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DIETARY FIBER UTILIZATION IN THE GUT: THE ROLE OF HUMAN GUT MICROBES IN THE DEGRADATION AND CONSUMPTION OF XYLOSE-BASED CARBOHYDRATES

by

Elizabeth Drey

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DIETARY FIBER UTILIZATION IN THE GUT: THE ROLE OF HUMAN GUT MICROBES IN THE DEGRADATION AND CONSUMPTION OF XYLOSE-BASED CARBOHYDRATES

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University of Nebraska, 2021

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Xylans are a family of xylose-based polysaccharides naturally present in fruits, vegetables, and cereal grains. Resistant to digestion by host enzymes, xylans reach the large intestine intact, where they are utilized by members of the gut microbiome. They are initially hydrolyzed by primary degraders that utilize extracellular xylanases to cleave xylan into smaller oligomers. These xylooligosaccharides (XOS) can then either be further hydrolyzed by primary degraders or can cross-feed secondary consumers, including *Bifidobacterium*. While several *Bifidobacterium* species have metabolic systems for XOS, studies have shown these species grow poorly on longer XOS and xylan substrates. In this thesis, we assessed the ability of *Bifidobacterium pseudocatenulatum* strains to grow on XOS and xylan. Two distinct phenotypes were observed: a xylan^{+} phenotype, where strains displayed growth on xylan and XOS regardless of chain length, and a xylan^{-} phenotype, where strains showed no growth on xylan and a preference for shorter XOS fractions. In-silico analysis revealed XOS-active gene clusters, with the presence or absence of key genes correlating to observed phenotypes. Importantly, only xylan^{+} strains contained an extracellular GH10 endo-1,4-beta-xylanase, a key component to primary degradation of xylan. The presence of an extracellular xylanase was confirmed by detection of xylan hydrolysates via TLC and

HPLC analysis. Furthermore, xylan$^-$ strains showed improved growth on xylan when combined with a xylan$^+$ strain supernatant, indicating crossfeeding capabilities. These results indicate some strains of *Bifidobacterium pseudocatenulatum* act as primary degraders of xylan through extracellular enzymatic degradation, a novel trait within the genus *Bifidobacterium*.

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Preface

This thesis is comprised of three chapters. The first chapter is a review of dietary fiber utilization in the gut, with an emphasis on role of primary degraders and secondary consumers in the consumption of the xylose-based carbohydrate, xylan. Chapter 2 describes our experimental analysis of the unique role of gut commensal, *Bifidobacterium pseudocatenulatum* in the utilization of xylan and xylan-based oligomers among the *Bifidobacterium* genus. Chapter 3 is a summary of findings and implications of research outlined in this thesis, as well as potential future research.

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Chapter 1: Xylan- Structure, Hydrolysis, and Role in the Human Gut Microbiome

Introduction

The trillions of microbes that reside in the human gastrointestinal tract depend on food constituents that are consumed by the host. Because simple sugars and other small molecules are digested and absorbed during transit through the stomach and small intestine, only large, complex, or otherwise recalcitrant molecules ultimately reach the colon. The ability of various gut microbes to degrade these substrates, including fiber, into smaller, more consumable products has a profound influence on the composition of the gut microbiome, with important implications for human health.

In this review, we provide an overview of the chemistry of this large group of complex, non-digestible molecules. We then describe industrial processing of the xylose-based fiber, xylan, and its conversion into xylooligosaccharides (XOS). Finally, we review the biochemical and metabolic steps required for utilization of xylan and XOS by primary degraders and secondary consumers in the human gut, with an emphasis on the role of *Bifidobacterium.*

Dietary Fiber: Definitions and Functional Properties

Definition. Establishing a clear and consistent definition for dietary fiber has challenged scientists for more than 70 years (DeVries, 2004). Dietary fibers are generally described as a broad category of plant-derived material resistant to digestion in the human gastrointestinal tract. They are typically found in plant cell walls. Historically, different national authorities have used varying definitions for what constitutes dietary fiber, where some include non-digestible carbohydrates (NDC's) and others include other plant cell wall-associated materials in addition to NDC's (i.e. lignin, cross-linked polyphenolic compounds that interacts with NDC's) (Jones, 2014; Kay, 1982; Makki et al., 2018**).** In

2009, a standardized definition was described by the Codex Alimentarius Commission. They stated that dietary fiber are comprised of carbohydrate polymers of greater than 3 monomeric units that are not hydrolyzed by endogenous intestinal enzymes from humans and included one of the following: 1) intrinsic non-digestible carbohydrates, naturally occurring in food as consumed, 2) carbohydrate polymers obtained from food raw material via physical, enzymatic or chemical means that offer a physiological benefit, or 3) synthetic carbohydrates that offer physiological benefit (Lupton, 2009). The U.S. has partially adopted this definition, as the Food and Drug Administration (FDA) currently defines dietary fiber as 'non-digestible soluble and insoluble carbohydrates (with 3 or more monomeric units), and lignin that are intrinsic and intact in plants; isolated or synthetic non-digestible carbohydrates (with 3 or more monomeric units) determined by FDA to have physiological effects that are beneficial to human health.' (21 CFR 101.9(c)(6)(i)). The latter clause – that a physiological benefit must exist, represents a recent, but very important addition to what had been mostly chemically-focused definitions.

Still, nearly all definitions of dietary fiber include non-digestible carbohydrates (NDC), indicating their centrality to the definition of dietary fiber. NDC's are the dietary oligo- and polysaccharides that cannot be degraded by human alimentary enzymes. Differences in solubility of these NDC's play a critical part in the role of these carbohydrates in the gastrointestinal tract. Soluble, non-digestible carbohydrates (which include pectins, gums, and some hemicelluloses) can be fermented by gut bacteria to produce metabolites such as short chain fatty acids (SCFA's). In contrast, insoluble non-digestible carbohydrates (i.e., cellulose and lignin) pass through the gut with minimal

degradation, and generally do not serve as substrates for gut microbes. However, these insoluble NDCs do provide other physiological effects, as discussed below (Capuano et al., 2017). Currently (as of 2021), the FDA recognizes a limited number of non-digestible carbohydrates (isolated or synthetic) including beta-glucan soluble fiber, psyllium husk, cellulose, guar gum, pectin, locust bean gum, and hydroxypropylmethylcellulose. Since initial identification of recognized non-digestible carbohydrates, ten additional isolated/synthetic fibers are intended to be added to the regulatory definition of dietary fiber in the U.S. as of this writing. These proposed fibers include: mixed plant cell wall fibers, arabinoxylan, alginate, inulin, high amylose starch (resistant starch 2), galactooligosaccharides, polydextrose, resistant maltodextrin/dextrin, cross linked phosphorylated resistant starch 4, glucomannan (FDA, 2018).

Functional properties and physiological effects. The function and effects of dietary fiber effect in the gut can vary considerably, depending on their structural properties. These structural properties can affect solubility, viscosity, bulking, and fermentability. Linear fibers, such as cellulose, are typically insoluble and non-fermentable, but are still a key component to normal gut function. The crystalline structure of insoluble fibers increase fecal bulk via increased water-holding capacity (water held within the fiber matrix). Improved fecal bulking allows for increased stool mass, as well as the alleviation of constipation and regularity (Dai et al., 2014).

Soluble dietary fibers can be degraded by members of the gut microbiome, a diverse microbial community resident to the colon. These microorganisms are known to have the genetic machinery to hydrolyze large polymers into smaller molecules. The latter can then be transported across cell membranes and metabolized into short chain

fatty acids (SCFA) and other end-products. This fermentation activity can be delayed by the completeness of the dietary fiber. Intrinsic dietary fibers that contain other cell wall components, such as lignins and cell wall-associated proteins, cross-link together, blocking enzymatic degradation, thereby slowing microbial fermentation (Moore et al., 2001).

Health Properties of Fiber

In addition to the chemical and structural properties associated with dietary fiber, most current interest is focused on their suggested health benefits. Indeed, as noted above, to be labeled as dietary fiber, the FDA now requires that they must provide a beneficial physiological effect to human health. Specifically, at least one of the following physiological benefits must be demonstrated by the fiber: lowered blood glucose, lowered cholesterol levels, lowered blood pressure, increased frequency of bowel movements, increased mineral absorption in the intestinal tract, or reduced energy intake (21 CFR 101.9(c)(6)(i)) **(Table 1).** Currently, these are the only physiological benefits listed by the FDA, although they are considered as "examples" and other potential health benefits could still be considered (FDA, 2020).

The fiber gap While not currently recognized as a beneficial physiological effect by the FDA, gut microbiome maintenance is a major factor in overall gut health. Furthermore, dietary fiber plays a profound role in regulating the gut microbiome, by acting as the major substrate for microbial fermentation. Unfortunately, there is a discrepancy between recommended fiber consumption and true fiber consumption in diets. This discrepancy is exacerbated by the low fiber, high-fat foods common to the 'Western' diet. This so-called 'fiber gap' has been suggested to lead to a depleted or dysbiotic gut microbiome. The

latter is not only associated with gastrointestinal disorders such as irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD), but also systemic metabolic diseases such as obesity, heart disease, and type 2 diabetes (Deehan & Walters, 2016).

Low-fiber diets lead to a cascade of potentially deleterious health outcomes. First, the absence of dietary fiber leads to insufficient substrate reaching the colon. This not only causes loss of species reliant upon the substrate, but also reduces production of fermentation end-products, such as short chain fatty acids, that reduce colonic pH and attenuate inflammation (Sonnenburg et al., 2014). Without this reduction in pH, proliferation of acid-labile opportunistic pathogens can occur, further disrupting intestinal health. Furthermore, when fibers are absent, some gut microbes shift their focus from metabolizing dietary fibers to consuming host-secreted mucus glycoproteins, or mucin (Desai et al., 2016). When mucin is degraded, it no longer acts as a protective barrier for epithelial cells, allowing for pathogenic invasion and inflammation of the colon (Qu et al., 2021).

Overall, these factors can result in a decrease in microbial diversity and beneficial bacteria. To minimize this issue, current scientific opinion has suggested increased consumption of dietary fibers, and supplementing other non-digestible oligo- and polysaccharides to low-fiber foods, to help restore microbiome diversity (Deehan & Walters, 2016). Among the most common fibers consumed by humans are the hemicelluloses, of which xylans are among the most abundant. In the next sections, the chemistry of the xylans and their derivatives and their effect on microbial fermentation and gut health will be reviewed.

Xylan and XOS Chemistry, Production and Utilization by Microbes

Chemistry. Xylans are considered hemicellulosic polysaccharides that are the main components of lignocellulosic biomass in the secondary cell walls of plants. They are also considered the third most abundant biopolymer on Earth, following cellulose and chitin (Beckers et al., 2020).

Xylans are comprised of linear polymers of ß-1,4 linked d-xylopyranose units typically substituted with acetyl, glucuronic acid, 4-*O*-methylglucuronic acid (MeGlcA), and arabinose residues. The location and degree of substitution to the xylan backbones depends on the species and tissue type of the plant source (Rennie et al., 2014).

The four major types of xylan found in nature are: linear homoxylan, glucuronoxylan, arabinoxylan, and glucuronoarabinoxylan (Bajpai 2014). Linear ß-1,4 linked homoxylan, while uncommon, can be found in tobacco stalk and esparto grass (**Fig. 1).** Glucuronoxylan is a heteroxylan consisting of the xylan backbone, typically acetylated, and substituted with 4-O-methyl-d-glucuronic acid or glucuronic acid **(Fig. 1)**. This xylan can be found in hardwoods, soybean hulls, legumes and at low levels in fruits and vegetables (Biely et al., 2016, Assor et al., 2013, Broxterman et al., 2018, Martin-Cabrejas et al., 1995).

Arabinoxylan is the major form of dietary xylan (and the only isolated xylan recognized as dietary fiber), which consists of the xylan backbone with single or double substitutions of α-l-arabinose to the xylose unit. Arabinoxylan is present at high levels in rye, barley, wheat, oats, and other cereal grains) and can have different branching patterns depending on the source **(Fig. 1).** As described below, these structural differences can lead to different routes of utilization by intestinal bacteria (Rennie et al., 2014). Finally, glucuronoarabinoxylans are simultaneously substituted by α-l-arabinose

and α-d-glucuronic acid and are typically found in perennial plants and dicot primary walls. Within this category are galactoglucuronoarabinoxylans, which are glucuronoarabinoxylans that also have a terminal ß-l-galactopyranosyl residue present on longer side chains to the xylan (Bajpai 2014). **(Fig. 1)**

Industrial production of XOS. Xylan backbones can be hydrolyzed into smaller oligomer fractions, known as xylooligosaccharides (XOS). The latter are now of considerable interest due to their prebiotic potential (Amorim et al., 2019). XOS can have a degree of polymerization (DP) ranging between 2 and 20, although most commercial XOS products are typically between DP2-6 (Moure et al., 2006, Kim et al., 2014, Boonchuay et al., 2016, Joshi et al., 2020).

XOS can be produced from xylan-containing lignocellulosic material by one of several methods (Amorim et al., 2019, Moure et al., 2006). Traditional hydrolysis of xylan is performed using a dilute mineral acid, such as dilute sulfuric or hydrochloric acid for short periods of time (Samanta et al., 2015). The acid breaks the covalent bond between linked xylose units, with the extent of hydrolysis dependent on acid concentration and treatment time (Mielenz, 2020; Niju et al., 2020). Autohydrolysis is a more recent and environmentally friendly method for xylan hydrolysis, as it only requires hot water or steam to induce hydrolysis (Carvalheiro et al., 2016). Enzymatic methods rely on xylanases, typically from fungal or bacterial origin, to degrade the xylan backbone into smaller XOS fragments. This method is ideal for the production of consistent XOS products, as many industry-selected xylanases produce XOS with a degree of polymerization between two and six, and rarely produce xylose (Sun et al., 2015; Liu et al., 2018). However enzymatic processing alone may be inefficient, as xylan

in its native form is typically bound to lignins and cellulose, inhibiting xylanase binding to xylan (Moreira et al., 2016). Therefore, the most common methods for XOS production include an chemical or auto-hydrolytic pretreatment, followed by enzymatic hydrolysis and purification, if necessary (Chen et al., 2016, Jang et al., 2021). This allows for consistency in the final product, and minimal undesired by-products. **(Fig. 2)**

How Microorganisms Degrade and Consume Xylan

Xylans are ubiquitous in nature due to their importance in plant cell wall structure. Therefore, the ability to break down compact polysaccharides appears to have evolved in fungi and bacteria in several ecological biospheres. For example, microbes isolated from seawaters (Zhan et al. 2020), alkaline hot springs (Jia et al. 2014), and mammalian digestive tracts (Dodd et al. 2010) all have significant xylan degradation abilities. In this next section, we will review the microbial enzymatic degradation mechanisms for XOS production from xylan, before describing relevant gut microorganisms associated with xylan breakdown and consumption in the gut.

Microbial enzymatic degradation of xylan. The complexity of xylans as shown above means that highly specialized enzymes are required for hydrolysis and metabolism by microbes. In general these include an extensive array of carbohydrate active enzymes (CAZy) for xylan breakdown, as they work synergistically for complete degradation (Lombard et al., 2013, Biely et al., 2016). The major enzymes involved in complete xylan degradation include both xylanases and accessory enzymes, or enzymes that remove decorations on the xylan backbone. **(Fig. 3)** The most important are the CAZy enzymes relevant to xylan degradation would be glycosyl hydrolases (GH), that hydrolyze

glycosidic linkages between sugars, and carboxyl esterases (CE), that act to hydrolyze ester linkages to carbohydrates (Lombard et al., 2013).

Accessory enzymes. Accessory enzymes are primarily involved in removal of side chains and can include: α-glucuronidases, α-L-arabinofuranosidases, acetylxylan esterases, feruloyl esterases (Biely et al., 2016). Glucuronidases (EC 3.2.1.139) belonging to GH67 and GH115 work to remove glucuronic acid and MeGlcA linked α-1,2 to the xylan backbone in glucuronoxylan. Arabinofuranosidases (EC 3.2.1.37) hydrolyze L-arabinose units linked α-1,2 or α-1,3 to the xylan backbone. Those active on xylan belong to glycosyl hydrolase families GH43, 51,54, and 62 (Malgas et al., 2019, Biely et al., 2016, Lagaert et al., 2014).

Acetylxylan esterases (3.1.1.72) work to remove acetic acid from the acetylated xylan backbone(Zhang et al., 2011). Acetylxylan esterases active on xylan can come from the carbohydrate esterase (CE) groups: CE1, CE4, CE5, CE6, CE16. For complete acetylated xylan degradation, acetylxylan esterases are a necessity to remove the acetic acid groups, though alkali treatment also can remove most acetic acid groups (Biely et al., 2016). Feruloyl esterases (EC. 3.1.1.73) liberate hydroxycinnamates, such as ferulic acid or *p*-coumaric acid from the xylan backbone, where they can be found in certain arabinoxylans (Wong et al., 2013). Substrate specificity for the associated CAZy accessory enzymes can be seen in **Table 2**.

Xylanases. In addition to accessory enzymes that remove substitutions from the xylan backbone, there are also enzymes specialized in cleaving the xylan backbone that are considered either exo- acting or endo- acting and can be classified as xylosidases, xylanases, or a special family of oligo-xylanases (Biely et al., 2016). Xylosidases achieve

exo- action on the non-reducing end of XOS, as they cleave monomer units of xylose, and can belong to glycosyl hydrolase families GH3, GH39, GH43, GH52, and GH120 (Lagaert et al., 2014). Another exo-acting xylanase, GH8 reducing-end-xylose releasing exo-oligoxylanase cleaves xylose and xylobiose from the reducing end of xylan hydrolysates, with preference for XOS (Valenzuela et al.,2016; Jimenez-Ortega et al., 2020; Hong et al., 2014).

Endo- acting xylan-active enzymes, known as endo- ß-1,4 xylanases, cleave the ß-1,4 linkage internally on the xylan backbone to produce XOS of varying chain lengths (Biely et al., 2016). These xylanases can belong to a variety of glycosyl hydrolase families, including: GH5, GH10, GH11, GH30, and GH98 (Biely et al., 2016). GH5 xylanases are active only on arabinose-substituted xylans and GH30 xylanases are only active on glucuronic acid-substituted xylans (Karlsson et al., 2018; Moreira et al., 2016; Biely et al., 2015).

Xylanases of highest interest belong to GH10 and GH11, as they do not require substitutions to cleave the xylan backbone (Biely et al., 2016; Saka et al., 2016). GH11 xylanases are low in molecular weight and have a larger substrate binding site, indicating the ability to degrade longer xylan chains (Bray et al. 1992). However, due to their large binding sites, they require highly unsubstituted xylan as a substrate, relying heavily on accessory enzymes to remove chain decorations (Song et al. 2013). Studies indicate GH11 xylanases are inefficient on XOS smaller than DP5 and produce fewer soluble end-products than other xylanase families (Paes et al., 2012; Nieto-Dominguez et al., 2017; Ravn et al., 2017).

