PRODUCTION OF PLANTARCIN BY *LACTOBACILLUS PLANTARUM* SR18

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ABSTRACT

Out of 86 lactobacilli previously screened in our laboratory, *Lactobacillus plantarum* SR18 isolated from yoghurt revealed the largest detected inhibition zone against the selected indicator *Streptococcus salivarius* 5. The obtained electrophoretic patterns revealed that *L. plantarum* SR18 was free from plasmids. Exposure of 6 h growing *L. plantarum* culture to T-8M, 3B ultraviolet B lamp (8w, 220v & 312 nm) for 2 h and subsequent growth for further 24 h resulted in an increase of cell-bound bacteriocin titer reached 2 fold at 12 h. Whereas bacteriocin secreted in the culture filtrate was not affected by UV irradiation. Plantarcin SR18 production was maximal (12800 AU/ml) between 12 and 18 h by incubation of the culture at 37°C and pH 5-7 in candle jar (CO_2). The bacteriocin bound to the cells and that secreted into the culture filtrate of *L. plantarum* SR18 were precipitated by 75% ammomium sulphate, dialysed and further purified by Gel filtration on Sephadex G-100. The specific activities (AU/mg protein) were increased by a factor of about 5.3 and 2.35 for plantarcins purified from proteins bound to the cell of *L. plantarum* SR18 (plantarcin SR18 a) and that secreted into the culture filtrate (plantarcin SR18 b), respectively. Gel filtration of plantarcin SR18a resulted in moderate antibacterial activity (3200 AU/ml) and very high activity (25600 AU/ml) of plantarcin SR18b.

Keywords: *Lactobacillus plantarum,* Bacteriocin, plasmid, UV induction

INTRODUCTION

Bacteriocins are regarded as the next generation of antibiotics on account of their narrow-spectrum bactericidal activities. Many attentions have been paid to colicins; those prototype bacteriocins produced by various members of the family *Enterobacteriaceae* because they are believed to be safe in regard to human body **(Li et al., 2010).** Bacteriocins as bactericidal polypeptides that are lethal against closely related species were identified **(Mills et al., 2011).** They are small molecular weight proteins, often heat-resistant and the structural genes encoding bacteriocins are usually plasmid-linked. The structure of bacteriocins consists of bacterial peptides with specific activity against competing species in addition to carbohydrate and/or lipid moieties **(Venugopal et al., 2011).** Generally, bacteriocins exert their antimicrobial action by interfering with the cell wall or the membrane of target organisms by binding to cell surface receptors **(Bharathi et al., 2011).** Their bactericidal mechanisms vary and may include pore formation **(Todorov, 2008)**, degradation of cellular DNA **(Bastos et al., 2010)**, disruption through specific cleavage of 16S rDNA, and inhibition of peptidoglycan synthesis subsequently resulting in death **(Sullivan et al., 2002)**.

The proteinaceous nature of these antimicrobial molecules as well as their natural occurrence in nature has promoted consideration for their use in foods to prevent microbial food borne diseases and bacterial food spoilage **(Nissen-Meyer et al., 2010).** Lactic Acid Bacteria (LAB) are considered as Generally Recognised as Safe (GRAS) microorganisms that have been used in the processing of fermented food for centuries **(Gharaei-Fathabad and Eslamifar, 2011)**. Many lactic acid bacteria produce broad-spectrum bacteriocins, some of which could provide valuable alternatives to traditional therapeutic antibiotics for the treatment of infectious diseases. Bacteriocins (antimicrobial peptides) have been produced from various strains which was isolated from different habitat such as: infant faeces **(Kawai et al., 2001)**, fermented meat products **(Valenzuela et al., 2009)**, milk **(Savadogo et al., 2006)**, cheese **(Nespolo and Brandelli, 2010)**, fermented cucumber **(Pal and Ramana, 2010)** and smoked salmon **(Todorov et al., 2010)**.

The objective of this work was to produce a bacteriocin from some *Lactobacillus* species isolated from different sources under different culture conditions, partial purification, characterization and testing the antimicrobial activity of the selected bacteriocin.

MATERIAL AND METHODS

Bacterial isolates

Out of 86 tested isolates of lactic acid bacteria cultivated on MRS medium containing bromocresol green, *Lactobacillus* Y18 obtained from yogurt was chosen as the highest bacteriocin producing bacterium and *Streptococcus salivarius* 5 was selected as the indicator strain. All indicator strains were provided from Microbiology Department, Faculty of Pharmacy, Tanta University, Egypt.

