Selective Isolation of Bifidobacterium From Human Faeces Using Pangenomics, Metagenomics, and Enzymology

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Bifidobacterium, an important genus for human health, is difficult to isolate. We applied metagenomics, pangenomics, and enzymology to determine the dominant glycoside hydrolase (GH) families of Bifidobacterium and designed selective medium for Bifidobacterium isolation. Pangenomics results showed that the GH13, GH3, GH42, and GH43 families were highly conserved in Bifidobacterium. Metagenomic analysis of GH families in human faecal samples was performed. The results indicated that Bifidobacterium contains core GHs for utilizing raffinose, D-trehalose anhydrous, D(+)cellobiose, melibiose, lactulose, lactose, D(+)sucrose, resistant starch, pullulan, xylan, and glucan. These carbohydrates as the main carbon sources were applied for selective media, which were more conducive to the growth of bifidobacteria. In the medium with lactose, raffinose and xylan as the main carbon sources, the ratio of cultivable bifidobacteria to cultivable microorganisms were 89.39% ± 2.50%, 71.45% ± 0.99%, and 53.95% ± 1.22%, respectively, whereas the ratio in the ordinary Gifu anaerobic medium was only 17.90% ± 0.58%. Furthermore, the species significantly (p < 0.05) varied among samples from different individuals. Results suggested that xylan might be a prebiotic that benefits host health, and it is feasible to screen and isolate bifidobacteria using the oligosaccharides corresponding to the specific GHs of bifidobacteria as the carbon sources of the selective media.

Keywords: Bifidobacterium, glycoside hydrolase, metagenomics, pangenomics, enzymology

INTRODUCTION

Among the numerous microbial communities that colonise the human body, the intestinal microbiome plays a major role in maintaining host health. The intestinal microbiome of the gastrointestinal ecosystem comprises a collective genome of trillions of microorganisms. Interactions between the host and the intestinal microbiota are complex. Changes in the intestinal microbiota might be critical to prevent or treat various intestinal and non-intestinal diseases.
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85 species
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et al., 2016), and today, the bifidobacteria group includes
the faeces of exclusively breastfed infants in 1899 (Riviere
2014; Jaglan et al., 2019).

resistance and regulation of diseases, bifidobacteria can promote
affects cognition in mice (Savignac et al., 2015). In addition to the
2015). Furthermore, *Bifidobacterium longum* 1714 positively
acts on hosts (Hsieh et al., 2015; Matson et al., 2018). The numbers
of bifidobacteria in the total colonic microbiota of normally
delivered breast-fed infants decrease from 90% to <5% in adults
(Riviere et al., 2016). The abundance of bifidobacteria in the
gastrointestinal tract is related to some pathologies, such as
intestinal (Grazul et al., 2016; Roškar et al., 2017) and mental
(Pinto-Sanchez et al., 2017) diseases. Bifidobacteria can also
improve the intestinal barrier function by producing salts of
short chain fatty acids such as butyrate (Rios-Covian et al.,
2015) and acetate (Hsieh et al., 2015) and reducing serum
FITC-dextran levels in a mouse model of colitis (Srutkova et al.,
2015). Furthermore, *Bifidobacterium longum* 1714 positively
affects cognition in mice (Savignac et al., 2015). In addition to the
resistance and regulation of diseases, bifidobacteria can promote
nutrient yield to enhance immunity (Kim et al., 2014; Yang et al.,
2014; Jaglan et al., 2019).

Tissier isolated the first strain of *Bifidobacterium* from the
faeces of exclusively breast-fed infants in 1899 (Riviere et al.,
2016), and today, the bifidobacteria group includes
85 species
1. However, the isolation of new *Bifidobacterium*
species or their cultivation is difficult because they are strictly
anaerobic and easily contaminated by other bacteria. Traditional
methods of separation and cultivation no longer meet actual
needs, and thus, new technologies are required to explore the
taxonomical and functional profiles of microbial DNA extracted
from microbial communities, which are constantly increasing
(Al-Masaudi et al., 2017). Lugli et al. (2019) proposed that
they could mine genetic information of related *Bifidobacterium*
strains based on whole metagenome shotgun sequencing to
design a reasonable selective medium based on GHs and
obtain target strains when screening new *Bifidobacterium* strains.
However, they only mined GH based on metagenomic data
to design the medium. Monosaccharides are limited in the
large intestine, which is inhabited by bifidobacteria. Therefore,
bifidobacteria possess various glycosidases and sugar transporters
that assimilate indigestible polysaccharides, oligosaccharides, and
complex carbohydrates (Garrido et al., 2012). The gene content of
the *Bifidobacterium* genome encodes numerous enzymes with
predicted roles in carbohydrate modifications (Pokusaeva et al.,
2011). To promote the growth of the target genera and inhibit
the growth of other microorganisms, selective medium can be
designed using omics to identify GHs and their corresponding
carbohydrates in samples with unique effects.