GH10 xylanases are typically higher in molecular weight with a small substrate binding site, active on shorter chains of xylan and XOS (Biely et al. 1997; Kim et al., 2014). Compared to GH11 xylanases, GH10 xylanases have lower activity on insoluble xylan but greater catalytic versatility, meaning they can more easily degrade substituted xylan, due to their smaller substrate binding site (Bhardwaj et al., 2019; Nordberg Karlsson et al.,2018). Products of GH10 xylanases range in size from xylobiose to xylohexaose, though a majority produce xylobiose and xylotriose (Kim et al., 2014; Teo et al., 2019; Joshi et al., 2020; Liu et al., 2018; Sun et al., 2015).

Utilization of Xylan and XOS in the Gut Microbiome

Xylan utilization in the human digestive tract relies on several families of gut bacteria that are known to use xylans and their hydrolyzed products. In this next section, we will describe the relevant gut bacteria associated with xylan and XOS degradation, emphasizing those microbes capable of degrading large xylan substrates (primary degraders) and those that consume smaller xylan degradation products (secondary consumers).

Primary Degraders. Only a few genera of bacteria are known to directly consume xylans. These primary degraders are found mainly in the genera, *Bacteroides, Prevotella* (phyla Bacteroidetes), *Roseburia, and Eubacterium (*phyla Firmicutes). Mechanisms for each differ, as Bacteroidetes are gram-negative bacteria, which means they have two cell membranes, with a periplasm in between, while Firmicutes are gram-positive with only one cell membrane (Ndeh & Gilbert, 2018). However, in general, all rely on the presence of anchored extracellular xylanases for external cleavage of xylans (Ndeh & Gilbert,

2018; Hong et al., 2014). Specific mechanisms for how these genera degrade xylans are briefly described below. **(Fig. 4A)**

Bacteroidetes. The Gram negative Bacteroidetes rely on polysaccharide utilizing loci (PULs) for xylan degradation. PULs are gene clusters containing a multitude of transporters, regulators, and CAZys relevant to specific glycans (Grondin et al., 2017). Among the most well-studied PULs are those involved in starch utilization (SUS), typified by gut commensal, *Bacteroides thetaiotaomicron* (Foley et al., 2016; Dodd et al., 2010). Collectively, the SUS system relies on the presence of a cell surface-anchored extracellular enzyme that hydrolyzes starch and generates oligosaccharides that are transported into the periplasm (Dodd et al., 2010; Ndeh & Gilbert, 2018; Tamura et al., 2021). Monomers are then formed by periplasmic xylanolytic enzymes and are transported into the cytoplasm for energy harvesting (Ndeh & Gilbert, 2018; Tamura et al., 2021). This is considered a 'selfish' capture, as the XOS is immediately transported to the periplasm, leaving minimal product extracellular (Briggs et al., 2021).

In addition to the SUS system for starch, some species of *Bacteroides* are also xylan primary degraders and xylan and XOS consumers. For example, in *Bacteroides ovatus* two xylan-active PULs were identified, one for simple xylans and another for complex glucuronoarabinoxylans. Furthermore, it appears that complex or decorated xylans are more closely associated with selfish capture, as the cleaved products are not easily assimilated by other microbes (Schwalm III & Groisman, 2017). For example, secondary consumers were not capable of growth in co-culture with *B. ovatus* on complex arabinoxylan compared to the simpler arabinoxylan (Rogowski et al., 2015).

Firmicutes: *Roseburia* and *Eubacterium*. Primary degraders from the Gram positive Firmicutes do not have SUS-systems, as they only contain one cell membrane and have no periplasm. These bacteria, instead, rely on gram-positive polysaccharide utilizing loci (gpPULs). Like PULs from Bacteroidetes, gpPULs also contain genes for membrane-bound extracellular glycosyl hydrolases and regulators. However, unlike PULs, they lack the SUS transport system. They instead rely on ATP-binding cassettes (ABC) transporters or PEP-phosphotransferase system (PTS) transporters (Ndeh & Gilbert, 2018). In this system, the extracellular glycosyl hydrolase cleaves the xylan into XOS that is recognized by a substrate binding protein associated with the ATP transport system, where it is transported intracellularly for cytoplasmic degradation and metabolism. The two major genera from Firmicutes that are associated with primary degradation of xylan are *Clostridium* cluster XIVa organisms, *Roseburia* and *Eubacterium* (Briggs et al., 2021). Both genera are also associated with production of the short-chain fatty acid, butyrate, from carbohydrates (Sheridan et al., 2016).

Secondary Consumers and XOS Metabolizers. As briefly mentioned above, not all primary degradation of xylan leads to selfish capture, especially from simpler xylan. This means that some cleaved XOS remains extracellular, free for consumption by whichever microbes have the genetic and biochemical wherewithal to metabolize xylan degradation products. Accordingly, these secondary consumers have highly specialized substrate recognition and transport systems for extracellular XOS. Many belong to the genera *Lactobacillus* and *Bifidobacterium* (Ndeh & Gilbert, 2018). In addition to these secondary consumers, primary degraders are also able, to some extent, to transport extracellular XOS as well (Tamura et al., 2021) **(Fig 4 B).**

Bifidobacterium. A wide range of oligosaccharides have been shown to be bifidogenic in the gastrointestinal tract. However, XOS is unique among these oligosaccharides as they are considered more selective than other commercial prebiotic oligosaccharides, often enriching for specific species or even strains (Kaplan and Hutkins, 2000; Rycroft et al., 2001; Mäkeläinen et al., 2010). Adult-type *Bifidobacterium* species, particularly *B. longum* ssp. *longum* and *B. adolescentis* are known to degrade XOS as they more frequently rely on dietary sources of plant fibers compared to infant-associated bifidobacteria that can grow on human milk oligosaccharides (Kelly et al., 2021). However, strains from *B. animalis lactis, B. pseudocatenulatum, B. catenulatum* have also shown XOS utilization (Gilad et al., 2010; Saito et al., 2020, Palframan et al., 2003).

The strain specificity of XOS utilization has been demonstrated in several studies. In a survey of twelve strains of *Bifidobacterium* belonging to *B. adolescentis, B. breve, B. longum ssp. infantis, B. animalis ssp. lactis,* and *B. longum ssp. longum*, only strains of *B. animalis ssp. lactis* and *B. adolescentis* were capable of growth on XOS (Mäkeläinen et al., 2010). In another study, *B. longum ssp. longum*, *B. adolescentis*, and *B. pseudocatenulatum* were isolated from fecal culture enriched with XOS, although only *B. longum ssp. longum* showed persistence when reintroduced to fresh fecal culture supplemented with XOS (Kok et a., 2019). In a pangenome and phenotyping analysis, only ten of twenty strains of *B. longum* showed growth on XOS (Arboleya et al., 2018). In general, these studies indicate that XOS utilization is both species and strain-dependent, with longer XOS even more specific.

The pathway for XOS metabolism has been recently described in *B. pseudocatenulatum* JCM1200 (Saito et al., 2020). In this strain, three major XOS/AXOS

active gene clusters were identified based on increased transcription levels in the presence of arabinoxylan hydrolysate substrate. Each cluster contained ABC transport systems with specific substrate binding proteins, along with intracellular glycosyl hydrolases, associated carboxyl esterases and transcriptional regulators. Each substrate binding protein had an affinity for specific decorated XOS/AXOS compounds, meaning they most likely only transported specific forms of XOS/AXOS, and all three were required for total XOS/AXOS transport (Saito et al., 2020). After the XOS/AXOS is transported intracellularly, they are broken down via GH43 xylosidases and arabinofuranosidases to their monomer components for metabolism (Saito et al., 2020). A similar system for XOS utilization was previously described in *B. animalis* ssp. *lactis* (Gilad et al., 2010).

Although *Bifidobacterium* is typically only associated with growth on XOS, more recent studies suggest that some species can grow on longer xylose-based substrates (Liu et al., 2021; Zhu et al., 2021). For xylans containing arabinose or other side chain moieties, those components must first be removed from the xylan backbone. This is done enzymatically by one GH8 reducing end xylose-releasing exo-oligoxylanase from *B. adolescentis, that* shows low activity in producing xylose from xylan (Arboleya et al., 2018; Lagaert et al., 2007). However, currently, there are no bifidobacterial strains known to grow well on the xylan backbone (Kelly et al., 2021). This corresponds with the rarity of GH10 xylanases in bifidobacteria, which are required for xylan degradation (Turroni et al., 2018). Still, recent in-silico research has identified a GH10 endo-xylanase present in some strains of *B. pseudocatenulatum*. However, biochemical evidence is

needed to confirm if this GH10 xylanase offer confers xylanolytic activity in this species (Liu et al., 2021).

Lactobacillus. Lactobacilli are frequently enriched in vitro after fecal fermentations with xylan and XOS (Chen et al., 2020; Hughes et al., 2007; Chen et al., 2015). However, like *Bifidobacterium*, growth on XOS by *Lactobacillus* is highly strain-dependent. In an earlier study with multiple strains from twelve species of *Lactobacillus,* only strains of the species *Lactobacillus brevis* utilized XOS for growth (Crittenden et al., 2002). More recently, strains from *L. casei, L. fermentum, L. plantarum, L. zeae* also were reported to grow on XOS, although high concentrations of XOS were necessary (Ratnadewi et al., 2020; Li et al., 2015). However, in vitro and in vivo studies suggested that XOS was more effective at stimulating growth of *Bifidobacteria* than *Lactobacillus* (Finegold et al., 2014; Li et al., 2015; Chapla et al., 2012). Furthermore, strains of lactobacilli that grow on XOS, generally prefer shorter chain length products, such as xylobiose, whereas *Bifidobacterium* strains can utilize varying XOS chain lengths (Moura et al., 2007). Transport and metabolism of XOS by lactobacilli is similar to *Bifidobacterium*, with an initial transport of XOS fragments via ABC transporters and internal hydrolysis (Illiev et al., 2020). Overall, *Lactobacillus* appear to be secondary consumers of xylan hydrolysates, though they are not as capable as *Bifidobacterium* in growth in pure culture.

Conclusions

Xylans are a diverse group of xylose-based polysaccharides that can be found in the diet. As a source of dietary fiber, these polysaccharides serve as an important substrate for microbes in the GI tract that ferment these carbohydrates and produce short chain fatty acids and other health-promoting metabolites. The breakdown of these fibers

into utilizable substrates by human gut microbes require a diverse array of xylanases and accessory enzymes, as described above. Primary degraders, usually members of the Bacteroidetes, produce extracellular xylanases that cleave xylan into xylooligosaccharides. The resulting XOS fragments from extracellular degradation can also be transported and metabolized directly by primary degraders, or they can cross-feed secondary consumers, including *Bifidobacterium*, whose enrichment is associated with gastrointestinal health. However, more research is needed to better understand the role each of these microbes play in xylan utilization.

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Table 1. Isolated and synthetic carbohydrates recognized as dietary fiber by the U.S. Food and Drug Administration

Dietary Fiber	Health Effect	Solubility	Approved
Beta-glucans	Attenuating blood cholesterol levels	Soluble	2016
Psyllium husk	Attenuating blood cholesterol levels	Soluble	2016
Cellulose	Improved laxation	Insoluble	2016
Guar gum	Attenuating blood cholesterol levels	Soluble	2016
Pectin	Attenuating blood cholesterol levels	Soluble	2016
Locust bean gum	Attenuating blood cholesterol levels	Partially Soluble	2016
Hydroxypropylmethylcellulose	Attenuating blood cholesterol levels	Soluble	2016
Proposed:			
Mixed plant cell wall fibers		N/A	2018
Arabinoxylan	Attenuate Blood Glucose and/or Insulin Levels	Soluble	2018
Alginate	Attenuate Blood Glucose and/or Insulin Levels	Soluble	2018
Inulin/Inulin-type fructans	Enhanced calcium absorption or bone calcium retention	Soluble (non-viscous)	2018
Resistant starch 2	Attenuate Blood Glucose and/or Insulin Levels	Soluble	2018
Galactooligosaccharides (GOS)	Enhanced calcium absorption or bone calcium retention	Soluble	2018
Polydextrose	Reduce energy intake	Soluble	2018
Resistant Maltodextrin/Dextrin	Enhanced calcium absorption or bone calcium retention	Soluble	2018
Resistant starch 4 (cross-linked, phosphorylated)	Attenuate Blood Glucose and/or Insulin Levels	Soluble	2019
Glucomannan	Attenuating blood cholesterol levels	Soluble	2020

Sources: FDA 2016, FDA 2018, Letter from FDA 2020

Table 2. Microbial carbohydrate-active enzymes (CAZy) associated with xylan and XOS degradation.

Enzyme Type	CAZy Family	Substrate Activity
Accessory Enzymes		
Glucuronidase	GH67	GXOS
	GH115	GX; GXOS; GAX
a-l-Arabinofuranosidase	GH43	Single or Double-substituted AX/AXOS
	GH51	Single- substituted AX/AXOS; Single or double-substituted AXOS
	GH54	Single-substituted AX; AXOS
	GH62	Single-substituted AX;AXOS
Acetylxylan esterases	CE1	Acetylated X/XOS
	CE4	Acetylated X/XOS
	CE5	Acetylated X/XOS
	CE6	Acetylated X/XOS
	CE16	GAX
Feruloyl esterases	CE1	Cleaves hydroxycinnamates
Xylanases		
Xylosidases	GH3	XOS
	GH39	XOS
	GH43	XOS
	GH52	XOS
	GH120	XOS
Reducing end xylose releasing exo-oligoxylanase	GH8	XOS; shorter xylan
Xylanases	GH5	AX; AXOS
	GH10	GX; AX; GAX; XOS
	GH11	X; XOS (DP > 5)
	GH30	GX
	GH98	Complex Xylan

Sources: Biely et al., 2016; Malgas et al., 2019; Lagaert et al., 2014; Rogowski et al., 2015
GX=glucuronoxylan
AX=arabinoxylan
GAX=glucuronoarabinoxylan
XOS=xylooligosaccharides
GXOS=Glucuronoxylooligosaccharides
AXOS= arabinoxylooligosaccharides

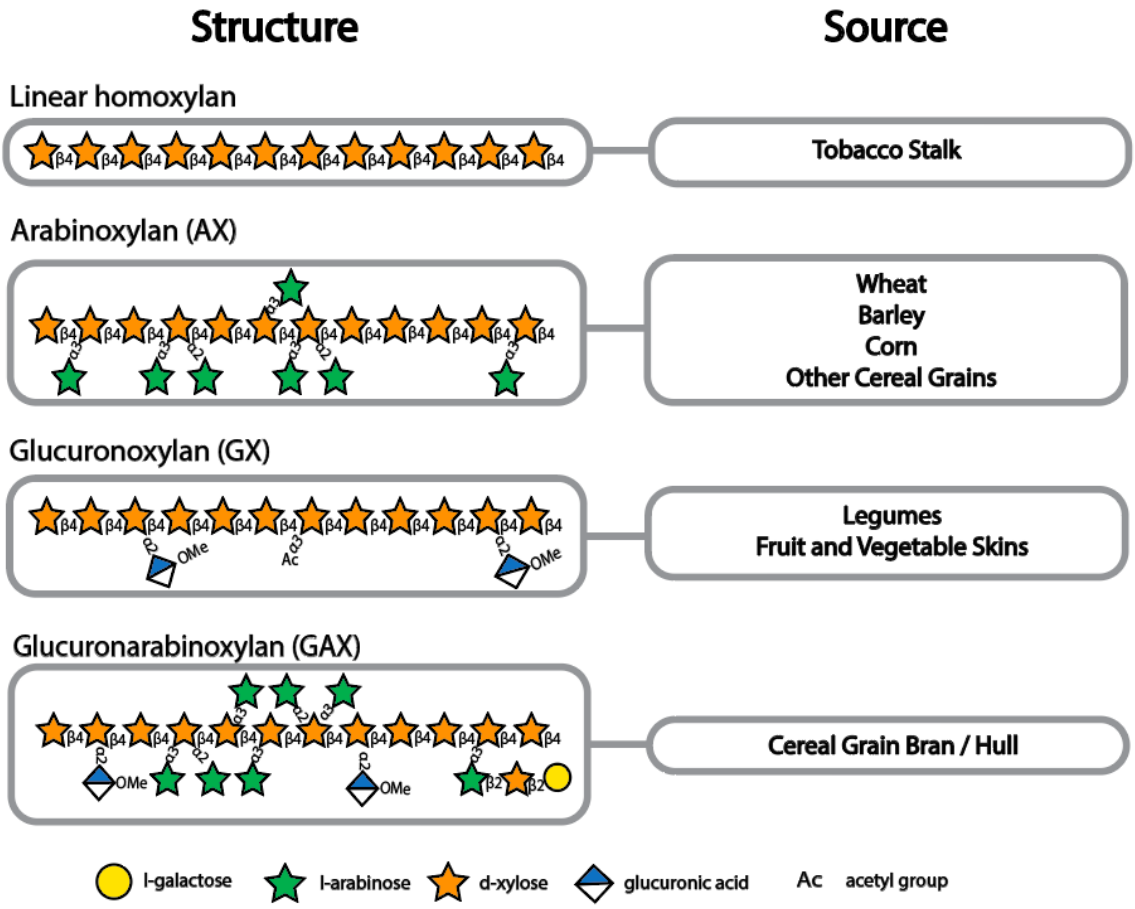

Figure 1. Xylan structure and sources. The structure of xylan varies depending on source material. Linear homoxylan is considered the xylan backbone, consisting of a linear polymer of ß-1,4 linked d-xylopyranose. Uncommon in nature, it is not found in foods, but can be found in tobacco stalk. Arabinoxylan consists of the xylan backbone substituted with arabinose units and is the most common dietary xylan and is primarily found in cereal grains. Glucuronoxylan consists of the xylan backbone substituted with glucuronic acid and 4-0-methyl-glucuonic acid and is also usually acetylated. In the diet, glucuronoxylan can be found at low levels in legumes and fruit and vegetable skins. The most complex xylan is glucuronoarabinoxylan, consisting of both glucuronic acid and arabinose substitutions, and it can be found in cereal grain bran and hulls, such as corn stalk.

