EXPERIMENTAL ARTICLES

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THE PROBIOTIC PROPERTIES OF *Lactiplantibacillus plantarum* ISOLATED FROM PLANT MATERIAL

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Aim. To identify and study the probiotic properties of a typical representative of lactic acid bacteria isolated from maize sourdough.

Methods. The sourdough was prepared by mixing flour with water, followed by incubation for 24 hours. The species identity of *Lactiplantibacillus plantarum* was determined based on phenotypic characteristics. Stress resistance was assessed by evaluating cell viability after exposure to artificial saliva, low-pH saline solution, and a mixture of bile and simulated duodenal juice. Antibiotic susceptibility was determined using the disk diffusion method with reference values, while autoaggregation ability was evaluated by cell sedimentation through centrifugation and absorbance measurement using a spectrophotometric method.

Results. The isolate was identified as *L. plantarum*. Its survival rates under simulated conditions of the oral cavity, stomach, and duodenum were 97.13±1.12%, 95.06±0.52%, and 91.67±1.66%, respectively. The strain was sensitive to erythromycin, ampicillin, and chloramphenicol, moderately sensitive to streptomycin and tetracycline, and resistant to benzylpenicillin and kanamycin. Autoaggregation levels after 2 and 24 hours were 6.88±0.1% and 41.83±0.4%, respectively.

Conclusions. *L. plantarum* isolated from maize sourdough demonstrated high-stress resistance, sensitivity to several antibiotics (although resistance to kanamycin and benzylpenicillin requires further investigation), and sufficient autoaggregation capacity for a probiotic strain.

Key words: probiotics, lactic acid bacteria, *Lactiplantibacillus plantarum*, maize, gastrointestinal tract survival, antibiotic resistance, autoaggregation.

The origins of probiotics can be traced back to the dawn of human civilization, to the times of the ancient Egyptians, Phoenicians, and Eastern peoples, as they are closely linked to the history of fermented foods — milk, bread, pickled vegetables, wine, and others. The term "probiotic" was first used by the German scientist W. Kollath in 1953. Still it gained a meaning closer to the modern one only in 1992, when Fuller defined it as "a live microbial feed supplement that beneficially affects the host animal by improving its intestinal microbial balance" [1].

Today, probiotics are considered microorganisms whose metabolic products exert a nonspecific positive effect on the

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human body as a whole rather than on a specific group of cells or tissues. This effect results from a combination of various characteristics inherent in the metabolic products of probiotic microorganisms, though none of these traits are unique to a single species. Due to their ability to adhere to the intestinal mucosa, competitively exclude pathogenic microorganisms, strengthen the epithelial barrier, and produce antimicrobial substances as metabolic byproducts, probiotic microorganisms colonize the human intestine and help the body combat potential pathogens. Additionally, some probiotics have immunomodulatory properties, meaning they can regulate immune cell activity and cytokine production, making them effective in treating inflammatory bowel diseases, gastric ulcers, urinary tract infections, and many other conditions [2]. It is even known that combining radiotherapy with the intake of certain probiotics has, to some extent, improved cancer treatment [3]. However, the beneficial effects of probiotic preparations are not limited to protecting the gastrointestinal tract (GIT) from harmful bacteria or viruses entering the body from external sources.

The metabolic products of probiotic microorganisms possess mechanisms that reduce total cholesterol and low-density lipoproteins, which affect the severity of symptoms in diabetes mellitus. By increasing serum vitamin D levels and enhancing the production of short-chain fatty acids, which contribute to bone formation, probiotics are effective in treating osteoporosis. Additionally, by preventing oxalate formation through its enzymatic degradation by specific bacterial enzymes, such as oxalyl-CoA decarboxylase or formyl-CoA transferase, chronic kidney diseases can be successfully treated [4]. Probiotics can even prevent or alleviate the progression of neurodegenerative diseases. Studies show that probiotic supplementation helps ease neurodegenerative symptoms in Parkinson's disease [5], Alzheimer's disease, and mild cognitive impairments [6].

Probiotic strains include yeasts and various bacteria, but mainly lactic acid bacteria (LAB). Sources of probiotic microorganisms include environmental objects (soil, water), humans and animals (saliva, breast milk, GIT), and food (milk, fermented dairy products, fruits, vegetables, fermented meat products, etc.) [7]. Sourdough starters of various types hold great potential for isolating probiotic candidates. Much attention has been given to isolating and studying microorganisms as potential probiotics from sourdoughs based on wheat, rye, and spelled flour.

However, the potential probiotic properties of microbial isolates from maize-based sourdoughs have not been studied [8], and the last research examining the functional properties of bacteria obtained from maize flour-based sourdough could be found dates back to 2008 [9].

