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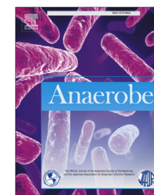

Clinical microbiology

Screening of *Propionibacterium* spp. for potential probiotic properties

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A B S T R A C T

The main topic of this paper is the evaluation of adhesion of propionibacteria to IPEC-J2 cells and the survival at pH 2.5 and with 0.3% bile salts added, bioactivity towards pathogens and antibiotic resistance of *Propionibacterium freudenreichii* subsp. *shermanii*, *Propionibacterium jensenii*, *Propionibacterium acidipropionici* and *Propionibacterium thoenii*.

Adhesion to IPEC-J2 cell lines was ca. 25–35% and significantly increased with $CaCl_2$. Moreover, propionibacteria showed a reduction of cell count of ca. 0.5% at pH 2.5 after 3 h, whereas cell count increased after 24 h with bile salts; finally, they significantly inhibited *Escherichia coli* O157:H7.

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1. Introduction

Propionibacteria belong to the Actinobacteria class with a high G + C content (64–68%); they are mesophilic, Gram-positive, catalase positive, non-motile pleomorphic rods, non-spore-forming, and anaerobic to aerotolerant bacteria. Some cells may be elongated, bifid or arranged in “Chinese characters” [1]. They grow at 15–40 °C and at pH 5.1–8.5; the optimal temperature for growth is 30 °C [1,2].

The current taxonomy describes 13 species that can be divided into two groups: “dairy or classical” and “cutaneous”; the classical propionibacteria (*Propionibacterium acidipropionici*, *Propionibacterium cyclohexanicum*, *Propionibacterium freudenreichii*, *Propionibacterium jensenii*, *Propionibacterium microaerophilum*, *Propionibacterium thoenii*) are generally isolated from milk and dairy environments, whereas the cutaneous propionibateria (*Propionibacterium acidifaciens*, *Propionibacterium acnes*, *Propionibacterium australiense*, *Propionibacterium avidum*, *Propionibacterium granulosum*, *Propionibacterium humerusii*, *Propionibacterium propionicus*) are from skin/intestine of human and animals. Propionibacteria metabolize different carbohydrates (glucose, galactose, lactose, fructose and others), alcohols (glycerol, erythritol and others) and organic acids (lactic and gluconic acids), and produce propionic and acetic acids and carbon dioxide as final products. In addition, bacteriocins and vitamins can be produced.

Due to their antimicrobial activity, propionibacteria are used to enhance the technological properties of various food products, e.g. they are used to prolong the shelf life of bread, cakes, cheeses, fruits, vegetables and tobacco, as they suppress the growth of moulds and spoilage microorganisms [1].

Propionibacteria also stimulate the immune system and limit cancer progression although the mechanism involved is not defined. Cousin et al. [2] reported that dairy propionibacteria are able to prevent infections and allergies, promote immune system maturation, and reduce the risk of cancer because they bind carcinogenic compounds (mycotoxins, plants lectins and heavy metals).

Cutaneous propionibacteria show a similar genetic and biochemical profile [3] and have been used as a pre-treatment in patients with colorectal carcinoma where they produced beneficial immunostimulation [2].

Propionibacteria persist transiently in the gut after ingestion for some weeks and exert positive effects on human health, because of their adaptability and high tolerance to digestive stress. Probiotic bacteria, delivered through food products, have to survive during the transit through the upper gastrointestinal tract, persist in the gut, and confer a health benefit [4,5]; on the other hand, the low pH of the stomach and secretion of bile salts into the small bowel could cause a bactericidal effect. Two desirable properties for probiotic bacteria are adhesion to intestinal mucosa and the antagonistic activity towards pathogens; EFSA (European Food Safety Agency) also requires the evaluation of antibiotic resistance as this trait could be a safety issue [6]. *P. freudenreichii* and *P. acidipropionici*,

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have been added to the QPS list (Qualified Presumption of Safety) [7].

Probiotics can be selected by using many criteria; the main aim of this paper was the evaluation of adhesion of propionibacteria to IPEC-J2 cells, used as a model of the ileal mucosa. Strains from a public collection (*P. freudenreichii* subsp. *shermanii*; ATCC 9614, *P. jensenii* ATCC 4870; *P. acidipropionici* ATCC 4875; *P. thoenii* ATCC 4874) were used as study subjects. As an additional aim, some other criteria were assessed (survival throughout the transit through the stomach and intestine, antagonistic activity towards some pathogens and resistance/susceptibility to antibiotics).

2. Material and methods

2.1. Microorganisms and growth conditions

P. freudenreichii subsp. *shermanii* DSM 20270 (ATCC 9614; source: cheese), *P. jensenii* DSM 20279 (ATCC 4870; source: emmental cheese), *P. acidipropionici* DSM 20272 (ATCC 4875; source: emmental cheese), *Propionibacterium thoeni* DSM 20276 (ATCC 4874; source: emmental cheese) were used in this research. Strains were purchased from DSM (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany) and grown in Propionibacterium medium (caseine peptone tryptic digest 10 g/L; yeast extract 5 g/L; Na-lactate 10 g/L; pH was adjusted to 7.0–7.02), with incubation at 30 °C for 7 days, as suggested by the depositor. Cells were then harvested by centrifugation (5000× *g* for 10 min) and suspended in MRS broth (Oxoid, Hampshire, United Kingdom) containing 0.05% (w/v) L-cysteine (Sigma–Aldrich, Milan) (cMRS) and incubated at 37 °C for 5 days. Propionibacteria were stored at −18 °C in cMRS containing 33% (v/v) sterile glycerol (J.T. Baker, Milan).

