Protective effect of *Lactobacillus casei*and *Lactobacillusfermentum* cell-free supernatants against verotoxin 1 of*E. coli* O157

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ABSTRACT

Verotoxin 1 (VT1) or Shiga-like toxin 1 (Stx-1) is an AB5 holotoxin with a potent inhibitor of protein synthesis and it is one of the major virulence factors of enterohemorrhagic*Escherichiacoli* (EHEC). This study is aimed to investigate the inhibitory effect of some lactic acid bacteria against the cytotoxicity of verotoxin 1. Cytotoxicity of verotoxin 1 (VT1) on the NCM425 cell line was evaluated with and without the presence of *Lactobacilluscasei* (Lc) and *Lactobacillusfermentum* (Lf) cell-free supernatants using 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay. Results indicated that VT1 exhibited a cytotoxic impact on NCM425 which was in a dose-dependent manner. The cytotoxic effects of VT1 were lessened by probiotic bacteria's cell-free supernatants at 25 and 50 µg/ml concentrations. No significant difference was recorded between the protective effect of Lc and Lf against VT1. In conclusion, *L. casei* and *L. fermentum* cell-free supernatants can suppress VT1-mediated cytotoxicity and promote NCM425 survival. The cell-free supernatants of *L. casei*and *L.fermentum* may be added to a wide range of foods and employed as additives to reduce VT-mediated cytotoxicity.

Keywords: Cell toxicity; *Lactobacillus casei*; *Lactobacillus fermentum*;probiotics; verotoxin 1
DOINumber:10.147042022/nq.20.8.NQ44954 **NeuroQuantology 2022; 20(8): 9333-9341**

Introduction

Verotoxins (VTs), are a subclass of cytotoxic proteins with comparable structural and functional properties commonly referred to as Shiga-like toxins (SLTs) (1).*E. coli*, which belongs to the family *Enterobacteriaceae*, produces VTs(2).A significant pathogenic subtype of these bacteria called enterohemorrhagic *E. coli*(EHEC) produces the Vero (Shiga) toxin(3).A cytotoxic A component (Stx1A) and a pentamer of carbohydrate-binding B components (Stx1B) comprise VT1(4).VT1 has been shown to harm the endothelial cell lining of the glomeruli as well as the epithelial cells of the renal

tubulesfurther to other body tissues that display the same toxin receptor, globotriaosylceramide (Gb3)(5).In various *E. coli* infections, including HUS, VT1 is considered to cause hemorrhagic diarrhea, brain damage, and apoptosis (6).Probiotics are living, non-pathogenic bacteria that give health advantages when taken in appropriate quantities(7). It has been observed that natural substances like glycosphingolipid (GSL) from cow's milk may be able to counteract the cytotoxic effects of VT(8). Another study suggested that a culture supernatant from a probiotic straindisplayed an inhibitory effect on VT by interfering with its binding with GB3 receptors on target cells(9). For several *Lactobacillus* strains, including *Lactobacilluscasie* and *Lactobacillusfermentum*, significant inhibitory effects against the development of VTEC have been shown which may be considered as an initial step to preventing VT production(10). Consuming the probiotic *L. casei* strain Shirota daily inhibited the colonization of VTEC in the GIT and decreased the levels of both VT1 and VT2 toxins (11).*L. casei* Shirota's protective effect resulted from the increase of the local immune response and the removal of VTEC cells, which therefore decreased toxin levels in the gut(12).The purpose of this study was to examine the protective characteristics of *Lactobacilluscasei* and *Lactobacillusfermentum* against the cytotoxicity of verotoxin 1 of *E. coli* O157.

