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Bacteriocins of Four *Lactobacilli* Species Isolated from Yogurt can Inhibit Growth and Verotoxins Production in *E. coli* O157: H7

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Lactic Acid bacteria **are associated with rich habitats in nutrients, such as various food products and plant materials; they can produce compounds such as organic acids, hydrogen peroxide, diacetyl and bacteriocin or bactericidal proteins. The aim of present study was to evaluate the inhibitory effect of yogurt *lactobacilli* bacteriocins on the growth and verotoxins production of *E.coli* O157: H7. four *Lactobacilli* species (*Lactobacillus acidophilus, Lactobacillus casei, Lactobacillus bulgaricus* and *Lactobacillus helveticus*) were isolated from local yogurts and their bacteriocins investigated by agar-well-diffusion,SDS page, Minimum Inhibitory Dilution (MID), Vero cell cytotoxicity and VTEC-RPLA assays. All the species had antibacterial activity against *E.coli* O157: H7and were effective on the inhibition of verotoxins production. Verotoxin production inhibited by *L.casei* and *L. acidophilus* in 1.32 and 1.64 dilutions, *L.bulgaricus* in 1.16, 1.32 and 1.64 and *L. helveticus* in 1.8, 1.16 and 1.32. Culture supernatant of *L.acidophilus, L.casei* and *L.bulgaricus* were resistant to heating at 56°C, 80°C and 100°C for 10, 30 and 60 min, but the bacteriocin produced by *L.helveticus* was inactivated in 100°C for 60 minutes. The former culture supernatants were stable between pH 3 and 10 but the latter bacteriocin (*L.helveticus*) was detected to be sensitive to pH 10. All the lactobacilli culture supernatants maintained full stability after storage for 30 days at –20°C and partial stability in 60 days at 4°C and no activity in 60 to 120 days at 37°C. All four lactobacilli produced a bacteriocin-like protein (10.4 KDa). Findings of this study shows that these bacteriocin-like substances might be useful as a starter culture and natural preservative. The inhibition of the bacterial growth or production of enterotoxins such as verotoxins by administrating *lactobacilli* bacteriocins would be of great importance.**

Key words: Enterohemorrhagic *Escherichia coli* O157:H7, *Lactobacillus*, Yogurt, minimum inhibitory dilution (MID).

Lactic Acid bacteria (LAB) are gram positive and have different morphologically from long, slender rods to short coccobacilli, which frequently form chains. Their metabolism is related to fermentation[1]. *LAB* are associated with rich habitats in nutrients, such as various food products and plant materials. They can be found in soil, water, manure, sewage, and silage and can ferment or rot the food. Particular *LAB* are inhabitants of the human oral cavity, the intestinal tract, the vagina, and have a beneficial influence on these human ecosystems. This inhabitant could be according to the production of inhibitory compounds such as organic acids, hydrogen

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peroxide, diacetyl and bacteriocin or bactericidal proteins during lactic fermentations[2, 3].

Many pathogenic *E. coli* are host-adapted and only a limited number of strains infecting animals are able to cause disease in humans. A typical example of zoonotic *E. coli* is the O157:H7 serotype that can be responsible for two severe syndromes in humans, haemorrhagic colitis (HC) and haemolytic uremic syndrome (HUS), and whose reservoir is the intestinal tract of healthy cattle and other ruminants. The organism is the most common serotype identified in the group of enteric pathogens variously referred to as enterohemorrhagic *E. coli*, verotoxin-producing *E. coli*, and Shiga-like toxin producing *E. coli*. The hallmark of these "enterohaemorrhagic *E. coli*" ("EHEC") strains is the production of cytotoxins called Vero(cyto)toxins (Vtx) or Shiga toxins (Stx), two synonyms for the same group of toxins referring either to their toxicity for Vero cells or to their homology with the Shiga toxin produced by *Shigella dysenteriae* type 1[4]. Anyway, certain subsets of this bacterial species have acquired genes that enable them to cause intestinal or extra intestinal disease[5]. The mechanism of infection remains unclear; so, preventive and therapeutic measures have not been established. These strains, however, are more likely to get the attention of the scientific community and of the news media.

