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TAXONOMIC RESEARCH, BIOLOGICAL PROPERTIES AND BIOSYNTHETIC ACTIVITY OF LACTIC ACID BACTERIA AND BIFIDOBACTERIA ISOLATED FROM VARIOUS NATURAL ECOLOGICAL NICHES

Lactic acid bacteria (LAB) and bifidobacteria play an important role in human health and have wide application in food industry. LAB propagation and high quantity of them in natural substrates testifies that many species of these microorganisms have adapted to the environmental conditions, and specific ecological niches. Lactic acid bacteria isolated from different ecological niches were identified using phenotypic and molecular–genetic methods. The species of Lactobacillus plantarum, Enterococcus faecium, E. durans, E. hirae, E. faecalis were identified among LAB. Bifidobacteria were majorly represented by Bifidobacterium infantis, B. breve, B. animalis, B. bifidum and B. dentium species. The intraspecific heterogeneity of LAB and bifidobacteria strains isolated from distal part of intestinal tract of people belonging to various age group was investigated. The role of teichoic acids in adhesion of the studied LAB strains to human's buccal epithelium was evaluated. Different biological activities, such as gastric juice resistance, tolerance to bile acids, adhesive properties to macroorganism's epithelial cells, sensitivity to clinically significant antibiotics were studied. All of these properties make specific LAB and bifidobacteria strains to be promising for probiotics and functional foods creation.

K e y w o r d s: lactic acid bacteria, bifidobacteria, probiotic properties, taxonomy.

Lactic acid bacteria (LAB) propagation and high quantity of them in natural substrates testifies that many species of these microorganisms have adapted to the environmental conditions, and specific ecological niches are LAB's native habitats. These issues are widely covered in the literature. However, for example, traditionally it was considered that bifidobacteria along with certain species of lactic acid bacteria present the microbiota of newborn children and infants [32]. In the meantime, the data obtained in the last several years testifies that lactic acid bacteria and bifidobacteria exist copiously in the alimentary tract of people of various age groups, especially among long–living persons [23, 31]. Furthermore, lactic acid bacteria and bifidobacteria inhabit the alimentary tract of farm livestock, insects, dolphins [33], plants' surfaces [39], etc.

Over the last years these groups of microorganisms attract special attention of researches all over the world. It is mainly caused by their positive effects on human health being used as components of probiotics and functional foods [4, 12, 19, 21].

In compliance with WHO's requirements probiotic microbial strains shall precisely match with the species name adopted by modern international list of microorganisms [24]. However, it is worth mentioning, that the determination of LAB's systematic position along with certain representatives

of *Bifidobacterium* genus is quite often a matter of argument. On the one hand, it is due to the live environment effect on these bacteria's physiology and, on the other hand, while adapting to certain conditions of ecological niches these microorganisms can modify some morphology–cultural characteristics and consequentially manifest heterogeneous properties, causing inaccuracy of species classification with microbiological methods [41, 9]. The use of API 20 Strep and API 50CHL test systems testified incorrectness of identification of closely related enterococci species and lactobacilli conducted based only on biochemical tests [2, 16, 18]. In the result, a demand arose for sensitive and fast methods of species-related identification of strains, isolated from ecological niches, as their species diversity is much more wide then can be determined with test-systems and existing schemes of traditional identification. Moreover the necessity for simultaneous use of several molecular-genetic methods became essential [1, 9].

Considering the fact that for the last years we have collected vast number of lactobacilli, enterococci, bifidobacteria and other LAB species' strains, isolated from various ecological niches, the necessity arose to use polyphase taxonomic principles while identifying the species. These principles include microbiologic, chemotaxonomic and various molecular-genetic methods [14, 23, 39]. Achievement of exact identification made it possible to perform strains screening to use them as components of probiotics and functional food.

