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Probiotic from Cabbage: Isolation, Characterization, and *In Vitro* Antimicrobial Evaluation of Intracellular and Extracellular Extracts

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بروبيوتيك من الملفوف: العزل، والتوصيف، والتقييم المختبري للنشاط المضاد للميكروبات للمكونات داخل الخلوية وخارج الخلوية

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Abstract:
The search for novel probiotic strains LAB, with potent broad-spectrum antimicrobial activity is crucial to combating antibiotic-resistant pathogens. This study details the isolation, phenotypic characterization, and *in vitro* probiotic safety profiles of *Lactobacillus* sp isolated from long-term naturally fermented cabbage (sauerkraut). Morphological and biochemical identification confirmed that bacteria is a Gram-positive, rod-shaped, catalase- and oxidase-negative, non-sporing, non-motile, and homofermentative bacterium. Probiotic characters were confirmed via standard survivability metrics; the strain demonstrated exceptional acid tolerance, remaining viable under simulated gastric juice at pH 3.0 for up to 180 min, and displayed halotolerance up to 6.5% NaCl. *In vitro* safety assessment confirmed a total lack of hemolytic activity, classifying the isolate as safer γ-hemolytic. Although crude neutralized cell-free supernatants exhibited no inhibition due to low standard metabolite concentrations, cold-methanolic extracts of both extracellular and intracellular compounds demonstrated substantial, concentration-dependent antibacterial efficacy. A well-diffusion assay revealed high sensitivity across seven clinical pathogens, with *Staphylococcus aureus* exhibiting the largest zone of inhibition (30 mm) for both extracts at 1000 mg/mL. Quantitative Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) screens against *S. aureus* proved that the extracellular extract possessed superior potency (MIC = 15.625 mg/mL; MBC = 31.25 mg/mL) compared to the intracellular framework (MIC ≤ 23.4 mg/mL; MBC ≈ 46.9 mg/mL). These results establish *L. plantarum* as a highly resilient probiotic candidate with strong biocontrol applications against multidrug-resistant pathogens including MRSA.

Keywords: Probiotic, Sauerkraut, LAB, *Lactobacillus* sp, MRSA.

الملخص
يُعدّ البحث عن سلالات (البروبيوتيك) ذات الفعالية المضادة للميكروبات واسعة الطيف وعالية الكفاءة أمراً بالغ الأهمية لمواجهة الممرضات المقاومة للمضادات الحيوية. في هذه الدراسة تم عزل بكتيريا العصيات اللبنية LAB (*Lactobacillus* sp.) من الملفوف المخمّر طبيعياً لفترات طويلة (الساوركراوت)، وتوصيفها المظهري، وتقييم أمانها كائن حيوي دقيق نافع خارج الجسم الحي (*in vitro*). أكّدت الصفات المورفولوجيا والكيميائية الحيوية أن العزلة البكتيرية موجبة لصبغة غرام، وعصوية الشكل، وسالبة

لإنزيمي الكاتالاز والأوكسيداز، وغير متبوّغة (لا مكونة للأبواغ)، وعديمة الحركة، ومتجانسة التخمر. كما أُثبتت سماتها كمعزز حيوي عبر مقاييس العكارة القياسية؛ إذ أبدت السلالة تحملاً استثنائياً للحموضة، محافظَةً على حيويتها في العصارة المعدية المحاكاة عند أس هيدروجيني (pH 3.0) لمدة تصل إلى 180 دقيقة، فضلاً عن إظهارها تحملاً للملوحة حتى تركيز 6.5% من كلوريد الصوديوم .(NaCl) وأكد تقييم السلامة خارج الجسم الحي الانعدام التام للنشاط الحالّ للدم، مما يصنّف العزلة ضمن نمط التحلل الدموي غاما (γ-hemolytic) الأكثر أماناً. وعلى الرغم من أن السائل الخالي من الخلايا البكتريا والمُعادل لم يُبدِ أي تثبيط نظراً لانخفاض تراكيز المستقلبات القياسية، فقد أظهرت المستخلصات الميثانولية الباردة لكل من المركبات خارج الخلوية وداخل الخلوية فعالية مضادة للبكتيريا الممرضة ومعتمدة على التركيز. وكشف اختبار الانتشار بالحفر عن حساسية مرتفعة لدى سبعة من الممرضات السريرية، حيث سجلت بكتيريا المكورات العنقودية الذهبية MRSA (*Staphylococcus aureus*) أكبر قطر لهالة التثبيط (30 ملم) لكلا المستخلصين عند تركيز 1000 ملغم/مل.

