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# Farm-raised tiger shrimp, *Penaeus monodon*, fed commercial feeds with added organic acids showed enhanced nutrient utilization, immune response and resistance to *Vibrio harveyi* challenge

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ABSTRACT

The threat of disease in the aquaculture industry, coupled with greater restrictions or ban on antibiotic use, has increased interest in evaluating antibiotic alternatives. A promising alternative is organic acids, which have been used for decades in the livestock feed industry as an antimicrobial and growth promoter. However, very limited information exists on their applications to the shrimp aquaculture industry. The aim of the current study was to examine the potential beneficial effects of a novel microencapsulated organic acid blend (OAB) to the production of tiger shrimp, *Penaeus monodon*, in earthen ponds at a commercial farm when fed commercial feeds without (Diet A) or with (Diet B) organic acid supplementation at 2% OAB throughout the grow-out period. Farm-raised shrimp were randomly sampled and transferred to the laboratory to examine any effects on nutrient utilization, resistance of shrimp to *Vibrio harveyi* and associated hepatopancreatic histopathology and phenoloxidase (PO) activity. Results showed that after 22 weeks of culture in ponds, shrimp growth was similar between treatments. Lower nitrite-N and nitrate-N concentrations in the pond water indicated potential improved protein utilization from shrimp fed Diet B. This was supported by data from the digestibility trial, demonstrating crude protein, but also dry matter, ash and phosphorous utilization was significantly enhanced ($P < 0.05$). Total viable bacteria and presumptive *Vibrio* spp. counts were lower at the end of the grow-out period in the pond water of shrimp fed Diet B. Shrimp fed Diet B showed significantly higher survival to *V. harveyi* challenge, likely due to enhanced PO activity and less hepatopancreatic damage. Total viable bacterial and *Vibrio* counts in the hepatopancreas of shrimp fed Diet B were significantly lower compared to Diet A. This study provides the first reported data on the use of dietary organic acids in a commercial shrimp farm setting. The enhancement to nutrient utilization may reduce feeding costs and improve water quality while the higher resistance of shrimp to pathogenic bacteria such as *Vibrio* spp. may provide shrimp farmers with an effective method to mitigate disease outbreaks in the global shrimp aquaculture industry.



## 1. Introduction

Diseases in the aquaculture industry are a significant limitation to productivity, and shrimp are certainly no exception with substantial losses due to various bacterial, fungal, parasitic and viral diseases (Flegel et al., 2008). The most recent disease to hit the global shrimp industry is the acute hepatopancreatic necrosis disease (AHPND) or more commonly known as early mortality syndrome (EMS) where shrimp mortalities can be 100% with certain strains of *Vibrio parahaemolyticus* being identified as the causative agents (Tran et al., 2013). In response, this has led to the abuse and overuse of antibiotics in the aquaculture industry. The uncontrolled use of antibiotics can negatively affect the host species, the environment as well as the health of human consumers which has led to greater restrictions or ban of such practices in many countries (Marshall and Levy, 2011). Moreover, even in areas where antibiotics are used, this can potentially depress crustacean growth (Bray et al., 2006) as well as lead to the emergence of antibiotic resistant bacteria, including *Vibrio harveyi*, and therefore reducing subsequent antibiotic efficacy (Karunasagar et al., 1994).

For these reasons, it is becoming increasingly imperative to identify suitable antibiotic alternatives. While various feed additives have been investigated for use in aquaculture feeds, organic acids are receiving increasing attention due to their strong antimicrobial and prophylactic properties against various pathogenic bacteria (da Silva et al., 2013; Defoirdt et al., 2006; Ng and Koh, 2011). Organic acids are "Generally Regarded as Safe" compounds often containing one or more carboxyl groups (–COOH) with antimicrobial properties (Defoirdt et al., 2009). Some of the most common are those with short chains (C1–C6) including, formic, lactic, propionic and citric acids and their salts. Many of

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these are known to inhibit various *Vibrio* strains *in vitro*, but are highly dependent on the type and level (da Silva et al., 2013; Defoirdt et al., 2006; Ng and Koh, 2011) when tested *in vivo* with various aquatic animal species. It is believed that the primary antimicrobial action of organic acids is by altering the cell cytoplasm pH of bacteria and those that are sensitive to such changes are inhibited, thus reducing harmful bacteria within the gastrointestinal tract of the host animal (reviewed by Booth and Stratford, 2003). Therefore a reduction to gastrointestinal bacteria, that often includes pathogenic species, may improve growth and disease resistance in the host animal. Meanwhile, organic acids can also lower dietary and gastrointestinal pH, which may chelate or solubilize minerals to make these more available for absorption, as well as break up antinutritional compounds (Hossain et al., 2007). The inclusion of organic acids in the diets of red drum, *Sciaenops ocellatus*, has been reported to increase the activity of several digestive enzymes (Castillo et al., 2014).

Generally, reports on organic acids improving growth performance and nutrient utilization in aquacultured animals have been positive, but appear to be dependent on the type of organic acids used and host species (reviewed by Ng and Koh, 2011). For example, Arctic charr, *Salvelinus alpinus*, fed sodium-propionate supplemented diets suppressed growth, while dietary sodium-lactate acted as a growth promoter (Ringø, 1991). Meanwhile, among different organic acid salts of acetate, propionate and butyrate in the diets of the white Pacific shrimp, *Litopenaeus vannamei*, sodium-propionate significantly increased apparent gross energy and phosphorus (P) digestibility, while sodium-acetate tended to lower P availability (da Silva et al., 2013). In a subsequent study, Da Silva et al. (in press) reported that white shrimp fed dietary sodium butyrate or proprionate showed a modified intestinal microbiota and improved growth performance. We recently showed in a series of laboratory-based feeding trials that a novel dietary microencapsulated organic acid blend (OAB) was able to enhance growth, nutrient utilization, immune response, hepatopancreatic integrity and resistance against *V. harveyi* in *L. vannamei* (Romano et al., 2015). Due to these encouraging laboratory results, field evaluation of the OAB product was deemed as an essential next step.

To the best of our knowledge, no information currently exists regarding the application of dietary organic acids in commercial shrimp feeds in a commercial farm. To investigate the practical applications of organic acids, a unique hybrid experimental design consisting of related experiments in commercial earthen shrimp ponds and in controlled replicated laboratory-based tanks was conducted. Tiger shrimp, *Penaeus monodon*, were cultured in commercial ponds and fed diets with or without supplementations of a novel microencapsulated organic acid blend. Over 22 weeks, shrimp growth and various water quality parameters of the ponds were measured. At various intervals during the grow-out period, these farm-raised shrimp were transferred to the laboratory and two separate experiments were performed to determine nutrient utilization efficiencies and subsequent resistance to *V. harveyi*. With the incorporation of more controlled laboratory-based experiments using farm-raised shrimp, this helped provide a broader complement of data to the pond trial and a unique opportunity to assess the potential of dietary organic acids to the shrimp aquaculture industry.

## 2. Materials and methods

### 2.1. Commercial farm site and pond

Two earthen ponds in a semi-intensive commercial tiger shrimp (*P. monodon*) farm located at Penang, Malaysia (5° 23′ 44″ N, 100° 11′ 46″ E) were used in the present study. The ponds were about 0.6 ha in size with a depth of approximately 1.2–1.5 m. Each pond was equipped with a properly screened water inlet connected to a reservoir, and water exchanges were performed via a central drain. Up to nine paddle-wheel aerators (1 hp) were provided in each pond to maintain strong water currents and continuous aeration.

