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e-mail: jamborkoann@ukr.net**BIOLOGICAL ACTIVITY OF LACTOBACILLI
FROM DIFFERENT ECOLOGICAL NICHES OF THE
SOUTHERN REGION OF UKRAINE**

***Aim** of the research was to study the antagonistic activity of lactobacilli isolated from different sources of the Southern region of Ukraine. **Methods.** The experiments used 13 strains of bacteria of the genus *Lactobacillus* isolated from different sources. The antagonistic activity of lactobacilli was determined by the well-diffusion method in the agar column using test cultures of pro- and eukaryotic microorganisms. Acid formation of the strains was evaluated by active and titrated acidity when cultured in milk. **Results.** All tested lactobacilli were active antagonists against gram-negative bacteria *Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enteritidis* and *Proteus vulgaris*, as well against gram-positive *Staphylococcus aureus* and *Staphylococcus saprophyticus*. Lactobacilli showed less antagonistic activity against *Bacillus subtilis* and *Klebsiella pneumoniae*. Lactobacilli isolated from meat (*Lactobacillus* spp. M2 and M3) were the most active antagonists. The active and titrated acidity of lactobacilli varied for different strains. Strain *Lactobacillus* sp. 175 isolated from child feces showed the highest titrated acidity. Using statistical methods (cluster analysis) made it possible to form clusters with high reliability by the level of antagonistic activity of investigated lactobacilli. The environmental factors have a definite influence on the formation of the general activity of lactobacilli strains, but the manifestation of each of the individual features is rather specific and depends on the capabilities of the certain microorganism. **Conclusions.** In the study of a number of properties that determine the antagonistic activity, five strains (*Lactobacillus* spp. O1, B4, 175, M2 and M3) have been selected. They can be recommended for further researches for creation of probiotic preparation and functional foods.*

Key words: lactobacilli, antagonistic activity, acid-producing activity, clustering.

The total number of lactic acid bacteria in the biosphere is steadily increasing. They inhabit various natural substrates and can build symbiotic relationships in the digestive tract of worms, insects, fish, domestic and wild birds, mammals and humans. In recent years, there is a large number of scientific works devoted to the study of biological properties, taxonomy, classification and identification of lactic acid bacteria isolated from various ecological niches [13, 17, 18]. Today the search for new probiotic strains is still on going despite the widespread popularity of prophylactic and therapeutic probiotics [16]. The effectiveness of probiotic above all depends on the properties of the bacterial strains. The list of probiotic



microorganisms that can positively affect various aspects of the macroorganism's functional state is quite numerous. Among them representatives of the genera *Lactobacillus* and *Bifidobacterium* are the most studied [6, 14], as well as the certain strains of some *Streptococcus*, *Lactococcus*, *Enterococcus*, *Bacillus* and *Saccharomyces* [12]. Since lactobacilli are one of the main microorganisms of the human body, study of their biological properties, and the antagonistic activity against pathogens and opportunistic pathogens, is one of the main criteria for their selection for the creation of probiotics.

Today at the modern market there is a large number of domestic and imported products based on lactobacilli for the correction of human microbiota. However, it is well known that use of microorganisms from specific ecological region for the maintenance of a normal level of metabolic processes and immunological reactivity of a macroorganism is required. Therefore, the selection of biologically active lactobacilli strains from ecological niches of the southern region of Ukraine is necessary for the creation of effective biological products and biological preparations of functional nutrition for the inhabitants of this ecological and geographical zone. The aim of our work was to study the antagonistic properties of *Lactobacillus* strains isolated from different sources.

Material and methods

Bacterial strains

Totally 13 strains of lactobacilli isolated from different sources were used in experiments: from auto-fermenting vegetables (*Lactobacillus sp.* B1, B3, B4, B5, B6, O1); from raw meat material (*Lactobacillus sp.* M1, M2, M3, M6); from feces of healthy children (*Lactobacillus sp.* 146, 275, 175).

Antagonistic activity

The antagonistic activity of lactobacilli was determined by the agar well diffusion method [4]. Yeast and bacteria strains were used as the test cultures: *Candida albicans* ONU 415, *C. utilis* ONU 413, *Escherichia coli* ONU 90, *Bacillus subtilis* ONU 24, *Pseudomonas aeruginosa* ONU 211, *Staphylococcus aureus* ONU 223, *Staphylococcus saprophyticus* ONU 537 M2, *Salmonella enteritidis* ONU 466, *Klebsiella pneumoniae* ONU 463 and *Proteus vulgaris* ONU 92. Test cultures were obtained from the Collection of microorganisms of the Department of Microbiology, Virology and Biotechnology of Odesa I.I. Mechnykov National University.

