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Characterization and bioactivities of *Lactobacillus plantarum* and *Pediococcus acidilactici* 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 *L. plantarum* and *P. acidilactici* strains. The results revealed that, 178 strains from 250 samples (71.2%) of *L. plantarum* (76=30.4%) and *P. acidilactici* (102 =40.8%) were isolated from the examined samples. All of them produce bacteriocin and biosurfactant that inhibited the growth of tested pathogenic bacteria. In addition, most isolated strains had the ability to perform biofilm which able to inhibit the biofilm formation of tested pathogenic strains. The amplification for *16S rRNA*, sequencing and phylogenetic tree construction of the obtained sequences with the closely related lactobacillus species were performed. Sequences for and are available in the GeneBank and NCBI with the accession numbers MK806485 and MK850564 for *L. plantarum* and MK871658, MK871674 for *P. acidilactici*.
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1. Introduction

Meat and meat products are the most susceptible food and effective deliverer of nutrients as protein, necessary amino acids, minerals and vitamins, but they are a very suitable substrates for the growth and multiplication of pathogenic bacteria like *Escherichia coli*, *Staphylococcus aureus*, Salmonella spp. and Listeria *monocytogenes*. Because of inadequate measures during the storage of fresh meat and meat products, post-processing, handling and cross contamination, it is necessary for maintaining them with high quality before consumption; hence, researchers are constantly looking for different methods to improve the quality and safety of them and prolonged their storage period through applying bio preservation techniques (**AlizadehSani *et al.*, 2017**). These have involved through introduction of a competitive microflora as lactic acid bacteria which have inhibitory effect against other microorganisms through production of antagonistic compounds like bacteriocins and biosurfactant **(Deegan *et al.*, 2006** and **Moldes *et al.*, 2007)** so act as a protective culture for meat products. From the populous Lactobacillus species, *Lactobacillus plantarum* and *Pediococcus acidilactici* are versatile strain with useful properties and usually found in numerous meat and food products (**Guidone *et al.*, 2014**).

Bacteriocins are small peptides or bioactive proteins, ribosomally synthesized by Gram-positive bacteria especially lactic acid bacteria and Gram-negative bacteria that extracellularly released **(Guerra and Pastrana, 2002).** These molecules have antimicrobial activity against food-borne pathogens and deteriorating bacteria, justifying their biotechnological potential (**Martinez *et al.*, 2013**). Besides extending the shelf-life, bacteriocins also reduce the risk of transmission of pathogenic microorganisms, permitting the reduction in the use of synthetic preservatives (**Allende *et al.*, 2007 and Castellano *et al.*, 2008**). In addition, an important advantage of them over classical antibiotics is that the digestive enzymes can destroy them **(Caplice and Fitzgerald, 1999)** and this will not alter digestive tract ecology and also overcome the risk from use of excessive antibiotics so it's known as "generally recognized as safe" (GRAS) products.

Microbial biosurfactants are amphiphilic metabolites with a pronounced surface activity with a broad range of chemical structures. They have several advantages over chemical surfactants, that is, low toxicity, biodegradable, and effective at different ranges of temperature and pH **(Saharan *et al.*, 2011).** Biosurfactant derived from various microorganisms have been reported for antimicrobial properties

(Sharma *et al.*, 2015). Gram-positive bacteria are more profound against the biosurfactants than Gram-negative ones, which were moderately inhibited. As it affects in the permeability of cellular plasma membranes.

Biosurfactants plays 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 *L. plantarum* and *P. acidilactici* strains of Lactobacillus species had useful properties and usually found in meat and products, so, the present study was conducted to throw light over their prevalence in meat and meat products beside phenotypic characterization; bioactivities and genes that code for the *16S rRNA* with sequence analysis for the bacterial phylogeny and diversity of them.

2. Materials and Methods

Samples

The present study was conducted on 250 random samples of meat and meat products (minced meat – kofta – beef burger- sausage) 50 samples each were collected from small retails and different supermarkets at Kaliobia Governorate, Egypt, for inspection of *L. plantarum* and *P. acidilactici* strains and studying the bioactivities of them.

Isolation and phenotypic characterization

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 De Man, Rogosa and Sharpe agar (MRS agar) and incubated aerobically at 30 °C for 24 to 48h **(Russo *et al.*, 2006).** The creamy white colonies were picked up and Catalase test was performed. The suspected colonies that gave catalase negative stored at -20 °C in MRS broth supplemented with 20% glycerol. The purified colonies were morphologically identified by Gram' s stain and biochemical tests (**Thoesen, 1994; Oliveira *et al.*, 2008 and De Vos *et al.*, 2009**) and confirmed by using MALDI-TOF mass spectrometry.