Medium

MRS agar medium was used for solid plates and MRS broth was used as a culture medium for detection and production of bacteriocin. It is based on formulation of de Man, Rogosa and Sharpe (MRS). This medium supports luxuriant growth of lactobacilli from oral, fecal, dairy and other sources **(De Man et al., 1960)**. The medium composed of (g/l); peptone 10 g, beef extract 10 g, yeast extract 5 g, glucose 20 g, sodium acetate 5g, tween 80 1ml, potassium phosphate 2 g, ammonium citrate 2 g, magnesium sulfate 0.1 g, manganese sulfate 0.05 g and agar 15g for solidification. The medium was adjusted at pH 6.5.

Screening for bacteriocin production

The recovered isolates were subjected to bacteriocin production test against different indicator strains by the double –layer method. The bacterial isolates to be tested were grown to form colonies on MRS solid medium. After overnight incubation, the bacterial growth was thoroughly removed by sterile cotton swab. The residual growth, if any, was killed by exposing the plates to chloroform vapors for 15 min then left to dry. The plates were then overlaid with 4 ml soft agar seeded with 0.5ml (10^7 CFU/ml) of the indicator strains. The cultured plates were further incubated for 24 h at 37 ºC in air and in a candle jar just for anaerobic indicator strains.

After incubation period, all plates were visually examined. The resultant growth inhibition zones around the colonies were measured using a ruler and considered an indicative of bacteriocin production **(Expert and Toussaint, 1985)**. The bacterial isolate which found to be most producing for bacteriocin was selected.

Characterization of the selected isolate

The selected isolate was subjected to different morphological, physiological and biochemical tests for identification to the species level according to the procedures described in the Bergey's Manual **(Klander and Regular, 1986)**.

Plasmid detection

Plasmids were prepared by a modified Brinbain and Doly's method **(Manniatis, 1982)**. Agarose gel electrophoresis was performed using 0.8% agarose. To avoid overheating and the resolve high molecular weight plasmids, a low salt buffer system (Tris acetate buffer) was used agarose in the same buffer was melted in a boiling water bath and mixed well before pouring into the electrophoresis tray to a depth of 7 mm. Sample of plasmid preparation of the tested bacteria and standard molecular weight plasmid were loaded into wells in the gel, made by a comb inserted during the casting of the gel. Electorphoresis was performed on a horizontal apparatus (Pharmacia, Sweden) and it was run at 60 volts for 6 hours or 15 volts overnights. The gel was stained with a solution of ethidium bromide (Sigma, USA), (0.5 µg/ml) in tris acetate buffer for 30 min at room temperature in a dark place. Plasmid DNA was visualized using a UV transilluminator. Photographs were taken by a polaroid camera with 667 instant films (Polaroid CO., USA), exposed through a tiffen 15 orange filter **(Meyers et al., 1976)**.

Factors affecting the production of bacteriocin

1. UV induction of bacteriocin synthesis

Bacteriocin of the producer isolate was examined for increasing of the biosynthesis by induction of a log-phase culture with UV light. Irradiation was carried out on cells that had been grown in MRS broth at 37ºC for 6 h., centrifuged and re-suspended in 1/10 volume of saline. Portions (5 ml, containing about 5×10^4 cells/ ml) were irradiated in open glass Petri dishes by using T-8M, 3B ultraviolet-B lamp (8 w, 220 v & 312 nm) at a distance of 25 cm for 2 hours. The irradiated suspension was diluted 10-fold into fresh broth and incubated at 37ºC for 24 hours. The cells were removed by centrifugation and bacteriocin was assayed by the agar well diffusion method **(Expert and Toussaint, 1985).**

2.   Effect of the growth phase on the production of bacteriocin

Growth experiments were performed in flask of containing 100 ml of (M6) (pH 6.5) at 37°C without shaking. An overnight pre-culture of tested strain was used for the inoculation of the MRS broth at initial cell density of 1×10^7 CFU ml^{-1}. At different time intervals, samples of 5ml were obtained from the culture, the final pH was detected and the culture was centrifuged at 4000Xg for 10 min. The supernatant was assayed for its bacteriocin contents by the agar well diffusion method and the precipitate was dried at 80 °C for 48 h ,then the dry weight was estimated **(Mezaini et al., 2009)**.

