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The potential use of prodigiosin as a shrimp feed additive and its dynamic influence on the shrimp gut microbial community – an *in vitro* gut model

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ABSTRACT

Aims: A simple *in vitro* model system was applied in this study assessing the dynamics of the microbial community associated with the shrimp gut system to understand the changes that influence dietary variables.
Methodology and results: The diversity and abundance of microbiome were monitored within two different treatment slurries inoculated with shrimp faecal samples as to mimic the effect of diet manipulation, and 16S rRNA gene of MiSeq Illumina-based sequencing was applied. The different diets tested were a commercial standard diet and a prodigiosin added diet. There was very clear separation between the commercial standard diet and prodigiosin added diet as revealed by the total viable counts (TVC) and sequencing data. It suggested that the microbial community of the shrimp gut system exhibited a dynamic response with the treatments and allochthonous bacterial present. The prodigiosin added diet was clearly separated from the commercial standard diet serving as a potential shrimp feed additive. The sequencing data analysis showed that members of the genera *Vibrio*, *Shigella* and *Photobacterium* became predominant on the commercial standard diet treatment. The prodigiosin-added diet treatments indicated an abundance of members of the genera *Micrococcus*, *Arthrobacter*, and *Shigella*.
Conclusion, significance and impact of study: *In vitro* model system-based testing of diets could be a useful method to determine the potential effect of diet manipulation on shrimp gut system microbiome members.

Keywords: Gut bacteria, microbial community, *in vitro* model system, 16S rRNA gene, prodigiosin

INTRODUCTION

Shrimp health and productivity can potentially improve the understanding of shrimp gut microbiota and how shrimp physiology is influenced by the gut microbiome (Cornejo-Granados *et al.*, 2017). Thus, it leads to better sustainability of the aquaculture industry and improves its productivity. Studies reveal that microorganisms associated with gut systems serve a variety of functions in the health and nutrition of fish by preventing pathogen colonization and promoting nutrient supply by immunomodulation (Nayak, 2010; Sørensen *et al.*, 2011). Usually shrimp gut microbiome and other marine animals such as fish are highly dynamic due to the open monogastric nature of the gut system (Zarkasi *et al.*, 2017). Therefore, microbial communities in the gut system can be changed over a short-term period under antibiotic application (Burridge *et al.*, 2010), environmental factors (Dehler *et al.*, 2017) and diet manipulation (Kongnum and Hongpattarakere, 2012; Luis-Villasenor *et al.*, 2013).

Previous studies determined that gut microbiome were greatly influenced by several factors including reflecting water surface temperature (Neuman *et al.*, 2016; Lau *et al.*, 2018), different geographical locations, ecosystem (Llewellyn *et al.*, 2016; Lyons *et al.*, 2016), weather seasons (Hovda *et al.*, 2012; Neuman *et al.*, 2016), faecal consistency (Zarkasi *et al.*, 2016), diet composition (Ringø *et al.*, 2015) and pollution levels (Khan Chowdhury *et al.*, 2009). Besides, external influences such as changes in salinity have not shown any effect on the gut microbiome community in euryhaline fish nor in shrimps,

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thus gut and seawater microbial communities may not be interconnected (Schmidt *et al.*, 2015). In shrimp farms, microbial communities are dominated by members of the *Vibrionaceae*, *Enterobacteriaceae* and *Pseudomonadaceae* (Xiong *et al.*, 2015; Cornejo-Granados *et al.*, 2017) that also include species commonly found in other marine fauna gut systems.

Gut system microorganism communities show different structural and physical changes when they are observed over time (Zarkasi *et al.*, 2016). This has been suggested by observations that changing feed formulations potentially influence the microbial communities associated with the gut. In addition, the bacterial present in the gut were likely the result of diet components during the general gut system process (Gajardo *et al.*, 2017). The *in vitro* model system has been chosen here deliberately as an uncomplicated basal nutrient medium in which feeds are suspended (Van den Abbeele *et al.*, 2013; Zarkasi *et al.*, 2017) to avoid working in complex biological natural systems. Moreover, *in vitro* model system promotes dynamic sampling over short-time intervals, improves reproducibility and amenability in an ethical sense. The *in vitro* model system is only a preliminary step before it is necessary to do further testing with live animals and could thus possibly be the initial test-bed for assessing new diet formulations in a cost-effective way (Van den Abbeele *et al.*, 2013; Amin *et al.*, 2017).

