Glycosidic linkage of rare and new-to-nature disaccharides reshapes gut microbiota *in vitro*

Stanley O. Onyango,[a] Koen Beerens,[b] Qiqiong Li, [a] John Van Camp,[c] Tom Desmet,[b] and Tom Van de Wiele[a#]

[a]Center for Microbial Ecology and Technology (CMET), Ghent University, Belgium

[b]Center for Synthetic Biology (CSB), Department of Biotechnology, Ghent University, Belgium

[c]Laboratory of Nutrition and Food Chemistry, Ghent University

Running title: Potential of Disaccharides as Prebiotics

#Address correspondence to Tom Van de Wiele, Tom.Vandewiele@ugent.be

CMET, Coupure Links 653, B-9000 Gent

Abstract

The impact of glycosidic linkage of seven rare and new-to-nature disaccharides on gut bacteria was assessed *in vitro*. The community shift of the inocula from four donors in response to 1% (w/v) disaccharide supplementation was captured by sequencing the 16S rRNA gene. A significant loss of bacterial alpha diversity, short lag time, low pH, and high total short-chain fatty acid displayed a faster fermentation of trehalose(Glc-α1,1α-Glc) and fibrulose(fructan, DP2-10). *Bacteroides* reduced in relative abundance under disaccharide supplementation suggesting a loss in complex carbohydrates metabolizing capacity. Fibrulose and L-arabinose glucoside(Glc-α1,3-L-Ara) significantly stimulated bifidobacteria but was suppressed with trehalose, ribose glucoside(Glc-α1,2-Rib), and 4'-epitrehalose(Glc-α1,1α-Gal) supplementation. Albeit insignificant, bifidobacteria increased with 4'-epikojibiose(Glc-α1,2-Gal), nigerose(Glc-α1,3-Glc), and kojibiose(Glc-α1,2-Glc). Prior conditioning of inoculum in kojibiose medium profoundly induced bifidobacteria by 44% and 55% upon reinoculation into kojibiose and fibrulose-supplemented media respectively. This study has demonstrated the importance of the disaccharide structure-function relationship in driving the gut bacterial community.

Keywords: Disaccharides, glycosidic linkage, kojibiose, trehalose, fibrulose, gut microbiota, prebiotics

1.1 Introduction

Gut microbiota contributes to energy harvesting from diets, particularly in the host colon (Flint, Scott, Duncan, Louis, & Forano, 2012). The activity and assembly of the gut microbiota are strongly influenced by diet and especially by carbohydrates (Flint et al., 2012; Payling, Fraser, Loveday, Sims, Roy, & McNabb, 2020) whose influence can override the host genetics (Kashyap et al., 2013). High absorption and metabolism of digestible carbohydrates mostly occur in the small intestine. However, approximately 5-30% of digestible carbohydrates still end up in the colon (Di Rienzi & Britton, 2019). The levels of these carbohydrates such as mono- and disaccharides reaching the colon largely depend on the absorption capacity of the small intestine and can either be passive, slow, or poor (Flint et al., 2012; Oku & Nakamura, 2000). A plethora of studies have reported on the impact of complex carbohydrates (i.e. dietary fibers) on gut microbiota (see extensive review (Flint et al., 2012)), but less on simple digestible carbohydrates.

The interest in dietary fibers and non-digestible food ingredients is not without merit largely driven by the increasing scientific clarity on their prebiotic effects. Prebiotics are food ingredients that can selectively stimulate health-promoting microbial bacteria such as bifidobacteria and lactobacilli in the host gut (Davani-Davari et al., 2019). It is worth noting that the selective stimulation is not exclusive to lactobacilli and bifidobacteria as other indigenous health-promoting strains can as well be supported to exert antimicrobial effects, or to modulate host immune response and offer colonization resistance against pathogens (Davani-Davari et al., 2019; Jia, Li, Zhao, & Nicholson, 2008). To this end, the use of

non-digestible oligosaccharides including inulin, galactooligosaccharides, lactulose, and resistant starch as prebiotics is well documented (Guarino et al., 2020). However, the prebiotic potential of disaccharides though not well explored cannot be underestimated. Digestible carbohydrates as opposed to complex carbohydrates are more energy-efficient carbon sources for the microbiota (Di Rienzi et al., 2019) and are therefore essential for their rapid growth and survival (Townsend et al., 2019). Evidence suggests that certain oligosaccharides, particularly those that have α1,2-, β1,2-, α1,3-, β1,4-, α1,6- glycosidic linkages tend to be prebiotic/bifidogenic (Sanz, Gibson, & Rastall, 2005) and have been demonstrated to selectively support the growth of lactobacilli and bifidobacteria (García-Cayuela et al., 2014).

The characterization and/or commercialization of these putative prebiotics has been hampered by their limited availability in nature besides their high production cost. Some breakthrough technologies have nevertheless made it possible to produce rare disaccharides kojibiose and nigerose, as well as the new-to-nature disaccharides 4'-epitrehalose (hereafter epitrehalose), 4'-epikojibiose (hereafter epikojibiose), ribose glucoside, and L-arabinose glucoside among others. The disaccharide epitrehalose is a trehalose analog in which one glucose has been substituted by galactose. Epikojibiose and ribose glucoside both are kojibiose analogs with galactose and ribose substituting glucose at the reducing end, respectively, while the L-arabinose glucoside is a nigerose analog with an L- arabinose at the reducing end.

Based on the monomeric composition and linkage of the digestible carbohydrates, gut bacteria can metabolically respond by releasing carbohydrate-active enzymes (Sonnenburg, Sonnenburg, Manchester, Hansen, Chiang, & Gordon, 2006) to ferment sugars predominantly yielding short-chain fatty acids; acetate, butyrate, propionate, and intermediary organic acids; lactate, formate, and succinate (Robayo-Torres, Quezada-Calvillo, & Nichols, 2006). Apart from providing energy to the enterocytes, these acids are substrates for metabolic interactions between gut microbiota (Robayo-Torres et al., 2006). Even though our diets consist of varied sources of dietary carbohydrates, it is not yet clear how intake of alternating dietary sugars can shape the gut community. This shift in a community can either be desirable or non-desirable by promoting the adaptation of commensals or pathogens, respectively (Di Rienzi et al., 2019).

We have investigated *in vitro* the impact of composition and glycosidic linkage on prebiotic potency of the new-to-nature and rare disaccharides in comparison to fibrulose, a fructan of low degree of polymerization (DP 2–10). We have in addition tested the concept of switching disaccharide intake on the gut microbial community.

1.2 Materials and Methods

1.2.1 Sugar substrates

Six of the seven disaccharides used in this study (Figure S1) were synthesized at the center for synthetic biology of Ghent University. Briefly, kojibiose and nigerose were produced as previously described by Beerens et al. (Beerens et al., 2017) and Franseus et al. (Franceus et al., 2019) using the engineered BaSP variants L341I/Q345S or R135Y/D342G/Y344Q/Q345F, respectively. Their analogs (epikojibiose, L-arabinose glucoside, and ribose glucoside) were biosynthesized using the stabilized *Bifidobacterium adolescentis* SP variant (BaSP_LNFI) as described by Dhaene et al. (Dhaene et al., 2022). For kojibiose 1.8 M sucrose and 0.2 M of glucose was mixed together with the L341I/Q345S BaSP variant. Glucose isomerase was added for fructos-to-glucose recycling. For nigerose production 1.5 M of both sucrose and glucose was mixed with the R135Y/D342G/Y344Q/Q345F variant. For the analogues, 1 M sucrose and the acceptor sugar (1 M galactose, 1 M L-arabinose, and 1 M ribose) were mixed with the thermostability engineered BaSP variant, which acted as a transglycosidase to form the mentioned products. The reactions were allowed to react until maximal conversion, after which spray-dried baker's yeast (Algist Bruggeman) was used to remove formed common sugars (e.g., maltose, glucose, fructose, …) and unreacted monosaccharide acceptors. Consequently, an anion-exchange step was applied to remove anions and finally the disaccharides were purified by preparative liquid chromatography (prepLC). Details can be found in the respective papers. The epitrehalose was synthesized from trehalose, galactose and inorganic phosphate with the use of a trehalose phosphorylase as described by Chen et al. (Chen, Desmet, Van der Borght, Lin, & Soetaert, 2012) In short, 200 mM of trehalose, 400 mM galactose and 30 mM phosphate buffer (pH 7.0) were incubated with trehalose phosphorylase originating from *Thermoanaerobacter brockii* (2 $g.L^{-1}$) at 60 °C for 72 h. The disaccharides were also recovered through enzymatic degradation and/or yeast treatment followed by subsequent preparative liquid chromatography as described earlier (Franceus et al., 2019). The evaluation of purities was also conducted using high-performance anion-exchange chromatography (HPAEC, Dionex ICS-3000, Thermo Scientific) and was as: epikojibiose (94%), kojibiose monohydrate (99%), L-arabinose glucoside (97%), nigerose (87%), ribose glucoside (98%), and epitrehalose (77%). Detailed NMR-based structural confirmation and purity analysis can be consulted in the respective publications describing the initial production of the rare disaccharides (Beerens et al., 2017; Dhaene et al., 2022; Franceus et al., 2019). Trehalose dihydrate (99%) and fibrulose (oligofructose DP=2-10) were kind donations from Cargill (Cargill R&D Centre Europe BV, Belgium, and Cosucra (Belgium), respectively.

