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Inhibitory Effect of Yogurt Lactobacilli Bacteriocins on Growth and Verotoxins Production of Enterohemorrhgic *Escherichia coli* O157:H7

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Abstract: The inhibitory effect of bacteriocins produced by yogurt Lactobacilli on the growth and verotoxins production of enterohemorrhagic *Escherichia coli* O157:H7 (EHEC) was investigated. Three Lactobacilli species (*Lactobacillus acidophilus*, *Lactobacillus bulgaricus* and *Lactobacillus helveticus*) were isolated from commercial yogurts. The antibacterial activity against *E. coli* O157: H7 was examined and the results showed that all the species were effective. They were also effective on the inhibition of verotoxins production. The inhibition against verotoxins was observed at the concentrations lower than the Minimum Inhibitory Dilution (MID) of each lactobacilli bacteriocin, indicating that these lactobacilli bacteriocins would preferentially prevent the production of verotoxins rather than have bactericidal effect on EHEC. We also found that bacteriocins of *L. acidophilus* and *L. bulgaricus* 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. Also the former bacteriocins were stable between pH 3 and 10 but the latter bacteriocin (*L. helveticus*) was considered to be sensitive to pH 10. It was observed that all the bacteriocins produced by the test isolates maintained full stability after storage for 30 days at -20°C and partial stability after storage for 60 days at 4°C while no activity was detected after storage for 60 to 120 days at 37°C.

Key words: *Lactobacillus*, bacteriocin, antibacterial activity, enterohemorrhagic *Escherichia coli* O157:H7

INTRODUCTION

Lactic acid bacteria are widely used as starter cultures and play an important role in food preservation and maintenance of the intestinal microbial ecosystem. They have been shown to possess inhibitory activity toward the growth of pathogenic bacteria such as *Listeria monocytogenes* (Ashenafi, 1999; Jacobsen *et al*., 1999), *enterohemorrhagic Escherichia coli* (EHEC) and *Salmonella* sp. (Drago *et al*., 1997). This inhibition could be due to the production of inhibitory compounds such as organic acids, hydrogen peroxide, diacetyl and bacteriocin or bactericidal proteins during lactic fermentations (Holzapfel *et al*., 2001; Hirano *et al*., 2003). Research on bacteriocins of *Lactobacilli* has been expanded during the last decades, including the use of bacteriocins or producer organisms as natural food preservatives (Ogunbanwo *et al*., 2003a, b). By definition, the term "bacteriocin" refers to the proteins of colicin type, characterized by lethal biosynthesis, intraspecific activity and adsorption to specific receptors. Those bacteriocins produced by gram-positive bacteria fit closely to the classical colicin model. *Lactobacillus* bacteriocins are found within each of the four major classes of antimicrobial proteins produced by lactic acid bacteria. Class I bacteriocins (antibiotics) were discovered in the *lactobacillaceae* by Mortvedt *et al*. (1991). These bacteriocins are small membrane-active peptides (<5 KD_a) containing an unusual amino acid, lanthionine. The class II bacteriocins are small heat-stable, non-lanthionine containing and membrane-active peptides (<10 KD_a). The class III bacteriocins, have been found in *Lactobacillus*, include heat labile proteins of large molecular mass. The class IV bacteriocins are a group of complex proteins, associated with other lipid or carbohydrate moieties, which appear to be required for activity. They are relatively hydrophobic and heat stable (Alpay *et al*., 2003).

Some bacteriocins produced by *Lactobacilli* have a broad spectrum of activity against the pathogenic bacteria. However, reports on the effect of *Lactobacilli* bacteriocins on growth and verotoxins production of *E. coli* O157:H7 have not yet appeared in the medical press.

Although *Escherichia coli* is a member of the normal flora of the human and animal gastrointestinal tract, several pathogenic types of *E. coli* can lead to diseases. *E. coli* O157: H7 and other Shiga toxin-producing *E. coli*

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(STEC) strains have emerged in recent years as important human pathogens associated with a spectrum of diseases ranging from diarrhea to hemorrhagic colitis and hemolytic-uremic syndrome (HUS). Due to the morbidities and mortalities associated with outbreaks and sporadic cases of STEC diseases, these pathogens are now considered as major public health problems of worldwide importance. *E. coli* O157: H7 is a serotype most frequently isolated from patients and shares a variety of virulence factors, including two Shiga toxins, Stx 1 and Stx 2 and a pathogenicity island, termed as the locus for enterocyte effacement, that encodes the proteins responsible for the intimate adherence of *E. coli* to epithelial cells .The production of Shiga toxins by *E. coli* O157: H7 has a major role in pathogenesis, particularly in the pathogenesis of HUS (Sakagami *et al*., 2001).

