



Nematicidal Activity of Lactic Acid Bacteria against Root-Knot Nematodes

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Research Article

Volume 7 Issue 1

Received Date: February 21, 2022

Published Date: March 11, 2022

DOI: 10.23880/oajmb-16000216

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Abstract

Root-knot nematodes are economically important obligate parasites of plant root that parasitize more than 3000 species of plant. Lactic Acid Bacteria (LAB) are well known as strong producers for wide range of organic acids that have been suggested recently to possess a remarkable lethal effect on root-knot nematodes. In the present 'study' 37 LAB strains were isolated from soil and agricultural wastes by using MRS medium and screened for their nematicidal activity under lab conditions. Four bacterial isolates, recorded lethal effect on second stage juveniles by more than 94%, were selected and identified by molecular method. Further, the nematicidal activity at two concentrations (i.e 50 and 10%) of the most promising ten LAB cultures was screened. The nematicidal activity of LAB was positively correlated to the concentration of their suspension cultures. 2S4 (*Pediococcus pentosaceus* MW558270), 2S5 (*Pediococcus pentosaceus* MW558883), 3S1 (*Pediococcus pentosaceus* MW558885) and 1A3 (*Pediococcus pentosaceus* MW558152) isolates showed significant mortality effect by 88.42, 87.37, 81.05 and 85.96% at the low concentration (10%), respectively. In order to confirm the relationship between organic acid production by LAB and their nematicidal activity, an artificial succinic acid, lactic acid, malic acid, acetic acid, and their mixtures were tested at concentration of 1% for their ability to inhibit juveniles vitality. The mixture of acids induced the maximum mortality effect by 100% followed by lactic acid which recorded 99.66% mortality effect. The microscopic studies and the malformation pattern of the juveniles indicating that the nematicidal activity of LAB may be derived mainly from their natural organic acids mainly lactic acid.

Keywords: Root-Knot Nematodes; Lactic Acid Bacteria; Bio-Control; Organic Acids

Abbreviations: LAB: Lactic Acid Bacteria; FDA: Food and Drug Administration; MRS: Man Rogosa and Sharpe; ANOVA: Analysis of Variance; NCBI: National Center for Biotechnology Information; GRAS: Generally Recognized as Safe; SDW: Sterilized Distilled Water.

Introduction

Plant-parasitic nematodes are considered one of the most important pests that infect many economic crops, causing many heavy losses. The losses were estimated at more than

\$77 billion a year worldwide [1,2]. The seriousness of these pests lies in the fact that the family range has a wide range [3-5]. These pests cause many problems in the cultivated fields, and the most important symptoms are wilting, stunting and yellowing, the appearance of scattered areas in the field devoid of plants [6]. Some symptoms also appear on the roots, which are ulcers, scaling of the roots and the appearance of some knots on the roots as a result of infection with the root-knot nematode [7]. All this leads to weak plants and reduced production [8]. One of the most common nematodes is the root-knot nematode, which includes more than 100 species, the most common of which are *Meloidogyne incognita*, *M. arenaria*, *M. javanica* and *M. hapla* [3,9,10]. Infection with root-knot nematodes causes knots on the roots, in addition, it allows the entry of other pathogens present in the soil [11]. All of this is reflected on the plant and the quality of the crop, which necessitates management against this pest. Control of RKNs (Root-knot nematodes) is difficult because they have short generation times and high reproduction rates [12]. As a result of the various harms of chemical control on human and animal health and the disruption of the ecosystem, concerns about public health and environmental safety are pushing for reducing chemical pesticides used in controlling plant diseases [13].

So scientists were forced to find safe alternative methods. Biological control strategies have been proposed as a safer and highly practical alternative to the management of phytonematodes. The term biological control is defined as the use of microorganisms for the purpose of reducing the population density of harmful pests [14]. These microorganisms can interact with 'pathogen' whether directly by antibiosis and competition for nutrients or indirectly by inducing plant resistance [15-17]. Biological control is one of the effective and safe ways to control many species of plant parasitic nematodes using some species of fungi and bacteria has led to an anti-nematode effect [18-20]. Lactic acid bacteria (LAB) are heterogeneous group of Gram-positive bacteria, non-respiring non-spore-forming, cocci or rods, and their importance is associated mainly with their safe metabolic activity [21]. LABS have been reported recently as an agricultural input for improvement of crop yields and protection of agriculturally important species against certain phytopathogens [22]. LAB has long been appreciated by proponents of holistic, ecologically oriented agricultural systems. The ease of growing wild LAB without the use of laboratory facilities or microbiological knowledge has led to the widespread use of these microbes by farmers [23].

