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Beta-glucan-enriched diets improve immune function, antioxidant activity, and survivability in challenged oysters

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ABSTRACT

Beta-glucans are bioactive polysaccharides with immunomodulatory and hydrocolloid properties. This study explores the potential of beta-glucans extracted from mushroom waste to enhance oyster (*Crassostrea gigas*) immunity and quality, providing a sustainable alternative to conventional aquaculture interventions. Beta-glucan supplementation led to a 100 % survival rate in treated groups compared to 70 % in the bacteria-only group ($p = 0.024$). Flow cytometry revealed a significant reduction in viable hemocytes in the beta-glucan group post-infection, suggesting modulation of inflammation. Protein content in gills, mantle, and digestive tissues increased by up to 2-fold in beta-glucan-fed oysters versus controls. Antioxidant activity, assessed by FRAP assay, was 25–40 % higher in the mantle and digestive tract if beta-glucan groups. Bacteriostatic assays demonstrated a marked reduction in *Vibrio* sp. Counts in tissues, particularly in the digestive tract, with bacterial load similar to uninfected controls. These findings highlight the dual functionality of beta-glucans as both immunomodulators and hydrocolloids, with implications for improving aquaculture sustainability and food safety. Further research should explore feeding behaviour, gut transit time, and nutrient absorption to optimize beta-glucan application in commercial shellfish production.

1. Introduction

The aquaculture industry is experiencing rapid growth and reported a total production of 17.74 million tonnes of bivalve shellfish in 2020, with 5.45 million tonnes exclusively for the *Crassostrea* bivalve species, popular for its high nutritional value and rich flavour (FAO, 2022). As a sustainable solution to the increasing global demand for protein, bivalve cultivation offers additional significant benefits. They provide high quantities of essential omega-3 fatty acids and a range of crucial micronutrients such as zinc, iron, and vitamins A and B_{12}. In addition, from an ecology perspective, bivalves are essentially ecosystem engineers that play key roles in water purification and habitat provision while also vital to fisheries and aquaculture. In recent years, the bivalve aquaculture industry has faced challenges including infectious disease outbreaks and harmful algal blooms, leading to significant bivalve mortality, industry closures, and health risks to consumers due to accumulated toxins and pathogens (FAO, 2023). Between 2010 and 2018, the oyster industry, particularly the cultivation of *Crassostrea gigas*, suffered significant financial losses due to widespread mortality events associated to *Vibrio* bacteria, Ostreid herpesvirus 1, and environmental factors (Alfaro et al., 2019; Barbosa Solomieu et al., 2015).

Antibiotics are not a viable option to treat infectious diseases in aquaculture. This overreliance accelerates the development of antimicrobial resistant strains which is a pressing concern in human health and

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the ecological balance, all while undermining the natural resistance of the organisms themselves (Alfaro et al., 2019; Barbosa Solomieu et al., 2015; Félix et al., 2020). Immunostimulants such as polysaccharides, by directly boosting immunity, present a promising alternative to antibiotics (Pogue et al., 2021). These substances are recognized through pathogen-associated molecular patterns, triggering immune responses without the adverse effects typically associated with infections. This approach strategically enhances immunity and circumvents the issues linked to the overuse of antibiotics.

Beta-glucans belong to a broad class of naturally occurring complex polysaccharides known for their immunomodulatory and bioactive properties. They exhibit varying structures and biological activities which are defined based on their source such as cereals, mushroom, seaweed and yeast (Friedman, 2016; Murphy, Rezoagli et al., 2020; Zhang et al., 2018). Beyond their bioactive properties, beta-glucans also function as hydrocolloids, influencing viscosity, gelation, and water-holding capacity in food matrices, with their rheological behaviour largely dependent on molecular weight, solubility, and linkage patterns (Chioru et al., 2023; Lante et al., 2023a; Xu et al., 2010).

The primary structure of all beta-glucans consists of a glucose backbone with 1,3 beta linkages crucial for their functionality (Adams et al., 2008). The branching patterns of beta-glucans differ, with some having multiple branches and others none, such as curdlan from *Agrobacterium* species (Kataoka et al., 2002). Beta-glucans can then be classified by this branching off the main 1,3 backbone as either 1,4 or 1,6 linked. Cereal-derived beta-glucans typically branch at the 1,4 positions, while those from fungi and yeast usually have 1,6 branches (Manners et al., 1973; Tosh et al., 2004; Yehia, 2022; Zeković et al., 2005). The effects of cereal-based beta-glucans are mainly metabolic, such as influencing gut microbiota and reducing cholesterol (Murphy, Rezoagli et al., 2020). In contrast, non-cereal beta-glucans are known for their impact on the immune system, including anti-inflammatory, anti--cancer, and anti-infective properties (Martins et al., 2024; Murphy et al., 2022; Murphy, Rezoagli et al., 2020; Thomas et al., 2022). Beta-glucans derived from mushroom fruiting bodies are effective immune modulators due to their 1,6-side branched structure. Exploring their use in animal health is promising avenue especially considering that these molecules are non-toxic and already a part of the food chain.

In an era focused on reducing waste, exploring industrial and agricultural food by-products as potential sources of functional beta-glucan bioactive molecules present a promising opportunity. This aligns with the growing trend of upcycling waste materials for valuable uses within a biorefinery and circular bioeconomy framework (Doroški et al., 2022; Thomas et al., 2022). In this study, beta-glucan was isolated from the mushrooms King Oyster and Maitake, which are known for their high beta-glucan content and biological activity. Utilizing methods previously effective in sepsis models involving antimicrobial resistance (AMR) and inflammation, these beta-glucans were extracted and characterized (Masterson et al., 2020; Murphy et al., 2022; Murphy, Masterson et al., 2020). A critical component of this process was confirming the beta-glucan content and structural integrity through beta-glucan content assays and Fourier-transform infrared spectroscopy (FTIR).

In pursuit of a sustainable circular bioeconomy, beta-glucans were extracted from morphologically irregular and consumer-rejected parts of mushrooms. These bioactive compounds were then administered to oysters that were exposed to two pathogens responsible for significant annual financial losses in the seafood industry. This innovative approach not only aims to reduce infections using a resource otherwise considered 'waste', but also addresses substantial economic challenges in aquaculture. By repurposing discarded materials for disease prevention, this strategy effectively merges ecological sustainability with tangible solutions to pressing industry issues. Beyond their immunomodulatory effects, beta-glucans also exhibit hydrocolloid properties that may influence their solubility, bioavailability, and interaction with oyster tissues, potentially enhancing their stability and functional efficacy in aquatic systems.

2. Materials and methods

2.1. Sustainable sourcing of mushrooms for beta-glucan extraction

In line with sustainable practices, the methodology reported in this paper involved using beta-glucans from mushroom parts that were unsuitable for sale due to their irregular shapes. These mushrooms were sourced as a waste product from Garryhinch Wood Exotics Ltd., Portarlington, Co. Offaly, Ireland. Fig. 1 demonstrates the distinction between commercially viable mushrooms and those selected for upcycling due to their non-standard morphology, which were repurposed for beta-glucan extraction.

2.2. Beta-glucan extraction

For the extraction of beta-glucans from mushroom fruiting bodies, the procedure outlined by Murphy et al. (2021) was adapted. Initially, the fruiting bodies were thoroughly washed and freeze dried, and then ground into a fine powder. Approximately 500 g of this powdered biomass was submerged in 1 L of water and subsequently sterilized through autoclaving at 121 °C/1.1 bar for 20 min. Following autoclaving, the polysaccharides were separated from the supernatant by the addition of 100 % ethanol, causing them to precipitate. These precipitates were then dried and milled for administration in the feeding trials.

