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In vitro evaluation of antimicrobial and antioxidant activities of algal extracts

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ABSTRACT

The aim of this study was to evaluate the antioxidant capacity, the antimicrobial proprieties of algae *Ascophyllum nodosum* and *Schizochytrium* spp. against one of major swine enteric pathogen *Escherichia coli* O138 by broth macro-dilution method in Luria–Bertani (LB) medium. The antimicrobial effect of the algal extracts at supplementation of 0.12%, 0.06% and 0.03% (v/v) on *E. coli* O138, genetically characterised by PCR, was evaluated by following the bacterial growth. The antioxidant activity was determined by the ABTS Radical Cation Decolorisation Assay. In particular, the $\log_{10}$ *E. coli* used as control resulted significantly higher than 0.12% at 3 hours (8.82 ± 0.07 and 8.18 ± 0.07 $\log_{10}$ cells/mL, respectively; $p < .01$) suggesting an inhibitory activity related to the dose. No effect activity was observed with *Schizochytrium* spp. against *E. coli* growth. *A. nodosum* and *Schizochytrium* spp. exhibited antioxidant capacity ($p < .05$). The combination of them (1:1) exhibited antioxidant activity suggesting a synergistic effect ($p < .05$). The different proprieties of algal species that can modulate the O138 *E. coli* growth, one of the major pathogen of swine species, together with the antioxidant capacity, make them a promising functional feed additive to improve the gut health, therefore further studies are needed to confirm these activities *in vivo*.

HIGHLIGHTS

- The aim of the study was to evaluate the antimicrobial and antioxidant proprieties of two species of algae: *Ascophyllum nodosum* and *Schizochytrium* spp.
- *Ascophyllum nodosum* revealed antimicrobial effect against *Escherichia coli* O138. Both algae exhibited antioxidant capacity also with a synergistic effect.

Introduction

One of today's challenges, in line with the One-Health principles, is to reduce the use of drugs and antibiotics in humans and livestock because of the rise of antibiotic resistance (EFSA and ECDC 2013; Dhama et al. 2013). In this context, pig farming is one of the most profitable agricultural practices; however, antibiotics have often been used in order to deal with critical phases of a pig's life, such as weaning. Post weaning diarrhoea (PWD), a gastrointestinal disease mainly associated with certain *Escherichia coli* strains, represents the most common indication for the antimicrobial prescription (Amezcua et al. 2002). In particular, the pathogroup of porcine verocytotoxin-producing *Escherichia coli*, belonging to serogroups O138, O139 and O141, is characterised by a virulence profile responsible for acute and severe enterotoxemia and for important economic losses (Verdonck et al. 2002; Rossi et al. 2014). Many factors, infectious and non-infectious, are involved in the outbreak of the PWD that is considered a multifactorial disease where nutrition plays a pivotal role (Rossi et al. 2013). The reduction of the use of antimicrobials in food-producing animals, replacing them where possible and re-thinking the livestock production system, is essential for the future of animal and public health (EFSA 2012;

Murphy et al. 2017). For these reasons, several functional feed additives need to be evaluated in the weaning phase in order to increase the health status and reduce the need for antibiotics (Windisch et al. 2008). In this scenario, algae, besides being a valid source of essential nutrients, can be a very interesting natural source of new compounds with biological activity that could be used as functional ingredients of pig rations (Makkar et al. 2016; Madeira et al. 2017).

Algae are a heterogeneous group of autotrophic, photosynthetic, aquatic organisms that ranges from single-celled microorganisms defined as microalgae, to huge complex seaweeds (Evans and Critchley 2014). *Schizochytrium* spp., a microalga from Thraustochytrids family, is one of the most commonly studied microalgae in animal nutrition because of its high content in n-3 and n-6 poly-unsaturated fatty acids with observed effects on animal's products quality and animal health status (Jiang et al. 2004; AbuGhazaleh et al. 2009; Madeira et al. 2017).

Macroalgae are macroscopic, multicellular plant-like organisms with a highly variable composition depending on species, habitat, temperature, light intensity, nutrients concentration of water (Makkar et al. 2016). Macroalgae can be divided into three groups: brown algae, red algae and green algae. Although brown algae are of lesser nutritional value than red and green algae, they contain important bioactive compounds. *Ascophyllum nodosum* is a large, common cold water brown alga and is one of the most used seaweed in animal nutrition, it is rich in minerals, particularly potassium and iodine (Combet et al. 2014) and contains polyphenols and phlorotannins, enlisted as bioactive compounds (Munir et al. 2013; Makkar et al. 2016). Algal biomass can also have a positive impact on food security and the environmental impact: in fact, they are cheap to harvest and grow exclusively in water, preventing the use of arable land. They also release oxygen into the atmosphere and, if included in the diet of ruminants, are able to reduce methane emissions (Fievez et al. 2007). Algal culture technology is similar to terrestrial plants agriculture; however, algae possess higher productivity. Plants are in a different scale level with an evolved organisation of tissues, but micro and macroalgae have some advantages compared to them: they are not cultivated in agricultural soils, they can grow in non-potable water, in coastal, arid and marginal areas unsuitable for agricultural purposes (Cardon et al. 2008; Gouveia and Oliveira 2009; Bochenski et al. 2019). Moreover, considering the challenges and the agrozootechnical scenario of the coming years, the enlargement of feed resource base through identification of novel feeds or development of new additives that enhance resources use efficiency would play an important role in sustainable development of the animal productions.

