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Characterization of surface layer proteins and its role in probiotic properties of three Lactobacillus strains

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ABSTRACT

The objective of this study was the characterization of the surface layer proteins (SLPs) and their functional role in the probiotic activity of *Lactobacillus helveticus* fb213, *L. acidophilus* fb116 and *L. acidophilus* fb214. SLPs were extracted and identified by SDS-PAGE, circular dichroism spectra and LC–MS analysis. The results revealed that the molecular masses of the three proteins were 49.7 kDa, 46.0 kDa and 44.6 kDa, respectively. The secondary structures and amino acid compositions of the three proteins were found to be similar. After removing SLPs, the survival of the three lactobacilli in simulated gastric and intestinal juices was reduced by 2–3 log as compared with survival of the intact cells. And the adhesion ability of the three strains to HT-29 cells decreased by 61%, 65% and 92%, respectively. SLPs also inhibited the adhesion and invasion of *Escherichia coli* ATCC 43893 to HT-29 cells. These results suggest that SLPs are advantageous barriers for lactobacilli in the gastrointestinal tract, and these proteins help make it possible for lactobacilli to serve their probiotic functions.

1. Introduction

The lactobacilli are members of the lactic acid bacteria group and common commensals in the gastrointestinal tracts of mammals. Due to their beneficial effects on immunomodulation and the alleviation or prevention of diverse intestinal disorders [1], lactobacilli are recognized as health-promoting microorganisms. In addition, lactobacilli are reported to prevent the adhesion, establishment or invasion of specific enteropathogens [2,3]. Several possible mechanisms have been proposed, such as contribution to mucosal barrier function, competitive exclusion, modulation of the immune response and co-aggregation with pathogens. Although the mechanisms for their beneficial effects are not well understood, many species of lactobacilli are widely used as probiotic ingredients in various food products.

Surface layers composed of one glycoprotein or protein, known as surface layer proteins (SLPs), are two-dimensional, regular, highly porous crystalline arrays of subunits that present the outermost layer of the cell wall in most bacteria, including many species of the genus *Lactobacillus* [4,5]. The molecular masses of lactobacilli SLPs are between 25 kDa and 71 kDa [6,7]. SLPs are highly basic proteins, with calculated pI values ranging from 9.35 to 10.40. Most of the lactobacilli SLPs are determined to be non-glycosylated [5]. To date, only the SLPs of *Lactobacillus buchneri* and *L. kefir* have been reported to be glycosylated. Although the functions of SLPs are not completely elucidated, several functions have been attributed to the different SLPs of lactobacilli, such as being protective agents against a hostile environment, acting as molecular sieves and ion trap molecules, maintaining cellular shape, controlling the transfer of nutrients and metabolites and playing an important role in the colonization of *Lactobacillus acidophilus* in the gastrointestinal tract [8,9]. SLPs are also involved in mediating adhesion to various extracellular matrix proteins and epithelial cells. The adhesive quality of intestinal epithelia allows lactobacilli to exert their probiotic effects for a longer period of time [8,10–12]. However, even SLPs from the same species appear to have a high heterogeneity. SLPs from aggregating and non-aggregating *L. kefir* strains show different capacities for antagonizing cytotoxic effects *in vitro* [13].

Due to the great diversity of SLPs, many have not been clearly investigated. In the present study, SLPs were extracted from three lactobacilli: *L. helveticus* fb213, *L. acidophilus* fb116 and *L. acidophilus* fb214. The main objective of this work was to investigate the physicochemical properties of the three SLPs and their functional role in the probiotic activity of lactobacilli. The researchers expected that the biological contribution of these SLPs would to be revealed, offering a theoretical basis for the application of SLPs and SLPs-carrying lactobacilli in food and pharmaceutical industries.

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2. Materials and methods

2.1. Chemicals

Pepsin, Pancreatin, and low molecular weight markers from 14.4 to 97.0 kDa (α-lactalbumin, trypsin inhibitor, carbonic anhydrase, ovalbumin, albumin and phosphorylase), were purchased from the China National Medicines Corporation (Shanghai, China). RPMI 1640 medium, penicillin, streptomycin and gentamicin were purchased from Gibco BRL, Life Technologies (NY, USA). Fetal bovine serum was purchased from Hyclone (Logan, UT, USA). TritonX-100 was purchased from Shanghai Chemical Reagent Ltd. (Shanghai, China). All of the other chemicals used were of the highest analytical grade, and were purchased from common sources.

2.2. Bacterial strains and growth conditions

L. helveticus fb213, *L. acidophilus* fb214 and *L. acidophilus* fb116 were provided by the Research Center of Food Biotechnology, Jiangnan University, Jiangsu, China. The lactobacilli were grown in De Man-Rogosa-Sharpe (MRS) broth (Oxoid) overnight for 18 h at 37 °C. *Escherichia coli* ATCC 43893 was grown in Luria-Bertani (LB) medium (10 g L^{-1} tryptone, 5 g L^{-1} yeast extract, 10 g L^{-1} NaCl, pH 7.2) overnight for 18 h at 37 °C.

2.3. HT-29 cell culture

HT-29 cells were obtained from Shanghai Institute of Cell Biology (China) and cultured in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 100 U/mL penicillin and 100 U/mL streptomycin in an atmosphere of 5% (v/v) CO_2 and 95% (v/v) air at 37 °C. HT-29 cells were grown in cell culture plates (Costa Inc., NY, USA) at a density of 3×10^5 per well.

