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Expanding the menu for New Zealand farmed abalone: dietary inclusion of insect meal and grape marc (effects on gastrointestinal microbiome, digestive morphology, and muscle metabolome)

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ABSTRACT

Limited supply of sustainable feed ingredients is a significant concern for future aquaculture practices. Alternative ingredients, such as insect meal and grape marc, are suitable for aquaculture nutrition due to their nutritional profile and more sustainable production methods. This study assessed the effect of dietary insect meal and grape marc on gut microbial composition, digestive system morphology, and muscle metabolome of *Haliotis iris*. Juvenile abalone were fed four encapsulated experimental diets with varying levels of insect meal/grape marc and a commercial feed for 165 days in a commercial farm. The gut health of abalone was evaluated through histopathology, and 16S rRNA amplicon libraries through Illumina *MiSeq* sequencing. The abalone metabolome was evaluated through gas chromatography-mass spectrometry (GC-MS). Results showed that animals had good intestinal condition across all dietary treatments. Microbiome analysis revealed increased relative abundances of bacteria (*Firmicutes and Fusobacterium*) associated with the breakdown of polysaccharides in diets including insect meal and grape marc. Metabolite profile variations indicate that the inclusion of insect meal and grape marc favoured fatty acid metabolism and amino acid catabolism, respectively. This study provides foundation to produce more sustainable feeds including insect meal and grape marc without affecting the gut and overall health of abalone.

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Abalone; microbiome; histology; metabolomics; insect meal; grape marc; gut health

Introduction

In the last two decades, significant efforts have been made to reduce the amount of fishmeal included in aquafeeds as a primary source of protein due to the unsustainability

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of wild pelagic stocks that provide this material (Tacon and Metian 2008). Many alternative ingredients, such as plant proteins and insect meals, have been shown to promote comparable growth to fishmeal in aquatic species (Bautista-Teruel et al. 2003; Cho 2010; Bonvini et al. 2018; Sankian et al. 2018; Belghit et al. 2019). However, the presence of antinutrients (substances that interfere with absorption of nutrients) in plant meals can jeopardise digestion and nutrient utilisation in aquatic species (Francis et al. 2001; Yu et al. 2022).

Abalone are herbivorous marine snails, which predominantly feed on seaweeds in the wild. Within land-based aquaculture farms, formulated feeds for abalone have generally included fishmeal as a main protein component (Troell et al. 2006, Bansemer et al. 2016), with around 30%–40% to expedite growth. Some abalone formulated feeds have include seaweeds as the main source of protein, resembling the natural preference of this natural food for abalone. However, regulations in certain countries in terms of seaweed supply from natural resources and continuous availability throughout the year hinder the industrial use of seaweed for abalone formulated feeds.

Insect-based sources have gained interest due to their superior nutritional characteristics (Makkar et al. 2014), and their production is more sustainable than fish and plant production (Van Huis 2015; Nugroho and Nur 2018; Ferrer et al. 2019). Insect meals have shown acceptable digestibility (Basto et al. 2020), and the chitin level (the most available polysaccharide in the exoskeletons of insect) is relatively high in content (Marono et al. 2015; Stenberg et al. 2019). To date, insect meals have not been included in abalone commercial formulated feeds. However, they have been used in feeds for other aquatic animals.. Insect meals have been shown to improve immune function in diets for mandarin fish (*Siniperca scherzeri*) (Sankian et al. 2018), white shrimp (*Litopenaeus vannamei*) (Choi et al. 2018), and rainbow trout (*Oncorhynchus mykiss*) (Bruni et al. 2018; Rimoldi et al. 2021). To date, only five species of insects have been approved for use in animal feed in the European Union (European Comission 2017). Among others, such as black soldier fly (*Hermetia illucens*) and house cricket (*Acheta domesticus*), mealworm (*Tenebrio molitor*) is considered to be the most promising species for industrial applications (Shin and Lee 2021).

Grape marc is another alternative ingredient that has been demonstrated to have beneficial effects on the gut health and immune capacity of aquatic species, such as rainbow trout (Pulgar et al. 2021) and grass carp (*Ctenopharyngodon idella*) (Souza et al. 2019). Grape marc is a waste by-product from the winery industry (*Vitis vinifera*) comprising the skins and seeds of grapes. Grape marc disposal has become problematic and represents a current global environmental concern (Gómez-Brandón et al. 2019). Grape marc is characterised by high levels of non-digestible carbohydrates, such as oligosaccharides (OS), which act as prebiotics (Bordiga et al. 2019; Sinrod et al. 2021). The supplementation of grape by-products, such as grape seed extract has shown positive effects on abalone survival and gene expression of certain genes which are related to oxidative defences and innate immunity (Shiel et al. 2017). However, grape marc supplementation is still limited. The addition of grape marc in feeds for rainbow trout (Pulgar et al. 2021), grass carp (Souza et al. 2019), and abalone (*Haliotis laevigata*) (Currie et al. 2019) has shown positive effects on growth performance and immunity status. A previous study has shown that the inclusion of

grape marc in feeds for abalone promotes comparable growth to formulated diets without grape marc (Bullon et al. 2023).

Numerous studies in aquaculture nutrition have focused on the inclusion of feed supplements for growth improvement (Macey and Coyne 2005; Ten Doeschate and Coyne 2008). However, studies that comprehensively evaluate the multi-factorial effects of nutrition on overall health, such as gut health and metabolome, are still limited. Evaluating the effects on the gastrointestinal tract (GIT) is a crucial aspect when new dietary ingredients are included, as they indicate the capacity and effectiveness to absorb nutrients. The digestive gland is the major site of nutrient absorption in molluscs, while the intestinal epithelium, formed by columnal supporting cells and secretory cells, also contribute to nutrient absorption (Maguire et al. 1998). The intestinal epithelium cells of molluscs perform multiple tasks, such as endocytosis and intracellular digestion, absorption of small molecules, storage of glycogen and lipid, and transport of nutrients to the underlying tissues (Lobo-da-Cunha 2019). The intestinal microbiome is another aspect of gut health which plays an important role in immunity, disease resistance, and growth of aquatic species (Hai 2015). In aquaculture, the enhancement of gut colonisation with beneficial bacteria is a common practice aimed at improving animal health and growth rates. The inclusion of black soldier flies in diets has been shown to benefit the lactic acid bacteria in the intestinal microbiota of salmonids (Bruni et al. 2018; Rangel et al. 2022). In addition, the inclusion of insect meal from *T. molitor* has increased the beneficial bacterial population of *Lactobacillus* and *Enterococcus* in the gut of rainbow trout (Józefiak et al. 2019). Beneficial bacteria in the gut can produce bacteriocins which inhibit pathogens in the gastrointestinal tract (Ringø et al. 2005; Dimitroglou et al. 2011). The increase of these beneficial communities has also been associated with the presence of chitin in insect meals (Antonopoulou et al. 2019; Terova et al. 2021). Dietary chitin has demonstrated improvement in beneficial enzyme-producing bacterial communities with antibacterial activities (Assan et al. 2022) acting as a prebiotic (Rangel et al. 2022). Conversely, detailed information on the specific effects of dietary grape marc on the microbiome of aquatic animals is still limited. However, micro-encapsulated grape marc extracts have increased the population of *Acetobacteraceae* and *Lactobacillaceae* in the gut of rainbow trout, following a positive impact on growth (Pulgar et al. 2021).

Changes in the intestinal tissue morphology and microbiome can modify metabolic processes, which can further affect the animal's health (Levy et al. 2017). The metabolome provides insights on metabolite fluctuations while exposed to different nutrients (Venter et al. 2019). The presence or absence of certain metabolites in abalone can suggest enriched metabolic pathways that can affect growth (Venter et al. 2019; Masoomi et al. 2023), immunity (Nguyen et al. 2018), and feed efficiency (Yu et al. 2022). In abalone, higher feed efficiency has highlighted the role of amino acid metabolism, causing the upregulation of arginine, tyrosine, and tryptophan pathways (Yu et al. 2022). Increments of end-products, such as DL-methionine, tyramine, and indoleacetic acid metabolites have been linked to an increased abundance of Proteobacteria in *Haliotis discus hannai* with high feed efficiency (Yu et al. 2022)

This study aimed to assess the effect of insect meal and grape marc feed inclusion on gut microbial composition, digestive system morphology, and adductor muscle metabolome (indicative of nutritional and health stress conditions) of *H. iris*. The key hypothesis

was that the dietary fibre content in grape marc and chitin in insect meal contribute to a better health status in abalone, evidenced by changes in microbiota and improved morphological state of the digestive tract. In addition, key signatures in metabolite profiles can reflect improved gut health.

Materials and methods

Experimental design and sample collection

This study was conducted within a commercial abalone (*H. iris*) farm (The New Zealand Abalone Company, Ocean Beach, Bluff, New Zealand). Juvenile abalones (17-month-old, n = 200) with initial mean weights (± SD) of 1.1 ± 0.5 g and shell lengths (± SD) of 21.5 ± 3.3 mm were randomly selected from the farm stock for the feeding trial. A total of 15 plastic tanks, each containing 200 juveniles, were stocked with filtered seawater (100-micron filter) at a volume of 90 L per tank. Out of the 200 abalone, 50 were tagged for the purpose of allometric measurements. For tagging, abalone were removed from the tanks with the aid of a flat, blunt knife and dried using paper towel. Numeric tags were attached to the dorsal exterior of the shells using a cyanoacrylate glue. The

Table 1. Proximate composition of experimental diets and a commercial feed, and ingredients used for experimental diets (g/100 g diet).

