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Relationship between the resistance to bile salts and low pH with exopolysaccharide (EPS) production of *Bifidobacterium* spp. isolated from infants feces and breast milk

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ABSTRACT

The purpose of this study was to investigate a possible relation between resistance to bile salts and low pH with exopolysaccharide (EPS) producing of *Bifidobacterium* spp. In this study, a total of 31 *Bifidobacterium* spp. were isolated from breast fed infants feces and breast milk samples. As a result of the identification tests, isolates were identified as *Bifidobacterium breve* (15 strains), *B. bifidum* (11 strains), *B. pseudocatenulatum* (3 strains) and *B. longum* (2 strains). *Bifidobacterium* spp. were determined exopolysaccharide (EPS) production. EPS productions observed at chance rations (38.00–97.64 mg/l) among of *Bifidobacterium* spp. Furthermore, *Bifidobacterium* spp. were determined resistance to bile salts and low pH. Positive correlations between production of exopolysaccharide and resistance to bile salts ($p < 0.01$) or low pH ($p < 0.01$) were found *Bifidobacterium* spp. This investigation showed that high EPS production of Bifidobacteria may be important in the selection of probiotic strains for resistance to bile salts and low pH.

1. Introduction

Bifidobacteria were first discovered in 1899 by Tissier at the Pasteur Institute, Paris, France. These bacteria were found to be a predominant component of the intestinal flora in breast fed infants [1]. Some *Bifidobacterium* strains can colonise the gastrointestinal tract (GIT), in which they occur at concentrations of 10^9–10^{10} cells/g of feces [2]. The significance of human origin has been debated recently, but most current successful strains are indicated to be of human origin. The genus of *Bifidobacterium* can generally be characterized as gram-positive, non-sporeforming, non-motile, anaerobes that are catalase-negative and sacchralytic [3]. They are played an important and beneficial role in the proper balance of normal intestinal flora [4]. Establishment of high numbers of *Bifidobacterium* is reported to from barriers against the proliferation of exogenous pathogens [5,6].

Many *Bifidobacterium*-containing dairy products have been developed due to their reported health-promoting effects. These organisms are employed to increase the beneficial properties of fermented milks, infant formulas, cheese, and ice cream [7–10]. The difference between the microbiota of breast fed and formula-fed infants lies in the numbers and the species composition of bifidobacteria. This can be explained by the presence of natural bifidogenic oligosaccharides in breast milk [11] and by the possibility that breast milk also contains bifidobacteria, in addition to the lactobacilli and other lactic acid bacteria as recently reported [12].

The production of EPS could also be an interesting property to consider for the selection of putative probiotic strains [13]. There are several possible benefits that cultures of Bifidobacteria intended for use as a probiotic might derive for production of an EPS. A protective coating of EPS may allow the bacterium to better withstand stomach acid and bile salts. In the intestinal tract, EPS production may improve adherence to the intestinal mucosa and increase longevity in the intestinal tract [14,15]. In addition, the health benefits of lactic acid bacteria and bifidobacteria have been attributed to the production of EPS, which has anti-tumor, immunomodulating and cholesterol-lowering activity [16,17]. EPS producing of *Bifidobacterium* may be performed as an initial step in establishing rational criteria for screening and selecting microorganisms with human probiotic properties.

Many studies have also focused on the survival of probiotic bacteria in dairy products. However, several studies have suggested that successful probiotic bacteria should be able to tolerance the acidic conditions in the stomach and bile in the small intestine [18,19]. The acidic environment of the stomach and the bile salts

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secreted in the duodenum are major impediments to the survival of ingested bacteria. The tolerance of bifidobacteria at pH values of gastric juices is considered to be generally low [20–23]. In the literature, there are several studies on tolerance to gastrointestinal conditions, whereas the relations between resistance to bile salts or tolerance to low pH and EPS production of *Bifidobacterium* spp. have never been studied.

The purpose of this study was to investigate a possible relation between EPS producing and resistance to bile salts or tolerance to low pH of *Bifidobacterium* spp. isolated from breast fed infants feces and mother's milk.

2. Materials and methods

2.1. Isolation of bifidobacteria

This study were isolated a total of 31 bifidobacteria on the brink of from 16 breast fed infants feces and 15 breast milk samples. Breast milk samples were collected by manual expression from 2 women. The regular consumption of probiotic preparations over the preceding month, defined as daily or nearly daily consumption, was obtained by interviews. Isolates studies were used to method of Hadadji et al. [24]. For each feces samples, about 1 g of freshly feces was transferred into flasks containing 9 ml of pre-reduced salt solution (NaCl, 0.9 %) containing 0.2% L-cysteine-HCl (Merck) and the suspension was homogenized for 2 min. After checking, serial dilutions were made and 0.1 ml of the suspension was inoculated in *Bifidobacterium* Selective Medium (BSM, Oxoid) including agar [25]. All plates were incubated anaerobically at 37 °C for 5 days, using oxoid gas jars and anaerobic gaspak (Oxoid, USA). The selective medium BSM was prepared by the addition of 50 mg mupirocin (Oxoid, USA). Bifidobacteria were enumerated using BSM solid medium. Colonies from the highest dilution of each sample were picked at random and inoculated into BSM solid medium. Colonies picked from countable plates were selected for gram reaction and morphology.

