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Effects of biosecure macroalgae in formulated feeds on the gut bacterial microbiome of abalone *Haliotis midae*

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Abstract

Dietary macroalgae are known to contribute directly and indirectly to the gut microflora of abalone. However, certain farms require macroalgae to be made biosecure to eliminate microbiota and reduce their risk of acting as a vector for potential disease before being included as a dietary ingredient in aquafeeds. The aim of this study was to assess the effect of biosecure macroalgae on the gut bacterial microbiome of cultured abalone (shell length of 55–65 mm). Fresh macroalgae: brown seaweed *Ecklonia maxima*, green seaweed *Ulva lacinulata*, and red seaweed *Gracilaria gracilis* were subjected to a combination of three biosecurity processing treatments: disinfection with povo-iodine solution, oven drying, and UVC treatment before being incorporated into feed and fed to abalone *Haliotis midae* for 150 days. Control diets that included no macroalgae or non-biosecure macroalgae were also included in the trial. A 16S rDNA next-generation sequencing (NGS) of the hypervariable V4 region demonstrated that the within-sample

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bacterial diversity did not differ significantly across all the tested diets ($p > 0.05$). No significant differences in the overall microbiome composition were detected, indicating a high degree of similarity of the bacterial communities across all the biosecure and non-biosecure macroalgae diets. A similar core microbiome (SAR324 clade [Marine group B], *Mycoplasma*, and *Psychrilyobacter*) was also observed in the digestive tracts of abalone fed with the biosecure and non-biosecure diets, with fine-scale differences between dietary treatments. It was concluded that the biosecure process previously shown to significantly reduce the abundance of bacteria, fungi/oomycetes, and viruses associated with each seaweed prior to incorporation into a formulated feed did not adversely affect other components of the seaweeds that may positively benefit the growth, health, and gut microbiome of the cultured abalone fed these feeds.

KEYWORDS
16S rDNA, *Ecklonia maxima*, next-generation sequencing, oven drying, povo-iodine solution, *Ulva lacinulata* and *Gracillaria gracilis*

1 | INTRODUCTION

The gastrointestinal microbiota of abalone contains a complex bacterial assemblage that is vital to the general health of these gastropods (Cicala et al., 2018). Studies conducted on abalone gut microbial flora have revealed the role of these microorganisms in food digestion and assimilation, providing crucial information to the abalone farming industry (Erasmus et al., 1997; Gobet et al., 2018; Guo, 2017), aquafeed nutritionists, and feed manufacturers. Furthermore, understanding the role of the microorganisms resident in the digestive tract of abalone is a crucial aspect for the improvement of growth rates and health, considering their role in digestive processes and gut health (Gobet et al., 2018).

The South African abalone industry is fast expanding and is the largest producer of cultured abalone outside Asia (FAO, 2004; Robertson-Andersson et al., 2008). Six haliotid species occur in South African waters (*Haliotis midae L.*, *Haliotis parvum L.*, *Haliotis spadicea* [Donovan], *Haliotis queketti* [Smith], *Haliotis speciosa* [Reeve], *Haliotis pustulata* [Reeve]). Of all the six species, only *H. midae* (Linnaeus, 1758), locally known as "perlemoen," is intensively cultured (Sales, 2001). The rest of the species are considered small and non-commercial species (Barkai & Griffiths, 1986). *Haliotis midae* is an opportunistic herbivore, feeding predominantly on kelp in the wild, but also accepting a wide range of diets under culture conditions (Troell et al., 2006). Consequently, many South African abalone farms feed their abalone with fresh brown seaweed *Ecklonia maxima*, green seaweed *Ulva lacinulata*, and red seaweed *Gracillaria gracilis*, as well as formulated feeds, with the latter shown to be crucial for improving the growth of farmed abalone (Britz, 1996). The seaweeds *G. gracilis* and *U. lacinulata* are not harvested from the wild but are grown in tanks or raceways on abalone farms as food for the abalone, often in abalone effluent in integrated multi-trophic aquaculture (IMTA) systems (De Jager et al., 2024). Seaweeds grown in IMTA present several benefits to the farm and the farmed animals fed the seaweeds, but the biosecurity risks associated with this practice need to be considered (Bolton et al., 2016).

The inclusion of both fresh and dried macroalgae or their extracts in aquafeed has been shown to have immunomodulatory and growth-enhancing effects on the fed animals (Gonçalves et al., 2022; Peixoto et al., 2019). This is partially attributed to the polysaccharides (particularly sulfated polysaccharides) and other bioactive compounds in macroalgae that have prebiotic effects and influence the intestinal microbiota by stimulating the growth of beneficial bacterial communities, thus exerting growth-promoting and health-improving effects on the host (Erasmus et al., 1997; Gonçalves et al., 2022; Macey & Coyne, 2005; Nel et al., 2017). In addition, fresh macroalgae have their own bacterial communities (De Jager et al., 2024; Singh & Reddy, 2016), and when digested, many of these bacteria become resident within the digestive tract of the animals but can also be transient. Either way, microorganisms contribute toward the digestion of dietary ingredients, particularly the complex polysaccharides found within seaweeds, and facilitate the assimilation of ingested feed ingredients.

Some consider the supplementation of diets with macroalgae the most promising method of controlling disease in farmed aquatic animals because of the immunostimulants they possess, the beneficial microorganisms they introduce, and the potential modulatory effect seaweeds and their associated microbiota may have on the gut microbiome (Califano et al., 2020; De Jager et al., 2024; Egan et al., 2013; Hindu et al., 2019; Newaj-Fyzul & Austin, 2015). For example, more stable gut bacterial communities were observed in the digestive tract of the South African abalone *H. midae* when fed a kelp-supplemented formulated diet (Nel et al., 2017). This was coupled with improved growth rates and feed conversion ratios (FCR), whereas animals fed a control diet that was not supplemented with seaweed exhibited a higher abundance of potentially opportunistic bacteria (Nel et al., 2017). The latter observations could have important implications for farmed animals, such as abalone, that are subjected to stress during their production cycle, something that is highly likely in intensive aquaculture. An improved understanding of the effects of various diets and dietary ingredients on the gut microbiome is therefore essential for improved management of stock health on aquafarms.

In most cases, macroalgae are supplemented in abalone diets either in a fresh state or after some form of processing, such as drying. However, no direct assessment of the impacts of these processing treatments on the gut microbial composition of the animals fed with the processed macroalga diets has been made. Previous studies have shown that some processing treatments can change the nutritional composition and phytochemical contents of seaweed, which in turn could compromise their functional benefits (Susanto et al., 2017). For example, the microbial diversity and antimicrobial properties of Irish edible brown seaweeds changed following heat treatment at 95°C for 15 min, with complete inactivation of surface microflora observed. In addition, heating the seaweed resulted in the reduction of the antimicrobial activity of the macroalga when compared to raw, untreated seaweed (Gupta et al., 2011). Such findings suggest that the processing of macroalgae could indirectly impact the gut microbiota of the animals fed with these diets.

To date, research on the impact of macroalgae as a supplementary diet or dietary ingredient on host-microbiome interactions in the South African abalone *H. midae* is limited. No experiment has tested the effects that biosecurity-treated macroalgae feed ingredients have on abalone growth and health. Moreover, a data gap exists on the effect of biosecurity processing of macroalgae as a dietary ingredient, besides drying macroalgae, on the gut microbial composition of this important commercial species. This study sets out to contribute new knowledge on the gut microbiome composition of the South African abalone *H. midae* fed varying seaweed-supplemented diets to support the production and associated benefits of formulated diets containing macroalgae.

