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Authors

Hejna, Monika

DellAnno, Matteo

Liu, Yanhong

et al.

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Assessment of the antibacterial and antioxidant activities of seaweed-derived extracts

Monika Hejna[1✉], Matteo Dell'Anno[2], Yanhong Liu[3], Luciana Rossi[2], Anna Aksmann[4], Grzegorz Pogorzelski[1] & Artur Jóźwik[1]

In swine farming, animals develop diseases that require the use of antibiotics. In-feed antibiotics as growth promoters have been banned due to the increasing concern of antimicrobial resistance. Seaweeds offer bioactive molecules with antibacterial and antioxidant properties. The aim was to estimate the in vitro properties of seaweed extracts: *Ascophyllum nodosum* (AN), *Palmaria palmata* (PP), *Ulva lactuca* (UL), and 1:1 mixes (ANPP, ANUL, PPUL). *Escherichia coli* strains were used to test for growth inhibitory activity, and chemical-based assays were performed for antioxidant properties. The treatments were 2 (with/without *Escherichia coli*) × 2 (F4 + and F18 +) × 5 doses (0, 1.44, 2.87, 5.75, 11.50, and 23.0 mg/mL). Bacteria were supplemented with seaweed extracts, and growth was monitored. The antioxidant activity was assessed with 6 doses (0, 1, 50, 100, 200, 500, and 600 mg/mL) × 6 compounds using two chemical assays. Data were evaluated through SAS. The results showed that AN and UL significantly inhibited ($p < 0.05$) the growth of F4 + and F18 +. PP and mixes did not display an inhibition of the bacteria growth. AN, PP, UL extracts, and mixes exhibited antioxidant activities, with AN showing the strongest dose–response. Thus, AN and UL seaweed extracts reveal promising antibacterial and antioxidant effects and may be candidates for in-feed additives.

Keywords Seaweeds extracts, Bioactive compounds, Antibacterial, Antioxidant, Livestock farming

Food production with swine farming ranks among the most profitable agricultural practices, often relying on the use of antibiotic drugs to manage a critical phase of a pig's development, such as weaning, which is exposed to stressors that cause multifactorial diseases[1]. Weaning stress is a main factor of diarrhea occurrence linked with Enterotoxigenic and Verotoxigenic *Escherichia coli* (ETEC and VTEC) strains[2]. Animals also experience oxidative stress, which can damage proteins, lipids and DNA[3]. Previously, in-feed antibiotics were commonly applied as preventive treatment for pig diseases. However, the increase in antimicrobial resistance (AMR)[4,5] poses a significant challenge to global health, with interconnections among human, animals and the environment. The primary cause of AMR is the overuse of antimicrobials, both in humans and animals, leading to the spread of resistance genes through the food chain or by direct human-animal contact[6]. Recent reports have highlighted *Escherichia coli* strains as important vectors for antibiotic-resistance genes with zoonotic spread[7]. Therefore, in-feed antibiotics as growth promoters have been banned in Europe[8] and mass veterinary medication applied to cope with infections has been also recently restricted[9,10]. In alignment with the One-Health and 3R (reduce, replace and rethink) approaches, today's challenges in food production require decreasing the use of antibiotics in livestock. This approach aims to improve profitability, increase the sustainability of agriculture, and mitigate the spread of antimicrobial resistance[11,12]. Novel bioactive sustainable feed-additives serving as substitutes to in-feed antibiotics are essential for improving of sustainability and reducing the antimicrobial resistance in the animal industry[4,13]. Among the various potential alternatives, seaweeds (macroalgae) emerge as promising natural sources of bioactive molecules for application as functional feed ingredients[14,15]. Seaweeds are rich in proteins, vitamins, polyphenols and pigments offering antioxidant potential[16–18], and in proteins, peptides, phlorotannins, polysaccharides and fatty acids demonstrating broad antibacterial action against pathogenic bacteria[19,20]. Brown algae, *Ascophyllum nodosum* (AN), even if is commonly used seaweed species in animal nutrition, though it has

[1]Department of Biotechnology and Nutrigenomics, Institute of Genetics and Animal Biotechnology of the Polish Academy of Sciences, Postępu 36A, 05-552 Jastrzębiec, Poland. [2]Department of Veterinary Medicine and Animal Sciences-DIVAS, Università degli Studi di Milano, Dell'Università 6, 26900 Lodi, Italy. [3]Department of Animal Science, University of California, 2251 Meyer Hall, One Shields Ave, Davis, CA 95616, USA. [4]Department of Plant Experimental Biology and Biotechnology, Faculty of Biology, University of Gdańsk, Wita Stwosza 59, 80-308 Gdańsk, Poland. ✉email: m.hejna@igbzpan.pl

not been fully exploited in pig feeding[21]. AN contains polyphenols and phlorotannins[15] with strong antioxidant potential[22] and antibacterial activity against enteric pathogens in pigs, respectively[23]. Fucoidan and laminarin extracted from AN reported to possess the antibacterial property[24,25]. Another option, red algae such as *Palmaria palmata* (PP), although rich in bioactive molecules such as lipids, fatty acids, polysaccharides and pigments[26–29], has not received much attention as a feed ingredient. Yuan et al.[26] proved that flavonoids extracted from PP exerted in vitro antioxidant property stronger than vitamin C and E. Moreover, Lopes et al. also proved that PP is abundant in fatty acids with strong antioxidant properties[27]. Lastly, green seaweed, such as *Ulva lactuca* (UL) contains ulvan with strong antioxidant effects[30] and phenols with antibacterial properties[31] thus has been demonstrated to be a promising additive for monogastric animals[32]. However, is not yet supplemented in swine feed' nutrition.

Furthermore, research related to the bio-accessibility and bioavailability of algae compounds after in vitro digestion, as well as the assessment of synergistic and complementary effects of different activities from combined algal extracts is limited[33]. In vitro digestion models are essential for studying (i) the physiology of specific segments of the digestive tract, and (ii) the digestive properties of algae. Further, the synergistic action of both agents is more effective than the action of a single one achieving the specific activity, while the complementary action of both agents combines two or more different actions[34]. Thus, testing the seaweed extracts in mixes (1:1) is important to establish their possible synergistic, and complementary interactions reinforcing their effectiveness and reducing the minimal effective dosage against infections using combinatory treatment.

Therefore, the rationale of selecting these seaweed species lies in their broad spectrum of bioactive molecules and diverse chemical compositions, which may enhance the synergistic effects. Additionally, their global geographical distribution facilitates further applications in the field. Thus, several approaches were employed to assess the biological activities of seaweed extracts and their mixes (1:1). The current study targeted algal extracts and their inhibitory activity against ETEC, and VTEC *Escherichia coli* strains (F4 + and F18 +), which are the most significant pathotypes responsible for post-weaning diarrhea, thereby increasing the utilization of antibiotic treatment in swine[35]. Besides, two chemical-based assays, including the 2,2-diphenyl-1-picrylhydrazyl (the DPPH) radical scavenging and reducing power assays (the RPA) were used to test the antioxidant property of algal extracts.

Results

Total polyphenol content in seaweed species

The TPC was evaluated in the powder of three seaweed species using tannic acid as a reference standard. The results disclosed that the total polyphenol content was the highest in *Ascophyllum nodosum* (4951.53 ± 152.47 µg TAE/g of sample). TPC in *Palmaria palmata*, and *Ulva lactuca* reached 896.84 ± 27.41 µg TAE/g of sample and 201.48 ± 36.29 µg TAE/g of sample, respectively.

