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ANTIMICROBIAL ACTIVITY OF BIFIDOBACTERIAL BACTERIOCIN-LIKE SUBSTANCES

Antagonistic activity of 13 bifidobacterial strains, isolated from humans, has been studied. It was shown that specific antagonistic activity in bifidobacteria is a strain characteristic and does not depend on species of these microorganisms. It was determined that bifidobacteria are able to produce bacteriocin-like substances against both gram-positive and gram-negative bacteria. Strains Bifidobacterium sp. 278 and B. bifidum 174 produced antimicrobial substances of wide spectrum of activity and manifested the highest antagonistic activity as compared to the rest of bifidobacterial strains studied. The maximal activity of bacteriocin production by strains B. bifidum 174 ma Bifidobacterium sp. 278 occurs between 8-16 hours of cultivation that is in the late logarithmic phase of growth. According to obtained characteristics the antimicrobial substances are complex peptides and belong to the 4th class of bacteriocins.

K e y w o r d s: bifidobacteria, antimicrobial activity, bacteriocins.

One of the basic properties of bifidobacteria, which determines their prolonged persistence in the human intestine, is their antagonistic activity [5; 14]. It was shown that bifidobacteria are able to produce specific antimicrobial substances of protein nature – bacteriocins, which inhibit the growth of other microorganims [2].

Nowadays bacteriocins of lactic acid bacteria are the most studied ones. According to modern classification offered in 1993 by Klaenhammer et al., bacteriocins of lactic acid bacteria are devided into the following classes: 1) membrane-active thermostable peptides, including lantibiotics; 2) non-lantibiotics; 3) thermostable peptides of high molecular weight; 4) complex peptides, including lipo- and glycoproteins [11].

On the other hand, there is no much information concerning bacteriocins produced by bifidobacteria. Anand et al. [3] described bifidin from *Bifidobacterium bifidum*, Kang et al. [10] informed about bifilong produced by *B. longum*. According to Abd El-Salam et al., strains *B. lactis* Bb-12 and *B. longum* Bb-46 produced bacteriocins bifilact Bb-12 and bifilong Bb-46, correspondingly [1]. There are data about bacteriocin production by bifidobacterial species *B. adolescentis, B. breve, B. thermacidophilum, B. animalis, B. infantis* [4, 6, 9, 13, 15]. In 1998 Yildirim and Johnson [16] described bifidocin B isolated from *B. bifidum* NCFB 1454, which is able to inhibit the growth of a wide spectrum of opportunistic microorganisms. A purified fraction of bacteriocin B consists of polypeptide chain of 36 aminoacid residues of molecular weight 4432.9 Da [17]. Nowadays bifidocin B is a unique bifidobacterial bacteriocin which molecular structure has been determined.

Studies for the ability of bifidobacteria to synthesize antimicrobial peptides, as well as research of their nature, are of great scientific interest. These compounds have a wide perspective of use both in medicine and food industry.

The aim of the study – screening of bifidobacterial strains which manifested specific antimicrobial activity against a wide spectrum of opportunistic microorganisms and characterization of the antimicrobial compounds of the most perspective strains.

Materials and Methods. The objects of the study were 13 bifidobacterial strains isolated from the intestinal tract of children (aged 1.5-2) and elderly people (aged 63-84) of both sexes (Table 1). Antimicrobial activity (AMA) of the bifidobacterial strains was determined by microtiter plate (MTP) method [8] with slight modifications. Cell-free supernatants (CFSs) were obtained from 24 h old cultures of bifidobacteria grown on MRSC broth (MRS medium supplied with 0.05% of cysteine hydrochloride) by centrifugation at 14 000 g for 15 min at 4 °C and filter sterilized (filters VWR international, USA, pore diameter 0.22 μm). After that 80 μl of CFSs (neutralized with 5M NaOH) were added into microtiter plate (TPP, Switzerland). For *C. difficile* inhibition study MTP with CFSs was preincubated overnight at anaerobic conditions at 37 °C.

