Assessment the Effect of *Lactobacillus Acidophilus* on *Escherichia Coli* Serotype O157:H7 with Detection of Some Virulence Factors

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Abstract

Aim: To evaluation the effect of *Lactobacillus acidophilus* on Enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 with detection of some virulence factors.

Methods: Two hundred and fifty specimens (stool) from children under five years for both sexes were collected from some hospitals. All isolates were diagnosed according to morphological characteristics, biochemical tests. Monoplex pattern of PCR was used also for detection different genes in (7) *Escherichia coli*)O157:H7 (isolates; include *16SrRNA, eae, lifA, Stx1,Stx2* that encoded for ribosomal RNA, intimin, lymphocyte inhibitory factor, shiga toxins. Three types of probiotics strains were obtained, *Lactobacillus fermentum*, *Lactobacillus plantarum* and *Lactobacillus acidophilus* (ATCC4356). Minimum inhibitory concentration (MIC) of cell free supernatants of *Lactobacillus acidophilus* was determined by employing different dilutions (1/2, 1/4, 1/8, 1/16, 1/32), to detect the concentration of probiotic that will inhibit *E.coli* (O157:H7) growth.

Results: Results showed, 210 (84%) samples were identified as *E.coli* from 250 samples. The result showed (7) isolates were identified as Enterohemorrhagic *Escherichia coli* (EHEC) serotype O157:H7 and showed all isolates of O157:H7 were positive for *16SrRNA* gene with (213bp) and *eae* with (741bp), *lifA* with (712bp), only *Stx1* gene appeared in all isolates with (446bp) and no bands with *Stx2.* Current result showed only cell free culture supernatant of *Lactobacillus acidophilus* has inhibitory activity against all *E.coli* (O157:H7) isolates with different dilutions (1/2, 1/4, 1/8, 1/16, 1/32), while *Lactobacillus fermentum*, *Lactobacillus plantarum* have no effect against *E.coli* (O157:H7). The result showed the bacteriocin has inhibitory effect against *E.coli* (O157:H7), while organic acids and hydrogen peroxide haven't any role in inhibition. The (MIC) value was (1/8) which inhibits the bacterial growth of isolates.

Key words: *E.coli (O157:H7), 16SrRNA, eae, lifA, STX1, MIC*

Introduction

Enterohemorrhagic *Escherichia coli* O157:H7 is a major foodborne pathogen causing severe disease in humans worldwide. The infections caused by EHEC range from asymptomatic to severe. Chronic renal pathology may persist at times among those that survive. Humans are known to acquire EHEC in multiple ways, for example, contaminated food and water and direct contact with infected animals and humans. Consumption of contaminated food like ground beef, dairy products, and fresh produce is responsible for the majority of the outbreaks [(1)]. The initial reported of EHEC serotype O157:H7 infection linked with hemolytic syndrome (HUS), also it was recognized in China as a reasons of bloody diarrhea, because of contamination of uncooked

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hamburger, emerging public health challenges of Shiga toxin (stx) producing *Escherichia coli* (STEC) include the occurrence of more frequent or severe disease and risk factors shifts associated with changes, often interconnected, in the pathogen, the population, and the environment [2]. Shiga toxin (STX) is the cardinal virulence factor of EHEC, so far, the pathophysiology of EHEC infection is not fully understood and more knowledge is needed to characterize the "auxiliary" factors that enable a STEC strain to cause disease in humans [3]. The most important pathogenic factor of Enterohemorrhagic *E. coli* O157:H7 (EHEC) is its primary attachment with intestinal epithelial cells that leads to A/E process. Since there is a bacteria clean up mechanism in intestinal epithelial cells, bacteria need special attachment system for passing through this barrier, this attachment factor is encoded by eae chromosomal gene which called Intimin. Intimin is the first attachment factor of the bacterium to enterocyte then Tir protein through it transfers to host cell [4,5]. Probiotics are live microorganisms intended to provide health benefits when consumed, generally by improving or restoring the gut flora, Probiotics are considered generally safe to consume, but may cause bacteria-host interactions and unwanted side effects in rare cases. Lactic acid bacteria (LAB), especially *Lactobacillus,* are the most commonly used microorganisms as probiotics, members of lactobacilli are "Generally Recognized As Safe" (GRAS) ingredients, and are desired members of the gastrointestinal tract (GIT) microflora, and contribute mainly in maintaining the GIT homeostasis [6]. From all the information that remembered above, this study was aimed to isolation and identification of *E. coli* O157:H7 from patients with diarrhea, detection the presence of some virulence determinants of *E. coli* O157:H7 and evalution the effect of cell-free supernatant of *Lactobacillus acidophilus* against Enterohemorrhagic *Escherichia coli* serotype O157:H7.