Even if the algae bioactivities have been recognised, considering the heterogeneity of the algae-based commercial products for feed and the intra- and interspecific differences among algae, it is necessary to evaluate their functional proprieties, define the suitable species and the possibility to combine them to enhance their effect. For these reasons, the aim of this study was to evaluate the *in vitro* antioxidant properties and the antibacterial effect against O138 *E. coli,* major pathogen in swine livestock, of *Ascophyllum nodosum* and *Schizochytrium* spp. in order to establish their possible further integration in weaned pigs' diets as functional feed additive and as possible alternative to antibiotic compounds.

Materials and methods

Evaluation of nutritional value

Algae dried meal samples were obtained from Italfeed S.r.l. (Milan, Italy). The samples were analysed for the main nutrient components (ether extract, crude proteins, fats, crude fibre, ash) according to AOAC (2005) 'Official methods of analysis' in double.

Dry matter (DM) was obtained by inserting samples in preweighted aluminium bags and dried in a forced-air oven at 105 °C for 24 h (AOAC method 930.15).

Ashes (Ash) were obtained using a muffle furnace at 550 °C (AOAC method 942.05). Crude protein (CP) was determined by a Kjeldahl method (AOAC method 2001.11). Ether extract (EE) was determined using ether extraction in the Soxtec system (DM 21/12/1998). Crude fibre (CF) was determined by filtering bag technique [AOCS (2009) method Ba 6a-05]. Mineral content was determined after sample mineralisation. In particular, pulverised samples (0.3 g) were digested by a microwave digestor system (Anton Paar MULTIWAVE-ECO) in Teflon tubes filled with 10 mL of 65% HNO_3 by applying a one-step temperature ramp (at 120 °C in 10 min and maintained for 10 min). The mineralised samples were cooled for 20 minutes and they were transferred into the polypropylene test tubes. Mineralised samples were diluted 1:100 with 0.3 M HNO_3 in MilliQ Water and the concentration of elements was measured by inductively coupled plasma atomic emission spectrophotometer (ICP; model: Optima 3300 XL, PerkinElmer Inc., Massachusetts, USA).

Table 1. Primers used for polymerase chain reaction (PCR).

Primers	Nucleotide sequences
FedF-5′	CCATGGCTACTCTACAAGTAGACAAGTCTGTTTC
FedF-3′	GAGCTCTTACTGTATCTCGAAAACAATGGGCACCG
VT2e-B subunit-5′	GGATCCATGAAGAAGATGTTTATAGCGG
VT2e-B subunit-3′	AACGGGTCCACTTCAAATGATTCTCGAG

FedF gene is an essential adhesion protein of F18 fimbriae, VT2eB is the gene codifying verocytotoxin type 2 variant B-subunit.

Escherichia coli characterisation

The O138 *E. coli* strain was obtained from the Lombardy and Emilia Romagna Experimental Zootechnic Institute (IZSLER, Italy).

The strain was genetically characterised for the presence of genes codifying for two virulence factors represented by the F18 adhesive fimbriae and the verocytotoxin (VT2e). In particular, specific oligonucleotides were designed for the detection of FedF gene essential for F18 adhesion fimbriae and the B subunit of VT2e, responsible for binding the toxin to the intestinal cell surface before the absorption (Table 1).

Genomic DNA was extracted using phenol/chloroform (1:1) from an overnight culture of *E. coli* strain and the quality of DNA was evaluated spectrophotometrically (260/280 ratio) and by agarose gel electrophoresis (0.8%, 10 V/cm 2 h) for quantification and to test for the presence of RNA or degraded DNA. The presence of FedF and VT2eB genes was evaluated by polymerase chain reaction (PCR) using specific primer-pairs. PCR was performed using the following experimental conditions: first denaturation 94 °C for 2 minutes, denaturation phase 94 °C for 1 minute, followed by annealing phase 55 °C for 2 minutes and elongation phase 72 °C for 2 minutes (the cycle was repeated 34 times). The volume of reaction mixture was 50 μL, with 5 μL of template (bacterial DNA) added to PCR mixture.

Antimicrobial assay

Antimicrobial extracts from algae *Ascophyllum nodosum* and *Schizochytrium* spp. were obtained according to Jiménez et al. (2010) method. Five grams of dried algal meal sample was dissolved in 150 mL of acetone and extracted using a Soxhlet apparatus for 6 hours. After the evaporation of the solvent under vacuum at 50 °C, the residue (120 mg) was resuspended in 20 mL of MilliQ water, filtered with 0.22 μm syringe filter and stored at −20 °C until the analysis.

