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Diversity and characterization of lactic acid bacteria from Common Carp (*Cyprinus carpio* L.) intestine in winter (Northern Kazakhstan)

Currently, in Kazakhstan, chemical agents and antibiotics are widely used for treatment and prevention of fish diseases at fish farms. The use of probiotics as an alternative to antibiotics can help reduce the spread of antibiotic resistance in this area. The aim of the present study was to isolate the intestinal lactic acid bacteria of wintering carps. We assume that such bacteria can have more adaptive properties and can be used as probiotics for growing carp juveniles at fish farms. A probiotic characteristic of 22 lactic acid bacteria isolated from Common carp intestines was studied. Universal primers were used to determine the sequence of 16S rRNA gene fragments of lactic acid bacteria (LAB). Phylogenetic relationships of the isolates were estimated using the neighbor-joining (NJ) method in Mega 6,0. All identified isolates can grow in temperature range from 10° C to 37° C and in presence of bile salt. The isolated bacteria were screened for antibacterial activity, resistance to bile, resistance to antibiotics and growth at low temperatures. All isolates were tested in vitro for their ability to inhibit the growth of Shewanella xiamenensis, Pseudomonas taiwanensis, Ps. aeruginosa and Aeromonas punctata. As a result, 7 isolates with strong antagonistic activity were selected. 16S rDNA gene sequencing identified 4 isolates as Lactobacillus fermentum, 2 - as L. casei/paracasei and 1 - as Pediococcus pentosaceus. Antibiotic resistance profile of selected strains was studied, too. This study is the first attempt for Kazakhstan to isolate and study the representatives of the normal intestinal microflora of commercial fish species. Selective strains could be potential probiotics for freshwater aquaculture practices in Kazakhstan.

The paper contains 3 Figures, 3 Tables and 36 References.

Key words: *Cyprinus carpio*; lactic acid bacteria (LAB); pathogen; aquaculture; probiotics.

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Introduction

Potential probiotic bacteria for use in aquaculture should exert antimicrobial activity and be regarded as safe not only for their hosts, but also for aquatic environment [1-3]. However, there is still no consensus on the species and generic composition of probiotic for aquaculture. Wu S. et al studied the taxonomic composition of intestine bacterial community of grass carp, based on OTU-s analysis [4]. As a result, they supposed that using the members of *Lactobacillus* genera in creation of a new probiotic for aquaculture is not rational, because *Lactobacillus* species have low abundance in the intestinal microbiota of carps. Their research shows that a better candidate can be antagonistic strains of *Pseudomonas* genera, which were highly abundant in the intestinal community. However, the authors did not deny that *Pseudomonas* spp. could be potential pathogens [4]. Therefore, most probiotics proposed for aquaculture now as biocontrollers and bioremediators belong to safe lactic acid bacteria (LAB) group [5-7]. Antagonistic strains of LAB can be good defense against infectious fish diseases and, probably, can replace antibiotics and chemotherapeutics in aquaculture [8-12]. Picchietti (2009) showed that the effectiveness of probiotics in fish is higher if the strains were previously isolated from the host belonging to the same species [13].

The basis of freshwater fisheries in Kazakhstan consists of various commercial species of carp family (*Cyprinidae*). The Caspian Sea in the west of Kazakhstan is the only location where carps loose commercial importance in comparison with valuable sturgeon species. Most of freshwater reservoirs of Northern and Central Kazakhstan are landlocked and shallow, and during cold seasons, fish have low survival rate. In order to maintain fisheries on these reservoirs, various measures must be taken, one of them is fish stocking. Common carp due to its good adaptability and high growth rate is a preferred species for stocking. There are several fish farms focused on the cultivation and maintenance of Common carp broodstock in Kazakhstan.

The aim of the research was to study the probiotic potential of LAB from the intestines of carps at the end of wintering period.

Materials and methods

Maybalyk farm, located on the Nura, tributary of the Ishim river, uses only natural feeding base of the river for fish growing without any commercial feed and additives. Therefore, the presence of LAB in carp intestines is original and not associated with feed additives and any human activity.

