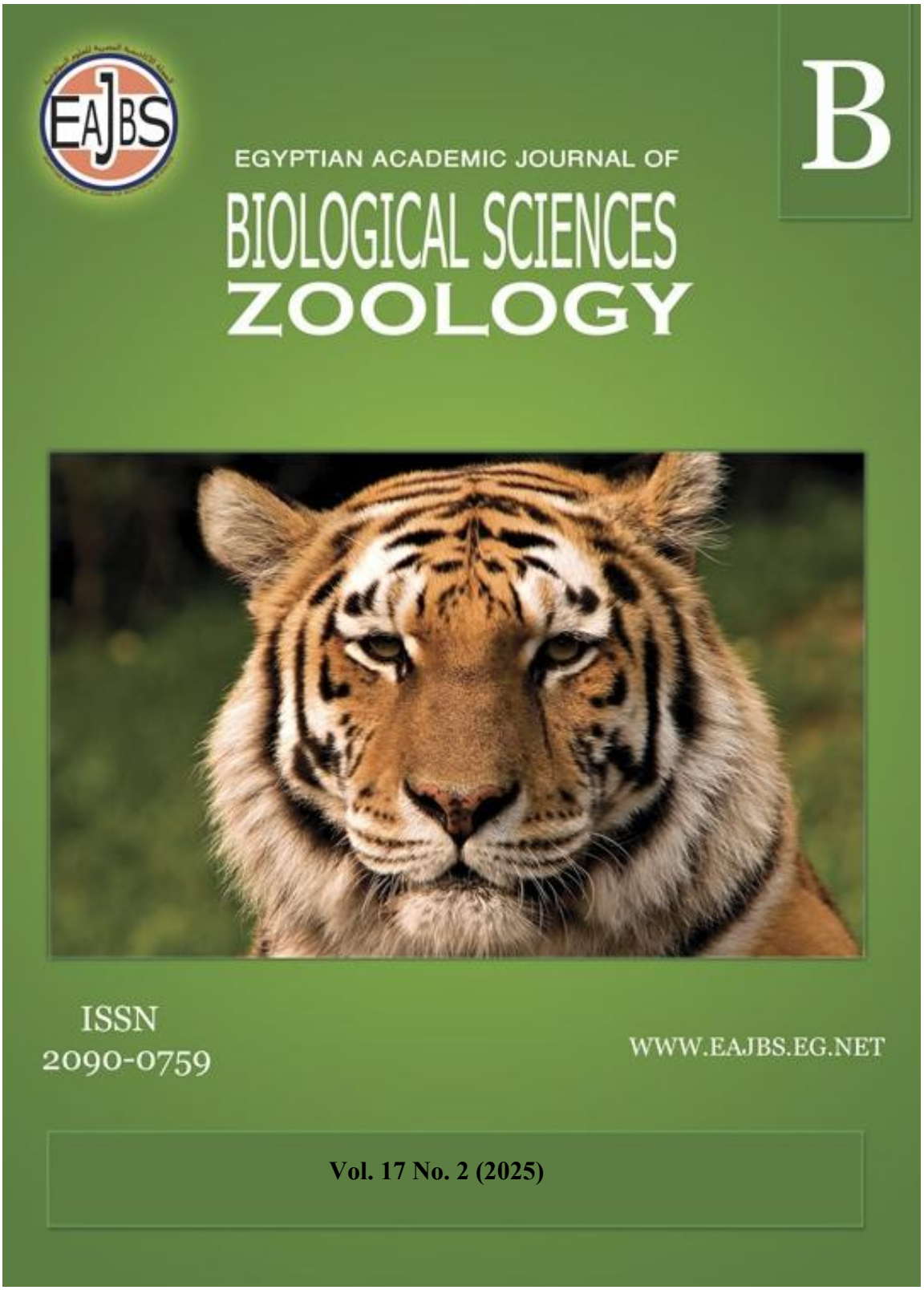

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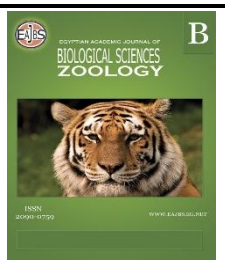

Probiotic Lactic Acid Bacteria Improve Growth Performance and Hormone in *Oreochromis mossambicus*

Pushpa T. A. Glory[1], Iyyadurai Vasudhevan[2], Tharmaraj R. J. JebaMalar[3], Ponnuswamy Vijayaraghavan[4*]

[1]Department of Zoology, Holy Cross College (Autonomous), Nagercoil, Affiliated to Manonmaniam Sundaranar University, Tirunelveli, India.
[2]Department of Zoology, Vivekananda College, Agasteeswaram, Affiliated to Manonmaniam Sundaranar University, Tirunelveli, India.
[3]PG and Research Department of Nutrition and Dietetics, Muslim Arts College, Thiruvithancode, Affiliated to ManonmaniumSundaranar University, Tirunelveli, India.
[4]Bioprocess Engineering Division, Smykon Biotech, Nagercoil, India.
*E-mail: vijayaraghavanp22@gmail.com

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Fish; Diet, Probiotics; Lactic acid bacteria; Growth hormone; Immune function.

