 doi:10.20944/preprints202106.0108.v1

Growth performance, immunity, and disease resistance of Pacific white shrimp fed dietary organic acid and their salts microencapsulated with four materials

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Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 3 June 2021 doi:10.20944/preprints202106.0108.v1

ABSTRACT

A 63-day trial with Pacific white shrimp (0.33g) was conducted to assess the effects of two forms of organic acid (COMP), free acids (OA) and acid salts (OS) microencapsulated (ENCAP) with hydrogenated fat (HF), hydrogenated fat + alginate (HA), wax esters – (WE), and double coating of HA and WE (HAWE) on their growth performance, nutrient utilization, digestive enzyme, immune response and resistance to pathogenic *Vibrio parahaemolyticus*. A high fishmeal (FM) diet containing 200 g/kg FM and a low FM diet containing 130 g/kg FM and 120 g/kg soybean meal were formulated as positive (PC) and negative control (NC), respectively. Eight other diets were formulated supplementing NC diet with microencapsulated OA (OAHF, OAHA, OAWE, OAHAWE) and OS (OSHF, OSHA, OSWE, OSHAWE). All diets were formulated to be iso-proteic (36.9% CP) and iso-energetic (16.1 MJ/g). Total 1600 shrimps were distributed to 40 tanks (40 shrimps/tank and 4 replicates/treatment). Shrimp fed diets containing OA showed significantly lower feed intake ($P = 0.017$) and FCR ($P = 0.012$), and higher protein retention efficiency ($P = 0.016$) than those fed the OS diets. Among the ENCAPs, significant difference was observed in serum malondialdehyde ($P = 0.026$) where HF showed the lowest level (6.4 ±0.3 mmol/L). Significant interactions between COMP and ENCAP were observed in lipid deposition ($P = 0.047$), serum alkaline phosphatase and acid phosphatase ($P < 0.0001$), and hepatopancreatic and serum phenol oxidase ($P < 0.0001$). Despite no differences, the 96-h mortality in all treatment diets (45% - 56%) was significantly lower compared to

 doi:10.20944/preprints202106.0108.v1

the NC diets (63%) under pathogenic *vibrio parahaemolyticus* challenge tests. Overall score combining the parameters shows that shrimp fed the OA diets with HF microencapsulation performed better compared to those fed diets with OS and other microencapsulation materials.

Key words

Organic acid; Digestive enzymes; Immune response; Microencapsulation; *Vibrio* sp.; Shrimp

1. Introduction

The global farmed shrimp industry is frequently plagued with disease outbreaks starting from yellow head (YHV) and white spot syndrome (WSSV) virus in the 1990s to more recently, acute hepatopancreatic necrosis disease (AHPND) (Flegel 2019, Ng et al. 2015). The frequent outbreaks led to an increased use of antibiotics as a metaphylactic or prophylactic to treat or prevent diseases, respectively or as antibiotic growth promoters (AGP) (Limbu et al. 2020). Reducing antibiotic use in farmed animals for disease control and banning as AGP is a global trend driven mainly by the increasing risk of antibiotic resistant bacteria (WHO 2017, Zhao et al. 2020).

Various alternatives to AGP such as, phytogenic compounds or plant derived essential oils (Kesselring et al., 2020, Yang et al. 2015), probiotic, prebiotic and synbiotic (Jamal et al. 2019, Jueliang et al. 2013), enzymes (Song et al. 2017, Yao et al. 2019), organic acids and their salts (Da Silva et al. 2013, Krome et al. 2018, Mine and Boopathy 2011, Ng et al. 2015, 2017, Nascimento et al. 2020) have been proposed

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in recent years. Organic acids are “Generally Regarded as Safe” compounds often containing one or more carboxyl groups (–COOH) (Defoirdt et al. 2009, Sarder et al. 2020). The most common are those with short chain (C1–C6) such as formic, lactic, propionic and citric acids, and their salts. Their probable mode of actions includes reducing the digesta pH, stimulating digestive enzyme secretion, promoting intestinal integrity, and regulating gut microbial populations. The efficacy of an acid in inhibiting microbes is dependent on its p*K*a value, which is the pH where 50% of the acid is dissociated. The p*Ka* of organic acids ranges from 3.02 for fumaric acid (COOHCH:CHCOOH) to as high as 6.4 for citric acid (COOHCH2C(OH)(COOH)CH2COOH) (Reviewed in Ng and Koh 2016).

Intestinal pH usually ranges from slightly acidic (>6.4) in the proximal intestine to full alkaline (>8.0) in the rest of the intestine, e.g., tilapia (Payne, 1978). In Pacific white shrimp, the pH remains above 8.0 throughout the gastrointestinal tract. The organic acids and their salts need to remain in undissociated form or for dissociated form, pH needs to be highly acidic to be effective against most pathogens (Eklund, 1983). The required high dosage (2-5 g/kg) to suppress intestinal pH induces high stress and costs significant energy to maintain homeostasis (Li et al. 2019; Yu et al. 2020). An alternative strategy is to encapsulate active ingredient to bypass the proximal intestine ensuring their release in the microbe rich hind gut.

Microencapsulation has been becoming one of the most popular and practical approaches to deliver bioactive compounds in the GI tract of farmed animals

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(Chitprasert and Sutaphanit 2014, Omonjio 2018, Piva et al. 2007, Yang et al. 2019). An ideal encapsulation should not only present the stability of the active compound but also release them in the target regions of the intestine (Chen et al. 2016). Many materials including polysaccharides (alginate and xanthan gum), starch, proteins (whey protein and gelatin) and lipids (milk fat and hydrogenated fat) have been used for encapsulation for effective delivery in the gut (El Asbahani et al. 2015, Fiorda et al. 2015, Romano et al. 2018, Tester et al. 2004, Udachan et al. 2012, Zhu 2017). Hydrogenated fat has been considered one of the most cost-effective materials for encapsulating bioactive compounds because of low cytotoxicity (Müller et al. 2000) and higher stability (Souto and Müller 2010). Alginate, a natural polymer derived from brown seaweed and a linear and anionic polysaccharide (Dragan 2014). At room temperature, alginate is soluble in water allowing the formation of gel without heating and cooling cycles, which make alginate as an attractive material for feed applications (Agüero et al. 2017, Benavides et al. 2016). The inclusion of alginate to the starch or hydrogenated fat matrix can improve the shape and surface properties that could be attributed to its remarkable crosslinking capability and excellent film-forming properties (Costa et al. 2018). Recently, an advanced lipid-based delivery system has been developed using edible wax as an encapsulation material (Soleimanian et al. 2020).