Chemical composition and in vitro digestion of algal powders

The chemical composition results revealed that crude proteins and lipids were under 10% of their value, except for crude proteins for *Ulva lactuca* (15.86 ± 0.60, Table 1). Moreover, a low content of crude fiber (< 10%) was observed in all species. However, higher content was observed in *Ascophyllum nodosum*, and *Ulva lactuca*. Additionally, all tested algae species displayed a high content of ash, and as a consequence, a high content of minerals (Table 1). Moreover, the in vitro digestibility analysis revealed that the digestibility of 100% algal extract ranged from 20.05% in AN and 37.09% in PP (Table 2). Regarding the algae 1:1 mixes the mixture of PPUL exerted the highest digestibility (41.34%; Table 2). The digestibility of the control samples (carbohydrate and protein sources) reached 90.0%.

Antibacterial activity through the *Escherichia coli* growth inhibitory assay

The results of algae species demonstrated that F4 + and F18 + were significantly sensitive to different doses of brown and green seaweed extracts, AN and UL, respectively. A significant ($p < 0.05$) dose-dependent effect was observed at time points T1 to time points T4 for AN and UL (Figs. 1A,B and 2A,B). The highest dose of AN and UL (23 mg/mL) resulted the significant maximum inhibitory activity against F4 + and F18 + growth at each time point. Red seaweed extract, PP did not exhibit the growth inhibitory effect from T4 to T6 for both strain of *Escherichia coli,* although the results exhibited significant differences in the highest dose of PP in both T1, T2 and T3 time points (Figs. 1C and 2C). However, algal extract mixes (ANUL, ANPP, PPUL) of seaweed extract show low inhibitory effects on F18 +, and F4 + strains (Figs. 3A,B and 4A,B). A significant dose-dependent effect

Algae species	DM (%)	CP (%)	EE (%)	CF (%)	Ash (%)
Ascophyllum nodosum	94.24 ± 0.07	4.37 ± 0.32	2.60 ± 0.02	5.57 ± 0.35	21.16 ± 0.85
Palmaria palmata	94.93 ± 0.09	9.68 ± 0.15	0.46 ± 0.06	1.14 ± 0.34	24.30 ± 0.20
Ulva lactuca	89.23 ± 0.08	15.86 ± 0.60	0.28 ± 0.10	6.30 ± 0.34	20.58 ± 0.35

Table 1. Percentage chemical composition on the dry matter basis of *Ascophyllum nodosum*, *Palmaria palmata* and *Ulva lactuca*. All values are expressed as mean and standard deviation of the mean (±) from triplicated samples. *DM* dry matter, *CP* crude protein, *EE* ether extract, *CF* crude fiber.

Digestibility (%)	
AN	20.05 ± 3.94
UL	36.53 ± 2.97
PP	37.09 ± 1.84
ANUL	22.92 ± 0.02
ANPP	22.88 ± 2.39
PPUL	41.34 ± 0.34

Table 2. Percentage of in vitro digestibility in all tested samples of algae species. All values are expressed as mean and standard error of the mean (±).

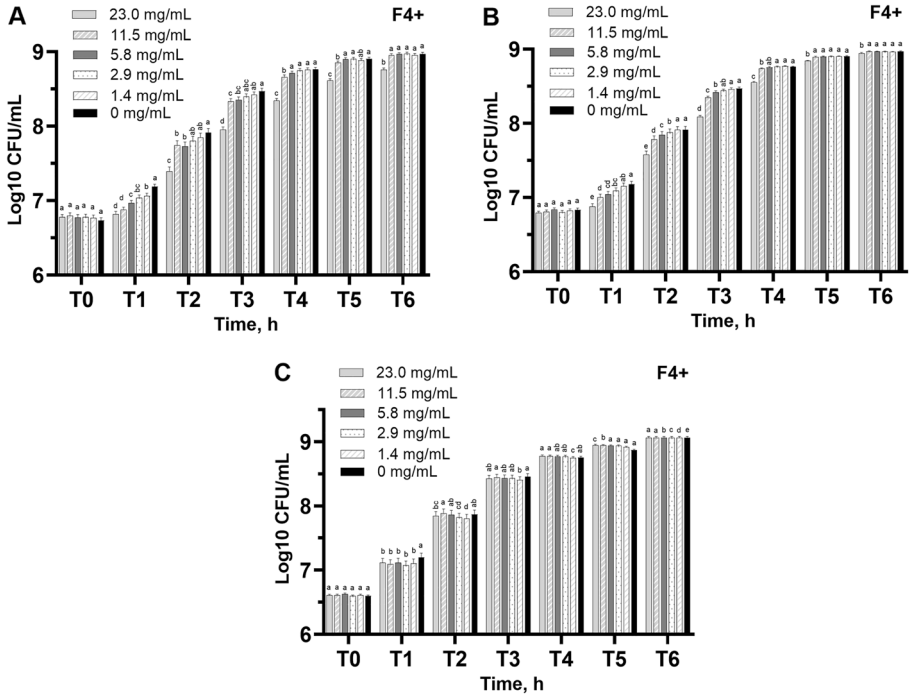

Fig. 1. The impact of different concentrations (0, 1.44, 2.87, 5.75, 11.50, and 23.00 mg/mL) of AN (**A**) UL (**B**) ant PP (**C**) on *Escherichia coli* F4 + growth in 60 min time interval points (T0, T1, T2, T3, T4, T5, T6). Data are expressed as $\log_{10}$ CFU/mL LSMEAN ± SEM (n = 3). Different superscript letters express significant differences at $p < 0.05$ among different concentrations within the same time point.

was observed only at time points T1, and T2 for ANUL, and ANPP, respectively. No dose-dependent effect was resulted for PPUL extract mix in each time point, excluding T2 for F18 + strain (Figs. 3C and 4C).

Antioxidant properties of algal species through chemical-based assays

The radical scavenging capacity and reducing power were used to define the antioxidant capacity of three different algal species (AN, UL, PP). Moreover, the synergic or combined outcome of antioxidant effects was also assessed by testing 1:1 algae extract mixes (ANUL, ANPP, PPUL) using the same chemical assays. A dose-dependent growth in radical scavenging activity in 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was detected in AN, PP and UL and their 1:1 extract mixes in the range of tested concentrations (Fig. 5A,B). The strongest response was noticed at the highest concentration (600 mg/mL) of each algae extract however, among all tested algae species AN, and its extract mixes (ANUL; ANPP) had the highest dose response starting from 50 mg/mL in comparison to UL and PP confirming the highest antioxidant capacity. Moreover, the maximal plateau was observed in the DPPH assay for *Ascophyllum nodosum*. All algal species also elicited increased reducing power in a dose-dependent manner, with the highest dose–response for AN and their 1:1 extract mixes (Fig. 6A,B). Moreover, the effective concentrations (EC) calculated on the basis of the DPPH assay, and exerted the highest EC_{10} for PPUL (504.57 mg/mL), and the lowest for AN (5.10 mg/mL), and ANUL (5.99 mg/mL). EC_{50} could be calculated only for three experimental variants, namely AN, ANUL and ANPP, with lowest EC_{50} value for AN (55.86 mg/mL; Table 3, Supplementary Fig. S1, Supplementary Tables S1–S6).