2.3. Beta-glucan quantification

Extracts were analysed for 1,3–1,6 beta-glucan content using the Megazyme yeast and mushroom kit (K-YBGL) (Megazyme Ltd., Bray, Co. Wicklow, Ireland). Assays were carried out according to manufacturer's instructions. After milling, samples were placed in 12 M H_2SO_4 at −4 °C for 2 h to solubilize the beta-glucans. Samples were then hydrolysed in 2 M H_2SO_4 at 100 °C for a further 2 h. Any remaining beta-glucan fragments were quantitatively hydrolysed to glucose using a mixture of exo-1,3-beta-glucanase and beta-glucosidase which gives a measurement of total beta-glucan content after substrate addition. The alpha-glucan content of the sample was determined by hydrolysing specifically to d-glucose and d-fructose. Glucose was measured with amyloglucosidase and invertase using a glucose oxidase peroxidase GOPOD reagent. Beta-glucan content was determined by the difference between the two measurements.

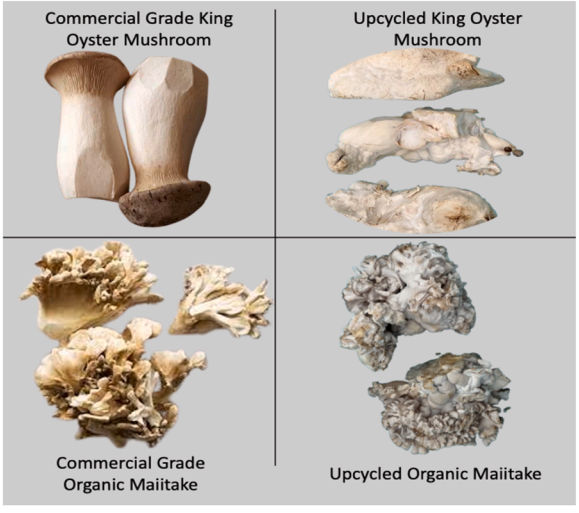

Fig. 1. Comparison of commercial and upcycled mushroom biomass for beta-glucan extraction. In line with sustainable practices, our methodology involved using beta-glucans from mushroom parts that were unsuitable for sale due to their irregular shapes.

2.4. Beta-glucan structural characterisation

In the analysis, ATR-FTIR was conducted using a Perkin Elmer Spectrum instrument equipped with a universal ATR sampling accessory (Perkin Elmer, USA). All measurements were taken at room temperature within the spectral range of 4000–650 cm^{-1}. Each sample underwent 16 scans, and a constant universal compression force of 80 N was applied. Post-analysis was performed using Spectrum software.

2.5. Oyster collection and acclimatization

The primary purpose of this study was to conduct a short-term, acute exposure trial to evaluate the immediate immunological and physiological responses of *Crassostrea gigas* to beta-glucan supplementation when challenged with common bacterial pathogens.

Depurated oysters (*Crassostrea gigas*) were obtained from a local certified producer and acclimatized for 24 h in sterile artificial seawater (Red Sea Salt, 35 g/L) at 16 °C, when normal shell activity was observed. The oysters were fed with 15 mL of a fresh microalgae culture at 5×10^4 cells/mL of *Spirulina platensis*, which was also used as basal feed during the entire experiment. Four setups were prepared each containing a total of 10 oysters, and a 48-hour exposure performed as follows. Group 1 - Control: oysters fed with *S. platensis*; Group 2 - Treatment: oysters fed with *S. platensis* and beta-glucan extract; Group 3 - Injury: oysters fed with *S. platensis* and insulted with bacteria; Group 4 – Injury and treatment; oysters fed with *S. platensis*, beta-glucan extract, and insulted with bacteria (4) as per Fig. 2.

Oysters were kept in sterile tanks containing 5 L of sterile artificial seawater at 16 °C with constant aeration. *Escherichia* sp. and *Vibrio* sp. were used to expose oysters to contamination due to its importance and number of intoxication cases associated to consumption of raw shellfish. A 10 mL blend of *Escherichia* sp. (4×10^6 CFU/mL) and *Vibrio* sp. (2.1×10^5 CFU/mL) was used to inoculate groups (3) and (4) at 0 and 24 h. Both bacteria were cultured in Luria Bertani broth for 24 h at 37 °C before inoculation. For the groups (2) and (4), beta-glucan was administered by directly homogenising 500 mg of extracted beta-glucan into the *Spirulina platensis* microalgae suspension used as basal feed. This mixture was freshly prepared and provided to oysters at the beginning of the experiment (0 h) and again at 24 h. The beta-glucan was not encapsulated or embedded in any carrier particles, instead, it was uniformly dispersed in the microalgae solution to ensure even intake. This method was chosen to reflect a practical and scalable approach suitable for short-term applications such as depuration or stress mitigation, while allowing for direct interaction of the bioactive compound with oyster digestive tissues.

2.5. Survival observations

The survival observation of *C. gigas* during the acclimation and experiments was assessed by appearance and shell activity. Any oyster with the shell open and no activity was prodded with a sterile pipette, and no response was considered non-viable as per S.E. . During the experimental period, the oysters were observed at 10:00 and 17:00 daily.

2.6. Sampling of hemolymph and excision of gills, mantle, and digestive tract

The complete procedure, including hemolymph extraction, and excision of gills, mantle, and digestive tract was performed within a timeframe of <100 s to prevent potential stress-induced effects on the oyster. First, oysters were shucked with an oyster knife and liquid present in the inner shell discarded. Hemolymph was extracted from the pericardial cavity using a 200 μL sterile tip. Hemolymph samples were analysed to quantify the viable hemocytes (VH). Gills, mantle, and digestive tract were dissected using a sterile razor blade and forceps. Dissected tissues were transferred to 1.5 mL tubes containing 500 μL of buffer (25 mM KH_2PO_4, 25 mM K_2HPO_4, pH 7) and stored at −80 °C until analysis. Tissues were further used to quantify the levels of protein, antioxidant activity, bacteriostatic activity, and 16S rRNA sequencing for microbiome analysis.

2.7. Oyster tissue homogenization

The dissected tissues were homogenized prior the assays of total protein quantification, antioxidant activity, bacteriostatic activity, and 16S rRNA sequencing. Dissected tissues were weighed and placed in 1.5 mL tubes with buffer (25 mM KH_2PO_4, 25 mM K_2HPO_4 pH 7) in ice bath. Two glass beads were added to each tube, vortexed for 2 min, and immediately placed back in ice. The tubes were then centrifuged at

