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Antimicrobial effect of isolated lactic acid bacteria and *Bacillus* spp.

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ABSTRACT

Lactic acid bacteria (LAB) have an effective antimicrobial effect against pathogenic bacteria linked to food-borne diseases, including *E.coli, Salmonella, Listeria,* and other human pathogenic bacteria. LAB produces antimicrobial substances, primarily lactic acid, which lowers pH and creates an acidic environment that inhibits the growth of harmful bacteria. Identified LAB strains, predominantly Lactobacillus species, exhibited probiotic potential and effectively inhibited various tested pathogens. The study collected twelve samples from natural dairy products, homemade pickles, and infant faeces. Catalase assays confirmed the isolates were catalase-negative. The findings revealed that raw milk contains a diverse microbial community rich in LAB, which possesses antagonistic properties. After the Antimicrobial assay of LAB species, the best results were LA2, LA4, LA5, LA7, and LA12. The most effective results after Optimization tests were LA2 and LA7 whose sources are Breast Milk and Infant faeces. Other LAB isolates that tested positive for catalase activity were identified as *Bacillus* species, exhibiting antifungal properties against different pathogenic fungi such as *Aspergillus, Trichoderma*, *Fusarium, and Rhizoctonia*. After fungal assay of *Bacillus* species, the best isolates were B1, B4, and B5. According to these results, we conducted Optimization tests. Selected isolates B1 and B4 displayed stability across different pH levels, temperatures, and incubation periods, with B4 giving more inhibition zone. *Bacillus* species produced soluble antibiotics, highlighting their potential in medical and pharmaceutical applications. Overall, LAB and *Bacillus* isolates present promising opportunities for biological control of diseases in humans, animals, and plants, suggesting their use as effective biological control agents.

Keywords: Lactic acid bacteria [LAB], *Bacillus*, Antibacterial, Antifungal, probiotics.

1. Introduction

Lactic acid bacteria (LAB) are a group of gram-positive, rod or coccus-shaped cells, catalase-negative, facultative anaerobes, non-motile, non-spore-forming bacteria that produce lactic acid as the final result of carbohydrate fermentation (Abid *et al.,* 2022). Probiotic LAB is classified as homofermentative or heterofermentative based on their metabolic pathways (Amany *et al.,* 2021). In homofermentative LAB, they ferment sugars to produce mainly lactic acid under anaerobic conditions. This metabolic pathway not only leads to the production of lactic acid but also results in a decrease in pH, which creates an acidic environment. This acidification plays a crucial role in food preservation by inhibiting the growth of spoilage and pathogenic microorganisms (Leroy and De Vuyst, 2004); (Axelsson, 2004); (Tavakoli *et al.,* 2017). In heterofermentative LAB, sugars are fermented to produce ethanol, CO2, and less lactic acid (Ayyash *et al.,* 2018). LAB does not constitute cytochrome and does not form spores. They belong to the order Eubacteriales under the Streptococcaceae and Lactobacillaceae families. The genera that comprise LAB are *Lactobacillus*, and *Bifidobacterium*, (Yerlikaya, 2019). LAB that is utilized as probiotics

has the following characteristics: nonpathogenic, viable in medium with low pH, able to grow on medium with high-concentrated bile salts, able to be adherent and colonize epithelial cells, have antibacterial activities, and have health benefits (Son *et al.,* 2017); (Ouwehand *et al.,* 2002). Lactic acid bacteria are proven to have an antibacterial effect against pathogenic bacteria that cause food poisoning such as *Salmonella sp., Helicobacter sp., Shigella sp., Staphylococcus sp.,* and *Escherichia coli sp.* (Parvez *et al.,* 2006). Probiotics containing LAB strains can help restore and maintain a healthy gut microbiota during and after antibiotic treatment. They can help prevent or alleviate antibiotic-associated diarrhea by competing with and inhibiting the growth of pathogens, promoting the restoration of beneficial bacteria, and modulating the immune system (Mack *et al.,* 1999); (Sanders *et al.,* 2010); (Riaz *et al.,* 2017). LAB strains such as *Lactobacillus* and *Bifidobacterium* are commonly used as probiotics and have been studied for their potential to mitigate the side effects of antibiotics, improve gut health, and reduce the risk of antibiotic resistance. However, it is important to note that the effectiveness of probiotics in the context of antibiotics can vary depending on the specific strains used, the antibiotic regimen, and individual factors (McFarland, 2006). In summary, while lactic acid bacteria are not directly involved in the production of antibiotics, certain LAB strains can be used as probiotics to support the restoration and maintenance of healthy gut microbiota during and after antibiotic treatment (Toghyani *et al.,* 2011). They can help mitigate the side effects of antibiotics and reduce the risk of antibiotic resistance (Fijan, 2014); (Reuben *et al.,* 2020). In the past decade, the antioxidant activity of LAB and LAB-related products has been frequently reported (Kim *et al.,* 2022). LAB can also produce antimicrobial compounds besides lactic and acetic acids, such as diacetyl and bacteriocins antagonistic to a broad spectrum of microorganisms and can inhibit food spoilage and pathogenic bacteria (Gänzle, 2015); (Ogunbanwo *et al.,* 2003); (Reis *et al.,* 2012). Those are the reasons why LAB is often used as an alternative to food preservation products. Bacteriocins are antimicrobial peptides produced by bactericidal or bacteriostatic bacteria. Bacteriocins produced by microorganisms do not have any inhibitory effect on the producing organisms. Bacteriocin can also inhibit the growth of contaminant bacteria that cause foodborne diseases (Cotter *et al.,* 2005); (Assari *et al.,* 2023).

Bacillus species are rod-shaped, endospore-forming aerobic or facultatively anaerobic, Gram-positive bacteria; in some species, cultures may turn Gram-negative with age and milky white color (Logan and De Vos, 2015); (Bahamdain *et al.,* 2015); (Setlow, Cowan and Setlow, 2003). The many species of the genus exhibit a wide range of physiologic abilities that allow them to live in every natural environment (Logan and De Vos, 2015). Only one endospore is formed per cell. The spores are resistant to heat, cold, radiation, desiccation, and disinfectants and Bacilli bacteria are present in substantial numbers in nearly all agricultural soils and other environments (Sansinenea and Ortiz, 2011). Among their other activities (biosynthesis of different enzymes), various strains of *Bacillus* spp. Exhibit antifungal abilities (Raaijmakers and Mazzola, 2012). Recently, it was shown that strains of bacilli inhibited different species of filamentous fungi: *Fusarium sp., Alternaria sp., Sclerotinia sp., and Charctonia solani*. (Elkahoui *at al.,* 2012); (Khan *et al.,* 2018); (Youcef *et al.,* 2014); (Perkins *et al.,* 1990). Bacilli are identified to yield more than 45 antimicrobial molecules, some of which are of clinical importance (Stein, 2005); (Hansen, Pschorn and Ristow, 1982). *Bacillus* species produce antibiotics in soluble protein form that they synthesize and secrete into the growing medium (Stein, 2005). So, the antibiotics they produce are cheaper and more effective, thus preferable in commercial production (Mardanova *et al.,* 2017); (Youcef *et al.,* 2014). Most *Bacillus* species are of remarkable significance because they produce antibiotics (Islam *et al.,* 2022). The capability of *Bacillus* species to synthesize a wide range of metabolites with antimicrobial activity in medicine and the pharmaceutical industry is significant, one of its potentials is to control different kinds of diseases in humans, animals, and plants when applied as a biological control agent (Graumann, 2007); (Borriss, 2011); (Petruţa *et al.,* 2003). *Bacillus* species that produce antibiotics include *B. Subtilis, B. Licheniformis, B. Brevis, B. Polymyxa, B. Circulans,* and *B. Cereus* (Logan and De Vos, 2015). Polypeptide antibiotics produced by *Bacillus* that are used in medical treatments are gramicidin, bacitracin, tyrotricidin, and polymyxin (Sharif *et al.,* 2016); (Islam *et al.,* 2022); (Stein, 2005). In our study, we examined the antimicrobial activity of *Bacillus* isolates with some species of fungi such as (*A.flavus, Trichoderma, A.niger, Alternaria, Fusarium*) and also with pathogenic plant bacteria such as (*EMB, Erwinia, Ralstonia*) and unidentified pathogen bacteria such as (12.4, 6.2), finally with yeasts such as (*Candida*