Materials and Methods

Isolation and Identification:

From (October 2018) to (February 2019) two hundred and fifty specimens (stool) from children under five years for both sexes, were collected in sterilized containers from hospitals. The diagnosis of *E.coli* was achieved according to their morphological properties on MacConkey agar, EMB medium, Sorbitol MacConkey agar, chrome agar O157, Oxidase production, Catalase production, Methyl red test, Indole production, Urease production, Vogeus Proskauer tests, Kligler iron agar and Citrate utilization, motility test [7].

Molecular study

DNA extracted and purified carry out using genomic DNA purification kit protocol (Geneaid Extraction Kit). From each DNA extracted sample, 2 µl was added to the specialized measuring lens of the Nano- drop system after swabbing the lens with D.W. wetted cotton swab to measure the concentration and purity of extracted DNA sample at 260 nm and 280 nm. The results were recorded computerization**.** The specific primers were designed according to Bio edit program and NCBI BLAST **(Table 1)**. The extracted DNA, primers and PCR master mix were mixed together. PCR mixture was set up in a total volume of 20µL included 5µl of PCR Green master mix,1µL of each primer, and 2µL of template DNA have been used, the rest volume was completed with sterile de-ionized distilled water, then vortexed. The PCR program includes 35 cycles were carried out: Initial denaturation at 95 ºC for 5min, denaturation 95 ºC for 30sec, extension 72 ºC for 40sec, final Extension 72 ºC for 5min, and annealing stage are changing, *16srRNA* at 59.2ºC for 30sec, *eae* at 58.5ºC for 40sec, *lifA* at 58ºC for 30sec, *Stx1* at 61.6ºC for 30sec. The PCR products were analyzed by using 2% agarose gel electrophoresis in 100 ml of 1x TBE buffer and melted.

Table 1: The primers and their sequences used in conventional PCR

	Primer name	Sequence 5' 3'	Product length	Origin
1.	16SrRNA	F:GATGACCAGCCACACTGGAA R:GGAGTTAGCCGGTGCTTCTT	213pb	New
2.	eae	F:GGGCGGTCAGATTCAGCATA R:CCATCACTGACTGTCGCACT	741bp	New
3.	LifA	F:TGGTCGGAGTCGTCCAGTAT R:GGACGATGACCGATTTTGCG	712bp	New
4.	Stx1	F:GTGTTGCAGGGATCAGTCGT R:GACTCTTCCATCTGCCGGAC	446bp	New
5.	Stx2	F:TCCGGAAGCACATTGCTGAT R:CCACCGGGCAGTTATTTTGC	500bp	New

Isolation and identification of *Lactobacillus spp.*

Three types of probiotics strains were obtained, *Lactobacillus fermentum*, *Lactobacillus plantarum* from department of biology and *Lactobacillus acidophilus* from (Holisherb-USA), *American Type Culture Collection* (ATCC4356). Samples diluted with normal saline by adding 1ml of N.S, and mixing for 5 minutes. Samples cultivation on MRS broth and incubated anaerobically at 37°C for 48 hrs. A loop full of the cultured broths was streaked on MRS agar plates and incubated under the same conditions. These bacteria cultivation on MRS broth and incubated anaerobically at 37°C for 48 hrs.

Antimicrobial activity of *Lactobacillus* isolates

Antimicrobial activity was carried out according to the agar well diffusion assay as described previously [8]. Diarrheagenic *E. coli* were cultured in nutrient broth for 24 hours, and then cultured on *nutrient agar*. *Lactobacillus* isolates were grown in MRS broth for 20 hours, cell free culture supernatants (CFCS) were obtained by centrifuging the culture broth at 10000 g for 10 minutes, 100 µL of the CFCS was placed into the wells of the nutrition agar and the nutrition agar plates were incubated at 37°C for 14 - 15 hours with sterile MRS broth was used as negative control**,** the diameter of the clear zones around each well was measured.