	Diet				
Proximate composition (%)	F	FI	FG	FIG	CF[4]
Protein	30.4 ± 0.1^{ab}	27.3 ± 0.3^{b}	30.8 ± 0.7^{ab}	26.4 ± 2.0^{b}	32.4 ± 2.7^{b}
Carbohydrate[1]	47.9	50.8	45.4	49.3	48.9
Carbohydrate – reducing sugars[2]	28.9 ± 1.3^{b}	32.0 ± 3.1^{ab}	8.9 ± 1.9^{c}	9.1 ± 1.1^{c}	39.1 ± 4.7^{a}
Total dietary fibre	9.0 ± 0.2^{b}	7.5 ± 0.1^{c}	16.1 ± 0.1^{a}	15.4 ± 0.3^{a}	3.7 ± 0.3^{d}
Lipid	4.0 ± 0.2^{c}	7.0 ± 0.6^{a}	5.3 ± 0.5^{b}	7.2 ± 0.3^{a}	1.2 ± 0.3^{b}
Ash	13.5 ± 0.1^{b}	11.9 ± 0.1^{c}	14.5 ± 0.3^{a}	12.9 ± 0.3^{b}	6.8 ± 0.4^{d}
Moisture	4.2 ± 0.01^{b}	3.0 ± 0.01^{b}	3.9 ± 0.03^{b}	4.2 ± 0.8^{b}	10.7 ± 0.1^{a}
Energy (KJ per g)[3]	18.1	20.4	18.9	20.2	15.9
Ingredients (g/100 g diet)					
Fishmeal[5]	35	25	35	25	–
Insect meal[6]	–	10	–	10	–
Corn meal	30	30	–	–	–
Grape marc[7]	–	–	30	30	–
Seaweed (dry) *Macrocystis pyrifera*[8]	4	4	4	4	–
Starch (Native maize flour)[9]	10	10	10	10	–

Notes: Data are represented by means ± standard deviation of three technical replicates. Significant differences are represented by different alphabetic superscripts (Tukey test, $p < 0.05$). Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc) and commercial feed (CF).

[1]Carbohydrate proportion was calculated by difference 100 − (moisture + protein + lipid + ash).

[2]Carbohydrate was determined using reducing sugar method Anthrone.

[3]Total energy was calculated based on the physiological values at 5.6 kCal g^{-1} protein, 9.5 kCal g^{-1} lipid and 4.1 kCal g^{-1} carbohydrates (Cho et al. 1982).

[4]Commercial feed used was Marifeed S34.

[5]Fishmeal supplied by Sandford, NZ.

[6]Insect meal supplied by Mahurangi Technical Institute (MTI).

[7]Grape marc supplied by Bragato Research Institute, NZ.

[8]Seaweed (*Macrocystis pyrifera*) supplied by Southern Clams.

[9]Starch supplied by New Zealand Starch.

tagging process lasted approximately 5 min per animal and the water temperature during the tagging process was 13°C.

Abalone were initially fed commercial feed (Marifeed S34) before being assigned to one of four experimental diets or the commercial feed, which were allocated in three replicates. The nutritional information of the commercial feed is shown in Table 1. The tanks were maintained using a flow-through water system with a water exchange of 40 times per day at a flow rate a rate of 1.5 L/min. Farming procedures were followed to clean the tanks by flushing out faeces, debris, and uneaten food residues with a water supply every other day. Animals remained with minimal disturbance in the tanks during cleaning and covered in total darkness during the whole feeding trial, except when feeding and cleaning activities were performed. During the feeding trial the water temperature ranged from 12.4–19.7°C and the dissolved oxygen varied from 86.3% to 104.1% oxygen saturation. Water temperature and dissolved oxygen were measured with a dissolved oxygen metre (Handy Polaris TCP, Denmark) which was calibrated before use using a 'air-saturated' seawater according to manufacturer's instructions.

During the 165-day feeding trial, abalone were fed 1.2%–2.2% of their body weight per day in the late afternoon (~1600 h). A set of 50 animals per tank were tagged and measured for growth at the beginning and end of the feeding trial according with Section 2.3. Prior to the dietary intervention, twenty individuals were selected, removed from their shells, and dissected for microbiome and histological analysis to provide baseline information on the initial condition of the animals. Samples for metabolomic analyses were not collected at this time point due to logistical constraints. At the end of the feeding trial, abalone were dissected for microbiome and metabolite profiling (n = 3) and histological analysis (n = 5) from each replicate tank (Figure 1). Microbiome and metabolomic samples were collected from the same animal. Microbiome samples were placed in 2 mL cryovials (Biostor™) that contained 0.5 mL RNA stabiliser (Qiagen, Germany) as per Section 2.4. Metabolomic samples were collected and placed into 2 mL cryovials (Biostor™) following Section 2.6. Both types of samples were then immersed in liquid nitrogen for 10 min and then stored in a −80°C freezer until further analysis. Histological samples were collected and analysed according to section 2.5.

Diet preparation and encapsulation

Four encapsulated diets were used as experimental diets and a commercial feed (Marifeed S34) was used as a control. Experimental diets were formulated as per Bullon et al. (2023) to contain graded levels of fishmeal (FM), insect meal (IM) and grape marc (GM) and they were designed as follows: diet F (only containing fishmeal as source of protein), FI (fishmeal + insect meal), FG (fishmeal + grape marc), and FIG (fishmeal + insect meal + grape marc) (Table 1). The commercial feed was not encapsulated, and the nutritional profile is also detailed in Table 1. Experimental diets were encapsulated according to the protocol detailed by Masoomi et al. (2022) with small modifications as detailed in Bullon et al. (2023). Dietary and proximate composition of the experimental diets is presented in Table 1. The nutritional determination method used has been detailed previously in Bullon et al. (2023).

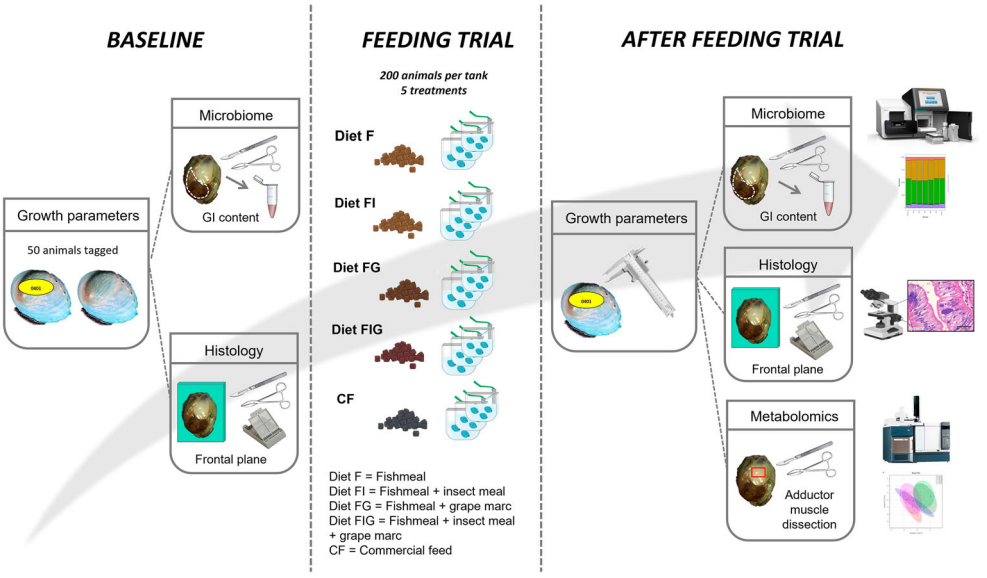

Figure 1. Summary of the experimental workflow.

Growth parameters

Tagged abalone were measured in their maximum shell length and width using a vernier calliper (Mitutoyo 0–125 mm, Warwickshire, UK) to the nearest 0.1 mm, and total and wet weights were measured with a digital balance to the nearest 0.1 g.

The growth performance was evaluated using the following parameters:

Abalone specific growth rate was calculated in total weight, shell length, and shell width as per equations (1), (2), and (3):

Specific growth rate total weight.

$$SGR\ TW\ \%\ day^{-1} = 100\ [(\ln final\ TW - \ln final\ \ TW)\ /\ 165\ days\ ] \quad (1)$$

Specific growth rate shell length

$$SGR\ SL\ \%\ day^{-1} = 100\ [(\ln final\ SL - \ln initial\ SL)/\ 165\ days] \quad (2)$$

Specific growth rate shell width.

$$SGR\ SW\ \%\ day^{-1} = 100\ [(\ln final\ SW - \ln initial\ SW)\ /\ 165\ days] \quad (3)$$

Where *ln final* is the natural log of the final total weight (TW), shell length (SL) or shell width (SW) and *ln initial* is the natural log of the initial TW, SL, and SW.

Daily increment in shell length (DISL, in μm d^{-1}) was calculated according to Dlaza et al. (2008) as per equation (4)

$$DISL\left(\frac{\mu m}{day}\right) = 1000\ x\ \left(\frac{SL_f - SL_i}{165\ days}\right) \quad (4)$$

Where SL_f is the final shell length and SL_i is the initial shell length.

Muscle yield (%) as per equation (5)

$$Muscle\ yield\ (\%) = (muscle\ weight\ /\ \ total\ body\ weight)\ x\ 100\% \quad (5)$$

Soft body: shell ratio (SB/S ratio) as per equation (6)

$$SB/S\ ratio = (Soft\ body\ weight\ /\ shell\ weight) \quad (6)$$

Microbiome sampling

Sample collection

Abalone bodies were disinfected with ethanol 70% and phosphate buffer solution (PBS) to remove surface biofouling. Stomach, digestive gland, and radula were aseptically excised from the abalone using sterile forceps and placed in 2 mL cryovials (Biostor™) with 0.5 mL RNA stabiliser. Vials were immersed in liquid nitrogen for 10 min and stored in −80°C freezer until processed for DNA analysis.

