Research Note: Effect of a phlorotannin extract of the brown seaweed *Ascophyllum nodosum* as a potential control strategy against *Campylobacter* in broilers

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ABSTRACT Poultry is generally recognized as the main source of human campylobacteriosis and *Campylobacter* is highly prevalent at the farm level. To reduce the relative risk of human campylobacteriosis attributable to broiler meat, it is necessary to reduce *Campylobacter* loads in broiler ceca but to date, no effective, reliable and practical strategy is available. The marine environment is a rich source of original natural compounds exhibiting different biological activities. The objective of this study was to test a phlorotannin extract of the brown seaweed *Ascophyllum nodosum* as a potential control strategy against *Campylobacter* in broilers. Bactericidal activity has been demonstrated *in vitro*, on several *Campylobacter* spp. strains at a range of 0.06 to 0.47 mg/mL. Therefore, an *in vivo* trial in experimental facilities was performed to evaluate addition of 0.2% (w/w) of an *A. nodosum* extract to feed distributed at the end of rearing from day 31 to day 35, and to assess the effect on artificial *Campylobacter jejuni* colonization. No statistical differences in *Campylobacter* enumeration were observed between the treated and control groups. Another trial was performed in a commercial broiler flock. Feed containing the extract at 0.2% (w/w) (2 kg/t) was distributed during the last 5 days of rearing (day 33–day 38). No significant effects on *Campylobacter* colonization and on growth parameters were observed compared to the control group. Additional studies are needed to assess whether active polyphenols are found in the cecum.

Key words: *Campylobacter*, *Ascophyllum nodosum*, control strategy, poultry

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INTRODUCTION

Campylobacter spp. are the causative agents of campylobacteriosis, the most common zoonosis in Europe with 220,682 reported cases in 2019 (EFSA, ECDC, 2021). Poultry is generally recognized as the main source of human campylobacteriosis and could be responsible for 50% to 80% of human cases (EFSA European Food Safety Authority, 2020). *Campylobacter* is highly prevalent at the broiler farm level in Europe and reducing *Campylobacter* spp. loads by 3-log10 in broiler ceca would reduce the public health risk by at least 58%, as estimated by EFSA experts (EFSA European Food Safety Authority, 2020). Moreover, a process hygiene criterion (**PHC**) for *Campylobacter* in broiler carcasses has been in place in Europe for food business operators since January 2018 (Commission Regulation (EU) 2017/1495; http://data.europa.eu/eli/reg/2017/1495/oj). This PHC sets a critical limit of 1,000 CFU/g on neck skin after chilling, and has led to stepping up of efforts to reduce *Campylobacter* on farms. To date, there is no effective, reliable and practical strategy available but according to the recent opinion of EFSA, bacteriophages, vaccination feed structure and feed or water additives are possible control options to reduce *Campylobacter* in the birds at primary production level (EFSA European Food Safety Authority, 2020). Several studies have tested different types of products such as organic acids, probiotics, prebiotics, and plant extracts, and some of them have appeared to be promising during in vivo trials in experimental facilities (Guyard-Nicodème et al., 2016). However, experimental testing during field trials is lacking or did not produce the same effect, making it difficult to draw conclusions on the actual potential of these compounds.

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The marine environment is a rich source of original natural compounds exhibiting different biological activities. *Ascophyllum nodosum* (*A. nodosum*) is a brown alga from the *Fucaceae* family exclusive to the North Atlantic (Catarino et al., 2017) and commonly found on the northwest coast of France. As a member of the *Fucaceae, A. nodosum* contains phlorotannins, a class of marine phenolic compounds that are oligomers of phloroglucinol, particularly found in brown algae. Phlorotannins can account for 20% of the dry matter in *A. nodosum* (Bocanegra et al., 2009). During the last few years, there has been increasing interest in phlorotannins as they exhibit various biological activities, such as antioxidant, anti-inflammatory, antidiabetic, and bactericidal activities (Catarino et al., 2017). An extract of *A. nodosum* demonstrated a beneficial effect in reducing the cecal bacterial load in 10-day-old chicks colonized with *C. jejuni* (Sweeney et al., 2016).

