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Isolation and Characterization of *Lactobacillus bulgaricus* and *Lactobacillus casei* from Various Foods

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Abstract: The aim of this study was to determine *Lactobacillus bulgaricus* and *Lactobacillus casei* isolated from yoghurt, different kinds of cheese and a traditional food named 'tarhana' (a fermented food made of a mixture of cereal, yoghurt and thyme), and to determine the antimicrobial activity and antibiotic resistance of these isolates. The identity of the culture was based on characteristics of the strains of *Lactobacillus* spp. as presented in Bergey's Manual of Determinative Bacteriology, carrying out microscopy (morphology), Gram straining, growth at 15 and 45 °C, and fermentation of different carbon sources and growth in 7.5% NaCl. On the basis of all of the identification tests one strain isolated from the cheese was identified as *Lactobacillus casei*, and the other strain isolated from the probiotic dairy product was identified as *L. bulgaricus*. The *L. casei* isolate was resistant to all of the antibiotic discs used in this study. Culture supernatants obtained from the 2 isolates of *Lactobacillus* spp. exhibited varying degrees of inhibitory activity against strains of *Escherichia coli, Staphylococcus aureus, Pseudomonas aeroginosa, Bacillus subtilis, Klebsiella pneumonia, Salmonella typhimurium,* and *Enterobacter cloacae.*

Key Words: *Lactobacillus bulgaricus*, *Lactobacillus casei*, antimicrobial effect, antibiotic resistance, isolation and identification

Çeşitli Gıdalardan İzole Edilen *Lactobacillus bulgaricus* ve *Lactobacillus casei*'nin İzolasyon ve Karakterizasyonu

Özet: Çalışmada yoğurt, değişik tür peynirlerden ve tarhanadan izole edilen *Lactobacillus bulgaricus* ve *Lactobacillus casei* izolatlarının identifikasyonu, antimikrobiyal aktivitesi ve antibiyotik dirençliliği çalışılmıştır. İzolatların karakterizasyonu Bergey's Manual of Determinative Bacteriology'e göre morfolojik özellikler, Gram boyama, 15 ve 45 °C' de büyüme, değişik karbon kaynaklarını fermente etme ve %7,5'luk NaCl' li ortamda gelişme özellikleri göz önünde bulundurularak yapılmıştır. İdentifikasyonu testlerine göre peynirden izole edilen bir suş *Lactobacillus casei*, probiotic yoğurttan izole edilen diğer suş *L. bulgaricus* olarak tanımlanmıştır. *Lactobacillus casei* izolatının çalışılan tüm antibiyotiklere dirençli olduğu tespit edilmiştir. İzole edilen kültürlerinin supernatantlarının *Escherichia coli, Staphylococcus aureus, Pseudomonas aeroginosa, Bacillus subtilis, Klebsiella pneumoniae, Salmonella typhimurium, Enterobacter cloacae* bakterilerine karşı değişik oranlarda inhibitör etkilerinin olduğu gözlenmiştir.

Anahtar Sözcükler: *Lactobacillus bulgaricus*, *Lactobacillus casei*, antimikrobiyal etki, antibiyotik direnç, izolasyon ve identifikasyon

Introduction

Lactobacilli have been used for many centuries in food fermentation processes. The majority of the new probiotic products contain bifidobacteria, strains of *L. acidophilus* or closely related species (the so-called *L. acidophilus* group). Strains of the so-called *L. casei* group comprising the species *L. casei*, *L. paracasei* subsp. *paracasei* and subsp. *tolerans* and *L. rhamnosus* are also being increasingly applied in novel-type yoghurts. The taxonomy of both *L. acidophilus* and *L. casei* group has been subjected to considerable changes during recent years and may have caused some confusion (1,2). *L. bulgaricus* is required for the production of yogurt and fermented milk, and it has an essential role in the development of the organoleptic, hygienic and perhaps probiotic qualities of these foods.