2 | MATERIALS AND METHODS

2.1 | Study site and experimental system

The study was conducted at Wild Coast Abalone Farm (Pty) Ltd., located on the east coast of South Africa (32°45′02.7″ S, 28°16′29.0″ E), and data were collected under full commercial production conditions. The farm

operates as a land-based, flow-through aquaculture system. All seawater for the production of abalone on this farm is pumped ashore into the abalone rearing tanks from the bordering Indian Ocean using eight pumps, which supply 6250 m^3 of seawater to the farm every hour. Water inflow to each experimental abalone rearing tank was maintained at 75.80 ± 0.53 L/min to allow seven full seawater exchanges per tank per day according to standard practices on the farm. Abalone rearing tanks, measuring 500 × 200 × 80 cm (length × width × height), were made of polyvinyl chloride (PVC) and supported by wooden frames.

The abalone in each tank were stocked in oyster mesh baskets (94 × 54 × 46 cm; length × width × height) that were suspended in the tanks. Each basket contained five vertically oriented square high-density polyethylene plates that formed a rack to increase the internal surface area of each basket. The vertically oriented rack was covered with a rectangular corrugated fiber-crete feeder plate (60 × 45 cm; length × width), which was placed horizontally on top of the vertical plates, approximately 10 cm below the surface of the water. These baskets were suspended in the rearing tanks, and each tank contained eight abalone baskets arranged in series.

2.2 | Experimental animals and diets

Subadult abalone (35–45 g (weight) and 55–65 mm [shell length]) from the same broodstock and offspring cohort were obtained from the farm grow-out facility. Approximately 10.5 kg of abalone (250–350) were stocked in each basket using the farm's standard practice. The animals were acclimated for a period of 2 weeks, during which time they were fed a formulated diet (Abfeed™, Marifeed, South Africa), and after which they were purged for 48 h before the feeding trial started. *Ecklonia maxima* was collected from the seashore in front of the Department of Forestry, Fisheries, and the Environment (DFFE) Marine Aquaculture Research Facility in Sea Point (33°55′6.492″ S, 18°22′52.572″ E), Western Cape, South Africa, while *U. lacinulata* and *G. gracilis* were harvested from production tanks on Wild Coast Abalone Farm Pty Ltd. in the Eastern Cape. A total of 20 kg of fresh macroalgae was collected for each of the macroalgal species. The macroalgae were first washed with clean water to remove any debris, then divided into two batches of 10 kg each. The first batch of macroalgae was subjected to a combination of three biosecurity processes: disinfection using povo-iodine solution (5000 mg/L) for 20 min, oven drying at 40°C for 48 h, and ultraviolet C (UVC) treatment for 10 min (Mwangudza, 2024). The remaining batch of macroalgae was only dried at 40°C for 48 h, which is standard procedure in formulated diet ingredient preparation, and these were considered as not biosecurity-treated (FF_nb; Table 1) and provided a control comparison for the biosecurity-treated macroalgae.

Isonitrogenous (29.6% ± 0.3% protein) and isolipidic (3.0% ± 0.2% fat) diets were manufactured using a commercial abalone feed formulation (Abfeed™, Marifeed Pty Ltd., Hermanus, South Africa). The macroalgae that either had

TABLE 1 A summary of the eight experimental diets tested in this study.

Diet	Diet code
Abfeed™ (formulated diet/control)	FF1
Formulated diet + biosecure *Gracilaria* (Abfeed_b_*Gracilaria*)	FF1_b_G
Formulated diet + non-biosecure *Gracilaria* (Abfeed_nb_*Gracilaria*)	FF1_nb_G
Formulated diet + biosecure *Ulva* (Abfeed_b_*Ulva*)	FF1_b_U
Formulated diet + non-biosecure *Ulva* (Abfeed_nb_*Ulva*)	FF1_nb_U
Formulated diet + biosecure kelp (Abfeed_b_kelp)	FF1_b_K
Formulated diet + non-biosecure kelp (Abfeed_nb_kelp)	FF1_nb_K
Formulated diet + fresh *Ulva* and *Gracilaria*	FF1_FS

Note: The macroalgae included in the diets were subjected to either a biosecure process (_b_) or not subjected to a biosecure process (_nb_). An additional treatment included the formulated diet supplemented with fresh macroalgae. All diets were fed to the abalone for 5 months.
Abbreviations: FF, formulated feed; FS, fresh seaweed; G, *Gracilaria*; K, kelp; U, *Ulva*.

or had not (i.e., the control group) been subjected to the biosecurity process were incorporated into the formulated feed at 3% macroalgae on a dry weight basis (Table 1). A control treatment included abalone fed the basal formulated diet with no macroalgae (Abfeed™ FF1, Table 1).

The seven dietary treatments were each fed to four replicate baskets of abalone using a randomized block design, with each treatment represented once in each tank to ensure standardized conditions. Feeding of the formulated diets was carried out daily between 15:00 and 17:00 using standard farm feeding rates of 0.3%–0.4% of body weight per day (Nel et al., 2017). The rearing tanks were cleaned twice weekly. The trial was conducted for 150 days between November 2020 and April 2021. An additional treatment of the standard farm diet was included to provide a comparison with the biosecure and non-biosecure macroalgae diet treatments. In this treatment, the animals were grown in the commercial standard farm setup and were fed a mixed diet consisting of the formulated diet Abfeed™ S34®, fresh *U. lacinulata*, and fresh *G. gracilis* (Abfeed™_FF1_FS, Table 1). The fresh macroalgae were fed at 0.29% of the body weight of the animals on alternative days as follows, which is standard farm practice: *U. lacinulata* twice a week and *G. gracilis* once weekly.

Water quality parameters were measured weekly in each of the abalone rearing tanks. The water temperature in the abalone tanks ranged between 18.5 ± 0.17 and 22.5 ± 0.22°C, and DO and pH in the abalone tanks averaged 8.37 ± 0.14 mg/L and 8.47 ± 0.07, respectively.

2.3 | Sampling and DNA extraction

At the end of the feeding trial, the animals were starved for 48 h before sampling to limit the presence of ingested food-associated (transient) bacteria in the digestive tract (Nel et al., 2017). One healthy animal was sampled from each replicate basket, resulting in a total of four abalone per treatment and a total of 32 animals for microbiome analysis. The sampled abalone were immediately euthanized by severing the cerebral ganglia with a sterile scalpel blade and then shucked. To shuck an animal, a sterile scalpel was used to cut the adductor muscle as close to the shell as possible without puncturing the digestive tract tissues (Harris et al., 1998). Following removal from the shell, the abalone was mounted on the pre-sterilized surface of a dissection tray using sterilized pins, and the entire digestive tract was carefully removed from the animal using a scalpel and tweezers. A scalpel was used to carefully dissect away the gills and other tissues before transferring the entire digestive tract to a sterile 15 mL centrifuge tube. Tubes containing tissue were immediately frozen at −20°C and then transported on ice to the South African Institute for Aquatic Biodiversity (SAIAB) laboratory for DNA extraction (within 12 h of dissecting). The dissection surface and all equipment used were rinsed, dried, and sterilized using 70% ethanol after each animal dissection to prevent cross-contamination of bacteria between samples.

Once in the laboratory at SAIAB, whole gut samples were ground to a fine powder in liquid nitrogen. An aliquot (∼10 mg) of the powdered tissue from each abalone was used for the isolation of total genomic DNA. Total genomic DNA was extracted using a QIAamp DNA Micro Kit (Qiagen, Hilden, Germany, Cat# 51304), following the manufacturer's protocols for the "Isolation of genomic DNA from tissues". The concentration of the isolated DNA from each sample was confirmed using a NanoDrop spectrophotometer (GENOVA NANO, JENWAY, Bibby Scientific Ltd., UK), and the DNA quality was assessed by determining the 260/280 ratio, with a ratio of 1.8 indicative of DNA that is pure and free of protein contaminants (Coasta & Roberts, 2014). The extracted DNA from the whole gut tissue samples ($n = 32$) was then stored at −20°C until needed for polymerase chain reaction (PCR) amplification and next-generation sequencing (NGS), as described below.

