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Propionibacterium acidipropionici CRL1198 influences the production of acids and the growth of bacterial genera stimulated by inulin in a murine model of cecal slurries

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ABSTRACT

Different attempts have been made to improve the health status of humans and animals by increasing the intestinal production of short-chain fatty acids (SCFA) derived from non-digestible carbohydrates fermentation. In this paper we investigate the *in vitro* production of short-chain fatty acids (SCFA) after addition of inulin, propionibacteria or a combination of both in an experimental model of mice cecal slurries. The development of bacterial genera which are usually stimulated by inulin addition was also investigated. According to our experimental data, acetic acid and butyric acids concentrations increased after incubation in slurries that had no supplements. By contrast, butyric acid concentrations remained in the basal value when supplements were used. Fermentation of only inulin did not increase the concentration of total SCFA. *Propionibacterium acidipropionici* CRL1198 improved the production of propionic acid in cecal slurries when it was added alone, but the effect was more noticeable in the combination with inulin. A modulation of the global fermentative activity of the cecal microbiota was evidenced by the increase on the ratio propionic acid/SCFA in supplementations with propionibacteria. Statistical analysis of data demonstrated that samples from homogenates with propionibacteria alone or combined with inulin belong to the same cluster. The presence of propionibacteria limited the growth of *Bacteroides fragilis* and *Clostridium hystoliticum* groups in slurries with and without inulin. The growth of *Bifidobacterium* was not modified and the stimulating effect of inulin on lactobacilli disappeared in the presence of propionibacteria. In conclusion, dairy propionibacteria are potential candidates to develop new functional foods helpful to ensure the intestinal production of SCFA during inulin supplementation and to control the overgrowth of bacteria belonging to *Bacteroides* and *Clostridium* genera.

1. Introduction

The large intestine of humans and monogastric animals is a complex ecosystem that harbors several hundreds of different bacterial species including autochthonous components and allochthonous members that pass through together with food and beverages [1]. In order to obtain the energy for growth and cellular maintenance, this microbiota metabolizes different exogenous carbohydrates, no digested by the host's intestinal enzymes, and endogenous substrates like mucus and epithelial or bacterial cells residues. The bacterial catabolism of carbohydrates produces organic acids like lactic acid and short-chain fatty acids (SCFA), mainly acetic, propionic and butyric acids. [2]. Concentration and ratio of SCFA are strongly influenced by the diet and the metabolism of bacterial genera that are in high number in the intestine.

Measurements of SCFA in the intestinal content only provide an indication of SCFA production as its concentration result from the equilibrium between the production by the metabolic activity of the microbiota and the absorption throughout the intestinal mucosa. The transport of SCFA from the intestinal lumen is responsible for Na and water absorption [3] in the colon. SCFA are also important energy sources for the colonocytes and have a trophic effect on the colonic mucosa [4]. Butyric acid induces cell differentiation and stimulates apoptosis of cancerous cells, as well as propionic acid [5–7]. Part of the SCFA absorbed reaches the general circulation but are rapidly cleared and metabolized in muscles and brain, providing additional energy, and in the liver where propionic acid interferes with the cholesterol synthesis. Given the beneficial effects of SCFA, different attempts have been made in order to increase the intestinal production of these acids

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throughout fermentation of dietary carbohydrates expecting to improve the health status of consumers. For this purpose, dietary supplementation with non-digestible carbohydrates like oligofructose and fructooligosaccharides has been evaluated [8,9].

In the last years, other strategies to increase SCFA concentrations in the intestinal environment have been also considered, such as the consumption of probiotic strains which release acetic, propionic or butyric acid as final products from carbohydrates fermentation. *Saccharomyces boulardii* [10] and anaerobic bacteria like *Faecalibacterium prausnitzii* or *Butyricicoccus pullicaecorum* have been proposed for this purpose [11]. In that sense, dairy propionibacteria may be suitable to be used as probiotics for improving intestinal SCFA concentrations as they are producer of acetic and propionic acid, they are considered GRAS microorganisms (Generally Recognized As Safe) and commonly used for dairy products elaboration. It has been demonstrated *in vitro*, in mice cecal slurries, that propionibacteria may influence the overall production of SCFA, as they enhance propionic acid concentration derived from fermentation of dietary residues, lactose or carbohydrates released from mucus by the bacterial glycosidases [12]. There is also *in vivo* evidence of the survival and metabolic activity of some strains of propionibacteria in the intestine of mice and rats [12,13], gastrointestinal tract of human microbiota-associated rats [14], and humans' intestine [15,16]. However there is no information about the benefits of a simultaneous administration of dairy propionibacteria and non-digestible carbohydrates like inulin-type fructans. Our interest was to determine if cross-feeding among propionibacteria and autochthonous bacteria that metabolize fructans promotes propionic acid production during inulin supplementation and if this interferes the development and fermentative activity of the resident bacteria. The interest was focused to lactobacilli, bifidobacteria and bacteroides, which have been reported as inulin consumers and clostridia that can utilize lactic acid [17–20]. Taking into account that the degradation of fibers show good correlation between man and murine models and that these models has been previously used to predict bacterial degradation of dietary fibers in man [21], we have performed our *in vitro* assays in an experimental model of mice.

