	0.6	3.4	0.1
Main effects															
HB, %															
0		477	102	42	11	6	125	4.0	715[a]	118	75	19	9	146	5.0
30		436	98	41	12	6	126	3.5	701[ab]	116	74	21	9	155	4.7
60		430	95	35	16	7	134	3.3	641[b]	118	72	22	9	142	4.6
BGase, %															
0		428	103[a]	38	12	5	130	3.3	665	114	77	20	9	139[b]	5.0
0.01		457	94[b]	42	14	8	125	3.8	674	123	76	22	10	144[ab]	4.7
0.1		458	99[ab]	38	13	6	130	3.7	718	115	68	19	8	161[a]	4.5
Probability															
HB		0.53	0.16	0.35	0.06	0.64	0.56	0.11	0.04	0.82	0.85	0.75	0.93	0.31	0.22
BGase		0.74	0.03	0.71	0.58	0.21	0.81	0.31	0.21	0.06	0.15	0.62	0.47	0.03	0.16
HB × BGase		0.96	0.41	0.77	0.42	0.42	0.76	0.98	0.28	0.61	0.93	0.73	0.28	0.74	0.32

[a-b] Means within a column not sharing a common superscript are significantly different ($P \leq 0.05$).
[1] SEM = pooled standard error of the mean ($n = 6$ birds per treatment).

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