XYLAN DEGRADATION MECHANISM OF HUMAN INTESTINAL BACTERIA

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ABSTRACT

XYLAN DEGRADATION MECHANISM OF HUMAN INTESTINAL BACTERIA

Xylan is the second most abundant plant cell wall polysaccharide after cellulose. The xylan rich lignocellulosic material obtained from agriculture, forestry and industrial wastes provides cost effective raw materials.

The degradation of xylan in the human body is an important process contributing to the continuation of the microbial communities living in the human colonic ecosystem. Due to its complex, long chain structure and the various chemical bonds it contains, xylan hydrolysis requires different enzymatic activities. Bacteria that live in the colon and are useful for human health, such as *Bifidobacterium* and *Lactobacillus* species can not perform xylan utilization. However, several types of xylan are utilized by the *Bacteroides* species, which have the second largest density in the colon.

In this study, different *Bifidabacterium* and *Bacteriodes* species were investigated for their ability to degrade beechwood xylan and corncob xylan. *Bifidabacterium* and *Bacteriodes* were cultured together in tubes containing xylan as the sole carbon source. It was observed that; the *B. animalis subsp. lactis*, which does not have the ability to use the xylan, could grow when cultured on xylan-containing medium with *Bacteroides* species. These showed that, the xylan in the media was degraded into xylooligosaccharides by the *Bacteroides* species and the XOS formed was used as a carbon source by both species. The short chain fatty acid and lactic and succinic acid production profiles of co-cultures were different than the mono cultures, indicating a positive effect of co-culturing.

This study showed that xylan is a potential prebiotic carbohydrate, which can selectively stimulate the growth of beneficial bacteria in the colon, as a result of possible cross feeding of different bacteria residing in the colon.

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ÖZET

İNSAN BAĞIRSAK BAKTERİLERİNİN KSİLAN PARÇALAMA MEKANİZMASI

Ksilan, selülozun yanında doğada en çok bulunan ikinci bitki hücre duvarı polisakkaritidir. Tarım, orman ve endüstriyel atıklardan elde edilen ksilanca zengin lignoselülozik materyal, ucuz ham madde eldesi sağlamaktadır.

Ksilan parçalanması, insan kolonik ekosisteminde yaşayan mikrobiyal toplulukların devamının sağlanmasına katkıda bulunan önemli bir süreçtir. Karmaşık, uzun zincirli yapısından ve içerdiği çeşitli kimyasal bağlardan dolayı, ksilan hidrolizi farklı enzimatik faaliyetler gerektirmektedir. Kolonda yaşayan, insan sağlığı açısından yararlı olan probiyotik türler, örneğin *Bifidobacterium* ve *Lactobacillus* türleri; ksilan parçalması gerçekleştirememektedir. Ancak, kolonda yaşayan ve kolonda ikinci büyük yoğunluğa sahip olan *Bacteroides* türleri tarafından çeşitli ksilan tipleri kullanılmaktadır.

Bu çalışmada farklı *Bifidabacterium* ve *Bacteriodes* türlerinin birlikte kültüre edildiklerinde kayın kerestesi ve mısır koçanı ksilanı parçalama yeteneklerini incelenmiştir. *Bacteroides* ve *Bifidobacterium*, karbon kaynağı olarak ksilanın bulunduğu tüpte kültüre edilmiştir. Ksilan'ı kullanma yeteneğine sahip olmayan *B. animalis subsp. lactis*'in, ksilan içeren ortamda *Bacteroides* türleri ile birlikte kültüre edildiğinde büyüyebildiği gözlenmiştir. Besiyerindeki ksilanın *Bacteroides* türleri tarafından KOS'a ayrıldığı ve oluşan KOS'un her iki tür tarafından bir karbon kaynağı olarak kullanıldığını göstermiştir. Birlikte kültür edilmiş bakterilerin, kısa zincirli yağ asidi ve laktik ve süksinik asit üretim profilleri tek kültürlerden farklı olmuştur, bu da birlikte kültürün olumlu bir etkisini göstermektedir.

Bu çalışma, kolonda bulunan farklı bakterilerin çapraz beslenmesinin bir sonucu olarak, ksilan'ın kolondaki yararlı bakterilerin büyümesini seçici olarak uyarabilen potansiyel bir prebiyotik karbonhidrat olduğunu göstermiştir.

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CHAPTER 1

INTRODUCTION

Xylan is the second most abundant plant cell wall polysaccharide after cellulose. Agricultural wastes such as straw, corn stalks and hardwood dough form an important source of xylan. The cheap raw material, agricultural forests and industrial wastes are rich lignocellulosic materials and this feature is a very good advantage. There are several types of xylan, because they are derived from different sources. For example, beechwood xylan, birchwood xylan, rye arabinoxylan, wheat arabinoxylan and oat spelt xylan. The disruption of xylan is a very important process in terms of maintaining the continuity of microbial communities living in human colon. However, xylan is a complex, long chain structure and contains various chemical bonds. Therefore, xylan hydrolysis requires different enzymatic activities. However, *Bacteroides* species, which have the second largest density in the column, use various types of xylan. It can be used by some bacterial species, including *Bacteroidetes* family, can not be used by prebiotic microorganisms. Probiotic species that are beneficial to human health, such as *Bifidobacterium* and *Lactobacillus* species; they do not have the ability to degrade xylan. The *Bifidobacterium* community is a group of potentially useful microorganisms that live in the large intestine. It is known that the carbohydrate mechanism of bacteria in this population differs from species to species and even from strains. And this feature emphasizes the importance of the choice of *Bifidobacterium* strain to be applied probiotically. As already mentioned above, *Bifidobacterium* species, which cannot utilize xylan, can metabolize Xylooligosaccharides (XOSs). XOS is obtained by enzymatic hydrolysis of xylan but this process is quite long and costly. For this reason, it is advantageous to use xylan instead of XOS, which is used to increase the probiotic microorganisms. In this study, the mechanism of xylan metabolism of bacteria living in the colon was tried to be explained. For this purpose, growths of *Bacteroides* and *Bifidobacterium* species on xylan and XOS were investigated. The cross-feeding phenomenon in the colon helps to explain the mechanism of the co-cultured bacteria.

CHAPTER 2

LITERATURE REVIEW

2.1. Human Intestinal Tract

As with other part of the body, gastrointestinal tract (GIT) of people is rapidly colonized from birth. The presence of bacterial population in the GI tract has been extensively researched because the mucosal surface of human intestine is more than 100 m^2 of habitable area (Korecka and Arulampalam, 2012). There are about 100 trillion bacteria in the GIT and cell density reach up 10^{12} cell ml^{-1} at the end of the gut. This is the highest recorded number for any microbial habitat up to now. The density and composition of the bacteria vary throughout the GIT. GIT tract is consisted of different parts, such as stomach, duodenum, jejunum etc. Bacterial numbers increase along the GIT. For example, luminal contnents in stomach and duodenum have 10^3 to 10^5 and ileum and large intestine have 10^9 to 10^{12} bacteria per mL. As a result, most dense part is large intestine part (Korecka and Arulampalam, 2012).

Large intestine or colon is one of the most considerable part of gastrointestinal tract. It includes complicated bacterial ecosystem. This system plays significant role in health and nutrition of human (Harmsen et al., 2002). The activities of colon microbiota have great effect on the nutrition and health of the host by the way of conversions of metabolites, supply of nutrients and interaction with host cell (Flint et al., 2007).

The large intestine consists of three different regions according to their differences, in nutrient availability and bacterial activity, namely the proximal or ascending colon, transversal colon and the distal or descending colon (Falony and Vuyst, 2009). Nutrients and nutrient residues that are not absorbed in small intestine are fermented by microbiota that are living in the large intestine. Protein degradation increases towards the end of the distal part of the colon, while carbohydrate fermentation occurs in the proximal colon (Macfarlane et al., 1992). The proximal colon has higher bacterial growth rates than transverse colon luminal pH has an average of approximately 6.2 (Flint et al., 2007).

2.2. Intestinal Microbiota

The human body contains a large number of microorganism such as bacteria, archaea viruses and unicellular eukaryotes. This microorganism community live in harmonious coexistence with their host and this is defined as microbiota, microflora or normal flora (Kunz et al., 2009). The structure and roles of bacteria, which are part of this community, have been studied intensively over the last few years, but the role of viruses, archaea and unicellular eukaryotes living in mammalian bodies is less known than bacteria. It is estimated that human microbiota contains 10 times more bacterial cells (about 10^{14}) than the number of human cells in our body (Ley et al., 2006). The microbiota has colonized almost all surfaces of the parts of human body that are in contact with the external environment, like our skin, genitourinary, gastrointestinal respiratory tracts. Until today, the most intensive colonized organ is GIT; it is estimated that only the colon contains more than 70% of all the microbes in the human body (Neish, 2009). In addition, it is the preferred site for colonization, because GIT is rich in molecules that can be used by microbes. The intestinal microbiota does not have homogeneous structure. The number of bacterial cells present in the mammalian gut is 10^1 to 10^3 bacteria per gram of contents in stomach and duodenum, 10^4 to 10^7 bacteria per gram in the jejunum and ileum and 10^{11} to 10^{12} cells per gram in the colon (Figure 2.1) (O'Hara and Shanahan, 2006).
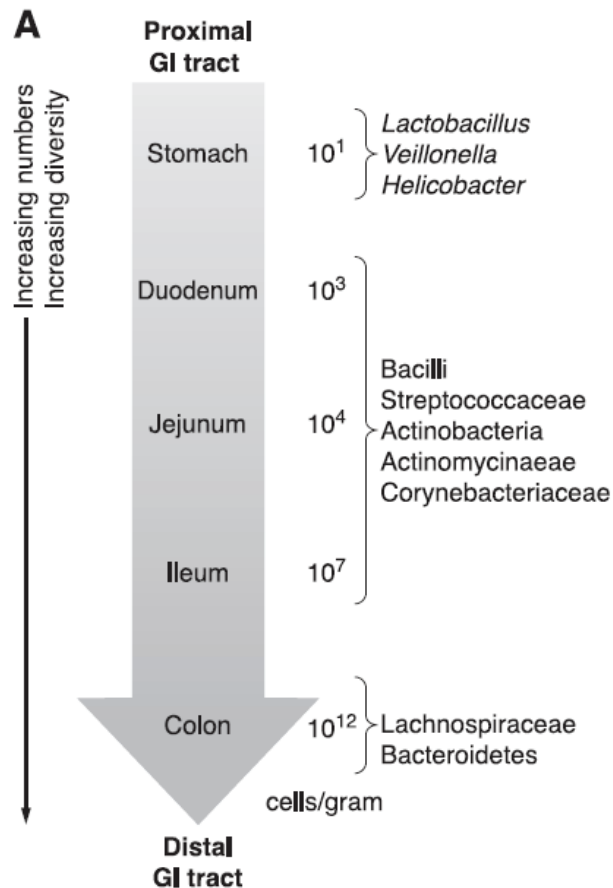
Figure 2.1. Microbial composition and structure of the gastrointestinal tract
(Source: O'Hara and Shanahan, 2006)

As mentioned above; it is known that there are many strains and species colonized in the gut, with species belonging to the genera *Bacteroides, Eubacteirum, Clostridium* and *Bifidobacterium* being amongst the dominant members of the faecal microflora (Harmsen et al., 2002).

Bacteroides and *Bifidobacterium* genera are found in the human large intestine and constitute 25% and 30% of the total cultivable gut flora (Mitsuoka, 1984; Salyer, 1984). Both of two bacteria are saccharolytic and obligatory anaerobes. Also, they are important the in the colonic system because they have ability to digest complex polymers that are resistant to hydrolysis by human digestive enzymes. From high numbers, it is obvious that, these bacteria play important role in degradation of polysaccharides in the fermentation system (Macfarlane et al., 1998). Major functions of intestinal microbiota are summarized in list below,

1. Normal gastrointestinal immune function (Hamer et al., 2008)

2. Energy harvest and nutrient supply (Turnbaugh and Stintzi, 2011)

3. Mucin Degradation (Derrien et al., 2004)

4. Cancer Prevention (Segain et al., 2000)

5. Inhibition of pathogen (Guarner and Malagelada, 2003)

6. Normal gut motility (Lewis and Heaton, 1997)

7. Cardiovascular health (Wikoff et al., 2009)

2.2.1. *Bifidobacteria*

Bifidobacterium is a member of the *Bifidobacteriaceae* family, the *Bifidobacteriales* order, and *Actinobacteria* phylum. It represents one of the largest bacterial taxonomic units (Stackebrandt, 2000). *Bifidobacteria* species are gram positive, heterofermentative non-spore forming, non-motile and catalase negative anaerobes. They have various shapes, such as club-shaped rods, bifurcated Y-shaped rods and curved rods (Sgorbati, 1995). *Bifidobacteria* are extremely important microorganisms in warm-blooded animals, because they take part in the active and complex ecosystem of the intestinal tract (Sgorbati, 1995). They are distributed in various ecological areas of the human GIT, and their exact rate is predominantly determined by diet and age. Babies' native microflora is dominated by *Bifidobacteria* shortly after birth. Since the number of *Bifidobacteria* decreases as age increases, they become third largest group after the genera *Bacteroides* and *Eubacterium* (Finegold et al., 1983). *B. longum, B. pseudolongum, B. animalis subsp. lactis, B. adolescentis, B. bifidum* and *B. breve* are prominent bifidobacterial species present in the human gut (Turroni et al., 2011).

Like most other bacteria in the colon, *Bifidobacteria* are saccharolytic microorganism and they play key role in carbohydrate fermentation in the colon. It is known that *Bifidobacterium* can actually ferment various complex carbon sources such as galacto-oligosaccharides, soy bean oligosaccharides, fructo-oligosaccharides, xylo-oligosaccharides and other plant derived oligosaccharides. However, it is clear that the ability to metabolize certain carbohydrates is dependent on species and strain (Vrese and Schrezenmeir, 2008). In the colon, intestinal bacteria degrade polymeric carbohydrates to low molecular weight oligosaccharides. And then these oligosaccharides can be degraded by the use of a wide variety of carbohydrate

degrading enzymes. These monosaccharides are converted to intermediates, and finally converted short chain fatty acids (SCFAs) and other organic compounds. A general schematic of the fermentation and SCFAs is shown in Figure 2.2. SCFAs and some other component are beneficial to host (Pokusaeva, 2011).
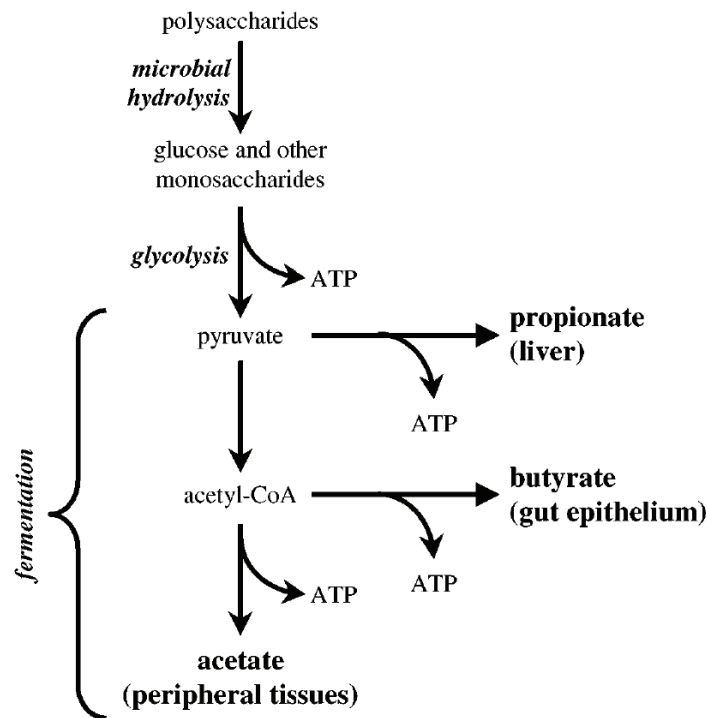
Figure 2.2. Bacterial fermentation in the intestine
(Source: Hooper et al., 2002)

Short chain fatty acids (SCFAs) are the most important end products of the metabolism of colonized bacteria in the human large intestine (Cummings and Macfarlane, 1991). The main SCFAs; acetate, propionate and butyrate resulting from carbohydrate and amino acid fermentation. The concentrations of SCFA are the highest in the proximal colon because of the greater amount of carbohydrate fermentation (Cummings et al., 1987).

SCFA is absorbed in the cecum and colon by passive diffusion along the epithelium. Each SCFA is absorbed by different organs and has different metabolic fates. Firstly, butyrate, which is metabolized by the intestinal epithelium, is converted to ketone body or oxidized to CO_2. The colonic epithelial energy requirement, 60-70% is from butyrate. Propionate is also transported to the liver via the portal vein. Due to the low rate of glucose uptake in the intestine, propionate is an important precursor for

gluconeogenesis. Little is known about the role of propionate metabolism in humans. Acetate that taken by peripheral tissues such as skeleton and heart muscle can be used by adipocytes for lipogenesis. In addition to the known nutritional values of SCAFs, SCFAs have significant effects on other aspects of intestinal physiology. For example, SCFAs are the dominant anion at the site. The water absorption is combined with sodium, chloride as well as SCFAs transport. SCFA is also associated with promoting intestinal blood flow. Especially butyrate, affect epithelial proliferation and differentiation. Based on all these beneficial effects, SCFAs are very important for people's health.

  Bifidobacteria found in the human intestine have been of interest to date, due to their potential probiotic properties. The definition of probiotics as universally accepted and recommended by the Food and Agriculture Organization (FAO) World Health Organization (WHO); "Probiotics are live microorganisms which when administrated in adequate amounts confer a health benefit on the host" *Bifidobacteria*, as mentioned above, play important role in human health (FAO/WHO, 2001). Probiotic strains have one or more capacities of positive activity. These activities are, modulation of the intestinal microbiota, immuno-modulation, reduction of allergic disease symptoms, reduction of lactose intolerance, reduction of intestinal inflammation, SCFA production and relieving of constipation (Ouwehand, 2002; Saxelin et al., 2005). Some *Bifidobacterium* species-strains that have been declared as probiotic activities have become economically important. These living bacteria are added to the preparations, which are called functional foods and have good health-related effects (Stanton, 2005). Intestinal bacteria and host interaction can be categorized as symbiosis (both partners derive benefit) and commensalism (one partner benefits but the other unaffected), that can be grouped as mutualism (Marco et al., 2006; Saxelin et al., 2005).

2.2.2. *Bacteroides*

The human colon has the largest bacterial population in the body. Anaerobes constitute the majority of these organism and 25% of them are *Bacteroides species* (Salyers, 1984). *Bacteroides species* are obligate anaerobic microorganisms and they are gram negative, bile resistant and non-spore forming rods. Many of *Bacteroides* species were isolated from human feces (Liu et al., 2003). *Bacteroides* exist part of the human flora at the earliest stages of life, because they can pass from mother to babies during vaginal birth (Reid, 2004). The bacteria usually have a complex relationship with the host, but they generally maintain a useful relationship (Xu and Gordon, 2003). *Bacteroides* species are a group characterized by their ability to ferment various carbohydrates. Nutritional requirements of colonized in gut of *Bacteroides* are simple. Volatile fatty acids resulting from carbohydrate fermentation of *Bacteroides* and other intestinal bacteria are reabsorbed through the large intestine, used as an energy source by the host, and a significant portion of the host's daily energy need is provided (Hooper et al., 2002).

