



Seaweed-derived mixed extracts exhibit immunomodulatory properties on porcine alveolar macrophages

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ARTICLE INFO

Keywords:

Seaweed species
Bioactive properties
Inflammation
Nutraceuticals
Gene expression
Porcine alveolar macrophages

ABSTRACT

Antimicrobial resistance is a growing global concern, prompting for antibiotic alternatives in animal production. Seaweed, abundant in bioactive compounds with anti-inflammatory properties, offers a natural substitute to synthetic compounds. Considering this, the objective of the present study was to evaluate the anti-inflammatory bioactivity of three seaweeds 1:1 combination of *Ascophyllum nodosum*, *Palmaria palmata*, and *Ulva lactuca*. Initially, polyphenol, flavonoid, and total phlorotannin content of the three seaweed species were assessed through colorimetric assays. Subsequently, the anti-inflammatory bioactivity was first evaluated through an inhibition protein precipitation assay and then confirmed *in vitro* through gene expression assays in LPS-stimulated porcine alveolar macrophages (PAMs). The evaluation of the bioactive molecules revealed a high content of TPC (1487.67 ± 40.39 and 1763.57 ± 69.01 mg TAE/100 mg of sample, respectively), as well as of TFC (95.68 ± 3.62 and 126.09 ± 7.34 mg CE/100 mg of sample) and TPhC (0.167 ± 0.02 and 0.23 ± 0.01 mg PGE/100 mg) for AN and UL, respectively. The assay for inhibiting protein precipitation disclosed that the extracts combining two algae species (ANUL, ANPP, PPUL) were more effective than the effect exhibited by each single extract. The assessment of anti-inflammatory bioactivity revealed a significant down-regulation of IL-1 β and TNF- α in the algae combination extracts. In contrast, TGF- β showed an increasing trend. These findings, along with confirmation of the high content of bioactive molecules, highlight the algae's anti-inflammatory potential, making them suitable as natural alternatives to antibiotics for disease prevention in the livestock sector. Therefore, future research should explore the specific bioactive compounds and validate their efficacy *in vivo* to confirm their potential use in animal production.

1. Introduction

The swine production system faces challenges from a wide range of pathogens posing significant implications to animal health and food safety. Since 1985, a total of 186 new variants of swine pathogens have been identified, emphasizing the dynamic nature of pathogen emergence in this sector (Fournie et al., 2012). Consequently, the complexity of managing swine health and the potential risks to human consumers are on the rise. Among various pathogens, *Escherichia coli* is a major concern due to its impact on animal health and its potential contribution to antimicrobial resistance (AMR) which threatens both animal and human health (Arbab et al., 2022). This swine bacterial pathogen could stimulate macrophages to secrete inflammatory cytokines (Kaper et al., 2004). Macrophages are pivotal cells of the innate immune system with

porcine alveolar macrophages (PAMs) serving as the first line of defense against bacterial infections and producing pro-inflammatory cytokines (tumor necrosis factor- α (TNF- α); interleukin-1 β (IL-1 β)), and anti-inflammatory cytokines (such as TGF- β transforming growth factor β) after lipopolysaccharide (LPS) stimulation (Li et al., 2023; Zhang and An, 2007). LPS stimulates nuclear factor-kB (NF-kB) and mitogen-activated protein kinases through various signal transduction pathways (Han et al., 2020). Thus, the triggering of isolated cells, such as PAMs with LPS is a common model of acute inflammation, mimicking the response to bacterial infection (Olsthooorn et al., 2021). Due to a key role in immune regulation, and their ability to produce both pro- and anti-inflammatory cytokines in response to stimuli, PAMs represent an ideal model for this study to evaluate the anti-inflammatory effects of bioactive compounds. Their well-characterized inflammatory signaling

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<https://doi.org/10.1016/j.tvj.2025.106358>

Accepted 12 April 2025

Available online 15 April 2025

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pathways, particularly those activated by LPS-induced NF- κ B and MAPK pathways, further enhance their sustainability for assessing the immunomodulatory effects of seaweed compounds. Furthermore, as key mediators of the innate immune response, PAMs closely mimic the *in vivo* inflammatory processes, providing valuable insights into the potential therapeutic applications of seaweed in management of inflammation (Li et al., 2018; Liu et al., 2012; Shen et al., 2023; Zhong et al., 2022).

Seaweeds are natural sources of bioactive compounds such as alginates, fucoidans, laminarins, and phlorotannin with great potential as dietary ingredients active against infections (Lomartire and Gonçalves, 2022; Sudharsan et al., 2015). Different studies have revealed that the inclusion of seaweeds in daily diet is associated with decreasing incidence of numerous diseases, improving digestive health, benefiting chronic diseases, and combatting bacterial and viral infections (Cao et al., 2016; Charoensiddhi et al., 2017; Peñalver et al., 2020). Multiple *in vitro* studies using cell lines have demonstrated that algae extracts and algae-derived compounds exhibit anti-inflammatory activity through the inhibition of pro-inflammatory cytokines and reduction of expression of pro-inflammatory genes (Lee et al., 2013; Robertson et al., 2015). Numerous health-promoting bioactive compounds of the brown seaweed extracts from *Ascophyllum nodosum* have been reported to display immune modulators (Bahar et al., 2012) and anti-inflammatory properties (Cumashi et al., 2007; Zhang et al., 2014) both *in vitro* and *in vivo*. Additionally, red seaweed, *Palmaria palmata*, and green algae, *Ulva lactuca*, demonstrated as health-promoting agents with anti-inflammatory activity (Robertson et al., 2015). Accordingly, evidence suggests that algae extracts may be beneficial as functional food ingredients to control inflammation also in piglets (Olsthooorn et al., 2021). Seaweeds are ideal candidates for replacing synthetic compounds with those of natural origin due to their non-toxic, edible, low-cost, and easy culturing properties (Lomartire and Gonçalves, 2022). Since inflammation plays a significant role in the development of many diseases, seaweeds offer considerable potential for applications in both medicine and the feed industry as a preventive strategy to reduce disease occurrence and, consequently, the reliance on antibiotics. For instance, our research group has demonstrated that seaweed administration in post-weaning piglets provided a protective effect against intestinal damage caused by *E. coli* F4 + infection, supporting the animals' growth, and enhancing overall health (Dell'Anno et al., 2024; Frazzini et al., 2024a). Hence, the incorporation of seaweed compounds into the formulation of novel natural drugs has become one of the aims in the field of marine pharmaceuticals and nutraceuticals, which has developed significantly over the last decades (Lomartire and Gonçalves, 2022). Moreover, despite the growing interest in seaweed bioactive compounds, studies assessing the synergistic and complementary effects of combined algae extracts remain limited. Although some evidence suggests that combining different algae species may enhance bioactivity, comprehensive research quantifying these interactions is still insufficient. Synergistic action, where the combined effect exceeds that of individual components, and complementary action, where different bioactive properties contribute to a broader therapeutic effect, have yet to be fully explored (Yang et al., 2017). Therefore, testing seaweed extracts in combination is crucial to determine whether these interactions can enhance effectiveness and reduce the minimal effective dosage of treatment.

