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Biological filters regulate water quality, modulate health status, immune indices and gut microbiota of freshwater crayfish, marron (*Cherax cainii*, Austin, 2002)

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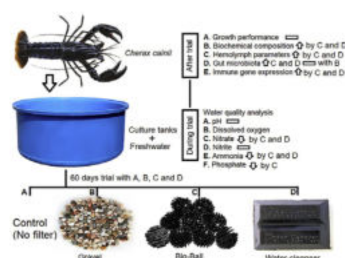
HIGHLIGHTS

- Biological filters, Bio-Ball (BB) and Water-cleanser (WC) improve the water quality.
- BB and WC improve the health and immune status of freshwater crayfish, marron.
- Biological filters significantly modulate the hindgut microbiota of marron.
- Up-regulation of genes associated with innate immune response was observed with BB and WC.
- BB and WC can be used for better water quality and health status of crayfish.

GRAPHICAL ABSTRACT



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ABSTRACT

Water quality has significant impacts on the health and immune responses of aquaculture species. This study aimed to analyse and compare the effects of two biological filters namely, gravel and, Bio-Ball with a recently developed filter called Water-cleanser on regulation of water quality parameters, health and immune response of marron reared in plastic tanks for 60 days. Results showed that addition of Bio-Ball significantly ($P < 0.05$) reduced the concentration of ammonia, nitrate and phosphate while Water-cleanser showed the ability to reduce ammonia and nitrate from water in aquaculture tanks. Although the biological filters had no significant effect on marron growth but inclusion of Bio-Ball and Water-cleanser positively influenced the biochemical composition of tail muscle and some haemolymph parameters of marron. The next generation sequence data demonstrated higher bacterial diversity in the hindgut of marron with Water-cleanser, followed by Bio-Ball and gravel, respectively. In addition, the predicted metabolic pathways revealed a significantly higher bacterial activity and gene function correlated to metabolism and biosynthesis of protein, energy and secondary metabolites in Bio-Ball and Water-cleanser. Bio-Ball and Water-cleanser were also associated with up-regulation of innate immune responsive genes of marron gut. Overall, Bio-Ball and Water-cleanser proved to have higher water

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remediation and immune response modulation capabilities, and therefore could be used as preferred filters for growth of beneficial bacteria in crayfish culture.

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1. Introduction

Globally, unplanned and unregulated expansion of aquaculture industry has negatively impacted on the aquatic environment (Roriz et al., 2017). At the same time, increase in aquaculture production by means of improving the water quality has become an utmost priority among the fish farmers. Regulation of temperature, pH, dissolved oxygen (DO), nitrogenous compounds including nitrate, nitrite and ammonia, phosphorus in form of phosphate, suspended solids (SS) are critical for the growth of aquatic organisms (Camargo and Alonso, 2006; Yildiz et al., 2017). Poor water quality in any stagnant or flow-through production systems can lead to the accumulation of organic waste, deterioration of the surrounding ecosystem, enhanced growth of pathogenic microbes, and increase in the cost of production (Gutierrez-wing and Malone, 2006). Recirculating aquaculture systems (RAS), on the other hand, can be a good solution for maintaining optimum water quality and enhanced environmental sustainability. However, high capital and operational cost of RAS are a few constraints for its economic viability (Ngoc et al., 2016). In addition, accumulation of nitrogenous wastes and organic load in any RAS can compromise the performance of the cultured species (Ngoc et al., 2016).

Some bacterial communities shown to have positive impact on the growth and immune performance of cultured species meditated through improving the water quality (Chun et al., 2018; Foysal et al., 2019a). However, there is a limited knowledge available on the performance of the key microbial species in water remediation. Different kinds of substrates and filters are used in aquaculture systems are known to establish a biofilm that has an ability to play a significant role in improving water quality (Cole et al., 2019; Khatoon et al., 2007; Lananan et al., 2014; Viau et al., 2012). Conventional biological filters using gravel and sand as substrates reported to have positive effects on water quality during the culture of various aquatic species (Huang et al., 2018; Rogers and Klemetson, 1985; Summerfelt, 2006). However, slow filtration, trapping of organic debris, manual hand cleaning and limited activity against nitrates and heavy metals are some of the major limitation of this type of filters (Odell, 2014). Conversely, some non-substrate based biological filters come with more surface area for the growth of probiotic and denitrifying bacteria that demonstrated to have positive effects on water quality (Cole et al., 2019; Foysal et al., 2019a). However, none of the studies have yet analysed the impacts of different biological filters on growth, gut microbiota, and immune indices of any crayfish under laboratory condition.

Marron (*Cherax cainii*) is the largest commercially farmed parasticid crayfish in Western Australia (Cole et al., 2019). In spite of the best intentions from the farmers, the total production of marron has remained stagnant (Cole et al., 2019; Foysal et al., 2019a). Excessive dissolved unionised ammonia, nitrite and nitrates have harmful physiological effects on crayfish including marron (Cole et al., 2019; Roessink et al., 2017; Svobodova et al., 2012). Although marron farming is devoid of any major diseases till today, the poor water quality can hasten accumulation and growth of pathogenic microbes including *Vibrio* species, a possible future pathogen (Ambas et al., 2013; Foysal et al., 2019a). Certain advances in molecular techniques have recently validated the positive claims made by a few biological filters on the water quality and the health of the farmed species (Andre et al., 2007; Castine et al., 2013; Cole et al., 2019; Foysal et al., 2019a; Permatasari et al., 2018). Hence, this study was conducted to evaluate the performance of three selected biological filters on the water quality, health and immune performance of the targeted host crayfish species, marron.

2. Materials and methods

2.1. Ethics statement

Ethics approval is not mandatory for the study performed with invertebrates at Curtin University, Australia. However, the laboratory trial, crayfish handling and sample collection were performed according to the guidelines of Animal Welfare Act, Western Australia and the Australian Code for the Care and Use of Animals for Scientific Purposes (2013).

