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Effect of dietary encapsulated organic salts (Na-acetate, Na-butyrate, Na-lactate and Na-propionate) on growth performance, haemolymph, antioxidant and digestive enzyme activities and gut microbiota of juvenile narrow clawed crayfish, *Astacus leptodactylus leptodactylus* Eschscholtz, 1823

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Abstract

A 63-day experiment was done to study the effects of four levels (5, 10, 20 and 50 g/kg) of encapsulated organic salts (Na-acetate, Na-butyrate, Na-lactate and Na-propionate) on the growth indices and haemato-immunological responses of crayfish *Astacus leptodactylus leptodactylus* (4.38 ± 0.08 g). Crayfish were distributed at 51 1,000-L tanks (17 treatments at triplicate). The highest values of final weight (27.86 g), specific growth rate (2.94% body weight per day) and survival rate (96%) were observed in the crayfish fed the 20 g/kg of encapsulated Na-propionate diet ($p < .05$). The highest activities of phenoloxidase (7.4 U/min), superoxide dismutase (7.80 U/min) and lysozyme (9.40 U/min) were observed in the gut of crayfish fed the 20 g/kg of encapsulated Na-propionate diet ($p < .05$), as well as the highest activities of alkaline protease (10.70 U/mg), lipase (9.10 U/mg), amylase (9.60 U/mg) and the lactobacillus count ($p < .05$). Broken line regression model of SGR and phenoloxidase activity suggested that the optimum dietary levels of encapsulated Na-acetate, Na-butyrate, Na-lactate and Na-propionate could be 30.7, 31.8, 31.4 and 33.5 g/kg, respectively, in crayfish reared in culture conditions.

KEYWORDS

antioxidant enzyme, crayfish, encapsulated organic acids, growth performance, gut microbiota, haemolymph

1 | INTRODUCTION

The intensification of the rearing systems poses serious challenges to aquaculture of shellfish and finfish worldwide, including the crustacean crayfish culture or astaciculture (FAO, 2018). In intensive practices, the spreading of diseases and infectious pathologies is facilitated and, along with the impoverishment of the water quality, negatively affects the health of the aquatic species (Assefa & Abunna, 2018). Optimal diet formulations are promising strategies to improve the production efficiency and the health condition of the farmed species, while limiting the use of antibiotics and chemicals, widely used to control mortality and infections (Glencross et al., 2007; Safari et al., 2014a). Using feed additives including prebiotics (Safari et al., 2014b), probiotics (Valipour et al., 2019), synbiotics (Safari & Paolucci, 2017a; Safari et al., 2017), nucleotides (Safari et al., 2015), L-carnitine (Safari et al., 2015) and phytogenics, plant-derived materials (Parrillo et al., 2017; Safari & Paolucci, 2017b) as generally recognized as safe (GRAS) materials

have shown positive effects on the growth performance, immunity and the stress resistance of crayfish. In this regard, organic acids (OAs), well known as acidifiers (ACIs) and short-chain fatty acids (SCFAs), were defined as by-products of substrate fermentation with gastrointestinal microbiome (Ng & Koh, 2017). Acetic, butyric, lactic and propionic acids are produced in the digestive tract (intestine) of animals via anaerobic microbial population (Llewellyn et al., 2014; Merrifield et al., 2014). Organic acids as feed additives in aquafeed production industry have been shown to have positive effects on the diet quality (reduction in pH and harmful bacteria intake), stomach functions (increment in enzyme activity and mineral solubility), intestine performance (increment in nutrient digestibility, mineral availability and gut health), faeces traits (reduction in phosphorus load and microbiota count) and, finally, the improvement of water quality (Ng & Koh, 2017).

Na-butyrate (20 g/kg) and Na-propionate (20 g/kg) administered in the diet of Pacific white shrimp, *Litopenaeus vannamei* (initial weight: 9.98 g for 14 days), improved feed intake compared with those fed the Na-acetate (20 g/kg) and the control group (da Silva et al., 2013). Also, feeding *L. vannamei* with diet containing Na-propionate showed the highest apparent digestibility coefficients (ADCs) of gross energy (ADC_{GE}) and phosphorus (ADC_{p}) (da Silva et al., 2013). Four-week feeding *L. vannamei* (5.34 g) with Na-butyrate (20 g/kg), probiotic (*Lactobacillus plantarum*; 1×10^7 CFU/kg) and the mixture (Na-butyrate + probiotic) did not improve final weight, feed conversion ratio and survival rate. However, total haemocyte count (THC) in shrimp fed with Na-butyrate diet was higher than those fed the diets containing probiotic and Na-butyrate + probiotic (Bolívar Ramírez et al., 2017). *L. vannamei* fed the Na-propionate (5, 10 and 20 g/kg) and Na-butyrate (5, 10 and 20 g/kg) for 47 days showed higher final weight, feed efficiency, survival rate and nitrogen retention when compared to control diet (da Silva et al., 2016). The inclusion of dietary Na-butyrate (20 g/kg) caused better growth performance in *L. vannamei* than Na-propionate (da Silva et al., 2016). The molecular weight of the organic acids, the number of carbons (C1-C18) existing in the molecular chain, the type of salts (calcium, potassium and sodium), the dose content (minimum and maximum), the inclusion method (encapsulated or non-encapsulated forms), the aquatic species (finfish and shellfish), the feeding time duration, the adaptation period and the types of measured biological responses are important parameters to take into consideration when selecting beneficial dietary organic acids in aquafeed production industry (Lückstädt, 2008; Ng & Koh, 2017; da Silva et al., 2013). In this regard, feeding behaviour of aquatics can affect the process of diet formulation and select aquafeed production technology (e.g. extrusion, expansion and cold-pressed) (Huntingford et al., 2012; Nates, 2016; Nazari et al., 2018; Ng & Koh, 2017; Safari et al., 2014a, 2016). To our best knowledge, there is no information in the literature about the use of organic salts as a supplement in the diet of Astacid crayfishes, especially *Astacus leptodactylus leptodactylus*. Therefore, the aim of the present study was to evaluate the dose responses of encapsulated organic salts (sodium (Na)-acetate, Na-butyrate, Na-lactate and Na-propionate) on the growth performance, nutritional efficiency indices, digestive enzyme activities, antioxidant and haemolymph responses, and the antioxidant status of juvenile narrow clawed crayfish (*Astacus leptodactylus leptodactylus*).

2 | MATERIALS AND METHODS

2.1 | Organic salt encapsulation and diet preparation

Four organic salts (Sigma-Aldrich Co.), sodium (Na)-acetate ($C_2H_3NaO_2$; 82.03 g/mol), sodium (Na)-butyrate ($C_4H_7NaO_2$; 110.09 g/mol), sodium (Na)-DL-lactate ($C_3H_5NaO_2$, 112.06) and sodium (Na)-propionate ($C_3H_5NaO_2$, 96.06 g/mol), were used at four levels (5, 10, 20 and 50 g/kg).

Briefly, organic salts were dissolved in double-distilled water (1:10 v:w) (Sarkheil et al., 2019). Then, 40 g gelatine (bovine skin, Merck Co.) was added to the solution, and the temperature was raised to 50°C, kept constant and stirred until achieving a clear solution. In another glass beaker, canola oil (Behpak Co., 50 ml) was mixed with 1 ml Span 80 (Merck Co.) as a non-ionic surfactant, stirred with a mechanical homogenizer (600 rpm for 5 min) and the temperature raised to 50°C. Afterwards, gelatine solution was gently added to the oil solution and stirred for 30 min at 50°C. Next, the solution was stirred until reaching the temperature of 25°C. The solution temperature was then decreased to 0–5°C through ice bath for 1 hr. The remaining oil was decanted, and the residual was washed with hexane solvent. Then, the solvent was decanted and GCs were washed with acetone solution and formaldehyde (35%) at 20–25°C for 30 min. Finally, the GCs were washed with cold water and acetone, and dried at room temperature.

A basal diet (384.1 g/kg, crude protein; 128.5 g/kg, crude fat; 14.93 Mj/kg, gross energy) as control diet (Safari et al., 2014b) was formulated with WUFFDA (Windows User-Friendly Feed Formulation, done again; University of Georgia) software (Table 1). GCs were replaced with carboxymethyl cellulose in the basal diet. Also, basal diet was supplemented with gelatine. After feedstuffs were ground to a particle size of <250 μm (Safari et al., 2014), the mash was processed by extrusion cooking technology (Fardan Machine Shargh Co) at 150°C with mesh size of 2 mm. Then, GCs containing organic salts and fish oil were coated over the pellet after extruding the diets, respectively, during decreasing pellet temperature in the mixer (stainless steel, 150 L), dried at 30°C, packed in three-layer waterproof nylon bags and maintained at −20°C until use.