To address this research gap, the objectives of this present study were to evaluate *in vitro* the anti-inflammatory and immunomodulatory bioactivities of 1:1 seaweed mixes (ANPP, ANUL, PPUL) of the three species (*Ascophyllum nodosum* (AN), *Palmaria palmata* (PP), *Ulva lactuca* (UL)) via testing their bioactive molecules and measuring the inflammatory gene expression in LPS-stimulated porcine alveolar macrophages (PAMs).

2. Materials and methods

2.1. Extraction of seaweed biomass

The standardized lyophilized powder (100 % pure) of *Palmaria palmata* (PP; catalog number: 10418) was commercially 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 experiment, the extraction was performed using ethanol as solvent following the procedures of López et al. (2011) and Mazur-Marzec et al. (2013) with some modifications. Briefly, mixed (in 1:1 ration) seaweed biomass (ANUL, ANPP, ULPP) powder was dissolved in 80 % ethanol at a 1:10 ratio, ground in the mortar, and vortexed for 3 min. Then, all samples were overnight frozen at -20°C , centrifuged at 5000 rpm for 20 minutes at 4°C , and the supernatant was decanted. Solid glass beads (3 mm) were then added to each sample. After homogenization for 30 s at 4.5 RPS (FastPrep-24 Classic homogenizer, MP Biomedicals, Irvine, CA, USA), all samples were centrifuged at 5000 rpm for 20 minutes at 4°C . The ethanol extraction procedure was repeated three times for the remaining pellet. The extraction solution was then evaporated by an evaporator (Rotary Evaporator Strike 300, Steroglass srl, Perugia, Italy) and dried residues were weighed to be weighed to determine the yield relative to the weight of the dry seaweed powder. Each residue was subsequently resuspended in sterile RPMI-1640 medium (Roswell Park Memorial Institute) to obtain desired concentrations, then filtered (using $0.22\ \mu\text{m}$ syringe filters) to dissolve any particles, all under sterile conditions, for further analysis.

2.2. Evaluation of bioactive compounds: total polyphenols content (TPC), total flavonoids content (TFC), and total phlorotannin content (TPhC)

The total content of polyphenols, flavonoids, and phlorotannin was evaluated on the extracts of AN, PP, and UL. Specifically, the quantification of the TPC was established by the Folin-Ciocalteu method, according to Attard (2013) and the results were expressed as mg of Tannic Acid Equivalents (TAE)/100 mg of sample. The quantification of the total flavonoid content (TFC) was obtained with the aluminum chloride (AlCl_3) colorimetric method described by Herald et al. (2012). The results were expressed as mg of catechin equivalent (CE)/100 mg of sample. Finally, TPhC was quantified according to the 2,4-dimethoxybenzaldehyde (DMBA) colorimetric method according to Lopes et al. (2012). The results were expressed as mg of phloroglucinol equivalents (PGE)/100 mg of sample. All assays (TFC, TPC and TPhC) were conducted in three technical triplicates and two biological replicates to ensure the experiment's reproducibility. This involved repeating the investigation from the sample extraction stage and performing the tests in additional sets using the same procedures. Evaluation of bioactive compounds: HPLC-Q-TOF-MS/MS metabolomic characterization

For the combined extract, metabolomic characterization was performed using high-performance liquid chromatography (HPLC) and mass spectrometric analyses (LC-MS2), as described in our previous study (Frazzini et al., 2024b), via an HPLC-Q-TOF-MS/MS system (Sciex, Massachusetts, USA). Briefly, 10 μL of the sample was injected and eluted through a Synergy Hydro-RP 80 Å, LC Column (250 mm 4.6 mm, 4 m) (Phenomenex, Lane Cove, NSW, Australia) with a binary mobile phase (A: 0.1 % formic acid in water; B: 0.1 % formic acid in acetonitrile) and a flow rate of 0.4 mL/min. Compounds were identified by comparing retention time (RT) and UV spectral information with those in the library of Natural Compounds and Metabolites (Sciex, Massachusetts, USA).

2.3. Inhibition of protein precipitation

Inhibition of protein precipitation was evaluated as a possible

marker of the inflammatory process, according to [Osman et al. \(2016\)](#). Briefly, 1 mL of each algal extract at different concentrations (200, 100, 50 and 25 µg/mL) was mixed with 450 µL of 5 % (w/v) of bovine serum albumin solution and 1.4 mL of phosphate-buffered saline. Afterward, the mixtures were incubated at 37°C for 15 min and then heated at 70°C for an additional 5 min. After cooling, the absorbance was measured spectrophotometrically at 660 nm (BioTek Epoch, Agilent, Santa Clara, CA, United States). Distilled water was used as a negative control, while ibuprofen was served as a positive control. The experiment was conducted in three technical triplicate and with two biological replicates and the percent inhibition for protein precipitation was calculated based on the formula:

$$\% \text{Inhibition of precipitation} = \left(\frac{1 - D}{C} \right) * 100$$

Where D is the absorbance of the sample, and C is the absorbance of the negative control.