### 2.2. Pond preparation

Prior to stocking, the ponds were completely drained and pond bottom cleaned through pressure washing to flush out wastes, debris and undesirable aquatic species followed by manually removing any leftover waste in the ponds. The ponds were left to dry in the sun for 7–14 days or until the soil cracked. After drying, calcium carbonate ($CaCO_3$) was spread uniformly on the pond bottom. The ponds were then filled with filtered seawater and tea seed powder (20–30 mg $L^{-1}$) was added to eliminate any foreign species in the pond. The filled pond was left for seven days before the shrimp post-larvae were stocked.

### 2.3. Source of tiger shrimp post-larvae and pond stocking

Tiger shrimp, *P. monodon* (specific pathogen free; SPF) post-larvae (PL-12) were purchased from a hatchery at Penang, Malaysia. Shrimp post-larvae were transported in plastic bags and upon arrival at the farm, slowly acclimated to the pond conditions for 1 h. After acclimation, the PL were released directly into the pond at a density of 160,000 per pond giving a stocking density of 26 PL $m^{-2}$. This was estimated by individually counting the PL from three randomly selected bags. Duplicate groups of 30 PL were individually measured and batch weighed for initial total length and average body weight, respectively.

### 2.4. Feeds and feeding

The proprietary OAB (Orgacids™-AQUA) was developed and produced in collaboration with Sunzen Feedtech Pte Ltd (Malaysia) and consisted of a blend of four different types of organic acids (formic, lactic, malic and citric acids) microencapsulated within a specialized lipid coating by high speed centrifugal spray chilling. The resultant spherical microcapsules were less than 250 μm in size and came in the form of a free-flowing white powder which can be easily incorporated into commercial shrimp feeds.

A series of five commercial tiger shrimp feed types (from crumble, 0.5–1.1 mm to pellet sizes of Ø1.8 × 2.0–3.5 mm) containing no added OAB (control, Diet A) or 2% OAB (Diet B), respectively, were produced and purchased from Gold Coin Specialities Ltd. (Malaysia) for use at various stages of the grow-out cycle (Table 1). The dietary OAB level of 2% was selected based on the optimal results obtained with this dietary

**Table 1**
Analyzed proximate composition (g 100 $g^{-1}$ dry weight) and pH of Gold Coin® classic-type commercial marine shrimp feeds[a].

| Commercial feed type (feed size, mm)[b] | Moisture | Ash | Protein | Lipid | pH |
|---|---|---|---|---|---|
| Diet A | | | | | |
| 901 (0.5–1.1) | 10.1 ± 0.1 | 13.8 ± 0.1 | 44.9 ± 0.7 | 10.3 ± 0.0 | 6.1 ± 0.0 |
| 902 (1.2–1.8) | 10.8 ± 0.0 | 13.8 ± 0.1 | 45.0 ± 0.3 | 10.3 ± 0.0 | 6.1 ± 0.0 |
| 903S (Ø1.2 × 2.0–3.5) | 10.9 ± 0.1 | 14.0 ± 0.0 | 44.2 ± 0.2 | 10.2 ± 0.1 | 6.2 ± 0.0 |
| 903P (Ø1.5 × 2.0–3.5) | 10.3 ± 0.1 | 13.4 ± 0.0 | 44.3 ± 0.2 | 10.2 ± 0.0 | 6.2 ± 0.0 |
| 904 (Ø1.8 × 2.0–3.5) | 10.5 ± 0.0 | 12.9 ± 0.0 | 45.0 ± 0.1 | 9.6 ± 0.1 | 6.2 ± 0.0 |
| Diet B | | | | | |
| 901 (0.5–1.1) | 9.6 ± 0.1 | 13.1 ± 0.0 | 44.1 ± 0.2 | 10.4 ± 0.1 | 6.2 ± 0.0 |
| 902 (1.2–1.8) | 10.1 ± 0.1 | 13.4 ± 0.1 | 44.4 ± 0.2 | 10.0 ± 0.0 | 6.2 ± 0.0 |
| 903S (Ø1.2 × 2.0–3.5) | 10.9 ± 0.0 | 13.2 ± 0.0 | 43.6 ± 0.3 | 10.6 ± 0.1 | 6.2 ± 0.0 |
| 903P (Ø1.5 × 2.0–3.5) | 10.1 ± 0.1 | 13.5 ± 0.1 | 44.0 ± 0.1 | 10.2 ± 0.1 | 6.2 ± 0.0 |
| 904 (Ø1.8 × 2.0–3.5) | 9.6 ± 0.0 | 13.2 ± 0.0 | 46.0 ± 0.1 | 9.6 ± 0.1 | 5.8 ± 0.0 |

[a] Values are mean ± standard error (n = 3).
[b] Diet A = without organic acid supplementation; Diet B = with added 2% Orgacids™-AQUA. Usage period of feed type #901, up to PL30; #902 from PL30 to 2.5 g; #903S, 2 to 7 g; #903P, 6 to 11 g; #904, >11 g average shrimp body weight.

inclusion level in laboratory-based feeding trials (Romano et al., 2015). All shrimp feed bags were clearly marked as A or B and neither the farmers nor the field researchers were informed of the dietary treatments until all the data were collected and processed to prevent any potential personal biases. The feeds were stored in a well-ventilated shed and used within three months from manufacturing date.

After stocking, shrimp were fed either Diet A or B in one of the two ponds. All feeds were manually dispersed to both groups five times daily at 0700, 1100, 1500, 1900 and 2230 h throughout the culture cycle. The feeding rate was adjusted daily through visual observations of the feeding trays 2 h after each feeding. The amount of feeds used in each pond was recorded throughout the grow-out period.

### 2.5. Water quality management and measurements

After 40 days of stocking, a 10–15% water exchange of the total pond volume was conducted once every three days and $CaCO_3$ was added when required. Every fortnight, water quality parameters, including pH, temperature, conductivity, dissolved oxygen, salinity and water transparency of both ponds were measured on site at 1300 h using a portable multi-probe CyberScan PCD 650 water quality meter (EuTech Instruments) at three different locations (~20 cm depth from the water surface). The salinity was determined using a hand held refractometer, while water transparency was measured using a Secchi disk. Meanwhile, the surface water samples from three locations in each pond were collected in 500 mL clean plastic bottles, wrapped with heavy-duty aluminum, kept on ice and transported to Universiti Sains Malaysia for further analysis.

Total suspended solids (TSS), nitrite-N, ammonium-N and chlorophyll *a* concentrations of pond water samples were measured following the Standard Methods for the Examination of Water and Wastewater (APHA, 1998), while orthophosphate of the filtered water samples was measured by ascorbic acid method (Boyd and Tucker, 1980). Nitrate-N was measured using commercial test kits (JBL GmbH & Co. KG, Neuhofen, Germany).

In the laboratory, the water samples were left to stand at room temperature. Half of the sample (250 mL) was filtered through a 1.2 μm preweighed Whatman™ glass microfiber filter paper (GF/C, 47 mm) using a vacuum-pump (Fisher Scientific, FB 70155), the paper was folded and then dried at 103 °C for 24 h or to a constant weight to determine the TSS. The filtered water was then used for measuring ammonia-N, nitrite-N and orthophosphate-P. The other half of the water sample (250 mL) was used to determine the chlorophyll *a* concentration. Briefly, the water sample was filtered through a Whatman™ glass microfiber filter paper to concentrate the algae cells. The filter paper was then folded and steeped in 3 mL of 90% aqueous acetone solution at 4 °C overnight in the dark. After 24 h, the sample was macerated mechanically at 500 rpm with a TFE tissue grinder, adjusted to 10 mL with 90% aqueous acetone solution, and clarified by centrifuging for 20 min at 500 *g*. The optical density (absorbance) of the clarified extract was analyzed with a spectrophotometer at 750, 664, 647 and 630 nm. The resulting absorbance measurements were then applied to the standard trichromatic equation, as follows,

$$\text{Chlorophyll } a\left(\mu g\ l^{-1}\right) = (Ca \times A)/B$$

where, A is the total volume of the extract supernatant after centrifuging, B is the volume of the sample filtered, and $Ca = 11.85(OD_{664nm}) - 1.54(OD_{647nm}) - 0.08(OD_{630nm})$.

