Effects of dietary supplementation with a laminarin-rich extract on the growth performance and gastrointestinal health in broilers

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ABSTRACT Restriction in antimicrobial use in broiler chicken production is driving the exploration of alternative feed additives that will support growth through the promotion of gastrointestinal health and development. The objective of this study was to determine the effects of dietary inclusion of laminarin on growth performance, the expression of nutrient transporters, markers of inflammation and intestinal integrity in the small intestine and composition of the caecal microbiota in broiler chickens. Two-hundred-and-forty day-old male Ross 308 broiler chicks (40.64 (3.43 SD) g) were randomly assigned to: (T1) basal diet (control); (T2) basal diet + 150 ppm laminarin; (T3) basal diet + 300 ppm laminarin (5 bird/pen; 16 pens/treatment). The basal diet was supplemented with a laminarin-rich *Laminaria* spp. extract (65% laminarin) to achieve the two laminarin inclusion levels (150 and 300 ppm). Chick weights and feed intake was recorded weekly. After 35 days of supplementation, one bird per pen from the control and best performing (300 ppm) laminarin groups were euthanized. Duodenal, jejunal and ileal tissues were collected for gene expression analysis. Caecal digesta was collected for microbiota analysis (high-throughput sequencing and QPCR). Dietary supplementation with 300 ppm laminarin increased both final body weight (2033 vs. 1906 ± 30.4, $P < 0.05$) and average daily gain (62.3 vs. 58.2 ± 0.95, $P < 0.05$) compared to the control group and average daily feed intake (114.1 vs. 106.0 and 104.5 ± 1.77, $P < 0.05$) compared to all other groups. Laminarin supplementation at 300 ppm increased the relative and absolute abundance of *Bifidobacterium* ($P < 0.05$) in the caecum. Laminarin supplementation increased the expression of interleukin 17A (***IL17A***) in the duodenum, claudin 1 (***CLDN1***) and toll-like receptor 2 (***TLR2***) in the jejunum and *IL17A, CLDN1* and *SLC15A1*/peptide transporter 1 (***SLC15A1/PepT1***) in the ileum ($P < 0.05$). In conclusion, supplementation with laminarin is a promising dietary strategy to enhance growth performance and 300 ppm was the optimal inclusion level with which to promote a beneficial profile of the gastrointestinal microbiota in broiler chickens.

Key words: laminarin, broiler, performance, microbiota, gastrointestinal health

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INTRODUCTION

In the past, broiler production was heavily reliant on the routine use of antimicrobial growth promoters (**AGP**) which have established benefits in terms of performance and feed efficiency (Dibner and Richards, 2005). However, the EU ban of AGP in 2006 (Regulation (EC) No 1831/2003) and the implementation of further restrictions regarding the use of antimicrobials in food-producing animals (Regulation (EU) No. 2019/6 and 2019/4) from 2022 have increased the need for alternative feed additives. Prebiotic non-digestible polysaccharides are promising with regard to improving broiler growth and health (Gadde et al., 2017). Brown macroalgae or seaweeds are a source of wide ranging and novel bioactives, including laminarins. These polysaccharides are (1,3)-β-D-glucans with varying degrees of β-(1,6) branching that have exhibited prebiotic and immunomodulatory activities (O'Sullivan et al., 2010; Sweeney et al., 2012; Murphy et al., 2013; Kadam et al., 2015; Cherry et al., 2019; Vigors et al., 2020). In previous studies on broiler chickens, *in ovo* injection with a seaweed extract containing predominantly laminarin increased growth parameters, as well as *Bifidobacterium* spp. and *Lactobacillus* spp. counts (Bednarczyk et al., 2016; Maiorano et al.,

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2017; Tavaniello et al., 2018). Furthermore, dietary supplementation with a highly purified laminarin extract derived from *Laminaria digitata* in chicks infected with *Campylobacter jejuni* not only improved overall performance but there were also improvements to villus architecture coupled with the upregulation of immunological markers (Sweeney et al., 2017). However, the concentration, structure and bioactivity of the macroalgal polysaccharides including laminarin varies with seaweed species, season of harvest and extraction method (Garcia-Vaquero et al., 2017).

Recently, dietary inclusion of a laminarin-rich *Laminaria* spp. extract (65% laminarin) at 300 ppm led to improved growth performance through the modulation of nutrient absorption and markers of inflammation and intestinal integrity in newly weaned pigs (Rattigan et al., 2020). Furthermore, this laminarin-rich extract promoted a more beneficial bacterial composition in the caecal and colonic microbiota of the supplemented pigs characterized by a reduction in the potentially pathogenic *Enterobacteriaceae* family and stimulation of bacterial populations associated with improved growth (Vigors et al., 2020). To date, no optimal dietary inclusion level of laminarin has previously been established for broiler chickens. Therefore, the objective of this study was to explore the effects of two inclusion levels of a 65% purified laminarin *Laminaria* spp. extract on the growth performance of broiler chickens. The second objective of this study was to investigate the effects of the optimal inclusion level of laminarin on selected gastrointestinal parameters that influence performance such as the expression of nutrient transporters and markers of inflammation and intestinal integrity in the small intestine and the composition of the caecal microbiota. It was hypothesized that dietary supplementation with a laminarin-rich extract would enhance growth performance by increasing nutrient absorption, improving the composition of the caecal microbiota and modulating the immune response and intestinal integrity.

MATERIALS AND METHODS

All animal experimental procedures described in this study were approved by the Nottingham Trent University ethics committee (ARE856) and all animal care met the guidelines approved by the institutional animal care and use committee (IACUC) and the guidelines outlined in Directives 2007/526/EC and 2010/63/EU for animal experimentation.

