



Ulvan from *Ulva armoricana* (Chlorophyta) activates the PI3K/Akt signalling pathway via TLR4 to induce intestinal cytokine production



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ABSTRACT

The biological activities of water-soluble sulfated polysaccharides of green algae (ulvans) have been explored for use as bioactive molecules for the benefit of human and animal health. A purified ulvan fraction was prepared from the green algae *Ulva armoricana* harvested in the Brittany (France) and tested for its capacity to stimulate the immune response of the gut using an *in vitro* system of porcine intestinal epithelial (IPEC-1) cells. RT-qPCR and ELISA analyses showed a significant increase in the mRNA and protein expression of cytokines such as CCL20, IL8, and TNF α . Using human embryonic kidney (HEK) 293 reporter cell lines for pattern recognition receptors, ulvan was found to primarily stimulate TLR4. We also examined the effect of the ulvan fraction on different signalling pathways involved in the activation of cytokine gene expression. Western blot analyses of ulvan-treated HEK293-TLR4 cells showed an increase in the phosphorylation of Akt and the p65 subunit of nuclear factor- κ B. Inhibition of Akt phosphorylation with the specific inhibitor abrogated the ulvan-mediated enhancement of IL-8 secretion. The overall results showed that ulvan is an immunostimulatory compound by itself, and furthermore, it could be used to effectively complex and deliver TLR ligands to relevant immune cells in vaccination strategies.

1. Introduction

Marine algal-originated compounds exhibit various biological activities with relevant applications in areas spanning the food, cosmetic and pharmaceutical industries to microbiology and biotechnology [1,2]. The biologically active metabolites produced by marine algae are mainly peptides, polyunsaturated fatty acids, pigments, polyphenols and polysaccharides [3,4]. Highly complex sulfated polysaccharides are commonly found in the cell wall matrix of three major marine algae including green, brown and red algae, which range from 4% to 76% of the dry weight [5–7]. The sulfated polysaccharides extracted from green, brown and red algae are referred to as ulvans, fucoidans and carrageenans respectively [5–9]. Ulvans from green algae such as *Ulva rigida* and *Enteromorpha* sp. are mainly composed of sulfated rhamnose residues linked to uronic acids, resulting in a repeated disaccharide unit β -D-glucuronosyl-(1,4)- α -L-rhamnose 3-sulfate, called aldobiuonic acid [10]. *In vitro* and *in vivo* studies have shown that ulvans, similarly fucans and carrageenans, exhibit a wide range of biological activities such

as anticoagulant, antiviral, antibacterial, anti-tumoural, anti-proliferative and immuno-modulatory activities [11–14]. Detailed screenings of the functions of low molecular weight ulvans have demonstrated that their interaction with immune cells triggers cellular and molecular events and leads to the activation of the immune system or the control of the inflammation process [15,16]. Recent studies showed that ulvan is able to enhance phagocytosis and other macrophage functions, such as the production of reactive oxygen species (ROS) and nitric oxide (NO) and the secretion of pro-inflammatory cytokines such as tumour necrosis factor (TNF α), the interleukins IL-1, IL-6, IL-8, IL-12, and interferon (IFN) [17–20]. In addition, ulvans have been explored for their anti-inflammatory effects using a macrophage cell line activated by lipopolysaccharide (LPS) and showed a significant reduction in the LPS-triggered secretion of pro-inflammatory cytokines, including IL-1 β , IL-6 and TNF- α , and NO production [21,22]. These data highlight that low molecular weight ulvans possess immunomodulatory activities, but the underlying molecular mechanisms involved in the immune response regulation by the ulvan have not been clearly elucidated. Nevertheless,

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marine immunomodulatory carrageenan and fucoidan polysaccharides, like those derived from plants, fungi or yeast, are able to regulate the innate immune response directly by binding to Toll-like receptors, such as TLR4, expressed on target cells and inducing signalling pathways that lead to the activation of the transcription factor NF-kappa B [23–25].

For example, red algae λ -carrageenan stimulated mouse T cell cultures in a Toll-like receptor-4 (TLR4)-dependent manner, generating a T helper 1 (Th1)-patterned cytokine response. However, splenocytes prepared from TLR4-deficient mice still retained some ability to produce interferon- γ in response to λ -carrageenan, suggesting that Pattern Recognition Receptors (PRRs) other than TLR4 were also involved [26]. Carrageenan has also been used as an adjuvant and has generated high antigen-specific immune responses *via* the TLR4 pathway in mice vaccinated with the human papillomavirus E7 peptide [27]. Brown algae fucoidan induced macrophage activation through the membrane receptors TLR4, CD14, and scavenger receptor class A and through MAPK signalling pathways [28]. Fucoidan also has immunostimulatory and maturing effects on bone marrow-derived dendritic cells *via* a pathway involving the transcription factor NF-kB. However, the receptor involved has not been identified [29].

The potential of marine algae harvested on France's shores to modulate the immune response has rarely been investigated. Recently, and for the first time, an aqueous extract of marine sulfated polysaccharides (MSPs) prepared from the green macroalgae *Ulva armoricana* harvested in 2012 from the shores of northern Brittany was evaluated for its antibacterial and immunostimulatory activities. We showed that this MSP extract was able to inhibit the bacterial growth of pathogenic bacteria and stimulate the mRNA expression of immune response mediators such as IL1 α , IL1 β , L-6, IL-8, TNF α , TGF β and CCL20 using an *in vitro* system of the porcine intestinal epithelial cell line IPEC-1 [30].

In the present study, a new MSP batch was prepared from algae that were harvested in 2013 in the same area and used for the purification of a low molecular weight ulvan fraction. We attempted first to evaluate the immunostimulatory activity of this ulvan fraction in comparison with the MSP extract and second to elucidate the molecular mechanisms underlying this biological activity. Thus, we investigated whether this ulvan fraction was able to stimulate cytokine expression using an IPEC-1 *in vitro* model and evaluated its interaction with HEK293 cell lines expressing a panel of PRRs to identify the target receptor. We have also undertaken the identification of the signalling pathway involved in cytokine expression after TLR stimulation. Understanding the mechanism of the ulvan-mediated immunostimulatory activity is very important for the design of bioactive polysaccharides as health-improving molecules for future preventive/therapeutic strategies to enhance the immune response of the host.