In 2003, the completion of sequence projects of *B. thetaiotaomicron* (Xu et al., 2003) and *B. fragilis* between 2004 and 2005 (Cerdeño-Tárraga et al., 2005 and Kuwahara et al., 2004), and proteomic analyzes carried out, obtained useful information for us to understand how these organisms adapt to the human intestine and how they develop. Some of the information obtained was as follows: (i) they interacted with the host immune system; (ii) their multiple pump systems were used to discard bacterial toxic substances; (iii) had complex systems to perceive their existing nutrients.

Although the relationship between *Bacteroides* and humans is called commensal in current articles, the new approach is that this relationship is mutualistic, meaning that both humans and bacteria benefit from this relationship (Bäckhed et al., 2005). Other organisms in the intestine that do not have the sequence of sugar-using enzymes of *Bacteroides* can benefit from the presence of *Bacteroides*, using sugars that they normally would not be able to use (Sonnenburg et al., 2004). For example, *Bifidobacterium longum* imports simple sugars better than *B. thetaiotaomicron*. However, *B. thetaiotaomicron* can break down a wide variety of glycosidic bonds that provide nutrients that *B. longum* can then use. Besides, studies with mice indicate that *B. thetaiotaomicron* may direct the ability to use carbohydrates to dietary host polysaccharides, depending on the presence of the nutrient (Sonnenburg et al., 2005).

In addition to the ability to break down wide range polysaccharides, association of intestinal *Bacteroides* with obesity has also been studied in various studies. These studies shown that, the number of *Bacteroides* species was found to be low in obese people and it was observed that weight loss was achieved when these species were increased (Turnbaugh et al., 2006).

Besides the useful properties of the B*acteroides* mentioned above, these organisms can be responsible for infections with important morbidity and mortality. The most well-known pathogenic species among the *Bacteroides* species is *B. fragilis*. It is the most common anaerobic pathogen isolated because of its strong virulence factors such as bacterial toxins, cell surface proteins, although it accounts for about 0.5% of the colonic flora (Polk and Kasper, 1977).

Bacteroides and *Roseburia* isolated from human faeces are essentially xylan-degrading species, which are the two main genera in human intestinal microbiota (Mirande et al., 2010). In previous studies, Salyers et al. have isolated a few *Bacteroides* strains from human excreta that could ferment xylan. The two major xylanolytic bacteria among these isolates are *Bacteroides fragilis* and *Bacteroides ovatus* (Salyers et al., 1977). After that isolated new species, *Bacteroides xylanisolvens* (Chassard et al. 2008). In addition, among the *Bacteroides* in the human intestine, *Bacteroides xylanisolvens* showed particular characteristics: Because this species cannot use the starch, it is specialized in xylan degradation and fermentation.

Studies have shown that *Bacteroides xylanisolvens* produced propionate, succinate and acetate as metabolites. In the most of the complex substrates, the main metabolite is propionate and this is also observed for *B. xylanisolvens* growing on xylan. Succinate is probably rapidly converted to propionate by intestinal microbiota under the physiological conditions of the intestine. It is likely that xylan fermentation of *B. xylanisolvens* mainly leads to the production of propionate and acetate (Mirande et al., 2010). In addition, butyrate and propionate producers may contribute to the potentially beneficial effects of SCFA. Butyrate plays a role in the prevention of intestinal pathologies, while propionate has been suggested to prevent lipogenesis (Wong et al., 2006).

2.3. Microbial Contribution to Carbohydrate Metabolism

Carbohydrates of human and animal diets are critical nutrients for host and microbiota. The mammals have good equipped that can easily absorb simple sugars such as glucose and galactose in the proximal part of the small intestine (Ferraris, 2002). In here, specific disaccharides such as sucrose, lactose and maltose are hydrolyzed to monosaccharides. However, the capacities of hydrolyzing and using other polysaccharides are usually limited. For this reason, large amounts of non-digestible carbohydrates pass to the distal part of the gastrointestinal tract and these are the polysaccharides in the components of plant cell walls. The utilization of carbohydrate is briefly examined in Figure 2.3. Simple sugars such as glucose and galactose are absorbed easily in the proximal part of the small intestine by active transport. However, mammals do not have the ability to absorb polysaccharides in this area. Undigested polysaccharides pass into the distal region of the small intestine and colon and they are degraded by colonized bacteria in this area (Hooper et al., 2002).
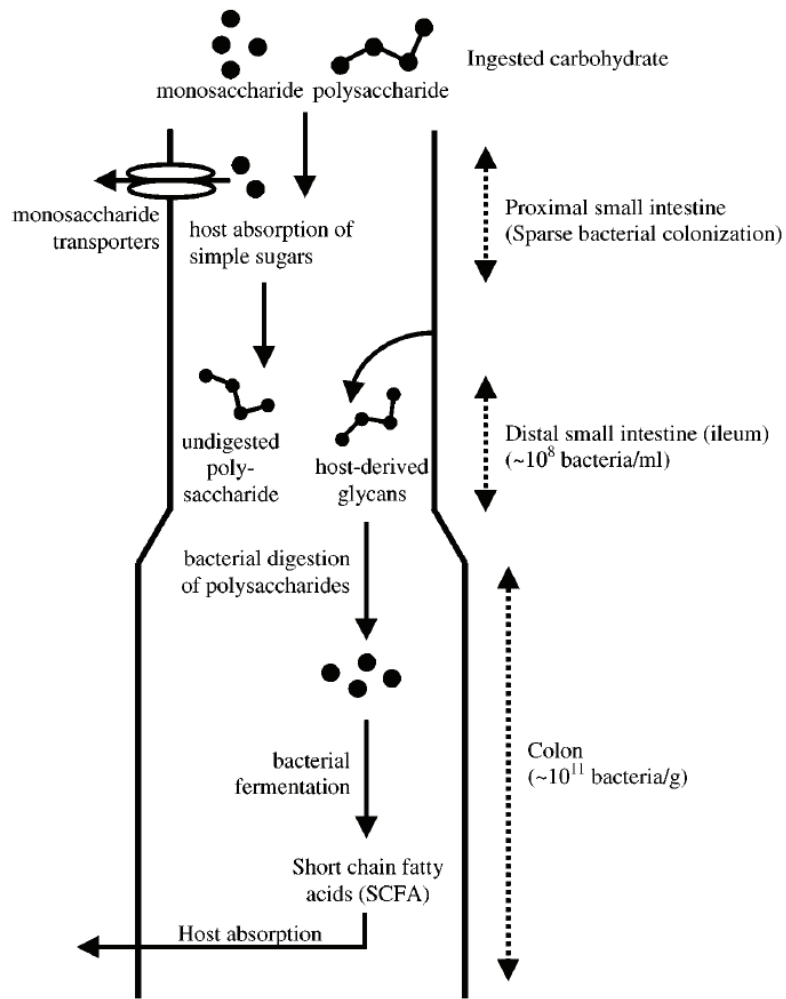
Figure 2.3. Utilization of carbohydrate in the gastrointestinal tract
(Source: Hooper et al., 2002)

2.3.1. Catabolism of Polysaccharides

Glycans can be used as generic naming for long chain polysaccharides. Glycans, a common component of our diets, are associated with a number of different plants. These diets include plant cell wall glycans like starchs and fructans and plant cell wall glycans. Cellulose is the most abundant of cell wall glycans, followed by hemicelluloses and pectins, two heterogeneous classes of polysaccharides. Hemicelluloses include galactoglucomannas, xyloglycan and xylan (Martens et al., 2009). The group in which xylan is also found is highly digestible by human intestinal microbiota, on the contrary to cellulose. In vitro fermentation experiments with fecal bacteria have shown that individual polysaccharides are used at different ratios. For example, starch and pectin

are degraded more rapidly than xylan or arabinogalactan (Englyst et al., 1987). Since this finding is related to the bacterial catabolic processes in the intestine, the substrate concentration greatly regulates the way the organisms compete for the fermentable substrate and the control mechanisms involved in the fermentation reactions (Macfarlane and Macfarlane, 2003).

In a study by Macfarlane et al., 1990, *B. ovatus* was grown in 12 different carbohydrates and was determined at specific growth rates. When comparing the growth rates on polymerized carbohydrates to simple sugars, they have found that polymerized carbohydrates have a faster rate of growth than simple sugars and this showed that the rate of polysaccharide depolymerization is not a limiting factor in growing. *Bacteroides ovatus*, as well as other *Bacteroides* species that can synthesize a wide range of polymer degrading enzymes. Xylanase activity is cell-associated in the human colonic *Bacteroides* species. This feature reveals a crucial question of how these bacteria efficiently utilize large polysaccharides in the absence of a comprehensive extracellular xylanase system. The answer to this problem lies in the *Bacteroides thetaiotaomicron* starch utilization system (SUS) that the Salyers et al. discovered. This system is a multi-protein system associated with a cell envelope that allows carbohydrates to bind and degrade. Subsequent microbial genome sequencing projects reveal that derivatives of this prototypical system ("Sus-like systems") are highly represented in the genome of many saccharolytic *Bacteroides* species (Martens et al., 2009). The Sus-like system is a group of proteins associated with a cell envelope that degrade a specific glycan. Sus like system highlights the two fundamental concepts. During catalysis, more than one protein works together and the genes encoding these compatible functions are usually linked genetically to distinct clusters. Thousands of different SUS like PULs in the *Bacteroidetes* form an incredibly diverse group of genes that regulating and directing the glycan catabolism (Dodd et al., 2011). The common feature of these Sus-like systems is the coordinated action of many gene products related to substrate binding and fragmentation. In this model, the activities of multiple gene products can be more sophisticated and elaborate than their individual and isolated functions. The Sus system is briefly summarized (Figure 2.4.), the carrier SusC, associated with TonB, works in concert with the starch-binding lipoprotein SusD, SusE, SusF and SusG; SusG is a glycoside hydrolase family 13 (GH13) a-amylase. Starch binding is initiated by SusD, SusE and SusF, initial degradation is carried out by SusG. Produced oligosaccharides are transported periplasmically via SusC concurrently with the inner membrane protein

TonB. Oligosaccharides are separated into monosaccharides or disaccharides by periplasmic glycans degrading enzymes such as Susa and SusB and the finally released saccharides serve as signals for transcriptional regulators that activate the expression of the polysaccharide utilization loci (PULs) gene. For example, in *B. thetaiotaomicron* VPI-5482, about 88 PULs. The use of other polysaccharides is explained by the Sus like system, but with slight differences. For example, in xylanolitic Bacteroidetes, Sus C and Sus D are homologues xus A, xus B, xus C and xus D. As shown in the predicted model for binding of xylan (Figure 2.5); the proteins, XusA and XusC, are the SusC homologs involved in oligosaccharide transport across the outer membrane of *B. thetaiotaomicron*. XusB and XusD are homologues of *B. thetaiotaomicron* SusD, that binds polysaccharides on outer membrane. XusE does not show any similarity to other characters in proteins. However, the presence of an N-terminal signal peptidase II cleavage site in this protein indicates that it is bound to the outer membrane. Xyn10C can represent a functional homolog of *B. thetaiotaomicron* SusG protein and catalyze the extracellular cleavage of xylan polymers and the transfer of the resulting oligosaccharides into the cell (Martens et al., 2009)
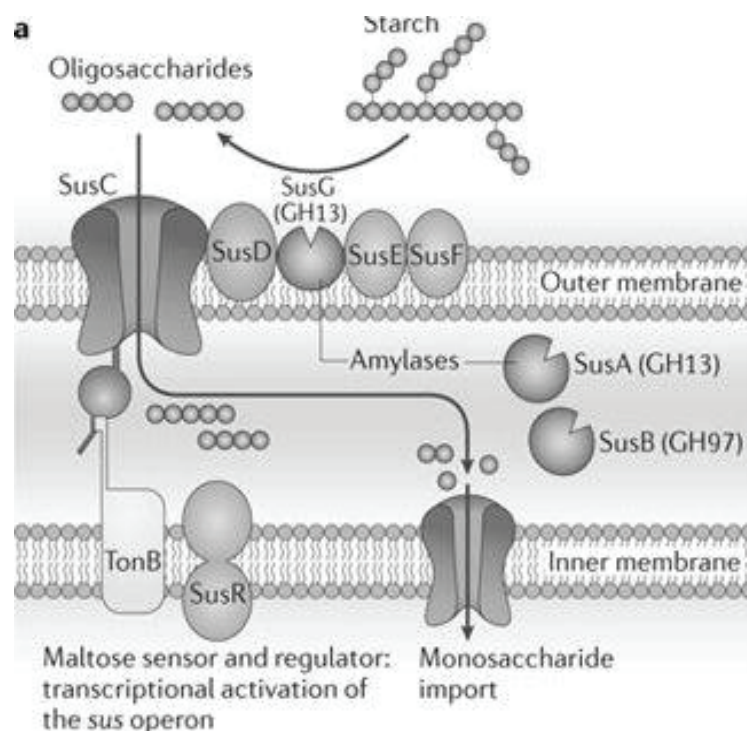
Figure 2.4. The Sus System of *Bacteroides thetaiotaomicron*
(Source: Koropatkin et al., 2012)
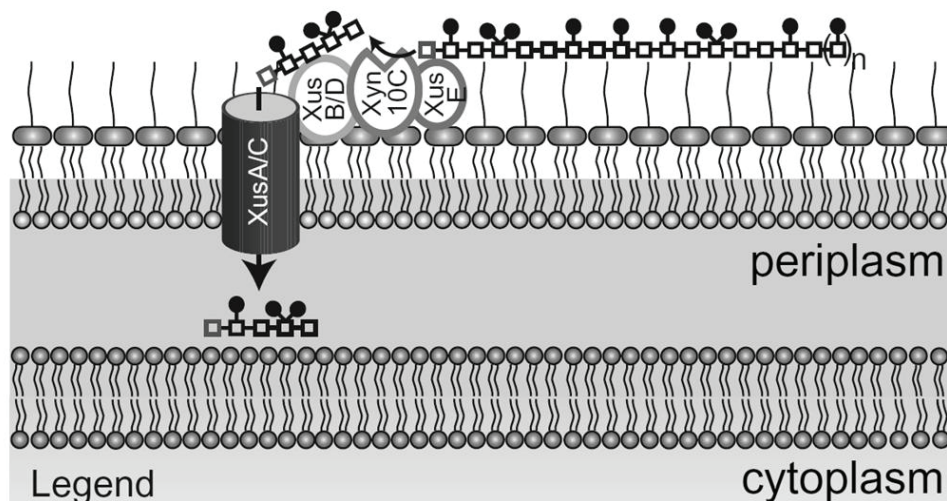
Figure 2.5. Model for the xylan utilization systemin xylanolytic Bacteroidetes
(Source: Dodd et al., 2011)

2.3.1.1. Xylan

Xylan is the second most abundant plant cell wall polysaccharides after cellulose. It consists of β-1,4 linked xylose backbone which can be substituted with other molecules such as acetyl groups, 4-O-methyl glucuronyl groups or arabinose at 2'-OH or 3'-OH. (Dodd et al., 2011). Chemical structure of xylan shown in figure 2.6 (Shallom and Shoham, 2003). Generally, xylans are classified based on the substituents (Figure 2.5) (Scheller and Ulskov, 2010; Ebringerova, 2006).
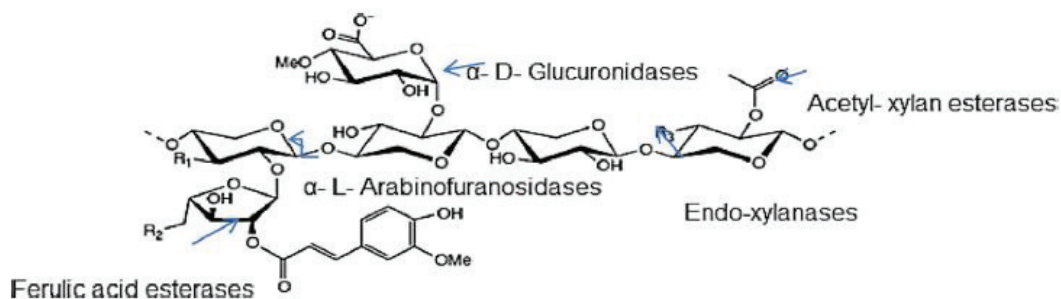
Figure 2.6. Chemical structure of Xylan
(Source: Ramkrishna et al., 2014)
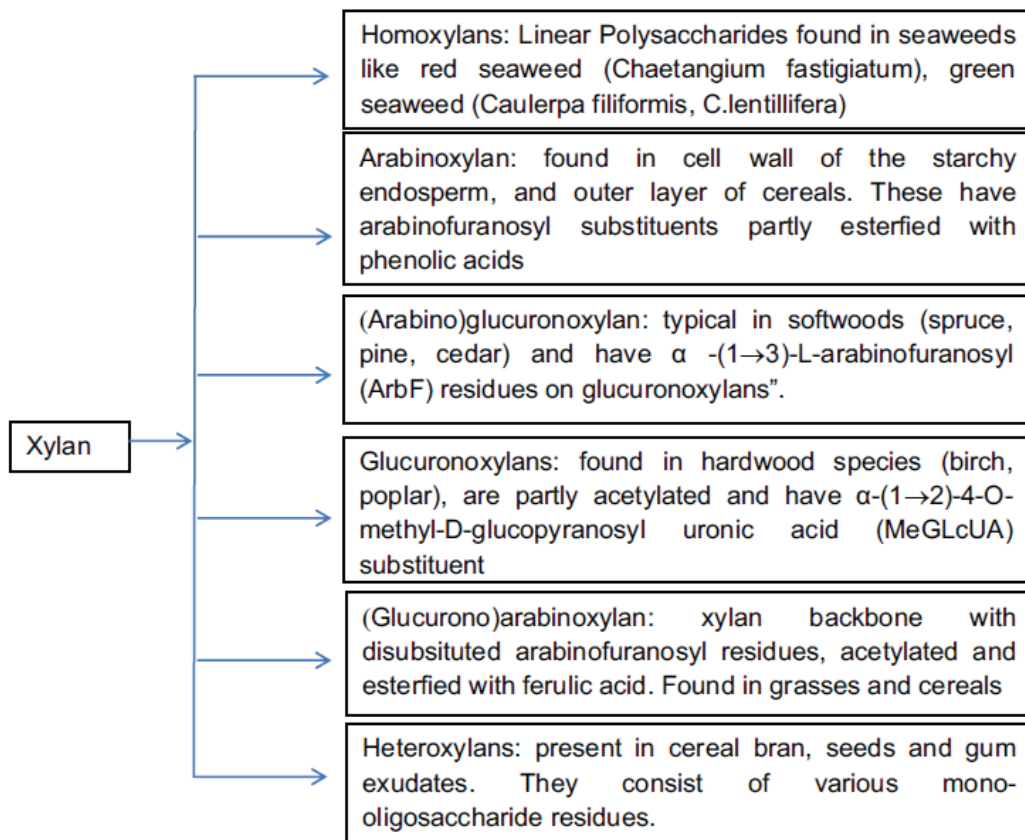
Figure 2.7. Classification of Xylan
(Source: Ramkrishna et al., 2014)