A liquid culture-based growth inhibition assay with *E. coli* O138 was performed to evaluate the ability of algae Soxhlet extracts to inhibit bacterial growth. An overnight culture of *E. coli* O138 in Luria–Bertani (LB)

liquid medium was used as the inoculum for the experiments.

The experiment was set up as follows: 10 mL of LB with 120 μL of *E. coli* culture in liquid LB medium without algal extract was used as a positive control in order to evaluate the bacterial growth without any external influence. Three concentrations of the algal extract were added to a 50-mL tube with 10 mL of LB, to obtain a final concentration of treatment of 0.12%, 0.06% and 0.03% respectively. These concentrations were tested to evaluate if lower concentrations, compared with previous literature (Jiménez et al. 2010), were able to exert antimicrobial activity also in order to optimise the possible future inclusion of algae in feed and considering their cost-effective.

120 μL of overnight LB culture of *E. coli* were then added to each 50-mL tube. The same algal extract concentrations were added to 10 mL of LB without adding 120 μL of *E. coli* culture for the negative controls. All the samples were then maintained at 37 °C in a shaking incubator for six hours. The growth rate of *E. coli* was estimated, every hour for six hours, measuring the absorbance with a spectrophotometer (V-630 UV-VIS Spectrophotometer, JASCO, Germany) at an optical density (OD) of 600 nm.

The measured OD was converted in $\log_{10}$ of the number of cells/mL considering 1 OD $= 1 \times 10^9$ cells/mL (Myers et al. 2013).

All assays were performed in technical duplicate and three biological replicates that are meant to verify the replicability of the experiment, using the same procedures repeating the experiment starting from the sample also repeating the test in different days.

Evaluation of antioxidant properties (ABTS assay)

To perform the antioxidant assay, dried meal of *Schizochytrium* spp. and *A. nodosum* were extracted using ethanol and water according the method proposed by Machu et al. (2015) with some adaptations. One gram of each algae was dissolved in 10 mL of solvent (pure ethanol or water) and stirred for 24 hours at 23 °C. A mixture of both algae was prepared using 1:1 (w/w) of *A. nodosum* and *Schizochytrium* spp. powder extracted with 10 mL of water or ethanol as solvents. The mixture was stirred for 24 hours at 23 °C.

Extracts were centrifuged for 10 min at 3000 rpm, the supernatants were collected and filtered with 0.45-μm syringe filter and stored at −20 °C until the analysis.

The antioxidant activity was tested by adopting an ABTS assay, according to Re et al. (1999).

The 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS$^{\bullet+}$) radical cation was generated by the reaction of 7 mM ABTS with 2.45 mM of K-persulfate. The reaction mixture was left to stand in the dark for 16 hours at room temperature and used within two days. Working solutions of ABTS$^{\bullet+}$ were obtained by diluting ABTS$^{\bullet+}$ in ethanol in order to obtain an absorbance of 0.700 ± 0.02 OD at 734 nm at room temperature. First, a calibration curve was obtained using different concentrations (2000 μM, 1500 μM, 1000 μM, 500 μM, 100 μM, 0 μM) of Trolox (6-hydroxy-2,5,7,8-tetramethychroman-2-carboxylic acid) as standard. The assay was performed using 10 μL of diluted sample added to 1 mL of working solution (ABTS$^{\bullet+}$). The absorbance was recorded from 1 to 6 minutes and all determinations were performed in triplicate.

The A. nodosum and Schizochytrium spp. extracts were diluted in their solvents (water and ethanol) and tested in the following concentrations: 10%, 5%, 2%. Secondary, in order to test the synergistic effect, the mixture with an equal concentration of both algae (1:1 w/w) was then tested with the same concentrations: 10%, 5%, 2%. The concentrations tested were prepared diluting the original extract 1:5 (v/v), 1:10 (v/v) and 1:20 (v/v) in line with the range tested by Jiménez et al. (2010) for DPPH assay. The dilutions were also necessary to obtain absorbances comparable with the calibration curve in order to quantify the final results that were expressed as equivalent concentration of Trolox/g after six minutes (TroloxEq/g).

The total antioxidant capacity was expressed as the percentage inhibition (PI), according to the equation: PI = [(AbsABTS$^{\bullet+}$ - Abs sample)/AbsABTS$^{\bullet+}$] × 100; here AbsABTS$^{\bullet+}$ denotes the initial absorbance of diluted ABTS$^{\bullet+}$, and Abs sample denotes the absorbance of the sample in every 6 min of reaction.

