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2′-Fucosyllactose as a prebiotic modulates the probiotic responses of *Bifidobacterium bifidum*

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ABSTRACT

2′-Fucosyllactose (2′-FL), one of the most representative oligosaccharides in human milk, is intimately linked to the enrichment of specific *Bifidobacterium* species. However, the efficacy of 2′-FL in modulating the probiotic responses of bifidobacterium has been rarely researched. Thereinto, three key issues have yet to be reported: the effects of 2′-FL hydrolysis on bifidobacterial growth, the protective effects of 2′-FL on bifidobacterium under gastrointestinal stress and the inhibitory activity of 2′-FL metabolites against *Cronobacter* spp. This work intended to address these concerns. 2′-FL dramatically accelerated the growth and proliferation of *Bifidobacterium bifidum* YH17 and *Bifidobacterium bifidum* BBI01. The glucose in lactose core on 2′-FL was preferable for *B. bifidum* to achieve substantial increases in biomass while the galactose was not readily available. Additionally, 2′-FL showed unique advantages in ameliorating the resistance of *B. bifidum* to gastrointestinal challenges. 2′-FL considerably improved the adhesive property of *B. bifidum*, thus facilitating the competitive elimination of *Cronobacter sakazakii* ATCC 29544 and *Cronobacter muytjensii* ATCC 51329 by B. *bifidum*. The growth inhibition of 2′-FL on the *Cronobacter* strains was mediated by promoting the secretion of antibacterial substances from *B. bifidum*. The inhibitory activity hinged on the *B. bifidum* strains. 2′-FL specifically induced *B. bifidum* BBI01 to produce some antibacterial substances that were proteinaceous, thermostable and relatively stable even at pH 8.0. These antibacterial substances played a key role in the inhibitory activity and had a synergistic effect with acidification. These observations provide a useful guideline for developing synbiotic supplements to intervene the infant gut microbiota.

1. Introduction

Establishing a salutary intestinal microbial ecosystem is vital to motivate the development of neonatal gastrointestinal tract (GIT) (Sela et al., 2008). A key premise for the maintenance of infant gut microbiota homeostasis is the ability of beneficial commensal microbes to survive in adequate amounts, colonize, and suppress the overgrowth of pathogens in GIT (Patel and Denning, 2015; Plaza-Diaz et al., 2019). Bifidobacterial presence and abundance have been widely regarded as a microbial biomarker which represents the positive succession of core infant beneficial microbiota (Milani et al., 2017; Turroni et al., 2022). However, the growth and probiotic properties of bifidobacterium are greatly limited by the highly microbial competition and complex stress conditions in early-life GIT (Fernández et al., 2013; Kanjan and Hongpattarakere, 2017; Matamoros et al., 2013).

The metabolic accessibility to prebiotic oligosaccharides in neonatal diet is a potent evolutionary force in shaping a uniquely bifidobacterium-rich gut ecosystem (Milani et al., 2016). It has been fully validated that supplementation with nondigestible prebiotic oligosaccharides can exert pronounced bifidogenic effects (Akkerman et al., 2019; Thomson et al., 2018; Wang et al., 2024). Galactooligosaccharides (GOS) were the most conventional prebiotic oligosaccharides. Unfortunately, even with the presence of GOS, powdered infant formula is still incriminated as the principal infection source of *Cronobacter* necrotizing enterocolitis (a bacterial infection that can be devastating to infants) in epidemiology (Feeney et al., 2014; Pagotto et al., 2003; Singh et al., 2015). By contrast, breast milk is the optimal diet to nourish and protect the developing neonatal microbiome (Walsh et al., 2020). Human milk oligosaccharides (HMOs) in breast milk are natural prebiotic carbohydrates composed of more than 200 structurally diverse oligosaccharides (Spicer et al., 2022). HMOs are famous for their unwavering support in constructing an ideal gut microbial consortium dominated by *Bifidobacterium* (Sela and Mills, 2010; Thomson et al., 2018; Walsh et al., 2020). Besides, multiple studies conducted in animal

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models and neonates have demonstrated that specific HMOs can reduce the morbidity and delay the progression of necrotizing enterocolitis (Nino et al., 2016).

2′-Fucosyllactose (2′-FL) accounts for around 30% of all HMOs, thereby being labeled as the most abundant oligosaccharide in human milk (Chen et al., 2021; He et al., 2016). An extra fucose unit is joined together with a lactose molecule working as the core by an α (1–2) glycosidic bond, which forms 2′-FL. It is a typical representative of HMOs that have strong bifidogenic effects (Natividad et al., 2022; Van den Abbeele et al., 2021). The underlying mechanisms by which 2′-FL exerts bifidogenic effects have been investigated mainly in three aspects: the growth of target bifidobacterium, the interaction among *Bifidobacterium* members and the maintenance of probiotic properties of bifidobacterium. Stimulation to the growth of target bifidobacterium and cross-feeding activities within *Bifidobacterium* communities have been the sharp focus of most research (Bunesova et al., 2016; Centanni et al., 2019; Egan et al., 2014; Garrido et al., 2016; Ruiz-Moyano et al., 2013; Ward et al., 2007; Yu et al., 2013). Until now, the evaluation with respect to the impact of 2′-FL and even other HMOs on probiotic properties of bifidobacterium has only considered a few features, namely the improvement of adhesion as well as the generation of short chain fatty acids (Centanni et al., 2019; James et al., 2019; Zabel et al., 2019; Zhang et al., 2020). Therefore, the effects of 2′-FL hydrolysis on bifidobacterial growth, the protective effects of 2′-FL on bifidobacterium throughout GIT conditions and the inhibition of 2′-FL metabolites on *Cronobacter* spp. have been less studied for years.

In this work, 2 strains of *Bifidobacterium bifidum* were screened out from 14 candidates as desired hosts for efficiently metabolizing 2′-FL. We paid close attention to the effects of 2′-FL hydrolysis on the growth of *B. bifidum*, the protective effects of 2′-FL on the survival of *B. bifidum* under simulated GIT conditions and the inhibition of 2′-FL metabolites against *Cronobacter* strains. Additionally, the effects of 2′-FL on the adhesion of *B. bifidum* were estimated.

2. Materials and methods

2.1. Bacterial strains, cells and culture conditions

Except for *B. longum* subsp. *longum* N58, *B. longum* subsp. *longum* L2 and *B. longum* subsp. *longum* A45 that were provided by our lab, the other eleven *Bifidobacterium* strains listed in Table 1 were obtained from Jiaxing Innocul-Probiotics Co. LtD (Jiaxing, Zhejiang, China). Bifidobacterial strains were routinely subcultured in deMan Rogosa Sharpe (MRS) broth (Oxide Ltd, Basingstoke, Hampshire, UK) and incubated anaerobically (90% N_2, 5% CO_2 and 5% H_2) at 37 °C for 24 h. All growth media were supplemented with 0.05% L-cysteine.

The pathogenic bacteria used in this study were *Cronobacter sakazakii* ATCC 29544 and *Cronobacter muytjensii* ATCC 51329. They were purchased from the American Type Culture Collection (ATCC). The pathogens were grown in Tryptic Soy Broth (Oxide Ltd, Basingstoke, Hampshire, UK) at 37 °C for 18 h with constant shaking (100 rpm).

Human enterocytelike Caco-2 cells (ATCC HTB-37, ATCC, Manassas, VA) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Gibco) at 37 °C in a humidified atmosphere containing 5% CO_2 (v/v). The medium was replaced every 2 days.

Table 1
Bifidobacterial strains used in this work.

Bifidobacterium	Strain	Origin
Bifidobacterium animalis subsp. *lactis*	UD03	Infant feces
Bifidobacterium animalis subsp. *lactis*	0704–2	Infant feces
Bifidobacterium animalis subsp. *animalis*	YH11	Infant feces
Bifidobacterium bifidum	YH17	Infant feces
Bifidobacterium bifidum	BBI01	Infant feces
Bifidobacterium breve	YH68	Infant feces
Bifidobacterium breve	UD04	Infant feces
Bifidobacterium longum subsp. *longum*	N58	Infant feces
Bifidobacterium longum subsp. *longum*	INO31	Infant feces
Bifidobacterium longum subsp. *longum*	YH295	Infant feces
Bifidobacterium longum subsp. *longum*	INO17	Infant feces
Bifidobacterium longum subsp. *longum*	L2	Infant feces
Bifidobacterium longum subsp. *longum*	A45	Infant feces
Bifidobacterium longum subsp. *longum*	INO10	Infant feces

2.2. Analysis of carbohydrates utilization

Bifidobacterial abilities to utilize each carbohydrate were evaluated according to the method described previously with some modifications (Thongaram et al., 2017). Exponential phase cultures were subcultured twice in MRS. Bacteria were harvested by centrifugation for 10 min at 10000 rpm at 4 °C. Cell pellets were washed twice with phosphate buffered saline (PBS) before being resuspended in 5 mL of carbohydrate-free semidefinite MRS (sMRS) (10^9 CFU/mL). Bacterial suspensions were separately inoculated (1%, v/v) into sMRS or sMRS supplemented with 1% (w/v) of glucose (Glu), galactose (Gal), fucose (Fuc), lactose (Lac), GOS, 2′-FL. With the exceptions of Fuc, GOS and 2′-FL purchased from Glycarbo Co. Ltd (Takamatsu, Kagawa Prefecture, Japan), the rest of the carbohydrates were purchased from Sigma-Aldrich (St. Louis, Mo., USA). 400 μL aliquots of bacterial inocula were injected into the honeycomb plates and then 50 μL aliquots of mineral oil were also added to seal the liquids. The incubation was carried out under anaerobic conditions at 37 °C for 72 h. A Bioscreen C machine (Oy Growth Curves Ab Ltd, Helsinki, Finland) was used to monitor the growth of bifidobacterium by capturing the changes in optical density at 600 nm (OD_{600}) every 30 min. Based on the maximum OD_{600} values obtained, the growth of given strains was classified into five phenotypes: no appreciable growth ($OD_{600} \leq 0.03$), limited growth ($0.3 \leq OD_{600} \leq 0.5$), moderate growth ($0.5 \leq OD_{600} \leq 0.8$), good growth ($0.8 \leq OD_{600} \leq 1.1$) and vigorous growth ($1.1 \leq OD_{600}$). The differences in OD_{600} values were visualized by heatmap and dendrogram to assess the capacity of each strain for growth on various carbohydrates.