For 'example' LAB showed significant antagonistic potential against phytopathogenic bacteria and is widely accepted by the United States Food and Drug Administration (FDA) as healthy (GRAS), rendering it suitable for the

production of bioprotective agents in fresh fruits and vegetables [24]. LAB produces a number of antimicrobial substances, including antifungal diketopiperazines, derivatives of fatty acid hydroxide, 3-phenyllactate, antibacterial bacteriocins and bacteriocin-like compounds, and general antimicrobials such as organic acids, hydrogen peroxide, diacetyl and reuterine (b-OH-propionic aldehyde) pyrrolidone-5-carboxylic acid [25]. Further, Seo, et al. [26] confirmed the vital role of natural organic acids produced by LAB in controlling root-knot nematodes. The main objective of the current study was to isolate novel LAB from different environmental sources and evaluate the antagonistic and suppression effects of their culture broths and culture filtrates on juveniles (J2s) of *M.incognita*. under laboratory conditions.

Material and Methods

Samples Collection

Four samples (250 g) from each environmental source soil (citrus, grapes and peanuts) and agricultural wastes (beet, dill, cabbage and lettuce) were collected. Samples were transported in an ice-box and directly tested in our microbiology lab upon arrival.

Isolation and Characterization of LAB

One gram of each sample (was suspended in 9ml of sterile distilled saline (0.9% NaCl).The isolates were obtained by plating a serial of nine-fold dilutions of each sample suspension on to de Man Rogosa and Sharpe (MRS) agar plates by pour plate method [27]. Nine- fold serial dilutions then made from the mixed solution and 1ml from the last 3 dilutions (10-7, 10-8, and 10-9) pipetted and plated on (MRS) agar plates by pour plate method [27]. LAB were isolated on de Man Rogosa and Sharpe (MRS) agar (LAB-M, Bury, UK) after an incubation with MRS broth supplemented with 2% NaCl for 12 h at 37°C. Plates were incubated at 37°C for 24 h aerobically. The Gram-staining and catalase tests were performed for pure colonies with morphological characters. Only the isolates that were Gram-positive, catalase-negative, positive growth at 15, 45°C and pH (5-7-9-11) were sub-cultured in MRS broth. Glycerol stocks (60% v/v) were used for preservation of each selected isolate and stored at - 80°C.

Safety Assessment

Susceptibility to Antibiotics: The antibiotic susceptibility of the selected isolates was determined against six antibiotics, namely: ampicillin, vancomycin, kanamycin, tetracycline, erythromycin and fusidic acid. Antibiotic discs were obtained from bioMerieux (Marcy l'Etoile, France). Antibiotic susceptibility was determined semi-quantitatively using the

agar overlay diffusion methods of the National Committee for Clinical Laboratory Standards [28]. The susceptibility to antibiotic was graded as either resistant or susceptible according to Kim, et al. [29].

Hemolytic Activity: In order to perform the hemolytic activity tests, the LAB strains were streaked on Columbia blood agar plates containing 5% of sheep blood. The plates were incubated for 48 h at 37°C. As suggested by Maragkoudakis, et al. [30], the strains that produced green-hued zones around the colonies or did not produce any effect on the blood plates were considered non hemolytic. The strains showed blood lysis zones around the colonies were classified as hemolytic (β -hemolysis).

Molecular Identification of Selected Isolates: Genomic DNA was extracted from pure cultures of the bacterial isolates using the DNeasy mini kit (Qiagen, CA, USA) according to the manufacturer's instructions. The primers used to amplify the 16S ribosomal RNA gene were 8F 5'AGT TGA TCC TGG CTC AG 3' and 1492R 5'TAC CTTGTT ACG ACT T 3', Rochelle, et al. [31]. DNA amplification was performed using 20ng of DNA template 250 mM each dNTPs [dATP, dCTP, dGTP, dTTP], 25 pmol each primer, 2.5mM MgCl₂ 10 μ l of 5x PCR buffer and 2.5 U Taq polymerase (promega) and added up to total volume of 50 μ l ddH₂O. The reactions were performed in an automatic thermal cycler (GeneAmp1 PCR System 9700, Perkin-Elmer) under the following conditions: initial denaturation at 94°C for 3 min; 35 cycles of 94°C for 50 s, 55°C for 1 min, 72°C for 1.30 min; final extension at 72°C for 7 min. An aliquot of 10 μ l PCR product were analyzed on 1% agarose gel. The purification of PCR product was done using high pure PCR purification kit (Qiagen). Purified PCR products were sent for sequencing at Macrogen (South Korea). The deduced sequences were submitted to the GenBank database and accession numbers were obtained. Further, identification of phylogenetic neighbours and calculation of pairwise similarity of these sequences were done by using the National Center for Biotechnology Information (NCBI) taxonomy tree building service, ClustalW and MEGA 5.2 software.

