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Screening of Wild Lactobacillus Plantarum Found in Brine Solution of Naturally Fermented Cucumbers

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ABSTRACT

Fermentation has been used as a simple technique for preserving vegetables since ancient days. This research work aimed to isolate, characterize, and identify wild Lactobacillus plantarum found in a brine solution (8% w/v NaCl) of naturally fermented cucumber. Cucumber samples were naturally fermented at ambient temperature for 14 days in a brine solution, in which lactic acid bacteria (LAB) were then cultured (37℃, 48 h) within 24 h for 3, 5, 7, 10, and 14 days. Seventeen different LAB strains were isolated and identified within the different time intervals of fermentation using morphological, biochemical API 50 CHL, PCR verification using Lactobacillus subspecies-specific genes primer, and 16S rRNA gene sequencing. The API confirmed strains were further verified by PCR to be L. plantarum subsp. plantarum M23 based on the amplified gene fragment separation by gel electrophoresis and gene sequencing. After 24 h of fermentation, the most dominant LAB-identified strains were Leuconostoc mesenteroides, Tetragenococcus halophilus, and L. plantarum M23, respectively. Starting from the fifth day of fermentation, L. plantarum M23 controlled the fermentation process as the dominant LAB. The drop in the pH value was significant (from 6.8 to 3.18) due to the variations in the LAB strains throughout the 14 days of fermentation. Further investigation will be carried out to study the production of plantaricin, which has great importance in food biopreservation.

Keywords: Lactobacillus plantarum, LAB, fermented cucumbers, planta ricin, API 50 CHL, PCR, gel electrophoresis.

INTRODUCTION

Fermentation is the world's oldest method of food preservation (Farnworth, 2008), owing to its practice since the dawn of civilization. Fermentation has not only enabled our ancestors to survive various epochs and climates; it was mainly used to preserve food and gave it different flavors and sensory attributes (Bangar *et al.,* 2022). Nowadays, fermented foods are produced in a variety of geographical areas, each with its own set of traditions and cultural preferences (Rodzi & Lee, 2021).

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Fermented foods are a rich supply of live and active microbes; in fact, fermented foods are heavily reliant on naturally occurring lactic acid bacteria (LAB), which are a common bacterial genus found in fermented food habitats as well as the gastrointestinal systems of human and other animals.

The majority of LABs are essential to the intrinsic properties of fermented food products superscript LAB present in raw and fermented products are mainly *Lactobacillus plantarum, L. pentosus, L. brevis, L. fermentum, L. casei, Leuconostoc mesenteroides, L. kimchi, L. fallax, Weissella confusa, W. koreenis, W. cibaria, and Pediococcus pentosaceus*, which are considered as a probiotic source in food. (Garcia-Gonzalez *et al*., 2021; Adams & Moss, 2000; Swain *et al.,* 2014).

L. plantarum strains have obtained special attention for their ability to produce bacteriocins known as plantaricins (Marco *et al.,* 2017). *L. plantarum* and their plantaricins have got great importance in different areas as a food biopreservative in dairy products such as cheese and yogurt, fermented meat and fish, sourdough, pickles, and kimchi (Kim *et al*., 2021).

The objective of this research work was to isolate, characterize, and identify wild *L. plantarum* M23 found in a brine solution (8% NaCl w/v) of naturally fermented cucumber using API 50 CHL carbohydrate identification, PCR technique, and 16S rRNA gene sequencing.

Materials and Methods

Cucumber Fermentation and LAB Cultures

Fresh cucumber samples were purchased from the local market. Samples were washed, sanitized with a food-grade food sanitizer, and then proceeded for fermentation in 8% w/v NaCl brine solution at ambient temperature for 14 days, in which the pH of the fermented samples was measured on a daily basis.

