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RESEARCH

Testing the inhibitory effects of different algal extracts on fish intestinal metalloproteases using *in vitro* assays

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Summary
Matrix metalloproteinases (MMPs) are a family of enzymes widely distributed across vertebrates, playing crucial roles in various physiological processes, particularly those related to tissue remodeling. As demonstrated in humans, MMPs are also implicated in a range of inflammatory disorders, including those affecting the intestine. In farmed species, undesirable intestinal inflammation is commonly observed when plant-based ingredients are incorporated into feed formulations. The hypothesis of the present study is that these negative effects may be mitigated by the inclusion of algal products with MMP-inhibitory properties in the diet. Therefore, a preliminary investigation was designed to evaluate the presence and effects of potential MMP inhibitors in various algal products on intestinal MMP activity across several fish species. The results revealed that extracts from different fresh and processed species of brown and red seaweeds exhibited significant inhibitory activity against intestinal MMPs in three carnivorous fish species: Atlantic salmon (*Salmo salar*), European seabass (*Dicentrarchus labrax*), and gilthead seabream (*Sparus aurata*). Furthermore, in vitro assays simulating the digestive conditions of two fish species demonstrated that experimental feeds containing two types of algal meals maintained inhibitory effects on intestinal MMPs.

Keywords Seaweeds · Algal extracts · Fish species · Metalloproteases · *In vitro* digestion

Introduction

The beneficial effects of various bioactive compounds present in macroalgae have been extensively studied in humans; however, their effects on aquatic animals, particularly fish, remain relatively underexplored (García-Vaquero and Hayes 2015). In recent years, there has been a growing interest in investigating the potential benefits of incorporating macroalgae or their derivatives into the diets of aquaculture species, either as sources of essential nutrients or bioactive compounds (Mwendwa et al. 2023). In terms of nutritional contribution, macroalgae generally offer limited value, as the concentrations of key nutrients, such as proteins and lipids, on a dry matter basis are typically low. As a result, algal meals currently lack economic viability as viable alternatives to terrestrial ingredients (Overland et al. 2019). Nonetheless, an expanding body of research underscores the potential of macroalgae, particularly through their bioactive compounds, which have demonstrated promising effects on the oxidative and immune status of fish when incorporated into feed formulations (Naiel et al. 2021).

Among the bioactive compounds present in macroalgae, matrix metalloproteinase inhibitors (MMPIs), primarily consisting of sulfated polysaccharides (e.g., galactans and fucoidans) and phlorotannins, have garnered increasing attention, with extensive research being conducted in humans (Thomas and Kim, 2014; Thomas et al. 2014; Jayawardhana et al. 2023; Liao et al. 2024). Matrix metalloproteinases (MMPs) are a family of Zn^{2+}-dependent neutral proteinases primarily responsible for the degradation and remodeling of the extracellular matrix (ECM) (Sternlicht and Werb 2001;

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Visse and Nagase 2003). These enzymes exhibit considerable structural diversity and are classified into various subgroups based on their substrate specificities, including collagenases, gelatinases, stromelysins, elastases, and membrane-type MMPs, among others (Page-McCaw et al. 2007). MMPs are predominantly secreted as latent, inactive zymogens by a wide range of stromal and epithelial cell types, such as mesenchymal cells, monocytes, T cells, macrophages, neutrophils, keratinocytes, and certain tumor cells. Due to their structural diversity, MMPs participate in numerous physiological processes, including tissue remodeling during development, growth, and wound healing. In addition, they are implicated in various pathological conditions, such as atherosclerotic plaque rupture, arthritis, tumor progression, and a range of inflammatory disorders (Medina and Radomski 2006; Zhang et al. 2008; Yoshida et al. 2009; Shoari 2024). Recent studies have increasingly focused not only on elucidating the role of MMPs in these pathological processes but also on investigating strategies to inhibit their activity through specific inhibitors, including those derived from algae (Kim et al. 2011; Thomas et al. 2014; Islam et al. 2024).

Elevated levels of matrix metalloproteinases (MMP)−2 and MMP-9 in human intestinal tissues have been implicated in various pathological conditions (Sengupta and MacDonald 2007). Evidence suggests that the functional roles of MMPs in teleost fish may parallel those observed in humans. For example, MMP-9 has been identified as a critical component of the innate immune response within the intestine of teleost fish, as demonstrated in studies of induced peritonitis in carp (Chadzinska et al. 2008). Furthermore, inflammatory conditions in the intestines of farmed fish share notable similarities with MMP-mediated inflammatory bowel diseases in humans. A prominent example is soybean meal-induced enteritis (SBMIE), a condition that predominantly affects the distal intestine of various fish species fed diets containing full-fat soybean meal. SBMIE is characterized by non-infectious, subacute enteritis, including shortening of mucosal folds, infiltration of inflammatory cells into the lamina propria, and a reduction in absorptive vacuoles within enterocytes (Krogdahl et al. 2015). Although the precise etiology of SBMIE remains uncertain, saponins and other bioactive components of legumes are hypothesized to contribute to its development (Krogdahl et al. 2023).

