Polyphenols from Brown Seaweeds as a Potential Antimicrobial Agent in Animal Feeds

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ABSTRACT: Seaweeds offer a natural source of antimicrobials that may help curb antibiotic resistance in livestock. The antibacterial activity of phlorotannin extracts isolated from two brown seaweeds Ascophyllum nodosum and Fucus serratus was tested. The mechanism of action of phlorotannin extracts against Escherichia coli O157, Salmonella agona, and Streptococcus suis was elucidated by observing cell membrane permeability and intracellular adenosine triphosphate (ATP). The two extracts were effective at killing three foodborne pathogens without negatively affecting the pig intestinal cells. A. nodosum minimum inhibitory concentration (MIC) range for the different pathogens was between 1.56 and 0.78 mg/mL, whereas F. serratus was 3.13 mg/mL for all pathogens tested. A. nodosum was found to be much more potent compared to F. serratus. The difference in potency in the seaweeds may be a result of the phlorotannins’ structural linkages. The antimicrobial properties of the seaweed extracts tested may provide alternative and complementary treatments to antibiotics and zinc oxide in animal feeds. The seasonal screening was performed on both species to assess the availability of phenolics throughout the year using two quantification methods, the Folin–Ciocalteu (FC) assay and quantitative nuclear magnetic resonance (NMR). The variation between the methods highlights the challenges involved in the quantification of complex phenolic structures. However, both methods show that the phenolics are subject to seasonal variation, which may prove problematic to the animal feed industry.

1. INTRODUCTION

Intensive large-scale pig farming has produced a plethora of issues, resulting in increased physiological and psychological stressors on animals.1 One major challenge that pig producers nowadays face is the increasing restriction on the use of antibiotics and metal ions, namely, the use of zinc to combat piglet predisposition toward acquiring Salmonella or Escherichia coli infection during weaning.2 The use of antibiotics in animal husbandry has since been shown to contribute to the antimicrobial resistance crisis,3 and increasing regulation and license laws have resulted in the ban of the use of antibiotics as a growth promoter in the European Union (EU) (Regulation (EU) No. 1831/2003). Since banning antibiotic growth promoters (e.g., avoparcin, zinc bacitracin), the pig feed industry has moved toward the use of zinc oxide in their formulations.3

Polyphenolic compounds from both marine and terrestrial plants have received significant interest as effective antimicrobial agents.4–8 Brown seaweed contains a unique class of polyphenolic compounds, called phlorotannins.9 These compounds are based on the monomer unit phloroglucinol (1,3,5-trihydroxybenzene) and polymerize either through ether (C–O) linkages or aryl–aryl (C–C) linkages.10 Structural elucidation of these compounds remains difficult owing to their variation in both the number of monomers present and their linkages,10,11 however, they are structurally less complex than terrestrial tannin counterparts.12 A review by Pérez et al. summarizes the studies to date, which have shown seaweed extracts to be effective at inhibiting several microorganisms, including E. coli and Salmonella.6 Most of the studies reviewed tested crude seaweed extracts of the compounds into either ethanol or methanol. From these studies, it is unclear if the antimicrobial activity is a result of single or multiple compounds acting in synergy. Some other studies have proposed both laminarin and fucoidan brown seaweeds as an alternative to in-feed antibiotics,13–15 as a natural therapeutic alternative to zinc oxide in pig diets,16 or as an antimicrobial...
agent. In these studies, both phlorotannins and sulfated polysaccharides have been cited as the active compounds that inhibit pathogens. Phlorotannins are known for their affinity to bind to proteins. In a pathogen-rich environment, it is thought that they bind to bacterial proteins through the OH groups of the phloroglucinol units and the NH groups of bacterial proteins by hydrogen bonding and hydrophobic interactions, causing cell lysis.

Phlorotannin characterization, much like those of terrestrial tannins, is very complex due to the large number of structurally similar isomeric compounds and differing sizes of the polymeric repeating structure. Some specific phlorotannins have been characterized although there are thousands that are still unelucidated and hence full structural assignment is difficult. Studies of this type also involve very large quantities of the starting material and multiple separation and purification steps for a full characterization of one compound; this approach would not be feasible in a systematic seasonal overview of the natural products in the selected seaweeds. To add to this, there are no commercial standards of seaweed phlorotannins available, so identification of any individual compounds can only be tentative unless individual full structural elucidation is done. However, purification methods, e.g., solid-phase extraction (SPE), can be used to produce an extract whereby the major phenolic constituents are phlorotannins. To date, there is no optimized extraction method for the isolation of phenolics from seaweeds.