2.2. Experimental set-up and sampling

A total of 84 marron with an average weight of 71.8 $\pm$ 0.7 g were procured from Blue Ridge Marron Farm, Manjimup, Western Australia (34.2019° S, 116.0170° E) and transported alive to Curtin Aquatic Research Laboratories (CARL), Turner Avenue, Bentley (32.0010° S, 115.9240° E). Marron were then randomly distributed into 16 different plastic tanks (80 cm diameter and 50 cm height), each of 200 L capacity, filled with a density of five marron per tank and acclimatized for two weeks prior to the start of the experiment. After acclimation, 16 tanks were randomly divided into four different treatment groups, namely, control, gravel, Bio-Ball and Water-cleanser. Gravel was collected from Bibra Lake Soils, WA, Australia (32.1023° S, 115.8240° E), while Bio-Ball (38 mm in diameter) and Water-cleanser (200g block size) were purchased from Serene Aquarium, NSW, Australia and Marine Easy Clean, WA, Australia, respectively. Gravel, Bio-Ball and Water-cleanser were added in 12 tanks according to manufacturer's instructions: 10 kg/150 L gravel, 1 gallon of Bio-Ball (approximate 60 pieces, 40 mm size) per 50 gallon of water, and one 200 g block Water-cleanser per tank (for <1000 L water). Control tanks had no filtration system. Constant aeration and optimum temperature for marron growth (22 $\pm$ 0.5 °C) were maintained using air stones (Aqua One, Perth, Australia) and submersible thermostat (Aqua One, Perth, Australia), respectively (Foysal et al., 2019a; Nugroho and Fotedar, 2013). To avoid cannibalism, each marron was reared inside a special cage made of plastic mesh (0.8–8.0 mm thickness). Throughout the trial including acclimation period, marron were fed fishmeal based basal diet (Glenn Forest, Perth, Australia) containing 29% protein, 45% carbohydrate, 8% lipid and 9% ash, every day at 6 p.m. and 1.5% of the total biomass per tank. The water quality in each tank was measured every day. The temperature and pH of the water in all tanks were monitored using portable waterproof °C/mV/pH meter (CyberScan pH 300, Eutch Instruments, Singapore). Dissolved oxygen (DO) concentration was measured using digital DO meter (YSI55, Perth Scientific, Australia). The concentration of nitrate, nitrite and ammonia of the water were monitored using Hach DR/890 Colorimeter (Hach, Loveland, CO, USA). Phosphate (PO_4^-) concentration was measured by ascorbic acid 4500-PE method, as previously described (Mai et al., 2010). The faecal waste of marron

in each cage and tank was removed carefully using a hand-held net once a week. To determine marron health indices and haemolymph parameters, two marron from each tank (n = 8, N = 32) were randomly sampled. For DNA extraction and microbiome analysis, two randomly selected marron from each tank (n = 8, N = 32) were used followed by careful aseptic excision of whole gut and separation of anterior, mid and hindgut inside a biological safety cabinet. The contents from two marron hindgut samples from each tank were then pooled together (N = 16), homogenized, and transferred to 1.5 mL Eppendorf tube for DNA extraction. Finally, two randomly selected marron from each tank (n = 8, N = 32) was used for the gene expression analysis with specific primers. The whole intestine samples were chopped into small pieces, suspended into RNA later solution (Sigma-Aldrich, Germany) and stored at −80 °C until further use. Same marron was used for health and haemolymph parameters as well as for gene expression analysis.

2.3. Analysis of growth, immune and biochemical parameters

A mid-term sampling was performed on day 30th and at the end of the trial on day 60th in order to calculate weight gain (WG), specific growth rate (SGR). However, haemolymph osmolality (HO), lysozymal activity, total haemocyte counts (THC) from haemolymph of marron were analysed at the end of trail as previously described (Ambas et al., 2017; Foysal et al., 2019b). For HO, haemolymph (0.1 mL) of marron was extracted carefully from the pericardial cavity using 0.5 mL syringe comprising of 0.1 mL of precooled anticoagulant (0.1% glutaraldehyde in 0.2 M sodium cacodylate, pH 7.0 ± 0.2). The osmolality of the anti-coagulant added mixture was then measured in a Cryoscopic Osmometer-Osmomet 030 (Gonotec, Berlin, Germany). The lysozyme activity in the haemolymph of marron was measured using turbidimetric assay method. First, anti-coagulant added haemolymph (50 μL) was added into 96-well microtiter-plate (Iwaki, Tokyo, Japan). After 15 min of incubation at room temperature (23 °C), 50 μL of *Micrococcus lysodeiktikus* (Sigma-Aldrich, St. Louis, MO, USA) suspended PBS (0.25 mg mL^{-1}) solution was added into different wells of the same plate. The absorbance of the wells was then measured at 1 min interval for 5 min at 450 nm wavelength in MS212 reader (Titertek Plus, Tecan, Grodig, Austria). The lysozyme activity unit (U mL^{-1}) was calculated based on the amount of enzyme required for a decrease in absorbance of 0.0001/min. For THC, 0.2 mL of haemolymph suspended in same volume of anti-coagulant was used to calculate total haemocyte under a Neubauer hemocytometer (Paul Marienfeld GmbH & Co. KG, Lauda-Königshofen, Germany) with 100× magnification. The amount of protein, energy and fat in the tail muscle was measured according to guidelines of the Association of Official Analytical Chemists, AOAC international (AOAC, 2006). The percentage of crude protein was measured using Kjeldahl method (N × 6.25) with aid of sulfuric acid (H_2SO_4) and copper catalyst tablets in Kjeltec Auto 1030 analyser (Foss Tecator, Höganäs, Sweden). The crude fat was analysed following Soxhlet ether extraction method using Soxtec System HT6 (Tecator, Höganäs, Sweden). The total gross energy (Mj kg^{-1}) in the tail muscle was measured by using bomb calorimeter (Heitersheim, Germany).

2.4. Microbiome study of hindgut

The hindgut was selected for bacterial diversity analysis due to its role in digestion, and immunity of crayfish (Foysal et al., 2019b). The bacterial DNA from the pooled sample was extracted using DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The quantification of extracted DNA was performed in NanoDrop spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) followed by dilution to 30 ng μL^{-1} final concertation. PCR amplification was performed with 50 μL final volume having 25 μL Hot Start 2× Master Mix (New England BioLabs Inc., Ipswich, MA, USA), 2 μL of template DNA, 1 μL of each V3−V4 sequencing primers, and 21 μL of DEPC treated water (Thermo Fisher Scientific, Waltham, MA, USA). A total of 30 cycles of amplification reactions were performed in a BioRad S100 Gradient Thermal Cycler (Bio-Rad Laboratories, Inc., Foster City, California, USA). The PCR products were separated using 1% agarose gel in electrophoresis (Bio-Rad Laboratories Inc., California, USA) and pictured under gel doc (FujiFilm LAS−4000 Image Analyser, Boston Inc., Foster City, California, USA). The 16 S rRNA PCR amplicon of each sample was barcoded via a secondary PCR according to the Illumina standard protocol (Part # 15044223 Rev. B). The samples were then sequenced up to 30,000 reads on an Illumina MiSeq platforms (Illumina Inc., San Diego, California, USA) at Harry Perkins Institute of Medical Research, Western Australia, using a v3 kit (600 cycles).

