Microencapsulation of Lactobacillus plantarum in the Alginate/Chitosan improves immunity, disease resistance, and growth of Nile tilapia (Oreochromis niloticus)

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Abstract

This study evaluated the effects of microencapsulation of L. plantarum (as a probiotic) with chitosan/alginate biopolymers (MLCA) on innate immune response, disease resistance, and growth performance of Nile tilapia (Oreochromis niloticus). Four hundred and eighty fish were randomly distributed in glass tanks (150 L) and fed with diets including Diet 1: Control; Diet 2: 10 g kg-1 microcapsules; Diet 3: 108 CFU g-1 L. plantarum ; and Diet 4: 10 g kg-1 MLCA for 60 days. The hematology and biochemical indices, lysozyme activity, alternative complement activities, respiratory burst, serum bactericidal activity as well as growth performance parameters (specific growth rate, feed conversion ratio) were assayed. White blood cells, plasma protein and globulin concentration, serum lysozyme, and respiratory burst activities of fish were significantly increased in the MLCA diet. A challenge test against Streptococcus agalactiae , at the end of the experiment, showed the highest survival rate of the fish fed with MLCA. Moreover, the fish fed with MLCA showed a significant improvement in SGR and FCR and had the highest growth performance. These results suggest longer stability of probiotics in the microcapsules, and their immunomodulatory effect can be considered a promising immunostimulant and growth enhancer in the Nile tilapia diet.

1. Introduction

The increasing world population and benefits of aquaculture products for human health caused the aquaculture industry to be one of the most viable and promising human food sources (Mohapatra et al. 2012; Van Alfen 2014). In aquaculture systems, a limited volume of water is used to achieve maximum productivity (Faggio et al. 2014). Therefore, intensive aquaculture has to fight many challenges such as the degradation of the water environment, the outbreak of diseases, and the significant economic losses (Carbone and Faggio 2016; Van Doan et al. 2016a). One of the most important obstacles to the development of aquaculture, which causes a lot of economic loss, is infectious diseases (Van Doan et al. 2016a). The widespread use of vaccination and antibiotic substances in the aquaculture sector to control these problems has not only led to the development of antibiotic-resistant bacterial strains but also posed adverse impacts on the environment and human health (Ai et al. 2011). Therefore, to mitigate these problems in the intensive aquaculture systems, health-promoting and growth-enhancing feed additives such as probiotics have been developed (Hoseinifar et al. 2018). Probiotics are viable cells or dead, or even a component of the microorganism that have beneficial effects on the host health (Okey et al. 2018).

Probiotics can act as promoting factors for the growth and immune response and inhibiting factors for pathogenic microorganisms and have an anti-mutagenic and anti-carcinogenic activity (Dawood et al. 2019). In aquaculture, common probiotics that are used in fish feeding include *Enterococcus*, *Lactobacillus*, *Bacillus*, and *Saccharomyces* (Ganguly et al. 2013). *Lactobacillus spp* found in the human's and animals' gut microbiota have been widely used in aquaculture as feed supplements and probiotics (Van Doan et al. 2020). Dietary administration of *Lactobacillus spp* can enhance the growth and innate immune responses, and suppress the growth of pathogens by releasing bacteriocin (Beck et al. 2015; Dawood et al. 2015). *Lactobacillus plantarum* is a potential probiotic candidate and one of the functional additives for fish that can produce antimicrobial substances like plantaricin against certain pathogens (Banerjee and Ray 2017). Although some beneficial effects of this probiotic bacteria in aquaculture are documented (Banerjee and Ray 2017), the problem of its stability during processing and gastrointestinal transit remains a concern. Adopting improved methods to enhance the survival and stability of *L. plantarum* (e.g., their protection in biopolymeric structures) can increase the delivery of viable bacteria in the fish intestine. Incorporation of chitosan in alginate is one of the best methods to improve encapsulation of various species of *Lactobacillus* (Krasaekoopt et al. 2004). Unique properties of alginate such as simplicity, nontoxicity, biocompatibility, biodegradability, pH-sensitive profile, and low cost caused this biopolymer to be one of the most frequently used biopolymers for encapsulating the cells and bioactive compounds (Anal and Singh 2007; Rokka and Rantamäki 2010). Sodium alginate obtained from the brown algae is a salt of alginic acid (Aprilliza 2017). This biopolymer has antibacterial and antioxidant properties with beneficial effects on innate immune response and growth performance in fish (Falkeborg et al. 2014; Hu et al. 2005; Van Doan et al. 2016b). To the best of our knowledge, there is no available information about the microencapsulation of *L. plantarum* with chitosan/ alginate biopolymers in fish culture. Therefore, the present study aimed to investigate effects of microencapsulation of *L. plantarum* with chitosan/alginate on growth performance, innate immune responses, and disease resistance of the Nile tilapia (*Oreochromisniloticus*) fingerlings.

2. Materials and methods

2.1. Bacterial strains

The *L. plantarum* strain used in this study was primarily identified based on the colony and cell morphology, gram staining, biochemical characteristics, and 16S rRNA gene sequencing (GenBank accession number EU520326) (Mohammadian et al. 2016). This strain was grown for 30 h at 37 ° C in MRS broth (BD Difco, Sparks, MD, USA).