Due to issues associated with the chemical complexity of phlorotannins, the literature largely focuses on assay quantification of phlorotannins with the Folin–Ciocalteu (FC) assay being the most widely used. These assays are redox reactions and therefore poorly sensitive to the seaweed extract of the overview of the structural linkages, which is compared to the seaweed extract’s biological activity.

Herein, assesses the antimicrobial potential of two purified phlorotannin extracts from *Ascophyllum nodosum* and *Fucus serratus*, two intertidal brown seaweeds. Both *A. nodosum* and *F. serratus* belong to the Fucaceae family of seaweeds, which are known to be a rich source of phlorotannin. The objective of the study was to assess the bactericidal effects of phlorotannin bioactive extracts on three foodborne pathogens (*E. coli* O157, *Salmonella agona*, and *Streptococcus suis*) that often colonize weaning piglets. Nuclear magnetic resonance (NMR) spectroscopy and the Folin–Ciocalteu assay were used to assess the seasonal availability of the phenolic compounds in crude seaweed extracts. ¹³C NMR was used to assess the difference in linkage profiles of the purified phenolic extracts in the different seaweed species.

### 2. RESULTS AND DISCUSSION

#### 2.1. Biological Analysis

The microbiological assays herein display a significant difference in both the minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) of the phlorotannin extracts. Table 1 shows the numerical data for the activity of phlorotannins isolated from *F. serratus* and *A. nodosum* against three common foodborne pathogens (*E. coli* O157, *S. agona*, and *S. suis*). *A. nodosum* was shown to have a much more potent antibacterial effect against these bacteria than *F. serratus*. The MIC represents the minimum concentration of phlorotannin extracts for each bacteria strain tested. The lowest MIC value of 0.781 mg/mL was found in the phlorotannin extract isolated from *A. nodosum* tested against both *E. coli* O157 and *S. suis*. The bactericidal activity of phlorotannins isolated from *F. serratus* was found to be the same for all three pathogens tested. *A. nodosum* was particularly potent against *S. suis* compared to *F. serratus*, which shows a MBC of 1.56 and 6.25 mg/mL, respectively. The effect of the phlorotannin extracts on the cell membrane permeability and adenosine triphosphate (ATP) levels was also investigated to provide an insight into the antimicrobial mechanism of the seaweed extracts against the three foodborne pathogens. In the cell membrane permeability and ATP tests, the MIC and MBC concentrations are shown in Table 1 were tested for each pathogen. There was no significant difference in electrical conductivity between seaweed species (*F. serratus* = 1.49, *P* = 0.231; Figure 2). However, a significant difference among pathogens (*F*₂,₅₄ = 47.86, *P* < 0.001; Figure 2) was observed in the order of *E. coli* O157 > *S. agona* > *S. suis*. There was also a highly significant difference among the three extract concentrations (control, MIC, MBC) (*F*₂,₅₄ = 2011.78, *P* < 0.001; Figure 2) in the order MBC > MIC > control. The statistically significant seaweed × pathogen, pathogen × extract, and seaweed × pathogen × extract interactions (*F*₂,₅₄ = 30.72, *P* < 0.001; *F*₅₄ = 9.604, *P* < 0.001; *F*₅₄ = 8.871; Figure 1) indicate that the differences in electrical conductivity across the two seaweeds, three pathogens, and three extracts were slightly variable in magnitude, however, this was clearly not biologically significant as the patterns were consistent in direction (see Figure 1). The intracellular ATP levels (Figure 2) of the three pathogens tested were found to significantly reduce (at both MIC and MBC concentrations) with respect to the media-only control. There were significantly higher ATP levels found in *A. nodosum* compared to those of *F. serratus* (*F*₂,₅₄ = 73.45, *P* < 0.001; Figure 2). A significant difference among pathogens (*F*₂,₅₄ = 31.38, *P* < 0.001) and extracts (*F*₂,₅₄ = 409.28, *P* < 0.001; Figure 2) was also found with respect to the levels of intracellular ATP. Fisher’s least significant difference (LSD) test revealed that there was no significant difference in the intercellular ATP between *E. coli* O157 and *S. suis* pathogens (*P* = 0.92). However, a significant difference was observed in the intercellular ATP between *S. agona* and *E. coli* O157 and *S. agona* and *S. suis* (*P* < 0.001). Similar to the above, the statistically significant seaweed × pathogen, pathogen × extract, and seaweed × pathogen × extract

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**Table 1. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of *F. serratus* and *A. nodosum* Polyphenol Extracts against Three Different Foodborne Pathogens (*S. agona*, *E. coli* O157, and *S. suis*)**

| seaweed species | microorganism | MIC (mg/mL) | MBC (mg/mL) |
|----------------|--------------|-------------|-------------|
| *F. serratus* | *S. agona*   | 3.125       | 6.25        |
|                | *E. coli* O157 | 3.125       | 6.25        |
|                | *S. suis*    | 3.125       | 6.25        |
| *A. nodosum*  | *S. agona*   | 1.56        | 3.125       |
|                | *E. coli* O157 | 0.781       | 3.125       |
|                | *S. suis*    | 0.781       | 1.56        |
interactions ($F_{2,54} = 16.76, P < 0.001$, $F_{2,54} = 12.80, P < 0.001$, $F_{4,54} = 17.62, P < 0.001$, respectively; Figure 2) indicate that magnitudes of ATP were variable across factors but patterns and directions were consistent, although clearly there were higher ATP levels associated with *As. nodosum* (Figure 2).