2. Materials and methods

2.1. Ulvan fraction preparation

Green tide algae *Ulva* sp. were collected on the beach at Plestin les Grèves (Bretagne, France, 48°39'28"N 3°37'47"W) in June 2013, Algae

were then washed with tap fresh water, drained, and deep-frozen. The algae were thawed, wet ground, and both liquid and solid phases were separated as part of an industrial process. The liquid was fractionated by tangential filtration (Tami Industries, Nyons, France) and concentrated on a single effect concentrator (EVA 1000, Pignat, Genas, France). The filtrate was dried by freeze-drying (Christ alpha 1–4 LSC, Fisher Scientific, France), and the freeze-dried material was ground to a powder using a bead-mill (MiniMill, Philips, France) with two zirconium bowls and four zirconium beads per bowl.

The desalting process of the MSP extract and the ulvan fraction purification were performed as described before [30,31]. Biochemical analyses, protein and sugar composition as well as the molecular weight of the ulvan fraction were also given in these two references [30,31].

The contamination of different fractions with LPS was evaluated using a commercially available kit (*E*-toxate Kit, Sigma). No LPS was detected by this assay in either preparation.

2.2. IPEC-1 culture, differentiation and stimulation with ulvan

The IPEC-1 cell line is a non-transformed epithelial cell line derived from the small intestine of a newborn unsuckled piglet [32]. They were grown, differentiated and stimulated with either the crude extract or the purified ulvan fraction as previously described [30]. Briefly, the cells were cultured in DMEM/HAMF-12 supplemented with 5% foetal calf serum, 1% insulin-transferrin-selenium, 2 mM L-glutamine, 5 ng/mL epidermal growth factor, 50 U/mL penicillin and 50 mg/mL streptomycin. IPEC-1 cells were grown for 2 days to reach total confluence, after which the cells were allowed to differentiate for 12 days in culture medium containing 10^{-7} M dexamethasone and lacking foetal calf serum. Before performing the stimulation assay, the cytotoxic effects of the MSP extracts (0–10 mg/mL) or ulvan (0–1 mg/mL) were examined using trypan blue staining and microscopic observation/counting as described previously [30]. The cell proliferation was not affected at a concentration of 500 μ g/mL even after 72 h of treatment. Therefore, the IPEC-1 cells were seeded in triplicate onto 4.2 cm² cell culture inserts (pore size of 0.4 μ m) at a density of 3.5×10^5 cells per insert and differentiated as described above. After 14 days of cell culture, a concentration of 500 μ g/mL and two serial decimal dilutions (50 and 5 μ g/mL) of the MSP extract and the ulvan fraction were used to stimulate the epithelial cells for 4 h in a 37 °C-humidified incubator with 5% CO₂.

2.3. Cytokine expression analysis upon IPEC-1 stimulation

The IL-8, TNF α and CCL20 expression in differentiated IPEC-1 cells was investigated at both the mRNA and protein level using quantitative real-time PCR (qPCR) and ELISA, respectively. The RNA extraction, cDNA synthesis and qPCR were conducted as previously described [30]. Briefly, after 4 h of IPEC-1 stimulation, high-quality RNA was purified using a Nucleospin RNA-L kit and 1 μ g of total RNA was reverse-transcribed using MuMLV reverse transcriptase. After heat inactivation at 93 °C for 5 min, the generated cDNA was subjected to qPCR using the Mesa Green qPCR Mastermix Plus for SYBR Assay (Eurogentec, Angers, France). The primers used to amplify the cytokines and housekeeping genes are shown in Table 1. The qPCR data were analysed using the

Table 1

Characteristics of the primer pairs used for RT-PCR analysis. Primer sequences (5' → 3'), annealing temperature of primer pairs (°C), expected PCR fragment sizes (bp), and PubMed accession numbers for the genes used for constructing the primers. The housekeeping genes used in this study are underlined.

Primer name	Sense sequence	Antisense sequence	Ann. temp. (°C)	Length (bp)	Accession no.
<u>RPL-19</u>	AAC1CCCGTCAGCAGATCC	AGTACCCTTCGGCTTACCG	60	147	AF435591
<u>HMBS2</u>	AGGATGGGCAACTCTACCTG	GATGGTGGCCTGCATAGTCT	58	83	DQ845174
<u>HPRT-1</u>	GGACTTGAATCATGTTTGTG	CAGATGTTTCCAACTCAAC	60	91	DQ845175
<u>CCL-20</u>	GTCCTGGCTGCTTTGATGTC	CATTGGCGAGCTGCTGTGTG	66	146	NM_001024589
IL-8	TCCTGCTTCTGCGAGCTCTC	GGGTGGAAGGTGTGGAATG	62	100	NM_213867
TNF α	CCAATGGCAGAGTGGGTATG	TGAAGAGGACCTGGGAGTAG	62	116	\times 54859

2 $\Delta\Delta$ Ct method, where the amount of the target gene, normalized to an endogenous reference and relative to an experimental control, is given by $2^{-\Delta\Delta Ct}$ [30]. The results were expressed as the relative fold change (Fc) in comparison with untreated control cells. For protein analysis, differentiated IPEC-1 cells were incubated only with 500 μ g/mL ulvan overnight at 37 °C in a humidified incubator with 5% CO₂. Both the apical and basolateral supernatants were then removed, and the IL-8 and TNF α production was measured by ELISA using commercial kits (R & D Systems, Lille, France).

2.4. Identification of the putative ulvan receptor

Five different human embryonic kidney (HEK293) reporter cell lines were purchased from Invivogen (Invivogen, Toulouse, France), each with a different inserted construct for human TLR4, TLR5, TLR9, NOD1, or NOD2. These cell lines are commonly used to determine TLR or NOD receptor activation upon ligand stimulation by assessing the IL-8 production in comparison with HEK293-null cells. HEK293-TLR4 cells also express the MD-2 and CD14 co-receptors needed for TLR activation. The cells were cultured in DMEM (Life Technologies Europe B.V.) containing 10% heat-inactivated bovine foetal serum, 2 mM L-glutamine, 4.5 g/L D-glucose, penicillin/streptomycin (50 U/mL and 50 mg/mL, respectively) and Normocin (100 mg/mL). The cells were maintained in 75-cm² tissue culture flasks (Greiner-BioOne, Germany) in incubators at 37 °C with 5% CO₂. For receptor identification experiments, the cells were plated at a density of 1.5×10^6 cells/well in six-well culture plates (Becton Dickinson Labware, Le Pont De Claix, France), cultured for 48 h and then stimulated for 16 h with the ulvan fraction at a concentration of 500 μ g/mL. The resulting supernatants were used to quantify the production of IL-8 by ELISA (Peprotech, Neuilly-sur-Seine, France). As positive controls, the recommended agonists, ultra-pure LPS O111B4 (100 ng/mL) for TLR4, flagellin (100 ng/mL) for TLR5, CPG-ODN 2395 (10 μ g/mL) for TLR9, iE-DAP (1 μ g/mL) for NOD1 and MDP (1 μ g/mL) for NOD2, were used. All agonists were purchased from Invivogen except LPS O111B4, which was obtained from Sigma.

