International Journal of Pharmacology

ISSN 1811-7775

science alert

ansinet
Asian Network for Scientific Information

International Journal of Pharmacology 11 (5): 440-447, 2015
ISSN 1811-7775

ansinet
Asian Network for Scientific Information

RESEARCH ARTICLE OPEN ACCESS

DOI: 10.3923/ijp.2015.440.447

Antibacterial Bioactivity of Selected Lactic Acid Bacterial Strains against some Human Pathogenic Bacteria

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ARTICLE INFO
Article History:
Received: February 26, 2015
Accepted: May 25, 2015

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ABSTRACT
Bacteriocin producing lactobacilli were obtained from different sources and were previously characterized outdoors. *Lactobacillus bulgaricus* 761N, *Lactobacillus fermentum* DSMZ 20049, *Lactobacillus delbrueckii* subsp. *bulgaricus* NCTC 12197 *T* and *Lactobacillus delbrueckii* subsp. *bulgaricus* DSMZ 20080T were the strains under study. Subcellular fractions were investigated for their proteolytic activity that results in production of bioactive peptides in the cell-free supernatant (bacteriocin). A single strain bacteriocin was selected depending upon cytotoxicity of the strain's bacteriocin and antibacterial capacity. The cell-free supernatants of the tested strains were tested for their antibacterial capacity against a broad spectrum of pathogens involving *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Salmonella* spp., *Escherichia coli* and *Shigella* spp. The selected candidate was *Lactobacillus bulgaricus* 761N. The bacteriocin was partially purified and assured via applying the semi-purified samples on SDS-PAGE. Advance to the purification process, the fractions selected were tested for their antibacterial capacity as well. Both fractions have been found to possess antibacterial activity but the lower molecular weight was over succeeded.

Key words: Bacteriocin, lactic acid bacteria, pathogenic bacteria, proteolytic activity

INTRODUCTION

Bacteriocins were differentially defined by many scientists. Bacteriocins are proteinaceous compounds that are inhibitory towards sensitive strains and are produced by both gram-positive and gram-negative bacteria (Tagg *et al*., 1976). They were also termed as the ribosomally produced cationic proteins inhibiting other bacteria living in the same ecological niche (Riaz *et al*., 2010). Five required criteria were identified to call a chemical substance as a bacteriocin involving the presence of a biologically active part of protein nature, a spectrum of inhibitive activity narrow and centered on the homologues, a mode of bactericidal action, the adsorption to specific receivers and nature plasmodic genetic determiners coding for the production of the bacteriocin and for the immunity in this one (Tagg *et al*., 1976). During the last few years, a large number of new bacteriocins produced by Lactic Acid Bacteria (LAB) have been identified and characterized. LAB-bacteriocins comprise a heterogeneous group of physicochemically diverse ribosomally-synthesized peptides or proteins showing a narrow or broad antimicrobial activity spectrum against gram-positive bacteria (Cintas *et al*., 2001). Other studies showed that bacteriocins have also antimicrobial capability against gram negative bacteria, for instance, *E. coli* and *Salmonella* but usually only when the integrity of the outer membrane has been compromised, for example after osmotic shock or low pH treatment, in the presence of a detergent or chelating agent, or after pulsed electric field or high-pressure treatment (Stevens *et al*., 1991). The resistance of gram negative pathogenic bacteria to bacteriocins is attributed to the particular nature of their cellular envelope besides the fact that the mechanisms of action described for bacteriocins is owing to the phenomenon of adsorption. According to Bhunia *et al*. (1991), the pediocin (bacteriocins produced by

Pediococcus acidilactici) interacts with lipoteichoic acids absent in gram negative bacteria. These variations of sensibility are due to the characteristics of indicators strains (presence or absence of receiving sites or immunoprotein) and thus in level of hurts caused by the inhibitive factor (Savadogo *et al*., 2004). Recently, It was proven that bacteriocins have the advantage over antibiotics even the third generation antibiotics owing to the fact that some microbial pathogens gain acquired immunity against these antibiotics due to their frequent uptake, for instance, bacteriocins produced by *Lactobacilli* strains isolated from yogurt was found to possess antimicrobial potential against cephalosporin resistant *E. coli* (Riaz *et al*., 2010). Also, bacteriocins obtained from *L. fermentum* were found to possess antimicrobial potential against methicillin resistant *S. aureus* (Nawaz *et al*., 2009). With respect to medical applications, antimicrobials produced by LAB might play a serious role during the in vivo interactions occurring in the human gastrointestinal tract, hence contributing to gut health (De Vuyst and Leroy, 2007). Thus, innovative approaches have been tried as alternative to antibiotics in treating gastrointestinal diseases and these include using live biotherapeutic agent (Daly and Davis, 1998; Soomro *et al*., 2002; Oyetayo *et al*., 2003).

