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Some bacteriological studies on bacteriocinogenic strains of lactic acid bacteria isolated from meat and meat products

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ABSTRACT

The present study was conducted on 250 random samples of meat and meat products (minced meat, kofta, beef burger and sausage, 50 for each) collected from small retails and different supermarkets at Kaliobia Governorate, Egypt for inspection of Lactobacillus (LAB) species and studying the bioactivities of them. The results revealed that 252 strains of LAB strains were recovered from 250 examined samples. *P. acidilactici* were the most isolated (102 = 40.5%) followed by *L. plantarum* (76=30.2%), *L. acidophilus* (38=15.1%); *L. brevis* (19=7.5%) and *P. pentosaceus* (17=6.7%). All of them produced bacteriocin and biosurfactant that inhibited the growth of tested pathogenic bacteria. In addition, most isolated strains had the ability for biofilm production which able to inhibit the biofilm formation of tested pathogenic strains. Moreover, the sequences obtained for 16S rRNA genes had accession numbers MK850930 and MK852180, MK852397 and MK852398, MK806485 and MK850564, MK871658 and MK871674, MK852683 and MK852691, and were 91% to 98% identical to the corresponding GenBank sequences.

1. INTRODUCTION

Lactic acid bacteria (LAB) produced bacteriocins are of particular interest to food industry, since these bacteria are GRAS (generally regarded as safe) (Altuntas *et al.*, 2010; Zendo, 2013). They have been used in food production as an effective method for extending shelf-life of food stuffs by simple fermentation (Galvez *et al*., 2008).

LAB are Gram-positive cocci or rods, able to ferment carbohydrates for energy and lactic acid production (Parada *et al*., 2007). From the genera lactobacilli (L.) species; *L. acidophilus*, *L. brevis*, *L. plantarum*, *Pediococcus* (*P.*) *acidilactici* and *P. pentosaceus* are versatile strains with useful properties and found abundantly in meat and food products (Guidone *et al*., 2014). They produce some antimicrobial compounds including; organic acids, hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde, reuterin, bacteriocins and biosurfactants (Gurakan, 2007; Cornea *et al.*, 2016).

Bacteriocins are proteinaceous antibiotics produced by LAB with bactericidal activity against Gram-positive and Gram-negative spoilage organisms and food-borne pathogens as *E. coli*, *L. monocytogenes*, *Staph. aureus* and *S. Typhimurium* (Altuntas *et al*., 2010; Martinez *et al*., 2013). Bacteriocins are commonly referred to be ribosomal synthesized antimicrobial peptides that usually display a high degree of target specificity against strains of bacteria closely related and/or broad range antimicrobial activity (Guerra and Pastrana, 2002). They are generally low molecular-weight proteins that gain entry into target cells by binding to cell surface receptors and which bactericidal mechanisms vary, including pore formation of the cytoplasmic membrane; by inhibiting synthesis of the cell wall, degradation of cellular DNA, disruption through specific cleavage of 16S rRNA and inhibition of peptidoglycan synthesis (Diep *et al*., 2006; Murua *et al*., 2013). Microbial biosurfactants are amphiphilic metabolites with a pronounced surface activity with a broad range of chemical structures. They have several advantages over chemical surfactants, including low toxicity, biodegradable, and effective at different ranges of temperature and pH (Saharan *et al*., 2011).

Biosurfactant derived from various microorganisms has been reported for antimicrobial properties (Sharma *et al*., 2015). Gram-positive bacteria are more profound against the biosurfactants than Gram-negative ones, which are moderately inhibited. As it affects in the permeability of cellular plasma membranes. Biosurfactants play role in prevent biofilm formation through the reduction of the interaction of bacteria with the surface by changing the wettability properties and charge of the surface (Banat *et al*., 2010). In addition, the biofilm formation by Lactobacillus spp., is considered a beneficial property because it could promote colonization and longer permanence in the mucosa of the host, avoiding colonization by pathogenic bacteria (Terraf *et al*., 2012). Proper identification and characterization of lactobacilli includes not only phenotypic but also molecular studies (Donelli *et al*., 2013). As lactobacillus species had useful properties and usually found in meat and products, the present study was conducted to throw light over their prevalence in meat and meat products beside phenotypic characterization, bioactivities and genes

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that code for the 16S ribosomal ribonucleic acid (rRNA) is sequencing and
analysis to study the bacterial phylogeny and diversity of them.