2.5. Western blot analysis of the signalling pathways

The Erk1/2 (extracellular signal-regulated kinases), p38 MAPK, and AMPK (AMP-activated protein kinase) MAPK signalling pathways as well as the PI3K/Akt1/2 (phosphatidylinositol 3-kinase) and NF- κ B pathways were investigated. For the receptor identification experiments, HEK-TLR4/MD-2 cells were plated at a density of 1.5×10^6 cells/well in six-well culture plates, cultured for 48 h and then stimulated with 500 μ g/mL ulvan fraction for 5, 10, 30 or 60 min, and the supernatants were harvested for IL-8 quantification by ELISA (Peprotech, USA). In addition, protein preparations and Western blot analyses were performed as previously described [33]. Briefly, the cells were disrupted using a lysis buffer containing protease and phosphatase inhibitors. Equal amounts of proteins were separated using SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked and incubated with the appropriate primary antibodies at a final dilution of 1:1000. After washing steps, the membranes were incubated for 2 h at room temperature with a horseradish peroxidase-conjugated secondary antibody (final dilution 1:10,000), and the proteins were detected by enhanced chemiluminescence (Western Lightning Plus-ECL, Perkin Elmer, Courtaboeuf, France) using a Syngene G:Box system (Ozyme, Saint-Quentin-en-Yvelines, France) with the GeneSnap software (Syngene UK, Cambridge, UK, release 7.09.17). The detected signals were quantified with the GeneTools software (Syngene UK, release 4.01.02), and the results were expressed as the signal intensity in arbitrary units after normalization as indicated in the figure legends.

2.6. Antibodies used in Western blot assays

Antibodies against p-Akt (Ser473), p-ERK1/2 (Thr202/Tyr204), p-p38 (Thr180/Tyr182) and p-NF- κ B p65 (Ser536) were purchased from Ozyme (Saint Quentin en Yvelines, France), while the p-AMPK (Thr172) antibody was obtained from Cell Signalling Technology, Netherlands. Monoclonal and polyclonal antibodies against total Akt, ERK1/2, p38, AMPK and NF- κ B were obtained from Tebu Bio (Le Perray-en-Yvelines, France) and Ozyme. All antibodies were used at a 1:1000 dilution in assays.

2.7. Inhibition of the Akt1/2 signalling pathway

For studying the involvement of Akt in cytokine expression, HEK293-TLR4 cells were pre-treated with LY294002, a pharmacological inhibitor of phosphatidylinositol 3-kinase (PI3K) (Sigma). The solution was prepared as 1000 \times concentrated stocks in dimethyl sulfoxide (DMSO) in order to ensure that the final concentration of DMSO in the culture medium did not exceed 0.1%. 1 h before stimulation, the HEK293-TLR4 cells were treated with the LY294002 inhibitor at a final concentration of 50 μ M to block the signalling pathway during 4 and 16 h of stimulation with ulvan. After the incubation step, the media were collected, and the concentration of IL-8 was measured by ELISA using a commercial kit (R & D) according to the manufacturer's instructions. The control group was cultured in DMEM with DMSO. We also examined whether the inhibition of the PI3K signalling pathway by LY294002 has any cytotoxic effects on HEK293-TLR4 cells. The cells were treated with either 50 μ M LY294002, DMSO or DMEM, and the cell viability was assessed using trypan blue staining and microscopic observation/counting as described previously [30].

2.8. Statistical analysis

The data for the comparison of differences in mRNA expression and protein production between treated and untreated cells were expressed as relative values. The data are expressed as the mean value of triplicates \pm SEM. Because the data are independent and non-normally distributed, they were analysed using the Kruskal-Wallis test followed by Bonferroni-Dunn post-test group comparison tests of means using GraphPad (GraphPad Prism version 4.00 for Windows; GraphPad Software, San Diego, CA, USA). The statistical differences between the various treatments were considered significant either when the *P*-values were < 0.05 (*) or < 0.01 (**).

3. Results

3.1. Composition of the ulvan fraction

The chemical composition of the crude extract and the purified ulvan fraction was determined by elemental and proximate analysis, monomeric sugar analysis and molecular weight. Table 2 shows that the purification step removed all salts and minerals to reduce the ash content from 32.9 to 2.9%. As shown by the proximate analysis, this procedure also enhanced the organic matter content to 92.5%, which was composed mainly of carbohydrate and proteins. The sugars composition analysis of the MSP extract yielded meaningless and unusable results, with low levels of sugars detected with high standard error values, which correlated to the low reproducibility of the method in the presence of a high content of salts. After an ultra-filtration step, which was aimed at removing salts from the sample, the deduced organic matter was 89.6%, of which 72.4% was carbohydrate, mostly neutral sugars and uronic acids. The yield of the ulvan fraction is calculated and showed a value of 20.5%.

The composition of the sugar residues was determined with a low standard deviation and could account for 70.9% of the dry weight, slightly lower than indicated by the proximate analysis. The deduced

Table 2

Composition of the low molecular weight aqueous MSP extract and the purified ulvan fraction used in this study. The analysis of the composition of sugar units was carried out directly on the purified ulvan fraction. n.d. means not determined.

Composition (%)	MSP extract	Ulvan fraction
Elemental		
C	21.14 ± 0.06	28.80 ± 0.07
H	4.73 ± 0.13	5.80 ± 0.003
N	2.29 ± 0.01	2.00 ± 0.10
O	33.1 ± 0.10	52.38 ± 0.09
S	4.33 ± 0.04	5.12 ± 0.01
Proximate (on DM basis)		
Ash	32.9 ± 0.1	2.9 ± 0.1
Fat	< 0.1	< 0.1
Proteins	10.2 ± 1.0	8.9 ± 0.3
Neutral sugars	11.7 ± 1.8	40.2 ± 1.4
Uronic acids	7.4 ± 2.4	32.2 ± 2.5
Sulfate groups	4.0 ± 1.0	8.3 ± 0.6
Monomeric sugars		
Arabinose	n.d.	n.d.
Galactose	n.d.	0.9 ± 0.09
Glucose	n.d.	1.8 ± 0.81
Xylose	n.d.	2.75 ± 0.08
Mannose	n.d.	1.46 ± 0.02
Rhamnose	n.d.	39.55 ± 1.56
Glucuronic acid	n.d.	24.4 ± 1.01

final composition of the ulvan fraction consisted of 39.55% rhamnose, 32.2% uronic acid, 24.4% glucuronic acid, 2.75% xylose and 8.3% sulfate. The weight-average (M_w) and the number-average (M_n) molecular weight were estimated as 3.2×10^3 and 2.9×10^3 Da, respectively, and the polydispersity index was calculated to be 1.1.