Table 1

Bifidobacterial strains used in the study

Species	Strain	Source (sample)*
Bifidobacterium bifidum	102	I 5
B. bifidum	152	I 8
B. bifidum	174	I 8
B. bifidum	272	I 15
B. bifidum	34s	S 36
B. bifidum	35s	S 36
B. longum	139	I 7
B. longum	142	I 7
B. longum	276	I 15
B. dentium	47s	S 37
Bifidobacterium sp. **	271	I 15
Bifidobacterium sp. **	278	I 15
Bifidobacterium sp. **	82D	D 33

* I – infants, S – seniors, D – seniors more than 80 years old.** - species was not determined

Antimicrobial activity was determined against 16 opportunistic microorganisms and 10 strains of bifidobacteria (Table 2). Indicator strains were grown on the appropriate medium during 18-24 hours at 37 °C , washed twice in sterile phosphate buffer saline (PBS, pH 7.2) and resuspended in PBS. The suspension of test-microorganism with optical density $A_{620\ nm}$ = 0.5 was prepared in the appropriate medium (5 times concentrated), after that 20 µl were added into the well of MTP. After 24-48 hours of incubation the intensity of the test-strain growth was determined using the microplate absorbance reader "BIO-RAD iMark" at a wavelength of 620 nm. Test-cultures grown in the appropriate medium without filtrate of cultural supernatant of bifidobacteria were used as a positive control. Test-cultures grown in MRSC were used as a negative control.

Table 2

Indicator strains used in the study

Strain	Medium*	Incubation conditions
Bacillus cereus LU 1	MH	Aerobic
Clostridium difficile 027	BHI	Anaerobic
C. difficile 2167	BHI	Anaerobic
Streptococcus sp. group A 036	BMH	Aerobic
Streptococcus sp. group A 037	BMH	Aerobic
Streptococcus sp. group B 038	BMH	Aerobic
Staphylococcus aureus LU 2	BMH	Aerobic
Enterococcus sp. LU 3	BMH	Aerobic
Pneumococcus sp. LU 4	BMH	Aerobic
Salmonella typhimurium LU 5	MH	Aerobic
Escherichia coli LU 6	MH	Aerobic
Citrobacter sp. LU 7	MH	Aerobic
Serratia sp. LU 8	MH	Aerobic
Stenotrophomonas sp. LU 9	MH	Aerobic
Pseudomonas aeruginosa LU 10	MH	Aerobic
Klebsiella pneumoniae LU 11	MH	Aerobic
B. bifidum 102	MRSC	Anaerobic
B. longum 139	MRSC	Anaerobic
B. longum 142	MRSC	Anaerobic
B. bifidum 152	MRSC	Anaerobic
Bifidobacterium sp. 271	MRSC	Anaerobic
B. longum 276	MRSC	Anaerobic
B. bifidum 34s	MRSC	Anaerobic
B. bifidum 35s	MRSC	Anaerobic
B. dentium 47s	MRSC	Anaerobic
Bifidobacterium sp. 50s	MRSC	Anaerobic

* MH - Muller Hinton agar, BHI – brain heart infusion , BMH - Muller Hinton agar with 5% sheep blood.

AMA was expressed by the formula:

AMA (%) = 100 - ($OD_{620\ nm}$ of test-stain culture with CFS/ $OD_{620\ nm}$ of test-stain culture in control*100%), where $OD_{620\ nm}$ – optical density at wave length 620 nm.

Production of specific antimicrobial substances at different cultural growth phases were determined by the method of *Cheikhyoussef* et al. [7] with slight modifications. The night culture of bifidobacteria in the amount of 1% (v/v) was inoculated into 200 ml of MRSC and incubated at 37 °C during 24 h. The culture samples (2ml) were picked out every 2 hours, the growth of the cells was measured by the suspension turbidity (optical density, OD) at a wavelength of 620 nm. Antimicrobial activity was determined by the method described above.

The effect of enzymes, thermal treatment and different pH rate on stability of specific antimicrobial substances of bifidobacteria was estimated by the method of *Cheikhyoussef* et al. [7]. Cell-free supernatant of bifidobacteria was treated with different enzymes (proteinase K, protease X, pepsin, tripsin, lipase A, alpha-amylase; all from Sigma, USA).

Stability of specific antimicrobial substances of bifidobacteria at thermal treatment was determined by measuring their antimicrobial activity after heating 2 ml of the sample at 80 °C during 15, 30 and 60 min, at 100°C – during 15 and 20 min, and after autoclaving at 121°C – during 10 and 20 min.

Their stability at different pH level was determined by incubation of the samples at pH from 3 to 11 (using 5M NaOH and 5M HCl) during 2 h at 25 °C. Native samples of cell-free neutralized supernatants were used as a control. Antimicrobial activity of the experimental and control samples was determined as described above.

The extraction of bacteriocin-like substances from cell-free supernatant was conducted using ammonium sulfate precipitation (85% saturation) at 4 °C during 12 h. The precipitate was dialyzed using a dialysis membrane with pore diameter 1000 Da (Spectrum©Laboratories, Inc.) in PBS pH 6.0 during 48 h at 4 °C. Antimicrobial activity of dialysates was determined as described above.