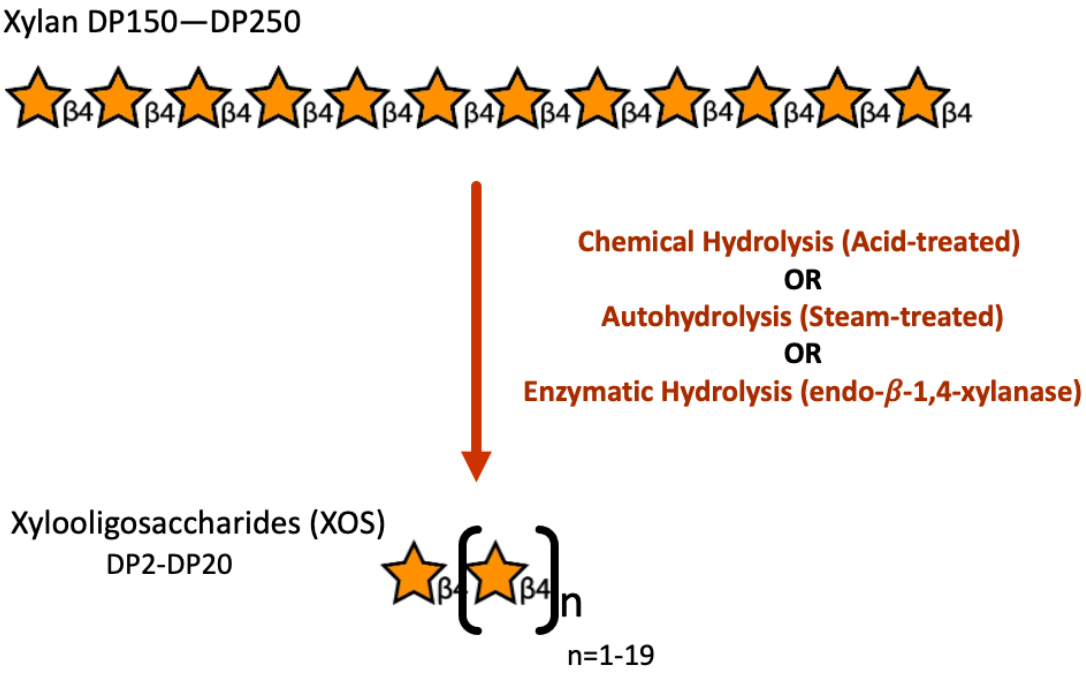

Figure 2. Industrial production of xylooligosaccharides from xylan. Industrial production of XOS occurs via one or a combination of three method. Chemical hydrolysis uses a dilute acid treatment (i.e., dilute hydrochloric or sulfuric acid), where the acid hydrolyzes beta-1,4 glycosidic linkages. Autohydrolysis relies on hydronium ions to act as acid catalysts and requires the direct addition of hot water or steam to the xylan. This solubilizes the backbone and releases acetyl groups to form acetic acid, which can drive additional hydronium production. Enzymatic degradation relies on a simple xylanase treatment, such as endo beta-1,4 xylanase to cleave the backbone distinctly, typically producing XOS products with a degree of polymerization between 2 and 6.

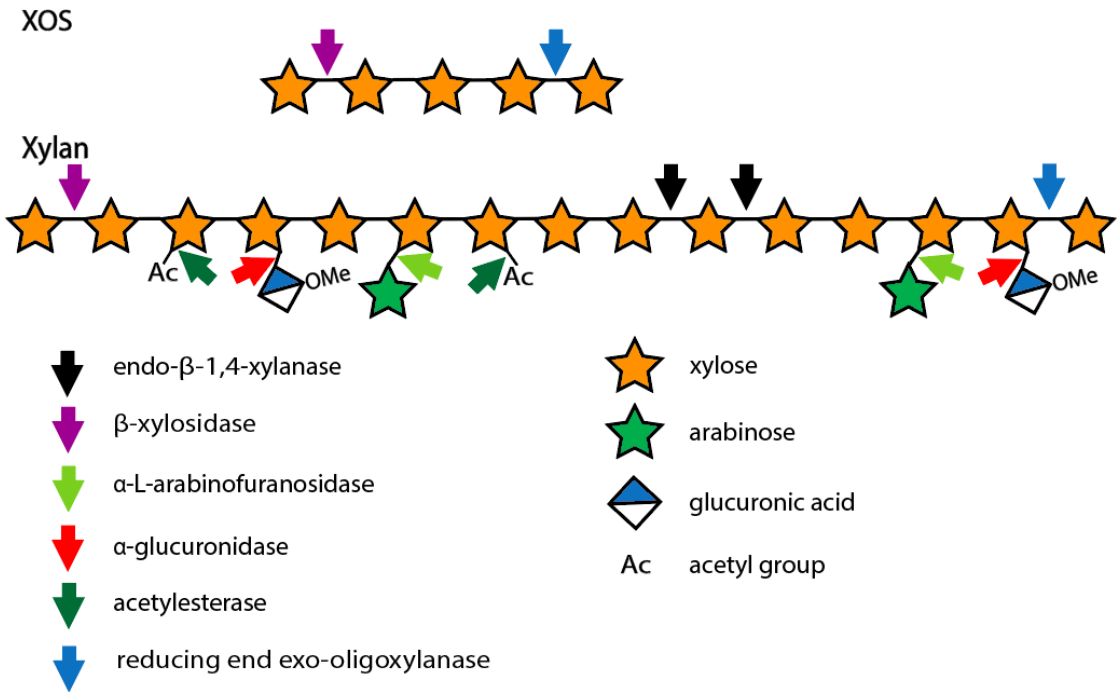

Figure 3 Microbial enzymatic degradation of XOS and xylan. Complex xylan structures rely on a diverse array of microbial carbohydrate active enzymes (CAZy) to either cleave the backbone, as xylanases, or remove substitutions to the backbone, as accessory enzymes. Accessory enzymes can include arabinofuranosidases that remove the arabinose units; glucuronidases that remove glucuronic acid units; or acetyl esterases that remove esterified acetyl groups from the backbone. Xylanases then act on the backbone to remove either single xylose units or xylooligosaccharides. Xylose releasing xylanases include xylosidase and reducing end exo-oligoxylanases, and XOS releasing enzymes include endo beta-1,4 xylanase.

Figure 4

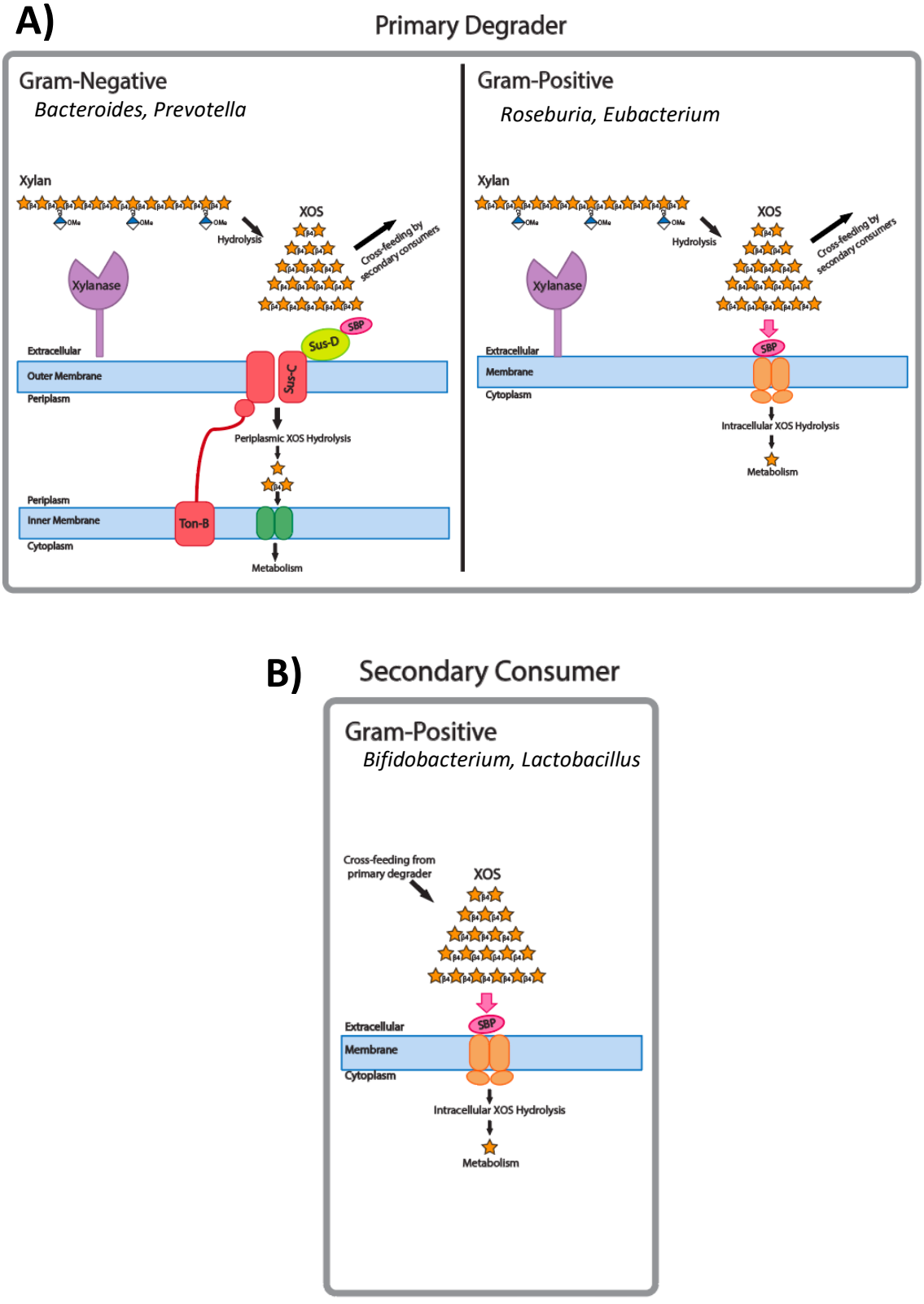
A)
Primary Degrader
Gram-Negative
Bacteroides, Prevotella
Xylan
Hydrolysis
XOS
Cross-feeding by secondary consumers
Xylanase
SBP
Sus-D
Sus-C
Extracellular
Outer Membrane
Periplasm
Periplasmic XOS Hydrolysis
Periplasm
Inner Membrane
Ton-B
Cytoplasm
Metabolism
Gram-Positive
Roseburia, Eubacterium
Xylan
Hydrolysis
XOS
Cross-feeding by secondary consumers
Xylanase
SBP
Extracellular
Membrane
Cytoplasm
Intracellular XOS Hydrolysis
Metabolism
B)
Secondary Consumer
Gram-Positive
Bifidobacterium, Lactobacillus
Cross-feeding from primary degrader
XOS
SBP
Extracellular
Membrane
Cytoplasm
Intracellular XOS Hydrolysis
Metabolism

Figure 4. Models for xylan hydrolysate utilization by members of the gut microbiome. Xylan utilization in the gut is achieved by primary degraders that cleave xylan extracellularly, and secondary consumers, that utilize the resulting XOS. There are two phyla associated with primary degradation, Bacteroidetes and Firmicutes. **(A)** Bacteroidetes, as gram-negative bacteria, use polysaccharide utilization loci (PUL) for xylan degradation and transport, which include an outer membrane-anchored extracellular xylanase, and a SusC/SusD-like transport system to identify and transport the resulting XOS into the periplasm for periplasmic XOS degradation. Xylose or xylobiose is then transported into the cytoplasm via a major facilitator superfamily transporter. While Firmicutes also utilize an anchored extracellular xylanase for primary degradation of xylan, as gram-positive bacteria, they only have one membrane, and therefore rely on specific substrate binding proteins and ATP-powered transporters and permeases. They also hydrolyze small XOS species intracellularly as opposed to periplasmically. Secondary consumers belong to the genera *Lactobacillus* (in Firmicutes) and *Bifidobacteria* (in Actinobacteria). **(B)** As gram-positive bacteria, they also rely on specific substrate binding proteins and ATP-powered transporters, but do not have an extracellular xylanase, requiring them to crossfeed from primary degraders.

Chapter 2: *Bifidobacterium pseudocatenulatum* as primary degraders of xylans and xylan-derived oligomers

Abstract

Xylans are a family of xylose-based polysaccharides found in fruits, vegetables, and cereal grains. They are resistant to digestion and reach the large intestine intact, where they are utilized by members of the gut microbiome. They are initially broken down by primary degraders that utilize extracellular xylanases to cleave xylan into smaller oligomers. These xylooligosaccharides (XOS), depending on degree of polymerization (DP) or linkages, can then either be further hydrolyzed by primary degraders or cross feed secondary consumers, including *Bifidobacterium*. While several *Bifidobacterium* species have metabolic systems for XOS, most grow poorly on long DP XOS and xylan substrates. However, we observed some bifidobacteria display growth on longer chain XOS, suggesting the XOS phenotype is strain specific. In this study, we explored the ability of five *Bifidobacterium pseudocatenulatum* strains to grow on XOS and xylan. Two distinct phenotypes were observed: a xylan^{+} phenotype, where strains grew moderately on xylan and robustly on XOS regardless of chain length, and a xylan^{-} phenotype, where strains showed no growth on xylan and moderate growth on XOS, with a preference for shorter fractions. In silico analysis found XOS-active clusters in all five strains, with the presence or absence of key genes correlating to observed phenotypes. Importantly, only xylan^{+} strains contained a putative extracellular GH10 endo-1,4-beta-xylanase, a key component for primary degradation of xylan. The presence of an extracellular xylanase was confirmed by the appearance of xylan hydrolysis products in cell-free supernatants. Extracellular xylanolytic activity was only detected in xylan^{+} strains, as indicated by the production of XOS fragments DP2-6 identified by TLC and HPLC. Furthermore, xylan^{-} strains had improved growth on xylan when combined with

supernatant from xylan$^+$ strains, indicating cross-feeding capabilities. Strains were then grown in stepwise fecal fermentations in the presence of xylan and XOS to assess persistence. The xylan$^+$ strain, ED02, persisted on both xylan and XOS in fecal culture, while the xylan$^-$ strain persisted only on XOS. These results indicate that xylan$^+$ *B. pseudocatenulatum* may have a competitive advantage in the complex environment of the gastrointestinal tract, due to their ability to act as primary degraders of xylan through extracellular enzymatic degradation.

Introduction

Xylans are considered a class of non-digestible polysaccharides that contain a xylose-based backbone with β-1,4 glycosyl linkages. Linear homoxylans are uncommon in foods, rather, they appear in the diet as heteroxylans in the form of arabinoxylans, glucuronoxylans, or glucuronoarabinoxylans (Bajpai 2014). These heteroxylans are decorated with a variety of other sugars, including arabinose and glucuronic acid, in the case of arabinoxylans and glucuronoxylans, respectively. These dietary fibers can be found predominantly in cereal grains, although they can also be found at low levels in the skins of fruits and vegetables (Rennie et al., 2014, Broxterman et al., 2018).

Xylans appear to have evolved as important substrates for fungi and bacteria in a variety of ecological biospheres, including seawater, alkaline hot springs, and the mammalian digestive tract (Zhan et al., 2020, Jia et al., 2014, Dodd et al., 2010). These microbes employ enzymatic machinery that includes a combination of xylanases and accessory enzymes that work synergistically to degrade heteroxylan. Xylanases are enzymes that cleave β-1,4 glycosyl linkages between consecutive xylose units in the xylan backbone, while accessory enzymes are enzymes that remove decorations from

substituted xylan. For substantial xylan degradation, an endo-xylanase is required to cleave the interior of the xylan backbone into xylooligomer fractions. These endo-xylanases, identified via the carbohydrate active enzyme (CAZy) database, typically are found in the glycoside hydrolase (GH) families GH5, GH10, GH11, GH30, and GH98 (Ndeh et al., 2018; Biely et al., 2016).

In humans, xylans are resistant to digestion by alimentary enzymes, and reach the large intestine intact, where they are subject to fermentation by members of the gut microbiome. These fermenters can be categorized as primary degraders and secondary consumers, based on their ability to degrade, and consume xylan. Primary degraders are capable of direct consumption of xylan; most belong to the phyla *Bacteroidetes* and *Firmicutes.* For primary degradation of xylan, *Bacteroidetes* express extracellular xylanases that are anchored to the outer membrane and near a SusC/SusD transport system. This xylanase degrades the xylan into small XOS species, which are then transported into the periplasm by the SusC/SusD receptor, where further dismantling of the oligosaccharide occurs. Similarly, primary degradation in *Firmicutes* also relies on an anchored extracellular xylanase, but these gram-positive bacteria utilize substrate binding proteins and ATP-requiring transport systems, such as ATP-binding cassettes (ABC) or PEP-phosphotransferase system (PTS) transporters to move XOS substrates directly into the cytoplasm (Ndeh and Gilbert, 2018).

Apart from these primary degraders, other organisms are known to take advantage of the xylooligosaccharide products produced by primary degraders via cross-feeding. These secondary consumers also gram-positive, like primary degraders in the phylum *Firmicutes*, but do not have the key extracellular xylanolytic activity for direct

degradation of xylan. The two major genera of secondary consumers are *Lactobacillus* and *Bifidobacterium,* which utilize highly specialized substrate recognition and transport systems for detection and transport of XOS (Ndeh and Gilbert, 2018). In vitro and in vivo studies suggested that XOS was more effective at stimulating growth of *Bifidobacteria* compared to *Lactobacillus* and is also considered far more selective than other prebiotic oligosaccharides studied (Finegold et al., 2014; Li et al., 2015; Chapla et al., 2012; Kaplan and Hutkins, 2000; Mäkeläinen et al., 2000). Even within *Bifidobacterium,* XOS utilization is highly specific, with many XOS-capable strains only able to grow on low degree of polymerization (DP) XOS fragments. Therefore, growth on longer XOS and xylan fragments is a rare trait in *Bifidobacterium*. In fact, no strain of *Bifidobacterium* has been shown to grow on the insoluble xylan backbone (Kelly et al., 2021).

Bifidobacterium pseudocatenulatum, is an adult-type bifidobacteria that is clustered in the *B. adolescentis* phylogenetic group and has been shown to ferment a diverse array of carbohydrate sources. (Lugli et al., 2018) Some strains have also been reported to have probiotic or health-promoting properties (Biagi et al., 2013; Cano et al., 2013; Peiroten et al., 2019). This species was of particular interest, as it has been isolated from in vitro and in vivo XOS enrichment studies (Kok et al., 2019; Finegold et al., 2014). Furthermore, genomic analysis of the reference genome *B. pseudocatenulatum* JCM1200 revealed the presence of three arabinoxylan hydrolysate active clusters, whose transcription was enhanced during growth on XOS (Saito et al., 2020). This study also identified substrate specificity for substrate binding proteins from each cluster, further elucidating XOS/AXOS utilization by *B. pseudocatenulatum.* However, when *B.*

pseudocatenulatum strains were grown on xylan or arabinoxylan, they did not show growth (Crittenden et al., 2002; Saito et al., 2020).

Recent research in our lab also led to identification of *B. pseudocatenulatum* strains that displayed growth on longer chain XOS and xylan, indicating a potentially novel phenotype in *Bifidobacterium*. In this study, we compared the ability of five *Bifidobacterium pseudocatenulatum* strains to grow on XOS and xylan, in order to further clarify the role of *B. pseudocatenulatum* as a consumer of xylan and xylan hydrolysates. We hypothesized that XOS and xylan phenotypes would be associated with expression of relevant enzymes as well as gene clusters encoding for these enzymes.