2.2. Microencapsulation of *L. plantarum* and properties

Microencapsulation of *L. plantarum* with chitosan/alginate (MLCA) was done according to the extrusion method (Jiang et al. 2013). Briefly, the mixture of *L.plantarum* (10^8 CFU g^{-1}), sodium alginate, and 15% (v/v) glycerol was dropped into 0.1 M $CaCl_2$ by passing through a cannula-like syringe in the presence of nitrogen gas pressure. The sodium alginate final concentration was 2% (w/v). Formed microcapsules were incubated for 30 min, then washed with 0.85% saline to remove unreacted $CaCl_2$. The chitosan (MW 10,000) solution 0.8% (w/v) was used to coat microcapsules for 30 min followed by two times washing. The microcapsules coated with chitosan-alginate were further coated with 0.1% (w/v) sodium alginate for 10 min followed by washing. Then microcapsules were stored at −75 ° C for 6 h and lyophilized for 18 h. The control microcapsules without bacteria were also prepared by the same procedure. The morphologic observation and size measurement of MLAC were performed by scanning electron microscopy(SEM) (Philips XL 20, Oregon, USA). The viability of free and encapsulated bacteria in simulated small intestinal fluid (SIF) was measured by the counting method for 0, 0.5, 1, 2, 4, 8, 12, and 24 h.

2.3. Diet preparation

In the present study, the used basal diet was modified based on the work of Van Doan et al (Van Doan et al. 2016a). Preparation of experimental diets by the inclusion of different additives was as the following: Diet 1 (Control), 10 g kg^{-1} microcapsules (Diet 2), 10^8 CFU g^{-1} *L. plantarum* (Diet 3), and 10 g kg^{-1} MLCA (Diet 4) (Table 1). The experimental diets were milled into powder and were thoroughly mixed with soybean oil, and then water was added to produce stiff dough. Then, the doughs were ground to form spaghetti-like pellets. The pallets were dried in the oven at 50 ° C until moisture levels were around 10% and stored at 4 ° C until use. To ensure high probiotic levels in the diet, fresh diets were prepared at 30 days intervals.

Table 1. Experimental diet formulation and proximate composition.

Components	Diet 1(g kg^{-1})	Diet 2(g kg^{-1})	Diet 3(g kg^{-1})	Diet 4(g kg^{-1})
Fish meal	300	300	300	300
Rice bran	240	240	240	240
Soybean meal	200	200	200	200
Corn meal	125	125	125	125
Wheat flour	60	60	60	60
Soybean oil	30	30	30	30
Cellulose	30	20	30	20
Premix	10	10	10	10
Vitamin and trace mineral mix	5	5	5	5
L.plantarum (CFU g^{-1})	0	0	10^8	0
MLCA[a]	0	0	0	10
Microencapsules	0	10	0	0
Proximate composition of the experimental diets(g.kg^{-1}dry matter basis)				
Gross energy(cal g-1)	4387	4419	4387	4419
Dry matter	931	9313	931	931.3
Crude Protein	353.1	353.6	353.1	353.6
Ash	114.4	115.2	114.4	115.2
Crude Lipid	100.5	99	100.5	99
Fibre	63.8	60.6	63.8	60.6

[a]:MLCA: microencapsulation of *L. plantarum* with chitosan / alginate

2.4. Experimental design

The healthy Nile tilapia (*Oreochromis niloticus*) fingerlings (n=480,) that had no previous history of parasitic infections and no signs of disease (gross and microscopic examination of gills, skin, and kidney tissues of representative samples) were obtained from Yazd fish farm, Yazd, Iran. The fish were transferred to the laboratory of the Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Iran and acclimatized to laboratory conditions for 2 weeks in 500-L plastic quarantine tanks at 27 ± 2 ° C and fed with the control diet. Then, the fish with a similar size (15.56 ± 0.02 g) were randomly divided into 150 L tanks for each diet treatment group. The experiment was laid out in a Completely Randomized Design with four replications. In each tank, approximately 30% of the water was exchanged daily, and 100% of the water was exchanged once a week. The fish *ad libitum* was twice a day at 7:00 a.m. and 6:00 p.m and diets were hand-fed to the fish. Measurement of basic Physico-chemical parameters of the water was done every week. The O_2 concentration was maintained at no less than 5 mg L^{-1} and pH ranged from 7.5 to 8.2 throughout the study period.

2.5. Blood and serum sample collection

Sampling was performed on day 30 and day 60 after probiotic feeding and a total of 12 fish (3 fish from each treatment diet) were randomly collected per treatment for immunological assays. After anesthetizing the fish with clove powder (200 ppm, 20 min), blood was collected from the caudal vein using a 1 mL syringe. Then the blood samples were transferred into Eppendorf tubes without anticoagulant and incubated at room temperature for 1 h and 4 ° C for 4 h. After coagulation, blood was centrifuged (2000 g, 10 min, 4 ° C), and serums were collected and stored at -20 ° C until used.

2.5.1. Leukocyte isolation

The peripheral blood leukocytes were isolated by the method modified by Chung & Secombes (Chung and Secombes 1988). First, 1 mL of blood samples was diluted with 2 mL of RPMI 1640 (Sigma-UK). Then, 3 mL of Histopaque (Sigma-UK) was carefully laid onto a 15 ml tube and centrifuged at 500 g for 20 min at room temperature. After that, a white buffy coat of leukocyte cells was carefully aspirated with a Pasteur pipette. The isolated leukocytes were washed twice with phosphate buffer solution (PBS, pH 7.4) using centrifugation at 250 g for 10 min. For assaying respiratory activities, the leukocytes were resuspended in the PBS and adjusted to the required cell numbers.

2.6. Hematology and biochemical indices

Determination of hematocrit values in the blood was performed by calibrating the centrifuged hematocrit pipettes, and the percentage of blood cells in the total volume of blood was expressed (Brown 1988). The differential leukocyte count was done based on Schäperclaus et al method (Schäperclaus et al.1992). Total protein and albumin (ALB) concentrations in serum were measured using a commercial kit (Zist Shimi kits) according to the manufacturer's instructions. Globulin (GLO) was calculated by subtracting albumin values from total protein values. The ratio of albumin-globulin was calculated by dividing albumin values by globulin values.