Determination of the indicator strain biomass accumulation in the presence of specific antimicrobial substances of bifidobacteria. Protein dialyzate of supernatant of bifidobacterial culture was added into the cultivation medium of the indicator strain (10% of total volume), incubated at 37 °C 24 h. Samples were selected every 3 h. Optical density of the indicator strain suspension was measured at the wavelength of 620 nm. The indicator strain without the strain-antagonist supernatant was used as a positive control.

Results and Discussion. As a result of research the neutralized supernatant of bifidobacteria showed antimicrobial activity. The results are presented in Tables 3 and 4. Bifidobacterial strains did not inhibit the growth of all pathogens, and this antagonistic activity was strain-dependent. All bifidobacteria inhibited the growth of *S. aureus*, *Citrobacter* sp., *S. typhimurium* and *E. coli*, but did not inhibit that of *P. aeruginosa* and *K. pneumoniae*. Only strain *Bifidobacterium* sp. 278 suppressed effectively the growth of *Pneumococcus* sp. and *C. difficile*. The studied strains of bifidobacteria did not inhibit the growth of test-strains of bifidobacteria (data are not shown). The results are in agreement with the data obtained by Anand [3] and Kang [10], who investigated specific antimicrobial substances which suppressed the growth of both gram-positive and gram-negative microorganisms.

An analysis of literature data shows that there are only a few data about the fact that bacteriocin-like substances of gram-positive bacteria exhibit antimicrobial activity against gram-negative bacteria [4]. Therefore, the ability to inhibit the growth of gram-negative bacteria, especially those which cause spoilage of foods and nutritional diseases, offers the prospect of applying bacteriocins of bifidobacteria as preservatives in the food industry.

We showed that bifidobacteria strains studied demonstrated specific antimicrobial activity, which depends on bifidobacterium strain as well as on the indicator culture. The strains *Bifidobacterium* sp. 82D, *Bifidobacterium* sp. 278, *B. bifidum 272* and *B. bifidum* 174 suppressed the growth of a wide range of pathogens and were selected for further research.

It is known [7] that the microorganism can produce simultaneously several different bacteriocin-like substances specific for different sensitive microorganisms. In addition, it may produce different bacteriocin-like substances at different stages of the culture growth. Therefore, we have used different types of pathogens in the investigation of production of specific antimicrobial substances. The given results are presented in Fig. 1 (a) and 1 (b).

Table 3

Growth inhibition of gram-positive opportunistic microorganisms by cell-free neutralized supernatants of bifidobacteria

Bifido-bacterial strains	Antimicrobial activity, %								
	B. cereus	*Enterococcus sp.*	*Streptococcus sp. GA 036*	*Streptococcus sp. GA*	*Streptococcus sp. GB*	*Pneumococcus sp.*	*S. aureus*	*C. difficile 027*	*C. difficile 2167*
102	-1.94±14.08	-4.91±7.74	-0.88±3.06	8.33±5,14	11.79±6.26	-23.35±1.88	21.00±2.71	-102.34±12.38	-273.41±30.26
152	-29.83±7.02	7.22±3.95	-17.55±4.33	25.33±5.20	25.91±8.24	-18.50±25.32	22.14±4,54	-146.72±12.68	-429.81±31.10
174	-1.94±6.20	16.64±7.08	7.55±4.21	31.23±6.13	51.24±5.23	-13.66±3.88	35.70±4.02	-95.60±17.15	-248.48±33.70
272	-10.67±9.33	24.60±4.38	60.20±1.58	30.44±6.05	50.02±3.50	-65.64±68.57	24.07±3.91	-134.65±37.99	-150.24±32.66
34s	-18.28±4.95	13.78±7.32	6.67±2.80	28.15±9.83	45.48±2.39	-13.22±3.62	32.58±1.03	-169.08±15.31	-192.77±15.65
35s	11.33±17.74	1.58±2.43	-2.25±4.55	6.10±9.06	9.00±12.45	-13.66±2.11	28.53±1.50	-176.18±22.08	-224.05±28.94
139	-14.67±5.56	-9.39±4.41	-3.04±3.85	14.93±2.31	24.02±8.64	-17.18±3.88	42.55±3.82	-131.81±20.21	-116.03±24.62
142	-21.17±10.73	22.28±4.57	4.41±3.29	11.98±7.28	24.29±3.87	-10.13±0.94	34.28±3.24	-130.74±21.43	-357.97±10.02
276	-6.94±8.14	-0.82±1.10	21.57±4.24	7.36±6.60	45.30±5.46	-0.88±2.79	43.34±2.25	-153.46±22.32	-193.26±42.03
47s	-17.22±10.41	14.48±7.30	3.43±2.50	19.12±8.30	37.47±1.84	-351.10±368.30	27.38±1.94	-188.96±23.03	-265.59±49.70
271	-37.61±8.93	7.60±3.56	19.80±2.63	14.14±4.91	9.45±5.63	-8.81±0.82	39.64±3.88	-100.57±9.35	-138.03±9.67
278	-1.72±15.28	24.98±4.23	11.86±5.03	29.69±8.40	45.84±5.10	-103.52±130.04	51.58±3.11	55.63±8.54	50.15±5.72
82D	-0.67±11.18	1471±2.59	85.39±0.69	20.04±5.94	22.72±7.04	34.80±2.31	35.93±5.86	-112.28±23.82	-215.74±26.57