The purpose of this study was to investigate the inhibitory effect of yogurt *Lactobacilli* bacteriocins on the growth and verotoxins production of *E. coli* O157: H7. Furthermore we sought to characterize bacteriocins for their structural properties.

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 as a M.Sc. thesis in Department of Microbiology, School of Medical Sciences in Tarbiat Modarres University (Iran, Tehran) in 2005.

Three lactic acid bacteria species (*L. acidophilus*, *L. bulgaricus* and *L. helveticus*) were isolated from commercial yogurts (Ogunbanwo *et al*., 2003b). 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 and 45°C in the de Man Rogosa Sharpe (MRS) broth as described in Bargey's book (Kandler and Weiss, 1986). Species were stored at -80°C in MRS broth with 15% glycerol.

Preparations of culture supernatants: *Lactobacilli* species were propagated anaerobically in 1000 m L MRS broth for 72 h at 30°C (Oxoid Gas Generating Kit). For extraction of the bacteriocins, a cell-free solution was obtained by centrifuging (10,000 rpm for 20 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. Inhibitory activity of hydrogen peroxide was eliminated by the addition of 5 mg mL^{-1} catalase (Boris *et al*., 2001; Alpay *et al*., 2003; Ogunbanwo *et al*., 2003b).

Inhibitory effect of bacteriocins on *Escherichia coli* O157: H7: Bacteriocin activity was performed by the agar-well-diffusion assay. Five millimeter diameter wells were made on the agar media, which preinoculated with *Escherichia coli* O157: H7. Each well was filled with 50 μL of the culture supernatant of *Lactobacilli* species. Adjustment of the cell free supernatants to pH 6.5-7.0 with 1NNaOH prevented the inhibitory effect of organic acids. Then the inhibition zones around the wells were measured and recorded (Mortvedt *et al*., 1991).

Determination of minimum inhibitory dilutions: 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 (Finegold and Barron, 1990).

Effects of sub-minimum inhibitory dilutions on the production of VT by *E. coil* O157:H7: A commercial reverse passive latex agglutination (RPLA) assay was used to evaluate the production of VT by *E. coil* 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 μm 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 equal volumes (25 μL) of latex particles sensitized 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 (Sakagami *et al*., 2001).

Sensitivity of the bacteriocins to heat: To test heat sensitivity, 100 μL of the culture supernatant was heated for 10 min at 56, 80 and 100°C. The agar-well-diffusion was performed to detect residual activity (Kelly *et al*., 1996). The resistant culture supernatants were further heated for 10, 30 and 60 min at 100°C.

Stability of the bacteriocins during storage: The culture supernatants were stored at -20, 4 and 37°C. At different time intervals, samples were taken out from the stored condition to determine the bacteriocins' activity by agar well diffusion method (Ogunbanwo *et al*., 2003b).

Sensitivity of the bacteriocins to different pH values: The pH of culture supernatants was adjusted to 3.0, 4.0, 7.0 and 10 with hydrochloric 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 (Alpay *et al*., 2003).

The effect of growth media on the bacteriocin production: Two commercial media, Rogosa SL and MRS were tested for their ability to support the production of bacteriocin. Both broth media were inoculated with bacteriocin producing *Lactobacilli* and incubated anaerobically at 30°C for 24 h. The bacteriocin activity of the culture supernatants was detected by the agar well diffusion assay (Ogunbanwo *et al*., 2003a).

RESULTS

Three *Lactobacilli* species (*L. acidophilus, L. bulgaricus* and *L. helveticus*) were isolated from commercial yogurts. The culture supernatants, obtained from three *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 (Table 2). The results showed that these bacteriocins play a significant role in the growth inhibition of *Escherichia coli* O157: H7. They also prevented the production of verotoxins (VT1 and VT2) at concentrations lower than the minimum inhibitory dilution (Table 3). Bacteriocins of *L. acidophilus* and *L. bulgaricus* 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. The former two bacteriocins were stable between pH 3 and 10 but the bacteriocin of *L. helveticus* was considered to be sensitive to pH 10 (Table 4).

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, partial stability after storage for 120 days at 4°C, while they revealed no activity after the storage for 60 to 120 days at 37°C (Table 5). The effects of media composition were also evaluated. We found no significant difference between the MRS and Rogosa SL media in terms of bacteriocin production.

