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Efficacy of synbiotic Jerusalem artichoke and *Lactobacillus rhamnosus* GG-supplemented diets on growth performance, serum biochemical parameters, intestinal morphology, immune parameters and protection against *Aeromonas veronii* in juvenile red tilapia *(Oreochromis* spp.)

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ABSTRACT

Synbiotics, a synergistic combination of probiotics and prebiotics, are currently regarded as one of the most practical nutritional supplements in tilapia farms. In this study, the effect of supplementing the diet of red tilapia *(Oreochromis* spp.) with Jerusalem artichoke (*Helianthus tuberosus*) and *Lactobacillus rhamnosus* GG (LGG) was evaluated. Growth performance, serum biochemical parameters, intestinal morphology, goblet cell counts, immune parameters and protection against *Aeromonas veronii* challenge were determined. The results showed that fish fed with synbiotic-supplemented diets had a significantly higher ($P < 0.05$) feed conversion ratio (FCR), specific growth rate (SGR), and average daily gain (ADG) than fish fed with a control diet. The synbiotic-supplemented diet increased glucose, total protein and the total cholesterol levels. The absorptive area of the proximal and distal intestine of fish fed on the synbiotic diet was significantly higher ($P < 0.05$) than in those fed with probiotics (LGG), prebiotic-supplemented diets (JA), and the control diet. Goblet cell counts revealed that the numbers of acid mucous cells, neutral mucous cells and double-staining mucous cells of fish fed the synbiotic-supplemented diet (JA + LGG) were significantly higher ($P < 0.05$) in the proximal and distal intestine. Fish fed the synbiotic-supplemented diets also exhibited significantly higher ($P < 0.05$) lysozyme activity. The cumulative mortalities of fish fed with a synbiotic-supplemented diet were significantly lower than those of fish fed other diets. The results suggested the beneficial effect of JA and LGG synbiotic diet on growth performance and health status of red tilapia. Direct administration of JA and LGG in fish feed can be used as a practical nutritional supplement in red tilapia.

1. Introduction

Red tilapia *(Oreochromis* spp.) is an important aquaculture species in Thailand. It is a popular source of protein worldwide, due to its attractive appearance, good flavor, fast growth and the ease with which it can be cultured [1]. Infectious diseases commonly occur in intensive red tilapia farming operations, and these diseases are one of the major causes of economic losses in tilapia culture [2]. Among bacterial diseases, the genus *Aeromonas* including *A. veronii, A. hydrophila,* and *A. bestiarum* has been related to septicemic conditions in various fish species including red tilapia. *Aeromonas* are gram-negative, anaerobic, oxidase positive bacteria that can be found in soil and water, a wide range of food products, and human and fish infections [3,4]. Recently, a disease outbreak caused by concurrent infections by *A. Veronii*, *Flavobacterium columnare* and *Iridovirus* resulting in high mortalities was reported from tilapia farms in Thailand. Clinical signs included hemorrhagic septicemia, red sore disease, and ulceration [5]. Disinfectants and antibiotics have been widely used for the treatment and control of disease outbreaks in red tilapia farms. However, the overuse and misuse of antibiotics can cause environmental hazards and food safety

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concerns [6,7]. Biotherapeutics such as prebiotics, probiotics and synbiotics have recently come to the fore as a potential alternative to chemical and antibiotic agents [8–11]. Several probiotics and functional food prebiotics have been intensively studied in aquafeed. Most probiotic microorganisms are lactic acid bacteria, which can improve the microbiota balance in the intestine of the host and act as a defensive barrier against enteric pathogens [12]. *Lactobacillus rhamnosus* GG (LGG) has been used as a probiotic in humans and fish [13–15], and has been reported to control bacterial infections such as *A. salmonicida*, *Vibrio anguillarum* and *Flavobacterium psychrophilum* in rainbow trout and Turbot [8], as well as *Edwardsiella tarda* infections in tilapia [16]. LGG was also found to promote the intestinal structure and mucosal immunity of Nile Tilapia [17]. Jerusalem artichoke (JA) or Kantawan (*Helianthus tuberosus*), a natural prebiotic enriched with inulin and fructooligosaccharide (FOS), has been used as one of the most common functional feed ingredients in human and animal feedstuffs [18,19]. An improvement in the growth performance and immune response parameters in fish, poultry and swine have been reported when using JA-supplemented feed [20–22]. Dietary inulin and FOS supplements can promote the proliferation of beneficial lactic acid bacteria in the gut [23–26]. A human-derived or food industry-derived probiotic and a prebiotic derived from natural source of oligofructose-enrich inulin would serve as a promising candidate synbiotic for sustainable use in red tilapia farms. However, the data on nutritional synbiotic supplements in tilapia feed is quite limited [27,28], as are studies regarding the potential benefits of JA and LGG synbiotics for red tilapia. In this study, the synbiotic effect of dietary JA and LGG was evaluated in juvenile red tilapia. The growth performance, serum biochemical profiles, and intestinal morphology were measured. In addition, immune parameters and defense against *A. veronii* challenge were also determined to better extend the beneficial effect of synbiotic supplementation on the health and disease status of the red tilapia.

2. Materials and methods

2.1. Probiotic and prebiotic preparation

The probiotic bacterium, *L. rhamonsus* GG (ATCC 53103) was cultured in a MRS broth at 37 °C for 48 h, centrifuged, and washed with sterile phosphate-buffered saline three times. Then, the density of the bacterial suspension in the phosphate-buffered saline was determined and bacteria were mixed into commercial dry pellets (10^8 CFU/g) in feed for probiotic group according to previous study [16]. JA samples were obtained from Phetchabun Research Station, Agro-Ecological System Research and Development Institute, Kasetsart University, Thailand. The JA tubers were cleaned and thin pieces were sliced. These slices were then ground into powder using a hammer grinder. The samples were dried at 50 °C for 24 h and kept at 4 °C until use. The proximate composition of JA was analyzed using the standard methods of AOAC (1990). JA consists of oligo-fructose compounds, protein, lipid, fiber, dry matter and ash [20].

