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Research Article

The use of dietary probiotics in long-term exposure to treat the effect of the nonsteroidal anti-inflammatory drug (NSAID) naproxen causes thyroid dysfunction in zebrafish (*Danio rerio*)

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Abstract

Naproxen (NPX) is a nonsteroidal anti-inflammatory drug (NSAID) that has been identified in aquatic environments. It has led to growing concerns about endocrine disruption in aquatic organisms exposed to its drug residues. This study aimed to evaluate the disruptive effects of NPX and the improving effects of probiotic nutrition on thyroid, and zebrafish growth. The fish were fed for 60 days by basic diet (TN, Control) basic diet with probiotics (TP, TPN) (*Lactobacillus reuteri*, CFU/1.5×10^8) and simultaneously exposed to NPX (100 μg/L) (TN, TPN). During the experiment, Triiodothyronine (T3), Thyroxine (T4), Thyroid-stimulating hormone (TSH), Iodothyronine Deiodinase-1 (DIO1), and Iodothyronine Deiodinase-2 (DIO2), in addition to growth rate factors were evaluated with ELISA and Quantitative real-time PCR assay. The results showed probiotic feeding and NPX exposure did not affect T3 levels (p>0.05), but decreased T4 (p<0.05). TSH gene transcription expression increased as a result of probiotic feeding and NPX exposure (p<0.05) while DIO1 and DIO2 gene expression decreased (p<0.05). Weight gain and growth rate were also observed as a result of probiotic feeding, while exposure to NPX decreased growth rate (p<0.05). Generally, the results showed that NPX increased the risk of thyroid dysfunction and reduced growth rate in zebrafish, while probiotic feeding improved these factors. Therefore, the use of probiotic supplements in rearing centers is recommended due to the continuous increase in the consumption and distribution of drugs. Prolonged exposure to drug concentrations causes thyroid dysfunction and consequently reduced growth in fish, which will lead to significant economic losses.

Keywords: Naproxen, *Lactobacillus reuteri*, Probiotic, Zebrafish, Thyroid Hormones

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Introduction

Water pollution by pharmaceutical contaminants is one of the most disturbing environmental problems (Brausch *et al.*, 2012; Li, 2014; Comber *et al.*, 2018). Increases in drug use and incomplete disposal in wastewater treatment plants lead to the continuous release of drugs into groundwater, surface water, and drinking water (Benotti *et al.*, 2009; Li, 2014; Comber *et al.*, 2018). To avoid potential risks, the removal of pharmaceuticals at sewage treatment plants before final release into receiving waters is utterly imperative. Meanwhile, the Technologies for the removal of pharmaceuticals have very high economic costs (Silva *et al.*, 2018).

Aquaculture or the farming of aquatic organisms (fish, crustaceans, molluscs, aquatic plants) is a rapidly growing food production sector at a rate of 6% per year in the world and is becoming the main source of protein for human nutrition, while in recent decades the contamination of waters with pharmaceuticals has caused significant economic losses in the aquaculture industry by disrupting growth performance and increasing losses (DDTTD *et al.*, 2021). Prolonged exposure can have several adverse effects on aquatic organisms (Fabbri and Franzellitti, 2016; Ebele *et al.*, 2017). Interference with the endocrine system and disruption of homeostasis in non-target organisms is one of the main concerns of drug contaminants (Ebele *et al.*, 2017). Nonsteroidal anti-inflammatory drugs (NSAIDs) are among the endocrine disruptors that have been reported to contaminate aquatic environments (Mezzelani *et al.*, 2016). Although knowledge of the NSAID's effects on aquatic organisms is limited, recent studies have reported dysfunction of the endocrine system (including the thyroid gland) in fish (Xu *et al.*, 2019). The thyroid is an important component of the endocrine system that has a significant impact on the growth and development of fish (Nelson and Habibi, 2009).

NPX is a derivative of bicyclic propionic acid and a known non-selective and non-steroidal anti-inflammatory drug (Dzionek *et al.*, 2018). It is one of the most common NSAIDs detected in aquatic environments. The action mechanism is based on the inhibition of two cyclooxygenase isoforms involved in the synthesis of prostaglandins, prostacyclin, and thromboxane from arachidonic acid (Angiolillo and Weisman, 2017; Barcella *et al.*, 2019). Previous studies have examined the adverse effects of NPX on antioxidant function (Stancová *et al.*, 2015; Neal and Moore, 2017; Sehonova *et al.*, 2017), reproduction (Kwak *et al.*, 2018), and endocrinology (Kwak *et al.*, 2018; Xu *et al.*, 2019) in fish.

Probiotics are live microbial feed additives that increase survival and growth rates by entering the host gastrointestinal tract and improving intestinal microbial flora (Liu *et al.*, 2018; Alavinezhad *et al.*, 2020). Probiotics also competitively eliminate harmful bacteria (Liu *et al.*, 2018; Mirabdollah Elahi *et al.*, 2020), improve immune function (Hoseinifar *et al.*, 2021), and improve physiological functions including endocrine system function (Kanwal and Tayyeb, 2019). Lactobacillus is one of the most widely used probiotics among probiotic bacteria. The genus Lactobacillus includes a large heterogeneous group of gram-positive, non-sporulating, and anaerobic bacteria that includes *lactobacillus acidophilus*, *lactobacillus rhamnosus*, *lactobacillus bulgaricus*, *lactobacillus casei,* and *lactobacillus reuteri* (Mu *et al.*, 2018). *L. reuteri* is a species of Lactobacillus that has been isolated from the gastrointestinal tract of humans and fish and Its beneficial effects on host health have been reported in several studies (Mu *et al.*, 2018; Ahmad *et al.*, 2022).

Based on the previous studies, it was hypothesized that exposure to NPX leads to impaired growth and production of thyroid hormones (TH) (Xu *et al.*, 2019). According to Kanwal and Tayyeb (2019), the use of probiotics improves the growth function and function of the thyroid gland. In this study, zebrafish was used as a model to investigate the effect of probiotic nutrition on reducing the adverse effects of NPX on growth and TH. The results of this study will show the risks of NPX, the benefits of using probiotics in the fish diet, and the process of recovery and thyroid dysfunction after feeding with probiotics and long-term exposure to NPX.

