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Effect of in vitro selected synbiotics (galactooligosaccharide and mannanoligosaccharide with or without *Enterococcus faecalis*) on growth performance, immune responses and intestinal microbiota of juvenile narrow clawed crayfish, *Astacus leptodactylus leptodactylus* Eschscholtz, 1823

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Abstract

The aim of this study was to determine the best synbiotic combination (based on growth and short-chain fatty acids production) between *Enterococcus faecalis* and eight prebiotics. Based on the results of in vitro studies, *E. faecalis* + galactooligosaccharide (EGOS) and *E. faecalis* + mannanoligosaccharide (EMOS) were selected as synbiotics. A 126-day feeding trial was conducted to compare the effects of prebiotics, probiotic and synbiotics on the growth indices, In vivo ADC of nutrients, digestive enzymes, hemolymph indices and finally, biological responses against 48-hr *Aeromonas hydrophila* exposure challenges of juvenile (4.13 ± 0.12 g) crayfish. The highest values of SGR (2.19% body weight day^{-1}), VFI (2.75% body weight day^{-1}), survival rate (96.67%) and the lowest FCR (2.33) were observed in the juvenile crayfish fed the EGOS- diet. The significantly ($p < .05$) highest means of in vivo ADC$_{OM}$, in vivo ADC$_{CP}$, in vivo ADC$_{CF}$ and in vivo ADC$_{GE}$ were measured in crayfish fed the EGOS- diet. The mean survival rate of *Aeromonas hydrophila*-injected crayfish fed the EGOS- diet (56%) was significantly ($p < .05$) higher than those of fed the control (8.67%) and other diets (22.67–35.32%). At the levels tested, 7.86 log CFU *E. faecalis* g^{-1} + 10 g kg^{-1} GOS in the diet was considered optimum.

KEYWORDS
challenge, crayfish, fermentation, growth performance, immunity, synbiotic

1 | INTRODUCTION

The gut microbiota of aquatic species (finfish and shellfish) as a complex and dynamic ecosystem has an important role in the health status and growth performance (Hoseinifar, Mirvaghefi, Amoozegar, Merrifield, & Ringø, 2015; Llewellyn, Boutin, Hoseinifar, & Derome, 2014; Nayak, 2010; Pérez et al., 2010; Ringø & Gatesoupe, 1998; Safari, Shahsavani, Paolucci, & Atash, 2014a; Vadstein et al., 2013). Genetic, nutritional and environmental factors have been proposed to modulate gut microbiota (Pérez et al., 2010). Using dietary additives (prebiotics, probiotics and synbiotics) in aquafeeds is considered as a typical appliance to balance gut microbiota, mainly towards lactic acid

bacteria (LAB) as profitable communities (Daniels & Hoseinifar, 2014; Lauzon, Dimitroglou, Merrifield, Ringø, & Davies, 2014; Merrifield et al., 2014; Newaj-Fyzul & Austin, 2015; Ringø, Dimitroglou, Hoseinifar, & Davies, 2014; Safari et al., 2014a; Ye, Wang, Li, & Sun, 2011). Synbiotics, the combined forms of probiotics and prebiotics, is critically considered as potential feed additives with growing interests and concerns to achieve a sustainable aquaculture industry (Cerezuela, Meseguer, & Esteban, 2011; Llewellyn et al., 2014). Synergistic effect of synbiotics on growth performance, nutritional efficiency indices, carcass composition, digestive enzymes activities, haemato-immunological parameters and stress resistance of cultivable aquatic species was reported in previous studies (Cerezuela et al., 2011; Ringø

et al., 2014; Rodriguez-Estrada, Satoh, Haga, Fushimi, & Sweetman, 2009). Regarding the importance of in vitro selection of a symbiotic compound, there is limited information in literature review (Hoseinifar et al., 2015; Rurangwa et al., 2009).

Enterococcus faecalis, a gram-positive cocci, can live in a wide range of physicochemical conditions (pH, temperature and osmotic pressure) (Vos et al., 2009), and it has been confirmed that the bacterium is able to adhere and colonize the gastrointestinal tract of aquatic species, and consequently, it has beneficial effects on growth performance, health status and haemato-immunological parameters (Allameh, Ringø, Yusoff, Daud, & Ideris, 2015; Rodriguez-Estrada, Satoh, Haga, Fushimi, & Sweetman, 2013; Rodriguez-Estrada et al., 2009). To our best knowledge, there is no information available on proper evaluation of prebiotics for *E. faecalis*.

Use of different prebiotics in the diet of crayfish showed interesting results. Inclusion of mannan-oligosaccharide (MOS; 3.0 g kg^{-1}) in the diet of juvenile *Astacus leptodactylus* showed the highest SGR and the lowest FCR (Mazlum, Yılmaz, Genç, & Guner, 2011). Using combination of 2.25 g kg^{-1} MOS and 1.5 g kg^{-1} fructo-oligosaccharide (FOS) in the diet of adult *A. leptodactylus leptodactylus* showed positive effects on the growth performance, feed utilization and immune responses against air and bacterial exposure challenges (Safari et al., 2014a). Addition of FOS (2.0 to 10.0 g kg^{-1}) in the diet of red swamp crayfish, *Procambarus clarkii*, increased immune responses including the activities of phenoloxidase and superoxide dismutase (Dong & Wang, 2013). Recently, *Aeromonas hydrophila* as a biological stressor led to increase the mortality rate of cultured crayfish (Longshaw, 2011). Supplemented diets with FOS and MOS increased the immunity responses and survival rate of *A. hydrophila*-injected crayfish (Safari et al., 2014a). Crayfish and crab haemocytes improve the immunity responses via different biological pathways including phagocytosis, cytotoxicity and prophenoloxidase (Johansson, Keyser, Sritunyalucksana, & Söderhäll, 2000). Due to the ever increasing need to improve aquaculture productivity to support the growing demand for shellfish, the use of synbiotics in shellfish culture warrants greater attention and particular consideration should be paid to detect the causative actions in order to optimize the overall efficacy of applications. The aims of this study were (1) to assess the best in vitro synbiotic combination between *E. faecalis* and eight different prebiotics and (2) to evaluate the in vivo effect of two selected synbiotics on the growth performance, nutrient digestibility, immune responses and stress resistance of juvenile narrow clawed crayfish (*Astacus leptodactylus leptodactylus*).

2 | MATERIALS AND METHODS

2.1 | In vitro studies

2.1.1 | Prebiotics tested as substrate and probiotic strain

The prebiotics used in this study were non-digestible, but fermentable carbohydrates (NDFs) with different degrees of polymerization (DP) include β-glucan (BGL; MacroGard®, Orffa Co., Netherlands; DP: 8),

chitosanoligosaccharide (COS; Dosic Co., USA; DP: 10), fructooligosaccharide (FOS; Raftilose®, Orafti Co., Belgium; DP: 4), galactooligosaccharide (GOS; Friesland Foods Domo Co., Netherland; DP: 5), inulin (INU; Raftilose®, Orafti Co., Belgium; DP: 25), isomaltooligosaccharide (IMO; Raftilose®, Orafti Co., Belgium; DP = 15), mannanoligosaccharide (MOS; International Commerce Corporation Co., USA; DP: 6) and xylooligosaccharide (XOS; Shandong Longlive Bio-Technology Co., China; DP: 3).

The probiotic strain used in this study was a lyophilized form of *E. faecalis* (Nichi Nichi Pharmaceutical Co., LTD, Japan). The strain was cultured for 24 hr at 30°C in De Man, Rogosa and Sharpe (MRS) broth (Merck, UK). Bacterial numbers were estimated by serial dilutions being plated in triplicate on MRS agar plates and counted after 24 hr of incubation at 30°C.