2. MATERIAL AND METHODS

2.1. Samples
The present study was conducted on 250 random samples of meat and meat products (minced meat, Kofta, beef burger and sausage), 50 of each, were collected from small retails and different supermarkets at Kaliobia Governorate, Egypt, for isolation of Lactobacillus species and studying the bioactivities of them.

2.2. Isolation and phenotypic characterization of lactic acid bacteria.
Twenty-five grams of each sample was aseptically weighed and pooled in 225 ml sterile 0.1% peptone water in sterile Stomacher bag and blended with stomacher for 2 min. One ml of prepared sample was inoculated into MRS agar and incubated anaerobically at 30 °C for 24-48 h (Russo *et al.*, 2006). The creamy white colonies were picked up and Catalase test was performed. The suspected colonies that gave catalase negative were taken and inoculated into MRS broth and were incubated aerobically at 35 °C for 48-72 h. The colonies were purified by further sub-culturing on MRS agar plates and incubated aerobically at 35 °C for 18 h and stored at -20 °C in MRS supplemented with 20% glycerol. The purified colonies were morphologically identified by Gram stain and biochemical tests (Oliveira *et al.*, 2008; De Vos *et al.*, 2009) and confirmed by using MALDI-TOF mass spectrometry.

2.3. Bacteriocins and Biosurfactants Extraction: (Bromberg et al., 2006; Gudina et al., 2010)
Each isolate was grown in 100 ml MRS broth at 37 °C for 48 h and centrifuged at 8000 rpm for 30 min at 4 °C for the extraction of bacteriocin. The cell free supernatant was adjusted to pH 6 with 1 M NaOH and heated at 80 °C for 10 min to inactivate extracellular proteases and hydrogen peroxide, then filter-sterilized and submitted to the critical dilution method in 10 mM phosphate buffered saline at pH 6.5 for the recovery of bacteriocins.
On the other hand, the biomass was washed twice with demineralized water, centrifuged (10,000 ×g, 15 min, 10 °C), resuspended in a volume of phosphate buffer saline (pH 7.0), incubated for 2 h at room temperature, and centrifuged at (10,000 ×g, 15 min, 10 °C) to take the cell-free supernatants which used for specific tests .

2.4. The antimicrobial activities of extracted bacteriocins and biosurfactants:
The inhibitory activities of bacteriocins and biosurfactants were performed using the disc diffusion assay method of Ochei and Kolhatkar (2008) against the following pathogenic strains (*L. monocytogenes* NCTC 13372, *E. coli* NCTC 12241 and *S. Typhimurium* NCTC 12023) obtained from Cairo-MIRCEN (Microbiology resource center). Faculty of Agriculture, Ain Shams University, Cairo, Egypt. Besides, filed isolated Methicillin resistant *Staph. aureus* (MRSA) from meat samples.

2.5. Detection of ability of isolated strains for biofilm formation.
The isolated strains were examined for the development of biofilm using tube method of Christensen *et al.* (1985).
The antibiofilm effects of isolated strains were detected by Sancineto *et al.* (2016) by inoculation of each tested pathogenic strain firstly confirmed to produce strong biofilm with each isolated LAB (*L. acidophilus, L. brevis, L. plantarum, P. acidilactici* and *P. pentosaceus*) strains in test tubes containing 10 ml of trypticase soy broth with 1 % glucose, then the tubes were incubated at 37 °C for 24 h and the ability of LAB to prevent biofilm formation was mentioned and recorded.

2.6. DNA extraction and 16S rRNA sequencing following (Syukur et al., 2014)
The isolated strains were confirmed using polymerase chain reaction (PCR) by using a pair of universal primers 27 F: (5'-AGAGTTTGATCCTGGCTAG-3') and 1525 R: (5'-AGAAAGGAGGTGATCCAGCC-3') for 16S rRNA. After amplifications at 1500 bp. The purified PCR product was sequenced using Sanger Dideoxy method (Sanger *et al.*, 1977). The sequences of the gene fragment of the isolates were compared with other bacterial sequences by using NCBI GenBank database using the BLAST program, available at website (http://blast.ncbi.nlm.nih.gov/Blast.cgi) for their pairwise identities. The phylogenetic tree was performed by using MEGA 6 program.