In such a way our research has been first and foremost focused on the study of phenotypic characteristics of the wide range of LAB and bifidobacteria strains using microbiological methods. The performance of species identification for these groups of microorganisms tended to be insufficient due to the manifestation of heterogenic properties among some species of lactic acid bacteria, enterococci and bifidobacteria. Moreover while comparing the results obtained with classic methods with API-testing results we've quite often observed gaps in certain properties [9, 25].

According to data received along with microbiological methods of LAB identification we've investigated chemotaxonomic features, in particular fatty acids profile of cellular lipids. Literature concerning the issue is insufficient [35], however in the recent years due to taxonomic methods improvement many researches have focused their attention on fatty acids profile of lactic acid bacteria, enterococci cellular lipids as differential species character when describing type strains [5, 7, 36–38].

Application of the method of combined gas chromatography and mass spectrometry made it possible to identify fatty acids of C14–C19 carbonic chain length (saturated – tetradecanoic (C14:0), pentadecanoic (C15:0), hexadecanoic (C16:0) and octadecanoic (C18:0), unsaturated – hexadecenoic (C16:1) and octadecenoic (cis and trans isomers C18:1), and also cyclopropanoic acid (C19:0cyc)) in the strains of enterococci subject to investigation. These acids have been identified in all *Enterococcus* species (Tabl), however, their presence was not species–specific. The only exception was the pentadecanoic acid absent in *Enterococcus faecalis* strains. This was proved with the analysis of similarity of the studied strains with regards to their quantitative and qualitative fatty acid content profile. We've used cluster analysis and built *Enterococcus* strains similarity dendrogram based on the fatty acid qualitative composition. We have also used cluster analysis while investigating their quantitative com-

Table

Fatty acids content in Enterococcus strains of different species

Fatty acid	species, fatty acid content, %				
	E. faecium	***E. durans***	***E. hirae***	***E. faecalis***	***Enterococcus* sp**
C14:0	2.3±0.24	2.29±0.49	1.88±0.33	2.77±1.36	3.27±1.13
C15:0	0.28±0.07	0.17±0.08	0.36±0.18	0	0.23±0.23
C16:0	32.07±1.54	31.44±1.71	33.07±1.53	22.56±0.66	32.53±1.05
C16:1	1.69±0.28	1.51±0.42	2.06±0.56	2.31±1.38	2.08±1.19
C18:0	2.59±0.17	2.74±0.34	2.76±0.21	2.08±0.61	2.40±0.30
cC18:1	15.39±1.27	15.97±1.42	14.61±1.09	28.12±6.24	14.11±1.53
тC18:1	1.14±0.19	1.28±0.21	1.54±0.43	3.4±0.37	1.52±0.16
C19:0cyclo	38.49±0.82	37.29±2.87	39.52±0.83	26.98±6.49	37.65±1.14

position. However, despite certain difference in fatty acids composition of various strains of *Enterococcus* species, it was not possible to differentiate them. Based on the data obtained it follows that cellular lipids' fatty acids profile is not differentiating characteristic of closely related species of enterococci [9].

Wide use of molecular genetic methods allowed coping with major deficiencies of phenotypic and chemotaxonomic identification when identifying taxonomic position of closely related species. Thus, while we have identified *Lactobacillus plantarum* species based on phenotypic properties [39], *E. faecium*, *E. durans*, *E. hirae*, *E. faecalis* [14], *B. infantis*, *B. breve, B. animalis, B. dentium* [23, 42], the molecular typing of LAB and bifidobacteria genera using various types of DNA markers with polymerase chain reaction (PCR) has shown high level of nucleotide sequence variability in the mentioned species of microorganisms. Mini- and microsatellite replication markers usage allowed identifying genome's optional portion variability. High level of variability of DNA sequence of the strains *B. longum* and *L. plantarum*, 90 % and 94 %, respectively, has been noted [42].

High level nucleotide sequence heterogeneity among *L. plantarum* strains is declarative of broad adaptability of this type to various conditions of living environment. PCR performed for enterococci with species-specific primers has confirmed phenotypic identification of species *E. hirae, E. faecium* and *E. durans* [14].