ABSTRACT

Probiotic bacteria are used for the development of probiotic fish diets to improve fish growth and stimulate immune function. In the present study, we analyzed the effects of *Lactobacillus acidophilus* and *Lactobacillus rhamnosus* on the growth performance of *Oreochromis mossambicus fingerlings.* The dried probiotic LAB were mixed with the artificial diet and mixed. The mixture was supplemented with a probiotic mixture (*L. acidophilus and L. rhamnosus*) at five different concentrations. The diet was divided into aliquots, and probiotic were added at concentrations of 2.5 $\times 10^8$ (T1), 5 $\times 10^8$ (T2), 7.5 $\times 10^8$ (T3), 10 $\times 10^8$ CFU/mL (T4) and T0 (without probiotics). The growth performance was analyzed for two months. The results revealed that the growth performance of the fish fed the probiotic diet at 10 $\times 10^8$ CFU/mL significantly improved. This concentration efficiently improved the feed conversion ratio and specific growth rate. The weight gain rate of *O. mossambicus* fingerlings fed diets with varying levels of probiotics was significantly greater than that of those fed the control diet. The probiotic diet improved growth hormone and thyroid-stimulating hormone levels. The results revealed that the combination of *L. acidophilus* and *L. rhamnosus* has an effective symbiotic effect on improving the growth and physiological activity of *Oreochromis mossambicus.*

INTRODUCTION

The requirement for high-quality protein is growing rapidly due to the growth of the world's population and the growing need for nutritious diets. Fish are valuable nutritional and economic resources, and they provide people with high-quality protein. However, there are numerous problems associated with extensive aquaculture farming, including low feed efficiency and environmental issues, which affect fish quality and food safety. Enhancing fish growth performance and meat quality is critical for aquaculture practices. The use of probiotics

for improving growth and enhancing the quality of meat has increased tremendously in recent years. Probiotics improved health in livestock, poultry, and aquatic species. Probiotic organisms support gut health and play crucial regulatory roles in fishes. Animal growth performance is positively influenced by probiotic organisms. Probiotics have been shown to improve feed utilization and feed conversion efficiency, which in turn enhances the growth performance of aquatic animals. In addition, probiotic organisms improved the gut microbial flora and the biosynthesis of enzymes. The growth performance of hybrid grouper (*Epinephelus fuscoguttatus* × *E. lanceolatus*) was considerably enhanced by *Bacillus* supplementation as a probiotic bacterium (Amoah *et al*., 2023). Supplementing a probiotic diet (*Lactobacillus acidophilus* or *L. bulgaricus*) with *Oncorhynchus mykiss* greatly improved growth performance and improved immune function (Mohammadian *et al*., 2019). Probiotics have shown promising effects in improving protein content in addition to growth performance and enzyme activities. In addition, probiotics have been shown to improve the amino acid and fatty acid contents of muscles and stimulate muscle protein synthesis (Giron *et al*., 2022). Probiotic bacteria also reduce fat accumulation and control the proportion of adipose tissue in muscles (Prokopidis *et al*., 2023). The probiotic *Clostridium butyricum* influences lipid metabolism, meat quality, and the composition of amino and fatty acids (Liu *et al*., 2018). Probiotic organisms alter the microbial community structure in the gut of animals and support the regulation of glucolipid metabolism (Luo *et al*., 2023). According to recent findings, probiotics affect the nutrient absorption and metabolism of the gut via interactions with intestinal epithelial cells and alter the composition of the gut microbiota (Sánchez *et al*., 2017; Alfarhan *et al*., 2024). Probiotic microbes can produce bioactive compounds that affect the host's metabolic functions either directly or indirectly (Wang *et al*., 2023). Probiotic bacteria produce short-chain fatty acids, which improve fat oxidation, improve insulin sensitivity, and reduce blood glucose levels (Wang Y. *et al*., 2020).

Tilapia (*Oreochromis mossambicus*) is one of the most widely acclaimed fish species worldwide because of its specific characteristics, such as rapid growth, resilience to environmental stressors, rapid growth in confined environments, and strong market demand. This species has been cultivated in more than 100 nations across the world and serves as a vital source of income to fishers in various developing nations. In modern aquaculture, intensive farming methods are often used to improve fish production within short areas. Nevertheless, the high prevalence of various bacterial diseases suggests the application of commercial antibiotics in the natural environment. For the past two decades, antibiotic usage has led to the development of drug-resistant microbial species. In addition, the unfair use of antibiotics has caused food safety issues. Additionally, the frequent use of antibiotics can easily lead to the development of drug-resistant bacteria in the gut of fish, resulting in gut dysbiosis, which may affect nutrient metabolism and the immune function of the fish.

