To control spoilage microflora, wheat bread manufacturers conventionally use chemical preservatives, which does not meet modern "clean label" principles and reduces the consumer value of the product.

This study has determined the possibility of using biologically active strains of lactic acid bacteria in the composition of sourdough starter to prolong the shelf life of wheat bread. The object of the research was the level and spectrum of antimicrobial, antifungal activity of lactic acid bacteria, as well as their effect on prolonging the shelf life of packaged wheat bread.

As a result of in vitro selection, Lactiplantibacillus pentosus XL 3440 and Limosilactobacillus reuteri XL 4024 were chosen, which were characterized by high antimicrobial activity against test strains of pathogenic, conditionally pathogenic bacteria: Escherichia coli K12 (NCTC 10538), Escherichia coli O11 (GISK 240170), Staphylococcus aureus ATCC 6538P, Staphylococcus aureus ATCC 25923, Bacillus subtilis 8-901, Bacillus mesentericus, Salmonella ssp.; fungicidal activity against yeast-like fungi Candida albicans ATCC 1918, Candida tropicalis Y-2473, Candida utilis Y-984; mold fungi Aspergillus brasiliensis 16404, Fusarium solani F-50718. In situ experiments showed that on day 6 of storage, bread made on a starter with these lactobacilli and infected with Aspergillus brasiliensis 16404 remained clean, without signs of fungal growth.

Based on the results, the shelf life of wheat bread was extended from 3 to 6 days. When combining such a starter with 0.1% calcium propionate, the shelf life of bread was extended to 8 days.

Therefore, the strains Lactiplantibacillus pentosus XL 3440 and Limosilactobacillus reuteri XL 4024 could be used as protective cultures for biopreservation of bread, thereby increasing its microbiological, fungicidal safety. The use of these cultures in bread making technologies could replace, compensate for the effect of chemical preservatives, and, therefore, warrant the quality and environmental friendliness of food products

Keywords: lactic acid bacteria, antimicrobial activity, fungicidal activity, wheat bread, shelf life

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DETERMINING THE EFFECT OF LACTIPLANTIBACILLUS PENTOSUS XL 3440 AND LIMOSILACTOBACILLUS REUTERI XL 4024 STRAINS WITH HIGH ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY ON PROLONGING THE SHELF-LIFE OF WHEAT BREAD

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1. Introduction

Bakery products are among the most popular food products consumed daily by people around the world. However, bakery products usually have a short shelf life. Spoilage of bakery products mainly occurs in the case of the development of mold fungi of the genera *Aspergillus*, *Penicillium*, *Fusarium*, *Mucor*, *Rhizopus*, *Candida*, *Endopyrrhiza*, and

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spore-forming bacteria of the genus *Bacillus*, in particular *B. subtilis* and *B. licheniformis*. As a result, the quality, aroma, taste, texture, and nutritional value of bakery products deteriorate, and their shelf life is shortened. In addition, many fungi of the genera *Aspergillus*, *Penicillium*, and *Fusarium* can produce mycotoxins, which pose a serious threat to human health [1].

To inhibit the growth of harmful bacteria and fungi in bakery products, various physicochemical methods are traditionally used: drying, microwave and infrared radiation treatment, addition of chemical preservatives, packaging in a modified atmosphere [2]. At the same time, such processing significantly reduces the consumer value of baked goods. For example, calcium propionate and other chemical preservatives are allowed to be added directly to baked goods in certain quantities, but their long-term consumption significantly increases the risks of the appearance and/or course of certain chronic diseases [3].

Scientific research in recent years has focused on the use of natural preservatives as a basis for the development of innovative food technologies. Manufacturers are also interested in reducing the use of synthetic additives because of their cost and growing consumer demand for products without chemical preservatives in their composition. Ukraine also participates in the EU program for the gradual transition to "clean label ingredients", that is, minimizing the use of chemical components in food products, including bread [4].

Scientific studies on the search for lactic acid bacteria (LAB) with high antagonistic activity is relevant as their use makes it possible to replace chemical preservatives and ensure the safety of bread.

2. Literature review and problem statement

The widespread use of LAB in the food industry is due to the fact that they are naturally present in the products from which they are typically isolated. Lactobacilli have a safety status, included in the list of Generally Recognized As Safe in the USA and in the list of Qualified Presumption of Safety in the European Union. Some strains of LAB have established high antimicrobial and antifungal activity. Due to these properties, LAB play an important role in the production of food products, in particular, bread. Sourdough fermentation is an environmentally friendly and biotechnological bread production technique.

Thus, work [5] established that it is appropriate to use LAB strains of different taxonomic groups, which are capable of producing a wide range of antifungal compounds. Specifically: organic acids, carbon dioxide, hydrogen peroxide, fatty acids, diacetyl and acetoin, cyclic dipeptides, non-protein antimicrobial substances of a wide spectrum of action, exopolysaccharides, etc. However, in [5], no attention was paid to the study of the antimicrobial activity of LAB strains.

In [6], it was shown that the dominant species in French spontaneous bread starters was *Lactobacillus sanfranciscensis*. In addition, strains of such species as *Lactobacillus xiangfangensis* and *Lactobacillus diolivorans* were isolated from these starters. At the same time, the authors report no studies on the antimicrobial and fungicidal activity of the isolated microorganisms.

In [7], spontaneous starter cultures produced in western Sicily (southern Italy) were analyzed. It was shown that the strains *Lactobacillus plantarum*, *Lactobacillus curvatus* and *Lactobacillus graminis* predominated. And the strain *L. plantarum* PON100148 was characterized by the ability to produce inhibitory substances similar to bacteriocins and exhibited antifungal activity. However, in [7], a limited range of test cultures was used to determine antimicrobial activity – only *Listeria innocua* 4202 and *Listeria monocytogenes* ATCC 19114. When determining antifungal activity, a wide range of test cultures *Alternaria alternata* Ra56, *Aspergillus carbonarius* Sc13, *Aspergillus ochraceus* Ra49, *Cladosporium cladosporioides* Sc71, *Penicillium roquefortii* R1 and *Penicillium verrucosum* Sc29 were used. However, the source of origin of these strains was not indicated, which limits the scientific value of the results of the work.

