Investigation of Spatial Distribution, Antimicrobial, and Histopathological Effects of Probiotics on *Zebrafish* Model Infected with *Aeromonas Hydrophila* Through Evaluation of IL-1β and TNF-α Genes Expression

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Abstract

Background

Usage of “probiotics” for treatment of food-borne pathogens associated diseases, makes a significant reduction in transmission of resistant bacteria, and antimicrobial resistance genes from aquaculture environments to humans. In this research, authors aim to evaluate immunomodulatory, and histological effects of two probiotic strains on *Zebrafish* model. Biometric tests, height, weight, and mortality rate of the fishes were assessed. Afterward, RT-PCR was conducted for bacterial existence of probiotic strains, and quantitative assessment of alterations in targeted immune genes. Subsequently, histological sampling was done for investigation of spatial distribution, and villus length in proximal, middle, and distal sections of intestinal tissues.

Results

Based on the results, there was an increased rate of goblet cells, and villus length in the middle and distal sections of intestinal tissue in case groups receiving both probiotic bacteria in 28th and 56th days. Furthermore, highest number of intraepithelial cells was observed in the proximal sections of intestinal tissue in groups receiving both probiotic bacteria in 56th days. The highest gene expression for *Lactobacillus bulgaricus* was found in groups only treated with this probiotic bacteria in 60th day. The highest gene expression for *Lactobacillus acidophilus* was found in case groups treated with both of probiotic bacteria in 28th day. The highest expression level of IL-1β, and TNF-α were found in control group (only infected by pathogen and receiving no probiotic). The highest survival rate was in case groups only treated with *Lactobacillus bulgaricus.*

Conclusion

To sum up, it seems that usage of probiotic for improvement of public health and fisheries industries can be helpful.

1. Introduction

As one (1) of the fastest growing food-producing sector, aquaculture is going to be drastically diversified. Due to increasing demands for having an environment-friendly aquaculture, it seems rational to do a comprehensive look on need for treatment of food-borne pathogens associated diseases, especially the ones related to fisheries industries (2–7). Fishes are considered as the most abundant seafood products consumed by a wide range of people. Hence, it can be summarized that controlling of infectious diseases in aquatic organisms can majorly contribute to promote healthiness of human beings (8), improve public health, and reduce the rate of Gastrointestinal (GI) disorders in marine organisms and humans like: colitis or Inflammatory Bowel Diseases (IBD) (6, 7, 9). Thereafter, needless to say how socioeconomically and

clinically significant this aforesaid issue could be for aquaculture scientists, food microbiologists, medical microbiologists, environmentalists, and public health managers.

The fish are susceptible to many bacterial, viral, fungal, and parasitic pathogens. In the genera of freshwater and brackish water fish, *Infectious Hematopoietic Necrosis Virus* (*IHNV*), *Lymphocystis Disease Virus* (*LCDV*), *Pseudomonas, Citrobacter*, *Proteus, Streptococcus, Edwardsiella, Staphylococcus*, and different species of *Vibrio*, are considered as the most life-threatening infections. One of the major diseases in the fish, is caused by *Aeromonas hydrophila*, which is colonized in GI tracts of the fish, leading to severe hemorrhagic septicemia and enteritis (10). Current therapeutic strategies for the fish mainly include: usage of chemotherapeutic drugs like antibiotics and vaccination (3, 5, 11). Despite of a wide range of reported efficacies, those aforementioned approaches have shown several major drawbacks. Alteration in gut microflora, inadequacy of vaccines on some fishes due to small size and high mortality rate compared with the big ones, emergence of antibiotic-resistant bacterial strains, ecological contamination in the fish habitats, all impose extortionate expenditure that make basic medical researchers and food science technologists toward more efficient approaches (2, 4, 11, 12).

One of the most newly introduced approaches goes back to the usage of “Probiotics” (Nobel prize awarded to *Metchnikoff* in 1910) as an alternative strategy for making a meaningful decrease in the usage of antibiotics (13), reduction in the re-emergence of antibiotic-resistant strains (as a direct consequence), significant decline in the emergence of new human pathogens (reduced transmission of resistant bacteria from aquaculture environments to humans, and even reduced transfer of antimicrobial resistance genes to human pathogens), as well (14). As Food and Agriculture Organization (FAO) and World Health Organization (WHO) defined probiotics (word with Greek origin) in the first decade of 21st century, “they are living microorganisms with beneficial effects on the health and well-being of the host when orally administered in the sufficient amounts” (3, 4, 6, 14–18). In case of the fish, definition of probiotics is as: “a live, dead, or inactivated microorganism, that can be recruited as whole form of microbial cell preparations or components of microbial cells, which is administered via the feed or to the rearing water, benefiting the host by improving disease resistance, via improving the microbial balance fort hosts, or via improving the microbial balance of the ambient environment” (14, 19, 20).

There are a wide array of researches that highly recommend dietary supplementation with digestible food additives like probiotic bacteria which are classified as health-promoting bacteria. These bacteria can attenuate pathogenicity of GI strains and exacerbate the antibody responses like Immunoglobulin A (IgA), and IgG for preventing infections by effecting on intestinal epithelial and Dendritic Cells (DCs). Otherwise, some strains can induce secretion of Interlukin-6 (IL-6) and Tumor Growth Factor-β (TGF-β) from DCs, enhance IL-12 secretion for Natural Killer (NK) cells activation, and/or inhibit the production of Tumor Necrosis Factor-α (TNF-α) through a Toll-Like Receptor-2 (TLR-2) dependent performance, leading to suppression of NF-κB activation. Among those bacteria, clinical efficacies of heat-inactivated Lactic Acid Bacteria (LAB) have been well-documented. It is worth-mentioning that some of the isolated LAB strains (acid-tolerant facultative anaerobes) with human intestinal tracts resource, have depicted an acceptable probiotic efficacy in animals (2, 7, 21–28).

In spite of the fact that alteration of microbiome through application of probiotics for animals is fairly a new concept, it seems that these probiotics can be of high prominence for aquatic organisms, drawing new horizons of microbial biotherapy for marine organisms, as well. In the fish farming, many published studies have demonstrated profound effects of probiotics for various fish species, including *rainbow trout* (27, 29, 30), common carp (5, 31), finfish (32), Mediterranean species (33), *Mozambique tilapia*, tilapia *(Oreochromis niloticus*) (34–41), juvenile hybrid tilapia (42), *Nile tilapia* (43, 44), *European Sea bass juveniles*, grouper fish (45), gilthead sea bream (*Sparus aurata*) (46), Caspian white fish (*Rutilus frisii kutum*) (47), striped catfish (*Pangasianodon hypophthalmus*) (48), African catfish (*Clarias gariepinus*) (49), Shabot (*Barbus grypus*, and *Tor grypus*) (50, 51), grass carp (52, 53), black swordtail (*Xiphophorus helleri*) (54), eastern oyster (*Crassostrea Virginica*) (55), *zebrafish* (*Danio rerio*) (56–60), and shrimp (6, 61–64). There are several well-characterized and potentiated strains which act as probiotics, altering the composition of intestinal microbiota to achieve favorable effects such as enhancing growth, reproduction, digestion, nutrient absorption and metabolism, intestinal mucosal morphology, competitive binding and blocking of adhesion sites, host defense and immunity against infections, and disease resistance of the host organism. Although, their usage and mechanisms of action are yet to be understood and completely legalized (2, 3, 11, 15, 23, 65, 66). Firstly, this issue is deeply rooted in benefits of probiotics which is beholden to doing researches in the large-scale aquaculture platforms. Additionally, on the one hand, successfulness of probiotics based strategies are highly variable. For instance, there are untackled obstacles including: difficulties in bacterial viability after ingestion, ability of residence in Gut Associated Lymphoid Tissues (GALT) like M cells and Peyer's patches, and amplification of probiotics interaction with GALT cells. Thereafter, advocating of such theories are almost impossible to be guaranteed (21–23). On the other hand, type of administration method (as water or food additives, single or combinative administration, duration of feeding, encapsulated or enriched ones, live or dead/inactivated bacteria, locality to the host or culturing environment) should not be underestimated (4, 20, 66, 67). Therefore, comprehensive researches in order to fully characterize the fish intestinal microbiota, and their mechanisms of action on intestinal ecosystem, immunity, fish health and performance, and optimizing the most efficient dosage as food additives are necessitated to manipulate gut microbiota. Unquestionably, validation of up-to-date and innovative molecular procedures to study of the gut microbiota through *in vitro*, *ex vivo* and *in vivo* models can decipher mentioned ambiguities, and qualify commercialization of aquaculture production.

Totally, in this experimental study, authors aimed to investigate the immunomodulatory properties and spatial distribution of probiotic bacteria (*Lactobacillus acidophilus* and *Lactobacillus bulgaricus*) on GI tissues of a *Zebrafish* model infected by *Aeromonas hydrophila* through histopathological sampling and quantitative assessment of targeted immune genes involved in immunomodulation (IL-1β and TNF-α).

2. Material And Methods

2.1. Ethical Considerations

This experimental study accessed ethics approval from the Science and Research Branch, Islamic Azad University Research Ethic's Committee (no.)

2.2. Study Design and Procedure:

2.2.1. Preparation of Aquarium for the Fish

In order to prepare the most suitable aquarium for our studied population of the fish, 12 aquarium and 12 air pump were bought. After washing, the aquarium container were watered and decholorized. Ventilation pumps were installed for all of them, as well. All requirement for acclimatization of the fish to the environment were done. Constant water temperature with continuous aeration was considered. Water was exchanged daily before feeding.

2.2.2. Preparation of *Zebrafish*

240 Zebrafish which were clinically healthy for follow-up experiments, were bought from fish farm, and kept inside the aquariums (Temperature: 26 ± 2, pH: 7-7.5, light: 12:12, and salinity: 0.25–0.75 ppt). Feeding was done by commercialized food (as a basal diet) commencing from one week before main test (during acclimatization period). Probiotic based food was considered in our study during our assay. All experiments on fish, were carried out based on the Regulations for Animal Experimentation at Jilin Agricultural University (JLAU08201409).

2.2.3. Preparation of Probiotic Bacterial Strains

Lactobacillus acidophilus and *Lactobacillus delbrueckii* strains subspecies *Lactobacillus bulgaricus* were dedicated by microorganism bank of Iranian Biological Resource Center (ATCC® 4356™, and ATCC® BAA2844™, respectively). After transferring those bacterial culture petri dishes into our well-equipped microbiology laboratory, isolation and suspension of both mentioned probiotic strains was made from culture of both strains. Then, a loop of both bacteria were inoculated into de Man, Rogosa and Sharpe (MRS) broth (as a selective medium) (Merck Co, Germany), and anaerobically incubated in 37ºC in an atmosphere of 5% CO2 for 24 hours on shaking incubator with 180 round per minute (rpm). After achieving enough turbidity and optimized concentration of bacterial growth, 100 milliliter (ml) of bacterial suspension was centrifuged for 15 minutes with 4000 rpm and supernatant was removed. Then, bacterial pellet was dissolved in normal saline (pH = 7.5). Thereafter, 0.5. McFarland Standard was prepared to adjust the turbidity of bacterial suspension (0.05 mL of 1.175% barium chloride dihydrate ($BaCl_2 \cdot 2H_2O$), with 9.95 mL of 1% sulfuric acid (H_2SO_4)). Here, viable counts of cultured bacteria (in the log or exponential phase) as Colony Forming Unit (CFU) measured at Optical Density $_{600nm}$ (OD_{600nm}) was equivalent to 1×10^8. They were aliquoted in 15cc falcons, and after adding glycerol 15% v/v (Merck Co, Germany), stored temporarily at freezer with − 20ºC until further usage in next steps.

