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Tea extract render probiotic *Lactobacillus helveticus* more resistant to oxygen exposure through lipid modification mechanism

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ABSTRACT

Exposure to oxygen can cause a decrease in growth rates or a complete inhibition of growth of oxygen-sensitive probiotic bacteria. A recent study in our laboratory demonstrated that the growth of an oxygen-sensitive strain, *Lactobacillus helveticus*, was stimulated, under aerobic conditions, when the culture medium was enriched with green tea extracts (GTE). However, information on the mechanism by which GTE influenced the growth, in the presence of oxygen, of that strain is limited. In the present work, the effects of GTE concentrations (0 to 2000 μg/mL) and exposure to oxygen on maximal populations of *L. helveticus* R0052 cells and bacterial lipids were evaluated using viable counts, infrared spectroscopy and gas chromatography analyses. Supplementation of the culture medium with 0 to 500 μg/mL GTE did not have an effect on the populations reached under microaerophilic conditions and on bacterial lipid structure and composition. However, at 2000 μg/mL GTE, high population levels were reached under microaerophilic conditions concomitant with an increase in lipid order and with important changes in fatty acid composition of the bacterial lipids. Interactions between GTE components and bacterial lipids were shown by spectroscopic results. Moreover, bacterial cells have adapted to the presence of 2000 μg/mL GTE in the growth medium by changing their lipid composition. To the best of our knowledge, this work is the first to establish a relationship between the effects of GTE at 2000 μg/mL on bacterial cell's lipids and a stimulation of growth under microaerophilic conditions of an oxygen-sensitive strain.

1. Introduction

Green tea (*Camellia sinensis*) contains polyphenols which have been reported to have many potential health benefits such as cancer chemopreventing properties, antimutagenic, antidiabetic, anti-inflammatory and antiviral activities (Cabrera, Artacho, & Giménez, 2006). These health-promoting properties have been attributed, in part, to strong antioxidative activities of tea catechins (Jaziri, Slama, Mhadhbi, Urdaci, & Hamdi, 2009). The antimicrobial activity of green tea extracts (GTE) containing catechins against pathogenic bacteria is also well known (Radji, Agustama, Elya, & Tjampakasari, 2013; Sharma, Gupta, Sarethy, Dang, & Gabrani, 2012). In contrast, green tea and catechins had no inhibitory effect on many beneficial bacteria (Almajano, Carbó, López Jiménez, & Gordon, 2008; Jaziri et al., 2009; Su, Henriksson, Nilsson, & Mitchell, 2008). They have also stimulated the growth of beneficial bacteria in in vitro (Vodnar, Ranga, Pop, & Socaciu, 2012) and in vivo human studies (Jin, Touyama, Hisada, & Benno, 2012). Moreover, a previous study in our laboratory demonstrated that a probiotic strain with oxygen sensitivity, *Lactobacillus helveticus* R0052, was able to grow in the presence of oxygen to the same extent than under anaerobic conditions when the culture medium was enriched with GTE at concentrations up to 500 μg/mL (Gaudreau, Champagne, Remondetto, Bazinet, & Subirade, 2013). Since exposure of oxygen-sensitive bacteria to dissolved oxygen generally results in a decrease of their growth rates or in a complete inhibition of their growth, the use of GTE could be interesting for commercial producers of oxygen-sensitive *Lactobacillus* probiotics.

At our knowledge, there is limited information on the mechanism by which GTE can influence the growth, in the presence of oxygen, of oxygen-sensitive probiotic strains. The important ability of tea catechins to scavenge oxygen species (Wiseman, Balentine, & Frei, 1997), to chelate metal ions known to catalyze many processes leading to the appearance of free radicals (Saija et al., 1995) and to decrease the oxido-reduction potential of the growth medium (Gaudreau et al., 2013) could explain, in part, the stimulation of growth under aerobic conditions observed in our previous study. Nevertheless, catechins can

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modulate the physical structure of lipids of bacterial cells (Bernal et al., 2010; Zhao & Shah, 2014b) and preliminary spectroscopic analyses suggested that the effects of GTE on bacterial growth in the presence of oxygen could also involve the cell's lipids (Gaudreau et al., 2013).