2.4. In vitro anti-inflammatory assays

2.4.1. Collection of porcine alveolar macrophages (PAMs)

PAMs were collected from healthy breeding animals, intended for commercial trade in the meat-processing industry in compliance with EU 2010/63/EU. Piglets used for PAM collection underwent both antemortem and post-mortem inspection by official veterinarian to ensure the absence of clinical symptoms or signs of infectious diseases. Consequently, the PAM cells were assembled from piglets at 10 weeks and approximately 22 kg of body weight through bronchoalveolar lavage following the procedures described by [Baarsch et al. \(1991\)](#). Briefly, lungs with intact trachea were removed immediately after euthanizing pigs, and 100 mL of ice-cold phosphate-buffered saline (PBS) without calcium or magnesium was poured through the trachea. After massaging, the lavage fluid was filtered through a sterile gauze into 50 mL conical centrifuge tubes and then pelleted by centrifuging at $400 \times g$ for 10 min at room temperature. The pelleted cells were added with 4 mL ACK lysing buffer (Thermo Fisher, Massachusetts, USA) to lyse red blood cells and then suspended in 5 mL of culture medium RPMI-1640 supplemented with 1 % antibiotics (100 mg/mL penicillin and 100 mg/mL streptomycin; Mediatech, Inc., Manassas, VA) and with 5 % fetal bovine serum (HyClone Laboratories, Inc., Logan, UT). Live cells were stained using trypan blue dye exclusion (Sigma-Aldrich Co., St. Louis, MO, USA) and were counted by a hemocytometer (Fisher Scientific, Inc., Pittsburgh, PA, USA). The final cell concentration was adjusted to 1×10^6 cells/mL with cell viability exceeding 97 %. In this study, the term “porcine alveolar macrophages” was used because the majority of bronchoalveolar lavage fluid cells are macrophages ([Dickie et al., 2009](#)).

2.4.2. Cell culture and experimental design

PAM cells were cultured at 10^6 cells/mL in 24-well cluster plates and incubated overnight at 37°C with 5 % CO₂ to allow macrophages to adhere to the bottom. The nonadherent cells were washed away, and the adhered macrophages were treated in duplicate with fresh pre-warmed RPMI-1640 medium containing different treatments. After 24 h incubation, cells were detached with Trypsin-EDTA Solution (Sigma-Aldrich Co., St. Louis, MO, USA), collected, and stored at −80°C for RNA extraction. As treatments, ANPP, ANUL, and PPUL were tested using the same experimental design, following a factorial arrangement in a randomized complete block design. The experiment included 2 LPS conditions (absence or presence of 1 µg/mL) $\times$ 3 seaweed mix concentrations (0, 100, and 200 µg/mL), resulting in a total of six treatment combinations. All conditions were tested in both technical and biological triplicates to ensure the reproducibility and reliability of the result. The negative control was the treatment without either seaweed or LPS, and the positive control was the treatment without seaweed but with LPS.

2.4.3. RNA extraction, retro-transcription, and quantitative real-time PCR (qRT-PCR)

Total RNA was isolated from PAMs using the RNeasy Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The RNA concentration and purity were established by measuring absorbance at wavelengths of 230, 260, and 280 nm with a NanoDrop™ 2000 Spectrophotometer (Thermo Fisher Scientific, Massachusetts, USA). The samples were stored at −80°C until further use. According to the manufacturer's instructions, 1 µg of total RNA from all the samples was reverse transcribed using iScript™ cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA, United States). The reverse transcription was performed in a 20 µL reaction mixture according to the following program: incubation (i) at 25°C for 5 min; (ii) at 46°C for 20 min; (iii) and step 3 at 95°C for 1 min. The qRT-PCR was carried out with the CFX Opus 96 (BioRAD, Richmond, CA, USA). The reaction mixes were optimized in a total of 20 µL volume containing 10 µL of 2X SsoAdvanced Universal SYBR Green Supermix (BioRad, Richmond, CA, USA), 500 nM of each primer ([Table 1](#)), and 10 ng of cDNA template. The qPCR cycling conditions were as follows: (i) pre-denaturation of 95°C for 30 s, (ii) 40 cycles of denaturation of 95°C for 15 s, (iii) and an annealing/extension step at 60°C for 30 s. The results were deemed negative when the target genes' cycle threshold (Ct) values were > 37. A sample, selected as a cell without LPS and treatment, was used as the control for gene expression assays. Gene expression quantification was performed using the $2^{-\Delta\Delta CT}$ method. GAPDH was included as the housekeeping gene, following the approach of [Oh et al. \(2023\)](#). Data normalization and analysis were conducted using the BioRad CFX Maestro V2.2 software (BioRad, Richmond, CA, USA). The baseline for comparisons was the control sample, without LPS or treatment. Gene expression analyses were conducted in technical triplicate, with biological replicates obtained from cell culture.

2.5. Statistical analysis

All the data were analyzed using GraphPad Prism, version 9.0.0 (Boston, MA, USA). Before performing the two-way analysis of variance (two-way ANOVA), the normality of the data distribution and residuals was assessed using the D'Agostino-Pearson test. The data were then analyzed to evaluate the effects of treatment, time, and their interaction. Post hoc pairwise comparisons were performed using Bonferroni Sidak's test. Data from qRT-PCR were analyzed using a one-way analysis of variance (one-way-ANOVA) for comparing the median of different groups. The data were reported as the mean $\pm$ standard deviation, and differences were considered to be statistically significant at $p \leq 0.05$.

3. Results

3.1. Bioactive compounds composition

The evaluation of the bioactive compounds of the three different algae species revealed that the concentrations of polyphenols, flavonoids, and phlorotannin were directly proportional to the dilution of the sample ([Fig. 1](#)). The results disclosed that UL carried the highest content of bioactive molecules among those evaluated in this study. Indeed, UL content in polyphenols was equal to 1764.00 ± 69.02 mg TAE/100 mg of sample, the TFC was equal to 0.445 ± 0.005 mg CE/100 mg of sample, and ThPC was about 0.23 ± 0.01 mg PGE/100 mg of sample. Also, AN showed a high content in TPC, TFC, and ThPC that were equal to 1488.00 ± 40.40 mg TAE/100 mg of sample, 0.334 ± 0.003 mg CE/100 mg of sample, and 0.167 ± 0.022 mg PGE/100 mg of sample, respectively. On the other hand, in PP extract, the concentration of bioactive molecules was lower, reaching the value of 143.70 ± 9.55 mg TAE/100 mg of sample for TPC, 0.135 ± 0.002 mg CE/100 mg of sample for TFC and 0.0068 ± 0.0001 mg PGE/100 mg of sample for ThPC.

