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Yeast β-glucans and microalgal extracts modulate the immune response and gut microbiome in Senegalese sole (*Solea senegalensis*)

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ABSTRACT

One bottleneck to sustainability of fish aquaculture is the control of infectious diseases. Current trends include the preventive application of immunostimulants and prebiotics such as polysaccharides. The present study investigated how yeast β-glucan (Y), microalgal polysaccharide-enriched extracts (MAe) and whole *Phaeodactylum tricornutum* cells (MA) modulated the gut microbiome and stimulated the immune system in Senegalese sole (*Solea senegalensis*) when administered by oral intubation. Blood, intestine and spleen samples were taken at 3 h, 24 h, 48 h and 7 days after treatment. The short-term response (within 48 h after treatment) consisted of up-regulation of *il1b* and *irf7* expression in the gut of the Y treated group. In contrast, administration of MAe decreased expression of *tnfa* and the chemokine *cxc10* in the gut and spleen. Both treatments down-regulated the expression of *irf3* with respect to the control group. Lysozyme activity in plasma decreased at 48 h only in the MAe-treated soles. Medium-term response consisted of the up-regulation of *clec* and *irf7* expression in the gut of the Y, MAe and MA groups and of *il1b* mRNAs in the spleen of the MA group compared to the control group. Microbiome analysis using 16S rDNA gene sequencing indicated that the intestine microbiome was dominated by bacteria of the *Vibrio* genus (> 95%). All the treatments decreased the relative proportion of *Vibrio* in the microbiome and Y and MAe decreased and MA increased diversity. Quantitative PCR confirmed the load of bacteria of the *Vibrio* genus was significantly decreased and this was most pronounced in Y treated fish. These data indicate that orally administrated insoluble yeast β-glucans acted locally in the gut modulating the immune response and controlling the *Vibrio* abundance. In contrast, the MAe slightly reduced the *Vibrio* load in the intestine and caused a transient systemic anti-inflammatory response. The results indicate that these polysaccharides are a promising source of prebiotics for the sole aquaculture industry.

1. Introduction

Prebiotics are important feed supplements in aquaculture due to their capacity to enhance health and prevent disease outbreaks. These ingredients are normally non-digestible molecules that selectively modulate the intestinal microbiome by promoting indigenous microbial populations and preventing pathogen proliferation [1,2]. Carbohydrates (poly- and oligosaccharides) such as mannan-, fructo- and galacto-oligosaccharides, inulin or β-glucans are widely recognized for their prebiotic activity. All these non-digestible sugars are fermented in the gut resulting in the enhanced production of short chain fatty acids (SCFA), mainly formate, acetate, propionate and butyrate [3,4]. These secondary metabolites modulate the production and release of cytokines and chemokines and leukocyte recruitment producing beneficial effects on diabetes, colon cancer, obesity and systemic inflammation in mammals [4,5]. In spite of the potential benefits of prebiotics for animal health, their use in aquaculture is still limited and their actions are far from understood [1].

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The β-glucans are a chemically heterogeneous family of polysaccharides commonly utilized as prebiotics and vaccine adjuvants in aquaculture [6,7]. These molecules are composed of a glucose backbone of varying lengths linked by either β-(1,3)-, β-(1,4) or β-(1,6) glycoside bonds and side chains linked by β-(1,4) or β-(1,6) [8,9]. β-glucans are ubiquitous in bacteria, algae, protozoans, chromistans and plants and act as bacterial aggregators, virulence factors, energy storage compounds or structural components of cell walls [9,10]. However, only β-(1,3;1,6)-glucans from fungi and algae have been used as prebiotics due to their action as microbiome modulators and immunosaccharides that bind to specific pathogen associated molecular pattern (PAMP) receptors to trigger an innate immune response [6,7]. The β-glucans have a wide spectrum of molecular sizes, solubility and degree of branching and polymerization, which explains their unique tertiary structures, physicochemical properties and specific bioactivity [11–14]. In human whole blood cultures, a specific cytokine response is stimulated by β-glucans with different chemical characteristics [11]. For this reason, the activity of each β-glucan as an immune modulator needs to be evaluated.

Yestimun® and chrysolaminarin are two different β-(1,3;1,6)-glucans obtained from brewers' yeast and microalgae, respectively. The former is particulate and insoluble with a branch-on-branch structure, high molecular weight (> 200 kDa) and high degree of polymerization [8,15]. In contrast, chrysolaminarin is a soluble, side-chain branched glucan of small size (1–40 kDa) containing between 20 and 30 linear residues and a low degree of branching [16]. Both types of β-glucans exhibit different antioxidant and antiproliferative capacities [17]. Studies in mammals indicate that particulate β-glucans interact with a C-type lectin receptor to activate the dectin-1 pathway whereas the soluble β-glucans act via the CR3 receptor to activate the complement pathway [18]. In aquaculture, the action of particulate β-glucans has been studied and they enhance non-specific and specific immune markers regardless of the route of administration [6,19]. However, the immunomodulatory action of chrysolaminarin-enriched extracts is still poorly characterized although we have shown that they trigger a transient acute inflammatory response when injected intraperitoneally [17].

A major threat to sustainable fish aquaculture is disease and for this reason there is high interest in prebiotics that can stimulate a robust immune response and improved disease resistance [20]. The rapid expansion of the microalgae industry has led to the development of new valuable biotechnological products [21,22]. The status and scale of biomass production for the diatom *Phaeodactylum tricornutum* has made this species a target for biorefinery feedstock and for high value-added bioactive compounds mainly fucoxanthin, chrysolaminarin and eicosapentaenoic acid [23]. A previous study in gilthead seabream using this microalga as a dietary supplement indicated they had enhanced immune parameters [24]. However, the use of whole microalgae cells or their extracts as feed supplements to modulate microbiota and the immune response still remains poorly explored and new studies are required to explore their biological actions and beneficial properties. The present study aimed to evaluate short- and medium-term effects of particulate yeast β-glucans and soluble microalgal extracts enriched in polysaccharides, when applied by oral intubation to Senegalese sole (*Solea senegalensis*). The outcome of the treatments was evaluated by assessing immune plasma activities, transcript expression in the intestine and spleen and the intestinal microbiota composition. The results provide insight into the immunomodulatory action of yeast β-glucans and chrysolaminarin on *Vibrio* communities and gut health and their potential as prebiotics for sole aquaculture.