Methods

Cell-free supernatants and Verotoxin 1

L. casei and *L.fermentum* wereprovided by The Department of Biology/College of Science/Baghdad University. They were activated twice by subculturing their stock solution in MRS broth (Oxoid, UK) and incubating at 37 ˚C for 24 and 48 h anaerobically. To make cell-free supernatant (CFS), 120 ml of MRS broth containing 1×10^8 CFU/ml of 24 h growing *L. casei* and *L. fermentum* cultureswere incubated anaerobically for 72 hours at 37 ˚C. Then each one was filtered using 0.22 m pore-size filter sheets (Microlab, UK) after centrifuging at 4000 rpm for 15 minutes, then lyophilized and stored at -20 C until use(13). VT1 was purchased from Biorbyt Ltd, UK

Cell viability assay

NCM425 cell line(RRID: CVCL_D876) was suspended in full RPMI-1640 media and propagated in flasks for 24 hours at 37°C in a humidified environment supplied with 5% CO_2(14). Five concentrations (3.1, 6.25, 12.5, 25, and 50 µg /ml) of each of VT1 alone and a combination of VT1 and cell-free supernatant of each probiotic isolate (1:1) were prepared after adding 0.5 ml of distilled water or PBS to the stock solution and by using the MTT kit (Intron Ltd kit, China) which was tested upon NCM425 cell cultures. Each concentration was cultivated in an RPMI1640 medium; the cells were then scraped with an EDTA/trypsin solution and resuspended in a medium containing 10^{-1} percent bovine serum albumin before being plated on a 96-well microtiter plate(15).Every experiment was done in triplicate. The MTT test was carried out to test cell viability and the results were read at 517 nm after 24 hours. The following formula was used to calculate thecell viability: %Viability = Mean OD $_{sample}$ / Mean OD $_{blank}$ ×100.

Statistical analysis

ANOVA and Dunnett's multiple comparisons tests were used to analyze the data. A statistically significant p-value of 0.05 was established. For all statistical studies, GraphPad Prism 8.4.3 was utilized.

Results

This experiment aimed to investigate the cellviabilityof NCM425 with various dosages of VT1 alone and then in combination with cell-free supernatants of the probiotic bacteria. Results showed that NCM425 viability was reduced when treated with VT1.The highest viability reduction recorded was 51.8% at 50 µg/ml which indicates that VT1 impact was in a dose-dependent manner incomparison to the control (Figure 1). On the other hand, NCM425 viability was reduced by 68.4% and 67.4% at 50 µg/ml when cells were treated with VT1 in combination with Lc and Lf respectively which indicates that *L. casei* and *L. fermentum*cell-free supernatants suppressed the cytotoxicity of VT1.The impact between VT1 alone and with Lc and Lf was significantly different at 25 and 50 µg/mlas revealed by statistical analysis. These findings were confirmed by measuring the half-maximal inhibitory concentration (IC_{50}) which is the concentration at which cell viability is reduced by 50%.Findingsrevealed that the IC_{50} value was 47.68 µg/ml after treatment with VT1 alone while the combination with supernatants of *L. casei* (Lc) and *L. fermentum* (Lf) raised the IC_{50} values to 125.20 and 107.50 µg/ml respectively which were significantlydifferent as revealed by statistical analysis as illustrated in Figure 2.Although minor differences between the reduction ability of Lc and Lf against VT1 cytotoxicity toward the NCM425 cell line, the outcomes displayed no significant differences between their effect (Figure 3).

