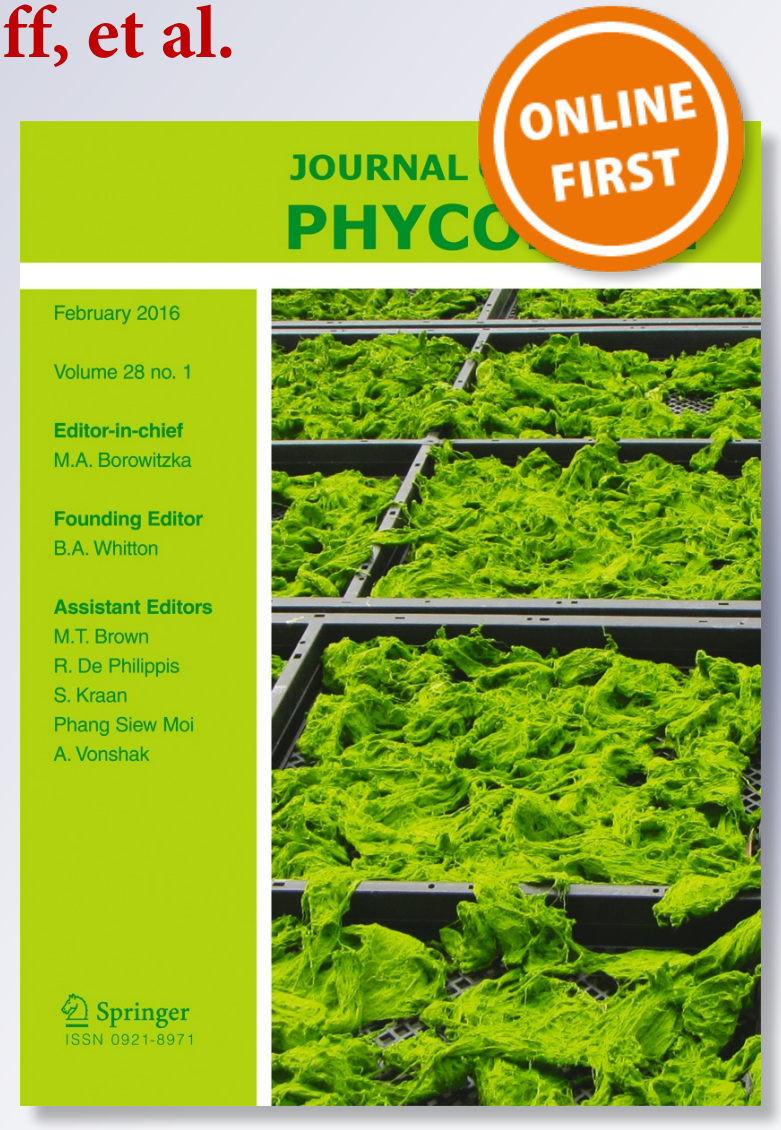
Marine-sulfated polysaccharides extract of Ulva armoricana *green algae exhibits an antimicrobial activity and stimulates cytokine expression by intestinal epithelial cells*

Mustapha Berri, Cindy Slugocki, Michel Olivier, Emmanuelle Helloin, Isabelle Jacques, Henri Salmon, Hervé Demais, Matthieu Le Goff, et al.

Journal of Applied Phycology

ISSN 0921-8971

J Appl Phycol
DOI 10.1007/s10811-016-0822-7



Springer

Your article is protected by copyright and all rights are held exclusively by Springer Science +Business Media Dordrecht. This e-offprint is for personal use only and shall not be self-archived in electronic repositories. If you wish to self-archive your article, please use the accepted manuscript version for posting on your own website. You may further deposit the accepted manuscript version in any repository, provided it is only made publicly available 12 months after official publication or later and provided acknowledgement is given to the original source of publication and a link is inserted to the published article on Springer's website. The link must be accompanied by the following text: "The final publication is available at link.springer.com".

Author's personal copy

J Appl Phycol
DOI 10.1007/s10811-016-0822-7

Marine-sulfated polysaccharides extract of *Ulva armoricana* green algae exhibits an antimicrobial activity and stimulates cytokine expression by intestinal epithelial cells

Mustapha Berri[1,2] · **Cindy Slugocki**[1,2] · **Michel Olivier**[1,2] · **Emmanuelle Helloin**[1,2] · **Isabelle Jacques**[1,2] · **Henri Salmon**[1,2] · **Hervé Demais**[3] · **Matthieu Le Goff**[4] · **Pi Nyvall Collen**[4]

Received: 8 January 2016 / Revised and accepted: 25 February 2016
© Springer Science+Business Media Dordrecht 2016

Abstract An aqueous marine-sulfated polysaccharide (MSP) extract, prepared from the green macroalga *Ulva armoricana*, was tested as an antibacterial compound against 42 bacterial strains and isolates found in livestock animals. Both Gram-positive and Gram-negative bacteria growths were affected. The most susceptible pathogens were *Pasteurella multocida*, *Mannheimia haemolytica*, *Erysipelothrix rhusiopathiae*, *Staphylococcus aureus*, and *Streptococcus suis*, with a minimum inhibitory concentration (MIC) ranging from 0.16 to 6.25 mg mL^{-1}. *Enterococcus cecorum*, *Streptococcus dysgalactiae*, *Corynebacterium*, *Trueperella pyogenes*, and *Bordetella bronchiseptica* strains were also susceptive to MSP with a complete inhibition recorded at MIC values of 25 and 50 mg mL^{-1}. The stimulation of the immune response mediators of the host's gut with the extract was evaluated using an in vitro system of differentiated porcine intestinal epithelial cells (IPEC-1). RT-qPCR analysis showed a significant increase of mRNA expression of cytokines such as IL1α, IL1β, L6, IL8, TNFα, and TGFβ as well as the chemokine CCL20. The extract also significantly induced the expression of PPARγ, a ligand-activated transcription factor, and TLR2 receptor.

Keywords Green algae · *Ulva armoricana* · Sulfated polysaccharides · Antibacterial activity · Intestinal epithelial cells · Cytokines

✉ Mustapha Berri
mberri@tours.inra.fr

[1] INRA, UMR 1282 Infectiologie et Santé Publique, 37380 Nouzilly, France

[2] UMR 1282 Infectiologie et Santé Publique, Université de Tours, 37000 Tours, France

[3] Biovet Conseil, 1 Route du Linès, 56700 Merlevenez, France

[4] Amadéite SAS "Pôle biotechnologique" du Haut du Bois, 56580 Bréhan, France

Introduction

Indiscriminate and excessive use of antimicrobial agents for decades as growth promoters in farm animal feed has created a continuous selective pressure. This promoted the development of resistant strains and resulted in a complete ban on the use of antimicrobial growth promoters in the European Union. Alternative prophylactic strategies able to stimulate the innate immune response and to limit farm animals' infection are needed. Marine algae are the fastest growing plant organisms in nature and have the ability to produce biologically active compounds which may find applications in industries such as pharmaceuticals, cosmetics, food, and animal feed (O'Sullivan et al. 2010; Jiménez-Escrig et al. 2011; Lee et al. 2013). Nutritional studies on marine algae indicated that green, brown, and red seaweeds possess good nutritional characteristics and could be used as an alternative source of dietary fiber, protein, vitamins, and minerals (Chojnacka et al. 2012; Raposo et al. 2013). In addition, detailed screening of micro- and macroalgae functions revealed new ranges of biological activities including anticoagulant, antiviral, antibacterial, antitumor, antiproliferative, and immunomodulatory activities. All of them could be of relevance in nutraceutical functional food (Wijesekara et al. 2011; Lee et al. 2013; Fedorov et al. 2013). The biologically active compounds of algae include proteins, polyunsaturated fatty acids, pigments, polyphenols, minerals, vitamins, and polysaccharides (Ngoa et al. 2011; Faulkner 2002). Indeed, green, brown, and red algae cell walls contain large amounts of sulfated polysaccharides named ulvan, fucan, and carrageenan respectively, and ranging

