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A synergistic blend of dietary organic acids, monoglycerides and phytobiotics enhance growth performance, intestinal mucosal height, and anti-viral immune gene expression in juvenile Barramundi (*Lates calcarifer*)

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ABSTRACT

Barramundi or Asian seabass, *Lates calcarifer*, is an important food fish species in tropical aquaculture, but intensive farming is associated with increased susceptibility to various bacterial, viral, and parasitic diseases. Recent findings suggest that functional diets provide a broader advantage over the pathogen-specific protection of vaccines by improving growth performance, gut health, and immunity in teleosts. In this study, we conducted a six-week feeding trial in juvenile *L. calcarifer* (50.1 ± 0.0 g) whereby growth and production performance on a diet containing 0.5 % of a synergistic blend of organic acids, monoglycerides esters of organic acids and phytobiotics (OMGP) was compared with a control diet (CTRL), using quadruplicate 500 L tanks with 30 fish per tank. At the end of six weeks, juveniles were injected with Poly(I:C) to evaluate if the OMGP blend feed additive stimulates anti-viral immune responses based on a comparative RNAseq study 24 hour (24 h) and 72 h post-challenge. Juveniles fed with the OMGP blend exhibited an 8 % higher average final body weight (147.5 ± 6.9 *vs.* 136.5 ± 4.6 g) and a 6 % higher overall final biomass (4087 ± 107 vs. 3855 ± 149 g) compared to CTRL group ($P < 0.05$). The addition of the OMGP blend resulted in significantly reduced fasting blood glucose levels, higher white blood cell counts and greater intestinal mucosal villi heights compared to fish fed CTRL diet. OMGP fed *L. calcarifer* exhibited an increased number and upregulation of differentially expressed genes associated with infectious disease related immune pathways, such as Toll-like receptor (TLR) and NOD-like (NLR) receptor signaling, and Chemokine signaling pathways. Incorporating organic acids, monoglyceride esters of organic acids and phytobiotics improved growth performance, increased intestinal mucosal villi heights, and modulate the anti-viral immune capacity of juvenile *L. calcarifer*.

1. Introduction

Barramundi or Asian seabass, *Lates calcarifer,* is a versatile and increasingly important food fish species in tropical aquaculture, contributing significantly to the global production of 105,000 tons in 2020 (FAO, 2022). However, intensive farming of the species raises susceptibility to a suite of bacterial (e.g., *Vibrio harveyi,* novel *Vibrio* sp. in 'Big Belly disease', *Streptococcus iniae*), viral (e.g., Scale Drop Disease Virus (SDDV), *L. calcarifer* herpesvirus (LCHV), Nervous Necrosis Virus (NNV)), and parasitic diseases, which can cause heavy mortalities of 80–100 % (Jahangiri et al., 2022); (Erfanmanesh et al., 2024; Gibson-Kueh, 2012; Gibson-Kueh et al., 2021; Irshath et al., 2023). While vaccines have proven efficacious against some diseases, they provide protection only against specific pathogens (Irshath et al., 2023). Increased intensity and scale of aquaculture have led to an increase in the use of antibiotics. Growing concerns about antimicrobial resistance have prompted research to find alternatives for prevention and treatment of diseases (Rico et al., 2013). Effective biosecurity, improved husbandry, and optimal nutrition are some considerations to boost general health, reduce production losses, and create more sustainable aquaculture with less use of chemicals (Austin, 2023).

Feed additives such as immunostimulants, probiotics and organic

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acids offer sustainable alternatives to antibiotics, as they stimulate the innate immune system and/or promote the growth of beneficial bacteria in the gut, addressing concerns related to antimicrobial resistance and environmental pollution (Dawood et al., 2018; Montero et al., 2023; Neuls et al., 2021; Yadav et al., 2020). Probiotics such as *Lactobacillus* and *Weissella* may be useful in counteracting loss of microbiome diversity and increased abundance of potential pathogens in guts of *L. calcarifer* with chronic bacterial enteritis or 'Big Belly' (Chew et al., 2023). Organic acids are produced via microbial fermentation of carbohydrates by various bacterial species under different metabolic pathways and conditions. Organic acids are able to cross bacterial cell membrane, kill by disrupting their metabolism, and enhance nutrient availability to fish hosts by lowering gut pH, increased pepsin activity and solubility of minerals. Organic acid salts in fish diet was found to improve growth, feed intake, specific growth rates (SGR) and feed conversion ratio (FCR) in various aquaculture species (Ng and Koh, 2017); such as citric acid, lactic acid and sorbic acid in rainbow trout (*Oncorhynchus mykiss*) (Hernández et al., 2012; Pandey and Satoh, 2008; Pelusio et al., 2020). Organic acids tend to be absorbed in the upper digestive tract, limiting their impact on bacteria in the lower digestive tract. Fatty acids such as monoglycerides are able to reach and exert their antimicrobial activity in the distal gut by virtue of a glycerol side chain that is removed only by lipases in the intestines (Rimoldi et al., 2018). A previous study showed that a blend of monoglycerides increased immune function and reduced growth of *Vibrio* spp in gastrointestinal tract of salmon and shrimp (Cordts and Quandt, 2021). In another study, a diet supplemented with monoglycerides in the form of a sodium salt of coconut fatty acid distillate enhanced the overall feed intake and growth rates of gilthead sea bream (*Sparus aurata*) (Simó-Mirabet et al., 2017). Phytobiotics, derived from various plant sources, are seen as a promising alternative to antibiotics due to the presence of various bioactive compounds that have the potential to enhance fish health (Kalaiselvan et al., 2024). Other studies show that dietary phytobiotics such as limonene found in citrus fruit peels have growth-promoting effects in Mozambique tilapia (*Oreochromis mossambicus*) (Acar et al., 2015) and Ningu (*Labeo victorianus*) (Ngugi et al., 2017)

Transcriptome profiling based on RNA-Seq is increasingly utilized to investigate host immune strategies against pathogens and comprehend how pathogens overcome host-mediated immune responses (Sudhagar et al., 2018). The fish kidney serves as a crucial immune organ with hematopoietic and lymphoid tissues, melanomacrophage centers, and a rich network of sinusoids capable of trapping foreign antigens (Ferguson, 2006; Stosik et al., 2019). The application of RNA-Seq in studying the kidney and other essential immune organs has contributed to an improved understanding of immune response in *L. calcarifer* (Domingos et al., 2021; Jiang et al., 2014; Liu et al., 2016; Xia et al., 2011, 2013). Polyinosinic:polycytidylic acid [Poly(I:C)] is a structural analog of double-stranded RNA (dsRNA) widely used in several studies to mimic immune responses to viral infections (Wan et al., 2023; Zhao et al., 2023; Zhou et al., 2014), as TLR3 agonist and to induce interferon (IFN) production (He et al., 2021). RNA-Seq analysis following Poly(I:C) challenge could elucidate the molecular pathways by which feed additives may improve growth and immunity in *L. calcarifer*.