The four treatment diets were as follows: a control diet (C), 10.0 g kg^{-1} JA-supplemented (JA) diet, 10^8 CFU g^{-1} LGG-supplemented (LGG) diet and 10.0 g kg^{-1} JA + 10^8 CFU g^{-1} LGG-supplemented (JA + LGG) diet. The concentration of JA was selected based on the results obtained in previous data. The previous data showed that 10.0 g kg^{-1} JA-supplemented (JA) diet improved the growth performance in juvenile Nile tilapia [29]. The proximate composition of the control diet was dry matter, crude protein, crude lipid, fiber, fructans and ash [29].

2.2. Fish culture

Two hundred and forty male fish (average body weight 14.05 ± 0.42 g) were obtained from a Good Aquaculture Practice certified farm, Thailand. The mono males of red tilapia in this experiment were produced by hormonal sex reversal. The fish were divided into eight 1000-L tanks (30 each) and allowed to acclimatize for two weeks. The water in the tanks was continuously aerated and had a continuous flow of water. Water temperature was maintained between 25 and 28 °C, and dissolved oxygen and pH levels were 5.24–5.98 mg $L^{-1}$and 7.48–8.16, respectively. Fish were hand-fed approximately 5% of their body weight twice a day. The experiment was carried out in duplicate (i.e. two tanks for each experimental diet). All protocols were approved by the ethics committee of Chulalongkorn University Animal Care and Use Committee (CUACUC; Approval No. 1731039).

2.3. Growth performance

After 30 days of feeding, the final weight, weight gain (WG), specific growth rate (SGR), and feed conversion ratio (FCR) were calculated according to standard formulae.

WG (%) = 100 x (final mean body weight - initial mean body weight) / initial mean body weight

SGR = [(ln (final body weight) – ln (initial body weight) / days] x 100

FCR = feed intake (g) / Weight gain

ADG = (% gain)/(number of days)

2.4. Blood collection and measurement of serum biochemical parameters

After 30 days of feeding, blood samples were collected from six fish from each tank. Samples were taken from the caudal vein using a hypodermic syringe. Blood samples were allowed to clot at 4 °C for at least 3 h and were centrifuged at 2600 × *g* for 10 min at room temperature to obtain serum samples. The samples were analyzed by using an automate chemistry analyzer (AU400, Olympus, Tokyo, Japan). The following parameters were measured: glucose, triglyceride, cholesterol, total protein, albumin, blood urea nitrogen (BUN), total bilirubin (T-bilirubin), direct bilirubin (D-bilirubin), serum alanine transaminase (ALT) and serum aspartate aminotransferase (AST).

2.5. Measurement of villous height, villous width, absorptive area and goblet cells

After 30 days of feeding, six fish from each tank were randomly sampled and anesthetized with clove oil. Three parts of the intestine, the foregut (after the pyloric part of the stomach to the spiral part of the intestines), the midgut (the spiral part of the intestines), and the hindgut (after the spiral part to 2 cm before the anus) were collected and fixed in neutral buffered 10% formalin. Samples were processed, embedded in paraffin, sectioned at 5 μm, stained with hematoxylin and eosin and examined by light microscopy [17].

Villous height and width were measured using I-Solutions DT software (Image & Microscope Technology Inc., USA). For the villus height measurement, the ten highest intact villi were selected per section and their height was measured from the tip to the bottom. The average was expressed as the mean villus height for each section [17]. The absorptive area was calculated using the following calculation: absorptive area = villous height x villous width [30].

The goblet cells in the intestine were counted and classified by special staining as Periodic Acid-Schiff (PAS) staining for neutral mucin, Alcian blue (AB) staining (pH 2.5) for acid mucin, and AB-PAS double-staining for mixed type. The five highest intact intestinal villi were randomly selected. The goblet cells from each section were counted using a high-power field (HPF; 400× magnification) and calculated (goblet cell numbers/HPF).

2.6. Immunological assay

Immune parameters, including lysozyme activity, alternative complement haemolytic 50 (ACH50) activity and total immunoglobulin (Ig) were measured. Using fish serum, lysozyme activity was estimated as described by Pitaksong et al. [31]. Lysozyme activity was evaluated by indicating the level of lysis of the Gram-positive bacterium *Micrococcus luteus* and absorbance was measured using the spectrophotometer at a wavelength of 450 nm.

ACH50 activity was measured as per Sunyer and Tort [32]. This involved diluting the test serum in GVB-EGTA to a final volume of 250 μL, to prepare a 2-fold serial dilution; 50 μL of goat red blood cells were added to the test serum and incubated for 90 min at room temperature. The supernatant was measured using spectrophotometer at a wavelength of 415 nm. The ACH50 activity was calculated as the volume of serum causing 50% lysis of the goat red blood cells.

Ig was estimated by using the Lowry method, incorporating the modifications described by Siwicki and Anderson [33]. Using this method, immunoglobulin was precipitated out of the plasma with polyethylene glycol and the remaining protein of the plasma was determined. The total immunoglobulin was calculated by subtracting the total plasma protein concentration from the remaining protein in the plasma concentration.

