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ChemTech

International Journal of ChemTech Research
CODEN (USA): IJCRGG ISSN : 0974-4290
Vol.6, No.4, pp 2507-2520, July-Aug 2014

Isolation and Characterization of BacteriocinProducing Lactic Acid Bacteria from some Syrian fermented foods

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Abstract: A total of 25 isolates of lactic acid bacteria from different Syrian dairy products, pickels and a dried plant samples were obtained and tested for their antibacterial activity against four pathogens one gram positive bacteria (*Staphylococcus aureus*) and three gram negative bacteria (*Klebsiella pneumoniae, Escherichiacoli* and *Pseudomonas aeruginosa*). The cell free supernatants of fifteen LAB isolates demonstrated significant antibacterial activity against the four tested pathogens. Results of the standard physiological and biochemical tests identified fourteen isolates as *Lactobacillus plantarum* and one isolate as *Lactobacillus fermentum*.All fifteen isolates were mesophilic and were able to grow in the presence of 4 % NaCl and in pH values ranging from 4-9.The antibacterial activity of the CFSsof the most active six isolates were significantly influenced after treatment with proteinase K and after neutralization, while no change occurred after heat treatment at 60°C and 80°C for 15 minutes.Two of the tested *L. plantarum*isolates (SH4 and CK56) had the structural genes for both bacteriocins plantaricin EF and plantaricin N, and two of them (CK57 and CP44) had the structural genes for plantaricin N only.
Key words: Lactic acid bacteria, Plantaricin, Antibacterial effect.

Introduction

Lactic acid bacteria (LAB) are among the most important groups of microorganisms used in food fermentation where they play an essential role and a wide variety of strains are routinely employed as starter cultures in the manufacture of dairy, meat, vegetable and bakery products[1,2]. One of the most important contributions of these microorganisms is the extended shelf life of the fermented products. Growth of spoilage and pathogenic bacteria in these foods is inhibited due to competition for nutrients and the presence of starter-derived inhibitors such as lactic acid, hydrogen peroxide, diacetyl and bacteriocins[1,3].

Bacteriocins are antimicrobial peptides or proteins produced by strains of diverse bacterial species. The antimicrobial activity of this group of natural substances against foodborne pathogens, as well as spoilage bacteria, has raised considerable interest for their application in food preservation[1,4,5]. In the past years, a lot of work has aimed to detect, purify and characterize bacteriocins, as well as their application in food preservation strategies. Application of bacteriocins may help reduce the use of chemical preservatives and/or the intensity of heat and other physical treatments, satisfying the demands of consumers for foods that are fresh tasting, ready to eat, and lightly preserved. In recent years, considerable effort has been made to develop food applications for many different bacteriocins using bacteriocinogenic strains[1,3,5,6].

According to the importance of bacteriocins as food preservatives we will focus in this study on the isolation and characterization of bacteriocin producing local lactic acid bacteria isolates, beside the activity of these strains against several spoilage and pathogenic bacteria, choosing the best isolate which which has the best antibacterial activity.

Materials and methods

Isolation and of lactic acid bacteria

Fifty one samples were collected from different sources of local foods including: Dairy products, pickles, dried plant. Samples were cultured using the dilution pour plate method, for this purpose, 2 grams of each sample were weighed aseptically and homogenized in 18 ml of sterile salt solution(0.1% NaCl) using the vortex. Then, sequential decimal dilutions of the homogenate were obtained. One ml of each aliquot dilution was used, for the isolation of LAB surface plated MRS agar and M17 agar were incubated anaerobically for 72 h at 37°C, plates with colony forming units (CFU) ranging from 30 and 300 were selected for enumeration[7].The colonies were randomly picked from plate and purified by successive streaking on MRS agar media before being subjected to characterization.

Gram-positive and catalase-negative isolates were isolated and characterized by phenotypic criteria[8]. The isolates were stocked on MRS agar slant at4°C and sub-cultured monthly.

Test micro organisms

Pathogens used for testing antibacterial activity were gram positive bacteria (*Staphylococcus aureus*) and gram negative bacteria (*Klebsiella pneumoniae, Escherichia coli* and *Pseudomonas aeruginosa*) received from the collection of microbiology laboratory (National Commission for Biotechnology, Syria).

Antibacterial activity of isolated LAB

The pathogenic bacteria were inoculated on nutrient blood agar then in nutrient broth for 24 hours and incubated at 37°C. Lactic acid bacteria were inoculated in MRS broth for bacilli and M17 for cocci for 48 hours at 37°C. Five microliters of each tested isolate were spotted on MRS/M17 agar, plates were incubate for 48 hours anaerobically at37°C, a layer of Mueller Hinton agar was poured and left till it could well solidify, plates were then left in the refrigerator (4°C) for about two hours to allow the antibacterial substance to disperse on the Mueller Hinton agar layer, the indictor (target) isolates were then spread with cotton swap on the Mueller Hinton agar. The plates were then incubate 24 hours aerobically at 37°C, bacteriocin-positive cultures displayed a halo of clearing in the lawn around the original button of growth, bacteriocin-positive isolates were selected for further study[9, 10].

Antibacterial Activity of the Cell Free Supernatant

Lactic acid bacteria were inoculated in MRS broth for 48 hours at 37°C. Cell free supernatants were obtained by centrifugation of the liquid culture (8000 rpm for 20 minutes at 4°C). For screening of the antibacterial activity of the cell free supernatants, they were tested for their antibacterial activity using the well diffusion method[11]with some modification. Five milliliters diameter wells were loaded with 250 µl of each of the cell free supernatants. Wells were prepared in the Mueller Hinton agar previously seeded with the test isolates. The plates were then incubated at 37°C for 24 h after which the diameter of inhibition zones was determined.