Materials and Methods

Isolation and identification of *Bifidobacterium* isolates. Fifteen putatively unique *Bifidobacterium* isolates from five adult subjects were used in this study. These isolates had been previously obtained (Kok et. al, 2019) from in-vitro fecal enrichment fermentations with 2% XOS (Prenexus Health, USA) and presumptively identified as *B. pseudocatenulatum* by 16S rRNA sequencing. To confirm species identity, DNA was extracted via the QIAamp DNA Mini Kit (Qiagen, USA), and qPCR was performed using *B. pseudocatenulatum*-specific primers (Junick and Blaut, 2012). One isolate from each subject was selected as the representative strain for further genomic and functional analysis. These strains were renamed as: ED01, ED02, ED03, CR16, and ED05. In addition to the *Bifidobacterium pseudocatenulatum* isolates, eleven *Bifidobacterium* strains from other species were assessed for their ability to utilize xylose-based glycans.

Growth characteristics of *Bifidobacterium* strains and isolates. To characterize the ability of *B. pseudocatenulatum* strains to utilize xylose-based glycans, growth studies

were performed in modified deMan, Rogosa, and Sharpe media (mMRS) where the glucose was replaced with various carbohydrates **(Table 1)**. Strains were first grown on

Table 1. List of substrates used in study.

Substrate	Label	Supplier	Composition
SXOS	XOS-NUSR	Prenexus Health	Short-chain XOS; enriched in DP≤4
LXOS	XOS-NUSP	Prenexus Health	Long-chain XOS; enriched in DP>4
PreneXOS	PreneXOS	Prenexus Health	Mixture of XOS and xylan
Xylan	Xylan (Beechwood)	Megazyme	Glucuronoxylan from beechwood
Arabinoxylan	AgriFiber BFG	AgriFiber	Soluble Prebiotic Corn Bran Fiber; DP17-24
Xylose	D-(+)-Xylose	Sigma	Xylose
Glucose	D-(+)-Glucose	Sigma-Aldrich	Glucose

MRS plates for 48 hrs., and single colonies were inoculated into MRS broth and incubated anaerobically at 37ºC for 24 hours in a Sheldon Bactron IV-900 anaerobic chamber (Cornelius, OR, USA). Cells were then transferred into MRS, and overnight cultures were used to inoculate mMRS containing 0.5% carbohydrate **(Table 1)**. Growth experiments were performed in quadruplicate, with a final volume of 200 uL, in 96-well microplates. Plates were incubated anaerobically, and optical density measurements (600 nm) were obtained every 20 minutes using a Tecan Sunrise microplate reader (Mannedorf, Switzerland) until stationary phase was reached.

To determine growth phenotypes on xylose-based substrates in the *Bifidobacterium* genus, a rapid, high throughput qualitative method was used. Isolates were added to mMRS containing the pH indicator bromocresol purple and xylan, LXOS, or xylose at 1% concentration. In total, 26 isolates were added to each substrate, in triplicate, in 200 uL microwell volumes. Color changes were noted over 48 hours, and a change from purple to yellow indicated strain growth on the respective substrate.

Co-cultures of xylan$^+$ and xylan$^-$ strains. To determine if growth on xylan in monoculture could predict growth in a more competitive environment, we paired a xylan$^+$ strain (ED03) and a xylan$^-$ strain (ED05), with the expectation that the xylan$^+$ strain

would out-compete the xylan$^-$ strain. This was done by equilibrating the concentrations of each strain, 1:1, based on optical density measurements at 600nm (OD_{600}). Then, each strain was inoculated, at equal cell densities, into mMRS containing 0.5% substrate. Cultures were incubated at 37^{o}C and portions were transferred at a100-fold dilution every 24 hours for 96 hours. Samples were taken every 24 hours, and DNA was subsequently extracted. Quantitative Real Time PCR was performed using strain-specific primers to quantify each strain. Results are based on relative abundance of the two strains.

DNA extraction. Genomic DNA from isolated strains and for pure culture experiments was extracted with the QIAamp DNA Mini-Kit using the manufacturer's protocol for gram-positive bacteria (Qiagen, Hilden, Germany). For fecal culture experiments, DNA was extracted using BioSprint 96 One-For-All Vet Kits (Qiagen, Hilden, Germany) according to manufacturer's protocol with modifications as described previously (Benson et al., 2014). Sample concentrations were quantified using the NanoDrop ND-1000 Spectrophotometer.

Whole genome sequencing, assembly, and annotation. Whole genome sequencing of the five selected *B. pseudocatenulatum* strains was performed by the Genomics Core Facility at Michigan State. Raw read sequences received from the Genomics Core Facility were first checked for read quality using FastQC (Andrews, 2010). The sequences were then trimmed using Sickle to remove low quality reads (Del Fabbro et al., 2013). The reads were assembled into contigs and combined to form scaffolds using SPAdes (Bankevich et al., 2012). Scaffolds were reassembled using Mauve with the reference genome *B. pseudocatenulatum* 12 retrieved from NCBI (Darling et al., 2004). The assembled genomes were annotated using PROKKA (Seemann, 2014). A summary

of features for each strain is shown in **Table 5.** Additional annotations against the carbohydrate active enzyme (CAZy) database were obtained using dbCAN2 to identify CAZy clusters (Zhang et al., 2018). The XOS-active CAZy clusters were then compared to known XOS/AXOS active clusters identified in *B. pseudocatenulatum* JCM1200 (Saito et al., 2020).

***B. pseudocatenulatum* pangenome analysis.** Pangenome analysis was performed using 86 *B. pseudocatenulatum* genomes. The genomes included the 5 strains reported in this study, and an 81 additional *B. pseudocatenulatum* genomes from the Prokaryotic RefSeq Genomes database (Li et al., 2021). Pangenomic analyses were performed with anvi-o (Delmont & Eren, 2018; Eren et al., 2015). Further annotations were performed using dbCAN2 to identify prevalence of XOS active clusters in the *B. pseudocatenulatum* pangenome (Zhang et al., 2018).

Prevalence of GH10 xylanase in *Bifidobacterium*. To determine the prevalence of GH10 xylanases in *Bifidobacterium*, 1355 non-redundant *Bifidobacterium* genomes were obtained from the Prokaryotic RefSeq Genomes database from NCBI (Li et al., 2021). These genomes were annotated with three CAZy databases, HMMER, DIAMOND, and Hotpep, using the dbCAN2 metaserver. (Wheeler &Eddy 2013; Buchfink et al., 2021; Busk et al., 2017; Zhang et al., 2018). A genome was considered to contain a GH10 if at least two CAZy databases concurred. Species level associations were then assigned.

Preparation of crude enzymatic supernatants. To determine if extracellular enzymes capable of hydrolyzing XOS or xylan were secreted, cell free supernatants were obtained from spent XOS culture. Strains were grown anaerobically at 37ºC in mMRS supplemented with 1% LXOS in 10 mL volumes for 24 hours. Cultures were centrifuged

(Eppendorf AG, Hamburg, Germany) at 3100 x g for 10 min. Supernatants were collected and re-inoculated at 1% with the same strain for an additional 24 hours in order to consume all remaining residual sugars. Cells were centrifuged a second time at 3100 x g for 10 min. and filter sterilized with a 0.22 uM filter syringe and divided into two aliquots. One aliquot was used directly (see below), and the second was heat-treated at 95^{o}C for 5 minutes to inactivate enzymes.

Identification of hydrolysis products. Basal mMRS containing 1% xylan or 1% LXOS was supplemented with 20% supernatant that was either heat treated, or not-heat treated. This media was incubated for 48 hours at 37^{o}C, with samples were taken at times 0 and 48 hours. All samples were then heat-treated, as above, after collection to halt further enzymatic activity and then stored at -20^{o}C. Identification of hydrolysis products was done using high-performance thin-layer chromatography silica gel 60 (Sigma-Aldrich, St. Louis, MO). Briefly, 8.5 uL of sample and 5 uL of XOS (15uM) standards DP1-DP6 (Megazyme, Cork, Ireland) were applied per lane. Plates were developed twice with a solvent containing 1-butanol/2-propanol/H_2O (3:12:4), dried, sprayed with 0.5% α-naphthol and 5% H_2SO_4 in ethanol.

For selected samples, analysis of xylan and XOS hydrolysis products was performed at the National Renewable Energy Laboratory (NREL) by HPLC. The HPLC system, Waters Acquity Ultra Performance LC (Waters Co., Milford, MA), was coupled with MS and an ELSD detector. The system ran with a Shodex Sugar SZ5532(Zinc) column (Showa Denko K.K., Japan) at 6 x 150 (mm), 6 µm particle size, using a ramped mobile phase of acetonitrile and water with .1% formic acid with a flow rate of 0.9 mL min^{-1} and a column temperature of 60 °C. DP fractions were identified via molecular

weight and RT matching from standards. Detector responses recorded enzymatic action observed by changes in area under the chromatogram curves from baseline to 48 hours.

Co-culture simulation of *B. pseudocatenulatum* strains. To assess if cross-feeding was occurring between strains, growth curves were performed using the enzymatic supernatants described above. Supernatants were added at 20% to fresh media containing 1% LXOS or xylan. Media was then inoculated with xylan non-fermenting strains and incubated for 48 hours. Growth was analyzed by comparing the area under the experimental growth curve between growth on supernatant-supplemented media and media alone using Growthcurver package in R and comparing the two via a two-sample t test (Sprouffske & Wagner, 2016).

***In vitro* establishment of *B. pseudocatenulatum* strains.** Fecal samples were obtained from five volunteers following UNL Institutional Board Review protocols (IRB 20160616139). Inclusion criteria included the following: at least 19 years of age, no known history of gastrointestinal diseases, no consumption of antibiotics or probiotics in the previous 6 months, and no regular consumption of yogurt. The fecal samples were then homogenized and stored in phosphate-buffered saline (PBS, pH 7.0), as previously described (Kok et al., 2019).

Sterile fermentation media was made as described in Yang and Rose (2014) with modifications. This included the addition of three substrate treatments at a 0.5% concentration: LXOS and xylan, and basal (no added sugars). In addition, there were three strain treatments: a xylan grower (ED02), a xylan non-grower (ED01), and no added strain (control). The test-of-persistence was performed in stepwise fecal fermentations as described previously (Kok et al., 2019; Gomez, unpublished). Briefly,

this involved diluting fecal slurries in PBS in a 1:10 ratio, then adding the slurry to the selected fermentation media in a 3:6 ratio (vol./vol.) in a total volume of 9.0 mL. Then, *B. pseudocatenulatum* strains ED01 and ED02, grown as previously described, were inoculated at 10^7 cfu/mL into the fecal fermentation media with respective substrate treatments. The fermentations were incubated anaerobically at 37ºC with subsequent dilutions performed in microplate format by transferring 10 uL of spent media into 990 μL fresh fermentation media every 24 hours. This process was repeated three times, with samples collected at 0 time, 24, 48, and 72 hours. The DNA was extracted from those samples to determine strain persistence by qRT-PCR. Samples were also used for 16S rRNA sequencing.

Strain-specific primer design. Strain-specific primers were designed using RUCS, 'rapid identification of PCR primers for unified sequences' (Thomsen et al., 2017). All five isolated *B. pseudocatenulatum* genomes and two complete *B. pseudocatenulatum* genomes from the NCBI genome database were used to identify unique target sequences. Primer specificity was confirmed using NCBI Primer BLAST against the NCBI RefSeq representative genome database for bacteria. No primers were accepted that gave hits for species present in human fecal samples. Primer annealing temperatures were identified using a gradient PCR program and primer specificity was confirmed by performing qPCR against all other *B. pseudocatenulatum* strains isolated.

Quantification using quantitative real-time PCR. To determine persistence of specific strains and total *Bifidobacterium* in fecal fermentations on different substrates, quantitative real-time PCR was performed using strain-specific and genus-specific primers (Table 2). Quantitative PCR was performed using these primers with the

Mastercycler Realplex2 (Eppendorf AG, Hamburg, Germany). The qPCR master mix contained 12.5 μL SYBR Green (Thermo Fisher Scientific, MA, USA), 0.5 μL forward primer, 0.5 μL reverse primer, 10.5 μL DNA grade water and 1 μL of sample, in a total volume of 25 μL per reaction. The qPCR conditions were as previously described (Junick & Blaut,2012).

Persistence of strains on respective substrates was statistically analyzed using a repeated measure mixed model previously designed in our lab (Gomez, unpublished). This model relegates subject as the random effect while prebiotic and time are fixed effects. Persistence was measured as the $\log_{10}$ difference in CFU/mL of strains between the 24- and 72-hour samples. Analysis was performed using SAS v.9.4 and p-values adjusted with Tukey adjustment.

Table 2. Bifidobacterium primers used in study.

Target Organism	Primer Direction	Primer Sequence (5' → 3')	Annealing Temp.(°C)	Source
B. pseudocatenulatum ED01	Forward	CAG CCA AGA AGA ACA CAC TGC	62 °C	This study
	Reverse	GCA TGG CAA CTG TCT TCG TTT		
B. pseudocatenulatum ED02	Forward	GCA GGT CAG AAT GTG AGA CGA	62 °C	This study
	Reverse	CGA ACC TCT GTC CAA CGA TGA		
B. pseudocatenulatum ED03	Forward	CTT GCT GGG AGA AGT CGT CTT	62 °C	This study
	Reverse	TCG ATT TGT TCC TCC GTT CGT		
B. pseudocatenulatum CR16	Forward	AAG GCT CGT GTT ATC CGG TTT	62 °C	This study
	Reverse	GCG CTT CGA AAT CCT GTG TAC		
B. pseudocatenulatum ED05	Forward	CGA TTC GAC AAC ACG TAC ACG	62 °C	This study
	Reverse	TCA GAT GTT TGT CCG CTT CGA		
B. pseudocatenulatum	Forward	AGC CAT CGT CAA GGA GCT TAT CGC AG	68 °C	Junick & Blaut, 2012
	Reverse	CAC GAC GTC CTG CTG AGA GCT CAC		
Bifidobacterium	Forward	TCG CGT CYG GTG TGA AAG	68 °C	Martinez et al., 2009
	Reverse	CCA CAT CCA GCR TCC AC		

16S rRNA amplicon sequencing and analysis. 16S rRNA sequencing was performed following procedures described previously (Kok et al., 2019). The V4 region of the 16S rRNA gene was amplified using primers described previously (Caporaso et al., 2010). Fecal fermentation samples at the 72-hr time point and the five fecal baselines were selected for analysis. Samples were sequenced on an Illumina MiSeq platform with

paired-end sequencing of 250 basepairs (bp). A total of 1,292,111 sequences were obtained, with a mean of 25,335 sequences per sample (maximum 73,706, minimum 7,061).

Sequence processing was performed using the DADA2 pipeline within QIIME2 2017.4 (Callahan et al., 2016; Bolyen et al., 2019). Raw sequence data was demultiplexed and denoised to remove chimeric sequences and trim forward and reverse reads to 220 bp and 160 bp, respectively. Amplicon sequence variants (ASVs) were grouped in each sample and taxonomy was assigned using the SILVA 16S database (Quast et al., 2013). To further refine output, samples with less than 1,500 reads and ASVs with fewer than 15 reads were removed.

Statistical analysis and visualization of community sequencing data was done in QIIME2 2017.4 and RStudio (ver. 4.0.0) (RStudio Team, 2020). Alpha diversity was measured via the Shannon Index and compared via a Wilcoxon rank sum test. Beta diversity was visualized using a principal coordinate (PCoA) plot of the Jaccard index with 95% confidence interval ellipses for the respective treatments. Changes in microbial composition from baseline to 72 hours was visualized by Log2 fold change of specific ASVs with a significance cutoff of $p<0.05$.

Results

Growth characteristics of *Bifidobacterium* strains and isolates. While all five sequenced *B. pseudocatenulatum* strains showed growth on glucose and minimal growth on basal media (OD < 0.10), differences were observed in the ability to grow on xylan and XOS. Two major phenotypes were defined among the five sequenced *B. pseudocatenulatum* strains based on their ability to grow on xylan. Strains ED02 and

ED03 were considered xylan-utilizing strains (xylan$^+$) due to their ability to grow moderately on xylan (OD > 0.30) and robustly on XOS (OD > 0.45), regardless of chain length, as typified by strain ED02 **(Fig. 1A, Fig. 1C).** Strains unable to grow on xylan (xylan$^-$) include ED01, CR16, and ED05. Despite the absence of growth on xylan (OD < 0.10), these strains had moderate growth on XOS, with a preference for SXOS enriched in low DP XOS, as shown for ED01 **(Fig. 1B, Fig. 1C).** All strains displayed robust growth on glucose and no growth on basal media. However, none of the strains indicated grew on arabinoxylan **(Fig. 1).** Strain growth phenotypes are summarized in **Figure 1C**.

In addition to the five characterized strains, a rapid colorimetric qualitative growth method was used to screen twenty-one other strains for growth phenotypes on xylan, LXOS, and xylose. These included ten additional *B. pseudocatenulatum* strains and eleven strains from other *Bifidobacterium* species. The *B. pseudocatenulatum* isolates showed similar phenotypes to the previously described xylan$^+$ and xylan$^-$ phenotypes **(Table 3).** However, none of the other species of *Bifidobacterium* grew on xylan, and only three of the eleven collection strains grew well on LXOS **(Table 4).**

Xylan$^+$ strains do not always outcompete xylan$^-$ strains in co-culture. When xylan$^+$ and xylan$^-$ strains *B. pseudocatenulatum* strains were grown in co-culture in a 1:1 ratio, varying results were observed, depending on substrate. While we hypothesized that xylan$^+$ strains would outcompete xylan$^-$ strains during growth on XOS and xylan, these phenotypes were not always predictive. Thus, while the xylan$^+$ strain ED03 outcompeted ED05 on SXOS **(Fig. 2A),** strain ED05 outcompeted strain ED03 on LXOS. **(Fig. 2B).**

Genomic evidence for the presence of GH10 xylanases in *Bifidobacterium*. In silico detection of carbohydrate active gene clusters (CGC's) in the sequenced *B.*

pseudocatenulatum genomes revealed the presence of three major clusters for XOS utilization. These three clusters contain substrate binding proteins, transporters and key glycosyl hydrolases. They are distinguished on the basis of substrate binding specificity for size and backbone substitution. As hypothesized, the presence or absence of key glycosyl hydrolases and transport systems also aligned with the observed phenotypes of our strains. All five strains contain genes associated with XOS-active cluster III. This cluster includes a substrate binding protein with substrate specificity to short XOS products, along with two ABC transporter permeases. In addition, this cluster includes two GH43 glycosyl hydrolases associated with monomer (either xylose or arabinose) production from XOS. Furthermore, cluster III also contains a transcriptional regulator and sugar metabolism genes **(Fig. 3, 4).**

Conversely, strains ED01 and CR16 are missing portions of XOS active cluster I. This includes the substrate binding protein, BpAXBP1, with substrate specificity for longer XOS. These xylan$^-$ strains are also missing the ABC transporter permeases and ATP-binding proteins associated with this substrate binding protein. They also are missing the GH43 and GH120 glycoside hydrolases with hypothesized xylosidase activity **(Fig. 3, 4).** Cluster I is completely present in strains ED02 and ED03.