2.3. Analysis of growth kinetic parameters

To confirm that the increases in OD_{600} values are associated with bacterial growth, bacterial cultures of *B. bifidum* YH17 and *B. bifidum* BBI01 were serially diluted and pour-plated into agar-Reinforced Clostridial Medium (Oxide Ltd, Basingstoke, Hampshire, UK). Under anaerobic conditions, the petri-dishes were incubated at 37 °C for 48 h. Whereafter, colonies were enumerated. The DMFit program worked out the kinetic parameters (lag phase and the maximum growth rate) by fitting the growth data into the Baranyi and Roberts mathematical model.

2.4. Determination of the concentrations of saccharides

The bacterial cultures of *B. bifidum* YH17 and *B. bifidum* BBI01 were centrifuged so that the supernatants were collected. All the limpid supernatants were filtered through 0.22 μm sterile filter membranes to prepare cell-free supernatants (CFSs). Thereafter, 10 μL of sample solution was injected into an Alliance E2695-HPLC system (Waters, Milford, Massachusetts, USA). A Dikma Polyamino HILIC column (Dikma, Beijing, China) coupled with an evaporative light-scattering detector was employed to separate and quantify the saccharides (Glu, Gal, Fuc, Lac and 2′-FL). The saccharides were eluted with a mixed solvent of phosphoric acid (1%), MiliQ water (24%) and acetonitrile (75%) at a flow rate of 1.0 mL/min. The column temperature was maintained at 30 °C. When plotting standard curves, a mixture of standards with varying concentrations (0.15, 0.3, 0.6 and 1.2 mg/mL) was prepared. The peak areas of the saccharides belonging to different samples were standardized by comparing them with those of standards, and the corrected values were used for statistical analysis.

2.5. Resistance to the chemically simulated GIT transit

The survival rates of *B. bifidum* YH17 and *B. bifidum* BBI01 suffering from the chemically simulated GIT transit were determined using a standardized laboratory digestion model with slight modifications (Brodkorb et al., 2019; Minekus et al., 2014). Both *B. bifidum* strains were cultivated for 24 h in sMRS containing either Glu, Lac, GOS or 2′-FL as the sole carbon source. Prior to being resuspended in aliquots of simulated gastric juice, bacterial cells were washed twice with sterile PBS. Afterwards, anaerobic incubation was performed at 37 °C for 120 min with swirling (200 rpm). This procedure was carried out again under otherwise identical conditions. To wit, the cells were incubated in aliquots of simulated intestinal juice. Viable cell counts were separately obtained before and after incubation in the chemically simulated gastrointestinal juices. The survival percentage was calculated from the logarithm of viable cell counts recovered after complete simulated GIT transit with respect to the logarithm of initial cell counts.

2.6. Resistance to lysozyme

The resistance of *B. bifidum* YH17 and *B. bifidum* BBI01 to lysozyme was assessed by the method of Dias et al. (2015) with minor modifications. *B. bifidum* strains were grown for 24 h in sMRS supplemented with either Glu, Lac, GOS or 2′-FL as the single carbohydrate. Bacterial cells were resuspended in aliquots of PBS containing 100 μg/mL lysozyme after being washed. Samples were incubated anaerobically at 37 °C for 2 h. The method for ciphering out the survival rate was identical to that described in 2.5.

2.7. Auto-aggregation

The auto-aggregation assay was conducted according to the method adopted by Anand et al. (2018). *B. bifidum* strains were cultivated for 24 h in sMRS added either Glu, Lac, GOS or 2′-FL as the sole carbon source. Cells were harvested, washed and resuspended in 4 mL aliquots of sterile PBS, respectively. The concentrations of cell suspensions were standardized to 10^8 CFU/mL by vortexing for 10 s. Samples were incubated anaerobically at 37 °C for 5 h. Whereafter, the upper most portion (1 mL) of the cell suspensions were carefully collected and the OD_{600} values were measured. The percentage was calculated according to the following equation:

$$\text{Auto-aggregation (\%)} = (1 - A_t / A_0) \times 100$$

where A_t represents the OD_{600} value at 5 h and A_0 represents the OD_{600} value at 0 h.

2.8. Adhesion to Caco-2 cells

The adhesive properties of *B. bifidum* YH17 and *B. bifidum* BBI01 to Caco-2 cells were examined referring to the methods of Xiao et al. (2023) and Parkar et al. (2008). Both *B. bifidum* strains were cultured for 24 h in sMRS containing either Glu, Lac, GOS or 2′-FL as the single carbon source. Bacteria were washed thrice with sterile PBS. DMEM without serum was used to prepare the bacterial cell suspensions (2×10^8 CFU/mL). Caco-2 cells were inoculated at a concentration of 2×10^5 cells/mL in 6-well tissue culture plates and cultivated until monolayers formed. Bacterial suspensions were subsequently added to the washed cell monolayers at a multiplicity of infection ratio of 100:1 (bacteria to Caco-2 cells). Two hours of incubation was carried out in a humidified atmosphere containing 5% CO_2 at 37 °C. Then the unattached bacteria were softly rinsed off the monolayers with PBS. Finally, cells were lysed for 15 min with 0.1% (v/v) Triton-X100 solutions. The number of adhered bacteria was enumerated by the plate count method. The ratio of adhered bacteria to those initially added was calculated.

2.9. Co-aggregation

The co-aggregation assay was performed in line with the method of Anand et al. (2018). *B. bifidum* YH17 and *B. bifidum* BBI01 were grown for 24 h in sMRS where either Glu, Lac, GOS or 2′-FL served as the single carbon source. *C. sakazakii* ATCC 29544 and *C. muytjensii* ATCC 51329 were grown in Tryptic Soy Broth for 18 h. Bifidobacterial cells and pathogenic bacteria were respectively harvested, washed and resuspended in sterile PBS. Every cell suspension was adjusted to about 10^8 CFU/mL. Each cell suspension of the *B. bifidum* strains (4 mL) was transferred in a sterile tube. Cell suspensions of *Cronobacter* strains (4 mL) were also transferred into sterile tubes separately. Each cell suspension of *B. bifidum* strains (2 mL) was mixed with the cell suspension of either *C. sakazakii* ATCC 29544 (2 mL) or *C. muytjensii* ATCC 51329 (2 mL) separately. Every sample was mixed well by vortexing for 10 s and then incubated for 5 h at 37 °C without agitation. After that, OD_{600} values of the upper most portion of the cell suspensions (1 mL) were measured. Co-aggregation percentage was calculated as follows:

$$\text{Co-aggregation (\%)} = \{(A_x + A_y)/2 - A_{x+y}\}/\{(A_x + A_y)/2\}$$

Where A_x represents the OD_{600} value of each cell suspension of either *B. bifidum* YH17 or *B. bifidum* BBI01, A_y represents the OD_{600} value of cell suspension of either *C. sakazakii* ATCC 29544 or *C. muytjensii* ATCC 51329, A_{x+y} represents the OD_{600} value of each mixed cell suspension.

2.10. Inhibitory activity against Cronobacter strains

The inhibitory activity of both *B. bifidum* strains cultivated with different carbohydrates against *C. sakazakii* ATCC 29544 and *C. muytjensii* ATCC 51329 was measured using agar well diffusion method described by Geng et al. (2023) with necessary modifications. CFSs deriving from the *B. bifidum* strains were prepared. Bacterial suspensions at the concentration of 10^8 CFU/mL were prepared after the pellets of both *B. bifidum* strains were washed. Analogously, bacterial suspensions of the two pathogens at the concentration of 10^8 CFU/mL were prepared using Tryptic Soy Broth. A petri-dish was pre-prepared by pouring 10 mL of 2% agar as the base. Three sterile Oxford cups (**Φ** 8.0 mm × 6 mm × 10 mm) were placed after the agar had solidified. Bacterial suspensions of the two *Cronobacter* strains were separately mixed well with sterile Tryptic Soy agar to a concentration of 10^7 CFU/mL. 15 mL of each mixture was poured into sterile pre-prepared plates. Oxford cups were taken off after the agar had solidified and each well was filled with 200 μL of either media, probiotic cell suspensions or CFSs before incubation at 37 °C for 24 h. The inhibitory activity was expressed as the diameter of the zone which was not colonized by pathogenic bacteria around each well.