2.1.2 | In vitro effect of prebiotics on *E. faecalis* growth under aerobic condition

Growth of *E. faecalis* was examined using eight prebiotics as substrate in a minimal MRS (mMRS) broth medium with omission of glucose (Rurangwa et al., 2009). After pH adjusting (5.6) media with 0.1 N HCl and autoclaving at 121°C for 15 min (not added any carbohydrate source), all prebiotics (BGL, COS, FOS, GOS, INU, IMO, MOS and XOS) were dissolved (1% w/v) in the modified media and filter-sterilized (0.22 μm) before inoculation of bacteria (Allameh et al., 2015; Hoseinifar et al., 2015). All culture media (50 mL) and control (50 mL) were inoculated with similar density of *E. faecalis* 10^8 CFU mL^{-1}. The cultures were incubated at 30°C for 24 hr in shaking incubator (KM65; Fan Azma Gostar, Tehran, Iran) at 150 rpm. Optical density (OD) was measured at 600 nm (OD$_{600}$) with a spectrophotometer (UV-160 A; Shimadzu, Kyoto, Japan) at 3-hr intervals.

2.1.3 | Batch culture fermentation

Anaerobic growth of *E. faecalis* was studied using the protocol described previously. To create an anaerobic condition, sodium thioglycolate (0.2%, w/v) was added to all media (Mandal, Sen, & Mandal, 2010) and test tubes were covered with seal layers of sterile paraffin (Elliott & Dole, 1947). After inoculating 10^8 CFU mL^{-1} of *E. faecalis*, the experimental cultures were incubated at 30°C for 24 hr. The growth trend of the probiotic strain was monitored by measuring OD$_{600}$s at 3-hr intervals postinoculation.

2.1.4 | Short-chain fatty acid (SCFA) analysis

During anaerobic growth, SCFAs (acetate, butyrate and lactate) production in different treatments (synbiotics and control) was measured at the end of exponential growth phase (18.5 hr after inoculation). To omit bacteria and particulate material, samples were centrifuged at 11000 g for 15 min and 20 μl was injected to HPLC (Model 1350T; Bio-Rad, Hemel Hempstead, UK) attached to a UV detector (Knauer, Type 298.00; Bedfordshire, UK) at 210 nm with flow rate of 0.6 mL min^{-1} (Rycroft, Jones, Gibson, & Rastall, 2001). Also, SCFAs quantification was carried out using external calibration standards for acetate, butyrate and lactate.

2.2 | In vivo studies

2.2.1 | Experimental diets

A basal diet (384.1 g kg^{-1}, crude protein; 128.5 g kg^{-1}, crude fat; 14.93 MJ kg^{-1}, Gross energy) as control diet (Safari et al., 2014a) was formulated with WUFFDA (windows-based user-friendly feed formulation and performed again; University of Georgia, Georgia, USA) software (Table 1). To prepare experimental diets, two selected prebiotics (GOS and MOS; each 10 g kg^{-1}) and *E. faecalis* (7.86 log CFU g^{-1}) were used as following: prebiotic diets (GOS, MOS), probiotic diet (*E. faecalis*) and synbiotic diets (GOS + *E. faecalis*, MOS + *E. faecalis*).

2.2.2 | Crayfish and sample collection

Six hundred healthy juvenile crayfish (4.13 ± 0.12 g) were obtained from the Shahid Yaghoobi reservoir (35°9´36″N 59°24´18″E, Khorasan Razavi Province, Iran) and stocked at a density of twenty-five crayfish per 1000-L tank (2 × 1 × 0.5 m) in a semi-recirculating system with daily water exchange rate of 35% at four replicates for each experimental diet. Each tank was fitted with 25 plastic tubes (4 cm diameter and 12 cm length), which served as hiding places for the animals. Unconsumed feed was collected three hours after feeding and weighed. Water temperature was maintained at 25.5°C throughout the feeding trial. DO (6.4 ± 0.19 mg L^{-1}), pH (7.08 ± 0.44), hardness (141 ± 5.7 mg L^{-1} as CaCO$_3$), unionized ammonia (<0.06 mg L^{-1}) and nitrite contents (<0.6 mg L^{-1}) were evaluated every week. Animals were held under L:D 14:10 hr. Briefly, 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 126 days. Biometry was carried out during first and last day of the experiment.

2.2.3 | Evaluation of growth performance and carcass quality

At the end of the feeding trial, each crayfish was individually weighed (±0.01) 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 (Glencross, Booth, & Allan, 2007; Safari et al., 2014a):

Specific Growth Rate (SGR; % day^{-1}) = [(lnW$_f$ −lnW$_i$)/t] × 100

Survival Rate (%) = (Final Individual Numbers/ Initial Individual Numbers) × 100

Voluntary Feed Intake (VFI; % body weight day-1)
= [(Feed$_{consumed\ (DM)}$)/(W$_{mean}$ × t)]

Feed Conversion Ratio (FCR) = (Feed$_{consumed}$/W$_{gain}$)

Protein Efficiency Ratio (PER) = (W$_{gain}$/Crude protein$_{consumed}$)

Protein Productive Value (PPV; %)
= 100 × [(Protein$_{retained}$)/(Protein$_{consumed}$)]

TABLE 1 Composition (g kg^{-1} dry matter) of the control diet fed juvenile crayfish (4.13 ± 0.12 g)

Ingredient	g kg^{-1} (dry-weight basis)
Menhaden fish meal[a]	147
Soybean meal[a]	175
Corn gluten[a]	112
Wheat flour[a]	289
Corn starch[b]	49
Fish oil[a]	42
Canola oil[a]	41
Soy lecithin[a]	50
Cholesterol[d]	5
Glucosamine[c]	10
Choline chloride[4] (70%)[d]	15
Vitamin C (stay)[d]	10
Vitamin premix[d,e]	20
Mineral premix[d,e]	15
Carboxymethyl cellulose[c]	19.9
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^{-1})	14.93
Crude fat/Crude protein	0.33

[a]Behparvar Aquafeed Co, Iran.
[b]Scharloo Chemical Co, Spain.
[c]Sigma, Germany.
[d]Kimia Roshd Co. Iran.
[e]Mineral premix contains (mg kg^{-1}) Mg, 100; Zn, 60; Fe, 40; Cu, 5; Co, 0.1; I, 0.1; Antioxidant (BHT), 100. Vitamin premix contains (mg kg^{-1}) 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.

In the above equations, W$_i$, W$_f$, W$_{mean}$ and W$_{gain}$, t and Feed$_{consumed}$ are initial weight, final weight, mean weight, weight increment (g), time period (day) and consumed feed (g), respectively.

2.2.4 | Calculation of In vivo apparent nutrient digestibility

In vivo ADCs of organic matter (ADC$_{OM}$), crude protein (ADC$_{CP}$), crude fat (ADC$_{CF}$) and gross energy (ADC$_{GE}$) of experimental diets were calculated according to the following equations (Safari, Shahsavani, Paolucci, & Atash Mehraban Sang, 2014b):

ADC$_{test}$ = 100 × (1-(Marker$_{test}$ × Nutrient$_{faeces}$/ Marker$_{faeces}$ × Nutrient$_{test}$))

In above equation, the terms "Marker$_{test}$" and "Marker$_{faeces}$" represent the marker (0.1 g kg^{-1} Ytterbium oxide, Yb$_2$O$_3$, in the diet) contents of the diet and faeces, respectively, and Nutrient$_{test}$ and Nutrient$_{faeces}$ represent the nutritional parameters of concern (e.g., protein or energy) in the diet and faeces, respectively.