### 2.6. Growth assessment

After six weeks of cultivation, shrimp from both ponds were randomly sampled fortnightly early in the morning at three to four different locations by using a feeding tray (week 6 to week 10), or a cast net (week 12 to week 22) to monitor growth performance. The sampled shrimp were then quickly weighed on a digital scale and the shrimp put back into their respective pond.

### 2.7. Bacterial enumeration of pond water samples

Every four weeks, water samples from three locations of each earthen pond were collected in sterile universal bottles at 1100 h and serially diluted (10 folds). Aliquots (0.1 mL) were spread plated onto thiosulfate citrate bile sucrose (TCBS) agar (Merck, Darmstadt, Germany) or tryptic soy agar (TSA, Merck, Darmstadt, Germany) to determine presumptive *Vibrio* spp. and total cultivable bacterial counts, respectively, after being incubated at room temperature for 24 h.

### 2.8. Partial harvest

It was the normal practice for this commercial farm to partially harvest the shrimp for sale to local retail markets and restaurants. In order to ensure similar biomass throughout the grow-out period, partial harvest was done alternately from the two ponds. This began by week 11, only from shrimp with an average body weight of 20–25 g, and at each partial harvest, their total harvest weights and average body weights were recorded.

### 2.9. Bacterial strain and inoculum preparation

*V. harveyi*, originally isolated from diseased *Talorchestia* sp. (ATCC 14126), was used in the disease challenge trial. The strain was maintained as a frozen stock in tryptic soy broth with 1.5% (w/v) sodium chloride (NaCl) (TSB+) containing 20% (v/v) glycerol (Qrec, New Zealand) and stored at −80 °C until needed.

For the challenge trial, frozen stock cultures were resuscitated in 50 mL of TSB+ within a 250 mL conical flask and incubated at 30 °C with orbital shaking at 200 rpm for 18–24 h. The bacterial broth was then streak plated onto tryptic soy agar with 1.5% NaCl (TSA+) plates for 18 h at 30 °C and this was followed by inoculating two loops full of pure bacterial colonies in 50 mL of TSB+ for 18 h at 30 °C and 200 rpm to achieve an optical density of approximately 0.6 at 545 nm (~$5 \times 10^8$ CFU $mL^{-1}$). A bacterial pellet from 10 mL of bacterial broth was then harvested by centrifugation at 10,000 rpm for 10 min, followed by two washes of sterile normal saline solution (0.85% NaCl), and resuspended in 10 mL of sterile normal saline solution. This bacterial suspension was used as the inoculum for the challenge trial. The bacterial concentration was determined by standard plate count method using TSA plates.

### 2.10. V. harveyi challenge trial

After 16 weeks of cultivation, adult tiger shrimp (approximately 19–21 g) were collected from both ponds and acclimatized for 15 days in a static system consisting of 10 glass aquaria [68.5 (L) × 29.0 (W) × 45.1 (H) cm] housed within a temperature controlled room. Each aquarium was filled with 60 L of sterilized artificial seawater (InstantOcean®) at a salinity of 20 ± 1‰ and continuous gentle aeration was provided *via* an individual air-stone. Each treatment was performed in five replicates with each aquarium containing six shrimp. Over the acclimatization period, all shrimp were hand-fed three times daily at 2.5% body weight with their respective diets at 1000, 1400 and 1800 h. Each aquarium received two daily 70% water exchanges (morning and evening) and the pH, dissolved oxygen, temperature, and ammonia-N levels were 7.30 ± 0.05, 5.78 ± 0.06 mg $L^{-1}$, 27.9 ± 0.1 °C and 1.03 ± 0.07 mg $L^{-1}$, respectively.

The disease challenge test was carried out by intramuscular injection, with a $LD_{50}$ of 20 μl ($4.94 \times 10^5$ CFU $g^{-1}$ shrimp) (Liu et al., 1996) of live bacterial suspension ($4.95 \times 10^8$ CFU $mL^{-1}$), into the shrimp between the third and fourth abdominal segments. Mortalities

were recorded for eight days and any dead shrimp were removed. During the challenge trial, the shrimp were fed three times daily, received a 50% water exchange in the morning and evening, respectively and the ammonia-N levels were at or below 1.07 ± 0.03 mg $L^{-1}$.

### 2.11. Hemolymph collection and phenoloxidase activity assay

Phenoloxidase activity (PO) was measured from shrimp after the bacterial challenge test according to Liu and Chen (2004) with slight modifications. To obtain the hemolymph, syringes were coated with anticoagulant (30 mM trisodium citrate, 0.34 M NaCl, pH 7.55 and glucose was added until 30‰ was reached), inserted in the ventral sinus and then 40 μl hemolymph was added to 360 μl of the anticoagulant. This was then centrifuged at 700 × *g* for 20 min., the supernatant was removed and the pellet was gently re-suspended with 200 μl cacodylate-citrate buffer, centrifuged a second time and re-suspended with 200 μl cacodylate buffer (0.01 M sodium cacodylate, 0.45 M sodium chloride, 0.01 M calcium chloride, 0.26 M magnesium chloride, pH 7.0). Each aliquot (100 μl) was incubated with 50 μl trypsin (1 mg $mL^{-1}$) for 30 min at room temperature followed by adding 50 μl of L-DOPA (1 mg $mL^{-1}$). Five minutes later, 800 μl cacodylate buffer was added and then measured at an optical density of 490 nm. A control was used to correct for background interference, which replaced 50 μl trypsin for cacodylate buffer. PO activity (unit of optical density at 490 nm) was expressed as dopachrome formation in 40 μl of hemolymph.

### 2.12. Histopathology assay

After the hemolymph was collected, the hepatopancreas was dissected for histological examination, immersion fixed in Bouin's solution for 18 h and then transferred to 70% (v/v) ethanol until processing. Sections (5–7 μm) were made using a rotary microtome, stained with hematoxylin and eosin and examined under a light microscope (×20 and ×40 magnification).

### 2.13. Digestibility trial

For the digestibility trial, the same commercial diets used in the pond trial were ground into powder and re-pelleted to include 0.5% chromic oxide ($Cr_2O_3$) as an indigestible marker. The mixing and diet preparation were similar to Ng et al. (2009). After 20 weeks of culture, shrimp (30–32 g) were collected from both ponds and transferred into eight 500 L fiberglass tanks (cone shaped bottom) in a closed recirculation aquaculture system (RAS). The RAS was fitted with a pump, aerators, sand filters, biofilter, foam fractionators and sedimentation tank housed in a hatchery under natural photoperiod as previously described (Romano et al., 2015). Each dietary treatment was performed in four replicates with each tank containing nine shrimp. All shrimp were hand-fed four times daily at 2.5% body weight with their respective diets at 0900, 1200, 1500 and 1800 h.

After a 5 day acclimatization period, the tanks were siphoned for any feces or uneaten food prior to first feeding. One hour after each feeding, freshly expelled shrimp feces were carefully siphoned and collected on a fine mesh net at intervals of 30 min until the next feeding. Feces were rinsed with freshwater to remove any residual seawater and only intact fecal strands were collected using tweezers and kept at −20 °C until analysis. Daily fecal collections were pooled according to each tank, oven dried and finely ground prior to analyzing their chemical composition and $Cr_2O_3$ concentration.

Throughout the digestibility trial, the pH, salinity, dissolved oxygen, temperature, and ammonia-N levels were measured twice weekly and maintained at 7.25 ± 0.04, 25 ± 1‰, 6.54 ± 0.08 mg $L^{-1}$, 29.9 ± 0.21 °C and 0.18 ± 0.05 mg $L^{-1}$, respectively. The water flow rate was fixed at about 1.92 ± 0.05 L $min^{-1}$, and the digestibility trial was conducted for four weeks.