Experimental Design and Diets

The experiment was a complete randomized design consisting of the following treatment groups: (T1) basal diet (Target Feeds, Whitchurch, UK) (control); (T2) basal diet + 150 ppm laminarin and (T3) basal diet + 300 ppm laminarin. Day old male Ross 308 broiler chicks (n = 240) with an average initial weight of 40.64 g (3.43 SD) were sourced from PD Hook Hatcheries Ltd (Cote, Bampton, UK). All chicks were vaccinated against Marek`s disease, infectious bronchitis and Newcastle disease at the hatchery. Chicks were allocated randomly in groups of five per pen. Each pen was assigned to one of the three treatments (16 replicates/treatment). Basal diets were mixed for the starter (d 0−21) and finisher (d 22−35) phases on a wheat-soya bean meal base and manufactured in house as mash. All diets were formulated to meet the nutrient requirements of broilers (National Research Council, 1994). The ingredient and nutrient composition of the starter and the finisher basal diet are presented in Table 1. The T2 and T3 diets were formulated from the basal diet through the addition of 150 and 300 ppm laminarin which were mixed for 5 min using a ribbon mixer (Rigal Bennett, Goole, UK). A laminarin-rich extract (BioAtlantis Ltd., Clash Industrial Estate, Tralee, Ireland) was obtained from *Laminaria* spp. using a hydrothermal-assisted extraction and pre-optimized conditions for maximum yield of laminarin as described previously (Garcia-Vaquero et al., 2019). The crude extract was partially purified to

Table 1. Ingredient and nutrient composition of the starter and finisher basal diets.

Ingredients (g/kg)	Starter basal diet (d 0-21)	Finisher basal diet (d 22-35)
Wheat	630.4	718.8
Soyabean meal (Hipro)[1]	300.0	206.0
Soya oil	32.0	44.0
Limestone flour	8.0	7.2
Dicalcium phosphate (18%)	13.1	11.1
Salt	1.6	1.8
Sodium bicarbonate	2.5	1.5
DL Methionine	3.9	2.3
Lysine HCl	3.6	2.8
L-Threonine	1.4	1.0
TMV premix[2]	3.5	3.5
Phytase[3]	0.1	0.1
Calculated compositional analysis		
Metabolizable energy (MJ/kg)[4]	12.58	13.39
Crude protein %	23.10	21.22
Calcium %[4]	0.95	0.80
Lysine %[4]	1.37	1.15
Methionine %[4]	0.51	0.46
Tryptophan %[4]	0.27	0.24
Threonine %[4]	0.88	0.81
Phytate P %[4]	0.24	0.23
Total phosphorus %[4]	0.62	0.54
Available phosphorus %[4]	0.48	0.41
Na %[4]	0.19	0.17
Cl %[4]	0.28	0.22
K %[4]	0.99	0.92
Analyzed compositional analysis		
Gross energy (MJ/kg)[4]	16.48	16.87
Dry matter %	87.64	87.64
Crude protein %	21.87	19.37
Ash %	4.68	3.89

[1]Crude protein content 48%.

[2]TMV: Trace minerals and vitamins. TMV premix content (per kg diet): Mn, 100 mg; Zn, 88 mg; Fe, 20 mg; Cu, 10 mg; I, 1 mg; Mb, 0.48 mg; Se, 0.2 mg; Vitamin A, 45,000 IU (retinol, 13.5 mg); Cholecalciferol, 3 mg; Vitamin E 50% adsorbate, 25 IU (dl-α-tocopheryl acetate, 25 mg); Menadione, 5 mg; Thiamine, 3 mg; Riboflavin, 10 mg; Pantothenic acid, 15 mg; Pyroxidine, 3.0 mg; Niacin, 60 mg; Cobalamin, 30 μg; Folic acid, 1.5 mg; Biotin, 125 μg.

[3]Quantum Blue Phytase (AB Vista Feed Ingredients, Marlborough, UK).

[4]Calculated for nutritional composition (Sauvant et al., 2004).

increase the relative polysaccharide content and to remove or reduce other constituents; proteins, polyphenols, mannitol and alginate. This was achieved through mixing the crude extract with pure ethanol (to remove polyphenols) followed by water (to remove protein) and calcium chloride (to remove alginates). The chemical composition (% w:w dry matter) of the laminarin-rich *Laminaria* spp. extract included 65% laminarin, 18.1% fucoidan, 6% mannitol, 2.8% alginate, 7.7% ash, and 0.4% phlorotannins. The basal diet was supplemented with the appropriate quantity of the laminarin-rich extract to achieve inclusion levels of 150 and 300 ppm.

Housing and Animal Management

Birds were housed in a 48-pen bird room similar to a commercial broiler setting at the Nottingham Trent University Poultry Research Unit (Brackenhurst Campus, Southwell, UK). Each pen (0.64 m^2) was bedded with clean wood shavings and equipped with a 30 cm trough and 2 nipple drinkers. Birds had *ad libitum* access to feed and water and care was taken to ensure birds ate and drank on d 1. Birds were kept under artificial light for 24 h on d 1 followed by an hour increase of darkness each day until d 6. Six hours of darkness were then maintained for the remainder of the study. The room was thermostatically controlled, and temperatures recorded daily from different areas of the unit. The initial temperature of 31°C on d 1 was gradually reduced to 21°C on d 21 and maintained at this temperature for the remainder of the study. Individual bird weight and feed consumption per pen was recorded on a weekly basis for the calculation of average daily gain (**ADG**), average daily feed intake (**ADFI**) and feed conversion ratio (**FCR**).

Feed Analysis

Dry matter of the feed was determined after drying for 5 d to stable weight at 105°C. Metabolizable and gross energy were determined using an adiabatic bomb calorimeter (Parr Instrument Company, Moline, IL). Ash content was determined after ignition of diet samples for 14 h at 650°C in a muffle furnace. (Nabertherm, Bremen, Germany). Nitrogen content of the diets was determined using a combustion analyzer (Dumatherm N Pro, Gerhardt Analytical Systems, Königswinter, Germany) and multiplied by 6.25 to derive the crude protein content.

