JJBS

Volume 16, Number 1,March 2023
ISSN 1995-6673
Pages 63 – 71
https://doi.org/10.54319/jjbs/160109

Jordan Journal of Biological Sciences

The ability of *Lactobacillus helveticus -13* (*Lh-13*) Isolate Isolated from Lactic Acid Products to form A biofilm by Applying Modern Microscopy Methods

Zhanerke Amirkhanova[1,2], Saule Akhmetova[2,*], Samat Kozhakhmetov[3], Almagul Kushugulova[3], Rahat Bodeeva[1], Leila Ahvlediani[4], Anara Turmukhambetova[2]

[1]Buketov Karaganda University, University Street, 28, Karaganda, Kazakhstan; [2]Karaganda medical university, Gogol Street, 40, Karaganda, Kazakhstan; [3]Laboratory of Human Microbiome and Longevity "National Laboratory Astana" Nazarbayev University, Kabanbay Batyr Ave., 53, Astana, Kazakhstan; [4]Batumi International University, Fridon khalvashi street, 237, Batumi, Georgia.

Received: May 30, 2022; Revised: October 8, 2022; Accepted: October 21, 2022

Abstract

This article presents the study of the morpho-cultural properties and biofilm formation of lactobacilli isolated from lactic acid products (irimchik, suzbe, cheese, ashygan kozhe) produced in different districts of the Karaganda region. The species of the isolated lactic acid bacillus isolates were identified by using a MALDI-TOF mass spectrometer.

The evaluation of the resistance of the isolates to various stress factors, such as the ability to survive at low acidic pH values and in the presence of bile, made it possible to identify some promising isolates – *Lactobacillus helveticus -13* (*Lh-13*) and *Lactiplantibacillus plantarum* – 5 (*Lpl-5*), for studying antimicrobial and biofilm-forming activity.

The antimicrobial activity of *Lh-13*, *Lpl-5* was closely studied. They have a certain level of antibacterial and fungicidal activities, which indicates their pronounced inhibition against indicator microorganisms.

Using a laboratory robot, *TecanEVolizer100 (Tecan)*, the optical density of *Lh-13*, *Lpl-5* was studied in the wells of polystyrene plates, reflecting the intensity of biofilm formation along their surface. Using laser capture microdissection, the images of cell morphology in biofilm of *Lh-13* isolates included in the matrix were obtained. The atomic force microscopy made it possible to study the two and three-dimensional morphological images of biofilm-forming isolates of *Lh-13*.

Keywords*: lactobacilli, mass spectrometry (MALDI-TOF MS), biofilm, laser capture microdissection, atomic force microscopy, antimicrobial activity.*

1. Introduction

The advances in the sciences of human and animal microbiota confirm the beneficial health effects of lactic acid foods and probiotics containing probiotic bacteria, which can adhere and colonize on the inner wall of the intestinal tract, form microbial communities, and regulate the balance of intestinal microflora, etc. (Hill *et al.,* 2014; George *et al.,* 2018; Yue *et al.,* 2020; Bechelaghem *et al.,* 2022).

There are many lactic acid products and probiotics on the market right now. They are used to provide alleged health benefits based on the probiotic properties of specific strains of lactic acid bacteria (Casey *et al.,* 2004; Mitropoulou *et al.,* 2013; Singh *et al.,* 2012). The effectiveness of probiotic agents and functional food products primarily depends on the properties of the species of various strains of lactic acid bacteria included in their composition. Lactobacilli are a key component of starter cultures for such products (Corona-Hernandez *et al.,* 2013; Taverniti *et al.,* 2014). Lactobacilli, along with other members of the normal microbiota of human and animal mucous membranes, plays an important role in the body's anti-infectious defense and the formation of microorganism colonization resistance (Ghosh *et al.,* 2019; Zeng *et al.,* 2020).

In the modern biomedical practice, they can be used as methods of personalized medicine: the probiotic culture of lactobacilli exhibits antimicrobial activity against the pathogenic flora of the patient as well as stimulates antagonism and is not inhibited by pathogens (Ejtahed *et al.,* 2017; Torres and Tovar, 2021; Schupack *et al.,* 2022).

The viability and survival of lactobacilli under stressful conditions are the most important parameters for ensuring therapeutic functions. This parameter is also considered an important criterion for the selection of an active probiotic strain. In addition, the beneficial properties of lactobacilli are in their ability to form biofilms, which allows them to withstand environmental conditions, resulting in successful colonization and maintenance of their population (Guarner *et al.,* 2005; Son *et al.,* 2017).

A biofilm, according to current concepts, is a community of microorganisms connected by the surface and enclosed in a matrix composed of extracellular polymeric substances synthesized by them, as well as polysaccharides and proteins. Biofilm formation by probiotic bacteria such as lactic acid bacteria is considered

* Corresponding author. e-mail: S.Ahmetova@qmu.kz.

a beneficial property as it can promote colonization and longer persistence in the host mucosa avoiding colonization by pathogenic bacteria (Salas-Jara *et al.,* 2016; Tatsaporn and Kornkanok , 2020).

The scientifically established phenomenon of the protective function of the lactobacilli's biofilm is promising for further use in the medical, pharmaceutical, food and agricultural industries, which is a priority for Kazakhstan (Aitzhanova *et al.,* 2020).

In this regard, the above mentioned conditions prompted the search for new unique candidates for probiotic cultures of lactobacilli with highly active probiotic properties having *the GRAS status* (*generally recognized as safe)* (Wu *et al.,* 2017).

The search and study of the probiotic properties of domestic competitive starter cultures isolated from traditionally natural ethnical products, such as those traditionally produced, is an effective way to obtain genetically stable strains of probiotic cultures as a constant source of the strains for the needs of the country to improve the quality of microbiological resources in the national stocks.

The present study is aimed at screening the probiotic properties (tolerance to acidic pH and bile) of lactic acid bacillus isolates isolated from national lactic acid products in order to assess the ability to form biofilms and to study the morphology of cells in biofilm using laser capture microdissection and atomic force microscopy.

2. Materials and methods

Materials. For the given study, the isolates of lactobacilli isolated from traditional home-made national lactic acid products produced in different districts of the Karaganda region, Kazakhstan were used (Table 1).

Table 1. National lactic acid products and number of samples from different regions.