Generally, farmed shrimp diets contain both fishmeal and fish oil because they contain the required amount of protein and lipids (Sargent and Tacon, 1999; Hulefeld *et al.*, 2018), and different diet compositions have been studied to observe the responses in fish and shrimp growth and microbial community diversity associated with marine animals (Silva *et al.*, 2011; Sørensen *et al.*, 2011; Ringø *et al.*, 2015). In addition, farm management and environmental complexity of current shrimp farming industries provide a great challenge to apply diet formulations and meet expectations of farm-based growth performance under different environmental conditions. Currently, there is not much development concerning the manipulation of shrimp diet formulation compared to fish diets that have been extensively manipulated as reported and considered successful in replacing fishmeal or fish oil with other feed additives (Bakke-McKellep *et al.*, 2007; Glencross, 2009; Molina-Poveda *et al.*, 2013). The diet manipulation and replacement of some diet components have improved farm cost and industry sustainability (Tacon and Metian, 2008).

However, information and research are still lacking on the potential of prodigiosin natural pigments serving as a bacterial inhibitor, and thus further studies need to be conducted. Moreover, previous studies found that these natural pigments contained antimicrobial activities against bacteria that may have the potential to inhibit the growth of certain bacterial species improving the shrimp health and productivity (Ibrahim *et al.*, 2014; Darshan and Manonmani, 2015). According to Ibrahim *et al.* (2014) prodigiosin was found to have an inhibitory effect on several bacteria species such as *Staphylococcus*, *Enterococcus*, *Vibrio* and *Bacillus* that can improve shrimp health. This natural pigment has the potential as a bacterial inhibitor and can be added as a feed additive for better shrimp health and productivity (Darshan and Manonmani, 2015). Although the effect of this change in the microbiome for the fish and shrimp health, further study is needed to better understand of its potential and significance (Bakke-McKellep *et al.*, 2007).

Shrimp bacterial diseases are one of the important issues that affect shrimp health and productivity. Currently, the infection of *Vibrio parahaemolyticus* is the major concerned in shrimp farmed since the bacteria is the causative agent of acute hepatopancreatic necrosis disease (AHPND) across the globe (Soto-Rodriguez *et al.*, 2014). This disease put a major threat for shrimp industry and cause a great loss mounted to million dollars. Current strategies need improvement, since farmers depend on antibiotic application and expensive management strategies to maintain shrimp health (Cornejo-Granados *et al.*, 2017). This practice will eventually lead to antibiotic abuse and increased the production cost. Due to this problem, this study exploring and investigate the suitability of prodigiosin as a means to control shrimp bacterial diseases associated with the shrimp and its gut system. Sustainable approach of carotenoid and secondary metabolites such as prodigiosin, recently shows a good potential to suppress the bacterial pathogens associated with the shrimp (Ibrahim *et al.*, 2014; Darshan and Manonmani, 2015). Prodigiosin extract use in this study is extracted from the marine microbes of *Serratia marcescens* IBRL USM84 isolated from the east coast of Malaysian waters, thus it sustainable for usage against bacterial pathogens in shrimp as well as fish (Ibrahim *et al.*, 2014). It has potential as an antibacterial but remains poorly understood.

This study aims to investigate a simple *in vitro* gut model system to determine how microorganisms associated with the shrimp gut system responded to diet formulations, and whether the microbial community structure varies in a simple system when a different diet formulation is applied. Formulations of diets that may lead to improved shrimp gut health and productivity can develop sustainable shrimp farm industries and improvement of aquaculture industries that serve as a food resource.

MATERIALS AND METHODS

Prodigiosin extraction

The red pigment of prodigiosin produced by the isolate *Serratia marcescens* IBRL USM 84 was extracted and analysed by a method developed by Ibrahim *et al.* (2014). The bacterial isolate of *Serratia marcescens* IBRL USM 84 was isolated from a coral reef in a coastal region of Terengganu, Malaysia. Three milliliters of acetone we added into 1.0 ml of a thick IBRL USM84-cell suspension and agitated at 150 rpm for 30 min at room temperature (30±2 °C). The mixture was centrifuged at 5000 *g* for 20

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min. The red supernatant was mixed with petroleum ether at the ratio of 1:2 in a separating funnel and shaken vigorously until the red pigment was in the petroleum ether. The extracted red coloured petroleum ether solution was evaporated using a rotary evaporator under reduced pressure until the dried red pigment was obtained and used for spectral analysis. Fifty micrograms of dried red pigment were dissolved in 10.0 mL of absolute ethanol and to obtain an acidic condition, 1.0 mL of 1.0 M HCl was added into the solution. To get an alkaline solution, 1.0 mL of 1.0 M NaOH was added and to acquire a neutral condition 1.0 mL of sterile distilled water was added into the solution. Spectral analyses of the pigment in acidic, alkaline and neutral solution were determined using a spectrophotometer (Spectronic Unicam, Genesys 10UV, United State) at 400-600 nm.

