

Production of bacteriocins from infant *Lactobacillus* spp. active against *Listeria monocytogenes*

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Abstract

Three *Lactobacillus* isolates, isolated from breast-feed infants feces belong to the species: *L. fermentum* (Lf), *L. acidophilus* (La), and *L. reuteri* (Lr). The three isolates are able to produce bacteriocins with antagonistic activity against indicator bacteria *Listeria monocytogenes* in agar – well diffusion technique. The cell-free extracts (supernatants) fractionated with 60% ammonium sulfate, and some of physicochemical properties were studied. They indicated acidic pH stability in a range of pHs 2- 6, and showed heat stability in a range of temperature 50 – 100 °C for 10 minutes. Enzymes sensitivity revealed inhibition of antilisterial activity after treatment with proteolysis enzymes, pepsin and trypsin, while lysozyme has no activity. Bacteriocins displayed complete antagonistic maintenance during storage at -18°C for a month.

Introduction

Lactobacillus gram-positive rods, colonize the gastrointestinal tract of mammals and human immediately after birth (Sevage, 1977). Gastric *Lactobacillus* contribute approximately 75% in gastric functions, they known to benefit health as natural predominant microflora (Tannoch, 1995; Duffy *et al.*, 1994; Perdigon *et al.*, 1995). The beneficial biological functions of gastric *Lactobacillus* include; reduction of serum cholesterol, amelioration of diarrhea or constipation, elimination of procarcinogens, synthesis of vitamin B, activation of immune system, improve of adhesive ability, and prevent gastrointestinal infections (Holzapfel *et al.*, 2001; Fooks and Gibson., 2002). *Lactobacilli* inhibitory action of gastric illnesses due to different mechanisms; compete with pathogenic viruses and bacteria for binding sites on epithelial cells and produce bioactive molecules such as, organic acids particularly lactic acid, hydrogen peroxide, diacetyl, and antimicrobial peptides, like bacteriocins (Hirano *et al.*, 2003; Ogunbanwo *et al.*, 2003a). By definition the term "bacteriocin" refers to the heterogeneous family of small, heat – stable peptides with potent antimicrobial activity, are produced by many bacterial species including *lactobacillus* strains. Bacteriocins producing by gram- positive bacteria have bacteriocidal or bacteriostatic effect on other species or genera, but activity is usually limited to other gram positives (Ogunbanwo *et al.*, 2003b; Sinead *et al.*, 2007). Lactic acid bacteria bacteriocins has received much attention in recent years and researches in this field have been expanded during the last decades, including the use of bacteriocins or producer organisms as novel food preservatives to reduce the use of chemicals, prevent foodborn diseases, and control bacterial diseases of human and animals, such strategies could potentially decrease antibiotic use and associated problems of antimicrobial resistance (Guven *et al.*, 2001).

Listeria monocytogenes has been recognized as the causative agent of food-born illness of which numerous outbreaks have occurred worldwide. The ingestion of products contaminated with this organism may be a potential health threat to high-risk population such as immune-suppressed, children, pregnant women, and the elders (Harres *et al.*, 1989 ; Tour *et al.*, 2003). *L.monocytogenes* shows the ability to survive adverse conditions such as vacuum, freezing, ultraviolet ray, and to resist conventional pasteurization (Gahan and Collins., 1991). Antilisterial bacteriocins production demonstrated in gastric lactobacilli and because of most of the bacteria in this group are reported as GRAS (generally regarded as safe) microorganisms and their bacteriocins are considered innocuous due to proteolytic degradation in gastrointestinal tract, human selected strains of probiotic Lactobacilli used as biological preservative in food industry (Jacobsen *et al.*, 1999).

The aims of this study was to present some data on antimicrobial activity of local human gastric *lactobacillus* isolates and study some of physicochemical features of produced bacteriocins, in respect with effect of pH, heat, sensitivity to some enzymes, and effect of storage temperature and time.

Materials and methods

Bacteria and cultural conditions

Fecal samples from 4-8 week old breast – feed infants analyzed for the presence of *Lactobacillus* bacteria in de Man Rogosa and Sharpe medium (MRS) supplemented with 5µg / ml erythromycin. Briefly : 1 gm of feces was inoculated in 10 ml MRS broth medium for enrichment of resident *Lactobacillus* , the tubes were incubated anaerobically (anaerobic jar and gas pack) at 37°C for 48 h. Cultures was streaked on MRS agar plates several times. The isolates were identified to genus level by: gram staining, nitrate reduction, gelatin liquefaction, oxidase and catalase test. The interesting isolates were identified to species level by carbohydrates fermentation pattern.

The *Lactobacillus* isolates were maintained in MRS broth with 20% glycerol at -18°C as stock culture.

Listeria monocytogenes obtained from biology dep. Baghdad university, used as test microorganism, propagated in brain heart infusion medium (BHI).