2.2 | Crayfish and sample collection

Nine hundred eighteen healthy juvenile crayfish *Astacus leptodactylus leptodactylus* (4.38 ± 0.08 g) were obtained from the Shahid

TABLE 1 Composition (g/kg dry matter) of the control diet-fed juvenile crayfish (4.38 ± 0.08 g)

Ingredient	g/kg (dry-weight basis)
Menhaden fishmeal[a]	90
Soybean meal[a]	278
Corn gluten[a]	99
Wheat flour[a]	267
Corn starch[b]	38
Fish oil[a]	42
Canola oil[a]	41
Soy lecithin[a]	50
Cholesterol [c,d]	5
Glucosamine[c]	10
Choline chloride (70%)[d]	15
Gelatine[c]	2
Vitamin C (stay)[d]	10
Vitamin premix[d,e]	20
Mineral premix[d,e]	15
Carboxymethyl cellulose[c]	17.9
Ytterbium oxide[c]	0.1
Chemical composition	
Dry matter	874.2
Crude protein	384.1
Crude fat	128.5
Crude fibre	28.9
Nitrogen-free extract	420.6
Ash	37.9
Gross energy (Mj/kg)	14.93
Crude fat/ crude protein	0.33

[a]Behparvar Aquafeed Co.
[b]Scharloo Chemical Co.
[c]Sigma.
[d]Kimia Roshd Co.
[e]Mineral premix contains (mg/kg) Mg, 100; Zn, 60; Fe, 40; Cu, 5; Co, 0.1; I, 0.1; and antioxidant (BHT), 100. vitamin premix contains (mg/kg) vitamin E, 30; vitamin K, 3; thiamine, 2; riboflavin, 7; pyridoxine, 3; pantothenic acid, 18; niacin, 40; folacin, 1.5; choline, 600; biotin, 0.7; and cyanocobalamin, 0.02.

Yaghoobi reservoir (35°9′36″N 59°24′18″E, Khorasan Razavi Province, Iran) and stocked at a density of eighteen crayfish per 1,000-L tank (2 × 1 × 0.5 m) in a semi-recirculating system with daily water exchange rate of 25% at three replicates for each experimental diet. Each tank was fitted with 18 plastic tubes (4 cm diameter and 12 cm length), which served as hiding places for the animals. Unconsumed feed was collected 3 hr after feeding by manual syphoning and weighed. Water temperature was maintained at 25.3°C throughout the feeding trial. DO (6.26 ± 0.78 mg/L), pH (7.31 ± 0.67), hardness (153 ± 5.9 mg/L as $CaCO_3$), unionized ammonia (<0.06 mg/L) and nitrite contents (<0.6 mg/L) were evaluated every week. The animals were held under L:D 13:11 hr. Each diet was randomly assigned to a tank of crayfish, and they were fed 4% body weight thrice daily (8:00, 14:00 and 20:00) for 63 days. Biometry was done during the first and the last day of the experiment.

2.3 | Evaluation of growth performance and carcass quality

At the end of the feeding trial, each crayfish was individually weighed (±0.01 g) on an electronic scale (AND, Japan). All parameters were corrected based on the ingested feed. Growth parameters, survival rate and nutrient efficiency indices were calculated as follows (Safari et al., 2014b):

$$\text{Specific growth rate (SGR; \%/day)} = \frac{\ln W_f - \ln W_i}{t} \times 100$$

$$\text{Survival rate (\%)} = \frac{\text{Final Individual Numbers}}{\text{Initial Individual Numbers}} \times 100$$

$$\text{Voluntary feed intake (VFI; \% body weight per day)} = \frac{\text{Feed}_{\text{consumed (DM)}}}{W_{\text{mean}} \times t}$$

$$\text{Feed conversion ratio (FCR)} = \frac{\text{Feed}_{\text{consumed}}}{W_{\text{gain}}}$$

$$\text{Protein efficiency ratio (PER)} = \frac{W_{\text{gain}}}{\text{Crude protein}_{\text{consumed}}}$$

In the above equations, W_i, W_f, W_{mean} and W_{gain}, t and $\text{Feed}_{consumed}$ are initial weight, final weight, mean weight, weight increment (g), time period (day) and consumed feed (g), respectively.

2.4 | Biochemical analyses

2.4.1 | Haemolymph indices

At the end of the feeding trial, six crayfish from each tank (18 crayfish per treatment) were killed 24 hr after the last meal. All assays were done one by one at triplicate with syringe 25 G, according to previously described protocol (Safari et al., 2014b). The haemolymph (125 μl) was stored in the tube (1 ml) without heparin as an anticoagulant. THC was determined with haemocytometer cell (Beco). To measure hyaline count (HC), semi-granular count and large-granular count (SGC and LGC, respectively), the haemolymph was extended at room temperature (25°C), fixed at methanol (1 min), stained with method of May–Grunwald–Giemsa and finally counted with light microscope.

2.4.2 | The activities of phenoloxidase, superoxide dismutase, lysozyme and nitric oxide synthase

The heparinized haemolymph (250 μl) was centrifuged at 700 *g* for 20 min at 4°C to separate the haemocytes from plasma, and the supernatant fluid was used for plasma determinations (Safari et al., 2014b). All activities of enzymes were standardized based on the protein concentration. Total plasma protein content was estimated using the biuret procedure. Phenoloxidase activity (PO) was assayed spectrophotometrically by recording the formation of dopachrome from L-dihydroxyphenylalanine (L-DOPA) at 490 nm (Hernández-López et al., 1996; Safari et al., 2014). Superoxide dismutase (SOD) activity was measured by observing the inhibition of ferricytochrome C reduction at 550 nm (Cooper et al., 2002). The lysozyme (LYZ) activity was determined with a decrease in absorbance compared to *Micrococcus lysodeikticus* suspension without plasma at 530 nm (Ellis, 1990). Nitric oxide synthase (NOS) activity was measured with the assay kit (Nanjing Jiancheng Bioengineering Institute) (Marzinzig et al., 1997).

2.4.3 | Digestive enzyme activities

The gut (nine crayfish per treatment) was quickly removed, rinsed with distilled water, dried with paper towel, homogenized (30 g/ 70 ml distilled water) using a homogenizer (DI 18 Disperser) and the homogenate was then centrifuged at 10,000 *g*, at 4°C, for 25 min. The supernatant was stored in liquid nitrogen. The measurement of digestive enzyme activities was explained elsewhere (Safari et al., 2014b). Briefly, the amylase activity was measured using starch as substrate at 550 nm with a UV-Vis spectrophotometer (Ultrospec 2000 Pharmacia Biotech) (Coccia et al., 2011). Lipase activity was measured using α-naphthyl caprylate as substrate at 540 nm (López-López et al., 2003). Alkaline protease activity was determined using azocasein as substrate at 366 nm (Fernández Gimenez et al., 2001). In this study, specific enzyme activity was defined as enzyme units (U) per mg of protein.

2.5 | Chemical analysis

Analysis of dry matter (oven drying, 105°C), crude protein ($N \times 6.25$, Kjeldahl System: Buchi Labortechnik AG), crude fat (Soxtec System HT 1,043: Foss Tecator, AB), ash (muffle furnace, 550°C), gross energy (Parr Bomb Calorimetry Model 1266, Parr Instrument Co.) and crude fibre (after digestion with H_2SO_4 and NaOH) contents of feedstuffs, diets and faeces was performed according to standard methods (AOAC, 2005). Nitrogen-free extract (NFE) was calculated by subtracting crude protein, crude fat, crude fibre and ash contents from the dry matter.