Genomic DNA extraction and amplicon sequencing

The laboratory workflow for 16S rRNA (V4 region of the gene) amplicon libraries followed the protocols published by Li et al. (2022) and consisted of four main steps. First, genomic DNA (gDNA) of abalone's gut content were extracted with the DNeasy PowerSoil Pro Kits (Qiagen, Category No. 12888-100, Germany) and quantified with a Qubit™ dsDNA HS Assay Kits (Thermo Fisher Scientific, Catalog No. Q32854). Second, gDNA was normalised to 4 nanograms/microliter (ng/μL) with molecular grade nuclease-free water and amplified through polymerase chain reactions (PCR) with a set of indexed 16S rRNA markers. The PCR amplification was conducted in triplicates, and subsequently they were pooled, purified with a customised bead solution, normalised to 2.5 ng/μL, and mixed into one microcentrifuge tube for library quantification via the Qubit assays and the Bioanalyzer High Sensitivity DNA Kit (Agilent, Catalog No. 5067-4626). Finally, the quality-controlled sample libraries were sequenced on an Illumina *MiSeq* platform using the V3 (600-cycle) sequencing kits (Illumina, Catalog No. MS-102-3003).

Histological evaluation of intestinal samples

Abalone shells were removed before dissection (Figure 2A). Specimens were sectioned transversally, cutting approximately 5 mm thick slices to maximise the chances of obtaining the required tissues (gills, foot, mantle, digestive tract, digestive gland, and left and right kidney) (Figure 2B). The sections were placed in histology cassettes prior to fixation using a 4% formaldehyde solution (1:9 v/v, 37% formaldehyde: filtered seawater) for 48 h. Histological cassettes were then immersed and stored in 70% ethanol until the slides were processed. Samples were sent to Gribbles Veterinary pathology, Christchurch, New Zealand for histological processing. In brief, samples went through the routine process of dehydration, embedding in paraffin and sectioning using a rotary microtome, and staining using haematoxylin and eosin (H&E).

The tissues were examined under a compound light microscope, Olympus BX40, at magnifications of 40x, 200x, 400x and 1000x and images were captured using cellsens (Olympus cellsens Standard 3.1 [build 21199]). Each of the tissues was screened using

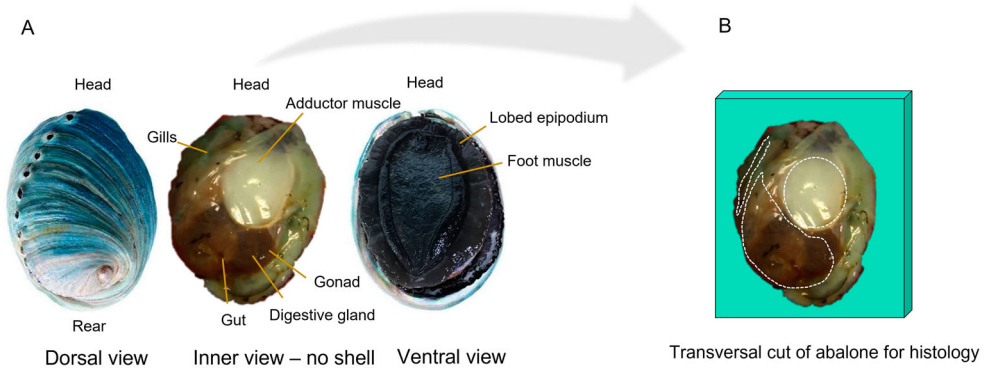

Figure 2. A, General diagram (juvenile abalone) indicating parts of interest for *H. iris*. **B,** Transversal cut plane for histological sectioning for *H. iris*.

semi quantitative criteria as described by Bignell et al. (2008), Costa et al. (2013) and Knowles et al. (2014) whereby deviation from normality were scored as: 0 No impact, 1: Mild, 2: Moderate, and 3: Severe impact on structural architecture of the tissue types when compared to baseline samples. For targeted gastrointestinal tract observations in histological section of abalone, observations were made on the mucosal layers' goblet cell types, alterations to the epithelial layers of the intestinal tract as well as the haemocytes in the connective tissues. The epithelial layer of the stomach and the posterior intestinal tract (rectum) were measured using the cellsens software (as above). A standardised section of stomach and rectum epithelium was selected and three measurements per individual were acquired. The three measurements were then averaged to produce a single measurement per individual. Those individuals that did not have the same section in the same orientation were not selected or measured (Figure 3B).

Metabolite profile

Adductor muscle tissue of approximately 1 cm^2 was obtained from abalone from each treatment. Tissue was snap frozen in liquid nitrogen, placed in 2 mL cryovials

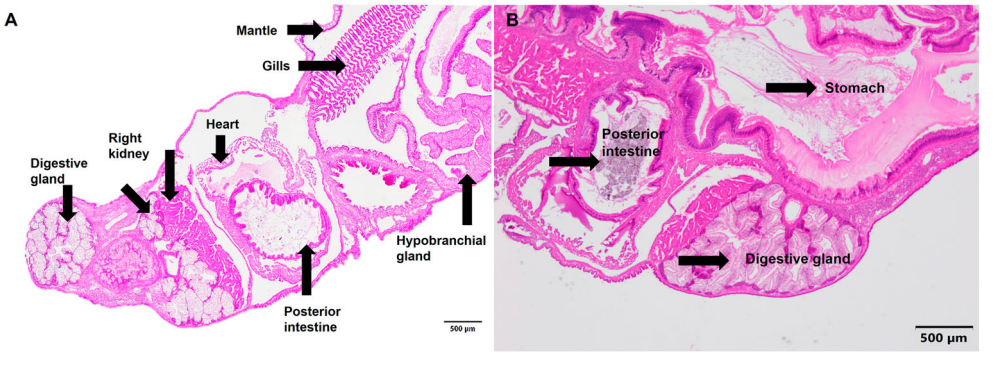

Figure 3. A, Histological image of tissues scored for *H. iris*, not shown in this image is the foot. **B,** Location of epithelial measurements.

(Biostor™), and stored in −80°C freezer until analysis. Before analysis, tissues were lyophilised for 48 h using a freezer dryer (−80°C, 0.001 mbar, Christ alpha series freeze dryer, Osterode am Harz, Germany) and pulverised using a mortar and pestle.

Metabolite extractions were performed using the cold-methanol water method (Villas-Bôas et al. 2011; Nguyen et al. 2018). Briefly, 7 – 8 mg of powdered tissues were mixed with 20 µL of d_4-alanine (10 mM) as an internal standard. Extractions were performed using 400 µL of cold methanol–water solution (50% v/v, 80% v/v, respectively). The supernatants were collected and dried in a SpeedVac (Labconco, USA) concentrator for 6 h.

Dried samples were derivatised using methyl chloroformate (MCF) (Nguyen et al. 2018). Derivatized samples were transferred into 2 mL amber GC-MS glass vials fitted with 300 µL inserts with bottom springs (Sigma-Aldrich, St. Louis, MO, USA) and then analysed on an Agilent 7890B gas chromatograph (GC) coupled to an Agilent MSD5977A mass spectrometer detector (Agilent Technologies, CA, USA) with an electron ionisation (EI) source operated at 70 eV. The system was equipped with a ZB-1701GC capillary column (30 m x 250 µm internal diameter x 0.15 µm film thickness with a 5 m guard column) (Phenomenex, Torrance, CA, USA). The instrument parameters were set according to Smart et al. (2010). Helium was used as the carrier gas and was held at a constant flow of 1 mL per min.

Different types of quality controls (QC) were used to guarantee reproducibility of GC-MS measurements, including d_4-alanine, blank samples, and pooled biological QC samples from all samples after extraction, as previously described by Nguyen et al. (2021) with small modifications. Blank samples contained only 20 µL of 10 mM d_4-alanine. Blank samples and pooled QC samples were extracted and derivatized with the other samples. For QC purposes, chloroform solvent and non-derivatized n-alkanes (C10–C40) were injected at the beginning of the analysis, followed by pooled QC samples, and blank. A random fashion injection of samples after QCs was followed. Injections of pooled QCs were repeated after every 5 samples. On the final day of the analysis, all pooled QC samples were run again to compare with the previous days.

Statistical analyses

Growth data were analysed by one-way analysis of variance (ANOVA) when a normal distribution was found (Kolmogorov–Smirnov test, $p < 0.05$), followed by pairwise comparisons with Tukey's post-hoc test. Nonparametric Kruskal Wallis test was used when data did not follow a normal distribution ($p < 0.05$), followed by Dunn post-hoc test for multiple comparisons. Homogeneity of variances was analysed using Levene's test when samples followed a normal distribution ($p < 0.05$). The fixed factor was dietary treatment (diet F, FI, FG, FIG, and commercial feed), and the random factor was tank. Significant differences were evaluated using the statistical software XLSTAT 2022.3.1 (Addinsoft, New York, USA).

For histology, chi square test was used to determine the difference in the width of stomach epithelium and intestine epithelium ($p < 0.05$). For microbiome, data were processed using established amplicon DNA sequence analysis workflow (Archer et al. 2020). Briefly, quality DNA sequence data were generated following a modified DADA2 and cutadapt v3.4 (Martin 2011) pipelines. The SILVA nr v134 database was used for the

taxonomic assignments of the unique amplicon sequence variants (ASVs). Comparisons of ASV and relative abundance at the microbial phylum level across dietary treatments before and after the feeding trial was performed using Mann–Whitney and Kruskal Wallis test ($p < 0.05$).

