Scientific Foundation SPIROSKI, Skopje, Republic of Macedonia
Open Access Macedonian Journal of Medical Sciences. 2020 Apr 25; 8(A):195-202.
https://doi.org/10.3889/oamjms.2020.3573
eISSN: 1857-9655
Category: *A - Basic Sciences*
Section: *Microbiology*

Screening and Characterization of the Antagonistic Properties of Microorganisms Isolated From Natural Sources

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Edited by: Sasho Stoleski
Citation: Le VM, Asyakina LK, Velichkovich NS, Kozlova OV, Milentyeva IS, Pozdnyakova AV, Prosekov AY. Screening and Characterization of the Antagonistic Properties of Microorganisms Isolated From Natural Sources. Open- Access Maced J Med Sci. 2020 Apr 25; 8(A):195-202. https://doi.org/10.3889/oamjms.2020.3573
Keywords: Pharmaceutical substance; Antimicrobial action; Microorganisms- antagonists; Metabolite; Bacteriocin; Cultivation; Biosynthesis; Inhibition; Screening
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Received: 23-Aug-2019
Revised: 06-Apr-2020
Accepted: 07-Apr-2020

Funding: Financial support for the research was provided by the Ministry of Education and Science of the Russian Federation as part of the implementation of the Federal Target Program "Research and Development in Priority Areas of the Scientific and Technological Complex of Russia for 2014–2020", the grant agreement No. 075-15-2019-1383 dated June 18, 2019 (unique project identifier RFMEFI57418X0207)
Competing Interests: The authors have declared that no competing interests exist

Abstract

BACKGROUND: Human infectious diseases caused by antibiotic-resistant bacterial pathogens present a serious problem for clinical medicine. Causative agents of nosocomial infections, such as *Escherichia coli, Klebsiella pneumoniae, Enterobacter* spp., are the most common among them. An active search for antimicrobial agents that can effectively combat drugresistant pathogens is underway. Antimicrobial substances of bacterial origin are of particular interest. Promising sources of microorganisms with antibiotic properties are natural sources: Soil, water, plants, etc.

AIM: The purpose of this work is to screen and characterize the antagonistic properties of microorganisms isolated from natural sources in connection with the creation of new pharmaceutical substances.

METHODS: The material for the isolation of microorganisms was the soil, water bodies, and plant objects of various municipal districts of the Kemerovo Region. Identification of the isolated microorganisms was carried out using the methods proposed in the directory "Bergey's Manual of Determinative Bacteriology" and in the monograph Nesterenko *et al*. The selection of strains from soil samples was carried out according to standard methods described in "Methods of soil microbiological control. Methodical recommendations," cultural-morphological properties of isolates were studied using conventional microbiological methods.

RESULTS: The following results are obtained: (1) Lactic acid bacteria and other microorganisms antagonists from natural sources were isolated: Soil, water bodies, and plant objects; 20 isolates were isolated, their cultural and morphological properties were studied; isolated microorganisms were found to belong presumably to the genera *Bacillus, Leuconostoc, Pedio-coccus, Lactobacillus,* and *Bacteroides*; (2) Antimicrobial properties of lactic acid bacteria and other antagonistic microorganisms isolated from natural sources on solid and liquid nutrient media were studied; (3) 12 strains of 20 isolates with maximum antimicrobial properties were selected for further studies.

CONCLUSION: Further research on the biochemical properties of lactic acid bacteria and other antagonist microorganisms isolated from natural sources, the study of antibiotic resistance of lactic acid bacteria and other antagonist microorganisms isolated from natural sources, as well as other more detailed studies will be conducted with selected 12 strains with maximum antimicrobial properties.

Introduction

The formation of antimicrobial resistance, such as antibiotics, antiseptics, and bacteriophages, is a natural adaptation mechanism of microorganisms and is a serious medical and social problem. Today, most clinically important pathogenic and opportunistic bacteria are able to develop resistance to antibacterial substances. There were strains of various types of microorganisms that are not sensitive to the action of almost all antibiotics used in medical practice, while the rate of obtaining new drugs has significantly decreased. In this regard, the creation of new antimicrobial agents or methods of their delivery is a priority area of biology, medicine, and veterinary medicine. Such an alternative may be bacteriocins, low-molecular peptides, having a highly specific antibacterial effect, aimed at strains of phylogenetically related or conamed species of bacteria, delivered to the target cells by conjugative transport – a natural way of intercellular communication of prokaryotic cells [1], [2], [3].

