



Short communication: Genotype-phenotype association analysis revealed different utilization ability of 2'-fucosyllactose in *Bifidobacterium* genus

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ABSTRACT

The oligosaccharide 2'-fucosyllactose (2'FL) in human breast milk selectively promotes the proliferation of bifidobacteria. One hundred fifty-one *Bifidobacterium* strains were evaluated for their capacity to utilize 2'FL based on the combination of phenotype and genotype association analysis. Through genotype analysis, 37 strains were predicted to have the ability to use 2'FL, including *Bifidobacterium bifidum*, *Bifidobacterium breve*, *Bifidobacterium longum* ssp. *longum*, *Bifidobacterium longum* ssp. *infantis*, and *Bifidobacterium dentium*, whereas *Bifidobacterium adolescentis*, *Bifidobacterium animalis*, *Bifidobacterium pseudocatenulatum*, and *Bifidobacterium angulatum* could not use 2'FL. For in vitro utilization, there were noteworthy differences for 2'FL usage among different species, which were 100% consistent with genotype prediction. The results indicated that 2'FL utilization ability differed even within the same species, and *Bifidobacterium* followed the currently well-known pathway to utilize 2'FL, which could provide guidance to develop personalized prebiotics for different bifidobacteria via gene-trait matching analysis.

Key words: *Bifidobacterium*, 2'-fucosyllactose, utilization, genotype, phenotype

Short Communication

Human milk oligosaccharides (HMO) are complex and mixed oligosaccharides in human breast milk; they are the second largest carbohydrate content in breast milk and play important roles in the development of

neonates (Kunz et al., 2000; Asakuma et al., 2011). The HMO are not digested by human digestive enzymes; therefore, ingested 2'-fucosyllactose (2'FL) arrives at the large intestine and is finally utilized and degraded by commensal bacteria (Gevers et al., 2012). Fucosylated HMO account for ~70% of total HMO (Weiss and Hennet, 2012), and 2'FL is one of the most copious HMO, made up of fucose and lactose via α -1,2 glycoside bonds and only found in secretor milk (Goehring et al., 2014). The HMO 2'FL has several health-associated benefits, such as inhibiting the pathogenesis of enteropathogens, preventing the adhesion of pathogenic bacteria to epithelial surface within gastrointestinal tract, and promoting the growth of some specific probiotics, especially particular *Bifidobacterium* species (Yu et al., 2013). In addition, gut microbiota in infants consuming 2'FL-enriched formula was similar to that in breastfed infants (Marriage et al., 2015).

Bifidobacteria are abundant bacteria in breastfed infants' intestines (Ruiz-Moyano et al., 2013; Arboleya et al., 2016; Kato et al., 2017). Most *Bifidobacterium* species cannot grow with 2'FL as a unique carbon source (LoCasco et al., 2010; Garrido et al., 2016), and *Bifidobacterium longum* ssp. *infantis* and *Bifidobacterium bifidum* are the major 2'FL consumers (Garrido et al., 2015; Arboleya et al., 2016; Bunesova et al., 2016). Degradation of 2'FL relies on a complex network of a specific ATP-binding cassette (ABC) transporter and glycosyl hydrolase (GH). Most bifidobacteria, such as *B. longum* ssp. *longum*, *B. longum* ssp. *infantis* and *Bifidobacterium breve*, first transfer 2'FL intracellularly, relying on an ABC transporter capable of importing 2'FL and then further hydrolyzes it by GH. *Bifidobacterium bifidum* directly degrades 2'FL extracellularly by secreting extracellular GH, and an ABC transporter is not required for 2'FL utilization (Sela et al., 2008; Turroni et al., 2014). Although some literature (Ruiz-Moyano

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et al., 2013; Yu et al., 2013) reported the utilization of 2'FL by some *Bifidobacterium* strains, the differences in 2'FL utilization ability among different bifidobacteria species have not been fully compared; the utilization mechanism shared among different species especially needs further investigation. The current study aimed to compare the capacity to utilize 2'FL of different *Bifidobacterium* via genotype-phenotype association analysis.

The 2'FL was donated by Domo (FrieslandCampina, Amersfoort, the Netherlands). All remaining chemicals used were analytical grade and can be purchased through commercial channels.

