Characterization of probiotic potential of lactic acid bacteria isolated from aquaculture objects of Uzbekistan

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Abstract. Lactic acid bacteria have a high potency to combat infections in the body. The range of lactic acid bacteria isolated from aquaculture in Uzbekistan has been described in this article. We found that *Lactobacillus delbrueckii, L. plantarum, L. sakei, L. brevis, Lactococcus lactis, Pediococcus acidilactici, P. pentosaceus, Enterococcus faecium, E. hirae, E. mundii, E.faecalis, Leuconostoc citreum*, and *Weisella sibaria* strains are represented in hydrobionts. Among them isolates *Lactobacillus delbrueckii R1, Lactobacillus plantarum Kr5, Pediococcus acidilactici B, Enterococcus faecium R2, Lactobacillus plantarum R3, Pediococcus pentosaceus R1* showed high antagonistic activity against aquaculture pathogens. Four strains: *Lactobacillus plantarum Kr5, Pediococcus acidilactici B, Enterococcus faecium R2,* and *Pediococcus pentosaceus R1*, meet all the criteria for probiotics and can be recommended as part of probiotic feed additives. **Keywords**: lactic acid bacteria, antagonistic properties, probiotic strains, aquaculture, pathogen.

1 Introduction

Autoflora, or normal microflora, is an open biocenosis of microorganisms in healthy people and animals [1]. The normal microflora of animals performs many vital functions. It has a particular qualitative and quantitative composition for each species and is a powerful barrier to pathogenic microorganisms. Any microbiological changes, for example, when rod-shaped bacteria begin to dominate in the microbiocenosis, or then some species replace others, are one of the first signals of an unfavorable state of the macroorganism. Studies conducted on aquacultures are limited. The importance of a healthy gut microbiome for fish lies in inhibiting the pathogenic microflora of the digestive tract, which is one of the main ways of spreading infections [2].

Lactic acid bacteria of the genus *Lactobacillus* in the intestines of fish are part of the normal microflora while not being the dominant species [3].

The probiotic culture's antagonistic activity and ability to colonize the mucosa are essential in the complex colonization resistance mechanisms [4, 5].

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A healthy microbiome is critical for fish farming since it is integral to keeping fish raised for healthy and productive food. To increase the productivity of industrial fish farming, special attention should be given to preventing diseases and treating fish. When fish are grown in recirculating water supply systems, conditionally pathogenic microflora in water can become a pathogenic, resistant form due to their excessive growth and cause diseases of various etiologies. The traditional approach for preventing the overgrowth of pathogenic and opportunistic microorganisms includes antibiotics application. However, due to antibiotic use, a particular problem is the spread of resistant forms of pathogenic microorganisms resistant to modern antibiotics and accumulating their active substances in the body. In addition, fish have a particular biological ability to concentrate many dangerous toxic substances from water. Their content in the fish body is much higher than in water [6]. A person can experience food poisoning and toxicosis when these products are used as food.

Probiotics have been actively investigated as an alternative to antibiotics [7] and contain living microorganisms of the normal intestinal microflora or microorganisms that contribute to its formation. Probiotics help absorb nutrients, promote post-stress adaptation, increase the resistance of the macroorganism to pathogenic microorganisms, and improve the digestive system's functioning due to the additional production of enzymes in the digestive system tract [8, 9].

Microorganisms intended as probiotics in aquaculture should exhibit antimicrobial activity and be considered safe for aquatic hosts, the environment, and humans [10].

The microflora of hydrobionts of aquaculture in Uzbekistan has yet to be investigated. This research aims to study the range of Lactic acid bacteria in the aquaculture of Uzbekistan and characterize their probiotic potential.

2 Materials and Methods

2.1 Isolation of lactic acid bacteria

Lactic acid bacteria were isolated from various organs of healthy fish (scales, abdominal cavities, gills, and intestines). Serial dilutions of samples were seeded on MRS agar (HiMedia) containing plates and incubated in aerobic and anaerobic conditions at 37oC for 24 - 48 hours.

Identification was carried out by MALDI-TOF mass spectrometry using the Biometric Analyzer VITEK MS.

All isolated strains are stored in the laboratory "Microbiology and Biotechnology of Lactic Acid Bacteria" of the Institute of Microbiology, Academy of Sciences of the Republic of Uzbekistan, in a frozen stock at -80 °C.

