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Changes in growth and survival of *Bifidobacterium* by coculture with *Propionibacterium* in soy milk, cow's milk, and modified MRS medium

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ABSTRACT

Bifidobacterium adolescentis Int57 (Int57) and *Propionibacterium freudenreichii* subsp. *shermanii* ATCC 13673 (ATCC 13673) were grown either in coculture or as pure cultures in different media, such as cow's milk, soybean milk, and modified MRS medium. The viable cell counts of bacteria, changes in pH, concentrations of organic acids, and contents of various sugars were analyzed during incubation up to 7 days. In soy milk, the survival of cocultured Int57 was six times higher than the monocultured cells, and ATCC 13673 cocultured with Int57 consumed 69.4% of lactic acid produced by Int57 at the end of fermentation. In cow's milk, coculture with ATCC 13673 increased the growth of Int57 from 24 h until 120 h by approximately tenfold and did not affect the survival of Int57 cells. After 96 h of fermentation of modified MRS, the survival of ATCC 13673 cells cocultured with Int57 increased by 3.2- to 7.4-folds as compared with ATCC 13673 monoculture, whereas the growth of Int57 cells was unaffected. The growth and metabolic patterns of two strains during coculture showed noticeable differences between food grade media and laboratory media. The consumption of stachyose in soy milk during coculture of Int57 with ATCC 13673 was increased by more than twice compared with Int57 monoculture, and completed within 24 h. The combinational use of *Bifidobacterium* and *Propionibacterium* could be applied to the development of fermented milk or soy milk products.

1. Introduction

Some representative strains of *Bifidobacterium* spp. are known for their positive effects in relieving symptoms of lactose intolerance, preventing and treating diarrhea, improving immunity by balancing the intestinal microflora, lowering serum cholesterol, and reducing the risk of colon cancer (Chandan, 1999; Parvez et al., 2006; Shah, 2007). There are also beneficial industrial applications of dairy *Propionibacterium* spp., including the production of vitamin B_{12}, manufacture of Swiss cheese, and production of propionic acid which is used as an antifungal agent (Mantere-Alhonen, 1995; Pérez Chaia et al., 1999; Thierry et al., 2004; Vorobjeva, 1999).

Several studies have reported on the interactions between bifidobacteria and dairy propionibacteria. *Propionibacterium freudenreichii*, *Propionibacterium shermanii*, *Propionibacterium acidipropionici* and *Propionibacterium jensenii* are known to produce BGS (bifidogenic growth stimulator) such as 1,4-dihydroxy-2-naphthoic acid (DHNA), which can increase the activities of intestinal microflora (Isawa et al., 2002; Thierry et al., 2011; Kaneko et al., 1994; Kouya et al., 2007; Mori et al., 1997). On the other hand *P. freudenreichii* prefer to utilize lactic acid, which is produced together with acetic acid by diverse *Bifidobacterium* species (Taniguchi et al., 1998; Taniguchi and Tanaka, 2004; Vorobjeva, 1999).

The growth and the organic acid production of *Bifidobacterium longum* in coculture with *P. freudenreichii* was investigated using the TPY medium containing either peptones or whey protein hydrolysate as nitrogen sources (Taniguchi et al., 1998). However, the effects of coculture of *Bifidobacterium* spp. with *Propionibacterium* spp. on the growth patterns and the production of organic acids in soy milk and cow's milk have not been reported yet.

Therefore, the purpose of this study was to characterize cocultures of *Bifidobacterium* and *Propionibacterium* in cow's milk and soybean milk. The growth and survival patterns of *Propionibacterium* and *Bifidobacterium* were analyzed and characterized, including changes in acid production, pH and sugar contents.

2. Materials and methods

2.1. Microorganisms

The description of *Bifidobacterium adolescentis* Int57 (Int57) was previously reported (Paik et al., 2007). *P. freudenreichii* subsp. *shermanii*

Abbreviations: Int57, *Bifidobacterium adolescentis* Int57; ATCC 13673, *Propionibacterium freudenreichii* subsp. *shermanii* ATCC 13673.