1.2.2 Preparation of fecal slurry, inoculation, and incubation

Ethical approval for working with human fecal material was given by the ethical committee of Ghent University (BE6702018363). Fecal slurries from four healthy donors who did not use antibiotics in the

preceding 3 months were prepared as described by De Paepe et al. (De Paepe, Verspreet, Courtin, & Van de Wiele, 2020). Before inoculation, the fecal slurry was washed twice with pre-reduced phosphate-buffered saline (PBS, pH 6.8) by centrifugation at 3000 x *g* for 5 minutes to remove the carryover nutrients. Inoculation was made to a final concentration of 1% (w/v) fecal material in Hungate tubes containing a carbon-limited medium (Table S1) supplemented with 1% (w/v) disaccharide or fibrulose. Medium without any disaccharide was used as a control. Upon inoculation, the Hungate tubes were immediately flushed with N_2 gas for 20 cycles to create an anaerobic condition and then incubated at 37°C in an orbital shaker at 120 rpm (KS 4000 i control, IKA, Staufen, Germany). The course of fermentation was monitored over 24 h with sampling at 0, 1, 2, 3, 4, 6, 12, and 24 h (Figure S2).

1.2.3 Impact of conditioning and re-exposure to disaccharides or fibrulose on the bacterial community

To test the concept of the influence of alternating sugar intake on microbial community and metabolic function, we selected epitrehalose with (galactose substitute) and the kojibiose analog containing ribose since they displayed recalcitrant behavior based on long lag time and pH. Besides, kojibiose was included and their prebiotic properties were benchmarked against fibrulose, a commercial prebiotic. We first conditioned the microbial community of D4 in the sugar-supplemented media for 24 h. This is the shortest time in humans that has been reported to have a diet-induced shift in the microbial community (David et al., 2014). Re-exposure was accomplished by inoculating the 24 h conditioned inoculum into the medium supplemented with epitrehalose, ribose glucoside, kojibiose, and fibrulose for a further 24 h incubation (Figure S2).

1.2.4 pH, gases, and short-chain fatty acid analysis

Gas production was analyzed by measuring the headspace pressure and the composition using gas chromatography fitted with a thermal conductivity detector. Next, the pH of the spent medium was measured using a pH meter (Consort SP28X, Turnhout, Belgium). To quantify the levels of short-chain fatty acids and lactic acid generated, 1.5 mL medium was filtered and analyzed accordingly on 930 compact IC flex Ion exchange chromatography (Metrohm, Switzerland) fitted with a Metrosep Organic acid 250/7.8 column and a Metrosep Organic acids Guard/4.6 guard column. The flow rate of 1 mM H_2SO_4 eluent was set at 0.8 mL/min.

1.2.5 Cell count based on flow cytometry

Microbial biomass during the 24 h course of incubation was determined using a benchtop Accuri C6+ cytometer (BD Biosciences, Belgium) as described by Van Nevel *et al*. (Van Nevel, Koetzsch, Weilenmann, Boon, & Hammes, 2013). Filtered (0.22 µm) pre-reduced phosphate-buffered saline (PBS, pH 7.2) was used to serially dilute cells to 10^4 times to get an even rate between 500 and 2000 per second. Samples were stained with 1:100 diluted Sybr Green Propidium Iodide (Thermo Scientific, Waltham, USA) to a final concentration of 1%. Finally, the plate was incubated at 37°C for 13 minutes. Cell counts were determined at fast speed with blue laser at 488 nm and bandpass filters on the FL-1

(533/30 nm) and FL-3 (670 nm LP). The intact and damaged cell counts were acquired by gating cell density plots to capture the events corresponding to the SYBR green and propidium iodide labeled cells. For the actual counts, the exported cell densities were corrected for the dilution factor. Microbial community shift during disaccharide incubation

During disaccharide and fibrulose fermentation, the changes in pH and organic acid became clearer at 12 h of incubation hence we sequenced these samples to decipher early microbial community shift in response to disaccharides and fibrulose.

1.2.6 FastPrep DNA extraction, library preparation, and high-throughput partial 16S rRNA gene sequencing (at 2x300 bp read length)

The luminal suspension of 250 µL was transferred into tubes, pelletized at 13000 x *g* for 10 minutes, and stored at -20°C until DNA extraction. The DNA extraction and quality control and sequencing were performed as earlier described (Onyango, De Clercq, Beerens, Van Camp, Desmet, & Van de Wiele, 2020). Briefly, hypervariable V3-V4 regions of the 16S rRNA gene libraries were prepared using primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 785R (5'-GACTACHVGGGTATCTAAKCC-3'). The libraries were sequenced on an Illumina MiSeq platform using 2 × 300 paired-end mode (Illumina, Hayward, California). Demultiplexed paired reads were analyzed using the DADA2 bioinformatic pipeline (Callahan, McMurdie, Rosen, Han, Johnson, & Holmes, 2016) within R (v 4.1.3) (R Core Team, 2019). Based on the calculated error rates, dereplicated reads were quality filtered before removal of chimeras from constructed sequence table using removeBimeraDenovo function. Taxonomic classification and assignment were based on a naive Bayesian classifier against the Silva v138 database at the genus level (97% identity). Although species-level assignment based on short reads (250 bp) can be ambiguous, an exact matching at species level where possible at 100% identity using species alignment database Silva V138 (Quast et al., 2013) was done (Table S3). Where species resolution was not possible, further classification via the RDP SeqMatch web tool (Cole et al., 2014) (version 3, accessed in March 2021) was made by restricting the search to type strains, near full-length and good quality sequences. Amplicon sequence variants (ASVs) of each sample with counts were generated. Using the Phyloseq package (v 1.40.0) (McMurdie & Holmes, 2013), the dataset was further filtered to only consider the ASVs with an abundance of at least 1% across all samples for subsequent analysis.

1.3 Data analysis

All the statistical analyses were performed in R. Based on the Richards model growth curves were modeled to obtain growth parameters including growth rate (h^{-1}), and lag time (h). The input data were the cell counts determined by the flow cytometry (Candry et al., 2018). All formal hypothesis tests were conducted and considered significant at $p<0.05$. A phyloseq package was used to compute relative abundances at the genus level and the results of the top 20 genera were plotted. Shannon diversity index was computed to determine the alpha diversity of bacteria within the donors and sugars during

the incubation. The taxonomic β-diversity was also calculated based on Bray-Curtis distance to explore the community structure between sugars and donors and their association with the treatment variables then plotted on a constrained ordination plot. The significance of the association was tested using the Adonis function in the vegan library based on 999 permutations following permutational multivariate analysis of variance (PERMANOVA) (Oksanen et al., 2015).

Detecting differentially abundant genera between disaccharides/fibrulose and control

Based on Wald tests, DESeq2 (v 1.28.1) analysis (Love, Huber, & Anders, 2014) was used to make a pairwise comparison between disaccharides/fibrulose and control. Bacterial genera with a log2 fold change absolute value above 1.5 and a false discovery rate (FDR) less than 0.05 as determined by Benjamini–Hochberg (BH) correction for multiple hypothesis testing were judged to be differentially abundant (Love et al., 2014). The design incorporated the sugar and donor factors. We also applied an empirical Bayes shrinkage correction for low counts. For graphical display, EnhancedVolcano plot package (v 1.7.16) was used to generate volcano plots showing the -log10 (adjusted p-value) as a function of the log2FoldChange while annotating the most pronounced ASVs (Blighe K, 2018).

1.4 Data availability

The supporting data for this study has been provided within this article and its Supplementary Information file. Raw sequencing data and the associated metadata have been deposited to NCBI Sequence Read Archive under accession PRJNA856717.

1.5 Results and Discussions

In vitro incubation was used to characterize the impact of glycosidic linkage of disaccharides on the bacterial community and metabolites of the gut bacteria from four healthy donors. To benchmark their prebiotic potential, we included a well-established and commercially available prebiotic soluble fiber fructan (fibrulose) of a low degree of polymerization (DP 2-10).

1.5.1 Gut microbiota exhibited a faster metabolism with fibrulose and trehalose

The estimated lag times based on flow cytometric cell counts ranged from 3.4±2.8 h for fibrulose to 19.9±1.7 h for the control medium. Notably, gut bacteria required the longest lag time with epitrehalose (9.5±27.0 h) among the disaccharides followed by ribose glucoside, L-Arabinose glucoside and epikojibiose (Table S2). Gut bacteria also exhibited a faster growth rate in trehalose-supplemented medium (0.9±0.2 h^{-1}) as opposed to other substrates that were < 0.3 h^{-1}. The drop in pH (Figure 1A) with fibrulose and trehalose was faster, indicating a rapid utilization by the *in vivo* derived gut bacteria. For epitrehalose, a delayed drop in pH was observed and only became apparent after 12 h of incubation (Figure 1A, B) revealing the importance of the galactose moiety in slowing epitrehalose fermentation. These results suggest that gut microbiota have higher efficiency in metabolizing the glucosidic linkages particularly of trehalose α1,1α- in comparison to the α1,2- and α1,3- linkages of kojibiose and nigerose respectively. In agreement with these findings, bacteria from both oral and gut have been shown to

