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Isolation and Molecular Identification Of Lactobacillus Bacteria And Evaluation Of Their Efficacy In Inhibiting The Pathogenic Fungus *Pythium aphanidermatum*

ABSTRACT

Twenty-eight isolates of Lactobacillus were isolated from the rhizosphere of pea cultivated in the fields of five districts in Salah al-Din, which included: Tikrit, Al-Alam, Al-Sharqat, Samarra and Al-Baiji. The isolates identified according to molecular diagnosis using the analysis of the 16S rRNA sequences and were divided into five species: 7 Isolates of *L. fermentum*, 3 isolates of *L. casei*, 9 isolates of *L. plantarum*, 4 isolates of *L. herbarum* and 5 isolates of *L. paralimentarius*. Antagonism tests were conducted for these isolates against the pathogen Pythium aphanidermatum, the highest rate of inhibition was by isolates of bacteria *L. paralimentarius* 1981 reached 2.7 mm, followed by isolate *L.plantarum* 1982 with an inhibition rate of 2.5 mm. In the antagonism test using bacterial filtrate of *L. paralimentarius* 1981, the concentration of 30% was the highest in inhibiting the pathogenic fungus, as the fungus colony diameter reached 3.22 cm, followed by the concentration of 20 and 25%, as the colony diameter reached 4.47 and 3.68 cm, respectively. *L. paralimentarius* 1081 was recorded in the databases of the World Genetic Bank within the accession number MW149434.1. This is the first record of this bacteria in Iraq. *L. paralimentarius* 1081 showed the highest production of the antibacterial active substances including *siderophores*, total phenols and bacteriocin reached to 61.24%, 1.05 mg/ml and 18.02 mg/ml, respectively, as well as pH decreased to 4.5. The results also showed there are a strong correlation of pathogenic fungus inhibition with high concentrations of *siderophores*, total phenols, *bacteriocin* and low pH in the bacterial filtrate.

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INTRODUCTION

Lactobacillus has been used for decades in food preservation due to its anti-microbial activity and has received scientific attention due to its anti-fungal activity (Rouse et al., 2008). It is useful for pathogen control, crop improvement, plant protection, germination rate and seedling emergence. These bacteria produce bacteriocin as an effective biological control agent to suppress soil-borne pathogens (Shrestha et al., 2009). Kirithiga et al., (2010) mentioned the possibility of using Lactobacillus bacteria as a biological control agent against a number of pathogens, while Lamont et al., (2017) mentioned the effectiveness of these bacteria as useful, renewable and safe agricultural inputs to improve plant growth. According to the Food and Drug Administration (FDA), the use of lactic acid bacteria is safe for humans and the environment and has many uses in the food, drug and nutritional supplement industries (Ghadoliya et al., 2020). Several bacterial isolates can solubilizing the minerals ,in previous study, 39 isolates able to phosphate solubilizing the phosphorus, isolate P.f16 showed a high capacity to produce indole acetic acid (IAA) amounting to 16.28 mg.ml -1 (Alkurtany et al. (2018a) Lactic acid bacteria have many properties such as tolerance to heavy metals, pesticides and soil salinity as well as its stimulating and encouraging plant growth

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properties such as the production of *phytohormones* , *siderophore*, hydrogen cyanide and ammonia as well as the production of *nitrogenase* enzyme, B complex vitamins and effective in dissolving phosphates and can be used as protective agents to improve plant and food safety from the pathogenic fungi and bacteria (Sivasakthi et al., 2014). Lactic acid bacteria ferment sugars and convert them to lactic as well as produce substances that are anti-bacterial and fungal pathogens like organic acids, hydrogen peroxide and hydrolytic enzymes, as they work to inhibit their growth or kill them (Sabina and Fijan, 2014). Damping off disease, a very important plant disease, which leads to the decay of seeds germination and killing of young seedlings, it is one of the most important restrictions facing plant cultivation, as most stages of plant growth are infected with this disease (Jay Ram et al., 2017). Damping off disease is caused by most species related to the genus *Pythium*, which are pathogenic fungi with a wide host range, there is very little geographical area around the world that does not suffer from these pathogens (Van Buyten and Hofte, 2013). Pythium spp. causes root rot, seed rot, seedling death and stem blackening, most species of Pythium are among the most fungal species that cause losses in economic plants (Parveen and Sharma, 2015). *Pythium spp*. cause great losses in crops with appropriate conditions such as environmental conditions and sensitive plant hosts, this fungus infects plants in different stages of growth as it infects seeds before germination or during the germination period. *P.aphanidermatum* infects the underground and aerial parts of plants. This fungus also attacks young seedlings pre and post emergence, attacking the root system of the plant feeding on its nutritional components, leading to weak germination and low yield in quantity and quality (McKellar and Nelson, 2003). Alkurtany et al. (2018b) reported that several bacteria like P. *fluorescens* isolates led to decrease in the infection severe of pathogen *Macrophomina phaseolina*, the interaction between the *P. fluorescens* and *Topsin* in present of the pathogen *M. phaseolina* induced the systemic resistance, this treatment gives highest specific activity of the peroxidase (11.2 unit.mg protein -1) and chitinase (180 unit.mg protein -1).

Owing to importance of the pathogenic fungus *P.aphanidermatum* in causing Damping off and root rot diseases of most economic plants, On the other hand, the importance of Lactobacillus bacteria as a biological control agent that possesses many properties against the pathogenic fungi, this study was conducted, which aims to isolation of these bacteria from the rhizosphere of pea plants from various geographical areas within Salah al-Din Governorate, identified by molecular methods and evaluation of its biological activity in inhibiting the pathogenic fungus *P.aphanidermatum.*

MATERIALS AND METHODS

The pathogenic fungus *Pythium aphanidermatum*

A highly pathogenic isolate of the pathogenic fungus *P.aphanidermatum* was obtained from the Plant Diseases Laboratory - Plant Protection Department - College of Agriculture - Tikrit University. This fungus isolated according to a previous study (Hassan and Hasan, 2020).