ATCC, Candida SP) to determine the type of its antimicrobial activity as antifungal, anti-yeast, or antibacterial activity (Emmert and Handelsman, 1999); (Basyony and Abo-Zaid, 2018); (Schallmey, Singh and Ward, 2004); (Horikoshi, 1999).

2. Material and methods

2.1. Isolation and Phenotypic Characterization of LAB

Twelve samples were collected from local markets and homes in Menofia and Gharbia. These samples included goat milk, mother's milk, raw cow milk, buttermilk (churn), olive pickles, old cheese, baby feces, yogurt, mesh, pickles, rayeb milk, and buffalo milk yogurt. The samples were randomly obtained in sterile corked plastic tubes and were immediately stored in a 4°C icebox for transportation to the laboratory. Upon arrival, the samples were examined for the presence of LAB. To identify LAB, 10 mL of each sample was enriched in 40 mL of de Man, Rogosa, and Sharpe (MRS) broth media (Sharif *et al.,* 2016; Wisd Laboratory Instruments, Menofia). The isolates that grew were retrieved and inoculated on MRS agar plates using an ose needle and streak method to obtain pure cultures.

Table 1: isolation sources of lactic acid bacteria

Source	Code of isolates	Dates of collection
Goat milk	LA1	4/10
Mother's milk	LA2	13/10
Raw's cow milk	LA3	15/10
Buttermilk "Churn"	LA4 & B1	20/10
Olive pickles	LA5 & B2	13/10
Old cheese	LA 6& B3	30/10
Baby faeces	LA7	1/11
Yogurt	LA8 & B4	5/11
Pickles	LA10 & B5	6/11
Rayeb milk	LA11 & B6	10/11
Buffalo milk yoghurt	LA12 & B7	12/11

(LA): meaning Lactic acid bacteria**, (B):** meaning *Bacillus* bacteria

These plates were incubated for 24-48 hours at 37°C under aerobic conditions. After incubation, individual colonies from each plate were selected and purified through three progressive exchanges on MRS agar. The pure isolates were characterized as LAB by Gram staining, cell morphology, and catalase test according to standard methods (Sharpe, 1979), as well as through the Methyl Ruddy (MR) test, Triple Sugar Press (TSI) test, and motility test. After this characterization, gram-positive, catalase-negative isolates were chosen for storage at −20°C in MRS broth additionally 28% glycerol (El Soda *et al*., 2003; Sharpe, 1979).

2.2. Morphological and Biochemical Characterization of lactic acid bacteria

2.2.1. Gram stain

A loopful of bacterial culture was collected and placed onto a glass slide suitable for microscopy. Crystal violet dye was then applied to the slide and allowed to react for one minute. The slide was rinsed with distilled water and allowed to air dry. Iodine drops were added next, and the slide was left to react for another minute. After the iodine treatment, the slide was rinsed with distilled water and air-dried again before being immersed in ethanol for 20 minutes. Finally, counterstaining was performed using safranin for 30 seconds, and the results were examined under a microscope at 100× magnification (Amelia and Philip, 2021).

2.2.2. Catalase test

Catalase is an enzyme produced by microorganisms that inhabit oxygen-rich environments, enabling them to neutralize harmful oxygen metabolites such as H2O2. A small quantity of bacterial colony was transferred to the surface of a clean, dry glass slide using a loop. A drop of 3% H2O2 was then added to the slide and mixed, as described by (Bhavi *et al*., 2020). A positive result is indicated by a rapid release of oxygen (within 5-10 seconds), which is observable through bubbling.

2.2.3. Motility test

We utilize Sulfide-indole-motility (SIM) medium (3, 5, and 16). To prepare, bring the total volume to 1 liter with distilled water and heat it to boiling to dissolve the agar, following the protocols outlined by (Ismail *et al*., 2018) ;(Bhavi *et al*. 2020). The medium is then dispensed in 5-ml aliquots into sterilized test tubes and autoclaved at 121°C under 15 psi pressure for 15 minutes. For motility testing, a sterile needle is used to pick a well-isolated colony and stab the medium to a depth of approximately 1 cm from the bottom of the tube. It is important to keep the needle aligned in the same direction during insertion and removal. The tubes are then incubated at 37°C for 18 hours or until growth is noticeable. The observation of the motility test is determined by the growth pattern of

the bacteria in the medium. If bacteria only grow around the point of insertion, it indicates a negative result. Conversely, if the bacteria grow on the surface of the medium or spread throughout it, this indicates a positive result.

2.2.4. TSI test

Utilize a straight inoculating needle to collect an isolated colony. Inoculate the TSI slant by first stabbing the needle into the butt of the medium all the way to the bottom, then withdraw the needle and streak the surface of the slant. Ensure to use a loosely fitting closure to allow air access. Read the results after incubating at 37°C for 18 to 24 hours. Three types of data may be obtained from the reactions, depending on the specific carbohydrate that has been fermented, as noted by (Lehman 2005).

2.2.5. MR test

The methyl red uses a standard media (MRVP broth) and an indicator reagent (methyl red), Inoculate MRVP broth with a pure culture of the organism. Incubate at 35°C-37°C for at least 48 hr. Include 5 or 6 drops of methyl red reagent per 5 mL of broth. Observe the color alteration in the broth medium. This test is utilized to decide the capacity of the organism to produce an expansive number of organic acids that incorporate formic acid, acetic acid, lactic acid, and succinic acid from glucose fermentation or not by changing the color of the PH indicator as in (Nayak *et al*., 2020).

2.3. Antimicrobial Assay

Antimicrobial assays were conducted utilizing the disk diffusion method, involving different Gram-negative bacteria such as *Escherichia coli, Listeria sp., Salmonella sp., Pseudomonas sp., Candida ATCC, Candida* sp., Additionally, Gram-positive bacteria included Staphylococcus. All isolates were too assessed for their antifungal properties against a wide range of fungi, including *Alternaria sp., Trichoderma sp., A. niger, and Fusarium* sp. In the disk diffusion method, pure cultures of the tested lactic acid bacteria were utilized to make disks, which were then added onto pure Petri plates settling different pathogenic bacteria utilizing sterilized forceps. All plates were incubated aerobically at 37 °C. The antibacterial activity was measured by the diameter of the clear zones of inhibition produced by the LAB disks. The second approach employed was the well diffusion method, where all LAB isolates were cultured in MRS broth media, and *Bacillus* isolates were grown in nutrient broth media. Following the incubation period, centrifugation of all isolates was performed at 6000 rpm, 4℃, 30 min to obtain a supernatant free of bacterial cells. The antimicrobial activity of the various LAB and *Bacillus* isolates was evaluated using the well diffusion method (Tejero-Sariñena *et al.*, 2012) against different pathogenic strains, and the diameter of the inhibition zones was recorded in millimeters.