2.7. Immunological measurements

2.7.1. Lysozyme activity assay

Serum lysozyme activity was determined according to the method described by Parry et al. The standard was compared with the equivalent unit of the activity of the sample and reported in mg ml^{-1} serum.

2.7.2. Alternative complement pathway activity assay

Complement pathway activity (ACH_{50}) was assayed based on the Yano et al method (Yano et al. 1988). Briefly, 50 µL of activated and inactivated serums were poured separately into sterile microtubes at different times, followed by 350 µL of PBS containing Ca2+ and Mg2+. Then, 5% washed rabbit red blood cells (100 µL) were added to each microtube and centrifuged at 200 g for 5 min. The supernatant of each microtube (100 µL) was collected and poured into a plate of 96 microplate chambers in an active serum column. In the side column at a certain time and the inactive serum was poured into the pits at the same time and read at 450 nm.

2.7.3. Respiratory burst activity

The respiratory burst activity of leucocytes was determined using the Secombes method with slight modification (Secombes 1990). The leukocyte samples (175 µL ,6Í10^6cells ml^{-1}) were added to the wells of 96-well microtiter plates. Then 25 µL of Nitro Blue Tetrazolium (NBT) (1 mg ml^{-1} concentration) was added to the wells and incubated at 25 ° C for 2 h. The supernatant was carefully discarded and 125 ml of 100% methanol was added to each well. After that, the supernatant was removed and each well washed again twice using 125 ml of 70% methanol. The supernatant of wells was carefully discarded and the plate was dried at room temperature for 30 min. Then 125 ml of 2 N KOH followed by 150 mL of DMSO were added to the wells. The spontaneous O_2^- production was calculated based on the following formula:

The spontaneous O_2^-= (Absorbance NBT reduction of the sample) - (Absorbance of blank)

2.7.4. Bactericidal activity of serum

The serum bactericidal activity was measured according to Yin et al (Yin et al. 1996), with some modifications. The bacteria culture (*Streptococcus iniae*) was pelleted (3000 g, 10 min) and washed 3 times with sterile PBS. A volume of 25 µL bacterial suspension (adjusted to 4×10^9 cells/ml) was added to 25 µL serum of fish in sterile Eppendorf tubes. Then, the tube was incubated at room temperature for 1 h. After that, plating the mixtures on TSA containing 1.5% NaCl was used to determine colony forming units (CFU)/ml.

2.7.5. Challenge study

Streptococcus agalactiae was purchased from the Pasteur Institute of Iran (IPI). The bacterium was cultured in Tryptic Soy Broth and incubated overnight in the rotation shaker at 110 rpm and 37° C. After obtaining sub-culture from the stock, 5 mL of stock was transferred into a 50 mL flask containing Tryptic Soy Broth and incubated at 37 ° C for 24 h. Duplicate sub-cultures were used under

similar conditions for the experiment. The optical density of 560 nm and plate counting in Tryptic Soy Agar was used for growth evaluation. The LD_{50} was determined based on Wang et al study (Wang et al. 2016). In the present study, 0.1 mL of 10^7 CFU ml^{-1} of *S. agalactiae* in normal saline solution (0.85%) was injected intraperitoneally into the healthy fish (n=20) from each treatment at the end of the experiment. Dead fish from the challenge tanks was daily removed and recorded and the bacterium was reisolated by culturing on TSA plates for 24 h at 27 ° C. *S. agalactiae* was confirmed by morphologically and biochemically tests of inoculated strain. After 15 days of the challenged test, the relative percentage of survival (RPS) was calculated:

RPS = 100 –(test mortality/control mortality)× 100

2.8. Growth performance

At the end of the feeding trial, 10 randomly selected fish in each replication were weighed on day 0, day 30, and day 60. The survival rate and growth performance of fish were calculated using the following equations:

Weight gain (WG)= final weight (g) - initial weight (g)

Specific growth rate (SGR%) = 100 - (ln final weight - ln initial weight)/Duration of experiment

Feed conversion ratio (FCR) = feed given (dried weight)/ weight gain (wet weight)

Survival rate (%) = (final fish number/ initial fish number) -100.

2.9. Statistical analysis

Before statistical analysis of data, their normality was determined using the Kolmogorov-Smirnov test. One-way and two-way ANOVA with Multiple Comparisons Test was used to compare the different groups, followed by Tukey's test ($P < 0.05$) and quantitative data were presented as mean ± standard deviation. All statistical analyses were performed using SPSS software (Version 24).

3. Results

Observation of microcapsulees indicated that the morphologies of empty microencapsulates and MLCA were as spherical shapes (Figure 1). There were no significant differences in the average sizes between empty microencapsules and MLCA ($P>0.05$).

3.1. Hematology and biochemical indices

No significant effects of different dietary supplementation on hemoglobin and hematocrit and red blood cells were observed compared to the control group ($P>0.05$)(Table 2). Whereas a significant increase in white blood cells after being fed with diet 4 (MLCA) was observed compared to the control diet ($p<0.05$)(Table 2).

There were no significant differences between albumin concentrations in fish under different diets ($P>0.05$)(Figure 2). Plasma protein concentration in fish fed with diet 3 (10^8 CFU g^{-1} L. Plantarum) and diet 4 (MLCA) significantly increased ($P<0.05$)(Figure 2). The plasma globulin concentration and albumin/globulin ratio in fish fed with diet 4 (MLCA) significantly increased and decreased, respectively($P<0.05$)(Figure 2).