2.2.4. Preparation of Pathogenic Bacterial Strain

Aeromonas hydrophila strain was dedicated by microorganism bank of Iranian Biological Resource Center (ATCC® 7966™). After transferring this bacterial culture petri dishes into our well-equipped

microbiology laboratory, isolation and suspension of mentioned strains was made from culture of strain. Then, a loop of bacteria was inoculated into Luria-Bertani (LB) Broth (as an enrichment medium) (Merck Co, Germany), and a serial dilution was made. Then, confirmed bacteria were cultured in Nutrient (NA) Agar (Merck Co, Germany). Thereafter, 0.5. McFarland Standard was prepared as mentioned earlier in order to adjust the turbidity of bacterial suspension. Here, viable counts of cultured bacteria (in the stationary phase) as CFU measured at OD_{600nm} was equivalent to 1.3×10^{8} CFU g^{-1}. Bacteria were resuspended and transferred to tanks at a final concentration of 1.3×10^{8} CFU g^{-1}. Of note, making an infectious model for studied population of *Zebrafish* was measured out 56 days after starting our study.

2.2.5. Feeding

Feeding was done through commercialized food of KOI & GOLDFISH (pellet) twice a day (11.00 AM and 17.00 PM) for control group. For case groups, feeding was done through probiotic based food twice a day (11.00 AM and 17.00 PM). The amount of food was estimated as 2% of overall weight of the fish in both of groups. Mean weight of the fish was assessed through M.T.ELECTRONIC BALANCE. The amount of needed food was calculated according to the number of the fish in each aquarium and mean weight of the fish in every round of feeding.

According to the weight of needed food, probiotics were added as 1%-2% of food which was calculated 0.0003g. (As one milliliter of bacteria is equivalent to 500 milligram (mg) of dried pellet of bacteria). To preserve the powdered phase of each pellet, skimmed milk was mixed with them. Formulation for preparation of each food is as following:

Three gram skimmed milk + 100 gram food pellet + 1–2 gram probiotics

It is worthy to mention that one of the variable in this study is detection and spatial distribution of probiotics in the intestinal tissues of the fish. So, fluorescent dying was formulated in preparation of the probiotic based food.

2.2.6. Case and Control Groups

In this study, a random distribution was done foe the fish. In each aquarium, 20 *Zebrafish* were considered. Hence, studied population of the fish were divided into four groups as following with three repetition:

A. Control group: *Zebrafish* infected with *Aeromonas hydrophila* and receiving no probiotic bacteria.
B. First Case group: *Zebrafish* infected with *Aeromonas hydrophila* and receiving 1×10^{8} CFU g^{-1} of both probiotic bacteria.
C. Second Case group: *Zebrafish* infected with *Aeromonas hydrophila* and receiving only 1×10^{8} CFU of *Lactobacillus acidophilus*.
D. Third Case group: *Zebrafish* infected with *Aeromonas hydrophila* and receiving only 1×10^{8} CFU of *Lactobacillus delbrueckii*.

2.2.7. Biometric Tests

From 0th day of the test, height and weight of the *Zebrafish* were measured by a ruler and M.T.ELECTRONIC BALANCE, respectively. Mortality rate of each treated group was registered since 0th day of starting our study.

2.2.8. Histopathological Sampling (Hematoxylin & Eosin (H&E))

For Investigation of Intestinal Villus through: By a random selection, three fish were collected from each treated groups. Under biological hood and aseptic condition, anatomical site of the intestine was dissected and intestinal tissues were separated. Samples were fixed with formalin 10% for 24 hours. In the next step, samples were put in ethanol for dehydration with ascending degrees as: 50 minutes in 70º, 50 minutes in 80º, 50 minutes in 90º, and 50 minutes in 100º, respectively. Next, xylol type I (45º) and II (pure) was used for substitution with ethanol for 40 minutes. Then, to remove xylol, samples were soaked and infiltrated in melted paraffin I and II (70ºC) for 40 minutes. After paraffin freezing, samples were placed in an embedded melted paraffin to be prepared for sectioning by microtome (5–10 micron). Afterward, to melt paraffin, prepared lams were put in oven 90ºC for 20 minutes. In order to have a transparent samples and removing paraffin of the samples, xylol type I (45º) and II (pure) was used for 10 minutes. Now, samples were put in ethanol, for hydration and removing ethanol with descending degrees as: one minute in 100, one minute in 90, one minute in 80, one minute in 70, and one minute in tap water. At the end, samples were dyed with Hematoxylin as basophil dye for 15 minutes and then were washed. At the end, they were put in Eosin for 10 seconds. In this stage, dehydration by ascending degrees of ethanol, and infiltration by xylol were done as mentioned earlier. After doing mountage with Entellan for sticking lam and lamels and removing air bubbles, lams were prepared for being investigated by fluorescent microscopes.

For detection of probiotics in the Intestinal Tissues

By a random selection, three fish were collected from each treated groups. Under biological hood and aseptic condition, anatomical site of the intestine was dissected and intestinal tissues were separated. Samples were fixed in formalin 10%. As it was mentioned earlier, fluorescent dying was used for detection of probiotics in the intestinal tissues and investigation of spatial distribution. Rhodamine (green for *Lactobacillus acidophilus*) and DiI (red for *Lactobacillus delbrueckii*) dyes were formulated in the preparation of each probiotic based food (wavelength = 520nm). Formulation for preparation of each dye in the food pellet is as following

1 µ of each dye + 1 µ of suspended bacteria + 1000 µ of Phosphate Buffer Saline (PBS)

After incubation of this solutions for five minutes in room temperature, dark vials containing mentioned solution were incubated for 20 minutes in the refrigerator temperature. Fluorescence microscopy based approaches were used for detection of formulated probiotic bacteria.

For Qualitative Detection of Probiotics through PCR

In order to quantitatively assess the expression levels of each probiotic in the intestinal tissues, sampling was done in the 28th, 56th, and 60th day of our assay.

Detection of Probiotics through PCR

Table 1 reveals used primers (forward and reverse primers) according to mentioned sequences for detection of probiotic bacteria through PCR assay.

Table 1
Forward and reverse primers used for detection of both probiotic bacteria through PCR.

Primer name	Sequence	Length (Amplicon)
Forward primer *L. acidophilus*	GAAAGAGCCCAAACCAAGTGATT	…. bp
Reverse primer *L. acidophilus*	CTTCCCAGATAATTCAACTATCGCTTA	
Forward primer *L. delbrueckii*	CACTTGTACGTTGAAAACTGAATATCTTAA	….. bp
Reverse primer *L. delbrueckii*	CGAACTCTCTCGGTCGCTTT	

For Quantitative Assessment of IL-1β and TNFα genes through RNA Extraction and Real-Time PCR (RT-PCR):

By a random selection, in the 28th, 56th, and 60th day of our assay, three *Zebrafish* were collected from each treated groups. Under biological hood and aseptic condition, anatomical site of the intestine was dissected and intestinal tissues were separated. Samples were fixed in formalin 10%, transferred into the molecular laboratory and kept inside RNase-free vials in the freezer − 80ºC until further use in the next steps (RNA extraction).

RNA Extraction for Quantitative Assessment of IL-1β and TNFα genes:

In the time of assay, samples were removed from freezer and 300 µl trizol was added to homogenated tissues (next to ice box). Then RNase-free micro tubes were vortexed for 10 seconds and kept in room temperature for five minutes. Then, under biological hood situation, 200 µl cold chloroform was added and content of micro tubes mixed gently for 15 seconds. Next, after keeping micro tubes in 4ºC for five minutes, they were centrifuged for 17 minutes and 8000 rpm in 4ºC. After watching a clear supernatant, cold isopropanol (volume equivalent to the supernatant) was added to the content of micro tubes and mixed gently. Then micro tubes were incubated in 4ºC for 10 minutes, which is followed by a centrifugation for 12 minutes and 8000 rpm in 4ºC and resulted in protein denaturation. After removing supernatants, 200 µl of ethanol 80% v/v was added to the pellet in order to precipitate RNA and micro tubes were vortexed for 10 seconds. In the next step, centrifugation was conducted for five minutes and

7500 rpm in 4ºC and supernatants were removed. In this step, a 15-minutes pause was needed to reach semi-wet pellets. Subsequent to reaching semi-wet pellets, 30 µl of Di Ethyl Pyro Carbonate (DEPC) water (free of RNase) was added in order to dissolve RNA precipitation and micro tubes were placed in water bath in 55ºC-60ºC for 15 minutes aimed at complete dissolving of RNA precipitation. Enzymatic properties of RNase-free DNase I was used for removing any DNA contamination. One microliter of $MgCl_2$ 10X buffer and 0.5 µl of RNase-free DNase I were added to one microliter of RNA solution and the volume optimized with DEPC water to 10 µl. After incubating in 37ºC for 30 minutes, one microliter of EDTA was added in order to neutralize RNase-free DNase I and samples were incubated in 65 ℃ for 10 minutes. Quantity, purity, and integrity of extracted RNA were tested using a spectrophotometer and by electrophoresis on 1% agarose gels as following.

cDNA Synthesis for Quantitative Assessment of IL-1β and TNFα genes

For quantitative investigation on concentration of total extracted RNA, nanodrop based spectrophotometry approach was measured out and concentration of RNA was calculated (as ng/µl) in the wavelengths of 260 and 280 nm (260/280). For cDNA synthesis, after confirming about sufficient concentration of RNA, 10 µl of cDNA synthesis reagent was added to 10 µl of treated RNA according to the manufacturer's instructions (Easy cDNA Synthesis kit, Pars tous Co). Then samples were incubated in room temperature (25ºC) for five minutes and subsequently in 60C for 60 minutes through thermocycler. Afterward, they were storage on ice boxes and freezed in -20ºC until further steps.

Designation of Forward and Reverse Primers for Quantitative Assessment of IL-1β and TNFα genes

In the time of assay, Real-Time PCR was recruited for the quantitative assessment of immune genes. Firstly, genome of Zebrafish was investigated and GAPDH gene was considered as standardized (reference) gene. Reverse and forward primers for immune genes (IL-1β and TNFα) were designated by Gen Runner application (version 6.5) and made by SINACOLON Company. The list of synthesized primers and amplified sequences (Table 2) are as following:

Table 2
Forward and reverse primers used for quantitative detection of reference, and immune genes.

Primer name	Sequence	Length (Amplicon)
Forward primer d-GAPDH	CAGAACATCATCCCAGCCTCC	152 bp
Reverse primer d-GAPDH	TTGGCAGGTTTCTCAAGACGG	
Forward primer d-IL-1β	ACAGCACACACACTGATGCAC	218 bp
Reverse primer d-IL-1β	AGAATAAGCAGCACTTGGGGA	
Forward primer d-TNF-α	TGGATTGTGAACGAAAGTGAG	108 bp
Reverse primer d-TNF-α	AGCAATGTTCAGATGTGTTGG	

In order to refrain from any homologies and complementary sequences with nucleotide sequences of other genes, mentioned primers were rechecked in BLAST search tool of National Center for Biotechnology Information (NCBI) website in (http://www.ncbi.nlm.nih.gov/Blast). For estimation of our target immune genes/reference gene ratio, efficiency for PCR is hypothesized 100%. So, this formula was considered:

$$Ratio = 2^{-\text{Error! Bookmark not defined.}}$$

RT-PCR for Quantitative Assessment of IL-1β and TNFα genes

Name of the reagents and their used volumes for each reagent was mentioned in the Table 3:

Table 3
Reagents and used volume for quantitative assessment of IL-1 and TNF genes.

βα	
Reagents	**Volume (Total Volume 10 µl)**
Real Q Plus 2x Master Mix Green (HIGH ROX) Including: KCl, MgCl2, dNTP, taq DNA polymerase	5 µl
cDNA	2 µl
Forward Primers	0.5 µl
Reverse Primers	0.5 µl
DEPC water	2 µl

Stages of RT-PCR (cycles and temperature) was mentioned below in Table 4.

Table 4
Cycles and temperature in RT-PCR assay for quantitative assessment of immune genes.