We designed 11 selective media based on data derived from
pangenomics, metagenomics, and enzymology to promote the
proliferation of *Bifidobacterium* while simultaneously reducing
the growth of other intestinal bacteria. Stool samples for
faecal bacterial transplantation were collected from the First
Affiliated Hospital of Guangdong Pharmaceutical University.
We also combined the results of a pangenomic analysis of
*Bifidobacterium* to suitable GHs to select a suitable carbon source
for selectively isolating this genus. The growth of *Bifidobacterium*
on different selective media was determined and analysed using
16S amplicon sequencing to determine the optimal carbon source
for *Bifidobacterium* isolation.

**Materials and Methods**

**Microbial Genome Sequences**

We retrieved complete genome sequences for 144 bifidobacteria
and 5 other genera (Supplementary Table 2 sheet 6) from the
National Center for Biotechnology Information (NCBI)
public database. These sequences were used as inputs for the
pangenomic analysis to identify specific GHs.

**Collection of Faecal Samples**

Stool samples were collected from three healthy donors of
faecal microbiota transplantation (FMT) at the First Affiliated
Hospital of Guangdong Pharmaceutical University. The study
was approved by the ethics committee of the First Affiliated
Hospital of Guangdong Pharmaceutical University (reference
2017-98). Healthy donors were recommended to not eat spicy
and greasy food at least the day before donating faeces. The
donors collected the faeces in a sterile container and provided it
to a professional for pre-treatment of the FMT. The pretreatment
steps were as follows. Install the faecal collection barrel into the
automated faecal bacteria separation system GenFMTer (FMT
technological, Nanjing, China), add physiological saline to stir, filter,
and purify, which can remove faecal residue and large particles.
And then collect the faecal bacteria suspension and repeatedly
washed three times. The supernatant was removed, and then
resuspended in physiological saline.

S17, S181, and S201 were human stool samples (donor S17,
S18, and S20) that had not been processed before FMT; the stool
samples S171 and S181 were washed to remove food residue
(donor S17 and S18), used for FMT in this hospital, and named
as S172 and S182. Approximately 2 g of faeces from the original
faeces and 2 g of faeces after pre-treatment were taken.

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1https://lpsn.dsmz.de/genus/bifidobacterium
DNA Extraction
Total faecal DNA and DNA used for 16S rRNA gene sequencing were extracted using QIAamp PowerFecal Pro FNA Kits (50) (Qiagen GmbH, Hilden, Germany), as per the manufacturer’s instructions. We extracted DNA from *Bifidobacterium* using gram-positive bacterial DNA extraction kits (Magen Biotech, Guangzhou, China). The approximate concentration and purity of all DNA samples were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, United States), and concentrations were accurately measured using a Qubit 3.0 fluorometer (Thermo Fisher Scientific Inc.).

Metagenome Sequencing and Data Analysis
A DNA library was prepared and sequenced using a NovaSeq 6000 system (Illumina, Inc., San Diego, CA, United States) at a commercial laboratory (Novogene Co., Ltd., Beijing, China). Barcodes were removed from the sequencing results using Practical Extraction and Reporting Language (Perl) script, low quality data were removed using Trimmomatic Version 0.39 (Bolger et al., 2014), and host DNA was removed using CLC Genomics Workbench (Qiagen). Thereafter, assembling and announcement were conducted using SPAdes Version 3.12.0 (Nurk et al., 2013) and Prokka (Version 1.13.7) (Seemann, 2014), respectively.

Pangenomic Analysis
A total of 144 *Bifidobacterium* genome sequences were downloaded from the NCBI database for pan-genome analysis. The sequences were then annotated and compared them with annotation files through a local server. We also annotated the 144 genomes using the dbCAN-seq database. Relative abundance annotation files through a local server. We also annotated the 144 genomes using the dbCAN-seq database. Relative abundance heat maps were prepared using Prism 8.2.1 (GraphPad Software Inc., San Diego, CA, United States) and stack columns of abundance were drawn using OriginPro® 8.5 (OriginLab Corporation, Northampton, MA, United States).