2.4. Bacteriocin Assay

The LAB candidates were subsequently inoculated into MRS broth media and incubated at 37 °C for 24 hours. Additionally, 1 ml of the bacterial culture was precipitated into pellet and supernatant fractions using a centrifuge at 6000 rpm, 4℃ for 10 minutes. The supernatant was then neutralized to a pH of 6.5 by adding 0.1 M NaOH. Following this, the supernatant was filtered through 0.22-micron filter membranes, resulting in a cell-free neutral supernatant (Sukmawati *et al.,* 2022).

2.5. Bile salt Resistance Assay

A resistance test was performed to evaluate the LAB isolates against salinity by culturing them from the stock into 6 ml of liquid MRS media. For the samples (LA2, LA4, LA5, LA7and LA12), each was inoculated with 100 µL into five falcon tubes containing varying concentrations of bile salt/sodium deoxycholate (NaDC) concentrations of 0.1%, 0.2%, 0.3%, 0.4%, and 0.5%, (Hu *et al.,* 2022). A control test tube containing MRS media without bile salt solution was also included. All tubes were incubated anaerobically at 37 °C for 48 hours.

2.6. Salinity Resistant Assay

A resistance test was performed to evaluate the LAB isolates against salinity by culturing them from the stock into 6 ml of liquid MRS media. For the samples (LA2, LA4, LA5, LA7and LA12), each was inoculated with 100 µL into six falcon tubes containing varying concentrations of salinity (0.5%, 1%, 5%, 10%, 15%, and 20%). The tubes were then incubated in a static incubator at 37 °C for 48 hours, following the methodology outlined by (Papadopoulou *et al.,* 2023).

2.7. Acid-resistant Assay

Using 6 ml of MRS broth, 100 µL of bacterial culture was inoculated and incubated at 37 °C for 48 hours. The pH of the media was adjusted to 4.5, 5.5,

6.2, and 8.5, respectively, using 0.5 N HCl or 1 N NaOH. The culture media consisted of MRS broth with these initial pH levels, as described in (Yang *et al.,* 2018).

2.8. Temperature-resistant Assay

Using 6 ml of MRS broth, 100 µL of bacterial culture was inoculated at various temperature settings (20 °C, 30 °C, and 40 °C) as outlined in (Yang *et al.*, 2018), and the antibacterial activity of the cultures was evaluated against pathogens.

2.9. Antimicrobial Assay of *Bacillus* spp.

The antimicrobial effect of *Bacillus* spp. was evaluated using the well diffusion method. For this, *Bacillus* cultures were centrifuged at 6000 rpm for 15 minutes to obtain a supernatant free of bacterial cells. This supernatant was then tested against various fungi, including *A. niger, A. flavus, Alternaria sp., Fusarium sp., and Trichoderma sp*., as well as plant pathogenic bacteria such as *Erwinia sp., Ralstonia sp.*

2.10. Optimization of antifungal agent produced by *Bacillus* spp.

2.10.1. **Effect of *Bacillus* spp. At different temperatures degrees**

A volume of 0.5 ml from each *Bacillus* isolate (B1, B4, B5) was inoculated into 20 ml of nutrient broth media and incubated at different temperatures (20 °C, 30 °C, 40 °C, and 55 °C) (Vásquez and Millones, 2023). The antimicrobial effect was then examined after an incubation period of 48 hours.

2.10.2. Effect of PH levels on *Bacillus* spp

A volume of 0.5 ml from each *Bacillus* isolate was inoculated into flasks containing 20 ml of nutrient broth media, with the pH adjusted to different levels (4, 5, 6, 7, and 8). The cultures were then incubated for 48 hours, following the methodology outlined by (Vásquez and Millones, 2023).

2.10.3. Time of optimization production of antifungal agents

This test was conducted to evaluate the effect of *Bacillus* bacteria on fungi over different incubation periods, given that these isolates exhibit antifungal properties. Three flasks, each containing 50 ml of nutrient broth media, were inoculated with 1 ml of *Bacillus* isolates (B1, B4, B5) and incubated at 30 °C in a shaker incubator for various durations (1, 4, 6, and 8 days). After each incubation period, samples were collected and stored in falcon tubes at -4 °C, as described by (Petruţa *et al.,* 2003). Following the different incubation periods, the falcon tubes were centrifuged at 6000 rpm for 15 minutes, and the antimicrobial effect of the *Bacillus* isolates was assessed using the well diffusion method against *A. niger.*

3. Results and discussion

3.1. Phenotypic Characterization of isolated LAB and *Bacillus* spp.

The isolated single colonies exhibited a variety of morphological shapes. It was noted that some colonies on the isolation plates could be categorized as round-shaped with smooth edges, a smooth and convex surface, white-beige coloration, and measuring 1.8 mm in diameter. These colonies displayed a bright color, which indicates they are lactic acid bacteria, as stated by (Amelia and Philip, 2021). In contrast, other colonies were characterized by a gray-white, round, opaque, flat, and dry appearance, with a medium size, which can be identified as a specific type of *Bacillus*. We used the sources that LAB can be found from Dairy products but by chance, we discovered another important *Bacillus* bacteria. We test the antimicrobial effect of LAB and *Bacillus* sp. which means the antibacterial and antifungal so we use some species of pathogenic bacteria such as (*E.coli* sp*., Erwinia* sp*., Ralstonia* sp*., Pseudomonas* sp*., Listeria sp., Staph. Salmonella* sp.) And species of fungi as (*A .flavus, Trichoderma* sp*., A.niger, Fusarium sp.*)And finally from yeast as (*Candida ATCC, Candida* Sp*).*

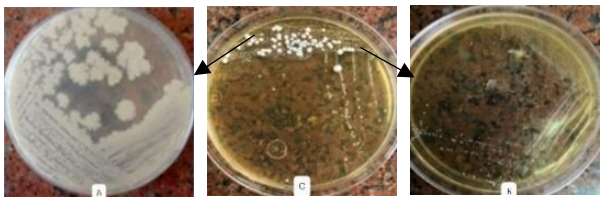


Figure 1: morphological shape of some isolates of LAB and *Bacillus* sp.
[A] Pure culture of *Bacillus* sp.
[B] pure culture of LAB
[C] mixed culture

3.2. Morphological and Biochemical characterization

3.2.1. Gram stain and catalase

All isolates of lactic acid bacteria (LAB) and *Bacillus* species were initially identified through morphological and several biochemical studies, including Gram staining, catalase, TSI, motility tests, and MR tests. The majority of the isolates displayed Gram-positive results, while LA10 was Gram-negative and LA2 exhibited a Gram-variable

characteristic with a cocci shape. Under a light microscope, the morphological shapes of the cell isolates varied between cocci and bacilli. The isolates were subjected to the catalase test, and the results indicated that some of them tested positive, suggesting they were *Bacillus* species. This aligns with the fact that *Bacillus* bacteria are aerobic or facultatively anaerobic, in contrast to lactic acid bacteria (LAB), which thrive in anaerobic conditions, as noted by (Amelia and Philip, 2021).