The metabolomic evaluation of the algae combination revealed that the extract combinations were abundant in long-chain fatty acids

Table 1
Oligonucleotide sequences used for the qRT-PCR assay.

Gene ^a	Nucleotide sequence	Accession number	Reference
IL-1β	F: TGCCACGTCGAGTCTATGG R: TGGGCCAGCCAGCACTAG	NM_214055	Smolinska et al. (2019)
TGF-β	F: TGATGTCACCGAGTTGTGC R: GGCCAGAATTGAACCCGTTA	NM_214015.2	Dell'Anno et al. (2024)
TNF-α	F: CCACGCTCTTCTGCCTACTGC R: CGACGGGCTTATCTGAGGTTTG	NM_214022	Chen et al. (2019)
GAPDH	F: ACCCAGAAGACTGTGGATGG R: AAGCAGGGATGATGTTCTGG	NM_001206359.1	Luo et al. (2018)

^a IL-1β: interleukin-1 β; TGF-β: transforming growth factor β; TNF-α: tumor necrosis factor-α; GAPDH: glyceraldehyde-3-phosphate dehydrogenase.

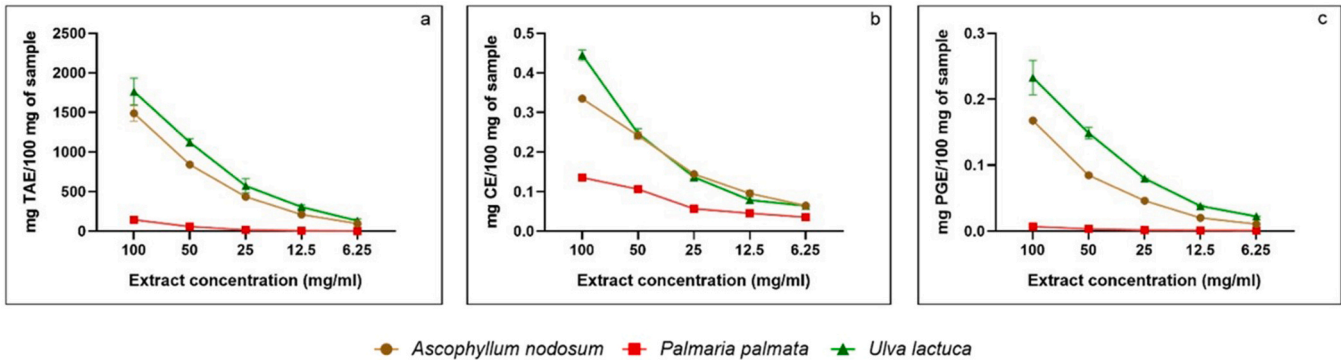


Fig. 1. Major bioactive compounds in *Ascophyllum nodosum*, *Palmaria palmata*, and *Ulva lactuca* extracts, with Total phenolic content (TPC, panel a); Total Flavonoid Content (TFC, panel b); Total Phloronin Content (TPhC, panel c). TAE: tannic acid equivalent; CE: catechin equivalent; PGE: phloroglucinol equivalent. The data are presented as the means $\pm$ standard deviations (SDs) ($n = 3$).

including α -linolenic and oleic acid (Table 2). Notably, ANPP combinations disclosed higher chromatographic peaks compared to other combinations, showing a pronounced presence of oleic acid with an area of 2,742,990, followed by stearic, linoleic, α -linolenic, and nervonic acid (722,598, 617,685, 476,957, and 401,821 of area, respectively). Additionally, to the content in long-chain fatty acids, the ANPP combination disclosed content in D-Xylose (320,148 of area). Furthermore, the analysis identified that the extract of algae combination highlighted the presence of 4-Hydroxybenzoic acid, a molecule derived from the phenol class, with areas of 890,859, 1,065,729, 686,174 respectively for ANPP, ANUL, PPUL.

3.2. Inhibition of protein precipitation

The addition of AN, PP, and UL extracts to the BSA-containing solution allowed the inhibition of protein precipitation. Analysis of the range of concentrations from 25 μ g/mL to 200 μ g/mL revealed that all three algal extracts were capable of inhibiting protein precipitation by approximately 30 % (Table 3). In contrast, the evaluation of the capacity of the algae combination to inhibit the protein precipitation disclosed that combinations containing *Ascophyllum nodosum*, particularly at the highest concentrations demonstrated a significant difference in protein precipitation inhibition percentage of 42.51 ± 0.15 % and 38.95 ± 0.16 % for ANPP at 200 and 100 μ g/mL, respectively; and 40.49 ± 0.27 % and 36.82 ± 0.11 % for ANUL at 200 and 100 μ g/mL, respectively. Moreover, PPUL also disclosed a significant difference at a concentration of 200 μ g/mL, where it showed a percentage of protein precipitation inhibition of 35.11 ± 0.18 %. Notably, the protein inhibition was observed to be dose-dependent, with higher concentrations exhibiting more pronounced effects. Specifically, for individual algae extracts the percentage of protein precipitation inhibition increased progressively with each dose, reaching statistical significance when comparing the two lower doses (25 and 50 μ g/mL) to the two higher doses (100 and 200 μ g/mL). In contrast, the extracts from seaweed

combination disclosed a statistically significant dose-dependent inhibition across all tested concentrations (Table 3).

