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RESEARCH

Growth performance, mucosal immunity and disease resistance in goldfish (*Carassius auratus*) orally administered with *Escherichia coli* Strain Nissle 1917

Katayoon Nofouzi · Najmeh Sheikhzadeh · Gholamreza Hamidian · Amir Ali Shahbazfar · Mehdi Soltani · Amin Marandi

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Abstract The current research aimed to shed light on the efficacy of *Escherichia coli* strain Nissle 1917 (EcN) on goldfish (……) growth, gut immunity, morphology, bacterial nutritional enzyme activity and resistance to *Aeromonas hydrophila* infection. Fish fed with EcN at 10^6, 10^7 and 10^8 CFU/g feed for 80 days showed an enhancement in growth better than control fish. The gut innate immunity in terms of lysozyme activity, immunoglobulin and total protein levels was increased in the treatment fish with the best result being observed in fish fed EcN at 10^8 CFU/ g. In addition, an increase was noted in the upregulation of immune-relevant genes, namely lysozyme, interleukin-1β, inducible nitric oxide synthase and tumor necrosis factor α of fish intestine. A marked surge in the number of proteolytic and heterotrophic bacteria was noted in the gut of fish nourished with the probiotic. Histological studies exhibited an improvement in the intestinal absorption surface area, intraepithelial lymphocyte count and goblet cell density. Significantly higher survival rate was obtained in fish fed EcN at 10^8 CFU/g compared with the fish fed with the basal diet. These data exhibited the beneficial effect of EcN on goldfish growth, digestive enzymes, intestine heterotrophic bacteria and resistance against *Aeromonas hydrophila* challenge. This study confirmed the favorable outcomes resulting from the administration of EcN at10^8 CFU/g.

Keywords *Escherichia coli* · Goldfish · *Aeromonas hydrophila* · Growth · Innate immunity

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s10695-024-01366-x.

Highlights
- Goldfish were fed with *Escherichia coli* strain Nissle 1917.
- This probiotic could enhance the growth performance.
- Gut immunity as well as immune-related gene expressions increased.

K. Nofouzi (✉) · A. A. Shahbazfar
Department of Pathobiology, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran
e-mail: nofouzi@tabrizu.ac.ir

N. Sheikhzadeh
Department of Food Hygiene and Aquatic Animals, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran

G. Hamidian
Department of Basic Sciences, Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran

M. Soltani · A. Marandi
Department of Aquatic Animal Health, Faculty of Veterinary Medicine, University of Tehran, Tehran, Iran

M. Soltani
Centre for Sustainable Aquatic Ecosystems, Harry Butler Institute, Murdoch University, Murdoch, WA, Australia

Introduction

Ornamental fish trading is popular in different countries. Goldfish, *Carassius auratus*, is one of the main and attractive aquarium fish due to their color, behavior and shape (Kumar et al. 2013). However, the intensive fish culture causes a stressful condition for this species by triggering disease outbreaks in the aquaculture industry (Lieke et al. 2020). Pathogens, such as *Aeromonas hydrophila*, are the usual causative agents of bacterial diseases in goldfish (Harikrishnan and Balasundaram 2005; Anjur et al. 2021). To overcome these infectious agents in farmed condition, various chemical compounds and antibiotics are usually used which negatively affect the host and surrounding environment (Dawood et al. 2018). Due to these health and environmental issues, the chemotherapy in aquaculture has been seriously highlighted by many researchers (Okocha et al. 2018; Lulijwa et al. 2020) and the recent applications of probiotic therapy have been strongly recommended as a novel approach to control infectious diseases in aquaculture (De et al. 2014; Pérez-Sánchez et al. 2014; Daniel and Nageswari 2017; Chauhan and Singh 2019; Van Doan et al. 2020). The administration of probiotics has major benefits for fish in several different ways, including improving the gut health, digestive enzyme activities, gut microbial community and the general health of farmed aquatic animals (Allameh et al. 2017; Hoseinifar et al. 2017; Mamun et al. 2019; Soltani et al. 2019; Dawood et al. 2020).

The selection of probiotics for fish species is indeed important since inconvenient microorganisms can disrupt the balance of bacterial population in animal gut which may subsequently impair the nutrient metabolism, immune system and animal health status (Lazado et al. 2015; Soltani et al. 2018, 2019). *Escherichia coli* strain Nissle 1917 (EcN) is the most frequent bacterial strain in medicine and veterinary with probiotic potential (Duncker et al. 2006; Sonnenborn and Schulze 2009; Sonnenborn 2016; Vlasova et al. 2016; Wassenaar 2016). It has been used as the active pharmaceutical ingredient in a licensed medicinal product in different countries. In humans, EcN improves gut immunity, ulcerative colitis and allergic reactions as well as strengthening the tight junctions of the intestinal barrier, thus reducing infection via the gut (Zyrek et al. 2007; Trebichavsky et al. 2010; Losurdo et al. 2015; Guo et al. 2019). Despite the importance of this bacterium in human gut health, very few efforts have been made in aquaculture industry. In only one study, dietary EcN supplementation could improve the immune responses of Nile tilapia (*Oreochromis niloticus*) (ZeinEddine et al. 2022). This study aimed to evaluate the beneficial effects of EcN on the growth, gut immunity, bacterial nutritional enzyme activity and histological structure of goldfish as well as appraising fish resistance to *A. hydrophila* challenge.

Materials and methods

Bacterial strains

EcN in the form of pure culture was prepared from Pharma-Zentrale Company (Herdecke, Germany). *A. hydrophila* (ATCC 7966) purchased from Iranian Biological Resource Center (IBRC) was used as the pathogenic bacterium.

