The synergistic effects of *Lactobacillus acidophillus ROO52* and *Lactobacillus bulgaricus LB-12* bacteriocins against *E.coli* O157 :H7 in milk

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Summary

The aims of this study are to evaluate the antibacterial potential of standard strains (*Lactobacillus acidophilus ROO52* and *Lactobacillus bulgaricus LB-12*) that produce bacteriocins against *E.coli* O157:H7 in raw milk. Thirty raw milk samples randomly were collected weekly from different markets of Baghdad city in order to investigate the prevalence of *E.coli* O157:H7 in milk. The prevalence of *E.coli* O157:H7 in raw milk samples was 6 out 30 (20%) by using the modern chromogenic media with serological latex agglutination test kit. The average diameter of the inhibition zone of crude bacteriocin against *E .coli* O157:H7 by using combination of 1:1 (v/v) of the *Lactobacillus acidophillus ROO52* and *Lactobacillus bulgaricus LB-12*was (14mm) ,while that produced by bacteriocins of *Lactobacillus acidophillus ROO52* and *Lactobacillus bulgaricus LB-12* independently were 16 and 12mm respectively .*L. delbrucekii ssp bulgaricus* showed significantly (P<0.05) low antimicrobial effect against *E.coli* O157 :H7 .While the strongest antimicrobial effect was shown by *Lactobacillus acidophilus ROO52*. The bacteriocins of the reference strains used in this study did not result in an increase in inhibition when used in combination of 1:1 (v/v).

Keywords: Synergistic effects, Bacteriocins, *E.coli* O157:H7.

Introduction

Escherichia coli is the most abundant facultative anaerobic gram-negative bacterium of the intestinal microflora which naturally colonize the mucous layer of the colon. Enterohemorrhagic *E. coli*, (EHEC) is responsible for outbreaks of hemolytic uremic syndrome (HUS) and bloody diarrhea. Intervention and treatment strategies for EHEC infections are quite controversial to that of conventional antibiotics which may be harmful by increasing the probability of patients developing hemolytic uremic syndrome (HUS) (1). Further, studies have shown that *Escherichia coli* is one of the important pathogens that may cause meningitis and may cross blood brain barrier to the central nervous system (CNS) without altering its integrity (2).

Lactic acid bacteria (LAB) are a group of gram-positive bacteria.The general description of the bacteria included in the group is gram-positive, non-spore forming, cocci or rods, which produce lactic acid as the major end product during the fermentation of carbohydrates. Lactic acid bacteria are nutritionally fastidious, requiring carbohydrates, amino acids, peptides, nucleic acids and vitamins. Taxonomic revisions suggest that the lactic acid bacteria: such as *Aerococcus, Carnobacterium, Enterococcus, Lactobacillus, Lactococcus, Leuconostoc, Pediococcus, Streptococcus, Tetragenococcus Vagococcus,* and *Weissella* (3). Bacteriocins are ribosomally synthesized antimicrobial compounds that are produced by many different bacterial species including many members of the lactic acid bacteria (4). Some bacteriocins produced by lactic acid bacteria, such as nisin, inhibit not only closely related species but are also effective against food-borne pathogens and many other gram-positive spoilage microorganisms (5). For this reason, bacteriocins have attracted considerable interest for use as natural food preservatives in recent years, which has led to the discovery of an ever increasing potential sources of these protein inhibitors. The target of the bacteriocins is the cytoplasmic membrane and because of the protective barrier provided by the Lipopolysaccharide of the outer membrane of gram-negative bacteria, they are generally only active against gram-positive cells.

The permeability of the outer membrane of gram-negative bacteria can be increased by sublethal injury including that which can occur

when using ultrahigh hydrostatic pressure (UHP) and pulsed electric field (PEF) as non-thermal methods of preservation (6). Bacteriocins are suitable for food preservation and studies conducted suggest that their use offers a lot of advantages such as extend shelf life, provide protection especially during times of temperature abuse, decrease the risk of transmission of food borne pathogens, decrease the losses due to food spoilage, reduce the application of chemical preservatives, and permit the application of less severe heat treatment without compromising food safety (Hurdle Concept). Bacteriocins are non-toxic to eukaryotic cells and hence pose no threat to human intestinal cells. Being proteinaceous in nature they are readily degraded by protelytic enzymes in human gastrointestinal tract. Moreover, they do not have any therapeutic application and are not known to cause allergies. Being of LAB origin they are probiotic in nature and also help in restoring the normal gut microflora (7 and 8). Applications of bacteriocins for the control of some pathogens and food spoilage organisms have been approved in a number of countries (9 - 11). Advances in bacteriocins research and combination treatment for food preservation will benefit both the producer and consumer (12). The aim of this study was to evaluate the antibacterial effect of crude bacteriocin of mono and co-cultures against *E.coli* O157:H7 in milk.

