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# Isolation of Lactobacillus Strains with Probiotic Activity to be used as a Fish Feed Supplement

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**ABSTRACT**

The paper reports the results of studies on the isolation of Lactobacillus strains with probiotic properties for use as a fish feed supplement. For this purpose, a pure culture of Lactobacillus strains with probiotic activity was isolated from the intestines of fish, and molecular genetic identification of the isolated Lactobacilli was carried out by 16S RNA sequencing following the Sanger method. The amplicons of 20 bacterial samples with a fragment length of 790bp were obtained. The results of nucleotide sequence decoding are as follows: two nucleotide sequences were not identified, and fish pathogens causing intestinal disorder belonging to three species (*Kurthia gibsonii*, *Kurthia zopfii*, and *Lactococcus garvieae*) were identified from eight sequences. The remaining 10 strains were identified as Lactobacillus belonging to two species: *Leuconostoc mesenteroides* and *Lactococcus lactis*. A phylogenetic tree was constructed using the nucleotide sequences. Catalase activity determination results confirm that the L. mesenteroides and L. lactis strains do not possess catalase activity. In contrast, air bubbles were formed when K. gibsonii strain was used, indicating catalase activity. These findings highlight the potential of *L. mesenteroides* and *L. lactis* as probiotic strains for developing functional fish feed supplements.

**Key words:** Lactobacillus Strains, Probiotic activity, Fish feed supplement, Nucleotide sequences.

## INTRODUCTION

Aquaculture is a fast-growing industry; with global population on the rise, there is an emerging requirement to enhance fish farming productivity in order to cope with the surging demand for seafood. However, the need for higher production presents many difficulties to the farmer, such as maintaining fish health and feed efficiency while lowering the environmental impact of fish farming. The use of functional feed additives in aquaculture has gained increasing attention in recent years due to their potential to enhance the growth performance and health of farmed fish.

Amidst its popularity as an alternative protein source, multiple research have focused on finding better ways to produce functional additives. A review by Hossain et al. (2024) addresses the need for functional feed additives (FUFA) from plant origin as a cost effective and ecological alternative in aquaculture. Hossain et al. (2024) cites FUFA as a solution to the rising cost of fishmeal, probiotic maintenance of aquaculture water and a conducive disease management strategy for aquatic animals.

Another review by Van Doan et al. (2023), highlights the production of functional feeds additives such as polysaccharides, carotenoids and polyunsaturated fatty acids from invertebrates of marine origin. Among these additives, symbiotics—combinations of prebiotics and probiotics—have emerged as a promising tool to improve gut health, nutrient absorption, and overall animal welfare. Probiotics are live beneficial microorganisms that, if given in sufficient amounts, confer health benefits to the host, whereas prebiotics are nondigestible food ingredients that selectively stimulate the growth and activity of gut bacteria. Combined, these two components interact in a symbiotic way, enabling the establishment of a healthy gut microbiota-an extremely important factor for nutrient absorption, resistance against disease, and welfare in fish. The use of symbiotics confers a number of advantages over administration with either probiotics or prebiotics alone.

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Symbiotics can promote gut health through the establishment of a healthy gut flora, enhancing feed efficiency and growth performance and feed conversion ratios; two major factors that determine profitability and sustainability in aquaculture (Martínez et al. 2012; Peredo et al. 2015; Phupaboon et al. 2024; Mirnawati et al. 2024). Symbiotics have also been observed to strengthen the immunity of fish by better equipping them against diseases and stressful factors occurring within aquaculture.

An appropriate balance in gut microbiota increases not only digestives and absorptions but also a barrier to harmful pathogens. Symbiotics may also improve intestinal health in farmed fish, decreasing the need for antibiotics and chemotherapy drugs generally linked to environmental hazards and the spread of antibiotic resistance.

In this context, Laice et al. (2021) conducted a study to investigate the effects of symbiotics on the growth performance of tilapia, one of the most widely farmed fish species. The study provides valuable insights into how dietary symbiotics can significantly influence key growth parameters in aquaculture. In their experiment, symbiotics were incorporated into the main diet of tilapia, and their impact on growth parameters was evaluated. The results showed significant improvements in the group of fish that consumed the symbiotics-enhanced diet compared to those that did not. Specifically, the final weight, weight gain, and specific growth rate (SGR) were all significantly higher in the fish fed with symbiotics.

These findings underscore the potential of symbiotics in aquaculture, suggesting that their inclusion in fish diets can significantly enhance growth performance, likely due to their role in improving gut health and nutrient absorption. The statistical significance of the results highlights the robustness of the observed effects. Thus, the study provides valuable insights for aquaculture practices, where improving feed efficiency and animal welfare are key goals.