2.2 Antimicrobial activity assay

The antimicrobial activity of isolated lactobacilli was determined by the agar spot method [11]. Test - cultures and pathogenic strains previously isolated from diseased fish and stored at frozen stock at -80oC. Before the experiment, the test cultures was refreshed by three subcultures in nutrient broth (HiMedia, India) and incubation at 25-28°C for 24 hours.

2.3 Determination of resistance to NaCl

The ability of the studied strains to grow at elevated concentrations of sodium chloride was determined by the method described in MUK 4.2.2602-10 [12].

2.4 Probiotic properties of selected strains.

Bile resistance of selected strains was determined according to MUK 4.2.2602-10 [12].

The study of resistance to gastric juice and the juice of the small intestine was studied according to the method of B.M. Corcoran [13].

The sensitivity to antibiotics was determined to the following antibiotics adsorbed on paper discs (Hi Media): cefotaxime (5 μg), gentamicin (10 μg), rifampicin (5 μg), kanamycin (30 μg), erythromycin (15 μg), levofloxacin (5 μg), amikacin (30 μg), vancomycin (30 μg), amoxiclav (30 μg), ofloxacin (5 μg) [14].

2.5 Study of the physiological and biochemical properties of strains.

The test for catalase activity for the production of lecithinase, hemolysin, gelatinase, and amylase was carried out according to MUK 4.2.2602-10 [12].

Casein proteolysis and lipolytic activity of the isolated strains was carried out according to Yegorov N.S. [15].

3 Results and Discussion

3.1 Isolation of potential probiotic bacteria and their identification

We isolated and identified 37 isolates, including 4 isolates of *L. delbrueckii*, 4 isolates of *E. Faecium*, 3 isolates of *E. hirae*, 1 isolates of *E. mundii*, 12 isolates of *L. plantarum*, 2 isolates of *P acidilactici*, 2 isolates *E. faecalis*, 3 isolates *Lactococcus lactis*, 2 isolates of *Lactobacillus sakei*, 1 isolates *Leuconostoc citreum*, 1 isolates *Lactobacillus brevis*, 1 isolates *Weissella cibaria*, 1 isolates *Pediococcus pentosaceus*. These isolates were further used to study antagonistic activity against aquaculture pathogens.

3.2 Study of antagonistic activity to aquaculture pathogens

To select potential probiotic candidates, we screened the antagonistic activity of lactic acid bacteria isolated from aquacultures to pathogenic and opportunistic microorganisms isolated from the same sources.

As a result, it was found that strains of *Lactobacillus delbrueckii* isolated from healthy fish, *Lactiplantibacillus plantarum* isolated from shrimp, and the *Pediococcus* species have a pronounced high activity. At the same time, the diameter of the inhibition zone of pathogens by these strains in some cases exceeds 40 and even 50 mm (Table 1). *Enterococcus hirae R1, and Enterococcus mundii R*. showed negligible antimicrobial potential.

All bacterial pathogens were observed to be sensitive to at least one lactic acid bacteria isolate. This observation indicates that lactic acid bacteria have a high potential for maintaining healthy aquaculture microbiocenosis.

Based on the most potent antimicrobial activity, 6 strains of lactobacilli were selected for further research: *Lactobacillus delbrueckii R1, Lactiplantibacillus plantarum Kr5, Pediococcus acidilactici B, Enterococcus faecium R2, Lactobacillus plantarum R3, Pediococcus pentosaceus R1.*

Table 1. Antagonistic activity of LAB isolates to pathogens of aquaculture.

