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The effects of laminarin derived from *Laminaria digitata* on measurements of gut health: selected bacterial populations, intestinal fermentation, mucin gene expression and cytokine gene expression in the pig

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Abstract

The aim of the present study was to establish the optimum inclusion level of laminarin derived from *Laminaria digitata* on selected microbial populations, intestinal fermentation, cytokine and mucin gene expression in the porcine ileum and colon. A total of twenty-one pigs (mean body weight 17·9 kg) were randomly assigned to one of three dietary treatments: T1 – basal (control) diet, T2 and T3 – basal diets supplemented with laminarin included at 300 and 600 parts per million (ppm), respectively. Selected intestinal bacterial populations and volatile fatty acid (VFA) concentrations were measured in the ileum and colon. Relative gene expression levels for specific cytokine and mucin genes were investigated in ileal and colonic tissue in the absence and presence of a lipopolysaccharide (LPS) challenge. There was an up-regulation of *MUC2* gene expression at the 300 ppm inclusion level in the ileum. In the colon, there was a significant reduction in the enterobacteriaceae population at the 300 ppm inclusion level ($P=0·0421$). Dietary supplementation of 600 ppm laminarin led to a significant increase in *MUC2* ($P=0·0365$) and *MUC4* ($P=0·0401$) expression in the colon, and in the total VFA concentration in the caecum ($P=0·0489$). A significant increase was also recorded in *IL-6* ($P=0·0289$) and *IL-8* gene expression ($P=0·0245$) in LPS-challenged colonic tissue at both laminarin inclusion levels. In conclusion, dietary inclusion of 300 ppm laminarin appears to be the optimum dose in the present study due to the reduction in the enterobacteriaceae populations and enhanced IL-6 and IL-8 cytokine expression in response to an *ex vivo* LPS challenge.

Key words: β-Glucan: Pigs: Colon: Ileum: Microbiota: Immunity: Mucin

While dietary supplementation with in-feed antibiotics promotes growth and feed efficiency in swine, the 2006 European Union widespread prohibition of such antibiotics has prompted the search for alternatives. The use of natural supplements, such as β-glucans, is currently under consideration as an alternative to in-feed antibiotics[1,2]. β-Glucans are a heterogeneous group of glucose polymers, which form the main constituent of the cell walls of cereals, fungi, macroalgae and a limited number of bacteria[3]. The two major types of polysaccharides in seaweeds are structural and storage polysaccharides[4]. After carbon, laminarin is the second major storage component in brown algae[5]. Laminarin is a seaweed-derived (1–3) β-D-glucan with a chemical structure consisting mainly of a linear β-(1–3)-linked glucan with some random β-(1–6)-linked side chains, which is dependent on the variety of seaweed[6]. Water-soluble β-glucans such as those derived from *Laminaria digitata* contain small numbers of β-(1–6)-linked side chains, while water-insoluble β-glucans such as those derived from *L. hyperborea* only contain linear β-(1–3)-linked residues[7]. Other sources of β-glucans extracted from barley and oats are similar in structure but differ in the ratio of β-(1–3):β-(1–4) linkages[8]. β-Glucans in yeast and macroalgae are polysaccharides characterised by a basic structure of chains of glucose molecules that have a β-(1–3)-linked backbone with (1–6)-linked branches.

As well as being considered as a source of dietary fibre in monogastric nutrition[9], β-glucans also have distinctive immunomodulatory characteristics[10]. They bind to mammalian non-Toll-like receptor (TLR) pattern-recognition receptors such as dectin-1, complement receptor 3, lactosylceramide and scavenger receptors, thereby stimulating innate immunity through the activation of macrophages, dendritic cells, neutrophils, natural killer cells and helper T-cells[11]. The activation/proliferation of these cells results in enhanced phagocytosis and oxidative burst, cytokine production, activation of the alternative complement pathway and release of lysosomal enzymes[12].

Abbreviations: cDNA, complementary DNA; LPS, lipopolysaccharide; ppm, parts per million; TLR, Toll-like receptor; VFA, volatile fatty acid.