The aim of this study was to evaluate the potential use of a phlorotannin extract of *A. nodosum* as a potential control strategy against *Campylobacter* in poultry. The minimum bactericidal concentration (**MBC**) was determined in vitro; then, in vivo trials were carried out in experimental facilities and in a conventional flock to determine the ability of the algal extract as a feed additive to reduce *C. jejuni* colonization in broilers at the end of rearing.

MATERIALS AND METHODS

Extract Preparation and Purification

Freshly harvested *A. nodosum* was soaked overnight in seawater for cleaning and removal of foreign bodies and epiphytic seaweed, then strained and rinsed with tap water. After additional draining, the seaweed was milled to 2 to 3 mm particles (coarse grinding with 66,708 grid, followed by fine grinding with 66,896 grid), using a Comitrol grinder (Urschel, Chesterton, IN).

Extraction was performed in an agitated tank, for 17 h at room temperature and pressure, after addition of a 60% (v:v) ethanol/water mixture to reach about 10% dry matter.

The spent biomass was separated from the extract on a 300 pm sieve (SWECO, Florence, KY). Alcohol was evaporated at low temperature in an evaporator (<40° C) (Brouillon Process, Sainte-Bazeille, France). The extract was further clarified using a filtration aid (FW12 - diatomaceous earth) and KDS12 filters (cellulose-based depth filter sheets) mounted on a Millipore frame, then subsequently ultra-filtered on a 1 kDa organic membrane (Millipore Burlington, MA) to concentrate the polyphenolic fraction and diafiltered on the same equipment with 5 volumes of water to remove minerals and small metabolites.

The concentrate was then mixed with maltodextrin (Avebe MD20) in a 1:4 ratio (dry weight) and freeze-dried. After grinding, the powder extract was frozen and protected from moisture, heat and light to avoid deterioration.

Table 1. Minimum bactericidal concentration (MBC) obtained for the *A. nodosum* extract.

Campylobacter spp. strain	Origin	Source	MBC (μg/mL)
C. jejuni 81-176	Human	ATCC	[230−470]
C. jejuni C97ANSES640	Broiler - Farm	ANSES	[230−470]
C. jejuni C230	Broiler - Retail	ANSES	[60−120]
C. coli C09MJL T12	Broiler - Retail	ANSES	[230−470]

Results in brackets (in μg/mL) indicate the minimum and maximum concentration obtained for the three replicates.

The polyphenolic content of the extract was determined colorimetrically using the Folin-Ciocalteu reagent and a modified Folin-Glombitza method (Stévant et al., 2017) in duplicate using a standard curve with phloroglucinol solutions (from 0 to 100 μg/mL). The polyphenol contents were expressed as gram phloroglucinol equivalent per 100 g of powder. A polyphenol content of 60.2% was measured for the extract used in preliminary experimental trials, and the extract used for the field trial contained 60.5% polyphenols.

Bacterial Strains and Culture Conditions

Campylobacter spp. strains used in this work (Table 1) were stored at −70°C in peptone broth containing 20% (v/v) glycerol. Strains were recovered from frozen stock after plating on selective modified charcoal cefoperazone deoxycholate agar (**mCCDA**) (Thermo Fisher Diagnostics, Dardilly, France) at 41.5°C for 48 h under a microaerobic atmosphere (85% N_2, 10% CO_2 and 5% O_2). For each strain, a bacterial suspension was prepared by picking one colony from the mCCDA plate to inoculate 10 mL Brucella broth (BD Biosciences, San Jose, CA) and incubating at 41.5°C for 24 h under a microaerobic atmosphere.

To determine the MBC, 100 μL of each bacterial suspension in Brucella broth were added to 10 mL of Dulbecco's Modified Eagle Medium (**DMEM**, Life Technologies, Grand Island, NY) and incubated at 41.5° C under a microaerobic atmosphere for 18 h.

The strain *C. jejuni* C97ANSES640 was used for broiler challenge in experimental facilities. Five days before animal challenge, the strain was recovered from frozen stock, as described previously. One colony was used to inoculate 10 mL Brucella broth (BD Biosciences, San Jose, CA). After 24 h at 41.5°C in a microaerobic atmosphere, 100 μL of the bacterial suspension were added to 10 mL Brucella broth. After 24 h at 41.5°C in a microaerobic atmosphere, the bacterial suspension was diluted to 10^5 CFU/mL in tryptone salt broth.