№	Microorganisms	*L.delbrueckii R1*	*L.delbrueckii R2*	*L.delbrueckii R3*	*L.delbrueckii R4*	*E.faecium R3*	*E. hirae R1*	*E.faecium R2*	*E.cassel R2*	*E.hirae R2*	*E.mundtii R*	*E.faecium R1*	*E.faecium K2*	*L.plantarum Kr1*	*L.plantarum Kr2*	*L.plantarum Kr3*	*L.plantarum Kr4*	*L.plantarum Kr5*	*Ped.acidilactici B*	*Ped.acidllactici S*
1	*Arthtr. Gan davensis R1*	14	11	-	-	20	-	14	10	8	-		-	17	13	18	18	14	15	15
2	*Psevdomonas jessenii*	25	26	-	-	25	15	12	11	14	-		12	24	23	19	26	24	23	23
3	*Aeromonas veronii R1*	27	21	-	-	25	11	20	15	20	-		14	23	14	19	24	19	19	20
4	*Acinobacter lwoffii*	35	36	-	12	38	22	32	28	25	-		-	42	10	43	43	41	43	42
5	*Aeromonas veronii R2*	38	36	-	25	15	15	25	-	28	-	23	26	30	31	30	30	28	30	35
6	*Staph. aureus R1*	24	29	-	-	-	-	23	-	20	-	13	14	24	26	25	32	25	28	30
7	*Staph. aureus R2*	29	20	-	11	-	-	15	-	14	-	14	21	24	23	22	35	24	29	33
8	*Staph. hominisR1*	40	40	25	15	-	-	24	-	23	-	18	19	28	29	23	35	27	27	30
9	*Arthtr. Gan davensis R2*	30	30	-	-	-	-	25	-	24	-	27	24	28	27	25	27	25	27	30
10	*Aeromonas veronii Hov*	35	30	-	28	15	-	24	-	29	-	25	29	31	35	36	30	28	30	31
11	*Aeromonas sobria2*	-	6	12	10	22	14	30	22	34	18	32	28	40	40	34	40	32	34	38
12	*Ps. libanensis1*	8	-	6	6	20	20	18	24	20	-	24	20	40	40	24	24	20	24	18
13	*Chryseobact gleum1*	-	-	-	-	12	8	26	-	22	-	26	22	28	26	23	30	24	27	26
14	*Bacillus mojavensis*	-	-	-	-	14	-	30	10	14	10	30	24	30	32	26	32	28	26	32
17	*Shewanella balticaR4*	14	34	32	26	24	32	26	24	26	18	44	18	22	26	42	42	40	40	28
19	*Ps. Libanensis2*	22	30	26	32	24	36	24	26	24	30	36	20	34	36	41	40	32	42	46
20	*Chryseobact gleum2*	-	-	-	-	24	44	36	20	21	24	44	22	34	48	40	44	34	42	45
21	*Shewanella balticaR7*	-	-	-	-	26	32	22		14	12	40	16	40	42	42	44	38	45	42
22	*Aeromonas salmonicida*	24	14	20	14	30	32	32	14	26	20	42	24	40	48	42				
23	*Hafnia alvei1*	-										43	35	34	40	42	42	40	40	45
24	*Ps. Brassi cacearum1*	14	15	6	25	40	30	40	34	46	24	32	50	50	50	50	64	50	64	50
26	*Pseudomonas fragi1*	44	40	-	40	30	26	40	24	34	36	34	50	50	50	34	40	40	40	40
28	*Flavobact flevense*	-	-	-	-	20	25	50	12	24	10	15	50	44	40	36	50	50	50	50
30	*Ps. japonica1*	-	-	-	-	28	27	48	28	32	30	30	54	50	52	50	54	50	45	50
31	*Glutamicib.rgerei*	-	-	-	-	20	20	48	20	24	20	24	50	50	50	50	50	45	50	50
32	*Aeromonas caviae*	-	-	-	-	22	22	36	22	24	30	38	40	38	42	44	42	44	45	44
47	*Bacillus cereus 1*	-	-	35	12	42	32	42	12	40	38	40	50	50	50	50	44	50	42	44
48	*Exiguobacterium sp 4*	-	-	-	-	50	50	50	50	50	60	45	∞							
58	*Aeromonas media4*	-	-	-	-	14	12	30	26	24	14	22	40	40	40	40	40	32	34	40
59	*Aeromonas bohiminis R*	40	40	32	38	40	40	50	50	50	50	50	50	50	50	50	50	50	40	50

3.3 Probiotic properties of selected strains of lactic acid bacteria.

We have studied the probiotic properties of 6 selected most antagonistically active lactic acid bacteria.

Concerning the resistance to gastric juice, all tested cultures showed resistance to pH=3 for 60 minutes - the most significant number of cells was observed in *Lactiplantibacillus plantarum Kr5, Lactobacillus delbrueckii R1, Enterococcus faecium R2, Pediococcus pentosaceus R1* - 10^6, 10^5, 10^5, 10^5 CFU/ml, respectively. *Pediococcus acidilactici* and *L.plantarum* were more sensitive to low pH, and in 60 min, the number of CFU of these cultures was 10^3 and 10^2, respectively.

With the cultivation time exceeding 60 minutes, the number of viable cells dropped to 10^2 - 10^3 CFU/ml for all strains except *Enterococcus faecium*, which was not stable for low pH after 60 min (Figure 1).

