in vitro evaluation of inhibitory activity of enteric *Bifidobacterium* isolates against Shiga toxin producing *E.coli* (STEC)O157:H7

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Abstract :

Six *Bifidobacterium* isolates, isolated from breast – feed infant faces on reduced de Man Rogosa and Sharp medium (MRS - C). Isolates identified to species level on the basis of : microscopical properties, biochemical tests, fructose-6-phosphate phosphoketolase enzyme(F6PPK) activity and carbohydrates fermentation profile. Results showed that *B. adolescentis* was the predominant species (B4,B5and B6),the other species were *B. breve*(B3),*B. longum* (B1), *B. dentium*(B2).

Strains were screened for their inhibitory effects against pathogenic bacteria shiga toxin producing *E.coli*(STEC)O157:H7 using agar – well diffusion method.B3 and B6 showed clear inhibitory actions toward STEC,22 mm and 15 mm diameter of inhibition zone srespectively. While the rest of isolates did not pronouncedany inhibitory activities. This indicate that the efficiency of probiotic bacterial strain specially *Bifidobacterium* spp. as antibacterial factor to treat or reduce bloody diarrhea and hemorrhagic urinary syndrome (HUS) as symptom of (STEC) infection .

Keywords: Inhibitory activity *in vitro* , *Bifidobacterium* , *E.coli* (STEC)O157:H7, hemorrhagic urinary syndrome

تقييم الفعالية التثبيطية لعزلات البفيدس المعوية تجاه بكتريا القولون البرازية الفارزه لسموم الشيكا *E.coli*(STEC)O157:H7 خارج الجسم الحي

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ألخلاصة :

عزلت ستة عزلات لبكتريا البفيدس المعويه من نماذج لبراز أطفال ذات الرضاعة الطبيعية بأستخدام وسط deMan Rogosa and Sharp mediumالمختزل (MRS – C) . أظهرت الفحوصات المجهريه والفحوصات البيوكيميائية وفحص نشاط الأنزيمF6PK ونمط تخمير السكريات سيادة النوع *B. Adolescentis* (B4,B5and B6)،اما بقية الانواع فكانت مايلي : *B. breve* (B3), *B. longum* (B1), *B. dentium* (B2) .أختبرت قدرة عزلات البفيدس على تثبيط بكتريا *E.coli* الفارزه لسموم الشيكا O157:H7(STEC)بطريقة الانتشار بحفر الأغار، كانـت العزلتين B3 و B 6 أكثر العزلات تثبيطاً لبكتريا STEC وبأقطار تثبيط 22 ملم و 15 ملم على التوالي و لم تظهر بقية عزلات البفيدس اي فعالية تثبيطية . تشير النتائج الى امكانية استخدام سلالات البكتريا الداعمة للحياة خاصة *Bifidobacterium* spp. كعامل مضاد للبكتريا لعلاج او تقليل من حدة حالات الاسهال الدموي و متلازمة البول الدموي في اعراض المصابين بال E. coliالمنتجة لسموم الشيكا (STEC) .

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Introduction:

Bifidobacterium is –gram positive, non – spore forming , non – motile anaerobic pleomorphic rods[1**]**. These bacteria are the prominent group of microorganisms in the human gut, comprising up to 3% of total fecal microflora of adult [2].They are more numerous in the infant gut, where they form up to 95% of total microflora in breast – feed babies, that being supported by bifidogenic factors presented in human milk, and up to 75% in formula – feed babies [3] . The genus *Bifidobacterium* are extensively used in the microflora – normalizing (probiotic) preparation[4]. The mechanism of probiotic activities attributed to *Bifiodobacterium* includes several beneficial effects on host health, such as; stimulation of intestinal cells growth, enhance immunity in host and training the immune system to respond only to pathogens[5]**,**elimination of procarcinogens, synthesis of vitamins, , deconjugation of bile acids and assimilate cholesterol, improve lactose utilization in malabsorbers, and protect the gastrointestinal tract from microbial infections[6]. Several mechanisms have been proposed to explain the efficacy of *Bifidobacterium* in preventing enteric infections, these mechanisms includes ; modulation the immune response of host intestinal mucosal epithelia, blocking the adhesion of pathogens and toxins to the intestinal epithelial cells, ,reduction of gut pH by the production of organic acids(acetic and lactic acids), competition for nutrient and adhesion sites, and secretion of antimicrobial substances like bacteriocins and bacteriocin – like peptides[7].