MATERIALS AND METHODS

Experimental Fish and Maintenance:

A total of 200 healthy *Oreochromis mossambicus* fingerlings, approximately 10 ± 1 g in weight and 7 ± 0.5 cm in length, were used for this study. The plants were maintained in a well-aerated round fiber tank. The other physiological conditions included a pH of 7.1, a temperature of 29 °C, and a dissolved oxygen level of 6.9 mg/L. To perform individual experiments, individual fish were weighed, and their length was measured. The fish were maintained in a well-aerated fiber tank, and an adequate number of fish were used for all experiments. Before all the experiments were performed, the stock fingerlings were acclimatized for fifteen days and used for analysis. An artificial pellet diet was used for all the fish, which were fed twice a day. The proximate composition of the artificial diet was 28% crude protein, 4% fat, 13% moisture, 18% ash and 8% crude fiber.

Lactobacillus and Culture Conditions:

Lactobacillus acidophilus (MTCC 447) and Lactobacillus rhamnosus (MTCC 5462) were used for the preparation of the probiotic diet. These two LABs were collected from the MTCC, Pune, India, and used until otherwise stated. Briefly, a loopful culture of individual cultures was inoculated in a 250 mL Erlenmeyer flask containing 100 mL of sterilized culture medium. The mixture was incubated for 24 h at 37 °C and used as inoculum. The cell density was 2×10^6 CFU/mL, and the mixture was lyophilized and used for this study (Al-Dhabi *et al.,* 2020). The dried probiotic LAB were mixed with the artificial diet and mixed. The mixture was supplemented with a probiotic mixture (L. acidophilus and L. rhamnosus) at five different concentrations. The diet was divided into five different aliquots, and probiotics were added at 2.5×10^8 (T1), 5×10^8 (T2), 7.5×10^8 (T3), 10×10^8 CFU/mL (T4) and T0 (without probiotics). For each experiment, 20 fish were maintained (n=20), and triplicate assays were performed. To the control, only the basal diet was added. The experiment was performed for 60 days, and growth performance was determined.

Analysis of the Fish Growth Profile:

The growth of the fish was analyzed on the basis of body length and body mass. The total length was measured via a scale, and the weight was measured via an electronic balance (Wang B. *et al.,* 2020). The growth profiles were analyzed via the following equation.

Specific growth rate = Log final weight – Log initial weight/Number of Days × 100

Feed conversion ratio (FCR) = Feed consumed (g)/Weight gain (g)

Protein efficiency ratio = weight gain (g)/protein intake (g) × 100

Analysis of Fish Protein and Lipid Contents:

After 60 days of the experimental trial, two fish from each group were randomly selected and further anesthetized with clove oil (100 µL/L). It was further sacrificed for the preparation of tissue samples and blood collection. The crude protein and lipid contents of the fish muscle were analyzed, and the results were compared with those of the control group. The total protein content of each sample was determined *via* the Kjeldahl method. The crude lipid content of each sample was determined *via* the chloroform:methanol extraction method.

Determination of Growth Hormones from Serum:

A blood sample (0.5 mL) was collected from the caudal fin of the fish and left undisturbed for 30 min. The sample was further centrifuged at 5000 rpm for 3 min, and the pale yellow solution was used for analysis. The amounts of growth hormone (GH) and thyroid stimulating hormone (TSH) were determined via ELISA (ELISA Kit, DiaMetra, Italy) (Farraj *et al*., 2024).

RESULTS AND DISCUSSION

Growth Performance of *O. Mossambicus* Fed a Probiotic Diet:

Figures 1 and 2 show that the number of juvenile *O. mossambicus* that were given probiotic diets increased with increasing levels of *Lactobacillus* species. Compared with the control diet (without probiotics), the weights and lengths of *L. acidophilus* and *L. rhamnosus* improved significantly ($p < 0.05$) (Fig. 1). Probiotics are beneficial microbes that pass through the esophagus to reach the intestines. Once nutrients are reached, they regulate the host's nutrient absorption and support immune function and metabolic processes. As a result, this improves the growth and overall health of the animals (Son *et al*., 2009). The probiotic diet containing *L. acidophilus* and *L.* rhamnosus used in our study can proliferate within the gut environment (Son *et al.,* 2009). Feeding experiments revealed that, in comparison with control diets, different levels of dietary probiotic supplementation significantly improved the growth performance of juvenile *O. mossambicus* and significantly increased in weight (Fig. 2).