### 2.14. Hepatopancreas and intestine bacterial enumeration

At the end of the digestibility trial, five shrimp were removed from each tank and sterilized with 70% ethanol to avoid cross contamination. The hepatopancreas and intestine were then aseptically dissected, weighed separately and then homogenized in a sterile mortar. The homogenates were transferred into sterilized 15 mL polypropylene tubes and diluted to 10 mL with sterile normal saline solution. The supernatants were serially diluted with 0.1 mL of the aliquots spread plated in duplicates onto TSA and TCBS agar plates and then incubated at room temperature for 24 to 48 h. Only plates that had between 30–300 colonies were used to determine the number of total cultivable bacteria and presumptive *Vibrio* spp. counts and the results are expressed as log CFU $g^{-1}$.

### 2.15. Chemical analysis

The chromic oxide ($Cr_2O_3$) content of the feces and feeds was determined according to the wet-acid digestion method described by Furukawa and Tsukahara (1966). The phosphorus concentrations of both the feces and feeds were determined by the molybdovanadate method and measured using a spectrophotometer (GENESYS 20, Thermo Electron Corp., Madison, WI, USA) at 450 nm (AOAC, 1997). The proximate composition (moisture, crude protein, crude lipid and ash) of the feeds and feces were determined using standard methods in AOAC (1997). To measure dietary pH, 5 g of each diet (finely ground) was mixed in 50 mL of distilled water for 5 min and then the water pH was measured using a digital pH meter after prior calibration with precision buffers.

The apparent digestibility coefficient (ADC) of dietary nutrients (dry weight basis) was calculated using $Cr_2O_3$ as an inert marker using the following equation,

$$\text{ADC } (\%) = 100 - [100 \times (D_i / F_i) \times (F / D)]$$

where, $D_i$ is the inert marker ($Cr_2O_3$) concentration in feed, $F_i$ is the inert maker concentration in feces, F the nutrient concentration in feces, and D is the nutrient concentration in feed.

### 2.16. Statistical analysis

No statistical analysis of the shrimp growth and water quality data collected from the field trial was performed as only one pond per treatment (no replication) was used. However, several measurements were made in each pond for the same parameter to determine the mean and range of values which are presented as mean ± standard deviation. For the related laboratory-based trials of the farmed raised shrimp, mortalities in the bacterial challenge test, digestibility data, PO activity and bacterial counts were all subjected to a one-way analysis of variance ANOVA after prior confirmation of variance using the SPSS statistical software (SPSS version 14.0 for Windows). Differences between means were determined by independent samples *t*-test and effects with a probability of $P < 0.05$ were considered statistically significant.

## 3. Results

### 3.1. Proximate composition and pH of shrimp feeds

The proximate composition of the two commercially-produced shrimp feeds are presented in Table 1 and showed a similar composition. The pH was also generally similar, except for the feed type 904 for Diet B which was supplemented with organic acids which showed a pH of 5.88 compared to 6.19 for Diet A which was not added with OAB.

### 3.2. Water quality parameters

The range and mean values of various water quality parameters of Pond A (shrimp fed Diet A) and Pond B (shrimp fed Diet B) are shown in Table 2. Over the course of 22 weeks, many of the water quality measurements were similar or followed no obvious pattern between Pond A and B. However, there were several notable differences, which included pH, chlorophyll *a*, and nitrogenous wastes, dissolved oxygen, and TSS. In the case of the pond water pH, which was initially similar at 8.08 and 8.15 in Pond A and B, respectively, by the second week the pH of Pond B became higher than that of Pond A and this trend continued until the end of the trial. Similarly, dissolved oxygen and TSS were initially similar, but by week 6 generally became consistently higher in the water of Pond B and Pond A, respectively.

Chlorophyll *a* was also initially similar between the ponds, however by week 14 and 16 there was a spike, which led to the farmers' adding algaecide to control these levels. From week 18 to 22, the chlorophyll *a* levels in Pond B became over two-fold lower than in Pond A. Meanwhile, the mean ammonia-N levels ranged from over two and 1500 folds higher in Pond A between weeks 8 and 14, but from week 16 onwards the ammonia-N levels in Pond B ranged between 4 and 19 folds higher than in Pond A. Nevertheless, the overall mean ammonia-N level in Pond B was higher than in Pond A since the discrepancy between the ponds in the final weeks were numerically higher (but not proportionally higher), than in the earlier weeks. On the other hand, both nitrite-N and nitrate-N were consistently higher in Pond A than in Pond B, leading to an overall mean difference of over six and five-fold, respectively (Table 2).

### 3.3. Bacterial count of pond water samples

The total bacterial count at weeks 8, 12 and 20 was significantly higher in the water of Pond A than of Pond B (Fig. 1), while presumptive *Vibrio* spp. counts in the water of Pond A was significantly higher at weeks 12 and 20 than in Pond B. The only time bacteria or *Vibrio* spp. counts were higher in Pond B was at week 4.

### 3.4. Growth performance

The initial size of the shrimp PL when stocked was approximately 0.012 g in both ponds. When the shrimp was first weighed at week 6, the mean weight was slightly higher for shrimp sampled from Pond A than Pond B. This trend continued from week 8 to 18, however, by the final weeks of the grow-out period, the mean weight of tiger shrimp between Ponds A and B became similar at 33.36 and 31.22 g, respectively (Fig. 2). Although the growth was similar, according to the shrimp farmer, the overall production costs and net profitability was slightly better in Pond B than Pond A (per. comm.). This was due mainly to the fact that less feeds were used in Pond B. Due to the partial harvesting of shrimp practiced by the farmer, it was unfortunate that we were not able to obtain accurate total shrimp harvest data.

**Table 2**
The range and mean (±SD) of various water quality parameters between the ponds.

| Parameter | Control (Pond A) | | Treatment (Pond B) | |
|---|---|---|---|---|
| | Range | Mean ± S.D. | Range | Mean ± S.D. |
| pH | 7.12–8.34 | 7.62 ± 0.33 | 7.70–8.31 | 8.07 ± 0.22 |
| Conductivity (mS $m^{-1}$) | 31.43–38.47 | 34.72 ± 2.27 | 32.34–39.78 | 35.66 ± 2.24 |
| Dissolved oxygen (mg $L^{-1}$) | 6.20–10.03 | 7.46 ± 1.08 | 5.90–10.35 | 8.28 ± 1.52 |
| Temperature (°C) | 30.2–33.0 | 31.3 ± 1.2 | 30.4–33.2 | 31.5 ± 1.1 |
| Turbidity (cm) | 15.5–49.3 | 24.3 ± 11.6 | 15.4–64.7 | 34.2 ± 14.9 |
| Salinity (‰) | 16–28 | 20 ± 4 | 16–29 | 20 ± 4 |
| Total suspended solids (mg $L^{-1}$) | 77.9–464.8 | 241.8 ± 107.4 | 62.0–355.8 | 160.1 ± 83.9 |
| Ammonia-N (mg $L^{-1}$) | 0.00–1.29 | 0.46 ± 0.45 | 0.00–4.77 | 0.98 ± 1.54 |
| Nitrite-N (mg $L^{-1}$) | 0.00–3.38 | 0.79 ± 1.15 | 0.00–0.54 | 0.12 ± 0.21 |
| Nitrate-N (mg $L^{-1}$) | <0.5–50 | 17.2 ± 17.6 | <0.5–15 | 3.1 ± 6.2 |
| Orthophosphate (mg $L^{-1}$) | 0.20–0.56 | 0.40 ± 0.10 | 0.21–0.60 | 0.40 ± 0.13 |
| Chlorophyll a (μg $L^{-1}$) | 3.7–103.7 | 45. 0 ± 32.1 | 3.0–247.5 | 59.3 ± 74.3 |

### 3.5. Bacterial challenge, phenoloxidase activity and hepatopancreatic histopathology

The shrimp fed Diet A had significantly higher mortalities (50%) than those fed Diet B (30%) after 8 days of *V. harveyi* challenge (Fig. 3). Meanwhile, after 8 days of *V. harveyi* challenge, shrimps fed Diet B had significantly higher PO activity of 0.125 ± 0.008 units compared to 0.088 ± 0.007 units from shrimp fed Diet A.