2.11. Sensitivity of B. bifidum BBI01 CFSs to pH, heat and enzymes

To determine the nature of the antibacterial substances in CFSs deriving from *B. bifidum* BBI01, the sensitivity of the *B. bifidum* BBI01 CFSs to pH, heat and enzymes were detected according to the methods described previously with some modifications (Hirasawa and Kurita-Ochiai, 2020; Lin and Pan, 2019). A pH meter was applied to measuring the pH values of Glu CFS, Lac CFS, GOS CFS and 2′-FL CFS every 2 h during 24 h of batch cultivation of *B. bifidum* BBI01. The *B. bifidum* BBI01 CFSs were adjusted to different pH values ranging from 2.0 to 10.0 using 1 mol/L HCl or 1 mol/L NaOH. As for heat treatments, Glu CFS, Lac CFS, GOS CFS and 2′-FL CFS of *B. bifidum* BBI01 were heated at 60 °C, 80 °C, 100 °C and 121 °C for 15 min, respectively. As for enzyme treatments, proteinase K, pepsin, trypsin and catalase (Sigma-Aldrich, St. Louis, MO, USA) were separately added to the *B. bifidum* BBI01 CFSs at a concentration of 1 mg/mL. The reaction mixtures were adjusted to the optimum pH for proteinase K (7.5), pepsin (2.0), trypsin (8.0) and catalase (7.0), respectively. The reaction mixtures were

incubated for 2 h at 37 °C. After the enzyme reactions, the reaction mixtures were separately adjusted to the initial pH values of the *B. bifidum* BBI01 CFSs. Finally, the enzymes were inactivated at 95 °C for 20 min. The inhibition zones of the treated *B. bifidum* BBI01 CFSs against *Cronobacter* strains were measured. The inhibition zones produced by the treated CFSs were normalized as a percentage of the inhibition zones produced by the untreated Glu CFS, Lac CFS, GOS CFS and 2′-FL CFS of *B. bifidum* BBI01. The data were subjected to One-way ANOVA followed by Dunnett's multiple comparisons test.

2.12. Statistical analysis

The heatmap and dendrogram were generated using Hiplot, a network utility (https://hiplot.com.cn/home/index.html) required for visualizing multivariate data clustering. Statistical analysis was implemented in GraphPad Prism 8.0 software. At least three replicates were performed in each program. The data were expressed as mean ± SD. Outliers were excluded after checking the normal distribution of the data sets. Unless emphasized otherwise, data were subjected to Two-way ANOVA followed by Tukey's multiple comparisons test. Statistical significance and the relative symbols were defined as follows: * ($P < 0.05$); ** ($P < 0.01$); *** ($P < 0.001$); and **** ($P < 0.0001$).

3. Results

3.1. B. bifidum YH17 and B. bifidum BBI01 were desired hosts for efficiently metabolizing 2′-FL

Differential ability to utilize the tested carbohydrates divided 14 bifidobacterial strains into three clusters (Fig. 1). All the strains did not display appreciable growth in sMRS without specific carbohydrate supplementation, whereas they were able to utilize either Glu, Lac or GOS to achieve good to vigorous growth. *B. animalis* subsp. *lactis* UD03、*B. animalis* subsp. *lactis* 0704-2 and *B. animalis* subsp. *animalis* YH11 constituted the first cluster. This cluster was characterized by the inability to metabolize Gal, Fuc as well as 2′-FL. The second cluster exhibited good to robust growth on all carbon sources except for Fuc and only included *B. bifidum* YH17 and *B. bifidum* BBI01. The third cluster consisted of 7 *B. longum* subsp. *longum* strains and 2 *B. breve* strains. Apart from *B. breve* YH68 that could attain limited growth on Fuc, the other 8 strains were incapable of any growth on Fuc. Even though 2′-FL was shown to be a poor substrate for the growth of the strains in this cluster, Gal was observed to support intense growth of these strains. In the light of these results, *B. bifidum* YH17 and *B. bifidum* BBI01 were obtained as desired hosts for efficiently metabolizing 2′-FL.

3.2. 2′-FL accelerated the growth and multiplication of B. bifidum

Both *B. bifidum* strains achieved substantial increases in biomass within 24 h when either Glu, Lac, GOS or 2′-FL served as the sole carbon source (Fig. 2A/B). During 24 h–72 h, the order of the OD_{600} values of both *B. bifidum* strains was Lac > 2′-FL > Glu > GOS ($P < 0.0001$). When either Glu, Lac or 2′-FL was employed as the single carbon source, no significant difference in the lag phase among the three groups was detected ($P > 0.05$) (Fig. 2C). By contrast, GOS distinctly shorten the lag phase of the *B. bifidum* strains compared with the other three carbon sources ($P < 0.05$). The order of the maximum growth rates of the two strains was Lac > Glu > 2′-FL (Fig. 2D), albeit with no considerable difference among the three groups ($P > 0.05$). The maximum growth rates of both *B. bifidum* strains grown in 2′-FL were substantially higher

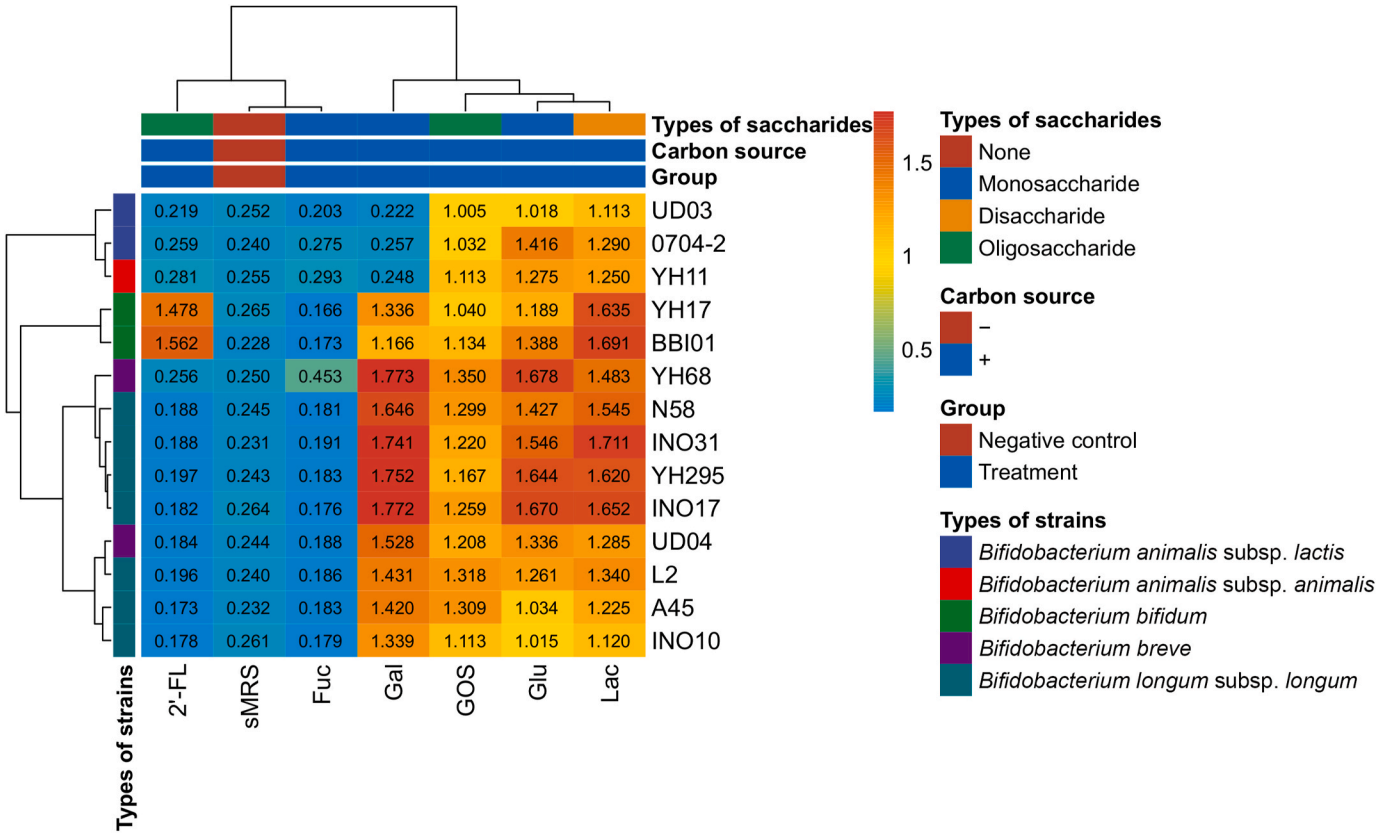

Fig. 1. Maximum growth of bifidobacterium using media with or without carbohydrate. Maximum growth was defined as the maximum optical density at 600 nm (OD_{600}) attained by bifidobacterium in carbohydrate-free semidefinite MRS (sMRS) or sMRS supplemented with 1% (w/v) glucose (Glu), galactose (Gal), fucose (Fuc), lactose (Lac), galactooligosaccharides (GOS) or 2′-fucosyllactose (2′-FL). No growth was observed in sMRS. The blank control wells without bacteria or carbohydrate were subtracted to correct the OD_{600} values. Relatedness of growth patterns was visualized by dendrogram.

