Effect of Lactobacillus acidophilus and Pediococcus pentosaceus on spvR gene expression of Salmonella enterica serovar Enteritidis (S.Enteritidis)

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Abstract
The study aimed to evaluate the inhibitory role of probiotics on Salmonella Enteritidis in molecular level. Twenty five stool specimens were collected from children under five years suffering from acute diarrhea hospitalized in Al-Zahrawi Hospital,Mosul city from 1/7/2021 till 1/9/2021. One isolate of S.enteritidis 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. Two probiotics were used in this study included Lactobacillus acidophilus and Pediococcus pentosaceus. Treatment of local isolate S.enteritidis with sub-MIC cell-free culture supernatant of the probiotics L.acidophilus and P.pentosaceus led to down regulation of the gene spvR which was tested by Real Time –quantitative PCR.
Keywords: Gene expression, *spvR, S.*Enteritidis, Probiotic.

1. Introduction

Salmonella represents a large genus of global public health significance and is the leading cause of foodborne illnesses responsible for thousands of deaths worldwide (1).
In recent years, studies on the global burden of non-typhoidal Salmonella (NTS) have shown an increasing incidence of NTS, for instance, one of these studies estimated that there are approximately 94 million cases of NTS gastroenteritis resulting in 155,000 deaths globally each year (2).
The pathogenicity of Salmonella is mediated by numerous genes comprising invA, fmA, stn, spvR, spvC, spiC and pipD (3). Different strains of Salmonella enterica often carry serotype-associated plasmids (SAPs) that encode a virulence operon that consists of five open reading frames designated spvR, spvA, spvB, spvC, and spvD (4). The spvR gene encodes a regulatory protein of the LysR family that, together with the chromosomally encoded regulatory gene rpoS, regulates the spvABCD genes (5).
Antimicrobial treatment may lead to development of antimicrobial resistance, which threatens public health as well as future treatment possibilities against the pathogens of calf diarrhea. Alternatives to antimicrobial treatment for control of the disease are highly needed. Inhibition of the virulence factors which enables the bacteria to cause diarrhea have been suggested as a possibility (6).
Lactic acid bacteria (LAB) are a group of Gram-positive, lactic acid producing Firmicutes. They have been used for centuries as starter or adjunct cultures in dairy fermentations. Milk proteins are a major source of bioactive peptides and an increasing number of bioactive peptides have been identified in milk protein hydrolysates and fermented dairy products
(7). the breakdown of milk proteins by LAB plays an important role in generating peptides and amino acids for bacterial growth and in the formation of metabolites that contribute to flavor formation of fermented products. Recent studies indicate that degradation products from certain LAB, as well as some Bifidobacterium strains affect expression of virulence-associated genes in specific pathogens. For instance, probiotic Lactobacillus acidophilus La-5 and Bifidobacterium
longum NCC2705 strains have the ability to downregulate virulence genes (ciaB and flaA) expression (8). Likewise, B.bifidum ATCC25921, B. bifidum BBA1, B. crudilactis FR/62/B/3, and L.acidophilus La-5 produce metabolites inhibiting virulence gene expression of enterohemorrhagic E.coli O157:H7 (9, 10, 11) and S. Typhimurium (11, 12). L.acidophilus GP1B has been shown to cause downregulation of virulence genes in Clostridium difficile (13) and L.bulgaricus NRRL B548, Lacticaseibacillus rhamnosus NRRL B442, L.paracasei DUP-13076, and L.helveticus LH-2 affect attachment and invasion of Salmonella in vitro and in vivo through significant inhibition of the virulence genes expression (14, 15, 16, 17).
The aim of this study was to investigate the protective effect of probiotics against spvR gene expression of S.Enteritidis

2. Materials and Methods

Sample Collection

Twenty fecal samples were collected from children

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(aged from
1 day - 5 years old) suffering from diarrhea from both sexes during the
period from July 2021 to Septemper 2021, in Al-Zahrawy Private hospital, Mosul,Iraq.