2. Materials and methods

2.1. Overview of experimental trial

The present study aims to investigate the effects of dietary monoglycerides, organic acids and phytobiotics synergistic blend on growth performance and immunomodulation in juvenile *L. calcarifer* in a 6-week feed trial, followed by intraperitoneal administration of Poly(I:C). A comparative transcriptomic study of kidney tissues post-Poly(I:C) challenge was used to study the effects of dietary supplementation on the regulation of key immune-related genes and regulatory pathways in *L. calcarifer*.

Juvenile *L. calcarifer* were sourced from Barramundi Group Ltd hatchery in Singapore. A feeding trial was carried out in the Aquaculture Research and Teaching Facility (ARTF) Recirculatory Aquaculture System (RAS) at James Cook University Singapore (JCUS), under Institutional Animal Care and Use Committee (IACUC) approval number 2021-A012. To minimize size variation across 500 L circular RAS experimental tanks, 40–60 g juvenile *L. calcarifer* were sedated with 5 ppm Aqui-S immersion bath, individually weighed and 30 fish (average body weight 50.1 ± 0.0 g) were equally distributed across eight tanks (P = 1). The experiment ran in quadruplicate tanks with two dietary treatments: a control group with fish fed a control basal diet (CTRL), or the basal diet supplemented with 0.5 % of a proprietary blend of organic acids, monoglycerides esters of organic acids and phytobiotics (OMGP) from Adisseo Group for 6 weeks.

The experimental diets (slow sinking) were made at the Singapore Food Agency Marine Aquaculture Centre (MAC) research feed mill, using Evolum 25 (Clextral, France) twin-screw extruder. Both CTRL and OMGP diets were sent to BV-AQ (Singapore) Pte. Ltd. for nutritional composition analysis. Results and formulations of the two experimental diets are presented in Table 1.

2.2. General husbandry

Fish were fed CTRL or OMGP diets to satiation thrice daily (10 am, 2 pm, 5 pm) for 6 weeks, and per tank feed consumption was recorded daily. Individual fish weight checks were conducted at the start and end of the trial, and average body weight (ABW) of fish derived based on total biomass and fish numbers in each tank every two weeks (weeks 2 and 4). Temperature of the water in the RAS system was recorded as 27.98 ± 0.25°C, DO above 5 mg/L, pH 8–8.2, salinity 30–33 ppt and total ammonia nitrogen and nitrite levels were < 1 mg/L throughout the 6 weeks, and individual tank flow rate was set at 350/L per hour.

Table 1
Dietary formulation of control and experimental diets.

	CTRL (%)	OMGP (%)
Ingredients		
Danish fish meal 71 % Crude Protein (CP)	20	20
Soybean meal 45.8 % CP	18.5	18.5
Wheat gluten 76 % CP	10.5	10.5
Soy protein concentrate 60.5 %	14	14
Wheat flour	32	31.5
Fish oil	2.3	2.3
Soybean oil	1	1
Soy lecithin	1	1
Vitamin Premix – marine fish	0.15	0.15
Mineral Premix – marine fish	0.15	0.15
Choline chloride, 60 %	0.1	0.1
Funginate FP (Norel) mold inhibitor	0.05	0.05
Antioxydant (haltox)	0.02	0.02
Vitamin C	0.05	0.05
L-Taurine	0.2	0.2
Organic acids, monoglycerides esters of organic acids and phytobiotics (OMGP) additive blend	-	0.5
Composition		
Dry Matter (%)	90.0	88.9
Energy (Kcal/100 g)	393	388
Ash (% m/m)	4.8	5.0
Carbohydrate (% m/m)	30.3	30.2
Fat Substrate Breakdown Ratio (SBR) (% m/m)	10.4	10.4
Moisture (% m/m)	10.0	11.1
Protein (% m/m)	44.5	43.3
Cholesterol (mg/100 g)	79	75
Salt (% m/m)	0.67	0.66
Phosphorus (mg/100 g)	624	629

2.3. Sample collection for hematology and gut mucosal height assessment at 0 and 6 weeks

Blood samples were collected from 6 fish at the start of the experimental trial and from 6 fish each from OMGP and CTRL groups at the end of 6 weeks. Fish were sedated with 5 ppm Aqui-S immersion bath for blood collection and body weight measurements. Blood was collected from the caudal vein of each fish into heparinized microtainers (BD) and kept chilled in a cooling block at 4°C until analyzed for blood glucose, total plasma protein, hematocrit, and total white blood cell counts on the same day, as described by Chew and Gibson-Kueh (2023).

After blood collection, the same 6 fish from both OMGP and CTRL groups at start and end of trial were euthanized in a prolonged bath with 40 ppm Aqui-S, and gut tissues fixed in 10 % buffered formalin for histoprocessing into hematoxylin and eosin (H&E) tissue sections to assess gut mucosal heights on an Olympus BX53 transmission light microscope, Digital Camera DP74, and CellSens™ Standard Imaging System (Olympus Corporation). Mucosal heights were recorded to reduce variability of measurements as it was often difficult to determine the base of villi at sections adjacent to intestinal crypts (Figs. 1B, 1C). Height from tip of villi to muscularis mucosa (mucosal layer) (Figs. 1B, 1C) were taken in H&E tissue sections in each of four segments of the gut: anterior gut (AG), anterior half of mid-gut (MG1), posterior half of mid-gut (MG2), and hind gut (HG) (Fig. 1A). Up to 5 measurements of mucosal heights were taken in each of 5 tissue sections (n = 25 measurements) of each segment of the gut for each fish.

2.4. Sample collection for transcriptomics at 24 h and 72 h post intraperitoneal injection with PBS and poly(I:C)

Thirty fish from OMGP and CTRL groups were each given 0.1 mL Poly(I:C) (100 μg/fish) (Sigma Aldrich), or 0.1 mL phosphate-buffered saline (PBS) intraperitoneal injections at the end of the 6 weeks feeding trial. Kidney tissues from 6 fish each from OMGP or CTRL group were sampled at 24 h and 72 h after injection with Poly(I:C) or PBS, for transcriptomics. Kidney tissues were snap frozen in liquid nitrogen and stored at −80°C until processed for analysis.

2.5. RNA isolation, library construction and sequencing

Total RNA was extracted from kidney tissues using a RNeasy® Plus Micro kit (Qiagen, 74034) according to the manufacturer's instructions. All RNA samples were treated with RNase-free DNase-I (M610A, Promega) to remove genomic DNA contamination. The quality and quantity of the total RNA were determined with an Agilent 2100 Bioanalyzer (RNA 6000 Nano chip assay) and a Qubit 3.0 (Quant-It dsRNA BR Assay). A total of 48 libraries of kidney samples (6 fish x 4 treatments x 2 time periods with challenges using PBS and poly(I:C)) were generated using the VAHTS mRNASeq V3 Library Prep Kit for Illumina (NR611, Vazyme) according to the manufacturer's instructions. Briefly, mRNA with poly(A) was enriched by mRNA Capture Beads and fragmented by heating. Short mRNA was reverse transcribed with random hexamer primers to generate the first cDNA, and the second cDNA was synthesized. cDNA fragments underwent an end repair process, followed by addition of a single 'A' base to the 3' end and ligation of the adapters. Products were purified and size selected (350 bp range). Final fragments were enriched by PCR amplification and purified using VAHTSTM DNA Clean Beads. The quality and quantity of the PCR product was assessed using the Agilent Bioanalyzer 2100 and Qubit 2.0 (Thermo). Finally, libraries were sequenced on an Illumina NovaSeq 6000 platform with 150 bp paired end reads at Omics Drive Pte Ltd (Singapore).