Fourier transform infrared (FTIR) spectroscopy can be employed to study interactions between a compound and membranes of living bacterial cells (Santivarangkna, Naumann, Kulozik, & Foerst, 2010), monitor properties of bacterial membranes in changing environments (Scherber, Schottel, & Aksan, 2009) and structural changes in bacterial cells after exposure to stress conditions (Alvarez-Ordóñez & Prieto, 2010; Kamnev, 2008). In this study, FTIR spectroscopy was employed to monitor the effects of GTE and dissolved oxygen exposure on the structure and order of lipids of *Lactobacillus* cells. Moreover, the composition of bacterial cell's lipids was studied by gas chromatography analyses. In this context, the objective of the present work was to employ viable counts, FTIR spectroscopy and fatty acid analysis to study the impacts of aeration conditions (microaerophilic versus anaerobic) and GTE concentration in the culture medium on *L. helveticus* R0052 populations and on the lipids of these bacterial cells.

2. Materials and methods

2.1. Bacterial strain and culture conditions

L. helveticus R0052 was kindly provided by Lallemand-Health Solutions Inc. (formerly Institut Rosell, Montreal, Qc, Canada). Stock cultures were prepared by mixing MRS + ascorbic acid (0.1%) grown culture with sterile 20% (w/v) glycerol and sterile 20% (w/v) reconstituted skim milk in a ratio 2:5:5 and freezing resulting solutions at −80 °C in 1 mL portions. Before each independent assay, fresh cultures were prepared by adding one thawed stock culture to 9 mL of lactobacilli MRS broth + ascorbic acid (0.1%), and incubating about 10 h at 37 °C in an anaerobic jar containing Anaerogen sachets (Oxoid) until a pH close to 4.5 was reached.

2.2. Preparation of green tea extract solutions

Aqueous green tea extract (GTE) solutions were prepared by solubilizing, in deionized water, a GTE purchased from Pharmacie des Vosges (Nice, France). These solutions were centrifuged (5804R, Eppendorf, Mississauga, ON, Canada) at 7822 ×*g* (10 min/room temperature) in order to remove insoluble components. Supernatant fluids were sterilized by filtration through 0.45 μm filter units (Acrodisc sterile syringe filters, Pall, Mississauga, ON, Canada). A solution of 50 mg/mL GTE contained 4.9 mg/mL epigallocatechin gallate, 3.5 mg/mL gallocatechin gallate, 2.3 mg/mL epigallocatechin, 1.4 mg/mL epicatechin gallate, 0.7 mg/mL epicatechin and 3.7 mg/mL caffeine (Rozoy, Bazinet, Araya-Farias, Guernec, & Saucier, 2013).

2.3. Biomass production

A 1% inoculum of fresh culture was grown at 37 °C in MRS broth (25 mL in 50 mL test tubes) supplemented with 0, 250, 500, 1000 and 2000 μg/mL GTE. These concentrations were selected because a previous study (Gaudreau et al., 2013) has shown that GTE had no negative effect on growth rates of *L. helveticus* R0052 at concentrations up to 500 μg/mL, but increased the biomass levels when up to 1000 μg/mL GTE was added. Microaerophilic broth cultures were continuously shaken at 140 rpm (Labline Orbit Environ-shaker, Melrose Park, IL). Anaerobic growth was performed in anaerobic jars containing Anaerogen sachets (Oxoid). Fermentations were stopped when the cultures had attained the beginning of the stationary growth phase, i.e. when the pH of the culture media was close to 4.5.

2.4. Analyses

Viable counts on samples taken at the end of fermentations were obtained by plating appropriate dilutions (0.1% sterile peptone) in MRS agar (EMD Millipore, Billerica, MA). Plates were incubated at 37 °C for 48 h in anaerobic jars containing Anaerogen sachets (Oxoid).