MATERIALS AND METHODS

Isolation and identification of lactic acid bacteria: *Lactobacillus delbrueckii* subsp. *bulgaricus* DSMZ 20080T and NCTC 12197 T *Lactobacillus fermentum* DSMZ 20049, *Lactobacillus acidophilus* DSMZ 20079 T were transferred from reference strains purchased from culture collection. Another strain of *Lactobacillus delbrueckii* subsp. *bulgaricus* 761N was isolated from yogourt sample and identified up to PCR level at the laboratory of microbial biochemistry of dairy microorganisms (LMB), Alexandria University, Egypt.

Target pathogens preparation: Pathogens used involves *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Burkholderia cepacia*, *Salmonella* spp., *Escherichia coli* and *Shigella* spp. were obtained from microbiology Dept. faculty of medicine-Alexandria University. They were next sub-cultured on their specific media involving mannitol salt agar, *Pseudomonas* citrimide agar, brilliant green agar, Eosin methylene blue agar and triple sugar iron. All cultures were incubated for 24-48 h at 37°C then kept at 2-8°C for not more than 1 month. Prior use they were enriched on tryptic soya broth.

Subcellular fractionation of cell lysate: (1) Total cellular extracts: Total cellular extracts were obtained as described by Atlan *et al*. (1989). (2) Cell wall and cytoplasmic extracts: Proteins were extracted from the bacterial cell wall of *Lactobacilli* using a modification of the procedure previously developed to release APII from *Lactobacillus delbrueckii* subsp. *bulgaricus* (Atlan *et al*., 1989). After 4 h growth in 100 mL MRS or milk medium (A_{600} = 0.8-1.0), cells were harvested by centrifugation (10000 g, 10 min, 6°C), washed in 25 Mm-KH, PO, 10 mM-$MgC1_2$, (pH 5.8) and resuspended in 10 mL of the same buffer supplemented with 0.6 M-sucrose and treated with 0.1 or 1 mg mL^{-1} (for cells grown in MRS or milk medium, respectively) of lysozyme from chicken egg white (Sigma) for 30 min at 25 or 37°C (for cells grown in MRS or milk medium, respectively) with gentle shaking. Bacterial suspensions were centrifuged (10000 g, 10 min, 6°C) and the supernatants are referred to as lysozyme fluids. Lysozyme-treated bacteria were osmotically shocked by suspending them in 10 mL cold distilled water and maintained on ice for 10 min. Shocked cells were centrifuged (10000 g, 10 min, 6°C) and these supernatants are referred to as osmotic fluids. The bacterial pellets were resuspended in 10 mL cold distilled water and disrupted with a pulse sonicator 6°C for 30 min. The supernatants obtained after centrifugation of the broken cells (25000 g, 20 min, 6°C) are referred to as soluble cytoplasmic fluids.

Protease activity assessment: The protease assay was done according to the method of Kunitz (1947).

Antibacterial assay: Antibacterial assay was evaluated by microplate reader assay method according to Bechert *et al*. (2000) with some modifications. Aliquot of 100 µL of preinoculated pathogens (10^6 CFU μL^{-1}) in LB broth was transferred to each well of 96 well plates, the same volume of extracellular lactic acid bacterial extracts added to each well in replica. The plates were incubated under microaerophilic conditions at 37°C for 24 h. After incubation, the absorbance of the plates was determined using automated ELIZA microplate reader adjusted at 620 nm. The inhibition percentage of lactic acid bacterial extracts was calculated according to the following equation:

$$\text{Inhibition percentage} = \frac{\text{A-A}_1}{\text{A}_0} \times 100$$

where, A is absorbance of the treatment group, A_1 is absorbance of the blank and A_0 is absorbance of the control group.

Cytotoxicity test: PBMC cells were plated onto 96-well plates at a cell count of 5×10^4 cell mL^{-1}. After 24 h cells incubation at 37°C and 5% CO_2 supply, the cells were sub-confluent. The medium was removed and a serial dilution of tested subcellular fraction in culture medium was added instead. The treated cells were then incubated for 3 days at 37°C and 5% CO_2. Cells were fixed with 100 µL fixing solution (0.5% formaldehyde and 1% $CaCl_2$) for 1 min then each well was supplemented with 100 µL solution of 50% ethanol with 1% acetic acid and shacked for 5 min.