Xylan$^+$ strains ED02 and ED03 are missing a majority of cluster II, while xylan$^-$ strains ED01 and CR16 contain the entire cluster. ED02 and ED03 are missing the substrate binding protein BpAXBP2, with specificity for arabinose substituted XOS fractions. Additionally, these xylan$^+$ strains are also missing associated transport permeases, and xylose-releasing glycosyl hydrolases. However, they still contain the GH8 reducing end xylose-releasing exo-oligoxylanase associated with cleaving xylose

from xylan and XOS. Moreover, the ED02 and ED03 genomes contain an alternate cluster II centered around the GH8 xylanase that includes a putative extracellular GH10 endo-β-1,4- xylanase. Strain ED05 is missing all genes in cluster II **(Fig. 3, 4).**

Pangenome analysis of 86 *B. pseudocatenulatum* genomes, including the 5 genomes from this study, indicated the presence of 5620 genes contributing to 1227 core genes, 2965 shell genes, and 1428 cloud genes. Among the 86 genomes, 19 contained a GH10, including the xylan$^+$ strains, ED02 and ED03 **(Fig. 5A).** Results indicated that some XOS-active gene clusters were more prevalent in the pangenome. For example, Cluster III, in its entirety, was present in all strains. Cluster I was the second most prevalent, with 64 of 86 genomes containing the complete cluster and all 86 genomes containing at least some genes in Cluster I **(Fig. 5A)**. Cluster II was the rarest, as it was complete in 33 of the 86 strains, and only 50 of the 86 genomes contained portions of Cluster II **(Fig. 5A).** All strains with an incomplete Cluster II contained the GH8 reducing-end xylose-releasing exo-oligoxylanase gene. Furthermore, many of these partial cluster II genomes were present in the GH10-containing genomes, that contain the consecutive GH8 and GH10 xylanases in alternate cluster II found in ED02 and ED03 **(Fig. 5B).**

Screening for GH10 xylanases in the *Bifidobacterium* genus using NCBI reference sequences revealed that a GH10 xylanase was present in only 29 of 1355 non-redundant *Bifidobacterium* genomes, or 2.14% of genomes. Of these twenty-nine strains, seventeen were classified as *B. pseudocatenulatum,* four belonged to *Bifidobacterium catenulatum* sp., three belonged to *Bifidobacterium pullorum,* and two genomes were

identified as *Bifidobacterium adolescentis*, and singular genomes of *B. reuteri, B. animalis* ssp. *lactis*, and an unknown *Bifidobacterium* genome **(Fig. 6).**

Hydrolysis products from xylan$^+$ strain supernatants display biochemical evidence of xylan$^+$ phenotype and offer cross-feeding opportunity. Both *in vitro* growth experiments and genome analysis indicated that the xylan$^+$ phenotype was dependent on an extracellular xylanolytic system. To confirm the presence of an extracellular xylanase, cell-free supernatants were collected as a putative source of this enzyme. When LXOS media was supplemented with supernatants from all five representative strains, hydrolysis products were only observed in media supplemented with ED02 or ED03 supernatant **(Fig. 7A)**. This same activity was also observed on xylan, where only the xylan media inoculated with the xylan$^+$ supernatant, ED02, resulted in xylan hydrolysis products. When the supernatant was heat-treated, no hydrolysis products were formed, indicating hydrolysis was due to the presence of extracellular xylanases **(Fig. 7B).**

The presence of an ED02 xylanase and its activity on XOS and xylan were further demonstrated by HPLC analysis of hydrolysis products. Results indicated an increase in concentration of DP2-4 XOS products and decrease in DP5-13 XOS fragments from LXOS, and the production of DP2-DP6 products from xylan (**Fig. 8).**

Finally, cell-free supernatant from ED02 was added to fresh media that was then inoculated with the xylan$^-$ strains, ED01. Compared to their growth on xylan and XOS alone, ED01 was able to grow on XOS and xylan in the presence of the ED02 xylanase **(Fig. 7C).**

Persistence of strains in fecal culture is consistent with phenotypes in pure culture. Persistence of strains during stepwise fecal fermentations containing five different donor

sample was generally consistent with their xylan and XOS phenotypes. The xylan$^+$ strain, ED02, persisted on XOS in all five subjects and was displaced during fermentation on basal media **(Fig. 9D, 9F).** On xylan, ED02 showed persistence in four subjects and an apparent washout for subject A35 **(Fig. 9B).** However, even including this data point from the subject on xylan, no significant difference was observed between XOS and xylan for cell count between 24 and 72 hours, while the change in both XOS and xylan were significantly different from basal media **(Fig. 9H).**

The xylan$^-$ strain, ED01 persisted on XOS for all five subjects and was displaced on xylan and basal media for all five subjects **(Fig. 9A, 9C, 9E).** All treatments were significantly different from each other, although both xylan and basal media had more than a 2-log reduction, confirming that this xylan$^-$ strain could not persist on xylan **(Fig. 9G).**

16S rRNA Analysis. Fecal fermentation samples from the 72-hour time point and from the five fecal baseline samples were sequenced via 16S rRNA amplicon sequencing. All strain treatment samples, including control treatment, saw a significant decrease in alpha diversity by Shannon Index from the fecal baseline with p-values less than 0.001 **(Fig. 11A).** Similarly, all substrate treatments were significantly different from the fecal baseline in alpha diversity by Shannon Index. Additionally, the XOS treatment was significantly less diverse than the basal treatment. ($p<0.01$**) (Fig. 11B).** PCoA analysis of Jaccard beta-diversity indicated clustering of substrate and strain treatments from the fecal baseline. No clustering was seen based on strain treatment, but there was clustering seen based on substrate treatment, where control samples were significantly different from XOS samples **(Fig 11C).**

Bifidobacterium was detected in all five fecal baseline samples and was present at greater than 5% relative abundance in subjects A33 and A35, initially. Three *Bifidobacterium*-associated ASV's were detected from the samples, with one associated with *B. pseudocatenulatum*.

Basal media, with no added carbohydrates, was used as a substrate control for this study, and the relative abundance of *Bifidobacterium* decreased for ED01, ED02, and control treatments. The basal control media was used to identify potential method effects. Based on log2fold change analysis comparing basal control fermentation to fecal baseline data, a method effect was suggested. Specifically, this method effect was observed as a consistent increase in various 'fast growing' taxa, including ASV's from the genera, *Escherichia-Shigella, Enterococcus, Streptococcus* and *Fusobacterium* **(Fig. 12A).** Other method effects include significant decreases in other fiber-associated bacteria, including *Roseburia, Akkermansia,* and *Prevotella* **(Fig. 12A).**

In contrast to the decrease in *Bifidobacterium* during fecal fermentations in the absence of carbohydrates, *Bifidobacterium* was highly enriched with XOS supplementation. In the absence of an inoculated strain, but with the addition of XOS, subjects A33 and A35, who initially had high bifidobacteria abundance had increases in *Bifidobacterium* relative abundance. Strains ED01 and ED02 also increased in relative abundance for all subjects **(Fig. 10B).** Statistically, this was confirmed as the strain-associated ASV increases significantly in both ED01 and ED02 supplemented XOS media, with a 7.7-fold increase and 9.6-fold increase, respectively **(Fig. 12B, 12D).**

On xylan, relative abundance of *Bifidobacterium* for all five subjects in both the control treatment and ED01 treatment decreased by the 72-hour time point **(Fig. 10A).**

This was statistically significant, as the two non-*pseudocatenulatum* strain associated ASV's decreased by 21.2-fold and 40.5-fold, respectively **(Fig. 12C).** In the ED02 treatment, there was an increase in relative abundance in subjects A31, A33, A34, and B39, but a decrease in subject A35. Statistically, based on Log2Fold changes, there was no significant change in the *B. pseudocatenulatum*-associated ASV, while there was a significant reduction in one of the baseline-associated ASV's, with a 21.7-fold reduction **(Fig. 12E).**

Discussion

The importance of xylans as dietary substrates for gut microbes has led researchers to identify and characterize strains having the biochemical and genetic means to metabolize these carbohydrates. In this study, we propose two major xylan phenotypes exist in the species *B. pseudocatenulatum.* Strains having a xylan$^+$ phenotype, represented by strain ED02, were further considered as primary degraders, as illustrated in **Fig 13A.** These strains rely on extracellular degradation of xylan by a GH10 xylanase, forming XOS fractions of varying DPs. For *B. pseudocatenulatum* ED02, xylanase products were mainly DP2-DP6, as identified by both TLC and HPLC. This is consistent with the activity of GH10 xylanases described previously, that typically produce` DP2-DP6 products from xylan (Liu et al., 2018; Falck et al., 2013; Boonchuay et al., 2016). Growth of ED02 directly on xylan indicated that this strain could also consume these oligosaccharides. Genome analysis of the xylan$^+$ strains revealed that they contain Cluster I and Cluster III transport systems, whose substrate binding affinities are associated with long XOS (> DP4) and short XOS ($\leq$ DP4), respectively (Saito et al., 2020). Other

glycoside hydrolases are also present in the XOS-active clusters that degrade the intracellular XOS into xylose, which enters the bifido shunt for subsequent metabolism.

In contrast to ED02, which was a primary degrader as well as consumer of xylan, other strains, such as ED01, had a xylan$^-$ phenotype, but could still grow on XOS. These strains were considered as secondary consumers. Thus, in a complex community where xylan is hydrolyzed by primary degraders, secondary consumers can still benefit through cross-feeding **(Fig. 13B)**. Experimentally, this was demonstrated by the improved growth of strain ED01 on LXOS and xylan in the presence of an extracellular xylanase produced by ED02. As described above for ED02, xylan$^-$ strains likely employ the same XOS transport systems, encoded in cluster III. The cluster III system is highly conserved in *Bifidobacterium,* as homologs of this cluster have also been found in *B. adolescentis, B. catenulatum,* and *B. longum* (Ojima et al., 2022; Liu et al., 2021).

We observed that all sequenced strains examined in this study grew better on XOS than on xylose. However, this was not entirely surprising, as *Bifidobacterium* are well-known to have adapted to the gut environment, where simple sugars are absent and oligosaccharides, including XOS, are more abundant. For example, *B. adolescentis* was reported to have higher specific growth rates on fructooligosaccharides than on their constitutive monomer, fructose (Amaretti et al., 2006). Likewise, a strain of *Bifidobacterium longum* subsp. *lactis* was also shown to grow better on human milk oligosaccharides than on glucose (Ward et al., 2006). These findings strongly suggest that the ability to grow on oligosaccharides is largely dependent on the presence of relevant transporters and associated transport machinery. Moreover, *Bifidobacterium* are known to have a large number of carbohydrate active enzymes, consistent with their ability to adapt

to the GI tract, where oligo- and polysaccharide components are more likely to be found (Pokusaeva et al, 2011; O'Callaghan & van Sinderen, 2016).

Substantial xylan utilization via xylan backbone degradation by *Bifidobacterium* has not been previously reported (Kelly et al., 2021). Growth on arabinoxylan has been identified in some strains of *B. adolescentis* and *B. longum*, but this growth appears to require accessory enzymes that cleave arabinose from the xylan backbone. (Lagaert et al., 2010; Riviere et al., 2018; Crittenden et al., 2002). In our study, none of the strains showed significant growth on arabinoxylan, which is consistent with previous studies of *B. pseudocatenulatum.* (Saito et al., 2020; Crittenden et al., 2002).

Interestingly, strains capable of growing on xylan and LXOS neither contained the cluster II substrate binding protein, *bp*AXBP2, nor did they contain the associated permeases. However, *bp*AXBP2 prefers highly substituted AXOS species, as previously suggested (Saito et al., 2020). As the XOS and xylan used for our study were not substituted with arabinose, this transport system was not necessary for growth on xylan. Furthermore, one recent study suggested that the associated substrate binding protein in cluster II was the least prevalent, as only 34% of strains contained *bp*AXBP2, while Cluster I SBP and Cluster III SBP were found in 62% and 92% of *B. pseudocatenulatum* strains surveyed. (Ojima et al., 2022).

Results from our qualitative, rapid screening of xylan and XOS growth by *Bifidobacterium* strains indicated that no other species grew on xylan. While the strains tested were biased towards species in our lab collection, these findings are consistent with more expansive screening studies of bifidobacteria (Crociani et al., 1994; Crittenden et al., 2002).

Our study is not the first report of a GH10 xylanase in *B. pseudocatenulatum.* Another recent study identified the alternate XOS cluster containing GH8 and GH10 xylanases present in 9 of 30 strains of *B. pseudocatenulatum* (Liu et al., 2021). In this latter study, which contained a total of 217 strains of *Bifidobacterium* from six human-origin species, the GH10 xylanase was found only in *B. pseudocatenulatum.* This mostly agrees with the results of our study, while also confirming the rarity of GH10 xylanases in *Bifidobacterium,* as reported in previous studies (Turroni et al., 2018). However, our analyses also identified other GH10 xylan-containing species, including *B. catenulatum, B. adolescentis* and *B. pullorum, B. animalis* ssp. *lactic, and B. reuteri. B. pullorum* is associated with chickens and *B. reuteri* is from marmosets, and therefore are not considered human-residential strains. In contrast, *B. catenulatum* and *B. adolescentis,* along with *B. pseudocatenulatum,* belong to the *B. adolescentis* phylogenetic group that are considered adult-type bifidobacteria (Kato et al., 2016). Thus, their ability to utilize dietary fiber is consistent with the presence of a GH10 xylanase (Lugli et al., 2014). Our pangenome analysis also revealed similar results to previous findings in *B. pseudocatenulatum,* with the detection and varied presence of three XOS-active gene clusters (Saito et al., 2021). However, our study is the first to biochemically define a xylan$^+$ phenotype in *B. pseudocatenulatum* that also correlates with the presence of a GH10 xylanase.

The ability of *B. pseudocatenulatum* ED02 to grow on xylan or LXOS might be predicted to confer a competitive advantage in mixed cultures or complex environments containing XOS as the carbohydrate source. However, when xylan$^+$ and xylan$^-$ strains were grown in co-culture in LXOS medium, the latter strain was able to out-compete the

xylan$^+$ strain. This observation demonstrated that cross-feeding, even between members of the same species, had occurred. It is also interesting to speculate that the *B. pseudocatenulatum* group may have evolved as a community, acting in concert to degrade and consume xylan-rich substrates and hydrolysis products. This possibility was further supported by the *in vitro* stepwise fermentations. Although ED01 failed to persist with xylan as a substrate, this strain persisted on XOS, suggesting that as long as primary xylan degraders are present and producing free XOS, XOS consumers could have sufficient substrate to grow and persist.

Finally, in the *in vitro* test-of-persistence fermentations, we also observed one of the donor samples failed to support growth of the test strain. Specifically, strain ED02 persisted in four of the donor samples during stepwise fermentation on xylan (based on both qPCR and 16S rRNA analyses) but was displaced in sample A35. Thus, A35 would be considered a non-responder to this synbiotic pairing, due to potential XOS/xylan - consuming competitors present in this sample.

Collectively, this research contributes to understanding the role of *B. pseudocatenulatum in* the degradation and utilization of xylan and xylan-derived oligosaccharides. To our knowledge, this is the first study that has described biochemical evidence for a GH10 xylanase for primary degradation of linear xylan by human-origin *Bifidobacteria*. Further in vitro and in vivo studies are needed to examine the role of xylan$^+$ *Bifidobacterium* in the gut on xylan.

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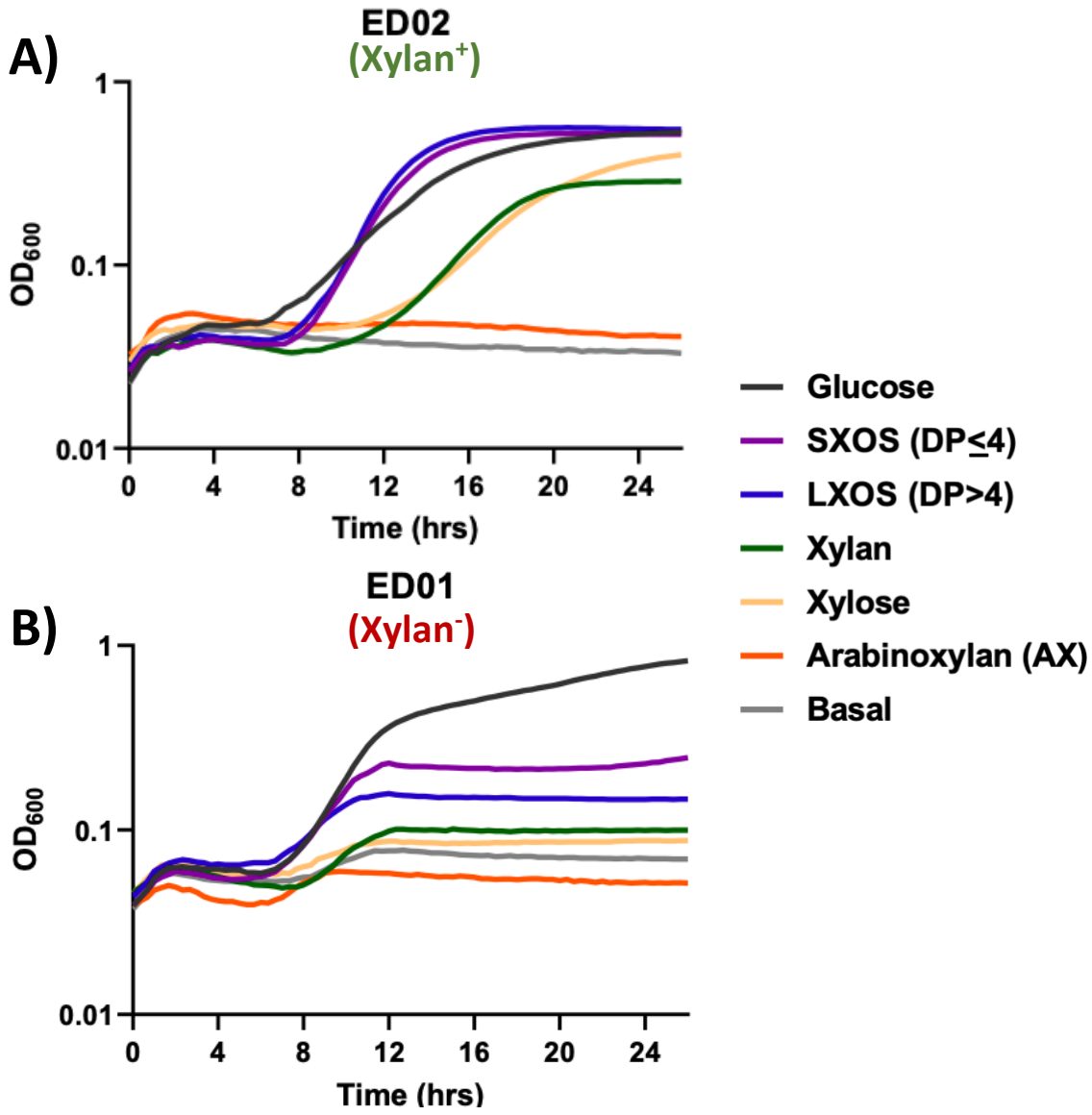
Figure 1



C)

	Strains				
Substrate	ED01	ED02	ED03	CR16	ED05
Glucose	+++*	+++	+++	+++	+++
Xylose	-	++	-	-	-
Xylan	-	++	++	-	-
Long XOS (DP>4)	+	+++	+++	+	+
Short XOS (DP≤4)	+	+++	+++	+	+
Arabinoxylan (AX)	-	-	-	-	-
Basal	-	-	-	-	-

Figure 1. Growth phenotypes of five *B. pseudocatenulatum* strains. Growth of *Bifidobacterium pseudocatenulatum* xylan$^+$ strain ED02 **(A)** and xylan$^-$ strain ED01 **(B)** on various xylose-based carbohydrates and growth phenotypes of all five *B. pseudocatenulatum* strains studied **(C).** Cells were grown in modified deMan, Rogosa, and Sharpe (mMRS) media supplemented with 0.5% carbohydrate. Basal media, with no added carbohydrate, was used as a negative control and glucose was used as a positive control. Growth phenotypes were classified based on change in optical density at 600nm (ΔOD_{600}), where (-) indicates no growth with $\Delta OD_{600} < 0.1$; (+) indicates weak growth with $0.1 < \Delta OD_{600} < 0.3$; (++) displays moderate growth with ($0.3<\Delta OD_{600}<0.45$); (+++) displays robust growth with $\Delta OD_{600}>0.45$.

