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Survival of probiotic lactic acid bacteria immobilized in different forms of bacterial cellulose in simulated gastric juices and bile salt solution

Karol Fijałkowski [a, *], Dorota Peitler [a], Rafał Rakoczy [b], Anna Żywicka [a]

[a] Department of Immunology, Microbiology and Physiological Chemistry, West Pomeranian University of Technology in Szczecin, Piastów 45, 70-311 Szczecin, Poland

[b] Institute of Chemical Engineering and Environmental Protection Processes, West Pomeranian University of Technology in Szczecin, Piastów 42, 70-311 Szczecin, Poland

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ABSTRACT

In this work, we discuss the possibilities of using bacterial cellulose (BC) produced by *Gluconacetobacter xylinus* as carrier support for the immobilization of probiotic strains of *Lactobacillus* spp. In our study, immobilization of the microorganisms was performed by the adsorption of bacterial cells on the surface of the synthetized BC and by a simultaneous cultivation of the probiotic bacteria with cellulose-synthetizing *G. xylinus*. Co-cultures were conducted in stationary cultures, in which BC was synthesized as pellicles on the medium surface and in shaken cultures in which BC was synthetized in the forms of beads. The experiments carried out also included the analysis of the survival of immobilized probiotic bacteria in the presence of gastric juices and bile salts solution. The results showed that immobilization of probiotic *Lactobacillus* in BC during co-culture with *G. xylinus* was the most effective immobilization method, providing high-level protection of the microorganisms against the influence of gastric juices and bile salts.

1. Introduction

Probiotics are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO, 2002). According to generally accepted definitions, 31 microbial species and genera are considered as probiotics (Holzapfel, Haberer, Snel, Schillinger, & Huis in't Veld, 1998). The microorganisms most frequently used as probiotic agents are lactic-acid producers, including in particular *Lactobacillus* species. *Lactobacillus* species are also known as non-pathogenic inhabitants of human and animal intestine and their presence is important for the maintenance of the intestinal microbial ecosystem (Ozyurt & Ötles, 2014). Probiotics are increasingly used in food and pharmaceutical applications to balance perturbed intestinal microflora and related dysfunctions of the gastrointestinal tract. In general, preparations containing probiotics are administered orally and are available in various forms such as food products, capsules, sachets or tablets (Weichselbaum, 2009). The basic principle of using probiotics is that the bacteria must survive in the food during its shelf life and for the period of transit in the acidic conditions of the stomach. To be able to influence the gut microbiota, probiotic microorganisms should also resist degradation by hydrolytic enzymes and bile salts in the intestine (De Vos, Faas, Spasojevic, & Sikkema, 2010). However, it has been indicated that the survival of probiotic bacteria in probiotic preparations during storage and in the gastro-intestinal system is often poor (Chávarri, Marañón, & Villarán, 2012; Kailasapathy, 2009).

Providing probiotic living cells with a physical barrier against adverse conditions is an approach currently receiving considerable interest (Borgogna, Bellich, Zorzin, Lapasin, & Cesŕro, 2010; Burgain, Gaiani, Linder, & Scher, 2011). Immobilized cells exhibit many advantages over free cells, including the maintenance of stable and active biocatalysts, high volumetric productivity, improved process control, protection of cells against damage and reduced susceptibility to contamination (Göksungur & Güvenç, 1999; Indira et al., 2015; Kourkoutasa, Bekatoroua, Banatb, Marchantb, & Koutinasa, 2004; Yun, Lin, & Cometabolism, 2009). However, immobilization of probiotic cells requires some specific processing steps which complicate the manufacture of the food product and increase its cost (Burgain et al., 2011). When considering the immobilization of bacterial cells, one should take into

* Corresponding author.
E-mail address: karol.fijalkowski@zut.edu.pl (K. Fijałkowski).

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account the probiotic strain selection, the quantity required for positive health benefits, stability of the probiotic cells during the processing steps or storage and finally, the effects on the sensory properties of the food (Burgain et al., 2011).

Recently, bacterial cellulose has gained particular interest owing to its suitability for immobilization processes. Among cellulose-producing bacteria, *Gluconacetobacter xylinus* has been considered a model microorganism for basic and applied studies on cellulose (Mikkelsen, Flanagan, Dykes, & Gidley, 2009). Cellulose ultrastructure and its physical and mechanical properties are strictly influenced by the culture method (Krystynowicz et al., 2002). Under stationary culture conditions, a thick, gelatinous membrane of BC is accumulated on the surface of the culture medium, whereas under agitated culture conditions, cellulose can be produced in the form of a fibrous suspension, irregular masses, pellets or spheres (Krystynowicz et al., 2002). BC does not require treatment to remove unwanted polymers and contaminants (lignin, hemicellulose) and it retains a high degree of polymerization. In its native state, BC also has a high hydration rate, holding over a hundred times its own weight in water. Thus, BC can be considered a good support for cell immobilization (Serafica, 1997). The cellulose synthetized by microorganisms is also characterized by the ability to form various shapes and textures that can be utilized in the food industry. Furthermore, BC used as a food ingredient does not adversely affect the sensory properties of the food such as flavor or color. Until now, BC has been used as a source of dietary fiber for the production of desserts, fruit cocktails and fruit jellies (Jagannath, Raju, & Bawa, 2010). However, a thorough review of the available literature has shown that so far no research concerning the evaluation of the usefulness of BC as a carrier support for immobilization of probiotic *Lactobacillus* has been performed. Therefore, the aim of the current study was to assess the possibility of using the BC naturally produced by *G. xylinus* as a carrier support for the immobilization of probiotic strains of *Lactobacillus* spp. and to evaluate its protective properties in the presence of simulated gastric juices and bile salt solution. BC in the forms of wet and dry pellicles or beads was used as immobilization carrier. Additionally, immobilization of *Lactobacillus* by simultaneous cultivation of cellulose-synthetizing *G. xylinus* was assessed. Furthermore, the study aimed to discover whether the efficiency of the immobilization process and the properties of the resulting material depend on the *Lactobacillus* strain used.

