المجلة العراقية
لبحوث السوق وحماية المستهلك

المجلد (6) العدد (2)
لسنة 2014

Comparison methods for the evaluation of bacteriocin potency against *E.coli* O157:H7 in milk

Zina Saab khudhir
College of Vet .Medicine/ University of Baghdad

تأريخ استلام البحث: 2013/11/12 تأريخ قبول النشر: 2014/3/4

Abstract

The main objective of the present study was to evaluate the antagonistic effect of reference strain *Lactobacillus acidophilus* ROO52 bacteriocin against*Escherichia coli.* O157:H7 with a comparison of disk diffusion agar, well diffusion agar ,spot method and modified agar.overlays methods. A total of 30 raw milk samples were collected randomly at weekly intervals from different local markets in Baghdad province during the period from the beginning of January 2013 till the end of April 2013). The prevalence of *E .coli* O157:H7 in raw milk samples was 20% by using the modern chromogenic media with serological latex agglutination test kit. The data revealed that the method applied had a significant (P < 0.05) effect on the antimicrobial potency of the crude bacteriocin against *E. coli* O157:H7. The average diameter of the inhibition zone of crude bacteriocin against*E .coli* O157:H7 by both, agar disk diffusion method and the agar well diffusion method was (10mm) respectively and showed a potency of (62.5%) while that produced by the agar spot diffusion method was (8mm) and showed a potency of (50%) while that produced by the modified agar overlays method was (16mm) which exhibited significantly (P< 0.05) the highest antimicrobial potency (100%) compared to that produced by the above mentioned three methods. Results showed that the modified agar overlays method was the most suitable and reliable method for assessing the antagonistic effect of the crude bacteriocin against *E. coli* O157:H7.

Key words: *E.coli* O157:H7, bacteriocin potency, milk.

المجلة العراقية
لبحوث السوق وحماية المستهلك

المجلد (6) العدد (2)
لسنة 2014

مقارنة طرائق تقييم فعالية البكتروسين ضد بكتريا الايشيريشيا القولونية المعوية في الحليب O157: H7 النزفية.

زينة صائب خضير
فرع الصحة العامة البيطرية– كلية الطب البيطري/ جامعة بغداد

الخلاصة

الهدف الرئيس للدراسة الحالية هو تقييم التاثير المضاد للبكتروسين الخام المنتج من سلالة القياسية ضد بكتريا الايشيريشيا ***Lactobacillus acidophilus*** **ROO52** القولونية المعوية النزفية وللمقارنة في تقييم الفعالية التثبيطية استعمل الاختبار الاحيائي للانتشار عن طريق الاقراص والحفر عبر الاكار والتنقيط والطريقة المحورة للانتشار في الحفر عبر الاكار. جمعت 30 عينة من الحليب الخام اسبوعيا بشكل عشوائي من اسواق البيع المختلفة في محافظة بغداد خلال مدة اربعة اشهر (من بداية كانون ثاني 2013 حتى نهاية/ شهر نيسان 2013. تواجدت بكتريا الايشيريشيا القولونية المعوية النزفية في عينات الحليب الخام بنسبة 20% شخصت البكتريا وباستخدام الاوساط الصباغية المتطورة مع عدة اختبار التلازن المصلي. اشارت نتائج الطرائق المستخدمة ان شدة الفعالية التثبيطية (القوة الفعالة) للبكتروسين الخام مؤثرة وبصورة معنوية وعلى مستوى ($P< 0.05$) ضد بكتريا الايشيريشيا القولونية المعوية النزفية. معدل قطر منطقة التثبيط للبكتروسين الخام المنتج من السلالة القياسية ضد بكتريا الايشيريشيا القولونية المعوية النزفية بكل من طريقة الاقراص والحفر عبر الاكار(10) ملليليتر وعلى التوالي واظهرت شدة الفعالية التثبيطية (62.5%) بينما بطريقة التنقيط (8) مليليتر واظهرت شدة الفعالية التثبيطية (50%) بينما بطريقة الانتشار عبر الاكار المحورة (16) ملليليتر وبمستوى معنوية ($P< 0.05$). اعلى شدة تثبيطية هي (100%) مقارنة بالطرائق المذكورة انفا. في النتيجة اثبتت هذة الدراسة ان طريقة الاختبار الاحيائي للانتشار في الحفر عبر الاكار المحورة هي الطريقة الاكثر مناسبة ووثوقا في دراسة الفعالية التثبيطية للبكتروسين الخام المنتج من السلالة القياسية ضد الايشيريشيا القولونية المعوية النزفية في الحليب.