Therefore, in this study, we aimed to identify and investigate the probiotic properties of LAB isolated from maize sourdough.

Materials and Methods

The preparation of sourdough was carried out according to the method described by McKenney et al. [10]. Maize flour (obtained from dried and ground *Zea mays* grains) and water were mixed in a 1:1 ratio (100 g : 100 g), after which the mixture was incubated for 24 hours at 30 °C.

The isolation of LAB was performed according to the method described by Taccari et al. [11], with modifications, namely: by suspending 10 g of the obtained sourdough in 100 g of peptone solution (8.5 g/L) and subsequently plating the resulting suspension onto the following media: modified MRS (mMRS) (De Man, Rogosa, Sharpe) and mMRS+AV (mMRS with ampicillin and vancomycin) according to [12], MRS with an indicator (bromothymol blue) according to [13], and acetate agar according to [14]. Microorganisms were isolated in liquid mMRS medium, followed by microscopy (1000×) and purification through repeated subculturing until a pure culture was obtained.

The purity of the culture was verified by microscopy after staining the smears with crystal violet for 2 minutes. The identification of LAB was confirmed through microscopy (cell morphology and Gram staining), a catalase test, the ability to ferment milk at 30 °C, 37 °C, and 45 °C, and gelatin liquefaction, following the methods described by Sharpe [15].

Phenotypic identification was performed by assessing the culture's ability to ferment a range of carbohydrates (glucose, lactose, sucrose, fructose, maltose, mannose, mannitol, glycerol, sorbitol, inositol, raffinose, rhamnose, galactose, ribose, trehalose, xylose). Additionally, the culture's gas production capability in liquid mMRS with glucose was tested using a Durham tube. Species identification was carried out using the ABIS

online service [16], designed for bacterial identification based on morphological and biochemical characteristics.

To assess probiotic properties, the isolated LAB strains were evaluated for stress resistance, antibiotic sensitivity, and autoaggregation ability. The assessment of survival in GIT conditions was conducted according to Kim et al. [17], with modifications. The experiment was performed in triplicate. The tested strain was cultivated in 10 mL of MRS at 37 °C for 24 hours, after which it was transferred into 100 mL of artificial saliva solution with the following composition (according to Pytko-Polonczyk et al. [18]): 100 mL 25 mM KH_2PO_4, 100 mL 24 mM Na_2HPO_4, 100 mL 150 mM $KHCO_3$, 100 mL 100 mM NaCl, 100 mL 1.5 mM $MgCl_2$, 6 mL 25 mM citric acid, 100 mL 15 mM $CaCl_2$. Samples from the obtained suspensions were taken, serially diluted, plated onto Petri dishes with MRS agar for colony counting, and incubated at 37 °C for 72 hours. The bacterial suspensions in artificial saliva were incubated in a thermostat at 37 °C for 30 minutes, after which additional samples were taken for plating, incubation for 72 hours, and colony counting.

After that, 10 mL of the saliva suspension was aseptically transferred into 100 mL of physiological saline solution (0.85% NaCl), with its pH adjusted to 2.5 by adding 14% HCl to simulate bacterial passage through the stomach. The suspensions were incubated at 37 °C for 1 hour, after which samples were retaken for colony counting (adjustments in dilutions were accounted for here and in subsequent steps).

Next, 10 mL of the suspension was again aseptically transferred into a bile solution with artificial duodenal juice. The solution was prepared as follows: 4 mL of a 10% purified dry bile solution was mixed with 17 mL of artificial duodenal juice (6.4 g/L $NaHCO_3$, 0.239 g/L KCl, 1.28 g/L NaCl; pH adjusted to 6.0). The suspensions were incubated at 37 °C for 2 hours, after which samples were taken for colony counting.

Survival was calculated after each stage of passage through the simulated gastrointestinal tract using the following formula:

$$SR = \frac{LgN_t}{LgN_{t0}} \times 100\%$$

where N_{t0} — CFU/mL before passage, N_t — CFU/mL at the end of the respective stage.

The determination of antibiotic susceptibility was performed using the disk diffusion method according to [19] with modifications. Specifically, 1 mL of the tested 24-hour culture was evenly spread onto MRS agar in Petri dishes, followed by the placement of antibiotic disks (ERY — erythromycin, AMP — ampicillin, LEV — levomycetin (chloramphenicol), STR — streptomycin, BPZ — benzylpenicillin (penicillin G), KAN —kanamycin, TET — tetracycline) and incubation at 37 °C for 24 hours. Each antibiotic was tested in triplicate. The results were measured using a ruler from the bottom side of the Petri dishes and categorized as follows: resistant — R, intermediate — I, susceptible — S. The study and interpretation of antibiotic susceptibility data were conducted following the guidelines developed by Charteris et al. [20] (Table 1).

