2'-Fucosyllactose promotes *Bifidobacterium bifidum* DNG6 adhesion to Caco-2 cells

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ABSTRACT

Adhesion to the intestinal mucosa is the prerequisite for bifidobacteria to colonize and exert biological functions, whereas the choice of carbon source affects the ability of bifidobacteria to adhere to and interact with intestinal epithelial cells. However, knowledge about the relationship between human milk oligosaccharide consumption by bifidobacteria and its adhesion is still limited. In this study, we aim to investigate the effect of 2'-fucosyllactose (2'-FL) as the carbon source on the growth and adhesion properties of *Bifidobacterium bifidum* DNG6, and make comparisons with galactooligosaccharides and glucose. We found that the growth and adhesion properties of *B. bifidum* DNG6 grown in different carbon sources were varied. The 2'-FL as a carbon source improves the adhesion ability of *B. bifidum* DNG6. The expression of adhesion-associated genes was significantly higher in *B. bifidum* DNG6 grown in 2'-FL after incubation with Caco-2 cells compared with that in galactooligosaccharides and glucose. Our results indicated that 2'-FL may promote *B. bifidum* DNG6 adhesion to Caco-2 cells through high expression of genes encoding adhesion proteins. The findings of this study contribute to a better understanding of the involvement of human milk oligosaccharides in the adhesion of bifidobacteria and further support the potential application of 2'-FL as a prebiotic in infant nutritional supplements.

Key words: *Bifidobacterium bifidum*, 2'-fucosyllactose, adhesion, surface proteins

INTRODUCTION

Bifidobacteria are heterofermentative gram-positive bacteria and are particularly enriched in the intestine of breastfed infants due to the large amounts of human milk oligosaccharides (HMO) in breast milk that

support the growth of *Bifidobacterium* (Turroni et al., 2012; Ruiz-Moyano et al., 2013; Matsuki et al., 2016; Yassour et al., 2016). As a probiotic, bifidobacteria contribute to the health of the host, including modulating the balance of intestinal microbiota, promoting the development of the immune system, maintaining the integrity of the mucosal barrier, and resisting the colonization of pathogens (Gareau et al., 2010; Sivan et al., 2015).

To achieve these beneficial effects, adhesion to the intestinal mucosa is the prerequisite for bifidobacteria colonization and persistence in the gastrointestinal tract (Riedel et al., 2006; Ventura et al., 2012). Cell surface proteins play an important role in the bifidobacteria to adhere to and interact with intestinal epithelial cells (Westermann et al., 2016), including pili or fimbriae and some secondary proteins such as elongation factor Tu (**EF-Tu**), chaperone protein DnaK and GroEL, bile salt hydrolase, glutamine synthetase, and so on (Candela et al., 2007; Duranti et al., 2014; Wei et al., 2016; Zhu et al., 2016). Many researchers have demonstrated that in addition to the primary catalytic effect, some enzymes of the bifid shunt are also involved in bacterial adhesion, including transaldolase (**Tal**), enolase (**Eno**), and glyceraldehyde phosphate dehydrogenase (Candela et al., 2009; Gilad et al., 2011; Liu et al., 2011; González-Rodríguez et al., 2012).

Human milk oligosaccharide, the second-largest carbohydrate component of human breast milk, plays a critical role in the establishment and composition of the initial intestinal microbiota, dominated by *Bifidobacterium* species (Thomson et al., 2018). 2'-Fucosyllactose (**2'-FL**, Fuca1–2Galβ1–4Glc), a major oligosaccharide in human milk, which is formed from lactose by fucosylation in α-1,4-glycosidic linkage at the terminal galactose (Ninonuevo et al., 2006; Bode and Jantscher-Krenn, 2012). The 2'-FL attributes many potential health benefits for infants, such as immune system modulation, host-microbe interactions, brain development, and cognitive functions (Vázquez et al., 2015; He et al., 2016; Matsuki et al., 2016). Galactooligosaccharides (**GOS**) and fructooligosaccharides are usually added to infant formula in a certain proportion

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to partly replace the function of HMO (Vandenplas et al., 2015), but they are much simpler in structure than HMO (Roberfroid and Slavin, 2000; Torres et al., 2010). Galactooligosaccharides and fructooligosaccharides are linear polymers of residues of galactose or fructose with terminal glucose, whereas HMO are characterized by a lactose core at the reducing end, and typically modified by a fucose or sialic acid residue at the nonreducing end (Ninonuevo et al., 2006; Bode and Jantscher-Krenn, 2012).

