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Pathogenesis and toxins

Acid-bile, antibiotic resistance and inhibitory properties of propionibacteria isolated from Turkish traditional home-made cheeses

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ABSTRACT

In this study, a total of 29 *Propionibacterium* spp. were isolated from traditional home-made Turkish cheese samples. As a result of the identification, isolates were identified as *Propionibacterium freudenreichii* subsp. *freudenreichii* (15 strains), *Propionibacterium jensenii* (12), and *Propionibacterium thoenii* (2). All isolates and 5 reference strains were examined for their abilities to survive at pH 2.0, 3.0, 4.0, 5.0 and in the presence of 0.06, 0.15 and 0.30% bile salts, their influence on the growth of food-borne and spoilage bacteria, as well as their sensitivity against 11 selected antibiotics. Only seven propionibacteria strains survived in both the acidic and bile salt environments. *Propionibacterium* spp. strains strongly inhibited growth of the *Escherichia coli* ATCC 11229 and *Shigella sonnei* Mu:57 strains (91%). All propionibacteria strains were sensitive to a majority of the antibiotics used in the investigations. Overall, dairy propionibacteria showed high antibacterial activity, resistance to pH 4.0, 5.0, high resistance to bile salts and will provide an alternative source to *Lactobacillus* and *Bifidobacterium* as probiotic culture.

1. Introduction

Dairy propionibacteria are important organisms in Emmental and in Swiss-type cheeses. The popionibacteria are essential in eye-formation and development of the characteristic sweet and nutty flavor in cheeses [1]. Among nearly 40 local cheeses that are peculiar to Turkey, there are lots of cheese types such as Balıkesir Manyas Dil Cheese, Izmir Tulum Cheese, Kars Gravyer Cheese, Kars Stager…etc. that have the whole hole structure as in the Swiss cheeses. Propionibacteria are gram-positive, non-motile, and anaerobic to aerotolerant bacteria. The genus *Propionibacterium* is generally divided into dairy (classic) and cutaneous groups. The dairy propionibacteria include the species *Propionibacterium freudenreichii, Propionibacterium jensenii, Propionibacterium thoenii* and *Propionibacterium acidopropionici.* These dairy species are not typical for human intestinal microflora [2].

Probiotics are defined as live microbial food supplements that beneficially affect the host health when ingested. Propionibacteria have been shown to display interesting probiotic potentialities and are used in human probiotic formulations [3]. Their main claimed effects are promotion of bifidobacterial growth [4,5], the production of propionic acid, bacteriocins, vitamin B12 [6,7], modulation of colon motility [8], supply of lactase activity [9] and antimicrobial activities [10]. They are also thought to stimulate the immune system and limit cancer progression [11,12], although the mechanisms involved are not fully defined. In this sense, the development of a cheese as carrier of beneficial microorganisms presents certain advantages. Its pH, higher than those of convential fermented dairy products, the fat content, and the matrix of the cheese may offer some protection to the probiotic bacteria during ripening and storage and through the gastrointestinal tract [9]. The technological qualities of dairy propionibacteria constitute a key advantage for their uses as probiotics. It is therefore necessary to screen dairy propionibacteria in order to select strains with the best potential for dedicated applications [13].

Probiotic bacteria that are delivered through food systems have to firstly survive during the transit through the upper gastrointestinal tract, and then persist in the gut to provide beneficial effects for the host [14]. In order to be used as potential probiotics, dairy propionibacteria strains need to be screened for their capacity of transit tolerance to the upper gastrointestinal tract conditions [15]. After ingestion, these bacteria must overcome two main biological barriers: (i) the acidic environment of the stomach and (ii) the bile secreted in the duodenum [16]. To guarantee their survival during passage through the gastrointestinal tract, new probiotic

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strains are primarily screened for their tolerance to an acid pH and to bile. Nonetheless, the viability of bifidobacteria at the pH values (≈2) of gastric juices is generally thought to be low [17]. As a consequence, several methods including those based on stress adaptation mechanisms, are being investigated as possible strategies for the enhancement of their acid resistance [18].

In this paper, we isolated 29 dairy propionibacteria from traditional home-made Turkish cheese. Some probiotic properties of these 29 strains were assessed along with five reference strains of dairy propionibacteria, by testing their resistance to low pH (2.0, 3.0, 4.0, 5.0), bile tolerance (0.06, 0.15 and 0.3% (*w/v*)), antibiotic resistance (11 different antibiotics), and antimicrobial activity against pathogens. These evaluations were performed as an initial step toward establishing rational criteria for screening and selecting food-borne microorganisms with human probiotic properties.

2. Materials and methods

2.1. Microorganisms and growth conditions

29 propionibacteria were isolated from the eight different traditional home-made Turkish cheese samples, that were collected from Turkey villages. Table 1 reports the species and strains of propionibacteria and their origin. Seventy-four substrate utilization was determined using the AP1 20 E, 20 A and 50 CH kits™ (bioMerieux, France) [19]. Morphological characteristics were determined with bright field microscopy of Gram-stained preparations, motility after 48 h in MRS medium and colony pigmentation after 10 d on Sodium Lactate Agar (SLA) (1% peptone, 1% yeast extract, 1% sodium lactate, 0.025% K_2HPO_4, 0.05% $MnSO_4$, 1.5% agar, pH 7.0) plates [1,20]. Gram reaction, morphological, physiological and API kits tests were compared with the reference strains (*P. freudenreichii* subsp. *shermanii* DSMZ 20270, *P. freudenreichii* subsp. *freudenreichii* DSMZ 20271, *P. acidopropionici* DSMZ 20272, *P. thoenii* DSMZ 20276, *P. jensenii* DSMZ 20235) in standard tests for identification. Propionibacteria species were cultured under anaerobic conditions for 48 h at 30 °C in Sodium Lactate Broth (SLB) medium. To prepare of active cultures for all experiments, propionibacteria strains were activated by three successive transfers every 48 h in the SLB at 30 °C [21]. The bacterial strains were stored frozen at −80 °C in 10% glycerol broth to supply a stable inoculum for this study and subcultured twice before use in the manipulations. In the experiments, 100 μL of cultures containing a final optical density (OD) of 0.6 at 600 nm (10^5–10^7 CFU/mL) were inoculated to test medium. All tests were carried out in three independent assays.