2.4. Extraction of cell surface proteins and whole-cell proteins, and SDS-PAGE analysis

SLPs from lactobacilli were extracted according to a routine procedure [14,15]. Lactobacilli cells were harvested by centrifugation (5000 × *g*, 10 min, 4 °C) and washed twice with phosphate buffered saline (PBS, pH 7.2). The washed cells were resuspended in 5 mol/L LiCl, and stirred for 30 min at room temperature. The supernatants containing surface layers were harvested after centrifugation (12,000 × *g*, 4 °C, 15 min) and dialyzed against distilled water at 4 °C. Then the surface layers were lyophilized (Labconco Corp., Kansas City, MO, USA) and stored at −20 °C.

Whole cell proteins were extracted by a sodium dodecyl sulphate (SDS) solution. The lactobacilli cells were collected by centrifugation (5000 × *g*, 10 min, 4 °C) and washed twice with phosphate buffered saline (PBS, pH 7.2). The washed cells were resuspended in 10 g L^{-1} of SDS solution and boiled for 10 min. The supernatants were harvested after centrifugation (12,000 × *g*, 4 °C, 15 min) and dialyzed against distilled water at 4 °C.

The supernatants were mixed with 2× loading buffer, boiled for 5 min and analyzed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% polyacrylamide gels. Protein bands were made visible by staining the gels with Comassie brilliant blue.

2.5. Amino acid content analysis of SLPs

A total of 17 amino acids were identified using the method described by Lu et al. [16].

2.6. Circular dichroism analysis

The secondary structure of SLPs was monitored by a MOS-450/AF-CD circular dichroism spectrometer (Bio-Logic, Grenoble, France). Samples were placed in a 1 mm pathlength quartz cuvette filled with the corresponding solvent during analysis, and scanned at protein concentrations of 0.1–0.2 mg mL^{-1}. The scanning wavelength range was between 190 nm and 250 nm, and the scanning rate was 100 nm min^{-1}. A buffer sample containing no protein was subtracted from all spectra to account for any background signal.

2.7. Survival of lactobacilli in simulated gastric and small intestinal juice

Simulated gastric and small intestinal juices were prepared according to Frece et al. [17]. Preparation of the simulated gastric juice involved dissolving pepsin (3 mg mL^{-1}) in a sterile sodium chloride solution (5 mg mL^{-1}), and adjusting the pH to 2.0. The simulated small intestinal juice was prepared by dissolving pancreatin (1 mg mL^{-1}) and bile salts (1.5 mg mL^{-1}, oxgall) in sterile sodium chloride solution (5 mg mL^{-1}) and adjusting the pH to 8.0.

The lactobacilli cells, before and after treatment with 5 mol/L LiCl, were mixed with the gastric and the small intestinal juices (1 mL each) and 0.3 mL NaCl (5 mg mL^{-1}). The changes in total viable cell counts were monitored during treatment with the gastric (4 h) and the small intestinal juices (4 h) by the pour plate method, using MRS agar incubated at 37 °C for 48 h.

2.8. Adhesion of lactobacilli to HT-29 cells

The adhesion ability of lactobacilli to HT-29 cells was examined according to a modified method described by Guglielmetti et al. [18]. The adhesion assays were performed on HT-29 cell monolayer in 6-well plates (Costa Inc., NY, USA). HT-29 cell monolayer was washed twice with sterile PBS and incubated with 1 ml RPMI-1640 medium and 1 mL lactobacilli suspension (10^8 CFU/mL) for 2 h at 37 °C in a 5% CO_2 and 95% air atmospheres. The cell monolayer was washed four times with sterile PBS and fixed with methanol for 30 min. Then, cells were stained and examined by light microscope. The adhesion ability of lactobacilli was measured by counting bacteria adhering to per 100 HT-29 cells.

2.9. E. coli adhesion and invasion assays

The ability of lactobacilli to inhibit the adhesion and invasion of *E. coli* to HT-29 cells was evaluated by a method with some modifications [9,19,20].

Control assays were performed as follows: HT-29 monolayers were washed twice with sterile PBS (pH 7.2) and then 1 mL *E. coli* suspension (10^8 CFU/well) and 1 mL RPMI-1640 medium were added to each well and incubated 2 h at 37 °C in a 5% CO_2 and 95% air atmospheres. The monolayers were washed four times with sterile PBS and lysed by adding 0.5 mL 0.5% (v/v) TritonX-100 for 8 min at 37 °C. Appropriate dilutions in 0.1% tryptone were plated and colony counts were performed in order to examine the number of associated (adhering plus invading) viable cells.

E. coli invasion was determined by counting only bacteria located into the HT-29 cells. To perform this experiment the monolayer was washed and incubated with *E. coli* as described above. Then, 50 μg mL^{-1} gentamicin was added to each well and the monolayer was incubated for 1 h at 37 °C before lysing and colony counts.

Different types of experiments were performed: (1) HT-29 cells were preincubated with 1 mL intact lactobacilli cells suspension (10^8 CFU/well) and washed twice with sterile PBS. Then, 1 mL *E. coli* suspension (10^8 CFU/well) and 1 mL RPMI-1640 medium