Evaluation of the Antimicrobial Activity, Starter Capability and Technological Properties of Some Probiotic Bacteria Isolated from Egyptian Pickles

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Abstract: The work employed in this paper was an endeavour to select and characterise a probiotic bacterial strains which could be used as starter and protective cultures for pickles fermentation. Sixty one bacterial isolates were isolated from Egyptian pickled vegetables and fruits onto both MRS and nutrient agar media. One isolate only of lactic acid bacteria growing on MRS agar: LPS10 and three isolates BPS4, BPS20 and BPS33 growing on nutrient agar inhibited other pathogenic and non-pathogenic bacteria. Additionally the LPS10 isolate inhibited *Candida albicans* fungus. The antimicrobial spectrum of LPS10 isolate was more than that obtained from other BPS4, BPS20 and BPS33 isolates. The LPS10 bactic acid bacterial isolate showed fast growth and decreased pH of the medium from 6.5 to 3.5 within 48 h, tolerated NaCl up to 11%, grew at wide range of pH (2.0-8.0) and temperatures (15-44°C). It showed interested probiotic capabilities such as production of acetoin, citrate utilization, oxalate hydrolysis and production of some important enzymes. The BPS4, BPS20 and BPS33 isolates grew in nutrient broth, at 4-7% NaCl; at initial pH 3.5-8.0; at 15°C-50°C, they produced acetoin and decomposed some polymers and consequently, could be used as probiotics and biocontrol agents during pickles fermentation. The bacterial isolates LPS10; BPS4; BPS20; BPS33 were identified by biochemical and molecular methods as belonging to *L. plantarum* LPS10' *B. acidicola*BPS4; *B. amyloliquefaciens* BPS20; *B. mycoides*BPS33 respectively.
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1. Introduction

Pickling is the processes of preserving food by fermentation in brine or vinegar. The resulting food is called a pickle **(Lee, 2004)**. Pickles are considered one of the popular foods in Egypt as they are cheap and available for all consumers **(Ismaiel *et al*., 2014)**. There are two ways of producing pickles. First are the brine process; inwhich pickles are made mainly by submerging vegetables or fruits in brine covered by plastic films and salt helps to withdraw nutrients (sugars) from the vegetables or fruits which in turn are fermented naturally by lactic acid bacteria. Second are the fresh-pack processes; inwhich pickles are produced without fermentation processes but are produced after immersing vegetables of fruits in brine for few hours and then immersed in a boiling solution of vinegar and pickling spices **(Dalmasso, 2001)**. In both cases salts decrease the free (unbound) water where pathogenic bacteria can not grow and beneficial lactic acid bacteria can grow **(Pundir and Jain, 2010; Enan *et al*., 2013b, c)**

Pickling made by brine processes using fermentation is useful since lactic acid bacteria used decrease, the pH of the medium to the acidic side (below 4.0) where many pathogens cannot grow. Hereby further research is needed to select and characterise lactic acid bacterial cultures to be used as starter and protective cultures for pickling **(Enan *et al*., 2013a,b)**. The use of lactic acid bacteria for pickling is a hazardous way because, these bacteria produce lactic acid,diacetyl, carbon dioxide and bacteriocins**(Enan, 2006; Enan and Amri, 2006; Enan *et al*., 2013a; Ouda *et al*., 2014)**. Consequently, these bacteria must be used as starter and protective cultures. The recent approach is to select a starter and protective lactic acid bacterial cultures with probiotic capability to make pickles with inhibition of food-borne pathogens and improving human health by the ability to acidify the medium, produce proteases, β-glucosidase and lactase **(Moreno *et al*., 2000; Buyukyouk *et al*., 2010)**. Many authors (**Doyle *et al*., 2001; Lee, 2004; Pundir and Jain, 2010)** found growth of pathogenic bacteria in this acidic conditions during pickling. Consequently, using of bacterial biocontrol agents is necessary to antagonise such pathogenic bacteria by producing antimicrobial substances **(Weagant *et al*., 1994; Jay *et al*., 2005; Enan *et al*., 2012; Enan *et al*., 2014)**. The present work was undertaken to select and characterise lactic acid bacteria to be used as starter, probiotic and protective cultures for pickles fermentation. Also, a strains of *Bacillus* bacteria were selected and

characterized to be biocontrol agents during pickling of Egyptian vegetables.

2.Material and Methods

Pickles samples and isolation of bacteria.

Ten types of Egyptian pickles were a subject of our study including carrot, cucumber, green olive, cabbage, cauliflower, lemon, pepper, turnip, beetroot and one mixed sample. Ten samples of each type of traditionally made pickles were bought from Egyptian markets; each was made in a 250 ml bottle. Samples were transported to the Laboratory immediately. 10 ml of brine and pickles were mixed by mixer (Monolex, Co.) and added to 90 ml sterile distilled water and were serially diluted **(Enan, 2006b)**. Lactic acid bacteria were isolated onto MRS agar **(DeMan *et al*., 1960)**. Other bacteria were isolated from the same pickles samples on nutrient agar (Oxoid). Pure representative colonies were purified on slope agar of the same media. Sixty one isolates were prepared assimilating 15 isolates of lactic acid bacteria, designated LPS1-LPS15 and 46 isolates of other bacteria which designated BPS1-BPS46.

