Received: 18 April 2021 | Revised: 8 September 2021 | Accepted: 19 September 2021

DOI: 10.1111/anu.13360

Aquaculture Nutrition WILEY

ORIGINAL ARTICLE

Dietary supplementation with powder and gelatine micro and nanoencapsulated sodium propionate: Influence on growth performance, digestive and antioxidant enzymes and mucosal immunity of koi carp (*Cyprinus carpio* koi)

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Funding information
Ferdowsi University of Mashhad, Grant/Award Number: 2/47519

Abstract

The current study evaluated the efficiency of three forms of dietary sodium propionate (SP) on growth performance, digestive and liver antioxidant enzymes activities, as well as skin mucus immune responses of koi carp (*Cyprinus carpio* koi) fingerlings. Gelatine micro and nanoparticles loaded with SP were synthetized and expressed as gelatine-SP MPs and gelatine-SP NPs, respectively. Basal diet was supplemented with gelatine microparticles, gelatine nanoparticles, powder-SP, gelatine-SP MPs and gelatine-SP NPs. Fish were distributed into 18 experimental units and fed with prepared diets for eight weeks. The results indicated that the growth performance improved in fish fed SP-supplemented diets in comparison with the controls. The highest growth performance was observed in gelatine-SP NPs followed by gelatine-SP MPs. The digestive enzymes activity including protease, trypsin, lipase and α-amylase in gelatine-encapsulated SP micro and nanoparticles dietary groups increased significantly in comparison with other dietary groups ($p < .05$). The use of SP-loaded micro and nanoparticles decreased the liver alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels in comparison to other groups ($p < .05$). Feeding fish with SP-supplemented diets led to increasing antioxidant enzymes of superoxide dismutase (SOD), glutathione (GSH) and catalase (CAT), as well as alkaline phosphatase (ALP) in the liver tissue in comparison to the controls ($p < .05$). The skin mucus lysozyme, alternative haemolytic complement activity (ACH50) and total immunoglobulin (Ig) levels were higher in SP-dietary groups than the controls; the highest levels were in gelatine-SP NPs dietary group ($p < .05$). The findings confirmed that the administration of SP loaded into micro and nanoparticles of gelatine could improve its efficiency in the diet of koi fish.

KEYWORDS
antioxidant enzyme, gelatine, koi carp, microencapsulation, mucus immune response, nanoencapsulation, sodium propionate

1 | INTRODUCTION

Nowadays, ornamental fish industry knows as a multibillion-dollar industry in the world (Raja et al., 2019). It is estimated that the ornamental fish trade is more than USD 15 billion and more than 125 countries involved the trade (Raja et al., 2019; Satam et al., 2018). Disease outbreaks is one the main problems in aquaculture (Bondad-Reantaso et al., 2005). Antibiotics and chemotherapeutics are often used to control and treatment infectious diseases in the aquaculture industry (Hernandez-Serrano, 2005; Ramesh & Souissi, 2018). The extensive usage of these chemical compounds has been limited because of the emergence of some problems, such as drug resistance in various pathogen microorganisms (Agersø et al., 2007; Miranda & Zemelman, 2002), the threat to human health (Heuer et al., 2009; Nawaz et al., 2001) and environmental hazards (Rico et al., 2012). Enhancement of the growth, health conditions and disease resistance of the fish are key factors in economic and sustainable production in the ornamental fish industry. The application of effective non-antibiotic compounds like acidifiers has attracted the attention of aquaculture researchers in the recent years (Ahmadniaye Motlagh et al., 2019; Ng & Koh, 2016; Safari et al., 2020).

Acidifiers are defined as short-chain organic acids (C1–C7) and their salts or mixtures (Ng & Koh, 2016). Some organic acids have traditionally been used as antibacterial agents in terrestrial livestock feeds (Van Immerseel et al., 2002, 2003). It has been reported that organic acids-supplemented diets have beneficial effects on feed intake, growth and health of terrestrial livestock (Kluge et al., 2006; Partanen & Mroz, 1999). The studies on utilization of organic acids as feed additives in aquatic animals have been intensified in the recent years but limited information is available regarding their efficacy in aquaculture. Some studies have demonstrated positive effect of some organic acids on feed palatability and feeding responses in aquatic animals. Silva et al. (2013) found that inclusion of propionate and butyrate salts in diet at the rate of 2 g/kg had function of feed attractants and increased feed intake in a commercial marine shrimp. Few studies have been investigated the effects of dietary organic acids and their salts on activities of digestive enzymes in aquatic animals. It was found that supplementation of diet with citric acid (10 g/kg) enhanced the protease activity in tilapia gut (Li et al., 2009). Castillo et al. (2014) also showed a significant elevation in lipase, trypsin and amylase activity of juvenile red drum (*Sciaenops ocellatus*) fed 15 g/kg of potassium diformate (KDF) and citric acid. The beneficial effects of dietary propionic acids and its salts have been reported on growth performance of Caspian white fish (*Rutilus frisii kutum*) fry (Hoseinifar et al., 2016) and immune status of common carp (*Cyprinus carpio*) fingerlings (Hoseinifar et al., 2017) and zebra fish (*Danio rerio*) (Safri et al., 2016). Hoseinifar et al. (2016) found that supplementation of diet with 0.25% and 0.5% SP improved growth performance, mucosal and non-specific immune responses of Caspian white fish (*Rutilus frisii* kutum) fry. Wassef et al. (2019) also reported that enrichment of diet with 0.2% or 0.3% improved European seabass (*Dicentrarchus labrax*) fry growth performance, immune response efficiency and intestinal function.

Highlights

- Gelatine-sodium propionate nanoparticles (G-SP NPs) with spherical shape and mean diameter of 98.78 ± 25.53 nm were synthetized.
- Growth performance improved in koi carps fed diets supplemented with three forms of SP at levels of 5 g/kg of dry diet.
- The highest digestive and liver antioxidant enzymes activity was observed in G-SP NPs dietary group.
- The diets supplemented with gelatine micro and nanoencapsulated SP enhanced the immune responses in koi fish.

However, most organic acids and their salts are easily soluble in water and leaching of them from the feed ingredients into the water is a significant problem for aquatic organisms (Ng & Koh, 2016). Silva et al. (2013) found that leaching of Na-acetate from shrimp pellets was 100% within 30 min. To improve the efficiency of dietary organic acids and eliminate the leaching of them into water, coating or encapsulation of organic acids has been developed recently (Ng & Koh, 2016).

The strategy of drug encapsulated into the nanocarrier or covalently attached to its surface is very important for slow and controlled drug delivery (Wilczewska et al., 2012). Nanoparticles because of higher intracellular uptake compared to larger carriers have gained more attention for delivery systems (Chen et al., 2012; Treuel et al., 2013). Polymers are commonly used in the fabrication of nanocarriers. Biopolymers (e.g., gelatine, collagen, chitosan, alginate, etc.) have several advantages such as biodegradability, high biocompatibility and low toxicity (Ahmad Khan & Schneider, 2013; Dasi et al., 2002). Gelatine as denatured animal collagen protein has been administrated for a long time in pharmaceuticals, cosmetics and food industry (Elzoghby, 2013). Gelatine nanoparticles (Gelatine NPs) are appropriate candidate for drug carrier because of their harmlessness, biocompatibility, biodegradability, recyclability, lack of immunogenicity, high content of amino acids, low cost and possess various active groups with special bonding capacity to attach to target molecules (Elzoghby et al., 2012; Subara et al., 2017; Weber et al., 2000). The impacts of dietary microencapsulated organic acids blend (OAB) and microencapsulated sodium butyrate have been reported in white shrimp, *Litopenaeus vannamei* (Romano et al., 2015) and juvenile common carp (*C. carpio*; Liu et al., 2014), respectively. The published data on administration of gelatine-based nanoparticles as drug delivery systems in aquatic animals are limited. Therefore, the current study was aimed to evaluate the potential impacts of sodium propionate loaded into gelatine micro and nanoparticles versus powder sodium propionate on immune parameters of skin mucus, liver and digestive enzymes activity, as well as growth performance of koi carp (*Cyprinus carpio* koi) fingerlings.

2 | EXPERIMENTAL

2.1 | Materials

Gelatine powder from bovine skin, sodium propionate ($C_3H_5NaO_2$; purity of 99%) and span 80 ($C_{24}H_{44}O_6$) as nonionic surfactant were purchased from Sigma-Aldrich Co. Solvents including hexane, acetone and formaldehyde were also obtained from Merck Co.