2.6. Gene differential expression, functional GO, and pathway enrichment analysis

High-quality clean data were produced from the raw data by

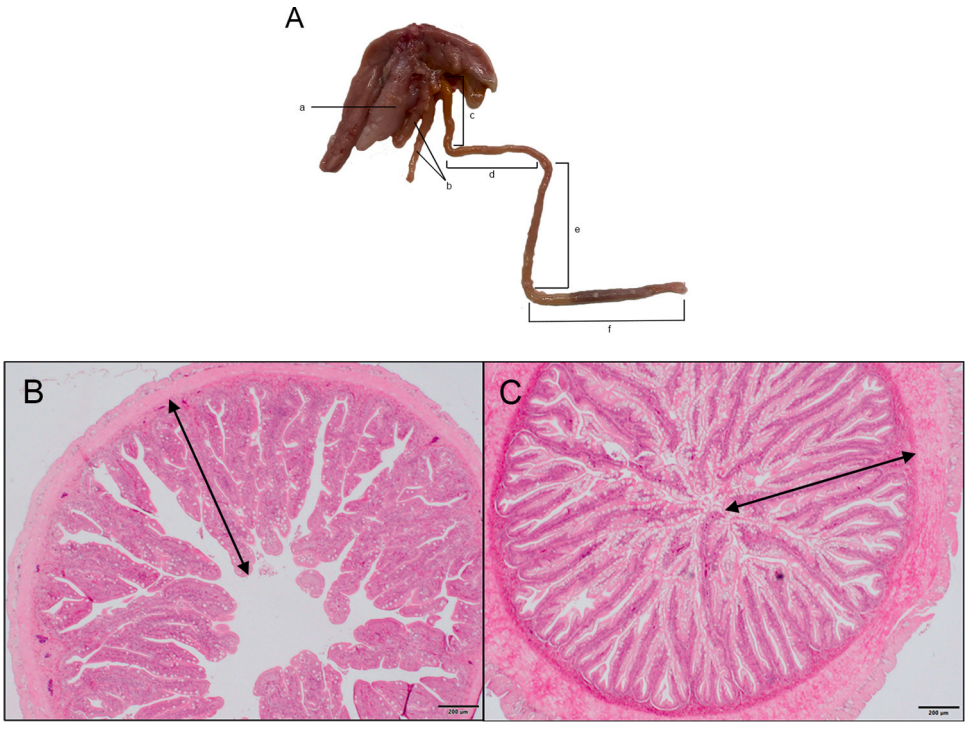

Fig. 1. Gross morphology of the L. calcarifer gut (A). (a) stomach, (b) pyloric caeca, (c) anterior gut, (d) mid gut 1, (e) mid gut 2, (f) hind gut. The mid gut is similar in morphology to anterior gut (B) except with progressively larger numbers of mucus cells in epithelium. The hindgut (C) is characterized by a marked increase in epithelial mucus cells. The height of tips of villi to the muscularis mucosa (double headed arrows, mucosal height) is measured in the anterior gut (B), anterior and posterior half of midgut, and the hindgut (C). Mucosal heights were taken in each of 6 fish from groups fed a control or organic acids, monoglycerides esters of organic acids and phytobiotics (OMGP) diets at the end of 6 weeks and presented in Fig. 2.

removing reads containing adapters, more than 10 unknown nucleotides, or more than 50 low-quality (Q ≤ 20) bases. Paired-end clean reads were aligned with the *L. calcarifer* reference genome using TopHat v2.0.12 (Kim et al., 2013). Genetic quantification of the gene expression level was determined with HTSeq v0.6.1 by counting the read numbers mapped to each gene (Anders et al., 2015). The expected number of fragments per kilobase of transcript sequence per million base pairs sequenced (FPKM) of each gene was calculated based on the length of the gene and read count mapped to this gene. To characterize differentially expressed genes (DEGs), the raw read number data sets were analyzed using the DESeq R package (1.18.0) (Wang et al., 2009). Genes with an adjusted P value < 0.05 and $|\log2FoldChange| \geq 1$ were assigned as the threshold for indicating significant differential expression. Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented by the GOseq R package, in which gene length bias was corrected (Young et al., 2010). GO terms with corrected P values < 0.05 were considered significantly enriched by differentially expressed genes. KOBAS software (Mao et al., 2005) was utilized to test the statistical enrichment of those differentially expressed genes in the Kyoto Encyclopedia of Genes and Genomes (KEGG, http://www.genome.jp/kegg/) pathways (Kanehisa et al., 2016).

2.7. Statistical analyses of growth performance and blood values

Statistical analyses for the effects of diet were conducted using the SPSS software package (IBM SPSS Statistics 23), where data were analyzed using ANOVA or t-tests. Data was assessed for normality using Kolmogorov-Smirnov test and homogeneity using Levene's test ($p > 0.05$). Tukey's HSD test was used after conducting ANOVA.

3. Results

3.1. Growth performance in fish fed CTRL and OMGP diets

Fish fed OMGP dietary blend for 6 weeks had significantly higher ABW (+ 8 %) compared to the CTRL group (two-tailed *t*-test, $P < 0.01$), in part associated with a higher feed consumption (two-tailed *t*-test, $P < 0.05$) (Table 2). FCR was relatively low (~1.20 – 1.22) and survival was relatively high (~96–99 %) for both groups, with no statistical differences between them. *L. calcarifer* final biomass, biomass gain, and specific growth rates between diets demonstrated a higher trend in the OMGP when compared with the CTRL diet (6 %, 10 % and 8 % higher values, respectively), however only approaching statistical significant difference for two-tailed *t*-test ($P = 0.06$–0.07).

3.2. Blood values

No significant difference in blood values was observed between fish fed OMGP and CTRL diets at the end of the trial. Comparative blood values post-trial was however significantly lower for fasting blood glucose levels ($P < 0.001$) and total plasma protein ($P < 0.05$), but higher for hemoglobin ($P < 0.01$) compared to pre-trial values (Table 3).

3.3. Height of intestinal mucosa

Intestinal mucosal heights across the four gut section AG, MG1 and HG were greater in fish fed diet OMGP than CTRL, although this was statistically significant ($P < 0.05$) only for MG1 and HG (Fig. 2).

3.4. Transcriptomics in kidney tissues at 24 h and 72 h post IP Poly(I:C) and PBS

The RNA-seq data showed raw read counts ranging from 80.6 to 112.4 million reads, with an average count of 93.6 million per sample. In addition, the average GC percentage was 47.3 %, and greater than 93.8 % of clean reads in all samples exceeded Q30, confirming the high quality of the sequencing (Supplementary Table S1). The pairwise comparisons of Poly(I:C) vs PBS at 24 hours post-injection (24-HPI) in each diet group revealed 391 DEGs (347 upregulated and 44 downregulated) in fish fed the CTRL diet (CTRL-Poly(I:C) vs CTRL-PBS) and 629 DEGs (401 upregulated and 228 downregulated) in fish fed the OMGP diet (OMGP-Poly(I:C) vs OMGP-PBS) (Fig. 3a). Additionally, fifty DEGs were detected in the CTRL-Poly(I:C) vs OMGP-Poly(I:C) treatment (Fig. 3a). Venn analysis showed 171 DEGs and 409 DEGs distinct from the CTRL group and OMGP group, respectively, with an overlap of 220 DEGs (Fig. 3b). Details of all genes which expression was significantly altered between group are presented in Supplementary Tables S2-S4.