Extraction of Nematode Juveniles: Eggs were extracted from galled tomato roots infected with *M. incognita* using 1 % sodium hypochlorite solution [32]. Eggs were collected on 500 Mesh sieve and transferred to a beaker containing tap water. Hatching of second stage juveniles was stimulated by aerating the egg suspension with oxygen in the dark for 7 days as described by Naserinasab, et al. [33]. Freshly hatched second-stage juveniles were separated from the un-hatched eggs using a modified Baermann technique [34] and used to evaluate the nematicidal potentiality of LAB.

Juveniles (J2) Mortality Bioassay: Thirty seven bacterial isolates and their culture filtrates were screened for their antagonistic activity against second stage juveniles (J2) of *M. incognita*. One ml from each bacterial culture and culture filtrate was transferred separately to wells of a 96-well Microtest™ Tissue Culture Plate, then one ml of water

suspension containing 100 of juveniles was added to each well and incubated at 28-30°C for 24 hours. The same number of juveniles received sterilized distilled water (SDW) and was served as control. Control and each treatment were replicated thrice. After 24 hours, the dead juveniles were counted in each well depending on their mobility [35]. Morphological changes and distortions noticed in treated juveniles were also studied by aid of compound microscope [36]. The mortality percentage was calculated according to Schneider-Orelli formula [37]: Mortality (%) = [(mortality percentage in treatment – mortality percentage in the negative control)/(100 – mortality percentage in the negative control)] × 100.

In vitro Nematicidal Activity of Two Concentrations of the Most Prominent LAB: Ten promising LAB isolates which showed distinct morphological and recorded significant mortality effect on J2 were selected, grown and adjusted to contain 108 CFU. Two concentrations (50 and 10%) of bacterial suspension culture soft he selected bacterial isolates were tested for their nematicidal activity at lower concentrations.

In vitro Nematicidal Activity of Some Artificial Organic Acids: According to Seo, et al. [26], in order to investigate the relationship between organic acid production potentiality of LAB and their nematicidal activity, four artificial edible organic acids, which are known to be produced naturally by LAB i. e.: succinic, lactic, malic, acetic acid, and their mixtures were screened at concentration of 1% for their nematicidal activity against *M. incognita*. The organic acid solutions were transferred to wells of a 96-well Microtest™ Tissue Culture Plate with three replications, into which about 100 J2 of *M. incognita* were placed. After 24 hours, the dead juveniles were counted in each well and morphological changes were studied by aid of compound microscope.

Statistical analysis: Experiments were carried out in a completely randomized design. Data were subjected to analysis of variance (ANOVA). Means were compared by Duncan's multiple range tests [38].

Results

Isolation and Presumptive characterization of LAB

In total, 40 LAB strains were isolated from the selected environmental sources (soil and agricultural crop wastes), based on their typical morphological appearance (small pinpointed and creamy white colonies), catalase and coagulase-negative and non-motile, coccus and rod-shaped characteristics (Table1).

Furthermore, the selected isolates were able to grow at different pH concentrations as shown at (Table 2). Most of the strains were able to survive in pH 11. The study of bacteria growth according to parameters by Bergey's

manual 9th edition was pH, temperature and salinity. For temperature, corresponds to the favorable temperature for optimal growth of lactic acid bacteria; so all strains are mix

between mesophilic and thermophilic, also, 17 soil isolate and 15 agriculture waste isolates were gram positive (Table 3).

Samples	Elevation	Colony texture and Edge	Colony color
1S 1	Raised	Coarse surface and rough	Creamy
1S 2	Raised	Coarse surface and rough	Creamy
1S 3	Convex	Shiny surface and rough	Whitish
1S 4	Raised	Shiny surface and rough	Creamy
1S 5	Convex	Shiny surface and smooth	Whitish
2S1	Raised	Shiny surface and smooth	Creamy
2S 2	Raised	Shiny surface and rough	Creamy
2S 3	Raised	Coarse surface and rough	Creamy
2S 4	Convex	Shiny surface and smooth	Whitish
2S 5	Raised	Coarse surface and rough	Creamy
3S1	Raised	Coarse surface and rough	Creamy
3S2	Raised	Shiny surface and smooth	Creamy
3S3	Raised	Shiny surface and rough	Creamy
3S4	Convex	Shiny surface and smooth	Whitish
3S5	Raised	Shiny surface and smooth	Creamy
4S1	Raised	Shiny surface and rough	Creamy
4S2	Raised	Shiny surface and rough	Creamy
4S3	Raised	Coarse surface and rough	Creamy
4S4	Convex	Shiny surface and smooth	Whitish
4S5	Convex	Shiny surface and smooth	Whitish
1A1	Raised	Shiny surface and rough	Creamy
1A2	Convex	Shiny surface and smooth	Whitish
1A3	Convex	Shiny surface and smooth	Whitish
1A4	Convex	Shiny surface and smooth	Whitish
1A5	Convex	Shiny surface and smooth	Whitish
2A1	Raised	Shiny surface and rough	Creamy
2A2	Convex	Shiny surface and smooth	Whitish
2A3	Convex	Shiny surface and smooth	Whitish
2A4	Convex	Shiny surface and smooth	Whitish
2A5	Convex	Shiny surface and smooth	Whitish
3A1	Raised	Shiny surface and rough	Creamy
3A2	Convex	Shiny surface and smooth	Whitish
3A3	Convex	Shiny surface and smooth	Whitish
3A4	Convex	Shiny surface and smooth	Whitish
3A5	Convex	Shiny surface and smooth	Whitish
4A1	Raised	Shiny surface and rough	Creamy
4A2	Convex	Shiny surface and smooth	Whitish
4A3	Convex	Shiny surface and smooth	Whitish
4A4	Convex	Shiny surface and smooth	Whitish
4A5	Convex	Shiny surface and smooth	Whitish