In vitro studies

Fish bile resistance assay

Bile tolerance test was performed in fresh goldfish bile obtained aseptically from the gall bladder of a healthy goldfish immediately after being euthanized in clove oil (50 µL/L). Suspensions of *A. hydrophila* and EcN prepared in phosphate-buffered saline (PBS) at OD 600 nm were set at 0.25. Then, 500 µl of the bacterial suspension was centrifuged at 3000 *g* for 10 min; this was followed by being re-suspended in PBS with or without 10% goldfish bile. The samples were incubated at 25°C for 1.5 h and the serial dilutions in PBS were cultured in tryptic soy agar and eosin methylene blue agar for a viable count of *A. hydrophila* and EcN, respectively (Nikoskelainen et al. 2001).

Water tolerance assay

Water tolerance test was performed directly on goldfish rearing water. Water solution was filtered through syringe filter (0.22 µm) before being mixed with an overnight culture of EcN and *A. hydrophila* at a ratio of 1:50. The viability of the bacterial species with the

initial approximate density of 2×10^9 CFU ml^{-1} was calculated by plate counting before and after 4 h incubation at 25°C in tryptic soy agar and eosin methylene blue agar for *A. hydrophila* and EcN, respectively (Feckaninova et al. 2019).

Growth inhibition by spent culture liquid

Initial screening of antagonism was evaluated by a method described previously (Uddin et al. 2008). In brief, EcN was cultured in 10 ml of tryptic soy broth overnight at 37°C. The bacterium was removed by centrifugation at 2000 *g* and the supernatant was sterilized using a filter (0.22 µm). After sterilization, half (5 mL) of the supernatant was neutralized with 5 M NaOH to prevent the inhibitory effect of acidic products.

A. hydrophila was cultured in 1 mL of tryptic soy broth overnight at 25°C, harvested by centrifugation at 2000 *g*, washed twice with PBS, and suspended in 1 mL of PBS. The suspension was transferred to tryptic soy agar plates. Four wells were made in each agar plate with a sterile Pasteur pipette; 50 µL of normal and 50 µL of neutralized spent culture supernatant from EcN were added to the wells. Neutralized tryptic soy broth and soy broth (pH 6.0) were added to the remaining wells to determine the possible inhibitory activity. The clearing zone was then determined 3 days post-incubation at 25°C (Nikoskelainen et al. 2001).

Experimental diet

An overnight culture of EcN in Luria–Bertani broth at 37 °C was centrifuged while being shaken for 15 min at 2500 *g* before the cells were collected in phosphate-buffered saline (PBS). The final probiotic concentrations of 10^6, 10^7 and 10^8 CFU/g (ZeinEddine et al. 2022) feed were adjusted according to Ahmadifar et al. (2020) (Table 1). To confirm these concentrations in the pellets, an amount of each prepared pellet was homogenized in sterile PBS before being cultured on Luria–Bertani broth using spread plate count at 37 °C for 24 h. The prepared pellets were dried at room temperature before being stored inside airtight packaging at 4 °C for weekly use. Besides, fresh batches of diets were prepared every other week to ensure the viability of EcN. The main stock of EcN frozen at − 80 °C was used for probiotic preparation to prevent the possibility of a genetic instability.

Table 1 Proximate composition of the basal diet used in this work

Ingredient	g kg^{-1}
Kilkafish meal[a]	300
Wheat flour	240
Soybean meal[b]	310
Cottonseed meal	100
Cellulose	10
Vitamin premix[c]	20
Mineral premix[d]	20
Chemical composition (% dry matter)	
Dry matter	88.50
Crude protein	37.07
Crude lipid	8.54
Ash	6.55
Gross energy (kcal kg^{-1})	4159.97

[a]Crude protein, 60.6%

[b]Crude protein, 44.2%

[c,d]Mixture detailed by Mousavi et al. (2020)

Fish

Goldfish with a mean weight of 1.81 g ± 0.01 were kept in a commercial fish farm in Marand, Iran. All fish were clinically monitored, then adapted to the experimental setup for 10 days and fed with a control diet (Table 1). During the adaptation period, fish were also being checked for the abnormal clinical signs. Afterwards, the fish were randomly allotted and placed into 12 glass tanks (300-L capacity) with four groups each in three replicates (25 fish/tank). They were fed with the experimental diets: basal diet (EcN0), 10^6 CFU/g (EcN1), 10^7 CFU/g (EcN2) and 10^8 CFU/g (EcN3) for 80 days up to apparent satiation (ZeinEddine et al. 2022). The physicochemical characteristics of the inlet water during this study were as follows: temperature 25.1 ± 0.7°C, pH 7.3 ± 0.2, ammonia < 0.01 mg L^{-1}, nitrite < 0.1 mg L^{-1}, hardness 280 mg L^{-1}, dissolved oxygen 7.3 ± 0.3 mg L^{-1}, with 50% of water being exchanged once a week.

Fish growth and sampling procedure

At the end of the experiment, the fish were anesthetized with clove oil (50 µL/L), and the weight and visceral weight of ten fish per tank were recorded followed by dry feed intake, feed conversion ratio, specific growth rate and survival rate according to following formula:

Feed conversion ratio	Total feed intake (g) / (Final body weight (g) − Initial body weight (g)).
Specific growth rate	(Ln final body weight − Ln initial body weight) / feeding period.
Survival rate (SR) (%)	(final number of goldfish / initial number of goldfish) × 100.