Devi et al. (2019) note that probiotic, prebiotic, or symbiotic supplements in the diet increase antioxidant properties, gene transcription of pro- and/or anti-inflammatory cytokines, innate adaptive immunity, growth rate, and feed digestibility with very low or no mortality in healthy fish (Oleinikova et al. 2024).

According to Ogunkalu (2019), probiotics inclusion in feed improves the feed conversion ratio and reduces mortality rate. Probiotics have been shown to enhance the response immunity of fish. Chen et al. (2022) have demonstrated that fish gut microbiota promotes feed adaptation and improves feed conversion in fish.

In aquaculture, probiotics have been extensively studied for their role in enhancing fish health, stress tolerance, and growth performance (Dimitroglou et al. 2011; Tamura et al. 2021). Probiotics, like Lactobacillus acidophilus and nisin-producing Lactococcus lactis, are increasingly being integrated into fish diets to promote better gut health and boost immune responses, ultimately leading to improved production outcomes. These studies highlight the varying effects of different probiotic strains on fish species, showcasing their potential to improve stress resilience and growth while maintaining gut integrity.

In a study conducted by Hoseinifar et al. (2018), the introduction of *Lactobacillus acidophilus* into the diet significantly enhanced the resistance of black swordtail (Xiphophorus helleri) to salinity-induced stress, as indicated by improved survival rates ($P<0.05$). Moreover, the dietary administration of varying amounts of *L. acidophilus* resulted in marked improvements in weight gain, specific growth rate (SGR), and feed conversion ratio (FCR) compared to fish on a non-supplemented diet ($P<0.05$). The optimal supplementation level was $6\times10^8$ $CFU.g^{-1}$, demonstrating the probiotic's beneficial effects on mucosal immunity, gut microbiota balance, stress tolerance, and overall growth performance in black swordtail.

Similarly, Moroni et al. (2021) explored the impact of dietary nisin-producing *Lactococcus lactis* on the growth and health of gilt-head bream (Sparus aurata). Although no significant differences were observed in the feed conversion ratio or specific growth rate between the experimental and control groups, the fish receiving the probiotic supplement achieved a significantly higher final body weight. Furthermore, histological analysis using a semi-quantitative scoring system revealed that the probiotic had no adverse effects on intestinal morphology and did not trigger inflammation, indicating the safety and potential growth-promoting properties of *L. lactis* in gilt-head bream.

These studies collectively illustrate the positive role that probiotics, such as *Lactobacillus acidophilus* and *Lactococcus lactis*, can play in improving stress tolerance, immune responses, and growth performance in fish, supporting their use as beneficial dietary supplements in aquaculture.

## MATERIALS AND METHODS

The objects of the study were frozen fish samples in the amount of 6 pcs (four crucian carps and two pikes), which were used to isolate Lactobacillus strains from fish intestines. Our study used a commercial nutrient medium MPC agar. According to the instructions provided by the manufacturers, the commercial nutrient medium was dissolved in distilled water in the required volume. The dissolved nutrient media were then placed in an autoclave for sterilization for 30min at 1atm. After autoclaving, the media were poured over a torch flame into sterile Petri dishes.

### Obtaining accumulative culture

The samples were grown by surface cultivation in Petri dishes on a solid nutrient medium – MPC agar. 50μL of the fish intestinal microflora sample were dissolved in 500μL of sterilized distilled water (1:10). Using a disposable pipette, 100μL of the suspension was added to the nutrient medium. Glass beads were used to achieve the uniform growth of isolated colonies. Eight to twelve glass beads (sterilized in advance) were dropped into a Petri dish with the inoculum. The cup was gently shaken for 1min. Afterward, the beads were removed and sent off for sterilization. The seeded Petri dishes were placed in a thermostat at 28°C for 16-18 hours until morphologically different colonies were formed.

### Isolation of pure culture

At the first stage, isolated colonies from the accumulative culture, different by culture characteristics, were sampled with a bacteriological loop and sown on solid nutrient medium in longitudinal strokes, 2-5 strokes per one Petri dish 12cm in diameter, which were then placed in a thermostat at 24.5°C for 16-18 hours.

**Microscopy**

The preparation was stained according to Gram's method. The fixed smear was stained with a basic stain (1min), iodide solution (1min), acetone-containing solution (until the effluent became clear), and safranin (1min). At each stage of staining, the smear was washed with distilled water. The fixed preparation was then dried with filter paper.