2.3 | Biochemical analyses

2.3.1 | Hemolymph indices

In the 126th day, six crayfish from each tank (24 crayfish per treatment) were killed after 24 hr of last feeding time. All assays were carried out one by one at four replicates. According to protocol previously described (Safari et al., 2014a), hemolymph was obtained from ventral sinus with needle (25G), pooled and stored via two following methods: (1) one millilitre microtube without anticoagulant agent (see section 2.3.2) and (2) one millilitre microtube containing 0.4 ml Alsever as an anticoagulant and then divided into two parts. Briefly, one part (125 μl) was used to measure the following hemolymph indices: THC with hemocytometer cell (Beco, Hamburg, Germany) (Jiang, Yu, & Zhou, 2004), hyaline count (HC), semi- and large granular count (SGC and LGC, respectively) via hemolymph extension at room temperature (25°C), fixation at methanol for 1 min, staining with method of May-Grunwald-Giemsa and then counting with light microscope (Pousti & Adib Moradi, 2004). Total plasma protein was estimated using the biuret procedure.

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

The remaining anticoagulated hemolymph (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., 2014a; Zhang et al., 2011). All activities of enzymes were standardized based on the protein concentration. Phenoloxidase activity (PO) was assayed spectrophotometrically by recording the formation of dopachrome from L-dihydroxyphenylalanine (L-DOPA) at final reading 490 nm (Hernández-López, Gollas-Galván, & Vargas-Albores, 1996; Safari et al., 2014a). Superoxide dismutase (SOD) activity was measured by observing the inhibition of ferricytochrome C reduction at final reading 550 nm (Cooper, Clough, Farwell, & West, 2002). The lysozyme (LYZ) activity was determined with a decrease in absorbance compared to *Micrococcus lysodeikticus* suspension without plasma at final reading 530 nm (Ellis, 1990). Nitric oxide synthase (NOS) activity was measured with the assay kit (Nanjing Jiancheng Bioengineering Institute, China) (Marzinzig et al., 1997).

2.3.3 | Digestive enzyme activities

The hepatopancreas (12 crayfish per treatment) was quickly removed, rinsed with distilled water, dried with paper towel and homogenized (30 g/70 ml distilled water) using a homogenizer (DI 18 Disperser), and the homogenate was then centrifuged at 10000 × g, 4°C, 25 min

and the supernatant stored in liquid nitrogen. The measurement of digestive enzyme activities was explained elsewhere (Safari et al., 2014a). Briefly, the amylase activity was measured using starch as substrate at final reading 550 nm with a UV/VS spectrophotometer (Ultro Spec 2000 Pharmacia Biotech) (Coccia et al., 2011). Lipase activity was measured using α-naphthyl caprylate as substrate at final reading 540 nm (López-López, Nolasco, & Vega-Villasante, 2003). Alkaline protease activity was determined using azocasein as substrate at final reading 366 nm (Fernández Gimenez, García-Carreño, Navarrete del Toro, & Fenucci, 2001). In this study, specific enzyme activity was defined as enzyme units (U) per mg of protein.

2.4 | Chemical analysis

Analysis of dry matter (oven drying, 105°C), crude protein (N × 6.25, Kjeldahl system: Buchi Labortechnik AG, Flawil, Switzerland), crude fat (Soxtec System HT 1043: Foss Tecator, AB), ash (muffle furnace, 550°C), gross energy (Parr bomb calorimetry model 1266, Parr Instrument Co., Moline, IL) and crude fibre (after digestion with H$_2$SO$_4$ and NaOH) analysis of feedstuffs, diets and faeces were performed according to standard methods (AOAC, 2005). Nitrogen-free extract (NFE) was calculated by subtraction dry matter minus crude protein, crude fat, crude fibre and ash contents. Organic matter was calculated by subtraction dry matter minus ash content. Ytterbium oxide was determined in diets and faeces by inductively coupled plasma atomic absorption spectrophotometry (ICP; GBC Integra XL, Australia).

2.5 | Bacteriological analysis

At the start of the feeding trial, total viable counts (TVC) of heterotrophic aerobic bacteria and presumptive lactic acid bacteria (LAB) levels in hepatopancreas were determined by random sampling of 20 crayfish from stock. As described previously (Safari et al., 2014a), at the end of the experiment, crayfish (12 individuals per a treatment) was transported alive to laboratory, anesthetized with ice, rinsed with benzalkonium chloride (0.1% for 60 min) and dissected with scalpel. Then, hepatopancreas was removed and homogenized with sodium chloride (0.9 w/v) using a homogenizer (DI 18 Disperser) and the homogenate was then centrifuged at 5000 × g, 4°C, 5 min. One hundred microlitres from the prepared samples were put on plate count agar (PCA; Merck Co) and de Man, Rogosa and Sharpe media (MRS; Merck Co) at four replicates to determine TVC and LAB, respectively. The plates were incubated at room temperature (25°C) for 5 days, and colony-forming units (CFU) g^{-1} were calculated from plates containing 30–300 colonies (Hoseinifar et al., 2015).

2.6 | Bacterial exposure challenge

Challenge test was initiated on day 127 of the feeding trial. Twelve crayfish from each test diet tank were injected with 1 × 10^8 cells ml^{-1} *Aeromonas hydrophila*, ATCC 49141 through the base of the fifth thoracic leg with 20 ml bacteria stock suspension (Safari et al., 2014a; Sang, Ky, & Fotedar, 2009). The injected crayfishes were marked

TABLE 2 The mean ($\pm$SD[1]) of maximum growth (OD_{600} nm) of *Enterococcus faecalis* grown in minimal MRS broth media supplemented with different prebiotics (BGL, COS, FOS, GOS, INU, IMO, MOS and XOS) under aerobic and anaerobic condition. TRMG indicates the time needed in hours to attain the maximum density (n = 3)[2]

Treatment	Aerobic culture		Anaerobic culture	
	Maximum growth (OD_{600} nm)	TRMG (hr)	Maximum growth (OD_{600} nm)	TRMG (hr)
Control	1.07 $\pm$ 0.02[a]	19.3	1.14 $\pm$ 0.01[a]	12.4
BGL[3]	3.01 $\pm$ 0.01[c]	18.9	1.95 $\pm$ 0.04[d]	12.1
COS[4]	3.47 $\pm$ 0.02[c]	18.9	2.03 $\pm$ 0.06[e]	12.5
FOS[5]	3.24 $\pm$ 0.06[c]	18.5	1.70 $\pm$ 0.02[b]	12.2
GOS[6]	5.35 $\pm$ 0.01[e]	18.2	4.26 $\pm$ 0.01 [g]	10.3
INU[7]	1.23 $\pm$ 0.02[a]	19.0	1.68 $\pm$ 0.01[b]	13.2
IMO[8]	1.47 $\pm$ 0.06[a]	19.1	1.77 $\pm$ 0.01[c]	12.4
MOS[9]	4.11 $\pm$ 0.02[d]	18.2	3.14 $\pm$ 0.01[f]	10.2
XOS[10]	2.15 $\pm$ 0.01[b]	19.1	1.75 $\pm$ 0.01[c]	12.5
p-value	.0001	–	.0001	–

[1]Standard deviation.
[2]Different superscripts within a column indicate significant differences at *p* > .05.
[3]β-glucan.
[4]Chitosanoligosaccharide.
[5]Fructooligosaccharide.
[6]Galactooligosaccharide.
[7]Inulin.
[8]Isomaltooligosaccharide.
[9]Mannanoligosaccharide.
[10]Xylooligosaccharide.

before releasing back into their original tanks to avoid repeat sampling. The infected crayfishes were monitored for survival rate, THC, HC, SGC and LGC after 48 hr of injection.

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 and Kolmogorov-Smirnov tests (Zar, 2007), respectively, ANOVA was used to compare the treatments at four replicates. Duncan test was applied to compare significant differences among the treatments (*p* < .05) with SPSS[TM] version 19. All results were given as mean $\pm$ SEM.

3 | RESULTS

3.1 | Growth of *E. faecalis* on different prebiotics under aerobic and anaerobic conditions

Maximum growth of *E. faecalis* on various prebiotics and control under aerobic and anaerobic conditions are presented in Table 2. Under aerobic condition, maximum growth (OD_{600}) of *E. faecalis* was 5.35 and 4.11 when GOS and MOS were added, respectively. The significantly (*p* < .05) lowest growth (1.07) of *E. faecalis* was observed on the control. The growth of *E. faecalis* on BGL, COS, FOS and IMO (3.01–3.47) was similar (Table 2) but significant (*p* < .05) different from XOS (2.15) and control.

