

Study Antibacterial Activity of *Lactobacillus brevis* Against *Escherichia coli* and *Enterobacter aerogenus* Causes Diarrhea Among Children in Holy City of Karbala.

دراسة الفعل المضاد لجرثومة *Lactobacillus brevis* ضد كل من بكتريا *Escherichia coli* و *Enterobacter aerogenus* المسببة لحالات الاسهال بين الأطفال في مدينة كربلاء المقدسة

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بحث مسـتـنـل

Abstract

The study included isolation and identification of pathogenic bacterial which responsible for diarrheal infection to 39 stool samples were collected from children under five years of age suffering from diarrhea attending to education children hospital for of education in the Holy Karbala during the period from May to August 2013, as it was obtained on 39 bacterial isolates from these samples, which have been diagnosed by using differential mediums, biochemical tests and by using Api 20 kits, and appeared that isolates belonging to *Escherichia coli* (43.58%) and *Enterobacter aerogenes* (30.76%) are the most common isolates that have been selected to complete the rest of the steps of the study.

A total of ten *Lactobacillus* spp. isolates have been obtained from different sources such as (Yoghurt ,Whey and Raw milk) ,and only one of them belong to *Lactobacillus brevis* showed ability to produce the cell free supernatant (biosurfactant) and showed antimicrobial activity against all pathogenic bacterial strains tested .The MIC and Sub-MIC of the cell free supernatant (biosurfactant) extracted from *Lactobacillus brevis* isolate was determined against studied pathogenic bacteria and the results showed that the MIC of the biosurfactant was 70 mg/ml and Sub-MIC was 60 mg/ml respectively, these results indicates that biosurfactant must be used in high concentration to inhibit the growth of pathogenic bacteria.

Key words: Probiotics ; Biosurfactant ; antimicrobial activity ; *Lactobacillus* spp.

الخلاصة

تضمنت الدراسة الحالية عزل وتشخيص البكتريا المرضية المسؤولة عن اصابات الاسهال لـ (39) عينة براز جمعت من أطفال دون سن الخامسة من العمر والراقدين في مستشفى الأطفال التعليمي في كربلاء المقدسة خلال الفترة من شهر مايو ولغاية شهر اغسطس لسنة 2013 . اذ تم الحصول على 39 عزلة بكتيرية مرضية من هذه العينات والتي تم تشخيصها باستخدام الأوساط التفريقية والاختبارات البايوكيميائية وعدة التشخيص Api 20 ، وظهرت ان العزلات العائدة لنوعي الـ *Escherichia coli* (43.58%) و *Enterobacter aerogenes* (30.76%) هي العزلات الأكثر شيوعاً لذلك تم اختيارها لأكمال بقية خطوات الدراسة.

تم الحصول على (10) عزلات جرثومية تابعة لبكتريا *Lactobacillus* من مصادر مختلفة مثل (اللبن الخاثر (اليوگرت)، الحليب الخام والشرش) وقد اظهرت واحدة منها فقط والتي تعود لـ *Lactobacillus brevis* القابلية العالية على انتاج المشتتات الحيوية .وقد اظهر الراشح الحيوي المستخلص من عزلة *Lactobacillus brevis* فعالية تثبيطية واضحة ضد نمو العزلات المرضيه قيد الدراسة . كما حدد التركيز المثبط الأدنى (MIC) والتركيز الأدنى منه (Sub-MIC) للمشتت الحيوي ضد البكتريا المدروسة والتي بلغت 70ملغرام/مللتر , 60 ملغرام/مللتر على التوالي , الشيء الذي يؤكد ضرورة استخدام المشتت الحيوي لهذه البكتريا بتراكيز عالية لتثبيط نمو الجراثيم الممرضة .