Re–identification of all of the collected strains has been done with PCR using species–specific primers, primers to nucleotide repeats. As a result of amplification 50 strains of lactic acid bacteria have been re–identified and identified being isolated from various ecological niches – alimentary tract of humans, and warm–blooded and cold–blooded animals, dairy and plant origin foods. These strains refer to *L. plantarum, L. acidophilus, L. fermentum, L. delbrueckii, L. casei* species.

We've investigated intraspecific heterogeneity of bifidobacteria strains collection isolated from distal portion of intestinal tract of people of various age groups. The strains belong to *B. longum, B. dentium, B. bifidum, B. animalis* subsp. *lactis* species. The mentioned species of bifidobacteria strains manifested high level of intraspecific cellular polymorphism [42]. In the meantime bifidobacteria have shown much higher degree of intraspecies variability than the three type's of lactic acid bacteria *L. plantarum, L. acidophilus, L. fermentum*.

Based on the conducted experiments oligonucleotide primers database was created for the purpose of LAB and bifidobacteria identification.

Biological properties and biosynthetic activity of industry–promising lactic acid bacteria and bifidobacteria

Major natural functions of LAB and bifidobacteria involve high biological activity, macroorganism protection from adverse effects of environmental factors, immune system stimulation. These properties have been taken as a basis for creation of probiotics and functional food using probiotics [3, 29].

One of the major features in LAB's properties is their acid tolerance. It allowed investigating these microorganisms' ability to survive in stomach conditions at low pH values of gastric juice. Hydrochloric acid and digestive enzymes are the most aggressive agents of gastric juice in regard to microorganisms. Of all those lactic acid bacteria subject to our research following 2 hours exposure to gastric juice 42 % of strains survived and 58 % were completely inhibited [40]. All bifidobacteria strains subject to the research tended to be sensitive to gastric juice effect. Data obtained confirm the data available from the literature testifying negative effect of acid environment on bifidobacteria viability and acid conditions tolerance most of the members of *Lactobacillus* genus.

An equally important issue related to the LAB and bifidobacteria biological properties is to the investigation of their ability to survive in bile presence. Literature data testify that bacteria being components of probiotics shall be tolerant to various concentrations of bile acids [6, 43]. These concentrations are within 0.15–0.30 % of bile range. Firstly, we've investigated bacterial viability under 0.3 % bile presence, and then its inhibiting concentration for each strain has been identified. Bile acids effect was species dependent. For instance, *L. paracasei* strains grew with 0.2 % bile in culture medium, *L. acidophilus* – with 0.15 %, and *L. delbrueckii* subsp. *bulgaricus* manifested weak growth with 0.1 % bile [22]. At the same time, *L. plantarum* strains tolerated bile acids in the best way: they grew in the medium containing 0.3 % bile acids [40].

As for bifidobacteria, their viability at bile acids presence depends both on the isolation source and on the strain. Strains isolated from children manifested the highest sensitivity to bile. Only 10 of 15 strains grew at 0.1 % bile concentration, and no growth was observed with higher concentrations. However, the strains received from mid–aged and elder people showed dynamic growth with 2 % bile concentration in the medium, except *B. dentium*. Strains showed weak growth with 0.3 % bile in the medium. At the same time *B. animalis* was the most tolerant to 0.3 % bile [22].

According to the literature data [6], bile acids tolerance is primarily observed with bacteria isolated from mammals' alimentary tract, but can also be manifested by strains isolated from other sources, in particular from dairy products [6, 40]. This is due to the fact that bile acids presence in habitat is natural for LAB strains isolated from mammals' alimentary tract.