3.3. Gene expression of pro- and anti-inflammatory cytokines on porcine alveolar macrophages with seaweed combinations

Different seaweed combinations were evaluated for the impact on the expression of selected cytokine genes based on the results of inhibition of protein precipitation. The evaluation of the relative expression of the selected cytokines revealed the effectiveness of LPS treatment in inducing cellular stimulation. The relative expression values of pro-inflammatory cytokines (IL-1 β and TNF- α) in samples stimulated with LPS but not treated with the extracts of the algae combinations were significantly higher ($p < 0.001$) compared to the control sample (Fig. 2a-f). An opposite trend was observed in the expression of the anti-inflammatory cytokine TGF- β (Fig. 2g-i). Focusing on the effect of the algae extracts, our results disclosed that treatments with extracts of the ANPP and ANUL combinations were able to down-regulate the IL-1 β gene expression to values comparable to those observed in the control, where cells were not stimulated (LPS) (Figs. 2a and 2b). Especially, treatment with the ANUL combination extract at the highest concentration (200 μ g/mL) significantly down-regulated IL-1 β compared to CTRL, disclosing a value of 7099.28 ± 166.79 AU ($p = 0.001$) (Fig. 2b). On the contrary, while the PPUL combination downregulated, even if not significantly ($p > 0.05$), the expression compared to the LPS sample without the extract, it was unable to restore the expression values of those observed in the CTRL sample, showing 35 % and 39 % higher values in cells treated with PPUL at a concentration of 100 μ g/mL and 200 μ g/mL, respectively (Fig. 2c). A similar trend was observed in TNF- α . In this case, all tested combinations at a concentration of 200 μ g/mL showed significantly ($p < 0.05$) downregulated TNF- α expression to values below those observed in the CTRL. A reduction in TNF- α expression that showed lower values than in the control was observed with the presence of the combinations of ANUL and PPUL at a

Table 2
Main molecules with bioactive properties present in the extract of ANPP, ANUL, PPUL combination found by HPLC-QTOF-MS/MS analysis.

Compound identified	Formula	Area	Retention Time
ANPP			
4-Hydroxybenzoic acid	C ₇ H ₆ O ₃	890,859	9.39
α-linolenic Acid	C ₁₈ H ₃₀ O ₂	476,957	31.09
D-sorbitol	C ₆ H ₁₄ O ₆	1,067,170	1.20
D-Xylose	C ₅ H ₁₀ O ₅	320,148	1.34
Erucic Acid	C ₂₂ H ₄₂ O ₂	93,454	36.87
Linoleic Acid	C ₁₈ H ₃₂ O ₂	617,685	32.05
Nervonic Acid	C ₂₄ H ₄₆ O ₂	401,821	33.66
Oleic Acid	C ₁₈ H ₃₄ O ₂	2,742,909	33.11
Pentadecanoic Acid	C ₁₅ H ₃₀ O ₂	49,804	32.13
Stearic Acid	C ₁₈ H ₃₆ O ₂	722,598	34.67
ANUL			
4-Hydroxybenzoic acid	C ₇ H ₆ O ₃	1,065,729	8.34
α-linolenic Acid	C ₁₈ H ₃₀ O ₂	176,183	31.14
D-sorbitol	C ₆ H ₁₄ O ₆	3,021,325	1.23
Erucic Acid	C ₂₂ H ₄₂ O ₂	45,392	36.97
Linoleic Acid	C ₁₈ H ₃₂ O ₂	296,379	32.08
Nervonic Acid	C ₂₄ H ₄₆ O ₂	310,284	33.70
Oleic Acid	C ₁₈ H ₃₄ O ₂	1,641,162	33.14
Stearic Acid	C ₁₈ H ₃₆ O ₂	566,940	34.70
PPUL			
4-Hydroxybenzoic acid	C ₇ H ₆ O ₃	686,174	9.16
α-linolenic Acid	C ₁₈ H ₃₀ O ₂	440,277	31.11
D-sorbitol	C ₆ H ₁₄ O ₆	1,724,731	1.19
D-Xylose	C ₅ H ₁₀ O ₅	297,295	1.33
Erucic Acid	C ₂₂ H ₄₂ O ₂	108,638	36.87
Linoleic Acid	C ₁₈ H ₃₂ O ₂	687,141	32.06
Nervonic Acid	C ₂₄ H ₄₆ O ₂	132,078	33.83
Oleic Acid	C ₁₈ H ₃₄ O ₂	2,585,534	33.11
Pentadecanoic Acid	C ₁₅ H ₃₀ O ₂	60,433	32.14
Stearic Acid	C ₁₈ H ₃₆ O ₂	681,931	34.66

ANPP: combination of *Ascophyllum nodosum* and *Palmaria palmata* (1:1); ANUL: combination of *Ascophyllum nodosum* and *Ulva lactuca* (1:1); PPUL: combination of *Palmaria palmata* and *Ulva lactuca* (1:1).

Table 3
Percentage of protein precipitation inhibition by the algae extracts.

	25 µg/mL	50 µg/mL	100 µg/mL	200 µg/mL
AN	29.43 ± 0.10 ^a _A	29.07 ± 0.51 ^a _A	30.91 ± 0.17 ^a _B	31.32 ± 0.10 ^a _B
PP	32.21 ± 0.10 ^b _A	32.75 ± 0.09 ^b _A	32.95 ± 0.08 ^b _B	33.39 ± 0.17 ^b _B
UL	32.17 ± 0.05 ^b _A	32.50 ± 0.17 ^b _{A, B}	32.65 ± 0.06 ^b _{A, B}	32.92 ± 0.10 ^b _B
ANPP	33.45 ± 0.10 ^c _A	33.98 ± 0.20 ^c _A	38.96 ± 0.27 ^c _B	42.51 ± 0.27 ^c _B
ANUL	31.73 ± 0.21 ^b _A	32.68 ± 0.31 ^b _B	36.83 ± 0.21 ^d _C	40.50 ± 0.46 ^d _D
PPUL	31.68 ± 0.11 ^b _A	32.56 ± 0.10 ^b _B	33.10 ± 0.09 ^b _{B, C}	35.11 ± 0.18 ^e _D
CTRL	40.91 ± 0.67 ^d _A	47.25 ± 0.17 ^d _B	52.58 ± 0.35 ^e _C	57.25 ± 0.71 ^f _D

AN: *Ascophyllum nodosum*; PP: *Palmaria palmata*; UL: *Ulva lactuca*; ANPP: combination of *Ascophyllum nodosum* and *Palmaria palmata* (1:1); ANUL: combination of *Ascophyllum nodosum* and *Ulva lactuca* (1:1); PPUL: combination of *Palmaria palmata* and *Ulva lactuca* (1:1); CTRL: control represented by ibuprofen.