3.   Effect of growth temperature on the bacteriocin production

The tested strain was grown in 5 ml sterile portions of (M6) tubes at different incubation temperature (28, 30, 37, 40 and 45°C) for 18 h in a candle jar. The final pH was detected, the treated cultures were centrifuged at 4000 X g for 10min.The supernatant was assayed for its bacteriocin content by the agar well diffusion method and the precipitate was dried at 80 °C for 48 h ,then the dry weight was estimated **(Abo-Kamar, 1992)**.

4. Effect of different pH values on bacteriocin production

The tested strain was cultivated in (M 6) tubes adjusted to pH values (4, 5, 6, 7, 8, 8.5 and 9) using predetermined microvolume of phosphoric acid and sodium hydroxide solutions. After incubation for 18hr at 37°C in a candle jar, the final pH was detected and the obtained cultures were readjusted to pH (6.5). The treated cultures were centrifuged at 4000×g for 10 min. The supernatant was assayed for its bacteriocin content by the agar well diffusion method and the precipitate was dried at 80 °C for 48 h, then the dry weight was estimated **(Abo-Kamar, 1992)**.

5. Effect of gaseous condition of incubation on bacteriocin production

The tested strain was grown in 5 ml sterile portion of (M6) tubes in air and in presence of 5% CO_2 (in candle jar) at 37°C for 18 h. After incubation period, The supernatant was assayed for its bacteriocin content by the agar well diffusion method and the precipitate was dried at 80 °C for 48 h, then the dry weight was estimated **(Abo-Kamar 1992)**.

Isolation and purification of the active bacteriocin

1. Ammonium sulphate precipitation

The bacteriocin was partially purified from two liters cultures of the tested organism. Cells were grown to stationary phase in MRS broth at 37ºC in a candle jar and then collected by centrifugation (2.500 x g at 4º C, for 10 min). The culture supernatant was filter-sterilized, ultrafiltered on 1.000 cut-off membrane to concentrate 10-fold. The supernatant (extracellular bacteriocin) purified directly but the precipitates (cells) were subjected to NaCl extraction of cell bound bacteriocin.

2. Extraction of cell-bound bacteriocin

The bacteriocin activity in the culture was also found in the cell pellet. The extracellular nature of the bacteriocin permitted the extraction of activity without disruption of the cells by simply suspending the cell pellet in 1 liter of 0.05 M KPO_4, pH 7.0, containing 1 M NaCl. The cells were mixed with the extractant for 1h, and the suspension centrifuged at 10.000 x g for 20 min. The milky supernatant fluid was decanted and sterilized by shaking with 5ml of chloroform. Usually, over 90% of the activity in the cell pellet was solubilized by this procedure. Residual bacteriocin could be solubilized by simply repeating the NaCl extraction **(Foulds, 1972)**.

Both cell bound and culture supernatant bacteriocins were treated with ammonium sulphate to 75% saturation with constant stirring under cooling. The preparation were then left overnight at 4º C and the precipitate was collected by centrifugation at 16.300 g for 20 min , re-dissolved in 5ml of 0.05M phosphate buffer (pH 7.2) and stored in a refrigerator, until used **(El-Shouny, 2006)**.

Statistical analysis

The obtained results were statistically analyzed using the two ways analysis of variance (ANOVA) to determine the degree of significance for the variations between the treatments, F test and the LSD at 0.05 level was calculated for treatment means and their interactions. All of the statistical methods were according to the method described by **Bishop (1983)**. Each value presented in the tables is the means of three readings ±the standard

deviation (SD).The values are highly significant (*) at P<0.001, significant at P=0.001 and insignificant at P> 0.001. The least significant difference is abbreviated as LSD.

RESULTS AND DISCUSSION

Selection and identification of bacteriocin producing bacterium

Out of 86 tested organisms, *Lactobacillus* sp. Y18 isolated from yoghurt revealed the largest detected inhibition zone against the selected indicator *Streptococcus salivarius* 5. Therefore, it was chosen as the most active bacteriocin producer in the current work. The result obtained from morphological, physiological and biochemical tests suggested that the bacterial isolate could be an isolate of *Lactobacillus plantarum* SR18 **(Klander and Regular, 1986, Ragy, 2012)** and the produced bacteriocin named as plantarcin SR18. Bacteriocin producing lactobacilli were previously isolated and identified; *L. plantarum* TF711 was isolated from raw Tenerife goats' cheese **(HernÃ¡ndez et al., 2005)** and *L. plantarum* AA135 was isolated from Egyptian home-made yogurt **(Abo-Amer, 2007)**.

