 doi:10.20944/preprints202106.0108.v2

Effects of microencapsulated organic acid and their salts on growth performance, immunity, and disease resistance of Pacific white shrimp *Litopenaeus vannamei*

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 doi:10.20944/preprints202106.0108.v2

Abstract

Use of antibiotics and other chemicals to combat disease outbreaks have been a bottleneck for the sustainable growth of shrimp industry. Among various replacement proposed, organic acid (OA) and their salts (OS) are commonly used by farmers and feed millers. However, in free forms, their requirement is very high (2-3 kg/MT) as they tend to disassociate before reaching the hindgut. The dosage can be reduced by microencapsulation of the ingredients. In this study, a 63-day trial was conducted to assess the effects of OA and OS (COMP) microencapsulated (ENCAP) with fat (HF), fat + alginate (HA), wax esters – (WE), and HA and WE (HAWE) on performance, digestive enzyme, immune, and resistance to *Vibrio parahaemolyticus*. A positive control (PC, 200 g/kg fishmeal - FM) and a negative control (NC, 130 g/kg FM) diet were formulated. Eight other diets were formulated supplementing NC diet with microencapsulated OA (OAHF, OAHA, OAWE, OAHAWE) and OS (OSHF, OSHA, OSWE, OSHAWE). 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, 96-h mortality during pathogenic *Vibrio parahaemolyticus* challenge in all treatment diets (45% - 56%) was lower compared to the NC diets (63%). In conclusion, use of HF microencapsulated OA diets could provide improved performance and disease resistance that could contribute to the reduction of antibiotic use by the shrimp industry.

 doi:10.20944/preprints202106.0108.v2

Key words

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

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) [1, 2]. 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) [3]. 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 [4,5].

Various alternatives to AGP such as, phytogenic compounds or plant derived essential oils [6,7] Antibiotic resistome in the livestock and aquaculture industries: Status and solutions), probiotic, prebiotic and synbiotic [8,9], enzymes [10,11], organic acids and their salts [2,292,13,14,15,16] have been proposed in recent years. Organic acids are “Generally Regarded as Safe” compounds often containing one or more carboxyl groups (–COOH) [17,18]. The most common are those with short chain (C1–C6) such as formic, lactic, propionic, 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

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50% of the acid is dissociated. The p*Ka* of organic acids ranges from 3.02 for fumaric acid to as high as 6.4 for citric acid [19].

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 [20]. 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 [21]. The required high dosage (2-5 g/kg) to suppress intestinal pH induces high stress and costs significant energy to maintain homeostasis (22,23). An alternative strategy is to encapsulate active ingredient to bypass the proximal intestine ensuring their release in the microbe rich hind gut.

Microencapsulation is one of the most popular and practical approaches to deliver bioactive compounds in the GI tract of farmed animals [24,25,26,27]. An ideal encapsulation should not only present the stability of the active compound but also release them in the target regions of the intestine (28). 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 [29,30,31,32,33]. Hydrogenated fat has been considered one of the most cost-effective materials for encapsulating bioactive compounds because of low cytotoxicity [34] and higher stability [35]. Alginate, derived from brown seaweed and a linear and anionic polysaccharide, is soluble in water in room temperature [36]. The ability to form gel without heating and cooling cycles makes alginate an attractive material for feed applications [37]. The inclusion of alginate to the starch or

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hydrogenated fat matrix improves the shape and surface properties that could be attributed to its remarkable crosslinking capability and excellent film-forming properties [38]. Another encapsulation material, the edible wax, has been recently used as lipid-based delivery system [39].

Both organic acids and their salts have been used in aquafeed for better performance and disease resistance of aquatic animals [40]. The blends of organic acids used in this study are fumaric acid, sorbic acid and citric acid. Salts of organic acids used are calcium propionate, calcium formate, and sodium acetate. Dietary fumaric acid (catfish) [41], fumaric and sorbic acid (*E. coli*) [42], citric acid (*E. coli*) [43], calcium propionate (tilapia [44] and silver catfish [45]), calcium formate (shrimp) [13], and sodium acetate (tilapia [46] and yellowfin seabream [47]) showed varying level of antimicrobial activity in-vitro and in various farmed species. Most studies to-date 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 acids or their salts.

The aim of this study is to find a better way to deliver alternative solutions to antibiotics and antibiotic growth promoters (AGP) such as organic acid or organic acid salts in the hindgut of shrimp. 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

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WE - HAWE on Pacific white shrimp performance, immune response and disease resistance were assessed.

Materials and methods

The experiment had two components: *in-vitro* microencapsulation stability tests 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).