Anaerobic growth of *E. faecalis* revealed lower maximum values (1.07–5.35) than those under aerobic condition (1.14–4.26);

the significantly (*p* < .05) highest growth was noticed when GOS (OD_{600} = 4.26) and FOS (OD_{600} = 3.14) were added. The lowest growth of *E. faecalis* was measured on control (OD_{600} = 1.14). The growth of *E. faecalis* on IMO and XOS was similar. Based on maximum OD_{600}, the treatments of *E. faecalis* + GOS and *E. faecalis* + MOS showed the lowest incubation time to obtain the maximum growth values and were significantly (*p* < .05) different compared with the other treatments (Table 2).

3.2 | Short-chain fatty acids (SCFA) production

The production of SCFA (acetate, propionate and butyrate) with *E. faecalis* on the different prebiotics during the anaerobic exponential growth phase is presented in Figure 1. The significantly (*p* < .05) highest acetate concentration (3.10–3.17 mmol L^{-1}) was revealed in the treatments of probiont with GOS and MOS. The treatments of control (no prebiotic) and XOS showed the lowest acetate production (2.03–2.13 mmol L^{-1}) (Figure 2). The significantly (*p* < .05) highest concentrations of propionate (111.67 mmol L^{-1}) and butyrate (355 mmol L^{-1}) were measured in the treatment of *E. faecalis* with GOS (Figure 2). The control treatment showed the significantly (*p* < .05) lowest propionate and butyrate (Figure 2).

3.3 | Growth performance and survival rate

Administration of prebiotics (GOS and MOS), probiotics (*E. faecalis* (EnF)) and synbiotics (*E. faecalis* + GOS (EGOS), *E. faecalis* + MOS (EMOS)) improved significantly (*p* < .05) growth performance (final weight, SGR, FCR, VFI), survival rate and nutritional efficiency indices
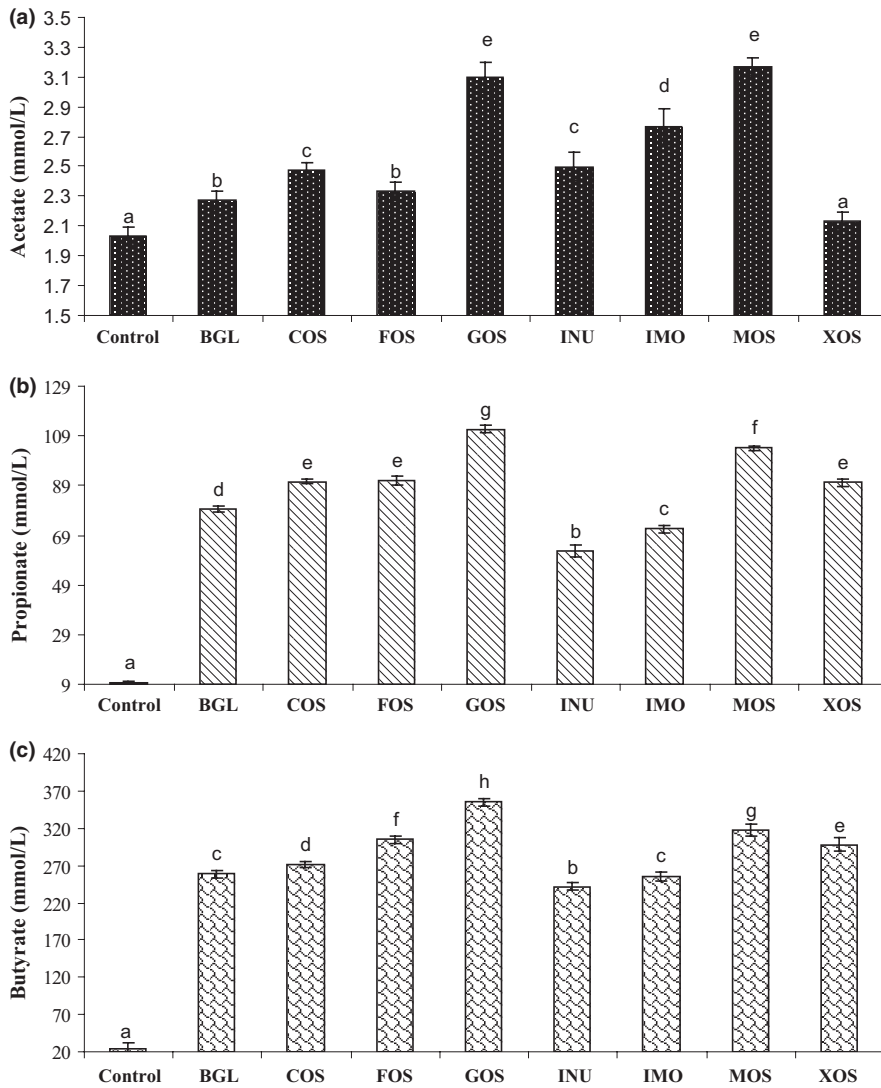
FIGURE 1 The mean (± *SD*) of (a) acetate, (b) propionate and (c) butyrate production at the end of exponential growth phase using different prebiotics (BGL, COS, FOS, GOS, INU, IMO, MOS and XOS) and control (no prebiotic) at three replicates. Bars assigned with different superscripts are significantly different (*p* < .05)

(PER and PPV) in juvenile crayfish compared with control-fed crayfish (Table 3). Juvenile crayfish fed the diet containing EGOS showed the significantly ($p < .05$) highest final weight (64.47 g), SGR (2.19% BW day^{-1}), VFI (2.75% BW day^{-1}), PER (3.27) and PPV (64.67%) and significantly ($p < .05$) lowest FCR (2.33) (Table 3). Survival rate of the crayfish fed the diets containing EGOS (96.67%) and EMOS (95.67%) was significantly ($p < .05$) higher than other treatments. The crayfish fed the diet containing EMOS placed in the second rank after the treatment EGOS based on the final weight (60.70 g), SGR (2.14% BW day^{-1}), FCR (2.38), VFI (2.70% BW day^{-1}), PER (3.15) and PPV (60%) (Table 3). Final weight and FCR in crayfish fed the diet containing GOS were similar to those fed the diet containing EnF. VFI in crayfish fed the diets containing GOS or MOS was significantly ($p < .05$) higher than crayfish fed the diet containing EnF (Table 3). Similar survival rate (86.67–87.67%) was observed in crayfish fed the diets containing GOS, MOS and EnF (Table 3). PER in crayfish fed the diet containing GOS (2.60) was significantly ($p < .05$) higher than those of fed the diets containing MOS (2.33) or EnF (2.30) (Table 3). PPV in crayfish fed the diets containing GOS (53%) and MOS (54%) was significantly ($p < .05$) lower than that of fed the diet containing EnF (56.33%) (Table 3).

3.4 | In vivo ADCs of organic matter, crude protein, crude fat and gross energy

The juvenile crayfish fed the diet containing EGOS showed the significantly ($p < .05$) highest values for in vivo ADC$_{OM}$ (86.67%), in vivo ADC$_{CP}$ (94.67%), In vivo ADC$_{CF}$ (92%) and In vivo ADC$_{GE}$ (88.67%) (Table 4). In this regard, the crayfish fed the control diet showed the lowest values for In vivo ADC$_{OM}$ (61%), In vivo ADC$_{CP}$ (77.33%), In vivo ADC$_{CF}$ (70%) and In vivo ADC$_{GE}$ (65%) (Table 4). The significantly ($p < .05$) similar values for In vivo ADC$_{OM}$, In vivo ADC$_{CP}$ and In vivo ADC$_{GE}$ were observed in crayfish fed the diets containing GOS, MOS and EnF. Crayfish fed these diets (GOS, MOS and EnF) showed significantly ($p < .05$) lower values compared with crayfish fed the diet containing EMOS (Table 4).

