Characterization of bacterocin-like substances produced by two local *Lactobacillus paracasei* subsp. *paracasei* strains

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ABSTRACT

The increasing consumer awareness of the risks derived not only from food borne pathogens, but also from the artificial chemical preservatives used to control them, has led to an increased interest in food -grade preservatives of biological origin. In this respect, special interest has been focused on the antimicrobial bacteriocins and the lactic acid bacteria producing them which are considered safe biopreservatives. In the present study, sixty nine lactobacillus strains isolated from retail samples of local foods and dairy products were screened for bacteriocin production

Of the 47 Lactobacillus isolates found to be bacteriocin-like substances producers, five best strains which showed the strongest antibacterial activity against *E.coli; Listeria monocytogenes*; *Bacillus cereus*; *Salmonella enteritides*; and *Staphylococcus aureus,* were selected and identified, among these isolates, two were found belong to *Lb. paracasei* subsp. *paracasei.* The bacteriocin-like substances (BLS) produced by these strains were tested for characteristics that could determine their usefulness as food biopreservatives. The two BLS retained most of their bacterial activity even after autoclaving and after extended refrigerated and freezing storage, as well as after exposure to organic solvents or surfactants, and they remained functional over a wide pH rang of 3.0 to 12.0 and tolerated up to 20.0% sodium chloride. In addition they exhibited broad antimicrobial spectra against not only G^+ bacteria but also against G^- bacteria and yeasts. These results suggest that BLS produced by two local *Lactobacillus* strains has an application potential as food biopreservatives, and may be used as alternatives or complimentary for antibiotics.

Keywords: Bacteriocins, *Lactobacillus paracasei* subsp. *paracasei*, antibiotics.

INTRODUCTION

Quality and safety of foods have always a significant public health concern. Although various methods are employed for the preservation of foods, an increasingly health conscious public may seek to avoid foods that have undergone extensive processing or which contain artificial chemical preservatives. This provided an increased interest in food- grade preservatives of biological origin. In this respect, special interest has been focused on bacteriocins produced by bacteria.

Bacteriocins are defined as ribosomally synthesized peptides or proteins with antagonistic activity against species genetically closely related **(De Vuyst and leroy, 1994),** and some of them are inhibitory towards food spoilage and food borne pathogenic bacteria **(Zamfir *et al.*, 1999).** The aim of using bacteriocins to improve quality and safety of foods has stimulated intensive research efforts in recent years **(Deegan , 2006).**

Although bacteriocins are produced by many Gram-positive and Gram-negative bacteria, those produced by lactic acid bacteria (LAB) are of particular

interest to the food industry **(Nettles and Barefoot, 1993).** Bacteriocins of LAB are considered safe biopreservatives, as it is assumed that bacteriocins are degraded by the proteases of the gastrointestinal tract and most LAB are considered GRAS (Generally Recognized as Safe) microorganisms **(Halzapfel *et al.*, 1998).** Furthermore, as the majority of bacteriocin-producing LAB are natural food isolates, they ideally suited to food applications. Lactobacilli are among the most important LAB used in food production and are gaining increasing attention in the area of probiotics **(Tannack, 2004).** Isolation and characterization of novel strains of Lactobacilli could have the twofold advantage of revealing taxonomic characteristics and obtaining strains with interesting functional traits that may be useful for technological and/or probiotics applications **(Ortu *et al.*, 2007).** According to **Deegan (2006),** continued research on bacteriocin will undoubtedly lead to our increased understanding; and with the emergence of new bacteriocins, new potential biopreservatives.

Therefore, keeping in view the above introduction, the present work was carried out to characterize bacteriocin-like substances (BLS) produced by two *Lactobacillus paracasei* subsp. *paracasei* strains isolated from Egyptian fermented milk and youghurt (Zabadi).

MATERIAL AND METHODS

Materials

Food samples

Samples of Raw milk, Fermented milk, Yoghurt, Karech cheese, Domiati cheese, Kechk, Boza, Meat products and mixed pickles were used to isolate Lactobacilli bacteria.

Indicator bacteria

Two Gram-negative bacteria (*Escherichia coli* ATCC 25922 and *Salmonella enteritidis* ATCC 13076) and three Gram-positive bacteria (*Listeria monocytogenes* ATCC 15313, *Bacillus cereus* ATCC 13753 and *Staphylococcus aureus* ATCC 8095) were used as indicator bacteria for detection of the antibacterial activity. To evaluate the antimicrobial spectra of Bacteriocin-Like Substances (BLS), $17G^{+}$, 11 and 2 yeast strains listed in Table 1 were used as indicator microorganisms. All strains mentioned above were obtained from the culture collection of Agricultural Microbiology Department, Faculty of Agriculture, Fayoum University.

Antibiotics

In *vitro* diagnostics discs (Pasteur LAB, Egypt) of 16 antibiotics were used to compare their antibacterial activity with those of BLS. The antibiotics are: Negram (30), Penecillin (10), Amiks (30), Claforan (30), Colistin sulfate (10), Rifadn (30), Pyopen (100), Rocephen (30), Augmentin (30), Geremycin (10), Zinnat (30), Chloramphenicol (30), Neomycin (30), Erythromycin (10), Ampicilline (10) and Streptomycin (10).

Carbohydrates fermentation kits

The API 50CHL kits (Biomèrieux, Marcy l'Etoile, France) were used to obtain the carbohydrate fermentation pattern of the selected *Lactobacillus* isolates.

Methods

Isolation of lactobacilli (Lb)

A food sample of 1g or 10 ml was added to $^1/_4$ - strength sterile Ringer's solution (50 ml) and shaken for 30 minutes. Ten -fold and 100-fold serial dilutions were prepared and 100 µl were plated onto MRS agar plates **(Atlas and Parks 1997)** and incubated (30°C, 48 hours), and colonies with a

Lactobacillus-like morphology (white, smooth, convex) were isolated and purified. Isolates found to possess general characters of Lactobacilli: Gram-positive, catalase-negative, non motile, non spore -forming rods, that able to colt milk and not able to produce indol or liquefying gelatin were considered Lactobacilli isolates.