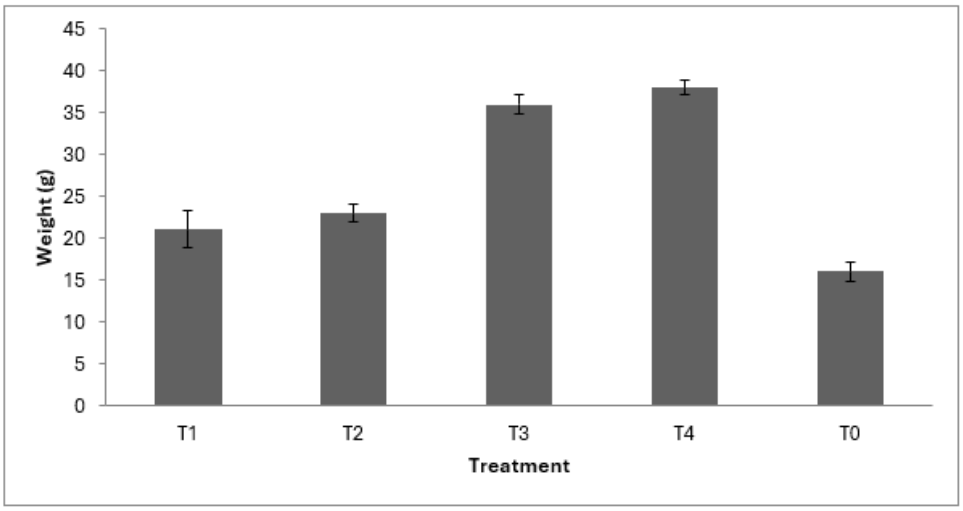

Fig. 1: Effects of a probiotic diet on the weight of *Oreochromismossambicus* fingerlings. Two probiotic bacteria (*Lactobacillus acidophilus and Lactobacillus rhamnosus)* were used for the preparation of the artificial fish diet. Five different types of diets were prepared with varying inoculum sizes (2.5 $\times 10^8$ CFU/mL (T1), 5$\times$ 10^8 CFU/mL (T2), 7.5 $\times$ 10^8 CFU/mL (T3), 10 $\times$ 10^8 CFU/mL (T4) and T0 (without probiotics)).

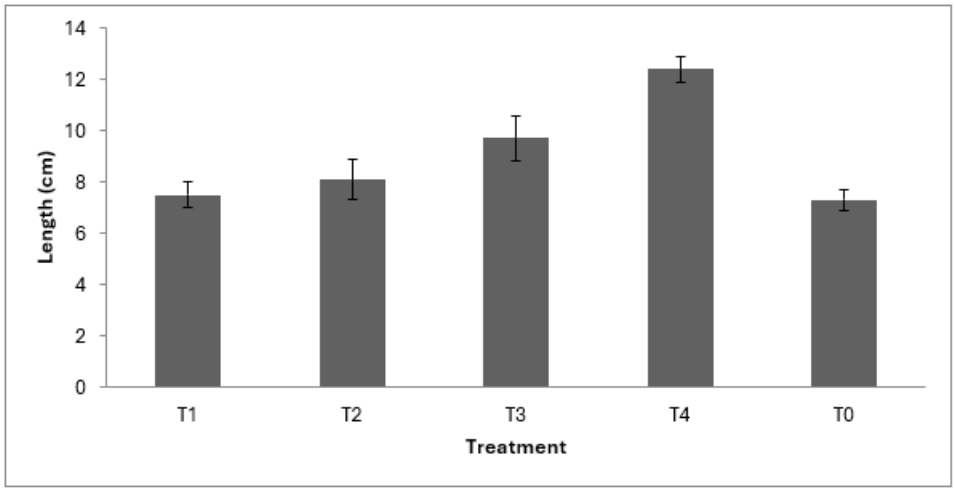

Fig. 2: Effects of a probiotic diet on the length of *Oreochromis mossambicus* fingerlings. Two probiotic bacteria (*Lactobacillus acidophilus and Lactobacillus rhamnosus*) were used for the preparation of the fish diet. Five different types of diets were prepared with varying inoculum sizes (2.5 $\times 10^8$ CFU/mL (T1), 5 $\times$ 10^8 CFU/mL (T2), 7.5 $\times$ 10^8 CFU/mL (T3), 10 $\times$ 10^8 CFU/mL (T4) and T0 (without probiotics)).

