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Effect of Local Lactic Acid Bacterial Isolates as Probiol for Microbiota Population Balance of Fish Intestine

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Abstract

The aim of this study is to understand the effect of some types of probiotics on the intestinal microbial flora of fish, Lactic acid bacteria strains isolated from various traditional and industry, fermented yogurt and local cheese sources based on the biochemical tests. The Antibacterial susceptibility and theantimicrobial activity of a Lactobacillus isolates against pathogenic bacteria such as *E. coli, Staph.spp* and *Klebsiellaspp* was determined. Among the Lactobacilli isolates, (76.92%) were resistant to ampicillin 10U, (76.92%) were sensitive to tetracycline 30 mcg and (7.69%) had shown intermediate resistance to nalidixic acid 30 mcg. All the isolated Lactobacillus strains exhibited average inhibition (3-9 mm) on the growth of test pathogens, but the LB3 isolate were the most effective ones in inhibiting the test pathogens (5-9 mm). The treatment fish group was fed a diet supplemented with live bacteria, while the second group was fed a basic feed. After six weeks the results indicated that, the fish treatment groups which received probiotics supplemented diets revealed significant improvements in gut bacteria screening before and after the addition of the probiotics and a decrease in the re-isolation of some pathogenic bacteria at the end of the experimental period.

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Introduction

Historically, the knowledge and discovery of probiotics are associated with commonly consumed fermented foods, and it was approved that probiotic strains have been transferred to us since the first food raw materials were subjected to fermentation process (Ołdak et al., 2020).

Cultured dairy products were probably the first functional food supplemented with probiotics. They are the best carriers of probiotic strains in the food industry (Khojah et al., 2022). *Lactic acid* bacteria (LAB) species are gram-positive, anaerobic, catalase-negative, and motile bacteria (Sharma et al., 2021). They are well-known as probiotics, and they play a key role in biotechnological products such as cheese, yogurt, and bread (Yerlikaya, 2019). LAB bacteria also play a significant role in food preservation by producing antimicrobial compounds such as lactic acid, diacetyl, hydrogen peroxide, and bacteriocins, which have been found in dairy starter cultures and could be used as food preservatives (Samet and Icen, 2022).

The use of biological products namely probiotic either alone or in combination with prebiotics is recently the goal of the disease biocontrol strategy in aquaculture as they improve the fish health and modify the fish-associated microbial community (Zoumpopoulou et al., 2018)

Furthermore, probiotic bacteria provide essential health benefits, such as enhancing gut microbiota balance and fighting pathogenic bacteria, stimulating the immune system and numerous studies showed that LAB isolated from different kinds of food can effectively inhibit the growth of Staphylococcus aureus (Ołdak et al., 2020) also lowering blood cholesterol levels, producing vitamins (particularly vitamin B group), and antibacterial action. (Hamad ,2022; Latif, 2023). The anti-bacterial activity of LAB is mainly connected with the pH lowering, organic acid production and also with the possibility of bacteriocin synthesis and other antimicrobial agents such as hydrogen peroxide, reuterin or reutericyclin, and peptidoglycan hydrolases (Ołdak et al., 2020).

Lactic acid bacteria (LAB also employed as therapeutic bacteria due to limitations in traditional cancer therapies and other disorders (Sedighi et al., 2019 :Singh et al., 2022). The most interesting application of modern *Lactococcus lactis* (*L. lactis*) is as an antigen factory, allowing the bacteria to behave as live vaccines. In the last two decades, *L. lactis* has emerged as a good alternative expression system to *E. coli* (Frelet-Barrand, 2022). The using LAB as vaccine carriers is tempting because they can generate mucosal and systemic immune responses (Aghamohammad,2022). Regarding vaccine development, *L. lactis*' ability to surface display antigens makes it the preferable host with higher immunogenicity than intracellularly produced counterparts (Song et al., 2017).

`*Lactococcus lactis spp* bacteria is commonly found in naturally fermented dairy products (Bandyopadhyay et al., 2022). This subspecies is of high economic value due to its wide application in the dairy industry. However, the genetic background and evolutionary history of *L. lactis spp*. are still poorly understood (Liu et al., 2022).