For metabolomic profiling, statistical analyses were performed using the integrated web-based platform MetaboAnalyst 5.0 (metaboanalyst.ca). Data were normalised by auto-scaling (mean-centred and divided by the standard deviation of each variable). Data were normalised by weight and internal standard. One-way ANOVA ($p < 0.05$; post-hoc analysis: Fisher's LSD) was used to compare effects of different dietary treatments on abalone metabolite profiles. Chemometric analysis via partial least squares-discriminant analysis (PLS-DA) was performed to assess the discrimination between dietary treatments and to facilitate visualisation of the major trends. A heatmap of detected metabolites in adductor muscle samples was generated to visualise variations among treatments.

Results

Growth performance

The inclusion of insect meal, grape marc, and both did not significantly affect the specific growth rates in weight and length of abalone (Table 2) compared to the diet free of insect meal and grape marc (diet F). However, there were significant effects on the shell width and shell length increment per day among the experimental diets. In addition, the inclusion of insect meal and grape marc did not significantly affect the muscle yield and the soft body/ shell ratio (SB/S).

Microbiome analysis

The total number of ASVs generated from the sequencing reads was 3916 from 65 samples. Comparisons were made in two areas: between the baseline group and commercial feed (before and after the feeding trial) (Figure 4A), and among the dietary treatments after the feeding trial (Figure 4B). The baseline abalone group (before dietary treatment) possessed 2404 and 2174 total and unique ASVs, respectively. After the feeding trial, no significant differences were observed among dietary treatments. Abalone fed the commercial feed possessed 553 and 323 total and unique ASVs,

Table 2. Growth indicators in *H. iris* fed with the experimental diets and a commercial diet.

	Diet				
Measured parameters	F	FI	FG	FIG	CF
SGR Total weight (%)	0.41 ± 0.1^{b}	0.41 ± 0.13^{b}	0.41 ± 0.13^{b}	0.42 ± 0.13^{b}	0.63 ± 0.11^{a}
SGR Shell length (%)	0.15 ± 0.07^{b}	0.16 ± 0.06^{b}	0.14 ± 0.05^{b}	0.14 ± 0.05^{b}	0.27 ± 0.09^{a}
SGR Shell width (%)	0.17 ± 0.09^{b}	0.17 ± 0.08^{b}	0.14 ± 0.05^{c}	0.14 ± 0.05^{bc}	0.30 ± 0.13^{a}
Length increment (um day^{-1})	37.7 ± 16.2^{bc}	33.5 ± 11.3^{c}	41.9 ± 17.6^{b}	33.5 ± 11.3^{bc}	72.6 ± 32.4^{a}
Muscle yield	68.5 ± 2.1^{a}	68.5 ± 2.2^{a}	68.6 ± 2.1^{a}	68.8 ± 2.7^{a}	67.8 ± 2.0^{a}
SB/S ratio	2.2 ± 0.2^{a}	2.2 ± 0.2^{a}	2.2 ± 0.2^{a}	2.2 ± 0.4^{a}	2.1 ± 0.2^{a}

Notes: Data are represented by means ± standard deviation (n = 750). Significant differences are represented by different alphabetic superscripts ($p < 0.05$). Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc) and commercial feed (CF).

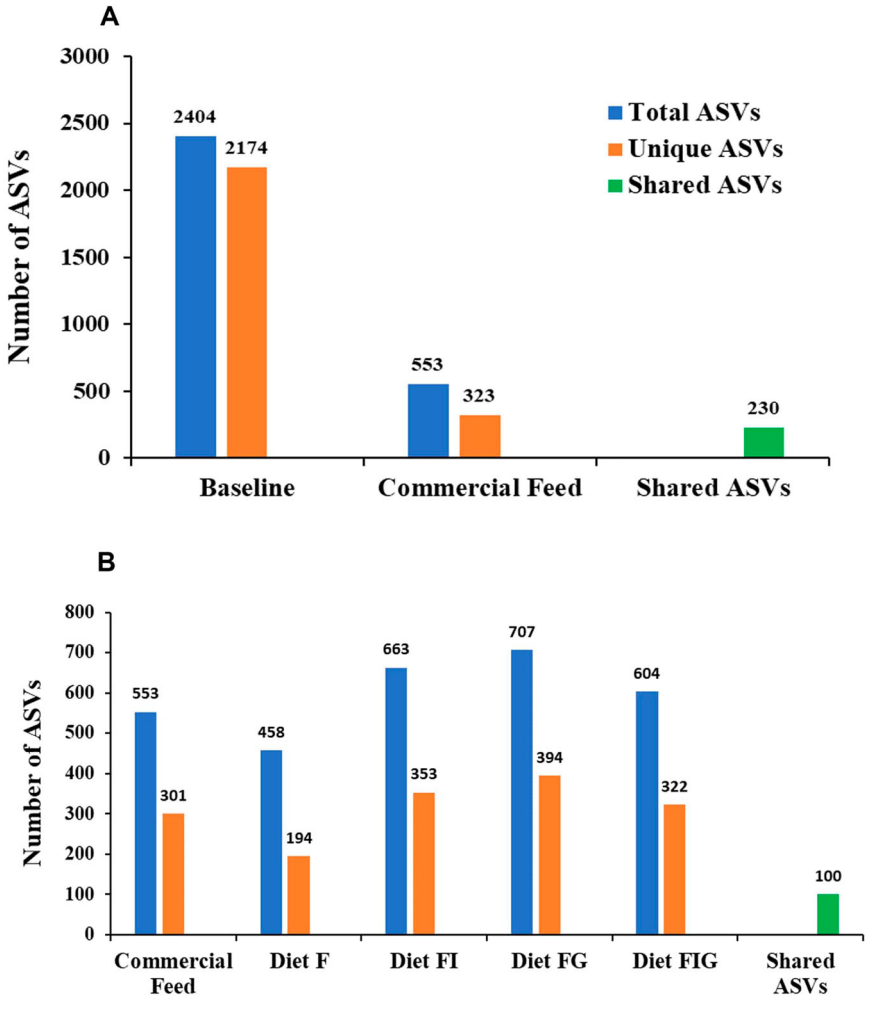

Figure 4. A, ASV Comparison between the baseline and commercial feed groups after a feeding trial (165 days). **B,** ASV Comparison among four formulated diets and commercial feed groups after a feeding trial (165 days). Abbreviation: CF, commercial feed; F, fishmeal based; FI, fishmeal + insect meal; FG, fishmeal + grape marc; FIG, fishmeal + insect meal + grape marc; ASV, amplicon sequence variant.

respectively. The insect meal/grape marc free diet (Diet F) showed 458 and 194 total and unique ASVs, respectively, while the diet including grape marc (Diet FG) showed the 707 and 304 total and unique ASVs, respectively. Only 100 ASVs were shared by all dietary treatments (Supplementary Table 1).

The taxonomic assignment returned 33 prokaryotic phyla of which 14 were shared by all diet groups (Supplementary Table 2). Phylum Deinococcota was unique to Diet F, and Phyla Armatimonadota, Dadabacteria, Nanoarchaeota, and NB1-j were only unique to the baseline group. Proteobacteria, Bacteroidota, and Fusobacteriota were the top three bacterial phyla in all dietary treatments. The relative abundance of Firmicutes was high in the baseline diet and Fusobacteria was high in diet FI and FG diets (Supplementary Table 2 and Figure 5).

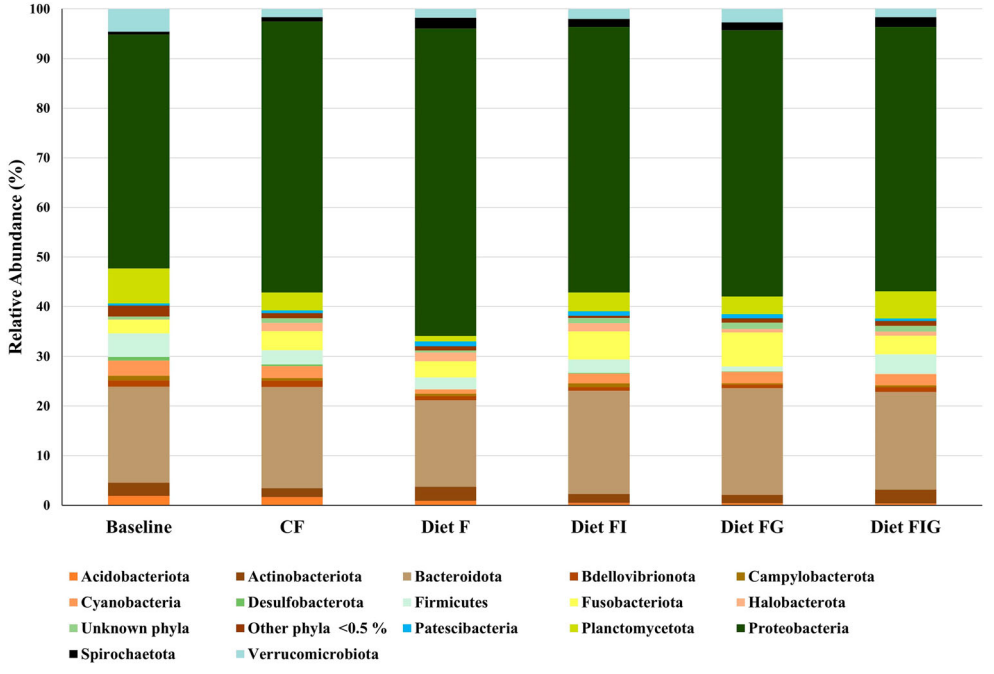

Figure 5. Relative abundance (%) of the most abundant prokaryotic phyla recovered from the farmed abalone gut content before and after dietary treatment with experimental diets and commercial feed. Abbreviation: CF, commercial feed; F, fishmeal based; FI, fishmeal + insect meal; FG, fishmeal + grape marc; FIG, fishmeal + insect meal + grape marc.