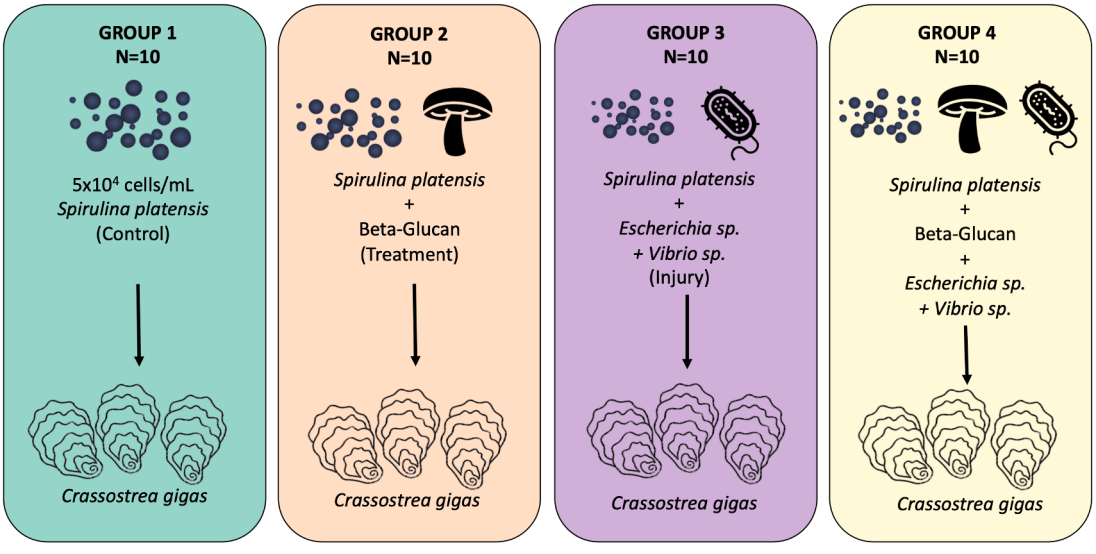

Fig. 2. Graphical representation of treatments or injuries within each group in a 48-hour experimental duration. Group 1 - Control: oysters fed with Spirulina platensis (5×10^4 cells/mL); Group 2 - Treatment: oysters fed with S. platensis and beta-glucan extract; Group 3 - Injury: oysters fed with S. platensis and insulted with bacteria; Group 4 – Injury and treatment; oysters fed with S. platensis, beta-glucan extract, and insulted with bacteria.

10,000 x g for 10 min at 4 °C, and the supernatant collected for each of the assays.

2.8. Flow cytometry analysis

A flow cytometer MACSQuant Analyser (Miltenyi Biotec, Germany) was used to analyse viable hemocytes (VH) in hemolymph, and viable bacteria in the bacteriostatic activity, performed as reported by Donaghy et al. (2009) and A. with modifications. The fluorescent dye SYBR® green I (Invitrogen) was used to stain the double-stranded DNA in VH, and viable bacteria in the bacteriostatic assessment. Hemolymph samples were diluted 10X in sterile artificial seawater (35 g/L) and incubated with SYBR® green for 10 min at room temperature. Samples were washed with flow buffer (FB) (Miltenyi Biotec, Germany), centrifuged at 1500 rpm for 3 min, and resuspended in 200 μL of FB prior analysis. A negative control was prepared sonicating diluted hemolymph samples for 10 min at room temperature and incubated with SYBR® as described previously. A non-stained control was also prepared, and both negative and non-stained controls were referenced on gating process of VH. First, cells were selected (Side scatter x Forward scatter), singlets separated from doublets (Area scatter x Forward scatter), and viable cells emitting green fluorescence from SYBR® green staining verified in channel FITC-A (FITC-A x Forward scatter). VH were expressed as cells per volume of sample, converted to cells per mL. For bacterial staining in bacteriostatic activity, samples were diluted 50X in phosphate saline solution (PBS), washed twice with 500 μL FB followed by centrifugation at 8000 rpm. The washed pellet was resuspended in 200 μL of running buffer and incubated with SYBR® green for 40 min at 30 °C. Non-stained and negative controls were also prepared. Negative control was sonicated for 40 min prior to analysis and stained as reported in this section. For non-stained samples, SYBR® green was replaced by PBS.

2.8. Total protein quantification

Total protein was quantified in supernatant from tissue homogenization using the Pierce BCA Protein assay kit (Thermo Scientific Pierce BCA protein assay kit. Pierce BCA, 449). A working solution (WS) was prepared mixing 50 parts of reagent A to 1 part of reagent B. Samples were quantified in 96 well plates, and 25 μL of sample pipetted with 200 μL of WS and incubated for 30 min at 37 °C. After incubation, absorbance was verified in spectrophotometer at 562 nm. A BSA standard curve with 6 concentrations ranging from 20 to 2000 μg/mL was prepared to calculate total protein in samples. All results were then standardized by wet weight (g) of tissue in the samples.

2.9. Antioxidant activity

The antioxidant content of samples supernatant from hemolymph, homogenised tissues of oyster gills, mantle, and digestive tract were analysed by ferric reducing antioxidant power (FRAP) assays according to the method described by I.F.F. . The assay reaction involves the reduction of Fe^{3+}—TPTZ (iron [III]−2,4,6-tripyridyl-S-triazine) to Fe^{2+}-TPTZ with an antioxidant present in the sample, resulting in an intense blue colour. In this assay, 10 μL of sample was mixed with 190 μL of FRAP Reagent. A blank was also prepared replacing the sample volume by distilled water. The 96 well-plates were incubated at room temperature in the dark for 20 min and measured at 593 nm. To calculate the ferrous sulphate equivalent, a calibration curve of ferrous sulphate was prepared at 1000, 500, 250, 125, 62.5, 31.25, 15.625 and 0 μM. The ferrous sulphate equivalent for each individual sample per gram of dry weight (FE mg/g dw) was calculated and used to determine the ferric reducing ability for each individual sample.

2.10. Bacteriostatic activity

For validating the presence of compounds within oyster tissues that might affect bacterial growth, supernatant from dissected and homogenised tissue samples were analysed. A 48 well plate was used in the experiments and 50 μL of tissue supernatant, 100 μL of Tryptic Soy Broth, and 150 μL of *Vibrio* sp. at 7.5×10^3 CFU/mL added to each well and incubated for 24 h at 37 °C. Samples were collected and centrifuged at 9000 x g for 5 min, washed twice with 500 μL of WB, and analysed by flow-cytometry as described in Section 2.8.

2.11. Next generation sequencing 16S rRNA

The 16S rRNA sequencing kit by MinION Nanopore was used to identify and compare bacterial diversity in the excised tissues, based on the presence of both highly variant regions (different across species) and highly conserved (adequate for universal primers/phylogenetic signal). For sample preparation, the tissue samples from gills, mantle, and digestive tract were homogenised by vortexing with two glass beads and 500 μL of buffer for 2 min. A volume of 200 μL of homogenised tissue was used to inoculate glass tubes containing Luria Bertani agar, with a stab strategy, allowing the development of aerobic and anaerobic microorganisms, and incubated for 24 h at 37 °C. A loop from both anaerobic and aerobic regions was collected and bacterial genomic DNA extracted as described on the GenElute kit, with modifications. Samples were incubated with 200 μL of lysozyme for 30 min at 37 °C, followed by a second incubation with 20 μL of proteinase K and 200 μL of lysis solution C for 10 min at 55 °C. The subsequent steps of column preparation, binding, and washing were performed following manufacturer instructions as described in the GenElute kit (Sigma-Aldrich). The DNA retained in the column was eluted with elution solution (10 mM Tris–HCl, 0.5 mM EDTA, pH 9.0) and DNA quantified in Qubit fluorometer. Gel electrophoresis was used to verify the purity of DNA extracts, stored at −20 °C until analysis. The protocol for 16S rRNA sequencing of bacteria using MinION Nanopore and 16S barcoding kit 1–24 was performed as described on the SQK-16S114.24 protocol, with steps involving quality check, library preparation, sequencing, and analysis.

2.12. Statistical analysis

All statistical analyses were conducted using Statistica software version 10 (StatSoft, USA). Data were tested for normality and homogeneity of variance prior analysis. Differences between treatment groups were assessed using Student's *t*-test for comparison of means when data met parametric assumptions. For multiple group comparisons of means, one-way ANOVA followed by Tukey's post hoc test was used to identify statistically significant differences ($p < 0.05$). Kaplan-Meier survival analysis with a log-rank test was applied to evaluate differences in oyster survival across treatment groups. All experiments were conducted with at least three biological replicates per treatment, and results are expressed as mean ± standard deviation unless otherwise stated. Statistical significance was set at $p < 0.05$.