Purification of the crude treatment: Skim milk medium containing the extracellular fraction (Crude enzyme that produces oligobioactive peptides) undergone ultrafiltration

(150 kDa, VRR 5-20). The retentate is discarded, while the permeate undergone ultrafiltration (10 kDa, VRR 5) and the hydro lysate contains the bioactive peptides according to Konrad and Kleinschmidt (2008). The hydrolysate undergone several processing steps. First crude enzyme is inactivated by heating at 80°C for 20 min, Second separation of hydrolysate by centrifugation at 10000xg for 30 min, third Desalting (Demineralization) is achieved by using a negatively charged sephadex gel G50, Forth fractionation of peptide fraction is done by using size exclusion chromatography using sephadex G200. The purification process is followed by bioactivity testing and confirmed by performing SDS-PAGE (Konrad and Kleinschmidt, 2008).

Bioactivity testing: Bioactivity testing involves antibacterial assessment by Micro-broth dilution method.

RESULTS

Protease activity results of subcellular fractions: The extracellular fraction has the highest activity for all strains in comparison with inter-and intracellular fractions as shown in Table 1. The selected fraction for the four strains would be assessed for antibacterial activity and cytotoxicity.

Antibacterial activity assessment (expressed in inhibition percent using microplate reader assay method): The inhibition percent for all extracts of selected LAB strains against pathogenic bacteria increases with the increase of the concentration. The antibacterial activity of *Lactobacillus bulgaricus* 761N extracellular extract against different pathogenic bacteria under study was illustrated in Table 2. The maximum inhibition was recorded for *Shigella* spp, where it was elevated from 0 at 50% concentration to 89 at 100% concentration of the extracellular extract. However, the antibacterial effect of the extracellular extract of *L. fermentum* DSMZ 20049, illustrated in Table 3, the inhibition percent varied from 0 at 50% concentration of the extracellular extract against *Shigella* spp., to 96.89 at 100% concentration of the extracellular extract. Moreover, the antibacterial activity of *L. delbrueckii* subsp. *bulgaricus* NCTC 12197 T extracellular extract, shown in Table 4, illustrated the inhibition percent was found to be ranging from 0 at 50% concentration against *P. aeruginosa* to 85.6 at 100% concentration. The least antibacterial activity was that of the extracellular extract of *L. delbrueckii* subsp. *bulgaricus* DSMZ 20080T, clarified in Table 5. The inhibition percentage was the highest (70.17%) at 100% concentration of the extracellular extract against *Salmonella* spp.

Table 1: Protease activity of subcellular fractions of the four different *Lactobacilli* strains

Bacterial strain	Subcellular fractions	Protease activity (U mL^{-1} min^{-1})	F-value	LSD
Lactobacillus bulgaricus 761N♦	Intracellular fraction*	39.5[c]	1183.40	1.2879
	Intercellular fraction**	42.1[b]		
	Extracellular fraction***	63.1[a]		
Lactobacillus fermentum DSMZ 20049♦	Intracellular fraction	83.1[b]	2326.32	1.7271
	Intercellular fraction	22.5[c]		
	Extracellular fraction	123.1[a]		
Lactobacillus delbrueckii subsp. *bulgaricus* NCTC 12197 T♦	Intracellular fraction	85.1[b]	2178.73	1.5928
	Intercellular fraction	63.1[c]		
	Extracellular fraction	106.1[a]		
Lactobacillus delbrueckii subsp. *bulgaricus* DSMZ 20080T♦	Intracellular fraction	66.1[c]	9213.97	2.5289
	Intercellular fraction	180.1[b]		
	Extracellular fraction	194.1[a]		

*Cytoplasmic fluids, **Osmotic fluids, ***Lysozyme fluids. Equation: y = 0.001x+0.0859, where, y = Test-control, x = Proteolytic activity (U mL^{-1} min^{-1}). ♦α-significance = 0.05, p<0.0001 (i.e., highly significant difference). [a, b, c]Symbols to express significant difference in the statistical analysis (i.e., similar symbols means no significant difference while different symbols means that these data are significantly different), values are given in Mean±SD

Table 2: Antibacterial assay for *Lactobacillus bulgaricus* 761N extracellular extract tested against different pathogenic bacteria