The ability to produce bacteriocins has Gram-positive and Gram-negative bacteria. However, bacteriocins synthesized by Gram-negative bacteria have a rather narrow spectrum of action and therefore have not been widely used as strains-producers. Gram-positive bacteriocins (lactic acid bacteria) have stronger antimicrobial properties and are currently promising for the study of the possibility of various kinds of bacteriocins [4], [5], [6]. Antimicrobial activity of bacteriocins of lactic acid bacteria is directly dependent on environmental conditions (temperature, pH and consistency of the medium, the composition of the medium, the concentration of metals, and other factors) [7], [8].

Bacteriocin Classification

Bacteriocins are divided into four classes. Division by classes is carried out according to the

methods of synthesis, physicochemical and amino acid composition, and antibacterial properties [9], [10].

The first class bacteriocins represent lantibiotic, low molecular weight peptides (<5 kDa), hydrophobic, and cationic nature [11, [12], [13], [14].

The most studied bacteriocin from the first class is nisin isolated from *Lactococcus lactis*. The action of nisin is based on the violation of the permeability of bacterial spores, thereby reducing their thermal stability [15], [16].

The second class of bacteriocins includes lanthionine-containing peptides of low molecular weight (<10 kDa), resistant to temperature, and active in a wide range of pH (3–9). Bacteriocins of this class do not cause a high immune response and are non-toxic to both humans and animals [17, [18], [19], [20], [21].

In turn, Class II bacteriocins are divided into subclasses IIa, IIb, and IIc depending on antimicrobial activity, methods of separation from the producing strain and chemical composition [22], [23], [24], [25], [26], [27].

Bacteriocins belonging to Class III (e.g., enterolysin A) are proteins with a high antimicrobial action and a molecular weight of more than 30 kDa, which are destroyed by high and low temperatures.

Class IV includes bacteriocins that have lipid and carbohydrate components in their composition, which gives them high activity [28], [29].

All four classes of bacteriocins in some cases have the same amino acid sequence, which allows a large number of strains of lactic acid bacteria to synthesize bacteriocins [30], [31].

Mechanism of Action of Bacteriocins

Bacteriocins affect the main functions of bacterial cells due to the huge variety of chemical structures. The action of bacteriocins is based on the change of cell membrane tension due to the formation of pores in it [32], [33].

The mechanism of pore formation in each class of bacteriocins is different [34], [35], [36].

Figure 1 shows the mechanism of action of the bacteriocin nisin. C-end of the nizin molecule reduces the potential difference in the cytoplasmic membrane and thus inhibits the development of cellular components and the action of intracellular.

Similar to nisin mechanism of action was observed in bacteriocins of Class IIa [11].

The action of bacteriocins mainly consists in the formation of pores in the cell membrane, leading to the destruction of the cell. In some cases, bacteriocins prevent cell wall formation [8]. Different