At 72 hours post-injection (72-HPI), a total of 66 DEGs (29 upregulated and 37 downregulated) were detected in the kidney between OMGP-Poly(I:C) and CTRL-Poly(I:C) (Supplementary Table S5). The number of DEGs detected between CTRL-Poly(I:C) vs CTRL-PBS (only 1 downregulated gene: *pcolce2*) and OMGP-Poly(I:C) vs OMGP-PBS (15 upregulated and 18 downregulated) (Supplementary Table S6) were significantly reduced when compared to 24 HPI. Venn analysis showed no overlapping DEGs between CTRL-Poly(I:C) vs CTRL-PBS and OMGP-Poly(I:C) vs OMGP_PBS, while 6 overlapping DEGs were identified between OMGP-Poly(I:C) vs CTRL-Poly(I:C) and OMGP-Poly(I:C) vs OMGP-PBS (Fig. 3c).

3.5. Immune-related genes in response to Poly(I:C) challenge in juvenile *L. calcarifer* fed with dietary OMGP

KEGG pathway functional analysis was performed for 171 differentially regulated specific DEGs in CTRL-Poly(I:C) vs CTRL-PBS and 409 specific DEGs in OMGP-Poly(I:C) vs OMGP-PBS at 24-HPI with Poly(I:C) (Fig. 3b). Subsequent investigations revealed an increased number of DEGs associated with immune and infectious disease related pathways, such as Toll-like receptor and NOD-like receptor receptor signaling pathways, Complement and coagulation cascades pathway and Chemokine signaling pathway, in fish fed OMGP diets compared to those on CTRL diets in response to Poly (I:C) (Table 4). Significantly overexpressed key immune response DEGs (OMGP-Poly(I:C) vs OMGP-PBS) included B-cell linker (*blnk*), NOD-like receptors (NLRs) (*nlrc12l, nlrc3*), leukocyte differentiation antigens (*cd22, cd209, cd276*), C-X-C chemokine receptor type 4 (*cxcr4*), toll-like receptor 13 (*tlr13*), integrin alpha 4 (*itga4*), macrophage colony-stimulating factor 1 receptor 2 (*csf1r2*), C-type lectin receptor (*mrc1l*), and histone H2A (*h2a*). In contrast, some DEGs were significantly downregulated in the above

Table 2
Growth performance in *L. calcarifer* fed organic acids, monoglycerides esters of organic acids and phytobiotics (OMGP) vs control (CTRL) diet for 6 weeks.

Diet	ABW Initial (g)	ABW Final (g)	Final Biomass (g)	Biomass Gain (g)	Feed intake (g)	Feed Conversion Ratio (FCR)	Specific Growth Rate (SGR)	Survival (%)
CTRL	50.1 ± 0.0	136.5 ± 4.6[a]	3855 ± 149	2351 ± 150	2820 ± 107[a]	1.20 ± 0.05	2.38 ± 0.08	99.2 ± 1.44
OMGP	50.1 ± 0.1	147.5 ± 6.9[b]	4087 ± 107	2584 ± 103	3160 + 192[b]	1.22 ± 0.05	2.57 ± 0.11	95.8 ± 3.63

Different letters denote a statistically significant difference for two-tailed *t*-test ($P < 0.05$).

Table 3
Pre-trial blood values of *L. calcarifer* taken at start of experimental trials and end of 6 weeks fed on diets organic acids, monoglycerides esters of organic acids and phytobiotics (OMGP) and control (CTRL) (n = 6 fish each), against established *mean values and reference intervals (RI) (Chew and Gibson-Kueh, 2023).

Diet	Glucose (mmol/L)	Hematocrit (%)	Hemoglobin (g/L)	Total Plasma Protein (g/L)	Total White Blood Cell Counts (x10^9/L)
Pre-trial	7.50 ± 3.18[a]	40.67 ± 7.20	75.3 ± 4.8[a]	90.0 ± 10.0[a]	9.86 ± 2.18
CTRL	3.27 ± 0.55[b]	42.67 ± 4.27	89.8 ± 4.6[b]	72.3 ± 5.0[b]	15.2 ± 5.1
OMGP	2.55 ± 0.50[b]	42.67 ± 5.47	92.2 ± 8.6[b]	76.3 ± 3.7[b]	14.3 ± 6.5
Mean values*	6.3–7.0	28.5–30.0	71.0–73.5	65.4–67.0	25.7–32.7
Ref Interval*	2.4–11.3	18.9–39.2	56.0–85.0	56.0–77.0	5.3–69.9

Different letters denote a statistically significant difference for ANOVA (P < 0.05).

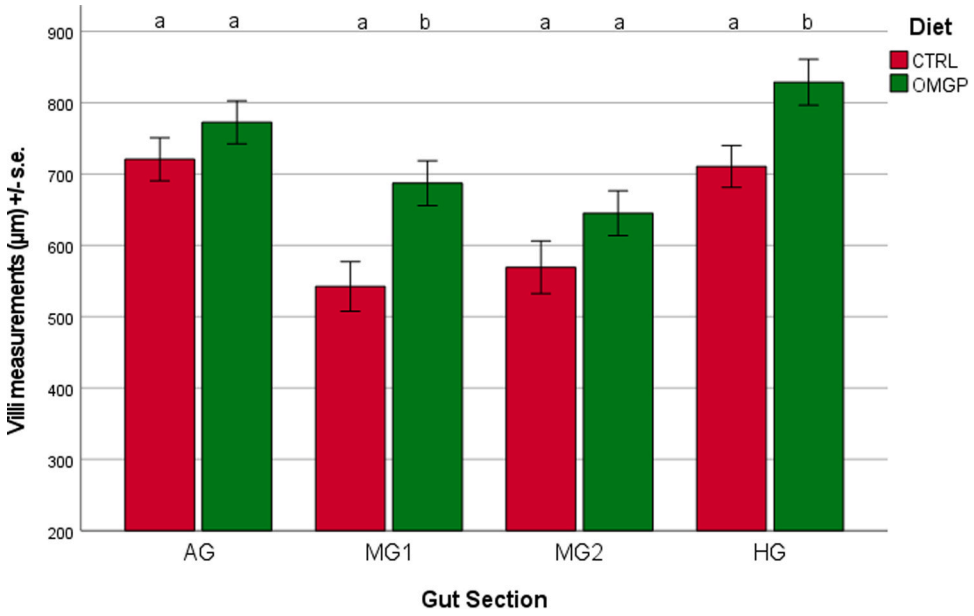

Fig. 2. Intestinal mucosal heights (µm) + /- s.e. in anterior gut (AG), mid-gut anterior half (MG1), mid-gut posterior half (MG2), and hind gut (HG). Different letters denote a statistically significant difference (P < 0.05) within gut sections between organic acids, monoglycerides esters of organic acids and phytobiotics (OMGP) and control (CTRL).