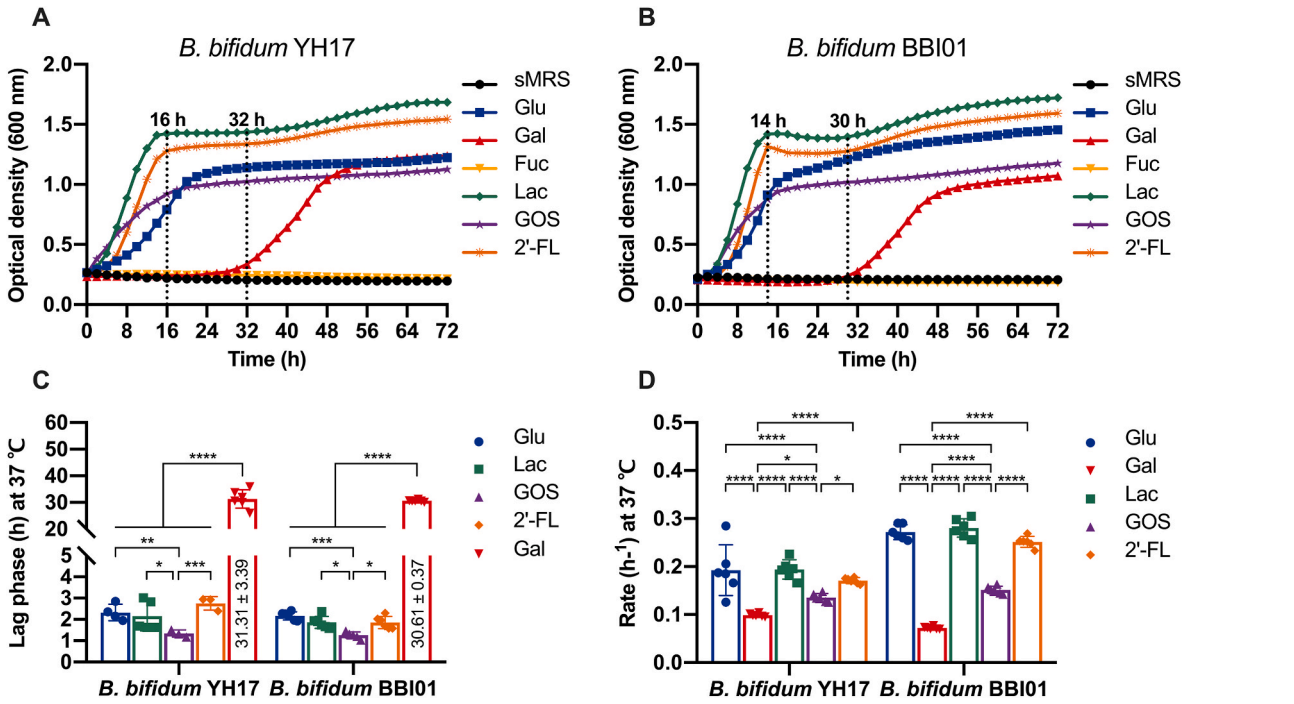

Fig. 2. Growth of *B. bifidum* YH17 and *B. bifidum* BBI01. (A) Growth profiles of *B. bifidum* YH17. (B) Growth profiles of *B. bifidum* BBI01. (C) Lag phases of *B. bifidum* YH17 and *B. bifidum* BBI01. (D) The maximum growth rates of *B. bifidum* YH17 and *B. bifidum* BBI01. *B. bifidum* strains were grown in carbohydrate-free semidefinite MRS (sMRS) or sMRS supplemented with 1% (w/v) glucose (Glu), galactose (Gal), fucose (Fuc), lactose (Lac), galactooligosaccharides (GOS) or 2′-fucosyllactose (2′-FL).

than those attained by growth in GOS ($P < 0.05$). On the basis of above data, 2′-FL notably accelerated the growth and multiplication of the two *B. bifidum* strains.

3.3. The surge in biomass of *B. bifidum* benefited from the hydrolysis of the lactose core on 2′-FL wherein the glucose was preferred to the galactose for the hosts

As shown in Table 2, Glu, Gal, Fuc and Lac are structural units of 2′-FL. Hereby, the growth profiles of the *B. bifidum* strains cultivated in these four different carbon sources were included for contrastive analysis. As shown in Fig. 2A/B, the growth patterns of both *B. bifidum* strains in 2′-FL almost perfectly replicated the ones obtained in Lac. Both strains did not show any sign of growth when Fuc was used as the sole carbon source. The growth kinetic parameters of the *B. bifidum* strains in Lac were not significantly different from those in 2′-FL ($P > 0.05$) (Fig. 2C/D). Early in the first logarithmic phase (0–6 h), the concentration of 2′-FL dropped drastically with a concomitant sharp increase in Fuc and Lac (Fig. 3B/D). At the mid-logarithmic phase (8–10 h), a spot of 2′-FL remaining in the spent media was depleted. The concentration of Fuc had remained constant ever since. When the cells entered the late logarithmic growth period, the concentration of Lac declined rapidly. Trace amounts of Lac declined to negligible levels during the stationary phase. These observations indicated that the surge in biomass of both

Table 2
Structures of lactose, 2′-fucosyllactose and galactooligosaccharides.

Saccharide [a]	Structure [a]
Lac	Gal β (1–4) Glu
2′-FL	Fuc α (1–2) Gal β (1–4) Glu
GOS	n [Gal β (1–2, 3, 4 or 6)] Glu [b]

[a] Abbreviations involved were as follows: lactose (Lac), galactose (Gal), glucose (Glu), 2′-fucosyllactose (2′-FL), fucose (Fuc), galactooligosaccharides (GOS).

[b] n represented the degree of polymerization (n = 2–8).

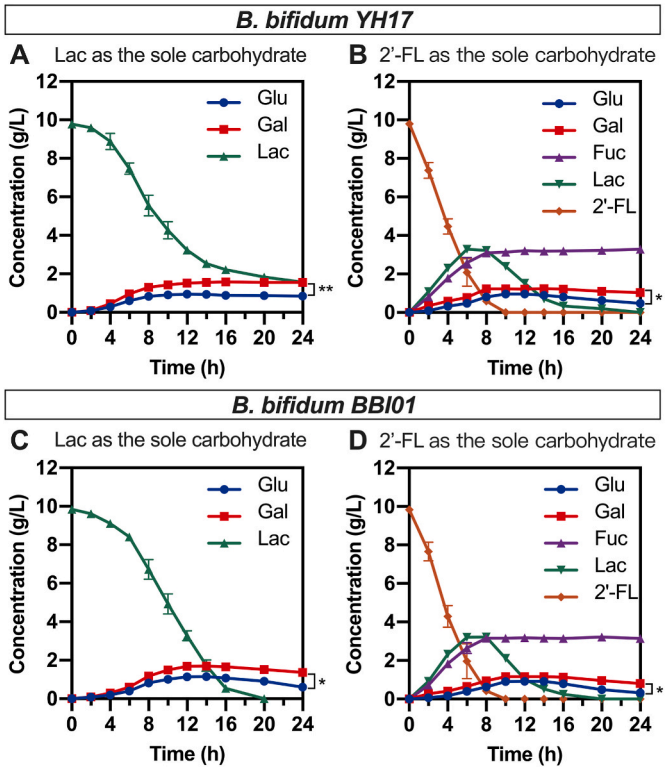

Fig. 3. Saccharide profiles of lactose (Lac) or 2′-fucosyllactose (2′-FL) degradation by *B. bifidum* YH17 and *B. bifidum* BBI01. (A) (C) Variation in concentrations of glucose (Glu), galactose (Gal) and Lac in media containing Lac as the sole carbon source. (B) (D) Variation in concentrations of Glu, Gal, fucose (Fuc), Lac and 2′-FL in media containing 2′-FL as the sole carbon source.

B. bifidum strains primarily benefited from the hydrolysis of the lactose core on 2′-FL.

Furthermore, Gal conferred both *B. bifidum* strains on the maximum lag phases and the minimum growth rates ($P < 0.05$) (Fig. 2). The end of the lag phase of either *B. bifidum* YH17 or *B. bifidum* BBI01 in Gal (31.31 ± 3.39 h, 30.61 ± 0.37 h) coincided virtually with the onset of the second small logarithmic growth phase (32 h, 30 h) in Lac and 2′-FL. The variation trends of Glu and Gal in 2′-FL spent media were similar to those in Lac spent media (Fig. 3). The concentration of Gal was always prominently higher than that of Glu within 24 h ($P < 0.05$). Even when the cells entered the stationary phase, the concentration of Glu declined rapidly with the occurrence of a slight and slow decrease in Gal. These findings in aggregate implied that the glucose in lactose core on 2′-FL was the much more favorable substrate for the *B. bifidum* strains to attain a sharp increase in biomass during the first logarithmic phase.