Materials and methods

Potential Probiotic Strain

Lactobacillus reuteri ATCC 23272 strain was prepared through the Iranian Biological Resource Center. This strain was then cultured aerobically in Man, Rosaga, and Sharpe (MRS) broth for 24h at 37°C. The broth culture medium was centrifuged at 8000 g for 15 min at 4°C. The initial bacterial concentration was then diluted using saline phosphate buffer (pH 7.4) to a concentration of 0.5 McFarland (1.5×10^8 colony forming units (CFU/mL)) (Giri *et al.*, 2018). The prepared suspensions were stored at -20°C after adding 20% glycerol until consumption (Barbour and Priest, 1986).

Experimental design

Zebrafish (average weight: 0.13±0.1 g) were purchased from an ornamental fish breeding center in Tehran. The fish were kept in aerated glass aquariums (120 L) and adapted to laboratory conditions for two weeks. During the adaptation period, the fish were fed with basic feed (Biomar, France) based on 2% of the body weight twice a day and about 30% of the aquarium water was changed daily. Water temperature, dissolved oxygen, and pH during the experimental period were 26±1°C, 6.9±0.5 mg/L, and 7±0.4, respectively. The light period was 12h of light to 12h of darkness. 600 fish were randomly divided into four groups (n=50 per group) with three replications to experiment. Control: Basic diet feeding; TP: Probiotic diet; TPN: Probiotic diet and NPX poisoning; TN: Basic diet and NPX poisoning. The total duration of the experiment was 60 days.

Preparation of feed

The probiotic diet was obtained by adding *L. reuteri* suspension to the basal diet with a final CFU/g feed concentration of 1.5×10^8 and then incubated in ice (for bacterial uptake) for 15 min. The basal diet was also prepared by combining commercial feed with sterile phosphate-buffered saline (PBS, Sigma-Aldrich) in an equivalent volume of bacterial suspension (Wang *et al.*, 2016). Food preparation was done daily and feeding was performed twice a day (at 9 and 17 o'clock) based on 2% of the bodyweight of the fish.

Exposure to NPX

Exposure to NPX (Razak Pharma Co., Iran) was performed with a slight change based on the method of Xu *et al.* (2019). In this study, the NPX test concentration was 100 μg/L. NPX stock solution was obtained using methanol, and equivalent methanol concentration was used in TP and Control groups (no drug poisoning). About 80% of the aquarium water was changed daily and the desired drug and methanol concentrations were renewed.

Lactobacillus reuteri genetic analysis

This phase was performed based on the methods of Alonso *et al.* (2019) and Shayan and Rahbari (2005) with some changes. At the end of the experiment, intestinal tissue samples of fish belonging to different groups were isolated under sterile conditions and transferred with ice to the laboratory. In the laboratory, the intestines were homogenized in 10 ml of sterile PBS using Stomacher for 5 min at room temperature. The obtained homogeneity with glass beads was dispersed in MRS agar containers with 1.5% NaCl and incubated at 30 °C for 3 to 7 days under aerobic conditions. After incubation, several colonies were randomly isolated based on their morphology, color, and brightness and stored at -80°C in 10% glycerol-MRS solution (v/v). DNA extraction was performed using a DNA isolation kit (MBST, Germany / Iran) and according to the manufacturer's instructions. The primers used to identify *L. reuteri* based on 16S rRNA gene sequences were retrieved from the National Biotechnology Information Center (NCBI) database (Table 1) and synthesized by SinaClon (Iran). PCR analysis and thermal program were adjusted based on Shayan and Rahbari (2005) method. PCR products were analyzed on 1.8% agarose gel in 0.5 times TBE buffer and observed using ethidium bromide and UV-illuminator.

Table 1: Primers specifications are based on 16S rDNA sequences used for *L. reuteri* (F=Forward primer, R=Reverse primer) (Kim *et al.*, 2020).

Target bacteria	Primer	Sequence (5' –3')	PCR product Size
Lactobacillus reuteri PCR assay	F-lacto R-lacto	GAT TGA CGA TGG ATC ACC AGT CAT CCC AGA GTG ATA GCC AA	161

Enzyme-linked immunosorbent assay (ELISA)

Three fish were randomly separated from each treatment on days 0, 30, and 60. They were packed inside a zip-keep bag after being euthanized using clove oil (Wong *et al.*, 2014) and transported with ice to the laboratory. Determination of T4 hormone from whole fish body homogeneity was performed by ELISA kit (Autobio Diagnostics, Co., China) with a sensitivity of 1.29 g/dL. Tissue hormone T3 was also determined using a commercial ELISA kit (Autobio Diagnostics, Co., China) with a sensitivity of 49.5 pg/mL according to the manufacturer's recommendations.

Quantitative real-time PCR assay

Three fish of each treatment were separated randomly at the end of the experiment (day 60). They were packed inside a zip-keep bag after being euthanized using clove oil (Wong *et al.*, 2014) and transported with ice to the laboratory. Isolated samples were kept at -80°C for gene expression of Thyroid-stimulating hormone (TSH), Iodothyronine Deiodinase-1 (DIO1), and Iodothyronine Deiodinase-2 (DIO2). First, the whole body of the fish was washed and homogenized twice with PBS (pH 7.4). Total RNA was extracted using TRIzol reagent (Invitrogen, USA) according to the manufacturer's instructions. Total RNA concentration was measured by spectrophotometry at 260 nm and RNA purity was confirmed at 280.260 nm ratios. RNA was then washed and used as a template for cDNA synthesis. Reverse transcription reactions for cDNA synthesis were performed using the cDNA Synthesis Kit (Thermo Scientific) according to the manufacturer's instructions. The primers used were retrieved from the National Biotechnology Information Center (NCBI) database (Table 2) and designed by Primer Express Version 2.0 (Applied Biosystems Inc.). It was then synthesized by the SINACOLON Company. Quantification of target genes was performed on Mastercycler® ep realplex (Eppendorf, Hamburg, Germany) using the SYBR Green PCR kit. Real-time PCR temperature conditions were initial denaturation at 95°C for 1 minute, followed by 40 cycles at 95°C for 15s and 60°C for 1 minute (Xu *et al.*, 2019). mRNA levels were calculated using $2^{-\Delta\Delta CT}$ method and β-actin was considered as endogenous reference (Livak and Schmittgen, 2001).