comparison: interleukin-8 (*il-8),* chemokine proteins (*ccl19a, cxcl9*), interferon regulatory factors (*irf7, irf3*) and several interferon-induced proteins (*ifi44, ifit1, ifih1, gimap7*) (Table 4). Fewer immune-related DEGs specific for CTRL-Poly(I:C) vs CTRL-PBS were detected, with increased mRNA abundance, including major histocompatibility complex (MHC) factors (*hla-f10a, h2-eb1)*, heat shock proteins (*hsp90aa1.2, hsf2*), complement factors (c4b, *c7a, c1r*), toll-like receptor 2 type-2 (*tlr2–2*), collagen alpha-1(I) chain-like (*col1a1*), interferon-induced proteins (*ifind-27 l*, *ifind-44 l*), and NACHT, LRR and PYD domains-containing protein 3 (*nlrp3*) (Table 4). A heatmap was generated to illustrate these immune-related DEGs detected in fish at both 24-HPI and 72-HPI (Fig. 3d). The transcriptome profile of the kidney in fish fed OMGP was markedly different from that in fish fed the CTRL diet in response to intraperitoneal injection of Poly(I:C) at 24-HPI. However, the dissimilarity in gene expression patterns between the two groups became less noticeable after 72-HPI (Fig. 3d).

4. Discussion

In this study, *L. calcarifer* fed the OMGP-supplemented diet consumed more feed and were 8 % heavier compared to fish on the basal control diet (CTRL) after a 6-week feeding trial, but with comparable FCR in both OMGP and CTRL groups. The OMGP diet was positively associated with increased intestinal mucosal heights throughout the *L. calcarifer* intestine, particularly in the anterior mid-gut and the hindgut sections. This parameter has been correlated with improved growth performance in various species, including fish (Libanori et al., 2023; Lin et al., 2023; Ramos et al., 2017), piglets, (Wang et al., 2020) and chickens (Amer et al., 2021).

Organic acids are known to enhance nutrient availability by activating pepsin through lowering gastric pH, increasing mineral solubility and absorption, and inhibiting the overgrowth of harmful gut microbes (Ng and Koh, 2017). Short-chain and medium-chain fatty acid monoglycerides serve as an energy source for enterocytes, thereby enhancing their functionality and potentially improving nutrient absorption (Rimoldi et al., 2018), such as amino acids, lipids and carbohydrates (Fabay et al., 2022; Watanabe and Tsujino, 2022; Zhang et al., 2020b). In gilthead sea bream (*Sparus aurata*), dietary monoglycerides positively modulated fish intestinal microbiota by increasing the abundance of beneficial lactic acid bacteria often associated with a healthy intestinal epithelium (Rimoldi et al., 2018). Other studies showed that a diet supplemented with 0.3 % medium-chain fatty acids enhanced growth rates and overall feed intake (Simó-Mirabet et al., 2017). A diet with 0.15 % glycerol monolaurate has been shown to improve growth performance, liver enzymes and lipid metabolism in juvenile cage-farmed pompano (*Trachinotus ovatus)* (Lin et al., 2022). Even at a low level of 0.5 %, incorporation of garlic peel (*Allium sativum L.*) as phytobiotic in feed was shown to enhance immune resistance of African catfish (*Clarias gariepinus*) to opportunistic bacterial pathogens such as *Aeromonas hydrophila* (Thanikachalam et al., 2010). Different fish species possess distinct digestive systems and gut microbiota compositions, and tailoring dietary interventions to their unique physiological and