3.4. 2′-FL ameliorated the resistance of B. bifidum to GIT challenges

Pre-cultivated in either Glu, Lac, GOS or 2′-FL for 24 h, the survival rates of both *B. bifidum* strains after exposure to GIT transit or lysozyme are presented in Fig. 4. *B. bifidum* YH17 and *B. bifidum* BBI01 subsisting on 2′-FL displayed the highest survival rates (70.2 ± 1.7%, 73.5 ± 1.1%) after being subjected to GIT transit. The survival percentages of both strains were remarkably lower when GOS was employed and still lower when Lac was used. As expected, both *B. bifidum* strains cultivated in the media containing Glu were least tolerant of GIT transit. There were systematic statistically significant differences among these results ($P < 0.05$). Moreover, the ability to withstand lysozyme determines the viability of bifidobacterium in GIT to some extent. Metabolism of 2′-FL endowed *B. bifidum* YH17 and *B. bifidum* BBI01 with the highest resistance to lysozyme (88.4 ± 2.8%, 92.8 ± 2.4%). The tolerance of both strains to lysozyme followed the same trend as their survival rates after exposure to GIT transit, to wit, 2′-FL > GOS > Lac > Glu ($P < 0.01$). These findings manifested that none of the three other carbon sources could provide such an effective protection to the persistence of target *B. bifidum* in GIT as 2′-FL.

3.5. 2′-FL improved the adhesion efficiency of B. bifidum

Adhesion to host cell surfaces is considered a necessary characteristic for bifidobacterium to colonize the intestinal mucosa. Auto-aggregation is postulated to be the first step in adhesion process and hence being identified as an important phenotype of adhesion (Krausova et al., 2019). Fig. 5A shows the percentages of auto-aggregation of both *B. bifidum* strains pre-cultivated in different carbon sources. Both strains exhibited the highest percentages of auto-aggregation when 2′-FL was employed as the single carbon source, followed by GOS, Lac and Glu in descending order ($P < 0.01$). Re et al. (2010) reported that the auto-aggregation of bacteria was positively correlated with their adhesive properties. The notion was verified by the percentage of the *B. bifidum* strains attached to Caco-2 cells (Fig. 5B). 2′-FL contributed to markedly higher binding efficiency of *B. bifidum* YH17 and *B. bifidum* BBI01 to Caco-2 cells (9.94 ± 1.38%, 12.20 ± 1.35%) than the other groups ($P < 0.05$). When the three other carbohydrates served as the sole carbon source, the order of the adhesive ability of both strains was GOS > Lac > Glu. Significant differences only existed between the group of GOS and Glu ($P < 0.05$). Overall, our findings further emphasized a distinct advantage of 2′-FL in improving the adhesion efficiency of the *B. bifidum* strains.

3.6. 2′-FL facilitated the competitive elimination of Cronobacter strains by B. bifidum

The co-aggregation of bifidobacterium and pathogenic bacteria will minimize the risk of infection by impeding pathogens from adhering to the intestinal mucosa. *B. bifidum* YH17 and *B. bifidum* BBI01 pre-cultured in 2′-FL had the highest co-aggregation percentage with *C. sakazakii* ATCC 29544 (38.20 ± 0.68%, 45.67 ± 0.92%), followed by the *B. bifidum* strains pre-cultured in GOS (32.31 ± 1.19%, 40.34 ± 0.94%), Lac (31.17 ± 1.25%, 38.40 ± 0.78%) and Glu (25.98 ± 1.30%, 34.17 ± 0.98%) (Fig. 6A). Likewise, the co-aggregation of the *B. bifidum* strains and *C. muytjensii* ATCC 51329 showed a uniform tendency. *B. bifidum* YH17 and *B. bifidum* BBI01 pre-cultured in 2′-FL had the highest co-aggregation percentage with *C. muytjensii* ATCC 51329 (36.58 ± 1.24%, 42.26 ± 0.98%), followed by both *B. bifidum* strains pre-cultured in GOS (35.46 ± 0.92%, 39.10 ± 0.81%), Lac (30.20 ± 1.04%, 36.61 ± 0.59%) and Glu (22.43 ± 1.51%, 32.08 ± 0.96%) (Fig. 6B). To sum up these observations and the data of adhesion, it was the enhanced adhesion characteristic of *B. bifidum* thanks to 2′-FL that remarkably elevated their competitive elimination of *Cronobacter* strains.

3.7. 2′-FL inhibited the growth of Cronobacter strains by promoting the secretion of antibacterial substances from B. bifidum

Prior to the fermentation of the *B. bifidum* strains, media supplemented with different carbon sources were incapable of any growth inhibition on *Cronobacter* strains (No inhibition zone). Regardless of the carbon source, bacterial cells of both *B. bifidum* strains could not result in the formation of inhibition zones. Conversely, CFSs deriving from either

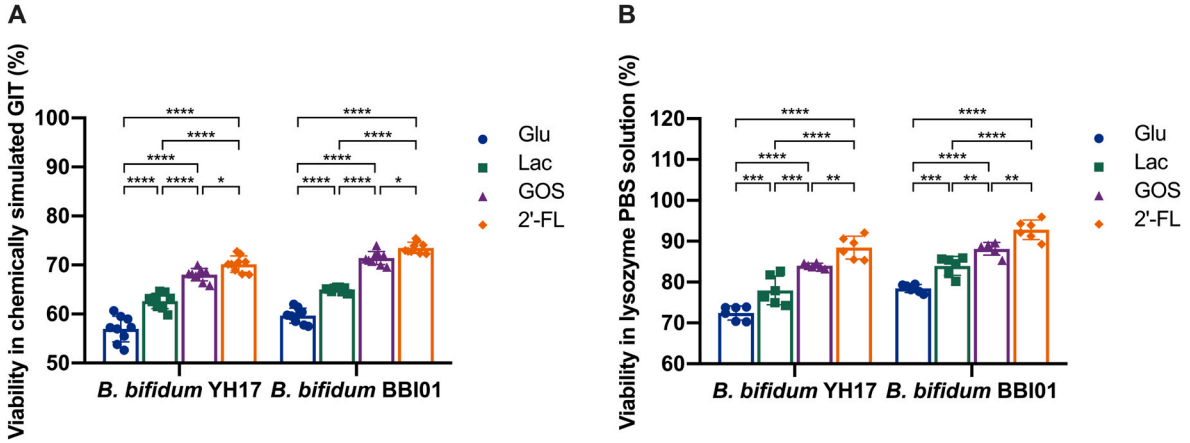

Fig. 4. Stress tolerance of *B. bifidum* YH17 and *B. bifidum* BBI01 pre-cultured in media containing either glucose (Glu), lactose (Lac), galactooligosaccharides (GOS) or 2′-fucosyllactose (2′-FL) as the sole carbon source. (A) Viability after chemically simulated gastrointestinal tract (GIT) transit. (B) Viability in PBS solution supplemented with lysozyme.

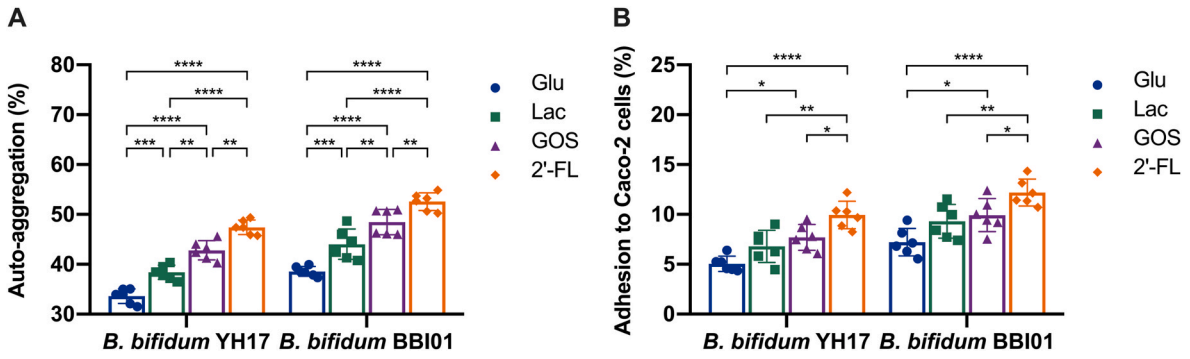

Fig. 5. Adhesive properties of *B. bifidum* YH17 and *B. bifidum* BBI01 pre-cultured in media containing either glucose (Glu), lactose (Lac), galactooligosaccharides (GOS) or 2′-fucosyllactose (2′-FL) as the sole carbon source. (A) Auto-aggregation. (B) Adhesion to the human epithelial intestinal cell line Caco-2.