3. RESULTS AND DISCUSSION

The results of bacteriological examination of examined meat and meat product samples, *in vitro* antimicrobial activities of extracted bacteriocins and biosurfactants, biofilm production and phylogenetic tree for the isolated strains were tabulated in tables (1-4) and figures (1-5).
Bacteriocins producing LAB strains are generally regarded as natural in meat and meat products that could ensure the safety and extend the shelf-life of these foods (Oliveira *et al*., 2008; Dhewa, 2012). The results of bacteriological examination of examined samples table (1) revealed that a total of 252 strains of LAB strains were recovered from 250 samples. All positive examined samples were mixed isolate cultures.

Table 1 Prevalence of lactic acid bacteria species in examined samples

	Fresh meat		Beef burger		Kofta		Minced meat		Sausage		Total	
Isolates	NO.	%*	NO.	%*	NO.	%*	NO.	%*	NO.	%*	NO.	%**
L. acidophilus	4	8.0	9	18.0	8	16.0	6	12.0	11	22.0	38	15.1
L. brevis	0	0.0	7	14.0	5	10.0	0	0.0	7	14.0	19	7.5
L. plantarum	8	16.0	20	40.0	12	24.0	12	24.0	24	48.0	76	30.2
P. acidilactici	11	22.0	26	52.0	22	44.0	17	34.0	26	52.0	102	40.5
P. pentosaceus	2	4.0	3	6.0	5	10.0	2	4.0	5	10.0	17	6.7
Total %***	25	9.9	65	25.8	52	20.6	37	14.7	73	29.0	252	100.0

%* Percentage in relation to total number of each sample (50). %** Percentage in relation to total number of isolates (252)

Moreover, *P. acidilactici* were the most isolated (102= 40.5 %) followed by *L. plantarum* (76= 30.2%), *L. acidophilus* (38= 15.1%), *L. brevis* (19= 7.5%) and *P. pentosaceus* (17= 6.7%). In addition, *L. acidophilus* was mostly isolated from sausage samples (11=22.0%) followed by beef burger (9= 18.0%), kofta (8= 16.0%), minced meat (6= 12.0%) and meat samples (4= 8.0%). *L. brevis* was isolated from beef burger and sausage samples (7= 14.0% for each) and kofta samples (5= 10.0%) but failed to be isolated from both minced meat and meat samples. *L. plantarum* was mostly isolated from sausage samples (24= 48.0%) followed by beef burger (20= 40.0%), kofta, minced meat (12= 24.0% for each) and meat samples (8= 16.0%). *P. acidilactici* was mostly isolated from sausage and beef burger samples (26= 52.0% for each) followed by kofta (22= 44.0%), minced meat (17=34.0%) and meat samples (11= 22.0%). Moreover, *P. pentosaceus* was mostly isolated from kofta and sausage samples (5= 10.0% for each) followed by beef burger (3= 6.0%) and meat, minced meat samples (2= 4.0% for each). Nearly similar results were recorded by Bromberg *et al*. (2004), Oliveira *et al*. (2008), Dhewa (2012), Miranda *et al.* (2014) and Kalschne *et al*. (2015), who isolated the same LAB strains and other ones from meat and meat products.

Regarding to the colonial appearance and the biochemical profile of LAB isolated strains, they were similar to those previously reported such as the fermentation of carbohydrates (Oliveira *et al*., 2008; De Vos *et al*., 2009; Dhewa, 2012; Naimi and Khaled, 2014).