Table 2: Primers specifications used for DIO1, DIO2, TSH, and beta-actin genes (F=Forward primer, R=Reverse primer).

Primers	Primer sequence
DIO1-F	5-GTTCAAACAGCTTGTCAAGGACT-3
DIO1-R	5-AGCAAGCCTCTCCTCCAAGTT-3
DIO2-F	5-GCATAGGCAGTCGCTCATTT-3
DIO2-R	5-TGTGGTCTCTCATCCAACCA-3
TSHß-F	5-GCAGATCCTCACTTCACCTACC-3
TSHß-R	5-GCACAGGTTTGGAGCATCTCA-3
ß-Actin-F	5-ATGGATGAGGAAATCGCTGCC-3
ß-Actin-R	5-CTCCCTGATGTCTGGGTCGTC-3

Growth measurements

The weight of 20 fish from each group was randomly recorded on days 0, 30, and 60 of the experimental period. Growth performance indices were then measured using formula:

Weight gain=Final Weight-Initial weight

Weight gain percentage (%)= Final Weight-Initial weight/Initial weight×100

Specific Growth Ratio = ((ln Final weight–ln Initial weight)/time in days) ×100

Data analysis

Data analysis was performed using SPSS 21 and Microsoft Office Excel 2013 software. All data were reported as Mean±SE. The normality of the data was determined using the Kolmogorov-Smirnov test. Significant differences between treatments were considered by one-way analysis of variance (One-way ANOVA). Duncan's test was used at a significant level of 0.05 to compare means.

Results

Lactobacillus reuteri genetic analysis

16S rDNA gene sequencing results indicated the identification of *L. reuteri* in the intestines of the study groups (Fig. 1).

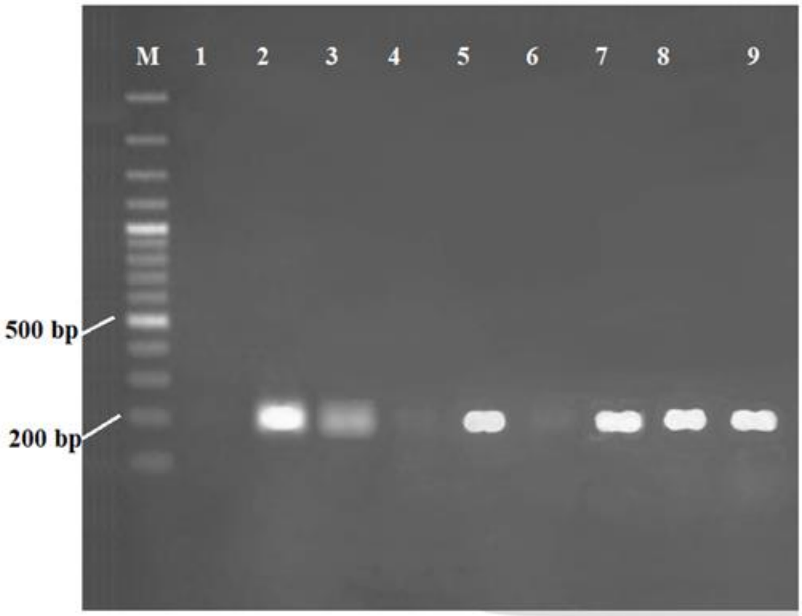

Figure 1: Left to right, 1: bp100 marker, 2-8: set-up samples at different temperatures. 9: Negative control of PCR, 10: Negative control of ctrl, 11: Positive control of *L. reuteri*, 12: TN, 13: TPN, 14: TP.

ELISA

Mean comparison results of Triiodothyronine (T3) showed that no significant difference was observed between different treatments on the experimental days (p>0.05) (Fig. 2). Mean comparison results of Thyroxine (T4) showed that no significant difference was observed between different treatments on day 0 of the experiment (p>0.05). On day 30 of the experiment, there was a significant difference between TPN treatment and TP and control treatments (p<0.05). On this day, the highest value was reported in the control treatment and the lowest value was reported in the TN treatment (p<0.05). Also, on this day, no significant difference was recorded between TPN and other treatment (p>0.05).