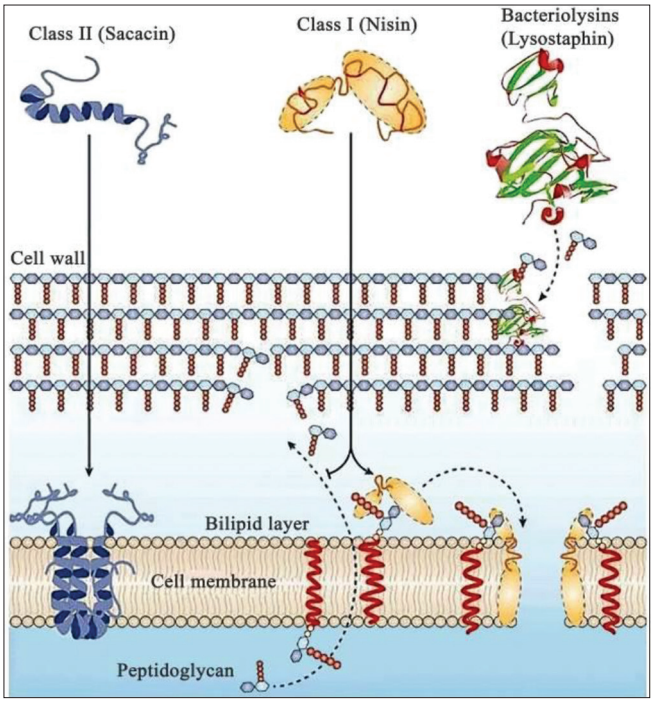

Figure 1: The mechanism of action of bacteriocins

kinds of bacteriocins are capable of forming lactic acid bacteria [37], [38], [39].

The practical use of bacteriocins is in the treatment of infectious diseases. They are also used as preservatives of food and fodder products.

In this regard, the selection, study of the properties and synthesis of new antibacterial and fungicidal antibiotics formed by lactic acid bacteria and other antagonist microorganisms, as well as the study of the prospects for their use in the pharmaceutical industry is of fundamental and practical interest.

The purpose of this work is screening and characterization of antagonistic properties of microorganisms isolated from natural sources in connection with the creation of new pharmaceutical substances.

Objectives

The objectives are as follows:

- Isolation of lactic acid bacteria and other antagonist microorganisms from natural sources: soil, water bodies, and plant objects;
- Study of antimicrobial properties of lactic acid bacteria and other antagonistic microorganisms isolated from natural sources on solid and liquid nutrient medium;
- Selection of strains with maximum antimicrobial properties for further research.

Research Objects and Methods

The soil, water bodies, and plant objects of various municipal districts of the Kemerovo region: Kemerovo, Prokopevsky, Mariinsky, Yaysky, and Guryevsky served as a material for the isolation of microorganisms.

Soil sampling was performed 3 times. Samples were selected in a checkerboard pattern, diagonally, through the envelope at a specific depth or horizon. A shovel, scoop, knife, and soil drill were used for soil sampling. Each object before taking a separate sample was thoroughly cleaned, wiped with a cotton swab with alcohol and burned. Samples were taken in sterile kraft paper.

Isolation of strains from soil samples was carried out according to standard methods described in the "Methods of microbiological soil control. Methodical recommendations" (app. Chief state sanitary doctor of the Russian Federation 24.12.2004 № TC/4022) as well as a workshop on soil biology edited by G. M. Zenova.

Bacterial cultures of microorganisms were grown for 48 h on dense nutrient media of the following composition:

- Agar GRM, g/l: acid hydrolysate of fish meal – 20, 0; glucose – 10, 0; and agar-agar – 15, 0;
- Meat-peptone agar (MPA), g/l: dry enzymatic pepton – 10, 0; meat extract – 11, 0; NaCl – 5, 0; agar-agar – 15, 0; and glucose – 10, 0; distilled water.

Identification of the isolated microorganisms was carried out using the methods proposed in the determinant of Berga bacteria and in the monograph by Nesterenko *et al*. Smears were stained with gram.

Cultural and morphological properties of isolates were examined using conventional microbiological methods, recorded the color, size, texture of the colonies in a dense environment, carried out staining smears for the Gram-stain, evaluated the motility of the cells in the preparation "crushed drop."

Evaluation of antimicrobial properties of lactic acid bacteria and other antagonistic microorganisms isolated from natural sources of the Kemerovo region was carried out in two ways: Diffusion method and method of determining the antagonistic activity in the liquid nutrient medium by optical density (OD).

The following pathogenic test strains were used:

Escherichia coli ATCC 25922; *Salmonella enterica* ATCC 14028; *Staphylococcus aureus* ATCC 25923; *Pseudomonas aeruginosa* B6643; *Bacillus mycoides* EMTC; *Alcaligenes faecalis* EMTC 1882; *Proteus vulgaris* ATCC 63; *Shigella flexneri* ATCC 12022; *Listeria monocytogenes* ATCC 7644; *Candida albicans* EMTC 34; *Aspergillus flavus* ATCC 9643; and *Penicillium citrinum* ATCC 9849.