Treatment concentration	Inhibition (%)					
	S. aureus	*Salmonella*	*P. aeruginosa*	*E. coli*	*Shigella* spp.	*B. cepacia*
100%	82.90±0.03[a]	66.98±0.02[a]	72.29±0.02[a]	72.24±0.04[a]	89.00±0.05[a]	79.76±0.06[a]
90%	81.74±0.03[b]	65.84±0.04[a]	66.69±0.01[b]	68.57±0.02[b]	86.40±0.02[b]	79.16±0.04[b]
80%	80.48±0.04[c]	29.50±0.02[b]	30.18±0.03[c]	54.12±0.02[c]	71.35±0.03[c]	76.31±0.01[c]
70%	76.39±0.01[d]	22.07±0.02[c]	24.42±0.02[d]	51.27±0.03[d]	69.85±0.04[d]	72.52±0.03[d]
60%	76.18±0.02[d]	13.13±0.03[d]	22.37±0.02[e]	49.31±0.01[e]	68.56±0.01[e]	64.60±0.03[e]
50%	75.55±0.05[e]	0[e]	08.39±0.03[f]	39.02±0.02[f]	66.52±0.02[f]	51.08±0.08[f]
F-value	218331	374458.3	490577.4	739294	382936	232799
LSD	0.22	0.15	0.04	0.04	0.06	0.08
p-value	*	**	<0.0001	<0.0001	<0.0001	<0.0001

*No significant difference was observed for the extracellular extract of *Lactobacillus bulgaricus* 761N at concentrations 60 and 70% against *Staphylococcus aureus* (p>0.005), i.e., in the case of using the crude extract as a treatment against *S. aureus* at treatment concentration 60% instead of 70%, it would be better owing to gaining a less side effects, for example cytotoxicity and roughly the same influence as that of the latter treatment concentration. **No significant difference was observed for the extracellular extract of *Lactobacillus bulgaricus* 761N at concentrations 90 and 100% against *Salmonella* spp. (p>0.005), i.e., using the crude treatment against *Salmonella* at a concentration of 90% is better than using it at 100% concentration achieving less negative effects and roughly the same influence as that of the latter concentration

Table 3: Antibacterial assay for *Lactobacillus fermentum* DSMZ 20049 extracellular extract against some pathogenic bacteria

Treatment concentration	Inhibition (%)					
	S. aureus	*Salmonella*	*P. aeruginosa*	*E. coli*	*Shigella* spp.	*B. cepacia*
100%	81.80±0.02[a]	50.87±0.02[a]	62.39±0.01[a]	61.60±0.02[a]	96.89±0.04[a]	79.69±0.07[a]
90%	80.70±0.03[b]	45.83±0.03[b]	54.33±0.03[b]	52.49±0.03[b]	94.50±0.02[b]	79.10±0.05[a]
80%	58.70±0.04[c]	21.50±0.08[c]	20.18±0.03[c]	39.30±0.03[c]	92.20±0.03[c]	76.21±0.02[b]
70%	53.20±0.02[d]	17.77±0.07[d]	15.49±0.03[d]	35.50±0.05[d]	71.80±0.02[d]	73.12±0.02[c]
60%	47.18±0.02[e]	08.03±0.03[e]	11.67±0.02[e]	12.87±0.03[e]	70.96±0.04[e]	65.32±0.03[d]
50%	27.60±0.04[f]	0[f]	02.11±0.01[f]	0[f]	70.40±0.03[e]	51.12±0.02[e]
F-value	145783.7	303240	330545.1	575861.3	520587	227329
LSD	0.5	0.8	0.42	0.51	0.58	0.67
p-value	<0.0001	<0.0001	<0.0001	<0.0001	*	**

a,b,c,d,e and f: Significant difference in the statistical analysis, *: No significant difference was observed for the extracellular extract of *Lactobacillus fermentum* DSMZ 20049 at concentrations 50 and 60% against *Shigella* spp. (p>0.005), **: No significant difference was observed for the extracellular extract of *Lactobacillus fermentum* DSMZ 20049 at concentrations 90 and 100% against *B. cepacia* (p>0.005)

Table 4: Antibacterial assay for *Lactobacillus delbrueckii* subsp. *bulgaricus* NCTC 12197 T extracellular extract against some pathogenic bacteria