Step	Temperature	Time	Cycles
Initiation	95	15 minutes	One
Denaturation	95	15 seconds	40
Ligation	60	30 seconds	40
Elongation	60	30 seconds	40

2.3. Bio-Statistical Analysis of Data

Prism software and ANOVA statistical test were used to analyze the obtained results. All tests were performed in triplicates and results were expressed as Mean plus minus Standard Deviation (Mean ± SD). Overall statistical significant difference level was considered as p value < 0.05.

3. Results

3.1 Histopathological Sampling of Proximal, Middle, and Distal Sections of Intestinal Tissues through H&E Dying in 0th, 28th, 56th, and 60th day of Assay

Figures (Fig. 1, 2, 3, 4) show results related to histopathological sampling of proximal, middle, and distal sections in intestinal tissues of *Zebrafish* models infected by *Aeromonas hydrophila* in all case and control groups (G1, G2, G3, and G4) in 0th, 28th, 56th, and 60th day of assay.

3.1.1. Histopathological Sampling of Proximal, Middle, and Distal Sections of Intestinal Tissues through H&E Dying in 0th day of Assay

Figure 1, depicts histopathological changes in proximal, middle, and distal sections in intestinal tissues of *Zebrafish* models infected by *Aeromonas hydrophila* in all case and control groups (G1, G2, G3, and G4) in 0th day of assay.

According to the Fig. 1, histopathological sampling of proximal, middle, and distal sections of intestinal tissues through H&E dying in 0th day of assay for all case and control groups, did not show any pathological signs.

3.1.2. Histopathological Sampling of Proximal, Middle, and Distal Sections of Intestinal Tissues through H&E Dying, and Villus Length in 28th day of Assay

Figure 2, reveals histopathological changes of proximal, middle, and distal sections in intestinal tissues of *Zebrafish* models infected by *Aeromonas hydrophila* in all case and control groups (G1, G2, G3, and G4) in 28th day of assay. For each section, villus length is also calculated in a separated graph.

According to the Fig. 2, there is a significant difference among all case and control groups (G1, G2, G3, and G4) in case of length of intestinal villus and spatial distribution in intra-epithelial lymphocytes and goblet cells, after 28 days of treatment with probiotics in *Zebrafish* models infected by *Aeromonas hydrophila*. There is an increased rate of goblet cells in proximal, middle, and distal sections in intestinal tissues of *Zebrafish* models infected by *Aeromonas hydrophila* in all case groups after 28 days of treatment with probiotics in comparison with control groups. To be more precise, there is an increased rate of goblet cells in proximal, middle, and distal sections in intestinal tissues of *Zebrafish* models infected by *Aeromonas hydrophila* in case groups treated with both mentioned probiotic strains (G2) for 28 days compared with control groups. Additionally, there is an increased rate of goblet cells in middle sections in intestinal tissues of *Zebrafish* models infected by *Aeromonas hydrophila* compared to proximal and distal sections.

For the length of villus in zero groups and normal groups, there is not found any significant difference between zero groups (not being infected by *Aeromonas hydrophila*) and normal groups of *Zebrafish* models (only being infected by *Aeromonas hydrophila* and not receiving any probiotics) after 28 days of assay. Meanwhile, of note, three is an increased rate of intestinal villus in proximal, and middle sections in intestinal tissues of *Zebrafish* models infected by *Aeromonas hydrophila* treated with probiotic bacterial strains for 28 days compared to distal sections.

On the one hand, to make a comparison between first case group (*Zebrafish* infected with *Aeromonas hydrophila* and receiving 1×10^8 CFU of both probiotic bacteria) and each of second and third case groups (receiving only one type of probiotic strains), there is an insignificant increased rate of intra-epithelial lymphocytes in first case group than second and third groups. On the other hand, there is a significant difference between first case group and second/third groups in proximal, and middle sections in intestinal tissues of *Zebrafish* models infected by *Aeromonas hydrophila*.

3.1.3. Histopathological Sampling of Proximal, Middle, and Distal Sections of Intestinal Tissues through H&E Dying, and Villus Length in 56th day of Assay

Figure 3, reveals histopathological changes of proximal, middle, and distal sections in intestinal tissues of *Zebrafish* models infected by *Aeromonas hydrophila* in all case and control groups (G1, G2, G3, and G4) in 56th day of assay. For each section, villus length is also calculated and reported in a separated graph.

According to Fig. 3, there is an increased rate of goblet cells in middle, and distal sections in intestinal tissues of *Zebrafish* models infected by *Aeromonas hydrophila* with a significant difference among second/third case groups (G3, and G4) and control groups (G1) after 56 days of assay in comparison with control groups.

In addition, there is an increased length of intestinal villus in *Zebrafish* models infected by *Aeromonas hydrophila* treated with probiotic bacteria (G2, G3, and G4) after 56 days of assay in comparison with zero and control groups (G1). There is a significant difference in of intestinal villus in *Zebrafish* models infected by *Aeromonas hydrophila* treated with both probiotic bacteria (G2) after 56 days of assay in comparison with zero and control groups.

There is an increased length of intestinal villus in proximal section of intestinal tissues in *Zebrafish* models infected by *Aeromonas hydrophila* treated with both probiotic bacteria (G2) after 56 days of assay in comparison with middle and distal sections of intestinal tissues.

There is a significant difference in intra-epithelial lymphocytes of proximal, middle, and distal sections of *Zebrafish* models infected by *Aeromonas hydrophila* treated with both probiotic bacteria (G2) after 56 days of assay in comparison with control groups.

3.1.4. Histopathological Sampling of Proximal, Middle, and Distal Sections of Intestinal Tissues through H&E Dying, and Villus Length in 60th day of assay

Figure 4, reveals histopathological changes of proximal, middle, and distal sections in intestinal tissues of *Zebrafish* models infected by *Aeromonas hydrophila* in all case and control groups (G1, G2, G3, and G4) in 60th day of assay.

For each section, villus length is also calculated in a separated graph.

According to Fig. 4, it has been demonstrated that there was a decreased rate of intestinal villus with a significant difference between control groups (G1) and probiotic receiving groups (G2, G3, and G4) after 60 days of assay. Also, there was a decreased rate of intestinal villus in Zebrafish models infected by *Aeromonas hydrophila* treated with probiotic bacteria (G3, G4) with a significant difference compared to zero groups after 60 days of assay. Interestingly, there is an increased rate of intra-epithelial lymphocytes in Zebrafish models infected by *Aeromonas hydrophila* treated with both probiotic bacteria (G2) with a significant difference in comparison with other groups after 60 days of assay. There is a decreased rate of goblet cells in proximal, middle, and distal sections of intestinal tissues of Zebrafish models infected by *Aeromonas hydrophila* treated with probiotic bacteria (G3, G4) in comparison with control groups (G1) three days after induction of infectious by *Aeromonas hydrophila*.

Totally, according to the acquired results, from assessment on 0th, 28th, 56th, and 60th day of treatment with probiotics in all control and case groups, it can be summarized that there is an increased rate of intestinal villus length in middle, and distal sections of intestinal tissues of Zebrafish models infected by *Aeromonas hydrophila* treated with both probiotic bacteria (G2) with a significant difference in comparison with other groups after 28 and 56 days of assay. It is worth-mentioning that there is an increased rate of intra-epithelial lymphocytes in proximal sections of intestinal tissues of Zebrafish models infected by *Aeromonas hydrophila* treated with both probiotic bacteria (G2) with a significant difference in comparison with other groups after 56 days of assay.

3.2. Fluorescent Dying for Investigation of Spatial Distribution

Figure 5, shows results related to investigation of spatial distribution after fluorescent dying (Rhodamine and Dil) for all case and control groups (G1, G2, G3, G4).

3.3 Quantitative Assessment of Both Probiotics in the Intestinal Tissues through Real-Time PCR in 28th, 56th, and 60th day of Assay

Figure 6, depicts results related to quantitative assessment of both probiotics in intestinal tissues of *Zebrafish* models infected by *Aeromonas hydrophila* in all case and control groups (G1, G2, G3, and G4) in 28th, 56th, and 60th day of assay. Here, all of the results were reported in comparison with control groups (G1).

On the one side, according to Fig. 6, results of quantitative assessment of *Lactobacillus acidophilus* in the intestinal tissues through Real-Time PCR in 28th day of assay, show that the existence of mentioned probiotic is not found (or very low existence) in zero group. On the other side, the most abundant existence of *Lactobacillus acidophilus* is found in intestinal tissues of *Zebrafish* models infected by *Aeromonas hydrophila* receiving both prebiotic bacteria (G2) in comparison with control and zero groups. There is not any significant differences among zero, control (G1), and cases groups receiving only one type of probiotic bacteria (G3, G4).

Results of quantitative assessment of *Lactobacillus delbrueckii* in the intestinal tissues through Real-Time PCR in 28th day of assay, show that the existence of mentioned probiotic is not found (or very low existence) in zero, control, and cases receiving only one type of probiotic bacteria group (G3) without any significant difference. The most abundant existence of *Lactobacillus delbrueckii* is found in intestinal tissues of *Zebrafish* models infected by *Aeromonas hydrophila* receiving both prebiotic bacteria (G2), and cases receiving only one type of probiotic bacteria group (G4) in comparison with control, zero, and cases receiving only one type of probiotic bacteria group (G3) groups. There is an increased rate for existence of *Lactobacillus delbrueckii* in intestinal tissues of *Zebrafish* models infected by *Aeromonas hydrophila* receiving both prebiotic bacteria (G2) compared to cases receiving only one type of probiotic bacteria group (G4) without any significant difference.

Results of quantitative assessment of *Lactobacillus acidophilus* in the intestinal tissues through Real-Time PCR in 56th day of assay, show that the existence of mentioned probiotic bacteria is not found (or very low existence) in zero, control (G1), and cases receiving only one type of probiotic bacteria group (G4) without any significant difference. Among aforesaid groups, cases receiving only one type of probiotic bacteria group (G4) depict an increased rate for mentioned probiotic bacteria compared to zero, and control (G1) groups without any significant difference. On the other side, the most abundant existence of *Lactobacillus acidophilus* is found in intestinal tissues of Zebrafish models infected by *Aeromonas hydrophila* receiving both prebiotic bacteria (G2), and cases receiving only one type of probiotic bacteria group (G3) with significant difference in comparison with three other groups. Between G2 and G3, there is not any significant difference for increased rate of *Lactobacillus acidophilus.*

Results of quantitative assessment of *Lactobacillus delbrueckii* in the intestinal tissues through Real-Time PCR in 56th day of assay, show that the existence of mentioned probiotic is not found (or very low existence) in zero, control, and cases receiving only one type of probiotic bacteria group (G3) without any significant difference. Among aforesaid groups, cases receiving only one type of probiotic bacteria group (G3) depict an increased rate for mentioned probiotic bacteria compared to zero, and control (G1) groups without any significant difference. The most abundant existence of *Lactobacillus delbrueckii* is found in intestinal tissues of *Zebrafish* models infected by *Aeromonas hydrophila* receiving both prebiotic bacteria (G2), and cases receiving only one type of probiotic bacteria group (G4) in comparison with control, zero, and cases receiving only one type of probiotic bacteria group (G3) groups with a significant difference. Between these two groups, there is an increased rate for existence of *Lactobacillus delbrueckii* in cases receiving only one type of probiotic bacteria group (G4) in comparison with cases receiving both prebiotic bacteria (G2) group without any significant difference.

Results of quantitative assessment of *Lactobacillus acidophilus* in the intestinal tissues through Real-Time PCR in 60th day of assay, show that the existence of mentioned probiotic bacteria is not found (or very low existence) in zero, control (G1), and cases receiving only one type of probiotic bacteria group (G4) without any significant difference. On the other side, the most abundant existence of *Lactobacillus acidophilus* is found in intestinal tissues of *Zebrafish* models infected by *Aeromonas hydrophila* receiving both prebiotic bacteria (G2), and cases receiving only one type of probiotic bacteria group (G3) without any significant difference. There is an increased rate for existence of *Lactobacillus acidophilus* in cases receiving both prebiotic bacteria (G2), in comparison with zero, control, and cases receiving only one type of probiotic bacteria (G4) group with significant difference.

