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Effect of the gastrointestinal environment on pH homeostasis of *Lactobacillus plantarum* and *Lactobacillus brevis* cells as measured by real-time fluorescence ratio-imaging microscopy

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Abstract

In the present work, an in vitro model of the gastrointestinal tract (GIT) was developed to obtain real-time observations of the pH homeostasis of single cells of probiotic *Lactobacillus* spp. strains as a measure of their physiological state. Changes in the intracellular pH (pH_i) were determined using fluorescence ratio imaging microscopy (FRIM) for potential probiotic strains of *Lactobacillus plantarum* UFLA CH3 and *Lactobacillus brevis* UFLA FFC199. Heterogeneous populations were observed, with pH_i values ranging from 6.5 to 7.5, 3.5 to 5.6 and 6.5 to 8.0 or higher during passage of saliva (pH 6.4), gastric (pH 3.5) and intestinal juices (pH 6.4), respectively. When nutrients were added to gastric juice, the isolate *L. brevis* significantly decreased its pH_i closer to the extracellular pH (pH_{ex}) than in gastric juice without nutrients. This was not the case for *L. plantarum.*

This study is the first to produce an in vitro GIT model enabling real-time monitoring of pH homeostasis of single cells in response to the wide range of pH_{ex} of the GIT. Furthermore, it was possible to observe the heterogeneous response of single cells. The technique can be used to determine the survival and physiological conditions of potential probiotics and other microorganisms during passage through the GIT.

Keywords: *Lactobacillus brevis*; *Lactobacillus plantarum*; In vitro gastro-intestinal model; pH_i; FRIM; Probiotic

1. Introduction

The interest in functional foods containing probiotic microorganisms has increased significantly during the most recent decade due to greater awareness of the benefits of probiotics for gut health, disease prevention and therapy [1,2]. Thus, before probiotic bacteria can even begin to fulfill their physiological role in the gut, the bacteria themselves must handle a number of stresses before they reach the target site [3,4]. Passage of probiotics through the gastrointestinal tract (GIT) is a hazardous journey, with stress stages which may affect cell viability. The principal stress is that of shifting pH encountered in the stomach, resulting from gastric acid as well as bile [5,6]. The ability of probiotic bacteria to survive the harsh environments encountered during processing and gastrointestinal transit has been a major factor in their selection criteria [4]. Studies evaluating probiotic survival during GIT transit have shown that the survival rate varies due to several factors, including the probiotic strain and physiologic growth state, as well as the food matrix [3,7,8].

Information on the survival and/or vitality of microorganisms in different environments has traditionally been obtained at the population level, typically using plate count techniques

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and others [9–15]. However, these experimental approaches do not take into account heterogeneity within an isogenic population of cells. Therefore, there increased interest is being paid to methods that can evaluate the physiological status (i.e., viability/vitality) of individual cells within a population [16–18]. Regulation of pH_i has previously been reported as a factor in induction of tolerance to stress, and fluorescence ratio imaging microscopy (FRIM) has been reported to be a method for assessment of the physiological condition of single cells by measurement of their pH_i [19,20]. FRIM has also been used to follow pH homeostasis during changing extrinsic conditions [16,21,22].

Several lactic acid bacteria have been recognized as probiotics due to their health-promoting and nutritional properties [1]. A correlation between a decrease in pH_i and loss of vitality has been found for these bacteria in standardized laboratory media [23,24], and regulation of pH_i has been suggested to be an important factor in induction of tolerance to acid and other stress factors [19]. However, how probiotic lactic acid bacteria regulate their pH_i under changing conditions exposed during passage of the GIT has not been previously investigated.

The aim of this work was to develop a small-scale GIT system for real-time monitoring of the effect of gastrointestinal conditions on the pH homeostasis of individual bacterial cells via FRIM. The effect of simulated gastrointestinal conditions on pH_i was determined for the potential probiotic strains *Lactobacillus plantarum* UFLA CH3 and *Lactobacillus brevis* UFLA FFC199.