Superscripts of lowercase letters (a, b, c, d, e, f) indicate the statistical difference between the treatments. Superscripts consisting of capital letters (A, B, C, D) indicate the statistical difference between different extract concentrations (p-value: treatment < 0.001; concentration < 0.001; treatment*concentration < 0.001).

concentration of 100 µg/mL (9014.05 ± 301.17 AU and 8338.25 ± 152.60 AU, respectively) (Fig. 2e and Fig. 2f). Conversely, ANPP extract at the same concentration down-regulated TNF-α; however, this effect did not reduce TNF-α to the values expressed by the CTRL sample (Fig. 2d). Unlike pro-inflammatory cytokines, the evaluation of gene expression for the anti-inflammatory cytokine TGF-β indicated that exposure of PAMs to algal extracts resulted in a slight up-regulation (p > 0.05) of TGF-β compared to the levels expressed in the sample stimulated with LPS but not treated with algal extracts. However, the expression remained lower than those of CTRL (Fig. 2h and Fig. 2i). Only the ANPP combination significantly (p < 0.001) up-regulated TGF-β compared with CTRL, showing values of 22,664.77 ± 737.46 AU and 962,685.20 ± 24,062.36 AU for the concentrations of 100 µg/mL and 200 µg/mL, respectively (Fig. 2g).

4. Discussion

With antimicrobial resistance (AMR) representing a major risk to public health, the zootechnical sector is increasingly exploring new nutritional strategies that incorporate functional ingredients to support critical phases of production cycles while reducing the reliance on antibiotics (Hernandez-Patlan et al., 2023; Shrivastava et al., 2015; Velazquez-Meza et al., 2022). In this context, seaweeds have gained increasing interest as natural feed ingredients due to their rich bioactive profile with antioxidant, antibacterial, and immunomodulatory properties. Thus, the present study aimed to focus on the combinations of three algae species (*Ascophyllum nodosum*, *Palmaria palmata*, and *Ulva lactuca*), which were previously found to display strong antioxidant and antibacterial capabilities (Hejna et al., 2024). The main purpose of this study was to access the biological molecules of the three seaweeds and to evaluate the anti-inflammatory bioactivity of 1:1 seaweed mixes (ANPP, ANUL, PPUL) in LPS-treated porcine alveolar macrophages (PAMs) *in vitro*.

The bioactive compound content in seaweeds can vary significantly due to multiple factors associated with harvest conditions. Environmental parameters such as water temperature, salinity, light exposure, and nutrient availability play a crucial role in influencing phenolic biosynthesis. Additionally, seasonal variations also affect the accumulation of bioactive compounds, with certain species exhibiting elevated phenol content during periods of heightened environmental stress, including UV exposure or temperature fluctuations (Kumar et al., 2021). Additionally, geographical location and local water quality can influence metabolite production (Olsson et al., 2020). These natural variations, combined with differences in post-harvest handling and extraction techniques, further contribute to the variability in phenolic content observed across seaweed samples (Rossi et al., 2024). Despite these variations, our results of the chemical evaluation of the bioactive molecules from the phenol classes confirmed, as extensively reported in the literature, the dose-dependent abundance in the tested seaweeds (Lomartire et al., 2021; Sadeghi et al., 2024). Castejón et al. (2021) also showed that the TPC in UL was abundant (1950.6 ± 109.5 µg GAE/g dry weight) in gallic acid equivalent. AN, PP, and UL are known for their high content of bioactive compounds classified as secondary metabolites. According to the literature, green, red, and brown seaweeds are abundant in polyphenols. Green and red seaweeds are typically rich in phenolic compounds, such as flavonoids, whereas brown seaweeds primarily contain phlorotannins and phenolic acids (Matos et al., 2024; Sardari et al., 2020). These compounds play a key role in the seaweed's biological activities on overall health (Palaniyappan et al., 2023). These phytochemicals include various polyphenols, flavonoids, and phlorotannins (Freile-Pelegrín and Robledo, 2013; Putra et al., 2024; Xu et al., 2024). Also the phenolic compounds, characterized by hydroxyl groups attached to aromatic rings, contribute significantly to the bioactivity of seaweed, providing antioxidant, antimicrobial, and anti-inflammatory properties (Cotas et al., 2020).