2.2 | Synthesis of gelatine-sodium propionate microparticles

Firstly, 1g of sodium propionate (SP) was dissolved in 10 ml of double distilled water under stirring condition. Then, 4 g of gelatine powder was added to the solution and the reaction mixture was maintained at 50°C with continuous stirring. Amounts of 50 ml of canola oil was poured into a 100 ml container and span 80 (1 ml) was added and mechanically stirred at 50°C. Afterwards, the prepared gelatine solution was added drop-wise to the canola oil solution and mechanically stirred for 30 min at 50°C. The temperature of reaction mixture was allowed to reach the room temperature under mechanical stirring. Following, the reaction mixture was mechanically stirred for 1 h in an ice bath (0–5°C). The oil of mixture was decanted and the residue washed with hexane. The gelatine particles were washed with acetone and then, 20 ml of formaldehyde 35% was added to them and mechanically stirred for 20 min at the room temperature. Finally, the product (gelatine-SP MPs) was rinsed with cooled distilled water and acetone, respectively, and dried in air. The gelatine particles without SP was also synthetized as control (G MPs).

2.3 | Synthesis of gelatine-sodium propionate nanoparticles

The gelatine and canola oil solutions were prepared based on the method described above. Then, the 2 ml of prepared gelatine solution was added drop-wise to the canola oil solution and homogenized using a homogenizer (IKA T_{25} model) at 18,684 *g* for 20 min at 50°C. Afterwards, the mixture was kept in an ice bath for 1 h. In following, the oil was decanted and the residue washed based on the method described in Section 2.2. The prepared particles were defined as gelatine-SP NPs. The gelatine particles without SP also prepared as control (gelatine NPs).

2.4 | Characterization of prepared gelatine-SP particles

The shapes, sizes and elemental composition of synthetized gelatine-SP particles were determined using field emission scanning electron microscopy (FE-SEM; MIRA3 TESCAN, Brno, Czech Republic) coupled with x-ray energy dispersive spectroscopy (EDS). Transmission electron microscopy (TEM; ZEISS LEO 912 AB) images were taken for gelatine-SP NPs sample. Average diameter and size distribution of particles on TEM and FE-SEM images were determined by measuring the diameter of 346 and 100 individual particles on TEM and FE-SEM images, respectively, using AxioVision digital image processing software (Release 4.8.2.0, Carl Zeiss Micro Imaging GmbH, Germany). Fourier transform infrared (FT-IR) spectroscopy (Bruker Alpha, Ettlingen, Germany) was used to identify the functional groups in synthetized gelatine-SP particles over range of 400–4000 cm^{-1}.

The amount of SP loaded into gelatine micro and nanoparticles was determined by measuring sodium (Na^+) element using ICP-OES (Spectro Acros, Germany). Briefly, 0.5 g of gelatine-SP particles were putted in a 50 ml round bottom balloon and 3 ml of HNO_3, 9 ml of HCL and 2 ml of H_2O_2 were added. The mixture was kept at room temperature for 24 h. Then, the mixture was heated at 95°C for 2 h. The digested sample was passed through a Whatman filter paper (0.45 μm) and diluted to 25 ml using double distilled water. The concentration of sodium in prepared solution was determined using inductively coupled plasma-optical emission spectrometry (ICP-OES). The measurement was done in triplicate.

2.5 | Preparation of experimental diets

The ingredients and proximate composition of the basal and experimental diets used for feeding of koi fish (*C. carpio* koi) are shown in Table 1. To prepare the experimental diets, sodium propionate (SP) in three forms of powder-SP (P-SP), gelatine-SP microparticles (gelatine-SP MPs) and gelatine-SP nanoparticles (gelatine-SP NPs) was added to the basal diet at the level of 5 g/kg of dry diet. The SP inclusion level was chosen according to the previous studies on dietary SP in different life stage of fish species (Hoseinifar et al., 2016; Wassef et al., 2019). The experimental diets were prepared through grinding the basal diet using a miller (IKA, M20 Universal, Germany) converting into a uniform paste by adding water and then adding different forms of SP. The pellets were obtained by passing the dough through a meat grinder (EC-10 model, Iran) with a mesh diameter of 2 mm and drying at 30°C for 24 h. The size of dried pellets was 2 mm and they were kept at −20°C until use. The SP content of the synthetized particles was measured and the administrated diets for fish based on the amounts of gelatine particles and SP added to the basal diet were as follows: (1) control; (2) gelatine microparticles (gelatine MPs) (52.47 g/kg); (3) gelatine nanoparticle (gelatine NPs) (48.76 g/kg); (4) P-SP (5 g/kg); (5) gelatine-SP MPs (57.47 g/kg); (6) gelatine-SP NPs (53.76 g/kg). Gelatine powder from bovine skin was added as the protein source to the basal diet to ensure equal amount of gelatine in the all experimental diets. The pH of experimental diets was measured based on the method described by Boland et al. (1981) using a pH meter (Crison, Basic 20^+, model).

TABLE 1 Ingredients and proximate composition of experimental diets

Ingredients (g kg^{-1} dry-weight basis)	Control	Gelatine MPs	Gelatine NPs	P-SP	Gelatine-SP MPs	Gelatine-SP NPs
Fish meal[a]	175	175	175	175	175	175
Wheat flour[a]	245	245	245	245	245	245
Soybean meal[a]	250	250	250	250	250	250
Corn gluten[a]	65	65	65	65	65	65
Gelatin[b]	60	7.52	11.24	60	2.53	6.24
Soybean oil[a]	25	25	25	25	25	25
Fish oil[a]	25	25	25	25	25	25
Mineral premix[c]	35	35	35	35	35	35
Vitamin premix[d]	35	35	35	35	35	35
Carboxymethyl cellulose[e]	64	64	64	64	64	64
Anti-fungi[e]	15	15	15	15	15	15
BHT[e]	1	1	1	1	1	1
Vit C[d]	5	5	5	5	5	5
Sodium propionate[i]	0	0	0	5	0	0
Gelatine microparticles	0	52.47	0	0	0	0
Gelatine nanoparticles	0	0	48.76	0	0	0
Gelatine-sodium propionate microparticles	0	0	0	0	57.47	0
Gelatine-sodium propionate nanoparticles	0	0	0	0	0	53.76
Proximate composition (g kg^{-1} dry-weight basis)						
Dry matter	967.3	967.3	967.3	967.3	967.3	967.3
Crude protein	340	340	340	340	340	340
Crude fat	50	50	50	50	50	50
Crude fibre	380	380	380	380	380	380
Ash	68	68	68	68	68	68
Cross energy (Mj/Kg)	15.80	15.80	15.80	15.80	15.80	15.80
pH	5.88	5.83	5.86	5.79	5.72	5.74

Abbreviations: Gelatine MPs, Gelatine microparticles; Gelatine NPs, Gelatine nanoparticles; P-SP, Powder sodium propionate; Gelatine-SP MPs, Gelatine-sodium propionate microparticles; Gelatine-SP NPs, Gelatine-sodium propionate nanoparticles. Amount of sodium propionate loaded into gelatine micro and nanoparticles were 8.76% and 9.30%, respectively.

4Vitamin premix contains (mg/kg): E, 30; 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 (Kimia Roshd Co, Gorgan, Iran).

[a]Behparvar Aquafeed Co, Iran.

[b]Sigma-Aldrich Co, Germany.

[c]Mineral premix contains (mg/kg): Mg, 100; Zn, 60; Fe, 40; Cu, 5; Co, 0.1 and I, 1 (Kimia Roshd Co, Gorgan, Iran).

[d]Sigma-Aldrich Co, Germany.

[e]Kimia Roshd Co, Gorgan, Iran.

2.6 | Feeding and fish culture

Four-hundred koi fish (*C. carpio* koi) fingerlings with initial weight of 1.56 ± 0.03 g were purchased from a local ornamental fish farm in Mashhad city, Rhazavi Khorasan province, eastern of Iran and transferred to the aquatic laboratory of Ferdowsi University of Mashhad (FUM). Fish were kept in two 500 L fiberglass tanks to acclimatize to laboratory condition and fed on the basal diet for two weeks. Afterwards, 18-glass aquarium (150 L) in the laboratory were filled with 130 L of Tap water and then, 20 fish were randomly distributed to each aquarium; triplicate for each experimental diet. During eight weeks of experimental period, fish were fed with the experimental diets three times daily (8:00, 12:00, and 16:00 hours) to apparent satiation. To keep water quality in suitable condition, 10% of the water in each aquarium was replaced with freshwater and uneaten feeds and faeces were siphoned daily at 6 p.m. The fish were kept under 16/8 photoperiod by using a white fluorescent lamp. Water quality was monitored daily using the portable multi-meter model AZ-8603. Water temperature, dissolved oxygen and pH were measured as 25 ± 2.5°C, 6.35 ± 0.75 mg/L and 7.42 ± 0.5, respectively.