The autoaggregation ability was assessed according to the method described by Collado et al. [21]. The cells of the studied culture were collected by centrifugation at 3,500 *g* for 15 minutes, then resuspended in phosphate-buffered saline (PBS) to a concentration of approximately 10^7–10^8 CFU/ml and incubated at 37 °C. Samples of the supernatant were taken at the beginning, as well as after 2 and 24 hours, and their absorption at a wavelength of 600 nm was measured using a spectrophotometer. All measurements were performed in triplicate. Autoaggregation was calculated using the formula:

$$A(\%) = \frac{A_0 - A_t}{A_0} \times 100\%$$

where A_0 — the absorption at 0 hours, A_t — the absorption at 2 and 24 hours.

Results and Discussion

During the isolation of a pure culture from maize sourdough, 16 isolates were obtained, which, based on their morphological, cultural, and physiological-biochemical characteristics, were identified as representatives of a single species — *L. plantarum*. The morphological and cultural characteristics of the isolates are presented in Table 2.

Analysis of the morphological and cultural characteristics of the 16 isolates revealed their morphological and cultural uniformity. Since no other morphological forms were detected, this indicates the predominance of a single variant of isolates. Therefore, isolate No. 1 was selected for further study (Fig. 1).

Table 1. **Diameters of inhibition zones for determining antibiotic susceptibility** (according to V. Charteris et al.) [20]

Antibiotic	Concentration in disk, μg	Zone diameter, mm		
		R	I	S
ERY	15	≤13	14–17	≥18
AMP	10	≤12	13–15	≥16
LEV	30	≤13	14–17	≥18
STR	10	≤11	12–14	≥15
BPZ	10	≤19	20–27	≥28
KAN	30	≤13	14–17	≥18
TET	30	≤14	15–18	≥19

Table 2. **Morphological and cultural characteristics of the isolates**

Cultivation medium	Isolate №	Cell morphology	Cultural properties of the isolated strain
mMRS	1	Straight or slightly curved small rods occurring singly, in pairs, or short chains. Gram-positive. Non-motile. Do not form spores, flagella, or capsules	Shape: round with smooth edges Colony diameter: d = 2–4 mm Color: opaque creamy Surface: smooth and shiny Elevation: convex Consistency: soft
MRS with an indicator	2		
mMRS+AV	3		
mMRS+AV	4		
Acetate agar	5		
mMRS	6		
Acetate agar	7		
mMRS+AV	8		
MRS with an indicator	9		
mMRS	10		
Acetate agar	11		
mMRS	12		
Acetate agar	13		
MRS with an indicator	14		
mMRS	15		
mMRS	16		

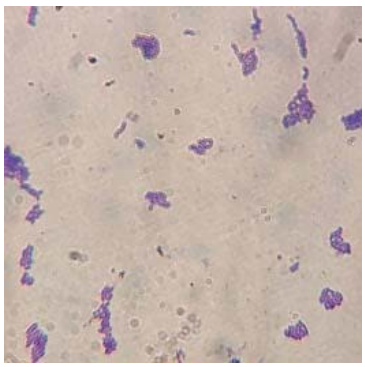

Fig. 1. **Isolated microorganism under a microscope (1000×)**

The isolated microorganism belongs to the group of facultatively heterofermentative LAB, which ferments hexoses (Table 3) into lactic acid via the Embden-Meyerhof-Parnas pathway. Additionally, they are capable of utilizing pentoses (in this case, ribose) as a carbon and energy source through the pentose phosphate pathway. The classification as facultative rather than obligate heterofermentative LAB is confirmed by the absence of gas production in liquid mMRS with glucose using a Durham tube during aerobic cultivation [22].

Based on the results of phenotypic tests entered into the ABIS online service, it was determined that the microorganism isolated from maize sourdough has a 96.9% similarity to *L. plantarum* and a 99.8% probability of belonging to this species. *L. plantarum* is frequently isolated from various flour-based products [8], including those made from maize flour [9] or a combination of maize and rye flour [23].