Analysis of Specific Growth Rates and Feed Conversion Ratios:

The weight gain rate of *O. mossambicus* fingerlings fed diets with varying levels of probiotics was significantly greater ($p < 0.05$) than that of those fed the control diet (Fig. 3). The feed conversion ratio for juveniles receiving different levels of probiotics was significantly lower ($p < 0.05$) than that for those consuming the control diet (Fig. 4). According to Zheng *et al*. (2018), a probiotic diet improved the growth of *O. mossambicus* fingerlings by increasing the activities of digestive enzymes. In addition, supplemented LAB produce various biochemicals that can be directly utilized as nutrients. Furthermore, LAB produce organic acids and vitamins that contributes to maintaining a balanced intestinal ecosystem and promoting growth (El-Saadony *et al*., 2021; Tran *et al*., 2022). Additionally, Giorgia *et al*. (2018) reported that LAB can improve feeding in Nile tilapia by increasing the levels of neuropeptide Y and

growth hormone-releasing peptide while reducing leptin levels, which in turn stimulates appetite and improves growth. The various effects of LAB on different aquatic species may vary in terms of species-specific responses and differences in the bioactive compounds produced by distinct strains of LAB.

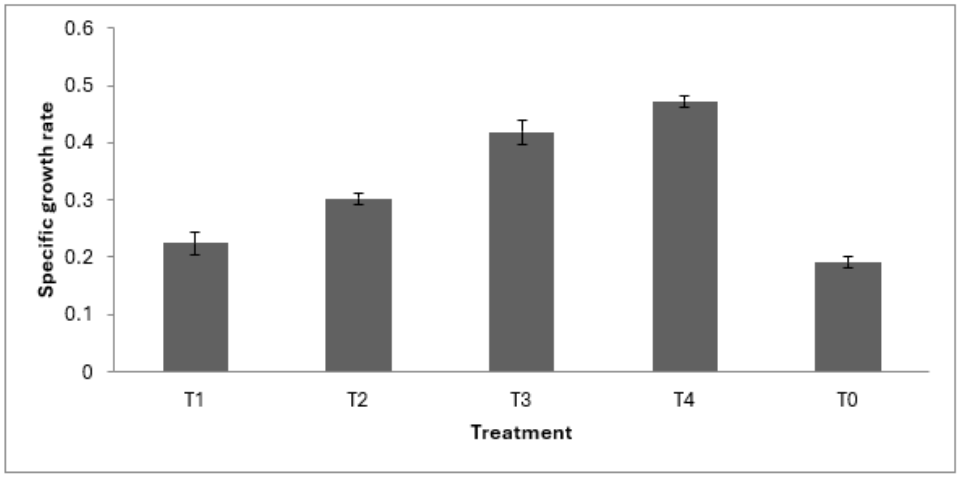

Fig. 3: Effects of a probiotic diet on the specific growth rate of *Oreochromis mossambicus* fingerlings. Two probiotic bacteria (*Lactobacillus acidophilus and Lactobacillus rhamnosus)* were used for the preparation of the fish diet. Five types of diets were prepared with varying inoculum sizes (2.5 ×10^8 CFU/mL (T1), 5 × 10^8 CFU/mL (T2), 7.5 × 10^8 CFU/mL (T3), 10 × 10^8 CFU/mL (T4) and T0 (without probiotics), and the specific growth rates were calculated.

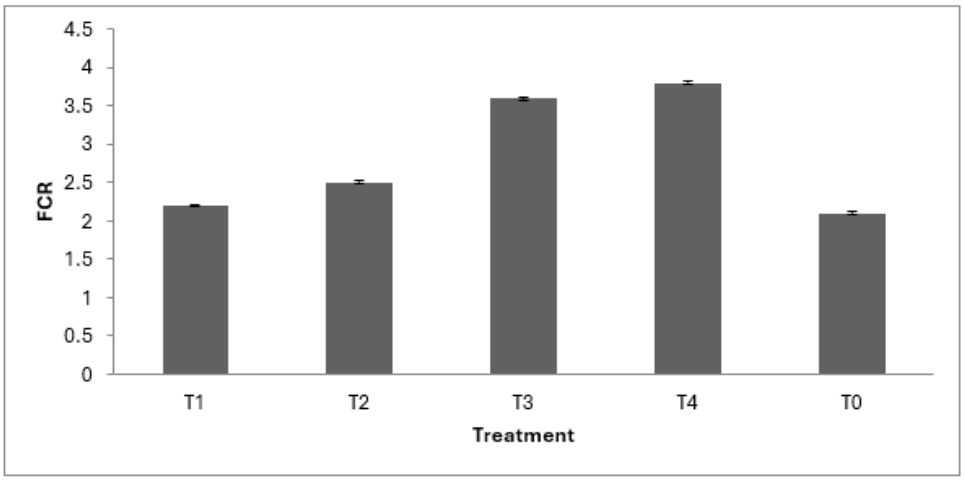

Fig. 4: Effects of a probiotic diet on the feed conversion ratio of *Oreochromis mossambicus* fingerlings. Five different types of diets were prepared using probiotic mixtures *(Lactobacillus acidophilus and Lactobacillus rhamnosus)* with varying inoculum sizes (2.5 ×10^8 CFU/mL (T1), 5× 10^8 CFU/mL (T2), 7.5 × 10^8 CFU/mL (T3), 10 × 10^8 CFU/mL (T4) and T0 (without probiotics).