Prodigiosin and commercial standard diet

A prodigiosin diet (PD) formulation was prepared including a commercial standard (CS) diet for this study. The prodigiosin diet formulation had prodigiosin added as a feed additive. The general composition of the prodigiosin diet (PD) and standard diet (CS) is shown in Table 1.

Table 1: The composition of diet ingredients and formulations applied in this study.

Diet group	PD	CS
Composition and energy:		
Protein (%)	45	45
Lipid (%)	25	25
Digestible energy (Mj/kg)	18.8	18.8
Protein to digestible energy ratio	23.9	23.9
Ingredients:		
Fishmeal (%)	63.5	63.5
Fish oil (%)	19.1	19.1
Wheat flour (%)	13.8	16.8
Vitamin/minerals premix (%)	0.5	0.5
Yttrium oxide (%)	0.1	0.1
Prodigiosin (%)	3.0	0

Shrimp faecal collection

Shrimp samples were collected during November 2017 from a shrimp farm at Sungai Pasir, Kedah, Malaysia and Balik Pulau, Penang, Malaysia. Samples were collected by randomly seining a group of shrimps (*Penaeus vannamei*) crowding the shrimp in the seine to minimise bias. Shrimp samples were immediately transferred on ice to the laboratory and processed within two hours. The faecal samples were collected from 60 apparently healthy shrimps by squeezing shrimp gut into sterile tubes (Zarkasi *et al.*, 2017).

***In vitro* gut model system**

In vitro gut model system was conducted in three replicates of the prodigiosin diet (PD) and commercial standard diet (CS) shown in Table 1 and a negative control (a sample of the inocula in the medium without feed added). The diets were crushed and suspended at 10 g/L as a slurry in the basal growth medium. The basal growth medium contained the following compounds: $NaHCO_3$, 4 g/L; K_2HPO_4, 0.5 g/L; KH_2PO_4, 0.5 g/L; $MgSO_4 \cdot 7H_2O$, 0.09 g/L; $CaCl_2$, 0.09 g/L; NaCl 30 g/L; resazurin, 0.5 mg/L; hemin, 10 mg/L (MP Biomedicals, Santa Ana, US); and sterile water, 1L (Zarkasi *et al.*, 2017). The faecal samples collected from 10 individual shrimps were pooled with equal contributions per shrimp (Hatje *et al.*, 2014; Neuman *et al.*, 2016). Then samples were homogenised and diluted in the ration 1:2 (wt/vol) in marine broth (Oxoid, Basingstoke, England). Then, 1 mL of faecal slurry was aseptically inoculated into the 1000 ml growth medium and incubated at 30 °C, with mixing periodically performed during incubation. The Anaerogen system produced an atmosphere containing approximately 90:10 N_2:CO_2 with O_2 content reduced below 0.1% within 1 h. The sampling time points were determined and set at time intervals of 0, 3, 6, 12 and 24 h by a prior pH analysis in a trial run where pH was found to decline and stabilise at the 24 h time point, the original inoculum had a pH 10.0. Samples (5 mL) were taken from the three-replicate growth mediums (PD and CS diet) and processed for microbial enumeration and DNA extraction.

DNA extraction and microbial enumeration

DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen Sciences, Germantown, MD, US) following the manufacturer's instruction and standard protocols (Zarkasi *et al.*, 2018). Samples collected from the *in vitro* fermentation at 0, 3, 6, 12 and 24 h were serially diluted using marine broth (Oxoid, Basingstoke, England) and plated onto marine agar (MA), thiosulfate-citrate-bile salts-sucrose (TCBS) agar, Eosin methylene blue (EMB) agar, and De Man-Rogosa-Sharpe (MRS) agar (Oxoid, Basingstoke, England) (Hovda *et al.*, 2007; Zarkasi and Nazari, 2018). The plates were incubated at 30 °C for 24-48 h in order to determine the total viable counts. The plates that possessed between 30 and 300 colonies were counted manually to obtain estimates of bacterial numbers (colony forming units/gram wet weight).