2.5. Gene expression analysis

Primers used for gene expression analysis in this study are listed in Table S1. Only those genes, associated with health and immunity of crayfish were selected (Dai et al., 2017; Foysal et al., 2019; Foysal et al., 2019b; Jiang et al., 2015; Liu et al., 2013). The gene used for expression analysis in this study were interleukins (IL-1β, IL-8, IL-10, IL-17F), tumour necrosis factor (TNF-α), cathepsin L (PcCTSL), prophenoloxidase (proPO) and cytosolic manganese superoxide dismutase (MnSOD). After post thawing of samples from −80 °C at 4 °C and air drying, the intestine tissues were grounded into fine powder using TissueLyser (Qiagen, Hilden, Germany). However, for analysis of proPO and MnSOD, 0.5 mL of haemolymph was diluted in 0.5 mL of anticoagulant and centrifuged immediately at 8000 rpm for 20 min to collect hemocytes (Liu et al., 2013). The collected haemocytes and 5 mg of post-thawed powder tissue samples were used for RNA extraction using RNeasy Mini Kit (Qiagen, Hilden, Germany) after following manufacturer's instructions. At a time of extraction, DNase-I (Qiagen, Hilden, Germany) was added to remove DNA impurities. Quality of extracted RNA was checked using 1% agarose gel whereas, quantity was analysed using NanoDrop spectrophotometer, (Thermo Fisher Scientific, USA). The cDNA library was prepared from the extracted RNA using Omnicript RT kit (Qiagen, Hilden, Germany). Quantitative real-time PCR was performed using PowerUp™ Cyber Green Master Mix (Thermo Scientific, USA) and gene primers with 7500 Real-Time PCR System (Applied Biosystems, USA). The relative expression level of each gene was calculated using the $2^{-\Delta\Delta CT}$ method, following normalisation against β-actin reference gene (Livak and Schmittgen, 2001). Duncan's multiple range test was performed to compare the differences in the expression level of immune gene of four different groups after trial.

2.6. Bioinformatics and statistics

The initial quality of 16 S rRNA sequences after extraction was checked using FastQC pipelines (Andrews, 2010). The sickle program was used for quality trimming of reads and following trimming, reads having less than 200 bp length were excluded (Joshi and Fass, 2011). For the merging of overlapping paired-end reads, MeFiT pipeline was used with default parameters (Parikh et al., 2016). Micca (version 1.7.0) pipeline was applied for filtering of chimeric sequences, *de novo* greedy clustering of 16 S sequences into operational taxonomic units (OTUs) at 97% similarity threshold and subtraction of singleton OTUs (Albanese et al., 2015).

Taxonomic assignment of the representative OTUs was performed using mica classify against SILVA 1.32 database clustered at 97% identity (Quast et al., 2013). Multiple sequence alignment of the representative OTUs was done using PASTA algorithm (Mirarab et al., 2015). The rarefaction depth value was set at 17,796 and consequent calculation of alpha and beta diversities were accomplished using QIIME (version 1.9.1) (Kuczynski et al., 2012). Concisely, alpha diversity was calculated based on the following metrics: species richness, Shannon, Chao1 indices and goods coverage. Non-parametric statistical analysis of the distance metric was performed using ANOSIM with 1000 permutations. One way ANOVA with Tukey's HSD was used to compare the water quality, health indices and alpha diversity measurements among the groups using agricolae, ggpubr, plyr, dplyr, vegan and ggplot2 packages in Rstudio. Beta dispersion in terms of non-metric multidimensional scaling (NMDS) and bacterial diversity at phylum and genus level were calculated and visualized using microbiomeSeq, phyloseq and ggplot2 packages in Rstudio. Venn diagram regarding bacterial diversity among groups at genus level was generated using FunRich tool (v3.1.3). To find out the indicator bacterial genus in different groups, Linear Discriminant Analysis Effect Size, LEfSe was applied with stringent LDA cut-off value of $\geq$3.5 (Segata et al., 2011). Differentially abundant metabolic pathways in four different groups were predicted from the 16 S rRNA sequence data using Piphillin algorithm (http://secondgenome.com/Piphillin) with supports of KEGG database, BioCyc (v21), and LEfSe (LDA 3.5) (Iwai et al., 2016; Segata et al., 2011). The numerical data for growth, health and immune indexes of marron were analysed using SPSS IBM (v23, 2017). At all stages alpha level of 0.05 was considered as statistically significant.

2.7. Calculations

$$\text{Weight gain (WG, g/fish)} = \left[\frac{\text{mean final body weight} - \text{mean initial body weight}}{\text{mean initial body weight}}\right] \text{Specific growth rate (SGR, \%/day)}$$

$$= \left[\frac{\ln\ (\text{final body weight}) - \ln\ (\text{pooled initial body weight})}{\text{days}}\right]$$

$$\times 100 \text{Total haemocyte counts (THC, cells/mL)}$$

$$= \left[\frac{\text{cells counted} \times \text{dilution factor} \times 1000}{\text{Volume of grid (0.1 mm3)}}\right]$$

3. Results

3.1. Water quality and marron health

Among some major water quality parameters such as pH, dissolved oxygen, nitrate, nitrite, ammonia and phosphate, significant ($P < 0.05$) reduction of nitrate and ammonia concentration were observed for both Bio-Ball and Water-cleanser treated tanks, and phosphate reduction noticed in Bio-Ball added tanks (Fig. 1). No significant ($P > 0.05$) impacts of different filters was recorded for weight gain (WG), specific growth rate (SGR), tail muscle protein and fat contents. However, one-way ANOVA revealed significant positive influence ($P < 0.05$) of Bio-Ball and Water-cleanser on the lysozymal activity and THC, and gross energy of tail muscle, compared to control and gravel (Fig. 2). Haemolymph osmolality (HO) was significantly ($P < 0.05$) influenced by Water-cleanser in relation to control, gravel and Bio-Ball. Gravel as a filter had no effects on marron health and immune response compared to any of the filters used in this study.