One study showed that HMO-grown *Bifidobacterium longum* ssp. *infantis* had significantly higher adhesion ability to Caco-2 cells compared with that in glucose and lactose (Wickramasinghe et al., 2015). The HMO utilization by *B. longum* ssp. *infantis* triggers the expression of surface adhesion proteins that interact with intestinal cells, and alters gene expression in Caco-2 cells in accordance with a protective regulatory mechanism in the host gut (Chichlowski et al., 2012). Kavanaugh et al. (2013) confirmed that *B. longum* ssp. *infantis* growth in 6'-sialyllactose also increased the adhesion ability to intestinal epithelial cells. In *Bifidobacterium adolescentis*, cell surface pili may play a role in its adhesion and their pilus gene clusters were differentially expressed depending on the carbon sources in the growth medium (Duranti et al., 2014).

It is evident that the types of carbon sources, especially the oligosaccharides with a specific structure, are crucial for selectively promoting bifidobacteria colonization while affecting the interaction of bifidobacteria with intestinal epithelial cells (Turroni et al., 2010; Nishiyama et al., 2017). However, the literature on the relationship between 2'-FL consumption by bifidobacteria and its adhesion is still limited.

In this study, we aim to investigate the effect of 2'-FL as a carbon source on the growth and adhesion properties of *B. bifidum* DNG6, and make comparisons with GOS and glucose. The results of this study may help us better understand the adhesion mechanism of bifidobacteria from the perspective of carbon source utilization. Furthermore, our data provide new insights into the design of symbiotic supplements and also support the potential application of 2'-FL as a prebiotic for infant nutritional supplements.

MATERIALS AND METHODS

Bacterial Strains and Culture Conditions

Bifidobacterium bifidum DNG6 (isolated from adult feces) and *Bifidobacterium animalis* ssp. *lactis* BB12 were obtained from the Key Laboratory of Dairy Science (Harbin, China). The strains were cultured in modified de Man, Rogosa, and Sharpe medium (BD

Difco, Detroit, MI) supplemented with 0.05% (wt/vol) L-cysteine at 37°C in an anaerobic incubator (Thermo Fisher Scientific, Waltham, MA).

Growth Characteristics of *B. bifidum* DNG6

Bifidobacterium bifidum DNG6 was inoculated (2%, vol/vol) into the tubes containing 10 mL of medium with the 2% (wt/vol) filter-sterilized glucose, 2% GOS, or 2% 2'-FL as the sole carbon source, then incubated for 36 h at 37°C in an anaerobic incubator (Thermo Fisher Scientific). The absorbance of the culture was monitored at 600 nm in a spectrophotometer DU 640 (Beckman, Fullerton, CA) every 2 h, and growth curves were prepared. Glucose and GOS were purchased from Sigma-Aldrich (Taufkirchen, Germany), and 2'-FL was purchased from Sharing Technologies (Shanghai, China).

Autoaggregation Assay

The autoaggregation assay was performed according to previous research with some modifications (González-Rodríguez et al., 2012). Briefly, bacterial cells were harvested by centrifugation at $6,000 \times g$ for 10 min at 4°C, washed twice, and resuspended in PBS (0.2 M $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ and 0.2 M $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, pH 7.4). Then, the A_{600} value of the suspension was adjusted to 0.8 ± 0.02 (A_0). Bacterial suspensions (10 mL, 1×10^8 cfu/mL) were mixed and incubated for 5 h at room temperature, and then the absorbance was measured at 600 nm (A_1). The autoaggregation rate was calculated as using the following formula: autoaggregation % = $(1 - A_1/A_0) \times 100$, where A_1 and A_0 represent absorbance values at 5 and 0 h, respectively.