Preparation of cell - free Lb culture supernatants

Lb isolates were grown in MRS broth at 32°C for 18h. The cultures were centrifuged at 10000 xg for 15 min at 4°C and the resulted supernatant was designated crude cell-free culture supernatant (CCFCS). To eliminate growth inhibition caused by organic acids and hydrogen peroxide, the pH of the CCFCS was adjusted to 6.5 and catalase (1mg mL^{-1}) was added, and the resulted supernatant was designated modified cell- free culture supernatant (MCFCS) and this represents the BLS solution. These supernatants were used immediately or stored at -20°C until needed. Concentrated MCFCSs (10 - and 20 - fold) were obtained using vacuum rotary evaporator at 40°C, and stored at -20°C until further use.

Antimicrobial activity assay agar well diffusion method

Agar well diffusions method was used as described by **Wolf and Gibbons (1996).** Briefly, 20 ml of molten agar medium were cooled at 47°C and seeded with 1% (v/v) overnight culture of the indicator organism. Seeded agar was then poured into sterile Petri dish and allowed to solidify at room temperature. Wells of 7 mm diameter were cut in the solidified agar using a sterile metal cork borer and filled with 100µl of sample. The plates were left at 4°C for 2h to allow diffusion of the substances and then incubated aerobically for 24h at temperature optimum for the indicator organisms. Absence or presence of inhibition zones as well their diameters were recorded. In case of antibiotics, LB agar plates were inoculated by swabbing overnight cultures onto the surface of agar plates which allowed standing at room temperature for 3 h before antibiotic discs were applied. The plates were incubated for 24 h at the optimum temperature for each bacterial strain. The antibacterial activities of antibiotics were assessed by measuring the zone of inhibition (mm) against the indicator bacteria. **(Patton *et al.*, 2006).**

Detection of lytic bacteriophages

A piece (3.0 mm in diameter) of agar from the inhibition zone was removed aseptically. This block of agar was homogenized, placed onto BHI agar and overlaid with 3.0 ml of BHI- soft agar inoculated with 0.1 ml of the indicator culture. After 48 h of incubation at 30°C, the plates were observed for the presence or absence of plaques **(Turner and Jordan, 1981)** the test was done in duplicate.

Identification of Lactobacilli isolates

The carbohydrate fermentation patterns of the selected five lactobacilli isolates were determined with API Rapid CH fermentation strips in CHL medium as specified by the manufactures instruction, and the data were analyzed by Biomèrieux program.

Characterization of BLS

(1) The effect of heating was determined by treating MCFCS in water bath at 60, 80 and 100°C for 10, 30, 60 min and at 121°C for 10, 15 and 30 min and cooled on ice before determination of activity. **(2)**To test pH influence, catalase treated CCFCS were adjusted to pH 3.0, 5.0, 7.0, 10.0 and 12.0 with HCl or NaOH, mixed, and allowed standing at room temperature for 2h. Before determination the residual activity, the pH of samples were readjusted to pH

6.5. **(3)**Sensitivity of MCFCS to enzymes was investigated by the addition of the proteolytic enzymes protease, pepsin and trypsin in addition of the α-amylase enzyme at final concentrations of 1.0 mg/ml. Samples with and without enzymes were incubated for 3h at 30°C and residual activity was determined. **(4)**The effect of extended storage on BLS activity was evaluated by placing MCFCS at 30 ± 2°C, 4°C and -20°C and after 7, 15, 30, 60 and 90 days, the residual activity was determined. **(5)**The effect of 3 organic solvents: chloroform, ethanol and n-hexane at concentration of 10, 15, 20, 30% (v/v) was determined. Samples with and without solvents as well as samples with solvents only were incubated at 30°C for 1,4,6, 24h, and thereafter the residual activity was determined. **(6)**The effect of 4 surfactants: EDTA, Urea, Tween 20 and Tween 80 (0.1, 1.0, 2.0, 5.0%) (v/v) was determined. Samples with and without surfactants as well as surfactants only incubated at 30°C for 2h, and thereafter the residual activity was determined.

Quantification of BLS antibacterial strength

BLS inhibitory strength was estimated by the critical dilution method, using serial twofold dilutions in the same medium used for the growth of the indicator strain. Activity was quantified by taking the reciprocal of the highest dilution that exhibited a clear zone of inhibition and was expressed as activity units (AU) per milliliter of BLS-containing solutions; AU ml^{-1} was calculated as (1000/V) X D, where D is the dilution factor and V is the volume of BLS solution pipetted in each well when using the agar well diffusion technique **(Parent *et al.*, 1994).**

Mode of BLC action

The mode of action of the BLS produced by the five selected Lb strains was tested by the method proposed by **Benkerroum *et al.*, (1993).** In this method, 20 ml of melted and tempered LB soft agar was seeded with 0.1 ml of an overnight indicator bacterial culture, poured in a sterile Petri dish and allowed to harden. It was then incubated at the appropriate temperature for growth of the indicator bacteria used until growth was evident. Wells of 7mm diameter were drilled into the plates and filled with 100 µl of concentrated MCFCS or partially purified BLS. Incubation of plates was continued and the bactericidal action was indicated by the lysis zone around the well.