16S rRNA gene of MiSeq Illumina-based sequencing

Sequencing of the 16S rRNA gene amplicon was applied to the 20 samples collected from the *in vitro* model system, to examine the microbial communities present in each of the samples, which were collected at the initial time of 0 h and at 24 h. Sequencing was carried out using the Illumina MiSeq platform. Pair-ended PCR amplification of the 16S rRNA gene V1-V3 region was carried out using 27F and 907R primers that possessed 12 bp barcode tags. FASTQ files generated were merged using PEAR, and were then trimmed to remove the primer, bar code and adapter regions using an internally developed algorithm. The seed sequence for each cluster was then sorted by length and clustered with a 3%

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divergence cut-off to create centroid clusters. Clusters containing only <2 sequences or <100 bp in length were then removed. Seed sequences were again clustered at a 3% divergence level using USearch to confirm whether any additional clusters appeared. Consensus sequences from these clusters were then accurately obtained using UPARSE (Edgar, 2013). Each consensus sequence and its clustered centroid of reads were then analysed to remove chimaeras utilising UCHIME in the de novo mode (Edgar *et al.*, 2011). After chimaera removal, each consensus sequence and its centroid cluster were denoised in UCHIME in which base position quality scores of >30 acted as the denoising criterion. Sequence de-replication and OUT demarcation were further performed in USEARCH and UPARSE to yield OTUs that were aligned using MUSCLE (Edgar, 2004) and FastTree (Price *et al.*, 2010) that infers the most approximate phylogenetic trees. OTUs were then classified using the RDP Classifier (Wang *et al.*, 2007) against the curated GreenGenes 16S rRNA gene database (DeSantis *et al.*, 2006; Hussin *et al.*, 2018).

Statistical analysis

PRIMER6 and PERMANOVA+ (version 6.1.12 and version 1.0.2; Primer-E, Ivybridge, UK) respectively were used for the analysis of similarities (ANOSIM), analysis of variance (ANOVA) (Anderson *et al.*, 2005), and Multidimensional scaling (MDS) (Zarkasi *et al.*, 2019) to assess the influence of prodigiosin as a feed additive on the microbial community compositions. ANOVA was conducted using default settings with 9999 permutations, while MDS was conducted using default settings. The ANOVA derived significance values were considered significant when $P<0.05$, while $0.01<P<0.05$ were considered only marginally significant (Zarkasi *et al.*, 2019).

RESULTS

Growth responses

The pH 10.0 was recorded in the beginning of the experiment that then dropped to a pH 8.5 at the end of this study (data not shown). Bacterial growth on marine agar, TCBS agar, EMB agar, and MRS agar is visualised in Figure 1. Bacterial growth reached log 7.2 CFU g/L within 24 h after 1-3 h adaptation to the lag phase. No growth was observed on the MRS agar and the negative control. According to the TVC results, growth was poorer by approximately 1-2 Log units on the PD diet (Log 3.0-6.5 CFU g/L), while the observable growth was good for the CS diet (Log 3.3-7.2 CFU g/L) (Figure 1). Overall, the TVC progression over time was consistent across the PD and CS diet for marine agar, TCBS agar and EMB agar. For TCBS agar, the TVC numbers were considered too low on the PD diet compared to the CS diet (Log 2.5-4.0 CFU g/L) (Figure 1).