Table 1: Inhibitory effect of three lactobacilli bacteriocins on growth of *E. coli* O157: H7

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

Diameter of the inhibition zone: ++, intermediate (13-20 mm); +++, strong (20-30 mm)

Table 2: Minimal inhibitory dilutions of lactobacilli bacteriocin

Lactobacilli species	Minimum inhibitory dilution
L. acidophilus	1/16
L. bulgaricus	1/16
L. helveticus	1/ 8

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

				L.a		L.b		L.h	
Sample	Latex	VT1	VT2						
Agglutination	control	control	control	1/32	1/64	1/32	1/64	1/16	1/32
VT_1	-	++++*	-**	-	-	-	-	-	+***
VT_2	-	-	++++	-	-	-	-	-	+

* Strong agglutination, ** Negative, *** Weak agglutination

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

	Resistance to heating (10 min) Temperature (°C)			Resistance to boiling (min)			Sensitivity to different pH values			
Lactobacilli strains	50	70	80	10	30	60	3.0	4.5	7.0	10
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	R	S

*Resistant, **Sensitive

Table 5: Effect of time and temperature of storage on bacteriocin activity

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

* Resistant, ** Moderately sensitive, *** Sensitive

DISCUSSION

It is generally believed that lactobacilli are protective in case of pathogenic infections and they can inhibit food-born and enteric pathogenic microorganisms (Pant *et al*., 1996; Heller, 2001). Earlier reports have shown that some bacteriocins produced by lactobacilli have a broad spectrum of activity. However, it has been generally observed that bacteriocin from the producer organism had no inhibitory effect on the organism produce it (Ogunbanwo *et al*., 2003b).

Ota (1999) have indicated that yogurt induces more lactic acid bacteria to colonize in the intestine, thereby protects human from being infected by EHEC.

Coconnier *et al*. (1997) have shown that *Lactobacillus acidophilus* was able to kill intracellular *Salmonella thyphimurium* in the human intestinal Caco-2 cell culture model. Sattari *et al*. (1999) have indicated that *Lactobacilli* of dairy products had inhibitory effect on growth of pathogenic *Salmonella*.

Itoh *et al*. (1995) have reported that Gassercin A, produced by *L. gasseri*, is one of the most active bacteriocins against enteric pathogens. All *Lactobacilli* species isolated from Turkish dairy products have

antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli* and *Yersinia entrocolitica* (Aslim *et al*., 2005). In the present study, bacteriocins produced by yogurt *Lactobacilli* were observed to have inhibitory effect on growth of *Escherichia coli* O157: H7 and it was deduced that the dilutions lower than minimum inhibitory dilution of each bacteriocin would inhibit the production process of verotoxins. The exact mechanism of the verotoxins production has been unresolved so far. It seems that *Lactobacilli* bacteriocins act directly or indirectly and interfere the transcriptional and/or translation steps, so reduce the toxins production. The findings described here suggest that administration of the *Lactobacilli* bacteriocins will prevent the production of verotoxin by EHEC in human intestines (Sakagami *et al*., 2001).

In recent years it has been indicated that some vaginal *Lactobacilli* bacteriocins are stable between pH 4.5 and 7.0 but sensitive to pH 9.0 (Alpay *et al*., 2003). In our study, bacteriocins of *L. acidophilus, L. bulgaricus* were stable between pH 3 and 10 and *L. helveticus* was found to be sensitive to pH 10. Antimicrobial activity of the bacteriocins, produced by the organisms in this study, was not lost after adjustment of pH to 7.0. According to a study, bacteriocins produced by *L. plantarum F1* and *L. brevis OG1* maintained full stability after the storage for 60 days at -20°C and partial stability after the storage for 120 days at 4°C, while no activity was detected after the storage for 80 to 120 days at 37°C (Ogunbanwo *et al*., 2003b).

In this study inhibitory compounds produced by the test isolates were found to be heat stable which is important if they are to be used as a food preservative, because many procedures of food preparation involve a heating step.

In a recent study it has been reported that *Lactobacillus plantarum ST194BZ* produces low bacteriocin activity when grown in BHI and M_{17} broth, but higher levels bacteriocin production were recorded in MRS broth (Todorov and Dicks, 2005). In the present study, we found no significant difference between Rogosa SL and MRS media in terms of the bacteriocins production. Therefore, it is concluded that the antimicrobial characteristics of *L. acidophilus*, *L. bulgaricus* and *L. helveticus* can have positive impact on their use as starter cultures for the traditional fermented foods, with a view to improving the hygiene and safety of the food products. In summer season, in which may increase the number of food-poisoning patients, 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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