3. Results

3.1. Beta-glucan quantification

The beta-glucan content in upcycled mushroom fruiting bodies is depicted in Fig. 3. A beta-glucan content of approximately 81 % w/w and 4 % alpha-glucan w/w was achieved. The extraction method yielded results consistent with those previously reported by Murphy et al. (2022), affirming that mushroom waste or by-products contain comparable levels of beta-glucan and can be extracted using similar techniques. Additionally, lower levels of alpha-glucan were observed, an important finding considering the potential antagonistic interaction between alpha-glucan and beta-glucan.

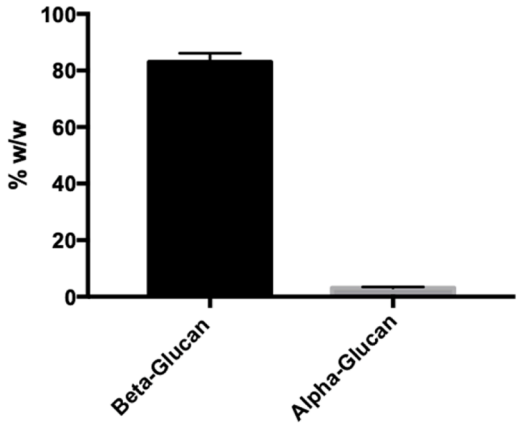

Fig. 3. Quantitative analysis of beta-glucan and alpha-glucan content in mushroom extracts from both Maiitake and King oyster mushroom using the Megazyme yeast and mushroom kit (K-YBGL).

3.2. Beta-glucan structural characterisation

The characterizations of beta-glucan extract are presented in Fig. 4. OH stretching vibrations of hydroxyls from the glucose structure in sugar ring and water was observed at 3233 cm^{-1}. Methyl (-CH_2) stretching vibrations of CH_2OH groups in sugars were assigned to 2922 cm^{-1}. At 1587.19 cm^{-1}, peak attributed to N—H bending vibrations indicates possible protein content. At 1403 cm^{-1}, characteristics peaks for CH_2 in-plane ring deformation including CH and OH bending modes in the polysaccharide structure were observed (Andriy Synytsya & Miroslav Novak, 2014; Novák et al., 2012). The intense peaks at 994 and 1040 cm^{-1} are typical for beta-glucans (CO)(CC). Last, alpha-glucans were observed at 530 cm^{-1} (Šandula et al., 1999).

3.3. Survival observations

Survival was assessed by Kaplan-Meier analysis and verified by visual inspection and shell activity (Fig. 5). No deaths were observed for the groups 1 (microalgae), 2 (microalgae + beta-glucan), and 4 (microalgae + bacteria + beta-glucan). The group 3 (microalgae + bacteria) showed a significantly lower survival rate as compared to the other 3 tested groups (microalgae + bacteria versus microalgae versus microalgae + beta-glucan versus microalgae + bacteria + beta-glucan; 70 % versus 100% versus 100 % versus 100 % survival; log-rank p-value=0.024).

3.4. Viable hemocytes

Viable hemocytes (VH) were assessed by flow cytometry analysis (Fig. 6). The highest count was identified in the treatment group 3 (microalgae + bacteria), statistically higher than groups 1 (microalgae), 2 (microalgae + beta-glucan), and 4 (microalgae + beta-glucan + bacteria). The group treated with microalgae and beta-glucan presented the lowest VH. This result indicates that adding bacteria to microalgae significantly boosts cell concentration, whereas adding beta-glucan alone has no substantial effect. Additionally, the simultaneous addition of both bacteria and beta-glucan does not significantly alter cell concentrations compared to the addition of bacteria alone. Results demonstrated that beta-glucan could reduce the number of inflammatory cells after infection.

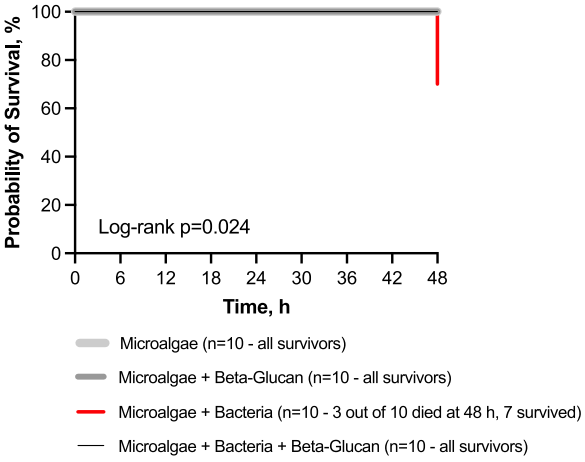

Fig. 5. Survival was explored by a Kaplan-Meier analysis and differences in survival between the groups were tested by log-rank test. Two-tailed p-value<0.05 was deemed significant. Survival was different between the 4 tested groups.

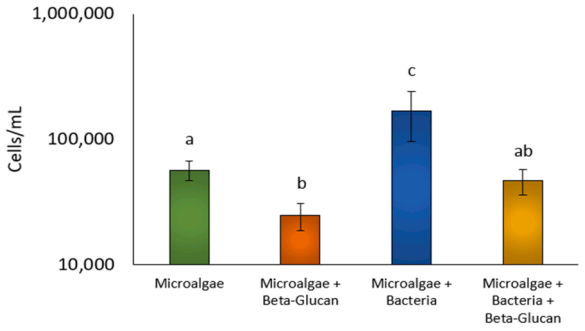

Fig. 6. Viable hemocytes in hemolymph measured by flow cytometry across four groups: group 1 (microalgae), 2 (microalgae + beta-glucan), 3 (microalgae + bacteria) and 4 (microalgae + bacteria + beta-glucan). Statistical significance is denoted by letters (p-value < 0.05).

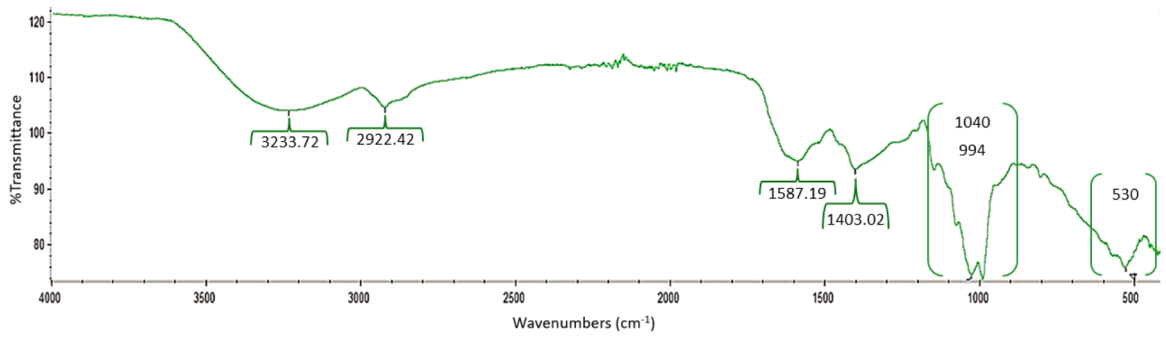

Fig. 4. Spectral analysis of beta-glucan extract from Maiitake and King Oyster mushrooms using ATR-FTIR was conducted using a Perkin Elmer Spectrum instrument equipped with a universal ATR sampling accessory (Perkin Elmer, USA).