Characterization of antimicrobial substances produced by *Lactobacillus* and determination of minimum inhibitory concentration (MIC)

The supernatant was aliquoted into five tubes: First tube was treated with 1 mg /mL trypsin to determine of bacteriocine production, second tube was adjusted to pH 6.5 ± 0.1 with NaOH, third tube was treated with 0.5 mg /mL catalase for 30 min at 25 °C to determine hydrogen peroxide production, fourth tube was adjusted to pH 6.5 ± 0.1, treated with catalase and treated with trypsin, fifth tube was used as positive control (non-treated). Minimum inhibitory concentrations (MICs) are defined as the lowest concentration of an antimicrobial that will inhibit the visible growth of a microorganism after overnight incubation. The experiment was carried out as follows: Serial dilutions were made from to several dilutions (1/2, 1/4, 1/8, 1/16, 1/32) of supernatant with sterile brain heart infusion broth within sterile tubes to complete the volume to (2ml). Each tube was inoculated with 200µl of 0.5 McFarland pathogenic *E.coli.* The tubes were incubated for 24hrs, at 37℃. Control tubes divided to positive control contain of broth with bacterial inoculum and negative control contain of broth only.

Results and Discussion

Results of the present study recorded, 210 (84%) samples were identified as *E.coli* from 250 samples. result showed (7) isolates were identified as Enterohemorrhagic

E. coli O157:H7. according to culturing on Chrome agar O157, the isolates gave mauve colonies. Results of the present study revealed that the highest percentage of *E.coli* isolates was in stool samples (84%) compare to other clinical isolates. The reason of differences in the isolate percentages may attributed to differences in season of collecting samples as well as used multi antibiotic that lead to confuse in isolated, also difference in the environment and the living area, as well as main reason, *E. coli* is a most type of bacteria that normally live in the intestines of people and animals [9]. Current study recorded (7) isolates were identified as (EHEC) serotype O157:H7, in children under five years, and agreement with other study when show that 40 children with diarrhea were examined, 10 cases were diagnosed as pathogenic *E.coli* [10]. Current study demonstrated the bloody diarrhea caused by *E. coli* O157:H7 under five years, patients' selection was restricted to those who had bloody diarrhea because *E.coli* O157 is mostly associated with this clinical feature.

Molecular study

The results revealed that the concentrations of DNA ranged from (88-450) ng/µl, while purity ranged from (1.8 - 2). Current results demonstrated that (100%) of Enterohemorrhagic *E. coli* O157:H7 isolates**,** had *16SrRNA*, *eae, lifA, stx1* with 213bp, 741bp, 712bp and 446bp, rrespectively, while no bands with *stx2* in all isolates, figures (1-4)**.** Sequencing technique has several advantages over phenotypic and biochemical identification. One of the advantages of using the *16SrRNA* gene analysis is that this gene is present in all bacteria; the lack of extensive mutations in this gene is another advantage of using this type of analysis; isolated by using *16SrRNA* is more accurate than bacteriological and biochemical assays[11]. Current study compatible with study in Northern Ireland about four-year 1997–2000, demonstrated that 80% of *E.coli* O157 isolates possessed the *eae* gene [12]. The results are compatible with previous study in University of Baghdad, Iraq; include, isolated 32 isolates of *E. coli* recovered from 350 fecal samples, and showed aproximately, (60%) isolates gave positive results with stx1primer [13]. Other results for a second study in Iraq, Basrah city, this study includes detection of the prevalence of Enterhemorrhagic *Escherichia coli* O157:H7and detection of *Stx1, Stx2,* results demonstrated, all the *E. coli* O157:H7 isolates were positive to *stx1* gene which was observed in 100% of isolates but none of the isolates were detected to having a *stx2* gene [14]. The production of shiga toxin (Stx) is a critical step in the establishment and progress of enterohemorrhagic *Escherichia coli* (EHEC) infections. The possible release of Stx from dead and dying bacteria, and the risk of resistance development have restricted the usage of antibiotics against EHEC. The pathogenesis of EHEC infections is associated with the production of Shiga toxins that are similar to the Shiga toxin that is produced by *Shigella dysenteriae* type 1 [15]. Enterohemorrhagic *E.coli* has *lifA* gene that act to inhibition lymphocyte, with strongest statistical association with diarrhea. Current results with high significant percentage of strains have *lifA* gene compatible with study in 2010,when demonstrated a strong statistical association between the presence of *lifA*-positive pathogenic *E.coli* and the presence of diarrhea that suggesting *efa1/lifA*-positive strains may be true diarrhea pathogens, and we observed positive strain are more causing severe diarrhea than other strains.