Despite the significant impact of SBMIE on aquaculture, research investigating the role of algal-derived MMP inhibitors (MMPIs) in mitigating inflammation in farmed fish is limited. To date, the only related study examined the use of an extract from the brown seaweed *Fucus vesiculosus* to reduce collagenase and gelatinase activity, thereby mitigating connective tissue degradation in Atlantic salmon fillets (Eysturskar et al. 2017). This finding highlights a potential avenue for exploring the application of algal MMPIs to counteract inflammatory effects associated with soybean meal-based diets in aquaculture. Such strategies could mirror approaches used in human intestinal disorders, where algal-derived compounds have shown promise in mitigating inflammation (Kim and Kim 2010).

This study was designed as a preliminary investigation to evaluate the potential effects of algal inhibitors on matrix metalloproteinases (MMPs) in the intestinal tissues of various fish species. The research was conducted in three distinct phases with the following objectives: (a) to quantify MMP inhibitory activity of various algal products against MMPs derived from *Clostridium histolyticum*; (b) to evaluate the sensitivity of MMPs from intestinal extracts of three carnivorous fish species—Atlantic salmon (*Salmo salar*), European seabass (*Dicentrarchus labrax*), and gilthead seabream (*Sparus aurata*)—to selected algal inhibitors; and (c) to assess the effects of the digestive process on inhibitors from two specific algal products on MMP activity in intestinal extracts of *S. salar* and *S. aurata* using *in vitro* digestibility assays.

The findings of this preliminary work may provide valuable insights into the potential benefits of incorporating algal meals into aquafeeds. Furthermore, they serve as a foundation for future *in vivo* studies to elucidate the biological responses of fish intestines to dietary algal MMP inhibitors, contributing to the development of innovative strategies for mitigating intestinal inflammation in aquaculture.

Materials and methods

Experimental design

The investigation into the inhibitory effects of various algal extracts on fish intestinal metalloproteases was structured into three distinct phases:

i) *Comparative assessment of inhibitory activity.* This phase involved evaluating the inhibitory potential of extracts derived from different macroalgae and algal products obtained via aqueous or ethanol extraction. The extracts were tested for their ability to inhibit matrix metalloproteases (MMPs) from *Clostridium histolyticum.*
ii) *Comparative assessment of inhibitory activity.* Algal extracts demonstrating significant inhibitory potential in the initial phase were further evaluated for their effect on MMP activity in intestinal extracts from three commercially important fish species: Atlantic salmon (*Salmo salar*), European seabass (*Dicentrarchus labrax*), and gilthead seabream (*Sparus aurata*).
iii) *In vitro assay under simulated digestive conditions.* In this phase, an *in vitro* assay was conducted to examine the inhibitory activity of selected algal products when

incorporated into a feed matrix. The assay simulated digestive conditions to evaluate the effect of algal inhibitors on MMP activity in intestinal extracts of *S. salar* and *S. aurata.* Simulations included species-specific enzyme extracts from different digestive tract regions (stomach and intestine) and accounted for pH variations representative of the digestive environment.

Algal products and active extracts

Various samples of brown seaweeds, including *Alaria esculenta* and *Saccharina latissima*, were provided by Lerøy Ocean Harvest's (Bergen, Norway) during the period from March to May 2023. All samples of these brown algae were harvested at different locations in Norway and processed as follows:

Fresh *Saccharina* and ***Alaria*** were harvested on 6 March 2024 in Trollsøy.
Acid processing was performed on material harvested 2May 2023 in Trollsøy by mixing 200 L of 3% formic acid with *Saccharina* in an intermediate bulk container (IBC; a standard container used for transport and storage of fluids and bulk materials).
Fermented *Saccharina* was prepared using fresh material harvested on 5 May 2023 from Flatøysundet and a bacterial inoculum containing *Lactobacillus sakei* combined with molasses. Specifically, the seaweed was placed in an IBC container equipped with an inner liner. Following this, 50 L of molasses and 250 g of bacterial inoculum dissolved in 1 L of seawater were added. A vacuum was created by removing air from the liner, and the container was sealed. The contents were then thoroughly mixed using an IBC mixer.
Auto-fermented *Saccharina* was processed on the same type of fresh material detailed above but without the addition of an external inoculum. The seaweed was placed in an IBC container with an inner liner, air was removed to create a vacuum, and the contents were mixed using an IBC mixer.

For all brown seaweed preparations—acid-treated, fermented and auto-fermented—approximately 690 kg of kelp was processed in each IBC container. All processes were certified by Debio, a Norwegian organic certification system that ensures producers adhere to national and international standards for organic production.