**Designed Selective Medium of Bifidobacterium**
Traditional GAM (Gifu anaerobic medium) consists of soytone (10 g), proteose peptone (10 g), bovine serum albumin (13.5 g), yeast extract (5 g), beef extract (2.2 g), KH₂PO₄ (2.5 g), liver extract (1.2 g), NaCl (3.0 g), L-cysteine (0.3 g), sodium thioglycolate (0.3 g), glucose (3 g), and soluble starch (5 g) in 1 L of water. Moreover, for the solid medium plates, agar was added (15 g/L). The selective medium was designed by eliminating glucose and soluble starch and replacing with raffinose, D-trehalose anhydrous, D(+)-cellobiose, melibiose, lactulose, lactose, D(+)-sucrose, resistant starch, pullulan, xylan, and glucan.

**16S rRNA Amplicon and Data Analysis**
A DNA library was prepared and sequenced using a Nova PE250 instrument (Illumina, Inc., San Diego, CA, United States) at a commercial laboratory (Genewiz, Inc., Suzhou, China). The original binary base-calling data obtained by sequencing were converted into PF (pass filtering) or raw sequence data using Illumina bcl2fastq software. Barcodes were removed from raw data using the Perl script and low-quality data were removed using Trimmomatic version 0.39 (Bolger et al., 2014). Relative abundance heat maps were prepared using Prism 8.2.1 (GraphPad Software Inc., San Diego, CA, United States) and stack columns of abundance were drawn using OriginPro® 8.5 (OriginLab Corporation, Northampton, MA, United States).

### Isolation and Identification of Bifidobacterium
The 11 carbohydrates were selected as the main carbon source, based on all analytical findings (Table 1 and Supplementary Table 1). Fresh faeces samples (0.5 g) were diluted 10⁻¹, 10⁻²,

### Table 1: Structure prediction of selected GHs and corresponding carbohydrates.

| GHs                          | Structure                                                                 | Structure prediction                     | Catalytic residues       | Carbohydrate                  |
|------------------------------|---------------------------------------------------------------------------|------------------------------------------|--------------------------|-------------------------------|
| Oligo-1,6-glucosidase (EC 3.2.1.10) | (β/α)β barrel structure, 4 β sheets                                         | Asp212, Pro274, and Ala335               | Raffinose, Dextran,         |
| Trehalose-6-phosphate hydrolase (EC 3.2.1.93) | (β/α)β barrel structure, 6 β sheets                                         | Asp205, Trp267, and Asp329              | Resistant starch, D-Trehalose anhydrous |
| Beta-glucosidase (EC 3.2.1.21) | Consisting of two chains                                                  |                                         | D(+)-Cellobiose, Melibiose  |
| β-galactosidase (EC 3.2.1.23) | (β/α)β barrel structure                                                   |                                         | Lactulose, Lactose         |
| Sucrose phosphorylase (EC 2.4.1.7) | Consisting of two chains, (β/α)β barrel structure, 4 β sheets              | Asp343, Glu375, and Asp466              | D(+)-Sucrose                |
| Pulullanase (EC 3.2.1.41) | The N-terminal and C-terminal were composed of 7 β sheets, and a (β/α)β barrel structure in the middle. |                                         | Pulullan                  |
| β-xylosidase (EC 3.2.1.37) | Consisting of two chains; almost all were β sheets                          |                                         | Xylan                     |
10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} in physiological saline (0.9%), and then, the 10^{-4}, 10^{-5}, and 10^{-6} dilutions were coated on the different designed medium plates, with each gradient performed in triplicate for each medium. After incubation at 37°C for 48–72 h in the anaerobic workstation, single colonies were inoculated and streaked on plates and cultured in the anaerobic workstation at 37°C for 48–72 h. This step was repeated. Finally, 16S rRNA gene sequences were amplified from DNA extracts of pure cultures using the primer pair 27F (5’-AGAGTTTGATCCTGGCTCAG-3’) and 1492R (5’-GGTTACCTTGTTACGACTT-3’), which targets the variable region between the 16S rRNA gene sequences. Bifidobacteria were phylogenetically analysed using MEGAX and iTOL.