have high efficiency in trehalose fermentation (Farag et al., 2020). Using brush border enzymes of rat origin and incubation with human intestinal Caco-2 cells, we recently showed that epikojibiose, ribose glucoside and L- arabinose glucoside exhibit a reduced digestibility and a limited impact on energy metabolism (Dhaene et al., 2022). Similarly, Hodoniczky and colleagues had previously demonstrated a partial resistance of α1,2- and α1,3- linked glucobioses; kojibiose and nigerose, respectively to both human and rat brush border enzymes when compared to trehalose (α1,1α) (Hodoniczky, Morris, & Rae, 2012). Therefore, a substantial amount of ingested nigerose and kojibiose and their analogs can escape the upper digestive tract for colonic microbial fermentation. These findings have given insight into the importance of structure rather than molecular weight in determining disaccharide digestibility. Despite the low purities of epitrehalose (77%) and nigerose (87%), this study has given an insight into their digestive profiles. Since the contaminants in epitrehalose and nigerose were mainly glucose and trehalose which may even have a faster fermentation profile (Figure 1A), their high purity might thus result in a slower hydrolysis and fermentation. Additional analysis is thus required to confirm this hypothesis. Consistent with the observed changes in pH, a significant amount of total acids (SCFAs, lactic and formic acid) was generated from each substrate over the control (p<0.05) (Figure 1C, Figure S3A). The differences were apparent after 6 h of incubation but were more pronounced after 12 h. At 12 h (Figure 1C, Figure S3B), we observed significantly high (p<0.05) total acid with all the substrates (14.6±10.3 mM - 46.9±8.0 mM) except with epitrehalose (14.6±10.3 mM) in comparison to control (4.7±2.3 mM) further revealing its recalcitrant behavior. Similarly, a high (p<0.05) amount of acetic acid was produced with all substrates except with kojibiose and trehalose (Figure 1C, Figure S3C). For formic acid, epitrehalose (3.7±4.9 mM) and fibrulose (2.3±1.7 mM) produced similar levels when compared to control (0.5±0.1 mM) (Figure 1C, Figure S3D) while levels generated from the rest of the disaccharides were higher (p<0.05). Despite a noticeable time-dependent increase in propionic (Figure S3E) and butyric acids (Figure S3F), the amounts formed were low with all the substrate amended incubations (Figure 1C). With regards to donors, there was no donor-dependent influence on both the profile and total SCFAs except for the high (p<0.05) propionic acid that was generated by D2 microbiota (Figure S4). Acetate synthesis is a widespread metabolic function in the human gut microbiota and can be synthesized via the oxidative decarboxylation of pyruvate during carbohydrate fermentation (Duncan, Louis, & Flint, 2004). This can explain the observed acetate dominance in the sugar/fibrulose fermentation despite the marked differences in microbial community during the sugar incubation. Moreover, the short incubation time might have ensured limited cross-feeding to yield propionate and butyrate (Flint et al., 2012).

1.5.2 Community shift is donor-dependent

The α and β diversity of gut microbiota were computed to examine microbial community structure. Based on alpha diversity (Figure 2A) fibrulose and trehalose demonstrated a similar but lower (p<0.05)

diversity index in comparison to control, inoculum, and the rest of the disaccharides. As for the donors, D3 (Figure 2B) exhibited the lowest diversity (p<0.05) than D1, D2, and D4. The coordination of Bray-Curtis distance (beta diversity) on the PCoA plot (Figure 2C) revealed a donor-dependent community clustering. Three separate clusters emerged which brought D2 and D4 together in one cluster and D1 and D3 in two more separate clusters. There was no clear separation between carbohydrate substrates. A beta dispersion in vegan was used to first test the homogeneity of dispersion among disaccharide/fibrulose and donors. It showed a similarity in bacterial communities among the disaccharide/fibrulose and the donors (p>0.05). Adonis test in vegan confirmed that the community structure between the donors was significantly different (p<0.05) but not the variation between the disaccharides/fibrulose.

The loss of alpha diversity in donor D3 (p<0.05) (Figure 2B) as compared to D1, D2, and D4 was marked by the dominance (p<0.05) of *Klebsiella* (ASV3) (57%) followed by *Lachnospiraceae* UCG-004 (ASV16) (15%) and a reduced *Parabacteriodes* (ASV5) (0.2%) (Figure 3, Table S4). D2 and D4 displayed a comparable microbial profile by sharing the most dominant genera. The results showed that *Escherichia-Shigella* (ASV1) was most abundant (p<0.05) in donor D4 (39%) and D2 (27%) when compared to donors D1 and D3. *Bacteroides* was also relatively more abundant (p<0.05) in D2 (30%) and D4 (27%) than in D1 and D3. D1 conversely displayed a dissimilar profile in which *Coprobacillus* (ASV12) (18%), and *Streptococcus* (ASV14) (15%) were the two most dominant genera. These results clearly demonstrate donor variability in sugar fermentation.

1.5.3 The disaccharide-dependent shift in bacterial community during sugar fermentation

Driven by donors D2 and D4, *Escherichia-Shigella* (ASV1) was enriched in all the *in vitro* incubations but was more pronounced with trehalose (34%) (Table S4). Driven by D3, it was also observed that *Klebsiella* (ASV3) which was identified as *K. granulomatis/pneumoniae* from less than 1% rose in relative abundance with and without (5%) substrate supplementation. It accounted for the second most dominant genus with trehalose (20%) but was less favored with fibrulose and epitrehalose. Based on D1, we also noted that only trehalose (19%) and fibrulose (13%) stimulated *Streptococcus* (ASV14) represented by *S. sinensis/salivarius/vestibularis/porcorum* (Table S4). From 3% in the inoculum, *Bifidobacterium* (ASV4) (33%) was stimulated with fibrulose to become the most dominant genus (Figure 3, Table S5). Notably, *Bifidobacterium* (ASV4) represented by *B. adolescentis/faecale/stercoris* also increased (p<0.05) with L-arabinose glucoside (8%), nigerose, and epikojibiose both at 6% when compared to control (Figure 4) displaying their bifidogenic potential. However, a small percentage (1%) increase in relative abundance was recorded with kojibiose. Contrastingly, bifidobacteria (*Bifidobacterium* (ASV4)) decreased in ribose glucoside, epitrehalose, and trehalose supplemented medium (Figure 3, Table S5) which demonstrated their poor bifidogenic properties.

Consistent with our finding, Sanz and coauthors reported that disaccharides with a β1,6- (gentiobiose) and 1,1-glycosidic linkage (αβ-trehalose and ββ-trehalose) did not increase the bifidobacteria upon incubation with fecal slurry for 12 h (Sanz et al., 2005). Moreover, the authors determined that disaccharides with α1,2-, β1,2-, α1,3-, β1,4-, α1,6- glycosidic linkages tended to have a high prebiotic score (Sanz et al., 2005) and that glucobioses kojibiose (α1,2), sophorose (β1,2), and nigerose (α-1,3), isomaltose (α-1,6), galactobioses (β-1,4, and β-1,6) and mannobiose (α-1,6), have a high prebiotic index able to support the growth of bifidobacteria and lactobacilli (García-Cayuela et al., 2014). The selectivity of prebiotics is not exclusive to the known beneficial bacteria as was observed with fibrulose and the disaccharides. In agreement with this study, Tungland and others also reported that apart from bifidobacteria, an increase in a few other familiar pathogens, such as *Salmonella*, *Escherichia coli*, *Yersinia pestis*, *Klebsiella*, and *Shigella,* are also incorporated with prebiotics (galactooligosaccharides) (Tungland, 2018). *Escherichia-Shigella* (ASV1) closely identified as *Pseudescherichia vulneris, Escherichia fergusonii/coli, Shigella sonnei/flexneri, Brenneria alni* and *Klebsiella granulomatis/pneumoniae* (ASV3) was also increased with all incubations but were more pronounced with trehalose. These species are known to be opportunistic pathogens, some of which are even multidrug-resistant (Chaudhury, Nath, Tikoo, & Sanyal, 1999). They are reported to be very competitive in taking up and fermenting digestible carbohydrates in the small intestine and may explain their robustness with the disaccharides. *In vivo* characterization of prebiotic candidates; kojibiose, L-arabinose glucoside, epikojibiose, and nigerose will help in resolving the intricate homeostatic balance between the pathogens and commensals in the host gut.

Parabacteroides (ASV5) represented by *P. distasonis* from 0.7%, was significantly enriched in all disaccharides but low in fibrulose-supplemented medium (Figure 3, Figure 4). *P. distasonis* is a common gut commensal and has been shown to alleviate obesity and obesity-related dysfunction by encouraging intestinal gluconeogenesis in mice (Wang et al., 2019). Some studies have further suggested that *P. distasonis* can suppress pro-inflammatory and tumorigenic activities that are likely mediated by the suppression of TLR4 and Akt signaling, besides promoting apoptosis (Koh et al., 2018). The candidacy of these disaccharides in selectively enriching *P. distasonis* can be further explored. Our findings further showed that the incubations with kojibiose, L-arabinose glucoside, ribose glucoside, nigerose, and epikojibiose increased *Erysipelatoclostridium* (ASV2) in abundance (Figure 3, Figure 4).The clostridial group including *Clostridium* sensu stricto 1 (ASV40) and *Clostridium innocuum* group (ASV57) were also enriched in trehalose medium (Figure 4), suggesting the metabolic preference of clostridial bacteria to trehalose. In fact, some studies have implicated trehalose with the enhancement of the virulence of certain *C. difficile* strains and infection prevalence (Collins, Danhof, & Britton, 2019; Collins et al., 2018).

The enrichment of *Coprobacillus* (ASV12) identified as *C. cateniformis* was also detected with ribose glucoside (12%), epikojibiose (7%), kojibiose (8%), nigerose (7%), and L-arabinose glucoside (6%), from non-detectable levels in the inoculum.