Published online: 05 March 2016

Author's personal copy

from 4 up to 76 % of dry weight (Holdt et al. 2011; Wijesinghe et al. 2011; Ferreira et al. 2012). The high content of these sulfated polysaccharides, their unusual structure, and their biological properties shed a new light on these compounds as promising natural products for medicinal and dietary applications (Rioux et al. 2007; Laurienzo et al. 2010). Ulvans represent the major water-soluble ramified acidic and sulfated polysaccharides found in green seaweed of the order Ulvales (*Ulva* and *Enteromorpha* spp.) with sulfate, rhamnose, xylose, iduronic, and glucuronic acids as the main constituents (Lahaye et al. 2007). In vitro and in vivo studies have shown that ulvans, like fucans and carrageenans, exhibited a wide range of biological activities such as antibacterial and immunomodulatory properties (Wijesekara et al. 2011; Silva et al. 2012; Fedorov et al. 2013). Promising antiprotozoal, antimycobacterial, and antibacterial activities were recently found in ulvans containing crude extracts isolated from several green algae collected from Turkey (Orhan et al. 2006) and from British freshwaters (Abd El-Baky et al. 2008; Spavieri et al. 2010). Moreover, ulvans purified from green algae collected mainly from Korea also seem to display immunomodulatory activities that might be of potential application in stimulating the immune response or in controlling the inflammatory process (Chen et al. 2008; Na et al. 2010; Kim et al. 2011; Karnjanapratum et al. 2012; Tabarsa et al. 2012). Studies evaluating the immunological properties of algae-derived polysaccharides were mainly carried out using primary culture of macrophages like cells lines (Chen et al. 2008; Jaswir et al. 2011). However, 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. They are therefore active participants in coordinating the mucosal immunity by producing a broad range of mediators involved in adjacent immune cells activation (Artis et al. 2008; Miron et al. 2012; Peterson et al. 2014).

The potential antimicrobial activities of marine algae harvested in the Brittany shores (France) have not been examined in depth (Hellio et al. 2000; 2001; 2004; Zubia et al. 2009), and their ability to modulate the immune response has never been investigated. The aim of this study was to assess the antibacterial activity of a low molecular weight aqueous extract from *Ulva armoricana* harvested from the shores of Brittany (France) on a large panel of 42 strains of pathogens isolated from infected farm animals. Moreover, the stimulation of the host's intestinal immune response mediators was evaluated using an in vitro system of intestinal epithelial cell line IPEC-1, spontaneously immortalized from the porcine intestine (Ganzalez-Vallina et al. 1996). Used as a feed additive, this marine-sulfated polysaccharide (MSP) extract may constitute a new prophylactic strategy to increase intestinal health of livestock animals, and thereby reducing the subsequent use of antibiotic treatment.

Materials and methods

Algae source and MSP extract preparation

Green tide algae *Ulva* sp. was collected on the beach at Plestin les Grèves (Bretagne, France; 48° 39′ 28″ N, 3° 37′ 47″ W) in June 2012. The algae were washed in 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.

Composition analysis

The extract composition was examined in triplicate using the following methods. Ash values were determined gravimetrically after incineration of samples at 550 °C for 6 h. The percentage elemental analysis (C, H, N, S, and O) was kindly performed by the Central Microanalysis Department of the CNRS (Gif-sur-Yvette, France) using elementar Analysensysteme GmbH-vario EL III Element Analyzer (Hanau, Germany) according to the manufacturer recommendations. This analysis allowed evaluation of carbon, nitrogen, sulfur, hydrogen, and oxygen in all organic and the majority of inorganic substances. Briefly, milligram amounts of samples are combusted (C, H, N, and S) or pyrolysed (O) at high temperature in a helium carrier gas. The measurable gases (CO_2, H_2O, N_2, SO_2, or CO) are separated on a chromatography column. The combustion products are measured quantitatively against known reference standards by means of a non-dispersive IR absorption detection system, except for the oxygen which is determined via a thermal conductivity detector (TC). Neutral sugars were determined using the phenol sulfuric acid method with anhydrous D-glucose used as standard (0–100 μg mL^{-1}) (DuBois et al. 1956). Uronic acids were determined using the meta-hydroxydiphenyl method (Blumenkrantz et al. 1973). The amount of sulfate groups present on the polysaccharides were determined by the Azure A method (Sigma-Aldrich, France) using 17 % of sulfated dextran as standard in the range of 0 to 100 μg mL^{-1} (Jaques et al. 1968). The molar ratio of monosaccharides was determined after acidic methanolysis of the MSP sample using 3 M L^{-1} of MeOH/HCl for 4 h at 100 °C, followed by gas chromatography (GC) analysis of the trimethylsilyl derivatives using the method of Montreuil et al. (1986). The myo-inositol (1 mg mL^{-1}) was used as the internal reference, nitrogen as a carrier gas and a non-polar column, type HP-5MS

Author's personal copy

(30 m, 0.25 mm internal diameter) for analysis. The temperature profile was programmed as follows: 120 °C for 1 min, followed by a gradient of 1.5 °C min^{-1} between 120 and 180 °C and 2 °C min^{-1} between 180 and 200 °C. Because of the high content of salt in the sample interfering with the reproducibility of the analyses, salts were eliminated by ultrafiltration using a 1-kDa cutoff Pall minimate membrane (Pall Corporation, France) and freeze-dried. Protein content was quantified by the bicinchinonic acid colorimetric method (BCA) using the Micro BC Assay Kit (Interchim, France) (Smith et al. 1985). The molecular weight distribution of the sample (0.5 mg mL^{-1}) was analyzed by size exclusion chromatography in 0.1 M sodium nitrate with 0.2 % sodium azide at a flow rate of 0.5 mL min^{-1}. The Shodex 802 and 803 columns in series and a multi-angle light scattering refractometer (Wyatt, 18 angles) for detection were used with a dn/dc of 0.150 mL g^{-1}.

Determination of antibacterial activity

Bacterial strains and culture conditions The antimicrobial activity of the MSP extract was investigated against 42 Gram-positive and Gram-negative bacterial strains. Thirty nine of them were isolated from clinical material of infected farm animals between 2007 and 2014, and deposited at the collection of the International Center for Microbial Resources dedicated to Pathogenic Bacteria (CIRM-BP, INRA-UMR ISP1282, Nouzilly, France). *Pasteurella multocida* strains subsp. *gallicida* and subsp. *multocida* (n° 3 and 4) were obtained from the University of Göteborg culture collection, while *Listeria monocytogenes* EGDe was kindly provided by Philippe Velge (INRA Val de Loire, Nouzilly, France). Table 1 indicates different bacterial strains and isolates, their CIRM-BP reference number, and their origin of isolation. The non-fastidious strains able to grow without special nutritional supplements such as *Escherichia coli*, *Salmonella*, and *Staphylococcus* strains as well as *Serratia marcescens* and *Klebsiella pneumoniae* were precultured overnight at 37 °C in brain-heart infusion (BHI) medium, and then respective colonies were isolated on BHI agar plates or on blood agar for *Staphylococcus* strains. The remaining strains considered as slow-growth and fastidious bacteria were precultured overnight at 37 °C in BHI medium containing 5 % of Gibco horse serum (Thermo Fisher Scientific, France) and then colonies were isolated on blood agar plates.