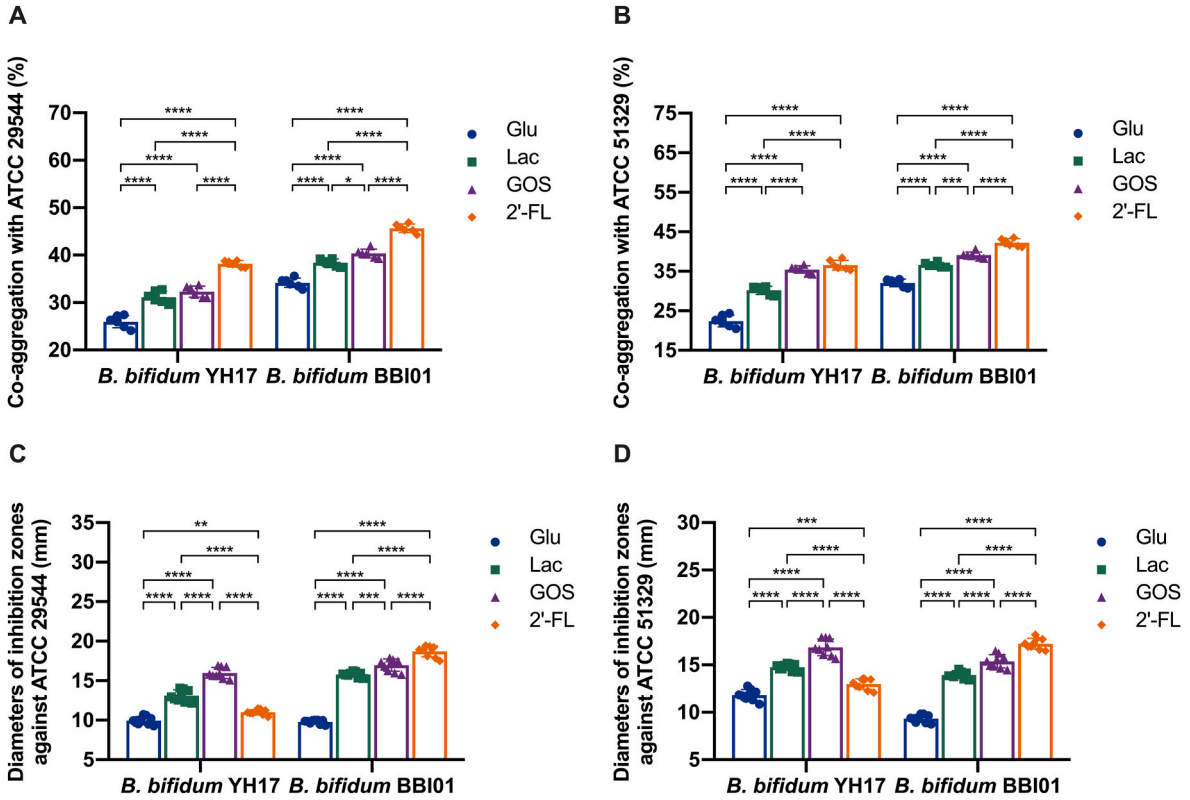

Fig. 6. Antagonism of *B. bifidum* YH17 and *B. bifidum* BBI01 against *Cronobacter* strains. (A) Co-aggregation with *C. sakazakii* ATCC 29544. (B) Co-aggregation with *C. muytjensii* ATCC 51329. (C) Inhibitory activity against *C. sakazakii* ATCC 29544. (D) Inhibitory activity against *C. muytjensii* ATCC 51329. Inhibitory activity was expressed as the inhibition zone diameter (mm). *B. bifidum* strains were cultivated in media containing either glucose (Glu), lactose (Lac), galactooligosaccharides (GOS) or 2′-fucosyllactose (2′-FL) as the sole carbon source.

B. bifidum YH17 or *B. bifidum* BBI01 displayed inhibitory activity against the *Cronobacter* strains (Fig. 6C/D). GOS CFS of *B. bifidum* YH17 produced the largest inhibition zones against the two *Cronobacter* strains, followed by Lac CFS, 2′-FL CFS and Glu CFS ($P < 0.01$). As for *B. bifidum* BBI01, the order of the inhibition zones against both *Cronobacter* strains was 2′-FL CFS > GOS CFS > Lac CFS > Glu CFS ($P < 0.001$). These data manifested that the growth inhibition of 2′-FL on *Cronobacter* strains was achieved by promoting the secretion of antibacterial substances from *B. bifidum*. And the inhibitory activity varied depending on the strains of *B. bifidum*. Obviously, *B. bifidum* BBI01was a more suitable host for producing antibacterial substances to inhibit the growth of the *Cronobacter* strains when 2′-FL served as the sole carbon source.

3.8. Some antibacterial substances in 2′-FL CFS of B. bifidum BBI01 played a key role in the inhibitory activity, kept relatively stable even at pH 8.0 and had a synergistic effect with acidification

As shown in Fig. 7B, after 24 h of batch cultivation, the pH values of *B. bifidum* BBI01 CFSs markedly increased in an order from Lac CFS (3.84 ± 0.02), 2′-FL CFS (4.05 ± 0.03), Glu CFS (4.15 ± 0.02) to GOS CFS (4.27 ± 0.02) ($P < 0.01$). The growth inhibition of *B. bifidum* BBI01 CFSs on *Cronobacter* strains (Fig. 6C/D) was obviously inconsistent with the acidity. Accordingly, acidification was not the sole determinant of the inhibitory activity of *B. bifidum* BBI01 CFSs.

As shown in Tables 3 and 4, when the pH value ranged from 4.0 to 2.0, the decrease in pH value endowed Glu CFS with the greatest percentage increase in the inhibitory activity against *Cronobacter* strains,

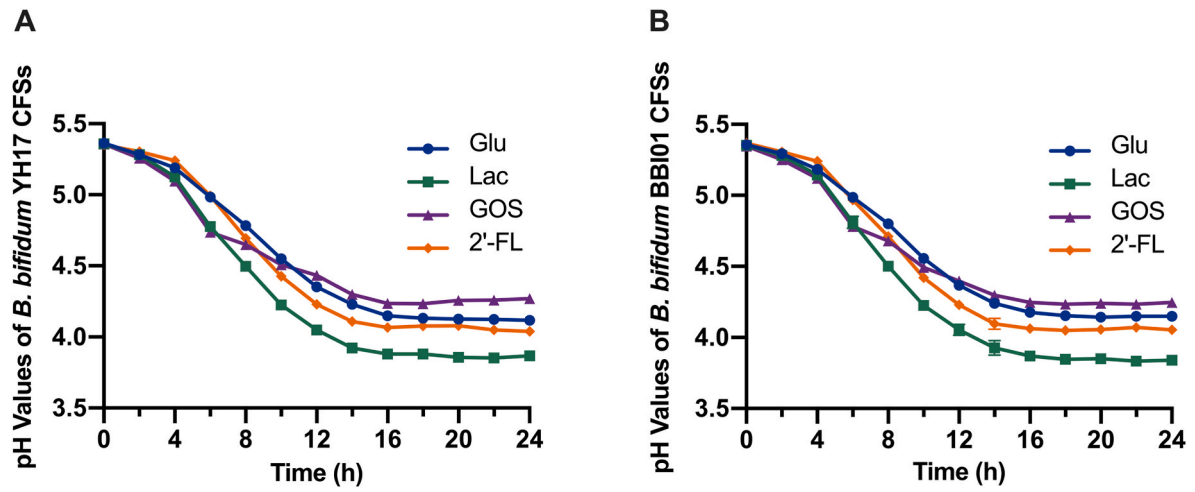

Fig. 7. pH values of cell-free supernatants (CFSs) deriving from media containing either glucose (Glu), lactose (Lac), galactooligosaccharides (GOS) or 2′-fucosyllactose (2′-FL) as the sole carbon source.

Table 3
Effects of pH, heat and enzymes on the inhibitory activity of *B. bifidum* BBI01 CFSs against *C. sakazakii* ATCC 29544.

Treatment conditions		The percentage of growth inhibition (%) [b]			
		Glu CFS	Lac CFS	GOS CFS	2′-FL CFS
Control [a]		100.00 ± 0.98	100.00 ± 2.14	100.00 ± 1.35	100.00 ± 1.20
pH	2	129.20 ± 1.67****	119.82 ± 0.59****	108.71 ± 0.83****	105.50 ± 0.61****
	3	112.58 ± 1.32****	108.75 ± 1.16****	105.60 ± 0.57****	104.25 ± 0.48***
	4	103.11 ± 2.28	96.74 ± 0.99**	101.43 ± 0.48	100.17 ± 1.40
	5	–	51.57 ± 0.39****	94.43 ± 0.68****	96.88 ± 0.90*
	6	–	–	80.12 ± 1.24****	76.98 ± 2.13****
	7	–	–	51.50 ± 0.82****	59.66 ± 0.71****
	8	–	–	–	50.27 ± 1.62****
	9	–	–	–	–
	10	–	–	–	–
Heat (15 min)	60 °C	100.31 ± 2.00	100.49 ± 0.73	99.44 ± 1.67	99.74 ± 0.84
	80 °C	100.48 ± 1.45	100.23 ± 0.74	99.29 ± 0.73	100.30 ± 1.14
	100 °C	99.96 ± 1.39	100.59 ± 0.83	100.20 ± 0.33	100.05 ± 0.52
	121 °C	99.86 ± 1.48	99.85 ± 1.35	100.00 ± 1.46	100.00 ± 0.85
Enzymes	Proteinase K	90.46 ± 1.65****	82.87 ± 0.85****	73.25 ± 0.97****	62.07 ± 0.67****
	Pepsin	91.93 ± 0.66****	85.81 ± 0.52****	75.83 ± 0.55****	61.10 ± 0.74****
	Trypsin	93.09 ± 1.97***	90.73 ± 1.36****	79.58 ± 0.77****	67.53 ± 0.71****
	Catalase	100.44 ± 1.74	99.57 ± 0.73	99.68 ± 0.51	100.40 ± 1.25

*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
– No inhibition zone.
[a] Untreated Glu CFS, Lac CFS, GOS CFS and 2′-FL CFS of *B. bifidum* BBI01 were used as control, respectively.
[b] The percentage of growth inhibition (%) = (Inhibition zone produced by treated CFS/Inhibition zone produced by untreated CFS) × 100.