Histology

Histological analysis of the gills, foot, mantle, gonad, digestive tract, and kidneys revealed well-structured tissue architecture (Figure 6A). There were no discernible differences in the tissue health and quality from animals among dietary treatments. Several abalone had enlarged digestive gland and right kidney lumina as well as patches of autolysed tissue.

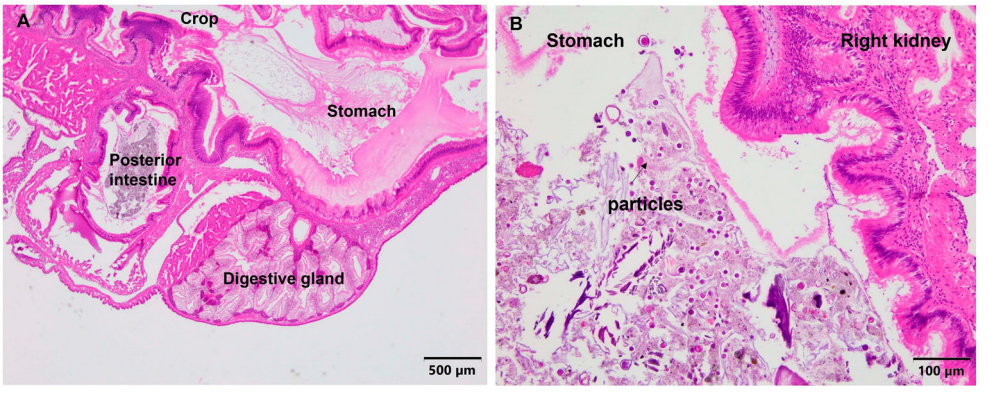

Figure 6. Gastrointestinal tract (stomach) regions showing examples of healthy individual *H. iris* from **A,** Diet F, and **B,** Example of particles observed in abalone fed encapsulated diets.

Table 3. Measurement of width of intestine and stomach epithelium of abalone among experimental diets, commercial feed and baseline.

Diet	Average intestine epi. thickness (μm)	Std dev	n	Average stomach epi. thickness (µm)	Std dev	n
F	43.5	15.7	3	65.2	12.5	11
FI	45.5	12.4	5	68.2	11.7	11
FG	41.0	6.3	4	69.0	8.5	13
FIG	46.0	7.4	4	75.1	7.7	14
CF	40.2	7.5	5	70.7	9.0	10
Baseline	35.5	11.2	2	69.3	5.6	3

Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc) and commercial feed (CF).

This condition did not, however, correspond exclusively with any diet regime. Most of the abalone appeared to have food in the digestive system at the time of sampling (Figure 6B). There were no differences in the width of the stomach epithelium ($\chi^2(4) = 5.04$, $p = 0.28$) and posterior intestine epithelium ($\chi^2(4) = 3.58$, $p = 0.47$) layers of the standardised section among the diet types (Table 3).

The digestive gland tubules were observed to be well structured with healthy epithelial architecture. The baseline (before feeding trial) samples were compared with the samples collected at the end of the experiment to identify any variations in quality of the digestive gland. The baseline samples showed enlarged digestive tubule lumina and increased vacuolation of the epithelial layer, suggesting lower glycogen reserves and appearance of increased spacing of the interstitial spaces (Figure 7A and B, Table 4). Those abalone fed the commercial feed appeared to have more densely packed epithelial layers which produced a more eosinophilic staining appearance, likely due to increased levels of stored glycogen-like and fatty acid-like materials compared to the experimental diets and baseline samples (before feeding trial). However, this has not been confirmed with other staining techniques, such as periodic acid-schiff (PAS) and PAS-D (with diastase) which specifically identifies glycogen-like substances (Figure 7C). Diets FG and FIG (Figure 7F, G and H) were associated with increased prevalence of vacuolation and depleted glycogen when compared to the other diets (Figure 7C, D and E). However, there was high variation among the diets as depicted in Figure 7G and H. Varying aspects and conditions of the digestive tissue architecture are described in Table 4.

Enlarged tubule lumens, increases in interstitial spaces, vacuolated epithelia (Figure 7) as well as thinning epithelia and sloughing can indicate either a potential health condition or general individual variation. Autolysis and sloughing appearance are likely to be an artefact of fixation, and bacteria were not observed in association with autolysed tissue.

Metabolite profiles

Metabolite identification yielded 86 annotated compounds from spectra of abalone muscle tissues. The multivariate data analysis via PLS-DA score plot revealed some clear separations among diets throughout the feeding trial (Figure 8A). Overall, samples from diet FIG were clearly distinct from samples from the commercial feed group. However, there was no good separation between diet F, FG, and FI. The first two components contributed 27.1% of the total variation. The PLS-DA model cross validation *via* LOOCV showed accuracy (two components) of 0.74, R^2 of 0.91 and Q^2 of 0.71, indicating a good prediction

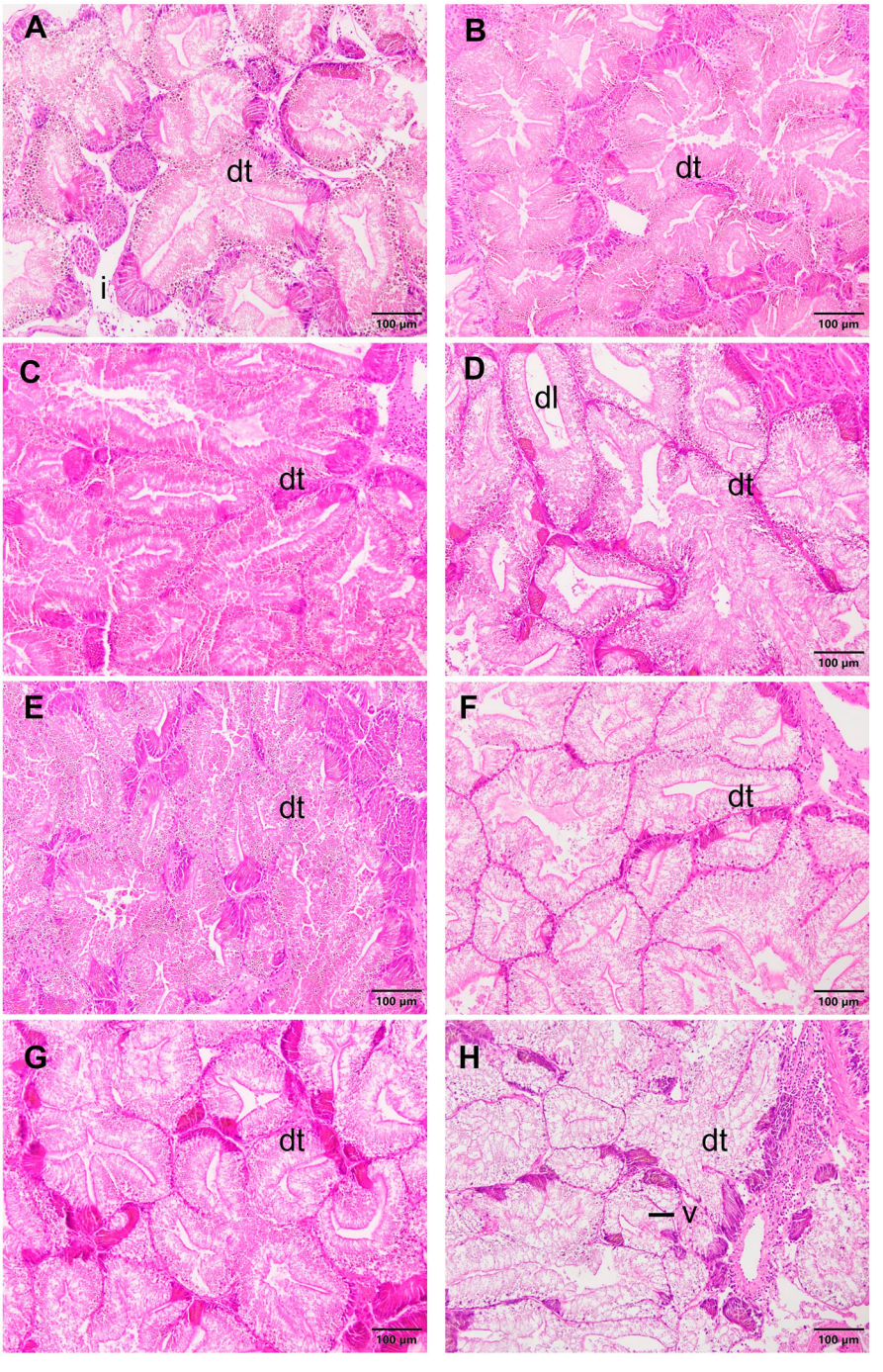

Figure 7. Histological images (haematoxylin and eosin) of digestive gland tissue from *H. iris* fed with experiment diets **A,** Baseline prior to diet treatment individual 1, **B,** Baseline prior to diet treatment individual 2, **C,** Commercial feed, **D,** diet F, **E,** Diet FI, **F,** Diet FIG, **G,** Diet FG individual one and **H,** Diet FG individual 2 as an example of the variation within the treatment. Interstitial space (i), digestive tubule (dt), digestive lumen (dl), vacuole (v). Scale 100 µm. Abbreviation: CF, commercial feed; F, fishmeal based; FI, fishmeal + insect meal; FG, fishmeal + grape marc; FIG, fishmeal + insect meal + grape marc.