Dissolved oxygen in broths was measured at the beginning and end of fermentations carried out under microaerophilic conditions using a VWR Symphony electrode (VWR Scientific Products, West Chester, PA) mounted with the specified membrane and filled with the supplied DO electrolyte solution. The electrode was connected to a VWR Symphony SP50D portable DO meter.

Total bacterial lipids were extracted according to the method developed by Lewis, Nichols, and McMeekin (2000) after adaptations. Cells, from 4 × 25 mL fresh cultures, were harvested by centrifugation (1548 ×*g*/5 min/4 °C) and washed with a 1% NaCl solution. Cell pellets were extracted for lipids into glass tubes to which a total of 11.4 mL were added in the sequence: chloroform, methanol and potassium phosphate buffer (0.05 M, pH 7.5) to achieve a final chloroform:methanol:phosphate buffer ratio of 1:2:0.8 (v/v/v). Samples were Vortex-mixed for 15 s following the addition of each solvent and allowed to stand in the darkness for about 18 h at 5 °C. The mixtures were filtrated with 2.5 μm pore size membranes (Scleicher and Schuell Bioscience Inc., Keene, NH) to remove residues. Bacterial lipids were then partitioned by addition of chloroform and saturated NaCl solution (3.5%) to obtain a final chloroform:methanol:aqueous phase ratio of 1:1:0.9. Lower organic phases were recovered and dried over anhydrous sodium sulfate (Anachemia Canada, Lachine, Qc, Canada). Total lipid extracts were dried under a gentle stream of nitrogen and dissolved with 1 mL hexane. Lipids were then methylated by addition of 500 μL 0.5 N sodium methoxide solution in methanol (Sigma-Aldrich, Oakville, ON, Canada) at 45 °C for 15 min. Lipids were partitioned by addition of chloroform (4 mL) and saturated saline (5 mL). Recovered lipid phases were dried over anhydrous sodium sulfate. Hexane (5 mL) was added and lipid phases were dried over anhydrous sodium sulfate. Resulting fatty acid methyl esters (FAME, 1 μL) were analyzed by gas chromatography using a Hewlett-Packard serie II (Mississauga, ON, Canada) gas chromatograph equipped with a BPX-70 capillary column (SGE, Melbourne, Australia, 60 m × 0.25 mm i.d., 0.25 μm film thickness) with a flame ionization (FID; at 250 °C) and a splitless detector. The column was operated at 60 °C for 1 min; then the temperature was increased at 10 °C/min up to 190 °C, held for 15 min, increased at 5 °C/min up to 200 °C and held for 14 min. Hydrogen was used as carrier gas under a constant head pressure of kPa. Identification was performed by comparison with spectra of a FAMEs standard mixture (GLC-607, Nu Chek Prep, Elysian, MN) analyzed under the same conditions. Identification of fatty acid methyl esters was verified and completed by GC–MS analysis using a Hewlett Packard HP 6890 model gas chromatograph and equipped with an Agilent (Mississauga, ON, Canada) mass selective detector model 5973N. The column used and the analysis conditions were as presented above for GC analyses except that the split ratio was 25:1. Mass spectra were recorded at an electron energy of 70 eV. The ion source, transfer line and quadrupole temperatures were 230 °C, 170 °C and 150 °C, respectively. The mass range used was 30 to 450 amu. Identification was performed by comparison with mass spectra of the NIST2005 MS database and of a BAME mixture (Sigma-Aldrich, Oakville, Canada) and CLA pure standards (Nu-Chek Prep, Elysian, MN) analyzed under the same conditions.

For FTIR analysis, cells were harvested after fermentation by centrifugation (1548 ×*g*/5 min/4 °C) and quickly washed two times with deionized water in order to prevent interferences on FTIR spectra from media components and bacterial metabolites. FTIR analyses were performed according to Gaudreau et al. (2013) except that each spectrum was appodized with a triangular function and that spectral processing was performed using Omnic software (version 7, Thermo Electron Corporation) and Varian Resolution Pro 4.0 (Agilent Technologies, Mississauga, ON, Canada).