The 151 strains of *Bifidobacterium* used in this study were deposited at Culture Collection of Food Microorganisms (Jiangnan University, Jiangnan, China) and included *B. bifidum*, *B. breve*, *B. longum* ssp. *longum*, *B. longum* ssp. *infantis*, *B. adolescentis*, *B. animalis*, *B. pseudocatenulatum*, *B. angulatum*, and *B. dentium* (Supplemental Table S1, <https://doi.org/10.3168/jds.2020-19013>). Each strain was subcultured in MRS broth plus 0.05% cysteine (mMRS) anaerobically (AW500SG, Don Whitley Scientific, Bingley, UK) at 37°C for 48 h, then inoculated into 5 mL of mMRS broth (2% inoculum) at 37°C for 24 h (Yang et al., 2017).

The prediction of carbohydrate active enzymes of bifidobacteria was done through the CAZY database (Matsuki et al., 2016). The putative 2'FL ABC transporters involved in 2'FL utilization were analyzed using the bi-directional best hit comparison method via the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (<http://www.genome.jp/tools/kaas/>) to perform KEGG orthology annotation (Matsuki et al., 2016). The results were displayed in heat map by HemI (Heatmap Illustrator, CUCKOO Workgroup, version 1.0).

The utilization in vitro was performed with 2'FL as the unique carbon source in 96-well microplates. The 2'FL aqueous solution was prepared by filtering it through a 0.22- μ m sterile filter (Navigator Lab Instrument Co., Ltd., Jiangsu, China). The bromocresol purple indicator was added to the medium and the sterile carbohydrate solution with 1% final concentration was added to the medium, replacing the presence of glucose as a carbon source (Ruiz-Moyano et al., 2013; Garrido et al., 2015). Glucose was used as a positive control, whereas noncarbohydrate MRS medium was used as negative control (Bunesova et al., 2016). The overnight culture was centrifuged at $3,381 \times g$ at 4°C for 3 min, then washed and resuspended with the assay medium. Four microliters of each resulting suspension were inoculated into 200 μ L of modified medium. The results were observed for color change after culturing in an anaerobic environment at 37°C for 24 h (Yu et al.,

2013); if the carbon source in the medium can be used by *Bifidobacterium*, the medium will turn yellow, and if it cannot be used, the medium will remain purple. The experiment was done 3 times with triplicate wells.

Alpha-L-fucosidase, an essential enzyme for 2'FL utilization, consisting of α -1,3/1,4-L-fucosidase (GH29) and α -1,2-L-fucosidase (GH95; Matsuki et al., 2016; Schwab et al., 2017; Thomson et al., 2018) has been completely identified in *B. longum* ssp. *infantis* ATCC15697 and *B. bifidum* JCM1254 (Asakuma et al., 2011; Garrido et al., 2016). In the current study, *B. breve* (11.11%), *B. longum* ssp. *longum* (8.33%), *B. longum* ssp. *infantis* (100%), and *B. dentium* (100%) contained the GH29 gene, and all of the *B. bifidum* contained both GH29 and GH95 genes, although the remaining species did not contain any α -L-fucosidase (Table 1). Simultaneously, GH29 was more widely present than GH95 in *Bifidobacterium* and played an important role in 2'FL utilization. This result was consistent with the previous literature (Ruiz-Moyano et al., 2013), recommending that the presence of GH29 endowed those strains with the capacity to utilize 2'FL.

A 2'FL ABC transporter coupled ATP hydrolysis with efficient internalization of sugars and appeared to represent the essential carbohydrate transportation frameworks for bifidobacteria (Davidson et al., 2008; Jojima et al., 2010; O'Callaghan and van Sinderen, 2016). These systems were broadly found in numerous microbes and had also been identified in most, but not all, bifidobacterial genomes (O'Callaghan and van Sinderen, 2016). Except in *B. bifidum*, the 2'FL-specific ABC transporter was essential for 2'FL utilization in *Bifidobacterium* (Thomson et al., 2018; Sakanaka et al., 2019), which was composed of nucleotide-binding domain, trans-membrane domain, and solute-binding protein (Verrier et al., 2008; Matsuki et al., 2016). Putative 2'FL ABC transporters (K02025, K02026, and K02027) were identified to play a vital role in 2'FL utilization through genomic analysis and gene knockout approaches in previous research (Matsuki et al., 2016). Through genetic determinants analysis, complete putative 2'FL ABC transporters were found in most *B. longum* ssp. *longum* (50%), *B. longum* ssp. *infantis* (90%), and *B. angulatum* (75%), which were significantly higher rates than those in *B. bifidum* (4.54%), *B. breve* (22.22%), *B. adolescentis* (5%), *B. pseudocatenulatum* (9.09%), and *B. dentium* (22.22%). No complete putative 2'FL ABC transporter system was identified in any *B. animalis* strains (Table 1). In addition, no obvious relationship was found between the existence of a carbohydrate-active enzyme and transporter system and the origin of those strains (e.g., infants, teenagers, adults; Supplemental Table S1, <https://doi.org/10.3168/jds.2020-19013>).