**Catalase activity**

The activity of bacterial extracellular catalase was determined titrometrically. The amount of enzyme catalyzing the decomposition of 1μM hydrogen peroxide ($H_2O_2$) (0.034 mg) in 1min was taken as a unit of catalase activity.

**Genetic identification of bacteria**

DNA extraction was conducted using the GeneJET PCR Purification Kit (Thermo Fisher Scientific, USA), following the manufacturers protocol for isolating DNA from bacterial samples. This procedure yielded DNA concentrations ranging from 5.4 to 34.8ng/μL, providing sufficient template for subsequent amplification steps. For the amplification of the 16S rRNA gene, which is commonly used in bacterial identification, a pair of universal primers targeting the 16S region were employed: the forward primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and the reverse primer 806R (5'-GGACTACCAGGGTATCTAAT-3'). These primers are widely recognized for their effectiveness in amplifying bacterial DNA across diverse species.

The amplification mixture was prepared in a total volume of 25μL, containing 25ng of the extracted DNA, 1U of DNA polymerase (Thermo Scientific, USA), 0.2mM of each deoxynucleotide triphosphate (dNTP), 1x PCR buffer, 2.5mM $MgCl_2$ to optimize enzyme activity, and 10pmol of each primer. The PCR amplification was performed using a SimpliAmp Thermal Cycler (Thermo Fisher Scientific, USA), a reliable platform for consistent thermal cycling. The PCR product was then verified by electrophoresis in a 1.5% agarose gel, conducted in a Max HU10 horizontal electrophoresis system with a Consort EV 243 current source. The electrophoresis was run using 1x TAE (Tris-acetate-EDTA) buffer, which served as the electrode buffer, facilitating the separation of DNA fragments based on their size.

Following successful amplification, the PCR products were subjected to Sanger sequencing to obtain high-resolution genetic data. The sequencing was carried out using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). A 25μL reaction mixture was prepared for each sample, consisting of 18μL $dH_2O$, 5μL 5x buffer, 0.5μL BigDye reagent, 0.5μL primer, and 1μL of the PCR product. To ensure sequencing accuracy and completeness, both forward and reverse primers (the same as used in the PCR step) were used for sequencing each fragment.

The sequencing reactions were analyzed using an ABI 3130XL Genetic Analyzer (Applied Biosystems, USA), a high-performance capillary electrophoresis system designed for DNA sequencing. The resulting chromatograms were inspected, edited, and analyzed using Sequencing Analysis Software 5.2 Patch 2 (Applied Biosystems, USA), which facilitated the accurate interpretation of the raw sequencing data. To further validate and contextualize the results, the obtained sequences were compared against known sequences in the National Center for Biotechnology Information (NCBI) database (www.ncbi.com), allowing for precise identification and analysis of the bacterial strains under investigation. This comprehensive approach ensured that the sequencing data were accurate, reproducible, and useful for downstream applications.

## RESULTS

**Research stages**

The fish were gutted and the intestines were cleaned and rinsed. The intestines were then cut across for better exit of microflora. Fecal residues were rinsed out of the intestines with distilled water. The separated material further used for bacterial isolation is shown in Fig. 1.