2.7. Mortality test

The mortality test was carried out by *A. veronii* isolated from naturally diseased Nile tilapia (*Oreochromis niloticus*) in Nong Khai province, northeastern Thailand [3]. A single colony of *A. veronii* and grown in 5 ml TSB, incubated at 30 °C for 18 h. The bacterial suspension was adjusted to OD600 equal 0.55 to 0.60. At the end of the experimental period (four weeks), 25 fish from each group received an intraperitoneal injection of 10^7 CFU/fish. The colony forming units (CFU) of *A. veronii* were calculated by the plate count method. The concentration of *A. veronii* was selected based on the results from previous study [3]. After the challenge, any clinical signs of infection or mortalities were recorded for 15 days. The cumulative mortality were calculated by the following calculation: (Total mortality in each treatment after challenge/Total number of fish challenged for same treatment) x 100 [34]. Moreover, the relative percentage of survival (RPS) was calculated using the following calculation: 100-[(test mortality/control mortality) x 100] [35].

2.8. Statistical analysis

Results were analyzed by one-way analysis of variance (ANOVA) using SPSS version 22 software for Windows (SPSS Inc., Chicago, USA). Statistically significant differences between the groups were determined by Duncan's multiple range tests with a significance level of $P < 0.05$.

Data of intestinal absorptive area and growth performance and feed efficiency were subjected to a correlation analysis (Pearson's correlation coefficient) in order to study the relationship between the different parameters. The significance level was also set at 5% ($P < 0.05$) using SPSS version 22 software for Windows (SPSS Inc., Chicago, USA).

3. Results

3.1. Growth performance

After feeding the fish for a month, the fish fed the JA + LGG diet were found to have the highest final weight, which was significantly higher ($P < 0.05$) than fish fed with the control diet (Table 1). The results showed that the WG, SGR and ADG of fish fed with the JA + LGG, LGG and JA diets were significantly ($P < 0.05$) higher than for fish fed the control diet. Fish fed with the synbiotic diet (JA + LGG) had the lowest FCR, significantly lower ($P < 0.05$) than fish fed with the control diet.

3.2. Serum biochemical parameters

The results of the blood serum biochemical parameters of juvenile red tilapia fed with the experimental diets for four weeks are presented in Table 2. The results showed that the fish from the synbiotic group (JA + LGG) had the highest total protein value, which was significantly ($P < 0.05$) higher than the control group. Fish fed with the JA + LGG, LGG and JA diets had a significantly ($P < 0.05$) higher glucose value than fish in the control group. The fish fed with the synbiotic diet (JA + LGG) had the lowest BUN value, which was significantly ($P < 0.05$) lower than fish in the control group. The fish from the control group had the highest T-bilirubin and D-bilirubin values, which were statistically significant ($P < 0.05$) when compared with the other groups. The fish fed with the JA diet had the lowest ALT value, which was significantly different from fish in the control group. There were no significant differences in triglyceride, albumin and AST among the groups.

3.3. Measurement of villous height, villous width, absorptive area and goblet cells

Villous height in the proximal, middle and distal parts of the intestine of fish fed JA + LGG and LGG diets were significantly higher ($P < 0.05$) than in fish fed the control and JA diets (Fig. 1). This was also the case for the villous width in the proximal part of the intestine (Fig. 2). The absorptive area of the proximal and distal intestine was found to be highest for fish fed with the JA + LGG diet and was significantly different ($P < 0.05$) when compared with the other groups (Fig. 3). In the middle intestine, the absorptive area of fish fed with JA + LGG and LGG was significantly higher ($P < 0.05$) than for fish fed with the control and JA diets. In comparison to the other three diets, fish fed the control diet had the lowest values ($P < 0.05$) for villous height, width and absorptive area (Figss. 1–3) in all parts of the intestine.

In the proximal intestine of red tilapia, the neutral and the mixed type mucous cells (Figs. 4 and 7) were significantly higher ($P < 0.05$) in fish fed with the JA + LGG diet, while the acid mucous cells were highest in fish fed with the LGG diet. The number of goblet cells in the middle intestine of red tilapia illustrated that the neutral and double-staining mucous cells of fish fed with the JA + LGG and LGG diets were significantly higher ($P < 0.05$) than for fish fed with the control and JA diets (Fig. 5). The number of goblet cells recorded in the distal intestine revealed that the acid, neutral and double-staining mucous cells of fish fed the JA + LGG diet was significantly higher ($P < 0.05$) than for the other groups (Figs. 6 and 8). The acid, neural and mixed type mucous cells was significantly lower in all intestinal parts of fish fed with the control diet.

3.4. Correlations among the growth performance and feed efficiency parameters and the intestinal absorptive area

Significant correlations were observed between the growth performance and feed efficiency parameters and the intestinal absorptive area of red tilapia fed the experimental diets (Table 3). The absorptive areas of the three parts of the intestine, proximal, middle and distal were positively correlated ($P < 0.05$) with the ADG and the SGR. However, no significant correlations ($P > 0.05$) were observed between the absorptive areas and the WG and FCR.

3.5. Immune parameters

The results showed that fish fed with the synbiotic-supplemented diets (JA + LGG) experienced a significant increase ($P < 0.05$) in

Table 1
Growth performance and feed utilization of red tilapia fed with the control diet, JA - 10.0 g kg^{-1} JA-supplemented diet, LGG - 10^8 CFU g^{-1} LGG-supplemented diet, and JA + LGG - 10.0 g kg^{-1} JA + 10^8 CFU g^{-1} LGG-supplemented diet for 30 days (mean ± SD, n = 6).