2.7 | Survival rate and growth performance

After 56 days of feeding, the fish with the experimental diets, the fish of each aquarium were collected and their final length and weight were individually measured. The survival rate, growth performance and feed utilization indices of fish were determined using the following equations:

$$\text{Survival rate}\,(\%) = \left(N_f/N_i\right) \times 100$$

$$\text{Weight gain}\,(\text{g}) = W_f - W_i$$

$$\text{Feed conversion ratio}\,(\text{FCR}) = \left(\text{Feed consumed}/W_{\text{gain}}\right)$$

$$\text{Specific growth rate}\,(\%) = \left(\text{SGR};\% \text{ Body weight day}^{-1}\right) = \left[\left(\text{Ln}W_f - \text{Ln}W_i\right)/\text{t}\right] \times 100$$

$$\text{Condition factor}\,(\text{CF}) = W_f/L_f^3 \times 100$$

$$\text{Daily growth index(DGI)}\,(\text{g}) = \left[\left(W_f - W_i\right)/\text{Time}\right]$$

Where N_i, N_f, W_i, W_f and L_f are initial number, final number, initial weight (g), final weight (g) and final length of fish (cm), respectively.

2.8 | Proximate analysis of diets and fish carcass composition

The chemical analysis of the experimental diets and the fish carcass was performed in triplicate according to the standard methods (AOAC, 2005). The crude protein, crude lipid, dry matter, ash and gross energy were measured by using kjeldahl system (Buchi Labortechnik AG, Flawil, Switzerland; N × 6.25), soxtec system HT 1043 (Foss Tecator, AB), oven drying at 105°C for 24 h, muffle furnace at 550°C for 12 h and parr bomb calorimeter (model 1266, Parr Instrument Co., Moline, IL), respectively. To determine the crude fibre, the diet samples were digested using H_2SO_4 (Merck Co.) and NaOH (Merck Co) solutions, the whole material was transferred into crucible and dried for 12 h at 120˚C. Afterwards, the crucible was placed into muffle furnace at 550˚C and then, weight of crucible was recorded.

2.9 | Digestive enzymes assays

Three starved fish for 24 h were randomly selected from each aquarium on 57th day of experiment. The sampled fish were individually anaesthetized using ground clove powder (500 mg/L) (Mohammadi Arani et al., 2019; Zou et al., 2016), and their entire intestine were removed and washed with cold distilled water at 4°C (Huang et al., 1999). The collected intestine was homogenized in the presence of 0.2 M NaCl (1:5; w/v) (Gawlicka et al., 2000) using a homogenizer (IKA T_{25} model). Then, the homogenized samples were centrifuged at 15,000×g, 4°C for 15 min and obtained supernatants were kept at −80°C for future analysis. The activities of digestive enzymes were determined using an ultraviolet visible spectrophotometer (DR 5000™ model, HACH CO., USA) and expressed as U mg^{-1} protein min^{-1}.

Protease enzyme activity was determined using the casein-hydrolysis method explained by Hidalgo et al. (1999). Briefly, the supernatant of centrifuged samples (0.05 ml) was blended with 0.125 ml of casein (1% w/v) and 0.125 ml of buffer (0.1 M Tris-HCl, pH 9.0). The mixture was incubated at 37°C for 1 h and then, the reaction was stopped by adding trichloroacetic acid (TCA) solution (8% w/v; 0.3 ml). The samples were stored at 4°C for 1 h and then centrifuged at 1800 × *g* for 10 min. The absorbance of supernatant was detected at optical density (OD) of 280 nm.

The activity of trypsin was determined using N-α-benzoyl-dl arginine-p nitroanilide (BAPNA) as substrate. The reaction mixture was prepared by mixing 1 mM BAPNA with 50 mM Tris-HCl, pH 8.2 and 20 mM $CaCl_2$. Then, the mixture was incubated with the enzyme extract at 25°C (Erlanger et al., 1961). The optical absorbance was read at 410 nm.

The α-amylase activity was measured according to the 3,5-dinitrosalicylic acid method at wavelength of 540 nm (Worthington, 1991). In this procedure, starch (1% w/v) diluted in a buffer (0.02 M Na_2HPO_4 and 0.006 M NaCl) at pH 6.9 was used as a substrate.

Lipase content of samples was determined using 0.4 mM of *p*-nitrophenyl myristate as a substrate at 25°C and a wavelength of 405 nm according to method explained by Gawlicka et al. (2000).

The activity of alkaline phosphatase (ALP) was measured using a commercial kit (Pars Azmoon Company, Iran) at OD of 405 nm.

2.10 | Immunological assays in skin mucus

At the end of feeding trial, skin mucus samples were collected based on the method explained by Subramanian et al. (2007) to analyse immune parameters. Briefly, three starved fish for 24 h were randomly sampled from each glass aquarium. The sampled fish were anaesthetized as described above and placed individually in a polyethylene bag that contained NaCl (50 mM; 5 ml/g fish; Merck, Germany). To collect skin mucus, fish was shaken gently inside the plastic bag for 1 min. Then, the mucus samples were immediately transferred into 15 ml sterile tube and centrifuged at 1500 × *g* for 10 min at 4°C. Finally, the collected supernatant was kept at −80°C for further analysis.

Total immunoglobulin (Ig) was measured based on the method explained by Siwicki et al. (1994). At first, the total protein content of each skin mucus sample was determined according to the standard protocol (Lowry et al., 1951). Then, the protein content was re-measured after precipitation down of Ig molecules by using 12% solution of polyethylene glycol. The difference between two measured protein levels was considered as the total Ig content of skin mucus (Siwicki et al., 1994).

The lysozyme activity in skin mucus was measured according to the lysis of the lysozyme-sensitive Gram-positive bacterium, *Micrococcus luteus* (Hoseinifar et al., 2016). In this method, 50 μl of prepared lysozyme-sensitive Gram-positive bacterium *Micrococcus luteus* (Sigma, USA) suspension using 0.02 M sodium acetate buffer with pH 5.8 (0.02 mg/L) was poured into a 96 well plate and 50 μl of skin mucus sample was placed in each well. Then, the well plates were incubated for 15 min at 30°C. The absorbance value of each sample was measured at 450 nm using a spectrophotometer (DR 5000™ model, HACH CO., USA). After 50 min, the second absorbance value of samples was measured. The change in absorbance value was calculated and the lysozyme activity was expressed as U/ml.

Alternative complement pathway haemolytic activity (ACH50) of the skin mucus was determined according to the method explained by Yano (1992). Briefly, the skin mucus samples were diluted from range of 50–250 μl and allotted into tube tests and the total volume of each tube was increased to 250 μl by adding barbitone buffer in the presence of ethylene glycol-bis (2-aminoethoxy)-tetraacetic acid (EGTA) and Mg^{2+}. Afterwards, New Zealand Rabbit Red Blood Cells (RaRBC) (100 μl) was poured into each tube and incubated at 20°C for 90 min. After adding NaCl (3.15 ml) to each tube, the samples were centrifuged at 1600 × g for 5 min and the absorbance of obtained supernatant was read at 414 nm. The skin mucus volume producing 50% ACH was used to calculate the number of ACH50 unit/ml.

2.11 | Liver enzymatic assessment

At the end of feeding experiment, the enzymatic activity was measured in liver of sampled fish. The liver of sampled fish as described earlier were removed and rinsed with ice-cold 0.95% saline. The liver samples were homogenized in ice-cold 0.1 M Tris HCL buffer (pH 7.1) with using a glass homogenizer. Then, the homogenate was centrifuged at 10,380 *g* for 10 min at 4°C. The obtained supernatant was kept at −80°C for further measurement of enzyme activity (Jindal et al., 2018).

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) activities were measured through colorimetric assay according to Frankel-Reitman method (Reitman & Frankel, 1957) using commercial kits (Ziest Chem Diagnostic Co., Tehran, Iran) at an OD of 505 nm.

The superoxide dismutase (SOD) activity was determined by the methodology of Marklund and Marklund (1974). The reaction mixture was a combination of 30 μl of homogenate supernatant and 2 ml of Tris-HCl (50 mM, pH 8.2). After few seconds, 20 μl of pyrogallol solution (10 mM, pH 7.4) was added to the reaction mixture. Pyrogallol autoxidation was determined compared to the control at OD of 420 nm. The SOD concentration was estimated by 50% inhibition of pyrogallol oxidation.