Results of quantitative assessment of *Lactobacillus delbrueckii* in the intestinal tissues through RT-PCR in 60th day of assay, show that the existence of mentioned probiotic is not found (or very low existence) in zero, control, and cases receiving only one type of probiotic bacteria group (G3) without any significant difference. Among aforesaid groups, cases receiving only one type of probiotic bacteria group (G3) depict an increased rate for mentioned probiotic bacteria compared to zero, and control (G1) groups without any significant difference. The most abundant existence of *Lactobacillus delbrueckii* is found in intestinal tissues of *Zebrafish* models infected by *Aeromonas hydrophila* receiving both prebiotic bacteria (G2), and cases receiving only one type of probiotic bacteria group (G4) in comparison with control, zero, and cases receiving only one type of probiotic bacteria group (G3) groups with a significant difference. Between these two groups (G2, and G4), there is an increased rate for existence of *Lactobacillus delbrueckii* in cases receiving only one type of probiotic bacteria group (G4) in comparison with all four groups (zero, control, cases receiving both prebiotic bacteria (G2) group without any significant difference.

3.4 Quantitative Assessment of IL-1β and TNF-α genes through RT-PCR in 28th, 56th, and 60th day of Assay

Figure 7, shows results related to quantitative assessment of IL-1β and TNF-α genes as immune target genes in intestinal tissues of *Zebrafish* models infected by *Aeromonas hydrophila* in all case and control

groups (G1, G2, G3, and G4) in 28th, 56th, and 60th day of assay. Here, results are considered as effects of probiotic bacteria on alterations in the expression levels of immune genes according to GAPDH as reference gene and in comparison with control groups.

According to Fig. 7, lower levels of IL-1β expression is found in zero, and cases receiving both probiotic bacteria groups in comparison with control, and cases receiving only one type of probiotic bacteria (G3, and G4) groups in 28th day of assay. The lowest and the highest levels of IL-1β expression are related to zero, and control groups, respectively. There is not found any significant difference for levels of IL-1β expression among control, cases receiving only one type of probiotic bacteria (G3, and G4) groups, and cases receiving both prebiotic bacteria (G2) groups, in 28th day of assay. In addition, there is found an increased level of IL-1β expression with a significant difference in control (G1), cases receiving only one type of probiotic bacteria (G3, and G4) groups compared to zero group in 28th day of assay.

Data related to 56th day of assay, revealed that lowest and the highest levels of IL-1β expression are related to zero, and control groups, respectively. There is an increased level of IL-1β expression in cases receiving only one type of probiotic bacteria (G3) groups compared to cases receiving only one type of probiotic bacteria (G4) groups, and cases receiving both prebiotic bacteria (G2) groups without any significant difference. Also, there is not any significant difference in the level of IL-1β expression among control, cases receiving only one type of probiotic bacteria (G3, and G4) groups, and cases receiving both prebiotic bacteria (G2) groups, in 56th day of assay. Only a significant difference in the level of IL-1β expression is found between zero, and control groups (p value ≤ 0.05).

Investigation of data acquired from 60th day of assay (three days after making our *Zebrafish* models infected by *Aeromonas hydrophila*) indicated that lowest and the highest levels of IL-1β expression are related to zero, and control groups, respectively. Among cases receiving only one type of probiotic bacteria (G3, and G4) groups, and cases receiving both prebiotic bacteria (G2) groups, highest levels of IL-1β expression is related to cases receiving only one type of probiotic bacteria (G4) groups and no significant difference is found. Similarly, there is not found any significant difference for levels of IL-1β expression among control, cases receiving only one type of probiotic bacteria (G3, and G4) groups, and cases receiving both prebiotic bacteria (G2) groups in 60th day of assay. Only, there is found a significant difference for levels of IL-1β expression between control, and cases receiving only one type of probiotic bacteria (G4) groups, with zero group in 60th day of assay.

Investigation of data acquired from quantitative assessment of TNF-α in 28th day of assay revealed that the lowest and the highest levels of TNF-α expression are related to zero, and control groups, respectively. Among cases receiving only one type of probiotic bacteria (G3, and G4) groups, and cases receiving both prebiotic bacteria (G2) groups, highest levels of TNF-α expression is related to cases receiving only one type of probiotic bacteria (G3) groups and no significant difference is found. Similarly, there is not found any significant difference for levels of TNF-α expression among zero, and control groups, with cases receiving only one type of probiotic bacteria (G3, and G4) groups, and cases receiving both prebiotic

bacteria (G2) groups in 28th day of assay. Totally, there is not found any significant difference for levels of TNF-α expression among all control and case groups in 28th day of assay.

In case of data acquired from quantitative assessment of TNF-α in 56th day of assay, it is demonstrated that that the lowest and the highest levels of TNF-α expression are related to cases receiving both prebiotic bacteria (G2) groups, and control groups, respectively. Among cases receiving only one type of probiotic bacteria (G3, and G4) groups, and cases receiving both prebiotic bacteria (G2) groups, highest levels of TNF-α expression is related to cases receiving only one type of probiotic bacteria (G3) groups and no significant difference is found. Identically, there is not found any significant difference for levels of TNF-α expression among zero, and cases receiving only one type of probiotic bacteria (G3, and G4) groups, and cases receiving both prebiotic bacteria (G2) groups in 56th day of assay. Of note, there is a significant difference for levels of TNF-α expression between control, and zero groups, and between control, and cases receiving only one type of probiotic bacteria (G4) groups, and cases receiving both prebiotic bacteria (G2) groups, as well.

With regard to data acquired from quantitative assessment of TNF-α in 60th day of assay, it is shown that the lowest and the highest levels of TNF-α expression with a significant difference are related to zero, and control groups, respectively. No significant difference is found among control groups, and cases receiving only one type of probiotic bacteria (G3, and G4) groups, and cases receiving both prebiotic bacteria (G2) groups in 60th day of assay. Among three receiving prebiotic bacteria (G2, G3, and G4), highest levels of TNF-α expression is related to cases receiving only one type of probiotic bacteria (G4) groups with no significant difference. Totally, usage of probiotics did not show significant effects on levels of TNF-α expression in intestinal tissues of *Zebrafish* models infected by *Aeromonas hydrophila* in 60th day of assay.

3.5 Biometric Assessment of the Zebrafish Models through Weekly Investigation of Mortality Rate, and Height, and Body Weight in 14th, 28th, and 56th day of Assay

Figure 8, and 9, show results related to biometric assessment of *Zebrafish* models infected by *Aeromonas hydrophila* through investigation of parameters as: mortality rate (weekly presented), height, and body weight in all case and control groups (G1, G2, G3, and G4) in 14th, 28th, and 56th day of assay.

3.5.1. Biometric Assessment of the *Zebrafish* through Investigation of Mortality Rate

Figure 8, shows results related to biometric assessment of *Zebrafish* models infected by *Aeromonas hydrophila* through investigation of parameters as: mortality rate (weekly presented), in all case and control groups (G1, G2, G3, and G4) in during nine weeks of assay.

As it is clear, through a nine-week investigation of mortality rate of *Zebrafish* models at the end of every week was recorded and results are presented by survival rate. The lowest survival rate of Zebrafish is related to control groups that infected by *Aeromonas hydrophila* and receiving no probiotic bacteria (G1). The lowest survival rate (the highest mortality rate) is observed in G1 groups in eight week before being

exposured with *Aeromonas hydrophila* and with the same trend in ninth week after being infected by it. Precisely, the highest survival rate (the lowest mortality rate) of Zebrafish is observed in receiving only one type of probiotic bacteria (G4) groups in eight week before being exposured with *Aeromonas hydrophila* and with the same trend in ninth week after being infected by it.

Totally, there is a decreasing trend for survival rate of *Zebrafish* models in all control and case groups at the end of every week.

3.5.2. Biometric Assessment of the Fish through Investigation of Height and Body Weight in 14th day of assay

Figure 9, shows results related to biometric assessment of *Zebrafish* models infected by *Aeromonas hydrophila* through investigation of parameters as: height, and body weight in all case and control groups (G1, G2, G3, and G4) in 14th, 28th, and 56th day of assay.

According to Fig. 9, in 14th day of assay, there is not any significant difference for height and body weight of the fish among all control and case groups, proving that we need more time for investigation of positive effects on height and body weight after administration of probiotics.

According to Fig. 9, in 28th day of assay, the highest rate of body weight is recorded for groups receiving only one type of probiotic bacteria (G4) with a significant difference in comparison with zero, control, cases receiving only one type of probiotic bacteria (G3) groups, and cases receiving both prebiotic bacteria (G2) groups ($p \leq 0.05$). But, there is not any significant difference among zero, normal, cases receiving only one type of probiotic bacteria (G3) groups, and cases receiving both prebiotic bacteria (G2) in case of body weight in 28th day of assay. In case of height, in spite of the fact that the highest rate of height is observed in control group in 28th day of assay, the highest rate of height is also observed in cases receiving only one type of probiotic bacteria (G3) groups, among cases receiving bacteria (G2, G3, and G4) groups. There is not any significant difference among normal, cases receiving only one type of probiotic bacteria (G3) groups, cases receiving only one type of probiotic bacteria (G4) groups, and cases receiving both prebiotic bacteria (G2) in case of height in 26th day of assay. Interestingly, there is a significant difference for height of the *Zebrafish* in 28th day of assay among zero, control, and both cases receiving only one type of probiotic bacteria (G3, and G4), proving profound effects of probiotics for the fish.

According to Fig. 9, in 56th day of assay, the highest rate of body weight is recorded for groups receiving only one type of probiotic bacteria (G4) with a significant difference in comparison with zero, and control groups ($p \leq 0.05$). But, there is not any significant difference among zero, normal, cases receiving only one type of probiotic bacteria (G3) groups, and cases receiving both prebiotic bacteria (G2) in case of body weight in 56th day of assay. In case of height, the highest rate of height is recorded for groups receiving only one type of probiotic bacteria (G4) with a significant difference in comparison with zero group ($p \leq 0.05$). In addition, there is not any significant difference among normal, cases receiving only

one type of probiotic bacteria (G3) groups, and cases receiving both prebiotic bacteria (G2) in case of height in 56th day of assay.

4. Discussion

It has been demonstrated that food-borne pathogens associated diseases are of environmental, socioeconomical, and clinical significance for aquaculture scientists, food microbiologists, medical microbiologists, environmentalists, and public health managers. Due to high rate of mortality for the fish, usage of antibiotic based approaches or other strategies with low clinical output, and high probability for transmission of resistant bacteria and antimicrobial resistance genes from aquaculture environments to humans, it seems that this is the exact time to change mind and shift our therapeutic strategies toward more efficient ones. Recently, theory of “Probiotics” has promisingly proved its clinical and environmental efficiency for human and fisheries industries through a wide array of biological mechanisms including: induction of anti-microbial products, stimulation of intestinal (mucosal) immunity, making a competitive relationship with pathogenic microorganisms existed in the intestinal tissues, change in the composition of intestinal microbiota (pathogen interference), inhibitory effects on expression of virulence genes, improved appetite through regulation of enzymatic secretions, symbiotic enhancement in phagocytic activities, synergistic enhancement in antibody secretion, reduction of severity of IBD and other intestinal disorders, and ecological improvement in marine environments through minimizing toxic gases and regulation of water microflora (68–70). One of the most important criteria for selection of a probiotic especially oral probiotic is choosing a Generally Recognized As Safe (GRAS) products which is defined by Food and Drug Administration (FDA). For sure, the next step would be characterization of isolate phenotype in the biofilm state for the selection of the most beneficial probiotic strains (21, 65, 71–76).