Arabinoxylans (AX) are part of the hemicelluloses predominant in grains, accounting for 30% of dry weight in grains and 5% of dry weight in vegetables (Cartano and Juliano, 1970). Although AX is the primary component of the walls surrounding plant cells in the starchy endosperm of most cereals, it is a major dietary source because some 64% of some nonendosperm tissues are AX (Obel et al., 2002). The cereal AX consists of the backbone chains of the β (1-4) linked D-xylopyranosyl residues to which the α-L-arabinofuranose units are attached as side chains. Although the majority of these side chains are monomeric, the oligomeric side chains in minor proportions are composed of two or more arabinosyl residues, which are linked via α (1-2), α (1-3) and α (1-5) linkages. The extent and distribution of the side chains are significant factors in the physicochemical properties of AX (Izydorczyk and Biliaderis 1995).
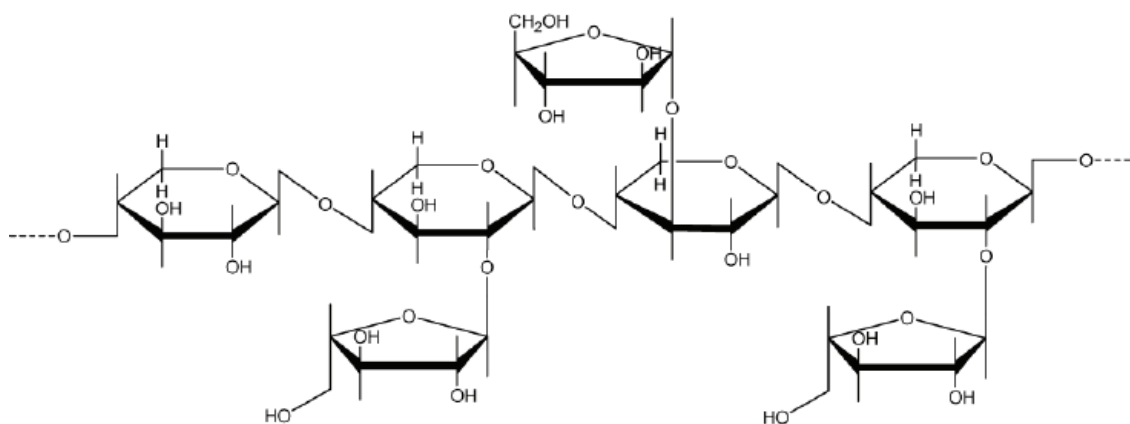
Figure 2.8. Partial structure of an arabinoxylan

A linear main chain formed by xylan (a pentosan consisting of D-xylose units connected by β(1→4) linkages), randomly attached to L-arabinofuranose residues by α(1→3) or α(1→2) linkages. Xylan is an abundant substrate, supporting microbial fermentation in gastrointestinal tract of humans. As mentioned under the heading *Bacteroides* above, different type of xylan and XOS are used by *Bacteroides species*. Table 2.1 shows these types of xylanolytic *Bacteroides* (Dodd et al., 2011).

Table 2.1. Xylanolytic gut *Bacteroides*
(Source: Dodd et al., 2011)

Organism	Gut environment	Type of xylan fermented [a]	Genome (GenBank Accession No.
B. cellulosilyticus DSM 148338	Colon	OSX (Robert et al., 2007)	Draft (NZ_ACCH00000000)
B. eggerthii DSM 20697	Colon	N.S. (Salyers et al., 1977)	Draft (NZ_ABVO00000000)
B. fragilis subsp. A[b]	Colon	N.S. (Salyers et al., 1977)	No
B.intestinalis DSM 17393	Colon	OSX (Robert et al., 2007)	Draft(NZ_ABJL00000000)
B.ovatus ATCC 8483	Colon	OSX (Weaver et al., 1992)	Draft(NZ_AAXF00000000)
B. fragilis AHN 11543	Colon	RAX (Crittenden et al., 2002)	No
B. fragilis RHN 3001	Colon	XOS, RAX (Crittenden et al., 2002)	No
B. fragilis AHN 2981	Colon	RAX (Crittenden et al., 2002)	No
B. fragilis AHN 2898	Colon	RAX (Crittenden et al., 2002)	No
B. thetaiotaomicron RHN 4171	Colon	XOS, RAX (Crittenden et al., 2002)	No
B. thetaiotaomicron AHN 1368	Colon	RAX (Crittenden et al., 2002)	No
B. vulgatus RHI 3621	Colon	RAX (Crittenden et al., 2002)	No
B. vulgatus AHN 413	Colon	RAX (Crittenden et al., 2002)	No
B. xylanisolvens XB1A	Colon	OSX, BWX (Chassard et al., 2008)	Draft (NZ_ADWO00000000)
a. Abbreviations are: N.S., not specified; OSX, oat spelt xylan; RAX, rye arabinoxylan; WAX, wheat arabinoxylan; BWX, birchwood xylan; XOS, xylo-oligosaccharides.			
b. Only certain strains of these species which were tested ferment xylan.			

2.3.2. Prebiotic Oligosaccharides

Prebiotics were first defined as "Nondigestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health" (Gibson and Roberfroid, 1995). This definition was then refined to include other fields that can benefit from the selective targeting of specific microorganisms; "A selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microflora that confer benefits" (Gibson et al., 2004). For a prebiotic is classified as a food ingredient, it is necessary to provide the features listed below (Gibson and Roberfroid, 1995).

1. It should be fermented by intestinal microflora.
2. It should be resistant to gastric acidity, hydrolysis by mammalian enzymes and absorption in the upper gastrointestinal tract.
3. It should selectively stimulate the growth and or activity of intestinal bacteria potentially associated with health.

Prebiotics have several benefits, either directly or indirectly, to human health. These benefits are listed below.

1. Reduce symptoms related to inflammation and bowel disease
2. Reduces the prevalence of infectious and antibiotic-associated diarrhea
3. Provides preventative effects on colon cancer
4. It increases bioavailability and the uptake of various minerals such as calcium, magnesium, iron.
5. It reduces some risk factors for cardiovascular diseases
6. It encourages satiety and weight loss and helps prevent obesity.

Examples of various prebiotic examples include: inulin, fructooligo- saccharides (FOS), galactooligosaccharides (GOS), isomaltooligo-saccharides, soybean oligosaccharides and lactulose (Macfarlane et al., 2006). Xylooligosaccharides (XOS) is a novel prebiotic class to consisting of xylobiose xylotriose and more (Vazquez et al., 2000 and Chapla et al., 2012).

Xylooligosaccahrides are hydrolysis product of xylan. They are oligomers, β-1,4-linked xylose residues with various substituents such as; phenolic, acetyl and uronic acid. In addition to fruits and vegetables, it is also found in xylan rich lignocellulosic material obtained from agriculture, forestal and industrial wastes (Vazquez et al., 2000; Kumaret al., 2012). XOSs are present in the form of a white powder containing from 2 to 10 xylose molecules. These oligosaccharides have a broad pH range of 2.5-8.0. It is stable at low gastric pH and temperatures up to 100 ° C. XOS with an average DP value of 3-5 is more susceptible to alkaline decomposition compared to long chain DP-15 XOS (Courtin et al., 2009). XOS has good thermal stability during pasteurization. FOS is more sensitive to autoclave sterilization at low pH compared to XOS (Wang et al., 2009 and Courtin et al., 2009). As a food ingradient, XOS is not carcinogenic, encourages the growth of bacteria and intestinal fermentation and enhances mineral absorption. It also has an effect on anti-oxidant, anti-allergic, antimicrobial, immunogenic and selective cytotoxic activity and also effect blood and skin health (Moure et al., 2006). XOS selectively stimulates growth or activity of certain bacterial groups and is useful for the prevention of various diseases, such as bowel infection and colon cancer (Macfarlane et al., 2006). It has been observed that there are some differences in microorganism flora fed with XOS and FOS. FOS primarily promotes the growth of lactobacilli and XOS has a bifidogenic effect, but both are known to contribute to the healthy colon environment. Table 2.2 also shows the utilization capability of XOS and xylan in these two bacterial strains (Crittenden, 2002).

Table 2.2. Gowth of Bifidobacterium and Lactobacillus on XOS, Xylan and Arabinoxylan (Crittenden, 2002)

Organism	Strain	Growth on xylan	Growth on Xylo-oligo 70	Growth on arabinoxylan
Lactobacillus acidophilus	VTT E-94507[a]	–	+ (–)*	–
Lactobacillus amylovorus	VTT E-981145[a]	–	+ (–)*	–
Lactobacillus brevis	PEL1[b]	–	+++ (+)*	–
Lactobacillus crispatus	VTT E-96729[a]	–	n	–
Lactobacillus fermentum	VTT E-78077[a]	–	++ (–)*	–
Lactobacillus fermentum	VTT E-71033[a]	–	+	–
Lactobacillus johnsonii	VTT E-97978[a]	–	+ (–)*	–
Lactobacillus johnsonii	VTT E-97797[a]	–	+ (–)*	–
Lactobacillus paracasei	VTT E-94510[a]	–	+	–
Lactobacillus plantarum	VTT E-71034[a]	–	+	–
Lactobacillus plantarum	VTT E-79098[a]	–	+	–
Lactobacillus plantarum	VTT E-78076[a]	–	+ (–)*	–
Lactobacillus reuteri	Ing 1[c]	–	+ (–)*	–
Lactobacillus rhamnosus	Lc 705[d]	–	+	–
Lactobacillus rhamnosus	VTT E-97800[a]	–	+ (–)*	–
Lactobacillus rhamnosus	VTT E-96666[a]	–	+ (–)*	–
Lactobacillus salivarius	VTT E-97798[a]	–	+ (–)*	–
Bifidobacterium infantis	Bb-02[g]	–	+	–
Bifidobacterium adolescentis	VTT E-981074[a]	–	++	–
Bifidobacterium adolescentis	VTT E-991436[a]	n	+++ (+)*	+
Bifidobacterium angulatum	ATCC 27535[e]	n	+++ (+)*	–
Bifidobacterium bifidum	VTT E-97795[a]	–	+	–
Bifidobacterium bifidum	Bb-11[g]	–	+	–
Bifidobacterium breve	VTT E-981075[a]	–	+	–
Bifidobacterium breve	CIP 64.68[f]	–	+ (–)*	–
Bifidobacterium catenulatum	ATCC 27539[e]	n	++ (+)*	–
Bifidobacterium gallicum	ATCC 49850[e]	n	++	–
Bifidobacterium infantis	VTT E-97796[a]	–	+	–
Bifidobacterium lactis	Bb-12[g]	–	+++ (+)*	–
Bifidobacterium lactis	VTT E-97847[a]	–	+++ (+)*	–
Bifidobacterium longum	VTT E-96664[a]	–	++ (+)*	+++
Bifidobacterium longum	VTT E-96702[a]	–	++ (+)*	+++
Bifidobacterium longum	CSCC 5532[h]	–	+ (–)*	+++
Bifidobacterium pseudocatenulatum	ATCC 27919[e]	n	++ (+)*	–
Bifidobacterium pseudolongum	ATCC 25526[e]	–	+++ (+)*	–

Relative growth yield compared to growth on glucose: '–' = no growth; '+' = 0–40% of the OD on glucose; '++' = 40–80% of the OD on glucose; '+++' = 80–120% of the growth on glucose; 'n' = not performed.

Glucose and inulin had more bifidogenic activity compared to the XOS. Moreover, XOS stimulates the growth of *Bifidobacteria* at higher rate than FOS. Lactate, acetate and other fatty acids produced by these probiotic bacteria cause a high concentration of acid, and they lower the intestinal pH and thus prevent pathogenic and infectious bacterial growth.

The prebiotic effect of XOS depends on the rate of bacterial strain tested as well as the degree of dependent polymerization, substitution and ratio of arabinose and xylose (Singh et al., 2015). For example, compared to DP 2-3 XOS and DP 4-5 XOS; DP 2-3 XOS has a faster usage of XOS (Gullon et al., 2008).

CHAPTER 3

MATERIALS AND METHODS

3.1. Materials

3.1.1 Chemicals

In this study, all chemicals were used in their analytical standards and no purification and other methods were performed. All information about chemicals used during the experiments is given in Table A.1. in appendix A. De-ionized water was used during the preparation of solutions.

3.1.2 Test Microorganisms

Bifidobacterium animalis subsp. lactis DSM-10140, *Bacteroides ovatus* DSM-1896 and *Bacteroides xylanisolvens* DSM-18836 were purchased from Leibniz Institute DSMZ – German Collection of Microorganisms and Cell Cultures. *B.bifidum* NRLL B-41410, *B. breve* NRLL B-41408*, B.infantis* NRLL B-41661, *B.longum* NRLL B-41409 which were obtained from Agricultural Research Service Culture Collection.

For inoculum preparation and storage purposes, strains were grown anaerobically in appropriate medium. Strains were stored at -80°C in the medium supplemented with 25% glycerol as a cryoprotectant.

3.1.3 Test Substrates

3.1.3.1 Beechwood Xylan

Beechwood Xylan was obtain from Megazyme (USA). Lot number: 141201a. Properties of xylan was; Xylose 82.3%, Glucuronic acid 12.8%, Other sugars 4.9%, Protein 0.2%, Ash 4.7%, Moisture 4.1%.

3.1.3.2 Corncob Xylan

Corncob xylan was obtained by applying alkali extraction to corncob. The corncob, used in xylan extraction, was obtained from Ministry of Food Agriculture Livestock Aegean Agricultural Research Institute.

Extraction of xylan from corncobs; 180 g corn cob was divided into six separate Erlenmeyer flasks, and 300 mL of 10% NaOH was added to each flask. The flasks were kept at 37 ° C for 3 h in a shaking incubator at 150 rpm. The liquid and the solid portions were separated from each other by filtration through cheesecloth and then centrifugation. pH of liquid part was then adjusted to about pH 6.5 – 7.0 using HCl. The pH adjusted liquid was treated with ethanol -3 times the volume of the liquid- to precipitate the solubilized xylan and kept in the fridge for 1 h. Subsequently, the sediment was separated from the liquid by centrifugation. The precipitated solid was washed once with 75% ethanol. Finally, the solid was dried at 60° C overnight. Moisture content of solid part was determined by drying a small portion of it at 104 ^{o}C for 24 h. All experiment with this substrate, as well as others, were reported on a dry weight basis.

Xylan, arabinose and xylose content of the solid obtained after the extraction were determined by HPLC. The dry samples were dissolved in water and 4% sulphuric acid was added to mixture. The mixture was hydrolyzed at 121°C for one h in an autoclave. After that, pH was adjusted to 5-6 by addition of $CaCO_3$. Finally, samples were centrifugated and filtered through membrane filters (pore size 0.45 μm) and analyzed in HPLC.

3.1.3.3 Xylooligosaccharide

Xylooligosaccharide (XOS) was obtain from Shandong Longlive Bio-Technology Co. Ltd. Lot number: 20150601 According to manufacturer the properties of XOS were; Ash 0.05%, Moisture 2.3 %, XOS_{2-7} content (DM) 95.9 %, XOS_{2-4} content (DM) 76.5 %.

3.2. Media

3.2.1. Pre-culturing Media

3.2.1.1. Reinforced Clostridial Medium

The liquid medium used for the growth of *Bifidobacterium* species was Reinforced Clostridial Medium (RCM). This medium is generally used for cultivating and enumerating anaerobic bacteria from foods and clinical specimens. It is a nonselective enrichment medium and grows diverse anaerobic and facultative bacteria when incubated anaerobically. It has been used to detect clostridia, *Bifidobacteria* and other anaerobes in fecal samples and food products. Peptone and beef extract are found in the medium as nitrogen source. Also, it contains nitrogen, vitamins and minerals. Yeast extract supplies B-complex vitamins which stimulate bacterial growth. Dextrose is the carbohydrate source. Sodium chloride maintains the osmotic balance. Cysteine HCl is used for the reducing agent. Sodium acetate acts as a buffer. The media contains small amount of agar, it makes the medium semisolid. The composition of Reinforced Clostridial Medium is shown Table B.1. in Appendix B.

3.2.1.2 Wilkins-Chalgren Anaerobe Broth

The liquid medium used for the growth of *B. ovatus* and *B.xylanisolvens* were Wilkins-Chalgren Anaerobe Broth. Wilkins Chalgren Anaerobic Broth Base, formulated by Wilkins and Chalgren (1976), is the preferred medium for susceptibility testing of anaerobes. This medium is also used for testing anaerobic bacteria (Hecht et al., 2007). Wilkins Chalgren Anaerobic Broth is similar to the agar medium, except the agar. The broth medium is particularly preferred in the broth micro-dilution tests. Wilkins Chalgren Broth media need to support the growth of certain anaerobic bacteria. Hemin and Menadione (Vitamin K$_3$) improves the growth of *Bacteroides* species and many other species of gram-negative anaerobic bacteria. Peptic digest of animal tissues and casein enzymic hydrolysate are used as a source of essential nutrients, including carbon and nitrogen. While pyridines are used as an amino acid source, pyruvate acts as an energy source. The composition of Wilkins-Chalgren Anaerobe Broth is shown Table B.2. in Appendix B.

3.2.1.3 Fermentation Medium

The Fermentation Medium which was used for growth both of *Bifidobacterium* and *Bacteroides species* was Basal Medium (Palframan et al., 2003). The composition of Basal Medium is shown Table B.3. in Appendix B.

3.3. Methods

3.3.1. Hungate Procedure

Before strictly anaerobic cultures can be inoculated, media must be oxygen free. This procedure requires multiple steps. Liquid media was prepared, except heat sensitive materials such as Heamin and Vitamine K1. Resazurin was added at concentration of 0.001 g/L. Resazurin is an oxygen indicator that monitor the redox potential of the media. Resazurin in the powder form is blue. The aqueous solution of it is pink in the presence of oxygen and it is colorless in the absence of oxygen. All strict anaerobes require a minimum redox potential (Eh, -110 mV) at which resazurin turns colorless. In this way, any oxygen contamination could be observed easily. The medium was heated for 20-30 minutes, while being stirred on a magnetic stirrer. The color of media slowly turned from blue to pink. At that point, L-cysteine HCL (0.5g/L) was added. Cysteine was used as a reducing agent. Medium was heated for 10 more minutes. The heater and stirrer were turned off when no more change was observed in color. Then medium was treated with high purity N_2 gas until medium became transparent. The medium (3-9 ml) was transferred to the hungate tubes (15 ml) under N_2 gas and the screw caps with septum were put on. Tubes containing the media were autoclaved at 121°C for 15 minutes. After the sterilization, the heat sensitive materials, which were sterilized by membrane filtration-, using Minisart filters (pore size, 0.2 µm), were added sterile hypodermic syringes.

This procedure allowed the incubation to be completed without alteration of the anaerobic environment. With the sterile syringe, any material could be added into the tubes and the sample could be taken.

3.3.2. Solid Medium Preparation Procedure

Reinforced Clostridial Medium with 1.5% agar was used for the growth of *B.animalis subsp. lactis* in solid medium.