The catalase (CAT) activity was assayed based on the method explained by Aebi (1984) using hydrogen peroxide (H_2O_2) as a substrate. Reaction solution was prepared by 50 mM H_2O_2 in 50 mM potassium phosphate buffer. The H_2O_2 decomposition by CAT was measured using spectrophotometer at a wavelength of 240 nm by keeping the pH at 7.0.

The Glutathione (GSH) activity was estimated by the method of Jollow et al. (1974) using 5,5-dithio-bis-[2-nitrobenzoic acid] (DTNB) reagent. Briefly, 500 μl of 4% sulphosalicylic acid was added to 100 μl of tissue homogenate and incubated at 4°C for 1 h. The mixture was centrifuged at 14,910 *g* for 15 at 4°C. Afterwards, 0.4 ml of supernatant was added to 2.2 ml of 0.05 M potassium phosphate (pH 7.4) and then mixed with 0.4 ml of DTNB. The colour of mixture was changed to yellow because of the reaction between DTNB and GSH. The OD was read at 412 nm.

The alkaline phosphatase (ALP) activity was monitored using a commercial kit (Pars Azmoon Company, Iran) at a wavelength of 405 nm.

2.12 | Statistical analysis

Data were shown as mean ± standard deviation (SD). Statistical analysis of data was assayed using SPSS software (Version, 19). The Kolmogorov–Smirnov test was performed to evaluate the normality assumption of data. One-way Analysis of Variance (ANOVA) followed by Duncan's New Multiple Range test was performed to test the significant differences between the means. The *p*-value of <.05 was employed to assay the significant differences.

3 | RESULTS

3.1 | Characterization of Gelatine-SP MPs and Gelatine-SP NPs

FE-SEM micrographs of gelatine-SP micro particles and gelatine-SP nanoparticles and EDS analysis are shown in Figure 1a–d. FE-SEM micrographs indicated that synthetized gelatine-SP particles had small spherical shape. The EDS analysis showed the presence of sodium (Na^+) element, which indicates the loading of sodium propionate into gelatine particles (Figure 1b, d). Based on the FE-SEM micrographs, the gelatine-SP micro particles had mean diameter of 45.20 ± 12.74 μm and size distribution ranged from 25.8 to 67.2 μm (Figure 1 a). TEM micrograph showed that the gelatine-SP nanoparticles had spherical shape with mean diameter of 98.78 ± 25.53 nm and size distribution of 42.75 to 190.16 nm (Figure 2a, b). The amounts of SP loaded into gelatine micro and nanoparticles were 8.70% and 9.30%, respectively.

The presence of gelatine as a natural organic polymer that can interact with sodium propionate can be inferred from FT-IR technique. Figure 3 show the spectrums of gelatine-SP micro and nanoparticles, and there are the following absorption bands characteristic for gelatine (Payne & Veis, 1988). The broad band at 3300–3429 cm^{-1} is imputed to N-H stretching vibrations of various amides in gelatine

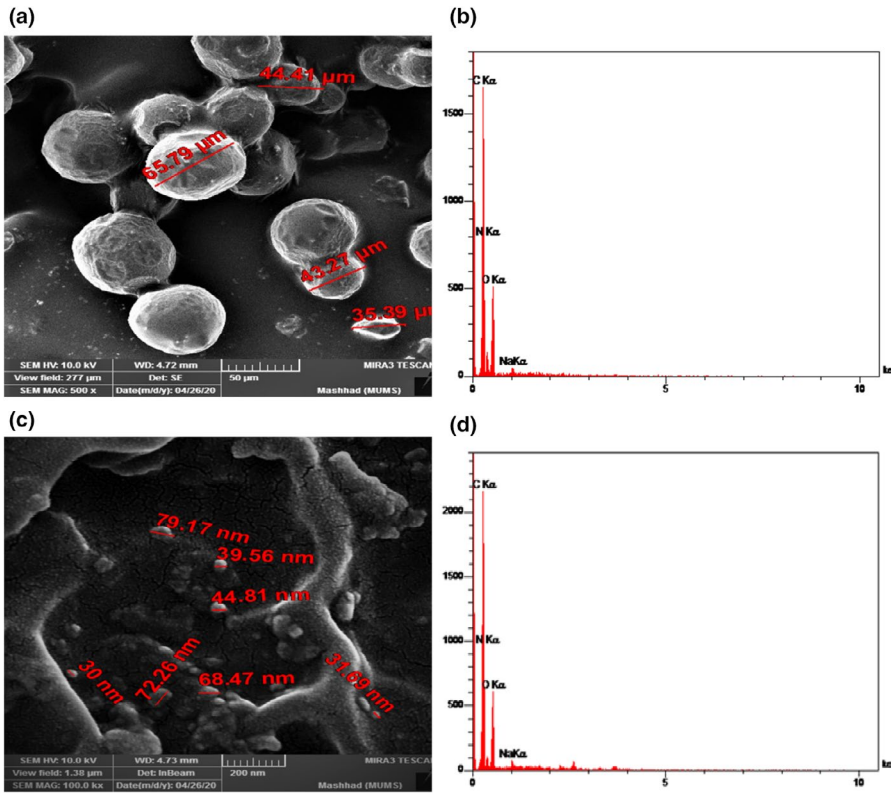

FIGURE 1 FE-SEM micrographs and EDS patterns of gelatine-SP microparticles (a,b) and gelatine-SP nanoparticles (c, d)

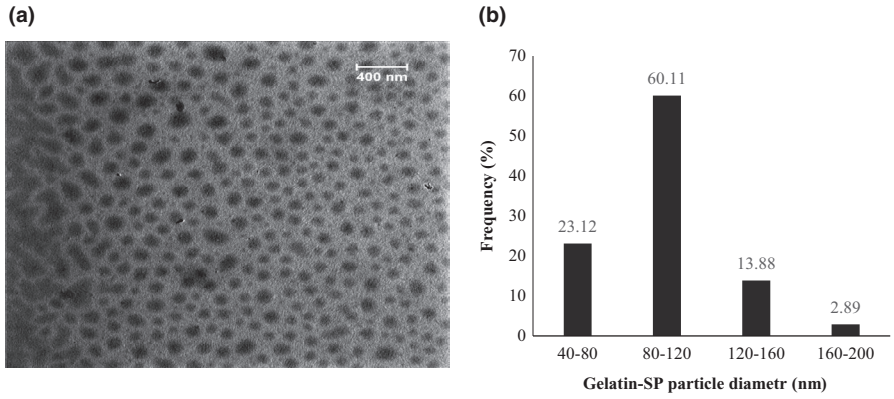

FIGURE 2 TEM image of gelatine-SP nanoparticles (a) and histogram for particle size distribution of the nanoparticles (b)

structure. In addition, the observed signal 2926 cm^{-1} is referred to C-H stretching. The band appeared at 1647 cm^{-1} is imputed to C=O stretching for the amide.

3.2 | Growth performance and survival rate

The growth performance parameters and survival rate of koi carp fingerlings fed on supplemented diets with different forms of sodium propionate (SP) for 56 days are presented in Table 2. The initial weight of fish was same between different dietary groups at the beginning of experiment ($p > .05$). The final weight of fish increased in gelatine-SP MPs and gelatine-SP NPs dietary groups compared to the control groups ($p < .05$). Feeding fish with SP-dietary groups increased the weight gain (WG) and specific growth rate (SGR%) of fish in comparison with the control groups ($p < .05$). The highest WG and SGR% were found in the gelatine-SP NPs dietary group ($p < .05$). Fish fed diets supplemented with SP showed lower feed conversion ratio (FCR) than the control groups ($p < .05$). The lowest FCR was found in the gelatine-SP NPs dietary group ($p < .05$). The condition factor (CF) of fish showed no significant difference between the dietary groups after 8 weeks of feeding trial ($p > .05$). The daily growth index (DGI) parameter increased in the fish fed on SP-supplemented diets ($p < .05$). The highest DGI value were recorded in the gelatine-SP NPs dietary group ($p < .05$). The WG, SGR%, FCR and DGI indices showed no significant differences between P-SP and gelatine-SP MPs dietary groups ($p > .05$). The survival rate (%) of fish was not significantly different between the dietary groups ($p > .05$).