Preparation of inoculum Individual 24-h colonies of each strain were suspended in sterile physiological saline solution (sodium chloride 0.85 %), and the bacterial suspension density was adjusted to equal that of the 0.5 McFarland standard. Standardization of each microorganism suspension was carried out using a spectrophotometer at 620 nm to match 0.5 on the McFarland scale, equivalent to 1×10^8 cfu mL^{-1}, followed by 1/10 dilution to a final concentration of 10^7 cfu mL^{-1}. The standardized inoculum was adjusted so that 10^4 cfu per spot are tested.

Antibacterial activity assay The antimicrobial activity of the MSP extract was determined using a Denley multipoint inoculator (Denley, England) which is less technically time-consuming than other assays, and allowed to test a total of 20 strains on the same agar plate. The MSP extract was firstly dissolved in ultra-pure water and sterilized by autoclaving. Twofold serial dilutions of MSP were made to obtain final concentrations ranging from 50 to 0.04 mg mL^{-1} in MH or MH with 5 % of horse serum (for fastidious bacteria) agar plates. Then bacterial suspension containing 10^4 cfu (2–5 μL of standardized inoculum) was inoculated onto the surface of the agar plates containing increased concentration of MSP. Gentamicin was used as a positive control at a final concentrations ranging from 10 to 0.08 mg mL^{-1}. The agar plates were incubated 24 h at 37 °C, and bacterial sensitivity to MSP was recorded. The minimal inhibitory concentration (MIC) value was determined as the lowest MSP concentration required to totally inhibit visible growth of tested microorganism after 24 h of incubation. Three replicates were made for each microorganism.

IPEC-1 cell culture

IPEC-1 cell culture and differentiation The non-transformed porcine intestinal epithelial cell line IPEC-1 was derived from the small intestine of a newborn unsuckled piglet (Ganzalez-Vallina et al. 1996), and grown as previously described (Zanello et al. 2011). Briefly, cells were cultured in DMEM/HAMF-12 medium (Invitrogen, France) supplemented with 5 % fetal calf serum, 1 % insulin-transferrin-selenium (Sigma-Aldrich, France), 2 mM L-glutamine, 5 ng mL^{-1} epidermal growth factor 50 U mL^{-1} penicillin and 50 mg mL^{-1} streptomycin (all from Invitrogen, France). IPEC-1 cells were grown for 2 days, a predetermined period that allowed reaching 100 % of confluence, and then differentiated for 10 days. For differentiation culture, the medium described above was modified with the omission of fetal calf serum and the addition of 10^{-7} M dexamethasone (Sigma-Aldrich, France). Differentiation culture medium was changed every 2 days, and once differentiated, IPEC-1 cells had an average cell density of 4×10^5 cells per well. IPEC-1 cells differentiation was evaluated using both transepithelial electrical resistance (TEER) measurement and immunohistochemistry as described previously (Zanello et al. 2011).

IPEC-1 cell stimulation For IPEC-1 stimulation, the cells were seeded in triplicate onto 4.2 cm^2 cell culture inserts (pore size of 0.4 μm, Becton Dickinson Labware, France) at 3.5×10^5 cells per insert, cultured in DMEM/HAMF-12

Author's personal copy

Table 1 Bacterial strains and isolates tested in this study, their CIRM-BP reference number, and the origin of isolation

Number	Strains	Strain reference	Origin
1	*Pasteurella multocida*	CIRMBP 969	Swine, lungs
2	*Pasteurella multocida* subsp. *septica*	CIRMBP 873	Rabbit, lungs
3	*Pasteurella multocida* subsp. *gallicida*	(CCUG 26985)	Cattle
4	*Pasteurella multocida* subsp. *multocida*	(CCUG 26985)	Swine
5	*Manheimia haemolytica*	CIRMBP 970	Goat, lungs
6	*Manheimia haemolytica*	CIRMBP 947	Bovin, uterus
7	*Erysipelothrix rhusiopathiae*	CIRMBP 973	Duck, intra cardiac blood
8	*Staphylococcus aureus*	CIRMBP 476	Cattle, milk
9	*Staphylococcus aureus*	CIRMBP 978	Cattle, milk
10	*Staphylococcus aureus*	CIRMBP 979	Chicken, articulations
11	*Staphylococcus aureus*	CIRMBP 980	Duck, yolk
12	*Staphylococcus chromogenes*	CIRMBP 552	Cattle, milk
13	*Streptococcus suis*	CIRMBP 985	Swine, brain
14	*Streptococcus uberis*	CIRMBP 984	Cattle, milk
15	*Streptococcus uberis*	CIRMBP 637	Cattle, milk
16	*Streptococcus dysgalactiae*	CIRMBP 982	Cattle, milk
17	*Streptococcus dysgalactiae*	CIRMBP 948	Cattle, uterus
18	*Streptococcus dysgalactiae*	CIRMBP 619	Cattle, milk
19	*Streptococcus dysgalactiae*	CIRMBP 635	Cattle, milk
20	*Enteroccocus cecorum*	CIRMBP 981	Duck, bone marrow
21	*Corynebacterium bovis*	CIRMBP 975	Cattle, milk
22	*Listeria monocytogenes*	EGDe	Rabbit, Tissue
23	*Listeria monocytogenes*	CIRMBP 976	Cattle, brain
24	*Listeria monocytogenes*	CIRMBP 974	Goat, brain
25	*Trueperella pyogenes*	CIRMBP 971	Swine, lungs
26	*Bordetella bronchiseptica*	CIRMBP 972	Swine, lungs
27	*Klebsiella pneumoniae*	CIRMBP 990	Cattle, milk
28	*Serratia marcescens*	CIRMBP 986	Cattle, milk
29	*Salmonella ser.* Enteritidis	CIRMBP 962	Chickens environment
30	*Salmonella ser.* Typhimurium	CIRMBP 963	Chickens environment
31	*Salmonella ser.* Hadar	CIRMBP 987	Chickens environment
32	*Salmonella ser.* Infantis	CIRMBP 988	Chickens environment
33	*Salmonella ser.* Virchow	CIRMBP 989	Chickens environment
34	*Escherichia coli* O2	CIRMBP 960	Duck, bone marrow
35	*Escherichia coli* O1	CIRMBP 961	Chicken, intra cardiac blood
36	*Escherichia coli* CS31A	CIRMBP 964	Cattle, feces
37	*Escherichia coli* K99	CIRMBP 965	Cattle, feces
38	*Escherichia coli* K85	CIRMBP 966	Swine, digestive contents
39	*Escherichia coli* F17	CIRMBP 967	Cattle, feces
40	*Escherichia coli* K88 (F4)	CIRMBP 968	Swine, feces
41	*Escherichia coli* O78K80	CIRMBP 959	Chicken, bone marrow
42	*Escherichia coli* O78K80	CIRMBP 949	Cattle, uterus

medium until 100 % of confluence, and then allowed to differentiate as described earlier. Cytotoxic effects of MSP extract were examined by incubating IPEC-1 cells with increasing amount of MSP (0.1, 0.5, 1, 5, and 10 mg mL^{-1}) for 72 h, and cell viability was determined by using trypan blue exclusion test. There is no significant sign of cytotoxicity and the cell proliferation was not affected at 1 mg mL^{-1} of MSP (data not shown). Therefore, after a period of 14 days of cell culture, the concentration of 1 mg mL^{-1} and two serial decimal dilutions (1, 0.1, and 0.01 mg mL^{-1}) were used to stimulate the epithelial cells for 4 h in a 37 °C humidified incubator with 5 % CO2. As controls, the cells were also treated in the same

Author's personal copy

conditions with 100 ng mL^{-1} of LPS from *E. coli* 0111:B4 (Sigma-Aldrich, France) or culture medium.