Table 2. Hematological characteristics of the Nile tilapia after feeding with Control (Diet 1), 10 g kg^{-1} microencapsules (Diet 2), 10^8 CFU g^{-1} L. plantarum (Diet 3), and 10 g kg^{-1} MLCA (Diet 4). Values are presented as the mean ± SD.

Parameters	Diet .1		Diet .2		Diet .3		Diet .4	
	Day 30	Day 60	Day 30	Day 60	Day 30	Day 60	Day 30	Day 60
Red Blood Cells (N/mm3)	1.5±0.2a	1.5±0.3 a	1.56±0.39a	1.52±0.12a	1.51±0.1a	1.54±0.3a	1.5±0.2a	1.55±0.4a
White Blood Cells(N/mm3)	16.1±1.5a	15.79±1.1a	15.68±2.7a	16.0±2.7a	15.9±2.7a	16.1±1.1a	17.9±1.3b	19.5±1.8b
Hemoglobin (%)	4.38±0.3a	4.48±0.3a	4.6±0.17a	4.55±0.2a	4.61±0.3a	4.49±0.1a	4.51±0.4a	4.51±0.3a
Hematocrit (%)	29.81±0.15a	30.1±0.1a	29.78±0.5a	29.84±0.4a	30.0±0.6a	30.1±0.3a	29. 9±0.5a	29.87±0.7a

Data assigned with different letters denote a significant difference in a row ($P < 0.05$).

3.2. Immune response

Dietary administration of MLCA significantly increased serum lysozyme activity and respiratory burst compared to the control ($P < 0.05$)(Figure 3). No significant differences in complement pathway activity (ACH50) and bactericidal activity of serum between different diets were observed ($P > 0.05$)(Figure 3).

3.3. Challenge test

The result of the challenge test indicated that the MLCA had the highest significant increase in the protection of tilapia, *O. niloticus* against *S. agalactiae* infection compared to the control (Figure 4). Dead fish were observed in the control group after 2 days post-challenge, while the dead fish in supplemented groups were observed respectively in diet 2 on 3th day, in diet 3, and diet 4 on day 4^{th} of infection. Dead fish revealed symptoms of *streptococcosis* such as darkness, exophthalmia, loss of appetite, pale liver, and pair-fins basal hemorrhage.

3.4. Growth performance

It was observed that fish fed with 108 CFU g-1 L. plantarum (Diet 3) and 10 g kg-1 MLCA (Diet 4) had a significantly higher final weight (FW), WG, SGR, and lower FCR than the control after 30 and 60 days of the feeding trial ($P < 0.05$) (Table 3). However, no significant difference in growth performance between the control diet and 10 g kg-1 microencapsules was observed ($P >0.05$) (Table 3).

Table 3. Growth performances and feed utilization of the Nile tilapia after feeding with Control (Diet 1), 10 g kg^{-1} microencapsules (Diet 2), 10^8 CFU g^{-1} L. plantarum (Diet 3), and 10 g kg^{-1} MLCA (Diet 4) . Values are presented as the mean ± SD

Days	Parameters	Diet 1	Diet 2	Diet 3	Diet 4
30	initial weight (g)	17.20±1.11 a	17.23±2.01 a	17.14±1.02 a	17.11±1.00 a
	Final weight (g)	53.67±8.05 a	53.94±6.65 a	60.25±3.01 b	68.25±1.05 c
	Weight gain (g)	36.47±4.21 a	36.71±2.11 a	43.11±1.3 b	51.14±0.9 c
	SGR (%)	1.21±0.3 a	1.19±0.2a	1.71±0.1 b	1.95±0. 2 c
	FCR	0.75±0.001 a	0.42±0.003 b	0.37±0.007 b	0.28±0.001 c
	Survivalrate (%)	100	100	100	100
60					
	initial weight (g)	17.20±1.11 a	17.23±2.01 a	17.14±1.02 a	17.11±1.00 a
	Final weight (g)	75.36±6.39 a	75.28±6.65 a	83.00±3.01 b	86.28±1.21 c
	Weight gain (g)	58.16±2.01 a	58.05±2.00 a	65.86±1.02 b	69.17±0.9 c
	SGR (%)	1. 82±0.01 a	2.33±0.3 b	2.28±0.21 b	3.12±0.18 c
	FCR	1.71±0.01 a	1.74±0.21 a	1.31±0.2 b	1.23±0.2 c
	Survivalrate (%)	100	100	100	100

Data assigned with different letters denote a significant difference in a row ($P < 0.05$).

4. Discussion

In the aquaculture industry, dietary administration of probiotics as immunostimulants has been used as an alternative strategy to control the disease (Carbone and Faggio 2016; Van Doan et al. 2016a). Live bacteria in probiotics can induce the immune system and act as alternatives to antibiotics and chemicals (Van Doan et al. 2016a). Probiotics have been proved to improve feed efficiency, and growth performance of fish and shellfish (Dawood and Koshio 2016). The present study was conducted to evaluate the effect of microencapsulation of *L. plantarum* with chitosan/alginate on growth performance and the health effects of Nile tilapia. The results indicated that the number of white blood cells among hematological parameters was significantly increased compared to other diets after feeding fish with *L. plantarum* microencapsulated with chitosan/alginate (MLCA). One of the most pathophysiological reflectors of the entire body is blood and the counts of hematological parameters can indicate the health status of fish by determining any abnormality occurring owing to the use of immunostimulants (Tewary and Patra 2011). The white blood cells are important in both innate and adaptive immune responses and the increasing abundance of these cells reflects an induced immune system (Opiyo et al. 2019). Other fish species(e.g., catfish (*Clarias macrocephalus*)(Hien et al. 2021) and Siberian Sturgeon (*Acipenser baerii*) (Pourgholam et al. 2017)) fed with *L. plantarum* also showed an increase in white blood cells, similar to the present study. Since the alginate coating technique enhances the stability and viability of microencapsulated *L. plantarum*, it seems that chitosan/alginate coatings probably reduced the porosity and decreased the leakage of encapsulated probiotics from microcapsules, and increased their stability in the fish intestinal system (Rather et al. 2017). Therefore, the MLCA diet has more effective than feeding with free *L. plantarum* diet.