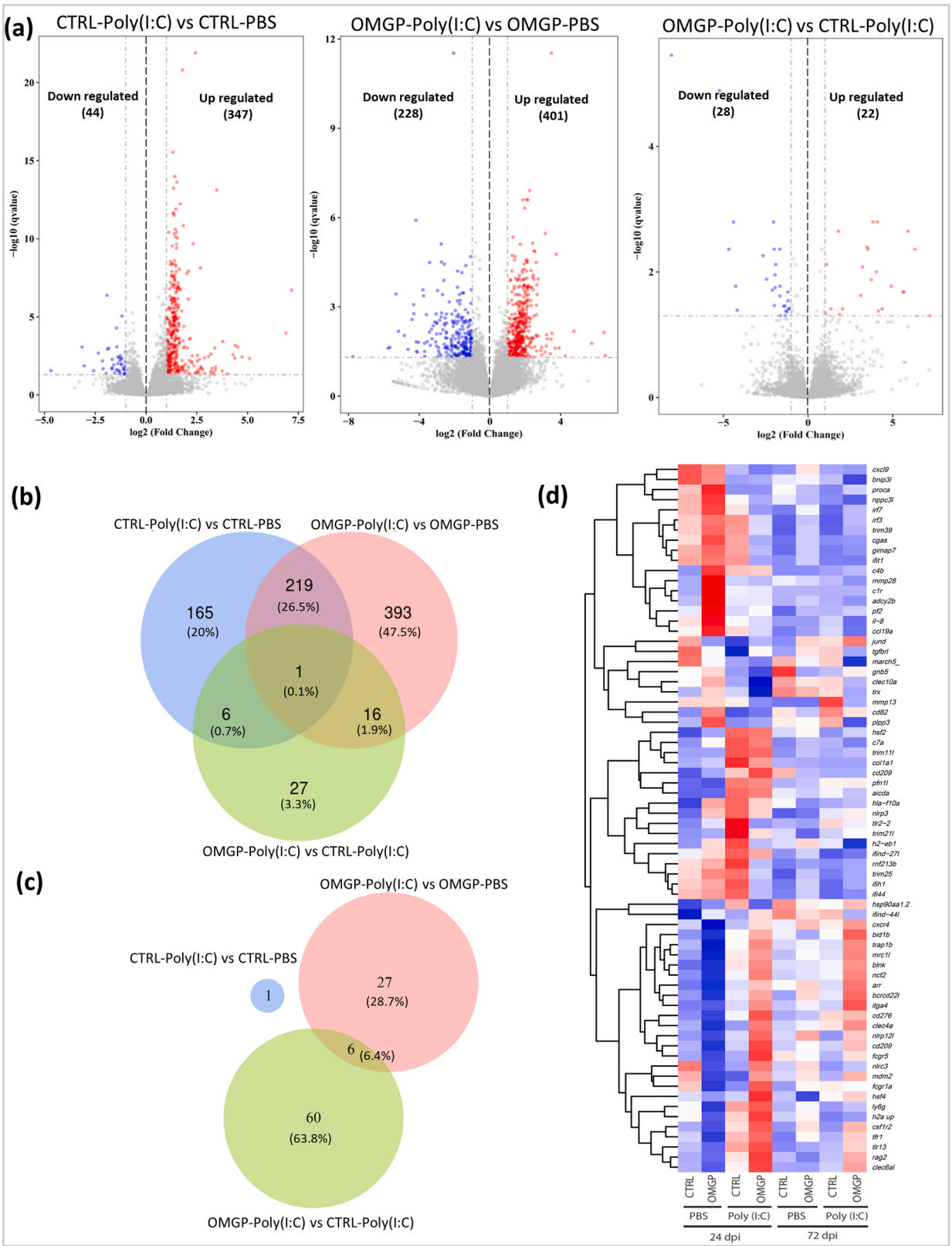

Fig. 3. (a) Volcano graph of all expressed genes in each pairwise comparison at 24 hours post-infection (24-HPI). The x-axis and y-axis present log-transformed fold change and log-transformed p-value, respectively. Each dot is a differentially expressed gene (DEG). (b) and (c) Venn chart of overlapping DEGs between (among) CTRL_PBS versus CTRL-Poly(I:C), OMGP_PBS versus OMGP-Poly(I:C) and CTRL-Poly(I:C) versus OMGP-Poly(I:C) pairwise at 24-HPI and 72-HPI. (d) Heatmap showing the expression profile of immune-related genes in fish fed the CTRL and OMGP diets at 24-HPI and 72-HPI with PBS and Poly(I:C). CTRL: control; OMGP: Organic acids, monoglycerides esters of organic acids and phytobiotics; PBS: Phosphate-buffered saline; Poly(I:C): Polyinosinic-polycytidylic acid; 72-HPI: 72 hours post-injection; 24-HPI: 24 hours post-injection.

Table 4
Summary of immune-relevant genes in L. calcarifer kidneys when fed control (CTRL) and organic acids, monoglycerides esters of organic acids and phytobiotics (OMGP) diets in response to 24 h post Poly(I:C) challenge.

Gene Family	Gene Name	Gene description	Log2 Fold change @24 h		Functional enrichment (Pathway analysis)	Gene ID
			CTRL-Poly I:C vs CTRL-PBS	OMGP-Poly I:C vs OMGP-PBS		
B7 family	*vtcn1l*	V-set domain-containing T-cell activation inhibitor 1-like	−2.6	3.2	T-cell receptor signaling pathway	LOC108885036
Pore-forming proteins	*pfn1l*	perforin−1-like	1.5	1.7	Natural killer cell-mediated cytotoxicity pathway; T cell receptor signaling pathway	LOC108880579
Chemokine	*cxcl9*	C-X-C motif chemokine 9	−2.8	−3.0	Chemokine signaling pathway	LOC108878497
Heat shock proteins	*hsf2*	heat shock transcription factor 2 binding protein	2.7	*1.2*	Protein processing in endoplasmic reticulum	hsf2bp
	hsp90aa1.2	heat shock protein 90, alpha (cytosolic), class A member 1, tandem duplicate 2	1.1	−0.5		LOC108896796
TRIM proteins	*trim11l*	E3 ubiquitin-protein ligase TRIM11-like	1.4	*1.2*	Ubiquitin mediated proteolysis pathway	LOC108877214
	trim21l	E3 ubiquitin-protein ligase TRIM21-like	1.2	0.4		LOC108880987
CD molecules	*cd209*	CD209 antigen-like protein E	3.3	*2.7*	Tuberculosis; Hepatitis C	LOC108889315
Collagen	*col1a1*	collagen alpha−1(I) chain-like	3.5	*1.2*	ECM-receptor interaction; PI3K-Akt signaling pathway	LOC108881445
Complement	*c1r*	complement C1r subcomponent	1.1	*−6.4*	Complement and coagulation cascades	LOC108880662
	c4b	complement 4B	1.7	*−3.1*		LOC108891077
	c7a	complement component 7a	2.1	0.8		LOC108882604
Serine protease	*proca*	protein C (inactivator of coagulation factors Va and VIIIa), a	−1.2	*−3.7*		proc
Major histocompatibility complex (MHC)	*hla-f10a*	class I histocompatibility antigen, F10 alpha chain	2.6	−0.4	Antigen processing and presentation; T cell receptor signaling pathway	LOC108888427
	h2-eb1	H−2 class II histocompatibility antigen, E-S beta chain-like	2.1	*−1.0*	Antigen processing and presentation; Th1 and Th2 cell differentiation	LOC108882204
Toll-like receptor	*tlr2–2*	toll-like receptor 2 type−2	1.8	−0.7	Inflammatory bowel disease; Toll-like receptor signaling pathway	LOC108885865
Interferon	*ifind-27l*	interferon alpha-inducible protein 27-like protein 2 A	2.4	0.0	Interferon pathway	LOC108898049
	ifind-44l	interferon-induced protein 44-like	3.4	0.3		LOC108879675
NLR (NOD-like receptor)	*nlrp3*	NACHT, LRR and PYD domains-containing protein 3	4.5	−0.8	NOD-like receptor signaling pathway	LOC108881849
AP−1 (activator protein 1) family	*jund*	transcription factor JunD	−1.0	−0.4	IL−17 signaling pathway	LOC108902773
Serine/threonine kinase receptors	*tgfbrl*	transforming growth factor beta receptor-like	−1.3	−0.3	TGF-beta signaling pathway; IL−17 signaling pathway	LOC108881978
B cell	*blnk*	B cell linker	0.7	1.5	B cell receptor signaling pathway	blnk
	bcrcd22l	B-cell receptor CD22-like	0.2	1.2		LOC108888182
Chemokine	*cxcr4*	C-X-C chemokine receptor type 4	−0.2	1.8	Chemokine signaling pathway	LOC108886893
	ccl19a	chemokine (C-C motif) ligand 19a, tandem duplicate 1	0.1	−1.8	Chemokine signaling pathway; T cell receptor signaling pathway	LOC108887152
CD molecules	*cd209*	CD209 antigen-like protein E	0.0	1.4	Antigen processing and presentation pathway	LOC108875780
	cd276	CD276 antigen	0.6	2.2	T cell receptor signaling pathway; B cell	LOC108892727
	cd82	CD82 antigen-like	−0.2	−1.1	receptor signaling pathway	LOC108894293
Heat shock proteins	*hsf4*	heat shock transcription factor 4	−0.2	2.0	MAPK signaling pathway; PI3K-Akt signaling pathway	hsf4
NLR (NOD-like receptor)	*nlrc3*	NLR family CARD domain-containing protein 3	*−1.9*	2.9	NOD-like receptor signaling pathway	LOC108879372
	nlrp12l	NACHT, LRR and PYD domains-containing protein 12-like	0.2	2.2		LOC108892802
Toll-like receptor	*tlr13*	toll-like receptor 13	0.6	1.9	Toll-like receptor signaling pathway	LOC108892776
Integrin	*itga4*	integrin alpha 4	0.3	1.3	PI3K-Akt signaling pathway	itga4
Interleukin	*il−8*	interleukin−8	−0.4	−1.8	Cytokine-cytokine receptor interaction	LOC108887160
MMP proteins	*mmp13*	matrix metallopeptidase 13	−0.4	−4.1	Collagen degradation pathway	mmp13
	mmp28	matrix metallopeptidase 28	0.2	−2.4		mmp28
C-type lectin	*clec10a*	C-type lectin domain family 10 member A	−0.6	−3.0	Collectin and complement pathways	LOC108892791
	clec4a	C-type lectin domain family 4 member A	−0.2	1.6		LOC108899434
	clec6al	C-type lectin domain family 6 member A-like	0.4	2.0		LOC108886975
	nppc3l	C-type natriuretic peptide 3-like	−0.6	−2.3		LOC108900485
	mrc1l	macrophage mannose receptor 1-like	0.5	1.6	Phagosome; C-type lectin family	LOC108883267