Parameters	Control	JA	LGG	JA + LGG
Initial weight (g)	14.68 ± 3.26	13.88 ± 3.61	13.56 ± 3.13	14.28 ± 3.33
Final weight (g)	24.52 ± 5.67^a	27.73 ± 6.18^b	27.46 ± 5.54^b	29.28 ± 6.16^b
WG (%)	67.03 ± 7.0^a	106.38 ± 13.79^b	104.12 ± 10.76^b	106.05 ± 7.39^b
FCR	2.32 ± 0.55^b	1.61 ± 0.35^a	1.62 ± 0.27^a	1.52 ± 0.32^a
SGR (% day^{-1})	1.76 ± 0.14^a	2.49 ± 0.23^b	2.46 ± 0.18^b	2.49 ± 0.12^b
ADG (% day^{-1})	2.31 ± 0.24^a	3.67 ± 0.48^b	3.59 ± 0.37^b	3.66 ± 0.25^b

Different letters indicate statistical significance ($P < 0.05$).
WG (%) = (final weight-initial weight)/(initial weight) × 100.
Feed conversion ratio = (dry feed fed)/(wet weight gain).
Specific growth rate = [ln(final weight)-ln(initial weight)]/(number of days) × 100.
Average daily gain = (% gain)/(number of days).

Table 2
Serum biochemical parameters of red tilapia fed with the control diet, JA - 10.0 g kg^{-1} JA-supplemented diet, LGG - 10^8 CFU g^{-1} LGG-supplemented diet, and JA + LGG - 10.0 g kg^{-1} JA + 10^8 CFU g^{-1} LGG-supplemented diet for 30 days (mean ± SD, n = 6).

Parameters	Control	JA	LGG	JA + LGG
Glucose (mg dl^{-1})	45.17 ± 7.49^a	50.67 ± 10.54^b	63.33 ± 19.68^b	51.33 ± 5.32^b
Triglycerides (mg dl^{-1})	200 ± 97.51	187 ± 48.53	137.33 ± 55.09	182.17 ± 46.59
Total cholesterol (mg dl^{-1})	161.33 ± 10.54^a	227.83 ± 24.60^c	184.33 ± 33.61^{ab}	196.00 ± 9.47^b
Total protein (g dl^{-1})	2.60 ± 0.22^a	2.83 ± 0.38^{ab}	2.80 ± 0.09^{ab}	3.12 ± 0.56^b
Albumin (g dl^{-1})	0.97 ± 0.27	0.97 ± 0.21	0.87 ± 0.08	1.00 ± 0.40
BUN^a (mg dl^{-1})	1.80 ± 0.40^b	1.50 ± 0.55^{ab}	1.50 ± 0.55^{ab}	1.17 ± 0.41^a
Total bilirrubin (mg dl^{-1})	0.02 ± 0.01^b	0.01 ± 0.00^a	0.01 ± 0.00^a	0.01 ± 0.00^a
Direct bilirrubin (mg dl^{-1}	0.015 ± 0.02^b	0.002 ± 0.00^a	0.003 ± 0.00^a	0.002 ± 0.00^a
ALT (IU l^{-1})	9.50 ± 6.53^b	3.33 ± 0.82^a	6.67 ± 3.44^{ab}	6.00 ± 1.90^{ab}
AST (IU l^{-1})	13.00 ± 2.76	17.33 ± 7.69	19.83 ± 10.53	11.60 ± 3.88

Values within the same row with different letters are significantly ($P < 0.05$) different.
BUN = blood urea nitrogen.
ALT = alanine transferase.
AST = aspartate aminotransferase.

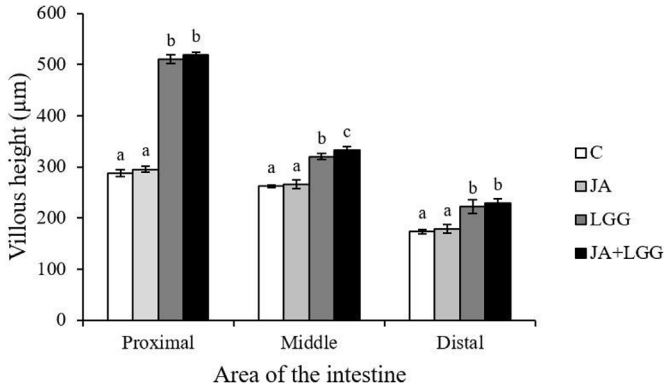

Fig. 1. V*illous height* in three parts of the intestine of red tilapia fed with the control diet, JA - 10.0 g kg^{-1} JA-supplemented diet, LGG - 10^8 CFU g^{-1} LGG-supplemented diet, and JA + LGG - 10.0 g kg^{-1} JA + 10^8 CFU g^{-1} LGG-supplemented diet for 30 days. Data represent the mean ± SD Bars assigned with different letters indicate statistical significance ($P < 0.05$, $N = 6$).

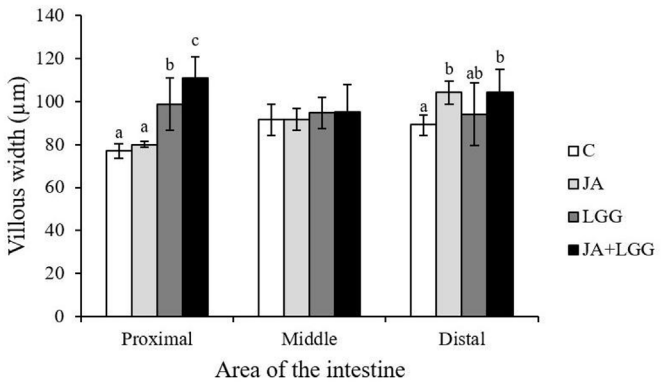

Fig. 2. Villous width in three parts of the intestine of red tilapia fed with the control diet, JA - 10.0 g kg^{-1} JA-supplemented diet, LGG - 10^8 CFU g^{-1} LGG-supplemented diet, and JA + LGG - 10.0 g kg^{-1} JA + 10^8 CFU g^{-1} LGG-supplemented diet for 30 days. Data represent the mean ± SD Bars assigned with different letters indicate statistical significance ($P < 0.05$, $N = 6$).

lysozyme activity in comparison with other groups (Fig. 9). There was no significant difference in ACH50 activity and Ig in fish fed with the different diets (Fig. 9).