2.6 | Bacteriological analysis

At the beginning of the feeding trial, total aerobic bacteria (TAB), lactic acid bacteria (LAB), *Escherichia coli* and fungi (yeast and mould) counts of the hepatopancreas were determined by random sampling 15 crayfish from the stock. As described previously (Safari & Paolucci, 2017a; Safari & Paolucci, 2017b; Safari & Paolucci, 2017c; Safari et al., 2014), at the end of the experiment, crayfish (15 individuals per a treatment) were transported alive to the laboratory, anaesthetized with ice, rinsed with benzalkonium chloride (0.1% for 60 min) and dissected with a scalpel. Then, the hepatopancreas was removed and homogenized with sodium chloride (0.9 w/v) using a homogenizer (DI 18 Disperser). The homogenate was centrifuged at 5,000 *g*, 4°C, 5 min, and the sample (100 μl) was put onto plate count agar (PCA; Merck Co.), de Man, Rogosa and Sharpe media (MRS; Merck Co.), MacConkey agar (Merck Co.) and potato dextrose agar (PDA; Merck Co.) at triplicates in order to determine TAB, LAB, *E. coli* and fungi counts, respectively. Colony-forming units (CFU)/g were calculated from plates containing 30–300 colonies (Safari & Paolucci, 2017a; Safari & Paolucci, 2017b; Safari & Paolucci, 2017c; Safari et al., 2014).

2.7 | Statistical analysis

All percentage data were transformed using arcsine method. After confirming the homogeneity of variance and normality of the data using Leaven's and Kolmogorov–Smirnov tests (Zar, 2007), respectively, ANOVA was used to compare the treatments at three replicates. Duncan's test was applied to compare significant differences among the treatments ($p < .05$) with SPSS™ version 19. Broken line regression model was used to determine the optimum dose of encapsulated organic acids for SGR value and PO activity of test animal with SPSS™ version 19. All results were given as mean ± *SD*.

3 | RESULTS

3.1 | Growth indices and survival rate

Administration of dietary encapsulated Na-acetate (20 and 50 g/kg), Na-butyrate (10, 20 and 50 g/kg), Na-lactate (5, 10, 20 and 50 g/kg) and Na-propionate (5, 10, 20 and 50 g/kg) improved significantly ($p < .05$) growth performance (final weight, SGR, FCR, VFI) and survival rate in juvenile crayfish compared with control-fed crayfish (Table 2). Nutritional efficiency indices (PER and PPV) increased significantly ($p < .05$) in crayfish fed the diets containing 5, 10, 20 and 50 g/kg of encapsulated Na-acetate, Na-butyrate, Na-lactate and Na-propionate compared with those fed the control diet (Table 3). Feeding crayfish with different levels of dietary encapsulated Na-lactate and Na-propionate improved significantly ($p < .05$) growth performance and nutritional efficiency indices compared with those fed the control, Na-acetate and Na-butyrate diets (Tables 2 and 3). Feeding crayfish with 20 g/kg of encapsulated Na-propionate diet showed the highest ($p < .05$) values of final weight (27.86 g), SGR (2.94% BW/day), survival rate (96%), PER (3.09) and PPV (68%) (Tables 2 and 3). The lowest FCR values were observed in crayfish

TABLE 2 The mean (±*SD*) of initial weight (g), final weight (g), specific growth rate (%/day), feed conversion ratio, survival rate (%), protein efficiency ratio and protein productive value (%) of crayfish fed the experimental diets containing different levels (5, 10, 20 and 50 g/kg) of encapsulated sodium (Na)-acetate, Na-butyrate, Na-lactate and Na-propionate after 63 days ($n = 3$)

	Initial weight (g)	Final weight (g)	Specific growth rate (%BW/day)	Feed conversion ratio	Survival rate (%)	Protein efficiency ratio	Protein productive value (%)
Control	4.38 ± 0.06^{a}	9.83 ± 0.61^{a}	1.28 ± 0.10^{a}	3.37 ± 0.17^{k}	39.00 ± 0.17^{b}	1.22 ± 0.03^{a}	41.00 ± 0.09^{a}
Na- acetate (g/kg)							
5	4.39 ± 0.06^{a}	10.08 ± 0.51^{a}	1.32 ± 0.68^{ab}	38.00 ± 0.16^{a}	3.26 ± 0.16^{ijk}	1.35 ± 0.03^{b}	42.00 ± 0.11^{b}
10	4.38 ± 0.01^{a}	10.93 ± 0.70^{abc}	1.45 ± 0.10^{bc}	49.00 ± 0.14^{d}	3.12 ± 0.14^{ijk}	1.66 ± 0.04^{d}	46.00 ± 0.12^{d}
20	4.38 ± 0.06^{a}	15.15 ± 1.05^{f}	1.97 ± 0.11^{f}	73.00 ± 0.15^{i}	2.67 ± 0.15^{def}	2.18 ± 0.05^{g}	53.00 ± 0.11^{i}
50	4.38 ± 0.06^{a}	12.25 ± 0.68^{cde}	1.63 ± 0.09^{d}	57.00 ± 0.17^{f}	2.90 ± 0.17^{fgh}	1.82 ± 0.05^{e}	48.00 ± 0.10^{f}
Na- butyrate (g/kg)							
5	4.38 ± 0.01^{a}	10.53 ± 0.61^{ab}	1.39 ± 0.09^{ab}	46.00 ± 0.15^{c}	3.19 ± 0.15^{ijk}	1.45 ± 0.04^{c}	45.00 ± 0.12^{c}
10	4.38 ± 0.06^{a}	11.73 ± 0.81^{bcd}	1.56 ± 0.11^{cd}	53.00 ± 0.13^{e}	3.01 ± 0.13^{ghi}	1.80 ± 0.04^{e}	47.00 ± 0.11^{e}
20	4.38 ± 0.06^{a}	13.48 ± 0.86^{e}	1.78 ± 0.10^{e}	65.00 ± 0.13^{h}	2.78 ± 0.13^{efg}	2.08 ± 0.05^{f}	51.00 ± 0.12^{h}
50	4.38 ± 0.01^{a}	12.54 ± 0.60^{de}	1.67 ± 0.08^{de}	61.00 ± 0.16^{g}	2.82 ± 0.16^{efg}	1.86 ± 0.05^{e}	50.00 ± 0.10^{g}
Na- lactate (g/kg)							
5	4.38 ± 0.01^{a}	16.33 ± 0.87^{f}	2.09 ± 0.09^{fg}	80.00 ± 0.13^{j}	2.64 ± 0.13^{def}	2.22 ± 0.06^{g}	57.00 ± 0.07^{j}
10	4.38 ± 0.06^{a}	19.53 ± 0.96^{h}	2.37 ± 0.08^{h}	86.00 ± 0.14^{l}	2.51 ± 0.14^{bcd}	2.44 ± 0.06^{h}	59.00 ± 0.09^{l}
20	4.38 ± 0.01^{a}	26.08 ± 0.03^{k}	2.83 ± 0.06^{j}	94.00 ± 0.14^{p}	2.29 ± 0.14^{ab}	2.86 ± 0.07^{j}	67.00 ± 0.11^{p}
50	4.38 ± 0.06^{a}	23.84 ± 0.08^{j}	2.69 ± 0.06^{i}	89.00 ± 0.11^{n}	2.45 ± 0.11^{abcd}	2.64 ± 0.07^{i}	65.00 ± 0.08^{n}
Na-propionate (g/kg)							
5	4.38 ± 0.01^{a}	17.66 ± 1.00^{g}	2.21 ± 0.09^{g}	84.00 ± 0.13^{k}	2.56 ± 0.13^{cde}	2.37 ± 0.06^{h}	58.00 ± 0.08^{k}
10	4.38 ± 0.01^{a}	22.41 ± 1.50^{i}	2.59 ± 0.11^{i}	87.00 ± 0.12^{m}	2.48 ± 0.12^{bcd}	2.55 ± 0.07^{i}	64.00 ± 0.10^{m}
20	4.38 ± 0.01^{a}	27.86 ± 0.21^{l}	2.94 ± 0.01^{k}	96.00 ± 0.13^{q}	2.21 ± 0.13^{a}	3.09 ± 0.08^{k}	68.00 ± 0.12^{q}
50	4.38 ± 0.06^{a}	23.98 ± 0.18^{j}	2.70 ± 0.01^{ij}	90.00 ± 0.15^{o}	2.34 ± 0.15^{abc}	2.83 ± 0.07^{j}	66.00 ± 0.09^{o}
p-value	.738	.0001	.0001	.0001	.0001	.0001	.0001
Mean of different levels (g/kg)							
Control	4.38 ± 0.06^{A}	9.83 ± 0.61^{A}	1.28 ± 0.10^{A}	3.37 ± 0.17^{C}	39.00 ± 0.17^{A}	1.22 ± 0.03^{A}	41.00 ± 0.09^{A}
Na-acetate	4.38 ± 0.08^{A}	12.11 ± 2.11^{A}	1.59 ± 0.27^{B}	2.99 ± 0.27^{B}	54.25 ± 13.32^{B}	1.75 ± 0.32^{B}	47.25 ± 4.14^{B}
Na-butyrate	4.38 ± 0.08^{A}	12.07 ± 1.29^{A}	1.60 ± 0.17^{B}	2.95 ± 0.21^{B}	56.25 ± 7.65^{B}	1.80 ± 0.24^{B}	48.25 ± 2.49^{B}
Na-lactate	4.38 ± 0.01^{A}	21.44 ± 3.98^{B}	2.50 ± 0.30^{C}	2.47 ± 0.17^{A}	87.25 ± 5.30^{C}	2.54 ± 0.25^{C}	62.00 ± 4.31^{C}
Na-propionate	4.38 ± 0.08^{A}	22.98 ± 3.90^{B}	2.61 ± 0.28^{C}	2.40 ± 0.18^{A}	89.25 ± 4.64^{C}	2.71 ± 0.29^{C}	64.00 ± 3.91^{C}
p-value	.932	.0001	.0001	.0001	.0001	.0001	.0001