3.2. Ulvan stimulated the mRNA expression of IL-8, TNF- α and CCL20 in IPEC-1 cells

Before investigating the stimulation effect of both extracts, the cytotoxic effects were examined by incubating IPEC-1 cells with increasing amounts of MSP extract (Fig. 1A) and ulvan (Fig. 1B), and the cell viability was determined using a trypan blue exclusion test. There was no significant sign of cytotoxicity and the cell proliferation was not affected at 0.5 mg/mL. Therefore, the capacity of the ulvan fraction to stimulate cytokine expression was first investigated at the mRNA level in comparison with the MSP crude extract after the exposure of differentiated IPEC-1 cells to concentrations of 500, 50 and 5 μ g/mL for 4 h. The relative quantification of mRNA, expressed as a fold change, showed that the 500 μ g/mL concentration of the MSP extract was able to significantly increase ($P < 0.01$) the relative expression of CCL20 (74.9), IL-8 ($\times 85.4$) and TNF- α ($\times 29.9$). The purification step did not

hamper the ulvan biological activity, and the activation of cytokine expression in IPEC-1 cells was retained. In fact, the mRNA expression analysis showed that the purified ulvan fraction increased the expression of CCL20 ($\times 61.5$), IL-8 ($\times 79.7$) and TNF- α ($\times 36.4$) to a similar extent as the MSP extract (Fig. 2A and B).

3.3. Ulvan induced the secretion of the IL-8 and TNF- α proteins in differentiated IPEC-1 cells

Having shown that differentiated IPEC-1 cells were able to express cytokines at the mRNA level in response to the purified ulvan fraction, we sought to confirm and extend this result by ELISA analysis. IPEC-1 cells were cultured on transwell filters and stimulated overnight by adding ulvan at a concentration of 500 μ g/mL, and the supernatants of the apical and basolateral compartments were analysed separately. As seen in Fig. 3, the ulvan fraction was able to stimulate the secretion of both the IL-8 and TNF α proteins into the collected supernatants, but at different levels depending on the compartment. Significantly increased amounts of IL-8 ($P < 0.01$) were detected in both the apical (5446 ± 308 pg/mL) and basolateral (1980 ± 190 pg/mL) compartments compared to untreated controls (Fig. 3). Ulvan treatment also stimulated TNF- α release from IPEC-1 cells in both compartments but at much lower level than IL-8 (125.5 ± 18.5 pg/mL versus 5446 ± 308 pg/mL and 7.8 ± 1.2 pg/mL versus 1980 ± 190 pg/mL). For the following experiments, only the ulvan fraction was evaluated.

3.4. Ulvan induced IL-8 protein expression in HEK293-TLR4 cells

To identify the receptor involved, the ulvan fraction was tested at the concentration of 500 μ g/mL in human embryonic kidney (HEK)293 cell lines stably expressing the receptors TLR4, TLR5, TLR9, NOD1 and NOD2. The ulvan/receptor interaction was evaluated by monitoring IL-8 release into the supernatant by ELISA. Our results showed that the purified ulvan fraction was able to activate TLR4 and induced the up-regulation of IL-8 production in the HEK293/TLR4-MD-2-CD14 cell line compared to HEK293-null cells. This fraction did not significantly activate any of the remaining receptors (Fig. 4).

3.5. Ulvan interaction with TLR4 mediated the activation of the PI3K/Akt and NF- κ B signalling pathway

We next examined the effect of the purified ulvan fraction on the activation of different protein kinases that are involved in cytokine gene expression. Thus, HEK293-TLR4 cells were incubated with ulvan (500 μ g/mL) for different lengths of time (5, 10, 30 and 60 min), and the phosphorylation status of MAPKs (ERK1/2 and p38), Akt, AMPK

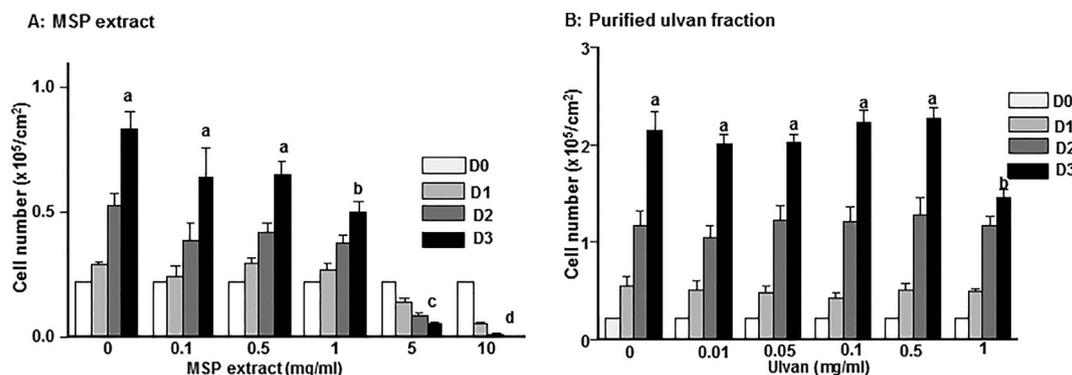


Fig. 1. IPEC-1 cells were treated with various concentrations of MSP extract (0–10 mg/mL) (A) or purified ulvan fraction (0–1 mg/mL) (B), and cell viability was assessed after 3 days (D0, D1, D2 and D3) using trypan blue staining and microscopic observation/counting. The data are expressed as the mean value \pm S.E.M. of triplicates. The results are compared with the untreated control after 3 days (D3) of incubation. Different letters indicate statistically significant differences (a, b, c and d) with $P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