Furthermore, shrimp fed Diet A appeared to have more histopathological damage to the hepatopancreas than shrimp fed Diet B. Damage included tubule degeneration and necrosis, sometimes leading to rupture, hemocyte infiltrations and reduced R-cell and B-cell prevalence (Fig. 4).

### 3.6. Apparent digestibility

The apparent digestibility coefficient (%) of dry mater, crude protein, crude lipid, ash and phosphorus of diets without (Diet A) or with (Diet B) organic acid blend (OAB) supplementations is shown in Fig. 5. With the exception of crude lipid, the apparent digestibility of all nutrients was significantly higher for Diet B compared with Diet A when fed to tiger shrimp.

### 3.7. Total cultivable bacteria and presumptive Vibrio spp. counts in the hepatopancreas and gut

The CFU counts of total cultivable bacteria in the hepatopancreas and gut were significantly lower in shrimp fed Diet B (Fig. 6). Similarly, presumptive *Vibrio* spp. counts in the hepatopancreas were significantly lower in shrimp fed Diet B. However, while the CFU counts of presumptive *Vibrio* spp. in the intestine of shrimp fed Diet A or Diet B were not significantly different, this was numerically higher for shrimp fed Diet A (Fig. 6).

## 4. Discussion

In the commercial farm feeding trial, after the shrimp were fed diets with (Diet B) or without (Diet A) organic acid supplementation from post-larvae to marketable size, there was no marked effect on the final shrimp size. Due to budget and logistical constraints, such as the supply of *P. monodon* PL from the same source, only one replicate pond per diet was used in the present study. Therefore, the results should be interpreted in consideration of the various other factors that can influence shrimp growth in a pond under commercial farming conditions. Under controlled laboratory conditions, we previously showed that a 2% dietary OAB inclusion in a soybean meal-based diet was able to significantly enhance the growth performance of white shrimp (Romano et al., 2015). When proprionate or butyrate salts were added at 0.5 to 2% in the diets of white shrimp, significant improvement in growth and feeding efficiencies was reported under laboratory conditions by da Silva et al. (in press).

Over the grow-out period of 22 weeks, Pond B often had the same or better water quality than Pond A. For example, total viable bacterial and *Vibrio* spp. counts in the water tended to be lower in Pond B, than Pond A, which may have implications for controlling bacterial diseases, including vibriosis. The water in Pond B often had higher pH than that in Pond A despite Diet B's having a slightly lower dietary pH due to the presence of organic acids, especially in the last grow-out feed type which was the main feed used, in terms of quantity. This appears to

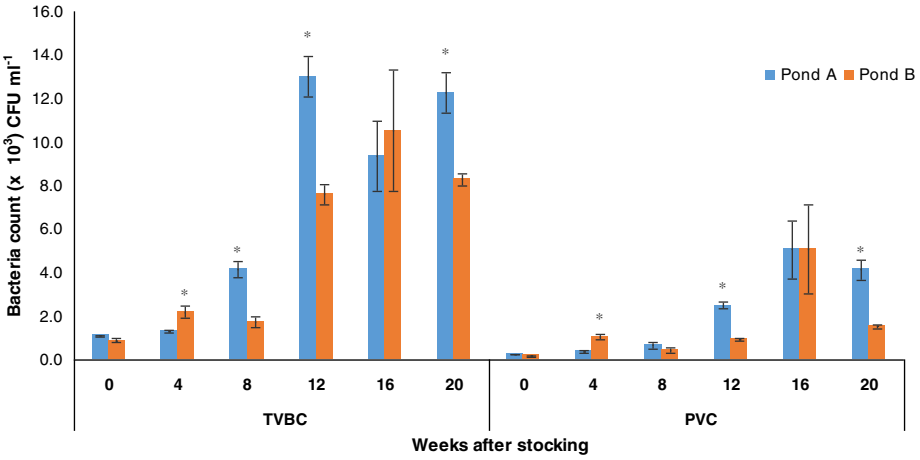


**Fig. 1.** The mean total viable bacterial count (TVBC) and presumptive *Vibrio* spp. count (PVC) ($\times 10^3$ CFU mL$^{-1}$) ($\pm$SE) in the water of Pond A (control) and Pond B (fed organic acid-added feed) over 20 weeks. Symbols indicate significant differences ($P < 0.05$).

indicate that the microencapsulation of the organic acid blend was effective in reducing the leaching of highly soluble dietary organic acids thereby ensuring that most of it got ingested by the shrimp. The use of coated organic acids is likely to be particularly important for shrimp that masticate feed, which increases nutrient leaching, as opposed to fish that ingest feed whole.

One of the most limiting factors in a shrimp farm, besides dissolved oxygen, is nitrogenous waste since this may reduce productivity as well as harm the surrounding environment (Romano and Zeng, 2013). While it was found that ammonia-N was higher in Pond B than Pond A which was due to the addition of an algaecide (zeolite) by the farmers in response to a microalgae level spike at week 16, both nitrite-N and nitrate-N levels were consistently lower in Pond B than Pond A. Taking into consideration this finding, along with ammonia-N possibly being influenced by microalgae levels, may indicate that dietary protein was better utilized by shrimp fed Diet B which would lead to less nitrogenous wastes being excreted. Indeed, this was later confirmed in the digestibility trial, showing that dietary protein was significantly better utilized in Diet B than Diet A by the farmed shrimp. Further studies using more replicate ponds are needed to better understand the potential benefits of dietary organic acid supplementation in commercial shrimp feeds as it relates to growth performance, feed utilization and water quality management. In the present commercial pond study, where replication and standardization were difficult, the pond data obtained should be considered in the light of the variability often present in ponds.

Similar to the results obtained from the replicated laboratory-based part of the present study, it has been previously reported that organic acids can improve protein, dry matter, and/or P digestibility in various other aquatic animals including, Indian major carp, *Labeo rohita* (Baruah et al., 2007), red sea bream, *Pagrus major* (Hossain et al., 2007; Sarker et al., 2007), red hybrid tilapia (Koh et al., in press; Ng et al., 2009), beluga sturgeon, *Huso huso* (Khajepour and Hosseini, 2012), rainbow trout, *Oncorhynchus mykiss* (Hernández et al., 2012; Pandey and Satoh, 2008), Atlantic salmon, *Salmo salar* (Lückstädt, 2008), yellowtail, *Seriola quinqueradiata* (Sarker et al., 2012) and *L. vannamei* (da Silva et al., 2013). However, this can be highly dependent on the organic acid type as well as the aquatic animal species. For example, da Silva et al. (2013) showed that propionate was more effective at improving nutrient utilization than acetate or butyrate when incorporated in feeds of *L. vannamei*. Meanwhile, Gao et al. (2011) found that formate or butyrate did not improve P availability to rainbow trout. It is believed that an improvement to nutrient availability by dietary organic acids is partly related to reducing gastrointestinal or dietary pH, which may then chelate minerals (thus making them more easily absorbed) or in the case of soybean meal-based diets, to dephosphorylate phytate (thus making P more easily absorbed) (Hossain et al., 2007). More recently, Castillo et al. (2014) reported that the inclusion