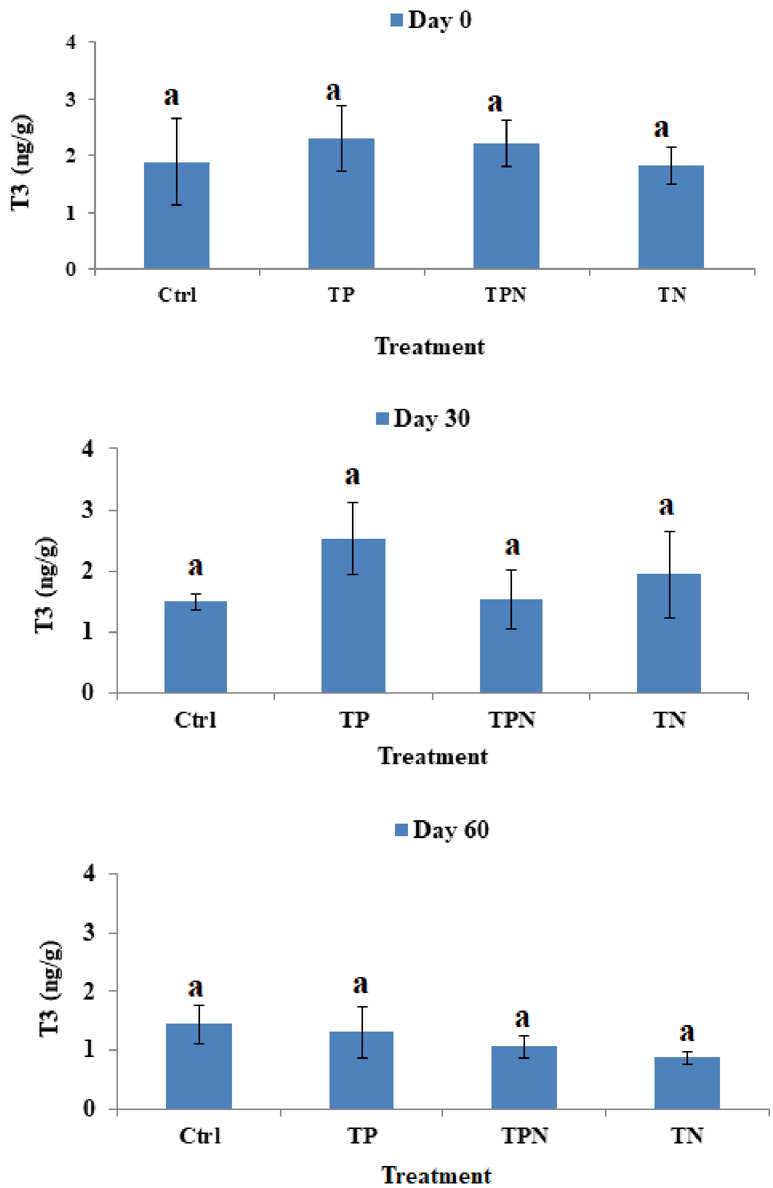

Figure 2: Levels of T3 in zebrafish after 0, 30 and 60 days of the experiment. Values are expressed as the mean±SD. The same letters mean no difference (p>0.05) and different letters mean a significant difference at the 5% level (p<0.05).

On day 60 of the experiment, there was a significant difference between TPN and TP treatments with TN and control treatments on day 60 (p<0.05). On this day, the highest value was reported in the control treatment and the lowest value was reported in the TN treatment (p<0.05). Also, on this day, no significant difference was recorded between TPN and TN (p>0.05) (Fig. 3).

Quantitative real-time PCR

The results of the mean comparison showed that there was a significant difference between all treatments (p<0.05). Based on these results, the highest value was recorded in TN and the lowest value was recorded in the control treatment (p<0.05). DIO1 treatments showed that there was a significant difference between TN and TPN treatments and control treatments (p<0.05).

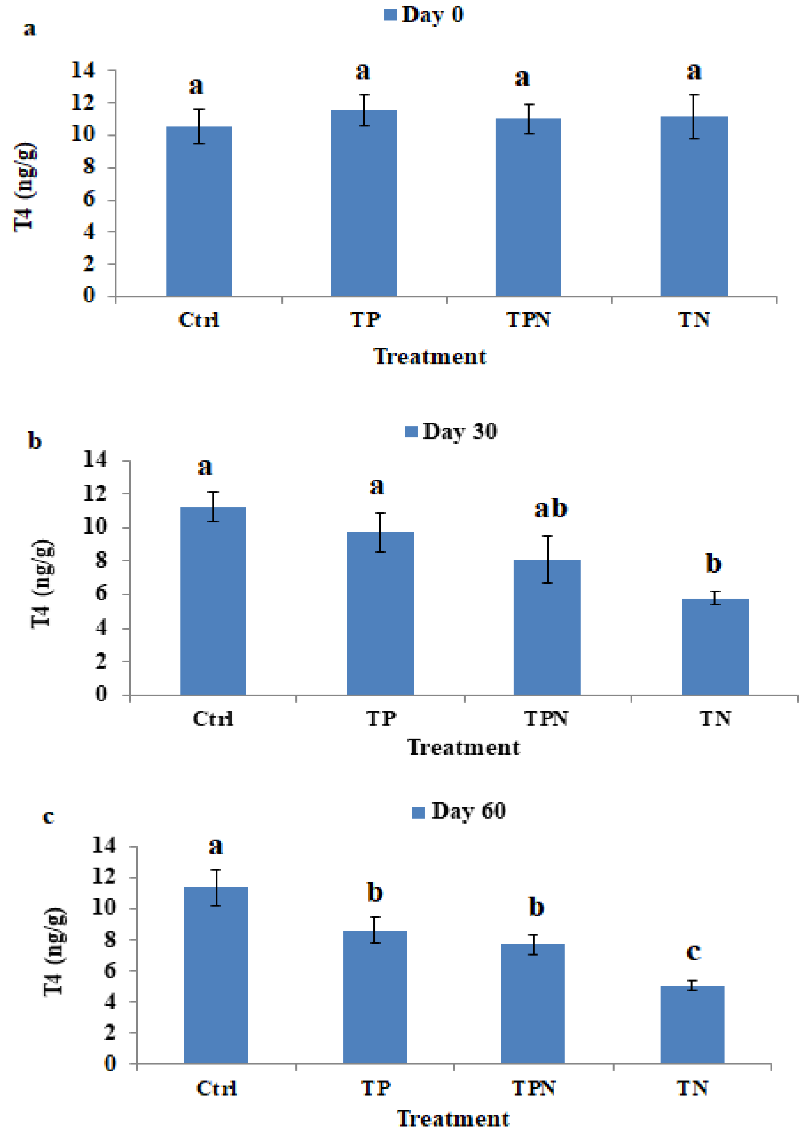

Figure 3: Levels T4 in zebrafish after 0, 30 and 60 days of the experiment. Values are expressed as the mean±SD. The same letters mean no difference (*p*>0.05) and different letters mean a significant difference at the 5% level (*p*<0.05).