Current therapy is limited to supportive treatment alone, because the use of antibiotics appears to increase the risk of systemic complications, such as acute renal failure occurring in hemolytic uremic syndrome, perhaps by promoting the release of toxin from the periplasm[6, 7].

In recent years Bacteriocins produced by LAB have received considerable attention for their possible use as preservatives in food, which results in reduction in the use of chemical preservatives. The aim of this study was to evaluate the inhibitory effect of yogurt lactobacilli bacteriocins on the growth and verotoxins production of *E.coli* O157: H7.

MATERIALS AND METHODS

Bacterial strains, media and growth conditions

Escherichia coli O157:H7 (EHEC) was obtained from the Reference Laboratory of Iran (Tehran, Iran). This study has been performed in Department of Microbiology, School of Medical Sciences in Tabriz University (Iran, Tabriz).

Four lactic acid bacteria species (*L. casei, L.acidophilus, L.bulgaricus and L. helveticus*) were isolated from commercial yogurts. The *Lactobacilli* species were identified on the basis of growth, cell morphology, gram staining, and catalase activity. Further identification of the species of these Lactobacilli was performed according to carbohydrate fermentation patterns and growth at 15^0 C and 45^0 C in the Man Rogosa Sharpe (MRS) broth as described in Bargey's book[8]. Species were stored at -80 ^{0} C in MRS broth with 15% glycerol.

Preparations of crude bacteriocin

The isolated strains were grown anaerobically in 1000 m L MRS broth for 24 h at 37 °C (Oxide Gas Generating Kit). For extraction of the bacteriocins, a cell-free solution was obtained by centrifuging (1500 rpm for 10 min. at 4 °C with Beckman L5050B) the culture and adjusted to PH 7.0 by the addition of 1NNaOH to exclude the antimicrobial effect of organic acids[7, 9, 10].

Inhibitory effect of bacteriocins on *Escherichia coli* O157: H7 by the agar-well-diffusion

As we have done in our previous study**,** bacteriocin activity was performed by the agar-well-diffusion assay. The supernatant of Lactobacilli species was filter sterilized by passage through 0.45 m pore size membrane filter. A liquots of the sterile supernatant were placed in five millimeter diameter wells that had been made on the Muller-Hinton agar media, which preinoculated with *Escherichia coli* O157: H7. Finally, the inhibition zones around the wells were measured and recorded[11].

Sensitivity of the bacteriocins to heat and different PH values

100µL of the culture supernatant was heated for 10 min at 56, 70 and 80° C. The resistant culture supernatants were further heated for 30 and 60 min at 100°C[12, 13].

The PH of culture supernatants was adjusted to 3.0, 4.5, 7.0 and 10 with hydrochloride acid (HCL) and sodium hydroxide (NaOH) and then they were incubated for 4 h at the room temperature. Residual activity was determined by the agar well diffusion method as described[10].

Sensitivity of the bacteriocins during storage

The culture supernatants were stored at -20 (60 days), 4 (120 days) and 37°C (60-120 days). Samples were taken out from the stored condition to determine the bacteriocins activity by agar well diffusion method[7].

SDS-PAGE analysis

Sodium dodecyl sulfate– polyacrylamide gel (SDS-PAGE) describes a collection of related techniques widely used in biochemistry, forensics, genetics and molecular biology to separate proteins according to their electrophoretic mobility. We use an AE-6200 electrophoresis unit (ATTO, Tokyo) to determine the size and number of structural proteins of the phage particles. Purified samples were boiled at 100°C for 10 min in SDS-PAGE buffer (125mM Tris-HCl [PH6.8], 10% 2-mercaptoethanol, 4% SDS, 10% glycerol, 0.004% bromophenol blue) to ensure denaturation before loading on the gel. Each well was loaded with 20 l of sample containing approximately 10 g of protein. After electrophoresis, proteins were stained with coomassie Ble 250-R, followed by destaining with 7.5% glacial acetic acid and 5% methanol, and then photographed[14, 15].

Determination of Minimum Inhibitory Dilutions

According to our last study, bacteriocins that showed antimicrobial activity were later tested to determine the Minimal Inhibitory Dilution (MID). The lowest concentration in the tube showing visual inhibition of growth was the Minimum Inhibitory Dilution[13] was also evaluated.