In [8], strains *L. plantarum* No. 122, *L. casei* No. 210, *L. curvatus* No. 51, *L. paracasei* No. 244 and *Lactobacillus coryniformis* No. 71 were isolated from spontaneous rye sourdough, which had the ability to inhibit the growth of 15 strains of pathogenic bacteria and 7 species of mold fungi from the collection of the Lithuanian University of Health Sciences (Kaunas, Lithuania). However, in [8], the antifungal activity of *Lactobacillus ssp.* strains was studied only by one "well" method, which does not fully reveal their antagonistic properties.

Analysis of the cited literature reveals that the effectiveness of spontaneous bakery starter as a multibacterial complex in inhibiting the growth of spoilage microorganisms varies significantly depending on the activity of individual strains of LAB. The possibility of using specially selected LAB strains as a part of starters in the production of bakery products has been investigated in a number of papers. Thus, in [9], a mixed culture was used for breeding wheat starter (produced by Böcker, Minden, Germany), consisting of one strain of *Lb. crispatus*, three strains of *Lb. pontis* and one strain of yeast *Saccharomyces cerevisiae*. It was found that wheat bread with 20% of such starter was distinguished by lower crumb hardness during 74 hours of storage. Relationships were established between crumb shrinkage and such indicators as hardness and elasticity, respectively; the Pearson correlation coefficient was 0.978 and – 0.983. In [9], there are no studies on the antagonistic properties of the sourdough microbiota and its effect on the microbiological indicators of bread during storage.

In [10], rye sourdough was fermented using starter cultures: *Saccharomyces cerevisiae*, *Candida milleri*, *Lactobacillus sanfranciscensis*, *Lactobacillus pontis*, as well as mixtures of *L. pontis* with *S. cerevisiae* or *C. milleri*. It was shown that the effect of starter cultures on the concentration of amino acids depended on their bacterial composition. In particular, the formation of ornithine was observed only in dough fermented with *L. pontis*, due to its ability to convert arginine to ornithine. This enhanced the taste of the finished product, giving it a touch of roasted wheat bread. In [10], there is no information on studies of the effect of starter cultures on the shelf life of bread.

In [11], 144 scientific papers were analyzed, which showed an increase in the consumer value of sourdough bread. The positive effect of sourdough on mineral bioavailability, phytic acid content, starch digestibility and glycemic index, protein digestibility, dietary fiber content, and vitamin and phenolic compound levels has been documented. However, [11] lacks studies on the effect of bakery sourdoughs with selected LAB strains on the long-term storage capacity of bread.

Thus, in [12], it was concluded that the determining factor influencing the quality and storage of bread is the composi-

tion of the microbiome of the sourdough starter, the spectrum of antifungal metabolites. Both monocultures of the species *L. plantarum*, *L. rossiae*, *L. amylovorus*, *L. hammessi*, *L. brevis*, *L. reuteri*, *L. delbrueckii subsp. bulgaricus*, *L. acidophilus*, *L. casei* and others, as well as their mixtures, were tested. It was shown that the shelf life of bread can be significantly extended depending on the composition (species, strain), the amount of starter added (10–30%), the type of flour and the conditions of bread storage. However, in [12] not enough attention was paid to the study of the antimicrobial properties of starters. Only the antagonistic effect on *Bacillus licheniformis*, *B. cereus*, *B. subtilis*, the spores of which are not destroyed during baking and can germinate in bread, forming mucus, was considered.

In [13], the antifungal potential of *Lactiplantibacillus plantarum* strains against *Aspergillus parasiticus* and *Penicillium expansum* was investigated in vitro. A peptide was isolated that inhibited the growth of *P. expansum* and *A. parasiticus* by 58% and 73%, respectively, in liquid medium after 48 hours of incubation with LAB. The disadvantage of the work is the lack of studies aimed at establishing the effect of LAB on extending the shelf life of bread.

In [14], the importance is noted of selecting LAB for the ability to inhibit not only the growth of *Bacillus subtilis*, a typical contaminant of bread products, but also *Staphylococcus aureus*, *Salmonella*. These pathogens can get into bread from personnel during raw material processing. The *Lactobacillus fermentum* C14 strain was isolated with a broad antimicrobial spectrum against gram-positive and gram-negative pathogenic bacteria, as well as activity against *Trichophyton rubrum*, *Penicillium digitatum* and *Mucor ssp.* fungi. When analyzing the antifungal effect of the strain, surface treatment of bread with a bacterial suspension and fungal spores was used. In [14], bread was not baked on sourdough with this LAB strain, which does not make it possible to assess its ability to function in the dough system.

In [15], it was shown that the use of sourdough with strains *Lactiplantibacillus plantarum*, *Lacticaseibacillus casei*, *Lactobacillus acidophilus* in bread technology was an effective alternative to synthetic preservatives. The starter with these LAB improved the physicochemical characteristics and extended the shelf life of wheat bread, inhibiting mold growth. However, these studies were conducted in a refrigerated chamber at 4°C, and under such conditions, the growth of extraneous microflora and mold is usually also slowed down. In addition, bakery products are conventionally stored at higher temperatures in the retail chain.

In [16], it was found that the sourdough fermented with the *Lactobacillus amylovorus* DSM19280 strain was effective in producing bread with improved nutritional value and long shelf life at a temperature of (20 ± 2) °C. It was reported that the production of antifungal compounds is a strain-specific property and depends on the substrate. When studying the effectiveness of the sourdough for prolonging the shelf life, scientists used a "gentle" method of infecting bread with mold fungi from the bakery environment. However, in [16], artificial infection of bread with a test strain of a certain type of fungus was not carried out, which limited the assessment of the antifungal potential of the sourdough.

