Effect of Lactobacillus Acidophilus and Pediococcus Pentosaceus on Ipah Gene Expression of Shigella Sonnei

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Abstract

The study aimed to evaluate the inhibitory role of probiotics on *Shigella sonnei* in molecular level. Twenty five stool specimens were collected from children under 5 years suffering from bloody acute diarrhea hospitalized in Al Zahrawi Private Hospital, Mosul city from July 2021 till September 2021. One isolate of *Sh.sonnei* was obtained from these specimens with incidence rate of (4%), it was identified according to cultural and morphological as well as biochemical characteristics measured by Vitek 2 System. Tow probiotics were used in this study included *Lactobacillus acidophilus and Pediococcus pentosaceus.* Treatment of local isolate *Sh.sonnei* with sub-MIC cell free culture supernatant of the probiotics *L.acidophilus* and *P.pentosaceus* led to down regulation of the gene *ipaH* which was tested by Real Time Quantification PCR.

Keywords: Gene expression, *ipaH* gene; *Shigella sonnei*; Probiotic.

1. Introduction

Diarrhea is the second major cause of death in children below 5 years of age, it kills nearly 3 million children per year (1). Every year, 1.5 billion cases occur of infections related to enteric system mostly diarrhea in children and they are also ranked at the fifth spot in causing deaths in people of almost all ages that accounts for approximately 2.2 billion on whole (2). Diarrhea arises from infectious diarrhea due to bacterial, viral and parasitic organisms, and non-infectious diarrhea, derived from malnutrition and food allergies (3). Escherichia coli, Salmonella enteritidis, Shigella and Staphylococcus aureus are the main common pathogens that cause bacterial diarrhea (4). Shigella is becoming a rising public health problem due to development of multiple antimicrobial resistance, frequently resulting in treatment failure, Worldwide shigellosis occurs in at least 80 million people and results in about 700,000 deaths a year (5). Shigella has four serogroups, Sh. dysenteriae, Sh. flexneri, Sh. boydii and Sh. sonnei, which cause more than 164,000 deaths per year, with 55,000 of these among children under 5 years of age. It is identified as one of the most important agents of diarrhea according to the World Health Organization, mainly in developing countries representing 11% of all deaths from diarrhea globally (6). Shigella causes shigellosis or bacillary dysentery, which is accompanied by a high fever, neurological disorders, and mucohemorrhagic dysentery (7), Several virulence factors have been associated with Shigella spp. Some virulence factors are involved in the invasion of intestinal cells such as invasion plasmid antigen H incoded by the gene ipaH which have multiple copies located both on plasmid and the chromosome (8). The spread of Shigella resistance to antibiotics has become an important problem in developing countries, so there is a need to develop alternative treatments to reduce its risk. Among the methods that have shown a preventive and curative effect for diarrhea are probiotics, including lactic acid bacteria (LAB), found in dairy products such as milk drinks, yogurt, cheeses (fresh and mature), meats and their products, and in certain vegetables, have been present in the human diet since ancient times (9). According to research conducted by (10), LAB belong to the phylum firmicutes, which distinguishes about 20 genres, the main ones of which are Lactococcus, Lactobacillus, Streptococcus, Leuconostoc, Pediococcus, Aerococcus, Carnobacterium, Enterococcus, Oenococcus, Tetragenococcus, Vagococcus, and Weisella; (10). LAB are characterized by the production of antimicrobial substances (lactic and acetic acids, metabolites) and bacteriocins, which are a family of antimicrobial peptides produced by bacteria through ribosomal synthesis that can act as helper peptides for probiotic strains in the gastrointestinal tract (11). Given these properties, the UN Food and Agriculture Organization (FAO) and the World Health Organization (WHO) have defined probiotics as "living microorganisms which, when administered in appropriate doses, confer beneficial effects on the health of the consumer" (12). The main study aim was to show the effect of L.acidophilus and P.pentosaceus on ipaH gene expression.

2. Materials and methods

Sample collection

Twenty-five fecal samples were collected from children (aged from 1 day - 5 years old) suffering from diarrhea from both sexes during the period from July 2021 to September2021, from AL-Zahrawi Private Hospital, Mosul, Iraq.