2. Material and methods

2.1. Yeast β-glucan and microalgal sources

An insoluble (1,3)-(1,6)-β-glucan, 85% pure from brewers' yeast (Yestimun® pure) was purchased from Quimivita (Barcelona, Spain; 42200P-030). Microalgal polysaccharide-enriched extract (MAe) was supplied by Fitoplancton Marino, S.L. using the microalgae, *Phaeodactylum tricornutum* from the Microalgae Culture Collection of (CCFM). Microalgae culture conditions and the preparation of the extracts is detailed in Carballo et al. [17]. In brief, microalgae were produced in outdoor seawater photobioreactors under natural environmental conditions for spring in Cadiz, Spain. The microalgae were harvested after sunrise, frozen at −20 °C and freeze-dried. The MAe was prepared using a warm-water extraction method [25] followed by protein precipitation using a pH-shift and then polysaccharides precipitation using ethanol. The final extract was estimated to contain 47.3% reducing sugar [17]. Whole-cells of *P. tricornutum* were supplied in freeze-dried packages.

2.2. Fish trial

All procedures were authorized by the Bioethics and Animal Welfare Committee of IFAPA and given the registration number 26–11–15-374 by the National authorities for the regulation of animal care and experimentation.

To test the effects of yeast β-glucans and MAe on the Senegalese sole, solutions were directly delivered to the intestine using an oral intubation method. To optimize this methodology and establish the timeframe of uptake, some preliminary trials were carried out. Intubation was performed using a flexible polypropylene catheter 1.3 mm in diameter, attached to a 1 mL syringe that was introduced into the mouth from the blind side of the sole. All the procedures were carried out using anesthetized fish (150 ppm phenoxythanol). To monitor the timeframe of uptake and dispersal after intra-intestinal delivery, phosphate-buffered saline (PBS) containing a blue food colouring (1.25% v:v) was used as a tracker. Once the intubation methodology was established, one hundred and twenty juvenile sole (19.2 ± 4.3 g, mean ± standard deviation) were randomly distributed between twelve rectangular tanks (500 L) in an open circuit at 21 ± 1 °C, oxygen saturation > 90% and salinity 42 ppt. Animals were adapted for one week to the experimental tanks and fed commercial pellets (2% biomass; Skretting) using belt-feeders. Before the intubation experiments sole were fasted for two days to avoid uncontrolled effects of the intestinal contents. Four groups were established in randomly chosen tanks (n = 10 fish/tank, three replicate tanks per group): "Control" (C), fish were intubated and provided with PBS (vehicle); "Yeast" (Y), fish were supplied with yeast β-glucans (1 mg/fish in PBS); "MAe", fish were supplied with microalgal extracts (1 mg/fish in PBS); and "MA", fish received a suspension of rehydrated microalgae *P. tricornutum* (10 mg/fish in PBS). The doses used for the Y and MAe groups were selected using the results of a previous study that revealed a pro-inflammatory response after the intraperitoneal injection of the microalgal extract [17]. The dose used for freeze-dried microalgae was estimated based on the 6.9–21.2% dry weight fraction of chrysolaminarin reported for *P. tricornutum* [26,27].

To investigate short-time responses fish were sampled at 3 h (n = 5 fish/group), 24 h (n = 8 fish/group) and 48 h (n = 5 fish/group) and for medium-term responses fish were sampled 7 days after oral intubation (n = 12/group). For sampling, fish were anesthetized (200 ppm phenoxyethanol) and blood was collected by puncture of the caudal vein. The anterior intestine and spleen were collected into RNA-later (Sigma-Aldrich) and a portion of the middle intestine was fixed in ice-cold 4% paraformaldehyde (PFA), pH 7.4. Blood samples were left to clot at room temperature for 3 h and then centrifuged at 2,000g for 10 min at 4 °C and the supernatant (serum) removed and stored at −20 °C until determination of the total protein content and enzymatic activities. Samples for RNA extraction (anterior intestine, and spleen) were fixed in RNA-later (Sigma-Aldrich), kept overnight at 4 °C and then stored at −80 °C. Samples for histology (middle intestine) were collected into ice-cold 4% paraformaldehyde (PFA), pH 7.4. Fixation

was carried out at 4 °C with agitation, samples were washed 3 times with PBS and once with sterile water and then stored in methanol at −20 °C, until processing for histology. At day 7 after intubation samples from the mid-gut were collected for microbiome analysis. Sample collection was carried out in a laminar flow cabinet, using sterilized dissection material, and tissue was transferred to RNA-later, incubated at 4 °C for 24 h and then stored at −20 °C until DNA extraction for the microbiome analysis.

2.3. Blood serum activity assays and histology

Serum samples were kept at −20 °C and used for immunoassays within 1 month of collection. Immune parameters were measured in serum collected from sole at 24 and 48 h and 7 days after oral intubation (n = 5/group). Total serum protein (mg mL^{-1}) was determined using the Bradford method and diluted serum samples (1:40) with a Bio-Rad protein assay dye reagent concentrate colorimetric assay (#500-0006, BioRad, USA) and measured at 595 nm using a microplate reader (BioTek Synergy 4, BioTek Instruments, Inc., USA). The total anti-protease activity was measured using the method reported by Ellis [28] and modified by Hanif et al. [29]. Total anti-protease activity was expressed based on the percentage of trypsin activity (calculated by comparison with the positive control) and normalized using the protein content of the serum (% of trypsin activity/mg protein). The lysozyme activity was carried out using the turbidimetric assay reported by Ellis [30]. The specific activity of serum lysozyme was expressed as U mg^{-1} protein and determined using a standard curve made with hen egg white lysozyme (HEWL, Sigma-Aldrich). One unit of lysozyme activity gave a reduction in absorbance of 0.001 per min.