Cell Surface Hydrophobicity Assay

Cell surface hydrophobicity assay was performed according to the method of bacterial adhesion to hydrocarbons as previously described (Pan et al., 2006) with some modifications. Bacterial cells were harvested by centrifugation at $6,000 \times g$ for 10 min at 4°C, washed twice in PBS, and resuspended in 0.1 M KNO_3 . Then, the suspension was adjusted to an optical density value of 0.8 ± 0.02 at 600 nm (A_0). Bacterial suspensions (15 mL) were mixed with xylene (5 mL) and incubated for 10 min at room temperature to form a 2-phase system. The system was vortexed for 2 min and then allowed to separate (aqueous phase and organic phase) by incubation for 20 min. The aqueous phase was carefully removed and the A_{600} was measured (A_1). The percentage of cell surface was calculated using the following formula: hydrophobicity % = $(1 - A_1/A_0) \times 100$, where

A₀ and A₁ were absorbance values before and after extraction with xylene, respectively.

Caco-2 Cell Culture

Enterocyte-like Caco-2 cells were obtained from the American Type Culture Collection (HTB-37, Manassas, VA). The Caco-2 cells were grown using in Dulbecco's modified Eagle medium (Hyclone, Logan, UT) supplemented with 20% (vol/vol) heat-inactivated fetal bovine serum, 1% antibiotics (100 IU/mL penicillin and 100 µg/mL streptomycin), 1% NEAA, and 2 mM L-glutamine (all from Hyclone). The cells were cultured in 25-cm² flasks at 37°C in a 5% CO₂ water-jacketed incubator (Thermo Fisher Scientific, Waltham, MA) and the culture medium was replaced every other day. For adhesion assay, Caco-2 cells were seeded in 6-well tissue culture plates (Corning, NY) at 1×10^5 cells per well and maintained for 2 wk after confluence.

Adhesion Assay

Adhesion to Caco-2 cells was assayed as reported previously with slight modifications (Bianchi et al., 2004). Bacterial cells were collected by centrifugation ($6,000 \times g$ for 10 min at 4°C) and washed twice with PBS. Then, 1 mmol/L CFDA-SE solution [5.0 mg 5(6)-carboxyfluorescein diacetate dissolved in 8.969 mL of dimethylsulfoxide; Beyotime, Shanghai, China] was added to the bacterial suspension at a final concentration of 20 µmol/L. Then the suspension was incubated in the dark at 37°C for 20 min, and centrifuged and washed again to remove excess dye. Two milliliters of CFDA-labeled bacteria (1×10^8 cfu/mL) was added to the confluent monolayer of Caco-2 cells followed by incubation at 37°C for 2 h. After incubation, unbound bacteria were removed by washing 3 times with PBS and the adhered bacteria cells were released by adding 250 µL of 0.25% trypsin-EDTA (0.25% Trypsin and 0.02% EDTA, Hyclone) for 2 min at 37°C. Then the fluorescence intensity was measured using a fluorescence spectrophotometer (F-4500, Hitachi, Tokyo, Japan) in the following conditions: excitation wavelength of 492 nm, emission wavelength of 517 nm, and slit width of 2.5 nm. The percentage of adhesion rate was represented by the ratio of the fluorescence intensity of bacteria adhesion to Caco-2 cells to the original fluorescence intensity of bacteria.

Treatments During Adhesion Assay

Bifidobacterium bifidum DNG6 was grown overnight anaerobically at 37°C in 300 mL of modified de Man,

Rogosa, and Sharpe medium with 2% (wt/vol) glucose, and then bacterial cells were harvested by centrifugation ($6,000 \times g$ for 10 min at 4°C) and washed twice with PBS. Then, 15 mL of lithium chloride (LiCl, 5 mol/L), sodium periodate (NaIO₄, 0.05 mol/L), proteinase K (0.5 mg/mL), or phenol (PhOH, 0.8 mol/L) was added to the above bacterial precipitate then incubated at 37°C for 30 min, respectively. After incubation, the bacterial cells were collected by centrifugation and used for adhesion assays. The bacterial adhesion rate was tested after different treatments as mentioned above, and the untreated bacteria were used as a control. The relative change of adhesion was calculated as a percentage (González-Rodríguez et al., 2012).