Additionally, two commercial samples of algae were included in the study: red alga (*Porphyra* sp., Nori) and brown alga (*Undaria* sp., Wakame), sourced from Algas Atlánticas ALGAMAR, S.L. (Pontevedra, Spain). A concentrated powder comprising a blend of various red algae species, produced by Alginor (Haugesund, Norway), was also included. All seaweed samples, regardless of their initial form, were dried at 60 °C for 24 hours (or until fully dehydrated) and subsequently ground into a fine powder that was used to prepare the working extracts use in the experimental assays. The extracts were prepared using two distinct protocols:

a) *Conventional extraction:* This method employed a solvent mixture of ethanol (1:1 v/v) and high temperature (60 °C), following the procedure described by Santos et al. (2019). This approach was used as a reference to estimate the maximum concentration of bioactive compounds present in the samples.
b) *Physiological extraction:* This method utilized phosphate-buffered saline (PBS) as the solvent at a low temperature (25 °C) to more closely mimic the physiological conditions of the fish gastrointestinal tract, as described by Brooke et al. (2015).

For both extraction protocols, a fixed concentration of algal powder (100 mg mL^{-1}) was subjected to continuous agitation for one hour. The resulting homogenates were then centrifuged at 4,000 × *g* for 15 min at 4 °C. Supernatants were carefully collected and stored at −20 °C until further analysis.

Determination of bioactive compounds and MMP inhibitors in algal extracts

The total phenolic content (TPC) in algal extracts was determined using the method described by Graça et al. (2005). Phlorotannin concentrations were quantified using the DMBA method as outlined by Stern et al. (1996). The antioxidant capacity, expressed as Trolox equivalent antioxidant capacity (TEAC), was assessed using the DPPH method following the procedure of Brand-Williams et al. (1995). All analyses were performed in triplicate for each algal product.

The presence of matrix metalloproteinase (MMP) inhibitors in the algal extracts was evaluated using the EnzChek Gelatinase/Collagenase Assay Kit, adhering to the manufacturer's protocol. In brief, the assay involved combining 20 µL of substrate solution, 100 µL of MMP solution from *Clostridium histolyticum*, and 80 µL of algal extract in a well of a 96-well black microplate. The following controls were included:

- **Substrate control:** 20 µL of substrate + 180 µL of buffer.
- **Positive control:** 20 µL of substrate + 100 µL of MMP enzyme + 80 µL of buffer.
- **Inhibitor control:** 20 µL of substrate + 100 µL of MMP enzyme + 80 µL of 1 mM 1,10-phenanthroline.

For activity assays, algal inhibitors were omitted from the reaction mixture. Fluorescence was measured at an excitation wavelength of 485 nm and an emission wavelength of 530 nm using a Cytation 5 multiplate reader (Biotek, USA). Results were expressed as relative fluorescence units per minute (RFU min^{-1}). Inhibition curves for both types of algal extracts were generated by incrementally increasing their concentrations while maintaining a fixed amount of substrate, enabling the assessment of their inhibitory potential.

Fish gut extracts

As described previously, three fish species were used in this study: Atlantic salmon (*S. salar*), obtained from one of Lerøy's commercial sea cages in Bergen, Norway, as well as European seabass (*D. labrax*) and gilthead seabream (*S. aurata*), both sourced from IFAPA 'Centro El Toruño' in Cádiz, Spain. For Atlantic salmon, fish were fed continuously during the light period, and sampling was performed at a specific time of the day. For European seabass and gilthead seabream, fish were sampled 4 hours post-feeding to maximize the presence of digestive enzymes in their gastrointestinal tracts. Fish were anesthetized and euthanized using species-specific protocols: Atlantic salmon with an overdose of Benzoak vet. (200 ppm) and European seabass and gilthead seabream with 2-phenoxyethanol (1000 ppm). Euthanized fish were immediately frozen at −20 °C until dissection. All experimental procedures complied with the European Union Council Guidelines for the protection of animals used for scientific purposes (2010/63/EU).

During dissection the entire digestive tract was carefully excised and separate extracts were prepared from the stomach and intestinal segments of multiple specimens. For Atlantic salmon, 11 individuals, each weighing approximately 700 g, were used. For European seabass and gilthead seabream, extracts were obtained from 8 and 10 specimens, with average body weights of 200 g and 1,200 g, respectively. The excised tissues were homogenized in cold distilled water using species-specific tissue-to-water ratios: 1:5 w/v for salmon and 1:3 w/v for seabream and seabass. The homogenates were centrifuged at 4,000 × *g* for 15 min at 4 °C. The resulting supernatants were used for enzymatic activity analyses. Two enzyme activities were assessed:

- *Acid protease activity* in stomach extracts, measured at pH 4 using hemoglobin as the substrate, according to the method of Anson (1938).
- *Matrix metalloproteinase (MMP) activity* in intestinal extracts, quantified using the EnzChek Gelatinase/Collagenase Assay Kit, as detailed above.