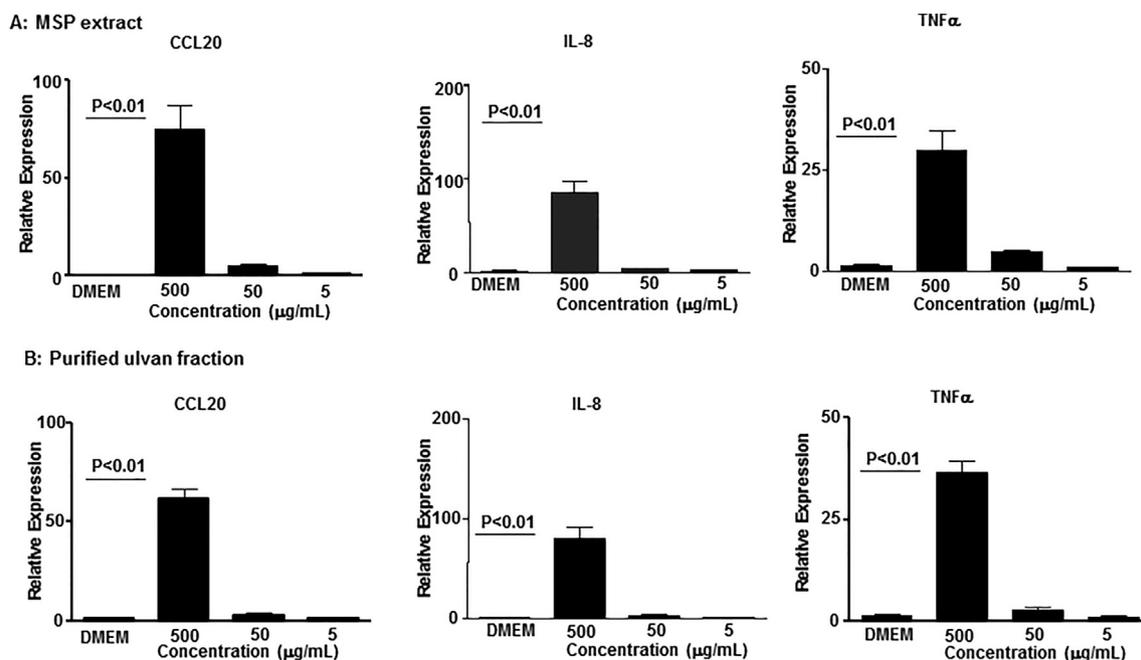


Fig. 2. Stimulation of the expression of IL-8, TNF α , and CCL20 in differentiated porcine epithelial (IPEC-1) cells using the MSP extract (A) and the purified ulvan fraction (B). The IPEC-1 cells were treated for 4 h with different concentrations (500, 50 and 5 μ g/mL) of the extracts, and the expression of target genes was determined as the fold change relative to controls. The data are expressed as the mean value \pm S.E.M. of triplicates, and the results were considered statistically significant at P -value < 0.01 ($P < 0.01$).

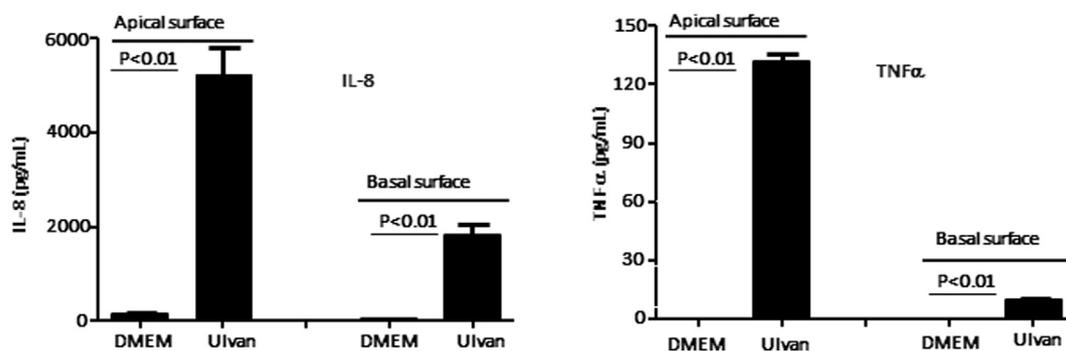


Fig. 3. Purified ulvan stimulated differentiated IPEC-1 cells to produce the TNF α and IL-8 proteins. IPEC-1 cells were incubated with 500 μ g/mL ulvan overnight at 37 $^{\circ}$ C, and the apical and basolateral supernatants were harvested separately and used for protein analysis using commercial ELISA kits. The results were considered statistically significant for P -values below 0.01 ($P < 0.01$).

and NF- κ B was evaluated using phospho-specific antibodies. Western blot results and a kinetic curve analysis of pAkt/Akt showed that the interaction of the ulvan fraction with TLR4 in HEK293 cells resulted in a significant increase in Akt phosphorylation that was detectable after a 5 min incubation compared to untreated cells, similar to the LPS-treated cells (Fig. 5A and B). The phosphorylation status analysis of MAPKs (Erk1/2 and p38) and AMP-activated kinase did not indicate any differences in the activation of these signalling pathways between different treatments (data not shown). Akt regulates the transcriptional activity of nuclear factor- κ B (NF- κ B) by inducing the phosphorylation and subsequent degradation of inhibitor of κ B (I κ B). We therefore analysed the phosphorylation status of p65 in HEK293-TLR4 cells and, as shown in Fig. 6 A and B, we observed that the ulvan fraction significantly increased the levels of phosphorylated p65.

3.6. Inhibition of the PI3K/Akt signalling pathway using the inhibitor LY294002

We first examined whether the pharmacological inhibitor LY294002 has any cytotoxic effects on the proliferation of HEK293-TLR4 cells. The treatment of cells with either LY294002 or DMSO did not affect the cell

viability in comparison with DMEM alone even after 16 h of incubation (Fig. 7A). The involvement of the PI3K/Akt signalling pathway was then verified using the inhibitor LY294002. The inhibitor, used at a final concentration of 50 μ M for 16 h, was able to block this signalling pathway and induce a > 65% decrease in IL-8 secretion (Fig. 7B). Similar results were obtained in the cells treated with LPS, while LY294002 did not affect IL-8 secretion in untreated cells (Fig. 7B).