Fish and isolation of lactic acid bacteria

22 males of Common carp (*Cyprinus carpio* L.) from Maybalyk farm, weighing from 850 grams to 1.2 kilograms, were transferred to the laboratory in 20-liter containers with river water in March 2019. After transporting, the fish were placed

in the freezer (-20° C) for a night, and we started isolation of LAB from the intestine next day. To do this, three segments of the intestine with a length of one centimeter from each fish were placed in saline solution and thoroughly shaken; after this, decimal dilutions were made. Next, 100 µl of the serial 10-delution of homogenate were spread on Man, Rogosa, Sharp (MRS, HiMedia) agar, a medium selective for *Lactobacillus* spp. The plates were incubated at 37 °C for 2 days. Pure cultures were obtained after following streaking technique again on plates with MRS agar and incubation. As a result, gram-positive bacteria, in the form of sticks and cocci, which gave circular colonies of white and cream colors, did not grow on meat-peptone agar, and did not produce catalase. These gram-positive bacteria were selected and stored in 25 % glycerol at -80 °C.

Test of Antagonistic activity of LAB

Agar well diffusion assay was used to determine the antagonistic activity of putative probiotic bacteria against the four indicator bacteria *Shewanella xiamenensis* AU 2R-1 B-RKM 0724*, Pseudomonas taiwanensis* CB 2R-1 B-RKM 0726*, Pseudomonas aeruginosa* G13 B-RKM 0427 and *Aeromonas punctata* G30 B-RKM 0287 which were deposited in the Republican Collection of Microorganisms, Ministry of Education and Science of the Republic of Kazakhstan (Astana, Kazakhstan). Culture density was determined using the colony-forming unit (CFU) method. After 24 hours of incubation in Nutrient Broth (NB, HiMedia, Mumbai, India) at 37 °C, 1 ml of indicator culture (10^8 CFU/ml) (McFarland standard set, HiMedia) was added to 15 ml of melted MRS agar (HiMedia) cooled to 43-44 °C. After solidification and drying for 15-20 min, wells were punched (diameter, 3 mm) and 50 µl of MRS-broth, containing a 2-day-old supposed probiotic culture, were added to wells in triplicates. Plates were incubated at 37 °C for 48 hours. After incubation, all plates were examined for the presence of the zone of inhibition around the wells [11, 14].

Bacteriocin-producing activity of LAB

The antimicrobial activity of supernatants of isolated lactic acid bacteria grown in MRS broth at 37 °C for 24 hours was determined also by an agar well-diffusion test as previously described with the same indicator strains. After cultivation, cell-free supernatants were obtained by centrifugation 4000×g at 4 °C for 5 min (Eppendorf Centrifuge 5810 R, Germany). Following this, the supernatants were passed through 0.22 µm filters (Millipore corp., USA). Approximately 35 µl of supernatant was placed into the first well of 3 mm in diameter cut into plates with cooled agar, previously seeded with pathogenic bacteria 0.1 ml (10^8 CFU/ml) (McFarland standard set, HiMedia). To eliminate inhibitory activity due to organic acids, the pH of the supernatant was adjusted to pH 6.0 with 1 M NaOH and that solution was added to the second well in the same volume. The plates were incubated for 1 day [15]. A positive result confirming the presence of bacteriocin in the supernatant was the appearance of inhibition zone of the target strain around the second well.

Identification of LAB and phylogenetic analysis

DNA was isolated from the 24-h cultures incubated at 37 °C on the MRS medium with reagent kit for DNA extraction “DNA-sorb-AM” (AmpliSens,

Russia) following the recommendations of the manufacturer (DNA-sorb-AM Manual). DNA concentration in the resulting samples was measured via Nano-Drop 2000 (Thermo Scientific, USA). Sequence analysis was performed in the Laboratory of Applied Genetics (National Center for Biotechnology, Astana, Kazakhstan). Briefly, the sequencing reaction was performed by using Big Dye® v 3.1 and primers. Universal primers 5'-AGAGTTTGATCCTGGCTCAG-3' (10F) and 5'GGACTACCAGGGTATCTAAT3' (806R) were used to determine the sequence of 16S rRNA gene fragments of antagonistic LAB. The obtained sequences were compared with the previously published data in GenBank and aligned with previously characterized sequences of closely related members of the genus, using ClustalW in Mega 6.0 multiple sequence alignments [16]. Phylogenetic relationships of the isolates were estimated using the neighbor-joining (NJ) method in Mega 6.0. Confidence in the NJ trees was determined by analyzing 1.000 bootstrap replicates using the Mega program.