الكلمات المفتاحية: بروبايوتيك، الساوركراوت، LAB، *Lactobacillus* sp, MRSA.

1-Introduction

Fermented foods have been part of the human diet for at least 10,000 years, and fermentation is one of the oldest methods of preserving food [28,26]. Among fermented vegetables, sauerkraut, the product of the spontaneous lactic acid fermentation of shredded and salted cabbage (Brassica oleracea), one of the oldest and most widely consumed [25,31]. Cabbage is an inexpensive, widely cultivated vegetable rich in vitamin C, vitamin K, polyphenols and glucosinolates, whose hydrolysis products are associated with antioxidant and chemoprotective effects [19]. LAB are Gram-positive, non-spore-forming, acid-tolerant bacteria that ferment carbohydrates mainly to lactic acid [27]. Besides lactic acid, LAB produce acetic acid, exopolysaccharides, vitamins and antimicrobial compounds such as bacteriocins, which contribute both to the safety of the product and to its potential health benefits [34]. The global rise of antimicrobial resistance. Long-term and indiscriminate use of antibiotics has contributed to resistant pathogens and to adverse effects such as disturbance of the gut microbiota [30] LAB and their metabolites are being investigated as natural alternatives to chemical preservatives and as possible sources of new antimicrobial agents [13, 34]. This study links a traditional food technology with applied microbiology, and to evaluate whether its metabolites inhibit clinically relevant and antibiotic-resistant bacteria. To the best of the author's knowledge, this study is among the first to incorporate these specific methods for making sauerkraut in Libya.

2- Material and methods.

2.1- Isolation, Purification, and Conservation of culture

According to the method of [19, 32] Lactic acid bacteria (LAB) were isolated from both a liquid 7-day low-temperature batch of white cabbage (2% salt, 4°C, pH 5.40) and long-term fermented white and red cabbage matrices (2% salt concentration, held at 25°C for 99 days, standard pH 3.45 and 3.46, respectively). Samples of homogenised the brine. were serially diluted up to a 10^{-2} factor in sterile 0.9% physiological saline solution. Spread over selective de Man, Rogosa, and Sharpe (MRS) agar plates, extracts (100 µL) from dilutions (10^{-2} to 10^{-1}) were anaerobically incubated at 37°C for 24–48 hours. Using repeated streaking on fresh MRS agar, isolated single colonies with characteristic LAB morphology were purified. Pure cultures were kept at -20°C in 20% glycerol.

2.2- Phenotypic and Biochemical Characterization

Bergey's Manual of Determinative Bacteriology [3] was used to characterise the purified isolates. Colony elevation, border, size, colour, consistency, and texture (smoothness vs. roughness, opacity, or mucoidity) were among the phenotypic criteria. Using oil-immersion microscopy and standard Gram-staining techniques, cellular morphology and Gram status were assessed. Catalase, oxidase, motility, and growth performance were assessed biochemically under a range of temperature limits (15°C and 45°C), pH ranges (3.0–4.5 and 9.6), and NaCl gradients (1.0%–6.5% and 10%)as [16]

2.3-Carbohydrate Fermentation and Gas Profiling

Using glucose broth supplemented with inverted Durham tubes[15], the patterns of glucose fermentation and gas production were assessed. For 24 to 48 hours, sealed culture tubes were incubated at 37°C. While homofermentative activity was validated by homogeneous acid development without gas accumulation, heterofermentative pathways were revealed by the existence of gas bubbles inside the Durham tube.