3.2.2. For MR test

All LAB isolates produced a yellow color, except for LA7 (Fig. 2.c), which exhibited a red color. The red coloration indicates that LA7 generates a significant amount of organic acids, including formic acid, acetic acid, lactic acid, and succinic acid, through glucose fermentation. Consequently, the broth medium remains red after adding methyl red, as the MR indicator remains red color at an acidic pH.

3.2.3. For Triple sugar iron (TSI)

All lactic acid isolates changed the color of both the butt and slant to yellow, indicating that the lactic acid bacteria possess the capability to ferment carbohydrates. TSI agar is a differential medium used to assess carbohydrate fermentation and H2S production. When any of the carbohydrates are fermented, a drop in pH occurs, causing the medium to shift from its original reddish-orange color to yellow. This color change in both the butt and slant **(B/S)** serves as an indication of sugar fermentation.

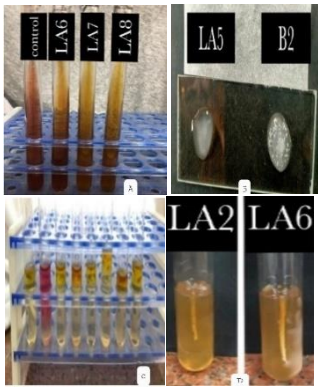
3.2.4. For Motility Test

All lactic acid isolates exhibited negative results, as LAB only grew in the vicinity of the inserted location and did not spread across the surface of the medium or throughout the media itself.us spp., according to (Lu *et al.,* 2018) ; (Pandav, 2021).

Table 2: Biochemical characterization of lactic acid bacteria.

Isolate codes	Gram stain test	Catalase test	MR test	TSI test B/S	Motility test
LA1	Positive cocci	Negative	Yellow	Y/Y	Negative
LA2	Variable bacilli	Negative	Yellow	Y/Y	Negative
LA3	Positive bacilli	Negative	Yellow	Y/Y	Negative
LA4	Positive bacilli	Negative	Yellow	Y/Y	Negative
LA5	Positive bacilli	Negative	Yellow	Y/Y	Negative
LA6	Positive bacilli	Negative	Yellow	Y/Y	Negative
LA7	Positive bacilli	Negative	Red	Y/Y	Negative
LA8	Positive bacilli	Negative	Yellow	Y/Y	Negative
LA9	-	-	-	-	-
LA10	Positive bacilli	Negative	Yellow	Y/Y	Negative
LA11	Positive bacilli	Negative	Yellow	Y/Y	Negative
LA12	Positive bacilli	Negative	Yellow	Y/Y	Negative

(B/S) meaning: Butt and slant, **(-)** meaning: Negative result, **(Y/Y)** meaning: Yellow to Yellow



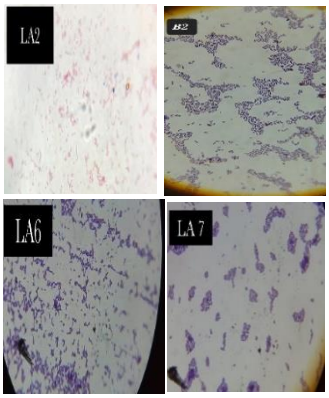


Figure 2: biochemical characterization of LAB. [A] TSI test [B] Catalase test [C] MR test [D] Motility test

[E] Gram stain test. LA2 show different shape and color from LA6, LA7 which mean that are different species of LAB

3.3. Antimicrobial activity

The results indicated that the largest inhibition zone was observed with Staphylococcus spp. at 23 mm, *Pseudomonas* spp. at 22 mm, *and Listeria* spp. at

20 mm. The zones of inhibition were categorized into four groups: weak (<5 mm), medium (5-10 mm), strong (>10-20 mm), and very strong (>20-30 mm). Consequently, the LAB's inhibitory activity against Pseudomonas spp. is classified as very strong. Additionally, a separate study found that L. acidophilus from fermented milk demonstrated antimicrobial effects against bacteria such as *E. coli*

and Staphylococcus spp. (Shafi *et al.,* 2019). The results also revealed that certain isolates, including LA2, LA4, LA5, LA7, and LA12, exhibited significant antimicrobial activity, selected based on their distinct isolation sources and high levels of antimicrobial effectiveness. Notably, all LAB isolates did not show any inhibitory effects against fungi or yeasts, indicating that our LAB isolates possess antibacterial properties against a broad spectrum of pathogenic bacteria.

Table 3: The Antibacterial effect of the most effective LAB isolates against different pathogenic bacteria.

Isolate codes	**Diameter of inhibition zone (mm) well diffusion method**									
	Erwinia sp.	*Ralstonia* sp.	*Listeria* sp.	*Pseudomonas aeruginosa. ATCC: 9027*	*E.coli. ATCC: 25922*	*Salmonella typhi ATTCC: 14028*	*Staph. aureus ATTC: 6538*	*E.coli*	*Klebsiella* sp.	*Enterobacter* .sp
LA2	15	-	-	19	-	-	22	10	-	11
LA4	20	23	20	15	7	10	20	7	15	14
LA5	13	-	15	12.5	-	7	21	9	9	5
LA7	12	-	15	22	6	7	23	-	15	12

(-) meaning: Negative result, **(mm)** meaning: a Diameter of inhibition zone in millimeters

Figure 3: the effect of LAB isolates on some pathogenic bacteria. Gram negative (A, B) and gram positive (c) by disk diffusion method

3.4. Bacteriocin Assay

The fact that none of the LAB isolates exhibited any antibacterial activity indicates that the bacteria's ability to produce one or more active metabolites, such as organic acids (lactic, acetic, formic, propionic, and butyric acids), which enhance their activity by lowering the pH of the media, is what accounts for the isolates' lack of efficacy when used in antibacterial assays.

3.5. Bile salt Resistance Assay

The impact of varying bile salt concentrations (0.1%, 0.2%, 0.3%, 0.4%, and 0.5%) on the antibacterial activity of LAB isolates was investigated. It was found that all five LAB isolates exhibited antibacterial activity against *pseudomonas* sp. up to a concentration of 0.3%; however, LA7 was the most effective LAB isolate against *pseudomonas* sp. at 0.1%, 0.2%, and 0.3%. At higher bile salt concentrations, there was no antibacterial effect. And LA7 had a decreasing impact on *Staph* from 0.1% to 0.4% (fig. 6), **(Tab 4)** Additionally, LA2 and LA5, had a substantial impact on *Staph.* At all concentrations and on *pseudomonas* sp. up to 0.4% bile salt concentration, respectively, showed antibacterial effects on *pseudomonas* sp. at all bile salt concentrations (fig. 4 and fig. 5). We can see that some isolates, such as LA7 and LA5, shown resistance to varying bile salt concentrations. This can be explained by possessing bile salt hydrolysis activity. The ability of probiotics to survive while traveling through the digestive system is crucial for preserving their health advantages. One of the indirect ways to lower cholesterol levels in the human body is by the action of bile salt hydrolysis (Sedláčková *et al.,* 2015). LAB strains that isolate from the gastrointestinal system, where bile salts are present, such as LA7, which isolates from baby feces, are more likely to include probiotic lactobacilli.