(*continued on next page*)

Table 4 (*continued*)

Gene Family	Gene Name	Gene description	Log2 Fold change @24 h		Functional enrichment (Pathway analysis)	Gene ID
			CTRL-Poly I:C vs CTRL-PBS	OMGP-Poly I:C vs OMGP-PBS		
Interferon proteins	*irf7*	interferon regulatory factor 7	−0.3	−1.0	Toll-like receptor signaling pathway; RIG-I-like receptor signaling pathway	irf7
	irf3	interferon regulatory factor 3	0.1	−1.8		LOC108884442
	ifih1	interferon induced with helicase C domain 1	0.4	−1.1		ifih1
	gmap7	interferon-induced GTP-binding protein Mx	−0.1	−2.3	Viral life cycle - HIV−1; Toll-like receptor signaling pathway	LOC108899664
	ifi44	interferon-induced protein 44	0.3	−1.4		LOC108896237
	ifit1	interferon-induced protein with tetratricopeptide repeats 1	0.0	−2.7		LOC108883428
Immunoglobulin (Ig) superfamily proteins	*fcgr1a*	Fc receptor-like A	−0.7	1.1	B cell receptor signaling pathway; Natural killer cell mediated cytotoxicity	LOC108874225
	fcgr5	Fc receptor-like protein 5	−0.1	1.6		LOC108880556
	ly6g	lymphocyte antigen 6G-like	0.4	2.2	leukocyte transendothelial migration	LOC108897845
Nucleotidyltransferase proteins	*cgas*	cyclic GMP-AMP synthase	0.0	−1.5	Herpes simplex virus 1 infection; Human immunodeficiency virus 1 infection	LOC108898898
G protein beta subunit	*gnb5*	guanine nucleotide binding protein (G protein), beta 5a	−0.4	−1.0	Chemokine signaling pathway	gnb5
Receptor tyrosine kinase	*csf1r2*	macrophage colony-stimulating factor 1 receptor 2	0.4	1.4	Cytokine-cytokine receptor interaction	LOC108873645
Transferrin receptor-like proteins	*tfr1*	transferrin receptor protein 1	0.3	1.1	HIF−1 signaling pathway; Phagosome	LOC108883855
Histone proteins	*h2a*	histone H2A	0.1	3.2	Neutrophil extracellular trap formation	LOC108901364
NADPH oxidase family	*ncf2*	neutrophil cytosolic factor 2	0.4	1.6	Neutrophil extracellular trap formation; Leukocyte transendothelial migration	LOC108898265
TRIM proteins	*trim25*	tripartite motif containing 25	0.3	−1.1	NF-kappa B signaling pathway; RIG-I signaling pathway	LOC108884561
	trim39	E3 ubiquitin-protein ligase TRIM39	0.2	−1.8	viral carcinogenesis; hepatitis B	LOC108889396

Note: The Log2Fold Change (italicized) indicates no significant difference ($p > 0.05$).

microbial characteristics are important (Ebrahimi et al., 2017; Romano et al., 2016). Overall, the incorporation of OMGP into the diet of juvenile *L. calcarifer* demonstrated promising benefits for both growth performance and gut health.

The pre-trial mean fasting blood glucose and total plasma protein levels were elevated relative to published reference intervals (Chew and Gibson-Kueh, 2023), likely due to the stress associated with handling and sampling, which can disrupt fish biochemistry and physiology (Barnett and Pankhurst, 1998; Bolasina, 2011; Bonga, 1997). At the end of the 6-week trial both OMGP and CTRL groups exhibited significantly reduced fasting blood glucose levels and total plasma protein concentrations, alongside increased hemoglobin concentrations compared to their pre-trial values. Although no significant differences were observed between diets, the OMGP-fed fish demonstrated lower fasting blood glucose levels (3.27 ± 0.55 vs 2.55 ± 0.50). This reduction typically signifies improved metabolic health and glucose homeostasis as also reported in diverse fish- and shrimp species (Ringø et al., 2022) and poultry (Shehata et al., 2022). Previous research has highlighted that hypoglycaemia can stimulate increased feed intake in rainbow trout (Polakof et al., 2012), potentially explaining the higher feed consumption in *L. calcarifer* fed OMGP diet. Although research on the response to monoglyceride supplementation in fish remains limited, monoglycerides have been shown to enhance feed palatability and intake in swine (Barbosa et al., 2023; Jackman et al., 2020; White et al., 2024).

In *L. calcarifer* aquaculture, viral pathogens (e.g., SDDV, LCHV) pose significant threats to production, underscoring the importance of enhancing fish immunity. Functional feeds enriched with immune-boosting supplements, such as organic acids, have demonstrated immunomodulatory effects in various fish species (Ng and Koh, 2017). This study investigated the effects of OMGP supplementation on the antiviral defense mechanisms of juvenile *L. calcarifer* challenged with Poly(I:C), an immunostimulant known to mimic viral infections. Poly(I:C) is recognized as a pathogen-associated molecular patterns by the immune system and activates immune pathways by engaging pattern recognition receptors such as TLR3 and RIG-I-like receptors, inducing the production of type I interferons and antiviral cytokines, thereby closely resembling the host's response to viral pathogens (Bao et al., 2022; Shen et al., 2024). Due to its ability to safely and reproducibly mimic viral infections, Poly(I:C) is widely used in studies to investigate the immune response across various fish species to viral challenges, including miiuy croaker (*Miichthys miiuy*) (Chu et al., 2015), Japanese flounder (*Paralichthys olivaceus*) (Thanasaksiri et al., 2015), lumpfish (*Cyclopterus lumpus*) (Emam et al., 2024; Rao et al., 2023), zebrafish (*Danio rerio*) (Ruyra et al., 2015), gibel carp (*Carassius auratus gibelio*) (Zhang et al., 2020a) and *L. calcarifer* (Sathyan et al., 2024). While Poly(I:C) is widely used to investigate immune responses, studies specifically exploring how dietary interventions, such as OMGP, influence antiviral defenses in fish species remain limited, highlighting the novelty of this research. In this study, an intraperitoneal injection of Poly(I:C) was used to mimic viral pathogen invasion and explore gene expression profiles in kidney of *L. calcarifer* at 24-HPI and 72-HPI through transcriptomic data. The results revealed an increased number of immune-related DEGs in the kidneys of OMGP-fed fish compared to those fed a control diet following Poly(I:C) challenge, suggesting that OMGP as a dietary additive may enhance antiviral immunity. Several toll-like receptors (TLRs) and NOD-like receptors (NLRs), pivotal components in fish innate immunity (Sahoo, 2020) were significantly affected in *L. calcarifer*. The observed upregulation of *tlr13,* a receptor involved in the innate immune system, in OMGP-fed fish indicated that Poly(I:C) stimulated antiviral immunity conferred by the additive, consistent with findings in other species such as Miiuy croaker (*Miichthys miiuy*) (Wang et al., 2016) and Dabry's sturgeon (*Acipenser dabryanus*) (Tang et al., 2020). Conversely, the upregulation of *tlr2–2* in the control diet group suggested a heightened innate immune response to bacterial components, potentially leading to increased inflammation, consistent with findings in large yellow croaker (*Larimichthys crocea*) (Ao et al., 2016; Fan et al.,