National lactic acid products (Number of samples)	Regions				
	Karkaraly	Zhanaarka	Aktogay	Bukhar-Zhyrau	Osakarov district
Irimchik	2	1	3	2	-
Suzbe	2	2	3	2	-
Cheese	-	-	-	3	1
Ashygan kozhe	1	-	2	-	-
Total	24				

Note: The results of the primary screening are published earlier in the given work: «Screening of Antimicrobial and Adhesive Activity of Lactobacilli Isolated from the National Food Products from Different Districts of the Karaganda Region (Kazakhstan)» Open Access Macedonian Journal of Medical Sciences. 2021 Sep 24; 9(A):827-832.

2.1. Isolation and identification of lactobacilli from dairy products

One of each sample (irimchik, suzbe, cheese, ashygan kozhe) was taken aseptically and transferred to a 10 ml phosphate buffered saline and then vortexed. The 1 ml of the produced homogenate was then inoculated in a 10 ml MRS broth contained in screw caped tube, and incubated under anaerobic conditions for 24 hours at 37°C. Further on, the serial dilutions prepared from each obtained culture were spread over the MRS (deMan, Rogosa, Sharpe) agar-1 plates and incubated anaerobically for 48 hours at 37° C (Lin *et al.,* 2006; Mulaw *et al.,* 2019). After incubation, the isolated colonies underwent both the Gram stain and catalase test. Only rod-shaped, gram-positive, catalase-negative isolates were selected for the tests. The individual colonies were subcultured in the MRS broth for 48 hours at 37°C, and the control smears were taken. The selected strains were stored in the MRS broth containing 20% glycerol at -22°C for further analysis. The isolates were activated in the MRS broth prior to each analysis (Wang *et al.,* 2016).

The pure cultures of lactobacilli were identified using MALDI-TOF MS (*Matrix Supported Laser Desorption / Ionization Flight Time Mass Spectrometry, Bruker, Germany*). The bacterial spectra were matched against the MALDI-TOF MS biotype reference library (Alatoom *et al.,* 2011; Schulthess *et al.,* 2014).

2.2. Tolerance to acidic pH and bile

The acid resistance of the bacterial isolate was observed by incubating lactobacilli at pH3 in the MRS broth. 9 ml of the acidified MRS broth (adjusted to pH3 by 5N HCl) and 1 ml of the MRS broth containing 10^9 CFU/ml of the test isolate of lactobacilli were added to each tube. The tubes with the suspension were further incubated at 37°C for 24 hours under anaerobic conditions. The growth of the isolates was monitored for 0h, 3h and 6h by measuring the absorbance of the culture broth at 492 nm using a spectrophotometer. The growth of lactobacilli in the MRS broth without HCl was used as a control (Kim *et al.,* 2019). The experiment was repeated three times.

The isolated isolates were tested for bile tolerance. To study the tolerance of lactic acid bacillus isolates to bile, a medicinal bile preparation containing natural gallbladder bile of cattle was used. Bile medicine tolerance of the isolated bacteria was examined by inoculating the freshly cultured isolates at 10^7 CFU / ml into the MRS broth containing bile medicine at different concentrations (0.3%; 0.5%; 1%). The medium was then incubated at 37°C in anaerobic conditions. The growth of the isolates was monitored at 0h and 3h by measuring the absorbance of the culture broth at 492 nm using a spectrophotometer. The growth of lactobacilli in the MRS broth without bile was used as a control (Tambekar and Bhutada, 2010). The experiment was repeated three times.

2.3. Antimicrobial properties isolates against indicator microorganisms

The study of the bactericidal and antifungal activity of antagonist strains in relation to test strains to opportunistic microorganisms of different groups was determined by *the deferred-antagonism method* (Bohora *et al.,* 2019).

The antimicrobial activity of the isolated lactic acid bacillus isolates was assessed using indicator microorganisms: *Staphylococcus aureus NCTC 12973, Escherichia coli NCTC 12923, Salmonella typhimurium NCTC 12023* and *Candida albicans NCPF 3179* (test strains from the *Laboratory of Human Microbiome and Longevity "National Laboratory Astana", Nazarbayev University (Nur-Sultan, Kazakhstan).* The antimicrobial

activity was assessed by the zone of no growth of test strains around the colony of the tested isolate of lactobacilli; the studies were repeated three times with the results expressed as an arithmetic mean.

2.4. Study of biofilm formation of strains

The ability of each individual strain to form a biofilm was assessed using the O'Toole method (O'Toole, 2010). The biofilm - synthesizing ability of *Lh-13*, *Lpl-5* was determined by a 96-well polystyrene plate (flat-bottomed, transparent, sterile, U-shaped bottom) for enzyme immunoassay. The sterile plastic plates were used. The 150 µl of the MRS broth and the prepared bacterial culture of lactobacilli were added to each well. The initial optical density of bacterial cultures in the medium was 0.1 unit (OD (490nm) = 0.1). At the same time, each strain was introduced into 4 wells, 50 µl each. The 50 µl of the MRS broth was used for further control. It was incubated for 48 hours at 37°C under anaerobic conditions. To assess the state of biofilms, the contents of the wells were removed by phosphate buffer pH = 7.2 three times, and dried in a thermostat at 60°C for 60 minutes. Further on, it was stained with a solution of gentian violet (2% crystal violet) in the volume = 150 µl for 15 minutes at room temperature. Afterwards, the dye solution was sucked out, washed off under a stream of distilled water, shake out and dried in the air at room temperature. The dye was eluted with 95% ethyl alcohol (150 µl per well). The plate was covered with a lid, left for 30 minutes and measured by a *TecanEVolizer100 laboratory robot (Tecan)* at a wavelength of 490 nm.

The results were interpreted in accordance with the optical density of the colored solvent. The efficiency of biofilm formation was assessed by the method (Stepanović *et al.*, 2007). The biofilm biomass formation: ODc≤ ~ ≤ 2*Odc – no/weak; 2*ODc <~ ≤ 4*Odc – moderate; > 4*Odc – dense. The experiment was repeated three times.

2.5. Study of biofilm formation by a laser capture microdissection

To visually assess the ability of *Lh-13* to form biofilms on the surface of the slide, the sterile Petri dishes with a diameter of 100 mm were used. A sterile glass slide was placed in the dish (Lenz *et al.*, 2008), a bacterial suspension with a density of 1.0 McF and a volume of 1 ml was applied to a sterile glass slide and placed in a thermostat at 37°C and kept for 4 hours for the cells to bind, then 5 ml MRS broth was added after 4 hours and incubated at 37°C for 24h and 48h respectively.