Statistical analysis

Data of antimicrobial and antioxidant assays were analysed using Proc GLIMMIX of SAS 9.4 (SAS Inst. Inc., Cary, NC) (SAS Institute 2009). The model included the fixed effect of treatments, time, the interaction between treatment and time. Within significant two-way interactions, slice option was used to separate means within a specific treatment and time and the results are reported as least squares means (LSMEANS) and standard error. Data of μmol TroloxEq/g were compared using Proc GLM. The model included the effect of algae and extraction methods (water and

Table 2. Chemical composition of dried algae samples of *A. nodosum* and *Schizochytrium* spp.

	Ascophyllum nodosum	Schizochytrium spp.
DM (%)	92.12	99.39
Ash (%)	21.41	5.26
CP (%)	8.25	0.00
EE (%)	3.31	25.33
CF (%)	3.57	0.00

All values are expressed as percentage of dry matter (% DM).
DM: Dry matter; Ash: Ashes; CP: Crude Protein; EE: Ether Extract; CF: Crude Fibre.

ethanol) and the interaction between algae and extraction methods. Means were considered different when $p < .05$ and tended to different if $.05 < p \leq .10$. Tukey–Kramer studentised adjustments were used to separate the means of extraction methods within the two-way interactions. Results are reported as means and standard deviations.

Results and discussion

In this study, the attention was focalised on the *in vitro* evaluation of nutraceutical properties of *Schizochytrium* spp. and *Ascophyllum nodosum* in order to establish their further use as functional additives.

The obtained results of chemical analysis of algae are in line with literature (Makkar et al. 2016) and the commercial feed label (Table 2). Both algae were freeze-dried meal with moisture content less than 8% in order to guarantee adequate storage conditions. In general, *A. nodosum* is characterised by a high content of minerals (more than 20% DM), in particular, it is characterised by a high content of calcium and a low content of phosphorous. This aspect should be considered in the diet's formulation in order to maintain a correct balance of these two elements. However, if algae are used as feed additives, they will be included less than 5% in the diet and this percentage should not constitute an important change in the mineral balance. In general, mineral premix additive is always included during the ration formulations, the amount of minerals contained in algae must be considered for their enclosure in the feed in order to respect the admitted levels of European Union regulation (Reg 1081/2003/EC) (EC 2003). In particular, some minerals are required as nutrients for the piglets, but they are frequently integrated in excess also to increase animal performances and this aspect could represent a risk for the environment (Hejna et al. 2018). Minerals such as cadmium (Cd) are undesirable compounds and Cd could become toxic in higher concentrations. Our results revealed an amount of Cd in line with permitted level of European regulation about feed additives (Directive 2002/32/EC). Other minerals like zinc (Zn),

copper (Cu) and selenium (Se) are useful to satisfy the requirements of animals, their concentration should be balanced with the mineral content of the ingredients utilised in animal feed, also respecting the safety of the environment (Table 3). On the contrary, *Schizochytrium* spp. contains a lower amount of minerals (5.25% DM), but it represents an important source of lipids. *A. nodosum* is characterised also by 8.25% (DM) of crude protein content with high biological value (Becker 2004). Even if the amount of protein is comparable to the corn meal, it should be considered that the presence of non-protein nitrogen in different algal species can affect the results slightly. In particular, the quantification of the crude protein using the standard conversion value for nitrogen (6.25) could lead to an overestimation of the protein content if non-protein nitrogen is present (Lourenço et al. 2004).

The antimicrobial properties were evaluated against O138 *E. coli,* one of the major enteric pathogens of weaned piglets responsible for postweaning enteritis and enterotoxaemia that causes significant morbidity

and mortality in pigs worldwide. PWD represents an important issue in swine farming that causes important economic losses, at the same time affecting the health of animals and leading to the consumption of antibiotic drugs (Rossi et al. 2013). The pathogenicity is usually influenced by the presence of virulence factors, such as VT2e toxin and the F18 adhesive fimbriae (Verdonck et al. 2002). The detection of FedF adhesion factor of F18 and of VTe2B gene confirmed the virulence profile of the O138 *E. coli* strain (Figure 1).
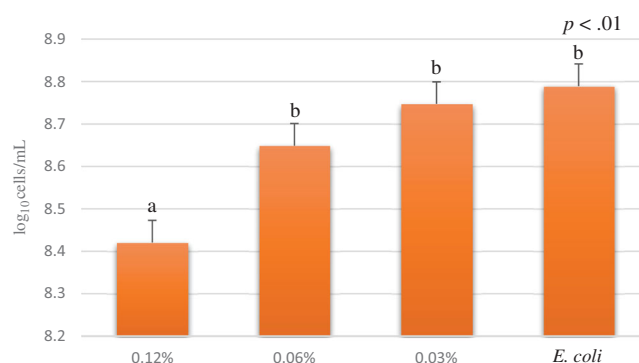
Significant differences in the bacterial growth were observed among the *A. nodosum* concentrations, respectively: 0.12%, 0.06%, 0.03% and 0% (*E. coli* without treatment) ($p<.01$) (Figure 2). In particular, *A. nodosum* disclosed that the most concentrated treatment (0.12%) exhibited the highest inhibition activity on O138 *E. coli* growth; on the contrary, the lowest concentrations (0.06% and 0.03%) revealed a bacterial growth comparable to the positive control, indicating that these concentrations did not influence *E. coli* growth.