Materials and Methods

The indicator organisms namely *E.coli O157:H7* was isolated from raw milk samples while the reference strains *Lactobacillus acidophilus* ROO52 received from Rosell Institut (Montreal, Canada) as freeze-dried powder and *Lactobacillus bulgaricus LB-12* Procured from the school of Animal sciences LSU Agriculture center (Louisiana State University).

All the *Lactobacillus acidophilus ROO52* and *Lactobacillus bulgaricus LB-12* cultures were maintained at 4°C in MRS broth while the pathogenic organisms were maintained at 4°C on Brain Heart Infusion broth. All the bacterial cultures were sub-cultured every 15 days interval. Prior to their use in the experiment, back cultures were subcultured in appropriate broth (13).

A total of 30 random freshly drawn morning cow's raw milk samples were collected at weekly intervals from different retail markets in Baghdad city during four months from the beginning of January to April 2013. Samples were collected in labeled sterile polyethylene sacs and kept in insulated ice box cooled (5°C) and were transported to the laboratory without delay. For each milk sample, tenfold serial dilutions (10^{-1} to 10^{-5}) were prepared in sterile 0.1% (wt/v) Peptone water as a diluent. Colonies of *E.coli* O157:H7 were isolated from raw milk samples by conventional methods and their identification were confirmed based on biochemical and both cultural and serological characteristics.

The antimicrobial activity of crude bacteriocin against indicator organism was determined after subjecting *E.coli* O *157: H7* to a stress condition at low refrigeration temperature (4°C) for six hours. *E.coli* O157:H7 was isolated and identified from raw milk samples after 24 hours of aerobic incubation at 37°C on chromogenic agar. Five identified colonies of *E.coli* O *157: H7* were selected and subcultured onto nutrient agar by streaking to obtain pure colonies by incubating at 37 °C for overnight then five colonies inoculated directly in 10 ml of sterile nutrient broth. The inoculated nutrient broth was incubated aerobically at 37 °C for 24 hours (13).

The crude bacteriocins were obtained from the bacteriocin producing strains *Lactobacillus acidophilus R0052* and *Lactobacillus bulgaricus LB-12* which were grown in MRS broth under anaerobic condition at 37 °C for 24 hrs and the supernatant fluid was separated from cells by centrifugation at 10000 rpm for 20 min. The supernatant was collected and pH was adjusted to 7 with sterile 1N NaOH so as to rule out inhibition through production of organic acids and filtered through a syringe filter with pore size of 0.45 µm , then heating for 5 min at 70 °C to prevent inactivation of antibacterial peptides by protease and killed cells and then stored at 4 °C in a refrigerator (14). Inhibitory activity of the crude bacteriocin against *E.coli* O157:H7 was

assayed according to the method of Food microbiology protocols (2001). Use of combination of bacteriocins :Crude bacteriocins preparation from both organisms were used alone and mixed in equal amount and assay performed as (the method of Food microbiology protocols 2001) by the modified agar overlays method: Two hundered µl of an overnight culture of *E.coli O157: H7* was mixed gently with 10 ml of molten Mueller-Hinton agar (MHA) top agar at 45 °C and the content was poured into a Petri dish containing 10 ml of solidified Mueller-Hinton agar (MHA) base agar 1.5% ,Wells of 6 mm in diameter were made with a sterile hollow punch. 50 µl of bacteriocin was added into each well and the plate was incubated for overnight at 37 °C (13).

Statistical methods include Mean and standard error of the mean were analyzed using SPSS (version 16.0, SPSS Inc, Chicago, IL, USA, 2007). The significant differences were determined at ($P<0.05$) among the different mean values. Each experiment was performed in three repeats.

Results and Discussion

Colonies of *E. coli* O157:H7 that were isolated from raw milk samples were confirmed based on biochemical; and both cultural and serological characteristics showed in (Tables, 1 and 2). *E. coli* O157:H7 was negative for potassium cyanide (no growth) and for both sorbitol and cellobiose fermentation.Typical colonies of *E. coli* O157:H7 on chromoagar appeared as mauve in color .Presumptive *E. coli* O157:H7 isolates obtained were further tested serologically for the presence of both the O157 and H7 antigens using the commercial latex agglutination kits. Commercial latex kits were available for O157 and H7 and tests were carried out according to the manufacturer's instructions and both positive and negative control organisms and control latex were incorporated. The isolate was tested for the presence of flagellar antigen. The antisera were raised to H antigen which required passage through motility medium (Table, 2).