Transmission Electron Microscopy Observation

The cell surface characteristics of bacteria were observed as described by Zhu et al. (2016). *Bifidobacterium bifidum* DNG6 was cultured anaerobically overnight at 37°C in an Erlenmeyer flask containing 200 mL of medium with 2% (wt/vol) glucose, and was harvested by centrifugation ($6,000 \times g$ for 10 min at 4°C) and washed twice with PBS. Subsequently, 10 mL of LiCl (5 mol/L) was added to the above bacterial cells, then incubated at 37°C for 30 min. After incubation, the bacterial precipitate was collected by centrifugation, and then fixed with 3% glutaraldehyde for 2 h at room temperature. After being washed 5 times with PBS, the samples were dipped into 1% phosphotungstic acid for 1 h, dehydrated by 30, 50, 70, 90, and 100% graded ethanol for 5 min each, and dried in a drying chamber. The specimens were observed under a transmission electron microscope (HT7700, Hitachi, Tokyo, Japan), and the untreated bacteria were used as a control.

Expression of Genes in *B. bifidum* DNG6 in Response to Incubation with Caco-2 Cells

The expression levels of target genes in *B. bifidum* DNG6 in the presence or absence of Caco-2 cells were analyzed by reverse transcription quantitative real-time PCR (RT-qPCR). The sequences of the primers are listed in Table 1. Bacterial cells were incubated with Caco-2 cells as described above. As a control group, the bacteria were incubated under the same conditions without Caco-2 cells. After 2 h of incubation, the coculture cells were washed 3 times with PBS and harvested in Trizol reagent (Invitrogen, Carlsbad, CA) followed by RNA extraction immediately. The RNA was extracted from bacterial cells using the RNeasy Midi Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. Complementary DNA was

prepared with 1 µg of total RNA as the template using the cDNA RT reagent kit (Takara, Kusatsu, Japan). The RNA and DNA concentrations were measured by an ND-1000 spectrophotometer (Thermo Fisher Scientific) at 260/280nm and the quality was further assessed by agarose gel electrophoresis. The RT-qPCR was performed using SYBRGreen Premix Ex Taq (TaKaRa, Kusatsu, Japan) in a LightCycler instrument (Applied Biosystems, Waltham, MA) according to the manufacturer's protocol. The reaction mixture volume totaled 20 µL containing 10 µL of SYBR Green PCR Master Mix (TaKaRa), 0.4 µL of ROX Reference Dye (TaKaRa), 1 µL of 10 µM each primer, 2 µL of cDNA, and 6.6 µL of RNase-free water. The amplification conditions suggested by the manufacturer was 3 min of activation at 95°C, followed by 40 cycles of denaturation at 95°C for 10 s and annealing at 60°C for 30 s. Three independent repetitions were performed for the test and the $2^{-\Delta\Delta C_t}$ method was used to calculate the relative expression levels of genes. The 16S rRNA was amplified as an internal standard to normalize gene expression (Matsuki et al., 2004).

Statistical Analysis

Data are presented as the means $\pm$ standard deviations of 3 independent experiments. Statistical significance was determined by Student's *t*-test or one-way ANOVA, and *P*-values <0.05 were considered significant.

RESULTS

Growth of *B. bifidum* DNG6 Grown in Different Carbon Sources

The growth curve is shown in Figure 1; the growth rates of *B. bifidum* DNG6 grown in different carbon sources were varied. When *B. bifidum* DNG6 was grown in 2'-FL, the lag phase (12 h) was significantly longer than that in glucose (4 h) and GOS (6 h; *P* < 0.05). After reaching the stationary phase, the maximum optical density value (1.3) of *B. bifidum* DNG6 grown in 2'-FL was significantly lower compared with that in glucose (1.7) and GOS (1.5; *P* < 0.05).

Autoaggregation of *B. bifidum* DNG6 Grown in Different Carbon Sources

Because autoaggregation has been demonstrated to be linked to bacteria adhesion (González-Rodríguez et al., 2012), we examined the aggregation of *B. bifidum* DNG6 grown in different carbon sources (Figure 2A).

Table 1. Primers used in reverse-transcription quantitative real-time PCR

Name	Sequence ¹ (5'-3')
16S rRNA	(F) AGAGTTTGATCCTGGCTCAG (R) TACGGTTACCTTGTACGA
<i>tal</i>	(F) GCTCAAGGAACCTCGGCAAGGTC (R) CGGAAGATGTGGTGGCGTTG
<i>eno</i>	(F) TCCGCCTACATGCTCGACTACTAC (R) CTTCCCAGCCTTCCTCGTTGAAC
<i>groL</i>	(F) GCCAAGAAGGTCATCGTCTCCAAG (R) GCAGCTTCTCACGGTCGTAATCG
<i>tuf</i>	(F) GTGCTGACCAAGGACGAAGGC (R) GTCGGTGGTGCGGAAGTAGAAC

¹F = forward; R = reverse.