Shigella Isolation

Stool samples were cultured by taking a full loop of feces and culturing them into sterile test tubes

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containing 5 ml of Brain Heart Infusion Broth. Then the tubes incubated at 37C° for 18-24 hours.After the incubation period and observation of growth, a ring filled with the bacterial suspension was cultured on the XLD agar.The dishes incubated at 37C° for 18-24 hours.The pink-colored colonies were recultured on the same medium to obtain pure isolates.Pure isolates were cultured on SS agar and suspicious Shigella colonies were cultured onto the solid MacConkey medium to complete the diagnosis(13), then the diagnosis was completing by microscopic examination, and confirmed by Vitek2 compact system.

Isolation and identification of Pediococcus pentosaceus

For the isolation of LAB, 1 gm of yogurt was taken and serially diluted using sterile distilled water. These diluted samples were plated using the spread plate technique on deMan Rogosa Sharpe (MRS) agar plates and incubated at 37 ◦C for 24 h. White and clear elevated colonies were selected(14), then the diagnosis was completing by microscopic examination and confirmed by Vitek2 compact system. Lactobacillus acidophilus isolate was obtained from Al-Ameen Center for Research and Advanced Biotechnology,Al-Najaf,Iraq.

Preparation of supernatants from Lactobacillus acidophilus and Pediococcus pentosaceus culture

Briefly, cultures of *L. acidophilus* and *P. pentosaceus* were grown in MRS broth (pH 5.5) at 37°C for 24 hr under microaerophilic conditions. Overnight bacterial cultures contained 2.5 × 108 colony-forming units were centrifuged at 10,000 g for 15 min at 4°C. The resulting supernatants were filtered through a 0.2-μm membrane filter to remove the remaining bacteria and debris (15).

Determination of minimum inhibitory concentration (MIC)

Serial dilutions were made from (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 *Sh.sonnei*. The tubes were incubated for 24hrs, at 37℃, control tubes divided to positive control contain broth with bacterial inoculum and negative control contain broth only (16).

Gene expression

The expression level of the virulence gene (*ipaH*) under stress with *Lactobacillus acidophilus* and *Pediococcus pentosaceus* at sub- MIC were determined for *Shigella sonnei* isolate as follow.

RNA extraction from *Sh. sonnei* isolate

RNA was isolated using an extraction kit (GENEzol TM TriRNA Pure Kit, Geneaid company,Taiwan) for total RNA isolation by mechanical disruption, according to the manufacturer's instructions.

Convert RNA to cDNA

The RNA was converted to the complementary nucleic acid strand by using a strandsynthesis kit (WizScript TM cDNA Synthesis Kit, Wizbio company, South Korea) under the following conditions: 25◦C for 10 min, 37◦C for 120 min, 85◦C for 5 min, and a cooling step to 4◦C for 5 min.

Estimate the concentration of cDNA

The Quantus Fluorometer was optimized with preprogrammed settings for Biosciences QuantiFluor Dye Systems (QuantiFluor dsDNA, ssDNA Systems, Canada. to quantitate nucleic acids and offers the flexibility to create customized methods and quantitation settings for other fluorescent dyes. For 1μL of cDNA and 199 μL of diluted Quanty Flour Dye were mixed, after 5min incubation at room temperature in dark place, cDNA concentration values were detected and then stored in -20◦C.

Designing of primer

The primer was provided in lyophilized form by Oligomer (Korea) designed special for this study, dissolved in nuclease free water to give a final concentration of 100 pmol/ μL and stored in deep freezer until used in Real Time Quantification PCR, (Table-1).

Table (1): The primers and their sequences used in Real Time Quantification PCR.

No.	Primer name	Sequence 5 '----3 '	Product length	Origin
1.	ipaH	F: GACGGACAACAGAATACACTCCATC R: ATGTTCAAAAGCATGCCATATCTGT	155	This study
2.	H. K	F: AACGTCAATGAGCAAAGGTATTAAR: TACGGGAGGCAGCAGTGG	140	(27)

Determination the expression of the gene *ipaH* using Real Time Quantification PCR.

Real Time Quantification PCR was performed using quantitative amplification kit (WizpureTM qPCR master mix, Wizbio company, South Korea). For each reaction,5 μl of cDNA was subjected to 1 μl of Forward primer, 1 μl of Reverse primer ,3 μl of Nuclease free water and 10 μl of qPCR master mix final volume containing 20 μl and mixed well by vortex, The following conditions were used for amplification: 1 cycle at 95°C for 600 sec. , 40 cycles at 95°C for 15 sec. , 40 cycles at 60°C for 60 sec. and 1 cycle at 60- 95°C for 5 sec. .