Physiological and biochemical characterizations of selectedLAB

All isolates active against tested pathogens were characterized by their carbohydrate fermentation pattern using the API 50 CHL strips according to the manufacturer's instructions (API system, Bio-Merieux, France). For the identification of isolates into species, resulting patterns were analysed with API computer program (Bio Merieux) which discriminates between species on the basis of a pattern matching principle [12, 13].

The strains were further tested for salt tolerance incubation for 48h at 37°C in MRS broth supplemented with 4% and 6.5% NaCl[14].

The Growth at different temperatures was observed in MRS broth after incubation for 7 days at 4 °C, 10 °C, 37°C, 40 °C and 45 °C. The determination of their fermentative type was also done in MRS broth with Durhambell[14].

Partial characterization of inhibitory substances in supernatant

Selected LAB strains were grown in MRS broth for 48 h at 37°Cand the cell free supernatant was obtained by centrifugation of the liquid culture (8000 rpm for 20 minutes at 4°C). Some samples were neutralized with 3M NaOH to pH 7.0 to eliminate the action of acid, and were then used for the antimicrobial

activity using the agar well diffusion method as described above, plates were then incubated at 37°C for 24 h after which the diameter of inhibition was determined[15].

In order to test the sensitivity of CFSs inhibitory substances to proteinase K CFSs were incubated for 24 h with the enzyme at a final concentration of (1mg/ml) at 37°C. The treated CFS samples were then tested for their antibacterial activity using the well diffusion method as described above[15].

The heat sensitivity was determined by heating aliquots of CFSs preparation (5ml) at 60, 80 and 100°C for 15 min prior to antibacterial activity evaluation[15].

Detection of Plantaricin Genes by PCR

Genomic DNA Isolation

Genomic DNA was prepared using Cardinal *et al* (1997)[16] procedure[17]. The method was modified by omitting the use of enzymes. Three ml overnight cultures were prepared in MB broth. Cells were harvested in a microfuge for 5 min at 6000 rpm. After that cells were resuspended in 500 µl TE buffer (pH 8) containing 10% SDS. The cell suspensions were then incubated for 2 h at 55 °C in a water bath. After incubation, chloroform extraction was performed twice using an equal volume of chloroform (chloroform/isoamyl alcohol:24/1), and samples were incubated for 0.5 h at -20° C then centrifuged for 10 min at 7000 rpm to eliminate cellular debris. The aqueous phase was transferred into a clean eppendorf tube and the genomic DNA was precipitated by the addition of one and half volume of ethanol 96% and incubated for 0.5 h at -20° C then centrifuged for 10 min at 7000 rpm. The precipitated DNA was washed once with 100 µl of 70% ethanol. DNA was pelleted by centrifugation for 10 min at 6000 rpm. Ethanol was removed and the pellets were dried for 30 min at 37°C. Dried DNA pellets were dissolved in 100 µl TE for 24 h at 37°C, after which they were stored at -20°C.

PCR amplification of plantaricin genes

For screening ofplantaricin structural genes, amplification by PCR was used to amplify genomic DNA of the six most active isolates of *L. plantarum*. Plantaricin genes were amplified in 25 µl volumes each containing 200 ng template DNA, 12.5 master mix and 25 pM of each forward and reverse primer. PCR amplification of the bacteriocin genes was carried out using the primers for the described plantaricin genes using the amplification conditions as shown in Table 1. The PCR reactions were performed with an initial denaturation step at 94 °C for 3 min, followed by 32 cycles of 94 °C for 1 min at a different primer annealing temperature for 1 min and 72 °C extension for 30 sec, followed by a final extension step at 72 °C for 6 min[18].

Table 1: PCR primers used for amplification of plantaricin genes

Target	Annealing temp (°C)	Amplicon size (bp)	Primer sequence
plnJK	56	306	F: ACG GGG TTG TTG GGG GAG GC R: TTA TAA TCC CTT GAA CCA CC
plnEF	60	365	F: GGT GGT TTT AAT CGG GGC GG R: ACT TGA TGG CTT GAA CTA TCC
plnW	58	387	F: CTA GTC GTC GTA AGA ATG CT R: CTT GGC ATT CAT GTG ACA AGG
plnNC8	56	344	F: CAA ATT GAG GGC GGA TCA GTC R: TAA TCA CAC TGA ACA TCT CTA A
pln1.25β	50	249	F: TTA GCA TTG ATT GAT GGA GGA R: GCA TCC TAT GTG AGG CTG CTG
plnS	54	460	F: ATG CTG TTA TCG GTG GGA A R: TCA TGC AAG GAG TGC CCA TGC
pln423	50	197	F: TAT GAT GAA AAA AAT TGA AAA AT R: CCA AAG ATA ATC CCC CCC CAT
plnN	50	160	F: GGG TTA GGT ATC GAA ATG G R: CTA ATA GCT GTT ATT TTT AAC C

Agarose gel-electrophoresis for PCR products

PCR products were separated by gel electrophoresis using a 2% (w/v) agarose gel. Two DNA ladders were used (50 bp and 100 bp) as molecular weight markers. Electrophoresis was performed at 60 V for 1.5 h. The stained gel was then documented using a UV transilluminator.

Statistical analysis

Analysis of variances and differences between means were evaluated using SPSS version 17 referring to triplicates.