2.4- *In Vitro* Safety Testing: Hemolytic Activity

Haemolytic activity [2,5] was assessed using a blood agar substrate enriched with 5% (w/v) defibrinated sheep blood in accordance with worldwide probiotic safety standards. After streaking the purified LAB isolate onto the

blood agar plates, it was anaerobically incubated for 24 to 48 hours at 37°C. Based on the area surrounding the colonies, haemolysis was divided into three groups: α-hemolysis: Partial lysis characterized by green zones. β-hemolysis: Complete lysis characterized by clear zones, and γ-hemolysis: No lysis or zone formation (deemed safe).

2.5- Acid Tolerance in Simulated Gastrointestinal Transit

Acid tolerance was measured using the parameters outlined by [18]. to evaluate gastric transit capacity. The purified strain's overnight MRS broth cultures were subcultured (3%, v/v) into new MRS media that had been dynamically adjusted to extreme gastric pH levels of 2.0 and 3.0 using 3.0 M hydrochloric acid (HCl). After 60 minutes (for pH 2.0) and up to 180 minutes (for pH 3.0) at 37°C, bacterial survival and viable cell counts were constantly monitored.

2.6- Target Pathogenic Indicator Strains

Seven important clinical pathogens—*Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli,* Methicillin-Resistant *Staphylococcus aureus* (MRSA), *Proteus* sp, antibiotic-resistant *Pseudomonas aeruginosa*, and antibiotic-sensitive *Pseudomonas aeruginosa*—were used to assess the antibacterial spectrum. Prior to bioassays, all indicator pathogens were obtained from the Alfa Zliten Medical Services Centre, grown at 37°C in Tryptone Soy Broth (TSB, Oxoid), and adjusted to a 0.5 McFarland turbidity standard (≈ 1.5×10^1 CFU/mL). Post-trial pathogen waste was autoclaved for 15 minutes at 121°C with 15 psi steam pressure to thoroughly deactivate it.

2.7-Extraction of Extracellular Active Compounds of *Lactobacillus* sp

For active compounds of Lactobacillus sp extract. The procedure described by [19,20] was used Lactobacillus sp isolate was grown in MRS broth at 35 C for 24 hours. The extraction of biologically active of secondary metabolites of bacterial cells, centrifugation of the broth culture was executed at 4,000 rpm for 15 minutes. Bacterial cells settled at the bottom rendering the supernatant to be cell-free. This supernatant was separated and mixed at a 1:3 ratios with 80% cold methanol (-20°C). for extracellular antimicrobial activity compounds extraction for overnight incubation. The solvents layer of extracts was evaporated by rotary evaporator, while the crude extract of bacteria was collected. This extracts were used for subsequent bioactive analysis. Stock concentration of 250, 200, 150, 100 mg/mL was prepared by adding 1% Dimethyl Sulfoxide (DMSO) to the crude extracts of the bacterial isolate. Working solutions concentrated of 50, 25, 20, 15, 10 mg/mL were also made by diluting the stock solutions in 1% DMSO. Both stock and working solutions were stored at -20 C around 7-10 days.

2.8- Agar Well-Diffusion and MIC/MBC Assays

According to [1], a well-diffusion assay was used to assess the antibacterial activity of *Lactobacillus* bacteria. McFarland turbidity of 0.5 were used to the chosen pathogenic bacterial cell suspensions. Mueller-Hinton agar plates were inoculated with each bacterial suspension. The agar was divided into 5 mm wells, and each well was filled with 100 µl of the extraction or supernatant. After allowing the supernatant to diffuse for one hour at room temperature, the plates were incubated overnight at 37˚C. The presence of released chemicals was responsible for the antimicrobial activity against the pathogenic indicator, as seen by the inhibition halos surrounding the wells. The negative control was MRS broth. To ensure that the inhibition was caused by antimicrobial compounds rather than lactic or other organic acids. The diameter of the zones of inhibition (mm) was determined following a 24-hour aerobic incubation period. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) assays were performed to evaluate the antibacterial activity of intracellular and extracellular bioactive compound extracts against *Staphylococcus aureus*.