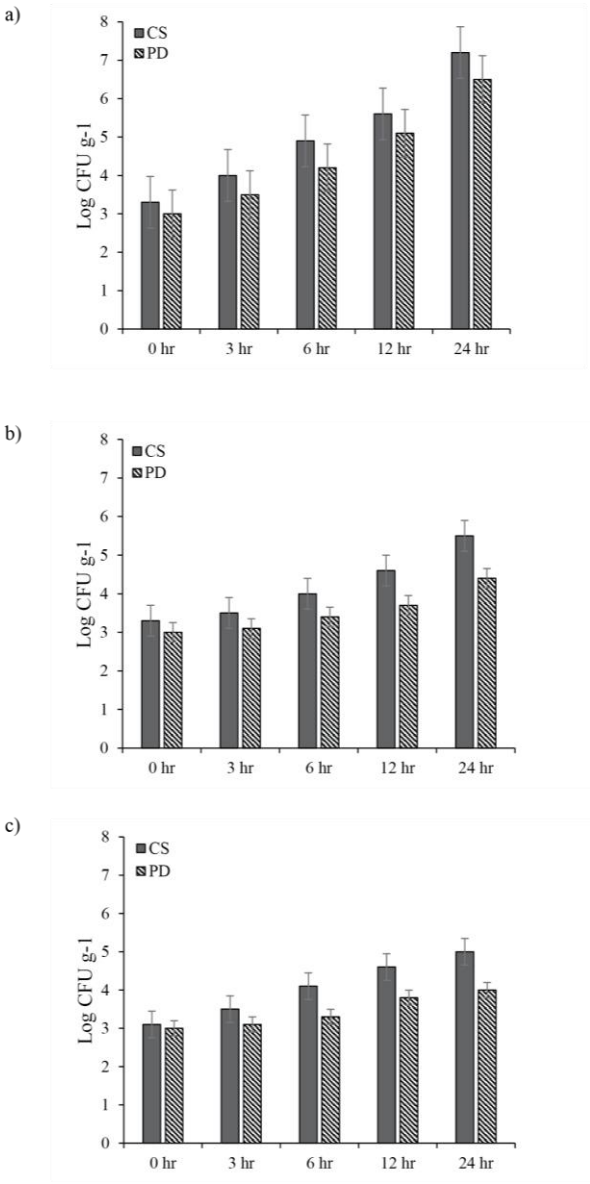

Figure 1: Total viable counts (TVC) in the *in vitro* model system experiment according to the time of sampling. TVC are derived from the colony numbers appearing on a) marine agar, b) TCBS agar and c) EMB agar (see Table 1 for abbreviations). The low TVC is indicative of the dilution effect.

ANOVA analysis

The ANOVA analysis indicated a significant difference which were clearly separated between the PD and CS diets ($P<0.01$) (Table 2). The sampling time factor also indicated a significant difference (0 h vs 24 h, $P<0.01$). However, there was no significant interaction between diet and sampling time factors ($P=0.06$, Table 2) which thus, indicated that bacteria growing within the system

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inevitably became predominant. Further analysis showed a strong separation between PD and CS diets as illustrated in MDS plots (Figure 2) which shows that clustering can be readily correlated on the basis of diets. Results showed that the bacterial community was dynamic and was influenced by the diet formulation and manipulation, and therefore prodigiosin has the potential to become a shrimp and fish feed additive.

Table 2: ANOVA table for comparison of microbial community structure with response to diet and sampling time.

Source	df	MS	*F*	*P*
Diet	1	2052.2	3.4200	0.0001
Sampling time	4	2044.0	2.0570	0.0032
Diet x Sampling time	4	2426.7	1.1327	0.0658
Total	20			

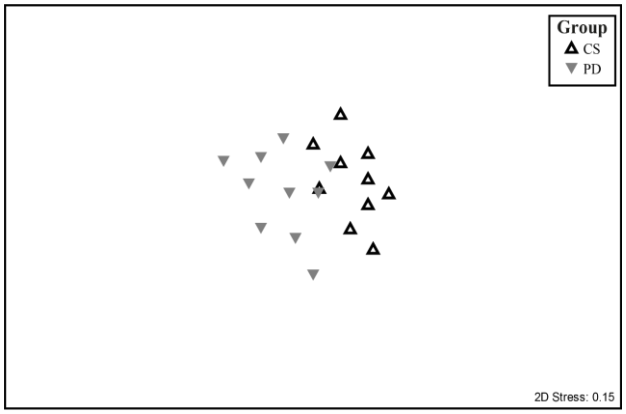

Figure 2: Multidimensional scaling plots showing faecal community similarity on the basis of diet (see Table 1).

Composition of the microbial community grown in the *in vitro* growth model system

In vitro samples from PD and CS diets in three replicates were analysed for bacterial composition by using the 16S rRNA sequencing of MiSeq Illumina-based platform. Results indicated that the inoculum microbial community was diverse but was mainly dominated by members of family of *Micrococcaceae* and *Vibrionaceae* (Figure 3). The inoculum communities also represented those likely associated with the feed itself (Salter *et al.*, 2014). Besides, the presence of certain taxa which are generally considered non-marine microbes' species that are relatively present due to the heavy dilution of faecal materials (cells added < 10^4 CFU g/L) against a feed background. Other bacteria present may have originated from the skin, organ or other parts of the gut microbiome of shrimp (Figure 3).

Following the *in vitro* model system of 24 h fermentation, CS diet samples were dominated by bacterial sequences that were affiliated with the family *Vibrionaceae* making up >65% of total reads (Figure 3). While, PD diet samples were dominated by bacterial sequences affiliated with the family *Micrococcaceae* making up >50% of total reads (Figure 3). In addition, feed-associated bacteria did not grow on the fish shrimp meal-containing diets and represented a very small proportion of reads after 24 h (Figure 3). According to the bacterial sequences identified down to the genus and species level, the CS diet formulation mainly supported the growth of *Vibrio* sp. (making up 46% of reads), *Shigella* sp. (10% of reads), *Photobacterium* sp. (10% of reads), *Enterobacter* sp. (5% of reads), *Micrococcus* sp. (3% of reads) (Figure 3). By comparing the diets, PD differed in containing mainly *Micrococcus* sp. (making up 35% of reads), *Shigella* sp. (14% of reads), *Arthrobacter* sp. (15% of reads), *Vibrio* sp. (12% of reads), and *Enterobacter* sp. (8% of reads) (Figure 3). Whereas other bacterial species that grew in the CS and PD diets; (based on the reads and TVCs increasing relative to the inoculum), included *Yersinia* sp., *Lactococcus* sp., and *Klebsiella* sp. (Figure 3). Results from this study observed a very small number of lactic acid bacteria that grew in the *in vitro* growth model system, and only comprised 1% of total reads in PD diet (Figure 3). MDS analysis of the sequence data (Figure 2) reiterated the outcomes of ANOVA analysis showing essentially similar statistical relationships between samples.