In vitro incubation led to a decrease in the abundance of some genera (Figure 3, Table S4). While the reduction of *Bacteroides* (ASV6) (38%) was observed with all incubations, the largest drop was recorded with trehalose (8%) while the least drop was recorded with nigerose and kojibiose (28%) incubations. The levels with epikojibiose and fibrulose were similar to the control (23%). Considering that *Bacteroides* have vast repertoire of CAZyme encoding genes (Kaoutari, Armougom, Gordon, Raoult, & Henrissat, 2013) their inhibition in the microbial community in the presence of disaccharides may reflect decreased capacity to metabolize complex carbohydrates which was more pronounced in trehalose incubations than in nigerose and kojibiose. A recent finding has at least demonstrated that the colonization of *Bacteroides,* and particularly *B. thetaiotaomicron*, is compromised in the presence of glucose and fructose which suppresses the regulator of colonization protein (Townsend et al., 2019) and may in part explain the lack of robustness in disaccharides/fibrulose incubations. *Faecalibacterium* (ASV23) identified as *F. prausnitzii* was not resilient either in all the incubations and dropped from 15% to less than 5%. *F. prausnitzii* is a primary contributor of butyrate production and is an important commensal bacteria in healthy adults, making up about 5% of the human microbiome (Tungland, 2018). This can also explain the observed low butyrate during the sugar fermentation. Similarly, *Agathobacter* (ASV31) identified as *A. ruminis* was reduced from 6% to below 2% in all the disaccharides and fibrulose. The low abundance of *Paeniclostridium* (ASV15) was also detected with fibrulose, nigerose, kojibiose, and trehalose amended incubations. Besides, the abundance of *Blautia* (ASV70) represented by *B. obeum/wexlerae* and *Alistipes* (ASV41) identified as *A. finegoldii/onderdonkii* decreased in the presence of nigerose, kojibiose, and fibrulose substrates. Since these bacteria are important in cross-feeding to produce the short-chain fatty acids (Payling et al., 2020), their low abundance signifies a loss in capacity to convert acetate and lactate to butyrate and propionate as was observed in this study.

Notably, some genera were selectively enriched in medium without sugar and included bacteria belonging to Lachnospiraceae UCG-004 (ASV16) (12%) represented by *Lachnoclostridium pacaense* and *Paeniclostridium* (ASV15) identified as *P. ghonii/sordellii* (20%) making it the second most dominant genus in the control medium. Epitrehalose similarly enriched *Lachnospiraceae UCG-004* (ASV16) (11%) (Figure 3, Table S5). The resilience of bacterial spp. belonging to *Lachnospiraceae UCG-004* suggests that they can remain transcriptionally active and increase in abundance even in starvation state was observed with other spp. including *Klebsiella* and *Neisseria* (Onyango et al., 2020).

Even though limited research on the impact of sugar-based diets on the gut microbiota has been conducted, sugar can profoundly induce shifts in gut microbial community as has been shown in this

and previous studies (Sen et al., 2017). Consistent with our results, available data suggests that oligosaccharides in particular tend to lower *Bacteroides* bacteria (Davis, Martínez, Walter, Goin, & Hutkins, 2011). Since *Bacteroides* normally occupy the distal colon where the pH is near neutral as opposed to the proximal colon characterized by the high rate of fermentation and moderate acidity, the drop in pH during the sugar fermentation contributed to their low dominance (Flint et al., 2012). The drop in pH in the colon may inhibit proliferation of opportunistic pathogens and is considered an important prebiotic mechanism (Payling et al., 2020). While using fluorescent in situ hybridization (FISH) technique, Sanz et al broadly studied the shift in four predominant gut bacterial groups; *Bacteroides*, *Clostridium*, *Bifidobacterium* and *Eubacterium,* and in the contrary observed a relatively stable *Bacteroides*, clostridia and eubacteria up to 12 h of incubation but decreased with extended incubation in disaccharides supplemented medium (Sanz et al., 2005). Previous analysis of fecal samples from healthy human volunteers who consumed galactooligosaccharides (GOS), also revealed overstimulation of *Faecalibacterium prausnitzii* and *Bifidobacterium* (Davis et al., 2011). We on the contrary observed a decrease *F. prausnitzii* which can be attributed to substrate specificity. Consistent with our study, we observed a substrate-specific influence on the bifidobacteria abundance. It is worth noting that prebiotic consumption results in shifts of diverse taxa and can be influenced by several factors. When a dynamic *in vitro* colon model and ^{13}C labeling was used, Maathuis et al. (2012) determined that GOS consumption enriched species that have been considered probiotics: *Bifidobacterium catenulatum, Bifidobacterium longum, Bifidobacterium bifidum, Ligilactobacillus salivarius,* and *Lactobacillus gasseri*. Nonetheless, the same authors reported an increased abundance of bacteria belonging to the Enterobacteriaceae family, including symbionts, and known opportunistic pathogens as earlier highlighted (Maathuis, van den Heuvel, Schoterman, & Venema, 2012). As regards oligosaccharides, some studies have reported that their prebiotic score is dependent on degree of polymerization where oligosaccharides with higher DP tends to have a poorer prebiotic score. For example, Sanz and others revealed that DP3 oligosaccharides exhibited the highest selectivity towards bifidobacteria while oligosaccharides above DP7 are non-bifidogenic (Sanz, Côté, Gibson, & Rastall, 2006). This suggests that lower DP such as disaccharides can be very energy-efficient prebiotics in comparison to complex or longer DP prebiotics. Future research on optimizing their linkages and monomers is still required to fully unlock their prebiotic potential.

1.5.4 Conditioning of gut microbiota to kojibiose increases *Bifidobacterium*

Table 1, Figure S5, shows the results for the impact of sugar alternation on the microbial community structure. Inoculum conditioning in a medium supplemented with kojibiose regardless of the subsequent substrate supplementation stimulated *Bifidobacterium* (ASV4) (18%). This was largely contributed by the high relative abundance (51%) observed when the inoculum was re-exposed to kojibiose. Interesting to note that the relative abundance of *Bifidobacterium* (ASV4) during conditioning

with kojibiose was at 7% (Table 1). As expected, fibrulose (22%) similarly stimulated *Bifidobacterium* (ASV4), particularly when inoculated with kojibiose inoculum. An increase in *Bifidobacterium* (ASV4) in ribose glucoside upon conditioning with kojibiose was also noted. In contrast, epitrehalose suppressed the *Bifidobacterium* (ASV4) from both kojibiose and fibrulose inoculum. This suggests that repeated exposure to kojibiose can be superior in spurring bifidogenic activities. Although the bifidogenic properties of bacteria are strain-specific species assignment using DADA2 closely classified *Bifidobacterium* (ASV4) as *Bifidobacterium adolescentis/faecale/stercoris*. *B. adolescentis* is considered a psychobiotic with the potential to modulate the gut-brain axis via the production of gamma-aminobutyric acid, a major inhibitory neurotransmitter in the central nervous system (Duranti et al., 2020). Conditioning with kojibiose and not fibrulose induced bifidobacteria in ribose glucoside supplemented medium. Moreover, epitrehalose exhibited a decreased abundance even when inoculated with kojibiose (4%) or fibrulose (3%) conditioned inoculum further revealing its poor bifidogenic property.

Even though conditioning with epitrehalose (23%) and fibrulose (20%) induced *Erysipelatoclostridium* (ASV2), a higher representation of *Erysipelatoclostridium* (ASV2) to over 70% was recorded when the ribose glucoside inoculum was re-exposed to ribose glucoside and kojibiose. *Erysipelatoclostridium* has been found to be enriched in low fiber, a diet closely linked to metabolic diseases. The consequence of this overrepresentation is intriguing and requires further investigation.

Meanwhile, *Enterococcus* (ASV22) represented by *Enterococcus azikeevi/casseliflavus/durans/faecalis/faecium/hirae/lactis/mundtii/raffinosus/ratti/rivorum/thailandicus/villorum* was induced whenever the inoculum was first conditioned with kojibiose and fibrulose. This was, however, more pronounced when the kojibiose inoculum was re-exposed to fibrulose (30%).

Epitrehalose favored *Parabacteroides* (ASV5) whether conditioning was done in epitrehalose, ribose glucoside, or kojibiose but not with fibrulose supplemented medium. Upon re-exposure, *Parabacteroides* (ASV5), which was identified as *Parabacteroide distasonis*, was most stimulated in epitrehalose and ribose glucoside amended incubations. *P. distasonis* is a common gut commensal and has been shown in mice to alleviate obesity and obesity-related dysfunction by encouraging intestinal gluconeogenesis (Wang et al., 2019). Some studies have further suggested that *P. distasonis* can suppress pro-inflammatory and tumorigenic activities that are likely mediated by the suppression of TLR4 and Akt signaling, besides promoting apoptosis (Koh et al., 2018). The candidacy of epitrehalose in selectively enriching *P. distasonis* needs more exploration. Conditioning with fibrulose induced *Escherichia-Shigella* (ASV1) in all the disaccharide-supplemented medium except with kojibiose. Conditioning with kojibiose, conversely, suppressed *Escherichia-Shigella* (ASV1) by over 16% in fibrulose supplemented medium further confirming the non-exclusive stimulation of only the health promoting

bacteria. Selectively, fibrulose supported the growth of *Mitsuokella* (ASV33) when inoculated with ribose glucoside (28%) and fibrulose (27%) conditioned inoculum. Notably, conditioning with kojibiose increased *Collinsella* (ASV28) from less than 1% to 16% upon re-exposure to kojibiose as opposed to other sugar conditioning (Table 1, Figure S5). *Bacteroides* (ASV6) identified as *Bacteroides uniformis* was found to be the most dominant in the kojibiose conditioned inoculum (43%) but declined in relative abundance upon re-exposure to ribose glucoside and fibrulose, which indicates a reduced complex carbohydrates metabolic capacity in subsequent intake of ribose glucoside or fibrulose.

Concerning the generated metabolites during re-exposure, a significant (p<0.05) amount of total acid was produced with kojibiose (40.7±9.3 mM) as compared to ribose glucoside (23.3±8.5 mM) and epitrehalose (22.2±8.4 mM) (Figure 5A). Even though the levels between fibrulose (31.4±2.8 mM) and kojibiose were not statistically significant, kojibiose was slightly more than fibrulose demonstrating the comparable prebiotic properties with fibrulose. The generated acids were mostly dominated by lactic and acetic acid (Figure 5A). Kojibiose also recorded a significant drop in pH which was consistent with the observed high level of total acids. The levels of CO_2 and H_2 could not be separated by the substrate (Figure 5B).