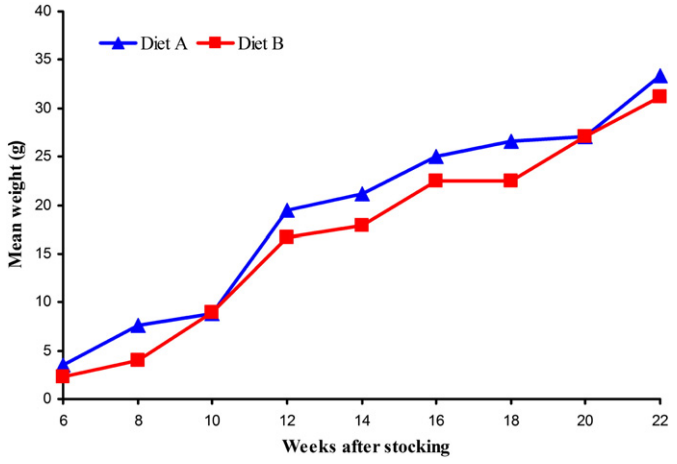


**Fig. 2.** The mean weight (g) of tiger shrimp, *Penaeus monodon*, between Pond A (control) and Pond B (fed organic acid-added feed) over 22 weeks.

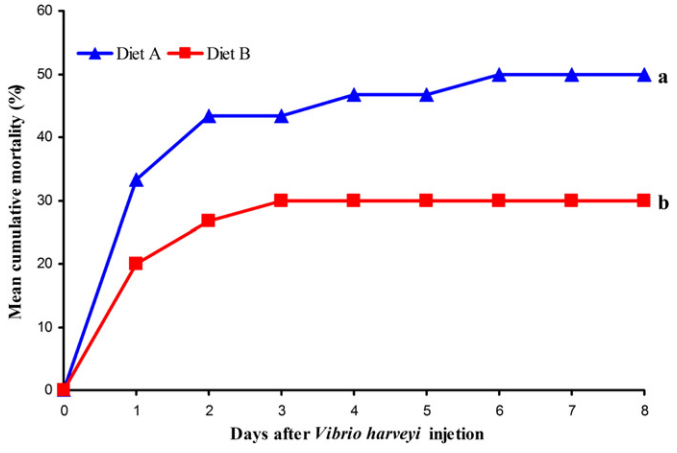


**Fig. 3.** The mean cumulative mortality (%) of tiger shrimp, *Penaeus monodon*, fed Diet A (control) or Diet B (added 2% organic acid blend) over eight days after *Vibrio harveyi* challenge. Different letters indicate significant difference ($P < 0.05$).

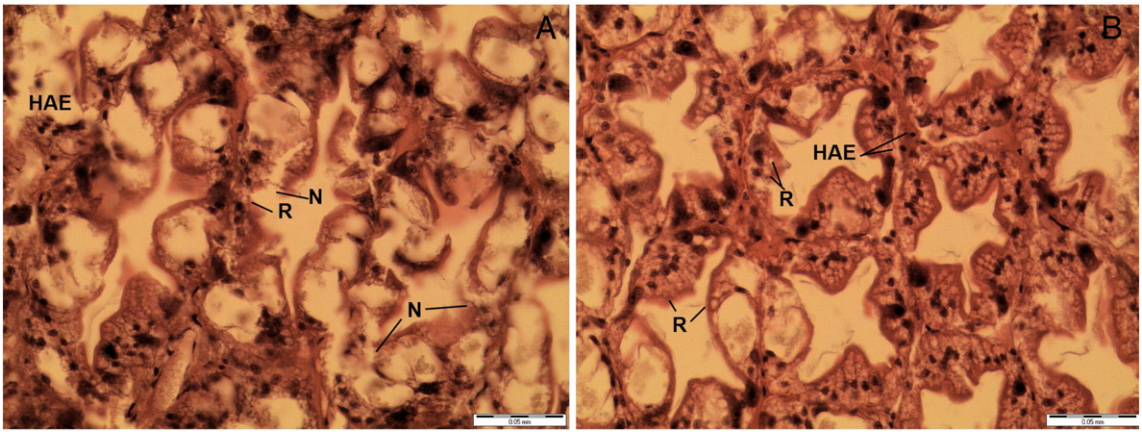


**Fig. 4.** Histopathology of the hepatopancreas from tiger shrimp, *Penaeus monodon*, fed Diet A (control) or Diet B (added with 2% organic acid blend) after 10 days of *Vibrio harveyi* challenge. More hepatopancreatic damage was observed from shrimp fed Diet A, including necrotic tissue (N), compromised tubules, higher hemocyte infiltrations (HAE) and less lipid storage R-cells (R).

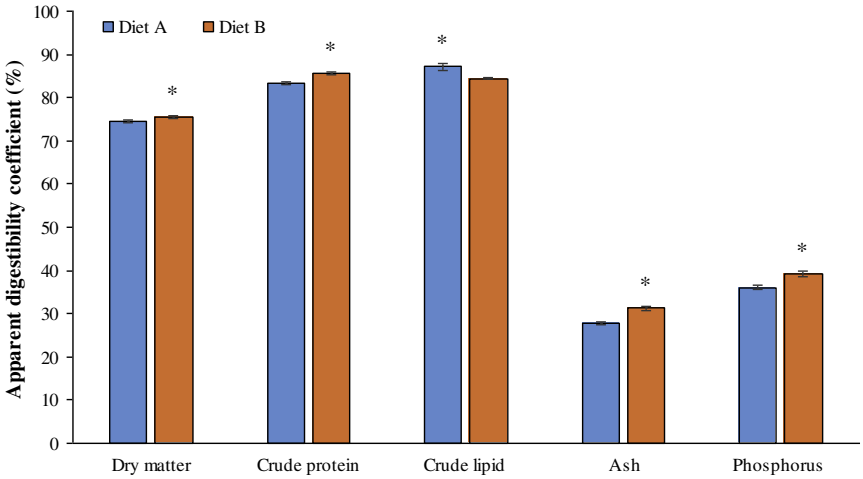


**Fig. 5.** Apparent digestibility coefficient (%) (±SE) of Diet A (control) or Diet B (added with 2% organic acid blend) fed to tiger shrimp, *Penaeus monodon*. Symbols indicate significant differences ($P < 0.05$).

of organic acids in the diets of red drum, *S. ocellatus*, was able to increase the activity of several digestive enzymes.

While the applications of organic acids in aquaculture have mostly focused on potential improvements to growth and/or nutrient utilization in fish, their prophylactic efficacy against pathogenic bacteria has been receiving increasing attention recently (Defoirdt et al., 2006; Koh et al., in press; Ng et al., 2009; Park et al., 2011; Romano et al., 2015). For example, when formic, acetic, propionic, butyric or valeric acid were added in equimolar concentrations to the culture water, the resistance of *Artemia franciscana* to the pathogenic bacterium *Vibrio campbelli* was significantly improved (Defoirdt et al., 2006). However, as noted by Defoirdt et al. (2006), on large scale commercial farm operations, the additions of organic acids to the culture water will likely become cost-prohibitive. More recently, the prophylactic effectiveness of dietary organic acids in fish has been successfully shown (Koh et al., in press; Ng et al., 2009; Park et al., 2011). Ng et al. (2009) demonstrated that dietary OAB or potassium diformate led to improved resistance of red hybrid tilapia to *Streptococcus agalactiae* and, similarly, Park et al. (2011) showed that the resistance of olive flounder, *Paralichthys olivaceus*, to *Edwardsiella tarda* was enhanced when fed OAB supplemented diets. Ng et al. (2009) suggested that this was likely due to the antimicrobial activity of the OAB substantially reducing the CFU counts within the intestine post-challenge, particularly since *S. agalactiae* was not detected in surviving tilapia. Later, Koh et al. (in press) reported that dietary OAB supplementation was as effective as conventional antibiotics such as oxytetracycline in the prevention of streptococcis in tilapia. In the present study with tiger shrimp, there was a clear correlation between the prophylactic effectiveness of Diet B with protecting the hepatopancreas from bacterial-induced damage as well as preventing PO activity reductions.

