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Safety aspects of *Lactobacillus plantarum* strains isolated from Siahmazgi cheese

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Abstract: In order to use lactic acid bacteria as starter and non-starter cultures in fermentative products, their safety qualities should be evaluated. The objective of the present study was to evaluate safety characteristics including antibiotic sensitivity pattern, antimicrobial effect, H_2O_2 production, and biogenic amine production by *Lactobacillus plantarum* strains isolated from Siahmazgi cheese. *Lactobacillus plantarum* strains were unable to produce tyramine except for SD6 strain. All strains in the current study were able to produce histamine but unable to decarboxylate neither lysine nor ornithine. *Lactobacillus plantarum* strains showed considerable antimicrobial activity against *Salmonella typhimurium* and *Listeria monocytogenes*. All *L. plantarum* strains showed stronger antimicrobial activity against *S. typhimurium* (3.5-42.55 mm) than *L. monocytogenes* (1.5-30.49 mm). The SC9 strain had the strongest inhibitory effect against both pathogens. After titering pH of the medium to approximately 6.5, no antimicrobial activity was noticed indicating that the antimicrobial activity of *L. plantarum* strains was contributed to their acid production and not to bacteriocin. All *L. plantarum* strains were capable of producing H_2O_2. SA32 and SD13 strains with 2.37 and 0.77 mmol/L were the strongest and the weakest strains regarding H_2O_2 production, respectively ($P< 0.05$). All *L. plantarum* strains were sensitive to chloramphenicol, erythromycin, rifampicin, and tetracycline, yet resistant against vancomycin, and norfloxacin. Four patterns of antibiotic resistance were observed among *L. plantarum* strains. Only two strains of SC9 and SE4 were resistant against four antibiotics. *L. plantarum* strains naturally found in Siahmazgi cheese do not generally possess dangerous characteristics to be used in fermentative dairy products.

Keywords: Artisanal cheese, food safety, lactic acid bacteria, *L. plantarum*, Siahmazgi cheese.

Introduction

With respect to metabolic characteristics of lactic acid bacteria (LAB), they are used to improve the taste, texture, nutritional value, and safety of fermentative foods (38). Production of traditional fermentative products is dependent on spontaneous fermentation by wild LAB. Manufacturing such products in industrial scale and under hygienic conditions necessitates formulating a new set of specifically chosen strains as a starter culture which can guarantee the quality, safety, and consistency of the product (32).

In order to use starter and non-starter cultures in fermentative products, their safety qualities should be evaluated. Indiscriminate use of antibiotics is leading to resistance, mutations in the genes of microorganisms or resistance gene transformation from other bacteria (5). Antibiotic resistance is a serious concern today due to the risk of gene transformation from LAB to human pathogenic bacteria. Numerous studies have been conducted on antibiotic sensitivity/resistance of pathogenic bacteria so far (12, 16, 26, 33). Cataloluk and Gogebakan (5) showed that manually produced cheese contained the highest number of antibiotic resistant lactobacilli.

Many researchers have proven that the LAB will lengthen the shelf life and improve the safety of the product by restriction the growth of unfavorable microorganisms (3, 29, 38). Some LAB produces metabolites (organic acids, H_2O_2, and bacteriocins) which act against bacteria including *Escherichia coli*, *Staphylococcus aureus*, and *Salmonella* species (14). LAB with ability to produce H_2O_2 restrict pathogens' growth in food and also can act as probiotic strains with beneficial health effects for consumers. Researchers have found a relationship between the presence of H_2O_2 producing Lactobacilli and decreased bacterial vaginosis, healing colitis and human immunodeficiency virus prevention (14, 36, 40).

Decarboxylation of amino acids histidine, tyrosine, ornithine, and lysine will lead to biogenic amine production including histamine, tyramine, putrescine, and cadaverine. Production of excessive amounts of such substances will stimulate secretion of adrenalin and noradrenalin, stimulate gastric acid secretion, cause

tachycardia and hypertension (10). In addition to all these, some people have histamine intolerance which causes diarrhea, headache, dizziness, cough, respiratory distress, hypotension, arrhythmia and heart rhythm disorders (17, 19, 21). The ability of biogenic amine production by LAB need to be evaluated before they are used in foods so as to make it possible to evaluate freshness or spoilage of the product by measuring the concentration of these substances (19). Biogenic amine producing a capacity of *Lactobacillus* species is not considered as a legal criterion for the selection of species used as a starter or probiotic culture (7).