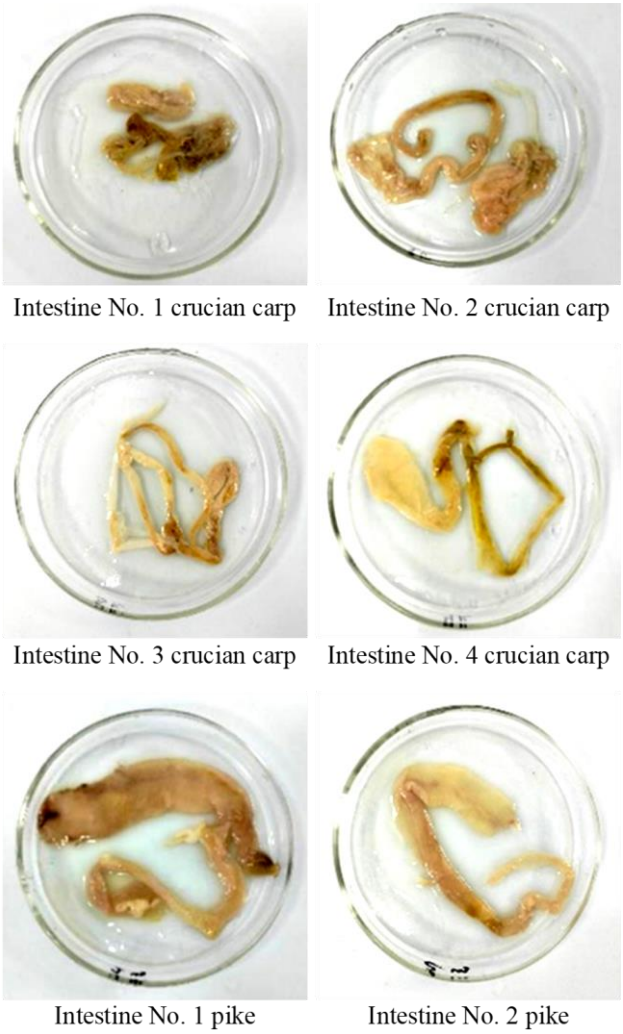


**Fig. 1:** Fish intestines.

After the preparation of fish intestines, bacterial culture accumulation was carried out according to conventional procedures. For this purpose, the intestines were transferred into vials with peptone water and cultivated for 14-16 hours in a thermostat at 28°C (Fig. 2).


**Fig. 2:** Cultivation of fish intestines in peptone water.

After the growing time had elapsed, the medium turned cloudy and a white turbid precipitate was formed. To isolate pure colonies of lactobacilli and grow them on solid nutrient media, the samples were diluted tenfold in sterile physiological saline.

Incubation was performed in a thermostat at 37°C for 24 hours. The results of cultivation are shown in Fig. 3. Similar methods were used to isolate Lactobacillus cultures from the fish gut by several authors and identified by PCR with further sequencing (Tamura and Nei 1993; Dhanasekaran et al. 2010; Kumaree et al. 2015; Alonso et al. 2019; Govindaraj et al. 2021). From the obtained Petri dishes, we selected 20 colonies differing in growth patterns, consistency, and size. The colonies were transferred to solid nutrient media, five colonies per dish (Fig. 4). Microscopy was performed following the Gram's staining method. The results are presented in Fig. 5.

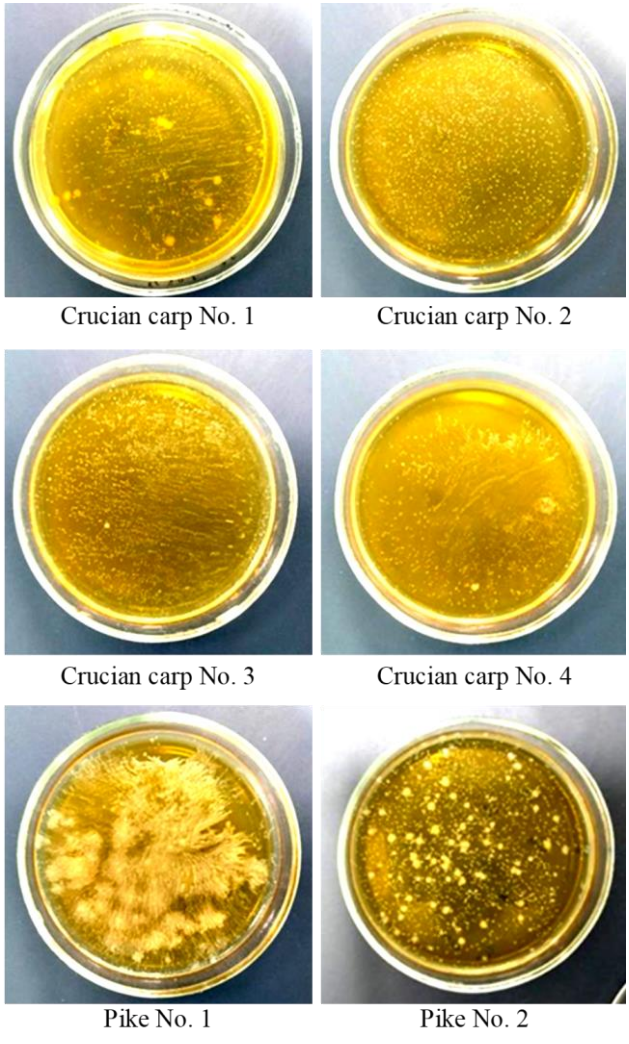


**Fig. 3:** Primary sowing of fish intestinal microflora

**Fig. 4:** Isolation of pure cultures from the initial sowing.

DNA extraction was performed using a ready-to-use kit *GeneJET PCR Purification Kit* (*Thermo Fisher Scientific*, *USA*). The results of PCR are presented in the form of an electrophoregram in Fig. 6.