Stability tests

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. [48]. 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

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124 acids corresponding to the active ingredients of the micro-encapsulated product was
125 used as a control. The pH of the supernatant was determined after filtration. Each
126 treatment was conducted in triplicates.
127
128 *Feeding trial*
129 The feeding trial was conducted for 63 days at the Guangdong Ocean University
130 field experimental station situated at Donghai Island, Zhanjiang of Guagdong province
131 of China. Experimental procedure and animal care were accomplished in accordance
132 with the ethical guidelines for the care and use of laboratory animals provided by the
133 Animal Care Committee of the Guangdong Ocean University.
134
135 *Experimental design and diet preparation*
136 Ten isoproteic (37.3 ±0.12 % CP) and isoenergetic (16.4 ±0.02 MJ/kg) diets were
137 prepared: diet 1 - positive control with 20% FM (PC); diet 2 - negative control with
138 13% fishmeal and 12% meat and bone meal (NC); diets 3-6 were manufactured by
139 supplementing NC with 0.75 mg/kg of OA microencapsulated with HF, HA, WE and
140 HAWE (OAHF, OAHA, OAWE and OAHAWE, respectively); and diets 7-10 were
141 manufactured by supplementing 0.85 mg/kg of OS microencapsulated with HF, HA,
142 WE and HAWE (OSHF, OSHA, OSWE and OSHAWE, respectively) (Table 1 & 2). It
143 was ensured that all microcapsulated products contained same amount of active
144 ingredients. The microencapsulated test products were supplied by Jefo Nutrition Inc.,

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145 Quebec, Canada. Diet composition and their proximate chemical composition including
146 amino acid profile are provided in Table 1 and 2, respectively.

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

153

154 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 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

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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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163 Table 2. Proximate chemical composition and calculated essential amino acid profile of the control and test diets (dry matter – DM basis)
164

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

165 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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166 *Experimental conditions*

167 Twenty-five thousand PL10 Pacific white shrimp *L. vannamei* postlarvae were
168 obtained from Allied Pacific Aquaculture Co., Ltd., Zhanjiang, Guangdong, China. The
169 shrimp were acclimatized in two cement pools for 40 days until the average body
170 weight reached 0.3 g. From the cement pools, a total of 1600 white shrimp (0.33 ±0.02g
171 ABW) were selected and 40 shrimp/tank were randomly distributed into 40 cone-
172 shaped tanks (350-L volume each) with four replicates per treatment.

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

176

177 *Sampling*

178 At the end of the experiment, shrimp were fasted for 24 hours before the final
179 sampling. From each treatment, 15 and 10 shrimps were randomly selected from each
180 tank for serum and hepatopancreatic analyses, respectively. Both analyses were not
181 conducted on same shrimp because of the possibility of influence of one sampling on
182 another. For serum, the blood was drawn using a dispensable 1 ml syringe into 1.5 ml
183 test-tube. The test-tubes were then stored at 4 ^{o}C overnight before being centrifuged at
184 5867 g for 10-min at 4 ^{o}C (3K30, Sigma, Germany). The supernatant was then collected
185 into 1.5 ml tube and stored at -80 ^{o}C for subsequent analyses. Hepatopancreas was
186 removed from each shrimp, immediately frozen in liquid nitrogen, and then stored at -

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80C for analyses. Another six shrimps from each tank were taken for body chemical composition, ground into slurry, lyophilized and kept at -20 °C until analyses.

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™ 8400, FOSS, Sweden), moisture by drying the samples at 105 °C under atmospheric pressure for 24 hours, crude lipid using a Soxhlet apparatus (Soxtec™ 2050, FOSS, Sweden), crude ash by burning the samples at 550 °C using a muffle furnace (Shanghai Boxun industry & Commerce Co., Ltd., Shanghai, China), and gross energy using a bomb calorimeter (Changsha Kaiyuan Instruments, Changsha, China).

The activity of acid (ACP) and alkaline (ALP) phosphatase, 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

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209 (OD_{sample}) on a Microplate Spectrophotometer (Multilskan spectrum, Thermo, USA) at
210 490 nm. At the same time, 300 µl of *L*-DOPA solution was placed in a 96-well plate
211 and absorbance of the blank control group was recorded (OD_{blank}). Enzymatic activity
212 for all assays was expressed as the change in absorbance/min.
213
214 *Resistance to Vibrio parahaemolyticus*
215 Resistance to the pathogen, *V. parahaemolyticus* was determined from the
216 cumulative mortality of shrimp in 96 h. For this, 10 shrimps for each replicate (3
217 replicates in each treatment) were used. After injecting each shrimp with 2.4×10^7
218 colony-forming units (CFU) of *V. parahaemolyticus*, the cumulative mortality in 96 h
219 was recorded.
220
221 *Scoring*
222 All variables from treatment 3-8 were grouped into three categories to determine
223 the most suitable composition (COMP: free acid vs acid-salt) and microencapsulation
224 (ENCAP: HF, HA, WE and HAWE), and scored ranging from 1-8. The scores assigned
225 from smallest to largest are as follows: growth performance (SGR – 1-8; FCR – 8-1;
226 and PER – 1-8), nutrient utilization (PRE – 1-8; LRE – 1-8; and amylase (1-8) and
227 lipase (1-8) activity), and immune response (serum SOD – 1-8, ALP – 1-8, ACP – 1-8,
228 PO – 1-8 and MDA – 8-1; and cumulative mortality – 8-1).
229
230 *Calculation*

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

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

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

234 Serum MDA (nmol/mL) = ($OD_{sample} - OD_{sample\ blank}$)/($OD_{standard} - OD_{standard\ blank}$) X

235 standard concentration (10nmol/ml) X sample dilution times before assay.

236 Serum T-SOD (U/mL) = ($OD_{contrast} - OD_{sample}$)/$OD_{contrast}$/50% X reaction system

237 dilute multiple X sample dilute multiple before assay.

