INVESTIGATIONS ON THE PREBIOTIC ACTIVITY OF XYLAN AND XYLOOLIGOSACCHARIDES USING IN VITRO MOUSE FECAL CULTURE AND EX VIVO MOUSE COLON MODEL

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Kevser SABANCI

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We approved the thesis of **Kevser SABANCI**

Examining Committee Members:

Prof. Dr. Ali Oğuz BÜYÜKKİLECİ
Department of Food Engineering, İzmir Insitute of Technology

Prof. Dr. Efe SEZGİN
Department of Food Engineering, İzmir Insitute of Technology

Assoc. Prof. Dr. Hakan GÜVELİ
Department of Nutrition and Dietetics, Bahçeşehir University

Assoc. Prof. Dr. Sibel UZUNER
Department of Food Engineering, İzmir Insitute of Technology

Assoc. Prof. Dr. Sırma YEĞİN
Department of Food Engineering, Ege University

06 December 2024

Prof. Dr. Ali Oğuz BÜYÜKKİLECİ
Supervisor, Department of Food Engineering
İzmir Insitute of Technology

Prof. Dr. Efe SEZGİN
Head of the Department of
Food Engineering

Prof. Dr. Mehtap EANES
Dean of the Graduate School of
Engineering and Sciences

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I dedicate this thesis to my precious daughter, Asya SABANCI, who was born at the beginning of my doctoral journey and is now five years old. Her energy and joy inspire me daily, and her presence brings purpose to all my efforts. My love for her knows no bounds, and I am grateful for the light she brings to my life.

ABSTRACT

INVESTIGATIONS ON THE PREBIOTIC ACTIVITY OF XYLAN AND XYLOOLIGOSACCHARIDES USING IN VITRO MOUSE FECAL CULTURE AND EX VIVO MOUSE COLON MODEL

Xylan (XY) and its hydrolysis products, xylooligosaccharides (XOS), are recognized for their prebiotic properties. It has been observed that XY is utilized more slowly than XOS and has distinct effects on the gut microbiota, However, research on the physiological effects of XY itself remains limited. This study was set up to investigate the utilization of XY in the colon and its impact on microbiota using mice-based *in vitro* and *ex vivo* models.

In vitro studies utilized BALB/c mice fecal inoculum to assess the effects of XOS, XY, INU, and combinations of XOS+XY and INU+XY. INU, a well-known prebiotic, was included in the study for comparison. Results demonstrated that all tested prebiotics significantly increased populations of *Bifidobacteria* and *Lactobacillus* while reducing *Enterococcus*, *Staphylococcus*, and *Clostridium sensu stricto* to varying extents. Oligomeric XOS notably enhanced *Bifidobacteria* and *Lactobacillus* populations, whereas polymeric XY primarily supported the growth of *Bacteroides*. The *ex vivo* model, employing sections of mice large intestine, examined the localized effects of XY and XOS in the cecum, proximal, and distal colon. Findings indicated that XY and XOS were metabolized in all sections, producing short-chain fatty acid and supporting *Bacteroides* and *Bifidobacteria* growth. XY exhibited higher *Bacteroides* growth across sections. The slow fermentation of XY in the cecum suggested that this polysaccharide could extend prebiotic activity to the distal colon section. These findings enhance our understanding of prebiotic dynamics and their potential applications in gut health.

ÖZET

KSİLAN VE KSİLOOLİGOSAKKARİTLERİN PREBİYOTİK AKTİVİTESİNİN İN VİTRO FARE DIŞKI KÜLTÜRÜ VE EX VİVO FARE KOLON MODELİ KULLANILARAK ARAŞTIRILMASI

Ksilan (KS) ve onun hidroliz ürünü olan ksilooligosakkaritler (KOS), prebiyotik özellikleriyle tanınmaktadır. KS'nin, KOS ve inüline (INU) göre daha yavaş bir şekilde kullanıldığı ve bağırsak mikrobiyotası üzerinde farklı etkiler gösterdiği gözlemlenmiştir. Ancak, KS'nin fizyolojik etkilerine yönelik araştırmalar hâlâ sınırlıdır. Bu çalışma, KS'nin kolondaki kullanımını ve mikrobiyota üzerindeki etkilerini, fare tabanlı *in vitro* ve *ex vivo* modeller kullanarak araştırmayı amaçlamıştır.