Note: Different superscripts (a-q and A-C) within columns indicate significant differences at $p < .05$.
Abbreviation: *SD*, Standard deviation.

fed the diets containing 20 and 50 g/kg of encapsulated Na-lactate and Na-propionate (Table 2). Based on the broken line regression model, the dietary requirements of encapsulated Na-acetate, Na-butyrate, Na-lactate and Na-propionate for maximum growth (SGR) of crayfish were estimated to be 31.3. 30.7, 31.8 and 31.7 g/kg, respectively (Figures 1 and 2).

3.2 | Haemolymph indices

Feeding the juvenile crayfish with the diets containing 10, 20 and 50 g/kg of encapsulated Na-acetate and Na-butyrate and 5, 10, 20 and 50 g/kg of encapsulated Na-lactate and Na-propionate improved significantly ($p < .05$) the THC value with respect to the control diet

TABLE 3 The mean (±*SD*) of total haemocyte count (THC, $\times 10^5$ cell/ml), hyaline count (HC, $\times 10^5$ cell/ml), semi-granular count (SGC, $\times 10^5$ cell/ml) and large-granular count (LGC, $\times 10^5$ cell/ml) of crayfish fed the experimental diets containing different levels (5, 10, 20 and 50 g/kg) of encapsulated sodium (Na)-acetate, Na-butyrate, Na-lactate and Na-propionate after 63 days ($n = 3$)

	THC ($\times 10^5$ cell/ml)	HC ($\times 10^5$ cell/ml)	SGC ($\times 10^5$ cell/ml)	LGC ($\times 10^5$ cell/ml)
Control	75.00 ± 5.00^{a}	70.00 ± 0.09^{a}	19.00 ± 0.09^{a}	18.00 ± 0.17^{a}
Acetic acid (g/kg)				
5	78.00 ± 6.00^{ab}	75.00 ± 0.11^{b}	21.00 ± 0.11^{b}	19.00 ± 0.16^{b}
10	87.00 ± 7.00^{bcd}	80.00 ± 0.12^{d}	25.00 ± 0.12^{d}	23.00 ± 0.14^{d}
20	98.00 ± 6.00^{defg}	95.00 ± 0.11^{i}	36.00 ± 0.12^{i}	32.00 ± 0.15^{h}
50	90.00 ± 7.00^{cde}	85.00 ± 0.10^{f}	30.00 ± 0.10^{f}	24.00 ± 0.17^{e}
Butyric acid (g/kg)				
5	84.00 ± 8.00^{abc}	78.00 ± 0.12^{c}	24.00 ± 0.12^{c}	21.00 ± 0.15^{c}
10	89.00 ± 8.00^{bcde}	83.00 ± 0.11^{e}	28.00 ± 0.11^{e}	23.00 ± 0.13^{d}
20	95.00 ± 5.00^{cdef}	92.00 ± 0.12^{h}	35.00 ± 0.12^{h}	28.00 ± 0.13^{g}
50	93.00 ± 6.00^{cdef}	89.00 ± 0.10^{g}	34.00 ± 0.10^{g}	26.00 ± 0.16^{f}
Lactic acid (g/kg)				
5	99.00 ± 8.00^{efgh}	98.00 ± 0.07^{j}	39.00 ± 0.07^{j}	34.00 ± 0.13^{i}
10	104.00 ± 6.00^{fghi}	103.00 ± 0.09^{l}	43.00 ± 0.09^{l}	37.00 ± 0.14^{k}
20	117.00 ± 6.00^{j}	109.00 ± 0.11^{p}	51.00 ± 0.11^{p}	49.00 ± 0.14^{o}
50	110.00 ± 5.00^{hij}	105.00 ± 0.08^{n}	47.00 ± 0.08^{n}	42.00 ± 0.11^{m}
Propionic acid (g/kg)				
5	100.00 ± 7.00^{efgh}	100.00 ± 0.08^{k}	42.00 ± 0.08^{k}	35.00 ± 0.13^{j}
10	108.00 ± 5.00^{hij}	104.00 ± 0.10^{m}	46.00 ± 0.10^{m}	39.00 ± 0.12^{l}
20	118.33 ± 2.89^{j}	110.00 ± 0.12^{q}	55.00 ± 0.12^{q}	59.00 ± 0.13^{p}
50	115.00 ± 7.00^{ij}	107.00 ± 0.09^{o}	49.00 ± 0.09^{o}	45.00 ± 0.15^{n}
p-value	.0001	.0001	.0001	.0001
Mean of different levels (g/kg)				
Control	75.00 ± 5.00^{A}	70.00 ± 0.09^{A}	19.00 ± 0.09^{A}	18.00 ± 0.17^{A}
Acetic acid	88.25 ± 9.31^{B}	83.75 ± 7.73^{B}	28.00 ± 5.86^{B}	24.50 ± 4.93^{A}
Butyric acid	90.25 ± 7.32^{B}	85.50 ± 5.65^{B}	30.25 ± 4.69^{B}	24.50 ± 2.82^{A}
Lactic acid	107.50 ± 8.87^{C}	103.75 ± 4.14^{C}	45.00 ± 4.67^{C}	40.50 ± 5.93^{B}
Propionic acid	110.33 ± 8.83^{C}	105.25 ± 3.87^{C}	48.00 ± 4.96^{C}	44.50 ± 9.50^{B}
p-value	.0001	.0001	.0001	.0001

Different superscripts (a-q and A-C) within columns indicate significant differences at $p < .05$.
Abbreviation: *SD*, Standard deviation.

(Table 4). The values of HC, SGC and LGC in juvenile crayfish fed the 5, 10, 20 and 50 g/kg of encapsulated Na-acetate, Na-butyrate, Na-lactate and Na-propionate were significantly ($p < .05$) higher than the control diet (Table 4). Feeding crayfish with different levels of dietary encapsulated Na-lactate and Na-propionate increased significantly ($p < .05$) the values of THC, HC and SGC compared with the control, encapsulated Na-acetate and Na-butyrate diets (Table 4). However, the values of LGC in crayfish fed the different levels of encapsulated Na-acetate and Na-butyrate and control diet were significantly ($p < .05$) lower than those fed the different levels of encapsulated Na-lactate and Na-propionate (Table 4). Feeding crayfish with 20 g/kg of encapsulated Na-propionate diet showed the highest ($p < .05$) values ($\times 10^5$ cell/ml) of HC (110), SGC (55) and LGC (59) (Table 4).