Protein Efficacy Analysis and Fish Muscle Protein and Fat Content Determination:

The crude muscle protein content in fingerlings that were given the formulated diet with LAB was significantly greater ($p < 0.05$) than that in those that received the control diet. The muscle crude protein content in juvenile *O. mossambicus* fed inoculum (7.5 × 10^8 CFU/mL (T3) or 10 × 10^8 CFU/mL (T4)) significantly improved protein efficacy, and the results are shown in Fig. 5. In addition, the amount of muscle protein and fat content were significantly greater in the T4 diet (10 × 10^8 CFU/mL) (Fig. 6). Similarly, the probiotic diet improved the fat content in the muscle (Fig. 7). The bioactive compounds from the culture of LAB are rapidly

absorbed by the gut mucosa, potentially stimulating the production of several digestive enzymes in the gastrointestinal tract. The release of these digestive enzymes in the body can lead to the digestion of nutrients in the feed into smaller, more easily absorbed molecules such as proteins, small peptides, and amino acids, which in turn enhances nutrient digestion and absorption, increases the nutritional value and nutrient utilization rate of the feed, enhances the overall condition of the fish, improves the appetite, and lowers the feed conversion ratio. This specifically enhances the growth performance of the body and positively influences the muscle protein content of fish. Mukherjee *et al*. (2019) reported that the application of *Bacillus* can have a positive effect on the body composition of Rohu (*Labeo rohita*).

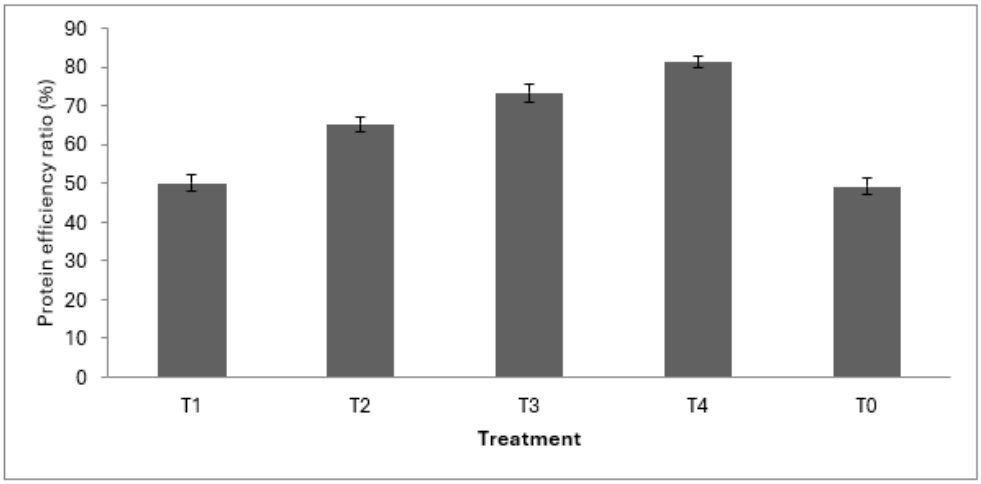

Fig. 5: Effects of a probiotic diet on the protein efficiency ratio of *Oreochromis mossambicus* fingerlings. Two probiotic bacteria (*Lactobacillus acidophilus and Lactobacillus rhamnosus)* were used for the preparation of the fish diet. Five different types of diets were prepared with varying inoculum sizes (2.5 $\times 10^8$ CFU/mL (T1), 5 $\times$ 10^8 CFU/mL (T2), 7.5 $\times$ 10^8 CFU/mL (T3), 10 $\times$ 10^8 CFU/mL (T4) and T0 (without probiotics)).