Our results are in line with other findings, demonstrating a significant decrease in bacterial growth *in vitro* (Dierick et al. 2010). Nevertheless, obtained results confirmed the antimicrobial activity also against a wild type strain characterised by a relevant virulence profile.
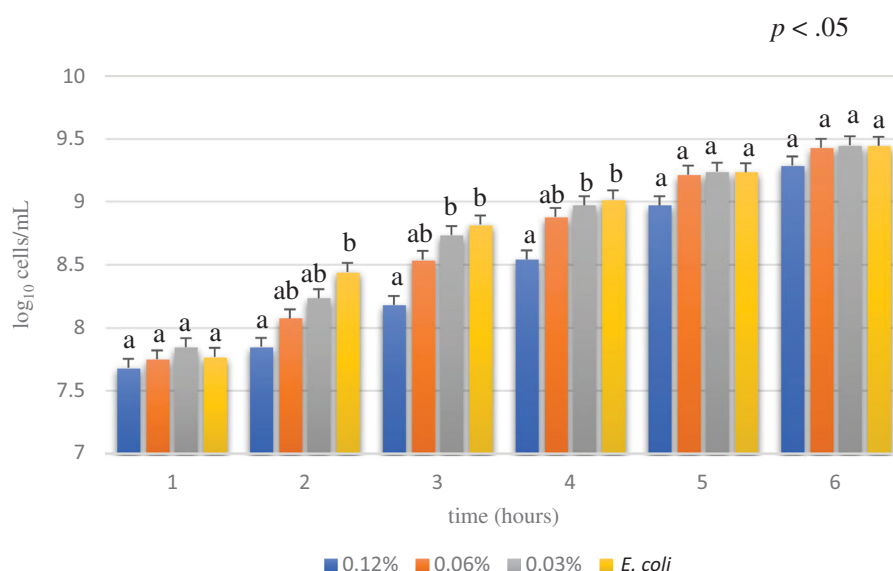
The inhibitory effect is probably attributed to phlorotannins, which are known to be powerful bacteriostatic and also a bactericidal component of brown algae (Wang et al. 2009). Other components of brown seaweed have demonstrated to possess an antibacterial activity, in particular polyphenols, which are a class

Table 3. Mineral composition of dried algae samples *A. nodosum* and *Schizochytrium* spp.

	A. nodosum	Schizochytrium spp.
Ca	10466.00	n.d.
Cd	0.49	0.36
Cu	4.19	n.d.
Fe	433.00	11.10
Mg	10546.20	15.40
Mn	132.00	10.10
P	51.90	48.50
Se	2.30	2.00
Zn	32.60	6.10

All values are expressed as mg/kg of fresh weight.
n.d.: not detectable

Figure 1. (A) Agarose gel (1%) one-dimensional electrophoresis of PCR products for the detection of FedF gene from genomic DNA of O138 *Escherichia coli* strain. Lane 1: marker 1 kb; lane 2: positive sample; lane 3: negative control sample. (B) Agarose gel (1.5%) one-dimensional electrophoresis of PCR products for the detection of VT2eB gene from genomic DNA of O138 *Escherichia coli* strain. Lane 1: marker 50 pb; lane 2: positive sample; lane 3: negative control sample.

Figure 2. Average of *E. coli* growth (log$_{10}$ cells/mL) of different concentrations of *A. nodosum* Soxhlet extract from time 1 to 6 hour. *A. nodosum* Soxhlet extract concentrations tested were 0.12%, 0.06%, 0.03% and positive control (*E. coli*). Data are shown as least squares means and standard errors. [a,b]means (*n* = 3) with different superscripts are significantly different (treatment *p* < .01).

Figure 3. Average of *E. coli* growth (log$_{10}$ cells/mL) of different concentration of *A. nodosum* Soxhlet extract through experimental time (from 1 to 6 hour). *A. nodosum* Soxhlet extract concentrations tested were 0.12%, 0.06%, 0.03% and positive control (*E. coli*). Data are shown as least squares means and standard errors. [a,b]means (*n* = 3) with different superscripts are significantly different, means are separated within treatment groups though the experimental time with Tukey (interaction treatment by time *p* < .05).

of secondary metabolites also known for their antimicrobial activity (Daglia 2012). It has been demonstrated that polyphenols form different sources are able to produce hydrogen peroxide in aerobic conditions, its production is also directly related to the content of the hydroxyl groups. Tannins possess affinity for bounding proteins and this capacity increases with the number of hydroxyl groups this could explain that phlorotannins exert higher antimicrobial activity compared with terrestrial tannins because they are readily oxidised upon exposure to air and contain a higher number of hydroxyl groups (Wang et al. 2009).

