Antibacterial activity of Bacteriocins isolated from infant Lactobacillus against Listeria monocytogenes

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Abstract

Three Lactobacillus isolates, isolated from breast-feed infants feces belongs to the species: L. fermentum, (L**f**), L. acidophilus (L**a**), and L. reuteri(L**r**) . 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 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.

الخلاصه

تم الحصول على ثلاث عزلات من بكتريا Lactobacillus من عينات براز لأطفال ألرضاعه ألطبيعيه ، والعزلات صنفت للأنواع : L. fermentum (L**f**)، و L. acidophilus (L**a**)، و L. reuteri (L**r**) .أظهرت العزلات الثلاث ألقدره على أ نتاج البكتريوسينات ضد بكتريا الأختبار Listeria monocytogenes بتقنية الأنتشار في حفرالأغار agar- well .diffusion جزئت البكتريوسينات المنتجه من العزلات المنتخبه بالترسيب بكبريتات الأمونيوم ،وبنسبة تشبع ٦٠% ، ودرست بعض الصفات الفيزيوكيميائيه لها ، ثبتت البكتريوسينات عند قيم الارقام الهايدروجينيه الحامضيه عند مدى pH (٢ – ٦) ، وأظهرت ثبوتيه حراريه عند مدى من درجات الحراره ٥٠ – ١٠٠ م° ولفترة تعريض ١٠ دقائق . فقدت

البكتريوسينات فعالياتها الضد 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 فعالياتها الضد مايكروبيه بدرجة حرارة التجميد عند -١٨ م° ولفترة خزن مدتها شهر .

Introduction

Lactobacillus gram-positive rods, colonize the gastrointestinal tract of mammals and human immediately after birth to a high population [(1)]. Gastric Lactobacillus contribute approximately 75% in gastric functions, they known to benefit health as natural predominant microflora [(2,3,4)]. 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[(5,6)] . 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 [(7,8)]. 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 [(9,10)]. 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 [(11)].

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 [12,13]. L.monocytogenes shows the ability to survive adverse conditions such as vacuum, freezing, ultraviolet ray, and to resist conventional pasteurization [14]. 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 [15].

The aim 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 proteolytsis enzymes, and effect of storage temperature and period.

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 inoculated in MRS broth medium for enrichment of resident Lactobacillus , tubes incubated anaerobically (anaerobic jar and gas pack) at 37°C for 48 h. Cultures streaked on MRS agar plates several times, isolates identified to genes 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^{o}C as stock culture.
Listeria monocytogenes obtained from biology dep. Baghdad university, used as indicator microorganism, propagated in brain heart infusion medium (BHI).

*Bacteriocin production screening

Bacteria were screened for their antilisterial activity by agar – well diffusion technique [16], they were grown in MRS broth at 37°C anaerobically 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).

*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 10^6cell /ml [17] , procedure continued as previous, and the diameters of inhibition zones around wells recorded after 24h incubation at37°C.
The antilisterial titer of tested bacteriocins was defined as the reciprocal of the highest dilution showing inhibition.

*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 o.o5 M phosphate buffer pH 7, dialyzed against same buffer[18], and the remaining activity was assayed against indicator bacteria.

Physicochemical characterization

The fractionated bacteriocins were characterized with respect to: pH sensitivity, thermal stability, sensitivity to lysis enzymes, and stability during storage [19].

*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 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 indicator 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 Lactobacilli 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 indicator 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 [20].

The isolates belongs to the species; Lactobacillus fermentum (L**f**), Lactobacillus acidophilus(L**a**), and Lactobacillus rutrie(L**r**).

Bacteriocins production varied among these three species, L**a** was more potent than the other two species, it exhibited larger inhibitory zone, 15mm when tested by agar-well diffusion technique, while the species L**f** and L**r**. 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 lactobacilli to their pH reduction, while other attributed it to the production of bacteriocins in addition to the pH reduction effects [21].