Inhibition of fish intestinal MMPs

A preliminary assay was conducted to evaluate the sensitivity of fish intestinal matrix metalloproteinases (MMPs) to inhibition. The assay followed a protocol similar to that described in Section "Determination of bioactive compounds and MMP inhibitors in algal extracts", with the key difference that intestinal extracts from the three fish species were used in place of the commercial *Clostridium histolyticum* MMP preparation provided in the assay kit. Inhibition curves were generated by combining 20 µL of substrate solution, 100 µL of intestinal enzyme extract, and 80 µL of algal product extract at varying concentrations (2.5–100 mg mL^{-1}). The reaction mixtures were incubated at 25 °C for 40 min, and fluorescence was measured using a Cytation 5 multiplate reader (Biotek, USA) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. The reduction in enzymatic activity was expressed as a percentage of inhibition, using the activity of the enzyme extract without the addition of algal products as the reference value.

In vitro assay

This assay aimed to evaluate the effects of inhibitors present in selected algal products on intestinal matrix metalloproteinases (MMPs) from *S. salar* and *S. aurata* when incorporated into a feed matrix, simulating the digestive process in both species. The experimental feed matrix was formulated using ingredients commonly employed in aquafeeds, with proportions approximating those of standard commercial formulations: fish meal (29%), soybean concentrate (29%), corn gluten (14%), and sunflower oil (18%). These components were combined with distilled water to create a moist paste (50% moisture), which served as the base for preparing three distinct experimental diets:

- *Feed S:* Containing 10% (dry weight basis) auto-fermented *Saccharina latissima*.
- *Feed A*: Containing 10% (dry weight basis) red algae concentrate produced by Alginor.
- *Feed C (Control):* Containing 10% (dry weight basis) wheat meal, substituting the algal products.

Following mixing, the experimental feeds were dried at 60 °C for 24 hours and subsequently finely ground to obtain a uniform texture suitable for *in vitro* digestion assays.

Enzyme extracts obtained from the respective tissues of each species were utilized at each stage of the assay. The activity of stomach proteases measured in the sampled individuals of both species was used as a reference to establish physiologically relevant enzyme-to-substrate ratios. These ratios were determined through the following procedure:

1. The average enzyme activity per gram of live fish was calculated using the enzyme activity data obtained from all individuals during the preparation of gut extracts.
2. The total amount of available enzyme extracts and the number of assays to be conducted were factored into these calculations.
3. A "model" fish size of 100 g was selected, and the corresponding enzyme activity for a fish of this size was introduced into the bioreactor.
4. The amount of substrate (feed) was calculated based on the theoretical intake per meal for a 100 g fish, in accordance with commercial feeding guidelines.

In vitro digestion assays were performed in two sequential stages to simulate the physiological processes of the stomach (acidic phase) and the intestine (alkaline phase), following the methodology described by Moyano et al. (2015). The digestions were conducted in 50-mL Falcon tubes, which served as closed reactors, placed in a wheel stirrer to maintain gentle and continuous agitation. The entire set of 36 experimental units was maintained at 25 °C within a thermal chamber. For the acidic phase (2 h of incubation), the bioreactors were prepared with the selected amount of feed matrix dissolved in acidified water and adjusted to pH 4.0 for *S. salar* or pH 5.0 for *S. aurata* by adding a few drops of 0.1 M HCl. These pH values were based on measurements taken from the stomachs during the preparation of the extracts (Section "Fish gut extracts"). The crude enzyme extract from the stomach of each respective species was also introduced into the system at this stage. For the alkaline phase (4 h of additional incubation), the pH of the bioreactor was raised to a value of 8.0 with a few drops of NaOH 0.05M and then adjusted using 0.1 M borate buffer, supplemented with 20 mM $CaCl_2$, 45 µM sodium taurocholate and 50 mM NaCl. Intestinal enzyme extracts were then added to the bioreactors. After the incubation period, the reactions were halted by placing the tubes at −20 °C for 10 min, followed by centrifugation at 4,000 × *g* for 15 min at 4 °C. The resulting supernatants were stored at −20 °C until further analysis of MMP activity, as outlined in Section "Determination of bioactive compounds and MMP inhibitors in algal extracts". The reduction in MMP activity observed in feeds S and A was expressed as a percentage of inhibition, with feed C serving as the control reference. The conditions for each stage of the simulation are summarized in Table 1.

Statistical analysis

Data normality was assessed using the Shapiro–Wilk test, and homoscedasticity was evaluated using the Brown–Forsythe test. Statistical comparisons were performed using one-way or two-way ANOVA, depending on the experimental design, followed by Fisher's Least Significant Difference (LSD) test for post-hoc comparisons, when appropriate. Statistical significance was determined at $p < 0.05$. Percentage data were arcsine transformed prior to analysis when necessary. All statistical analyses were conducted using Statgraphics Centurion software (Statgraphics Corp., USA) for Windows.