S.enteritidis Isolation

Stool sample cultured direct on MaCconkey agar incubated at 37°C
for 18-24 hours and after incubation period, non-lactose ferment colonies
were cultured on XLD and SS agar at 37°C overnight (18), after this period Salmonella suspect colonies cultured on nutrient agar plates, Vitek 2 compact system was used to confirm identification of the isolate.

P.pentosaceus Isolation

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 (19). white and clear elevated colonies were selected then identified with Vitek 2 compact system, An isolate of *L.acidophillus* was obtained from the Al-Ameen Center for Research and Advanced Biotechnology, Al-Najaf, Iraq.

Preparation of cell-free culture supernatant

Briefly, cultures of Lactic acid bacteria were grown in MRS broth and incubated at 37°C for 24 hr under microaerophilic conditions.Overnight bacterial cultures contained 2.5 × 108 colony-forming units, and these cultures 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, then stored at -20°C (20).

Determination of minimum inhibitory concentration (MIC)

The experiment was carried out as follows

1.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).
2.Each tube was inoculated with 200µl of 0.5 McFarland pathogenic S.enteritidis. The tubes were incubated for 24hrs, at 37°C.
3.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 regulator gene (*spvR*) under stress with *Lactobacillus acidophilus* and *Pediococcus pentosaceus* at sub- MIC were determined for *Salmonella enteritidis* isolate as follow.

RNA extraction from *S.enteritidis* isolate

RNA was isolated using an extraction kit (GENEzol ™ TriRNA Pure Kit) supplied by Geneaid,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 using a strand-synthesis kit (WizScript ™ cDNA Synthesis Kit) supllied by (Wizbio, 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 extracted cDNA

The Quantus Fluorometer was optimized with preprogrammed settings for Bioscience QuantiFluor Dye Systems (QuantiFluor dsDNA and 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 -20C◦.

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 cocentration of 100 pmol / µl and stored in deep freezer until used in Real Time – quantitative PCR (Table 1).

Table 1: The primers and their sequences used in Real Time –quantitative PCR :

No.	Primer name	Sequence 5---3	Product length	Origin
1.	spvR	F: TTACCCGACCAAGAAACGGG	174	This study
		R: GCCGTGAATACAGGTGTTGC		
2.	16SrRNA	F: CGGGGAGGAAGGTGTTGTG	178	(37)
		R: GAGCCCGGGGATTTCACATC		

Determination the expression of the gene *spvR* using Real Time quantitative PCR

Real Time-quantitative PCR was performed using quantitative amplification kit (Wizpure™ qPCR master mix) supplied by Wizbio, 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 mix 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 °Cfor60 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
Δ Ct = Ct of tested gene – Ct of housekeeping gene(16 SrRNA)
ΔΔCt = ΔCt (sample) – ΔCt (control)
Folding = 2-ΔΔCt
Ct ---- Cycle number
Δ Ct ---- Cycle threshold

3. Results and Discussion

Sample collection

Infectious diarrhea in under-five year children is commonly caused by Shigella, Salmonella, Campylobacter species and diarrheogenic E. coli (22). Higher rates of Salmonella infections were observed in the young children, elder adults and people with weakened immune systems are the most likely to have severe Salmonella infections (23).
In present study, S.enteritidis are isolate with incidence rate of 4% from all diarrheal samples,the results of Salmonella isolates are approximate with many studies performed in several locations in Iraq in which the proportions of Salmonella isolation were ranged from 1.07% to 16% during the years 2008 – 2017. The highest percentage was reported in Al-Hawijah city, and the lowest percentage was reported in Mosul city (24, 25, 26, 27, 28, 29, 30).

Isolation of S.Enteritidis

Salmonella growth was appeared on XLD agar: small, smooth, rounded colonies red in color with black center (figure 1a); on MaConkey agar (MAC): none lactose ferment, smooth, colorless colonies (figure 1b) ; on SS agar small, smooth with black center (figure 1c), The bacteria was appeared under light microscope (100x) as gram negative bacilli, single cells. The diagnosis is confirmed by vitek 2 compact system.