الكلمات المفتاحية :الايشيريشيا القولونية المعوية النزفية، فعالية البكتروسين، الحليب.

Introduction

Escherichia coli. is commonly found in the intestines of all animals, including humans. The *E. coli* serotype O157:H7 produced large quantities of strong toxins called verotoxin or shiga- like toxin that caused severe damage to the lining of the intestine (17) .Since the first reported outbreak in the US in 1982 (18). *E. coli* O157:H7 infections have been reported most frequently in developed countries, although illness due to *E.coli*O157:H7 has been reported in over 30 countries on six continents (17). *E.coli*O157:H7 was considered an emerging disease pathogen (16). The majority of transmission was through eating of undercooked contaminated ground meat and consumption of raw milk, raw vegetables, fruits contaminated by water, cheese and also through consumption of sprouts, lettuce and juice. In developing countries of the world, where there is still an alarming rate of insanitary conditions, malnutrition and poor health facilities, there is an urgent need to study this organism and its characteristics with an aim to reduce the human hazard caused by this emerging pathogen (4). The use of lactic acid bacteria (LAB) especially the isolates from dairy products such as *L. acidophilus*and/ or their antagonistic metabolism such as bacteriocin was an example of biopreservation(8). The use of friend microorganisms or their natural products was preferred to chemical in the field of food preservation due to their antagonistic activity against many food borne pathogens and spoilage bacteria(19). Natural antimicrobials were derived from many sources, ranging from animal (chitosan, lysozyme, and lactoperoxidase) to plant (essential oils, aldehydes, esters, herbs and spices) and to microbial origin bacteriocin(nisin) (27) .Several methods have been applied to measure the *in vitro* susceptibility of bacteria to bacteriocin, such as agar dilution, broth microdilution, and disk diffusion, which were standard methods recommended by the Clinical and Laboratories Standards Institute (CLSI) for measuring the in *vitro* susceptibility of bacteria to antimicrobial agents used in clinical settings (3; 7).The aim of this study was to compare different methods in order to determine the most reliable method for evaluation the antibacterial activity of reference strain *Lactobacillus acidophilus ROO52*bacteriocin against *E.coli* O157:H7 in raw milk .

Materials and Methods

Microorganisms:

The tested organism namely *E.coli O157:H7* was isolated from raw milk samples while the reference strain *Lactobacillus acidophilu*s ROO52 from RosellInstitut (Montreal, Canada) as freeze- dried powder Procured from the school of Animal sciences LSU Agriculture center (Louisiana State University) by Dr. Najim Hadi Najim**.**

Maintenance of microorganisms:

All the *Lactobacillus acidophilus ROO52* 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 daysintervals.Prior to their use in the experiment, cultures were subcultured in appropriate broth (15).

Samples collection:

Milk samples: A total of 30 random freshly drawn morning cow**'**s raw milk samples were collected at weekly intervals from different retail markets in Baghdad province during the period from the beginning of January 2013 till the end of April 2013 .

Samples were collected in labeled sterile polyethylene sacs, kept in ice box cooled (5°C) and transported to the laboratory without delay.

Sample preparation: For each sample, tenfold serial dilutions (10^{-1} to 10^{-6}) were prepared in sterile 0.1% (wt/v)Peptone water as a diluent.

Isolation of *E.coli*O157 :H7 bacteria from raw milk samples:

Colonies of *E. coli* O157 :H7were 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 using different methods after subjecting *E.coli*O *157: H7* to a stress condition at low refrigeration temperature (4°C) for six hours. *E. coli* O*157: 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 streak 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 (6).

Preparation of bacteriocin:

The crude bacteriocins were obtained from the bacteriocin producing strains *Lactobacillus acidophilus* R0052 which was 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 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 (22). Inhibitory activity of crude bacteriocin against *E.coli* O157 :H7 was assayed according to the Method of Food Microbiology Protocols (2001). In order to choose the best methodology for detection of antibacterial activity of bacteriocin against *E.coli O157 :H7* the following methods were examined .