In 24 hours and 48 hours after incubation, the culture medium was carefully removed with a dispenser, the glass surface was washed three times with 1.15 M phosphate buffer, fixed with 96°alcohol, dried, stained with gentian violet solution (2% crystal violet) (Azeredo *et al.*, 2017) for 2 minutes at room temperature, after which washed with a phosphate buffer. After staining, the samples were covered with coverslips and embedded in the vitrogel mounting medium (12–005, Biovitrum). Further on, the samples were microscoped by laser capture microdissection *ArcturusXT ™ LaserCapture Microdissection (LCM)* in the Differential Interference Contrast mode.

The laser capture microdissection studies were carried out in the shared laboratory of the NCJSC Medical University of Karaganda. After obtaining the images of the cell surface, the data was processed using *Arcturus XT* software.

2.6. Study of biofilm formation by an atomic force microscope (AFM)

A microscope slide was used as a support. The *Lh-13* isolate was cultivated on a glass slide according to the above-mentioned method, the material was fixed using a 2.5% glutaraldehyde solution (Chao *et al.*, 2011) with an exposure for 2 hours at a temperature of 4°C, after which the prepared agent was thoroughly washed with bi-distilled water and dried in the air at room temperature. The biofilm formation ability of *Lh-13* isolate isolated from lactic acid products of the Karaganda region was assessed using the method of atomic force microscopy in a semi-contact mode. The advantage of the semi-contact scanning method is that a cantilever does not touch its surface for the most of the oscillation period and, therefore, does not damage the object under study.

The atomic force microscope was used to carry out the research in the laboratory of solid-state Physics of the Astana branch of the Institute of Nuclear Physics. The AFM samples were measured using an *AIST-NT SmartSPM* microscope in a semi-contact mode with an amplitude of 50 nm. The high-resolution silicon AFM cantilevers of the NSG10 series with a resonance frequency of about 210 kHz were used as a probe. After obtaining images of the cell surface, the data was processed using the *IAPro 3.2.2* software.

2.7. Statistical analysis

The obtained results are presented as mean ± standard error, obtained as a result of three-fold repetition of the experiment (Statistica 8.0 software was used) (Kabanikhin *et al.*, 2021).

3. Results

3.1. Isolation of lactobacilli and identification

Thirteen isolates of lactobacilli were isolated from 24 samples of irimchik, suzbe, cheese, ashygan kozhe produced by the traditional home-made method from different districts of the Karaganda region (Kazakhstan). All 13 isolated isolates are gram-positive with the cells located: singly, in pairs, chain-shaped. The isolates are immobile and catalase-negative. They do not form spores (lactobacilli do not have catalase thus, no gas formation in the sample with hydrogen peroxide was observed). On the MRS agar-1, the colonies are small, white, round, not pigmented, with the smooth edges, 1-3 mm in diameter, grow well at 37°C for 48 hours under anaerobic conditions. The figure illustrates the morphological (Figure 1 a, b, c, d) and cultural (Figure 1. e, f, g, h) characteristics of some isolated lactobaccili isolates.

As a result of the identification of isolates on the mass spectrometer, the following isolates were detected: *Lactobacillus helveticus (6 strains), Lacticaseibacillus rhamnosus (2 strains), Lactiplantibacillus plantarum (1 strains), Lacticaseibacillus paracasei (3 strains), Limosilactobacillus fermentum (1 strains).*

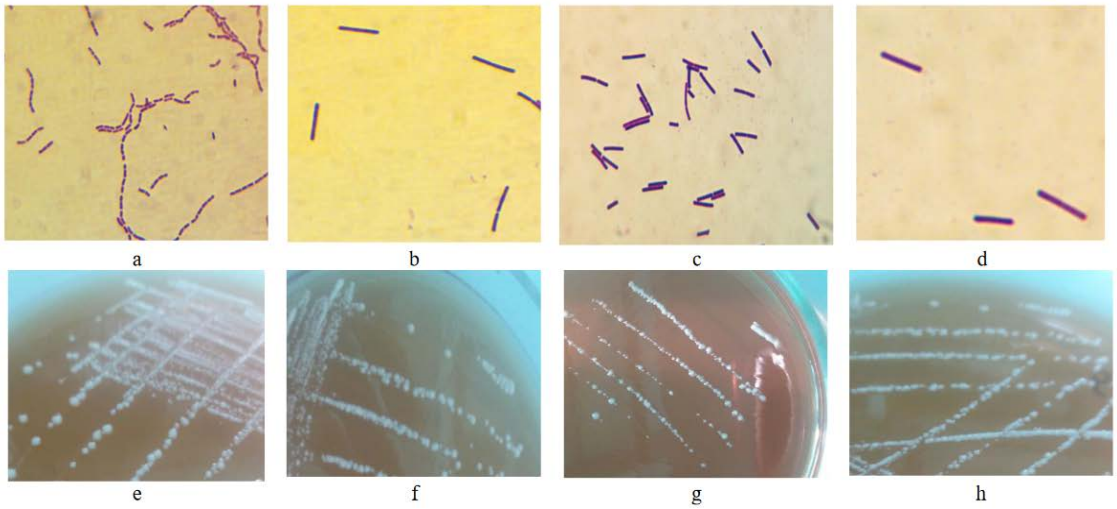

Figure 1. Morphological (100x objective) and cultural properties of the isolated lactobacilli: a-e) *Lpc-5/1*; b-f) *Lpl-5*; c-g) *Lh-13*; d-h) *Lrh-24.*

3.2. Tolerance to low acidic pH and bile salts

All thirteen lactobacilli isolates were tested for acidic pH resistance. As a result of the experiment, not all strains were resistant to acidic pH. The results demonstrated that 9 of 13 lactic acid bacillus isolates showed resistance to pH 3 for 0h, 3h and 6h despite differences in viability (growth with marked turbidity of the broth).

The isolates of *Lactobacillus helveticus-13 (Lh-13), Limosilactobacillus fermentum-18 (Lf-18), Lacticaseibacillus rhamnosus-24 (Lrh-24), Lacticaseibacillus paracasei -5/1 (Lpc-5/1), Lactiplantibacillus plantarum -5 (Lpl-5)* were found to be more resistant to acid stress. However, the isolates *Lactobacillus helveticus-14 (Lh-14), Lactobacillus helveticus -22 (Lh-22), Lacticaseibacillus paracasei -12 (Lpc-12), Lacticaseibacillus paracasei – 44 (Lpc-44)* did not tolerate low pH, yet some growth of isolates was observed (Figure 2).