3.3 | The activities of phenoloxidase (PO), superoxide dismutase (SOD), lysozyme (LYZ) and nitric oxide synthase (NOS)

After 63-day feeding trial, the juvenile crayfish fed the 10, 20 and 50 g/kg of encapsulated Na-acetate diet and 5, 10, 20 and 50 g/kg of encapsulated Na-butyrate, Na-lactate and Na-propionate diets

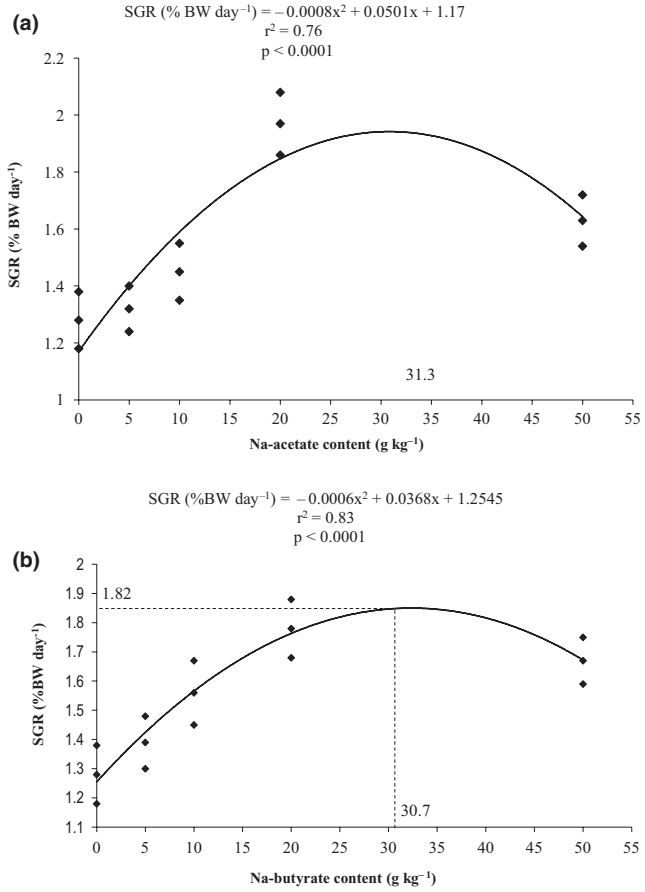

FIGURE 1 Polynomial model fitting specific growth rate (% bodyweight per day) to different levels of encapsulated (a) sodium (Na)-acetate content (g/kg) and (b) sodium (Na)-butyrate content (g/kg) in crayfish fed with experimental diets at three replicates

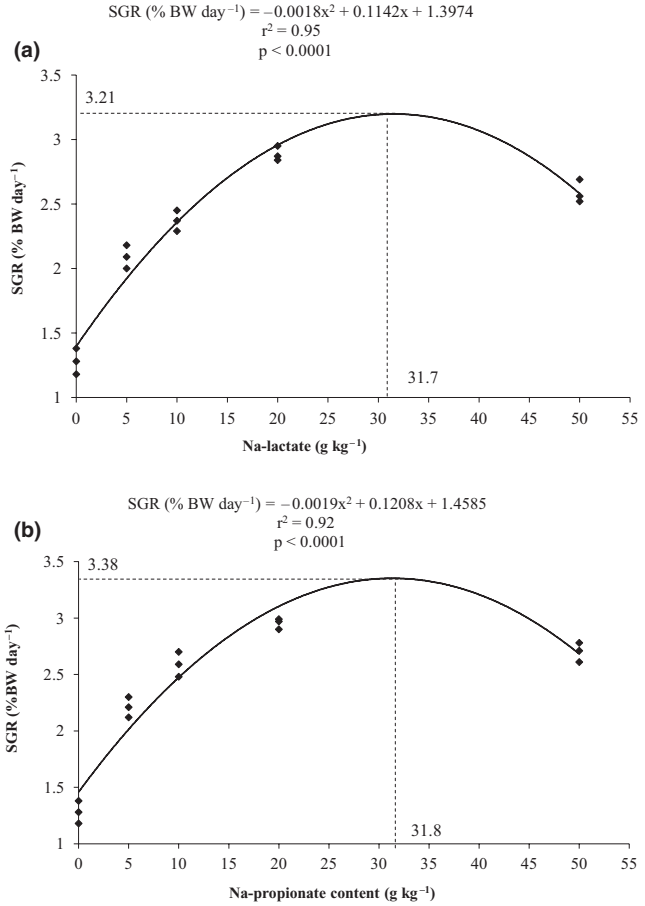

FIGURE 2 Polynomial model fitting specific growth rate (% bodyweight per day) to different levels of encapsulated (a) sodium (Na)-lactate content (g/kg) and (b) sodium (Na)-propionate content (g/kg) in crayfish fed with experimental diets at three replicates

showed significantly ($p < .05$) higher activities (U/min) of PO, SOD, LYZ and NOS with respect to the control diet (Table 5). The activities of PO, SOD, LYZ and NOS in crayfish fed the diets containing different levels of encapsulated Na-lactate and Na-propionate were significantly ($p < .05$) higher than the control and encapsulated Na-acetate and Na-butyrate diets (Table 5). Feeding crayfish with 20 g/kg of encapsulated Na-propionate diet showed the highest ($p < .05$) activities of PO (7.40 U/min), SOD (7.80 U/min) and LYZ (9.40 U/min) (Table 5). Based on the broken line regression model, the dietary requirements of encapsulated Na-acetate, Na-butyrate, Na-lactate and Na-propionate for maximum PO activity of crayfish were estimated to be 31.6. 33.5, 31.4 and 31.4 g/kg, respectively (Figures 3 and 4).

3.4 | Digestive enzyme activities

The alkaline protease and amylase activities (U/mg) in the hepatopancreas of juvenile crayfish fed the diets containing 5, 10, 20 and 50 g/kg of encapsulated Na-acetate, Na-butyrate, Na-lactate and Na-propionate were significantly ($p < .05$) higher than the control (Table 5). Lipase activity in the hepatopancreas of crayfish fed the 5 g/kg of encapsulated Na-acetate did not show significant difference with the control. However, lipase activities in crayfish fed the diets containing 10, 20 and 50 g/kg of encapsulated Na-acetate and 5, 10, 20 and 50 g/kg of encapsulated Na-butyrate, Na-lactate and Na-propionate were significantly ($p < .05$) higher than the control (Table 5).

3.5 | Microbiological analysis

Total aerobic bacteria (TAB; CFU/g) and fungi counts in the hepatopancreas of the crayfish fed the experimental diets containing different levels of organic acids did not show the significant values compared with those fed the control diet (Figures 5 and 6). The crayfish fed the 20 g/kg Na-propionate showed the significantly ($p < .05$) highest lactic acid bacteria count and the lowest *E. coli* count (Figures 5 and 6).

4 | DISCUSSION

Organic acids or their salts with different origins including internal (by microbiota activity) or external (via along inclusion in diet) are regarded as non-antibiotic growth promoters (Lückstädt, 2008; da

TABLE 4 The mean (±*SD*) activities (U/min) of phenoloxidase (PO), superoxide dismutase (SOD), lysozyme (LYZ) and nitric oxide synthase (NOS) of crayfish fed the experimental diets containing different levels (5, 10, 20 and 50 g/kg) of encapsulated sodium (Na)-acetate, Na-butyrate, Na-lactate and Na-propionate after 63 days ($n = 3$)