RESULTS AND DISCUSSION

Isolation and screening of microorganisms from naturally occurring processes have always been the most powerful means of obtaining useful cultures for scientific and commercial purposes. This is certainly true for lactic acid bacteria (LAB), which play an important role in a large number of various traditional food fermentations **(El Soda., 2003).**

Among LAB, Lactobacilli are a fruitful source of antibacterial substances including organic acids, hydrogen peroxide and bacteriocins **(Miteva *et al.*, 1998).** A number of well characterized bacteriocins with different properties have already been described within these genera **(Nettles and Barefoot, 1993).** In Egypt, few studies detected Bacteriocin-Like Substance (BLS) in cultures of local LAB (eg. **Abou Donia *et al.*, 1993 and El Soda., 2003)** and rare studies characterized BLS produced by local LAB isolates (e.g. **Abo-Amer, 2007).**

The objective of this work was to find local bacteriocin- producing *Lactobacillus* (Lb) isolates with unique characteristics that could determine

their usefulness as food biopreservatives. In order to achieve this objective, Lactobacilli were isolated from retail samples of different foods.

Isolation of lactobacilli bacteria

A total of 128 LAB strains were isolated from samples of different local foods. Of these isolates, 69 were Gram- positive, catalase negative, negative for both of indol production and gelatin liquefaction, non- motile and non- spore forming rods which indicated that they belong to genus *Lactobacillus* (Lb).

Screening Lb isolates for antibacterial activity and bacteriocin production

The 69 Lb isolates were screened *in vitro* for antibacterial activity, using an indicator panel of five bacteria: *E. coli*, *L. monocytogenes*, *B. cereus*, *S. enteritidis* and *S. aureus*. Lb Isolates exhibited antibacterial activity (AA) against at least one of the indicator bacteria were 58. Those 58 Lb isolates were rescreened for antibacterial activity of their crude cell- free culture supernatants (CCFCSs) after adjustment to pH 6.5 and elimination of hydrogen peroxide and diagnosed modified cell -free culture supernatants (MCFCSs).

The number of Lb isolates which their MCFCS showed bacteriocin - like activity is 47 represents 68% of total Lb isolates. This percentage seems to be high when compared with results of similar studies. It was reported that the frequency of isolating bacteriocin- producing LAB from foods is rather low. **Ennahar *et al.*, (1996)** showed antagonistic effects caused by antimicrobial substance other than organic acids just from six isolates of 1962 LAB from Münster cheese, whereas **Rodriguez *et al.*, (2000)** found that 24% of the LAB isolates from raw milk showed inhibitory activity after neutralization and treatment with catalase. Of the 47 assumed bacteriocinogenic Lb isolates, 42, 42, 38, 31 and 32 isolates inhibited *E. coli*, *L. monocytogenes*, *B. cereus*, *S. enteritidis* or *S. aureus*, respectively. Among the 47 Lb, isolates the five best isolates showed the strongest antibacterial activities and broad antibacterial spectra were selected for further study.

When the five selected Lb isolates were tested for inhibition by the MCFCS they produce, it was found that they were not sensitive to its own antibacterial agents in the MCFCSs. These indicate that the antibacterial activities in these MCFCSs are unrelated to production of a toxic nonspecific metabolic by- product **(Ennahar *et al.*, (1996).** Also, no plaques were observed from homogenates of the inhibition zones caused by the MCFCS on lawns of sensitive indicator bacteria. This indicated that the antibacterial activity of these MCFCSs was not caused by bacteriophages.

Sensitivity to enzymes

Investigating the sensitivity of MCFCSs for proteolytic enzymes (Pronase, Pepsin and Trypsin) and for α- amylase enzyme revealed that the antibacterial activity exhibited by the substances produced by the two strains was inactivated completely by treatment with proteolytic enzymes. This result suggests the proteinaceous nature of the antibacterial substances in the MCFCS of these strains. However, **Hardy, (1987)** had reported that bacteriocin may be either simple protein or protein linked to carbohydrate. This was the case of BLS of the two *Lactobacillus paracasei* subsp. *paracasei* strains which was partially sensitive for amylase. These results suggest that a carbohydrate moiety could be important for its full antibacterial activity **(Miteva *et al.*, 1998).**

On the basis of the above mentioned results and observations, the antimicrobial substances found in the CCFCS of the two selected *Lactobacillus* strains after excluding antibacterial activity due to both acids and hydrogen peroxide, and bacteriophages in addition to their proteinaceous nature can be

considered bacteriocins **(Tagg *et al.*, 1976 and Jack *et al.*, 1995).** However because those antibacterial substances have not yet been characterized for amino-acid sequence and/ or the nucleotide sequence of the corresponding structural gene, they well be referred to as Bacteriocin-Like Substances (BLS) **(Schillinger and Holzapfel, 1996).**

Identification of Lb isolates producing BLS

Identification of the two bacteriocinogenic *Lactobacillus* isolates to species level was carried out on the basis of their carbohydrate fermentation patterns obtained by API 50 CHL kits. According to the API database correlation, the identification of the two isolates Eg - FM3 and Eg - YO1 were found to be belonging to *Lactobacillus paracasei* subsp. *paracasei* and designated *Lactobacillus paracasei* subsp *paracasei* Eg-YO1. The BLS produced by the two Lb strains were designated, lactobacin Eg-FM3 (from *Lactobacillus paracasei* subsp. *paracasei* Eg-FM3), lactobacin Eg-YO1 (from *Lactobacillus paracasei* subsp *paracasei* Eg-YO1).

Characteristics of BLS

The bacteriocins possess many salient features important for application in food production and in other products of human interest **(Larsen *et al.*, 1993).** Therefore, the BLS produced by the two Lb strains were tested for characteristics such as temperature stability, pH resistance, storage at refrigeration and freezing temperatures, salt, organic solvents and surfactants tolerance. In general, the effects of the studied factors were varied according to the tested BLS, the indicator bacteria used, the degree or concentration of the studied factor and the time of exposure (Table 1).