Results and Discussion

Isolation of Lactic Acid Bacteria

Ninety bacterial isolates were obtained from 51 food samples, 25 isolates of them shared characteristics of lactic acid bacteria which are gram positive, catalase negative, none motile, none sporulating and anaerobic bacteria. Twenty three of these isolates appeared under the microscope as bacilli, while only two were cocci (Table 2).

Antibacterial activity of isolated LAB

A total of twenty two isolates were shown to produce inhibition zones against the pathogenic bacteria used in this study. Seven isolates had weak antibacterial activity while fifteen showed good activity (Table 3).

Antibacterial Activity of the Cell Free Supernatant

All the CFSs were found to produce inhibition zones against pathogenic bacteria in this study,significant differences among the isolates appeared as can be observed by the data mentioned in Table 4 (Fig 2).

The CFSs of the selected isolates inhibited the growth of *K. pneumoniae, S.aureus,E. coli* and *P. aeruginosa* similar results were reported by[4, 6, 19, 20].

Table 2: Morphology of isolated LAB isolates.

Isolates Sources	Isolate Code	Identification	Shape of Cell
Cheese	C35	*Lactobacillus* ssp.	Bacilli
Cheese	C2	*Lactobacillus* ssp.	Bacilli
Cheese	C4	*Lactobacillus* ssp.	Bacilli
Cheese	C12	*Lactobacillus* ssp.	Bacilli
Cheese	C20	*Lactobacillus* ssp.	Bacilli
Cheese	C25	*Lactobacillus* ssp.	Bacilli
Milk	H2	*Lactobacillus* ssp.	Bacilli
Jamid	J1	*Lactobacillus* ssp.	Bacilli
Kishk	K3	*Lactobacillus* ssp.	Bacilli
Shanglish	SH1	*Lactobacillus* ssp.	Bacilli
Shanglish	SH2	*Lactobacillus* ssp.	Bacilli
Shanglish	SH4	*Lactobacillus* ssp.	Bacilli
Cucumber Pickle	CP1	*Lactobacillus* ssp.	Bacilli
Cucumber Pickle	CP10	*Lactobacillus* ssp.	Bacilli
Cucumber Pickle	CP11	*Lactobacillus* ssp.	Bacilli
Cucumber Pickle	CP44	*Lactobacillus* ssp.	Bacilli
Cucumber Pickle	CP46	*Lactobacillus* ssp.	Bacilli
Cucumber Pickle	CP48	*Lactobacillus* ssp.	Bacilli
Cucumber Pickle	CP50	*Lactobacillus* ssp.	Bacilli
Cucumber Pickle	CP58	*Lactobacillus* ssp.	Bacilli
Pepper Pickle	PP33	*Lactobacillus* ssp.	Bacilli
Pepper Pickle	PP34	-	Cocci
Pepper Pickle	PP35	-	Cocci
Karkadeh	CK56	*Lactobacillus* ssp.	Bacilli
Karkadeh	CK57	*Lactobacillus* ssp.	Bacilli

Table 3: Antibacterial activity of lactic acid bacteria isolates against tested bacteria

Inhibition zone diameter (cm)				
P.aeruginosa	*E.coli*	*S.aureus*	*K.pneumoniae*	Isolate Code
2.6	2.7	2.5	2.6	CP46
2.7	2.6	2.7	2.5	CP50
2.8	2.7	2.5	2.5	CK57
2.5	2.6	2.6	2.5	J1
2.7	2.6	2.5	2.5	CP11
2.4	2.5	2.2	2.7	K3
2.2	2	2.5	2.3	CK56
2.4	2.5	2	2.3	PP33
2.2	2	2.5	2.3	CP58
2.4	2.3	2.2	2.4	C4
2.5	2	2.3	2.5	SH2
2.2	2.1	2.3	2.3	SH4
2.5	2	2	2.3	CP44
1.7	1.6	1.6	1.7	SH1
1.7	1.6	1.6	1.7	C12
W	W	W	W	C1
W	W	W	W	C2
W	W	W	W	C20
W	W	W	W	C25
W	W	W	W	H2
W	W	W	W	CP48
W	W	W	W	PP34
N	N	N	N	CP1
N	N	N	N	CP10
N	N	N	N	PP35

W: week antibacterial activity, N: no antibacterial activity

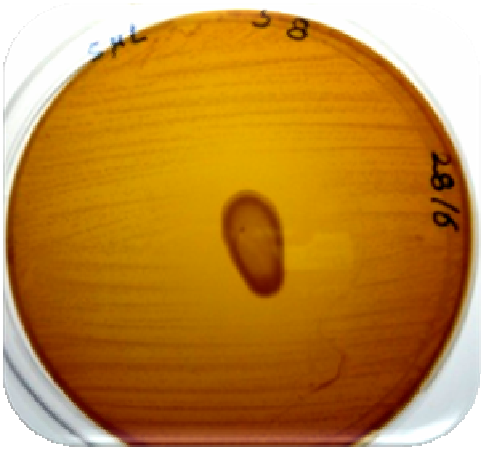

Figure 1: Inhibition zone of *E. coli* by isolate CP58.