Acid-producing activity

Acid production of the strains was evaluated as active and titratable acidity during cultivation in sterile skimmed milk [5]. The ability of the bacterial cultures to ferment milk and form a clot was studied. Acidity of milk was determined by the titrimetric method and expressed in Turner's degrees (°T).

Statistical analyses

The study was conducted in triplicates. Statistical analysis of the results were performed using Excel and free software environment R 3.4.0. Values were reported as the mean \pm standard error of the mean (SEM). The Wilcoxon's test was used during the comparative analysis of the results. The degree of uniformity of indicators within the group was determined using the non-parametric Kruskal-Wallis test. Kruskal-Wallis test by rank is a non-parametric alternative to one-way ANOVA



test, which extends the two-samples Wilcoxon test in the situation where there are more than two groups. It's recommended when the assumptions of one-way ANOVA test are not met. The p-value < 0.05 was considered as statistically significant [7, 11].

Results and discussion

The perspective direction of microbiology and biotechnology is the search for new active strains among lactic acid bacteria for the production of probiotics and products of functional nutrition [1, 3, 6]. The antagonistic activity of lactobacilli is one of the main requirements for bacterial strains – candidates in probiotics. The study of this feature for 13 lactobacilli strains isolated from different sources showed that they were all antagonists against yeast and bacteria strains at different levels (Fig. 1).

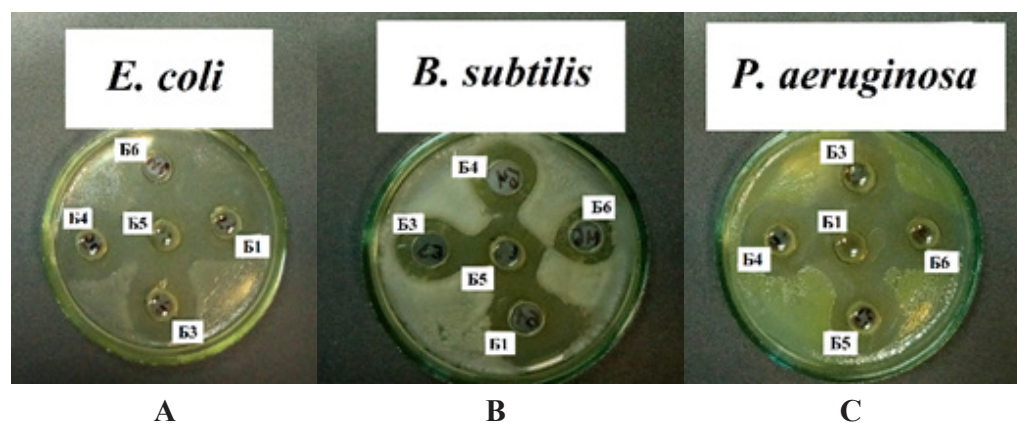


Fig. 1. The most sensitive test cultures to lactobacilli isolated from auto-fermenting vegetables: A – test-culture *E. coli* ONU 90; B – test-culture *B. subtilis* ONU 24; C – test-culture *P. aeruginosa* ONU 211

The major antagonistic activity of lactobacilli was observed against prokaryotic microorganisms. It was expressed in a greater number of strains-antagonists and in the sizes of growth inhibition zones of indicator strains (Fig. 1). *S. saprophyticus* ONU 537 M2 was most sensitive to metabolites of lactobacilli. Its growth was suppressed by all examined strains, and the diameter of growth inhibition zones was greater than 25 mm in almost all cases. Lactobacilli were found less active for the test-cultures *B. subtilis* and *K. pneumonia*. Only strains isolated from raw meat material (*Lactobacillus* sp. M1, M2, M3 and M6) showed antagonistic activity for *B. subtilis*. The greatest antagonistic activity was observed by strain *Lactobacillus* sp. M6; the diameter of growth inhibition zones of *B. subtilis* reached 23.0 ± 2.31 mm. *K. pneumoniae* ONU 463 was the most resistant to metabolites of lactobacilli. Its growth was suppressed by only 5 lactobacilli strains (isolated from different sources), and the diameter of growth inhibition zones did not exceed 25 mm. *Lactobacillus* sp. 175 was the most active antagonist for *K. pneumonia* (growth inhibition zones was 22.0 ± 1.21 mm).