3.5 | Hemolymph indices

Feeding the juvenile crayfish with the diets containing prebiotics, probiotic and synbiotics improved (×10^5 cell ml^{-1}) significantly ($p < .05$) the THC (149.66.67–191), HC (86.33–99), SGC (30–41.67)
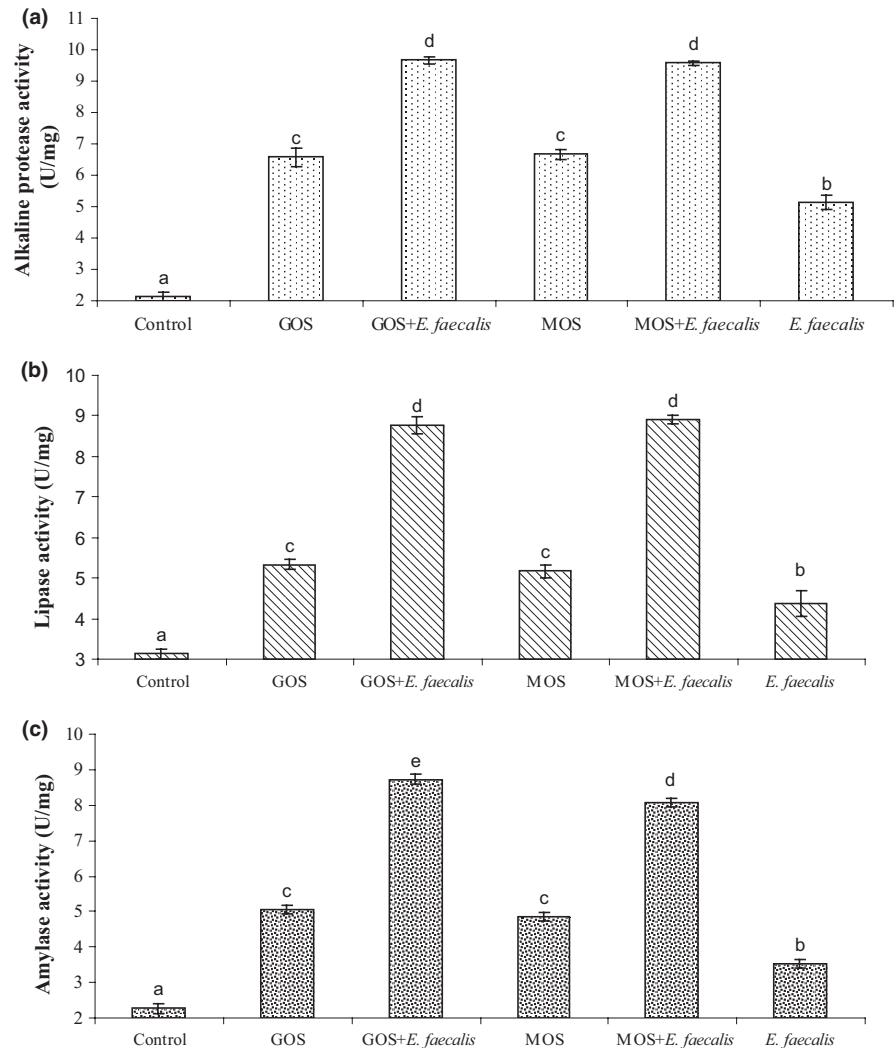
FIGURE 2 The mean (± SD) activities (U mg^{-1}) of (a) alkaline protease, (b) lipase and (c) amylase in the hepatopancreas of crayfish fed the experimental diets after 126 days at four replicates (GOS: Galactooligosaccharide; MOS: Mannanoligosaccharide). Different letters indicate significant differences ($p < .05$)

TABLE 3 The mean (± SD^1) of initial weight (g), final weight (g), specific growth rate (% day^{-1}), voluntary feed intake (% BW day^{-1}), feed conversion ratio, survival rate (%), protein efficiency ratio (PER) and protein productive value (PPV, %) of crayfish fed the experimental diets after 126 days ($n = 4$)^2

	Control	GOS^3	GOS + E. faecalis (EGOS)	MOS^4	MOS + E. faecalis (EMOS)	E. faecalis (EnF)	p-value
Initial weight (g)	4.14 ± 0.06[a]	4.13 ± 0.01[a]	4.14 ± 0.01[a]	4.13 ± 0.01[a]	4.14 ± 0.06[a]	4.14 ± 0.01[a]	.753
Final weight (g)	30.72 ± 2.25[a]	54.50 ± 2.50[c]	64.47 ± 2.18[e]	49.33 ± 2.65[b]	60.70 ± 2.13[d]	54.00 ± 2.10[c]	.0001
Specific growth rate (% BW day^{-1})	1.59 ± 0.62[a]	2.08 ± 0.73[d]	2.19 ± 0.94[f]	1.98 ± 0.61[b]	2.14 ± 0.82[e]	2.04 ± 0.94[c]	.0001
Voluntary feed intake (% BW day^{-1})	1.98 ± 0.63[a]	2.42 ± 0.91[c]	2.75 ± 0.82[e]	2.38 ± 0.83[c]	2.70 ± 0.96[d]	2.33 ± 0.96[b]	.0001
Feed conversion ratio	3.11 ± 0.61[e]	2.51 ± 0.73[c]	2.33 ± 0.90[a]	2.55 ± 0.91[d]	2.38 ± 0.94[b]	2.50 ± 0.91[c]	.0001
Survival rate (%)	43.00 ± 2.05[a]	86.67 ± 2.36[b]	96.67 ± 2.66[c]	87.00 ± 2.90[b]	95.67 ± 2.58[c]	87.67 ± 2.58[b]	.0001
Protein efficiency ratio	1.19 ± 0.41[a]	2.60 ± 0.51[c]	3.27 ± 0.66[e]	2.33 ± 0.86[b]	3.15 ± 0.98[d]	2.30 ± 0.90[b]	0.0001
Protein productive value (%)	44.00 ± 2.50[a]	53.00 ± 2.71[b]	64.67 ± 2.58[e]	54.00 ± 2.80[b]	60.00 ± 3.70[d]	56.33 ± 3.88[c]	.0001

GOS, galactooligosaccharide; MOS, mannanoligosaccharide.
[1]Standard deviation.
[2]Different superscripts within a row indicate significant differences at $p > .05$.

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TABLE 4 The mean (± SD[1]) of In vivo ADCs of organic matter (ADC$_{OM}$, %), crude protein (ADC$_{CP}$, %), crude fat (ADC$_{CF}$, %) and gross energy (ADC$_{GE}$, %) of crayfish fed experimental diets after 126 days (n = 4)[2]

	Control	GOS[3]	GOS + *E. faecalis* (EGOS)	MOS[4]	MOS + *E. faecalis* (EMOS)	*E. faecalis* (EnF)	p-value
In vivo ADC$_{OM}$	61.00 ± 2.10[a]	72.00 ± 2.08[b]	86.67 ± 2.88[d]	72.67 ± 2.98[b]	83.00 ± 2.76[c]	72.33 ± 3.35[b]	.0001
In vivo ADC$_{CP}$	77.33 ± 2.58[a]	82.67 ± 2.58[b]	94.67 ± 2.58[d]	81.67 ± 2.58[b]	90.33 ± 2.58[c]	82.67 ± 2.58[b]	.0001
In vivo ADC$_{CF}$	70.00 ± 3.00[a]	72.67 ± 3.58[bc]	92.00 ± 3.20[e]	73.67 ± 3.58[c]	87.67 ± 3.58[d]	72.00 ± 3.00[b]	.0001
In vivo ADC$_{GE}$	65.00 ± 3.00[a]	73.67 ± 3.58[b]	88.67 ± 3.58[d]	73.67 ± 3.58[b]	86.67 ± 3.58[c]	73.00 ± 3.00[b]	.0001

GOS, galactooligosaccharide; MOS, mannanoligosaccharide.
[1]Standard deviation.
[2]Different superscripts within a row indicate significant differences at p > .05.

and LGC (33–51) than those of control diet (111.67, 73, 19 and 19.67, respectively) (Table 5). Juvenile crayfish fed the EGOS showed the significantly (p < .05) highest values for THC, HC and LGC. Also, based on the effect on hemolymph indices (THC, HC and LGC), EMOS- diet ranked in the second place. The effect of diets containing GOS, MOS and EnF on HC and LGC of test crayfish was significantly (p < .05) similar.