Assessment of antibiotic susceptibility

Antibiotic resistance of the isolated LAB was studied by agar disk diffusion method according to the CLSI [17-18]. Antibiotic resistance profiles were obtained using amoxicillin (AMO, 10 mg), ampicillin (AM, 10 mg), cefazolin (CF, 30 mg), kanamycin (K, 30 mg), gentamycin (GE, 10 mg), vancomycin (VA, 30 mg) and tetracycline (TE, 30 mg). The bacterial suspension (10^7 CFU/ml) was inoculated into MRS agar (HiMedia, India) plates using swabbing technique. Then, antibiotics disks were deposited on the plates. To check the quality of the disks, 2 reference microorganisms *E.coli* ATCC 25922 and *S. aureus* ATCC 25923 were tested for susceptibility to the respective antibiotics.

Bile Tolerance

The modified method of Arihara et al. (1998) method, described by Buntin et al. (2008) was used to determine bile tolerance of LAB. Before testing for bile tolerance, LAB strains were grown at 37 °C for 24 hours in MRS broth [19-20]. After this, 1 ml of the culture broth was poured onto MRS agar with bile salt (HiMedia, India) concentrations of 2000, 3000 and 4000 ppm. Bacterial growth was determined after incubation at 37 °C for 48 hours [20].

Growth tests

Antagonistic active isolates were inoculated into 10 ml of MRS liquid medium, and then incubated for 24 hours at 37 °C. One hundred microliters of the culture (about 10^6-10^7 CFU/ml) was then inoculated into 10 ml fresh MRS liquid medium and incubated at 10, 15, 20, 30 and 37 °C. The growth was monitored by measuring optical density (OD) 600 nm after 24 hours of incubation [21].

Statistical analysis

Experiments of this study were performed in triplicate and the results developed as mean ± standard deviation ($M \pm SD$). Statistical significance was assessed by Student's ¼ s t test. Results are considered significant at $p \leq 0.05$.

Results and Discussion

Isolation and identification of antagonistic LAB strains

The intestinal microbiome of fish has a great scientific interest now. In several reviews based on metagenome analysis, the composition, features and formation of intestinal microflora of various commercial fish species of North America, East and Southeast Asia and Europe have been presented. The results of all these studies showed that lactic acid bacteria (LAB) are a common microbial group of intestinal microbiota of fish [24-30]. Earlier studies, based on isolation of LAB from the intestines of fish, did not give a complete picture of their appearance and role in this ecosystem [31, 32].

For the isolation of potential probiotic bacteria, the basic and common selection methods and medium were used. It was essential for us to obtain strains, whose cultivation and production will not require excessive efforts and costs in the future. A total number of bacterial isolates obtained from the intestines of carps was 22. All isolates presumptively corresponded to LAB based on phenotypic characteristics. It is known that LAB can produce several antimicrobial compounds, such as organic acids, diacetyl, hydrogen peroxide, ethanol, reuterin and other bacteriocins [22]. By the investigation of total antagonistic activity of 22 isolates, seven were tolerant for two (*Ps. aeruginosa* G13 B-RKM 0427*, A. punctata* G30 B-RKM 0287) of four test microorganisms and eight isolates showed a low level of antagonistic activity (diameter of the zone ≤ 10 mm). Only seven isolates had a high ability to inhibit all indicator bacteria (clear zones around the wells were larger than 10 mm). Bacteriocin-producing activity of cell-free culture supernatants of seven active antagonistic isolates was studied next. For this experiment, the same test microorganisms were used. As a result, bacteriocins providing inhibitor activity of cell-free supernatants of all tested isolates against 3 (*Sh. xiamenensis* AU 2R-1 B-RKM 0724*, Ps. taiwanensis* CB 2R-1 B-RKM 0726*, Ps. aeruginosa* G13 B-RKM 0427) out of 4 indicator bacteria were proven. The results of the experiments are shown in Table 1.