1.6 Conclusion

With considerable donor variability in the microbial community, this study has demonstrated the profound impact of glycosidic linkage and monomeric constituent of disaccharides on the structure of the microbial community. Although our results still require confirmation and refinement with *in vivo* studies, they open interesting prospects for developing future prebiotics by exploring the structure-function relationship. Given the simple structure of disaccharides, their chemical synthesis is much more feasible than conventional prebiotics which are mostly complex in structure. When implemented via synbiotics, for example, these ready-to-digest prebiotics can in small doses stimulate the growth or maintain the function of beneficial microbes including bifidobacteria besides suppressing host pathogens. Optimizing the bifidogenic potential of kojibiose requires re-exposure to kojibiose or fibrulose. This can be achieved via nutritional planning in which constant intake of kojibiose in combination with fibrulose can be encouraged. Conversely, the use of trehalose as a prebiotic is doubtful considering the overstimulation of pathobionts and under-stimulation of health-promoting bacteria such as bifidobacteria.

1.7 Acknowledgments

This work was supported by Fonds Wetenschappelijk Onderzoek FWO/SBO GlycoProFit project grant no. S003617N and FWO (G0B2719N).

List of tables

Table 1. Changes in microbial community structure following 24 h conditioning and re-exposure to medium containing ribose glucoside, epitrehalose, kojibiose, and fibrulose (reference prebiotic). The conditioning incubations were used as the inoculum for the re-exposure incubation. The values in re-exposure are the mean relative abundance (±SD) of the community following re-exposure, therefore, depicting the general community as driven by each carbohydrate substrate irrespective of the conditioning substrate.

Genus	*Community structure in conditioning*				*Community structure in re-exposure to*			
	Ribose glucoside	Epitrehalose	Fibrulose	Kojibiose	Ribose glucoside	Epitrehalose	Fibrulose	Kojibiose
Escherichia-Shigella (ASV1)	7.7	19.8	51.8	17.4	16.4±14.5	27.7±24.6	17.9±15.2	14.3±12.1
Senegalimassilia (ASV104)	0.0	0.0	0.2	0.2	0.2±0.2	0.3±0.3	0.4±0.5	0.2±0.1
Lachnospiraceae_uncl (ASV126)	0.0	0.7	0.0	0.0	0.2±0.3	0.2±0.3	0.3±0.6	0.3±0.6
Streptococcus (ASV14)	0.2	0.0	1.8	0.1			0.5±1.0	0.1±0.2
Erysipelatoclostridium (ASV2)	10.2	0.0	1.6	4.2	31.5±28.1	5.3±5.5	4.2±7.4	25±31.5
Enterococcus (ASV22)	1.0	0.0	0.1	0.0	2.7±3.4	3.5±3.3	8.6±14.3	3.1±3.8
Faecalibacterium (ASV23)	2.1	2.1	3.3	8.1	0.1±0.1			0.1±0.1
Collinsella (ASV28)	0.2	0.1	0.4	0.6	0.4±0.5	0.7±0.5	2.1±2.4	4.3±7.9
Mitsuokella (ASV33)	8.5	0.0	3.2	0.0	4.1±5.7		13.9±15.9	0.2±0.3
Enterobacter (ASV34)	12.0	0.0	0.5	1.3	1.4±1.0	1.0±1.1	1.1±2.2	0.9±1.2
Bifidobacterium (ASV4)	1.0	0.2	13.4	6.9	5.7±6.5	1.8±2.1	21.9±27.7	17±23.6
Clostridium sen_ str_ 1 (ASV40)	0.0	0.0	0.0	0.0			1.1±2.2	0.1±0.2
Lachnoclostridium (ASV44)	1.2	4.6	0.0	0.5	1.6±2.5	3.6±4.8	1.5±2.1	1.4±1.8
Enterobacteriaceae_uncl (ASV47)	12.6	0.2	0.8	2.3	1.8±1.7	1.0±1.2	1.3±2.7	0.7±1.2
Parabacteroides (ASV5)	22.1	53.1	0.1	2.9	24.5±20	44.3±19.3	16.1±23.2	18.2±23.2
Bacteroides (ASV6)	13.8	8.4	15.0	42.9	5.2±4.5	5.1±3.4	6.7±6.8	12.0±12.5
Lachnospiraceae NK4A136 group (ASV65)	0.2	2.3	0.2	0.4	0.3±0.7	0.6±1.0	0.8±1.5	0.6±1.2
Acidaminococcus (ASV67)	0.0	0.0	0.0	0.0	0.9±1.0	2.4±4.4	0.3±0.5	0.1±0.1
Lactococcus (ASV86)	0.1	0.4	0.0	0.0	0.5±0.8	0.7±0.8	0.4±0.6	0.7±1.3
Tannerellaceae_uncl (ASV95)	0.2	1.8	0.0	0.2	0.3±0.4	0.5±0.4	0.3±0.5	0.2±0.3
Other	7.0	6.4	7.5	12.1	2.3±1.6	1.5±0.7	0.5±0.3	0.6±0.4

List of figures

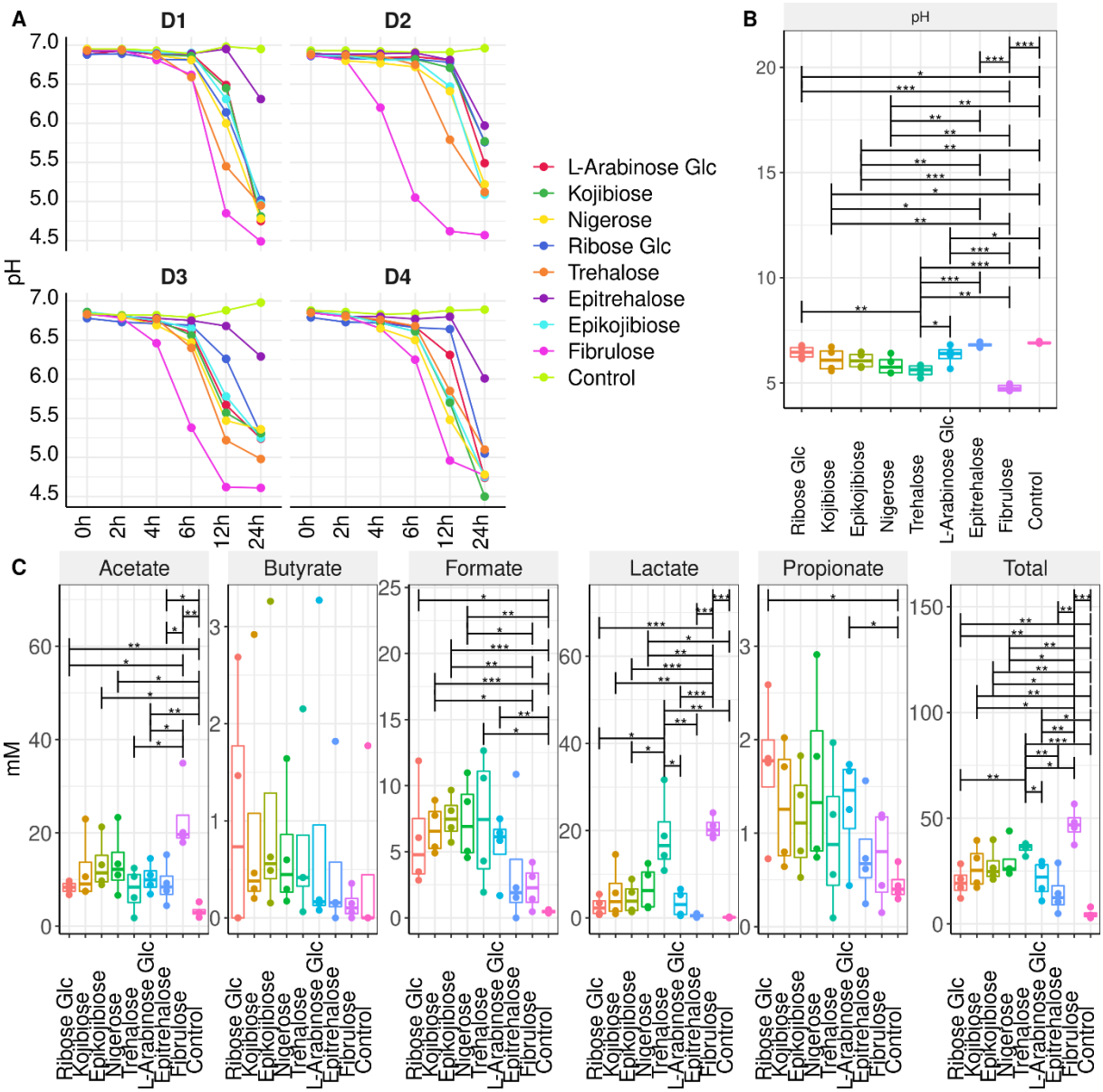

Figure 1. (A) A course change in pH during fermentation of disaccharides and fibrulose (fructan DP 2-10) by in vivo derived gut microbiota from four healthy donors (D1-D4). (B) A boxplot of a pairwise comparison of pH change at 12 h of incubation. (C) Lactic, formic, and short-chain fatty acids are generated during disaccharide and fibrulose fermentation at 12 h of incubation. The total represents the sum of lactic, formic, and short-chain fatty acids from four donors. Control- represents incubation with a medium without sugar. The statistical significance follows $p < 0$ '***' 0.001 '**' 0.01 '*' 0.05.