Bacteriocin production screening

Bacteria were screened for their antilisterial activity by agar – well diffusion technique, the isolates were grown in MRS broth anaerobically at 37°C for 48h, cell free solution were prepared by, centrifugation of grown cultures (6000 rpm for 30 min. at 4°C), followed filtration through 0.2 µm pore size filter, and the obtained supernatants were adjusted to PH 7. BHI agar was seeded with overnight culture of *L. monocytogenes* at a final concentration 10^6 cell / ml, poured into sterile Petri dishes and allowed to solidify at room temperature, wells 5 mm were cut in agar using a sterile cork borer, the wells were filled with 50µL of tested supernatants separately and allowed to diffused into agar for 6 h at 4°C, plates incubated at 37°C for 48h, and formed inhibition zones were recorded in millimeters after subtraction of 5mm (well diameter) (Barefoot and Kalaenhammer., 1989).

Determination of Bacteriocins titer

Titer of bacteriocins was quantified with the assay of minimum inhibitory concentration (MIC). Serial dilution (2 – fold) of bacterial supernatants in physiological saline were prepared and 50µL from each dilution filled into wells in BHI agar plates seeded with *L. monocytogenes* at a final concentration 10^6 cell /ml, procedure continued as previous, and the diameters of inhibition zones around wells recorded after 24h incubation at 37°C. The antilisterial titer of tested bacteriocins was defined as the reciprocal of the highest dilution showing inhibition of growth (Mayer-Harting *et al.*, 1972)

Fractionation of Bacteriocines

The obtained bacterial supernatants fractionated by precipitation with solid ammonium sulfate to 20, 40, 60, and 80 % saturation, the precipitate at different saturation were collected and dissolved separately in 0.05 M phosphate buffer pH 7, dialyzed against same buffer and the remaining activity was assayed against test bacteria. (Todorov and Dicks., 2004)

Physicochemical characterization

The fractionated bacteriocins were characterized with respect to:

pH sensitivity, thermal stability, sensitivity to lysis enzymes, and stability during storage

(De Vuyst and Leory., 2007).

Sensitivity to pH and heat

500µL of culture supernatants adjusted to pHs between 2 to 8 and incubated for 5h. at 37°C , residual activity was assayed by agar – well diffusion method against *L. monocytogenes*. Heat sensitivity measured by exposition 500µL of supernatants to various treatments 50, 60, 70, 80, and 100°C for 10 min in water bath, samples were cooled immediately after and remaining activity assayed.

Sensitivity to lysis enzymes

500µL of bacteriocin samples were treated with the enzymes; pepsin, trypsin, and lysozyme at a final concentration of 0.5 mg /ml. The samples were incubated for 3h. at 37°C, except for samples containing trypsin were incubated at 25°C, the remaining activities were assayed against test bacteria by agar-well diffusion method .

Stability during storage

Bacteriocins stored at three different temperature (– 18, 4, and 25°C) for one month, after the antilisterial activity was determined by previously described technique.

Results and Discussion

Twenty *Lactobacillus* isolates being isolated from breast – feed infant feces on MRS medium supplemented with 5µg /ml erythromycin. All isolates tested for bacteriocins production by agar-well diffusion technique, antagonistic activities

against test bacteria *L. monocytogenes* were detected in three isolates (15%), which were shown clear inhibition zones, these three candidates identified to the species level according to the sugar fermentation pattern of human strains and compared with sugar fermentation scheme described in Bergey's manual of systematic bacteriology (Kanlder and Welles., 1986).

The isolates belongs to the species; *Lactobacillus fermentum* (Lf), *Lactobacillus acidophilus* (La), and *Lactobacillus rutrie* (Lr) .

Bacteriocins production varied among these three species, La was more potent than the other two species, it exhibited larger inhibitory zone, 15mm when tested by agar-well diffusion technique, while the species Lf and Lr. showed 9 and 7.5 mm diameter inhibition zones respectively (Fig.1). In general infants *Lactobacillus* strains in this study showed higher antagonistic activity against food-born invasive *L. monocytogenes*, and their antagonistic effects related to the production of bacteriocins , since the bacterial supernatants pHs adjusted to 7 prior to detection , some studies have attributed the antagonistic activity of *Lactobacillus* to their pH reduction, while other attributed it to the production of bacteriocins in addition to the pH reduction effects (Aslim *et al.*, 2005).

Using pH controlling batch culture Gibson and Wang proved that organic acid production is not the sole mechanism responsible for inhibition of microbial growth by different *lactobacillus* strains, human isolated *lactobacillus* appeared to produce antibacterial substances, this was confirmed when various pathogens were inhibited by the addition of pH- neutralizing supernatant of lactobacilli (Gibson and Wang, 1994., De Vuyst and Leory, 2007).

