Zhanerke Talgatkyzy Amirkhanova

ABSTRACT
Of the academic dissertation for the degree of Doctor of Philosophy (PhD) in 6D060700 Biology

Impact of Dietary Fiber on the Biological Properties of Lactobacilli

Research Rationale. Currently, the modern market of Kazakhstan offers a wide range of domestic and foreign dairy products and probiotics with added dietary fibers. These products claim to have health benefits based on the probiotic characteristics of selected strains of lactic acid bacteria.

The effectiveness of synbiotic preparations and functional food products primarily depends on the biological properties of various strains of lactobacilli included in their composition. One of the main components of starter cultures for such products and preparations are often lactobacilli, which have demonstrated specific positive effects on the macroorganism and prebiotic substances, which enhance the selective advantages of the gastrointestinal tract's normal flora and its biological activity.

Along with other representatives of the normal microbiota of the human mucous membranes, lactobacilli play a key role in the body's anti-infectious defense and in the formation of colonization resistance in the macroorganism. In modern biomedical practice, methods of personalized medicine can be used: probiotic culture of lactic acid bacteria exhibits antimicrobial activity against the patient's pathogenic flora, stimulates antagonism, and is not inhibited by pathogens. In addition, one of the beneficial properties of lactobacilli is their ability to form biofilms, which allows them to withstand environmental conditions and successfully colonize and maintain their population. According to current understanding, a biofilm is a community of microorganisms attached to each other and enclosed in a matrix composed of extracellular polymeric substances, including polysaccharides and proteins synthesized by the microorganisms themselves. The formation of a biofilm by probiotic bacteria such as lactic acid bacteria is considered a beneficial property as it can facilitate colonization and longer persistence in the host's mucous membranes, thereby preventing colonization by pathogenic bacteria. The scientifically established phenomenon of the protective function of lactobacilli biofilms holds promising applications in the medical, pharmaceutical, food, and agricultural industries, which are priorities for the Republic of Kazakhstan.

In light of this, the search and study of probiotic properties in domestically competitive starter cultures isolated from traditionally natural ethnic products, including those produced by traditional artisanal methods, are an effective approach for obtaining genetically stable strains of probiotic cultures. This serves as a sustainable source of culture strains for the country's needs in order to enhance the quality of microbiological resources in collections.

In the production of synbiotic preparations and fermented dairy products, it is important to consider the influence of prebiotic substances on the growth, proliferation, and biological properties of starter cultures.

Currently, the starter cultures and prebiotic substances are primarily selected based on empirical methods, relying on years of accumulated experience. Therefore, studying the biological properties of genetically stable, domestically competitive starter cultures, including their behavior in the presence of prebiotic substances, is a vital and interesting task. It sets the research priorities for planning and conducting the current dissertation work.

Research aim is to study the impact of dietary fibers on the biological properties of lactobacilli *(in vitro)*.

To achieve the given aim, the following objectives were set forth:

1. To identify and isolate pure cultures of lactobacilli strains from artisanal dairy products produced in different regions of the Karaganda region as well as to sort out lactobacilli isolates with the highest capability for exopolysaccharide synthesis.

2. To evaluate the biofilm-forming ability and spectrum of antimicrobial activity of lactobacilli isolates as well as to study the morphology of lactobacillus cells in the biofilm using laser capture microdissection.

3. To explore the impact of soluble and insoluble dietary fibers on the growth of lactobacilli isolates, to determine the viability of lactobacilli cells when stored in a nutrient medium supplemented with soluble and insoluble dietary fibers.

4. To determine the adhesive properties of lactobacilli isolates as well as to investigate the resistance of lactobacilli isolates to low pH values in the environment.

5. To study the biofilm-forming properties of lactobacilli isolates using the micro-cultivation method in a modified nutrient medium, employing atomic force microscopy.