The 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) results shown in Figure 3 show the cell viability of intestinal porcine epithelial cells (IPECs), IPEC-J2 exposed to six different concentrations of each phlorotannin extract. In both the extracts, the viability of IPEC-J2 increased in a dose-dependent manner with the exception of an anomaly in *As. nodosum* at a concentration of 3.125 mg/mL, which could have been caused by inhomogeneity in the type of phlorotannin compounds present. Replicate MTT cell viability data (Figure 3) for both seaweed species tested at each dosage concentration were examined statistically. None of the concentrations tested was found to be cytotoxic, with cell proliferation increasing with respect to an increase in concentration; however, this increase was not always statistically significant. Although, there was a statistically significant difference between species ($F_{1,42} = 17.08, P < 0.001$; Figure 3) and phlorotannin extract concentration ($F_{6,42} = 55.92, P < 0.001$; Figure 3). The cell viability at lower concentrations of 0.78 and 1.56 mg/mL did not increase significantly compared to the control group ($P > 0.05$) in either species. At higher concentrations (>1.56 mg/mL), the increase in viability within each species with respect to the control was highly significant ($P < 0.001$). A significant species × concentration interaction was also observed ($F_{6,42} = 4.97, P < 0.001$; Figure 3), suggesting different effects of concentration on the cell viability across seaweed species. When directly comparing species at specific concentrations, *As. nodosum* phlorotannin extracts were found to have a more pronounced effect on the cell proliferation compared to *F. serratus* extracts at concentrations of 0.78, 3.125, and 25 mg/mL, which was statistically significant ($P < 0.05$).

The potency of the phlorotannin extracts was dependent on the specific mechanism of cell death or inhibition of the pathogens. In the cell membrane permeability study, no difference was found between the potency of the extract.
whereas in the intracellular ATP study, A. nodosum was found to be more potent. In both studies, interactions were observed between the species and the pathogens with respect to the mechanism of death or inhibition. The findings support the hypothesis that the potency of the extract was species- and pathogen-dependent, although the effects are relatively subtle. Several previous studies have reported the potency of A. nodosum against E. coli O157; to the authors’ knowledge, however, no studies have reported it against S. agona and S. suis, which are other common foodborne pathogens known to colonize piglets during weaning. Kadam et al. reported that the phenolic extract at concentrations of 0.110–0.156 mg phloroglucinol equivalents (PGE)/g isolated from A. nodosum using different extraction techniques showed either no inhibition or a MIC of 43.2 and 596.8 mg/mL against E. coli DSM 1103. This is significantly higher than the MIC for A. nodosum reported in this study of 0.781 mg/mL against E. coli O157. However, the phenolic extract in Kadam et al. study contained a mixed extract of polysaccharide with only a small amount of polyphenol present, as opposed to pure phlorotannin. That study also used the FC assay to analyze the phenolic content in the extracts, which is not selective for phlorotannin. This study also used the FC assay to analyze the phenolic content in the extracts, which is not selective for the phlorotannin compounds. Herein, purified polyphenolic phlorotannin mixtures isolated from A. nodosum and F. serratus were used to identify MIC and MBC limits. Phytochemicals are considered to have significant antimicrobial activity when their MIC is below 10 μM/mL and moderate when their MIC is <100 μM/mL. The significant reduction in MIC in this study compared to Kadam et al.’s study is a result of the purification of phlorotannins from other phenolic-like compounds in the seaweed extract.

Several studies have reported that phytochemicals can cause severe bacterial cell membrane damage through the disruption of the membrane integrity. After the membrane has been damaged, the active phytochemicals can enter the bacteria and interfere with DNA, RNA, protein, or polysaccharide production causing bacterial cell inactivation. In this study, the exposure of the pathogens to the MIC and MBC concentrations of the phlorotannin extracts resulted in a significant increase in the electrical conductivity in comparison to the control, which suggests that the bacterial cell membrane has become permeable and electrolytes are released into the environment. Recently, phenolic compounds from nonmarine sources, such as black pepper and pine needles, were shown to increase the permeability of the cell membrane of E. coli and Staphylococcus aureus at different concentrations. The results in this study also showed that the two phlorotannin extracts significantly reduced the intracellular ATP levels of all three pathogens tested. The reduction of ATP levels could be due to an increased rate of ATP hydrolysis in the cell or due to the outflow caused by the permeable cell membrane. Exposure of E. coli O157, Listeria monocytogenes, and Lactobacillus sakei to carvacrol, a phenolic compound abundant in oregano, was also shown to decrease their intracellular ATP levels.