Adhesive properties of LAB and bifidobacteria are the criterion for their selection as probiotic cultures, providing their efficiency when used as components of biopharmaceuticals [24]. In the aftermath of the research it became apparent that all of the studied bifidobacteria strains are able to adhere to human buccal epithelial cells, while the adhesiveness index varied within a wide range from от 1.76 to 48.1. Adhesive activity depended neither on bifidobacteria species relation, nor on the isolation source, but was strain specific. *B. animalis*

subsp *lactis* 99 (IAM 48.1), *B. longum* 144 (IAM 34.7), *B. bifidum* 278 (IAM 28.7), *B. bifidum* 345 (IAM 27.31) and *B. catenulatum* 460 (IAM 27.34) strains were the ones with the highest adhesive properties. *B. longum* 62A (IAM 3.30), strain 145 (IAM 2.93) and strain 143 (IAM 1.76) had weak adhesive activity. Five of the studied *B. dentium* strains had not manifested high adhesive properties – oral cavity microbiota representatives, causing dental caries. Possibly, it is associated with their localization directly inside tooth caries and does not expand on the whole oral cavity.

All studied strains of lactobacilli have also manifested adhesive properties. However, their adhesive activity in human buccal epithelial cells was much weaker; it did not depend on the isolation source and was of strain–specific character. It was shown that all 26 strains of *L. plantarum* subject to the study adhered to human buccal epithelial cells at various degrees. Low adhesiveness index was manifested by 50 % strains (IAM from 3.63 to 9.95), average – by 37 % strains (IAM from 10.2 to 14.58). High adhesiveness (IAM from 17.97 to 30.11) was manifested by three (12.5 %) strains only [40]. Ability to adhere to epithelium cells causes LAB survival in various habitats within a macroorganism and possibility to create biofilms thus performing passive antagonism to pathogenic flora. Also, the lactic acid bacteria adhesion to specific biotope's epithelium surface allows them to perform continuous monitoring of nutrients content in the habitat, and reproduce forming protective monolayers creating barriers for pathogen penetration [27].

Microbial cell surface layer components, in particular teichoic acids, are actively involved in the process of adhesion. Teichoic acids participation in this process facilitates the manifestation probiotic functions. Teichoic acids structure is highly strain–specific, and these functions' manifestation depends on their specific features [27].

To evaluate the role of teichoic acids in adhesion of the studied LAB strains from which these acids were isolated, we've treated the targeted epithelium cells. It was found that LAB's teichoic acids preliminary contact with buccal cells causes a decrease of the mean values of adhesion. Moreover, *L. plantarum* teichoic acid preparation decreased the adhesiveness index of the given strain for 29.1 %. Likewise the number of buccal cells involved in adhesive process also decreased along with the percentage of cells with a big number of adhered bacteria. It suggests that teichoic acids prevent the adhesion of lactobacilli strains from which they were isolated [26].

An important issue in this context is to identify the mechanisms of manifestation of probiotic properties by lactic acid bacteria. This process can incorporate the aspects which are of major importance for probiotic properties, such as cell surface hydrophobic behavior, sensitivity to antibiotics and autolytic activity [30]. Bacteria cell surface hydrophobic behavior means its adhesiveness to the epithelial cells and is probiotic strains viability factor in the conditions of various macroorganism biotopes. Hydrophobic behavior properties are related to the degree of cell wall polarity and its charge and depend on its components' properties [11, 28]. In this regard it seemed relevant to study the relationship between the phosphates content (phosphorus containing teichoic acids) in the medium and the degree of microbial cell hydrophobicity. Solvent agents of various donor–acceptor properties and polarity were used for the study. The study of hydrophobic behavior of *L. plantarum* strains identified differences

between strains under cells treatment with chloroform, hexane, ethyl acetate. Under *L. plantarum* 11/16 strain treatment with chloroform, the hydrophobic value was 25.8 %, and *L. plantarum* 195D strain – 53.9 %; and with hexane treatment – 23.6 % for 11/16 strain and 38.2 % for 195D strain.

Analysis of hydrophobic properties of lactobacilli cells in conditions with various content of inorganic phosphates as one of the major components required for teichoic acids synthesis has shown that inorganic phosphorus content in culture medium effected lactobacilli cell hydrophobic properties along with the solvent agent: chloroform and hexane reduced the hydrophobic value to 11–15 %, and inorganic phosphate absence in the medium increased the hydrophobic value up to 64.8 % [28].