Both organic acids and their salts have been used in aquafeed for better performance and disease resistance of aquatic animals (Huan et al. 2018). The blends of organic acids used in this study are fumaric acid, sorbic acid and citric acid. Salts of organic

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acids used are calcium propionate, calcium formate, and sodium acetate. Dietary fumaric acid (catfish, Omosowone et al. 2015), fumaric and sorbic acid (*E. coli*, Lu et al. 2011), citric acid (*E. coli*, Allende et al. 2009), calcium propionate (tilapia, Reda et al. 2016; silver catfish, Pereira et al. 2018), calcium formate (shrimp Da silva et al. 2013) and sodium acetate (tilapia, Li et al. 2020; yellowfin seabream, Sangari et al. 2020) showed varying level of antimicrobial activity in-vitro and in various farmed species. Most of these studies tested a single compound in free-form and rarely in combination of two or more compounds. In addition, there are very few studies with shrimp using dietary microencapsulated blend of organic acid or salt. To address this gap, in this study, the effects of blends of organic acids (fumaric acid, sorbic acid and citric acid) and organic acid salts (calcium propionate, calcium formate and sodium acetate) encapsulated with hydrogenated fat - HF, a mixture of HF and alginate - HA, wax esters - WE, and double coating with HA and WE - HAWE on Pacific white shrimp performance, immune response and disease resistance were assessed.

2. Materials and methods

The experiment had two components: in-vitro stability tests of the microencapsulation materials and in-vivo feeding trial with Pacific white shrimp fed diets supplemented with microencapsulated blends of fumaric, sorbic and citric acids (OA) and calcium propionate, calcium formate and sodium acetate (OS).

2.1. Stability tests

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Four microencapsulation products using hydrogenated fat (HF), HF and alginate (HA), wax esters (WE) and double coating with HA followed by WE (HAWE) as encapsulation materials were tested to determine solubility or leaching of the active ingredient. All four products were prepared by spray drying and congealing where active ingredients are dispersed in HF, HA, WE, and for the double coated HAWE, the process was conducted first with HA and them repeated with WE using a process slightly modified from Jyothi et al. (2010). In brief, active ingredients are dispersed in a solution and spray-dried where the material solidifies onto the particles of active ingredients such that the microcapsules obtained are of matrix type.

For solubility, 10 g of each test product was mixed with 200 ml of deionized water, then stirred for 6 hrs at 100 rpm at 19 ^{o}C. After 6 hrs, the supernatant was filtered, and insoluble active ingredient from the filtrate was dried and weighed. A mix of organic acids corresponding to the active ingredients of the micro-encapsulated product was used as a control. The pH of the solution was determine following the same protocol mentioned above. The pH of the supernatant was determined after filtration. Each treatment was conducted in triplicates.

2.2. Feeding trial

The feeding trial was conducted for 63 days at the Guangdong Ocean University field experimental station situated at Donghai Island, Zhanjiang of Guagdong province of China. Experimental procedure and animal care were accomplished in accordance

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with the ethical guidelines for the care and use of laboratory animals provided by the Animal Care Committee of the Guangdong Ocean University.

2.2.1. Experimental design and diet preparation

Ten isoproteic (37.3 ±0.12 % CP) and isoenergetic (16.4 ±0.02 MJ/kg) diets were prepared: diet 1 - positive control with 20% FM (PC); diet 2 - negative control with 13% fishmeal and 12% meat and bone meal (NC); diets 3-6 were manufactured by supplementing NC with 0.75 mg/kg of OA microencapsulated with HF, HA, WE and HAWE (OAHF, OAHA, OAWE and OAHAWE, respectively); and diets 7-10 were manufactured by supplementing 0.85 mg/kg of OS microencapsulated with HF, HA, WE and HAWE (OSHF, OSHA, OSWE and OSHAWE, respectively) (Table 1 & 2). It was ensured that all microcapsulated products contain same amount of active ingredients. The microencapsulated test products were supplied by Jefo Nutrition Inc., Quebec, Canada. Diet composition and their proximate chemical composition including amino acid profile are provided in Table 1 and 2, respectively.

All feed ingredients were ground, sieved through 80 mesh screens, mixed with a V-type mixer (Shanghai Tianxiang & Chentai Pharmaceutical Co., Ltd., Shanghai, China), pelleted with a screw pelletizer (South China university of technology, Guangzhou, China) after adding 30% water, air-dried, and then stored at -20 ^{o}C until used. Pellets of two different sizes, 1.0- and 1.5-mm diameter, were produced for the trial.

2.2.2. Experimental conditions

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Twenty-five thousand PL8-10 Pacific white shrimp *L. vannamei* postlarvae were obtained from Allied Pacific Aquaculture Co., Ltd., Zhanjiang, Guangdong, China. The shrimp were acclimatized in two cement pools for 40 days until the average body weight reached 0.3 g. From the cement pools, a total of 1600 white shrimp (0.33 ±0.02g ABW) were selected and 40 shrimp/tank were randomly distributed into 40 cone-shaped tanks (350-L volume each) with four replicates per treatment.

The shrimp were fed the experimental diets four times daily (7:00, 11:00, 17:00 and 21:00 h) at 8%-10% of their body weight. The water was completely exchanged once in every 2-3 days from 1st to 4th week and once daily from 5th to 9th week.

2.2.3. Sampling

At the end of the experiment, shrimp were fasted for 24 hours before the final sampling. From each treatment, 15 and 10 shrimps were randomly selected from each tank for serum and hepatopancreatic analyses, respectively. Both analyses were not conducted on same shrimp because of possibility of influence of one sampling on another. For serum, the blood was drawn using a dispensable 1 ml syringe into 1.5 ml test-tube. The test-tubes were then stored at 4 ^{o}C overnight before being centrifuged at 5867 g for 10-min at 4 ^{o}C (3K30, Sigma, Germany). The supernatant was then collected into 1.5 ml tube and stored at -80 ^{o}C for subsequent analyses. Hepatopancreas was removed from each shrimp, immediately frozen in liquid nitrogen, and then stored at -80C for analyses. Another six shrimps from each tank were taken for body chemical composition, ground into slurry, lyophilized and kept at -20 ^{o}C until analyses.

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2.3. Chemical analyses and enzymatic assay

Diets, ingredients, body chemical composition were analyzed following AOAC (1995). Nitrogen for crude protein (CP, %N×6.25) was analyzed using a Kjeldahl apparatus (Kjeltec^{TM} 8400, FOSS, Sweden), moisture by drying the samples at 105 ^{o}C under atmospheric pressure for 24 hours, crude lipid using a Soxhlet apparatus (Soxtec^{TM} 2050, FOSS, Sweden), crude ash by burning the samples at 550 ^{o}C using a muffle furnace (Shanghai Boxun industry & Commerce Co., Ltd., Shanghai,China), and gross energy using a bomb calorimeter (Changsha Kaiyuan Instuments, Changsha, China).

