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Effects of *Ascophyllum nodosum* supplementation on *Campylobacter jejuni* colonisation, performance and gut health following an experimental challenge in 10 day old chicks

T. Sweeney [a,*], H. Meredith [a], M.T. Ryan [a], V. Gath [a], K. Thornton [a], J.V. O'Doherty [b]

[a] School of Veterinary Medicine, Belfield, University College Dublin, UCD, Ireland
[b] School of Agriculture and Food Science, Belfield, University College Dublin, UCD, Ireland

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ABSTRACT

The objective was to determine the effects of nutritional supplementation of an extract of *Ascophylum nodosum* on *Campylobacter jejuni* colonisation and performance following an experimental challenge in 10 day old chicks. The experiment consisted of three diets: 1) basal diet, 2) basal diet + 500 ppm extract and 3) basal diet + 1000 ppm extract. Day old Ross chicks (n = 135) were housed in groups of three, with 15 replicates per treatment group. On day three, all chicks were orally gavaged with 0.1 ml 10^6 colony forming units of *C. jejuni*. Following humane sacrifice on day 10, caecal, ileal and faecal samples were collected for analysis. While supplementation with *A. nodosum* reduced *C. jejuni* counts in the caecum of the chicks at both concentrations ($P < 0.05$), decreases in growth parameters were also observed ($P < 0.05$). Supplementation with 500 ppm had no effect on the gut morphology in the ileum ($P < 0.05$), while potential disruptive effects were observed at 1000 ppm. However, there were significant increases in the expression of tight-junction genes *OCLN* and *CLND-1* alongside increases in *MUC2 and CCND1* expression ($P < 0.05$). In conclusion, an extract of *A. nodosum* is beneficial in reducing the caecal bacterial load in chickens colonised with *C. jejuni*. The negative impact on growth parameters at 10 days is a concern, however optimisation of both the timing of administration and dosage of this seaweed extract could circumvent the negative impact on growth, while capitalising on its antimicrobial effects.
Industrial relevance: The subject of this paper is of relevance to the Poultry Industry, as the described seaweed extracts decreased campylobacter levels in the caecum. As campylobacter is concentrated in the caecum it can pose a significant carcass contamination risk during processing. This paper is also relevant to the seaweed industry as it highlights more applications for natural seaweed products.

1. Introduction

Campylobacteriosis is the most prevalent foodborne gastroenteritic disease in humans in industrialised societies. Up to 80% of human cases of *Campylobacteriosis* in humans are caused by *Campylobacter jejuni*, a zoonotic gram-negative commensal microorganism which occurs naturally in poultry (Tauxe, 2002). Infected birds are known to harbour very high *C. jejuni* loads in their gastrointestinal (GI) tract, and in particular in the caecum. Inevitably, during processing, the risk of transmitting *C. jejuni* from the GI tract to the carcass itself is high, hence, reducing or limiting *C. jejuni* present in the GI tract prior to slaughter is a strategy which could have significant impact on its control and prevalence at the processing level (Diarra and Malouin, 2014).

In the past *C. jejuni* was controlled by the prophylactic use of antibiotic growth promoters (AGPs). These substances have been banned in EU member states from 1st January 2006 (Huyghebaert, Ducatelle, and Van Immerseel, 2011). Enhanced efforts are now being made to find natural alternative to AGPs. To date the number of alternatives to AGPs that has been explored is diverse and includes exogenous enzymes, probiotics, prebiotics and plant derived bioactive compounds (Diaz-Sanchez, D'Souza, Biswas, and Hanning, 2015; Huyghebaert et al., 2011). With respect to *C. jejuni*, several natural compounds have been explored with the aim of achieving reductions within the GI tract including trans-cinnamaldehyde (Hermans et al., 2011), thymol (Epps et al., 2015) extracts of plant phenolic compounds, (Klancnik, Mozina, and Zhang, 2012) and benzyl isothiocyanate (Dufour, Stahl, Rosenfeld, Stintzi, and Baysse, 2013), all with varying success.