Effects of sub-Minimum Inhibitory Dilutions on the production of VT by *E. coli* O157:H7 using Vero cell cytotoxicity

Vero cells (National Cell Bank, Pasteur Institute of Iran) were grown in RPMI 1640 medium (Biosera), pH 7.4, supplemented with 5% heat-inactivated fetal bovine serum, (FBS) 1% (w/v) penicillin, and 1% (w/v) streptomycin at 37 °C in a 5% CO_2 atmosphere. Vero cells with a concentration of 15×10^4 cell/mL were plated on 96-well cluster plates and incubated to grow in complete RPMI 1640 medium. After 24 h, sub-minimum inhibition dilutions of bacteriocins were added to the prepared medium, and cells were incubated for an additional 16 h. Then, the viability of cells was assessed using inverted microscope[16].

Effects of sub-minimum inhibitory dilutions on the production of VT by *E. coli* O157:H7 using VTEC-RPLA assay

A commercial reverse passive latex agglutination (RPLA) assay was used to evaluate the production of VT by *E.coli* O157: H7. One milliliter of 10^6 organisms mL^{-1} of EHEC was inoculated in tubes containing 1 mL TSB, supplemented with sub-minimum inhibition dilutions of bacteriocins. After 24 h incubation, the supernatants were filtered through 0.22 m pore size membrane filters (Millipore Corp., Bedford, Mass.). The VTEC-RPLA assay (Denka Seiken Co., Ltd., Tokyo, Japan) was performed according to the manufacturer's instructions. By using 96 well V-bottom microtiter Plates (Gamedium, Ricany, Czech Republic), serial dilutions of culture filtrates were mixed with rabbit polyclonal anti-VT1 or anti-VT2 immunoglobulin G antibody. The plates were then covered, incubated at room temperature and examined for latex agglutination after 20 to 24 h. The positive and negative controls included in the kit (purified VT1 and VT2 and latex particles sensitized with normal rabbit immunoglobulin G, respectively) were also run with the assay[14].

Statistical analysis

The data were analyzed using General Linear Model (GLM). Student's t-test was used to compare means .The results were presented as the means with SEM (standard error of the mean). Significance level for the comparison of the group means was set at $P<0.05$. Statistical analyses were carried out using the statistical program of SPSS for Windows (version, 10.0).

RESULTS

Four *Lactobacilli* species (*L. acidophilus, L. bulgaricus, L. helveticus and L. casei*) were isolated from commercial yogurts. The culture supernatants, obtained from four lactobacilli isolates, were tested for antibacterial activity against *Escherichia coli* O157: H7 (Table 1). The bacteriocins that showed antimicrobial activity were later tested to determine minimum inhibitory dilution using serial dilution method. It was reported that bacteriocins play a significant role in the growth inhibition of *Escherichia coli* O157: H7 and the least effective bacteriocin was related to *L.bulgaricus and L.helveticus*. They also prevented the production of verotoxins (VT1 and VT2) at dilutions lower than the Minimum Inhibitory

Dilution. Vero cell cytotoxicity and VTEC-RPLA assay were used to evaluate the production of VT by *E.coli* O157: H7 after exposure to dilutions lower than Minimum Inhibitory Dilutions (Fig. 1, 2). Verotoxin production inhibited by L.casei in 1.32 and 1.64 dilutions, *L.bulgaricus, L. acidophilus* in 1.16, 1.32 and 1.64. Also results showed that verotoxin production inhibited regarding to *L. helveticus* in 1.8, 1.16 and 1.32. Results were the same in Vero cell cytotoxicity and VTEC-RPLA assay (Table 2, 3).

Table 1. Inhibitory effect of *Lactobacilli* species on growth of *E.coli* O157:H7.Diameter of inhibition: +, weak (5-12mm); ++, intermediate (13-20mm); +++, strong (20-30mm)

E.coli O157:H7	Lactobacilli Species
++	*L. acidophilus*
++	*L. bulgaricus*
+	*L. helveticus*
+++	*L. casei*

Bacteriocins of *L. acidophilus, L.bulgaricus and L. casei* were resistant to heating at 56, 80 and 100°C for 10, 30 and 60 min, but the bacteriocin produced by *L. helveticus* was inactivated by heating at 100° C for 30 and 60 min. Three *Lactobacilli* bacteriocins were stable between pH 3 and 10 but the bacteriocin of *L. helveticus* was considered to be sensitive to PH 10 (Table 4). Maximum bacteriocin production was noted at 30°C, and pH 6.0.