Table 4: Antimicrobial activity of cell free supernatant against tested isolates

Inhibition zone diameter (cm)					
P.aeruginosa	*E.coli*	*S.aureus*	*K.pneumoniae*	Isolate Code	No.
1.9 ±00 [a]	2 ±00 [a]	1.9 ± 00 [a]	1.8 ± 0.10 [a]	CK56	1
1.86 ±0.057 [ab]	1.86 ±0.057 [b]	1.83 ±0.057 [a]	1.8 ± 00 [a]	SH4	2
1.8 ±00 [b]	1.86 ±0.057 [b]	1.73 ±0.057 [b]	1.8 ± 0.10 [a]	CK57	3
1.66 ±0.057 [c]	1.66 ±0.057 [c]	1.63 ±0.057 [c]	1.4 ±0.10 [bc]	CP50	4
1.66 ±0.057 [c]	1.6 ±00 [c]	1.66 ±0.057 [bc]	1.5 ±0.10 [b]	CP58	5
1.66 ±0.057 [c]	1.63 ±0.057 [c]	1.6 ±00 [c]	1.46 ±0.057 [b]	CP44	6
1.43 ±0.057 [d]	1.4 ±00 [de]	1.46 ±0.057 [d]	1.46 ±0.057 [b]	CP11	7
1.33 ±0.057 [ef]	1.46 ±0.057 [d]	1.43 ±0.057 [d]	1.33 ±0.115 [cd]	PP33	8
1.36 ±0.057 [de]	1.46 ±0.057 [d]	1.33 ±0.057 [e]	1.26 ±0.057 [d]	C4	9
1.3 ±00 [ef]	1.33 ±0.057 [e]	1.23 ±0.057 [f]	1.4 ±00[bc]	K3	10
1.26 ±0.057 [f]	1.2 ±00 [f]	1.13 ±0.057 [g]	1.4 ±0.10[bc]	CP46	11
1.36 ±0.057 [de]	1.23 ±0.057 [f]	1.2 ±00 [fg]	1.3 ±00 [cd]	J1	12
1.16 ±0.057 [g]	1.16 ±0.057 [f]	1.13 ±0.057 [g]	1.33 ±0.057 [cd]	SH2	13
1.03 ±0.057 [h]	0.93 ±0.057 [g]	1.03 ±0.057 [h]	1.06 ±0.057 [e]	SH1	14
0.63 ±0.057[i]	0.63 ±0.057[h]	0.83 ±0.057[i]	0.66 ±0.057 [f]	C12	15

Values followed with different letters in superscript on the same column are significantly different (P < 0.05).

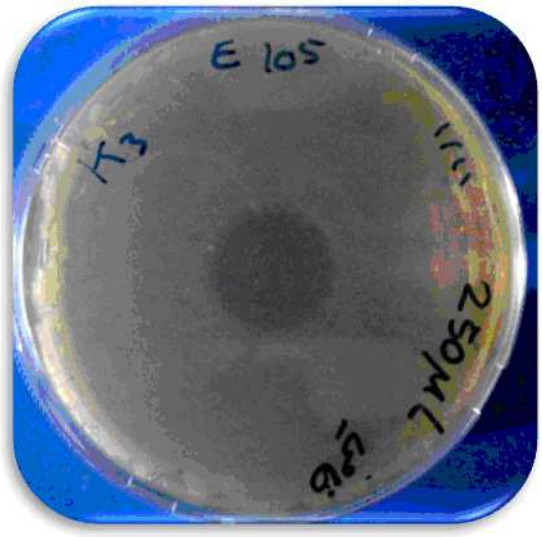

Figure 2: Inhibition zone formed by K3 *L. plantarum* CFS on the growth of *E. coli*

Physiological and biochemical characterizations of selected LAB

Identification of Selected Isolates

Positive sugar fermentation results indicated that 14 of the isolates were *Lactobacillus plantarum* and one isolatewas*L.fermentum* as shown in Table 5.The API 50 CHL classification system proved to be reliable because all of the Lactobacilli isolates were well classified.

Lactobacillus plantarum bacteria in this study were isolated from cheese, cucumber pickles, pepper pickles, jamid, Kishk, Shanglish and karkadeh. It has been isolated from different sources of food, from "Jiaoke" a traditional fermented cream from China[4], from Tenerife goats' cheese[19], from fermented cucumber[21],fromItalian ewe cheeses[22], and from raw goats' milk[23].The dominant isolated Bacillus genus from Koopeh Cheese was *Lactobacillus plantarum* (58% of lactobacilli population)as reported by[2].

Table 5: Biochemical Identification and Classification of Selected Isolates

API 50 CHL ID %	Identification	Isolates
99.9	*L. plantarum*	C4
99.7	*L .fermentum*	C12
99.9	*L .plantarum*	J1
99.9	*L .plantarum*	K3
99.9	*L .plantarum*	SH1
99.9	*L .plantarum*	SH2
99.9	*L .plantarum*	SH4
99.9	*L .plantarum*	CP11
99.9	*L .plantarum*	CP44
99.9	*L .plantarum*	CP46
99.9	*L .plantarum*	CP50
99.9	*L .plantarum*	CP58
99.9	*L .plantarum*	PP33
99.9	*L .plantarum*	CK56
99.8	*L .plantarum*	CK57

Growth at Different Temperatures

Fifteen isolates were mesophilic as those isolated by[20,23,24,25]. It was also observed that all 15 isolates were able to grow at 10°C and 40°C, (Table 6), while two *L. plantarum* isolates isolated by[24]weren't able to grow at the mentioned temperatures. Two isolates *L. plantarum*K3 and *L. plantarum*SH4 and *L. fermentum* C12 were able to grow at 45°C, while 12 isolates weren't able to grow at the same temperature (Table 6). *L. plantarum* isolates isolated by[20,24,25] weren't able to grow at 45°C. None of the isolates were able to grow at 4°C (Table 4), the same result was reported by [20,23,24,25].

Growth at Different NaCl Concentrations

As shown in (Table 6) 15 isolates were able to grow in the presence of 4% NaCl, while they weren't able to grow in the presence of 6.5% NaCl. The growth of two *L. plantarum* isolates isolated by[24] showed very weak growth in the presence of4% NaCl.