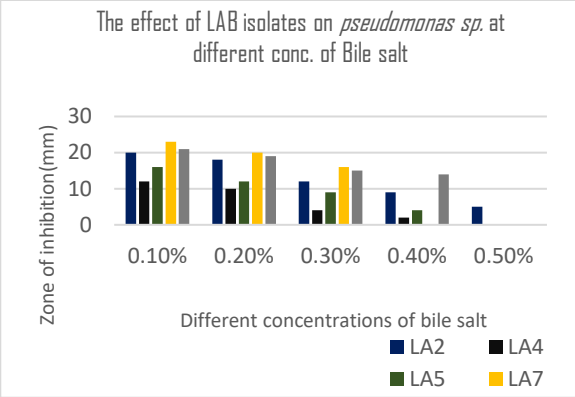


Figure 4: Effect of different bile salt concentrations on the antibacterial activity of The LAB isolates on *Pseudomonas* sp**.**

Figure 5: Effect of different bile salt concentrations on the antibacterial activity of LAB isolates on *Staph***.**

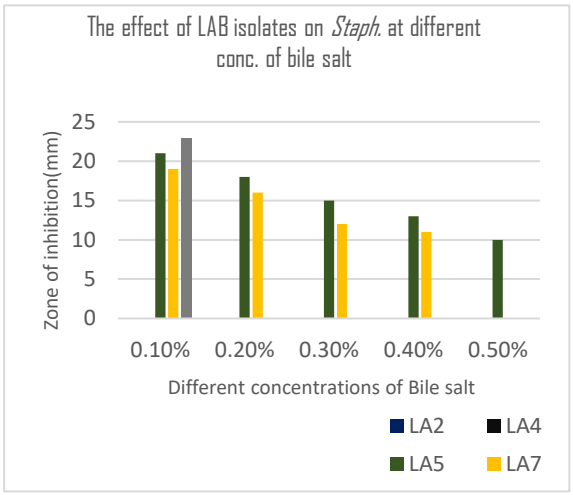


Figure 6: Antibacterial activity of LA7 on *Staph.* at different bile salt concentrations

Table 4: Anti-bacterial effect of LAB isolates at different bile salt concentrations.

Isolate Codes	Pathogenic Bacteria	Diameter of inhibition zone at different Bile salt concentration % in (mm)				
		0.1	0.2	0.3	0.4	0.5
LA2	*Erwinia* sp.	-	-	-	-	-
	Pseudomonas ATTCC	20	18	12	9	5
	Staph ATTCC	-	-	-	-	-
LA4	*Erwinia* sp.	-	-	-	-	-
	Pseudomonas ATTCC	12	16	4	2	-
	Staph ATTCC	-	-	-	-	-
LA5	*Erwinia* sp.	5	-	-	-	-
	Pseudomonas ATTCC	16	12	9	4	-
	Staph ATTCC	21	18	15	13	10
LA7	*Erwinia* sp.	7	5	3	-	-
	Pseudomonas ATTCC	23	20	16	-	-
	Staph ATTCC	19	16	12	11	-
LA12	*Erwinia* sp.	16	13	9	-	-
	Pseudomonas ATTCC	21	19	15	14	-
	Staph ATTCC	23	-	-	-	-

(mm) meaning: a Diameter of the inhibition zone in millimeters, **(-)** meaning: Negative result

3.6. Salinity Resistant Assay

The goal of the study was to obtain microorganisms that could metabolize saline substrates and create high titer lactic acid while withstanding salinity. For LAB isolates, various NaCl concentrations (0.5%, 1%, 5%, 10%, and 20%) were investigated. It has been noted that at high salt concentrations, the antibacterial activity of LAB isolates decreases. For example, at 15% and 20%, no isolate exhibits an antibacterial action against pathogenic bacteria. Antibacterial activity was demonstrated by all isolates at 0.5%, 1%, and 5%. Except for LA5, which showed no inhibitory effect at 1% concentration. LA2, on the other hand, was thought to be the most effective isolate against *Pseudomonas* sp. because it demonstrated an antibacterial effect against the bacterium up to 10% concentration and displayed an inhibition zone (13 mm) at 10% concentration (Fig8.B). Additionally, LA7 exhibited antibacterial activity against *Pseudomonas* sp. However, LA2 exhibited a greater antibacterial effect, indicating that it was more resistant to various salt concentrations than other LAB

isolates. (Figure 7) also demonstrated the decline in the antibacterial effect of LAB isolates against Pseudomonas sp. for plant pathogenic bacteria *Erwinia* sp. Based on the finding that LA4 (fig.8.A) and LA12 were the most effective LAB isolates, we may calculate the antibacterial rate of our isolate to be (≥10%). Additionally, many LAB strains can endure because they may use the salt content as a substrate in the glucose to lactic acid fermentation process, which results in a salt solution that creates osmotic pressure. This study demonstrated the impact of various salt concentrations on the rate of lactic acid production by

LAB strains and other parameters tested on kohlrabi. It did this by allowing nutrients and water in the raw materials to diffuse into the environment, creating a substrate for lactic acid bacteria to work (Hien *et al.,* 2022). Based on the results, we may classify our LAB isolates as mild halophiles (Khanal, **2023)** because they cannot exhibit any activity at salt concentrations of 15% and 20%.

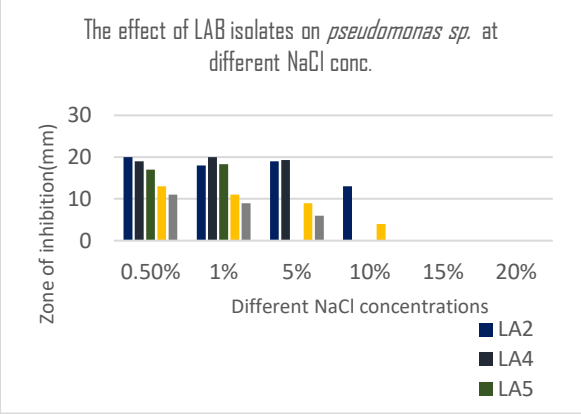


Figure 7: Effect of different NaCl concentrations on the Antibacterial effect of LAB isolates on *Pseudomonas*

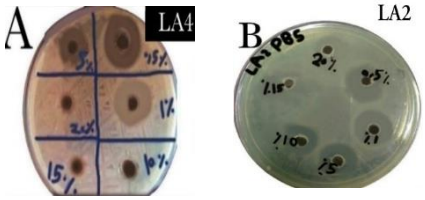


Figure 8: [A] Antibacterial effect of LA4 on Erwinia sp. [B]Antibacterial effect of LA2 on pseudomonas sp.