3.2. Microbial diversity in marron gut

The study generated 577,808 raw reads from 16 samples from four different treatment groups. After removing single-tone, the reads were classified into 348 OTUs, 12 phyla and 149 genera. The rarefaction curve indicated that the 16 S rRNA sequence captured enough depth and diversity for 16 samples of four different treatment groups (Fig. 3A). The curve revealed significant influences of Bio-Ball and Water-cleanser on bacterial communities in the marron hindgut. ANOVA measurements of alpha diversity showed significantly higher species richness, Shannon and Chao1 indices in Bio-Ball and Water-cleanser groups compared to control and gravel (Fig. 3B–D). However, the number of genera and unshared genera were higher with Water-cleanser compared to other groups (Fig. S1). The goods coverage index of 0.95–0.99 suggested that the coverage degree of sequences were high and anticipant (Table S2). The non-metric multidirectional scaling (NMDS) plot based on PERMANOVA analysis of rarefied data exhibited a distinct clustering of bacterial OTUs, and R value of 0.8221 and P value of 0.002 revealed significant effects of biological filters on marron gut microbiota (Fig. 4A). Proteobacteria was found to be more abundant in control (77.7%) and gravel (92.1%) while Firmicutes profusion was observed for Bio-Ball (55.7%) and Water-cleanser (46.7%) at phylum level (Fig. 4B). Whereas at genus level, *Citrobacter* (86.9%), *Vibrio* (61%), *Clostridium* (49.2%) and *Streptococcus* (49.6%) were recorded to be the most abundant bacteria in the hindgut of marron in control, gravel, Bio-Ball and Water-cleanser groups, respectively (Fig. 4C).

3.3. Indicator bacterial groups and metabolic pathways

Linear discriminant analysis revealed 20 significantly abundant signature bacterial genera in four different treatments at strict LDA cut-off value of $\geq$3.0. Out of 20, 14 enriched in Water-cleanser group followed by three, two and one from control, Bio-Ball and gravel groups, respectively. In control, *Citrobacter* was found to be the most enriched signature bacteria (LDA $\geq$ 5.0) while *Vibrio, Clostridium* and *Streptococcus* were the indicator genera for gravel, Bio-Ball and Water-cleanser, respectively (Fig. 5A). Out of significantly abundant 52 taxa in all four treatment groups, 39 were found to be enriched with Water-cleanser (Fig. 5B). The predicted KEGG metabolic pathways indicated that Water-cleanser stimulated the biosynthetic pathways for amino acids, amino-acyl tRNA, antibiotics, secondary metabolites etc. Relative to Water-cleanser, the substrate in gravel filter up-regulated the expression of two-component system, biofilm formation (*Vibrio cholerae*) and bacterial chemotaxis. In control group, the significantly overexpressed

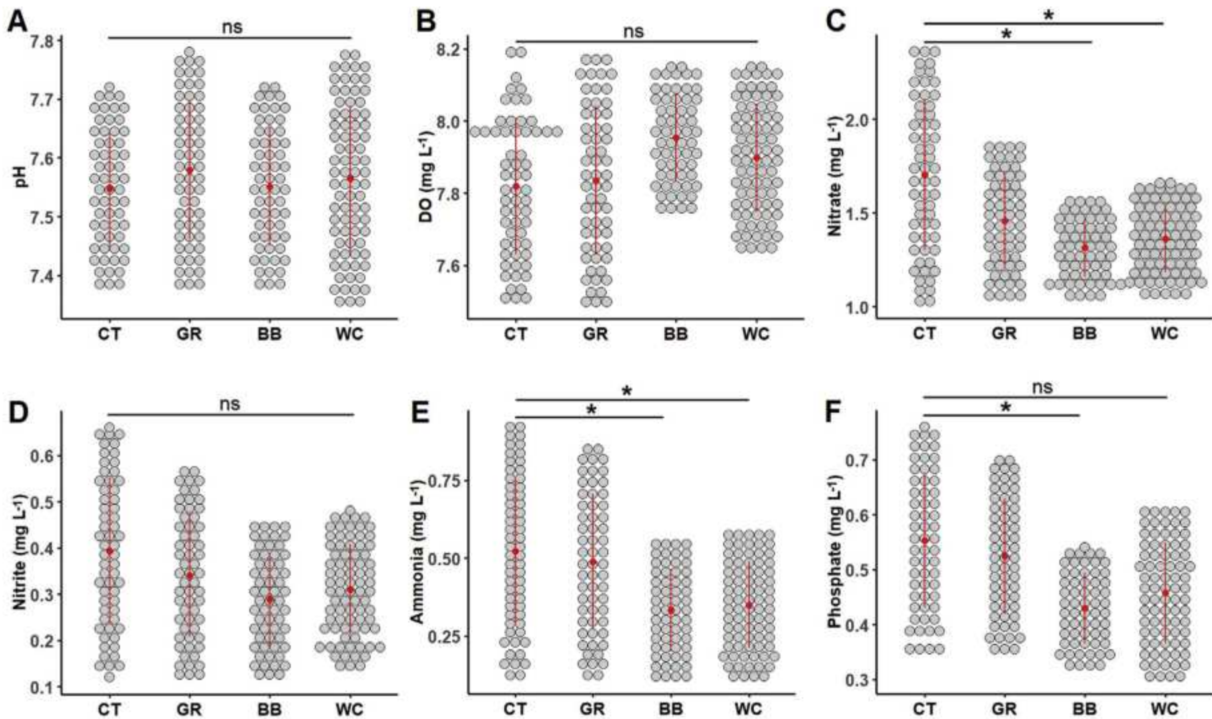

Fig. 1. Water quality parameters of tanks for four different treatment groups. (**A**) pH; (**B**) Dissolved oxygen; (**C**) Nitrate; (**D**) Nitrite; (**E**) Ammonia; (**F**) Phosphate. Data gathered from four tanks of each group are expressed as mean ± SE for 60 days. Abbreviation: BB = Bio-Ball; CT = Control; GR = Gravel; WC = Water-cleanser. *Significant at α-level of 0.05.