In vitro çalışmalar, BALB/c fare dışkı inokulumu kullanılarak KOS, KS, INU ve KOS+KS ile INU+KS kombinasyonlarının etkilerini değerlendirmiştir. İyi bilinen bir prebiyotik olan INU, karşılaştırma amacıyla çalışmaya dahil edilmiştir. Sonuçlar, test edilen tüm prebiyotiklerin *Bifidobacteria* ve *Lactobacillus* popülasyonlarını önemli ölçüde artırırken, *Enterococcus*, *Staphylococcus* ve *Clostridium sensu stricto* popülasyonlarını çeşitli oranlarda azalttığını göstermiştir. Oligomerik KOS, özellikle *Bifidobacteria* ve *Lactobacillus* popülasyonlarını artırırken, polimerik KS daha çok *Bacteroides* türlerinin büyümesini desteklemiştir. *Ex vivo* modelde ise farelerin sekum, proksimal ve distal kolon bölümlerinde KS ve KOS'un lokalize etkileri incelenmiştir. Bulgular, her iki prebiyotiğin tüm bağırsak segmentlerinde metabolize edilerek kısa zincirli yağ asidi üretimine yol açtığını ve *Bacteroides* ile *Bifidobacteria* popülasyonlarını desteklediğini ortaya koymuştur. KS, tüm bağırsak bölümlerinde daha yüksek *Bacteroides* büyümesi ile ilişkilendirilmiş olup, sekumda yavaş fermantasyon göstererek prebiyotik aktivitenin distal kolona kadar uzanabileceğine işaret etmektedir. Bu bulgular, prebiyotiklerin bağırsak sağlığında potansiyel uygulamaları ile prebiyotik dinamiklerinin anlaşılmasına önemli katkılar sunmaktadır.

Dedicated to my precious daughter, Asya

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LIST OF SYMBOLS

%	Percent
×g	G-force
°C	Celcius degree
µL	Microliter
µm	Micrometer
Da	Dalton
g	Gram
kcal	Kilocalories
kDa	Kilodalton
min	Minutes
mL	Milliliters
mm	Millimeter
mM	Milli molar
s	Second
v/v	Volume per volume
W	Watt
w/v	Weight per volume
w/w	Weight per weight

LIST OF ABBREVIATIONS

a*	Redness value in color analysis
ANOVA	Analysis of variance
AX	Arabinoxylan
b*	Yellowness value in color analysis
CC	Corncob
DC	Distal Colon
D-Gal	Galactose
D-GlcA	D-glucuronic acid
EC	Emulsification capacity
EFSA	European Food Safety Authority
ES	Emulsion stability
EU	European union
FDA	Food and Drug Administration
FOS	Fructooligosaccharides
FC	Foaming capacity
FS180min	Foam stability at 180th minutes
FS30min	Foam stability at 30th minutes
GIT	Gastrointestinal tract
GOS	Galactooligosaccharides
GRAS	Generally recognized as safe
HPLC	High-performance liquid chromatography
INU	Inulin
L*	Luminous value in color analysis
L-Ara	L-arabinofuranose
MW	Molecular weight
n.d.	Not determined
OHC	Oil holding capacity
O-4 Me	O-4 methyl
D-GlcA	D-glucuronic acid
PC	Proximal Colon
RID	Refractive index detector
SCFA	Short Chain Fatty Acid
SHIME	Simulator of Human Intestinal Microbial Ecosystem
TIM	TNO gastrointestinal model
UV	Ultraviolet
WHC	Water holding capacity
XOS	Xylooligosaccharides
XY	Xylan

CHAPTER 1

INTRODUCTION

The intestinal microbiota plays a fundamental role in various host processes, including metabolic, physiological, nutritional, and immunological functions. Dysbiosis has been associated with a range of diseases through the disruption of normal gut function. As a response, functional foods have been developed to modulate the gut environment, particularly through probiotic and prebiotic supplementation aimed at restoring or enhancing microbial equilibrium (Obayomi, Olaniran, and Owa 2024). Evidence from ongoing research and randomized clinical trials has demonstrated that probiotics and prebiotics exert beneficial effects on host health (Pavlidou et al. 2022). Prebiotics—non-digestible, plant-derived oligo- and polysaccharides—are selectively fermented by specific members of the gut microbiota, promoting the growth of beneficial bacterial species in the large intestine and conferring a range of health benefits (Gibson and Roberfroid 1995). These prebiotics pass undigested through the upper gastrointestinal tract to the colon, where they act as substrates for certain microbial populations, leading to physiological effects that support gut health (Gong and Yang, 2012). One of the primary outcomes of prebiotic fermentation is the production of short-chain fatty acids (SCFAs), including acetate, propionate, and butyrate. These SCFAs, which constitute 90-95% of SCFAs in the colon, are absorbed by the intestinal cells and enter systemic circulation, contributing to metabolic health and immune regulation (Carlson et al. 2017; Jayamanohar et al. 2019; Jue Wang et al. 2019).