Importantly, the phlorotannin extracts had no adverse effects on the pig intestinal cells when exposed to the same concentration or higher (up to 25 mg/mL) than those that were shown to inhibit bacterial growth. The increase in cell viability can be explained by an enhanced mitochondrial activity due to the exposure to the two extracts, since the measurement of cell viability is correlated to the mitochondrial metabolic capacity of the gut cells. Similarly, a study by Di Nunzio et al. in 2018 reported that an increased cell viability of Caco-2 cells was observed after treatment with different concentrations of polyphenols derived from olive pomace.

2.2. Chemical Analysis. The presence of phlorotannins in the phenolics extract (acetone/water, 7:3, v/v) of A. nodosum and F. serratus was determined by UV-vis spectroscopy (FC assay), 1H NMR and 13C NMR spectroscopy. The 1H NMR and 13C NMR analyses allowed for quantitative and qualitative analyses of the total phenolic content (TPC) and the linkages between phlorotannins present in the seaweed biomass that was then used for the extractions and purification by solid-phase extraction. The quantitative NMR (qNMR) method expresses the phenolic content as a measurement of milligram of polyphenols per gram of seaweed (mg/g). Whereas the FC assay is expressed in phloroglucinol equivalents per gram of seaweed (PGE/g). The concentration of phenolics in A. nodosum was found to be significantly higher than F. serratus, at 37.35 and 17.00 mg/g, respectively (Figure 4a) according to the 1H NMR analysis. Whereas, with the FC assay showed, the reverse trend was found, whereby the phenolics in A. nodosum was found to be 30.68 (±0.55) PGE/g compared to 36.68 (±1.33) PGE/g in F. serratus (Figure 4b).

1H qNMR was used to analyze phenolics in the whole seaweed biomass to understand the concentration of phenolics in the seaweed overall and how this changes throughout the season.

13C NMR spectra of the phenolic extract prepared using SPE from each species were compared to calculate the difference in linkages of the phenolics between seaweed species. This overview of the extracts was deemed the most useful tool in displaying chemical differences in the bonding of the phenolic extracts of the samples. The 13C NMR data collected was of the same samples analyzed in the Section 4.5, therefore directly compares the linkages of the extracts used for biological testing.

Figure 5 shows the effective removal of sugar and nonphenolic compounds from the extract between 2.5 and
4.5 ppm in $^1$H NMR. The extracts from the SPE processing were used for the biological testing and were further chemically characterized by $^{13}$C NMR to determine differences between linkages of the phenolic compounds. $^{13}$C inverse-gated decoupled NMR was used to identify the different linkages and chemical structures in the phenolic extracts. Different chemical shifts were observed for C–OH (151–160 ppm), C–O–C (121–128 and 156–158 ppm), C–C (99–103 ppm), and C–H (92–97 ppm) bonds and can be related to the phlorotannin linkages. From the intensity of these peaks on the $^{13}$C NMR spectra, the ratio of one linkage to another can be measured when using inverse-gated decoupling experiments. This specific acquisition of data from the NMR allows for a quantitative analysis of the $^{13}$C spectrum due to the removal of nuclear Overhauser enhancement (NOE) interactions in the experimental parameters.

$^{13}$C NMR cannot be used quantitatively due to polarization transfer of $^1$H to $^{13}$C via NOE; however, when these interactions are removed and $^1$H decoupling is only applied in the acquisition period, then the intensity of the $^{13}$C peaks can be used quantitatively. C–O–C linkages are measured from the integration between 121 and 128 ppm. Analysis of linkages in phlorotannins has been done this way previously for Laminaria digitata but not for A. nodosum or F. serratus.25

The phlorotannin compounds are linked by C–C or C–O–C bonds. The nature of the linkage will affect the overall structure of the phlorotannin and also possibly its reactivity. Little is known about the effect of the linkages present overall in the phenolic mixture and their biological activity. The research done herein suggests that the biological activity could be affected by the linkage-type present. The $^{13}$C inverse-gated decoupled NMR spectrum displayed in Figure 6 shows the differences in the ratios of the C–C to the C–O–C linkages of A. nodosum when compared to F. serratus.25