Wilkins-Chalgren Anaerobe Broth with 1.5% agar and 5% defibrinated horse blood was used for the growth of *B. ovatus* and *B.xylanisolvens* in solid medium. To prepare Non-selective medium for Bacteroides species, suspended Wilkins-Chalgren Anaerobe Broth with 1.5% agar was dissolved in distilled water, and sterilized 121°C for 15 minutes in the autoclave. After, cooling to 50-60 ^{o}C sterile 5% horse blood was added aseptically. The medium was mixed gently, and poured into sterile 9 cm petri dishes.

Anaerobic culturing system (Anoxamat, MART Microbiology, USA) was used to create anaerobic environment for petri dishes (Figure 3.1.). Anoxamat can create rapidly and automatically anaerobic condition in an anaerobic jar. The system evacuates a portion of the jar content and refills the jar with an anaerobic gas mixture consisting of nitrogen (80%), CO_2 (10%) and hydrogen (10%). During this procedure, the oxygen concentration in the air is rarefied. In this process, a catalyst attached to the lid was used to obtain and maintain absolute anaerobiosis. According to the manufacturer, a mere 0.16% of oxygen still remains in the jar when using the anaerobic recipe. Catalyst ensures the complete removal of oxygen.
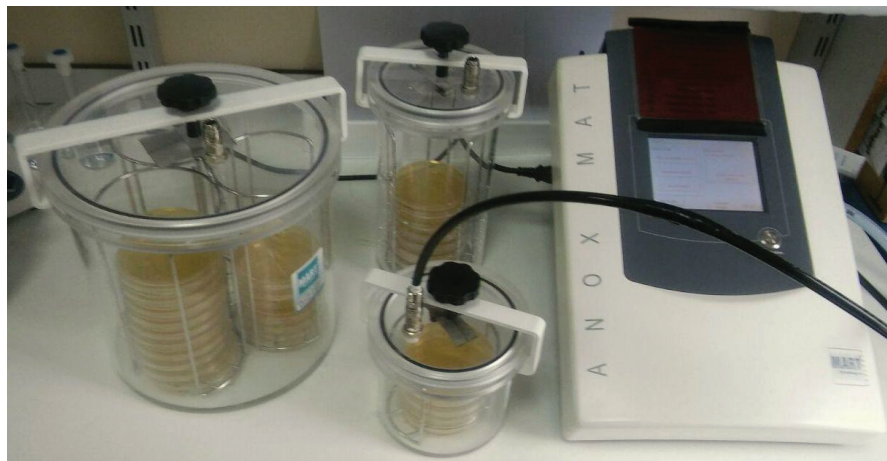

Figure 3.1. Anoxamat system

3.4. Fermentation Condition

The fermentation temperature kept constant at 37° C in all cases. pH was set to 6.8-7.0 before sterilization in the autoclave, using solution of NaOH and H_3PO_4. In order to control the continuity of the oxygen-free environment during the fermentation, it was checked for color change.

Fermentation medium was prepared except Heamin and Vitamine K_1 with anaerobic hungate procedure. After autoclaving, heat sensitive materials, which were sterilized with membrane filter, were added aseptically. While XOS were sterilized by membrane filter, beechwood xylan and corncob xylan were sterilized by autoclaving. Later they were added aseptically. Beechwood and corncob xylan had a viscous structure so that they could not pass through the membrane filters. Finally, 1% inoculum was added. Fermentations were monitored for 48 h; samples were taken at regular time intervals for further analysis.

3.5. Analysis

3.5.1. Cell Concentration

Growth was followed during all fermentations by plating samples on the selective agar media. Plates were incubated for 24-48 h in Anoxamat jar under anaerobic conditions. In order to follow the growth of the bacteria and to make a growth chart, selective media were used. The selectivity comes from gram positive and negative structure of bacteria. As mentioned before, *Bacteroides* species is gram negative *Bifidobacterium species* is gram positive bacteria.

Wilkins-Chalgren Anaerobe Medium supplemented with 1.5% agar, 5% defibrinated horse blood and gram negative anaerobic supplement. Gram negative anaerobic supplement, was used to enumerate *Bacteroides* species. Gram negative anaerobic supplements contains two antibiotics; Nalidixic acid and Vancomycin. These antibiotics act against gram positive bacteria like *Bifidobacterium species*. When samples from co-culture were spread on WCA, this selective medium only allowed to grow *Bacteroides*.

Bifidobacterium species were enumerated on RCA, which was prepared by adding 1.5% agar to RCM. When co-culture samples were cultivated only *Bifidobacterium* species can grow in this medium (Falony et al., 2009).

In order to test the selectivity of RCA, *Bacteroides* species were inoculated on this medium and checked whether they formed colonies. The absence of colonies after 48 h of incubation at 37°C showed that the RCA was selective towards *Bifidobacterium*. In the same way, WCA was found to be selective towards *Bacteroides*. Plate counting was used for enumeration of the bacteria. Plate counting is bacterial dilution with a diluent solution such as sterile 0.9% NaCl, until the bacteria are diluted enough to count correctly when spreading to a plate. The assumption is that each living bacterial cell forms a single colony. Bacterial cell numbers should be reduced by dilution because it is possible to produce colonies that are very close together (over 300) on a standard 9 cm plate. Plates with colonies between 30 and 300 are accepted in the countable range. However, plates containing fewer or more colonies are not suitable for counting. Samples that taken regular times were diluted to the range that could be counted and 0.1 ml of appropriate dilutions were spread on the agar plate using a sterile bacterial spreader. After 24-48 h incubation, the colonies were counted, and the result was calculated in terms of colony forming unit (cfu) per ml of culture.

3.5.2. Carbohydrate Concentration

High Performance Liquid Chromatography (HPLC) was used to determine concentrations of subsrates (Figure 3.2). Calibration curves given in Appendix C were obtained using standard solutions at certain concentrations. Samples that taken regular times were centrifugated and filtered by membrane filters with a pore size 0.45 μm (Sartorius). Samples were diluted 5 times with ultra-pure water.

The procedure and the operational conditions listed below;

HPLC system: Perkin Elmer, Series 200 (Shelton, USA)

Column: Rezex RPM-monosaccharides (Phenomenex, USA)

Mobile phase: Ultra-pure water

Injection volume: 20 μL

Flow rate of mobile phase: 0.6 mL min^{-1}

Column temperature: 80°C

Detector: Refractive index (RI)

Long chain sugars such as xylan and XOS could not be analyzed directly in HPLC. However, it was possible to quantify their concentrations indirectly, by measuring the amount xylose in their structures. Firstly, polymeric or oligomeric sugars were converted into the monomeric form using acid hydrolysis and analyzed by HPLC. For converting into monomeric sugar, samples were hydrolyzed with 4% sulphuric acid for one h at 121°C in the autoclave. After autoclaving, $CaCO_3$ was added to increase the pH to 5-6. Finally, samples were centrifugated and filtered by Sartorius membrane filters (pore size 0.45 μm) before HPLC analysis. Calibration curves given in Appendix C were obtained using standard xylose solutions at range of concentrations.

Figure 3.2. The HPLC equipment for carbohydrate analysis

Calculation of total XOS and Xylan is shown below;

$$= \frac{\text{Concentration of Detected by HPLC}}{\text{Known Concentration of Standard}} \times \text{Anhidro Factor (0.88 for xylose)}$$

3.5.3. Organic Acid Production

Organic acids excreted into the fermentation medium were quantified using HPLC (Figure 3.3). Calibration curves given in Appendix D were obtained using standard organic acid solutions at certain concentrations. With these calibration curves organic acid concentrations in samples were calculated from the peak areas in the chromatogram. Samples that taken regular times were centrifugated and filtered membrane filters (pore size 0.45 μm).

Figure 3.3. The HPLC equipment for organic acid analysis

The operational conditions and the procedure used for organic acid analysis in HPLC were listed;

HPCL system: Thermo Fisher Scientific

Column: BIORAD Aminex HPX-87H (300 x 7.8 mm)

Mobile phase: 5mM H_2SO_4

Injection volume: 20 μL

Flow rate of mobile phase: 0.6 mL min^{-1}

Column temperature: $50^{o}C$

Detector: UV 210 nm

CHAPTER 4

RESULTS & DISCUSSION

4.1. Characterization of Xylan from Corncob

Characterization of the solid obtained by the alkali extraction procedure in section 3.1.3.2, was carried out using HPLC. The amount of solid obtained from 360 g corncob was 66.87 g. In order to determine the amount of xylan in the solid obtained, solid was mixed with water to a concentration of 10 g/L and the mixture was analyzed for xylose and arabinose after acid hydrolysis. Before the hydrolysis the mixture was applied one of the following treatments:

i) Centrifugation

ii) Autoclave at 121°C for 15 min

iii) No Treatment (Analyzed directly)

iv) Autoclave at 121°C for 15 min and Centrifugation

The amounts of xylan and arabinose in the liquid were determined by HPLC (Table 4.1).

Table 4.1. Xylan concentaration of solid obtained from corncob

Treatments	Xylan Concentration
Centrifugation	2.8 g / L
Autoclave at 121°C for 15 min	5.8 g / L
No treatment	5.7 g / L
Autoclaved at 121°C for 15 min and centrifugation	5.3 g / L

The amount of xylan of the centrifuged xylan solution was 2.8 g / L, indicating that the insoluble xylan was precipitated. Since the solution was not centrifuged and autoclaved; as a result of the decomposition of the insoluble xylan during the acid hydrolysis, the amount of xylan of the untreated xylan solution was 5.7 g/L. According to the results of second and fourth treatment, the autoclave followed by centrifugation

again showed that some amount of xylan was precipitated. However, the results of the only autoclaved solution was almost similar to that value. In addition to xylan, the amount of arabinose was 0.8 g /L in autoclave and centrifugation treatments.

As mentioned in the preparation procedure for fermentation broth, corn cob xylan was sterilized by autoclave because the viscosity of the xylan does not pass through the membrane. Therefore, the amount of xylan in the fluid which has been autoclaved and centrifuged -for clarity- has been used in experiments. It also showed a low amount of xylan in the non-autoclaved solids; the solids could not be completely dissolved in the liquid and this resolution could be increased with the help of autoclave. The calculation of the net xylan in the solid is as follows:

$$(Amount\ of\ Xylan + Amount\ of\ Arabinose) \times Anhidro\ factor for\ xylan\ (0.88)$$

As a result; the xylan content of the solid obtained from corn cob was 53.6%.

4.2. Mono-culture Fermentation on Different Carbon Sources

Crittenden et al., (2002) showed previously that, some *Bifidobacteria* species grew to high cell concentration on XOS but they did not grow well on xylose. This shows that some *Bifidobacteria* import XOS before hydrolyzing and they do not have effective membrane transport mechanism for free xylose. Similar phenomena have been observed in *Bifidobacteria* for other non-digestible oligosaccharides and monosaccharides.

In contrast to that, it was shown that some *Bifidobacterium* species could grow on xylose as well. This was attributed to the different carbohydrate utilization mechanism even in different strains of the same species (Vrese and Schrezenmeir, 2008). It was concluded that the XOS and xylose metabolism in *Bifidobacteria* is species or strain specific, so that one should be careful in interpreting the observations.

The results obtained by growing the selected microorganisms on different sugars are shown in Table 4.2. Glucose was used as a control substrate in these experiments. In the fermentation carried out in the Hungate tubes, the growth was evaluated by checking the turbidity and the pH value in the tubes. The results showed that, two species of *Bacteriodes* (*B.xylanisolvens* and *B.ovatus*) showed good growth on corncob and beechwood xylan, XOS and xylose. Among the *Bifidobacterium* species, only *B.*

animalis subsp. lactis was found to grow on the XOS. None of the remaining *Bifidobacteirum* species were able to grow on these sugars. While *B. animalis subsp. lactis* could grow on XOS, it did not grow on xylose.

Table 4.2. Mono-culture fermentation on different carbon sources

Strain	Beechwood xylan	Corncob xylan	XOS	Xylose
B.xylansolvens	+	+	+	+
B.ovatus	+	+	+	+
B.animalis subps. lactis	-	-	+	-
B.bifidum	-	-	-	-
B.breve	-	-	-	-
B.infantis	-	-	-	-
B.longum	-	-	-	-

+: growth   - : no growth

4.2.1. Mono-culture Fermentation on XOS

B.animalis subsp. lactis, *B.xylanisolvens* and *B.ovatus* were selected as a result of experiments that was conducted to check bacterial growth on different carbon sources (Table 4.2). These bacteria were cultured in the hungate tubes, and analyzed. *B.animalis subsp. lactis* and *Bacteroides* species, previously grown in RCM and WCM respectively, were grown overnight in Basal medium containing XOS as the pre-culture and 1% inoculum was inoculated into the same medium. All the fermentation conditions mentioned in Section 3.4 were provided. Fermentation continued for 48 h. Samples were taken at regular times.

Cell Concentration and XOS degradation: Samples taken at specific time intervals were plated on RCA for *Bifidobacteria*, on WCA for *Bacteroides* species medium with necessary dilutions. The colonies formed at the end of 48 h were counted.
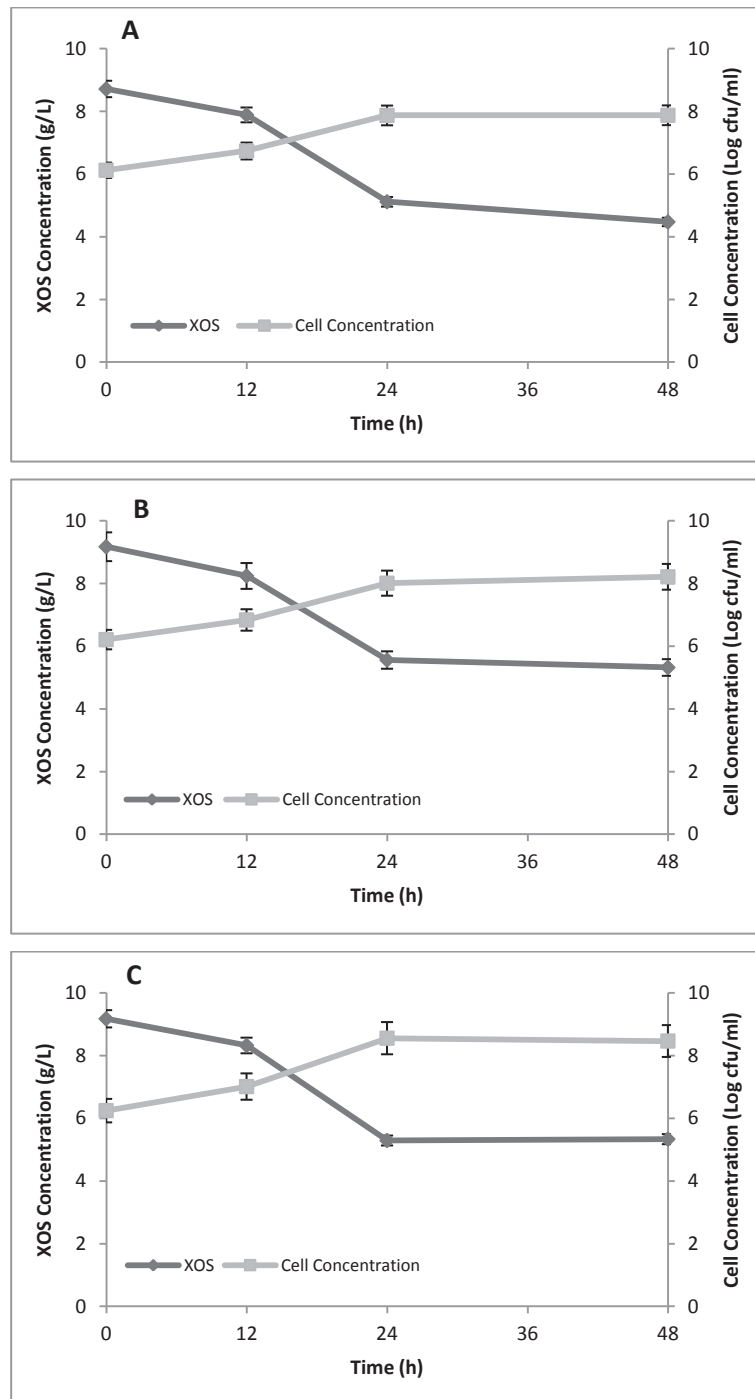
Figure 4.1. Mono-cultured growth and XOS consumption by **a**) *Bifidobacterium animalis subsp. lactis* **b**) *Bacteroides xylanisolvens* **c**) *Bacteroides ovatus*

In the figure 4.1., starting amount of each bacterium was the same. They grew for 24 h and then entered the stationary phase. XOS consumption was also negligible after 24 h. So, XOS consumption and cell concentrations were parallel. Although their growths on XOS were close to each other, *B. ovatus* grew slightly more. It was concluded that XOS was a good carbon source for these three organisms.

In the in-vitro studies XOS were utilised well by some *Bacteroides* isolates in addition to *Bifidobacteria* (Crittenden et al., 2002). However generally XOS are selectively and preferentailly fermented by *Bifidobacteria* and *Lactobacilli*. The ability of microorganism to metabolise XOS depends on their xylanolytic enzyme systems. A xylosidase and other few enzymes have been reported in some *Bifidobacteria* (Chapla et al., 2012).
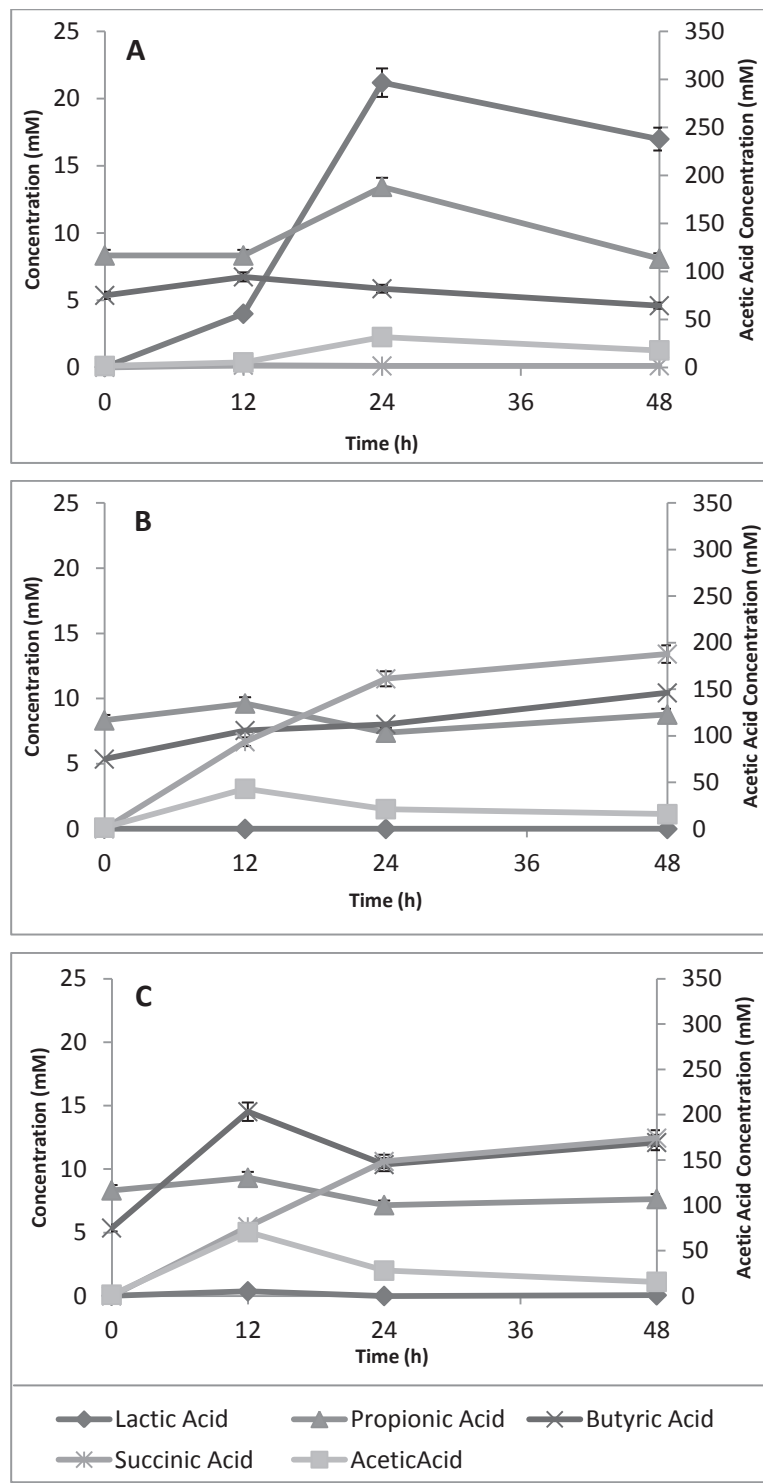
Figure 4.2. Organic acid production on mono-cultured with XOS **a**) *Bifidobacterium animalis subsp. lactis* **b**) *Bacteroides xylanisolvens* **c**) *Bacteroides ovatus*

B.animalis subsp. lactis grown on XOS produced acetic and lactic acid and propionic acid Figure 4.2.a. The highest level of lactic acid (21.18 mM) was obtained in 24 h. Acetic acid increased rapidly between 12 and 24 h after that, decreased slightly. The highest level of acetic acid was 30.18 mM. No increase in butyric acid was observed, succcinic acid was not produced at all. The maximum amounts of organic acids were usually in 24 h, since cells did not show any appreciable growth after 24 h. After that they either remained stable or increased only slightly.