Table 3. **Analysis of the phenotypic characteristics of the isolated strain**

Characteristic		Result
Growth at different temperatures	15 °C	+
	45 °C	–
Milk fermentation at different temperatures	30 °C	+
	37 °C	+
	45 °C	–
Carbohydrate	Glucose	+
	Lactose	+
	Sucrose	+
	Fructose	+
	Maltose	+
	Mannose	+
	Mannitol	+
	Glycerin	–
	Sorbitol	+
	Inositol	–
	Raffinose	+
	Rhamnose	–
	Galactose	+
	Ribose	+
	Trehalose	+
	Xylose	–
Gelatin hydrolysis		–
Arginine hydrolysis		–
Gas production		–

The survival rate of the studied isolate during passage through the simulated GIT, specifically through artificial saliva solution, physiological saline with pH 2.5, and a bile mixture with synthetic duodenal juice, was 97.13±1.12%, 95.06±0.52%, and 91.67±0.88%, respectively (Fig. 2).

When comparing the survival results of this strain with those of other *L. plantarum* strains tested using the same methods — specifically *L. plantarum* ST63HK and ST66HK, as studied by Kim et al. [17] — the examined isolate demonstrated significantly higher survival under low pH conditions (approximately 50% and 72% survival for ST63HK and ST66HK, respectively) and in bile solution (approximately 50% and 56% survival, respectively).

A high survival rate of *L. plantarum* after 1 hour of incubation at pH 2.5 has also been reported by other authors: Li et al. [24] described five similar strains isolated from wheat sourdough, while Bartkiene et al. [25] studied an acid-tolerant *L. plantarum* strain isolated from rye sourdough, among others.

The survival rate characteristic of cultures used in commercial probiotic preparations, as determined through a multistage *in vitro* study [26], ranges from 45.45 % to 91.07 % and is a strain-specific trait. Given these values, it can be concluded that the isolate under study exhibits superior stress resistance, making it noteworthy even when compared to commercially available strains.

The measured diameters of antibiotic susceptibility zones are presented in Fig. 3.

The studied isolate is sensitive to erythromycin, ampicillin, and chloramphenicol, moderately sensitive to streptomycin