The activity of acid (ACP) and alkaline phosphatase (ALP), total superoxide dismutase (T-SOD), malondialdehyde (MDA), lipase and amylase were determined using diagnostic kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). Prophenoloxidase (PO) activity was measured spectrophotometrically by recording the formation of dopachrome produced from *L*-di-hydroxy-phenylalanine (*L*-DOPA) following a procedure slightly modified from Huang et al. (2010). In brief, 3 mg/ml *L*-DOPA solution was prepared by using 1 L of 0.1M potassium phosphate buffer (0.1 M $K_2HPO_4 \cdot 3H_2O$, 0.1 M KH_2PO_4, adjusted to pH 6.6). Shrimp serum (20µl) was mixed thoroughly with 980 µl *L*-DOPA solution. A 300 µl of the mixture was placed in a 96-well plate and incubated at room temperature. The absorbance was recorded after 6 min (OD_{sample}) on a Microplate Spectrophotometer (Multilskan spectrum, Thermo, USA) at 490 nm. At the same time, 300 µl of *L*-DOPA solution was placed in a 96-well plate

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and absorbance of the blank control group was recorded (OD_{blank}). Enzymatic activity for all assays was expressed as the change in absorbance/min.

2.4. Resistance to Vibrio parahaemolyticus

Resistance to the pathogen, *V. parahaemolyticus* was determined from the cumulative mortality of shrimp in 96 h. For this, 10 shrimps for each replicate (3 replicates in each treatment) were used. After injecting each shrimp with 2.4×10^7 colony-forming units (CFU) of *V. parahaemolyticus*, the cumulative mortality in 96 h was recorded.

2.5. Scoring

All variables from treatment 3-8 were grouped into three categories to determine the most suitable composition (COMP: free acid vs acid-salt) and microencapsulation (ENCAP: HF, HA, WE and HAWE), and scored ranging from 1-8. The scores assigned from smallest to largest are as follows: growth performance (SGR – 1-8; FCR – 8-1; and PER – 1-8), nutrient utilization (PRE – 1-8; LRE – 1-8; and amylase (1-8) and lipase (1-8) activity), and immune response (serum SOD – 1-8, ALP – 1-8, ACP – 1-8, PO – 1-8 and MDA – 8-1; and cumulative mortality – 8-1).

2.6. Calculation

Specific growth rate (SGR) = [(ln final weight – ln initial weight)/Days] X 100.

Feed conversion ratio (FCR) = Feed intake/Weight gain.

Protein efficiency ratio (PER) = (Weight gain/Protein intake) X 100.

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Serum MDA (nmol/mL) = ($OD_{sample} - OD_{sample\ blank}$)/($OD_{standard} - OD_{standard\ blank}$) X standard concentration (10nmol/ml) X sample dilution times before assay.

Serum T-SOD (U/mL) = ($OD_{contrast} - OD_{sample}$)/$OD_{contrast}$/50% X reaction system dilute multiple X sample dilute multiple before assay.

Hepatopancreas T-SOD (U/mL) = ($OD_{contrast} - OD_{sample}$)/$OD_{contrast}$/50% X reaction system dilute multiple/protein content in hepatopancreas (mgprot/mL).

Serum ACP (King U/100mL) = ($OD_{sample} - OD_{blank}$)/($OD_{standard} - OD_{blank}$) X standard concentration (0.1 mg/mL) X 100 mL X sample dilution times before assay.

Hepatopancreas ACP (King U/gprot) = ($OD_{sample} - OD_{blank}$)/($OD_{standard} - OD_{blank}$) X standard concentration (0.1 mg/mL)/protein content in hepatopancreas (gprot/mL).

Serum ALP (King U/100mL) = ($OD_{sample} - OD_{blank}$)/($OD_{standard} - OD_{blank}$) X standard concentration (0.1 mg/mL) X 100 mL X sample dilution times before assay.

Hepatopancreas ALP (King U/gprot) = ($OD_{sample} - OD_{blank}$)/($OD_{standard} - OD_{blank}$) X standard concentration (0.1 mg/mL)/protein content in hepatopancreas (gprot/mL).

PO (U/mL) = ($OD_{sample} - OD_{blank}$) /6 X 1000 X 1000/20.

Amylase (U/gprot) = ($OD_{blank} - OD_{assay}$)/OD_{blank} X 80/[volume of sample (0.1mL) X protein concentration (mgprot/mL)] X 1000

Lipase (U/gprot) = ($A_{sample1}$-$A_{sample2}$)/$A_{standard}$ X Standard tube concentration (454 umol/L) X Sample dilution times in reaction system/Reaction time length (10 min)/Protein concentration in sample homogenate (gprot/L)

2.7 Statistical analysis

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All data were expressed as the mean ±SD (standard deviation) and subjected to one-way ANOVA (SPSS 17.0, Chicago, IL, USA). Percentage data were arcsine-square root transformed before statistical analysis. Multiple comparison analyses were performed using Duncan's multiple-range tests. Statistically significant differences were considered at $P < 0.05$.

3. Results

During the feeding trial, the water temperature was ranged between 28 ^{o}C and 34 ^{o}C, and salinity, dissolved oxygen and total ammonia nitrogen content were maintained at 27-28 g/L, >7 mg/L, and <0.03 mg/L, respectively. Feed intake was normal, and survival was not affected by the dietary treatments.

3.1 Stability test of the microencapsulation materials

The pH-value were similar among the non-protected acids, HF and HA microencapsulation (2.8-2.9) which slightly increased with WE (3.2) and HAWE (3.5) microencapsulation (Figure 1A). All four microencapsulation materials showed significantly higher recovery than the free acid. Corresponding to the pH values, the recovery was significantly higher for WE (95%) and HAWE (97%) compared to HF (74%) and HA (77%) (Figure 1B).

3.2 Growth Performance and body composition

Feed intake and growth were normal and similar to the studies conducted at the laboratory. Effects of the microencapsulated OA and OS on body chemical composition and final body weight, specific growth rate (SGR), feed conversion ratio (FCR), and

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protein efficiency ratio (PER) are presented in tables 3 and 4, respectively. The form of organic acids (free or salt) significantly affected the feed intake and FCR where shrimp fed diets with OA showed lower FCR and feed intake compared to those fed the OS diets ($P < 0.05$). There were no differences ($P > 0.05$) in body chemical composition among the treatments.

3.3 Nutrient utilization and hepatopancreatic enzyme activity

Either the form of organic acid (COMP) or the microencapsulation (ENCAP) did not affect ($P > 0.05$) protein deposition, lipid retention efficiency, and hepatopancreatic amylase and lipase activity (Table 5). However, protein retention efficiency of shrimp fed diets supplemented with OA (0.29) was significantly higher ($P = 0.016$) than those fed the OS (0.28) diets. Significant interaction (COMP*ENCAP) was observed in lipid deposition where OS (0.27) and HAWE (0.28) was higher compared to OA (0.26) and HF (0.24), HA (0.27) and WE (0.25) ($P = 0.047$).