When *B.xylanisolvens* was grown on XOS (Figure 4.2.b), the succinic acid reached 11.51 mM in 24 h and continued to increase in small quantities. Butyric acid concentration was 2.62 mM in 24 h. Acetic acid reached 42.10 mM in 12 h, which decreased to 20.25 mM in 24 h and remained constant until 48 h. But still acetic acid was the dominant SCFA in the medium. Lactic acid was not produced, whereas propionic acid only reached 1.3 mM in 12 h and then decreased.

As shown in the Figure 4.2.c, in *B.ovatus* culture, the succinic acid reached 12.44 mM in 48 h and butyric acid concentration was 5.01 mM in 24 h and continued to increase in small quantities. Acetic acid was the dominant SCFA in the medium. It reached 69.08 mM in the 12 h, which decreased to 26.61 mM in 24 h. Lactic acid was not produced, whereas propionic acid only reached 0.99 mM in 12 h and then decreased.

Comparisons between of these three bacteria growing on XOS can be made as follows. The cell concentration and XOS degradation were almost the same in all bacteria but differences were observed in organic acid production as expected. Organic acid profiles of *B.xylanisolvens* were different compared to *B. animalis subsp. lactis*. Butyric acid was produced by both *Bacteroides* species, the amount was higher in *B.ovatus* than *B. xylanisolvens* and there was no lactic acid production. Propionic acid production was observed in *B.animalis subsp. lactis* cultures, but no propionic acid production was observed in two *Bacteroides* species. While succinic acid was not produced in *B.animalis subsp. lactis*, whereas in *Bacteroides* species it was produced in close values. Finally, the acetic acid was produced by all bacteria but the highest value was seen in *B.ovatus* culture. In *Bacteroides* cultures, acetic acid produced at earlier stage (12h) compared to *Bifidobacterium* culture. Also, its concentration started to decrease after 24h in all cultures. These profiles were later used to evaluate the co-cuultures.

4.2.2. Mono-culture Fermentation on Beechwood Xylan

Bacteroides species, previously grown in WCB, was grown overnight in Basal medium containing glucose as pre-culture medium and 1% inoculum was inoculated into the hungate tubes containing Basal medium with beechwood xylan. All the fermentation conditions mentioned in Section 3.4 were provided. Fermentation continued for 48 hours. Samples were taken at regular times.
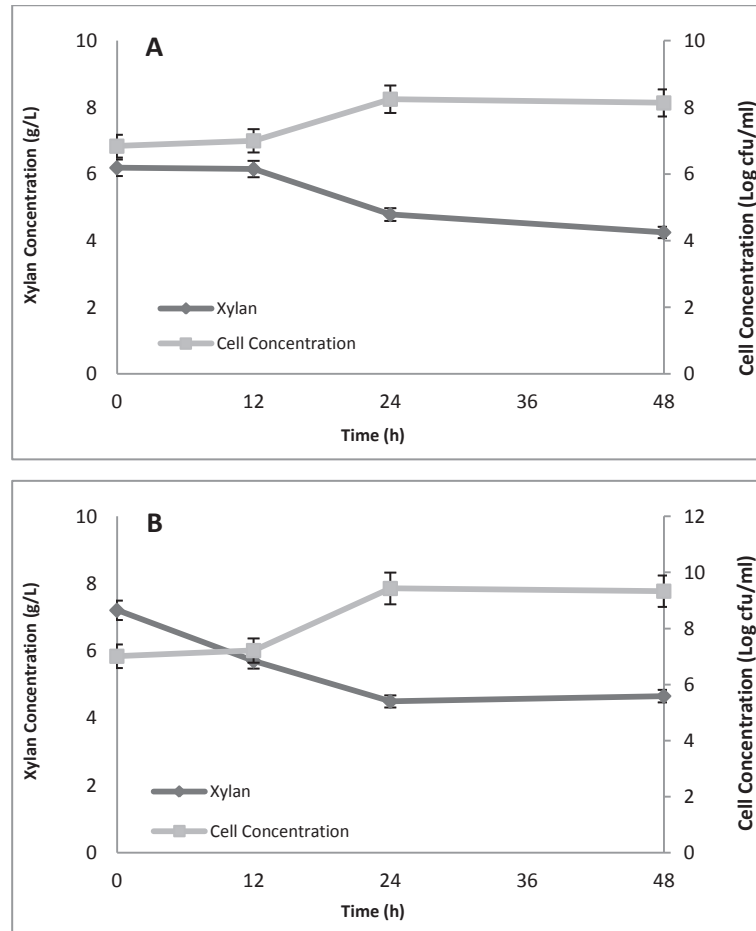
Figure 4.3. Mono-cultured growth and beechwood xylan consumption by **a**) *Bacteroides xylanisolvens* **b**) *Bacteroides ovatus*

Both *Bacteroides* species grew on xylan rapidly until 24 h. After that two bacteria entered the stationary phase. In the same way xylan consumption was negligible after 24 h. Comparing cell concentration and xylan consumption of two bacteria, it was observed that the *B. ovatus* had higher cell concentration and xylan consumption.

In the human colon, xylanolytic community is present at high levels. Especially *Bacteroides* genus plays a major role in the degradation of xylan (Chassard et al., 2008). The main xylan degrading species isolated from human faeces were *Bacteroides* and *Roseburia* (Chassard et al., 2007). The major xylanolytic bacteria among the isolates were *Bacteroides fragilis* and *Bacteroides ovatus*. However, new xylanolytic species were found in later studies (Chassard et al., 2008). *B. xylanisolvens* is important among these species. because it is more specialized in xylan degradation and fermentation (Mirande et al., 2010).

Figure 4.4. Organic Acid Production on mono-cultured with beechwood xylan **a**)*Bacteroides xylanisolvens* **b)***Bacteroides ovatus*

Lactic acid and propionic acid production was not observed in the two *Bacteroides* species. While the production of butyric acid was slightly higher in *B. xylanisolvens*, whereas the production of acetic acid was greater in *B. ovatus*. Lastly; succinic acid production was observed at close values (16.57 and 17.16mM) in both bacteria (Figure 4.4).

In the colon, *Bacteroides* species generally produced propionic, succinic and acetic acid. Fermentation of xylan by *B. xylanisolvens* leads mainly propionic and acetic acid (Mirande et al., 2009). In current study, *B. xylanisolvens* grown in beechwood xylan produced 43.99 mM acetic acid but did not produce propionic acid on the contrary. The same situation was seen in *B. ovatus*. It produced 69.89 mM acetic acid.

4.2.3. Mono-culture Fermentation on Corncob Xylan

Bacteroides species, previously grown in WCB, was grown overnight in Basal medium containing glucose as pre-culture medium and 1% inoculum was inoculated into the hungate tubes containing Basal medium with corncob xylan. All the fermentation conditions mentioned in Section 3.4 were provided. Fermentation continued for 48 hours. Samples were taken at regular times.
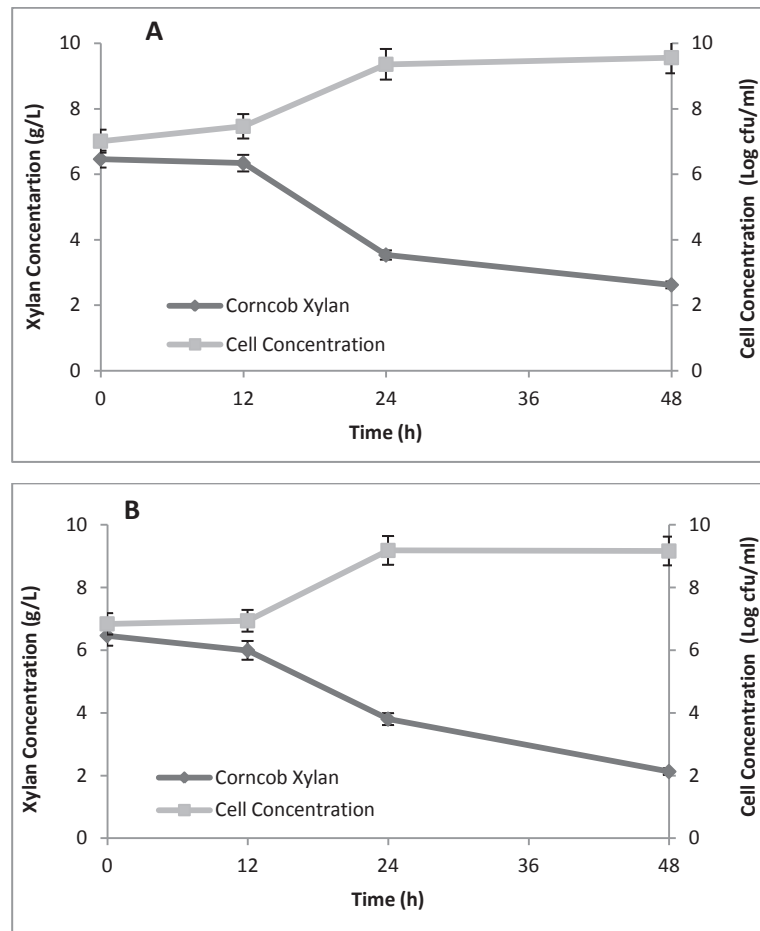
Figure 4.5. Mono cultured growth and corncob xylan consumption by **a**) *Bacteroides xylanisolvens* **b**) *Bacteroides ovatus*

Both *Bacteroides* species grew on xylan rapidly until 24 h. *B. xylanisolvens B.ovatus* grew about 2.33 and 2.55 log respectively. After that two bacteria entered stationary phase. In contrast xylan consumption continued slightly until 48h (Figure 4.5).
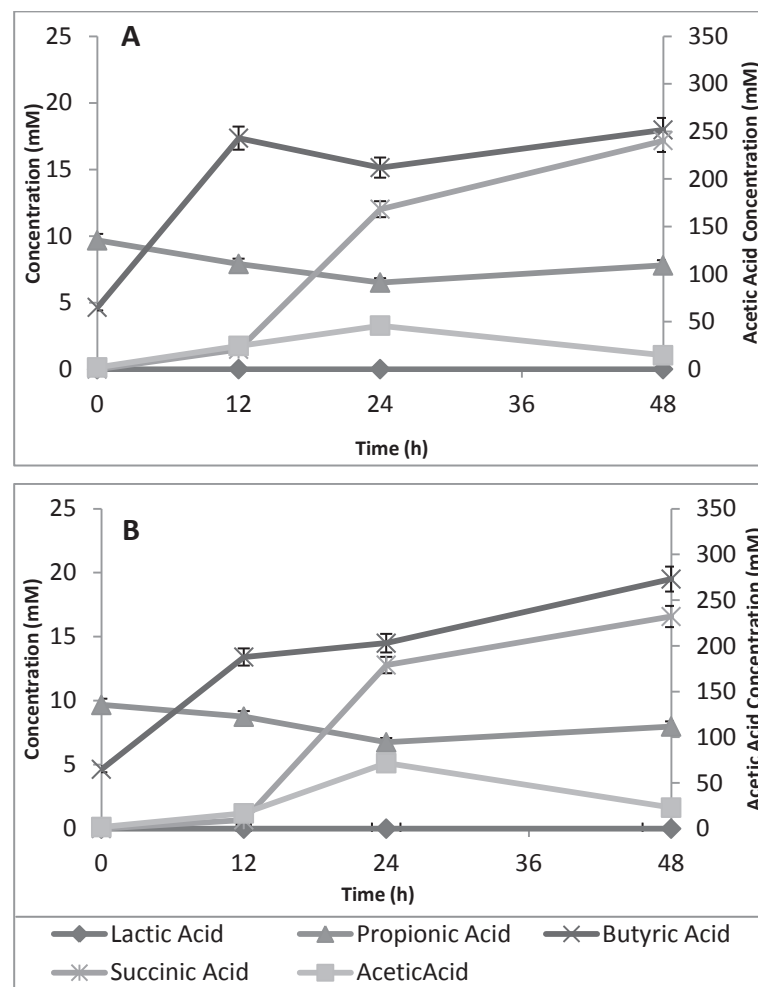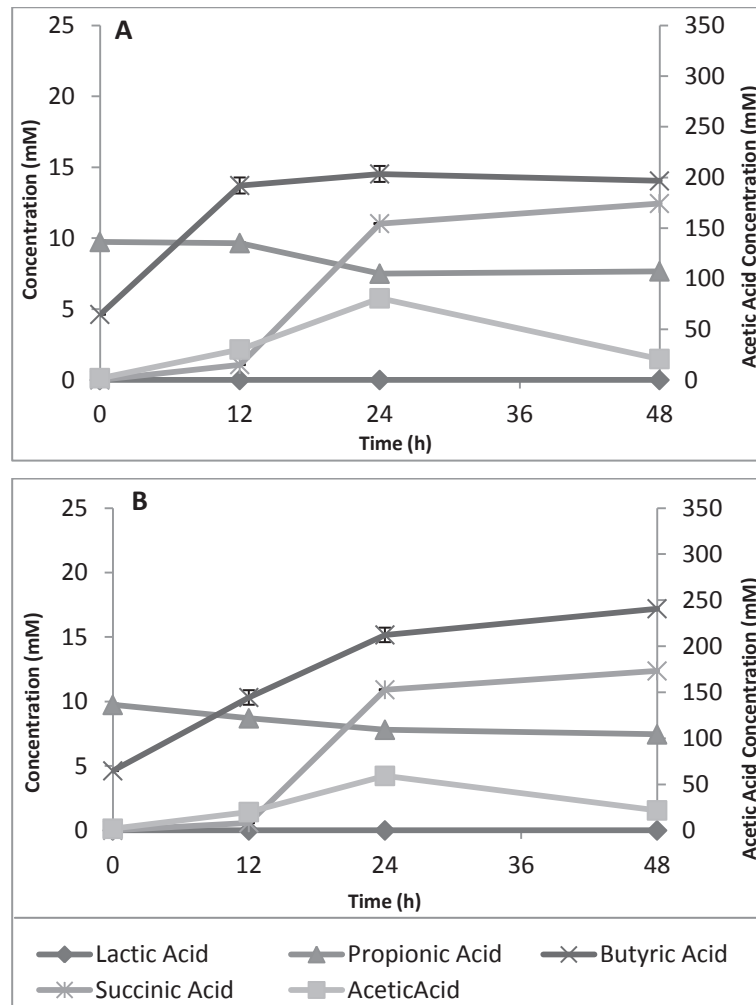
Figure 4.6. Organic Acid Production on Mono-cultured with Corncob Xylan **a)***Bacteroides xylanisolvens* **b)** *Bacteroides ovatus*

Lactic acid and propionic acid production was not observed in the two *Bacteroides* species. While the production of butyric acid was slightly higher in *B. ovatus*, whereas the production of acetic acid was greater in *B. xylanisolvens*. Succinic acid production was observed at close values (12.37 mM and 12.44 mM) in both bacteria (Figure 4.6).

The use of xylan and cell concentrations of *Bacteroides* species grown on corncob xylan were close to each other. Compared with beechwood xylan, it was observed that the *Bacteroides* species were grown on corncob more and thus the xylan degradation was higher. However, it was interesting to observe that this difference did not have an appreciable difference in organic acid production.

As in beechwood xylan, no production of lactic acid and propionic acid has been observed in corncob xylan. The values of other produced organic acids were close to each other.

4.3. Co-culture Fermentation on Different Type of Xylan

Bacteroides species and *B. animalis subsp. lactis* previously grown in WCB and RCM respectively, were grown overnight in Basal medium containing glucose as pre-culture medium and equal amounts of the overnight cultures of *B. lanimalis* subsp. *lactis* and one of the two Bacteroides species were inoculated (1%) simultaneously into the hungate tubes. All the fermentation conditions mentioned in Section 3.4. were provided. Fermentation continued for 48 h. Samples were taken at regular times.

## 4.3.1 Co-culture Fermentation on Beechwood Xylan

Cell Concentration: Samples taken at specific time intervals, were plated on WCA with defibrinated horse blood and gram negative anaerobic supplement medium for enumeration of *Bacteroides* species. The colonies formed after 48 h were counted. The colonies formed in the WCA medium were morphologically round, flat-edged, raised, flat surface, bright yellow, moist and homogenous. *Bifidobacterium animalis subsp. lactis* colonies formed in the RCA medium were morphologically round, raised, flat surface, smaller colonies compare with *Bacteroides* species, bright yellow-white, moist and homogenous (Figure 4.7).
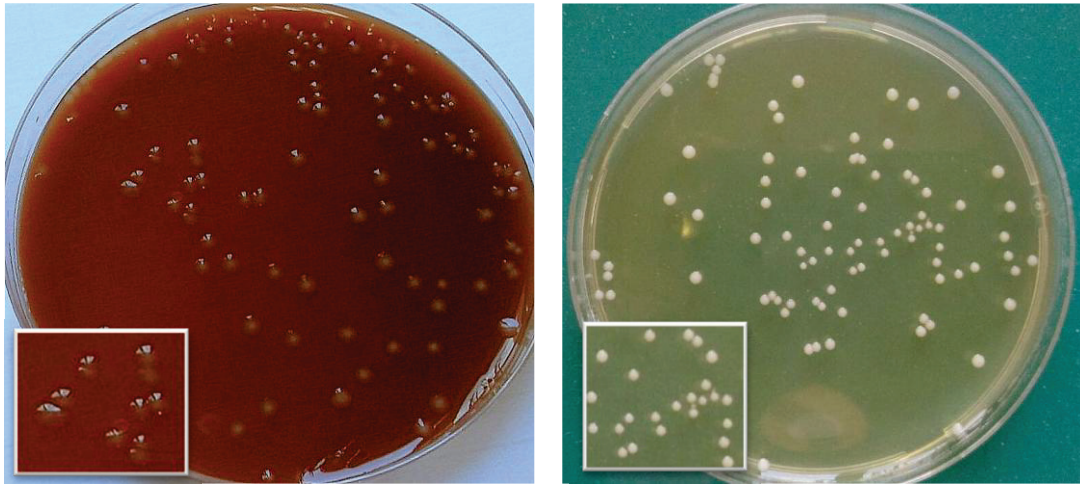
A.   B.