2. Materials and methods

2.1. Strains and growth conditions

Strains *L. plantarum* UFLA CH3 and *L. brevis* UFLA FFC199 isolated from fermented foods and previously described as potential probiotic [25] were employed in the present study. They were stored at −80 °C in Man Rogosa Sharpe broth (MRS, Merck, Darmstadt, Germany) with 20% (v/v) glycerol, streaked onto MRS agar and incubated for 48 h at 37 °C. A loopful of cells was transferred to 10 mL MRS broth and incubated for 24 h (37 °C), after which 10 μL were transferred to 10 mL MRS broth and incubated for 18 h (37 °C), obtaining cells at log phase to be used in the assays. The temperature (37 °C) was used to grow the cells at the same temperature as the experiment (simulating the body temperature).

2.2. Fluorescence labeling of L. plantarum and L. brevis strains

Following propagation as described above, *L. plantarum* and *L. brevis* strains were fluorescently labeled with one of the two pH-sensitive probes, CFDA-SE or CDCFDA-SE (both from Molecular Probes Inc., Eugene, OR, USA), using a modified version of a previously published protocol [24]. Briefly, cells (1 mL) were harvested by centrifugation at 10,000× *g* for 2 min and 30 s and resuspended in sterile-filtered (0.22 μm; Millipore, Billerica, MA, USA) citric acid phosphate buffer (pH 5.8). Glucose and one of the two probes were added to a final concentration of 10 mM (glucose), 10 μM CFDA-SE and 39 μM CDCFDA-SE, respectively, and cells were incubated at 37 °C for 30 min. Fluorescently labeled cells were harvested by centrifugation (10,000× *g*, 2 min and 30 s) and re-suspended in 1 mL of MRS broth.

2.3. Immobilization of cells for microscopy analysis

Cells were immobilized on the surface of a glass coverslip which had been cleaned with ethanol/HCL, thoroughly rinsed with sterile MilliQ-water and allowed to air-dry. To facilitate immobilization of the microorganisms, each coverslip was coated with 30 μL poly-L-lysine solution (0.1% [w/v] aqueous solution, Sigma, St. Louis, MO, USA) for 5 min, rinsed in sterile MilliQ-water and allowed to air-dry. A CoverWell™ perfusion chamber gasket (1 chamber per slide, dimensions 19 × 6 × 0.5 mm; Molecular Probes) was placed on top of each coverslip, and the chambers were then mounted as previously described [26]. The stained cells (70 μL) were added to the chamber and centrifuged (2000× *g*, 30 s) to facilitate cell attachment.

2.4. Determination of the intracellular pH of immobilized L. plantarum and L. brevis cells

The pH_i of individual *L. plantarum* UFLA CH3 and *L. brevis* UFLA FFC199 cells stained with either CFDA-SE or CDCFDA-SE was measured by FRIM as previously described by Kastbjerg et al. [22]. The set-up consisted of an inverted epifluorescence microscope (Axiovert 135 TV; Zeiss, Birkerød, Denmark) equipped with a Zeiss Fluar 100× objective (1.3 numerical aperture), a dichroic mirror (510 nm) and an emission band-pass filter (515 nm–565 nm). Bacterial cells were excited at 488 nm and 435 nm with an exposure time of 3000 ms by a monochromator equipped with a 75 W xenon lamp (Monochromator B; TILL Photonics GmbH, Planegg, Germany). Fluorescence emission was collected with a cool charge-coupled device camera (Coolsnap FX; Photometrics, Roper Scientific, Planegg, Germany), and images were analyzed with Metavue 7.1 software (Molecular Devices, Dowington, PA). A region near each cell but without a cell was subtracted for each cell as background. The ratio R488/435 for each examined cell was obtained by dividing the fluorescence intensity at 488 nm (pH-sensitive wavelength) by the fluorescence intensity at 435 nm (pH-insensitive wavelength). pH_i measurements were based on the ratio R488/435 and were therefore independent of the intracellular concentration of the probes.

2.5. Standard curves for pH_i measurements

Standard curves were constructed for each strain and probe as described previously [22]. If a cell membrane is