

The Use of Cellobiose and Fructooligosaccharide on Growth and Stability of *Bifidobacterium infantis* in Fermented Milk

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ABSTRACT

The effects of cellobiose, fructooligosaccharide and their combination on fermentation of skim milk by probiotic *Bifidobacterium infantis* were evaluated using mean doubling time as a parameter for sustaining growth. The lowest mean doubling time was observed for 2% cellobiose, followed by a combination of 2% fructooligosaccharide (FOS) with 2% cellobiose, while during storage at 4°C for 4 weeks of fermented milk, no significant differences were observed between fermented milk supplemented with 2% cellobiose and 2% FOS. The highest viability retention during storage was observed for the combination of the two prebiotics, cellobiose and FOS. The results indicate that, in milk supplemented with cellobiose or a combination of cellobiose and FOS, *Bifidobacterium infantis* remain viable during 4 weeks of storage, suggesting the usefulness of cellobiose as a prebiotic ingredient in fermented products involving bifidobacteria.

Keywords: Bifidobacteria; Cellobiose; Synbiotic; Fermented Milk; Viability

1. Introduction

Dairy functional products containing probiotics, especially fermented milk, have attracted increased interest for prevention of gastrointestinal disorders [1]. Consumption of probiotic dairy products has been shown to exert benefits to human health [2,3]. Probiotics are defined as “live microorganisms which when administered in sufficient numbers, confer a health benefit to the host” [4]. Thus, probiotics (*Lactobacillus* spp. and *Bifidobacterium* spp.) are more and more frequently used for production of fermented dairy products. To provide potential therapeutic benefits, probiotics should remain viable at concentrations $\geq 6 \log \text{CFU} \cdot \text{g}^{-1}$ before consumption [5]. During production and storage of fermented dairy products, the probiotics are exposed to several stresses such as low pH, presence of oxygen, cold and osmotic stress [6-8].

The improvement of growth and viability in fermented foods may be achieved by the use of growth enhancers

such as prebiotics [9-11].

Prebiotics are defined as “non-digestible carbohydrates that beneficially affect the host by selectively stimulating the growth and/or activity of colonic microflora” [12]. Several groups of oligosaccharides have been used for improving the survival of probiotics [13-15]. In addition to their effect on viability retention of probiotics, prebiotics have been shown to increase the growth of lactobacilli and bifidobacteria in the caecum and large intestine. Furthermore, the prevention of travelers’ diarrhea, alleviation of irritable bowel syndrome symptoms and reduction of risk factors for colon cancer have been documented to be related to prebiotics intake [16-18].

Fructooligosaccharides are widely used as prebiotics to improve the growth and viability of bifidobacteria in dairy foods [8,9,19]. Recently, interest in new potential prebiotics such as cellobiosaccharides has increased [20,21].

Bioavailability of cellobiose in humans has been evaluated using cellobiose tolerance tests and breath hydrogen excretion [22]; it has been observed that after inges-

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tion, cellobiose can be fermented by gut microflora. Furthermore, the ingested cellobiose could not be hydrolyzed by the enzymes in the small intestine, reaching the colon undigested [23-25].

Few studies have appeared on the effect of cellobiose on growth rates of *Bifidobacterium* spp. [20]. According to these reports, cellobiose has a higher prebiotic index than FOS [26].

To the best of our knowledge, no study has been reported on the effect of cellobiose on growth and viability of probiotics in fermented milk.

The aim of the present study was to investigate the effects of cellobiose as food ingredient on the growth and fermentation profiles of *Bifidobacterium infantis* UV16PR in comparison to fructooligosaccharide as widely used prebiotic. In addition, the effect of prebiotics alone or in combination on cell viability retention during storage was investigated.

2. Materials and Methods

2.1. Bacterial Growth and Culture Preparation

Bifidobacterium infantis (UV16PR) was kindly by Medipharma (Kågeröd, Sweden). The cells were activated and grown in Reinforced Clostridial Medium (RCM, Oxoid Ltd., Hampshire, UK) by incubation at 37°C in an anaerobic jar using Anaerogen kits (Oxoid Ltd., Hampshire, UK).