$p \leq 0.05$

Footnote: negative values of antimicrobial activity were received in cases of stimulation of growth of test-strains by cell-free neutralized supernatants of bifidobacteria

Table4

Growth inhibition of gram-negative opportunistic microorganisms by cell-free neutralized supernatants of bifidobacteria

Bifido-bacterial strains	Antimicrobial activity, %						
	Serratia sp.	*Stenotrophomonas sp.*	*P. aeruginosa*	*K. pneumoniae*	*Citrobacter sp.*	*S. typhimurium*	*E. coli*
102	-9.05±2.62	29.69±25.80	-37.75±9.26	-4.03±2.37	29.26±2.00	27.74±6.02	27.38±3.14
152	-28.43±1.63	-8.10±9.21	-67.07±14.62	-4.28±5.73	27.17±0.24	26.15±6.73	36.48±6.99
174	-17.01±4.90	7.74±8.43	-76.59±18.96	-3.17±7.06	30.30±1.41	30.93±0.81	49.34±8.53
272	-16.54±2.08	45.75±10.92	-102.18±16.68	-3.24±5.05	29.26±1.39	34.19±5.46	44.71±4.31
34s	18.88±20.35	24.20±9.23	-79.07±7.23	-2.45±4.88	27.87±1.32	33.32±3.79	43.63±8.19
35s	-19.72±2.66	7.82±18.83	-103.11±17.14	-7.49±2.75	26.94±1.17	38.89±3.24	24.09±6.63
139	-21.70±8.39	31.30±10.40	-92.67±10.69	-6.08±4.22	30.01±1.35	33.56±1.89	30.41±3.12
142	-19.33±1.94	15.56±16.23	-80.24±14.66	-2.56±4.85	29.95±2.37	34.99±2.37	31.82±3.15
276	3.77±22.72	27.87±8.39	-72.74±21.72	-6.80±3.44	29.78±1.15	33.88±1.09	30.09±1.02
47s	14.05±15.64	-22.45±13.68	-74.49±15.03	-22.35±8.39	23.76±3.77	23.28±1.11	27.78±3.28
271	-22.17±5.14	39.19±11.55	-86.65±33.96	-5.08±4.33	31.17±1.30	36.98±4.00	27.60±3.17
278	-18.40±1.44	10.96±10.39	-107.00±3.67	-4.46±8.96	24.22±3.90	29.57±8.52	32.07±2.03
82D	-5.88±18.40	-2.36±11.42	-60.12±1.75	-2.99±5.34	31.98±4.35	36.42±1,87	50.67±2.87

$p \leq 0.05$

Footnote: negative values of antimicrobial activity were received in cases of stimulation of growth of test-strains by cell-free neutralized supernatants of bifidobacteria