Table 5: Antibacterial effect of LAB isolates at NaCl concentrations

Isolate Codes	Pathogenic Bacteria	Diameter of inhibition zone at different NaCl concentration % in (mm)					
		0.5	**1**	**5**	**10**	**15**	**20**
LA2	***Erwinia* sp.**	5	-	-	-	-	-
	***Pseudomonas* ATTCC**	20	18	19	13	-	-
	***Staph* ATTCC**	-	-	-	-	-	-
LA4	***Erwinia* sp.**	14	13	12	-	-	-
	***Pseudomonas* ATTCC**	19	20	19,3	-	-	-
	***Staph* ATTCC**	-	-	-	-	-	-
LA5	***Erwinia* sp.**	11	-	-	-	-	-
	***Pseudomonas* ATTCC**	17	18,3	-	-	-	-
	***Staph* ATTCC**	11	-	-	-	-	-
LA7	***Erwinia* sp.**	-	-	-	-	-	-
	***Pseudomonas* ATTCC**	13	11	9	4	-	-
	***Staph* ATTCC**	11	10	-	-	-	-
LA12	***Erwinia* sp.**	16	15	6	-	-	-
	***Pseudomonas* ATTCC**	11	9	6	-	-	-
	***Staph* ATTCC**	23	20	13	10	-	-

(mm) meaning: a Diameter of the inhibition zone in millimeters, **(-)** meaning: Negative result

3.7. Acid-resistant Assay

We studied LAB isolates at four different PH values (3.5, 4.5, 6.2, and 8.5). All the LAB isolates at these PH values demonstrated strong antibacterial activity against *Pseudomonas* sp., but we found that LA2 and LA12 had the highest antibiotic activity at PH 6.2 with an inhibition zone (20 mm) and that LA2 had the highest inhibitory effect on *Staph*. At these PH values (fig. 10. B). As shown in **(Tab.6)** at PH 8.5, LA5 was the strain most affecting plant pathogenic bacteria *Erwinia* sp. with an inhibition zone of 23.7 mm. LA7 also had a good antibacterial effect with *Erwinia* sp. as shown in (fig. 10.D). LA7 and LA2 were the most effective isolates on Pseudomonas sp. with a 17 mm inhibition zone. According to the findings, lactic acid bacteria's (LAB) capacity to ferment sugars in lactic acid is one of its primary characteristics. This ability helps explain why

probiotic strains in the digestive tract survive in acidic environments, as reported by (Champomier-Verges *et al.,* 2002). Additionally, the kind of media used is crucial in the synthesis of organic acids by LAB because MRS broth media contains glucose as a carbon source, which is more hydrolyzable than other types of media, which contain starch as a carbon source, making them less hydrolyzable (Śliżewska and Chlebicz-Wójcik, 2020).

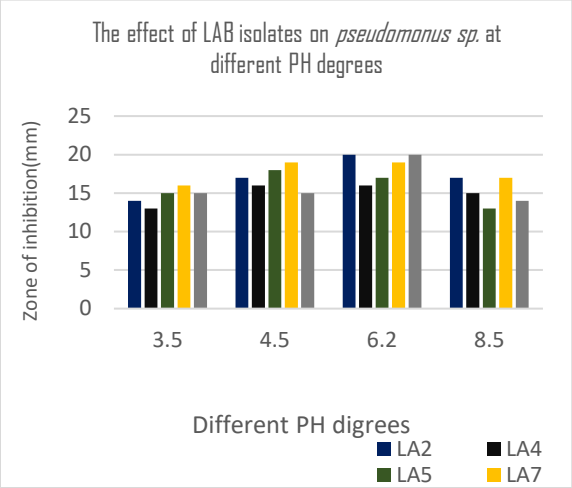


Figure 9: Effect of different PH degrees on the Antibacterial effect of LAB isolates on *Pseudomonas* sp.

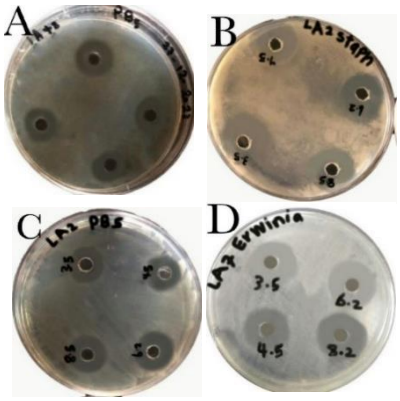


Figure 10: Effect of LAB isolates on pathogenic bacteria *Staph., Erwinia* sp., *Pseudomonas* sp. at different PH degrees

Table 6: Antibacterial effect of LAB isolates at different PH degrees**.**

Isolate codes	Pathogenic Bacteria	Diameter of inhibition zone at different PH degrees in (mm)			
		3.5	**4.5**	**6.2**	**8.5**
LA2	***Erwinia* sp.**	14.3	20	22	15,7
	***Pseudomonas* ATTCC**	14	17	20	17
	***Staph* ATTCC**	20	21	22	20
LA4	***Erwinia* sp.**	19,3	17	16,3	20
	***Pseudomonas* ATTCC**	13	16	16	15
	***Staph* ATTCC**	-	-	-	-
LA5	***Erwinia* sp.**	20	23	25	23,7
	***Pseudomonas* ATTCC**	15	18	17	13
	***Staph* ATTCC**	14	18	18	14
LA7	***Erwinia* sp.**	22,7	21	19,7	21
	***Pseudomonas* ATTCC**	16	19	19	17
	***Staph* ATTCC**	-	12	12	10
LA12	***Erwinia* sp.**	-	23	21	21,3
	***Pseudomonas* ATTCC**	15	15	20	14
	***Staph* ATTCC**	16	18	17	17

(mm) meaning: a Diameter of the inhibition zone in millimeters, **(-)** meaning: Negative result

3.8. Temperature-resistant Assay

One of the most significant physical factors influencing bacterial cell activity and behavior is temperature. The effect of temperature on the production of antibacterial agents revealed that the optimal temperature for LAB isolates to produce antibacterial agents was 30 °C. It was observed that all LAB isolates, except LA4, had the strongest antibacterial effect on *Pseudomonas* sp. at this temperature. The results also showed that the antibacterial effect of LA2 was unaffected by temperature changes, as the range of its inhibitory effect between different temperature degrees remained relatively constant, in contrast to other isolates whose antibacterial activity decreased at lower temperatures (20 °C). (figur11) At 30°C, LA7 was the most effective isolate against Staph. Its inhibition zone was measured at 23 mm. Additionally, LA2 demonstrated a strong antibacterial effect against *Staph*. At various temperature points. It was also noted that *Erwinia* sp. was impacted by LA7 (fig. 12. B), LA12 (incubated at 40°C), and LA4 (incubated at 30°C) with an inhibition zone measured at 20 mm **(Tab 7)**. The findings indicated that the range of temperatures between 20

and 40 degrees Celsius was ideal for the growth of lactic acid bacteria and that temperature variations are a key factor in distinguishing between LAB strains.