4. Discussion

Green algae have emerged in recent years as a rich and important source of bioactive natural compounds that could be used as a new generation of growth enhancers and natural antibiotic alternatives to potentiate the immune function and limit the infections of farm animals and therefore improve animal health [34,35]. However, further research is needed to identify the bioactive components, mechanisms of action, and *in vivo* biological effects to ultimately adapt the findings for agronomic use. In this context, we recently prepared an aqueous marine sulfated polysaccharides (MSP) crude extract from the green macroalgae *Ulva armoricana* and showed its capacity to stimulate the mRNA expression of a broad range of cytokines and chemokines using an *in*

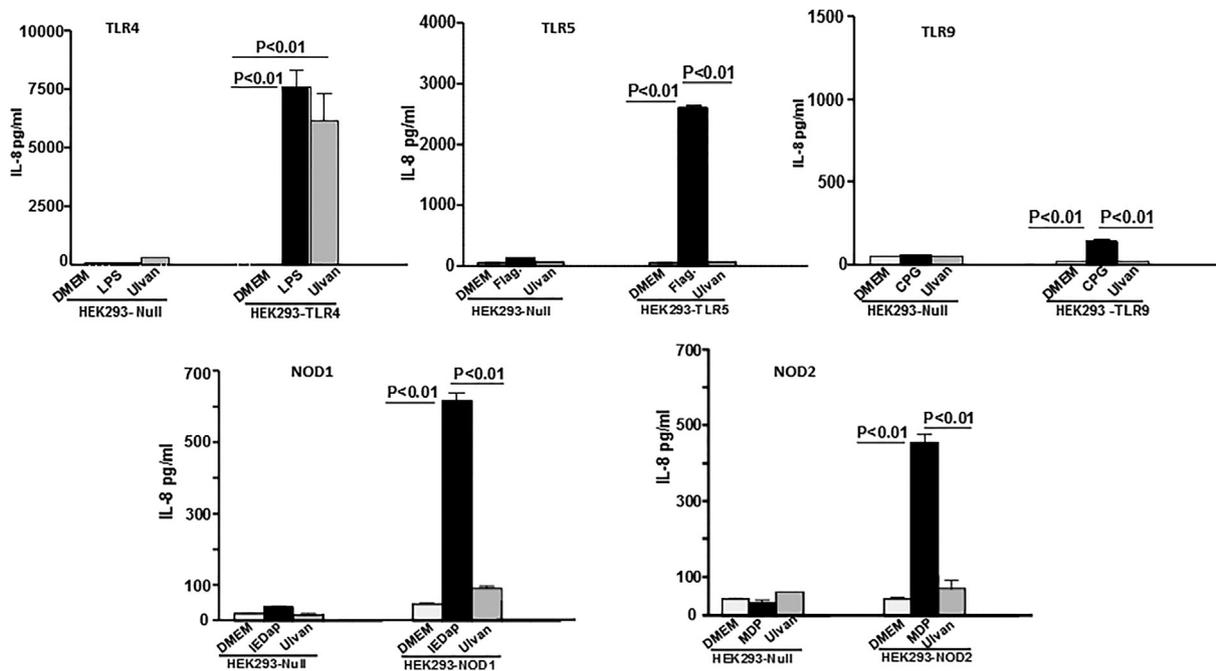


Fig. 4. Purified ulvan stimulates only HEK293-TLR4 cells to produce the IL-8 protein. HEK293-TLR4, TLR5, TLR9, NOD1 and NOD2 cells were plated at a density of 1.5×10^6 cells/well in six-well culture plates and stimulated for 16 h either with the ulvan fraction at a concentration of 500 μ g/mL or the recommended agonists as positive controls. The agonists ultra-pure LPS O111B4 (100 ng/mL) for TLR4, flagellin (100 ng/mL) for TLR5, CPG-ODN 2395 (10 μ g/mL) for TLR9, iE-DAP (1 μ g/mL) for NOD1 and MDP (1 μ g/mL) for NOD2 were used. The data are expressed as the mean value \pm S.E.M. of triplicates, and the IL-8 production was considered statistically significant for P -values below 0.01 ($P < 0.01$).

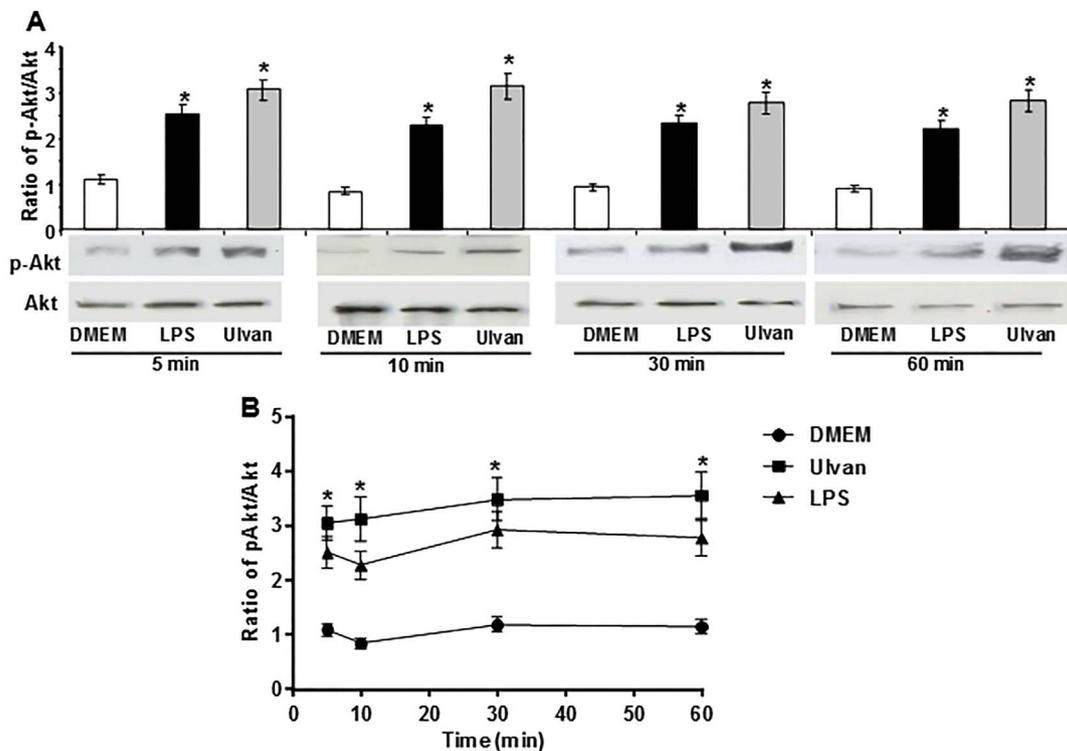


Fig. 5. Ulvan fraction induced the phosphorylation of Akt in HEK293 cells expressing TLR4. HEK293-TLR4 cells were stimulated with 500 μ g/mL of ulvan fraction for 5, 10, 30 or 60 min, and the cell lysates were analysed for the expression of both Akt and phospho-Akt (p-Akt). Western blot (A) and kinetic curve results of pAkt/Akt (B) are expressed as the mean value \pm S.E.M. of triplicates, and $*P < 0.05$ indicates significant differences between the treatments and the DMEM control.

