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# Survival and metabolic activity of probiotic bacteria in green tea

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ABSTRACT

The aim of this study was to determine the enzymatic activity and survival of three probiotic strains (*Lactobacillus paracasei* LAFTI-L26, *Lactobacillus acidophilus* LAFTI-L10 and *Bifidobacterium animalis ssp. lactis* LAFTI-B94) during incubation in six different varieties of green tea extracts. The polyphenol content, antioxidant, antimicrobial and antihypertensive properties in green tea (variety *Wu Lu Mountain*) and in two standards (epigallocatechin-3-gallate and rutin) were also investigated before and after incubation with *B. animalis B94*. The green tea extracts permitted the survival of the selected probiotic strains better than the saline solution, with *B. animalis* B94 maintaining the highest levels of viable cells. These selected probiotic bacteria exhibited $\beta$-glucosidase, $\beta$-galactosidase and $\alpha$-rhamnosidase activity. The antioxidant and antihypertensive properties of standard solutions of epigallocatechin-3-gallate and rutin increased after incubation with *B. animalis* B94, caused by polyphenol content reduction and the formation of other more highly biologically active metabolites. However, during the incubation of green tea with *B. animalis* B94, changes in the concentration of the most abundant green tea polyphenols did not enhance the biological activity.

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## 1. Introduction

Green tea is produced with unfermented leaves and buds from the plant *Camellia sinensis* L. These components are rich in flavan-3-ols (epicatechin, catechin and galloylated derivates), which are responsible for the majority of the beneficial properties of green tea. Other major polyphenols present in green tea are the flavonols, mainly derivatives of quercetin and kaempferol (principally glycosylated). Green tea also contains other compounds like tea pigments, amino acids, vitamins, carbohydrates, minerals and purine alkaloids (Graham, 1992). The beneficial effects of green tea have been attributed to all these compounds, but principally to phenolic compounds because of their high antioxidant properties (Bolling, Chen, & Blumberg, 2009; Weisburger & Chung, 2002).

Probiotic bacteria, with a wide variety of positive health effects, are principally members of the *Bifidobacterium* and *Lactobacillus* family. Probiotics have been extensively studied and explored commercially in many different products, including dairy and non-dairy products (Champagne, 2009; Soccol et al., 2010). Probiotic microorganisms contribute to intestinal balance, play a role in maintaining health and can even improve the quality of some products formulated with them (Soccol et al., 2010).

The fermentation of foods with probiotic microorganisms could improve or add additional beneficial properties to the product. This is because some probiotic bacteria possess certain enzymatic activities (such as deglycosylation, ring-fission, dehydroxylation, etc.) (Aura, 2008), that are capable, in some cases, of transforming polyphenols into compounds which are even more bioavailable or bioactive than the original polyphenol (Donkor & Shah, 2008; Tsangalis, Ashton, McGill, & Shah, 2002; Uskova, Kravchenko, Avrenjeva, & Tutelyan, 2010). However, despite the beneficial properties contained in the composition of green tea, green tea has rarely been used as a food matrix for probiotics. Furthermore, no published reports have been found that deal with the effect of the probiotics selected for this study (*Lactobacillus acidophilus* LAFTI-L10®, *Bifidobacterium animalis spp. lactis* LAFTI-B94® and *Lactobacillus paracasei* LAFTI-L26®) on green tea polyphenols. The aim of this work was to study the survival of the probiotic bacteria in different varieties of green tea extracts and determine their enzymatic activity. The capability of one of this probiotics, *B. animalis* B94, to transform polyphenols while maintaining the initial properties of the tea was also tested. In addition, the survival of *B. animalis* B94 in two different polyphenol solutions and their transformation by the probiotic strain was studied.