In this study, plasma protein concentration in fish fed with diet 3 (10^8 CFU g^{-1} *L. plantarum*) and diet 4 (MLCA) was significantly higher than in the control diet. In addition, plasma globulin concentration and albumin/globulin ratio in fish fed with diet 4 (MLCA) were increased and decreased, respectively, compared to the control diet. Some previous studies in African Catfish (*Clarias gariepinus*)(Al-Dohail et al. 2009) and in rainbow trout (*Oncorhynchus mykiss*)(Panigrahi et al. 2010) reported similar results after feeding with lactobacillus bacteria. It seems that encapsulated probiotics can increase humoral components of the non-specific defense mechanism in fish via the increase in total blood protein and globulin (Gerwick et al. 2002; Shu and Gill 2002). These proteins in fish blood after being fed with MLCA include the stimulation of immunoglobulin A, various nutrients, neo nutrients, growth and clotting factors, and production of antioxidants (Gerwick et al. 2002). The increase in total plasma proteins can be an adaptive response that contributes to regaining homeostasis after tissue injury or infection in fish (Panigrahi et al. 2010).

In this study, enhanced serum lysozyme activity and respiratory burst were observed after fish feeding with MLCA. In the fish immune system, lysozyme is an important component. This enzyme has bactericidal activity via hydrolyzing bacterial cell wall peptidoglycans and is also known to act as opsonin (Magnadóttir 2006). Probably, probiotic stability in microcapsules more induces lysozyme activity than in other diets. It is confirmed that probiotics can modulate the innate and humoral responses and stimulate host resistance and then facilitate the exclusion of potential pathogens (Hoseinifar et al. 2015a). It is assumed that when probiotics enter the fish body, immunosaccharides can directly activate the non-specific immune system via contact with PRRs, such as b-glucan or dentin-1, expressed on macrophage receptors (Brown et al. 2002). These ligand-receptor interactions can activate signal transduction molecules, such as NF-kB, activating immune cells and respiratory bursts in these cells (Yadav and Schorey 2006). In addition, the molecular patterns of bacteria, such as teichoic acid, glycosylated protein, peptidoglycan, and the capsular polysaccharide can be detected by immune cells and thus inspire the immune response (Song et al. 2014). Therefore, microencapsulated probiotics can improve immune system activity in two different ways. They may directly stimulate the non-specific immune system or growth stimulation of beneficial bacteria (Hoseinifar et al. 2015a). The MLCA diet used in the present study may have the same mechanisms as the above-mentioned.

In this study, the fish fed with the MLCA diet showed higher survival compared to other diets during the pathogen challenge test. One of the effective ways for enhancing disease resistance in fish based on recent studies is nutritional manipulation (Hoseinifar et al. 2015b). The fish fed with the MLCA diet in this study showed higher survival compared to other diets during the pathogen challenge test. This result indicated that dietary administration of probiotics and MLCA in *O. niloticus* significantly increased resistance to *Streptococcus agalactiae* . These results were in agreement with previous studies on freshwater fish, *Labeo rohita* (Giri et al. 2013), and triangular bream, *Megalobrama terminalis* (Son et al. 2009), and in contrast with the study on gilthead sea bream, *Sparus aurata* (Cerezuela et al. 2012). The improvement of the survival rate may be a result of innate immune defense activation in fish via both chitosan/alginate microcapsule and *L. plantarum* as probiotics. The ability of *L. plantarum* to produce antimicrobial substances like plantaricin and the presence of cell wall components, such as surface-bound proteins, lipoteichoic acid, and peptidoglycans, play critical roles in the prevention and treatment of intestinal inflammatory diseases (Baik et al. 2015; Liu et al. 2016). *L. plantarum* can modify the composition of the intestinal microbiota, thereby protecting against pathogenic microorganisms (Gao et al. 2016). In addition, stimulator effects of sodium alginate from seaweed to improve fish immunity response and pathogen resistance have been well documented (Yeh et al. 2008).

The fish fed with MLCA and fish fed with free *L. plantarum* showed a significant improvement in specific growth rate (SGR), weight gain (WG), and feed conversion ratio (FCR) compared with the control. Similarly, significant improvements in these growth parameters by administration of probiotics and prebiotics have been reported in previous studies on gilthead seabream, *Sparus aurata* (Guzmán-Villanueva et al. 2014), Nile tilapia, *Oreochromis niloticus* (Hassaan et al. 2014; Van Doan et al. 2016b) and snakehead, *Channa striata* (Talpur et al. 2014). Some previous documents showed that Lactobacilli as probiotics could produce short-chain fatty acids (SCFAs) (as a by-product of carbohydrate metabolism) in the fish digestive tract. Intestinal epithelial cells can use these SCFAs as major sources of energy and hence possibly play a critical role in increasing the villi height of the digestive tract, which can provide more absorptive surface area and improve nutrient absorption (Pirarat et al. 2011). In addition, Lactobacilli can improve growth performance via modulating the transcription of genes that have an important role in vital tissue differentiation and maturation (Avella et al. 2012; Gioacchini et al. 2012). In contrast, in shrimp and carp fed with prebiotic or probiotic diets, no significant effects on the SGR and FCR were observed (Van Hai and Fotedar 2009). Instability of selected probiotics in the gut and incorrect selection of associated prebiotics as substrate can be reasons for growth inefficiency (Hoseinifar et al. 2015b; Wang et al. 2018). The stability and increased survival of probiotics in the microcapsules of chitosan is due to alginate. On the one hand, the bioactive peptides of microcapsules can stimulate digestion process in the intestine and consequently increase growth rates (Van Doan et al. 2016a). On the other hand, the protection of probiotics in the MLCA can improve microbial balance, which in turn enhances nutrient absorption and utilization (Lara-Flores et al. 2003).