3.6. Mortality test

Cumulative mortality was significantly lower in the synbiotic (JA + LGG) group (4%) than in the control group (56%). No significant difference in cumulative mortality was observed between fish fed the JA and LGG diets (Fig. 10). In addition, the relative percent of survival (RPS) the red tilapia fed synbiotic diet (86.43 ± 9.09) was higher than those of fish fed prebiotic (69.29 ± 13.13) and probiotic (76.43 ± 23.24) supplemented diet but these values did not differ statistically ($P > 0.05$). All dead fish exhibited a pale body surface, fin rot, cloudy eyes, haemorrhage in liver and spleen, and a swollen intestine with an accumulation of yellow liquid. The cause of death for the mortalities observed during the challenge trials was caused by *A. veronii* as determined by bacterial isolation from the spleen and liver (data not shown).

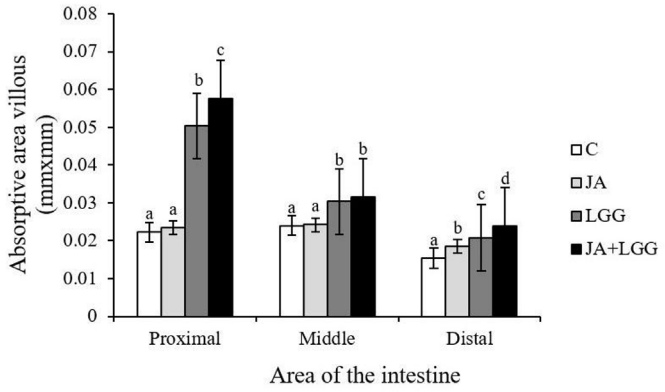

Fig. 3. Absorptive area of three parts of the intestine of red tilapia fed with the control diet, JA - 10.0 g kg^{-1} JA-supplemented diet, LGG - 10^8 CFU g^{-1} LGG-supplemented diet, and JA + LGG - 10.0 g kg^{-1} JA + 10^8 CFU g^{-1} LGG-supplemented diet for 30 days. Data represent the mean ± SD Bars assigned with different letters indicate statistical significance ($P < 0.05$, $N = 6$).

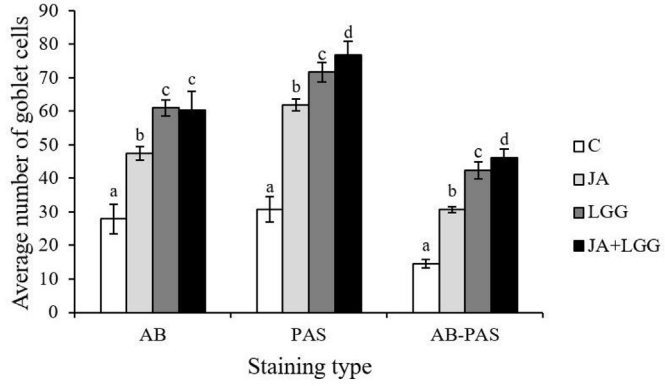

Fig. 4. The average number of goblet cells in the proximal intestine of red tilapia fed with the control diet, JA - 10.0 g kg^{-1} JA-supplemented diet, LGG - 10^8 CFU g^{-1} LGG-supplemented diet, and JA + LGG - 10.0 g kg^{-1} JA + 10^8 CFU g^{-1} LGG-supplemented diet for 30 days after three types of staining: **a** AB staining, **b** PAS staining, and **c** AB-PAS double-staining. Data represent the mean ± SD Bars assigned with different letters indicate statistical significance ($P < 0.05$, $N = 6$).

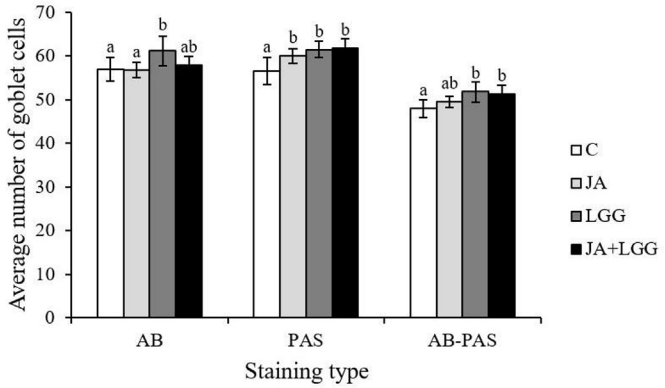

Fig. 5. The average number of goblet cells in the middle intestine of red tilapia fed with the control diet, JA - 10.0 g kg^{-1} JA-supplemented diet, LGG - 10^8 CFU g^{-1} LGG-supplemented diet, and JA + LGG - 10.0 g kg^{-1} JA + 10^8 CFU g^{-1} LGG-supplemented diet for 30 days after three types of staining: **a** AB staining, **b** PAS staining, and **c** AB-PAS double-staining. Data represent the mean ± SD Bars assigned with different letters indicate statistical significance ($P < 0.05$, $N = 6$).