3.4 Immune response and disease resistance

No differences in serum SOD, hepatopancreatic ALP, ACP and MDA (Table 6), and cumulative 96-h mortality (Figure 2) when challenged with *Vibrio parahaemolyticus* was observed with either main effects of COMP, ENCAP or their interaction (Table 6). Significant interaction was observed for serum ALP ($P < 0.0001$), ACP ($P < 0.0001$), and hepatopancreatic and serum phenol oxidase level ($P < 0.0001$). Significantly lower serum MDA level ($P < 0.026$) was observed in HF (6.4) compared to the other microencapsulation (HA = 7.7, WE = 6.9 and HAWE = 7.7).

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3.5 Scoring

Shrimp fed the OA diets showed higher scores in growth performance (58 vs 38), nutrient utilization (67 vs 57) and immune response (112 vs 96) than those fed the OS diets with a combined score of 237 compared to 191 (Table 7). Among the four microencapsulation materials, the overall scores of HF and HA (118 and 117, respectively) were higher than WE (95) and HAWE (98) ($P < 0.05$) (Table 7).

4. Discussion

This study investigated the efficacy of dietary organic acids (free or salt) microencapsulated with hydrogenated fat (HF), hydrogenated fat + alginate (HA), wax esters (WE), and the double coating of HAWE (first coated with HA followed by WE) on performance of Pacific white shrimp. The organic acid blend contains fumaric acid (p*K*a = 3.03), sorbic acid (p*K*a = 4.75) and citric acid (p*K*a = 2.92-5.21). Whereas, the organic acid salt blend contains Ca-propionate, Ca-formate and Na-acetate.

Organic acids are low molecular weight aldehyde containing compounds with one or more carboxyl groups. They are used as dietary supplement to reduce gastrointestinal tract pH and inhibit the growth of gram-negative bacteria through the disassociation of the acids and production of anions in bacterial cells (Hosseinifar et al. 2016). As weak acids, the p*K*a values or the disassociation constant of organic acids are higher than the strong acids such as HCl or H_2SO_4 (Soames et al. 2018). These acids do not dissociate in the highly acidic stomach pH but tend to dissociate quickly in the proximal intestine as pH increases and the condition becomes alkaline. Shrimp are slow-eating animals

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taking 1-2 hours for holding and chewing the pellets. In free-form, organic acid or their salts have considerable risk of leaching in water preventing them from reaching the hepatopancreas and gut in undissociated form (Romano et al. 2015). Coating or encapsulation may significantly reduce leaching and consequently, can remain effective at lower dosage (Yao et al. 2019). For example, micro-encapsulated organic acid salt blend used by Yao et al. (2019) and in this study, is much lower (835 mg/kg) than in their free form (2000-6000 mg/kg) reported in various studies (Chuchird et al. 2015; Su et al. 2014). Micro-encapsulation provides better protection than simple coating that may prevent or reduce the loss of active ingredient in case of breakage of the prills as active ingredients are embedded in the matrix of coating material (Bakry et al. 2015).

Microencapsulation of easily degradable bioactive compounds has been becoming a popular and practical approach to mask unpleasant characteristics of the compounds and deliver them at the intended location of the gastrointestinal tract (Chen et al. 2017; Piva et al. 2007). In this study, despite their lower solubility and recovery, both HF and HA (118 and 117, respectively) had higher total performance scores in-vivo compared to WE and HAWE (95 and 98, respectively (Table 7). However, between HF and HA, growth performance score was higher for HA but lower for immune response than those for HF. No differences in the nutrient utilization score was observed between the two materials. Omnojio et al. (2018) tested both HF and HA *in vitro* and observed well-timed release of the active ingredient. Timely release of active ingredient at the intended location of the digestive tract is utterly important for their efficacy. Hydrogenated fat

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can be easily digested by intestinal lipase thus guaranteeing the slow release of the active ingredient along the GI tract. In a recent study, Ndiyae et al. (2020) reported efficacy of HF based microencapsulated aluminum and iron sulfate in in-situ chelation of undigestible phosphorus in the hind gut of rainbow trout. The study confirms the release of the active ingredient in the hindgut where it was intended to bind with phosphorus thus reducing the risk of eutrophication of the surrounding environment. Relatively poor performance of shrimp fed WE diets compared to those fed other treatment diets may be attributed to low solubility and higher retention of active ingredient than hydrogenated fat (Figure 1). Wax based solid lipid matrix provides better physical stability and more protection against chemical reaction (Soleimanian et al. 2020). The positive characteristics such as slower degradation and mass transfer rate may not be suitable for shrimp for their short gut-transit time (~2 hours) to release the active ingredient.

Blends of organic acids and their salts in free or microencapsulated forms have shown to improve growth performance of fish (Huan et al. 2018; Moradi 2015; Sherif and Doaa 2013) and shrimp (Ng et al. 2015; Romano et al. 2015; Rombenso et al. 2020; Yao et al. 2019) as well as antioxidant status (He et al. 2017). Several studies reported improved growth performance, nutrient utilization and immune response in crustacean fed microencapsulated blend of organic acid or acid salts. Safari et al. (2020) reported efficacy of encapsulated blend of Na-butyrate, Na-lactate and Na-propionate on growth performance and survival of crawfish at 20g/kg. The OS blend used in the present study

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contains Ca-propionate, Ca-formate and Na-acetate and showed higher feed intake compared to those fed the OA diets. Yao et al. (2019) also reported improved weight gain and FCR in Pacific white shrimp compared to NC diet with the same OS blend. When compared between the OA and OS treatments of this study, shrimps fed the OA diets showed improved FCR, protein retention, and immune response i.e., higher ALP and PO compared to the OS blend (Table 4-6). This is in accordance with the findings of Romano et al. (2015), who reported improved growth performance of Pacific white shrimp with 1-4% microencapsulated OA (blend of formic, lactic, malic and citric acids).

In an in-vitro study, Mine and Boopathy (2011) demonstrated EC50 values of 0.023%, 0.041%, 0.03%, and 0.066% for formic, acetic, propionic and butyric acid, respectively against *Vibrio harveyi*. Romano et al. (2015) showed similar efficacy in *V. harveyi* resistance when fed OA supplemented diets. Efficacy of organic acid in combination with essential oil against *Vibrio* sp. infections also demonstrated by He et al. (2017), where a microencapsulated blend of organic acid (citric acid and sorbic acid) and essential oils (thymol and vanillin) showed significantly higher survival in Pacific white shrimp challenged with *V. parahaemolyticus* after 48-h compared to those fed the control diets. These are in accordance with the findings of the present study where treatments containing microencapsulated organic acid and organic acid salt blends showed significantly lower cumulative 96-h mortality ranging from 45 to 56%

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compared to 63% for those fed the NC diets when challenged with pathogenic *V. parahaemolyticus* (Figure 2).