The effect of time and temperature of storage on bacteriocin activity was also investigated. It was observed that all the bacteriocins, produced by the test isolates, maintained full stability after storage for 60 days at -20°C. *L. acidophilus, L.bulgaricus and L. casei* had partial stability after storage for 120 days at 4^0 C, while *L.helveticus* was sensitive. Also they had no activity after the storage for 60 to 120 days at 37°C (Table 5). According to sodium dodecyl sulfate– polyacrylamide gel (SDS-PAGE) all four *lactobacilli* produce protein with a molecular mass of approximately 10.4 KDa (Fig 3). The effects of media composition were also evaluated.

Table 2. Effect of sub-minimal inhibitory dilutions on production of VT by *E .coli O157:H7* using VTEC-RPLA assay

Sample Agglutination	Latex Control	*VT1* Control	*VT2* Control	*L. casei*		*L. acidophilus*			*L. bulgaricus*			*L. helveticus*			
				1/32	1/64	1/16	1/32	1/64	1/16	1/32	1/64	1/8	1/16	1/32	1/64
VT1	-	++++	-	-	++	-	-	++	-	-	++	-	+	+	++
VT2	-	-	++++	-	++	-	-	++	-	-	++	-	+	+	++

Table 3. Effect of sub-minimal inhibitory dilutions on production of VT by *E.coli* O157:H7 using Vero cells

Dilution	*L. casei*		*L. acidophilus*			*L. bulgaricus*			*L. helveticus*			
	1/32	1/64	1/16	1/32	1/64	1/16	1/32	1/64	1/8	1/16	1/32	1/64
CPE	-	++	-	-	++	-	-	++	-	+	+	++

Table 4. Sensitivity of bacteriocins to different pH values and heating

R: Resistant, S: Sensitive

Lactobacilli Strain	Resistence to heating(10min) Temperature(°C)			Resistence to boiling(min)		Sensitivity to different pH values			
	56	70	80	10	30	60	3	4.5	7	10
L.casei	R	R	R	R	R	R	R	R	R	R
L.acidophilus	R	R	R	R	R	R	R	R	R	R
L.bulgaricus	R	R	R	R	R	R	R	R	R	R
L.helveticus	R	R	R	R	S	S	R	R	S	S

DISCUSSION

The present investigation highlights the isolation, characterization and activity of bacteriocin produced by yogurt *lactobacilli*. It is rich in nutrient and organic matter. To state that, the isolate Lactobacilli strains tested for antibacterial activity against *Escherichia coli O157:H7.*

E. coli is a genetically heterogeneous group of bacteria whose members are typically nonpathogens that are a part of the normal microflora of the intestinal tract of humans and animals[16]. Most of the time humans become infected with Shiga toxin-producing *E.coli* (STEC) by ingestion of contaminated food or water or by direct contact with animals, resulting in sporadic cases of disease or outbreaks, involving up to several thousand individuals[17, 18]. *Lactic acid* bacteria are widely used as starter cultures and play an important role in food preservation, microbiological stability and production of aroma compounds. Many of these lactic acid bacteria produce bacteriocins. By definition, bacteriocins are small proteins with bactericidal or bacteriostatic activity against genetically closely related species[19].

