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Characterization of two strains of lactic acid bacteria with promising starter and probiotic capabilities.

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ABSTRACT

This work focused on selection of lactic acid bacteria(LAB) strains to be used as starter and protective cultures for food fermentations.Two LAB isolates only namely PO24 ; CK28 isolated from pickled olives;Kareesh cheese inhibited many food-borne pathogens and were identified by both biochemical and molecular methods as belonging to *Lactobacillus plantarum* and designated *L. plantarum* PO24 ; *L.plantarum* CK28 respectively. The PO24 and CK28 strains showed NaCl tolerance up to 13% ; acidified growth medium up to pH 3.1 within 24h ,grew at wide PH (2.0-8.0) and temperature ranges (15-44^{O}C) and produced protease ,amylase ,β-galactosidase , acid phosphatase and decarboxylase and ,therefore, could be used as probiotic cultures and starter bacteria for food fermentations with protective capabilities.

Keywords: Lactic acid bacteria (LAB) ,Probiotics ,Starter cultures ,Identification.

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INTRODUCTION

Lactic acid bacteria (LAB) are a group of Gram-positive bacteria that are widely used as starter cultures in the manufacture of fermented food products including fermented meat,vegetables, fruits and dairy products and also in intestinal,genital and respiratory tracts of men and animal [1]. They give a characteristic flavor to fermented foods and increase their shelf life [2,3]. They possessed certain interest, due to their use as probiotic bacteria since they improve the nutritional benefits for health as they have been reported to antagonize pathogenic bacteria in human gut by their antimicrobial metabolites such as bacteriocins, diacetyl, hydrogen peroxide, acetaldehyde, ethanol, organic acid and carbon dioxide [4,5] They were reported also to produce enzymes such as cellulase, protease and hydroxylase which are involved in conversion of precipitated bile salts and cholesterol derivatives to soluble compounds to be easily excreted by kidney .Therefore,they are an ideal probiotics [6,7,8] .

LAB were showed to consume sugars from foods, giving lactic acid and other organic acids and decrease pH of the medium reaching pH 2.5 within 24h and all such properties enable these organisms to be starter cultures for food fermentations [9,10].

The present work was undertaken to select ,characterise and identify some LAB isolates which could be used as a probiotic ,starter and protective cultures for food fermentations.

MATERIALS AND METHODS

Food samples and bacterial strains:

Different types of pickled vegetables ,fermented dairy products including yoghurt and kareesh cheese,hony bee,sausage and fermented fish samples were transported in sterile copped bottles to the laboratory , LAB were isolated from these foods onto specific MRS medium [11].Different pathogenic bacteria were used as an indicator cultures.The sensitive pathogenic bacteria and their sources are given in table 1

Bioassay for inhibitory activity produced by bacterial isolates:

The antibacterial spectrum of 33 LAB cultures were studied against different pathogenic bacteria by the agar disc diffusion assay [12,13] . A 1% v/v suspension of log phase cells of the LAB to be tested to produce inhibitory activity was inoculated and spread onto MRS agar plates and incubated for48h at 35^{o}C.Agar discs were made by 7mm sterile corckporer and transferred onto the surface of soft agar top layer seeded with lawns of the indicator organisms used.Plates were incubated for 4h at 4^{o}C to allow diffusion of the inhibitory substance(s).The plates were then incubated at 30^{o}C for 48h and were tested for appearance of inhibition zones around agar discs of LAB studied.

Temperature and pH growth range :

10 ml aliquots of MRS broth were inoculated by 1% v/v of suspension of log phase cells of LAB tested .Each inoculated tube was incubated at certain temperature (15-44^{o}C). Growth was tested (OD 600 nm) after 24h and 48h of incubation . MRS medium in other different test tubes were adjusted at different pH values (2.0-12.0), inoculated by the LAB tested (1% v/v)and were incubated at the optimum temperature appeared for each LAB for 48h.Growth (OD 600nm)was tested after 24h and 48h of incubation [14] **.**

Growth and medium acidification :

The inhibitory activity producing LAB bacteria viz. CK28,PO24 were tested for their growth and medium acidification according to [15] **.**MRS broths media were inoculated by 2x10^{4} CFU/mL final concentration ,incubated at 37^{o}C as this temperature was appeared preliminary to be the optimum growth temperature of the LAB bacterial isolates tested . Incubation continued 2 days and whilst samples were removed for analysis for growth (CFU/mL) and final pH value by pH meter (New Brunswek Scientific Co.) .