Such relative resistance of *K. pneumoniae* and *B. subtilis* to lactobacilli antagonistic metabolites can be partly explained by the ability to form resistance factors such as the polysaccharide capsule (for *K. pneumoniae*) and endospores (for *B. subtilis*).

Data in literature on the impact of lactobacilli to *K. pneumoniae* and *B. subtilis* are quite controversial [3, 10].

The high resistance of eukaryotic microorganisms of the genus *Candida* to the metabolites of lactobacilli is known from the publications of many researchers of lactic acid bacteria [8, 9, 14].

The studies have found that the representatives of the genus *Candida* were resistant to the action of lactobacilli inhibitory substances (Table 1). The growth of *C. albicans* was suppressed by only 4 strains of *Lactobacillus*, three of which were isolated from raw meat materials. Growth of *C. utilis* was suppressed by 7 lactobacilli strains from auto-fermenting vegetables (*Lactobacillus spp.* B1, B5, O1), raw meat material (*Lactobacillus sp.* M2) and feces of children (*Lactobacillus spp.* 146, 275, 175). *Lactobacillus sp.* B4 was the most active against *C. albicans*, *Lactobacillus sp.* B1 against *C. utilis*, the diameters of growth inhibition zones were 25.0 ± 1.14 mm and 26.0 ± 1.13 , respectively.

In the literature there are data on the dependence of the manifestation of the antagonistic activity of lactobacilli on the source of isolation. Thus, in the work of Vasilyuk O.M. with co-authors (2014) it was found that *L. plantarum* strains isolated from fermented milk products are antagonistically more active against opportunistic pathogenic microorganisms than strains isolated from fermented vegetables. In this case, *L. plantarum* strains from fermented vegetables are the best antagonists of phytopathogens [3].

In the work of Mezaini A. et al. (2009) also have affirmative data on the effect of the source of isolation on the antagonistic activity of lactobacilli [15]. In other works, for example, Bissenova N.M. and others (2007) found no effect of the source of lactobacilli isolation on the manifestation of antagonistic activity; all strains were highly antagonistically active [2].

According to the results of the cluster analysis, it has been shown that the most sensitive to the action of the investigated lactobacilli strains was *S. saprophyticus* ONU 537 M2. The following indicator strains – *E. coli*, *P. aeruginosa*, *S. enteritidis*, *S. aureus* and *P. vulgaris* – share the same cluster with *S. saprophyticus* ONU 537 M2 (Fig. 2).

Indicator strains *B. subtilis*, *C. albicans*, *K. pneumonia* and *C. utilis* belong to a different cluster (Fig. 2) depending on the levels of resistance to the metabolites of lactobacilli. Indicators of probability are given at each internal node of the dendrogram and confirm the authenticity of the formed group. The results of the cluster analysis conducted to group the investigated lactobacilli by their total antagonistic activity are shown at the figure 3.

Obviously, all the examined strains were grouped in two clusters, and the second cluster has two subcluster. It should be noted that to the first cluster with high probability (values are indicated at each internal node) belong strains isolated from the raw meat material (*Lactobacillus spp.* M3, M6, M1, M2), which revealed to be the most active against *B. subtilis*, *K. pneumonia* and partially to *Candida sp.*



Table 1
Antagonistic activity of the investigated lactobacilli against yeast and opportunistic test-cultures