Isolation of *Lactobacillus*

Lactobacillus bacteria were isolated from five areas within Salah Al-Din Governorate using the dilution method by mixing 1 g of the Rhizosphere soil of pea plants for each of the five samples separately, with 9 ml of distilled water to get a dilution of 10^{-1} then 1 ml was taken from this dilution mixing with 9 ml of distilled water to obtain a 10^{-2} dilution, 1 ml of the last diluted solution was placed in a Petri dish, then the sterile and cooled Man Rogosa Sharpe (MRS Agar), a selective medium for *Lactobacillus*, was poured before it solidified and the dishes were incubated at 30ºC for 24-72 hours with observation of bacterial growth. After the incubation, *Lactobacillus* isolates were phenotypically identified depending on the size, texture, color and edges of the colonies, and microscopically using gram stain and some other characteristics according to the related references (Holt et al., 1994: Mac Faddin, 2000). Finally, Lactobacillus isolates were identified at the species level using molecular methods.

Preparation of Bacterial Cell Suspesion

Bacterial isolates were grown separately in nutrient broth (NB) media by transferring a swab of the newly grown colony (24 hours' age) into flask containing 100 ml of sterile NB medium, then

incubated at 30°C for 48 hours with continuous stirring. The bacterial account in 1 ml of the bacterial suspension was 6.62 x 10^8 cells/ml, and this suspension was used in subsequent experiments.

Antagonistic Test

Petridishes contain Potato dextrose agar (PDA) medium without adding any antibiotics were inoculated with pathogenic fungus *P.aphanidermatum* (5 mm diameter from a fresh colony-5 days old) , then the medium inoculated with 0.1 ml of the bacterial suspension which put in 3 lines about 3 cm long and 3 cm from the fungus disc, while in the fourth line was consists of 0.1 ml of the sterile NB medium that was not cultured with bacteria (as control line), the plates were incubated at 25°C. When the growth of the fungal colony reached the control line, the area of inhibition was measured from the edge of the pathogenic fungus colony to the bacterial lines using a digital ruler as inhibition zone (Hassan et al., 2017).

Molecular Indentification of *Lactobacillus* Isolates

The bacterial isolate that exhibit highest pathogenicity against *P.aphanidermatium* was diagnosed at the species level according to the molecular method based on the analysis of nucleotide sequences of 16S rRNA gene.

Extraction of Genomic DNA

Genomic DNA was extracted using the ready-made ZR Fungal / Bacterial / Yeast DNA Mini Prep TM kit supplied by the American company ZR. The genomic DNA was extracted from a swab (100 mg) of a newly grown colony of bacterial isolate according to the manufacturer's instructions. Gel electrophoresis of genomic DNA extraction from the bacterial isolates was carried out in 1.5% agarose gel at 5 vol /cm for 1:15 hours.

Polymerase Chain Reaction (PCR)

Maxime PCR PreMix kit (i-Taq) 20µlrxn (Cat. No. 25025) extraction kit was used for the amplification of the 16S rRNA gene by PCR, using the universal primer which sequences were shown in Table (1). Primers set supplied by IDT (Integrated DNA Technologies company, Canada.).

Table (1) Sequence universal primer of 16S rRNA gene

Primer	Sequence	Molecular size (bp)	Reference
Forward	5'AGATTGACCTGGCTCAG-3'	1250	Miller et al., 2013
Revers	5'AGATTGACCTGGCTCAG-3'		

PCR technique was used to amplify the ITS1 and ITS4 region in DNA through the additions shown in Table (2) and depending on the attached leaflet of the Premix reaction mixture. Table (3) shows the components of the reaction mixture with their concentrations.

Table (2) The Components of the Maxime PCR PreMix kit (i-Taq)

Material	Volume
i-Taq DNA Polymerase	5U/ µl
DNTPS	2.5mM
Reaction buffer (10X)	1X
Gel loading buffer	1X

Table (3) Mixture of the PCR

Components	Concentration
Taq PCR PreMix	5 µl
Forward primer	10picomol/ml (1 µl)
Reverse primer	10picomol/ml(1 µl)
DNA	1.5 µl
Distill water	16.5µl
Final volume	25 µl

Electrophoresis of pcr products

An Applied Biosystem Gene-amp PCR System 9700 Thermocycler Apparatus was used in the mentioned gene amplification. Table (4) included the standard reaction conditions. The PCR

product was separated by using electrophoresis on agarose gel (1.5%), then PCR product bands were demonstrated using UV rays with a wavelength (302nm) after treating them with the dye (Intron Korea red stain).

Table (4) The optimum condition of PCR

No.	Phase	Tm(C)	Time	No.of cycle
1	Initial Denaturation	95 ˚C	5min	1 cycle
2	Denaturation	95 ˚C	45sec	35 cycle
3	Annealing	58 ˚C	45sec	
4	Extension -1	72 ˚C	45sec	
5	Extension -2	72 ˚C	7min	1 Cycle

Nucleotide Sequencing Analysis

The nucleotide sequences of the PCR-amplified gene were determined immediately after obtaining the 16S rRNA gene amplification by sending a volume of 25 µl of PCR product and a volume of 10 µl (10 pcm concentration) of each primer to the Korean company Biotechnology Lab (used device Applied Biosystem 3730XL, DNA Sequencer) .The results were compared by a computer program connected to the web (Basic Local Alignment Search Tool (BLAST) with the database at the National Center for Biotechnology Information (NCBI). The process of alignment and matching was carried out with the strains registered in the World Genetic Bank. The most efficient isolate in inhibiting the pathogenic fungus *P.aphanidermatium* were registered in the World Genetic Bank.

Preparation of Cell-Free Bacterial Filtrate

Bacterial isolates were grown in nutrient broth (NB) media by transferring a swab of the newly grown colony (24 hours of age) into flask containing 100 ml of sterile NB medium, then incubated at 30°C for 48 hours, filtered using filter paper (Whatman NO.1). The filtrate was centrifuged at 5000 rpm for 10 minutes to obtain a filtrate free of bacteria cells.