Using pH controlling batch culture Gibson and Wang ,1994[22] 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 [23].

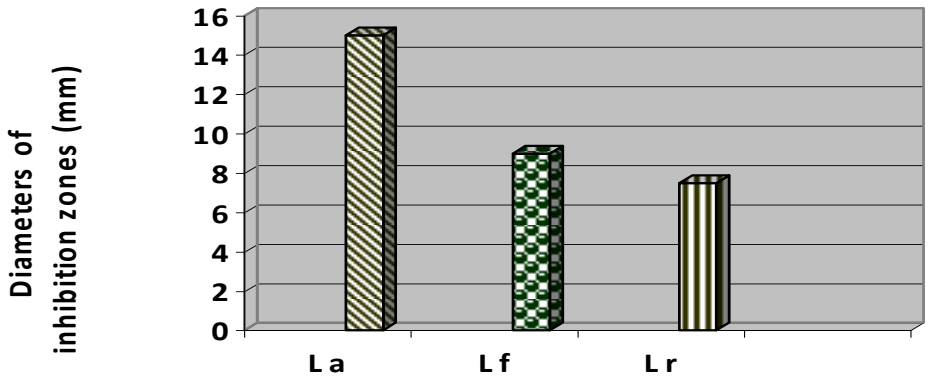

Figure (1): Inhibition zones of *Lactobacillus* isolates (L***a***, L***f***, L***r***) **against *L. monocytogenes***

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

The MIC for L***f*** and L***r*** bacteriocins was 32, and for L***a*** bacteriocin was 16.

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

Bacteriocins	Minimum inhibitory concentration
L***f***	32
L***a***	16
L***r***	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 lactobacilli bacteriocins precipitate at 80% saturation with ammonium sulfate [24], but agreed with the bifidiocin produced by *Bifidobacterium bifidum* , which precipitate from growth medium at 60% saturation of ammonium sulfate [25].

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 strain L***f*** was stable at pH 4 – 6, while the strains L***a*** and L***r*** 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* [26].

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

Bacteriocine of isolates	2	3	4	5	6	7	8
L***f***	-	-	+	+	+	-	-
L***a***	+	+	+	+	+	-	-
L***r***	+	+	+	+	+	-	-

+ Resistant, - Sensitive.

Results showed that the bacteriocin samples are heat stable, the bacteriocin produced by the isolate L***a*** 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 L***f*** and L***r***, 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 [27, 28]. 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.

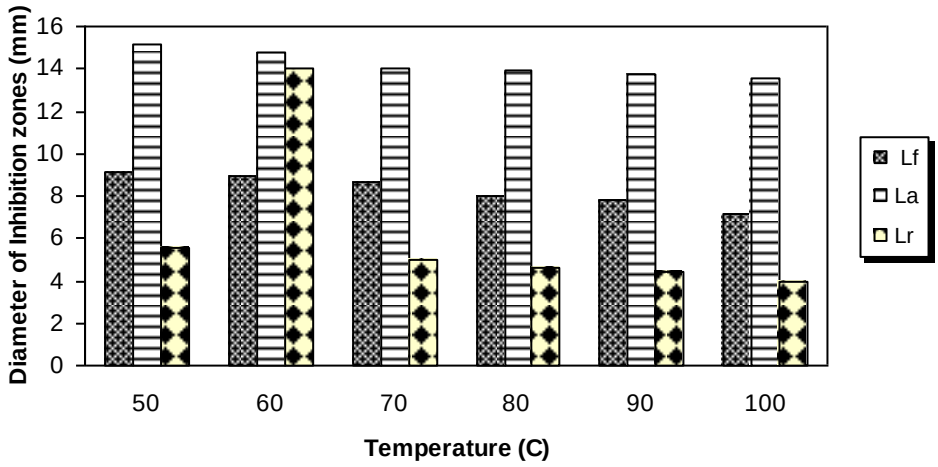

Figure (2) : Heat sensitivity of bacteriocins produced by Lactobacillus isolates

The antimicrobial activity of all three bacteriocins against indicator bacteria 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 *et al*, 1994 [29]. Poplished in that *Lactobacillus* compete with other bacteria by secreting antagonistic proteinaceous compounds with different molecular mass affected surrounding microbial cells viability.