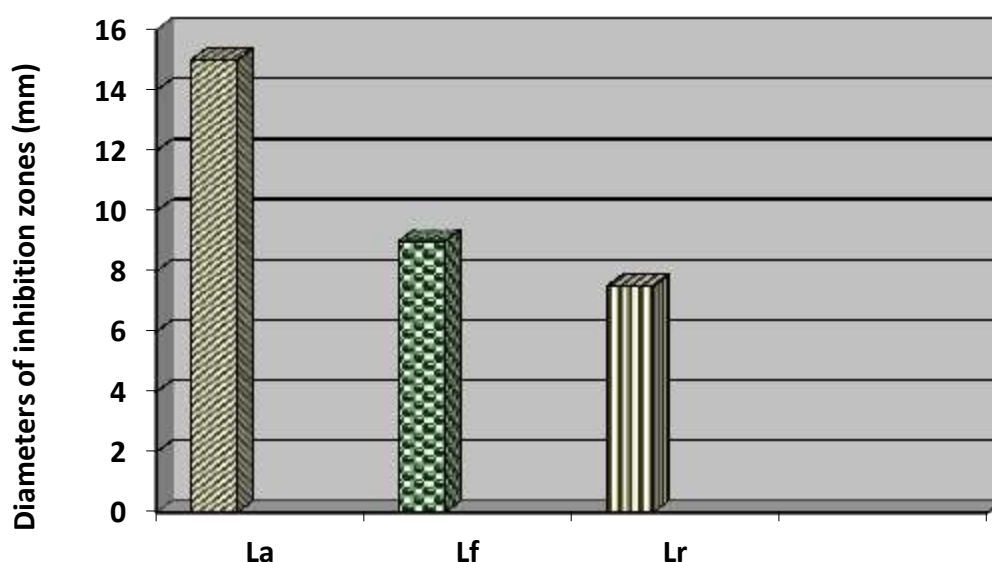


Figure (1): Inhibition zones of *Lactobacillus* isolates (La, Lf, Lr) against *Listeria monocytogenes*

The MIC of Bacteriocins which appeared activity against test bacteria was varied among the three tested strains (Table-1).

The MIC for *Lf* and *Lr* bacteriocins was 32, and for *La* bacteriocin was 16.

Table (1): Minimum inhibitory concentration of *Lactobacillus* spp. bacteriocins

Bacteriocins	Minimum inhibitory concentration
<i>Lf</i>	32
<i>La</i>	16
<i>Lr</i>	32

During fractionation procedure with solid ammonium sulfate, all studied bacteriocins recovered antilisterial activity in the pellet at 60% saturation. This not agreed with the finding of researchers in respect with this subject, which found that most *Lactobacillus* bacteriocins precipitate at 80% saturation with ammonium sulfate (Lievine *et al.*, 2000), but agreed with the bifidiocin produced by *Bifidobacterium bifidum*, which precipitate from growth medium at 60% saturation of ammonium sulfate (Meghrour *et al.*, 1990).

The inhibitory effect of bacteriosins against *L. monocytogenes* appeared pH dependent; they remained stable and exhibited highest activity in acidic pH range 2 – 6. Bacteriocin of isolate *Lf* was stable at pH 4 – 6, while the isolates *La* and *Lr* bacteriocins were the most stable, since they remained active over the pH 2 – 6, and all three bacteriocins lost antilisterial activity after incubation at pH over 6 (Table-2). Similar phenomenon of acid stability has also been demonstrated for bacteriocines; plantaricin, bulgaricin, and lactobulgaricin produced by human *Lactobacillus* (Yildirim *et al.*, 1999).

Table (2): Sensitivity of bacteriocins to different PH values

Bacteriocine of isolates	PH values						
	2	3	4	5	6	7	8
<i>Lf</i>	-	-	+	+	+	-	-
<i>La</i>	+	+	+	+	+	-	-
<i>Lr</i>	+	+	+	+	+	-	-

(+ Resistant, - Sensitive)

Results showed that the bacteriocin samples are heat stable, the bacteriocin produced by the isolate *La* considered to be more heat stable, as it retained almost of its activity (89.5%) after heating at 100°C for 10 min compared with bacteriocins of *Lf* and *Lr*, since the former lost about 21% of its antimicrobial activity at same temperature within 10 min, while the latter lost 29% of its activity (Fig-2).

Heat stability of *Lactobacillus* bacteriocins have been confirmed by researchers (Olukoya *et al.*, 1993; Alpay *et al.*, 2003). The heat stability is an advantage and important parameter if the bacteriocins are to be used as food preservative because many processing procedure involve heating steps.