Whether the extracts from the individual algae disclosed an

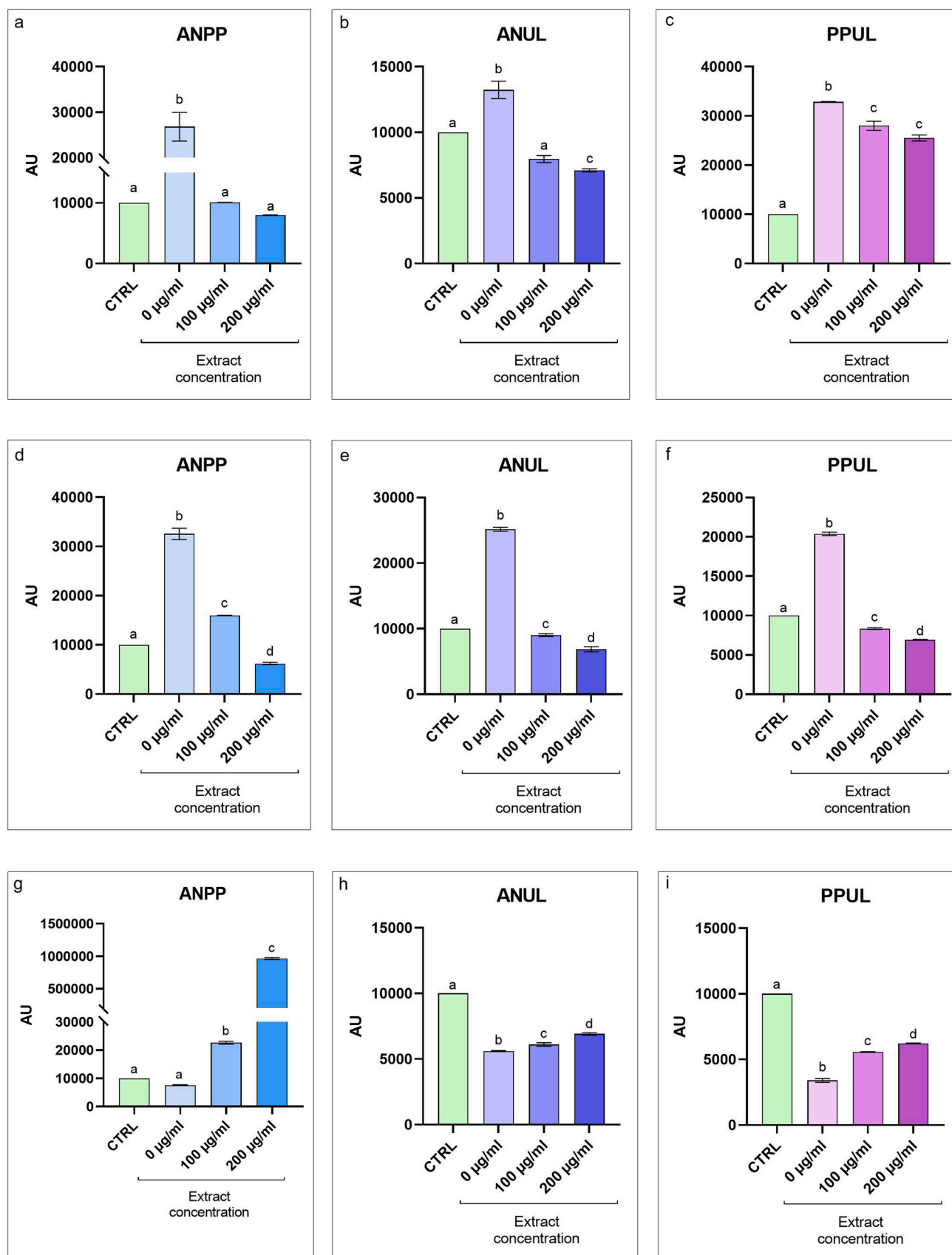


Fig. 2. Expression of cytokines involved in the inflammatory process in PAMs by different algae combinations: (a-c) expression of IL-1 β by ANPP, ANUL, and PPUL, respectively; (d-f) expression of TNF- α by ANPP, ANUL, and PPUL, respectively; and (g-i) expression of TGF- β by ANPP, ANUL, and PPUL, respectively. AU: arbitrary units. The data are shown as the means and standard errors. ^{a, b, c, d} Means with different superscript letters indicate statistically significant differences ($p < 0.05$).

undisputed bioactive potential, the extracts from the algae combination could exhibit enhanced bioactive activity due to the synergistic interactions among various bioactive molecules when they interact with one another, as demonstrated in our previous study as well as studies reported in the literature (Ebrahimi et al., 2023; Frazzini et al., 2022; Thangsiri et al., 2024).

In the context of anti-inflammatory activity, extracts of seaweed species have gained significant attention for their properties, highlighting their potential as promising candidates for treating inflammation-related diseases (Ghallab et al., 2024). In fact, as metabolomics results highlight, the algal compounds analyzed are rich in long-chain fatty acids. These compounds exhibit significant anti-inflammatory effects since they modulate immune responses by incorporating into cell membranes, thereby altering membrane fluidity and displacing pro-inflammatory arachidonic acid. Furthermore, long-chain fatty acids downregulate the production of inflammatory cytokines and inhibit key signaling pathways such as NF- κ B, ultimately contributing to reduced systemic inflammation and improved immune regulation (Calder, 2012; Hidalgo et al., 2021).

In line with what resulted from the bioactive composition of the algal extracts, the results of the present study revealed that the use of seaweed mixed extracts on the LPS-stimulated macrophages resulted in a decrease of gene expression of pro-inflammatory cytokines such as IL-1 β and TNF- α while enhancing the expression of TGF- β , a well-known cytokine involved in anti-inflammatory processes. Inflammation is a natural protective response that aims to eliminate dead or damaged host cells in the event of tissue injury or infection. This inflammatory response triggers an influx of blood leukocytes, oxidative burst, and the release of cytokines (Chen et al., 2018). However, during inflammation, some cytokines may be overproduced (Marshall et al., 2018). Therefore, the anti-inflammatory mechanisms of algae are crucial and multifaceted, involving the inhibition of pro-inflammatory cytokines and down-regulation of the inflammatory genes (Elbandy, 2022). Initially, this study highlighted the anti-inflammatory efficacy of algal extracts by assessing their ability to inhibit protein precipitation, a key indicator of anti-inflammatory potential. Indeed, proteins are essential components of cells, necessary for their proper functioning. When proteins become damaged or misfolded, can trigger and sustain inflammation (Chaiya et al., 2022). Additionally, to further support the anti-inflammatory potential, the protein inhibition assay demonstrated that at high concentrations (100 μ g/mL and 200 μ g/mL), the individual algal extracts (AN, PP, and UL) exhibited a limited ability to prevent protein denaturation. In contrast, when these extracts were combined, their inhibitory effect was significantly enhanced. This suggests a possible synergistic interaction among the compounds present in the different algae, which may work together to stabilize protein structures and more effectively counteract denaturation processes associated with inflammation (Ebrahimi et al., 2023; Lee et al., 2025). The anti-inflammatory effect of the algae combinations used in this study (ANPP, ANUL, and PPUL) was further confirmed by evaluation of the gene expression of two pro-inflammatory cytokines (IL-1 β and TNF- α) and one anti-inflammatory cytokine (TGF- β), on LPS-stimulated PAMs, which showed down-regulation and up-regulation of these genes, respectively.

Our study also highlighted that PPUL combination was less effective in downregulating IL-1 β compared to other evaluated combination (ANUL, ANPP). While existing literature does not report on the effects of these specific combinations in regulating the IL-1 β , these results may be attributed to the chemical composition and interactions among various bioactive components. Notably, the most effective combinations included AN, recognized for its high content of bioactive compounds with strong anti-inflammatory properties (Generalić Mekinić et al., 2019). In addition, synergistic interactions among the extracts could play a key role, as the inclusion of *Ascophyllum nodosum* could enhance the anti-inflammatory effect of other algae species (Frazzini et al., 2022). Finally, variations in bioavailability and metabolic interactions and regulations within the cell culture system might have further

influenced the lower efficacy of the PPUL combination compared with the other combinations (Lomartire and Gonçalves, 2022).