238 Hepatopancreas T-SOD (U/mL) = ($OD_{contrast} - OD_{sample}$)/$OD_{contrast}$/50% X reaction

239 system dilute multiple/protein content in hepatopancreas (mgprot/mL).

240 Serum ACP (King U/100mL) = ($OD_{sample} - OD_{blank}$)/($OD_{standard} - OD_{blank}$) X

241 standard concentration (0.1 mg/mL) X 100 mL X sample dilution times before assay.

242 Hepatopancreas ACP (King U/gprot) = ($OD_{sample} - OD_{blank}$)/($OD_{standard} - OD_{blank}$)

243 X standard concentration (0.1 mg/mL)/protein content in hepatopancreas (gprot/mL).

244 Serum ALP (King U/100mL) = ($OD_{sample} - OD_{blank}$)/($OD_{standard} - OD_{blank}$) X

245 standard concentration (0.1 mg/mL) X 100 mL X sample dilution times before assay.

246 Hepatopancreas ALP (King U/gprot) = ($OD_{sample} - OD_{blank}$)/($OD_{standard} - OD_{blank}$)

247 X standard concentration (0.1 mg/mL)/protein content in hepatopancreas (gprot/mL).

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

249 Amylase (U/gprot) = ($OD_{blank} - OD_{assay}$)/OD_{blank} X 80/[volume of sample (0.1mL)

250 X protein concentration (mgprot/mL)] X 1000

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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)

Statistical analysis

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. If there is a difference, multiple comparison analyses were performed using Duncan's multiple-range tests. Statistically significant differences were considered at $P < 0.05$.

Results

During the feeding trial, the water temperature was ranged between 28 °C and 34 °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.

Stability 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

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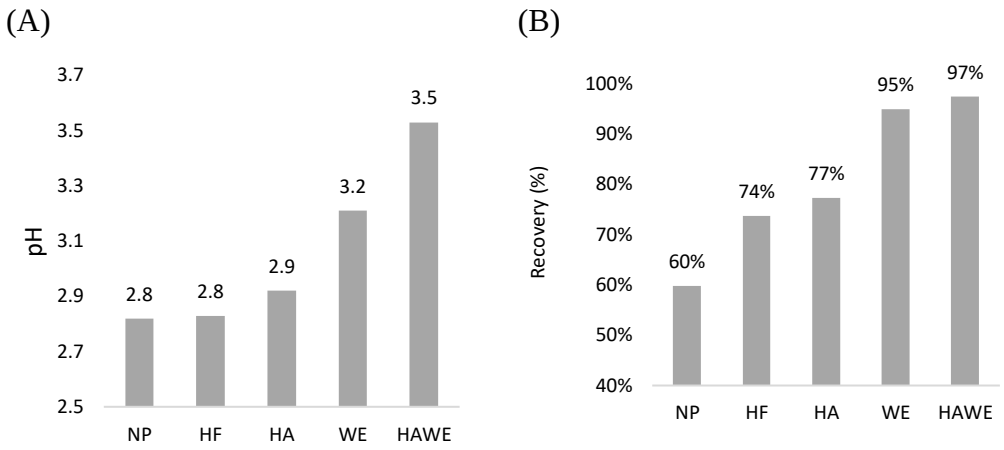
recovery was significantly higher for WE (95%) and HAWE (97%) compared to HF (74%) and HA (77%) (Figure 1B).

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

Growth Performance and body composition

Feed intake and growth were normal 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 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.

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Table 3. Whole body chemical composition of Pacific white shrimp 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	FBW (g)	SGR	FI (g/shrimp)	FCR	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: FBW – Final body weight, SGR – Specific growth rate, FI – Feed intake, FCR – Feed conversion ratio, PER – Protein efficiency ratio, 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.

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

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fed the OS (0.28) diets. Significant interaction (COMP*ENCAP) was also 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).

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

Treatments	**PD (g)**	**LD (g)**	**PRE (%)**	**LRE (%)**	**HP Amylase (U/gprot)**	**HP 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: PD – Protein deposition, LD – Lipid deposition, PRE – Protein retention efficiency, LRE – Lipid retention efficiency, HP – Hepatopancreatic, 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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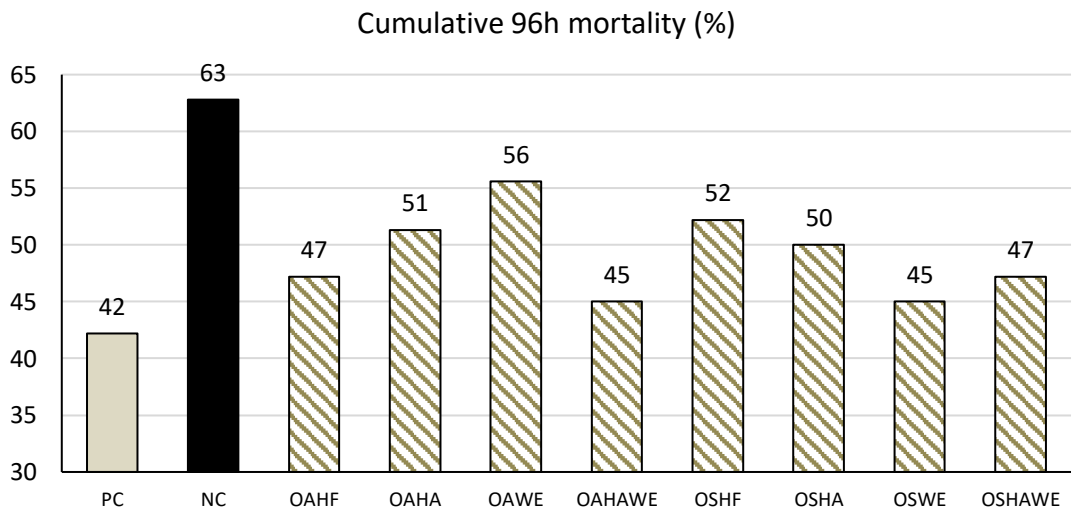
325