Table 1 Conditions used in the in vitro digestive simulation of *S. salar* and *S. aurata*

Conditions	*Sparus aurata*	*Salmo salar*
Temperature (ºC)	25	
Amount of feed (mg)	625	
Acid stage		
E:S ratio (Units protease g^{-1} feed)	1.73	3.13
Liquid medium	Distilled water adjusted to the required pH with HCl	
pH	5	4
Time (h)	2	
Number of samplings	1	
Time of samplings (min)	120	
Alkaline stage		
E:S ratio (Units MMP mg^{-1} algal product)	0.48	0.49
Liquid medium	Borate buffer + salts	
pH	8	
Time (h)	4	
Number of samplings	2	
Time of samplings (min)	240 and 360	

Results

Comparative contents of bioactive compounds

The concentrations of select bioactive compounds, including total phenolics and phlorotannins, as well as antioxidant capacity in the algal products, extracted with PBS as indicated in section "Algal products and active extracts". are presented in Table 2. *Porphyra* sp. ("Nori") exhibited significantly higher levels of total phenolics compared to the other samples, while *Saccharina* displayed the lowest concentrations. However, different treatments applied to *Saccharina*, such as acid preservation and fermentation, significantly enhanced the extractable phenolic content. Concerning phlorotannins, the Alginor concentrate demonstrated a significantly higher content than any of the other samples. The highest antioxidant capacity was observed in auto-fermented, acid-, and fermented conservation methods of *Saccharina*.

MMP Inhibitors present in algal products

The inhibition curves generated by extracts from algal products prepared using either PBS or 50% ethanol on the MMP activity of *Clostridium histolyticum* are presented in Fig. 1. A significant effect of relative concentration was observed in all cases ($p < 0.05$), with notable variability in the concentrations required to achieve 100% inhibition among the different algal products. Significant differences ($p < 0.001$) were also observed between ethanol and PBS extracts, with ethanol extracts generally displaying higher MMP inhibitory activity, except for *Undaria* sp. ("Wakame"). Furthermore, all treated samples of *Saccharina* exhibited higher inhibition compared to the untreated sample, which is consistent with previously reported results on their phenolic and phlorotannin contents (see Table 2).

Inhibition of fish intestinal MMP

The inhibitory effects of PBS extracts from three selected algal products (autofermented *Saccharina*, red algae concentrate and Wakame) on the MMP activity present in intestinal extracts from three fish species (*S. salar*, *S. aurata*, and *D. labrax*) are shown in Fig. 2. Although no significant differences were observed among the responses of different fish species, a significant effect of algal product was detected ($p < 0.001$). This was particularly evident in the strong inhibition at low concentrations induced by the red algae concentrate, in contrast to the comparatively low inhibition observed with autofermented *Saccharina*.

In vitro assays

The effects of MMP inhibitors released from feed matrices containing algal products on intestinal MMP activity in *S. salar* and *S. aurata* during *in vitro* digestive simulations are presented in Table 3. Significant differences were observed in both species. In *S. salar*, the feed containing red algae concentrate (A) resulted in a significantly higher percentage of MMP inhibition compared to the control feed. In contrast, in *S. aurata*, the feed containing auto-fermented *Saccharina* (S) led to significantly higher MMP inhibition than the control feed, while experimental feed A did not induce any inhibitory effect in this species.

Discussion

The primary objective of the present study was to evaluate the potential of MMP inhibitors present in algal products to effectively inhibit MMP activity in the fish intestine. As outlined in the introduction, the initial hypothesis posited that MMPs play a role in several intestinal inflammation processes in vertebrates, often manifesting as enteritis. It was further hypothesized that the inclusion of algal meals in aquaculture feeds could serve as a potential solution to alleviate such inflammatory issues, particularly in carnivorous

Table 2 Bioactive compounds of the algal products used in this study using a conventional extraction described in Section "Algal products and active extracts". Values are presented as mean ± SD (n = 3 samples). For each parameter, values not sharing common superscripts are significantly different with $p < 0.05$

Algal product	g total phenolics per 100 g product		mmol TEAC per 100g product		g phlorotannins per 100g product	
Alaria	0.411	$\pm 0.018^{bc}$	0.254	$\pm 0.092^{b}$	0.016	$\pm 0.000^{c}$
Saccharina	0.349	$\pm 0.018^{cde}$	0.183	$\pm 0.074^{b}$	0.007	$\pm 0.001^{e}$
Acid conserved *Saccharina*	0.378	$\pm 0.019^{bcd}$	0.746	$\pm 0.060^{a}$	0.008	$\pm 0.001^{e}$
Fermented *Saccharina*	0.441	$\pm 0.018^{b}$	0.654	$\pm 0.029^{a}$	0.008	$\pm 0.001^{e}$
Auto-fermented *Saccharina*	0.435	$\pm 0.021^{b}$	0.795	$\pm 0.064^{a}$	0.008	$\pm 0.000^{e}$
Red seaweed concentrate Alginor	0.341	$\pm 0.021^{de}$	0.229	$\pm 0.035^{b}$	0.026	$\pm 0.002^{a}$
Wakame	0.281	$\pm 0.012^{e}$	0.385	$\pm 0.088^{b}$	0.011	$\pm 0.000^{d}$
Nori	0.634	$\pm 0.016^{a}$	0.179	$\pm 0.022^{b}$	0.019	$\pm 0.001^{b}$