Bioassay for inhibitory activity produced by some bacterial isolates.

The antibacterial spectrum of the bacterial isolates LPS10, BPS4, BPS20 and BPS33 against different microorganisms was studied by the agar disc diffusion assay **(Bello *et al*., 2012; Abdel-Shafi *et al*., 2014)**. A 1% v/v suspension of log phase cells of the bacteria to be tested to produce inhibitory activity was inoculated and spreaded onto either MRS agar (for LPS10) or on nutrient agar (for other bacteria). Agar plates were incubated for 48h at 35°C.Agar discs were made by 7 mm corckporer and transferred onto surface of soft agar top layer seeded with lawns of the indicator organisms used. Plates were incubated for 4h at 4°C to allow diffusion of the inhibitory substance(s). The plates were then incubated at 30°C for 48h and were examined for appearance of inhibition zones around agar discs of the inhibitory activity producing bacteria.

Temperature and pH growth range.

A series oftest tubes, each containing 10 ml broth medium, were inoculated by 1% v/v of suspension of log phase cells of the bacteria tested. Each inoculated tube was incubated at certain temperatures (15°C-50°C). Growth was rested (OD 600 nm.) after 24h and 48h of incubation. Broth media in other different test tubes were adjusted at different pH values (2.0-12.0), inoculated by the bacteria tested (1% v/v) and were incubated at the optimum temperature appeared for each bacterium for 48 h. growth (OD 600 nm) was tested after 24h and 48h of incubation **(Enan *et al*., 2013c)**.

Growth and medium acidification.

The inhibitory activity producing bacteria viz. LPS10, BPS4, BPS20 and BPS33 were tested for their growth and medium acidification according to **Chammas *et al.* (2006)**. Broth media were inoculated by $2x10^4$ CFU/ml final concentration, incubated at 35°C for the bacterial isolates LPS10, BPS4 and BPS20 and at 30°C for the bacterial isolate BPS33 as these temperatures were appeared preliminary to be the optimum growth temperatures of the bacterial isolates tested. Incubation continued 4 days and whilst samples were removed after appropriate time intervals and were analysed for growth (CFU/ml) and final pH value by pH-meter (New Brunswek Scientific Co.).

Phenotypic and biochemical characteristics and some probiotic capabilities.

Four isolates only (LPS10, BPS4, BPS20 and BPS33) which showed the highest values of growth, medium acidification and inhibited some pathogenic bacteria were characterised. The carbohydrate fermentation profiles were examined using API20.Strep tests following the manufacturer's instructions (Biomerieux, Montalieu-Verciue, France). Enzyme activities were assayed using API ZYM system (Biomerieux, France) according to the manufacturer's instruction Isomers of lactic acid were studied by enzymatic method **(Gawehn and Bergmeyer, 1974)**. Arginine hydrolysis by the bacterial isolate LPS10was tested by the method of **Hebert *et al.* (2000)**. Growth of bacterial isolates in different NaCl concentrations was tested in MRS broth for isolate LPS10 and in nutrient broth for other isolates **(Enan, 2006a; Enan *et al*., 2013c).** Other biochemical tests regarding catalase test, citrate utilization, hippurate and oxalate hydrolysis, oxidase test, reduction of nitrate to nitrite and H_2S production were in addition to the ones obtained by API20 further assayed following **Cappuccino and Sherman, 1995; Harrigan, 1998)**.

Molecular identification of bacterial isolates.

The five isolates employed herein viz. LPS10, BPS4, BPS20, BPS33 were further identified via 16S rRNA cataloging analysis. Total DNA samples were extracted from exponentially growing bacterial cells **(Sambrook and Russel, 2001)**. The 16S rRNA gene was amplified for each isolate by PCR using the foreward primer: 5′-AGAGTT GATCCT- GGCTCAG-3′ and also using the reverse primer 3′-TTCAGCATTGTTCC-ATTG-5′**(Turner *et al*., 1999; Chenbey *et al*., 2000)**. The PCR products were subjected to agarose gel electrophoresis **(Sambrook and Russel, 2001)** and bands of amplified 16S rRNA gene were cleaned up using Gene Purification Kit (Fermentas). Amplified DNA fragments were partially sequenced at GATC Biotech AG (Konstanz, Germany) using ABI 373 OXI DNA sequencer. Sequences analysis and their comparison to

deposited data in Gene Bank was made using local Aligment Search Tool (BLAST) programme at http://ncbi.nlm.gov/blast **(Altschull *et al*., 1997)**.