Strains of the investigated lactobacilli	<i>B. subtilis</i>	<i>S. aureus</i>	<i>S. saprophyticus</i>	<i>C. albicans</i>	<i>C. utilis</i>	<i>P. aeuruginosa</i>	<i>S. enteritidis</i>	<i>K. pneumoniae</i>	<i>P. vulgaris</i>	<i>E. coli</i>
175	0.0±0.00	20.6±2.01	30.9±3.02	0.0±0.00	19.6±1.91	21.6±2.11	23.7±2.31	22.6±1.21	20.6±1.01	23.7±2.31
275	0.0±0.00	22.7±2.35	33.1±1.42	0.0±0.00	21.7±1.25	24.8±1.45	23.8±1.12	0.0±0.00	20.7±2.14	23.8±1.46
146	0.0±0.00	22.4±2.01	34.7±1.15	0.0±0.00	17.4±1.57	19.4±1.76	22.4±2.04	0.0±0.00	19.3±0.76	18.4±1.67
O1	0.0±0.00	20.5±1.98	31.7±1.06	0.0±0.00	18.4±0.78	22.5±1.18	25.6±1.47	20.5±0.98	19.4±0.88	21.5±1.08
B1	0.0±0.00	23.9±1.27	34.3±1.26	0.0±0.00	26.0±1.13	25.0±1.17	20.8±0.97	0.0±0.00	18.7±0.78	29.1±1.76
B3	0.0±0.00	23.5±1.79	26.6±1.29	0.0±0.00	0.0±0.00	22.5±0.93	23.5±1.58	18.4±1.02	19.4±1.58	23.5±1.02
B4	0.0±0.00	31.0±2.57	28.9±2.40	25.0±1.14	0.0±0.00	20.7±1.71	21.7±1.80	0.0±0.00	19.6±1.63	32.0±2.66
B5	0.0±0.00	23.5±1.77	22.5±1.70	0.0±0.00	19.4±1.46	26.6±2.00	20.5±1.54	21.5±1.62	18.4±1.39	32.7±2.46
B6	0.0±0.00	24.3±1.93	21.3±1.69	0.0±0.00	0.0±0.00	23.3±1.85	19.3±1.53	0.0±0.00	19.3±1.53	26.3±1.09
M1	21.5±1.35	23.5±1.48	29.6±1.87	0.0±0.00	0.0±0.00	24.5±1.54	25.6±1.61	0.0±0.00	21.5±1.35	24.5±1.54
M2	20.5±1.46	23.6±1.67	29.8±2.11	21.6±1.53	20.5±1.46	24.6±1.75	21.6±1.53	0.0±0.00	23.6±1.67	21.6±1.53
M3	21.4±1.71	22.4±1.80	27.5±2.20	22.4±1.80	0.0±0.00	23.5±1.88	23.5±1.88	20.4±1.63	22.4±1.80	21.4±1.71
M6	22.4±1.74	23.4±1.82	31.6±1.45	20.4±1.58	0.0±0.00	22.4±1.74	23.4±1.82	20.4±1.58	22.4±1.74	21.4±1.66

Notes: p ≤ 0.05.



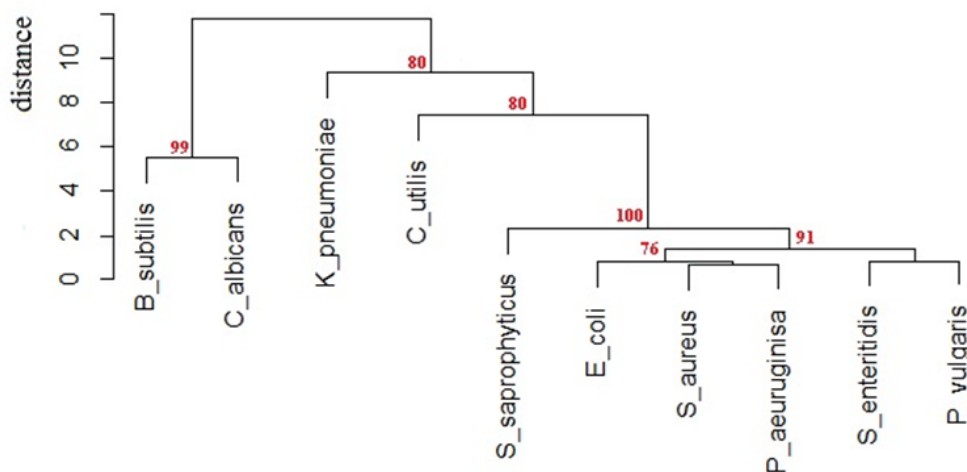


Fig. 2. Dendrogram of the clustering results of the indicator strains that resistance to the investigated lactobacilli. Note: clustering of data was performed using the function pvclust at nboot = 1000 (distance matrix – method "canberra", mode of "aggregation" – method "complete")