Effect of Heat

Heat stability is considered an important character not only that it makes them attractive in food industry but also it an important factor in classifying bacteriocins. The BLS of the two Egyptian Lb strains were found to tolerate heating temperature up to 100°C for 60 min and autoclaving treatment (121°C, 15 min) (Table 1). Based on the bacteriocin classification made by **Klaenhammer (1993),** the two BLS tested could be placed in class II with lactocin 27, lactocin B and gassercin A **(Toba *et al.*, 1991).**

pH resistance

The antibacterial activity of some of the BLS studied was detected over a wide pH range from 3 to 10, with greatest activity at pH 7.0 (Table 1, photo 1d). **Gonzalez and Kunka, (1987)** found that pediocin PA-1, a bacteriocin produced by *Pediococcus acidilactici* was active over a pH range of 4.0 to 7.0 with a complete loss of activity at pH 3.0, 9.0, and 10.0. This stability of BLS produced in the present study over a wide range of pH values may be useful if those BLS are used in fermented foods or other products.

Effect of NaCl

Sodium chloride, an important constituent of food, has been reported to affect the antimicrobial activity of bacteriocins. In the present study, BLS generally tolerated salt concentrations up to 10% for 4 weeks (the experimental period). However, some of these BLS retained some of their activity at 30% salt concentration, while all BLS losted completely their activity at 35% concentration during the period between one and four weeks (Table 1).

Organic solvents tolerance

Treatment of the BLS with organic solvents completely abolished antibacterial activity when used in 30% concentration. Whereas, concentrations 10 and 15% resulted in approximately 40% loss in antibacterial activity (Table 1).

Surfactants tolerance

Various surfactants have a profound effect on the bacteriocin activity **(Tagg *et al.*, 1976).** In the present study, the tested BLS almost retained its activity after treatment with EDTA, Urea, Tween 20 and Tween 80 .Similar observation has been reported by **Blackborn *et al.*, (1998).** Results (Table 1) showed that in of case of lactobacin EG- FM3 enhanced activity was observed. It has been suggested that the dispersion of the bacteriocin complex into active subunits ultimately resulting in more lethal hits and consequently enhanced antibacterial activity **(Muriana and Klaenhammer, 1991).** Also, **Stevens *et al.*, (1991)** provided an additional explanation for this result. They implied that the increase in activity of bacteriocin with a surfactant could also be due to the elimination of some of the natural defenses of the indicator cells. In view of their interesting antimicrobial spectra and their technological properties, the BLS produced by some Egyptian *Lactobacillus* strains studied in this thesis have an application potential as food biopreservatives.

Antimicrobial spectra of the tested BLS

Considering antimicrobial spectrum of bacteriocin, there is a classic type, which has a spectrum of activity only against closely related species, and a second type, less common, which shows activity against a wide range of Gram-positive microorganisms **(Parada *et al.*, 2007).**

In the present study, the antimicrobial spectra were evaluated using a panel of 29 indicator microorganisms in addition of the producer strain (Table 1). Although varied in their antimicrobial activity (diameter of inhibition zone, mm), the two BLS inhibited all the 29 indicator organisms, and the tested BLS had no inhibitory effect on the Lb strains producing them. According to the definition of **Klaenhammer, (1988)** bacteriocins of LAB are active against closely related bacteria. The BLS produced by the two *Lactobacillus paracaseo* subsp. *paracasei* strains in the present study seems to be exception to the rule.

The results also revealed two interesting points. **The first** is that the two BLS inhibited all the 11 Gram- negative bacteria used as indicators. Bacteriocins are not frequently active against Gram -negative bacteria. However, some studies have already reported bacteriocins activity against this group of bacteria. Examples are plantricin 35d **(Messi, 2001)** and bacteriocin ST151BR **(Torodov and Dicks, 2004). The second** interesting point is that the two indicator yeasts (*Candida albicans* and *Saccharomyces cerevisiae*) were inhibited by all the two BLS, since these yeasts, particularly *C.albicans* resist the antimicrobial action of most bacteriocins.

The results of this study indicate that the tested BLS possess a very important character, that none of them is limited by the extremely narrow antimicrobial spectrum reported for most bacteriocins produced by lactic acid bacteria. Bacteriocin may process a bacteriocidal or bacteriostatic mode of action on sensitive cells and this distinction being greatly influenced by several factors as bacteriocin dose and degree of purification, physiological state of the indicator cells and experimental conditions **(Cintas *et al.*, 2001).** In the present study, the mode of action of the 20- fold concentrated BLS on five indicator bacteria: *E. coli, S. enteritidis, B. cereus, L. monocytogenes* and *S. aureus* was investigated. The results (Table 1, photo 1b) indicated that all five BLSs showed a bactericidal action on the five indicator bacteria, except in case of lactobacin Eg- FM3 which not form lysis zones in plates of both *S. enteritidis* and *B. cereus.*

Contribution of antibacterial activity of different antibacterial agents (%) in the total antibacterial activity of CCFCS of the two *Lactobacillus* strains on growth of three indicator bacteria

The contribution of antibacterial activity of the three main antibacterial substances acids, H_2O_2 and bacteriocins usually produced in LAB cultures in the total antibacterial activity (TAA) exhibited by the crude cell-free culture supernatants of the two Lb strains were investigated (Table 1, photo 1a). It was found, in general, that antibacterial activity (AA) due to each substance varied according the Lb culture and the used indicator bacteria. The AA due to bacteriocin showed the highest contribution in TAA of the CCFCS, and represents in average 75% whereas the contribution of both acids and H_2O_2 are approximately equal and each represents 15% of TAA. In case of BLS produced by strain Eg-YO1 it was observed a contribution of other substances (about 7%) aside of bacteriocins, acids and H_2O_2. This may be due to the production of diacetyl or other inhibitory substances by this strain. Diacetyl (2, 3- butanediol, biacetyl) was found to be lethal for Gram-negative bacteria and generally inhibitory for Gram- positive bacteria **(Jay, 1982).**

Inhibitory strength (IS) of BLS

The inhibitory strength of 20- fold concentrated BLS is shown in **(Table 1, photo 1c)**. The lactobacin Eg- FM3 showed a higher IS of 80 AU ml^{-1} 20- fold concentrated BLS, compared to 40 AU ml^{-1} for lactobacin Eg-YO1.