Gas Production from Glucose

Three *L. plantarum* isolates (K3, SH2, CP58) and *L. fermentum* isolate C12 were heterofermentative as deduced by gas production, while 11 *L. plantarum* isolates were homofermentative since they didn't produce CO2 as show in Table 6. All isolates of *L.plantarum* isolated by[2,20,23,24,25] were homo-fermentative.

Table 6: Physiological characteristics of the fifteen local isolates

Gas	pH 9	pH 8	pH 7	pH 6	pH 5	pH 4	pH 3	pH 2	45°C	40°C	37°C	10°C	4°C	NaCl 6.5%	NaCl 4%	Isolate Code
_	+	+	+	+	+	+	_	_	_	+	+	+	_	_	+	C4
+	+	+	+	+	+	+	+	_	+	+	+	+	_	_	+	C12
_	+	+	+	+	+	+	_	_	_	+	+	+	_	_	+	J1
+	+	+	+	+	+	+	_	_	+	+	+	+	_	_	+	K3
_	+	+	+	+	+	+	+	_	_	+	+	+	_	_	+	SH1
+	+	+	+	+	+	+	_	_	_	+	+	+	_	_	+	SH2
_	+	+	+	+	+	+	_	_	+	+	+	+	_	_	+	SH4
_	+	+	+	+	+	+	_	_	_	+	+	+	_	_	+	CP11
_	+	+	+	+	+	+	_	_	_	+	+	+	_	_	+	CP44
_	+	+	+	+	+	+	_	_	_	+	+	+	_	_	+	CP46

_	+	+	+	+	+	+	+	_	_	+	+	+	_	_	+	CP50
+	+	+	+	+	+	+	+	_	_	+	+	+	_	_	+	CP58
_	+	+	+	+	+	+	_	_	_	+	+	+	_	_	+	PP33
_	+	+	+	+	+	+	_	_	_	+	+	+	_	_	+	CK56
_	+	+	+	+	+	+	_	_	_	+	+	+	_	_	+	Ck57

Each value is represented by the mean of triplicate

Growth at different pH values

All of the 15 studied isolates were able to grow at pH ranging from 4 to 9, but only 3 *L. plantarum* isolates (SH1, CP50 and CP58) and the *L. fermentum* isolate C12 were able to grow at pH 3 as shown in Table 6.The growth at pH 4.4 was weak[24], while it was good at pH 3.9 - 4.8[20].

Partial characterization of inhibitory substances in supernatants

Antibacterial activities of selected LAB-CFS were significantly influenced by pH as shown in Figures (3-6). In this respect, it was observed that the activities at pH 4.5 were significantly higher than those at pH 7.0 suggesting an inhibition effect of acidity on the growth of *S. aureus, K. pneumoniae, P.aeruginosa* and *E. coli*. Most of LAB excrete acid that has been shown to inhibit growth of pathogens. These observations are in agreement with those reported by [15].On the other hand, it was observed a residual activity at pH 7.0 suggesting that compounds other than acids inhibit the growth of the tested pathogens. These observations are in agreement with those reported by[18]who showed that *L. plantarum* excreted other compounds such as bacteriocins that inhibited the growth of pathogens.

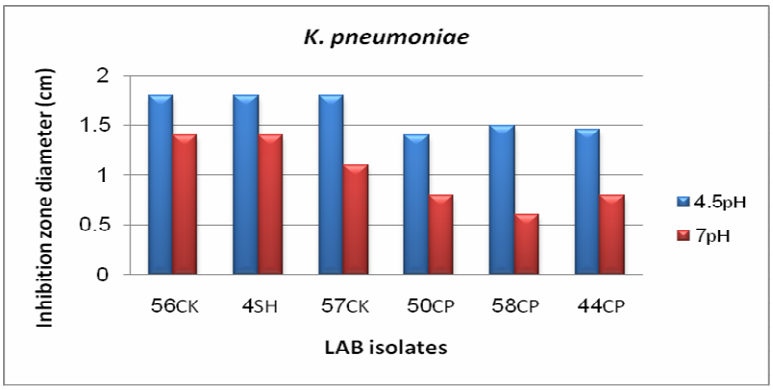

Figure 3: Effect of pH change on CFSs antibacterial activity against *K. pneumoniae*.

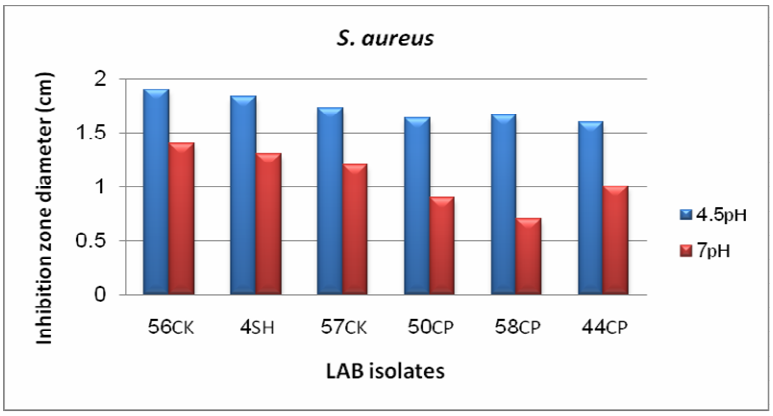

Figure 4: Effect of pH change on CFSs antibacterial activity against *S. aureus.*