Table 3. Summary of growth phenotypes of fifteen unique Bifidobacterium pseudocatenulatum strains and detection of GH10 xylanase gene via qPCR.

	Substrate			qPCR Identification
Isolate	**Xylan**	**XOS**	**Xylose**	**GH10**
A1-2	-	w	+	N
A1-3	-	w	-	N
A1-5	-	w	-	N
A1-7	-	w	+	N
ED01 (A1-10)	-	w	-	N
ED02 (A4-10)	+	+	+	Y
ED03 (A5-3)	+	+	-	Y
A7-4	-	w	+	N
CR16 (A7-5)	-	w	-	N
A7-6	-	w	+	N
A8-2	+	+	+	Y
A8-3	-	w	+	N
A8-4	+	+	+	Y
A8-7	+	+	+	Y
ED05 (A8-10)	-	w	-	N

- = no growth; purple; pH > 6.2
w = weak growth; transitional color; 5.2<pH<6.2
+ = growth; yellow; pH<5.2
N = Gene not detected via qPCR
Y= Gene detected via qPCR

Table 4. Summary of Bifidobacterium collection strain phenotypes on xylose-based glycans from bromocresol purple assay.

	Substrate		
Strain	**Xylan**	**XOS**	**Xylose**
Bifidobacterium longum ssp. infantis ATCC 17930	-	-	+
Bifidobacterium longum ssp. longum ATCC 15707	-	-	+
Bifidobacterium longum ssp. longum DJ010A	-	-	+
Bifidobacterium longum ssp. longum AH1206	-	-	+
Bifidobacterium longum ssp. longum UCD VEN 272	-	-	-
Bifidobacterium longum ssp. longum JDM301	-	w	+
Bifidobacterium longum ssp. suis	-	+	+
Bifidobacterium adolescentis ATCC 15703	-	w	+
Bifidobacterium adolescentis IVS-1	-	-	-
Bifidobacterium animalis ssp. lactis BB-12	-	+	+
Bifidobacterium pullorum	-	+	+

- = no growth; purple; pH > 6.2
w = weak growth; transitional color; 5.2<pH<6.2
+ = growth; yellow; pH<5.2

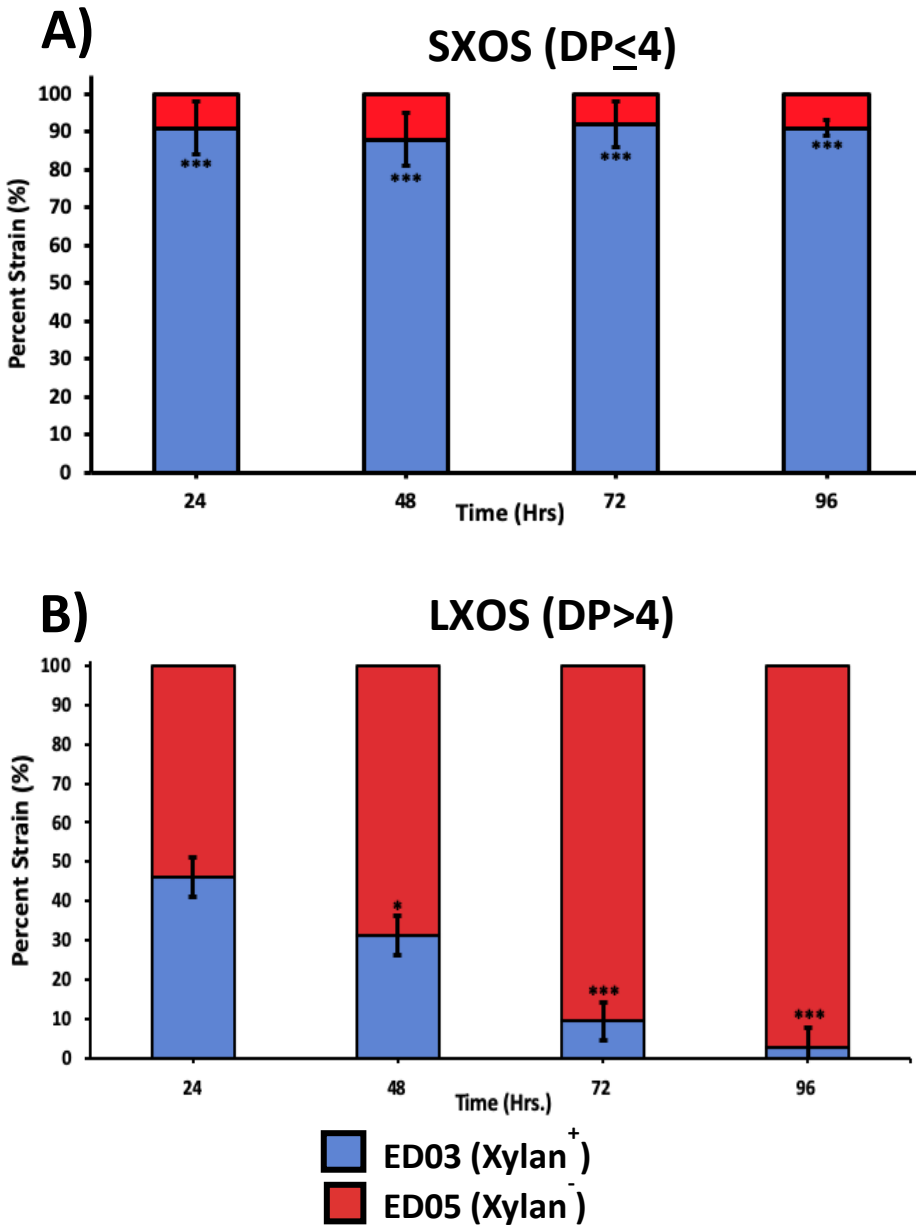


Figure 2. Co-culture competition results. Relative abundance of xylan^{+} strain, ED03 (blue), and xylan^{-} strain, ED05 (red), on XOS of varying chain lengths during co-culture competitions. Samples were diluted every 24 hours into fresh media for each time point. SXOS is shorter chain XOS, enriched in majority DP<4 and LXOS is long-chain XOS, enriched in majority DP>4. A two sample-t test was performed to determine if strain relative abundances were significantly different from each other, and significance is indicated by asterisks. (* = $p < 0.05$; **= $p<0.01$; ***= $p<0.001$)

	note	annotation	JCM1200	ED01	CR16	ED05	ED02	ED03
Cluster I	BpAXBP1	ABC transporter substrate-binding protein	■			■	■	■
		ABC transporter permease	■			■	■	■
		ABC transporter permease	■			■	■	■
		ABC transporter ATP-binding protein	■			■	■	■
		ABC transporter ATP-binding protein	■			■	■	■
	GH43_11	Beta-xylosidase	■			■	■	■
	GH120	hypothetical protein	■	■	■	■	■	■
	GH8	Reducing end xylose-releasing exo-oligoxylanase	■	■	■	■	■	■
	GH43_10	Beta-xylosidase	■	■	■	■	■	■
	CE10	Para-nitrobenzyl esterase	■	■	■	■	■	■
Cluster II	BpAXBP2	carbohydrate ABC transporter substrate-binding protein	■	■	■			
		sugar ABC transporter permease	■	■	■			
		carbohydrate ABC transporter permease	■	■	■			
	GH120	right-handed parallel beta-helix repeat-containing protein	■	■	■			
	GH43_11	glycoside hydrolase family 43 protein	■	■	■			
		hypothetical protein	■	■	■		■	■
	GH8	Reducing end xylose-releasing exo-oligoxylanase	■	■	■		■	■
Cluster III		xylose isomerase	■	■	■	■	■	■
	GH43_12	glycoside hydrolase family 43 protein	■	■	■	■	■	■
		LacI family transcriptional regulator	■	■	■	■	■	■
	BpAXBP3	extracellular solute-binding protein	■	■	■	■	■	■
		sugar ABC transporter permease	■	■	■	■	■	■
		carbohydrate ABC transporter permease	■	■	■	■	■	■
	GH43_11	glycoside hydrolase family 43 protein	■	■	■	■	■	■
		9-O-acetylesterase	■	■	■	■	■	■
		sugar O-acetyltransferase	■	■	■	■	■	■
		xylulokinase	■	■	■	■	■	■

Figure 3. Genomic evidence of xylan$^+$ phenotype. The presence or absence of three XOS-active gene clusters in reference genome JCM1200 and five representative *B. pseudocatenulatum* strains, as defined previously by Saito et al., 2020 are shown. Gene presence is indicated by a filled square, and absence by an open square. Map is color-coded by gene cluster, with pink for cluster I, orange for cluster II, and green for cluster III.

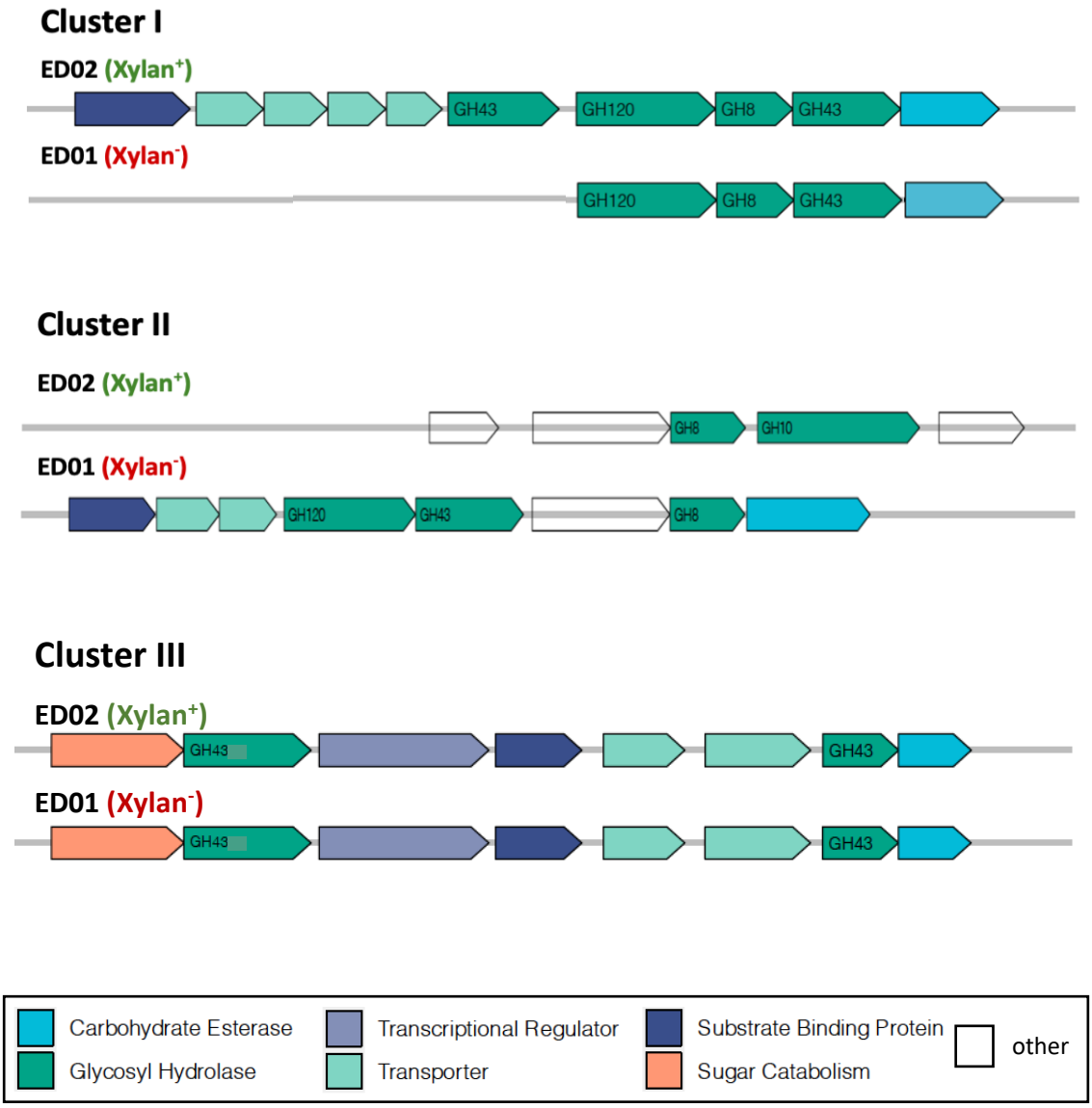


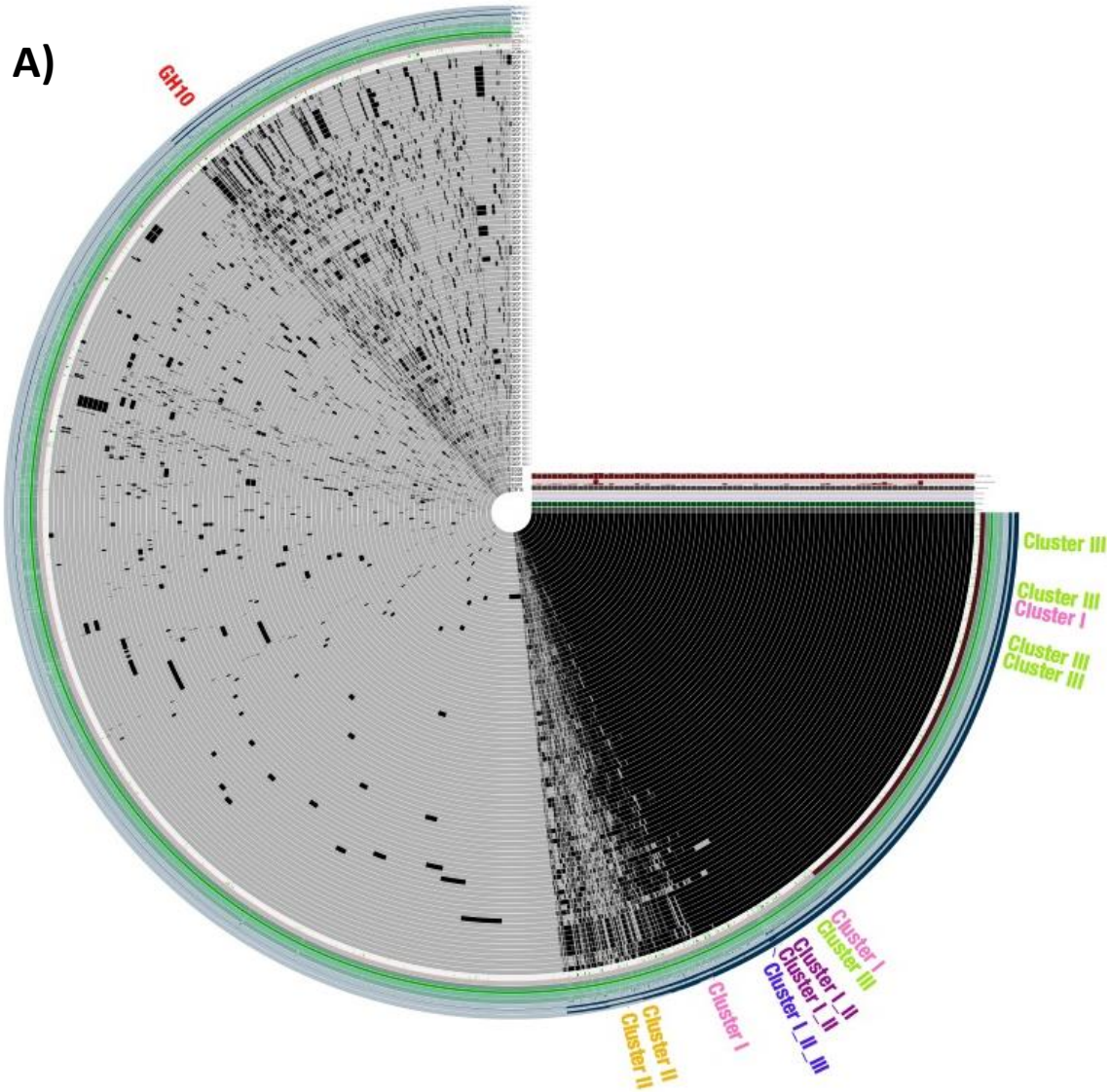
Figure 4. XOS-active gene clusters differ between xylan$^+$ and xylan- phenotypes. Genes encoding for XOS-active clusters I, II and III are aligned according to their phenotype, color-coded for relevant carbohydrate-active function.

Table 5. Complete genome features of the five Bifidobacterium pseudocatenulatum strains used in this study.

Strain	Contigs	Genome length (bp)	Number of Predicted Genes	tRNA	GC content(%)
ED01	44	2,222,613	1825	55	56.46
ED02	25	2,202,781	1836	54	56.6
ED03	42	2,219,299	1849	55	56.53
CR16	27	2,314,087	1935	55	56.37
ED05	35	2,267,139	1898	57	56.61

Figure 5

A)



B)

	note	annotation	ED02	ED03	CA-D29	TF07-45	TF07-23	TF05-2AC	TF05-19AC	OM05-2	OF05-12	AF02-36-1	AM43-10	AM08-25	AM08-2	LH_14	1001287H_170206_E3	J1101437_171009_A10	MGYG-HGUT-03683	LH_13	100112758_160808_D7
			GH10-containing genomes																		
Cluster II	BpAXBP2	carbohydrate ABC transporter substrate-binding protein																			
		sugar ABC transporter permease																			
		carbohydrate ABC transporter permease																			
	GH120	right-handed parallel beta-helix repeat-containing protein																			
	GH43_11	glycoside hydrolase family 43 protein																			
		hypothetical protein																			
	GH8	Reducing end xylose-releasing exo-oligoxylanase																			

Figure 5. Pangenome results for eighty-six strains of *B. pseudocatenulatum*.