2.2. Growth of *Bifidobacterium infantis* in Skim Milk in the Presence of Glucose or Prebiotics

Cellobiose and FOS were used as prebiotics. Glucose was used as reference. The prebiotics were added to 12% (w/v) skim milk, reconstituted according to the manufacturer's instructions, to give a final concentration of 2% (w/v). The supplemented reconstituted skim milk (RSM) was divided in sterile tubes and pasteurized at 70°C for 15 min, according to the method described earlier [19]. Control samples did not contain glucose or prebiotics. A 5% inoculation of cells was used to determine the growth. The inoculated samples were incubated under anaerobic conditions in an anaerobic chamber using Anaerogen kits at 37°C for 48 h. For determination of growth, aliquots of 1 ml were withdrawn immediately after inoculation (time 0 h, baseline) and after 12 h, 24 h, and 48 h. Growth was measured by enumeration of cells using pour plate count methods. Aliquots of 1 ml were serially diluted and then spread onto Reinforced Clostridial Agar (RCA) plates, incubated anaerobically at 37°C for 48 h - 72 h. The mean doubling time (T_d) was calculated using Equation (1) according to Shin *et al.* [19] with minor modification.

$$T_d = \ln 2 / \mu \quad (1)$$

where μ -specific growth rate is calculated using Equation (2) as follows:

$$\mu = (\ln C_2 - \ln C_1) / (t_2 - t_1) \quad (2)$$

where

C_2 —colony forming units per milliliter (CFU/ml) determined at time t_2 ;

C_1 —colony forming units per milliliter (CFU/ml) determined at time t_1 .

2.3. Stability of Cells during Storage

Bifidobacterium infantis cells were cultured in RCM supplemented with 2% cellobiose, 2% FOS or a combination of 2% cellobiose and 2% FOS; after 24 h fermentation in anaerobic conditions, the samples were stored at 4°C for 4 weeks. Aliquots of 1 ml were serially diluted using 9 ml of sterile phosphate buffer. Viable cells were determined after 24 h fermentation (before storage) and after 4 weeks of storage, using plate count methods. The viability during storage was expressed as percent, calculated using Equation (3) as follows:

$$\text{Viability [\%]} = N * 100 / N_0 \quad (3)$$

N —number of viable cells after 4 weeks of storage (CFU/ml);

N_0 —number of viable cells before storage (CFU/ml).

2.4. pH Measurement

pH was determined before and after 24 h fermentation and after 4 weeks of storage at 4°C, using a pH meter (Hanna Instruments, Woonsocket, RI, USA), calibrated with fresh pH 4.0 and 7.0 standard buffers.

2.5. Statistical Analysis

All experiments were performed in triplicate. Statistical analysis was performed using GraphPad Prism (GraphPad Software, San-Diego, USA) by one-way ANOVA, and Tukey test cross-comparing all study groups. Values of $p < 0.05$ were considered significant.

3. Results and Discussion

3.1. Growth of *Bifidobacterium infantis* in Skim Milk Supplemented with Glucose or Prebiotics

The effectiveness of prebiotics toward growth promotion of *Bifidobacterium infantis* was evaluated by measuring mean doubling time. The mean doubling times (expressed in minutes) of *Bifidobacterium infantis* in RSM containing 2% (w/v) cellobiose, 2% FOS, or a combination of 2% (w/v) cellobiose and 2% (w/v) FOS are shown in **Figure 1**.

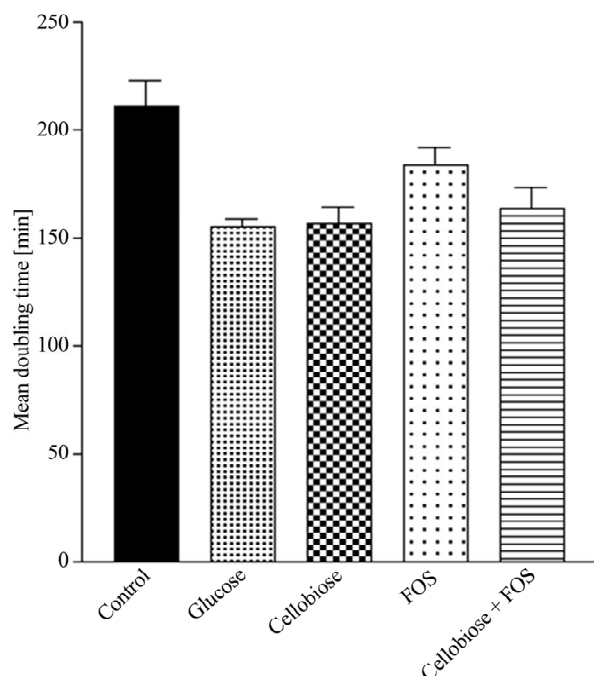


Figure 1. Mean doubling times (in minutes) of *Bifidobacterium infantis* grown in skim milk containing prebiotics (cellobiose and fructooligosaccharides-FOS) or glucose. Control represents samples without addition of any carbon source. Each bar represent means \pm standard deviation, $n = 3$. Determined by Tukey test cross-comparing all study groups. Values of $p < 0.05$ were considered significant.

This is the first study to show the effectiveness of cellobiose-supplemented milk on the growth of probiotics. The fructooligosaccharides have been shown to reduce the mean doubling times of several *Bifidobacterium* spp. and *Lactobacillus* spp. [9,19,27].