This is one of the first reports comparing the effects of OA and OS on performance, nutrient utilization, immune response and disease resistance of Pacific white shrimp as well as comparing different microencapsulation materials and techniques in the same study. Based on the findings, it can be concluded that organic acid blend microencapsulated with hydrogenated fat or hydrogenated fat + alginate may provide better responses in Pacific white shrimp and can be used as an effective strategy to improve immune response and disease resistance. Further studies are recommended to investigate the effects of microencapsulated organic acid compounds on intestinal health, metabolic response and gut microbiome of farmed Pacific white shrimp.

Author contribution

Xiao-Hui Dong and Mohiuddin Amirul Kabir Chowdhury designed the experiment, prepared the first draft of the manuscript, Hong-Li Song conducted the feeding trial, laboratory assays and statistical analysis, Jean-Daniel Bunod conducted the in-vitro tests and reviewed the manuscript, Xiao-Hui Dong supervised the trial and reviewed the manuscript, and Liu Yao participated in the feeding trial and reviewed the manuscript.

Conflict of interest

The authors declared no conflict of interest.

Funding and Acknowledgement

This study was supported by the Special Fund for Agroscientific Research of Public Interest (No. 201003020), by Guangdong Ocean University. We would like to extend our sincere thanks to Ze-Zhong Tian and Qiu-Long Liu for their assistance during the study.

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Table 1. Ingredient composition of the control and test diets

Ingredient (g/kg)	PC	NC	OAHF	OAHA	OAWE	OAHAWE	OSHF	OSHA	OSWE	OSHAWE
Fish meal, 70% CP	200.0	130.0	130.0	130.0	130.0	130.0	130.0	130.0	130.0	130.0
Shrimp head meal, 46% CP	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0	30.0
Soybean meal 50% CP	30.0	120.0	120.0	120.0	120.0	120.0	120.0	120.0	120.0	120.0
Corn gluten meal, 61% CP	60.0	60.0	60.0	60.0	60.0	60.0	60.0	60.0	60.0	60.0
Peanut meal, 41% CP	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0	75.0
Soybean meal, 52% CP	120.0	120.0	120.0	120.0	120.0	120.0	120.0	120.0	120.0	120.0
Wheat flour	318.0	318.0	318.0	318.0	318.0	318.0	318.0	318.0	318.0	318.0
Fish oil	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0
Soy lecithin	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
Soybean oil	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0	15.0
Lysine-HCl	0.0	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3
Methionine	0.0	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2	1.2
Choline chloride	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0	5.0
Di-calcium phosphate	10.8	10.8	10.8	10.8	10.8	10.8	10.8	10.8	10.8	10.8
Mineral premix[a]	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0	8.0
Vitamin premix[b]	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0	2.0
Antioxidant	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
Microencapsulated OA or OS	0.0	0.0	0.75	0.75	0.75	0.8	0.850	0.9	0.9	0.9
Cellulose	99.4	75.9	75.2	75.2	75.2	75.2	75.1	75.1	75.1	75.1
Vitamin C	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Attractant	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Total	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0	1000.0

Note: PC - positive control, NC = negative control, OA - organic acid, OS - organic acid salt, HF - hydrogenated fat, HA - HA + alginate, WE - wax ester, HAWE - double coating with HA and WE.

[a]Contained the following (per kg of mineral premix): KIO_4 0.03 g, $CoCl_2 \cdot 6H_2O$ 4.07 g, $CuSO_4 \cdot 5H_2O$ 19.84 g, ferric citrate 13.71 g, $ZnSO_4 \cdot 7H_2O$ 28.28 g, $MgSO_4 \cdot 7H_2O$ 0.12 g, CaH_2PO_4 80 g, $MnSO_4 \cdot H_2O$ 12.43 g, KCl 15.33 g, Na_2SeO_3 2 g, zeolite power 824.19 g.

[b]Contained the following (per kg of vitamin premix): Vit-A 10 g, Vit-D3 50 g , Vit-E 99 g, Vit-K 5.0 g, Vit-B_1 25.50 g, Vit-B_2 25 g, Vit-B_6 50 g, Vit-B_{12} 0.1 g, calcium pantothenate 61 g, nicotinic acid 101 g, biotin 25 g, inositol 153.06 g, folic acid 6.25 g, cellulose 389.09 g.

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Table 2. Proximate chemical composition and calculated essential amino acid profile of the control and test diets (dry matter – DM basis)

Proximate composition, DM basis	PC	NC	OAHF	OAHA	OAWE	OAHAWE	OSHF	OSHA	OSWE	OSHAWE
Dry matter, %	91.3	91.6	91.5	91.6	91.6	91.5	91.8	91.6	91.5	91.5
Crude protein, %	37.2	37.2	37.2	37.4	37.1	37.4	37.3	37.4	37.4	37.4
Crude lipid, %	8.0	8.0	7.9	7.9	7.9	8.0	7.9	8.0	8.0	8.0
Crude ash, %	8.0	8.0	7.9	7.9	7.9	8.0	8.0	7.9	8.0	8.0
Gross energy, MJ/kg	16.4	16.4	16.4	16.4	16.4	16.4	16.5	16.5	16.4	16.4
Digestible EAA, %										
Methionine, %	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Cystine, %	0.47	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45	0.45
Methionine + Cystine, %	1.27	1.24	1.24	1.24	1.24	1.24	1.24	1.24	1.24	1.24
Lysine, %	2.17	2.17	2.17	2.17	2.17	2.17	2.17	2.17	2.17	2.17
Tryptophan, %	0.39	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35	0.35
Threonine, %	1.31	1.28	1.28	1.28	1.28	1.28	1.28	1.28	1.28	1.28
Isoleucine, %	1.33	1.28	1.28	1.28	1.28	1.28	1.28	1.28	1.28	1.28
Histidine, %	0.88	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90	0.90
Valine, %	2.08	1.91	1.91	1.91	1.91	1.91	1.91	1.91	1.91	1.91
Leucine, %	2.52	2.45	2.45	2.45	2.45	2.45	2.45	2.45	2.45	2.45
Arginine, %	2.41	2.28	2.28	2.28	2.28	2.28	2.28	2.28	2.28	2.28
Phenylalanine, %	1.41	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40	1.40
Tyrosine, %	0.86	0.71	0.71	0.71	0.71	0.71	0.71	0.71	0.71	0.71
Phenylalanine + Tyrosine, %	2.27	2.11	2.11	2.11	2.11	2.11	2.11	2.11	2.11	2.11

Note: PC - positive control, NC = negative control, OA - organic acid, OS - organic acid salt, HF - hydrogenated fat, HA - HA + alginate, WE - wax ester, HAWE - double coating with HA and WE