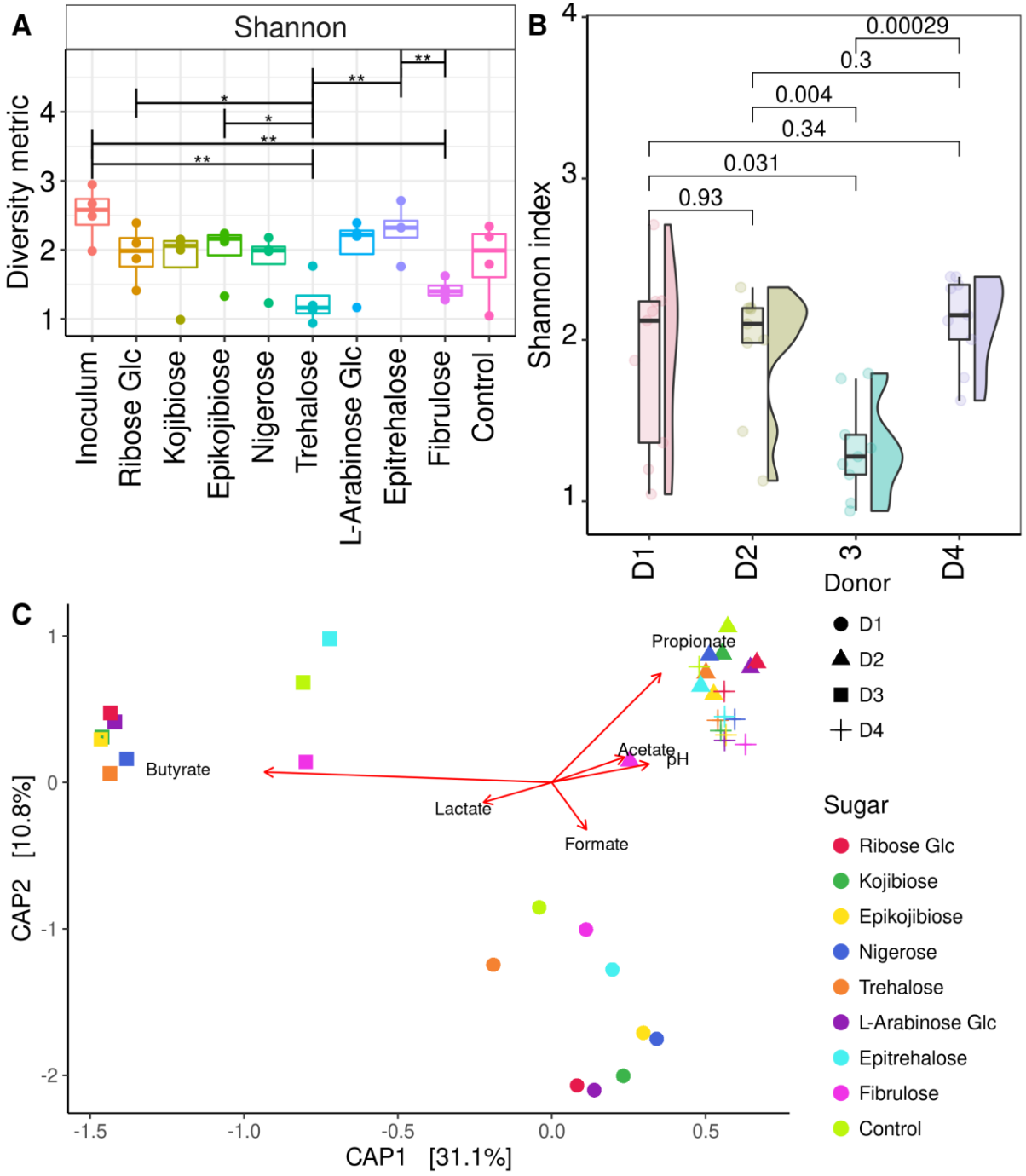

Figure 2. Pair-wise comparison of α diversity; Shannon index within microbial communities in (A) donors and (B) disaccharides and fibrulose amended incubations. Constrained canonical analysis of principal coordinates (CAP) based on Bray-Curtis distance showing the association between bacterial community structure and the treatment variables. The association significance (p=0.001) was tested using Adonis function in the vegan package. The significance codes denote *p*< 0 '***' 0.001 '**' 0.01 '*' 0.05.

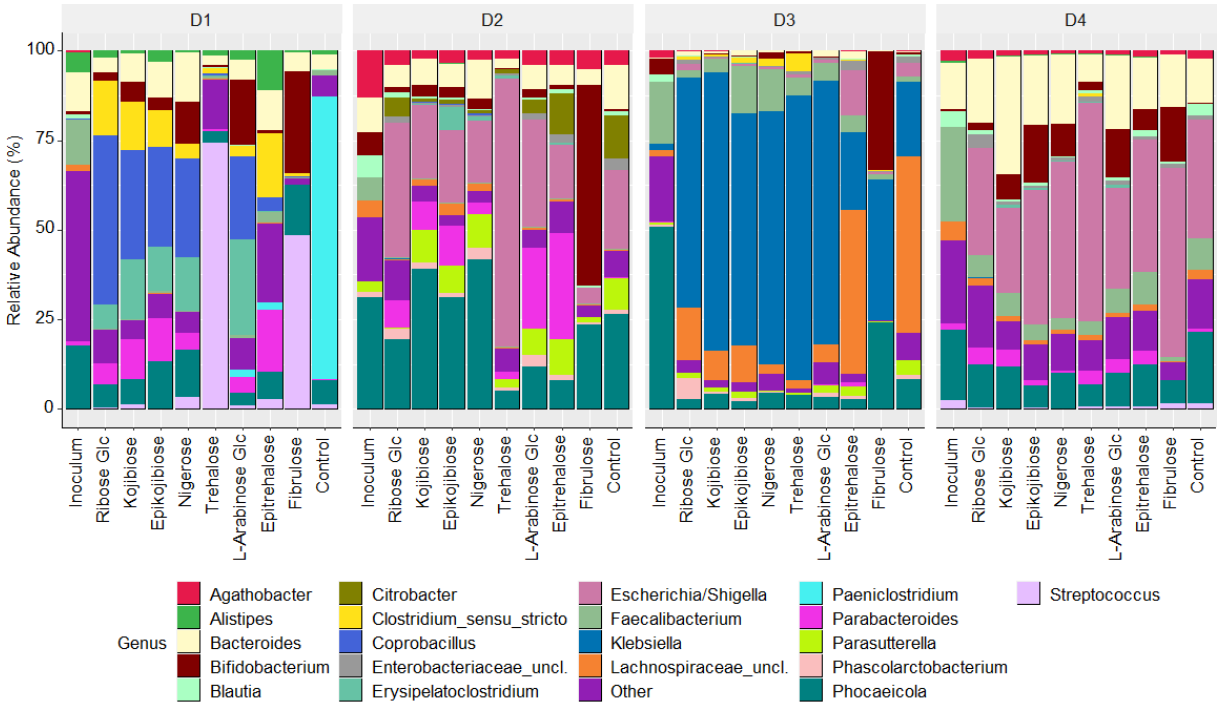

Figure 3. Genus-level microbial community signatures in seven rare and new-to-nature disaccharides and fibrulose (DP2-10) during a 12 h incubation with fecal inoculum from four healthy individuals. The data represent the relative abundance of bacterial genomes of the top 20 genera constituting >0.1% and "other genera" accounting for all other genera <0.1%. The abbreviations D1-D4 represent donors. Family level taxa appearing in the genus level plot, are unclassified genus belonging to the respective family.