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Phenotypic and biochemical characterisitics and some probiotic capabilities of LAB:

Two isolates (PO24, CK28) which showed the highest growth value, medium acidification and inhibited some pathogenic bacteria were characterized. The carbohydrate fermentation profiles were examined using API 20 Strep tests following the manufacture's instructions (Biomerieux,Montalieu-Vereiue, 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 [16].Growth of LAB isolates in different NaCl concentrations was tested in MRS broth [5,14]. Other biochemical tests regarding catalase test, citrate utilization, hippurate and oxalate hydrolysis were in addition to the ones obtained by API 20 ,further assayed [17,18].

Molecular identification of bacterial isolates:

The PO24, CK28 LAB isolates were further identified via 16S rRNA cataloging analysis .Total DNA samples were extracted from exponentially growing bacterial cells [19] The 16S rRNA gene was amplified for each isolate by PCR using the foreward primer : 5'-AGAGTTGATCCT-GGCTCAG-3' and also using the reverse primer 3'-TTCAGCATTGTTCC-ATTG-5' [20,21]. The PCR products were subjected to agarose gel electrophoresis [19] 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 . Sequances analysis and their comparison to deposited data in Gene Bank was made using Basic Local Alignment Search Tool (BLAST) programme at http://ncbi.nlm.gov/blast [22].

RESULTS

Thirty three LAB isolates were obtained as all food samples tested showed LAB count . The obtained Thirty Three bacterial cultures were bioassayed for their ability to Produce antimicrobial substances . Agar discs of LAB cultures grown on specific MRS agar were made by sterile test tubes and were transferred by sterile inoculation needles on BHI agar previously seeded with the Indicator bacteria. Results were taken after Incubation at 37°C for 48h . Only two LAB (PO24 and CK28) Isolates inhibited indicator pathogenic bacteria used . LAB isolate PO24 inhibited three Indicator pathogenic bacteria used and diameter of inhibition zones were 25mm ,23mm ,25mm against *S.aureus ,St.pyogenes , L . monocytogenes* respectively. Also the LAB isolate CK28 inhibited the all indicator strains used (Table 1) .Fast growth and medium acidification are two important properties for any LAB organism to be starter culture for food fermentation. Therefore, the two experimental LAB isolates (PO24, CK28) were grown in MRS broths adjusted initially at PH 6.8 and at inocula sizes of about $2x10^4$CFU/mL for both isolates. After incubation for 48h, results were taken and are given in Table 2 .Growth values of isolates PO24 ;CK28 were $2.1x10^9$CFU/mL ; $7.3x10^8$CFU/mL respectively. Final pH values were 3.6 for the former and 3.1 for the latter (Table 2).

Because both PO24 and CK28 isolates inhibited many food –borne pathogens and showed fast growth and medium acidification , these isolates were subjected to identification using biochemical and molecular methods. The biochemical characteristics of both PO24 and CK28 are given in Table 3. Both LAB isolates (PO24 and CK28) were Gram positive rods and were catalase negative. They were non- spore formers and produced DL- isomers of lactic acid. Except for raffinose, rhamnose, inositol, salicin, xylose and glycerol which were not utilized by both LAB isolates, all other carbothydrates used were homofermented. They grew in MRS broth adjusted initially at pH range 2.0-8.0 and grew at temperatures ranges from 15°C to 40°C. They showed tolerance to NaCl up to 13%. Additionally both PO24 and CK28 showed positive results regarding Voges proskauer test, acetoin production, citrate utilization, oxalate hydrolysis, and positive activity of amylase, acid phosphatase, esterase, protease, β & α- glucosidase, β- galactosidase, protease; but showed negative results regarding production of ammonia from arginine, hippurate hydrolysis, pyrolidonyl- arylamidase and lucin arylamidase .

Following the criteria reported by [23] and surveying [24] ,both LAB isolates PO24, CK28 could be identified as a strains belonging to *L. plantarum* and designated *L . plantarum* PO24 ; *L. plantarum* CK28 respectively.

It was necessary to confirm the biochemical identification of *L. plantarum* PO24 and *L. plantarum* CK28. Hence, molecular identification of bacterial isolates was carried out by 16S rRNA cataloging analysis.