Probiotics have various action mechanisms, such as inhibiting pathogens by producing organic acids and antibacterial substances such as hydrogen peroxide, bacteriocins, and defensins (Dai et al.,2022). Probiotics compete with pathogenic bacteria on intestinal epithelial binding sites and essential nutrients (Dawood et al., 2020). they enhance the immune response by releasing regulatory T cells, effector T and B cells, and antigen-presenting cells (Frelet-Barrand et al., 2022).

Probiotics have been regarded as one of the most potential eco-friendly feed additives that enhance fish production (Chowdhury and Roy, 2020). By enhancing the performance of various digestive enzymes, probiotics also enhance feed utilization by promoting the accessibility of nutrients (Daoud et al., 2020). This study aimed to determine the influence of lactobacillus probiotic performance on the growth and intestinal microbial flora pupulation of common carp (*Cyprinus carpio L*.).

Materials and Methods

Bacterial isolation and identification

This studied was carried out in the Industrial Microbiology Dept-Directorate of Agricultural Research from May 2021 to March 2022. Samples were collected from different traditional and industry fermented yogurt and cheese sources purchased from Al -Karada local market in Baghdad. Traditional samples as local yogurt (homemade) and local cheese (homemade) whereas industry samples as Activia yogurt, canon yogurt, and cheese. 10% diluted samples were plated on MRS agar plates containing 3% $CaCO_3$ (w/vol). The plates were incubated at 37 ºC for 24–48h. The colonies with distinct morphology were picked and purified by further sub-culturing morphological and physiological assessment to identify potential LAB strains (Sharma et al., 2021). The morphological characterization for the strains was performed through a Gram staining kit (Hi-Media, India). The cultures were examined under a bright field microscope (Olympus, Japan) and observed according to Bergey's manual of determinative bacteriology The strains were then investigated for the presence of catalase enzyme using 3% H_2O_2 (Reiner, 2010 ;Shalsh et al.,2023).

Antibiotic susceptibility test

Antibacterial susceptibility test is widely performed using the disk diffusion method. The activated cultures were swabbed onto the agar plates. We have used antibiotics in the form of dodeca discs. The zones of inhibition were noted after incubation at 37 C for 24 h. Resistance and sensitivity pattern data were interpreted as per the Clinical Laboratory Standard Institute (CLSI, 2017).

Survival rates (%) = [3 h viable counts (CFU/mL)/0 h viable counts (CFU/mL)] ×100%.

Antagonistic Activity of Lactic Acid Bacteria against pathogenic microorganisms.

The antimicrobial activity of all pooled Lactobacillus isolates against test pathogens was determined by the agar spread method according to Ashraf et al., 2009. E. coli, Staph.spp and Klebsiella spp were obtained from the Laboratory of Microbiology- Biotechnology of Agriculture Researches Centre and used as pathogen testers. 100 µL of cell-free supernatant from each Lactobacillus isolate was filled into 7mm well nutrient agar containing experimental pathogens. The diameter of the clear area of fixation was observed after 24 hours of incubation. The experiment was performed in triplicate.

Experimental scheme

A laboratory experiment was conducted for 40 days (6 weeks) in the laboratories of the Agricultural Research Center / department of biotechnology to find out the effect of the probiotic lactic acid bacteria on the microbial balance in the intestines of common carp. Young fish were obtained. from a private farm in Baghdad. The weight of the fish ranged from 120 to 160 g. The process of sterilization with salt solutions at the concentration of 3% for a period of three to five minutes before were transferred into glass aquaria with dimensions of 30 cm x 30 cm x 60 cm those were filled with 40L of clean tap water and acclimatized for 15 days before the implementation of the experiment. the fish were divide randomly to treatment group with three duplicates. The average temperature was 27.5 ◦C (± 0.2). The aquaria pH range was set between 7.0 and 7.8. The aquaria were cleaned every daily to reduce the potential risk of bacterial growth and ammonia toxicity.