Histology of the middle intestine was performed to evaluate possible inflammatory effects of the treatments. Paraffin embedded tissue blocks were prepared by dehydration of tissue through a graded ethanol series (70–100%), followed by saturation in xylene and inclusion in low melting point paraffin wax. Serial transverse sections (5 μm) of the embedded middle intestine were prepared using a rotary microtome (Leica RM 2135, Germany). Sections were mounted on poly-L-lysine (Sigma-Aldrich) coated slides and dried overnight in an oven at 37 °C. Dewaxed and rehydrated sections were stained using haematoxylin and eosin and were mounted in DPX (Fluka, Germany). Slides were examined with a Leica DM2000 microscope and images were captured with a digital camera (Leica DFC480) coupled to a computer.

2.4. RNA isolation and RT-qPCR analysis

Samples from the anterior intestine (n = 3; ~50 mg) and spleen (n = 3–5; ~15 mg) were suspended in 1 ml of TRI-Reagent (Sigma-Aldrich) and homogenized in a Fast-prep FG120 instrument (Bio101) using Lysing Matrix D (Q-Bio-Gene) for 60 s at speed setting 6. After adding chloroform (0.2 ml) and centrifuging at 14.000 rpm for 15 min, the aqueous phase was recovered and transferred to a column of the Isolate II RNA Mini Kit (Bioline). Contaminating genomic DNA was removed from total RNAs by treating twice for 30 min with DNase I following the manufacturer's protocols. Total RNA quality was checked by agarose gel electrophoresis and a Nanodrop ND-8000 (Thermo Scientific) was used to determine the concentration. Total RNA (1 μg) was reverse transcribed using an iScript™ cDNA Synthesis kit (Bio-Rad) according to the manufacturer's protocol.

Real-time PCR analysis (qPCR) was carried out for samples taken at 3 h, 24 h, 48 h and 7d after oral intubation. The number of specimens analysed ranged between 3 and 5 for each sampling time. All qPCR assays were run in duplicate on a CFX96™ Real-Time System (Bio-Rad). The final reaction volume was 10 μL and it contained cDNA generated from 10 ng of the original RNA template, 300 nM each of the specific forward and reverse primers, and 10 μl of SsoAdvanced™ Universal SYBR® Green Supermix (Bio-Rad). The genes for the pro-inflammatory cytokines, interleukin 1b (*il1b*) and tumor necrosis factor alpha (*tnfa*) and the chemokine *cxc10*, interferon-related factors *irf3* and *irf7*, glucan receptor c-type lectin (*clec*), g-type lysozyme (*lysg*), antimicrobial peptide hepcidin (*hamp1*) and cell surface markers of T lymphocytes (T helper *cd4* and T cytotoxic *cd8* [31]) were analysed. Primers for Senegalese sole *il1b, tnfa, clec, cxc10, irf3, irf7, hamp1* and *lysg* have previously been published [17,32–34]. The qPCR amplification protocol was as follows: 7 min for denaturation and enzyme activation at 95 °C followed by 40 cycles of 30 s at 95 °C and 1 min at 60 °C. Data were normalized using the geometric mean of ubiquitin (*ubi*) and β-actin (*actb2*) [35] and the relative mRNA expression was calculated using the comparative Ct method [36].

2.5. DNA isolation, microbiome analysis and vibrio quantification

DNA was extracted from 20 to 35 mg of middle intestine collected 7 days after intubation (n = 5 individuals/treatment) using a DNeasy Blood & Tissue Kit (Qiagen). Tissue was subjected to mechanical disruption with 0.1 mm zirconia/silica beads (Biospec) and by applying three cycles of 20 s at 6,800 rpm in a Bertin Precellys 24 homogenizer. DNA extraction followed the manufacturer's instructions and included pre-digestion with 20 mg/ml lysozyme (BioChemica, PanReact, AppliChem) and RNAse treatment (0.3 mg/ml, Thermo Fisher). The concentration, quality and integrity of DNA was analyzed by agarose gel electrophoresis and spectroscopy (Nanodrop ND-8000). 50 ng of DNA was generated per treatment group by pooling an equal concentration of DNA/fish (n = 5) and four microbiome libraries, C, Y, MAe and MA, were generated. A 16S Metagenomic Sequencing Library Preparation protocol for Illumina MiSeq was used for library production using primers targeting the hypervariable V3 and V4 regions of the 16S rRNA gene [37]. Libraries were sequenced at Lifesequencing S.L.-ADM (Valencia, Spain) on an Illumina MiSeq instrument. Bioinformatics analyses were carried out using a pipeline developed by Lifesequencing S.L.-ADM, as previously described [38]. These analyses included adaptor trimming, cleaning and merging of the reads (with a minimum of Q20 and final quality of 36.6), chimera removal, identification and classification of operational taxonomic units (OTU) by comparison with the NCBI 16S rRNA database (using a minimum identity of 97%) and hierarchical clustering based on microbial composition. The Chao1 index was determined as a measure of species richness and the Shannon index as an indicator of species diversity.

To obtain further insight into *Vibrio* and *Mycoplasma* in the collected samples, qPCR using optimized genus-specific primers targeting the 16S rRNA gene [39,40] was performed using as the template the individual DNAs used to generate the pools for treatment-specific library construction. Duplicate qPCR reactions were carried out as previously described [41] in a 10 μL volume containing 20–50 ng DNA, 300 nM of each primer and the Sso Fast EvaGreen Supermix (Bio-Rad). The calculated copy number of *Vibrio* or *Mycoplasma* bacteria was normalized in relation to the micrograms of total DNA extracted from the mid-gut.