2.4 | PCR amplification of the bacterial 16S rDNA V4 gene region

The V4 hypervariable region (360–370 base pairs) of the 16S rDNA gene was amplified using forward (515F) and reverse (806R) primers: 515F:5′TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA and 806R:5′GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGGACTACNVGGGTWTCTAAT, where the

underlined sequences are the adaptor overhang nucleotide sequences used in the Illumina 16S metagenomics workflow. The PCR reactions were prepared in a final volume of 25 μL with 1.0 μL of genomic DNA (5 ng/μL), 200 nM of each primer (0.5 μL), 12.5 μL of 2 × KAPA HiFi HotStart Ready mix (Roche), and 10.5 μL of PCR-grade water. The reactions were run in duplicate to allow for the amplicons to be pooled before amplicon purification and DNA sequencing. The PCR thermal cycling conditions were optimized to a "touchdown" PCR protocol to improve primer specificity. The PCR cycling conditions were as follows: initial denaturation at 95°C for 5 min followed by 10 cycles of touchdown PCR (30 s at 95°C, 30 s at 65°C, with a 1°C per cycle decrement, and 30 s at 72°C), followed by an additional 25 cycles of PCR (30 s at 95°C, 30 s at 55°C, and 30 s at 72°C), and a final extension step for 10 min at 72°C. A non-template control (PCR-grade H_2O instead of genomic DNA) was included during each PCR amplification run to ensure that the reagents were not contaminated and there was only amplification of the bacterial genomic DNA in samples.

2.5 | Purification of amplicons

The PCR products were purified using a PureLink PCR purification kit (Life Technologies Cat# K3100-01 and K3100-02). Binding buffer B2 (200 μL) was added to the combined (50 μL) amplified PCR products from each sample and mixed well by inverting the tubes several times. Samples were carefully transferred into separate PureLink spin columns that had been placed in a sterile collection tube before centrifuging the columns at 10,000 *g* for 1 min. The flow-through from each sample was discarded following centrifugation before re-inserting each column into its respective collecting tube and adding 650 μL wash buffer containing ethanol to each column. Samples were centrifuged as before, the flow-through discarded, and each column returned to its collection tube. Columns were centrifuged at maximum speed for 3 min; the collecting tubes were discarded, and each column was then transferred to a clean 1.5 mL microcentrifuge tube (eluting), and 50 μL eluting buffer was added at the center of the column. Columns were incubated at room temperature for 1 min before being centrifuged at maximum speed for 2 min. The purified PCR products in each eluting tube were transferred to clean sterile 1.5 mL microcentrifuge tubes and stored at 4°C. The quality and size of the purified PCR products, including the negative control, were verified by performing 0.8% agarose gel electrophoresis. The resultant PCR products, including the negative control, were electrophoresed, and the PCR products were visualized using UV illumination.

2.6 | Library preparation and NGS

Purified PCR products were used to amplify the 16S rDNA V4 hypervariable region before the addition of Illumina sequencing adaptors and dual-index barcodes, using the Nextera XT Index Kit (Illumina, Cat# FC-131-1001). Following the addition of the indices and sequencing adapters, the library was cleaned using the Mag-Bind Total Pure NGS magnetic bead (OMEGA) kit, and the final library was validated on a Bioanalyzer DNA 1000 chip. The DNA concentrations of the final libraries were determined using an Agilent 2100 Bioanalyzer system (Agilent Technologies, Waldbronn, Germany), and the library concentrations were adjusted to 4 nM by diluting with 10 mM Tris (pH 8.5). Five microliter aliquots of the diluted DNA from each library that had a unique index were pooled. Pooled libraries were denatured with 0.2 N NaOH, diluted with hybridization buffer (HT1), and heat-denatured. Sequencing was conducted using paired-end (PE) Illumina MiSeq sequencing (Illumina Inc., San Diego, CA, USA) according to the manufacturer's instructions. The Illumina MiSeq uses 2 × 300 bp PE v3 chemistry, which allows for the overlap and stitching together of paired amplicon reads into one full-length read of higher quality (Comeau et al., 2017). A minimum of 5% PhiX was included in each run to serve as an internal control for lower diversity libraries. Sequences were generated using the 16S-based metagenomics workflow of MiSeq Reporter v2.3 (Illumina), and the resulting raw sequences were demultiplexed based on index sequences and converted to FASTQ files with corresponding Phred quality scores (Tawfik et al., 2018).

2.7 | Raw data processing and taxonomic assignment

The raw sequence reads were analyzed using Quantitative Insights into Microbial Ecology 2 (QIIME2; version 2022.2; http://qiime2.org/) software (Caporaso et al., 2010), where raw reads were imported using the Casava input format. Computations were performed using facilities provided by the University of Cape Town's ICTS High Performance Computing team (hpc.uct.ac.za). The QIIME2 open-source software was used to remove low-quality reads (Phred quality score < Q20) and reads with low abundance (n < 10 counts) using the Divisive Amplicon Denoising Algorithm 2 (DADA2) plugin in QIIME (Callahan et al., 2016). This plugin was also used to merge forward and reverse reads and to screen for and remove chimeric sequences (Table S1). Taxonomy was assigned to each Amplicon Sequence Variant (ASV) by mapping sequences using a naïve Bayes classifier trained on the SILVA database (release 132; Glockner et al., 2017; Quast et al., 2013).

2.8 | Data analysis

MicrobiomeAnalyst, a web-based tool for comprehensive statistical, visual, and meta-analysis of microbiome data, was used to assess the taxonomic diversity and composition of the abalone digestive tract samples in this study (Dhariwal et al., 2017). The data were filtered to exclude low-count reads (minimum 2 counts) for within-sample assessments and low variance reads of 10% based on standard deviation for between-sample assessments. The latter step aims to exclude features that are mostly low quality or uninformative to improve downstream statistical analysis and reduce the effect of multiple testing (Dhariwal et al., 2017). Subsequently, the filtered data were used to generate rarefaction curves in MicrobiomeAnalyst by randomly sampling a fixed number of reads and assessing the species diversity of each sampling event to show the depth of the sequencing. Alpha diversity (within sample) statistics and the overall abundance of taxa were also assessed using a filtered dataset as described above. The data were normalized using relative log expression (RLE) transformation (Hawinkel, 2015) to correct the variability in sampling depth and the sparsity of the data to enable more biologically meaningful comparisons when performing multivariate beta diversity (between-sample) tests and univariate differential abundance analysis.

Alpha (within-sample) bacterial diversity was assessed using the R *phyloseq* (McMurdie & Holmes, 2013) and *vegan* packages (Dixon, 2003) at the genus taxonomic level, as implemented in MicrobiomeAnalyst. These diversity measures included the Chao1 (Chao, 1984) and Shannon (Shannon, 1948) indices. The statistically significant differences between the samples were analyzed by an analysis of variance (ANOVA; statistical significance at p < 0.05).

Beta (between-sample) diversity assessments were carried out using the same R packages as described above, using the normalized data set. A non-metric multidimensional scaling (NMDS) analysis using a Bray–Curtis dissimilarity matrix was performed using the non-rarefied dataset to visualize the bacterial community structure between samples of abalone fed the biosecure and non-biosecure macroalgae diets and those fed a mixed diet of formulated diet with fresh seaweed. To test the statistical significance (p < 0.05) of differences observed between samples, a permutational multivariate analysis of variance (PERMANOVA; Anderson, 2001) and an analysis of similarities (ANOSIM; Clarke, 1993) were performed. Venn diagrams were drawn using the online web tool, Jvenn (Bardou et al., 2014), to show the number of shared genera between the biosecure and non-biosecure macroalgae diets, the diet containing fresh seaweed, and the control diet samples.

MicrobiomeAnalyst was also used to visualize the relative (%) taxonomic abundance by constructing abundance plots at the genus level. The biological replicates were merged, and only the top 30 taxa were included; all the rest were merged as "Others." Differential abundance of ASVs across the samples collected from abalone fed different diets was assessed using the univariate method, DESeq2 (Love et al., 2014), with false discovery rate (FDR) corrected p-values to reduce the possibility of type I errors (Benjamini & Hochberg, 1995). The core microbial taxa were also identified using a global exact test, which determines the set of taxa detected in a high fraction of the samples above a given abundance threshold (20%).