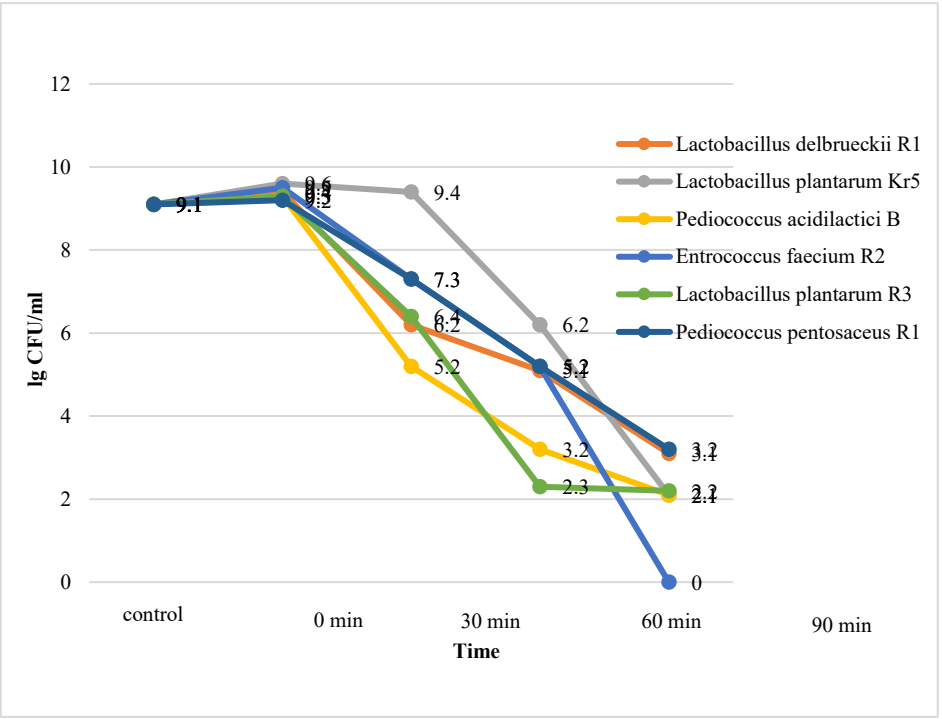

Fig. 1. Resistance of selected Lactic acid bacteria to simulated gastric juce.

Resistance to NaCl determines the survival of probiotic strains of microorganisms during processing and in the finished dosage form.

The Lactobacteria cells grown in various salt concentrations and the number of viable cells were counted to study salt tolerance. It was shown that all isolates are resistant to the presence of 0%, 2%, 4%, and 6.5% NaCl in the medium, and the viable cell number was not lower than 10^7 cfu/ml. It should be noted that in the presence of 6% NaCl in the medium, the cell number decreases by only one logarithm for *Lactobacillus delbrueckii R2, Lactiplantibacillus plantarum Kr5, Pediococcus acidilactici B, Lactobacillus plantarum R3*; and decreases by two logarithms for *Entrococcus faecium R2 and Pediococcus pentosaceus R1* (Figure 2).

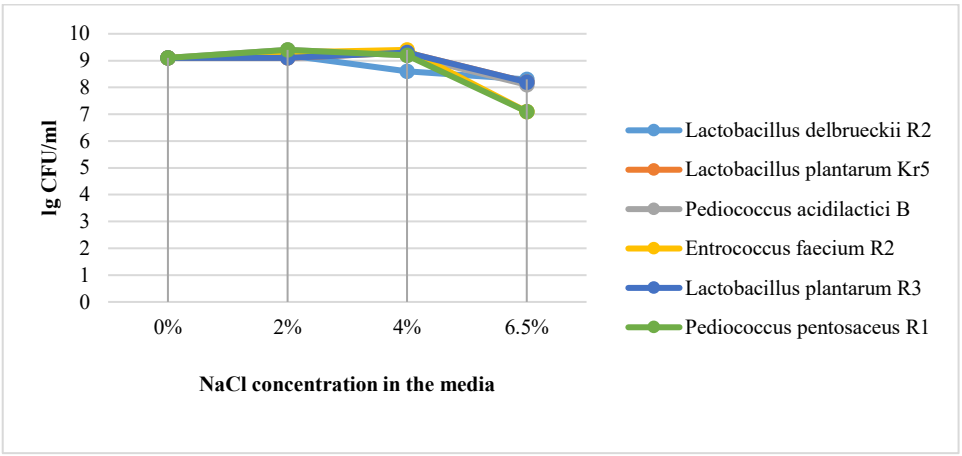

Fig. 2. Viability of LAB in different concentrations of NaCl.