Lactic acid bacteria (LAB) have been used successfully, with few adverse effects, to prevent antibiotic associated diarrhea, to treat acute infantile diarrhea and recurrent *Clostridium difficile* disease and to treat various diarrheal illnesses (3-5). The antagonistic property is attributed to the lowered pH, the undissociated acids and production of

other primary and secondary antimicrobial metabolites produced by LAB. The metabolites produced by the fermentation process, except the volatile ones, are kept in the foods and result in growth inhibition of food spoilage or poisoning bacteria and detoxification of noxious compounds of plant origin (6,7). The primary antimicrobial effect exerted by LAB is the production of lactic acid and reduction of pH (8). In addition, LAB produce various antimicrobial compounds, which can be classified as low-molecular-mass (LMM) compounds such as hydrogen peroxide (H_2O_2), carbon dioxide (CO_2), diacetyl (2,3-butanedione), uncharacterized compounds, and high-molecular-mass (HMM) compounds like bacteriocin (9-12). All of these can antagonize the growth of some spoilage and pathogenic bacteria in foods.

Most of the probiotic lactobacilli in human foods are supplied in highly concentrated forms containing more than 10^{10} cfu/g. These concentrates are usually freeze dried, spray dried or microencapsulated. These lactobacilli are typically incorporated in fermented milks (13-16), cheeses (17-23), and ice creams (24).

The aim of this work was to determine the strains of *Lactobacillus* spp. isolated from yoghurt, different kinds of cheese and a traditional food named 'tarhana' (a fermented food made of a mixture of cereal, yoghurt and thyme), and to determine the antimicrobial activity and antibiotic resistance of these isolates.

Materials and Methods

Total mesophilic aerobic bacteria in food samples

A 25 g sample of each food sample was taken aseptically and transferred to sterile plastic bags and then homogenized in 225 ml of sterile buffered peptone water (BPW). Five 10-fold dilutions of the homogenates were then prepared and these were inoculated on plates of Nutrient Agar (Difco).

Isolation and phenotypic characterization

A 25 g sample of each food sample was taken aseptically. They were transferred to sterile plastic bags and then homogenized in 225 ml of sterile buffered peptone water (BPW). Five 10-fold dilutions of the homogenates were then prepared and these were inoculated on plates of MRS agar (Oxoid), acidified with glacial acetic acid to pH 5.7 and incubated anaerobically for 48 h at 32 °C. Colonies with typical characteristics were randomly selected from plates and tested for Gram stain, cell morphology, and catalase and oxidase reaction before further sugar fermentation and characterization tests (25). During the test the cultures were kept in MRS agar stabs at refrigeration temperature.

Biochemical characterization and presumptive identification

Growth at 8 and 15 °C in tubes containing MRS broth, growth in 7.5% NaCl, and fermentation of carbohydrates were determined as described by Schillinger and Lücke (26) and Sneath et al. (27). The carbohydrates tested were D(+) cellobiose (Difco, Detroit, MI, USA), D(+) galactose (Difco), inulin (Difco), lactose (Difco), fructose (Difco), maltose l-hydrate (Difco), D mannitol (Difco), D(+) melezitose (Difco), melibiose (Difco), D(-) raffinose (Difco), rhamnose (Difco), ribose (Difco), sorbitol (Difco), D(+) trehalose (Difco), and D(+) xylose (Merck, Darmstadt, Germany), and glucose (Difco) and sterile water were used as positive and negative controls. Gas production from glucose, dextran production from saccharose and hydrolysis of arginine were tested in MRS broth without glucose and meat extract but containing 0.3% arginine and 0.2% sodium citrate replacing ammonium citrate. Ammonia was detected using Nessler's reagent as described by Schillinger and Lücke (26) with the exception of adding glucose to the final concentration of 0.3 g/l to test NH_3 production from arginine. Production of acetoin was detected by the Voges-Proskauer test (28).

Determination of antibiotic resistance of the isolates

In the study, 6 antibiotic discs were used to determine the antibiotic resistance of lactobacilli strains. These antibiotic discs (Oxoid, Hampshire, England) were as follows: ampicillin (10 µg), vancomycin (30 µg), oxacillin (1 µg), cephalothin (30 µg), cefodizime (30 µg) and tobramycin (10 µg).

Activated cultures were grown on slopes and the bacterial cells were removed from the surface with saline. Cell suspensions (0.5 on the McFarland scale) were inoculated to Mueller-Hinton agar plates (Oxoid) containing horse blood and glucose (29).