Siahmazgi cheese is an artisanal cheese produced from raw sheep and goat milk without addition of starter culture in the spring. The cheese ripens for a six month period in bags made of sheepskin called Khik and ripening is carried out by wild LAB normally found in raw milk, Khik or those naturally added to the product during handling in the production process. Partovi et al. (30) studied the microbial and chemical properties of Siahmazgi cheese for the first time and identified the majority of the LAB found using biochemical methods and 16s rDNA analysis. *Lactobacillus plantarum* was the major strain isolated from Siahmazgi cheese with 41.6 % occurrence among the total LAB (34). Technological properties of *L. plantarum* strains isolated from Siahmazgi cheese have also been evaluated (31).

The objective of the present study is to evaluate safety characteristics including antibiotic sensitivity/ resistance pattern, antimicrobial effect, H_2O_2 production, and biogenic amine production by *L. plantarum* strains isolated from Siahmazgi cheese.

Material and Methods

***Strains selection and preparation*:** Ten strains of *L. plantarum* were selected from the strains isolated from Siahmazgi cheese and were identified by means of biochemical tests and 16s rDNA analysis. The strains were lyophilized and stored in the Food Hygiene department of the Faculty of Veterinary Medicine of the University of Tehran (30).

***Biogenic amine production*:** Moeller Decarboxylase broth base (Merck, Darmstadt, Germany) was used to evaluate biogenic amine production by *L. plantarum* strains. An amount of 1 % amino acid (histidine, lysine, tyrosine and ornithine) was added to the medium and sterilized at 110 °C for 10 minutes. Bacterial culture (in De Man, Rogosa and Sharpe (MRS) broth (Merck, Darmstadt, Germany) at 30 °C for 48h) was used in order to inoculate the medium and the surface of the medium was covered with sterile liquid paraffin. Test tubes were then incubated at 30 °C for 1-4 days and evaluated daily. A control test tube was dedicated to each *L. plantaru*m strain containing every item except the amino acid (20).

***Antimicrobial activity*:** The antimicrobial activity of *L. plantarum* strains isolated from Siahmazgi cheese was evaluated using well diffusion method. Two reference strains (*Listeria monocytogenes* ATCC 7644 and *Salmonella enterica* subsp. *enterica* serotype Typhimurium (*S. typhimurium* hereafter) ATCC 14028) were used to check sensitivity to the antimicrobial substances produced by *L. plantarum* strains. Indicator strains were grown in Brain Heart Infusion (BHI) broth (Merck, Darmstadt, Germany) at 37 °C for 24h. These indicator strain cultures were added to sterile MRS agar (100 µl/L) and poured into petri-dishes. Then wells were made with a sterile pipette. *L. plantarum* strains were initially grown in MRS broth at 30 °C for 24h and they were adjusted to OD_{600} = 0.5 and then 50 µl of the cultures were added to the wells. The plates were held at 4 °C for 2h and incubated at 30 °C for 24h and were subsequently examined for zones of inhibition. Then the diameter of inhibition zones (mm) around the agar wells were measured. *Lactobacillus plantarum* strains were cultured in MRS broth at 30 °C for 24h and then centrifuged at 12000 g for 15 minutes to remove cells. The supernatant fluid was adjusted to pH=6.5 with sterilized 1 N NaOH and antimicrobial activity was checked by a well diffusion assay after excluding inhibition due to organic acids (8).

***H_2O_2 production*:** In order to evaluate H_2O_2 production (mmol/L) by *L. plantarum* strains, 25 ml of MRS broth supernatant from each strain was transmitted to 150 ml flask. Twenty-five ml of newly made sulfuric acid was then added to it and tittered using 0.1 N potassium permanganate ($KMnO_4$). Becoming colorless is the end point of titration (13).