Treatment concentration	Inhibition (%)					
	S. aureus	*Salmonella*	*P. aeruginosa*	*E. coli*	*Shigella* spp.	*B. cepacia*
100%	60.30±0.03[a]	80.87±0.02[a]	85.60±0.02[a]	42.10±0.01[a]	40.60±0.05[a]	34.50±0.02[a]
90%	52.69±0.02[b]	72.34±0.04[b]	80.54±0.04[b]	37.29±0.04[b]	40.22±0.02[a]	31.12±0.03[b]
80%	42.20±0.04[c]	43.50±0.03[c]	76.30±0.03[c]	32.80±0.05[c]	39.90±0.02[a]	23.51±0.01[c]
70%	36.70±0.03[d]	27.77±0.04[d]	53.70±0.01[d]	26.80±0.03[d]	33.40±0.04[b]	17.02±0.02[d]
60%	30.18±0.03[e]	21.09±0.02[e]	29.76±0.02[e]	10.63±0.03[e]	15.96±0.01[c]	10.32±0.04[e]
50%	27.60±0.02[f]	16.21±0.01[f]	5.40±0.02[f]	0[f]	0[d]	0[f]
F-value	88126.8	692935	702931.2	303171	101311.1	89653.8
LSD	0.5	0.42	0.4	0.9	0.73	0.4
p-value	<0.0001	<0.0001	<0.0001	<0.0001	*	<0.0001

a,b,c,d,e and f: Significant difference in the statistical analysis, *: No significant difference was observed for the extracellular extract of *Lactobacillus delbrueckii* subsp. *bulgaricus* NCTC 12197 Tat concentration 80, 90 and 100% against *Shigella* spp. (p>0.005). Thus, using the crude extract as a treatment at 80% would be better than 100% treatment concentration for both safer utility and a nearly close *anti-shigella* activity

Table 5: Antibacterial assay for *Lactobacillus delbrueckii* subsp. *bulgaricus* DSMZ 20080T extracellular extract tested against some pathogenic bacteria

Treatment concentration	Inhibition (%)					
	S. aureus	*Salmonella*	*P. aeruginosa*	*E. coli*	*Shigella* spp.	*B. cepacia*
100%	66.30±0.02[a]	70.17±0.02[a]	67.90±0.05[a]	19.90±0.05[a]	17.60±0.02[a]	0[a]
90%	56.23±0.03[b]	69.96±0.01[ab]	66.33±0.03[b]	19.10±0.03[a]	8.22±0.02[b]	0[a]
80%	49.50±0.05[c]	69.60±0.04[b]	65.28±0.03[c]	16.40±0.03[b]	0[c]	0[a]
70%	47.60±0.02[d]	65.90±0.03[d]	15.50±0.02[d]	14.80±0.06[c]	0[c]	0[a]
60%	47.00±0.06[de]	64.70±0.02[e]	0[e]	12.50±0.01[d]	0[c]	0[a]
50%	46.40±0.04[e]	63.21±0.02[f]	0[e]	7.10±0.01[e]	0[c]	0[a]
F-value	116391	436162	444785.7	49543.7	50430	….
LSD	0.67	0.4	0.45	0.86	0.22	0
p-value	*	**	<0.0001	***	****	….

a,b,c,d,e and f: Significant difference in the statistical analysis, *: No significant difference was observed for the extracellular extract of *Lactobacillus delbrueckii* subsp. *bulgaricus* DSMZ 20080T between concentration 60 and 70% and between concentrations 50 and 60% against *S. aureus* (p>0.005), **: No significant difference was observed for the extracellular extract of *Lactobacillus delbrueckii* subsp. *bulgaricus* DSMZ 20080T between concentration 90 and 100% and between concentrations 80 and 90% against *Salmonella* spp. (p>0.005), ***: No significant difference was observed for the extracellular extract of *Lactobacillus delbrueckii* subsp. *bulgaricus* DSMZ 20080T at concentration 90 and 100% against *E. coli* (p>0.005), ****: No significant difference was observed for the extracellular extract of *Lactobacillus delbrueckii* subsp. *bulgaricus* DSMZ 20080T at concentration 50, 60, 70 and 80% against *Shigella* spp. (p>0.005)

Table 6: Cytotoxicity assay on human peripheral blood mononuclear cells

Treatments	Cytotoxicity (%)								
	2	6	8	10	12	14	16	18	20
Lactobacillus bulgaricus 761N	1.66±0.5	3.70±0.9	4.47±1.2	5.18±1.1	7.32±1.5	8.95±1.02	10.10±0.11	11.12±1.0	11.97±1.5
Lactobacillus fermentum DSMZ 20049	1.45±0.85	2.90±1.2	3.48±1.5	9.10±0.5	23.37±1.0	27.43±2.10	30.48±1.80	32.80±2.3	35.70±2.5
Lactobacillus delbrueckii subsp. *bulgaricus* NCTC 12197T	1.27±0.7	3.04±1.0	4.79±1.5	5.91±1.2	10.70±0.5	11.82±0.90	16.93±1.80	26.52±2.5	33.71±2.0
Lactobacillus delbrueckii subsp. *bulgaricus* DSMZ 20080T	2.59±1.0	4.07±0.8	7.41±1.0	8.89±1.1	9.11±1.0	9.94±0.9	10.74±1.5	11.59±1.2	12.01±2.1