**Milk souring**

For this experiment, we used skim milk. The milk was poured into sterile tubes in the amount of 10mL. The inoculum introduced amounted to 5% of the total volume. Fermentation was carried out at 37°C in a thermostat for 16-20 hours. The results of fermentation are reported in Fig. 8. The main organoleptic indices of the soured milk are provided in Table 1.

*L. mesenteroides* (subsp. *cremoris*). The cells are globular or lenticular, (0.5-0.7) (0.7-1.2) µm in size, connected in pairs or short chains. The optimum growth temperature is 22-25°C, the minimum temperature is about 5°C. Leuconostocia can grow in milk when growth factors (yeast or corn extract) are added. The limiting acidity does not exceed 40-50°T. After the pH of the medium decreases to 5.0-4.5, it forms diacetyl, which is why this species is used in multispecies starters for cheese and cultured butter production in combination with *L. lactis* (lactic acid streptococcus). The cells are spherical or oval measuring (0.5-1.2 µm) (0.5-1.5 µm), connected in pairs (diplococci) or short chains. The optimum temperature for development is 28-32°C. Active strains of this species curdle milk in 4-6h, forming a smooth, dense clot. The limiting acidity (after 5-7 days of development in milk) reaches 125°T. The strains of this species are included in the composition of starters for sour milk drinks, cottage cheese, sour cream, cultured butter, and cheese with a low second heating temperature.