This is because all lactic acid bacteria share an optimum temperature, which aids in distinguishing them from one another. After all, temperature regulates bacterial growth. Since every species has a different optimal growth period, temperature influences the time it takes for bacteria to generate during different phases of growth. It was thoroughly examined by (Ahmed *et al.,* 2006), leading to the classification of LAB as mesophilic bacteria growth that demonstrated. activity between 10 and 45

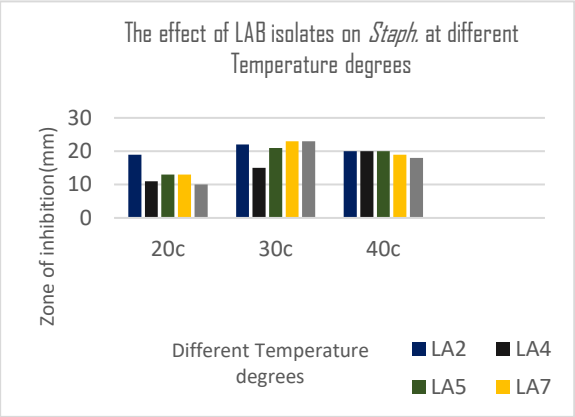


Figure11: The effect of different Temperature degrees on the Antibacterial effect of LAB isolates on *Staph.*

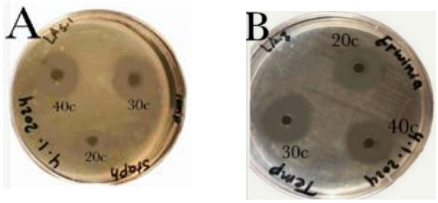


Figure 12: [A] Antibacterial effect of LA5 on staph. [B] Antibacterial effect of LA7 on Erwinia Sp**.**

Table 7: Antibacterial effect of LAB isolates at different temperatures in Celsius degrees

Isolate codes	Pathogenic Bacteria	Diameter of inhibition zone at different Temperature degrees in (mm)		
		20 ℃	**30** ℃	**40** ℃
LA2	***Erwinia* sp.**	-	-	-
	***Pseudomonas* ATTCC**	14	20	19
	***Staph* ATTCC**	19	22	20
LA4	***Erwinia* sp.**	12	6	11
	***Pseudomonas* ATTCC**	20	18	15
	***Staph* ATTCC**	19	10	20
LA5	***Erwinia* sp.**	8	13	10
	***Pseudomonas* ATTCC**	7	19	12
	***Staph* ATTCC**	13	21	20
LA7	***Erwinia* sp.**	18	12	20
	***Pseudomonas* ATTCC**	-	15	-
	***Staph* ATTCC**	13	23	19
LA12	***Erwinia* sp.**	-	7	20
	***Pseudomonas* ATTCC**	6	9	13
	***Staph* ATTCC**	10	23	18

(mm)meaning: a Diameter of the inhibition zone in millimeters, **(-)** meaning: Negative result

3.9. Antimicrobial Assay of *Bacillus* spp.

Bacillus spp. Demonstrated a significant antifungal effect on a variety of fungi species. The results indicated that three *Bacillus* isolates (B1, B4, and B5) exhibited antimicrobial activity against fungi and yeasts (*candida ATCC, candida* sp.). It was observed that the most effective *Bacillus* isolates on fungi are B1 and B4, as they have antimicrobial activity against (*A.niger, A.flavus, Alternaria* sp*., and Trichoderma* sp.). Consequently, based on the results of the antimicrobial assay, we selected the most effective isolates (B1, B4, and B5) to investigate their antimicrobial effect under various conditions of temperature, PH, and extended incubation periods.

Figure 13: The antimicrobial effect of *Bacillus* spp. On *Alternaria* sp*., A.niger, A.flavus*

Table 8: The Antimicrobial activity of *Bacillus* sp

Isolate codes	*A.flavus*	*Trichoderma* SP.	*A.niger*	*Alternaria* SP.	*Fusarium* SP.	*Candida* Sp.	*Candida ATCC*
B1	19	20	18	20	-	17	10
B2	-	-	-	-	-	-	-
B3	-	18	-	-	-	-	-
B4	-	19	20	22	-	-	6
B5	-	13	-	-	13	-	-
B6	20	-	7	18	-	-	-
B7	18	-	5	-	-	-	-

(mm) meaning: a Diameter of the inhibition zone in millimeters,
(-) meaning: Negative result

3.10. Optimization of antifungal agent produced by *Bacillus* spp

3.10.1. Effect of *Bacillus* spp. at different temperature degrees

It was observed that (B1 – B4 – B5) showed antimicrobial activity at temperature 20c and that (B5) was the most effective isolate on *Trichoderma* sp. with inhibition zone (17mm) (fig 14) and (Tab 9). The optimal temperature for *Bacillus* isolates was 30c, and there was no antimicrobial activity at higher temperatures 40c and 55c. The most effective isolate was B4, which showed antimicrobial activity with fungi such as *A. niger* at 30c and its inhibition zone was (20mm) shown (fig 15). According to (Vásquez and Millones, 2023) the ideal temperature range for *Bacillus* antimicrobial activity is between 20 and 30 degrees Celsius. *Bacillus* isolates did not exhibit any antimicrobial activity at temperatures between 40 and 55 degrees Celsius. The ideal temperature range for growth is between 20 and 50 degrees Celsius. However, in our investigation, the *Bacillus* isolates grew between 20 and 40 degrees Celsius, and they only slightly expanded at 55 degrees. Based on the results, it is possible that some of the active ingredients produced by the *Bacillus* species—such as lipases, bacteriocins, and proteases—are sensitive to high temperatures, which accounts for the decrease in antimicrobial activity of the isolates when the temperature rises. These substances' enzymatic activity may be impacted by high temperatures. High temperatures can cause certain enzymes to lose their function completely or become less active, which reduces the efficiency of antimicrobials. Moreover, at high temperatures, antimicrobial substances like proteins and peptides can denaturize. Proteins that have undergone denaturation have lost their functional characteristics due to the rupture of their tertiary or secondary structure. The antibacterial activity may be reduced as a result of this structural change.

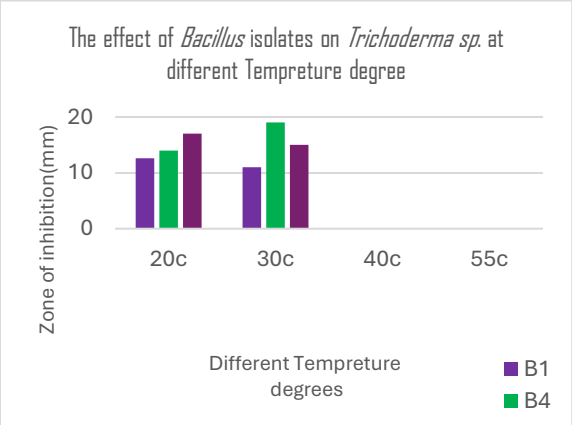


Figure 14: The effect of different temperature degrees on the antimicrobial effect of *bacillus* isolates on *Trichoderma* sp

Figure 15: Antifungal effect of supernatant for B4 isolate which incubated at different temperature degrees against *A.niger*

Table 9: Antimicrobial effect of *Bacillus* isolates at different temperatures in Celsius degrees.

Isolate codes	Pathogenic Bacteria	Diameter of inhibition zone in (mm) at different temperatures in Celsius degrees			
		20℃	**30**℃	**40**℃	**50**℃
B1	***A.niger***	15	15,3	-	-
	***Trichoderma* sp**	12,6	11	-	-
B4	***A.niger***	16	20	-	-
	***Trichoderma* sp**	14	19	-	-
B5	***A.niger***	-	-	-	-
	***Trichoderma* sp**	17	15	-	-

(mm) meaning: a Diameter of the inhibition zone in millimeters, **(-)** meaning: Negative result

3.10.2. Effect of PH level on *Bacillus* spp

It was found that the antimicrobial effects of (B1, B4, and B5) decreased at PH 8 with *Trichoderma* sp. as shown in (fig 16). B5 had the greatest antimicrobial effect against *Trichoderma* sp. at PH 5 with an inhibition zone (25mm). Additionally, we found that *Bacillus* spp. can grow at PH degrees (4, 5, 6, 7, and 8) and also showed an antimicrobial effect at different PH degrees, even at alkaline PH.