The Disk Diffusion agar method:

The bacterial inoculum was adjusted to certain concentration, inoculated onto the entire surface of a Mueller-Hinton agar (MHA) plate with a sterile cotton- tipped swab to form aneven lawn. The paperdisks (6 mm in diameter) impregnated with crude bacteriocin (50 µl) were placed on the surface of each MHA plate using a sterile forceps. Then the plates were incubated aerobically and the diameter of theinhibition zonewas measured by a ruler or caliper (6)**.**

The well Diffusion agar method:

Petri dishes are filled with an appropriate Mueller-Hinton agar to a thickness of 15 mm, and various numbers of holes were punched out of the agar, by using a cork borer of 6 mm diameter. The base of each hole was sealed with a drop (0.05 ml) of melted Mueller-Hinton agar, and then standardized quantities ofbacteriocin preparations (50 µl)were added to the appropriate wells (22).

The spot Diffusion method:

Two hundred µl of *E. coli* O157 :H7 culture in the broth was mixed with 15 ml of Mueller- Hinton soft agar and poured on the plate. Then, 50 µl of crude bacteriocinwere dropped onto the solidified agar. The plates were incubated for 24hrs**.** Bacteriocin inhibition was indicated by a clear zone surrounding each spot in the soft agar layer(10)**.**

The modified agar overlays method:

Two hundred µl of an overnight culture of *E.coli* O*157: 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 agar1.5% ,Wells of 6 mm in diameter were made with a sterile hollow punch.50 µl of bacteriocinwas added into each well.The plate was incubated overnight at 37 °C (9).

Statistical analysis:

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 andDiscussion:

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(table, 1). Results obtained in this study revealed that six isolates (20%) were identified as*E. coli* O157:H7 from the 30 raw milk samples.

Table (1): The Prevalence level (%) of *E.coli*O157:H7 isolatedfrom raw milk samples:

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

Colonies of *E. coli* O157:H7 that were isolated from raw milk samples were confirmed based on biochemical and both cultural and serological characteristics are shown in (table, 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 appeared 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 available latex agglutination kits or antisera(Table, 2).

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

Medium	Reaction
Trypton broth (Indol test)	Positive
Potassium cyanide (KCN)	Negative (No growth)
Motility test medium	Motile
Cellobiose fermentation	Negative
Sorbitol fermentation	Negative
Chromogar™ *O15:H7*	Mauve colonies(Positive)
O antiserum	Agglutination
H antiserum	Agglutination

The antimicrobial activity of the crude bacteriocin was evaluated by the addition of crude bacteriocin directly into impregnated disk or as a spot or well (6mm) inside the soft agar seeded with *E. coli* O157 :H7 .After incubating the agar plates, Circular clear inhibition zone of no growth appeared around each well, disk and spot.The average diameters of all the inhibition areas were measured using the standard ruler and the presence of 2mm or more clear inhibition zones around the wells, disks and spots was regarded as a positive results.The antimicrobial spectrum exhibited by the crude bacteriocin against *E. coli* O157 :H7 is shown in (table, 3). The average diameter of the inhibition zone of crude bacteriocin against *E. coli* O157 :H7 by both the agar disk and well diffusion methods was 10 mm while that produced by the spot diffusion methods was 8 mm while by the modified agar over lay method was 16mm exhibited which significantly ($P < 0.05$) the highest antimicrobial activity effectiveness compared to that produced by the above mentioned three methods.

Table (3): The antimicrobial activity of the crude bacteriocinagainst *E. coli*O157 :H7:

Methods used in this study	Inhibition Zone diameter (mm) Mean± S.E	% of potency
Disk Diffusion agar method	10± 0.02 **b**	62.5%
well Diffusion agar method	10± 0.02**b**	62.5%
spot Diffusion method	8± 0.01 **c**	50 %
modified agar overlays method	16 ± 0.06**a**	100%

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

Methods used in this study to demonstrate antagonism are referred to generally the simultaneous (or direct) antagonism procedures based on diffusion of inhibitory substance in the agar medium (26). It may be stated that the size of inhibition zones depends not only on sensitivity of the target strain on antimicrobial compounds produced by *Lactobacillus acidophilus* ROO52 but also on the method used for detection. The most reliable results of antimicrobial activity of *Lactobacillus acidophilus* ROO52 bacteriocin were observed in case of three methods: modified agar overlays method, the well diffusion assay and disk diffusion agar method respectively .The highest mean value of inhibition zones (12 mm). In recent years much attention has received the application of biopreservation. Biopreservation refers to extended shelf life and enhanced safety of foods obtained by using the natural or added microflora and their antimicrobial products(21). Extensive work has been carried out on bacteriocins and bacteriocin producing strains of Lactic acid bacteria (LAB) for their potential use as biopreservatives(20). Over the past decade, levels of bacterial resistance to antibiotics have risendramatically, in the context of bacterial antibiotic resistance, the non- bacteriocin, antibiotic-like molecules produced by selected lactobacilli and bifidobacteria strains are of interest in terms of innovative antimicrobial therapy. There are several methods used to detect antimicrobial activity(1). Generally,