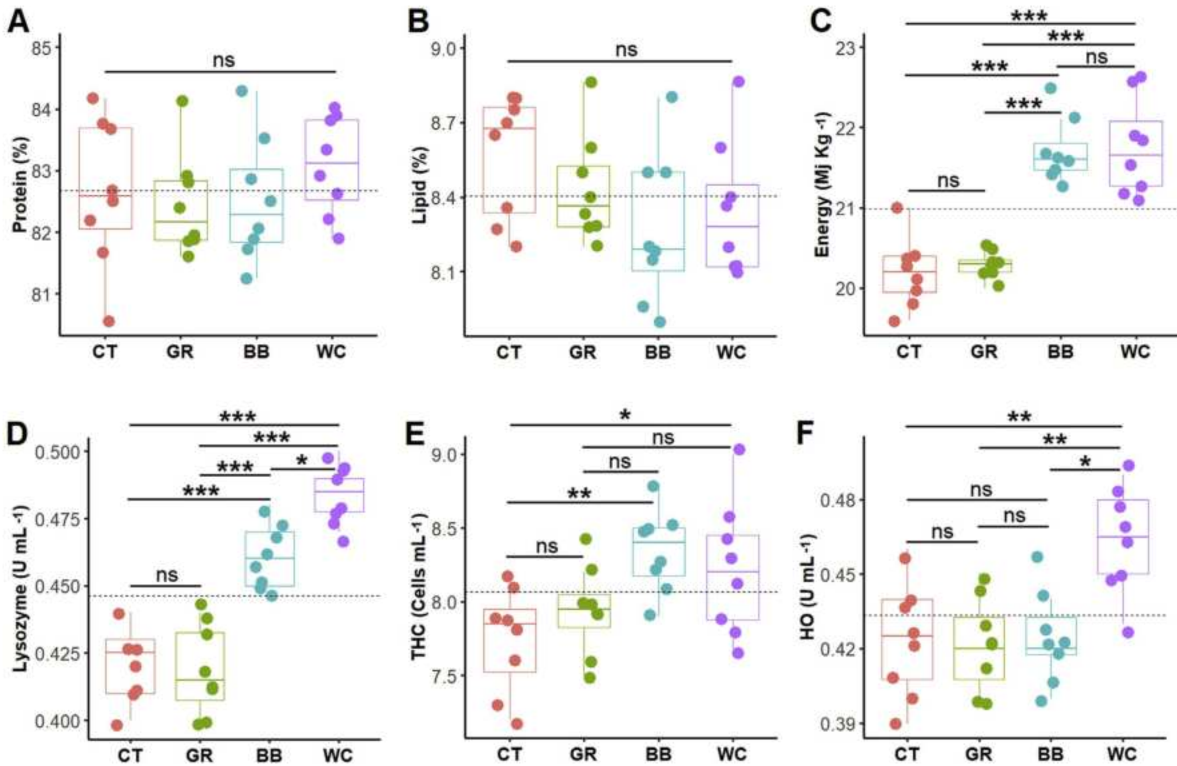

Fig. 2. Effects of biological filters on growth performance, health and immune indices of marron. (**A**) Protein; (**B**) Lipid; (**C**) Gross energy in tail muscle; (**D**) Lysozyme activity in haemolymph; (**E**) Total haemocyte count in haemolymph; (**F**) Haemolymph osmolality in haemolymph. Data expressed as mean ± SE. *Significant at α-level of 0.05; **Significant at α-level of 0.005; ***Significant at α-level of 0.001. Abbreviation: BB = Bio-Ball; CT = Control; GR = Gravel; WC = Water-cleanser.

predicted pathways were flagellar assembly, microbial metabolism in diverse environment and ABC transporter. Compared to these three, no pathway was found to be up-regulate with Bio-Ball in this study (Fig. 6A).

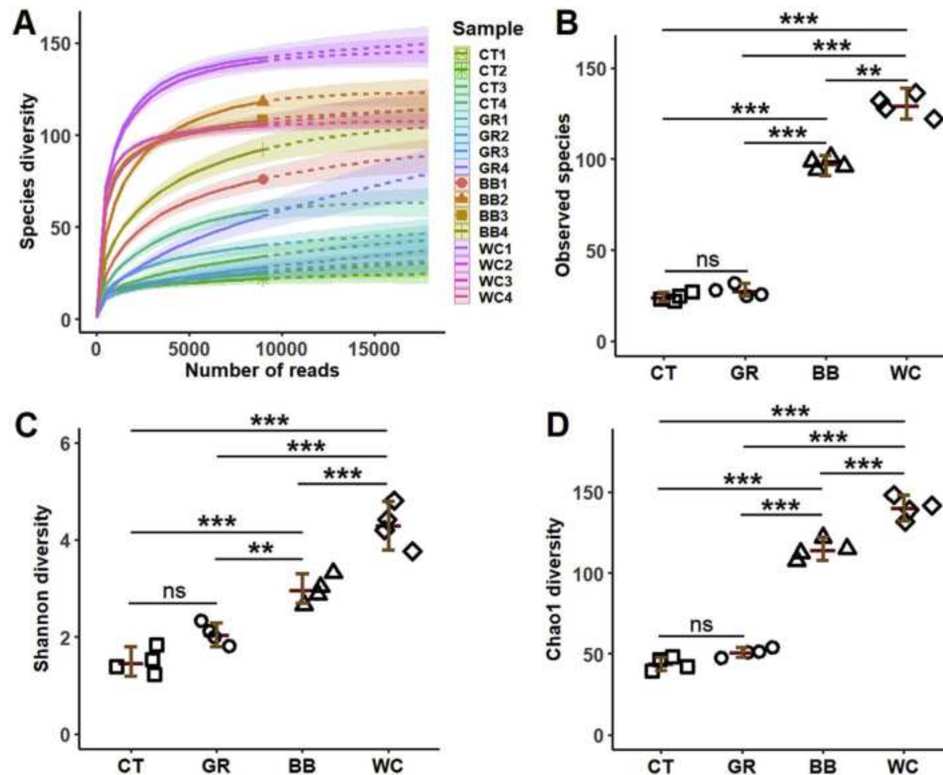

Fig. 3. Alpha diversity measurements of samples in four different treatment groups. (**A**) Rarefaction curve showing the depth of sequencing in terms of species diversity; (**B**) Observed species; (**C**) Shannon index; (**D**) Chao1 index. Data expressed as mean ± SE. *Significant at α-level of 0.05; **Significant at α-level of 0.005; ***Significant at α-level of 0.001. Abbreviation: BB = Bio-Ball; CT = Control; GR = Gravel; WC = Water-cleanser.

3.4. Impacts on immune gene expression

Relative to control, the results of qRT-PCR showed the up-regulation of innate immune responsive genes in the Bio-Ball and Water-cleanser groups. The expression level of immune genes in the marron with gravel had almost similar patterns to the control treatment. There was 3.0 and 2.8 fold increase in the expression of pro-inflammatory cytokine, interleukin-17F (IL-17F); 1.6 and 1.8 fold increase in the expression of anti-inflammatory cytokine, interleukin 10 (IL-10); 2.0 and 1.8 fold up-regulation of prophenoloxidase (proPO); both 1.6 fold increase expression for cathepsin L (PcCTSL); 2.3 and 2.5 fold increase expression for cytosolic manganese superoxide dismutase (cytMnSOD) with Bio-Ball and Water-cleanser, respectively (Fig. 6B). Compared to control and gravel, the relative expression level of interleukin 1β (IL-1β), interleukin 8 (IL-8) and tumour necrosis factor (TNF-α) genes were relatively static with Bio-Ball and Water-cleanser group.