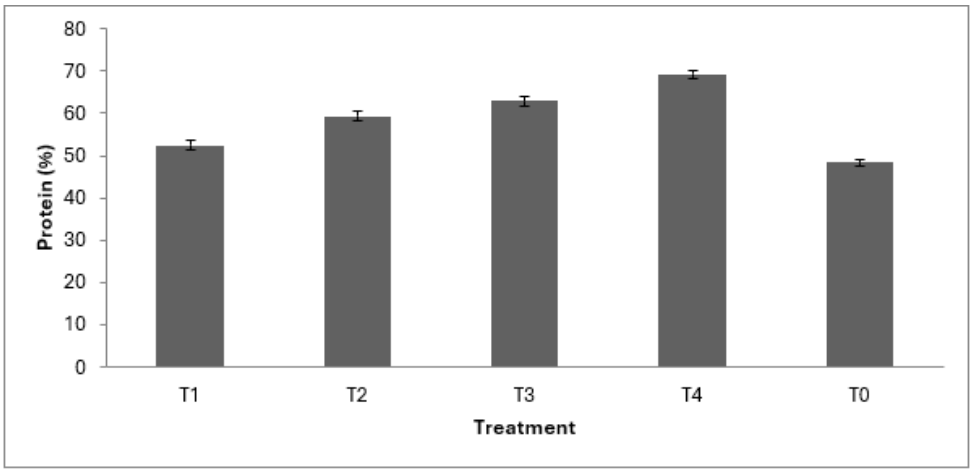

Fig. 6: Effects of a probiotic diet on the protein content of *Oreochromis mossambicus* fingerlings. Five different types of diets were prepared with varying inoculum sizes (2.5 $\times 10^8$ CFU/mL (T1), 5 $\times$ 10^8 CFU/mL (T2), 7.5 $\times$ 10^8 CFU/mL (T3), 10 $\times$ 10^8 CFU/mL (T4) and T0 (without probiotics)).

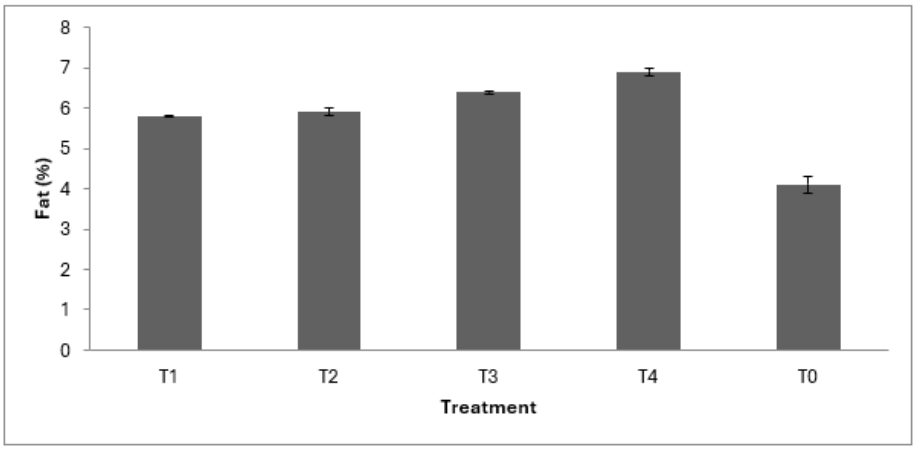

Fig. 7: Effects of a probiotic diet on fat content in *Oreochromis mossambicus* fingerlings. Two probiotic bacteria (*Lactobacillus acidophilus and Lactobacillus rhamnosus*) were used for the preparation of the fish diet. Five different types of diets were prepared with varying inoculum sizes (2.5 $\times 10^8$ CFU/mL (T1), 5 $\times$ 10^8 CFU/mL (T2), 7.5 $\times$ 10^8 CFU/mL (T3), 10 $\times$ 10^8 CFU/mL (T4) and T0 (without probiotics)).

Improvement of Growth Hormone and Thyroid-Stimulating Hormone by a Probiotic Diet:

The LAB-supplemented diet improved the serum GH levels. The serum GH concentration exhibited a substantial linear increase ($p < 0.05$) among the treated groups with increasing concentrations of LAB in the diet. The amount of growth hormone increased significantly in the experimental group compared with the control group (Fig. 8). In addition, the level of TSH was significantly greater in the experimental fish than in the control fish (Fig. 9). GH plays crucial roles in the body, including growth and immune system regulation (Franz *et al*., 2016). The supplementation of probiotics led to a significant increase in serum GH levels. Thyroid hormones are crucial for numerous physiological processes, including growth, development, behavior, and response to stress. In our study, the supplemented diet improved the level of thyroid stimulating hormone in the groups treated with probiotics. Following the administration of prebiotics, thyroid hormone levels significantly improved. Various studies have indicated that thyroid hormones play crucial roles in metabolic processes that are closely linked to growth-promoting factors (Biji *et al*., 2016; Wang B. *et al*., 2020).