Table 2 shows the difference in the peak integration between C–C bonding and C–O–C bonding. In the table, the C–H bond is set to a ratio of 1.0 and the intensity of the other peaks is used relative to the C–H integration. From the results more, C–C linkages are observed in F. serratus than A. nodosum. This suggests that the structures of the phlorotannins in the two respective seaweed species are different. There was also a stark contrast in the C–H to C–O–C ratio from A. nodosum and F. serratus, showing a ratio of 1.63 and 0.85, respectively. This suggests that there is a much higher ratio of C–O–C-type linkages in the phenolic extract of A. nodosum compared to F. serratus. The differences in these structures shown in the $^{13}$C NMR and the differences in chemical linkages in the different seaweed extracts could account for the differences in the biological activity between the two species of brown seaweed. This study shows that the phlorotannins in the two brown seaweeds A. nodosum and F. serratus differ in terms of their structure. The structural differences observable in their $^{13}$C NMR spectra have been analyzed to show differences in the abundance of C–C and C–O linkages between species. The subtly higher ratio of C–H bonds observed in F. serratus would suggest a higher number of small compounds and terminal units in the phlorotannins present in this species of seaweed, which support findings from mass spectra data from the previous studies.25 F. serratus has also been shown to have a higher concentration of the low-molecular-weight phlorotannins in extracts, which could account for the higher average number of C–H bonds on the ring.16 Other species belonging to the Fucus genus have also been shown to have a lower

![Figure 5. From top to bottom: $^1$H NMR of seaweed extracts: (purple) A. nodosum crude extract, (blue) F. serratus crude extract, (green) A. nodosum after SPE, (red) F. serratus after SPE.](image)

![Figure 6. $^{13}$C NMR data showing differences in structural linkage types between F. serratus (green) and A. nodosum (red). Ether linkage peaks C–O–C bonds are shown in blue and orange and aryl–aryl (C–C) linkages are shown in green. C–H bonds are also labeled on the spectra.](image)

| seaweed species | ratio of intensity of chemical bond measured by $^{13}$C NMR spectroscopy |
|-----------------|-----------------------------|
|                 | C–O–C | C–C | C–H |
| A. nodosum      | 1.63  | 0.23| 1.0 |
| F. serratus     | 0.85  | 0.35| 1.0 |

ACS Omega 2020, 5, 9093–9103
degree of polymerization of the phlorotannins compared to *A. nodosum* and *Pelvetia canaliculata*.

### 2.3. Seasonal Results

The seasonal profile of the phenolic compounds in the brown seaweed species *A. nodosum* and *F. serratus* was measured. The results of the FC assay compared to the qNMR method (Figure 7) show some variation between methods. For *A. nodosum*, the FC assay shows an overestimation of phenolic compounds from February to April and then an underestimation from May to July. The results showed May to have the highest phenolic content for *A. nodosum* from both the FC assay and qNMR. *F. serratus* showed the highest phenolic content for July, when analyzed by qNMR spectroscopy; however, the highest phenolic content was observed in February when analyzed by the FC assay.

The seasonal profile of the 1H NMR spectra displays a large variation in the polyphenol content for *A. nodosum*, shown in Figure 7. A seasonal dip in the phenolic content was observed between March and April. *F. serratus* displays a much less seasonal variation in terms of phenolic content measured by quantitative NMR spectroscopy when compared to *A. nodosum*. The extraction process was repeated in triplicate and combined for NMR analysis, which means that error bars are not generated as the NMR spectrum is an average of three extractions.

To measure the seasonal variation in phenolic compounds, the FC assay was used. This is a colorimetric assay, which is used to determine the TPC in plants, seaweeds, and extracts. Although it is widely used, the concept of the assay is based on redox chemistry, meaning anything with a redox potential is able to react with the reagent and cause a color change. Seaweeds, when extracted with solvents or water, contain a multitude of different compounds, which could potentially react with the FC reagent such as reducing sugars, amino acids, metal salts, and fats. This may explain the overestimation of phenolic compounds observed from February to April in these results, when compared to the qNMR results.

The change in color observed by the FC assay was used with a calibration curve to measure the total phenolic content. The resulting unit from the FC assay was in phloroglucinol equivalents and not a direct measurement of the TPC in the sample. This makes the qNMR and the FC assay incomparable because they use different units, as reported in previous studies. The qNMR method is a direct measurement and does not use phloroglucinol to express the TPC. The NMR method is therefore more selective for direct measurement of the phlorotannin compounds, whereas as stated above, the FC assay can also react with other compounds, so it is less specific for phlorotannin recognition and detection.