RNA preparation and RT-qPCR analysis

RNA purification and cDNA first strand synthesis After 4 h of stimulation, IPEC-1 cells were washed three times with sterile PBS, and total RNA was purified using the Nucleospin RNA-L kit (Macherey–Nagel, Germany) according to the manufacturer's protocol, and RNA concentration was determined by optical density at 260 nm (OD_{260}). To minimize sample variations, we used high quality RNA which was assessed by calculating OD_{260}/OD_{280} using a NanoDrop spectrophotometer analysis (NanoDrop Technologies, USA) and also by capillary electrophoresis (Agilent 2100 Bioanalyzer, Agilent Technologies, USA). One microgram of total RNA was reverse-transcribed for 90 min at 37 °C in a 20-μL volume containing 0.25 mM dNTP, 2.5 μM of oligodT, 25 U μL^{-1} of MuMLV reverse transcriptase in its buffer (Eurogentec, Liege, Belgium). After a heat inactivation at 93 °C for 5 min, generated cDNA was stored at −80 °C until use.

Real-time PCR assays Real time assays (qPCR) were run on a Bio-Rad Chromo 4 (Bio-Rad, USA) using the Mesa Green qPCR Mastermix Plus for SYBR Assay (Eurogentec, Belgium) as previously described (Zanello et al. 2011; Meurens et al. 2007). Briefly, primers of various genes involved in the immune response and the three housekeeping genes were designed, their annealing temperature calculated using the Clone Manager 9 software (Scientific & Educational Software, USA), and purchased from Eurogentec (Liège, Belgium). The nucleotide sequences (5′ to 3′) of the forward and reverse primers, the annealing temperature and the amplicon sizes are described in Table 2. The thermal cycle conditions for SYBR Green assay were 95 °C for 5 min, followed by 38 cycles with a denaturation step at 95 °C for 15 s, and annealing/elongation for 45 s. Each reaction was performed in duplicate from three replicates of cell culture and repeated two times. The quantitative RT-PCR data were analyzed using the $2^{\Delta\Delta Ct}$ method, where the amount of target, normalized to an endogenous references and relative to an experimental control, is given by $2^{-\Delta\Delta Ct}$ (Livak et al. 2001). For all qPCR assays, the expression level of target genes were normalized internally using simultaneously the average cycle quantification (Cq) of hydroxymethylbilane synthase 2 (HMBS2), hypoxanthine phosphoribosyltransferase-1 (HPRT-1), and ribosomal protein L-19 (RPL-19) using a standard curve method. These three reference genes were selected for their stable expression in IPEC-1 cell line as described previously (Bruel et al. 2010). The results were expressed as relative fold change (Fc) in comparison with untreated control cells.

Statistical analysis

The data for the comparison of difference in relative messenger RNA (mRNA) expression between treated and untreated IPEC-1 cells were expressed as relative values. Data are expressed as the mean value ± S.E.M. of triplicate assays. Because the data are independent and not normally distributed, they were analyzed using the Kruskal-Wallis test followed by Bonferroni-Dunn post-test group comparison tests of means using the GraphPad (GraphPad Prism version 4.00 for Windows; GraphPad Software, USA). Statistical differences between the various treatments were considered significant when P values are <0.01.

Results

Chemical composition analysis of the MSP extract

The composition analysis of the algal fraction before and after demineralization step is described in detail in the "Materials and methods" section. This chemical composition is performed by elemental analysis, proximate analysis, unitary sugars analysis, and molecular weight are reported in Table 3. The initial MSP extract contained 30.6 % of ash corresponding to mineral matter and 69.4 % of deduced organic matter, composed mainly of carbohydrate and proteins. Only 50 % of the deduced organic matter was identified by the proximate analysis. Sugars composition analysis of the MSP extract yielded inexploitable results with low levels of sugars detected and with high standard error values, correlated to the low reproducibility of the method in presence of high content of salts. After ultra-filtration, aiming at removing salts from the sample, the deduced organic matter was 93.4 %, and the majority was identified as organic matter by the proximate analysis (82.5 %) whereof 58.1 % as carbohydrate. The composition of the sugar residues was determined with low standard deviation and could account for 45 % of the dry weight, slightly lower than the proximate analysis. The main residues observed were rhamnose, xylose, and glucuronic acids which are all involved in the composition of ulvan and represented about 99 % of the sugars identified in the sample. The occurrence of sulfate observed both by colorimetric assay and elemental analyses suggested the occurrence of a sulfated component such as ulvan. Therefore, it is likely that the sugar content estimated in MSP prior ultrafiltration could be attributed essentially to ulvan. Based on the measured neutral sugars, uronic acid residues, and sulfate groups, ulvan represented about 27 % of the dry matter. After desalting the MSP extract, the fraction of carbohydrate increased from about 24 (neutral and uronic residues) to 58.1 % with little effect on the relative ratios in the elemental composition and the protein content constant at about 7.5 %. This suggests that the high

Author's personal copy

Table 2 Primer sequences (5′→3′), annealing temperature of primers set (°C), expected PCR fragment sizes (bp), and accession numbers of the PubMed database

Primer name	Sense sequence	Antisens sequence	Ann. temp (°C)	Length (bp)	Accession no.
RPL-19	AACTCCCGTCAGCAGATCC	AGTACCCTTCCGCTTACCG	60	147	AF435591
HMBS2	AGGATGGGCAACTCTACCTG	GATGGTGGCCTGCATAGTCT	58	83	/
HPRT-1	GGACTTGAATCATGTTTGTG	CAGATGTTTCCAAACTCAAC	60	91	/
CCL20	GCTCCTGGCTGCTTTGATGTC	CATTGGCGAGCTGCTGTGTG	66	146	NM_001024589
CCL25	GCCTACCACAGCCACATTAAG	GCTTCCCGCACACCATCTT	64	136	NM_001025214
CCL28	GCTGCTGCACTGAGGTTTC	TGAGGGCTGACACAGATTC	62	144	NM_001024695
IL-1α	CCCGTCAGGTCAATACCTC	GCAACACGGGTTCGTCTTC	60	170	NM_214029
IL-1β	AGAAGAGCCCATCGTCCTTG	GAGAGCCTTCAGCTCATGTG	62	139	NM_001005149
IL-6	ATCAGGAGACCTGCTTGATG	TGGTGGCTTTGTCTGGATTC	62	177	NM_214399
IL-8	TCCTGCTTTCTGCAGCTCTC	GGGTGGAAAGGTGTGGAATG	62	100	NM_213867
IL-10	ACCAGATGGGCGACTTGTTG	TCTCTGCCTTCGGCATTACG	65	123	NM_214041
PPARγ	AAGACGGGGTCCTCATCTCC	CGCCAGGTCGCTGTCATCT	62	149	/
TGFβ	GAAGCGCATCGAGGCCATTC	GGCTCCGGTTCGACACTTTC	64	162	NM_214015
TNFα	CCAATGGCAGAGTGGGTATG	TGAAGAGGACCTGGGAGTAG	62	116	X54859
TLR2	ACGGACTGTGGTGCATGAAG	GGACACGAAAGCGTCATAGC	62	101	NM_213761
TLR4	TGTGCGTGTGAACACCAGAC	AGGTGGCGTTCCTGAAACTC	62	136	NM_001113039