3.Results

Bacteria were isolated from 100 samples of Egyptian pickles (vegetables and fruits) and the isolation produces resulted in selection of one isolate only of lactic acid bacteria (LPS10) and four isolates of other bacteria which grew well on nutrient agar, with good inhibitory activity against other pathogenic and non-pathogenic microorganisms (Table 1). Except for *Pseudomonas aeruginosa* LMG8029, the LPS10 lactic acid bacterium inhibited all indicator microorganisms used. The BPS4; BPS20; BPS33 isolates inhibited *Klebsiellaoxytoca* and *Escherichia coli*; *Listeria monocytogenes* and *Escherichia coli; Bacillus subtilis, Escherichia coli* and *Lactococcuslactis* respectively (Table 1). Consequently the producers of inhibitory activity could be used as ideal probiotics after pickles ingestion with neutralization of bacterial pathogens in human gut.

Studies were concentrated to check the growth and medium acidification by the experimental bacterial isolates (Table 2). The lactic acid bacterium LPS10 showed the highest values of growth and medium acidification; reaching $3.6x10^6$ CFU/ml and final pH of about 3.8 respectively after 24h; reaching $8.3x10^8$ CFU/ml and final pH 3.5 after 48 respectively. Its growth and medium acidification didn't increase by further incubation. Other bacterial isolates (BPS4, BPS20 and BPS33) showed moderate growth values reaching three log cycles within 3 days; they decreased final pH from 6.8 to 5.2 within 3 days. It was concluded that the LPS10 isolate could be used as starter and probiotic bacterium as it was the better isolate in growth and medium acidification; however the bacterial isolates BPS4, BPS20 and BPS33 could be used as biocontrol strains during pickles fermentation.

Cultural and biochemical characteristics of the lactic acid bacterium LPS10 is given in Table 3. It was Gram positive rods, non-spore former and produced DL-isomers of lactic acid. Except for raffinose, rhamnose, inositol, xylose and glycerol, all other sugars used were fermented.Its pH-growth range; temperature growth range was 2.0-8.0; 15°C-44°C respectively. This showed that the LPS10 strain could be used as starter culture for pickles fermentation at wide range of both pH and temperature. It showed NaCl tolerance up to 11%. It showed also some interested properties which are useful for starter and probiotic capabilities. It showed positive results regarding to Voges-proskauer test, acetoin production, citrate utilization, oxalate hydrolysis and positive activities of amylase, acid phosphatase, esterase, glucosidase, β-galactosidase, protease but showed negative results in view of other tests given in Table 3. Following the criteria reported by **Krieg and Holt (1984)** and surveying Bergey's Mannual of Systematic Bacteriology **(Holt *et al*., 1999)**; the LPS10 isolate could be identified as belonging to *Lactobacillus plantarum* and designated *L. plantarum* LPS10.

The cultural and biochemical characteristics of bacterial isolates BPS4, BPS20 and BPS33 are given in Table 4. All of them were catalase positive, Gram positive rods, spore-formers; shwoed positive results regarding gelatin hydrolysis, casein hydrolysis, starch decomposition, Vogesproskauer test, lysine decarboxylase, citrate utilization and fermentation of glucose, fructose, lactose and galactose; but showed negative results regarding urease test, H_2S production, indole production and arabinose utilization. They showed temperature growth range of about 14-45°C; 20-50°C; 25-40°C and showed a pH growth range of about 3.5-6.9; 4.0-8.0; 5.0-10.0 for the bacterial isolates BPS4; BPS20; BPS33 respectively. NaCl tolerance was upto 4%, >7%; 4% for these isolates respectively. Except for glucose, fructose, galactose and lactose which were fermented by these bacterial isolates and arabinose which was not assimilated by the three bacterial isolates, other sugars tested showed variable results (Table 4). By surveying, literature and following Bergey'sMannual of Systematic Bacteriology **(Krieg and Holt, 1984)**. The bacterial isolates BPS4; BPS20; BPS33 were idnetfiied as strains belonging to *Bacillus acidicola; Bacillus amyloliquefaciens; Bacillus mycoides* respectively and designated *B. acidicola*BPS4; *B. amyloliquefaciens* BPS20; *B. mycoides*BPS33 respectively. These bacterial strains could be considered as industrial isolates and could be used as probiotic and biocontrol agents since they inhibited other bacterial pathogens, hydrolysed gelatin, casein and starch and produced acetoin and utilized lactose and grew at 4-7% NaCl.

