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The effect of pectins on survival of probiotic *Lactobacillus* spp. in gastrointestinal juices is related to their structure and physical properties

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ABSTRACT

Pectins are plant polysaccharides used in food industry as gelling and stabilizing agents. This study investigated the ability of pectins to improve survival of probiotic species *Lactobacillus fermentum* PCC, *L. reuteri* RC-14, *L. rhamnosus* LGG and *L. paracasei* F-19 in simulated gastric solution in relationship to their structural and physical properties. Electrostatic interactions between pectins and bacteria were evaluated by the Zeta-potential approach. Bacterial survival was assessed by flow cytometry and plate counting. *L. fermentum* PCC and *L. reuteri* RC-14 were more resistant to gastric conditions; their survival rate was further improved in the presence of five out of ten tested pectins. Additionally, two of the pectins had a positive effect on viability of the less resistant *L. rhamnosus* LGG and *L. paracasei* F-19. The beneficial effect was generally observed for the high-methoxylated pectins, indicating that substituted polygalacturonic acid in the backbone is essential for bacterial protection. Other pectin features associated with improved survival, included less negative Zeta-potential, higher molecular weight, as well as lower values of hydrodynamic sizes, viscosity and degree of branching. The study indicates that pectins have a potential to protect probiotic bacteria through the gastro-intestinal transit and identifies the features linked to their functionality.

1. Introduction

Probiotic *Lactobacillus* species encounter various stress conditions during the passage through the gastro-intestinal (GI) tract, including acid stress, exposure to digestive enzymes and bile. Survival and establishment of probiotic bacteria in the intestine can be improved by usage of synbiotic formulations, containing a mixture of pro- and prebiotics (Kolida and Gibson, 2011). Prebiotics are lately defined as substrates, selectively utilized by host microorganisms, conferring a health benefit (Gibson et al., 2017). Pectins are referred as emerging prebiotics which potential is currently being investigated (Gómez et al., 2014). Pectins are cell wall polysaccharides from fruit peel and vegetables, commonly used in the food industry as emulsifier, gelling or stabilizing agents (Gullón et al., 2013; Ridley et al., 2001). The functional properties and bioactivity of pectins and pectic oligosaccharides (POS) are known to be influenced by their physicochemical and structural features (Naqash et al., 2017; Wicker et al., 2014). The major structural units of pectin molecules consist of homogalacturonan (HG) or α-1, 4-linked units of D-galacturonic acid (GalA); rhamnogalacturonan I (RGI), containing repeating units of α-(1,4)-galacturonosyl and α-(1,2)-rhamnosyl with arabinan/galactan side chains; and rhamnogalacturonan II (RGII) substituted with various monosaccharides. The polygalacturonic acid is partly esterified with methyl groups, having different degree of esterification (DE). Accordingly, pectins are classified as low-methoxyl pectins (DE < 50%) and high-methoxyl pectins (DE < 50%) (Sila et al., 2009).

In-vitro studies by Pak et al. (2013) indicated that high-methoxyl pectins support survival of probiotic lactobacilli exposed to bile. Gelling and emulsion capacities make pectin-based preparations a

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good entrapment media for bacterial cells for targeted delivery to the colon (Naqash et al., 2017). Several studies introduced pectin-containing matrices for encapsulation of *Lactobacillus* and *Bifidobacterium* as an approach to increase bacterial survival at intestinal conditions. The authors suggested different procedures of capsule preparation including spray-drying of pectin-based dispersions (Oliveira et al., 2007) and ionotropic gelation of pectin microparticles (Gebara et al., 2013). It has been reported that synbiotic matrices supplemented with POS had a higher protective effect on *B. breve*, *L. acidophilus* and *L. reuteri* compared to alginate alone (Chaluvadi et al., 2012). In a recent study, Khorasani and Shojaosadati (2016) described a pectin-containing formulation used to entrap probiotic *Bacillus coagulans* and increase its survival rate in GI fluids.

The beneficial effects of prebiotics are governed by different types of interactions between prebiotic macromolecules and bacterial cells, including electrostatic forces, hydrogen bonding and steric hindrance as described elsewhere (Sila et al., 2009). Pectins are anionic in nature due the dominance of carboxyl groups in polygalacturonan backbone, which, thereby, may be involved in electrostatic interactions. Furthermore, the neutral carbohydrates in the pectin side chains ("hairy" regions) may provide steric hindrance effects. It has been demonstrated that charge density and charge distribution within the backbone are important for the binding capacity of pectins (Lutz et al., 2009; Wicker et al., 2014). The surface charge of lactobacilli is negative at physiological pH and highly influenced by the number of ionized phosphoryl and carboxylate groups in the teichoic acids present in peptidoglycan cell wall and the virtue of the cell surface proteins (Halder et al., 2015; Wilson et al., 2015). Zeta-potential (Zp) is often used as an approach to describe the surface charges of the particles and evaluate the electrostatic interactions between bacteria and surrounding macromolecules. Zp is defined as potential at a shear plane of the electrical double layer, encompassing polyelectrolyte molecules (Poortinga et al., 2002). Additionally, Zp is related to steric hindrance, since conformation of polyelectrolytes will be affected by surface charges.