Using sterile stomacher bags (Seward Stomacher® 400 classic strainer bag), 100 g of 5 composited fermented cucumber samples (including brine solution) were homogenized by using a laboratory stomacher (Interscience BagMixer, Model No. BabMixer 400 CC) under aseptic conditions. The homogenized solution was then used for microbiological analysis.Differe cimal serial (0.1ml inocuandm of, 10^{-3} , 10^{-4}, 10^{-5} an superscript dilutions of fermented samples were spread plated on LAB de Man Rogosa and Sharpe (MRS) agar plates (Oxoid, CM0361, USA). LAB was cultured at different time intervals: 24 h, 3, 5, 7 d, 10, and 14 days of fermentation. LAB plates were incubated for 48 h at 37°C.

Isolation and Screening of LAB

LAB colonies were selected based on their morphological appearance (typical colonies). Presumptive LAB was then isolated using the streaking technique on MRS agar and incubated for 48 h at 37°C. Isolated LAB colonies were morphologically investigated (Gram staining), examined for oxidase and catalase tests, and then confirmed by using the API 50 CHL system (bioMerieux, France) and PCR techniques.

API 50 CHL System

The API 50 CHL carbohydrate identification kit (API 50 CHL, BioMerieux, France) recognized the carbohydrate fermentation patterns of the different isolated LAB strains. According to the manufacturer's instructions, the pure isolated inoculum was aseptically transferred from MRS agar into a CHL Medium API 50 (10.0 mL). After mixing the suspension, 150 µL of the inoculated API 50 CHL medium suspensions were transferred into the API wells using a sterilized micropipette. The API strips were then incubated at 37°C and LAB was biochemically screened after 48 h of incubation.

Color variations were detected in every well and according to the manufacturer’s instructions. The positive result was verified by a change in the color of the bromocresol purple indicator from purple to yellow. As a control, the first well on the strip was employed. A lack of color change suggested a negative result. To identify Lactobacillus species, the results (+/-) were analyzed using the api-webTM identification software database (Biomérieux, France, V 5.1).

PCR Verification Using Lactobacillus Specific Genes

The API confirmed *L. plantarum* M23 strains were further verified by 16S rRNA-specific primers and *L. plantarum* subspecies-specific primers (Table 1). The genomic DNA of the selected strains was extracted by The Wizard® Genomic DNA Purification Kit (Promega, USA).

Table 1. Gene sequence primers used in PCR reaction

Primer sequence (5' – 3')	Product length	Gene	Reference
F*16S rRNA1*: GCAGCAGTAGGGAATCTTCCA **R*16S rRNA1***: GCATTCCACCGCTACACATG	349 bp	*16S rRNA* (LAB)	(Castillo et al., 2006)
F*16S rRNA3*: AAGGGTTTCGGCTCGTAAAA **R*16S rRNA3***: TGCACTCAAGTTTCCCAGTT	247 bp	*16S rRNA*	(Wu et al., 2016)
***plantarum* F**: CGG CAA CAA GCC ACT AAA CT ***plantarum* R**: GAT AAT TAG CGG CTG CCT GA	120 bp	*L. plantarum* subsp. *plantarum*	(kim et al., 2020)

The PCR mix reaction was composed of 1.3 µM of each primer, 210 µl of template were pelleted and resuspended in 3.5 mL double-distilled H_2O, and 7.5 µl of 2x EasyTaq PCR SuperMix (TRANS, CHINA). The PCR amplification conditions were conducted with initial denaturation at 95℃ for 5 min, followed by 35 cycles of denaturation at 95℃ for 30 secplantains at 50℃ for 30 secs, extension at 72℃ for 1 min, and final extension at 72℃ for 10 min. The amplified fragments were then separated by electrophoresis (1.8% agarose gel), with the first well containing 5 µl of 50 bp DNA Ladder RTU (GeneDirex, USA).

DNA extraction, Sequencing, and phylogenetic tree

The genomic DNA (PCR products) of M23 bacterial isolates were purified using an E.Z.N.A.® DNA Extraction Kit according to the manufacturer's protocol (Spin Protocol).

The genomic DNA of M23 extracted from agarose gel was then quantified and qualified using the next-generation NanoDrop™ (Thermo Scientific, USA).