According to the previous reports (Turroni et al., 2014; Thomson et al., 2018), *B. bifidum* degraded 2'FL extracellularly, which was catalyzed by GH directly. Therefore, 2'FL utilization ability in *B. bifidum* only depends the presence of α -L-fucosidase, whereas for other *Bifidobacterium*, a 2'FL-specific ABC transporter and α -L-fucosidase were both essential for 2'FL utilization (James et al., 2019; Sakanaka et al., 2019). Hence, only 37 out of 151 strains were predicted to have the ability to utilize 2'FL, including 22 *B. bifidum*, 2 *B. breve*, 2 *B. longum* ssp. *longum*, 9 *B. longum* ssp. *infantis*, and 2 *B. dentium* (Table 1).

All the strains were loaded into an in vitro carbohydrate utilization test. The results showed all the *B. bifidum* could grow well with 2'FL as the unique carbon source, and most of *B. longum* ssp. *infantis* (90%) can utilize 2'FL (Table 1) as well, which was consistent with previous reports (Ruiz-Moyano et al., 2013; Yu et al., 2013), probably because *B. longum* ssp. *infantis* and *B. bifidum* are the major bifidobacteria in the gut of breastfed infants. However, only a few strains of *B. breve* (11%), *B. longum* ssp. *longum* (8%), and *B. dentium* (22%) showed the ability to use 2'FL (Table 1). Notably, those data were consistent with the current knowledge on bifidobacterial 2'FL utilization abilities, whereas the relatively few *B. longum* ssp. *longum*, *B. breve*, and *B. dentium* strains tested showed less adaptation to the substrate (Ruiz-Moyano et al., 2013; Duranti et al., 2017). On the contrary, *B. adolescentis*, *B. animalis*, *B. pseudocatenulatum*, and *B. angulatum* cannot grow with 2'FL as the unique carbon source

(Table 1), consistent with previous results (Schell et al., 2002; Yu et al., 2013).

Among those 2'FL utilizers, most strains were isolated from neonates and infants (67.57%) (Supplemental Table S1, <https://doi.org/10.3168/jds.2020-19013>). Similar results were found in a previous report, in which 19 bifidobacterial isolates from Kenyan infants could grow well with 2'FL as the carbon source (Bunesova et al., 2016). Only a few adult-derived *B. bifidum* and *B. dentium* strains showed the ability to utilize 2'FL (Supplemental Table S1). The capacity to utilize 2'FL was hence recognized as a characteristic of specific bifidobacteria species. *B. longum* ssp. *suis* and *B. kashiwanohense* have not been considered as infant-dominant bifidobacteria; yet, some strains from the species showed the ability to utilize fucosyllactose (Bunesova et al., 2016). It can be speculated that 2'FL was more conducive to the growth of bifidobacteria in infant intestine as previously reported (Thongaram et al., 2017; Lawson et al., 2020).

For in vitro utilization, only the strains consisting of both complete putative 2'FL ABC transporter and α -L-fucosidase could utilize 2'FL (Figure 1A and 1B), which was consistent with current reports (Matsuki et al., 2016; Thomson et al., 2018). In *B. bifidum*, only M130R01M51 possessed a complete putative 2'FL ABC transporter, which was a prerequisite for the ability to utilize 2'FL in other bifidobacteria species, and their α -L-fucosidase (GH29 or GH95) could be secreted extracellularly to degrade 2'FL directly without transferring it into the cell (Turroni et al., 2010; Thomson et