Discussion

In the current study, we demonstrated that VT1 has a cytotoxic effect against the NCM425 cell line. Numerous reports have shown that VT1 can trigger death and antiproliferative effect against target cells(16–18).VT1 cytotoxicity on mammalian cells had been reported when VT1 was found to be able to bind to the normal tissue of the colon(19). Furthermore, protein production was suppressed in a dose-dependent pattern by VT1 in both pediatric and adult human glomerular microvascular endothelial cells HGMVECs(20).A lesser IC_{50} value indicates that the agent is more effective in inhibiting cell viability, and vice-versa. Here we showed that cell-free supernatant of two probiotics bacteria *L. casei* and *L. fermentum*had a protective effect against VT1 toxicity evidenced by rising the IC_{50} value of VT1 which led to a decreasedcytotoxicity mediated by the toxin. Several studies linked probiotics with the detoxification of verotoxin, for instance, it had been shown that *L. acidophilus*cell lysate was capable of reducing the cytotoxic influence of VT2 when pre-incubated with Vero cells(21). Earlier study demonstrated that probiotics belonging to *Bifidobacterium* spp. and their supernatant decreased cell toxicity of VT1, VT2, and a mixture of both on Vero cells(22).Another study suggested that *Bifidobacteriumlongum* HY8001culture supernatant displayed an inhibitory effect on VT by interfering with its binding with GB3 receptors on target cells(9).*Bifidobacterium longum* HY8001culture supernatantreduced the cytopathic impact of the VTs. This indicates that the culture supernatant of *B. longum* HY8001 contains soluble VT-neutralizing substance(s) and that might be used to treat VT-producing *E. coli* ADDIN CSL_CITATION {"citationItems":[{"id":"ITEM-1","itemData":{"ISSN":"02572389","abstract":"Prophylactic effects of Bifidobacterium longum HY8001, Korean isolate, against Escherichia coli O157:H7 and Salmonella typhimurium DT104

enteric infection were examined at four groups of specific pathogen free(SFP)-ICR mouse for each pathogen. B. longum HY8001+S. typhimurium DT104+B. longum HY8001 (BL+ST+BL)group and B. longum HY8001+E. coli O157:H7+B. longum HY8001(BL+E+BL)group were fed with B. longum HY8001 before and after E. coli O157:H7 or S. typhimurium DT104 challenge, while B. longum HY8001+S. typhimurium DT104(BL+ST) and B. longum HY8001+E. coli O157:H7(BL+E) groups were fed with B. longum HY8001 only before E. coli O157:H7 or S. typhimurium DT104 challenge. E. coli O157:H7(E) and S. typhimurium DT104(ST) groups were challenged with each pathogen without B. longum HY8001 administration and control groups were administered with phosphate buffered solution(PBS). After the oral administration with B. longum HY8001(109 cfu), the mice were challenged with E. coli O157:H7(2 x 1010 cfu) or S. typhimurium DT104(108 cfu) and the mortality rate and the fecal shedding of challenged pathogen were also examined to define the reactivity of the B. longum HY8001. Production of toxin neutralizing substance(s) of B. longum HY8001 was determined by cell cytotoxicity assay using Vero cells. Fecal shedding of the S. typhimurium DT104 was significantly decreased in BL+ST+BL group fed with B. longum HY8001 before and after challenge($p<0.05$), while the fecal sheddings of S. typhimurium DT104 in BL+ST and ST groups remained more than 106 cfu. The protective effect of the B. longum HY8001 against E. coli O157:H7 was significantly high only in BL+E+BL group fed with B. longum HY8001 before and after E. coli O157:H7 challenge from the result of fecal E. coli O157:H7 isolation rate, mortality rate, and intestinal contents culture to detect E. coli O157:H7. The mortality rate of the BL+E and E groups was 20% and 30% respectively, when that of the BL+E+BL group was 0%. The isolation rates of E. coli O157:H7 from the intestinal contents in BL+E+BL, BL+E, and E group resulted in 50%, 87.5%, and 86%, respectively. However, the E. coli O157:H7 isolation rate from the feces of BL+E+BL group was not lower than those of BL+E and E groups. The cytopathic effect (CPE) of the Vero cytotoxin (Shiga like toxin I and II) in Vero cell was neutralized in B. longum HY8001 culture supernatant added wells which indicate the presence of soluble Vero cytotoxin neutralizing substance(s) in B. l…","author":[{"dropping-particle":"","family":"Yang","given":"S. J.","non-dropping-particle":"","parse-names":false,"suffix":""},{"dropping-particle":"","family":"Yoon","given":"J. W.","non-dropping-particle":"","parse-names":false,"suffix":""},{"dropping-particle":"","family":"Seo","given":"K. S.","non-dropping-particle":"","parse-names":false,"suffix":""},{"dropping-particle":"","family":"Koo","given":"H. C.","non-dropping-particle":"","parse-names":false,"suffix":""},{"dropping-particle":"","family":"Kim","given":"S. H.","non-dropping-particle":"","parse-names":false,"suffix":""},{"dropping-particle":"","family":"Bae","given":"H. S.","non-dropping-particle":"","parse-names":false,"suffix":""},{"dropping-particle":"","family":"Baek","given":"J.","non-dropping-particle":"","parse-names":false,"suffix":""},{"dropping-particle":"","family":"Park","given":"Y. H.","non-dropping-particle":"","parse-names":false,"suffix":""}],"container-title":"Korean Journal of Applied Microbiology