Bacteriocins and Biosurfactants Extraction following **Bromberg *et al.* (2006)** and **Gudina *et al.* (2010)**

Each isolate was grown in 100 ml MRS broth at 37^{o}C for 48h and centrifuged at 8000 rpm for 30 minutes at 4^{o}C for the extraction of bacteriocin. The cell free supernatant (CFS) was adjusted to pH 6 with 1M NaoH and heated at 80 °C for 10min to inactivate extracellular proteases and hydrogen peroxide, then filter-sterilized and submitted to the critical dilution method in 10 mM phosphate buffered saline (PBS) at pH 6.5 for the recovery of bacteriocins.

On the other hand, the biomass was washed twice with demineralized water, centrifuged (10,000 xg, 15min, 10^{o}C), resuspended in a volume of phosphate buffer saline (PBS; pH 7.0), incubated for 2 h at room temperature, and centrifuged at (10,000 g, 15 min, 10^{o}C) to take the cell- free supernatants that contain biosurfactants which used for specific tests**.**

In-Vitro 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 *S. aureus* (MRSA) from meat samples.

Detection of biofilm formation of isolated strains and the antibiofilm effects of them

The isolated strains were examined for the development of biofilm using tube method (**Christensen *et al.*, 1985).**

The antibiofilm effects of isolated strains were detected following **Sancineto *et al.* (2016)** by inoculation of each tested pathogenic strain firstly confirmed to produce strong biofilm with each isolated LAB (*L. plantarum* and *P. acidilactici*) strains in test tubes containing 10 ml of trypticase soy broth with 1 % glucose, then incubated at 37 o C for 24hr and the ability of LAB to prevent biofilm formation is mentioned and recorded.

DNA extraction and 16S rRNA sequencing

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.*** The method was performed with initial denaturation at 95°C for 3 min, and with 30 cycles of denaturation at 95°C for 1 min; annealing at 55°C for 1,5 min and extension at 72°C for 1,5 m (BioRad thermocycler). The DNA was analyzed by using 1.5% (w/v) agarose gel electrophoresis (BioRad Gel Electrophoresis) in 1x TBE buffer at 100 V for 30 min; and was examined under UV light (**Syukur *et al.*, 2014).** 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 phylogenetic tree was performed by using MEGA 6 program.

3. Results

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 (1-3) and Figures (1-2).

Table (1): Prevalence of *Lactobacillus plantarum* and *Pediococcus acidilactici* species in examined samples

Samples	Fresh meat		Beef burger		Kofta		Minced meat		Sausage		TOTAL	
Isolates	no.	%*	no.	%*	no.	%*	no.	%*	no.	%*	no.	%**
L. plantarum	**8**	**16.0**	**20**	**40.0**	**12**	**24.0**	**12**	**24.0**	**24**	**48.0**	**76**	**30.4**
P.acidilactici	**11**	**22.0**	**26**	**52.0**	**22**	**44.0**	**17**	**34.0**	**26**	**52.0**	**102**	**40.8**
TOTAL	19	38.0	46	92.0	34	68.0	29	58.0	50	100.0	178	71.2

%* Percentage in relation to total number of each sample (50) %**Percentage in relation to total number of samples (250)

Table (2): The diameter of the inhibition zone of bacteriocins and biosurfactant against different tested pathogenic bacteria (measured by mm).

Strains	*L. monocytogenes*		MRSA		*E. coli*		*S. Typhimurium*	
	Bacteriocins	Biosurfactant	Bacteriocins	Biosurfactant	Bacteriocins	biosurfactant	Bacteriocins	biosurfactant
L. plantarum	**9-12**	**5-7**	**6-8**	**2-4**	**4-8**	**0**	**2-4**	**1-2**
P. acidilactici	**9- 15**	**5-9**	**7-11**	**1-3**	**2-5**	**0**	**4-7**	**0**

Table (3): Prevalence of Biofilm produced isolates

Isolates	No. of isolates	Biofilm produced isolates					
		Strong		Medium		Negative	
		no.	%*	no.	%*	no.	%*
L. plantarum	76	46	60.5	14	18.4	16	21.1
P.acidilactici	102	52	51.0	23	22.5	27	26.5