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DNA samples of the experimental bacterial isolates (PO24, CK28) were isolated using Maniatis protocol as given in Material and Methods. The extracted DNA samples were purified from RNA residues, The purified chromosomal DNA samples were prepared for PCR technique ; they were mixed with 16S rRNA gene forward and reverse primers as given in Materials and Methods. PCR technique was carriedout successfully and the PCR products were electrophoresed via agarose gel as shown in Figure 1. The agarose gel elecrophoresis profiles of PCR products showed the amplified 16 S r RNA of both isolates PO24 and CK28 ; it was obvious that the 16 S r RNA gene of bacterial isolates was amplified successfully as sharp bands of DNA of about 1500 bp were showed obviously.

DNA sample assimilating the amplified 16S rRNA genes of bacterial isolates (PO24 and CK28) tested were cut from agarose gel using Gene Purification kit (Promega). These amplified DNA samples were sent to Brotech , knostans , Germany and were subjected to nucleotide sequences using ABI 3730XI DNA sequencer. Nucleotide sequences of 16S rRNA genes of the both bacterial isolates PO24 and CK28 are given in Figures 2a,b respectively. The nucleotide sequences appeared in figures 2a,b of both isolates PO24 and CK28 were sent to Gene Bank under the accession numbersPO3780202, CK3790201 with their sources (to be stored in Gene Bank) including pickles samples;kareesh cheese in Egypt respectively . Using the Basic Local Search Tool Programe , pairwise similarity of the 16 S rRNA gene(s) sequences employed herein with the ones stored in Gene Bank was done using the available data obtained from Gene Bank.

For clearance and for more explanations we used the all capabilities and tools in the Basic Local Search Tool Programme(BLAST). Consequently a cluster analysis was designed and gave a dendrogram analysis for each bacterium. The phylogenetic tree for *L . plantarum* PO24 and *L . plantarum* CK28 are given in figure 3a,b resperctively. It was shown that both PO24, CK28 isolates showed > 99% similarity to *L. plantarum* cluster and proved the success of their biochemical identification.

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

Sensetive organism	Source and code	Diameter of inhibition zone (mm) obtained by the producer strains	
		CK28	PO24
Bacillus cereus	**ATCC 14579**	**22**	**0**
Streptococcus pyogenes	**Our strain collection**	**28**	**25**
Staphylococcus aureus	**DMS 1104**	**15**	**23**
Listeria monocytogenes	**LMG 10410**	**14**	**25**
Escherichia coli	**LMG 3223**	**25**	**0**

LMG: Laboratory VoorMicrobiology, Gent Culture Collection, University Ghent, Belgium.
DMS: Deutsche "Sammtug Von Mikroorganissemen and Zellkulturen GmbH;Braunschweig,Germany.
ATCC: American Type Culture Collection, Rockville, Maryland, USA, IP, Institute,
Pasteur Paris, France

Table 2:Growth (CFU/mL)and medium acidification (final pH) obtained by the experimental bacteria isolates after 48h of incubation.

Bacterial isolate	Growth (CFU/ml)	Medium acidification(final PH)
PO24	$2.1X10^9$	3.6
CK28	$7.3X10^8$	3.1

Table3:Cultural and biochemical characteristics of the LAB PO24, CK28 isolated from food samples.

-Test and characterisitics	Results	
	PO24	CK28
-Cell morphology	Rod shaped	Rod shaped
-Gram staining	+	+

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-Spore formation	-	-
-Production of D and L-isomers of lactic acid	+	+
-carbohydrate fermentation profile		
Amygdalin	+	+
L-Arabinose	+	+
D-Cellobiose	+	+
Esculine	+	+
D-Fructose	+	+
D-Galactose	+	+
D-Lactose	+	+
Maltose	+	+
D-Mannose	+	+
Melibiose	+	+
Rafinose	-	-
Rhamnose	-	-
Ribose	+	+
Sucrose	+	+
Inositol	-	-
Trehalose	+	+
Xylose	-	-
Sorbose	+	+
Glycerol	-	-
Salicin	-	-
-Production of ammonia from arginine	-	-
-Voges proskeur test	+	+
-Catalase test	-	-
-Acetoin production	+	+
-Citrate utilization	+	+
-Hippurate hydrolysis	-	-
-Oxalate hydrolysis	+	+
-NaCl tolerance	Up to 13%	Up to 13%
-Temperature growth range	15-44°C	15-44°C
-pH growth range	2.0-8.0	2.0-8.0
-Amylase activity	+	+
-Protease activity	+	+
-β-Glucosidase activity	+	+
-β-Galactosidase activity	+	+
-α-Glucosidase activity	+	+
-Glutamate decarboxylase	+	+
-Pyrrolidonyle-arylamidase	-	-
-Lucinarylamidase	-	-
-Acid phosphatase	+	+