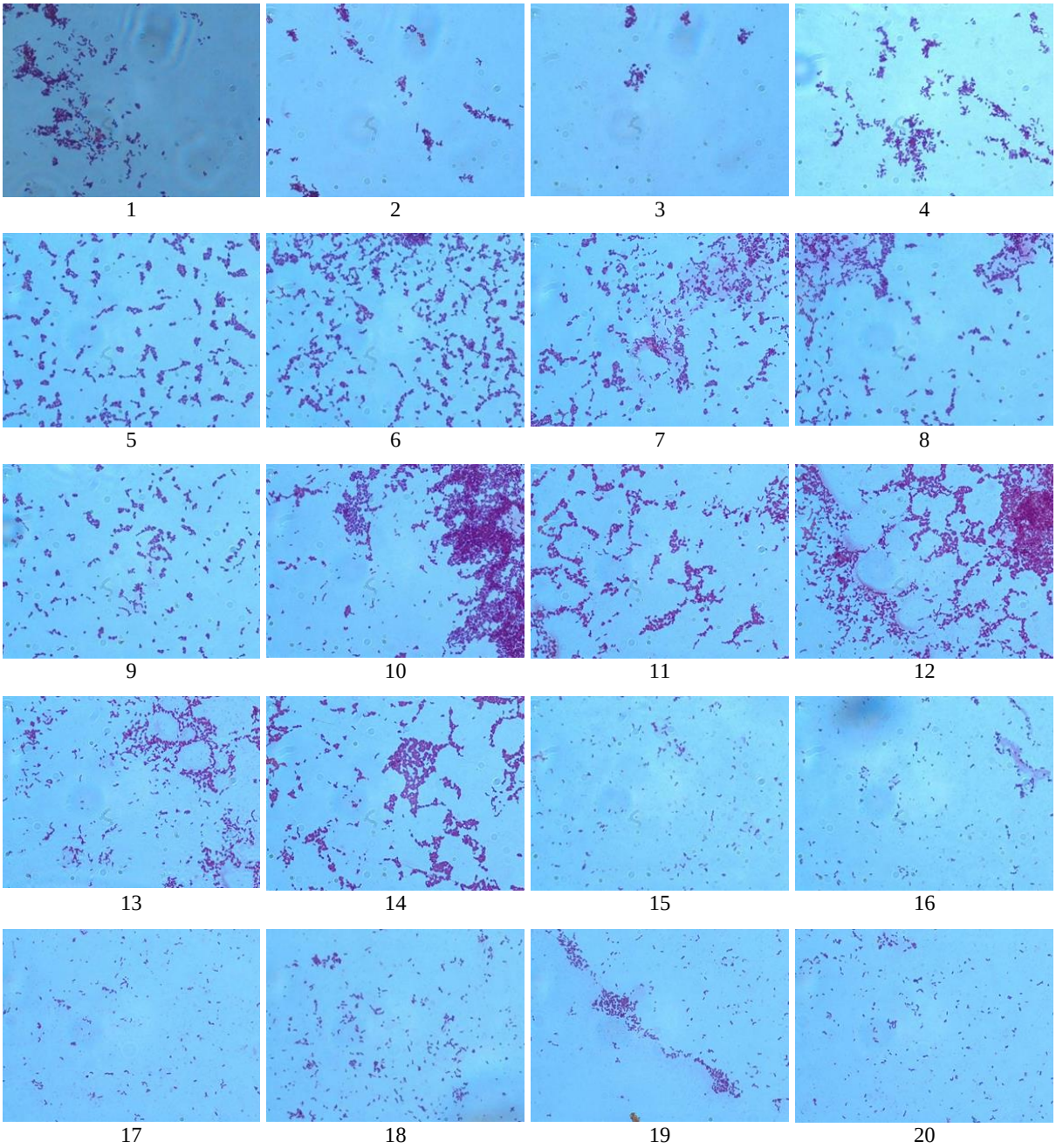


**Fig. 5:** Microscopy of pure cultures.

| | |
|---|---|
| Sample 1 | *Leuconostoc mesenteroides* - spherical cells arranged in short chains, Gram-positive. |
| Sample 2 | *Leuconostoc mesenteroides* - globular cells, showing uniform staining, Gram-positive. |
| Sample 3 | *Leuconostoc mesenteroides* - elongated spherical cells, visible pairs, Gram-positive. |
| Sample 4 | *Leuconostoc mesenteroides* - small cocci forming irregular clusters, Gram-positive. |
| Sample 5 | *Leuconostoc mesenteroides* - oval-shaped cells, forming chains, Gram-positive. |
| Sample 6 | *Lactococcus lactis* - spherical cells, arranged in diplococci or short chains, Gram-positive. |
| Sample 7 | *Lactococcus lactis* - oval cells, dense arrangement, Gram-positive. |
| Sample 8 | *Lactococcus lactis* - pairs of spherical cells, clearly stained, Gram-positive. |
| Sample 9 | *Lactococcus lactis* - spherical or oval cells in uniform chains, Gram-positive. |
| Sample 10 | *Lactococcus lactis* - cells showing strong uniform staining in pairs, Gram-positive. |
| Sample 11 | *Kurthia gibsonii* - rod-shaped cells, forming chains, catalase-positive. |
| Sample 12 | *Kurthia gibsonii* - irregular rods with visible catalase activity, Gram-variable. |
| Sample 13 | *Kurthia gibsonii* - short rods with scattered arrangement, Gram-variable. |
| Sample 14 | *Kurthia gibsonii* - elongated rods showing catalase activity, Gram-variable. |
| Sample 15 | *Kurthia zopfii* - thin rods forming single cells or short chains, Gram-variable. |
| Sample 16 | *Kurthia zopfii* - dispersed rod-shaped cells, irregularly stained, Gram-variable. |
| Sample 17 | *Kurthia zopfii* - rod-shaped cells arranged in single lines, weak catalase activity, Gram-variable. |
| Sample 18 | *Lactococcus garvieae* - cocci forming clusters, associated with pathogenic traits, Gram-positive. |
| Sample 19 | *Lactococcus garvieae* - densely packed spherical cells, showing uniform staining, Gram-positive. |
| Sample 20 | *Lactococcus garvieae* - cocci in irregular arrangements, indicative of pathogenic properties, Gram-positive. |

**Table 1:** Organoleptic indices of soured milk

| No. | Strain | Smell | Color | Gas formation | Texture | pH |
|---|---|---|---|---|---|---|
| 1 | *Leuconostoc mesenteroides* | curd-like | no changes | absent | homogenous, creamy | 5.5 |
| 2 | *Leuconostoc mesenteroides* | curd-like | no changes | present | separated, flaky | 6.0 |
| 3 | *Leuconostoc mesenteroides* | curd-like | no changes | absent | separated, flaky | 5.0 |
| 4 | *Leuconostoc mesenteroides* | curd-like | no changes | absent | homogenous, creamy | 5.0 |
| 5 | *Lactococcus lactis* | curd-like | no changes | absent | homogenous, creamy | 5.5 |
| 6 | *Lactococcus lactis* | curd-like, sour | no changes | present | separated, flaky | 5.5 |
| 7 | *Lactococcus lactis* | curd-like | no changes | absent | homogenous, creamy | 5.5 |
| 8 | *Lactococcus lactis* | curd-like | no changes | absent | homogenous, creamy | 5.5 |
| 9 | *Lactococcus lactis* | curd-like | no changes | absent | homogenous, creamy | 5.5 |
| 10 | *Lactococcus lactis* | curd-like | no changes | absent | homogenous, creamy | 5.5 |
| 11 | *Lactococcus lactis* | curd-like | no changes | absent | homogenous, creamy | 5.5 |
| 12 | *Lactococcus lactis* | curd-like | no changes | absent | homogenous, creamy | 5.5 |
| 13 | *Lactococcus lactis* | curd-like | no changes | absent | homogenous, creamy | 6.0 |
| 14 | *Lactococcus lactis* | curd-like | no changes | absent | separated, flaky | 5.0 |
| 15 | *Lactococcus lactis* | curd-like | no changes | absent | homogenous, creamy | 5.5 |
| 16 | *Lactococcus lactis* | curd-like, sour | no changes | present | separated, flaky | 5.5 |
| 17 | *Lactococcus lactis* | curd-like | no changes | absent | homogenous, creamy | 5.5 |
| 18 | *Lactococcus lactis* | curd-like, sour | no changes | present | separated, flaky | 5.0 |
| 19 | *Lactococcus lactis* | curd-like | no changes | absent | homogenous, creamy | 5.5 |
| 20 | *Lactococcus lactis* | curd-like | no changes | absent | homogenous, creamy | 5.5 |

