UNIVERSITI PUTRA MALAYSIA

EFFECTS OF TRASH FISH AND PELLETED FEED ON GROWTH, MICROBIOTA, AND INTESTINAL HISTOLOGY OF THE JUVENILE AUSTRALIAN RED CLAW CRAYFISH, CHERAX QUADRICARINATUS (VON MARTENS, 1868)

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CERTIFICATIONS

It is hereby certified that we have read this project paper entitled "Effects of Trash Fish and Pelleted Feed on Growth, Microbiota, and Intestinal Histology of the Juvenile Australian Red Claw Crayfish, *Cherax quadricarinatus* (Von Martens, 1868)" by Leong Yuan Hang and in our opinions, it is satisfactory in terms of scope, quality and presentation as partial fulfilment of the requirement of the course VPD 4999-Project.

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ABBREVIATIONS

AB 2.5	Alcian Blue pH 2.5
ADWG	Average Daily Weight Gain
ADLG	Average Daily Length Gain
CD	Crypt Depth
DNA	Deoxyribonucleic Acid
FAO	Food and Agriculture Organization
FCR	Feed Conversion Ratio
GC	Goblet Cell Number
gDNA	Genomic Deoxyribonucleic Acid
H&E	Haematoxylin & Eosin
LG	Length Gain
LPT	Lamina Propria Thickness
rDNA	Recombinant Deoxyribonucleic Acid
RNA	Ribonucleic Acid
PCR	Polymerase Chain Reaction
PAS	Periodic Acid-Schiff
SGRL	Specific Growth Rate of Length
SGRW	Specific Growth Rate of Weight
SR	Survival Rate
TMT	Tunica Muscularis Thickness
VH	Villus Height
VW	Villus Width
WG	Weight Gain

ABSTRAK

Abstrak daripada kertas projek yang dikemukakan kepada Fakulti Perubatan Veterinar untuk memenuhi sebahagian daripada keperluan kursus VPD 4901 - Projek.

KESAN IKAN BAJA DAN MAKANAN PELET PADA PERTUMBUHAN, MIKROBIOTA, DAN HISTOLOGI USUS DALAM BENIH UDANG KARA CAKAR MERAH AUSTRALIA, CHERAX QUADRICARINATUS (VON MARTENS, 1868)

oleh

LEONG YUAN HANG

2023

Penyelia: Dr. Mohd Akmal Mohd Noor

Pada 1 tahun belakangan ini, udang kara cakar merah Australia (*Cherax quadricarinatus*) telah mendapat perhatian dalam industri akuakultur tempatan sebagai spesies yang berpotensi dalam akuakultur tempatan disebabkan oleh pertumbuhan yang pantas dan penyesuaian yang luar biasa, menjadikannya fokus penelitian akuakultur. Sebagaimana dengan spesies akuakultur yang lain, pemakanan adalah faktor kritis yang mempengaruhi penternakan udang kara. Dalam penternakan udang kara tempatan, rejim pemberian makanan termasuk komersial makanan yang diformulasi, makanan pelet, dan sumber tradisional seperti ikan baja. Walau bagaimanapun, penggunaan ikan baja adalah kontroversial disebabkan oleh kesannya terhadap keberlanjutan penternakan dan potensi masalah kesihatan. Oleh demikian, kajian tersebut bertujuan untuk menyelidiki kesan ikan baja dan makanan pallet pada pertumbuhan, analisis mikrobiota usus, dan histologi usus dalam benih udang kara ini. Sebanyak 40 ekor udang kara diaklimatisasi selama satu

minggu dan diternakkan selama empat minggu dalam dua kumpulan (Kumpulan A dan Kumpulan B). Kumpulan A sebagai kumpulan kawalan, diberi makanan udang TIGER saiz (TT663) sebagai pelet, dan Kumpulan B sebagai kumpulan rawatan, diberi sardin pelangi (*Dussumieria acuta*) sebagai ikan baja. Indeks prestasi pertumbuhan telah dicatat dan dikira. Kadar kehidupan dalam Kumpulan A adalah signifikansi lebih tinggi ($p < 0.05$) daripada Kumpulan B. Namun, tiada signifikansi perbezaan ($p > 0.05$) dalam indeks prestasi pertumbuhan antara kedua-dua kumpulan. Teknik sekuensing berkecepatan tinggi digunakan untuk menganalisis perubahan dalam komposisi mikrobiota usus. Filum dominan dalam usus kedua-dua kumpulan adalah Proteobacteria dan Firmicutes. Kekayaan spesies dan keragaman mikrobiota dalam Kumpulan A lebih tinggi daripada Kumpulan B. Histologi usus dievaluasi menggunakan Analisis Gambar dan perisian komputer ImageJ. Indeks histologi usus, termasuk tinggi vili, rasio tinggi vili terhadap kedalaman kripta, luas permukaan vili, ketebalan lamina propria, dan jumlah sel goblet dalam Kumpulan A, adalah signifikansi berbeza ($p < 0.05$) daripada Kumpulan B. Kesimpulannya, udang kara yang diberi makanan pelet memiliki kadar kehidupan yang signifikansi lebih tinggi, kesihatan usus yang lebih baik, dan populasi mikroba yang substansial dibandingkan dengan udang kara yang diberi makan ikan baja. Pertemuan ini menegaskan kepentingan nutrisi yang disesuaikan dalam akuakultur, memperjuangkan adopsi diet yang diformulasikan seperti makanan pelet untuk mengoptimalkan produksi udang kara.

Kata Kunci: udang kara cakar merah Australia, akuakultur, ikan baja

ABSTRACT

Abstract of the project paper presented to the Faculty of Veterinary Medicine in partial fulfilment of the course VPD 4901 - Project.

EFFECTS OF TRASH FISH AND PELLETED FEED ON GROWTH, MICROBIOTA, AND INTESTINAL HISTOLOGY OF THE JUVENILE AUSTRALIAN RED CLAW CRAYFISH, CHERAX QUADRICARINATUS (VON MARTENS, 1868)

by

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2023

Supervisor: Dr. Mohd Akmal Mohd Noor

In the past decade, Australian red claw crayfish (*Cherax quadricarinatus*) has gained attention as a promising species in local aquaculture due to its rapid growth and exceptional adaptability, making it a focal point of aquaculture research. Similar to the trend with other aquaculture species, feed input is a critical factor affecting crayfish farming. In local crayfish cultivation, the feeding regime incorporates a commercially formulated diet, pelleted feed, and traditional sources like trash fish. However, the use of trash fish is controversial due to its impact on farming sustainability and potential health issues. Therefore, the study was to investigate the effects of trash fish and pelleted feed on growth performance, gut microbiome analysis, and the intestinal histology of juvenile crayfish. A total of 40 crayfish were acclimatised for one week and cultured for four weeks in two groups (group A and group B). Group A served as the control group, fed with TIGER

prawn feed size (TT663) as pellet, and Group B acted as the treatment group, fed with rainbow sardine (*Dussumieria acuta*) as trash fish. Growth performance indices were recorded and calculated. The survival rate in Group A was significantly higher ($p < 0.05$) than those in Group B. However, there were no significant differences ($p > 0.05$) in other growth performance indices between the two groups. High-throughput sequencing techniques were employed to analyse the alterations in intestinal microbiota composition. The dominant phyla in the intestines of both groups were Proteobacteria, and Firmicutes. The species richness and diversity of the microbiota in Group A were higher than in Group B. Intestinal histology was evaluated using Image Analyser and ImageJ software. Intestinal histological indices, including villus height, villus height to crypt depth ratio, villus surface area, lamina propria thickness, and goblet cells number in Group A, were significantly different ($p < 0.05$) than in Group B. In conclusion, pellet-fed crayfish had a significantly higher survivability, improved intestinal health and substantial microbial population compared to trash fish-fed crayfish. These findings underscore the significance of tailored nutrition in aquaculture, advocating for the adoption of formulated diets like pelleted feed to optimise crayfish production.

Keywords: Australian red claw crayfish, aquaculture, trash fish

1.0 INTRODUCTION

1.1 Background

Freshwater crayfish encompass a diverse group of over 600 species found naturally across the globe, with the exception of continental Africa, the Indian subcontinent, and Antarctica. Simultaneously, ongoing discovery of new species continues each year, that documented by Crandall and Buhay (2008), Loughman *et al*., (2017), Lukhaup *et al*., (2017), McCormack and Ahyong (2017), and Schuster and Kendrick (2017). The global landscape of crayfish aquaculture has experienced a significant upswing in the last six decades, which indicated by Crandall and Buhay (2008) and Lodge *et al*., (2012). Over recent decades, the success of freshwater crayfish aquaculture on a global scale has been attributed to the availability of species possessing favourable characteristics for cultivation and commercial endeavours (Viau & Rodríguez, 2010). Fatihah *et al*., (2020) emphasised that, out of the extensive catalogue of more than 100 Australian crayfish species, only three species from the Cherax genus, namely *Cherax tenuimanus* (Smith), *Cherax destructor* (Clark), and *Cherax quadricarinatus* (von Martens), are presently being cultivated due to their substantial market potential.

Australian red claw crayfish, scientifically known as *Cherax quadricarinatus* (von Martens, 1868), is a member of the Parastacidae family, a freshwater crayfish group exclusively found in the Southern Hemisphere (Jones, 1990). According to Jones (1997), the species originates from tropical regions in Queensland, northern Australia, and the southeastern part of Papua New Guinea. Awangku *et al*., (2016) reported that the

Australian red claw crayfish is locally referred to as the freshwater lobster due to its lobster-like features and habitat.