%* Percentage in relation to total number of *L. plantarum* (76) and *P. acidilactici*

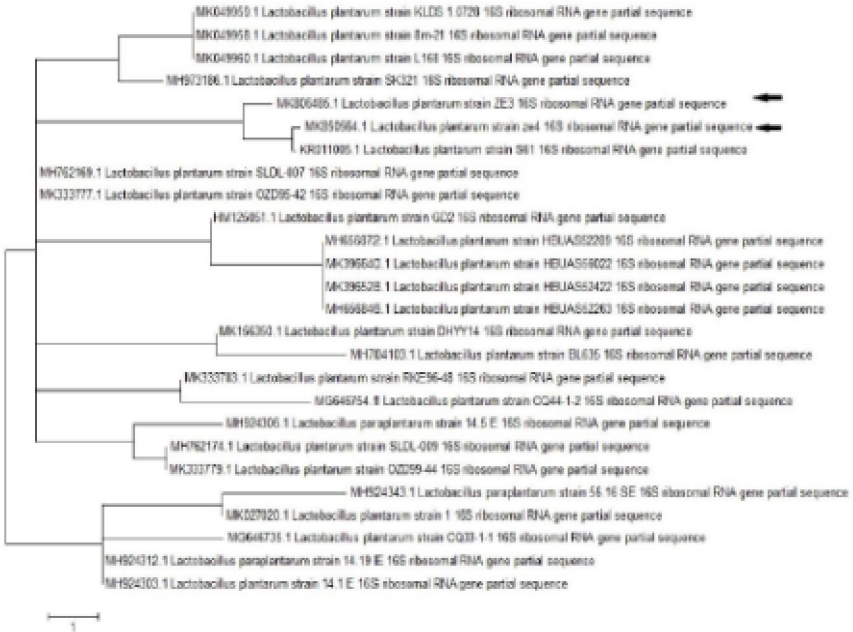

Fig 1: The phylogenetic analysis for the strains related to the isolated *L. plantarum*

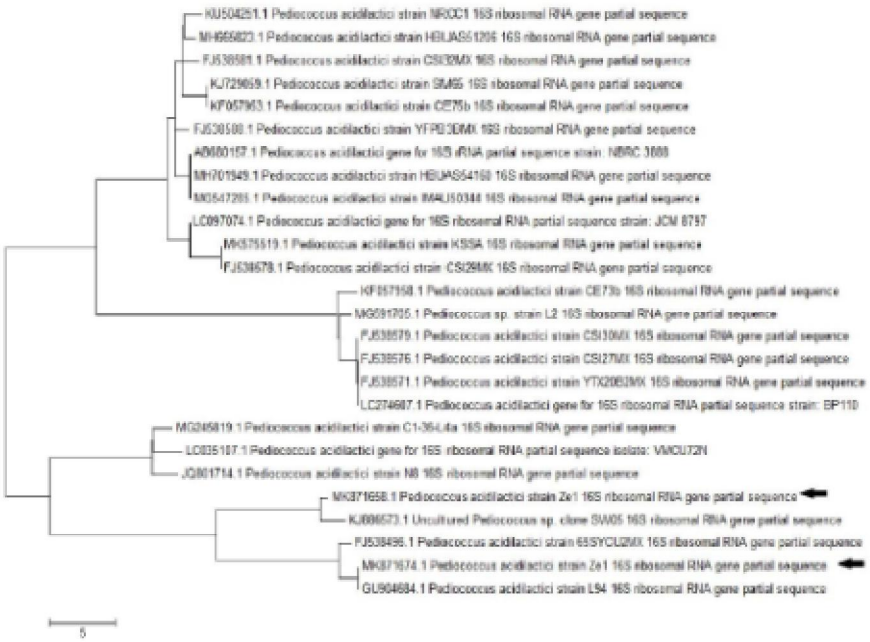

Fig 2: The phylogenetic tree for the strains related to the isolated *P. acidilactici*

4. Discussion

Bacteriocins producing *L. plantarum* and *P. acidilactici* 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 and Dhewa, 2012**).

The results of bacteriological examination of examined meat and meat products samples (Table 1) revealed that, a total of 178 strains (71.2%) of *L. plantarum* (76=30.4%) and *P. acidilactici* (102 =40.8%) were recovered from 250 examined samples. All positive examined samples showed mixed isolate cultures. Moreover, *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%). In addition, *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%). These results agree with those of **Bromberg *et al*. (2004); Oliveira *et al*. (2008); Dhewa (2012) and Kalschne *et al*. (2015)** who isolated *L. plantarum* and *P. acidilactici* beside other LAB strains from meat and meat products.