Antibacterial activities of the strains of *Lactobacillus* spp. isolates

Antimicrobial effects of presumptive strains of *Lactobacillus* spp. on *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeroginosa* ATCC 9027, *Bacillus subtilis* ATCC 6633, *Klebsiella pneumonia* ATCC 18833, *Salmonella typhimurium* ATCC 13311, *Enterobacter cloacae* ATCC 13047 were determined by the agar diffusion method (30). The test bacteria were obtained from the Pharmaceutical Microbiology and Hygiene Laboratory, University of Antwerp, Belgium.

The test bacteria were incubated in nutrient broth at appropriate temperature for 24 h. Approximately 10^5-10^7 cfu/ml of the bacteria to be tested for sensitivity (indicator bacteria) were inoculated (1%) into 20 ml of nutrient agar and poured in the Petri dishes.

For the detection of antibacterial activity of the strains of *Lactobacillus* spp., MRS containing only 0.2% glucose (MRS-0.2) was used. Ten milliliters of broth was inoculated with each strain of *Lactobacillus* spp. and were incubated at 35 °C for 48 h. After incubation, a cell-free solution was obtained by centrifuging (6000 x g for 15 min) the culture, followed by filtration of the supernatant through a 0.2 µm pore size (Schleicher & Schuell, Germany) cellulose acetate filter. Some supernatants were neutralized with 1 N NaOH to pH 6.5, and the inhibitory effect of the hydrogen peroxide was eliminated by the addition of catalase (5 mg/ml). Unneutralized (general inhibitory effect) and neutralized (bacteriocin and bacteriocin-like metabolites) supernatants of the strains of *Lactobacillus* spp. were checked for antibacterial activity against pathogenic bacteria in inoculated nutrient agar (6,31). Then 100 ml of cell free supernatants was filled in 8-mm diameter sealed wells cut in the nutrient agar. Once solidified, the dishes were stored for 2 h in a refrigerator. The inoculated plates were incubated for 24 h at 37 °C, and the diameter of the inhibition zone was measured with calipers in millimeters (32).

Results

Twenty-one presumptive *Lactobacillus* strains were isolated from 3 different yoghurts, from 2 probiotic dairy products, 3 different cheeses and 2 different tarhana samples. The mean pH values of these food samples were 5.52, 5.59, 5.78 and 4.49, respectively. All isolates were catalase-negative, Gram positive and oxidase negative rods producing no gas from glucose. On the basis of all of the identification tests one strain isolated from the cheese was identified as *Lactobacillus casei*, and the other strain isolated from the probiotic dairy product was identified as *Lactobacillus bulgaricus*.

The results concerning the total mesophilic aerobic bacteria in yoghurts, probiotic dairy products, cheese and tarhana were 4, 5, 5.78 and 5 log CFU/g, respectively.

Metabolic characteristics and presumptive identification of *Lactobacillus casei* and *Lactobacillus bulgaricus*, isolated from 3 different yoghurts, 2 probiotic dairy products, 3 different cheese samples and 2 different tarhana samples are shown in Table 1.

The *Lactobacillus casei* isolate was resistant to all of the antibiotic discs used in this study. Results concerning the determination of antibiotic resistance of the isolates are given in Table 2.

The bacterial activities exhibited by *Lactobacillus casei* and *Lactobacillus bulgaricus* are presented in Table 3. Culture supernatants (CFF) obtained from *Lactobacillus casei* and *Lactobacillus bulgaricus* exhibited varying degrees of inhibitory activity against strains of *Escherichia coli* ATCC 8739, *Staphylococcus aureus* ATCC 6538, *Pseudomonas aeroginosa* ATCC 9027, *Bacillus subtilis* ATCC 6633, *Klebsiella pneumonia* ATCC 18833, *Salmonella typhimurium* ATCC 13311, and *Enterobacter cloacae* ATCC 13047.