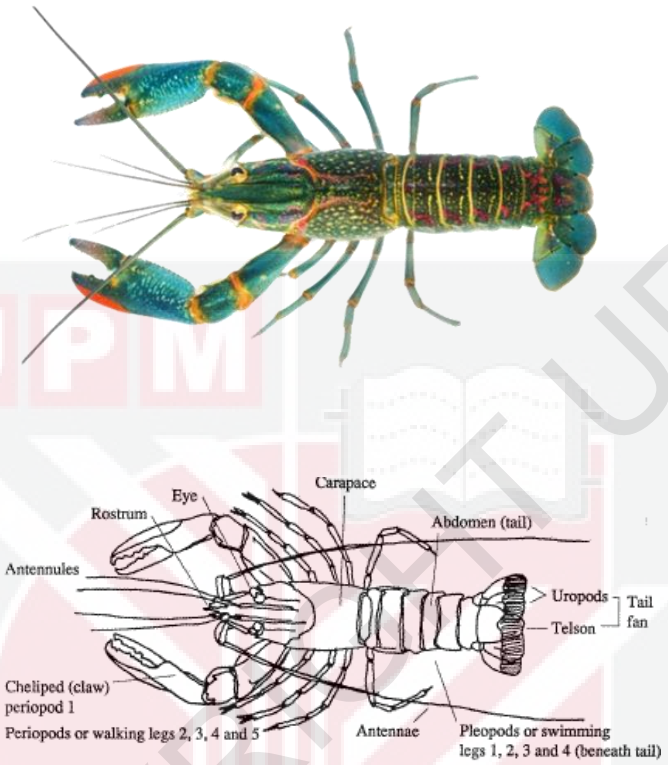


Figure 1: Australian red claw crayfish, *Cherax quadricarinatus*, von Martens, 1868. Courtesy photograph by Tan Heok Hui & Tinaroo Environmental Education Centre

Although the precise year of the Australian red claw's introduction to Malaysia remains uncertain, records indicate commercial-scale cultivation has been taking place since 2003 in the southern region of the Malaysian peninsula (Alimon, 2003). With its substantial commercial potential, the Australian red claw crayfish has been cultivated for several decades in Australia and various other countries, evolving into a key species in aquaculture globally (Medley *et al*., 1994; Karplus *et al*., 2003; Rodgers *et al*., 2006). As highlighted by Awangku *et al*., (2016), the local community has taken a keen interest in

Australian red claw crayfish farming as a means of generating additional income. Renowned for its ideal characteristics as an aquaculture species, such as rapid growth, high adaptability, tolerance to diverse water quality and environmental conditions, ease of production, and significant market value due to its large size, flavour, and texture, the Australian red claw crayfish has become a focus of aquaculture endeavours (Jones, 1990; Masser & Rouse, 1997; Viau & Rodríguez, 2010). Additionally, its vibrant colours make it highly suitable for the aquarium industry, contributing to its widespread translocation as an economically important ornamental trade (Lawrence & Jones, 2002). While local cultures primarily target sizes of 6 to 8 inches for food consumption, there is a heightened demand for juveniles measuring 1 to 2 inches, leading to increased emphasis on breeding rather than grow-out activities (Awangku *et al*., 2016).

Figure 2: A bulk of trash fish, Rainbow sardine, *Dussumieria acuta.*

The precise definition of trash fish or low-valued fish lacks consensus within the academic discourse, as acknowledged by previous researchers (Hasan *et al*., 2007; De Silva & Turchini 2009). Identifying trash fish is challenging due to the absence of a dedicated

fishery, with its presence primarily attributed to by-catch from trawling or capture in artisanal coastal and/or commercial inshore fisheries (Edwards *et al*., 2004, Hasan & Halwart 2009).

Trash fish is commonly associated with small-sized fish species characterised by lower economic value, perishability, and relatively poor edible qualities, often not intended for direct human consumption (Hasan & Halwart 2009). The term "Trash fish" encompasses a diverse range of non-economically significant fish species caught in fisheries, including inedible low-value marine fishes and commercially valuable fish juveniles typically discarded as waste (Kasthuri *et al*., 2021). Often referred to as fishmeal or low-value fishmeal, trash fish represents a collective group of marine life with small to medium sizes that fall short of market standards (Gomez *et al*., 2010). Despite a limited understanding of the overall global bycatch situation, it constitutes a substantial portion, approximately 40.4%, of global marine catches, highlighting systemic deficiencies in fisheries policy and management (Davies *et al.*, 2009). The United Nations agency (FAO, 2010) observes a global trend shifting towards the enhanced utilisation of non-commercial discarded elements, encompassing bycatch and trash fish. Recognized as the most crucial fish product in terms of both weight and value, certain trash fish species are undergoing transformation into value-added products (Edwards *et al*., 2004). The composition of trash fish includes anchovy, pilchards, sardines, mackerel, small shrimp, and squid (Gomez *et al*., 2010), with the specific selection of Rainbow sardine (*Dussumieria acuta*) for this research purposes (Kasthuri *et al*., 2021).

1.2 Problem Statement and Justification

The heavy reliance on trash fish as a primary feed source in the aquaculture industry raises concerns about its reliability, efficacy and safety as highlighted by Gomez *et al*., (2010). There are issues surrounding the use of trash fish as a feed source including nutritional considerations and potential indirect sources of diseases (Gomez *et al*., 2010). According to Kasthuri *et al*., (2021), the nutritional value of discarded trash fishes has not been thoroughly understood. Factors like the lower cost of trash fish compared to commercial pelleted feed further incentivize its use, particularly for species without readily available pelleted feed options (Sim, 2006; Kongkeo *et al*., 2010). In addition, the concerns shift towards potential health issues and infectious disease outbreaks in indoor cultivation of the Australian red claw crayfish, given its susceptibility to mixed infections from bacteria, fungi, and viruses that might be carried by trash fish (Kim *et al*., 2007; Syahidah, 2020).

However, scant attention has been given to how trash fish and pelleted feed impact the relative abundance and function of intestinal microbiota in Australian red claw crayfish during long-term feeding (Alimon *et al*., 2003). Furthermore, the influence of different diets on intestinal histomorphology and its correlation with the overall health status of Australian red claw crayfish remains underexplored (Wan *et al*., 2022). The absence of reports on the gut microbiota composition of Australian red claw crayfish after prolonged culture with trash fish underscores the need for a comprehensive understanding of intestinal bacterial dynamics under conditions of nutritional stress, crucial for optimising crayfish health in cultivation (Wan *et al*., 2021). Therefore, investigating the effects of diverse feed sources on the growth, microbiota, and intestinal histology of juvenile

crayfish holds promise for refining aquaculture feeding strategies, ultimately enhancing the overall performance and health of the species (Jones, 2000). This review emphasises the crucial aspects of growth and intestinal health in the Australian red claw crayfish, with a specific focus on the differential effects of feeding diets, trash fish, and pelleted feed. Despite limited existing literature on this topic compared to other farmed crustacea (Alimon *et al*., 2003), the research aims to fill this gap and provide essential insights into the effects of trash fish and pelleted feed on the growth, microbiota, and intestinal histology of the Australian red claw crayfish, offering valuable information for the sustainable production of this species.

1.3 Objectives and Hypothesis

The present study is to investigate the effects of trash fish and pelleted feed on growth and intestinal health in juvenile Australian red claw crayfish. To achieve this objective, the study evaluated effects of feeding diets on the growth performance, intestinal microbiota composition and intestinal histomorphological changes of juvenile Australian red claw crayfish. The findings offer valuable insights into the correlation between feeding diets and the intestinal health of the crayfish species. The study posits two hypotheses, namely the null and alternative hypotheses:

Null Hypothesis (H_0): There are no significant differences in the effects of different feeding diets (trash fish and pelleted feed) on the growth performance, gut microbiota, and intestinal histology of the Australian red claw crayfish.

Alternative Hypothesis (H_A): There are significant differences in the effects of different feeding diets (trash fish and pelleted feed) on growth performance, gut microbiota, and intestinal histology of the Australian red claw crayfish.

2.0 LITERATURE REVIEW

2.1 Aquaculture and Feeding Regime

Aquaculture presently contributes almost half of global seafood consumption, with Asia at the forefront, contributing over 85% to the worldwide output (Subasinghe *et al*., 2009). While production remains predominantly Asian and largely comprises small-scale operations, there is a widespread consensus that aquaculture holds the potential to address the escalating global demand for nutritious seafood, fostering national economic growth, and supporting sustainable livelihoods in various communities (FAO, 2006). Feeding emerges as a critical aspect in aquaculture as it will contribute up to 70% of the cost production. In addition, it will exert a significant influence on the growth, health, and survival of aquatic organisms, including Australian red claw crayfish, where feed input plays a pivotal role in performance and production (Wan *et al*., 2022). The current aquaculture feeding regimen incorporates conventional sources like trash fish and commercial pelleted feed, tailored to meet species-specific nutritional requirements (Hasan & Halwart, 2009). Numerous studies have undertaken comparative analyses between trash fish and formulated diet feeding in fish and crustaceans (Chaitanawisuti *et al*., 2011; Han *et al*., 2021), with the latter commonly employed as a diet for carnivorous fish and crustaceans in aquaculture. However, concerns have been raised about the potential impediments to future aquaculture expansion due to an escalating reliance on low-value marine "trash fish" and fish meal (Kasthuri *et al*., 2021). The use of trash fish has become controversial, posing challenges to aquaculture sustainability, nutritional balance, and environmental integrity (Bunlipatanon *et al*., 2012).