There were significantly lower total cultivable bacteria and presumptive *Vibrio* spp. counts within both the hepatopancreas and intestine of shrimp fed Diet B in the present study. In crustaceans, the hepatopancreas is known to be targeted by *V. harveyi* (Lavilla Pitogo et al., 1998) as well as inhibiting/altering PO activity (Amparyup et al., 2009; Huang et al., 2013), which are believed to be the main contributors to *Vibrio* spp. induced mortalities. In the present study, it was demonstrated that shrimp fed Diet B had significantly higher survival to *V. harveyi* challenge than those fed Diet A. It is likely that the significantly higher PO activity and less hepatopancreatic histopathological changes, including hemocyte infiltrations, tubule damage/rupture, necrosis and higher R-cell prevalence, which is responsible for lipid storage (Vogt et al., 1985), for shrimp fed Diet B than those fed Diet A led to this result. A similar observation was reported for Pacific white shrimp in our earlier disease challenge trials (Romano et al., 2015). Considering that *Vibrio* spp. can lead to significant mortalities, particularly in the hatchery stage or for more advanced post-larvae during times of stress (Karunasagar et al., 1994; Lavilla Pitogo et al., 1998; Sung et al., 2001), these findings may have important implications to controlling the spread of bacterial disease in the shrimp aquaculture industry.

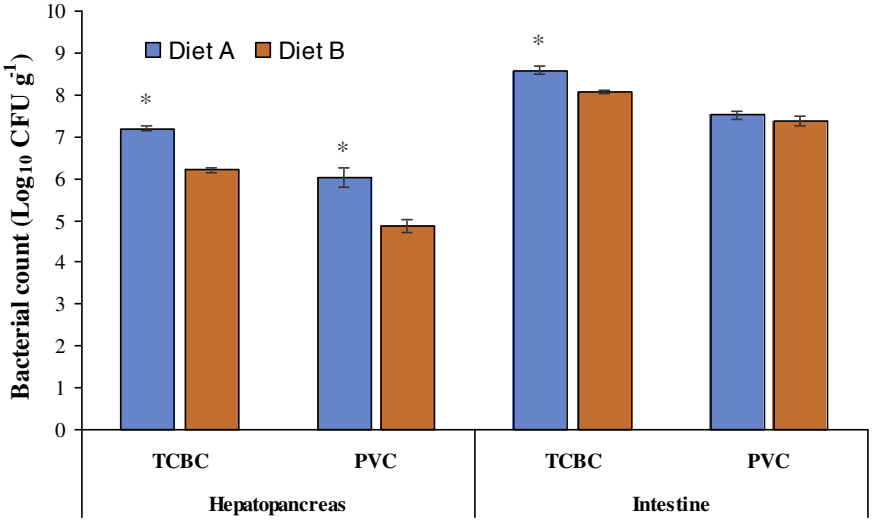


**Fig. 6.** Total cultivable bacteria count (TCBC) and presumptive *Vibrio* spp. count (PVC) (±SE) in the hepatopancreas and intestine of tiger shrimp, *Penaeus monodon*, fed Diet A (control) or Diet B (added 2% organic acid blend). Symbols indicate significant differences ($P < 0.05$).

Moreover, AHPND/EMS which can lead to mass mortalities during the early stages of shrimp grow-out, has been attributed to specific strains of a *Vibrio* bacterium, *V. parahaemolyticus* (Tran et al., 2013). While the resistance to this pathogen was not tested, it is worth noting that the antimicrobial effectiveness of a blend of organic acids may provide a broader spectrum of antimicrobial protection than the sole use of one organic acid type (Chaveerach et al., 2002; Thompson and Hinton, 1997). It is therefore conceivable that the antimicrobial actions of the tested OAB extends to other pathogenic bacteria, such as *V. parahaemolyticus*, and certainly points to interesting research directions.

In conclusion, several important benefits were demonstrated in both the pond and laboratory trials. The lower bacterial count as well as the generally lower nitrogenous waste in the water of Pond B indicate that organic acids can play a role in expanding the aquaculture industry more sustainably. This finding may be due, in part, to improved protein utilization, as demonstrated in the digestibility trial. Meanwhile, the improved protein, dry matter, ash and P utilization may have implications for reducing feeding costs as well as mitigating excessive P discharges to the surrounding environment that cause eutrophication. Whether the improved feed utilization efficiencies will translate into better FCR and growth in a commercial farm setting remains to be further investigated. Finally, the significantly higher resistance of shrimp fed Diet B to *V. harveyi* challenge demonstrates the effective prophylactic properties of the tested OAB. It is anticipated that should shrimp farmers encounter a disease outbreak such as *vibriosis*, the role of the tested OAB may mitigate productivity losses. This is based on significantly better results obtained from the laboratory-based disease challenge tests and further confirmed by the healthier hepatopancreas observed in histological sections. Considering that organic acids pose no health risks to the environment, host animal or human consumers, the use of this functional feed additive may help reduce the reliance on antibiotics in the aquaculture industry.

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## References

Amparyup, P., Charoensapsri, W., Tassanakajon, A., 2009. Two prophenoloxidases are important for the survival of *Vibrio harveyi* challenged shrimp *Penaeus monodon*. Develop. Comp. Immunol. 33, 247–256.

AOAC, 1997. Official Methods of Analysis of AOAC International. 16th ed. AOAC International, Arlington, Virginia.

APHA, 1998. Standard Methods for the Examination of Water and Wastewater. 20th ed. APHA, Washington, USA.

Baruah, K., Sahu, N.P., Pal, A.K., Jain, K.K., Debnath, D., Mukherjee, S.C., 2007. Dietary microbial phytase and citric acid synergistically enhances nutrient digestibility and growth performance of *Labeo rohita* (Hamilton) juveniles at sub-optimal protein level. Aquac. Res. 38, 109–120.

Booth, I.R., Stratford, M., 2003. Acidulants and low pH. In: Russel, N.J., Gould, G.W. (Eds.), Food Preservatives. Kluwer Academic/Plenum Publishers, New York, pp. 25–47.

Boyd, C.E., Tucker, L., 1980. Determination of filterable orthophosphate in water from fish ponds. Trans. Am. Fish. Soc. 109, 314–318.

Bray, W.A., Williams, R.R., Lightner, D.V., Lawrence, A.L., 2006. Growth, survival and histological responses of the marine shrimp, *Litopenaeus vannamei*, to three dosage levels of oxytetracycline. Aquaculture 258, 97–108.

Castillo, S., Rosales, M., Pohlenz, C., Gatlin, D.M., 2014. Effects of organic acids on growth performance and digestive enzyme activities of juvenile red drum *Sciaenops ocellatus*. Aquaculture 433, 6–12.

Chaveerach, P., Keuzenkamp, D.A., Urlings, H.A.P., Lipman, L.J.A., van Knapen, F., 2002. *In vitro* study on the effect of organic acids on *Campylobacter jejuni/coli* populations in mixtures of water and feed. Poult. Sci. 81, 621–628.

da Silva, B.C., Vieira, F.N., Mouriño, J.L.P., Ferreira, G.S., Seiffert, W.Q., 2013. Salts of organic acids selection by multiple characteristics for marine shrimp nutrition. Aquaculture 384–387, 104–110.

da Silva, B.C., Vieira, F.N., Mouriño, J.L.P., Bolivar, N., Seiffert, W.Q., 2015. Butyrate and propionate improve the growth performance of *Litopenaeus vannamei*. Aquacult. Res. http://dx.doi.org/10.1111/are.12520 (in press).