Sicne*L. plantarum* LPS10 showed interested qualified characteristics such as inhibition of many food-borne pathogens, medium acidification, fast growth, production of acetoin and production of enzymes which are good criteria for this strain to be starter, probiotic and protective culture with making safe pickles; and because other *Bacillus* spp. identified inhibited other pathogenic bacteria and produced proteases, amylases and lactase enzyme which are certain qualifications for these *Bacillus* spp. to be used as biocontroil agents during pickles making with good probiotic capabilities after pickles ingestion. Studies were further concentrated to confirm their identification by 16S rRNA cataloging analysis. DNA samples were extracted from these bacteria and

primers for 16Sgene given in Material and Methods were added to these DNA samples and then PCR was carried out. The PCR products of the amplified 16SrRNA gene were electrophoresed via agarose gel. The results given in Figure 1 showed that the 16S rRNA gene of the bacteria under identification studies was amplified successfully as clear bands were cut from agarose gel by gene clean kits and were sequenced and 16S rRNA sequences are given in Figure 2. These sequences of isolates LPS10; BPS4; BPS20; BPS33 were sent to Gene Bank under the accession numbers KF656725; KF656721; KF65624; KF656723 respectively. The Basic Local Alignment Search Tool Programme was used to obtain the similarity of the above sequences with the stored ones in data base. As given in Figure 3, the cluster analysis showed a similarity of the above isolates to *L. plantarum; B. acidicola; B. amyloliquiaciens; B. myoides* respectively. Consequently, these strains were kept in our culture collection for further studies.

Table 1: Antibacterial activity of some bacteria isolated from pickles against sensitive microorganisms.

Sensitive organism	Source and code	Diameter of inhibition zone (mm) obtained by the producer strains			
		LPS10	BPS4	BPS20	BPS33
Bacillu cereus	ATCC 14579	17	-ve	-ve	-ve
Bacillus subtilis	Our strain collection	11	-ve	-ve	25
Streptococcus pyogenes	Our strain collection	18	-ve	-ve	-ve
Staphylococus aureus	DMS 1104	20	-ve	-ve	-ve
Listeria monocytogenes	LMG 10410	26	-ve	18	-ve
Salmonella typhi	LMG 10395	10	-ve	-ve	-ve
Klebsiellaoxytoca	LMG 3055	15	20	-ve	-ve
Pseudomonas aeruginosa	LMG 8029	0	-ve	-ve	-ve
Escherichia coli	LMG 3223	10	20	15	25
Candida albicans	Our strain collection	16	-ve	-ve	-ve
Lactobacillus plantarum	LMG 8155	25	-ve	-ve	-ve
Lactobacillus sake	FRC 706	20	-ve	-ve	-ve
Lactococuslactis	ATCC 11454	19	-ve	-ve	-ve

LMG: Laboratory VoorMirobiologie, Gent Culture Collection, Universiteit Ghent, Belgium.
DMS: Deutsche "Sammtug Von Mikroorganisemen and Zellkulturen GmbH; Braunschweig, Germany.
ATCC: American Type Culture Collection, Rockville, Maryland, USA, IP, Institute, Pasteur Paris, France.
FRC: Federal Research Center for Nutrition, Institute for Hygiene and Toxicology, Engesserstrasse 20, 76131, Karlsruhe, Germany.

Table 2: Growth (CFU/ml) and medium acidification (final pH) obtained by the experimental bacterial isolates.

Time (h)	Bacterial isolate	Growth (CFU/ml)	Medium acidification (final pH)
0	LPS10	2×10^4	6.5
	BPS4	2×10^4	6.8
	BPS20	2×10^4	6.8
	BPS33	2×10^4	6.8
12	LPS10	7.0×10^4	4.6
	BPS4	3.2×10^4	6.6
	BPS20	8.0×10^4	6.4
	BPS33	6.0×10^4	6.6
24	LPS10	3.6×10^6	3.8
	BPS4	7.1×10^5	6.0
	BPS20	1.2×10^5	6.01
	BPS33	6.0×10^5	6.0
48	LPS10	8.3×10^8	3.5
	BPS4	6.0×10^6	5.6
	BPS20	8.3×10^6	5.5
	BPS33	7.2×10^6	5.3
72	LPS10	7.2×10^8	3.5
	BPS4	3.3×10^7	5.01
	BPS20	2.0×10^7	5.0
	BPS33	7.5×10^7	5.1

Table 3: Cultural and biochemical characteristics of the lactic acid bacterium LPS10 isolated fro pickles.

Tests and characteristics	Results
- Cell morphology	Rod-shaped
- Gram staining	+
- Spore formation	-
- Production of D and L-isomers of lactic acid	+
- Carbohydate fermentation profile	
Amygdalin	+
L-Arabinose	+
D-Cellobiose	+
Esculin	+
D-Fructose	+
D-Galactose	+
D-Lactose	+
Maltose	+
D-Mannose	+
Melibiose	+
Rafinose	-
Rhamnose	-
Ribose	+
Sucrose	+
Inositol	-
Trehaloe	+
Xylose	-
Sorbose	+
Glycerol	-
Salicin	-
- Production of ammonia from oginine	-
- Voges-prokauer test	+
- Catalase test	-
- Acetoin production	+
- Citrate utilization	+
- Hippurate hydrolysis	-
- Oxalate hydrolysis	+
- NaCl tolerance	Up to 1%
- Temperature growth range	15-44°C
- pH growth range	2.0-8.0
- Amylase activity	+
- Protease activity	+
- β-Glucosidase activity	+
- β-Galactosidase activity	+
- α-Glucosidase activity	+
- Glutamate decarboixylase	+
- Pyrrolidonyl-orylamidase	-
- Lucinarylamidase	-
- Acid phosphatase	+

+ Positive result
- Negative results

Table 4: Biological and biochemical characteristics of Gram positive and catalase positive bacteria obtained from pickles of Egypt.