Values are given in Mean±SD

Cytotoxicity assay: The extracellular extract of *Lactobacillus bulgaricus* 761N was observed to possess the least cytotoxicity on PBMC (Table 6) and that 16% concentration would be the selected non-toxic dose.

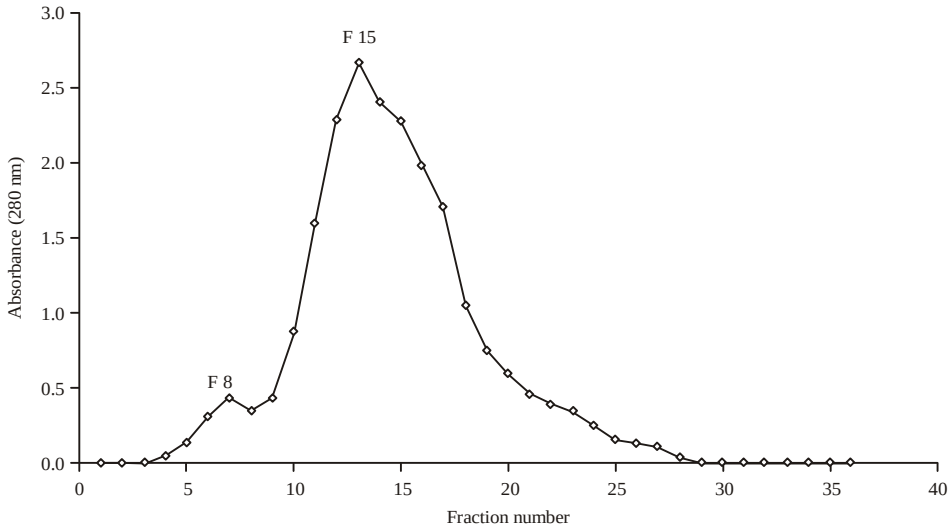

Fig. 1: Bacteriocin fractionation by size exclusion chromatography (El-Adawi *et al*., 2015)

Table 7: Antibacterial activity of fraction No. 8 against different pathogenic bacteria

Treatment concentration	Inhibition (%)					
	S. aureus	*Salmonella*	*P. aeruginosa*	*E. coli*	*Shigella* spp.	*B. cepacia*
100	80.61±0.02[a]	92.67±0.04[a]	75.49±0.03[a]	88.92±0.02[a]	96.80±0.02[a]	77.87±0.02[a]
90	70.99±0.03[b]	90.98±0.03[b]	71.39±0.02[b]	87.70±0.02[b]	90.42±0.02[b]	74.23±0.02[b]
80	67.28±0.04[c]	89.52±0.03[c]	67.29±0.02[c]	86.07±0.07[c]	85.94±0.02[c]	70.13±0.02[c]
70	56.83±0.03[d]	88.28±0.05[d]	64.13±0.03[d]	85.18±0.07[d]	82.27±0.02[d]	68.58±0.02[d]
60	54.3±0.02[e]	85.91±0.01[e]	61.32±0.02[e]	82.66±0.04[e]	77.96±0.02[e]	67.21±0.02[e]
50	33.73±0.03[f]	78.35±0.02[f]	61.06±0.06[e]	71.34±0.03[f]	73.00±0.02[f]	64.39±0.02[f]
F-value	139220.9	729533	540521	863053	519738	204016
LSD	0.5	0.32	0.41	0.8	0.91	0.82
p-value	<0.0001	<0.0001	*	<0.0001	<0.0001	<0.0001

a,b,c,d,e and f: Significant difference in the statistical analysis, *No significant difference was observed for the fraction No. 8 (obtained from purification process of extracellular extract of *Lactobacillus bulgaricus* 761N) against *P. aeruginosa* at concentrations 50 and 60% (p>0.005)

Fractionation of extracellular extract by size exclusion column chromatography: Two fractions were obtained following the fractionation of the semi-purified extracellular extract of *L. bulgaricus* 761N using size exclusion chromatography which was fraction No. 8 (F8) and fraction No. 15 (F15) as reported previously by our group (El-Adawi *et al*., 2015) (Fig. 1). Both fractions were subjected to SDS-PAGE and their molecular weights are 14.4 and 45 kDa, respectively.