Experimental scheme

Experimental diets used with three repetitions for each treatment Table (1). The use of the mixture of useful bacterial colonies as probiotics and enzymes as in the Table (2). All dry components were measured and thoroughly combined with molasses using a food processor, along with a mixture of probiotic bacteria and 750 mg/Kg of diet supplements. To create the dough, cold distilled water was added. The dough was then formed into wet tablets and dried in the sun for two days. It was then placed in nylon bags, numbered, and used to feed fish in the appropriate ratios. The fingerlings were fed daily at 5 % of the total body weight with a control diet at 9.00 AM and 5.00 PM.

Table 1. Effective Parameters and Their Levels Were Used in Taguchi's Experimental Design.

Ingredients (%)	Control group Diet	Treatment group Diet
Formulation		
*Protein complex (Provimi)	10	10
Soybean meal	25	25
Wheat bran	10	10
Molasses	5	5
**Vitamin-Mineral premix	2	2
Probiotic (g /kg)	0	0.75
Proximate composition		
Moisture (%)	12.09	12.11
Crude lipid (%)	7.45	7.12
Ash (%)	8.46	8.37
Crude fibre (%)	5.20	4.88
carbohydrate		

Table 2 Bacterial colonies as probiotic.

Probiotic	Colony size
Lactic acid bacteria LB1	6.73×10^{10} CFU
Lactic acid bacteria LB2	4.98×10^{10} CFU
Lactic acid bacteria LB3	8.43×10^{10} CFU
Lactic acid bacteria LB4	5.64×10^{10} CFU

Bacterial analysis withdrawal of samples from the intestines of fish, each treatment by 25 gm, and adding it to 225ml Ringer Broth medium, A series of dilutions was carried out to isolate and count the bacteria, and the dishes were incubated at a temperature of 37 °C for 48 hours. *Klebsiella* characteristics on MacConkey Agar and mantiol salt agar for staphylococcus and *E. coli*.

Data statistics

Data of all variables were run into one-way analysis of variance (ANOVA) and presented as mean ± standard deviation (SD). The significance of the difference between means was found out by Duncan Multiple Range Test at a 5% significant level ($p < 0.05$).

Results and Discussion

Bacterial isolation and identification

Four LAB strains were isolated from traditional and industrial fermented yogurt in Baghdad. Based on the biochemical tests, all isolates showed Gram-positive, catalase-

negative characteristics (Fig.1). All isolates were studied for their morphological, physiological characteristics. Based on the temperature (37 ºC) and pH (5).
lactic acid bacteria strains were isolated: -
1-Lactobacillus LB1 showed Gram-positive, catalase-negative, cream color, round, edges colony morphology and single or paired, short chain microscopy.
2- Lactococcus LC2 showed Gram-positive, catalase-negative and cream color, round, edges clear colony morphology and spherical or ovoid cells microscopy.
3-Lactobacillus LB3 showed Gram-positive, catalase-negative, yellowish color, round, edges colony morphology and single or paired, short chain microscopy.
4- Lactobacillus LB4 showed Gram-positive, catalase-negative, slightly yellowish color, round, edges colony morphology and single or paired, short chain microscopy.