The intestinal tissues (anterior, mid and posterior parts) were aseptically obtained from three fish per tank, rinsed with sterile PBS before being fixed with 10% buffered neutral formalin for histological examination. The bacterial nutritional enzyme activity was carried out using intestinal tissues of four fish per tank, with each being individually mixed with two volumes of PBS and homogenized mechanically for 10 min. The intestine samples of four fish per tank were homogenized mechanically and stored in Tris–HCl buffer (pH 7.2) for immune assays and meanwhile, the homogenized intestine samples of the four fish per tank were individually placed in liquid nitrogen for real-time PCR assay.

Immune assays

Prior to the immune assays, the protein concentration in each intestinal homogenate was determined by the method of Bradford (1976) to calculate the immune-related enzyme activities. For lysozyme activity, fish homogenates (25 µL) were well mixed with the suspension (75 µg mL^{-1}) of the bacterium *Micrococcus lysodeikticus* (175 µL). The turbidity was recorded at 450 nm continuously for 6 min using a microplate reader (Hiperion, Germany). The specific quantity of lysozyme enzyme that triggered a fall in the absorbance at 0.001 per minute was considered a single unit of lysozyme activity per g of fish intestine (Ahmadifar et al. 2020).

Complement titer in the intestine homogenates was measured according to Andani et al. (2012). Rabbit red blood cells (2×10^8 cells mL^{-1}) prepared in veronal solution (0.01 M, pH 7) were mixed with diluted homogenates (250 µL). After incubation for 90 min at 20 °C, NaCl solution (0.85%) was added and centrifugation was performed for 10 min at 1600 *g*. The amount of intestinal homogenate that induced 50% haemolysis of rabbit red blood cells was considered a unit of fish complement titer per gram of intestine.

Immunoglobulin level in the intestine homogenates was measured according to Siwicki et al. (1994). Fish homogenates (100 µL) were mixed with an equal volume of polyethylene glycol (Sigma Aldrich, USA). After incubation at 25°C for 2 h, centrifugation was performed at 5000 *g* for 10 min. The difference between the initial and final protein levels by Bradford method was considered the immunoglobulin concentration in fish intestine and expressed as unit per gram of fish intestine.

Real-time PCR

Gene All reagent (Gene All Biotechnology, Korea) was deployed for extracting the whole RNA from fish intestine. The quantity and integrity of the purified RNA were checked by the spectrophotometer (Bio-Rad, CA, USA) and 1% agarose gel, respectively and then reverse-transcribed with a transcription Kit (Thermo Fisher Scientific, USA). Analysis of the target genes, including lysozyme, *interleukin (IL)-1β*, *inducible nitric oxide synthase* (*iNOS*) and *tumor necrosis factor α* (*TNF α*), was conducted by quantitative real-time reverse-transcription-polymerase chain (q-RT-PCR) reaction and using SYBR® Premix (Takara Biotechnology Company, China) (Table 2). The 20 µL qPCR reaction buffer contained 1 µL cDNA, 20 pmol forward and reverse primers, 10 µL of PCR Mix and nuclease free water. The *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) gene also served as the reference gene for internal control. The thermal profile for all reactions was as follows: holding step 3 min at 95 °C; cycling step 40 cycles of 20 s at 95 °C, 30 s at 60 °C; melting step 20 s at

Table 2 Primers used for assessing the expression of immune-related genes

Genes	Primer	Accession no	Efficiency value (%)
Lysozyme	F: 5′ GCCGGAAATGTCCTGAATAA 3′ R: 5′ GTGGTCCTGGCATCGATATT 3′	KM100713.1	98.43
IL-1β	F: 5′ TTCATTTGAAGGCAGTGACG 3′ R: 5′ TAAGCTGTGCCCGTCTCTTT 3′	AJ249136.1	96.45
iNOS	F: 5′ TTGGTACATGGGCACTGAGATT 3′ R: 5′ CCAACCCGCTCAAGAACATT 3′	AY904362	94.54
TNF-α	F: 5′ CATTCCTACGGATGGCATTTACTT 3′ R: 5′ CCTCAGGAATGTCAGTCTTGCAT 3′	EU069817	95.68
GAPDH	F: 5′ TGATGCTGGTGCCCTGTATGTAGT 3′ R: 5′ TGTCCTGGTTGACTCCCATCACAA 3′	AY641443.1	93.43

72 °C. The fold change of expression for the target genes was estimated by $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen 2001). The PCR reactions were run three times to decrease the errors.

Digestive enzyme producing bacteria

The enumeration of bacteria related to digestion, including amylolytic, lipolytic and proteolytic activities, was performed in various agar mediums. The homogenate gut samples were first serially diluted before being spread on tryptic soy agar (total viable heterotrophic aerobic bacteria), starch agar (amylolytic activity), tryptic soy agar with 1% Tween 80 and 0.001% $CaCl_2$ $2H_2O$ (lipolytic activity) and tryptic soy agar with 1% skim milk (proteolytic activity). The grown colonies were quantified by producing clear zone surrounding colony and presented as the colony-forming units (CFU) per intestine sample after a 5-day incubation of the plates at 25 °C (Asaduzzaman et al. 2018).

Histological examination

The fixed gut tissues were processed with automatic tissue processor and stained by standard hematoxylin and eosin method. For every single sampled tissue, five microscopic fields in each tissue section and ten sections per goldfish were studied using a light microscope (Olympus, BX-60) and a digital micro camera (Olympus, DP 12). For histomorphological studies, 20–22 sections from each block were randomly selected and scrutinized via stereo-investigator system, ver. 9 (MBF Bioscience, Germany). In each intestinal segment, different indices, namely tunica mascularis thickness (μm), villus height (μm), villus width (μm), absorption surface area (mm^2/villi), goblet cell density ((#/mm^2) and intraepithelial lymphocyte distribution (IEL) (#/100 entrocytes) were measured according to Hamidian et al. (2018).

