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Isolation of *Enterococcus* Species from Food Sources and Its Antibacterial Activity against *Staphylococcus Aureus*

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Abstract

The microbial production of substances that have the ability to inhibit the growth of other microorganisms is possibly the most common defense strategy developed in nature. Microorganisms produce a variable collection of microbial defense systems, which include antibiotics, metabolic by-products, lytic agents, bacteriocins and others. The aim of the present study was to isolate and identify *Enterococcus spp.* and its most prevalent species from food samples and determine its antibacterial activity against *Staphylococcus aureus* isolates. A total of 50 food samples from different sources (dairy products (20 samples) and vegetables and fish (15 samples each)) were collected from different local markets in Baghdad and cultured. *Enterococcus spp.* were isolated from only 32 food samples. *E. faecium* was the most predominant species which was recovered from 20 samples (62.5 %), 10 dairies, 7 vegetables, and 2 fish. *E. faecalis* was found in 8 samples (25 %), 5 vegetables and 3 fish. *E. avium* was recovered 6.25% as well as *E. gallinarium* (2 samples for each) *Enterococcus avium* were all isolated from dairy products but *Enterococcus gallinarium* one sample isolated from dairies and the other from fish. This study indicates the presence of *Enterococcus spp.* in the food samples and the ability of these bacteria to produce antibacterial substances which are active against closely related clinical isolates.

Keywords: bacteriocins, *Enterococcus faecium*, *Enterococcus faecalis*, *Staphylococcus aureus*, antibacterial activity.

عزل بكتيريا *Enterococcus spp.* من مصادر غذائية والتحري عن فعاليتها ضد بكتيرية ضد بكتيريا المكورات العنقوية

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الخلاصة

تعد قابلية الاحياء المجهرية لانتاج مواد لها القدرة على تثبيط نمو احياء مجهرية اخرى، احدى اهم الاستراتيجيات الدفاعية في الطبيعة، الاحياء المجهرية لها القدرة على انتاج مجموعة متباينة من الوسائط الدفاعية كالمضادات الحيوية، منتجات التمثيل الغذائي، عوامل محللة، البكتيريوسينات وغيرها. الهدف من هذه الدراسة هو عزل وتشخيص بكتيريا *Enterococcus spp.* من مصادر غذائية وتحديد النوع الأكثر

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انتشارا والتحري عن قابليته الضد بكتيرية ضد عزلات سريرية. تم جمع 50 عينة غذائية من الاسواق المحلية في بغداد ومن مصادر مختلفة (مشتقات الالبان 20 عينة, الخضروات والاسماك 15 عينة من كل مصدر) تم زرعها لغرض التحري عن وجود بكتيريا *Enterococci*, 32 عينة فقط كانت تحتوي على هذا النوع من البكتيريا. النوع الغالب هو *Enterococcus faecium* 62.5% (20 عينة) 10 من مشتقات الالبان, 7 من الخضروات و2 من مصدر السمك), يليه النوع *Enterococcus faecalis* 25% (8 عينات) 5 تم عزلهم من الخضروات وما تبقى تم عزله من السمك) اما النوعين *Enterococcus avium*, *Enterococcus gallinarium* بنسبة 6.5% (2 عذلة لكل نوع). كلا العزلتين تم عزلهما من مشتقات الالبان بينما *Enterococcus gallinarium* تم عزل احدى العزلتين من مشتقات الالبان والاخرى من السمك.