2.2 Trash Fish as Aquaculture Feeding Diet

Trash fish, beyond the conventional use of fishmeal and fish oil in compound and farm-made aquafeeds, serves as a complete or supplementary feed for farmed fish, crustaceans, and select molluscan species globally (Hasan & Halwart, 2009). The direct input of trash fishes or incorporation in artificial feed was considered a better alternative (Kasthuri *et al*., 2021). In the traditions of Asia-Pacific mariculture, trash fish holds a central role, predominantly sourced from commercial fish landings or artisanal fisheries, acting as the primary food source in various practices (Bunlipatanon *et al*., 2012). The evolution of the aquaculture industry intensifies the demand for trash fish, particularly in Asia, where its affordability and mass availability contribute to its widespread utilisation (Gomez *et al*., 2010; Bunlipatanon *et al.*, 2012). The most recent application of trash fish involves coastal aquaculture, crucial for the industry's development, with some stakeholders asserting that "it is impossible to practise aquaculture without trash fish" (Edwards *et al*., 2004). The global usage of trash fish as direct feed in aquaculture is estimated at approximately 5 to 6 million tonnes, with a significant focus on carnivorous fish species in China and several Southeast Asian nations, as well as crustaceans (Tacon *et al*., 2006). A recent estimate places the annual Asian utilisation of trash fish as fish feed between 1.6 to 2.8 million tonnes, with low and high predictions for 2010 ranging from 2.2 to 3.9 million tonnes of trash fish or low-value fish as direct feed inputs (FAO, 2008). The dependence of finfish and crustacean aquaculture on capture fisheries is evident, as they rely on sourcing feed inputs from fishmeal, fish oil, trash fish, or other marine resources (Hasan & Halwart, 2009).

The utilisation of trash fish as a dietary component in aquaculture has given rise to numerous concerns, including issues of source instability, uncontrollable quality, and nutritional imbalances, as highlighted by Bunlipatanon *et al*., (2012). The controversial nature of using trash fish in aquaculture extends to its potential impact on sustainability and environmental degradation, thereby raising significant apprehensions (Bunlipatanon *et al.*, 2012). The debate intensifies when considering the employment of raw, unprocessed fish, commonly known as trash fish, primarily as feed in aquaculture, even though numerous environmental concerns have been raised in this regard, the scientific substantiation for many of these concerns remains inconclusive, as noted by the World Wild Life Mediterranean Programme in 2005. Prolonged use of trash fish in aquaculture is anticipated to exacerbate water quality issues, potentially leading to environmental problems such as eutrophication, as indicated by Xu *et al*., (2007). Additionally, concerns about the nutritional inadequacy of trash fish compared to commercial pellets have been voiced by Gomez *et al*., (2010). Additionally, the quality of trash fish emerges as a significant worry, with concerns about a rapid decline in quality during preservation on board ships using only ice or chilled water, particularly problematic for offshore fisheries where boats may be at sea for extended periods (Edwards *et al*., 2004). Some farmers face challenges in providing formulated feed due to initial high costs, leading to the risk of malnutrition or stunted growth in fishes (Shingare *et al*., 2020). According to Bunlipatanon *et al*., (2012), farmer perceptions, grounded in the belief that the use of trash fish results in superior performance in cultured stocks, play a role in sustaining the tradition, which especially evident in the case of marine species that pose challenges in transitioning to dry commercial pelleted feed. Studies also underscore the potential of

trash fish to serve as an incidental source of diseases in cultured fish, as reported by Gomez *et al*., (2010). The presence of pathogenic bacterial and viral agents in trash fish, causing severe diseases in farmed aquatic species, has been documented by Kim *et al*., (2007). Notably, the use of frozen trash fish in aquaculture feed has been associated with the presence of bacteria and viruses such as Streptococcus and Iridovirus (Kim *et al*., 2007). Despite these concerns, traditional feed sources like trash fish persist as prevalent alternatives, recognized for their cost-effectiveness and sustainability when compared to pelleted feed (Bunlipatanon *et al*., 2012).

2.3 Effects of Feeding Diets on Aquaculture Species

In the pursuit of more sustainable and environmentally friendly feeding practices in aquaculture, there has been a discernible shift from conventional methods to alternatives such as incorporating trash fish and pelleted feed. The choice of feed may significantly influence the growth performance, gut microbiota, and intestinal histology of aquaculture species. Studies have shown that replacing trash fish with a formulated diet can enhance the survival and growth performance of pre-adult Gazami crab, *Portunus trituberculatus* (Hou *et al*., 2016). Besides, the gut microbiota plays a vital role in resisting environmental stress and maintaining physiological equilibrium in crustaceans, which is crucial for nutrient absorption, immune system regulation, and disease prevention in aquatic animals (Peled and Livney, 2021). Formulated diets have been linked to improved digestive capacity and immune function in various aquatic species, such as juvenile hybrid grouper. (Ye *et al*., 2020). Djunaidah *et al*., (2003) proposed that the utilisation of artificial diets in mud crab (Scylla paramamosain) achieved reproductive success comparable to fresh food,

yielding larvae of superior quality by enhancing the nutritional composition of the artificial diets. Probiotics, as feed additives, have been employed to enhance intestinal microbial balance and promote growth and disease resistance (Haque et al., 2021).

3.0 MATERIALS AND METHODS

3.1 Experimental Animals

Juvenile Australian red claw crayfish (*Cherax quadricarinatus*) with uniform sizes (50.66 ± 5.32 mm in body length; 3.60 ± 1.26 g in body weight) were procured from a local commercial crayfish hatchery, FW Crustacean, located in Kuala Selangor, Malaysia. Acclimatisation to laboratory conditions was conducted in glass aquaria (42 cm x 28 cm x 30 cm, water height: 25 cm) with recirculating freshwater over a 7-day period. Aeration was provided in each tank to maintain dissolved oxygen levels, and the experimental design included two study groups: pelleted feed (control) and trash fish (treatment). Tanks for each group were duplicated and randomly arranged. Ventilated plastic boards were affixed to the tops of the tanks to prevent the entry of foreign objects and the escape of crayfish. Each tank housed 10 juvenile Australian red claw crayfish. Additionally, a glass tank (81 L) served as a water reservoir filled with tap water for experimental water needs. The tanks were maintained at a room temperature of 26 ± 2 °C with an 8-hour light : 16-hour dark photoperiod. Throughout this period, the crayfish were exclusively fed pelleted feed. Ethical approval for this study was obtained from the Institutional Animal Care & Use Committee (IACUC) (UPM/IACUC/AUP-U007-2023).

3.2 Experimental Feeds

The crayfish cultivation employed two study experimental feeds, namely TIGER prawn feed size (TT663) as pelleted feed and rainbow sardine (*Dussumieria acuta*) as trash fish. Pelleted feed was purchased from the hatchery, while trash fish was procured from a wet

market in Pasar Borong Selangor, Malaysia. The pelleted feed was stored in a dry environment at room temperature, whereas the trash fish underwent fillet processing, being cut into cube-shaped portions for ease of consumption by juvenile crayfish. The processed trash fish was refrigerated at a low temperature of 4 ± 2 °C to maintain freshness. Both experimental feeds underwent nutritional testing for proximate composition, encompassing moisture (M), dry matter (DM), crude protein (CP), and crude fat (CF), in accordance with AOAC standards (2005).

3.3 Experimental Design and Management

A total of 40 juvenile crayfish, with an average body weight of 3.60 ± 1.26 g, were randomly allocated to four glass tanks (42 cm x 28 cm x 30 cm, water height: 25 cm), with each tank containing two replicates of 10 crayfish for both the control (pelleted feed) and treatment (trash fish) groups. To mitigate aggressive behaviours among crayfish, three housing structures, constructed with a double layer of PVC pipes (total length: 22cm; PVC pipe diameter: 2cm), were positioned in the middle of each tank to serve as shelters. Crayfish were fed twice daily (08:30 and 18:30) until satiation, ensuring no residual diet after feeding. The feeding trial, spanning four weeks from August to September, took place at the Aquatic Laboratory in the Faculty of Veterinary Medicine, Universiti Putra Malaysia. The recirculating aquaculture system maintained aerated filtered water, with daily monitoring of water quality parameters, including temperature, dissolved oxygen, total dissolved solids, salinity, pH, and ammoniacal nitrogen. Additionally, ammonia, nitrite, and nitrate levels were assessed using an electronic handheld multiparameter water quality field instrument (YSI ProQuatro) and a water test

kit (API Fresh Water Master Test Kit).

3.4 Sampling

Following a four-week period, the total length, body weight, and crayfish count were measured and recorded following a 72-hour fasting period. The final measurement of the crayfish's body weight and total length were recorded by using an electronic digital scale and digital calliper. Total length was measured between rostrum and the end of telson of the crayfish (Sedik *et al*., 2018). Subsequently, nine crayfish per group underwent anaesthesia in an ice bath for 15 minutes. Following the administration of anaesthesia in an ice bath, intestinal samples were obtained using dissecting forceps and iris scissors that have been sterilised through autoclaving. Of these, the intestines from six crayfish (approximately 1.0 cm long) were preserved in Davidson's solution for 24 hours to facilitate histological examination. Simultaneously, intestines from the remaining three crayfish were fixed in the lysis buffer for 24 hours and transported to Patriot Biotech Sdn. Bhd. (Selangor, Malaysia) for analysis of the intestinal microbiome.