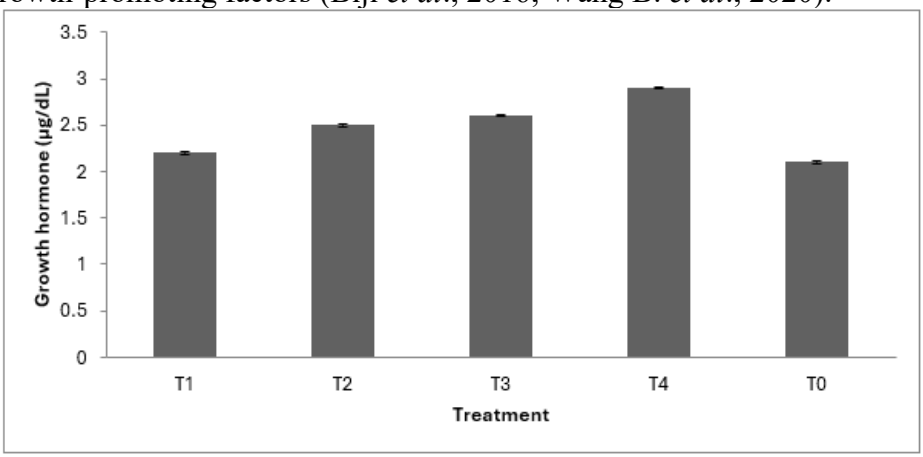

Fig. 8: Effect of a probiotic diet on the improvement of growth hormone in *Oreochromis mossambicus* fingerlings. Two probiotic bacteria (*Lactobacillus acidophilus and Lactobacillus rhamnosus)* were used for the preparation of the fish diet. Five different types of diets were prepared with varying inoculum sizes (2.5 $\times 10^8$ CFU/mL (T1), 5 $\times$ 10^8 CFU/mL (T2), 7.5 $\times$ 10^8 CFU/mL (T3), 10 $\times$ 10^8 CFU/mL (T4) and T0 (without probiotics)).

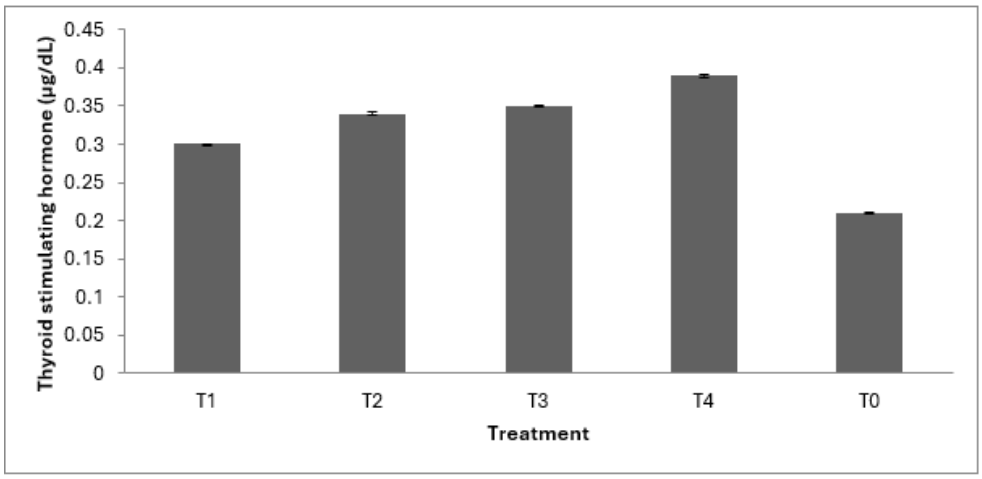

Fig. 9: Effect of a probiotic diet on the improvement of thyroid-stimulating hormone in *Oreochromis mossambicus* fingerlings. Two probiotic bacteria (*Lactobacillus acidophilus and Lactobacillus rhamnosus)* were used for the preparation of the fish diet. Five different types of diets were prepared with varying inoculum sizes (2.5 $\times 10^8$ CFU/mL (T1), 5 $\times$ 10^8 CFU/mL (T2), 7.5 $\times$ 10^8 CFU/mL (T3), 10 $\times$ 10^8 CFU/mL (T4) and T0 (without probiotics)).

Conclusions

The present findings revealed that dietary supplementation with probiotic LAB improved the gut function, growth and immune function of tilapia fingerlings (*Oreochromis mossambicus*). Two probiotic bacteria (*Lactobacillus acidophilus and Lactobacillus rhamnosus)* were mixed with an artificial pellet diet and fed to experimental animals. LAB improved growth, FCR, muscle protein content and fat content. In addition, it improved growth hormone and thyroid hormone levels. The LAB species used in this study represent a promising approach for improving growth performance and gastrointestinal function and stimulating growth hormones.

Declarations:

Ethical Approval: Not applicable.

Competing interests: The authors declare that they have no competing interests.

Author's Contributions: All the authors are equal in their contributions.

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Availability of Data and Materials: The data presented in this study are available upon fair request from the corresponding author.

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