Table 1. Presence of glycosyl hydrolases (GH) and ABC transporter in *Bifidobacterium* and the in vitro utilization of 2'FL. The presence/absence of glucose is used as a positive/negative control¹

Species	GH29	GH95	ABC transporter	2'FL	Glucose	Glucose-free
<i>B. bifidum</i>	22/22	22/22	1/22	22/22	22/22	0/22
<i>B. breve</i> ²	2/18	0/18	3/18	2/18	18/18	0/18
<i>B. longum</i> ssp. <i>longum</i> ³	2/24	0/24	12/24	2/24	24/24	0/24
<i>B. longum</i> ssp. <i>infantis</i> ⁴	10/10	1/10	9/10	9/10	10/10	0/10
<i>B. adolescentis</i>	0/20	0/20	1/20	0/20	20/20	0/20
<i>B. animalis</i>	0/22	0/22	0/22	0/22	22/22	0/22
<i>B. pseudocatenulatum</i>	0/22	0/22	2/22	0/22	22/22	0/22
<i>B. angulatum</i>	0/4	0/4	3/4	0/4	4/4	0/4
<i>B. dentium</i> ⁵	9/9	0/9	2/9	2/9	9/9	0/9

¹Values indicate the number of isolates able to grow moderately or vigorously on substrate.

²The 2 GH29 positive *B. breve* strains (HuNCS6M1 and HuNCS1M5) were also positive for putative 2'FL ABC transporter and for growth on 2'FL.

³The 2 GH29 positive *B. longum* ssp. *longum* strains (CCFM752 and CCFM688) were also positive for putative 2'FL ABC transporter and positive for growth on 2'FL.

⁴The 9 GH29 positive *B. longum* ssp. *infantis* strains (FGZ23_I1_M2, FHuNCS6M8, FJND16M4, HeNJZ8M1, JSSZ7M7, SDZC2M4, JSWX3M1, FJSYZ1M3 and ATCC15697) were also positive for putative 2'FL ABC transporter and positive for growth on 2'FL. *B. longum* ssp. *infantis* ATCC15697 was the unique strain possessing GH95 gene.

⁵The 2 GH29 positive *B. dentium* strains (FGDLZ36M8 and FJSWXJ29M2) were also positive for putative 2'FL ABC transporter and positive for growth on 2'FL.