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Recent studies have indicated that the inclusion of laminarin derived from *Laminaria* spp. in pig diets has ameliorated feed efficiency and growth performance[13–15] and reduced *Enterobacterium* spp.[15,16]. Dietary laminarin inclusion also affects the quality and quantity of mucin production in the jejunum, ileum, caecum and colon in the murine model[17]. Mucins are a family of large heterogeneous, complex glycoproteins that are subdivided into two main groups; secreted gel-forming (MUC2, MUC5AC, MUC5B, MUC6 and MUC19) and non-gel-forming (MUC7) and membrane-bound mucins (MUC1, MUC3A/B, MUC4, MUC12, MUC13, MUC15, MUC16, MUC17 and MUC20)[18,19]. Within the gastrointestinal tract, mucins are an integral rheological component of the mucous gel layer, which acts as a biophysical barrier to protect the underlying epithelium from biological, mechanical and chemical insult and pathogenic invasion[19]. However, it is clear that β-glucans vary in their structure and chemical composition, which may modulate their effect on animal performance and gastrointestinal health and hence the optimal dietary inclusion level[1,2,20–22]. Therefore, the aim of the present study was to establish the optimum inclusion level of laminarin derived from *L. digitata* on piglet performance, selected bacterial populations, volatile fatty acid (VFA) production, as well as mucin gene expression and cytokine expression in both unchallenged and lipopolysaccharide (LPS)-challenged ileal and colonic tissues.

Materials and methods

Experimental design and animal diets

All procedures described in this experiment were conducted under experimental licence from the Irish Department of Health in accordance with the Cruelty to Animals Act, 1876, and the European Communities (Amendments of the Cruelty to Animals Act, 1876) Regulations[23].

This experiment was designed as a complete randomised design. A total of twenty-one 56-d-old pigs, previously weaned at 26 d with an initial live weight of 17·9 (SEM 2·2) kg, were blocked on the basis of live weight. Then, seven pigs were randomly assigned to one of the three dietary treatments as follows: T1 – basal diet (control: *n* 7); T2 – basal diet supplemented with 300 parts per million (ppm) laminarin from *L. digitata* (*n* 7); and T3 – basal diet supplemented with 600 ppm laminarin from *L. digitata* (*n* 7). Experimental feeding continued for 21 d *ad libitium*. Purified laminarin (990 g/kg laminarin) was sourced from Bioatlantis Limited, Tralee, County Kerry, Republic of Ireland, and extracted according to the procedure described by Lynch *et al.*[16]. The molecular weight of laminarin was measured using the method of Friedlaender *et al.*[24] and was calculated to be less than 5000 Da. The diets were formulated to have similar digestible energy (14·4 MJ/kg) and ileal digestible lysine (12·5 g/kg) contents. The ingredient composition and chemical analysis of the dietary treatments are presented in Table 1.

Animals and management

Initially, twenty-one pigs were housed individually in fully slated pens (1·7 m × 1·2 m) and were allowed a 10 d dietary adaptation period after which time they were weighed and transferred to individual metabolism crates, which facilitated total but separate collection of urine and faeces. The pigs were given a further 5 d to adapt to the metabolism crates before collections begun. A 5 d collection period followed to facilitate the nutrient digestibility study. The daily food allowance (digestible energy intake in MJ/d) was calculated as $3{\cdot}44 \times \text{live weight}^{0{\cdot}54}$ according to Close[25] and was divided over two meals each day. Water was provided with the meals on a 1:1 ratio, and between meals, fresh water was provided *ad libitium*. The metabolism crates were located in an environmentally controlled room, which was maintained at a constant temperature of 22 ± 1·5°C throughout the experiment. During collections, total faeces weight was recorded daily. At the end of the collection period, faecal samples were pooled and a subsample retained for laboratory analysis. The pigs were then rehoused to their respective pens and diets until they were humanely killed by euthanol injection (pentobarbitone sodium BP) at a rate of 1 ml/1·4 kg body weight.

Table 1. Diet composition and chemical analysis of experimental diets (g/kg, unless otherwise indicated)

Dietary composition and analysis	Treatment 1 (basal diet)	Treatment 2 (basal diet + 300 ppm)	Treatment 3 (basal diet + 600 ppm)
Composition (g/kg)			
Wheat	686·7	686·7	686·7
Soya bean meal	260·0	260·0	260·0
Soya oil	24·8	24·8	24·8
Minerals and vitamins*	23·0	23·0	23·0
Lys HCl	3·4	3·4	3·4
L-Thr	1·3	1·3	1·3
DL-Met	0·8	0·8	0·8
Laminarin extract (ppm)	0	300	600
Analysed composition (g/kg)			
DM	864·7	836·2	818·6
Crude protein (*n* × 6·25)	189·6	208·3	206·4
Neutral-detergent fibre	112·6	123·2	131·1
Ash	45·50	46·45	45·34
Gross energy (MJ/kg)			
Ca	6·90	6·60	6·82
P	4·35	4·55	4·17
Lys†	10·0	10·0	10·0
Met and Cys†	6·0	6·0	6·0
Thr†	6·5	6·5	6·5
Try†	1·8	1·8	1·8

ppm, Parts per million.