Table 4. Prevalence of digestive gland conditions among each of the diet treatments. Data represent % prevalence rounded to 1 decimal place.

Digestive gland condition (% prevalence)	Baseline	Diet				
		F	FI	FG	FIG	CF
Good tubule architecture	55.6	28.6	20.0	46.7	53.3	33.3
Sloughing into lumen	0.0	0.0	20.0	0.0	0.0	26.7
Increased interstitial space	22.2	7.1	6.7	20.0	0.0	0.0
Enlarged lumina space	11.1	28.6	20.0	13.3	6.7	13.3
Enlarged lumina space and vacuolated	0.0	7.1	0.0	0.0	0.0	0.0
Large patches of autolysis in otherwise healthy tissues	11.1	21.4	20.0	0.0	0.0	26.7
Thinning epithelia	0.0	0.0	6.7	0.0	0.0	0.0
Vacuolated epithelia	0.0	0.0	0.0	20.0	40.0	0.0
No DG tissue available	0.0	7.1	6.7	0.0	0.0	0.0

Abbreviations: Diet F (fishmeal based), FI (fishmeal + insect meal), FG (fishmeal + grape marc), FIG (fishmeal + insect meal + grape marc) and commercial feed (CF). Dg = digestive gland.

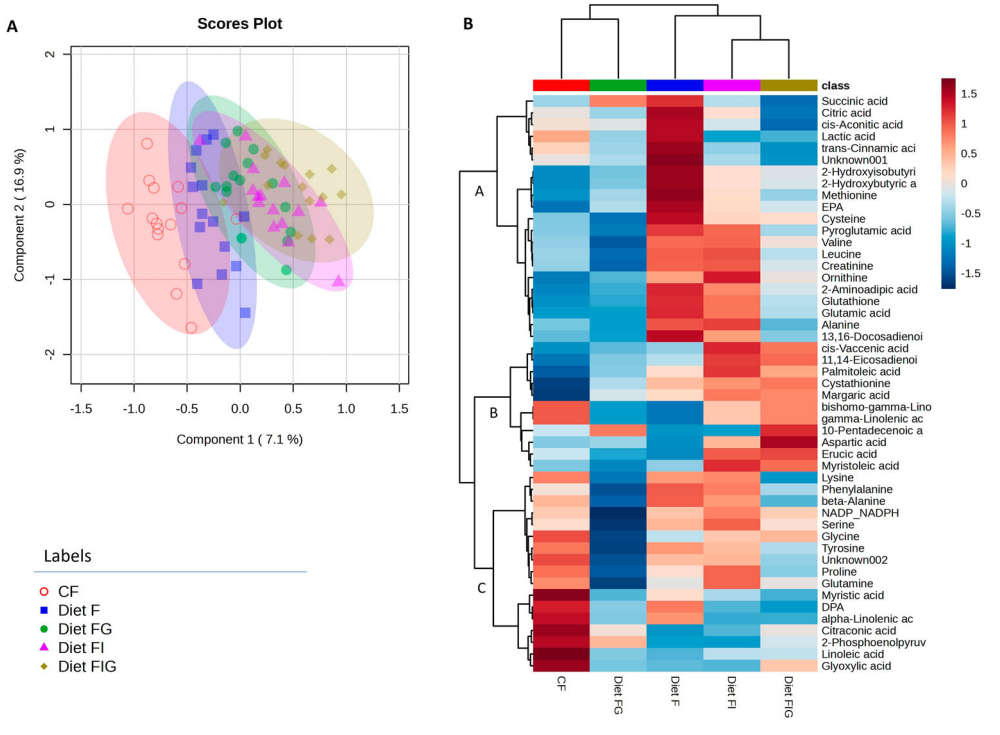

Figure 8. Effects of different feeding diet on metabolite profiles of abalone. **A,** PLS-DA score plot. **B,** Heatmap of 49 metabolites significantly different between treatments (One-way ANOVA, $p < 0.05$). Abbreviation: CF, commercial feed; F, fishmeal based; FI, fishmeal + insect meal; FG, fishmeal + grape marc; FIG, fishmeal + insect meal + grape marc.

model. Furthermore, PLS-DA analysis also identified 15 metabolites with VIP scores greater than 1 which are important classifiers (Supplementary Table 3).

The univariate data analysis *via* one-way ANOVA identified 49 metabolites that were significantly different by the diet ($p < 0.05$) and none of them by the tank component (Supplementary Table 4). The greatest difference was observed between diet FIG and commercial feed, with 19 significantly different compounds, such as myristic acid,

linoleic acid, eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA), aspartic acid, 2-phosphoenolpyruvic acid and α-linolenic acid (ALA). Among the experimental diets, major differences were observed between diet F and FIG, with 28 significantly different compounds. Other significant differences were observed between diet F and FG with compounds, such as in myristic acid, DPA, palmitoleic acid, aspartic acid, cystathionine, ALA, linolenic acid (LA), lactic acid, and lysine. A heatmap of these metabolites was generated to visualise the detail differences (Figure 8B), which divided the metabolites into 3 main clusters (A, B and C). Metabolites in cluster A are mostly amino acids, while most of the fatty acids are in cluster B. Cluster C is a mix of fatty acids, amino acids, and organic acids.

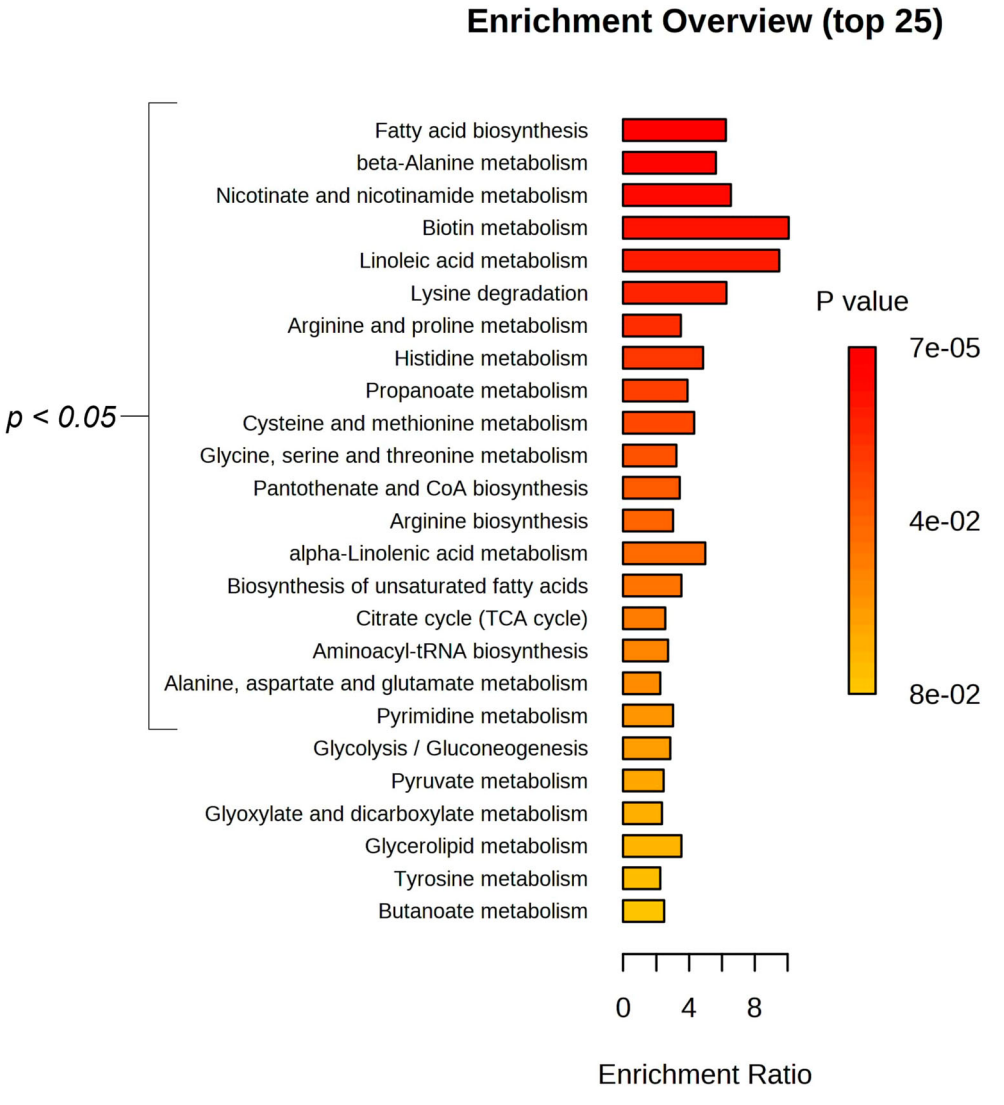

Figure 9. List of pathways (top 25) affected in abalone muscle fed on diet FIG and commercial feed. Abbreviation: CF, commercial feed; FI, fishmeal + insect meal.

The enrichment analysis between diet FIG and commercial feed (the best separated diets based on a PLS-DA analysis) showed that 19 pathways were significantly enriched and impacted by diets (Figure 9 and Supplementary Table 5). The majority of pathways were amino acid metabolism. Fatty acid biosynthesis was the most affected pathway while biotin metabolism and linoleic were the most enriched pathways.