Scientific Novelty of Research. There has been a collection of domestically competitive starter cultures of lactobacilli set up and comprised of the lactobacilli isolated from natural ethnic dairy products, particularly those produced by artisanal methods in the regions of the Karaganda region. The majority of the isolated lactobacilli strains has demonstrated the ability to synthesize exopolysaccharides.

The laser microdissection was employed for the first time to study the biofilm-forming properties of the isolated lactobacilli isolates.

New experimental data were obtained regarding the influence of soluble and insoluble dietary fibers on the adhesive properties of lactobacilli. The impact index of inulin was found to be IAM 14.2±0.39, pectin exhibited an influence index of IAM 13.1±0.43, flaxseed fiber had an influence index of IAM 19.4±0.37, and flaxseeds had an influence index of IAM 17.5±0.27.

The laser microdissection method allowed for visualization through images to see that insoluble dietary fibers with the highest impact index (flaxseed fiber and flaxseeds) on which lactobacilli were cultivated led to *Lh13* isolate cells' auto-aggregation (the ability to form clusters or aggregates). Coaggregation (cell clumping) of the *Lh13* isolate was observed on flaxseeds. Both aggregation and coaggregation contribute to the formation of a biofilm.

The atomic force microscopy technique was used for the first time to visualize the biofilm surface roughness profile, which determines the morphological properties of the biomass in the modified nutrient medium.

Theoretical and practical significance of the findings. The conducted research confirms the theoretical and experimental significance of the obtained results. Building the basis upon the identification of lactobacilli with the highest exopolysaccharide production, a working collection of isolates with active biological properties was generated with the results in the spectrum of antimicrobial activity, the resistance of lactobacilli to low pH values, biofilm-forming activity, and the influence of dietary fibers on adhesive properties *(the collection has been officially registered and incorporated into the State Register of Copyrights and implemented in the educational process under registration number No.8863 as of March 17, 2020 (as indicated in the Appendix 2)).* The obtained results are important for the development of microbiology, as well as experimental, food, and veterinary microbiology as they open up prospects for wide practical application and further study of lactobacilli.

The working collection of microorganisms at the Biological-Geographical Faculty of Karaganda State University named after E.A. Buketov, NJSC and the Department of Biomedicine of KMU, NJSC has been supplemented with 34 isolates of lactobacilli: six isolates of *Lactobacillus helveticus* (*Lh-13, Lh-14, Lh-15, Lh-17, Lh-20, Lh-22*), seven isolates of *Lacticaseibacillus rhamnosus* (*Lrh-1, Lrh-1/1, Lrh-2э, Lrh-3э, Lrh-24, Lrh-39, Lrh-40),* three isolates of *Lactiplantibacillus plantarum* (*Lpl-2/1, Lpl-5, Lpl-89*), ten isolates of *Lacticaseibacillus paracasei* (*Lpc-4, Lpc-5/1, Lpc-28, Lpc-32, Lpc-34, Lpc-35, Lpc-36, Lpc-38, Lpc-37, Lpc-48*), three isolates of *Limosilactobacillus fermentum* (*Lf-18, Lf-38/1, Lf-81*), three isolates of *Lactobacillus acidophilus* (*Lac-50, Lac-52, Lac-79*), and two isolates of *Lactobacillus delbrueckii (Lacd-53, Lacd-54*).

Based on the obtained data, an atlas of probiotic cultures has been created (*Certificate of Inclusion in the State Register of Rights to Objects Protected by Copyright under No. 30964 dated December 9, 2022*) with implementation into the production of *Nateezhe Sut Fabrikasy LLC* (Refer to the Appendix 3).

A new method for preserving the working collection of lactobacilli cultures in a fermentative medium with added dietary fibers has been proposed (*Certificate of Inclusion in the State Register of Rights to Objects Protected by Copyright under No. 35559 dated May 17, 2023*).