2.6. Statistical analysis

Prior to statistical analyses, all data were checked for normality using a Kolmogorov-Smirnov test and homogeneity of variance using a Bartlett's test and, when necessary, they were log-transformed. To assess statistical differences in serum activities and the short-term transcriptional response in the intestine, a two-way analysis of variance (two-way ANOVA) using treatment and time as fixed factors was carried out. A Duncan's post-hoc test was carried out when the factor, treatment, was significant. For the short-term transcriptional response in the spleen and medium-term expression profiles in the intestine and spleen, a one-way ANOVA was performed. Differences in *Vibrio* and *Mycoplasma* abundance in each treatment with respect to the control were assessed with a student's t-test. Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were carried out using SPSS v21 software (IBM) with statistical significance set at

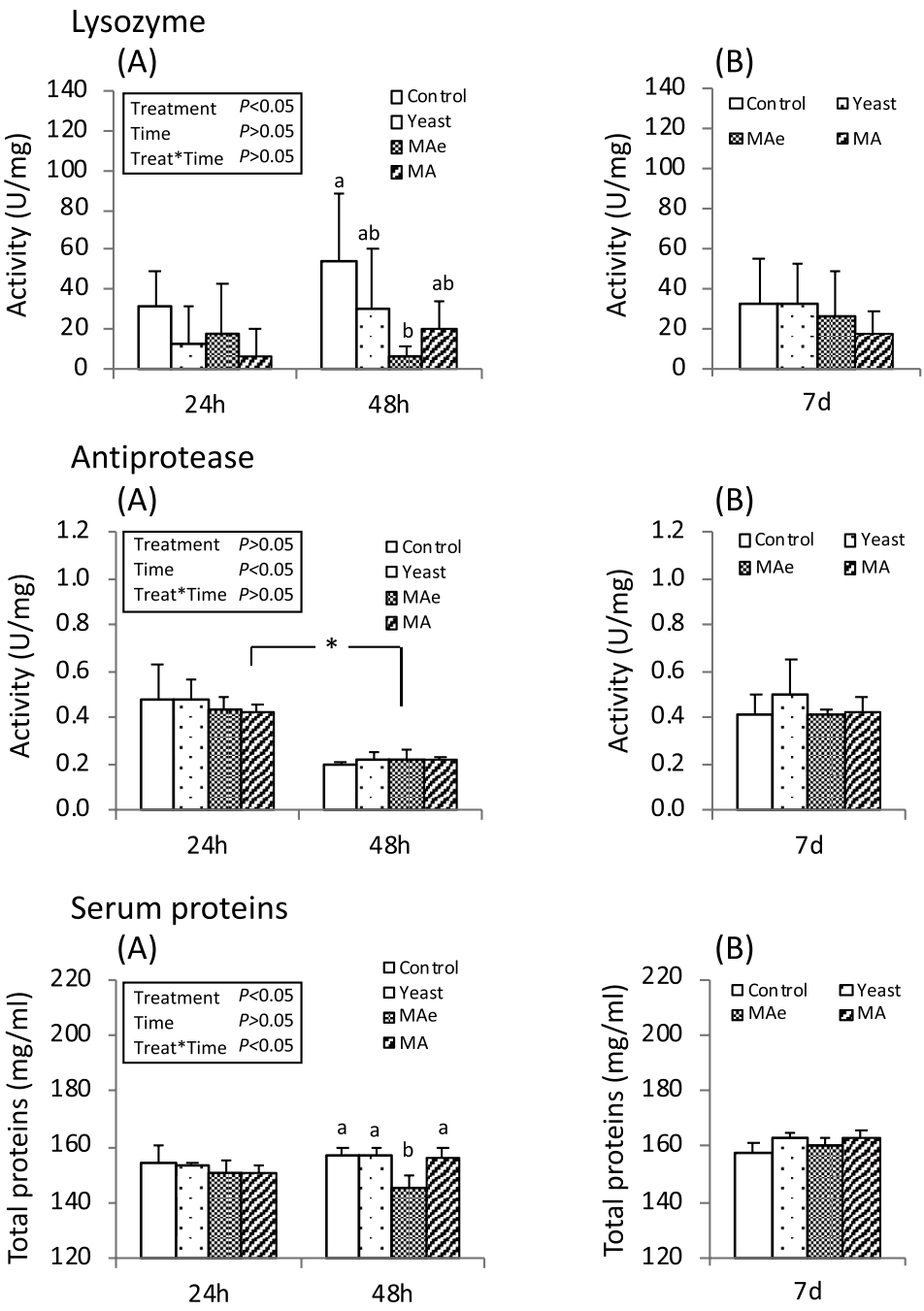

Fig. 1. Plasma activities and protein levels. Samples of fish from the control group (control), treated with insoluble yeast β-glucans (Y), microalgae extracts (MAe) and whole-cell microalgae (MA) were sampled at 24 and 48 h (A) or 7d (B) after oral intubation. The results are shown as the mean ± SEM and expressed as $U.mg^{-1}$ for lysozyme and anti-protease activity and as $mg.mL^{-1}$ for total proteins. Different letters indicate significant differences between oral intubation treatments at a specific time point. Asterisks indicate significant differences between samples at different times post-intubation in the short-term response. Significant differences were set at $P < 0.05$ (Two-way ANOVA).

$P < 0.05$.

3. Results

3.1. Blood serum activities and histology

Short-term responses after Y, MAe and MA treatments revealed significant differences in the lysozyme activity and total serum proteins ($P < 0.05$; Fig. 1). For lysozyme, the MAe group had a significantly lower activity than the C group 48 h after intubation ($P < 0.05$), while no significant differences were observed with respect to the Y or MA groups. The antiprotease activity did not differ between groups but differed significantly across time ($P < 0.05$). Fish from the MAe group had significantly reduced levels of total serum proteins compared to the C, Y and MA groups at 48 h ($P < 0.05$; Fig. 1). No significant differences in total serum proteins or enzyme activities was observed between any of the groups in the medium-term response after 7 days. Histological analysis of the middle-gut at 24 h and 7d after treatments did not reveal any notable changes in the morphology of the middle intestine of the treated and control fish (see Supplementary file 1).