3- Results and Discussion

3.1. Phenotypic Architecture and Biochemical Validation

In highly acidic substrates, the isolates LAB sp [12]. demonstrated remarkable growth capability. Colonies were characterized morphologically as round, smooth, elevated, unclear, and milky white on solid MRS agar (Table 1). Typical *Lactobacilli* morphotypes, figure (1) such as uniform Gram-positive rods with smooth, rounded ends, were validated by microscopic analysis. Biochemical analysis established a classic homofermentative functional profile The isolate was non-motile, did not form endospores, and lacked the enzymes catalase and oxidase. LAB isolate was non-motile, did not form endospores, and lacked the enzymes catalase and oxidase. The growth rates of LAB isolates were quite variable; it responded to various temperature ranges (15°C to 45°C), acidic borders (pH 3.0–4.5), and saline frameworks up to 6.5% NaCl, but it was inhibited at pH 9.6 and 10% NaCl [17]. Its classification as a homofermentative lactic acid bacterium was further confirmed by the glucose fermentation assay in Durham tubes [32]., which produced positive acid development without any discernible gas accumulation.

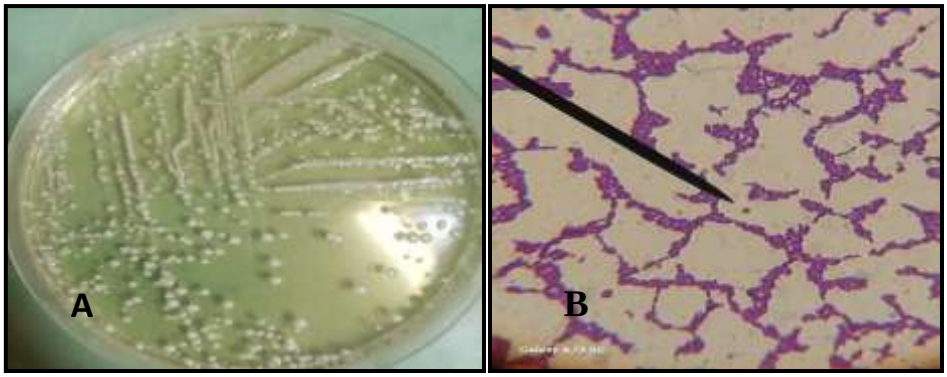


Figure-1 (A) Morphology of *Lactobacillus* sp colonies on MRS agar. (B) Microscopic view (100x oil immersion) displaying regular Gram-positive rod-shaped bacterial cells arranged individually or in short chains

Table 1 Physiological and biochemical characteristics of LAB isolates**.**

Characteristic	Test Method	Results	Interpretation
Cell Shape	Microscopic Examination	Rod	Typical morphology of Lactobacilli
Gram Staining	Gram Staining	+	Gram-positive bacteria
Catalase	Biochemical Testing	-	Lack of catalase enzyme
Oxidase	Biochemical Testing	-	Lack of oxidase enzyme
Spore Forming	Microscopic Observation	-	Non-sporing bacteria
Motility	Microscopic Observation	-	Non-motile bacteria
Anaerobic Growth	Incubation under Anaerobic Conditions	+	Facultative anaerobe / aerotolerant
Growth at 15°C	Incubation at Specified Temp	+	Capable of psychrotrophic growth
Growth at 45°C	Incubation at Specified Temp	+	Capable of thermotolerant growth
Hemolytic Activity	Blood Agar Base (5% Sheep Blood)	γ	No hemolysis (Safe profile)
Growth at pH 3.0–4.5	Growth in Acidified MRS Broth	+	High acid tolerance
Growth at pH 9.6	Growth in Alkaline MRS Broth	-	Inhibited by alkaline conditions
Growth in 1.0–6.5% NaCl	NaCl Tolerance Test	+	Salt tolerant up to 6.5%
Growth in 10% NaCl	NaCl Tolerance Test	-	Inhibited by 10% NaCl
Gas from Glucose	Durham Tube Assay	-	Homofermentative mechanism
Glucose Fermentation	Carbohydrate Fermentation Test	+	Positive acid production from glucose