3.5. Total protein in tissues (Gills, mantle and digestive tract)

Fig. 7 presents total protein levels in the dissected gills (1), mantle (2), and digestive tract (3). The data reveal that the addition of beta-glucan alone enhances protein content. When combined with bacteria, beta-glucan reduces protein content. The simultaneous combination of microalgae, bacteria, and beta-glucan results in protein levels similar to those observed with microalgae alone, indicating that beta-glucan may counteract the detrimental effects of bacteria. Panel 3 shows a similar modulation trend, potentially indicating a regulatory effect of beta-glucan on protein levels in response to bacterial presence.

3.6. Antioxidant activity in tissues (Gills, mantle and digestive tract) and hemolymph

Fig. 8 and 9 displays the antioxidant activity observed in the tissue samples and in the hemolymph, respectively, determined by FRAP assay. Antioxidant activity was highest in the gills when oysters were fed only with microalgae. In the mantle and digestive tract, the highest levels of antioxidant activity were observed with beta-glucan alone. While antioxidant potential in the gills decreased, it remained consistent

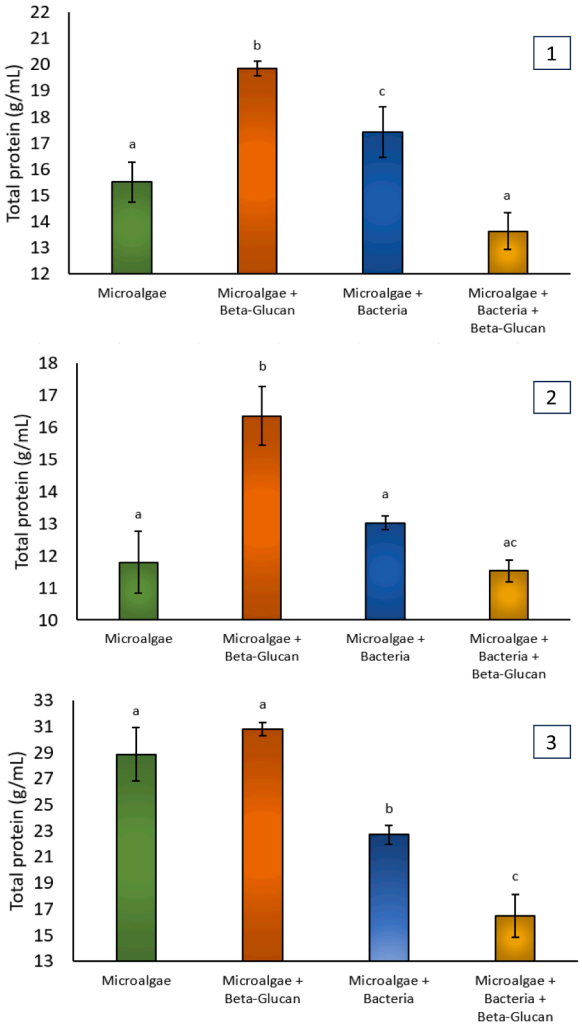

Fig. 7. Comparative analysis of total protein content in tissues following exposure to various treatments is illustrated across three panels. Group 1 (microalgae), 2 (microalgae + beta-glucan), 3 (microalgae + bacteria) and 4 (microalgae + bacteria + beta-glucan). The protein content was assessed in three different tissues: Panel 1 for gills, Panel 2 for mantle, and Panel 3 for the digestive tract. Letters indicate statistical significance (p-value < 0.05).

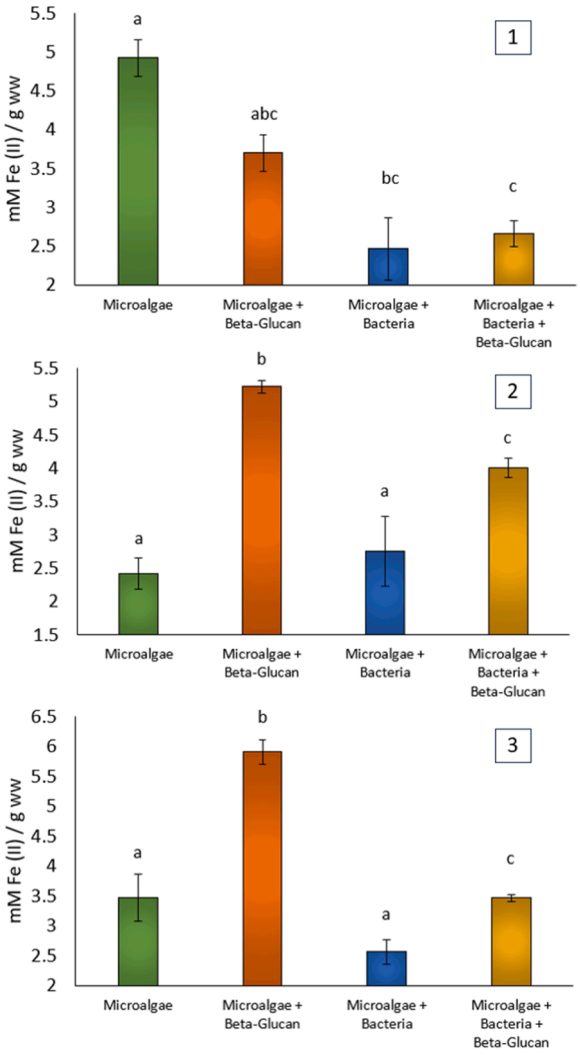

Fig. 8. Comparative analysis of antioxidant activity in tissues as determined by FRAP in Group 1 (microalgae), 2 (microalgae + beta-glucan), 3 (microalgae + bacteria) and 4 (microalgae + bacteria + beta-glucan) with results displayed across three panels for different tissues: Panel 1 for gills, Panel 2 for mantle, and Panel 3 for the digestive tract. Letters indicate statistical significance (p-value < 0.05).

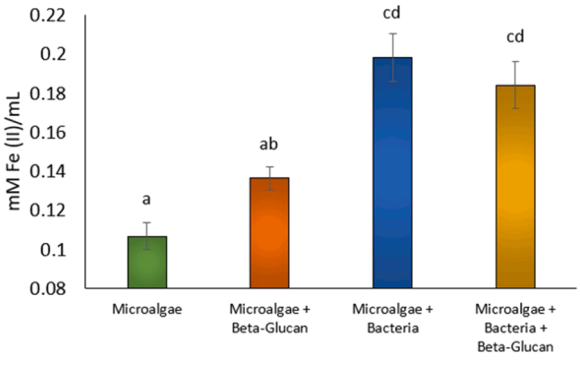

Fig. 9. Comparative analysis of antioxidant activity in the hemolymph as determined by FRAP in Group 1 (microalgae), 2 (microalgae + beta-glucan), 3 (microalgae + bacteria) and 4 (microalgae + bacteria + beta-glucan). Letters indicate statistical significance (p-value < 0.05).

with the levels observed in microalgae alone in both the mantle and digestive tract. Notably, beta-glucan enhanced antioxidant activity in the mantle and digestive tracts when combined with an injury-inducing treatment. In the hemolymph, beta-glucan alone did not raise antioxidant activity levels, and it did not alter these levels when combined with bacteria.