Table 5. Effect of time and temperature on bacteriocin activity

R: Resistant, S: Sensitive, R/S: Moderately Sensitive

Lactobacilli Species	Storage at -20°C 60 days	Storage at 4°C 120days	Storage at 37°C 60-120 days
L.casei	R	R/S	S
L.acidophilus	R	R/S	S
L.bulgaricus	R	R/S	S
L.helveticus	R	S	S

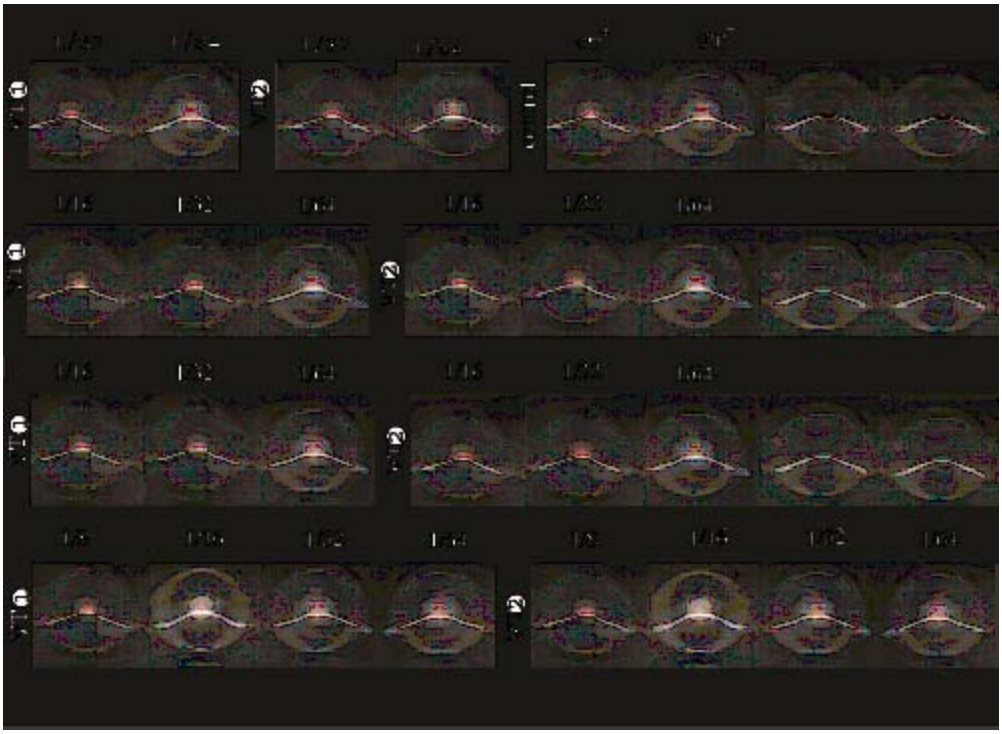

Fig. 1. The results of inhibitory effects of concentrations lower than the minimum inhibitory dilution of lactobacilli culture supernatants using VTEC-RPLA kit. Respectively, from top to bottom after exposure to *L. Casei, L. Acidophilus, L. Bulgaricus and L. helveticus*

Ota *et al.* [20] showed that yogurt induces more lactic acid bacteria to colonize in the intestine, thereby protects human from being infected by EHEC. Hirano *et al*.[21] have indicated that *lactobacillus rhamnosus* prevents the growth of Escherichia coli.

Coconnier *et al*.[22] have indicated that *Lactobacillus acidophilus* could kill intracellular *Salmonella thyphimurium* in the human intestinal Caco-2 cell culture model. Sattari *et al*.[23] have shown that Lactobacilli of dairy products could inhibit the growth of pathogenic *Salmonella*. Itoh *et al*.[24] have stated that Gassercin A which is produced by *L. gasseri* is one of the most active bacteriocins against enteric pathogens. All of the Lactobacilli sorts isolated from Turkish dairy products have antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* and *Yersinia entrocolitica*[25].

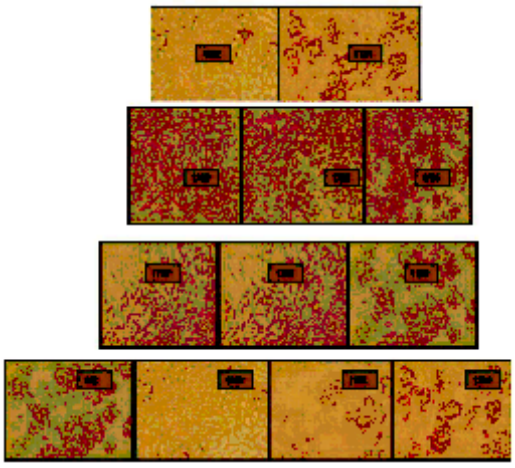

Fig. 2. The results of inhibitory effects of dilutions lower than the minimum inhibitory dilution of lactobacilli culture supernatants using Vero cells. Respectively, from top to bottom after exposure to *L. Casei, L. Acidophilus, L. Bulgaricus and L. helveticus*