Bioactivity testing: After the fractionation process, the antibacterial activity of the bacteriocin extracts of *L. bulgaricus* 761N for both fractions (F8 and F15) were tested. In case of fraction 8, the inhibition against four (*Salmonella*, *P. aeruginosa*, *E. coli* and *Shigella* spp.) out of six pathogenic strains was elevated (Table 7). In case of fraction 15, the inhibition against only two (*Salmonella* and *E. coli*) out of six pathogenic strains was elevated (Table 8).

DISCUSSION

The proteolytic system of lactobacilli is complex and is composed of proteinases and exopeptidases with different subcellular locations (Atlan *et al*., 1989). It was found that the extracellular fraction for all strains under study possessed the highest proteolytic activity. This is in agreement with Kok and Venema (1988), who reported that the cell-wall-bound proteinases of LAB perform the first steps in the hydrolysis of casein. Seo *et al*. (2007), however, observed that the first step in casein degradation is mediated by proteases in the cell membrane. Further degradation into smaller peptides and amino acids that can pass through cell membrane, is achieved by peptidases of LAB (Wohlrab and Bockelmann, 1992; Shihata and Shah, 2000). The findings of Kabadjova-Hristova *et al*. (2006) approved that *Lactobacillus* (*L. kefir* DR22x) had cell-wall-bound proteinases with an extracellular location.

The antibacterial activity of extracellular extract referring to bacteriocins was mentioned in many literatures. Earlier reports (Tagg *et al*., 1976; Daeschel and Klaenhamner, 1985; Sanni *et al*., 1999) have shown that some bacteriocins produced by gram-positive bacteria have a broad spectrum of activity. However, it was generally observed that bacteriocin from the producer organism had no inhibitory effect on the organism producing it. Tufail *et al*. (2011), have observed that culture supernatants that were obtained from the sixty out of

Table 8: Antibacterial activity of fraction No. 15 against different pathogenic bacteria

Treatment concentration	Inhibition (%)					
	S. aureus	*Salmonella*	*P. aeruginosa*	*E. coli*	*Shigella* spp.	*B. cepacia*
100	52±0.05[a]	91.66±0.02[a]	72.25±0.01[a]	88.86±0.04[a]	62.46±0.02[a]	69.67±0.07[a]
90	49.24±0.04[b]	91.54±0.04[a]	70.2±0.03[b]	87.42±0.02[b]	62.14±0.04[a]	68.67±0.05[b]
80	35.91±0.01[c]	89.06±0.04[b]	66.95±0.05[c]	85.18±0.05[c]	54.95±0.04[b]	66.67±0.02[c]
70	35.08±0.07[c]	88.05±0.05[c]	55.85±0.02[d]	78.26±0.06[d]	49.04±0.02[c]	64.48±0.03[d]
60	33.39±0.06[d]	86.13±0.02[d]	48.51±0.01[e]	65.23±0.03[e]	47.92±0.02[d]	55.46±0.03[e]
50	27.82±0.02[e]	68.66±0.06[e]	31.51±0.02[f]	46.27±0.04[f]	18.21±0.02[e]	32.15±0.02[f]
F-value	49090.3	704323	500521	859098	240375.5	170105
LSD	0.9	0.23	0.6	0.7	0.45	0.7
p-value	*	**	<0.0001	<0.0001	***	<0.0001

a,b,c,d,e and f: Significant difference in the statistical analysis, *: No significant difference was observed for the fraction No. 8 (obtained from purification process of extracellular extract of *Lactobacillus bulgaricus* 761N) against *S. aureus* at concentrations 70 and 80% (p>0.005), **: No significant difference was observed for the fraction No. 8 (obtained from purification process of extracellular extract of *Lactobacillus bulgaricus* 761N) against *Salmonella* at concentrations 90 and 100% (p>0.005), ***: No significant difference was observed for the fraction No. 8 (obtained from purification process of extracellular extract of *Lactobacillus bulgaricus* 761N) against *Shigella* spp. at concentrations 90 and 100% (p>0.005)