Minimum Bactericidal Concentration (MBC)

Lyophilized extract of *A. nodosum* was diluted at 30 mg/mL in DMEM medium, as precipitation was observed in Brucella broth. Serial dilutions (1/2) were performed in 96-well microtiter plates to obtain final concentrations from $1.5.10^4$ μg/mL (m/v) to 7 μg/mL (m/v) (9.10^3 μg/mL (m/v) to 4.2 μg/mL (m/v)

polyphenols expressed as phloroglucinol). A control well using only DMEM broth was used. Bacterial suspension (10^5 CFU/mL in DMEM) was added to each well. Plates were incubated for 16 h hours at 41.5°C in microaerobic atmosphere. After incubation, no visible growth was observed in any well due to the presence of the algal extract; therefore, minimum inhibitory concentrations could not be determined. Decimal dilution of each well was performed and 10 μL of each dilution was deposited on an mCCDA plate. After incubation for 48 h at 41.5°C in microaerobic atmosphere, growth was observed, and the MBC was determined as the lowest concentration of *A. nodosum* extract reducing the viable cell population by 99.9% compared to the control well. At least three replicates were performed.

Animals and Experimental Design

Experimental Trial The experimental trial was carried out at the Animal Biosafety Level 2 (**ABL2**) facilities of the ANSES Laboratory of Ploufragan-Plouzané-Niort (Ploufragan, France), an approved establishment for animal experimentation (No. D-22-745-1). The trial was conducted in accordance with the principles and specific guidelines presented in the Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010). The protocol was reviewed and approved by the committee for ethics in animal testing No. 016 at the French Ministry for Education, Higher Education and Research before the start of the trials (APAFIS#12329-201711241346758 v2). Experimental facilities (room, pens, feeding and drinking systems) were cleaned and disinfected before the beginning of the trial. A total of 60 day-of-hatch Ross PM3 broilers (males and females), purchased from a commercial hatchery, were included in the study. Chicks were vaccinated against infectious bronchitis. The trial lasted 35 d from receipt of the chicks to the last sampling procedure. Birds were kept in 3.42 m^2 floor pens, with unused wood shaving litter as desbribed in Guyard-Nicodème et al., 2016. Chicks were randomly assigned to each experimental groups (n = 30 chicks per group). Feed was weighed and manually distributed. Feed (specific for broilers and adapted to their age) and water were available *ad libitum* during the rearing period. On D1, and at each sampling time, each animal was individually weighed and feed intake per pen was recorded. Environmental swabbing from facilities and transport crates was carried out and five randomly selected chicks were ethically euthanized upon arrival in order to confirm the absence of *Campylobacter* colonization. Analyses were carried out according to the NF EN ISO 10272−1:2017 Standard. Two groups of chicks were used, the control group and the treated group, receiving the algal extract added in feed at 0.2% (w/w) (2 kg/t) *ad libitum* from D31 to D35. At D18, all the birds in each group were orally challenged with 5 logCFU/ml of *C. jejuni* C97ANSES640 in a 100 μL suspension of tryptone salt broth by oral gavage. During the trial, animals were anesthetized by electronarcosis before being ethically euthanized by exsanguination. Broilers were sampled at D21 (14 broilers per group) and at D35 (14 broilers in control group and 15 in treated group), and ceca were recovered for *Campylobacter* analysis.

Field Trial The field trial involved a commercial flock with recurrent *Campylobacter* spp. contamination to perform the trial from May to June 2019. Broilers (Ross 308, males, vaccinated against infectious bronchitis and Gumboro disease) were reared conventionally until D31 in a house containing 22,000 birds. At D31, feces sampling was performed to check for the presence of *Campylobacter*, according to the NF EN ISO 10272−1:2017 Standard, before distributing the treatment. At D32, ten pens of 2 m^2 each were set up in the house; 300 broilers were randomly selected, individually weighed and identified, and then assigned to either of the 2 groups and placed in different pens (30 broilers per pen). Feed containing the extract at 0.2% (w/w) (2 kg/t) was distributed ad libitum to five pens from D33 to the end of the trial at D38. The other 5 pens corresponded to the control group and received the feed without the extract. At the end of the trial, remaining feed and the 300 broilers were weighed. Ninety broilers from the study (45 from each group) were ethically euthanized, and ceca were removed and analyzed for *Campylobacter* enumeration. No antibiotic or biocide treatments were applied in the flock during the trial.