After bacterial exposure challenge, the crayfish fed the EGOS- diet showed the significantly (p < .05) highest values for survival rate (56%) and hemolymph indices (×10^5 cell ml^{-1}) including THC (221.92), HC (73.77) and LGC (20.83) (Table 6). The calculated SGC in crayfish fed the EGOS- and EnF- diets was significantly (p < .05) higher than those of fed the other diets. LGC in crayfish fed the MOS- diet was significantly (p < .05) higher than those of fed the GOS and EnF (Table 6). After challenge, crayfish fed the MOS- diet showed higher survival rate than crayfish fed the EnF diet (27.33% versus 22.67%) (Table 6).

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

After 126-day feeding trial, the juvenile crayfish fed the control diet showed the significantly (p < .05) lowest activities (U min^{-1}) of PO (2.0), SOD (1.90), LYZ (3.97) and NOS (1.97) (Table 5). The juvenile crayfish fed the diets containing EGOS and EMOS showed significantly (p < .05) higher activities of PO, SOD, LYZ and NOS than those of fed the other diets (Table 5). The crayfish fed the diets containing GOS, MOS and EnF had significantly (p < .05) similar effects on PO (3.07–3.17 U min^{-1}) and SOD (2.33–2.47 U min^{-1}) (Table 5). The MOS- and EnF- diets showed significantly (p < .05) similar effect on LYZ (6.80–6.97 U min^{-1}) of test crayfish (Table 5). Also, the GOS- and EnF- diets revealed the significantly (p < .05) similar effect on NOS (3.10–3.20 U min^{-1}).

3.7 | Digestive enzyme activities

The digestive enzyme activities (U mg^{-1}) including alkaline protease (5.13–6.57), lipase (4.37–8.90) and amylase (3.53–8.73) in the juvenile crayfish fed the prebiotic, probiotic and synbiotics diets were significantly (p < .05) higher than those of control diet (2.13, 3.13 and 2.27,

respectively) (Figure 2a–c). The significantly (p < .05) highest activities of alkaline protease and lipase appeared in crayfish fed the EGOS- and EMOS- diets (Figure 2a,b). The significantly (p < .05) highest amylase activity was observed in crayfish fed the EGOS- diet (Figure 2c). The crayfish fed the MOS- and GOS- diets showed higher activities of alkaline protease, lipase and amylase compared with crayfish fed the EnF- diet (Figure 2a–c).

3.8 | Microbiological analysis

The presumptive autochthonous lactic acid bacteria (LAB) count (CFU g^{-1}) to total viable heterotrophic aerobic bacteria count (CFU g^{-1}) ratios (%) in the extracted hepatopancreas in the juvenile crayfish fed the EGOS- diet showed the significantly (p < .05) highest value (67.58%) (Figure 3). The GOS-, MOS- and EnF- diets had the significantly (p < .05) similar values for this ratio (44.44–45.33%); however, these treatments showed significantly (p < .05) lower values than that of fed the EMOS- diet (Figure 3).

4 | DISCUSSION

4.1 | In vitro fermentation and synbiotic selection

The present study revealed maximum in vitro growth of *E. faecalis* under aerobic and anaerobic conditions when GOS (DP = 5) and MOS (DP = 6) were added to the growth medium. Degree of polymerization of prebiotic affects on the fermentation process (Hoseinifar et al., 2015; Kolida, Tuohy, & Gibson, 2002; Safari, 2011; Safari et al., 2014a). The lower DP leads to increase fermentability (Kolida et al., 2002). In accordance with this, the results of the present study revealed lower in vitro growth of *E. faecalis* when IMO (DP = 15) and INU (DP = 25) used as substrate, similar growth as control (medium not supplemented with prebiotic). Inulin (with high DP) was not fermented with *Carnobacterium* (piscicola) *maltaromaticum* LMG 9839 (Khouiti & Simon, 1997). However, the previous studies (Hoseinifar et al., 2015; Kolida et al., 2002) did not explore what is efficient DP range to maximize bacterial growth. In spite of lower DP (≤4) of test prebiotics including XOS (DP = 3) and FOS (DP = 4), higher in vitro growth of *E. faecalis* did not observed. Based on the DP definition, the number of monomeric units in a macromolecule or polymer or

TABLE 5 The mean (± SD[1]) of total haemocyte count (THC, ×10⁵ cell ml⁻¹), hyaline count (HC, ×10⁵ cell ml⁻¹), semi-granular count (SGC, ×10⁵ cell ml⁻¹), large granular count (LGC, ×10⁵ cell ml⁻¹), phenoloxidase activity (PO, U min⁻¹), superoxide dismutase activity (SOD, U min⁻¹), lysozyme activity (LYZ, U min⁻¹) and nitric oxide synthase activity (NOS, U min⁻¹) of crayfish fed experimental diets after 126 days ($n = 4$)[2]

	Control	GOS[3]	GOS + *E. faecalis* (EGOS)	MOS[4]	MOS + *E. faecalis* (EMOS)	*E. faecalis* (EnF)	*p*-value
THC (×10⁵ cell ml⁻¹)	111.67 ± 3.00ᵃ	150.33 ± 3.58ᵇᶜ	191.00 ± 4.00ᵉ	149.66 ± 3.58ᵇ	187.00 ± 4.00ᵈ	151.67 ± 3.58ᶜ	.0001
HC (×10⁵ cell ml⁻¹)	73.00 ± 5.00ᵃ	87.33 ± 4.58ᵇ	99.00 ± 5.00ᵈ	86.33 ± 4.58ᵇ	97.00 ± 5.00ᶜ	86.67 ± 4.58ᵇ	.0001
SGC (×10⁵ cell ml⁻¹)	19.00 ± 5.00ᵃ	30.00 ± 5.00ᵇ	41.00 ± 5.00ᵈ	29.00 ± 5.00ᵇ	41.67 ± 4.58ᵈ	32.00 ± 5.00ᶜ	.0001
LGC (×10⁵ cell ml⁻¹)	19.67 ± 4.58ᵃ	33.00 ± 4.00ᵇ	51.00 ± 5.00ᵈ	34.33 ± 4.58ᵇ	48.33 ± 4.58ᶜ	33.00 ± 4.00ᵇ	.0001
Phenoloxidase activity (U min⁻¹)	2.00 ± 4.10ᵃ	3.10 ± 4.10ᵇ	5.63 ± 4.15ᵈ	3.17 ± 4.21ᵇ	4.93 ± 4.12ᶜ	3.07 ± 4.12ᵇ	.0001
Superoxide dismutase activity (U min⁻¹)	1.90 ± 4.15ᵃ	2.33 ± 4.06ᵇ	5.13 ± 4.12ᵈ	2.37 ± 4.06ᵇ	4.57 ± 4.12ᶜ	2.47 ± 4.06ᵇ	.0001
Lysozyme activity (U min⁻¹)	3.97 ± 4.06ᵃ	7.17 ± 5.21ᶜ	9.20 ± 5.20ᵉ	6.80 ± 5.10ᵇ	8.30 ± 5.10ᵈ	6.97 ± 5.06ᵇᶜ	.0001
Nitric oxide synthase activity (U min⁻¹)	1.97 ± 4.06ᵃ	3.20 ± 4.10ᵇᶜ	4.67 ± 5.06ᵉ	3.30 ± 5.10ᶜ	4.40 ± 5.10ᵈ	3.10 ± 5.10ᵇ	.0001

GOS, galactooligosaccharide; MOS, mannanoligosaccharide.
[1]Standard deviation.
[2]Different superscripts within a row indicate significant differences at $p > .05$.