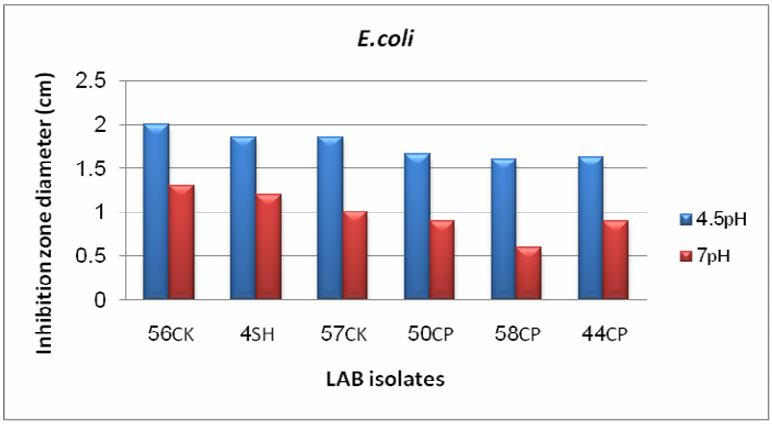

Figure 5: Effect of pH change on CFSs antibacterial activity against *E.coli.*

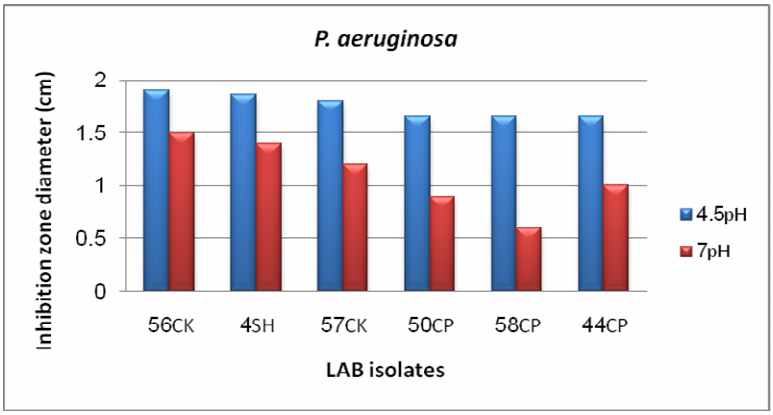

Figure 6: Effect of pH change on CFSs antibacterial activity against *P. aeruginosa.*

CFSs antibacterial activity after treatment with proteinase K

As shown in Figure (7-10) the treatment of cell free supernatants with the proteolytic enzyme, proteinase K, at pH 7 resulted in a significant reduction of the antibacterial against test pathogens. This result suggested that the antibacterial activity was associated with peptide inhibiting molecules, usually known as bacteriocin, these observations are in agreement with those reported by[15, 19, 21, 26, 27].

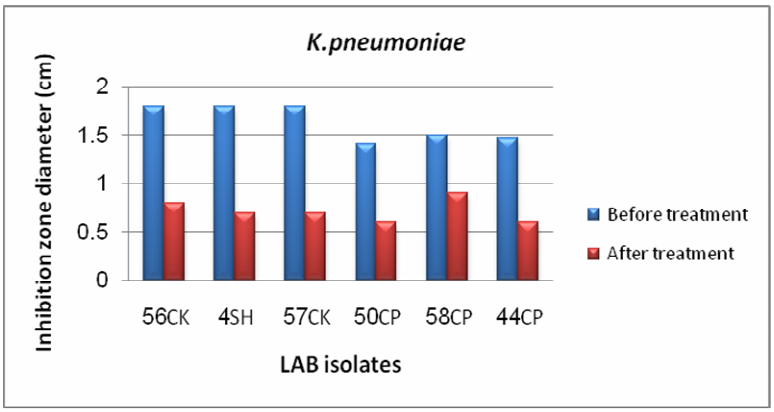

Figure 7: Antibacterial activity before and after treatment with proteinase K against*K. pneumoniae*.

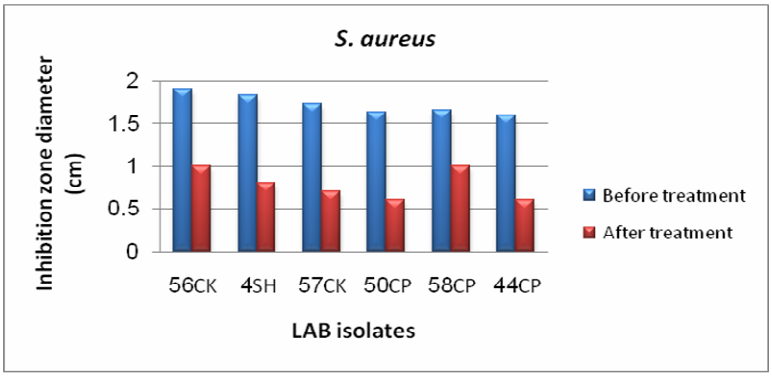

Figure 8: Antibacterial activity before and after treatment with proteinase K against *S. aureus*.