Cultivation of test strains of microorganisms.

Strain *E. coli* ATCC 25922 cultured on a nutrient medium composition: trypton – 10 g, yeast extract – 5 g, sodium chloride – 10 g, and water – 1 l. pH 7.5–8.0. The Cultivation temperature is 37°C.

S. enterica ATCC 14028 strain was cultured on the nutrient medium of the composition: Peptic digestion of animal tissue – 10 g, meat extract – 5 g, glucose – 5 g, sodium hydrophosphate – 4 g, iron sulfate – 0.3 g, bismuth sulfite – 8 g, diamond green – 0.025 g, agar – 20 g, and water – 1 l. pH of the medium 7.5–7.9. The culture temperature is 35°C.

Strain of *S. aureus* ATCC 25923 was cultured on the nutrient medium of the composition: Casein hydrolysate – 10 g, yeast extract – 2.5 g, gelatin – 30 g, D-mannitol – 10 g, sodium chloride – 55 g, ammonium sulfate – 75 g, potassium hydrophosphate – 5 g, agar – 15 g, and water – 1 l. pH of the medium 6.8–7.2. The cultivation temperature is 30°C.

Strain *P. aeruginosa* B6643 was cultured on the nutrient medium composition: Meat water – 1 l, NaCl – 5 g, pepton – 10 g, and pH 6.8–7.0. The cultivation temperature is 37°C.

Strain *B. mycoides* EMTC 9 was cultured on the nutrient medium of the composition: Casein hydrolysate – 10 g, yeast extract – 2.5 g, glucose – 5 g, potassium hydrophosphate – 2.5 g, agar-agar – 3 g, and water – 1 l. pH of the medium 7.2–7.6. The cultivation temperature is 30°C.

The strain of *A. faecalis* EMTC 1882 was cultured on the nutrient medium of the composition: Special pepton – 10 g, sodium chloride – 5 g, sodium azide – 0.3 g, chromogenic mixture – 0.06 g, twin 80 – 2 g, sodium hydrophosphate – 1.25 g, agar – 15 g, and water – 1 l. pH of the medium 7.3–7.5. The cultivation temperature is 37°C.

Strain *P. vulgaris* ATCC 63 was cultured on the nutrient medium of the composition: Pepton – 8 g, sodium chloride – 5 g, sodium deoxycholate – 1 g, chromogenic mixture – 1.5 g, propylene glycol – 10.5 g, agar-agar – 15 g, and water – 1 l. pH 7.1–7.5. The cultivation temperature is 37°C.

Strain *S. flexneri* ATCC 12022 cultured on a nutrient medium composition: Peptic digestion of animal tissue – 5 g, meat extract – 5 g, lactose – 10 g, a mixture of bile acids – 8.5 g, sodium citrate – 10 g, sodium thiosulfate – 8.5 g, iron citrate – 1 g, diamond green – 0.00033 g, neutral red – 0.025 g, agar – 15 g, and water – 1 l. pH environment 6.8–7.2. The cultivation temperature is 37°C.

The strain of *L. monocytogenes* ATCC 7644 was cultured on the nutrient medium of the composition: Peptic digestion of animal tissue – 23 g, starch – 1 g, sodium chloride 5 g, D-mannitol – 10 g, iron ammonium citrate – 0.5 g, esculin – 0.8 g, glucose – 0.5 g, lithium chloride – 15 g, phenolic red – 0.08 g, agar – 13 g,

and water – 1 l. pH of the medium 6.8–7.2. The culture temperature is 35°C.

Strain *C. albicans* EMTC 34 was cultured on the nutrient medium composition: Glucose – 20 g, pepton – 10 g, yeast extract – 5 g, and water – 1 l. The temperature of cultivation is 30°C.