3.5 Feed Proximate Composition

The proximate composition of the feed was assessed following the standardised procedures outlined in the AOAC (2005) guidelines. The determination of feed moisture content involved weighing a finely ground sample (0.5 to 1g) in a pre-weighed porcelain crucible, followed by subjecting it to oven-drying at 105 °C until a constant weight was attained after approximately 15 hours. The crucible, containing the now moisture-free sample, was then allowed to cool to room temperature in a desiccator. Subsequent to

cooling, the dried sample within the crucible was precisely re-weighed, enabling the computation of moisture loss as a percentage based on the initial and final weights. The dry matter content was subsequently calculated by deducting the moisture percentage from 100%. The analysis of crude protein in feed samples involved the application of the Kjeldahl nitrogen method. In this procedure, a precisely weighed 0.25g sample was transferred to a Kjeldahl flask, to which a teaspoon of digestion mixer (referred to as a catalyst mixture) and 5 ml of concentrated H_2SO_4 were added. The Kjeldahl flask was then positioned on a digestion apparatus and heated until the solution became clear. Following this, the solution was allowed to cool. Subsequently, a series of 250ml conical flasks were each treated with 25 ml of a 4% boric acid solution and placed on a distillation apparatus. To each flask, 5 ml of distilled water was added. The conical flask and Kjeldahl tube were then attached to the distillation unit and preheated. Distillation was continued until 100ml of a solution containing boric acid and ammonia was obtained. The flask containing boric acid was titrated with 0.1N H_2SO_4.

The determination of crude lipid content in feed samples employed a Soxtherm apparatus. Initially, 1 g of the dry sample was precisely weighed and placed in an asbestos thimble. Subsequently, fat-extracting beakers were meticulously cleaned and weighed. To these beakers, 310 ml of acetone was added after ensuring their thorough drying. The beakers, along with the sample tube, were affixed to the fat-extraction apparatus and heated for a duration of 4-5 hours at a temperature of 60 °C. Following the extraction process, the thimble containing the extracted sample was removed, and the fat-extracting beakers were transferred to a vacuum oven set at 80 °C. The beakers were then re-

weighed. The proximate compositions of the experimental feeds were computed using the corresponding formula, as detailed below.

Moisture (M, %) = (sample fresh weight with crucible - crucible weight) - (sample dry weight with crucible - crucible weight) / sample fresh weight x 100

Dry Matter (DM, %) = 100 - moisture %

Crude Protein (CP, %) = (burette reading, ml x 0.1 normality of H2SO4 x 8.75) / (weight of sample x dry matter, %)

Crude Fat (CF, %) = (weight of fat / weight of dry sample x dry matter) x 100

3.6 Growth Performance and Physical Indices

In order to assess the growth performance of juvenile Australian red claw crayfish under various feeding regimens (pelleted feed and trash fish), the physical indices of the crayfish in this investigation were calculated as follows:

Weight gain (WG, g) = final weight (g) - initial weight (g)

Average daily weight gain (ADWG, g) = body weight gain (g) / number of days

Specific growth rate of weight (SGRw, %.day-1) = 100 x [ln final weight (g) - ln initial weight (g)] / days of the experiment (d)

Length gain (LG, mm) = final length (mm) - initial length (mm)

Average daily length gain (ADLG, mm) = body length gain (g) / number of days

Specific growth rate of length (SGRw, %.day-1) = 100 x [ln final length (mm) - ln initial length (mm)] / days of the experiment (d)

Survival rate (SR, %) = 100 x final number of crayfish / initial number of crayfish

Feed conversion ratio (FCR) = feed intake (g) / weight gain (g)

3.7 PCR Intestinal Microbiota Analysis

For DNA extraction, approximately 0.25 mg or 250 µL of sample were homogenised in DNA/RNA shield followed by DNA purification using the Zymobiomics DNA Extraction Kit according to the manufacturer's instructions. For library preparation and sequencing, the bacterial 16S rRNA V3 hypervariable region was amplified from provided gDNA template using the primers 341F:CCTACGGGNGGCWGCAG and 518R:ATTACCGCGGCTGCTGG (Klindworth *et al*., 2013; García-López *et al*., 2020). An additional 5 bases of inline barcode were incorporated at the 5' end of the primers to enable inline barcoding (Glenn *et al*., 2019). Different samples were amplified using different combinations of the forward and reverse inline primers. PCR was performed using SolarBio PCR mastermix (SolarBio, China) using the following PCR amplification steps: denaturing stage (95°C for 3 minutes followed by 30 cycles of 95°C for 15 s), annealing stage (50°C for 20s) and extending stage (72°C for 10s). The barcoded amplicons were subsequently visualised on gel, normalised and pooled based on their intensity and purified with 0.8 X vol of SPRI bead. The purified pooled amplicons were subsequently processed with the NEB Ultra II Library preparation kit to incorporate Illumina adapter and dual-index barcodes. The constructed library was quantified with Denovix high sensitivity assay and sequenced on a NovaSEQ6000 (Illumina, San Diego) for 2 x 150 paired-end sequencing.

3.8 Intestinal Histological Examination

Subsequently, the harvested intestines underwent fixation in Davidson's solution for a 24-hour period, following standard histological procedures. Subsequent to fixation, 4 μm thick tissue slices were acquired using a microtome, and half of these sections were stained with Haematoxylin and Eosin (H&E) for examination under an Image Analyser. The remaining tissue slices were subjected to staining with Periodic Acid-Schiff (PAS) and Alcian blue pH 2.5 (AB 2.5) reagents, facilitating the visualisation and enumeration of goblet cell presence. PAS imparts a magenta colour to neutral mucins, whereas AB 2.5 is used to dye acidic mucins blue. (Yamabayashi, 1987) The intestinal histological indices, including villus height (VH), villus width (VW), crypt depth (CD), lamina propria thickness (LPT), tunica muscularis thickness (TMT), and goblet cell number (GC) in both experimental groups, were meticulously analysed and quantified using the Image Processing and Analysis in Java (Image-J) software. Figure 3 illustrates the measurement of the intestinal histological indices under various staining techniques. Furthermore, the villus height to crypt depth ratio (VC) and villus surface area (VSA) were calculated in this study, as shown below.

Villus height to crypt depth ratio (VC) = villus height (μm) / crypt depth (μm)

Villus surface area (VSA, μm²) = villus height (μm) x villus width (μm)

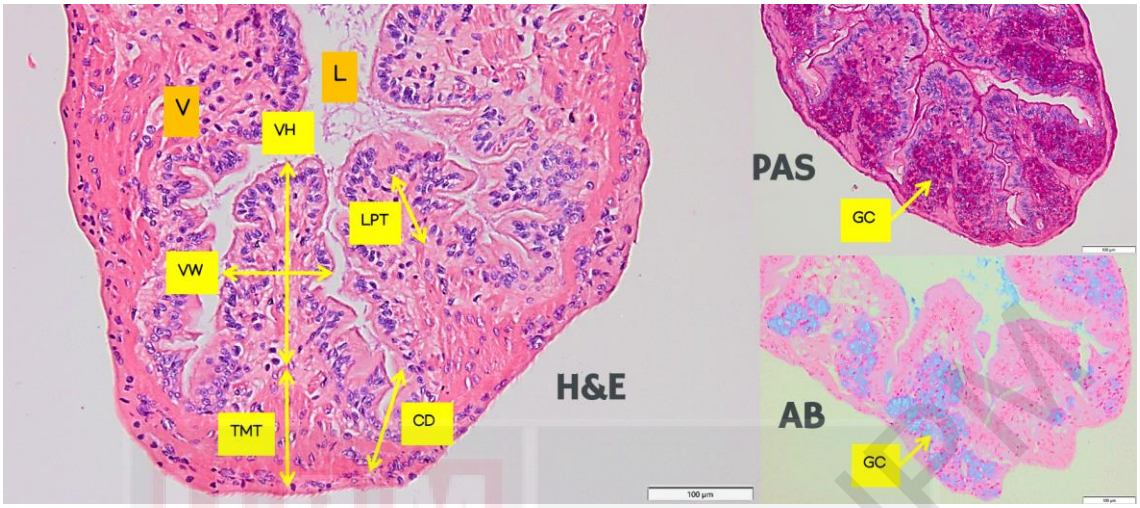


Figure 3. Intestinal histological indices of juvenile Australian red claw crayfish under different staining; H&E: Haematoxylin and Eosin; PAS: Periodic Acid-Schiff; AB: Alcian blue pH 2.5. Scale bar: 100 μm. Uppercase highlighted letters indicate labelling of intestinal structures; L: lumen; V: villus; VH: villus height; VW: villus width; CD: crypt depth; LPT: lamina propria thickness; TMT: tunica muscularis thickness; GC: goblet cell number.

3.9 Statistical Analysis

Test data were processed in Microsoft Excel 2021, and then statistical analysis were performed using IBM SPSS Statistics 26 for growth performance and intestinal histomorphological changes. The significance of difference between two groups was analysed by Independent T-test for parametric data and Mann-Whitney U-test for non-parametric data. All data are presented as mean ± SD.

For gut microbiome analysis, paired-end reads were overlapped using fastp v0.21 (Chen *et al*., 2018). Demultiplexing and primer removal of the merged reads used Cutadapt v1.18 (Martin 2011). The demultiplexed and trimmed reads were imported into QIIME2 v.2021.4 (Bolyen *et al*., 2019) and subsequently denoised with dada2 (Callahan *et al*., 2016). Taxonomic assignment of the ASV used q2-feature-classifier (Bokulich *et al*.,

2018) trained on the latest GTDB release (R07-RS207) 16S rRNA database (trimmed to only retain the V3 hypervariable region) that comprises 311,480 bacterial and 6,062 archeal genomes (Parks *et al*., 2020). ASVs with taxonomic assignments at least to the phylum level were selected for subsequent analysis. Both ASV table and taxonomic classification table were exported using QIIME2 tools into tab-separated values (tsv format) and manually formatted to generate MicrobiomeAnalyst-compatible input (Chong *et al*., 2020) that can perform SparCC co-occurrence network construction (Friedman and Alm 2012) and statistical analysis using the linear discriminant analysis (LDA) effect size (LEfSe) method (Segata *et al*., 2011). Alpha- and beta-diversity was calculated using the QIIME2 plug-ins. The filtered relative abundance table was also used as the input to generate Krona plots for intuitive exploration of relative abundances within the hierarchies of taxonomic classifications (Ondov, Bergman, and Phillippy 2011). PiCRUSt2 analysis was also carried out to predict the function of each ASV that can be subsequently visualised and analysed statistically in the STAMP software (Douglas *et al*., 2020; Parks *et al*., 2014).