Note: +, positive reaction; -, negative reaction; γ, no hemolysis**.**

3.2. Probiotic Functionality: Gastrointestinal Survival and Safety

Bacteria require gastric transit by resisting the stomach's low pH [7,8] and the small intestine's high bile concentrations in order to be a useful probiotic [11]. In this investigation, the lab isolate showed strong resistance characteristics, keeping high biological viability for up to 180 minutes in an extreme pH 3.0 environment [3]. This performance indicates that the LAB isolate has the acidic pathways required to survive gastric passage, which is consistent with observations by [21]. To make sure isolates don't have virulent characteristics, safety validation is still the essential requirement for probiotic selection. On sheep blood agar, LAB isolate clearly demonstrated γ-hemolytic activity [5], resulting in no lysis zones surrounding the colonies. The complete lack of haemolysin inhibits opportunistic pathogenicity, meeting worldwide safety standards for the selection of dietary adjuncts [23].

3.3 Intracellular and Extracellular Potency of Antimicrobial Activity

It's important to observe that crude cell-free supernatants (CFS) with pH adjustments of 6.5 showed zero zone clearance against the target pathogens [10]. This absence of activity in crude supernatants may indicate that the main inhibitory mechanism was neutralized by organic acid or that easily diffusible extracellular metabolites were present at amounts below the detection threshold. Nevertheless, these active fractions were effectively concentrated by cold-methanolic extraction, exhibiting strong, dose-dependent antibacterial activity in all seven clinical indications (Table 2).

Table 2 *In vitro* antimicrobial activity of extracellular and intracellular extracts from LAB isolate against target pathogens.

Pathogenic Indicator Strains	Extract Type	1000 mg/mL	250 mg/mL	200 mg/mL	150 mg/mL	100 mg/mL
E. coli	Extracellular	25	16	15	14	13
	Intracellular	25	18	17	16	15
S. aureus	Extracellular	30	16	14	13	12
	Intracellular	30	20	16	15	15
K. pneumoniae	Extracellular	26	16	13	12	11
	Intracellular	25	17	15	14	12
P. aeruginosa (Resistant)	Extracellular	25	13	13	11	10.5
	Intracellular	23	18	15	13	14
P. aeruginosa (Sensitive)	Extracellular	28	16	15	14	13
	Intracellular	30	23	17.5	16	15
MRSA	Extracellular	25	16	15	15	15
	Intracellular	24	21	20	18	16
Proteus spp.	Extracellular	28	18	22	20	18
	Intracellular	26	20	20	18	16

Values represent the mean Inhibition Zone Diameter (IZD) in mm.

With a 30-mm zone of inhibition at 1000 mg/mL, both extracts demonstrated strong effectiveness against Gram-positive *S. aureus* (18.37 mm), (figure 2, 3). Antibiotic-sensitive *Pseudomonas aeruginosa* showed the highest susceptibility as shown (figure 4, 5) among gram-negative organisms, with inhibition zones of 28 mm and 30 mm for external and intracellular extracts, respectively. Cell-bound bacteriocins or concentrated secondary metabolites (such functional peptides or cyclic compounds) [29] were probably recovered during the extraction procedure, allowing for the detection of their antimicrobial activity.



Figure -2 Inhibition zones produced by the extracellular extract of *Lactobacillus* sp against *Staphylococcus aureus* on Mueller Hinton agar.



Figure-3 Inhibition zones produced by the intracellular extract of *Lactobacillus* sp against *Staphylococcus aureus* on Mueller Hinton agar.



Figure- 4 Inhibition zones produced by the extracellular extract of *Lactobacillus* sp *Pseudomonas aeruginosa* antibiotic-sensitive on Mueller Hinton agar.