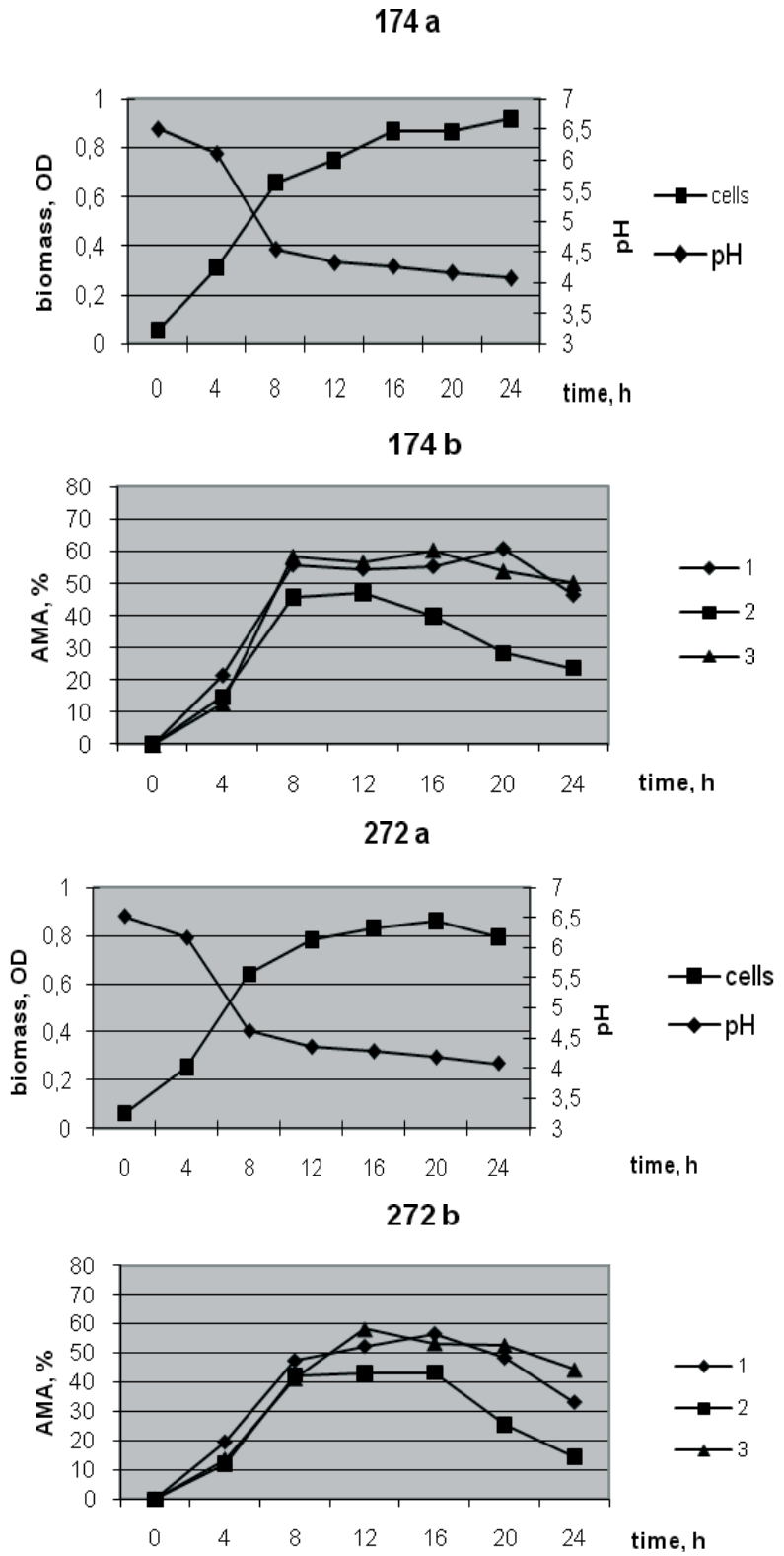

Fig. 1(a). Production of bacteriocin-like substances by bifidobacteria depending on their growth phase (a – biomass accumulation, b – antimicrobial activity). 1- *Streptococcus* sp., 2- *Stenotrophomonas* sp., 3 - *S. typhimurium*.