4. Discussion

Many intensive aquaculture practices result in accumulation of organic wastes that are linked to water quality deterioration and environmental pollution. Poor water quality favours the growth of pathogenic microbes in the aquatic environment that can be transmitted to aquatic species (Bhateria and Jain, 2016; Foysal et al., 2019a; Yildiz et al., 2017). Therefore, a cost effective approach for maintaining optimum water quality is a major challenge for the farmers and researchers. Biological filter system has been successful in treating organic waste from both fresh and marine farming operations (Foysal et al., 2019a; Gutierrez-wing and Malone, 2006; Permatasari et al., 2018). The three different filters used in present study (gravel, Bio-Ball and Water-cleanser) were reported to be effective in improving the quality of aquaculture water (Castine et al., 2013; Cole et al., 2019; Foysal et al., 2019a). Studies also reported the augmentation of bacterial communities in water with two commercial biological filters, Bio-Ball and Water-cleanser (Cole et al., 2019; Foysal et al., 2019a). However, none of the above study analyse the impacts of commercial substrate and non-substrate based biological filters on the gut microbiota health and immune indices of any aquatic species.

Both Bio-Ball and Water-cleanser improved the water quality parameters, whereas marron growth was not influenced by any filter. Both of the biological filters are claimed to provide substrates for the growth of denitrifying bacteria that can remove nitrogenous compound and reactive phosphorus from water (Cole et al., 2019; Foysal et al., 2019a; Permatasari et al., 2018). Earlier studies with Bio-Ball, reported significant improvement of bacterial diversity of Firmicutes, a phylum that is known to be involved in denitrification process in the aquatic environment (Foysal et al., 2019a; Sharmin et al., 2013). The bacterial abundance at phylum level in this study showed significant enrichment of Firmicutes in Bio-Ball and Water-cleanser treatments, similar to previous reports where Firmicutes mediated waste water treatment established (Sharmin et al., 2013). The information relating to the impacts of biological filters on growth performance of crayfish is very limited, however, a most recent study found no significant differences in weight gain of marron with Water-cleanser (Cole et al., 2019). Previous studies on marron with different diets reported insignificant growth changes in laboratory trial for 56–60 days (Ambas et al., 2017, 2013; Foysal et al., 2019). Alike previous studies, present study also lasted for two months– a very short time to achieve significant growth rate for a species having fairly long life cycle under farming conditions. However, some water quality parameters in this study were positively impacted after application of Bio-Ball and Water-cleanser,

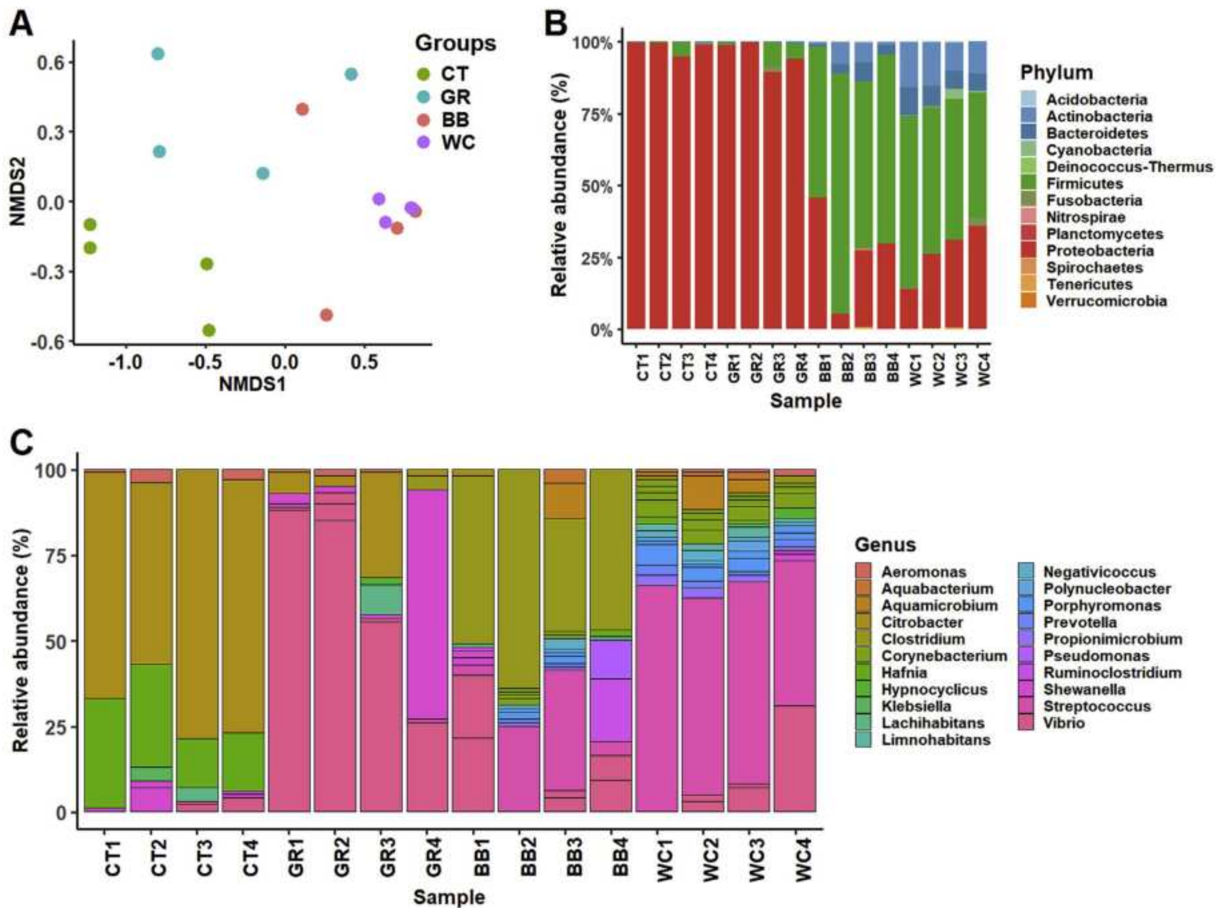

Fig. 4. Beta diversity and bacterial communities in four different treatment groups. (**A**) Non-metric multidimensional scaling (nMDS) plot showing clustering of marron gut samples; (**B**) Relative abundance of bacteria at phylum level in the hindgut of marron; (**C**) Relative abundance of bacteria at genus level in the hindgut of marron. Abbreviation: BB = Bio-Ball; CT = Control; GR = Gravel; WC = Water-cleanser.