4.0 Results

4.1 Feed Proximate Composition

The experimental diets for juvenile Australian red claw crayfish encompassed pelleted feed (TIGER prawn feed size TT663) as the control and trash fish, specifically rainbow sardine (*Dussumieria acuta*), as the treatment. The compositional analysis of both the pellets and the tissues of the trash fish included assessments of moisture, dry matter, crude protein, and crude fat (AOAC, 2005). Notably, the trash fish tissue exhibited a higher moisture content (80%) compared to the pelleted feed (11%), whereas the dry matter composition showed an inverse relationship between the two groups. The pelleted feed displayed elevated levels of crude protein (40%) and crude fat (5%) in contrast to the trash fish's lower values of crude protein (12%) and crude fat (1%), indicating superior nutritional content in the pelleted feed. A comprehensive breakdown of the proximate composition details for the two experimental feeds is provided in Table 1, with values presented as percentages.

Table 1. Feed proximate composition. Values are percentages.

Proximate composition (%)	TIGER Prawn Feed	Rainbow Sardine
Moisture	11	80
Dry matter (DM)	89	20
Crude protein	40	12
Crude Fat	5	1

4.2 Water Quality Parameters

No discernible distinctions were evident in the water quality parameters between the two feeding diets of pelleted feed and trash fish for juvenile Australian red claw crayfish. These parameters encompassed temperature, dissolved oxygen, total dissolved solids, salinity, pH, ammoniacal nitrogen, ammonia, nitrite, and nitrate. All recorded values for these water quality parameters fell within the recommended range for optimal Australian red claw crayfish aquaculture conditions (Jones, 2000). The details of the water quality parameters are provided in Table 2, with the data values presented as ranges.

Table 2. Water quality parameters. Values are range.

Water quality parameters	Pelleted feed	Trash fish
Temperature (°C)	25.70 - 27.70	25.70 - 27.70
Dissolved Oxygen (mg/L)	4.26 - 6.12	4.27 - 6.12
Total Dissolved Solid (mg/L)	104.46 - 249.15	104.46 - 283.14
Salinity (ppt)	0.08 - 0.18	0.08 - 0.21
pH	7.90 - 8.08	7.81 - 8.02
Ammoniacal Nitrogen (mg/L)	0.07 - 0.35	0.07 - 0.19
Ammonia (ppm)	0.00 - 0.25	0.00 - 0.25
Nitrite (ppm)	0.00 - 0.25	0.00 - 0.25
Nitrate (ppm)	10.00 - 20.00	20.00 - 40.00

4.3 Growth Performance

Juvenile Australian red claw crayfish subjected to the pelleted feed exhibited a significantly higher survival rate (SR) compared to those receiving trash fish. However, no statistically significant disparities were observed in terms of weight gain (WG), average daily weight gain (ADWG), specific growth rate of weight (SGRW), length gain (LG), average daily length gain (ADLG), specific growth rate of length (SGRL), and feed conversion rate (FCR) between the two experimental diets. The physical indices are detailed in Table 3, with data values presented as mean ± SD.

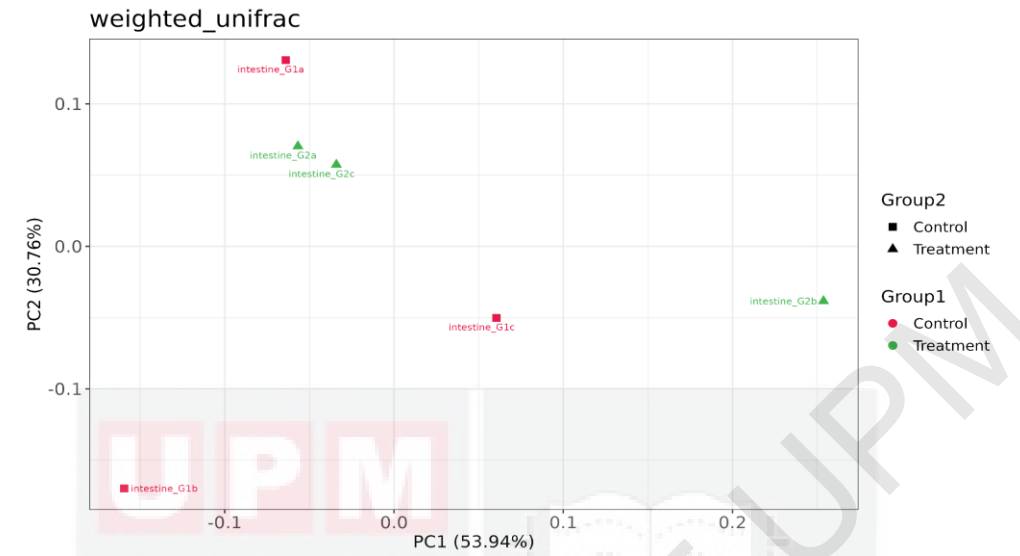
Table 3. Measurement of growth physical indices of juvenile Australian red claw crayfish under different feeding diets: pelleted feed and trash fish. Values are mean ± SD. Values with asterisk in the same row indicate a significant different (*,*; $p < 0.05$).

Physical Indices	Pelleted feed	Trash fish
Weight Gain (g)	1.00 ± 1.13	0.88 ± 1.60
Average Daily Weight Gain (g)	0.40 ± 0.40	0.30 ± 0.60
Specific Growth Rate of Weight (% / day)	0.95 ± 0.93	0.70 ± 1.24
Length Gain (mm)	4.75 ± 4.79	2.40 ± 7.00
Average Daily Length Gain (mm)	0.17 ± 0.17	0.09 ± 0.25
Specific Growth Rate of Length (% / day)	0.32 ± 0.30	0.14 ± 0.46
Survival Rate (%)	1.00 ± 0.00*	0.75 ± 0.44*
Feed Conversion Ratio	4.32 ± 1.47	4.68 ± 1.23

4.4 Intestinal Microbiota

Juvenile Australian red claw crayfish subjected to a diet comprising pelleted feed, designated as the control group, exhibited greater microbial species richness and diversity in comparison to counterparts fed trash fish, serving as the treatment group. The discernible variation in microbial composition between the two dietary groups was highlighted through beta diversity, as evidenced by the Principal Coordinate Analysis plot. Distances between subjects were determined using both the weighted and unweighted UniFrac metrics (Fig. 4a & 4b). The trash fish group displayed a more closely clustered arrangement of samples, suggesting a similarity in the microbial composition within the intestines of Juvenile Australian red claw crayfish fed this alternative diet. The microbial species richness and diversity are detailed in Figure 4.

(a)



(b)

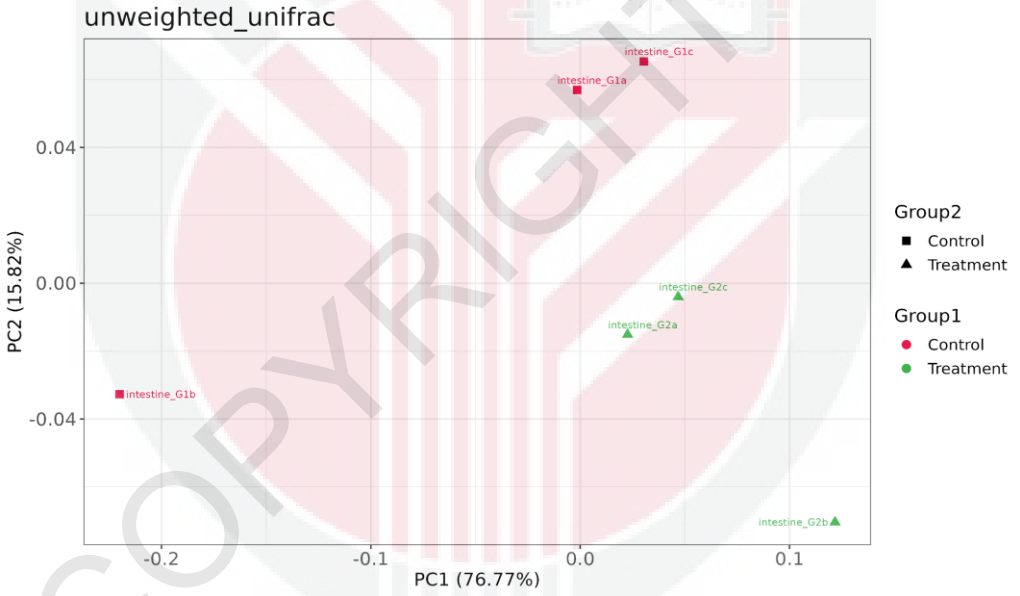


Figure 4: Beta diversity represented by the Principal Coordinate Analysis plot with distances between subjects determined by: (a) weighted UniFrac metric; (b) unweighted UniFrac metric. Each plot point represents a single sample and was coloured and shaped by group according to the legend. Samples that clustered close together are more likely to share the similar microbial composition.