In this study, we hypothesized that resistance of probiotic *Lactobacillus* spp. to GI stresses might be improved by treatment with specific pectins. The main objective was to investigate the impact of pectins on survival of probiotic species *L. fermentum* PCC, *L. reuteri* RC-14, *L. rhamnosus* LGG and *L. paracasei* F-19 in simulated gastric solution (GS). Pectins from various citrus fruits and sugar beet were included in the study, and their structural and physical properties were characterized in relation to the effect on bacterial viability in GS. Zeta-potential approach was used to evaluate the surface charges and electrostatic interactions between bacteria and pectins.

2. Methods

2.1. Bacterial strains and growth conditions

Bacterial strains *Lactobacillus fermentum* PCC®, *Lactobacillus reuteri* RC-14®, *Lactobacillus paracasei* subsp. *paracasei* F-19® and *Lactobacillus rhamnosus* LGG® were provided by Chr. Hansen A/S (Hoersholm, Denmark) and maintained in 20% (v/v) glycerol at −80 °C for long-term storage. Before the experiments bacterial strains were spread on Man Rogosa Sharpe (MRS) agar and incubated anaerobically (AnaeroGen sachets; Oxoid A/S, Roskilde, Denmark) at 37 °C for 48 h. Cultures for the survival experiments were prepared from a single colony incubated in MRS broth at 37 °C overnight (18 h). MRS broth and agar were purchased from Becton Dickinson A/S (Albertslund, Denmark). Other reagents were of highest purity and from Merck A/S (Hellerup, Denmark) and Sigma-Aldrich Aps (Broendby, Denmark) unless otherwise stated.

2.2. Pectins

Pectins were produced by CP Kelco ApS (Lille Skensved, Denmark), using different extraction and modification procedures, as presented in Table 1: harsh extracted pectin from orange (P1), mild extracted pectin from lemon (P2), differently extracted pectin from lemon (P3), harsh extracted from sugar beet (P4), harsh extracted LM pectin from lime (P5), mild extracted LM pectin from lemon (P6), harsh extracted amidated pectin from lime (P7), harsh extracted amidated pectin from orange (P8), harsh extracted pectin from lemon (P9) and rhamnogalactorunan RGI (P10).

2.3. Tests of bacterial survival in simulated gastric solution

The simulated gastric solution was prepared according to the composition of the stomach compartment in TIM1 intestinal model (TNO, the Netherlands) as described before (Barker et al., 2014). The stock solutions were (i) Gastric Electrolyte Solution (GES, pH 6.5) containing 3.1 g sodium chloride, 1.1 g potassium chloride, 0.6 g sodium bicarbonate and 0.6 g calcium chloride dehydrate dissolved in MilliQ water (total volume of 1 L) and autoclaved (121 °C, 20 min); (ii) 2% (w/v) pectin stock suspensions in MilliQ water; and (iii) enzyme solution in 100 ml GES, containing 8 U/ml lipase from porcine pancreas and 120 U/ml pepsin from porcine gastric mucosa prepared before use. Simulated gastric solution (GS) contained 20 ml citrate/phosphate buffer (pH 2.6, 0.5 M), 4 ml enzyme solution and GES added to the total volume of 100 ml. GS was mixed (1:1 vol ratio) with pectin stocks to the final concentration of 1% (w/v) (test samples) or MilliQ water (controls) and pH was adjusted to 2.5 (±0.01) with 0.1 M HCl. Bacterial cells were collected from overnight cultures in MRS broth, washed and re-suspended in saline solution (0.85% (w/v) NaCl) to the cell density of 10^{10} CFU/ml. Cells suspensions (1 ml) were added to 9 ml GS (test samples and controls) and incubated at 37 °C and shaking (220 rpm) for 6 h. Bacterial cells were withdrawn during incubation and analyzed by flow cytometry and standard plating to determine survival rate.

2.4. Flow cytometric analysis

Bacterial cells were collected by centrifugation (10,000 × g, 5 min) from 0.5 ml suspensions, washed twice and re-suspended in 0.5 ml saline solution. Cells were stained with fluorescent markers (Life Technologies Europe BV, Denmark) SYTO®13 and propidium iodide (PI) added into each sample in a volume of 1 μl and 5 μl, respectively. For negative controls cell suspensions were treated with 70% ethanol (30 min) prior staining to get permeabilized populations. Cell suspensions with fluorescent dyes were incubated in the dark at room temperature for 20 min and analyzed by a BD FACS Jazz Cell Sorter (BD Biosciences, USA). Green fluorescence emitted from SYTO®13 stained cells was monitored at wavelength of 530 ± 20 nm, and the red fluorescence emitted from PI stained cells was recorded at 692 ± 20 nm. In total 100,000 events per sample were acquired. Cell subpopulations were estimated in four quadrants (Q1, Q2, Q3 and Q4) on the dot plot images (cytograms). Experiments were performed in triplicate and the data were analysed using the FlowJo software (version 10, Tree Star Inc. USA).

2.5. Determination of viability counts by plate counting

Standard plate counting technique was applied to validate the FC results. Samples for plate counting were withdrawn after 4 h incubation of strains PCC and RC-14 and after 1 h incubation of strains LGG and F-19 in test and control experiments conducted