326 *Immune response and disease resistance*

327 No differences in cumulative 96-h mortality when challenged with *Vibrio*
328 *parahaemolyticus* (Figure 2) and serum SOD, hepatopancreatic ALP, ACP and MDA
329 (Table 6) were observed with either main effects of COMP, ENCAP or their interaction
330 (Table 6). Significant interaction was observed for serum ALP ($P < 0.0001$), ACP ($P <$
331 0.0001), and hepatopancreatic and serum phenol oxidase level ($P < 0.0001$).
332 Significantly lower serum MDA level ($P < 0.026$) was observed in HF (6.4) compared
333 to the other ENCAP (HA = 7.7, WE = 6.9 and HAWE = 7.7).

334

335 Figure 2. Cumulative 96-h mortality under pathogenic *Vibrio parahaemolyticus*
336 challenge of shrimp fed the control and test diets.
337



338

339 Note: PC - positive control, NC = negative control, OA - organic acid, OS - organic acid salt, HF - hydrogenated fat, HA - HA +
340 alginate, WE - wax ester, HAWE - double coating with HA and WE.
341

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343 Table 6. Antioxidant capacity, immune response, and cumulative 96-h mortality under pathogenic *Vibrio parahaemolyticus* challenge of shrimp
344 fed the control and test diets.
345

Treatments	SOD (unit/ml)	ALP (unit/ml)		ACP (unit/ml)		PO (unit/ml)		MDA (mmol/L)		CM (%)
	Serum	Hepatopancreas	Serum	Hepatopancreas	Serum	Hepatopancreas	Serum	Hepatopancreas	Serum	
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

346 Note: SOD – Superoxide dismutase, ALP – Alkaline phosphatase, ACP – Acid phosphatase, PO – Phenol oxidase, MDA – Malondialdehyde, CM – Cumulative mortality, PC - positive control, NC = negative control,
347 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
348 column with different superscripts are significantly different from each other ($P < 0.05$). P-values in bold are significant.

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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 ENCAP, the overall scores of HF and HA (118 and 117, respectively) were higher than WE (95) and HAWE (98) ($P < 0.05$) (Table 7).

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 + alginate 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 utilization	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 ($P < 0.05$).

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

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370 (p*K*a = 3.03), sorbic acid (p*K*a = 4.75) and citric acid (p*K*a = 2.92-5.21). The organic
371 acid salt blend contained Ca-propionate, Ca-formate and Na-acetate.

372 Organic acids are low molecular weight aldehyde containing compounds with one
373 or more carboxyl groups. They are used as dietary supplement to reduce gastrointestinal
374 tract pH and inhibit the growth of gram-negative bacteria through the disassociation of
375 the acids and production of anions in bacterial cells [49]. As weak acids, the p*K*a values
376 or the disassociation constant of organic acids are higher than the strong acids such as
377 HCl or H_2SO_4 [50]. These acids do not dissociate in the highly acidic stomach pH but
378 tend to dissociate quickly in the proximal intestine as pH increases and the condition
379 becomes alkaline. Shrimp are slow-eating animals taking 1-2 hours for holding and
380 chewing the pellets. In free-form, organic acid or their salts have considerable risk of
381 leaching in water preventing them from reaching the hepatopancreas and gut in
382 undissociated form [51]. Coating or encapsulation may significantly reduce leaching
383 and consequently, can remain effective at lower dosage [11]. For example, micro-
384 encapsulated organic acid salt blend used by Yao et al. [11] and in this study, is much
385 lower (835 mg/kg) than in their free form (2000-6000 mg/kg) reported in various
386 studies [52,53]. Micro-encapsulation provides better protection than simple coating that
387 may prevent or reduce the loss of active ingredient in case of breakage of the prills as
388 active ingredients are embedded in the matrix of coating material [54].

389 Microencapsulation of easily degradable bioactive compounds has been becoming
390 a popular and practical approach to mask unpleasant characteristics of the compounds

 doi:10.20944/preprints202106.0108.v2

and deliver them at the intended location of the gastrointestinal tract [24,55]. 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 scores were observed between the two materials. Both HF and HA were tested *in vitro* by Omnojio et al. [26] 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 can be easily digested by intestinal lipase thus guaranteeing the slow release of the active ingredient along the GI tract. In a recent study, efficacy of HF based microencapsulated aluminum and iron sulfate in in-situ chelation of undigestible phosphorus in the hind gut of rainbow trout were also reported by Ndiyae et al. [56]. 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 [39]. The positive characteristics such as slower degradation and mass transfer

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411 rate may not be suitable for shrimp for their short gut-transit time (~2 hours) to release
412 the active ingredient.