5. Conclusion

From the results of this study, it is logical to conclude that the microencapsulation of *L. plantarum* with chitosan/alginate biopolymers in the Nile tilapia diet can improve innate immunity, protection against infection, and growth. The longer stability of probiotics in the microcapsules and their immunomodulatory effect may make the safe and effective method for oral delivery for various probiotic supplements in aquaculture research and the fish farming industry. However, a more accrued study in the future is needed to discover relationships and mechanisms of action of MLCA on immune response and disease resistance.

Declarations

Ethical approval

All handling of fish was carried out following the guidelings for control and supervision of experiments on animals by the Government of Iran and approved by Institutional Animal Ethics Committee.

Conflict of interest

The authors declare no competing interests.

Code availability

Note applicable

Consent for publication

Not applicable

Data availability

Not applicable

Consent to participate

Not applicable

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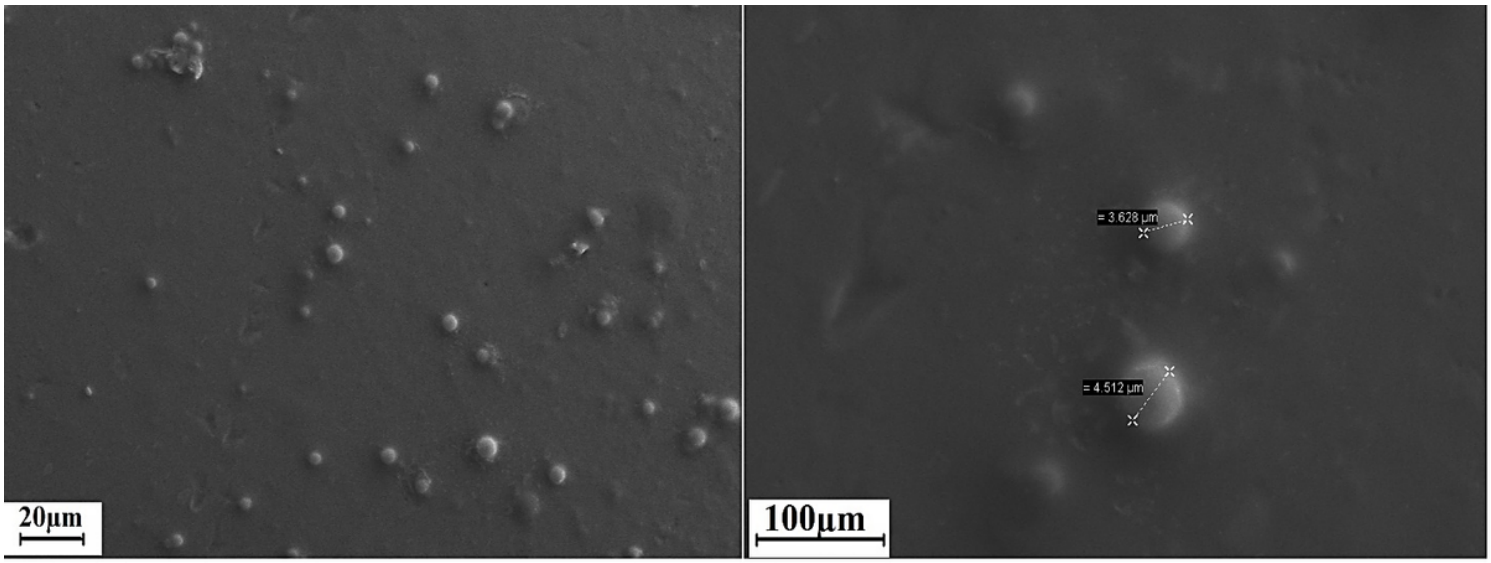
Figures



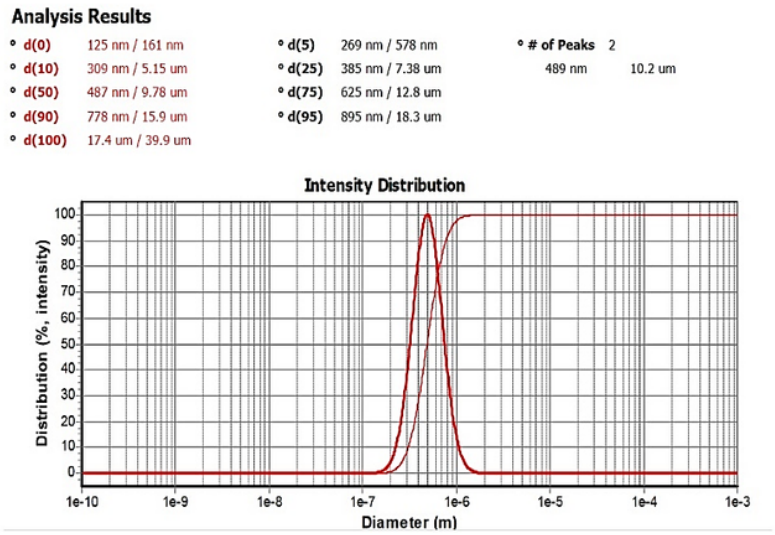


Figure 1

Analysis and Morphologies of empty microencapsules and MLCA by SEM.