Several researchers mentioned that the isolation of LAB from various organs of fish was linked with some difficulties: from preparation of composite mediums to prolongation of the primary cultivation period to seven or even more days [29]. In our studies, we used simple techniques and commercial mediums MRS (agar and broth) which are selective for lactobacilli (solid and liquid), and the cultivation period did not exceed 48 hours. According to our observations, when the cultivation period was up to 96 hours on broth, the species diversity of LAB was poor due to the elimination of one type of microorganisms by another, and coccal lactobacteria were dominant in this case.

Strong antagonistic properties of LAB allow them to be considered as an alternative to antibiotics and chemotherapeutic agents of disease control. The pathogenic inhibitory effect of LAB, in general, is due to the action of either acid or bacteriocins, as well as their combination.

Table 1

Diameter of the inhibition zone (mm) caused by antimicrobial activity of LAB strains against test microorganisms (*M*±*SD*)

Test micro-organisms LAB strains	*Pseudomonas aeruginosa* G13 B-RKM 0427			*Shewanella ximenensis* AU 2R-1 B-RKM 0724			*Pseudomonas taiwanensis* CB 2R-1 B-RKM 0726			*Aeromonas punctata* G30 B-RKM 0287		
	LC	CFS	CFH	LC	CFS	CFH	LC	CFS	CFH	LC	CFS	CFH
Lactobacillus fermentum 22-1c	18.0±2.0	17.7 ±1.6	0	24.7±3.2	21.7±2.1	0	19.2±1.3	17.7±1.2	0	11.0±1.7	0	0
L. fermentum 24 c	19.4±1.6	18.3±1.2	17.3±2.1	31.2±1.0	29.7±2.5	19±1.0	21.2±1.5	19.7±1.5	16±1.0	11.3±2.1	0	0
L. fermentum 13-1c	20.0±3.6	19.7±1.5	15.0±1.7	24.7±3.2	24.7±1.1	19.4±1.6	18.4 ±1.8	16.8±1.7	16.3±2.5	15.3±1.2	0	0
L. fermentum 2c	15.3±1.2	14.0±1.0	13.6±0.6*	18.0±2.0	16.8±1.2	16.3±2.1	16.8±1.7	15.3±2.5	13.3±2.5	11.7±2.5	0	0
L. casei 9c	17.3±2.1	15.0±1.7	14.3±0.6*	26.5±0.29*	25.3±1.5	18.0±2.0	21.2±1.5	20±1.7	16.8±1.7	18.7±2.1	0	0
L. casei 12-2c	15.3±2.5	13.3±1.5	11.3±1.5	17.8±0.75*	16.3±2.1	14.7±0.6*	15.3 ±1.2	14.7±1.2	12.3±2.1	11.3±1.2	0	0
Pediococcus pentosaceus 10-9k	18.4±1.8	16.3±2.1	14.3±2.0	23.0±0.41*	21.2±1.5	18.0±2.0	19.2±1.3	16.8±1.7	15.7±1.2	10.6±1.3	0	0

Note. LC - Live culture; CFS - Culture cell free supernatant; CFH - Culture cell free supernatant adjusted to pH 6.5-7 with 1M NaOH

Inhibition zones: 0 mm - No activity; ≤ 5mm - Doubtful; 5-15mm - Weak; 15-20 mm - Moderate; 20-25 mm - Strong; ≥ 25 mm - Very strong. *$p < 0.05$

The absence of the antibacterial activity of hydrogen peroxide produced by LAB has been confirmed experimentally in some studies [20, 31]. We selected seven isolates because of the data of total antagonistic activity against fish pathogens, as well as the antibacterial activity of cell-free culture supernatants of isolates. The detection of the antibacterial activity of cell-free supernatants showed inhibitory effect of three out four test strains. Inhibitory activity of cell-free supernatants of 7 selected isolates both pure and neutralized by NaOH against *Aeromonas punctata* G30 B-RKM 0287 were not detected. On the contrary, live cultures of isolates showed good inhibition of growth of *A. punctata* G30 B-RKM 0287. Neutralization of the acid with NaOH in the supernatants removes its antibiotic ability; however we cannot explain the ineffectiveness of the pure supernatant in this case (Table 1).