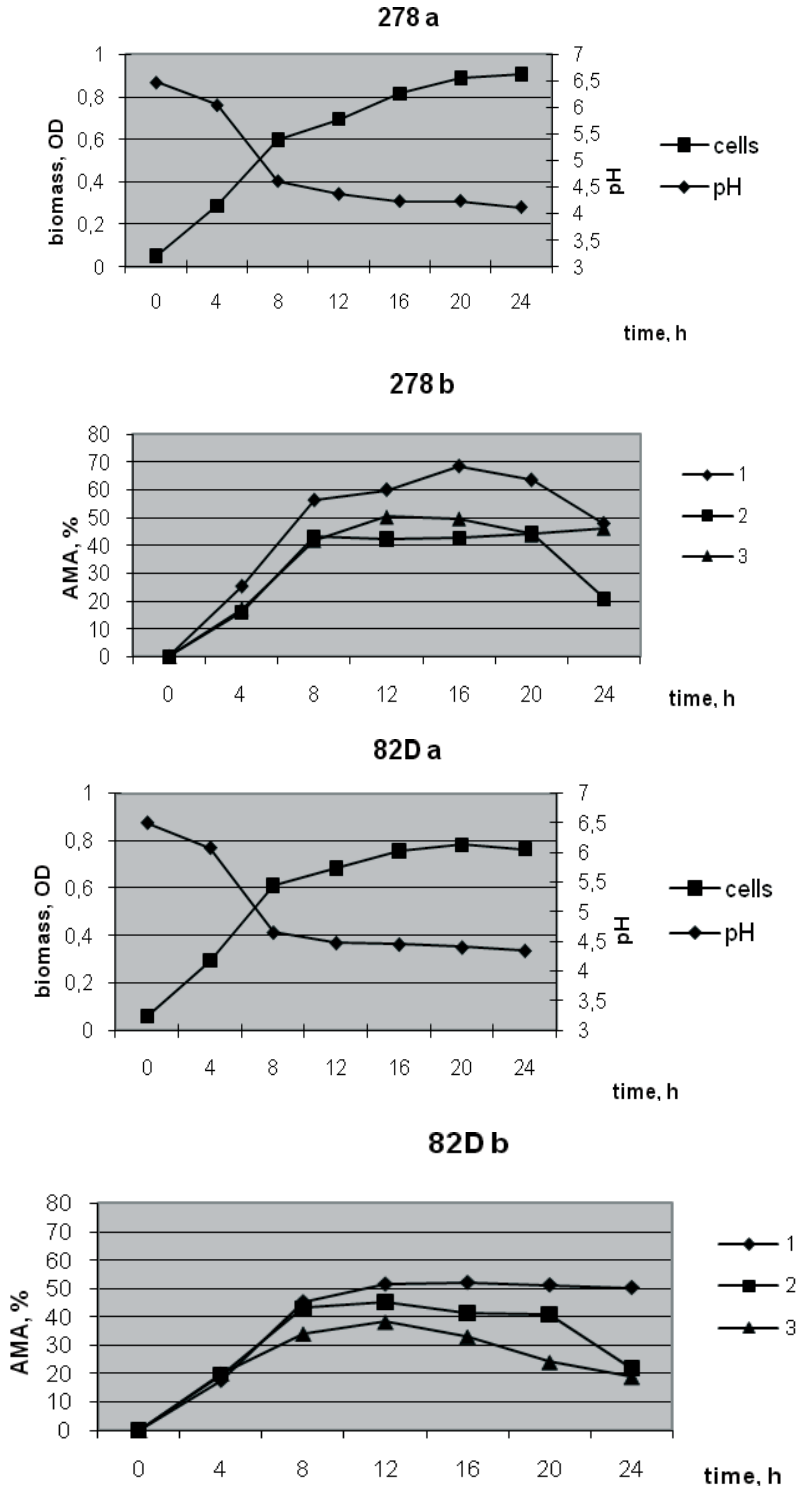

Fig. 1(b) Production of bacteriocin-like substances by bifidobacteria depending on their growth phase (a – biomass accumulation, b – antimicrobial activity). 1- *Streptococcus* sp., 2- *Stenotrophomonas* sp., 3 - *S. typhimurium*.

It has been found that the production activity of specific antimicrobial agents depends on the growth phase of the culture of bifidobacteria. Maximum production activity was observed at 12-16 hours of the culture growth and the highest biomass accumulation was observed after 18 hours of cultivation. Thus, the maximum production of bacteriocin substances of bifidobacteria occurs

in the late logarithmic growth phase, confirming the data of other authors obtained for lactic acid bacteria and bifidobacteria [7;12;18].

On the other hand, the duration of production of antimicrobial substances by the studied cultures differed depending on the strain of bifidobacteria and the indicator culture. Thus, a decrease in production of bacteriocins by strains *B.bifidum* 174 and *B.bifidum* 272 was observed after 16 hours of the cultivation. The strain *Bifidobacterium* sp. 278 exhibited the decrease in production of bacteriocin(s) only against *Streptococcus* sp. and *S. typhimurium*, and strains of *Bifidobacterium* sp. 82D - against *Streptococcus* sp. and *Stenotrophomonas* sp. The strain *B. bifidum* 278 kept producing of antimicrobial substance against *Stenotrophomonas* sp. up to 24 hours of cultivation, and *Bifidobacterium* sp. 82D - against *Streptococcus* sp. up to 20 hours of cultivation.

For further work we have selected the strains *B. bifidum* 174 and *Bifidobacterium* sp. 278 as a model because of the fact that they showed the highest specific antimicrobial activity in the previous experiment. We used *Streptococcus* sp. and *S. typhimurium* as the indicator cultures since they are the most sensitive test microorganisms.