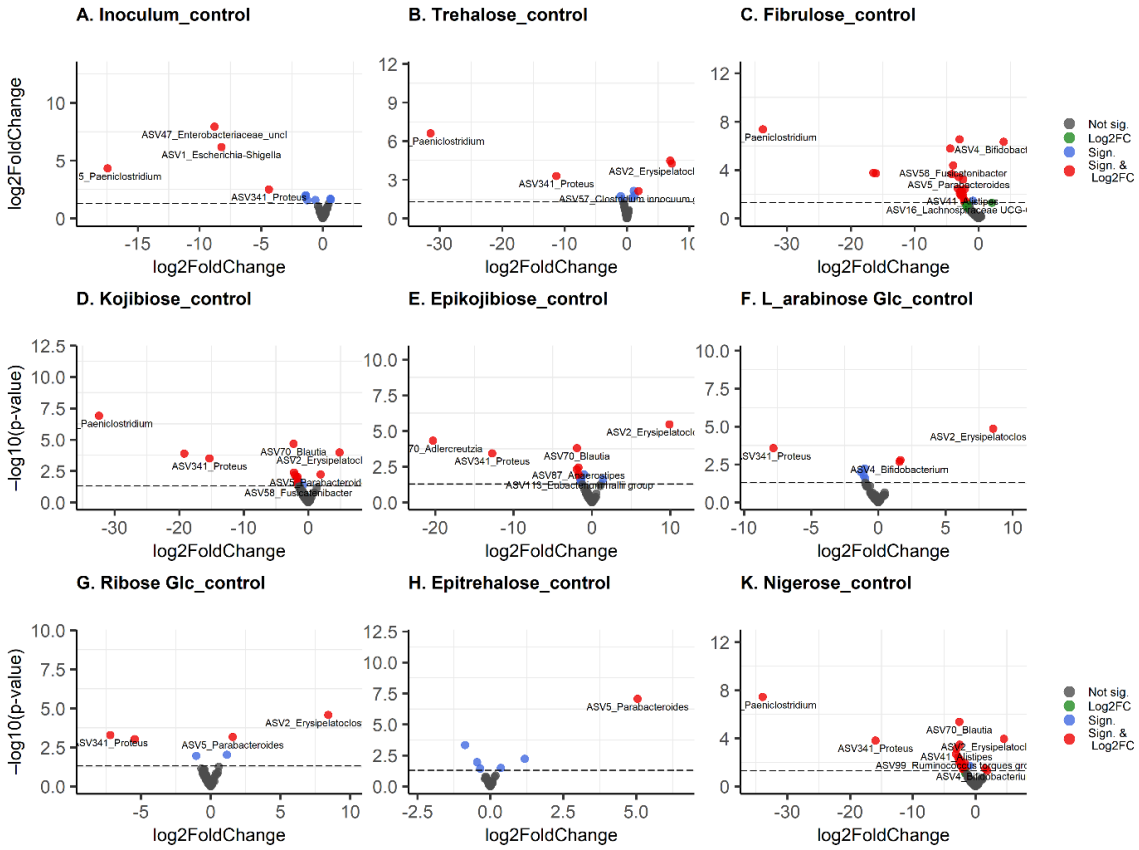

Figure 4. Volcano plots (A-K) as assessed by DESeq2 analysis showing Log2 Fold Change of normalized genus abundances following sugar incubations. A positive log2FoldChange indicates genera that are significantly stimulated by the respective sugar as compared to the control incubation. Control was incubated with fecal suspension without sugar supplementation in a carbon-limited medium. The red dots display genera with an absolute log2 Fold Change value exceeding 1.5 and a high statistical significance (-log10 of p-value, y-axis). The blue dots are genera with points above the horizontal line having $p < 0.05$, the gray dots represent genus having $p > 0.05$ while the green dots are genera with large log2 fold change exceeding $p > 0.05$ and below the horizontal line.

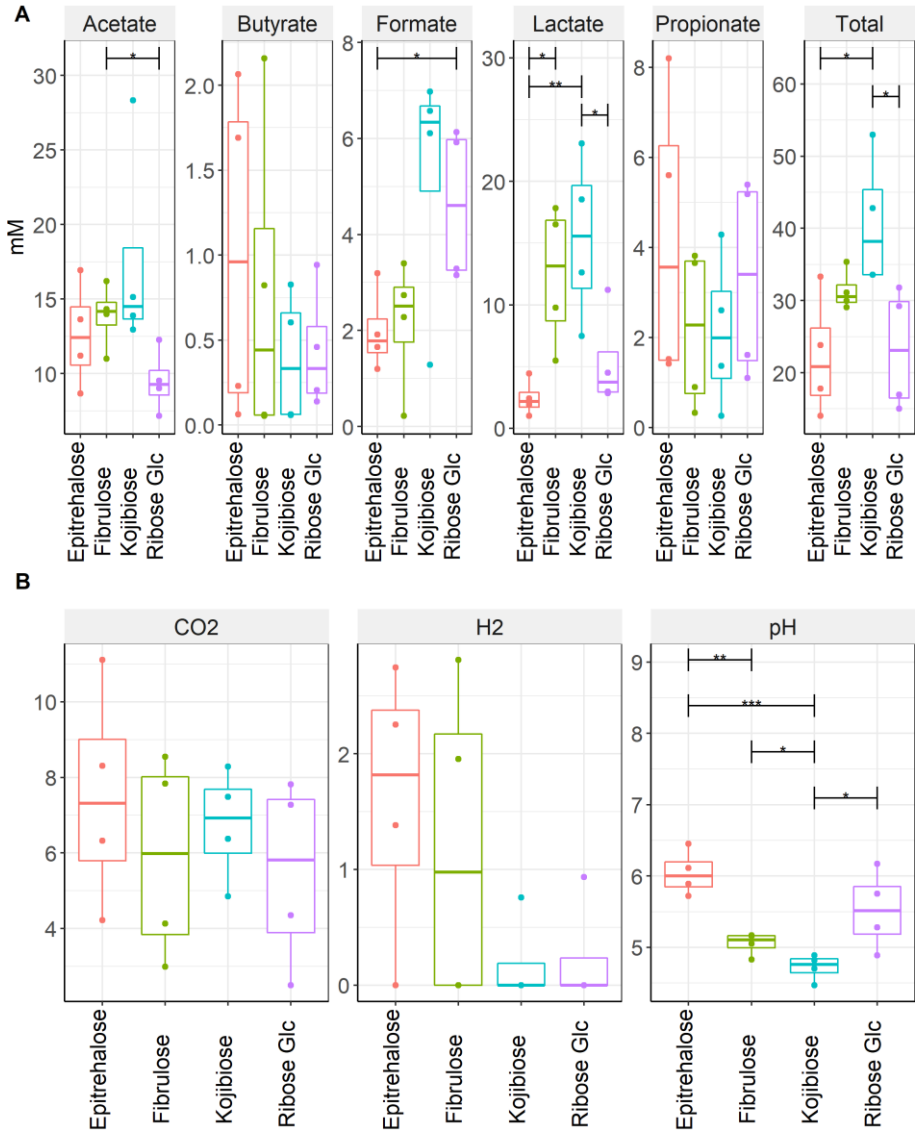

Figure 5. A boxplot of a pairwise comparison of (A) Lactic, formic and short-chain fatty acids generated after re-exposure to each disaccharide and fibrulose at 24 h of incubation. The total represents the sum of lactic, formic, and short-chain fatty acids. (B) Percentage generated CO_2, and H_2, and pH change at 24 h of incubation. The statistical significance follows $p< 0$ '***' 0.001 '**' 0.01 '*' 0.05.

References

Beerens, K., De Winter, K., Van de Walle, D., Grootaert, C., Kamiloglu, S., Miclotte, L., . . . Desmet, T. (2017). Biocatalytic Synthesis of the Rare Sugar Kojibiose: Process Scale-Up and Application Testing. *J Agric Food Chem, 65*(29), 6030-6041. https://doi.org/10.1021/acs.jafc.7b02258.

Blighe K, S. R., and M Lewis. (2018). EnhancedVolcano: Publication-ready volcano plots with enhanced colouring and labeling. github.com.

Callahan, B. J., McMurdie, P. J., Rosen, M. J., Han, A. W., Johnson, A. J. A., & Holmes, S. P. (2016). DADA2: High-resolution sample inference from Illumina amplicon data. *Nature Methods, 13*(7), 581-583. https://doi.org/10.1038/nmeth.3869.

Candry, P., Van Daele, T., Denis, K., Amerlinck, Y., Andersen, S. J., Ganigué, R., . . . Rabaey, K. (2018). A novel high-throughput method for kinetic characterisation of anaerobic bioproduction strains, applied to Clostridium kluyveri. *Scientific reports, 8*(1), 9724. https://doi.org/10.1038/s41598-018-27594-9.

Chaudhury, A., Nath, G., Tikoo, A., & Sanyal, S. C. (1999). Enteropathogenicity and Antimicrobial Susceptibility of New Escherichia Spp. *Journal of Diarrhoeal Diseases Research, 17*(2), 85-87. http://www.jstor.org/stable/23498636.

Chen, C., Desmet, T., Van der Borght, J., Lin, S. K. C., & Soetaert, W. (2012). Adsorption–desorption of trehalose analogues from a bioconversion mixture using activated carbon. *Separation and Purification Technology, 96*, 161-167. https://doi.org/https://doi.org/10.1016/j.seppur.2012.05.032.

Cole, J. R., Wang, Q., Fish, J. A., Chai, B., McGarrell, D. M., Sun, Y., . . . Tiedje, J. M. (2014). Ribosomal Database Project: data and tools for high throughput rRNA analysis. *Nucleic Acids Research, 42*(Database issue), D633-D642. https://doi.org/10.1093/nar/gkt1244.

Collins, J., Danhof, H., & Britton, R. A. (2019). The role of trehalose in the global spread of epidemic Clostridium difficile. *Gut microbes, 10*(2), 204-209. https://doi.org/10.1080/19490976.2018.1491266.

Collins, J., Robinson, C., Danhof, H., Knetsch, C. W., van Leeuwen, H. C., Lawley, T. D., . . . Britton, R. A. (2018). Dietary trehalose enhances virulence of epidemic Clostridium difficile. *Nature, 553*(7688), 291-294. https://doi.org/10.1038/nature25178.

Davani-Davari, D., Negahdaripour, M., Karimzadeh, I., Seifan, M., Mohkam, M., Masoumi, S. J., . . . Ghasemi, Y. (2019). Prebiotics: Definition, Types, Sources, Mechanisms, and Clinical Applications. *Foods, 8*(3). https://doi.org/10.3390/foods8030092.

David, L. A., Materna, A. C., Friedman, J., Campos-Baptista, M. I., Blackburn, M. C., Perrotta, A., . . . Alm, E. J. (2014). Host lifestyle affects human microbiota on daily timescales. *Genome biology, 15*(7), R89. https://doi.org/10.1186/gb-2014-15-7-r89.

Davis, L. M. G., Martínez, I., Walter, J., Goin, C., & Hutkins, R. W. (2011). Barcoded Pyrosequencing Reveals That Consumption of Galactooligosaccharides Results in a Highly Specific Bifidogenic Response in Humans. *PLOS ONE, 6*(9), e25200. https://doi.org/10.1371/journal.pone.0025200.

De Paepe, K., Verspreet, J., Courtin, C. M., & Van de Wiele, T. (2020). Microbial succession during wheat bran fermentation and colonisation by human faecal microbiota as a result of niche diversification. *The ISME Journal, 14*(2), 584-596. https://doi.org/10.1038/s41396-019-0550-5.