Bifidobacterium bifidum DNG6 cultured in 2'-FL exhibited the highest percentage of autoaggregation ($34.32 \pm 2.53\%$), followed by that in GOS ($31.11 \pm 3.62\%$) and glucose ($29.63 \pm 1.79\%$), but no significant difference in autoaggregation was identified between the 3 groups (*P* > 0.05).

Hydrophobicity of *B. bifidum* DNG6 Grown in Different Carbon Sources

Hydrophobicity probably has a certain influence on nonspecific interactions of bacteria adhesion (Pan et al., 2006); the hydrophobicity of *B. bifidum* DNG6 grown in different carbon sources was studied (Figure

Figure 1. Growth curve of *Bifidobacterium bifidum* DNG6 grown in different carbon sources: glucose (GL), galactooligosaccharide (GOS), and 2'-fucosyllactose (2'-FL), measured as the optical density (OD) at 600 nm in different culture times. The strain was controlled at 37°C in an anaerobic incubator.

Figure 2. Cell surface properties of *Bifidobacterium bifidum* DNG6 grown in different carbon sources: glucose (GL), galactooligosaccharide (GOS), and 2'-fucosyllactose (2'-FL). Data are expressed as means $\pm$ SD of 3 biological and technical replicates. Bars with different letters (a–c) differ significantly ($P < 0.05$). (A) Autoaggregation; (B) hydrophobicity.

2B). *Bifidobacterium bifidum* DNG6 cultured in 2'-FL exhibited the highest percentage of hydrophobicity ($88.24 \pm 3.02\%$), followed by that cultured in glucose ($83.27 \pm 5.47\%$) and GOS ($82.96 \pm 2.51\%$). Similar to autoaggregation results, the hydrophobicity values also did not differ significantly between the 3 groups ($P > 0.05$).

Adhesion of *B. bifidum* DNG6 Grown in Different Carbon Sources

The Caco-2 cells are widely used as an important in vitro model for studying the interaction between bacteria and intestinal epithelial cells (Pinto et al., 1983). As shown in Figure 3, the adhesion of *B. bifidum* DNG6 grown in glucose was $7.08 \pm 0.27\%$, whereas the adhesion of the reference strain *B. animalis* ssp. *lactis* BB12 grown in glucose was $6.53 \pm 0.34\%$ ($P > 0.05$). The adhesion of *B. bifidum* DNG6 to Caco-2 cells ranged from $6.64 \pm 0.36\%$ to $9.34 \pm 0.31\%$ depending on the carbon sources. *Bifidobacterium bifidum* DNG6 grown in 2'-FL showed the highest adhesion rate ($9.34 \pm 0.31\%$) compared with that in GOS ($6.64 \pm 0.36\%$) and glucose ($7.08 \pm 0.27\%$; $P < 0.01$).

Effect of Chemical Treatments on *B. bifidum* DNG6 Adhesion to Caco-2 Cells

Different treatments were performed to determine the involvement of carbohydrates (NaIO_4), proteins (proteinase K and LiCl), and teichoic acids (PhOH) on the cell surface of *B. bifidum* DNG6 adhesion to Caco-2 cells (Figure 4). All treatments inhibited the adhe-

sion ability of *B. bifidum* DNG6 ($P < 0.01$). Among them, the adhesion rate of *B. bifidum* DNG6 was significantly decreased by 62.92% and 58.96% after LiCl and proteinase K treatment ($P < 0.01$), respectively, and the adhesion rate was decreased by 48.38% after PhOH treatment, whereas the adhesion of *B. bifidum*

Figure 3. Adhesion of *Bifidobacterium bifidum* DNG6 to Caco-2 cells after culture with different carbon sources: glucose (GL), galactooligosaccharide (GOS), and 2'-fucosyllactose (2'-FL). *Bifidobacterium animalis* BB12 was used as the reference strain. Data are expressed as means $\pm$ SD of 3 biological and technical replicates. Different letters (a, b) above bars indicate statistical differences ($P < 0.01$).