Challenge experiment

A. hydrophila (ATCC 7966) purchased from Iranian Biological Resource Center (IBRC) was grown in tryptic soy broth for 24 h at 25 °C. After the 80-day feeding trial, the remaining fish in each tank ($n = 10$) were intraperitoneally injected with 0.1 ml of *A. hydrophila* (1×10^8 CFU/mL) (Soltanian and Fereidouni 2016) while the control fish received 0.1 ml of PBS. The fish were kept for 14 days and fed with the same experimental diets. The daily mortality was recorded and concurrently, the cause of death was confirmed by re-isolation of *A. hydrophila* from the kidney or spleen of affected fish on tryptic soy agar. During this challenge test, the water in each tank was controlled to be the same as the feeding trial.

Statistical analysis

The obtained data in form of mean ± standard error (SE) was analyzed by one-way analysis of variance followed by Tukey's post hoc using SPSS which was used to analyze the whole data. Cumulative survival (%) was plotted by Kaplan–Meier method and then analyzed by the log-rank test. The p value less than 0.05 was considered the level of acceptance.

Results

In vitro

Bile resistance

Both EcN and *A. hydrophila* exhibited resistance to 10% bile exposure for 90 min but no significant difference was seen in the total viable count between the treatments and control.

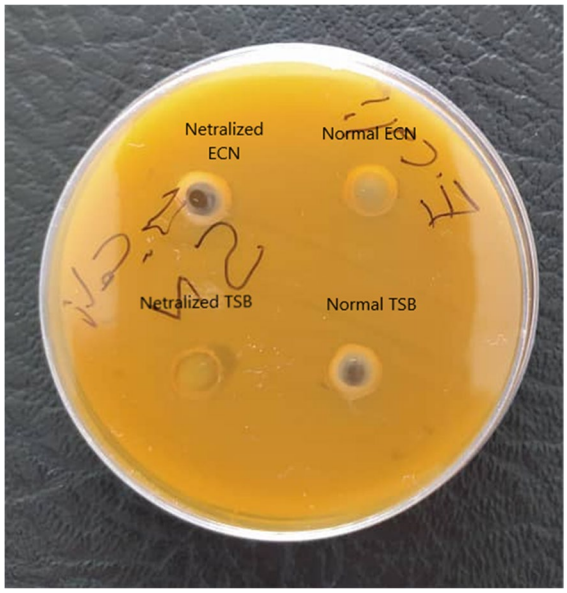

Fig. 1 Inhibition of *Aeromonas hydrophila* by neutralized and non-neutralized *E coli* N1917. After the incubation of *A. hydrophila* on tryptic soy agar plate, no measurable clear zone was noted around the wells with both normal and neutralized spent culture supernatants of EcN

Water tolerance

In the natural water condition, EcN and *A. hydrophila* growth reached 12×10^8 and 3×10^8 CFU/mL in 4-h incubation, respectively.

Growth inhibition by spent culture liquid

After the incubation of *A. hydrophila* on tryptic soy agar plate, no measurable clear zone was noted around the wells with both normal and neutralized spent culture supernatants of EcN (Fig. 1).

In vitro

Fish growth performance

Initial body weight did not show significant differences between all groups ($p>0.05$). After feeding this probiotic, the final body and visceral weights, specific growth rate and feed conversion ratio soared remarkably in the fish fed with EcN2- and EcN3-supplemented diets compared to control group ($p<0.05$). No difference was seen in fish survival between treatments and control during the feeding trial ($p>0.05$) (Table 3).

Immunological parameters

Lysozyme activity was higher in fish receiving EcN2 and EcN3 than the control group ($p<0.001$). Immunoglobulin and total protein levels were almost identical in treatments despite being significantly higher than those in the control group ($p<0.01$). Conversely, complement activity did not change following the

Table 3 Growth performance of goldfish administrated with dietary probiotic *Escherichia coli* strain Nissle 1917 (EcN) at the end of the feeding trial

Parameters	EcN0	EcN1	EcN2	EcN3
Final weight (g)	3.71 ± 0.23^{a}	4.10 ± 0.31^{ab}	4.54 ± 0.30^{b}	4.60 ± 0.28^{b}
Final length (cm)	5.62 ± 0.21^{a}	5.99 ± 0.29^{ab}	6.37 ± 0.09^{b}	6.45 ± 0.13^{b}
Fillet weight (g)	3.01 ± 0.20^{a}	3.20 ± 0.22^{ab}	3.55 ± 0.25^{ab}	3.71 ± 0.26^{b}
Visceral weight (g)	0.548 ± 0.033^{a}	0.639 ± 0.049^{ab}	0.708 ± 0.065^{b}	0.670 ± 0.048^{ab}
Feed conversion ratio	2.79 ± 0.11^{a}	2.70 ± 0.10^{a}	2.49 ± 0.08^{b}	2.40 ± 0.09^{b}
Specific growth rate	1.19 ± 0.13^{a}	1.30 ± 0.08^{ab}	1.41 ± 0.10^{b}	1.45 ± 0.09^{b}
Survival rate (%)	100	100	100	100

Data are mean ± SEM. Those within a row superscripted by different letters are significantly different ($p<0.05$)