## 2. Materials and methods

### 2.1. Chemicals

Standard polyphenol compounds: (−)-epigallocatechin gallate (EGCG, CAS: 989-51-5) and rutin (CAS: 153-18-4), (−)-epigalloca

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techin (EGC, CAS: 970-74-1), (+)-catequin (C, CAS:154-23-4), (−)-epicatequin (EC, CAS: 490-46-0), (−)-epicatechin-3-gallate (ECG, CAS: 1257-08-5), Hyperoside (CAS: 482-36-0), quercetin-3-*O*-glucoside (CAS: 482-35-9), kaempferol-3-*O*-rutinoside (CAS: 17650-84-9), kaempferol-3-*O*-glucoside (CAS: 480-10-4) were purchased from Extrasynthese (Genay, Cedex, France).

Reagents for enzyme activity assay: *ρ*-nitrophenyl-*β*-D-glucopyranoside, *ρ*-nitrophenyl-*β*-D-galactopyranoside, *ρ*-nitrophenyl-*α*-D-rhamnopyranoside, *ρ*-nitrophenol were provided by Sigma–Aldrich (Stenheim, Germany).

HPLC-MS: HPLC grade methanol, acetonitrile and formic acid (VWR international, Inc., Barcelona, Spain). HPLC grade water was prepared from distilled water using a Milli-Q system (Millipore Laboratory Bedford, MA).

Antioxidant activity: FRAP: (2,4,6-tripyridyl-*s*-triazine), $FeCl_3$ and $FeSO_4$ (Sigma–Aldrich, St. Louis, Mo., USA). ABTS: [2,2′-azino-*bis*-(3-ethylbenzothiazoline-6-sulfonic acid)], potassium persulphate, Vitamin C (Sigma–Aldrich, St. Louis, Mo., USA).

Reagents for antihypertensive activity: HHL (Hippuryl-L-Histidyl-L-Leucine), ACE (CAS: 9015-82-1), potassium phosphate buffer, containing 300 mM NaCl, pH 8.3 (Sigma–Aldrich, St. Louis, Mo, USA).

### *2.2. Bacterial strains and growth conditions*

Lyophilised strains of *L. paracasei* LAFT-L26® (L26), *L. acidophilus* LAFTI-L10® (L10) and *B. animalis ssp. lactis* LAFTI-B94® (B94) were purchased from DSM (DSM Food Specialties Ltd., Sydney, Australia) and kept at −20 °C until use. To obtain colonies, probiotics were dissolved in MRS broth and then in MRS agar medium, in both cases at 37 °C for 24 h. For B94, MRS broth and agar were supplemented with 0.05 g/100 g cysteine and incubated under anaerobiosis.

### *2.3. Enzyme activity assay of probiotic strains*

Activity assays were carried out using the method of Ávila et al. (2009), but with some modifications. Probiotic bacteria were grown as explained above and then the cells were harvested by centrifugation (6000*g*, 10 min, 5 °C) and washed twice with 50 mmol/L sodium phosphate buffer, pH = 7. The harvested cells were suspended in the same buffer to an $OD_{620}$ ∼0.6. *β*-Glucosidase, *β*-galactosidase and *α*-rhamnosidase activity was measured in triplicate with *ρ*-nitrophenyl-*β*-D-glucopyranoside, *ρ*-nitrophenyl-*β*-D-galactopyranoside and *ρ*-nitrophenyl-*α*-D-rhamnopyranoside respectively as substrate. This was done first by mixing 450 μL of 5 mmol/L substrate prepared in 50 mmol/L sodium phosphate buffer, pH 7, with 50 μL of the cell's suspension and incubated at 37 °C for 110 min. Then the reactions were stopped on ice by adding 500 μL of $Na_2CO_3$ 0.2 mol/L and the amount of *ρ*-nitrophenol released was measured at 410 nm spectrophotometerically. One unit of enzyme activity was defined as the amount of *β*-glucosidase or *β*-galactosidase or *α*-rhamnosidase that 1 nmol of *ρ*-nitrophenol released from the substrate/mL/min under the assay conditions (Otieno, Ashton, & Shah, 2005).