Biofilm formation by LAB strains provides their viability in adverse conditions of macroorganism's alimentary tract. We've studies the ability to create biofilm by 24 strains of *L. plantarum*, isolated from various fermented foods. Of all the strains subject to the research 3.7 % manifested high ability for biofilm creation. 79 % of strains were characterized by an average ration of its biofilm formation and only one strain of all the studied ones was practically incapable of creating a film. Biofilm creation process did not depend on strain isolation source. However, it is worth mentioning that two strains of *L. plantarum*, featured with the highest coefficient of biofilm formation, were isolated from the curd cheese [40].

Lactic acid bacteria sensitivity to antibiotics effect is one of the manifestations of its probiotic properties. As probiotic agents are often used on an antibiotic treatment background, it was useful to study the sensitivity to antibiotics of lactobacilli, enterococci and bifidobacteria monocultures subject to the study. We've established that bifidobacteria strains manifested sensitivity to all inhibitors of cell wall synthesis, except ceftibuten, to which *B. animalis* was resistant [33]. Lactobacilli have also manifested sensitivity to the majority of this range of antibiotics, however, *L. paracasei* was resistant to vancomycin and ceftibuten, *L. delbrueckii* subsp *bulgaricus* tolerates ceftazidime. Moreover, all lactobacilli strains showed resistance to cefepime [22].

Antibiotics inhibiting protein synthesis exhibit various action on the cultures subject to study. *L. plantarum* strains were resistant to aminoglycosides and fluoroquinolones, which is caused by the absence of an action target of these antibiotics in lactobacilli. Besides that, lactobacilli antibiotic sensitivity was of strain–specific nature and did not depend on its isolation source [40]. Bifidobacteria were also resistant to aminoglycosides action, which is caused by their anaerobic metabolic pattern [17]. Nitrofuran preparations exhibited various effects both on lactobacilli and on bifidobacteria. All strains of *B. aimalis*, *L. paracasei*, *L. acidophilus* and *L. delbrueckii* subsp *bulgaricus* were resistant to fusidin [22].

Nucleic acids transcription and synthesis inhibitors also exhibited various effects on the strains subject to investigation. They were sensitive to rifampicin and ofloxacin. *L. acidophilus*, *L. delbrueckii* subsp. *bulgaricus*, *B. animalis* strains were resistant to lomefloxacin and norfloxacin, *L. paracasei* manifested intermediate sensitivity to lomefloxacin [22].

Data received as a result of studying 58 strains of enterococci isolated from long–livers are to be referred to important research of antibiotic susceptibility. 9 % of the strains subject to the study were not resistant to clinically–significant

antibiotics, 12 % of strains manifested intermediate susceptibility and 79 % of strains were resistant to clinically–significant antibiotics [13].

Therefore, identification of the taxonomic position of lactobacilli, enterococci and bifidobacteria isolated from various ecological niches in compliance with modern taxonomy has shown that the isolated strains refer to *L. plantarum, E. faecium, E. durans, E. hirae, E. faecalis* species. Bifidobacteria were mainly represented by *B. infantis, B. breve, B. animalis, B. bifidum* and *B. dentium* species.

Enterococcus spp. cellular lipids' fatty acid analysis did not allow to identify clearly the systematic position of closely related species.

LAB and bifidobacteria genome molecular typing with various types of DNA markers using PCR has identified high level of nucleotide sequence variability within this genus of microorganisms.

All strains subject to this researh have manifested different biological activity: gastric juice resistance, tolerance to various concentrations of bile acids, adhesive properties to macroorganism's epithelial cells, and participation of performance and teichoic acids in this process, sensitivity to clinically significant antibiotics. All these properties make specific strains to be promising for probiotics and functional foods creation.