Regarding to the bioactivities of LAB isolated strains, the results revealed that, all isolated LAB strains were found to produce bacteriocin. In addition, the *in vitro* antimicrobial activity of the extracted bacteriocins of isolated LAB strains in table (2) declared that, they inhibited the growth of tested pathogenic bacteria and the diameters of the inhibition zones were varied from 2-15 mm. Meanwhile, extracted bacteriocins from both *L. brevis* and *P. pentosaceus* strains had no effects on *E. coli* strains. Moreover, the bacteriocins were more effective against Gram-positive bacteria (*L. monocytogenes*; *S. aureus* and MRSA) than Gram-negative ones (*E. coli* and *S. Typhimurium*). The resistance pattern of Gram-negative bacteria is attributed to the protective barrier provided by the LPS of their outer cellular envelope. Nearly similar results were recorded by Bromberg *et al*. (2004), Oliveira *et al*. (2008), Dhewa (2012) and Isa and Razavi (2018).

Regarding biosurfactant extraction, the results revealed that, all isolated LAB strains were found to produce biosurfactant. In addition, the *in vitro* antimicrobial activity of the extracted biosurfactant of isolated LAB strains (Table 3) showed that, biosurfactant of *L. acidophilus* inhibited the growth of tested pathogenic bacteria except *S. Typhimurium* and the diameters of the inhibition zones were 1-7 mm; of *L. brevis*, it inhibited the growth of *L .monocytogenes* and *S. Typhimurium* strains only and the diameters of the inhibition zones were 2-7 mm; of *L. plantarum* it inhibited the growth of tested pathogenic bacteria except *E. coli* and the diameters of the inhibition zones were 1-7 mm; of *P. acidilactici* it inhibited the growth of *S. aureus*; MRSA and *L .monocytogenes* strains only and the diameters of the inhibition zones were 1-9 mm and biosurfactant of *P. pentosaceus* inhibited the growth of tested pathogenic bacteria except *E. coli* and the diameters of the inhibition zones were 1-7 mm. Nearly similar results were recorded by Gudina *et al.* (2011) and Cornea *et al.* (2016), who recorded that, the antimicrobial activity of the microbial surfactants is due to the adhesion property of these surface-active agents to the cell surfaces instigating decline of cell membrane integrity leads to subsequent collapse of the nutrition cycle. Also, able to form pores and disrupt the plasma membrane. As a result of their action, several biosurfactants are recognized to have therapeutic claims as antifungal, antibacterial, and antiviral complexes.

Moreover, the results of biofilm formation for isolated LAB strains (Table 4) showed that, most isolated bacteria had the ability for biofilm production that was clearly marked by a visible film lined the wall and the bottom of the tubes and according to the intensity of stain colour , 9 *L. acidophilus* (23.7 %) showed strong biofilm, 25 (65.8%) medium biofilm and 4 (10.5 %) isolates failed to produce biofilms; for *L. brevis* 2 (10.5%) had strong biofilm, 13 (68.4%) medium biofilm and 4 (21.1%) isolates failed to produce biofilms; for *L. plantarum* 46 (60.5%) had strong biofilm, 14 (18.4%) medium biofilm and 16 (21.1%) isolates failed to produce biofilms; for *P. acidilactici* 52 (51.0%) had strong biofilm, 23 (22.5%) medium biofilm and 27 (26.5%) isolates failed to produce biofilms; meanwhile, only 3 *P. pentosaceus* (17.6%) produce medium biofilm and 14 (82.4%) isolates failed to produce biofilms.

Table 2 *In vitro* antimicrobial activity of the extracted bacteriocins

LAB strains	Bacteriocin production	Efficiency of bacteriocins (diameter measure by mm)				
		Staph. aureus	MRSA	*L. monocytogenes*	*E. coli*	*S. Typhimurium*
L. acidophilus	+	4-8	3-7	3-7	5-8	2-3
L. brevis	+	3-8	3-7	3-5	0	1-4
L. plantarum	+	7-9	6-8	9-12	4-8	2-4
P. acidilactici	+	7-13	7-11	9- 15	2-5	4-7
P. pentosacus	+	5-10	4-8	1-3	0	4-8

Table 3 *In vitro* antimicrobial activity of the extracted biosurfactant

LAB strains	Biosurfactant production	Efficiency of biosurfactant (diameter measure by mm)				
		Staph. aureus	MRSA	*L. monocytogenes*	*E. coli*	*S. Typhimurium*
L. acidophilus	+	4-7	4-6	3-6	1-2	0
L. brevis	+	0	0	4-7	0	2-5
L. plantarum	+	3-5	2-4	5-7	0	1-2
P. acidilactici	+	2-4	1-3	5-9	0	0
P. pentosacus	+	3-6	3-5	5-9	0	2-5