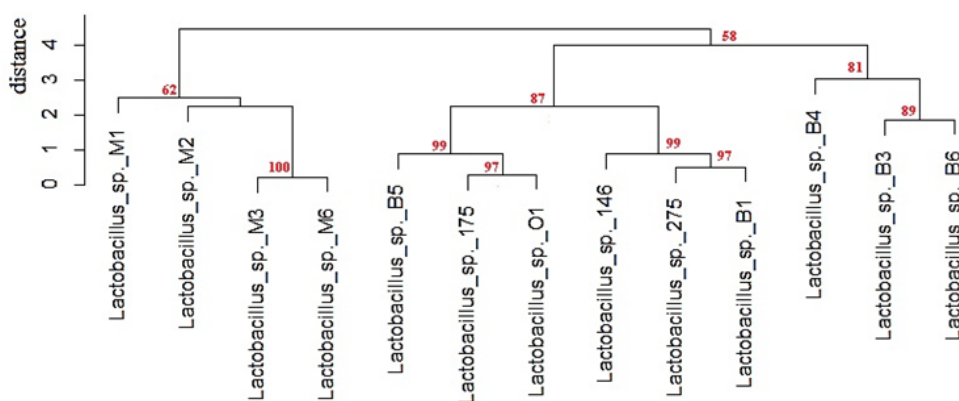


Fig. 3. Dendrogram of the clustering results on the levels of antagonistic activity of investigated lactobacilli. Note: clustering of data was performed using the function pvclust at nboot = 1000 (distance matrix – method "canberra", mode of "aggregation" – method "complete")

Similarly, the first subcluster of the second cluster includes lactobacilli isolated from auto-fermenting vegetables (*Lactobacillus spp.* B4, B3, B6). The second subcluster contains strains isolated from auto-fermenting vegetables (*Lactobacillus spp.* B5, B1, O1) and children feces (*Lactobacillus spp.* 146, 275, 175).

As was been shown the level of antagonistic activity depends on the strain and possibly depends on the strain's place isolation. So, result of ANOVA, carried out using Kruskal-Wallis test, corresponded to 28.8 at $p = 0.0042$, which indicates the heterogeneity of the studied parameter. However, at the same time, a similar analysis conducted on the basis strain's place isolation showed a much lower degree of heterogeneity of the studied parameter. In accordance with the results

obtained, the values of the criterion indicated an alternative hypothesis only for a sample formed from strains isolated from auto-fermenting vegetables (KW=11,26, p=0,043). The other two samples (“raw meat material” and “children feces”) that were formed on the basis of the grouping characteristic “place of isolation” were characterized by more homogeneity of the indicator of antagonistic activity in relation to yeast and opportunistic test-strains – KW= 6.59 (p=0.086) and KW =4.35 (p=0.113), respectively.

As was shown, when performing an equal analysis on the levels of antagonistic activity between samples, in general, the tested indicators are similar to each other in terms of the level of influence on yeast and conditionally pathogenic test strains (Table 2).

Table 2
Comparison of antagonistic activity of investigated samples of lactobacilli formed on the basis of the grouping characteristic “place of isolation” according to the Wilcoxon criteria

Wilcoxon criteria	Auto-fermenting vegetables	Raw meat material	Children feces
Auto-fermenting vegetables	W = 174, p = 1.0	W = 99, p = 0.18	W = 84, p = 0.75
Raw meat material	W = 99, p = 0.18	W = 80, p = 1.0	W = 74, p= 0.094
Children feces	W = 84, p = 0.75	W = 74, p = 0.094	W = 45, p=1.0

The antagonistic activity of lactic acid bacteria is due, first of all, to the production of organic acids that have bactericidal action, and, in addition, reduce the pH of the environment, which is unfavorable to many types of microorganisms. The determination of activity of acid producing is an important characteristic of probiotic lactobacilli strains of the definition of active and titratable acidity of 72-hour lactobacilli cultures showed that these indices were diverged for different strains (Table 3).

The titratable acidity levels varied from 39.67 ± 1.31 °T to 168.33 ± 0.65 °T. The highest titratable acidity levels were marked for *Lactobacillus sp.* 175 and B1 (168.33 ± 0.65 and 166.33 ± 0.65 °T, respectively). The least acid-producing ability was demonstrated by *Lactobacillus spp.* 275, O1, M1 and M6. The clots forming these strains were dense and homogeneous; *Lactobacillus sp.* M4 formed a loose clot.