Introduction

Gastrointestinal diseases can be considered as common illnesses and the second most common cause for visits to physicians, and a major factor in morbidity and mortality worldwide (*especially in children under 5 age years*). Diarrhea is the most common form of gastrointestinal infection, and definition as partial disability of intestinal movements that leads to pass large amount of fluid through colon in contrast to absorb fluid by other organs and because these large amount of fluid absorb by colon more need for actual human being, so develop diarrhea case in human. It was found that diarrhea is produced by several factors, such as pathogenic mechanism (which attack the proximal small intestine), or toxins causing overabundance of secretion intestinal tract without any damage the fabric mucous intestinal. The portion of the bowel in which more than 90% of physiologic net fluid absorption occurs [1][2]. Many causes are involved in the pathological mechanism of causing diarrhea, the most common causes are several viruses (which are involving rotavirus, norovirus, cytomegalovirus, herpes simplex virus, and viral hepatitis), most important members of pathogenic *Enterobacteriaceae* (include *Escherichia coli*, *Salmonella spp.*, *Shigella spp.*, *Vibrio cholera*), Parasites and reaction to medicines [3]. *Escherichia coli* bacteria, gram-negative, rod-shaped bacteria which found in healthy intestine of humans, are a that causing such extraintestinal infections (such as diarrhea) carry on array of virulence factors, including different toxins, colonization factors (fimbriae, capsules), haemolysin and aerobactin [4][5]. The nosocomial pathogen *Enterobacter* (rod-shaped gram-negative bacteria) are part of the commensal enteric flora and are sometime being stabilmente for minor infection but recently has become one of the most common pathogens that responsible for hospital-acquired infection. They express type 1 and type 3 fimbriae (involved adhesion process), aerobactin-mediated iron uptake systems, adhesive characteristics and in addition, their outer membrane protein, OmpX (may be act as pathogenic factor), for that they are known to act as opportunistic pathogens and produces gastrointestinal infections and bacteremia in both hospitalized and nonhospitalized patients [6][7]. Recent years have seen, from the last century and the beginning of the present century, considerable development in industrial production and vital treatment methods, and a broad directed toward the use of microorganisms and their metabolic products in treating some cases of disease. Because of the prevalence of the negative effects of antibiotic treatment on both human and environmental So the alternative medicine dependent on antagonism between microorganism becomes the best solution to get rid of bacterial infection and maintain public health of humans [8]. Most of *Lactobacillus* strains considered as probiotic bacteria, and play important role in improvement human health as a result of its ability to produce many of the antimicrobial agents such as biosurfactants that play important therapeutic roles like many antibacterial activity, control diarrheal diseases, synthesis and enhancing the bioavailability of nutrients. And the positive effect of the interaction between the antimicrobial activity of these bacteria and the ability of pathogen strains to adhere on the host epithelial cells tracts such as stimulating protective immune response, prevention faecal enzymatic activity, producing short chain fatty acids allowing an advisable acidification of the gut [9] [10]. The ability of probiotic *Lactobacillus* bacteria was found to produce biosurfactants (which is a complex biological mixture) in the mid-exponential and stationary growth phases [11][12]. Moreover, the release of these biological surface-active material by these bacteria *in vivo* play benefit roles in decrease virulence of different pathogenic bacteria and act as a strong antibacterial, antifungal and antiviral activity which considered as weather proof material for settlement of pathogenic bacteria on the gastrointestinal tract and urinary tract as well as medical equipment [13] [14]. Therefore, the current study aimed to find therapeutic alternatives from natural sources or symbiotic bacteria in the human body as probiotics instead of the commonly used antibiotics which are characterized by its dangerous side effects on human health, and to achieve this aim.

Materials and methods

Stool Specimen Collection

Collection of stool samples was carried out from thirty-nine children with diarrhea infection aged from (1) month for (5) years, attending children general hospital in the Holy city of Karbala during the period from May to August 2013. Stool specimens were collected according to [15]. Isolated bacteria were identified according to morphological and cultural properties and by using biochemical tests depending on [16] [17], then by using Api 20E. To detect the sensitivity of isolates pathogenic

bacteria, six antibiotic disks (Amoxicilline-Clavulanicacid, Ampicillin, Ceftazidime, Trimethoprim Sulfamethoxazole, Gentamicin) have been used in this test by using the agar overlay diffusion method according to [18], and the diameter of the inhibition zone was measured according to the Clinical and laboratory standards Institute [19].