Strain *A. flavus* ATCC 9643 was cultured on the nutrient medium of sucrose – 30 g, sodium nitrate – 2 g, potassium hydrophosphate – 1 g, magnesium sulfate – 0.5 g, potassium chloride – 0.5 g, iron sulfate – 0.01 g, agar – 15 g, and water – 1 l. pH of medium 7.1–7.5. The cultivation temperature is 30°C.

Strain of *P. citrinum* ATCC 9849 was cultured on the nutrient medium of dextrose – 40 g, a mixture of peptic digestion of animal tissue and pancreatic casein hydrolyzate (1:1) – 10 g, agar-agar – 15 g, and water – 1 l. pH of the medium 5.4–5.8. the cultivation temperature is 30°C.

Table 1: Morphological properties of isolates from soils, water bodies, and plant objects of the Kemerovo region

Sample, No	Indicator			
	Sporulation	Motility	Form	Gram-stain
Isolate No 1	−	+	Rod-shaped	Gram-negative
Isolate No 2	−	+	Yeast-like fungi	–
Isolate No 3	+	−	Yeast-like fungi	Gram-positive
Isolate No 4	+	+	Rod-shaped	Gram-positive
Isolate No 5	−	−	Yeast-like fungi	–
Isolate No 6	+	−	Spherical	Gram-positive
Isolate No 7	+	+	Rod-shaped	Gram-positive
Isolate No 8	−	−	Spherical	Gram-positive
Isolate No 9	+	+	Spherical	Gram-positive
Isolate No 10	−	−	Spherical	Gram-positive
Isolate No 11	−	−	Spherical	Gram-positive
Isolate No 12	−	−	Rod-shaped	Gram-positive
Isolate No 13	−	−	Rod-shaped	Gram-positive
Isolate No 14	−	+	Rod-shaped	Gram-negative
Isolate No 15	−	+	Rod-shaped	Gram-negative
Isolate No 16	−	−	Spherical	Gram-positive
Isolate No 17	−	+	Rod-shaped	Gram-negative
Isolate No 18	+	−	Rod-shaped	Gram-positive
Isolate No 19	+	+	Rod-shaped	Gram-positive
Isolate No 20	+	+	Rod-shaped	Gram-positive

For work, a suspension of night broth crops grown on standard nutrient media was taken. The number of microorganisms (titer) in the suspension was determined by the OD at a wavelength of 595 nm.

Research Results and Discussion

Isolation of lactic acid bacteria and other antagonistic microorganisms from natural sources: Soil, water bodies, and plant objects

As a result of the research, 20 isolates, different in morphological (Table 1) and phenotypic characteristics, were isolated from soils, reservoirs, and plant objects of the Kemerovo region (Table 2).

Preliminary analysis of phenotypic and morphological characteristics suggests the presence of the following genera in the analyzed samples: *Bacillus*, *Leuconostoc*, *Pediococcus*, *Lactobacillus*, and *Bacteroides*. The results of the identification of microorganisms isolated from natural objects of the Kemerovo region, based on the study of their cultural and morphological properties will be supplemented after genetic analysis, as well as the study of biochemical characteristics and antibiotic resistance.

Study of antimicrobial properties of lactic acid bacteria and other antagonistic microorganisms isolated from natural sources on solid and liquid nutrient media; selection of strains with maximum antimicrobial properties for further research

Table 2: Phenotypic properties of isolates isolated from soils, water bodies, and plant objects of the Kemerovo region