Estimation of The Active Compounds Produced by Bacteria Estimation of Siderophore

Siderophore was estimated by Chrome Azurol Sulphonate (CAS) method reported by Ghosh et al., (2015) by adding 0.5 ml of cell-free bacterial filtrate to 0.5 ml of CAS solution, the reaction mixture was incubated at 25°C for 20 minutes. The absorbance at a wavelength of 630 nm was measured, then Siderophore estimated in Percent Unit (PSU) according to the following equation:

$PSU = (Ar-As)/Ar \times 100$

Whereas represents the absorbance of the sample (CAS + bacterial filtrate)

Ar represents the absorbance of the blank solution (CAS + unculturable bacteria medium)

Determination of Total Phenols

The mixture of reaction consists of 1 ml of alcoholic extract, 1 ml of 0.05 N HCL, 1 ml of Arnaud's solution, 10 ml of distilled water, and 2 ml of 4% NaOH solution. The absorbance of this mixture was measured at a wavelength of 515 nm against the blank with which includes all solutions included in the experiment except for the sample, total phenols estimated from the standard curve consisting of different concentrations of catechol versus the absorbance values at wavelength 515 nm for each concentration (Mahadevan and Sridhar, 1986).

Determination of Bacteriocin

Crude Bacteriocin Extraction

The method mentioned in Kanatani et al. (1995) was used as follows- :

Bacterial isolates were grown in test tubes containing 10 ml of MRS liquid medium at a temperature of 30 °C for 24 hours. At the end of the incubation period, the liquid culture was transferred to a flask containing 250 ml of MRS liquid medium with a pH equal to 6.5 and incubated at 30°C for 48 hours. After the incubation period, the cultures were centrifuged in a refrigerated centrifuge at 4°C at 5000 rpm for 10 minutes. Collect the filtrate and adjust the pH to 7 using 1M sodium hydroxide solution, then sterilize the filtrate with Millipore filter (0.22 microns).

Determination of Bacteriocin in Terms of Protein Concentration

The Bayuret method was used to determine the protein (bacteriocin), 1 ml of crude bacteriocin solution was added to test tubes, then 4 ml of Bayuret solution was added to it, they were mixed well and the tubes were incubated for 20 minutes at 37°C, and the absorbance was measured at a wavelength of 540 nm. To determination of bacteriocin concentration, the standard curve for protein determination was adopted (Mahadevan and Sridhar, 1986).

Estimation of Ph

The pH of the bacterial filtrate was estimated at 25°C using a pH meter.

Effect of Bacterial Filtrate on the Growth of Pathogenic Fungi and The Growth of *Lactobacillus*

The effect of bacterial filtrate concentrations 0-30% (V:V) in the PDA media was tested by poison plate method. In case of the filtrate effect on the growth of pathogenic fungus *P.aphanidermatum*, petridishes contained PDA media were inoculated by placing a piece of pathogenic fungus colony with a diameter of 0.5 cm, (age 5 Days) by using a cork piercing in the center of each dish, then the dishes were incubated at 25°C, then the diameters of the developing fungus colonies were measured upon completion of fungus growth in the control (concentration zero).

The effect of the filtrate on the growth of the bacteria cells themselves, it was carried out using the above concentrations (0-30%) mixed homogeneously in the nutrient agar medium (NA) at each concentration separately.1 ml of the bacterial suspension was placed at a concentration of 6.62 x 10^8cells/ml in petri dishes, then nutrient medium has been poured, mixed well, and incubated at 30°C for 24 hours, then the bacterial numbers were calculated.

Statistical Analysis

Factorial experiments were carried out in this study and analysis of variance was conducted using the program (SPSS). The means were compared according to the Least Significant Deference (LSD) test at a level of significance of 0.05 (Al-Rawi and Khalaf Allah, 1980).

RESULTS AND DISCUSSION

Isolation of *Lactobacillus*

28 bacteria isolates were isolated from the rhizosphere of pea plants from five regions within Salah El-Din Governorate. Table (5) shows the number of isolates of lactic acid bacteria from each region. It is noted that there is a difference in the numbers and types of bacterial isolates according to the different geographical area. The reason for this discrepancy can be attributed to the fact that there are different isolates of bacteria that adapt without others according to the regions in which they are located, as well as the type of soil, its content of nutrients and its climate, which have a significant impact on the developing bacteria (Gourmelon et al., 2016).

Table (5) Number of *Lactobacillus* isolates according to the isolated areas

The region	N0. of isolates
Tikrit	6
Samrara	4
Al- Alam	9
Byjii	2
Al Sherqat	7

Inhibition Effect of the Pathogenic Fungus *P.Aphanidermatum* by *Lactobacillus* Isolates

Figure (1) shows the inhibition of the pathogenic fungus *P.aphanidermatum* by *Lactobacillus* isolates. The results showed all isolates showed inhibition against the pathogenic fungus and the highest inhibition was recorded by *L. paralimentarius* (isolate 1981), in which the area of the fungus growth inhibition was 2.7 mm (Fig. 2) followed by *L.plantarum* (isolate 1982), *L.herbarum* (isolate 1980), *L. fermentum* (isolate 1979) and *L.casei* (isolate 1978) with inhibition zone of 2.5, 2, 1.8 and 1.5 mm, respectively, while the lowest inhibition was 0.5 mm recorded by *L. fermentum* (Isolate 1990).