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Conclusions

In a conclusion, the results obtained in this study suggest thatVT1 possesses a dose-dependent cytotoxic impact on the normal colonic cell line of humans. The results also showed that *L. casei* and *L. fermentum* cell-free supernatants have a protective impact against the cytotoxicity of VT1 which could be exploited in various applications such as food detoxification.

Acknowledgments: The authors thank Dr. QasimHadi Kareem from Al-Farabi University College/ Computer Engineering Department for his great support.

Conflict of interest

The authors declare that they have no competing interests.

Funding

This research received no external funding.

Consent for publication

Not applicable

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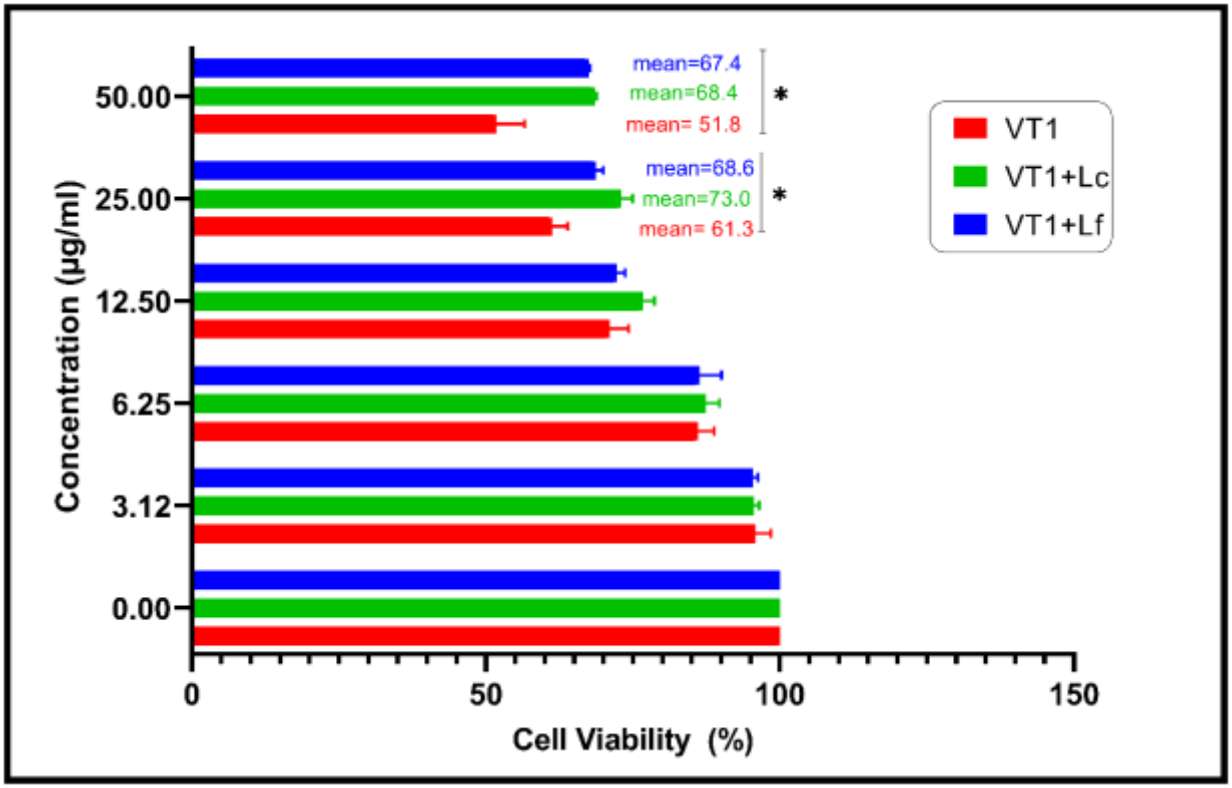
Figure1. Cell viability of NCM425 after treatment with verotoxin 1 (VT1) alone, combined with *Lactobacilluscasei* cell-free supernatant, and combined with *Lactobacillusfermentum* cell-free supernatant: The supernatants of *L. casei* (Lc) (green) and *L. fermentum* (Lf) (blue) suppressed cytotoxicity of VT1 (red) at concentrations 25 and 50 µg/ml; results are presented as mean ± standard deviations.