Figure 4. Percent changes in adhesion to Caco-2 cells of *Bifidobacterium bifidum* DNG6 after different chemical treatments. Data are presented as means $\pm$ SD of 3 independent experiments. *indicates a statistically significant difference compared with the untreated bacteria (** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$).

DNG6 was reduced by only 27.34% after treatment with NaIO₄. At the same time, we observed the surface morphology of bacterial by TEM. The *B. bifidum* DNG6 showed a rough outer surface layer (Figure 5, arrowhead), whereas the surface protein of cell was removed and the cell surface became smooth after LiCl treatment; this result suggested that surface-exposed proteins in *B. bifidum* DNG6 were mainly involved in the adhesion process.

Expression of Adhesion-Associated Genes in *B. bifidum* DNG6 in Response to Incubation with Caco-2 Cells

To further analyze the effect of carbon source utilization on bacterial adhesion, we tested the expression levels of genes encoding Tal, Eno, GroEL, and EF-Tu. As shown in Figure 6, the expression levels of tested genes (*tal*, *eno*, *groL*, and *tuf*) were significantly increased in *B. bifidum* DNG6 cultivated with Caco-2 cells ($P < 0.01$), indicating that these genes may be involved in the adhesion of bacteria to the host. However, these genes were expressed differently in *B. bifidum* DNG6 grown on different carbon sources. The expression of all genes was significantly higher in *B. bifidum* DNG6 grown in 2'-FL compared with that in glucose and GOS ($P < 0.01$). Among them, the highest expression levels (15.2-fold) were observed in *tal*, followed by *eno* (10.8-fold), *tuf* (9.2-fold), and *groL* (8.6-fold). These results suggest that 2'-FL can promote the expression of adhesion-associated genes in *B. bifidum* DNG6 in response to contact with Caco-2 cells. Additionally, the expression of *tal* and *tuf* in *B. bifidum* DNG6 was significantly higher in GOS than in the glucose ($P < 0.01$).

DISCUSSION

Adhesion to the intestinal mucosa plays a pivotal role in bifidobacteria colonization and modulation of the host immune system (Ventura et al., 2012). Evi-

Figure 5. Transmission electron microscopy micrographs of *Bifidobacterium bifidum* DNG6. (A) Control, and (B) treatment with LiCl (magnification, $\times 60,000$). Arrow indicates rough outer surface layer in control.

dence suggests that the choice of carbon source affects the ability of bifidobacteria to adhere to and interact with epithelial cells (Riedel et al., 2006; Chichlowski et al., 2012). Many enzymes of carbon catabolism have been demonstrated that are cell wall associated and are involved in adhesion progress (Wei et al., 2014; Nishiyama et al., 2017). Thus, it is necessary to study the effect of carbon source utilization in the adhesion of bifidobacteria to intestinal epithelial cells.

The ability of bifidobacteria to use fucosyllactose varies among strains and species (Bunesova et al., 2016). Sakanaka et al. (2019) revealed an evolutionary adaptation of bifidobacteria to HMO structures; the fucosyllactose transporter-2 in *B. longum* ssp. *infantis* is a key factor for fucosyllactose utilization. Ruiz-Moyano et al. (2013) found that a specific GH29 fucosidase contributed to the ability of *Bifidobacterium breve* to use 2'-FL. In this study, we found that *B. bifidum* DNG6 could grow in medium containing 2'-FL, indicating the strain has a specific enzyme system to hydrolyze fucosylated HMO. In addition, we observed that the growth rate of *B. bifidum* DNG6 cultured in 2'-FL was significantly lower compared with that in GOS, probably because the structure of 2'-FL is more complex than GOS and 2'-FL is decorated with a fucose residue attached to

the lactose core, whereas GOS are short-chain linear polymers containing residues of galactose terminating with glucose, which requires more glycosidases and carbohydrate transporters to be used (Torres et al., 2010; Bode and Jantscher-Krenn, 2012).

Our results showed that the adhesion ability of *B. bifidum* DNG6 to Caco-2 cells changed according to the carbon source supplied in their growth medium. 2'-Fucosyllactose-grown *B. bifidum* DNG6 had significantly higher adhesion ability to Caco-2 cells compared with that grown in glucose and GOS. This result is similar to those of Wickramasinghe et al. (2015) who showed that growth of *B. longum* ssp. *infantis* on HMO also resulted in increased adherence to intestinal epithelial cells.