	PO activity (U/min)	SOD activity (U/min)	LYZ activity (U/min)	NOS activity (U/min)
Control	2.30 ± 0.26^{a}	1.90 ± 0.09^{a}	4.30 ± 0.09^{a}	2.30 ± 0.26^{a}
Na-acetate (g/kg)				
5	2.50 ± 0.27^{ab}	2.30 ± 0.11^{b}	4.50 ± 0.10^{b}	2.50 ± 0.27^{ab}
10	2.90 ± 0.26^{bc}	2.80 ± 0.12^{d}	5.00 ± 0.09^{d}	3.00 ± 0.26^{cd}
20	4.30 ± 0.26^{gh}	4.50 ± 0.11^{i}	6.40 ± 0.10^{h}	4.00 ± 0.26^{fg}
50	3.40 ± 0.27^{de}	3.80 ± 0.10^{f}	5.30 ± 0.12^{e}	3.40 ± 0.27^{de}
Na-butyrate (g/kg)				
5	2.70 ± 0.27^{ab}	2.50 ± 0.12^{c}	4.80 ± 0.08^{c}	2.80 ± 0.27^{bc}
10	3.20 ± 0.24^{cd}	3.40 ± 0.11^{e}	5.20 ± 0.11^{e}	3.40 ± 0.24^{de}
20	3.90 ± 0.25^{fg}	4.30 ± 0.12^{h}	6.20 ± 0.11^{g}	3.60 ± 0.25^{ef}
50	3.80 ± 0.26^{ef}	4.00 ± 0.10^{g}	5.90 ± 0.12^{f}	3.50 ± 0.26^{e}
Na-lactate (g/kg)				
5	4.60 ± 0.20^{hi}	5.00 ± 0.07^{j}	6.80 ± 0.10^{i}	4.30 ± 0.20^{gh}
10	5.30 ± 0.23^{jk}	6.80 ± 0.09^{l}	7.40 ± 0.11^{j}	4.70 ± 0.23^{hij}
20	6.90 ± 0.25^{n}	7.40 ± 0.11^{n}	9.00 ± 0.10^{m}	5.40 ± 0.25^{kl}
50	5.90 ± 0.19^{lm}	7.00 ± 0.08^{m}	8.50 ± 0.08^{l}	5.00 ± 0.19^{ijk}
Na-propionate (g/kg)				
5	4.80 ± 0.21^{ij}	5.70 ± 0.08^{k}	7.10 ± 0.12^{j}	4.60 ± 0.21^{hi}
10	5.70 ± 0.22^{kl}	6.90 ± 0.10^{lm}	8.30 ± 0.07^{k}	5.00 ± 0.22^{ijk}
20	7.40 ± 0.25^{o}	7.80 ± 0.12^{o}	9.40 ± 0.08^{n}	5.50 ± 0.25^{l}
50	6.30 ± 0.24^{m}	7.30 ± 0.09^{n}	8.90 ± 0.09^{m}	5.10 ± 0.24^{jkl}
p-value	.0001	.0001	.0001	.0001
Mean of different levels (g/kg)				
Control	2.30 ± 0.26^{A}	1.90 ± 0.09^{A}	4.30 ± 0.09^{A}	2.30 ± 0.26^{A}
Na-acetate	3.28 ± 0.74^{B}	3.35 ± 0.90^{B}	5.30 ± 0.73^{B}	3.23 ± 0.62^{B}
Na-butyrate	3.40 ± 0.55^{B}	3.55 ± 0.72^{B}	5.53 ± 0.59^{B}	3.33 ± 0.39^{B}
Na-lactate	5.68 ± 0.91^{C}	6.55 ± 0.96^{C}	7.93 ± 0.91^{C}	4.85 ± 0.46^{C}
Na-propionate	6.05 ± 1.01^{C}	6.93 ± 0.81^{C}	8.43 ± 0.90^{C}	5.05 ± 0.39^{C}
p-value	.0001	.0001	.0001	.0001

Different superscripts (a–q and A–C) within columns indicate significant differences at $p < .05$.
Abbreviation: *SD*, Standard deviation.

Silva et al., 2016). Similar to the symbiotic effects on biological indices of aquatic species, organic acids act through the modification of gastrointestinal microbiota (Ahmadniaye Motlagh et al., 2019; De Schryver et al., 2010; Defoirdt et al., 2011; Lückstädt, 2008; Mine & Boopathy, 2011), the proliferation of gut epithelial cell (Ng & Koh, 2017), the action of digestive enzymes (Ahmadniaye Motlagh et al., 2019; da Silva et al., 2016), the improvement of nutrition efficiency indices (Sarker et al., 2012a, 2012b), lipid synthesis (Marcil et al., 2002) and bioenergetic pathways such as the routes of citric and carboxylic acids (Lückstädt, 2008; Ng & Koh, 2017). In this regard, feeding behaviour of aquatics can affect the process of diet formulation and select aquafeed production technology (e.g. extrusion, expansion and cold-pressed) (Huntingford et al., 2012; Nates, 2016; Nazari et al., 2018; Ng & Koh, 2017; Safari et al., 2014a, 2016). Due to the very slow feeding behaviour of crustaceans, especially crayfish, leading to nutrient leaching (Ringø, 1991), it is recommended to use encapsulated diets (Gatlin et al., 2007; Liu et al., 2014; Ng & Koh, 2017; Safari, et al., 2015). However, the rate of releasing of Na-organic salts into water body was not measured in the present study, but the improvement of biological indices in crayfish fed the experimental diets containing organic acids, totally, maybe confirmed this matter. In this context, the protection of feed additives, especially organic salts, against water leaching and the kinds of oxidation, merits further investigations to generate executive instructions.

4.1 | Growth indices

Administration of encapsulated Na-organic salts (acetate, butyrate, lactate and propionate) in the diet of juvenile crayfish improved

TABLE 5 The mean (±*SD*) activities (U/mg) of alkaline protease, lipase and amylase in the gut of crayfish fed the experimental diets containing different levels (5, 10, 20 and 50 g/kg) of encapsulated sodium (Na)-acetate, Na-butyrate, Na-lactate and Na-propionate after 63 days ($n = 3$)

	Alkaline protease (U/mg)	Lipase (U/mg)	Amylase (U/mg)
Control	2.50 ± 0.18^{a}	3.60 ± 0.17^{a}	2.40 ± 0.26^{a}
Na-acetate (g/kg)			
5	3.60 ± 0.21^{b}	3.70 ± 0.16^{ab}	3.70 ± 0.26^{b}
10	5.90 ± 0.21^{d}	4.30 ± 0.14^{c}	4.60 ± 0.23^{c}
20	7.90 ± 0.21^{g}	5.80 ± 0.15^{f}	6.40 ± 0.25^{f}
50	6.50 ± 0.22^{e}	4.90 ± 0.17^{de}	5.80 ± 0.29^{e}
Na-butyrate (g/kg)			
5	4.70 ± 0.20^{c}	3.90 ± 0.15^{b}	4.30 ± 0.23^{c}
10	6.20 ± 0.22^{de}	4.70 ± 0.13^{d}	5.20 ± 0.24^{d}
20	7.30 ± 0.23^{f}	5.70 ± 0.13^{f}	6.30 ± 0.24^{f}
50	7.20 ± 0.22^{f}	5.10 ± 0.16^{e}	5.70 ± 0.28^{e}
Na-lactate (g/kg)			
5	8.10 ± 0.17^{gh}	6.10 ± 0.13^{g}	6.50 ± 0.23^{f}
10	8.60 ± 0.20^{i}	7.00 ± 0.14^{i}	8.90 ± 0.25^{h}
20	10.50 ± 0.21^{m}	8.70 ± 0.14^{m}	9.70 ± 0.24^{j}
50	9.60 ± 0.16^{k}	7.90 ± 0.11^{k}	9.20 ± 0.19^{hi}
Na-propionate (g/kg)			
5	8.30 ± 0.20^{hi}	6.60 ± 0.13^{h}	7.60 ± 0.25^{g}
10	9.20 ± 0.17^{j}	7.30 ± 0.12^{j}	9.10 ± 0.19^{h}
20	10.70 ± 0.20^{m}	9.10 ± 0.13^{n}	9.90 ± 0.21^{j}
50	10.10 ± 0.18^{l}	8.30 ± 0.15^{l}	9.60 ± 0.24^{ij}
p-value	.0001	.0001	.0001
Mean of different levels (g/kg)			
Control	2.50 ± 0.18^{A}	3.60 ± 0.17^{A}	2.40 ± 0.26^{A}
Na-acetate	5.98 ± 1.63^{B}	4.68 ± 0.82^{B}	5.13 ± 1.16^{B}
Na-butyrate	6.35 ± 1.11^{B}	4.85 ± 0.69^{B}	5.38 ± 0.79^{B}
Na-lactate	9.20 ± 0.98^{C}	7.43 ± 1.02^{C}	8.58 ± 1.30^{C}
Na-propionate	9.58 ± 0.96^{C}	7.83 ± 1.01^{C}	9.05 ± 0.94^{C}
p-value	.0001	.0001	.0001

Different superscripts (a–q) within columns indicate significant differences at $p < .05$.
Abbreviation: *SD*, Standard deviation.

growth performance (SGR, FCR and VFI), survival rate and nutrition efficiency indices (PER and PPV) compared with the control. The dietary administration of encapsulated Na-lactate (C3) and Na-propionate (C3) improved the growth performance, nutrition efficiency indices and survival rate of crayfish compared with those fed the Na-acetate (C2) and Na-butyrate (C4). The positive effects of SCFAs in aquafeeds on the improvement of in vivo apparent digestibility coefficients of nutrients (Hoseinifar et al., 2015; da Silva et al., 2013) and growth performance, immunity and survival rate (Cummings & Macfarlane, 2002; Hoseinifar et al., 2015; Maslowski & Mackay, 2011; Scheppach, 1994; Schley & Field, 2002) were reported in the literature. Although the inclusion of dietary single or mixed organic salts in aquafeeds was reported in previous studies (Ng & Koh, 2017), there is little information available in the literature regarding the comparative effects of organic acids and the choice of the best organic acid (Ringø, 1991). The research on the dietary additives (organic salts) for crayfish compared with finfish is still in the early stage.