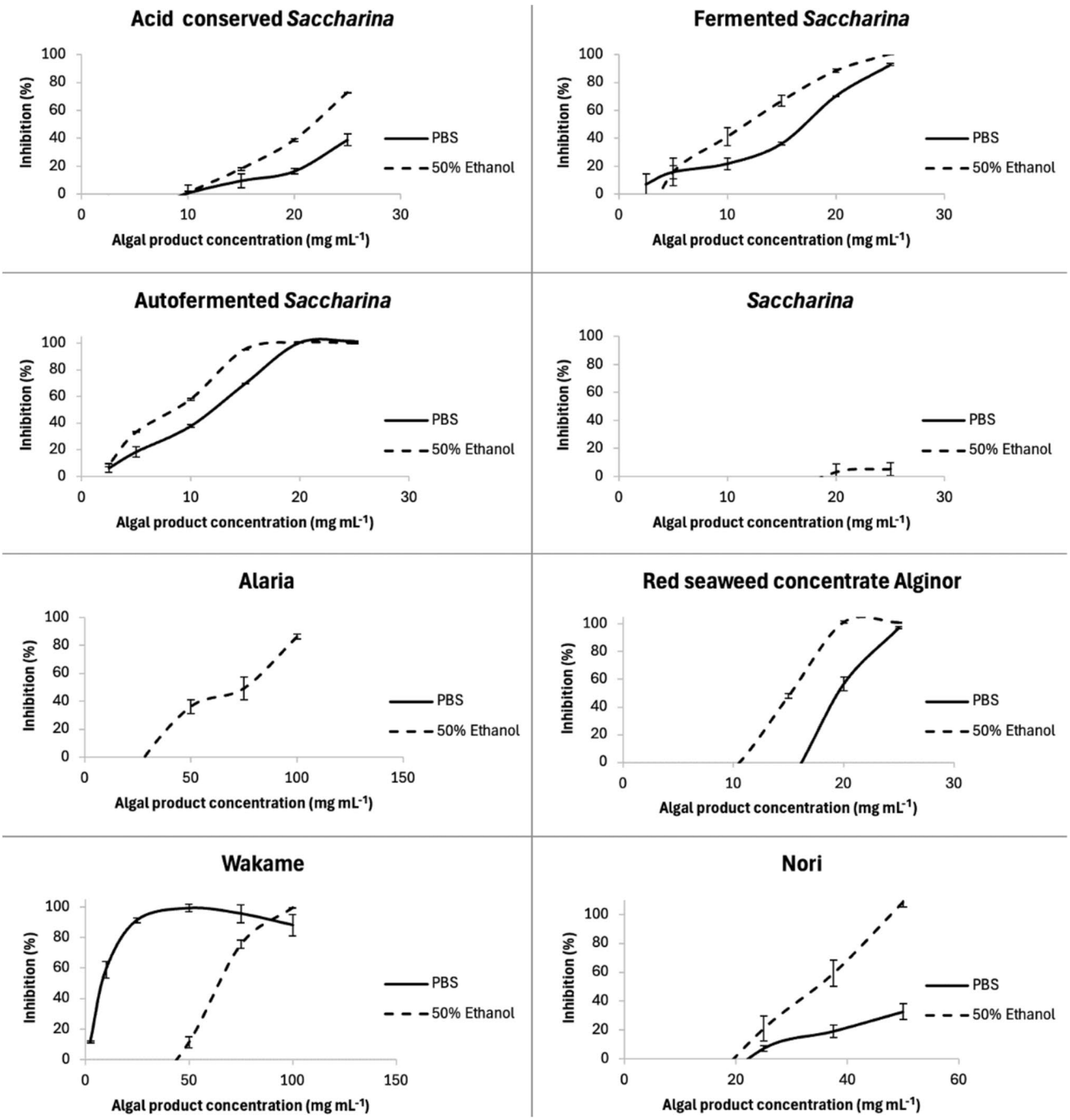

Fig. 1 Quantification of MMPI present in different algal products obtained either by PBS or ethanol 50 % extraction against MMP activity of *Clostridium hystoliticum*. Values are presented as mean ± SD (n = 3 samples per point)

fish species fed diets containing soybean meal (Chadzinska et al. 2008; Seibel et al. 2022; Krogdahl et al. 2023).