The cell envelope of bifidobacteria as the structural interface is critical for bacterial attachment and colonization because the surface-associated molecules directly affect host-microbe interactions (Desvaux et al., 2006; Nishiyama et al., 2017). In this study, the adhesion ability of *B. bifidum* DNG6 was significantly decreased after treatment with proteinase K and LiCl, indicating that the surface-associated proteins of *B. bifidum* DNG6 play a key role in adhesion to Caco-2 cells. This result is consistent with Guglielmetti et al. (2008) who

Figure 6. Relative expression of the adhesion-associated genes in *Bifidobacterium bifidum* DNG6 grown on different carbon sources in response to incubation with Caco-2 cells. As a control group, the strain was incubated under the same conditions without Caco-2 cells. The fold changes were calculated by dividing the gene expression values of the experimental group by that of the control group. Data are normalized against the internal reference gene of 16S rRNA and expressed as means $\pm$ SD of 3 biological replicates. Different letters (a–c) above bars indicate statistical significance ($P < 0.01$). GL = glucose; GOS = galactooligosaccharide; 2'-FL = 2'-fucosyllactose.

demonstrated that BopA, a surface protein, is directly involved in the adhesion of *B. bifidum* to Caco-2 cells, and the strain excited a strongly adhesive phenotype depending on the environmental conditions (the presence of sugars and pH). In addition, we observed that NaIO₄ and PhOH treatment inhibited the adhesion of *B. bifidum* DNG6, suggesting that carbohydrates and teichoic acids, in part, may also be involved in the adhesion of *B. bifidum* DNG6 to Caco-2 cells, which is in agreement with previous findings (Guglielmetti et al., 2009).

In this study, all the 4 tested genes (*tal*, *eno*, *groL*, and *tuf*) were expressed at higher levels in *B. bifidum* DNG6 following contact with Caco-2 cells. This agrees with the study by Wei et al. (2014), who concluded that these genes are involved in bifidobacterial adhesion. Furthermore, the expression of all genes was significantly higher in *B. bifidum* DNG6 grown in 2'-FL. These results suggest that 2'-FL may promote the interaction of bifidobacteria and intestinal epithelial cells by enhancing the expression of adhesion molecules.

Cell surface proteins have a great influence on the bifidobacterial adhesion through facilitating close contact with the gut epithelial cells (Desvaux et al., 2006). Acting as a cell surface receptor for human plasminogen, Eno plays a significant role in the interactions of bifidobacteria with the host (Candela et al., 2009). Tal, like other enzymes of the bifid shunt, has also been detected on the cell surface of several *B. bifidum* strains. González-Rodríguez et al. (2012) demonstrated that Tal can serve as an important adhesion factor favoring the colonization of *B. bifidum* in the intestinal tract.

The EF-Tu is a secondary protein, which in addition to the basic function in protein synthesis, also has the ability to bind plasminogen (Candela et al., 2007). Wei et al. (2014) indicated that EF-Tu as surface receptors for *Bifidobacterium longum* binding human plasminogen, is involved in bifidobacterial adhesion. Just like EF-Tu, GroEL is a kind of surface-associated proteins of bifidobacteria and contributed to its adhesion ability (Sun et al., 2016).

Our results demonstrated that 2'-FL can improve the adhesion ability of *B. bifidum* DNG6 in vitro. Laparra and Sanz (2009) reported that bacterial strains exhibit different adhesion ability in different in vitro models of intestinal epithelia, showing preferences for intestinal mucin. Therefore, further in vivo studies will be required to verify the in vitro results because in vitro models cannot fully mimic the mucosa-bacteria interactions, which is important for bacterial adhesion in vivo. Moreover, considering that the current results are based on the gene level, follow-up studies are needed at the protein level to elucidate specific surface proteins

and their role in bifidobacteria adhesion to intestinal epithelial cells.

CONCLUSIONS

In this study, the growth and adhesion properties of *B. bifidum* DNG6 grown on different carbon sources differed. We show that 2'-FL improves the adhesion ability of *B. bifidum* DNG6 through high expression of adhesion-associated genes. These results provide more information about the involvement of 2'-FL as a carbon source in the adhesion of bifidobacteria. Our results suggest that the design of probiotic supplements may need to choose appropriate prebiotics, thereby effectively promoting the colonization of bifidobacteria.

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