tests for antagonism are performed on solid media and involve the detection of inhibition of growth of an indicator strain caused by the test culture, methods which are used to evaluate the activity of antimicrobial agent are divided into *in vitro* and *in vivo* (application test) (1). The former may be termed "screening methods" and might include any test in which the compound is not applied directly to the product under use conditions(11). Generally, these tests provide preliminary information to determined potential usefulness of the test compound ,the second type includes those tests in which an antimicrobial is applied directly to a product(11). In vitroscreening methods are subdivided into endpoint and descriptive tests, endpoint tests are those in which a microorganism is challenged for an arbitrary period. The results reflect the inhibitory power of a compound only for the time specified, in descriptive test the microorganism is also challenged but periodic sampling is carried out to determine changes in viable cell number over time. Among the methods the disk diffusion has been the most popular one used to examine the antimicrobial activity of natural antimicrobials including chitosan(14). The disk diffusion method allows for the simultaneous testing of a large number of antimicrobials in a relatively easy and flexible manner(14). Disadvantages of this method are unable to generate the MIC value (i.e., not quantitative) and difficult to examine the susceptibility of fastidious and slow-growing bacteria. disk diffusion is labor-intensive and time-consuming (12). The well diffusion assay was suitable for aqueous extracts because they were difficult to dry on paper discs However, the leaking of sample under the agar layer must be considered (24) .*Klewickaet al*. (1999), (13) also suggested that the most reliable results were obtained with two methods: the dual culture overlay assay and the agar slab method. Moreover, it was found in one study (23), that *Lactobacillus* grown on agar medium was able to synthetize other inhibitory substances– bacteriocins in significantly greater amount than that in liquid culture production and release of antimicrobial molecules by *Lactobacillus* was known to be variable with factors such as cell density and population kinetics(5; 2). Such differences could account for the failure to detect inhibition zones in well-diffusion tests (2). Growth of pathogens was less inhibited by cell-free extracts from *L. rhamnosus* than by live culture of *L. rhamnosus* ,calculating mean values and standard error for diameters of inhibitory zones and making variance analysis showed that in comparison to other

methods with the agar slab technique the most consistent and reproducible results were obtained. These results are in agreement with another study in which three methods were compared: paper disc, double layer and agar slab test (25). While in this study i concluded that the modified agar overlays method was the most suitable and reliable method for assessing the antagonistic effect of the crude bacteriocinagainst *E .coli* O157:H7.

References

1. Ammor, S.; Tauveron, G.; Dufour, E. and Chevallier I. (2006). Antibacterial activity of lactic acid bacteria against spoilage and pathogenic bacteria isolated from the same meat small- scale facility. 1- Screening and characterization of the antibacterial compounds. Food Control .17:454- 461.

2. Chen H. and Hoover D. G. (2003). Bacteriocins and their food safe 2:82-100. applications. Compr. Rev. Food.

3. Clinical and Laboratory Standards Institude (NCCL). (2009). "Performance standards for antimicrobial disk susceptibility tests; approved standard- tenth edition M02- A10. National Committee for Clinical Laboratory Standards 29.

4. 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.

5. Delgado, A.; Arroyo, López, F. N.; Bito, D.; Peres, C.; Fevereiro P. and Garrido- Fernández A. (2007). Optimum bacteriocinproduction by *lactobacillu splantarum* 17. 2b requires absence of NaCl and apparently follows a mixed metabolite kinetics. J. Biotechnol. 130: 193- 201.

6. Doyle, M. P. (2001). Food Microbiology: Fundamentals and Frontiers 2 nd. Edn., ASM Press, Washillgton.

7. Drider, D.; Fimland, G.; Hechard, Y.; Mc Mullen, L. M and Prevost, H. (2006). The continuing story of class IIabacteriocins. Microbiol. Mol. Biol. Rev. 70: 564- 582.