Feeding arctic charr (*Salvelinus alpinus*; initial weight: 311 g) for 84 days with supplemented diet containing 10 g/kg Na-lactate improved the final weight compared with Na-propionate (Ringø, 1991). In the present study, the optimum content of dietary encapsulated Na-organic salts based on the broken line regression model of SGR data was 30–32 g/kg. However, the optimum content of Na-propionate in the diet of *L. vannamei* was reported 20 g/kg (da Silva et al., 2016). This difference can be related to the species, the physicochemical properties of water and the used method to estimate the optimum dose content. Butyrate (C4) as a major energy source of colonic epithelial cells improves the process of epithelium maintenance (Maslowski & Mackay, 2011), activates the immune

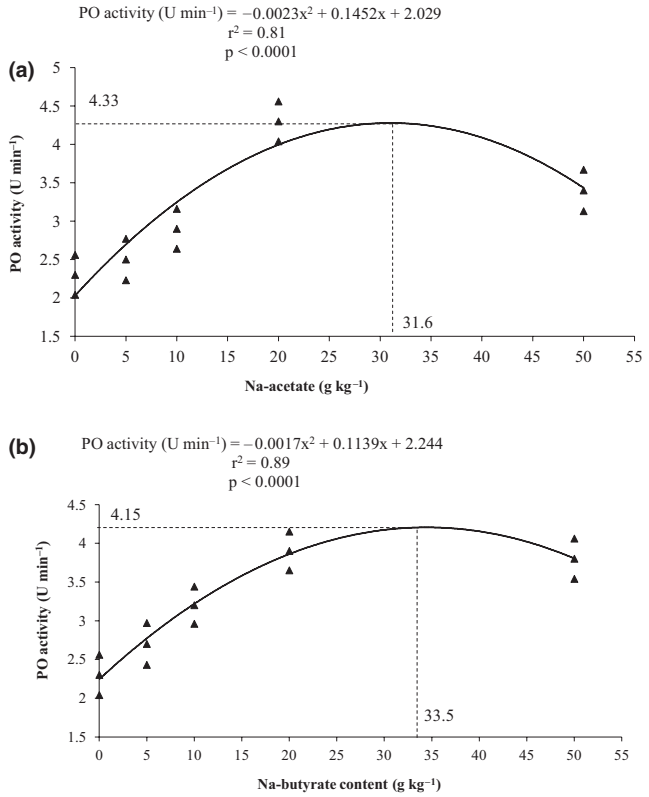

FIGURE 3 Polynomial model fitting phenoloxidase activity (U/min) to different levels of encapsulated (a) sodium (Na)-acetate content (g/kg) and (b) sodium (Na)-butyrate content (g/kg) in the hepatopancreas of crayfish fed with experimental diets at three replicates

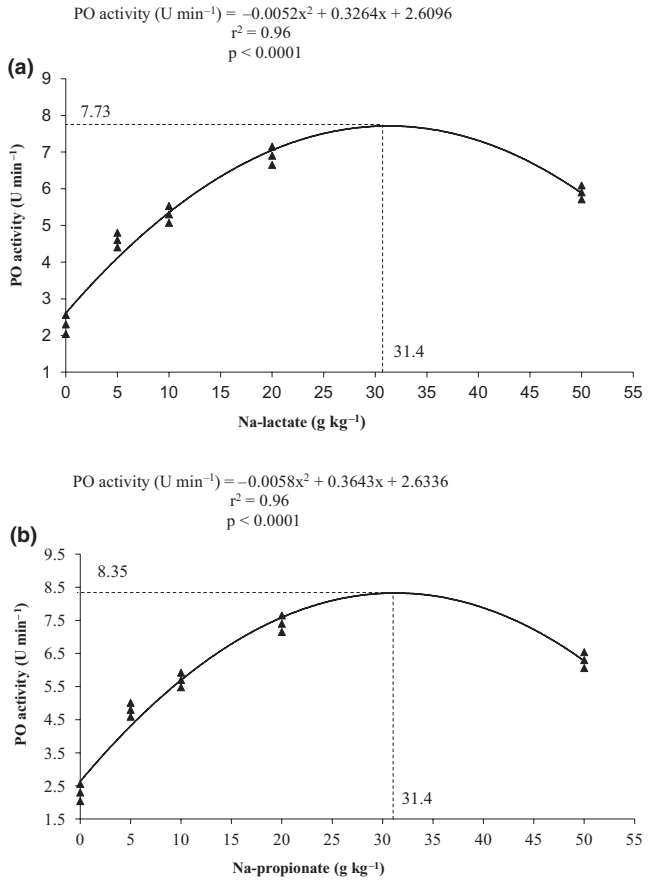

FIGURE 4 Polynomial model fitting phenoloxidase activity (U/min) to different levels of encapsulated (a) sodium (Na)-acetate content (g/kg) and (b) sodium (Na)-butyrate content (g/kg) in the hepatopancreas of crayfish fed with experimental diets at three replicates

system, increases the stress resistance (Maslowski & Mackay, 2011) and suppresses the expression of pathogenic genes (e.g. *Salmonella* sp.) (Van Immerseel et al., 2006). It is reported in the literature that butyrate as an energy substrate over glucose inhibits the glycolysis cycle and the oxidation of some amino acids and enhances the absorption rate of some essential amino acids and nucleotide in the fish gut (Ng & Koh, 2017; Robles et al., 2013). The improvement of growth performance of crayfish fed the diets supplemented with encapsulated Na-lactate and Na-propionate compared to encapsulated Na-acetate and Na-butyrate may be associated with nutrient flow and retention, and, mainly, carbohydrate synthesis (Halver & Hardy, 2002). The present data showed that crayfish fed the C3-organic acid-supplemented diets had higher weight gain (based on the FCR and final weight) and nutrition efficiency indices compared with those fed the C2- and C4-organic acid ones. The generally accepted level of dietary carbohydrate is about 200–300 g/kg (Wang et al., 2016). However, the carbohydrate metabolism in crustaceans is similar to mammals and finfish, but the glucose-regulating hormones are different (Wang et al., 2016). It is recommended to use nutrigenomic and metabolomic studies in order to confirm the preferred carbon sources in crayfish nutrition. However, further studies need to clarify these results.

Higher digestive enzyme activities (alkaline protease, lipase and amylase) in the hepatopancreas extracted from crayfish fed the different encapsulated Na-organic salts were observed compared with those fed the control diet. Increment in digestive enzyme activities can enhance the hydrolysis of nutrients and the lactic acid bacteria count in the hepatopancreas and, subsequently, lead to an increment in NEIs (e.g. PER and PPV). However, there is no sufficient evidence about the specific effect of organic acids on the activities of digestive enzymes (e.g. alkaline protease, lipase and amylase), but some researchers believed that organic acids penetrate the Gram-negative bacteria cell wall, free protons, diminish the intracellular pH of bacterial cytoplasm and, finally, cause cell death (Ahmadniaye Motlagh et al., 2019; Lückstädt, 2008; da Silva et al., 2013). In this regard, the growth inhibition of *Vibrio cambelli* exposed to different organic acids (e.g. acetic, butyric, formic and valeric) was reported at pH range from 5 to 6 (da Silva et al., 2013). Organic acids are classified based on the pKa value, molecular mass, odour and corrosiveness rate (Ng & Koh, 2017). Small pKa values lead to strengthen acidic properties. Nonetheless, the balance between dissociate and undissociate forms depended on pKa value (Ng & Koh, 2017; da Silva et al., 2013). The pKa values of acetic acid, butyric acid, lactic acid and propionic acid are reported to be 4.6, 4.81, 3.86 and 4.88, respectively (Ng & Koh, 2017).