3.8. Bacteriostatic activity

Supernatant from dissected and homogenised gills, mantle, and digestive tract tissues were incubated in nutrient media with *Vibrio* sp. at 37 °C for 24 h. Bacterial levels in Groups 1 and 2, not previously incubated with the bacterial blend, presented similar loads in the gills and mantle. Cell counts were lower in its digestive tract, while Group 3 treated with bacteria and incubated a second time with *Vibrio* sp. presented the highest counts in the experiment. Group 4, also previously incubated with bacteria but with the addition of beta-glucan, presented the lowest counts in the gills, mantle, and not statistically different than Groups 1 and 2 for digestive tracts samples (Fig. 10).

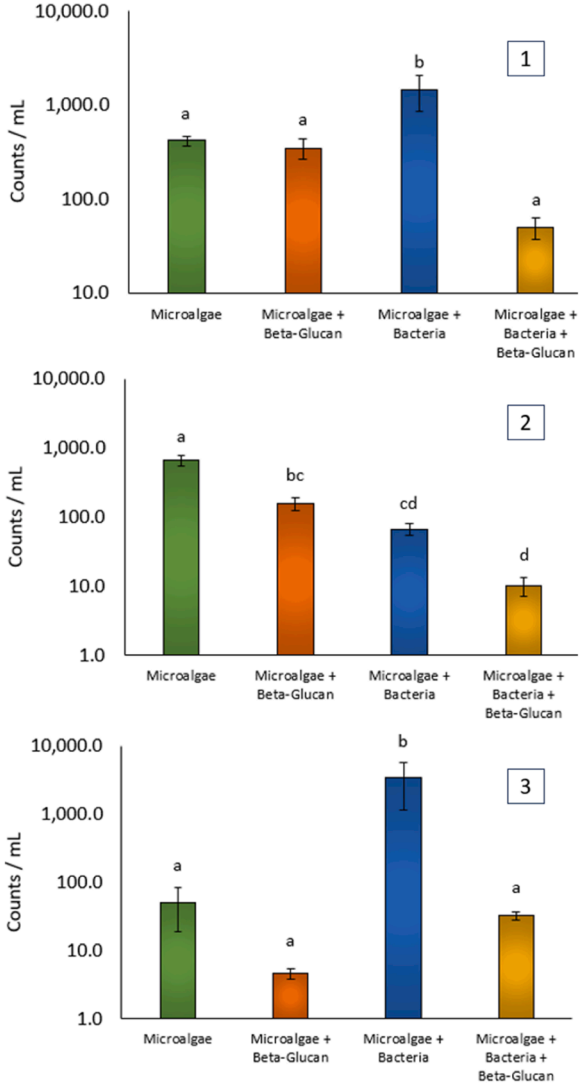

Fig. 10. Bacterial cell counting post-treatment after 24 H of incubation with *Vibrio* sp. This figure details the bacterial counts in three distinct tissues: Panel 1 - Gills, Panel 2 - Mantle, and Panel 3 - Digestive Tract. Group 1 (microalgae), 2 (microalgae + beta-glucan), 3 (microalgae + bacteria) and 4 (microalgae + bacteria + beta-glucan). Letters indicate statistical significance (p-value < 0.05).

3.9. Next generation sequencing determination

Bacterial diversity identified by 16S rRNA sequencing in the excised tissues is presented in Fig. 11 (Group 3) and Fig. 12 (Group 4). *Escherichia* was predominant in both groups, and the following Genuses were observed for both groups with slightly different frequencies: *Kosakonia, Citrobacter, Shigella, Serratia, Vibrio, Enterobacter, Sodalis, Xenorhabdus, Shimwellia, Erwinia,* and *Mixta.*

4. Discussion

Infectious bacterial diseases have long been a significant challenge impeding the sustainable growth of the aquaculture industry (Baert et al., 2015; Thitamadee et al., 2016). Traditional antibiotics, while somewhat effective, have had limited success in treating these diseases (Defoirdt et al., 2011). Moreover, the misuse of antibiotics has raised serious concerns due to the emergence of antibiotic-resistant strains, posing a substantial risk to human health (Stanton, 2013). Immunostimulants are increasingly used in aquatic farming to boost natural immune defences and tackle antibiotic-resistant pathogens.They complement vaccination and selective breeding in disease prevention, enhancing phagocytic cells' performance for more effective bacteria elimination (Loor et al., 2022; Pogue et al., 2021; Takahashi et al., 2017). While the beneficial impact of beta-glucans on the immune system in humans and animals is well-known, the effectiveness of bioactives isolated from upcycled waste in comparison remains less explored. Therefore, to achieve greater circularity is crucial to investigate whether bioactives derived from upcycled materials yield the same beneficial effects as those sourced from non-waste origins. This exploration is key to advancing sustainable practices and maximizing resource efficiency. Thus, the overall purpose of this study was to determine if beta-glucans isolated from upcycled mushroom waste could induce a protective effect in oysters insulted with pathogenic bacteria.

Equally under-investigated is the influence of mushroom-derived beta-glucans on oysters, particularly regarding their impact on key factors such as protein levels, antimicrobial properties, antioxidant levels, and the balance of commensal and pathogenic bacteria. This study measured these parameters in both the presence and absence of pathogenic bacteria. This approach aimed to assess the priming capabilities of beta-glucan on its own, as well as its modulation properties. This study demonstrates that comparable levels of beta-glucan can be isolated from commercial mushrooms and mushroom waste, with the beta-glucan from waste exhibiting similar bioactivity. Many publications on beta-glucans do not fully disclose extraction techniques or provide detailed analysis of structure and purity. To address this issue, this study details the extraction process and evaluates the purity and structure of the isolated beta-glucans. Purity was assessed using the

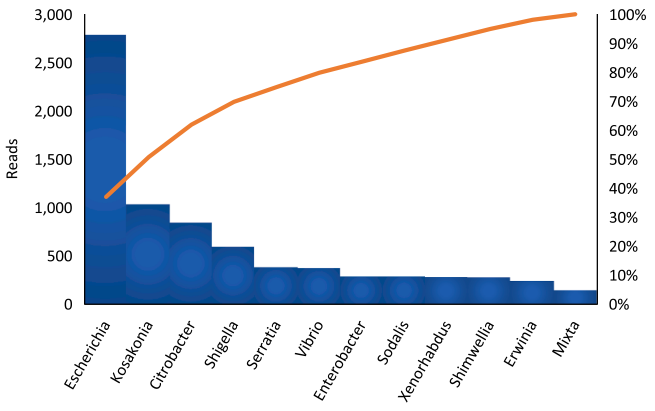

Fig. 11. Pareto chart of 16S rRNA sequencing results of Group 3 (microalgae + bacteria) samples ranked by Genus's level.

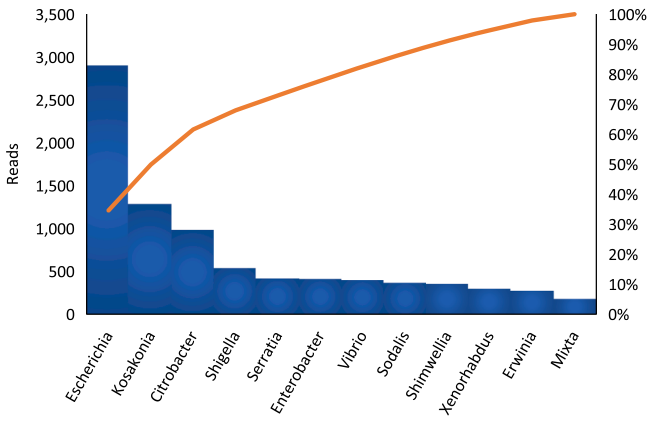

Fig. 12. Pareto chart of 16S rRNA sequencing results of Group 4 (microalgae + bacteria + glucan) samples ranked by Genus's level.