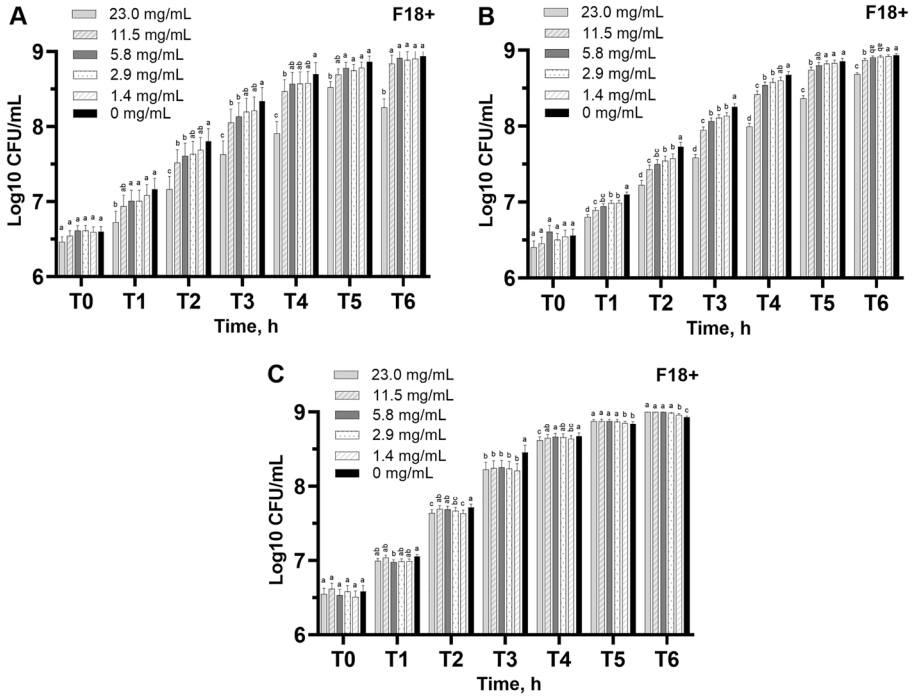

Fig. 2. The impact of different concentrations (0, 1.44, 2.87, 5.75, 11.50, and 23.00 mg/mL) of AN (**A**) UL (**B**) and PP (**C**) on *Escherichia coli* F18 + growth in 60 min time interval points (T0, T1, T2, T3, T4, T5, T6). Data are expressed as $\log_{10}$ CFU/mL LSMEAN ± SEM (n = 3). Different superscript letters express significant differences at $p < 0.05$ among different concentrations within the same time point.

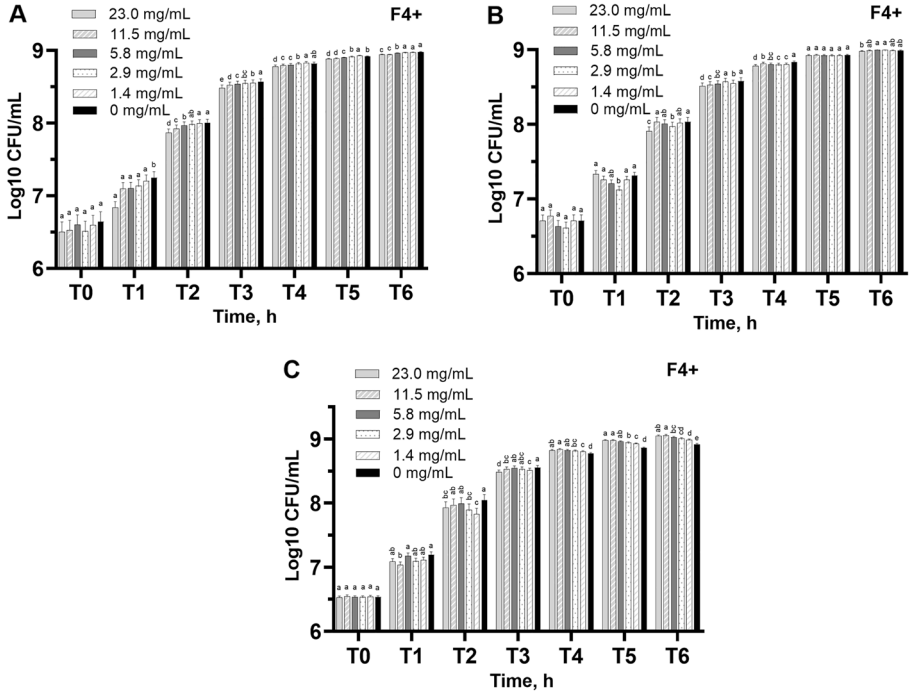

Fig. 3. The impact of different concentrations (0, 1.44, 2.87, 5.75, 11.50, and 23.00 mg/mL) of ANUL (**A**) ANPP (**B**) and PPUL (**C**) on *Escherichia coli* F4 + growth in 60 min time interval points (T0, T1, T2, T3, T4, T5, T6). Data are expressed as $\log_{10}$ CFU/mL LSMEAN ± SEM (n = 3). Different superscript letters express significant differences at $p < 0.05$ among different concentrations within the same time point.

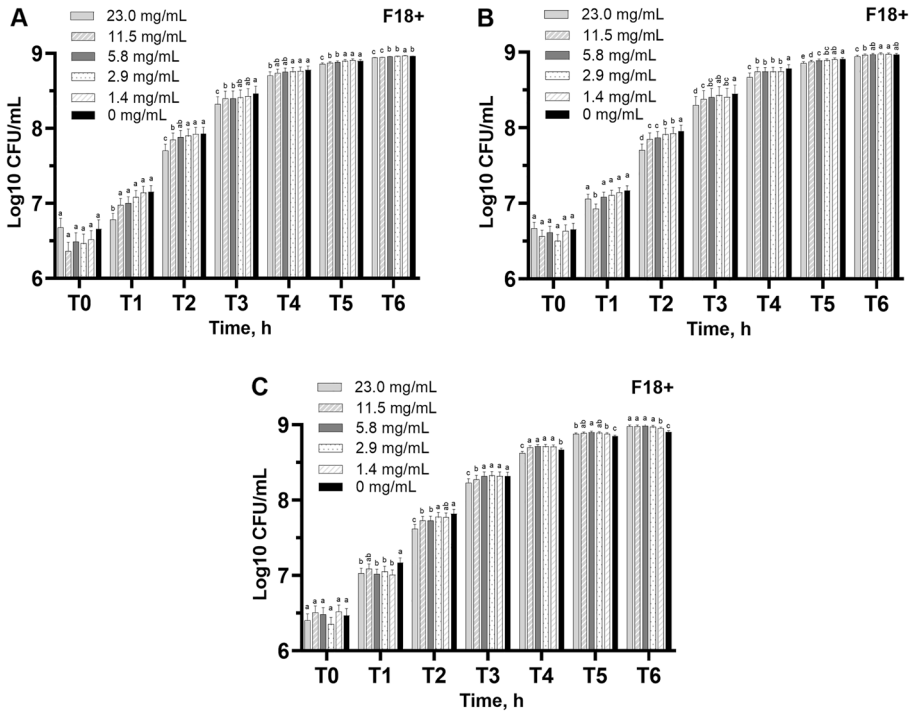

Fig. 4. The impact of different concentrations (0, 1.44, 2.87, 5.75, 11.50, and 23.00 mg/mL) of ANUL (**A**) ANPP (**B**) and PPUL (**C**) on *Escherichia coli* F18 + growth in 60 min time interval points (T0, T1, T2, T3, T4, T5, T6). Data are expressed as $\log_{10}$ CFU/mL LSMEAN ± SEM (n = 3). Different superscript letters express significant differences at $p < 0.05$ among different concentrations within the same time point.