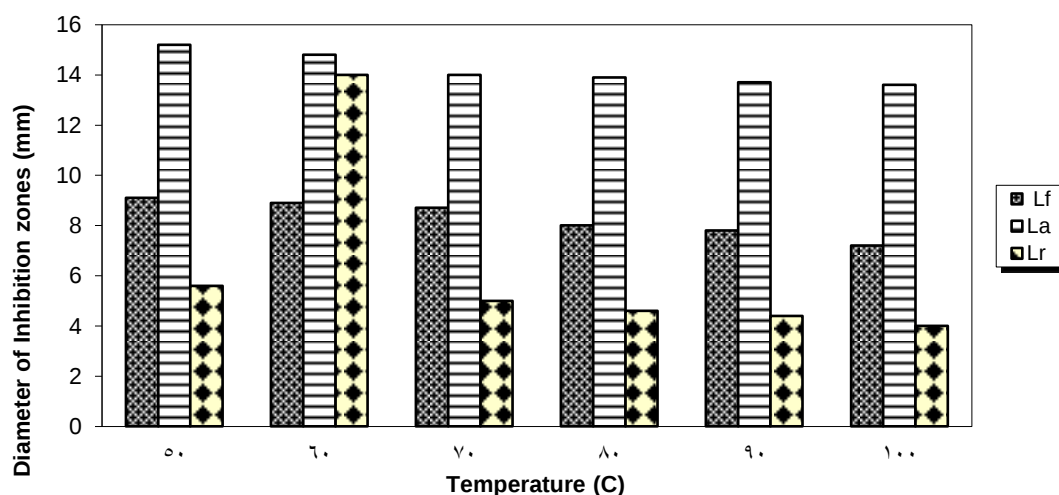


Figure (2) : Heat sensitivity of bacteriocins produced by *Lactobacillus* isolates

The antimicrobial activity of all three bacteriocins against *L. monocytogenes* lost completely when treated with proteolysis enzymes pepsin and trypsin, while this activity appeared unrelated to lysozyme, as their activity not lost after treatment.

Since antilisterial activity destroyed after treatment with pepsin and trypsin and not affected with lysozyme, so the bacteriocins have probably a proteinaceous nature. This was in accordance with Gonzaleze, who Poplished in that *Lactobacillus* compete with other bacteria by secreting antagonistic proteinaceous compounds with different molecular mass affected surrounding microbial cells viability (Gonzaleze *et al*, 1994).

Antagonistic activity of bacteriocins were relatively stable under a series of storage conditions, the antilisterial activity become lower but not lost by long term storage, as all bacteriocins produced by the test isolates maintained full stability after storage for a month at -18°C, and they revealed almost half activity after storage for month at 4°C, while no inhibition was found in storage at room temperature 25°C (Table-3) Similar results were confirmed for bacteriocins produced by *Pediococcus acidilactic* (Nieto – Lozano *et al.*, 2002).

The stability of tested bacteriocins during prolonged storage makes them superior as biopreservative and indicating that the cold temperature may be the most appropriate preservation technique.

Table (3) Effect of storage temperature for a month on bacteriocins activity

Bacteriocin	-18°C	4°C	25°C
<i>Lf</i>	+	±	–

La	+	±	–
Lr	+	±	–

(+ Resistant, ± Moderately sensitive, - Sensitive)

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أنتاج بكتريوسينات من عزلات *Lactobacillus spp.* لأطفال رضع فعاله ضد بكتريا

Listeria monocytogenes

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الخلاصة

تم الحصول على ثلاث عزلات من بكتريا *Lactobacillus* من عينات براز لأطفال الرضاعة الطبيعيه ، والعزلات صنفّت للأنواع : *L. fermentum* (Lf) ، و *L. acidophilus* (Lc) ، و *L. reuteri* (Lr) . أظهرت العزلات الثلاث ألقدره على أنتاج البكتريوسينات ضد بكتريا الأختبار *Listeria monocytogenes* بتقنية الانتشار في حفر الأغار (agar – well diffusion) ، جزئت البكتريوسينات المنتجة من العزلات المنتخبة بالترسيب بكبريتات الأمونيوم ، ونسبة تشبع 60% ، ودرست بعض الصفات الفيزيوكيميائية لها ، ثبتت البكتريوسينات عند قيم الارقام الهايدروجينية الحامضية عند مدى pH (2 – 6) ، وأظهرت ثبوتيه حراريه عند مدى من درجات الحراره 50 – 100 م° ولفترة تعريض 10 دقائق . فقدت البكتريوسينات فعاليتها ضد مايكروبيه عند المعامله مع الانزيمات المحلله للبروتينات، الببسين والتربسين ، في حين لم تتثبط هذه الفعاليه عند المعامله مع انزيم اللايسوزايم ، وحافظت عينات البكتريوسينات الثلاث على فعاليتها ضد مايكروبيه بدرجة حرارة التجميد عند -18 م° ولفترة خزن مدتها شهر .