The prevalence of *E.coli* O157:H7 in raw milk samples by using the modern chromogenic media with serological latex agglutination test kit is shown in Tables 3. Results obtained in this study revealed that 6 isolates (20%) were identified as *E. coli* O157:H7 out of 30 raw milk samples as shown in (Table, 3).

Table, 1: Cultural characteristics of *E.coliO157:H7* and *lactobacilli*:

microorganisms	Growth medium	Temp. (°C)	Positive reaction
E.coli O157:H7	Chromo agar™ *O157:H7*	37	Mauve colonies
	Motility test medium		Motile
	Trypton broth (Indol test)		Red ring
Lactobacillus acidophillus ROO52	de man Regosa Sharp (MRS) broth	37	Turbidity
Lactobacillus bulgaricusLB-12	de man Regosa Sharp (MRS) broth	37	Turbidity

Table, 2: Serological and biochemical characteristics of *E. coli* O157:H7

Potassium cyanide (KCN)	Negative (No growth)
Sorbitol fermentation	Negative
Cellobiose fermentation	Negative
O antiserum	Agglutination (positive)
H antiserum	Agglutination (positive)

Table, 3: prevalence of E. coli O157:H7 from raw milk samples

Number of samples	Number of positive samples	Percentage % of isolation
30	6	20

The antimicrobial spectrum exhibited by the crude bacteriocins of references strains against *E. coli* O157:H7 is shown in (Table, 4). The average diameter of the inhibition zone of crude bacteriocins of *Lactobacillus acidophillus ROO52* and *Lactobacillus bulgaricus LB-12* against *E. coli O157:H7* independently was 16 mm and 12 mm respectively, while the average diameter of the inhibition zone of crude bacteriocins of *Lactobacillus acidophillus R0052 and Lactobacillus bulgaricus LB-12* in combination. 1:1(v/v) was 14mm.

Table, 4: Zone of inhibition of indicator organisms upon the use of bacteriocins of *Lactobacillus acidophilus ROO52* and *Lactobacillus bulgaricus LB-12* independently and in combination.

Crude bacteriocin of reference strains	Zone of inhibition (mm)	
	independently Mean± S.E (mm)	combination 1:1 v/v Mean± S.E (mm)
L .acidophillus ROO52	16 ± 0.06 a	14±0.04 b
L.bulgaricus LB-12	12±0.02 c	

Different letters in columns revealed significant differences (P <0.05) between the diameters of the inhibition zone.SE = Standard error.

Antimicrobial peptides (AMP's) are produced and excreted by bacteria, fungi, plants, insects and vertebrata. Bacteriocins are a subgroup of AMP's. They are small peptides encoded by ribosomal DNA or plasmids characterized by antibacterial activity, most frequently against bacteria which are closely related phylogenetically (narrow spectrum of activity) or against other microbes (wide spectrum of activity). The connection with the increasing frequency of occurrence of pathogenic microbes resistance to antibiotics, there is a higher and higher interest in using probiotics or purified bacteriocins as alternative medical preparations (14 and 15). The major classes of bacteriocins produced by LAB include: Class Ilantibiotics, Class II small heat stable peptides, Class III large heat labile proteins and Class IV complex proteins whose activity requires the association of carbohydrates or lipid moieties. Out of these, first two groups have received increased attention as food biopreservatives (16).

Class II bacteriocins represent the largest, most diverse group of LAB bacteriocins to date. *Lactobacilli, streptococci, lactococci, enterococci, pediococci, and leuconostoc* all produce at least one class II bacteriocin (17). Members of the class IIb bacteriocins require the complementary action of two peptides to achieve full inhibitory activity. In some cases, each peptide may be inactive or confer very little antimicrobial activity (18). Synergy in biology is when two or more substances work together to achieve an enhanced effect that would not be produced singly, bacteriocins which belong to different categories and with different mode of actions are likely to exhibit synergistic effect (19).Anumber of studies are being conducted to enhance the antimicrobial spectrum of the bacteriocins for greater application in food systems. Use of more than one bacteriocin is one such approach. The two bacteriocin preparations prepared in the present study were mixed in equal volume. Combination of these bacteriocins in equal ratio did not result in any appreciable increase in antibacterial activity in this investigation. Mainly synergistic effects have been reported between pairs of bacteriocins from lactic acid bacteria.