Based on the proportional representation of taxa, the pelleted feed group exhibited three dominant phyla, including Proteobacteria (50.57%) Firmicutes (28.09%), and Fusobacteriota (10.70%) accounted for 99.99% of the total phylotypes (Fig. 5a). Likewise, the trash fish group demonstrated Proteobacteria (63.72%), Firmicutes (14.9%), and Fusobacteriota (10.79%), collectively accounting for 100% of all phylotypes (Fig. 5a).

At the class level, the pelleted feed group featured Gammaproteobacteria (37.52%), Bacilli (24.97%), and Alphaproteobacteria (13.05%) as the top three taxa, while the trash fish group exhibited Gammaproteobacteria (50.05%), Bacilli (14.43%), and Alphaproteobacteria (13.67%) (Fig. 5a).

Moving to the order level, Pseudomonadales (23.37%), Mycoplasmatales (14.42%), and Fusobacteriia (10.70%) were the principal taxa in the pelleted feed group, whereas Pseudomonadales (20.55%), Mycoplasmatales (10.87%), and Fusobacteriia (10.79%) were prominent in the trash fish group (Fig. 5b).

At the family level, dominant taxa in the pelleted feed group included Pseudomonadaceae (21.34%), Fusobacteriia (10.70%), and Hepatoplasmataceae (9.04%), while the trash fish group featured Pseudomonadaceae (15.57%), Fusobacteriia (10.79%), and Rhodobacteraceae (10.47%) (Fig. 5c).

Moving to the genus level, *Pseudomonadaceae* (19.46%), *Fusobacteriia* (10.70%) and *Hepatoplasma* (9.04%) were the top 3 bacterial taxa in the pelleted feed group, and *Pseudomonadaceae* (14.89%), *Fusobacteriia* (10.79%), *Arenimonas* (9.69%) and *Vibrio* (9.09%) were the dominant bacterial taxa in the trash fish group (Fig. 5d).

At species level, the dominant taxa were *Pseudomonadaceae* (19.46%), *Fusobacteriia* (10.70%) and *Hepatoplasma crinochetorum* (9.04%) in the pelleted feed group, and *Pseudomonadaceae* (19.46%), *Fusobacteriia* (10.70%), *Arenimonas maotaiensis* (9.69%) and *Vibrio* (9.10%) in the trash fish group (Fig. 5e).

Concurrently, the outcomes revealed distinct prevailing bacterial communities in juvenile Australian red claw crayfish that were nourished with pelleted feed as opposed to those receiving trash fish as their dietary source.

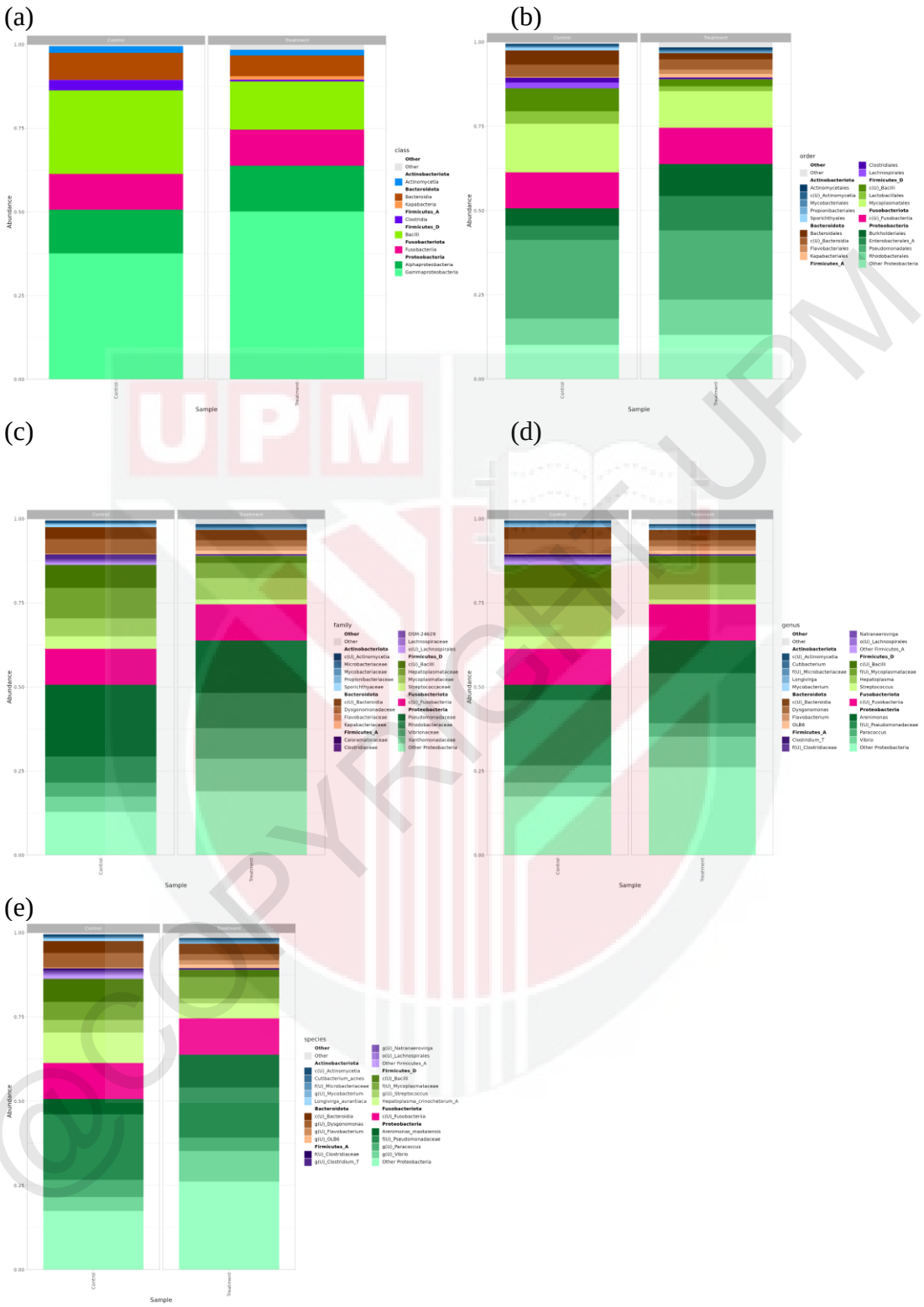


Figure 5: Barplot representing the relative abundance (proportion) of the most frequent bacterial: (a) class; (b) order; (c) family; (d) genus; (e) species. Each bar represents the merged and average relative abundance of each group.

4.5 Intestinal Histology

Crayfish fed pelleted feed exhibited significantly elevated villus height (VH), villus height to crypt depth ratio (VC), and villus surface area (VSA) compared to those fed trash fish. However, the differences were not statistically significant in crypt depth (CD), villus width (VW), and tunica muscularis thickness (TMT). In contrast to the trash fish group, crayfish fed the pelleted feed demonstrated a significantly lower lamina propria thickness (LPT). Furthermore, the goblet cell numbers, observed through both Periodic Acid-Schiff (PAS) and Alcian blue pH 2.5 (AB 2.5) staining, were significantly higher in crayfish fed the pelleted feed compared to those fed trash fish. The results of the histomorphological analysis of the crayfish intestine under different diets are presented in Table 4 and Figure 7, with data values expressed as mean ± SD.

Table 4. Measurement of intestinal histological indices of juvenile Australian red claw crayfish under different feeding diets: pelleted feed and trash fish. Values are mean ± SD. Values with asterisk in the same row indicate a significant different (*,*; $p < 0.05$).

Histological indices	Pelleted feed	Trash fish
Villus Height (μm)	224.69 ± 10.75*	190.28 ± 20.05*
Crypt Depth (μm)	67.59 ± 6.94	82.64 ± 18.94
Villus Height to Crypt Depth Ratio	3.36 ± 0.42*	2.43 ± 0.72*
Villus Width	204.08 ±18.67	179.76 ± 17.34
Villus Surface Area (μm²)	45759.15 ± 3428.87*	33968.04 ± 2275.90*
Lamina Propria Thickness (μm)	33.16 ± 4.55*	62.87 ± 8.33*
Tunica Muscularis Thickness (μm)	72.08 ± 4.37	64.32 ± 18.06
Goblet cell number, PAS stain	422.00 ± 42.22*	186.00 ± 28.19*
Goblet cell number, AB stain	350.00 ± 144.81*	160.00 ± 42.93*