Samples were sequenced in both sense and antisense directions by Marcogene Humanizing Genomics sequencing laboratory (KR) using Applied Biosystems Big-Dye ver 3.1 chemistry on an Applied Biosystems model 3730 automated capillary DNA sequencer (Applied Biosystems).

The obtained sequences were joined using CLC Genomics Workbench 22.0.2, and then, the similarity search of sequences was performed by conducting a Homology search of 16S rRNA sequences with the National Center for Biotechnology Information (NCBI) for the blast database. The phylogenetic tree was constructed using MEGA 11.0.

Statistical Analysis

Response Surface Methodology (RSM) was used to conduct data analysis for bacterial growth patterns using statistical analysis software (SAS Inc., USA).

Results and Discussion

Isolation and Identification of LAB

Seventeen bacterial strains were isolated throughout the fermentation process of cucumber and were identified using the metabolic profiles of the carbohydrate fermentation obtained by the API 50 CHL System. Further verification was carried out by 16S rRNA-specific primers and *L. plantarum* subspecies-specific primers (Table 1).

The Morphological and biochemical characteristic features of the LAB isolates are shown in Table 2. All isolates gave a positive reaction to Gram staining, in which five isolates were coccus in shape and organized in pairs or tetrads. Their colonies on MRS agar were round, low convex, with a complete edge, and creamy in color. The rest twelve LAB isolates were rod-in shape and organized in pairs or chains using Gram reaction.

Table 2. Morphological and biochemical characteristic of the isolated LAB

Fermentation interval	Colony Pigmentation	Gram staining	Morphology	Catalase test	Oxidase test
24 h	Creamy	+	Coccoid	-	-
	White	+	Rod	-	-
	Milky-white	+	Coccus	-	-
Day 3	White	+	Rod	-	-
	Transparent	+	Coccus	-	-
	White	+	Rod	-	-
	Creamy	+	Coccoid	-	-
Day 5	White	+	Rod	-	-
	White	+	Rod	-	-
	White	+	Rod	-	-
	White	+	Rod	-	-
Day 7	Creamy	+	Coccoid	-	-
	White	+	Rod	-	-
	White	+	Rod	-	-
	White	+	Rod	-	-
Day 10	White	+	Rod	-	-
Day 14	White	+	Rod	-	-

As shown in Table 3, the API CHL diagnosis tests confirmed eight LAB isolates as *L. plantarum*, three isolates as *L. mesenteroides*, two isolates as *L. pentosus*, two isolates as *L. brevis,* and two isolates as *Tetragenococcus halophilus*.

Table 3. Biochemical identification (%) of LAB isolates using API 50 CHL api-web™ software database (Biomérieux, France, V 5.1).

Fermentation Interval	Colony Pigmentation	Morphology	Significant taxa	% ID
24 h	*Creamy*	Coccoid	*Leuconostoc mesenteroides*	99.9%
	White	Rod	*Lactobacillus plantarum*	99.6%
	Milky-white	Coccus	*Tetragenococcus halophilus*	92.2%
Day 3	White	Rod	*Lactobacillus plantarum*	99.9%
	Transparent	Coccus	*Tetragenococcus halophilus*	99.8%
	White	Rod	*Lactobacillus brevis*	98.2%
	Creamy	Coccoid	*Leuconostoc mesenteroides*	99.9%
Day 5	White	Rod	*Lactobacillus plantarum*	99.8%
	White	Rod	*Lactobacillus plantarum*	99.9%
	White	Rod	*Lactobacillus brevis*	98.6%
	White	Rod	*Lactobacillus pentosus*	99.8%
Day 7	Creamy	Coccoid	*Leuconostoc mesenteroides*	98.2%
	White	Rod	*Lactobacillus plantarum*	99.9%
	White	Rod	*Lactobacillus plantarum*	99.9%
	White	Rod	*Lactobacillus pentosus*	99.8%
Day 10	White	Rod	*Lactobacillus plantarum*	98.6%
Day 14	White	Rod	*Lactobacillus plantarum*	99.9%

All confirmed *L. plantarum* strains were selected according to the morphological, biochemical, and metabolic profiles of the carbohydrates obtained by the API 50 CHL system, they were then verified by 16S rRNA-specific primers and *L. plantarum* subspecies-specific primer (Table 1) and separated by gel electrophoresis (Figure 1).