Figure 2. The half-maximal inhibitory concentration (IC_{50}) of Verotoxin 1 (VT1) alone (red), in combination with *Lactobacilluscasei* cell-free supernatant (Lc) (green), and *Lactobacillusfermentum* cell-free supernatant (Lf) (blue) against NCM425 cell line: rising in IC_{50} value of VT1 after combining with supernatants of *L. casei* and *L. fermentum* indicating a neutralizing effect against VT1 toxicity.

Figure 3. Comparison between *Lactobacilluscasei* (in green) and *Lactobacillusfermentum* (in blue) cell-free supernatants reduction ability against verotoxin 1 (VT1) toxicity toward NCM425 cell line; results are presented as mean ± standard deviations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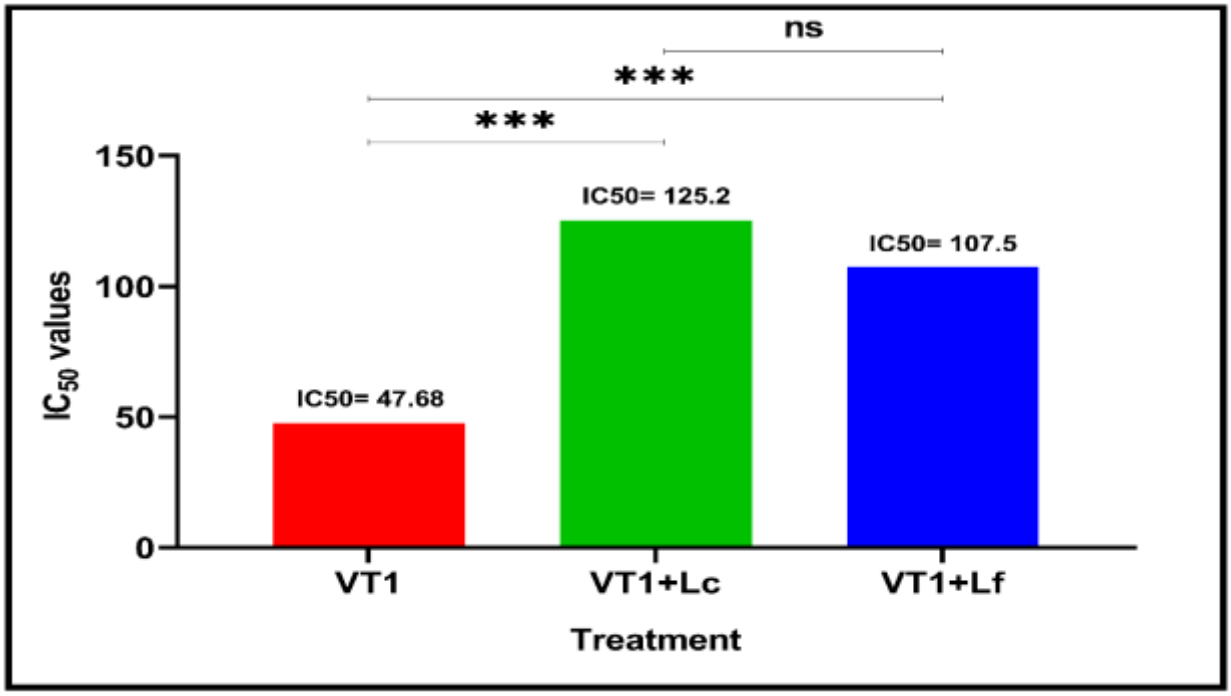
mean=68.4
mean= 51.8
*
mean=68.6
mean=73.0
mean= 61.3
*
VT1
VT1+Lc
VT1+Lf
Concentration (µg/ml)
50.00
25.00
12.50
6.25
3.12
0.00
0
50
100
150
Cell Viability (%)