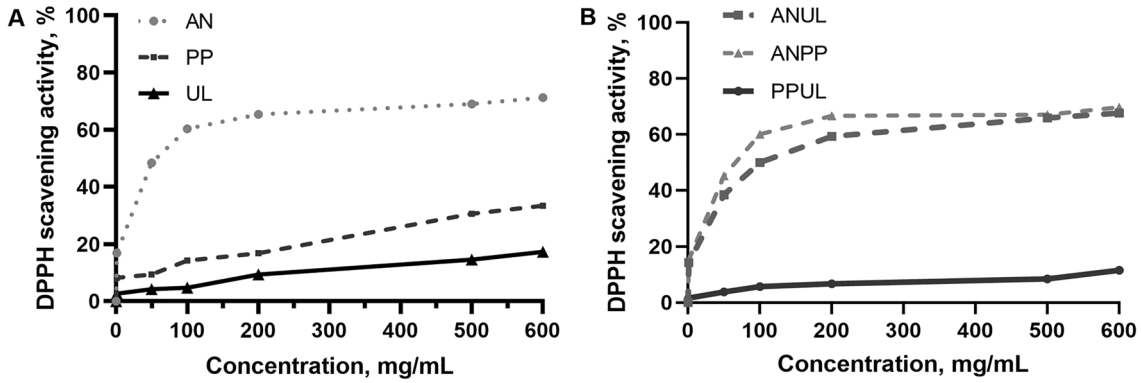

Fig. 5. Dose response of (**A**) *Ascophyllum nodosum* (AN), *Palmaria palmata* (PP) and *Ulva lactuca* (UL) algae species and (**B**) their 1:1 extract mixes using the 2,2- diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity. Data are presented as the mean of 5 observations.

Discussion

The main purposes of this study were to assess the in vitro biological activities of bioactive molecules from *Ascophyllum nodosum* (AN), *Palmaria palmata* (PP), and *Ulva lactuca* (UL), and their 1:1 extract mixes. Their inhibitory activity against *Escherichia coli* F4 + , and F18 + of algae extracts, and their antioxidant effects were evaluated. We also determined the total polyphenol content, the chemical composition, and in vitro digestibility of these compounds and their 1:1 extract mixes. These assessments were crucial to establish the further use of algae as functional additives to counteract antibiotic overuse in food-producing animals.

Seaweeds typically demonstrate a highly diverse chemical composition (protein, polysaccharide, mineral, and lipid contents) influenced by various environmental features, such as season of harvest, water temperature or light and nutrient availability in the water[36,37]. However, the results regarding the chemical composition of algal powders, including the variation observed, largely align with existing literature, and product labels[19,38]. The analysis revealed a high percentage of minerals (over 20%) in each algae species to their predisposition to mineral accumulation from seawater, in line with numerous findings in the literature[36,39]. Thus, these species

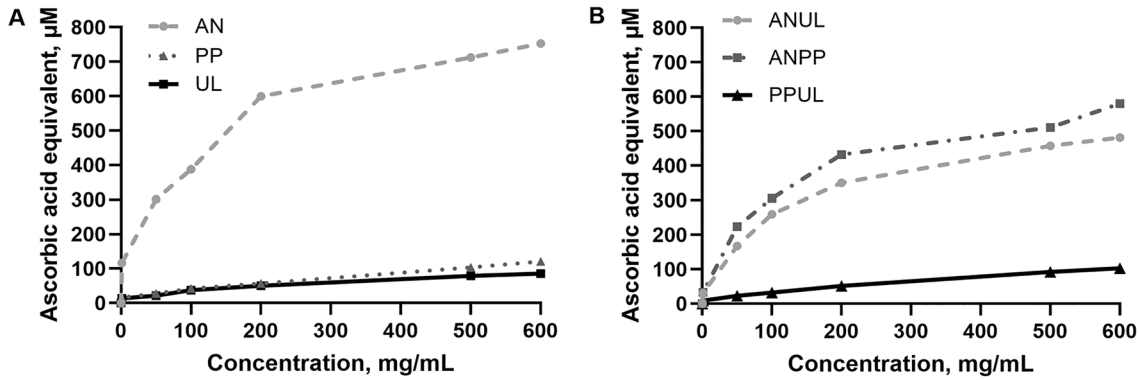

Fig. 6. Dose response of (**A**) *Ascophyllum nodosum* (AN), *Palmaria palmata* (PP) and *Ulva lactuca* (UL) algae species and (**B**) their 1:1 extract mixes using reducing power assay. Data are presented as the mean of 5 observations.

	2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity		
Assay	EC_{10} (mg/mL)	EC_{20} (mg/mL)	EC_{50} (mg/mL)
AN	5.10 [3.00–6.61]*	13.48 [12.01–15.02]	55.86 [52.25–60.06]
UL	313.06 [296.70–329.73]	–	–
PP	84.69 [59.46–105.88]	307.39 [293.09–321.92]	–
ANUL	5.99 [1.80–9.61]	22.05 [18.62–25.23]	111.05 [103.30–119.52]
ANPP	6.12 [3.00–8.41]	16.08 [13.81–18.62]	70.43 [64.26–76.88]
PPUL	504.57 [478.08–535.14]	–	–

Table 3. The values of effective concentrations (EC_{10} EC_{20} and EC_{50}; mg/mL) of different algae extracts measured by chemical-based antioxidant activity assays. *95% lower and upper confidence interval.

can serve as rich sources of essential minerals for livestock nutrition[19], contributing to a balanced diet, that typically contains less than 5% inclusion of minerals[40]. However, seaweed can also accumulate non-essential heavy metals from the marine environment, which may pose significant risks to animal health, especially gut health[41]. Heavy metals such as mercury, lead, cadmium, and arsenic can significantly alter the composition and function of the gut microbiota[42]. The populations of beneficial gut bacteria such as *Lactobacillus* and *Bifidobacterium* are sensitive to heavy metals, thus can decline with their exposure[43]. Heavy metals can induce oxidative stress and inflammation in the gut, prolonged exposure can cause damage to the epithelial cells lining the gut, resulting in increased intestinal permeability[42]. Moreover, the presence of heavy metals can trigger an inflammatory response, resulting in chronic gut inflammation, which can further exacerbate gut health issues. Heavy metals absorbed through the gut can also accumulate in various organs, causing damage not only to the gastrointestinal tract but also to the liver, kidneys, and brain[44]. Consequently, algae commercialized for feed production must have low levels of contaminants, such as heavy metals to ensure both safety and effectiveness[45], thus many seaweed species are commercially cultivated in controlled conditions. Additionally, water blanching can be used as one potential method to reduce the total ash content and consequently heavy metals from seaweeds[46]. Moreover, the bioavailability of minerals may be influenced by the fiber content of macroalgae, which can lead to the formation of insoluble chemical complexes and colloidal structures with minerals, thus reducing their bioavailability[47]. However, we observed a low content of crude fiber in the analysed samples, especially in *Palmaria palmata* species, suggesting these species may not influence mineral' bioavailability. Nonetheless, further studies are essential to explore the diverse interactions of dietary fiber in bio-absorption within the gastrointestinal tract[48]. Moreover, our results indicated a relative level of protein content in *Ulva lactuca* (15.86%) and *Palmaria palmata* (9.68%). The values were in line with literature reports, where *Ulva lactuca* contains from 7.06 to 23.1% of protein on a dry matter basis[19]. Instead, the protein content found in *Palmaria palmata* was slightly lower than reported range of 15.1–31.4% of dry mass in the literature[19]. However, the results highlighting these species may be valuable protein sources in farm animals[36]. Our analysis of the total polyphenol content (TPC) in *Ascophyllum nodosum* aligned with findings reported in the literature[19]. However, TPC in *Palmaria palmata* and *Ulva lactuca* was lower than the levels reported in some literature cases. Castejón et al. discovered that the TPC in PP and UL were 1850.5 ± 121.5 (µg GAE/g dry weigh) and 1950.6 ± 109.5 (µg GAE/g dry weigh) in gallic acid equivalent, using hot water extraction, respectively[49]. The total polyphenol content of detected seaweeds can vary significantly based on the extraction method used, the type of seaweed and the environmental conditions in which seaweeds are grown. Despite these variations, our results disclosed high polyphenol content in three algae *Palmaria* species. According to literature, brown, red and green seaweeds are rich in polyphenols. Phlorotannins and phenolic acids are the most abundant types of phenolic compounds found in brown seaweeds,