+Positive result, - Negative result

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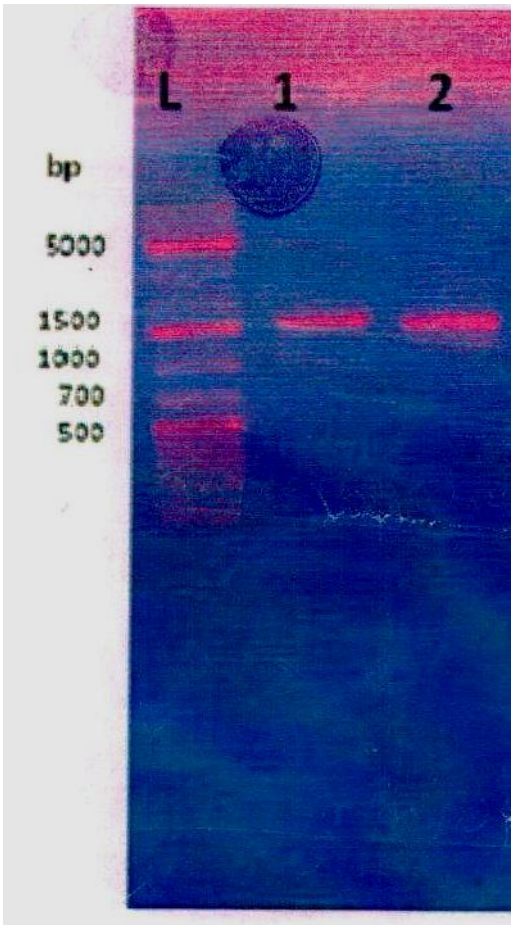

Figure1: Agarose gel electrophoresis of PCR products including the amplified 16S rRNA genes of certain bacterial isolates.

Lanes 1,2 refer to the PO24, CK28 respectively. L ;DNA marker of known molecular size by base pair.

GGCACGGACAATCATGGCGACTTACTGAACTGGTATCCTTACAGGAACGCCTGCCTGCAAAAACTCGGGAAGAAACTGCC
CGGCGCTGATCCACATGGAAACGATGCTAACTACCGCGTAGCAACTTGCACCCAGATCCAAATTGAAAATGGCTTTCAGA
TATTACTTATTGGAGGTTCGCACCTCGTTGTACCATCCGTTGGTACACGTGGTACCCAGGCATAAGGGGCATGATGATTGA
CGTCATCCCCACCTTCCTCCGGTTGTCACCCGCAGTCTCACCAGAGGCCCAACTTAATGCTGCCAACTGGACAATAAGGGT
TGCTGATGCGGGACAACCCACATCTCAAGACACGAGCTGACGACCCTGCACCACCTGATCCTGTCAAAGGGAACGTCTAA
TCTCTTCATATTTGCACTAGTATGTCAAGACCTGGTAAGGTTGCTTCGCGTAGCTTCTAATTAAACGGCATGCTCCACCGCT
TGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTATCCTTGCTGATGTACTCCCCAGGCGGAATGCTTAATGAAGTAGCTGCA
GAACTGAAGGGCGGAAACGCTCACAACACTGTAATTCCTCGTGTACGGATGGAATGCGGATATCTATCAAGATTGCTACT
GATACTTTCGACGTCTGCGTCAGTTACAGACCAGAAGCCGCCTTCGCACTGGTGTTCTTCCATATATCTACGCATTTCACCG
CTACACATGGAGTTACTGCCTCGTCTGCACTCAGAGTTTCCCAGTTTCCAATGCACATTCTTCGGTTGAGCCGAAGGTTTCA
CATCAGACTTAGAAAACCGCCTGCGCTGCTTTACGCCCATAAATCCGGACAACTTGTGCACCTACTTATAACGCGGCGCTG
CACGTAGGTAGCGGCTTTTCTGGTAAATACCAGAACCCAGTTACTTTAGATATGTTCTTAAAGTTTTCGAACTTGTAGCGTA
GCCTCAACCTTTTCGTGAGATGGCCCAGATCCGGACCTCGGCACGACCGACGAA