The housekeeping genes used in this study are set in italics

Table 3 Composition of the low molecular weight aqueous MSP extract used in this study for biological activities

Composition	Crude extract	Demineralised extract
Elemental (%)		
C	20.58 ± 0.03	27.00 ± 0.08
H	4.44 ± 0.02	5.85 ± 0.01
N	1.62 ± 0.11	0.96 ± 0.05
O	39.06 ± 0.10	54.48 ± 0.05
S	3.69 ± 0.03	5.15 ± 0.10
Proximate (%)		
Ash	30.6 ± 0.1	6.6 ± 0.1
Neutral sugars	11.6 ± 1.6	30.6 ± 5.2
Proteins	7.3 ± 1.1	7.5 ± 1.3
Uronic acids	12.2 ± 3.5	28.5 ± 5.2
Sulfate groups	3.6 ± 1.5	16.4 ± 3.4
Unitary sugars (%)		
Arabinose	n.d.	0.17 ± 0.04
Galactose	n.d.	0.6 ± 0.3
Glucose	n.d.	0.05 ± 0.0008
Xylose	n.d.	1.69 ± 0.05
Manose	n.d.	0.17 ± 0.03
Rhamnose	n.d.	30.9 ± 3.8
Glucuronic acid	n.d.	11.3 ± 1.6
Molecular weight		
Mw (Da)	n.d.	4.4×10^3
Mn (Da)	n.d.	3.4×10^3
Ip (Mw/Mn)	n.d.	1.4

n.d. not determined

concentration of salts affect the proximate analysis and specifically those dependent on hydrolysis before colorimetric analysis.

Antibacterial activity of MSP and MIC determination

In the present investigation, the antimicrobial activity spectrum of the MSP and the MIC values against pathogenic strains were determined using multispot inoculation assay. The MIC values used to determine the bacteria susceptibility to MSP are shown in Table 4. The results indicated that the MSP incorporation into a nutrient agar medium exhibited selective inhibition of tested microorganisms' growth with MICs values varying from 0.156 to 50 mg mL^{-1}. Both Gram-positive and Gram-negative bacterial strains were inhibited by the bioactive MSP. This extract displayed the most important spectrum of activity against 12 tested bacterial strains with very low recorded MIC values ranging from 0.156 to 6.25 mg mL^{-1}. *Pasteurella multocida*, *Staphylococcus aureus*, *Mannheimia haemolytica*, *Erysipelothrix rhusiopathiae*, and *Streptococcus suis* strains were the most sensitive pathogens. However, antimicrobial activity of MSP was different between species within the same genus of bacteria. Indeed, *Streptococcus chromogenes*, *Streptococcus uberis*, and *Streptococcus dysgalactiae* were less sensitive to the MSP activity compared to *Streptococcus suis*. The obtained results showed also that *Enterococcus cecorum* strain was susceptive to the MSP extract and its growth was completely inhibited at MIC value of 25 mg mL^{-1}. Finally, we found that MSP exhibited a limited antibacterial activity against

Author's personal copy

Table 4 Marine-sulfated polysaccharides extract were active against all tested bacteria. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of MSP preparation (mg mL^{-1}) inhibiting visible growth of bacteria for 24 h. The bacteria were classified according to their sensitivity to the MSP effect. Data are expressed as the mean value ± S.E.M. of triplicate

Number	Strains	MIC (mg mL^{-1})
2	*Pasteurella multocida* subsp. *septica*	0.156
3	*Pasteurella multocida* subsp. *gallicida*	0.391
5	*Manheimia haemolytica*	0.391
1	*Pasteurella multocida*	1.56
4	*Pasteurella multocida* subsp. *multocida*	3.125
6	*Manheimia haemolytica*	3.125
7	*Erysipelothrix rhusiopathiae*	3.125
8	*Staphylococcus aureus*	3.125
9	*Staphylococcus aureus*	3.125
10	*Staphylococcus aureus*	3.125
11	*Staphylococcus aureus*	3.125
13	*Streptococcus suis*	6.25
20	*Enteroccocus cecorum*	25
12	*Staphylococcus chromogenes*	50
16	*Streptococcus dysgalactiae*	50
17	*Streptococcus dysgalactiae*	50
18	*Streptococcus dysgalactiae*	50
19	*Streptococcus dysgalactiae*	50
21	*Corynebacterium bovis*	50
25	*Trueperella pyogenes*	50
26	*Bordetella bronchiseptica*	50
22	*Listeria monocytogenes*	>50
23	*Listeria monocytogenes*	>50
24	*Listeria monocytogenes*	>50
14	*Streptococcus uberis*	>50
15	*Streptococcus uberis*	>50
27	*Klebsiella pneumoniae*	>50
28	*Serratia marcescens*	>50
29	*Salmonella ser.* Enteritidis	>50
30	*Salmonella ser.* Typhimurium	>50
31	*Salmonella ser.* Hadar	>50
32	*Salmonella ser.* Infantis	>50
33	*Salmonella ser.* Virchow	>50
34	*Escherichia coli* O2	>50
35	*Escherichia coli* O1	>50
36	*Escherichia coli* CS31A	>50
37	*Escherichia coli* K99	>50
38	*Escherichia coli* K85	>50
39	*Escherichia coli* F17	>50
40	*Escherichia coli* K88 (F4)	>50
41	*Escherichia coli* O78K80	>50
42	*Escherichia coli* O78K80	>50

Corynebacterium bovis, *Trueperella pyogenes*, and all tested *E. coli*, *Salmonella*, and *Listeria* strains as well as *Klebsiella pneumoniae* and *Serratia marcecsens* (Table 4).