The difference in the qNMR and FC assay results in February for *F. serratus* could be due to a higher presence of reducing sugars in the seaweed or metal contaminants in the water where the seaweeds were collected, which are able to interfere with the FC assay. The FC assay is sensitive to anything in the solution with a redox potential, therefore the presence of other compounds or ions in the solution could give a false peak in the phenolic content when using this assay. A peak in TPC was observed in the FC assay for both species of seaweeds, however, this peak in TPC is not observed in the qNMR data for either species. This could suggest that there may be a contaminant in the water (e.g., metal ions), which could be causing this peak. The two species of seaweed tested were collected from the same area of water.

The seasonality study for *A. nodosum* displays a dip in the phenolic content present in the seaweed between March and April, and this coincides with the reproductive phase of the seaweed. The seasonal profile displayed herein for *A. nodosum* shows an earlier peak in the phenolic content compared to the previous work by Parys et al., which reported the peak in the phenolic content occurring in July, in the *A. nodosum* they collected in Oban, Scotland in 2005. May 2005 was very cold, compared to May 2018. Tulloch Bridge (U.K.) recorded a temperature of −6.3 °C on 18th May 2005, the lowest temperature recorded since 1956, whereas 2018 (the year seaweed was collected for this study) was a very warm summer, with hot weather from April. The mean temperature in May for 2018 was 12.7 °C, which makes it the second hottest May since 1910 in the U.K. This difference in the local weather could be one reason for the shift in the maximum phenolic content. Seaweeds have been shown to be very seasonal plants in previous studies and are very dependent on their local climatic and environmental conditions. In addition, high temperatures are tied to changes in the bacterial surroundings, which is likely to enhance production of the seaweed’s secondary metabolites, such as polyphenols, to protect itself from bacterial biofilm formation.

No previous studies have reported the seasonal profile of *F. serratus* by 1H qNMR spectroscopy; to the authors’ knowledge, therefore, it is not possible to compare these results to those shown in the literature. Studies using conventional FC analysis have shown that *F. serratus* and *A. nodosum* (both belonging to the Fucaceae family) have higher phenolic content than that of *Laminaria spp.*

### 3. CONCLUSIONS

The qNMR and FC seasonal results have clearly demonstrated that not only is there variation between samples collected each month, there is also significant variation between the results from each method. Further, method development is required to optimize the identification of phenolics and more specifically phlorotannin compounds to better understand
the effects of seasonality and also to help identify the bioactive responsible for killing the pathogen tested.

In addition, further identification and purification of the bioactive phlorotannins within this phlorotannin fraction of *A. nodosum* could yield one or more highly potent phytochemicals with significant importance to the animal feed industry. It is also noteworthy that a previous study on the antibacterial activity of marine sponges reported a synergistic effect from crude extracts, which resulted in lower MIC values compared to purified fractions. \(^55\) Therefore, it would be worth exploring in the future, different fractions of phlorotannins, in addition to pure extracts, to determine the optimum antimicrobial agent to target these specific pathogens.

Research findings from this study suggest that the antibacterial activity of the phlorotannin compounds could be dependent on the chemical structure. The structures of the phlorotannins are shown to be different depending on the species of seaweed used for extraction and the biological activities are also shown to be different depending on the species and structure. The antibacterial properties of the phlorotannins from *A. nodosum* were shown to be much more potent for inactivating and inhibiting bacterial growth than those of *F. serratus*. The cell viability was also carried out on a porcine intestinal cell line; the cells responded well to these conditions (37 °C with 5% CO2). The medium was then removed, first by rotary evaporation to remove acetone and then by lyophilization. The extraction process was repeated in triplicate and combined for NMR analysis.

4.4. Phenolic Seaweed Extracts for qNMR. Seaweed biomass (0.1 g) was extracted with a mixture of acetone/water (7:3, v/v, 6 mL) at room temperature for 3 h. Extracted liquid was then removed, first by rotary evaporation to remove acetone and then by lyophilization. The extraction process was repeated in triplicate and combined for NMR analysis.

4.5. Biological Analysis. For all of the biological testing, seaweed collected from July was chosen, as summer months are known to be higher in the phenolic content for the analysis of brown seaweed. \(^56\) Chemical analysis including qNMR, \(^13\)C NMR and the FC assay were used to test the extracts from the biological studies.

4.5.1. Bacterial Strains. *E. coli* O157:H7 EDL 933, *S. agona* (AFBI collection; food isolate), and *S. suis* (DSM 9682) were maintained in tryptone soya agar plus 0.6% yeast extract (TSAYE, Oxoid, U.K.) slopes at 4 °C. When necessary, they were cultured in Mueller–Hinton broth (MHB) at 37 °C for 24 h.

4.5.2. Cell Culture. Intestinal porcine epithelial cells (IPEC-J2) from a nontransformed intestinal cell line \(^56\) were grown at 37 °C and 5% CO2 in a humidified incubator. The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM)-high glucose (Sigma-Aldrich, U.K.) medium supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, U.K.). The IPEC-J2 cell line was produced from the DSMZ, Leibniz Institute, Germany.