413 Blends of organic acids and their salts in free or microencapsulated forms have
414 shown to improve growth performance of fish (40,57,58) and shrimp (2,11,33,59) as
415 well as antioxidant status [60]. Several studies reported improved growth performance,
416 nutrient utilization and immune response in crustacean fed microencapsulated blend of
417 organic acid or acid salts. Safari et al. [61] reported efficacy of encapsulated blend of
418 Na-butyrate, Na-lactate and Na-propionate on growth performance and survival of
419 crawfish at 20g/kg. The OS blend used in the present study contains Ca-propionate, Ca-
420 formate and Na-acetate and showed higher feed intake compared to those fed the OA
421 diets. Yao et al. [11] also reported improved weight gain and FCR in Pacific white
422 shrimp compared to NC diet with the same OS blend. When compared between the OA
423 and OS treatments of this study, shrimps fed the OA diets showed improved FCR,
424 protein retention, and immune response i.e., higher ALP and PO compared to the OS
425 blend (Table 4-6). This is in accordance with the findings of Romano et al. (2015), who
426 reported improved growth performance of Pacific white shrimp with 1-4%
427 microencapsulated OA (blend of formic, lactic, malic, and citric acids).

428 In an in-vitro study, Mine and Boopathy [12] demonstrated EC50 values of 0.023%,
429 0.041%, 0.03%, and 0.066% for formic, acetic, propionic, and butyric acid, respectively
430 against *Vibrio harveyi*. Romano et al. [33] reported similar efficacy in *V. harveyi*
431 resistance when fed OA supplemented diets. Efficacy of organic acid in combination

 doi:10.20944/preprints202106.0108.v2

with essential oil against *Vibrio* sp. infections also demonstrated by He et al. [60], 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% compared to 63% for those fed the NC diets when challenged with pathogenic *V. parahaemolyticus* (Figure 2).

Conclusions

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. Finding an effective microencapsulation strategy along with the effective composition of organic acid or their salts is important for sustainable development of the industry.

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

Conceptualization: M.A.K.C.; Methodology: X.D. and H.S.; Investigation: X.D., H.S. and Y.L.; Chemical Analysis: H.S.; Product Preparation: J.B.; Formal Analysis: M.A.K.C.; Writing – Original Draft Preparation: M.A.K.C. and J.B.; Writing – Review & Editing: X.D. and J.B.

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.

Reference

1. Flegel, T.W. A future vision for disease control in shrimp aquaculture. J. World Aquacul. Soc. **2019**, 50, 249-266.
2. Ng, W-K; Koh, C-K; Teoh, C-Y; Romano, N. Farm-raised tiger shrimp, *Penaeus monodon*, fed commercial feeds with added organic acids showed enhanced

472 nutrient utilization, immune response and resistance to *Vibrio harveyi* challenge.
473 Aquacult. **2015**, 449, 69-77.
474 3. Limbu, S.M.; Chen, L-Q; Zhang, M-L; Du, Z-Y. A global analysis on the systemic
475 effects of antibiotics in cultured fish and their potential human health risk: a review.
476 Rev. Aquacult. **2020**, https://doi.org/10.1111/raq.1251.
477 4. World Health Organization (WHO). WHO Guidelines on Use of Medically
478 Important Antimicrobials in Food-producing Animals, WHO, Geneva, Switzerland,
479 2017, 68pp.
480 5. Zhao, Y; Yang, Q.E.; Zhou, X.; Wang, F-H.; Muurinen, J.; Virta, M.P.; Brandt,
481 K.K.; Zhu, Y-G. Antibiotic resistome in the livestock and aquaculture industries:
482 Status and solutions, Crit. Rev. Env. Sci. Tech. **2020**, DOI:
483 10.1080/10643389.2020.1777815.
484 6. Kesselring, J.; Gruber, C.; Standen, B.; Wein, S. Effect of a phytogenic feed
485 additive on the growth performance and immunity of Pacific white leg shrimp,
486 *Litopenaeus vannamei*, fed a low fishmeal diet. J. World Aquacult. Soc. **2020**, 1–
487 13.
488 7. Yang, C.; Chowdhury, M.A.K.; Hou, Y.; Gong, J. Phytogenic compounds as
489 alternatives to in-feed antibiotics: potentials and challenges in application.
490 Pathogens, **2015**, 4, 137-156.