Sample Collection

All birds from the control and the best performing laminarin group, 300 ppm inclusion level, were euthanized on d 35 by cervical dislocation and one bird per pen was randomly selected for sample collection. Euthanasia was completed by a trained individual in a separate room away from sight and sound of the other birds. For gene expression analysis, duodenal, jejunal and ileal tissue samples were rinsed in sterile phosphate buffer saline (Sigma-Aldrich, St. Louis, MO), cut into 1 cm^2 tissue sections using a sterile scalpel. These were stored overnight at room temperature in RNAlater solution (Sigma-Aldrich, St. Louis, MO), and subsequently at −20°C prior to RNA extraction. Digesta were collected using gentle digital pressure from both caeca per bird and placed into sterile containers (Sarstedt, Nümbrecht, Germany). This was then snap frozen on dry ice and stored at −80°C for subsequent microbial analysis (QPCR and high-throughput sequencing).

Gene Expression in the Small Intestine

Total RNA was extracted from duodenal, jejunal and ileal tissue using TRI Reagent (Sigma-Aldrich, St. Louis, MO) and purified using GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO) and a DNase removal step using On-Column DNase I Digestion Set (Sigma-Aldrich, St. Louis, MO) according to the manufacturers' instructions. The quantity and purity (260:280 nm absorbance ratio) of the total RNA, were determined using a Nanodrop-ND1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). The cDNA was synthesized from 2 μg of total RNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA) with the total reaction volume (20 μL) adjusted to 400 μL using nuclease-free water. The QPCR reaction mix (20 μL) contained 10 μL GoTaq qPCR Master Mix (Promega, Madison, WI), 1.2 μl forward and reverse primers (5μM), 3.8 μL nuclease-free water and 5μL cDNA. All QPCR reactions were carried out in duplicate on the 7500 ABI Prism Sequence detection System (Applied Biosystems, Foster City, CA) with the following cycling conditions; 95°C for 10 min, 95°C for 15 s for 40 cycles and 60°C for 1 min. All primers were designed using the Primer Express software (Applied Biosystems, Foster City, CA) and synthesized by MWG Biotech UK Ltd (Milton Keynes, UK) and are presented in Table 2. The sequences of the forward and reverse primers have been described and validated previously (Sweeney et al., 2016; Sweeney et al., 2017) except for *SLC1A4, FABP2, AMY2A, SLC15A1/PepT1, SLC15A2/PepT2* genes which were newly designed and their specificity was verified *in silico* using Primer Basic Local Alignment Search Tool (Primer-BLAST, https://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi). QPCR assays exhibiting 90 to 110% efficiency and single and specific products based on the generated dissociation curves were solely used in this study. Normalized relative quantities were obtained using the qbase PLUS software (Biogazelle, Ghent, Belgium) from two stable housekeeping reference genes; *GAPDH* and *PPIA* for duodenum and *GAPDH* and *ACTB* for jejunum and ileum. These genes were selected as reference genes due to their lowest stability M value (<1.5) generated by the GeNorm application.

Table 2. Panel of target genes evaluated in the different regions of small intestine.

Target gene	Accession No	Forward primer (5′-3′)Reverse primer (5′-3′)	Amplicon length (bp)	Tm (°C)
Nutrient transporters and digestive enzymes				
SLC2A1/ GLUT1	NM_205209	F: AACCGCAATGAGGAGAACAAA	73	55.9
		R: GATCGCTGCTGACATCTGTTG		59.8
SLC2A5/ GLUT5	XM_004947446	F: GGATCAATGCAGTCTTCTACTATGCA	93	61.6
		R: CACCTATGGACACGGTGACATACT		62.7
SLC5A1/ SGLT1	NM_001293240	F: AGCATTTCAGCATGGTGTGTCT	88	58.4
		R: GAGGTGACACCAACTTTATTGAGATG		61.6
SLC1A4	XM_001232899.5	F: CCAACGATCTCTCCTTGATATTAGC	81	61.3
		R: GGGCATCCCCTTCCACAT		58.2
FABP2	NM_001007923.1	F: CTTGGAAAATAGAGAAAAATGAGAACTATG	83	59.9
		R: GGCTCCTAACTTTCTTTTCATCACA		59.7
AMY2A	XM_025152793.1	F: CACGGGCACCCACTCAAC	66	60.5
		R: GGCACAGCGGGAAAATCTC		58.8
SLC15A1/ PepT1	NM_204365.1	F: GACAACTTTTCTACAGCCATCTACCA	76	61.6
		R: CAATGAGCGCTCCCAGGAT		58.8
SLC15A2/ PepT2	XM_025152206.1	F: CGAAACTCTGTGGCTCCAACT	67	59.8
		R: CGCTCGCAGAACTCGTTCA		58.8
Inflammatory markers				
IL6	XM_015281283.1	F: CTCCTCGCCAATCTGAAGTC	100	59.4
		R: CCCTCACGGTCTTCTCCATA		59.4
CXCL8	NM_205498.1	F: CTGGAGTGCTCTAGTATG	112	53.7
		R: TCTTACAGATTCTGCAGGTTAGATTTCT		60.7
IL10	NM_001004414.2	F: GCTGCGCTTCTACACAGATGAG	73	62.1
		R: GCCCATGCTCTGCTGATGA		58.8
IL17A	NM_204460.1	F: CCATTCCAGGTGCGTGAAC	64	58.8
		R: TTTGATGGGCACGGAGTTG		56.7
LITAF	NM_204267.1	F: CCCCTACCCTGTCCCACAA	67	61.0
		R: TGAGTACTGCGGAGGGTTCAT		59.8
TGFB1	JQ423909.1	F: GGTTATATGGCCAACTTCTGCAT	102	58.9
		R: CCCCGGGTTGTGTTGGT		57.6
IFNG	FJ788637.1	F: AAGCCGCGAATGAACTCTTC	80	57.3
		R: CTGAGACTGGCTCCTTTTCCTTT		60.6
Toll-like receptors				
TLR2	NM_001161650.1	F: TCTGCAAAAGGCTGTGAACCT	78	57.9
		R: CCAAACGAGTCCTCATCTATGGA		60.6
TLR4	NM_001030693.1	F: GAGCTCTGTGGTTGTCTGTAGCA	91	62.4
		R: CGTTCATCCTCATATCTCTTCACTGA		61.6
Tight Junctions				
CLDN1	NM_001013611.2	F: GCCCTTGGCCAATACATTACA	71	57.9
		R: TGGCACCAGGGAGATATGC		58.8
OCLN	NM_205128.1	F: CCCAGAAGACGCGCAGTAAG	61	61.4
		R: GCGCGGTCCCAGTAGATG		60.5
Mucins				
MUC2	XM_001234581.3	F: CTGATTGTCACTCACGCCTTAATC	147	61.0
		R: GCCGGCCACCTGCAT		52.0
Cell apoptosis				
TNFRSF1A	NM_001030779.1	F: GACCCCATGCACCCAAAAG	75	58.8
		R: AAATCAGCCTCGCCAAACTG		57.3
Reference genes				
GAPDH	NM_204305.1	F: GGTGCTAAGCGTGTTATCATCTCA	70	61.0
		R: CATGGTTGACACCCATCACAA		57.9
ACTB	NM_205518.1	F: TGCTGCGCTCGTTGTTGA	60	56.0
		R: TCGTCCCCGGCGAAA		50.0
PPIA	NM_001166326.1	F: CCTGCTTCCACCGGATCAT	64	58.8
		R: CCGTTGTGGCGCGTAAA		55.2