3.3 | Carcass proximate composition of fish

The results of proximate analysis of carcass of koi carp fingerlings fed on diets supplemented with different forms of sodium propionate (SP)

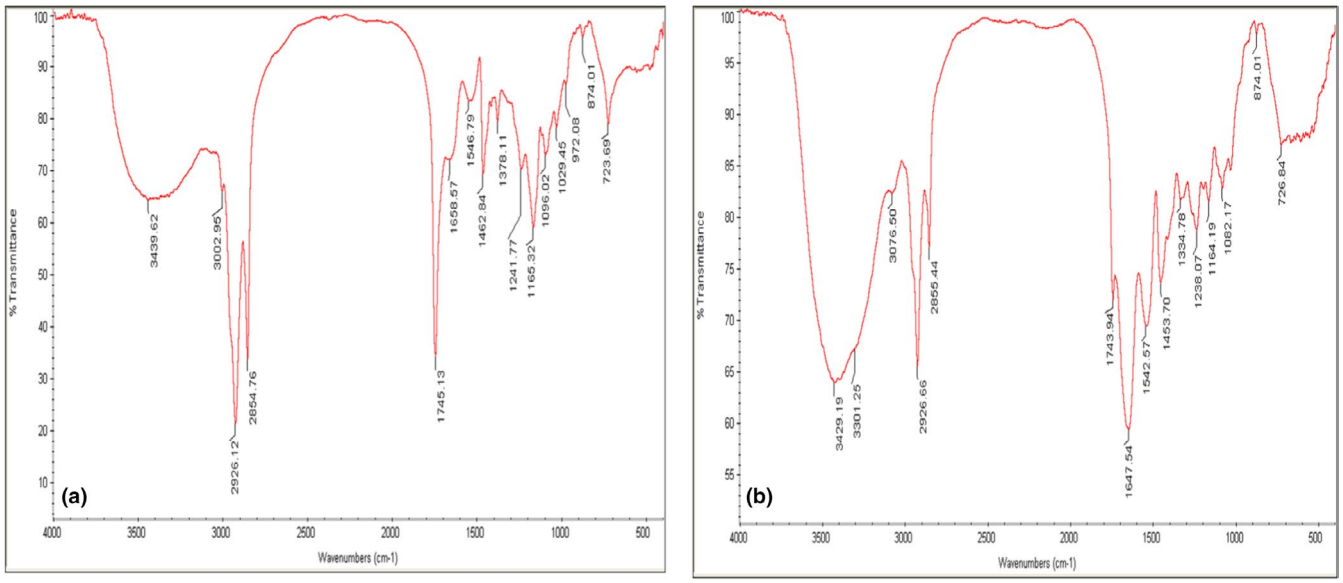

FIGURE 3 FT-IR spectra of gelatine-SP microparticles (a) and gelatine-SP nanoparticles (b)

TABLE 2 Growth performance, feed utilization parameters and survival rate of koi fish (*C. carpio* koi) fingerlings fed on diets supplemented with different forms of sodium propionate for 8 weeks (mean ± SD, $n = 3$)

	Dietary sodium propionate groups					
Parameters	Control	Gelatine MPs	Gelatine NPs	P-SP	Gelatine-SP MPs	Gelatine-SP NPs
Initial weight (g)	1.57 ± 0.01^{a}	1.58 ± 0.05^{a}	1.56 ± 0.02^{a}	1.54 ± 0.05^{a}	1.57 ± 0.06^{a}	1.55 ± 0.03^{a}
Final weight (g)	9.59 ± 0.35^{a}	9.39 ± 0.31^{a}	9.17 ± 0.14^{a}	11.20 ± 0.64^{ab}	11.91 ± 0.13^{b}	12.72 ± 0.66^{b}
Weight gain (g)	8.02 ± 0.35^{a}	7.81 ± 0.26^{a}	7.61 ± 0.18^{a}	9.66 ± 0.63^{b}	10.34 ± 0.11^{b}	11.17 ± 0.66^{c}
SGR (%BW/day)	3.22 ± 0.07^{a}	3.17 ± 0.003^{a}	3.19 ± 0.05^{a}	3.53 ± 0.11^{b}	3.61 ± 0.06^{bc}	3.75 ± 0.09^{c}
FCR	2.94 ± 0.17^{c}	2.90 ± 0.03^{c}	2.88 ± 0.07^{c}	2.35 ± 0.13^{b}	2.14 ± 0.02^{ab}	1.99 ± 0.11^{a}
CF%	1.58 ± 0.15^{a}	1.49 ± 0.13^{a}	1.53 ± 0.10^{a}	1.64 ± 0.23^{a}	1.59 ± 0.11^{a}	1.68 ± 0.05^{a}
DGI (g)	0.14 ± 0.006^{a}	0.13 ± 0.003^{a}	0.14 ± 0.002^{a}	0.17 ± 0.01^{b}	0.18 ± 0.002^{b}	0.20 ± 0.01^{c}
Survival rate (%)	98.14 ± 3.20^{a}	98.14 ± 3.20^{a}	96.29 ± 3.21^{a}	98.14 ± 3.20^{a}	100 ± 0.00^{a}	100 ± 0.00^{a}

Note: The values with different letters in the same row are significantly different (ANOVA, $p < .05$).

Abbreviations: Gelatine MPs, Gelatine microparticles; Gelatine NPs, Gelatine nanoparticles; P-SP, Powder sodium propionate; Gelatine-SP MPs, Gelatine-sodium propionate microparticles; Gelatine-SP NPs, Gelatine-sodium propionate nanoparticles. SGR, specific growth rate; FCR, feed conversion ratio; CF, condition factor; DGI, daily growth index.

for 56 days are shown in Table 3. The results revealed that the crude protein content and ash content increased in fish fed SP-supplemented diets compared to the controls ($p < .05$). The highest crude protein content was observed in gelatine-SP NPs dietary group ($p < .05$). Fish fed diets supplemented with SP had lower crude lipid content than the control groups ($p < .05$). There were no significant differences in the crude lipid content and the ash content between SP-dietary groups ($p > .05$). No significant difference in the dry matter content was observed between different dietary groups ($p > .05$).

3.4 | Digestive enzyme analyses

The digestive enzyme activities in fish fed on diets containing different forms of SP are presented in Table 4. The activity of enzymes increased significantly in SP-dietary groups compared to the control groups ($p < .05$). The enzyme activities were higher in gelatine-SP MPs and gelatine-SP NPs groups than P-SP-dietary group ($p < .05$). The highest enzyme activities was recorded in gelatine-SP NPs group ($p < .05$).

3.5 | Liver enzymatic analyses

The enzyme activities in liver of koi carps fed on SP-supplemented diets are shown in Figure 4a–g. The aspartate aminotransferase (AST) and alanine aminotransferase (ALT) and levels in the gelatine-SP MPs and gelatine-SP NPs dietary groups decreased compared to the controls and P-SP-dietary groups ($p < .05$; Figure 4a, b) The catalase (CAT), superoxide dismutase (SOD), glutathione (GSH)

TABLE 3 Carcass proximate composition of koi fish (*C. carpio* koi) fingerlings fed on diets supplemented with different forms of sodium propionate for 8 weeks (mean ± SD, $n = 3$)

	Dietary sodium propionate groups					
Parameter	Control	Gelatine MPs	Gelatine NPs	P-SP	Gelatine-SP MPs	Gelatine-SP NPs
Dry matter (%)	26.90 ± 0.76^{a}	26.94 ± 0.55^{a}	26.97 ± 0.52^{a}	27.07 ± 0.73^{a}	27.10 ± 0.64^{a}	27.25 ± 0.88^{a}
Crude protein (%)	14.67 ± 0.17^{a}	14.81 ± 0.22^{a}	14.74 ± 0.20^{a}	16.52 ± 0.25^{b}	16.85 ± 0.23^{bc}	17.14 ± 0.30^{c}
Crude lipid (%)	4.20 ± 0.10^{b}	4.18 ± 0.12^{b}	4.23 ± 0.15^{b}	3.73 ± 0.11^{a}	3.70 ± 0.09^{a}	3.68 ± 0.14^{a}
Ash (%)	4.69 ± 0.05^{a}	4.72 ± 0.08^{a}	4.68 ± 0.03^{a}	5.50 ± 0.07^{b}	5.54 ± 0.13^{b}	5.61 ± 0.19^{b}

Note: The data with different letters in the same row are significantly different (ANOVA, $p < .05$).

Abbreviations: Gelatine MPs, Gelatine microparticles; Gelatine NPs, Gelatine nanoparticles; P-SP, Powder sodium propionate; Gelatine-SP MPs, Gelatine-sodium propionate microparticles; Gelatine-SP NPs, Gelatine-sodium propionate nanoparticles.