Table 1: Phenotypic characteristics of lactic acid bacteria (LAB) isolates.

Isolates No.	Control pH 7/37°C cfu/ml10 ⁻⁷	pH 5	pH 9	pH 11	15°C	30°C	45°C
		5-Oct	7-Oct	7-Oct	5-Oct	7-Oct	5-Oct
1S 1	132±0.3	++	++	++	++	++	+
1S 2	112±0.2	+++	+++	+++	+++	+++	++
1S 3	123±0.1	+++	+++	+++	+++	+++	++
1S 4	130±0.2	+++	+++	+++	+++	+++	++
1S 5	43±0.3	+++	+++	+++	+++	+++	++
2S1	132±0.2	++	++	++	++	++	+
2S 2	45±0.3	++	++	++	++	++	+
2S 3	137±0.2	+	++	++	+	++	+
2S 4	51±0.3	+++	+++	+++	+++	+++	++
2S 5	129±0.2	++	++	++	++	++	+
3S1	58±0.3	+++	+++	+++	+++	+++	++
3S2	67±0.3	++	++	++	++	++	+
3S3	129±0.2	++	++	++	++	++	+
3S4	188±0.3	+++	+++	+++	+++	+++	++
3S5	120±0.3	++	++	++	++	++	++
4S1	138±0.2	+	+	++	+	+	+
4S2	144±2.0	++	++	++	++	++	+
4S3	98±0.3	++	++	++	++	++	+
4S4	121±0.2	+++	+++	+++	+++	+++	++
4S5	130±0.1	+++	+++	+++	+++	+++	++
1A1	121±0.1	+++	++	++	+++	++	++
1A2	180±0.2	+++	+++	+++	+++	+++	++
1A3	123±0.3	++	++	++	++	++	+
1A4	170±0.2	+++	+++	+++	+++	+++	++
1A5	121±0.2	+++	++	++	+++	++	++
2A1	145±0.3	++	++	++	++	++	++
2A2	137±0.2	+	++	++	+	++	+
2A3	145±0.2	++	++	++	++	++	+
2A4	137±0.1	++	++	++	++	++	+
2A5	121±0.2	+++	+	++	+++	+	++
3A1	180±0.2	+++	+++	+++	+++	+++	++
3A2	123±0.1	++	++	++	++	++	+
3A3	170±0.2	+++	+++	+++	+++	+++	++
3A4	180±0.2	+++	+++	+++	+++	+++	++
3A5	123±0.3	++	++	++	++	++	+
4A1	170±0.1	+++	+++	+++	+++	+++	++
4A2	121±0.2	+++	++	++	+++	++	++
4A3	180±0.2	+++	+++	+++	+++	+++	++
4A4	123±0.1	++	++	++	++	++	+
4A5	170±0.1	+++	+++	+++	+++	+++	++

Table 2: Growth of lactic acid bacteria (LAB) at different pH concentrations and different temperatures. Isolates.

Isolate code	(VA 30µg)	(FA 10µg)	(E 15µg)	(TE 30µg)	(K 30µg)	(AMP 10µg)
1S2	R	S	R	S	I	R
2S4	R	I	R	S	R	R
2S5	R	I	R	S	R	I
3S1	R	I	R	R	R	I
4S4	R	I	R	S	R	I
1A2	R	I	R	S	R	S
1A3	R	R	R	S	R	I
2A5	R	I	R	S	I	S
3A2	R	R	R	I	R	S
4A5	R	I	R	S	R	I

VA: Vancomycin; FA: Fusidic Acid; E.: Erythromycine; TE: Tetracycline; K: Kanamycin; and; Amp: Ampicillin; (Inhibition zone with mm, resistant (R), intermediate (I), and susceptible (S), S: ≥ 21 mm, R: ≤ 15 mm; I: 16-20 mm.