***Antibiotic susceptibility*:** Disc diffusion method was used to evaluate antibiotic susceptibility of *L. plantarum* strains (8). Müller-Hinton medium (Merck, Darmstadt, Germany) with a pH value between 7.2 and 7.4 was used. Inoculation dose of each strain was initially calculated at the wavelength of 600 nm and then a microbial suspension containing 1×10^8 cfu/ml of *L. plantarum* was prepared. Antibiotic susceptibility test was then followed by inoculating the bacteria on the medium and antibiotic discs were placed on the medium using a sterile forceps 15 min after inoculation. The plate was then incubated at 30 °C for 48h after which the diameter of growth inhibition area was measured in millimeters and the sensitivity of each strain against antibiotics was determined as sensitive, semi-sensitive, and resistant. The sensitivity of *L. plantarum* strains against 11 antibiotics chloramphenicol, gentamicin, vancomycin, ampicillin, penicillin, streptomycin, norfloxacin, erythromycin, rifampicin, tetracycline, and kanamycin was evaluated.

***Statistical analysis*:** Analysis of variance (ANOVA test) with the Bonferroni test was used to determine significant differences in H_2O_2 production between different *L. plantarum* strains. All results were expressed as Mean ± SD. Statistical analyses were performed using the SPSS version 22 software. $P< 0.05$ was considered as statistically significant.

Results

Amino acid decarboxylation activity of *L. plantarum* strains has been shown in Table 1. *L. plantarum* strains isolated from Siahmazgi cheese were unable to produce tyramine except for SD6 strain. All strains in this study were able to produce histamine. *L. plantarum* strains were unable to decarboxylate either lysine or ornithine.

As it is depicted in Table 1, *L. plantarum* strains isolated from Siahmazgi cheese showed considerable antimicrobial activity against *S. typhimurium* and *L. monocytogenes*. All *L. plantarum* strains showed stronger antimicrobial activity against *S. typhimurium* (3.5-42.55 mm) than *L. monocytogenes* (1.5-30.49 mm). The SC9 strain had the strongest inhibitory effect against both pathogens with 49.5 and 55.5 mm of inhibition area for *L. monocytogenes* and *S. typhimurium* respectively. After tittering pH to approximately 6.5, no antimicrobial activity was noticed indicating that the antimicrobial activity of *L. plantarum* strains was contributed to their acid production and that these strains were unable to produce bacteriocin.

All *L. plantarum* strains were capable of producing H_2O_2 (Table 1). There are significant differences among *L. plantarum* strains in the present study regarding H_2O_2 production (P value=0.0001). SA32 and SD13 strains with 2.37 and 0.77 mmol/L were the strongest and the weakest strains regarding H_2O_2 production, respectively and were significantly different from other strains.

Antibiotic sensitivity profile of *L. plantarum* strains against 11 antibiotics is depicted in Table 2. All *L. plantarum* strains isolated from Siahmazgi cheese were sensitive to chloramphenicol, erythromycin, rifampicin, and tetracycline, yet resistant against vancomycin, and norfloxacin. Four patterns of antibiotic resistance were observed among *L. plantarum* strains (Table 3). Only two strains of SC9 and SE4 were resistant against four antibiotics which belong to vancomycin, streptomycin, norfloxacin, kanamycin and gentamicin, vancomycin, ampicillin, norfloxacin patterns, respectively and 50 % of *L. plantarum* strains were resistant against three antibiotics vancomycin, norfloxacin and penicillin.

Table 1. Decarboxylation activity, H_2O_2 production and antimicrobial activity of *L. plantarum* strains isolated from Siahmazgi cheese against *L. monocytogenes and S. typhimurium.*

Strains of *L.plantarum*	**Decarboxylation activity***				**Antimicrobial activity****		**H_2O_2 production (mmol/L)*** **
	Ornithine	**Tyrosine**	**Lysine**	**Histidine**	***L.monocytogenes***	***S.typhimurium***	
SC9	-	-	-	+	49.5	55.5	1.45±0.04^{af}
SD6	-	+	-	+	34.7	54.5	1.90±0.01^{b}
SA32	-	-	-	+	37.5	49.5	2.37±0.09^{c}
SC6	-	-	-	+	35.1	51.2	1.93±0.04^{bd}
SD5	-	-	-	+	35.3	55.2	1.86±0.04^{ab}
SC4	-	-	-	+	43.5	47.5	1.66±0.04^{bf}
SD13	-	-	-	+	32.6	45.2	0.77±0.04^{e}
SE4	-	-	-	+	31.1	42.3	1.27±0.20^{f}
SD11	-	-	-	+	30.1	51.2	1.76±0.09^{ab}
SD12	-	-	-	+	37.7	43.7	1.21±0.11^{f}
P-value							<0.001