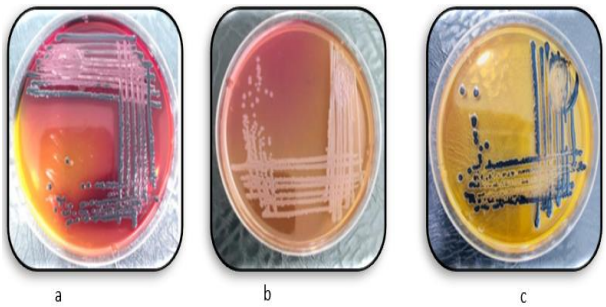

Figure 1 S.Enteritidis on differents media

a: XLD agar
b: MacConkey agar
c: SS agar

Isolation of P.pentosaceus

Lactic acid bacteria were isolated from the overnight yogurt samples. A total of 20 isolates of lactic acid bacteria were selected through preliminary screening. From those,one isolated cultures were Gram positive, cocci, arranged in tetrads and pairs, and catalase-negative **(figure 2 a and figure 2b),** The diagnosis is confirmed by vitek 2 compact system.

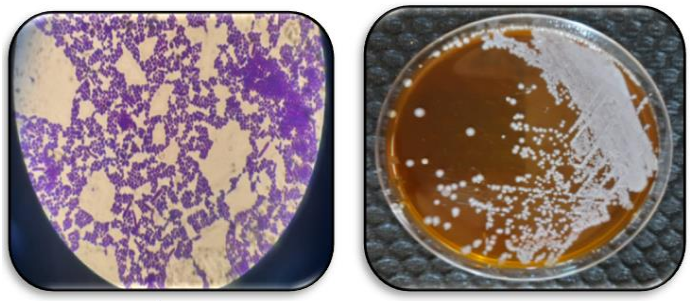

Figure 2 P.pentosaceus :

a: on MRS agar
b: under light microscope

Expression level of *spvR* gene in *S.*Enteritidis under stress with *L.acidophilus* and *P.pentosaceus* using quantitative reverse transcription PCR

The results demonstrated that expression level of *spvR* gene in *S.enteritidis* was decreased after being under stress with supernatant of probiotic species *L.acidophilus* and *P.pentosaceus* (Table 2,3). In Iraq, there is no previous study about the effect of *L.acidophilus* and *P.pentosaceus* on expression level of *spvR* gene of *S.*Enteritidis

Table (2): Gene expression level of spvR in S.Enteritidis treated with cell free culture supernatant of L.acidophilus :

S.Enteritidis	H.K Ct	spvR Ct	Δ Ct	Δ Δ Ct	Folding
Not treated	20.46	26.70	6.24	11.43	1.00
Treated	21.04	26.63	5.59	2.83	0.14

Table (3): Gene expression level of spvR in S.Enteritidis treated with cell free culture supernatant of P.pentosaceus :

Isolates	H.K Ct	SpvR Ct	Δ Ct	Δ Δ Ct	Folding
Not treated	22.03	5.03	2.90	0.14	0.91
Treated	0.90	24.93	3.92	1.16	0.44

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 affect other virulence factors of E.coli, L.caesi reduced the amount of gene expression in compares to L.plantarum, and demonstrated that Lactobacilli reduced the expression of stx1 gene more than stx2 (31).
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:H7 infection (32).

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 stx1 and stx2 genes, which are a component of bacterial pathogenicity. Also, these two genes are known to be useful in the infection caused by this pathogen (33, 31).

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 (34).

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. RT-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 RT-PCR analysis result, the genes that showed statistically significant downregulation were, ler, tir, espA, fliC, espD, hlyB, and qseA (9).

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. Almost ler utilize works by this pathway. In 2005, a study detected the mechanism of ler gene through the process of infections and explained over expression of ler. When the HEp-2 cells in culture were infected, Ler expression found to be only working during the early stages of the EPEC infection and using the real-time PCR the study revealed that the transcription of the EPEC LEE3, LEE4, and LEE5 operons was higher after 3 h post infection, in contrast the expression of LEE1, carrying ler, was low within the same period (35, 36).

4. Conclusion

The cell-free culture supernatants of L.acidophilus and P.pentosaceus act at lower inhibitory concentrations to reduce the gene expression of spvR gene of S.Enteritidis.

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