The ANOVA by Kruskal-Wallis has confirmed that the investigated lactobacilli strains were formed in two independent groups on the basis of their acid-producing activity (Fig. 4).

As shown on the data presented in Figure 4, the indicators of the level of active acidity of investigated lactobacilli were heterogeneous (KW=35.79, p-value = 0.000349). However, when we analyzed samples conducted on the basis strain's place isolation were shown that strains isolated from auto-fermenting vegetables (KW=8.31, p-value = 0.13) and children feces (KW=5.6, p-value = 0.061) practically do not differ among themselves in the level of active acidity and form homogeneous groups. The Wilcoxon criterion confirms the similarity of indexes in the level active acid-forming activity between these groups (W = 43.5, p-value = 0.13).



Table 3

Acid-producing ability of the investigated lactobacilli

<i>Lactobacillus</i> strains	Active acidity, pH	Titratable acidity, °T
B3	4.55	146.00±1.13
B4	4.68	154.67±1.31
M3	4.68	157.67±1.31
B6	4.69	151.67±1.31
B5	4.70	158.00±1.13
M2	4.70	148.33±0.65
B1	4.73	166.33±0.65
175	4.76	168.33±0.65
M6	6.01	48.7±1.13
O1	6.02	39.67±1.31
275	6.02	54.33±0.65
146	6.04	43.00±1.13
M1	6.04	42.33±0.65

Note: $p \leq 0.05$.

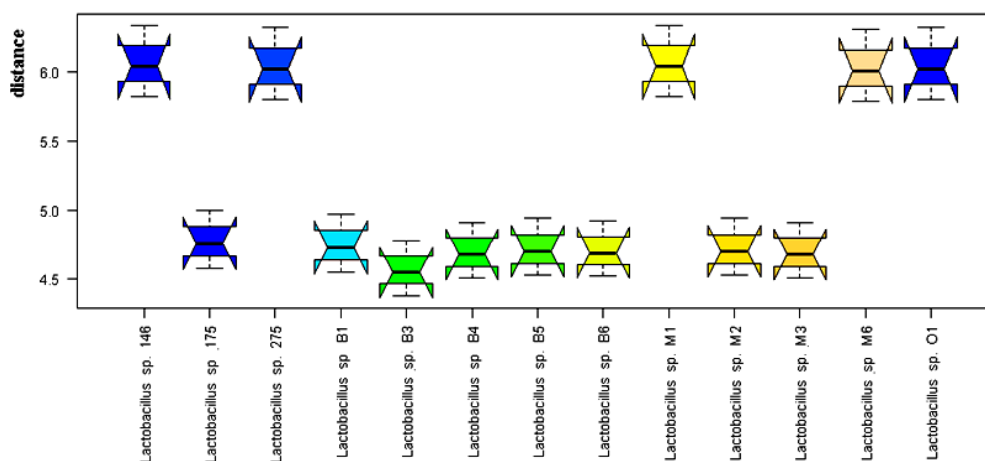


Fig. 4. Results of the one-factor dispersion analysis on the basis of the active acid-forming activity of the examined strains (Kruskal-Wallis chi-squared = 35.79, df = 12, p-value = 0.000349). Note: the boxplot lists the minimum values of active acidity, the value of the first quartile (Q1), the median, the value of the third quartile (Q3), the maximal values of the active acidity

Strains isolated from meat were more heterogeneous in active acid-forming activity regard (KW=8.53, p-value = 0.03).

During charting a binary dendrogram based on the parameters of antagonistic activity and the ability to active acid production, it was observed that *Lactobacillus spp.* M6, M1, B4, B1 and B5 formed a coupled lines between the vertices of both trees, which could serve as an indirect proof of the relationship between these indices for the listed strains (Fig. 5).