The results of experimental studies have shown that the degree of resistance to acidic pH does not depend on the species; they differ among strains of the same species. These results are consistent with those obtained in the previous similar in vitro studies. The previous studies showed the ability of strains of lactobacilli to maintain their viability when exposed to the pH values of 3 (Mishra and Prasad , 2005; Guo *et al.*, 2010; Grosu-Tudor *et al.*, 2012; Menconi *et al.*, 2014).

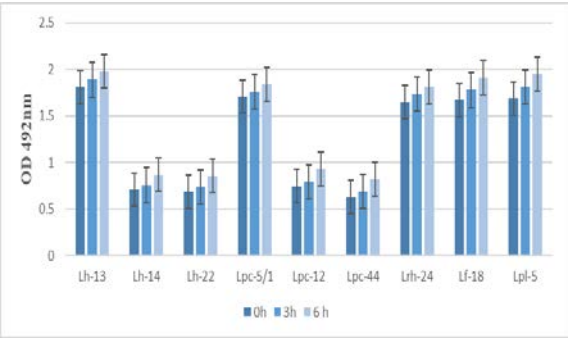

Figure 2. Acid resistance of lactobacilli isolates (mean±SD, three replicates).

The survival of 5 isolates of lactobacilli in the MRS broth containing 0.3, 0.5, 1% bovine bile was studied after incubation for 3 hours, and the results of optical density (OD) varied. According to the results (refer to Figure 3), lactic acid bacillus isolates retained their viability after exposure to 0.3-0.5% bovine bile for 3 hours (which reflects the time spent in the small intestine). The isolates of *Lh-13, Lpl-5* showed maximum growth at 0.3-0.5% bovine bile concentration compared to *Lf-18, Lrh-24, Lpc-5/1* (despite their acid resistance).

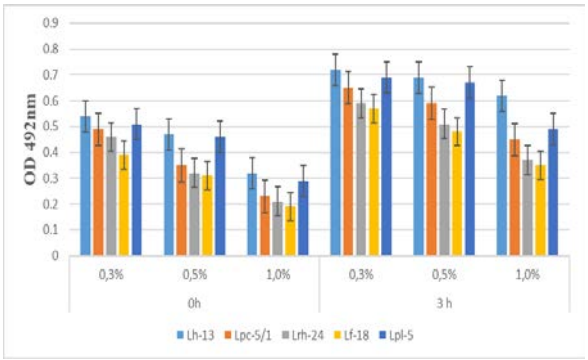

Figure 3. Survival of selected *Lh-13, Lf-18, Lrh-24, Lpc-5/1, Lpl-5* isolates in the MRS broth supplemented with 0.3%, 0.5%, 1.0% bovine bile (mean±SD, three replicates).

One could observe stable growth in *Lh-13, Lpl-5* at 1% bovine bile concentration, and optical density (OD) varied slightly. Despite the observed properties, such as acid resistance, the growth rate of the isolates of *Lf-18, Lrh-24, Lpc-5/1* decreased with an increase in the concentration of 1% bovine bile.

As a result of screening, *Lh-13, Lpl-5* have shown resistance to bovine bile and have been further tested for probiotic properties *in vitro.*

3.3. Detection of antibacterial activity of the isolates

The isolates of *Lh-13, Lpl-5* were tested for antimicrobial activity against gram-positive, gram-negative and yeast indicator microorganisms. The carried-out studies made it possible to establish that the *Lh-13, Lpl-5* showed antibacterial activity against indicator microorganisms, but the zone of inhibition of the test

strains varied. The antifungal activity was found in only one *Lh-13* isolate (Table 2).

Table 2. Antibacterial and antifungal activity of *Lh-13*, *Lpl-5* isolates

Isolated Lactobacilli isolates (symbols)	S. aureus NCTC 12973	E. coli NCTC 12923	S. typhimurium NCTC 12023	C. albicans NCPF 3179
	Zone of inhibition (diameter in mm)			
Lh-13	13.0±1.0	17.0±2.0	15.0±2.0	8.0±2.6
Lpl-5	14.0±3.0	15.0±2.0	16.0±1.0	0±0

Note: the diameters of inhibition zones (in mm) are presented as mean + SD, the research is repeated three times.

Thus, *Lh-13* possesses a certain level of antibacterial and fungicidal activity, which indicates their pronounced antimicrobial properties in relation to some opportunistic test strains.

3.4. Study of biofilm formation of isolated isolates

According to the study, the tested *Lh-13*, *Lpl-5* after 48 hours of incubation at 37°C in the standard MRS broth medium used to form biofilms on lactic acid bacteria on plastic plates varied in optical density (Figure 4).

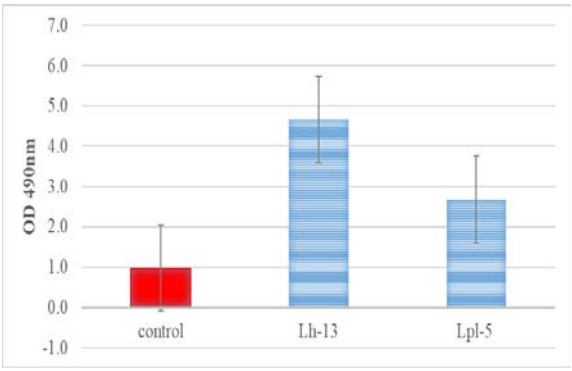

Figure 4. Assessment of the optical density of biofilm on plastic plates: Average optical density of the dye in the wells of polystyrene plates, reflecting the intensity of biofilm formation on their surface, by *Lh-13*, *Lpl-5* strains isolated from the national lactic acid products of the Karaganda region (mean±SD, three replicates).

According to the studied parameters, the intensity of biofilm formation shown by the *Lpl-5* (OD_{490nm}-2.686) expressed moderate intensity, and the one shown by the *Lh-13* strain expressed high intensity.

Microscopy is the simplest and fastest method to observe morphology of microorganisms attached to a surface. This observation allows researchers to compare the morphology of bacteria in a biofilm. In this regard, indicators reflecting the high intensity of *Lh-13* (OD_{490nm}-4.672) biofilm formation strains were studied by using a laser capture microdissection and an atomic force microscope.

3.5. Microscopy of the morphology of lactobacilli cells in biofilm using a laser capture microdissection

As a result of the study, in the morphology of the cells in the biofilm of the *Lh-13* by a laser capture microdissection in the DIC mode after 24 hours of incubation, one can observe some adhesion of single rod-shaped bacteria (Figure 5a). The bacteria adhere to a glass slide the most intensively with further compact microcolonies formed as they are united by an extracellular polymer matrix-mucus. Microcolonies gradually increase in size.