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Table 3. Whole body chemical composition of shrimps fed the control and test diets (dry matter basis)

Treatments	Dry matter (%)	Crude protein (%)	Crude lipid (%)	Crude ash (%)
PC	22.9 ±0.72	73.2 ±0.32	8.7 ±0.57	13.5 ±0.35
NC	22.6 ±0.98	73.9 ±1.11	8.5 ±0.49	13.2 ±0.67
OAHF	22.3 ±0.84	73.4 ±1.83	8.2 ±0.77	13.3 ±1.05
OAHA	23.2 ±0.58	74.6 ±1.06	8.7 ±0.30	13.1 ±0.21
OAWE	22.8 ±0.53	74.3 ±0.15	8.2 ±0.82	13.7 ±0.69
OAHAWE	22.9 ±0.77	74.0 ±0.91	8.5 ±0.62	13.5 ±0.63
OSHF	22.5 ±0.78	73.7 ±0.98	8.1 ±0.45	13.5 ±0.30
OSHA	23.2 ±0.77	73.6 ±0.30	8.9 ±0.62	13.2 ±0.76
OSWE	23.1 ±0.58	73.4 ±1.47	9.0 ±0.46	13.2 ±0.72
OSHAWE	22.8 ±0.65	73.7 ±0.77	8.8 ±0.68	14.0 ±0.70
COMP				
OA	22.7 ±0.36	13.3 ±0.28	74.0 ±0.49	8.4 ±0.24
OS	22.9 ±0.29	13.3 ±0.15	73.7 ±0.28	8.6 ±0.39
ENCAP				
HF	22.4 ±0.13	13.4 ±0.13	73.6 ±0.20	8.2 ±0.07
HA	23.2 ±0.00	13.2 ±0.08	74.1 ±0.67	8.8 ±0.18
WE	23.0 ±0.17	13.5 ±0.39	73.8 ±0.63	8.6 ±0.57
HAWE	22.9 ±0.07	13.7 ±0.38	73.9 ±0.25	8.7 ±0.17
P-Value				
COMP	NS	NS	NS	NS
ENCAP	NS	NS	NS	NS
COMP*ENCAP	NS	NS	NS	NS

Note: PC - positive control, NC = negative control, OA - organic acid, OS - organic acid salt, HF - hydrogenated fat, HA - HA + alginate, WE - wax ester, HAWE - double coating with HA and WE; COMP – composition; ENCAP – microencapsulation.

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Table 4. Growth performance (final body weight, specific growth rate, feed intake, feed conversion ratio, protein efficiency ratio) of shrimp fed the control and test diets.

Treatments	Final body weight - FBW (g)	Specific growth rate - SGR	Feed intake (g/shrimp)	Feed conversion ratio - FCR	Protein efficiency ratio - PER
PC	13.0 ±1.9ab	5.7 ±0.2ab	20.9 ±2.8ab	1.65 ±0.04ab	1.63 ±0.04ab
NC	12.3 ±0.6b	5.6 ±0.1b	20.5 ±0.5ab	1.72 ±0.05a	1.57 ±0.04b
OAHF	13.1 ±1.3ab	5.7 ±0.2ab	19.8 ±1.8b	1.56 ±0.10b	1.73 ±0.11a
OAHA	13.3 ±1.0ab	5.8 ±0.1ab	19.9 ±1.7b	1.54 ±0.05b	1.74 ±0.06a
OAWE	12.4 ±1.3ab	5.7 ±0.2ab	18.7 ±2.0b	1.55 ±0.00b	1.74 ±0.00a
OAHAWE	14.0 ±2.2ab	5.8 ±0.2ab	21.8 ±3.6ab	1.60 ±0.03ab	1.67 ±0.04ab
OSHF	13.0 ±1.6ab	5.7 ±0.2ab	20.4 ±2.3ab	1.62 ±0.14ab	1.67 ±0.14ab
OSHA	13.7 ±1.0ab	5.8 ±0.1ab	21.8 ±0.6ab	1.63 ±0.08ab	1.64 ±0.08ab
OSWE	13.8 ±0.3ab	5.8 ±0.0ab	22.2 ±1.4ab	1.65 ±0.07ab	1.63 ±0.07ab
OSHAWE	14.6 ±1.0a	5.9 ±0.1a	23.6 ±2.7a	1.65 ±0.09ab	1.62 ±0.09ab
COMP					
OA	13.2 ±0.66	5.8 ±0.06	20.1 ±1.29b	1.56 ±0.03b	1.72 ±0.03a
OS	13.8 ±0.66	5.8 ±0.08	22.0 ±1.32a	1.63 ±0.02a	1.64 ±0.02b
ENCAP					
HF	13.1 ±0.07	5.7 ±0.07	20.1 ±0.42	1.59 ±0.04	1.70 ±0.04
HA	13.5 ±0.28	5.8 ±0.00	20.9 ±1.34	1.59 ±0.06	1.69 ±0.07
WE	13.1 ±0.99	5.8 ±0.07	20.5 ±2.47	1.60 ±0.06	1.69 ±0.08
HAWE	14.3 ±0.42	5.9 ±0.07	22.7 ±1.27	1.63 ±0.04	1.65 ±0.04
P-Value					
COMP	NS	NS	**0.017**	**0.012**	NS
ENCAP	NS	NS	NS	NS	NS
COMP*ENCAP	NS	NS	NS	NS	NS

Note: PC - positive control, NC = negative control, OA - organic acid, OS - organic acid salt, HF - hydrogenated fat, HA - HA + alginate, WE - wax ester, HAWE - double coating with HA and WE; COMP – composition; ENCAP – microencapsulation. Values in a column with different superscripts are significantly different from each other ($P < 0.05$). P-values in bold are significant.

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Table 5. Nutrient utilization and digestive enzyme (amylase and lipase) activity in shrimps fed the control and test diets.