Bacteriocin production was strongly dependent on pH, nutrients source and temperature as claimed by Todorov and Dicks[19]. Also in a study in Turkey it has been indicated that some vaginal Lactobacilli bacteriocins are stable between PH 4.5 and 7.0 but sensitive to PH 9.0[10].

In the current study, bacteriocins of *L. acidophilus, L.bulgaricus* and *L. casei* were stable between PH 3 and 10 and *L. helveticus* was found to be sensitive to PH 10. Results also revealed the antimicrobial activity of the bacteriocins, produced by the organisms, was not lost after adjustment of PH to 7.0. From the results proved that bacteriocins could be used in acidic food like yogurt. It might be secondary metabolites.

According to another study, bacteriocins produced by *L. plantarum F1* and *L. brevis OG1* maintained full stability after the storage for 60 days at -20^0 C and partial stability after the storage for 120 days at 4^0 C, while no activity was detected after the storage for 80 to 120 days at 37^0 C (7). In a study by Rajaram *et al*. it was deduced that various physicochemical factors affect bacteriocin production as well as its activity. Maximum bacteriocin production was observed at 30°C , pH 6.0 and 1.5% sodium chloride solution[26].

The discovery of VTs and the establishment of the assay for their detection has assisted in the subsequent linkage of these toxins to hemorrhagic colitis (HC) and hemolytic uremic

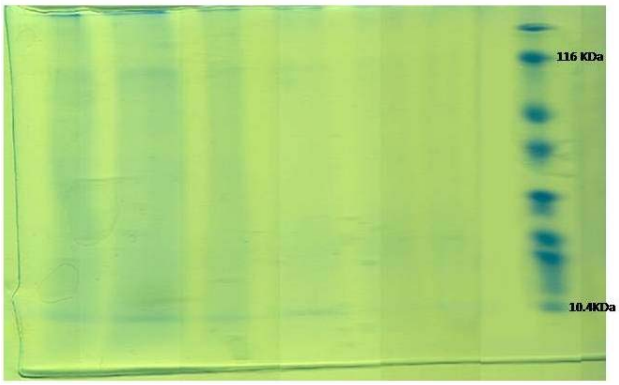

Fig. 3. SDS gel electrophoresis of bacteriocins from left to right respectively, *L. casei, L. acidophilus, L. bulgaricus, L. helveticus* and marker protein 10.4-116KD$_a$

syndrome (HUS)[26]. Especially important is serotype 0157:H7, responsible for numerous illness outbreaks. Therefore, the characterization of VTs and the elucidation of their role in the pathogenesis of these diseases have been the subjects of numerous studies that followed the discovery of VT[16]. In this study, we considered bacteriocins dilution less than MID for verotoxin production by Vero cell cytotoxicity and VTEC-RPLA assay. Verotoxin production inhibited by *L.casei* in 1.64, *L. acidophilus*, *L.bulgaricus* in 1.64. Also results showed verotoxin production inhibited regarding to *L. helveticus* in 1.16, 1.32 and1.64. From the results proved that they could be used as starter cultures for the traditional fermented foods, with a view to improving the hygiene and safety of the food products. SDS-PAGE of whole soluble cell proteins successfully differentiated between all *Lactobacillus* species. We precipitated the crude bacteriocins to verify the presence of inhibitory compounds in the supernatant of each protein *lactobacilli* with 80% ammonium sulphate and dialyzed sedimentation. Protein bands on the SDS-PAGE gel were observed in the culture supernatant. According to SDS-PAGE the molecular weight produced by all four *lactobacilli* was calculated to be about 10.4 KDa. These bacteriocin-like substances might be useful as a starter culture and natural preservative. The inhibition of the bacterial growth or production of enterotoxins such as verotoxins by administrating *lactobacilli* bacteriocins would be of great importance.

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