TABLE 4 Digestive enzymes activity (U mg protein^{-1} min^{-1}) of koi carp (*C. carpio* koi) fed on diets supplemented with different forms of sodium propionate for 8 weeks (mean ± SD, $n = 3$)

	Dietary sodium propionate groups					
Enzyme	Control	Gelatine MPs	Gelatine NPs	P-SP	Gelatine-SP MPs	Gelatine-SP NPs
Protease	1.26 ± 0.08^{a}	1.27 ± 0.08^{a}	1.28 ± 0.07^{a}	1.77 ± 0.09^{b}	1.88 ± 0.09^{c}	2.0 ± 0.07^{d}
Trypsin	0.36 ± 0.02^{a}	0.40 ± 0.01^{b}	0.39 ± 0.02^{b}	0.57 ± 0.01^{c}	0.75 ± 0.02^{d}	0.81 ± 0.03^{e}
Lipase	0.97 ± 0.04^{a}	0.96 ± 0.04^{a}	0.98 ± 0.03^{a}	1.13 ± 0.1^{b}	1.24 ± 0.06^{c}	1.28 ± 0.04^{c}
α-amylase	2.57 ± 0.03^{a}	2.59 ± 0.04^{a}	2.60 ± 0.03^{a}	2.91 ± 0.04^{b}	3.15 ± 0.06^{c}	3.26 ± 0.05^{d}
Alkaline phosphatase	1.09 ± 0.01^{a}	1.11 ± 0.02^{a}	1.13 ± 0.02^{a}	1.24 ± 0.03^{b}	1.27 ± 0.005^{b}	1.35 ± 0.03^{c}

Note: The data with different letters in the same row are significantly different (ANOVA, $p < .05$).

Abbreviations: Gelatine MPs, Gelatine microparticles; Gelatine NPs, Gelatine nanoparticles; P-SP, Powder sodium propionate; Gelatine-SP MPs, Gelatine-sodium propionate microparticles; Gelatine-SP NPs, Gelatine-sodium propionate nanoparticles.

and alkaline Phosphatase (ALP) enzymes activities in SP-dietary groups increased significantly in comparison to the control groups ($p < .05$; Figure 4 c-f). Fish fed on gelatine-SP MPs and gelatine-SP NPs-supplemented diets showed the higher activity of these enzymes than those fed on P-SP diet ($p < .05$). The highest levels of these enzymes were observed in gelatine-SP NPs dietary group ($p < .05$).

3.6 | Skin mucus immune indices

The variations of skin mucus immune indices in fish fed diets supplemented with different forms of SP are shown in Figure 5a–c. The alternative haemolytic complement activity (ACH50), lysozyme (LYZ) and total immunoglobulin (Ig) levels in SP-supplemented dietary groups were significantly higher than the control groups ($p < .05$). The LYZ and ACH50 activities increased in the skin mucus of gelatine-SP MPs and gelatine-SP NPs dietary groups in comparison with P-SP-dietary group ($p < .05$). The fish fed on gelatine-SP NPs-supplemented diet showed the highest LYZ and ACH50 activities ($p < .05$) (Figure 5a, b). The total Ig activity was not significantly different between the P-SP and gelatine-SP MPs dietary groups ($p > .05$). The highest Ig level was found in gelatine-SP NPs dietary group ($p < .05$) (Figure 5c).

4 | DISCUSSION

In this study, we evaluated the impacts of three forms of dietary sodium propionate (SP) including powder sodium propionate (P-SP), gelatine-sodium propionate microparticles (gelatine-SP MPs) and gelatine-sodium propionate nanoparticles (gelatine-SP NPs) on the survival, growth performance, liver and digestive enzymes activities and immune status of koi carp (*C. carpio* koi) fingerlings. The results of characterization methods of the gelatine-SP MPs and gelatine-SP NPs samples indicated that gelatine micro and nanoparticle were properly synthetized and loaded with SP. The results revealed that the growth performance parameters including weight gain (WG), specific growth rate (SGR%), feed conversion ratio (FCR) and daily growth index (DGI) significantly improved in fish fed on diets supplemented with SP at level of 5 g/kg of dry diet for 8 weeks. Hoseinifar et al. (2016) found that the final weight, WG and SGR% indices increased in Caspian white fish (*Rutilus frisii* kutum) fry fed on diet supplemented with 0.25% SP in comparison with the control. Feeding common carp (*C. carpio*) juveniles with SP-supplemented diet (10 and 20 g/kg) upregulated insulin-like growth factor 1 (IGF1) and growth hormone (GH) genes in brain and liver, respectively (Safari et al., 2017). Inclusion of organic acids and their salts in diet of aquatic animals has been shown to change the population and composition of microbiota in the intestinal tract, which

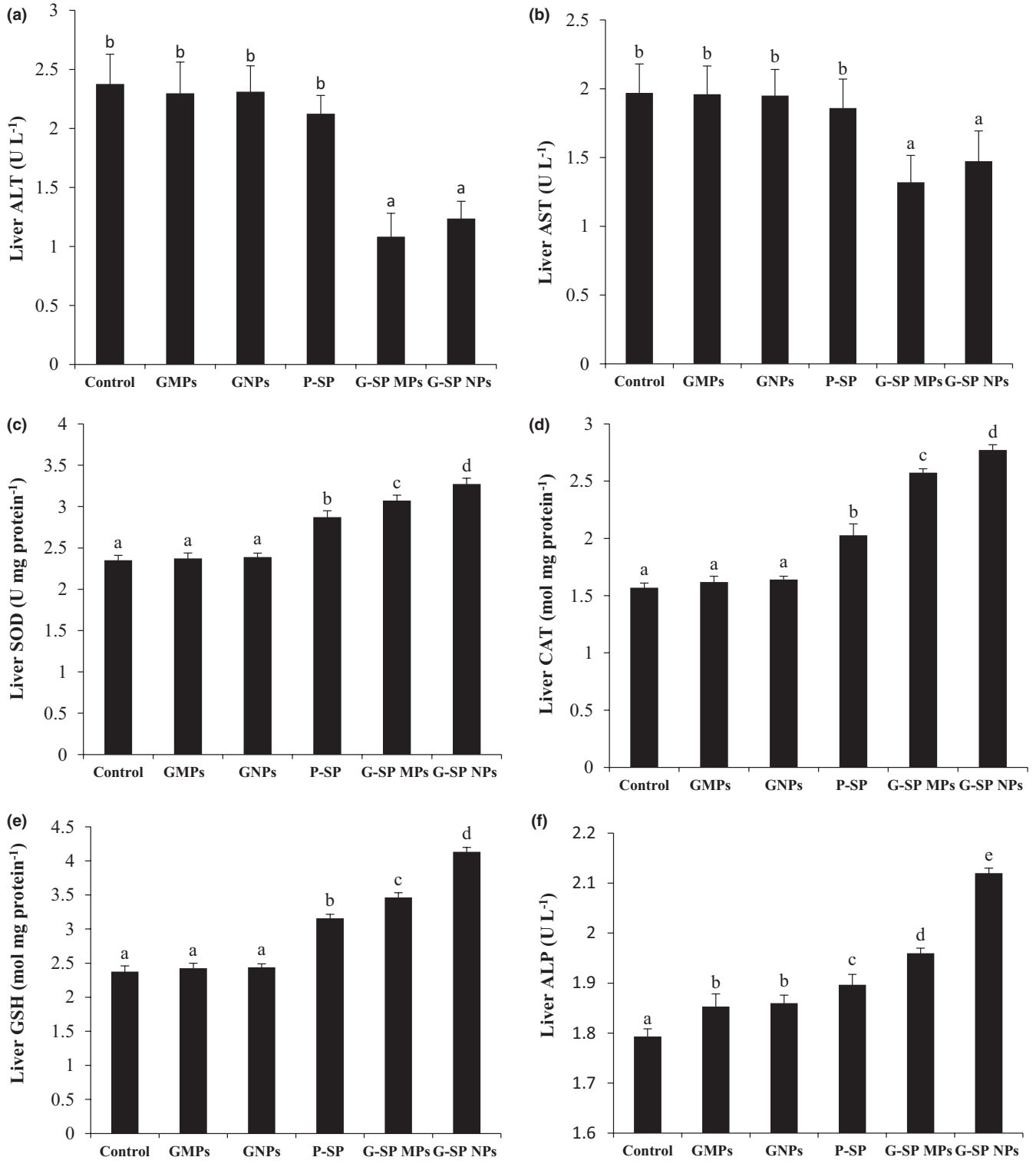

FIGURE 4 Alanine aminotransferase (ALT) (a), aspartate aminotransferase (AST) (b), superoxide dismutase (SOD) (c), catalase (CAT) (d), glutathione (GSH) (e) and alkaline phosphatase (ALP) (f) levels in liver of koi carp (*C. carpio* koi) fed on diets supplemented with different forms of sodium propionate for 8 weeks. Bars with different letters are significantly different (mean ± SD; ANOVA, $p < .05$; $n = 3$). Gelatine MPs: Gelatine microparticles; Gelatine NPs: Gelatine nanoparticles; P-SP: Powder sodium propionate; G-SP MPs: Gelatine-sodium propionate microparticles; G-SP NPs: Gelatine-sodium propionate nanoparticles

can have significant effect on nutrient utilization and growth performance (Ng & Koh, 2016). Organic acids can also act as an attractant in diets and increasing feed intake and subsequently improving growth performance (Da Silva et al., 2015; Omosowone et al., 2018). In the current study, supplementation of diet with gelatine-SP NPs led to significant increase in WG, SGR%, FCR and DGI parameters