* Vitamin and mineral inclusion (per kg diet): 3 mg retinol, 0·05 mg cholecalciferol, 40 mg α-tocopherol, 25 mg Cu as copper (II) sulphate, 100 mg Fe as iron (II) sulphate, 100 mg Zn as zinc oxide, 0·3 mg Se as sodium selenite, 25 mg Mn as manganese oxide and 0·2 mg iodine as calcium iodate on a calcium sulphate/calcium carbonate carrier.

† Calculated from proximate analysis[52].

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Chemical analysis for nutrient digestibility

Both concentrates and faeces were analysed for N, DM, ash, gross energy and neutral-detergent fibre. Following collection, faeces were dried at 100°C for 48 h. The concentrates and dried faeces samples were milled through a 1 mm screen (Christy and Norris hammer mill). The DM of faeces and feed was determined after drying overnight at 103°C. Ash was determined after ignition of a known weight of concentrates or faeces in a muffle furnace (Nabertherm, Bremen, Germany) at 500°C. The N content of both feed and faeces was determined using the LECO FP 528 instrument (Leco Instruments, UK Limited, Hazel Grove, Stockport, Cheshire, UK). The neutral-detergent fibre was determined using a Fibertec extraction unit (Tecator, Hoganas, Sweden). The gross energy of the feed and faeces was determined using a Parr 1201 oxygen bomb calorimeter (Parr, Moline, IL, USA). The total laminarin content in the diets was determined using a Megazyme kit (Megazyme, Bray, County Wicklow, Republic of Ireland).

Microbial and volatile fatty acid analysis

Immediately after slaughter, the entire digestive tract was removed by blunt dissection, and digesta (approximately 10 (SEM 1) g was removed from the ileum, 10 cm from the ileocaecal valve and from the second loop of the ascending colon. These digesta samples were removed and stored in sterile containers (Sarstedt, County Wexford, Republic of Ireland) on ice and transported to the laboratory immediately. Bifidobacteria, lactobacilli and enterobacteriaceae were isolated according to the methods described by Pierce *et al.*[26]. In brief, a 1·0 g sample was removed from each digesta sample, serially diluted (1:10) in 9·0 ml aliquots of maximum recovery diluent (Oxoid, Basingstoke, UK) and spread plated (0·1 ml aliquots) onto selective agars as follows: lactobacilli and bifidobacteria were isolated on de Man, Rogosa and Sharpe agar (Oxoid). Lactobacilli were incubated overnight (18–24 h) at 37°C in a microaerophilic (5 % CO_2) environment, and the bifidobacteria cultures were incubated anaerobically at 37°C for 72 h according to the manufacturer's instructions (Oxoid). An API 50 CHL kit (BioMerieux, Craponne, France) was used to confirm the presence of lactobacilli, and a Gram stain was used to distinguish bifidobacteria from lactobacilli based on colony appearance and rod size and shape. Enterobacteriaceae were isolated on MacConkey agar (Oxoid), following aerobic incubation at 37°C for 18–24 h. Positive enterobacteriaceae colonies were confirmed with API 20E (BioMerieux). Microbial colonies from each plate were counted, and bacterial numbers were presented as $\log_{10}$ colony-forming units/g of digesta. Digesta samples were collected from the ileum, caecum and colon, and were mixed with sodium benzoate and phenylmethylsulphonyl fluoride, in order to stop any bacterial activity and minimise the effects of post-thawing fermentation on resulting VFA concentrations. The VFA analysis was performed using GLC according to the method described by Pierce *et al.*[26]. All pH measurements were made on a Mettler Toledo MP220 pH meter. Distilled water was added to very viscous digesta samples to allow their pH to be read.

Collection of tissue samples and tissue challenge procedure

Ileal and colonic tissues were sampled from the same location as described for microbiological samples. Excised tissues were emptied by dissecting them along the mesentery and rinsing them using sterile phosphate-buffered saline (Oxoid). Tissue sections of 1 cm^3, which had been stripped of the overlying smooth muscle, were cut from each tissue. Two sections from each tissue were placed in 1 ml of Dulbecco's modified Eagle's medium (Gibco; Invitrogen Corporation, San Diego, CA, USA), one in the presence of bacterial LPS (Sigma-Aldrich Corporation, St Louis, MO, USA) at a concentration of 10 μg/ml. The other tissue sample was used as a control and incubated in sterile Dulbecco's modified Eagle's medium in the absence of LPS. Both challenged and unchallenged tissues were incubated at 37°C for 90 min before being removed, blotted dry and weighed. Approximately, 1000–2000 mg of the porcine ileum and colon tissues were cut into small pieces and stored in 15 ml of RNAlater® (Applied Biosystems, Foster City, CA, USA) overnight at 4°C. The RNAlater® was then removed before storing the samples at −80°C.