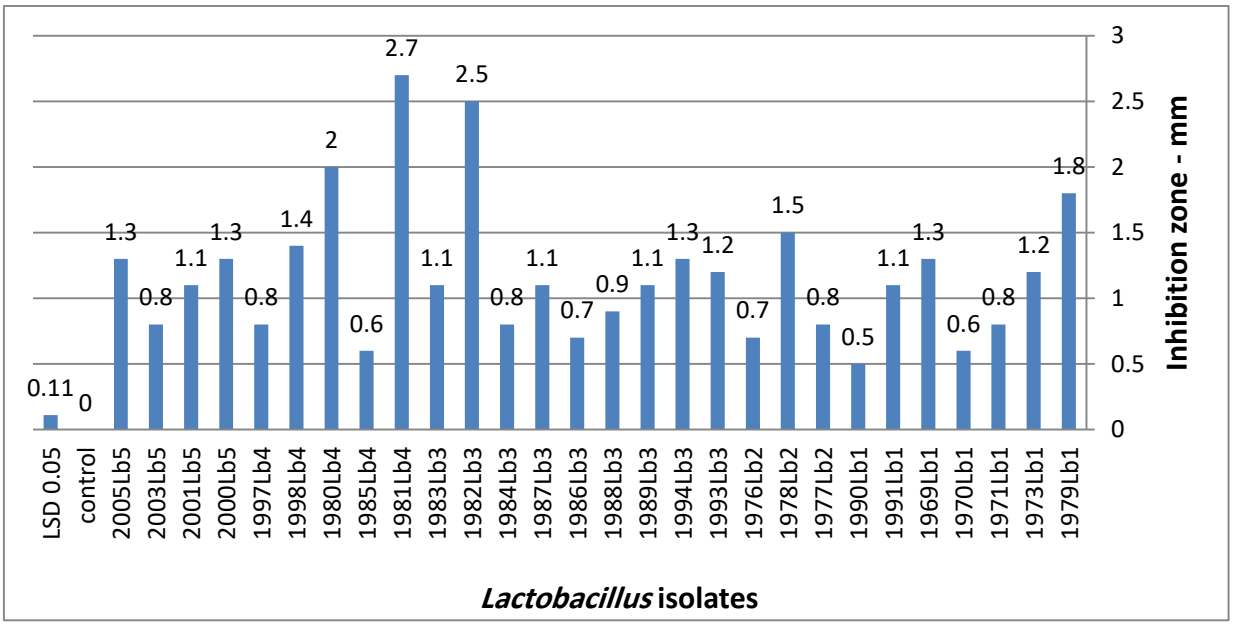

Figure (1) Inhibition of the pathogenic fungus P.aphanidermatum by Lactobacillus isolates

Figure (2) Inhibition of the pathogenic fungus P.aphanidermatum by L. paralimentarius isolate (isolate 1981)

The different antagonisms of different *Lactobacillus* isolates can be explained by the variation in their genetic composition, which was reflected on their bio-active substances, metabolic activities, as well as the active substances against the growth of pathogens. These bacteria produce several types of anti-pathogenic compounds, especially against the pathogenic fungi, Majeed and Al-Shammari, (2016) showed the strains of *Lactobacillus* produce sorbitol, manitol and trehalose that act as antifungal activity. The study of Vuyst et al., (2007) confirmed that strains of *Lactobacillus* have inhibitory activity against microorganisms, including fungi through production of lactic acid, peroxide hydrogen and bacteriocin. Our results showed the superiority of the isolate *L. paralimentarius* in inhibiting the pathogenic fungus *P. aphanidermatum*, perhaps due to its superiority in the production of organic acids and metabolic materials, more than other isolates, such compounds regard as antifungal substance according to the study of Husain et al., (2017). These results are consistent with the study of Canpolat et al., (2018), which confirmed the effectiveness of some species of lactic acid bacteria in inhibiting the pathogenic fungi *Pythium* sp. , *Macrophomina* sp. , *Pestalotiopis* sp. , *Rhizoctonia* sp. , *Fusarium* sp. , *Botrytis* sp. and *Cylindrocarpon* sp. the areas of inhibition were 12.7, 9.7, 9.3, 7.0, 6.0, 4.0, 1.3, respectively. Lv et al., (2018) mentioned the inhibition of *Lactobacillus* bacteria against the pathogenic fungus *Trichothecium roseum* that causes red rot on watermelons, as the highest inhibition rate was for the strains *L.plantarum, L.rhamnosus, L.helveticum* and *L.sakei*, which amounted to 93.93, 89.69, 83.93 and 78.29%, respectively.

Molecular Identification of *Lactobacillus* Species

The diagnosis of the five species of the most efficient isolates of *Lactobacillus* bacteria that showed the highest inhibition against the pathogenic fungus was confirmed depending on the molecular diagnosis to the level of the species according to the nucleotide sequencing test for the

16S rRNA gene. Figure (3A) shows the bands arising from the electrophoresis of genomic DNA, as the figure shows the presence of single band for each isolate, which indicates the purity of DNA and the accuracy of extraction. Figure (3B) shows the electrophoresis of the PCR product using a universal primer to amplified the 16S rRNA gene, the results show the appearance of bands at the size of 1250 base pairs, which is the expected size, as this size conclusively proves that the resulting bands related to the bacterial taxa, and this is evidence of the accuracy of the PCR .

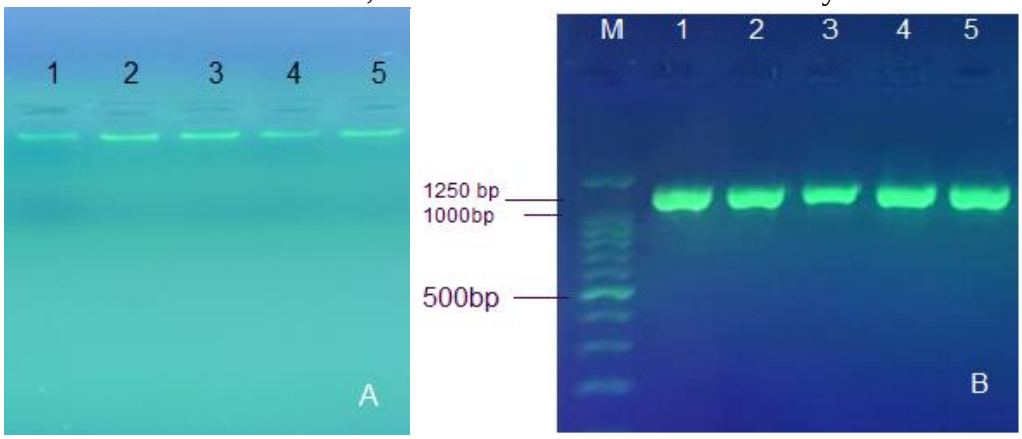

Figure (3) (A) Genomic DNA electrophoresis of Lactobacillus isolates, (B) Polymerase chain reaction using a universal primer for 16S rRNA gene amplification of *Lactobacillus* isolates.
1: *L. casei* (1078),2: *L. fermentum* (1079),3: *L. paralimentarius* (1081), 4: *L. herbarum* (1080) , 5: *L. plantarum* (1982)