Figure 4.7. Colony morphology **a)** *Bacteroides xylanisolvens* **b)** *Bifidobacterium animalis subsp. lactis*

Bacteria grown in selective medium were stained with "Gram stain" and examined under microscope for control purposes. Gram positive *Bifidobacteria* are observed purple while gram negative *Bacteroides* are pink (Figure 4.8).
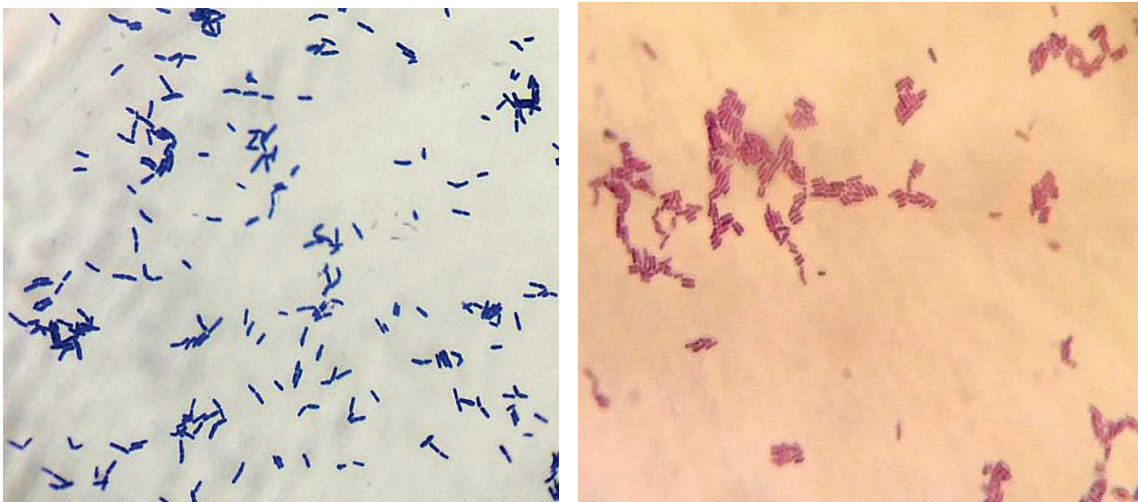
A.   B.

Figure 4.8. Microscopic morphology **a)** *Bifidobacterium animalis subsp. lactis* **b)***Bacteroides xylanisolvens*
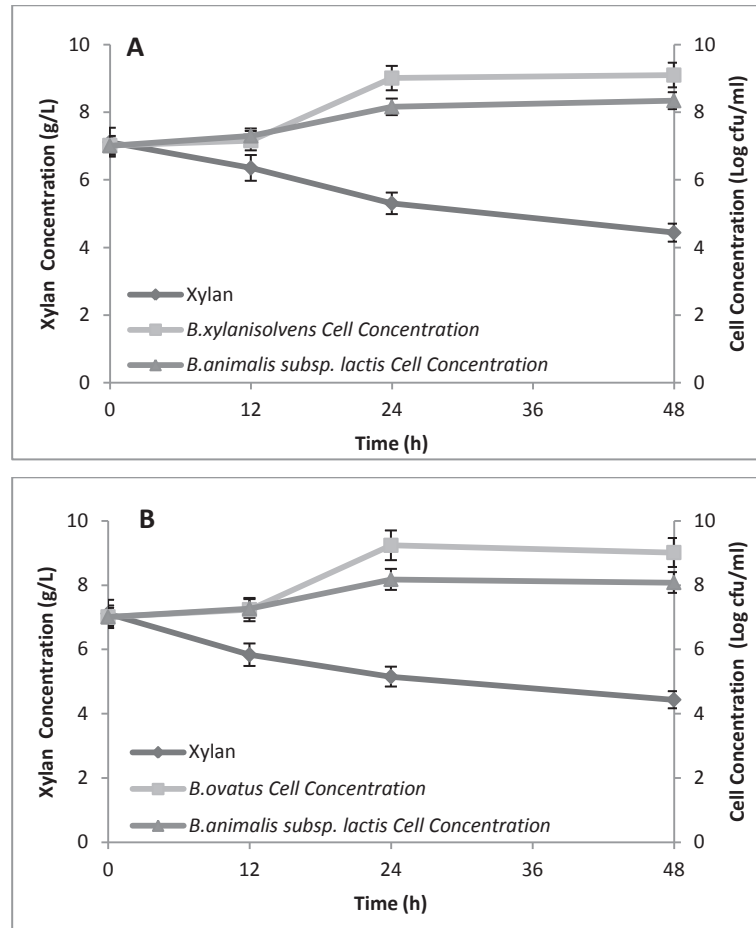
Figure 4.9. Co-cultured growth and beechwood xylan consumption by **a)**_Bifidobacterium animalis subsp. lactis_ with _Bacteroides xylanisolvens_ **b)**_Bifidobacterium animalis subsp. lactis_ with _Bacteroides ovatus_

B. animalis subsp. lactis did not grow on beechwood xylan (Table 4.2), but when co-cultured with _Bacteroides_ species, it grew 1.5-2.0 log in 24h (Figure 4.9). There were no significant differences in xylan degradation and _Bacteroides_ growth in the two co-cultures on beechwood xylan. _Bacteroides_ cell concentrations in both cultures were higher than in _B.animalis subsp. lactis._ Although cell concentrations were at the same level at 12 h in both bacterial species, _Bacteroides_ species have increased the difference until 24 h.

When _Bacteroides_ species was grown as mono culture in beechwood xylan, xylan concentration was lower at the end of 48 h compared to co-culturing. This may be due to the rapid pH decreases from the organic acids that will be discussed later.
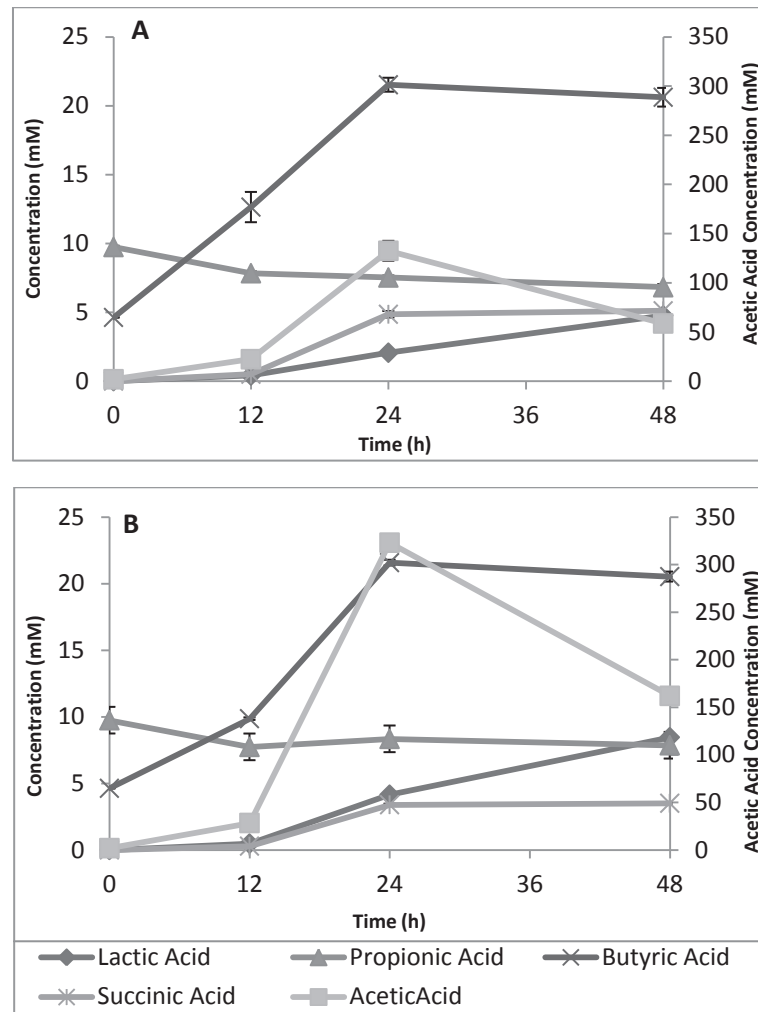
Figure 4.10. Organic acid production on co-cultured with beechwood xylan
a)*Bifidobacterium animalis subsp. lactis* with *Bacteroides xylanisolvens*
b)*Bifidobacterium animalis subsp. lactis* with *Bacteroides ovatus*

As seen in Figure 4.10, co-culture of *B.xylanisolvens* and *B.animalis subsp. lactis* growing on beechwood xylan produced acetic and lactic acid succinic and butyric acid. Acetic acid was high concentration. The highest level of lactic acid was produced in 24 h, 128.3 mM. Acetic acid concentration reached 128.3 mM in the 24 h, whereas it decreased to 57.8 mM after 48h. The highest level of butyric acid and succinic acid was produced in 24 h, 12.14 mM and 5.17 mM respectively.

When compared with mono-culture results of *B. xylanisolvens*, the amount of acetic acid was 127.64 in co-culture fermentation, 43.99 mM in mono-culture. Butyric acid concentration was comparable in mono and co-culture an it was measured as 13.33 mM in mono-culture as 17.18 mM in the co-culture. While lactic acid is not produced in

the mono-culture at all, in the co-culture it was 4.83 mM in 24 h. Briefly, lactic acid, butyric acid and acetic acid were produced more in co-culture than mono-cultures and the amount of succinic acid decreased. There was no change in the propionic acid values.

In the mono-culture of *B.animalis subsp. lactis* growing on XOS, the dominant organic acids were lactic and acetic acids. Thus, the significant acetic acid concentration in co-culture fermentation may have been due to production of this acid by *B.animalis subsp. lactis.*

As seen in Figure 4.10 b*,* the co-culture of *B. ovatus* and *B.animalis subsp. lactis* growing on beechwood xylan produced acetic, lactic succinic and butyric acid. Similar to the previous co-colture, acetic acid concentration was very high. The highest level of acetic acid was 319.38 mM after 24 h. The acetic and lactic acid value were higher compared co-culture *B.xylanisolvens* and *B.animalis subsp. lactis*.

Compared to mono-culture of *B. ovatus*, butyric acid, acetic acid and lactic acid values were increased in co-culture. There was no change in the propionic acid values and succinic acid value was reduced.

In addition to monitoring the use of xylan, oligosaccharide analyzes were performed by HPLC. In this analysis oligosaccharides present in the medium from xylobiose to xylohexaose can be analyzed. However, no oligosaccharides were observed in samples taken from co-cultured tubes. This may indicate that the oligosaccharides formed by *Bacteroides* species as a result of xylan hydrolysis may have been immediately catabolized by the *B. animalis* subsp. *lactis,* as soon as they were formed.

4.3.2 Co-culture Fermentation on Corncob Xylan

Arabinoxylan and arabinoxylooligosaccharides have the potential to reveal beneficial effects in humans. So far, some attempts have been made to investigate how the AXOS compounds will act prebiotic. Strategic research on different AXOS compounds is highly encouraging in terms of health promotion and development (Grootaert et al., 2007).
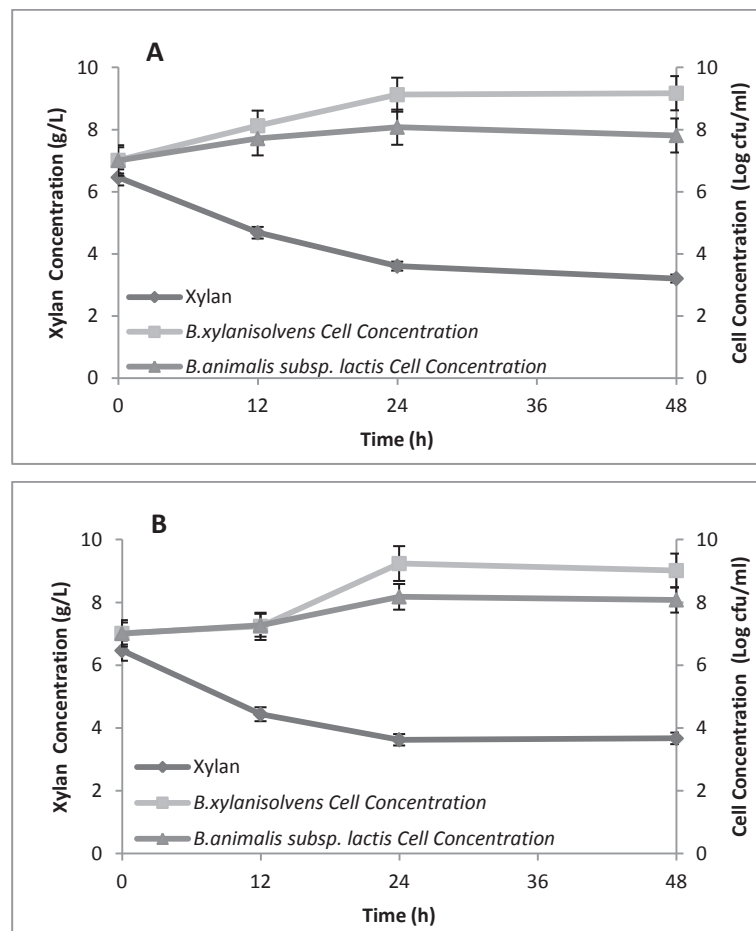

Figure 4.11. Co-cultured Growth and Corncob Xylan Consumption by **a)***Bifidobacterium animalis subsp. lactis* with *Bacteroides xylanisolvens* **b)***Bifidobacterium animalis subsp. lactis* with *Bacteroides ovatus*

B. animalis subsp. lactis which did not grow on corncob xylan, grew by about 1.5-2.0 log in 24h when co-cultured with Bacteroides species. There were no significant differences in xylan degradation and growth in the two co-cultures on corncob xylan. However, when compared to beechwood xylan, xylan degradation was observed to be slightly faster. Corncob xylan was utilized more rapidly than beechwood xylan in the mono-cultured as well.

The increase in the number of *Bifidobacterium* in co-cultured with beechwood and corncob xylan was clearly observed. The increasing in the number of *Bifidobacterium* is very beneficial in terms of colon health. When *Bifidobacterium* species concentration increase in colon, they are replaced by other bacteria. This shift in the intestinal microbiota is associated with improved overall health, better absorption of minerals, reduced gut infections and suppression of colon cancer initiation (MacFarlane et al., 2006).

Arabinoxylan was not fermented by *Bifidobacteria* species (except *B. longum*) or by any of the *Lactobacilli* (Crittenden et al., 2002). Importantly, it was not fermented by the potentially detrimental intestinal bacteria such as, *E. coli, C. perfingens and C. difficile*. Arabinoxylan can be regarded as an applicable polysaccharide to complement *B. animalis subsp. lactis* and *Bacteroides* species in symbiotic combination.

In terms of health functionality, larger and more slowly fermentable polysaccharides may provide more advantages than rapidly fermented oligosaccharides, because they provide a carbohydrate source for distal part of colon (Crittenden et al., 2002). It may be an advantage to digest slower due to the longer structure of arabinoxylan. It may be able to reach the distal part of that colon and increase the number of *Bifidocterium*.
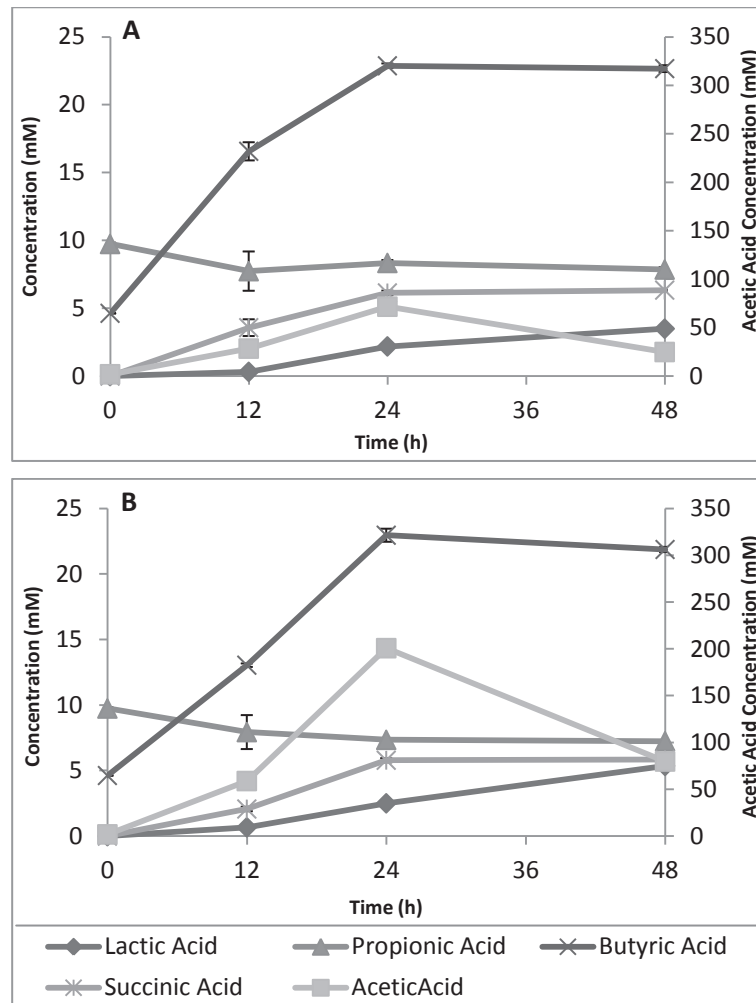
Figure 4.12. Organic Acid Production on Co-cultured with Corncob Xylan **a)***Bifidobacterium animalis subsp. lactis* with *Bacteroides xylanisolvens* **b)***Bifidobacterium animalis subsp. lactis* with *Bacteroides ovatus*

All acids except propionic acid were produced in the co-culture of *B. ovatus* and *B. animalis* subsp. *lactis* (Figure 4.12) . Compared to the mono-culture of *B. ovatus*, the amount of lactic acid and butyric acid increased while the amount of succinic acid decreased to half. Production of propionic acid was not observed as in other cultures.