In [17], the possibility of using fermentates containing inactivated LAB cells, their metabolites and culture medium as alternative antimicrobial agents was considered. It was also shown that the sourdough at a concentration of 20–30% demonstrated effective antimicrobial properties. Puratos company proposed using sourdough products such as Fidelio, Traviata, Aroldo, and Durum to improve bread quality. However, work [17] does not provide data on the component composition of these sourdoughs, the magnitude and spectrum of antagonistic activity of the microbiota, which limits the scientific and practical significance of the work.

Therefore, the prospects of using bakery starters with selected strains of LAB in bread technologies are beyond doubt. At the same time, the reason for the limited number of LAB strains that are promising for the biopreservation of bread may be objective factors associated with the pronounced species- and even strain-specific variability of antimicrobial and antifungal activity of LAB. The use of starters to extend the shelf life of bread requires a more thorough study of the antagonistic potential of the bakery microbiota; expanding the spectrum of test cultures of bacteria, yeasts, and molds used. Therefore, the issue of selecting biologically active strains of LAB of different taxonomic groups for use in bread technologies to extend its shelf life requires a more detailed study both *in vitro* and *in situ* experiments.

Thus, it is advisable to conduct research on the selection of LAB strains with high antimicrobial and antifungal potential, effective for prolonging the shelf life of bread.

3. The aim and objectives of the study

The aim of our study is to determine the effect of LAB on pathogenic and conditionally pathogenic bacteria, yeasts, and mold fungi, which will make it possible to use the most active strains for biopreservation of wheat bread.

To achieve this goal, the following tasks were set:

– to investigate the antibacterial activity of LAB *in vitro*;

– to investigate the antifungal activity of LAB *in vitro*;

– to determine the shelf life of sourdough bread with LAB strains with high antibacterial and antifungal activity.

4. The study materials and methods

4. 1. Research hypothesis

The object of our research is the level and spectrum of antagonistic activity of LAB and their effect on the extension of the shelf life of packaged wheat bread.

The principal hypothesis assumes that to extend the shelf life of wheat bread, it is advisable to use strains of *Lactobacillus ssp.* with high antimicrobial and antifungal activity in the composition of the sourdough.

It is expected that the use of strains of *Lactobacillus ssp.* in the composition of the baking sourdough would ensure the predicted stability of microbiological indicators of bread during long-term storage through their high antimicrobial and fungicidal activity.

When planning and conducting the research, the following assumptions were adopted:

– it was assumed that the shelf life of bread, given the experience of scientists, is affected by bacterial and fungal contamination of the product;

– it was believed that determining the antifungal activity of the microbiota of baking sourdough is one of the priorities for predicting the shelf life of bread.

The following simplifications were accepted:

– during the study, the emphasis was determining the microbiological indicators of the effect of LAB on test cultures

of pathogenic and conditionally pathogenic bacteria, yeast, fungi in *vitro*, and organoleptic indicators in model samples of bread *in situ*. The determination of the physicochemical indicators of bread during storage was not taken into account;

– the possibility of biopreservation was assessed by determining the absence of fungal mycelium growth in samples of sourdough bread with LAB strains with antifungal activity during storage compared to yeast bread and the addition of calcium propionate.

4. 2. Materials studied

Lactic acid bacteria were isolated from whole grain wheat starter by surface plating of tenfold dilutions on modified mmRS agar medium (Merck, Germany) with the addition of 20 g glucose and 0.1 g cycloheximide per 1 dm^3 of medium, pH 5.6. The plates were incubated at 30°C for 72 hours. Colonies were isolated into liquid mmRS medium, incubated at 30°C for 48 hours and pure cultures were selected. The morphological homogeneity of the cultures was examined by microscopy, by Gram staining. Microscopy was performed using a Motic microscope (Fischer Bioblock) with a built-in TopView video camera with a magnification of 1000 times. All isolates considered for further analysis were $Gram^+$, catalase-negative, nonmotile rods or cocci capable of acidifying the medium. Pure cultures of LAB were subcultured twice in liquid mmRS (1% inoculum, 24 hours, 30°C) before use.

Test strains. The work used test strains of pathogenic and conditionally pathogenic bacteria from a collection at the L. V. Gromashevsky Institute of Epidemiology and Infectious Diseases, the Ministry of Health of Ukraine: *Escherichia coli* K12 (NCTC 10538), *Escherichia coli* O11 (GISK 240170), *Staphylococcus aureus* ATCC 6538P, *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* 8-901, *Bacillus mesentericus*, *Salmonella ssp.*; yeasts – *Candida albicans* ATCC 1918, *Candida tropicalis* Y-2473, *Candida utilis* Y-984; molds – *Aspergillus brasiliensis* 16404, *Fusarium solani* F-50718. The test strains of bacteria were grown in MPB (meat-peptone nutrient broth) (HiMedia, India) at 30°C for 24 hours. Test strains of yeast and mold fungi were grown, respectively, on slanted SDA agar (Merck, Germany) with the addition of chloramphenicol 0.1 g/dm^3 and on potato-dextrose agar PDA (Merck, Germany) for 3–5 days. Several loops of inoculum were removed from the agar surface and transferred to sterile peptone water (0.2%), shaken.

The number of microorganisms was determined by plating serial dilutions on agar nutrient media. LAB were plated on mmRS medium and cultured at 30°C for 72 hours. Yeasts were plated on SDA medium and cultured at 30°C for 3–5 days. Molds were plated on potato-dextrose PDA medium and cultured at 30°C for 5–7 days.

4. 3. Research methodology and methods

The antagonistic activity of the strains was studied by the "well" method, the size of the zone of no growth of the test strain on a solid nutrient medium was measured in mm. 20 cm^3 of a certain medium was poured into Petri dishes: MPA, meat-peptone nutrient agar – for bacteria, SDA – for yeast. The dishes were dried in a thermostat at 30°C to remove excess condensate. The density of the suspensions of LAB, test strains of bacteria and yeast was adjusted to 0.5 according to the McFarland turbidity standard in saline, which corresponded to a concentration of $(1.5 – 2.0) \times 10^6$ CFU/cm^3 according to seeding into the appropriate agar media. The suspensions of test strains prepared in this way in an amount of 0.1 cm^3 were evenly distributed with a sterile Drygalsky spatula over the surface of the appropriate agar. 10–15 min after seeding, wells with a diameter of 6 mm were made in the agar with a sterile metal tube. 0.1 cm^3 of a suspension of one of the LAB cultures was added to each well, the plates were incubated in a thermostat for 24–72 h at 30°C. As control No. 1, the *Lactobacillus casei* B-7017 strain from a collection at the Institute of Food Resources of the NAAS of Ukraine was used, control No. 2 was a 0.5% solution of dihydroquercetin (TOV BioFlavid, Ukraine), prepared in sterile distilled water.