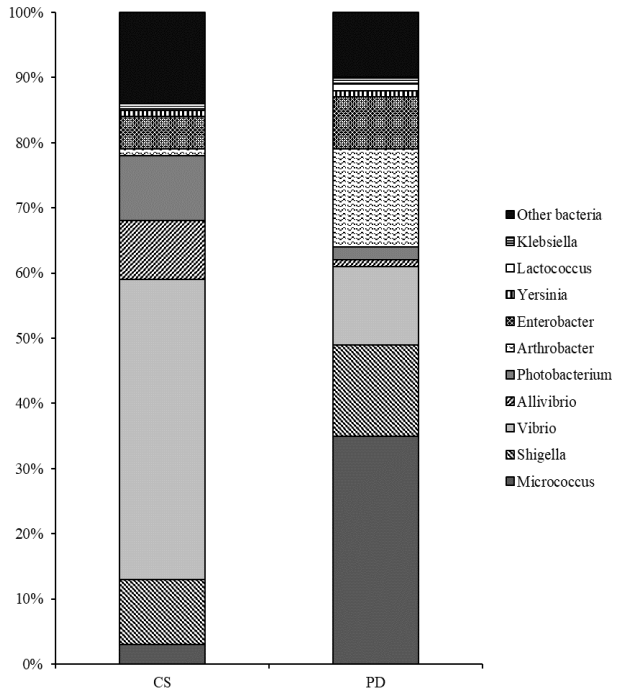

Figure. 3: Relative abundance of the bacterial species presents in the *in vitro* model system shown as average percentile. Community composition was determined by 16S rRNA sequencing of MiSeq Illumina-based analysis.

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In vitro growth model system of prodigiosin as a feed additive

This study results indicated that the PD diet showed the least amount of growth among the bacteria originating in the shrimp gut system which included members of the Vibrionaceae family as compared to the CS diet. The most abundant bacterial species detected were *Micrococcus* spp. which represented 35% and *Shigella* spp. 14% of the total identified bacterial species. The abundant the bacterial species belongs to the family of Vibrionaceae includes *Vibrio* spp., *Aliivibrio* spp., and *Photobacterium* spp., were lowered in PD diet (15% of total reads) compared to CS diet (65% of total reads) indicated the ability of prodigiosin to suppress this bacterial species (Figure 3). The interesting part is the numbers of bacterial species belongs to Vibrionaceae is lowered but still available in significant number and not totally inhibit. Overall, this result interestingly correlated with the findings from the ANOVA and MDS analysis (Table 2, Figure 2).

DISCUSSION

The growth response of the shrimp gut system affected by different diet formulations was investigated and analysed using a simple *in vitro* growth model system. The *in vitro* growth model system study aims to understand the microbial shrimp gut communities *in vitro* and to understand the potential use of prodigiosin as a shrimp feed additive. This experiment is not to attempt to replicate the shrimp gut system, the study is based on the principal that bacterial growth is controlled largely by several basic criteria including oxygen availability, salinity, pH, temperature, nutrient content (Skrodenyte-Arbaciauskiene *et al.*, 2008; Hovda *et al.*, 2012; Hatje *et al.*, 2014; Schmidt *et al.*, 2015; Neuman *et al.*, 2016). Moreover, the nutrient regime *in vitro* is different from the *in situ* gut system conditions, thus bacteria need to adapt to the diet slurries used in this study. In fact, the bacteria *in vitro* are directly exposed to diet while bacteria *in situ* exposed to gut secretions, mucous and different nutrient profiles rather than directly exposed to the diets (Austreng, 1978).

Besides, The *in vivo* approach requires considerable time and physical resources; thus, application of *in vitro* growth model system is interesting and relevant to study microbial communities and its dynamisms (Askarian *et al.*, 2012; Amin *et al.*, 2017; Zarkasi *et al.*, 2017). *In vitro* growth model system is a simple, fast and cheap model system that can be used by the shrimp farming industry to determine the microbial communities associated with shrimp and its influence based on several factors such as environments, diet as well as management practices (Kongnum and Hongpattarakere, 2012; Amin *et al.*, 2017). It also can play a vital role in the pre-application process in *in vitro* trials before they proceed to the shrimp farms.