Characteristics	Bacterial organism		
	BPS4	BPS20	BPS33
- Cell morphology	Rod-shaped	Rod-shaped	Rod-shaped
- Spore formation	+	+	+
- Gram staining	+	+	+
- Catalase test	+	+	+
- Urease test	-	-	-
- Oxidase test	-	-	-
- Reduction of nitrate to nitrite	-	+	+
- Temperature growth range (°C)	15-45	20-50	25-40.0
- pH growth range	3.5-6.9	4.0-8.0	5.0-10.0
- H_2S production	-	-	-
- Hydrolysis of			
• Casein	+	+	+
• Gelatin	+	+	+
• Starch	+	+	+
- Vogesproskauer test	+	+	+
- Indole production	-	-	-
- Lysine decarboylase	+	+	+
- Asimilation of:			
• Citrate	+	+	+
•Propiomate	-	-	-
- Growth in NaCl at			
• 2%	+	+	+
• 4%	+	+	+
• 7%	-	+	-
- Carbohydrate utilization			
• D-Glucose	+	+	+
• D-Mannitol	+	+	-
• D-Xylose	+	+	+
• L-Sorbose	-	-	+
• D-Sorbitol	-	-	+
• D-Lactose	+	+	+
• L-Rhamnose	-	-	+
• D-Cellolose	+	+	+
• D-Ribose	+	+	+
• D-Mannose	+	-	-
• D-Fructose	+	+	+
• L-Arabinose	-	-	-
• D-Glactose	+	+	+

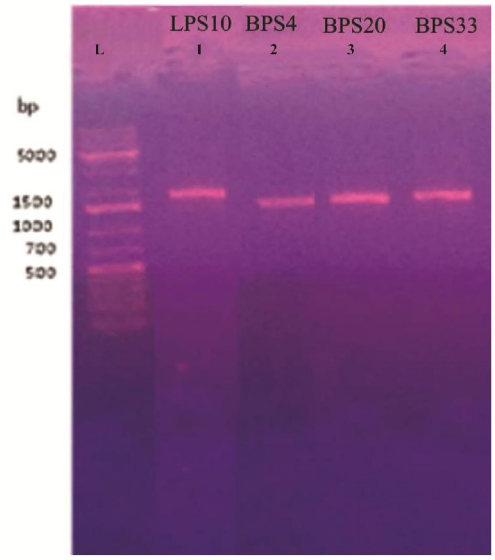


Figure 1: Agarose gel electrophoresis of PCR products including the amplified 16S rRNA genes of certain bacterial isolates. Lanes 1,2,3,4 refer to the the BPS4, BPS20, BPS33, LPS10 respectively. L ; DNA marker of known molecular size by base pair

CGTGGGAAACCTGCCCAGAAGCGGGGGATAACACCTGGAAACAGATGCTAATACCGCATA
ACAACTTGGACCGCATGGTCCGAGTTTGAAAGATGGCTTCGGCTATCACTTTTGGATGGT
CCCGCGGCGTATTAGCTAGATGGTGGGGTAACGGCTCACCATGGCAATGATACGTAGCCG
ACCTGAGAGGGTAATCGGCCACATTGGGACTGAGACACGGCCCAAACTCCTACGGGAGGC
AGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAACGCCGCGTGAGTGAA
GAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAGAACATATCTGAGAGTAACTGTT
CAGGTATTGACGGTATTTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTA
ATACGTAGGTGGCAAGCGTTGTCCGGATTTATTGGGCGTAAAGCGAGCGCAGGCGGTTTT
TTAAGTCTGATGTGAAAGCCTTCGGCTCAACCGAAGAAGTGCATCGGAAACTGGGAAACT
TGAGTGCAGAAGAGGACAGTGGAACTCCATGTGTAGCGGTGAAATGCGTAGATATATGGA
AGAACACCAGTGGCGAAGGCGGCTGTCTGGTCTGTAACTGACGCTGAGGCTCGAAAGTAT
GGGTAGCAAACAGGATTAGATACCCTGGTAGTCCATACCGTAAACGATGAATGCTAAGTG
TTGGAGGGTTTCCGCCCTTCAGTGCTGCAGCTAACGCATTAAGCATTCCGCCTGGGGAGT
ACGGCCGCAAGGCTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATG
TGGTTTAATTCGAAGCTACGCGAAGAACCTTACCAGGTCTTGACATACTATGCAAATCTA
AGAGATTAGACGTTCCCTTCGGGGACATGGATACAGGTGGTGCATGGTTGTCGTCAGCTC
GTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTATTATCAGTTGCCAG
CATTAAGTTGGGCACTCTGGTGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGAC
GTCAAATCATCATGCCCCTTATGAC
(A) (LPS10 isolate)

GACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACTTCTTCCTCCGCATGGGG
GRATATTGAAAGATGGCTTCGGCTATCACTTACAGATGGACCCGCGGCGCATTAGCTAGT
TGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCC
ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGC
AATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAA
ACTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGTACCTTGACGGTACCTA
ACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGT
TGTCCGGAATTATTGGGCGTAAAGCGCGCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGC
CCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAGAG
TGGAATTCCACGTGTAGCGGTGAAATGCGTAGATATGTGGAGGAACACCAGTGGCGAAGG
CGACTCTCTGGTCTGTAACTGACGCTGAGGCGCGAAAGCGTGGGGAGCGAACAGGATTAG
ATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTT
TAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAAC
TCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAAC
GCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTAGAGATAGGGCTTTCCCCT
TCGGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTTG
GGTTAAGTCCCGCAACGAGCGCAACCCTTGACCTTAGTTGCCAGCATTCAGTTGGGCACT
CTAGGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCATGCC
CCTTATGACCTGGGCTACACACGTGCTACAATGGATGGTACAAAGGGCTGCAAGACCGCG
AGGTTTAGCCAATCCCATAAAACCATTCTCAGTTCGGATTGTAGGCTGC
(B) (BPS4 isolate)

Figure 2: Nucleotide sequences of 16S rRNA gene of the purified PCR products of isolates (A), LPS10; (B) BPS4; (C), BPS20; (D), BPS33.

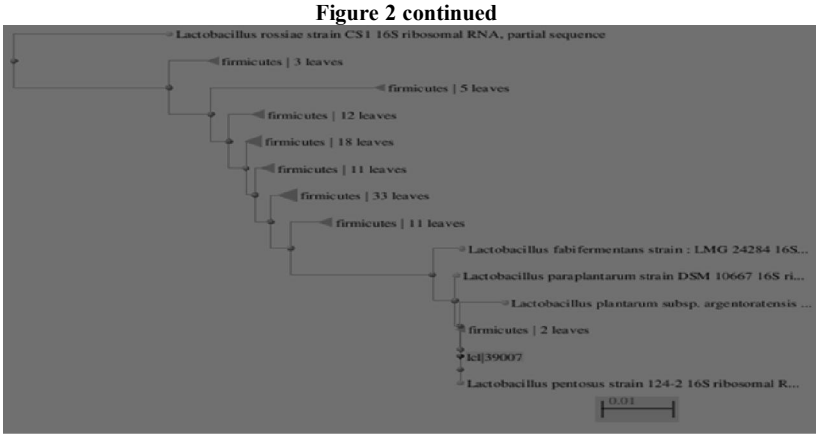
TTTGAACCGCATGGTTCAGACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCG
CGGCGCATTAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCT
GAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA
GTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAG
GTTTTCGGATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCCGTTCAAATAGGGCGGC
ACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATA
CGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTA
AGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGA
GTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAGGA
ACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGG
GAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTA
GGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACG
GTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGG
TTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAATCCTAGA
GATAGGACGTCCCCTTCGGGGGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGT
GTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCA
TTCAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGT
CAAATCATCATGCCCCTTATGACCTGGGCTACACACGTGCTACA

(BPS 20 isolate) (C)

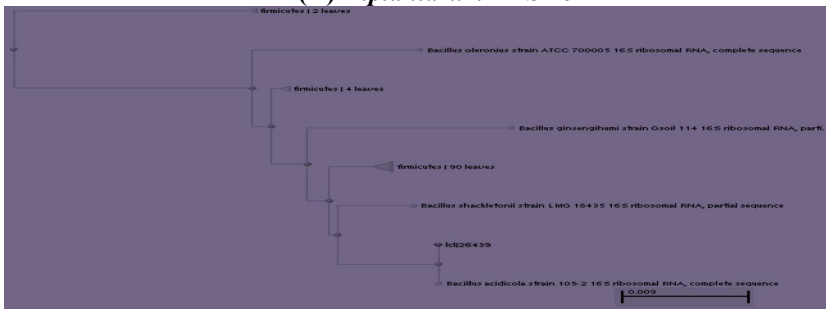
ATTTTGAACTGCATAGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCATTAGCT
AGTTGGTGAGGTAACGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGAC
TGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGC
AACGCCGCGTGAGTGATGAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAACAAGTGCTAGTTGAATA
AGCTGGCACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGT
GGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGCGCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCCACG
GCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGC
GGTGAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGC
GCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTA
GAGGGTTTCCGCCCTTTAGTGCTGAAGTTAACGCATTAAGCACTCCGCCTGGGGGTACGGCCGCAAGGCTGAAA
CTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTT
ACCAGGTCTTGACATCCTCTGAAAACTCTAGAGATAGGCTTCTCCTTCGGGAGCAGAGTGACAGGTGGTGCATG
GTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATC
ATTAAGTTGGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCA

(D) (BPS 33 isolate)

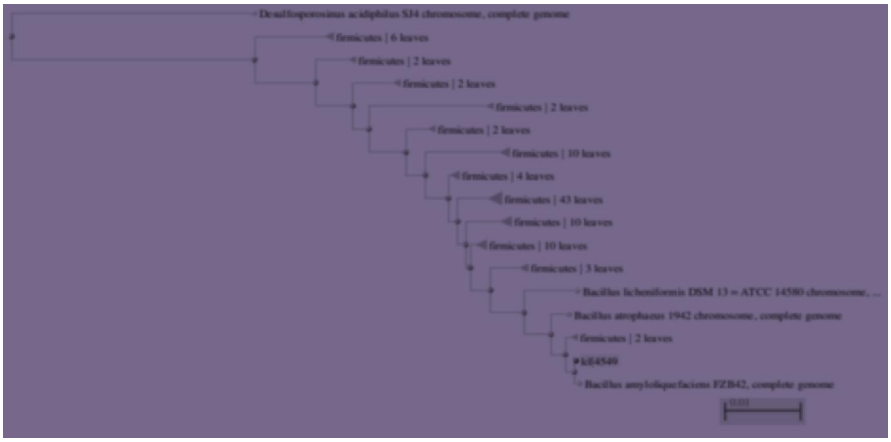
Figure 2 continued



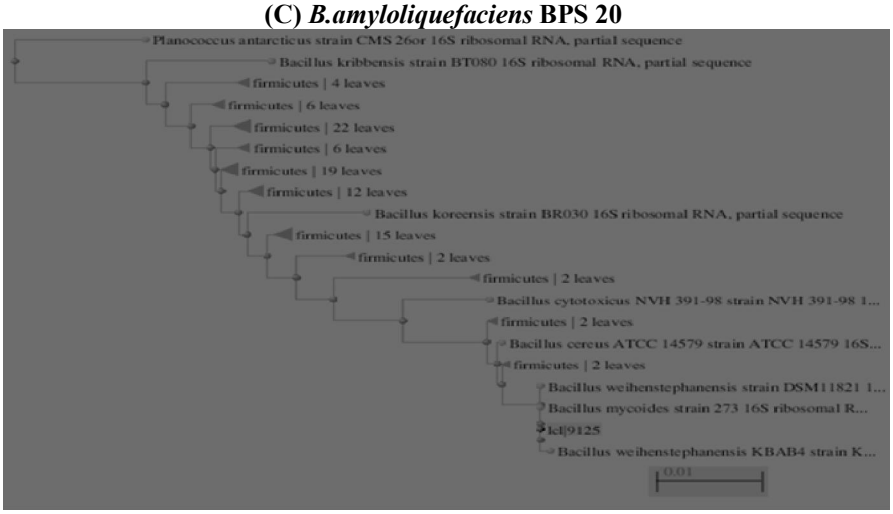
(A) *L.plantarum* LPS 10



(B) *B.acidicola* BPS4



(C) *B.amyloliquefaciens* BPS 20



(D) *B.mycoides* BPS 33

Figure 3: Phylogenetic tree showing cluster analysis and dendrogram of (A), *L. plantarum*LPS10; (B), *B. acidicola*BPS4; (C), *B. amyloliquifaciens*BPS20; (D), *B. mycoides*BPS33.

4.Discussion

The recent technology focuses on making hazardous pickles. This technology uses the brine process in pickles fermentation, in which lactic acid bacteria are used to ferment vegetables or fruits in brine conditions (≤12% NaCl) where pathogenic bacteria can not grow **(Ghonaimy, 1998)**. Currently certain interested characteristics should be possessed by the starter lactic acid bacteria such as growth, medium acidification, production of inhibitory substances and enzyme production **(Michaylova, 2012; Enan *et al*., 2013c)**. Consequently, the present work was an endeavour to select, starter, protective and probiotic cultures to make pickled vegetables or fruits with extended shelf life.

One fast growing organism, grew well on MRS agar **(DeMan *et al*., 1960)**. It was isolated from pickled pepper and was identified as one strain of *Lactobacullusplantarum* **(Krieg and Holt, 1984)** and designated *L. plantarum* LPS10. Many previous studies showed that *L. plantarum* was isolated from pickles **(Islami, 2009)**. When both salt concentration and temperature are low, *Leuconostocmesenteroides* dominates, producing a mix of acids and alcohols. At higher temperatures like Egyptian conditions in summer (≤40°C) *Lactobacillus plantarum* dominants and produces primarily lactic acid **(Islami, 2009)**. *L. plantarum*LPS10 showed interested properties such as fast growth and medium acidification which enable this organism to be ideal starter culture for pickles fermentation **(Hoover, 2000)**. It inhibited many food-borne pathogens and, therefore, it could be considered protective culture. The inhibitory activity of *Lactobacillusplantarum* could be due to lactic acid, diacetyl, CO_2, acetaldehyde or H_2O_2 **(Powell *et al*., 2007; Enan *et al*., 2013a,b; Enan *et al*., 2014)**. The LPS10 strain showed wide pH (2.0-8.0) and temperature (15-44°C) growth ranges and grew in MRS broth supplemented with up to 11% NaCl. This organism could be used, therefore, in fermentation of pickles at wide ranges of pH and temperature and at halophytic conditions. Brine processes with high salt