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-2).
Similar results were confirmed for bacteriocins produced by Pediococcus acidilactic [30].
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

Bacteriocins	-18°C	4°C	25°C
L*f*	+	±	_
L*a*	+	±	_
L*r*	+	±	_

+ Resistant, ± Moderately sensitive, - Sensitive

REFERANCES

(1) Sevage, D.C. (**1977**). Microbial ecology of the gastrointestinal tract. Rev. Microbiol. 31: 107 – 123.
(2) Tannock, G. W., Dashkvicz, M. P. and Feighner, S.D. (**1989**). Lactobacillus and bile salt hydrolyse in the murine intestinal tract. Appl. Environ. Microbiol. 55: 1848 – 1851.
(3) Perdigon, G., Alvarez, S., Rachid, M., Aguero, G. and Gobbato, N. (**1995**) Immune stimulation by probiotics. J. Dairy. Sci. 78:1597 - 1605.
(4) Duffy, L.C., Zielenzny, M.A. and Riepenhoff – Talty, M. (**1994**). Reduction of virus shedding by B. bifidum in experimentally induced MRV infection. Dig Dis. Sci. 39: 2334 – 2340.
(5) Fooks, L.G. and Gibson, G.R. (**2002**). Probiotic as modulator of gut flora. Brit. J. Nutri. 88:39 – 49.
(6) Holzapfel, W.H., Habere, P., Geisen, R., Bjorkroth, J. and Ulrich,

S. (**200**1). Taxonomy and important features of probiotic microbes in food and nutrition. Am. Clin. Nutr. 37: 365 – 373.
(7) Hirano, J., Yoshida, T., Sogiyama, N., Koid, I. and Yokochi, T. (**2003**). The effect of L. rhamnosus on enterohemorrhagic E. coli Infection of human intestinal cells in vitro. Microbio. Immunol. 97 : 405 – 409.
(8) Ogunbanwo, S.T., Sanni, A. I. and Onilude, A. A. (**2003a**). Influence of cultural conditions on the production of bacteriocin by L. brevis O . Afr. J. Biotechnol. 2: 179 – 184.
(9) Ogunbanwo, S.T., Sanni, A. I. and Onilude, A. A. (**2003b**). Characterization of bacteriocins produced L. plantarum F and L**.** brevis O. Afr. J . Biotechnol. 2: 219 – 227.
(10) Sinead, C.C., Christian, Y.L., Paul, W., Colin, H. and Cormac, G.M. (**2007**). Bacteriocin production as a mechanism for the anti-infective activity of Lactobacillus salivarius UCC118. Acad sci. USA. 104 (18) : 7617 – 7621.
(11) Guven, U., Semsek, H. and Maras, Y. (**2001**). The inhibitory effect of L. casei and L. helveticus on Bacillus species isolated from raw milk in various salt concentration. Intl. J . dairy Technol. 54: 146 – 153.
(12) Tour, R., Kheader, K., Moroni, O. and Fiss, I. (**2003**). Production of antimicrobial substances by Bifidobacterial isolates from infant stool active against Listeria. 95: 1058 – 1069.
(13) Harres, L.J., Daeschal, M.A., Stiles, M.E. and Klaenhammer, T.R. (**1989**). Antimicrobial activity of Lactic acid bacteria against Listeria monocytogenes. J. of Food Protec. 52: 384 – 387.
(14) Gahan, C.G. and Collins, J. K. (**1991**). Listeriosis: biology and Implication for the food industry. Trend in food science and Technol. 2: 89 – 93.
(15) Jacobsen, C.N., Nielsen, V.R., Hyferd, A.E., Moller, K.F., Michelson, A., Parregaard, b. and Jakokobes, M. (**1999**). Screening of probiotic activities of 47 strain of Lactobacillus spp. By in – vetro technique and evaluation of colonization ability of the five selected strains in human. Appl. Environ. Microbiol. 68: 4949 – 4956.
(16) Barefoot, S.F. and Kalaenhammer, T.R. (**1989**). Detection and the activity of lacticin B, a bacteriocin produced by L. acidophilus acidapohilus. Appl. Environ. Microbiol. 45: 1808 – 1815.
(17) Mayer-Harting, A., Hedges, A.J. and Berkely, R.W.(**1972**). Methods of study bacteriocins. Methods.Microbiol. 7:315-422.
(18) Todorov, S.D**.** and Dicks, L.M.T. (**2004)**. Partial characterization of bacteriocins produced by four lactic acid bacteria isolated from