RNA extraction and complementary DNA synthesis

RNA was extracted from approximately 50 mg tissue samples using the GenElute Mammalian Total RNA Miniprep Kit (Sigma Aldrich Corporation) according to the manufacturer's instructions. The purity of the total RNA was analysed using 1 μl of total RNA on a NanoDrop spectrophotometer ND1000 (Thermo Scientific, Wilmington, DE, USA), and samples with a 260:280 ratio ≥ 2·0 were considered suitable for complementary DNA (cDNA) synthesis. Total RNA integrity (i.e. quality and quantity) was also assessed by analysing 1 μl of total RNA using the Agilent 2100 Bioanalyser version A.02.12 (Agilent Technologies, Inc., Santa Clara, CA, USA) using RNA Nano LabChips® (Caliper Technologies Corporation, Mountain View, MA, USA).

The cDNA synthesis was performed using 1 μg of total RNA and oligo$(dT)_{20}$ primers in a final reaction volume of 20 μl using a Superscript™ III First-Strand synthesis Kit (Invitrogen Life Technologies, Carlsbad, CA, USA) following the manufacturer's instructions.

Real-time quantitative PCR and normalisation of quantitative PCR data

All primers for selected cytokines; genes such as interferon γ, *IL-1α*, *IL-6*, *IL-8*, *IL-10*, *IL-17*, *TNF-α*, the mucin genes

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Table 2. Quantitative PCR primer details

	Accession no.	Forward primer (5′–3′)	Melting temperature (°C)	Reverse primer (5′–3′)	Melting temperature (°C)	Product length (bp)	Efficiency (%)	R^2 of PCR efficiency
Reference genes								
ACTB	XM_001928093.1	GCACGGCATCATCACCAA	52·75	CCGGAGCTCGTTGTAGAAGGT	55·99	70	95·02	1
Cyclophilin	NM_214353.1	CGGGTCCTGGCATCTTGT	62·1	TGGCAGTGCAAATGAAAACT	60·7	75	100·26	1
GAPDH	AF017079.1	CAGCAATGCCTCCTGTACCA	62·2	ACGATGCCGAAGTTGTCATG	62·1	72	104·15	1
Cytokine genes								
IFN-γ	NM_213948.1	TCTAACCTAAGAAAGCGGAAGAGAA	61·12	TTGCAGGCAGGATGACAATTA	61·54	81	94·4	0·99
IL-1α	NM_214029.1	CAGCCAACGGGAAGATTCTG	63·0	ATGGCTTCCAGGTCGTCAT	60·49	76	106·6	0·98
IL-6	AB194100	AGACAAAGCCACCACCCCTAA	55·27	CTCGTTCTGTGACTGCAGCTTATC	59·92	69	99·99	0·86
IL-8	NM_213867.1	TGCACTTACTCTTGCCAGAACTG	61·9	CAAACTGGCTGTTGCCTTCTT	61·7	82	95·7	0·98
IL-10	NM_214041.1	GCCTTCGGCCCAGTGAA	63·4	AGAGACCCGGTCAGCAACAA	63·1	71	95·7	0·98
TNF-α	NM_214022.1	TGGCCCCTTGAGCATCA	62·5	CGGGCTTATCTGAGGTTTGAGA	62·8	68	91·5	1
Mucin genes								
MUC1	XM_001926883.1	ACACCCATGGGCGCTATGT	63·6	GCCTGCAGAAACCTGCTCAT	62·8	68	105·63	1
MUC2	AK231524	CAACGGCCTCTCCTTCTCTGT	63·1	GCCACACTGGCCCTTTGT	62·1	70	88·57	0·99
MUC4	XM_001926442.1	GATGCCCTGGCCACAGAA	63·3	TGATTCAAGGTAGCATTCATTTGC	62·4	89	93·57	0·98
MUC5AC	AF054584	CCCCCTCGTCTCCTTTTACC	62·1	GGATGTCGCCAGAGACTGAGTA	61·7	71	96·99	1
MUC12	FJ715471	GACTAACAAGAATTTCACAAAAGAGCTAA	60·8	GCCATCTGAGTCTTGAACTTTTGA	61·97	87	92	1
MUC13A	NM_001105293.1	AAGCTCCCCAGGATTGTTCA	61·9	TGCATGCTATCAACTCAAAGC	59·1	63	110·02	1
MUC20	NM_001113440	AGGCAGTTACAACATCCACAGAAG	61·8	CTGTAGACCATGGCCGAGAAC	61·97	82	84·24	1