Similar results were recorded by Jalilsood *et al*. (2015) and Laavanya-Kumar *et al*. (2017). In addition, all isolated LAB were able to inhibit the biofilm formation of tested pathogenic strains (*L. monocytogenes*; *S. aureus*; MRSA; *E. coli* and *S. Typhimurium*) where no biofilms were produced after inoculation of both tested pathogenic strain with isolated LAB strains in test tubes i.e., they had antibiofilm effects on pathogenic strains. These results came in harmony with those reported by Fernández *et al.* (2015), Gómez *et al.* (2016) and Laavanya-Kumar *et al*. (2017), who reported that the antibiofilm effects could be attributed to their inhibition mechanism through organic acid production as well as the mechanisms of pathogens exclusion through their trapping (killing of cells embedded in biofilms).

Table 4 Biofilm formation of isolated lactic acid bacteria

LAB strains	Number of strains	Biofilm formation					
		Strong		Medium		No biofilm	
		No.	%*	No.	%*	No.	%*
L. acidophilus	38	9	23.7	25	65.8	4	10.5
L. brevis	19	2	10.5	13	68.4	4	21.1
L. plantarum	76	46	60.5	14	18.4	16	21.1
P. acidilactici	102	52	51.0	23	22.5	27	26.5
P. pentosaceus	17	0	0.0	3	17.6	14	82.4

%* Percentage in relation to total number of each isolated strain

The phenotypic analysis for LAB did not allow the identification of the species strains so, identification was confirmed by using MALDI-TOF mass spectrometry and the molecular analysis. Because of this, identification of the LAB species selected was performed by DNA sequences of the 16S rRNA. So, the genomic DNA of LAB tested strains using specific 27F and 1525R primers for the 16SrRNA gene (Fig. 1) cleared that, the 16SrRNA gene was amplified in all 5 studied strains giving product of 1500 bp. and these results were correlated with those obtained by MALDI-TOF mass spectrometry for the isolates and they identified as *L. acidophilus, L. brevis, L. plantarum, P. acidiolactici* and *P. pentosaceus*.

Regarding the sequence detection of 16S rRNA gene in isolated LAB strains, the sequences obtained for *L. acidophilus* with provided in Gene Bank with accession number MK850930 and MK852180 (phylogenetic tree, Fig. 2) seemed to be identical by 97% identity with the strains of *L. acidophilus* with the following Gene Bank sequences (HQ293108.1 for Nawaz *et al*. (2011); JN188382.1 for Shokryazdan *et al.* (2014) and KU922757.1 for Gao (2016) Also, the sequences obtained for *L. brevis* with provided in Gene Bank with accession number MK852397 and MK852398 (phylogenetic tree, Fig., 3) seemed to be identical by 93% identity with the strains of *L. brevis* with the following Gene Bank sequences (MH844891.1 for Kim *et al*. (2018); MK614499.1, MK614498.1 and MK614488.1 for Abdeslam-Ait (2019); and MK408481.1 for Jianwen *et al.* (2019). Meanwhile, the sequences obtained for *L. plantarum* with provided Gene Bank accession number MK806485 and MK850564 (phylogenetic tree, Fig., 4) seemed to be identical by 95% to 96.3% identity with the strains of *L. plantarum* with the following Gene Bank sequences (KR011005.1 for Gulluce *et al*. (2015); MH762174.1 and MH762169.1 for Rajawardana *et al.* (2018) and MK156350.1 for Sharma *et al.* (2018). In addition, the sequences obtained for *P. acidilactici* with provided Gene Bank accession number MK871658 and MK871674 (phylogenetic tree, Fig., 5) seemed to be identical by 98% identity with the strains of *P. acidilactici* with the following Gene Bank sequences (FJ538571.1, FJ538588.1, FJ538581.1 and FJ538576.1 for Rodriguez-Buenfil *et al.* (2009) and JQ801714.1 for Adimpong *et al.* (2012). Moreover, the sequences obtained for *P. pentosaceus* with provided Gene Bank accession number MK852683 and MK852691 (phylogenetic tree, Fig., 6) seemed to be identical by 91% identity with the strains of *P. pentosaceus* with the following Gene Bank sequences (AB236939.1 and AB236938.1 for Chen *et al.* (2006) MH844906.1 and MH844907.1 for Kim *et al*. (2018) and NR_042058.1 for Heinz et al. (2019).