*+ = positive; - = negative, **average diameter of growth inhibition zone of two replicates, ***Values are means ± SD of two replicates, ****The different superscripts a, b, c, d, e, f in the same column indicate significant differences (P< 0.05).

Table 2. Antibiotic sensitivity/resistance properties of *L. plantarum* strains isolated from Siahmazgi cheese.

Strains of *L.plantarum*	**Chloramphenicol**	**Gentamicin**	**Vancomycin**	**Ampicillin**	**Penicillin**	**Streptomycin**	**Norfloxacin**	**Erythromycin**	**Rifampicin**	**Kanamycin**	**Tetracycline**
SC9	+++*	+++	R	+++	+++	R	R	+++	+++	R	+++
SD6	+++	+++	R	+++	R	+	R	+++	+++	++	++
SA32	+++	+++	R	+++	R	+	R	+++	+++	++	+++
SC6	+++	+++	R	+++	R	+	R	+++	+++	+++	+++
SD5	+++	+++	R	+++	+++	+++	R	+++	+++	+++	+++
SC4	+++	+++	R	++	R	+++	R	+++	+++	+++	+++
SD13	+++	+++	R	+++	+++	+	R	+++	+++	++	+
SE4	+++	R	R	R	+++	+++	R	+++	+++	+++	+++
SD11	+++	+++	R	+++	+++	+	R	+++	+++	++	++
SD12	+++	+++	R	+++	R	+	R	+++	+++	+++	+++

*+++: 15-20 mm, ++: 10-14 mm, +: 1-9 mm, R: resistant.

Table 3. Multiple drug resistance (MDR) patterns in *L. plantarum* strains isolated from Siahmazgi cheese.

MDR patterns	Percentage of resistant isolates (%)
Van/Str/Nor/Kan	10
Van/Pen/Nor	50
Van/Nor	30
Gen/Van/Amp/Nor	10

Van: vancomycin, Str: streptomycin, Nor: norfloxacin, Kan: kanamycin, Pen: penicillin, Gen: gentamicin, Amp: ampicillin

Discussion and Conclusion

Similar to the results of the current study, Moreno-Arribas et al. (24) and Landete et al. (18) reported that none of the *L. plantarum* strains were capable of producing putrescine or tyramine. Halasz et al. (11) showed that 20 %, 40 % and none of *L. plantarum* strains isolated from dairy products were capable of producing tyramine, cadaverine, and putrescine, respectively. A number of researchers have noticed considerable concentrations of histamine in Goda cheese which is presumably produced by the Lactobacilli in rennet (19). *Lactobacillus acidophilus* isolated from yoghurt in Nigeria possessed characteristics exactly contrary to those found in the *L. plantarum* strains in the current study as was capable of lysine, ornithine, and tyrosine decarboxylation but unable to produce histamine (27). This diversity in results regarding biogenic amine production suggests that this quality is strain dependent, and also it is affected by other factors including nutrient availability, production process, type of milk used, ripening time, growth condition, and pH (10, 19). The inability to decarboxylate amino acids is a favorable quality in selecting strains as starter or adjunct cultures.