The brown seaweed *Ascophylum nodosum* contains a number of bioactive compounds such as polysaccharides including mannitol, laminarin and fucoidan, polyphenols including phlorotannins, vitamins and trace minerals (MacArtain, Gill, Brooks, Campbell, and Rowland, 2007). Studies on specific extracts of *A. nodosum* have highlighted a variety of potential bioactive properties, including bactericidal (Gardiner et al., 2008; Jiménez, O'Connell, Lyons, Bradley, & Hall, 2010), anti-inflammatory and immunomodulatory (Bahar, O'Doherty, Smyth,

* Corresponding author.
E-mail address: torres.sweeney@ucd.ie (T. Sweeney).

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Ahmed, & Sweeney, (submitted)), antioxidant (Apostolidis and Lee, 2010; Jiménez et al., 2010), anticoagulant (Mauray et al., 1995) and anti-metastatic (Abu et al., 2015). Of relevance to the development of natural compounds that can reduce *C. jejuni* counts in chicks is the anti-bacterial and immunomodulatory properties. Numerous mechanisms for the anti-bacterial properties of seaweeds have been proposed (Smith et al., 2011; Sweeney et al., 2012), with some studies focussing on the directly bactericidal properties of the seaweed and others focussing on the immunomodulatory effects of the seaweed on receptive cells in the host. Many components within the seaweed are directly bactericidal in-vitro and in-vivo, including phlorotannins (Braden, Blanton, Allen, Pond, and Miller, 2004; Hierholtzer, Chatellard, Kierans, Akunna, and Collier, 2013; Wang, Xu, Bach, and McAllister, 2009). In addition, other components, such as β-glucans and fucoidans have immunemodulatory effects within host tissues and are capable of binding to receptors which have the capacity to modify downstream immune responses (Bahar et al., (submitted)). In fact, the *A. nodosum* animal feed supplement Tasco® has been reported to modulate GI microbiota through direct activation of the immune system (Kandasamy, Khan, Evans, Critchley, and Prithiviraj, 2012).

Hence the main objective of this study was to determine if an extract of *A. nodosum* that has anti-*C. jejuni* activity in-vitro can reduce *C. jejuni* numbers in the caecum of experimentally infected chicks. In addition, the effects of the *A. nodosum* extract on overall gut health and immune parameters were examined in the growing chicks.

2. Materials and methods

2.1. Experimental design

The extract of *A. nodosum* used in this experiment (BioAtlantis Ltd., Tralee, Co. Kerry, Ireland) inhibited *C. jejuni* growth in-vitro (zone of inhibition 18 mm) in a Mueller–Hinton agar spot assay (pH 7.2 and 7.4). The extract (w/w) contained the following known bioactives: Fucoidin 10.73%, Phlorotannin 7.4%, Mannitol 4.72%, Laminaran 2.48%, and Alginate 2.11%.

The experiment was a complete randomised design consisting of three treatment groups: 1) basal diet; 2) basal diet supplemented with 500 ppm *A. nodosum* and 3) basal diet supplemented with 1000 ppm *A. nodosum* (BioAtlantis Ltd., Tralee, Co. Kerry, Ireland). Day old Ross chicks (n = 135) were sourced from Carton Bros (Monaghan, Ireland). Chicks had an initial weight of 47 g (s.d. ± 4.11 g). Chicks were penned in groups of three, with 15 replicates per treatment group. Each group of chicks had ad libitum access to feed and water. Food consumption and refusal were recorded daily throughout the trial. The house temperature was maintained at 30 °C for the first 7 days and then reduced to approximately 28 °C for the remainder of the trial.

2.2. Preparation of Campylobacter inoculum

Five poultry *C. jejuni* isolates obtained from poultry abattoirs were cultured in 30 ml Hunts Broth (Nutrient broth (Oxoid, Basingstoke, UK) and Yeast Extract (Oxoid, Basingstoke, UK), 5% lysed horse blood and *Campylobacter* growth supplement (Oxoid, Basingstoke, UK) and incubated at 42 °C for 48 h in microaerobic (5% O_2, 10% CO_2 and 85% N_2) conditions using gas generating kits (Biomerieux, Marcy l'Etoile, France). From the resultant suspensions, 1 ml samples were re-suspended in 100 ml volumes of Hunt's Broth, and incubated at 42 °C for 24 h. The *C. jejuni* was recovered by centrifugation at 8000 rpm for 10 min, the pellet was washed 3 times in 9 ml PBS and finally re-suspended in 50 ml PBS, combined and diluted to 500 ml *Campylobacter* suspension.