In the present study, it was observed that in the presence of cellobiose, or FOS, or their combination the mean doubling time was significantly lower compared to the control ($p < 0.001$).

The 2% supplementation with cellobiose was shown to be the most effective for growth of *B. infantis* in milk, with a mean doubling time of 157.2 min, followed by a combination of cellobiose and FOS with 163.85 min and FOS with a mean doubling time of 183.66 min.

Mean doubling time in cellobiose supplemented milk was significantly lower compared to FOS ($p < 0.05$), but no significant difference was observed compared to the combination of cellobiose and FOS ($p > 0.05$).

The effect of FOS and cellobiose on the growth of *Bifidobacterium* spp. in milk during 48 h of fermentation was shown in **Figure 2**. To the best of our knowledge, the effect of cellobiose on the growth of *Bifidobacterium* spp. in milk has not been studied so far. The cellobiose and cellobioses have been shown to increase the growth of *Bifidobacterium* spp. to different extents in growth medium [20,28].

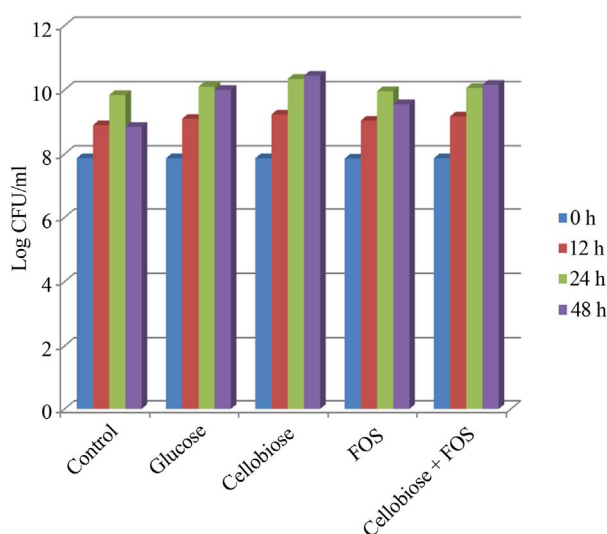


Figure 2. Growth of *Bifidobacterium infantis* in skim milk containing glucose, cellobiose, fructooligosaccharide (FOS), or a combination of cellobiose and FOS, during 48 h of incubation.

Even though the mean doubling time with cellobiose was significantly lower compared to FOS, the differences decreased during storage. This can be attributed to the higher substrate preferences for cellobiose compared to FOS which is in accordance to our previous work (data not shown).

Thus, fermented milk with *Bifidobacterium infantis* in the presence of cellobiose can be used as a functional food product.

Differences in carbohydrate utilization can be attributed to differences in enzyme activities, responsible for metabolizing the specific carbohydrates [20,29].

Similar results were reported earlier [16], who found that carbohydrates with short chains are metabolized faster than longer chain oligosaccharides.

The differences observed on the growth-promoting properties of different carbohydrates on various probiotics can be attributed to differences in transport systems available, to the bacterial cells and to the presence and localization of enzymes responsible for fermentation of certain carbohydrates [30]. Furthermore the type of the linkage between monomers and the degree of polymerization of the carbohydrate influence the growth of the probiotics [31].

3.2. Viability of Cells during Storage in Fermented Milk

The viability of *Bifidobacterium infantis* was decreased during storage in fermented milk (**Figure 3**).

As shown in the figure, the presence of prebiotics significantly increased the viability of cells during storage. These results are in accordance to those previous re-

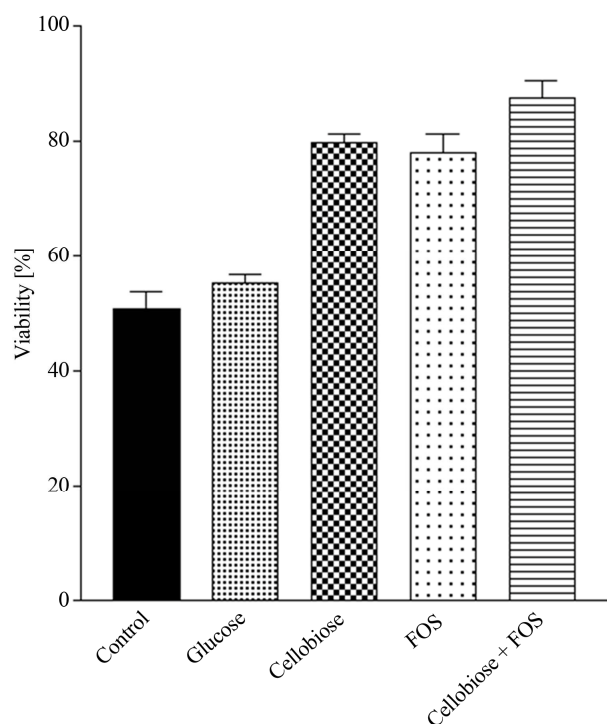


Figure 3. Viability of *Bifidobacterium infantis* grown in skim milk supplemented with glucose, cellobiose, fructooligosaccharide (FOS) or a combination of cellobiose and FOS, after storage for 4 weeks at 4°C. Each bar represent means \pm standard deviation, $n = 3$. Determined by Tukey test cross-comparing all study groups. Values of $p < 0.05$ were considered significant.