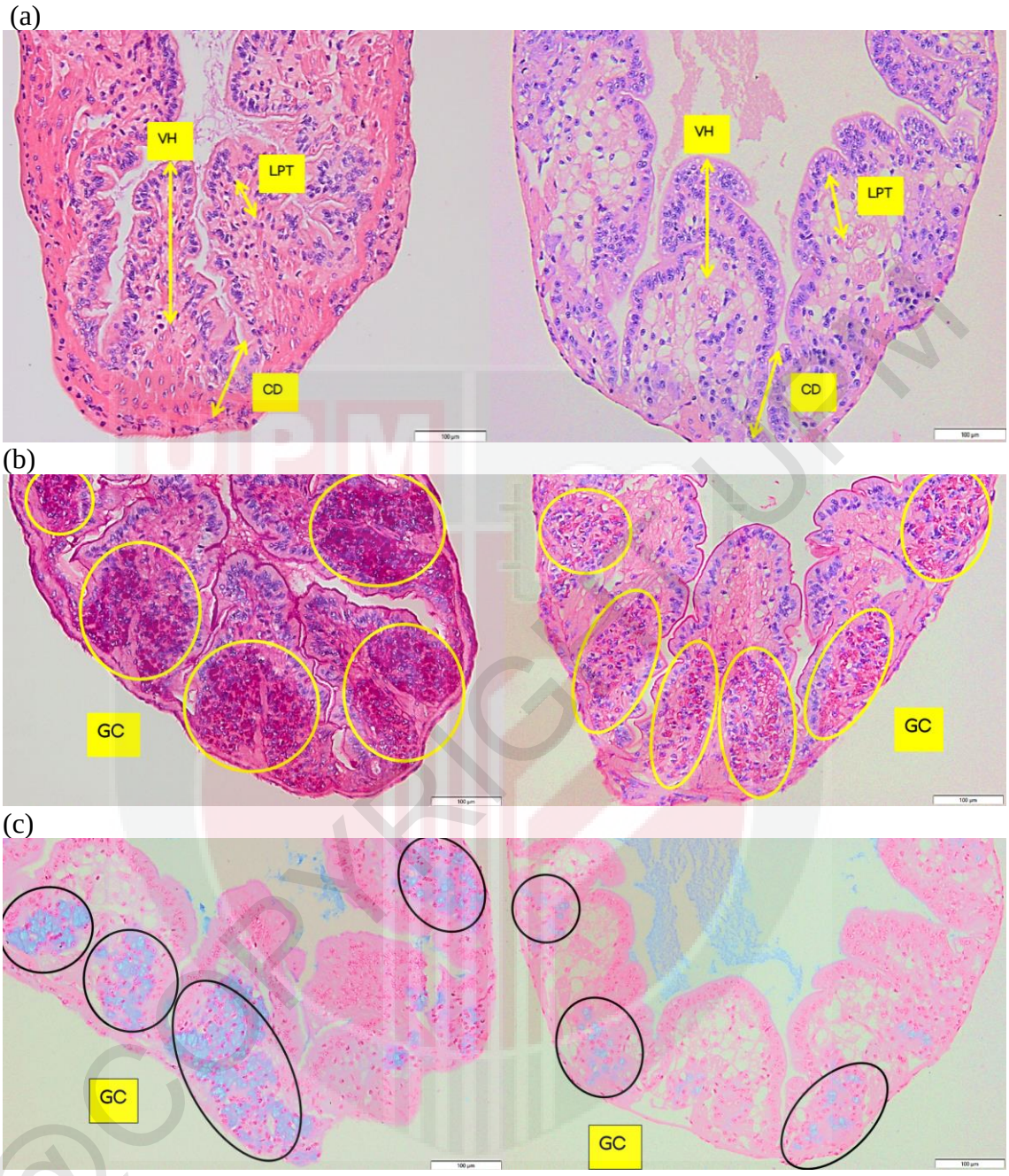


Figure 6. Histological sections of the intestines of juvenile Australian red claw crayfish. Scale bar: 100 µm; left: pelleted feed (control); right: trash fish (treatment); (a) H&E stain (10x); VH: villus height; CD: crypt depth; LPT: lamina propria thickness; (b) PAS stain (10x); yellow circle: goblet cell stained magenta; GC: goblet cell number; (c) AB stain (10x); black circle: goblet cell stained blue; GC: goblet cell number.

5.0 DISCUSSION

Current aquaculture feeding practices involve utilising conventional feed sources like trash fish and commercially formulated pelleted feed tailored to meet species-specific nutritional requirements (Hasan & Halwart, 2009). Trash fish, owing to its cost-effectiveness and widespread availability, is commonly employed as a protein source in aquaculture (Edwards *et al*., 2004). Nevertheless, criticism has been directed towards the use of trash fish as a feed source due to its potential adverse environmental impact and negative effects on the health and growth of cultured species (Bunlipatanon *et al*., 2012). This study suggests that employing pelleted feed as a dietary source for red claw crayfish may positively influence growth performance, gut microbiota, and intestinal histology. Such findings hold implications for the advancement of more sustainable and economical feeding strategies in red claw crayfish aquaculture.

First, our findings indicate that pelleted feed exhibits superior proximate composition in terms of nutritional value compared to trash fish, with particular emphasis on elevated levels of crude protein and crude fat. The nutritional quality of the diet plays a pivotal role in influencing the growth performance of the juvenile Australian red claw crayfish (Wan *et al*., 2022). However, it is noteworthy that our study incorporated meticulous control measures, including formulated and standardised variables such as feed ingredient composition, nutrient weight, and feeding duration. These controls were implemented to ensure a fair and unbiased assessment of growth performance outcomes (Han *et al*., 2021). Specifically, the formulated nature of the pelleted feed allowed for a more precise regulation of essential nutrients, contributing to its enhanced nutritional profile (Shapawi *et al*., 2011). The higher levels of crude protein and crude fat in the pelleted feed suggest

that it serves as a more nutritionally comprehensive dietary source, fostering optimal conditions for growth in the juvenile Australian red claw crayfish (Jones, 2000). Contrastingly, our results indicate that feeding with trash fish did not significantly influence the growth performance of the juvenile Australian red claw crayfish. This outcome underscores the importance of discerning the nutritional disparities between alternative dietary sources. While trash fish may be a common and accessible feed option (Bunlipatanon *et al*., 2012), our study suggests that its nutritional composition may not be as conducive to the growth of the juvenile Australian red claw crayfish. The bioavailability of protein in trash fish is influenced by several factors, including the types and amino acid profiles of proteins present, the presence of antinutritional factors that may hinder nutrient absorption, and the crucial aspect of digestibility influenced by factors such as the fish's age, species, and processing methods (Gasco *et al*., 2018). Additionally, the low dry matter content in trash fish, referring to the portion remaining after water removal, may impact overall nutritional density by potentially diluting essential nutrient concentrations, particularly protein (Gasco *et al*., 2018). The inclusion of processed animal-by-products in formulated feed presents a substantial potential as a protein source in contrast to trash fish (Hasan *et al*., 2009). A notable benefit of utilising formulated feed in the aquaculture sector lies in its capacity for manual formulation and adjustment to meet specific nutritional demands (Hasan *et al*., 2009). Consequently, an optimal diet, such as pelleted feed can be customised, reducing dependence on trash fish and concurrently promoting the diversification of gut microbiota.

Second, our findings demonstrate that key water quality parameters, including temperature, dissolved oxygen, total dissolved solids, salinity, pH, ammoniacal nitrogen, ammonia,

nitrite, and nitrate, were maintained at optimal levels for the thriving of juvenile Australian red claw crayfish, as supported by the works of Jones (2000) and Fatihah *et al*., (2020). This alignment with established optimal ranges attests to the creation of an environment conducive to the growth and well-being of the examined crayfish species. Our research emphasizes that incorporating trash fish into aquaculture feeding practices has no adverse impact on water quality and does not contribute to eutrophication. This is in stark contrast to the conclusions drawn in a prior study (Xu *et al*., 2007), underscoring the environmentally responsible aspects associated with the utilization of trash fish in aquaculture. Furthermore, the negative impact of a specific stress-inducing condition on juvenile Australian red claw crayfish is highlighted in another study, which shown the combination of relatively low temperature and high salinity significantly reduces growth, indicating that this particular juxtaposition of factors induces stress in the juvenile Australian red claw crayfish (Prymaczok *et al*., 2012). Understanding and managing such stressors are critical for ensuring optimal conditions for the successful cultivation of the juvenile Australian red claw crayfish. Additionally, the study underscores the importance of adequate nutrition and oxygen availability in determining the growth performance of the juvenile Australian red claw crayfish. Nutrition, as a key component in body metabolism, enhances oxygen intake when properly regulated (Thompson *et al*., 2005). Sufficient oxygen levels mitigate stress conditions, as organisms expend less energy on regulation and adaptation in stressful environments. This, in turn, reduces the need for increased feed intake and helps maintain the growth performance of the juvenile Australian red claw crayfish (Luo *et al*., 2021). Beyond water quality, the influence of population density on growth performance is acknowledged. An increase in population density results in reduced

growth performance due to heightened competition for essential resources, including nutrition, oxygen, space, and opportunities for natural behaviour (Fatihah *et al*., 2020). The intensified competition for basic needs creates a stressful environment, rendering the juvenile Australian red claw crayfish population more susceptible to adverse conditions (Viau & Rodríguez, 2010).