concentration are beneficial, because salts decrease the free (unbound) water allowing inhibition of pathogenic bacteria; and diffusion of sugars outside pickles to be easily fermented by lactic acid bacteria (**Dalmasso, 200; Enan, 2006b)**. The LPS10 organism produced protease, β-glycosidase, amylase and esterase and consequently considered ideal probiotic and these enzymatic activities are qualified properties for any organism to be used as probiotic bacterium **(Hebert *et al*., 2000)**. Oxalate, citrate were utilized by the LPS10 organism and acetoin was also produced by the LPS10 organim and this can give flavors and aroma for the pickled vegetable or fruits **(Simova *et al*., 2008)**. The LPS10 lactic acid bacterium could be used as starter, protective and probiotic bacterium.

Three *Bacillus* isolates were isolated herein from pickled vegetables of Egypt. They were characterized **(Enan *et al*., 2012; Abdel-Shafi *et al*., 2013)** and identified according to Bergey' Mannual of Systematic Bacteriology **(Krig and Holt, 1984)** as strains of *B. acidicola, B. amyloliquefaciens* and *B. mycoides* and designated *B. aidicola* BPS4, *B. amyloliqufacien* BPS20 and *B. mycoides* BPS33, respectively. Similar *Bacillus* spp. were isolated previously from pickled vegetables of Egypt **(Ghonaimy, 1998)**. It was found that the identification processes based on cultural and biochemical characteristics which can give ambigous and speculative results **(Kelly *et al*., 2010)**. Also *Bacillus amyloliquefaciens* is quite similar to *Bacillus subtilis* in biochemical properties **(Priest *et al*., 1987)**; and also *Bacillus acidicola* is similar in biochemical properties to *Bacillus aleronius*. Consquently, molecular identification by 16S rRNA cataloging analysis of the experimental isolates was mandatory **(Garde, 1999)**. The 16S rRNA cataloging analysis and Basic Local Alignment Search Tool Programme **(Altschul *et al*., 1999; Turner *et al*., 1999)** confirmed the identification and approved on a successful identification of the strains:*L. plantarum* LPS10, *B. acidicola* BPS4, *B. amyloliquifaciens* BPS20 and *B. mycoides* BPS33.

The three *Bacillus* stains: BPS4; BPS20 and BPS33 are non-toxic **(Goodwin, 1994; Stratford *et al*., 2013)** and appeared herein to grow at wide ranges of pH (3.5-10.0) and temperature (15-50°C) and at 4-7% NaCl and such properties enable these organisms to grow well in pickles. They inhibited other pathogenic bacteria and this enable these bacteria to be biocontrol agents during pickles fermentation. It was reported that *B. amyloliquifaciens* produce subtilin antibiotic **(Priest *et al*., 1987)**; *B. mycoides*inhibited the growth of harmful bacteria and fungi **(Stratford *et al*., 2013);***Bacilluacidicola* is acidophilic species and decreases pH of the medium to the acidic side preventing other pathogenic bacteria to grow **(Albert *et al*., 2005)**. The proliferation of pathogenic bacteria like *E. coli*O157 in pickles at acidic conditions **(Lee, 2004)** make interest to add *Bacillus* spp. As biocontrolagents such as *B. amyloliquefaciens* BPS20, *B. acidicola*BPS4 and *B. mycoides* BPS33 to pickles to increase their shelf life. These three *Bacillus* spp. identified herein produced acetoin which can give flavor to pickles **(Hansen, 2002)** and decomposed citrate, casein, gelatin and starch and these activities are qualified properties for these strains to be probiotic bacteria **(Hebert *et al*., 2000)** thoroughly with biocontrol agents.

Further work will be necessary for characterization of the biocontrol agents, quantitative study of the starter and probiotic capabilities.

Conclusion

L. plantarum LPS10, *B. acidicola* BPS4, *B. amyloliquifaciens* BPS20 and *B. mycoides* BPS33 were isolated from Egyptian pickles, characterized and identified by biochemical and molecular methods. The LPS10 strain inhibited many food-borne pathogens, produced good values of growth and medium acidification, grew at 11% NaCl concentration and produced many enzymes. Consequently it could be used as probiotic, starter and protective culture during pickles fermentation. The BPS4, BPS20 and BPS33 inhibited few pathogens, grew up to 4-7% NaCl concentrations, decomposed citrate, gelation, casein and starch. Hence, they could be used as probiotic and biocontrol agents in pickles.

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