Defoirdt, T., Halet, D., Sorgeloos, P., Bossier, P., Verstraete, W., 2006. Short-chain fatty acids protect gnotobiotic *Artemia franciscana* from pathogenic *Vibrio campbelli*. Aquaculture 261, 804–808.

Defoirdt, T., Boon, N., Sorgeloos, P., Verstraete, W., Bossier, P., 2009. Short-chain fatty acids and poly-β-hydroxyalkanoates: (new) biocontrol agents for a sustainable animal production. Biotech. Adv. 27, 680–685.

Flegel, T.W., Lightner, D.V., Lo, C.F., Owens, L., 2008. Shrimp disease control: past, present and future. In: Bondad-Reantaso, M.G., Mohan, C.V., Crumlish, M., Subasinghe, R.P. (Eds.), Diseases in Asian Aquaculture VI. Fish Health Section Asian Fish Soc, Manila, Philippines, p. 505.

Furukawa, H., Tsukahara, H., 1966. On the acid digestion method for determination of chromic oxide as an index substance in the study of digestibility of fish feed. Bull. Jpn. Soc. Sci. Fish. 32, 207–217.

Gao, Y., Storebakken, T., Shearer, K.D., Penn, M., Øverland, M., 2011. Supplementation of fishmeal and plant-protein based diets for rainbow trout with a mixture of sodium formate and butyrate. Aquaculture 211, 233–240.
Hernández, A.J., Satoh, S., Kiron, V., 2012. Supplementation of citric acid and amino acid chelated trace elements in low-fish meal diet for rainbow trout affect growth and phosphorus utilization. J. World Aquac. Soc. 43, 688–696.
Hossain, M.A., Pandey, A., Satoh, S., 2007. Effects of organic acids on growth and phosphorus utilization in red sea bream *Pagrus major*. Fish. Sci. 73, 1309–1317.
Huang, H.H., Liu, X.L., Xiang, J.H., Wang, P., 2013. Immune response of *Litopenaeus vannamei* after infection with *Vibrio harveyi*. Aquaculture 406–407, 115–120.
Karunasagar, I., Pai, R., Malathi, G.R., Karunasagar, I., 1994. Mass mortality of *Penaeus monodon* larvae due to antibiotic-resistant *Vibrio harveyi* infection. Aquaculture 128, 203–209.
Khajepour, F., Hosseini, S.A., 2012. Citric acid improves growth performance and phosphorus digestibility in beluga (*Huso huso*) fed diets where soybean meal partly replaced fish meal. Anim. Feed Sci. Tech. 171, 68–73.
Koh, C.B., Romano, N., Siti-Zahrah, A., Ng, W.K., 2015. Effects of a dietary organic acids blend and oxytetracycline on the growth, nutrient utilization and total cultivable gut microbiota of the red hybrid tilapia, *Oreochromis* sp., and resistance to *Streptococcus agalactiae*. Aquac. Res. http://dx.doi.org/10.1111/are.12492 (in press).
Lavilla Pitogo, C.R., Leaño, E.M., Paner, M.G., 1998. Mortalities of pond-cultured juvenile shrimp, *Penaeus monodon*, associated with dominance of luminescent *Vibrios* in the rearing environment. Aquaculture 164, 337–349.
Liu, C.H., Chen, J.C., 2004. Effect of ammonia on the immune response of white shrimp *Litopenaeus vannamei* and its susceptibility to *Vibrio alginolyticus*. Fish Shellfish Immunol. 16, 321–334.
Liu, P.C., Lee, K.K., Chen, S.N., 1996. Pathogenicity of different isolates of *Vibrio harveyi* in tiger shrimp, *Penaeus monodon*. Lett. Appl. Microbiol. 22, 413–416.
Lückstädt, C., 2008. Effect of dietary potassium diformate on the growth and digestibility of Atlantic salmon *Salmo salar*. Proceedings of the Thirteenth International Symposium on Fish Nutrition and Feeding, Florianopolis, Brazil, p. 179.
Marshall, B.M., Levy, S.B., 2011. Food animals and antimicrobials: impacts on human health. Clin. Microbiol. Rev. 24, 718–733.
Ng, W.K., Koh, C.B., 2011. Application of organic acids in aquafeeds: impacts on fish growth, nutrient utilisation and disease resistance. In: Luckstadt, C. (Ed.), Standards for Acidifiers — Principles for the Use of Organic Acids in Animal Nutrition. Nottingham University Press, Nottingham, United Kingdom, pp. 49–58.
Ng, W.K., Koh, C.B., Sudesh, K., Siti-Zahrah, A., 2009. Effects of dietary organic acids on growth, nutrient digestibility and gut microflora of red hybrid tilapia, *Oreochromis* sp., and subsequent survival during a challenge test with *Streptococcus agalactiae*. Aquac. Res. 40, 1490–1500.
Pandey, A., Satoh, S., 2008. Effects of organic acids on growth and phosphorus utilization in rainbow trout *Oncorhynchus mykiss*. Fish. Sci. 74, 867–874.
Park, G.H., Lee, J.H., Yun, H.H., Browdy, C.L., Bharadwaj, A.S., Bai, S.C.C., 2011. Effects of two different organic acid blends in olive flounder. Korean J. Org. Agric. 19, 39–42.
Ringø, E., 1991. Effects of dietary lactate and propionate on growth and digesta in Arctic charr, *Salvelinus alpinus* (L). Aquaculture 96, 321–333.
Romano, N., Zeng, C., 2013. Toxic effects of ammonia, nitrite, and nitrate to decapod crustaceans: a review on factors influencing their toxicity, physiological consequences, and coping mechanisms. Rev. Fish. Sci. 21, 1–21.
Romano, N., Koh, C.B., Ng, W.K., 2015. Dietary microencapsulated organic acids blend enhances growth, phosphorus utilization, immune response, hepatopancreatic integrity and resistance against *Vibrio harveyi* in white shrimp, *Litopenaeus vannamei*. Aquaculture 435, 228–236.
Sarker, M.S.A., Satoh, S., Kiron, V., 2007. Inclusion of citric acid and/or amino acid-chelated trace elements in alternate plant protein source diets affects growth and excretion of nitrogen and phosphorus in red sea bream *Pagrus major*. Aquaculture 262, 436–443.
Sarker, M.S.A., Satoh, S., Kamata, K., Haga, Y., Yamamoto, Y., 2012. Supplementation effect(s) of organic acids and/or lipid to plant protein-based diets on juvenile yellowtail, *Seriola quinqueradiata* Temminck et Schlegel 1845, growth and, nitrogen and phosphorous excretion. Aquac. Res. 43, 538–554.
Sung, H.H., Hsu, S.F., Chen, C.K., Ting, Y.Y., Chao, W.L., 2001. Relationships between disease outbreak in cultured tiger shrimp (*Penaeus monodon*) and the composition of *Vibrio* communities in pond water and shrimp hepatopancreas during cultivation. Aquaculture 192, 101–110.
Thompson, J.L., Hinton, M., 1997. Antibacterial activity of formic and propionic acids in the diet of hens on salmonellas in the crop. Br. Poult. Sci. 38, 59–65.
Tran, L., Nunan, L., Redman, R.M., Mohney, L.L., Pantoja, C.R., Fitzsimmons, K., Lightner, D.V., 2013. Determination of the infectious nature of the agent of acute hepatopancreatic necrosis syndrome affecting penaeid shrimp. Dis. Aquat. Org. 105, 45–55.
Vogt, G., Storch, V., Quinitio, E.T., Pascual, F.P., 1985. Midgut gland as monitor organ for the nutritional value of diets in *Penaeus monodon* (Decapoda). Aquaculture 48, 1–12.