Isolation of *Lactobacillus* bacteria.

A total of 30 samples of fermented milk products (Yoghurt), Raw milk and Whey was collected, and isolation of *Lactobacillus* was done by using serial dilutions, then culture onto selective media (MRS agar), then incubated at 37°C for 24 hr., in the presence of CO₂ by using Candle Jar. Isolates were identified depending on morphological and biochemical tests as compared with identification scheme described by [20].

Antibacterial activity of *Lactobacillus* species against *E. coli* and *Enterobacter*

Ten isolates of *Lactobacillus* species were used to study their inhibitory effect on most common causes of diarrheal infection *E.coli* and *Enterobacter* according to [21] methods.

Cell-free supernatants (Biosurfactant) preparation

➤

Detection the ability of *Lactobacillus* strains to produce biosurfactant in supernatant

The blood agar method is used to screen for biosurfactant production according to [22].

➤

Extraction of cell free supernatant (biosurfactant)

The biosurfactant was extracted according to [23].

➤

Surface activity determination

In order to test whether the biosurfactant material found in the cell free supernatants were able to decrease the surface tension between water and hydrophobic surface , The ability to collapse a droplet of water was tested according to [22].

➤

Determine minimum inhibitory concentration (MIC)

Stock solution for crude biosurfactant was prepared by concentration reached 100 mg/ml. Other concentration were prepared(100 to 10 mg /ml) which was used against bacteria to detect MIC of the extract according to the National Committee for Clinical Laboratory Standards [24].

➤

Inhibition of pathogenic bacteria by cell free supernatant

To study the inhibitory effect of cell free(biosurfactant) on *E. coli* and *Enterobacter* isolates according to [25].

Results and Discussion

Identification of pathogenic bacteria

In this study, several bacterial species were isolated from children are infected with diarrhea ,only 43.58% were *E. coli* and 30.76% were shown to contain *E. aerogenes* , but other isolates were distributed between *Serratia marcescens* 12.82% , *Proteus mirabilis* 10.25% and *Pseudomonas aeruginosa* 2.56% as shown in figure (1).This results explain clearly *E. coli* and *E. aerogenes* were the most common isolates bacterial pathogens there for these isolates had been chosen to continue and complete other steps of this study.

The results shown in table(1)indicate that sensitivity to antibiotics was widely distributed among the chosen isolates ,and was found that the highest sensitivity of the chosen isolates was to Imipenem (100%). followed by Gentamicin (50%), Trimethoprim-Sulfammethoxazole (30%) . Results also shown that all isolates (100%) were resistant to Ampicillin and Amoxicilline-Clavulanic acid and Ceftazidime . In the present study , all the tested isolates are resistant to a minimum of three classes of antibiotic that used in this study, Therefore all tested isolates are considered multidrug resistant [26].The resistance to antibiotics attributed as well as to acquired genes responsible for the resistance mounted on each of the bacterial chromosome and the plasmid where the resistance gene on the plasmid have the ability to move from the bacterial cell to another through the processes of natural genetic exchange as conjugation, transduction and transformation[27].