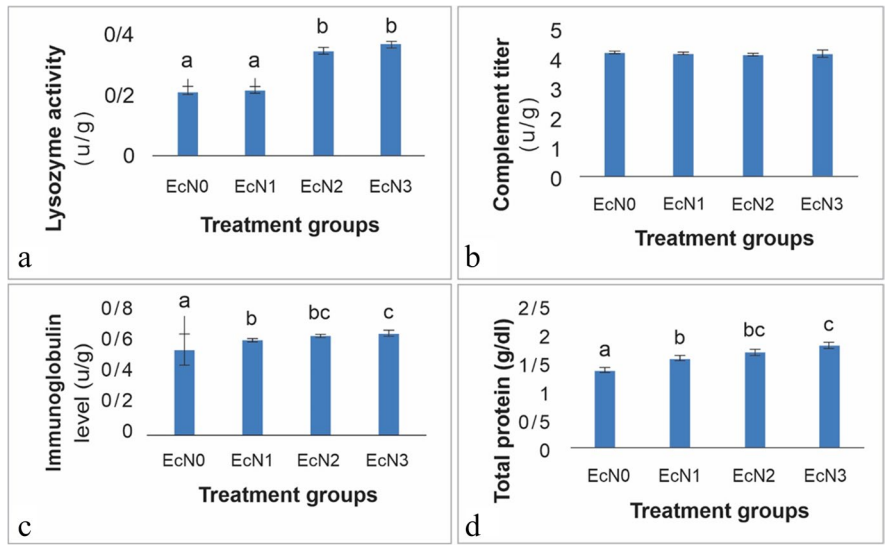

Fig. 2 Immune parameters, including lysozyme activity (**a**), complement titer (**b**), immunogolobulin level (**c**) and total protein (**d**) of goldfish administrated with dietary probiotic *Escherichia coli* strain Nissle 1917 at 0 (EcN0), 10^6 CFU/ g (EcN1), 10^7 CFU/ g (EcN2) and $10.^8$ CFU/ g (EcN3) for 80 days. Data are mean ± SEM. Those within a row superscripted by different letters are significantly different ($p < 0.05$)

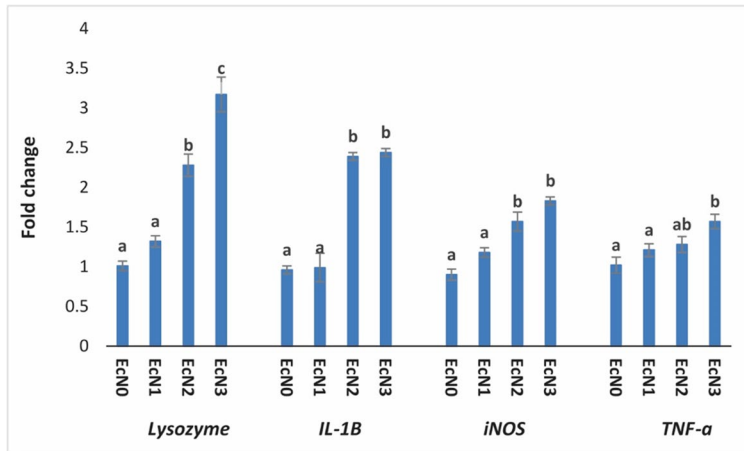

Fig. 3 Relative fold change of immune-related genes in goldfish administrated with dietary probiotic *Escherichia coli* strain Nissle 1917 at 0 (EcN0), 10^6 CFU/g (EcN1), 10^7 CFU/g (EcN2) and $10.^8$ CFU/g (EcN3) for 80 days. Data are mean ± SEM. Those within a row superscripted by different letters are significantly different ($p < 0.05$)

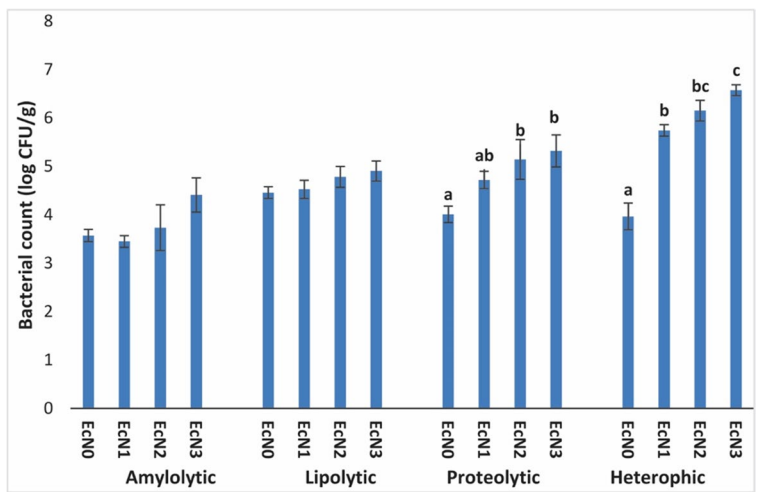

Fig. 4 Amylolytic, lipolytic, proteolytic and total heterotrophic bacterial counts of the gut microbiota in goldfish administrated with dietary probiotic *Escherichia coli* strain Nissle 1917 at 0 (EcN0), 10^6 CFU/ g (EcN1), 10^7 CFU/ g (EcN2) and $10.^8$ CFU/ g (EcN3) for 80 days. Data are mean ± SEM. Those within a row superscripted by different letters are significantly different ($p < 0.05$)

administration of EcN in comparison with the control group ($p > 0.05$) (Fig. 2).

Immune-relevant gene expression

An increase in lysozyme, *IL-1β* and *iNOS* gene expression of fish administrated with EcN2 and EcN3 was noted compared to the control group ($p < 0.001$); however, a higher upregulation of *TNF-α* gene expression was seen only in the EcN3 group than in the control group ($p < 0.001$) (Fig. 3).