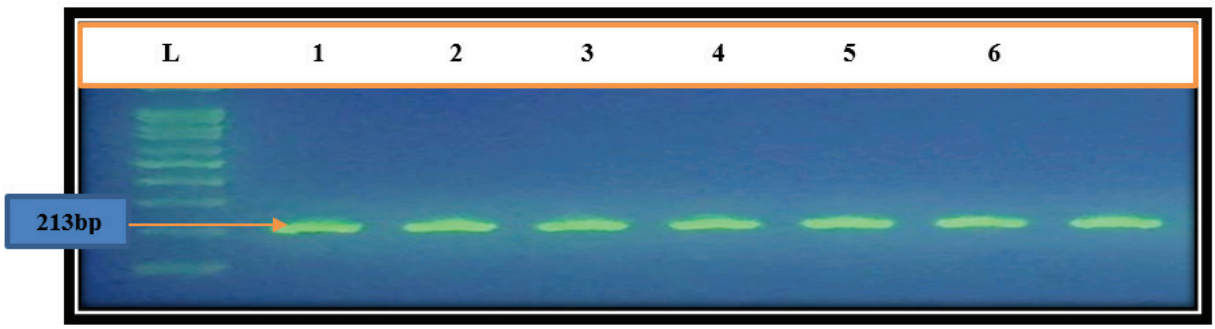

Figure (1): Gel electrophoresis of amplified *16sRNA* (213bp) in *E. coli* isolates on agarose (2%), TBE buffer (1x), 70 volt for 1 hrs. stained with red safe L: DNA ladder (100 bp).

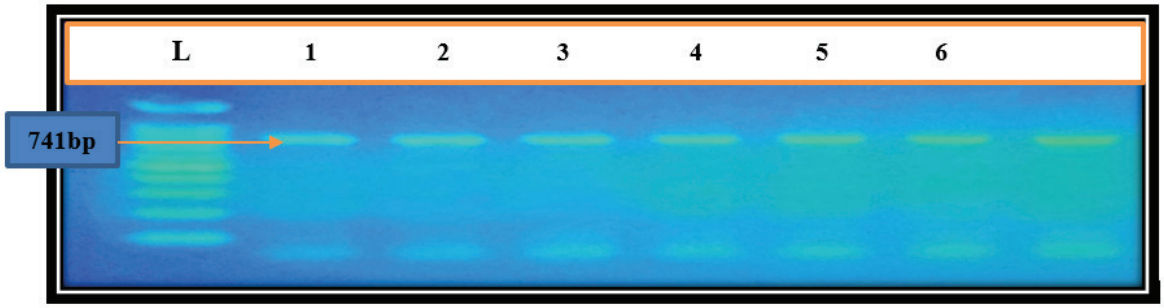

Figure (2) Gel electrophoresis of amplified *eae* (741bp) in *E. coli* isolates on agarose (2%), TBE buffer (1x), 70 volt for 1 hrs. stained with red safe L: DNA ladder (100 bp); Lanes 1-8were positive.