Based on analysis of the 16S rRNA gene sequence, six of active LAB strains were assigned to the genus *Lactobacillus* (2c, 9c, 12-2c, 13-1c, 22-1 c, 24c) (Fig.1) and one to *Pediococcus* (10-9k) (Fig. 2).

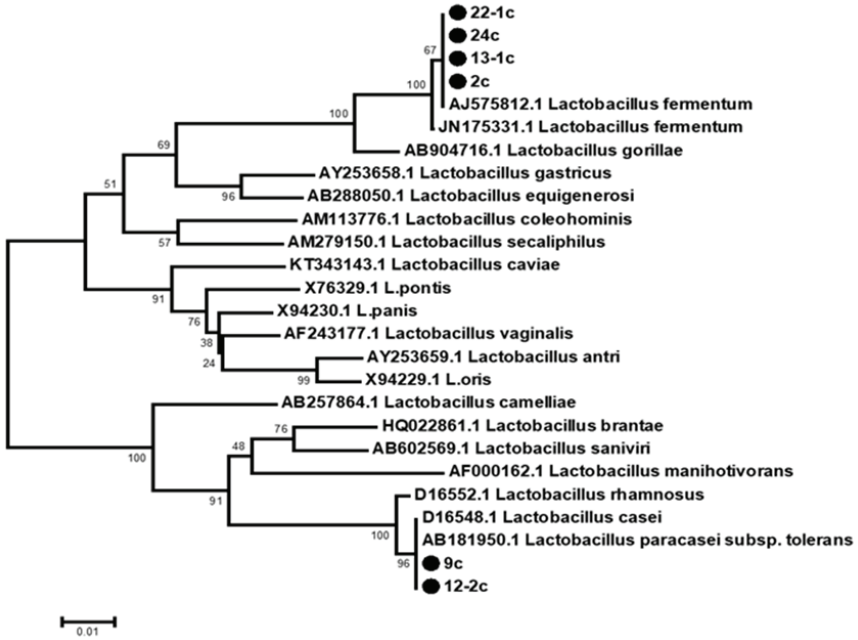

Fig. 1. Neighbor-joining Tree based on 16S rRNA Gene Sequences of Strains 22-1c, 24c, 13-1c and 2c (*Lactobacillus fermentum*), 9c and 12-2c (*L. casei*) and Other Related Taxa. (Bar, 0.01 substitutions per nucleotide position)

The 16S rRNA gene sequence from isolates 22-1c, 24c, 13-1c and 2c showed ≥99.9% sequence similarity with *Lactobacillus fermentum* (GenBank acc #AJ5758121, JN1753311), isolates 9c and 12-2c showed 100% similarity with

Lactobacillus casei/paracasei (GenBank acc #D165481, AB1819501), and isolate 10-9k showed 100% similarity with *Pediococcus pentosaceus* (GenBank acc #KP1198191) (Fig. 1 and 2)

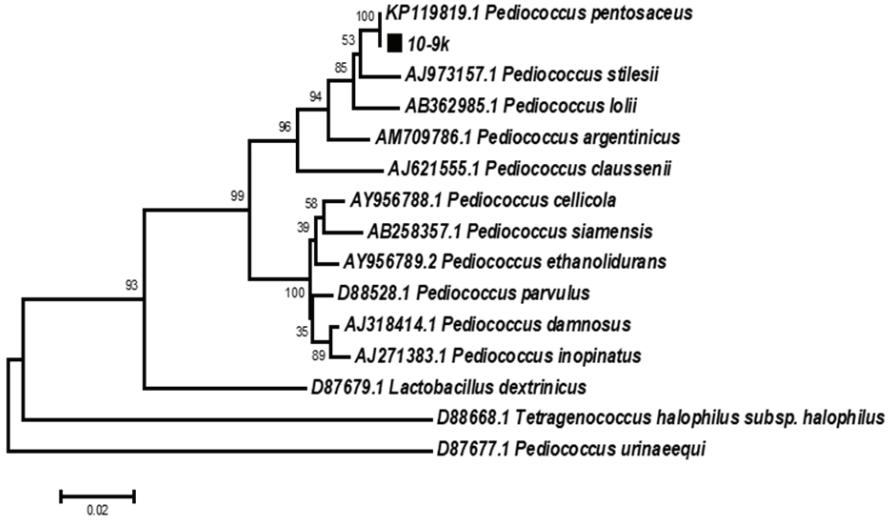

Fig. 2. Neighbor-joining Tree based on 16S rRNA Gene Sequences of Strain 10-9k (*Pediococcus pentosaceus*) and Other Related Taxa.
(Bar, 0.02 substitutions per nucleotide position)