Table 3: Antibiotic susceptibility profiles of the selected LAB isolates.

Nematicidal Activity of LAB Isolated from Soil and Agricultural Waste *In Vitro*

Four soil samples were used to isolate LAB. According to the morphological characters and growth pattern, seventeen bacterial isolates were selected for the current assay. It was noticed that all different isolations gave suppressive effect on juveniles activity. The maximum percentage of juveniles mortality was recorded with bacterial culture of isolate 2S4,

2S5, 3S1 with percentage of 100% mortality followed by 4S4 (98.63%) then 1S2 (95.9%). However the bacterial culture of isolate 1S1 gave the lowest percentage of juveniles mortality (72.01%). As for culture filtrate, isolates 2S4, 2S5 and 3S1 also showed 100 % of juveniles mortality followed by 4S4, 2S1, and 1S2 with percentages of (97.95, 95.22 and 81.57%), respectively (Table 4). In most isolates, we can conduct that bacterial culture showed best result than culture filtrate.

Isolate code	Juveniles Mortality %			
	Treatment type			
	Bacterial Culture		Culture Filtrate	
	No. of dead juveniles	Mortality %	No. of dead juveniles	Mortality %
1S1	72.66 ⁱ	72.01	65.33 ^{ef}	64.5
1S2	96.00 ^{abc}	95.9	82.00 ^{bc}	81.57
1S3	87.33 ^{efgh}	87.03	78.66 ^{cd}	78.15
1S4	90.00 ^{defg}	89.76	70.66 ^{ef}	69.96
2S1	95.33 ^{abcd}	95.22	95.33 ^{ab}	95.22
2S2	93.33 ^{bcd}	93.17	82.00 ^{bc}	81.57
2S3	91.33 ^{cdef}	91.1	82.66 ^{bc}	82.25
2S4	100.00 ^a	100	100.00 ^a	100
2S5	100.00 ^a	100	100.00 ^a	100
3S1	100.00 ^a	100	100.00 ^a	100
3S4	82.66 ⁱ	82.25	78.33 ^{cd}	77.81
3S5	85.33 ^{gh}	84.98	84.00 ^{bc}	83.62
4S1	86.00 ^{fgh}	85.67	81.00 ^{bcd}	80.55
4S2	92.00 ^{cde}	91.81	84.66 ^{bc}	84.29
4S3	91.00 ^{cdef}	90.78	82.00 ^{bc}	81.57
4S4	98.66 ^{ab}	98.63	98.00 ^a	97.95
4S5	82.66 ^h	82.25	74.00 ^{de}	73.38
Control	2.33 ^j	--	2.33 ^f	--
L.S.D at 0.05	5.01	-	6.54	-

Means in each column followed by the same letter(s) did not differ at $P \leq 0.05$ according to Duncan's multiple range test.

Table 4: Nematicidal activity of the tested lactic acid bacteria (LAB) isolated from soil against root-knot nematode (*Meloidogyne incognita*) under laboratory conditions.

Data in (Table 5) illustrate the nematicidal activity of fifteen bacterial isolates from agricultural waste on juveniles mortality of *M.incognita*. It was obvious that all different bacterial culture isolates showed approximately 100 % of

nematode mortality. Similar trend was noticed with culture filtrate except the isolate 1A5. Five bacterial isolates encoded, 1A2, 1A3, 2A5, 3A2 and 4A5 recorded the maximum mortality effect on J2of *M.incognita*. Were selected for further study.

Isolate code	Juveniles Mortality %			
	Treatment type			
	Bacterial Culture		Culture Filtrate	
	No. of dead juveniles	Mortality %	No. of dead juveniles	Mortality %
1A1	100.00 ^a	100	98.00 ^a	97.95
1A2	100.00 ^a	100	98.66 ^a	98.63
1A3	100.00 ^a	100	100.00 ^a	100
1A4	100.00 ^a	100	99.33 ^a	99.31
1A5	100.00 ^a	100	92.66 ^b	92.48
2A1	99.33 ^a	99.31	98.66 ^a	98.63
2A2	100.00 ^a	100	99.33 ^a	99.31
2A3	99.33 ^a	99.31	99.33 ^a	99.31
2A4	100.00 ^a	100	100.00 ^a	100
2A5	100.00 ^a	100	100.00 ^a	100
3A1	100.00 ^a	100	99.33 ^a	99.31
3A2	100.00 ^a	100	100.00 ^a	100
3A3	99.33 ^a	99.31	98.00 ^a	97.95
4A2	100.00 ^a	100	100.00 ^a	100
4A5	100.00 ^a	100	96.66 ^a	96.58
Control	2.30 ^b	--	2.33 ^c	--
L.S.D at 0.05	1.46	-	3.08	-

Means in each column followed by the same letter(s) did not differ at $P \leq 0.05$ according to Duncan's multiple range test.