***Campylobacter* Enumerations in Cecal Contents** *Campylobacter* enumerations were determined in cecal contents after direct plating and following the decimal dilution method. Ceca were weighed and diluted 1:10 (w/v) in tryptone salt broth (BioMérieux, Marcy-l'Étoile, France). After 1 min of homogenization in a stomacher (Interscience, Saint-Nom-la-Bretèche, France), 10-fold serial dilutions were carried out in tryptone salt broth and 50 μL of the dilutions were spread on mCCDA plates using an automatic easySpiral Dilute plater (Interscience). After 48 h of incubation at 41.5°C under microaerobic conditions, colonies showing *C. jejuni* morphology were enumerated. The detection limit for enumeration of *Campylobacter* was 2 log10 CFU/g of cecal content.

Statistical Analysis

The statistical unit was the animal for *Campylobacter* counts and body weight or the pen for feed conversion ratio, and mortality during the field trial. After a logarithmic transformation, the *Campylobacter* counts were compared between the two groups by a *t*-test. The final weight was analyzed by an ANOVA, taking into account the initial weight as a covariate. The average daily weight gain and feed conversion ratio were analyzed by a nonparametric Mann−Whitney test. A P-value <0.05 was used to indicate whether differences were statistically significant.

RESULTS AND DISCUSSION

The bactericidal effect of the *A. nodosum* extract against *Campylobacter* was studied in vitro.

MBCs of the *A. nodosum* extract obtained for several strains of *Campylobacter* are presented in Table 1. MBC ranged from 60 to 470 μg/mL, depending on the strain. Strain *C. jejuni* C230 appeared to be sensitive to lower concentrations of extract than other tested strains. The bactericidal activity of phlorotannins from brown algae has previously been demonstrated. In particular, *A. nodosum* phlorotannin exhibits bactericidal activity against *Escherichia coli* O157:H7 strains (Wang et al., 2009). On the other hand, in vitro bactericidal activity of phlorotannins from the brown algae *Ecklonia kurome* was demonstrated against *Campylobacter* (Nagayama et al., 2002). The mode of action against bacteria is not clear, but interaction between phlorotannins and bacterial proteins may play an important role (Wang et al., 2009); moreover, disruption of cell membranes has also been observed (Hierholtzer et al., 2013).

A trial was performed in animal facilities to determine the effect of distributing the *A. nodosum* extract in feed on broiler safety and on *Campylobacter* colonization. Feed additives are one of the 12 control options considered by EFSA to have a higher probability of achieving reduction of at least 10% of the incidence of campylobacteriosis in humans (EFSA European Food Safety Authority 2020). The main advantage of feed additives is that they are easy to administer to the birds. An in vivo trial was performed to explore efficacy in broilers artificially contaminated with a *C. jejuni* strain (C97ANSES640) originating from a poultry product and previously used in colonization studies (Guyard-Nicodème et al., 2016). Distribution of the algal extract did not influence bird mortality. The body weight of each animal was measured at each sampling time and results are presented in Table 2. The algal treatment in feed did not exert any detrimental effect on bird body weight at the tested dose. Moreover, it did not affect *Campylobacter* colonization in broilers neither at D21, nor at D35 ($P = 0.156$ and $P = 0.962$, respectively) (Table 2). Importantly, at D35 a mean of 6.12 ± 0.41 logCFU/g was observed for the control group and 6.15 ± 0.54 logCFU/g for the treated group. A slight (0.7 log) but significant reduction of *Campylobacter* was previously observed by Sweeney et al. (2016) during an experimental trial with *A. nodosum* supplementation in chickens. However, during this previous work, 3-day-old chicks were artificially contaminated with *C. jejuni* and cecal contamination was evaluated at D10, which did not represent commercial rearing conditions.