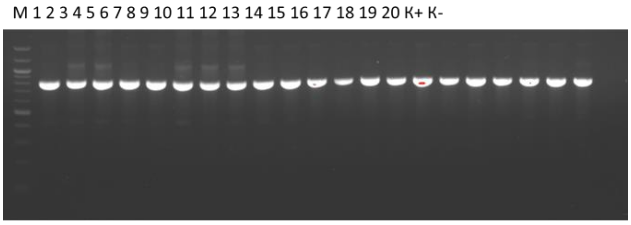


**Fig. 6:** Electrophoregram results: M – marker, 1-20 – bacterial samples studied, K+ – positive reaction control, K- – negative reaction control.

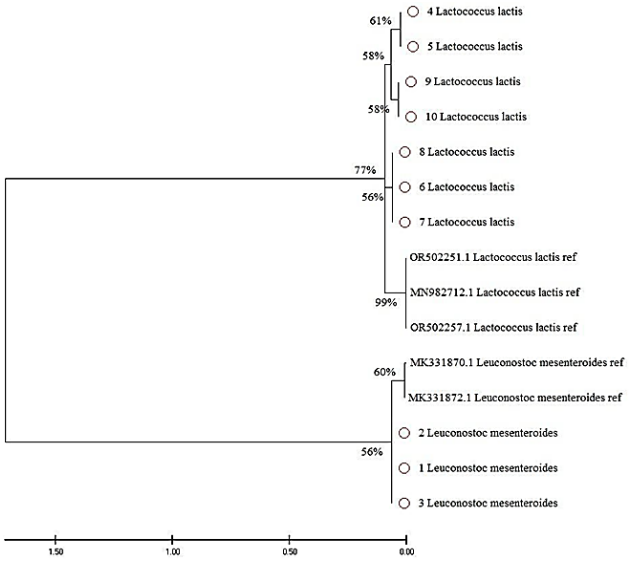


**Fig. 7:** Phylogenetic analysis of the isolated strains of lactic acid bacteria.

**Fig. 8:** Results of milk fermentation and pH measurements.

In the determination of catalase activity, we used a cell suspension of lactobacilli cells (*L. mesenteroides*, *L. lactis*). The cell suspension and 3% hydrogen peroxide were dripped onto the slide glass at a ratio of 1:1. A pathogenic strain causing intestinal disruption fish was used as a positive control (*K. gibsonii).*

As a result of catalase activity determination, we established that the strains of *L. mesenteroides* and *L. lactis* do not have catalase activity, whereas using the *K. gibsonii* strain led to the formation of bubbles indicating catalase activity.

## DISCUSSION

Our study focuses on isolating lactobacillus strains from the intestines of two fish species with probiotic activity to implement as a functional fish feed supplement. Following current research trends in aquaculture and probiotics, our research isolates lactobacillus strain from crucian carp and pike. Our method of cultivation, medium, conditions, and dilution rate agree with the work of Mirzabekyan et al. (2023), who studied the extraction of lactobacillus and *E. coli* from Salmo sp. fishes. These sources were picked as they provide for better adaptation in the specific gut environment.

The fish raised on the symbiotics diet exhibited an average final weight of 77.28±0.61g, a weight gain of 46.79±0.64g, and an SGR of 2.33±0.03%. In contrast, the fish that did not receive symbiotics had a final weight of 73.31±0.73g, a weight gain of 42.54±0.77g, and an SGR of 2.17±0.04.