The amplified PRC products that corresponded to *L. plantarum* subsp *plantarum* showed identical length size as the primers used, according to the DNA Ladder used the band with a size of 349 bp was identical to (Castillo *et al.,* 2006) 16s rRNA-specific primer, was used to quantify lactobacilli population in pig digesta study, 247 bp band was similar to (Wu *et al.,* 2016) 16s rRNA-specific primer, that was adapted to study the gene expression of *L. plantarum* FS5-5 in response to salt stress, and 120bp band was in line with (Kim et al., 2020) subspecies-specific primer, which was used in this research to detect *L. plantarum* group identified by comparative genomics. Both 16S rRNA and API diagnosis tests gave the same verification results for the isolated *L. plantarum* M23 strains. (Figure 4)

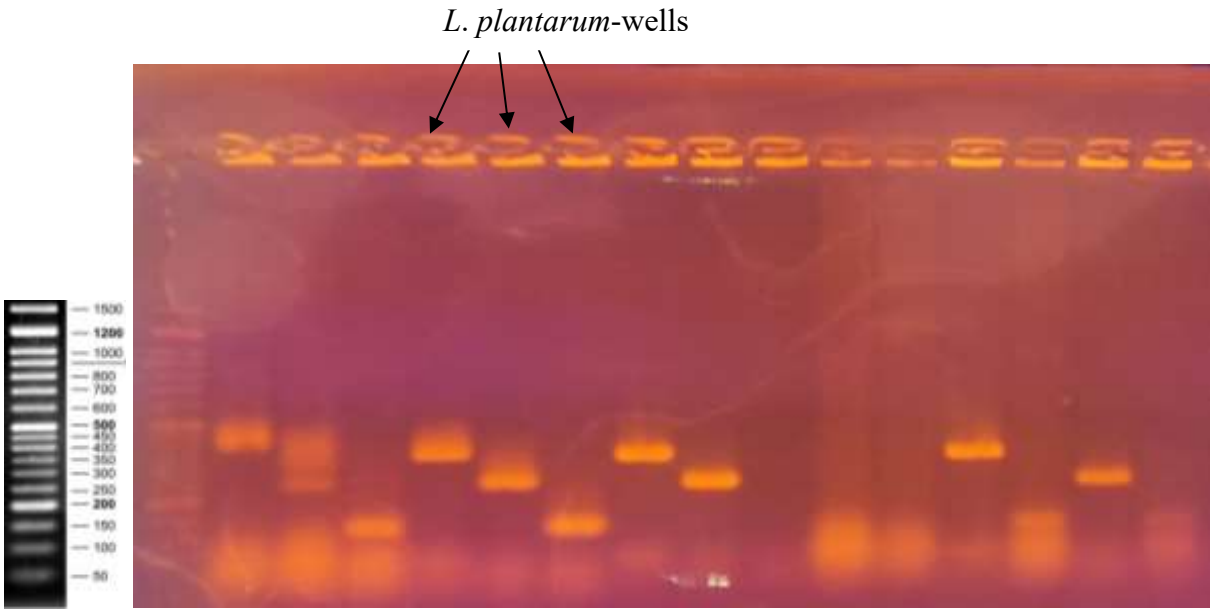

Figure 1. Amplified gene fragmentation using gel electrophoresis under UV light.

The bacterial growth curve (Log CFU/ mL) and pH changes during the fermentation process are shown in Figure 2. After 24 hours of fermentation, the most dominant identified LABs were *L. mesenteroides*, *T. halophilus,* and *L. plantarum* M23, respectively. This is inconsistent with what (Lennox & and Efiuvwevwere, 2013) found that several LABs engaged in cucumber fermentation based on their carbohydrate fermentation capacities. *L. mesenteroides* started development faster than any other LAB, in which it produced carbon dioxide and organic acids, which then started dropping the pH.