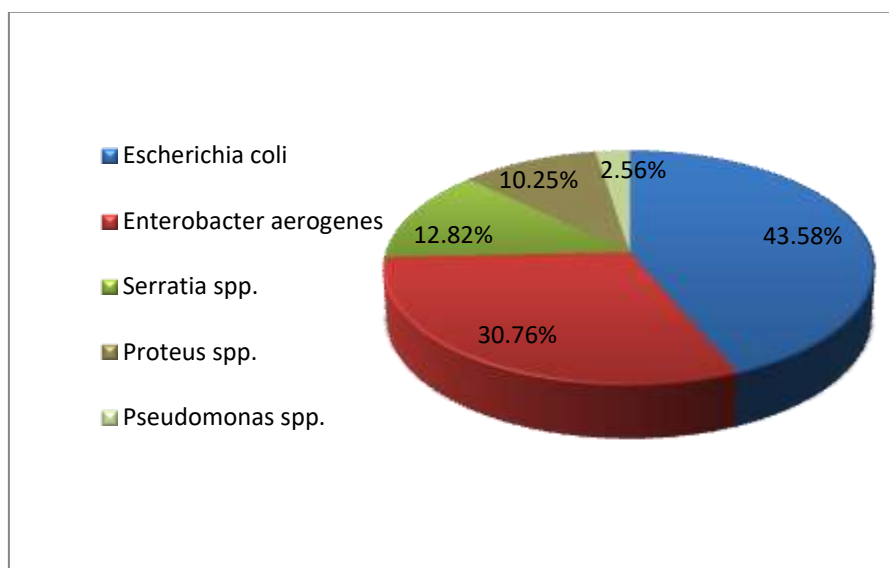


Figure (1): Percentage of isolates bacteria.

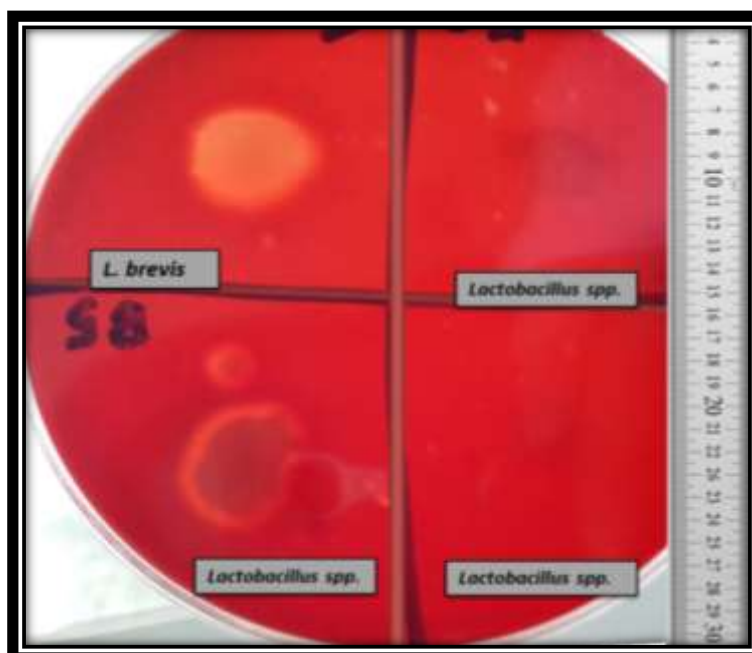
Table (1): Susceptibility of bacterial isolates to some antibiotics.

Antibiotic Disks	Isolates No.			
	<i>Eco-13</i>	<i>Eco-30</i>	<i>Eco-157</i>	<i>Ent-15</i>
Amoxicilline-Clavulanic acid	R	R	R	R
Ampicillin	R	R	R	R
Ceftazidime	R	R	R	R
Imipenem	S	S	S	S
Trimethoprim-Sulfammethoxazole	R	R	R	S
Gentamicin	R	R	S	S

R= resistant. S= sensitive.

Isolation and identification of *Lactobacillus* spp.