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ATCC 13673 (ATCC 13673) was obtained from the Korean Culture Center of Microorganisms (Seoul, Korea). Int57 and ATCC 13673 were activated by two successive precultures in MRS medium (Difco™, Becton–Dickinson and Company, Sparks) with 0.05% (w/v) cysteine–HCl (Sigma, St. Louis, MO, USA). Int57 and ATCC 13673 were grown under anaerobic conditions at 37 °C for 18 h and at 30 °C for 40 h, respectively. Enumerations were performed with YEL agar (Hettinga et al., 1968; Jan et al., 2000; Moussavi and Adams, 2010).

2.2. Preparation of media and fermentation conditions

The preparation of soy milk medium was conducted as previously described (Wang et al., 2002) with some modifications. Whole soybeans were washed and soaked in distilled water at 25 °C for 8 h. The water was then decanted and the soaked soybeans were ground for 3 min in distilled water (10 times the dry weight of soybeans). The resulting soybean slurry was filtered through a double-layered cheesecloth to produce the soy milk.

Cow's milk (Seoul Milk™) was purchased from the local market (Seoul, Korea). According to the supplier, the contents of fat, protein, carbohydrate, and calcium in Seoul Milk™ were 4%, 5%, 3%, and 0.1% (w/v), respectively. Modified MRS was prepared as the formulation of Lactobacilli MRS medium (De Man et al., 1960) without sodium acetate, which might interfere with the results of the subsequent analysis of organic acids. Soy milk and modified MRS medium were autoclaved at 121 °C for 15 min, while the cow's milk medium was autoclaved at 90 °C for 30 min. All three media were supplemented with 0.05% (w/v) cysteine–HCl and the pH of each medium was adjusted to pH 6.4 ± 0.2 prior to the fermentation.

Based on the preliminary experiments, 33 °C was determined as the optimum coculture temperature. Experimental bacteria were inoculated to monocultures or cocultures and samples were taken at 0, 6, 12, 24, 48, 72, 96, 120, 144, and 168 h. These samples were used to evaluate pH, organic acid contents, contents of various sugars, and viable cell counts.

Because the growth rate of ATCC 13673 was relatively slower than that of Int57, we inoculated modified MRS media with different inoculation volumes of ATCC 13673 and investigated the initial cell numbers of ATCC 13673 which were not inhibited by the growth of Int57 cells during coculture. Among the tested inoculation ratios of each strain including 1:1, 1:2, 1:5, and 1:8 (v/v, Int57:ATCC 13673), the ratio of 1:5 (v/v) was the most appropriate inoculation volume of each strain in coculture. When we inoculated soy milk, cow's milk, and MRS medium with 1% (v/v) of Int57 seed culture and 5% (v/v) of ATCC 13673 seed culture, the initial cell counts of Int57 and ATCC 13673 at 0 h were ranged between 6.20 and 6.35 $\log_{10}$ CFU/ml and 8.20 and 8.22 $\log_{10}$ CFU/ml, respectively.

2.3. Viable cell counts

Bacterial cell numbers were determined using the surface plating method with YEL agar, which allowed Int57 and ATCC 13673 to be readily distinguished. Confirmed colonies were counted after anaerobic culture at 33 °C for 6 days. Results are expressed as $\log_{10}$ CFU/ml of viable cell count.

2.4. pH measurement

Changes in pH of each culture medium were measured using a pH meter (model 730P, Istek, Inc., Seoul) throughout the seven day incubation period.

2.5. Determination of organic acids and sugars by HPLC

The 1.5 ml samples were centrifuged at 13,000 ×g for 30 min and the supernatants were filtered through 0.2 μm filters.

