Effect of Lactobacillus acidophilus and Pediococcus pentosaceus on sigA 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 cltuture supernatant of the probiotics *L.acidophilus* and *P.pentosaceus* led to down regulation of the gene *SigA* which was tested by Real Time Quantification PCR.
Keywords: Gene expression sigA gene; Shigella sonnei; Probiotic.

1. Introduction

Shigellosis is one of the most important causes of diarrhea worldwide. (1) The annual number of deaths due to shigellosis has been estimated at 1.1 million, and shigellosis associated mortality is particularly prevalent among countries. (2) Shigellosis can be caused by four Shigella species: Sh. dysenteriae, Sh. flexneri, Sh. boydii, and Sh. sonnei. Sh. sonnei is the predominant serogroup in developed countries, while Sh. flexneri is the main serogroup found in developing countries. (3) However, the frequency of Sh. sonnei has recently increased in several areas that have undergone rapid socioeconomic improvements. (4,5). Shigella belongs to Enterobacteriaceae family, class of gamma proteobacteria in the phylum of proteobacteria. The natural habitat of Shigella is in the intestine of humans,apes, and monkeys (6). All Shigella species are Slender rod-shaped bacteria and are found in coccobacillary forms in young culture.Shigella species are non-spore forming,nonmotile,gram negative,oxidase negative,facultative anaerobic bacteria.Their colonies are convex and round and have intact edges.The colonies have a diameter of about 2mm within 24 hours (7). Shigella divides into four serogroups depending on the O-antigen structure (A, B,C and D)The serogroup A,known as Sh.dysenteriae,contains 16 serotypes.The serogroup B known as Sh. flexneri contains 19 serotypes.The serogroup C known as Sh. boydii contains 20 serotypes and the serogroup D known as Sh. sonnei has one serotype. With the exception of the serogroup D which can be distinguished on the basis of chemical metabolism assays,the serogroups A,B,C are physiologically similar(8).The infectious dose of 10-100 germ cells is sufficient to cause shigellosis disease in adults (9).The infection usually occurs in young children who do not care about hygiene after the toilet.Shigellosis is more prevalent in day care centers where children meet from different areas. Transmission is also through foodand water (10). Finally, the genes encoding serine protease autotransporters of Enterobacteriaceae (SPATE) are phylogenetically classified into two main classes: class 1 SPATE genes, comprising the Shigella IgA-like protease homolog (sigA) and secreted autotransporter toxin (sat); and class 2 SPATE genes, comprising Shigella extracellular protein A (sepA) and protease involved in intestinal colonization (pic), (11).

Probiotics are defined as live microorganisms, which when administered in adequate amounts, confer a health benefit on the host. Health benefits have mainly been demonstrated for specific probiotic strains of the following genera: Lactobacillus, Bifidobacterium, Saccharomyces, Enterococcus, Streptococcus, Pediococcus, Leuconostoc, Bacillus, Escherichia coli (12). Probiotics possess inhibitory activities on the growth of pathogenic bacteria. The altered pH values by producing organic acids, producing bacteriocin, producing hydrogen peroxide, and competitive adhesion to the epithelium could result in the inhibitory effect which exerts probiotic activity (13). The effective probiotic microorganisms should be safe, viable, antimicrobial characteristics, acid, bile salt, and gastric juices tolerant, able to survive through the gastrointestinal tract, adherence to intestinal epithelial cells (14). Moreover, the capability of a bacterial strain to adhere to the digestive tract is a prerequisite for colonization (15). In the recent past, various investigations on lactic acid bacteria have reported a wide range of health stimulating properties inducing the host gut microbial balance (16, 17). Therefore, the present study aims for the effect of L.acidophilus and P.pentosaceus on sigA gene expression. The main study aim was to show the effect of

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L.acidophilus and P.pentosaceus on sigA 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 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(18), 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(19), 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 (20).

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 (21).

Gene expression

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

RNA extraction from Sh. sonnei isolate

RNA was isolated using an extraction kit (GENEzol ™ 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 ™ 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.	sigA	F: GAAACCGTACTGCGTGATGC R: CGAATTTCGGCGTTGAGACC	170	This study
2.	H. K	F: AACGTCAATGAGCAAAGGTATTAA R: TACGGGAGGCAGCAGTGG	140	(32)

Determination the expression of the gene *sigA* using Real Time Quantification PCR

Real Time Quantification PCR was performed using quantitative amplification kit (Wizpure™ 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 $SrRNA$)

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

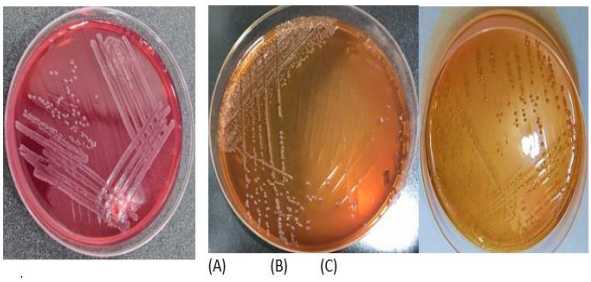
$Folding = 2^{-}\Delta\Delta ct$

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

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 (22). 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(23).

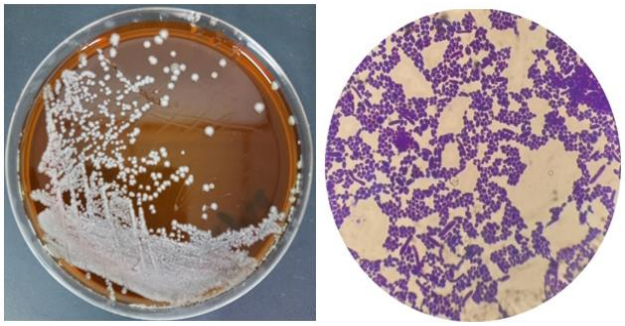


Figure (2): P.pentosaceus isolate colonies on MRS agar. Figure (3): microscopic images of P.pentosaceus.

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 sigA gene in Sh.sonnei under stress with L. acidophilus and P.pentosaceus using quantitative reverse transcription PCR

The result demonstrated that expression level of *sigA* 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 *sigA* in *Sh.sonnei* treated with cell free culture supernatant of *L. acidophilus.*

Sh.sonnei	H.K Ct	sigA Ct	ΔCt	ΔΔCt	Folding
not treated	22.65	2.76	19.89	0.00	1.00
treated	18.04	21.63	3.59	0.83	0.56

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

Sh.sonnei	H.K Ct	sigA Ct	ΔCt	ΔΔCt	Folding
not treated	2.23	5.03	2.80	0.04	0.97
treated	9.00	10.10	1.10	0.44	0.73

The current results showed an inhibition in gene expression value of the *sigA* gene when treated with *L.acidophilus* cell free culture at sub minimum inhibitory concentration, which reached 0.56 compared to the value of the standard gene activity 1.00, and inhibition in the gene expression value of the *sigA* gene when treated with *P.pentosaceus* cell free culture at sub minimum inhibitory concentration, which reached 0.73 compared to the value of the standard gene activity 0.97 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 *sigA* 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 *stx*1 and *stx*2 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 *stx*1 gene more than stx2 (24).

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 (25).

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. coli*O157 by reducing the production of AL-2 molecules in *E. coli* O157, which leads to reduce the expression of *stx*1and *stx*2genes, which are a component of bacterial pathogenicity. Also, these two genes are known to be useful in the infection caused by this bacterium (26, 24). 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 (27).

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* (28)

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 (29, 28).

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 (30, 31).

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 *sigA* gene of *Sh.sonnei* causing bacillary dysentery.

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