MSP stimulated the expression of the immune response mediators by IPEC-1 cells

The immunomodulatory capacity of MSP was investigated using differentiated porcine epithelial cells IPEC-1 incubated with 1, 0.1, and 0.01 mg mL^{-1} concentrations. The relative quantitation of mRNA expression of a panel of immune response mediators was analyzed by quantitative reverse transcription PCR (RT-qPCR), and the results were expressed as a fold change of expression levels. Figure 1 shows that MSP was able to increase significantly ($P<0.01$) the relative expression of cytokines and chemokines mRNA such as IL-8 (Fc = 313.53 ± 47.5), CCL20 (Fc = 159.44 ± 42.5), TNFα (Fc = 82.63 ± 15.8), and IL-1α (Fc = 22.96 ± 3.2) compared to the control at the concentration of 1 mg mL^{-1}. Furthermore, MSP increased significantly ($P<0.01$) the relative expression of IL-6 (Fc = 30.58 ± 7), IL-1β (Fc = 4.92 ± 1.6), and TGFβ (Fc = 4.83 ± 0.7 Fc) mRNA (Table 5). Toll-like receptor TLR2 (Fc = 11.29 ± 1.7) and peroxisome proliferator-activated receptor (PPARγ) mRNA (Fc = 3.71 ± 0.8) were also significantly ($P<0.01$) upregulated when the MSP extract was added to differentiated IPEC-1 cells. However, MSP did not produce any significant change on the relative gene expression of IL-10 and IL-12 cytokines mRNA, CCL25, and CCL28 chemokines as well as TLR4 receptor (Table 5). Therefore, lipopolysaccharide (LPS) did not induce the expression of any tested genes in IPEC-1 cells compared to the MSP-treated cells.

Discussion

The data obtained in this study revealed that MSP exhibited selective inhibition of tested microorganisms' growth with MICs values varying from 0.156 to 50 mg mL^{-1}. Both Gram-positive and Gram-negative bacteria were inhibited by bioactive MSP. *Pasteurella multocida*, *Staphylococcus aureus*, *Mannheimia haemolytica*, *Erysipelothrix rhusiopathiae*, *Streptococcus suis*, and *Enterococcus cecorum* strains were the most sensitive pathogens to the MSP exposure. These pathogens are known to cause illnesses like mastitis, reproduction disorders, pneumonia, and diarrhea leading to high livestock mortality and considerable economic loss (Economou et al. 2015). Therefore, MSP is a potential source of bioactive compounds and the obtained results brings a new insight toward the development of natural antimicrobial agents to be used against infectious pathogens in farm animal breeding. Nevertheless, the exact mechanism of this antibacterial activity is still unknown, and further studies are needed to clarify the MSP action.

The biological activity of MSP was also investigated by testing its capacity to stimulate the host immune response using an in vitro system of porcine intestinal epithelial cells (IPEC-1), mimicking an oral distribution of the extract. The

Author's personal copy

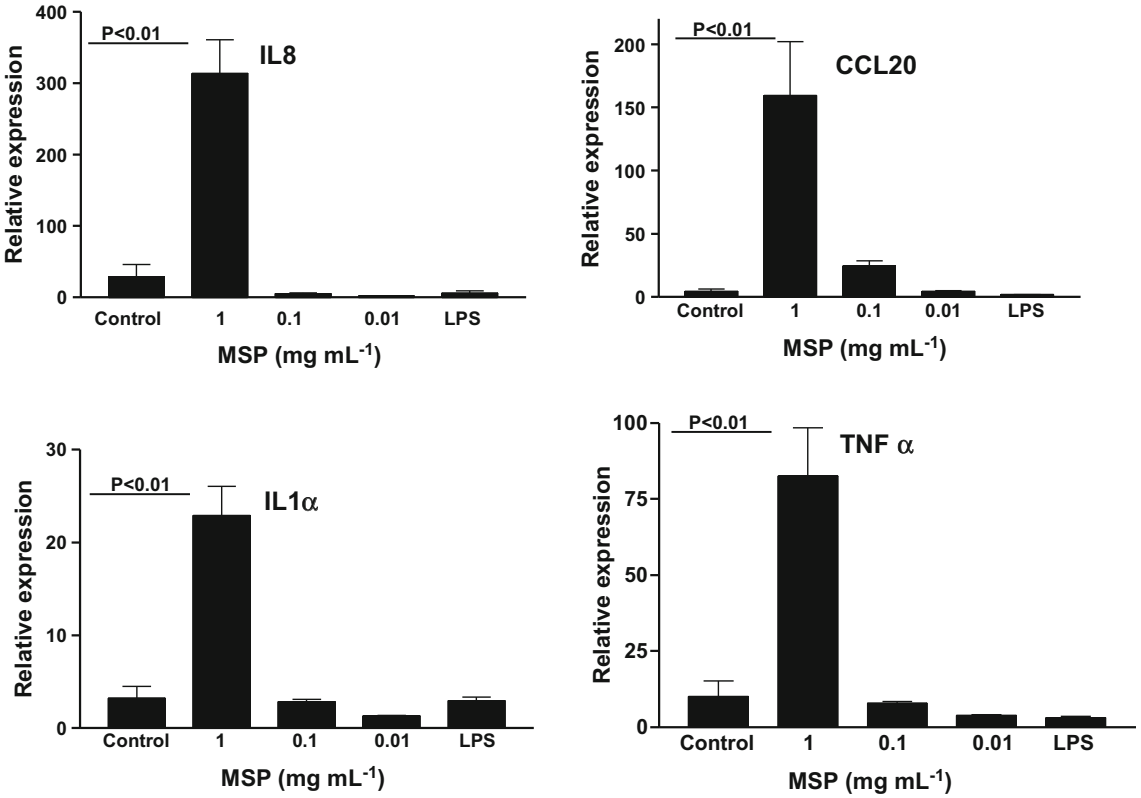


Fig. 1 Stimulation of the IL8, IL1α, TNFα, and CCL20 expression by differentiated porcine epithelial cells IPEC-1 using marine-sulfated polysaccharide (*MSP*) extract. IPEC-1 cells were treated for 4 h with different concentrations of MSP (1, 0.1, and 0.01 mg mL^{-1}), and the expression of targeted genes was defined as fold change relative controls. Data are expressed as the mean value ± S.E.M. of triplicate

Table 5 Relative fold change (Fc) ratio of targeted immune mediator between polarized IPEC-1 cells treated with 1 mg mL^{-1} of MSP and untreated control cells. Data are expressed as the mean value ± S.E.M. of triplicate assays

Genes	Fc	Significance
TNFα	82.63 ± 15.79	$P < 0.01$
IL1α	22.96 ± 3.16	$P < 0.01$
IL8	313.53 ± 47.54	$P < 0.01$
CCL20	159.44 ± 42.52	$P < 0.01$
IL6	30.58 ± 7.03	$P < 0.01$
IL1β	4.92 ± 1.63	$P < 0.01$
IL12p40	3.88 ± 0.66	$P < 0.01$
TGFβ	4.83 ± 0.66	$P < 0.01$
PPARγ	3.71 ± 0.78	$P < 0.01$
IL12p35	4.19 ± 0.63	NS
IL10	2.54 ± 0.61	NS
CCL25	6.20 ± 2.47	NS
CCL28	3.24 ± 1.11	NS
TLR2	11.29 ± 1.66	$P < 0.01$
TLR4	3.24 ± 0.54	NS

$P < 0.01$, statistical significance level

NS not significant

treatment of polarized IPEC-1 cells with MSP induced an upregulation of broad range of cytokines mRNA such as IL-1α, IL-1β, IL-6, IL-8, and TNFα. These results suggested that MSP might be able to activate intestinal epithelial cells to produce cell-mediated immune response cytokines that initiate and amplify protective immune responses of the host, and regulated mucosal immunity against intestinal pathogens (Stadnyk et al. 2002; Oswald et al. 2006; Leppkes et al. 2014). Interestingly, CCL20 (also known as MIP3α) chemokine was also highly upregulated by MSP. The CCR6/CCL20 axis is known to be involved in the recruitment of dendritic cells, B and T lymphocytes, and regulating several aspects of intestinal immunity under both physiological and inflammatory conditions (Williams 2006; Ito et al. 2011). MSP induced also a significant increase of mRNA of TGFβ suggesting that this extract might be effective to participate to IgA humoral immune response and to antiinflammatory process regulation within the gut epithelial tissue (Takenoshita et al. 2002; Walia et al. 2003; Stavnezer et al. 2009). MSP enhanced also mRNA expression of PPARγ, a transcription factors involved in the immune response through its ability to inhibit the expression of inflammatory cytokines and to direct the differentiation of immune cells toward antiinflammatory phenotypes (Tyagi et al. 2011). Similarly, in vitro and in vivo previous evaluations have shown that algae-derived polysaccharides extract played

Author's personal copy

two-edged roles and exhibited immunomodulatory activities that may be of potential application either in stimulating the immune response or in controlling the inflammation (Chen et al. 2008; Vo et al. 2012).