Our research revealed two main probiotic species, *Lactococcus lactis* and *Leuconostoc mesenteroides*, which exhibited no catalase activity due to their anaerobic or microaerophilic nature. This finding is also supported by the work of Sunny et al. (2022), who also reported the negative catalase activity in *Lactococcus lactis.* Bandyopadhyay et al. (2022) reinforced the benefits of *Lactococcus lactis* and its potential to be combined in symbiotics for human and animal feed. In addition, Abdul-Malik et al. (2023) highlight the combination of catalase-positive and negative probiotic strains in fish feed and more

research is needed on the possible implications and benefits of this inclusion.

Our study agrees with the recent focus on probiotics' role in improving aquaculture health and growth. Our study agrees with the works of Chattaraj et al. (2022), who explored the efficacy of bacterial probiotics such as *Lactobacillus casei* and *Lactococcus lactis* in controlling viral pathogens prevalent in aquaculture. Xia et al. (2024) reported that when *Leuconostoc mesenteroides (DH strain)* was used as a dietary supplement in feeding *Misgurnus anguillicaudatus*, there was a significant antibacterial effect on eight pathogens and a noticeable overall development in the juvenile fish. The works of Feng et al. (2022) displayed the effects of dietary *Lactococcus spp*. on growth performance, glucose absorption, and metabolism of common carp, Cyprinus carpio L. Their research reported that the fishes fed with *Lactococcus spp*. had enhanced glucose absorption and glycogen content, and there was an improvement in growth performance and a regulation of glucose absorption and metabolism.

However, this research (Feng et al. 2022) highlighted the benefits of *Lactococcus spp*. as a beneficial probiotic due to its effect on gut health. It employs a consortia of different strains of *Lactococcus spp.*, which does not agree with our research goal, but it adds value by focusing on just *Lactococcus lactis.* The benefits of using lactic bacteria probiotics, as highlighted in our study, are also supported by the works of Stover et al. (2023), who reported on the effect of *Lactococcus lactis* and *Leuconostoc mesenteroides* on the increase in lifespan of aquatic animals as shown in their experiments using *Caenorhabditis elegans*, nutrient uptake and the reduction of stress levels.

Probiotics' role in enhancing fish's immune response has been widely documented. While our study did not measure immune markers directly, our study supports using *lactobacteria* species for this purpose.

*One of* the dangers of using *L. lactis* as a probiotic is the existence of its pathogenic potential. The works of Wu et al. (2023) show that there have been records of the isolation of pathogenic *L. lactis* from various clinical cases, including milk, urine, and blood samples. This indicates an underlying risk that can be addressed through further research. The results of our study align with the findings of recent research, indicating the potential of *L. mesenteroides* and *L. lactis* as effective probiotics for aquaculture. These strains not only improve gut health but also hold promise in enhancing immune responses and growth performance. However, future studies should investigate the long-term effects of these probiotics under varying environmental conditions and in different fish species to optimize their application in aquaculture.

## Conclusion

In the course of the research, we achieved the following results:

1. The amplicons of 20 bacterial samples with a fragment length of 790bp were isolated.
2. Two nucleotide sequences were not identified. Fish pathogens causing gut disorders belonging to three species – *K. gibsonii*, *K. zopfii*, and *L. garvieae* – were identified from eight sequences. The remaining 10 strains were identified as lactobacilli belonging to two species: *L. mesenteroides* and *L. lactis*.
3. A phylogenetic tree was constructed based on nucleotide sequences.
4. Results on the catalase activity of the isolated lactobacilli strains indicate that the *L. mesenteroides* and *L. lactis* strains lack catalase activity, whereas *K. gibsonii* shows catalase activity.

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**Conflicts of Interest:** The authors declare no conflict of interest.

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**Authors Contribution:** A.Ye. Paritova: conceptualization, research design, and data collection. G.K. Murzakayeva: data analysis, statistical work. Y.A. Balji: methodological development. Zh.B. Kuanchaleyev, G.A. Abulgazimova: literature review, manuscript writing, interpretation of results. D. Askambayeva: data acquisition, technical support. G.B. Kuzembekova, K.K. Ashimova: funding acquisition, project administration, supervision. A.A. Zhanabayev: data interpretation, critical revisions, overall study supervision. Ye. Mayer: provided technical support, participated in data analysis, and assisted with manuscript preparation. B.Zh. Aytkozhina: compiling data, the methodological aspects. A.Ye. Paritova: reviewing the manuscript, editing and providing key resources and materials. All authors reviewed the article and confirmed after correction.

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