Bacteriocins may have bacteriostatic o rbactericidal mode of action on different pathogens; this distinction is being greatly influenced by several factors such as bacteriocin dose and degree of purification, physiological state of target cell and experimental conditions (20). They could not assign any reason for antagonism of different pairs of bacteriocins. Increased antibacterial activity of combination than when used alone had been reported by (21). Antimicrobial action of bacteriocins occurs in steps - adsorption of the bacteriocin on cell wall, its transport across the cell membrane and finally its action within the cytoplasm. Bacteriocins are cationic proteins and their primary receptors are anionic lipids (22). Presence of receptors on cell surface plays a role in bacteriocin specificity (23). Synergistic effect occurs when receptor for one bacteriocin is not present but receptor for another bacteriocin is available for antibacterial action. Antagonism can occur when the bacteriocin producers compete for the same receptors on indicator

cell surface. Bacteriocins which belong to different categories and with different mode of actions are likely to exhibit synergistic effect (24).

Authors (25) suggested using combination of bacteriocins belonging to different classes to obtain enhanced activity. Both the bacteriocins used in this study belong to the same class. Further work for understanding the mechanism of interaction of bacteriocins is in progress. Use of combination of other bacteriocins from same class and different classes is being pursued. Amino acid sequencing of highly purified form of the bacteriocins can give a greater insight into the nature of interaction with each other. In conclusion *L. delbrucekii* ssp *bulgaricus* that showed significantly ($P<0.05$) low antimicrobial effect against indicator strain while the strongest antimicrobial effect was shown by *L. acidophilus ROO52*.The bacteriocins of reference strains used in this study did not result in increased in inhibition when used in combination of 1:1 (v/v).

References

1. Kaper, J.B; Nataro, J.P and Mobley, H.L. (2004). Pathogenic *Escherichia coli*. Nat. Rev. Microbiol., 2: 123-140.
2. Stins, M.F; Badger, J.L and Kim, K.S. (2001). Physiology and Pathology of the blood-brain barrier: Implications for microbial pathogenesis. Microb. Pathog., 30: 19-28.
3. Stiles, M. E. (1996). Biopreservation by lactic acid bacteria. Antonie van Leeuwenhoek. 70:331-345.
4. Settani, L. and Corsetti, A. (2008). Rev: Application of bacteriocins in vegetable food biopreservation Int. J. Food Microbiol., 121:123-138.
5. Ross, R. P.; Morgan, S. and Hill, C. (2002). Preservation and fermentation: past, present and future. Int. J. Food Microbiol., 79:3-16.
6. Abriouel, H.; Franz, C.; Omar, N. B and Galvez, A. (2011). Diversity and applications of *Bacillus* bacteriocins. FEMS Microbiol. Rev., 35:201-232.
7. Thomas, L.V; Clarkson, M. R and Delves-Broughton, J. (2000). Nisin. In: Naidu, AS (Ed), Natural Food antimicrobials system. CRC Press, Boca-Raton, FL. Pp: 463-524.
8. Kim, J. S. and Kim, Y. H. (2007). "The inhibitory effect of natural bioactives on the growth of pathogenic bacteria."Nutr. Res. Prac. 1: 273-278.
9. Cotter, P.D; Hill, C and Ross, R.P. (2005). Batceriocins: Developing innate immunity for food. Nat. Rev. Microbiol., 3:777-788.
10. Fimland, G.; Johnsen, L. Dalhus, B and Nissen-Meyer, J. (2005). Pediocin like antimicrobial peptides (class IIa bacteriocins) and their immunity proteins: biosynthesis, structure and mode of action. J. Peptide Sci., 11:688-696.
11. Deegan, L. H; Cotter, P. D.; Hill, C. and Ross, P. (2006). Bacteriocins: biological tools for biopreservation and shelf life extension. Int. Dairy J., 16:1058-1071.
12. Mayachiew, P.; Devahastin, S.; Mackey, B. M. and Niranjan, K. (2010). Effects of drying methods and conditions on antimicrobial activity of edible chitosan films enriched with galangal extract." Food Res. Int., 43: 125-132.
13. Khudhier, Z. S. (2011). Antibacterial activity of *Lactobacillus acidophilus* bacteroicin against *E.coli* O157:H7 in raw milk. PhD. Thesis. Veterinary Medicine College, University of Baghdad.
14. Sergio, A.; Fernando, J. M.; Aida, A. and Raul, R. (2001). Food Microbiology Protocols .Food Microbiol. Bio., 14:141-146.
15. Heng, N.C; Ragland, N. L.; Swe, P. M.; Baird, H. J.; Inglis, M. A.; Tagg, J. R. and Jack, R. W. (2006). Dysgalacticin: a novel, plasmid-encoded antimicrobial protein (bacteriocin) produced by *Streptococcus dysgalactiae* subsp. equisimilis. Microbiol., 152: 1991–2001.
16. Chen, H. and Hoover, D.G. (2003). Bacteriocins and their food applications. CRFSFS., 2: 82- 100.
17. Diep, D. B.; Johnsborg, O.; Risoen, P. A. and Nes, I. F. (2001). Evidence for dual functionality of the operon pln ABCD in the regulation of bacteriocin production in *lactobacillus plantarum*. Mol Microbiol., 41:633-644