Our findings highlight the need to use the highest concentration in order to guarantee the antimicrobial effect. In particular, considering that the extract used in our study was obtained using 5 g of alga in 150 mL

of acetone, obtaining 120 mg of solid extract that was then dissolved in 20 mL of water, the final concentration of algal extract resuspended in water was 0.6%. Our study is in line with Gardiner et al. (2008), which used an inclusion percentage of *A. nodosum* extract in piglet's diet ranging from 0.3% to 0.9% of dry matter.

The effects of the different concentrations of algae tested to evaluate their inhibition capacity over time (Figure 3) revealed that after the first hour, the inhibition capacities of the tested concentrations were comparable to the positive control (*E. coli*). After 2, 3 and 4 hours, there was a significant difference among the most concentrated treatment (0.12%) and the other samples, which were comparable to the positive control (*E. coli*) (*p* < .05). The maximum inhibitory effect of algal extract was observed after 3 hours, where the

log_{10} cells/mL of control (*E. coli*) and 0.12% concentration were significantly different, respectively 8.82 ± 0.07 and 8.18 ± 0.07 ($p<.01$). No differences were observed at 5 and 6 hours, suggesting an exhaustion of the bioactivity due to algal degradation or to the development of bacterial resistance. According to Zoetendal et al. (2008) the bacterial resistance of *E. coli* against condensed tannins seems to be related to the activation the BaeSR two-component regulatory system. Phlorotannins contained in *A. nodosum* possesses similar property to polyphenols, main constituent of tannins, characterised by antimicrobial activity.

Even if the effect was observed only for a short period, it is important to consider the transit time of the feed in a pig's gastrointestinal tract, which lasts 4 hours, this guaranties the effect throughout the digestion tract.

Results of ABTS assay revealed that *A. nodosum* ethanol extract antioxidant activity (0.75 ± 0.31 µmol TroloxEq/g) is lower compared to the water extract

(*p*<.05) (Table 4) suggesting that this algal species contains a good amount of hydro-soluble antioxidant substances which are easily released in water (Figure 4). In line with our results, the study conducted by Machu et al. (2015) disclosed the highest antioxidant capacity adopting the water extraction for brown algae. Machu et al. (2015) tested *Eisenia bicyclis* that is a brown alga rich in phlorotannins that revealed also antimicrobial capacities against streptomycin resistant *Listeria monocitogenes* (Kim et al. 2018).

The results have also displayed that the antioxidant activity of *A. nodosum* water extract exhibited an antioxidant activity of 55.54 ± 16.05 µmol TroloxEq/g after 6 minutes of reaction (Table 4) (Figure 5). In general, free antioxidant substances react immediately when the sample is added to an ABTS$^{•+}$ reaction mixture in a similar way to the effect of standard Trolox. Other antioxidants that are not immediately available may require time to be released in order to exert their effect against radicals, for this reason the assay was conducted in six minutes.

The *Schizochytrium* spp. ethanol extract exhibited an antioxidant capacity of 2.56 ± 0.53 µmol TroloxEq/g calculated after six minutes of reaction (Table 4) (Figure 6). The *Schizochytrium* spp. water extract did not display any activity; in fact, we obtained an opaque extract which was not able to inhibit the ABTS$^{•+}$ radical. Furthermore, this extract when added to the working solution increased the turbidity of the mixture making the spectrophotometer reading inaccurate. These findings could be due to the high content of lipids of *Schizochytrium* spp. (Table 2) that are known

Table 4. Results of ABTS assay of *A. nodosum*, *Schizochytrium* spp. and the algal mixture (1:1 w/w) in response to the different extraction methods (water and ethanol).

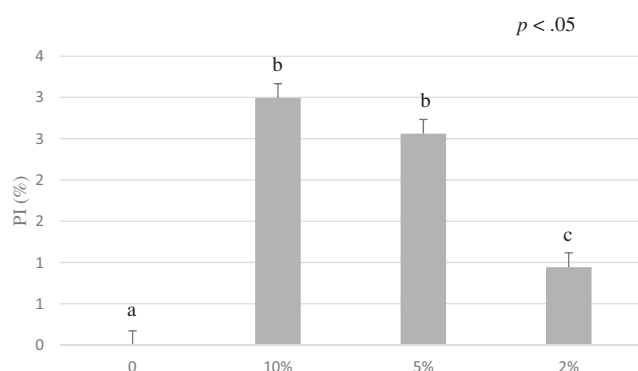|	Extraction methods	
	Water	Ethanol
A. nodosum	55.54 ± 16.05[a]	0.75 ± 0.31[b]
Schizochytrium spp.	n.d.	2.56 ± 0.53
Mixture (1:1 w/w)	10.06 ± 2.73[a]	3.18 ± 0.57[b]