It has been specified that LAB (majorly *lactobacillus*) have shown advantageous properties for induction of immunomodulatory and histologic effects on the fish as probiotic bacteria. Interestingly, they exist as intestinal microflora that makes researchers to look up probiotics as supplementary food additives for the fish, and their crosstalk between probiotic based diets and immune system (32, 71, 77–79).

As *Zebrafish* are the highly accepted animal for investigation of developmental biology, most practical and well-established models for studying biological effects of probiotic bacteria, researches related to aquatic nutrition, and intestinal inflammation, they are selected to be our animal model. Having a short-generation intervals, an easy-handling, a small size, huge numbers of eggs per brood and data point, perfect and functional organs for reporting immune reactions, an ability to be fed with a wide range of commercial and live foods, early hatching, and an approved ability to be alive for five days without feeding during larval period through consumption of the yolk, all are enumerated as advantages of *Zebrafish* to be served as a model for studies with aforesaid aims (80–82).

As it has said before, *Aeromonas hydrophila* are oxidase-positive, facultative anaerobic, and gram-negative bacteria. They are considered as one of the most causative pathogen for intestinal diseases in the fish.

Hence, in this experimental study, authors selected *Lactobacillus acidophilus* and *Lactobacillus bulgaricus* (*subsp. Lactobacillus delbrueckii*) as dairy probiotics to evaluate their immunobiological and histological effects on *Zebrafish* models infected by *Aeromonas hydrophila*. There are several studies that they investigated immunomodulatory and histological effects of probiotic strains on health promotion of the fish and here, we will review the results, discuss the findings, and compare the probable similarities and differences.

In an experimental study done by *Qin et el*, development and immunity response on *Zebrafish* treated with *Lactobacillus casei BL23* was investigated 14 and 35 days post-fertilation. Whole transcriptome sequencing (mRNAseq) was used for investigation of differentially expressed genes. They concluded that higher final body weight was observed at 14, and 35 days post-fertilation (($P < 0.05$), and ($P < 0.01$), respectively) (83).

Another newly conducted experimental study by *Taida Juliana Adorian et al.*, was aimed at investigation of growth performance, digestive enzyme activity, and hematological parameters of *Asian Sea Bass* (*Lates calcarifer* (*Bloch*)) after treatment with different doses of two species of *Bacillus* (*Bacillus licheniformis* and *Bacillus subtilis*) for a 56-day period. They totally reported that case groups including fish fed with supplementary diets including 1×10^6 CFU g^{-1} (the middest concentration) of both aforementioned probiotic bacteria showed significantly better growth, higher weight, higher length, higher weight gain, and higher protein levels than those fed the basal diet (control). Regardless of concentration of administered probiotics, higher survival rate was observed in the case group including fish fed with supplementary diets of both probiotic. Additionally, they reported that administration of 1×10^6 CFU g^{-1} of both probiotic Bacillus drastically reduced hepatic inflammation through lowering levels of hepatic enzymes (AST, ALT, ALP) (1). As it is clear, they did not measured out their experimental study on marine organisms infected by food-borne associated pathogens. They believe that further studies are needed focusing on immune mechanisms, stress responses, and resistance against fish pathogens for exploring the feasibility of both probiotic bacteria being used as commercial applications in fish farming industry.

Another recently done experimental study by *Hai-peng Zhang et al.*, was aimed at evaluation of growth, immunity, and disease resistance in *koi carp* infected by *Aeromonas veronii TH0426* after treatment with different concentrations of *Lactobacillus plantarum C20015*, for a six-week period. They totally reported specific growth rate, higher percentage of weight gain for the fish, promoted food intake, higher appetite of the fish, exacerbated enzymatic activation of superoxide dismutase (SOD), peroxidase (POD), lysozyme (LZM), and more levels of IgM antibody especially after 28 days of assay (totally reduced levels of bacterial inflammation) in the experimental groups supplementarily fed with sprayed *Lactobacillus plantarum C20015* than in the control. These improved activities, especially enzymatic activity, percentage of weight gain, and higher survival rate (reduced surface bleeding, abdominal swelling, and severe skin ulcers) were reached in the concentration of 1×10^8 CFU g^{-1} (the middest concentration) in comparison with cases fed with other concentrations of probiotic, and control groups (84). As it has been reported, they represented concentration of 1×10^8 CFU g^{-1} of *Lactobacillus plantarum C20015* as an

acceptable probiotic candidate with enhanced immunoprotective efficacy, and improved non-specific and specific immunity in *koi carp* to be used in fisheries industries.

Another experimental study conducted by *M. Soltani et al.*, aimed at investigation of growth, performance, and hematological indices after administration of *Lactobacillus plantarum* (*kc426951*) done on *Juvenile rainbow trout* (*Oncorhynchus mykiss*) for a 60-days of assessment. Results of this study indicated highest levels in variables including: hematological indices, weight gain, final weight, condition factor, feed utilization, thermal unit growth coefficient, and survival rate, and the lowest food conversion ratio in groups that were fed with 1×10^{8} CFU g^{-1} of *Lactobacillus plantarum* and administered normal diet (vaccinated with *streptococcosis and lactococcosis*) in the same time. In other words, they approved a synergistic effect in the vaccinated *Juvenile rainbow trout* fed with the probiotic (85). They guess that the reason behind increased growth condition by usage of probiotic bacteria in vaccinated, goes back to improved function of lysozyme, complement component, and phagocytosis. They believe that this type of administration leads to an enhancement in fish growth, physiology, and hematological parameters.

According to another experimental study, that *Chao Li et al.*, investigated growth, immune responses, survival rate, inflammatory activities of enzymes, and expression levels of (Heat Shock Proteins (*HSP60*, *HSP70* and *HSP90*)) genes on *Juvenile sea cucumber* (*Apostichopus japonicus Selenka*) after treatment with four strains of LAB including: with 1×10^{9} CFU g^{-1} of *Lactobacillus plantarum LL11* and other three strains for a 30-day assessment. They reported a significant increase in all mentioned variables in groups treated with *Lactobacillus plantarum LL11* compared to control group (fed with formulated with sea mud and seaweed powder without LAB supplementation), totally indicating enhanced immunity, and cytoprotection in intestinal tissues against oxidative stress and inflammation. Also, survival rate was dramatically increased in groups treated with LAB after being infected by *Vibrio splendius* challenge (86).

Interestingly, another study conducted *Y. Wang. et al.*, by done on *Zebrafish* infected with *Aeromonas hydrophila*, they reported there are immunoprotective roles as reduced degranulation of mast cells, remarkable decrease in expression of IL-1β and increase in IL-10 expression (post-challenge related data), restored intestinal morphology, sustainable barrier function, and higher percentage of intraepithelial lymphocytes related to usage of highly adhesive strains of *Bacillus coagulans 09.712*, and *Lactobacillus plantarum 08.923* when they are orally administrated (87). They suggest that aforementioned LAB strains are potentiated probiotic candidate to limit inflammation caused by acute infection (*Aeromonas hydrophila*) through down-regulation of IL-1β and upregulation of IL-10, and regenerate epithelial barriers by up-regulation in mucosal expression of TNF-α and more involvement of intraepithelial lymphocytes.

Accordingly, one experimental study conducted by *Yu-Sheng Lin et al.*, aimed at investigation of dietary supplementation with 1×10^{6} CFU g^{-1} of *Bacillus amyloliquefaciens R8*, whether they affect nutrient metabolism, hepatic oxidative stress, and innate immunity against pathogen in *Zebrafish* model for a 30-day period of assessment. The fish were infected with 1×10^{5} CFU per fish of each *Streptococcus agalactiae* (as a causative pathogen for meningoencephalitis and hemorrhagic septicemia, leading to high mortality rate in many marine and freshwater fish species), and *Aeromonas hydrophila*.

Biochemically, investigation of nutrient metabolism was assessed through hydrolytic enzymes like: xylanase. The treated fish showed significant increases in activity of intestinal xylanase, mRNA expressions of glycolysis-related genes, anti-apoptotic genes, and enzymatic activities associated with fatty acid β-oxidation, and mitochondrial integrity. Mentioned case group showed decreased mRNA expressions of oxidative stress-related and apoptotic genes. Before challenging with infections, there was increased expression of innate immune-related genes (*IL-1β, IL-6, IL-21*, and *TNF-α*). Results of post-challenging showed a higher survival rate than control fish (88). Totally, they reported reduced oxidative stress by prevented hepatic apoptosis, and lowered expression levels of hepatic *SOD*, *Gpx1a*, *NOS2a*, and *HSP70*.

Similarly, in one study, *Lactobacillus acidophilus* induced macrophage mediated inflammatory responses against *Aeromonas hydrophila*, significant increase in TNF-α expression, and attenuated *Aeromonas hydrophila* induced apoptosis was reported in in freshwater carp *Catla catla* after being fed with *Lactobacillus acidophilus* as probiotic bacteria (89).

Identically, results from another experimental study done on gold fish (*Carassius auratus gibelio*), aimed at investigation of being fed with supplemented diet including 6×10^8 CFU g^{-1} of *Lactobacillus acidophilus* for eight weeks, showed an up-regulation in TNF-1α, TNF-2α, and appetite related genes expression (90). Conclusively, there are a wide range of studies, indicating beneficial immunomodulatory effects on immune responses, weight gain, pro-inflammatory cytokines (down-regulatory effects on TNF-α, IL-1β, IL-6, and IL-12 expression, and up-regulatory effects on IL-10 and TGF-β expression), and increased survival rate by LAB (*Lactococcus lactis, Lactobacillus fermentum, Lactobacillus acidophilus, Bacillus subtilis* strains) on food-borne pathogens like *Aeromonas hydrophila* infecting common carp fish (*Cyprinus carpio*), suggesting usage of such potentiated probiotics in aquaculture (77, 91, 92). Totally, results of all mentioned studies, apart from type of probiotic and the fish, are in accordance with our results in case of improvement in body weight, immune responses against infection, length of intestinal villus, and survival rate through usage of probiotic bacteria used for fish industries.

5. Future Directions And Conclusion:

According to the high mortality and morbidity rate, and unsuccessfulness of antibiotics or other therapeutic strategies for the fish, food microbiologists, and health managers will look up for more efficient approaches. One of the criteria for choosing them, is not having any disadvantage for health situation. We believe that usage of probiotics, as live microorganisms with prove immunomodulatory effects on intestinal tissues, can pave the path for reduction in microbial infections in fisheries, increase in survival rate, decrease in emergence of antibiotic resistance and their direct consequences, and diminution in transmission of resistant pathogens from marine environments to human gut through food chain.

Declarations

Ethics approval and consent to participate

This experimental study accessed ethics approval from the Science and Research Branch, Islamic Azad University Research Ethics Committee (NO. IR.IAU.SRB.REC.1400.078).

Consent for publication

Not applicable.

Availability of data and materials

Not applicable.

Competing interests

All of the authors and co-authors declare that there is no financial conflict of interest relevant to the concept (subject matter) and publication of this article. There are not any non-financial and other competing interest's disclosures.

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Author Contribution

All of the authors and co-authors who were listed on the title page, greatly helped to accomplishment of this study with unsparing efforts. *Ehsannia. Sheida*, helped to preparation of the last version of manuscript, and literature review. She also contributed to conducting experiment, data curation, designation of images and tables, and final arrangement of the data making structure of the study. *Ahari. Hamed*, mainly did project supervision and corresponding, and verified the manuscript by the definitive approval for submission. He also academically and grammatically did peer revision, designation of methodological strategies and major conceptualization qualifying. *Anvar. Seyed Amirali*, did scientific consultations, critical revision of the manuscript for important intellectual content according to the journal's instruction, and designation of methodological strategies. *Kakoolaki. Sh,* helped for conceptualization, revision, methodology, and revision. *Yousefi. Shima*, helped for validation of data, edition, and visualization.