The overall antimicrobial and immunomodulation activities assessed from the above in vitro results indicate the presence of active compounds in MSP extract which can be exploited for the production of natural bioactive molecules to improve farm animal resistance against infectious disease. However, the MSP extract evaluated in this study was not fractionated, and the observed biological activities could be attributed to several compounds that needed to be purified. In fact, it is well documented that the biologically active compounds transferred from the biomass of algae to the liquid phase included polysaccharides, proteins, polyunsaturated fatty acids, polyamines, or minerals (Chonjnacka et al. 2012). Nevertheless, the chemical composition analysis indicated that the main component of the extract is ulvan, a sulfated polysaccharide showing structural similarity with glycosaminoglycan (sulfated polysaccharides composed with glucuronic and iduronic residues). Low molecular weight fractions of ulvan have already demonstrated biological activities in both in vitro and in vivo models (Leiro et al. 2007; Qi et al. 2011; Aguilar-Briseño et al. 2015). This component is regarded as the main candidate for activity, although further purification of the sample is needed for final confirmation. Finally, the immunomodulatory effect of MSP could not be attributed to LPS as MSP analysis using E-Toxate kit did not reveal any indication of LPS contamination (data not shown).

The biological relevance of this in vitro data needs to be ascertained using alternative approaches such as the three-dimensional coculture (MSP, epithelial, and immune cells), ex vivo model of intestinal explant culture or directly in vivo. In addition, the mechanisms and the pathways used by the MSP extract to stimulate the immune response of IPEC-1 epithelial cells are yet to be identified. Taken all together, our results showed that this MSP preparation may be used in animal diets to inhibit the pathogens growth and to stimulate the immune response, thereby to reduce the occurrence of infections in animal herds and the subsequent use of antibiotics. In the current study, it seems that MSP may constitute a potential adjuvant in the design of mucosal vaccine approaches to enhance immune cells recruitment and activation to improve both innate and adaptive immunity.

Acknowledgments The authors are grateful to Dr. Nathalie Guriec, CHU de Brest, for her constructive comments on the article. We also thank Claire Boisset (chromatography platform, CERMAV, Grenoble, France) and Marie-Francoise Bricot (Service de Microanalyse, Institut de Chimie des Substances Naturelles, CNRS UPR2301, Gif-sur-Yvette cedex France) for elementary analyses. This work was made possible by the support granted by Banque Publique d'Investissement (BPI, France) to the company Amadeite, Bréhan (France), via the ISI ULVANS project.

References

Abd El-Baky HH, Hussein MM, El-Baroty GS (2008) Algal extracts improve antioxidant defense abilities and salt tolerance of wheat plant irrigated with sea water. Afr J Biochem Res 2:151–164

Aguilar-Briseño JA, Cruz-Suarez LE, Sassi J-F, Ricque-Marie D, Zapata-Benavides P, Mendoza-Gamboa E, Rodríguez-Padilla C, Trejo-Avila LM (2015) Sulphated polysaccharides from *Ulva clathrata* and *Cladosiphon okamuranus* seaweeds both inhibit viral attachment/entry and cell-cell fusion, in NDV Infection. Mar Drugs 13:697–712

Artis D, Grencis RK (2008) The intestinal epithelium: sensors to effectors in nematode infection. Mucosal Immunol 1:252–64

Blumenkrantz N, Asboe-Hansen G (1973) New method for quantitative determination of uronic acids. Anal Biochem 54:484–489

Bruel T, Guibon R, Melo S, Guillen N, Salmon H, Girard-Misguich F, Meurens F (2010) Epithelial induction of porcine suppressor of cytokine signaling 2 (SOCS2) gene expression in response to *Entamoeba histolytica*. Dev Com Immunol 34:562–571

Chen D, Wu XZ, Wen ZY (2008) Sulphated polysaccharides and immune response: promotor or inhibitor? Panminerva Med 50:177–183

Chojnacka K, Saeid A, Witkowska Z, Tuhy L (2012) Biologically active compounds in seaweed extracts—the prospects for the application. Open Conf Proceed J 3(1-M4):20–28

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colorimetric method for determination of sugars and related substances. Anal Chem 28:350–356

Economou V, Gousia P (2015) Agriculture and food animals as a source of antimicrobial-resistant bacteria. Infect Drug Resist 8:49–61

Faulkner DJ (2002) Marine natural products. Nat Prod Rep 19:1–48

Fedorov SN, Ermakova SP, Zvyagintseva TN, Stonik VA (2013) Anticancer and cancer preventive properties of marine polysaccharides: some results and prospects. Mar Drugs 11:4876–4901

Ferreira LG, Noseda MD, Goncalves AG, Ducatti DRB, Fujii MT, Duarte MER (2012) Chemical structure of the complex pyruvylated and sulfated agaran from the red seaweed *Palisada flagellifera* (Ceramiales, Rhodophyta). Carbohydr Res 347:83–94

Gonzalez-Vallina R, Wang H, Zhan R, Berschneider HM, Lee RM, Davidson NO, Black DD (1996) Lipoprotein and apolipoprotein secretion by a newborn piglet intestinal cell line (IPEC-1). Am J Physiol 271:249–259

Hellio C, Bremer G, Pons AM, Le Gal Y, Bourgougnon N (2000) Inhibition of the development of microorganisms (bacteria and fungi) by extracts of marine algae from Brittany (France). Appl Microb Biotechnol 54:543–549

Hellio C, De La Broise D, Dufosse L, Le Gal Y, Bourgougnon N (2001) Inhibition of marine bacteria by extracts of macroalgae: potential use for environmentally friendly antifouling paints. Mar Environ Res 52:231–247

Hellio C, Marechal JP, Veron B, Bremer G, Clare AS, Yves Le Gal Y (2004) Seasonal variation of antifouling activities of marine algae from the Brittany Coast (France). Mar Biotechnol 6:67–82

Holdt S, Kraan S (2011) Bioactive compounds in seaweed; functional food applications and legislation. Rev Artic J Appl Phycol 23: 543–597

Ito T, Carson WF, Cavassani KA, Connett JM, Kunkel SL (2011) CCR6 as a mediator of immunity in the lung and gut. Exp Cell Res 317:613–9

Jaques LB, Ballieux RE, Dietrich CP, Kavanagh LW (1968) A microelectrophoresis method for heparin. Can J Physiol Pharmacol 46:351–360

Jaswir I, Monsur HA (2011) Anti-inflammatory compounds of macro algae origin: a review. J Med Plants Res 5:7146–7154