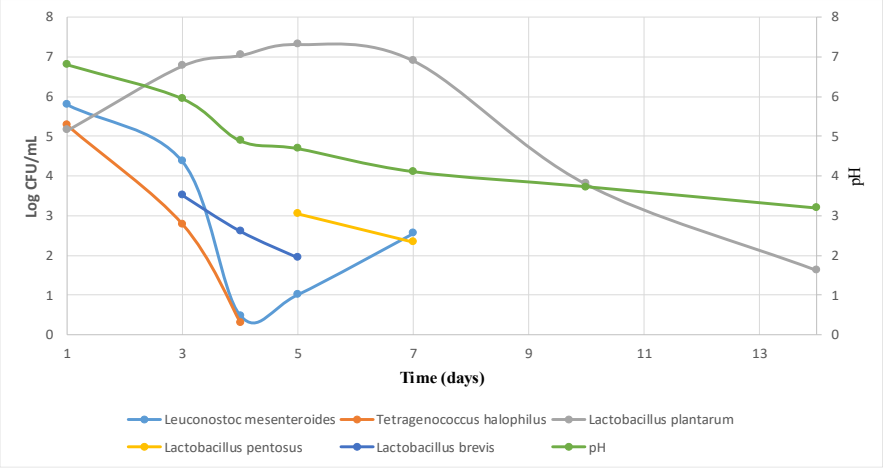

Figure 2. Bacterial growth curve (Log CFU/mL) and pH changes during the fermentation process

Uchida *et al*. (2014) demonstrated that halophilic lactic acid bacteria subsp. *T. halophilus* strains were capable of growing in highly salted cultures (10-15 % w/v NaCl). Guindo et al. (2022) also demonstrated that *T. halophilus* bacterium was found in miso and later found in a variety of salty foods, including soy sauce, anchovy brines, fermented foods, and smoked fish eggs, this might confirm the presence of this bacterium during the beginning of fermentation process of cucumber samples in our research work.

Starting from the fifth day of fermentation, the drop in the pH was significant (4.68-3.18), additionally, the dominant isolated LAB was *L. plantarum* M23 which controlled the fermentation process.

The drop in pH and production of fermentation byproducts such as bacteriocins might have a deactivation effect against other microorganisms found in brine solution.

It is well-reported that bacteriocins play a key role in reducing pathogenic and undesirable flora, as well as in the formation of beneficial bacterial populations (Collins *et al.,* 2010).

L. plantarum is known for its capability to produce the bacteriocin planta ricin at pH 4.8-4.1 (Lopetuso *et al.,* 2019)., in which we found that at this stage of fermentation (pH 4.8-4.1) *L. plantarum* was dominant.

The ability of *L. plantarum* to produce a variety of inhibitory compounds, such as organic acids (lactate, acetate, propionate, etc.), diacetyl, hydrogen peroxide, free fatty acids, and phenyl lactic acid, could have an inhibitory effect against other brine microflora during fermentation process and this may extend the shelf life of the fermented cucumbers by inhibiting a wide range of food spoilage microflora (Azizi *et al.,* 2017; Todorov, 2008; Corsetti & Gobbetti, 2018).

However, these antimicrobials compounds produced by *L. plantarum* are generally recognized as safe, food-grade molecules, and, widely accepted for food preservation (Ahmad *et al*., 2017).

Lennox & Efiuvwevwere, (2015) demonstrated the same, in which *L. plantarum* was the dominant strain during fermentation.

L. pentosus became involved in the fermentation process as it progressed, however, *L. plantarum* became the dominant LAB once the pH reached a certain threshold (4.68-4.1), in which just lactic acid was produced by *L. plantarum* as a homo-fermenter LAB. This goes in parallel with what (Grosu-Tudor & Zamfir, 2011) reported that several lactobacillus strains were identified from fermented vegetables, however, *L. plantarum* was the dominant species in spontaneous cucumber fermentation.