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ТАКСОНОМІЧНІ ДОСЛІДЖЕННЯ, БІОЛОГІЧНІ ВЛАСТИВОСТІ Й БІОСИНТЕТИЧНА АКТИВНІСТЬ МОЛОЧНОКИСЛИХ БАКТЕРІЙ І БІФІДОБАКТЕРІЙ, ІЗОЛЬОВАНИХ З РІЗНИХ ПРИРОДНИХ ЕКОНІШ

Резюме

Молочнокислі бактерії (МКБ) і біфідобактерії відіграють важливу роль у здоров'ї людини та мають широке практичне застосування в харчовій промисловості. Розповсюдження молочнокислих бактерій та їх висока чисельність у природних субстратах свідчать про те, що багато видів цих мікроорганізмів адаптувались до умов оточуючого середовища та окремих еконіш. Молочнокислі бактерії, ізольовані з різних екологічних ніш, були ідентифіковані з використанням фенотипових і молекулярно-генетичних методів. Серед МКБ, в основному, були ідентифіковані види *Lactobacillus plantarum, Enterococcus faecium, E. durans, E. hirae, E. faecalis*. Біфідобактерії були в основному представлені видами *Bifidobacterium infantis, B. breve, B. animalis, B. bifidum* і *B. dentium*. Була вивчена внутрішньовидова гетерогенність штамів МКБ і біфідобактерій, ізольованих з дистального відділу кишкового тракту людей різних вікових груп. Оцінена роль тейхоєвих кислот в адгезії штамів МКБ до букального епітелію людини. Вивчені різні біологічні активності, такі як стійкість до шлункового соку, стійкість до жовчних кислот, адгезивні властивості до епітеліальних клітин макроорганізму, чутливість до клінічно важливих антибіотиків. Всі ці властивості роблять окремі штами МКБ і біфідобактерій перспективними для створення пробіотичних препаратів і продуктів функціонального харчування.

Ключові слова: молочнокислі бактерії, біфідобактерії, пробіотичні властивості, таксономія.

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ТАКСОНОМИЧЕСКИЕ ИССЛЕДОВАНИЯ, БИОЛОГИЧЕСКИЕ СВОЙСТВА И БИОСИНТЕТИЧЕСКАЯ АКТИВНОСТЬ МОЛОЧНОКИСЛЫХ БАКТЕРИЙ И БИФИДОБАКТЕРИЙ, ВЫДЕЛЕННЫХ ИЗ РАЗЛИЧНЫХ ПРИРОДНЫХ ЭКОНИШ

Резюме

Молочнокислые бактерии (МКБ) и бифидобактерии играют важную роль в здоровье человека и имеют широкое практическое применение в пищевой промышленности. Распространение молочнокислых бактерий и их высокая численность в природных субстратах свидетельствуют о том, что многие виды этих микроорганизмов адаптировались к окружающим условиям и отдельным эконишам. Молочнокислые бактерии, изолированные из различных экологических ниш, были идентифицированы с использованием фенотипических и молекулярно-генетических методов. Среди МКБ, в основном, были идентифицированы виды *Lactobacillus plantarum, Enterococcus faecium, E. durans, E. hirae, E. faecalis*. Бифидобактерии были в основном представлены видами *Bifidobacterium infantis, B. breve, B. animalis, B. bifidum* и *B. dentium*. Была изучена внутривидовая гетерогенность штаммов МКБ и бифидобактерий, выделенных из дистального отдела кишечного тракта людей разных возрастных групп. Оценена роль тейхоевых кислот в адгезии штаммов МКБ к буккальному эпителию человека. Изучены различные биологические активности, такие как устойчивость к желудочному соку, устойчивость к желчным кислотам, адгезивные свойства к эпителиальным клеткам макроорганизма, чувствительность к клинически важным антибиотиками. Все эти свойства делают отдельные штаммы МКБ и бифидобактерий перспективными для создания пробиотических препаратов и продуктов функционального питания.

Ключевые слова: молочнокислые бактерии, бифидобактерии, пробиотические свойства, таксономия.

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