hundred isolates of *Lactobacillus* spp. involving *L. bulgaricus* isolated from yogurt exhibited varying degrees of inhibitory activity against strains of *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 10536, *Salmonella typhi* ATCC 19430, *Staphylococcus aureus* ATCC 6538 and *Vibrio cholerae* ATCC 25870. This is in agreement with the current research findings that observed a significant antibacterial capability against a range of pathogenic bacteria involving *E. coli*, *S. aureus*, *Pseudomonas aeruginosa*, *Salmonella* sp., *Burkholderia cepacia* and *Shigella* sp. These results are also in agreement with previous work carried out by Erdogrul and Erbilir (2006) but on different strain types, in which supernatants obtained from *Lactobacillus casei* and *L. bulgaricus* exhibited varying degrees of inhibitory activity against strains of *E. coli* ATCC 8739, *S. aureus* ATCC 6538, *Pseudomonas aeruginosa* ATCC 9027, *B. subtilis* ATCC 6633, *Klebsiella pneumoniae* ATCC 18833, *Salmonella typhimurium* ATCC 13311 and *Enterobacter cloacae* ATCC 13047. The probiotic potential of these bacteria is also vastly investigated (Gilliland, 1990; Cleveland *et al*., 2001; Mojgani and Ashtiani, 2006; Diez-Gonzalez, 2007).

Akpinar *et al*. (2011) have shown that *L. bulgaricus* produces a bacteriocin called Bulgarican that is inhibitory towards both gram-positive and gram-negative bacteria. Some inhibitory compounds against *Staphylococcus* and *Clostridium* species have also been found (Erkus, 2007). Ocana *et al*. (1999) observed by using transmission electron microscopy that the bacteriocin obtained from *L. salivarius* subsp. *salivarius* CRL 1328 causes vesiculization of protoplasm, formation of pores and complete disintegration of cells of *Enterococcus faecalis*. According to Marteau *et al*. (2001), the electrostatic interactions between the LAB and the negative charged phosphate groups on target cell membranes are thought to contribute to the initial binding, forming pores and killing the bacterial cells after causing lethal damage and autolysin activation to digest the microbial cellular wall.

Regarding the cytotoxicity, bacteriocins obtained from LAB has been mentioned in many literatures to have a very low cytotoxicity that agree with the current research findings. EL-Adawi *et al*. (2012) observed that extracellular extract of several LAB were safe when conducted the cytotoxicity assay on PBMC and had no TC50 even in the maximum concentrations which agreed with the current study results. Low molecular weight bioactive proteins, termed as lactoferrin, that was tested for its cytotoxicity on both HepG2 and PBMC, was found to be safe (El-Fakharany *et al*., 2008) indicating a very low cytotoxic effect. These findings approved our study results that these low molecular weight bioactive peptides contained in the extracellular extract exert a low cytotoxicity on PBMC.

The investigations of the current study concerning the purification process of the crude extract revealed the presence of two semi purified fractions whose molecular weights 14.4 and 45 kDa. These low molecular weight peptides are produced owing to the proteolytic activity of *L. bulgaricus* 761N performed in a casein-derived media. This agreed with the findings of (Kim *et al*., 2004) who reported that *L. bulgaricus* produced a bacteriocin of molecular weight 14.4 kDa, determined by SDS-PAGE and overlay method and that the mode of action for the antibacterial activity was bacteriocidal.

These later results approved our findings in that the bacteriocin obtained from the fractionation process was 14.4 kDa which is nearly the same as that obtained by Kim *et al*. (2004) and in possessing High antibacterial activity as well (Table 7). However, the other semi-purified fraction of molecular weight 45 kDa was not mentioned before has lower antibacterial activity (Table 8). Such variation in the potentiality of antibacterial activity between the small peptide (14.4 kDa) and a long peptide (45 kDa) might be explained if we take the three tables together, Table 2, 7 and 8. In case of gram-positive bacteria where the peptidoglycan makes up as much as 90% of the compact cell wall, the penetration of bacteriocin through the cell wall is much easier than penetration through the gram-negative bacteria where the cell wall is more chemically complex and less permeable. Accordingly, the antibacterial activity in case of small peptide fraction No.8 is higher against gram-ve bacteria (*Salmonella*, *P. aeruginosa*, *E. coli* and *Shigella* spp.) than both extracts, the crude extract (Table 2) and the fraction No. 15 (Table 8). On

the other hand, the antibacterial activity against gram-+ve bacteria (*S. aureus*) is nearly the same for the crude extract (Table 2) and both fraction extracts (Table 7 and 8).

In brief, for broad antibacterial activity spectrum against both gram-ve and gram+ve bacteria, we suggest the small peptide fraction No.8 (14.4 kDa) as a promising candidate for such purpose.

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