Table (7) shows the percentages of similarity of *Lactobacillus* isolates and their matching with the strains of bacterial species registered globally and their accession numbers in the genetic bank database. The five bacteria isolates selected as the best isolates for inhibiting the pathogenic fungus *P.aphanidermatum,* which included 1078, 1079, 1981, 1980 and 1982, were divided into five species: *Lactobacillus casei, L. fermentum, L. paralimentarius, L. herbarum and L. plantarum,* with a proportion of nucleotide sequences matching with species recorded in the World Genetic Bank 99.02, 97.16, 99.16, 99.32 and 99.10%, respectively. The more effeicnt *L. paralimentarius* (isolate 1981), against the pathogen *P. aphanidermatium* was registered on the NCBI website under the accession number MW149434.1, and this record is the first for this bacteria in Iraq. Although the isolated bacteria in this study are identical within the registered isolates at a rate of 97.17-99.32%, they can be considered genetically different isolates because there is no match (100%), and this can be attributed to the occurrence of many genetic variations due to environmental factors and continuous spraying of chemical pesticides and the environmental pollution factors, which leads to the occurrence of genetic mutations and thus genetic variation occurs which was reflected on the physiological factors of these species (Martins et al., 2013; Cadet and Wagner, 2014).

Table (6) Molecular identification of bacterial isolates based on the percentage of identical 16S rRNA gene sequences with bacterial strains in the World Genebank

Code of *Lactobacillus* isolates	Highest match bacteria	Accession Number	Country	Similarity (%)
1078	*Lactobacillus casei*	NR_118976.1	U. K	99.02
1079	*Lactobacillus fermentum*	NR_118978.1	U. K	97.16
1081	*Lactobacillus paralimentarius**	NR_043096.1	Spain	99.16
1080	*Lactobacillus herbarum*	NR_145899.1	China	99.32
1982	*Lactobacillus plantarum*	NR_042394.1	China	99.10

*Registered in the World Genetic Bank under the accession number MW149434.1

Biological Activities of *Lactobacillus* Filtrate

Siderophore Activity

The results shown in Figure (4) showed the siderophore activity of *Lactobacillus* species against the growth of the pathogenic fungus *P.aphanidermatum*. All the *Lactobacillus* isolates showed Siderophore activity, both *L. plantarum* and *L.paralimentarius* were significantly superior to other isolates resulting in 61.24 and 60.69%. respectively, while the lowest activity of Siderophore was recorded by *L. fermentum* bacteria, which amounted to 40.89%. Loper and Henkels, (1997) and Whipps, (2001) reported that the emission of iron carriers by plant growth-promoting bacteria present in the rhizosphere facilitates the action of PGPB to effectively gain iron in the form of the ferric ion that is readily available to them. In addition to the effectiveness of siderophores produced by bacteria to obtain iron, siderophores will prevent the pathogen from utilizing the iron that is important for their survival (Pedraza et al., 2007).

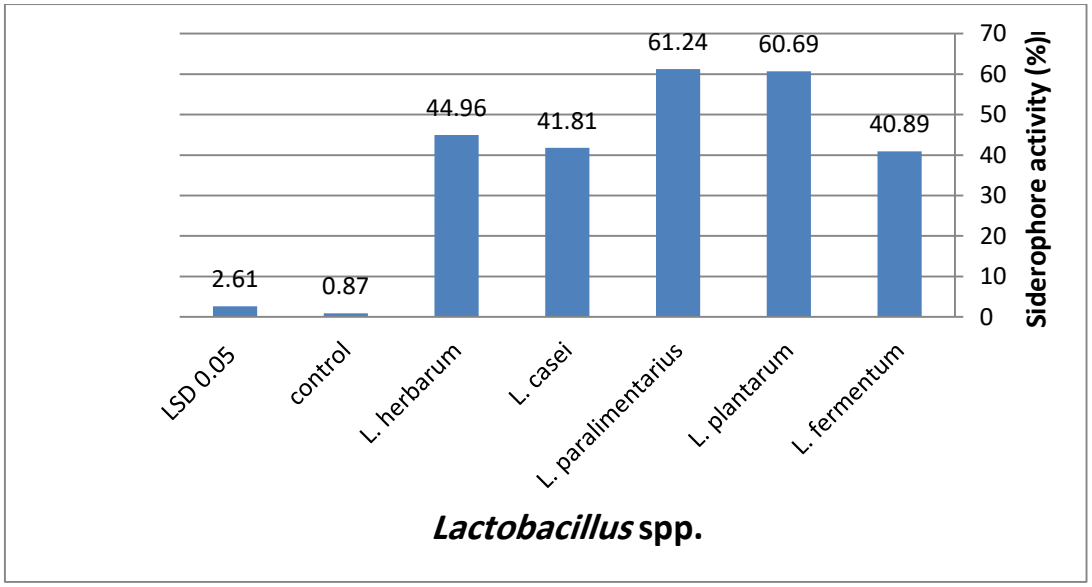

Figure (4) Siderophore activity of *Lactobacillus* species

Total Phenols

Figure (5) shows the total phenol concentrations in the filtrate of *Lactobacillus* species, the results showed that all isolates produced total phenols in varying proportions. *L. paralimentarius* showed the highest concentration of phenols, reaching 1.05 mg/ ml followed by *L. plantarum*, which reached 0.93 mg/ml, while the lowest concentration was 0.37 mg/ml by *L. casei*. Lv et al., (2018) demonstrated the effectiveness of *L. plantarum* C10 bacteria in stimulating the watermelon plant to produce total phenols against the pathogenic fungus *Trichothicium roseum* , after 3 days of treatment, the plant produced the highest percentage of phenols (6.3 : OD_{325}/ FW), about 5.0% higher than the control treatment which was 1.45: OD_{325}/FW, this increase in phenol content was considered as a plant defense reaction against pathogenic fungi.