Table 4
Effects of pH, heat and enzymes on the inhibitory activity of *B. bifidum* BBI01 CFSs against *C. muytjensii* ATCC 51329.

Treatment conditions		The percentage of growth inhibition (%) [b]			
		Glu CFS	Lac CFS	GOS CFS	2′-FL CFS
Control [a]		100.00 ± 1.44	100.00 ± 0.80	100.00 ± 1.24	100.00 ± 1.24
pH	2	123.64 ± 1.87****	114.38 ± 0.48****	106.43 ± 0.42****	104.12 ± 0.82****
	3	119.25 ± 1.14****	106.44 ± 0.47****	104.01 ± 0.95***	102.84 ± 0.71**
	4	108.81 ± 0.85****	95.06 ± 0.95****	102.90 ± 0.88**	100.77 ± 0.64
	5	–	45.48 ± 0.63****	92.81 ± 1.61****	94.46 ± 0.54****
	6	–	–	77.12 ± 1.37****	74.81 ± 0.61****
	7	–	–	52.23 ± 0.48****	57.59 ± 0.41****
	8	–	–	–	52.43 ± 0.84****
	9	–	–	–	–
	10	–	–	–	–
Heat (15 min)	60 °C	100.35 ± 1.13	99.30 ± 0.47	99.37 ± 0.82	99.88 ± 0.99
	80 °C	99.83 ± 1.30	99.53 ± 0.90	99.96 ± 0.71	99.69 ± 1.22
	100 °C	99.57 ± 2.09	99.89 ± 1.29	99.78 ± 0.76	100.04 ± 0.58
	121 °C	100.14 ± 1.39	99.43 ± 1.15	100.00 ± 1.04	100.39 ± 0.26
Enzymes	Proteinase K	91.15 ± 1.91****	82.52 ± 1.74****	72.73 ± 0.79****	64.49 ± 0.72****
	Pepsin	93.49 ± 1.14***	84.10 ± 0.56****	74.50 ± 0.75****	67.56 ± 0.51****
	Trypsin	94.12 ± 1.90***	81.49 ± 0.43****	79.22 ± 1.15****	72.08 ± 1.00****
	Catalase	100.18 ± 0.96	99.68 ± 1.10	99.53 ± 0.78	100.48 ± 0.76

*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.
– No inhibition zone.
[a] Untreated Glu CFS, Lac CFS, GOS CFS and 2′-FL CFS of *B. bifidum* BBI01 were used as control, respectively.
[b] The percentage of growth inhibition (%) = (Inhibition zone produced by treated CFS/Inhibition zone produced by untreated CFS) × 100.

followed by Lac CFS, GOS CFS and 2′-FL CFS ($P < 0.01$). Once the pH value exceeded 4.0, Glu CFS completely lost the inhibitory activity. When the pH value ranged from 4.0 to 7.0, the increase in pH value weakened the inhibitory activity of Lac CFS, GOS CFS and 2′-FL CFS to varying degrees ($P < 0.05$). As for Lac CFS, a portion of the inhibitory activity was retained at pH 5.0, while the inhibitory activity was

completely abolished at pH 6.0. In sharp contrast to Glu CFS and Lac CFS, GOS CFS and 2′-FL CFS retained the bulk of the inhibitory activity (more than 70%) when the pH value ranged from 5.0 to 6.0. At pH 7.0, GOS CFS and 2′-FL CFS retained more than 50% of the inhibitory activity, respectively. Intriguingly, even at pH 8.0, 2′-FL CFS still retained more than 50% of the inhibitory activity, whereas the inhibitory activity of GOS CFS completely disappeared.

These results indicated that acidification contributed the most to the inhibitory activity of Glu CFS, followed by Lac CFS, GOS CFS and 2′-FL CFS. The inhibitory activity of GOS CFS and 2′-FL CFS benefited from the synergistic effect of acidification and other antibacterial substances. Antibacterial substances other than acidification played a key role in the inhibitory activity of GOS CFS and 2′-FL CFS. The antibacterial substances in 2′-FL CFS could keep relatively stable over a wider pH range compared with those in GOS CFS.

3.9. The antibacterial substances in 2′-FL CFS of B. bifidum BBI01 were proteinaceous, thermostable and independent of hydrogen peroxide

As shown in Tables 3 and 4, high temperatures (even at 121 °C for 15 min) could not weaken the inhibitory activity of the four *B. bifidum* BBI01 CFSs against *Cronobacter* strains ($P > 0.05$). These results manifested that the key antibacterial substances in the four *B. bifidum* BBI01 CFSs are thermostable.

Treatment with either proteinase K, pepsin or trypsin decreased the inhibitory activity of 2′-FL CFS to the utmost extent, followed by GOS CFS, Lac CFS and Glu CFS ($P < 0.0001$). These results suggested that the antibacterial substances other than acidification in 2′-FL CFS had proteinaceous characteristics. And the antibacterial substances with proteinaceous characteristics in 2′-FL CFS contributed more to the inhibitory activity compared with those in GOS CFS.

Treatment with catalase had no significant effect on the inhibitory activity of the *B. bifidum* BBI01 CFSs, which proved that the growth inhibition of the *B. bifidum* BBI01 CFSs on *Cronobacter* strains did not depend on hydrogen peroxide.

4. Discussion

To establish a stable intestinal microbiota in infants, it is of great significance for bifidobacterium to survive in sufficient amounts, colonize and suppress the invasion of pathogens in GIT (Milani et al., 2017; Patel and Denning, 2015; Plaza-Diaz et al., 2019). A growing body of research has revealed that HMOs in breast milk are responsible for the selective enrichment of bifidobacterial species in infant GIT (Sela and Mills, 2010; Walsh et al., 2020). 2′-FL plays a prominent role in this process (Natividad et al., 2022; Van den Abbeele et al., 2021). However, scientists have been dedicated to exploring the relationship between 2′-FL and the growth phenotypic diversity of different members in *Bifidobacterium* (Bunesova et al., 2016; Centanni et al., 2019; Egan et al., 2014; Garrido et al., 2016; Ruiz-Moyano et al., 2013; Ward et al., 2007; Yu et al., 2013). The effects of 2′-FL as a prebiotic substrate on the probiotic responses of bifidobacterium have not been fully examined. Our work primarily shed light on this concern in three aspects: the effects of 2′-FL hydrolysis on bifidobacterial growth, the protective effects of 2′-FL on bifidobacterium under gastrointestinal stress and the inhibitory activity of 2′-FL metabolites against *Cronobacter* spp. We found that 2′-FL distinctly accelerated the growth and proliferation of *B. bifidum*. The glucose in lactose core on 2′-FL was preferable for supporting the surge in biomass of *B. bifidum* while the galactose was not readily available. On top of that, 2′-FL showed a unique superiority in enhancing the resistance of *B. bifidum* to GIT challenges. 2′-FL manipulated *B. bifidum* to antagonize *Cronobacter* strains relying on two strategies. 2′-FL significantly improved the adhesive ability of the *B. bifidum* strains to intestinal epithelial cells, thereby promoting the competitive elimination of *Cronobacter* strains by the target probiotics. The growth inhibition of 2′-FL on *Cronobacter* strains was mediated by promoting the secretion of antibacterial substances from *B. bifidum*. Nevertheless, this strategy was highly dependent on the strains of *B. bifidum*. We found that 2′-FL specifically induced *B. bifidum* BBI01 to produce some antibacterial substances. These antibacterial substances played a key role in the inhibitory activity of 2′-FL CFS and had a synergistic effect with acidification. The antibacterial substances were proteinaceous, thermostable, relatively stable even at pH 8.0 and independent of hydrogen peroxide.