RESULTS

Pangenomic and Genomic Analyses Identify Specific GHs in Bifidobacterium

A total of 144 bifidobacterial genome sequences were downloaded from NCBI for pangenomic analysis (Figure 1A and Supplementary Table 2 sheets 1–3). The total number of genes increased as the genomes increased, whereas conserved genes began to plateau at 20 genomes and remained constant thereafter. We used these whole genomes to find GHs, which are abundant in the pan-genome, and used the results for comparative analysis (Figure 1C). The proportion of these eight enzyme classes in Bifidobacterium was significantly higher than other five selected genus. The search results of carbohydrate active enzyme database and UniProt database were combined to analyse the hydrolysis characteristics of these enzymes (Supplementary Table 1). We further combined the metagenomics results to determine the optimal carbon source for growth.

Metagenomic Analyses Reveal Specific GHs in Samples

Whole metagenome shotgun sequencing of faecal bacterial samples from transplanted donor stools produced 32–40 million paired reads, with an average approximate length of 150 bp. Species significantly differed among samples from different individuals. The content of Bifidobacterium in all samples was in the top five (Figure 2A), and the proportion of Bifidobacterium in S201 was the highest, followed by that in S171 and S172, and was lowest in S181 and S182 (Figure 2B). Due to the low abundance of Bifidobacterium in S181 and S182, and no significant difference between S171 and S172, in which S172 is the stool after removing food residue from sample S171, only S201 and S171 were used in the next analysis and experiment to screen for bifidobacteria.

The pangenomic results of Bifidobacterium showed that β-galactosidase, β-glucosidase, trehalose-6-phosphate hydrolase, sucrose phosphorylase, α-amylase, pullulanase, β-xylosidase, and oligo-1,6-glucosidase were abundant in Bifidobacterium and the hydrolytic properties of eight specific GHs classes with corresponding organism are shown in Supplementary Table 1. The amounts of these GHs in the two metagenomic groups were essentially identical (Figure 2C).

Based on the pangenomic and metagenomic analysis, we found several GHs in Bifidobacterium that were prominent and important for carbohydrate decomposition (Supplementary Table 1). Therefore, we speculated as to whether oligosaccharides or polysaccharides differ in the selective separation and cultivation of Bifidobacterium.

Selective Medium Designed for Bifidobacterium Isolation

As shown in Table 1, the raffinose, D-trehalose anhydroys, D(+)-cellobiose, melibiose, lactulose, lactose, D(+)-sucrose, resistant starch, pullulan, xylan, and glucan were selected according to the hydrolysis characteristics of the selected GHs. Based on the results of 16S amplicon sequencing of clones collected from sample S171 that were cultured on selective media, in the medium with lactose, raffinose, and xylan as the main carbon sources, the ratio of cultivable bifidobacteria to cultivable microorganisms were 89.39 ± 2.50%, 71.45 ± 0.99%, and 53.95 ± 1.22%, respectively, whereas for the ordinary GAM, it was only 17.90 ± 0.58% (Figure 3, Table 2, and Supplementary Table 2 sheet 7). Except for that in the medium with lactulose as the carbon source, the
relative abundance of *Bifidobacterium* in the remaining 10 media was higher than that in ordinary GAM (Figure 3B). However, only half of S201 achieved this result, and the metagenomic results revealed more *Bifidobacteria* in S201 than in S171 (Figure 2B), and the abundance of enzymes in GH classes was basically the same (Figure 2C). The heat map (Figure 3D) also showed significantly higher abundances of *Bifidobacterium* in S171 than in S201, and lower abundances of *Shigella* in S171. Lactose had the best selectivity for *Bifidobacterium*, followed by raffinose in S171, and the selectivity of lactulose was the
worst. Xylan was more effective than glucan. Raffinose and xylan exhibited the best selectivity for S201.

**Pure Culture Identification**

The results of 16S amplicon sequencing showed that the bacterial composition significantly differed between the S171 and S201 faecal samples. We investigated the details of *Bifidobacterium* from different sources based on 76 colonies. We then purified these colonies and assessed 16S rDNA. We identified 28 (37%) *Bifidobacterium* strains (**Table 3**), and **Figure 4** shows the results of the phylogenetic analysis. These strains belong to the three branches of *B. longum*, *Bifidobacterium pseudocatenulatum*, and *Bifidobacterium bifidum*.

**DISCUSSION**

Many studies have associated gut microbes with human health. *Bifidobacteria* among intestinal microbes are beneficial microorganisms (Jaglan et al., 2019) and have
attracted the most attention. However, strict requirements for growth have caused difficulties with their isolation and purification. The carbon source is an indispensable factor for the growth of microorganisms, and it can be used to screen these bacteria. The distribution of glycoside hydrolases in Bifidobacterium as the key enzymes for carbohydrate hydrolysis is important for bacterial culture and functional analysis. Therefore, pangenomic analysis of the whole genome sequence of 144 Bifidobacterium species downloaded from NCBI and the dbCAN-seq database revealed the common GH families, GH13, GH3, GH42, and GH43, in Bifidobacterium.