The bacterial population in the beginning were based on the total viable counts (TVC) as seen from this research is 10^7-10^9 CFU g/L, however after 24 h the TVCs count was higher than the initial count (typically 10^6 to 10^7 CFU g/L). The reasons behind this is the dilution factor because bacteria need to adapt to the diet slurries due to nutrient regime changes which are different from *in situ* gut system conditions. In addition, the microbes rely on pre-digestion of the feeds and simpler substrates that lead to the inefficient growth. Based on the endpoint (24 h) results, the microbial community associated with shrimp gut system was influenced to a degree by specific diets according to the MDS and ANOVA analysis (Table 2, Figure 2). The abundance of bacterial species mainly belonged to the members of the *Vibrionaceae* family in diet slurries that seemed to reflect the shrimp gut system microbial community since the group of bacteria were previously found abundant in shrimp (Aguirre-Guzmán *et al.*, 2010; Joshi *et al.*, 2014; Soto-Rodriguez *et al.*, 2014; Xiong *et al.*, 2015; Cornejo-Granados *et al.*, 2017). According to Egerton *et al.* (2018) the predominate bacterial family up to 70% in most faecal samples of fishes and shrimps are Vibrionaceae, reaching densities of 10^8-10^9 CFU/mL. However, other bacterial species may also become predominant for reasons that cannot yet to be explained.

The complete commercial diet (CS) produced outcomes that were expected and which were in fact slightly similar to previous research (Zarkasi *et al.*, 2017). However, there is a significant difference between the CS diet and the PD diet suggesting a marked effect on the growth of different microbial species in the *in vitro* model system that were influenced by the different diet treatments. The manipulation of shrimp feed did have a demonstrable effect on the outcomes of this study because it presumed that the prodigiosins were able to supress several microorganisms with their antimicrobial capability (Ibrahim *et al.*, 2014). The results of CS showed high numbers of *Vibrionaceae* (> 65% of reads) that is typical of the *Vibrionaceae* composition in shrimp gut system as reported by previous studies (Moss *et al.*, 2000; Luis-Villasenor *et al.*, 2013; Rungrassamee *et al.*, 2014; Cornejo-Granados *et al.*, 2017). The results for PD showed low numbers of *Vibrionaceae* (> 12% of reads) that is indicative that prodigiosin may have a potential to inhibit certain bacterial species and pathogens (Ibrahim *et al.*, 2014). Prevention of the growth of bacterial pathogens in shrimp through the use of prodigiosin would provide an advantage to the beneficial bacteria.

The result indicated that the PD diet may be able to lower the number of *Vibrionaceae* that are linked to vibriosis diseases in fish and shrimp leading to production loss in the aquaculture industry, since vibriosis is a major concern among the aquaculture industry in Asia and other parts of the world. According to the latest finding, early mortality syndrome (EMS) in shrimp were caused by *Vibrio* spp. (De Schryver and Vadstein, 2014; Raja *et al.*, 2017), thus making vibriosis an important disease that needs to be addressed. Recent studies highlighted the prodigiosin had the potential to prevent vibriosis and other bacterial diseases, which in turn can improve fish and shrimp health and their production.

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Micrococcus spp. was one of the most abundant bacterial species found in prodigiosin-added (PD) content diets. This bacterial species is normally found in shrimp products (Jeyasekaran *et al.*, 2006; Okonko *et al.*, 2008), and previously found abundant in the shrimp gut system too (Rungrassamee *et al.*, 2014). In the CS diet, the most abundant bacterial species were *Vibrio* spp. together with *Photobacterium* spp., and *Shigella* spp. *Vibrio* spp. such as *Vibrio tasmaniensis*, *V. ichthyoenteri*, *V. scophthalmi*, *V. aestuarianus* and *V. splendidus*, appeared to be normal microbiota in the shrimp gut systems, since they have also been observed in other marine organisms such as fish (Hovda *et al.*, 2007). Other bacterial species detected in this study included *Photobacterium* spp., *Allivibrio* spp., *Arthrobacter* spp., *Enterobacter* spp., *Yersinia* spp., *Lactococcus* spp., and *Klebsiella* spp. Those bacterial genera are commonly found and previously isolated from the shrimp gut system (Moss *et al.*, 2000; Yousuf *et al.*, 2008; Rungrassamee *et al.*, 2014; Cornejo-Granados *et al.*, 2017).