ported [9]. The loss of viability during storage can be attributed the presence of oxygen, low pH, cold, and osmotic stresses [6].

In skim milk without prebiotics, the viability of cells was 50%, which is not in accordance to the results shown by [32] who observed an 83% loss of viability during 4 weeks of storage of *Bifidobacterium infantis*. Furthermore, studies reported earlier observed 29.66% viability of cells during storage of *Bifidobacterium infantis* [9]. These differences may be attributed to the different subspecies of *Bifidobacterium infantis* used. In our study, the selected strain of *Bifidobacterium infantis* was shown to exert high β -galactosidase activity that is important for utilization of the lactose present in milk.

These results suggest that the appropriate prebiotic for a single probiotic strain depends on the enzyme activity toward certain prebiotics.

No significant differences were observed in viability during storage in the presence of 2% FOS or 2% cellobiose ($p > 0.05$), namely 79.9% and 77.4%, respectively, while significantly higher viability was observed in the presence of the combination cellobiose + FOS ($p < 0.01$), namely 87.6%.

Cellobiose was shown to be significantly more effec-

tive compared to FOS on the growth of *B. infantis* during fermentation of skim milk, while no significant differences in viability during storage were observed. In the present study, FOS was shown to be effectively enhancing the growth of *B. infantis*, but the utilization is slower compared to cellobiose so that its effect is more evident during storage. The rate of utilization of carbohydrates is influenced by the degree of polymerization of oligosaccharides and the type of linkage between monomer units in the sugar [31].

Some of the differences in enzyme activities responsible for metabolizing the specific carbohydrates, leading to differences in carbohydrate utilization, have been described [20,29].

The pH values of the milk were determined before fermentation, after 24 h of fermentation and after 4 weeks of storage of fermented milk at 4°C. The pH values are shown in **Figure 4**.

Before fermentation the pH values ranged from 6.25 - 6.27.

During fermentation a strong decrease of pH was observed, with values ranging from 4.39 - 4.59 due to the formation of acetic and lactic acids which are the products of sugar metabolism [9,19]. The lower decrease during the storage can be attributed to lower metabolic activity of cells under the storage conditions (low pH and 4°C), resulting in values from 4.09 - 4.29. Similar results were observed on previous reported studies [9,19].

4. Conclusion

The presence of cellobiose and FOS supports the growth of *Bifidobacterium infantis* in skim milk. Supplementation with cellobiose led to significantly lower mean doubling times compared to controls, sucrose and FOS, while

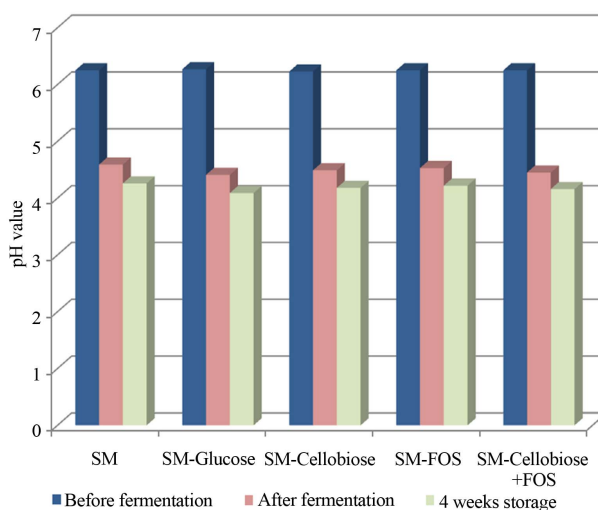


Figure 4. The changes of pH value after fermentation and storage at 4°C of skim milk (SM) fermented by *B. infantis*, in the presence of different carbon sources.

during storage of fermented milk their effectiveness on viability was shown to be comparable. Thus cellobiose alone or in combination with FOS is suitable for synbiotic combinations with *Bifidobacterium infantis* for enhancement of quality of fermented milk.

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Abbreviations

FOS: Fructooligosaccharide
 RCM: Reinforced Clostridial Medium
 RCA: Reinforced Clostridial Agar