TABLE 6 The mean (± SD[1]) of total haemocyte count (THC, ×10⁵ cell ml⁻¹), hyaline count (HC, ×10⁵ cell ml⁻¹), semi-granular count (SGC, ×10⁵ cell ml⁻¹), large granular count (LGC, ×10⁵ cell ml⁻¹) and survival rate (%) of crayfish exposed to bacterial exposure challenge ($n = 4$)[2]

	Control	GOS[3]	GOS + *E. faecalis* (EGOS)	MOS[4]	MOS + *E. faecalis* (EMOS)	*E. faecalis* (EnF)	*p*-value
THC (×10⁵ cell ml⁻¹)	115.60 ± 8.03ᵃ	162.96 ± 8.10ᶜ	221.92 ± 8.15ᵉ	158.03 ± 8.12ᵇ	212.84 ± 8.07ᵈ	163.35 ± 8.21ᶜ	.0001
HC (×10⁵ cell ml⁻¹)	73.77 ± 7.15ᵃ	90.81 ± 7.09ᶜ	110.36 ± 6.55ᵉ	90.13 ± 7.32ᵇ	106.11 ± 7.20ᵈ	91.08 ± 7.29ᶜ	.0001
SGC (×10⁵ cell ml⁻¹)	21.00 ± 4.20ᵃ	36.05 ± 3.15ᵇ	54.13 ± 4.15ᵉ	35.64 ± 3.31ᵇ	52.82 ± 4.95ᵈ	37.53 ± 3.43ᵉ	.0001
LGC (×10⁵ cell ml⁻¹)	20.83 ± 3.21ᵃ	36.10 ± 3.21ᶜ	57.43 ± 4.40ᶠ	32.26 ± 3.21ᵈ	53.91 ± 4.13ᵉ	34.74 ± 4.43ᵇ	.0001
Survival rate (%)	8.67 ± 5.58ᵃ	24.67 ± 6.16ᵇᶜ	56.00 ± 7.00ᵉ	27.33 ± 6.16ᶜ	35.32 ± 6.16ᵈ	22.67 ± 6.31ᵇ	.0001

GOS, galactooligosaccharide; MOS, mannanoligosaccharide.
[1]Standard deviation.
[2]Different superscripts within a row indicate significant differences at $p > .05$.

oligomer molecule, it is considered that probionts need a minimum DP (or molecule weight) of prebiotics to ferment substrate and to boost growth. This agreed well with the finding of Hoseinifar et al., 2015, who found *Peidiococcus acidilactici* fermented efficiently GOS (DP = 5) and XOS (DP = 3) compared with FOS (DP = 4) and INU (DP = 25). To the author's knowledge, there is no available information on SCFA production with *E. faecalis* after in vitro fermentation of prebiotics or other carbohydrates. Data from this study showed that the major SCFA produced was butyrate in control and symbiotic treatments. In contrast to our results, acetate was the major SCFA of β-glucan, XOS and arabinoxylooligosaccharide, INU and FOS with *C. maltaromaticum* LMG 9839, *Lactobacillus plantarum* LMG 9211 and *L. delbrueckii* subsp. *Lactis* (Rurangwa et al., 2009). Feeding mice with the diet containing *P. acidilactici* + FOS led to butyrate production in intestine (Juśkiewicz et al., 2007). A likely explantation of this conflicting finding may be attributed to difference in prebiotic origin (fungi or plant), chemical structure, degree of polymerization (DP), supplement dose and probiotic strain used (Merrifield et al., 2014;

Ringø et al., 2014; Safari et al., 2014a). However, further studies need to clarify this.

4.2 | In vivo studies

The final products of prebiotics fermentation, SCFA (acetic, propionic and butyrate acids), are considered as the main factors in the synbiotic effects on growth indices (weight gain, SGR and FCR), immune responses and survival rate (Cummings & Macfarlane, 2002; Hoseinifar et al., 2015; Maslowski & Mackay, 2011; Scheppach, 1994; Schley & Field, 2002). The functional role of butyrate is regarded as a main energy source for colonic epithelial cells and epithelium maintenance (Maslowski & Mackay, 2011). As a result, this led increased in vivo ADCs of organic matter, crude protein, crude fat and gross energy (Safari et al., 2014a; Ye et al., 2011) and improved feed efficiency in the gastrointestinal tract (Buentello, Neill, & Gatlin, 2010). Additionally, butyrate has been proposed to activate the immune responses and also, to resist host on stressors (Maslowski & Mackay, 2011). Butyrate
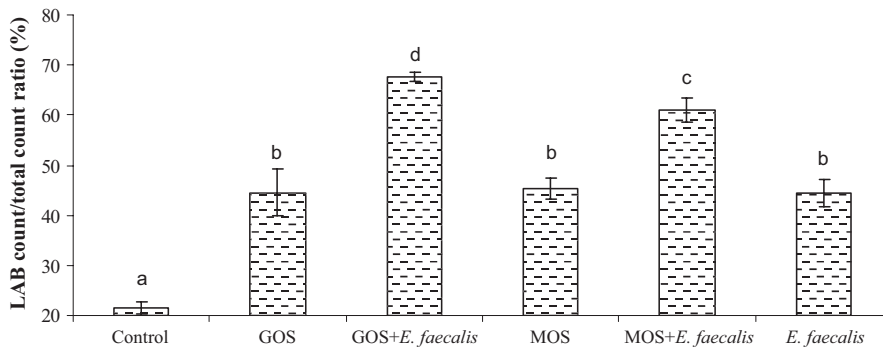
FIGURE 3 The mean (± SD) of presumptive autochthonous lactic acid bacteria (LAB) count (CFU g^{-1}) to total viable heterotrophic aerobic bacteria count (CFU g^{-1}) ratio (%) of hepatopancreas extracted from crayfish fed the experimental diets after 126 days at four replicates (GOS: Galactooligosaccharide; MOS: Mannanoligosaccharide). Different letters indicate significant differences ($p < .05$)

as an increasing factor of disease resistance could downregulate the expression of invasion genes in *Salmonella* sp. (Van Immerseel et al., 2006). In the present study, a significant in vitro increment of butyrate production was observed in the selected treatments (*E. faecalis* + GOS, *E. faecalis* + MOS); thus, these synbiotic combinations might have the potential to stimulate the hemolymph indices (THC, HC, SGC and LGC), antioxidant enzymes (PO, SOD and NOS) and immune responses (LYZ) of crayfish and to increase resistance against different stressors. However, this issue merits further investigations.

Compared to progress made with fish, research on the dietary additives (synbiotics) for crayfish is still in its infancy. To our best knowledge, this is the first time to study the effects of synbiotics in the diet of crayfish. This agreed well with the previous findings on *Penaeus japonicus* fed with Bacillus (*B. licheniformis* and *B. subtilis*) + isomaltooligosaccharide (Zhang et al., 2011); *Litopenaeus vannamei* fed with Bacillus sp + the oligosaccharides (Munaeni, Yuhana, & Widanarni, 2014); *L. vannamei* fed with *B. subtilis* + *P. acidilactici* and β-glucan (Wongsasak, Chaijamrus, Kumkhong, & Boonanuntanasarn, 2015); *L. vannamei* fed with *Vibrio alginolyticus* + oligosaccharide (Nurhayati & Yuhana, 2015); ovate pompano fed with *B. subtilis* + fructooligosaccharide (Zhang et al., 2014); hybrid surubim fed with inulin + *Weissella cibaria* (Mourino et al., 2012); and rainbow trout fed with *E. faecalis* + MOS (Rodriguez-Estrada et al., 2009) and *E. faecalis* + FOS (Mehrabi, Firouzbakhsh, & Jafarpour, 2012). Critically considering differences between prebiotic origin, probiotic variety, hydrolysis conditions (in vitro and in vivo), physicochemical conditions in the habitats and also, test animal properties including dominant feeding regime, gastrointestinal evacuation rate, diet formulation, initial weight, nutritional history and feeding period may help to interpret results.