The high level of Vibrionaceae (mean 50% of reads) especially in CS diet, however, typical composition of bacterial communities associated with the shrimp gut system (Moss *et al.*, 2000; Hatje *et al.*, 2014) thus the results are also very likely affected by this factor. This would inevitably provide a large advantage to this of species given they have fast growth rates. Therefore, the level of Vibrionaceae in PD diet is relatively low compared to CS diet indicated the Vibrionaceae may supressed by other microbes. In addition, the 24 h time frame and the period of the lag phase of the study was possibly not long enough for some taxa to adapt and grow.

The PD diet treatment was added with prodigiosin produced bacterial strains of *Serratia marcescens* IBRL USM 84 that were produced naturally (Ibrahim *et al.*, 2014). This ingredient was capable of inhibiting certain bacterial species that are unwanted in the shrimp gut system or pathogens as reported by other studies (Ibrahim *et al.*, 2014; Darshan and Manonmani, 2015). It has an antibacterial activity that remains poorly understood even though it has been demonstrated as a potential way of inhibiting the growth of certain bacterial species including that of *Vibrio* spp., *Bacillus* spp., *Streptococcus* spp., and *Clostrodium* spp. (Ibrahim *et al.*, 2014). This study determined the potential of prodigiosin as a feed additive not only for shrimp diets but also fish diets that can improve the shrimp/fish gut health and productivity. This could include other substances such as melanin (Rani *et al.*, 2013; Pavitra *et al.*, 2017), astaxanthin (Weeratunge and Perera, 2016), *Pisum sativum* (Saeed *et al.,* 2005) and flavonoids that usually have generalised antimicrobial properties (Ganguly, 2013).

The prodigiosin added as a diet supplement is still unclear whether it has the capacity to promote or inhibit a certain genus of microbes, but the data raises this possibility. According to the results, prodigiosin potentially supress the bacterial species belongs to the family of Vibrionaceae, but it is still unclear its potential as prebiotics, since the Vibrionaceae bacterial species doesn't completely inhibit but present in low numbers compared to the commercial diet. Further study and analyses are required to better understand its properties and potential or any role it may play in the gut microbiome of the shrimp gut system. Furthermore, in vivo and life trial suggested to apply for better understanding the potential of prodigiosin. In addition, Luis-Villasenor *et al.* (2013) revealed that manipulation of diet appeared to potentially boost bacterial proliferation, promote good bacteria and improve shrimp gut system health as well as productivity. Future experiments should examine the potential of this diet by its application on live shrimp trials, including testing on the number of additives added, application of mixing, overall culture volumes, pre-digestion of diets and control of pH.

Prodigiosin applied in this study is safe for human and animal consumption since their toxicity was removed in the final extracted product as reported by Ibrahim *et al*. (2014). In addition, this experiment chooses prodigiosin since it extracted from bacterial species of *Serratia marcescens* IBRL USM84 who been isolated from a coral in Malaysia's east coastal area. Previous studies reported that microorganism pigments such as prodigiosin can serve as an alternative source to replace synthetic pigments used in the food industry (Namazkar and Ahmad, 2013). Hence, spray-dried microcapsules containing prodigiosin were produced using kappa-carrageenan and maltodextrin as encapsulation agents after optimising the effect of spray-drying parameters on the encapsulation yield (EY), moisture content, particle size, and colour intensity of the prodigiosin microcapsules. The particles were successfully applied to yogurt, milk and carbonated drinks (Namazkar and Ahmad, 2013).

The study presented an examination of the feeds effect with different diet manipulations on the growth of shrimp gut system microbiome using a simple *in vitro* model system. The previous research that conducted slightly similar approach produced interesting results for understanding microbial community diversity when different treatments were applied (Askarian *et al.*, 2012; Zarkasi *et al.*, 2017). A further extension of the present study would be able to correlate microbial community observation with diet digestibility and other nutritional performance criteria (Glencross *et al.*, 2007). The additional study potentially be conduction in testing of different diet formulation and the use other potential diet additives such as prebiotics, probiotics, phytogenic additives. This model system of *in vitro* model system is valuable in the testing of different diet formulations, other diet additives used and different forms of the core ingredients.

CONCLUSION

Results obtained from this study suggested that in vitro model systems could provide an option for specific diet formulation screening to determine how they influence and affect the gut system community structure before the specific diet formulations are applied to shrimp or fish live

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trials. The data revealed the dynamic of shrimp gut system's microbial community members in which they were influenced by the manipulation of additives and treatments. This study provides useful information that could be used to develop predictable outcomes of diet manipulations, feed ingredients and feed additives on gut system's microbiomes of shrimp. However, more studies are needed to further understand the potential of in vitro model system applications for aquaculture and agriculture industries.

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