The antifungal activity of LAB strains was studied by various methods:

1) *in vitro:*

– by the agar diffusion method according to [18] with modification. 10 cm^3 of PDA medium with 1.5% agar was poured into Petri dishes – the lower layer; then the dishes were dried. 10 cm^3 of the same medium with 0.7% agar was poured on top of the lower layer, containing the fungal inoculum at a concentration of about 10^4 spores/cm^3 – the upper layer. To prepare the inoculum, the spore content in the initial suspensions of different fungal species was standardized by diluting with sterile 0.2% peptone water. The spore concentration was controlled by seeding on PDA agar, the crops were cultivated at 30°C for 5 days. Holes with a diameter of 6 mm were made in the agar with a sterile metal tube. Each well was added with 0.1 cm^3 of a suspension of one of the experimental strains of LAB, which were prepared as described above to test antagonistic activity. Control No. 1 used 0.3% calcium propionate solution (TOV Chemlaborreaktiv, China); control No. 2 – 0.5% dihydroquercetin solution prepared in sterile distilled water. The test plates were kept for a 5-hour pre-incubation period at room temperature for diffusion of antifungal substances into the agar, then at 30°C. The growth of fungi and the degree of inhibition were assessed visually for 5 days as follows: (–) – no inhibition, (+) – a clear zone around the well of 1 mm; (++) – a clear zone around the well of 2 mm; (+++) – a clear zone around the well of more than 2 mm;

– double cultivation method according to [19] with modification. 20 cm^3 of mmRS agar medium was poured into Petri dishes, the dishes were dried. Surface cultures of each strain of LAB were made in two parallel lines 2 cm long; the dishes were incubated at 30°C for 72 hours. Then, in each dish on the other side of the LAB, a well was made, into which 0.1 cm^3 of the fungus inoculum was introduced at a concentration of about 104 spores/cm^3. The dishes were incubated in a thermostat for 7 days at 25°C. As a control, dishes with a well inoculated with the fungus without LAB inoculum were used;

2) in situ:

– by the method according to [20] with modification. The control bread sample was prepared from 100 g wheat flour, 2 g yeast, 1.8 g NaCl and 2.0 g sugar, 3.0 g oil, and water. The appropriate amount of water for dough preparation was regulated using a Brabender farinograph (FE022N, Germany). The test bread sample No. 1 was prepared similarly to the control, but 25% (wt./wt.) of wheat sourdough fermented with a bacterial composition of *Lactiplantibacillus pentosus* XL 3440 and *Limosilactobacillus reuteri* XL 4024 strains, taken 1:1, was added to the dough at the mixing stage. The test bread sample No. 2 was prepared as the test sample No. 1, but 0.1% calcium propionate was additionally added. Then all samples were prepared in the same way. The above ingredients were placed in a laboratory dough mixer (EB124101, Germany),

then they were mixed at 60 rpm for 20 min. Then the dough was left for initial fermentation at (30 ± 1) °C and 75% relative humidity for 30 min, then divided into pieces of 280 g per mold. The dough pieces were aged at (45 ± 1) °C and 85% relative humidity for 90 min (final aging), then baked in an electric oven (Leisure, Italy) at (220 ± 5) °C for 15 min. After that, the bread samples were cooled to room temperature of 22°C for 3 h under sanitary conditions. Samples of chilled bread were infected with Aspergillus brasiliensis 16404. For this purpose, 0.1 cm^3 of the fungal inoculum (10^4 spores/cm^3) was dropped into the center of each bread sample. The bread was then packaged in a sterile plastic bag, heat-sealed, leaving a small hole open, and a pipette tip inserted into it to provide model aerobic conditions in each bag. *In situ* antifungal activity was monitored at 25°C for 3–8 days by determining the presence/absence of fungal mycelium growth in subsequent bread samples. Test sample No. 1 – sourdough bread with a composition of strains *Lactiplantibacillus pentosus* XL 3440 and *Limosilactobacillus reuteri* XL 4024. Test sample No. 2 – bread with the same sourdough with the addition of 0.1% calcium propionate. The control sample was bread without leaven, without calcium propionate. The intensity of visible fungal growth on the surface of the bread was visually assessed as follows: (–) – no visible colonies, (+) – one colony, (++) – two, (+++) – three or more.

The repeatability of the studies was 5 times. The results were considered significant at $p < 0.05$.

5. Results of investigating the antagonistic and fungicidal activity of lactic acid bacteria

5. 1. Results of determining the antagonistic activity of lactic acid bacteria

It is known that baker's sourdough is a natural source of isolation of biologically active strains of LAB [21]. Therefore, spontaneous wheat sourdough was prepared according to the traditional protocol, without the use of starter cultures or commercial baker's yeast. 115 gram-positive, catalase-negative, non-spore-forming bacteria were isolated from the mature sourdough. Of which, based on cultural, morphological, physiological, and biochemical characteristics, 12 cultures were identified to the species according to Bergey's Manual of Systematic Bacteriology 2009 tests, and using API 50 CHL test systems (bioMerieux, France). The species affiliation of the strains is represented according to the classification from [22].

The study on the manifestation of antagonistic activity in the isolated cultures against test strains of pathogenic, conditionally pathogenic bacteria, and yeasts in *in vitro* experiments was conducted. To compare the level of antagonistic activity in the isolated isolates, the biotechnologically active strain *Lactobacillus casei* B-7017 from the IPR collection was taken [23] as a positive control. In addition, the intensity of the manifestation of LAB antimicrobial activity was compared with dihydroquercetin – an antioxidant of plant origin. It was established that LAB strains exhibited antagonistic activity against all test strains to one degree or another. The results of our study are given in Table 1.