During the development process, a new method of sample preparation has been developed for studying the morphology of lactobacilli and their biofilm-forming properties using laser capture microdissection. This method has been registered in the *State Register of Copyrights under No. 23592 dated February 14, 2022.* It has been successfully implemented in the educational process of the Department of Biomedicine of KMU, NJSC (*Refer to the Appendix 2) and has obtained a utility model patent with the number No.8042 dated May 05, 2023).*

Research Objects. The study utilized isolates of lactobacilli obtained from traditionally homemade fermented products, specifically those produced in the Karaganda region (Table 1, Nos. 1-7), collection strains (Table 1, Nos. 8-10), and reference strains from the American Type Culture Collection (ATCC), which were

provided by the Laboratory of Human Microbiome and Longevity at the National Laboratory Astana of Nazarbayev University (Table 2, Nos. 1-4). The commercial dietary fibers were obtained from *Nateezhe Sut Fabrikasy LLC* (Table 3).

Table 1 - The lactobacilli cultures under study

№	Name	Source of isolates
1	*Lactobacillus helveticus (6 isolates)*	Naturally fermented ethnic dairy products produced by traditional methods
2	*Lacticaseibacillus rhamnosus (7 isolates)*	
3	*Lactiplantibacillus plantarum (3 isolates)*	
4	*Lacticaseibacillus paracasei (10 isolates)*	
5	*Limosilactobacillus fermentum (3 isolates)*	
6	*Lactobacillus acidophilus (3 isolates)*	
7	*Lactobacillus delbrueckii (2 isolates)*	
8	*Lactobacillus rhamnosus V300*	National BioResource Center, All-Russian Collection of Industrial Microorganisms – Kurchatov Institute, National Research Center for Genetics.
9	*Lactobacillus acidophilus*	
10	*Lactobacillus delbrueckii subsp. bulgaricus 86*	

Table 2 - Test strains

№	Name	Laboratory of microbiome, Center for Life Sciences, "National Laboratory Astana" PI, Nazarbayev university
1	*Staphylococcus aureus* ATCC 29213	
2	*Escherichia coli* ATCC 8739	
3	*Salmonella typhimurium* ATCC 14028	
4	*Candida albicans* ATCC 10231	

Table 3 - Fibers

Name	Technical Specifications
Inulin	TS 9164-030-00493534-2007
Pectin	TS 10.963.2.27
Flax fiber	TS 19009841.005-2011
Flaxseeds	TS 9198-130-80324188-15

Research methods

Microbiological research methods:

- Collecting samples from the studied specimens, isolating pure cultures of lactic acid bacteria, examining morphological properties (serial dilution method, Gram staining method, light microscope microscopy);
- Studying cultural (bacteriological method) and biochemical properties of lactobacilli (determining sugar-utilization properties, catalase test, assessing acid production activity of isolated lactobacillus isolates);
- Identifying lactobacilli by using the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (*MALDI-TOF*);
- Testing for exopolysaccharide formation;

- Examining biofilm-forming properties of isolated lactobacillus strains on a polystyrene plate *(O'Toole method, using a TecanEVolizer100 laboratory robot);*
- Studying cell morphology of lactobacilli in biofilms using laser capture microdissection;
- Determining the resistance of the isolated lactobacillus isolates to low pH levels in the environment;
- Assessing the spectrum of antimicrobial activity (delayed antagonism method);
- Studying the effect of 1.5% inulin, 1% pectin, 15% flaxseed fiber and 10% flaxseed on the growth intensity of lactobacillus isolates (bacteriological method, cultivation on dietary fibers);
- Determining the viability (Koch method) of working cultures of lactobacilli during storage in a fermentative medium with added dietary fibers;
- Examining adhesive activity (on buccal epithelial cells);
- Studying biofilm-forming properties using the micro-cultivation method on a modified nutrient medium using atomic force microscopy.