in vitro system of the porcine intestinal epithelial cell line IPEC-1 [30]. The chemical composition analysis indicated that the main component of the extract is ulvan. This component is regarded as the main candidate for the immunostimulatory activity, although further purification of the sample is needed for final confirmation. In the current study, we prepared a new MSP batch from algae that were harvested in the same area

during the summer of 2013, and an ulvan fraction was purified to evaluate its immunomodulation potential in comparison with the crude MSP extract. The composition analysis of this new MSP batch showed similar components to those of the MSP extract that was prepared and tested previously [30]. The purification process allowed us to obtain an ulvan fraction with a high content of organic matter (up to 89.6%), of

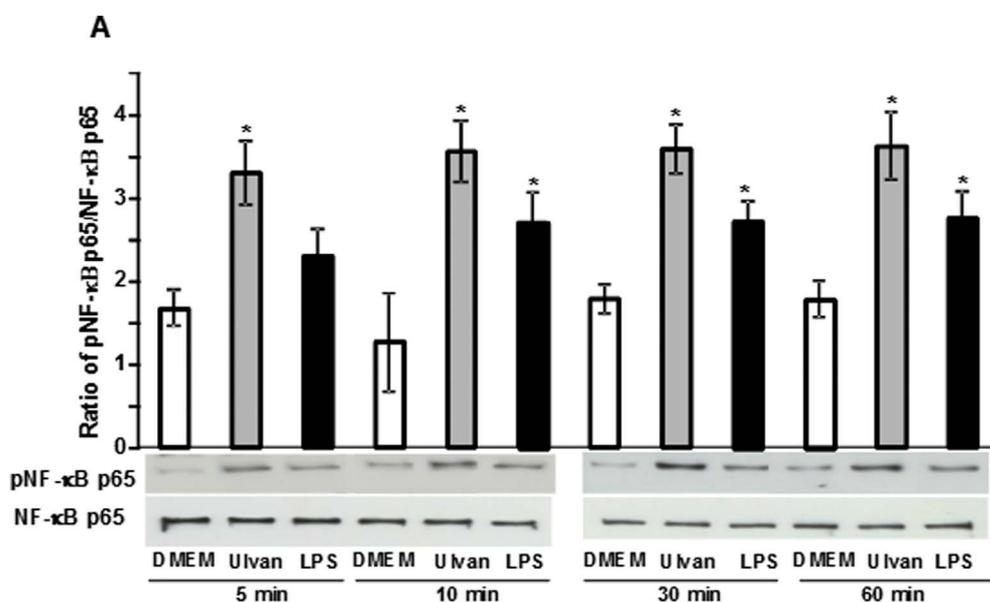


Fig. 6. Ulvan fraction activated the NF-κB signalling pathway in HEK293 cells expressing TLR4. Western blot analysis (A) and kinetic curve results (B) of extracts from untreated HEK293-TLR4 cells (DMEM) or stimulated with ulvan or LPS for different periods of time (5, 10, 30 or 60 min) using an anti-phospho-NF-κB p65 (Ser536) antibody. The data are expressed as the mean value ± S.E.M. of triplicates, and **P* < 0.05 indicates significant differences between the treatments and the DMEM control.

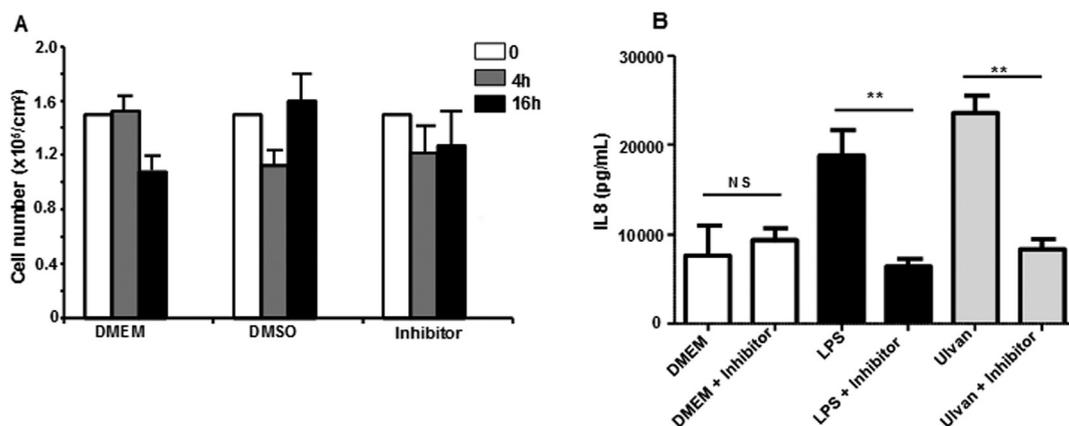
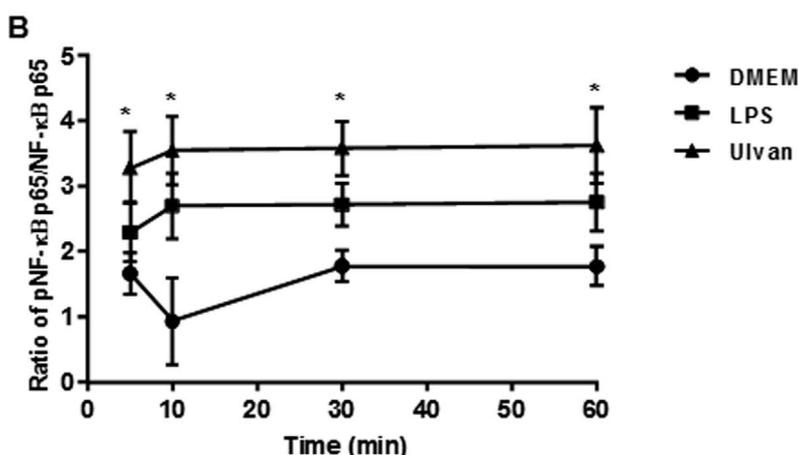


Fig. 7. LY294002, a pharmacological inhibitor of the PI3K/Akt signalling pathway, abrogates the production of IL-8 induced by the ulvan fraction in HEK293-TLR4 cells without affecting the cell viability. (A) HEK293-TLR4 cells were treated with either 50 μM LY294002, DMSO or DMEM, and the cell viability was determined by trypan blue staining. (B) HEK293-TLR4 cells were pre-treated with LY294002 before stimulation with the purified ulvan fraction (500 μg/mL) or LPS as a positive control. The IL-8 concentration and cell number/cm² are expressed as the mean value ± S.E.M. of triplicates. ***P* < 0.01 indicates significant differences between different treatments. NS: not significant. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

which 72.4% was carbohydrate, primarily neutral sugars and uronic acids. The deduced final composition of the ulvan fraction was 39.6% rhamnose, 32.2% uronic acid, 24.4% glucuronic acid, 2.8% xylose, 8.3% sulfate and 8.9% protein, which represent the typical main

constituents found in green algae ulvan. In addition, an elemental analysis also revealed the presence of proteins, which is in agreement with the fact that sulfated polysaccharides are closely associated with proteins within the cell wall structure of green algae as previously

described [10,36].