For organic acid analysis, samples were diluted with 0.0083 N H_2SO_4 solution (1:4, v:v) and injected into a Younglin series HPLC system equipped with a vacuum degasser and mixer, gradient pump, auto sampler, and a UV/Vis detector. Analytes were separated with an Aminex HPX-87H Column (300 × 7.8 mm, Bio-Rad Laboratories, Inc., USA) connected to a Micro-Guard pre-column (Bio-Rad). The mobile solution was 0.008 N H_2SO_4 and the flow rate was 0.6 ml/min. The UV detector wavelength was 210 nm. Standard calibration curves were prepared using serial dilutions of lactic acid, acetic acid and propionic acid.

For sugar analysis, 500 μl of the filtered samples was transferred to a 25 ml volumetric flask for protein precipitation using the Chávez-Servín method (Chávez-Servín et al., 2004). Each sample was diluted from 1:12 to 1:50, depending on the sugar contents of the sample. Prior to dilution, the sample was dissolved in 1:1 (v:v) ethanol–water and placed in a 60 °C water bath for 1 h. Then, 250 μl of Cazzels I and II was added to the sample successively, followed by stirring and incubating in the water bath for 2 h. The supernatant was passed through a C_{18} Sep-pak plus cartridge (Waters Co., Milford, MA), filtered through a 0.2 μm nylon filter and injected into a HPLC system.

For MRS and milk analysis, the HPLC system (Younglin Instrument Co., Ltd., Anyang) was equipped with a vacuum degasser and value module, solvent delivery pump, UV/Vis detector, column oven, automated sample injector, and an ELSD 800 (Alltech Associates, Inc., Derfield, IL). The column was a high performance carbohydrate (4 μm, 4.6 × 250 mm) (Waters corporation, Milford, MA). The mobile solution was acetonitrile–water (80:20, v:v), the flow rate was 1.5 ml/min, and the column temperature was 40 °C. The sugar standard for MRS was glucose, while for milk it was lactose at 0.5–1.5 mg/ml. In the soy milk analysis, the column was changed to a prevail carbohydrate ES 5u (250 × 4.6 mm) (Alltech Associates, Inc., Derfield, IL) with a gradient elution mobile phase of acetonitrile:water (from 81:19 to 50:50 in 20 min, v:v). The flow rate was 1 ml/min and the sugar standards were stachyose, raffinose and sucrose. All samples were obtained from three independent fermentations.

2.6. Statistical analysis

The mean values and the standard deviation were calculated by the triplicate independent trials. One-way analysis of variance (ANOVA) was used for statistical analysis (SPSS Statistics 17.0, SPSS Inc., Chicago, IL, USA). The significant differences (P value < 0.05) were determined by the Duncan multiple range test.

3. Results

3.1. Changes in the growth and survival of *Bifidobacterium* and *Propionibacterium* during the coculture period

The growth patterns and changes in pH during the monoculture or coculture of Int57 and ATCC 13673 are shown in Fig. 1. The growth of Int57 in soy milk (Fig. 1A) showed different patterns compared to other media such as cow's milk (Fig. 1B) and modified MRS (Fig. 1C). Until 24 h of soy milk fermentation, the viable microbial counts of Int57 in both monoculture and coculture increased by 8.78 ± 0.31 $\log_{10}$ CFU/ml and 9.04 ± 0.14 $\log_{10}$ CFU/ml, respectively (Fig. 1A). There was a clear decrease in the viability of Int57 after 24 h of soy milk fermentation, which was not seen in neither cow's milk nor modified MRS medium at this time point. Between 12 and 48 h, the viable counts of Int57 in coculture with ATCC 13673 were maintained at a slightly higher level than in the Int57 monoculture. At the end of fermentation, Int57 in coculture survived by maintaining the initial viable cell counts at a level of 6.30 ± 0.25 $\log_{10}$ CFU/ml, compared to 5.50 ± 0.12 $\log_{10}$ CFU/ml in Int57 monoculture (Fig. 1A). In the case of ATCC 13673, there was no significant difference in viability between the monoculture and coculture until