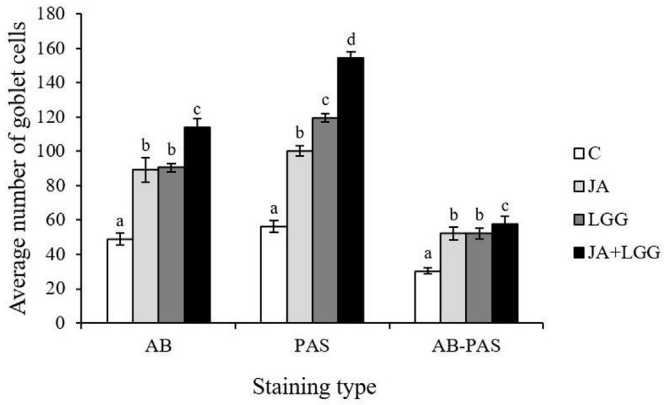

Fig. 6. The average number of goblet cells in the distal intestine of red tilapia fed with the control diet, JA - 10.0 g kg^{-1} JA-supplemented diet, LGG - 10^8 CFU g^{-1} LGG-supplemented diet, and JA + LGG - 10.0 g kg^{-1} JA + 10^8 CFU g^{-1} LGG-supplemented diet for 30 days after three types of staining: **a** AB staining, **b** PAS staining, and **c** AB-PAS double-staining. Data represent the mean ± SD Bars assigned with different letters indicate statistical significance ($P < 0.05$, $N = 6$).

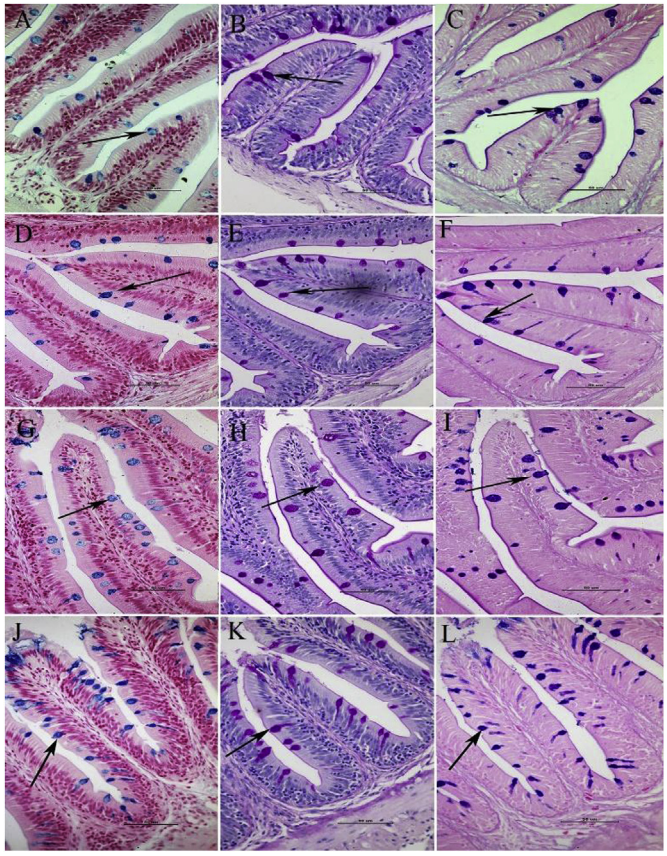

Fig. 7. Proximal intestinal goblet cells (arrows) of red tilapia fed with the control diet (A, B and C), JA - 10.0 g kg^{-1} JA-supplemented diet (D, E and F), LGG - 10^8 CFU g^{-1} LGG-supplemented diet (G, H and I), and JA + LGG - 10.0 g kg^{-1} JA + 10^8 CFU g^{-1} LGG-supplemented diet (J, K and L) for 30 days after three types of staining: AB staining (A, D, G and J), PAS staining (B, E, H and K), and AB-PAS double-staining (C, F, I and L). Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

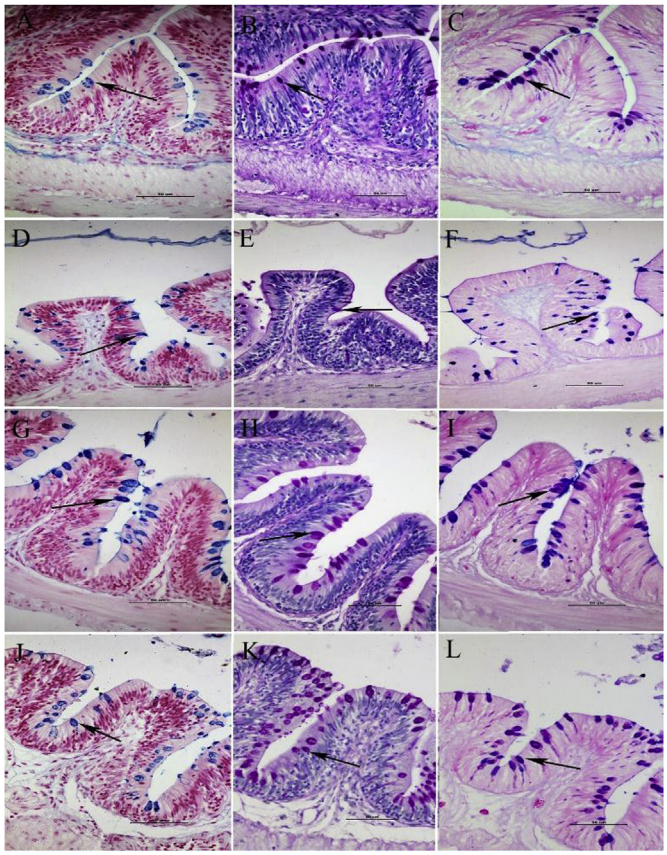

Fig. 8. Distal intestinal goblet cells (arrows) of red tilapia fed with the control diet (A, B and C), JA - 10.0 g kg^{-1} JA-supplemented diet (D, E and F), LGG - 10^8 CFU g^{-1} LGG-supplemented diet (G, H and I), and JA + LGG - 10.0 g kg^{-1} JA + 10^8 CFU g^{-1} LGG-supplemented diet (J, K and L) for 30 days after three types of staining: AB staining (A, D, G and J), PAS staining (B, E, H and K), and AB-PAS double-staining (C, F, I and L). Scale bar = 50 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 3
Correlations (r) among the growth performance and feed efficiency parameters and the intestinal absorptive area of red tilapia fed with the control diet, JA - 10.0 g kg^{-1} JA-supplemented diet, LGG - 10^8 CFU g^{-1} LGG-supplemented diet, and JA + LGG - 10.0 g kg^{-1} JA + 10^8 CFU g^{-1} LGG-supplemented diet for 30 days.