Thus, all the studied isolates were resistant to 6.5% NaCl in the medium, where the viable cells were at least $1x10^7$ cfu/ml.

According to Fuller (1992) [16] bile, even at low concentrations, can inhibit the growth of microorganisms in vitro. Gilliland et al. (1984) [17] reported that a concentration of 0.4% is critical for screening for resistant strains. In our study, all strains grew in the presence of 0.4% and 0.6% bile. The most significant resistance was shown by cultures of *Lactobacillus plantarum Kr5, Pediococcus acidilactici B, Lactiplantibacillus plantarum R3*, in which, at 0.3% bile, growth did not differ from the control with no bile. In the presence of 0.6%, it decreased for one logarithm. Slightly less resistance was shown in cultures of *Lactobacillus delbrueckii R1, Entrococcus faecium R2 and Pediococcus pentosaceus R1*, in which the number of cells decreased for one logarithm in the presence of 0.3% bile in the medium (Table 3). *L. plantarum* appears to be the most resistant to bile lactic acid bacteria in aquaculture. In previous studies, Ramos et al. (2013) demonstrated similar results [18], where *L. plantarum* strains showed the highest resistance to bile (Fifure 3).

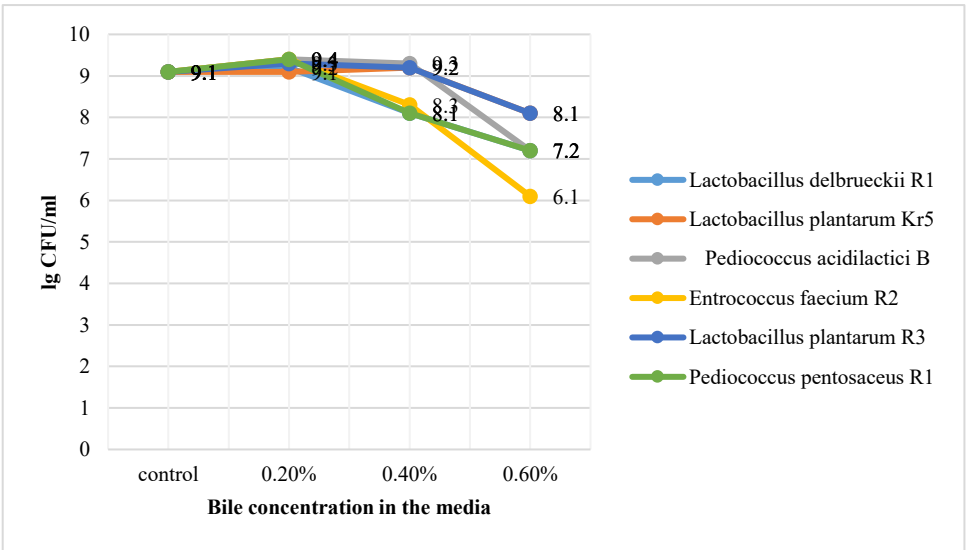

Fig. 3. Viability of LAB in different bile concentrations.

Before recommending LAB strains for use, it is necessary to determine their sensitivity to antibiotics. Antibiotic-resistant strains can harm the human or animal body by transferring resistance genes to pathogenic bacteria. In our study, we analyzed the sensitivity of lactobacilli to 10 antibiotics. The results showed that all cultures are sensitive to gentamicin. All cultures are resistant to vancomycin and most to ofloxacin. *Lactiplantibacillus plantarum R3* and *Lactobacillus delbrueckii R2* are resistant to many antibiotics studied (8 and 6 out of 10, respectively), which requires special attention to avoid potential risks (Table 2).

Table 2. The sensitivity of LAB to antibiotics.

№	Antibiotics	Amount in paper unit, mkg	Diameter of growth inhibition zone, mm/sensitivity to antibiotic					
			Lactobacillus delbrueckii R2	*Lactobacillus plantarum Kr5*	*Pediococcus acidilactici B*	*Entrococcus faecium R2*	*Lactobacillus plantarum R3*	*Pediococcus pentosaceus R1*
1	Kanamycin	30	0	30/S	38/S	30/S	0	7/R
2	Erythromycin	15	36/S	28/S	36/S	26/S	0	28/S
3	Rifampicin	5	35/S	34/S	36/S	30/S	0	30/S
4	cefotaxime	5	0	36/S	12/SR	30/SR	0	32/S
5	Levofloxacin	5	20/S	0	0	12/SR	0	10/SR
6	Amikacin	30	0	7/R	12/R	10/SR	0	8/R
7	Vancomycin	30	0	0	7/R	0	0	0
8	Amoxiclav	30	0	40/S	40/S	34/S	42/S	36/S
9	Gentamicin	10	20/S	12/R	16/S	12/SR	16/S	14/SR
10	Ofloxacin	5	0	38/S	0	0	0	0