After 48 hours, homogeneous (rod-shaped) microbial communities were observed in the preparation; the structure on the outside is presented in the form of a protective film that unites all cells into a single system (Figure 5b). The increased thickness of the biofilm formed its specific structures, namely depressions and voids between them.

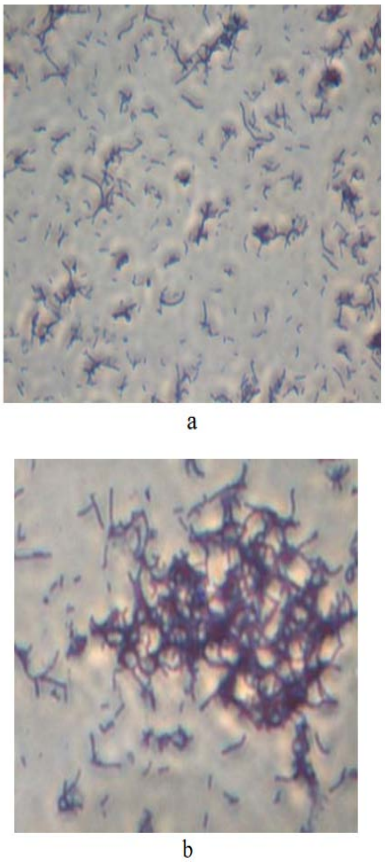

Figure 5. Cell morphology of *Lh-13* isolate in the biofilm (a) *Lh-13* after 24-hour incubation, (b) *Lh-13* after 48-hour incubation, a laser capture microdissection microscopy, homogeneous (rod-shaped) microbial communities, 60x objective.

Thus, using the resources of the laser capture microdissection allowed viewing the images of microcolony morphology and macro-colony of *Lh-13* isolate.

3.6. Study of the morphology of lactobacillus cells in the biofilm using an atomic force microscopy (AFM)

The AFM images obtained by scanning in the semi-contact mode made it possible to establish the morphology of cells in the biofilm. The advantage of the semi-contact scanning method of the sample is that a cantilever does not touch its surface for most of the oscillation period and, therefore, does not damage the object under study.

As a result of the study, one could observe adhesion to the surface of the glass slide and the distribution of the cell mass of the *L. hel-13* isolate after 24 hours. The bacterial cells are rod-shaped, homogeneously accumulated with sharp dips, which refer to depressions and voids between

them (Figure 6a). After 48 hours of incubation, one could observe some dense accumulations of rod-shaped biomass of *Lh-13* cells linked to each other. The architectonics of the adhered cells of the *Lh-13* isolate is supported by an exopolysaccharide matrix of microcolonies accompanied by the formation of clusters with the round channels around them. The microcolonies got increased largely (Figure 6c).

AFM provides 3D imaging of surface ultrastructures with molecular resolution in real time and under physiological conditions. Using the AFM resources, it was possible to get a clearer visualization to present the sample in a 3D image (Figure 6 b, d). The biofilm surface is not smooth, having numerous irregularities; *Lh-13* isolate is located in space randomly, but uniformly.

With the help of AFM, a biofilm surface roughness profile is constructed (Figure 6 e-j). The biofilm surface roughness is a set of surface irregularities. The roughness parameters were determined from the obtained images of surfaces with a microscopic field size of 20 × 20 μm. The differences in the roughness of the biofilm surface in their linear dimensions are associated with the relative position of the cells of the *Lh-13* isolate and matrix.

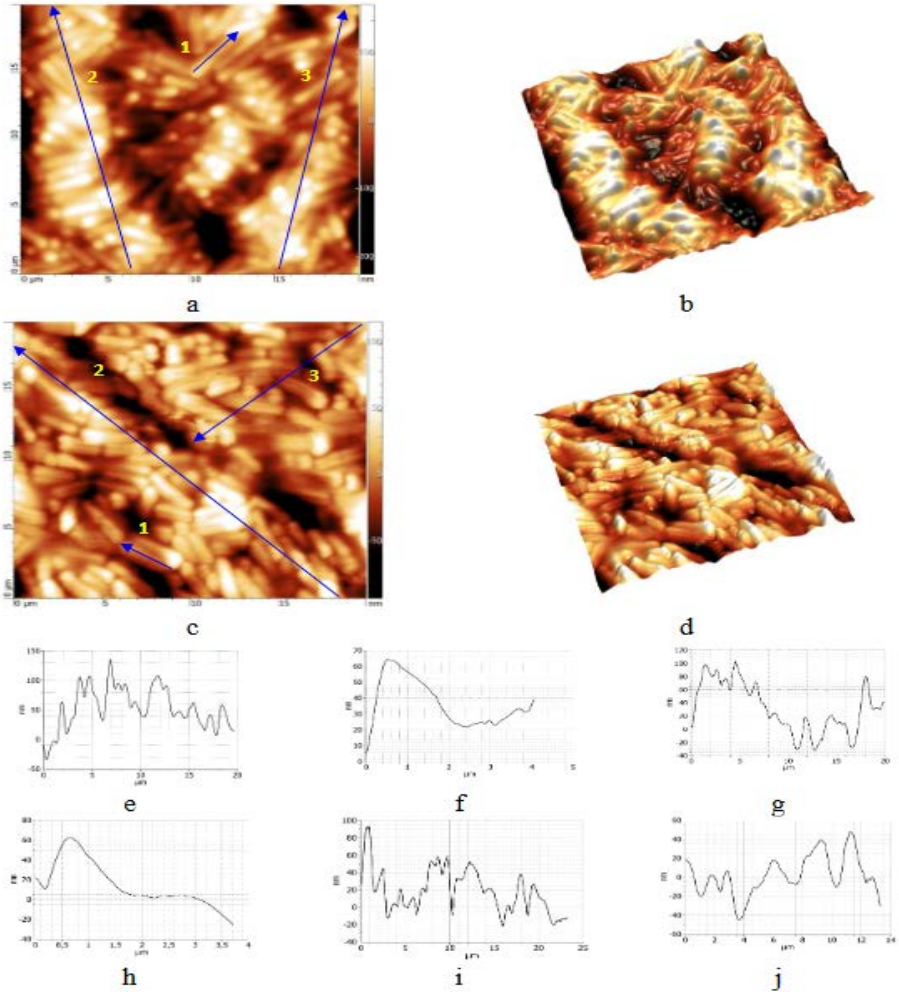

Figure 6. Cell morphology of *Lh-13* isolate in the biofilm, atomic force microscopy: a-b) 2D and 3D images of *Lh-13* after 24 hours of incubation; c-d) 2D and 3D images of *Lh-13* -13 after 48 hours of incubation; e-j) biofilm surface roughness profile. The biofilm surface roughness profile was measured by plotting the surface profile along the line: (e, h) directed along the maximum cell length; (f, i) where the long biomass of the cell accumulation of *Lh-13* is formed; (g, j) where a large number of the *Lh-13* aggregates (dense layers) is attached.