Test of plasmid presence in *Lactobacillus plantarum* SR18

Gel electrophoresis of plasmid DNA extracted from strain *Lactobacillus plantarum* SR18 was performed to study the plasmid profiles of such selected isolate. The obtained electrophoretic patterns (Fig. 1) revealed that *Lactobacillus plantarum* SR18 was free from plasmids which indicated that the antagonistic compound's production is encoded by chromosomal genes. There were many findings which agreed with our result; plantaricin produced by *L. plantarum* ST13BR **(Todorov and Dick, 2004),** plantaricin produced by *L. plantarum* AA135 **(Abo-Amer, 2007)**, plantaricin ST31 produced by *L. plantarum* ST31 **(Todorov and Franco, 2010)** as well as bacteriocins produced by *L.brevis* NM 24 and *L. fermentum* NM 332 **(Mogani et al., 2009).**
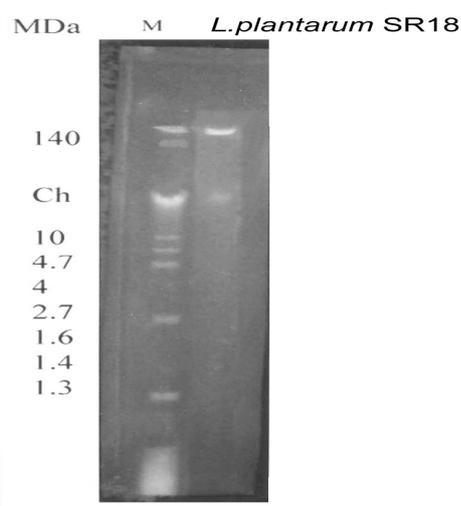
Figure 1 Agarose gel electrophoresis of plasmid DNA of the selected *Lactobacillus plantarum* SR18 and the standard marker

Factors affecting the production of plantarcin SR18

1. Induction of plantarcin SR18 synthesis

The activities of Plantarcin SR18 (Cell-bound and Culture filtrate) assayed from non induced cultures of *L. plantarum* SR18 grown in (M 6) were ranged between 400 and 12800 AU/ml. Plantarcin SR18 production was enhanced by UV irradiation relative to the control culture. Exposure of 6 h growing culture to T-8M, 3B ultraviolet B lamp ((8w, 220v & 312 nm) for 2 h and subsequent growth for further 24 h resulted in an increase of cell-bound bacteriocin titer reached 2 fold at 12 h. Whereas bacteriocin secreted in the culture filtrate was not affected by UV irradiation except an early production of bacteriocin after 8 h of incubation (Table 1). Similar results were obtained by **Expert and Toussaint (1985)** who greatly improved the biosynthesis of bacteriocin of *Erwinia carotovora* and *Erwinia uredovora* to 4-8 fold by the optimal dose of UV light 100-150 erg/mm2. On the other hand, **Ogunbanwo et al. (2003)** found that induction by UV-light did not affect the activity of bacteriocin produced by *L. plantarum* F1 and *L. brevis* OG1.

Table 1 Induction of plantarcin SR18 synthesis

Induction	Source of bacteriocin	Plantarcin activity (AU/ml) at various time after induction (h)					
		4h	6h	12h	16h	18h	24h
None	Cell-bound	0	0	400	400	800	400
None	Culture filtrate	0	0	12000	12000	12800	6400
UV	Cell-bound	0	0	800	800	800	400
UV	Culture filtrate	0	800	12000	12000	12800	6400

Two ways analysis of variances			
Source	**DF**	**F Value**	**P**
Induction	1	7135.42*	0.0001
Source of bacteriocin	1	924.75*	0.0001
Various time	5	6155.87*	0.0001
Induction x Source of bacteriocin	1	6304.86*	0.0001
Induction x Various time	5	2164.03*	0.0001
Source of bacteriocin x Various time	5	217.49	0.0001
Induction x Source of bacteriocin x Various time	5	2220.54	0.0001
Least Significant Difference = 105.84			

Legend: *Highly significant at $P < 0.001$
Each value is the mean of three readings

2. Effect of growth phase of Lactobacillus plantarum SR18

Quantitative production of the plantarcin SR18 from induced *Lactobacillus plantarum* SR18 was assessed at different time intervals during the different growth phases of the producing organisms as presented in table (2). The growth of the organism was followed by measurement of the dry weight, while the activity of bacteriocin was determined by the agar well diffusion.