Dhaene, S., Van Laar, A., De Doncker, M., De Beul, E., Beerens, K., Grootaert, C., . . . Desmet, T. (2022). Sweet Biotechnology: Enzymatic Production and Digestibility Screening of Novel Kojibiose and Nigerose Analogues. *Journal of Agricultural and Food Chemistry, 70*(11), 3502-3511. https://doi.org/10.1021/acs.jafc.1c07709.

Di Rienzi, S. C., & Britton, R. A. (2019). Adaptation of the Gut Microbiota to Modern Dietary Sugars and Sweeteners. *Advances in Nutrition*. https://doi.org/10.1093/advances/nmz118.

Duncan, S. H., Louis, P., & Flint, H. J. (2004). Lactate-utilizing bacteria, isolated from human feces, that produce butyrate as a major fermentation product. *Appl Environ Microbiol, 70*(10), 5810-5817. https://doi.org/10.1128/aem.70.10.5810-5817.2004.

Duranti, S., Ruiz, L., Lugli, G. A., Tames, H., Milani, C., Mancabelli, L., . . . Turroni, F. (2020). Bifidobacterium adolescentis as a key member of the human gut microbiota in the production of GABA. *Scientific Reports, 10*(1), 14112. https://doi.org/10.1038/s41598-020-70986-z.

Farag, M. A., Abdelwareth, A., Sallam, I. E., el Shorbagi, M., Jehmlich, N., Fritz-Wallace, K., . . . von Bergen, M. (2020). Metabolomics reveals impact of seven functional foods on metabolic pathways in a gut microbiota model. *Journal of Advanced Research, 23*, 47-59. https://doi.org/https://doi.org/10.1016/j.jare.2020.01.001.

Flint, H. J., Scott, K. P., Duncan, S. H., Louis, P., & Forano, E. (2012). Microbial degradation of complex carbohydrates in the gut. *Gut microbes, 3*(4), 289-306. https://doi.org/10.4161/gmic.19897.

Franceus, J., Dhaene, S., Decadt, H., Vandepitte, J., Caroen, J., Van der Eycken, J., . . . Desmet, T. (2019). Rational design of an improved transglucosylase for production of the rare sugar nigerose. *Chemical Communications, 55*(31), 4531-4533. https://doi.org/10.1039/C9CC01587F.

García-Cayuela, T., Díez-Municio, M., Herrero, M., Martínez-Cuesta, M. C., Peláez, C., Requena, T., & Moreno, F. J. (2014). Selective fermentation of potential prebiotic lactose-derived oligosaccharides by probiotic bacteria. *International Dairy Journal, 38*(1), 11-15. https://doi.org/https://doi.org/10.1016/j.idairyj.2014.03.012.

Guarino, M. P. L., Altomare, A., Emerenziani, S., Di Rosa, C., Ribolsi, M., Balestrieri, P., . . . Cicala, M. (2020). Mechanisms of Action of Prebiotics and Their Effects on Gastro-Intestinal Disorders in Adults. *Nutrients, 12*(4), 1037. https://doi.org/10.3390/nu12041037.

Hodoniczky, J., Morris, C. A., & Rae, A. L. (2012). Oral and intestinal digestion of oligosaccharides as potential sweeteners: A systematic evaluation. *Food Chemistry, 132*(4), 1951-1958. https://doi.org/https://doi.org/10.1016/j.foodchem.2011.12.031.

Jia, W., Li, H., Zhao, L., & Nicholson, J. K. (2008). Gut microbiota: a potential new territory for drug targeting. *Nature Reviews Drug Discovery, 7*(2), 123-129. https://doi.org/10.1038/nrd2505.

Kaoutari, A. E., Armougom, F., Gordon, J. I., Raoult, D., & Henrissat, B. (2013). The abundance and variety of carbohydrate-active enzymes in the human gut microbiota. *Nature Reviews Microbiology, 11*(7), 497-504. https://doi.org/10.1038/nrmicro3050.

Kashyap, P. C., Marcobal, A., Ursell, L. K., Smits, S. A., Sonnenburg, E. D., Costello, E. K., . . . Sonnenburg, J. L. (2013). Genetically dictated change in host mucus carbohydrate landscape exerts a diet-dependent effect on the gut microbiota. *Proceedings of the National Academy of Sciences, 110*(42), 17059-17064. https://doi.org/10.1073/pnas.1306070110.

Koh, G. Y., Kane, A., Lee, K., Xu, Q., Wu, X., Roper, J., . . . Crott, J. W. (2018). Parabacteroides distasonis attenuates toll-like receptor 4 signaling and Akt activation and blocks colon tumor formation in high-fat diet-fed azoxymethane-treated mice. *International Journal of Cancer, 143*(7), 1797-1805. https://doi.org/https://doi.org/10.1002/ijc.31559.

Love, M. I., Huber, W., & Anders, S. (2014). Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biology, 15*(12), 550. https://doi.org/10.1186/s13059-014-0550-8.

Maathuis, A. J. H., van den Heuvel, E. G., Schoterman, M. H. C., & Venema, K. (2012). Galacto-Oligosaccharides Have Prebiotic Activity in a Dynamic In Vitro Colon Model Using a 13C-Labeling Technique. *The Journal of Nutrition, 142*(7), 1205-1212. https://doi.org/10.3945/jn.111.157420.

McMurdie, P. J., & Holmes, S. (2013). phyloseq: An R Package for Reproducible Interactive Analysis and Graphics of Microbiome Census Data. *PLOS ONE, 8*(4), e61217. https://doi.org/10.1371/journal.pone.0061217.

Oksanen, J., Blanchet, F. G., Kindt, R., Legendre, P., Minchin, P. R., O'Hara, R. B., . . . Wagner, H. (2015). *Vegan: community ecology package. R package vegan, vers. 2.2-1*.

Oku, T., & Nakamura, S. (2000). Estimation of intestinal trehalase activity from a laxative threshold of trehalose and lactulose on healthy female subjects. *European Journal of Clinical Nutrition, 54*(10), 783-788. https://doi.org/10.1038/sj.ejcn.1601091.

Onyango, S. O., De Clercq, N., Beerens, K., Van Camp, J., Desmet, T., & Van de Wiele, T. (2020). Oral microbiota display profound differential metabolic kinetics and community shifts upon incubation with sucrose, trehalose, kojibiose and xylitol. *Applied and Environmental Microbiology*, AEM.01170-01120. https://doi.org/10.1128/AEM.01170-20.

Payling, L., Fraser, K., Loveday, S. M., Sims, I., Roy, N., & McNabb, W. (2020). The effects of carbohydrate structure on the composition and functionality of the human gut microbiota. *Trends in Food Science & Technology, 97*, 233-248. https://doi.org/https://doi.org/10.1016/j.tifs.2020.01.009.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., . . . Glöckner, F. O. (2013). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Research, 41*(D1), D590-D596. https://doi.org/10.1093/nar/gks1219.

R Core Team. (2019). R: A language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing.

Robayo-Torres, C. C., Quezada-Calvillo, R., & Nichols, B. L. (2006). Disaccharide digestion: clinical and molecular aspects. *Clinical gastroenterology and hepatology : the official clinical practice journal of the American Gastroenterological Association, 4*(3), 276-287. https://doi.org/10.1016/j.cgh.2005.12.023.

Sanz, M. L., Côté, G. L., Gibson, G. R., & Rastall, R. A. (2006). Influence of Glycosidic Linkages and Molecular Weight on the Fermentation of Maltose-Based Oligosaccharides by Human Gut Bacteria. *Journal of Agricultural and Food Chemistry, 54*(26), 9779-9784. https://doi.org/10.1021/jf061894v.

Sanz, M. L., Gibson, G. R., & Rastall, R. A. (2005). Influence of Disaccharide Structure on Prebiotic Selectivity in Vitro. *Journal of Agricultural and Food Chemistry, 53*(13), 5192-5199. https://doi.org/10.1021/jf050276w.

Sen, T., Cawthon, C. R., Ihde, B. T., Hajnal, A., DiLorenzo, P. M., de La Serre, C. B., & Czaja, K. (2017). Diet-driven microbiota dysbiosis is associated with vagal remodeling and obesity. *Physiol Behav, 173*, 305-317. https://doi.org/10.1016/j.physbeh.2017.02.027.

Sonnenburg, E. D., Sonnenburg, J. L., Manchester, J. K., Hansen, E. E., Chiang, H. C., & Gordon, J. I. (2006). A hybrid two-component system protein of a prominent human gut symbiont couples glycan sensing in vivo to carbohydrate metabolism. *Proceedings of the National Academy of Sciences, 103*(23), 8834. https://doi.org/10.1073/pnas.0603249103.

Townsend, G. E., Han, W., Schwalm, N. D., Raghavan, V., Barry, N. A., Goodman, A. L., & Groisman, E. A. (2019). Dietary sugar silences a colonization factor in a mammalian gut symbiont. *Proceedings of the National Academy of Sciences, 116*(1), 233. https://doi.org/10.1073/pnas.1813780115.

Tungland, B. (2018). Chapter 7 - Overview of Prebiotics: Membership, Physiological Effects and their Health Attributes. In *Human Microbiota in Health and Disease* (pp. 289-348): Academic Press.

Van Nevel, S., Koetzsch, S., Weilenmann, H.-U., Boon, N., & Hammes, F. (2013). Routine bacterial analysis with automated flow cytometry. *Journal of Microbiological Methods, 94*(2), 73-76. https://doi.org/https://doi.org/10.1016/j.mimet.2013.05.007.

Wang, K., Liao, M., Zhou, N., Bao, L., Ma, K., Zheng, Z., . . . Liu, H. (2019). Parabacteroides distasonis Alleviates Obesity and Metabolic Dysfunctions via Production of Succinate and Secondary Bile Acids. *Cell Rep, 26*(1), 222-235.e225. https://doi.org/10.1016/j.celrep.2018.12.028.