Thus, the study of the biofilm by an atomic force microscopy made it possible to study the morphology of the biofilm-forming strain in 2D and 3D projections, displaying the relief and topographic image of the biofilm of the *Lh-13* isolate.

4. Discussion

The isolates isolated by morpho-cultural characters and identification (Zheng *et al.*, 2020) by MALDI-TOF MS indicate that they refer to: *Lactobacillus helveticus (6 strains), Lacticaseibacillus rhamnosus (2 strains), Lactiplantibacillus plantarum (1 strain), Lacticaseibacillus paracasei (3 strains), Limosilactobacillus fermentum (1 strain).*

The mimicking conditions of the gastrointestinal tract made it possible to identify promising isolates of *Lh-13*, *Lpl-5* to study antimicrobial and biofilm-forming activity. The study of antimicrobial activity under conditions of intermicrobial relations enabled to witness the antimicrobial activity of *Lh-13* as the result the production of antimicrobial substances.

A detailed screening of *Lh-13* and *Lpl-5* isolates was carried out by the *TecanEVolizer100 (Tecan)* operating system. During such screening, *Lh-13* showed a high level of density (OD_{490nm}-4.672) during biofilm formation in the wells of polystyrene plates.

There is a large number of methods for visualizing the image of the obtained biofilms and studying their properties: from measuring the optical density to various options for light and electron microscopy (Relucenti *et al.,* 2021). Thanks to the use of lasers, highly sensitive light detectors, digital photography and computer technology, the methods of studying the cell's morphology and biofilm formation of bacteria have been transformed to provide objective studies in microscopy. A special role in forming this methodology was played by computer methods for processing and analyzing digital images. They made it possible not only to simplify, accelerate and automate many of the existing microscopy methods, but also ensured the acquisition of previously inaccessible images (Chang *et al.,* 2012; Babu and Singh, 2014).

The specialized microscopy bank made it possible to visualize the image and compare two methods: a laser capture microdissection and an atomic force microscopy, using the example of *Lh-13*. Both methods recorded the homogeneity of morphological properties with equal efficiency. The laser capture microdissection showed that *Lh-13* cells were rod-shaped, and the bacterial cells were in the extracellular matrix in the form of a homogeneous massive accumulation. Working in the mode of the investigated method, a distinctive feature is the use of gentian violet staining performed according to the above-mentioned technique, which gives a volumetric image, focusing on the clear lines and boundaries of the accumulation of microorganisms as the enveloping effect of biofilm.

The atomic force microscopy allows studying biological objects and processes without using complex fixation methods as well as obtaining images of bacterial cells with high resolution (Alsteens, 2012). By using an atomic force microscope, the two and three-dimensional morphological relief images were accurately studied, with an indication of the profile of surface roughness, a homogeneous massive accumulation is visually observed, reflecting the active biofilm formation of lactobacilli, 100% preserving the true topography.

The microscopic methods used, namely a laser capture microdissection and an atomic force microscopy, complement each other in the visualization of the experiment.

The expediency of using microscopic research methods is based on the fact that only visual control is able to assess the morphological state of both the strains of lactobacilli themselves and the matrix of biofilms.

The results of our own research and literature reviews (Flemming and Wingender, 2010; Gavrilova *et al.,* 2019) allow stating that the biofilm of lactobacilli is a community of cells attached to the surface and united by the extracellular matrix. In addition to adhesins, mucoid capsules and surface polysaccharides of bacterial cell walls can stimulate the formation of biofilms on the surface of solid substrates. After colonization, bacteria begin to actively secrete exopolysaccharides, which ensure the formation of a biofilm while filling the intercellular space (Spangler *et al.,* 2019; Barzegari *et al.,* 2020). In this case, communicative connections are regulated by means of special substances released into the environment, namely autoregulators with an established chemical structure. It is known that microbial cells in biofilms produce exopolysaccharides, mainly composed of polysaccharides, proteins and nucleic acids, which form a protective gel-like matrix around the cells (Aoudia *et al.,* 2016). According to the literature, the *L. helveticus* strain has a probiotic ability, in particular antimicrobial activity, and is able to produce mucus-binding proteins and proteins of the surface layer, having the properties of EPS formation and cell aggregation, proteolytic activity and bacteriocin production (Li *et al.,* 2015; Gómez *et al.,* 2016; Fontana *et al.,* 2019).

It is experimentally confirmed that *Lh-13* isolate can form biofilms in vitro on the surface of the glass slide, indicating their ability to colonize, having tolerance to acidic pH and bovine bile, and antimicrobial activity.

The given study has several limitations. When studying the cell morphology of *Lh-13* isolate in biofilm using the atomic force microscopy (AFM), an integrated approach was not taken into consideration. This approach includes three methods: semi-contact, mismatch, phase imaging, morphometric parameters of the cell (height, width, length, diameter), adhesive properties of the substrate, bacteria, biofilm (determination of adhesion forces between biofilm and substrate, as well as adhesion strength). These studies are in perspective.

We were able to visualize biofilms on a substrate (a microscope slide) using atomic force microscopy; due to the uniqueness of the biofilm creation approach; the image was augmented by a laser microdissection method. Because there was no scanning microscope in the lab, a stained image of the morphology and topography of the *Lh-13* cells in both singular and biofilm form was obtained.

This investigation enabled the selection of a biologically active (biofilm-forming) *Lh-13* isolate with antibacterial capabilities as a probiotic candidate; nevertheless, further studies of this isolate's probiotic properties are required.

5. Conclusions

The work's results established the groundwork for further study on the probiotic characteristics of isolate *Lh-13*. More research is needed to establish how biofilms and their active chemicals suppress foodborne pathogens, adhesive activity, antibiotic resistance, and so on.

These physiologically active strains of *Lh-13* will hopefully be used in vivo as probiotics for the nutritional needs of both people and animals in the near future.