Abbreviations: ACTB, actin beta; AMY2A, amylase alpha 2A; bp, base pairs; CLDN1, claudin 1; FABP2, fatty acid binding protein 2; IL6, interleukin 6; CXCL8, C-X-C motif chemokine ligand 8; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IL10, interleukin 10; IL17A, interleukin 17A; IFNG, interferon gamma; LITAF, lipopolysaccharide induced TNF factor; MUC2, mucin 2; OCLN, occludin; PPIA, peptidylprolyl isomerase A; SLC15A2/ PepT2, peptide transporter 2; SLC15A1/PepT1, peptide transporter 1; SLC2A1/GLUT1, glucose transporter 1; SLC2A5/GLUT5, glucose transporter 5; SLC5A1/SGLT1, sodium-glucose cotransporter 1; SLC1A4, glutamate and neutral amino acid transporter; TNFRSF1A, TNF receptor superfamily member 1A; TGFB1, transforming growth factor beta 1; TLR2, toll-like receptor 2; TLR4, toll-like receptor 4; Tm, melting temperature.

Quantification of Selected Bacterial Groups Using QPCR

Microbial genomic DNA was extracted using QIAamp PowerFecal Pro DNA Kit (Qiagen, West Sussex, UK) according to the manufacturer's instructions. The DNA quantity and quality were evaluated using a Nanodrop ND-1000 Spectrophotometer. The domain-, function-, family- or genus-specific primers for the selected bacterial groups were available in the literature and are provided in Table 3. The 16S rRNA gene was targeted for most bacterial groups except for the butyrate-producing bacteria where the gene butyryl-CoA:acetate CoA-transferase (**B-CoA**) associated with this function was selected (Louis and Flint, 2007; Metzler-Zebeli et al., 2010). Primers were designed using two tools, Primer3 program (https://primer3.org/) for larger amplicons (>150 bp) and Primer Express software

Table 3. List of forward and reverse primers used for the bacterial quantification by QPCR.

Target bacterial group	Forward primer (5′ - 3′)Reverse primer (5′ - 3′)	Amplicon length (bp)	Tm (°C)	References
Total bacteria	F: GTGCCAGCMGCCGCGGTAA R: GACTACCAGGGTATCTAAT	291	64.2 52.4	Frank et al. (2007)
Lactobacillus spp.	F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG	341	54.5 55.2	Metzler-Zebeli et al. (2010)
Bifidobacterium spp.	F: GCGTGCTTAACACATGCAAGTC R: CACCCGTTTCCAGGAGCTATT	125	60.3 59.8	Penders et al. (2005)
Enterobacteriaceae	F: ATGTTACAACCAAAGCGTACA R: TTACCYTGACGCTTAACTGC	185	54.0 56.3	Takahashi et al. (2017)
Butyryl-CoA:acetate CoA-transferase (B-CoA)	F: GCIGAICATTTCACITGGAAYW-SITGGCAYATG R			

CCTGCCTTTGCAATRTCIACRAANGC53067.0
64.0Louis and Flint (2007)
Abbreviations: bp, base pairs; Tm, melting temperature.

for smaller amplicons (<125 bp), and their specificity was verified *in silico* using Primer-BLAST. For the absolute quantification of the selected bacterial groups using QPCR, specific plasmids (total bacteria, *Lactobacillus* spp., *Bifidobacterium* spp., *Enterobacteriaceae*) were prepared as described by Venardou et al. (2021). The plasmid containing the B-CoA gene was additionally prepared using the genomic DNA of *Faecalibacterium prausnitzii* (DSMZ 17677) that was purchased from Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany). The primers and genomic locations of all targeted genes that were incorporated into plasmids are outlined in Table S1. The QPCR reaction volume (20 μL) included 3 μL template DNA, 1 or 2 μL (for B-CoA) of each primer (10 μM), 5 or 3 μL (for B-CoA) nuclease-free water and 10 μL of GoTaq qPCR Master Mix. All QPCR reactions were carried out in duplicate on the ABI 7500 Fast PCR System (Applied Biosystems, Foster City, CA) with the following cycling conditions; 95°C for 10 mis, 95°C for 15 s for 40 cycles and 60°C for 1 min. The specificity of the resulting PCR products was confirmed by the generation of dissociation curves and visualization on an agarose gel stained with 1% ethidium bromide. To ensure a 90 to 110% QPCR reaction efficiency, the cycling threshold (**CT**) values derived from 5-fold serial dilutions of the plasmid were plotted against their arbitrary quantities. Bacterial counts were determined using a standard curve derived from the mean CT values and the log transformed gene copy number of the plasmid and expressed as log transformed gene copy number per gram of digesta (logGCN/g digesta).