while other phenolic compounds such as flavonoids are commonly found in green and red seaweeds[50–52]. These phytochemicals contribute to their antioxidant properties and may help protect the body against oxidative stress and damage caused by free radicals[53].

Further, a three-step in vitro digestibility test was proceeded to simulate the digestive characteristics of algae, the physiology of certain segments, and their bio-accessibility and bioavailability[33]. Our results demonstrated that among the tested seaweed extracts, AN had the lowest digestibility (20.05%), and PPUL (41.34%) extract mix and PP (37.09%) showed the highest digestibility. Algae digestibility is influenced by various factors, including the physical characteristics and chemical composition of the algae[54]. In brown seaweeds, polymers such as alginates and sulphated fucoidans might account for the decrease in digestibility[55,56]. Alginates form viscous gels that can hinder nutrient breakdown and absorption by reducing the digestive process and decreasing enzyme activity[57]. Fucoidans instead, can inhibit digestive enzymes and reduce nutrient absorption[58]. Aside from the well-known polymers such as alginates and fucoidans that affect digestibility, other components in brown seaweeds, such as pigments (fucoxanthin), polysaccharides (laminarin), and polyphenols (tannins), can also reduce the digestibility of brown seaweeds[19,25]. Fucoxanthin in terms of bioaccessibility is complex due to its non-polar, hydrophobic, and water-insoluble nature. Thus, emulsification and colloid dispersion are necessary to enhance its solubility and adsorption capability. Consequently, the fucoxanthin from *Ascophyllum nodosum* affects metabolic processes and nutrient absorption[59]. Moreover, laminarins, a class of polysaccharides found in brown algae, may decrease digestibility due to their resistance to human digestive enzymes[60]. Additionally, tannins, a type of polyphenol present in brown algae, can bind the proteins, form stable complexes and cause depression of digestive capacity in the small intestine[61].

Moreover, the differences in in vitro digestibility could be attributed to the fiber content and the specific dietary fiber characteristics of each algae species. Generally, brown algae are more concentrated in neutral detergent fiber (NDF) and acid detergent fiber (ADF) fractions[62]. The cell wall of seaweed is rich in various polysaccharides, which can form stable complexes with proteins, making them inaccessible to proteolytic enzymes and thereby reducing the digestibility of seaweed protein[37,63]. Furthermore, studies have indicated that phlorotannins mostly from brown algae can negatively affect digestibility by binding with other macromolecules such as poorly digestible polysaccharides and proteins, consequently leading to different effects on digestibility[37,64]. Additionally, phenolic compounds can vary significantly within the same species due to seasonal effects, and the chemical composition of seaweeds. Moreover, to mitigate the decrease in digestibility in the animal feed, polyethylene glycol may be used to counteract the effect on digestibility[65].

The literature widely highlights the diversity in in vitro digestibility, nutrient composition, and nutritional values among seaweed species[37]. Moreover, it has been observed that the nutritional value and digestibility patterns differ among seaweed species and across harvesting seasons[39]. Hence, the rational use of seaweeds in animal diets and diet formulation will necessitate adequate chemical analysis of each batch of algae biomass to determine its nutrient composition, rather than relying solely on standardized percentage inclusion in the diet[36]. Likewise, cultivation of the seaweed could be of interest, potentially leading to higher yields with a desired and potentially more consistent or predictable chemical composition[36,66]. Further study is required to fully recognize the digestibility of different compounds and fully characterize the nutritional value originated in seaweeds to regulate the overall effect of seaweeds on pig feed.

Antibiotics have been widely applied in animal husbandry to treat the bacterial diseases, however, their overuse during the past decades has led to the rise of antibiotic-resistant bacteria in both humans and animals. The overuse has resulted in a diminishing effectiveness of antibiotics, and an increased risk of transmission of antibiotic-resistant pathogens to humans[67,68]. Hence, there is a pressing need to reduce the reliance on antibiotics and explore novel alternatives such as phytochemicals. In this study, we aimed to estimate the antimicrobial potential of algal extracts against ETEC, and VTEC *Escherichia coli*, targeting pathogenic strains with two different adhesive fimbriae (F4 + and F18 +), which are responsible for the bacterial adhesive abilities of these strains. *Escherichia coli* is among the most common pathogens in swine farming, where antibiotics are becoming increasingly ineffective against bacteria. Therefore, it is crucial to avoid the onset of its diseases initiated by this pathogen.

The growth inhibitory activity of *Escherichia coli* F4 + and F18 + indicated that *Ascophyllum nodosum*, and *Ulva lactuca* inhibited the growth of *Escherichia coli* at varying concentrations (1.44, 2.87, 5.75, 11.50, and 23.00 mg/mL), and time points (1, 2, 3, 4 and 5 h). In contrast, *Palmaria palmata* exhibited growth inhibitory effects from T1 to T3 time points. Notably, *Ascophyllum nodosum* and *Ulva lactuca* displayed the highest inhibitory activity against VTEC F18 +. Higher concentrations were not evaluated due to potential interference from the color of the extracts, which could affect the absorbance readings and lead to inaccurate results. These findings underscore the importance of employing the highest concentrations to ensure a significant antibacterial effect on the growth of *Escherichia coli* strains.

Numerous literature studies have confirmed the significant growth inhibition of seaweeds against various bacterial pathogens. Studies have reported their growth inhibition and antibacterial property against *Pseudomonas aeruginosa*[69], *Staphylococcus aureus*[70], and *Escherichia coli*[71]. For example, Dell'Anno et al.[23] observed that *Ascophyllum nodosum* (0.12%, 0.06%, 0.03% of inclusion) exhibited antibacterial property against O138 *Escherichia coli*, and Frazzini et al.[40] also revealed inhibitory activity of *Ascophyllum nodosum* at different doses. Similarly, laminarin from the Irish brown seaweed *Ascophyllum nodosum* also showed significant inhibition of *Escherichia coli* growth[25] while methanol extracted phlorotannins from the same species displayed bactericidal activity against *Escherichia coli*[72]. Besides, ascophyllan extracted from *Ascophyllum nodosum* resulted in in vitro antibacterial activities against the pathogenic *Escherichia coli*[73]. Moreover, *Ulva lactuca* ethanol extracts had high antibacterial activity against the *Escherichia coli* strain, decreasing its growth of 69.5% (at 500 µg/mL) attributed to its higher mineral concentration of metals, including copper, zinc, silver and mercury[74,75]. Tan et al.[76] also found that *Ulva lactuca* consistently formed compounds with activity against various bacteria, indicating that the antibacterial compounds were present seasonably, with the highest production detected in the autumn and

winter months. *Palmaria palmata* displayed moderate antimicrobial activity against pathogenic *L. monocytogenes* (62.09%), and weak activity against food spoilage *E. faecalis*[77]. However, ethanol extraction increased inhibition to 100%. These findings align with our results.