The initial evaluation of various algal products provided compelling evidence that MMP inhibitors are present not only in raw macroalgae but also in processed products such as fermented, hydrolyzed, or concentrated algal biomass. Results indicated that these processed products, which are being developed and tested as potential feed ingredients in various forms (Siddik et al. 2023), not only retain MMP inhibitory activity but may even exhibit enhanced levels of inhibition following treatments designed to partially hydrolyze complex carbohydrates or increase protein content.

Most studies quantifying bioactive compounds in seaweeds, including potential MMP inhibitors such as

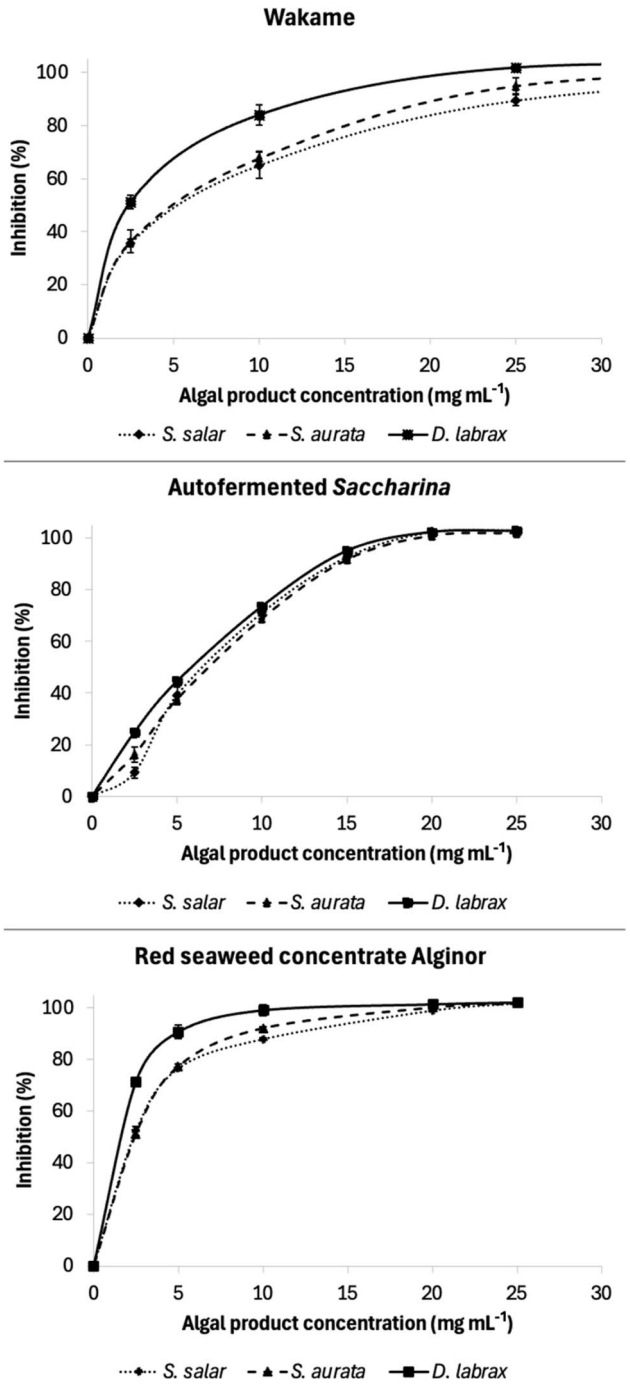

Fig. 2 Comparison of the sensitivity of MMP present in intestinal extracts of three fish species to inhibitors present in different algal products. Values are presented as mean ± SD

phlorotannins, polyphenols, and certain carbohydrate fractions, often utilize strong solvents like ethanol and high temperatures for extraction (Siddik et al. 2023). These methods are designed to maximize yields in the context of industrial-scale extraction processes; however, they differ significantly from the physiological conditions present in the

Table 3 Effect of MMPIs present in experimental feed matrix on the activity of MMPs present in intestinal extracts of *S. salar* and *S. aurata* using an in vitro digestive simulation. The reduction in activity was expressed as % inhibition, using the control feed as a reference. Values are presented as mean % inhibition ± SD for n = 3. For each species, values not sharing a common letter differ significantly with $p < 0.05$