3.2. Short-term expression profiles in the intestine and spleen

The short-term response to the treatments included a significant ($P < 0.05$, Two-way ANOVA) change in *il1b*, *tnfa*, *cxc10*, *irf3* and *irf7* expression levels. The *il1b* mRNAs were up-regulated in fish from the Y group compared to the C, MAe and MA groups ($P < 0.05$; Fig. 2). Moreover, *irf7* transcripts were increased in the Y group compared to the C and MAe groups. In contrast, *tnfa* and *cxc10* appeared significantly down-regulated ($P < 0.05$) in fish from the MAe group compared to the other three groups. The most pronounced change in expression was observed at 3 and 24 h after intubation. The *irf3* mRNAs

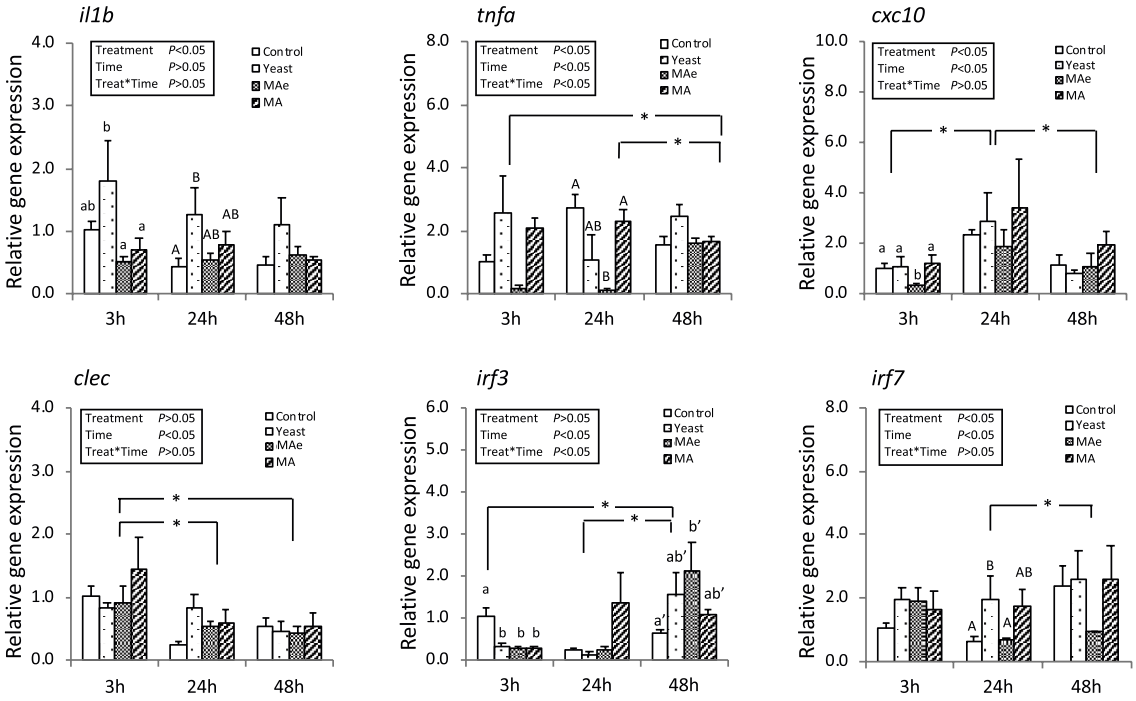

Fig. 2. Relative gene expression levels in the intestine at 3, 24, and 48 h after oral intubation. The four experimental groups are indicated: control, treated with insoluble yeast β-glucans (Yeast), microalgal extracts (MAe) and whole-cell microalgae (MA). Data are expressed as the mean fold change (mean ± SEM, n = 3) from the calibrator (control 3 h after oral intubation). A two-way ANOVA was used to determine statistical differences. Different letters indicate significant differences between oral intubation treatments for a specific time point. Asterisks denote significant differences between samples at different times post-intubation. Significant differences were set at $P < 0.05$ (Two-way ANOVA).

showed a clear interaction between factors, and were down-regulated in Y, MAe and MA groups with respect to the C group after 3 h and an inverse response was obtained at 48 h (Fig. 2). An effect of time was observed for *tnfa*, *cxc*, *irf3*, *irf7* and *clec*. No significant changes in gene expression of *lysg* and *hamp1* were observed (data not shown).

In the spleen, expression levels were only analyzed 24 h after the intubation treatments (Fig. 3). A down-regulation of *il1b*, *tnfa*, *cxc10*, *lysg*, *irf3* and *cd4* mRNA levels occurred in the MAe group and *tnfa*, *irf3* and *cd4* were down-regulated in the Y group relative to the C group. Moreover, *il1b* was up-regulated in the spleen of fish from MA group compared to the other three groups.

3.3. Medium-term expression profiles in the intestine and spleen

The gene expression profiles in the intestine 7 days after intubation revealed an up-regulation of *clec* and *irf7* in the Y, MAe and MA groups relative to the C group. In the spleen, only *il1b* was significantly up-regulated in the MA group compared to the C group (Table 1).

3.4. Microbiome modulation

To evaluate the effect of treatments on microbiome composition, more than 200,000 reads for the amplified 16S rRNA gene, which yielded an average of 23,457 good-quality reads per library (Supplementary file 2), were analysed. Rarefaction analysis (Supplementary file 3) confirmed that most libraries were close to saturation at the sequencing depth utilised and indicated that most of the bacterial diversity was covered. The results indicated that the intestinal microbiome had low complexity, with around 150 OTU genera detected in the C group and a lower number of OTUs in the treatment groups. A Chao1 index of 313 for the C group, 191 for the MAe, 155 for the Y and 265 for the MA groups also indicated that treatments decreased the microbiome species richness. The Shannon diversity indexes were 1.7 for the C group, 1.9 for MA, 1.3 for MAe and 1.1 for Y, and reinforced the notion of reduced bacterial diversity in the sole gut for MAe and Y treatments.