Treatments	Protein deposition PD (g)	Lipid deposition LD (g)	Protein retention efficiency PRE (%)	Lipid retention efficiency LRE (%)	Hepatopancreatic amylase (U/gprot)	Hepatopancreatic lipase (U/gprot)
PC	2.13 ±0.36	0.25 ±0.05abc	27.3 ±1.2ab	15.2 ±1.2ab	54.1 ±12.1a	21.5 ±3.2a
NC	1.99 ±0.16	0.23 ±0.03bc	26.1 ±1.5b	13.8 ±1.3b	41.6 ±6.0b	8.9 ±0.7e
OAHF	2.15 ±0.27	0.24 ±0.04abc	29.1 ±3.2ab	15.4 ±2.0ab	51.2 ±6.0ab	16.6 ±4.5bcd
OAHA	2.23 ±0.15	0.26 ±0.02abc	30.0 ±1.5a	16.5 ±0.5a	47.4 ±5.9ab	12.1 ±1.5de
OAWE	2.05 ±0.26	0.23 ±0.03c	29.5 ±0.6a	15.1 ±1.6ab	49.1 ±7.2ab	15.7 ±3.2bcd
OAHAWE	2.33 ±0.47	0.27 ±0.04abc	28.4 ±1.7ab	15.2 ±0.5ab	47.8 ±1.8ab	14.6 ±2.4bcd
OSHF	2.10 ±0.30	0.23 ±0.03abc	27.7 ±3.1ab	14.3 ±1.0ab	47.8 ±3.7ab	18.3 ±4.0ab
OSHA	2.28 ±0.14	0.28 ±0.02abc	27.9 ±1.1ab	15.9 ±1.3ab	49.6 ±7.3ab	17.7 ±2.8abc
OSWE	2.25 ±.0.06	0.27 ±0.01abc	27.2 ±1.7ab	15.6 ±1.2ab	47.6 ±2.0ab	14.2 ±2.3bcd
OSHAWE	2.39 ±0.18	0.28 ±0.01a	27.2 ±2.0ab	15.1 ±1.7ab	49.1 ±5.9ab	14.8 ±3.2bcd
COMP						
OA	2.19 ±0.12	0.26 ±0.0	0.29 ±0.68a	15.6 ±0.65	48.9 ±1.71	14.8 ±1.95
OS	2.26 ±0.12	0.27 ±0.02	0.28 ±0.36b	15.2 ±0.70	48.5 ±0.98	16.3 ±2.05
ENCAP						
HF	2.12 ±0.04	0.24 ±0.01	28.4 ±0.99	14.9±0.78	49.5±1.40	17.5±1.20
HA	2.25 ±0.04	0.27 ±0.01	29.0 ±1.48	16.2 ±0.42	49.5 ±1.56	14.9 ±3.96
WE	2.15 ±0.14	0.25 ±0.03	28.4 ±1.63	15.4 ±0.35	48.4 ±1.06	15.0 ±1.06
HAWE	2.36 ±0.04	0.28 ±0.01	27.8 ±0.85	15.2 ±0.07	48.5 ±0.92	14.7 ±0.14
P-Value						
COMP	NS	NS	**0.016**	NS	NS	NS
ENCAP	NS	**0.047**	NS	NS	NS	NS
COMP*ENCAP	NS	**0.047**	NS	NS	NS	NS

Note: PC - positive control, NC = negative control, OA - organic acid, OS - organic acid salt, HF - hydrogenated fat, HA - HA + alginate, WE - wax ester, HAWE - double coating with HA and WE; COMP – composition; ENCAP – microencapsulation. Values in a column with different superscripts are significantly different from each other ($P < 0.05$). P-values in bold are significant.

 doi:10.20944/preprints202106.0108.v1

Table 6. Antioxidant capacity, immune response and cumulative 96-h mortality under pathogenic *Vibrio parahaemolyticus* challenge of shrimp fed the control and test diets.

	Superoxide dismutase - SOD (unit/ml))	Alkaline phosphatase - ALP (unit/ml)		Acid phosphatase - ACP (unit/ml)		Phenol oxidase - PO (unit/ml)		Malonaldehyde - MDA (mmol/L)		Cumulative
Treatments	Serum	Hepatopancreas	Serum	Hepatopancreas	Serum	Hepatopancreas	Serum	Hepatopancreas	Serum	mortality (%)
PC	339.1 ±23.9a	493.5 ±8.8a	17.4 ±3.3ab	493.5±8.8a	62.9 ±1.3a	885.4 ±46.8ab	761.5 ±14.2a	2.3 ±0.2abc	7.1 ±0.7bcd	42.2 ±1.8d
NC	264.4 ±31.8c	431.6 ±5.4b	7.2 ±0.9f	431.6 ±5.4b	19.8 ±0.4e	535.4 ±68.8f	427.4 ±21.9e	2.5 ±0.2abc	9.3 ±0.7a	62.8 ±5.9a
OAHF	316.4 ±42.6ab	475.9 ±15.3ab	16.8 ±1.7b	475.9 ±15.3ab	38.6 ±2.9b	800.0 ±10.8abcd	694.1 ±79.7ab	2.4 ±0.3abc	6.6 ±0.8cd	47.2 ±2.3bcd
OAHA	296.5 ±19.6abc	491.2 ±77.5a	7.8 ±0.3ef	491.2 ±77.5a	21.2 ±0.7de	704.2 ±87.3de	715.3 ±47.8ab	2.3 ±0.4abc	7.8 ±1.5bc	51.3 ±9.2bc
OAWE	306.1 ±18.0abc	518.3 ±21.9a	9.3 ±0.9def	518.3 ±22.0a	14.0 ±0.2f	820.8 ±138.5abcd	625.0 ±88.8bc	2.2 ±0.3c	6.8 ±0.4bcd	55.6 ±9.1ab
OAHAWE	291.5 ±39.1bc	503.0 ±27.1a	14.6 ±0.4c	503.0 ±27.1a	36.8 ±4.5b	718.8 ±90.1cd	460.4 ±42.7de	2.2 ±0.2c	7.5 ±1.4bcd	45.0 ±4.1cd
OSHF	300.6 ±20.5abc	511.8 ±37.5a	8.6 ±0.0def	511.8 ±37.5a	25.7 ±3.7c	600.0 ±64.2ef	464.6 ±20.8de	2.2 ±0.3c	6.2 ±0.7d	52.2 ±6.4bc
OSHA	323.5 ±26.7abc	513.2 ±37.9a	18.8 ±1.0a	513.2 ±37.9a	25.6 ±1.3c	906.3 ±61.4a	537.5 ±110.8cd	2.3 ±0.1bc	7.5 ±0.8bcd	50.0 ±4.5bcd
OSWE	296.8 ±8.0abc	463.0 ±27.3ab	14.7 ±0.4c	463.0 ±27.3ab	13.4 ±0.9f	779.2 ±10.8bcd	431.9 ±14.2e	2.7 ±0.1ab	7.0 ±0.7bcd	45.0 ±4.1cd
OSHAWE	303.2 ±26.8abc	472.3 ±36.9ab	10.1 ±0.4d	472.3 ±36.9ab	23.1 ±1.9cd	829.2 ±54.3abc	437.5 ±3.4e	2.4 ±0.4abc	7.9 ±0.9abc	47.2 ±2.3bcd
COMP										
OA	302.6 ±11.0	497.1 ±18.0	12.1 ±4.27b	497.1 ±18.9	27.7 ±12.0	761.0 ±58.0	623.7 ±115.5	2.3 ±0.10	7.2 ±0.57	49.8 ±4.68
OS	306.0 ±11.9	490.1 ±26.2	13.1 ±4.63a	490.1 ±26.2	22.0 ±5.8	778.7 ±130.1	467.9 ±48.6	2.4 ±0.22	7.2 ±0.73	48.6 ±3.15
ENCAP										
HF	308.5 ±11.2	493.9 ±25.4	12.7 ±5.80ab	493.9 ±25.4	32.15 ±9.1a	700.0 ±141.4b	579.3 ±162.7ab	2.3 ±0.14	6.4 ±0.28c	49.7 ±3.54
HA	310.0 ±19.1	502.2 ±15.6	13.3 ±7.78a	502.2 ±15.6	23.4 ±3.1b	805.3 ±142.9a	626.4 ±125.7a	2.3 ±0.00	7.7 ±0.21a	50.7 ±0.92
WE	301.5 ±6.6	490.7 ±39.1	12.0 ±3.82b	490.7 ±39.1	13.7 ±0.4b	800.0 ±29.4a	528.5 ±136.5b	2.5 ±0.35	6.9 ±0.14b	50.3 ±7.50
HAWE	297.4 ±8.3	487.7 ±21.7	12.4 ±3.18ab	487.7 ±21.7	30.0 ±5.7ab	774.0 ±78.1ab	449.0 ±16.2c	2.2 ±0.03	7.7 ±0.28a	46.1 ±1.56
P-Value										
COMP	NS	NS	**0.004**	NS	**<0.0001**	**<0.0001**	**<0.0001**	NS	NS	NS
ENCAP	NS	NS	**<0.0001**	NS	**<0.0001**	**0.039**	**<0.0001**	NS	**0.026**	NS
COMP*ENCAP	NS	NS	**<0.0001**	NS	**<0.0001**	**<0.0001**	**<0.0001**	NS	NS	NS