Digestive enzymatic and heterotrophic bacteria

No significant change was seen in amylolitic and lipolytic bacterial counts between treatments and control fish ($p > 0.05$); however, proteolytic and heterotrophic bacterial counts demonstrated higher numbers than control fish ($p < 0.05$) (Fig. 4).

Intestinal histology

No pathological alterations were observed in both treatments and control fish (Fig. 5). Details of histomorphological and stereological studies in three segments of fish gut are presented in Table 4. Absorption surface area ($p < 0.001$), goblet cell density ($p < 0.001$) and IEL percentage ($p < 0.001$) in the anterior part of intestine showed a significant increase in the treated fish compared to the control fish, whereas villus height increase was just noted in the fish receiving EcN3 rather than the control group ($p < 0.001$). A notable increase in the size of villus height ($p < 0.01$), absorption surface area ($p < 0.01$), goblet cell density ($p < 0.001$) and IEL percentage ($p < 0.001$) in the mid part of the intestine of the treated fish was noted as against the control fish. In the posterior intestine, with higher villus height ($p < 0.05$) and width ($p < 0.05$), no significant changes in absorption surface area ($p > 0.05$) were noted in probiotic-treated fish in comparison

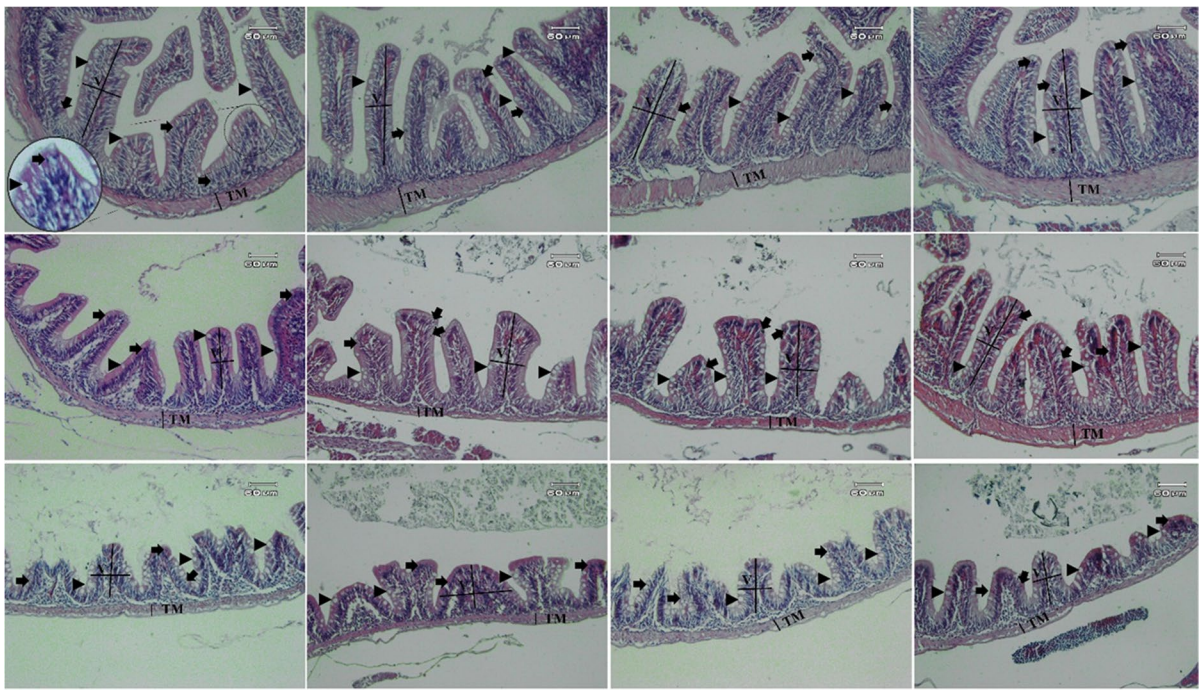

Fig. 5 Histological structure of the intestine segments in goldfish administrated with dietary probiotic *Escherichia coli* strain Nissle 1917 at 0 (EcN0), 10^6 CFU/g (EcN1), 10^7 CFU/g (EcN2) and $10.^8$ CFU/g (EcN3) for 80 days (H&E, ×200). Histological examination showed a normal structure in all groups. However, histomorphological changes (tunica mascularis thickness (TM); villus height and width (V); goblet cell (head arrow) density and intraepithelial lymphocyte (arrow) distribution) were noted in treatment groups depicted in Table 4

Table 4 Histomorphology of goldfish intestine administrated with dietary probiotic *Escherichia coli* strain Nissle 1917 (EcN) at the end of the feeding trial