Table 1

Antagonistic activity of isolated LAB cultures, *well method*

Test strain	Diameter of the growth inhibition zone of the test strain, mm			
	Enterococcus ssp, 2 PCS.	*Lactobacillus ssp*, 10 PCS	Control No. 1*	Control No. 2**
Escherichia coli K12	10–16	19–27	24 ± 2	23 ± 2
Escherichia coli 0111	12–21	18–28	25 ± 2	24 ± 2
Staphylococcus aureus ATCC 6538*P*	9–15	10–25	22 ± 2	20 ± 2
Staphylococcus aureus ATCC 25923	10–18	11–27	24 ± 2	21 ± 2
Salmonella ssp.	7–10	14–16	12 ± 2	11 ± 2
Bacillus subtilis 8-901	10–22	18–29	23 ± 2	22 ± 2
Bacillus mesentericus	10–21	17–30	25 ± 2	22 ± 2
Candida albicans ATCC 1918	5–10	10–15	12 ± 2	10 ± 1
Candida tropicalis Y-2473	4–5	7–9	7 ± 1	5 ± 1
Candida utilis Y-984	4–5	10–12	8 ± 1	6 ± 1

Note: – L. casei B-7017;** – dihydroquercetin, 0.5%.*

It was found that strains of *Lactobacillus ssp.* were characterized by a higher level of antagonistic effect compared to *Enterococcus ssp.* Thus, the diameter ranges of growth inhibition of pathogenic and conditionally pathogenic bacteria *Escherichia coli* K12, *Escherichia coli* 0111, *Staphylococcus aureus* ATCC 6538P, *Staphylococcus aureus* ATCC 25923 and *Salmonella ssp.* were, respectively, 19–27 mm, 18–28 mm, 10–25 mm, 11–27 mm, and 14–16 mm. Spore-forming bacteria *Bacillus mesentericus* and *Bacillus subtilis* 8-901, which, at a significant content, can reduce the quality of the finished product and cause spoilage of bakery products [24], were characterized by the greatest sensitivity. The diameter range of their growth inhibition under the influence of *Lactobacillus ssp.* was, respectively, 17–30 mm and 18–29 mm. The most sensitive of the three tested yeast species was *Candida albicans* ATCC 1918 – the range of the diameter of inhibition of their growth was 10–15 mm (Table 1).

The obtained data on the high antagonistic activity of *Lactobacillus ssp.*, especially against the yeast *C. albicans* and the spore-forming bacteria *B. subtilis* indicate the high biological potential of the selected strains to prevent spoilage of bakery products.

5. 2. Results of investigating the antifungal activity of lactic acid bacteria strains in vitro

Agar diffusion method. The antifungal activity of 7 strains of the genus *Lactobacillus*, selected at the previous stage for the highest level of antagonistic activity against a wide range of pathogenic and conditionally pathogenic bacteria, yeasts, was studied. The antifungal activity of LAB was tested against *Aspergillus brasiliensis* 16404 and *Fusarium solani* F-50718 – molds that cause spoilage of a wide range of food products and can produce mycotoxins [1]. The results are given in Table 2, Fig. 1.

As a result of our analysis of the size of the zones of inhibition of fungal growth, LABs were divided into three groups: group I – lack of antifungal activity; group II – weak antifungal activity and group III – strong antifungal activity. It was found that 28.6% of LAB strains were ineffective against the applied test strains of fungi, that is, they did not inhibit the growth of both species of fungi (group I). In contrast, the majority of the analyzed LABs (42.8%) inhibited the growth of both species of fungi, but with moderate intensity (group II). And only two LAB strains demonstrated high antifungal activity (group III) (Table 2).

Table 2

Antifungal activity of isolated LAB cultures, agar diffusion method

LAB, strains	Fungi, test strains			
	Aspergillus brasiliensis 16404		*Fusarium solani* F-50718	
	Degree of inhibition*	LAB group	Degree of inhibition*	LAB group
Lactiplantibacillus pentosus XL 3440	+++	III	+++	III
Lacticaseibacillus casei XL 3320	–	I	–	I
Lactiplantibacillus plantarum XL 3225	++	II	++	II
Lacticaseibacillus rhamnosus XL 3332	–	I	–	I
Limosilactobacillus reuteri XL 4024	+++	III	+++	III
Levilactobacillus brevis XL 3911	++	II	++	II
Lentilactobacillus buchneri XL 4126	++	II	++	II
Control No. 1**	+++	III	+++	III
Control No. 2***	–	I	–	I

Note: (–) – no inhibition; (++) – clear zone around well 2; (+++) – clear zone around well more than 2 mm;** – Ca propionate, 0.3%;*** – dihydroquercetin, 0.5%.*

As can be seen in Fig. 1, the largest zones of inhibition of the growth of *Aspergillus brasiliensis* 16404 fungi were detected by strains *Lactiplantibacillus pentosus* XL 3440 and *Limosilactobacillus reuteri* XL 4024 – more than 2 mm, which was similar to the fungicidal activity of Ca propionate, taken as control No. 1.