3 | RESULTS

3.1 | Data processing

A total of 3,793,834 raw reads were generated across the 32 samples included in this study, with an average of 118,557 ± 4985 reads per sample. After the reads were mapped to the Silva reference database, a total of 1198 ASVs were identified across the 32 samples. A total of 652 ASVs had less than two counts (<2) and were removed from the dataset. Subsequent data filtering resulted in an additional 248 low-abundance features/ASVs being removed based on low prevalence (10%) prior to performing within-sample diversity assessments, and a total of 41 low-variance features were removed based on standard deviation (SD) prior to performing between-sample diversity assessments. All samples reached a plateau after rarefaction analysis, indicating a good sequencing depth (Figure S1). A total of 363 ASVs remained after data filtering steps, with the observed ASVs assigned to 152 family- and 249 genus-level taxa.

3.2 | Alpha diversity

The richness estimator Chao 1 (which indicates the number of different kinds of bacteria present), as well as the evenness indicator Shannon (which indicates the uniformity of the population size of each species present in a sample) was generally higher for the formulated feed supplemented with either bio-secure or non-biosecure macroalgae (with the exception of the bio-secure kelp treatment), when compared with the formulated feed supplemented with the fresh seaweeds, *Ulva* and *Gracilaria* (Figure 1). However, no significant difference in either of the two diversity measures was recorded across samples at the genus level ($p > 0.05$).

3.3 | Beta diversity

The NMDS plots at the genus level for all the abalone samples combined showed a large degree of overlap for the majority of the dietary treatments, indicating that there are no major differences in the bacterial compositions associated with the varying feeds/treatments. However, a separation was observed between the Abfeed™ control diet and

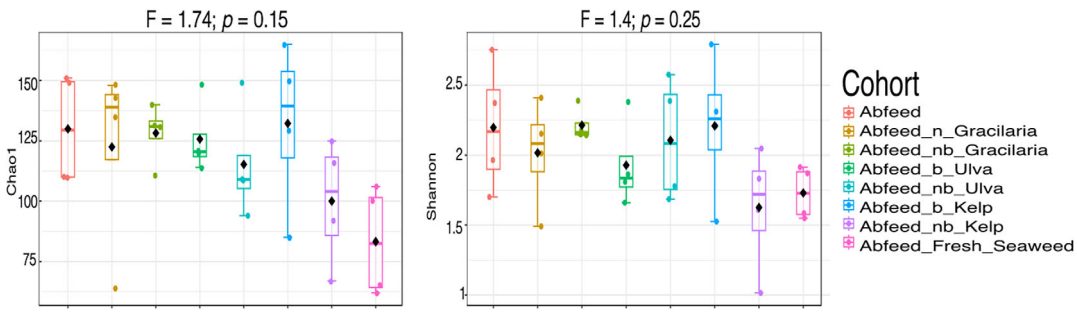


FIGURE 1 An overview of the alpha diversity (Chao1; Shannon) statistics for samples obtained from the digestive tract of abalone *Haliotis midae* fed with the biosecure (b) and non-biosecure (nb) macroalgae diets, control (Abfeed™). and the mixed diets consisting of Abfeed™ and fresh macroalgae (*Ulva lacinulata* and *Gracilaria gracilis*) at the genus level. The ANOVA *F*-values and *p*-values are indicated for each diversity index across each dietary treatment ($n = 4$ per dietary treatment).

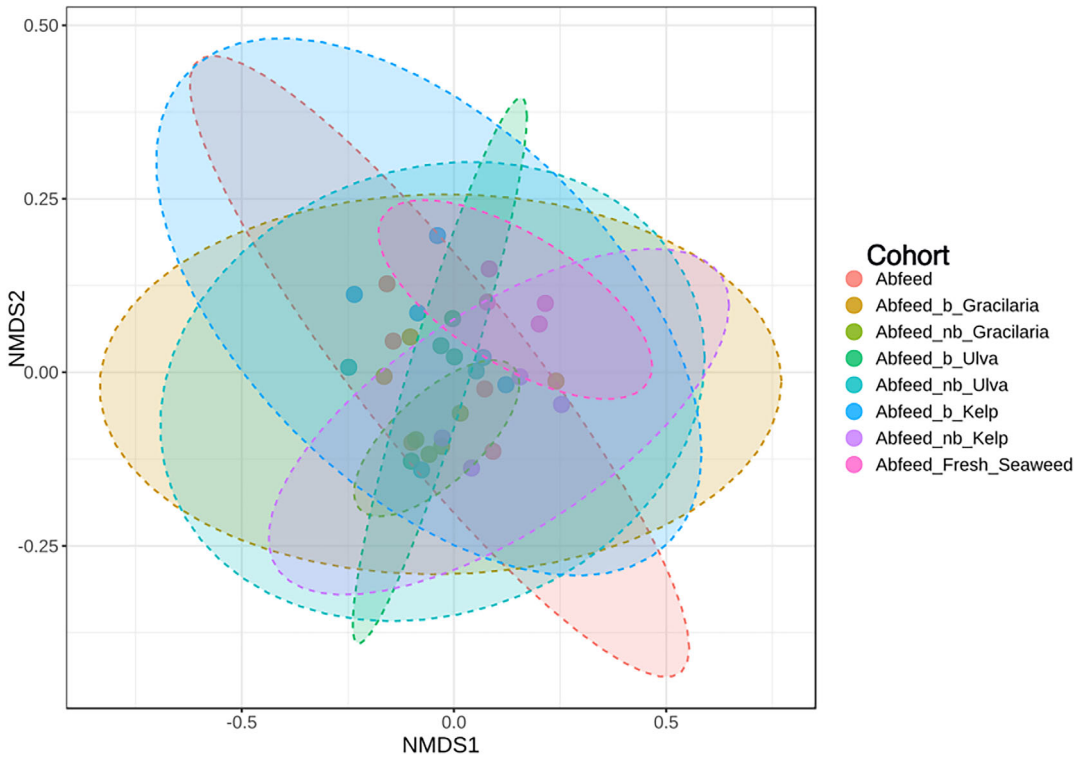


FIGURE 2 Beta diversity analysis (non-metric multidimensional scaling (NMDS)) of the microbial communities associated with the digestive tract of abalone *Haliotis midae* fed with formulated diet containing biosecure (b) and non-biosecure (nb) macroalgae and mixed diet consisting of Abfeed™ and fresh macroalgae (*Ulva lacinulata* and *Gracilaria gracilis*).

the Abfeed_nb_Gracilaria, fresh seaweed diet, and the *Gracilaria* diets, and Abfeed_nb_*Ulva* with Abfeed_nb_*Gracilaria* as indicated by the pairwise PERMANOVA ($p < 0.05$; Figure 2).

Specific comparisons of the bacterial composition of abalone digestive tract samples associated with non-biosecure and biosecure diets for each macroalgae with Abfeed™ as the control showed a similar large degree of overlap in *Ulva* and kelp diets (Figure 3b,d). This was also confirmed by the PERMANOVA and ANOSIM statistical analyses (p-values > 0.05, Table 2). A distinct clustering pattern was observed in the abalone gut samples associated with biosecure *Gracilaria* versus non-biosecure *Gracilaria* versus the control diet Abfeed™ (Figure 3a). This observation was supported by the PERMANOVA and ANOSIM statistical results ($p < 0.05$; Table 2), suggesting a higher degree of similarity between these diets than what was observed for the others.