(*MUC 1, 2, 4, 5AC, 12, 13* and *20*) and three porcine reference genes, β-actin (*ACTB*), glyceraldehyde-3-phosphate dehydrogenase and peptidylprolyl isomerase A, were designed using Primer Express™ (PE Applied Biosystems) and synthesised by MWG Biotech (Milton Keynes, UK). Specificity was established *in silico* using BLAST and confirmed by examining the dissociation curves for each primer set. The efficiencies of all primer sets were established using a semi-log curve of quantity *v.* C_t of twofold serial dilutions of cDNA (Table 2). Quantitative PCR was then carried out on cDNA using the ABI PRISM 7500 Fast sequence detection system for ninety-six-well plates (Applied Biosystems). All samples were prepared in duplicate using SYBR Green Fast PCR Master Mix (Applied Biosystems), cDNA as the template and specific primers for the genes selected. For each reaction, 5 μl cDNA, 1·2 μl forward and reverse primer mix (5 μM) and 10 μl Fast SYBR Green PCR Master Mix (PE Applied Biosystems) were added and made up to a final volume of 20 μl. The two-step PCR programme was as follows: 95°C for 10 min for one cycle, followed by 95°C for 15 s and 60°C for 1 min for forty cycles.

The raw C_t values for the reference genes were converted to relative quantities using the formula $Q = E\Delta C_t$, where E is the PCR efficiency of the assay and ΔC_t is the value calculated for the difference between the lowest C_t value and the C_t value of the sample in question for each gene. The relative quantities of the endogenous controls were then analysed for stability in geNorm[27]. The stability '*M*' value generated by the geNorm application for the selected endogenous controls (β-actin, glyceraldehyde-3-phosphate dehydrogenase and peptidylprolyl isomerase A), which was less than 1·5, indicated their suitability as endogenous controls for these intestinal samples. The geometric mean of the relative quantities for β-actin, glyceraldehyde-3-phosphate dehydrogenase and peptidylprolyl isomerase A (normalisation factor) was then calculated using geNorm. The relative quantities were divided by the normalisation factor (obtained in geNorm) for that sample to give the final normalised relative expression for each target gene. Results for both the unchallenged and challenged experiments are presented in Tables 3 and 4 as the least-square means of fold change of normalised relative gene expression with their standard errors.

Statistical analysis

All data from the experiment were analysed as a complete randomised design using the General Linear Model procedure of the Statistical Analysis Systems Institute (1985, version 6.11; SAS Institute, Cary, NC, USA)[28]. The statistical model used for animal performance and nutrient digestibility, microbiology and cytokine gene expression data analysis included both the linear and quadratic effects of laminarin inclusion levels. Metabolic live weight (live weight$^{0·75}$) was included as a covariate in the models. All

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Table 3. Effect of increasing inclusion levels of laminarin extracted from *Laminaria digitata* on the immune response in unchallenged ileum and colon tissues

(Least-square means of fold change of normalised relative gene expression with their standard errors; *n* 7 animals)

L. digitata inclusion level	0 ppm	300 ppm	600 ppm	SEM	Significance*	
					Linear	Quadratic
Ileum						
IFN-γ	1·000	1·278	0·841	0·233	NS	NS
IL-1α	1·000	1·141	0·597	0·148	NS	NS
IL-6	1·000	1·681	0·908	0·252	NS	NS
IL-8	1·000	1·003	0·475	0·245	NS	NS
IL-10	1·000	1·128	0·646	0·229	NS	NS
TNF-α	1·000	1·023	0·805	0·133	NS	NS
Colon						
IFN-γ	1·000	1·217	1·148	0·286	NS	NS
IL-1α	1·000	0·716	0·851	0·144	NS	NS
IL-6	1·000	1·579	1·788	0·434	NS	NS
IL-8	1·000	1·245	1·137	0·224	NS	NS
IL-10	1·000	1·029	0·843	0·285	NS	NS
TNF-α	1·000	1·400	1·446	0·301	NS	NS

ppm, Parts per million; IFN, interferon.
* Probability of significance: NS ($P>0·05$).

the data were checked initially for outliers and normality using the PROC Univariate procedure of the SAS Institute (version 6.11; SAS Institute)[28]. The microbial counts were log-transformed before statistical analysis. The least-square difference was used to separate means. The data in the tables are presented as least-square interaction means with their standard errors. The probability value, which denotes significance, is $P<0·05$.

Results and discussion

It is imperative that dietary supplementation does not negatively influence either performance traits or gut health. It would also be beneficial if the supplement could exhibit biological properties that may benefit the host in times of pathogen invasion. In the present study, 300 ppm laminarin did not affect performance or nutrient digestibility. It did not affect the selected bacteria in the ileum, but decreased enterobacteriaceae in the colon. Of particular interest in the present study was the fact that 300 ppm laminarin increased *IL-6* and *IL-8* cytokine gene expression in colonic tissue following an LPS challenge. This supports the concept of collaborative signalling between TLR and non-TLR pattern-recognition receptors in the intracellular response to laminarin[29].