The antibacterial activity of seaweeds arises from various mechanisms, including inhibition of oxidative phosphorylation and the presence of functional groups that act on different levels with the bacterial cell wall. These mechanisms enhance the permeability of the cytoplasmic membrane, resulting in damages of cell membranes, enzyme inhibition, and DNA intercalation, and cell lysis[78]. Seaweeds represent a promising innovation for animal feed due to their high content of functional molecules. Phenolic compounds, found abundantly in seaweeds, are responsible for their broad spectrum antibacterial activity against various pathogenic microorganisms, such as *Escherichia coli*[19,20,56]. Algae are rich sources of phenolic bioactive components such as polyphenols, phlorotannins, bromophenols, alginates, and peptides[40,72].

Furthermore, it is crucial to test the algal extracts in combinations mix (1:1) to establish their possible synergistic and complementary interactions, reinforcing their effectiveness and potentially lowering the minimal effective dosage against infections using combinatory treatment. The synergistic outcome of both agents is more effective than the action of a single agent in performing a specific activity[34]. However, our results indicated that F4 + and F18 + were not sensitive to different doses of algal extract mixes in all-time points (ANUL, ANPP, PPUL). A dose-dependent effect was observed only at points T1, and T2 for ANUL, and ANPP.

Although, our results elicited that AN may have a combined action, increasing the effect of *Palmaria palmata* or *Ulva lactuca* in line with literature findings[40]. The absence of *Escherichia coli* growth inhibition in the ULPP and ANPP combinations has been observed and these findings may be attributed to several factors, including antagonistic interactions, insufficient effectiveness of active compounds, interference from complex matrices and the extraction efficiency[79,80]. Our study demonstrated that PP alone did not exhibit inhibitory activity, and combining PP with AN or UL could lead to a lack of interactions. Compounds from PP might antagonistically interact with UL and AN, preventing them from exerting antibacterial effects. As a result, some components may decrease the effectiveness of others, flattening the dose–response curve. These findings may be due to characteristics of seaweed compounds such as proteins, polysaccharides, and lipids. Antimicrobial proteins in PP might be highly potent, achieving maximum activity at low concentrations by inserting into bacterial membranes and causing cell lysis without requiring higher doses[81]. In fact, our study presented a significant difference in *Escherichia coli* growth inhibition in ANUL at lower doses (1.4 mg/mL) at time points T1 and T2. Proteins and peptides could also degrade or denature at higher concentrations, leading to a plateau in antibacterial activity[82]. Furthermore, polysaccharides may affect the dose–response relationship through barrier function and viscosity effect[83] by forming protective barriers or disrupting bacterial adhesion, potentially exhibiting a threshold effect[84]. Once this threshold is reached, further increases in concentration may not improve the barrier properties. Additionally, high polysaccharide concentrations can increase viscosity which may limit their diffusion and interaction with bacterial cells[83].

Another factor that could potentially affect inhibition is the extraction efficiency of the compounds. In our study, we used ethanol as the extraction solvent. Ethanol typically dissolves free sugars, amino acids, some phenols, low molecular weight compounds[85], and lipids and other lipid-soluble compounds[86]. Although ethanol is less efficient than water for extracting polysaccharides, proteins and peptides[87]. Using ethanol as a solvent to extract compounds from *Palmaria palmata* might thus not recover all bioactive compounds, possibly explaining the lack of a dose–response. Future studies should involve fractionation and targeted isolation of components to identify specific compounds and assess their antibacterial activity. In conclusion, although PP is rich in polysaccharides, its lack of dose–response effect on antibacterial activity may result from interactions among its proteins, polysaccharides, and other bioactive compounds. To our knowledge, no existing literature confirms our findings on the lack of growth inhibition in these seaweed combinations. Further research is needed to identify the specific compounds responsible for antibacterial effects and their interactions. Moreover, although the data from this study require further validation through additional research to evaluate the in vivo effect of tested seaweed extracts in an animal model, our findings are highly promising. They indicate that these seaweed extracts alone should be examined more comprehensively in the pig farming sector due to their potential as phytochemicals and antibacterial agents.

The overproduction of free radicals triggers oxidative stress, resulting in cell damage and cell death[88]. Antioxidants, capable of slowing or retarding oxidation are essential for human and animal health[89–91]. The mechanism of action of the antioxidant effects of antioxidants derived from seaweed species include scavenging free radicals and chelating metals. Consequently, these substances can delay the formation of free radicals, and hamper the autoxidation process[11].

Macroalgae exhibit antioxidant effects due to the abundance of bioactive molecules such as (i) polysaccharides (fucoidan, alginate, laminarin) in brown algae, ulvan in green algae, and carrageenan in red algae; (ii) phenolic compounds, tannins and phlorotannins and (iii) carotenoid fucoxanthin which influences antioxidant status[92]. Various literature cases have disclosed the potent antioxidant effects of algal species, thus protecting animals from oxidative stress and cellular damage induced by free radicals[19,40,93].

Chemical-based antioxidant assays are cost-efficient and reliable methods for screening for the antioxidant capacity of seaweeds. One of these techniques involves the DPPH radical scavenging analysis. The DPPH exhibits a stable and vibrant violet color, which diminishes upon mixing its solution with a substance capable of donating a hydrogen atom. The formation of hydrazine (DPPH-H) as a result of radical reduction by hydrogen atom transfer from antioxidants causes the change of the solution color from violet to pale yellow. The color change can be easily verified by UV–vis spectroscopy[94]. The other commonly used method, named reducing power assay (RPA), estimates the capacity of electron donation by assessing the effectiveness of reducing the ferric cyanide complex (Fe^{3+}) to the ferrous cyanide form (Fe^{2+}) which is a detrimental of antioxidant activity of the analyzed material[95].

As expected, all seaweed extracts from three species exhibited consistent antioxidative activity, consistent with various literature findings[96–99]. Moreover, the extract from brown seaweed, *Ascophyllum nodosum* and its extract mixes with PP and UL displayed the highest increase in DPPH radical scavenging capacity assay as well as the highest capacity of electron donation assessed by the RPA method. The antioxidant capacity of seaweeds relies on their chemical compositions. Brown algae usually exhibit better antioxidant activity compared with green and red algae[100–102]. Brown algae, *Ascophyllum nodosum* contains various bioactive compounds such as laminarin, fucoidans and phlorotannin, which have been reported to have strong antioxidant capacity[52]. The concentrations of laminarin, fucoidans, phenols and phlorotannins in *Ascophyllum nodosum* reach 5.82% DM, 41.7% (417.6 ± 4.1 mg/g DW) and 12–14% DM, respectively[96,103,104]. These bioactive compounds serve as electron donors, binding free radicals ions and consequently reducing oxidative damage[100]. However, even though *Palmaria palmata* and *Ulva lactuca* elicited lower antioxidant capacity in our study compared to *Ascophyllum nodosum*, these algae species also possess antioxidant activity. Many studies have confirmed their bioactive potential to inhibit the oxidation process[19,97].