Regarding to the colonial appearance and the biochemical profile of *L. plantarum* and *P. acidilactici* isolated, they were similar to those previously reported such as the fermentation of carbohydrates (**Guessas *et al*. (2007); Oliveira *et al*., 2008; De Vos *et al*., 2009; Dhewa, 2012 and Naimi and Khaled, 2014**) and they were confirmed by using MALDI-TOF mass spectrometry.

All isolated 178 strains of *L. plantarum* and *P. acidilactici* were found to produce bacteriocins and biosurfactant like substances. In addition, the in- vitro antimicrobial activities of extracted bacteriocins for isolated strains (Table 2) revealed that, they inhibited the growth of tested pathogenic bacteria and the diameters of the inhibition zones were varied from 2 – 15 mm. They were more effective on Gram-positive bacteria (*L. monocytogenes* 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 **De Martinis *et al*. (2001); Bromberg *et al*. (2004); Rodrigues *et al*. (2006); Oliveira *et al*. (2008); Karska-Wysocki *et al*. (2010)** and **Dhewa (2012).** Moreover, the present results revealed that, biosurfactants for isolated strains had inhibitory activities against tested Gram-positive bacteria and the diameters of the inhibition zones were varied from 1- 9 mm for biosurfactants. Meanwhile, they had no inhibitory effects on Gram- negative ones. Nearly similar results were recorded by **Rodrigues *et al*. (2006); Karska-Wysocki *et al*. (2010)** and **Gudina *et al*. (2011**).

In addition, the results of (Table 3) cleared that, most isolated *L. plantarum* and *P. acidilactici* strains had the ability for biofilm production that was clearly marked by a visible film lined the wall and the bottom of the tube where 60 *L. plantarum* (78.9%) produce biofilm; 46(60.5%) strong biofilms, 14(18.4%) medium ones and 16(21.1%) isolates failed to produce

biofilms. Meanwhile, 75 *P. acidilactici* (73.5%) produce biofilm; 52(51.0%) strong biofilms, 23(22.5%) medium ones and 27(26.5%) isolates failed to produce biofilms. Similar results were recorded by **Høiby *et al.* (2010); Jalilsood *et al.* (2015)** and **Laavanya-Kumar *et al.* (2017)**. In addition, they were able to inhibit the biofilm formation of tested pathogenic strains where no biofilms 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 **Guessas *et al.* (2007); Guerrieri *et al.* (2009)**; **Radovanovic and Katic (2009)** and **Laavanya-Kumar *et al.* (2017)** who reported that the antibiofilm effects could be attributed to their inhibition mechanism through organic acid production and influence of EPS (exopolysaccharide). And the ability to co-aggregate with pathogenic strains and so interfere with the ability of the pathogenic species to colonize and form biofilm.

Regarding to the sequence detection of **16S *rRNA*** gene in isolated *L. plantarum* and *P. acidilactici* strains, the sequences obtained for *L. plantarum* provided in GeneBank with accession number MK806485 and MK850564 were 95.53%to 96.02% identity with the strains of *L. plantarum* with the following GeneBank sequences (accession numbers HM125051.1, KR011005.1, MH924343.1, MH924312.1, MH924306.1, MH924303.1, MK156350.1, MK049960.1, MK049959.1, MK049958.1, MK027020.1, MH973186.1, MH762174.1, MH762169.1, MH704103.1, and MK396640.1). Meanwhile, the sequences obtained for *P. acidilactici* were provided in GeneBank with accession number MK871658 and MK871674 were 98% identity with the strains of *P. acidilactici* with the following Gene Bank sequences (accession numbers FJ538571.1, LC274607.1, KU504251.1, LC097074.1, KJ729059.1, KJ886573.1, KF057958.1, KF057953.1, JQ801714.1, AB680157.1, FJ538588.1, FJ538581.1, FJ538576.1, FJ538496.1, GU904684.1, MK575519.1, MG245819.1, LC035107.1, MH701949.1, MH665823.1, MG547285.1, MG591705.1, FJ538579.1, FJ538578.1).

Finally, due to synergistic properties of *L. plantarum* and *P. acidilactici* bioactivities through production of bacteriocin, biosurfactant with antimicrobial activities and biofilms, 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.

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