Assembled pangenome for eighty-six strains of *B. pseudocatenulatum,* ordered by presence-absence in genomes. **(A)** The circle phylogram is annotated for GH10 xylanase and XOS-active clusters, color-coordinated by their respective gene cluster (cluster I in pink, Cluster II in orange, and cluster III in green). Two genes, belonging to CAZy families GH8 and GH120, are present in both clusters I and II and are identified in purple as Cluster I_II. One gene, a GH43_11 xylosidase, is present in all three clusters and is denoted in blue, with the name Cluster I_II_III. Presence of genes in cluster II is further shown for genomes containing GH10. **(B)**

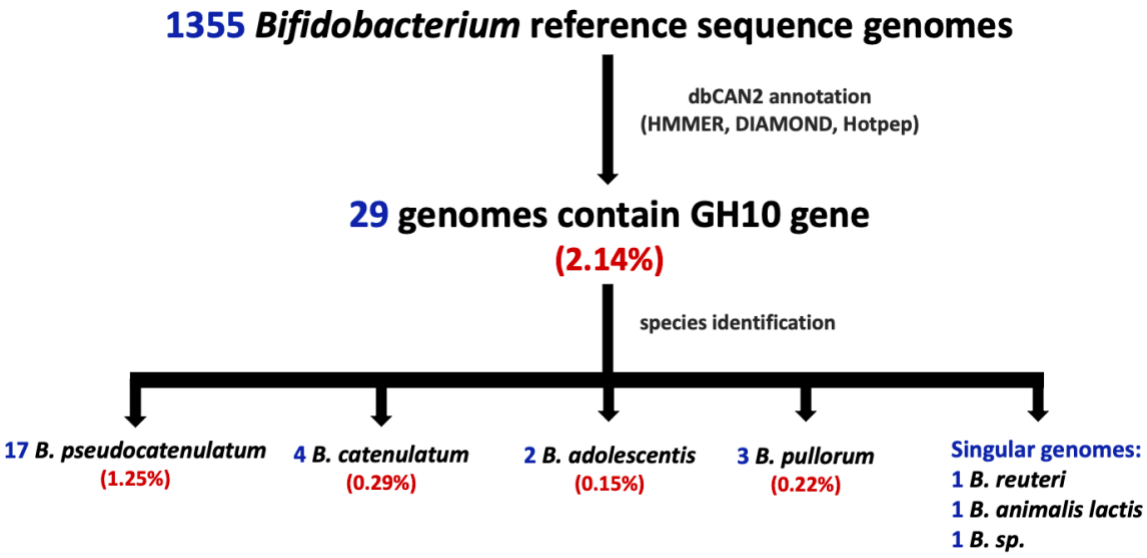


Figure 6. Prevalence of GH10 gene in *Bifidobacterium* genus. 1355 *Bifidobacterium* genomes obtained from NCBI's refseq database were screened for GH10 presence using dbCAN2, with 29 genomes identified to contain at least one GH10 gene. The species for these genomes include *B. pseudocatenulatum, B. catenulatum, B. adolescentis,* and *B. pullorum, B. reuteri, B. animalis ssp. lactis, and B. sp*.

Figure 7

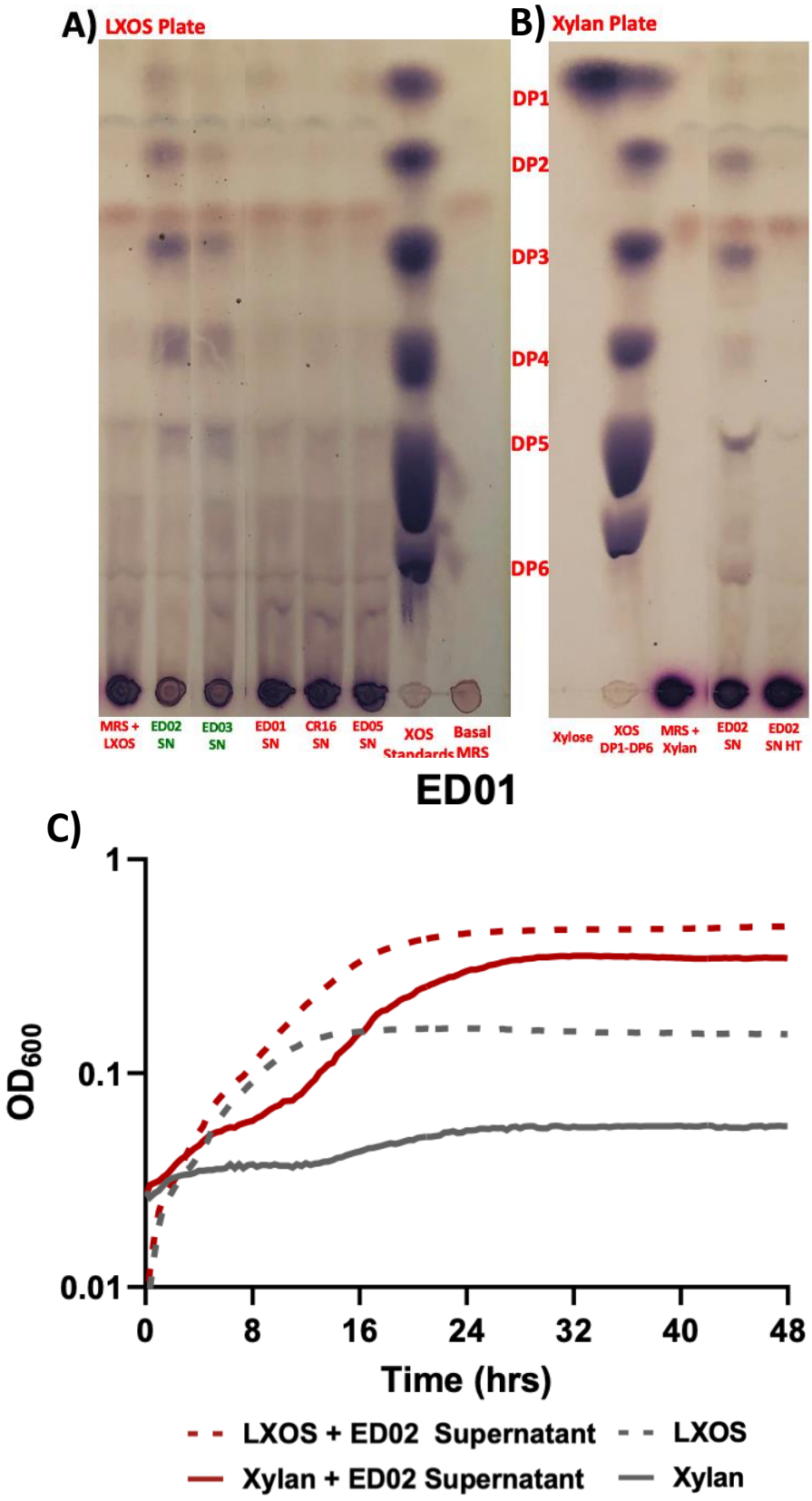
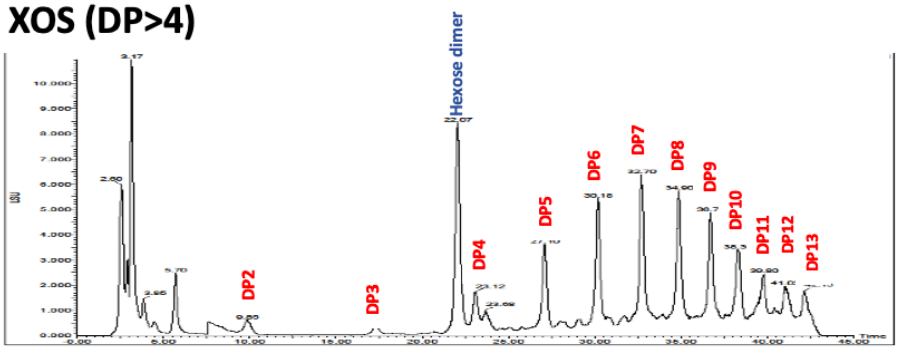

A) LXOS Plate
B) Xylan Plate
DP1
DP2
DP3
DP4
DP5
DP6
MRS + LXOS
ED02 SN
ED03 SN
ED01 SN
CR16 SN
ED05 SN
XOS Standards
Basal MRS
Xylose
XOS DP1-DP6
MRS + Xylan
ED02 SN
ED02 SN HT
ED01
C)
OD600
1
0.1
0.01
0
8
16
24
32
40
48
Time (hrs)
LXOS + ED02 Supernatant
LXOS
Xylan + ED02 Supernatant
Xylan

Figure 7. **Extracellular xylanase activity improves growth of xylan$^-$ strains.** Thin-layer chromatography (TLC) results of supernatant inoculated media containing either: **(A)** LXOS (lane 1: LXOS media, lanes 2-6: LXOS media inoculated with respective strain supernatant, lane 7: 15mM XOS standards DP1-DP6, Lane 7: Basal media) or **(B)** Xylan (lane 1: xylose, lane 2: 15mM XOS standards DP1-DP6, lane 3: xylan media, lane 4: xylan media inoculated with ED02 supernatant, lane 5: xylan media inoculated with heat-treated ED02 supernatant. Growth curve results from xylan$^-$ strains grown in the presence or absence of ED02 supernatant-inoculated LXOS and xylan. Growth curves in grey indicate growth on substrate alone, while curves in red indicate growth together with ED02 supernatant Substrate is indicated by the line pattern, with the dashed line indicating LXOS and complete line indicating xylan. **(C)**

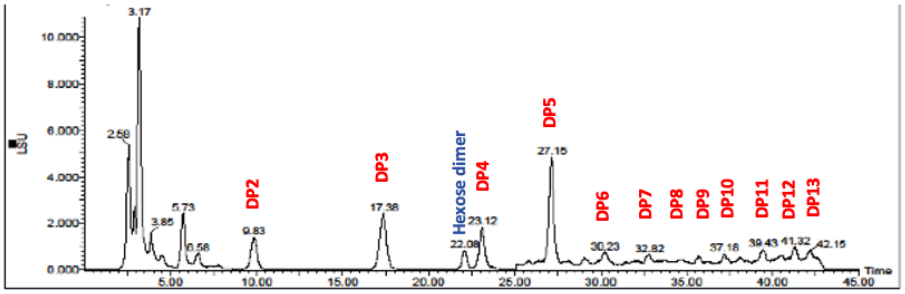
Figure 8

A)

XOS (DP>4)



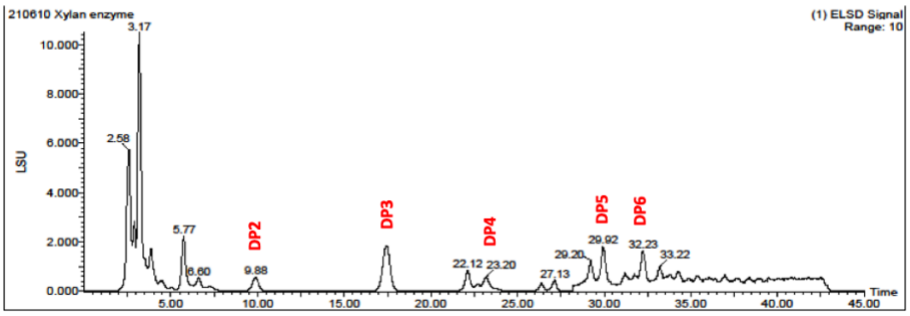
XOS (DP>4)+ ED02 Supernatant



	Trend
DP2	↑
DP3	↑
DP4	↑
DP5	↓
DP6	↓
DP7	↓
DP8	↓
DP9	↓
DP10	↓
DP11	↓
DP12	↓
DP13	↓

B)

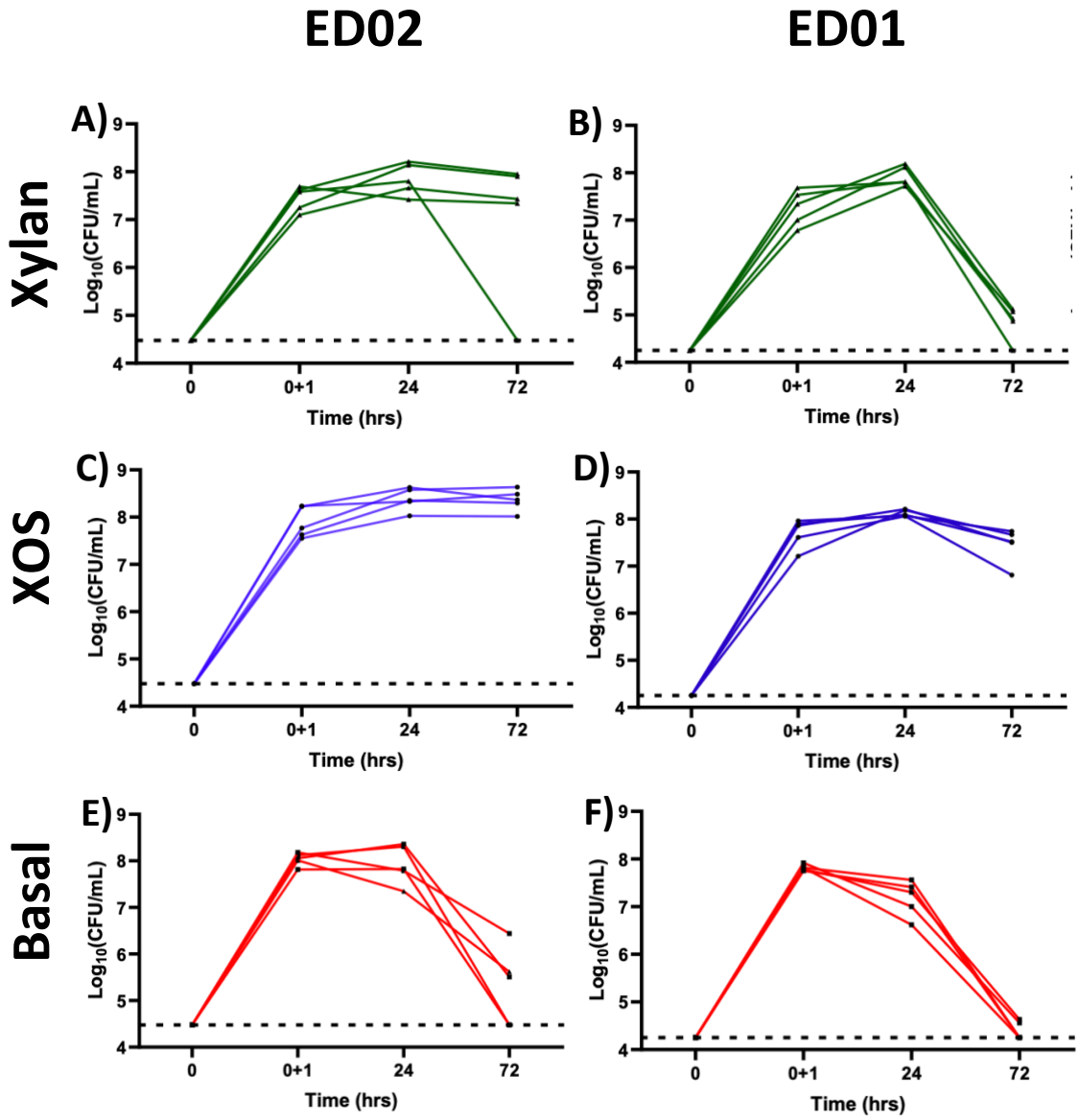
Xylan + ED02 Supernatant



	Trend
DP2	↑
DP3	↑
DP4	↑
DP5	↑
DP6	↑
DP7	-
DP8	-
DP9	-
DP10	-
DP11	-
DP12	-
DP13	-

Figure 8. High-performance liquid chromatography indicates endo-xylanase activity by ED02. Degradation products by ED02 supernatants after 48 h incubation with XOS (A) and xylan (B) were separated and quantified by HPLC. In the XOS media, DP2-4 increased, and DP5-13 decreased. In contrast, on xylan, an increase in DP2-6 xylooligomers was observed. Trends are indicated by arrows, displaying change in area under the curve from time 0 to 48 hour.

Figure 9



G) ED02

	Δ Log (CFU/mL)$_{72h-24h}$	Grouping (α<0.05)
XOS	-0.023	A
Xylan	-0.839	A
Basal	-2.664	B

H) ED01

	Δ Log (CFU/mL)$_{72h-24h}$	Grouping (α<0.05)
XOS	-0.677	A
Xylan	-2.698	B
Basal	-3.083	C

F**igure 9. Test of persistence of *Bifidobacterium pseudocatenulatum* strains.** Strain ED01 on Xylan **(A),** XOS **(C)**, and basal media **(E)**. Test of persistence of *Bifidobacterium pseudocatenulatum* strain ED02 on xylan **(B),** XOS **(D),** and basal media **(F)** in fecal fermentations by qPCR in 5 subjects. Statistical grouping of the average change in $\log_{10}$(CFU/mL) between the 72 hour and 24-hour time points by substrate is indicated by separate letters for strain ED01 **(G)** and ED02 **(H)**. Detection limit for each strain via qPCR is shown as a black dotted line.