Comparing antibacterial activities of BLS with those of antibiotics

The antibacterial activities (AAs) of lactobacin Eg - FM3 and lactobacin Eg-YO1 were compared with AAs of 16 antibiotics using *E. coli* , *L. monocytogenes* and *S. enteritidis* as indicators. Results show that the two BLS exhibited higher AAs than those of some antibiotics (Fig.1). For example, lactobacin Eg-YO1 exhibited higher AA than AAs of 14 of the 16 antibiotics tested against *L. monocytogenes*. Studies on comparing AAs of bacteriocins with those of antibiotics are not available to the authors. However, there are continuing attempts are being made to find applications of bacteriocin in veterinary and medicine **(Minahk *et al.*, 2004).** Typical examples are Nisin and lacticin 3147, which have proved to be effective agents in the treatments of mastitis **(Delves– Broughton *et al.*, 1996)** and Nisin is being considered for the treatment of stomach ulcers caused by *Helicobacter pylori* **(Hancock and Chapple, 1999).** On the other hand, **Minahk *et al.*, (2004)** found that the bacteriostatic activity of three clinical antibiotics: erythromycin, chloramphenicol and tetracycline, was strongly increased in the presence of sub-lethal concentrations of enterocin CRL35. Therefore, the BLS characterized in the present study may find application in veterinary and medicine.

Numbers of characteristics of the studied BLS make them interesting as a food preservative and for other applications. **First**, they inhibited the growth of all Gram-negative bacteria tested as the Gram-positive bacteria particularly the pathogens such as *L.monocytogenes* and *S. enteritidis* and showed a fungistatic effect on both *C.albicans* and *Saccharomyces cerevisiae*. **Second**, the BLS appear to stable under a wide variety of conditions. Their thermostability indicates that they can be used in pasteurized products. They are also stable over a wide pH range indicating that they may be useful in acidic as well as non acidic foods. They are different from Nisin, the most widely studied and commercially available bacteriocin produced by *L. lactis* subsp. *lactis* which unstable at natural and alkaline pH values and not active against Gram-negative bacteria only when used with EDTA. Also stability of these BLS for long time at refrigeration temperature (4°C) may therefore

protect refrigerated foods from the foodborne pathogenic bacteria such as *L. monocytogenes.*

In conclusion, the findings of this work contribute to a screening, identification and knowledge of the BLS with a wide range of antimicrobial activity by LB strain isolated. These BLS or Lb strains producing them could have a considerable interest to enhance the hygienic quality for the manufacture of foods and products.

Table (1). Characterization of BLS produced by two local *Lactobacillus paracasei* subsp. *paracasei* strains.

Item		Lactobacin Eg-FM3	Lactobacin Eg-YO1
1. Producer		*Lb. paracasei* subsp. *paracsei*	*Lb. paracasei* subsp. *paracsei*
2. Origin		Fermented milk	Yoghurt (Zabady)
3. Chemical nature		Glycoprotein	Glycoprotein
4. AA (%) due to different antibacterial substances in CCFCS.			
• Bacteriocin		74%	75 %
• Acids		12%	11%
• H_2O_2		14%	7%
• Others		0 %	7%
5. Antibacterial strength (AU ml^{-1}) of 20 - fold concentrated bacteriocin		80	40
6. Mode of action against:			
E.coli		Cidal	Cidal
S. enteritidis		Static	Cidal
B. cereus		Static	Cidal
S. aureus		Cidal	Cidal
L. monocytogenes		Cidal	Cidal
7. Characteristics of BLS*			
7.1. Effect of Enzymes (1mg ml^{-1})			
a. Pronase		- **	-
b. Pepsin		-	-
c. Trypsin		-	-
d. α - amylase		++++**	++++
7.2. Effect of Heating			
60°C	10 min	+++	++++
	30 min	+++	++++
	60 min	+++	++++
80°C	10 min	+++	++++
	30 min	+++	++++
	60 min	+++	-
100°C	10 min	+++	+++
	30 min	+++	+++
	60 min	++	-
121°C	15 min	-	+++
	30 min	-	+++
7.3. Effect of pH			
	3	+++	+++++
	5	+++	++++++
	7	+++	++++++
	10	++	++++++
	12	++	++++

Table (1) Continued

Item		Lactobacin Eg- FM3	Lactobacin Eg- YO1
7.4. Effect of NaCl			
5%	1 week	++++	+++++
	4 weeks	++++	+++++
10%	1 week	++++	++++
	4 weeks	++++	++++
20%	1 week	+++	+++
	4 weeks	+++	+++
30%	1 week	++	-
	4 weeks	++	-
35%	1 week	-	-
	4 weeks	-	-
7.5. Effect of organic solvents			
Chloroform			
10%	1 hour	++++	++++
	4 hours	+++	+++
	6 hours	+++	+++
	24 hours	+++	++
15%	1 hour	++++	+++
	4 hours	++	+++
	6 hours	++	++
	24 hours	++	++
20%	1 hour	-	++
	4 hours	-	-
	6 hours	-	-
	24 hours	-	-
30%	1 hour	-	-
	4 hours	-	-
	6 hours	-	-
	24 hours	-	-
Ethanol			
10%	1 hour	++++	+++++
	4 hours	++++	++++
	6 hours	++++	++++
	24 hours	+++	++++
15%	1 hour	+++	++++
	4 hours	+++	+++
	6 hours	++	+++
	24 hours	-	++
20%	1 hour	-	++++
	4 hours	-	+++
	6 hours	-	+++
	24 hours	-	-
30%	1 hour	-	+++
	4 hours	-	++
	6 hours	-	-
	24 hours	-	-