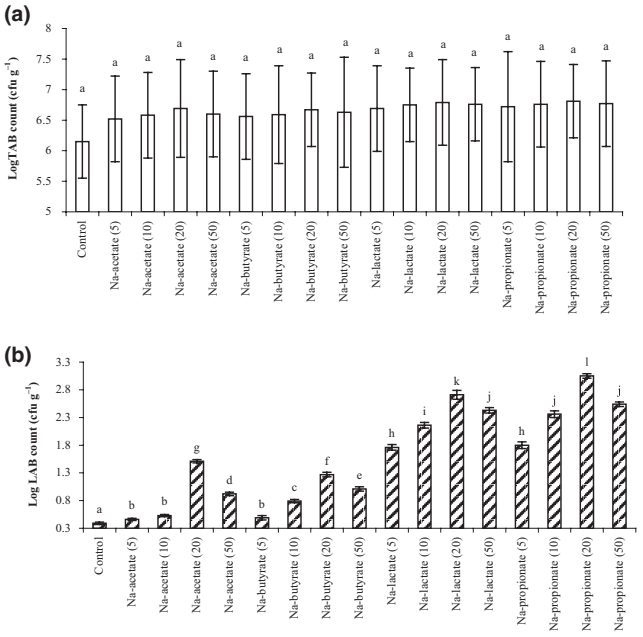

FIGURE 5 The mean (±*SD*) of (a) total aerobic bacteria count (TAB; CFU/g) and (b) lactobacillus count (LAB; CFU/g) of hepatopancreas extracted from crayfish fed the experimental diets containing different levels (5, 10, 20 and 50 g/kg) of encapsulated sodium (Na)-acetate, Na-butyrate, Na-lactate and Na-propionate after 63 days at three replicates. Different letters indicate significant differences ($p < .05$)

However, further studies need to focus on the effect of buffer capacity of the feed ingredients and diets and organic acids on the digestive enzyme activities.

Feeding crayfish with different synbiotics (galactooligosaccharide + *Enterococcus faecalis* and galactooligosaccharide + *Pediococcus acidilactici*) improved immune responses and stress resistance compared with those fed the diets containing only probiotics or prebiotics (Safari & Paolucci, 2017a; Safari & Paolucci, 2017b; Safari & Paolucci, 2017c; Safari et al., 2017). The positive effects of synbiotics can be related to the increase in the dose content and/ or produce a mixture of SCFAs and, mainly, butyrate in the digestive tract. Regarding the fermentation of different probiotic strains on prebiotics with various degrees of polymerization produces diverse SCFA content, therefore, it is impossible to trace the efficiency of single effective compounds. Therefore, using single organic acids in different dose contents in aquafeeds can help to estimate the minimum effective dose in order to achieve the maximum biological response. However, there is a lack of information about the combination effects of organic acids in aquafeeds. Therefore, as this has not been elucidated the topic merits further investigations.

4.2 | Haemolymph indices and microbial responses

One of the key roles of total haemocyte count (THC) and different haemocyte counts (HC, SGC and LGC) is to improve the well-being of shellfish species, through cytotoxicity and the storage and release of the prophenoloxidase system (Johansson et al., 2000). Crayfish, similarly to other crustacean species (e.g. shrimp and prawn), depend only on the innate immune response against the microbial invasion (Zhang et al., 2011). The juvenile crayfish fed the Na-lactate and Na-propionate diets showed the highest values of haemolymph indices (THC, HC and SGC), immunity (LYZ) and antioxidant enzymes (PO, SOD and NOS) after a 63-day feeding trial. Feeding with the Na-acetate and Na-butyrate diets ranked in the second place based on the haemolymph, immune responses and antioxidant enzymes. This outcome is in agreement with the administration of feed additives (e.g. probiotics, prebiotics, synbiotics and organic acids) that can increase the animal resistance through pathogenic inhibition routes in digestive tract including competition for territory, reduction in pH value and release beneficial compounds from microbial population (Li et al., 2007; Manning & Gibson, 2004). Feeding black tiger shrimp (*Penaeus monodon*) with a probiotic bacterium diet (Rengpipat et al., 2000) and a β-1,3-glucan diet (Chang et al., 2000) and also feeding western king prawn (*Penaeus latisulcatus*) with diets containing *Pseudomonas synxantha* and *P. aeruginosa* (Hai et al., 2009) increased haemolymph counts and the activities of immune responses and antioxidant defence enzymes.

Interestingly, crayfish fed the 20 g/kg of encapsulated Na-propionate diet had the highest values of growth indices including final weight (27.86 g), SGR (2.94% BW/day), survival rate (96%), PER (3.09) and PPV (68%) and immune responses such as the activities of PO (7.40 U/min), SOD (7.80 U/min) and LYZ (9.40 U/min). Concomitantly, the highest count of lactic acid bacteria (CFU/g) and the lowest count of *E. coli* (CFU/g) were measured in the extracted hepatopancreas of crayfish fed the 20 g/kg of encapsulated Na-propionate diets. The improvement of growth performance and immune responses in crayfish fed the Na-lactate and Na-propionate diets can be attributed to the structure–function relationship of Na-propionate and Na-lactate to modulate the beneficial microbiota of the gastrointestinal tract, the lipid synthesis (Marcil et al., 2002) and, finally, the metabolic pathways. Additional research requires to explore the mechanism(s) of immunomodulation between different organic acids (or their salts) and immune system interactions in crayfish. Such studies can show a new avenue to use different organic salts in the aquafeeds to improve the efficiency of digestion and absorption processes in the digestive tract.

5 | CONCLUSION

In the current experiment, juvenile crayfish fed the diets containing different levels (5, 10, 20 and 50 g/kg) of encapsulated Na-propionate and Na-lactate exhibited the highest values of SGR (1.59%–1.60% BW/day), survival rate (54.25%–56.25%), PER (1.75–1.80) and PPV (47.25%–48.25%). The juvenile crayfish fed the 20 g/kg of encapsulated Na-propionate diet showed the highest values of

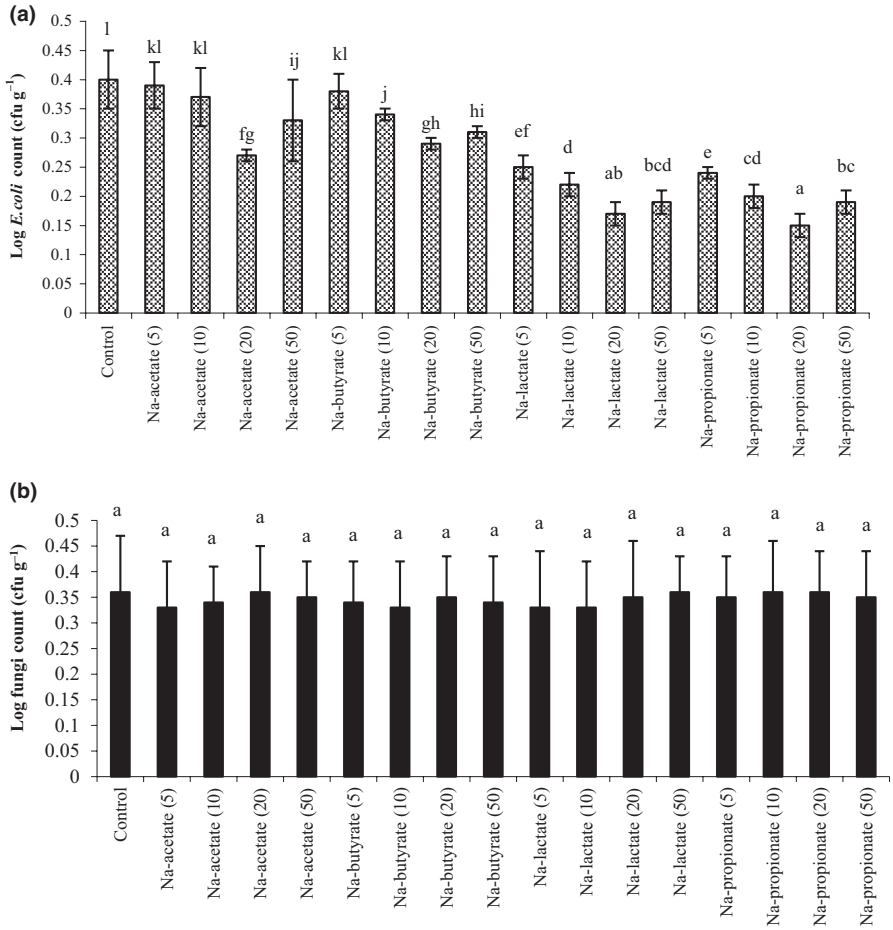

FIGURE 6 The mean (±*SD*) of (a) *E. coli* count (CFU/g) and (b) fungi count (CFU/g) of hepatopancreas extracted from crayfish fed the experimental diets containing different levels (5, 10, 20 and 50 g/kg) of encapsulated sodium (Na)-acetate, Na-butyrate, Na-lactate and Na-propionate after 63 days at three replicates. Different letters indicate significant differences ($p < .05$)

growth indices (SGR, final weight and survival rate) and activities of antioxidant defence enzymes including phenoloxidase, superoxide dismutase and lysozyme.

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