Shiga toxin producing *E.coli*(STEC) or Enterohemorrhagic *E.coli* (EHEC) cause bloody diarrhea and hemolytic – uremic syndrome. STEC or EHEC *E.coli* cause foodborn disease ranging from uncomplicated mild diarrhea to life threatening complication such as haemorrhagic colitis and heamolytic-uraemic syndrome HUS[8]. Shiga toxin is the major virulence factor(bacteriophage – mediated) of O157:H7 strains and is more responsible for the more severe symptoms of infection. STEC can produce one or both of two antigenically distinct forms of shiga toxins, shiga toxin l (STx1) and shiga toxin ll (STx2**)** [9]. Epidemological studies , together with *in vitro* and *in vivo* experiments have revealed that STx2 is the most important virulence factor associated with severe human disease **,** in deed STx2 is 1000 times more cytotoxic than STx1 to human renal endothelial cells**.** Animal models suggest that the severity of the disease is correlated to the amount of STx produced in the gut during infection [10].Current therapy is limited to supportive treatment with dialysis alone[11].The use of antibiotic therapy for STEC infection is controversial. Certain antibiotic may stimulate complication, such as acute renal failure occurring in HUS. In prospective cohort study of 71 children's hospitalized with O157:H7 diarrhea, antibiotic treatment significantly increased the risk of developing HUS [12]. Attention attracted in the last decade on the alternative therapies as promising approach. One of recommended treatment strategies include using of selected probiotic bacterial strains, that have demonstrated considerable potential for promoting rapid recovery from STEC causing diarrhea. A meta – analysis of randomized controlled trails provided evidence of the efficacy of lactic acid producing bacteria(*Lactobacillus* and *Bifidobacterium*) for both prevention and treatment of acute diarrhea in infants and young children[12].

The aim of this study was to present some data on isolation and identification of enteric *Bifidobacterium*, and *In vitro* study of inhibitory activities of isolated strains against test candidate bacteria STEC.

Materials and methods :

Bacteria and cultural conditions

Fecal samples from 4-8 week old healthy breast – feed infants analyzed for the presence of *Bifidobacterium* bacteria in reduced de Man Rogosa and Sharpe agar medium (MRS – C) broth (MRS supplemented with0.05 % w/v L – cysteine – HCl).Was inoculated in MRS-C broth. Briefly : 1 gm of feces was inoculated in 10 ml MRS broth supplemented with 20µg / ml gentamycin sulfate, 3 µg/ml nalidixic acid and 600 µg/ml lithium chloride, for enrichment of resident *Bifidobacterium*, the tubes were incubated anaerobically (anaerobic jar and gas pack) at 37°C for 48 h. Cultures were streaked on MRS – C plates several times to obtain pure culture. The isolates were identified to genus level by: gram staining, colonies morphology, biochemical tests, and fructose 6 – phosphate phosphoketolase (F6PPK) enzyme activity in cellular extract . F6PPK enzyme detection procedure briefly : bacterial cells were grown in MRS – C broth at 37 °C for 24h. and harvested by cool centrifugation (500 rpm for 10 min. 4°C). The pellet washed twice with reduced phosphate buffer(0.05 M sodium phosphate , pH 6.5 supplemented with 0.5 % w/v L – cysteine – HCl) and disrupted by

ultrasonication3 min. at 0°C(0.5 min on and 0.5min off). Crud cells extract centrifuged (500rpm for 10 min.4 °C),250 µl of supernatant mixed with the reagents (6mg/ml NaF, 10mg/ml sodium idoacetate and 80 mg/ml fructose – 6 – phosphate F6P), incubated 30min. 37°C, reaction stopped by adding 1.5 ml of hydroxylamine – HCl, 10 min later 1 ml of 15% w/v trichloroacetic acid and 1ml of 5% w/v $FeCl_3$. $6H_2O$ were added. The assay is based on production of erythrose-4 phosphate and acetyl phosphate from F6P. Acetyl phosphate reacts with hydroxylamine which in turn reacts with Fe^{3+} to form purple – colored complex [13,14].

The interesting isolates were identified to species level by sugars fermentation profile of human strains and compared with sugars fermentation scheme described in Bergey's manual of systematic bacteriology [15].

The *Bifidobacterium* isolates were maintained in MRS broth with 15 % glycerol at -18 °C as stock culture .Shiga toxin producing *E. coli* STEC used as test microorganism, obtained from central lab.. Baghdad /Iraq, confirmed as O157:H7 serotype by culturing on Sorbitol MacConkey agar (SMAC) and propagated on brain heart infusion medium (BHI**)**throughout the study**.**

Inhibitory activities screening :

Bifidobacterium isolates were analyzed for their antagonistic activities against indicator bacteria STEC O157:H7 by agar – well diffusion assay [16]. Overnight bifidobacterial culture suspensionsin MRS - C broth prepared , melted BHI agar seeded with overnight culture of STEC at a final concentration 10^6 cell/ ml, poured into sterile petri dishes and allowed to solidify at room temperature, wells 5mm were hollowed out in agar using a sterile cork borer, a volume of 50µL of bifidobacterial tested broth were dropped separately in each well, and incubated at 4°C for 6h to facilitate diffusion into agar, plates finally were incubated at 37°C for 48h, formed inhibition zones around the wells were measured and recorded in millimeter after subtraction 5mm (wells diameter)[16].