PO24 isolate

GGGAAAGATCGGGATTATAACCTAGTATCCTTAGCTTACTCCTGCCTGCTATTCTCGTGAGCCATTTTTCCCCTACTTCCCTC
TGACCTCATATGCTAATACCGCATAACAACTTGGACCGCATGGTCCGAGCTTGAAAGATGGCTTCAGCTATCTTTTGGATG
GTCCCGCGGCGTATTACTATGGTGGGGTAACGGCTCACCTGGCAATGATACGTACCGACCTGATAGGGTAATCCGCCACA
TTGGGACTGAACCGGCCCAAACTCCTACGGAGGCAGCAGTAGGGAATCTTCCACAATGGACGAAAGTCTGATGGAGCAA
CGCCGCGTGAGTGAAGAAGGGTTTCGGCTCGTAAAACTCTGTTGTTAAAGAAAACATATCTGACAGTAACTGTTCAGGTA

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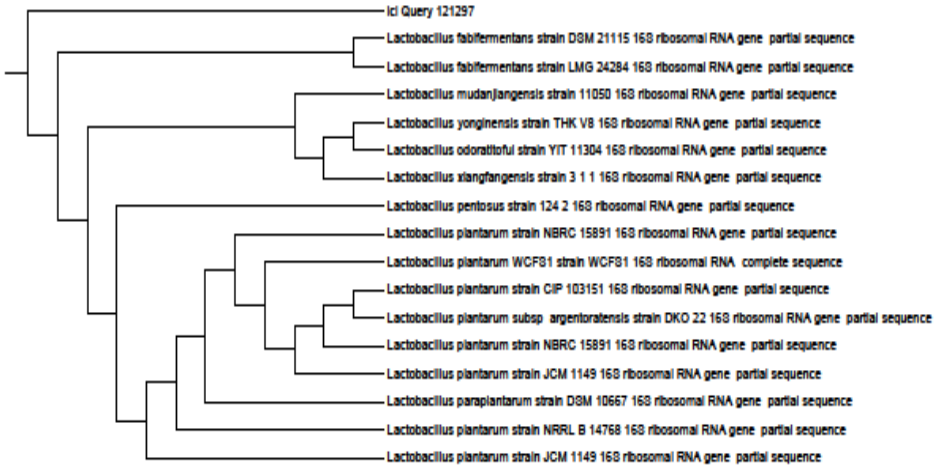
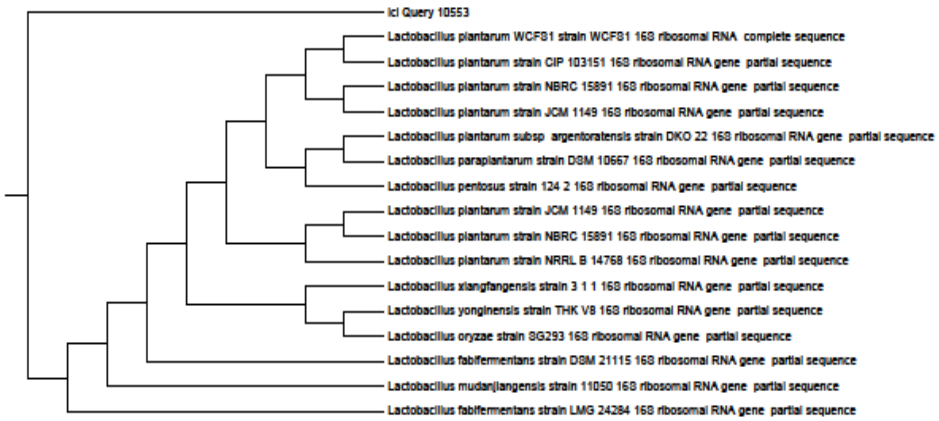
TTGACGGTATTTAACCAAAAAGCCACGGATAACTACTGCCATCAGCCGCGGTAATACTTAGTGGCAAGCGTTGTCCGGAT
TTATTGCCCGTAAAGCGAGCGCAGGCGGTTTTTTAAGTCTGATGTGAAAGCCTTCGGCTCGACCAAAGAAGTGCATCAAA
CTGGGAACTTGATGCTCAAGAGGACAGTGTCCATGTGTAGGGTGAAATGCGGATATCTAGCAAGACCCTGCAATGCGGT
CTGGCTGTCTGACGACGCTCGCGGCTGGGTCGATTAATCCCTGGTGCCCCGCCCCAGGAGGAGCTGGCTGCAGGTTCCGT
CAGTGGAGTACTCGGTGACGTAACTGAACCCCGCGCAACTCTTTGGCTAAGAAGCTTGGCTAATATACCCGCACTGATCC
GGGCTTTGGCACGGCATACGTCCCCTTACTCTCAGATTTGCTTGAGTTTTAAAGCGGAAA

CK28 isolate

Figure 2: Nucleotide sequence of 16SrRNA gene of the purified PCR products of isolates(a), PO24; (b), CK28.