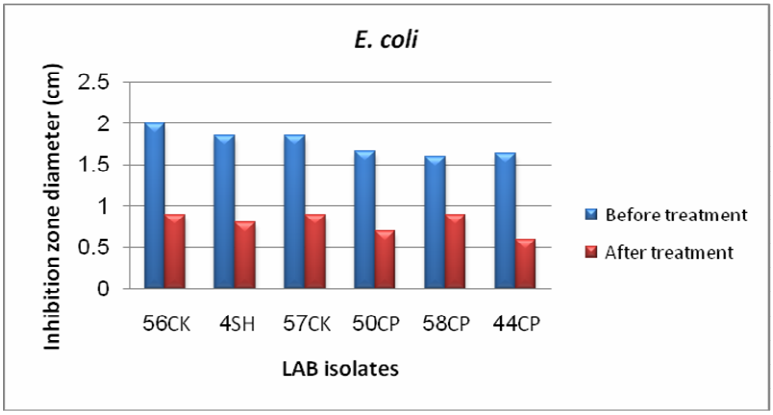

Figure 9: Antibacterial activity before and after treatment with proteinase K against*E. coli*,

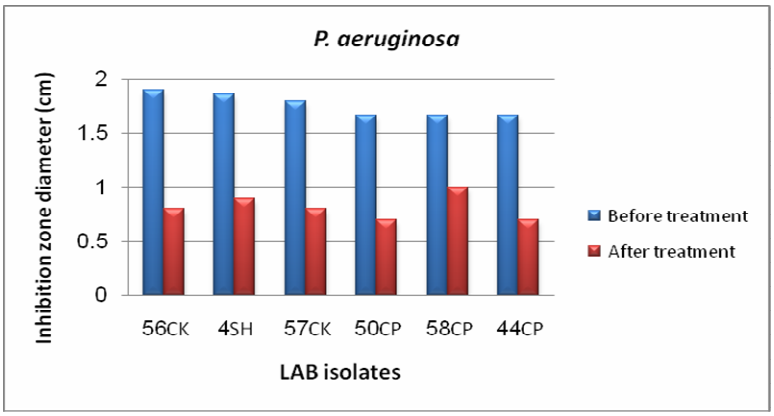

Figure 10: Antibacterial activity before and after treatment with proteinase K against*P. Aeruginosa*

Effect of heat on CFSs antibacterial activity

Antibacterial activity of cell free supernatants was heat stable after treatment at 60°C, 80 °C and 100 °C for 15 minutes, there was no significant reduction in the antibacterial activity after heat treatment(Fig. 11-14).Bacteriocin was heat stable even at autoclaving temperature (121°C for 15 min) and that antibacterial activity was stable after heat treatment at 100 °C for 30 min[28],it is also applicable to[3] who reported thatthe antibacterial agents produced by LAB were generally heat stable.

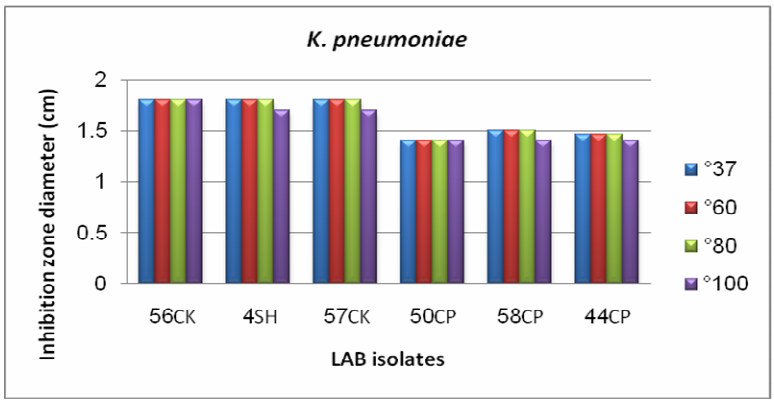

Figure 11: Effect of heat treatment on antibacterial activity of CFSs against *K. pneumoniae*.

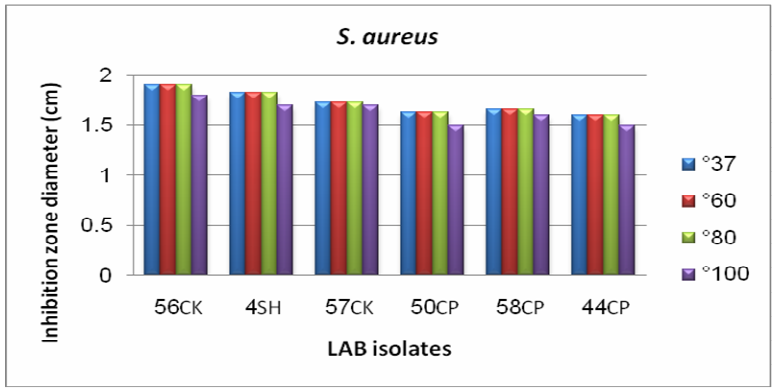

Figure 12: Effect of heat treatment on antibacterial activity of CFSs against *S. aureus*

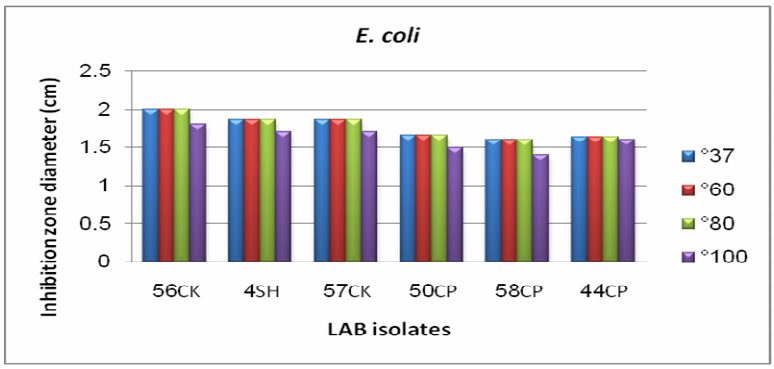

Figure 13: Effect of heat treatment on antibacterial activity of CFSs against *E. coli*