Bacterial Relative Abundance Using Illumina Sequencing

Extraction of the bacterial DNA from the caecal digesta samples and high-throughput sequencing of the V3−V4 hypervariable region of the bacterial 16S rRNA gene on an Illumina MiSeq platform were performed according to their standard protocols (Eurofins Genomics, Ebersberg, Germany). Briefly, the V3−V4 region was PCR-amplified using universal primers containing adapter overhang nucleotide sequences for forward and reverse index primers. Amplicons were purified using AMPure XP beads (Beckman Coulter, Indianapolis, IN) and set up for the index PCR with Nextera XT index primers (Illumina, San Diego, CA). The indexed samples were purified using AMPure XP beads, quantified using a fragment analyzer (Agilent, Santa Clara, CA), and equal quantities from each sample were pooled. The resulting pooled library was quantified using the Bioanalyzer 7500 DNA kit (Agilent, Santa Clara, CA) and sequenced using the v3 chemistry (2 × 300 bp paired-end reads).

Bioinformatic and Statistical Analysis of 16S rRNA Gene Sequencing Data

The bioinformatic analysis of the resulting sequences was performed by Eurofins Genomics (Ebersberg, Germany) using the open source software package (version 1.9.1) Quantitative Insights into Microbial Ecology (Qiime) (Caporaso et al., 2010). All raw reads passing the standard Illumina chastity filter were demultiplexed according to their index sequences (read quality score >30). The primer sequences were clipped from the starts of the raw forward and reverse reads. If primer sequences were not perfectly matched, read pairs were removed to retain only high-quality reads. Paired-end reads were then merged if possible, to obtain a single, longer read that covers the full target region using the software FLASH 2.2.00 (Magoč and Salzberg, 2011). Pairs were merged with a minimum overlap size of 10 bp to reduce false-positive merges. The forward read was only retained for the subsequent analysis steps when merging was not possible. Merged reads were quality filtered according to the expected length and known length variations of the V3−V4 region (ca. 445 bp). The ends of retained forward reads were clipped to a total read length of 285 bp to remove low quality bases. Merged and retained reads containing ambiguous bases were discarded. The filtered reads (merged and quality clipped retained forward reads) were used for the microbiome

profiling. Chimeric reads were identified and removed based on the de-novo algorithm of UCHIME (Edgar et al., 2011) as implemented in the VSEARCH package (Rognes et al., 2016). The remaining set of high-quality reads was processed using minimum entropy decomposition (**MED**) to partition reads to operational taxonomic units (**OTU**) (Eren et al., 2013; Eren et al., 2015). DC-MEGABLAST alignments of cluster representative sequences to the NCBI nucleotide sequence database were performed for taxonomic assignment (from phylum to species) of each OTU. A sequence identity of 70% across at least 80% of the representative sequence was the minimal requirement for considering reference sequences. Abundances of bacterial taxonomic units were normalized using lineage-specific copy numbers of the relevant marker genes to improve estimates (Angly et al., 2014).

The normalized OTU table combined with the phenotype metadata and phylogenetic tree comprised the data matrix. This matrix was then input into the phyloseq package within R (http://www.r-project.org; version 3.5.0). The dynamics of richness and diversity in the chicken's microbiota were computed with the observed, Chao1, ACE, Shannon, Simpson, inverse Simpson and Fisher indices. The Simpson and Shannon indices of diversity account for both richness and evenness parameters. To estimate beta diversity measurements, which are a measure of separation of the phylogenetic structure of the OTU in one sample compared with all other samples, the data was normalized to make taxonomic feature counts comparable across samples. Several distance metrics were considered, in order to calculate the distance matrix of the different multidimensional reduction methods. These included weighted/unweighted UniFrac distance and non-phylogenetic distance metrics (i.e., Bray−Curtis, Jensen−Shannon divergence and Euclidian) using phyloseq in R (Hamady et al., 2010; McMurdie and Holmes, 2013). Taxonomy and diversity plots were produced using graphics tailored for phylogenetic analysis using the R package ggplot2 (Wickham, 2009). Differential abundance testing was performed on tables extracted from the phyloseq object at phylum, family, genus and species level. The data were analyzed using the PROC GLIMMIX procedure of Statistical Analysis Software (**SAS**) 9.4 (SAS Institute, Cary, NC). The model assessed the effect of treatment with the bird being the experimental unit. Fourteen birds per treatment group were used for the statistical analysis of the relative bacterial abundances. Results are presented using Benjamini−Hochberg (BH) adjusted *P*-values.

Statistical Analysis of Data on Performance, Bacterial Populations by QPCR and Gene Expression

All data were initially tested for normality using PROC UNIVARIATE procedure of SAS. The performance data was analyzed by repeated measures analysis using PROC MIXED procedure (Littell et al., 2006). The model included the fixed effects of treatment and time and their associated interactions with the initial weight used as a covariate and the pen being the experimental unit. The data on gene expression and bacterial populations by QPCR were analyzed using PROC GLM procedure. The model assessed the effect of treatment with the bird being the experimental unit. An outlier from the 300 ppm laminarin-supplemented group was removed from the analysis of the data on bacterial populations by QPCR as it consistently had lower logGCN/g digesta for each bacterial group tested that was associated with problematic bacterial DNA extraction. Probability values of <0.05 denote statistical significance. Results are presented as least-square mean values ± standard error of the means.