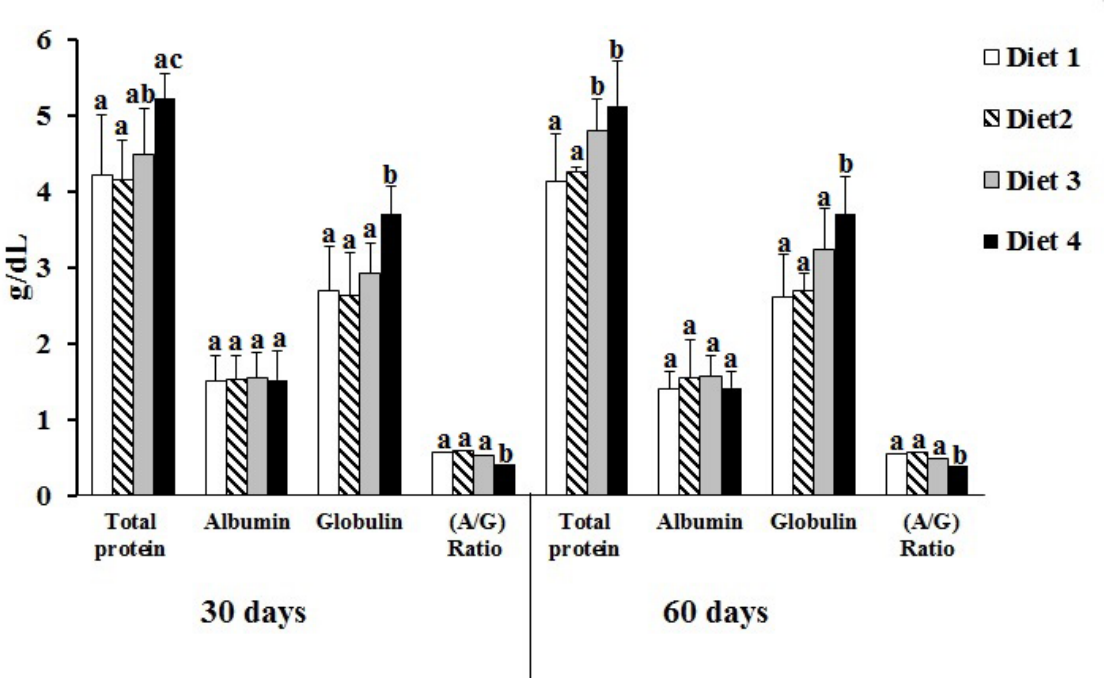


Figure 2

Serum total protein, albumin, globulin, and albumin/globulin ratio of *O. niloticus* , after feeding with Control (Diet 1), 10 g kg^{-1} microencapsules (Diet 2), 10^8 CFU g^{-1} L. plantarum (Diet 3) and 10 g kg^{-1} MLCA (Diet 4) . Values (means ±SD) with different letters in the same body index were significantly different at $P < 0.05$.