The results also demonstrated that the presence of *L. mesenteroides* decreased as the fermentation phase advanced, this might be due to acid production (lactic acid). Fleming & McFeeters, (1981) demonstrated that *L. plantarum* was primarily responsible for the brine acidity of the fermented cucumbers and that other typical forms of LAB, such as *Leuconostoc* species or *Lactobacillus* gas-producing species, did not contribute to acid generation during the fermentation process. Ammor & May, (2007) confirmed the capacity of some LAB species, to rapidly drop the pH value which is important in food preservation.

The previous findings were confirmed by conducting Response Surface Methodology (RSM) for data analysis, RSM was built with three variables to study bacteria growth patterns; microbial growth Log CFU/mL, pH value, and time (Figure 3). The central composite design was developed using pH value and time, all of which have a significant impact on the microbial growth of each species. The quadratic equation describing the connection between the microbial growth of each species and the two parameters pH value and time is as follows:

Microbial growth *(L. mesenteroides)*: 1.28 x time
+ 1.08 x pH
+ time x (pH x (-0.41))

Microbial growth *(T. halophilus)*: -1.86 x time
+ 0.87 x pH
+ time x (pH x 0.18)

Microbial growth *(L. plantarum* M23*)*: -2.04 x time
+ 0.41 x pH
+ time x (pH x 0.64)
Microbial growth *(L. pentosus)*: 0.55 x time
+ (-0.02) x pH
+ time x (pH x (-0.03))
Microbial growth *(L. brevis)*: -3.58 x time
+ (-0.35) x pH
+ time x (pH x 0.93)

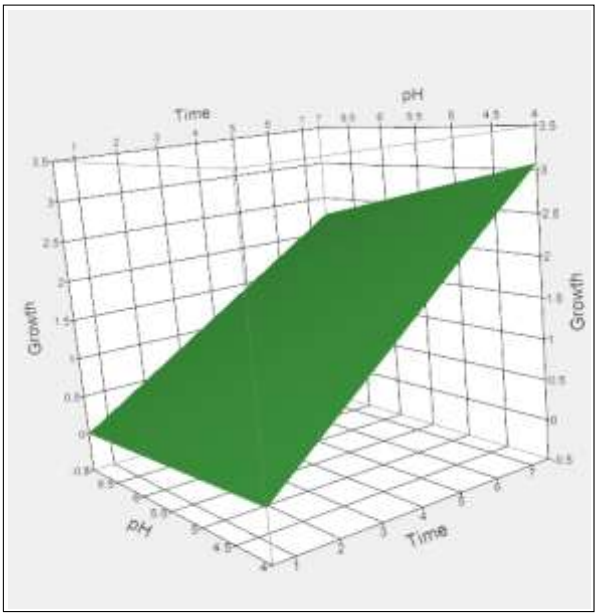

Figure 3. Response surface plot showing the effects of pH and time on microbial growth pattern of *L. plantarum* M23