Table 5: Nematicidal activity of the tested lactic acid bacteria (LAB) isolated from agricultural waste against root-knot nematode (*Meloidogyne* spp.) under laboratory conditions.

Isolate code	Juveniles Mortality %			
	Concentration (%) of bacterial culture			
	50%		10%	
	No. of dead juveniles	Mortality %	No. of dead juveniles	Mortality %
1S2	91.66 ^b	91.22	67.66 ^b	65.96
2S4	100.00 ^a	100	89.00 ^a	88.42
2S5	100.00 ^a	100	88.00 ^a	87.37
3S1	100.00 ^a	100	82.00 ^a	81.05
4S4	86.33 ^b	85.61	61.00 ^{bc}	58.95
1A2	87.00 ^b	86.32	64.66 ^b	62.8
1A3	100.00 ^a	100	86.66 ^a	85.96
2A5	87.00 ^b	86.32	67.00 ^b	65.26
3A2	87.66 ^b	87.01	46.33 ^d	43.51
4A5	86.66 ^b	85.96	54.66 ^{cd}	52.27
Control	5.00 ^c	--	5.00 ^e	--
L.S.D at 0.05	4.79	-	8.83	-

Means in each column followed by the same letter(s) did not differ at $P \leq 0.05$ according to Duncan's multiple range test.

Table 6: Comparative study of two bacterial culture concentrations of the most promising lactic acid bacteria (LAB) against root-knot juveniles vitality.

Through the results shown in (Tables 4 & 5), isolates (1S2, 2S4, 2S5, 3S1 and 4S4) from soil and isolates (1A2, 1A3, 2A5, 3A2 and 4A5) showed the best results in increasing the mortality rate of nematodes, so we compared two bacterial culture concentrations (10 and 50%) of these isolates on survival of nematodes under laboratory conditions (Table 6). Either at a concentration of 50 or 10%, it was found that the best results were obtained by using isolates 2S4, 2S5, 3S1 and 1A3 which gave a mortality percentage 100% at a concentration of 50% and >80% at concentration of 10%.

Nematicidal Activity of Different Artificial Organic Acids

Through some previous studies, some scientists have shown that lactic acid bacteria secrete some organic acids such as Succinic, lactic, Malic and Acetic acid. Therefore, in this study, we evaluated the effect of these acids artificially either individually or in a mixture on the death of nematode juveniles to examine the potential of organic acids as main products of LAB as nematicidal compounds. In general all acids effect significantly on survival of juveniles. Acids mixture gave (100 %) of nematode mortality followed by lactic acid (99.66%) and acetic acid (98.33%) (Table 7).

Artificial acids (1%)	No. of dead juveniles	Mortality %
Succinic acid	65.33 ^c	65.33
Lactic acid	99.66 ^a	99.66
Maleic acid	92.66 ^b	92.66
Acetic acid	98.33 ^{ab}	98.33
Acids mixture	100.0 ^a	100
Control	0.0 ^d	0
L.S.D	5.88	----

Means in each column followed by the same letter(s) did not differ at $P \leq 0.05$ according to Duncan's multiple range test.

Table 7: Nematicidal activity of succinic, lactic, malic, and acetic acid and their mixture on the mortality of juveniles of *Meloidogyne incognita* under laboratory conditions

Treatment with artificial acids at concentration 1% and their mixture look like that juveniles of *M. incognita* were shown to be dead with rigid and straight body. Similar trend was noticed with treatment by lactic acid bacteria (LAB) isolates. However the juveniles in the untreated control were vital and still moving. The intestinal tract of juveniles treated whether with LAB isolates or their artificial acids showed pathological changes such as vamp and the structures were clearly visible, after those destroying internal organs which are followed by creating cavities comparing to the control (Figure 1).