Reaction setup and thermal cycling protocol

Calculate the amount of change in the level of gene expression as shown by the following equations:

$\Delta ct = ct\ of\ tested\ gene - ct\ of\ house\ keeping\ gene$ $(16\ SrRNA)$

$\Delta\Delta CT = \Delta ct(sample) - \Delta ct(control)$

$Folding = 2{-}\Delta\Delta ct$

$ct \rightarrow cycle\ number, \Delta ct \rightarrow cycle\ threshold$

3. Results and Discussion

Isolation and Identification of *Sh.sonnei*

Twenty-five stool samples of acute diarrhea cases for children under five years old were collected from the auditors of Al-Zahrawi Private Hospital in Mosul, Iraq. one *Sh.sonnei* isolate was obtained with an isolate rate of 4%. The Shigella isolates were identified by examination of colonial morphology on each culture media. Shigella isolate on XLD was appeared as translucent, convex and colorless to pale pink colonies without H_2S production (Figure1-A). On SS agar were appeared as small, pale and colorless colonies (Figure1-B). On MacConkey agar were appeared as nonlactose fermenter translucent pale colonies (Figure1-C). The bacteria appeared under light microscope (100X) as gram negative, coccobacilli, short rods, single cell (17). VITEK2 system was used for identification isolates.One isolate of Shigella was identified, belong to the *Sh.sonnei* which recorded a high probability ranging 99%.

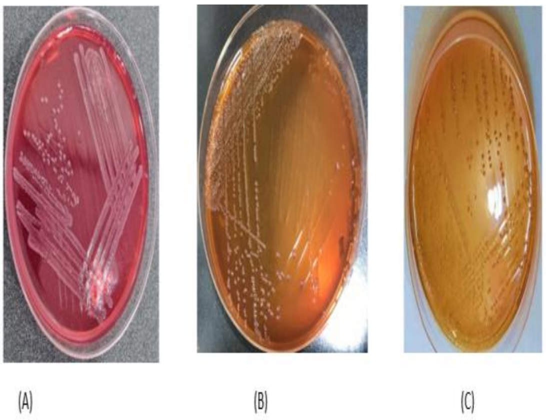

Figure (1) A: Shigella isolate colonies on XLD agar; B: Shigella isolate colonies on SS agar; C: Shigella isolate colonies on MacConkey agar.

Isolation and Identification of P.pentosaceus

Lactic acid bacteria were isolated from the yogurt samples. A total of 20 isolates of lactic acid bacteria were selected through preliminary screening. From those, one isolate was optained, it was Gram positive, cocci, arranged in tetrads and pairs, and catalase negative. (Figure 2 and Figure 3) show the results of the morphological and cultural characteristics of the isolated strain. The isolate was identified as P.pentosaceus using Vitek2 compact system(18).

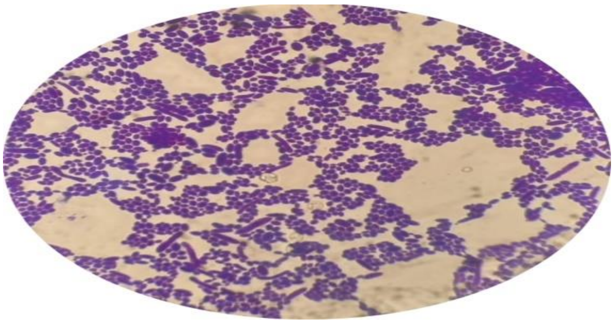

Figure (2): P.pentosaceus isolate colonies on MRS agar.
Figure (3): microscopic images of P.pentosaceus.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration and the sub minimum inhibitory concentration were determined for the cell free culture supernatant of the tow probiotics *L.acidophilus* and *P.pentosaceus* on the growth of *Sh.sonnei*, the results showed that the 1/8 dilution is the minimum inhibitory concentration and the 1/16 dilution is the sub minimum inhibitory concentration for both probiotics cell free culture supernatant.

Expression level of *ipaH* gene in *Sh.sonnei* under stress with *L. acidophilus* and *P.pentosaceus* using quantitative reverse transcription PCR .

The result demonstrated that expression level of *ipaH* gene in *Sh. sonnei* was decreased under stress with supernatant of the tow probiotics *L. acidophilus and P.pentosaceus* (Table2,3)

Table (2): Gene expression level of ipaH in Sh.sonnei treated with cell free culture supernatant of L. acidophilus.