Based on these results, the highest value was recorded in control and the lowest value was recorded in the TN group (p<0.05). Also, no significant difference was recorded between TP, TPN and TN and between control and TP treatment

(p>0.05). DIO2 treatments also showed that there was a significant difference between TN treatment and control treatment (p<0.05). Based on these results, the highest value was recorded in control and the lowest value was recorded in the TN treatment (p<0.05). Also, no significant difference was recorded between TP, TPN and TN and between control, TP and TPN treatment (p>0.05) (Fig. 4).

Growth analysis

Mean comparison results of weight showed that no significant difference was observed between different treatments on day 0 of the experiment (p>0.05).

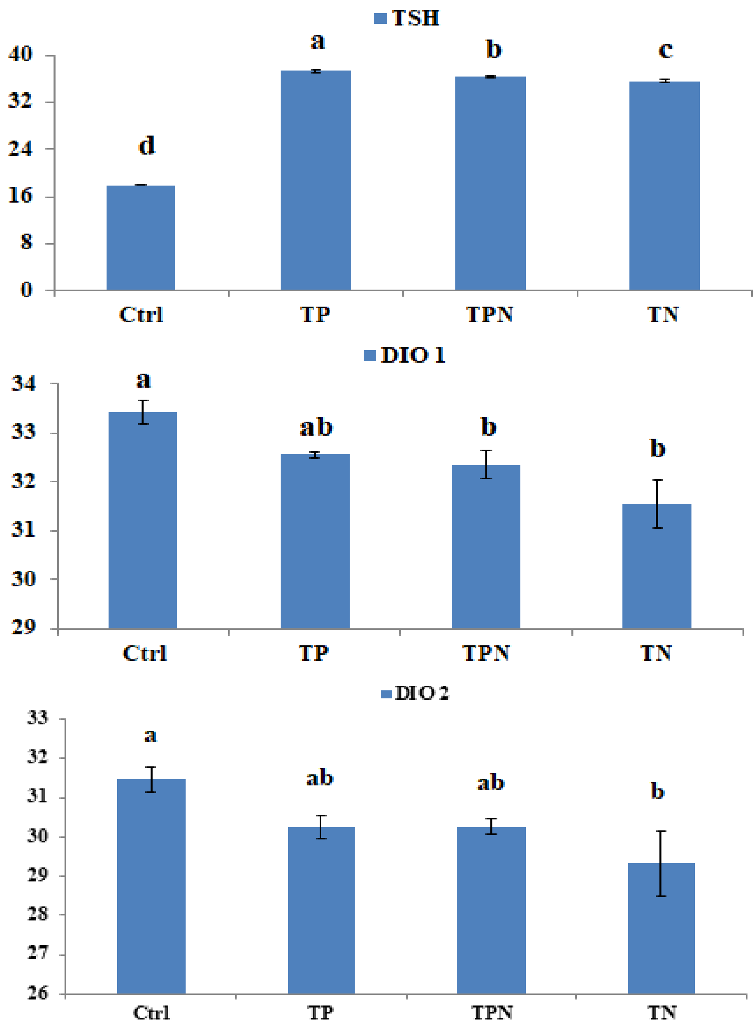

Figure 4: Levels of TSH, DIO1, and DIO2 in zebrafish after 60 days of the experiment. Values are expressed as the mean±SD. The same letters mean no difference (*p*>0.05) and different letters mean a significant difference at the 5% level (*p*<0.05)

On day 30 of the experiment, there was a significant difference between TP treatment and TN and control treatments (p<0.05). On this day, the highest value was reported in the TP treatment and the lowest value was reported in the Control treatment ($p<0.05$). Also, on this day, no significant difference was recorded between Control, TPN and TP and between TPN and TN ($p>0.05$). On day 60 of the experiment, there was a significant difference between TP treatment with TN and control treatments ($p<0.05$). On this day, the highest value was reported in the TP treatment and the lowest value was reported in the Control treatment ($p<0.05$). Also, on this day, no significant difference was recorded between Control, TPN and TP and between TPN and TN ($p>0.05$) (Figs. 5 to 9). The results of Weight Gain (g) and Percentage weight gain (%) after 60 days and Specific Growth Rate (g) after 30 and 60 days showed that significant difference between TP and TN and control treatments ($p<0.05$).