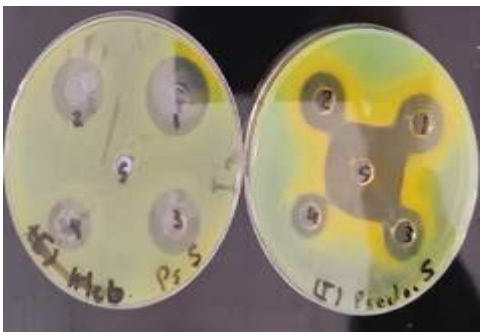


Figure- 5 Inhibition zones produced by the intracellular extract of *Lactobacillus* sp *Pseudomonas aeruginosa* antibiotic sensitive on Mueller Hinton agar.

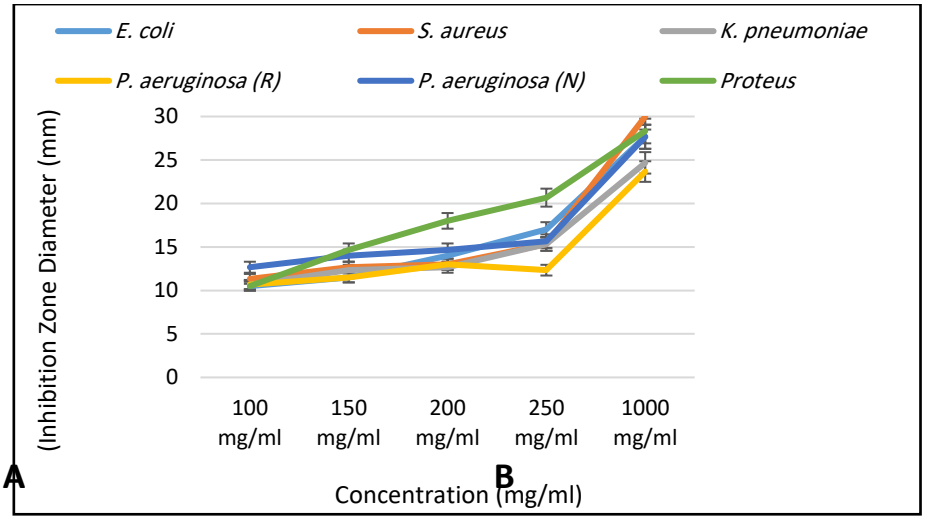


Figure (6) - Concentration-dependent inhibition zone diameters (mm) for Extracellular extract**.**

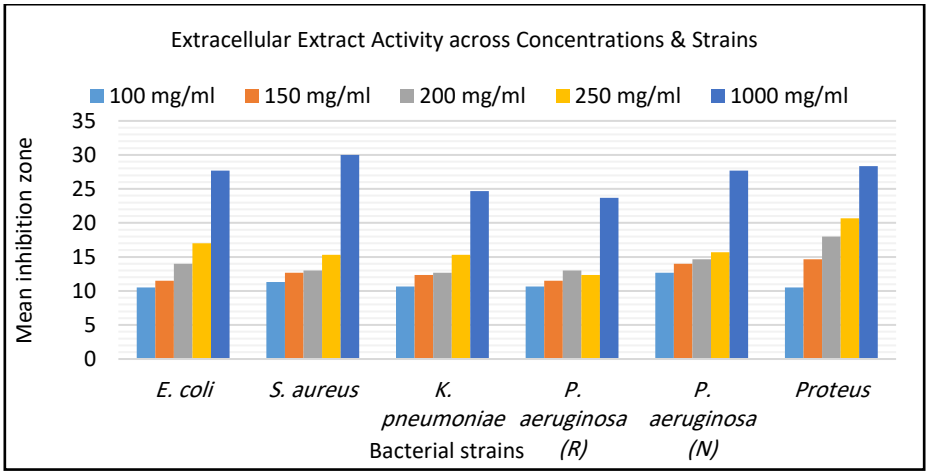


Figure (7)- Comparative activity across bacterial strains at each concentration level to Extracellular Extract**.**

3.4 Determination of Minimum inhibition concentration and Minimal Bactericidal Concentration:

The extracellular extract has more potency than the intracellular extract, according to micro-dilution profiling against *S. aureus*. There was no discernible turbidity in the first six tested dilutions of the extracellular extract.