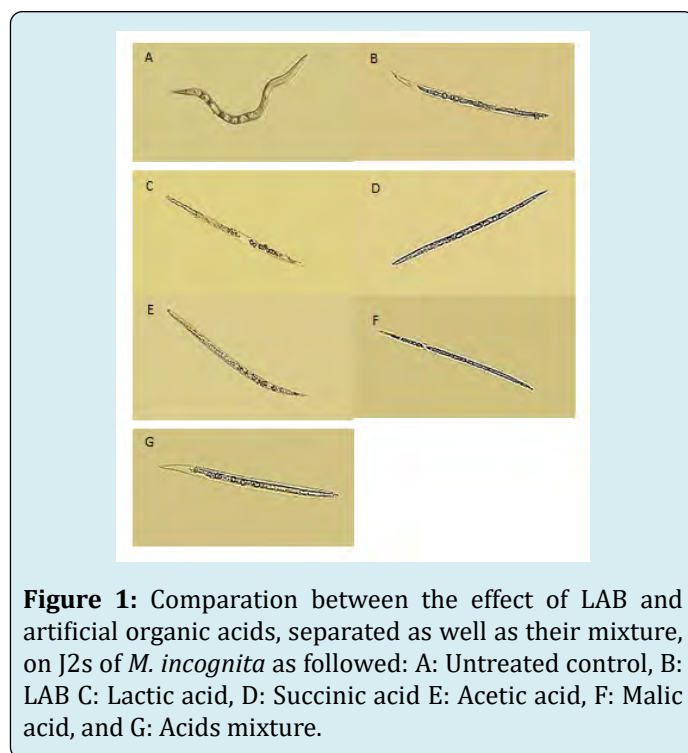


Figure 1: Comparison between the effect of LAB and artificial organic acids, separated as well as their mixture, on J2s of *M. incognita* as followed: A: Untreated control, B: LAB C: Lactic acid, D: Succinic acid E: Acetic acid, F: Malic acid, and G: Acids mixture.

Safety Assessment of LAB

Susceptibility to Antibiotics: Antibiotic susceptibility profiles of the ten tested isolates were shown at Table 3. There was resistance of 2 antibiotics used (Vancomycin 30 μ g and Erythromycin 15 μ g) for all isolates but eight isolates recorded high sensitivity for Tetracycline 30 μ g. Also the strains showed variance effects against Ampicillin, Kanamycin and Fusidic acid respectively. The results of strains showed safe properties for human use and susceptible to all of the tested antibiotics showing clear inhibition zones of diameters ranging between 9 and 30 mm.

Hemolytic Activity: There was no change in the color surrounding colonies of the selected isolates when tested on blood agar. The absence of β -hemolysis in any of the tested strains is an indication of their safe and GRAS properties. Historically, LAB associated with food has been generally recognized as safe, GRAS [39].

Molecular Identification of the Most Potent Strains and Phylogenetic Tree: PCR products of approximately 1500 bp amplified with the 8F/ 1498R primers and corresponding to the 16S rDNA region were obtained from all bacterial isolates. Sequence alignments of the four bacterial isolates showed identities over 99% with *Pediococcus pentosaceus* strains. Alignment analysis using 1500 bp of each identified fragment sequence was displayed against the corresponding top five homologous sequences that were determined from the NCBI database. Bacterial isolates sequences have been

submitted to GenBank database and accession numbers were obtained (2S4 (MW558270), 2S5 (MW558883), 3S1 (MW558885) and 1A3 (MW558152). Mega-alignment and the phylogenetic tree were generated (Figure 2) among the four nucleotide sequences that were indicated as *Pediococcus pentosaceus*.

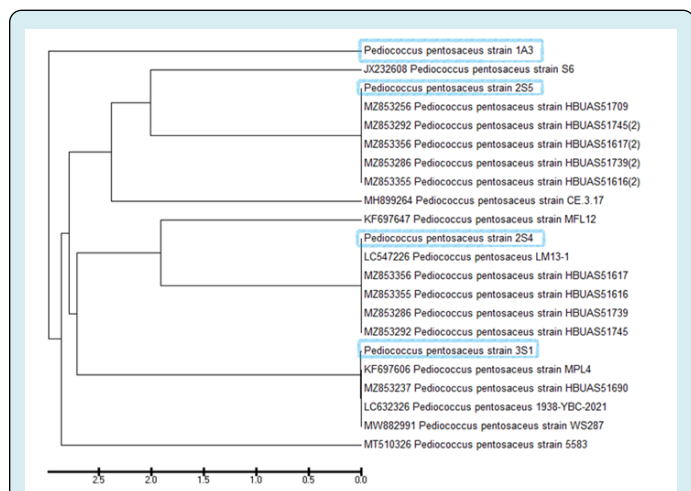


Figure 2: Phylogenetic tree based on the 16S rDNA gene sequences and relationship among the four accession numbers of identified strains (blue squared) and the top homologous on the NCBI data base.

Discussion

Lactic acid bacteria are considered one of the most important types of bacteria that improve the nutritional value of many foods, as well as resist some intestinal diseases for humans [40,41]. It is also considered one of the bacterial types used in the manufacture of many medicines because of its anti-effect to some fungi and pathogenic bacteria. For this it became possible to use it against some pathogens of the plant [42,22]. There is a basic group consisting of four genera, which are *Lactobacillus*, *Streptococcus*, *Pediococcus* and *Leuconostoc*. The modern methods of examination and classification have proven the existence of other genera which are *Tetragenococcus*, *Aerococcus*, *Carnobacterium*, *Alloicoccus*, *Dolosigranulum*, *Enterococcus*, *Lactococcus*, *Vagococcus*, *Globicatella*, *Oenococcus*, and *Weissella* [43]. Bourdichon, et al. [44] explained that this group of bacteria is important due to its metabolic activity during growth, as it uses available sugar to produce organic acids, and its safe presence in many foods has led to it being considered suitable as GRAS (Generally Recognized as Safe) for human use. Kim, et al. [45] reported that LAB isolates induce such morphological modifications in several pathogenic microbes.