The changes in the ratios of presumptive autochthonous lactic acid bacteria count to total viable heterotrophic aerobic bacteria count of hepatopancreas extracted from crayfish fed the experimental diets noted in the present study may have benefited the crayfish, possibly by increasing non-specific immune responses and by increasing concentrations and/or production of volatile fatty acids and butyrate as by-products of fermentation process in GIT. Additional research is being conducted to find mechanism(s) of immunomodulation between different synbiotics and immune system interactions in crayfish. Such studies explore applicable strategies to use different synbiotics in the diets in order to maximize the efficiency of digestion and absorption processes in GIT. Interestingly, crayfish fed the EGOS- diet had the highest values

for final weight (64.47 g), SGR (2.19% day^{-1}), VFI (2.75% BW day^{-1}), survival rate (96.67%), PER (3.27) and PPV (64.67) and the lowest values for FCR (2.33). One of the most important scenarios to diminish environmental impacts of aquaculture is to increase nutrient retention efficiency. In this regard, the broodstock selection and diet formulation (with emphasis on ideal protein, dietary energy levels or supplements) can be considered to increase nutrient retention (Dabrowski & Guderley, 2002; Safari et al., 2014a). It has been confirmed that SCFA can modulate lipid synthesis and maybe improve metabolic pathways related to nutrient retention (Marcil et al., 2002). Data from this study showed that there was a synergistic effect of *E. faecalis* + GOS included in the experimental diet on the growth performance and some biological indices. A likely explanation of this finding may be attributed to the structure–function relationship of the selected synbiotic to modulate beneficial microbiota of gastrointestinal tract (GIT) in aquatic species (Buentello et al., 2010; Safari et al., 2014a; Ye et al., 2011).

The mean of in vivo ADC$_{OM}$ (86.67%), ADC$_{CP}$ (94.67%) and ADC$_{CF}$ (92.00%) and ADC$_{GE}$ (88.67%) of crayfish fed the EGOS- diet was higher than those of fed control diet. The crayfish fed the EGOS- diet showed the highest presumptive autochthonous lactic acid bacteria count to total viable heterotrophic aerobic bacteria count ratio (67.58%), and the lowest ones was for crayfish fed the control diet (21.49%). As a result, data from the present study can confirm that *E. faecalis* + GOS via selective fermentation affected the composition of intestinal microflora by stimulating *Bifidobacteria* and *Lactobacilli*, which are present in the hepatopancreatic bacterial flora. Increment of in vivo ADCs could be likely regarded to upregulate the activities of specific digestive enzymes (Buentello et al., 2010; Ringø, Strøm, & Tabachek, 1995; Safari et al., 2014a). In the present study, the activities of digestive enzymes (alkaline protease, lipase and amylase) in crayfish fed EGOS- and EMOS- diets were higher than those of control. Feeding Japanese flounder (*Paralichthys olivaceus*) with the diet containing *B. clausii* + FOS + MOS increased the activities of digestive enzymes (protease and amylase) (Ye et al., 2011). The positive effects of synbiotics on growth performance may be associated with improved nutrient digestibility, which could be a result of a boosting of the digestive enzymes that would allow the host to degrade more nutrients (Cerezuela et al., 2011; Ringø et al., 1995; Safari et al., 2014a; Ye et al., 2011).

THC and different haemocyte counts (HC, SGC and LGC) have important roles in the health of shellfish species (Jussila, Jago, Tsvetnenko,

Dunstan, & Evans, 1997) including cytotoxicity and the storage and release of the prophenoloxidase system (Johansson et al., 2000). The juvenile crayfish fed the EGOS- and EMOS- diets showed the highest values of hemolymph indices (THC, HC, SGC and LGC), immunity (LYZ) and antioxidant enzymes (PO, SOD and NOS) after a 126-day feeding trial and also, a 48-hr bacterial exposure challenge. Dietary manipulations using feed additives (e.g., probiotics, prebiotics and synbiotics) can increase the animal resistance through pathogen inhibition pathways in GIT such as competition for territory in GIT, reduction in pH and release of natural antibiotics from beneficial microbial populations (Li et al., 2007; Manning & Gibson, 2004). Feeding black tiger shrimp, *Penaeus monodon* fed the diet containing a probiont bacterium increased the innate immune system (Rengpipat, Rukpratanporn, Piyatiratitivorakul, & Menasaveta, 2000). β-1,3-glucan increased haemocyte phagocytic activity and superoxide anion production and lowered clotting time in black tiger shrimp (Chang, Chen, Su, & Liao, 2000) and also promoted immune system against white spot syndrome virus (Chang, Su, Chen, & Liao, 2003). Feeding western king prawn (*Penaeus latisulcatus*) with test diets containing probiotics (*Pseudomonas synxantha* and *P. aeruginosa*) decreased HC proportion and increased SGC and THC than those of control diet (Hai, Buller, & Fotedar, 2009). In this trial, the incorporation of EGOS in diet may stimulate and enhance the proliferation rate of juvenile crayfish haemocytes to compensate for the loss of haemocytes due to *Aeromonas hydrophila* injection. Biological exposure challenge of blue crab, *Callinectes sapidus* (Johnson, 1976), and lobster, *Homarus americanus* (Stewart, Cornick, & Dingle, 1967), with the harmful bacteria decreased THC content. The decreasing trend in THCs of Chinese shrimp, *Fenneropenaeus chinensis*, and marron, *Cherax tenuimanus*, after injection with *Vibrio anguillarum* and *V. mimicus*, respectively, was reported (Sang et al., 2009; Thaithongnum, Ratanama, Weeradechapol, Sukhoom, & Vuddhakul, 2006). Hemolymph indices of juvenile crayfish fed the EGOS- diet did not decrease after the *A. hydrophila* injection. The survival rate of juvenile crayfish fed the EGOS- diet after injection was higher than that of control diet. Decapods species (shrimp, prawn and crayfish) only rely on innate immune responses against microbial invasion (Zhang et al., 2011). Defence activities could be started via hemolymph migration to the injection site and lysis of haemocyte (Sang et al., 2009). Injection of kuruma prawns with a lipopolysaccharide increased the haemocyte proliferation rate three times (Sequeira, Tavares, & Arala-Chaves, 1996). In this regard, life history, food intake, disease outbreaks, pollutants and environmental stress can affect on the quantity and quality of circulating haemocyte count (Sang et al., 2009). Finally, with the aim of sustainable crayfish culture, higher survival rate of injected crayfish fed the diets containing EGOS (56%) and EMOS (35.3%) confirmed the positive effects of synbiotics. However, further studies need to determine the best synbiotic, dose, feed formulation and manufacturing.

5 | CONCLUSION

In the current trial, juvenile crayfish fed the diet containing 7.86 log CFU *E. faecalis* g^{-1} + 10 g kg^{-1} GOS (EGOS) exhibited high growth

indices including final weight (64.47 g), SGR (2.19% day^{-1}) and survival rate (96.67%) and highest values for PER (3.27) and PPV (64.67%). The juvenile crayfish fed the EGOS- diet showed higher hemolymph indices (THC, HC, SGC and LGC) than those of fed the control diet. The results indicated that EGOS had the capacity to accelerate juvenile crayfish immune response of *Astacus leptodactylus leptodactylus* against *Aeromonas hydrophila* injection.

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