Results are expressed as µmol TroloxEq/g.
Data are shown as means and standard deviations.
[a,b]Means ($n = 3$) with different superscripts are significantly different (Treatment $p<.05$).
n.d.: not detectable
ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid).
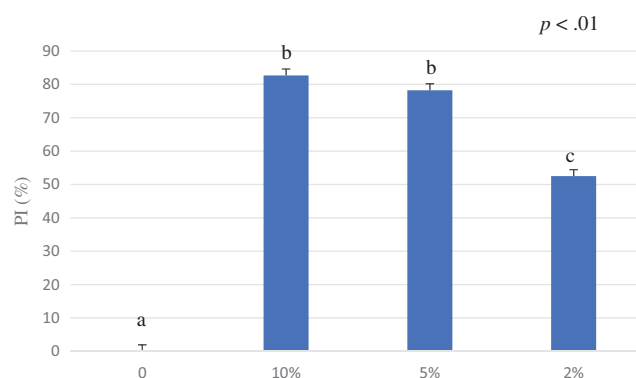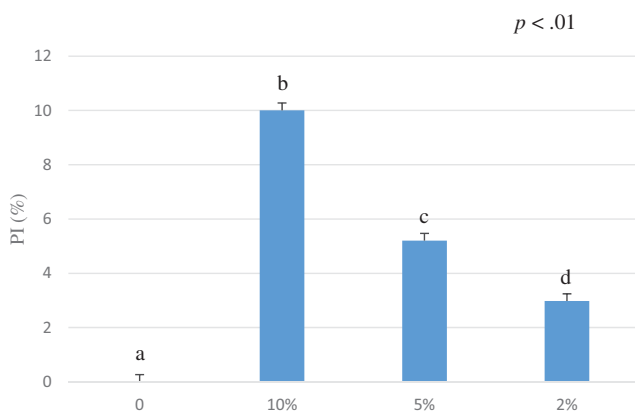

$p < .05$

Figure 4. Average of percentage of inhibition (PI%) of *A. nodosum* ethanol extract from 0 to 6 minutes. The ABTS antioxidant assay tested different concentrations of *A. nodosum* ethanol extract 10%, 5%, 2% and blank. Data are shown as least squares means and standard errors. [a,b]means ($n = 3$) with different superscripts are significantly different (treatment $p<.05$). ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid).

$p < .01$

Figure 5. Average of percentage of inhibition (PI%) of *A. nodosum* water extract from 0 to 6 minutes. The ABTS antioxidant assay tested different concentrations of *A. nodosum* water extract 10%, 5%, 2% and blank. Data are shown as least squares means and standard errors. [a,b]means ($n = 3$) with different superscripts are significantly different (treatment $p<.01$). ABTS: 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid).
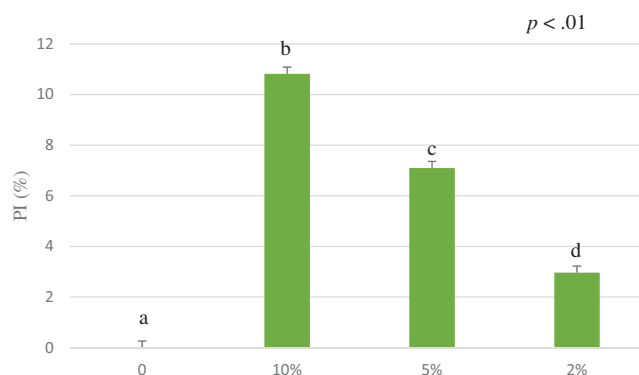
Figure 6. Average of percentage of inhibition (PI%) of *Schizochytrium* spp. ethanol extract from 0 to 6 minutes. The ABTS antioxidant assay tested different concentrations of *Schizochytrium* spp. ethanol extract 10%, 5%, 2% and blank. Data are shown as least squares means and standard errors. [a,b]means ($n = 3$) with different superscripts are significantly different (treatment $p < .01$). ABTS: 2,2′-azino-bis (3-ethylbenzo-thiazoline-6-sulfonic acid).
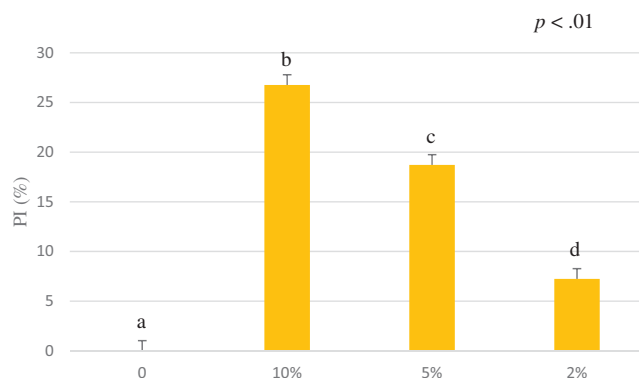
to be not hydro-soluble and water was not able to extract non-polar substances.