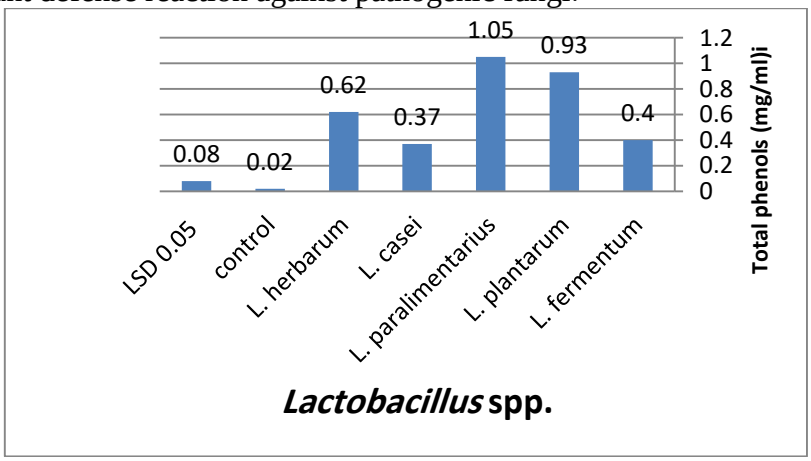

Figure (5) Total phenols produced in *Lactobacillus* spp. filtrate

Activity of Bacteriocin

The results listed in Figure (6) indicate to all isolates of *Lactobacillus* bacteria showed production of bacteriocin in varying proportions, the highest concentration was by *L. paralimentarius* resulting in 18.02 mg/ml, followed by *L. plantarum* with a concentration of 17.68 mg/ml, and the lowest bacteriocin production was by *L. casei* bacteria reached 9.51 mg/ml. LAB bacteria have the ability to produce chemical compounds that inhibit and prevent the growth of pathogenic fungi, among these compounds are bacteriocins, which are promising alternatives to antibiotics with strong activities in the field and laboratory (Caly et al., 2017; Seddik et al., 2017). The reason for the inhibitory or lethal activity of bacteriocin is due to the possibility of its binding to special receptors located on the plasma membranes of pathogens. Tumbarski et al., (2018) reported the effectiveness of bacteriocins produced by *Lactobacillus* bacteria against the pathogenic fungi *Fusarium* sp., *Aspergillus* and *Penicillum* sp .

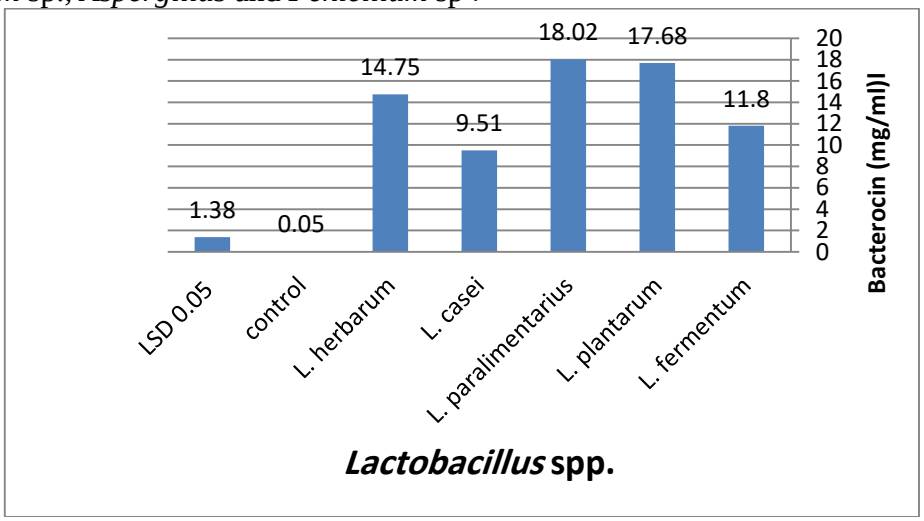

Figure (6) Bacterocin produced in *Lactobacillus* spp. filtrate

Ph in *Lactobacillus* Spp. Filtrate

The results in Figure (7) showed the pH values of *Lactobacillus* spp. filtrates, clearly, all isolates showed decrease in pH, the highest pH was recorded by *L. fermentum*, which was 5.5, and the lowest by *L. paralimentarius*, resulting in 4.5. The organic acids that are produced from the metabolism of carbohydrates by *Lactobacillus* bacteria, including: Lactic acid, Acetic acid and Propioinic acid, lead to decrease in the pH, low pH in turn leads to prevents the growth of various microorganisms and kills pathogenic bacteria and fungi. At the same time, they are considered safe materials for plants and humans (Stoyanova et al., 2012). The effect of organic acids and the decrease in pH could be explained by the acids diffuse indiscriminately over the cell membrane of the pathogen and then separate inside the cytoplasm through the release of positive hydrogen ions, and the undissociated form of the acids works on the breakdown of the electrochemical gradient of the proton and then the death of the pathogen (Desmazeaud et al., 1991).

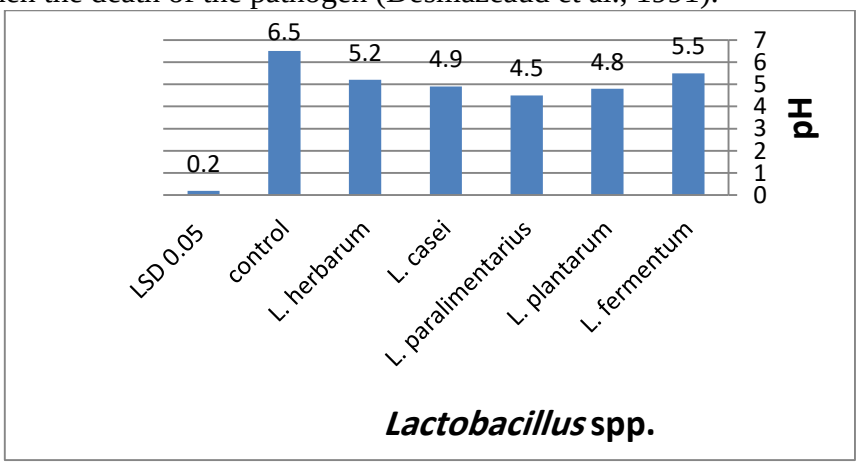

Figure (7) pH values of *Lactobacillus* filtrate

Correlation Coefficient Between The Inhibition of The Pathogenic Fungus *P.Aphanidermatum* And Biological Activity of *Lactobacillus* Isolates