Megazyme beta-glucan kit which measures both beta-glucan and alpha-glucan content. Structural properties were analysed through Fourier-transform infrared spectroscopy (FTIR), comparing them to literature results. This approach enhances the transparency and reproducibility of our results.

The structural details of beta-glucans are essential, as they are intricately tied to their biological functions. Additionally, the biological impacts of crude extracts can substantially vary from those of more refined samples, largely due to differences in their linkage patterns. Our results revealed a significant beta-glucan concentration, approximately 80 % w/w, with a minimal presence of alpha-glucan (Fig. 3). Structurally, the beta-glucan extracted from King Oyster and Maitake closely matched with FTIR results (Fig. 4) for beta-glucans extracts from mushroom such as *Pleurotus* and *Schizophyllum* reported in the literature (Nasir et al., 2021). Therefore, biological activity could be correlated to 1,3–1,6 beta-glucan from upcycled mushroom material.

Mushroom and cereal beta-glucans differ in structure and function. Mushroom beta-glucans have a beta−1,3 backbone with beta−1,6 branches, forming a branched structure, while cereal beta-glucans are linear with beta−1,3/1,4 linkages and no beta−1,6 branches (Cerletti et al., 2021). These structural differences effect their molecular behaviour—mushroom beta-glucans form triple-helical structures, making them semi-rigid, highly viscous, and stable, whereas cereal beta-glucans remain flexible random coils in solution (Du et al., 2019). All beta-glucans are hydrophilic due to abundant hydroxyl (-OH) groups, giving them high water-binding capacity, which increases viscosity in aqueous systems (Kaur et al., 2019). This property may enhance their functionality in aquaculture feeding by improving bioavailability, nutrient uptake, and immune interactions in oysters (Lante et al., 2023b; Sun et al., 2021). Their viscosity also influences dispersibility, ingestion efficiency, and digestive processing. In this study, beta-glucan administration increased protein content in oyster tissues (Fig. 7) and modulated hemocyte viability (Fig. 6), suggesting effects on metabolism and immune response. These outcomes may be linked to solubility and gel-forming properties, which could impact stress mitigation, nutrient retention, and bacteriostatic activity (Lante et al., 2023b; Sun et al., 2021). Both molecular weight and conformation determine beta-glucan viscosity. Long-chain, high-MW beta-glucans impart greater viscosity (Chioru et al., 2023; Kaur et al., 2019) while the triple-helix structure of mushroom beta-glucans further enhances viscosity, as seen with lentinan, which remains highly viscous even at low concentrations (Xu et al., 2010). Hot water extraction used in this study preserves the triple-helix, preventing its denaturation. When helices are disrupted by alkali or DMSO, chains transition to single coils, reducing viscosity, highlighting the role of structural integrity in rheological properties (Manabe & Yamaguchi, 2021).

In addition to fungal-derived beta-glucans, those extracted from *Saccharomyces cerevisiae* (yeast) and certain bacterial species share similar structural features, particularly beta-(1,3) backbones with beta-(1,6) size chains. These have been widely studied for their immunomodulatory effects in aquatic species While the core structure is conserved, subtle differences in branching degree, solubility, and molecular weight can influence their receptor binding affinity and downstream immune responses (Andriy Synytsya & Miroslav Novak, 2014). The effects observed in our study are in line with these known immune-stimulatory activities, suggesting that mushroom-derived beta-glucans may activate similar pathways, such as dectin-1 receptor signalling and complement-mediated responses. Although not the primary focus of this study, alpha-glucans were also detected in our beta-glucan preparations. While alpha-glucans such as dextran or glycogen are generally not considered strong immune activators, they may still play roles in supporting gut microbial health or as fermentable carbohydrates with prebiotic potential (Miao et al., 2016).

Regarding survival, oysters fed microalgae combined with bacteria exhibited a markedly lower survival rate compared to the other three groups. This suggests a protective effect of beta-glucan against pathogenic bacteria, as evidenced by complete survival in the beta-glucan group. Similarly, a study by Loor et al. (2022) found that oysters fed a diet containing the yeast cell wall Δmnn9 mutant (50A50Y) demonstrated significantly higher survival rates following a *Vibrio coralliilyticus* bacterial challenge. This was in contrast to groups fed with wild-type yeast (50A50WT) or a standard algae-based diet (100A), highlighting the potential protective role of specific yeast cell wall components in enhancing oyster survival against bacterial infections. Do-Huu et al. (2024) reported a higher survival rate for *Gnathanodon speciosus* fed with beta-glucan and challenged with *Streptococcus iniae*. To reduce stress cause by low-salinity, Qiao et al. (2022) assessed the effect of a diet with beta-glucan to *Litopenaeus vannamei*. Higher survival rates were reported in groups treated with 0.1 and 0.4 % beta-glucan, when compared to control, however not statistically significant. These results suggest that beta-glucan might be an interesting alternative to support highly contaminated shellfish purge/recovery in depuration plants. Further research is needed to evaluate its effectiveness in depuration plants.

Hemocytes are key components of the immune system and are involved in the response to pathogens, toxins, and other stressors. The increase in the number of cells in the hemolymph of oysters after bacteria administration can be attributed to the activation of various immune responses (Impellitteri et al., 2022). The highest level of viable hemocytes (VH) observed in the group fed with microalgae and bacteria may indicate a higher stress level and response to the bacteria, in the same group which the survival rate was significantly lower than others. Beta-glucan added to feeding reduced the VH, and statistically lower than control with microalgae. The beta-glucan administration with bacteria reduced the VH back to the same count as control. This potentially indicates a modulation activity of the beta-glucan. Other research has shown that the administration of beta-glucan can lead to an increase in the number of circulating hemocytes in the Eastern oyster (*Crassostrea virginica*) (Anderson et al., 2011). Hemocytes, crucial for defence against pathogens, were observed after beta 1,3 glucan injections, including zymosan A and MacroGard. The study found that MacroGard significantly increased the total circulating hemocytes for three days, while zymosan A's effect was not significant. Both treatments increased granulocytes, the most immunologically active hemocytes, and caused hemocytes aggregation, indicative of cell activation. This suggests yeast derived beta-glucans enhance oyster immune response by increasing available hemocytes, shifting the hemocyte profile towards more active cells, and promoting aggregation (Anderson et al., 2011). The differences observed between this study and others for TVL count could be correlated to beta-glucan structure. Yeast and mushroom contain similar structures in that they are 1,3–1,6 linked. However, side chain lengths and degrees of polymerisation may vary which would influence different bioactivity profiles.