Isolate	Indicator						
	Nature of the edge contour	Profile	Surface	Color	Structure	Consistency	Transparency
Soil of Kemerovo region (Pesherka village)							
Isolate No 1	Even	Flat	Smooth	Light yellow	Homogeneous	Dense	Transparent
Isolate No 2	Even	Waved	Small wrinkled	White	Homogeneous	Dense	Mat
Soil of Prokopyevsky District (Kara-Chumysh village)							
Isolate No 3	Uneven	Convex	Hairy	From white to brownish green	Heterogeneous	Dense	Mat
Isolate No 4	Uneven	Convex	Smooth	Greyish white	Heterogeneous	Viscous	Mat
Rhizosphere of plants of the Mariinsky District (Suslovo village)							
Isolate No 5	Even	Waved	Furrowed	Greyish white	Heterogeneous	Dense	Mat
Rhizosphere of plants of the Yaysky district (Voznesenka village)							
Isolate No 6	Uneven	Waved	Small wrinkled	From white to cream	Homogeneous	Dense	Mat
Isolate No 7	Uneven	Flat	Smooth	Greyish white	Heterogeneous	Viscous	Mat
Isolate No 8	Even	Convex	Smooth	Grey	Homogeneous	Soft	Transparent
Rhizosphere of plants from Guryevsky district (Ursk settlement)							
Isolate No 9	Uneven	Convex	Smooth	White	Homogeneous	Soft	Transparent
Isolate No 10	Even	Convex	Smooth	Orange green	Granular	Dense	Mat
Plant wastes of JSC "Sukhovsky" (Kemerovo)							
Isolate No 11	Even	Convex	Rough	Skin	Homogeneous	Dense	Mat
Isolate No 12	Even	Flat	Smooth	White	Homogeneous	Dense	Mat
Plant waste LLC Niva (Guryevsky district, and Gorskino settlement)							
Isolate No 13	Even	Flat	Smooth	White	Homogeneous	Dense	Mat
Isolate No 14	Even	Convex	Rough	Gray white	Granular	Dense	Translucent
Plant waste of LLC "Veles" (Yaysky district, and Yaya settlement)							
Isolate No 15	Even	Convex	Plicated	Gray white	Homogeneous	Soft	Mat
Isolate No 16	Uneven	Convex	Rough	Skin	Homogeneous	Soft	Mat
Bottom sediments of Lake Urskoye (Guryevskiy district)							
Isolate No 17	Even	Flat	Fine-grained	Yellowish	Homogeneous	Dense	Transparent
Isolate No 18	Even	Flat	Shiny	Cream	Granular	Soft	Mat
Bottom sediments of the Kara-Chumysh reservoir (Prokopyevsky district)							
Isolate No 19	Even	Convex	Shiny	White	Homogeneous	Pasty	Translucent
Bottom sediments of Lake Uday (Mariinsky District)							
Isolate No 20	Even	Flat	Shiny	White	Homogeneous	Soft	Mat