Figure 10

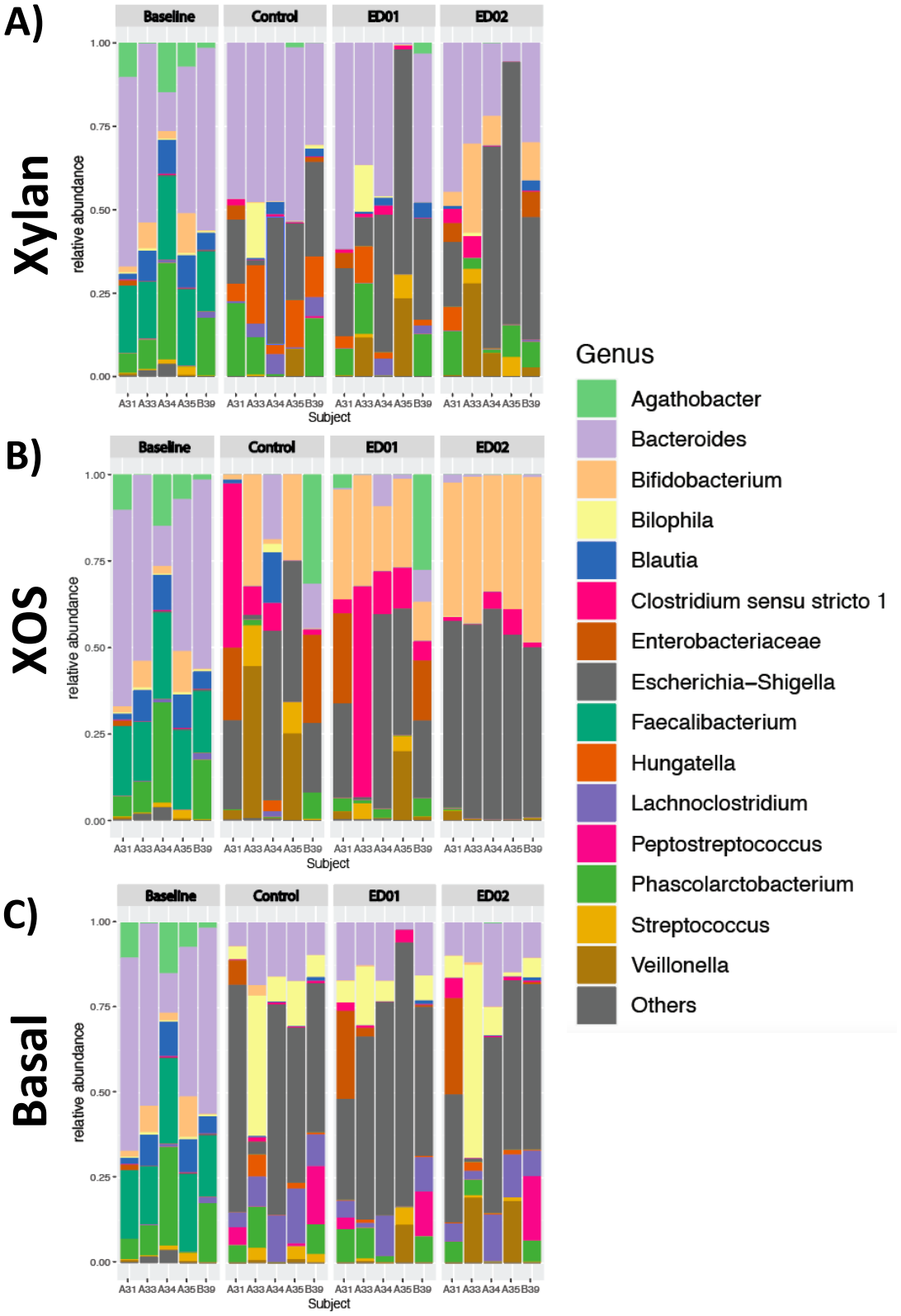

A)
Xylan
B)
XOS
C)
Basal
Baseline
Control
ED01
ED02
relative abundance
0.00
0.25
0.50
0.75
1.00
A31 A33 A34 A35 B39
Subject
Genus
Agathobacter
Bacteroides
Bifidobacterium
Bilophila
Blautia
Clostridium sensu stricto 1
Enterobacteriaceae
Escherichia–Shigella
Faecalibacterium
Hungatella
Lachnoclostridium
Peptostreptococcus
Phascolarctobacterium
Streptococcus
Veillonella
Others

Figure 10. Relative abundance of top twenty bacterial taxa for xylan (A), XOS (B) and no substrate (C) treatments from the 72-hour time point of fecal fermentations by 16S rRNA. Five fecal baseline samples are on far left of each figure, with basal treatment, ED01, and ED02 strain treatments adjacent. Each cluster shows subject-specific results and *Bifidobacterium* is shown in light peach.

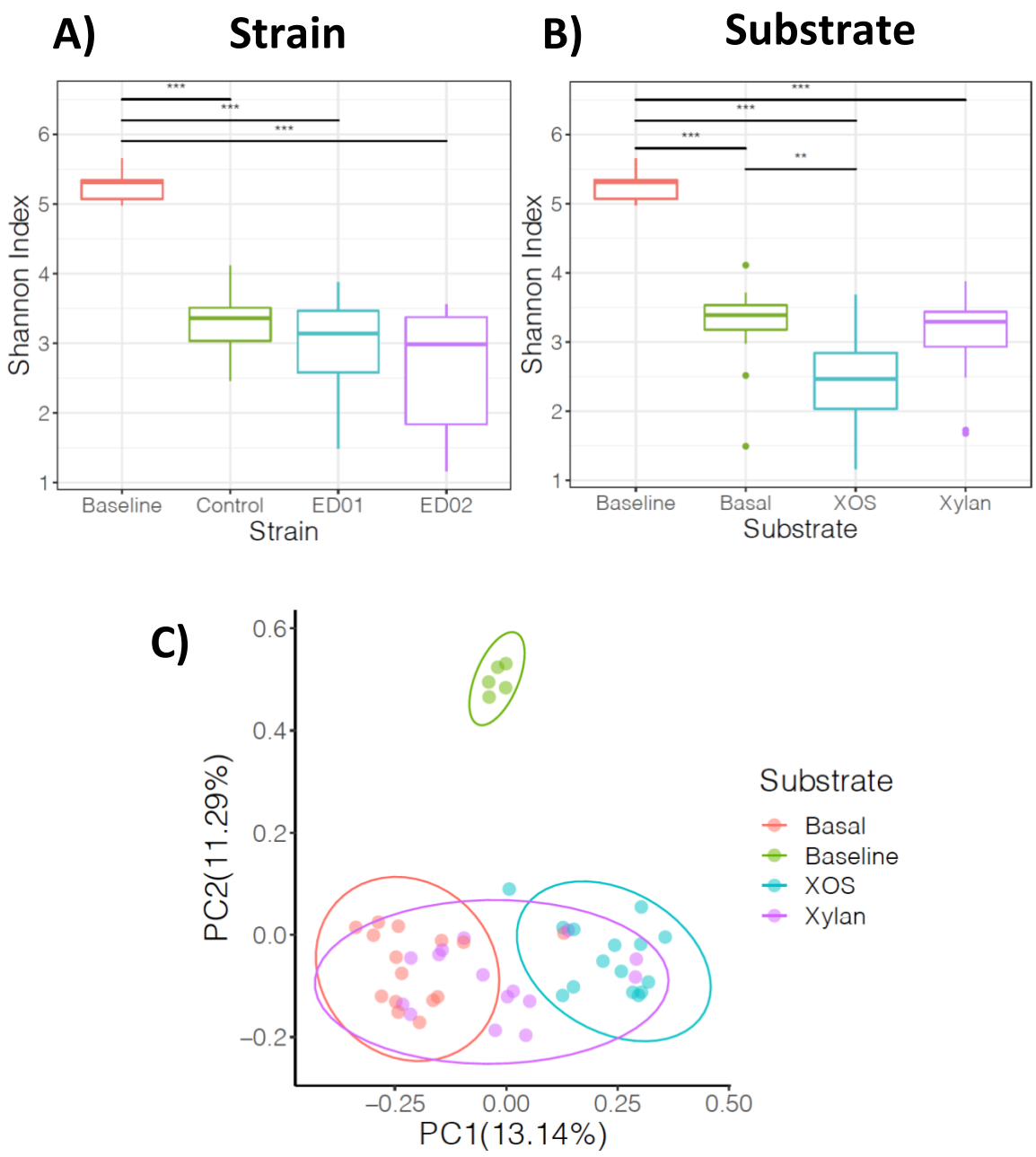


Figure 11. **Alpha and beta-diversity of fecal fermentations.** Alpha-diversity by Shannon Index metric for strain **(A)** and substrate **(B)** effect. Significant differences between values indicated by asterisk (* = p<0.05; ** = p<0.01; *** = p<0.001). Principal Coordinate Analysis (PCoA) of beta-diversity by Jaccard Index of all 72-hour samples by substrate. **(C)** 95% confidence intervals of substrate treatment are contained within ellipses.

Figure 12

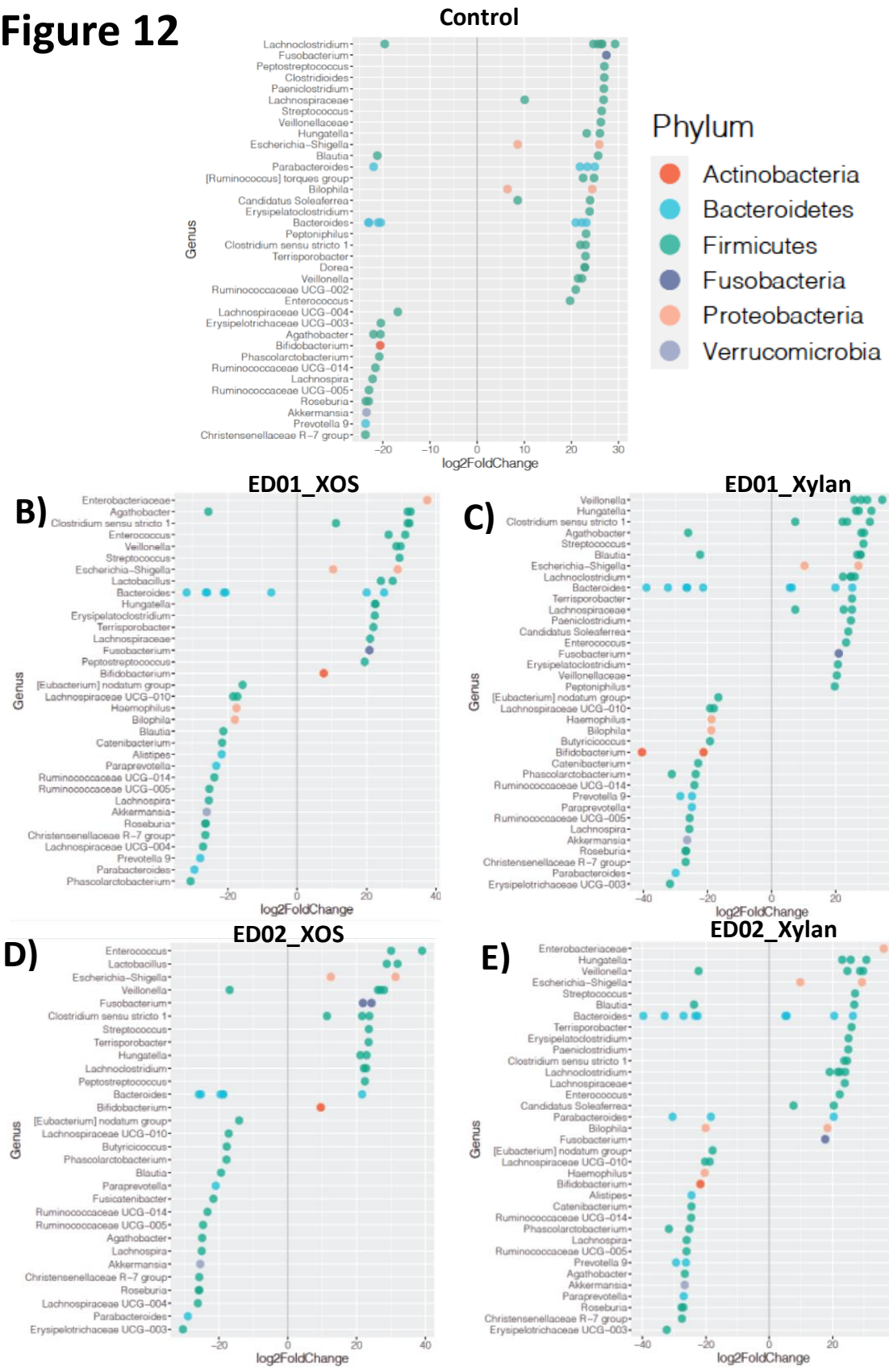

Control
Genus
log2FoldChange
Phylum
Actinobacteria
Bacteroidetes
Firmicutes
Fusobacteria
Proteobacteria
Verrucomicrobia
B)
ED01_XOS
C)
ED01_Xylan
D)
ED02_XOS
E)
ED02_Xylan

Figure 12. Log2Fold change data for fecal fermentations comparing between baseline samples and 72-hour samples for: basal control treatment **(A)**, ED01 + XOS treatment **(B)**, ED01 + xylan treatment **(C)**, ED02 + XOS treatment **(D)** and ED02 + xylan treatment **(E)**. Each dot represents a unique ASV at the associated genus level, and data points are color-coded by phylum. Data points in red belong to *Bifidobacterium* genus.

Figure 13

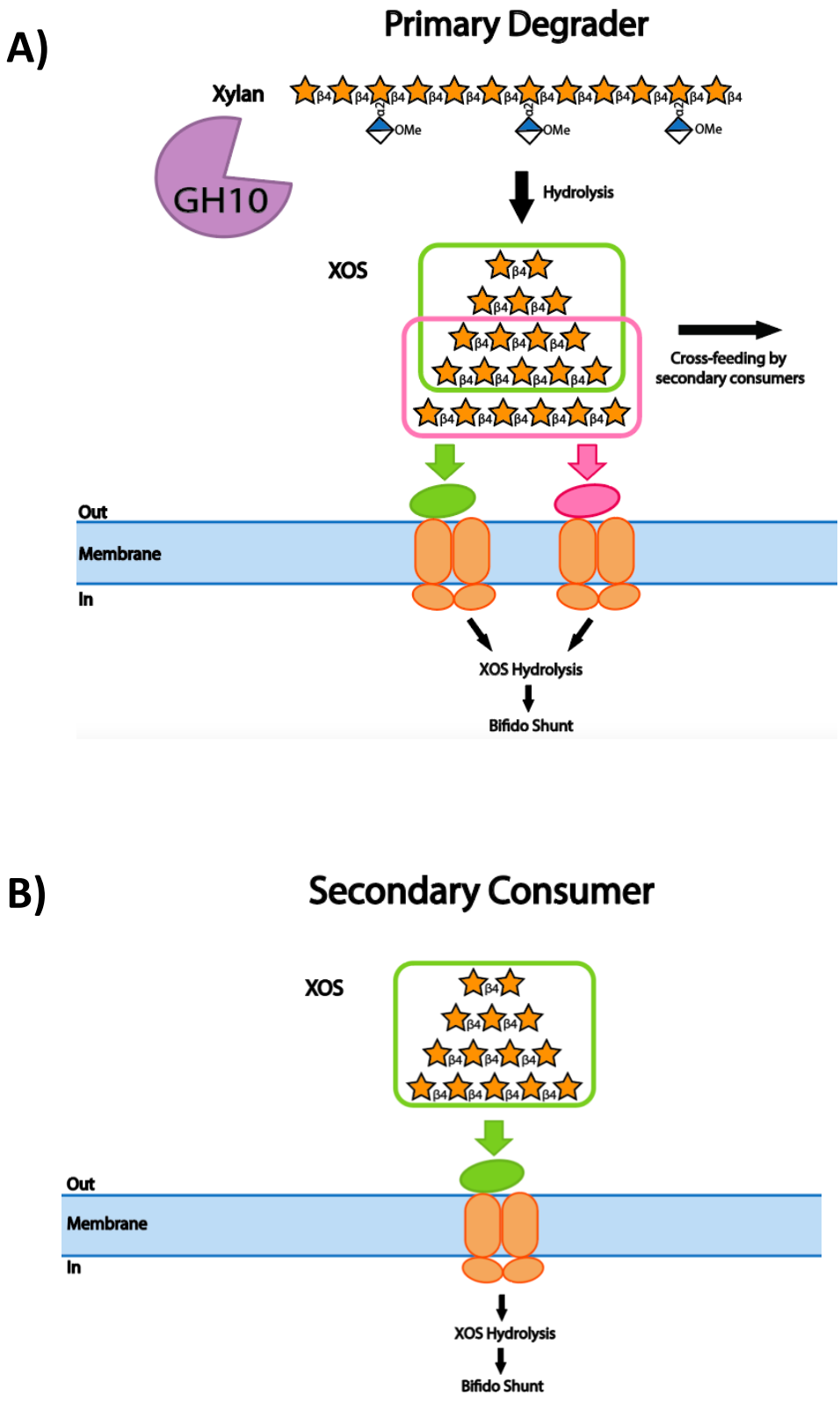

A)
Primary Degrader
Xylan
β4
α2
OMe
GH10
Hydrolysis
XOS
Cross-feeding by
secondary consumers
Out
Membrane
In
XOS Hydrolysis
Bifido Shunt
B)
Secondary Consumer
XOS
Out
Membrane
In
XOS Hydrolysis
Bifido Shunt

Figure 13. Model for xylan and XOS utilization by *Bifidobacterium pseudocatenulatum*. The xylan$^+$ phenotype is implicated as a primary degrader due to the secretion of an extracellular xylanase that degrades xylan into smaller oligomers. The latter are available for transport by the primary degrader, or they can crossfeed other organisms **(A).** In contrast, strains having a xylan$^-$ phenotype may still be secondary consumers. Although such strains do not have the extracellular enzymatic machinery or transporters to consumer larger XOS and xylan molecules, they instead rely on other members of the microbial community to degrade xylans into smaller oligosaccharides that can serve as substrates for relevant transporters **(B).**

Chapter 3: Conclusions, Implications and Future Research

The beneficial health effects of dietary fiber are now well established. Moreover, lack of fiber consumption can lead to deleterious health outcomes and is associated with an increase in metabolic diseases. This so-called 'fiber gap' can have a profound impact on members of the gut microbiome, leading to a disrupted or dysbiotic microbiota. Therefore, understanding the mechanisms by which keystone bacterial species in the gut utilize dietary fibers may provide a basis for developing strategies to restore a dysbiotic gut microbiome. In this research, we focused on the xylose-based polysaccharide, xylan, and its associated hydrolysis products. Current understanding of xylan utilization in the gut separates xylan fermenting microbes into two groups: primary degraders and secondary consumers. In general, primary degraders utilize extracellular enzymes to degrade xylan into utilizable xylooligosaccharides (XOS), that can then be consumed by the primary degraders, or those XOS products can cross-feed secondary consumers. In general, *Bifidobacterium* are considered secondary consumers, as many species can grow on XOS, but typically do not show growth on longer XOS or xylan itself. In fact, as of this year, no *Bifidobacterium* strain has been shown to grow on the insoluble xylan backbone.

In the research described in this thesis, we have identified, for the first time, strains of the gut commensal, *Bifidobacterium pseudocatenulatum,* that, have the genetic and biochemical basis for growth on longer XOS and xylan substrates. We also identified a potential pathway for xylan assimilation in these xylan^{+} strains. The key findings of this research are listed below:

- Xylan utilization is a rare phenotype in most bifidobacteria and appears to be a strain-specific trait within *B. pseudocatenulatum.*
- All *B. pseudocatenulatum* strains studied displayed growth on XOS and indicated stronger growth on XOS than on xylose.
- The presence or absence of key transporters and glycosyl hydrolases reflect observed phenotypes of our strains.
- Xylan^{+} *B. pseudocatenulatum* strains utilize an extracellular xylanase to produce XOS products available for transport and metabolism.
- Xylan^{-} strains saw improved growth XOS products from xylan^{+} strains, implicating potential for cross-feeding.
- Only the xylan^{+} strain persisted on xylan in stepwise fecal fermentations, indicating this trait may enhance persistence of the strain in a more complex environment.

Overall, the findings of this research indicate that some strains of *B. pseudocatenulatum* may act as primary degraders of xylan, a novel phenotype in this important group of gut microbes. Future research should focus on identifying potential health properties associated with the xylan^{+} strains, as their ability to utilize a variety of XOS and xylan compounds may indicate potential for synbiotic usage. In addition, extending the persistence studies into mouse models could confirm that the xylan and XOS phenotypes provide competitive advantages in a complex gastrointestinal environment. Therefore, if low-fiber diets are associated with depletion of keystone

microbes, then introducing xylan^{+} *B. pseudocatenulatum* strains combined with increased consumption of xylan fibers could enhance the functionality of the microbiome