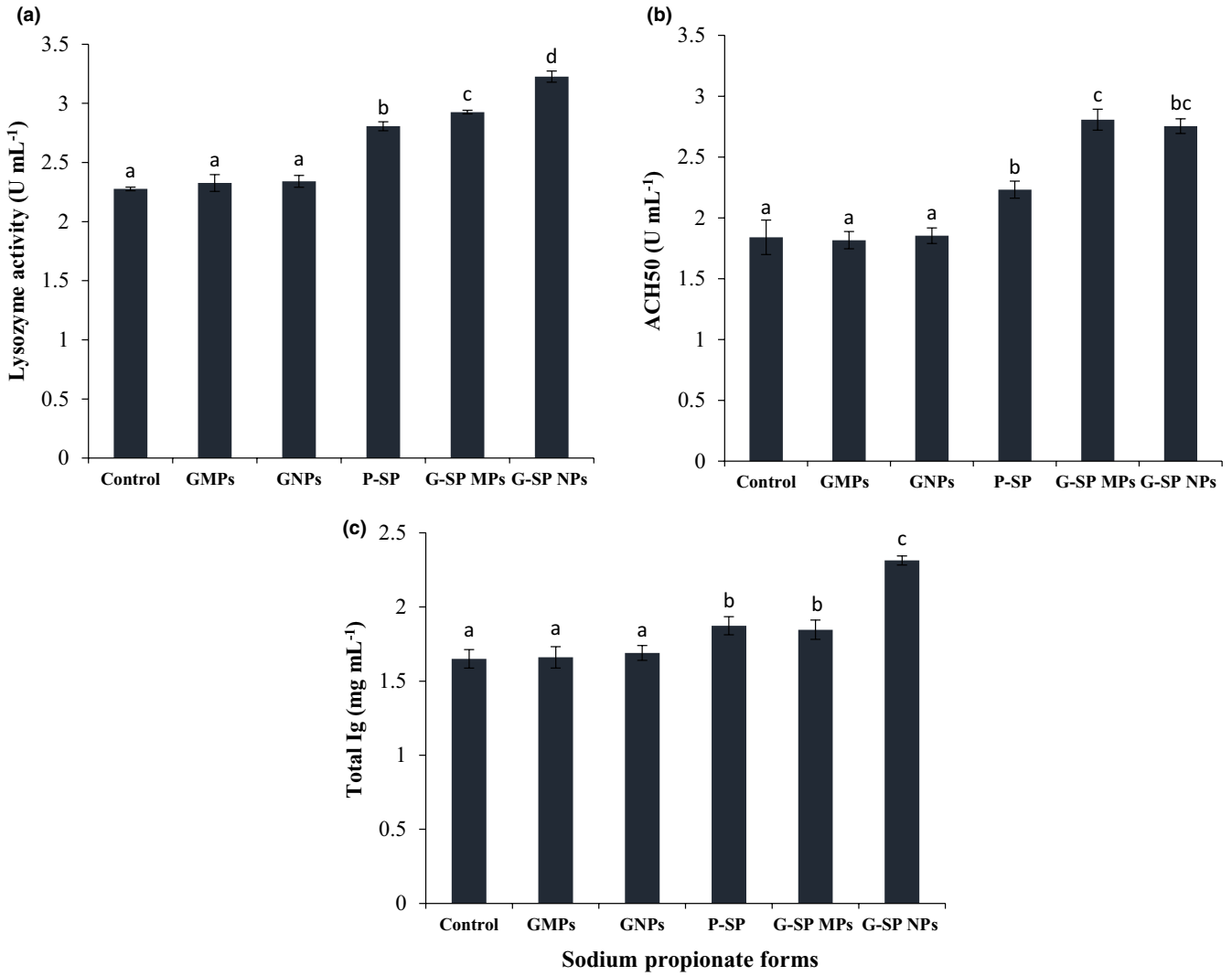

FIGURE 5 Lysozyme activity (a), alternative haemolytic complement activity (ACH50) (b) and total immunoglobulin (Ig) (c) levels in skin mucus of koi carp (*C. carpio* koi) fed on diets supplemented with different forms of sodium propionate for 8 weeks. Bars with different letters are significantly different (mean ± SD; ANOVA, $p < .05$; $n = 3$). Gelatine MPs, Gelatine microparticles; Gelatine NPs, Gelatine nanoparticles; P-SP, Powder sodium propionate; G-SP MPs, Gelatine-sodium propionate microparticles; G-SP NPs, Gelatine-sodium propionate nanoparticles

compared to the powder-SP and gelatine-SP MPs-supplemented diets. No significant differences in growth performance parameters were found between powder-SP and gelatine-SP MPs dietary groups. This result may be attributed to higher uptake of SP included into gelatine NPs by tissue cells. These nanoparticles are mainly absorbed by cells through endocytosis. Acidification of the endo-lysosomal complex causes the degradation of nanoparticles and release of their contents into the cytoplasm, resulting in pharmacological action (Kommareddy et al., 2005). Information on administration of dietary organic acids and their salts encapsulated into micro or nanocarriers in aquaculture is scarce. Liu et al. (2014) found that administration of sustained-release microencapsulated sodium butyrate (MSB) products increased WG and reduced FCR in juvenile common carp (*C. carpio*). Microencapsulation technologies exhibit the controlled release properties of active ingredients that improve the bioavailability of delivered active ingredients (Dong et al., 2008; Paramera et al., 2011). Different mechanisms including pH, temperature, degradation, diffusion, and swelling are involved in the active ingredients release at controlled rates over prolonged periods of time (Desai & Park, 2005; Jeyakumari et al., 2016). Chow et al. (2017) found that feeding hybrid catfish (*Clarias microcephalus* × *Clarias gariepinus*) with diet containing the encapsulated butyric acid (ButiPEARL) enhanced the body weight gain and improved the FCR value. They stated that the butyric acid is slowly released in the gastrointestinal tract, which make it more accessible in the small intestine to improve the villi growth and subsequently better digestibility of nutrient and growth performance of fish. Gelatine NPs are biodegradable and can easily clear from body (Yasmin et al., 2017). The findings of the current study also indicated that the growth performance parameters had no significant differences between fish fed diets supplemented with gelatine MPs and gelatine NPs and those fed diet without gelatine particles.

The improvement of growth performance may be related to the higher absorption of different nutrients in gastrointestinal tract of host due to the enhancement of digestive enzyme activity (Eslamloo et al., 2012; Haroun et al., 2006). In the current study, the activities of protease, trypsin, α-amylase, lipase and alkaline phosphatase enzymes were higher in the SP-supplemented dietary groups than the control groups. It has been shown that the organic acids through acidification of gut increase the secretion of secretin and subsequently stimulate the activity of pancreatic enzymes (Ng & Koh, 2016). Tian et al. (2017) showed that supplementation of diet with powder sodium butyrate (PSB) and microencapsulated sodium butyrate (MSB) improved the growth performance and the trypsin, chymotrypsin, amylase and lipase activities in young grass carp (*Ctenopharyngodon idella*). They also found that MSB (1000 mg/kg) was superior to PSB (1000 mg/kg) on improving enzyme activities. The current results also showed that the digestive enzyme activities except the alkaline phosphatase were higher in gelatine-SP MPs and gelatine-SP NPs dietary groups than the powder-SP group.