All of the authors and co-authors attest to the fact that they have approved the final version of the article, and they have received an electronic copy of the manuscript. Also, they attest to the validity and legitimacy of data, and agreed to submit this manuscript to this journal.

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Figures

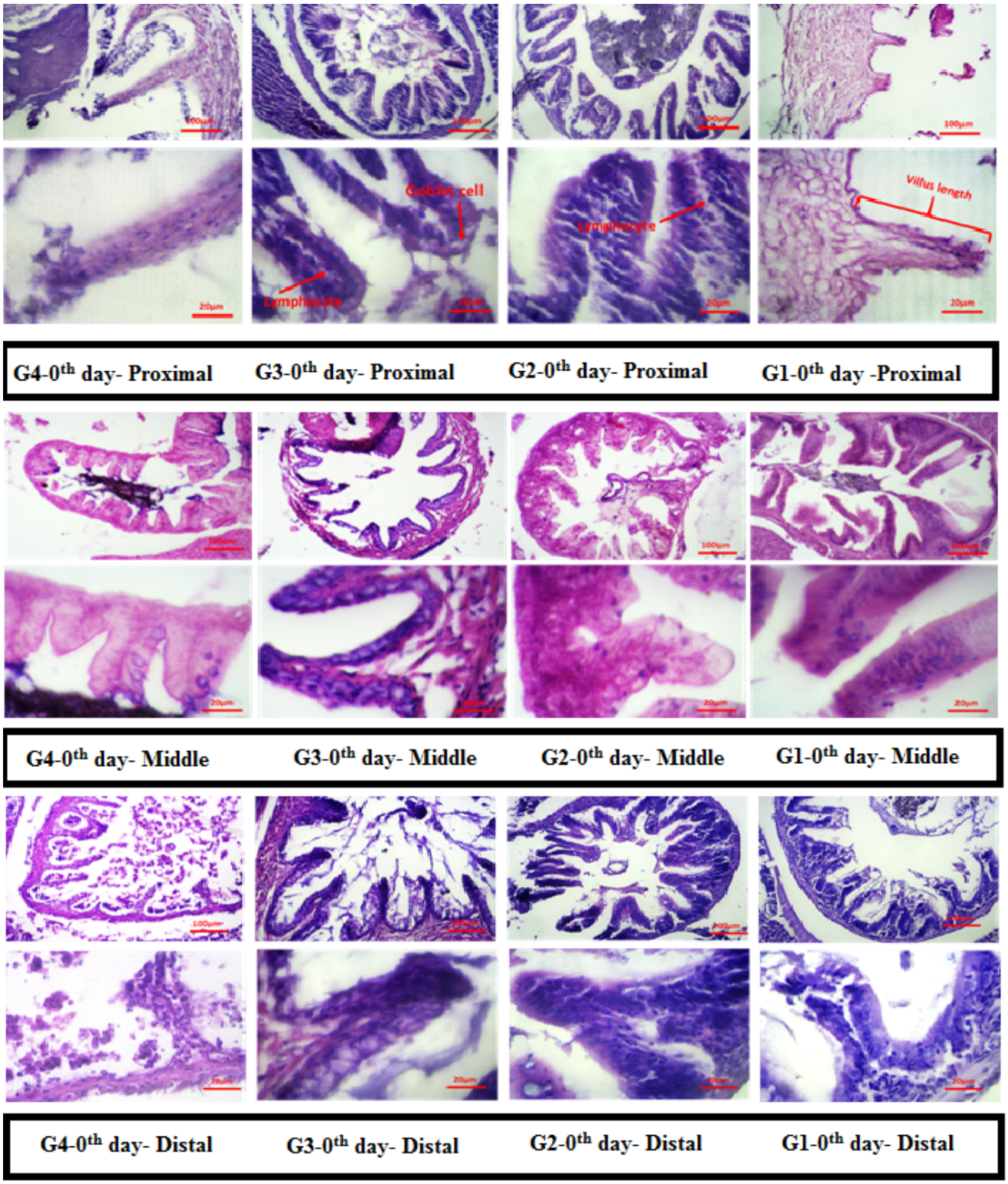

Figure 1

Histopathological changes in intestinal tissues of Zebrafish in 0th day of assay.

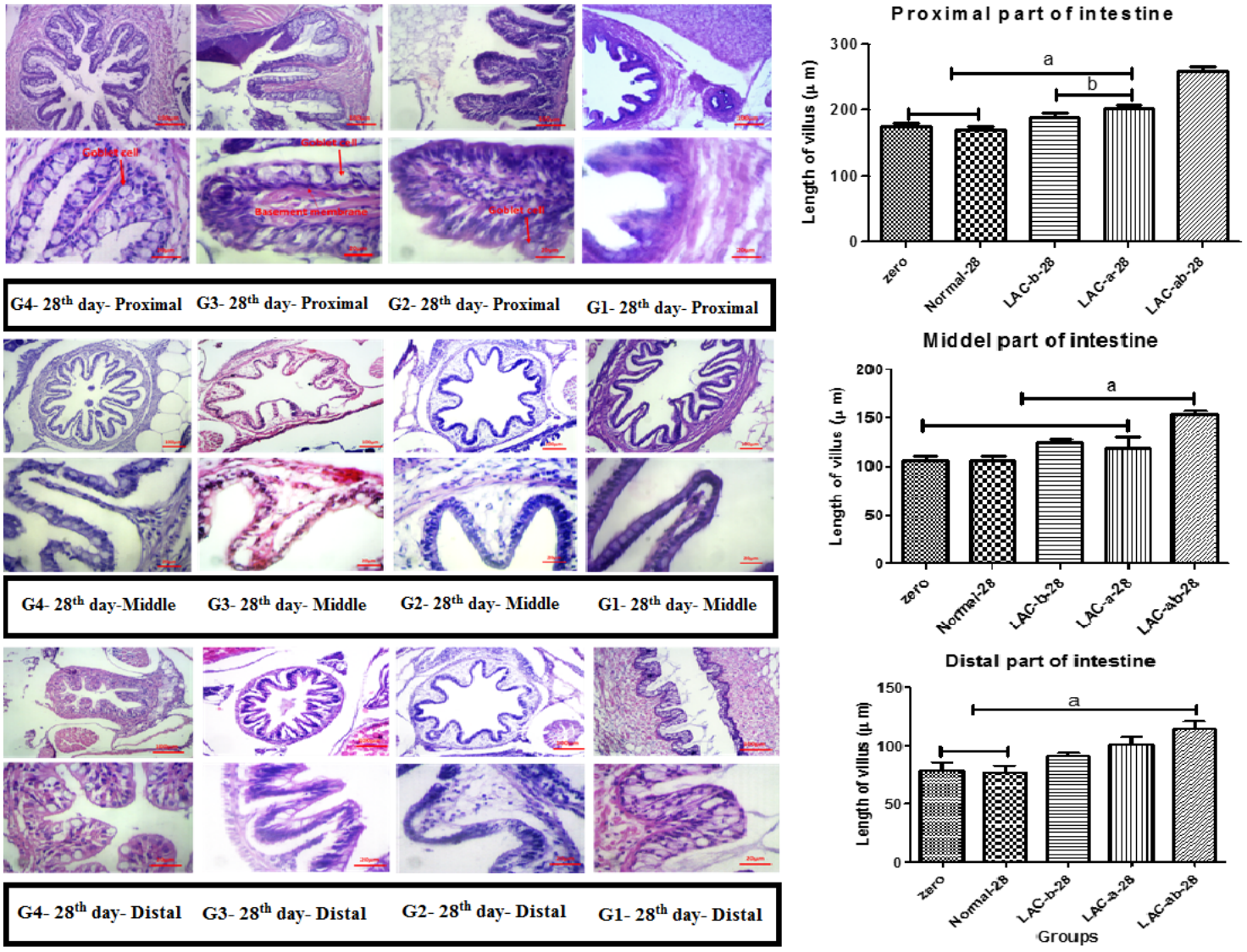

Figure 2

Histopathological changes and villus length in intestinal tissues of Zebrafish in 28th day.

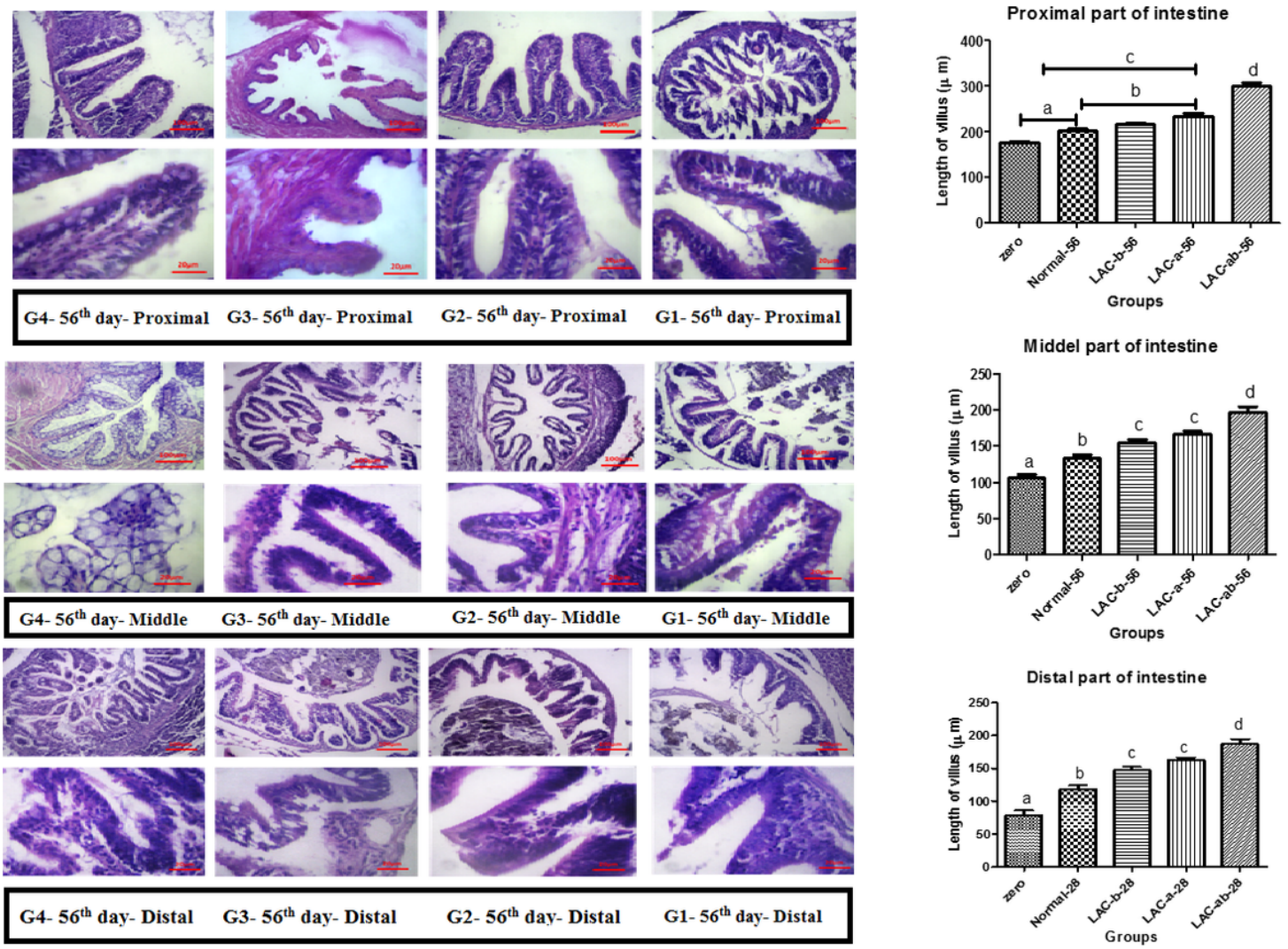

Figure 3

Histopathological changes and villus length in intestinal tissues of Zebrafish in 56th day.