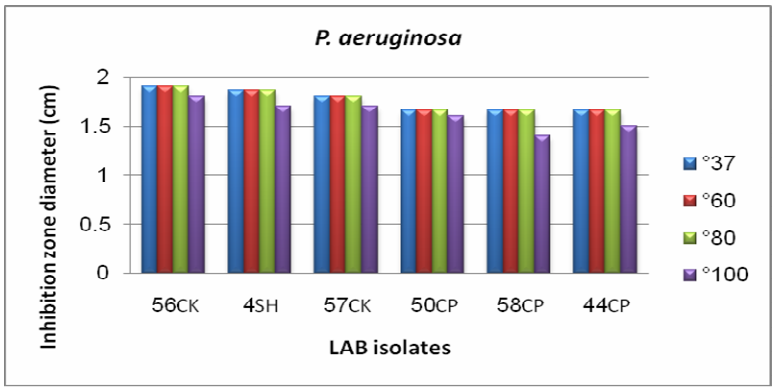

Figure 14: Effect of heat treatment on antibacterial activity of CFSs against *P. aeruginosa*

PCR amplification of plantaricin genes

Two of the *L. plantarum* isolates SH4 and CK56 contained the structural genes for both plantaricin EF and plantaricin N, and two of the *L. plantarum* isolates CK57 and CP44 contained the structural genes for plantaricin N only(Fig 15, Fig 16).It wasmentioned that*L. plantarum* strains BFE 5092 and 299V strains contained the structural genes for the plantaricin EF, plantaricin JK, and plantaricin N, while *L. plantarum* strain PC S20 contained the structural genes for plantaricin EF only[18]. Moghadam*et al* (2010)[29] reported that combination of plantaricin EF and plantaricin W structural genes were successfully amplified in all six strains he studied, while Noonpakdee*et al* (2009)[28] mentioned that *L. plantarum* PMU33 contained the structural genes for plantaricin W only.

Primers used for amplification of plantaricins genes were selected according to their size since the longer the primer is the more specific the result will be. Amplicon sizes of the amplified sequences were close to those obtained by Cho *et al* (2010)[18]who reported that the amplicon size of the amplified sequence of pln N was 160 bp, while ofpln EF it was 360 bp.

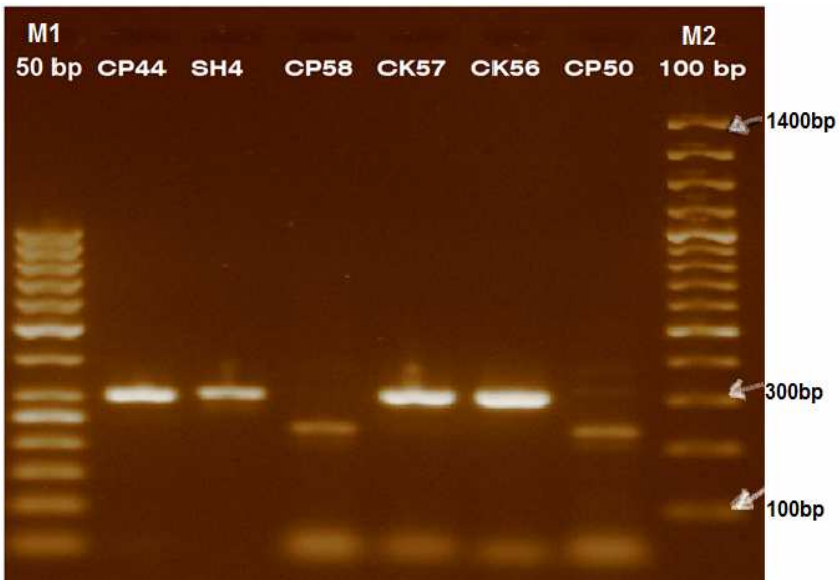

Figure 15: Amplification products from the most active six isolates with primer plnEF.
M1: Ruler DNA 50bp, M2: Ruler DNA 100bp. Agarose gel 2 % at 60 volt for 1.5 h.

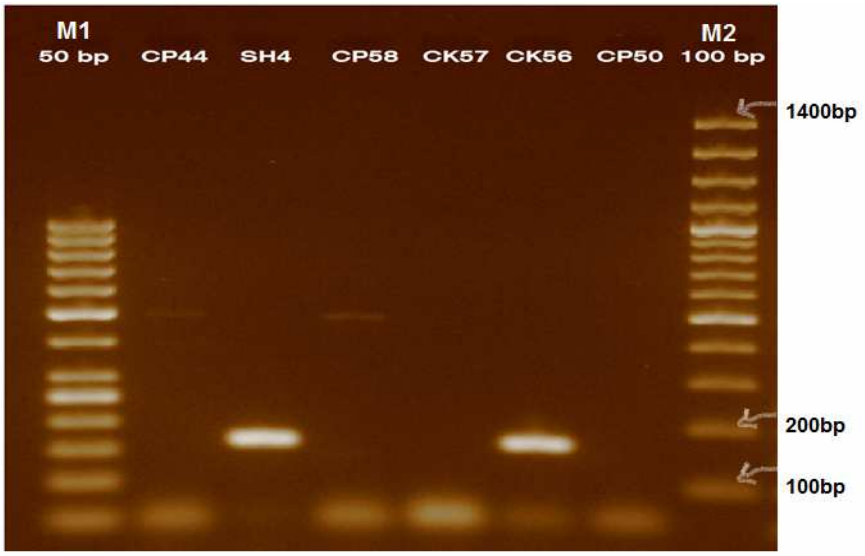

Figure 16: Amplification products from the most active six isolates with primer plnN.
M1: Ruler DNA 50bp, M2: Ruler DNA 100bp.Agarose gel 2 % at 60 volt for 1.5 h.

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