Animal performance and nutrient digestibility

There was no effect on animal performance (food intake, daily gain or food conversion ratio) or digestibility coefficients of DM, organic matter, ash, N or gross energy with increasing dietary inclusion levels of laminarin ($P>0·05$; results not shown). This lack of an effect of laminarin on any of these performance parameters is important, as any natural alternative feed supplement in the porcine diet should not compromise the host in any way. Gardiner *et al.*[20] reported linear decreases in daily gain, as the level of the *Ascophyllum nodosum* extract increased in healthy grower–finisher pigs. However, this extract also contained phenolic compounds and alginates and led the authors to conclude that unless the process of extraction removed these components, its use as a commercial feed additive would be limited. As a result, the seaweed extract used in the current experiment contained only laminarin.

Microbiology and volatile fatty acids

As a large number of intestinal bacterial species are unculturable[30], bifidobacteria and lactobacilli were enumerated as a reflection of changes in the population structure of beneficial bacteria. The relevance of measuring enterobacteriaceae populations as an indicator of pathogenic bacteria is debated; however, increased coliform counts were recorded in the intestine of scouring pigs[31] and the density of coliforms in the gastrointestinal tract is used as an indicator of *Salmonella* and *Escherichia coli* in pigs[32–34]. Hence, coliform reductions due to dietary intervention are, within limits, considered by many to be beneficial[20,32,35].

Increasing the level of laminarin from 0 to 600 ppm had no effect on the bifidobacteria, lactobacilli or enterobacteriaceae populations in the ileum ($P>0·05$; Table 5). While there was no effect on bifidobacteria or lactobacilli populations with increasing levels of laminarin in the colon, there was a decrease at 300 ppm (quadratic; $P=0·0421$) in enterobacteriaceae populations with no further decrease at 600 ppm. While the majority of enterobacteriaceae are commensals, the potential to reduce harmful enterobacteriaceae

Table 4. Effect of increasing levels of laminarin extracted from *Laminaria digitata* on the immune response in the ileum and colon following an *ex vivo* lipopolysaccharide tissue challenge

(Least-square means of fold change of normalised relative gene expression with their standard errors; *n* 7 animals)

L. digitata inclusion level	0 ppm	300 ppm	600 ppm	SEM	Significance*	
					Linear	Quadratic
Ileum						
IFN-γ	1·000	0·927	1·098	0·223	NS	NS
IL-1α	1·000	1·072	1·039	0·161	NS	NS
IL-6	1·000	1·352	1·143	0·266	NS	NS
IL-8	1·000	1·186	0·903	0·362	NS	NS
IL-10	1·000	1·198	1·076	0·160	NS	NS
TNF-α	1·000	0·819	0·921	0·108	NS	NS
Colon						
IFN-γ	1·000	2·051	1·614	0·385	NS	NS
IL-1α	1·000	0·983	1·242	0·199	NS	NS
IL-6	1·000	1·846	0·830	0·272	0·0369	0·0289
IL-8	1·000	1·590	1·948	0·303	0·0245	NS
IL-10	1·000	0·936	1·039	0·256	NS	NS
TNF-α	1·000	1·557	0·938	0·250	NS	NS

ppm, Parts per million; IFN, interferon.
* Probability of significance: NS ($P>0·05$).

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Table 5. Effect of increasing levels of seaweed extract on selected bacterial populations in the ileum, proximal and distal colon (log_{10} colony-forming units/g digesta with their standard errors) and total volatile fatty acids (VFA) in the ileum, caecum and proximal colon of the pig

(Least-square means with their standard errors; *n* 7 animals)

					Significance*	
Laminarin inclusion level	Control	300 ppm	600 ppm	SEM	Linear	Quadratic
Ileum						
Bifidobacteria	4·94	5·44	5·51	0·708	NS	NS
Lactobacilli	4·14	5·15	5·40	0·565	NS	NS
Enterobacteriaceae	2·24	3·35	2·86	0·839	NS	NS
Colon						
Bifidobacteria	7·37	7·29	7·46	0·365	NS	NS
Lactobacilli	7·89	8·16	8·19	0·221	NS	NS
Enterobacteriaceae	5·42	3·87	4·24	0·358	0·0345	0·0421
Total VFA						
Ileum	10·47	14·84	14·26	2·60	NS	NS
Caecum	173·5	189·2	194·4	9·70	0·0489	NS
Colon	185·4	146·4	161·8	13·79	NS	NS

ppm, Parts per million.
* Probability of significance: NS ($P>0·05$).

strains without influencing bifidobacteria and lactobacilli numbers is of great significance to the pig industry. The fact that this laminarin source had no effect on bifidobacteria and lactobacilli numbers is supported by the results of Lynch *et al.*[16] who supplemented the diet with a similar laminarin source. These data are in contrast to previous studies using crude extracts of seaweeds including *L. digitata*, a combination of *L. digitata* and *L. hyperborea*[36], and *A. nodosum*[20] which all resulted in a general anti-microbial effect on all bacterial species examined.