Introduction

Enterococci comprise a large genus of Gram-positive, catalase-negative, and oxidase-negative bacteria that produce lactic acid as a major end product of glucose fermentation. They are facultative anaerobic cocci that usually appear in pairs or short chains [1]. This genus was separated from the genus *Streptococci* in the 1980s because of DNA hybridization data showing that they were not closely related. Accordingly, they were moved into their own genus under group D *streptococci*. *Enterococci* are now comprised of more than 20 species, of which *E. faecalis* and *E. faecium* are the most common that are readily found in the feces of mammals. Fecal *Enterococci* are classified as indicators of fecal bacteria and unsanitary processing of foods. Because of their association with animals, *Enterococci* are found worldwide in a large variety of fresh and prepared foods, including vegetables, dairy milk, meats and meat products [2]. *Enterococci* are commensals in the animal and human intestinal tract; they are believed to play an important role in balancing the microbiota, thereby showing great potential as probiotics [3]. On the other hand, *Staphylococci* are a group of bacteria that cause diverse diseases ranging from minor skin infections to life-threatening diseases such as bacteremia. They are considered as a major cause of both hospitalized and community-acquired infections worldwide. One of the most common opportunistic pathogens of this genus is *Staphylococcus aureus* [4]. *S. aureus* strains are gram-positive facultative anaerobic, chemoorganotrophic cocci arranged in grape like clusters, with a respiratory and fermentative metabolism at an optimal temperature of 37°C. Also, they are non-motile, catalase positive, and found as pathogens or commensal organisms in both humans and animals. In addition, milk and its products can harbor a variety of microorganisms and can be regarded as an important source of food borne pathogens. One of these important pathogens is *Staphylococcus aureus* [5]. The current study aimed to detect the anti-staphylococcal activity of food-originated Enterococcal bacteriocin.

Materials and Methods

Isolation of bacteria

Fifty fresh food samples (dairy milk, vegetables and fish) were collected randomly from Baghdad markets, Iraq during the period from September to November 2019. All the collected samples were inoculated on Bile esculin-azide agar and incubated at 37°C for 24 hours. The ability to hydrolyze esculin in the presence of bile is a characteristic of *Enterococci* and group D *streptococci* [6].

Identification of isolates

All collected samples were identified according to the morphological features on culture medium and biochemical test [7]. VITEK2 was employed for the results confirmation. The identification of *Enterococcus* was performed by direct inoculating of the samples on Bile esculin-azide agar at 37°C for 24 hr. A positive result was indicated by converting the color of the media to black coffee brown around the colonies (due to the hydrolysis of esculin). The ABC streaking method was performed on brain heart infusion (BHI) agar to produce pure colonies. The samples were then suspended in 5 ml normal saline, and the results were read after 9 hr. *Enterococcus faecium* was the most predominant species, being recovered in 20 samples (62.5%), 10 from dairies, 7 from vegetables, and 2 from fish. It was followed by *Enterococcus faecalis* which was found in 8 samples (25%), 5 from vegetables, and 3 from fish. While *Enterococcus avium* was recovered 6.25% as well as *Enterococcus gallinarium* (2 samples for each). *E. avium* were all isolated from dairy products but *E. gallinarium* one sample isolated from dairies and the other from fish.

Isolation of pathogenic bacteria

Six different clinical isolates of *Staphylococcus* were obtained from wounds of different patients hospitalized in AL-Amerat Private Hospital. The identification of *S. aureus* was performed by direct inoculating of the clinical isolates on mannitol salt agar (MSA) at 37C° for 24 hr. The positive result was indicated by converting the media from red to yellow color. ABC streaking method on BHI agar was performed to obtain a pure single colony, which was then re-inoculated onto MSA to confirm the results [7].

Antibacterial activity

The detection of Enterococcal antibacterial activity was performed using two methods. The first method was the well diffusion method; *Enterococcus spp.* were grown for 24 hr. in BHI broth at 37 C°. One milliliter of the overnight culture was centrifuged for 3 min at 12,000 g and the supernatant was passed through a 0.2 micron pore-size filter to remove bacteria. Antibacterial activity was evaluated on Muller Hinton agar (MHA) plates inoculated with *S. aureus* under aseptic conditions. The wells (diameter = 8 mm) were filled with 50 µl of the filtered and sterilized supernatant and incubated at 37 C° for 24 hr. The diameter of the growth inhibition zones was then measured [8]