As a result of studying the effect of enzymes on cell-free supernatants of bifidobacteria it has been shown that inactivation of specific antimicrobial activity was observed after their treatment with proteinase K, protease X, pepsin and trypsin. In addition, the influence of lipase and alpha-amylase on the expression of antimicrobial action by neutralized supernatants of the studied strains has been observed. Cell-free neutralized supernatant of the strains *B.bifidum* 174 and *Bifidobacterium* sp. 278 retained their activity against test strains after heat treatment: they were stable in a wide range of high temperatures - from heating at 80 °C and autoclaving for 20 min at 121 °C. Cell-free neutralized supernatants retained their activity in the pH range from 4 to 10.

The given results indicate the complex nature of the bacteriocin-like substances of the studied strains of bifidobacteria. These substances are complex peptides with lipid and carbohydrate components. Furthermore, because of the fact that in the study of nature of bacteriocin-like substances the same strain of bifidobacteria manifested different effects, depending on the indicator culture, we can assume the presence of several antimicrobial substances in the strains *B. bifidum* 174 and *Bifidobacterium* sp. 278.

As a result of saturation of bifidobacterial supernatants with ammonium sulfate and their subsequent dialysis we received their protein dialysates. These native dialysates of bifidobacteria did not exhibit any antagonistic activity against opportunistic microorganisms. Their 10, 100 and 500-fold dilution allowed to inhibit the indicator strains; the dilution 1:10 (AMA 46.44-65.62%) manifested the highest antimicrobial activity and dilution 1:500 (AMA 11.96 – 45.60%) – the lowest one (Fig. 2). The absence of antimicrobial activity in the native protein dialysates of bifidobacteria can be explained by the fact that in such concentrated solution bacteriocins are bound and inactive.

The addition of dialysates of cell-free supernatants of *B. bifidum* 174 and *Bifidobacterium* sp. 278 into the culture medium of *Streptococcus* sp. and *S. typhimurium* led to a decrease in biomass accumulation of the indicator strains (Fig. 3). The inhibition of *Streptococcus* sp. growth was about 74% in the presence of dialysate *B. bifidum* 174 and 66% in the presence of *Bifidobacterium* sp. 278. The inhibition of *S. typhimurium* growth was about 66% in the presence of dialysate *B. bifidum* 174 and 62% in the presence of *Bifidobacterium* sp. 278, which corresponds to the results obtained in the previous study.

In conclusion, the study has shown that the presence of specific antimicrobial activity in bifidobacteria is strain-dependent characteristic and does not depend on the species of test-microorganisms as well. Bifidobacteria are able to produce bacteriocin-like substances against a wide range of gram-positive and gram-negative microorganisms.

The strains *B. bifidum* 174 and *Bifidobacterium* sp. 278 produce antimicrobial substances with broad-spectrum of activity and exhibit higher antimicrobial activity as compared to other investigated strains of bifidobacteria. Maximum bacteriocin-producing activity of the strains *B. bifidum* 174 and *Bifidobacterium* sp. 278 occurs between 8-16 h of cultivation, namely in the late logarithmic growth phase. According to given data the antimicrobial substances belong to Class 4 bacteriocins and are complex peptides (according to klaenhammer et al. [11]).

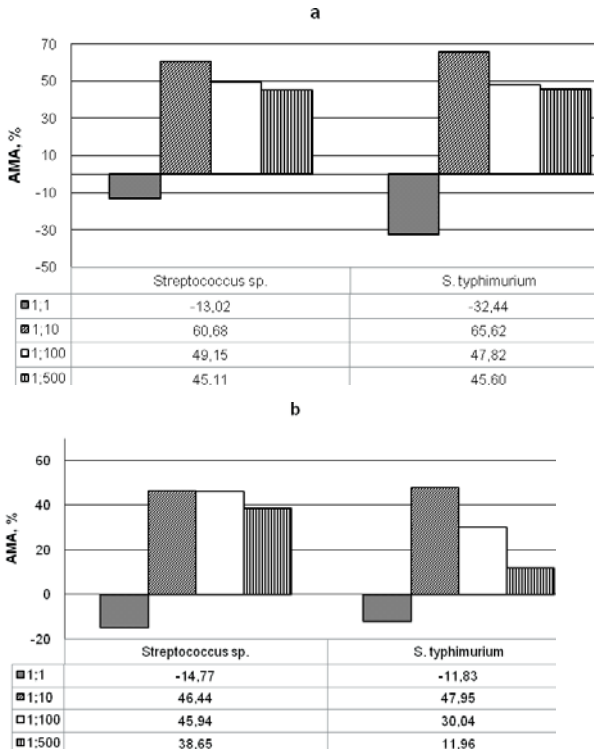

Fig. 2 Antimicrobial activity of dialysates of cell-free neutralized supernatants of bifidobacteria (a – *B. bifidum* 174, b – *Bifidobacterium* sp. *278*). $p \leq 0.05$