As the amount and composition of the resident microbiota and fermentable substrate affect the quantity and composition of VFA produced in the large intestine[37], VFA and digesta pH were analysed as a further indicator of gut health. Seaweed-derived polysaccharides are considered to be a source of dietary fibre as they are resistant to hydrolysis by digestive enzymes in the upper gastrointestinal tract[9], which explains the low concentration of VFA in the ileum of laminarin-supplemented pigs. There was a significant increase in total VFA with increasing levels of laminarin in the caecum (linear; $P=0·0489$). These results concur with Reilly *et al.*[36] who reported that the highest total VFA concentration was in the caecum with *L. digitata*-derived seaweed extracts. There was no significant alteration in digesta pH recorded from any of the intestinal regions sampled (data not shown).

Mucin gene expression

Dietary factors such as fibre, protein and anti-nutritional factors are known to directly influence the synthesis and secretion of mucin from goblet cells and the recovery of mucin in digesta[38–40]. As mucin composition can be influenced by post-translational events such as bacterial fermentation, the direct impact of laminarin ingestion was examined by looking at the gene expression profile of key mucin genes (Table 6).

All seven mucin gene transcripts were reliably detected in the porcine colon, but only five of the seven gene transcripts were accurately quantifiable in the ileum (*MUC1* and *MUC5AC* were undetectable). An increase in *MUC2* was observed in the ileum of pigs supplemented with 300 ppm laminarin (quadratic; $P=0·05$) but not at the higher dietary inclusion level. Laminarin supplementation had no effect on the remaining detectable mucin genes (*MUC4*, *MUC12*, *MUC13* and *MUC20*) in the ileum. In the colon, dietary supplementation with laminarin at 600 ppm significantly increased *MUC2* ($P=0·0365$) and *MUC4* ($P=0·0401$) expression compared with the control and the 300 ppm laminarin-supplemented animals. The fact that the 600 ppm laminarin inclusion level increased the expression of two genes involved with mucin synthesis is of interest.

In human subjects, maintaining a balance in mucin production and excretion in the gut is important. A number of hypotheses are proposed as the exact mechanisms by which laminarin exerts its affects on mucin gene expression are, as yet, unknown. The induced gene up-regulation of *MUC2* in the ileum and *MUC2* and *MUC4* in the colon may be attributed to the solubility of laminarin[7] allowing it to exert a direct effect at a cellular level, because laminarin delivered orally can bind directly and be internalised by intestinal epithelial cells and gut-associated lymphoid tissue cells in the murine model[41]. In addition to other colonic functions such as water and mineral absorption, indigestible dietary fibres are also broken down in the colon by resident microbiota. Also, since laminarin is regarded as a source of dietary fibre, the increase in *MUC2* and *MUC4* gene expression may be due to direct stimulation of the colonic mucosa by the increased level of dietary fibre at 600 ppm. This

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Table 6. Effect of increasing levels of laminarin extracted from *Laminaria digitata* on mucin gene expression in the ileum and colon

(Least-square means of fold change of normalised relative gene expression with their standard errors; *n* 7 animals)

L. digitata inclusion level	0 ppm	300 ppm	600 ppm	SEM	Significance*	
					Linear	Quadratic
Ileum						
MUC2	1·000	1·910	0·823	0·317	0·0421	0·0256
MUC4	1·000	2·287	1·697	0·637	NS	NS
MUC12	1·000	1·096	2·311	0·776	NS	NS
MUC13	1·000	0·558	1·062	0·326	NS	NS
MUC20	1·000	1·115	0·870	0·227	NS	NS
Colon						
MUC1	1·000	0·902	1·270	0·098	NS	NS
MUC2	1·000	0·726	1·207	0·145	0·0461	0·0365
MUC4	1·000	0·981	1·351	0·097	0·0482	0·0401
MUC5AC	1·000	2·134	0·285	0·349	NS	NS
MUC12	1·000	0·957	1·120	0·193	NS	NS
MUC13	1·000	0·924	1·077	0·272	NS	NS
MUC20	1·000	1·107	1·063	0·122	NS	NS

ppm, Parts per million.
* Probability of significance: NS ($P>0·05$).

up-regulation is consistent with the protective role of mucins in the formation of a 'gut barrier' after mucosal stimulation by dietary fibres[42]. Additionally, alteration in intestinal microbiota may also influence mucin synthesis and secretion, as adherence of beneficial bacteria to mucosal epithelia stimulates up-regulation of colonic *MUC2* expression *in vivo* [43] and *in vitro* [44]. Therefore, laminarin supplementation may have increased *MUC2* and *MUC4* expression indirectly by acting as a substrate for the resident microbiota, which, in turn, up-regulated mucin production.