Figure (8) - **A** Minimum inhibitory concentration (MIC)



Figure (9)- B Minimum bactericidal concentration (MBC) of Extracellular active compounds extract against *Staphylococcus aureus*

As shown in figure 8 and 9 when these extracts were culture onto solid Muller Hinton Agar plates, the first five plates showed total bactericidal clearance, but the sixth plate showed thick bacterial regrowth. This determines the extracellular extract's MIC of 15.625 mg/mL (Tube 6) and MBC of 31.25 mg/mL (Tube 5). There was no discernible turbidity in the first five tubes containing the intracellular extract. The first four agar plates showed no growth after subculturing, while the fifth plate showed colony regrowth. This translates to an MBC of 46.9 mg/mL (Tube 4) and a MIC of 23.4 mg/mL (Tube 5). The extracellular extract's lower MIC and MBC thresholds show that lab isolates mainly release their active antimicrobial components into the surrounding environment [33]. These bioactive substances have the potential to be natural biocontrol agents because they probably affect membrane permeability, damage cell wall construction, or interfere with vital metabolic pathways in target pathogens.

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host [14] Isolate *Lactobacillus* sp was a Gram-positive, catalase- and oxidase-negative, non-motile, non-spore-forming rod that fermented glucose without gasafter prolonged fermentation at 25 °C, a stage at which *L. plantarum* is expected to dominate the community [24] . In contrast, methanolic extracts from both the supernatant (extracellular) and the cell pellet (intracellular) produced inhibition zones of 10.5–30 mm against all six pathogens, and the zone diameter generally increased with extract concentration. However, the extracts were not compared with an extract of uninoculated MRS broth. Bacteriocins of several *Lactobacillus plantarum* species are known to remain adsorbed to the producer cell surface under acidic conditions [4,6], and LAB also produce hydrophobic secondary metabolites, such as fatty acids and cyclic compounds, that are poorly recovered in aqueous supernatants but extracted by organic solvents [22,9].

At the highest concentration tested, the largest zones were obtained against *S. aureus* (30 mm for both extracts) and antibiotic-sensitive *P. aeruginosa* (28–30 mm). This contrasts with the frequent observation that LAB are more active against Gram-positive than Gram-negative bacteria [15] antibiotic-sensitive *P. aeruginosa* with the intracellular extract (19.53 mm), whereas antibiotic-resistant *P. aeruginosa* had the smallest mean zone with both extracts (14.23 and 16.00 mm).

The MIC and MBC of the extracellular extract against *S. aureus* were 15.625 and 31.25 mg/mL, respectively, the extracellular extract was more potent in broth (four-fold lower MIC) [13] whereas the intracellular extract gave larger zones at low concentrations on agar; this apparent discrepancy may reflect differences in how readily the compounds in each extract diffuse through agar compared with their activity in liquid medium. LAB isolate whose crude methanolic extracts showed bactericidal activity against *S. aureus* and inhibit Gram-negative pathogens, including antibiotic-resistant *P. aeruginosa*. These findings support the potential of sauerkraut isolates as starter cultures and as sources of natural antimicrobials, subject to molecular identification and further functional characterization.

Conclusion

Important *in vitro* range conditions for beneficial probiotics are present in LAB, which was separated from naturally fermented sauerkraut. The isolates show better biological safety profile (γ-hemolysis), strong environmental tolerance, and high PH tolerance in stomach acid-like circumstances. Methanolic extraction of both intracellular and extracellular extracts demonstrated broad-spectrum antimicrobial efficacy against clinical infections, including MRSA and resistant Pseudomonas strains, but raw supernatants shown modest crude activity. This LAB isolate effectively secretes bioactive metabolites, as evidenced by the increased potency of the extracellular extract (MIC = 15.625 mg/mL). These results imply that LAB extracted from cabbage has a great deal of potential for growth as a natural food antibacterial or therapeutic probiotic aid

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