The second method was the spot on the lawn method; the intended isolates for antibacterial substances production were grown under appropriate conditions on MHA. During the growth, the antibacterial substances were released from the producing cells into the solid agar, after 18 hr. of incubation at 37C°, the growing bacteria was scraped from the surface of the medium using a microscopic slide. The plates were then exposed to a chloroform vapor. To ensure that there are no cells remaining and all were killed, a filter paper was laid in the middle of the glass petri dish then saturated with 1 ml chloroform. It was ensured that the chloroform could not drain away from the filter paper into the petri dish. The scraped plates were inverted and laid over the glass petri dish so that the agar plates were fully exposed to chloroform vapor for about 15 min at room temperature. After 15 min, the plates were still inverted but left open over their own lids for additional 15 min in a 37 C° incubator to ensure a complete removal of the remaining chloroform vapor [9, 10]. Then the plates were streaked with the tested bacteria (*S. aureus*).

Results and discussion

Fifty food samples (dairy milk, vegetables and fish) were collected randomly from Baghdad markets, Iraq. Thirty-two samples (64%) were considered as *Enterococcus*. Isolates were identified depending on microscopical properties as well as biochemical tests, as shown in Table-1.

Table 1-Biochemical and microscopical tests and their results for 32 *Enterococcus spp* isolates

Test	Result
40% Bile	Tolerant
Esculin hydrolysis	Positive
Azide tolerance	Positive
Gram stain	Gram positive (cocci appear in pairs or short chain)
Catalase	Negative
Oxidase	Negative
Glucose fermentation	Lactic acid producers

The identification of *Enterococcus* was performed by direct inoculating of the samples on Bile esculin-azide agar at 37C° for 24 hr. The positive result was indicated by converting the media to black coffee brown color around the colonies (due to the hydrolysis of esculin). The ABC streaking method on BHI agar was performed to obtain pure colonies (Figure-1).

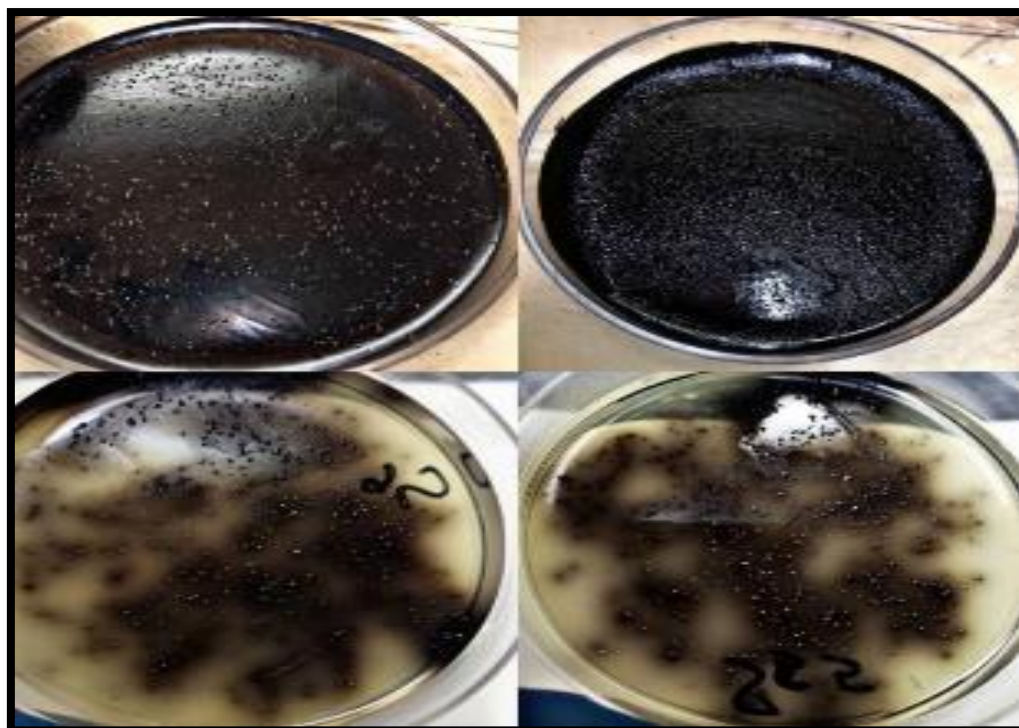


Figure 1-Enterococcus spp. colonies on Bile-Esculin azide agar. sculin) after 24 hr. at 37C°.