Figure (8-A) shows the correlation between the inhibition of the pathogenic fungus *P.aphanidermatum* by the five isolates of *Lactobacillus* bacteria and the siderophore activity of this bacteria. However, there is a close relationship between the effectiveness of siderophore and the inhibition of the growth of pathogenic fungi. The results of Figure (8-B) showed the correlation between the concentration of total phenols of the five isolates of *Lactobacillus* filtrate and the inhibition of the pathogenic fungus, as the correlation coefficient reached 0.889, which indicates a close correlation between the growth inhibition of the pathogenic fungus *P.aphanidermatum* and the total phenols. Figure (8-C) also shows the correlation between the bacteriocin concentration of five isolates of *Lactobacillus* bacteria with the inhibition of the pathogenic fungus *P.aphanidermatum*, which amounted to 0.989, which indicates the strong correlation between bacteriocin and the inhibition of the pathogenic fungus. The results shown in Figure (8-D) showed the correlation between the pH of five isolats of *Lactobacillus* bacteria and the inhibition of the pathogenic fungus *P.aphanidermatum*, in which the correlation coefficient reached 0.8481, and this indicates a close correlation between the pH and the inhibition of pathogenic fungi.

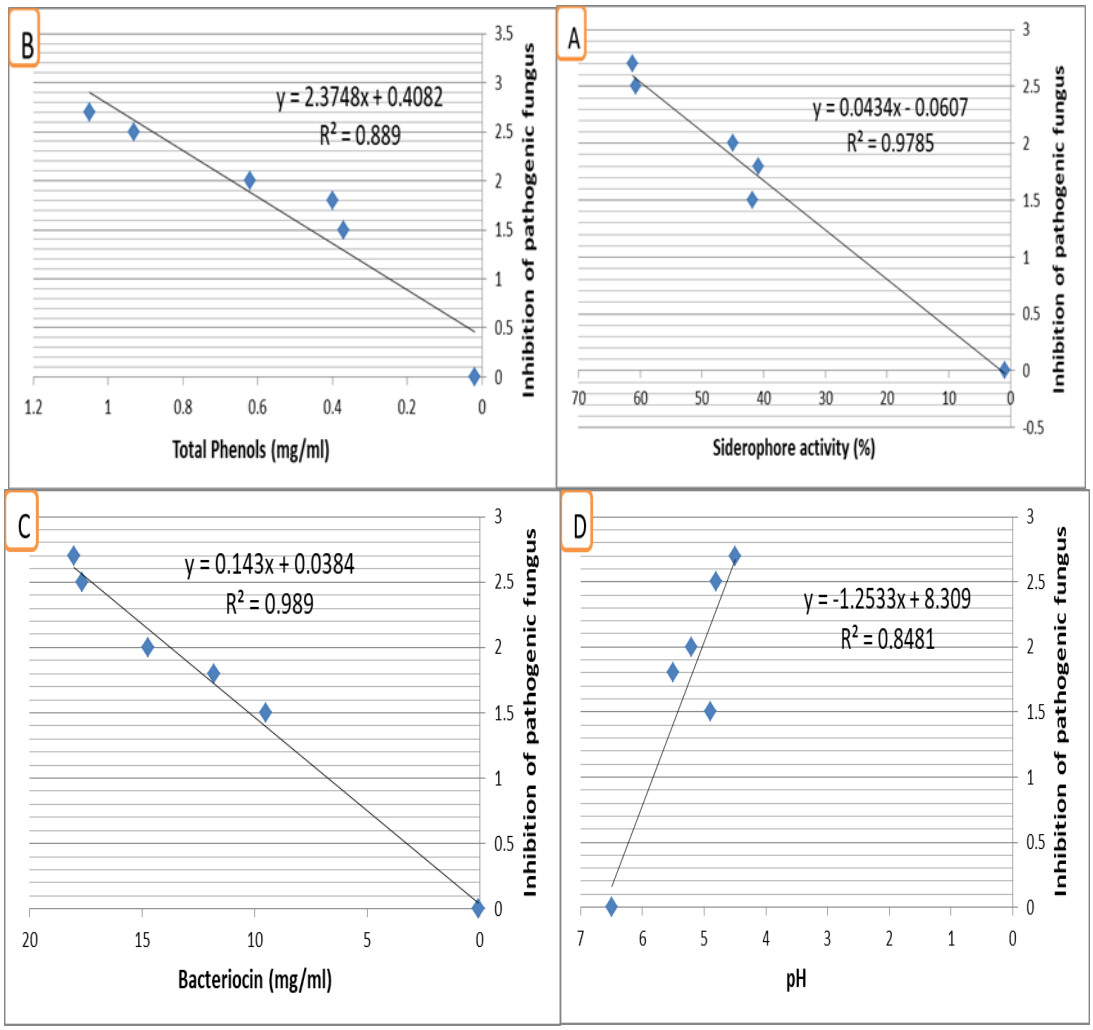

Figure (8) Correlation relationship between the biological activity of *Lactobacillus* spp with the inhibition of the pathogenic fungus *P.aphanidermatum* (A with sidrophore activity, B with total phenols, C with bacteriocin, D with pH)

Effect of *L.Paralimentarius* Filtrate on The Growth of *L.Paralimentarius* Cells and The Growth of Pathogenic Fungus *P.Aphanidermatum*

The results listed in Table (7) showed no significant effect of *L. Paralimentarius* filtrate on the growth of the same bacteria up to a concentration of 25%, in which the bacterial account was 5.93 x 10^8 cells/ml, then the number of bacterial cells started to gradually decrease, reaching the lowest number of 4.12 x 10^8 cells/ml. The results also showed there is an inhibitory effect of all concentrations of *L. paralimentarius* filter against pathogenic fungus *P. aphanidermatum*, the minimum diameter of the fungus colony was 3.22 cm at 30% of bacterial filtrate (Fig. 9). According to these results, the concentration of 20-25% of *L. paralimentarius* filtrate was the best concentration when applying in the field experiments, because this concentration did not have a significant effect on inhibiting the growth of the bacteria themselves, as well as a significant inhibition of the growth of pathogenic fungi.