Footnote: negative values of antimicrobial activity were received in cases of stimulation of growth of test-strains by cell-free neutralized supernatants of bifidobacteria

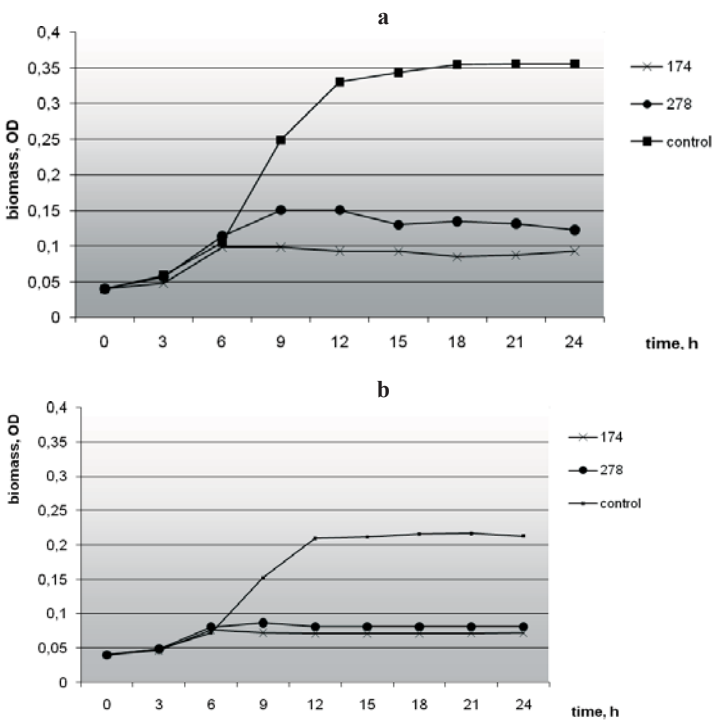

Fig. 3. Dynamics of microbial biomass accumulation of *Streptococcus* sp. (a) and *S. typhimurium* (b)

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АНТИМІКРОБНА АКТИВНІСТЬ БАКТЕРІОЦИНОПОДІБНИХ РЕЧОВИН БІФІДОБАКТЕРІЙ

Р е з ю м е

Досліджено антагоністичну активність 13 штамів біфідобактерій, ізольованих від людини. Показано, що наявність специфічної антимікробної активності у біфідобактерій є штамовою характеристикою і не залежить від виду цих мікроорганізмів. Встановлено, що біфідобактерії продукують бактеріоциноподібні речовини як проти грампозитивних, так і проти грамнегативних бактерій. Штами *Bifidobacterium* sp. 278 та *B. bifidum* 174 продукують антимікробні речовини широкого спектру дії і проявляють найвищу антимікробну активність, порівняно з іншими дослідженими штамами біфідобактерій. Максимальна активність продукування бактеріоцинів штамами *B. bifidum* 174 та *Bifidobacterium* sp. 278 відбувається між 8–16 год культивування, а саме у пізній логарифмічній фазі росту. За отриманими характеристиками дані антимікробні речовини відносяться до 4 класу бактеріоцинів і являють собою складні пептиди.

К л ю ч о в і с л о в а: біфідобактерії, антимікробна активність, бактеріоцини.

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АНТИМИКРОБНАЯ АКТИВНОСТЬ БАКТЕРИОЦИНОПОДОБНЫХ ВЕЩЕСТВ БИФИДОБАКТЕРИЙ

Р е з ю м е

Исследована антагонистическая активность 13 штаммов бифидобактерий, изолированных от человека. Показано, что наличие специфической антимикробной активности у бифидобактерий является штаммовой характеристикой и не зависит от вида этих микроорганизмов. Установлено, что бифидобактерии продуцируют бактериоциноподобные вещества как против грамположительных, так и против грамотрицательных бактерий. Штаммы *Bifidobacterium* sp. 278 и *B. bifidum* 174 продуцируют антимикробные вещества широкого спектра действия и проявляют наивысшую антимикробную активность по сравнению с другими исследованными штаммами бифидобактерий. Максимальная активность продуцирования бактериоцинов штаммами *B. bifidum* 174 и *Bifidobacterium* sp. 278 происходит между 8–16 ч культивирования, а именно в поздней логарифмической фазе роста. По полученным характеристикам данные антимикробные вещества относятся к 4 классу бактериоцинов и представляют собой сложные пептиды.

К л ю ч е в ы е с л о в а: бифидобактерии, антимикробная активность, бактериоцины.

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