The mixture of *A. nodosum* and *Schizochytrium* spp. exhibited an antioxidant capacity of $3.18 \pm 0.57\,\mu$mol TroloxEq/g for ethanol extract and 10.06 ± 2.73 for water extract (Table 4) (Figures 7 and 8). The antioxidant capacity (μmol TroloxEq/g) of the mixture was lower than the antioxidant capacity of *A. nodosum* water extract alone, but the ethanol extracted mixture ($3.18 \pm 0.57\,\mu$mol TroloxEq/g) was comparable to the sum of the antioxidant capacity of both ethanol extracted algae (2.56 ± 0.53 and $0.75 \pm 0.31\,\mu$mol TroloxEq/g) suggesting that could be possible a synergic effect. Significant differences were observed in a dose-dependent way among the concentration tested expressed as percentage of inhibition (PI%) ($p < .01$).

Even if in this study *Schizochytrium* spp. demonstrated lower antioxidant capacity compared to brown algae, several *in vivo* studies have been largely

Figure 7. Average of percentage of inhibition (PI%) of *A. nodosum* and *Schizochytrium* spp. mixture (1:1 w/w) ethanol extract from 0 to 6 minutes. The ABTS antioxidant assay tested different concentrations of *A. nodosum* and *Schizochytrium* spp. mixture ethanol extract 10%, 5%, 2% and blank. Data are shown as least squares means and standard errors. [a,b]means ($n = 3$) with different superscripts are significantly different (treatment $p < .01$). ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid).

Figure 8. Average of percentage of inhibition (PI%) of *A. nodosum* and *Schizochytrium* spp. mixture (1:1 w/w) water extract from 0 to 6 minutes. The ABTS antioxidant assay tested different concentrations of *A. nodosum* and *Schizochytrium* spp. mixture water extract 10%, 5%, 2% and blank. Data are shown as least squares means and standard errors. [a,b]means ($n = 3$) with different superscripts are significantly different (treatment $p < .01$). ABTS: 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid).

demonstrated the capacity of *Schizochytrium* spp. to improve health status and production efficiency of livestock animals (Mata et al. 2010; Lv et al. 2015). The content of polyphenols, tocopherols and other minor components acts against oxidation (Pandey and Rizvi 2009), in the last decade, interest in the potential health benefits of dietary plant polyphenols as antioxidants has increased rapidly. *Schizochytrium* spp. contains a large amount of DHA that have many beneficial effects. However, high concentrations of n-3 polyunsaturated fatty acids may increase lipid peroxidation and subsequently induce oxidative stress (Vericel et al. 2003). This may explain the lower antioxidant capacity of *Schizochytrium* spp. compared to *Ascophyllum nodosum*.

The antioxidant activity of *A. nodosum* may also be related mainly to phlorotannins, which possess strong antioxidant capacity (Sathya et al. 2017). The antioxidant activity of $55.54 \pm 16.05\,\mu$mol TroloxEq/g observed for *A. nodosum* is comparable with a strawberry antioxidant capacity (Castrica et al. 2019). Other studies also confirm the antioxidant activity of two polysaccharide groups, laminarin and fucoidans which are both present in brown algae (Kadam et al. 2015). Phenolic compounds such as flavonoids, phenolic acids, and tannins are considered to be major contributors to the antioxidant capacity of plants. These antioxidants also possess diverse biological activities, such as anti-inflammatory, anti-atherosclerotic and anti-carcinogenic activities. These activities may be related to their antioxidant activity that consists in the electron-transfer capacity from their molecules to the oxidised radical by scavenging the free radicals (Li et al. 2007).

Considering that *E. coli* diseases are commonly multifactorial, all the stressors during the weaning can reduce the immune defences. Antibacterial and antioxidant compounds could help young animals to maintain the health status, improving defences and thus increasing performance. *A. nodosum* and *Schizochytrium* spp. have displayed suitable characteristics for animal nutrition also revealing interesting bioactivities. The future trends will be the inclusion of algae as ingredient in feed evaluating the optimal level of inclusion related to their bioactivities *in vivo* and define their cost opportunity.

Conclusions

Considering that algae represent several advantages form agronomic and environmental point of view, they could become one of the valuable sources of food and feed.

A. nodosum and *Schizochytrium* spp. revealed interesting bioactivities *in vitro* and they are promising as future feed additives. Indeed, despite only an antibacterial effect was observed for a limited time, is interesting to notice the synergistic effect supplied from the complementary characteristic of these algal species. *A. nodosum* can modulate the *E. coli* growth, *Schizochytrium* spp. possesses antioxidant capacity and the combination of these two species can enhance the antioxidant power. With the urgent need of innovative functional feed additives as alternative to antibiotics, these algal species should be considered in animal breeding.

Therefore, more studies on the argument are needed to confirm the *in vitro* obtained results also in livestock conditions. Obtained findings revealed that *A. nodosum* and *Schizochytrium* spp. possess antimicrobial and antioxidant activities, these aspects should be considered as promising for innovative feed additives for functional animal nutrition, since each algal species has displayed different effects that could be used in complementary way.

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