Experimental feed	*S. salar*		*S. aurata*	
Control	0.00	± 5.28 ^{c}	0.00	± 6.65 ^{b}
S	8.20	± 3.54^{ab}	14.43	± 3.63 ^{a}
A	10.00	± 3.28^{a}	0.00	± 3.45 ^{b}

fish digestive tract. Therefore, in this study, solubility and potential bioavailability of MMP inhibitors were assessed using PBS extracts, which more closely resemble the aqueous environment found within the fish gut. The results demonstrated that MMP inhibitors were present in most samples in aqueous PBS extracts obtained at 25°C. Moreover, there were notable differences in the release of inhibitory compounds across species. For instance, extracts from fresh samples of brown algae, such as *Alaria* and *Saccharina*, did not exhibit detectable MMP inhibition, whereas extracts from *Undaria*, *Porphyra*, fermented *Saccharina*, and processed products like the Alginor red algae concentrate displayed significant levels of MMP inhibitory activity. These variations are likely influenced by differences in tissue structure and the nature of the inhibitors present in different algal species. Additionally, the release of inhibitory compounds appears to be enhanced in certain species through partial hydrolysis of the tissue matrix, as has been previously reported for other bioactive compounds (Visse and Nagase 2003; Kim et al. 2011; Thomas et al. 2014). From a practical application standpoint, these findings suggest the value of conducting such preliminary screenings to identify the most suitable algal sources of MMP inhibitors for incorporation into aquafeeds.

Results from the evaluation of MMP inhibitors on intestinal MMP activity in the three fish species confirmed the previously noted differences in relation to the algal source. However, no species-specific differences were observed. Despite these similarities, the inhibition curves generated for the three fish species indicated a higher sensitivity to MMP inhibitors compared to the microbial enzyme. For instance, the PBS extract of the Alginor concentrate required a concentration of 50 mg mL^{-1} to achieve 100% inhibition of the *C. histolyticum* MMP used as the reference in the assay kit. In contrast, only 10–20 mg mL^{-1} was needed to inhibit MMPs from any of the fish species. Such differences in the sensitivity of various MMPs to inhibitors have been reported previously (Visse and Nagase 2003; Medina and Radomski 2006). From a practical standpoint, the heightened sensitivity of MMPs in fish

intestinal tissue is an advantage, particularly considering that due to their high content of undigestible compounds, seaweed meals are typically limited to 10–12% (or less) of the total dry weight in aquafeed formulations (Silva et al. 2014; Abdel-Warith et al. 2016; Vizcaíno et al. 2016; Siddik et al. 2023).

For any potential effects to occur at the intestinal level, MMP inhibitors present in algal sources must remain bioavailable in sufficient quantities during or after the digestion process. Therefore, the present study aimed to assess the bioavailability of intestinal MMP inhibitors present in two selected algal sources (autofermented *Saccharina* and red seaweed protein concentrate) using an *in vitro* simulation of digestive conditions in two fish species. Unlike previous assays that involved mixing pure algal extracts with a simple aqueous medium, the *in vitro* simulation was designed to consider additional factors that would provide a more comprehensive and realistic scenario for potential application. These factors included: i) possible interactions with other feed ingredients, ii) stability across changing pH levels in the stomach and intestine, iii) the presence of a complex ionic environment during the intestinal phase, and iv) resistance to proteolytic degradation by the fish digestive enzymes. This type of *in vitro* simulation of fish digestive conditions has been successfully applied in recent studies to assess the bioavailability of nutrients and bioactive compounds (Gilannejad et al. 2017; Martínez-Antequera et al. 2023). The results not only confirmed substantial differences in the inhibitory efficacy of different algal products, but also demonstrated that significant amounts of MMP inhibitors can remain active during or after the digestion process. These effects were shown to be species-specific. Consequently, *in vitro* assays represent a valuable screening tool for selecting the most effective MMP inhibitors on a species-specific basis.

A recent study by Rehman et al. (2023) demonstrated that intestinal inflammation induced by soybean meal in zebrafish was partially alleviated by the inclusion of algae-derived alginate oligosaccharides (AOS). The anti-inflammatory properties of AOS have been attributed to several mechanisms, including the reduction of nitric oxide and prostaglandin E2 production, inhibition of nuclear factor κ B, enhancement of antioxidant enzyme activity, and improvements in intestinal morphology (e.g., increased villi length, goblet cell number, and mucin-2 expression). Additionally, dietary AOS may influence intestinal morphology and barrier function, as reported by Wan et al. (2018). The results of the present study suggest that compounds with MMP inhibitory activity could complement and reinforce the positive effects described above, being then aligned with the responses observed *in vivo*.

Conclusion

To the best of our knowledge, the present study is the first to investigate the effect of algal-derived inhibitors on fish intestinal MMPs as a preliminary step in evaluating their potential to mitigate diet-induced intestinal inflammation. Based on these findings, an *in vivo* study has been designed to assess the ability of algal MMP inhibitors to counteract the negative effects at the intestinal level in fish challenged by an inflammation-promoting diet. This study is currently in progress.

Author contributions FJM, JM-S, GN and JM contributed to the conception and design of the study. FM-A, JM-S, GN, SM-L, MN, MED and HS contributed to biological experiment. FM-A and FM organized the database. FM-A and FM performed the statistical analysis. FM-A wrote the first draft of the manuscript. GN, FJM, and JM wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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Data availability Data will be made available on request.

Declarations

Conflict of interest The authors declare no conflict of interest.

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