FIGURE 3 | Relative abundance of bacterial community compositions in selective medium plates at family (A), genus (B), or species (C) levels; (D) The genus-level relative abundance hot map of 16S amplicon sequencing. (E) The alpha diversity of 16S amplicon sequencing results. ns, not significant. *P < 0.05, **P < 0.01, ***P < 0.001. S1701: S17 represents sample 171 and 01 represents raffinose, 02 represents trehalose, 03 represents cellobiose, 04 represents melibiose, 05 represents lactulose, 06 represents lactose, 07 represents sucrose, 08 represents resistant starch, 09 represents pullulan, 10 represents xylan, 11 represents dextran, and 12 represents the ordinary GAM (glucose and soluble starch). The same is for S2001–S2012, only S20 represents sample 201.
The abundance of *Bifidobacterium* obviously differed among samples, and it was the most abundant in S201. The pangenomic findings of *Bifidobacterium* showed that GH13, GH3, GH42, and GH43 were abundant, and the abundance of enzymes in GH classes was basically the same in the metagenomic results of S171 and S201. Based on the hydrolysis characteristics of these enzymes, the corresponding carbohydrates (Table 1) were selected as main carbon source of the selective medium. The abundance of bifidobacteria in sample S201 is higher, so the abundance of bifidobacteria on the solid medium of S201 should be higher, but in fact sample S171 has a higher cultivable abundance of bifidobacteria in sample S201 is higher, so the proportion of bifidobacteria on the solid medium of S201 should be higher, but in fact sample S171 has a higher cultivable abundance of bifidobacteria on almost selective plates.

The selectivity of raffinose and xylan for bifidobacteria in both samples was effective based on the 16S amplicon sequencing analysis. Lactose was significantly selective for S171. Not only did the number of bifidobacteria surpass that of the control group (the ordinary GAM), but few other bacterial strains proliferated. Raffinose is an established prebiotic (Ose et al., 2018). Adding raffinose to the medium increases the production of short-chain fatty acids and carbon dioxide, and reduces the final pH and ammonia concentration in the medium (Amorim et al., 2020).

In addition, raffinose can increased the relative abundance of probiotics ($p < 0.05$), and decreased that of pathogenic bacteria (Pacifici et al., 2017). Therefore, xylan might also have similar or even more powerful prebiotic effects, but this needs to be further verified by animal experiments and high-performance liquid chromatography (HPLC). Animal experiments were done to verify whether xylan can regulate the intestinal microbiota of mice, i.e., whether there is a difference between the intestinal flora of mice on a diet containing xylan and a diet without xylan and whether mice fed on a xylan-containing diet have more probiotics in their gut microbiota than mice on a xylan-free diet.
**CONCLUSION**

Glycoside hydrolase 13, GH3, GH42, and GH43 are prevalent in *Bifidobacterium*, and the corresponding carbohydrates substrate can serve as the main carbon source in medium to selectively isolate and cultivate *Bifidobacterium*. Xylan might be a prebiotic that benefits host health, but its effects might differ among individuals. Metagenomics and pangenomics allow the accumulation of more information that can facilitate the isolation and cultivation of *Bifidobacterium*. The abundance of *Bifidobacterium* in samples might not mean that more bifidobacteria can be isolated, but it also depends on the characteristics of the *Bifidobacterium* species in samples. In the future, we will further verify the properties of specific GHs and the prebiotic properties of related oligosaccharides.

**DATA AVAILABILITY STATEMENT**

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI: BioProject ID PRJNA695860 for 16S rDNA amplicon sequencing and PRJNA695407 for Metagenome.
ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of The First Affiliated Hospital of Guangdong Pharmaceutical University (reference 2017-98). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JZ, WC, XX, and QW conceived and designed the experiments. XX, SY, MD, LL, and LY conducted the experiments. SY, JM, XH, andYL participated in data collection and processing. SY and XX drafted the manuscript. JZ and XX reviewed the manuscript. All authors read and approved submission of the final version of the manuscript for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/10.3389/fmicb.2021.649698/full#supplementary-material
Bifidobacterium Selectively Isolated From Faeces

Yang et al.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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