In earlier studies, LAB of the genera *Lactobacillus, Lactococcus, Streptococcus, Enterococcus, Pediococcus* and *Carnobacterium* were isolated from carp intestines [14, 28, 30]. The genetic identification of our isolates has determined that they belong to three species of *L. fermentum, L. casei/paracasei* and *P. pentosaceus*. It is low rate for species diversity. Perhaps, it is due to the fact that we made isolation of LAB in winter and used only MRS-broth and MRS-agar for bacteria isolation.

Assessment of antibiotic susceptibility

All test microorganisms showed resistance to glicopeptide vancomycin and to aminoglycoside antibiotic kanamycin. Lactobacilli strains were resistant to aminoglycoside gentamycin. *P. pentosaceus* 10/9к were susceptible to this antibiotic. All strains showed susceptibility to β-lactam group (amoxicillin, ampicillin, carbenicillin and cefazolin) and tetracycline. The pictures of susceptibility and resistance of all lactobacilli strains to examined antibiotics are the same. They are resistant to three of eight checked antibiotics. One strain *P. pentosaceus* 10/9k have resistance to two of eight antibiotics (Table 2).

Recent studies have revealed the presence and expression of antibiotic resistant genes in the probiotics used in food and aquaculture [33-36]. It is accepted that the possibility of transfer is related to the genetic basis of the resistance mechanism,

whether the resistance is acquired as a result of chromosomal mutations (intrinsic) or by horizontal gene transfer. The good example of intrinsic mechanisms is vancomycin resistance of LAB. In addition, it was shown that 70% of LAB were intrinsically resistant to gentamycin when the Minimal Inhibition Concentration (MIC) breakpoints of the Scientific Committee of Animal Nutrition (SCAN) were used [34].

Table 2

The assessment of antibiotic susceptibility in LAB

Antibiotic LAB strain	AMO	AM	CB	CF	GE	K	VA	TE
Lactobacillus fermentum 22-1c	S	S	S	S	R	R	R	S
L. fermentum 24 c	S	S	S	S	R	R	R	S
L. fermentum 13-1c	S	S	S	S	R	R	R	S
L. fermentum 2c	S	S	S	S	R	R	R	S
L. casei 9c	S	S	S	S	R	R	R	S
L. casei 12-2c	S	S	S	S	R	R	R	S
Pediococcus pentosaceus 10-9k	S	S	S	S	S	R	R	S

Note. Antibiotics: AMO - Amoxicillin (10 mg), AM - Ampicillin (10 mg), CB - Carbenicillin (25 mg), CF - Cefazolin (30 mg), GE - Gentamycin (10 mg), K - Kanamycin (30 mg), VA - Vancomycin (30 mg), TE - Tetracycline (30 mg). S - Susceptible, diameter of the zone of inhibition ≥ 17 mm; R - Resistant, diameter of the zone of inhibition ≤ 13; Intermediate, diameter of the zone of inhibition 13-17 mm.

Opposite, *tet* genes responsible for resistance of lactobacilli to tetracycline have a high transfer risk, which can be carried within plasmids. In the present study intrinsic susceptibility of all tested LAB toward the inhibitors of cell wall synthesis, such as ampicillin, amoxicillin, cefazolin and inhibitors of protein synthesis, such as tetracycline was in accordance with bibliographic data [35, 36]. Vancomycin, kanamycin, gentamycin resistant phenotype had been obtained for all tested lactobacilli. It was mentioned that commercial feed additives that may contain antibiotics are not used at Maybalyk fish farm. This strategy allowed to limit the spectrum of antibiotic resistance in representatives of normal microflora of fish intestines.

