Anaerobe 30 (2014) 137–145

Contents lists available at ScienceDirect

Anaerobe

journal homepage: www.elsevier.com/locate/anaerobe

Clinical microbiology

Study on bioactivity of cell-free filtrates from dairy propionibacteria

Alfonso Filippone, Milena Sinigaglia, Clelia Altieri*

Department of the Science of Agriculture, Food and Environment, University of Foggia, Via Napoli 25, 71122 Foggia, Italy

ARTICLE INFO

Article history:
Received 25 June 2014
Received in revised form
15 September 2014
Accepted 19 September 2014
Available online 30 September 2014

Keywords:
Bioactivity
Cell-free filtrate
Propionibacteria
Probiotic stability time

ABSTRACT

In this paper, the bioactivity of cell-free extracts obtained by dairy propionibacteria strains was investigated. Probiotic bifidobacteria and lactobacilli were used as microbial targets. The extracellular filtrates were added as ingredient (1% v/v) into the growth medium; the effect of cell-free filtrates was evaluated through viable count of microbial targets on appropriate media, monitoring the response of target microorganisms both in growth and death phase. The Gompertz equation was used to model the experimental data. Kinetics and time parameters were estimated in order to quantify the effectiveness of cell-free filtrates effect.

To emphasize the results about the bioactivity of cell-free extract, supporting a complete picture of response, a new approach was developed: Probiotic Stability Time was calculated.

This temporal parameter, defined as the time over that the cell load preserve a living value upper than 10^7 cfu ml^{-1}, was very useful to evaluate the probiotic capability and effectiveness.

A stimulant effect was registered on growth and a positive one was recordered on survival of both bifidobacteria and lactobacilli strains, and the results obtained suggest that a prebiotic activity by dairy propionibacteria cell-free filtrates could be supposed.

The cell-free filtrate obtained from *Propionibacterium freudenreichii* subsp. *shermanii* was the most effective, in our experimental conditions.

Although bifidobacteria were the most sensitive to the effect of cell-free filtrates, lactobacilli have been showed a similar probiotic stability time, showing a high sensitivity to the filtrates.

This paper is the first report of a positive bioactivity by propionibacteria cell-free filtrates on lactobacilli.

1. Introduction

Propionic acid bacteria (PAB) are gram-positive, nonsporing, nonmotile, pleomorphic rods. Although some strains may be relatively aerotolerant, they are basically anaerobes, producing propionic acid, acetic acid, and CO_2 as their main fermentation products. Their optimal growth temperature is between 30 and 37 °C. PAB are divided into two main groups: the classical or dairy PAB and the cutaneous PAB [1].

The classical PAB are commonly used as starter cultures in the dairy industry. Freudenreich and Jensen [1] first described propionibacteria when studying propionic acid fermentation in Emmental cheese. The dairy species are *Propionibacterium acidipropionici*, *Propionibacterium jensenii*, *Propionibacterium thoenii*, and both subspecies of *Propionibacterium freudenreichii*. PAB play an important role in the production of flavour compounds and in the ripening of Swiss type cheese.

The literature concerning the potential probiotic properties of PAB is very limited compared to that about lactobacilli and bifidobacteria. However, in recent years an increasing number of reports on the potential health benefits of PAB have been published; the dairy PAB have a number of properties that make them good probiotic candidates. Altieri et al. [2] observed that dairy propionibacteria strains performed very good adhesion percentages to IPEC-J2 cells, were able to tolerate low pH and bile salts and showed a significant antimicrobial activity against *E. coli* O157:H7 and competitive capabilities against *Staphylococcus aureus*.

Dairy propionibacteria can produce changes in the composition of intestinal microflora, in fact, in mice feeding on dairy PAB has been observed a modulation of the fecal microflora, such as a reduction in the coliforms levels [3], and an increase in fecal bifidobacteria and lactobacilli and a decrease in enterobacteria and staphylococci were observed in children with microflora abnormalities and treated with propionibacteria [4].

* Corresponding author. Tel.: +39 0881 589235.
E-mail addresses: clelia.altieri@unifg.it, cleliaaltieri@libero.it (C. Altieri).

http://dx.doi.org/10.1016/j.anaerobe.2014.09.016
1075-9964/

P. freudenreichii subsp. *shermanii* JS and *P. acidipropionici* can reduce fecal enzyme activity and remove aflatoxins, in fact, it was observed that *P. acidipropionici* CRL1198 reduced the fecal β-glucoronidase in mice [3].

PAB have been observed to be able to produce conjugated linoleic acid and biologically active components; in fact, in the formation of propionic acid, PAB use enzymes containing several specific cofactors such as vitamin B_{12}, folic acid, and biotin [5].

Besides, dairy PAB reduce serum cholesterol levels [6] exerting cholesterol-lowering effects, and modulate the immune response.

Organic acids, and in particular propionic acid, have been observed to stimulate the growth of bifidobacteria. In fact, propionate is often added to media for the selective enumeration of bifidobacteria.

The ability of PAB to stimulate the growth of bifidobacteria was first observed by Kaneko et al. [7]; they found a novel bifidogenic growth stimulator (BGS) produced by *P. freudenreichii*, and described as a mixture of 1,4-dihydroxy-2-naphtoic acid (DHNA), 2-amino-3-carboxy-1,4-napthoquinone (ACNQ), and other unknown compounds. DHNA is a precursor of menaquinone (vitamin K) [8] [9]. Yamasaki et al. [10] hypothesized ACNQ functions, as a mediator of electron transfer from NAD(P)H to O_2 and H_2O_2 in bifidobacteria cells. They also speculated that the exogenous oxidation of NADH is an efficient way for bifidobacteria to store pyruvate and to generate ATP [11]. A very interesting finding was reported by Satomi et al. [12], who observed a significant increase in the number and in the frequency of occurrence of bifidobacteria in fecal samples of healthy human adults, during the intake period of culture powder of *P. freudenreichii*.