2. Materials and methods

2.1. Bacterial strain and culture conditions

Propionibacterium acidipropionici CRL1198 (CRL: Centro de Referencia para Lactobacilos, CERELA, Tucumán, Argentina), a strain resistant to the gastrointestinal tract conditions [22,23], was selected for the present study. The strain was stored at −20 °C in 10% (w/v) reconstituted non-fat milk (NFM) supplemented with 0.5% yeast extract. Before each experiment, it was inoculated in Laptg broth [24] with the following composition: 15 g/L peptone, 10 g/L tryptone, 10 g/L yeast extract, 10 g/L glucose, 1 mL polysorbate 80, pH 6.50. It was transferred twice to fresh medium after incubations for 24 h at 37 °C.

2.2. Growth on inulin

To study the ability of *P. acidipropionici* CRL1198 to growth in media with inulin, the strain activated in Laptg broth medium was then propagated for 24 h at 37 °C in a modified Laptg broth containing 1% fructose instead of glucose. Afterwards, the cells were harvested by centrifugation (10,000 × g, 10 min, 4 °C), suspended in one tenth of the original volume of culture in fresh Lapt base medium without sugars and used to inoculate the same medium with and without 10 g/L filter-sterilized inulin (Sigma–Aldrich, Buenos Aires, Argentina). The suspension volume used to inoculate the media was 1% (v/v). The cultures were incubated at 37 °C for 12 h in an anaerobic glove box (Anaerobic System model 1024, Forma Scientific, Marietta, USA) with atmosphere of 100% N_2. The growth was followed by Absorbance at 560 nm.

2.3. Preparation of cecal slurries and in vitro fermentation experiments

A culture of *P. acidipropionici* propagated in Laptg broth medium was harvested by centrifugation (3000 × g, 10 min, 4 °C), washed with sterile saline solution (9 g/L NaCl) and suspended in one fifth of the original volume of culture in the same solution. This suspension was used to inoculate mice cecal homogenates when it was necessary.

Twelve male 6-weeks-old BALB/c mice, each weighing 25–30 g, from the inbreed colony of CERELA were housed in metal cages, three animals per each, and maintained in a room under controlled environmental conditions of 25 °C and a 12-h light-dark cycle. They were allowed free access to a conventional solid balanced diet (*Cooperación*, Asociación de Cooperativas Unidas, San Nicolás, Buenos Aires, Argentina) and water and at the day of the experiment were sacrificed by cervical dislocation. Four slurries containing a pool of the cecal content of three animals each one were obtained as previously reported [12]. They were weighed and diluted in pre-reduced sterile saline solution to adequate volume to give 5% (w/v) suspensions. Sterilized glass beads (diameter 3 mm) were added to the suspensions, which were homogenized on a vortex mixer for 2 min under anaerobic conditions. The homogenates were transferred to sterile glass bottles and then supplemented with sterile saline solution, sterile inulin to final concentration of 10 g/L of suspension (0.2 g per gram of wet weight of cecal content), bacterial suspension up to approximately 5×10^9 propionibacteria per gram of wet weight of cecal content, or a combination of propionibacteria and inulin to obtain four different fermentation mixtures. Afterwards, they were incubated without stirring for 10 h at 37 °C in the anaerobic glove box and samples were withdrawn at regular time intervals. Counts of bacteroides, clostridia, lactobacilli, bifidobacteria and *P. acidipropionici*, pH values and the concentration of fermentation products were determined.

The CERELA Committee of Ethics approved the protocol used for animal studies.

2.4. Organic acids analysis

Samples taken for organic acids analyses were acidified with H_2SO_4 0.01 mol/L, centrifuged for 30 min (3000 × *g*; 4 °C) and filtered onto 0.2 μm pore size membranes (Millipore, Massachusetts, USA). Ethanol, SCFA and lactic acid produced during fermentations were determined using a 300 × 7.8 mm Rezex ROA-organic acids column (Phenomenex, Torrance, USA) operated at 55 °C with H_2SO_4 0.01 mol/L as the mobile phase. Products concentrations were reported as mmol/g of cecal content.

2.5. Enumeration of total bacteria in cecal contents

For enumeration of bacterial populations, samples were prepared in a way similar to other reports [25–28]. Cecal homogenates were centrifuged at 700 × g for 1 min to remove large particles from the suspensions and 300 μL of sample supernatants were diluted with 900 μL of freshly prepared cold 4% paraformaldehyde solution (PFA) in 130 mmol/L NaCl, 10 mmol/L phosphate buffer pH 7.2 (PBS). Samples were fixed for 16 h at 4 °C, centrifuged (10,000 × g; 5 min; 4 °C), washed twice and suspended