RESULTS

Growth Performance

The effects of increasing the inclusion level of laminarin on final body weight (**BW**), ADG, ADFI, and FCR are presented in Table 4. Dietary supplementation with 150 and 300 ppm laminarin had no effect on the final BW, ADG, ADFI, and FCR during d 0 to 21 (starter phase) and d 22 to 35 (finisher phase). However, during the overall 35-d experimental period, birds supplemented with 300 ppm laminarin had increased final BW and ADG compared to the control group ($P < 0.05$) and a higher ADFI compared to all other groups ($P < 0.05$). There was no difference in FCR between different treatment groups throughout the experiment.

Effects of Laminarin Supplementation on the Caecal Microbiota

Bacterial Richness and Diversity. Dietary supplementation with 300 ppm laminarin had no effect on the measures of alpha diversity (Table S2) and on beta diversity (Figure S1) in the caecal digesta.

Differential Bacterial Abundance Analysis. There were three bacterial phyla identified in the caecal digesta with Firmicutes being the predominant (~94−97%) followed by Actinobacteria (~1.6−3.6%) and Proteobacteria (~1.3−2.7%). All data on bacterial abundances at phylum, family, genus and species level is provided in Tables S3 to 6. Only differentially abundant bacterial taxa at phylum, family, genus and species level in response to laminarin supplementation are presented in Table 5.

At the phylum level, dietary supplementation with 300 ppm laminarin increased the relative abundance of Actinobacteria and Proteobacteria in the caecal digesta ($P < 0.05$). At the family level, dietary supplementation with 300 ppm laminarin increased the relative abundance of *Bifidobacteriaceae* within the Actinobacteria

Table 4. Effects of increasing laminarin inclusion level on growth performance (Least-square mean values ± standard errors).

	Laminarin Inclusion Level (ppm)[1]								Laminarin Inclusion Level (ppm)[1]			P-value		
	0		150		300		SEM	0	150	300	SEM	Treatment	Time	Treatment × Time
	d 0-21	d 22-35	d 0-21	d 22-35	d 0-21	d 22-35		d 0-35	d 0-35	d 0-35				
Final BW (g)	735	1906	766	1979	786	2033	30.4	1906[b]	1979[ab]	2033[a]	30.4	0.016	<0.001	0.457
ADG (g)	33.1	83.4	34.5	86.6	35.5	89.0	1.35	58.2[b]	60.6[ab]	62.3[a]	0.95	0.014	<0.001	0.495
ADFI (g)[2]	60.0	148.9	62.2	149.9	65.2	163.0	2.51	104.5[b]	106.0[b]	114.1[a]	1.77	<0.001	<0.001	0.096
FCR[2]	1.37	1.77	1.35	1.73	1.38	1.84	0.043	1.57	1.54	1.61	0.030	0.284	<0.001	0.572

Abbreviations: ADG, average daily gain; ADFI, average daily feed intake; BW, body weight; FCR, feed conversion ratio.
[a,b]Mean values within a row for the different laminarin inclusion levels from d 0 to 35 with different superscript letter indicate significant differences between treatments ($P < 0.05$).
[1]A total of 16 replicates were used per treatment group except for 300 ppm whereby 14 replicates were used (replicate = pen, 5 birds/pen).
[2]Mean values were calculated using data collected from d 7 to 35, as feed intake is considered inaccurate the first 7 d post-hatching.

Table 5. Differential relative abundance of bacterial taxa at the phylum, family, genus and species level in the caecal digesta of chickens supplemented with 300 ppm laminarin (mean % relative abundance ± standard errors).

	Laminarin inclusion level (ppm)[1]			
	0	300	SEM	Adjusted *P*-value
Phylum				
Actinobacteria	1.60	3.56	0.419	0.005
Proteobacteria	1.29	2.66	0.368	0.020
Family				
Bifidobacteriaceae	1.53	3.53	0.416	0.003
Genus				
Bifidobacterium	1.48	3.48	0.410	0.004
Sporobacter	2.76	1.51	0.389	0.034
Species				
Bifidobacterium pseudolongum	1.22	2.71	0.367	0.011
Sporobacter termitidis	3.06	1.63	0.404	0.023
Streptococcus alactolyticus	1.06	2.23	0.337	0.026

[1]A total of 14 replicates were used for the control and 300 ppm laminarin treatment respectively (replicate = bird).

phylum in the caecal digesta ($P < 0.05$). At the genus level, dietary supplementation with 300 ppm laminarin increased the relative abundance of *Bifidobacterium* within the *Bifidobacteriaceae* family and decreased the relative abundance of *Sporobacter* within the Firmicutes phylum in the caecal digesta ($P < 0.05$). At the species level, dietary supplementation with 300 ppm laminarin increased the relative abundance of *Bifidobacterium pseudolongum* and *Streptococcus alactolyticus* and decreased the relative abundance of *Sporobacter termitidis* in the caecal digesta ($P < 0.05$).

Selected Bacterial Populations Quantified by QPCR. The effects of dietary supplementation with 300 ppm laminarin on selected bacterial populations in the caecal digesta of broiler chickens are presented in Table 6. Birds supplemented with 300 ppm laminarin had increased caecal *Bifidobacterium* spp. counts compared to the control group ($P = 0.05$). The inclusion of 300 ppm laminarin had no effects on the counts of total bacteria, *Lactobacillus* spp., *Enterobacteriaceae* and butyrate-producing bacteria in the caecum.

Table 6. Effects of 300 ppm laminarin on selected caecal bacterial populations measured by QPCR (Least-square mean values ± standard errors).