When considering differences in the antioxidant capabilities of individual algae species, it should also considering that the biological activity of seaweeds may be influenced not only from variations in algae origin, cultivation conditions, and environmental conditions, but also from differences in the extraction methods and solvents used to obtain tested compounds[105]. Therefore, the selection of an appropriate and effective extraction method should be considered when considering species of algae, the target compounds to be extracted, and environmental features[40,99].

An additional important issue related to the practical use of algae as antioxidants is the potential combination of two or more species to enhance their effect. Thus, to investigate the synergistic and complementary interactions of algae species, their extract mixes were also tested in this study. The combined effect was estimated on 1:1 extract mixes, and the results from both the DPPH and RPA showed that synergistic or complementary interactions were observed in *Ascophyllum nodosum* combinations with both *Palmaria palmata* and *Ulva lactuca*, enhancing the antioxidant activity of compared with single UL and PP extracts.

The data highlighted that the sum of the antioxidant capacity of the single AN or UL extracts was lower compared to the extracts mix of algae, indicating a potential complementary effect[40]. Literature studies have shown that the combination of diverse antioxidant sources could improve their effect in scavenging radicals[106,107]. Therefore, AN species may lower the minimal effective dosage against oxidative stress using combinatory treatment. However, additional studies are needed to fully understand the mechanism of action of the algal combinations in studies of antioxidant properties.

To conclude, this study investigated the in vitro antioxidant activity of three algal species and their extract mixes, with *Ascophyllum nodosum* exhibiting the strongest antioxidant effect. All tested seaweeds may have promising relevance as feed additives due to their antioxidant activities. However, it is crucial to note that the concentration of antioxidants in algal extracts used in chemical-based assays may not represent their physiological levels when directly administered in the diet in vivo, and chemical-based assays cannot measure indirect antioxidant assets such as alter intracellular antioxidant enzymes in a living organism[108]. Therefore, further in vitro studies using cell-based tests or in vivo studies would be necessary to fully elucidate the antioxidant properties of tested seaweeds in animal models.

Conclusion

As in-feed antibiotics, and mass veterinary medications have been banned and recently restricted in swine production, novel bioactive feed-additives as plant extracts are of interest to enhance animal disease resistance. Seaweeds may serve as promising sources of bioactive molecules and phytochemicals used as feed additives. Nevertheless, due to the wide variety of algal species and their distinct characteristics, it is essential to assess their individual activities. Thus, we assessed the antioxidant and antibacterial characteristic of three seaweed species extracts and their extract mixes. In this study, we demonstrated the presence of bioactive molecules, such as polyphenols in all tested seaweeds. Further, our study confirmed the antioxidant and some antibacterial activity of the selected seaweed extracts, which may reduce the amount of antibiotics using during the animal's infection, even if these algae had a lower digestibility level. In the recent study, the brown macroalgae *Ascophyllum nodosum* extracts were the most effective in terms of antioxidant activity, and antibacterial activity, while AN and their extract mixes were also most efficient in terms of antioxidant activity. The output of this analysis indicated that active molecules derived from *Ascophyllum nodosum* have a strong inhibitory effect on F4 + and F18 + *E. coli* strains. Moreover, AN in combinations mix (1:1) may induce a complementary effect with PP and UL. Thus, tested algae may be able to (i) decrease the risk of bacterial infection and (ii) reduce oxidative stress, and may significantly impact the development of new functional nutritional strategies to reduce reliance on antibiotic treatment in swine farming, and provide further guidelines for significantly improving sustainability. Additional research is needed to further explore the therapeutic potential, and perspectives of algae-derived compounds in addressing multifactorial diseases in the pig industry.

Materials and methods

Materials and experimental design

Lyophilized powder (100% pure) of *Palmaria palmata* (PP; catalog number: 10418) was purchased from Alganex Gmbh (Berlin, Germany), while *Ascophyllum nodosum* (AN; catalog number: SX 009776) and *Ulva lactuca* (UL; catalog number: SZ 009874) were purchased from Italfeed Srl (Milan, Italy) in line with European safety requirements. Before further antibacterial and antioxidant experiments, the extraction method of 100% pure lyophilized algal powders of different seaweed and their mixes based on ethanol was proceeded. The extraction method for Total polyphenol content evaluation differs from extraction for subsequent antibacterial and antioxidant

experiments. In the antibacterial assays, some of the extracted algal extracts were dissolved in DMSO (˜1%) and then all of them were further resuspended in Luria Bertani broth (LB), and filtered with 0.22 µm syringe filters prior to microbiological assay. In the antioxidant assays, algal extracts were prepared by diluting the stock of the extracted algae solutions with methanol (w/v).

The experimental design for the antibacterial assay comprised a factorial arrangement with 2 (with or without *E. coli*) × 2 (F4 + and F18 +) × 5 doses (0, 1.44, 2.87, 5.75, 11.50, and 23.00 mg/mL of seaweed extracts) × 6 compounds (AN, PP, UL and their 1:1 extract mixes). The experiment design for antioxidant assays comprised 6 doses (0, 1, 50, 100, 200, 500, and 600 mg/mL of seaweed extracts) × 6 compounds (AN, PP, UL and their 1:1 extract mixes).

Evaluation of total polyphenol content (TPC)

Firstly, *Ascophyllum nodosum*, *Palmaria palmat*a and *Ulva lactuca* were extracted according to Attard et al.[109]. Briefly, 5 g of algae powder were suspended with 30 mL of methanol and left stirred for 48 h at room temperature. The obtained mixtures were centrifuged (5000 rpm for 10 min) and filtered (0.45 µm), and the filtrates were diluted with deionized water in a 1:1 ratio. Subsequently, the TPC of *Ascophyllum nodosum*, *Palmaria palmata* and *Ulva lactuca* was evaluated by the Folin-Ciocalteu microtiter plate method based on Attard[109], and measured using a spectrophotometer at 630 nm (BioTek Synergy HTX, Agilent Technologies, Santa Clara, CA, USA). Calibration curves were prepared in five 1:2 dilutions ranging from 960 to 60 µg/mL, with tannic acid as the standard (Sigma Aldrich, St. Louis, MO, USA). Each sample and standard were analyzed in triplicate (n = 3). For *Ulva lactuca* a proper blank was included according to Attard et al.[109] for correcting for the strong background color. The TPC was expressed as µg Tannic Acid Equivalents (TAE) per g of algal powders (µg TAE/ g).

Extraction of algal biomass

Seaweed biomass of the tested species were extracted using ethanol as a solvent, following the literature[110,111] with some adaptations. Briefly, algal biomass powder was dissolved in 80% ethanol (1:10 ratio), rubbed in the mortar, and vortexed (3 min). Then, all samples were overnight frozen (– 20 °C) to maximize the efficiency of extraction procedure, centrifuged (5000 rpm × 20 min, 4 °C), supernatant was decanted, and the solid glass beads (3 mm) were added to each sample. The glass beads-solution were then homogenized 30 s × 4.5 RPS (FastPrep-24 classic homogenizer, MP Biomedical, Irvine, CA, USA) and all samples were centrifuged (5 000 rpm × 20 min, 4 °C). Ethanol extraction procedure for the remaining pellet was tripled. The extraction solution was then evaporated by an evaporator (Rotary Evaporator Strike 300, Steroglass srl, Perugia, Italy) at the temperature lower than 50°C, and dried residues were weighed, and the yield was determined considering the weight of the dry algae powder. Each residue was suspended in an appropriate medium for further analysis.