9340

Fig **1**


ns
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***
150
100
50
0
IC50= 125.2
IC50= 107.5
IC50= 47.68
IC50 values
VT1
VT1+Lc
VT1+Lf
Treatment

Fig 2

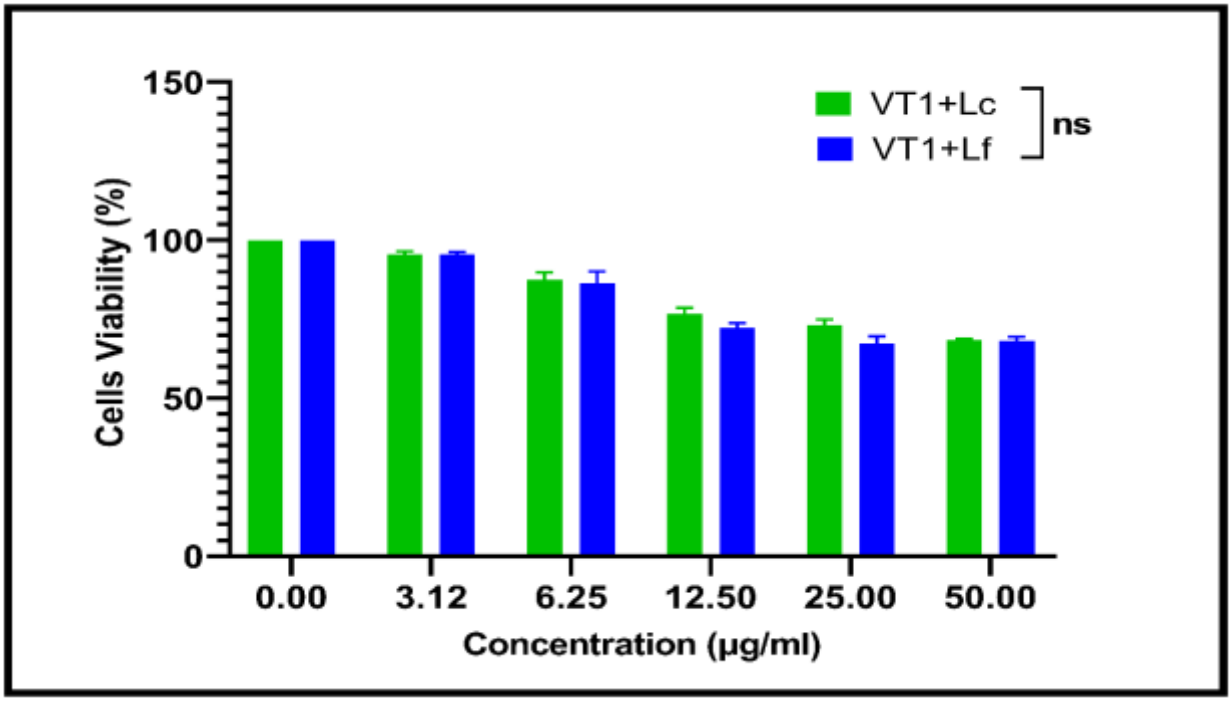
150
100
50
0
Cells Viability (%)
VT1+Lc
VT1+Lf
ns
0.00
3.12
6.25
12.50
25.00
50.00
Concentration (µg/ml)

Fig 3