The Venn diagrams showed overall high numbers of shared genera between the biosecure, non-biosecure, and control diet samples (143–153), which is in congruence with the NMDS plots (Figures 2 and 3). Notably, the number of genera exclusively associated with biosecure diets (11–14) was higher than that of the non-biosecure diets (6–10; Figure 4). A total of 144 genera of the abalone gut bacteria were shared between the *Gracilaria* and control diet, while 19 genera were common to the biosecure and non-biosecure *Gracilaria* diets (Figure 4c). In the abalone fed with the *Ulva* diets, 23 genera out of a total of 232 were shared between the biosecure and non-biosecure diets of this seaweed type (Figure 4b). Venn diagrams of all the biosecure and non-biosecure diets combined showed that only 3 genera (*Psychroserpens*, a genus belonging to the family *Isosphaeraceae*, and *Pedobacter*) were unique to the Abfeed_b_kelp fed abalone, while the rest of the genera were shared between the different associated diets

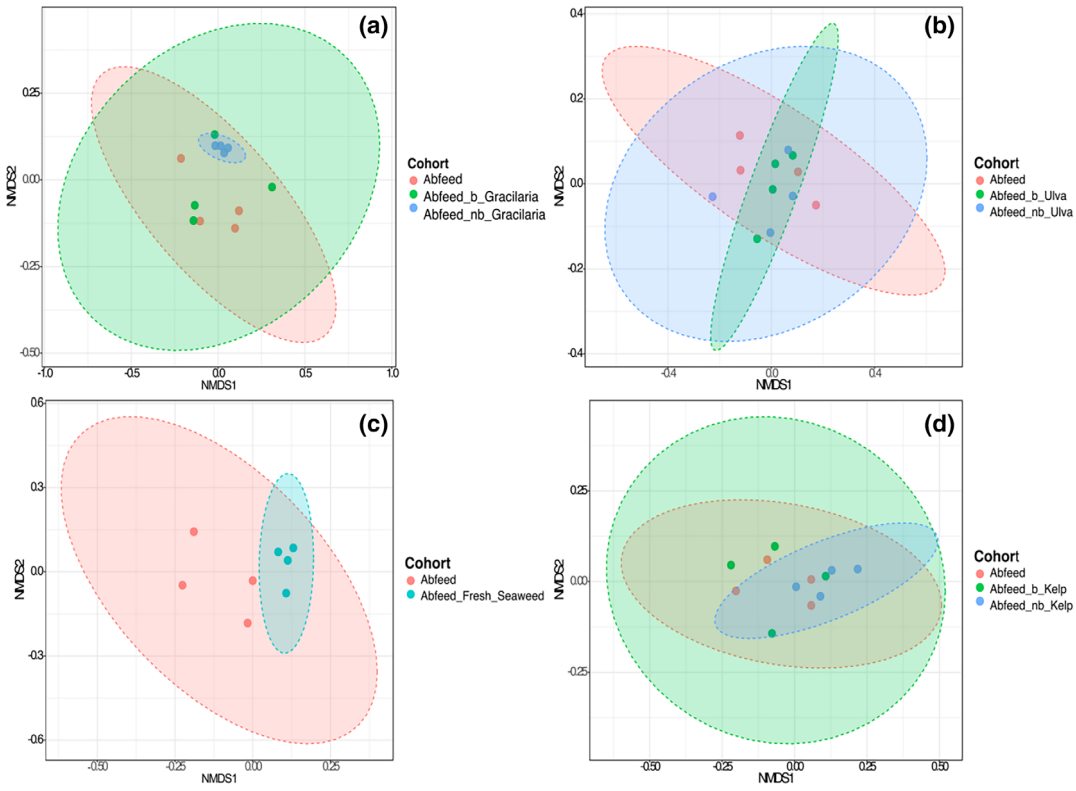


FIGURE 3 Beta diversity analysis (non-metric multidimensional scaling (NMDS)) of the microbial communities at genus level associated with abalone *Haliotis midae* digestive tract samples obtained from animals fed with formulated diets containing (a) biosecure (b) and non-biosecure (nb) *G. gracilis* with the control (Abfeed™); (b) biosecure (b) and non-biosecure (nb) *Ulva lacinulata* with the control (Abfeed™); (c) a mixed diet of the formulated feed (Abfeed™) with fresh macroalgae (*Ulva lacinulata* and *Gracilaria gracilis*) and (d) biosecure (b) and non-biosecure (nb) kelp with the control (Abfeed™).

TABLE 2 Beta diversity statistical tests (PERMANOVA and ANOSIM) for biosecure and non-biosecure macroalgae when compared to the control formulated feed at the genus level.

	Macroalgae			
Analysis	***Gracilaria***	***Ulva***	**Kelp**	**Fresh seaweed**
PERMANOVA; R^2	0.302 (0.050)	0.214 (0.299)	0.085 (0.928)	0.257 (0.127)
ANOSIM; *R*	0.219 (0.037)	0.049 (0.293)	0.206 (0.985)	0.197 (0.118)
NMDS stress	0.082949	0.1398	0.1017	0.0612

Note: Corresponding *p*-values are indicated in the brackets, and NMDS stress values corresponding to Figure 3 are indicated for each comparison.
Abbreviations: ANOSIM, analysis of similarities; NMDS, non-metric multidimensional scaling; PERMANOVA, permutational multivariate analysis of variance.

(Figure S2). Lastly, 138 genera were shared between the gut bacteria of abalone fed with the mixed diet containing fresh seaweed and the control diet Abfeed™ (Figure 4d). It was also observed that the fresh seaweed diet had fewer genera (153) compared to the rest of the dietary treatments.

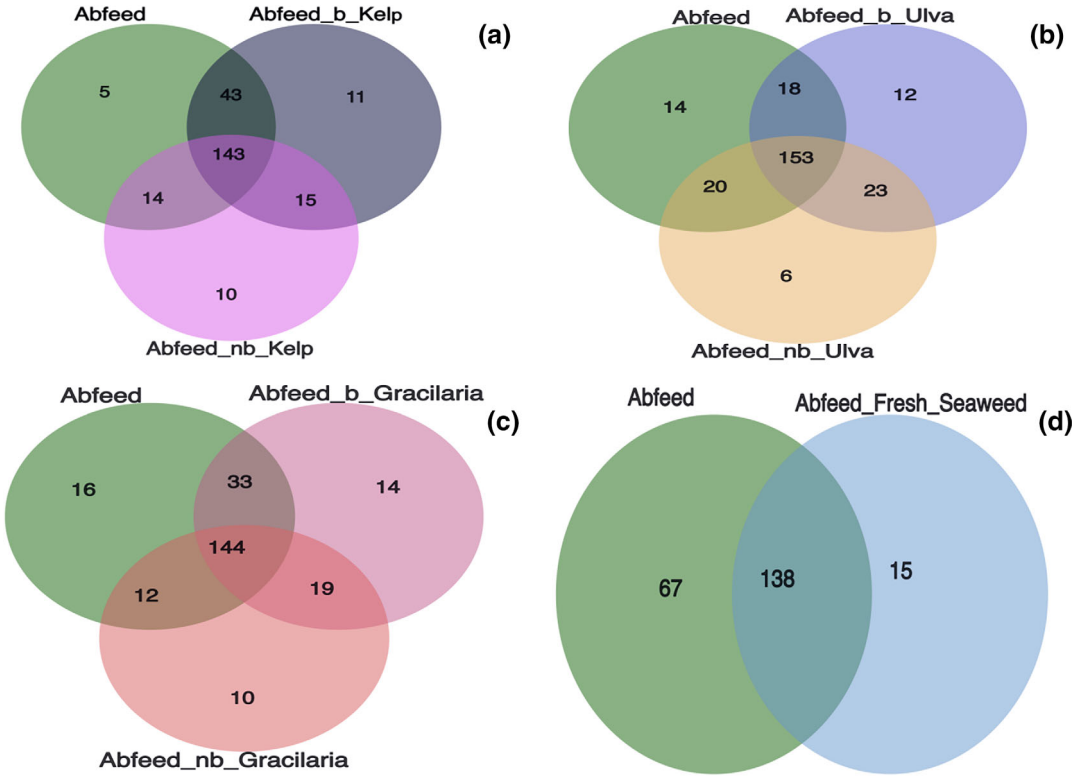


FIGURE 4 Venn diagrams showing the number of shared amplicon sequence variants (ASVs) between biosecure (b) and non-biosecure (nb) macroalgae diets with the control Abfeed™ diet. (a) biosecure (b) and non-biosecure (nb) Kelp diet with the control Abfeed™; (b) biosecure (b) and non-biosecure (nb) *Ulva diet* with the control Abfeed™; (c) biosecure (b) and non-biosecure (nb) *Gracilaria diet* with the control Abfeed™; (d) shared ASVs between a mixed diet of formulated feed with fresh seaweed with the control Abfeed™.