Over the past few decades, only Zhang et al. (2020) made a comparative analysis on the growth data of bifidobacterium in GOS and 2′-FL. In accordance with the changes in optical density, they found that 2′-FL brought about a growth retardation and a decrease in the maximum OD_{600} values of *B. bifidum* DNG6 compared with GOS. Surprisingly, our growth kinetics analysis of *B. bifidum* yielded diametrically opposite results. Heterogeneity in strains should be partially responsible for the conflicting results. However, easy availability of hydrolysis products is the proximate cause why 2′-FL is a preferred prebiotic substrate for the *B. bifidum* strains. Specifically, previous studies have only roughly indicated that *B. bifidum* can benefit from the lactose on 2′-FL (Garrido et al., 2015; Ward et al., 2007). In this work, we further expanded these observations. We found that Gal resulted in severe growth retardation of the *B. bifidum* strains (Fig. 2). And compared with Glu, a significant accumulation of Gal occurred in 2′-FL and Lac spent media (Fig. 3). These findings underscored the fact that the galactose in lactose core on 2′-FL was not readily available while the glucose was preferred for the *B. bifidum* strains. Accordingly, compared with 2′-FL, the assimilation of large amounts of Gal in GOS molecules (Table 2) was extremely likely to restrain the *B. bifidum* strains from growing rapidly. Besides, *B. bifidum* have been proved to rely on a membrane-bound α-L-fucosidase outside the cell to unmask the lactose core on 2′-FL (Sakanaka et al., 2019). Even so, our contrastive analysis manifested that this behavior did not lead to significant differences in the growth kinetics of the *B. bifidum* strains grown in either Lac or 2′-FL (Fig. 2). These observations further hinted that the expression of β-galactosidases and Gal transport may be the primary causes preventing GOS from being a preferred prebiotic for the *B. bifidum* strains.

The survival of bifidobacterium in GIT is a prerequisite for the occurrence of their colonization. We found that 2′-FL was the best carbohydrate source for *B. bifidum* YH17 and *B. bifidum* BBI01 to resist GIT stress in comparison with the other three carbon sources. As far as we know, this is also the first report that HMOs can lead to improved resistance of bifidobacterium to the simulated GIT environment. The enhanced GIT stress tolerance of the two strains might be highly correlated with the variation of protein expression patterns. It has been proposed that inulin as a prebiotic oligosaccharide has no obvious effect on the growth of *Lactobacillus acidophilus* DSM 20079 compared with Glu (Nazzaro et al., 2012). But their work manifested that inulin could induce active expression of proteins involved in cell protection to increase the resistance of the strain under GIT stress. Succi et al. (2017) demonstrated that the biochemical adaptation to the slow fermentation of prebiotics, as a pre-stress, could activate the expression of specific stress proteins by *Lactobacillus rhamnosus* GG, consequently improving the persistence of the strain throughout simulated GIT transit. Herein, although the low pH value of Lac fermentation liquor and the slow fermentation of GOS respectively caused acid and starvation stress, 2′-FL endowed the *B. bifidum* strains with notably higher GIT stress tolerance. The Fuc released by 2′-FL existed in free form in the spent media, which naturally built up some high osmotic pressure outside the *B. bifidum* cells. The hyperosmosis coupled with the low pH value of 2′-FL fermentation liquor (Fig. 7A/B) presumably brought about a cross-stress to induce the high expression of related stress proteins.

Currently, Salli et al. (2021) took the lead in linking 2′-FL with the growth of *Cronobacter* spp. They noted that 2′-FL neither supported the growth of *C. sakazakii* DSM4458 nor *C. sakazakii* BAA-894. However, whether 2′-FL can antagonize *Cronobacter* spp. and how 2′-FL antagonizes these pathogens have yet to be explored. Encouragingly, we revealed two strategies for the antagonism of 2′-FL against *C. sakazakii*

ATCC 29544 and *C. muytjensii* ATCC 51329. As the carbon source, 2′-FL prominently improved the adhesion characteristic of the *B. bifidum* strains, which facilitated the competitive elimination of *Cronobacter* strains by the probiotics. And the growth inhibition of 2′-FL on the *Cronobacter* strains was mediated by promoting the secretion of antibacterial substances from the *B. bifidum* strains.

Previous studies have revealed that the metabolism of 2′-FL by bifidobacterium produced copious lactate and short chain fatty acids. These metabolites created an acidic environment wherein the growth of other *Enterobacteriaceae* pathogens was hindered (Li et al., 2020; Salli et al., 2019; Van den Abbeele et al., 2021). In our work, acidification only contributed a portion to the inhibitory activity of 2′-FL CFS deriving from *B. bifidum* BBI01 against *Cronobacter* strains. Intriguingly, we found that some antibacterial substances had a synergistic effect with acidification and played a key role in the inhibitory activity of 2′-FL CFS. Awaisheh et al. (2013) suggested that the neutralized MRS CFS (Glu served as the carbon source) of *Lactobacillus acidophilus* and *Lactobacillus casei* had the inhibitory activity against *C. sakazakii*. They considered that the antibacterial substances other than acidification were heat labile bacteriocins with proteinaceous characteristics. Jamwal et al. (2019) also reported that the neutralized MRS CFSs of six lactic acid bacteria could inhibit the growth of *C. sakazakii* relying on some thermostable antibacterial substances other than acidification. In striking contrast to the bacteriocins and antibacterial substances deriving from those lactic acid bacteria, the thermostable antibacterial substances other than acidification in 2′-FL CFS not only had proteinaceous characteristics but also kept biological activity even at pH 8.0. Previously, two thermostable bacteriocins with proteinaceous characteristics separately produced by *B. bifidum* NCFB 1454 and *B. animalis* BB04, namely bifidocin B and bifidocin A, could retain biological activity after exposure to pH 2.0 to 10.0 (Liu et al., 2015; Yildirim and Johnson, 1998). Another thermostable bacteriocin with proteinaceous characteristics (bifidin I) produced by *B. infantis* BCRC 14602 also could keep relatively stable after exposure to pH 4.0 to 10.0 (Cheikhyoussef et al., 2010). On these grounds, it is highly possible that the unique antibacterial substances in 2′-FL CFS are one or some bacteriocins specifically expressed by *B. bifidum* BBI01. More interestingly, compared with Glu CFS, Lac CFS and GOS CFS, these unique antibacterial substances only existed in 2′-FL CFS of *B. bifidum* BBI01 (Tables 3 and 4). These results indicated that these unique antibacterial substances of *B. bifidum* BBI01 were specifically induced by 2′-FL *in vitro*.

It is worth mentioning that *Cronobacter* spp. could not thrive on the Fuc released in gut (Ng et al., 2013; Salli et al., 2021). In consequence, we cannot entirely rule out the possibility that the growth inhibition of 2′-FL CFSs on *Cronobacter* strains are correlated with the dissociative Fuc.

5. Conclusions

In conclusion, the results achieved in this work indicated that 2′-FL mediated excellent probiotic responses of *B. bifidum* YH17 and *B. bifidum* BBI01. 2′-FL substantially accelerated the growth and multiplication of *B. bifidum*. The glucose in lactose core on 2′-FL was the much more favorable substrate for *B. bifidum* to attain a sharp increase in biomass while the galactose was not readily available. It is remarkable that the uptake of 2′-FL could elevate the resistance of *B. bifidum* to harsh GIT conditions. In addition, 2′-FL served as the carbon source dramatically improved the adhesive property of *B. bifidum*. This characteristic not only facilitated the colonization of intestinal epithelial cells by *B. bifidum*, but also conferred a competitive advantage on *B. bifidum* in excluding *C. sakazakii* ATCC 29544 and *C. muytjensii* ATCC 51329. And the growth inhibition of 2′-FL on the pathogens was mediated by promoting the secretion of antibacterial metabolites from *B. bifidum*. The inhibitory activity varied depending on the *B. bifidum* strains. 2′-FL specifically induced *B. bifidum* BBI01 to produce one or some thermostable antibacterial substances with proteinaceous characteristics. These unique antibacterial substances in 2′-FL CFS of *B. bifidum* BBI01 played a key role in the inhibitory activity, kept relatively stable even at pH 8.0 and had a synergistic effect with acidification.

The findings presented here provide a new insight into the potential mechanisms by which 2′-FL and even other HMOs exert bifidogenic effects in breast-fed newborns. Meanwhile, this work advances some important complementary properties of HMOs that have yet to be reported. As we have stated, the free Fuc in 2′-FL CFSs appeared to have a positive impact on the probiotic responses of *B. bifidum*. Therefore, understanding how the dissociative Fuc works might be a key point in futher elucidating the precise mechanism by which 2′-FL modulates the probiotic responses of *B. bifidum*.

CRediT authorship contribution statement

Jingfang Du: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft. **Hong Yang:** Conceptualization, Resources, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Hong Yang reports financial support was provided by Shanghai Industry-University Joint Research Program [grant number HUCXY-2016-010]. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Abbreviations

2′-FL	2′-Fucosyllactose
GIT	Gastrointestinal tract
HMO	Human milk oligosaccharide
Glu	Glucose
Gal	Galactose
Lac	Lactose
GOS	Galactooligosaccharides
MRS	deMan Rogosa Sharpe
ATCC	American Type Culture Collection
DMEM	Dulbecco's Modified Eagle's Medium
PBS	Phosphate buffered saline
sMRS	Carbohydrate-free semidefinite MRS

(*continued on next page*)

(*continued*)

Fuc	Fucose
OD	Optical density
CFS	Cell-free supernatant
CFU	Colony forming units

Data availability

Data will be made available on request.

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