The treatment of differentiated IPEC-1 cells with this new MSP batch as well as the purified ulvan fraction induced the upregulation of CCL20, TNF α and IL-8 mRNA. In addition, the purified ulvan fraction was able to stimulate cytokine secretion in both the basal and apical compartments as analysed by ELISA. These results are similar to those obtained previously, showing that the extraction process is suitable to prepare MSP extracts with reproducible analytical compositions and immunostimulatory activities [30]. Moreover, the purification step was particularly useful for improving the immunostimulatory activity, which could be attributed to the ulvan fraction. The stimulation of the intestinal cytokines production of the MSP extract as well as the ulvan fraction could not be attributed to endotoxin contamination, because no endotoxin compounds were detected within the crude and fractionated polysaccharides by an *E-Toxate* kit analysis (data not shown). This investigation is of practical significance for determining the optimal extraction processing in order to produce natural bioactive molecules with an effective immunostimulatory activity to be used in a livestock diet to improve the immune response of animals and thereby enhance their resistance against infectious diseases.

Studies evaluating the immunological properties of algae-derived polysaccharides were mainly carried out using macrophage cell lines such as murine RAW264.7, and rarely tested on intestinal epithelial cells. Like fucoidans and carageenans, ulvans were tested directly on macrophages cell line or after challenging them with LPS to test the immunostimulatory or the anti-inflammatory responses respectively [15,16]. Although macrophages are essential effector cells of innate immunity, intestinal epithelial cells are also of interest since they express pattern-recognition receptors (PRRs) that enable them to act as dynamic sensors of microbial environment and foreign antigens. Thus, differentiated IPEC-1 cells used in our study is a relevant and suitable *in vitro* model that will allow to test and evaluate the effect of dietary bioactive compounds intake such as marine sulfated polysaccharides to stimulate the intestinal immune response. It has become clear that intestinal epithelial cells are crucial mediators of intestinal homeostasis, reinforcing the barrier function and participating in the coordination of appropriate mucosal immune responses by producing a broad range of cytokines involved in the activation, trafficking and function of adjacent immune cells [37–39]. Because of their ability to recognize specific carbohydrate moieties and elicit immune responses, we hypothesized that PRRs, and more specifically TLRs and NODs, are activated by the ulvan fraction which leads, through signalling cascades, to the stimulation of cytokine production. Thus, we used an HEK cell model to target a large panel of TLR and NOD receptors and showed that the ulvan fraction was able to activate immune signalling in HEK cells expressing TLR4. This implies that ulvan can act directly on target cells, including intestinal or immune cells, *via* TLR4 and differentially affect the expression of immunological parameters. We also analysed the activation of signalling pathways, and showed that the interaction of ulvan with TLR4 mediated the activation of the PI3K/Akt signalling pathway, which regulates the transcriptional activity of NF- κ B. The involvement of the PI3K/Akt pathway was confirmed using the specific pharmacological inhibitor LY294002. In a previous report, normal human colonic epithelial cell line NCM460 were exposed to sulfated polygalactose carrageenan (CGN) purified from red algae and showed a stimulation of IL-8 production. Similarly to our study, this CGN was able to recognize TLR4 receptor and induce B cell lymphoma-NF κ B activation to mediate cytokine production [40].

The activation of the PI3K/Akt pathway has been reported to be associated with TLR2, TLR3, TLR4, and TLR5 in different cells [41,42] and plays both pro-inflammatory and anti-inflammatory roles in TLR signalling [43,44]. Thus, this ulvan fraction may play dual roles and exhibit immunomodulatory activities that may be of potential application either in stimulating the immune response or in controlling inflammation, as was reported for other algae-derived polysaccharide extracts [15,21].

Overall, these *in vitro* data provide novel insights into the molecular mechanism used by marine sulfated polysaccharides to stimulate the production of soluble cytokines and chemokines and suggest that this signalling can be achieved upon the interaction with TLR4, inducing the activation of the PI3K/Akt and NF- κ B pathway. Although these *in vitro* results help to explain the molecular mechanisms used by ulvan to stimulate the expression of cytokines, we cannot rule out the involvement of other receptors and alternative signalling pathways through a mechanism that is likely more complex at the intestine surface. Natural TLR ligands (TLRLs) and their mimetics are increasingly being applied in immunotherapeutic strategies. As TLRLs are potent inducers of innate immune responses, they have been applied as adjuvants to stimulate adaptive immune responses [45]. The orientation of the pharmaceutical industry towards naturally derived polymers as multifunctional materials in drug delivery applications has become a subject of increasing interest, driving the continuous exploitation of such compounds [46]. Algal sulfated polysaccharides have recently been used in applications in the production of nanoparticles and microparticles, mainly owing to their ionic nature [47]. Thus, ligands for TLR can be encapsulated within biodegradable nanoparticles of ulvan to be delivered to relevant target immune cells in vaccination strategies. However, further studies are needed using *ex vivo* and *in vivo* models to confirm the activity of marine sulfated polysaccharides on the stimulation of the immune response under physiological conditions and during infectious or inflammatory processes.

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Author's contributions

M.B. conceived, coordinated the study and wrote the manuscript, M.O. performed all the experiments of IPEC-1 cells stimulation and RT-qPCR, S.H. performed the experiments related to the identification of the ulvan receptor, J.D. performed the western blot analysis and signalling pathways identification, H.D. and M.L. participated to the experiments design, P.N.C. participated to the study design and supervised the MSP and the ulvan preparation.

All the authors read and approved the manuscript.

Competing financial interests

The authors declare no competing financial interests.

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

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