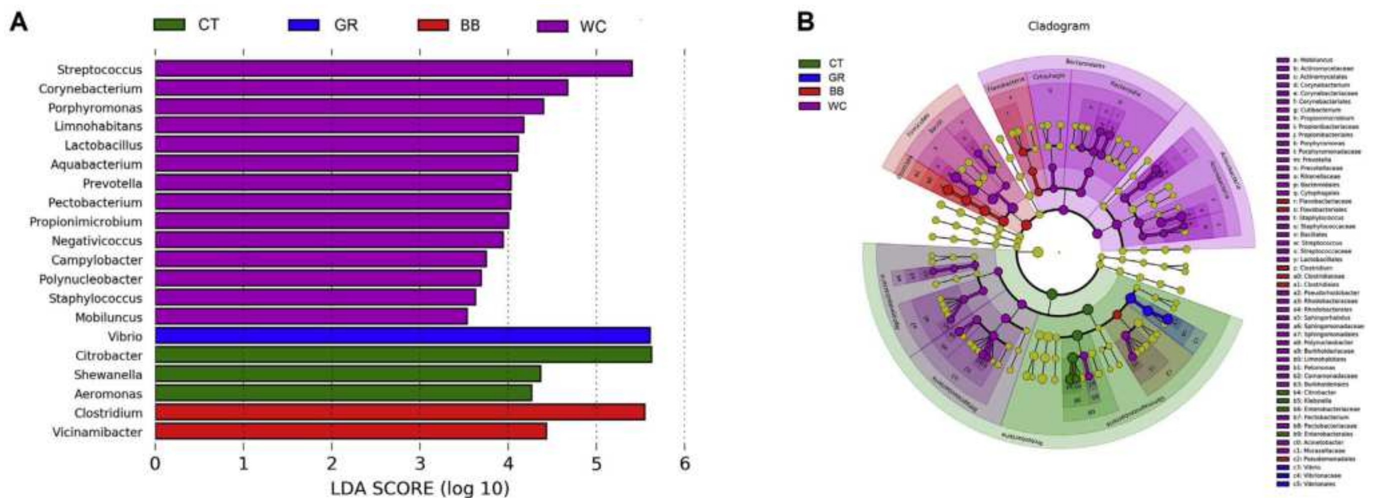

Fig. 5. Indicator microbial groups in the hindgut of marron in four different treatment groups. (**A**) Differentially abundant bacteria at genus level with strict LDA cut-off value of 3.5 and above and at 0.05 level of significance, (n = 4). **(B)** Circular LEfSe cladogram representing the phylogenetic distribution of bacterial lineage. The lineage with LDA scores of 3.5 or above are displayed here. The red, green, blue and purple colour indicate Bio-Ball, control, gravel and Water-cleanser, respectively. The dot at centre represents the OTUs at phylum level while the outer circle of dots denotes OTUs at genus level. The order, family, and genus that are significantly different between two groups are given in the upper right corner with respective colour codes. Abbreviation: BB = Bio-Ball; CT = Control; GR = Gravel; WC = Water-cleanser. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

identical to recent studies where nitrate and ammonia reduction from aquaculture water were reported (Foysal et al., 2019a; Permatasari et al., 2018).

The gut microbiota of aquatic species are commonly dominated

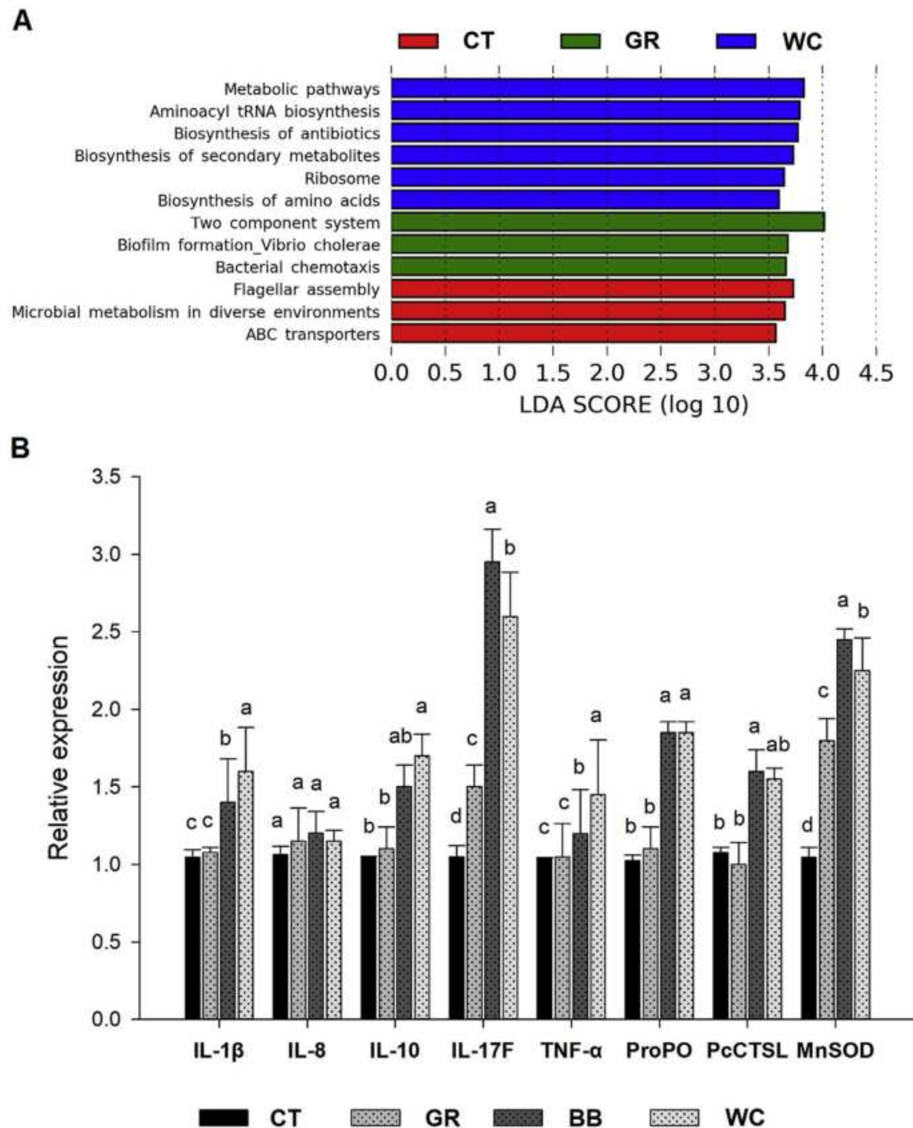

Fig. 6. Metabolic functions and immune response in hindgut of marron after trial. (**A**) Predicted differentially expressed KEGG metabolic pathway in four different treatment groups identified by Piphillin and linear discriminant analysis (LEfSe) (LDA>3.5, $P < 0.05$). No significant up-regulated pathway was found for the Bio-Ball group. (**B**) Relative expression profile of genes from the intestine tissue and haemolymph associated with innate immune response of marron in different treatment groups. Means with different superscripts are statistically significant at α-level of 0.05. Data expressed as mean ± SE. Abbreviation: BB = Bio-Ball; CT = Control; GR = Gravel; WC = Water-cleanser.

