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Two potential probiotic lactobacillus strains isolated from olive microbiota exhibit adhesion and anti-proliferative effects in cancer cell lines

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ABSTRACT

The beneficial effects of two potential probiotic lactobacillus strains, namely *Lactobacillus pentosus* B281 and *L. plantarum* B282 were examined and the mechanisms of action were investigated. Quantitative analysis and confocal microscopy showed that both strains exhibited a significant higher adherence to Caco-2 cells in comparison to the reference strain *L. casei* ATCC 393. Treatment with conditioned media (CM) of the two strains caused significant reduction of cell proliferation, as demonstrated by Sulforhodamine B (SRB) and clonogenic assays. Moreover, the CM of the two strains induced a G1-phase arrest and down-regulation of specific cyclin genes, as indicated by flow cytometry and real-time PCR analysis. To begin elucidating the nature of the bacterial components conveying these responses, the anti-proliferative effect of heat-treated CM was analysed. The anti-proliferative activity of heat-treated CM was similar to the non-heated CM in a time- and dose-dependent manner, indicating the presence of thermostable bioactive compounds.

1. Introduction

The term probiotic is a compound word that consists of the Latin word pro and the Greek word bios, and it literally means for life. In this context, the Food and Agriculture Organization of United Nations and the World Health Organization have defined probiotics as 'live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (FAO/WHO, 2002). The beneficial effects of probiotics

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may include management of severe digestive disorders (Del Carmen et al., 2011; Deshpande, Rao, Patole, & Bulsara, 2010), prevention of osteoporosis (Britton et al., 2014; Ohlsson et al., 2014), attenuation of the symptoms of stress-related diseases, such as anxiety and depression (Bravo et al., 2011), and prevention of the early stages of colon cancer development (Uccello et al., 2012; Zhong, Zhang, & Covasa, 2014).

Most probiotic microorganisms are lactic acid bacteria (LAB), among them lactobacilli represent one of the major microbial groups. They have been introduced in a wide range of food products, including yogurts, ice creams and other dairy products as well as non-dairy products, such as fruit juices and fermented sausages (Granato, Branco, Gomes Cruz, de Assis Fonseca Faria, & Shah, 2010; Rivera-Espinoza & Gallardo-Navarro, 2010). In addition, recent studies demonstrated the production of Spanish-style green olives with the employment of two potential probiotic LAB strains, namely *Lactobacillus pentosus* B281 and *L. plantarum* B282, as starter cultures (Argyri, Nisiotou, Mallouchos, Panagou, & Tassou, 2014; Blana, Grounta, Tassou, Nychas, & Panagou, 2014). The two strains were isolated previously from olive microbiota (Doulgeraki et al., 2013) and were found to possess desirable probiotic properties in a series of established *in vitro* tests (Argyri et al., 2013). However, the molecular and cellular mechanisms by which the two strains exert their probiotic effects have not yet been examined.

It is generally believed that probiotics act via alteration of the intestinal microflora, enhancement of the host's immune response, cell cycle arrest, promotion of apoptosis and induction of short-chain fatty acid (SCFA) production (Faghfoori, Gargari, Gharamaleki, Bagherpoure, & Khosroushahi, 2015; Kahouli, Tomaro-Duchesneau, & Prakash, 2013). In addition, the ability of probiotic cells to adhere on the intestinal mucosa is a critical parameter to probiotic action. It has been shown that specific probiotic strains exert anti-proliferative effects via synergic actions between adhesion to colon cancer cells and production of short-chain fatty acids, mainly butyric and propionic acids (Thirabunyanon & Hongwittayakorn, 2013). The adhesive probiotic cells may also act competitively and inhibit adhesion and invasion of pathogens on the intestine and/or stimulate the host's immune response. For example, it has been shown that adhesive lactobacillus strains on HT-29 colon cancer cells exert antagonistic activity against *Campylobacter jejuni in vitro* (Wang et al., 2014). Similarly, adhesion of specific LAB strains on Caco-2 cells inhibited *Salmonella enteritidis* adhesion and invasion to Caco-2 cells by induced expression of tumour necrosis factor-α and interleukin-12 (Feng, Liu, & Zhao, 2015).