Table (1) Continued

Item		Lactobacin Eg- FM3	Lactobacin Eg- YO1
n- Hexane			
10%	1 hour	++++	++++
	4 hours	+++	+++
	6 hours	+++	+++
	24 hours	+++	+++
15%	1 hour	++++	++++
	4 hours	+++	+++
	6 hours	+++	+++
	24 hours	+++	+++
20%	1 hour	++++	++++
	4 hours	++	+++
	6 hours	++	++
	24 hours	++	++
30%	1 hour	+++	+++
	4 hours	-	++
	6 hours	-	-
	24 hours	-	-
Item		Lactobacin Eg- FM3	Lactobacin Eg- YO1
7.6 Effect of surfactants			
EDTA	0.1%	+++++	+++++
	1.0%	++++	++++
	2.0%	++++	++++
	5.0%	++++	++++
UREA	0.1%	++++	++++
	1.0%	++++	++++
	2.0%	++++	++++
	5.0%	++++	++++
Tween 20	0.1%	+++++	+++++
	1.0%	+++++	+++++
	2.0%	+++++	+++++
	5.0%	+++++	+++++
Tween 80	0.1%	+++++	++++
	1.0%	++++	++++
	2.0%	++++	++++
	5.0%	++++	++++
7.7.Storage, period & temperature			
30±2°C	7 days	+++++	++++
	15 days	++++	++++
	30 days	+++	+++
	60 days	+++	-
	90 days	++	-
4°C	7 days	+++++	++++
	15 days	++++	++++
	30 days	++++	++++
	60 days	+++	++++
	90 days	++	+++
20°C	7 days	+++++	+++++
	15 days	+++++	+++++
	30 days	++++	+++++
	60 days	++++	+++++
	90 days	++++	++++

Table (1) Continued

Antimicrobial activity against:		Lactobacin Eg-FM3	Lactobacin Eg-YO1
8.1.Gram - positive LAB			
1	*Lactobacillus* sp. Eg- DCH1	17	25
2	*L. rhamnosus* Eg- YO1	24	0
3	*L. paracasei* subsp. *paracasei* Eg-FM3	0	27
4	*L. paracasei* subsp *paracasei* Eg-RM2	12	36
5	*L. rhamnosus* Eg- FM2	23	27
6	*Streptococcus* sp.1	32	20
7	*Streptococcus* sp.2	15	21
8	*Streptococcus* sp. 3	19	18
9	*Streptococcus* sp.4	36	12
10	*Streptococcus* sp.5	20	13
8.2.Gram - positive non LAB			
1	*Listeria monocytogenes*	32	35
2	*Staphylococcus aureus* 1	32	30
3	*Staphylococcus aureus* 2	25	27
4	*Bacillus cereus*	27	35
5	*Bacillus subtilis*	20	36
6	*Brochothrix thermophactum*	17	16
7	*Sarcina* sp	19	18
8.3.Gram - negative bacteria			
1	*E.coli* Top 10	20	28
2	*E.coli* O157:H	28	27
3	*Salmonella enteritidis*	24	17
4	*Salmonella* sp. 1	19	17
5	*Salmonella* sp. 2	17	18
6	*Pseudomonas* sp. 1	15	13
7	*Pseudomonas* sp. 2	19	16
8	*Pseudomonas* sp. 3	14	15
9	*Pseudomonas* sp. 4	21	22
10	*Pseudomonas* sp. 5	20	17
11	*Proteus* sp.	30	15
8.4.yeast			
1	*Candida albicans*	19	13
2	*Saccharomyces cerevisiae*	16	23
Antimicrobial spectrum		29/30 Broad	29/30 Broad

* BLS: CCFCS neutralized and treated with Catalase.
* Acids: CCFCS treated with Catalase and Pronase.
* H_2O_2: CCFCS neutralized and treated with Pronase.
* Others: CCFCS neutralized and treated with Catalase and Pronase.
** Residual antibacterial activity (%) using *E. coli* : >100% (+++++), 100% (++++), 80 - <100% (++++), 60 - <80% (+++), 40 - <60% (++), <40% (+), Zero (-).