Then, the VITEK system was adopted for further species identification, as illustrated in Table-2. The isolated Enterococcal species with the isolation percentage of each species are shown in (Figure-2).

Table 2-The species of Enterococcal isolates **identified** depending on VITEK system.

Isolate	Species
E1-E20	<i>Enterococcus faecium</i>
E21& E22	<i>Enterococcus gallinarium</i>
E23 & E24	<i>Enterococcus avium</i>
E25-E32	<i>Enterococcus faecalis</i>

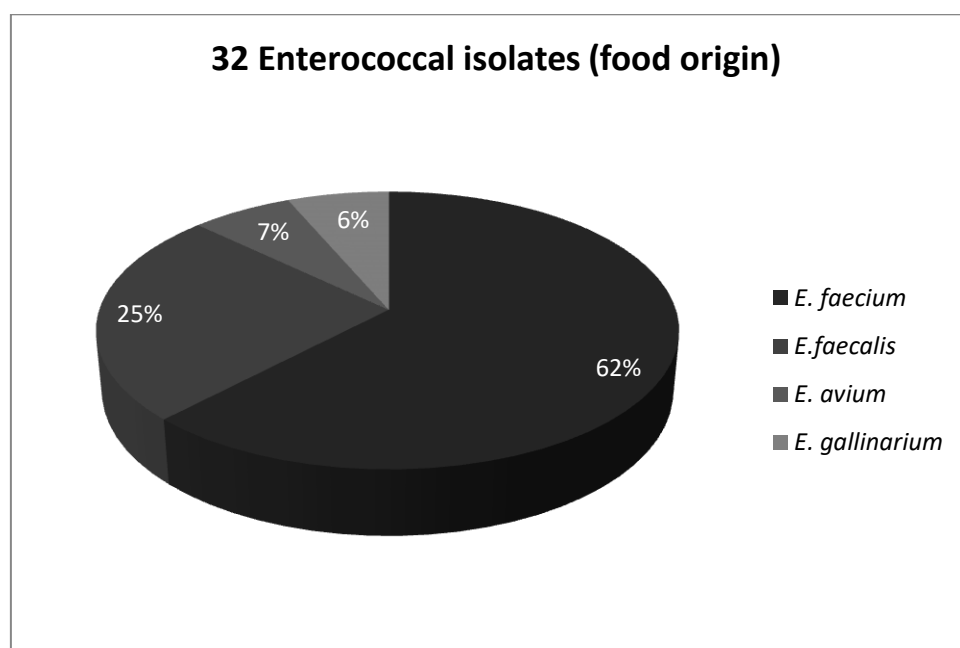


Figure 2-The isolated Enterococcal species with the isolation percentage of each species.

The identification of *Enterococcus spp.* by VITEK revealed that *E. faecium* was the most predominant species recovered in 62.5% (20 samples), followed by *Enterococcus faecalis* 25% (8 samples), and *E. avium* recovered 6.25% as well as *E.gallinarium* (2 samples for each). The identification of *S. aureus* was performed by direct inoculating the clinical isolates on MSA at 37°C for 24 hr. The positive result was indicated by converting the media from red to yellow color (because of its component: the mannitol sugar and the phenol red as an indicator). The ABC streaking method on BHI agar was performed to obtain pure colonies, which then re-inoculated onto MSA to confirm the results. The antibacterial activity tests were conducted for all *Enterococcus spp.* (32 isolates) using two methods, namely the well diffusion method and the spot on the lawn method, to examine the presence of 6 different *S. aureus* isolates.