3.4 Enzymatic activity

The digestive tract and enzyme physiology depend on dietary ingredients, which ultimately affect the health and growth of fish [19, 20]. Fish can adapt their metabolic functions to substrates through the regulation of enzyme secretion in order to utilize nutrient ingredients. Probiotics have a beneficial effect on the digestion of aquatic organisms because they synthesize extracellular enzymes such as proteases, amylases, and lipases and, in addition, provide growth factors such as vitamins, fatty acids, and amino acids [21]. Therefore, nutrients are absorbed more efficiently when meals are accompanied by probiotics [22].

Enzymatic activity was studied in lactic acid bacteria to evaluate their role in improving the degradation of feed ingredients. It has been shown that a small number of LABs can synthesize amylolytic and proteolytic enzymes; however, this activity was found in some of them. Thus, *L. delbrueckii R1* showed amylolytic activity, and *L. plantarum Kr2, L. plantarum Kr3, L. plantarum Kr4* showed the production of proteolytic enzymes (Table 3).

Table 3. Amylolytic and proteolytic activity of LAB isolates.

№	Lactic acid bacteria	Amylase	Protease
1	*L. delbrueckii R1*	+(26mm)	-
2	*L. delbrueckii R2*	-	-
3	*L. delbrueckii R3*	-	-
4	*L. delbrueckii R4*	-	-
5	*E. faecium R3*	-	-
6	*E. hirae R1*	-	-
7	*E. casse R1*	-	-
8	*E.faecium R2*	-	-
9	*E. casse R2*	-	-
10	*E. hirae R2*	-	-
11	*E. mundtii R*	-	-
12	*E.faecium R1*	-	-
13	*E.faecium K2*	-	-
14	*L.plantarum Kr1*	-	-
15	*L.plantarum Kr2*	-	*+(6mm)*
16	*L.plantarum Kr3*	-	*+(6mm)*
17	*L.plantarum Kr5*	-	-
18	*P. acidolactis B*	-	-
19	*P. acidolactis S*	-	-
20	*L.plantarum Kr4*	-	*+(6mm)*

Amylolytic lactic acid bacteria (ALAB) can utilize starch as the sole source of carbohydrates and can convert it into various products (mainly lactic acid) in a single fermentation step. ALABs have been used for many years for food preservation and have another role in food production: they partially hydrolyze starch, making it easier to digest [23].

Thus, the amylolytic activity of *L. delbrueckii R1* isolated from healthy fish is interesting for feed additives and food products.

4 Conclusion

As a result of the work done, it was shown that 37 isolates of non-pathogenic bacteria - potential probiotics - were isolated from healthy fish and other aquacultures. The predominance of bacteria of the genera *Lactobacillus* (22 isolates) and *Enterococcus* (9 isolates) was shown. At the same time, among *Lactobacilli*, most isolated cultures are representative of the *L. plantarum* (12 isolates).

As a result, it was found that strains of *Lactobacillus delbrueckii* isolated from healthy fish, *Lactiplantibacillus plantarum* isolated from shrimp, and representatives of the *Pediococcus* species have high antimicrobial activity. At the same time, the diameter of the zone of suppression of pathogens by these strains in some cases exceeds 40 and even 50 mm, which indicates a very high antimicrobial potential against susceptible pathogens.

In terms of the combination of probiotic properties of the culture, *Lactiplantibacillus plantarum Kr5, Pediococcus acidilactici B, Entrococcus faecium R2, Pediococcus pentosaceus R1* meet all the criteria for probiotic strains, while *Lactobacillus delbrueckii R2* and *Lactiplantibacillus plantarum R3* carry antibiotic resistance genes that are not encoded on the chromosomes, and, therefore pose a risk in terms of the spread of antibiotic resistance among bacteria. In this regard, their use in the composition of probiotic feed additives is not recommended.

A potential probiotic *L. delbrueckii R1*, possessing high amylolytic activity, was selected, which is of value for inclusion in the composition of feed additives.

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