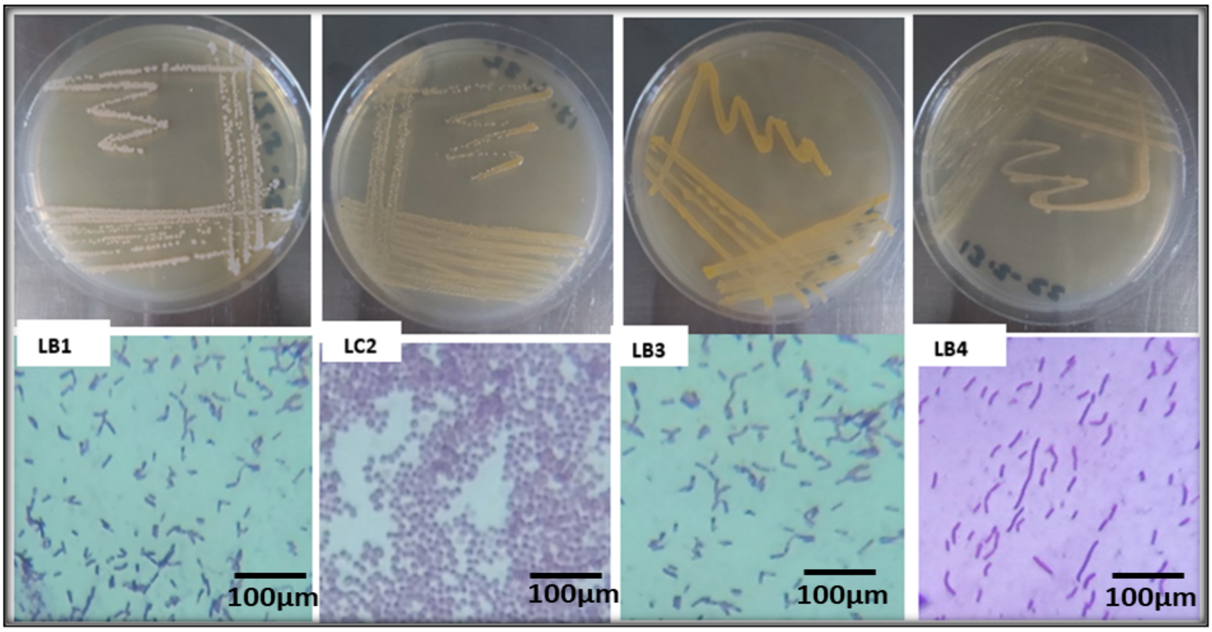

Fig. 1. Examination colonies and microscopy of strains on MRS screening medium.

Sensitivity to antibiotics

Sensitivity to antibiotics test was carried out on c Lactobacillus spp (Fig2). against antibiotics nalidixic acid, tetracycline and penicillin. Among the Lactobacilli isolates, (76.92%) were resistant to ampicillin 10U, (76.92%) were sensitive to tetracycline30 mcg and (7.69%) had shown intermediate resistance to nalidixic ascid 30 mcg.

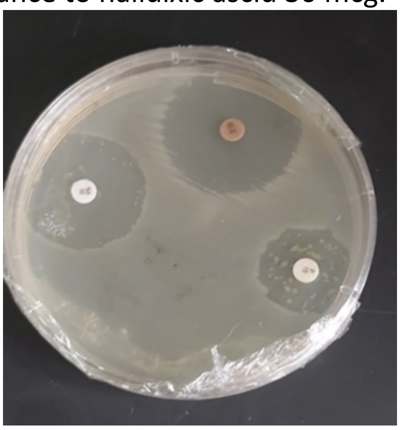

Fig 2: Sensitivity to antibiotics

Antagonistic Activity of Lactic Acid Bacteria against pathogenic microorganisms

The four lactic acid bacteria's antagonistic activity was primarily assessed using an agar well diffusion assay with cell-free supernatant (CFS) against E. coli, Staph.spp and Klebsiella. The results are shown in Fig.1 as a summary. All four examined Lactobacillus strains had antagonistic effects on the indicator bacteria, however the intensity of the effects differed. All the isolated Lactobacillus strains exhibited average inhibition (3-9 mm) on the growth of test pathogens, but the LB3 isolate were the most effective in inhibiting the test pathogens (5-9 mm) (Fig.3 Table .3).

Table 3: Inhibition zone produced against the test strains in agar diffusion assay.

Isolate	*E. coli*	*Staph.spp*	*Klebsiella*
L.B1	3.45±1.16	3.23±1.23	2.32±1.56
L.C2	5.34±0.76	4.34±0.16	7.23±2.16
L.B3	8.57±0.23	5.18±2.19	9.23±1.56
L.B4	3.23±0.11	4.23±1.54	4.64±1.22