These morphological modifications may be due to deviations in membrane lipogenesis, altered membrane

fluidity and/or membrane integrity resulting in cell wall lysis and loss of dense intracellular substance [46]. Bajpai, et al. [47] studied the antibacterial potential of lactic acid bacteria *Pediococcus pentosaceus* against *Staphylococcus aureus* and *Escherichia coli*. The cell free supernatant of *P. pentosaceus* revealed its antibacterial action against *S. aureus* and *E. coli* on membrane integrity as established by increased potassium ion release, reduced absorption at 260-nm, reduction in cell viability, and increased relative electrical conductivity. GC-mass analysis for this bacteria identified major compounds included organic acid, amino acid and fatty acid. Anas, et al. [48] showed that the antimicrobial activities may be related to the various compounds, such as H₂O₂, organic acids and bacteriocin-like substances. From this standpoint, the lactic acid bacteria drew attention to their use in the agricultural field as alternative method to chemical pesticides to reduce the harmful effect caused by them, in addition to the percentage of pesticide residues in the fruits.

In this study, some types of bacteria producing lactic acid were isolated from soil (17 isolates) and agricultural waste (15 isolates) and their effect against root-knot nematodes were studied under laboratory conditions. Most of isolates were able to survive in pH 11. This result is disagreed with Trias, et al. [22] who reported that lactic acid bacteria can grow well and survives at low pH such as fruit where it is a good source for sugar and nutrients needed for bacterial growth. In our study, we noticed that the nematicidal activity of lactic acid bacteria isolates showed remarkable death at root-knot nematode juveniles. The findings of Borgo, et al. [49] reported that the ability of *Enterococcus italicus* and *Lactococcus garvieae* to kill the nematode *Caenorhabditis elegans* and they suggested that this was due to the production of hydrogen peroxide (H₂O₂) by these bacteria. In another study on the same species of nematode *Caenorhabditis elegans* by Zanni, et al. [50], *Leuconostoc lactis*, *Lactobacillus fermentum* and *L. delbrueckii*, were found as the main species and shown to display different colonization capacity at worm gut. Moreover, they noticed that lactic acid bacteria supplementation decreased nematode lifespan. Also Fasseas, et al. [51] reported that *Lactobacillus reuteri* and *Pediococcus acidilactici* reduced the lifespan of wild-type and short-lived daf-16 worms of *C. elegans*. Gustin, et al. [52] conducted that some bacteria produced H₂O₂ could kill nematodes due to its toxicity. Ralmi, et al. [53] studied the effect of H₂O₂ against root-knot nematode infected *Ficus deltoidea* and they reported that no root gall formation.

There is little information on the use of lactic acid as a biological control against plant parasitic nematode. Takei, et al. [54] explained the possibility of using some capsules that contain lactic acid bacteria against root-knot nematodes. Seo, et al. [55] also showed that the use of lactic acid bacteria (*Lactobacillus farraginis*) with concomitant with *Bacillus*

cereus and *B. thuringiensis* can reduce the number of egg masses on the roots of *Cucumis melo* without *phytotoxicity*. The impact of four artificial organic acids (succinic, lactic, acetic and maleic acid) and their mixture was determined at 1% concentration on the survival of root-knot nematode juveniles. All treatments showed significant influence on juveniles mortality. Treatment with organic acids displayed the appearance of some gaps in the bodies of dead juveniles. This result is supported by the findings of Seo and Kim [56] who reported that incidence 100% mortality to juveniles at all concentrations of acetic Acid and mixture of acetic and lactic acid and only at 1.0% and 0.5% of Lactic Acid. Also Yeon, et al. [26] reported that Maleic acid showed 100% second stage juveniles mortality. Seo, et al. [26] conducted that the nematicidal activity of the culture filtrate of *L. brevis* WiKim0069 referred to organic acids such as acetic acid, lactic acid, malic acid, and succinic acid produced by bacteria [57-62].

Conclusion

Based on this discussion, it can be summarized that using lactic acid bacteria can be used as ecofriendly method to control root-knot nematode. Nevertheless, greenhouse and field experiments are needed on a large scale to confirm the results. Besides, isolation and identification of secondary metabolites compounds from these bacteria are required for further studies under laboratory and greenhouse conditions to understand their mode of action and nematicidal activities and to approve their effectiveness under field conditions.

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