A total of 243 different genera were identified but only five genera were present at more than 1% in the intestinal microbiome (Supplementary file 4). The dominant bacterial genera in the intestine of the C group or in treated sole was *Vibrio*, which accounted for 93% of the control microbiome. Lower proportions of *Vibrio* were detected in the Y and MAe groups (86% and 88% *Vibrio,* respectively) followed by the MA group with 73% *Vibrio* (Fig. 4A; Supplementary file 4). At the species level, a non-pathogenic *Vibrio scophthalmi* cluster, was the most abundant species and accounted for 56–74% of the annotated bacterial reads across the treatments (not shown). The second most abundant bacterial genus in the microbiome was the Vibrionaceae *Catenococcus*, present at 0.6% of the C group but increased to 6.7, 2.5 and 17.4% in the Y, MAe and MA groups, respectively. Finally, the genera *Brevinema*, *Shewanella* and *Mycoplasma* comprised up to 5.7% of the intestine microbiomes but varied relatively little between the treatment groups (Fig. 4A; Supplementary file 4). Hierarchal clustering analysis separated the polysaccharide treatments (Y and MAe) from the C group. The MA treatment had the most divergent microbiome composition relative to the other groups (Fig. 4A). The significant decrease ($P < 0.05$) in the load of bacteria from the *Vibrio* genus in the intestine of juvenile sole from the MAe and Y groups compared to the C group was confirmed by qPCR (Fig. 4B). No significant differences were detected in the load of *Mycoplasma* bacteria between the treatment groups and C group (not shown).

4. Discussion

Research into prebiotics and immunostimulants is a fast-developing field in aquaculture as it is a sustainable way to enhance fish health with less regulatory restrictions in relation to food safety than other methods such as probiotics. The β-glucans are among the polysaccharides with high potential as a feed supplement in fish production

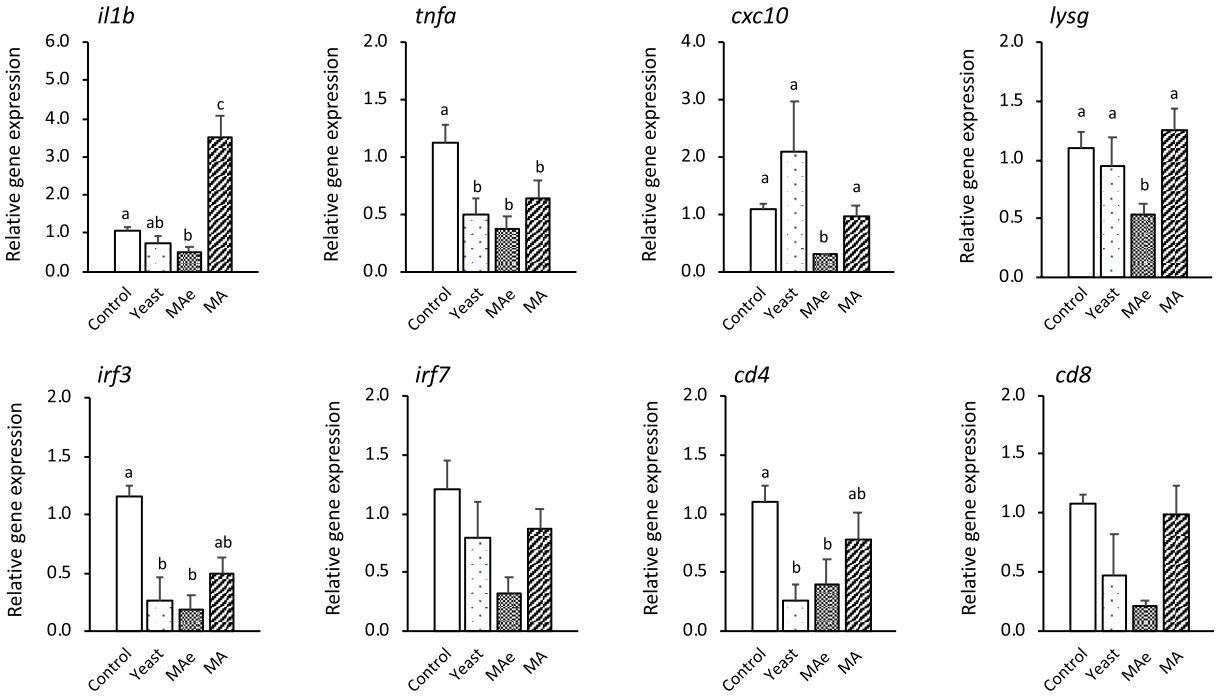

Fig. 3. Relative gene expression levels in the spleen 24 h after oral intubation. The four experimental groups are indicated: control, treated with insoluble yeast β-glucans (Yeast), microalgal extracts (MAe) and whole-cell microalgae (MA). Data are expressed as the mean fold change (mean ± SEM, n = 3) from the calibrator (control 3 h after oral intubation). Different letters indicate significant differences between oral intubation treatments. Significant differences were set at $P < 0.05$ (one-way ANOVA).

because they can act as both a) modulators of microbiota-host interactions and b) recruit and activate macrophages by binding to surface receptors [6,7,42]. An important consideration when using β-glucans as a prebiotic is the effect of source organism and the preparation method (both influence the conformation and chemical properties) on their specific immunomodulatory actions. In the present study, a particulate β-glucan from yeast and a MAe enriched in polysaccharides were evaluated using a novel and optimized oral intubation approach in a flatfish, the Senegalese sole. This intubation approach was chosen to reduce the interference from multiple feed components and to reveal more clearly the specific actions of the administered substances [43]. A comprehensive study of the effects of prebiotics on the gut microbiome and modulation of regulatory pathways of the innate immune system was done. Moreover, lysozyme and protease inhibitor activities, markers of the host anti-bacterial and anti-inflammatory response to pathogenic bacteria [44,45], were also characterized.