Lactobacillus plantarum **WCFS1 strain WCFS1 (PO24)**



Lactobacillus plantarum **strain CIP 103151 (CK28)**

Figure 3: Phylogenetic tree showing cluster analysis and dendogram of (A), L.plantarum PO24; (B), L.plantarum CK28 .

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DISCUSSION

The recent technology focuses on selection of LAB cultures to be used as starter and protective cultures for food fermentations [14] this is to make hazardous food ; fermented by LAB such as pickles , cheese zabady and sausage in which acidic media are made by ability of LAB to grow and produce lactic acid . in this acidic media pathogenic bacteria can not grow [25] . consequently this study was endeavour to select starter and protective LAB to be probiotic cultures to make foods with extended shelf life.

Thirty three LAB cultures were isolated from fermented foods on MRS agar medium [11] ; a specific medium used for isolation of LAB . They bioassayed for inhibition of some food -borne pathogens . Two LAB isolates namely PO24 , CK28 isolated from pickles , kareesh cheese respectively were the more active organisms and inhibited some food-borne pathogens .The PO24 isolate inhibited *St . pyogene , S . aureus* and *L . monocytogenes* while the CK28 isolate inhibited the same pathogenic bacteria and both *B . cereus* and *E . coli* . It was reported previously that LAB cultures inhibit pathogenic bacteria by many metabolites such as lactic acid , H_2O_2 , diacetyl and or bacteroicin [26,27] . These two isolates (PO24, CK28) were assayed for medium acidification ; because acid production is the second property desired for any LAB culturesto be an ideal probiotics [14] ; certain other interested characteristic should be possessed by LAB cultures such as inhibition of inhibitory activity , growth and production of enzymes [28,29] . These two LAB isolates (PO24 & CK28) decreased pH of the medium , reaching 3.1- 3.6 within 24 h and this is interesting result for using of these two isolates as starter cultures for food fermentations with inhibition of food - borne pathogens. This is coupled with previous work in this respect [30,31]**.** Due to the inhibitory activity of both LAB isolates : PO24 and CK28 and based on their distinctive ability to acidify growth medium , they were subjected to biochemical identification using the possible characteristics reported by [23,24] . Both PO24 and CK28 were identified as two strains belonging to *Lactobacillus plantarum* and designated *L . plantraum* PO24 *, L . plantarum* CK28 respectively . It was reported that biochemical identification could give elusive identification [14,32] . Therefore , their identification were confirmed by 16S rRNA cataloging analysis [21] . The molecular fingerprinting of those two LAB strains by their rRNA cataloging analysis proved a correct biochemical identification [13,14,21,32]**.**

Many previos studies showed that *L . plantarum* was isolated from pickles [33]. When salt concentration in pickles are below 10 %, *Leuconostoc mesenteroides* dominates producing a mix of acids and alcohol in pickles At higher salt concentration and at higher temperatures (≤ 40 °C) , *L . plantarum* dominates producing primarily lactic acid [33,34] . *L . plantarum*_CK28 was isolated from kareesh cheese in this study . It was reported previously that *L. plantarum* was isolated from vegetable fermentations [35,36] isolate *L . plantarum* strain from sausage . *L . plantarum* was recently isolated from fermented dairy products.

The two *L . plantarum* strains (PO24 & CK28) showed interested properties such as inhibition of some food - borne pathogens and medium acidification and such two criteria enable those two organisms to be used as starter cultures for food fermentations [26,31] They also showed wide pH range (2.0 – 8.0) and wide temperature growth ranges (15 – 44 °C) and grew in MRS broth [11] supplemented up to 13% NaCl concentrations and , hence they could be used in fermentations of processed meat and making pickles at halophytic conditions [37,38] **.**

The PO24 and CK28 produced amylase , esterase , protease , β – glucosidase and decarboxylase , consequently they considered an ideal probiotic organisms as they can improve many gut conditions with balance of pathogenic microflora [38,39]. Oxalate and citrate were utilized by these two organisms (PO24 & CK28) and this can give flavor and aroma for the produced fermenated food [34,40].

CONCLUSION

Two LAB strains : *L. plantarum* PO24 and *L. plantarum* CK28 were isolated from food samples , characterized and identified in this study . Both of them showed promising probiotic capabilities . They could ,also, be used as starter and protective cultures as they acidified growth medium rapidly and inhibited many food –borne pathogens .

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