From different sources, 30 samples were collected (10 fermented milk products (Yoghurt),10 Whey,10 Raw milk).Five isolates were obtained from Yoghurt ,but only 3 isolates from local milk products and 2 isolates from Whey , ten isolated bacteria were diagnosed according to morphology and biochemical tests as *Lactobacillus* spp.[20] . Among the ten *Lactobacillus* strains isolates ,only two showed clearing zone in the blood agar as shown in figure (2) .The isolate that was given large clearing zone diameter (Lac--20) has been chosen to complete other steps of the present study and depending on biochemical and carbohydrates fermentation pattern these chosen isolate belong to *L. brevis* using diagram described by [28]. Several study found an association between hemolytic activity and biosurfactant concentration and they recommended the use of blood agar lysis as a primary method to screen for biosurfactant activity[22]. Screening of biosurfactant producing bacteria by blood agar hemolysis method based on the fact that biosurfactants are able to haemolysis the red blood cell present in blood. This method is adopted by in many studies , including [29][30].



Figure(2):Blood agar screening method results for *Lactobacillus* spp.

Antagonist effect of *L. brevis* against *E. coli* and *E. aerogenes* isolates

The *L. brevis* isolate was shown inhibition activity against three isolates of *E. coli* (*Eco-13*, *Eco-30* and *Eco-157*) and one isolate of *E. aerogenes* (*Ent-15*) as shown in table (2), these isolates were chosen among all bacterial isolates depending on their ability to explain most tested virulence factor. These findings were conforming to those of [31] who found that *Lactobacillus* spp. showed antagonistic activity against *E. coli* and *E. aerogenes* bacteria, and the inhibition produced varied between 15 to 24 mm. Several studies showed that the antibacterial mechanisms of *Lactobacillus* spp. may be due to a number of factors such as their symbiosis with potential pathogens and production of biosurfactants that inhibit pathogen adherence, decreased pH levels, competition for substrates, production of hydrogen peroxide (H_2O_2), lactic acid and bactericidal or bacteriostatic substances, involving diacetyl and small heat stable inhibitory peptides (bacteriocins) [32], also [33] found that antimicrobial activity of probiotic bacteria has been attributed to various antimicrobial agents released by these bacteria such as biosurfactants.

Table (2): Antagonistic activity of *Lactobacillus* strains against pathogens bacteria.

pathogens bacteria	Inhibition of indicator microorganisms
	Cells of <i>Lactobacillus</i> in fresh MRS broth
<i>Eco-13</i>	+
<i>Eco-30</i>	++
<i>Eco-157</i>	+
<i>Ent-15</i>	+

(+ : inhibition zone between 2 and 10 mm ; ++ : inhibition zone between 10 and 20 mm .Results are averages of three experiments).

Production and detection of cell free supernatant (Biosurfactant)

The result of this study illustrated that the isolated crude biosurfactant showed antimicrobial activity against all the bacterial strains tested (*Eco-13*, *Eco-30* and *Eco-157* and *Ent-15*). This finding is similar to other study, [34] who reported that *Lactobacilli* spp. released glycosyldiglycerides as biosurfactant which is a mixture of various components show a direct antimicrobial action and inhibited pathogen adhesion. Typical biosurfactant released by *Lactobacilli* spp. are named surlactine is a mixture of various components have a glycoproteinaceous character with antibacterial and antibiofilm activity [35][36][37][38]. Biosurfactant is a mixture of various components show a direct antimicrobial action and inhibition [39], and [22] observed that biosurfactant is highly effective in reducing the pathogenesis of bacteria without inhibition of cell growth, a finding that confirmed a direct impact on the bacterial surface overlap, through the changes in surface tension and charge of cell wall of the bacteria, while [40] stressing the importance of biosurfactant in inflating overlap between cell to another cell and between cell to different cell surfaces.

Depended on a modified oil collapse method, the surface effectiveness of biosurfactant secreted by the *Lactobacillus* chosen strains was determined. The result of this study was illustrated clearly that cell free supernatant (Biosurfactant) was collapsed and showed like spread drops on the oily surface and drops diameter was at least 0.5 mm larger than the diameter of water did not collapse and appeared as a bead, which was used as negative control. The diameter of the sample supernatant was increased with biosurfactant concentration increase, due to the fact that the high concentration of biosurfactant will aid the drops of supernatant to spread over the oily surface, in comparison with that little concentration drops that remain beaded because the hydrophobicity for the oil that keeps the drops grouped [22].