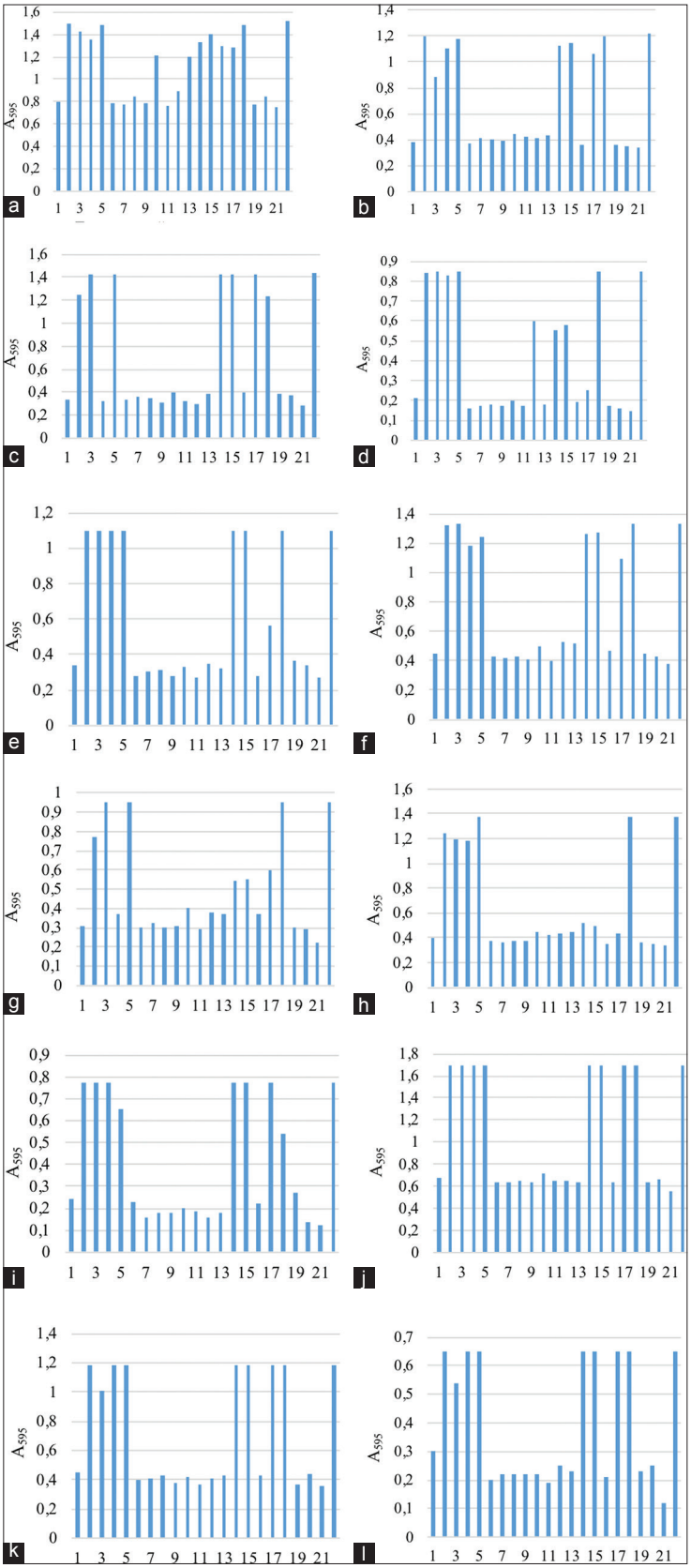

Figure 2: Results of the determination of antimicrobial activity of microorganisms isolated from natural sources of Kemerovo region in liquid nutrient medium: 1–20 – isolates 1–20, 21 – ciprofloxacin, 22 – control. Tested microorganism – (a) Escherichia coli ATCC 25922, (b) Salmonella enterica ATCC 14028, (c) Staphylococcus aureus ATCC 25923, (d) Pseudomonas aeruginosa B6643, (e) Bacillus mycoides ЭМТК 9, (f) Alcaligenes faecalis ЭМТК 1882, (g) Proteus vulgaris ATCC 63, (h) Shigella flexneri ATCC 12022, (i) Listeria monocytogenes ATCC 7644, (j) Candida albicans EMTK 34, (k) A. flavus ATCC 9643, (l) Penicillium citrinum ATCC 9849

Table 3: Results of determination of antimicrobial activity of microorganisms isolated from natural sources of the Kemerovo region by diffusion method (on solid nutrient medium)

Microorganism, isolated from natural sources of Kemerovo region	Diameter of the lysis zone, mm											
	Escherichia coli ATCC 25922$^-$	*Salmonella enterica* ATCC 14028$^-$	*Staphylococcus aureus* ATCC 25923$^+$	*Pseudomonas aeruginosa B6643*$^-$	*Bacillus mycoides* ЭМТК 9$^+$	*Alcaligenes faecalis* ЭМТК 1882$^-$	*Proteus vulgaris* ATCC 63$^-$	*Shigella flexneri* ATCC 12022$^-$	*Listeria monocytogenes* ATCC 7644$^+$	*Candida albicans* ЭМТК 34	*Aspergillus flavus* ATCC 9643	*Penicillium citrinum* ATCC 9849
Control	0	0	0	0	0	0	0	0	0	0	0	0
Ciprofloxacin (C)	21	24	19	22	25	23	20	22	24	18	25	21
Isolate 1	18	20	17	20	22	20	17	18	19	15	21	15
Isolate 2	0	0	5	0	0	0	7	4	0	0	0	0
Isolate 3	6	10	0	0	0	0	0	8	0	0	4	5
Isolate 4	12	5	18	0	0	17	15	10	0	0	0	0
Isolate 5	0	0	0	0	0	6	0	0	4	0	0	0
Isolate 6	20	22	17	21	24	21	18	20	21	16	23	19
Isolate 7	19	18	15	19	22	21	17	20	23	16	22	18
Isolate 8	17	20	16	19	22	20	18	19	21	15	21	18
Isolate 9	20	21	17	19	23	21	18	19	21	16	24	18
Isolate 10	15	18	14	17	20	17	15	16	20	13	20	18
Isolate 11	21	20	17	19	24	22	18	18	21	16	24	19
Isolate 12	18	19	15	10	20	17	15	17	22	16	22	17
Isolate 13	15	18	14	19	21	17	15	16	21	15	20	18
Isolate 14	12	10	0	14	0	9	11	13	0	0	0	0
Isolate 15	7	11	0	12	0	7	10	15	0	0	0	0
Isolate 16	17	22	16	20	23	19	18	21	20	16	21	18
Isolate 17	15	13	0	16	0	12	14	17	0	0	0	0
Isolate 18	0	0	11	0	14	0	0	0	10	0	0	0
Isolate 19	20	22	18	19	22	20	18	21	19	16	24	18
Isolate 20	18	23	17	20	21	22	19	21	23	15	20	17