### *2.4. Infusion preparation*

Six kinds of green teas were tested in this study: *Japan Sencha Makinohara*, *Japan Gyokuro Asahi*, *Cinnamon Green Tea*, *Lung Ching*, *China White Hair* and *Wu Lu Mountain*. All these green teas were purchased from a specialised local shop. The preparation of green tea extract was carried out according to the method described by López de Lacey (2012). Briefly, 35 g of each leaves was pulverised in a Osterizer blender (Oster®, Sunbean Products Inc., Boca Raton, FL, USA) and mixed with 350 mL of water (Milli-Q) at 80 °C for 30 min, with constant shaking and was then centrifuged at 12,000*g* for 10 min at 5 °C. The supernatant was filtered two times through Whatman N°1 filter papers. The infusion filtered was stored at −20 °C before the analysis.

### *2.5. Survival of bacterial strains in green tea extracts*

First the strains (*L. paracasei* L26, *L. acidophilus* L10 and *B. animalis* B94) were grown as previously explained. The cells were then harvested by centrifugation (6000*g*, 10 min, 5 °C), washed twice with 0.9 g/100 g NaCl solution and suspended in the same solution 0.9 g/100 g NaCl to an $OD_{620}$ ∼0.6. One aliquot of 50 μL of harvested cells adjusted to $OD_{620}$ ∼0.6 was mixed with 950 μL of green tea extract to study the survival rate of bacteria in green tea extracts. 50 μL of 0.9 g/100 g NaCl solution was used as control. The bacterial strains were incubated at 37 °C for 72 h as mentioned above. The final concentration of bacteria was approximately $10^6$ CFU/mL.

The viable cells were determined at 0, 24, 48 and 72 h of incubation in the green tea extracts by analysing 100 μL of each culture with the microdot method (Strahsburger, Baeza, Monasterio, & Lagos, 2005) on MRS agar for the *Lactobacillus* strain and 0.05 g/100 g cysteine MRS agar for *B. animalis* B94. All the counts were performed at least in triplicate.

### *2.6. Survival of B. animalis B94 in two separately prepared polyphenol standard solutions: epigallocatechin-3-gallate and rutin*

Determining the survival of *B. animalis* B94 in two standard polyphenol solutions was performed, first by mixing 1 mL of harvested cells adjusted to $OD_{620}$ ∼0.6 in 19 mL of 0.9 g/100 g NaCl solution. The standards were firstly dissolved in HPLC grade methanol before being added to the NaCl solution. The same concentration of standards was used in all the samples in order to compare the effect on probiotic survival of each compound. The final concentration of the bacteria was approximately $10^6$ CFU/mL and the final concentration of each standard in this solution was 25 μg/mL. *B. animalis* B94 growth was not affected by the methanol. The incubation conditions and the counting of *B. animalis* B94 was carried out as explained above. 0.9 g/100 g NaCl sterile solution containing the bacteria was used as control.

### *2.7. Changes in green tea polyphenols and standard solutions by B. animalis B94*

The study of polyphenol changes derived from the incubation with *B. animalis* B94 was performed in the *Wu Lu Mountain* green tea variety and in two separately prepared polyphenol standard solutions, epigallocatechin-3-gallate and rutin. Both, green tea and standard solution samples, incubated with the bacteria, were prepared exactly as described in the preceding section. In the case of green tea extract, 1 mL of harvested cells adjusted to $OD_{620}$ ∼0.6 were mixed with 19 mL of *Wu Lu Mountain* infusion. One aliquot of the solution was taken for the different analyses (counts, HPLC-MS, antioxidant activity and antimicrobial activity) at 0, 24, 48 and 72 h of incubation at 37 °C. The viable cells were measured using the microdot method explained above. After counting, each aliquot was centrifuged for 10 min at 6000*g*, 5 °C and the supernatant was kept for the rest of the analyses. All the analyses were performed at least in triplicate, except for the HPLC-MS analysis which was carried out in duplicate.

### *2.8. Quantification of polyphenols by HPLC-MS*

Reverse-phase high performance liquid chromatography was performed to analyse phenolic compounds present in the aqueous

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