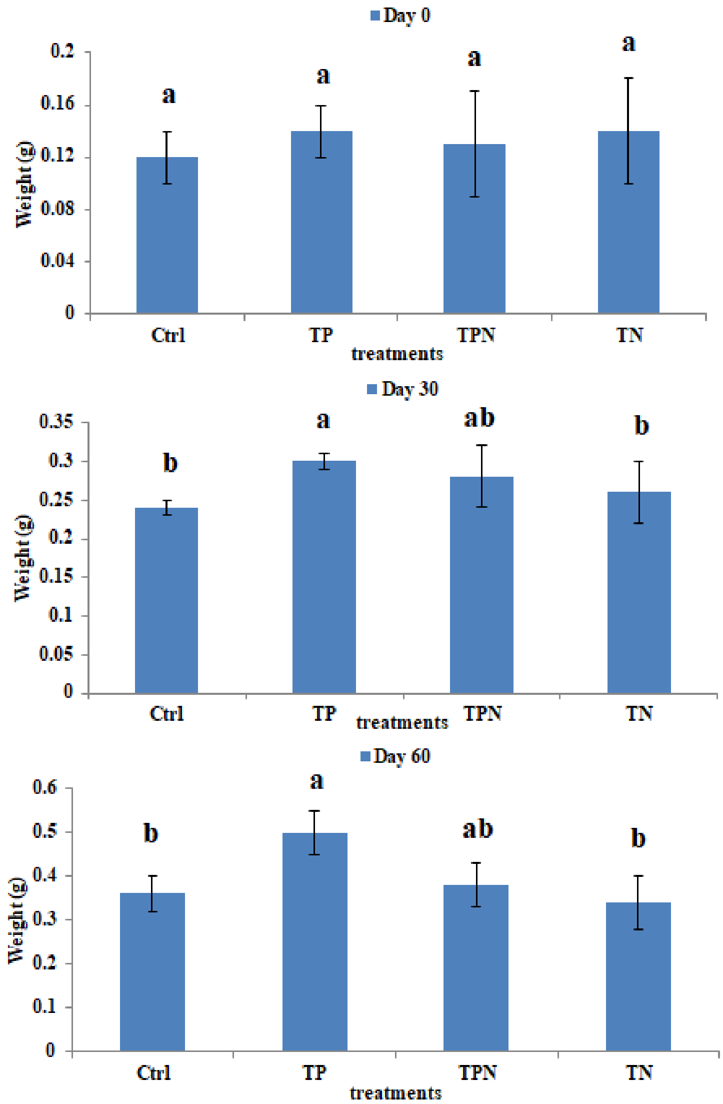

Figure 5: Weight (g) in zebrafish after 0, 30 and 60 days of the experiment. Values are expressed as the mean±SD. The different letters mean a significant difference at the 5% level ($p<0.05$).

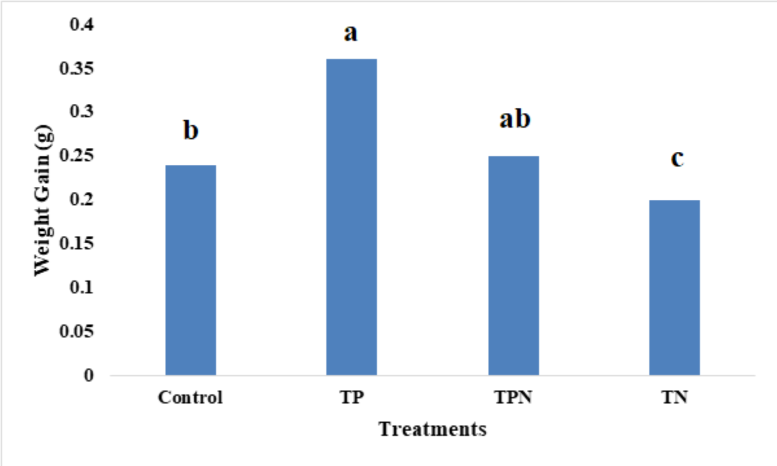

Figure 6: Weight Gain (g) in zebrafish after 60 days of the experiment. Values are expressed as the mean±SD. The different letters mean a significant difference at the 5% level (*p*<0.05).

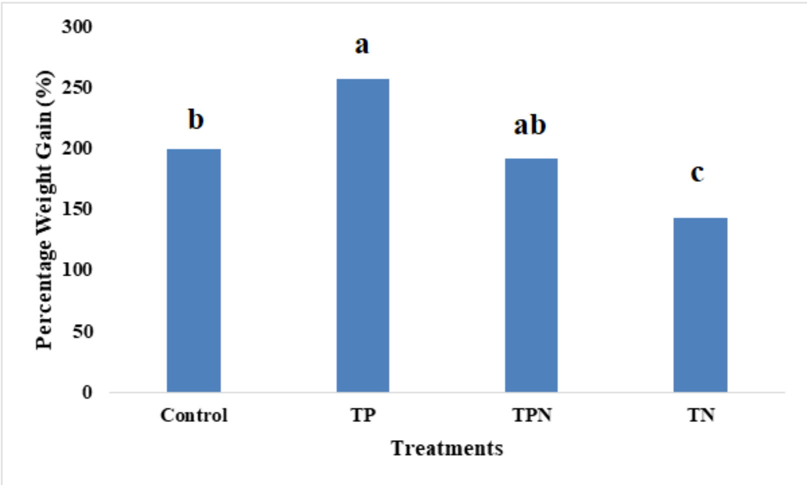

Figure 7: Percentage weight gain (%) in zebrafish after 60 days of the experiment. Values are expressed as the mean±SD. The different letters mean a significant difference at the 5% level (*p*<0.05).

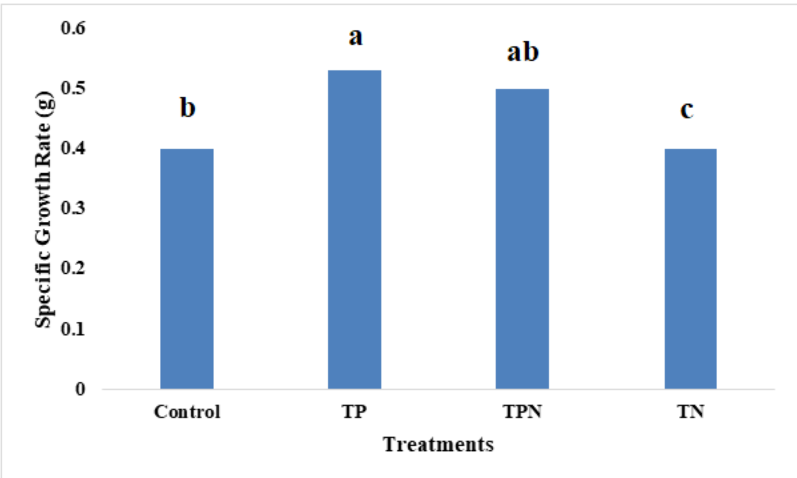

Figure 8: Specific Growth Rate in zebrafish after 30 days of the experiment. Values are expressed as the mean±SD. The different letters mean a significant difference at the 5% level (*p*<0.05).