2.2. Resistance to low pH and bile salts

pH of SLB was adjusted to 2.0, 3.0, 4.0, 5.0 and 7.0 (control) using 4 N HCl and also, the effects of bile salts were examined in SLB medium (pH 7.0) by oxgall (Sigma) to a final concentration of 0.06, 0.15 and 0.30% (*w/v*). 100 μL of cultures (a final optical density of 0.6 at 600 nm) were inoculated and growth for 4 d at 30 °C under anaerobic conditions. The cell growth was measured spectrophometrically (Digilab Hitachi U−1800) at 600 nm [22]. Results were given as OD. Experiments were made in triplicate.

2.3. Influence of Propionibacterium cultures on pathogens

Antimicrobial effects of all strains on *Escherichia coli* ATCC 11229 (American Type Culture Collection), *E. coli* ATCC 35218, *E. coli* O157:H7, *Staphylococcus aureus* ATCC 25923, *Listeria monocytogenes* ATCC 7644, *Salmonella enteritidis* ATCC 13076, *Bacillus cereus* RSKK 863 (Refik Saydam Culture Collection), *Shigella sonnei* Mu:57, *Micrococcus luteus* NRLL B-4375 (Northern Utilization Research and Development Division) and *Pseudomonas aeruginosa* ATCC 27853 were determined by the agar diffusion method [23]. Test microorganisms were propagated twice and then grown for 18–24 h in 10 mL of appropriate growth media. Turbidity of the culture broth was compared with McFarland tubes to give an estimate of bacterial population (10^6–10^7 CFU/mL). Propionibacteria species were inoculated (a final optical density of 0.6 at 600 nm) in reconstituted skim milk powder supplemented with 1% yeast extract and 0.5% glucose (pH: 6.8) at 30 °C for 8 d under anaerobic conditions. Activated cultures were centrifuged at 4000 rpm for 15 min and the clear supernatant was sterilized by filtration (0.45 μL) thus obtaining cell-free filtrates. Petri dishes with 20 mL of Nutrient agar (Oxoid) were prepared, previously inoculated with 0.1 mL of

Table 1
Propionibacterium spp. isolates and their identification based on API AP1 20 E, 20 A and 50 CH kits and source of cheeses used in this study.

Source of Cheese and Strains	Strains	Species identification by API	Similarity %
Balıkesir Manyas Dil Cheese	BDP2	*P. freudenreichii* subsp. *freudenreichii*	86
	BDP4	*P. freudenreichii* subsp. *freudenreichii*	86
	BDP5	*P. jensenii*	80
	BDP6	*P. jensenii*	80
	BDP7	*P. jensenii*	79
	BDP11	*P. jensenii*	77
Izmir Tulum Cheese	ITP1	*P. freudenreichii* subsp. *freudenreichii*	82
	ITP2	*P. jensenii*	75
	ITP3	*P. freudenreichii* subsp. *freudenreichii*	86
Kars Gravyer	DO2	*P. freudenreichii* subsp. *freudenreichii*	88
	DO5	*P. freudenreichii* subsp. *freudenreichii*	86
	DO1	*P. jensenii*	80
	DO3	*P. jensenii*	80
	DO4	*P. jensenii*	70
Kars Stager	DO9	*P. freudenreichii* subsp. *freudenreichii*	86
Mihalic Cheese	DO7	*P. freudenreichii* subsp. *freudenreichii*	88
	DO8	*P. freudenreichii* subsp. *freudenreichii*	85
	DO6	*P. jensenii*	79
Sepet Cheese	SP1	*P. freudenreichii* subsp. *freudenreichii*	89
	SP2	*P. freudenreichii* subsp. *freudenreichii*	85
	SP3	*P. freudenreichii* subsp. *freudenreichii*	85
	SP4	*P. freudenreichii* subsp. *freudenreichii*	85
	SP5	*P. freudenreichii* subsp. *freudenreichii*	88
	SP9	*P. freudenreichii* subsp. *freudenreichii*	91
	SP7	*P. jensenii*	82
	SP6	*P. jensenii*	88
	SP8	*P. jensenii*	83
Afyon Dinar Village Cheese	AKP1	*P. thoenii*	80
Mihalic Kelle Cheese	SMP1	*P. thoenii*	71
Cheese	DSMZ 20270	*P. freudenreichii* subsp. *shermanii*	Reference strain
Swiss Cheese	DSMZ 20271	*P. freudenreichii* subsp. *freudenreichii*	Reference strain
Buttermilk	DSMZ 20235	*P. jensenii*	Reference strain
Emmental cheese	DSMZ 20272	*P. acidopropionici*	Reference strain
Emmental Cheese	DSMZ 20276	*P. thoenii*	Reference strain