The results of well diffusion method

No inhibition zones were observed when this method was utilized in the current study. Several reasons can explain these results; the successful application of the technique for antibacterial substances determination depends on the agar-medium employed in the experiment, not only on the sensitivity of microorganisms. The agar well diffusion method is considered as a preliminary screening for the antibacterial activity by using a cell free supernatant. However, this supernatant may include other medium components and/or intracellular compounds. These interfering molecules are accidentally released during cell free supernatant preparation and may affect the results [11]. The molecular weight of the antibacterial substance may also affect the results of inhibition in the well diffusion method by affecting the infusion rate [12].

The results of spot on the lawn method:

Thirty two isolates of *E. spp.* were tested for their ability to produce antibacterial substances against *S. aureus* isolates. Each *Enterococcus spp* isolate was tested against six *S. aureus* isolates. *Enterococcus spp.* were cultured on Muller Hinton agar. After 18 hr. of incubation at 37°C, the producer cells were scraped from the surface of the medium. After that, the bacteria were mechanically removed from the solid media and the remaining viable cells were killed by exposure to chloroform vapor. The plates were then prepared for streaking with the clinical isolates.

All the results of the detection of the antibacterial substances-producing isolates are illustrated in Table- 3 and Figure-3). The results showed that most of *Enterococcus spp.* produced antibacterial substances against *S. aureus*, with the exception of E12, E18, E19, E22, E23, E30, E31, and E32, which had no effect on all the tested isolates.

Table 3-Antibacterial activity of *Enterococcus spp.* against *S. aureus* isolated from patients

<i>Enterococcus</i> isolates	<i>Staphylococcus aureus</i> isolates					
	S1	S2	S3	S4	S5	S6
E1	-	+	+	+	+	+
E2	-	+	+	+	+	+
E3	+	+	+	+	+	+
E4	+	+	+	+	+	+
E5	-	+	+	+	+	+
E6	+	+	+	+	+	+
E7	-	-	+	+	+	+
E8	-	+	+	+	+	+
E9	+	+	+	+	+	+
E10	-	-	+	+	-	-
E11	+	+	+	+	+	-
E12	-	-	-	-	-	-
E13	+	+	-	+	+	-
E14	-	-	+	+	+	+

E15	-	-	+	+	-	-
E16	+	+	+	+	+	+
E17	-	-	+	+	+	-
E18	-	-	-	-	-	-
E19	-	-	-	-	-	-
E20	-	+	+	+	-	-
E21	-	-	-	-	+	-
E22	-	-	-	-	-	-
E23	-	-	-	-	-	-
E24	-	+	+	+	+	+
E25	+	+	+	+	+	+
E26	+	+	+	+	+	+
E27	-	-	-	-	-	-
E28	-	-	-	-	-	-
E29	-	-	+	+	-	+
E30	-	-	-	-	-	-
E31	-	-	-	-	-	-
E32	-	-	-	-	-	-