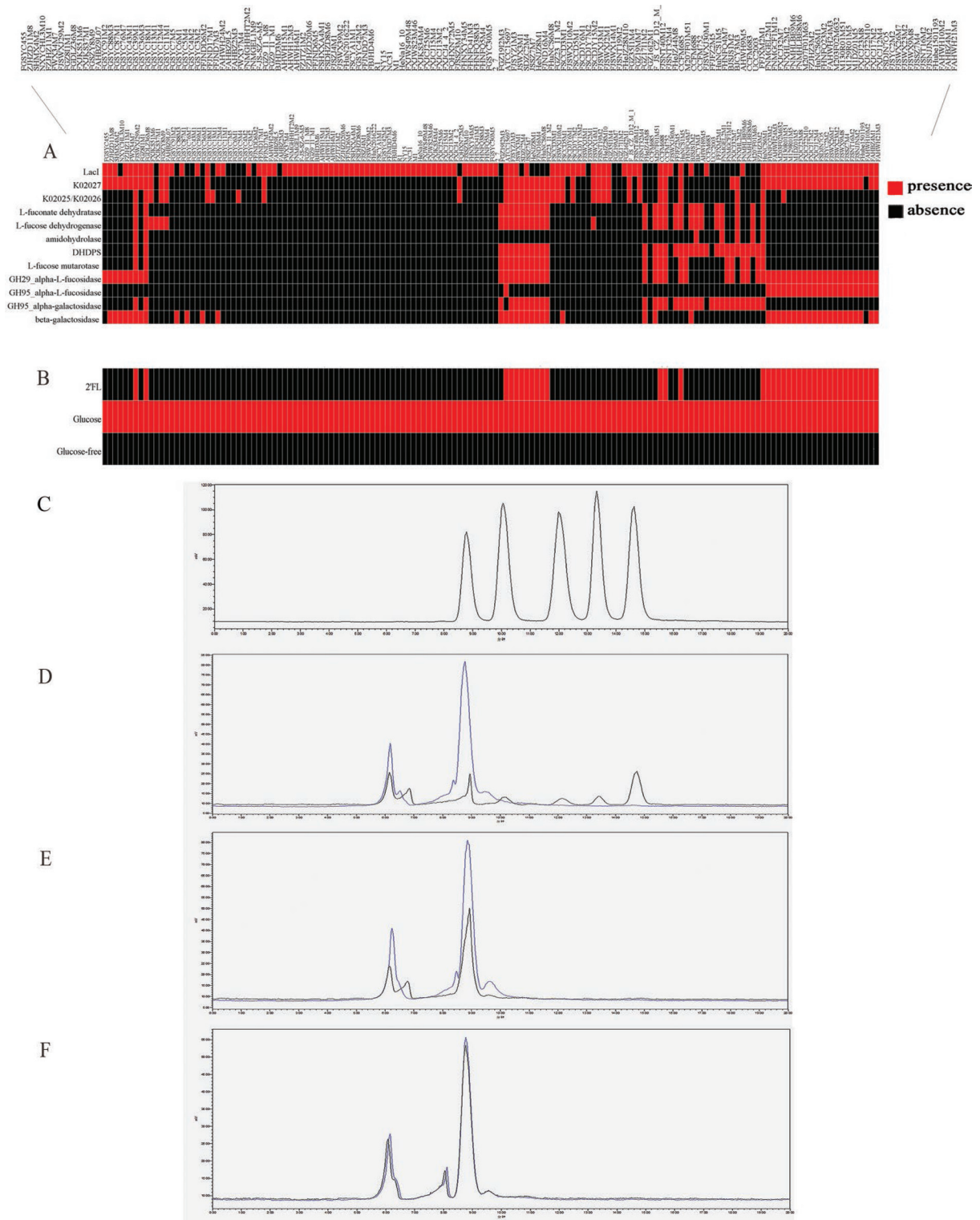


Figure 1. Bifidobacterial 2'FL utilization. (A) Genotype prediction. Heat map representation of the presence/absence of putative genes for 2'FL hydrolysis and transport red and black squares represent the presence and absence of genes, respectively. (B) 2'FL degradation capabilities of bifidobacterial strains. Black indicates that the strain cannot grow with 2'FL as the carbon source, and red indicates that it can grow. (C) Standard of 2'FL and its major metabolites. (D) The spectrum of 2'FL and its metabolites in *Bifidobacterium bifidum* M130R01M51 (a 2'FL consumer). (E) The spectrum of 2'FL and its metabolites in *Bifidobacterium longum* ssp. *infantis* SDZC2M4 (a 2'FL consumer). (F) The spectrum of 2'FL and its metabolites in *Bifidobacterium breve* FZJHZ3M2 (a non-2'FL consumer). The blue/black curves in the above picture represent 0 and 48 h, respectively.

al., 2018). Therefore, for *B. bifidum*, all the strains can grow with 2'FL as the unique carbon source, as all of them contained α -L-fucosidase (GH29 and GH95; Figure 1A and 1B). For example, this result revealed that growth of *B. bifidum* M130R01M51 on 2'FL resulted in the accumulation of L-fucose in the growth medium, which is a well-known metabolite of fucosyllactose in bifidobacteria. However, similar results were not observed in *B. longum* ssp. *infantis* SDZC2M4, suggesting that a common pathway for 2'FL metabolism was absent, which was consistent with previous report (Thomson et al., 2018). More detailed descriptions were added and shown in Figure 1C–F, in which the nontarget peaks that appeared in the spectrum were the miscellaneous peaks in the medium.

In the current study, a large-scale phenotype and genotype matching analysis was performed to explore 2'FL utilization capabilities in different *Bifidobacterium* species. By correlating the presence or absence of genes and the growth or nongrowth patterns of 151 *Bifidobacterium* strains with 2'FL as unique carbon source, the genotype prediction results were all consistent with the phenotype experiment results. The usage of 2'FL by *Bifidobacterium* followed the known utilization mechanism that *B. bifidum* only required α -L-fucosidase, whereas for other *Bifidobacterium* strains, both a 2'FL-specific ABC transporter and α -L-fucosidase were required. In conclusion, genotype-phenotype association enabled the identification of genes involved in and responsible for transportation and metabolization of specific carbon sources, finally providing some guidance for personalized prebiotics development for different probiotics.

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


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