Finally, LAB strains were isolated from studied meat and meat products and due to synergistic properties of their bioactivities through production of bacteriocin, biosurfactant with antimicrobial activities and biofilms, so their use in the food industry can help reduce the addition of chemical preservatives. This can be an alternative to satisfy the increasing consumer's demands for safe, meat and their products. Further work to evaluate the applicability of these substances in bio-preservation techniques for meats is in progress

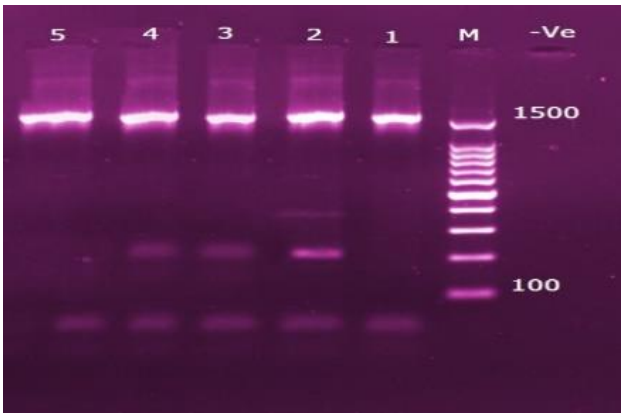

Fig. 1 PCR amplification of specific gene of Lactic acid bacteria (16SrRNA) on agarose gel. Lane L: 100-1500 bp. DNA Ladder. Lane -Ve: Negative control (has no product). Lane 1: *L. acidophilus* strain. Lane 2: *L. brevis* strain. Lane 3: *L. plantarum* strain. Lane 4: *P. acidilactici* strain. Lane 5: *P. pentosaceus* strain

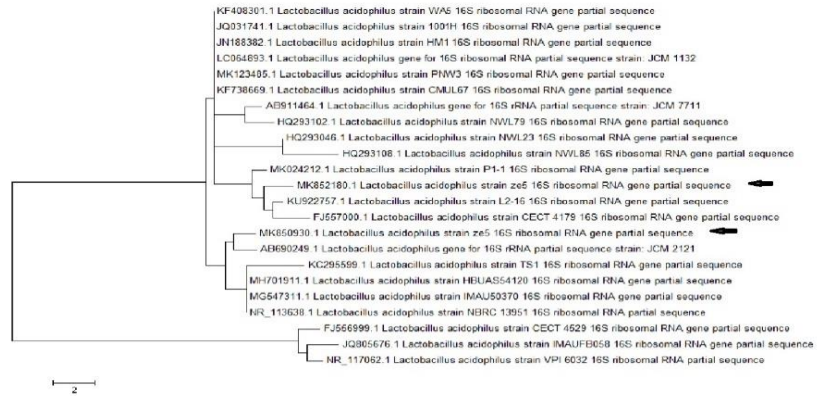

Fig. 2 The phylogenetic tree for the strains related to the isolated *L. acidophilus*