Compared to the co-culture of this bacteria on beechwood xylan, all acid's concentration except acetic acid were close each other. However, acetic acid did not show increase as in co-culture on beechwood xylan, and this value (68.01 mM) was close to the mono-culture on corncob xylan (74.76 mM).

Similar to other cultures, lactic, butyric and acetic acid values were increased compared to mono-culture. However, there was an extreme increase in the acetic acid as well as in the co-culture with beechwood xylan. Acetic acid was expected to be high

compared to others, because acetic acid was main product of xylan breakdown (Macfarlane, 2003).

It was observed that the amounts of acetic lactic and butyric acids increased in co-cultures either on corncob or beechwood xylan. These SCFAs and lactic acid are very beneficial in terms of human health. For example, these help regulate sodium and water absorption; they act to lower colonic pH so it can inhibit growth of potential pathogens, they promote the growth of beneficial bacteria such as *Bifidobacterium* and *Lactobacilli* (Slavin, 2013).

Observing butyric acid increase in co-culturing with corncob and beechwood xylan is very important for human health. Because butyric acid is a preferred energy source for colonocytes (Roediger, 1982), stimulates colon epithelial cells, thus increasing the absorptive capacity of the epithelium (Topping and Clifton, 2001). Additionally, it inhibits the growth of colonic carcinoma cells (Scheppach et al., 1995).

There was no lactic acid production on mono-culture except growth of *B. animalis subsp. lactis* on XOS. Although produced in co-cultures, it did not show any significant increase. In the same way, Macfarlane et. al. (2003), also shown that lactic acid generally was not produced in significant quantities from xylan degradation.

Table 2.4. shows that other *Bifidobacterium* species namely; *B. bifidum*, *B. breve*, *B. infantis* and *B. longum* did not grow on XOS, xylose and xylan. Yet, co-culturing of these species with *Bacteroides* species was tested. As excepted, there was no *Bifidobacteria* dedected on solid cultures. In other words, among the *Bifidobacteria* species tested in the study only *B. animalis subsp. lactis* was able to grow on xylan when co-cultured with *Bacteroides* species. It was showed that, even if *Bacteroides* species could form XOS in the medium as intermediates, *Bifidobacterium* species except *B.animalis subsp. lactis* could not grow because lack of ability to use XOS in those bacteria.

pH was monitored during the experiments and the pH of mono-cultured and co-cultured bacteria were compared. In all mono-cultures, the pH was measured around 5.30-5.50 after 48 h, while in the co-cultures the pH was measured as around 5.30 on after 24 h and decreased to 4.50 after 48 h. Observing the same situation in every mono and co-culture suggests that this difference may be due to the amount of organic acids produced. It should be noted that, either mono or co-cultures may behave differently in terms of kinetics of cell and organic acid productions and carbonhydrate consumptions when pH is not allowed to decrease and maintained at physiological levels.

In this study, we examined how *Bacteroides* and *Bifidobacterium* responded to two different xylan, as a result of cross-feeding. This phenomenon has previously been studied on different bacteria and substrates. Cross-feeding interactions among members of the microbiota of the colon is a common phenomenon. In general, intestinal ecosystems have two different mechanisms of cross-feeding, metabolic cross-feeding and substrate cross-feeding. Metabolic cross-feeding is the consumption of fermentation end products by other bacteria and substrate cross-feeding is the utilization of the partially cleaved intermediate produced by bacteria during the process of degrading a complex carbohydrate. This cross feeding example can be given by Cotta and Zeltwanger's work (1995). They used xylan degrading *Butyrivibrio fibrisolvens* and the XOS utilizing *Selenomonas ruminantium.* Study of Li et. al. (2014) can be given as one of the examples of cross-feeding. In that study, *Bifidobacterium* could not use agarose oligosaccharide because it had high degree of polymerization, however the *Bacteroides* species could utilize carbohydrates with high degree of polymerization. Thanks to cross feeding, the number of *Bifidobacterium* species was increased when *Bifidobacterium* species were co-cultured with *Bacteroide*s species on agarose oligosaccharide. Falony et al. (2009) investigated inulin-type fructan degradation capacities of four *Bifidobacteria* strains. These *Bifidobacteria* species have different carbohydrate mechanism on inulin and oligofructose. All *Bifidobacteria* strains were grown in co-culture fermentation with *Bacteroides thetaiotaomicron* which able to metabolize both oligofructose and inulin. They observed that some of the some *Bifidobacterium* species grew, thanks to the *Bacteroide*s species could break down inulin and formed oligosaccharides in different degree of polymerization.

Although the probiotic term is more associated with lactic acid bacteria such as *Lactobacillus* and *Bifidobacterium*, it may also spread to other microorganisms. For example, *Bacillus* species have been used as a probiotic for at least 50 years in a commercial Italian product. One of the benefits of using *Bacillus* strains is that they are resistant to antibiotics. Therefore, it is recommended that probiotic formulations containing some *Bacillus* species be used together with antibiotics. Although, in Italy and the UK, the use of *B. subtilis* as a supplement has been approved, the microorganism should be allowed if it has special properties for probiotic strains (Soccol et al., 2010). In addition to *Bacillus* strains, many different types of species are probiotic. For example, *Enterococcus, Streptococcus, Aspergillus* and *Saccharomyces* are used probiotic microorganisms in animal preparations (Fox, 19889). Although

theoretically, probiotics cause side effects such as systemic infections, harmful metabolic activities, excessive immune stimulation and gene transfer in susceptible individuals, these side effects are uncommon in *Bifidobacterium* and *Lactobacillus* species. For this reason, these two species are most commonly used commercial. A variety of studies can be conducted to assess the safety of a probiotic strain. For example; i) intrinsic features of the strains, ii) interactions between host and strain, and iii) the pharmacokinetics of the strain such as survival in the digestive tract, intestinal activity, dose-response relationships, fecal and mucosal recovery (Soccol et al., 2010). These studies on *Bacteroides* in the future can better explain their potential.

Although there are pathogens among the *Bacteroides* species, such as, *B. fragilis*, these species also have important human health benefits. In recent studies, it is thought that certain strains of *Bacteroides* are involved in the prevention of infection with *Clostridium difficile* (Hopkins and Macfarlane, 2002). Some *Bacteroides* species, which can produce antibacterial peptides, can prevent colonization of pathogens in the colon (Hooper et al., 2003).

In addition to increasing the number of probiotic bacteria that are beneficial to human health by using xylan, increasing the number of *Bacteroides* species using this subsurate can also provide a two-way advantage. It is also an advantage that xylan, as a substrate, is cost effective material. Because, many agriricultural, forestal, and food industrial residues and by products contain substantial amount of xylan in their structure. Using conventional and novel methods, xylan can be extracted in its polymeric form and can be utilized in various applications, including as a functional food additive. Moreover, xylan is a long chain carbohydrate. This can be considered as another advantage, because generally carbohydrate fermentation takes place in the proximal part (first part of the colon). As a result of fermentation, SCFAs and lactic acid are formed, the pH of the column decreases and a beneficial gut environment is formed. This way, colon cancer and infection risks are reduced. In contrast, protein degradation occurs in the distal part of the colon and as a result of this; ammonia, polyamines, polyphenols and other metabolites are formed. The arabinoxylan and arabinoxylan oligosaccharides can be graded towards the last part of the column (distal part), because the fermentations are suppressed by the other easier-to-use substrates. The increase in carbohydrate fermentation in the distal colon is thought to reduce the risk of colon cancer (Grootaert et al., 2007).

Studies on the ability of *Bifidobacteria* to ferment XOS were carried out both in vitro and in vivo. *Bifidobacteria* can ferment XOS, but many bacterial species such as *Clostridium* spp. and *Klebsiella pneumoniae* can also ferment XOS (Van Laere et al., 2000). Specificity is a desirable feature of having prebiotic substrates and this feature seems more suitable for xylan or arabinoxylan, which are more difficult to be utilized and not easily used by every bacterium species.

CHAPTER 5

CONCLUSION

This study was conducted to investigate the utilization of xylan and its hydrolysis products such as XOS and xylose, by two bacterial species, which are dominant in homan colon. *Bifidobacterium* species has been known to exert beneficial effects on human health, whereas *Bacteroides* species are able to utilize polysaccharides. In this study, firstly, it was shown that Bifidobacteria are unable to utilize xylan. Amoung the tested *Bifidobacteria* only *B. animalis* subsp. *lactis* could grow on XOS while none of the species could grow on xylose. *Bacteroides*, on the other hand, was successful in utilizing all tested carbohydrates. Since xylan is an abundant and cost effective carbohydrate, utilization of that by the beneficial bacteria, such as *Bifidobacterium* species, are of great importance. In an effort to prolifirate *Bifidobacterium* species on xylan, they were grown in co-cultures with one of the two *Bacteroides* species.

B. animalis subsp. *lactis* was the only *Bifidobacterium* species that could grow on xylan in the co-cultures with Bacteroides, either *B. xylanisolvens* or *B. ovatus*. This was attributed to the ability of this *Bifidobacterium* species to utilize XOS. Because, the xylan, which was probably hydrolyzed by the enzymatic system of *Bacteroides* species, may have been utilized by *B. animalis* subsp. *lactis*. This way, a beneficial microorganism could be grown on an abundant substrate. The symbiotic (or mutalistic) interaction shown in this study between the *Bacteroides* and *Bifidobacteria*, could be valid in the human colon, as well. This may be showing that xylan is a potential prebiotic, so that more emphasis should be given to xylan as a functional carbohydrate. Another possible advantage of xylan is that, it could reach the distal part of the colon, since it could be catabolized only slowly in the proximal parts, as a result of its polymeric and complex sturucture compared to oligomeric carbohydrates.

The SCFAs and lactic and succinic acids productions in the mono and co-cultures showed that *B. animalis* subsp. *lactis* not only could grow in co-cultures, but also could function normally and produce the respective acids. This phenomenon is important for the benefical effects of this bacterium regarding its SCFA and lactic acid productions.

It should be noted that, due to the acid production in the mono and co-cultures and consequent pH decrease, the cultures were stopped immaturely, while there was still available carbon sources. Therefore, it is necessary to conduct constant pH cultures in order to clarify the extent of growth and metabolite productions as well as carbohydrate utilizations. Further *in-vitro* and *in-vivo* studies are required to evaluate the effects of the interaction between these two bacterial species, as well as some other bacterial species with beneficial effects.

REFERENCES

Bäckhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., & Gordon, J. I. (2005). Host-bacterial mutualism in the human intestine. *science*, *307*(5717), 1915-1920.

Cartano, A. V., & Juliano, B. O. (1970). Hemicelluloses of milled rice. *Journal of agricultural and food chemistry*, *18*(1), 40-42.

Cerdeño-Tárraga, A. M., Patrick, S., Crossman, L. C., Blakely, G., Abratt, V., Lennard, N., ... & Barron, A. (2005). Extensive DNA inversions in the B. fragilis genome control variable gene expression. *Science*, *307*(5714), 1463-1465.

Chapla, D., Pandit, P., & Shah, A. (2012). Production of xylooligosaccharides from corncob xylan by fungal xylanase and their utilization by probiotics. *Bioresource Technology*, *115*, 215-221.

Chassard, C., Delmas, E., Lawson, P. A., & Bernalier-Donadille, A. (2008). Bacteroides xylanisolvens sp. nov., a xylan-degrading bacterium isolated from human faeces. *International journal of systematic and evolutionary microbiology*, *58*(4), 1008-1013.

Chassard, C., Goumy, V., Leclerc, M., Del'homme, C., & Bernalier-Donadille, A. (2007). Characterization of the xylan-degrading microbial community from human faeces. *FEMS microbiology ecology*, *61*(1), 121-131.

Crittenden, R., Karppinen, S., Ojanen, S., Tenkanen, M., Fagerström, R., Mättö, J., ... & Poutanen, K. (2002). In vitro fermentation of cereal dietary fibre carbohydrates by probiotic and intestinal bacteria. *Journal of the Science of Food and Agriculture*, *82*(8), 781-789.

Courtin, C. M., Swennen, K., Verjans, P., & Delcour, J. A. (2009). Heat and pH stability of prebiotic arabinoxylooligosaccharides, xylooligosaccharides and fructooligosaccharides. *Food Chemistry*, *112*(4), 831-837.

Cummings, J. H., & Macfarlane, G. T. (1991). The control and consequences of bacterial fermentation in the human colon. *Journal of Applied Bacteriology*, *70*(6), 443-459.

Cummings, J., Pomare, E. W., Branch, W. J., Naylor, C. P., & Macfarlane, G. T. (1987). Short chain fatty acids in human large intestine, portal, hepatic and venous blood. *Gut*, *28*(10), 1221-1227.

de Vrese, M., & Schrezenmeir, J. (2008). Probiotics, prebiotics, and synbiotics. In *Food biotechnology* (pp. 1-66). Springer Berlin Heidelberg.

Pokusaeva, K., Fitzgerald, G. F., & van Sinderen, D. (2011). Carbohydrate metabolism in Bifidobacteria. *Genes & nutrition*, *6*(3), 285-306.

Dodd, D., Mackie, R. I., & Cann, I. K. (2011). Xylan degradation, a metabolic property shared by rumen and human colonic Bacteroidetes. *Molecular microbiology*, *79*(2), 292-304.

Duncan, S. H., Louis, P., & Flint, H. J. (2007). Cultivable bacterial diversity from the human colon. *Letters in applied microbiology*, *44*(4), 343-350.

Ebringerová, A. (2005, December). Structural diversity and application potential of hemicelluloses. In *Macromolecular Symposia* (Vol. 232, No. 1, pp. 1-12). WILEY-VCH Verlag.

Englyst, H. N., Hay, S., & Macfarlane, G. T. (1987). Polysaccharide breakdown by mixed populations of human faecal bacteria. *FEMS Microbiology Ecology*, *3*(3), 163-171.

Falony, G., Calmeyn, T., Leroy, F., & De Vuyst, L. (2009). Coculture fermentations of Bifidobacterium species and Bacteroides thetaiotaomicron reveal a mechanistic insight into the prebiotic effect of inulin-type fructans. *Applied and environmental microbiology*, *75*(8), 2312-2319.

Falony, G., & De Vuyst, L. (2009). Ecological interactions of bacteria in the human gut. In *Prebiotics and Probiotics Science and Technology* (pp. 639-679). Springer New York.

Ferraris, R. P. (2001). Dietary and developmental regulation of intestinal sugar transport. *Biochemical Journal*, *360*(2), 265-276.

Finegold, S. M., Sutter, V. L., & Mathisen, G. E. (1983). Normal indigenous intestinal flora. *Human intestinal microflora in health and disease*, *1*, 3-31.

Fox, S. M. (1988). Probiotics: intestinal inoculants for production animals. *Veterinary medicine (USA)*.

Gebbers, J. O., & Laissue, J. A. (1988). Immunologic structures and functions of the gut. *Schweizer Archiv fur Tierheilkunde*, *131*(5), 221-238.

Gibson, G. R., & Roberfroid, M. B. (1995). Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *The Journal of nutrition*, *125*(6), 1401.

Gibson, G. R., Probert, H. M., Van Loo, J., Rastall, R. A., & Roberfroid, M. B. (2004). Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutrition research reviews*, *17*(02), 259-275.

Gomes, A. M., & Malcata, F. X. (1999). Bifidobacterium spp. and Lactobacillus acidophilus: biological, biochemical, technological and therapeutical properties relevant for use as probiotics. *Trends in Food Science & Technology*, *10*(4), 139-157.

Grootaert, C., Delcour, J. A., Courtin, C. M., Broekaert, W. F., Verstraete, W., & Van de Wiele, T. (2007). Microbial metabolism and prebiotic potency of arabinoxylan oligosaccharides in the human intestine. *Trends in Food Science & Technology*, *18*(2), 64-71.

Gullón, P., Moura, P., Esteves, M. P., Girio, F. M., Domínguez, H., & Parajó, J. C. (2008). Assessment on the fermentability of xylooligosaccharides from rice husks by probiotic bacteria. *Journal of agricultural and food chemistry*, *56*(16), 7482-7487.

Harmsen, H. J., Raangs, G. C., He, T., Degener, J. E., & Welling, G. W. (2002). Extensive set of 16S rRNA-based probes for detection of bacteria in human feces. *Applied and environmental microbiology*, *68*(6), 2982-2990.

Hecht, D. W., Citron, D. M., & Cox, M. (2007). *Methods for antimicrobial susceptibility testing of anaerobic bacteria; approved standard*. Clinical and Laboratory Standards Institute.

Hooper, L. V., Midtvedt, T., & Gordon, J. I. (2002). How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annual review of nutrition*, *22*(1), 283-307.

Hooper, L. V., Stappenbeck, T. S., Hong, C. V., & Gordon, J. I. (2003). Angiogenins: a new class of microbicidal proteins involved in innate immunity. *Nature immunology*, *4*(3), 269-273.

Hopkins, M. J., & Macfarlane, G. T. (2002). Changes in predominant bacterial populations in human faeces with age and with Clostridium difficile infection. *Journal of medical microbiology*, *51*(5), 448-454.

Hopkins, M. J., & Macfarlane, G. T. (2003). Nondigestible oligosaccharides enhance bacterial colonization resistance against Clostridium difficile in vitro. *Applied and environmental microbiology*, *69*(4), 1920-1927.

Izydorczyk, M. S., & Biliaderis, C. G. (1995). Cereal arabinoxylans: advances in structure and physicochemical properties. *Carbohydrate Polymers*, *28*(1), 33-48.

Korecka, A., & Arulampalam, V. (2012). The gut microbiome: scourge, sentinel or spectator?. *Journal of oral microbiology*, *4*.

Koropatkin, N. M., Cameron, E. A., & Martens, E. C. (2012). How glycan metabolism shapes the human gut microbiota. *Nature reviews Microbiology*, *10*(5), 323-335.

Kumar, G. P., Pushpa, A., & Prabha, H. (2012). A review on xylooligosaccharides. *International research journal of pharmacy*, *8*(3), 71-74.

Kunz, C., Kuntz, S., & Rudloff, S. (2009). Intestinal flora. In *Breast-Feeding: Early Influences on Later Health* (pp. 67-79). Springer Netherlands.

Kuwahara, T., Yamashita, A., Hirakawa, H., Nakayama, H., Toh, H., Okada, N., ... & Ohnishi, Y. (2004). Genomic analysis of Bacteroides fragilis reveals extensive DNA inversions regulating cell surface adaptation. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(41), 14919-14924.

Ley, R. E., Peterson, D. A., & Gordon, J. I. (2006). Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell*, *124*(4), 837-848.

Li, M., Li, G., Zhu, L., Yin, Y., Zhao, X., Xiang, C., ... & Wang, X. (2014). Isolation and characterization of an agaro-oligosaccharide (AO)-hydrolyzing bacterium from the gut microflora of Chinese individuals. *PloS one*, *9*(3), e91106.

Liu, C., Song, Y., McTeague, M., Vu, A. W., Wexler, H., & Finegold, S. M. (2003). Rapid identification of the species of the Bacteroides fragilis group by multiplex PCR assays using group-and species-specific primers. *FEMS microbiology letters*, *222*(1), 9-16.

Macfarlane, G. T., Gibson, G. R., & Cummings, J. H. (1992). Comparison of fermentation reactions in different regions of the human colon. *Journal of Applied Bacteriology*, *72*(1), 57-64.