4.5.3. Cell Viability Assay. Cell viability was determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay (Roche, Sigma-Aldrich, U.K.). Briefly, 2 × 10^4 cells were incubated in 96-well plates for 18 h to allow cell attachment. The plates were incubated under the same conditions (37 °C with 5% CO2). The medium was then aspirated and replaced with 100 μL of the fresh medium
containing the various concentrations of the phlorotannin extracts. Each concentration of the extract was added in triplicate (n = 3). The control wells contained only a fresh DMEM medium with no extract. After 3 h of incubation, the media containing extracts were removed. The cells were then washed once with 100 μL of the fresh medium and replenished with 100 μL of the fresh medium. Cell survival was evaluated by adding 10 μL of the MTT reagent (0.5 mg MTT/mL) to each well and incubating for an additional 3 h. This medium was then removed, and 100 μL of the solubilization solution was added to dissolve the MTT formazan. The plate was incubated overnight at 37 °C with 5% CO2. The absorbance of the MTT purple color was measured on a multiwell plate reader (FLUOstar Omega, BMG Labtech, U.K.) using a 570 nm filter. Cell viability was expressed as a percentage of control. Data is shown as the mean of triplicate cultures with standard deviation. The calculation of cell viability was done as follows:

\[ \text{cell viability (%) } = \frac{(\text{OD}_{\text{treatment group}} - \text{OD}_{\text{blank group}})}{(\text{OD}_{\text{control group}} - \text{OD}_{\text{blank group}})} \times 100 \]  

(1)

4.5.4. Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of Bioactive Extracts. To determine the lowest concentration of the two phlorotannin extracts that can inhibit the growth of bacteria (MIC) and the lowest concentration that leads to bacterial death (MBC), the twofold tube dilution method was employed.33 The individual phlorotannin extracts were diluted (50–0.78% mg/mL) in MHB. Separate overnight bacterial cultures for each pathogen were harvested by centrifugation, washed with phosphate-buffered saline (PBS), and diluted to approximately 1 × 10^6 CFU/mL in MHB (washing was repeated three times). Afterward, each tube was inoculated with approx. 5 × 10^8 CFU/mL of the respective bacterial culture. Noninoculated tubes containing the same growth medium were used as negative controls and tubes inoculated with individual bacterial cultures in MHB with no added phlorotannin extract were used as positive controls. Tubes were incubated at 37 °C for 24 h. All tubes for all three pathogens were plated out and the pathogens were enumerated. Tubes without visible growth were considered as the MIC. One hundred milliliters from the tubes that showed no growth was spread-plated onto Mueller–Hinton agar (MHA) plates. The highest dilution showing no microbial growth was considered as the MBC. Each assay was repeated three times.

4.5.5. Determination of Cell Membrane Permeability. Pathogen cell permeability was also determined by monitoring changes in electrical conductivity, using a conductivity meter (Jenway 4200, U.K.) after exposure to the MIC and MBC concentrations determined by the MIC and MBC studies for the two phlorotannin extracts and a control with no extract for 6 h.37 After incubation at 37 °C for 24 h, each of the three pathogen cultures was centrifuged (5000 rpm for 10 min) and bacteria were harvested. Subsequently, the bacteria were washed with 5% of glucose until their electric conductivity was near to that of 5% glucose (isotonic bacteria). The phlorotannin extracts were added to 5% glucose (MIC level), and the electric conductivities of the mixtures were determined (Lp). The same concentration of individual phlorotannin extracts was also added into the isotonic bacteria, and the conductivities of the individual cultures were measured after 6 h incubation at 37 °C (Li). The conductivity of bacterial cells in 5% glucose treated in boiling water for 5 min was used as a control (L0). The permeability of the cell membrane was calculated using the equation:

\[ \text{relative electric conductivity (%) } = 100 \times \frac{(L_0 - L_i)}{L_0} \]  

(2)

4.5.6. Determination of Intracellular Adenosine Triphosphate (ATP) Levels. To determine the effect of the phlorotannin extracts on the intracellular ATP levels of the pathogens after treatment with phlorotannin extracts, a method by Shi et al. was used.58 Overnight cultures of the bacteria were centrifuged for 5 min at 5000g, with subsequent removal of the supernatant. The cell pellets were washed three times with PBS and each time the cells were collected by centrifugation. One milliliter of the individual cell suspension (approx. 10^9 CFU/mL) was placed into Eppendorf tubes containing the individual phlorotannin extracts was tested at a concentration of 0 mg/mL (control) and their respective MIC and MBC concentrations. Subsequently, the samples were maintained at 37 °C for 6 h. The intracellular ATP was extracted by centrifuging the cells and treating them with a lysis buffer (Roche, U.K.) for 5 min at room temperature. The cell suspension was centrifuged again and the intracellular ATP level was measured by means of an ATP assay kit (ATP bioluminescence assay kit HS II, Roche, U.K.) using white 96-well plates. The ATP concentrations were measured with an automatic microplate reader (FLUOstar Omega, BMG Labtech, U.K.).