Table 2 Production of plantarcin from *Lactobacillus plantarum* SR18
at different incubation periods

Time (h)	Final pH	Growth D.wt. (g/l)	Activity of plantarcin (AU/ml)
0	6.5±0	0 ± 0	0 ± 0
6	6±0.2	0.34±0.01	800 ± 20
12	4.5±0.17	1.56±0.02	12000±600
18	3.8±0.15	2.5±0.1	12800±100
24	3.6±0.12	2.9±0.1	6400±200
30	3.4±0.12	2.9±0.1	3200±100
36	3.2±0.15	2.9±0	1600±50
42	3±0.17	2.9±0.1	800±30
48	3±0.16	2.9±0.1	400±10
F value	416.93*	748.39*	*1200.18
P	0.0001	0.0001	0.0001
L.S.D	0.2598	0.1761	510.076

Legend: *Highly significant at P< 0.001
Each value is the mean of three readings ± SD

Generally, plantarcin SR18 production started at the pre-and early exponential growth phases and reached a maximum level at the stationary phase. Accumulation in the medium was maximal between 12 and 18 h incubation. The bioactivity dropped 50% after 24 h and 90% after 48 h incubation. During 48 h of growth, the pH of the medium significantly decreased from pH=6.5 to 3.0 and the cell dry weight showed significant increase from 0 to 2.9 (g/l). Early production was supported by **Chin et al. (2001)** for plantaricin Y and **Todorov and Dicks (2004)** for bacteriocin of *L. plantarum* ST11BR. Thus, we can conclude that extended growth does not necessarily lead to increased production or activity levels. A similar decrease in the plantarcin activity with the increase of incubation time has also been observed for plantarcin ST13BR **(Todorov and Dicks, 2004)** and **(Leroy and De Vuyst, 2002)**.

3. Effect of growth temperature on plantarcin SR18 production

The production of plantarcin SR18 under test was measured at 28, 30, 37, 40 and 45 °C after18 hours of incubation. Within the range tested, increasing the temperature increased the plantarcin yield. From table (3), it is clear that the maximum plantarcin activity was obtained at 37°C (12800 AU/ml). After, 40°C, biomass and plantarcin SR18 activity decreased constantly with the increase of temperature. **Ogunbanwo et al. (2003)** concluded that the use of constituted medium at 30ºC incubation temperature fostered the best

production of bacteriocin by *Lactobacillus brevis* OG1. At 45ºC, neither bacterial growth nor bacteriocin production was detected. Also, **Abo-Kamar (1992)** mentioned that viridin production by *Streptococcus* isolates increased by increasing temperature between 34 and 37ºC. At 40ºC, neither bacterial growth nor viridian production was detected. However, **Karthikeyan et al. (2009)** reported that the maximum arbitrary unit of bacteriocin produced by *L. plantarum* was measured at 40°C.

Table 3 Effect of incubation temperature on plantarcin SR18 production by *Lactobacillus plantarum* SR18

Incubation temperature ºC	Final pH	Growth D.wt. (g/l)	Plantarcin activity (AU/ml)
28	3.8±0.1	2.55±0.05	3200±100
30	3.7±0.1	2.8±0.1	6400±200
37	3.6±0.1	2.9±0.1	12800±100
40	3.6±0.1	2.7±0.1	6400±50
45	4.1±0.1	1.19±0.05	800±40
F Value	10.50*	212.43*	4762.56*
P	0.0013	0.0001	0.0001
L.S.D	0.2588	0.2165	292.99

Legend: *Highly significant at P< 0.001
Each value is the mean of three readings ± SD

4. Effect of different pH values on plantarcin SR18 production

The data shown in table (4) indicated that the bacterial growth and plantarcin production by the producer *L. plantarum* SR18 took place at pH 4-8 but not higher than pH 8.5. The highest yield (12800 AU/ml) of plantarcin SR18 activity was recorded at pH 5-7. Lowest level of plantarcin activity (approximately 400 AU/ml) was detected at pH 8.5, while no production of plantarcin was observed at pH 9 as the growth sharply decreased. Similarly, **Todorov and Dicks (2005)** reported that in MRS broth adjusted to pH=5.5, 6.0 or 6.5, maximum bacteriocin production (12800 AU/ml) was recorded. However, at pH=4.5 low levels of bacteriocin ST194BZ (3200 AU/ml) were obtained.