regional south Africa barly beer**. Annals of Microbiol, 54: 404-413**
(19) De Vuyst, L. and Leory, F. (**2007**). Bacteriocins from lactic acid Lactic acid bacteria : Production , Purification, and food application J.mol. Microbiol. Biotechnol. 13: 149 – 199.
(20) Kanlder, O. and Weless, N. (**1986**). Regular non- sporing Gram Positive rods In: Bergey's Manual of Systematic Bacteriology. Vol 2. sectin 14: 1208 – 1260.
(21) Aslim, B., Yuksekdag, Z.N., Sarikagu, E. and Bayatti, Y. (**2005**). Determination of the bacteriocin – like substences produced by some lactic acid bacteria isolated from Turkish dairy products. Food. Sci. Technol. 33: 691 – 694.
(22) Gibson, G.R. and Wang, X. (**1994**). Regulatory effects of Bifidus on growth of other colonic bacteria. J. Appl. Bacteriol. 77: 412 – 420
(23) De Vuyst, L. and Leory, F. (**2007**). Bacteriocins from lactic acid Lactic acid bacteria : Production , Purification, and food application J.mol. Microbiol. Biotechnol. 13: 149 – 199.
(24) Lievine, V., Peiffer, I,. Hudault, S., Rochat., F., Barassat, D., Neeser, J.R . and Servin, A. (**2000**). Bafidobacterium strains from resident infant human gastrointestinal microflora exert antimicrobial activity Gut. 47: 646 – 652.
(25) Meghrour, J., Eulogy, P., Junellus ., A., Ballobu, J. and peptitemang, H, (**1990**). Screening of Bafidobacteria strains for bacteriocins production . Biotechnol. Letters. 12: 575 – 586.
(26) Yildrin, Z., Winter, D. and Jonson, M. (**1999**). Purification , amino acids sequence and mode of action of bifidiocin B by B, bifidus N CFB . J. Appl. Microbiol. 86: 45 – 51.
(27) Alpay, S., Aydin, F. and kolich, S.S. (**2003**). Antimicrobial activity and characteristics of bacteriocins produced by vaginal Lactobacilli. Turk. J. Med. Sci. 33: 7 – 13 .
(28) Olukoya, D.K., Tichazek, P.S., Butch, A., Vogel, R.F. and hummas, W, P. (**1993**). Characterization of bacteriocins produced by Lactococcus pentosis DK7 isolated from ogi. And L. plantarum DK, from fufu. Chem. Microbiol. Technol. 15: 65 – 68.
(29) Gonzalez, B., Araca, P. and Suareg, J.E. (**1994**). Detection and Partial characterization of plantaricin C1, a bacteriocin produced by Lactobacillus plantarum strain of dairy origin . Appl. Environ. Microbiol. 60: 2158 – 2163
(30) Nieto – Lozano J.C., Regura – Useros J.I., Pelaez – Martinez M.C., and De La TorreA.H. (**2002**) . Bacteriocinigenic activity from starter culture used in Spanish meat industry . Meat sci. 62: 237