3.4 | Taxonomic profiling

At the family level, SAR324 clade (Marine group B), Mycoplasmataceae, Fusobacteriaceae, and Vibrionaceae were the most abundant ASVs across all the dietary treatments, except for the abalone that were fed the Abfeed™ diet supplemented with the two fresh macroalgae, where bacteria belonging to the family Vibrionaceae had a lower relative abundance. Specifically, among the top four bacterial families identified in the various dietary treatment groups, the family Fusobacteriaceae was the most abundant in the digestive tract of abalone fed the biosecure and non-biosecure *Gracilaria* supplemented diets (27.5% and 30.3%, respectively; Table S2, Figure S3). Members of the Deltaproteobacteria SAR324 clade (Marine group B) were the most abundant family-level classification observed in the digestive tract of abalone fed the formulated diet supplemented with the biosecure and non-biosecure kelp, non-biosecure *Ulva*, fresh macroalgae, and Abfeed™.

The genus *Mycoplasma* had the highest relative abundance (42%) in the digestive tract of abalone fed with the biosecure *Ulva*. *Psychrilyobacter* dominated in the digestive tract of abalone fed the *Gracilaria* diets (30%, 27%), while bacteria belonging to the SAR324 clade (Marine group B) were found in higher abundance (28%–36%) in the digestive tract of abalone fed the remaining diets tested in this study (Table S2; Figure 5). The genus *Blastopirellula*, which was among the dominant genera (10%) recorded in the digestive tract of abalone fed Abfeed™ supplemented with fresh seaweeds (Abfeed_Fresh seaweed), was the only statistically significantly differentially abundant genus among the dominant genera (DESeq2; FDR-corrected $p < 0.05$). The dominant genera described here also formed part of

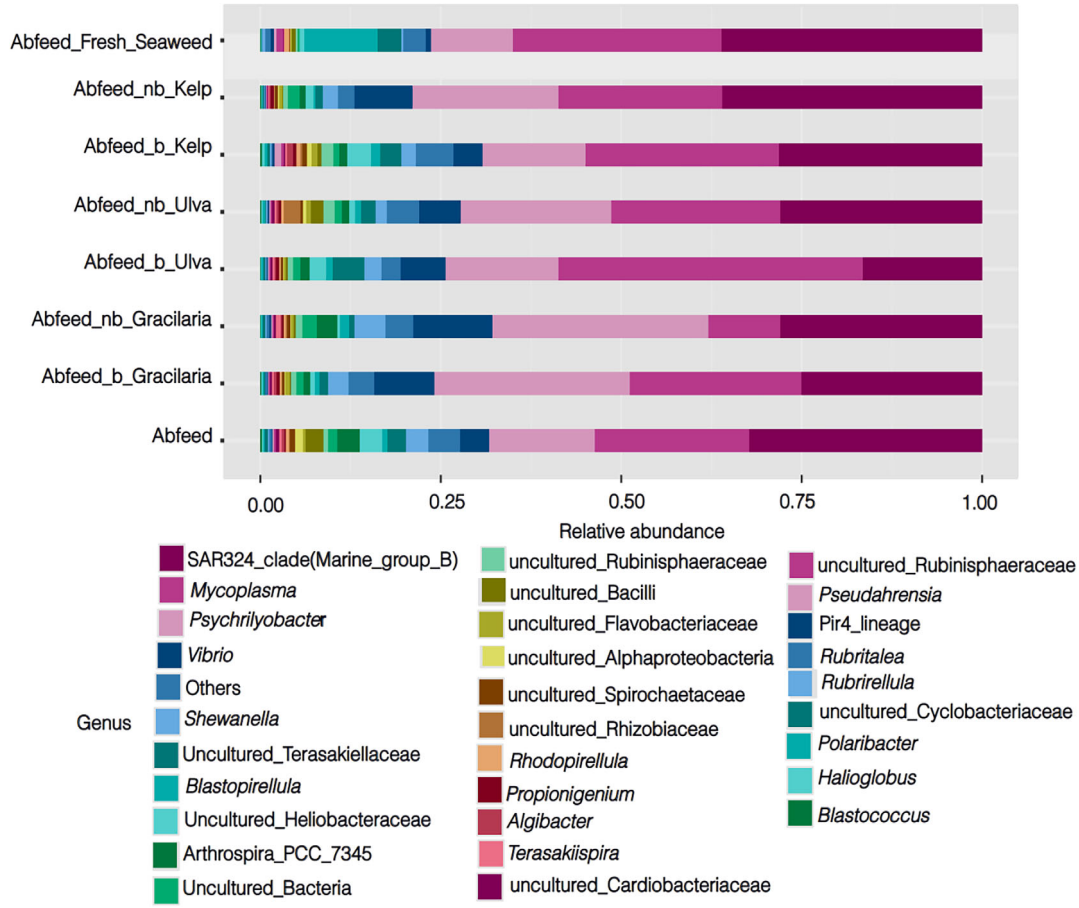


FIGURE 5 A stacked column bar graph of the top 30 most abundant taxa at the genus level associated with abalone *Haliotis midae* digestive tract samples ($n = 4$ per dietary treatment) obtained from animals fed with the formulated diet containing biosecure (b) and non-biosecure (nb) macroalgae and formulated diet (Abfeed™) supplemented with fresh macroalgae.

the core microbiome when all samples were analyzed together (Figure 6). These genera stayed stable between the biosecure and non-biosecure diets. However, differential abundance analyses identified 19 differentially abundant ASVs (Table S3; Figure 7), indicating that there are fine-scale differences between the digestive tract bacterial communities of abalone fed different diets.

4 | DISCUSSION

Processing, as opposed to the use of fresh ingredients or seaweeds, is thought to have a major impact on the microbiota associated with the ingredients/feeds that, in turn, could impact the gut microbiome and associated health and growth of the animals feeding on the processed feeds (Del Olmo et al., 2018; Zhang & Li, 2018). Processing of feed or feed ingredients often exerts a major effect on their constituents. Some studies reported that heat treatment altered the nutritional composition and phytochemical contents of seaweed, which in turn compromised their functional benefits (Cox et al., 2012).

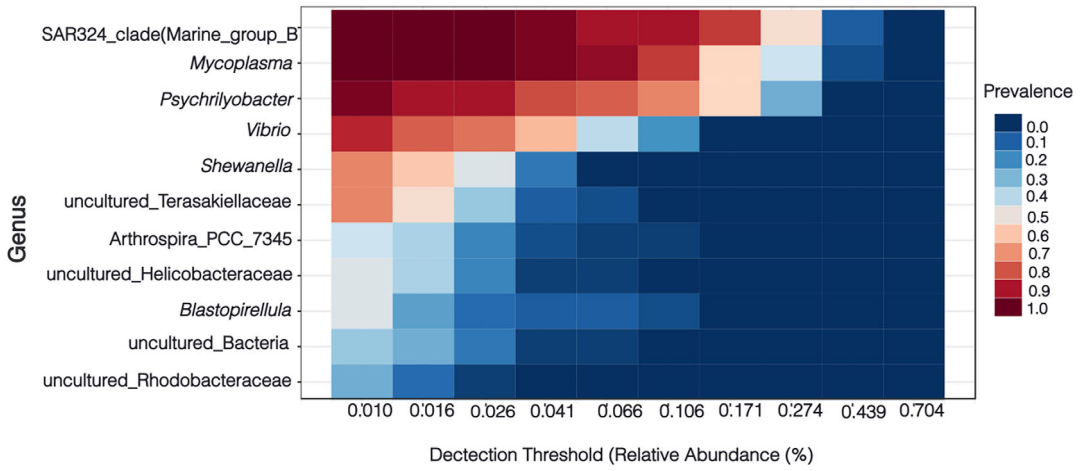


FIGURE 6 Composition of the core bacteria at the genus level in a combined dataset, determined using a global exact test, in abalone *Haliotis midae* digestive tract samples obtained from animals fed with the formulated diet (Abfeed™) containing biosecure (b) and non-biosecure (nb) macroalgae (*U. lacinulata* and *G. gracilis*), and the formulated diet supplemented with the same fresh macroalgae.