The SC9 strain had the strongest inhibitory effect against both pathogens. As it was stated in the previous study, SC9 strain was the strongest strain regarding acid production (31). The antimicrobial activity against *L. monocytogenes* is of paramount importance as this pathogen is ubiquitously found in the environment, and is resistant to refrigeration, acidity and high salt concentrations (29). A considerable number of listeriosis outbreaks worldwide have been contributed to raw milk and cheese consumption. The environment of ripened cheeses is suitable for the growth of *L. monocytogenes* because of lactate consumption by microorganisms and amine production and also increased pH. Therefore, *L. monocytogenes* is considered a concern in cheeses such as Siahmazgi that undergo ripening for six months (28). Fifty-eight percent of *L. plantarum* strains isolated from fermented sausages showed antimicrobial effects against *L. monocytogenes* with growth inhibition areas of 2-10 mm of diameter (29). Klinberg et al. (15) and Nieto-Lozano et al. (25) also have proven the antimicrobial activity of *L. plantarum* strains against *L. monocytogenes*. One of the most important pathways for *Salmonella* infection in human is consumption of raw milk or non-pasteurized dairy products (28). All *L. plantarum* strains isolated from traditional salted meat in Tunisia had antimicrobial activity against *Staphylococcus aureus* and *S. enterica* subsp. *arizonae* and most of them inhibited growth of *E. coli* and *Pseudomonas aeruginosa* (8). Contrary to the results of the present study, Nieto-Lozano et al. (25) showed that *L. plantarum* was ineffective against *Salmonella* species. The results of the current study verified those of Essid et al. (8) while are in contrast with those of Messi et al. (23) and Aymerich et al. (2) who have proven bacteriocin production by *L. plantarum* strains. Contrary to the results of the current study, Albano et al. (1) showed that *L. plantarum* strains are more effective against Gram positive than Gram negative bacteria, because bacteriocin producing strains are ineffective against Gram negative bacteria.

All *L. plantarum* strains isolated from Siahmazgi cheese were able to produce H_2O_2. SA32 strain with 2.37 mmol/L was the strongest regarding H_2O_2 production. Sakamoto and Komagata (35) showed that *L. delbrueckii* subsp. *delbrueckii* produced up to 4.9 mmol/L of H_2O_2. *L. acidophilus* strains isolated from dairy products produced 1.62 mmol/L of H_2O_2 (6).

A significant number of strains of *Lactobacillus salivarius*, *Lactobacillus casei*, *L. plantarum*, *Lactobacillus leichmannii*, and *L. acidophilus* possess a gene causing natural resistance against vancomycin (22). In a study conducted by Beyan et al. (4) all LAB isolated from traditionally fermented milk were sensitive to penicillin and erythromycin. Beyan et al. (4) reported that 98.2% of the LAB strains isolated from traditionally fermented milk were sensitive to tetracycline and 80.7% were resistant against norfloxacin which showed significant similarity with the results of the current study. *Lactobacillus plantarum* strains isolated from raw camel milk were sensitive to tetracycline, vancomycin, erythromycin, ampicillin, kanamycin and resistant against rifampicin (9). Vancomycin is prescribed in the treatment of severe infections caused by *Enterococcus* and *Staphylococcus* species. Some *Lactobacillus* species including *L. casei*, *Lactobacillus rhamnosus*, *Lactobacillus curvatus*, *L. plantarum*, *Lactobacillus coryniformis*, *L. brevis*, and *Lactobacillus fermentum* are naturally resistant against vancomycin (42). Drug resistance can be transferred to other pathogens by transformation and conjugative plasmids or

transposons in gastrointestinal tract (34, 37, 39). It may become dangerous if there is the probability of resistance gene against vancomycin being transferred to *Enterococcus* species. Furthermore, some *Enterococcus* species possess the resistance gene against other antibiotics which may cause serious consequences in *Enterococcus* infections (42). This is not a concern per se, because these bacteria are sensitive to other antibiotics and have been safely used for long periods. The majority of *L. plantarum* strains in the current study were sensitive to most antibiotics. Absence of antibiotic resistance implies the absence of naturally occurring antibiotic resistance genes and also indicates that such genes have not been acquired by the bacteria from the environment (4). Generally, lactic acid bacteria isolated from dairy products show less commonly found antibiotic resistance in comparison to bacteria isolated from clinical or environmental sources (41).

Lactobacillus plantarum strains naturally found in Siahmazgi cheese do not generally possess dangerous characteristics to be used in fermentative dairy products. Considering that technological properties and safety aspects of *L. plantarum* strains from Siahmazgi cheese have been identified, it is recommended to produce fermented dairy products with original organoleptic properties using these strains as starter or adjunct cultures.

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Conflict of Interest

The authors are declared that there is no conflict of interest.

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