Acknowledgements

The authors express their sincere gratitude to the employee of the laboratory of solid-state Physics of the Astana branch of the Nuclear Physics Institute, Shlimas I. Dmitriy, for assisting during the research with an atomic force microscope and to the Head of the laboratory of the NPJSC Medical University of Karaganda, Akhmaltdinova L. Lyudmila, for her active assistance during the research with the laser capture microdissection.

References

Aitzhanova AA, Oleinikova EA, Saubenova MG, Daugalieva ST, and Berzhanova, RZ. (2020). Selection of antagonistically active strains of lactic acid bacteria from milk of various animal species. *Bull. KazNU. Biol. series*, **83(2)**:72-81.

Alatoom AA, Cunningham SA, Ihde SM, Mandrekar J, Patel R. (2011). Comparison of direct colony method versus extraction method for identification of gram-positive cocci by use of Bruker biotyper matrix-assisted laser desorption ionization-time of flight mass spectrometry. *J Clin Microbiol.*, **49(8)**:2868-2873.

Alsteens D. (2012). Microbial cells analysis by atomic force microscopy. *Methods Enzymol.*, **506**:3-17.

Aoudia N, Rieu A, Briandet R, Deschamps J., Chluba J, Jego G, Garrido C, Guzzo J. (2016). Biofilms of Lactobacillus plantarum and Lactobacillus fermentum: Effect on stress responses, antagonistic effects on pathogen growth and immunomodulatory properties. *Food Microbiol.*, **53** (Pt A):51-59.

Azeredo J, Azevedo NF, Briandet R, Cerca N, Coenye T, Costa AR, Desvaux M, Di Bonaventura G, Hébraud M, Jaglic Z, Kačániová M, Knøchel S, Lourenço A, Mergulhão F, Meyer RL, Nychas G, Simões M, Tresse O and Sternberg C. (2017). Critical review on biofilm methods. *Crit Rev Microbiol.*, **43(3)**:313-351.

Babu R and Singh E. (2014). Atomic Force Microscopy: A source of investigation in biomedicine. *Int. J. Energy Environ. Eng.*, **7(1):** 59-66.

Barzegari A, Kheyrolahzadeh K, Mahdi S, Khatibi H, Sharifi S, Memar MY and Vahed SZ. (2020). The battle of probiotics and their derivatives against biofilms. *Infect Drug Resist.*, **13**:659-672.

Bechelaghem N, Djibaoui R, Ergün Y, Ettalhi M. (2022). *Lactobacillus reuteri* and *Lactobacillus salivarius*, two prevalent vaginal species isolated from healthy women in Western Algeria. *Jordan J. Biol. Sci.*,**15(1)**:133-137.

Bohora AA, Kokate SR, Khedkar S and Vankudre A. (2019). Antimicrobial activity of probiotics against endodontic pathogens: - A preliminary study. *Indian J Med Microbiol.*, **37(1):**5-11.

Casey PG, Casey GD, Gardiner GE, Tangney M, Stanton C, Ross RP, Hill C and Fitzgerald GF. (2004). Isolation and characterization of anti-Salmonella lactic acid bacteria from the porcine gastrointestinal tract. *Lett Appl Microbiol.*, **39(5)**:431-438.

Chang KC, Chiang YW, Yang CH and Liou JW. (2012). Atomic force microscopy in biology and biomedicine. *Tzu Chi Med. J.*, **24(4)**: 162-169.

Chao Y and Zhang T. (2011). Optimization of fixation methods for observation of bacterial cell morphology and surface ultrastructures by atomic force microscopy. *Appl Microbiol Biotechnol.*, **92(2)**:381-392.

Corona-Hernandez RI, Alvarez-Parrilla E, Lizardi-Mendoza J, Islas-Rubio AR, de la Rosa LA, Wall-Medrano A. (2013). Structural stability and viability of microencapsulated probiotic bacteria: A review. *Compr Rev Food Sci Food Saf.,* **12(6):** 614-628.

Ejtahed HS, Hasani-Ranjbar S, Larijani B. (2017). Human microbiome as an approach to personalized medicine. *Altern Ther Health Med.*, **23(6)**:8-9.

Flemming HC and Wingender J. (2010). The biofilm matrix. *Nat Rev Microbiol.*, **8(9)**:623-633.

Fontana A, Falasconi I, Molinari P, Treu L, Basile A, Vezzi A, Campanaro S, Morelli L. (2019). Genomic comparison of Lactobacillus helveticus strains highlights probiotic potential. *Front. Microbiol.*, **10:**1380.

Gavrilova E, Anisimova E, Gabdelkhadieva A, Nikitina E, Vafina A, Yarullina D, Bogachev M, Kayumov A. (2019). Newly isolated lactic acid bacteria from silage targeting biofilms of foodborne pathogens during milk fermentation. *BMC Microbiol.*, **19(1)**:248.

George Kerry R, Patra JK, Gouda S, Park Y, Shin HS, Das G. (2018). Benefaction of probiotics for human health: A review. *J Food Drug Anal.*, **26(3)**:927-939.

Ghosh C, Sarkar P, Issa R, Haldar J. (2019). Alternatives to conventional antibiotics in the era of antimicrobial resistance. *Trends Microbiol.*, **27(4)**:323-338.

Gomez NC, Ramiro JM, Quecan BX, de Melo Franco BD. (2016). Use of potential probiotic lactic acid bacteria (LAB) biofilms for the control of Listeria monocytogenes, Salmonella typhimurium, and Escherichia coli O157: H7 biofilms formation. *Front. Microbiol.*,**7:863:**

Grosu-Tudor SS and Zamfir M. (2012). Probiotic potential of some lactic acid bacteria isolated from Romanian fermented vegetables. *Ann Rom Soc Cell Biol.,* **17(1)**:234-239.

Guarner F, Perdigon G, Corthier G, Salminen S, Koletzko B, Morelli L. (2005). Should yoghurt cultures be considered probiotic? *Br J Nutr.*, **93(6)**:783-786.

Guo XH, Kim JM, Nam HM, Park SY, Kim JM. (2010). Screening lactic acid bacteria from swine origins for multistrain probiotics based on in vitro functional properties. *Anaerobe*, **6(4)**:321-326.

Hill C, Guarner F, Reid G, Gibson GR, Merenstein DJ, Pot B, Morelli L, Canani RB, Flint HJ, Salminen S, Calder PC, Sanders ME. (2014). Expert consensus document: The international scientific association for probiotics and prebiotics consensus statement on the scope and appropriate use of the term probiotic. *Nat Rev Gastroenterol Hepatol*., **11(8)**:506-514.