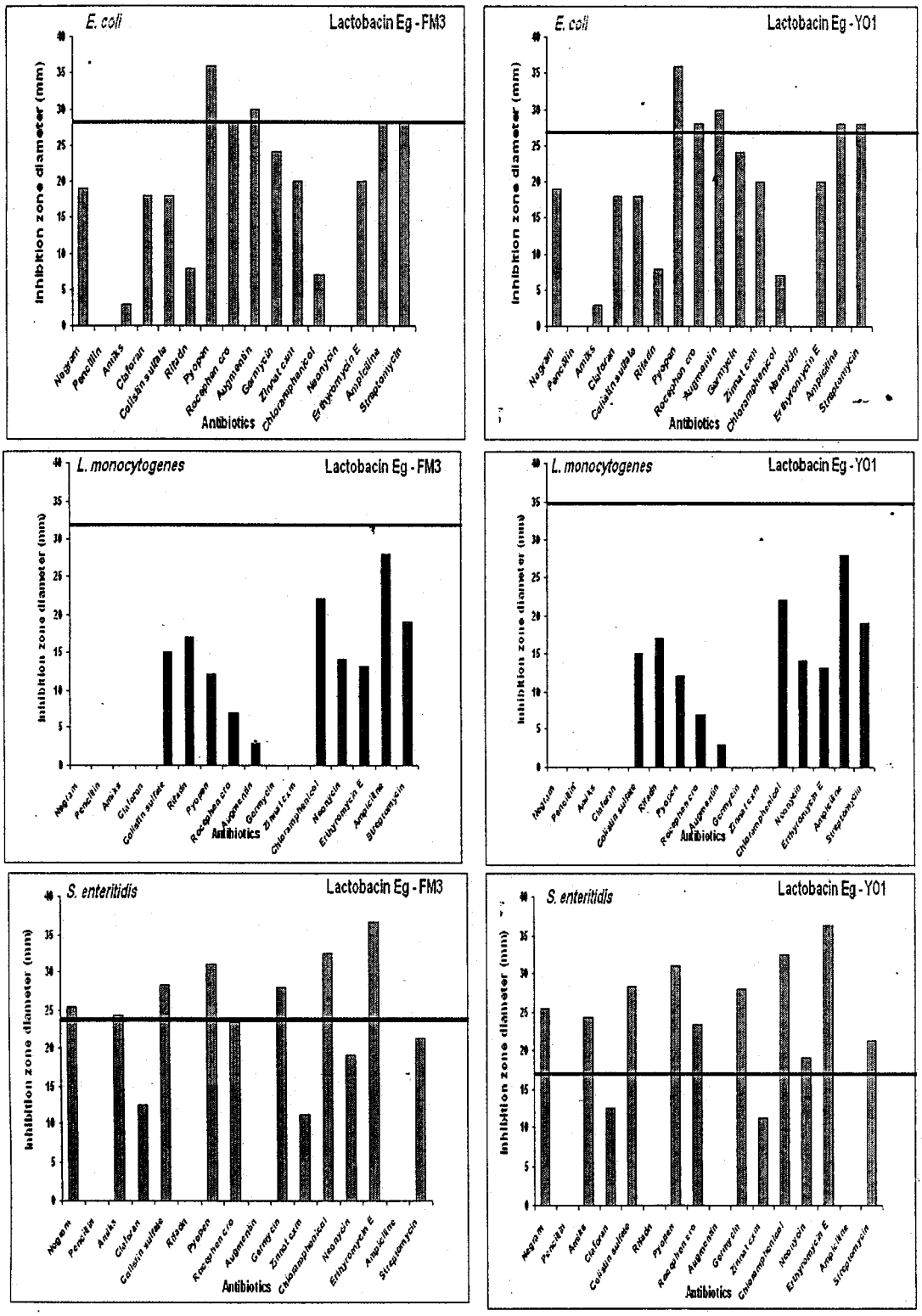
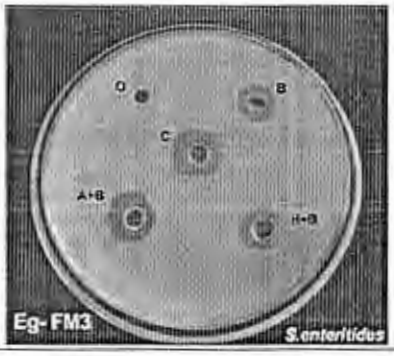


Fig. (1) Comparing antibacterial activities of 20 - fold concentrated lactobacin Eg- FM3 and lactobacin Eg-YO1 with those of antibiotics against *E. coli, L. monocytogenes* and *S. enteritidis*.



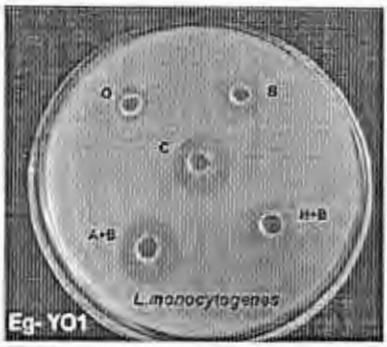
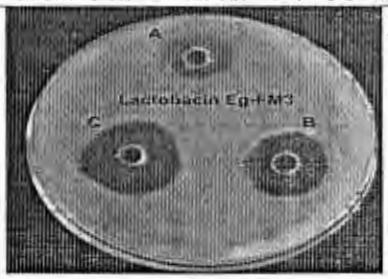
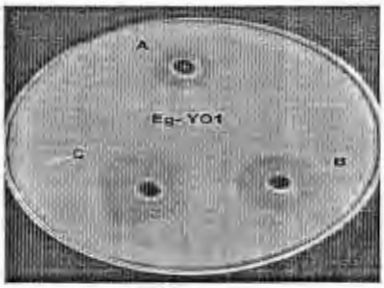
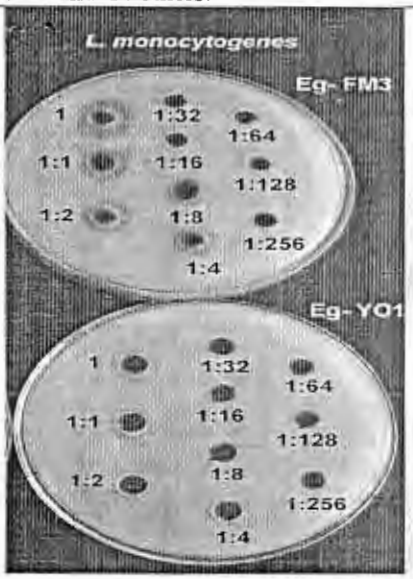


Photo 1a: Contribution of different antibacterial agents in antibacterial activity of CCFCS of *Lactobacillus* strains **A:** Acids **C**: CCFCS, **H**: H_2O_2, **B**: BLS and **O**: others.