Third, the assessment of growth performance stands as a crucial parameter in evaluating the effectiveness of various feeding regimes for juvenile Australian red claw crayfish. Common indicators used to assess growth performance include weight gain (WG), average daily weight gain (ADWG), specific growth rate of weight (SGRW), length gain (LG), average daily length gain (ADLG), specific growth rate of length (SGRL), feed conversion rate (FCR), and survival rate (SR) (Wan *et al*., 2022). Our study focused on comparing the growth performance of crayfish fed pelleted feed and trash fish, and the results demonstrate that the utilisation of pelleted feed yielded superior growth rates compared to trash fish, with survival rate being the only parameter showing a significant difference. Various factors such as animal species, age, and feed compositions can influence growth performance responses (Luo *et al*., 2021). Additionally, multiple factors, including seasonal effects, nutrition availability, population density, age, and species, can collectively influence the growth performance of the juvenile Australian red claw crayfish (Luo *et al*., 2021). Notably, our findings revealed a significantly higher survival rate in crayfish fed pelleted feed, achieving 100% survivability, while crayfish fed trash fish experienced increased mortality rates, attributed to cannibalism during moulting and a preference for meat-based diets (Thompson *et al*., 2005). Cannibalism, a prevalent cause of low survival in aquaculture species, especially during the juvenile or larvae stage, poses

a significant challenge to the industry (Fatihah *et al*., 2020). Previous studies have highlighted improved survival rates in juvenile crustaceans with a diet of artificially formulated pellets, emphasising the importance of satiety in reducing cannibalistic tendencies (Feng *et al*., 2021; Wan *et al*., 2022). Furthermore, crayfish fed pelleted feed exhibited a lower feed conversion ratio (FCR) compared to those fed trash fish, indicating higher feed digestibility, better nutrient utilisation, and more gut nutrient contents (Han *et al*., 2021). Calculating FCR helps assess the efficiency of feed utilisation, reinforcing the idea that using trash fish as a feed source may lead to the wastage of high-cost feed and financial resources (Shapawi *et al*., 2011). Moreover, crayfish fed pelleted feed demonstrated higher values across various growth performance parameters, including weight gain, average daily weight gain, specific growth rate of weight, length gain, average daily length gain, and specific growth rate of length compared to crayfish fed trash fish. These findings underscore the significance of tailoring feed strategies to different growth stages to optimise growth, size uniformity, and overall productivity while minimising feed costs and waste (Jones, 2000). The observed lack of linkage between feeding trash fish and the overall growth performance, except for survival rate, suggests a nuanced relationship that warrants further investigation for a holistic understanding of the feeding dynamics in crayfish aquaculture.

Forth, the composition of the intestinal microbiota in juvenile Australian red claw crayfish is subject to modification based on dietary factors, particularly in relation to the choice between pelleted feed and trash fish. In this investigation, it was observed that the utilisation of pelleted feed resulted in a heightened microbial species richness and diversity in comparison to trash fish. The predominant phyla identified in both groups were

Proteobacteria, Firmicutes, and Fusobacteriota, with pelleted feed demonstrating a superior relative abundance. Feeding on formulated feed not only fosters the stimulation of innate immunity but also mitigates the risk of infections, as evidenced by previous research (Egerton *et al*., 2018). The heightened diversity of gut bacteria is of paramount importance as it enhances nutrient digestion through the enzymatic breakdown facilitated by microbes. This enzymatic breakdown transforms complex organic matter into simpler forms, thereby optimising digestibility performance (Huang *et al*., 2020). Physiologically, the adoption of formulated feed plays a pivotal role in maintaining the health of the gastrointestinal tract, thereby contributing significantly to both nutrition and immunity development (Egerton *et al*., 2018). Additionally, the use of formulated feed minimises the risk of infective diseases such as Vibriosis (Zhang *et al*., 2020). Conversely, trash fish, when employed as a dietary component, significantly alters the composition of gut bacteria. Trash fish, notorious for its association with *Vibrio cholera*, poses a potential pathway for gut infections, a phenomenon particularly relevant to Vibrio harveyi, which has broad-spectrum implications for host health and immunosuppression, leading to diseases or infections (Zhang *et al*., 2020). Trash fish, acting as a carrier of bacteria and viruses such as streptococcosis, *Iridovirus*, and *Betanodavirus*, may contribute to the transmission of pathogens (Kim *et al*., 2007). This underscores the importance of carefully considering the dietary components in the context of promoting optimal gut health in juvenile Australian red claw crayfish.

Noteworthy is the consistent finding that different feeds can differentially affect intestinal microbes in crustaceans (Chen *et al*., 2021). Our study further corroborates this by revealing that Proteobacteria is the dominant phylum in the intestines of juvenile Australian

red claw crayfish, and its relative abundance decreases with pelleted feed, aligning with previous crustacean studies (Zhang *et al*., 2014; Sun *et al*., 2018). Sun *et al.,* (2018) postulated that an increase in Proteobacteria abundance may correlate with poorer growth performance in crustaceans, thereby emphasising the potential impact of dietary choices on overall crayfish health. Our results also highlight Firmicutes as another dominant phylum positively influenced by pelleted feed. Reports in the literature consistently identify Firmicutes and Fusobacteria as prevalent members in crustaceans (Zhang *et al*., 2020; Liu *et al*., 2021). Fusobacteria's capacity to produce butyric acid is particularly noteworthy, as it regulates transepithelial transport, reinforces the mucosal barrier, enhances oxidative and inflammatory status, and inhibits colon carcinoma (Sossai, 2012). Furthermore, Firmicutes play a pivotal role in carbohydrate breakdown through fermentation, providing additional benefits in digestion and immune status (Liu *et al*., 2020). Probiotics within Firmicutes, such as *Lactobacillus*, *Enterococcus*, and *Bacillus*, can counteract the effects of pathogenic bacteria, contributing to improved digestion and immune health (Costantini *et al*., 2017). Consequently, a higher abundance of Firmicutes, as observed in crayfish fed pelleted feed, may confer significant benefits to intestinal health. Similar trends have been noted in other aquatic species, such as juvenile hybrid grouper, where formulated diet groups exhibited greater relative Firmicutes abundance compared to those fed chilled trash fish (Ye *et al*., 2020). These findings collectively underscore the nuanced relationship between dietary choices, gut microbiota, and the overall health of juvenile Australian red claw crayfish.

Last, this research employed various histological indices to evaluate the intestinal health of juvenile Australian red claw crayfish under distinct feeding regimes. These indices encompassed villus height (VH), crypt depth (CD), villus height to crypt depth ratio (VC), villus width (VW), villus surface area (VSA), lamina propria thickness (LPT), and tunica muscularis thickness (TMT), analysed using three histological stains, such as H&E, PAS, and AB 2.5 (Deplancke & Gaskins, 2001; Chen *et al*., 2020; Wan *et al*., 2022). H&E staining was employed to examine the histological structure of the intestinal villi, facilitating the distinct identification of each structure through a purple-pink coloration (Fischer *et al*., 2008). PAS and AB 2.5 stains were utilised to evaluate the presence of goblet cells, with PAS exhibiting a magenta colour and AB 2.5 indicating a blue colour (Yamabayashi, 1987). PAS is particularly effective in detecting polysaccharides and mucosubstances in tissues, while AB 2.5 visualises acidic epithelial and connective tissue mucins (Yamabayashi, 1987). Our findings reveal that the pelleted feed group exhibited superior intestinal histology compared to those fed trash fish. These histological improvements likely contribute to the observed superior growth performance in the pelleted feed group." Specifically, pelleted feed resulted in a significantly higher villus height, indicative of robust intestinal health and improved nutrient absorption, which leads to increasing in average daily weight gain (Wan *et al*., 2022). Villus height correlates directly with the enteric and absorptive capacity of the intestine (Nordi *et al*., 2013). Additionally, the pelleted feed group displayed a lower crypt depth, yielding a higher villus height to crypt depth ratio, underscoring the favourable health condition of the intestine and an increased presence of beneficial lactic acid bacteria, such as lactobacillus that lower the feed conversion ratio (Yaqoob *et al*., 2021). Moreover, the pelleted feed group

showcased a higher villus width, contributing to an expanded villus surface area. This increased surface area enhances the efficiency of nutrient absorption, which leads to higher specific growth rate in terms of weight and length (Chen *et al*., 2020). Conversely, the trash fish group exhibited a thicker lamina propria, potentially signalling inflammation, nutritional stress, reduced digestibility, and diminished nutrient absorption (Zhang *et al*., 2020). Nutritional stress has been linked to extended lamina propria in shrimp intestines (Chen *et al*., 2020). Notably, the pelleted feed group demonstrated a higher tunica muscularis thickness, implying that the intestinal morphology became thicker due to an augmented surface area. This enhancement likely improves nutrient absorption efficiency and improve peristalsis, aligning with findings in other studies (Chen *et al*., 2020). Additionally, the pelleted feed group manifested a significantly higher goblet cell number, observed in both PAS and AB 2.5 staining. Goblet cells play a pivotal role in maintaining intestinal health by secreting mucins, forming a protective mucus layer that acts as a mechanical, immune, and biological barrier (Deplancke & Gaskins, 2001). This layer aids in resisting the invasion of foreign bacteria, regulating microbial immune responses, and contributing to intestinal lubrication and protection, particularly crucial in aquatic animal digestion (Corfield *et al*., 2001; Berillis & Mente, 2017). Our thorough histological assessment indicates that juvenile Australian red claw crayfish fed pelleted feed exhibit superior intestinal health and histological parameters compared to those fed trash fish. These findings underscore the substantial influence of diet on the morphological and functional aspects of the intestine, with potential implications for overall health, digestion, and nutrient absorption in crustaceans.

6.0 CONCLUSION

In summary, the growth performance of juvenile Australian red claw crayfish appears unaffected by various feeding regimens, with the exception of their survival rate. Crayfish fed with pelleted feed exhibited significantly higher survivability, improved intestinal health, and a more substantial microbial population when compared to those fed with trash fish. These results emphasise the importance of customised nutrition in aquaculture, advocating for the adoption of formulated diets, such as pelleted feed, to enhance crayfish production.

7.0 RECOMMENDATION

As a recommendation, we propose further exploration through additional studies encompassing Australian red claw crayfish (*Cherax quadricarinatus*) and other crustacean species, including whiteleg shrimp (*Litopenaeus vannamei*), tiger shrimp (*Penaeus monodon*), Giant mud crab (*Scylla serrata*), and other locally promising aquaculture species. Additionally, future research could concentrate on other vital organs, such as the hepatopancreas, which plays a crucial role in nutrient storage and energy metabolism in crustaceans. Ensuring optimal water quality parameters is also crucial to prevent environmental stress on the specimens. Furthermore, extending the study period and increasing the sample size could yield more comprehensive data and results, thereby enhancing the accuracy and robustness of the findings.

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