Discussion

L. casei and *L. bulgaricus* isolates showed weak (<12 mm zone of inhibition) antibacterial activity against *E. coli*, *S. aureus*, *P. aeroginosa*, *B. subtilis*, *K. pneumonia*, *S. typhimurium*, and *E. cloacae*. The antimicrobial effect exerted by LAB is the production of lactic acid and reduction of pH, and acetic acid, diacetyl, hydrogen peroxide, fatty acids, aldehydes and other compounds (8,9). Schillinger and Lücke (6) and Toksoy et al. (33) reported that some *L. plantarum* and *L. sake* strains from meat and meat products had inhibitory effects against several bacteria. In addition, Xanthopoulos et al. (34) indicated that *L. paracasei* subsp. *paracasei* and *L. acidophilus* strains isolated from infant feces had weak antibacterial activity on *Escherichia coli* and *Yersinia enterocolitica*. Alexandre et al. (35) reported that 192

Table 1. Metabolic characteristics and presumptive identification of *Lactobacillus casei* and *Lactobacillus bulgaricus*, isolated from 3 different yoghurts, 2 probiotic dairy products, 3 different cheese samples and 2 different tarhana samples.

Presumptive identification	Number of isolates	Fermented carbohydrates															
		Cellobiose se	Galactose se	Lacto se	Melezito se	Melibiose se	Malto se	Manni tol	Raffino se	Rhamno se	Ribose	Sucro se	Trehalose se	Xylo se	Fructose se	Sorbi tol	Argini ne
L. casei	1	+	+	+	+	+	+	+	–	–	+	+	+	–	+	+	–
L. bulgaricus	1	–	–	–	–	–	+	–	+	–	+	–	+	–	–	–	–

Table 2. Determination of antibiotic resistance of the isolates (diameter of inhibition zone: mm).

	ampicillin (10 µg)	vancomycin (30 µg)	oxacillin (1 µg)	cephalothin (30 µg)	cefodizime (30 µg)	tobramycin (10 µg)
L. casei	-[a]	-	-	-	-	-
L. bulgaricus	10.5	15	10.5	10	10.5	24

[a]: resistant

Table 3. Antimicrobial activity of *L. casei* and *L. bulgaricus* against *E. coli*, *S. aureus*, *P. aeroginosa*, *B. subtilis*, *K. pneumonia*, *S. typhimurium*, and *E. cloacae* (diameter of inhibition zone: mm).

	E. coli	*S. aureus*	*P. aeroginosa*	*B. subtilis*	*K. pneumonia*	*S. typhimurium*	*E. cloacae*
L. casei	8	9	8	-	7	8	8
L. bulgaricus	8	8	8	-	7	7	8

strains of lactic acid bacteria were isolated from 5 samples of Artisanal minas cheese. The results of direct inhibition test indicated that 48 strains inhibited the in vitro growth of the indicator microorganisms: *S. aureus* and *Listeria monocytogenes*. Aroutcheva et al. (36) revealed that no correlation was found between bacteriocin activity, lactic acid and hydrogen peroxide production. They found that 3 Lactobacillus strains produced H_2O_2 but did not demonstrate any inhibitory effect. Yüksekdağ et al. (37) reported that *Lactococcus lactis* subsp. *cremoris* Z20S strain produced maximum lactic acid but did not produce H_2O_2. Moreover, the strain had an inhibitory effect against *S. aureus* but no inhibitory effect against *E. coli* and *P. aeruginosa*. In a study by Tadesse et al. (38), LAB involved in the fermentation of traditional beverages had an antimicrobial property against various food-borne pathogens and the inhibitory products were extracellular and diffusible. The observed inhibitory property of LAB was influenced by the medium they grew in.

Many LAB are resistant to antibiotics. This resistance's attributes are often intrinsic and nontransmissible (39). On the other hand, intrinsically antibiotic-resistant probiotic strains may benefit patients whose normal intestinal microbiota has become unbalanced or greatly reduced in numbers due to the administration of various antimicrobial agents (40). Among antibiotic resistances, vancomycin resistance is of major concern because vancomycin is one of the last antibiotics broadly efficacious against clinical infections caused by multi drug-resistant pathogens (41). *L. casei* was resistant to all of the antibiotics used in this study but the *L. bulgaricus* isolate was susceptible to all of the antibiotics. *L. bulgaricus* is vancomycin sensitive, in contrast to most other lactobacilli (42). Vancomycin resistance was a general characteristic of bifidobacteria (39).

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