 doi:10.20944/preprints202106.0108.v2

8. Jamal, M.T.; Abdulrahman, I.A.; Al-Harbi, M.; Chithambaran, S. Probiotics as alternative control measures in shrimp aquaculture: A review. Appl. Biol .Biotech. **2019**, 7,69-77.
9. Jueliang, P.; Chuchird, N.; Limsuwan, C. The effects of probiotic, β-1,3-glucan and organic acid on Pacific white shrimp's (*Litopenaeus vannamei*) immune system and survival upon challenge with *Vibrio harveyi*. Fish. Env. **2013**, 3, 25-37.
10. Song, H-L.; Tan, B-P.; Chi, S-Y.; Liu, Y.; Chowdhury, M.A.K.; Dong, X-H. The effects of a dietary protease-complex on performance, digestive and immune enzyme activity, and disease resistance of *Litopenaeus vannamei* fed high plant protein diets. Aquac. Res. **2017**, 48,2550-2560.
11. Yao, W.; Li. X.; Chowdhury, M.A.K.; Wang, J.; Leng, X-J. Dietary protease, carbohydrase and micro-encapsulated organic acid salts individually or in-combination improved growth, feed utilization and intestinal histology of Pacific white shrimp. Aquac. **2019**, 503, 88-95.
12. Mine, S.; Boopathy, R. Effect of organic acids on shrimp Ppathogen, *Vibrio harveyi*. Curr. Microbiol. **2011**, 63, 1–7.
13. DaSilva, B.C.; Vieira, F.N.; Mourino, J.L.P.; Ferreira, G.S.; Seiffert, W.Q. Salts of organic acids selection by multiple characteristics for marine shrimp nutrition. Aquac. **2013**, 384-387, 104-110.
14. Ng, W-K.; Lim, C-L.; Romano, N.; Kua, B-C. Dietary short-chain organic acids enhanced resistance to bacterial infection and hepatopancreatic structural integrity

512 of the giant freshwater prawn, *Macrobrachium rosenbergii*. Intl. Aquat. Res. **2017**,
513 9, 293-302.
514 15. Krome, C.; Schuele, F.; Jauncey, K.; Focken, U. Influence of a sodium
515 formate/formic acid mixture on growth of juvenile common carp (*Cyprinus carpio*)
516 fed different fishmeal replacement levels of detoxified *Jatropha curcas* kernel meal
517 in practical, mixed diets. J. Appl. Aquac. **2018**, 30, 137-156.
518 16. Nascimento, M.S.; Mattos, B.O.; Bussons, M.R.F.M.; Oliveira, A.T.; Liebi, A.R.S.;
519 Carvalho, T.B. Supplementation of citric acid in plant protein- based diets for
520 juvenile tambaqui, *Colossoma macropomum*. J. World Aquac. Soc. **2020**, DOI:
521 10.1111/jwas.12735.
522 17. Defoirdt, T.; Boon, N.; Sorgeloos, P.; Verstraete, W.; Bossier, P. Short-chain fatty
523 acids and poly-β-hydroxyalkanoates: (new) biocontrol agents for a sustainable
524 animal production. Biotechnol. Adv. **2009**, 27,680–685.
525 18. Sarder, P.; Shamna, N.; Sahu, N.P. Acidifiers in aquafeed as an alternate growth
526 promoter: a short review. Anim. Nut. Feed Tech. **2020**,20, 253-366.
527 19. Ng, W-K.; Koh, C-B. The utilization and mode of action of organic acids in the
528 feeds of cultured aquatic animals. Rev. Aquac. **2016**, 9, 342–368.
529 20. Payne, A.I. Gut pH and digestive strategies in estuarine grey mullet (Mugilidae)
530 and tilapia (Cichlidae). Fish. Biol. **1978**, 13, 627-629.
531 21. Eklund, T. The antimicrobial effect of dissociated and undissociated sorbic acid at
532 different pH levels. J. Appl. Bacteriol. **1983**, 54, 383-389.

 doi:10.20944/preprints202106.0108.v2

533 22. Li, H.; Ren, C.; Jiang, X.; Cheng, C.; Ruan, Y.; Zhang, X.; Huang, W.; Chen, T.;
534 Hu, C. Na^+/H^+exchanger (NHE) in Pacific white shrimp (*Litopenaeus vannamei*):
535 Molecular cloning, transcriptional response to acidity stress, and physiological roles
536 in pH homeostasis. PLoS ONE **2019**, 14, e0212887.
537 23. Yu, Q.; Xie, J.; Huang, M.; Chen, C.; Qian, D.; Qin, J.G.; Chen, L.; Jia, Y.; Li, E.
538 Growth and health responses to a long-term pH stress in Pacific white
539 shrimp *Litopenaeus vannamei*. Aquac. Rep. **2020**, 16, 100280.
540 24. Piva, A.; Pizzamiglio, V.; Mauro, M.; Tedeshchi, M.; Piva, G. Lipid
541 microencapsulation allows slow release of organic acids and natural identical
542 flavors along the swine intestine. Anim. Sci. **2007**, 85, 486-493.
543 25. Chitprasert, P.; Sutaphanit, P. Holy basil (*Ocimum sanctum* Linn.) Essential oil
544 delivery to swine gastrointestinal tract using gelatin microcapsules coated with
545 aluminum carboxymethyl cellulose and beeswax. Agri. Food. Chem. **2014**, 62,
546 12641–12648.
547 26. Omonjio, F.A. Microencapsulation for effective delivery of essential oils to
548 improve gut health in pigs. MSc thesis, University of Manitoba, Manitoba, Canada,
549 2018, 126p.
550 27. Yang, X.; Liu, Y.; Yan, F.; Yang, C.; Yang, X. Effects of encapsulated organic
551 acids and essential oils on intestinal barrier, microbial count, and bacterial
552 metabolites in broiler chickens. Poult. Sci. **2019**, 98, 2858–2865. DOI:
553 10.3382/ps/pez031.