Macfarlane, S. M. G. T., & Macfarlane, G. T. (1995). Proteolysis and amino acid fermentation. *Human Colonic Bacteria: Role in nutrition, physiology and pathology*, 75-100.

Macfarlane, S. M. G. T., Macfarlane, G. T., & Cummings, J. T. (2006). Review article: prebiotics in the gastrointestinal tract. *Alimentary pharmacology & therapeutics*, *24*(5), 701-714

Macfarlane, G. T., Hay, S., Macfarlane, S., & Gibson, G. R. (1990). Effect of different carbohydrates on growth, polysaccharidase and glycosidase production by Bacteroides ovatus, in batch and continuous culture. *Journal of Applied Bacteriology*, *68*(2), 179-187.

Macfarlane, S., Quigley, M. E., Hopkins, M. J., Newton, D. F., & Macfarlane, G. T. (1998). Polysaccharide degradation by human intestinal bacteria during growth under multi-substrate limiting conditions in a three-stage continuous culture system. *FEMS Microbiology Ecology*, *26*(3), 231-243.

Macfarlane, S., & Macfarlane, G. T. (2003). Regulation of short-chain fatty acid production. *Proceedings of the Nutrition Society*, *62*(01), 67-72.

Marco, M. L., Pavan, S., & Kleerebezem, M. (2006). Towards understanding molecular modes of probiotic action. *Current opinion in biotechnology*, *17*(2), 204-210.

Martens, E. C., Koropatkin, N. M., Smith, T. J., & Gordon, J. I. (2009). Complex glycan catabolism by the human gut microbiota: the Bacteroidetes Sus-like paradigm. *Journal of Biological Chemistry*, *284*(37), 24673-24677.

Mirande, C., Kadlecikova, E., Matulova, M., Capek, P., Bernalier-Donadille, A., Forano, E., & Béra-Maillet, C. (2010). Dietary fibre degradation and fermentation by two xylanolytic bacteria Bacteroides xylanisolvens XB1AT and Roseburia intestinalis XB6B4 from the human intestine. *Journal of applied microbiology*, *109*(2), 451-460.

MITSUOKA, T. (1984). Taxonomy and ecology of bifidobacteria. *Bifidobacteria and Microflora*, *3*(1), 11-28.

Moure, A., Gullón, P., Domínguez, H., & Parajó, J. C. (2006). Advances in the manufacture, purification and applications of xylo-oligosaccharides as food additives and nutraceuticals. *Process Biochemistry*, *41*(9), 1913-1923

Neish, A. S. (2009). Microbes in gastrointestinal health and disease. *Gastroenterology*, *136*(1), 65-80.

Obel, N., Porchia, A. C., & Scheller, H. V. (2002). Dynamic changes in cell wall polysaccharides during wheat seedling development. *Phytochemistry*, *60*(6), 603-610.

O'Hara, A. M., & Shanahan, F. (2006). The gut flora as a forgotten organ. *EMBO reports*, *7*(7), 688-693.

Ouwehand, A. C., Salminen, S., & Isolauri, E. (2002). Probiotics: an overview of beneficial effects. In *Lactic Acid Bacteria: Genetics, Metabolism and Applications* (pp. 279-289). Springer Netherlands.

Palframan, R. J., Gibson, G. R., & Rastall, R. A. (2003). Carbohydrate preferences of Bifidobacterium species isolated from the human gut. *Current issues in intestinal microbiology*, *4*(2), 71-75.

Reid, G. (2004). When microbe meets human. *Clinical infectious diseases*, *39*(6), 827-830.

Roediger, W. E. W. (1982). Utilization of nutrients by isolated epithelial cells of the rat colon. *Gastroenterology*, *83*(2), 424-429.

Salyers, A. A. (1984). Bacteroides of the human lower intestinal tract. *Annual Reviews in Microbiology*, *38*(1), 293-313.

Salyers, A. A., Vercellotti, J. R., West, S. E., & Wilkins, T. D. (1977). Fermentation of mucin and plant polysaccharides by strains of Bacteroides from the human colon. *Applied and environmental microbiology*, *33*(2), 319-322.

Saxelin, M., Tynkkynen, S., Mattila-Sandholm, T., & de Vos, W. M. (2005). Probiotic and other functional microbes: from markets to mechanisms. *Current opinion in biotechnology*, *16*(2), 204-211.

Scheller, H. V., & Ulvskov, P. (2010). Hemicelluloses. *Annual review of plant biology*, *61*, 263-289.

Scheppach, W., Bartram, H. P., & Richter, F. (1995). Role of short-chain fatty acids in the prevention of colorectal cancer. *European Journal of Cancer*, *31*(7-8), 1077-1080.

Shallom, D., & Shoham, Y. (2003). Microbial hemicellulases. *Current opinion in microbiology*, *6*(3), 219-228.

Sgorbati, B., Biavati, B., & Palenzona, D. (1995). The genus Bifidobacterium. In *The genera of lactic acid bacteria* (pp. 279-306). Springer US.

Singh, R. D., Banerjee, J., & Arora, A. (2015). Prebiotic potential of oligosaccharides: a focus on xylan derived oligosaccharides. *Bioactive Carbohydrates and Dietary Fibre*, *5*(1), 19-30.

Slavin, J. (2013). Fiber and prebiotics: mechanisms and health benefits. *Nutrients*, *5*(4), 1417-1435.

Soccol, C. R., Vandenberghe, L. P. D. S., Spier, M. R., Medeiros, A. B. P., Yamaguishi, C. T., Lindner, J. D. D., ... & Thomaz-Soccol, V. (2010). The potential of probiotics: a review. *Food Technology and Biotechnology*, *48*(4), 413-434.

Sonnenburg, J. L., Angenent, L. T., & Gordon, J. I. (2004). Getting a grip on things: how do communities of bacterial symbionts become established in our intestine?. *Nature immunology*, *5*(6), 569-573.

Sonnenburg, J. L., Xu, J., Leip, D. D., Chen, C. H., Westover, B. P., Weatherford, J., ... & Gordon, J. I. (2005). Glycan foraging in vivo by an intestine-adapted bacterial symbiont. *Science*, *307*(5717), 1955-1959.

Stackebrandt, S. P. (2000). The prokaryotes: an evolving electronic resource for the microbiological community. Defining taxonomic ranks. Springer, New York, 29-57.

Stanton, C., Ross, R. P., Fitzgerald, G. F., & Van Sinderen, D. (2005). Fermented functional foods based on probiotics and their biogenic metabolites. *Current opinion in biotechnology*, *16*(2), 198-203.

Topping, D. L., & Clifton, P. M. (2001). Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. *Physiological reviews*, *81*(3), 1031-1064.

Turnbaugh, P. J., Ley, R. E., Mahowald, M. A., Magrini, V., Mardis, E. R., & Gordon, J. I. (2006). An obesity-associated gut microbiome with increased capacity for energy harvest. *nature*, *444*(7122), 1027-131.

Turroni, F., Van Sinderen, D., & Ventura, M. (2011). Genomics and ecological overview of the genus Bifidobacterium. *International journal of food microbiology*, *149*(1), 37-44

Van Laere, K. M., Hartemink, R., Bosveld, M., Schols, H. A., & Voragen, A. G. (2000). Fermentation of plant cell wall derived polysaccharides and their corresponding oligosaccharides by intestinal bacteria. *Journal of Agricultural and Food Chemistry*, *48*(5), 1644-1652.

Vazquez, M. J., Alonso, J. L., Domınguez, H., & Parajo, J. C. (2000). Xylooligosaccharides: manufacture and applications. *Trends in Food Science & Technology*, *11*(11), 387-393.

Wang, J., Sun, B., Cao, Y., Tian, Y., & Wang, C. (2009). Enzymatic preparation of wheat bran xylooligosaccharides and their stability during pasteurization and autoclave sterilization at low pH. *Carbohydrate polymers*, *77*(4), 816-821.

Wilkins, T. D., & Chalgren, S. (1976). Medium for use in antibiotic susceptibility testing of anaerobic bacteria. *Antimicrobial agents and chemotherapy*, *10*(6), 926-928.

Wong, J. M., De Souza, R., Kendall, C. W., Emam, A., & Jenkins, D. J. (2006). Colonic health: fermentation and short chain fatty acids. *Journal of clinical gastroenterology*, *40*(3), 235-243.

Xu, J., Bjursell, M. K., Himrod, J., Deng, S., Carmichael, L. K., Chiang, H. C., ... & Gordon, J. I. (2003). A genomic view of the human-Bacteroides thetaiotaomicron symbiosis. *Science*, *299*(5615), 2074-2076.

Xu, J., & Gordon, J. I. (2003). Honor thy symbionts. *Proceedings of the National Academy of Sciences*, *100*(18), 10452-10459.

APPENDIX A

CHEMICAL LIST

Chemical	Company	Chemical	Company
Agar	Applichem	Resazurin sodium salt	Sigma-Aldrich
Peptone from casein	Applichem	Reinforced clostridial medium	Oxoid
Peptone water	Oxoid	Wilkins-Chalgren Anerobe Medium	Oxoid
Tryptone	Applichem	Sodium chloride	Sigma-Aldrich
Ethanol (96%)	Merck	Vitamin K_1	Sigma-Aldrich
Tween-80	Fluka	Hemin	Sigma-Aldrich
Acetic acid	Sigma Aldrich	Defibrinated Horse Blood	Sigma-Aldrich
Butyric Acid	Sigma Aldrich	Xylobiose	Megazyme
Xylan, from beechwood	Sigma Aldrich	Xylotriose	Megazyme
D-(-)- Arabinose	Sigma Aldrich	Xylotetrose	Megazyme
D-(+)- Xylose	Sigma Aldrich	Xylopentose	Megazyme
Yeast extract	Merck	Xylohexose	Megazyme
Peptone	BD	Gr (-) anaerobic supplement	Oxoid
Potassium phosphate dibasic	Sigma-Aldrich	XOS	Longlive
Xylan, from beechwood	Sigma-Aldrich	Disodium hydrogen phosphate heptahydrate	Merck
D-(-)- Arabinose	Sigma-Aldrich	Sodium hydrogen phosphate dihydrate	Merck
Yeast extract	Merck	Ammonium chloride	Sigma-Aldrich
Peptone	BD	Bile salt	Oxoid
Potassium phosphate dibasic	Sigma-Aldrich	Peptone from soybean	Applichem
Potassium phosphate monobasic	Sigma-Aldrich	Calcium chloride	Applichem
D-(+)-Glucose monohydrate	Sigma-Aldrich	L-cysteine hydrochloride anhydrous	Sigma-Aldrich
Magnesium sulfate heptahydrate	Sigma-Aldrich	Magnesium chloride hexahydrate	Applichem
Calcium carbonate	Sigma-Aldrich	Sodium hydroxide	Sigma-Aldrich

APPENDIX B

MEDIA COMPOSITION

Table B.1. Reinforced Clostridial Medium composition

Component	Concentration g/L
Yeast Extract	13.0
Peptone	10.0
Glucose	5.0
Soluble strach	1.0
Sodium chloride	5.0
Sodium acetate	3.0
Cysteine hydrochloride	0.5
Agar	0.5

Table B.2. Wilkins-Chalgren Anaerobe Broth composition

Component	Concentration g/L
Tryptone	10.0
Gelatine peptone	10.0
Yeast Extract	5.0
Glucose	1.0
Sodium chloride	5.0
L-arginine	1.0
Sodium pyruvate	1.0
Menadione	0.0005
Haemin	0.005

APPENDIX B

MEDIA COMPOSITION

Table B.3. Basal Medium composition

Component	Concentration g/L
Peptone water	2.0
Yeast extract	2.0
Sodium chloride	0.1
Potassium phosphate dibasic	0.04
Potassium phosphate monobasic	0.04
Magnesium sulfate heptahydrate	0.01
Calcium Chloride hexahydrate	0.01
Sodium bicarbonate	2
Tween 80	2 μL
Heamin	0.05
Vitamine K_1	10 μL
L-cysteine hydrochloride	0.5
Resazurin	0.001
Bile salt	0.5

APPENDIX C

STANDARD CALIBRATION GRAPH FOR XYLOSE
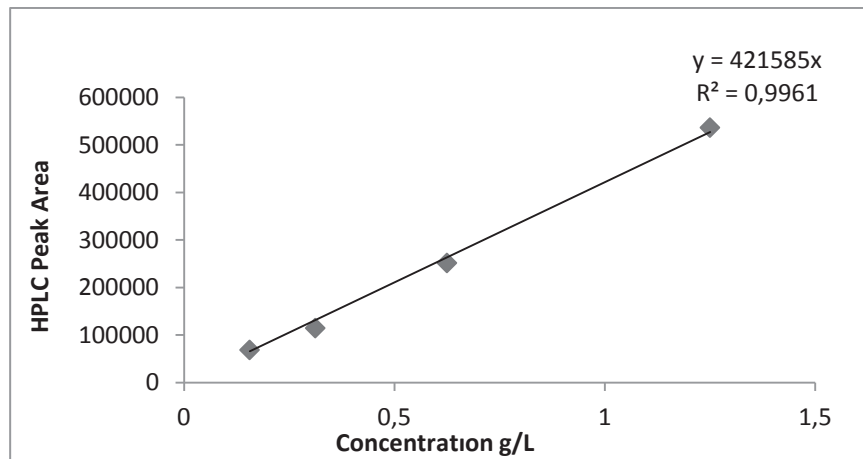
Figure C.1. Xylose standard curve for HPLC

APPENDIX D

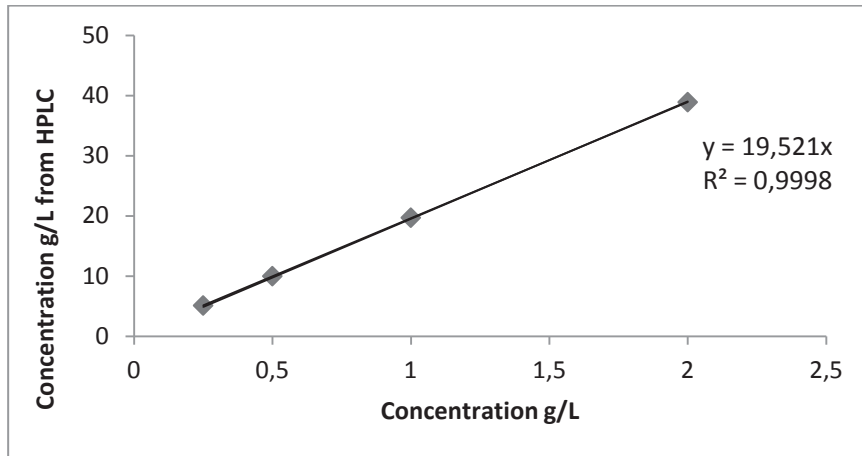# STANDARD CALIBRATION GRAPH FOR
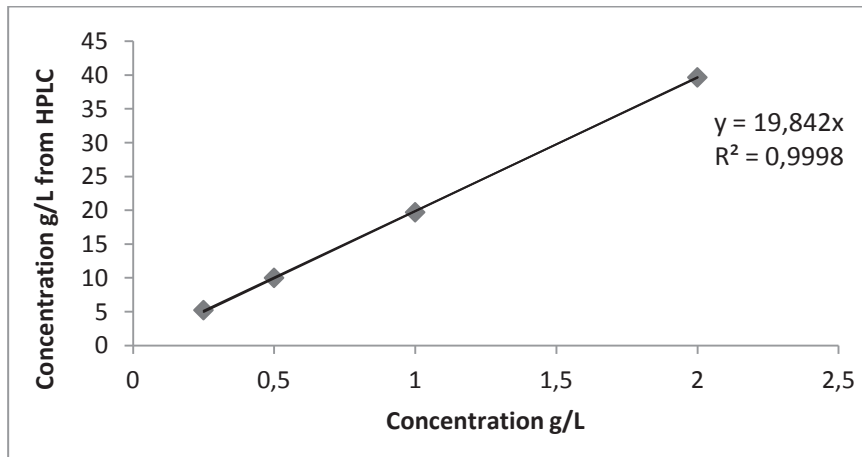# EACH ORGANIC ACIDS

Figure D.1. Lactic Acid standard curve for HPLC

Figure D.2. Acetic Acid standard curve for HPLC

APPENDIX D

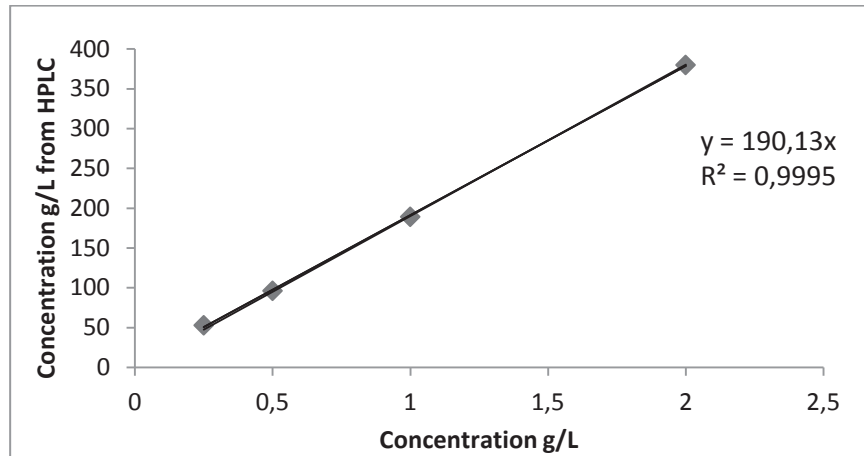# STANDARD CALIBRATION GRAPH FOR
EACH ORGANIC ACIDS

$$y = 190{,}13x$$
$$R^2 = 0{,}9995$$

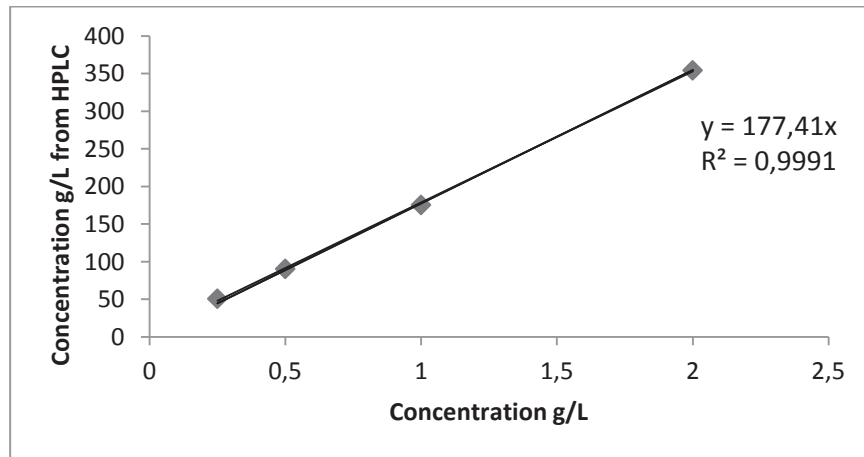Figure D.3. Propionic Acid standard curve for HPLC

$$y = 177{,}41x$$
$$R^2 = 0{,}9991$$

Figure D.4. Butyric Acid standard curve for HPLC

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