2.2. Culture media

Bifidobacterium species were cultured under anaerobic conditions for 24–48 h at 37 °C in modify de Man, Rogosa and Sharpe medium (MRS, Merck) broth supplemented with 0.05% w/v L-cysteine-HCl (Merck) [26]. To prepare of active cultures for all experiments, *Bifidobacterium* species were grown in MRSC (MRS with 0.05 % L-cystein-HCl) broth for 24–48 h at 37 °C. The bacterial strains were stored frozen at −80 °C in 10% glycerol broth to supply a stable inoculum for this study and subcultured twice before use in the manipulations.

2.3. Identification of Bifidobacteria

The isolates considering belong to the genus *Bifidobacterium* were identified by the detection of fructose-6-phosphate phosphoketolase (F6PPK) enzyme in cellular extracts as described by Bibiloni et al. [24] and Hadadji et al. [25]. Cells were grown in 10 ml of MRSC broth at 37 °C for 18 h and harvested by centrifugation at 5000 g for 10 min. The pellet was washed twice with 5 ml of 0.5 g/l phosphate cysteine buffer. After centrifugation, the pellet was collected in 1 ml of buffer; 0.25 ml of reagents (6 mg/ml NaF, 10 mg/ml sodium iodoacetate and 80 mg/ml fructose-6-phosphate) was added to the cells extract. The reaction was started by incubation 30 min at 37 °C and stopped by adding 1.5 ml of hydroxylamine-HCl (13.9%). After 10 min, 1 ml of trichloroacetic acid (15%) and 1 ml of $FeCl_3$.6H2O (5%) were added. The presence of fructose-6-phosphate phosphoketolase enzyme was revealed by the appearance of red and purple colors [3,24,25].

The carbohydrates fermentation was determined on MRSC broth containing bromocresol purple (0.04 g/l) as a pH indicator, and supplemented with 1% of the following carbohydrates: lactose, sucrose, xylose, arabinose, sorbitol, fructose, galactose, mannose, cellobiose, raffinose, melizitose and melibiose [27].

All strains were initially submitted to Gram staining, the catalase test and spore formation. Gram reaction, morphological, physiological and biochemical tests were compared with the reference strains (*B. bifidum* DSM20456, *B. breve* DSM20213, *B. longum* DSM20088, *B. pseudocatenulatum* DSM20438) in standard texts for identification. Gas production from glucose was determined in MRSC broth containing inverted Durham.

2.4. EPS of Bifidobacterium spp.

After inoculation, broth cultures were incubated anaerobically at 37 °C for 18 h. The cultures were boiled at 100 °C for 10 min. After cooling, they were treated with 17 % (v/v) of 85 % trichloracetic acid solution and centrifuged [28]. Removal of cells and protein was done by centrifugation. EPS was precipitated with ethanol (100% v/v). It was recovered by centrifugation 14 000 × g for 20 min. Total EPS (expressed as mg/l) was estimated in each sample by phenol–sulphuric method [29] using glucose as standard [30]. In study was done in three independent experiments and mean values were calculated.

2.5. Resistance to bile salts and low pH

One hundred microlitres of cultures containing a final optical density of 0.6 at 600 nm were transferred to fresh MRSC medium. pH was adjusted to 3.0, 3.5, 4.0 and 6.2 (control) using 4 N HCl and the effects of bile salts were examined in MRSC medium (pH 6.2) by oxgall (Sigma) to a final concentration of 0.15 and 0.3 % (w/v). Cultures were growth for 24 h at 37 °C for. The cell growth was measured spectrophometrically (Digilab Hitachi U–1800) at 620 nm. Results were given as optical density (OD). Experiments were made in triplicate [31].

2.6. Statistical analysis

Results are average of at least three independent trials. Statistical analysis was performed by SPSS (Version, 16.0 for Windows). Pearson's correlation was used for determine any significant differences between EPS production amount and bile salts resistance of the strains and between EPS production amount and low pH of the strains. The level of significance was defined at $p < 0.01$.

3. Results

3.1. Isolation and identification of Bifidobacteria

59 bacteria were isolated by breast milk and breast-fed infant feces. 31 of 59 purified isolates were gram-positive bacteria and negative for oxidase, and catalase in our study. Moreover, 31 isolates obtained are pleomorphic rods, positive for gram reaction and the F-6-PPK test. As a result of the identification tests, 31 *Bifidobacterium* spp. were identified as 15 *B. breve,* 11 *B. bifidum*, 3 *B. pseudocatenulatum*, 2 *B. longum*. Identified strains and isolation places observed in Table 1.

3.2. Exopolysaccharide (EPS) production of Bifidobacteria

In this study was determined EPS production of Bifidobacteria. The results are show in Table 2. In our study was a selected high and low EPS production strains in accordance with their capacity of EPS