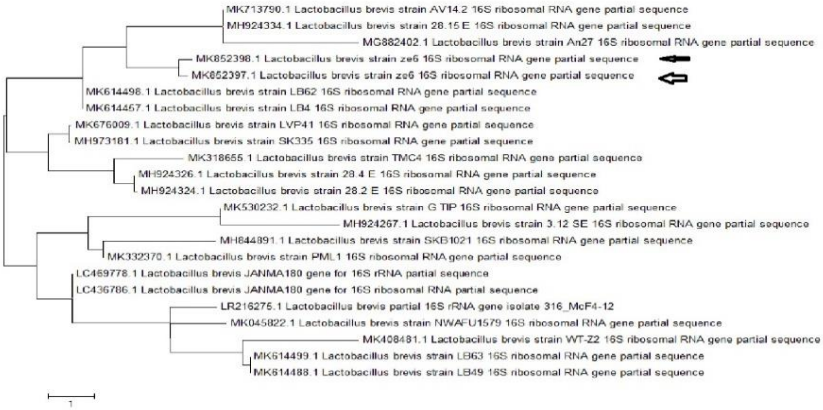

Fig. 3 The phylogenetic tree for strains related to the isolated *L. brevis*

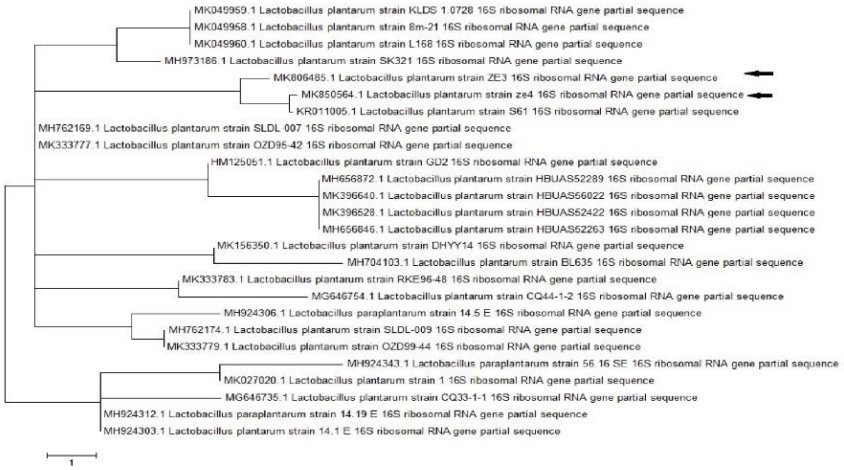

Fig 4 The phylogenetic analysis for the strains related to the isolated *L. plantarum*

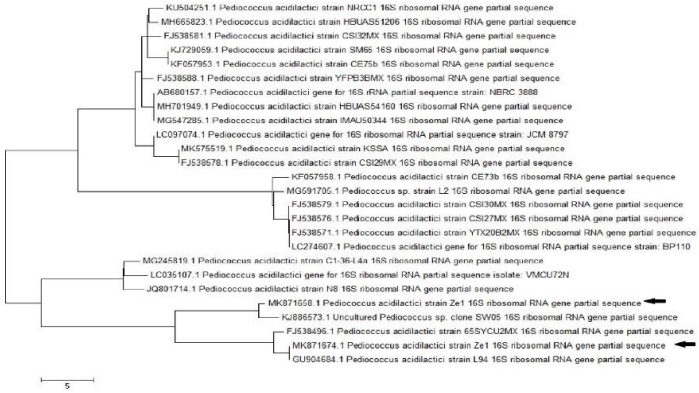

Fig 5 The phylogenetic tree for the strains related to the isolated *P. acidilactici*

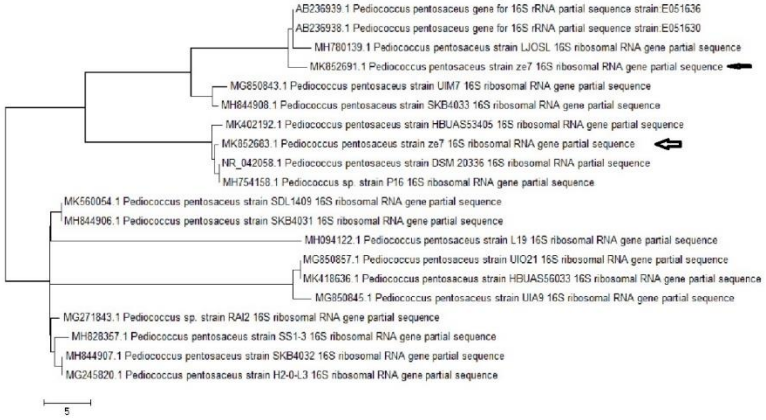

Fig. 6 The phylogenetic analysis for the strains related to the isolated *P. pentosaceus*

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