Statistical Analyses

All data have been processed by using MS Office Excel 2010 and Statistica version 8.0. All data are presented as mean values with their standard errors (M±m). To analyze differences between control and sample values, the Student's t-test was used with a significance level of $p<0.05$. For the statistical analysis and interpretation of the research data, the Pearson correlation coefficient and Spearman rank correlation coefficient were applied. Multiple comparisons were conducted using the Dunnett method, and regression equations were employed for modeling purposes.

Main Conclusions for Defense

1. A total of 34 (100%) isolates of lactobacilli were obtained from natural fermented ethnic dairy products. Using a mass spectrometric analysis of protein profiles, the species of lactobacilli were identified as follows: *Lactobacillus helveticus, Lacticaseibacillus rhamnosus, Lactiplantibacillus plantarum, Lacticaseibacillus paracasei, Limosilactobacillus fermentum, Lactobacillus acidophilus, and Lactobacillus delbrueckii*. The lactobacilli were screened to confirm their phenotypic ability to synthesize exopolysaccharides.
2. The biofilm-forming abilities of the lactobacilli were determined. The morphology of the micro- and macrocolonies of the lactobacilli in the biofilm was visualized for the first time by using laser capture microdissection.
3. During the preservation of cultures in the nutrient media for a month, the "self-preservation" function is performed by insoluble dietary fibers. This has been demonstrated in the experiments during cultivation, where the selective action of lactobacilli with soluble (inulin, pectin) and insoluble dietary fibers (flaxseed fiber, flax seeds) was observed. It is the latter that prolongs the shelf life of lactobacilli cultures when added to the formulation in the nutrient medium.
4. Antagonism of lactobacilli isolates against test strains was established, and

the SPA (surface protein aggregation) of adhesive properties of lactobacilli on buccal epithelial cells was determined. It was observed that the processes of aggregation and coaggregation influence the metabolism of lactobacilli and enhance their resistance to low pH values.

5. The atomic force microscope studies have shown that the roughness of lactobacilli cells in the biofilm differs in a modified medium with the addition of 15% flax fiber from lactobacilli without any added flax fiber, which is due to the differences in the initial thickness of the biofilm (the greater the thickness of the biofilm (nm), the higher the roughness is).

Declaration of the author's personal participation. The author of this dissertation participated in all the stages of the research: in the development of the research design, implementation of all the main theoretical and experimental studies, sampling, transportation, conducting microbiological studies in microbiological laboratories, interpreting and documenting the results, preparing manuscripts for publication. She wrote the dissertation and its abstract. The author's individual contribution to the given research is 90%.

Volume and structure of dissertation work. The dissertation work is presented across 147 pages and comprises sections including abbreviations, introduction, literature review, materials and methods, results and discussions, conclusion, and a reference list with 272 sources. The dissertation includes 20 tables, 23 figures, and 5 appendices.

Approval and publications. The dissertation has been presented and published in 9 printed works, including 2 articles in internationally recognized journals indexed in Scopus, and 3 articles recommended by the Committee for Control of Education and Science of the Ministry of Education and Science of the Republic of Kazakhstan. One article was published in the Bulletin of the Medical Center of the Presidential Administration of the Republic of Kazakhstan (2019). Several works have also been presented and published in the proceedings of international conferences held in Kazakhstan, such as «*Current Problems in Biology and Ecology»* (the city of Karaganda, 2018), «*Integration of Science, Education, and Industry as the Basis for Implementing the National Plan»* (the city of Karaganda, 2019), and «*The World of Farabi»* (the city of Almaty, 2020). As a result of the research, two methodological recommendations have been published in the Kazakh and Russian languages. Implementation certificates have been obtained for integrating the research outcomes into the educational process at the universities of Karaganda Medical University NJSC and Karaganda State University named after E.A. Buketov, NJSC. An implementation certificate has also been obtained for the application of the obtained research outcomes in the production of Nateezhe Sut Fabrikasy LLC. Furthermore, four certificates of inclusion in the State Register of objects protected by copyright and one utility model patent have been obtained.