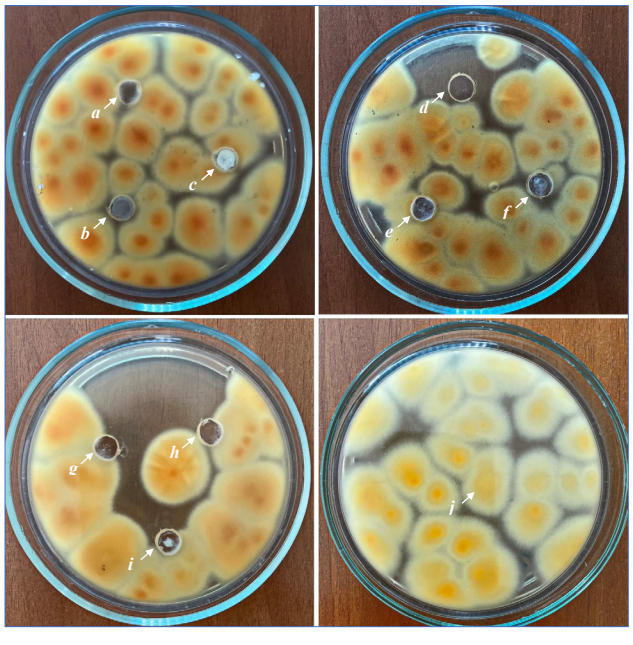


Fig. 1. Antifungal effect of *Lactobacillus ssp.* strains on *Aspergillus brasiliensis* 16404, well method, view from the bottom of the Petri dish: *a – L. casei; b – L. plantarum; c* – dihydroquercetin (0.5%); *d – L. buchneri; e – L. rhamnosus; f – L. brevis; g – L. pentosus; h – L. reuteri; i* – Ca propionate (0.3%); *j – Aspergillus brasiliensis*

Weak fungicidal activity was exhibited by strains *Lactiplantibacillus plantarum* XL 3225, *Levilactobacillus brevis* XL 3911 and *Lentilactobacillus buchneri* XL 4126. On the other hand, strains *Lacticaseibacillus casei* XL 3320, *Lacticaseibacillus rhamnosus* XL 3332 and dihydroquercetin (0.5%), taken as control No. 2, were unable to inhibit the growth of fungi.

Double cultivation method. For a more detailed characterization of the antifungal activity of LAB strains selected by the agar diffusion method, the double cultivation method with *Aspergillus brasiliensis* 16404 was used. It was found that strains *Lactiplantibacillus pentosus* XL 3440 and *Limosilactobacillus reuteri* XL 4024 also exhibited the best antifungal activity under the conditions of this experiment, as well as during the previous testing by the agar diffusion method. On plates with surface inoculation with two parallel lines of each of these LAB strains and the inoculum of *Aspergillus brasiliensis* 16404 introduced into the well on the other side of the plate, after 5 days of cultivation, the absence of fungal growth was recorded (Fig. 2, *d, e*).

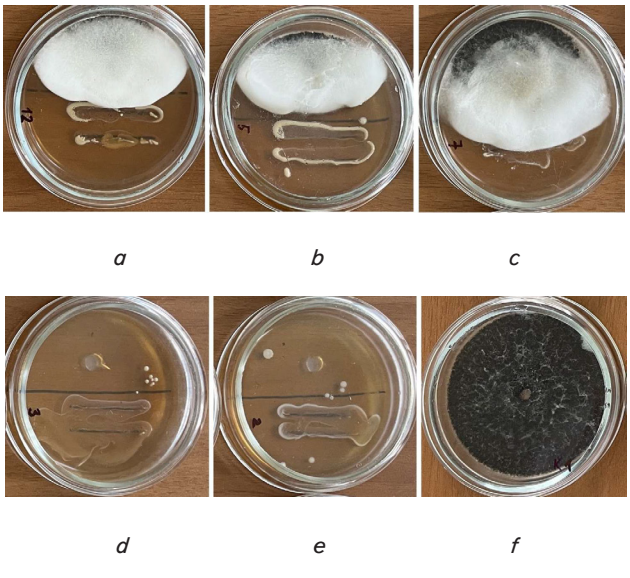


Fig. 2. Antifungal effect of *Lactobacillus ssp.* strains on *Aspergillus brasiliensis* 16404, double cultivation method, view from the upper side of the Petri dish: *a – L. plantarum; b – L. buchneri; c – L. brevis; d – L. pentosus; e – L. reuteri; f – Aspergillus brasiliensis*

The strains *Lactiplantibacillus plantarum* XL 3225, *Lentilactobacillus buchneri* XL 4126, and *Levilactobacillus brevis* XL 3911 exhibited a moderate degree of antifungal activity. It was shown that on ~ 60%, 55%, and 40% of the area of Petri dishes with surface inoculation, respectively, *L. plantarum*, *L. buchneri*, and *L. brevis* there was no growth of fungi (Fig. 2, *a–c*). While on 100% of the area of the Petri dish without LAB inoculation, continuous

growth of the fungus *Aspergillus brasiliensis* 16404 was recorded (Fig. 2, *e*).

5. 3. Results of determining the shelf life of bread on sourdough with LAB strains with high antibacterial and antifungal activity

Strains *Lactiplantibacillus pentosus* XL 3440 and *Limosilactobacillus reuteri* XL 4024, as the most active cultures with antifungal activity, were deposited in the Depository of the Institute of Microbiology, the National Academy of Sciences of Ukraine. From them, a bacterial composition was formed in a ratio between strains as 1:1 for baking sourdough, which was used for baking wheat bread.

During 8 days of storage at 25°C of bread infected with *Aspergillus brasiliensis* 16404, the appearance/absence of fungal colonies on the surface of the products was monitored. The results are given in Table 3.

Table 3

Effect of a sourdough starter with antifungal activity on the shelf life of bread infected with *Aspergillus brasiliensis* 16404*

Bread samples	Shelf life, days							
	1	2	3	4	5	6	7	8
Control sample of unleavened bread, without calcium propionate	–	–	–	+	++	+++	+++	+++
Test sample No. 1 of sourdough bread	–	–	–	–	–	–	+	+
Experimental sample No. 2 of sourdough bread with 0.1% Ca propionate	–	–	–	–	–	–	–	+

Note: (–) – no visible fungal colonies; (+) – one fungal colony; (++) – two fungal colonies; (+++) – three colonies or more.*

As shown in Table 3, already on the 4th day of storage in the central zone of the surface of the control sample of bread made without leaven and calcium propionate, the appearance of the fungus *Aspergillus brasiliensis* 16404 was recorded. That is, in the case of artificial infection of bread, colonies of the fungus sprouted after 4 days of storage, and their growth intensity further increased. Thus, on the 8th day, visible colonies of the fungus were observed even on the sides of the loaf; and at the site of infection – the largest zone of fungal growth with a diameter of up to 28 ± 1 mm (Fig. 3, *a*).