Although only a few studies in the literature currently report the efficacy of seaweed combinations against inflammatory processes, several studies have demonstrated that specific algae extracts can effectively modulate the secretion of key cytokines involved in inflammatory responses. Our experiments conducted in LPS-stimulated macrophages disclosed both pro-inflammatory and anti-inflammatory effects of various seaweed extracts, highlighting the complexity of the immune modulation processes.

The LPS, a component of the outer membrane of Gram-negative bacteria, is known to trigger robust immune responses, particularly in macrophages, resulting in the production of pro-inflammatory cytokines such as TNF- α and IL-1 (Ochando et al., 2023; Page et al., 2022). These cytokines, primarily produced by macrophages, are key regulators of the host immune response and play a crucial role in the inflammatory response; therefore, their regulation is vital for maintaining immune homeostasis (Mantovani et al., 2019; Zhao et al., 2021). Regarding these cytokines, Yoon et al. (2011) demonstrated that *Hizikia fusiforme*, administered as water extracts at a concentration of 50 mg/kg body weight under LPS stimulation, was able to enhance the production of IL-1 β and TNF- α . Additionally, *Sargassum hemiphyllum* exhibits an anti-inflammatory effect by reducing IL-1 β secretion in LPS-triggered RAW 264.7 macrophages, although the precise mechanisms remain to be fully elucidated (Hwang et al., 2011). Moreover, certain algal lipopolysaccharides, such as those derived from *Porphyra palmata* and *Pavlova lutheri*, demonstrated to inhibit TNF- α release from macrophages (Robertson et al., 2015). Among the various cytokines involved in inflammatory pathways, TGF- β plays a key role, as a multifunctional cytokine, often displaying both pro-inflammatory and anti-inflammatory effects (McCartney-Francis and Wahl, 2001). Research indicates that TGF- β boosts the production of nitric oxide (NO) in macrophages, which is essential for their cytotoxic functions against pathogens. However, the imbalance between TGF- β immunosuppressive and pro-inflammatory activities may result in chronic inflammation (Lin et al., 1995). Therefore, the incorporation of seaweed extracts may help to modulate this balance. Nutritional therapeutics that utilize dietary components such as algae have demonstrated the potential to prevent or treat inflammatory diseases by influencing cytokine production and immune responses (Naik et al., 2024). By modulating the immune response, algae may enhance the beneficial effects of TGF- β , promoting a more balanced immune environment. This modulation could involve the downregulation of pro-inflammatory cytokine production while simultaneously supporting the TGF- β role in promoting NO production and other anti-inflammatory pathways (Gonfa et al., 2023).

Although the effect of different algae species can vary, and many of the inflammatory mechanisms still require specific investigation, the immunomodulatory effects of algae are largely attributed to their bioactive compounds, including polyphenols, omega-3 polyunsaturated fatty acids (n-3 PUFA), and chlorophyll a. Among these, polyphenols play a crucial role due to their potent antioxidant and anti-inflammatory properties, which contribute significantly to immune regulation. These compounds inhibited the production of pro-inflammatory cytokines in macrophages (Robertson et al., 2015). Specifically, docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), both omega-3 fatty acids present in algae, reduced IL-1 β production, further supporting the anti-inflammatory potential of algal extracts (Wann et al., 2010). Moreover, lipid extracts from various algae, such as red seaweeds and microalgae, have demonstrated the ability to inhibit LPS-induced inflammatory pathways in macrophages. These extracts may down-regulate the expression of pro-inflammatory genes and inhibit signaling pathways mediated by Toll-like receptors and nuclear factor kappa-light-chain-enhancer of activated B cells (NF- κ B) (Robertson et al., 2015).

In conclusion, while the data from the current study require further validation and additional studies to assess the immunomodulatory effect

of seaweed extracts, these findings indicate that dietary supplementation with seaweed extracts could serve as a natural alternative to support animal health. In fact, algae could be used for preventive purposes by reducing the occurrence of diseases and consequently limiting the reliance on antibiotics. Such insights will contribute to bridging the gap between fundamental research and practical applications supporting broader efforts to combat antimicrobial resistance.

5. Conclusion

Amid the significant public health risk posed by antimicrobial resistance, the zootechnical sector is searching for new nutritional strategies based on the incorporation of functional ingredients to support critical phases of production. Thus, this study aimed to investigate the effect of bioactive compounds of three algae species combinations (*Ascophyllum nodosum*, *Palmaria palmata*, *Ulva lactuca*) on inflammation. Firstly, this study identified the abundance of bioactive molecules including polyphenols, flavonoids, and phlorotannins in tested seaweed extracts. Then, we tested the anti-inflammatory bioactivity of the tested algae. The use of their mixed extracts was demonstrated to reduce gene expression of pro-inflammatory cytokines such as IL-1 β and TNF- α while enhancing the expression of TGF- β , a well-known anti-inflammatory cytokine. These findings provide a solid basis for further *in vitro* and *in vivo* studies to confirm the anti-inflammatory effects of different seaweed extract combinations. Future research will focus on *in vivo* trials using animal models, such as swine, to assess their impact on immunity, gut health, and performance.

CRediT authorship contribution statement

Turin Lauretta: Writing – review & editing, Supervision. **Vanosi Graziella:** Writing – review & editing, Visualization. **Frazzini Sara:** Writing – review & editing, Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation. **Rossi Luciana:** Writing – review & editing, Validation, Supervision, Resources, Conceptualization. **Hejna Monika:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Founding sources

This project has received partially funding from the European Union's Horizon 2020 Research and Innovation Programme under the Marie Skłodowska-Curie grant agreement No 847639 and from the Polish Ministry of Education and Science. Moreover, this project also received partially funding from the International Cooperation Office of the Polish Academy of Sciences, Poland, and the Accademia Nazionale dei Lincei (ANL), Roma, Italy.

Declaration of Generative AI and AI-assisted technologies in the writing process

The authors did not use any artificial intelligence assisted technologies in the writing process.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

The authors would like to thank Biotecnologie BT s.r.l. for their valuable support in performing the metabolomic analyses that

contributed to this study.

Data availability

The data presented in this study are not deposited in an official repository. Data are available within the article and from the corresponding author upon reasonable request

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