The variation on protein levels in shellfish might be associated to a

response to stress factors. Elevated protein levels often reflect immunocompetence, and the presence and abundance of specific proteins can provide additional information on shellfish condition (Coates & Söderhäll, 2021). In this study, protein levels were measured in three tissues – gill, mantle, and digestive tract. Group 1, which was fed only microalgae and not exposed to bacteria, showed consistent protein levels in the gills and mantle, with levels in the digestive tract being twice as high. Group 2, which received microalgae and beta-glucan, displayed the highest protein levels observed in the study, significantly exceeding those in Group 4, which was treated with microalgae, beta-glucan, and bacteria. Group 3, fed with microalgae and bacteria, showed protein levels higher than those in Group 4 across all tissues but lower than those in Group 2 for gills and mantle, and lower than Group 1 for the digestive tract. Using Group 1 as a baseline representing normal physiological conditions, it was observed that beta-glucan alone increases protein levels in tissues, except for the digestive tract. Given that beta-glucans from mushrooms are digested in the gut and interact with macrophages in Peyer's patches (De Jesus et al., 2014; Hashimoto et al., 1991; Murphy, Rezoagli et al., 2020). It's plausible that a similar process occurs here, whereby beta-glucans are transported to other tissues, potentially inducing inflammation, or recruiting other immune cells. Beta-glucan acts as a priming agent as it is recognised as a pathogen associated molecular patterns on its own but exhibits a different activity profile in the presence of bacteria (Krishnan et al., 2022). These results mirror our previous mechanisms in macrophages: in the absence of injury, mushroom beta-glucans can induce inflammation, evidenced by increased IL-8, an inflammatory mediator. Conversely, in the presence of injury, these beta-glucans seem to suppress IL-8 secretion. While the underlying mechanism remains unclear, this study confirms a similar response pattern in shellfish, suggesting that beta-glucans might modulate immune responses similarly across different species (Murphy et al., 2022).

Nguyen et al. (2019) examined tissue-specific immune responses to *Vibrio sp*. infection in the mussel *Perna canaliculus*, noting significant tissue-dependent differences. These variations were potentially associated with changes in itaconic acid, amino acids, and fatty acids, likely linked to osmotic stress, amino acid metabolism shifts, oxidative stress, and altered protein synthesis in the immune system. Given the differences between shellfish species, a more detailed study on amino acids and protein transcriptome is essential to confirm these proteins as stress markers for *Crassostrea gigas*.

Antioxidant activity in shellfish tissues provides valuable information about the organism's health, environmental exposure, nutritional value, adaptation to habitat conditions, and the effectiveness of aquaculture management practices (Freitas et al., 2020). In this study, *C. gigas* fed with beta-glucan (Group 2) presented a higher antioxidant activity than negative control (Group 1) in mantle and digestive tract, and when exposed to bacteria, Group 4 fed with beta-glucan had the highest antioxidant activity than Group 3 in all three tissues. Beta-glucan stimulated the production of antioxidant compounds, providing a higher protection against adversities and stress factors such as the bacterial assessed in this study. A different pattern was observed for antioxidant activity in the hemolymph. Highest antioxidant activity was observed in the Group 3 exposed to bacteria, followed by Group 4 (beta-glucan, microalgae, and bacteria), Group 2 (microalgae and beta-glucan), and Group 1 (microalgae). This could be correlated to how beta-glucan is digested and dispersed in the oyster. Moreover, the observed increase in antioxidant activity and protein content is consistent with in vivo effects seen in *Haliotis diversicolor* (Wu et al., 2016), where beta-glucan administration elevated multiple immune and antioxidant parameters.

Bacteriostatic activity is the ability of inhibiting the growth or reproduction of bacteria without necessarily killing them, working by interfering with essential bacterial processes, such as protein synthesis, or cell wall formation. It differs from bactericidal where the agent directly kills bacteria (Pankey & Sabath, 2004). Bacteriostatic agents are also potential alternatives to extend the 'shelf time' of seafood products and decrease infection process. At the oyster industry, most of the fresh shellfish commercialized is retained in depuration plants until bacterial and viral levels are within stablished by regulatory agencies (Fehrenbach et al., 2023). A bacteriostatic agent might support this process and stimulate oyster immune system improving its resistance to bacterial contaminations and stress factors. Bacteriostatic activity was observed in tissues previous exposed to bacteria and fed with beta-glucan, with lower counts in gills and mantle, as Group 2 presented the lowest counts in the digestive tract. Group 3 fed with microalgae and bacteria, and subsequently incubated with *Vibrio* sp., presented the highest counts in gills and digestive tract. The bacteriostatic activity detected in oyster tissues following beta-glucan supplementation also parallels the antimicrobial modulation noted in brine shrimp (Han et al., 2020), where beta-glucans enhanced resistance against *Vibrio harveyi*.

The tissues of healthy marine invertebrates host diverse microbial communities, playing important roles in the host's immune response (Yu et al., 2019). While antimicrobial compounds of bacterial origin can be determined in the hemolymph, the microbiota present in oyster tissues have been understudied due challenges such as culture-dependent methods. Traditional cultivation and molecular fingerprinting methods may not accurately reflect community diversity and composition. Next-generation sequencing offers a more detailed characterization of microbial communities. For example, sequencing studies have revealed higher diversity in oyster gut and gill microbiomes, challenging previous notions that the oyster microbiota are dominated by pseudomonads and vibrios. Sequencing and profiling might support tracking oysters' origin and ensuring quality in seafood industry. For example, Singh et al. (2022) reported a distinct microbiome signature when characterizing the microbiome composition and diversity of same oyster species collected from two distinct geographical regions in United States. Microbial sequencing was conducted exclusively on Groups 3 and 4, as the primary aim of this analysis was to investigate the impact of beta-glucan supplementation on the oyster microbiome under pathogenic challenge. In this study, the microbiome in gills, mantle, and digestive tract was assessed to verify the effect on bacterial diversity when feeding and exposing to *Escherichia coli* and *Vibrio* sp. contamination. A high diversity of species was detected in Groups 3 and 4, with a similar predominance as presented by Genus in Fig. 11 and 12. Exposing for 48 h might not have been enough to induce differentiations at microbiome level, and longer periods of experimentation would be necessary. This parameter should be investigated in further studies.

5. Conclusion

Shellfish mortality is a major issue in aquaculture, raising concerns about food security. This study examined the use of beta-glucans from mushroom waste as a sustainable and cost-effective approach to improve oyster health. The extraction and characterization of these beta-glucans highlight their potential to support shellfish survival while also reducing waste in aquaculture.

Beta-glucan treatment improved oyster survival under stress, reduced viable hemocytes, and increased protein levels, suggesting an effect on immune function. Treated oysters also showed higher antioxidant activity, which may help protect against environmental stress. The observed bacteriostatic activity could have applications for seafood shelf-life and disease resistance in aquaculture.

Further research is needed to understand how beta-glucans affect feeding behaviour, gut transit time, and nutrient absorption in oysters. This study was conducted in a controlled setting, and field trials in commercial aquaculture are necessary to confirm these effects. Future work should also assess optimal dosing, long-term impacts, and interactions with other dietary components.

Ethics statement

This study was conducted in accordance with ethical guidelines for the care and use of aquatic organisms in research. All experimental procedures involving oysters (Crassostrea gigas) complied with relevant institutional and national regulations on animal welfare and aquaculture research. No procedures involved vertebrate animals or human participants, and no ethical approval was required. The research adhered to best practices for sustainable aquaculture and environmental responsibility.

CRediT authorship contribution statement

Gustavo Waltzer Fehrenbach: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **João Rui Tanoeiro:** Writing – original draft, Data curation. **Robert Pogue:** Writing – review & editing, Writing – original draft, Supervision, Data curation, Conceptualization. **Frank Carter:** Writing – review & editing, Writing – original draft, Supervision, Data curation, Conceptualization. **Marco F.L. Lemos:** Writing – review & editing, Writing – original draft, Data curation. **Patrick Murray:** Writing – original draft. **Declan Devine:** Writing – review & editing, Writing – original draft, Data curation, Conceptualization. **Emanuele Rezoagli:** Writing – original draft, Data curation. **Ian Major:** Writing – review & editing, Writing – original draft, Supervision, Funding acquisition, Data curation, Conceptualization. **Emma J. Murphy:** Writing – review & editing, Writing – original draft, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

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

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Data availability

Data will be made available on request.

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