Intestinal absorptive area (mm^2)	Growth performance and feed efficiency parameters			
	WG (%)	ADG (% day^{-1})	FCR	SGR (% day^{-1})
Proximal intestine	0.025	0.519*	−0.153	0.560*
Middle intestine	0.18207	0.49661*	0.098	0.53367*
Distal intestine	0.17575	0.72741*	−0.14596	0.74708*

Growth performance and feed efficiency parameters nomenclature as in Table 1. *Indicates a significant correlation ($P < 0.05$) (Pearson's correlation coefficient).

4. Discussion

Natural synbiotics, the synergistic combination of naturally derived-probiotics and prebiotics, are now being highlighted as one of the most practical nutritional supplements in eco-friendly sustainable fish farms [36]. It is expected that natural synbiotics will become a common alternative for the prevention and control of bacterial diseases in fish farms [37]. Previous studies have reported that including a dietary synbiotic supplement in aqua feed enhances the growth performance of fish [9,36,38–43]. In the present study, red tilapia fed a diet supplemented with synbiotic JA + LGG for four weeks showed a significantly higher weight gain, specific growth rate, and average daily gain in comparison with a previous study where Pangasius Catfish (*Pangasius bocourti*, Sauvage 1880) were fed a synbiotic (JA and *L. plantarum*) diet for 90 days [9], extending the beneficial improvement the growth performance and feed utilization by the JA and *Lactobacili* synbiotics in fish. Fish fed the synbiotic diet exhibited a greater villus height and villus width in all parts of the intestines, with significant differences in the proximal intestine. There was a positive correlation between the feed efficiency parameters (ADG and SGR) and the intestinal absorptive area of red tilapia fed the synbiotic diets. The stimulation of LGG growth by functional food ingredients such as inulin and FOS from a JA prebiotic [46,47] increases villi height and width resulting in a larger absorptive area, higher absorption of available nutrients and better growth performance [44,45,48]. The effect of supplementation with functional prebiotics on growth performance varies among fish species and prebiotic types [49]. The results of the current study revealed that red tilapia fed with the JA supplemented diet had a significantly better growth performance than the control group. Tiengtam et al. [29] reported that supplementation with JA for 8 weeks increased the villus height in the proximal and middle intestine in juvenile Nile tilapia. In contrast, there was no statistical difference in villus height in the proximal and middle intestine of red tilapia supplemented with JA for 1 month in the current study, suggesting that the feeding duration of JA prebiotic and fish species influence the feed efficiency and gut morphometry [54]. The beneficial effect of JA on growth performance may be involved in the result of JA compounds such as inulin, FOS, carbohydrate, protein, Vitamin C, and minerals [50]. Moreover, JA tubers contain natural antioxidants such as polyphenols involved in protecting against oxidative stress [51,52], which could ultimately have a positive effect on the growth performance [53].

Blood chemical parameter is one of the most common factors to assess the nutritional and health status of red tilapia. The JA, LGG and JA + LGG supplemented diet increased the glucose, total cholesterol and total protein levels of red tilapia. These results suggest that synbiotic-supplemented diets are related with the energy and protein contribution in red tilapia [58–60]. Probiotic and Prebiotic can enhance the intestinal digestive enzyme activities such as amylase, protease and lipase [55–57]. In addition, this study revealed that LGG and JA + LGG increased the intestinal absorptive area of red tilapia. Increased intestinal digestive enzyme activities and intestinal absorptive area would affect to the glucose, total cholesterol and total protein levels in serum. Fish from the control group had the highest T-bilirubin D-bilirubin and BUN values, which were significantly ($P < 0.05$) higher than the synbiotic group (JA + LGG). This may indicate that synbiotic-supplemented diets had effects in protecting the liver and kidney cells [61], as in the case of liver damage during which T-bilirubin and D-bilirubin ALT are released into the blood, allowing for the early detection of liver problems [62,63]. The results showed no significant differences in triglyceride, albumin and AST levels between fish fed the control diet and those fed the synbiotic supplemented diets, indicating that the experimental diets had no effect on the majority of blood serum biochemical parameters in juvenile red tilapia [64].

The immune system of teleosts, or bony fish, is composed of innate and adaptive immune responses. Mucus cells are a crucial first line of gut mucosal defense against pathogenic bacterial infection. Mucous goblet cells are composed of acid and neutral mucins, which aid in lubricating, trapping and removing pathogens [65,66]. Acid mucins are further differentiated based on their histochemical properties into sulfate-containing mucins (sulfomucins) and sialicacid-containing mucins (sialomucins) [67]. In this study, histochemistry with combined AB-PAS staining proved a useful tool for classifying different mucous cell populations on the basis of the type of mucins produced [68], to determine if there were any changes associated with the different diets. Fish fed the synbiotic- and probiotic-supplemented diets had