a *b* *c*

Fig. 3. Antifungal activity of sourdough on strains of *Lactobacillus ssp.* against *Aspergillus brasiliensis* 16404 *in situ* after 8 days of storage: *a* — bread without sourdough, without Ca propionate; *b* — sourdough bread; *c* — sourdough bread with 0.1% Ca propionate

The experimental sample of bread No. 1, prepared on sourdough with antifungal strains, remained clean, without signs of fungal growth during a longer storage period compared to the control. Only on the 7th day of storage did a colony of the fungus *Aspergillus brasiliensis* 16404 appear on it (Table 3). As can be seen in Fig. 3, *b*, on the 8th day of storage of the experimental sample of bread No. 1, fungal sporulation was significantly suppressed, the formation of white mycelium was recorded in the area of artificial damage with a diameter of 16 ± 1 mm.

In contrast, in the center of the experimental sample of bread No. 2, prepared on sourdough with the addition of 0.1% calcium propionate, the appearance of growth of the fungus *Aspergillus brasiliensis* 16404 was recorded only on the 8th day (Table 3). And the growth of fungi was significantly suppressed compared to both the control and experimental sample No. 1 (Fig. 3, *c*).

6. Discussion of results based on investigating the antagonistic activity of lactic acid bacteria and their effect on the prolongation of the shelf life of bread

As a result of our analysis of the test data (Table 1), strains of the genus *Lactobacillus* were selected, which demonstrated the highest level of antagonistic activity. They caused the formation of growth inhibition zones of all used test strains of pathogenic and conditionally pathogenic bacteria *Escherichia coli* K12, *Escherichia coli* O11, *Staphylococcus aureus* ATCC 6538P, *Staphylococcus aureus* ATCC 25923, *Salmonella ssp.* and spore-forming bacteria *Bacillus subtilis* 8-901, *Bacillus mesentericus* significantly greater than controls ($n = 5$, $p < 0.05$).

Our results are consistent with the literature data on the high strain specificity of the level and spectrum of antagonistic activity of *Lactobacillus ssp*. For example, in [25], strains of LAB were selected that demonstrated the greatest inhibitory effect against *Escherichia coli*, medium activity against *Staphylococcus aureus*, and low inhibitory effect against *Bacillus subtilis*. And scientists in [26] isolated LAB from 12 traditional Bulgarian starter cultures that suppressed bread spoilage caused by *Bacillus subtilis*, similar to our work.

It should be noted that the intensity of the manifestation of antimicrobial activity of the probiotic strain *Lactobacillus casei* B-7017 and dihydroquercetin, taken as positive controls, was almost at the same high level (Table 1). This indicates the effectiveness of using the antioxidant dihydroquercetin to control extraneous microflora of food products. As reported by other researchers [27], the antioxidant capacity of quercetin also significantly reduced the total phenolic content of bread. Therefore, quercetin fortification could be a promising way to produce functional bread that helps consumers prevent glycation-related diseases.

To assess the antifungal activity of lactobacilli *in vitro*, the yeast-like fungus strain *Candida albicans* ATCC 1918 was initially used as a reference strain according to literature data. A higher level of inhibition of these fungi under the influence of strains of the genus *Lactobacillus* was established compared to *Enterococcus ssp* (Table 1), which was also demonstrated by other researchers. In particular, in [28], a higher antifungal activity of strains of *Lactobacillus spp.* was recorded than in *Leuconostoc spp*. The authors explained this by the slower growth of the latter, which did not allow them to fully demonstrate their antifungal potential. And in [29] it was shown that the strain *Lactobacillus reuteri* RC14 alone or in combination with *Lactobacillus rhamnosus* GR1 can significantly reduce the population of *Candida albicans in vitro*.

Testing the activity of LAB against *Aspergillus brasiliensis* 16404 and *Fusarium solani* F-50718 showed that the growth inhibition of both types of molds was approximately at the same level (Table 2). Therefore, the antifungal activity was further investigated in more detail using only *Aspergillus brasiliensis* 16404, which was classified as *Aspergillus niger* before the taxonomy revision in 2007. The use of this test strain is very common for assessing the effectiveness of preservatives in cosmetic and pharmaceutical preparations, controlling the growth properties and sterility of nutrient media, and assessing the fungicidal activity of chemical disinfectants and antiseptics [30].

It was found that the selected strains *Lactiplantibacillus pentosus* XL 3440 and *Limosilactobacillus reuteri* XL 4024 were characterized by the highest antifungal activity against *Aspergillus brasiliensis* 16404. Similar high antifungal activity of LAB was also recorded by other researchers. Thus, among 60 strains of *Lactobacillus ssp.*, *Lactiplantibacillus pentosus* ŁOCK 0979 was selected as one of the most promising antifungal strains against *Aspergillus brassicicola* and *Aspergillus niger* [31]. In another work, the strains *Lacticaseibacillus paracasei* subsp. *paracasei* CCMA 1764, *Levilactobacillus brevis* CCMA 1762 and *Lactiplantibacillus pentosus* CCMA 1768 were selected, which demonstrated the strongest antifungal activity against *Aspergillus flavus*, *Aspergillus carbonarius*, *Penicillium nordicum* and *Penicillium expansum*. The strains inhibited mycelial growth, resulting in a significant reduction in the production of aflatoxin B1, ochratoxin A, and patulin [32].

In our work, it was found that the intensity of antifungal activity of *Lactiplantibacillus pentosus* XL 3440 and *Limosilactobacillus reuteri* XL 4024 was on a par with the effect of Ca propionate (0.3%), which is conventionally added to bread formulations in this concentration as a chemical inhibitor of mold growth (agar diffusion method, Fig. 1; double cultivation method, Fig. 2). However, chemical preservatives, with prolonged consumption, can accumulate in the human body and cause chronic diseases. In addition, many fungal strains are not affected by physicochemical treatment due to various resistance mechanisms that they have acquired over time. Therefore, it is so important to conduct research aimed at finding alternative inhibitory agents of natural origin – LAB to replace synthetic ones.