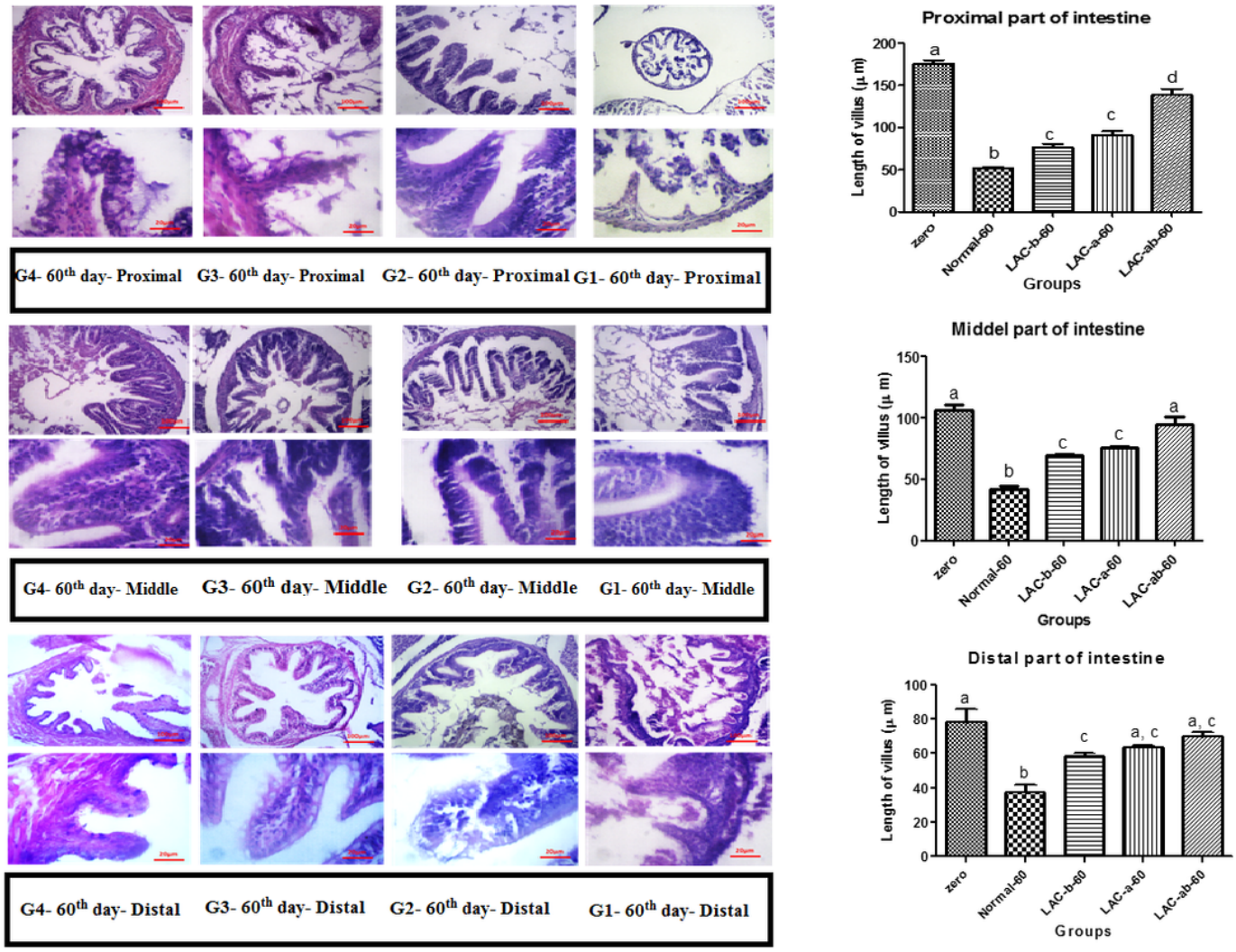

Figure 4

Histopathological changes and villus length in intestinal tissues of Zebrafish in 60th day.

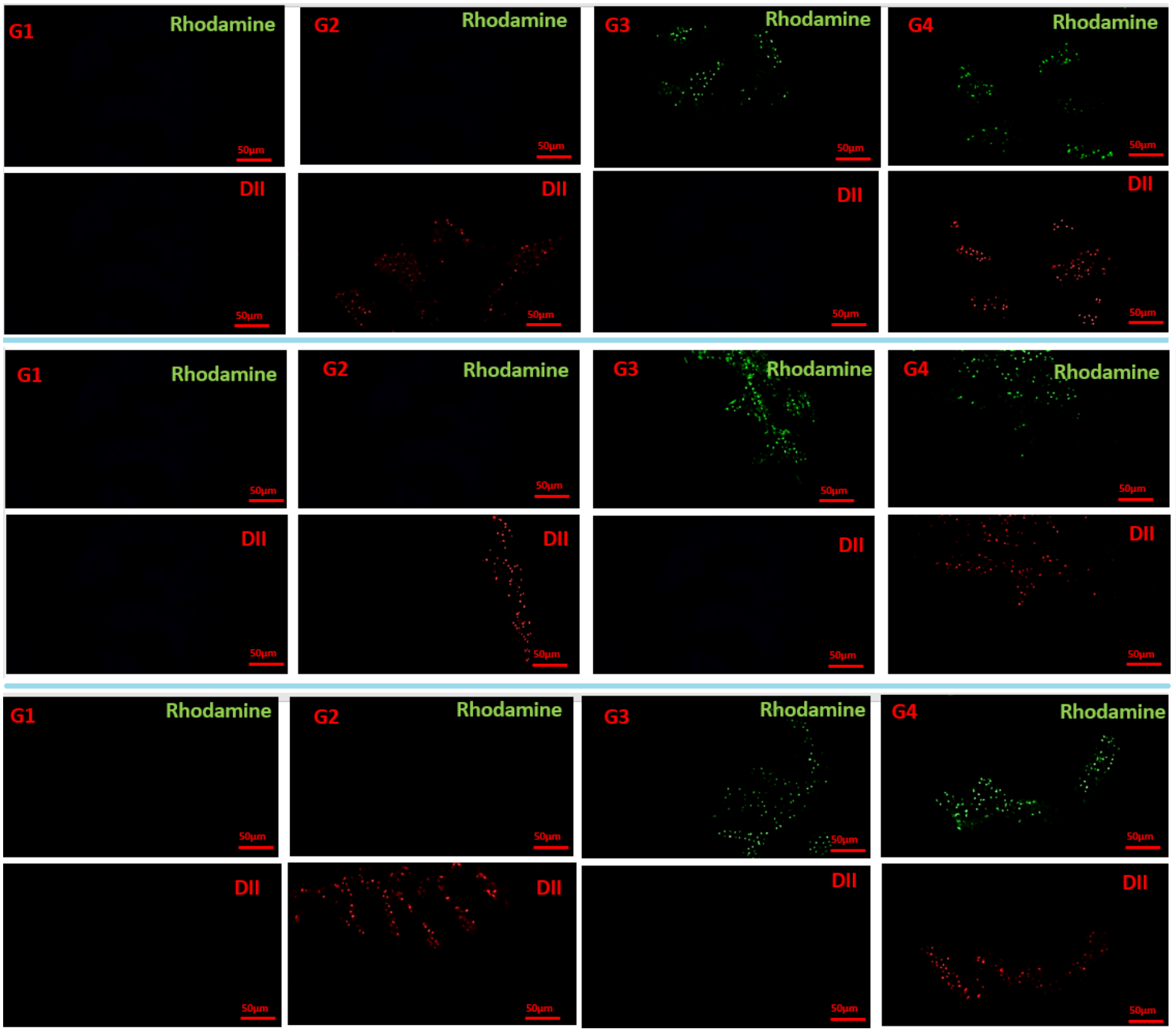

Figure 5

Rhodamine and DiI for all gropus in 28th, 56th, and 60th day of assay.

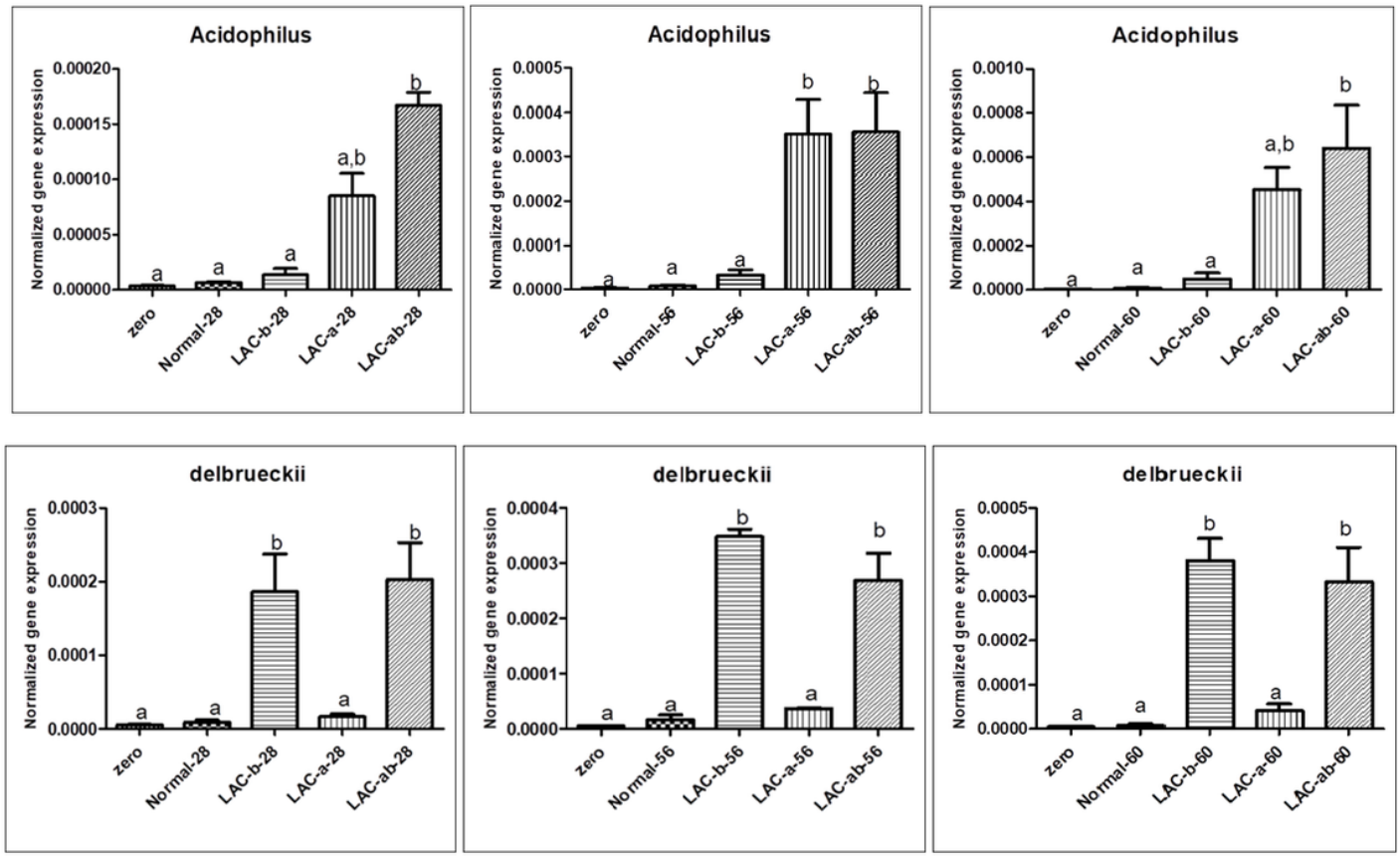

Figure 6

Quantitative investigation of probiotics in the 28th, 56th, and 60th day of assay.