Chemical composition of algal powders

The samples of algae powders were analyzed in triplicate based on the official analysis methods[112] for their principal composition, including dry matter, ether extract (EE), crude protein (CP), crude fiber (CF), and total ash contents. Dry matter was determined by forced-air oven at 65 °C for 24 h (AOAC, 930.15). Lipid content (ether extract, EE) was determined using petroleum ether extraction (AOAC, 2003.05). Crude protein content (CP) was measured according to the Kjeldahl method using 6.25 as a nitrogen conversion factor (AOAC, 2001.11), and crude fiber (CF) was assessed using the filtering bags technique (AOCS, Ba 6a-05). Total ash content was measured after incinerating samples at 550 °C for 3 h (AOAC, 942.05).

In vitro digestion of seaweed powders

Escherichia coli growth inhibitory of algal powders was measured by the described procedures[113,114] with few adaptations. Briefly, 1 g of each algal powder was mixed with distilled H_2O (20 mL) and shaken (150 rpm, 5 min). The control samples including digestion blanks (enzymes) and standard protein and carbohydrate sources, have been included. The process involved three phases. In the oral phase, 150 mg α-amylase (Sigma-Aldrich, Burlington, MA, USA) in 1 mL of 1 mM $CaCl_2$, pH 7 was added, and then the samples were incubated (30 min, 37 °C). In the gastric phase, the pH was decreased to 2 with 6 M HCl and 100 mg of pepsin (Sigma-Aldrich, Burlington, MA, USA) was added in 2 mL of 0.1 M HCl, and incubated (120 min at 37 °C). In the small intestine phase, the pH was increased to 7 with 6 M NaOH, and 200 mg pancreatin (Sigma-Aldrich, Burlington, MA, USA), and 50 g bile extract (Sigma-Aldrich, Burlington, MA, USA), were added with 2 mL of 0.5 M $NaHCO_3$, and incubated (180 min at 37 °C). Samples were then filtered on paper filters for the determination of digestibility (Whatman filters 54). Before further analysis of the antibacterial assay, aliquots were maintained at − 20 °C. Digestibility was calculated based on the formula:

$$Digestibility(\%) = \frac{(DM\ of\ sample - Undigested\ fraction(g))}{DM\ of\ sample} \times 100$$

Measurement of antibacterial activity through *Escherichia* coli growth inhibitory assay

A liquid culture-based of F4 + and F18 + *E. coli* growth inhibition assay was completed to estimate the inhibitory activity of previously extracted (from subchapter 5.3.) algal biomass at different concentrations. Two *Escherichia coli* strains, harboring F4 +, and F18 + adhesive fimbriae, were acquired from a collection of the University of Milan and formerly defined[23,115]. The bacteria were cultured overnight for 12 h at 37 °C with agitation (150 × rpm) in lysogeny broth (LB) medium under an aerobic conditions, serving as the inoculum for all subsequent experiments. Overnight-grown F4 + and F18 + cultures were inoculated in 96 microplates wells of containing 100 µL

of LB medium supplemented with different doses of extracts (0, 1.44, 2.87, 5.75, 11.50, and 23.0 mg/mL) of algal powders, respectively. Before inoculation, bacterial cultures were standardized to initial density (0.05 ± 0.02 OD when read against LB medium) by spectrophotometer (600 nm wavelength). Microplates were incubated aerobically with shaking (150 × rpm) at 37 °C. The bacterial growth was measured via measurement of the optical density of each culture at 620 nm (OD620) at 60 min intervals in a spectrophotometer (ScanReady P-800, Life Real, Zhejiang, China). Bacteria-free wells with equivalent concentrations of algal powders were used as blanks to subtract the background turbidity caused by algal-protein interactions[33,116]. All data acquired from the optical density measurement were converted to log-transformed based cell count (CFU/mL) using a calibration curve (considering 1 OD = 10^9 cells/mL). The assay was performed in three biological replicates and four technical replicates. The increase in absorbance determined bacterial growth. The following formula estimated the inhibition rate was calculated based on the formula:

$$Inhibitionrate(\%) = 100 * \left(\frac{OD_{CTRL^-} - OD_{sample}}{OD_{CTRL^-} - OD_{blank}} \right)$$

Measurement of the antioxidant activity of algal species through chemical-based assays

DPPH radical scavenging capacity and reducing power assays were implemented to estimate the antioxidant activity of previously extracted (from Sect. 5.3) algal biomass. AN, PP, UL, and their 1:1 extract mixes were tested at different doses of extracts: 0, 1, 50, 100, 200, 500, and 600 mg/mL. All assays were repeated with five technical replications.

DPPH radical scavenging capacity assay

The scavenging capacity of algal-based powders against 2,2-diphenyl-1-picrylhydrazyl (DPPH, Sigma, St. Louis, MO, USA) radical was assessed based on Zhou et al.[117] and Wu et al.[108]. Briefly, samples were mixed with DPPH solution (25 g/mL in methanol) at a ratio of 1:39 (v/v). The optical density (OD) was determined at 540 nm (Synergy 4 Microplate Reader, BioTek, Winooski, VT, USA). The scavenging capacity of each of the algal powders was calculated based on the below equation. A lower EC indicated a higher radical scavenging capacity. Effective concentrations (EC_{10}, EC_{20} and EC_{50}, mg/mL) of each of the algal powder extracts were defined as the concentrations that cause 10, 20 or 50% reduction of the DPPH radical[118,119], and were calculated from the curves fitted to the experimental data (for details see Supplementary graphs S1).

$$\text{DPPH} + \text{scavenging capacity } (\%) = \frac{(A_{blank} - A_{test})}{A_{blank}} \times 100$$

where A_{blank} was the absorbance of the blank sample, and A_{test} was the absorbance of the test sample.

Reducing power assay

The ferric iron reducing capacity of algal powders was measured by the procedures of Chung et al.[120] and Bhalodia et al.[121], with minor modifications according to Wu et al.[108]. Briefly, equal volumes of test sample, 2 M phosphate-buffered saline solution (PBS, pH 6.6), and 1% potassium ferricyanide (Sigma, St. Louis, MO, USA) were thoroughly mixed. Ascorbic acid prepared at different concentrations (0, 1, 5, 10, 50, 100 and 200 µM) was used as the standard sample. The optical density (OD) was measured at 540 nm (Synergy 4 Microplate Reader, BioTek, Winooski, VT, USA). Higher absorbance indicates higher reducing power. The ferric reducing capacity was calculated as the ascorbic acid equivalent.

Statistical analysis

All data generated from different assays were analyzed by ANOVA using the MIXED procedure (SAS 9.4, SAS Institute Inc., Cary, NC, USA) with different statistical models. *Escherichia coli* growth data were log10 transformed (normalization) prior to statistical analysis. The model included treatments, time, and time × treatment as fixed effects and block as a random effect. Data from antibacterial assays are presented as least-squares means and the standard error of the means. Data from antioxidant assays are presented as means and standard errors. The data from TPC are presented as means and standard deviation of the mean (n = 3). Probability values of ≤ 0.05 were considered to be significant.

Data availability

All data generated or analysed during this study are available from the corresponding author upon reasonable request.

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Author contributions

M. Hejna: conceptualization, data curation, formal analysis, funding acquisition, investigation, methodology, project administration, resources, software, supervision, validation, visualization, roles/writing—original draft, writing—review and editing; M. Dell'Anno: methodology, writing—review and editing; Y. Liu: formal analysis,

writing—review and editing; L. Rossi: conceptualization, writing—review and editing; A. Aksmann: formal analysis, writing—review and editing; G. Pogorzelski: writing—review and editing; A. Jóźwik: writing—review and editing.

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Competing interests

The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to M.H.

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