Bile tolerance and growth temperature

Potential probiotic strains for using in aquaculture should possess certain characteristics such as bile tolerance and ability to grow in a wide range of temperatures. To select bile-tolerant strains, antagonistic active isolates 2c, 9c, 12-2c, 13-1c, 22-1 c, 24c, 10-9k were tested for their abilities to grow at the bile salt levels of 2000, 3000 and 4000 ppm. All of them were able to grow in the presence of bile salt in concentration 2000 ppm and 3000 ppm, too. Only one isolate 10-9k (*P. pentosaceus)* was able to grow in 4000 ppm bile salt concentration (Table 3).

Table 3

Bile salt tolerance of LAB isolated from Common Carp

Bile Salt Concentration (ppm)	*Lactobacillus fermentum* 22-1c	*L. fermentum* 24 c	*L. fermentum* 13-1c	*L. fermentum* 2c	*L. casei* 9c	*L. casei* 12-2c	*Pediococcus pentosaceus* 10-9k
2000	++	++	++	++	++	++	++
3000	+	+	+	+	+	+	++
4000	-	-	-	-	-	-	+

Note. ++ Good growth; + Visible growth; - No growth.

In addition, all isolates were able to grow in temperature range from 10 °C to 37 °C. Better growth was observed in all isolates beginning from 20 °C to 37 °C (Fig. 3). It should be noted that spring in Northern Kazakhstan can be cold, even in May, and water temperature can be lower than 10 °C.

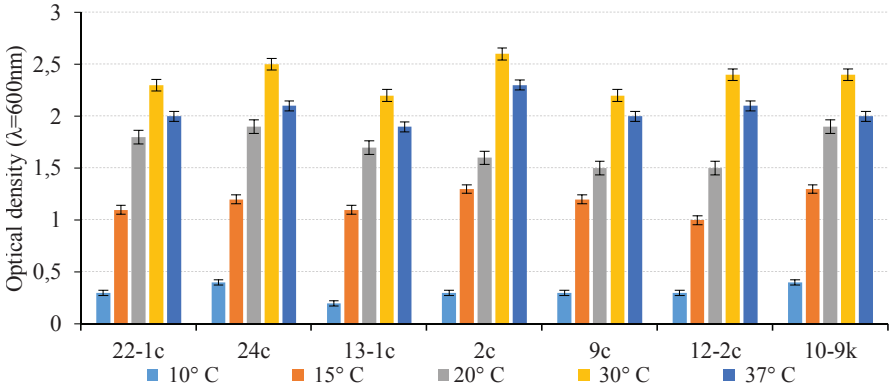

Fig. 3. Growth of lactic acid bacteria strains at different temperatures after 24h of incubation ($M \pm SD$)

The selection of different types of LAB depending on different seasons was well shown in the works of Hagi et al. (2004, 2009) on carps from Ibaraki Fisheries Station on Kasumigaura lake. Authors showed that such species as *Lactococus lactis* and *Lactobacillus fuchuensis* dominate during the summer period, while *Lactobacillus raffinolactis, Lactobacillus sakei, Leuconostoc gelidum* are dominant in winter. They also revealed that not only the composition of predominant LAB but also the composition of cholic acid-resistant LAB changed seasonally [21, 32]. The climate of Central and Northern Kazakhstan is severe and it is characterized by a cold winter lasting 5.5-6 months, sometimes rivers are covered with ice until the end of April. Long wintering is a stress for the whole organism of an animal, including its microflora. During the wintering period, Common Carp does not feed; the composition of their intestinal microflora becomes poorer. The dominance of the species *L. fermentum, L. casei/paracasei*

and *P. pentosaceus* among intestinal LAB during this period should be due to their specific characteristics, such as resistance to low temperatures and unfavorable environmental factors.

Conclusion

In this work, the composition and probiotic characteristics of representatives of lactic acid bacteria from the intestines of cultured carps in winter were studied. Despite the extremely adverse environmental conditions in winter in Northern Kazakhstan, lactic acid bacteria in the intestines of carps show high viability and retain most of their properties. The study showed the dominance of three species of lactic acid bacteria in this period: *Lactobacillus fermentum, L. casei/paracasei* and *Pediococcus pentosaceus.* The profiles of antibiotic resistance, resistance to bile and various temperatures of cultivation were studied in seven strains with high antagonistic activity. All strains showed growth at 10 °C and on the mediums with high concentrations of bile salt. These factors make them optimal candidates for use as probiotics in aquaculture of Kazakhstan.

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