4.6. Chemical Analysis. 4.6.1. Quantitative Nuclear Magnetic Resonance (qNMR) Seasonality Study. Phenolic extracts were prepared as per Section 4.4.2 for analysis with qNMR. NMR characterization was carried out on a Bruker DPX600 spectrometer. 1H chemical shifts are reported in parts per million (ppm). An inverse-gated decoupling program (Section 4.4.2) was then redissolved in a mixture of deuterated methanol/deuterium oxide (8:2, v/v, 2 mL), which was spiked with an internal standard of trimesic acid to a concentration of 2 mg/mL. Integration of the internal standard was compared to the integration of the aromatic peaks (between δ 5.7 and 7.5) using eq 3.

\[ \text{Total phlorotannin content was calculated based on eq 3} \]

\[ \text{TPC (mg/g)} = \frac{I_{\text{std}}N_{\text{std}}M_{\text{std}}P_{\text{std}}}{I_{\text{anl}}N_{\text{anl}}M_{\text{anl}}P_{\text{anl}}S \ g} \]  

(3)

where m is the weighted sample (mg); I is the integration; N is the number of H on the ring; N_{anl} = 1.7; N_{std} = 3; M is the molar mass; M_{anl} = 210.14; M_{std} = 124; anl is the analyte; std is the standard; and S is the mass of seaweed (g).

4.6.2. 13Carbon Nuclear Magnetic Resonance (13C NMR) Spectroscopy. Purified extracts were prepared using SPE as per Section 4.4.1. 13C NMR spectroscopy was carried out on a Bruker DPX600 spectrometer. Chemical shifts are reported in parts per million (ppm). An inverse-gated decoupling program was used in the 13C NMR experiments. In this method, proton decoupling is only applied during the acquisition period. Therefore, the effect of the polarization transfer from 1H to 13C...
via nuclear Overhauser enhancement (NOE) is removed and the resulting $^1$H coupled $^{13}$C spectra can be used for quantitative $^{13}$C analysis. To run these experiments, the pulse program “zgig30” was used on a Bruker DPX600 spectrometer.

4.6.3. Folin–Ciocalteu (FC) Assay. A 1 mL volume of the diluted phenolic extract solution (prepared as per Section 4.4.3) was then added to the FC reagent diluted 1:1 with deionized water (0.5 mL); the solution was left to stand for 5 min after which 20% aqueous sodium carbonate was added (2.5 mL). Solutions were then left in the dark for 40 min to develop color and the absorbance was recorded at 755 nm on a Jenway 6305 fixed wavelength spectrophotometer. The absorbances were converted to a concentration in phloroglucinol equivalents using a calibration curve of phloroglucinol, which had a good linear fit $R^2 > 0.95$. This analysis was performed in triplicate.

4.7. Statistical Analysis. A three-way ANOVA was applied to study the significance of the three parameters (factor 1 = seaweed species ($A. nodosum$ and $F. serrata$), factor 2 = pathogen ($S. agona, E. coli$ O157, and $S. suis$), and factor 3 = extract type (control, MIC, and MBC)) and their interactions on (1) cell membrane permeability and (2) intercellular ATP. The pathogen cell membrane permeability was determined in terms of percentage relative electrical conductivity. A least significant difference (LDS) post hoc test with $\alpha = 0.05$ was then applied. If any treatment effects were significant, and then pairwise differences between individual treatment levels were assessed using Fisher’s LDS test. The statistical analysis was used to test the hypothesis that the potency of the extract was species- and pathogen-dependent. For cell viability data, a comparison of parameters was achieved by a two-way ANOVA (factor 1 = species and factor 2 = extract concentration). In addition, a two-sample $t$-test was used to compare the species for each concentration separately. It was hypothesized that cell viability would be species-dependent. Statistical calculations were performed using IMB SPSS v25 (SPSS Inc., Chicago, IL).

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Notes
The authors declare no competing financial interest.

ACKNOWLEDGMENTS

This research was funded by Agri-Food Quest Competence Centre program (INI Agri-Food QUEST-11-01-17-003-AFQCC), which is funded by Invest NI and Industry (JMW Farms, Devenish Nutrition, and Finnebrogue). Thanks to Stephen Hull for editorial proofreading of this manuscript.

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