Bacterial group (logGCN/g digesta)	Laminarin inclusion level (ppm)[1]			
	0	300	SEM	***P*-value**
Total bacteria	11.24	11.37	0.049	0.075
Lactobacillus spp.	10.22	10.34	0.095	0.409
Bifidobacterium spp.	8.88	9.36	0.163	0.050
Enterobacteriaceae	7.64	7.81	0.166	0.462
B-CoA	6.30	6.46	0.167	0.505

Abbreviations: GCN, gene copy number; B-CoA, Butyryl-CoA:acetate CoA-transferase.
[1]A total of 16 and 13 replicates were used for the control and 300 ppm laminarin treatment respectively (replicate = bird).

Table 7. Differential expression of nutrient transporters, immunological markers and intestinal integrity markers in the small intestine of chickens supplemented with 300 ppm laminarin (Least-square mean values ± standard errors).

		Laminarin inclusion level (ppm)[1]			
Region	Target gene	0	300	SEM	*P*-value
Nutrient transporters					
Ileum	SLC15A1/PepT1	0.90	1.14	0.061	0.009
Markers of immune response					
Duodenum	IL17A	0.88	1.43	0.176	0.035
Jejunum	TLR2	0.93	1.17	0.123	0.036
Ileum	IL17A	0.86	1.54	0.222	0.041
Markers of intestinal integrity					
Jejunum	CLDN1	0.94	1.13	0.060	0.029
Ileum	CLDN1	0.93	1.19	0.089	0.042

Abbreviations: CLDN1, claudin 1; IL17A, interleukin 17A; SLC15A1/PepT1, peptide transporter 1; TLR2, toll-like receptor 2.
[1]A total of 16 and 14 replicates were used for the control and 300 ppm laminarin treatment respectively (replicate = bird).

Gene Expression in the Small Intestine

The effects of laminarin supplementation on the expression of genes associated with the functionality and health in the small intestine of broiler chickens were evaluated. All gene expression data is presented in Table S7. The differentially expressed genes in the different parts of the small intestines are presented in Table 7 and highlighted in this section:

Nutrient Transporters. Dietary supplementation with 300 ppm laminarin upregulated the expression of *SLC15A1*/peptide transporter 1 (***SLC15A1/PepT1***, $P < 0.05$) in the ileum.

Immunological Markers. Dietary supplementation with 300 ppm laminarin upregulated the expression of interleukin 17A (***IL17A***, $P < 0.05$) in the duodenum and ileum and the expression of toll-like receptor 2 (***TLR2***, $P < 0.05$) in the jejunum.

Intestinal Integrity. Dietary supplementation with 300 ppm laminarin upregulated the expression of claudin 1 (***CLDN1***, $P < 0.05$) in the jejunum and ileum.

DISCUSSION

In this study, it was hypothesized that dietary supplementation with a laminarin-rich extract would enhance the performance of broiler chickens by upregulating the expression of nutrient transporters, promoting a beneficial caecal microbiota and enhancing intestinal integrity. The optimal inclusion level identified was 300 ppm laminarin as it led to the greatest broiler performance. Further investigation of the effects of 300 ppm laminarin on the gastrointestinal system identified an increase in the absolute and relative abundance of the caecal *Bifidobacterium* spp. and an increase in the expression of a protein transporter gene (*SLC15A1/PepT1*), a tight junction protein gene (*CLDN1*) and the inflammatory genes *IL17A* and *TLR2*.

Laminarin supplemented at 300 ppm increased feed intake, final BW and ADG during the 35-d supplementation period, whereas that was not evident at the lower inclusion level of 150 ppm. A supplement that increases feed intake is of major significance to the broiler industry as it directly influences growth rate, nutrient uptake and utilization, and subsequently gastrointestinal integrity and development (Ferket and Gernat, 2006; Maiorka et al., 2006; Yegani and Korver, 2008). Interestingly, an earlier study has provided some supporting evidence to suggest that laminarin ingestion stimulates feed intake; as chicks that were supplemented with seaweed extracts (containing >30% laminarin) in water for the first 7 d post-hatching compared to *in ovo* had an increase in feed intake (Bednarczyk et al., 2016). Based on the above, the treatment group supplemented with 300 ppm laminarin was solely selected for further analysis along with the control group. In the current study, an increase in the expression of *SLC15A1/PepT1* in the ileum was identified in the laminarin-supplemented birds. PepT1 is a proton-dependent, low-affinity/high capacity di- and tripeptide transporter that is predominantly expressed in the small intestine in chickens (Daniel and Kottra, 2004; Zwarycz and Wong, 2013). The upregulation of *SLC15A1/PepT1* expression may have led to improved absorption of the oligopeptides produced after the digestion of dietary protein. Thus, the enhanced growth in the laminarin-supplemented chicks in the current study may be attributed to increased feed intake and the concomitant increased absorption of dietary protein. Interestingly, *SLC15A1/PepT1* was also among the nutrient transporters that were stimulated in weaned pigs supplemented with the same laminarin-rich extract (Rattigan et al., 2020).

Promoting a beneficial microbial composition in the caecum of broiler chickens via dietary interventions may improve productivity and health and confer colonization resistance to pathogens (Gaggia et al., 2010; Gadde et al., 2017). The major change in the overall composition of the caecal microbiota, as determined by 16S rRNA gene sequencing, in response to laminarin supplementation was the increase in the relative abundance of *Bifidobacterium* spp. (Actinobacteria phylum, *Bifidobacteriaceae* family). This result was subsequently confirmed by QPCR. In previous studies, laminarin supplementation increased the counts of *Bifidobacterium* spp. and selected strains of this genus in batch fermentation and pure cultures (Zhao and Cheung, 2011; Seong et al., 2019), while an increase in this bacterial group was also observed in day-old chicks after *in ovo* injection of a seaweed extract (containing >30% laminarin) (Bednarczyk et al., 2016). The ability of