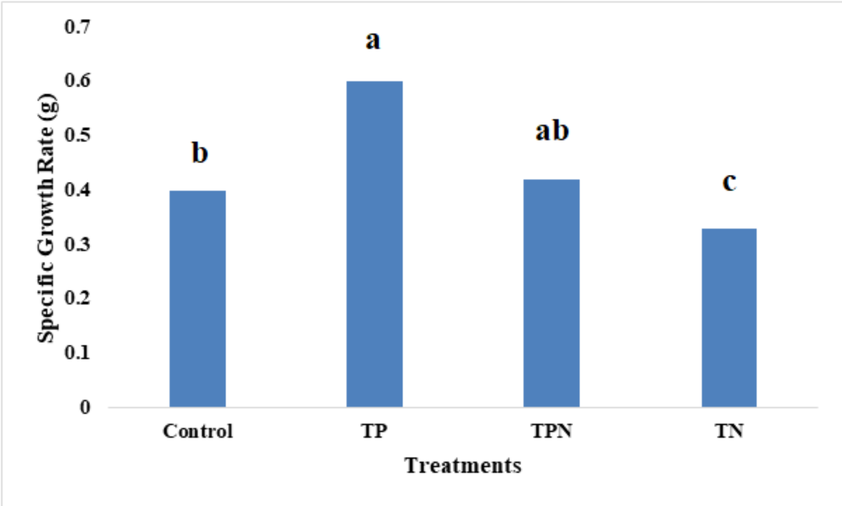

Figure 9: Specific Growth Rate (g) in zebrafish after 60 days of the experiment. Values are expressed as the mean±SD. The different letters mean a significant difference at the 5% level (*p*<0.05).

The highest value was reported in the TP and the lowest value was reported in the TN treatment ($p<0.05$). Also, no significant difference was recorded between Control, and TPN.

Discussion

TH is secreted from the hypothalamic-pituitary-thyroid (HPT) axis and is involved in the body's metabolism, growth, behavior, immune regulation, and stress response in fish (Shkil *et al.*, 2019). Therefore, changes in TH (T3, T4, and TSH) and factors involved in the metabolism of these hormones (DIO1, DIO2) are effective in the biological function (like metabolism of the body, protein synthesis, carbohydrate and fat metabolism, neural development) of fish. So far, no study has been done on the effect of using probiotic supplements on reducing the adverse effects of drug poisoning in fish. The present study was performed to investigate the role of *L. reuteri* probiotic and NPX exposure on thyroid and growth rate in zebrafish.

TH results did not show significant changes in NPX exposure at T3 levels but decreased T4 levels and increased TSH gene transcription were observed in fish. Published articles on thyroid dysfunction due to drug poisoning in aquatic organisms are limited, but the effects of some NSAIDs on fish thyroid disorder have been published (Saravanan *et al.*, 2014; Xu *et al.*, 2019; Zloh *et al.*, 2016). In similar studies, decreased T4 levels and increased TSH gene transcription were reported as a result of prolonged exposure to NPX in zebrafish (Xu *et al.*, 2019). Saravanan *et al.* (2014) reported decreased T4 levels and increased TSH levels in *Cirrhinus mrigala* after prolonged exposure to NSAIDs including clofibric acid and diclofenac (Saravanan *et al.*, 2014). According to the study of Xu *et al.* (2019), the reduction of T4-negative feedback is due to the positive regulation of tshβ genes in fish exposed to NPX, All these results are consistent with our study. In this regard, due to the limited studies on the effects of NPX on TH in fish, the mechanisms involved in the effect of NSAIDs on mammalian thyroid will be investigated. According to the model used (zebrafish), its genetic similarities with humans, and the importance of this fish in evaluating the effects of drugs (Chakraborty *et al.*, 2009), it is necessary to review the relevant articles. The first mechanism is due to the structure of many NSAIDs, which include carboxylic acid and is the site of metabolism and formation of acyl glucuronides (esters). These ester glucuronides are rapidly hydrolyzed after contact with intestinal microbiota to release aglycone. This process damages the intestinal mucosa in animals and causes the toxic action of NSAIDs (Wilson and Nicholson, 2017). Wilson and Nicholson (2017) recommend the study of intestinal mucosal histopathology in fish exposed to NPX. Alterations in intestinal microbiotas (such as reduced lactobacilli) have been reported in humans and mice due to NSAIDs (Mäkivuokko *et al.*, 2010; Liang *et al.*, 2015). The effect of intestinal microbiota and metabolites on TH regulation has been demonstrated by studying the effect on the intestinal-brain, intestinal-thyroid, and hypothalamic-pituitary (HPA) axes (Huo *et al.*, 2021), The mentioned items can be the reason mechanisms in the results of our study.

On the other hand, in this study, T3 levels did not change significantly due to probiotic feeding. T4 levels decreased and TSH transcription increased, which is consistent with the results of Kanwal and Tayyeb (2019). Kanwal and Tayyeb (2019) reported decreased T4 levels and increased TSH regulation in *Labeo rohita* as a result of feeding with commercial probiotics. Probiotics increase TSH in the host by reducing the production of bile acids. In this regard, there have also been limited studies on the mechanism of probiotic action on fish thyroid. Based on our reviews, the most important and first mechanism of probiotic action is the regulation of microbiota and intestinal metabolites. This mechanism improves thyroid function, reduces T3, and T4, and increases TSH in the host by affecting the intestinal-brain and intestinal-thyroid axes (Huo *et al.*, 2021). As, Huo *et al.* (2021) observed the presence of *Lactobacillus reuteri* in the intestinal microbial flora, and expressed that probiotics enhance the secretion of short-chain fatty acids (SCFAs) by regulating intestinal microbiota. SCFA also affects host neurotransmitters (such as dopamine) in the brain. in such a way that regulates HPA and increases TSH by decreasing the concentration of thyrotropin receptor antibody (TRAb) (Farzi *et al.*, 2018). In addition, the effect of probiotics on TH levels can be attributed to their effect on regulating blood iron levels. Thyroid dysfunction is associated with abnormal levels of this mineral (Huo *et al.*, 2021). All the mentioned cases can be the causes of the observed changes in the level of TH in the present study, and the authors recommend that they be taken into consideration in the next investigations.