18. Haas, W.; Shepard, B. D. and Gilmore, M. S. (2002). Two-component regulator of *Enterococcus faecalis* cytolysin responds to *quorum*-sensing auto induction. Nature. 415:84-87.

19. Vignolo, G.; Palacios, J.; Farias, M.; Sesma, F.; Schillinger, U.; Holzapfel, W. and Oliver, G. (2000). Combined effect of bacteriocins on the survival of various *Listeria* species in broth and meat system. Curr.Microbiol., 41:410-416.

20. Cintas, L. M.; Herranz, P. C.; Hernández, E.; Casaus, M. P. and Nes, L. F. (2001). Review: Bacteriocins of lactic acid bacteria. Food Sci. Tech. Int., 7: 281-305.

21. 21-Hanlin, M. B; Kalchayanand, N. P. and Ray, B. (1993). Bacteriocins of Lactic acid bacteria in combination have greater activity. J. Food Prot., 56:252-255.

22. O`Sullivan, L.; Ross, R. P.; and Hill, C. (2002). Potential of bacteriocin producing Lactic acid bacteria for improvements in food safety and quality.Biochimie. 84:593-604.

23. Drider, D.; Fimland, G.; Hechard, Y.; McMullen, L. M. and Prevost, H. (2006). The continuing story of class IIa bacteriocins. Microbiol., Mol. Biol. Rev., 70:564-582.

24. Dalie, D. K. D.; Deschamps, A. M and Richard-Forget, F. (2010). Lactic acid bacteria-Potential for control of mould growth and mycotoxins: a review. Food control. 21:370-380.

25. Tiwari, S. K. and Srivastava, S. (2008). Characterization of bacteriocin from *lactobacillus plantarum* strain LR/14. Food Bioethanol. 22:247-261.

الفعل التآزري للبكتروسينات المنتجة من *Lactobacillus acidophillus ROO52* و *Lactobacillus bulgaricus LB-12* ضد بكتريا الايشيريشيا القولونية المعوية النزفية O157: H7 في الحليب

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الخلاصة

صممت الدراسة الحالية لتقييم الفعل التازري لسلالات القياسية *Lactobacillus acidophillus ROO52* و *Lactobacillus bulgaricus LB-12* المنتجة للبكتروسين ضد الايشيريشيا القولونية المعوية النزفية في الحليب الخام .جمعت 30 عينة من الحليب الخام اسبوعيا بشكل عشوائي من اسواق البيع المختلفة في مدينة بغداد خلال مدة اربعة اشهر (من كانون ثاني 2013 الى شهرنيسان 2013). تواجدت بكتريا الايشيريشيا القولونية المعوية النزفية في 6 عينات من الحليب الخام من اصل 30 عينة وبنسبة 20% وبإستعمال الاوساط الصباغية المتطورة مع عدة اختبار التلازن المصلي . معدل قطر منطقة التثبيط للبكتروسين الخام المنتج من السلالات القياسية سويا وبنسبة 1:1 ضد بكتريا الايشيريشيا القولونية المعوية النزفية(14 مليمتير) . بينما كان معدل قطر منطقة التثبيط للبكتروسين الخام المنتج من السلالات *Lactobacillus acidophillus ROO52* و *Lactobacillus bulgaricusLB-12* كلا على حده 16و12 مليمتيروعلى التوالي. استنتج ان سلالة *Lactobacillus bulgaricusLB-12* اظهرت شدة فعالية منخفضة وبصورة معنوية وعلى مستوى ($P< 0.05$) ضد بكتريا الايشيريشيا القولونية المعوية النزفية بينما اظهرت سلالة *Lactobacillus acidophillus ROO52* شدة فعالية اقوى، ان البكتروسينات المنتجة من السلالات القياسية في هذة الدراسة لم تظهر شدة فعالية اقوى عند مزجهما سويا وبنسبة 1:1.

الكلمات المفتاحية : الفعل التآزري, البكتروسين، بكتريا الايشيريشيا القولونية المعوية النزفية.