Kabanikhin S, Krivorotko O, Takuadina A, Andornaya D, Zhang S. (2021). Geo-information system of tuberculosis spread based on inversion and prediction. *J. Inverse Ill-Posed Probl.,* **29(1)**:1-20.

Kim JH and Baik SH. (2019). Probiotic properties of Lactobacillus strains with high cinnamoyl esterase activity isolated from jeot-gal, a high-salt fermented seafood. *Ann. Microbiol.,* **69:**407–417.

Lenz AP, Williamson KS, Pitts B, Stewart PS, Franklin MJ. (2008). Localized gene expression in Pseudomonas aeruginosa biofilms. *Appl Environ Microbiol.*, **74(14)**:4463-4471.

Li W, Xia X, Chen X, Rui X, Jiang M, Zhang Q, Zhou J, Dong M. (2015). Complete genome sequence of Lactobacillus helveticus MB2-1, a probiotic bacterium producing exopolysaccharides. *J Biotechnol.,* **209**:14-15.

Menconi A, Kallapura G, Latorre JD, Morgan MJ, Pumford NR, Hargis BM, Tellez G. (2014). Identification and characterization of lactic acid bacteria in a commercial probiotic culture. *Biosci Microbiota Food Health*, **33(1)**:25-30.

Mishra V and Prasad DN. (2005). Application of in vitro methods for selection of Lactobacillus casei strains as potential probiotics. *Int J Food Microbiol.*, **103(1)**:109-115.

Mitropoulou G, Nedovic V, Goyal A, Kourkoutas Y. (2013). Immobilization technologies in probiotic food production. *J Nutr Metab*, **2013:**1-15.

O'Toole GA. (2010). Microtiter dish Biofilm formation assay. *J. Vis. Exp.,* **(47)**:2437.

Relucenti M, Familiari G, Donfrancesco O, Taurino M, Li X, Chen R, Artini M, Papa R, Selan L. (2021). Microscopy methods for biofilm imaging: Focus on sem and VP-SEM pros and cons. *Biology*, **10(1)**:51.

Salas-Jara MJ, Ilabaca A, Vega M, García A. (2016). Biofilm forming lactobacillus: new challenges for the development of probiotics. *Microorganisms*, **4(3)**:35.

Schulthess B, Bloemberg GV, Zbinden R, Böttger EC, Hombach M. (2014). Evaluation of the bruker MALDI biotyper for identification of gram-positive rods: Development of a diagnostic algorithm for the clinical laboratory. *J Clin Microbiol*., **52(4)**:1089-1097.

Schupack DA, Mars RAT, Voelker DH, Abeykoon JP, Kashyap PC. (2022). The promise of the gut microbiome as part of individualized treatment strategies. *Nat Rev Gastroenterol Hepatol***., 19(1)**:7-25.

Singh TP, Kaur G, Malik RK, Schillinger U, Guigas C, Kapila S. (2012). Characterization of Intestinal Lactobacillus reuteri Strains as Potential Probiotics. *Probiotics Antimicrob. Proteins*, **4(1)**:47-58.

Son SH, Jeon HL, Jeon EB, Lee NK, Park YS, Kang DK, Paik HD. (2017). Potential probiotic Lactobacillus plantarum Ln4 from kimchi: Evaluation of β-galactosidase and antioxidant activities. *Food Sci. Technol.,* **85** (A):181-186.

Spangler JR, Dean SN, Leary DH, Walper SA. (2019). Response of lactobacillus plantarum WCFS1 to the gram-negative pathogen-associated quorum sensing molecule N-3-oxododecanoyl homoserine lactone. *Front. Microbiol*., **10**:715.

Stepanović S, Vuković D, Hola V, Di Bonaventura G, Djukić S, Cirković I, Ruzicka F. (2007). Quantification of biofilm in microtiter plates: Overview of testing conditions and practical recommendations for assessment of biofilm production by staphylococci. *APMIS*, **115(8)**:891-899.

Tambekar DH and Bhutada SA. (2010). Studies on antimicrobial activity and characteristics of bacteriocins produced by lactobacillus strains isolated from milk of domestic animals. *Internet J. Microbiol*.,**8(2)**:1-5.

Tatsaporn T and Kornkanok K. (2020). Using Potential Lactic Acid Bacteria Biofilms and their Compounds to control biofilms of foodborne pathogens. *Appl. Biotechnol. Rep.,* **26** (e00477):1-8.

Taverniti V, Scabiosi C, Arioli S, Mora D, Guglielmetti S. (2014). Short-term daily intake of 6 billion live probiotic cells can be insufficient in healthy adults to modulate the intestinal Bifidobacteria and Lactobacilli. *J Funct. Food.,* **6(1):** 482-491

Torres N and Tovar AR. (2021). The present and future of personalized nutrition. *Rev Invest Clin*, **73(5)**:321-325.

Wang D, Liu W, Ren Y, De L, Zhang D, Yang Y, Bao Q, Zhang H, Menghe B. (2016). Isolation and identification of Lactic acid bacteria from traditional dairy products in Baotou and Bayannur of Midwestern Inner Mongolia and q-PCR analysis of predominant Species. *Korean J Food Sci Anim Resour*, **36(4)**:499-507.

Wu C, Huang J, Zhou R. (2017). Genomics of lactic acid bacteria: Current status and potential applications. *Crit Rev Microbiol*., **43(4)**:393-404.

Yue Y, Xu X, Yang B, Lu J, Zhang S, Liu L, Nassar K, Zhang C, Zhang M, Pang X, Lv J. (2020). Stable colonization of orally administered Lactobacillus casei SY13 Alters the Gut Microbiota. *Biomed Res Int.,* **2020**:1-8.

Zeng Y, Li Y, Wu QP, Zhang JM, Xie XQ, Ding Y, Cai SZ, Ye QH, Chen MT, Xue L, Wu S, Zeng HY, Yang XJ, Wang J. (2020). Evaluation of the Antibacterial Activity and Probiotic Potential of Lactobacillus plantarum Isolated from Chinese Homemade Pickles. *Can. J. Infect. Dis. Med. Microbiol.,* **2020**:1-11.

Zheng J, Wittouck S, Salvetti E, Franz CMAP, Harris HMB, Mattarelli P. et al. (2020.) A taxonomic note on the genus Lactobacillus: Description of 23 novel genera, emended description of the genus Lactobacillus Beijerinck 1901, and union of Lactobacillaceae and Leuconostocaceae. *Int. J. Syst. Evol. Microbiol.* **70(2)**:2782–2858.