In the present study the modes of action and the potential beneficial effects of *L. pentosus* B281 and *L. plantarum* B282 were investigated to establish further the probiotic character of the two strains. Evaluation of their adhesion properties and anti-proliferative effects on human colorectal cancer cells and their involvement on cell cycle distribution and on the expression levels of specific cell cycle related genes was performed. Finally, preliminary characterization of the nature of the bioactive molecules present in the conditioned media of the two strains was also conducted.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The strains *L. pentosus* B281 and *L. plantarum* B282 were isolated from naturally fermented olives of cv. Conservolea and Halkidiki (Argyri et al., 2013). *L. rhamnosus* GG ATCC 53103 and *L. casei* ATCC 393 strains were obtained from DSMZ (Braunschweig, Germany). All LAB strains were grown anaerobically at 37 °C on MRS broth (Merck, Darmstadt, Germany).

2.2. Preparation of conditioned media

For the preparation of *L. pentosus* B281 or *L. plantarum* B282 CM, the strains were grown in MRS broth (Fluka, Buchs, Switzerland) at 37 °C for 16 hr. The cultures were diluted and used to inoculate RPMI-1640 (Biosera, Boussens France) containing 10% FBS (Biosera) and 25 mM Hepes (Biosera) and were grown anaerobically for 24 hr at 37 °C. Culture supernatants were collected by centrifugation at 4000 × g for 10 min, and filtered twice through a 0.22 μm pore-size filter. For testing their thermostability, CM of *L. pentosus* B281 and *L. plantarum* B282 were prepared as described above and then subjected to heat inactivation for 30 min at 100 °C.

2.3. Cancer cell lines

The human colon adenocarcinoma cell lines Caco-2 and HT-29 were purchased from the American Type Culture Collection (ATCC). They were cultured in RPMI-1640 medium with stable glutamine (Biosera), supplemented with 10% FBS (Biosera), 100 U/mL penicillin (Biosera), and 100 μg/mL streptomycin (Biosera). Both cell lines were cultured at 37 °C, 5% CO_2 in a humidified atmosphere.

2.4. Determination of bacterial cell adherence by quantitative analysis and confocal fluorescent microscopy

The quantitative analysis of bacterial adhesion to Caco-2 cells was performed as described previously (Argyri et al., 2013) with minor modifications. In brief, 2×10^5 Caco-2 cells/well were seeded in 24-well microplates and cultured in RPMI-1640 containing 10% FBS and 1% penicillin/streptomycin (all from Biosera) for 24 h at 37 °C, 5% CO_2 in humidified atmosphere. Bacterial cultures were grown in MRS overnight at 37 °C. 10^7 bacteria cells were added to each well, with each strain being tested in triplicates. After 4 h of co-incubation at 37 °C, the cells were washed twice with PBS, lysed with 1% Triton X-100 and the lysates were serially diluted, plated on MRS agar and incubated at 37 °C, for 72 h. Adhesion values (%) were calculated as follows: % Adhesion = $(V_1 / V_0) \times 100$, where V_0 is the initial viable count of bacteria tested and V_1 is the viable bacteria count obtained from the Caco-2 cells, at the end of the experiment.

For the detection of bacterial cell adhesion by confocal fluorescent microscopy, *L. pentosus* B281, *L. plantarum* B282 and *L. casei* ATCC 393 were stained with 20 μM CFSE (Thermo Fisher Scientific, Waltham, MA, USA). Caco-2 cells (5×10^4 cells/well) were seeded on 8-well μ-slide (IBIDI, Martinsried, Germany), grown for 24 hours at 37 °C, 5% CO_2 and then were incubated