Jiménez-Escrig A, Gómez-Ordóñez E, Rupérez P (2011) Seaweed as a source of novel nutraceuticals: sulfated polysaccharides and peptides. Adv Food Nutr Res 64:325–37

Author's personal copy

Karnjanapratum S, Tabarsa M, MyoungLae C, You S (2012) Characterization and immunomodulatory activities of sulfated polysaccharides from *Capsosiphon fulvescens*. Int J Biol Macromol 51: 720–729

Kim JK, Cho ML, Karnjanapratum S, Shin IS, You SG (2011) *In vitro* and *in vivo* immunomodulatory activity of sulfated polysaccharides from *Enteromorpha prolifera*. Int J Biol Macromol 49:1051–1058

Lahaye M, Robic A (2007) Structure and functional properties of ulvan, a polysaccharide from green seaweeds. Biomacromolecules 8:1765–1774

Laurienzo P (2010) Marine polysaccharides in pharmaceutical applications: an overview. Mar Drugs 8:2435–2465

Lee JC, Hou MF, Huang HW, Chang FR, Yeh CC, Tang JY, Chang HW (2013) Marine algal natural products with anti-oxidative, anti-inflammatory, and anti-cancer properties. Cancer Cell Int 13:55. doi: 10.1186/1475-2867-13-55

Leiro JM, Castro R, Arranz JA, Lamas J (2007) Immunomodulating activities of acidic sulphated polysaccharides obtained from the seaweed *Ulva rigida* C. Agardh. Int Immunopharmacol 7:879–888

Leppkes M, Roulis M, Neurath MF, Kollias G, Becker C (2014) Pleiotropic functions of TNF-α in the regulation of the intestinal epithelial response to inflammation. Int Immunol 26:509–15

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using realtime quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25:402–408

Meurens F, Berri M, Siggers RH, Willing BP, Salmon H, Van Kessel AG, Gerdts V (2007) Commensal bacteria and expression of two major intestinal chemokines, TECK/CCL25 and MEC/CCL28, and their receptors. PLoS ONE 2:e677

Miron N, Cristea V (2012) Enterocytes: active cells in tolerance to food and microbial antigens in the gut. Clin Exp Immunol 167:405–12

Montreuil, J., Bouquelet, S., Debray, H., Fournet, B., Spik, G. and Strecker, G. (1986) Glycoproteins. In carbohydrate analysis: a practical approach. Chaplin MF and Kennedy JF (eds). New York Oxford University Press, pp143–204

Na WJ, Kim SM, Kim JW, Park SM, Lee SO, Kim SS, Synytsya A, Park Y (2010) Purification, characterization and immunostimulating activity of water-soluble polysaccharide isolated from *Capsosiphon fulvescens*. Int Immunopharmacol 10:364–370

Ngoa DH, Wijesekara I, Voa TS, Ta QV, Kim SK (2011) Marine food-derived functional ingredients as potential antioxidants in the food industry: an overview. Food Res Int 44:523–529

Orhan I, Sener B, Brun R, Perozzo R, Tasdemir D (2006) Turkish freshwater and marine macrophyte extracts show in vitro antiprotozoal activity and inhibit FabI, a key enzyme of *Plasmodium falciparum* fatty acid biosynthesis. Phytomedicine 13:388–393

O'Sullivan L, Murphy B, McLoughlin P, Duggan P, Lawlor PG, Hughes H, Gardiner GE (2010) Prebiotics from marine macroalgae for human and animal health applications. Mar Drugs 8:2038–2064

Oswald IP (2006) Role of intestinal epithelial cells in the innate immune defense of the pig intestine. Vet Res 37:359–68

Peterson LW, Artis D (2014) Intestinal epithelial cells: regulators of barrier function and immune homeostasis. Nat Rev Immunol 14:141–153

Qi H, Huang L, Liu X, Liu D, Zhang Q, Liu S (2011) Antihyperlipidemic activity of high sulfate content derivative of polysaccharide extracted from *Ulva pertusa* (Chlorophyta). Carbohydr Polym 87:1637–1640

Raposo MFJ, Morais RMSC, Morais AMMB (2013) Health applications of bioactive compounds from marine microalgae. Life Sci 93:479–486

Rioux LE, Turgeon SL, Beaulieu M (2007) Characterization of polysaccharides extracted from brown seaweeds. Carbohydr Polym 69: 530–537

Silva TH, Alves A, Pop EG, Reys LL, Gomes ME, Sousa RA, Silva SS, Mano JF, Reis RL (2012) Marine algae sulfated polysaccharides for tissue engineering and drug delivery approaches. Biomatter 2:278–289

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC (1985) Measurement of protein using bicinchoninic acid. Anal Biochem 150:76–85

Spavieri J, Kaiser M, Casey R, Hingley-Wilson S, Lalvani A, Blunden G, Tasdemir D (2010) Antiprotozoal, antimycobacterial and cytotoxic potential of some British green algae. Phytother Res 24:1095–1098

Stadnyk AW (2002) Intestinal epithelial cells as a source of inflammatory cytokines and chemokines. Can J Gastroenterol 16:241–246

Stavnezer J, Kang J (2009) The surprising discovery that TGF specifically induces the IgA class switch. J Immunol 182:5–7

Tabarsa M, Rezaei M, Ramezanpour Z, Waaland JR (2012) Chemical compositions of the marine algae *Gracilaria salicornia* (Rhodophyta) and *Ulva lactuca* (Chlorophyta) as a potential food source. J Sci Food Agric 92:2500–2506

Takenoshita S, Fukushima T, Kumamoto K, Iwadate M (2002) The role of TGF-β in digestive organ disease. J Gastroenterol 37:991–999

Tyagi S, Gupta P, Saini AS, Kaushal C, Sharma S (2011) The peroxisome proliferator-activated receptor: a family of nuclear receptors role in various diseases. J Adv Pharm Technol Res 2:236–40

Vo TS, Ngo D, Kim SK (2012) Potential targets for anti inflammatory and anti-allergic activities of marine algae: an overview inflammation and allergy. Drug Targets 11:90–101

Walia B, Wang L, Merlin D, Sitaraman SV (2003) TGF-β down-regulates IL-6 signaling in intestinal epithelial cells: critical role of SMAD-2. FASEB 17:2130–2132

Wijesekara I, Pangestuti R, Kima SK (2011) Biological activities and potential health benefits of sulfated polysaccharides derived from marine algae. Carbohydr Polym 84:14–21

Wijesinghe WAJP, Jeon YJ (2011) Biological activities and potential cosmeceutical applications of bioactive components from brown seaweeds: a review. Phytochem Rev 10:431–443

Williams IR (2006) CCR6 and CCL20: partners in intestinal immunity and lymphorganogenesis. Ann N Y Acad Sci 1072:52–61

Zanello G, Berri M, Dupont J, Sizaret PY, D'Inca R, Salmon H, Meurens F (2011) *Saccharomyces cerevisiae* modulates immune gene expressions and inhibits ETEC-mediated ERK1/2 and p38 signaling pathways in intestinal epithelial cells. PLoS One 6(4), e18573

Zubia M, Fabre MS, Kerjean V, Deslandes E (2009) Antioxidant and cytotoxic activities of some red algae (Rhodophyta) from Brittany coasts (France). Bot Mar 52:268–277