by Proteobacteria, Firmicutes and Bacteroidetes (Ghanbari et al., 2015; Huang et al., 2014; Michl et al., 2017). Firmicutes and Bacteroidetes are mostly beneficial bacteria for both human and animals including crayfish (Egerton et al., 2018; Rajilić-Stojanović et al., 2007). Firmicutes can improve the digestibility, health and immune performance of fish and marron, and counteract the damaging effects of pathogenic bacteria in the gut (Costantini et al., 2017; Foysal et al., 2019). Present study revealed significant enrichment of phylum Firmicutes and genus *Clostridium* in the marron gut with the Bio-Ball. *Clostridium butyricum*, a probiotic can modulate the gut microbiota and immune gene expression of marron (*C. cainii*) and white pacific leg shrimp (*Litopenaeus Vannamei*) where significant increase in the relative abundance of Firmicutes and Bacteroidetes (Duan et al., 2018; Foysal et al., 2019b). Water-cleanser on the hand favoured the growth of microbes from diverse genera in the marron gut, mostly from lactic acid bacteria (LAB). *Streptococcus* is one of the core bacterial group in the marron gut while *Lactobacillus* enrichment in the fish and crustacean gut plays a key role in health and immunity of *L. vannamei* and *Cyprinus carpio* (Foysal et al., 2019; Giri et al., 2018; Vieira et al., 2008). Increase in relative abundance of *Corynebacterium*, *Porphyromonas* and *Limnohabitans* were associated with better health and immune response of marron fed black soldier fly (*Hermetica illucens*) supplemented with poultry-by-product meal (Foysal et al., 2019).

Besides, the bacterial communities, information on metabolic pathway could be an effective way to understand the health condition of animals. The Water-cleanser and Bio-Ball as bio-filters enhanced the metabolic capability of marron and up-regulated the pathways for the synthesis of amino acids, antibiotics, secondary metabolites. Previous reports demonstrated *Firmicutes* driven up-regulation of amino acid biosynthesis and metabolic pathways in fish, mostly by lactic acid bacteria (LAB) *Streptococcus* and *Lactobacillus* (Besten et al., 2013; Bhute et al., 2017; Dai et al., 2014). The significant enrichment of LAB in marron gut with Water-cleanser, therefore associated with the modulation of these

metabolic pathways. The predicted up-regulated pathways in the gravel are mostly stress response elements that protect bacteria against host immune defence, antimicrobials, and adverse physiological and environmental conditions (Tiwari et al., 2017; Wang et al., 2013). The conditions created by two-component systems and biofilm pathways enforced the host cell to respond quickly to a changed environmental condition resulting release of more energy, and help bacteria in chemotaxis, cell wall invasion– thereby flagging the immune response of host (Falke et al., 2010; Rutherford and Bassler, 2012). Finally, flagellar assembly, ABC transporter and microbial metabolism in control marron commonly play an important role in regular metabolic process of aquatic species including adhesion, various metabolic processes including transport, metabolism, drug resistance under different environmental conditions (Tripathy et al., 2014; Wang et al., 2015; Zhou et al., 2009). The use of Bio-Ball and Water-cleanser is therefore beneficial for digestion and immunity of marron in compared to gravel.

The intestinal mucosal membrane plays a significant role in immunity of fish and crayfish (Ángeles Esteban, 2012; Lieschke and Trede, 2009). Among the various factors, cytokines (interferon, interleukin, and tumour necrosis factors), lysosomal peptidase (pcCTSL), and innate immune response genes, prophenoloxidase (prePO) and cytosolic manganese superoxide dismutase (cytMnSOD) play a pivotal role in regulation of the immune response of crayfish (Dai et al., 2017; Foysal et al., 2019; Jiang et al., 2015; Liu et al., 2013). Over-expression of IL-1B in fish modulates the expression of IL-17F, a gene essential for antibacterial defence (Wang et al., 2014). Administration of IL-1B in rainbow trout (*Oncorhynchus mykiss*) also known to be associated with antibody production thereby improving immunity (Taechavasonyoo et al., 2013; Yin and Kwang, 2000). Upregulated expression of pro-inflammatory cytokine (IL-1B, IL-17F) regulates the expression of anti-inflammatory cytokines in order to respond quickly to prevent the damages associated with inflammation (Foysal et al., 2019; Miao et al., 2018). Hence in the present study, over-expression of IL-10 could be linked to the expression profile of major cytokine genes. The gene PcCTSL have been shown to boost the growth, lysosomal activity and facilitate in antigen processing (Dai et al., 2017). The enhanced lysosomal activity in the Bio-Ball and Water-cleanser group is in accordance with the results of previous study. Prophenoloxidase system helps to improve the innate immune response of crayfish by controlling the growth of pathogens whereas cytMnSOD is an antioxidant that transport oxygen in the haemolymph (Gómez-anduro et al., 2012; Liu et al., 2013).

5. Conclusion

Addition of Bio-Ball and Water-cleanser into the culture system resulted in significant reduction of crayfish pathogen *Vibrio*. Due to the huge species diversity of microbial communities, the pathogenic properties and virulence of the respective species are dictated by the species type, species abundance and available stressors. Hence, the pathogenicity of any selected microbial species in the gut is difficult to predict without any *in vivo* challenge test under the culture conditions. Still, the overall results suggested that, Bio-Ball and Water-cleanser could be used as a potential water treatment biological filters in aquaculture tanks for better health and immune status of marron. However, the physiological interactive mechanism between microbes in biological filters and crayfish gut is a study of future. The surface area of biological filters could play a crucial role in augmentation of microbial community in water by producing bacterial biofilm that can hasten the process of organic waste decomposition. Therefore, extensive research studies are needed to characterize the microbial communities at species level, and *in vivo* challenge tests. The enrichment of beneficial bacteria on the biofilm is our next phase of research.

Data availability

The raw sequence data in FASTQ files are currently available at National Centre for Biotechnology Information (NCBI) BioProject under the accession number PRJNA549032.

Declaration of competing interest

The authors declare no conflict of interest.

CRediT authorship contribution statement

Md Javed Foysal: Conceptualization, Methodology, Formal analysis, Validation, Data curation, Software, Funding acquisition, Writing - original draft. **Ravi Fotedar:** Supervision, Conceptualization, Methodology, Validation, Writing - review & editing. **Chin-Yen Tay:** Methodology, Validation, Writing - review & editing. **Sanjay K. Gupta:** Investigation, Writing - review & editing.

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Appendix A. Supplementary data

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