554 28. Chen, H.; Ma, D.; Li, Y.; Liu, Y.; Wang, Y. Optimization the process of
555 microencapsulation of *Bifidobacterium bifidum* BB01 by Box-Behnken design.
556 Acta Universitatis Cibiniensis. Series E: Food. Tech. **2016**, 20, 17-28.
557 29. Tester, R.F.; Karkalas, J.; Qi, X. Starch composition, fine structure and architecture.
558 Cereal. Sci. **2004**, 39, 151-165.
559 30. Udachan, I.S.; Sahu, A.K.; Hend, F.M. Extraction and characterization of sorghum
560 (*Sorghum bicolor* L. Moench) starch. Intl. Food Res. J. **2012**, 19, 315-319.
561 31. Fiorda, F.A.; Soares Jr., M.S.; DaSilva, F.A.; DeMoura, C.M.A.; Grossmann,
562 M.V.E. Physical quality of snacks and technological properties of pre-gelatinized
563 flours formulated with cassava starch and dehydrated cassava bagasse as a function
564 of extrusion variables. LWT-Food Sci. Tech. **2015**, 62, 1112-1119.
565 32. Zhu, F. Encapsulation and delivery of food ingredients using starch based systems.
566 Food Chem. **2017**, 229:542-552.
567 33. Romano, N.; Mobili, P.; Zuniga-Hansen, M.E.; Gomez-Zavaglia, A. Physico-
568 chemical and structural properties of crystalline inulin explain the stability of
569 *Lactobacillus plantarum* during spray-drying and storage. Food Res. Intl. **2018**,
570 113, 167-174.
571 34. Müller, R.H.; Radtke, M.M.; Wissing, S.A. Nanostructured lipid matrices for
572 improved microencapsulation of drug. Intl. J. Pharm. **2000**, 242, 121-128.
573 35. Souto, E.B.; Müller, R.H. Lipid nanoparticles: effect on bioavailability and
574 pharmacokinetic changes. Handb. Exp. Pharmacol. **2010**, 197, 115-141.

575 36. Dragan, E.S. Design and applications of interpenetrating polymer network
576 hydrogels. A review. Chemical Eng. **2014**; 243, 572-590.
577 37. Benavides, S.; Cortes, P.; Parada, J.; Franco, W. Development of alginate
578 microspheres containing thyme essential oil using ionic gelation. Food Chem. **2016**,
579 204, 77-83.
580 38. Costa, R.S.; Teixeria, C.; Alves, T.V.G.; Ribeiro-Costa, R.M.; Casazza, A.A.;
581 Aliakbarian, B.; Coverti, A.; Silva Jr., J.O.C; Perego, P. Optimization of spray
582 drying conditions to microencapsulate cupuassu (*Theobroma grandiflorum*) seed
583 by-product extract. Nat. Prod. Res. **2018**, 33, 2600-2608.
584 39. Soleimanian, Y.; Goli, S.A.H.; Shirvani, A.; Elmizadfeh, A.; Marangoni, A.G.
585 Wax-based delivery systems: Preparation, characterization, and food applications.
586 Comp. Rev. Food Sci. Food Saf. **2020**, DOI:10.1111/1541-4337.12614.
587 40. Huan, D.; Li, X.; Chowdhury, M.A.K.; Yang, H.; Liang, G.; Leng, X.J. Organic
588 acid salts, protease and their combination in fish meal-free diets improved growth,
589 nutrient retention and digestibility of tilapia (*Oreochromis niloticus* × *O. aureus*).
590 Aquac. Nut. **2018**, 24, 1813-1821.
591 41. Omosowone, O.; Dada, A.; Adeparusi, E. Effects of dietary supplementation of
592 fumaric acid on growth performance of African catfish *Clarius gariepinus* and
593 *Aeromonas sobria* challenge. Croat. J. Fish. **2015**, 73, 13-19.

42. Lu, H.J.; Breidt, F.; Perez-Diaz, I.M.; Osborne, J.A. Antimicrobial effects of weak Acids on the survival of *Escherichia coli* O157:H7 under anaerobic conditions. J. Food Protect. **2011**, 74, 893-898.

43. Allende, A.; McEvoy, J.; Tao, Y.; Luo, Y. Antimicrobial effect of acidified sodium chlorite, sodium chlorite, sodium hypochlorite, and citric acid on Escherichia coli O157:H7 and natural microflora of fresh-cut cilantro. Food Control **2009**, 20, 230-234.

44. Reda, R.M.; Mahmoud, R.; Selim, K.M.; El-Araby, I.E. Effects of dietary acidifiers on growth, hematology, immune response and disease resistance of Nile tilapia, *Oreochromis niloticus*. Fish Shellfish Immun. **2016**, 50, 255-262.

45. Pereira, S.A.; Oliveira, H.M.; Jesus, G.F.A; Adam, K.G.S; Silva, B.C.; Yamashita, M.M.; Lehmann, N.B.; Martins, M.L.; Mourinho, J.L.P. Can the minerals calcium and sodium, chelated to propionic acid, influence the health and zootechnical parameters of native silver catfish *Rhamdia quelen*? Aquac. **2018**, 496, 88-95.

46. Li, M.; Hu, F-C.; Qiao, F.; Du, Z-Y.; Zhang, M-L. Sodium acetate alleviated high-carbohydrate induced intestinal inflammation by suppressing MAPK and NF-κB signaling pathways in Nile tilapia (*Oreochromis niloticus*). Fish Shellfish Immun. **2020**, 98, 758-765.