Regarding the simultaneous effect of feeding with probiotics and exposure to NPX, the results of this study showed that T4 levels and TSH gene expression were higher in the probiotic-fed group compared with the basal-fed and NPX-exposed groups. These results were more similar to the non-toxic groups, which shows the positive effect of feeding with probiotics on endocrine function during poisoning. In this regard, the benefits of using probiotics to prevent NSAID-induced disorders in host have been reported in several studies (Mäkivuokko *et al.*, 2010). The effect of NSAID poisoning on intestinal mucosal damage (Wilson and Nicholson, 2017) and changes in intestinal microbiota (Mäkivuokko *et al.*, 2010; Liang *et al.*, 2015) has been reported. On the other hand, based on previous studies the use of probiotics improves the function of the microbial flora and intestinal structure in fish (Alavinezhad *et al.*, 2020; Mirabdollah Elahi *et al.*, 2020). Which can be the cause of the observed changes in T4 level and TSH gene expression in our study. As Farzi *et al.* (2018) and Huo *et al.* (2021) and emphasized the significant effect of microbial flora on the thyroid. Of course, as it is clear in the results section the modification of the intestinal microbial flora of zebrafish in this study was due to feeding with *L. reuteri* as probiotic.

Deiodinases are important regulators of thyroid hormone levels in vertebrates. Their transcriptional level is considered a sensitive biomarker in thyroid disorders due to the sensitivity of deiodinases to environmental chemicals (Picard-Aitken *et al.*, 2007). In the present study, the results of DIO1 and DIO2 did not show significant changes due to probiotic feeding, while they decreased during exposure to NPX. Probiotic feeding improved DIO1 and DIO2 levels compared to basal feeding, and the data were more normal. Xu *et al.* (2019) reported a decrease in the transcription levels of DIO1 and DIO2 as a result of prolonged exposure to NPX in zebrafish, which was consistent with our results. The conversion of T4 to biologically active T3 in fish is mainly controlled by the activities of DIO1 and DIO2 (Xu *et al.*, 2019). DIO2 plays a major role in thyroid hormone homeostasis and active T3 production (Yu *et al.*, 2010). In this study, Not seeing significant changes in T3 levels at the time of NPX exposure were due to a decrease in deiodinases.

Also, an increase in weight index and growth factors was observed in the groups fed with *L. reuteri*. Numerous studies have reported an increase in the weight and growth rate of Lactobacillus-fed zebrafish (Falcinelli *et al.*, 2015; Mohammadian *et al.*, 2019; Alavinezhad *et al.*, 2020). In stating the reason for the above observations various studies have been performed on the mechanisms involved in increasing probiotic-induced growth enhancement, all of which can be the reasons for increased growth as a result of feeding with probiotics in the present study. Eleraky *et al.* (2014) reported an increase in fat and total protein content in probiotic-fed *Cyprinus carpio* fish. The increased growth rate in probiotic-fed fish can be attributed to better digestion (Kanwal and Tayyeb, 2019). According to El-Haroun *et al.* (2006), the use of the commercial probiotic *B. Subtilis* had a positive effect on the production of digestive enzymes (lipase, amylase, and protease) in Nile tilapia and increased its growth rate. These mechanisms were not investigated in our study, but it is interesting to investigate them in zebrafish fed with *L. reuteri*. in future studies.

On the other hand according to the results, long-term exposure to NPX reduced the growth rate in zebrafish. The inhibitory effect of exposure to drug residues on growth in aquatic organisms has been reported in previous studies (Wang *et al.*, 2021). Weight loss of zebrafish exposed to NPX has been reported in the study of Xu *et al.* (2019). Xu *et al.* (2019) identified thyroid dysfunction due to prolonged exposure to NPX as a possible reason for growth inhibition in zebrafish. Toxins affect the levels of TH and lead to significant impairment of zebrafish growth (Tu *et al.*, 2016; Cheng *et al.*, 2017; Xu *et al.*, 2019). The importance of thyroid hormones in fish metabolism and growth (Shkil *et al.*, 2019) and the obtained results in our study about TH confirm this theory.

Furthermore, the increase in growth was significantly higher in the probiotic-fed group compared with the basal-fed group exposed to NPX. Probiotic nutrition improves intestinal bacterial flora, increases feed intake (Eleraky *et al.*, 2014), and improves digestion (Kanwal and Tayyeb, 2019). While, exposure to NPX (as an NSAID) changes the gut microbiota and reduces lactobacilli (Mäkivuokko *et al.*, 2010). Therefore, an increase in the growth rate of fish-fed probiotics compared to fish-fed basal diets exposed to NPX in our study will not be unexpected. In this study, probiotic feeding improved TH levels exposed to NPX. These results justify the increase in growth due to the effect of thyroid hormones on growth factors such as epidermal growth factor (EGF), nerve growth factor (NGF) and growth hormone (Cabello and Wrutniak, 1989). Generally, probiotic nutrition improved thyroid function and reduced the adverse effects of NPX on this endocrine gland. Also, the probiotics used increased growth factors and improved growth rate by increasing consumption and digestion of food.

In conclusion, the identification of NPX in surface waters and the adverse effects of drug residues on the biological and physiological functions of fish have made it necessary to introduce dietary supplements to reduce these effects. The results showed that long-term exposure to low concentrations of NPX could cause significant thyroid dysfunction and growth inhibition in zebrafish. NPX poisoning disrupted gene transcription and thyroid hormone levels which were consistent with the observed effects on growth. While, Probiotic-fed groups improved thyroid function and growth rate in fish. These results indicate the importance of evaluating the thyroid disorder of aquatic organisms exposed to a variety of medicinal compounds in the environment. This study also shows the need to use dietary supplements (including probiotics) in fish farms contaminated with drug residues. Finally, it is recommended to study effective mechanisms in thyroid function and growth regulation in probiotic-fed fish exposed to NPX in future studies. On the other hand zebrafish is an animal model with genetic compatibility with humans. Due to the identification of NPX in drinking water (Wojcieszyńska and Guzik, 2020), and the adverse effects of drug residue in humans, including development of drug resistance, disruption of normal intestinal flora, drug hypersensitivity reaction, mutagenic, carcinogenic, and teratogenic effects (Okocha *et al.*, 2018), the results obtained can be generalized to humans and the use of oregano essential oil in humans as a functional food supplement is recommended to reduce the adverse effects of drug residues on endocrine function.

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