The promotion of the specific growth of bifidobacteria by BGS produced by propionic acid bacterium and thereby its health-promoting effects were considered to be due to a mechanism quit different from that exerted by oligosaccharides.

In a recent study, Kouya et al. [13] investigated the production of a bifidogenic growth stimulator by anaerobic and aerobic cultivations of several dairy propionibacterial strains. In order to measure the PAB extracellular BGS concentration, they evaluated the effects of bioassay conditions using only *Bifidobacterium longum* as a test microorganism by measuring the growth-stimulation zone.

Only one report [7] has been realized about cell-free filtrate production from propionibacteria strains, and no studies were conducted on propionibacteria cell-free extract bioactivity on lactobacilli.

In this study, the bioactivity of cell-free filtrates obtained from dairy propionibacteria strains on growth and death of bifidobacteria and lactobacilli was investigated through growth and death parameters, focussing on temporal parameters.

The analysis of these ones are considered a new approach to investigate the effect (positive or negative) of cell-free filtrates using PAB extracellular filtrates as well as ingredient into the growth medium because potential or not potential probiotic capability are emphasized.

2. Materials and methods

2.1. Microorganisms

P. freudenreichii subsp. *shermanii* DSM 20270 and *P. jensenii* DSM 20279 were examined throughout this study for the bioactivity of their cell-free extract.

Both strains were purchased from DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Germany) and stocked at −80 °C in Sodium Lactate Medium, as recommended by the producer (www.dsmz.de).

The influence of the PAB cell-free extract was tested against the following probiotic strains: *Lactobacillus plantarum* c1, *L. plantarum* c83 and *L. plantarum* c99, isolated from table olives "*Bella di Cerignola*" [14], *Bifidobacterium animalis* DSM 10140, *Bifidobacterium subtile* DSM 20096 and *Bifidobacterium breve* DSM 20213, purchased from DSMZ collection, all the strains were stocked at −80 °C in appropriate media.

Lactobacilli strains are wild strains and they could be possible multifunctional starters for Bella di Cerignola olives for mainly their probiotic properties and technological characteristics [15].

2.2. Purification of cell-free filtrate from Propionibacterium spp. cultures

P. freudenreichii subsp. *shermanii* and *P. jensenii* cells were grown in MRS broth (Oxoid, Milan, Italy) added with cysteine (0.05 w/v) (cMRS) (Sigma–Aldrich, Milan, Italy), at 37 °C for 72 h. The cells were harvested from the broth by centrifugation at 5000 rpm at 5 °C, for 15 min and then washed twice with sterile saline solution (NaCl 9 g/l). The supernatant was filtered through a 0.45 μm Millipore filter (Whatman, Dassel, Germany). This filtrate represented the cell-free hydrosoluble fraction [7]. Viable count was evaluated on all filtrates produced and no microbial cells were found.

2.3. Assay of the cell-free fraction

Frozen cultures of *L. plantarum* c1, *L. plantarum* c83 and *L. plantarum* c99, were thawed and pre-cultured in MRS broth at 30 °C for 48 h; *B. animalis*, *B. subtile*, *B. breve*, were thawed and pre-cultured in cMRS broth at 37 °C for 48 h. Serial dilutions of cell suspensions were carried out to get the desired inoculum level (10^2 cfu ml^{-1}).

The assays were performed in 250 ml Erlenmeyers, containing 100 ml of MRS broth or cMRS broth, for lactobacilli and bifidobacteria, respectively. The cell-free filtrate from propionibacteria, was added as medium ingredient (1% v/v).

The samples were inoculated with lactobacilli or bifidobacteria initial inocula (10^2 cfu ml^{-1}) and for each assay pure cultures of the microbial targets, without cell-free filtrate, were used as controls. The samples were incubated at 37 °C in anaerobic condition using paraffin layer, and the viable count was periodically evaluated on MRS agar, incubated at 30 °C, and cMRS agar, incubated at 37 °C until the phase of cell death, for lactobacilli and bifibobacteria, respectively. The plates were incubated in anaerobic condition using Anaerobic Gas Generating Kit (Oxoid, Milan, Italy).

3. Modelling

The cell loads recorded for each microbial group were modelled according to Gompertz equation modified by Zwietering et al. [16]:

$$y = k + A\exp\{-\exp[(\mu_{max} \times e/A) \times (\lambda - t) + 1]\}, \quad (1)$$

where y is log (cfu ml^{-1}), k is the initial level of the dependent variable to be modelled [log (cfu ml^{-1})], A is the maximum increase of bacterial load attained at stationary phase [log (cfu ml^{-1})], μ_{max} is the maximal growth rate [Δlog (cfu ml^{-1}) $hours^{-1}$], λ is the lag time (hours) and t is the time (hours).

A negative Gompertz equation was used to model the cell load data of each microbial group in death phase:

$$y = n_0 - \Delta\exp\{-\exp[(d_{max} \times e/\Delta) \times (\alpha - t) + 1]\}, \quad (2)$$

where y is log (cfu ml^{-1}), n_0 is the initial level of the dependent variable to be modelled (corresponding to maximum bacterial