2. Materials and methods

2.1. Microorganisms

Three probiotic strains of *Lactobacillus delbrueckii* PKM 490, *Lactobacillus plantarum* DSM 13273 and *Lactobacillus casei* ATCC 393 and a cellulose producing strain of *G. xylinus* (Deutsche Sammlung von Mikroorganismen und Zellkulturen – DSM 46604) were used in this study.

2.2. Immobilization procedures

Immobilization of *Lactobacillus* in BC was performed using two different methods.

Method 1: For the immobilization of *Lactobacillus* in BC by adsorption-incubation method, *G. xylinus* bacteria were cultured in Hestrin-Schramm (H–S) medium composed of bacto-pepton – 0.5 w/v% (Oxoid, UK), glucose – 2 w/v%, yeast extract – 0.5 w/v%, citric acid – 0.115 w/v%, Na_2HPO_4 – 0.27 w/v%, $MgSO_4 \cdot 7H_2O$ – 0.05 w/v% and ethanol – 1 v/v% (Poch, Poland) added after sterilization of the base (Ciechańska, Struszczyk, & Gruzińska, 1998) at 28 °C. In this experiment two different types of *G. xylinus* cultures were performed: (a) *G. xylinus* was incubated under stationary conditions (Galaxy+, RS Biotech, Ireland) in 24-well plates for 6 days (Corning Costar cell culture plate, Sigma–Aldrich, Germany) in order to obtain pellicle discs of BC, (b) *G. xylinus* was incubated under agitated conditions at 180 rpm (ES–20/60, Biosan, Latvia) in 100 mL Erlenmeyer flasks (Sorbex, Poland) for 6 days to obtain BC in the form of beads. Prior to the experiment, synthetized cellulose samples were removed from the culture medium, weighed, washed with deionized water, purified in 0.1 N NaOH and sterilized in an autoclave at 121 °C for 15 min. Half of the cellulose material obtained was subjected to drying at 60 °C, whereas the rest of the hydrated samples were stored in a refrigerator. In the next step, *Lactobacillus* cells from 24 h cultures in MRS medium (deMan, Rogosa and Sharpe, Biocorp, Poland) were separated, washed and suspended in isotonic phosphate buffered saline (PBS, Sigma–Aldrich) to obtain bacteria concentration of 1°McFarland (3×10^8 CFU/mL). For the immobilization process, the cellulose discs and the beads were incubated in *Lactobacillus* suspension for 12 h at 25 °C with shaking at 180 rpm. The average diameter of BC used for immobilization by adsorption-incubation method was 1.5 cm for pellicles and 0.4 cm for beads. The average weight was approximately 1 g and 25 mg, respectively for wet and dry BC pellicles and 0.5 g and 15 mg respectively for a single wet and dry BC bead. The difference between the weights of wet and dry form of BC was mainly due to the water absorbed in wet BC. After incubation, the cellulose samples were washed in deionized water and digested by incubation with cellulase (100 μL/1 mL 0.05 M citrate buffer, pH 4.8, Sigma–Aldrich).

Method 2: For immobilization of *Lactobacillus* during co-incubation with *G. xylinus*, the H–S medium was simultaneously inoculated with *G. xylinus* and *Lactobacillus* at a ratio1:100, respectively. The concentration of each microorganism amounted to 0.5°McFarland (1.5×10^8 CFU/mL). In this experiment two different cultures were prepared: (a) stationary cultures which were carried out in 50 mL tubes (Polypropylene Conical Centrifuge Tube, Becton Dickinson and Company) in which BC pellicles were synthesized as a pellicle on the medium surface, (b) shaken cultures (180 rpm) carried out in 100 mL Erlenmeyer flasks (Sorbex) in which BC was synthetized in the forms of beads. The cultures were incubated for 6 days at 28 °C. After incubation, the synthetized BC was washed in deionized water and digested with the cellulase enzyme (Sigma Aldrich).

2.3. Microscopic examination

In order to visualize the immobilized bacteria, BC samples were stained with Amido Black staining solution (Sigma–Aldrich) and examined under an optical microscope (Axioskop 2 plus microscope, Zeiss, Oberkochen, Germany) at magnification × 1000. In the case of immobilization of *Lactobacillus*, during co-incubation with *G. xylinus,* the staining procedure was preceded by cutting off the top layer of BC.

2.4. Determination of the number of immobilized Lactobacillus cells

Bacterial cells suspensions obtained after digestion with cellulase were washed 3 times with 10 mL PBS and suspended in 1 mL PBS. The number of immobilized microorganisms was determined by two methods: (a) by spectrophotometric readings of the optical density (OD) of microorganism suspension at 600 nm (Infinite 200 PRO NanoQuant, Tecan, Switzerland) and (b) by performing quantitative plating on MRSagar (Biocorp). After incubation for 24 h at 37 °C, the grown colonies were counted and the number of Colony Forming Units (CFU) per 1 g of cellulose was determined. The OD of bacterial cultures indirectly reflects the number of viable and dead