Table 4 Effect of pH on the plantarcin SR18 production by *Lactobacillus plantarum* SR18

Initial pH	Final pH	Growth D.wt. (g/l)	Plantarcin activity (AU/ml)
4	3.7±0.1	2.35±0.08	6400±200
5	3.7±0.1	2.7±0.1	12800±100
6	3.6±0.04	2.7±0.1	12800±100
7	3.6±0.1	2.8±0.1	12800±200
8	5±0.15	2.5±0.1	3200±100
8.5	8.4±0.25	1.2±0.1	400±10
9	8.8±0.2	0.34±0.01	0±0
F Value	730.64*	330.70*	6619.44*
P	0.0001	0.0001	0.0001
L.S.D.	0.3644	0.2184	304.83

Legend: *Highly significant at P< 0.001
Each value is the mean of three readings ± SD

5. Effect of gaseous condition of incubation on plantarcin SR18 production

The productivity of the selected isolate changed by incubation under aerobic condition and partially anaerobic (candle jar) as shown in table (5). The maximum production (12800 AU/ml) was obtained on incubation in candle jar (CO_2). But lower production (6400AU/ml) was recorded on incubation in air. **Leal-Sánchez et al. (2002)** reported that the best conditions for bacteriocin production by *L. plantarum* LPCO10 occurred with no aeration. Also, **Karthikeyan and Santosh (2009)** observed that the maximum arbitrary unit of bacteriocin produced by *L. plantarum* was measured on incubation at optimum conditions without aeration.

Table 5 Effect of gaseous condition of incubation on the production of plantarcin SR18 at 37ºC by *Lactobacillus plantarum* SR18

Incubation condition	Final pH	Growth D.wt. (g/l)	Plantarcin activity (AU/ml)
In air	3.6±0.1	2.4±0.1	6400±200
In candle jar (CO_2)	3.6±0.05	2.7±0.1	12800±100
F Value	0.0*	28.61*	2457.60 *
P	1.0	0.0005	0.0001
L.S.D.	0.2972	0.3759	594.39

Legend: *Highly significant at P< 0.001
Each value is the mean of three readings ± SD

Isolation and partial purification of the active plantarcin SR18

The bacteriocin bound to the cells and that secreted into the culture filtrate of *L. plantarum* SR18 were precipitated by 75% ammomium sulphate, dialysed and further purified by Gel filtration on Sephadex G-100. Table (6) summarizes the purification steps. The

specific activities (AU/mg protein) were increased by a factor of about 5.3 and 2.35 for plantarcins purified from proteins bound to the cell of *L. plantarum* SR18 (plantarcin SR18 a) and that secreted into the culture filtrate (plantarcin SR18 b), respectively.

Gel filtration of plantarcin SR18a resulted in two peaks for the precipitated cell bound proteins. The large peak showed antibacterial activity at fractions number (16-21) with moderate activity (3200 AU/ml) but the small one did not. From two peaks obtained from gel filtration of plantarcin SR18b (culture filtrate proteins), only one peak at fractions number (10-15) showed bacteriocin with very high activity (25600 AU/ml). This is in agreement with the results of **Jones et al. (2008)** as they concluded that the bacteriocin produced by *Lactococcus lactis* appeared to be either cell-associated or molecules released extracellularly.

Table 6 Purification of plantarcin from *Lactobacillus plantarum* SR18

Purification step	Volume (ml)	Plantarcin Activity AU/ml	Protein mg/ml	Specific Activity AU/ mg protein	Purification fold
Cell bound plantarcin					
NaCl extract	50	800	4.5	177	1.0
Amm. sulphate (75%)	5	400	ND	ND	ND
Sephadex G-100	3	3200	3.4	941	5.3
Culture filtrate plantarcin					
Crude filtrate	50	12800	27	474.07	1.0
Amm. Sulphate (75%) G-100	5	6400	82.1	77.95	0.16
Sephadex G-100	3	25600	23	1113.04	2.35

Legend: Amm. Sulphate- ammonium sulfate

ND- not detected

CONCLUSION

As *L. plantarum* SR18 is a non-pathogenic bacterium therefore, the produced plantarcin SR18 would be investigated as food preservative and as a therapeutic agent. Further confirmatory studies on the purity, characterization and safety of the herein obtained plantracin are carries out in our laboratory.

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