	Parameters	EcN0	EcN1	EcN2	EcN3
Anterior intestine	Tunica muscularis thickness (μm)	31.40 ± 1.57	30.60 ± 1.17	31.20 ± 1.07	34.40 ± 1.36
	Villus height (μm)	178.00 ± 7.52^{a}	189.00 ± 5.10^{a}	202.00 ± 7.35^{a}	232.00 ± 5.15^{b}
	Villus width (μm)	58.00 ± 2.55	53.00 ± 2.55	52.00 ± 2.00	52.00 ± 1.22
	Absorption surface area (mm^2/villi)	12.29 ± 0.21^{a}	14.33 ± 0.36^{b}	15.55 ± 0.18^{c}	17.87 ± 0.23^{d}
	Goblet cell density (#/mm^2)	14.80 ± 0.80^{a}	21.40 ± 0.81^{b}	24.40 ± 0.93^{b}	30.00 ± 0.95^{c}
	IEL (#/100 enterocytes)	10.00 ± 0.55^{a}	17.20 ± 0.80^{b}	20.80 ± 0.58^{c}	24.40 ± 0.98^{d}
	Tunica muscularis thickness (μm)	31.60 ± 1.50	31.60 ± 1.03	32.20 ± 0.59	33.40 ± 1.21
	Villus height (μm)	126.00 ± 4.30^{a}	141.00 ± 2.45^{b}	155.00 ± 1.58^{c}	167.00 ± 2.00^{d}
Midintestine	Villus width (μm)	65.00 ± 1.58	66.00 ± 1.58	68.00 ± 2.55	63.00 ± 1.22
	Absorption surface area (mm^2/villi)	7.75 ± 0.18^{a}	9.05 ± 0.26^{b}	9.17 ± 0.36^{b}	10.61 ± 0.11^{c}
	Goblet cell density (#/mm^2)	28.00 ± 1.14^{a}	36.60 ± 1.21^{b}	41.20 ± 0.58^{c}	45.60 ± 1.17^{d}
	IEL (#/100 enterocytes)	4.60 ± 0.51^{a}	9.40 ± 0.81^{b}	13.60 ± 0.60^{c}	17.40 ± 0.75^{d}
	Tunica muscularis thickness (μm)	36.20 ± 1.07	35.40 ± 0.75	36.00 ± 1.48	41.00 ± 2.00
	Villus height (μm)	101.00 ± 1.87^{a}	112.00 ± 2.55^{b}	111.00 ± 2.92^{b}	115.00 ± 1.58^{b}
Posterior intestine	Villus width (μm)	84.00 ± 1.85^{a}	95.00 ± 1.58^{b}	92.00 ± 2.00^{b}	91.00 ± 1.87^{ab}
	Absorption surface area (mm^2/villi)	4.82 ± 0.17	4.72 ± 0.17	4.84 ± 0.22	5.06 ± 0.10
	Goblet cell density (#/mm^2)	37.40 ± 1.36^{a}	49.40 ± 0.87^{b}	51.00 ± 1.38^{bc}	56.00 ± 1.76^{c}
	IEL (#/100 enterocytes)	22.00 ± 0.95^{a}	33.40 ± 1.44^{b}	36.80 ± 1.36^{b}	49.03 ± 1.22^{c}

Data are mean ± SEM. Those within a row superscripted by different letters are significantly different ($p < 0.05$)

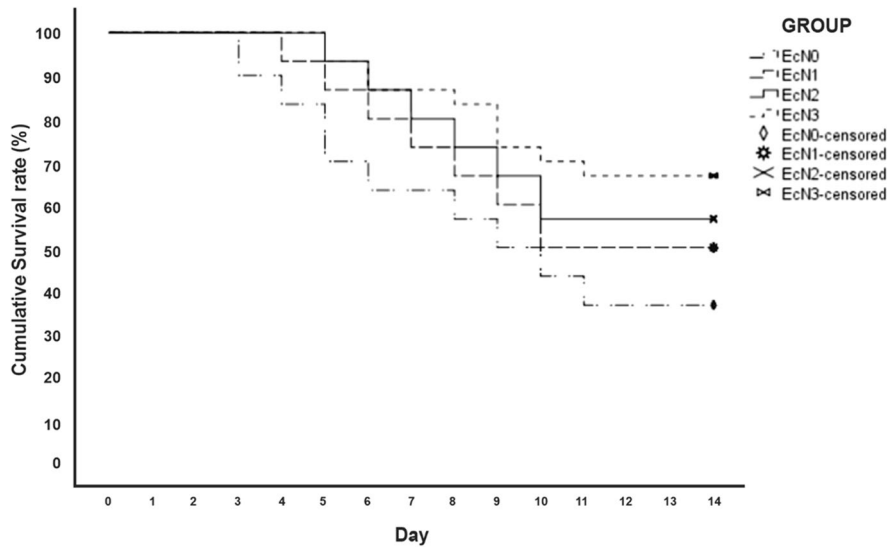

Fig. 6 Survival rate (%) in goldfish fed probiotic *Escherichia coli* strain Nissle 1917 at 10^6 CFU/g (EcN1), 10^7 CFU/g (EcN2) and 10^8 CFU/g (EcN3) and challenged with *Aeromonas hydrophila* infection

to the control group. Moreover, goblet cell density ($p < 0.001$) and IEL percentage ($p < 0.001$) grew meaningfully in treatment groups better than the control group (Table 4).

Disease resistance

Fish challenged with *A. hydrophila* exhibited some clinical signs including haemorrhages on the body, scale protrusion and dropsy a few days after

challenge. Cumulative survival rates of 50.0%, 56.7% and 66.7% were obtained in EcN1, EcN2 and EcN3, respectively, compared to 36.7% in control fish. There was no significant difference among cumulative survivals in EcN2 and EcN1 and control fish ($p > 0.05$), but the cumulative survivals in EcN3 was significantly higher in other treatments and control fish ($p < 0.05$) (Fig. 6).