Note: PC - positive control, NC = negative control, OA - organic acid, OS - organic acid salt, HF - hydrogenated fat, HA - HA + alginate, WE - wax ester, HAWE - double coating with HA and WE; COMP – composition; ENCAP – microencapsulation. Values in a column with different superscripts are significantly different from each other ($P < 0.05$). P-values in bold are significant.

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Table 7. Performance score of "COMP" (organic acid and organic acid salts) and "ENCAP" (hydrogenated fat, hydrogenated fat + alginate, wax ester, and double coating with hydrogenated fat + aligante and wax ester) based on growth performance, nutrient utilization and immune response of shrimps fed the control and test diets.

Factors	Type	Growth performance	Nutrient utlization	Immune response	Total score
COMP	OA	58[b]	67[b]	112	**237[b]**
	OS	38[a]	57[a]	96	**191[a]**
ENCAP	HF	22	35[b]	61[b]	**118[b]**
	HA	27	37[b]	53[ab]	**117[b]**
	WE	22	27[a]	46[a]	**95[a]**
	HAWE	25	25[a]	48[a]	**98[a]**

Note: PC - positive control, NC = negative control, OA - organic acid, OS - organic acid salt, HF - hydrogenated fat, HA - HA + alginate, WE - wax ester, HAWE - double coating with HA and WE. Values in a column with different superscripts are significantly different from each other ($P < 0.05$).

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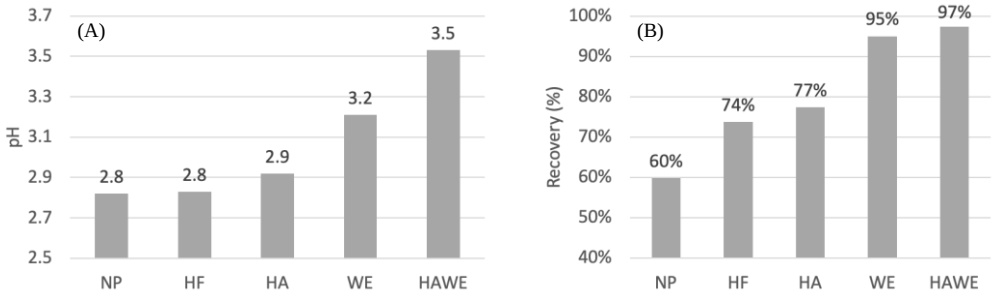
Figure 1. The pH value and recovery of the active ingredient during the *in vitro* solubility test of four microencapsulation (HF, HA, WE and HAWE) compared the non-protected product (NP).



Note: NP = unprotected, HF - hydrogenated fat, HA - HA + alginate, WE - wax ester, HAWE - double coating with HA and WE

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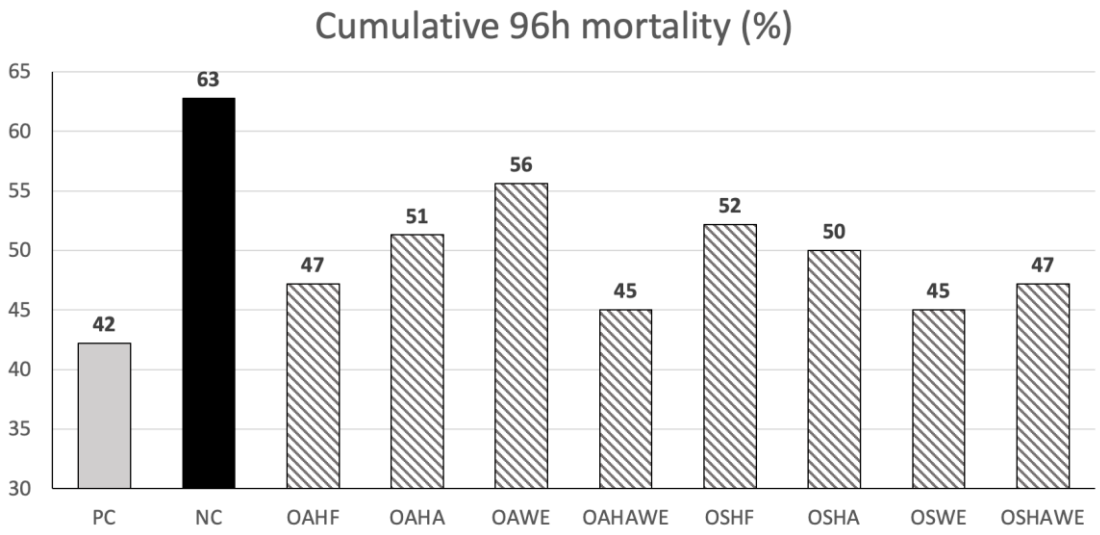
Figure 2. Cumulative 96-h mortality under pathogenic *Vibrio parahaemolyticus* challenge of shrimp fed the control and test diets.



Note: PC - positive control, NC = negative control, OA - organic acid, OS - organic acid salt, HF - hydrogenated fat, HA - HA + alginate, WE - wax ester, HAWE - double coating with HA and WE.