Our results demonstrated that the tested yeast β-glucans and MAe had a different effect on the microbiota composition and the expression of innate immune genes in both the intestine and spleen. These data support the notion that solubility is a key factor for β-glucan bioactivity and that they may act through different signalling pathways [18,46] with the particulate β-glucans having a major local effect on the gut and its microbiota. In mammals, particulate yeast β-glucans act as dectin-1 agonists and activate neutrophils, dendritic cells (DCs) and macrophages via two independent signalling pathways, Syk and Raf-1, which converge at the level of NF-*k*B [18,46]. Activation via dectin-1 triggers the production and release of proinflammatory cytokines (IL1β, IL6 and TNF-α), phagocytosis, production of reactive oxygen species (ROS) and T-cell differentiation to control adaptive immunity [47,48]. In fish, particulate β-glucans up-regulate the expression of *il1b, il6* and *il11* in carp macrophages and they have been proposed as good markers of macrophage activation [49]. The activation of *il1b* has also been reported in Atlantic salmon orally intubated to supply MacroGard [43]. In our experiments, yeast β-glucans triggered a fast and transient

Table 1
Relative expression levels at 7 days after oral intubation. The fold-changes between the control (Ctrl) group with respect to yeast β-glucans (Yeast), microalgal polysaccharide-enriched extract (MAe) and whole-cell microalgae (MA) are indicated. A one-way ANOVA was carried out followed by Tukey's posthoc test (significance at $P < 0.05$ is indicated by "*"; ns, not significant). The "-" denotes that expression was not quantified.

Gene name	Gene description	Intestine						spleen					
		Ctrl *vs* Y		Ctrl *vs* MAe		Ctrl *vs* MA		Ctrl *vs* Y		Ctrl *vs* MAe		Ctrl *vs* MA	
il1b	Interleukin 1b	0.60 ± 0.11	ns	0.80 ± 0.17	ns	1.17 ± 0.27	ns	2.13 ± 1.05	ns	2.17 ± 0.69	ns	3.23 ± 0.54	*
tnfa	Tumor necrosis factor α	1.41 ± 0.11	ns	1.90 ± 0.56	ns	1.90 ± 0.41	ns	0.98 ± 0.35	ns	0.58 ± 0.09	ns	0.46 ± 0.19	ns
lysg	g-type lysozyme	1.17 ± 0.06	ns	1.11 ± 0.33	ns	1.18 ± 0.10	ns	1.74 ± 0.25	ns	1.84 ± 0.35	ns	1.90 ± 0.55	ns
cxc10	Chemokine cxc10	1.19 ± 0.11	ns	1.45 ± 0.91	ns	3.46 ± 1.20	ns	1.24 ± 0.21	ns	1.92 ± 0.59	ns	3.27 ± 1.00	ns
irf3	Interferon regulatory factor 3	1.07 ± 0.37	ns	1.34 ± 0.69	ns	2.88 ± 1.60	ns	1.01 ± 0.21	ns	0.60 ± 0.23	ns	0.60 ± 0.18	ns
irf7	Interferon regulatory factor 7	2.81 ± 0.80	*	4.36 ± 1.37	*	4.74 ± 1.67	*	1.02 ± 0.14	ns	0.93 ± 0.33	ns	1.10 ± 0.25	ns
hamp1	hepcidin	0.82 ± 0.06	ns	0.82 ± 0.09	ns	1.04 ± 0.24	ns	-		-		-	
clec	c-type lectin	1.85 ± 0.27	*	1.87 ± 0.39	*	2.97 ± 0.17	*	-		-		-	
cd4	cluster of differentiation 4	-		-		-		1.04 ± 0.33	ns	0.48 ± 0.16	ns	1.30 ± 0.35	ns
cd8a	cluster of differentiation 8a	-		-		-		0.91 ± 0.13	ns	1.65 ± 0.25	ns	0.85 ± 0.17	ns

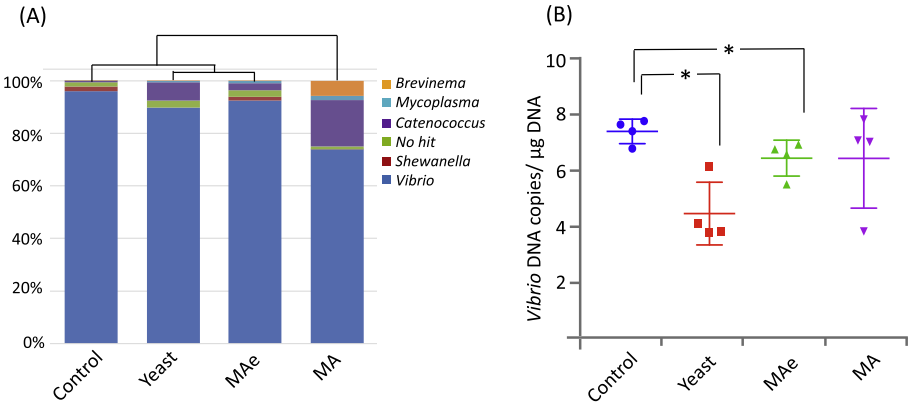

Fig. 4. A- Intestinal microbiome composition (in percentage) for the main bacterial genera, present at > 1% representation. The scheme represents the hierarchal clustering of the main bacterial genera in the gut of the four experimental groups: control, treated with insoluble yeast β-glucans (Yeast), microalgal extracts (MAe) and whole-cell microalgae (MA). B- Quantification of *Vibrio* DNA in the intestine of fish (n = 5) from each treatment group by qPCR with genera-specific primers and normalized using µg of DNA used in each PCR (bars represent the mean ± SEM). * indicate significant differences compared to the control with $P < 0.01$(Student's t-test).

induction of *il1b* in the intestine (but no change in *tnfa* and *cxc10a* genes were observed) and a shift in the microbiota composition, particularly the abundance of *Vibrio*, a major component of the fish intestine microbiome [50,51]. The results of the present study are in agreement with previous data in carp that reported a local stimulatory action of yeast β-glucans in the intestine [52] and a specific up-regulation of cytokine production indicative of glucan-mediated activation of macrophages [49]. Although with our experimental design it was not possible to establish a robust regulatory pathway model involving the pro-inflammatory cytokine *il1b*, the opposing response of the antiviral defence *irf3* gene is similar to the response of juvenile sole after intraperitoneally injection of *P. tricornutum* extracts [17]. These data suggest there is cross-talk between the type I interferon and interleukin-1 cytokine pathways to modulate the inflammatory responses and bacterial populations [53].