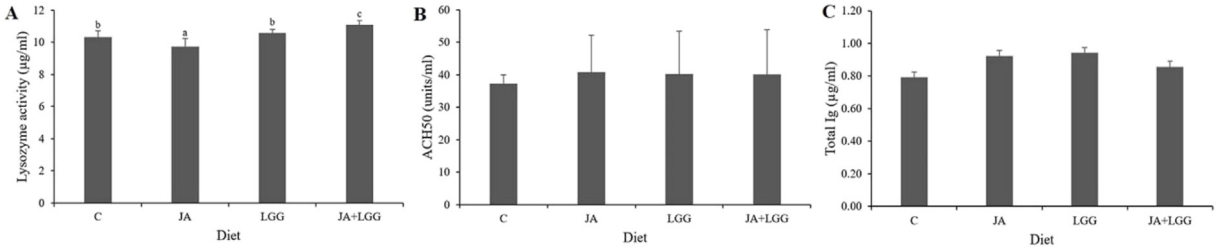

Fig. 9. Lysozyme activity (A), Alternative complement haemolytic 50 (ACH50) activity (B) and total immunoglobulin (C) of red tilapia fed with the control diet, JA - 10.0 g kg^{-1} JA-supplemented diet, LGG - 10^8 CFU g^{-1} LGG-supplemented diet, and JA + LGG - 10.0 g kg^{-1} JA + 10^8 CFU g^{-1} LGG-supplemented diet for 30 days. Data represent the mean ± SD Bars assigned with different letters indicate statistical significance ($P < 0.05$, $N = 6$).

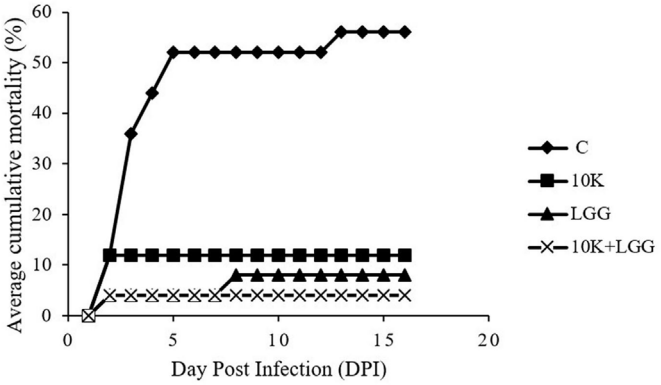

Fig. 10. The average cumulative mortality of red tilapia fed with the control diet, JA - 10.0 g kg^{-1} JA-supplemented diet, LGG - 10^8 CFU g^{-1} LGG-supplemented diet, and JA + LGG - 10.0 g kg^{-1} JA + 10^8 CFU g^{-1} LGG-supplemented diet for 30 days following *Aeromonas veronii* challenge. Data represent the mean ± SD Bars assigned with different letters indicate statistical significance ($P < 0.05$, $N = 6$).

significantly higher numbers of goblet cells in comparison with the other groups. Similar results were reported from studies where Nile tilapia was fed a *L. rhamnosus* supplemented-diet [17,68], and a multi-species (*Bacillus* sp., *Pediococcus* sp., *Enterococcus* sp. and *Lactobacillus* sp.) supplemented-diet [69]. It has been suggested that neutral mucin is related to the digestion processes [70]. Fish fed the synbiotic-supplemented diet exhibited significantly higher numbers of neutral mucous cells and double-staining mucous cells in the proximal intestine, confirming active digestion and feed utilization by JA + LGG. However, the dominant mucous cell recorded in all parts of the intestine of fish fed with the synbiotic and probiotic diets was the acid type. The acidic mucin is mainly associated with protection against bacterial translocation [71]. Current data state that acidic mucin carbohydrate chains that contain sialic acid residues and sulfate groups contribute to the adhesion of *Lactobacillus* (including LGG) to mucin [72]. Therefore, LGG adhesion to acidic mucins is advantageous to surviving transit to colonization of the intestinal tract of red tilapia. It could be implied that synbiotic LGG adapt to the constantly changing intestinal environment of red tilapia, maintaining the intestinal barrier function and allowing them to colonize the host while concurrently avoiding competition with other bacteria as suggested by several authors [72,73].

The current study revealed that synbiotic-supplemented diets enhance the innate immune response of red tilapia as indicated by increased levels of lysozyme activity. Similarly, a JA and *Lactobacillus plantarum* synbiotic-supplemented diet was reported to increase levels of lysozyme activity on Pangasius Catfish after 90 days [9], confirming the enhanced activity of digestive enzymes against peptidoglycan layers of bacteria. However, there was no change in lysozyme activity for rainbow trout (*Oncorhynchus mykiss*) fed with FOS and *Lactobacillus rhamnosus* supplemented diets for 30 days [74]. The lack of differences in the total immunoglobulin and ACH50 activity obtained in the present study is in agreement with results obtained in the oral administration of *Lactobacillus rhamnosus* supplemented diets (10^9 CFU g^{-1}) in rainbow trout (*Oncorhynchus mykiss*) for 30 days [75]. On the contrary, the difference in ACH50 activity was significant in rainbow trout fed the high dose of *L. rhamnosus* supplemented diet (10^{11} CFU g^{-1}), suggesting the beneficial effects of synbiotics on immune modulation might vary among fish species, level of supplementation, and duration of feeding.

Cumulative mortality levels were significantly lower for fish fed the synbiotic-supplemented diets than for fish fed the control diet and single prebiotic or probiotic diet. Similar results were observed for Pangasius catfish fed with symbiotic, JA and *L. plantarum*, supplemented-diets and challenged with *A. hydrophila* [9]. A JA prebiotic supplemented-diet showed protection against *A. hydrophila* infection in Asian seabass (*Lates calcarifer*) [76]. It has been reported that the low cumulative mortalities of fish fed synbiotic-supplemented diets may be caused by the synergistic effect between the prebiotic and the probiotic [77]. Prebiotics stimulate the growth of probiotics, and these reduce the presence of pathogens in the host by inhibiting their adherence and colonization [78,79], enhancing the host response to diseases [80,81].

Taken together, the data confirmed the beneficial effect of JA and LGG symbiotic diet on growth performance and health status of red tilapia. Direct administration of JA and LGG in fish feed can be used as a practical nutritional supplement in red tilapia.

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