47. Sangari, M.; Sotoudeh, E.; Bagheri, D.; Morammazi, S.; Torfi, M. Growth, body composition, and hematology of yellowfin seabream (*Acanthopagrus latus*) given

614 feeds supplemented with organic acid salts (sodium acetate and sodium propionate).
615 Aquac. Intl. **2021**,29, 261-273.
616 48. Jyothi, N.V.N.; Prasanna, P.M.; Sarkerkar, S.N.; Prabha, K.S.; Ramaiah, P.S.;
617 Srawan, G.Y. Microencapsulation techniques, factors influencing encapsulation
618 efficiency. Microencap. **2010**, 27, 187–197.
619 49. Hosseinifar, S.H.; Sun, Y-Z.; Caipang, C.M. Short-chain fatty acids as feed
620 supplements for sustainable aquaculture: an updated view. Aquac. Res. **2016**, 48,
621 1380-1391.
622 50. Soames, A.; Iglauer, S.; Barifcani, A.; Gubner, R. Acid dissociation constant (pKa)
623 of common monoethylene glycol (MEG) regeneration organic acids and
624 methyldiethanolamine at varying MEG concentration, temperature, and ionic
625 strength. Chem. Eng. Dat. **2018**, 63, 2904–2913.
626 51. Romano, N.; Koh, C-B.; Ng, W-K. Dietary microencapsulated organic acids blend
627 enhances growth, phosphorus utilization, immune response, hep- atopancreatic
628 integrity and resistance against *Vibrio harveyi* in white shrimp, *Litopenaeus*
629 *vannamei*. Aquac. **2015**, 435, 228–236.
630 52. Su, X.; Li, X.; Leng, X.; Tan, C.; Liu, B.; Chai, X.; Guo, T. The improvement of
631 growth, digestive enzyme activity and disease resistance of white shrimp by the
632 dietary citric acid. Aquac. Intl. **2014**, 22, 1823-1835.

633 53. Chuchird, N.; Rorkwiree, P.; Rairat, T. Effect of dietary formic acid and astaxanthin
634 on the survival and growth of Pacific white shrimp (*Litopenaeus vannamei*) and
635 their resistance to *Vibrio patahaemolyticus*. Spring. Plus **2015**, 4, 440.
636 54. Bakry, A.M.; Abbas, S.; Ali, B.; Majeed, H.; Abouelwafa, M.Y.; Mousa, A.; Li, L.
637 Microencapsulation of oils: a comprehensive review of benefits, techniques, and
638 applications. Comp. Rev. Food Sci. Safety **2015**, 15, 143-182.
639 55. Chen, J.; Wang, Q.; Liu, C.M.; Gong, J. Issues deserve attention in encapsulating
640 probiotics: critical review of existing literatures. Crit. Rev. Food Sci. Nut. **2017**, 57,
641 1228-1238.
642 56. Ndiaye, W.N.; Deschamps, M-H.; Comeau, Y.; Chowdhury, K.; Bunod, J-D.;
643 Letourneau-Montminy, M-P.; Vandenberg, G. *In situ* chelation of phosphorus using
644 microencapsulated aluminum and iron sulfate to bind intestinal phosphorus in
645 rainbow trout (*Oncorhynchus mykiss*). Anim. Feed Sci. Tech. **2020**,
646 https://doi.org/10.1016/j.anifeedsci.2020.114675.
647 57. Moradi, S. Effect of Feeding Diets Containing Organic Acid (Propionic Acid and
648 Formic Acid) on Growth Indices, Salinity Stress Resistance and Intestine
649 Microbiota in Common Carp (*Cyprinus carpio*). Master Thesis, Gorgan University
650 of Agricultural Sciences and Natural Resources, pp 70.
651 58. Sherif, A.H.; Doaa, M.G. Studies on the effect of acidifier on cultured *Oreochromis*
652 *niloticus* fish. J. Arab Aquac. Soc. **2013**, 8, 229–236.

 doi:10.20944/preprints202106.0108.v2

653 59. Rombenso, A.N.; Truong, H.; Simon, C. Dietary butyrate alone or in combination
654 with succinate and fumarate improved survival, feed intake, growth and nutrient
655 retention efficiency of juvenile *Penaeus monodon*. Aquac. **2020**, 528:735492.
656 60. He, W.; Rahimnejad, S.; Wang, L.; Song, K.; Lu, K.; Zhang, C. Effects of organic
657 acids and essential oils blend on growth, gut microbiota, immune response and
658 disease resistance of Pacific white shrimp (*Litopenaeus vannamei*) against *Vibrio*
659 *parahaemolyticus*. Fish Shell. Immun. **2017**, 70, 164-173.
660 61. Safari, O.; Paolucci, M.; Motlagh, H.M. Effect of dietary encapsulated organic salts
661 (Na-acetate, Na-butyrate, Na-lactate and Na-propionate) on growth performance,
662 haemolymph, antioxidant and digestive enzyme activities and gut microbiota of
663 juvenile narrow clawed crayfish, *Astacus leptodactylus* Eschscholtz, 1823. Aquac.
664 Nut. **2021**, 27, 91-104.