Results and Discussion :

Traditionally *Bifidobacterium* species have been identified on the basis of cell and colony morphology, biochemical analysis, detection of F6PPK enzyme and the ability to utilize various carbohydrates substrates. The application of these approaches have proved useful tools in the classification and identification of *Bifidobactrium* up to 99% [17]. All the bacterial isolates were pleomorphic nonsporulated, Gram positive. The bacterial colonies on MRS – C agar are circular, regular edges, convex , and glistening color. The tested isolates had F6PPK activity. *Bifidobacterium* genus can be distinguish from other bacteria occurring in human intestine by a peculiar pathway: "bifidus shunt", whose key enzyme is F6PPK. The demonstration of F6PPK activity serves as a taxonomic tool in the identification of the genus, due to *Bifidibacterium* are the only intestinal bacteria known to utilize this fermentation rout [18].These characters beside other shown in **Table. 1** confirmed that the majority of bacterial isolates are identified as belonging to the *Bifidobacterium* genus.

Table 1- Biochemical characteristics of *Bifidobacterium* isolates

Test	Strains					
	B1	**B2**	**B3**	**B4**	**B5**	**B6**
Catalase	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-
F6PPK	+	+	+	+	+	+
Nitrate reduction	-	-	-	-	-	-
Gelatinase	-	-	-	-	-	-
Gas from glucose	-	-	-	-	-	-

Bifidobacterium isolates differentiated to the species level on the base of sugars fermentation profile of human strains as a classic means of differentiation table-2.

Table 2- Sugars fermentation profile of *Bifidobacterium*

Bacterial isolates	Glucose	Galactose	fructose	lactose	Arabinose	Mannose	trehalose	Xylose	Raffinose	Sorbitol	Suggested species
B1	+	+	+	+	+	+	+	-	+	-	***B. longum***
B2	+	+	+	+	+	+	+	+	+	-	***B. dentium***
B3	+	+	+	+	+	+	+	-	+	±	***B. breve***
B4	+	+	+	+	+	+	+	+	+	+	***B. adolescntis***
B5	+	+	+	+	+	+	+	+	+	+	***B. adolescntis***
B6	+	+	+	+	+	+	+	+	+	+	***B. adolescntis***

(+Positive reaction, -Negative reaction, ± variable reaction)

As a result showed *Bifidobacterium adolescntis* was the predominant strain as its present 50% of the total stool samples isolated strain while *B. longum, B. dentium* and *B. breve* each present 16.67%. Biochemical tests for the identification of members of the genus *Bifidobactertium* are now superseded by the use of molecular techniques such as genus – specific PCR primers [19].

Sorbitol MacConkey agar SMAC used for confirming the diagnosis of *E.coli* obtained strain as serotype O157:H7, The cultivated strain after 24h. incubation on SMAC agar produced colorless colonies, in contrast other tested *E.coli* ferment sorbitol and formed pink colonies, figure-1.STEC is indistinguishable from other lactose – fermenting *E.coli* strains on standard MacConkey agar containing lactose. Unlike most *E.coli* strains, serotype O157:H7 do not ferment sorbitol (SOR^-), therefore, the efficacy of MacConkey agar containing sorbitol instead of lactose as selective and differential medium for detection of SOR^- *E.coli* serotypes O157:H7 is recommended[20]. Differentiation of enteric microorganisms is achieved by the combination of sorbitol and neutral red indicator. Colorless or pink to red colonies are produced upon the ability of the isolate to ferment sorbitol. The medium also included with 0.05 mg/l cefixime inhibits *proteus* spp. and 2 mg/l tellurite inhibits non – O157:H7 *E.coli* , thus improving the selectivity for O157:H7 serotypes[21].