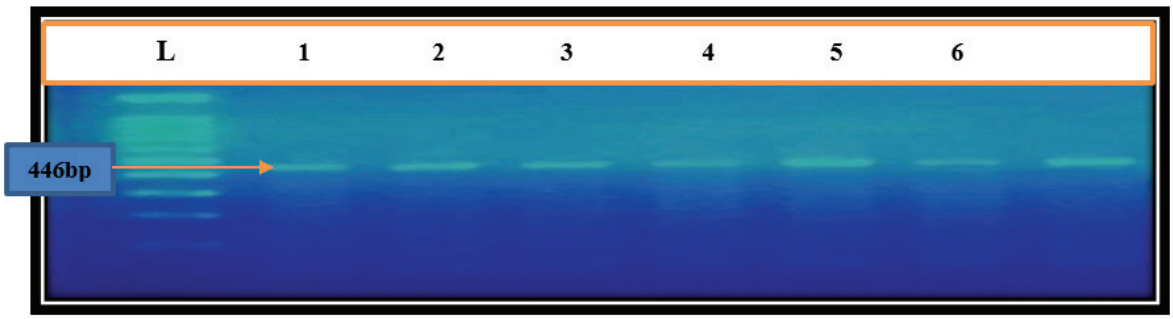

Figure (3): Gel electrophoresis of amplified *STX1* (446bp) in *E. coli* isolates on agarose (2%), TBE buffer (1x), 70 volt for 1 hrs. stained with red safe L: DNA ladder (100 bp).

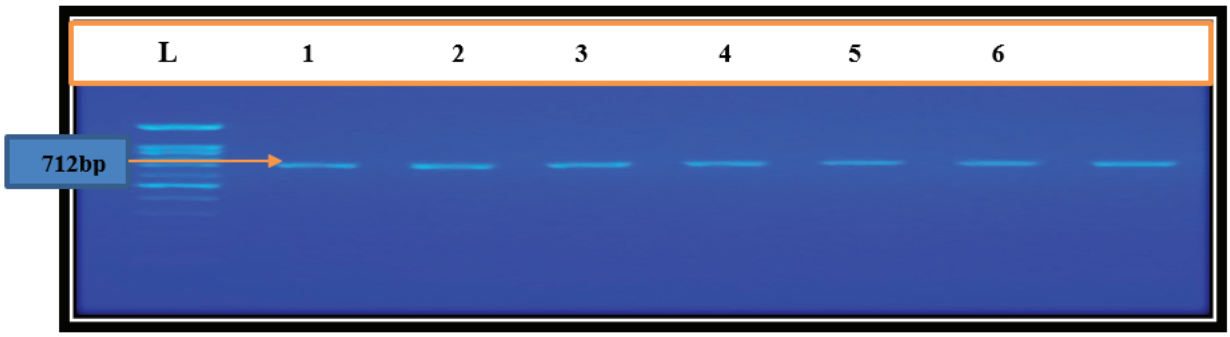

Figure (4): Gel electrophoresis of amplified *lifA* (712bp) in *E. coli* isolates on agarose (2%), TBE buffer (1x), 70 volt for 1 hrs. stained with red safe L: DNA ladder (100bp); Lanes1-7 were positive isolates.

Isolation and identification of *Lactobacillus spp.*

Three types of probiotics strains were obtained, *Lactobacillus fermentum*, *Lactobacillus plantarum* from University of Baghdad with confirmed identification (Sequencing 99% identify) and *Lactobacillus acidophilus* from (Holisherb-USA), *American Type Culture Collection* (ATCC4356). Pellet diluted with normal saline by adding 1ml of normal saline, and mixing for five minutes, all the samples were cultured primarily in MRS broth at 37ºC for 48 hr. All positive isolates on MRS agar were tested for several biochemical tests.

Antimicrobial activity of *Lactobacillus* isolates

Antimicrobial activity of the cell free culture supernatants (CFCS) was tested against the all pathogenic bacteria. Result showed only cell free culture supernatant of *Lactobacillus acidophilus* has inhibitory activity against all (E.coli O157: H7) isolates with

different dilutions (1/2, 1/4, 1/8, 1/16, 1/32), and the effect was differ according to isolates and concentration, while current result showed no effect by , *Lactobacillus fermentum*, *Lactobacillus plantarum* against pathogenic *E.coli.* Current study compitable with study that demonstrated *lactobacillus* have a mild inhibitory activity against the diarrheagenic *E. coli* and these strains may be useful as probiotic candidates in prevention of intestinal infections caused by diarrheagenic *E. coli* [16].

Conclusion

E. coli O157:H7 isolates were found Less than five years of patients, which have the ability to produce *Stx1* gene, and the cell-free supernatant of *Lactobacillus acidophilus* can inhibit the bacterial growth of *E. coli* O157:H7.

Ethical Clearance: The Research Ethical Committee at scientific research by ethical approval of both environmental and health and higher education and scientific research ministries in Iraq

Conflict of Interest: mThe authors declare that they have no conflict of interest.

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