Determination of the minimum inhibition concentration(MIC)

The derived biosurfactant was dissolved in PBS at dilution concentration ranging from 100 -10 mg/ml of detect MIC of the extracts and show the antibacterial activity of the isolate crude biosurfactant against bacterial strains tested (*Eco-13*, *Eco-30* and *Eco-157* and *Ent-15*).The result of this study showed that the MIC of the biosurfactant was 70 mg/ml and Sub-MIC was 60 mg/ml ,these results indicates that biosurfactant must be used in high concentration to inhibit the growth of pathogenic bacteria .The MIC and Sub-MIC were detected in this study gave inhibition zone when used against studied strains as shown in figure (3)and table (3), this study approached with [12] as they noticed the antibacterial activity of biosurfactant against pathogenic bacteria needed high concentration that must reach to 25 mg/ml ,while [37] found the absences of any antibacterial activity of biosurfactant against pathogen even when its concentration reached 100 mg/ml .

Table (3):Antimicrobial activity of biosurfactant against pathogens bacteria.

pathogens bacteria	Inhibition of indicator microorganisms	
	Sub-MIC of biosurfactant	MIC of biosurfactant
<i>Eco-13</i>	+++	+++
<i>Eco-30</i>	+++	+++
<i>Eco-157</i>	++	+++
<i>Ent-15</i>	+++	+++

(+ : inhibition zone between 2 and 10 mm ; ++ : inhibition zone between 10 and 20 mm . +++: inhibition zone larger than 20 mm .Results are averages of three experiments).

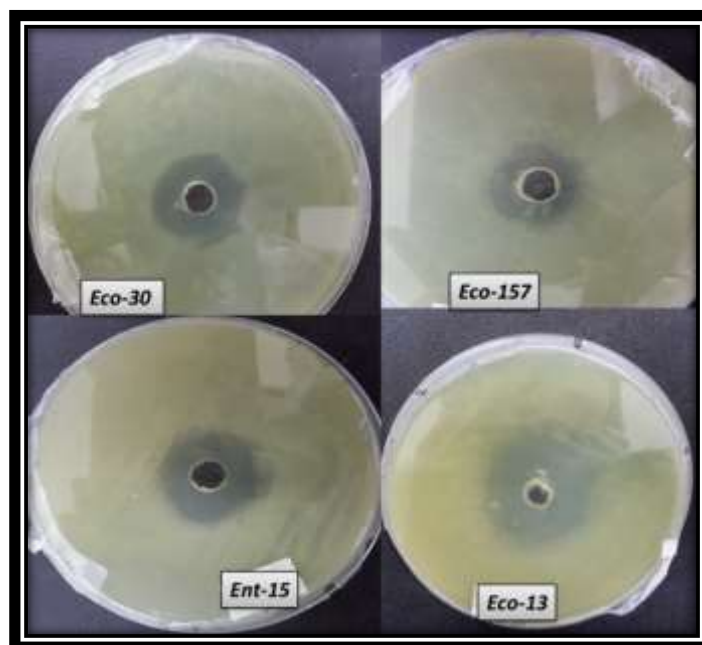


Figure (3): Antimicrobial effect of *L. brevis* biosurfactant against pathogens.

Conclusions

- *E. coli* and *E. aerogenes* bacteria the most common isolates causes diarrheal infection of children in Holy Karbala city that have many virulence factors which are responsible for pathogenicity.
- The high inhibition ability of the cell free supernatant that produced from *Lactobacillus* spp. bacteria against *E. coli* and *E. aerogenes* bacteria open the way to study its as probiotics for treating these pathogenic bacteria which are resistant for most antibiotic.
- The ability effect of *Lactobacillus* spp. bacteria and the biosurfactant that produced from it on pathogenic bacteria and largely effect on the ability of the studied bacteria to form biofilm and adhesion on the epithelial cell.

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