The current study compared the gut microbiome composition of the South African abalone *H. midae* fed a formulated feed (Abfeed™) supplemented with biosecure and non-biosecure treated macroalgae and compared this with abalone fed the formulated diet alone or the formulated diet provided together with two fresh macroalgae species, *U. lacinulata* and *G. gracilis*. It was observed that neither the biosecurity process nor the macroalgae type, when included as a dry ingredient (3% w/w) in formulated feed or provided as an additional fresh feed with the formulated feed, significantly changed the diversity or the composition of the gut microbiome of *H. midae* fed the various diets. Similar findings have been reported for the European abalone *Haliotis tuberculata*, fed with four monospecific algal diets: the brown algae *Laminaria digitata* and *Saccharina latissimi*, the red algae *Palmaria palmata*, and the green algae *Ulva lactuca*. Variations in the composition of the microbiome were shown to be more linked to seasonal variations than to the specific macroalgal diet (Gobet et al., 2018). In another study, a diet with 2.5% or 5% *G. gracilis* or its extract did not alter the richness or diversity of the European seabass gut microbiome (Gonçalves et al., 2022). On the contrary, a higher richness of seabream intestinal communities was observed when fish were fed formulated feeds where the fish meal was partially substituted with dried *Gracilaria* or *Ulva* biomass (15% w/w) (Rico et al., 2016). The discrepancy in results from different studies infers that microbiome modulation by algae is a complex phenomenon and could be highly dependent on various factors such as the host and algae species, inclusion rate, duration of feeding, age of the host and season, among other factors, reinforcing the complexity of the gut microbial community and the associated host-microbiome interactions (Danckert et al., 2020; Gonçalves et al., 2022). Assessing the impact of diet on the host-microbiome on a case-by-case basis is therefore imperative.

In the current study, the diets containing biosecure and non-biosecure macroalgae were isolipidic and isonitrogenous. Both the biosecure and non-biosecure feeds contained macroalgae that were dried, with the exception of the Abfeed_fresh seaweed treatment. The biosecure treatment was previously shown to significantly reduce the abundance of bacteria, fungi/oomycetes, and viruses associated with each seaweed prior to its incorporation into the formulated feed (Mwangudza, 2024). This may have contributed to the similarity observed in the gut microbiomes across treatments, although some fine-scale differences were noted. Moreover, the biosecure and non-biosecure macroalgae were included at only 3% (w/w) in the formulated feed, a lower level of inclusion compared with some related studies, possibly contributing to the non-significant change to the overall abalone gut microbiome. For example, Cyrus et al. (2014), Cyrus, Bolton, Sholtz, and Macey (2015), Cyrus, Bolton, and Macey (2015) showed

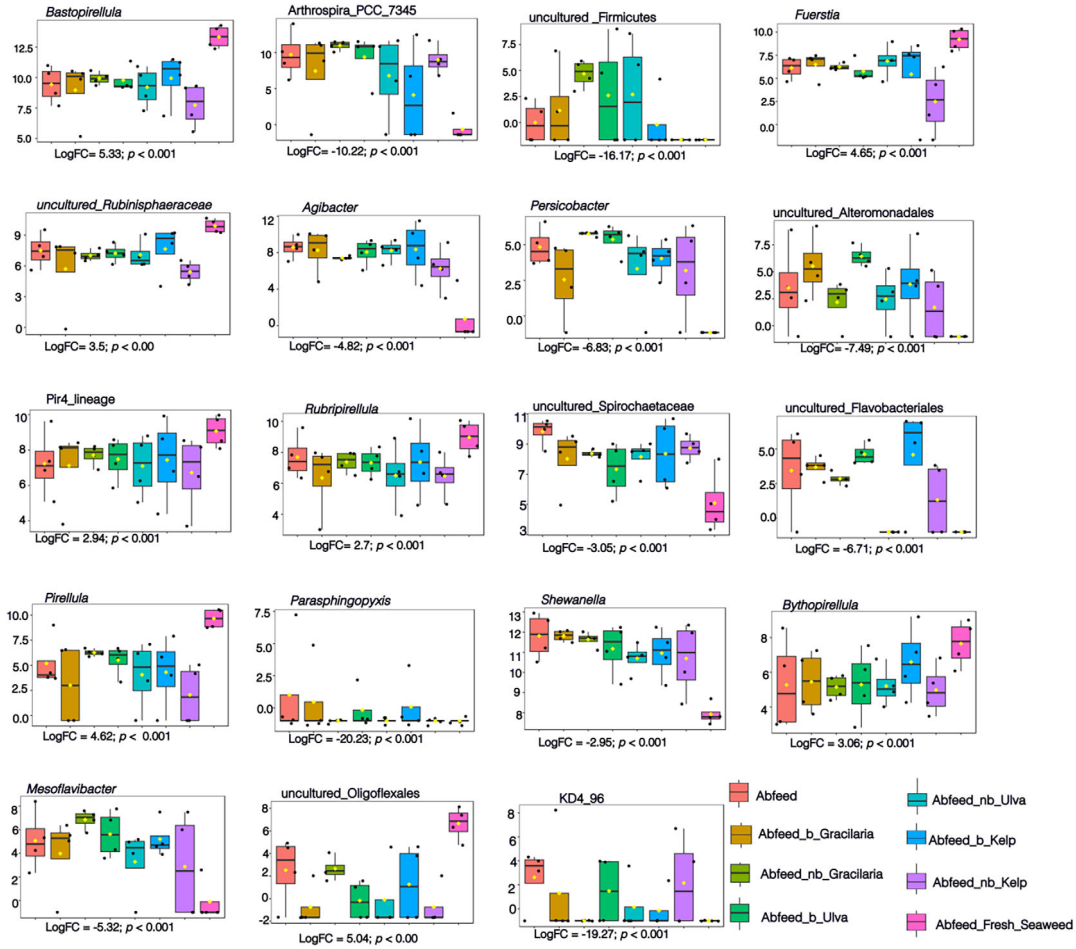


FIGURE 7 Differentially abundant amplified sequence variants (ASVs) at the genus level, associated with the digestive tract of abalone *Haliotis midae* fed a formulated feed (Abfeed™) supplemented with the biosecure (b) and non-biosecure (nb) macroalgae (*G. gracilis* and *U. lacinulata*), control (Abfeed™) diet, and the Abfeed™ diet.

that *Ulva* needed to be included in a formulated feed at a level above 15% (w/w), on a dry weight basis, before it had a significant impact on gonad quality, protein and energy efficiency, and consumption, among other factors. Although the latter authors did not assess impacts on the gut microbiome, the benefits they observed from dietary supplementation with seaweed may in part be attributed to the impact of the seaweed on the gut microbiome. This notion is supported by the study of Brand (2023), who demonstrated that abalone *H. midae* fed diets supplemented with 10% dried *Ulva*, or lower levels of *Ulva* constituents (1% Ulvan and 0.1% glucuronic acid), or with fresh *Ulva*, had a significant impact on the gut microbiome community, differing significantly from that of abalone fed Abfeed™ alone. Collectively, these observations support the notion that dietary inclusion levels as well as macroalgae type may be important in shaping the microbiome.