For work, a suspension of night broth crops grown on standard nutrient media was taken. The OD at a wavelength of 595 nm determined the number of microorganisms (titer) in the suspension. To assess the antibacterial effect of metabolites of microorganisms isolated from natural sources of the Kemerovo region, a joint incubation of test strains with the studied metabolites was carried out in 96-well plates for cultivation.

An analysis of the data presented in Table 3 and Figure 2 led to the following conclusions. From 20 tested microorganisms, 12 isolates (Isolate 1, Isolate 6, Isolate 7, Isolate 8, Isolate 9, Isolate 10, Isolate 11, Isolate 12, Isolate 13, Isolate 16, Isolate 19, and Isolate 20) show high antimicrobial activity against all test strains pathogenic and conditionally pathogenic microorganisms. For strains Isolate 14, Isolate 15, and Isolate 17, there was a slight antimicrobial activity against Gram-negative test strains, while Isolate 18 has an inhibitory effect only on Gram-positive species. Isolate 2 has a slight antimicrobial effect on *S. aureus*, *Proteus vulgaris*, *S. flexneri*; Isolate 3 – test strains *E. coli*, *S. enterica*, *S. flexneri*, *A. flavus*, and *P. citrinum*; and Isolate 5 – test strains *Alcaligenes faecalis* and *L. monocytogenes*.

Thus, for further studies, the following isolates showing maximum antimicrobial activity against pathogenic and conditionally pathogenic test strains were selected: Isolate 1, Isolate 6, Isolate 7, Isolate 8, Isolate 9, Isolate 10, Isolate 11, Isolate 12, Isolate 13, Isolate 16, Isolate 19, and Isolate 20.

Conclusion

The following results are obtained:

- Lactic acid bacteria and other microorganisms-antagonists from natural sources were isolated: Soil, water bodies, and plant objects; 20 isolates were isolated, their cultural and morphological properties were studied; isolated microorganisms were found to belong presumably to the genera *Bacillus*, *Leuconostoc*, *Pedio-coccus*, *Lactobacillus*, and Bacteroides;
- Antimicrobial properties of lactic acid bacteria and other antagonistic microorganisms isolated from natural sources on solid and liquid nutrient media were studied;
- 12 strains of 20 isolates with maximum antimicrobial properties were selected for further studies.

Further research on the biochemical properties of lactic acid bacteria and other antagonist microorganisms isolated from natural sources, the study of antibiotic resistance of lactic acid bacteria and other antagonist microorganisms isolated from natural sources, as well as

other more detailed studies will be conducted with selected 12 strains with maximum antimicrobial properties.

Acknowledgments

Financial support for the research was provided by the Ministry of Education and Science of the Russian Federation as part of the implementation of the Federal Target Program “Research and Development in Priority Areas of the Scientific and Technological Complex of Russia for 2014–2020”, the grant agreement No. 075-15-2019-1383 dated June 18, 2019 (unique project identifier RFMEFI57418X0207).

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