Sh.sonnei	H.K Ct	ipaH Ct	ΔCt	ΔΔCt	Folding
not treated	19.89	20.56	0.67	0.00	1.00
treated	18.96	23.48	4.52	1.76	0.29

Table (3): Gene expression level of *ipaH* in *Sh.sonnei* treated with cell free culture supernatant of *P.pentosaceus*

Sh.sonnei	H.K Ct	ipaH Ct	ΔCt	ΔΔCt	Folding
not treated	19.89	22.65	2.76	0.00	1.00
treated	19.86	23.28	3.42	0.66	0.63

The current results showed an inhibition in gene expression value of the ipaH gene when treated with L.acidophilus cell free culture at sub minimum inhibitory concentration, which reached 0.29

compared to the value of the standard gene activity 1.00, and inhibition in the gene expression value of the ipaH gene when treated with P.pentosaceus cell free culture at sub minimum inhibitory concentration, which reached 0.63compared to the value of the standard gene activity 1.00 meaning that the treatment led to the inhibition of the gene's activity as shown in(Table2,3).

In Iraq, there is no previous study about the effect of L. acidophilus or P.pentosaceus on expression level of ipaH gene in Sh.sonnei . On the other hand the result compatible with study about evaluation the effect of other probiotics strains on the expression of stx1 and stx2 genes in verotoxigenic E. coli (VTEC) and demonstrated that L. casei and L. plantarum decrease the expression of Shiga toxins, and can effect on other virulence factors of E. coli, L. caesi reduced the amount of gene expression in compares to L. plantarum, and demonstrated Lactobacilli reduced the expression of stx1 gene more than stx2 (19).

A study carried on University of Baghdad, referred to the ability of using non-pathogenic E. coli as probiotic strain, and the results showed strong reduction in the EHEC numbers (CFU) and also had effect on O157:H7. This study from both in vitro and in vivo data, suggests that the nonpathogenic E. coli probiotic could offer strong inhibitory effects on the growth and Shiga toxin gene expression of E. coli O157:H7 and might be useful to fight against O157:H7infection (20).

The production of an autoinducer-2(AI-2) that is used for bacterial interspecies relationships has been found in EHEC O157. One of the strategies used by L. acidophilus is the interfering with QS regulation of pathogens such as E. coliO157 by reducing the production of AL-2 molecules in E. coli O157, which leads to reduce the expression of stx1and stx2genes, which are a component of bacterial pathogenicity. Also, these two genes are known to be useful in the infection caused by this bacterium (21, 19). but some studies outside of Iraq demonstrate many inputs controlling ler gene expression in EHEC and EPEC and found that ler is stimulated in response to environmental signals such as temperature, pH, iron, ammonium, calcium, bicarbonate, and quorum-sensing signaling (22).

In a compatible study about the effect of L. acidophilus on the expression of virulence-related genes in EHEC O157, the study reported the presence of the probiotic L. acidophilus that could have negative effects on the expression of LEE operons. Real Time Quantification PCR was used to measure the expression of virulence-related genes of EHEC O157 and found to be reduced in the presence of 10% L. acidophilus. From the Real Time Quantification PCR analysis result, the genes that showed statistically significant downregulation were, ler, tir, espA , fliC, espD, hlyB, and qseA (23)

The expression level of ler gene in EHEC could be decreased in response to the effect on quorum-sensing signaling. The bacterial communication with each other and with their surrounding environment is achieved through chemical signaling molecules called auto-inducers. The phenomenon quorum sensing use cell-to-cell signaling mechanism in ease the regulation of many important behaviour of the enteric microbes, these mechanisms allow them to colonize and/or initiate infection in their host successfully. Quorum sensing signaling in E. coli O157 could be inhibited by a secretive molecule produced by L. acidophilus, the molecules work by decreasing the expression of ler gene or directly interfere with bacterial transcription of the genes that participate in colonization and thus, bacterial toxicity is inhibited (24, 23).

EPEC may be use the important biological pathway called fitness cost when expose to stress by probiotics. The cost to the 'fitness' of an organism is its ability to replicate and survive in a competitive environment and able to decrease some genes and in same period increase expression level for others. In this study, when bacteria expose to stress, bacteria start to inhibit all secondary genes and increase the expression level of substantial genes for highest level to survive in a competitive environment (25,26).

4. Conclusion

Shigella sonnei is one of the pathogenic agents that cause diarrhea because it has virulence factors that enable it to do so, the L.acidophilus and P. pentosaceus cell free culture supernatant act at sub minimum inhibitory concentration to reduce the gene expression of ipaH gene of Sh.sonnei causing bacillary dysentery.

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