2.2. Adhesion assay

This assay was performed in Denmark (Department of Food Science, Faculty of Life Sciences, University of Copenhagen). IPEC-J2 cells were grown at 37 °C in a 5% CO_2, 95% air-humidified incubator in a medium containing a 1:1 mixture of Dulbecco's modified Eagle medium (DMEM; Sigma–Aldrich) and F12 (Sigma–Aldrich) supplemented with 100 mg/L streptomycin (Fluka Chemie GmbH, Steinheim, Switzerland), 100 mg/L penicillin (Sigma–Aldrich), 2 mmol/L L-glutamine (Sigma–Aldrich), 1 mmol/L pyruvate (Sigma–Aldrich) and 10% fetal bovine serum (Cambrex Bio Science, Verviers, Belgium). Experiments were performed in DMEM medium containing F12 but without antibiotics.

IPEC-J2 cells were seeded at a concentration of 5×10^5 cells *per* well (ca. 1×10^5 *per* cm^2) in 12-well tissue culture plates (Corninc Inc, Corning, NY, USA) and grown to 100% confluence. Cell culture medium was changed every two days. cMRS broth (10 mL) was inoculated with propionibacteria and incubated at 37 °C for 24 h; these cultures were used to inoculate 2 mL of cMRS broth containing 100 μL of metabolic radiolabelling 2 Mq/mL L-[*methyl*-^{3}H] methionine (Amersham Biosciences, Uppsala, Sweden) and incubated at 37 °C for 21 h. Bacteria were then centrifuged (6000 rpm for 2 min), washed three times with sterile PBS (9.0 g/L NaCl, 0.3 g Na_2HPO_4 *$2H_2O$, pH 7) and suspended in DMEM containing F12 (7 log CFU/mL). The adhesion assay was performed by adding 1 mL of propionibacteria suspension separately to three wells containing an IPEC-J2 monolayer. After 1 h at 37 °C, IPEC-J2 cells were washed three times with DMEM containing F12 and left overnight with 500 μL of NaOH/SDS solution (0.1 mmol/L NaOH, 1% w/v SDS-Amersham Biosciences) to lyse cells. Cells were then treated with a buffer solution of Na_2HPO_4 *$2H_2O$/citric (Na_2HPO_4 *$2H_2O$ 0.1 M; citric acid 0.1 M; pH 3) and added to 4.5 mL scintillation liquid OptiPhase '*Hisafe*' 2 (Fisher Chemicals, Louhghsborough Leisc., UK); radioactivity was measured through a scintillation counter (Wallac 1414 WinSpectral, Turku, Finland). Adhesion was expressed as the ratio of the radioactivity of lysed cells to the radioactivity of the labeled bacterial suspension. *Lactobacillus reuteri* 12002 [8] was used as a positive control. Each assay was performed in triplicate. A second assay was performed to analyze the effect of Ca^{2+}; the protocol was modified as follows: the experiment was performed by adding 950 μL of propionibacteria labeled with L-[*methyl*-^{3}H] methionine and 50 μL of 200 mM of $CaCl_2$ solution to wells containing IPEC-J2 monolayers, and the adhesion assay was performed as above.

2.3. Effect of acidic pH and bile salts

The effect of pH and bile salts was assessed on propionibacteria during stationary phase. cMRS acidified to pH 2.5 with HCl 5.0 mol/L or containing 0.3% (w/v) bile salts (Oxoid) was inoculated to 7 log CFU/mL and incubated at 37 °C for 3 or 24 h; viable counts were assessed by pour-plating on cMRS agar, with incubation at 37 °C for 5 days under anaerobic conditions. cMRS broth (pH 6.0) inoculated with propionibacteria was used as a control. The experiments were performed on two independent batches; for each batch the analyses were performed twice.

Data were modeled as *viability loss* (*V.L.*) (effect of pH) and *growth index* (ΔlogN) (effect of bile salts):

$$V.L. = (1 - \log Nt/\log N_0)*100$$

$$\Delta\log N = \log Nt - \log N_0$$

where *Nt* is cell count after 3 or 24 h and N_0 the initial cell count.

2.4. Bioactivity against foodborne pathogens

Listeria monocytogenes and *Escherichia coli* O157:H7, belonging to the Culture Collection of the Laboratory of Predictive Microbiology-Department of Science of Agriculture, Food and Environment (SAFE, University of Foggia, Italy) and isolated respectively from dairy and meat products, were used as test pathogens.

Pathogens were grown in Nutrient Broth (Oxoid) (37 °C for 24–48 h); aliquots of 100 μL of each pathogen (7 log CFU/mL) were plated on cMRS agar; thereafter, disks (9 mm) (Schleicher & Schuell Microscience, Dassel, Germany) were placed onto the surface of the agar and inoculated with 20 μL of the following suspensions:

a) propionibacteria cell cultures (pH 4.5);
b) propionibacteria cell cultures, adjusted to pH 6.5;
c) supernatant of propionibacteria;
d) supernatant of propionibacteria adjusted to pH 6.5.

After the incubation (37 °C for 48 h), a clear halo indicated the antimicrobial activity of propionibacteria against *L. monocytogenes* or *E. coli* O157:H7; the diameter of the inhibition halo was measured [8]. The experiments were performed on 3 different batches.

2.5. Susceptibility of Propionibacterium strains to antibiotics

Susceptibility testing was performed as reported by NCCLS (*National Committee for Clinical Laboratory Standards*, 1993) [9]. Propionibacteria strains were grown in cMRS broth and inoculated onto the surface of cMRS agar. Antibiotic discs (Neo Sensitabs®, Taastrup, Denmark) (Table 1) were placed on inoculated plates and

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