Discussion

The purpose of this study was to investigate the partial replacement of fishmeal with insect meal and inclusion of grape marc to promote better health in juvenile *H. iris* within farm conditions. Fishmeal is generally included in abalone feeds at 30%–40% to promote faster growth (Bansemer et al. 2016). However, its use in aquafeeds has been questioned mainly due to sustainability issues. In this study, insect meal was included as a more sustainable protein substitute to achieve good nutritional profile, and grape marc was included as a carbohydrate source due to the antioxidant effect and the content of dietary fibre that can be beneficial for the gut microflora. The inclusion of both ingredients would promote a more sustainable aquaculture in terms of reduced fishmeal use. In addition, it considers the animal's overall health, which integrates the intestinal microbiota interactions and gastrointestinal morphology. An integrated and comprehensive analyses of such factors can be used as a more effective assessment and indicator of fish/shellfish welfare.

The survival was maintained at 99% in all treatments, indicating good abalone overall health despite the ingredient inclusion. The inclusion of insect meal did not affect the protein levels, but affected the reducing sugars, dietary fibre, lipid, and ash content of the experimental diets. The inclusion of grape marc did not affect the protein level, but significantly affected the dietary fibre content, lipid, and ash content of the experimental diets, compared to the fishmeal/grape marc free diet (diet F). The inclusion of insect meal and grape marc did not affect abalone shell length and weight. However, the inclusion of grape marc significantly affected abalone shell width. The muscle yield, and the soft body/shell ratio (SB/S) were similar among abalone fed the experimental diets and the commercial feed, suggesting a similar growth regardless of the inclusion or diet.

The commercial feed was used as a positive control to compare against abalone fed with the experimental encapsulated diets. The limited knowledge of the ingredients on the commercial feed did not allow accurate performance comparison between commercial feed and experimental diets. Considering this, single experimental diets can be compared to one another based on the whole diet, not the ingredient differences. Commercial feeds usually contain highly palatable and digestible dietary ingredients, which include a variety of plant meals, fishmeal, cereal grains, feed enhancers, oilseeds, minerals, and vitamins, which are carefully formulated to optimise growth (Bansemer et al. 2016). For this reason, abalone fed with the commercial feed exhibited significantly superior biomass gain, SGR, and length increment compared to animals fed any of the experimental diets. The SGR (total weight) values of the experimental diets in this study coincide with the ones reported previously by Allen et al. (2006) and Bullon et al. (2023) with around 0.45% and 0.42%, respectively for *Haliotis iris*.

In this study, abalone were fed commercial feed prior to the commencement of the study (since 6–8 months old). The commercial feed may have shaped digestion and palatability favouring commercial feed. The preference of the diet received after weaning over introduced diets has been documented in abalone (Hernández et al. 2009; Mulvaney et al. 2013). The introduction of experimental diets with alternative ingredients may have had implications in growth promotion. Further studies need to consider the effect of the previous received diet on the feed intake of introduced diets.

Microbiome

In this study, the gut microbiome of abalone fed with four experimental diets and a commercial feed revealed four dominant bacterial phyla that comprised the majority: Bacteroidota, Fusobacteria, Proteobacteria and Firmicutes. The presence of these phyla coincides with what has been reported for *H. midae* (Erasmus et al. 1997), *H. discus hannai* (Tanaka et al. 2004), *Haliotis tuberculata* (Gobet et al. 2018), and *Haliotis diversicolor* (Zhao et al. 2012).

In this study, results showed that the inclusion of insect meal (diet FI) and grape marc (diet FG) did not significantly increase the diversity of microbial communities compared to the insect meal/grape marc free diet (diet F). One possible explanation for this finding is that the percentage of insect meal and grape marc used in this study may have not contributed to significant changes in the microbiome of abalone. A previous study by Terova et al. (2019) has documented that the percentage of insect meal inclusion determines the significance and degree of positive change in the microbiome of rainbow trout. Another possible explanation is that insect meal from *T. molitor* is highly acceptable by abalone. A previous study has documented that the inclusion of insect meal from the black soldier flies *H. illucens* at 50% replacement increased microbial diversity in rainbow trout and Atlantic salmon (*Salmo salar*) (Bruni et al. 2018; Weththasinghe et al. 2022) highlighting the role of insects in the normal diets of salmonid fish. However, a study by Terova et al. (2021) included insect meal from *T.molitor* at 100% replacement, without significant differences in the microbial diversity and richness in the gut of rainbow trout. These differences may be associated with nutritional profiles of the insect species themselves (Antonopoulou et al. 2019), and the fact that they possess their own microbiomes.

The inclusion of insect meal and grape marc produced an increased relative abundance of Fusobacterium and decreased abundance of Proteobacteria. The high levels of indigestible fibre, such as chitin in insect meal and lignin in grape marc, may have contributed to these fluctuations as bacteria in Fusobacterium phyla have been associated with complex polysaccharide fermentation (Gobet et al. 2018) and the production of short chain fatty acids (SCFAs) (Levy et al. 2017; Parker-Graham et al. 2020). Microbial SCFAs are metabolic end products of microbial fermentation which enhance the renewal of gut epithelia (Vielma and Lall 1997; Pardesi et al. 2022), regulate the diversity of intestinal microbiota (Li et al. 2019), and reduce intestinal inflammation in aquatic species (Liu et al. 2019). Chitin, the prebiotic oligosaccharide found in insect meals (Vogel et al. 2018; Banerjee et al. 2021) may have contributed to a more diverse bacterial microbiome in those abalone fed diets including insect meal, which were also found to be reduced in Proteobacteria, compared to diet F (insect meal/ grape marc free diet). In fact, chitin has been shown to have a significant effect on the reduction of pathogenic

bacteria belonging to Proteobacteria, such as *Vibrio* (Terova et al. 2019; Rimoldi et al. 2021), *Shewanella, Aeromonas, Citrobacter*, and *Kluyvera* (Cai et al. 2006; Jiang et al. 2013; Rimoldi et al. 2019).

In this study, the inclusion of both insect meal and grape marc promoted a relative increase in Firmicutes abundance compared to an insect meal/grape marc free diet (diet F). Previous studies have documented that dietary insect meals have promoted more abundance of Firmicutes in rainbow trout, mostly associated with beneficial lactic acid bacteria species, such as *Lactobacillus* and *Bacillus* (Rimoldi et al. 2021). Lactic acid bacteria increase the digestibility of indigestible products producing higher levels of short chain fatty acids in the digestive tract of aquatic animals (Burr et al. 2008; Soltani et al. 2019) and have antimicrobial effects (Newaj-Fyzul et al. 2007; Ringø et al. 2012). In addition, the polyphenols available in grape marc (anthocyanins, catechins, flavonols, and phenolic acids) have been shown to contribute to increase beneficial bacteria in rainbow trout, such as *Bifidobacterium* and *Lactobacillus* (Pulgar et al. 2021; Zorraquín et al. 2020). In previous studies, the increased presence of Firmicutes has been linked to higher levels of butyrate, which is the most preferred short chain fatty acid used by colonocytes, promoting growth in sea bream (Robles et al. 2013). The results in this study showed general trends on microbial diversity across different dietary treatments with and without insect meal and grape marc. Further studies which include genus or species taxonomic classification are recommended for further association between beneficial bacterial species and alternative ingredients.

The results in this study showed that animals had significantly higher diversity of microbial communities before the feeding trial (2404 ASVs) compared to after the feeding trial (553 ASVs). The relative abundance after the feeding trial showed a decreased abundance of Bacteroidota and Firmicutes, an increased abundance of Proteobacteria and varying levels of Fusobacteria compared to the baseline samples (before feeding trial). Although variations were present, the predominance of these phyla remained over time. In this study, Proteobacteria has been identified as the predominant bacterial phylum, corroborating their importance in carbohydrate metabolism and digestibility of nutrients in *Haliotis* species (Gobet et al. 2018; Zhao et al. 2018; Parker-Graham et al. 2020; Choi et al. 2021; Wang et al. 2021). The increase in Proteobacteria has been associated with an attempt of abalone to improve feed efficiency (Yu et al. 2022) and the biosynthesis of amino acids (Xiong et al. 2022) to expedite abalone grow (Zhao et al. 2012). The differences found before and after the feeding trial are not conclusive and may be explained by additional factors that shape the intestinal microbiome in aquatic animals, such as seasons (Gobet et al. 2018; Danckert et al. 2021), developmental stage (Zhao et al. 2012), and the quality of the surrounding seawater (Medina-Félix et al. 2022). Further investigations are needed to reveal the contribution of these components on the microbiome of abalone.

Histology

In this study, no inflammatory and/or degenerative changes were observed in any of the dietary treatments. The presence of antinutritional factors in insect meals, such as phytate, tannins, and polyphenols and lignin in grape marc may not have caused any signs of intestinal disease at the percentage included in the experimental diets.

Antinutritional factors (ANF) are substances inherent to some ingredients that interfere with food utilisation, causing intestinal inflammation and affecting the health of animals (Francis et al. 2001) and constitute one of the main obstacles for inclusion of alternative ingredients in aquafeeds. Insect meals have been reported to have low levels of antinutritional factors (ANFs) compared to plant-based proteins, such as tannins (affect protein utilisation and digestion), oxalate, and phytate (which affect mineral utilisation and digestion) (Francis et al. 2001; Idowu et al. 2019). Therefore, inclusion of insect meals up to 75% in aquafeeds has demonstrated no gastrointestinal inflammatory effects for clownfish (*Amphiprion ocellaris*) and Atlantic salmon (Lock et al. 2016; Vargas-Abúndez et al. 2019). Conversely, grape marc is physically or chemically treated to remove potential antinutrients, such as lignin, which interfere with nutrient digestion (Moate et al. 2014) and reduce feed intake (Amyot et al. 2018). In this study, the inclusion of grape marc did not cause significant changes of the gastrointestinal epithelia and digestive gland. Therefore, it is presumed that percentages of 30% of grape marc can be included in abalone diets without further intestinal compromise.