The increase in mucin gene expression in the present study was interpreted as being beneficial for these animals. Other dietary inclusion studies with different varieties of brown seaweeds (such as *A. nodosum*) have been shown to increase digesta viscosity, leading to decreased diffusion and efficacy of digestive enzymes, thus leading to an overall decrease in average daily gain in pigs[20]. As there was no reduction in average daily gain and all animals remained healthy throughout the course of the study, we propose that increased mucin gene expression may serve to ameliorate mucin turnover, thus maintaining and enhancing the protective mucosal layer in the gastrointestinal tract of these animals. However, further studies measuring mucosal viscosity and/or thickness are required to substantiate this theory.

Cytokine gene expression

There were no effects of the laminarin inclusion level in unchallenged ileum or colon tissue for any of the cytokines analysed (Table 3). This overall lack of an effect on these inflammatory markers, similar to the lack of effect on animal performance, implies that the presence of laminarin in the diet did not elicit any deleterious effects on either of these two parameters.

To mimic the response of the ileal and colonic tissues of animals exposed to laminarin to a microbial challenge, these tissues were subsequently incubated with LPS *ex vivo*. While no effect was observed in the ileum (Table 3), a significant challenge effect was observed for *IL-6* and *IL-8* gene expression in the colon of LPS-challenged tissue (Table 4). Dietary laminarin inclusion led to an increase in *IL-6* expression (quadratic; $P=0·0289$), while a linear increase in *IL-8* gene expression was observed ($P=0·0245$). These data suggest that inclusion of laminarin in the diet could enhance the pro-inflammatory response to a microbial challenge. The potential benefit of this enhanced gene up-regulation of IL-6 and IL-8 cytokines following the LPS challenge is significant for the host, as IL-6 is a pro-inflammatory cytokine that plays an important role in acute inflammation in the early immune response[45]. Similarly, chemokine IL-8 also plays an important role in acute inflammation and is responsible for neutrophil recruitment and activation to the initial site of infection[46].

While dietary exposure to laminarin did not stimulate pro-inflammatory cytokine production in the gastric mucosa, it enhanced the LPS-induced pro-inflammatory cytokine production in colonic tissue. These data are of biological interest as they support the concept of collaborative signalling between TLR and non-TLR pattern-recognition receptors in immune stimulation[29]. LPS induces pro-inflammatory cytokine production via TLR4, while laminarins bind to non-TLR pattern-recognition receptors such as dectin-1, complement receptor 3, lactosylceramide and scavenger receptors[47]. The dectin-1 receptor pathway can collaborate via the Syk kinase pathway with a number of MyD88-coupled TLR, including TLR4, to enhance the production of pro-inflammatory cytokines in response to pathogen recognition[48]. Hence, the activation of the dectin-1 pathway by laminarin, coupled

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with the activation of TLR4 by LPS, would explain the enhanced IL-6/IL-8 response. Notwithstanding the fact that the dectin-1 laminarin receptor has recently been identified and characterised in this model[49], Kikkert *et al.*[50] found that small β-glucan molecules, such as laminarin, while having co-stimulatory effects, were not acting through the dectin-1 pathway, and CR3 has been identified as the major receptor for β-glucan molecules on human neutrophils[51].

Conclusions

Laminarin extracted from the brown seaweed, *L. digitata*, at both dietary inclusion levels of 300 and 600 ppm, exerted no deleterious effects on animal performance or immune function in the unchallenged subjects, as all animals remained healthy throughout the course of the present study. Both inclusion levels also increased mucin gene expression and enhanced IL-6 and IL-8 cytokine expression in response to an *ex vivo* LPS challenge. However, the dietary inclusion of 300 ppm laminarin had the added benefit of reducing potential pathogenic enterobacteriaceae populations in the colon. Therefore, we conclude that 300 ppm was more beneficial than the 600 ppm inclusion level for the animals in the present study. Future studies using several laminarin concentrations will assess the potential of laminarin dietary supplementation to suppress a deliberate *in vivo* pathogen challenge.

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