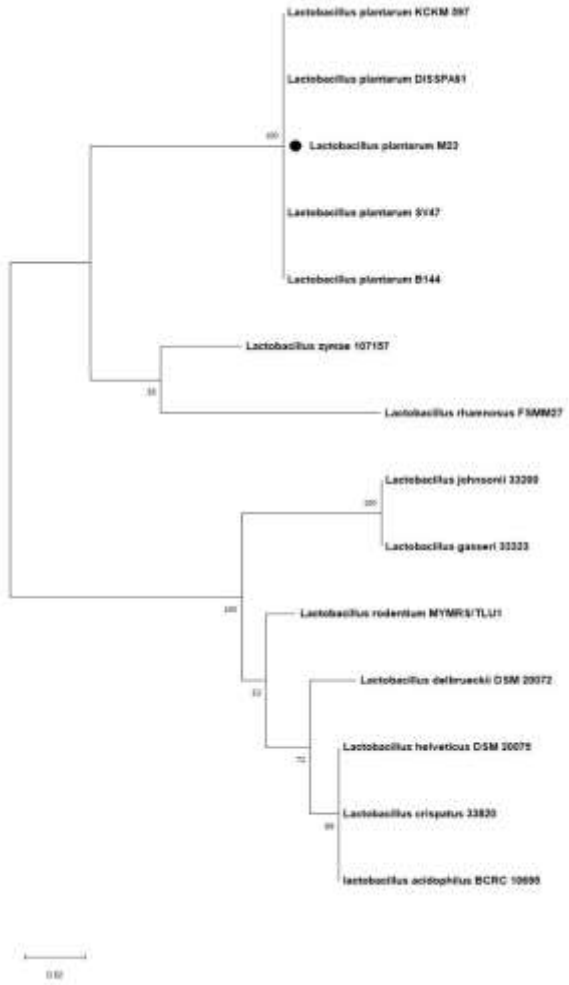

Figure 4: phylogenetic tree of 16S rRNA gene of *Lactobacillus plantarum* M23 strain

Conclusion

Seventeen wild LAB strains were isolated and identified during the fermentation of fresh cucumber in 8% brine solution at ambient temperature. After 24 h of fermentation, the most dominant LAB-identified strains were *L. mesenteroides*, *T. halophilus,* and *L. plantarum* M23, respectively. Starting from the fifth day of fermentation, *L. plantarum* M23 controlled the

fermentation process as the dominant LAB. The drop in pH during the fermentation process (4.8-4.1), production of organic acids, and other byproduct metabolites such as plantaricins may have an intrinsic effect on LAB strains variation present in the fermented brine solution, causing l plantarum to be dominant and to improve shelf-life and sensory attributes of fermented cucumbers as well.

Further investigation is to purify the peptide byproduct plantaricin produced by *L. plantarum* M23 to be used as an antimicrobial biopreservative.

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Declaration of Conflict of Interest

The authors declare no conflict of interest in the preparation of this work.

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فحص بكتيريا لاكتوباسيلاس بلانتيرام البرية الموجودة في محلول ملحي للخيار المخمر بشكل طبيعي

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ملخص

تم استخدام التخمير كتقنية بسيطة لحفظ الخضراوات منذ الايام القديمة. تهدف هذه الدراسة الى عزل وتوصيف وتعريف بكتيريا حمض اللاكتيك الموجودة طبيعيا في محلول ملحي بنسبة 8% للخيار المخمر طبيعيا حيث تم تخمير الخيار بشكل طبيعي لمدة 14 يوما في محلول ملحي في درجة حرارة الغرفة العادية, ثم تم زراعة العينات خلال 24 ساعة لمدد 3,5,7,10,14 يوما ثم تم عزل وتحديد 17 سلالة بكتيرية للحمض اللاكتيكي من مراحل مختلفة من التخمير باستخدام نظام التحاليل البيوكيماوية ونظام API 50 CHL. تم تاكيد Lactobacillus plantarum M23 بواسطة نظام الجينات التمهيدية الخاصة والتسلسل الجيني S rRNA16, حيث ان السلالة التي تم التاكد منها بواسطة PCR هي Lactobacillus plantarum subsp plantarum M23 حيث كانت هي السلالة المسيطرة والمسؤولة عن التخمير خلال اليوم الخامس من عملية التخمير بناء على الرحلان الكهربائي الهلامي والتسلسل الجيني. وبعد 24 ساعة من التخمير، كان أكثر انواع LAB انتشارا هي Leuconostoc mesenteroides وTetragenococcus halophilus و L. plantarum M23 على التوالي كان الانخفاض في قيمة الرقم الهيدروجيني معنويا بسبب الاختلافات في عدد انواع البكتيريا الحية الدقيقة اثناء عملية التخمير خلال 14 يوما. يتم اجراء مزيد من الابحاث لانتاج مركب البانترسير وهو منتج ثانوي من بكتيريا L. plantarum والذي يعتبر مضادا للميكروبات والذي اكتسب اهمية كبيرة في حفظ الاغذية.

الكلمات الدالة: Lactobacillus plantarum، بكتيرية اللاكتيكية، الخيار المخمر، البلانتا رايسينAPI 50 CHL، PCR، الرحلان الكهربائي الهلامي.

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