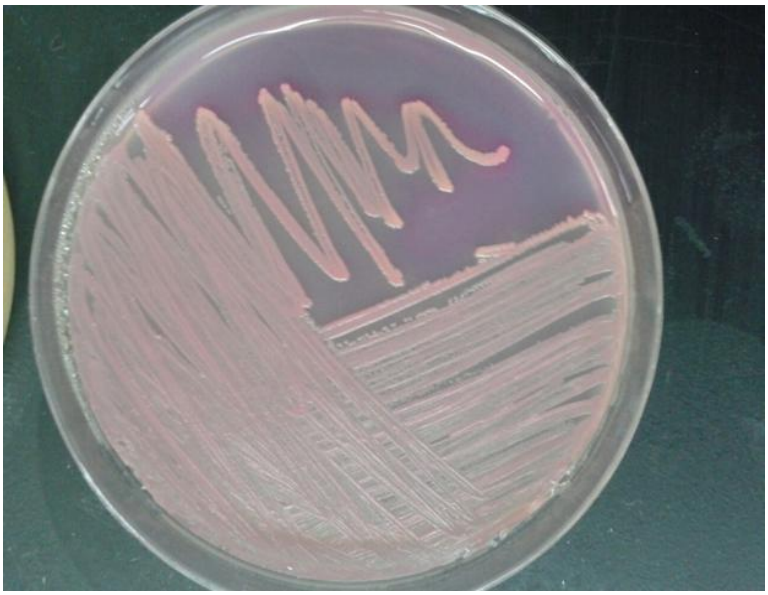

Figure 1- *E.coli* (STEC) H157:O7 on Sorbitol MacConkey agar

Bifidobacterial isolates were screened for inhibitory activity against indicator bacterium STEC by agar – well diffusion assay, only two isolates B3 and B6 (33.33%) were showed antagonistic activity detected by zones of inhibition 22 mm and 15 mm respectively (Figure. 2) , they were selected as potential antibacterial compounds producers. This effect also demonstrated by Wang *et al.*(2004) that yogurt containing *B. lactis* Bb12 had a suppressive effect (bacteriostatic effect) on *H. pylori* as it showed significantly decreased gastritis activity and *H. pylori* Density[22], and *B. themophilum*, *B. infantis* and *B. longum* active against *E.coli* and many other pathogenic bacteria *in vitro* as mentioned by Zinedine *et al.* (2007) using agar diffusion test [23].

The mechanisms underlying the antimicrobial activity (antagonism) of *Bifidobactrium* strains appear to be multifactorial and includes, lowering the pH by the production of acetic and lactic acids, as Scardovi (1986) reported that *Bifidobacterium* species did not produce butyric and propionic acids, but produce lactic and acetic acids. Such acids are responsible for the decrease of pH in intestines and the inhibition of the growth of pathogenic bacteria[15] ,production of antibacterial compounds, including bactriocins and nonbactriocins peptides and production of peroxides H_2O_2[24]. Adherence inhibition is a critical factor in antagonistic activity as Gagnon *et al.* (2004) mentioned the ability of *Bifidobacteria* isolated from infant feces to inhibit enterohemorrhagic *Escherichia coli* serotype O157:H7 *in vitro* and reduce its adhesion to human enterocyte-like Caco-2 cells ,this effect was dependent on bifidobacterial cell concentration[25].

The inhibitory activity of *Bifidobacterium* strains towered enteropathogenic *E.coli* for some extent is combined with lower pH and higher acetate concentration production[4].

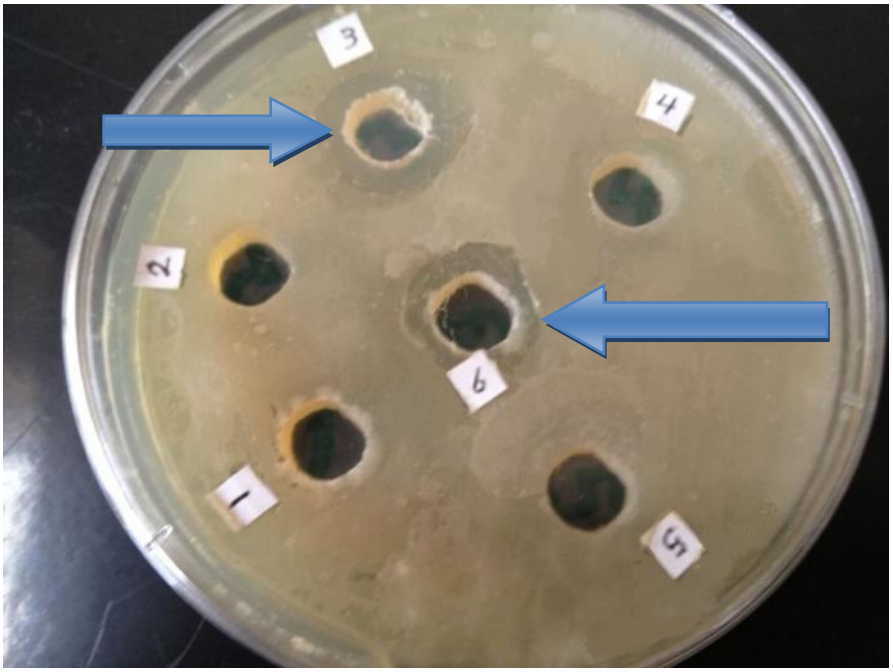

Figure 2- Inhibitory effect of *Bifidobacterium* isolates against STEC H157:O7

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