Discussion

When goldfish was fed the probiotic at different dosages, fish growth was enhanced particularly at 10^8 CFU/ g feed. It is widely known that certain probiotics can induce a positive influence on fish growth through several ways, including providing nutrients or vitamins for the host, enhancing feed digestibility via production of digestive enzymes, improving the morphology of mucosal layers of fish intestine and fine-tuning fish innate immune system that can reduce the level of stress in animal (Pérez-Sánchez et al. 2014; Soltani et al. 2018, 2019; Ringø et al. 2020). In the current study, the proteolytic bacterial count increased in the intestine of fish nourished with the probiotic. These bacteria are able to hydrolyze proteins into smaller peptides or amino acid units which are essential for nitrogenous compounds (Haetami et al. 2019). An increase in the activity of proteolytic enzymes of goldfish fed the probiotic might lead to higher protein digestibility and finally better growth performance. On the other hand, an increase in intestine villi of the treated goldfish could result in the higher fish growth performance.

Manipulation of fish mucosal surfaces is an effective and a ground-breaking means of disease control where outbreak is a persisting concern (Soltani et al. 2019, Ringo et al. 2020); thus, modulating animal immune responses by bio-ingredients such as probiotics is a well-known alternative tool for the protection of target aquatic organisms (Caipang and Lazado 2015; Hoseinifar et al. 2017; Mamun et al. 2019; Dawood et al. 2020; Van Doan et al. 2020). Administration of EcN, in our study, led to enhanced goldfish immunoglobulin and total protein in the intestine, supporting the positive effect of the probiotic on fish immunity. Furthermore, the intestinal immune cells of the treated goldfish showed an increase in the intestinal regions, suggesting the possible dependence of improved mucosal immunity on the presence of higher cells of immune system, e.g. macrophages, lymphocytes, granulocytes and plasma cells in the lamina propria that can play pivotal functions in gut mucosal surface defense system (Hamidian et al. 2018). Similarly, previous reports revealed that feeding fish with probiotics such as *Pediococcus acidilactici* could enhance the presence of IELs in intestine epithelial (Sheikhzadeh et al. 2019; Standen et al. 2013; Nofouzi et al. 2016). Therefore, higher intestinal immune cells in conjunction with the immune-related proteins can increase animal resistance towards potential pathogens. In this study, heightened lysozyme activity was observed in the fish fed with probiotic EcN. In parallel, a higher expression of *lysozyme* gene was observed in the treated fish with probiotic. The iNOS catalyzes the formation of nitric oxide which plays a vital role in different types of immune responses (Singh et al. 2019). It is assumed that higher expression of immune-related genes as well as improved immune indices can result in goldfish protection during the exposure to pathogenic agents.

An enormous amount of goblet cells in fish gut epithelium was noticed after administering the probiotic. What our results unveiled was the heightened goblet density in all three intestinal regions of probiotic-treated fish compared with the control group. The mucus secreted by goblet cells protects intestinal epithelium against physicochemical damages as well as harmful antigens and molecules; it also provides lubricated surface, and prevents the adherence of various pathogens. In addition, this mucus is proved to be more impervious to bacterial glycosidase activity and, thus, defends the intestinal epithelium against bacterial translocation and other infectious pathogens based on its nature (Ringø et al. 2003). Therefore, enhancement of goblet cell density as well as improved immune parameters bolsters the defense mechanism against pathogens in goldfish. This result is in full alignment with the previous study (ZeinEddine et al. 2022) that reported the systemic immune enhancement after feeding Nile tilapia the probiotic EcN.

There are different mechanisms by which various probiotics can affect pathogenic bacteria. A competition among nutrients, assessed by co-culture growth inhibition, was observed for EcN probiotic

to significantly reduce the growth of *A. hydrophila*. However, the probiotic could not produce strong antimicrobial substances against *A. hydrophila* in the culture liquid as was indicated by spent culture. Meanwhile, EcN was able to tolerate fish bile status for the better establishment of the probiotic in the gut of the target host. In fact, when the probiotics are less sensitive to bile, they are more likely to survive passage through the gastrointestinal tract and may easily colonize. Stabilization of a normal gut microflora is another mechanism by which probiotic can affect pathogens. In the current study, total gut bacteria were affected and augmented by the inclusion of probiotic in fish feed which was in alignment with the previous findings that indicated an enhancement in the biodiversity of intestinal microbiota (Ghanbari et al. 2015; Hoseinifar et al. 2017; Ringø et al. 2020). However, other probiotic mechanisms against pathogenic bacteria such as binding of bacterial toxins and penetration through the mucus layer and colonizing the fish intestine were not assessed here. According to the in vivo test, a significant increase in the survival rate following the administration of EcN was also shown. Bacterial enteritis caused by *A. hydrophila* as well as changes in the intestinal tissue and microbiota of fish species were shown previously (Zhou et al. 2020). Therefore, it seems that improved intestinal structure and bacterial count and higher expression of immune-related genes as well as improved immune indices after feeding EcN could result in better disease resistance in goldfish. Nevertheless, more studies are warranted to prove the exact mechanism of action of this probiotic against pathogenic *A. hydrophila* in fish species.

Overall, these data indicated EcN can modulate goldfish immunity and resistance against *A. hydrophila* and improve fish growth status. However, the detailed mechanism of action warrants further research.

Author contribution K. Nofouzi: designing the study, experimental work, funding acquisition, manuscript writing.

N. Sheikhzadeh: designing the study, experimental work, manuscript writing.

G. Hamidian: experimental work, manuscript writing.

A.A. Shahbazfar: experimental work.

M. Soltani: manuscript writing, editing the manuscript.

A. Marandi: field trial.

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Data availability The date supporting the findings of the present study are available under reasonable request.

Declarations

Ethical approval The fish handling procedures were in accordance with the Animal Experimentation Committee of the University of Tabriz (IR.TABRIZU.REC.1398.035).

Data storage and documentation All data that support the findings presented in the manuscript are available within the manuscript.

Competing interests The authors declare no competing interests.

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