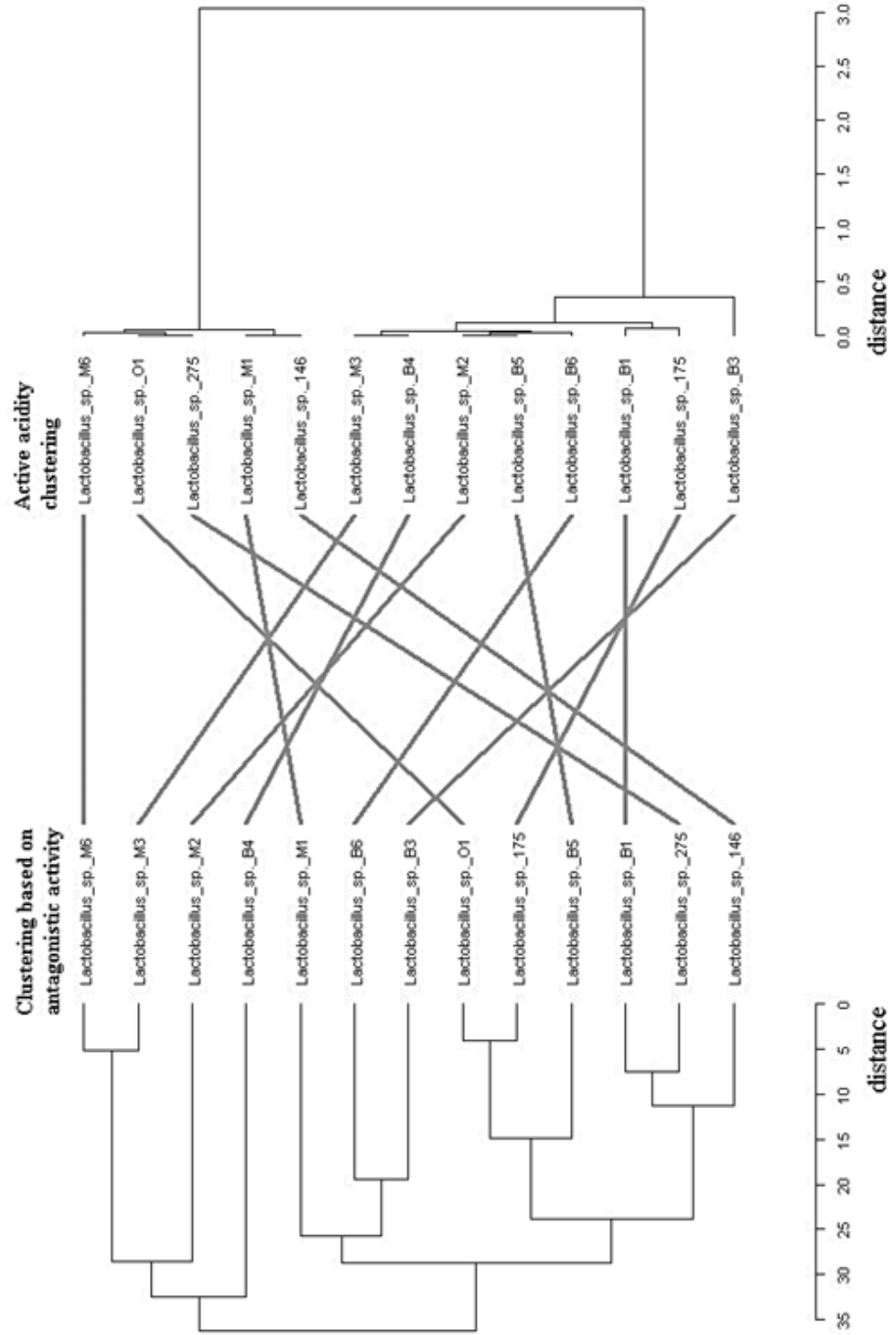


Fig. 5. Binary dendrogram illustrating the trees topology constructed on the basis of the results of clustering of the parameters of antagonistic activity and ability to active acid formation (distance matrix – "canberra", mode of aggregation – "complete")



Quite interesting is the fact that the connection between the level of antagonistic activity and the level of acid formation was found only for the strains isolated from raw meat materials and self-fermenting vegetables.

So, antagonistic properties of 13 lactobacilli isolated from different ecological niches have been investigated. Lactobacilli strains have been shown to inhibited the growth of pro- and eukaryotic microorganisms in different level. At the same time, the quantitative indicators of the synthesis of organic acids were different and depended on the certain strain. The levels of the studied abilities depended on the strain origin. During the cluster analysis of the set of investigated lactobacilli features has been revealed that the trend of grouping of the strains depending on their primary source of isolation. Thus, in the study of a number of properties that determine antagonistic activity, five lactobacilli (*Lactobacillus* spp. O1, B4, 175, M2 and M3) have been selected. They can be recommended for further researches for creation of probiotic preparation and functional foods.