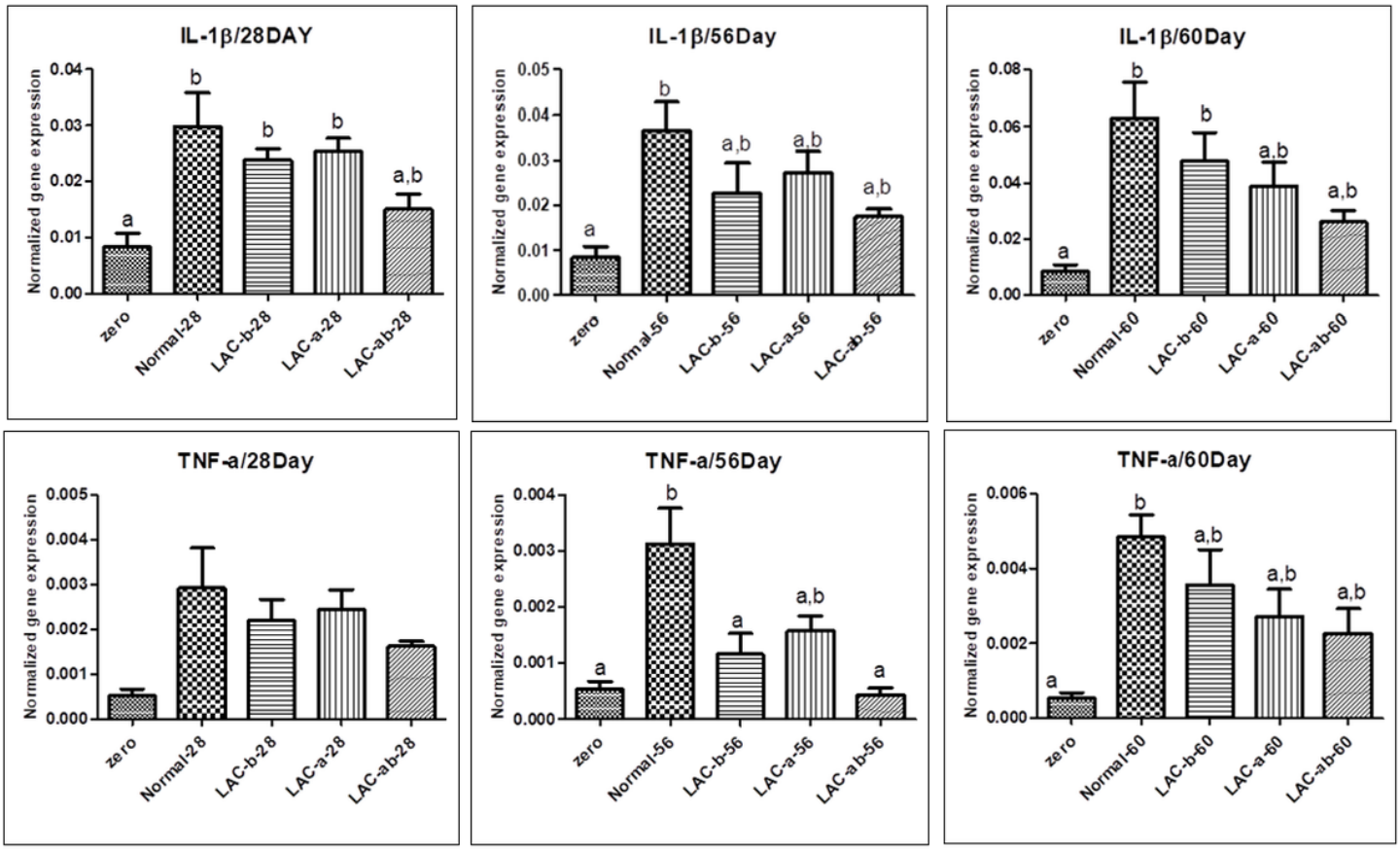

Figure 7

Quantative investigation of immune genes in the 28th, 56th, and 60th day of assay.

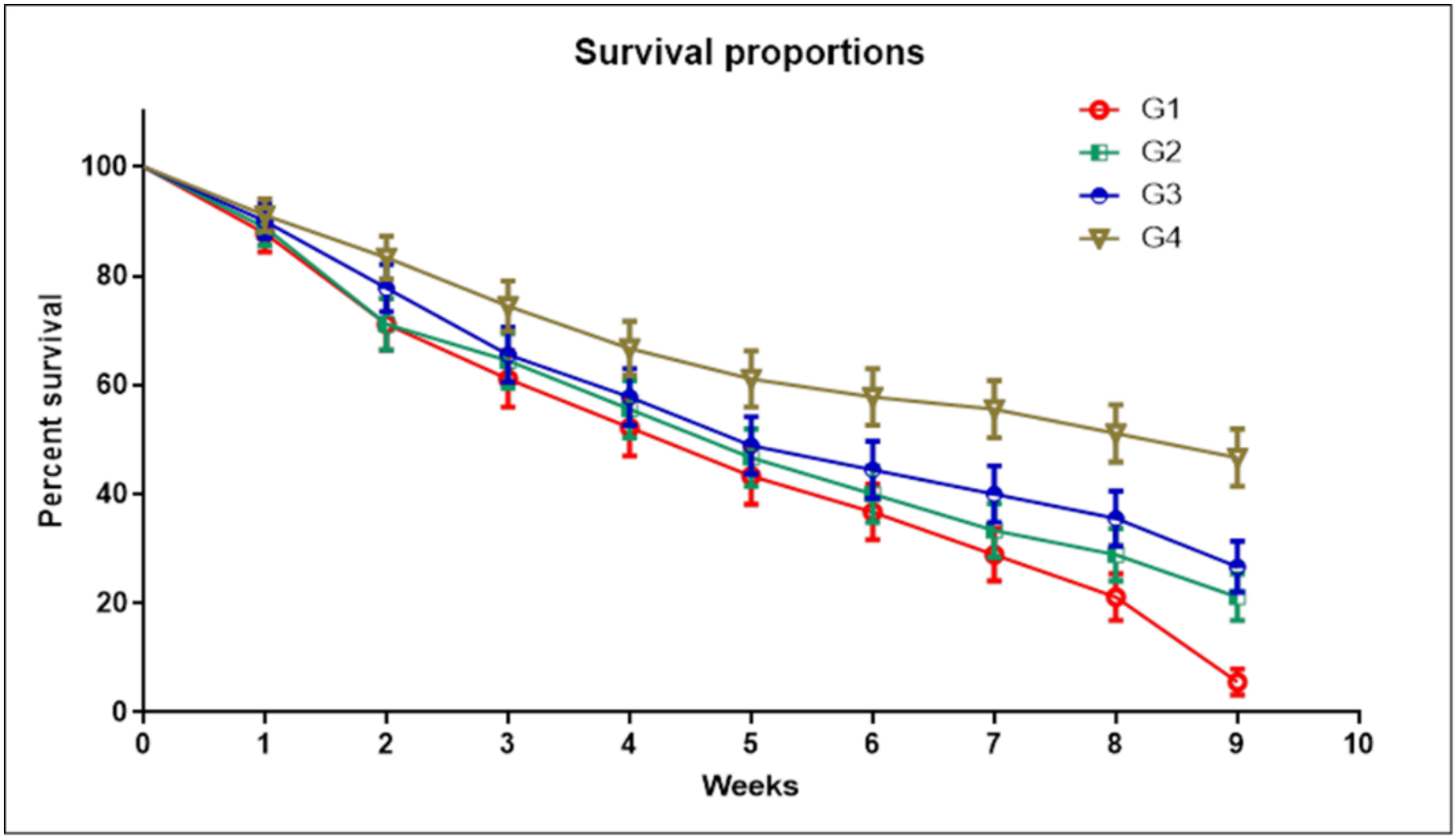

Figure 8

Investigation of survival proportions in the fish during nine weeks of assay.

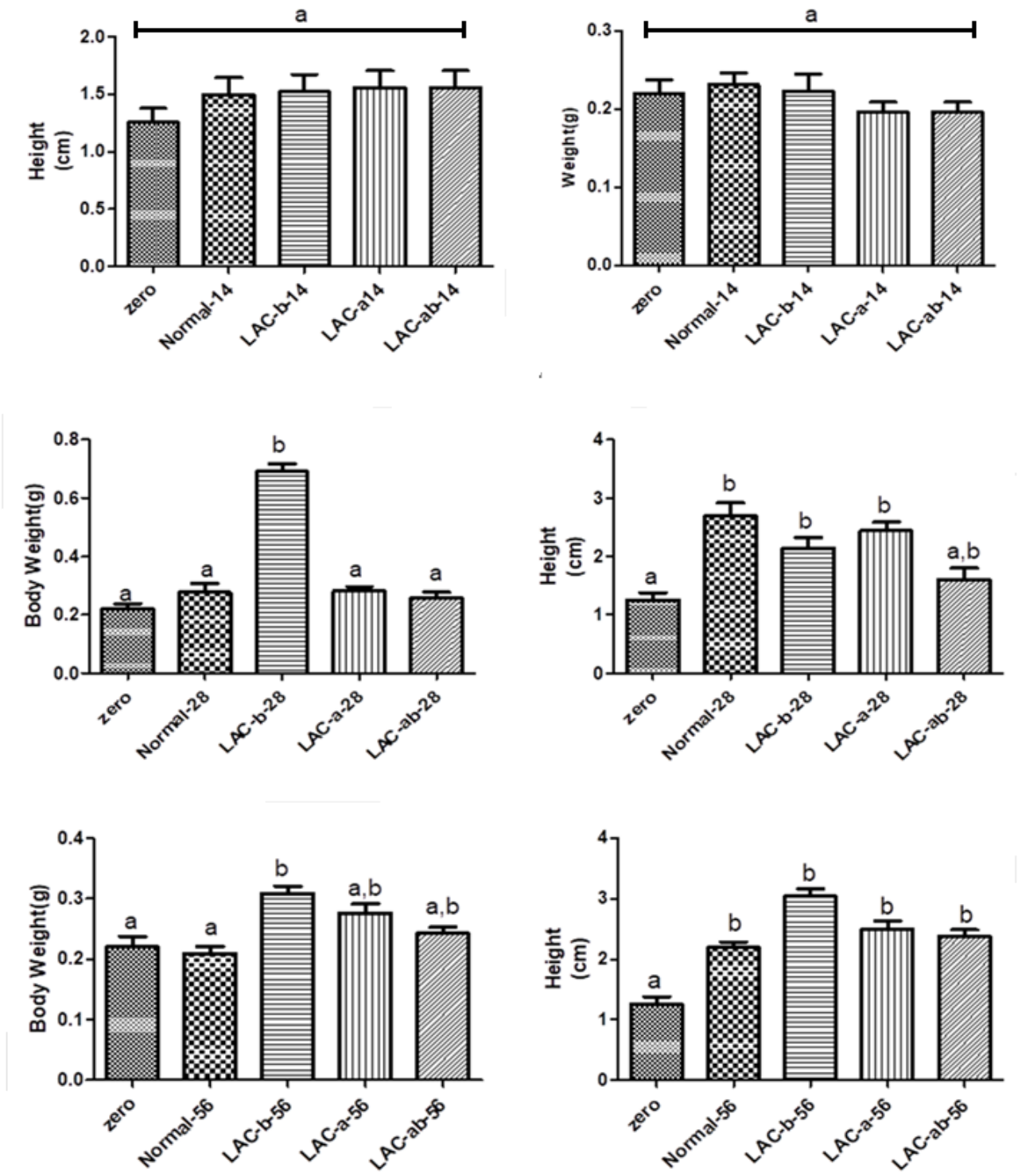

Figure 9

Investigation of height, and body weight of the Fish in three days of assay.

Supplementary Files

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