The effect of bacterial filtrate in inhibiting pathogenic fungi may be due to the presence of active substances such as organic and fatty acids, bacteriocin, phenols, hydrogen peroxide and digestive enzymes like the chitinase, protease and lipase, these active substances had a slight effect on the bacteria themselves and significantly on the fungus, as it was proven in this study, may be due to the structure of bacteria spatially, bacterial cell wall, is different from the fungi. These results are in line with the study of Zebboudj et al., (2014), which proved the effectiveness of *Lactobacillus* bacteria and its filtrate in inhibiting the fungus *Fusarium oxysporum* that causes Bayoud Disesse on palm, this study also showed highest inhibition of the pathogenic fungus was on MRS medium (41.17-100%) and from 13.51- 40.29% on PDA using *L. lactis* bacteria. Hasan and Ahmed (2015) isolated many species of bacteria from the compost, which proved to have an important role in inducing systemic resistance of maize plant and encouraging its vegetative and productive growth, as well as decreasing its infection with the pathogenic fungus *Macrophomina phaseolina* The study of Alkurtany et al. (2018b) showed the *P. fluorescens* (P.f 16) isolate inhibited 87% of the growth of pathogenic fungus *M. phaseolina*.

Table (7) Effect of cell-free *L. paralimentarius* filtrate on growth of *L. paralimentarius* and growth of pathogenic fungus *P.aphanidermatum*

Filtrate concentration (%)	*L. paralimentarius* account (× 10^8 cell/ml)	Colony diameter of *P.aphanidermatum (cm)*
0	6.62	7.8
5	6.6	7.31
10	6.55	6.55
15	6.47	5.21
20	6.33	4.47
25	5.93	3.68
30	5.76	3.22
LSD $_{0.05}$	0.31	0.47

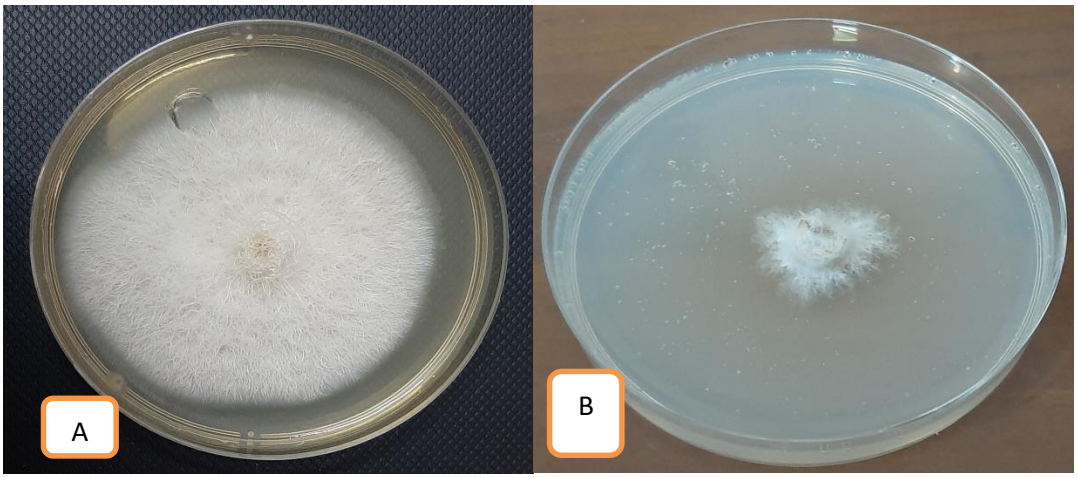

Figure (9) Effect of *L. paralimentarius* filtrate on growth of pathogenic fungus *P.aphanidermatum*. A: control, B: the effect of bacterial filtrate at a concentration of 30%.

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العزل والتشخيص الجزيئي لبكتيريا *Lactobacillus* وتقييم فاعليتها في تثبيط الفطر الممرض *Pythium aphanidermatum*

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الخلاصة

عُزلت 28 عزلة من بكتيريا *Lactobacillus* من منطقة المحيط الجذري لنباتات بازلاء مزروعة في حقول خمسة اقضية في صلاح الدين والتي شملت: تكريت، العلم، الشرقاط، سامراء والبيجي، شخصت حسب التشخيص الجزيئي باستعمال تحليل التتابعات النيوكلوتيدية للجين 16S rRNAوتوزعت العزلات الى خمسة انواع بواقع 7 عزلات تعود للنوع *L. fermentum* و 3 عزلات تعود للنوع *L. casei* و 9 عزلات تعود للنوع *L. plantarum* و 4 عزلات للنوع *L. herbarum* و 5 عزلات للنوع *L. paralimentarius*. اجريت اختبارات التضاد لعزلات البكتريا مع المسبب المرضي *Pythium aphanidermatum* والتي ادت الى تثبيط الفطر الممرض من قبل جميع العزلات وبلغ اعلى معدل تثبيط في عزلة البكتريا 1981 *L.paralimentarius* إذ بلغت منطقة التثبيط 2.7 ملم , تليها العزلة 1982 *L.plantarum* بمعدل تثبيط بلغ 2.5 ملم. وفي اختبار التضاد باستخدام راشح البكتريا للعزلة 1981 *L.paralimentarius* كان التركيز 30% هو الاعلى في تثبيط الفطر الممرض إذ بلغ قطر مستعمرة الفطر 3.22 سم يليه التركيز 20 و 25 % إذ بلغ قطر المستعمرة 4.47 و 3.68 سم , على التوالي. سُجلت العزلة 1081 *L.paralimentarius* في قواعد البيانات للبنك الوراثي العالمي ضمن الرقم العالمي MW149434.1 ويُعد هذا التسجيل الاول لهذهِ البكتريا في العراق والتي استخدمت في التجربة الحقلية لكونها اكفأ العزلات في تأثيرها ضد الفطر الممرض *P. aphanidermatum* .

الكلمات المفتاحية:
بكتريا حامض اللاكتيك، الفطر الممرض *Pythium aphanidermatum*، المقاومة الحيوية، التشخيص الجزيئي، مواد مضادة للفطريات.