Interestingly, the lowest number of ASVs at family and genus levels was observed in abalone fed with the diet supplemented with fresh macroalgae. Previous literature suggested that some macroalgae polysaccharides (i.e., agar and carrageenan) have inhibitory effects on specific/certain microorganisms in the digestive tract of the animals feeding on the macroalgae (Bullon et al., 2022; Elizondo-González et al., 2020; Nel et al., 2017; O'Sullivan et al., 2010). Moreover, macroalgae are known for their ability to defend themselves against other species, including

bacteria, in the environment by producing a variety of secondary metabolites, including alkaloids, polyketides, cyclic peptides, quinones, and lipids, with a broad range of biological benefits (Al-Saif et al., 2014; Habbu et al., 2016). In addition to protecting macroalgae against herbivores and environmental stressors, these metabolites are capable of impacting/modulating microorganisms that colonize their surfaces, the immediate (micro-) environment, or the digestive tract of the animals digesting them (Bullon et al., 2022; Singh et al., 2011). Moreover, reactive oxygen species (ROS; superoxide ions and hydrogen peroxide) are released by macroalgae as a defense mechanism against bacteria, thereby reducing the number of bacteria associated with them (Egan et al., 2013; Weinberger, 2007; Zhao et al., 2021). The low ASV numbers observed in the digestive tracts of abalone fed with fresh macroalgae could therefore be attributed to the macroalgae or the microorganisms associated with them that are inhibiting the growth of bacteria in the abalone digestive tract.

In this study, the genera SAR324 clade (Marine group B), *Mycoplasma*, *Psychrilyobacter*, and *Vibrio* dominated the gut bacterial community of abalone in almost all the dietary treatments, whereas *Blastopirellula* was also observed in higher abundances in animals fed the mixed diet of Abfeed™ and fresh macroalgae. These genera were shown to form part of the core microbiome of the abalone. Except for the SAR324 clade (Marine group B) and *Blastopirellula*, the other dominant genera *Mycoplasma*, *Psychrilyobacter*, and *Vibrio* have previously been reported to dominate within the digestive tract and form part of the core microbiome of several abalone species (*H. tuberculate*, *H. laevigata*, *and H. midae*) fed a variety of diets and sampled from different environments (Cicala et al., 2018; Danckert et al., 2020; Gobet et al., 2018; Huang et al., 2010; Iehata et al., 2014; Nel et al., 2017).

The genus *Vibrio* was shown to have a high relative abundance in the digestive tract of *H. midae* for all dietary treatments, except for abalone fed the mixed diet of Abfeed™ and the two fresh macroalgal species. It was also noted that the non-biosecure diets had a higher abundance of the *Vibrio* compared to the biosecure diets, except for the *Ulva* diets. Certain members of the genus *Vibrio* have been linked to high mortalities, especially during summer months, of several marine cultured species, including oysters, shrimp, various fish species, and abalone (Austin & Zhang, 2006; Cai et al., 2007; Cardinaud et al., 2014; Ina-Salwany et al., 2019; Liu et al., 2000). However, the high abundance of *Vibrio* spp. in the gut of several healthy abalone species (Danckert et al., 2020; Nel et al., 2017) suggests a strong positive association between this bacterium and the host. Members of the genus *Vibrio* are known to produce exogenous digestive enzymes that aid in the fermentation of a variety of seaweed polysaccharides and have been shown to contribute to the digestion of both kelp and formulated feeds (Erasmus et al., 1997; Macey & Coyne, 2005; Sawabe, 2006; Sawabe et al., 2003). Specific species, such as *V. halioticoli*, have also been isolated from a variety of Haliotid species, including *H. midae*, and have been shown to play an important role in fermentation in the lower intestine of abalone and the production of short-chain fatty acids that are important for gut health, gut development, energy metabolism, and immunity (Sawabe et al., 2003). This could explain the high abundance of this genus in both the macroalgae diets and the control (formulated feed) diet in this study. Interestingly, a clear reduction in the abundance of the genus *Vibrio* and an associated increase in the abundance of *Blastopirellula* spp., of the phylum Planctomycetota, was observed in animals fed the mixed diet of the formulated feed and fresh macroalgae in this study. These results are consistent with other studies that showed a lower abundance of *Vibrio* spp. in the digestive tracts of abalone fed macroalgae diets, including, for example, European seabass fed a diet with dried *G. gracilis* or an extract of the seaweed (Gonçalves et al., 2022). Furthermore, a lower abundance of *Vibrio* spp. has also been observed in the surrounding water of IMTA systems cultivating *Ulva* (De Jager et al., 2024).

The presence of *Blastopirellula* as one of the dominant genera in this study, particularly in abalone fed a mixed diet of Abfeed™ and fresh seaweeds, suggests that these bacteria may be associated with either of the two seaweeds, *G. gracilis* or *U. lacinulata*, fed to the abalone in this study. *Blastopirellula* has previously been isolated from the gut of the sea urchin *Abatus agassizii* (Schwob et al., 2020). Members of the genera *Blastopirellula* were associated with having a significant role in the breakdown of sulfated polymeric carbon by sulfatase enzyme activity (Wegner et al., 2013). The sulfated compounds found in marine photosynthetic organisms like microalgae and seaweeds were likely to have been deposited on the seafloor and ingested by the host together with the sediments (Schwob et al., 2020).

The presence of the SAR324 clade (Marine group B) as a dominant ASV in the digestive tract of abalone in this study appears to be a unique case, as this taxon has not been reported in other abalone gut microbiome studies. Although members of the SAR324 clade (Marine group B) genus, belonging to the phylum Deltaproteobacteria, have been shown to inhabit the entire water column, from the surface to the deep interior of the ocean, they have been found to make up a substantial part of the microbial community in oxygen minimum zones (Boeuf et al., 2021). The high abundance of this genus in the current study could be attributed to its presence in the seawater pumped into the abalone rearing tanks from the bordering Indian Ocean. In marine ecosystems, the seawater microbiome has been reported to colonize the gut of marine organisms. For example, the gut microflora of juvenile abalone fed on diatoms was almost identical to the surrounding seawater (Tanaka et al., 2003). The fact that this group of bacteria survives in oxygen-minimum environments may explain their abundance in the digestive tract of abalone, where oxygen levels are much lower than in the water column, as observed in this study. Harris et al. (1998) revealed that the oxygen level in the gut of abalone *Haliotis laevigata* was below the limit of detection and suggested anaerobic conditions. Nonetheless, future studies should be conducted to establish the role of the SAR324 clade (Marine group B) in the guts of abalone.

5 | CONCLUSION

The bacterial diversity and composition of the abalone gut microbiome did not differ significantly between the abalone fed formulated diets with biosecure and non-biosecure treated macroalgae inclusion. Furthermore, the same dominant bacterial genera, SAR324 clade (Marine group B), *Mycoplasma*, *Psychrilyobacter*, and *Vibrio*, were observed across all samples. This study shows that the biosecurity treatments to which the macroalgae were subjected prior to being included in the formulated diet, previously shown to significantly reduce the abundance of bacteria, fungi/oomycetes, and viruses associated with each seaweed (Mwangudza, 2024), did not affect the gut microbiome of the abalone. However, supplementation of the formulated diet with fresh macroalgae did result in a shift in the relative abundance of the dominant genera, and some fine-scale ASV differences were noted between the other treatment groups. Microbiome modulation by algae and their components is a complex phenomenon and could be highly dependent on a variety of factors such as the host and algae species, algae inclusion rate, duration of feeding, age of the host, and season, among other factors, reinforcing the complexity of the gut microbial community and its relationships. Future studies should, therefore, consider combining these different aspects to get a more holistic view of the effect of varying diets on abalone health, physiology, and microbiome.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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