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БІОЛОГІЧНА АКТИВНІСТЬ ЛАКТОБАЦИЛ РІЗНИХ ЕКОЛОГІЧНИХ НІШ ПІВДЕННОГО РЕГІОНУ УКРАЇНИ

Реферат

Метою дослідження було вивчення антагоністичної активності лактобацил, виділених з різних джерел Південного регіону України. **Методи.** В експериментах використано 13 штамів бактерій роду *Lactobacillus*. Антагоністичну активність лактобацил визначали лунково-дифузійним методом в товщі агару, використовуючи тест-культури про-і еукаріотних мікроорганізмів. Кислотоутворення штамів оцінювали за активною та титрованою кислотністю при культивуванні в молоці. **Результати.** Усі досліджені лактобактерії були активними антагоністами грамнегативних (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enteritidis* і *Proteus vulgaris*), а також грампозитивних (*Staphylococcus aureus* і *Staphylococcus saprophyticus*) бактерій. Лактобактерії виявляли меншу антагоністичну активність щодо *Vacillus subtilis* та *Klebsiella pneumoniae*. Найбільш активними антагоністами були лактобактерії, виділені з м'яса (*Lactobacillus* spp. M2 і M3). Активна і титрована кислотність лактобацил варіювала для різних штамів. Штам *Lactobacillus* sp. 175, виділений з фекалій дітей, характеризувався найвищою титрованою кислотністю. Використання статистичних методів (кластерний аналіз) дозволило сформувати кластери з високою вірогідністю за рівнем антагоністичної активності досліджуваних лактобактерій. Фактори навколишнього середовища мають певний вплив на формування загальної активності штамів лактобактерій, але прояв кожної з окремих особливостей є досить специфічним і залежить від особливостей певного мікроорганізму. **Висновки.** При дослідженні низки властивостей, що визначають антагоністичну активність, було відібрано п'ять штамів лактобактерій



(*Lactobacillus* spp. O1, B4, 175, M2 та M3). Вони можуть бути рекомендовані для подальших досліджень для створення пробіотичного препарату та функціональних продуктів харчування.

Ключові слова: лактобактерії, антагоністична активність, кислототворна активність, кластеризація.

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БИОЛОГИЧЕСКАЯ АКТИВНОСТЬ ЛАКТОБАЦИЛЛ РАЗНЫХ ЭКОЛОГИЧЕСКИХ НИШ ЮЖНОГО РЕГИОНА УКРАИНЫ

Реферат

Целью исследования было изучение антагонистической активности лактобацилл, выделенных из различных источников Южного региона Украины. **Методы.** В экспериментах использовано 13 штаммов бактерий рода *Lactobacillus*. Антагонистическую активность лактобацилл определяли луночно-диффузным методом в толще агара, используя тест-культуры про- и эукариотических микроорганизмов. Кислообразование штаммов оценивали по изучению активной и титруемой кислотности при культивировании в молоке. **Результаты.** Все исследованные лактобактерии были активными антагонистами грамотрицательных (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella enteritidis* и *Proteus vulgaris*), а также грамположительных (*Staphylococcus aureus* и *Staphylococcus saprophyticus*) бактерий. Лактобактерии проявляли меньшую антагонистическую активность в отношении *Bacillus subtilis* и *Klebsiella pneumoniae*. Наиболее активными антагонистами были лактобактерии, выделенные из мяса (*Lactobacillus* spp. M2 и M3). Активная и титруемая кислотность лактобацилл варьировала для разных штаммов. Штамм *Lactobacillus* sp. 175, выделенный из фекалий детей, характеризовался наибольшей титруемой кислотностью. Использование статистических методов (кластерный анализ) позволило сформировать кластеры с высокой вероятностью по уровню антагонистической активности исследуемых лактобактерий. Факторы окружающей среды оказывают определенное влияние на формирование общей активности штаммов лактобактерий, но проявление каждой из отдельных особенностей является достаточно специфическим и зависит от особенностей определенного микроорганизма. **Выводы.** При исследовании ряда свойств, определяющих антагонистическую активность, были отобраны пять штаммов лактобактерий (*Lactobacillus* spp. O1, B4, 175, M2 и M3). Они могут быть рекомендованы для дальнейших исследований для создания пробиотического препарата и функциональных продуктов питания.

Ключевые слова: лактобактерии, антагонистическая активность, кислотообразующая активность, кластеризация.



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