Bifidobacterium spp. to utilize laminarin as a substrate is further supported by the presence of relevant carbohydrate-degrading enzymes such as β-glycosidases in their genome (Zhao and Cheung, 2011; Seong et al., 2019). *In ovo* injection with various *Bifidobacterium* spp. strains, particularly *B. bifidum*, resulted in improved performance and villus architecture in the ileum, stimulation of humoral immunity and beneficial changes in the composition of the ileal microbiota (Abdel-Moneim et al., 2020; El-Moneim et al., 2020). A positive correlation of bird weight and *Bifidobacterium* spp. relative abundance in ileum, caecum and trachea early in the life of broiler chicks has previously been reported (Johnson et al., 2018). Furthermore, anti-*C. jejuni* activity was observed for several *Bifidobacterium* spp. strains *in vitro* with *B. longum* also reducing *C. jejuni* counts in the feces of broiler chickens following oral supplementation (Santini et al., 2010; Baffoni et al., 2012). Thus, laminarin supplementation may have simultaneously promoted growth and gastrointestinal development and increased colonization resistance to pathogens by stimulating the beneficial *Bifidobacterium* spp. population, particularly during the first weeks post-hatching.

Laminarin supplementation also led to an increase in the relative abundance of Proteobacteria, which is considered an indicator of dysbiosis (Shin et al., 2015). However, the relative abundance of Proteobacteria was below 3% and no specific families, genera or species linked to compromised health or zoonotic pathogens were differentially abundant following laminarin supplementation. Furthermore, members of the Proteobacteria phylum such as *Sutterella* have previously been correlated with improved body weight (Johnson et al., 2018). Additionally, there was a reduction in the relative abundance of *Sporobacter* genus and *Sporobacter termitidis,* and an increase in the relative abundance of *Streptococcus alactolyticus* in response to laminarin supplementation. The observed reduction in *Sporobacter* may be indirectly affected by laminarin supplementation as a result of the increases in *Bifidobacterium* spp. In a separate study, the relative abundance of this genus was reduced following supplementation with a mixture of *Bifidobacterium* spp. strains in mice (Wang et al., 2019). *Sporobacter* higher abundance in pathological conditions such as constipation in mice (Wang et al., 2019) and ETEC infection in weaned pigs (Zhang et al., 2017) indicates that it possibly acts as an opportunistic pathogen in animals. *Streptococcus alactolyticus* is the dominant species within the commensal streptococcal population in the chicken caecum (Czerwinski et al., 2010). *Streptococcus alactolyticus* (*Streptococcus bovis*/*Streptococcus equinus* complex) can degrade β-glucans including laminarin, which supports the observed increase in relative abundance (Schlegel et al., 2003; Beckmann et al., 2006).

Markers of intestinal integrity were also influenced by laminarin supplementation with increased expression of *CLDN1* in the jejunum and ileum, an effect that supports a similar observation in a previous study (Sweeney et al., 2017). Claudins are major components of the tight junctions that control the paracellular permeability between adjacent epithelial cells and, thus, control the diffusion of dietary antigens and nutrients and the translocation of microorganisms (Ulluwishewa et al., 2011). For instance, upregulation of claudin 1 and occludin in the jejunum of *Salmonella enterica* subsp. *enterica* serotype *Typhimurium*-infected chickens was associated with reduced pathogen translocation (Shao et al., 2013).

Nutritional immunomodulation is a promising strategy to confer resistance to important pathogens while minimizing the negative impact of the inflammatory response on broiler performance (Korver, 2012; Suresh et al., 2018; Kim and Lillehoj, 2019). In this study, laminarin supplementation upregulated the expression of *IL17A* and *TLR2* in the small intestine. β-glucans have previously been identified as a stimulant of genes within the *IL17* family, via dectin-1 (*CLEC7a*) signaling (Werner et al., 2011; Kamiya et al., 2018). IL17A is among the major cytokines associated with a protective inflammatory response against pathogens (Iwakura et al., 2011). *IL17A* is highly expressed in the small and large intestine in chickens (Kim et al., 2012) and is involved in the immune response against various infectious agents such as *Eimeria* spp. (Kim et al., 2008; Zhang et al., 2012), *C. jejuni* (Connerton et al., 2018), *S* Enteritidis (Crhanova et al., 2011; Karaffova et al., 2015) and *Clostridium perfringens* (Emami et al., 2019). Previously, oral supplementation of mice with laminarin increased *TLR2* expression in the small intestine, like the current study (Rice et al., 2005). TLR2 is a member of the transmembrane Toll-like receptors involved in pathogen recognition and immune response initiation, that is predominantly involved in the recognition of Gram-positive bacteria but also Gram-negative bacteria, fungi, viruses, and parasites (Keestra et al., 2013; Nawab et al., 2019). *S.* Enteritidis infection in chickens led to the suppression of the inflammatory response including *TLR2* downregulation that was associated with increased *S.* Enteritidis counts in the caecum and crop, *S.* Enteritidis invasion in extraintestinal tissues and susceptibility to infection (Gou et al., 2012; Quinteiro-Filho et al., 2017). Therefore, the observed upregulation of *IL17A* and *TLR2* solely due to laminarin supplementation might be indicative of a more controlled and potentially protective immune response without compromising the growth performance of the laminarin-supplemented birds.

CONCLUSION

Dietary supplementation with a laminarin-rich extract at 300 ppm was associated with increased BW, ADG and ADFI. Further analysis of various aspects of gastrointestinal functionality and health identified an upregulation of genes associated with nutrient absorption, intestinal integrity and protective immune responses against pathogens and an alteration in the composition of the microbiota characterized

predominantly by a beneficial increase in *Bifidobacterium* spp. Thus, laminarin supplementation is a promising dietary supplement to improve performance and support gastrointestinal development and health in broiler chickens.

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DISCLOSURES

The authors have no conflict of interest to declare.

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.psj.2021.101179.

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