2.3. Chick challenge trial

All chicks (n = 135) were orally gavaged with PBS containing 0.1 ml 10^6 colony forming units (CFU) of *C. jejuni* using a ball point needle on day three post-hatching. Fresh faecal samples were taken from the chicks two days post challenge (PC). The chicks were humanely sacrificed on day 10 by cervical dislocation.

2.4. Gut morphological analysis

Directly following slaughter, the digestive tract was removed and sections of the ileum were excised and fixed in 10% phosphate-buffered formalin from 15 chicks (one chick was taken at random from the groups of three). The preserved samples were prepared using standard paraffin-embedding techniques, sectioned at 5 μm thickness, and stained with haematoxylin and eosin for morphological assessment. Villus height (VH), villus width (VW) and crypt depth (CD) measurements were attained in the stained sections (4× objective) from fifteen well-orientated and intact villi using a light microscope fitted with an image analyser (Image-Pro Plus; Media Cybernetics). Measurements of fifteen well-orientated and intact villi and crypts were taken for each segment. VH was measured from the crypt–villus junction to the tip of the villus, and CD was measured from the crypt–villus junction to the base. Results are expressed as mean VH or CD in μm.

2.5. Microbial analysis

Faeces and digesta from the caecum were recovered and immediately stored at −20 °C (n = 135/45 per group, respectively). Genomic DNA was extracted using the QIAamp stool kit (Qiagen, West Sussex, UK) in accordance with manufacturer's instructions. The quantity and quality of DNA were assessed using the Nanodrop™ spectrophotometer (Thermo Scientific, Wilminton, DE, USA).

2.6. Generation of standards for absolute quantification of C. jejuni

A 1171 bp fragment within the *C. jejuni* cCon gene was amplified from genomic sequence genbank: gi|384440681, region (1400963-1402133). The primer pairs were designed using Primer 3 (http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/); Forward: CCCGCAATCCACATTGAAGC and Reverse: TCGGCATTGTTGGTATGGCT; cycling conditions; 95 °C, 2 min followed by 30 cycles; 95 °C for 30 s, 58° for 30 s and 72 °C for 2 min and a final extension step of 72 °C for 5 min. The amplified fragment was ligated into a vector using TOPO® Cloning kit (Invitrogen Corp., San Diego, CA, USA) and was transformed into One Shot® *Escherichia coli* (Invitrogen Corp., San Diego, CA, USA) which was then cultured overnight at 37 °C on LB agar containing Ampicillin (100 μg/ml). Colonies were screened directly for the presence of the insert using PCR conditions outlined above and positive clones were then re-cultured in a shacking incubator at 37 °C overnight in 100 ml LB broth containing Ampicillin (100 μg/ml). The transformed vector was then purified using the QIAGEN Plasmid *Plus* Maxi kit (Qiagen Ltd., Crawley, UK)). The purified plasmid (250 ng) was linearised using APA1 restriction enzyme (New England Biolabs, Ipswich, MA, USA). The linearised vector was electrophoresed on an ethidium bromide stained 2% agarose gel and visualised to confirm complete linearisation. The linearised plasmid was further purified using GenElute™ PCR clean-up kit (Sigma-Aldrich Corp., St. Louis, MO, USA) and re-quantified. The spectrometrically measured quantities were converted to copy number/μl using the online tool (http://cels.uri.edu/gsc/cndna.html) which employs the formula: number of copies = (amount ∗ 6.022×10^{23}) / (length ∗ 1×10^9 ∗ 650), where the total length of the vector and insert was 5102 bp. Ten-fold serial dilutions ranging from 10^8 to 10^1 were then prepared using Tris-EDTA buffer containing 5 ng/μl of Lambda DNA were then used as standards to enable the absolute quantification of the unknown samples.

Primers used for the quantification of the cCon gene were designed on Primer Express™ (Applied Biosystems, Foster City, CA) (Table 1). The primer pairs and all QPCR reactions were performed in duplicate in 96-well optical plates on 7500 ABI Prism Sequence Detection System