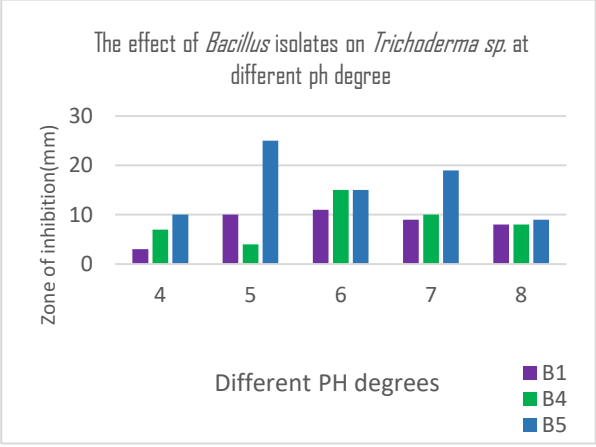


Figure 16: Effect of PH degrees on the antimicrobial effect of *Bacillus* isolates on *Trichoderma* sp.

This indicates that *Bacillus* isolates have a specific antimicrobial component that is responsible for its inhibitory effect rather than producing organic acids (Ramachandran *et al.,* 2014). B4 is the most effective isolate for *A. niger* at different PH degrees, with inhibition zones 14mm to 18mm from PH 8 to PH 4, respectively **(Tab 10)**.PH levels on *Bacillus* spp.

Table 10: Antimicrobial effect of *Bacillus* isolates at different PH degrees.

Isolate codes	Pathogenic Bacteria	Diameter of inhibition zone in (mm)at different PH degrees				
		4	**5**	**6**	**7**	**8**
B1	***A.niger***	10	16,6	15	13	11
	***Trichoderma* sp**	3	10	11	9	8
B4	***A.niger***	18	19	16	15	14
	***Trichoderma* sp**	7	4	15	10	8
B5	***A.niger***	-	-	-	-	-
	***Trichoderma* sp**	10	25	15	19	9

(mm) meaning: a Diameter of the inhibition zone in millimeters, **(-)** meaning: Negative result, the different degrees of PH didn't affect these species.

3.10.3. Time of optimization production of antifungal agents

The length of the incubation period is a crucial factor that affects the generation of antimicrobial compounds economically. A study involving varying incubation periods (one day, four days, six days, and eight days) revealed that the antimicrobial activity of B4 increased with the lengthening of incubation periods. For example, after one day, B4's antimicrobial activity on *A. niger* was 17 mm; after four days, this increased to 19.6 mm; after six days, it increased to 23.3 mm; and after eight days, B4's inhibition zone was 18.6 mm with A. niger (fig. 18), **(Tab11)** Furthermore, as per the research conducted by (Saraswathy, 2013) and (Kumar *et al.,* 2008), specific active ingredients such lytic extracellular enzymes are accountable for the inhibiting impact generated by specific *Bacillus* species. As protease enzyme, which demonstrated that these enzymes' activity increased with longer incubation times, in contrast to B1, whose antimicrobial activity decreased with longer incubation times.

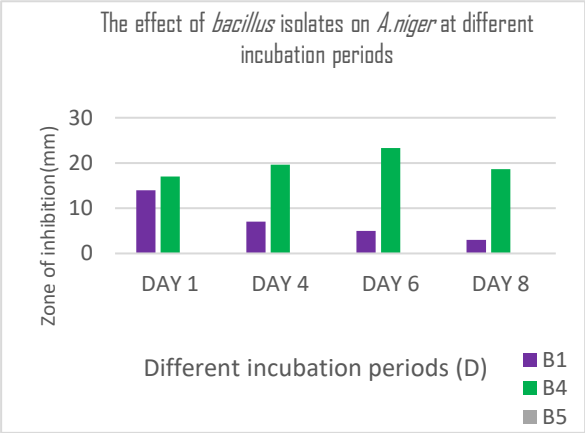


Figure 17: Effect of different incubation periods on the antimicrobial activity of *Bacillus* Isolates on *A.niger*

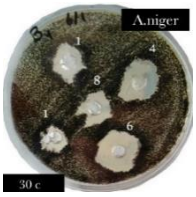


Figure 18: Antifungal effect of supernatant for B4 isolate which incubated at different incubation periods against *A. niger*

Table 11: Antimicrobial effect of *Bacillus* isolates at different incubation periods.

Isolate codes	Pathogenic Fungi	Diameter of inhibition zone in (mm)at different Incubation periods at 30 ℃			
		DAY 1	DAY 4	DAY 6	DAY 8
B1	*A.niger*	14	7	5	3
B4		17	19.6	23.3	18.6
B5		-	-	-	-

(mm) meaning: a Diameter of the inhibition zone in millimeters, **(-)** meaning: Negative result

4. Conclusion

The biochemical characteristics and characterization of lactic acid bacteria (LAB) with probiotic potential are the main topics of this study. The antibacterial qualities of LAB are widely recognized, as is their capacity to impede the development of harmful bacteria. These isolates demonstrated a strong antibacterial activity against several gram-positive pathogenic bacterial species, including *Staph*. Furthermore with gram-negative pathogenic bacteria such as *Erwinia* sp. *and pseudomonas* sp. We conducted experiments on LAB isolates to examine their antibacterial activity under various conditions, including temperature, salinity, bile salt concentrations, and PH levels. We found that LA7 showed a significant antibacterial effect against both gram-positive and gram-negative pathogenic bacteria, that LA2 showed high antibacterial activity, particularly at different temperature degrees and salt concentrations, and that we obtained another type of bacteria (*Bacillus* spp.).

Our study also revealed that the main reason for the antibacterial effect caused by LAB isolates is their ability to change the PH of the medium by making it more acidic due to the sugar fermentation advantage, giving Lactic acid bacteria an industrial importance that can be used as a probiotic. Which were isolated from the same sources as the *Bacillus* and LAB isolates, demonstrated a remarkable antimicrobial effect, particularly when combined with fungi like *Trichoderma* sp. and *A. niger* from the *Bacillus* isolates. From these isolates, we obtained B4, which exhibited antimicrobial activity at both alkaline pH and long incubation periods, indicating the presence of an active component that is responsible for the inhibitory effect. The purpose of this study was to determine whether LAB and *Bacillus* isolated from different food sources might stop the growth of prevalent foodborne pathogens including *Staph and Pseudomonas* sp. A viable substitute or addition to conventional antibiotics for reducing microbial contamination and hazards in the food business is the use of beneficial bacteria that do not produce antibiotics, such as Lactobacillus acidophilus (LAB) and *Bacillus* spp. Due to their antibacterial, antifungal, and anti yeast metabolites, LAB and *Bacillus* spp. Show promise as natural biocontrol agents in other sectors such as agriculture, aquaculture, and medicine. Further optimization and application of these natural bio-preservatives could help reduce dependency on antibiotics. For instance, whether used as probiotics or biopesticides, LAB and *Bacillus* species may aid in the control of diseases that affect plants and aquaculture. Additionally, LAB can be used in the medical.

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