Despite the absence of *Campylobacter* reduction during the experimental trial in animal facilities, the *A. nodosum* extract was also tested in feed distributed at the end of the rearing period in a commercial broiler flock to evaluate its impact on natural *Campylobacter* contamination. Results from experimental studies are difficult to extrapolate to field conditions, as there are multiple sources and dissemination routes for *Campylobacter* spp., as well as different stocking densities and housing conditions on broiler farms. The algal extract was added to the feed used for the trial and phenolic determination was performed on the feed. Findings corresponded to the expected level (data not shown). The trial was performed in a flock with recurrent *Campylobacter* contamination. Before distributing the treatment, *Campylobacter* detection was performed at D31 on fresh feces and revealed that the flock was positive for *Campylobacter*. At the end of the trial, *Campylobacter* enumeration was performed on 45 ceca from each group but did not revealed any significant differences ($P = 0.245$), as a mean of 6.12 ± 0.41 logCFU/g was observed for the control group and 5.03 ± 0.48 logCFU/g for the treated group (Table 2). Zootechnical parameters were also determined. At the end of the trial, mortality in the control group was 4.0% (6 birds) and 1.3% (2 birds) in the treated group, but this difference was not statistically significant ($P = 0.282$). No statistical differences were observed for mean body weight ($P = 0.380$), average daily weight gain ($P = 0.508$), or the feed conversion ratio ($P = 0.465$) (Table 2). During this field trial, the *A. nodosum* extract added to feed did not reduce *Campylobacter* colonization. However, it did not negatively affect commercial broiler growth, mortality, or feed intake. To the best of our knowledge, this is the first time that *A. nodosum* extract was used in a field trial. This trial demonstrated that the algal extract was easily added at the desired polyphenol rate, and despite thermal feed treatment. Higher mortality and feed

Table 2. *Campylobacter* loads and various zootechnical parameters measured during the experimental and field trials.

	Experimental trial						
	Campylobacter loads (CFU/g)		Body weight (g)				
	D21	D35	D1	D14	D21	D31	D35
Control	6.87 ± 0.32 (n = 14)	6.12 ± 0.41 (n = 14)	43.7 ± 2.5 (n = 30)	365.7 ± 40.5 (n = 30)	778.9 ± 68.6 (n = 29)	1629.5 ± 132.7 (n = 15)	1993.9 ± 166.5 (n = 15)
Treated	7.42 ± 0.20 (n = 14)	6.15 ± 0.54 (n = 15)	42.7 ± 3.5 (n = 30)	354.5 ± 58.5 (n = 30)	760.4 ± 116.2 (n = 30)	1573.5 ± 230.8 (n = 15)	1969.9 ± 272.4 (n = 15)

	Field trial				
	Campylobacter loads (CFU/g)	Mean body weight (kg)		Average daily weight gain	Feed conversion ratio[1] (n = 5)
		D32	D37		
Control	5.45 ± 0.51 (n = 45)	1.99 ± 0.11 (n = 150)	2.39 ± 0.16 (n = 144)	88 g/d (n = 144)	3.00
Treated	5.03 ± 0.48 (n = 45)	2.00 ± 0.11 (n = 150)	2.40 ± 0.11 (n = 148)	83 g/d (n = 148)	2.31

Means ± standard error are presented for each day of measure. No statistically significant differences were observed between the control and treated groups for the different parameters and for the different days.

[1]Median values are presented as nonparametric statistical tests were performed.

conversion ratios (not statistically significant) were observed in the control group, so another trial using higher doses of the extract could be performed to assess whether these differences can be replicated and better results achieved. Additional work is needed to study the bioavailability of the extract to check whether active polyphenols can reach the cecum. Moreover, it would be interesting to study whether the extract exerts modulation on gastrointestinal microbiota, or if it is able to reduce other bacterial pathogens in poultry. Additional work could also be performed to test the effect of the whole seaweed to take advantage of its rich source of various bioactive compounds.

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DISCLOSURES

The authors declare no conflict of interest.

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