2015). The NOD-like receptors (NLRs) further highlighted the immunoregulatory effects of OMGP. NLRs are known for their anti-inflammatory properties, which reduce pro-inflammatory cytokine production (Uchimura et al., 2018). The *nlrc3* and *nlrp12l*, both upregulated in *L. calcarifer* receiving the OMGP diet, act as negative regulators of inflammation by inhibiting NF-κB and type I interferon signaling pathways, thus promoting immune homeostasis. While *nlrp3*, a marker of inflammasome activation, was significantly upregulated in control diet-fed fish after the challenge, highlighting its role in inflammasome-mediated inflammation crucial for pathogen defense, whereas OMGP-fed fish exhibited no significant changes in *nlrp3* expression, implying a potential regulatory effect of OMGP on inflammation.

Interleukin-8 (*il8*), referred to as "CXCL8 or neutrophil-activating peptide (NAP-1)", is a pro-inflammatory chemokine that plays a crucial role in neutrophil activation and the regulation of inflammation (Bickel, 1993). In this study, *il8* was significantly downregulated in fish fed with OMGP diet in response to Poly(I:C) challenge, suggesting potential anti-inflammatory properties that may modulate immune responses to balance effective virus control with minimized tissue damage. In addition, the downregulation of *mmp13* and *mmp28* in OMGP-fed fish following Poly(I:C) challenge suggested a potential modulation of inflammatory responses and extracellular matrix (ECM) remodeling. These mmps play key roles in ECM degradation (Jiang et al., 2010) and immune cell migration during inflammation, with excessive activity often leading to tissue damage. Their reduced expression in OMGP-fed fish may indicate a potential reduction in inflammation-associated tissue remodeling, which is particularly beneficial in aquaculture settings, where prolonged inflammation can compromise fish health and growth. Several interferon-related genes, including *irf7, irf3, ifih1, gimap7, ifi44*, and *ifit1*, were downregulated in OMGP-fed fish following the Poly(I:C) challenge. Interferon regulatory factors (IRFs) such as *irf3* and *irf7* are crucial for activating type I interferon responses essential for antiviral defense (Zhang and Gui, 2012). The *ifih1* gene detects viral RNA and triggers interferon activation, while *gimap7, ifi44,* and *ifit1* are interferon-stimulated genes that contribute to antiviral defense mechanisms (Lazarte et al., 2019; Nitta et al., 2006; Qiao et al., 2022; Zhou et al., 2013). Their downregulation in OGMP-fed fish at 24-HPI may indicate modulation of interferon signaling pathways, potentially mitigating excessive inflammatory responses, and optimizing antiviral defense, in agreement with previous reports (Andresen et al., 2020; Hori et al., 2012). Furthermore, the NADPH oxidase family plays a vital role for fish immune response due to its role in producing reactive oxygen species (ROS), which are important for pathogen elimination (Vermot et al., 2021). The upregulation of NADPH oxidase family member *ncf2* in OMGP-fed fish challenged with Poly(I:C) indicated enhanced ROS production to combat viral infections, aligning with findings from other fish species' responses to Nervous Necrosis Virus (NNV) (Toubanaki et al., 2022).

The temporal dynamics of immune responses observed in this study provided valuable insights into the impact of OMGP supplementation. At 24-HPI, the transcriptomic differences were most pronounced, with a greater number of DEGs unique to the OMGP-Poly(I:C) group compared to the CTRL-Poly(I:C) group (Fig. 3B), suggesting enhanced immune activation relative to the control diet group. Additionally, comparison of 72-HPI Poly(I:C) samples to 72-HPI PBS samples revealed fewer DEGs responsive to Poly(I:C) across all diets when compared to 24-HPI samples. This observation suggests that either the transcriptomic effects Poly(I:C) are reduced after 3 days, or that the OMGP diet may affect the timing of the gene expression response to viral infection. Overall, these findings underscore the promise of synergistic blend of organic acids, monoglycerides and phytobiotics as a dietary supplementation to potentially enhance host immunity against viral diseases in aquaculture. By mitigating excessive inflammation and reducing tissue damage, OMGP supplementation could support fish health and productivity while decreasing reliance on antibiotics. To validate its efficacy and mechanisms of action, future studies should include viral challenge trials (e.g., SDDV, Infectious Spleen and Kidney Necrosis Virus (ISKNV), or LCHV) and assess its long-term impacts under commercial farming conditions. Such investigations are critical to realizing the full potential of OMGP for sustainable *L. calcarifer* aquaculture practices.

5. Conclusion

The present study demonstrated that dietary supplementation with OMGP exerted a positive impact on growth performance in juvenile *L. calcarifer* and may potentially enhance the immune response to viral diseases. This is evidenced by increased gut mucosal heights and a greater number of immune-related DEGs in fish fed the OMGP diet compared to those on the CTRL diet when challenged with Poly(I:C) as a viral response marker. These findings suggest that OMGP blend could serve as an important feed additive for sustainable aquaculture of *L. calcarifer* under commercial culture conditions. Nevertheless, further research with actual viral challenges, and additional histological assessments of goblet and epithelial cell integrity is needed to confirm whether OMGP blend may boost fish immunity to a level in which it confers higher survival.

Ethics declarations

This experiment was approved by the James Cook University Singapore Institutional Animal Care and Use Committee (IACUC approval 2021-A012).

Authors' contributions

This study was designed and conceived by Jose A. Domingos, David Bal, I-Tung Chen, Susan Gibson-Kueh and Xueyan Shen. Charlene Goh conducted the feeding trials, sampling, blood, and histological analysis. Transcriptome analysis was conducted by Xueyan Shen. Xueyan Shen, Charlene Goh, Susan Gibson-Kueh, and Jose A. Domingos wrote, reviewed, and edited the manuscript. David Bal, I-Tung Chen and Waldo Nuez-Ortín reviewed and edited the manuscript. All authors provided final approval for publication while agreeing to be responsible for the work's integrity and content.

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CRediT authorship contribution statement

Goh Charlene: Writing – review & editing, Writing – original draft, Project administration, Formal analysis, Data curation. **Bal David:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization. **Gibson-Kueh Susan:** Writing – review & editing, Writing – original draft, Supervision, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. **Shen Xueyan:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Domingos Jose A.:** Writing – review & editing, Writing – original draft, Methodology, Formal analysis, Conceptualization. **Nuez-Ortín Waldo:** Writing – review & editing. **Chen I-Tung:** Writing – review & editing, Methodology, Funding acquisition, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Susan Gibson-Kueh reports financial support was provided by Adisseo Asia-Pacific Pte Ltd. If there are other authors, they declare that they have no known competing financial interests or personal relationships

that could have appeared to influence the work reported in this paper.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.aqrep.2025.102692.

Data availability

I have shared the link to my data in the manuscript.

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