In contrast to the short-term response reported for yeast β-glucans, oral delivery of MAe was associated with a more systemic response with a reduction in blood lysozyme activity and the down-regulation of *tnfa* and *cxc* mRNAs in the intestine and spleen and *il1b* and g-type lysozyme transcripts in spleen. These results contrast with the rapid inflammatory response observed when MAe was injected intraperitoneally (i.p.) [17]. In the latter case induction of *il1b* in kidney, spleen and intestine was rapid and followed by the absence of a response to subsequent i.p. exposure to the same extracts. Such route-specific differences in the immunomodulatory response suggests a role for the microbiota and/or intestinal enzymes in the shift of MAe activity. In rainbow trout, dietary β-glucans differentially regulated the response of vaccinated trout after a microbial challenge and caused a down-regulation of pro-inflammatory, acute phase and lysozyme related genes in the head kidney [54]. In carp, dietary β-glucans also reduced the expression of inflammation-related cytokines in the gut, but not in the kidney, after a challenge with *A. salmonicida* [55]. In the rainbow trout, dietary β-glucans decreased the expression of genes involved in the acute inflammatory response after injection of bacterial lipopolysaccharide (LPS) in the spleen [56]. These data indicate that the regulation of immune-regulatory genes by β-glucans is dependent on the presence or absence of a microbial stimulus [57] and based on our data, we propose that the gut microbiota may play a role in this differential response. We should note that due to the complexity of our MA extract it was not possible to associate the observed effects to specific components of the extract, particularly since one of the likely contaminants, laminarin has been shown to act as a Dectin-1 antagonist or agonist depending on its form eg. soluble or aggregated [58]. Nonetheless, our results support an immunomodulatory and anti-inflammatory action for MAe as has previously been reported for crude polysaccharide extracts of *P. tricornutum* [59].

In the present study of Senegalese sole supplied with Y and MAe there was an apparent decrease in the global microbial richness and diversity of the intestine, which included both allochthonous microbiota of the intestinal content and the autochthonous microbiota of the intestine itself. The significant reduction in bacteria from the *Vibrio* genus, the most represented taxonomic group in the Senegalese sole microbiome [60], was confirmed by qPCR in the Y and MAe groups. Modulation of the gut microbiota by β-glucans has also been reported in carp fed diets supplemented with insoluble yeast β-glucans and the number of OTUs and the species richness of the allochthonous microbiota was reduced [61]. In seabass β-glucans caused a transient reduction in the most abundant groups of microorganisms in the intestine due to a shift in the relative abundance of OTUs in the autochthonous microbial community [62]. Dietary supplementation with laminarin extracts also had an inhibitory effect on the counts of *E. coli* in the faeces in piglets [63,64]. The more intense reduction in *Vibrio* caused by yeast β-glucan is compatible with a mainly local action of insoluble β-glucans on the intestine. Considering the high impact of *Vibrio* on sole disease outbreaks [65], the present results indicate β-glucans from yeast or microalgae are interesting candidate prebiotics for the health of the intestine in sole. Intriguingly, the *Shewanella* genus that is considered a probiotic in sole [66] and which increases when sole juveniles are fed polychaeta [60], was absent in the Y group. The failure to detect the *Shewanella* genus reveals there is a need for more studies to understand the factors that determine the gut microbiome and its modulation by pre-biotics. This knowledge will be essential for the application of pre-biotics to modify fish performance and to confer disease protection.

The supply of whole microalgae cells (MA treatment) decreased the relative abundance of *Vibrio* but in contrast to the Y and MAe groups, it also caused a slight increase in bacterial diversity. Microalgae are routinely used as a feed source for fish larvae [67,68] and experimental diets using whole-cell microalgae as a supplement for fish larval rearing also demonstrated a greater level of microbial diversity [69]. The nutritional value of the microalga presumably favours enrichment of the intestinal microbial community with organisms that can utilize nutrients from microalga. The use of the diatom *P. tricornutum* as a dietary supplement has been shown to enhance immune parameters such as, phagocytic haemolytic complement activities in the gilthead seabream [24]. We hypothesize that the up-regulation of *il1b* in the spleen of sole treated with MA for 7 days could be a result of the time needed for the shift in the microbiota and the associated delivery of metabolites that have an immunostimulant effect.

In summary, the results of the present study demonstrate that oral administration of insoluble β-glucans had a major action on the intestine and was associated with rapid up-regulation of *il1b* followed by a significant decrease in intestinal *Vibrio* abundance. In contrast, administration of soluble MAe enriched in polysaccharides had a limited effect on the intestine and caused only a slight reduction in the *Vibrio* load. The moderate response of the gut to MAe exposure coincided with a transient down-regulation of pro-inflammatory cytokines and g-type lysozyme transcripts in the spleen and lysozyme activity in the blood

serum. The oral supply of whole microalgae had the biggest effect on the intestinal microbial diversity presumably due to their nutrient content and this treatment was also associated with a delayed activation of *il1b*. The results obtained in the present study will form the basis for the design of new β-glucan enriched diets to control *Vibrio* populations in the gut, one of the major challenges for sole aquaculture.

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Appendix A. Supplementary data

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