In this study, insect meal replaced fishmeal to a maximum of 10%, thus maintaining fishmeal as the primary protein source in all experimental diets. This proportion of fishmeal may have provided enough nutritional requirements to maintain a healthy gut condition. In diets where grape marc was included, only corn meal was completely replaced, but the main protein components (insect meal, fishmeal, or both) were maintained. Therefore, although insect meal and grape marc are foreign ingredients in the abalone diet, the levels included in our study may have been low enough to cause no detectable inflammation or disrupted intestinal morphology. Abalone fed on diets with grape marc may have adapted to this ingredient without much difficulty, considering that there was a proportion of digestible carbohydrates in the whole formulation. Based on to the above-mentioned studies, there is evidence to support that the final mixture of ingredients and nutrients, regardless of the antinutritional factors may provide a balanced and positive aquafeed nutritional profile for growth.

A critical factor in the development of aquafeeds is the level of inclusion of alternative ingredients. For instance, studies have shown that when soybean meal is included in high levels (more than 50% of the protein component), signs of enteritis can appear in the distal intestine of the rainbow trout (Kumar et al. 2021). Conversely, when a mixture of plant-based protein replaces a maximum of 30%, there are no signs of intestinal inflammation in the European sea bass (*Dicentrarchus labrax*) (Bonvini et al. 2018). Compared to plant-based meals, insect meals seem to be more suitable to aquatic nutrition due to the presence of essential fatty acids (Belforti et al. 2015) and immunomodulatory factors, such as chitin (Ringø et al. 2012) which contribute to reducing proinflammatory substances in the intestinal tract of aquatic species (Kumar et al. 2021; Li et al. 2019).

Another possible explanation for the absence of gastrointestinal morphology differences among diets may be that abalone adapt easily to nutritional sources. Abalone are highly adaptable species which, due to their limited capacity to move geographically, need to adapt quickly to available sources of food to survive (Agrawal 2001; Schaefer et al. 2013). Since the digestive gland (site of nutrient absorption and storage), and the epithelial layer (provides insights into potential nutrient deficiencies) did not present any

significant changes, it can be concluded that the digestive tract of abalone responded effectively to the introduction of insect meal and grape marc.

The stomach and the crop were targeted in histological observations due to their involvement in feed digestion. Typically, macroalgae are partially digested in the crop before moving into the stomach and spiral caecum. Although the stomach has half the capacity of the crop it has the same peristaltic contraction to move food to the caecum and intestine. The stomach was targeted due to its proximity to both the digestive gland and the intestine, its ability to move food into the caecum and as the main site of digestion (Campbell 1965; Crofts 1929; Kemp 2001). The intestine was selected to observe whether there was any fibrous material making it through the whole gut tract and whether this was causing any damage. Additional sampling as the abalone developed (i.e. 1 year old) would have been beneficial to observe any future changes from the dietary alterations. Although these epithelial layer measurements can be subjective, they are worth recording to build a knowledge base for future research. Further studies that evaluate gradual increments of insect meal and grape marc on gastrointestinal morphology are recommended.

Metabolite profiles

The GC-MS based metabolomics approach revealed significant differences in metabolic profiles of abalone fed on experimental feeds and the commercial diet. The dietary effects resulted in significant 49 metabolites and the enrichment analysis identified 19 pathways that were significantly impacted between diet FIG compared to commercial feed. This reflects the tissue-specific response of abalone to new dietary ingredients (Grandiosa et al. 2018; Venter et al. 2019; Yu et al. 2022). The up- and down-regulation of metabolites provide metabolic signatures of the relevance of fatty acid and amino acid products and metabolism in abalone growth and gut health.

In the present study, it was observed that the metabolite profile among the experimental diets varied significantly with the inclusion of insect meal and grape marc. The metabolite profiles of animals fed on insect meal (diet FI) showed significantly higher levels of palmitoleic acid and myristoleic acid compared to animals fed on the insect meal/grape marc free diet (F). The elevated levels of these compounds in abalone muscle may be associated with elevated levels of the fatty acid precursors, which are abundant in insect meals, such as palmitic acid and myristic acid (Belforti et al. 2015; Gasco et al. 2016; Sankian et al. 2018). The presence of myristoleic and palmitoleic acid in the abalone muscle fed on the diet with insect meal (FI) may be attributed to the abalone's ability to desaturate the precursors myristic and palmitic acid (Tocher 2003), which are highly abundant in *T. molitor* species (Belforti et al. 2015; Dreassi et al. 2017).

The feed containing insect meal promoted elevated levels of proline, glutamic acid, aspartic acid, and erucic acid. The elevated levels of proline in diet FI support the fact that when dietary lipids are high (in diets with insect meal), proline regulates fatty acid oxidation (Venter et al. 2019). The high levels of aspartic acid and glutamic acid in diet FI may suggest an increase in amino acid catabolism caused by insect meal inclusion, promoting the conversion from aspartate to glutamate, and glutamate to alpha-ketoglutarate (Fabrikov et al. 2020). As catabolism is increased, the utilisation of glucogenic amino acids (arginine, glutamate, glutamine, glycine, histidine, methionine,

proline, and serine) to produce energy was possibly heightened and therefore found to be reduced in abalone muscle (Jarak et al. 2018; Venter et al. 2018). Reduced levels of these amino acids may suggest that abalone are using the low levels of digestible carbohydrates found in diets FI and FIG for energy metabolism. The utilisation of glucogenic amino acids for energy may have contributed to a slow growth, as amino acids are ideally allocated for growth rather than energy fuel.

The insect meal/grape marc free diet (F) has shown to produce high levels of essential amino acids, such as methionine, valine, leucine, alanine, lysine, phenylalanine, intermediates of the tricarboxylic citric acid (TCA), such as succinic acid and citric acid and important fatty acids, such as eicosapentaenoic acid (C20:5n-3, EPA) and docosapentaenoic acid (C22:5n-3, DPA). Abalone fed on diet F showed elevated levels of these compounds, which may be due to the high levels usually available in fishmeal. However, the growth rates were similar to the other experimental diets that included insect meal and grape marc. This finding may corroborate that growth in abalone is not limited to essential amino acid provision. As abalone are herbivorous species, its metabolism mainly digest carbohydrates (Thongrod et al. 2003; Wang et al. 2009), moderate levels of protein and low levels of lipids (Lee et al. 2019). Abalone do not possess enzymes to metabolise fatty acids, and therefore an excess of dietary lipids might translate into slow growth (Lee et al. 2019).

The results showed that the accumulation of alpha-linolenic acid (C18:3n-3, ALA) and linoleic acid (C18:2n-6, LA) in abalone muscle may have been linked with high levels of dietary digestible carbohydrates, resulting in animals with faster growth. As the lipid metabolism is closely involved with that of carbohydrates, an excess in carbohydrate consumption results in the formation of acetyl CoA which is the substrate for synthesising fatty acids (Wang et al. 2009). The diets with substantially higher levels of digestible carbohydrates (F and CF) promoted abalone muscle with higher levels of docosapentaenoic acid (C22:5n-3, DPA), alpha-linolenic acid (C18:3n-3, ALA), gamma-linolenic acid (C18:3n-6, GLA), and linoleic acid (C18:2n-6, LA). Many of these fatty acids and their derived lipids, such as arachidonic acid (C20:4n-6, ARA), docosahexaenoic acid (C22:6n-3, DHA), and eicosapentaenoic acid (C20:5n-3, EPA) cannot be synthesised *de novo* by abalone and play a critical role in cell signalling, gene expression (Liu et al. 2023), growth promotion, and overall health (Mai et al. 1996; Toledo-Agüero and Viana 2009; Bautista-Teruel et al. 2011). Studies have shown that animals with higher growth upregulate fatty acid metabolism (Venter et al. 2022). However, further elucidation on the effect of specific dietary fatty acids on abalone is still limited.

Conclusions

The findings of this study indicate that the inclusion of insect meal and grape marc did not significantly affect the diversity of gut bacteria of abalone. In addition, the inclusion of insect meal and grape marc increased the relative abundance of gut bacteria possibly associated with beneficial species in the gastrointestinal tract. The absence of signs of intestinal inflammation in abalone fed with both alternative ingredients suggest that abalone have a natural ability to utilise these substrates to maintain a healthy condition. Moreover, the metabolite profile showed increased essential and non-essential fatty acid synthesis, which may be associated with higher growth rates. These findings have

significant implications for understanding the benefits of alternative ingredients in abalone diets, resulting in a more affordable final price and lower carbon footprint.

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Disclosure statement

No potential conflict of interest was reported by the author(s).

Data availability statement

The datasets generated and/or analysed during the current study are available from the corresponding authors on reasonable request.

Author contributions

Conceptualisation: NB, AA, AS; **Methodology:** NB, AA, AS; **Software:** NB, JG, JC, TN; **Validation:** NB, JG, JC, TN; **Formal analysis:** NB, JG, JC, TN; **Resources:** AA; **Writing-original draft:** NB; **Writing-review editing:** NB, JG, JC, TN, AA; **Supervision:** AA, AS; **Funding acquisition:** AA, AS.

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