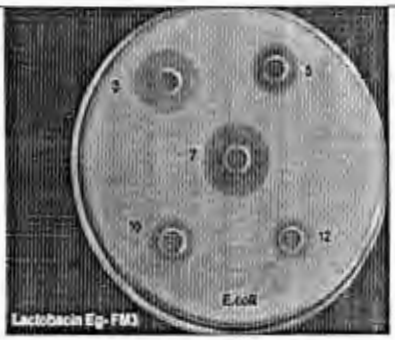


1b



1c

Photo 1b: Antibacterial activity of **A**: BLS; **B:** 10-fold conc. BLS, **C**: 20-fold conc. BLS
Photo 1c: Determination of BLS inhibitory strength (AU ML^{-1}) using *L. monocytogenes*



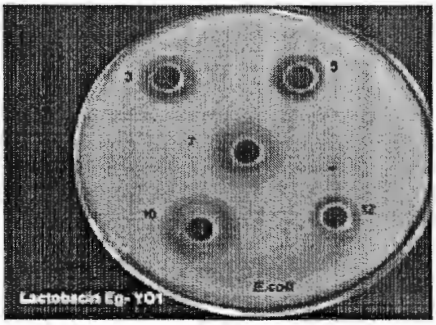


Photo 1d: Effect of pH on BLS activity against *E.coli*

Photo 1: Examples of testes carried out on BLS.

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خصائص مواد شبيهة بالبكتريوسين منتجة من سلالتين محليتين من بكتريا *Lactobacillus paracasei* subsp. *paracasei*

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في هذه الدراسه، تم عزل تسعة وستون عزله تنتمي إلي جنس اللاكتوبسيلس من عينات محلية مختلفة من الاغذية والألبان و منتجاتها واختبرت قدرتها على إنتاج البكتريوسين وجد ان هناك عدد ٤٧ عزلة من عزلات اللاكتوباسيلس تنتج في مزارعها مواد مشابهه للبكتريوسين Bacteriocin-Like Substances (BLS)، ومن بين هذه العزلات اختيرت العزلتان Eg - FM3 , Eg -YO1 المعزولتان من اللبن الرايب والزبادي على اساس قوة نشاطهم المضاد لنمو جميع بكتريا الاختبار الخمس: *E.coli, Listeria monocytogenes, Bacillus cereus, Staphylococcus aureus, Salmonella enteritidis* على التوالي. وتعريف العزلتان المختارتان اظهر انهما تنتميان الي بكتريا *Lactobacillus paracasei* subsp. *paracasei* .

تم إختبار العديد من الخصائص والصفات للمواد المشابهه للبكتريوسين التي يمكن أن تحدد مدى إمكانيةاستخدامها كمواد حافظة حيوية للاغذية ومن تلك الصفات تأثر نشاطها المضاد للميكروبات بالحفظ على درجات حرارة مختلفه ولفترات مختلفه، ودرجة ثباتها حراريا، وتأثرها بدرجات ال pH، وبتركيزات مختلفة من ملح الطعام، وكذلك تأثرها بالمذيبات العضوية و مواد التوتر السطحي.

و قد أظهرت نتائج هذة المرحلة أن ال BLS التي تنتجها العزلتان تتميز بعدة خصائص هامة:

– ان هذه المواد تحتفظ بنسبة كبيرة من نشاطها المضاد للميكروبات عند حفظها على درجة حرارة الثلاجة (٤ درجة مئوية) او درجة حرارة التجميد (-٢٠ درجة مئويه) لمده لا تقل عن ٦٠ يوما في الحالة الاولى ولمدة لا تقل عن ٩٠ يوما في الحالة الثانية.

– ان هذه المواد تتحمل التسخين علي درجة حرارة ١٠٠ درجة مئوية لمدة ٦٠ دقيقة (مدة التجربة) بدون تغير كبير في نشاطها و أكثر من ذلك فان BLS لم يتاثر كثيرا بالتعقيم بالأوتوكلاف عند درجة حرارة ١٢١ درجة مئويه لمدة ١٥ دقيقة.

– ان هذه المواد تظهر نشاطا مضادا للميكروبات في مدى و اسع من درجات الـ pH من (١٢– ٣) وتكون في أقصى نشاطها عند درجة pH 7.0.

– ان هذه المواد تتحمل تأثير ملح الطعام بتركيز يصل الى ٢٠ % لمدة لا تقل عن ٣٠ يوما

– ان هذه المواد لم تتأثر كثيرا بالمعاملة بمواد التوتر السطحي مثل EDTA, Urea, Tween 20, Tween 80

– ان هذه المواد احتفظت بحوالي ٦٠% من نشاطها عند تعرضها لبعض المذيبات العضوية مثل n- Hexane –Ethanol , Chloroform , بتركيزات حتى ١٥% لمدة ٦ ساعات.

– أختبرت الـ BLS لمدى قدرتها على تضاد نمو عدد ٣٠ ميكروب عبارة عن عدد ١٠ بكتريا حامض لاكتيك موجبة لجرام، عدد ٧ بكتريا اخرى موجبة لجرام، عدد ١١ بكتريا اخرى سالبة لجرام بالاضافة الى ٢ من الخمائر و أظهرت النتائج ان جميع الـ BLS أظهرت تضاداً لجميع ميكروبات الاختبار الثلاثين ما عدا سلالات Lb المنتجة لها.

مجمل نتائج الدراسة يبين أن من بين سلالات بكتريا اللاكتوبسيلس المحليه توجد سلالات منتجة لمواد شبيهة بالبكتريوسين ذات مدى و اسع و قوي فى تضاد بعض من الميكروبات الهامة الممرضة للانسان والبكتيريا التي تسبب فساد الأغذية وكذلك ذات قدرة على تحمل بعض الظروف والمعاملات التى قد تتعرض لها الأغذية أثناء تصنيعها أو حفظها مما يجعلها مواداً واعدة بإمكانية استخدامها كمواد حافظة بيولوجية آمنة بديلا للمواد الحافظة المصنعة كيميائيا المستخدمة حاليا في حفظ الأغذية. كما قد تفيد ايضا في استخدامها كبدائل آمنة للمضادات الحيوية لبعض أمراض الانسان و الحيوان.