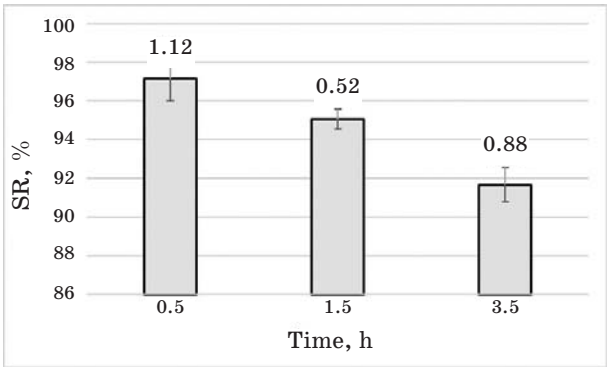

Fig. 2. **Survival of *L. plantarum* during passage through the simulated GIT**

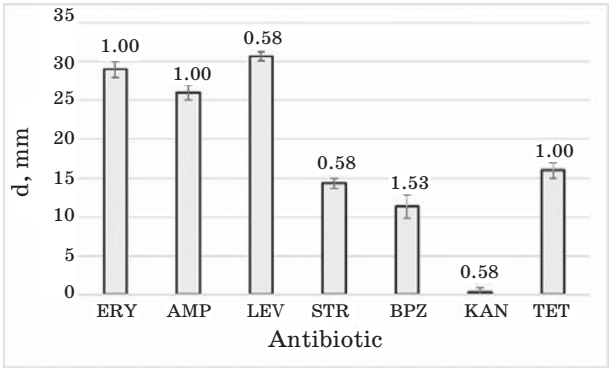

Fig. 3. **Sensitivity of *L. plantarum* to selected antibiotics**

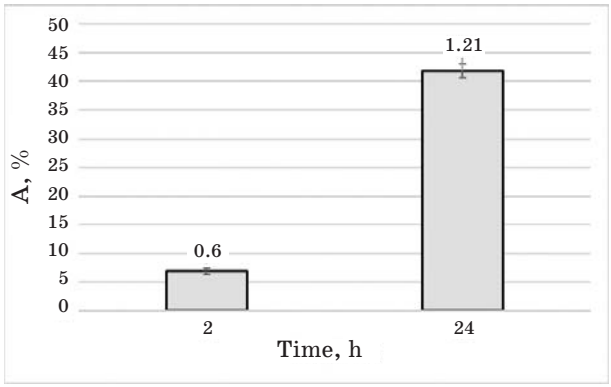

Fig. 4. **Autoaggregation of *L. plantarum***

and tetracycline, and resistant to benzylpenicillin and kanamycin. According to the recommendations of the European Food Safety Authority (EFSA) [27], microorganisms that enter the human GIT should be sensitive to critically essential antimicrobials due to the potential risk of transferring antibiotic resistance genes. *L. plantarum* exhibits good sensitivity, except for kanamycin, to which it is resistant (sensitivity to benzylpenicillin is not a requirement by EFSA). However, numerous studies [15, 28, 29] indicate that isolating microorganisms that fully comply with antibiotic susceptibility requirements is rare, though not impossible, including for other *L. plantarum* strains [30]. Moreover, the study by Feng et al. [31] suggests that kanamycin resistance genes in *L. plantarum* are highly unlikely to be located on mobile genetic elements, eliminating the risk of horizontal gene transfer. All of the above indicates that while there are some concerns regarding the antibiotic susceptibility of the studied microorganism, further investigation and evaluation are necessary.

The calculated autoaggregation after 2 and 24 hours was 6.88±0.6 % and 41.83±1.21%, respectively (Fig. 4). When compared to the values obtained for commercial probiotic strains by Collado et al. [21], it is evident that these values are significantly lower than those for *L. plantarum* Lp-115 (21.7±5.5% at 2 hours and 76.4±8.3% at 24 hours). However, they are similar to those of other microorganisms, such as *Lacticaseibacillus rhamnosus* LC-705 (7.4±1.3% at 2 hours and 38.7±8.8% at 24 hours). Similar or even lower values have also been observed in representatives of the *L. plantarum* species. For instance, in strains isolated from cheeses made with raw milk, autoaggregation after 24 hours varies from 29.32±1.46% to 59.51±1.51% [32].

From this, it can be concluded that in terms of autoaggregation, the studied isolate performs significantly worse than many other LABs with potential or confirmed probiotic properties. Despite this, it demonstrates a sufficient level to be of both scientific and practical interest.

Conclusions

The study examined the stress resistance, antibiotic susceptibility, and autoaggregation ability of *L. plantarum* isolated from maize sourdough. The strain demonstrated a significantly high level of stress resistance, which is an essential probiotic characteristic. It also exhibited a high level of sensitivity to several antibiotics, except for kanamycin and benzylpenicillin, to which it was resistant. This phenomenon requires further investigation, as it may either indicate the strain's ineligibility for further use if resistance genes are located on mobile genetic elements or, conversely, pose no obstacles. The calculated autoaggregation values for the isolate were moderate. *L. plantarum* isolated from maize sourdough shows excellent potential for use as a probiotic but requires further research and evaluation.

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Conflict
There was no conflict of interest.

Authors' contribution
All authors contributed equally to the conception and design of the study, data collection, analysis, and interpretation. Each author participated in drafting and revising the manuscript and approved the final version for submission.

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ПРОБІОТИЧНІ ВЛАСТИВОСТІ *Lactiplantibacillus plantarum*, ВИДІЛЕНОГО З РОСЛИННОЇ СИРОВИНИ

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Мета. Ідентифікація та вивчення пробіотичних властивостей типового представника молочнокислих бактерій, виділеного з кукурудзяної закваски.

Методи. Закваску отримували шляхом змішування борошна й води та інкубації упродовж 24 год. Видову належність культури *Lactiplantibacillus plantarum* визначали за фенотиповими характеристиками. Стресостійкість оцінювали за виживаністю клітин після оброблення їх штучною слиною, фізіологічним розчином з низьким значенням pH і сумішшю жовчі зі штучним соком дванадцятипалої кишки. Чутливість до антибіотиків визначали диско-дифузійним методом із використанням референтних значень, здатність до автоагрегації — шляхом осадження клітин центрифугуванням та оцінюання значень абсорбції спектрофотометричним методом.

Результати. Ізолят належить до виду *L. plantarum*. Його виживаність за модельованими умовами ротової порожнини, шлунку та дванадцятипалої кишки становила 97,13±1,12 %, 95,06±0,52% і 91,67±1,66% відповідно. Встановлено чутливість до еритроміцину, ампіциліну та хлорамфеніколу, помірну чутливість до стрептоміцину й тетрацикліну та стійкість до бензилпеніциліну та канаміцину. Автоагрегація через 2 та 24 год становила 6,88±0,6% і 41,83±1,21% відповідно.

Висновки. *L. plantarum*, виділений із кукурудзяної закваски, має високі показники стресостійкості, чутливість до низки антибіотиків (хоча стійкість до канаміцину та бензилпеніциліну вимагає подальшого вивчення) та достатні для пробіотика показники автоагрегації.

Ключові слова: пробіотики, молочнокислі бактерії, *Lactiplantibacillus plantarum*, кукурудза, виживання у шлунково-кишковому тракті, антибіотикостійкість, автоагрегація.