8. Keen, J. E.; Wittum, T. E.; Dunn, J. R.; Bonno, J. L. and Durso, L. M. (2006). Shigatoxigenic *Escherichia coli* O157 in agricultural fair livestock, United States. E*merg. Infect. Dis.*, 12: 780- 786.

9. 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.

10. Kili, A. O.; Pavlova, S. I.; Ma W. and Tao L. (1996) .Analysis of *lactobacillus*phages and bacteriocins in American dairy products and characterization of a phages isolated from yogurt. Appl Environ Microbial 62: 2111- 2116.

11. Kim, J. S. and Kim, Y. H. (2007). "The in hibitory effect of naturalbioactiveson the growth of pathogenic bacteria. Nutrition Research and Practice.1: 273-278.

12. Klancnik, A.; Piskernik, S.; Jersek, B. and Mozina, S. S. (2010). "Evaluation of diffusion and dilution methods to determinetheanti bacterial activity of plant extracts."J Microbiol Methods 81: 121-126.

13. Klewicka, E.; Libudzisz, Z.; Czajka D. and Kuc K. (1999). Antagonistic activity of lactic acid bacteria *Lactobacillus acidophilus* [Antagonistyc znaakty wnośćba kteriifer menta cjimle kowej *Lactobacillus acidophilus*. Żywność 4 (21): 168–175.

14. 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 withgalangal extract." Food Research International 43: 125-132.

15. Mirlohi, M.; soleimaninan- Zad, S. and Sheikh- Zeinoddin. R. (2008). Species- Specific Identification of wild type *L. rhamnosus*. Strains with potential probiotic properties. poster presented in 10th Congress of Iranian Genetics Tehran.

16. Nataro, J. P and Kaper, J. B. (1998). Diarrheagenic *Escherichia coli*. *Clin. Microbiol. Rev.* 11:142- 14.

17. Raji, M.; Minga, U and Machangu, R. (2006). Current epidemiological status of enterohemorrhagic *Escherichia coli* O157:H7 in Africa. *Clin Med. J.* 119: 217-222.

18. Riley, L. W.; Remis, R. S.; Helgerson, S. D.; McGee, H. B.; Wells, J. G., Davis; B.R., Helbert; R. J., Olcott; E. S., Johnson; L. M., Hargrett; N. T., Blake; P. A. and Cohen, M. L. (1983). Hemorrhagic colitis associated with a rare *Escherichia coli* serotype. *N. Engl. J. Med*. 308: 681- 685.

19. Ross, R. P.; Morgan, S. and Hill, C. (2002). Preservation and Fermentation: past, present and future international Journal of Food Microbiology. 79: 3-16.

20. SavadogoAly; OuttaraCheik A. T; BassoleImael H. N and Traore S. Alfred. (2006). Bacteriocins and Lacticacid Bacteria. *African Journal of Biotechnology*.5 (9): 678- 683.

21. Schnürer, J. and Magnusson J. (2005). Antifungal lactic acid bacteria as biopreser vatives. Trends Food Sci. Technol. 16:70- 78.

22. Sergio, A.; Fernando, J. M.; Aida, A. and Raul, R. (2001). Food Microbiology Protocols. Food Microbiology Bio 14:141-146.

23. Sip, A. (1999). Bacteriocin production by lactic acid Produkcjaba kteriocyny przezbakteriemle kowe.Biotechnologia . 2: 145–165.

24. Stoyanova, L. G. and Levina N. A. (2006). Components of fermentationmedium regulate bacteriocinsynthesis by the recombinant strain *Lactococcuslactis*subsp. Lactis F- 116. Microbiol. 75: 86- 291.

25. Strus, M. (1998). A new method for evaluation of the antagonistic action of bacterial lactic acid (LAB) on selected pathogenic indicator bacteria. Nowameto daocenyantagonistyc znegod ziałaniaba kterii kwasumle kowego (LAB) nawybrane, choro botwórczeb akteriewska źnikowe. Med. Dośw. Mikrobiol. 50: 123- 130.

26. Tagg J. R.; Dajani, A. S. and Wannamaker L. W. (1976). Bacteriocins of Gram- positive bacteria. Bacteriol. Rev. 40: 722–756.

27. Tiwari, B. K.; Valdramidis, V. P.; O' Donnell, C. P.; Muthukumarappan, K.; Bourke, P. and Cullen, P. J. (2009). "Application of natural antimicrobials for food preservation." J Agric Food Chem 57: 5987- 6000.