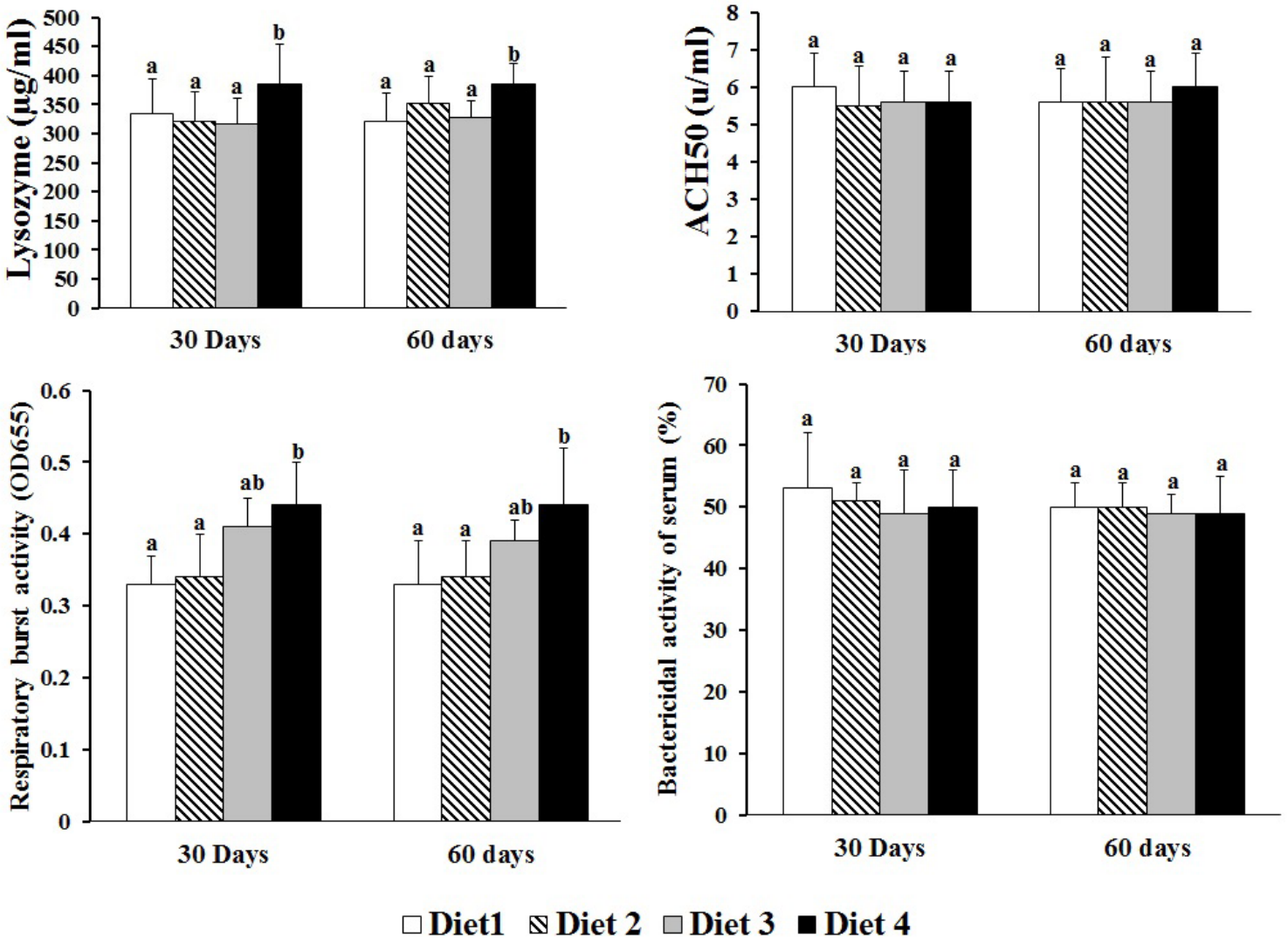


Figure 3

Serum lysozyme activity, alternative complement activity (ACH50), respiratory burst activity, and bactericidal activity of serum of the Nile tilapia, *O. niloticus* fed with different diets Control (Diet 1), 10 g kg^{-1} microencapsules (Diet 2), 10^8 CFU g^{-1} L. plantarum (Diet 3) and 10 g kg^{-1} MLCA (Diet 4) . Values (means ±SD) with different letters in the same body index were significantly different at $P < 0.05$.

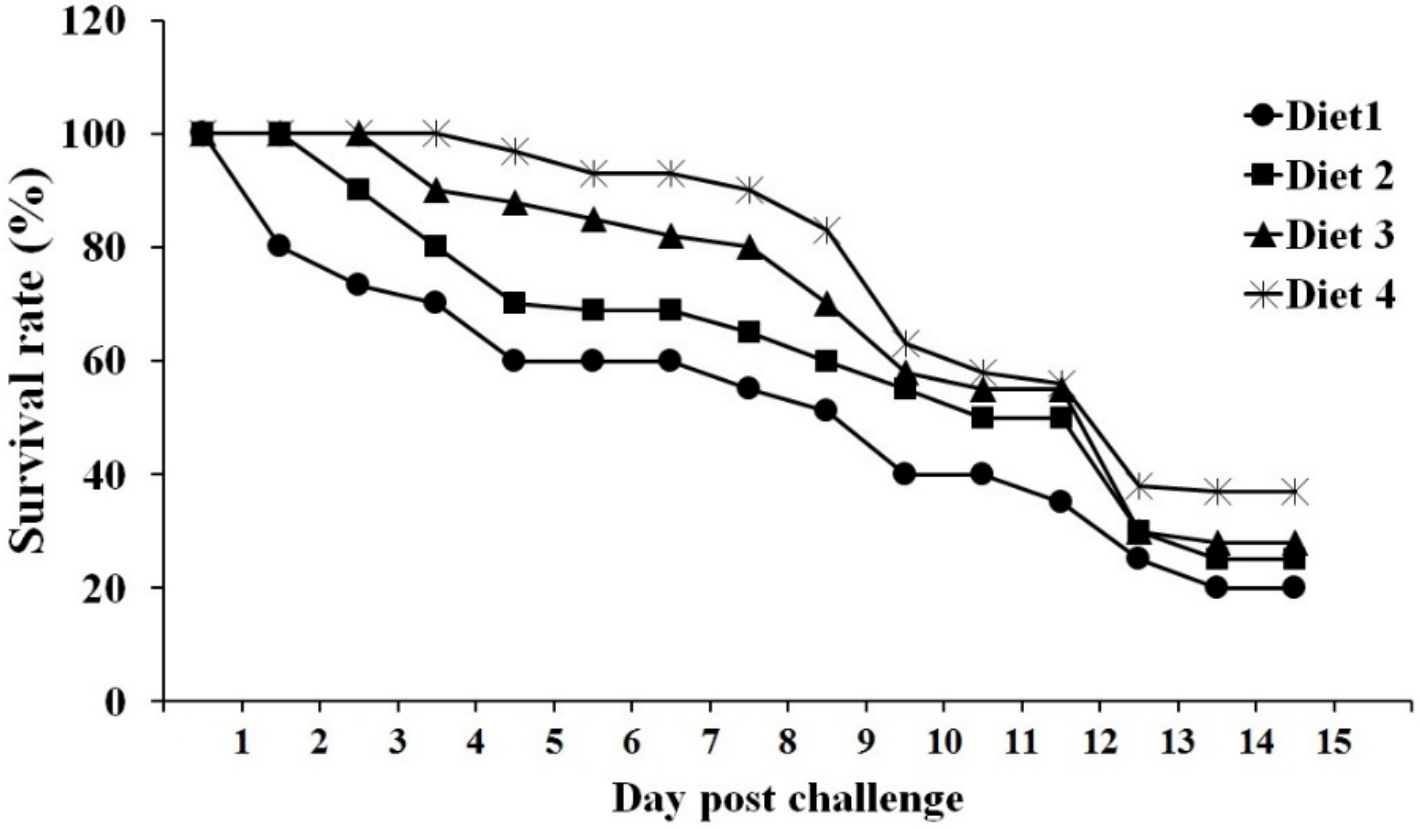


Figure 4

The survival rate of tilapia, *O. niloticus* fed with different diets including Control (Diet 1), 10 g kg^{-1} microencapsules (Diet 2), 10^8 CFU g^{-1} L. plantarum (Diet 3), and 10 g kg^{-1} MLCA (Diet 4).