*positive (+) means inhibition *negative (-) means no inhibition

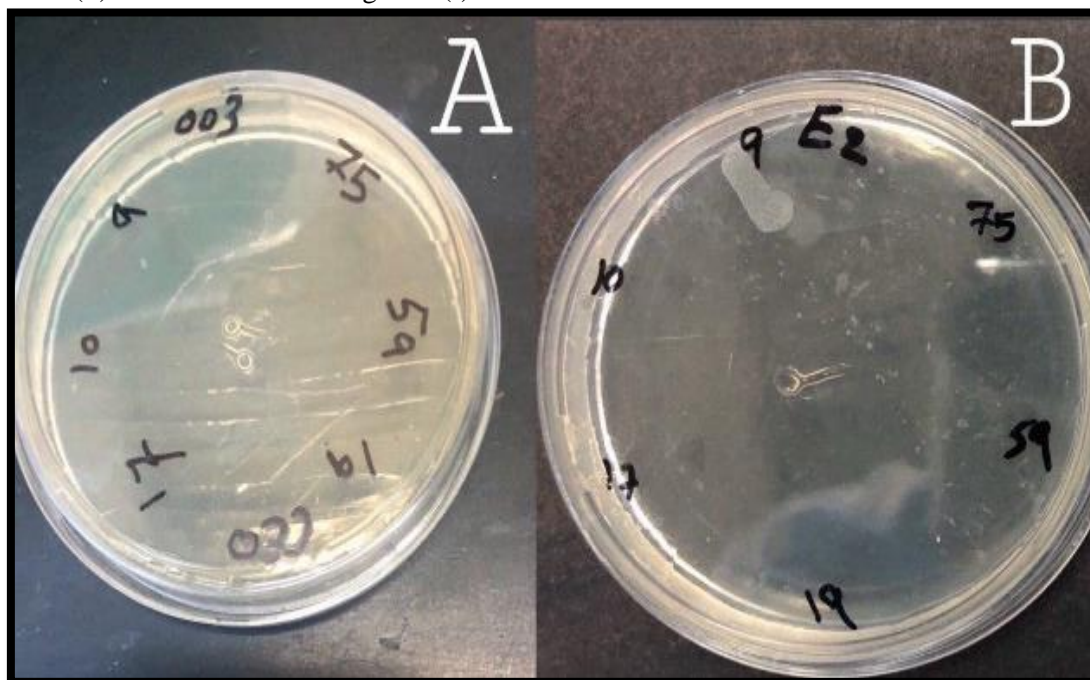


Figure 3- Antibacterial activity of *Enterococcus* spp. against *Staphylococcus aureus*
 *S1=9, S2=10, S3=17, S4=19, S5=59, S6=75 represent *Staphylococcus aureus* isolates used in this experiment

From the figure above, it is noticed in part (A) of the figure which represents sample E3, that *Enterococcus* produced antibacterial substances that inhibit the growth of *Staphylococcus aureus*. While part (B) which represents sample E2, shows that *Enterococcus* inhibited the growth of most *Staphylococcus aureus* isolates, except isolate number (9), which refers to S1 *Staphylococcus* isolate. In addition, both E2 and E3 isolated represented *Enterococcus faecium*, as shown in Table-2. The

antibacterial activity of *Enterococcus spp.*, especially *E. faecium*, had an obvious inhibitory effect on *S. aureus*. This is in agreement with previous results [13] which stated that *Enterococcus* contain antibacterial substances called enterocins that inhibit the growth of closely related foodborne organisms. Another study described the antibacterial effect of *E. faecium* against *S. aureus* [14]. The antibacterial activity of food-originated *Enterococcus spp.* isolates against other bacterial isolates from different sources may be due to several factors, one of which is the release of enterocins which is considered as the main antibacterial substance produced by *Enterococcus spp.* [15]. Dairy products represent excellent growth media for many pathogenic microorganisms, such as *S. aureus*, *Salmonella spp.*, *Listeria monocytogenes* and *Escherichia coli O157:H7*. These microorganisms are the most common pathogens associated with milk or dairy products and considered as the main microbiological hazards linked to raw milk and raw cheese [16]. The antibacterial activity of food-borne *Enterococcus spp.* against other pathogenic isolates could provide them with a possible role as sources of bacteriocin preparations to aid in preservation of many types of foods [17].

Conclusions

Lactic acid bacteria have continued to gain a remarkable amount of interest among an increasing number of research groups due to their major application potential, both in food and pharmaceutical industries. In the food industry, bacteriocins have long been proposed as a solution to the problems of food spoilage and food-borne infections. This study indicates the presence of *Enterococcus spp.* in the food samples and the ability of these bacteria to produce antibacterial substances which are active against closely related clinical isolates. Enterocins are one of those antibacterial substances that are produced by *Enterococcus spp.* and may play a possible role in the food industry as natural preservatives. The application of the produced antimicrobial compounds as a natural barrier against pathogens and food spoilage caused by bacterial agents has been proven to be efficient and the use of Lactic acid bacteria and their metabolic products is generally considered as safe.

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