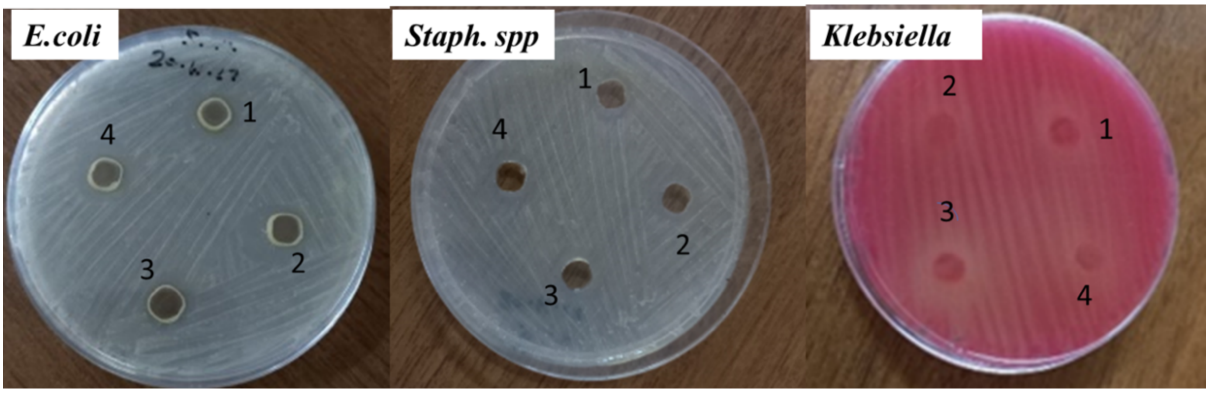

Fig. 3. Antagonistic of LB1. LC2, LB3, and LB4

A large microbial community that is present in fish's digestive tract has a significant impact on the immune system, nutrient assimilation, and a variety of other host behaviours. The dietary supplementation of L. plantarum @ 109 cfu/g diet is effective in improving water quality, growth and proximate composition of Cyprinus carpio.(Amit et al.,2021).

The gut microbiota can interact with each other in a positively or negatively way. The interactions that occur between the microbiota's constituents can have significant effects on fish health. (Mirzabekyan, *et. al.*, 2023). Also, these results could be attributed to the bactericidal or the bacteriostatic substances produced by lactobacillus that inhibit the growth of other bacteria as reported by Dai *et. al.*, (2022).

Experimental diets

At the end of the experimental period the results revealed that group that received probiotic-supplemented diets showed higher growth rate than those kept on a basal diet, suggesting that the addition of probiotics enhanced the growth performance, agrees with (Pereira et al., 2022). The results showed that the treatment with the presence of the probiotic caused a decrease in the bacterial population compared to the control treatment, and it was recorded without probiotic 2×1013, 2×106 and 2×107 for bacteria E. coli, Staphylococcus spp. and Klebsiella. spp respectively. The values of bacteria species decreased by 3×106, 2×103, and 6×103 respectively compared to the control treated(Table 4).

Table 4: The number of bacteria under study in the intestines of young carps

Fish group	***E. coli***	***Staph. spp***	***Klebsiella. Spp***	**Intimal weight**	**Weight gain**	**Specific growth rate (g/day)**
Control (without probiotic)	2×10^{13}	2×10^{6}	2×10^{7}	25 ± 0.75^{a}	4.8 ± 0.25^{b}	0.0046
Treatment (with probiotic)	3×10^{6}	2×10^{3}	6×10^{3}	23.6 ± 1.43^{b}	9.7 ± 1.48^{a}	0.0074

Values with different superscript letters in a row differ significantly ($p < 0.05$) among the treatments.

Conclusion

Fish cultures are being developed to address global protein shortages, but fish suffer from severe effects and microbial pathogens, often treated with antibiotics. The recent disease biocontrol strategy in aquaculture aims to improve fish health and modify the microbial community by the parallel use of biological products named probiotic either alone or in combination with prebiotics. The study focuses on the selection of probiotic strains based on their hydrophobicity and antibacterial activity against pathogenic bacteria. The results show that probiotic-supplemented diets resulted in higher growth rates than basal diets, indicating that the adding of probiotics enhances growth performance. Further research is needed to understand the physicochemical cell surface and adhesion properties of the specific probiotic strains.

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