In situ experiments showed that bread made with sourdough with antifungal strains *Lactiplantibacillus pentosus* XL 3440 and *Limosilactobacillus reuteri* XL 4024 remained clean after infection with *Aspergillus brasiliensis* 16404, without signs of fungal growth during 6 days of storage (Table 3). According to the literature, the delay time of mold growth can be considered a direct indicator for determining the shelf life of bread. Based on the experimental data obtained, the shelf life of wheat bread made with sourdough with antifungal strains was prolonged from 3 to 6 days.

Our results are consistent with the data by other scientists. In particular, in [33] it was shown that when *Limosilactobacillus reuteri* CRL100 was used as part of a mixed culture with antifungal properties, the shelf life of bread was extended to 5 days. A maximum shelf life of 8 days was established for bread with 0.4% Ca propionate, or with a combination of 0.2% Ca propionate and sourdough [33]. As a result of another study, the use of a mixed culture of four LAB strains (1:1:1:1:1) resulted in a reduction in the use of calcium propionate to 0.15% and an extension of the shelf life of bread to 5 days [34]. No visible fungal colonies were detected on bread fermented with *Lactiplantibacillus plantarum* AR524 up to 7 days. The shelf life of bread was extended from 3 to 5 days; and when the sourdough was combined with 0.1% Ca propionate, the shelf life of bread was extended to 7 days compared to yeast bread. The *Lactiplantibacillus plantarum* AR524 strain was recognized as a promising protective culture for bakery products, replacing traditional chemical additives [35].

In this work, the shelf life of bread was extended to 8 days when combining antifungal starter with 0.1% Ca propionate (Table 3). These differences may be due to the properties of the LAB strains used.

The limitations of the study are associated with the use of only one bakery starter with two selected strains of the genus *Lactobacillus*, which makes it impossible to extrapolate scientific results to starters with a different bacterial composition. In addition, our study concerns only wheat bread; when using a different flour matrix, the results of the studies may differ from those given in the article.

The shortcomings of the work include the lack of determining the content of metabolic activity products of lactic acid bacteria during the technological process, which makes it difficult to establish a reliable relationship between the activity of the starter microbiota and the shelf life of wheat bread.

Further research might aim at investigating the effect of sourdough with strains *Lactiplantibacillus pentosus* HL 3440 and *Limosilactobacillus reuteri* HL 4024 on the formation of biologically active compounds and rheological characteristics of bread.

7. Conclusions

1. As a result of *in vitro* analysis using the "well" method, strains of *Lactobacillus ssp.* were selected with high antimicrobial activity against pathogenic, conditionally pathogenic bacteria *Escherichia coli* K12, *Escherichia coli* O11, *Staphylococcus aureus* ATCC 6538P, *Staphylococcus aureus* ATCC 25923, *Salmonella ssp.*; spore-forming bacteria *Bacillus subtilis* 8-901, *Bacillus mesentericus* and yeast *Candida albicans* ATCC 1918, *Candida tropicalis* Y-2473, *Candida utilis* Y-984. *Lactobacillus ssp.* strains caused the formation of growth inhibition zones of all used test strains significantly larger than the controls: the biotechnologically active strain *Lactobacillus casei* B-7017 from the IPR collection and dihydroquercetin – an antioxidant of plant origin ($n = 5$, $p < 0.05$). It was recorded that the intensity of antimicrobial activity of the *Lactobacillus casei* B-7017 strain and dihydroquercetin was at the same high level. This justifies the feasibility of using the antioxidant dihydroquercetin to control extraneous microflora in food products.

2. As a result of *in vitro* analysis by the "well" method and diffusion into agar, it was found that the intensity of antifungal activity of strains *Lactiplantibacillus pentosus* XL 3440 and *Limosilactobacillus reuteri* XL 4024 was at the same level as the effect of Ca propionate (0.3%) – a chemical inhibitor of the growth of mold fungi. The strains were deposited at the Depository of the Institute of Microbiology, the National Academy of Sciences of Ukraine, which allows them to be used in food technologies. A bacterial composition was formed from them at a ratio between strains of 1:1 for baking sourdough.

3. It was found that the use of sourdough starter on pure cultures of *Lactiplantibacillus pentosus* XL 3440 and *Limosilactobacillus reuteri* XL 4024 ensured microbiological, fungicidal purity, and extended the shelf life of wheat bread to 6 days. With this dough preparation technique, the shelf life of wheat bread increased by 3 days compared to the traditional protocol using yeast. It was found that the shelf life of bread on antifungal starter and with the addition of 0.1% Ca propionate can be extended to 8 days.

Conflicts of interest

The authors declare that they have no conflicts of interest in relation to the current study, including financial, personal, authorship, or any other, that could affect the study and the results reported in this paper.

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Data availability

All data are available, either in numerical or graphical form, in the main text of the manuscript.

Use of artificial intelligence

The authors confirm that they did not use artificial intelligence technologies when creating the current work.

Author contributions

Oksana Naumenko: Conceptualization, Formal analysis, Investigation, Resources, Data Curation, Writing – original draft, Writing – review & editing, Visualization, Supervision, Project administration; **Ivanna Lukianchuk:** Conceptualization, Investigation, Resources, Data Curation, Writing – review & editing, Visualization, Project administration; **Valentyna Chyzh:** Formal analysis, Investigation, Data Curation, Writing – review & editing, Visualization; **Tsvitana Korol:** Conceptualization, Software, Data Curation, Writing – review & editing; **Natalia Shulga:** Investigation, Writing – review & editing, Visualization; **Sergiy Narizhnyy:** Formal analysis, Data Curation, Writing – review & editing; **Sergiy Bobokalo:** Formal analysis, Resources, Data Curation; **Nataliia Demianchuk:** Formal analysis, Data Curation, Writing – review & editing.

All authors have read and agreed with the published version of the manuscript and are jointly responsible for the reliability, accuracy, and integrity of the data and research results reported in the paper.

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