Administration of SP-supplemented diets significantly affected body protein and lipid contents in koi carp fingerlings. Factors such as changes in the rates of synthesis, deposition in muscle and fish growth at different ages can also affect the body protein and lipid content (Abdel-Tawwab et al., 2008). According to our results, the protein deposition in the carcass increased in the fish fed with diets supplemented with three forms of SP. The highest body protein content was observed in fish fed with gelatine-SP NPs, probably due to higher absorption of SP by tissue cells in the form of encapsulation into gelatine nanoparticles. Omosowone et al. (2018) reported that carcass protein content increased in *Clarias gariepinus* fed with butyric acid at level of 2 g/kg for 12 weeks. The lowest lipid content of whole-body was observed in fish fed with diets supplemented with different forms of SP. Nile tilapia (*Oreochromis niloticus*) fed diet supplemented by 0.5% of 1:1 Na-acetate + Ca-lactate blend showed the highest protein and ash content and the lowest lipid content (Agouz et al., 2015). In the present study, use of SP-supplemented diets increased the whole-body ash content of koi carp fingerlings. Similar results also reported by Vielma et al. (1999), who found that acidification of diet increased whole-body ash content of rainbow trout (*Oncorhynchus.mykiss*).

Alanine aminotransferase (ALT) and aspartate amino transferase (AST) enzymes are found in various bodily tissues but are most commonly localized within the liver tissue (Rastiannasab et al., 2016). These enzymes commonly elevate as a result of damage to tissue cells in the liver and blood circulation (Jeney et al., 1992; Rather, 2015). Therefore, these enzymes are measured as biomarkers for liver health (Rastiannasab et al., 2016). In the current study, AST and ALT were investigated in the liver of koi carp fed on diets supplemented with SP. The levels of these enzymes decreased in the carps fed on diets containing gelatine-encapsulated SP micro and nanoparticles compared to the control groups. Fish fed on powder-SP-supplemented diet also showed no significant alterations in the ALT and AST levels in comparison to the controls. These findings indicated that the koi carps could tolerate different forms of dietary SP without any detrimental impact on function of liver. Agouz et al. (2015) also reported that dietary two organic acid salts (calcium lactate + sodium acetate, 1:1) blend had no significant effect on ALT and AST levels in the blood of Nile tilapia *O. niloticus* in comparison to the control.

The finding of this study revealed that dietary SP enhanced the activity of antioxidant enzymes in the liver of koi carps. Safari et al. (2017) found that the expression of antioxidant enzyme genes including glutathione-disulphide reductase (GSR), glutathione peroxidase (GPx) and glutathione S-transferase (GSTA) upregulated in liver of common carp (*Cyprinus carpio)* fed on diet containing SP at levels of 1% and 2% for 8 weeks. Ma et al. (2018) also reported that sodium butyrate improved antioxidant capability in dairy goats through increasing mRNA expression of antioxidant genes. Administration of diet supplemented with sodium alginate at levels of 1.0 or 2.0 g kg^{-1} increased the SOD activity in juvenile grouper, *Epinephelus fuscoguttatus* (Chiu et al., 2008). In contrast, feeding of zebra fish (*Danio rerio*) with diets supplemented with 5, 10 and 20 g/kg of SP resulted in down-regulation of SOD and CAT genes in the liver tissue (Safari et al., 2016). Effectiveness of antioxidant compounds may be limited because of difficulties to cross the cell membranes, poor absorption by cells and degradation during delivery, which ultimately leads to limited bioavailability. The administration of nanoparticles as antioxidant delivery vehicle is effective method in enhancing the antioxidant activity of molecules through increasing the bioavailability and controlled release of the antioxidant (Khalil et al., 2020). Gelatine nanoparticles have been commonly used as a protein carrier to deliver antioxidants like curcumin, tannic acid and theaflavin to human cells (Shutava et al., 2009). Our findings revealed that the utilization of gelatine-SP MPs and gelatine-SP NPs was more effective than powder-SP on improving on the activities of SOD, CAT and GSH enzymes in liver of koi fish. The highest antioxidant enzymes activity was observed in gelatine-SP NPs dietary group.

Alkaline phosphatase as a lysosomal enzyme is found in cells of various tissues especially in liver and bone cells. This enzyme plays a vital role in the various metabolic functions such as the transport of metabolites across the membranes (Samanta et al., 2014), growth, protein synthesis (Ram & Sathyanesan, 1985) and skeleton mineralization of the aquatic animals (Labarrere et al., 2013). He et al. (2017) showed the increase of serum ALP activity in pacific white shrimp (*Litopenaeus vannamei*) fed diet supplemented with a blend of organic acid may result from facilitated absorption and transport of minerals. The elevated ALP activity was also reported in Nile tilapia (*Oreochromis niloticus*) fed malic acid (Hassaan et al., 2017). In the current study, the highest liver ALP activity was found in gelatine-SP NPs dietary group followed by gelatine-SP MPs and powder-SP groups, respectively.

Mucosal immunity is first line of defence system against the infectious microorganisms in aquatic vertebrates (Rombout et al., 2011; Woof & Mestecky, 2005). Several substances with biocidal activity such as lysozyme, complement, antimicrobial peptides, haemolysins and immunoglobulins have been identified in the skin mucus of fish (Palaksha et al., 2008; Whyte, 2007). Recently, the

research on the impacts of feed additives on fish mucosal immunity has been considered. Some studies have been investigated the impact of dietary organic acids on the humoral and mucosal immune responses in various fish species (Liu et al., 2014; Reda et al., 2016; Safari et al., 2016). The current results showed elevation of the total immunoglobulin (Ig) level, lysozyme (LYZ) and ACH50 activities in the skin mucus of koi carps fed diets containing various forms of SP. In line with our finding, Hoseinifar et al. (2016) found that use of diets supplemented with SP, especially at levels of 0.25% and 0.5% significantly enhanced the skin mucus Ig level, LYZ and protease activities in Caspian white fish (*Rutilus frisii kutum*) fry. Feeding common carp (*C. carpio*) with diets supplemented with different levels of SP (0.5%, 1% and 2%) also significantly increased the Ig level, LYZ activity and immune-related genes expression including interleukin 8 [IL-8], interleukin 1 b [IL1b], tumour necrosis factor a [TNF-a] and LYZ in the skin mucus after 8 weeks (Safari et al., 2017). Some studies have been reported a correlation between microbial communities in gastrointestinal tract of fish and immune responses (Abu Elala & Ragaa, 2014; Balcazar et al., 2007). For example, Abu Elala and Ragaa. (2014) showed that activation of cellular and humeral innate immunity of tilapia (*Oreochromis niloticus*) was related to stimulation of beneficial intestinal flora in gut by dietary 0.3% potassium diformate (KDF). The use of SP in the forms of gelatine-SP MPs and gelatine-SP MPs had more increasing effect on the mucosal immune responses compared to the powder-SP form in koi carps. Chow et al. (2017) reported that the administration of diet containing 0.5 kg/t encapsulated butyric acid (ButiPEARL) had positive effects on immune responses in hybrid catfish (*C. macrocephalus* ×C. *gariepinus*).

5 | CONCLUSION

Recently, the commercial administration of organic acids in feeds of aquatic animals to improve both growth and health has been considered. The finding of this study showed that supplementation of diets with three forms of SP beneficially influenced the growth performance and enhanced the digestive and antioxidant enzymes activity as well as immune responses in koi fish. According to the results, the best SP form for inclusion in the diet was gelatine-SP NPs followed by gelatine-SP MPs. Therefore, encapsulation of organic acids could be an effective strategy to enhance their efficiency in aquafeeds.

ACKNOWLEDGEMENT

The authors appreciate the staff of the Faculty of Natural Resources and Environment of Ferdowsi University of Mashhad for their kind help in conducting this research. The research of the corresponding author was supported by a grant from Ferdowsi University of Mashhad (No. 2/47519).

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

ETHICS APPROVAL

Ferdowsi University of Mashhad (FUM) animal ethic right and Directive 2010/63/EU of the European Parliament and of the Council of 22 September 2010 on the protection of animals were applied to the all experiments on fish.

DATA AVAILABILITY STATEMENT

Data will be available on request from the authors.

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How to cite this article: Sarkheil, M., Kordestani, D., & Safari, O. (2021). Dietary supplementation with powder and gelatine micro and nanoencapsulated sodium propionate: Influence on growth performance, digestive and antioxidant enzymes and mucosal immunity of koi carp (*Cyprinus carpio* koi). *Aquaculture Nutrition*, 00, 1–16. https://doi.org/10.1111/anu.13360