Among prebiotic substrates, low-molecular-weight carbohydrates such as fructooligosaccharides (FOS), galactooligosaccharides (GOS), and xylooligosaccharides (XOS) are rapidly fermented by gut bacteria, proliferate beneficial microorganisms in the first part of the colon (proximal colon), with limited effects on the end of the colon (distal colon) (Bhatia et al. 2024; Ravindra Kumar, Næss, and Sørensen 2024). In contrast, longer-chain carbohydrates like inulin (INU) are fermented more slowly, allowing their effects to extend further along the large intestine, including the distal part of the colon (Sheng, Ji, and Zhang 2023). This differential fermentation rate is significant, as it may

provide sustained microbial support and health benefits across a broader section of the gut.

Xylan (XY) and its hydrolysis product, XOS, are recognized for their significant prebiotic attributes and their extensive natural occurrence within plant cell walls. XY's molecular structure primarily comprises β-(1,4)-linked xylose units, which may include various substituent groups, such as acetyl, (4-O-methyl) glucuronyl, and arabinose. Additionally, XY may contain ferulic and p-coumaric acid residues, adding complexity to its structure. This intricate composition not only contributes to the robustness of XY and XOS, allowing them to remain stable under the conditions of the upper gastrointestinal tract but also enables them to transit through to the colon largely intact, thereby enhancing their efficacy as prebiotics (La Rosa et al., 2019; Rashid and Sohail, 2021; Samanta et al., 2015).

Once in the colon, XY was targeted by specific bacterial genera, such as *Bacteroides* and *Roseburia*, which produce extracellular xylanase enzymes capable of breaking down XY. Meanwhile, certain *Bifidobacteria* species, notable for their prebiotic activity, can directly utilize XOS. This metabolic capability fosters a process known as cross-feeding, wherein *Bifidobacteria* spp. indirectly benefits from the oligosaccharide fragments generated by the degradation of XY other species. Through this mechanism, *Bifidobacteria* spp. contributes to a bifidogenic effect, promoting a balanced and health-promoting microbial community in the colon (Falony et al. 2006; Zeybek, Rastall, and Buyukkileci 2020). Research on co-cultures, such as those including *Bifidobacterium animalis* subsp. *lactis* in combination with *Bacteroides ovatus* and *Bacteroides xylanisolvens*, has provided insight into these cooperative interactions. These studies illustrate how different bacterial species can synergistically metabolize XY, enhancing prebiotic utilization and demonstrating a complex microbial interaction that is central to the effectiveness of XY-based prebiotics (Zeybek, Rastall, and Buyukkileci 2020).

To investigate the effects of prebiotics on gut microbiota and host health, a variety of model systems—including *in vitro*, *in vivo* (animal), *ex vivo*, and clinical studies—are employed. *In vitro* models, which typically use fecal inoculates in controlled environments, offer ease of setup and a cost-effective approach for studying prebiotic fermentation. These models can observe microbial composition changes over time, allowing for a detailed examination of specific prebiotics on microbial communities. Single or multiple reactor types are dynamic *in vitro* human digestive system simulator models. Advanced versions such as SHIME (Simulator of Human Intestinal Microbial

Ecosystem) and TIM (TNO gastrointestinal model) systems have been developed, incorporating additional parameters such as stomach, pancreatic, and bile fluids, and mucus (Lemmens et al. 2021; Verhoeckx et al. 2015). Although many models have been designed to simulate microbiota activity, these models cannot fully represent some of the gastrointestinal tract's complex physicochemical and physiological properties including microorganism adhesion, colonization, and localization (Bajury et al. 2018).

In vivo studies using animal models, often involving rodents, pigs, or fish, allow researchers to investigate prebiotic effects within a complete, living organism. These studies can reveal systemic impacts of prebiotics and provide insights into host-microbiota interactions. However, *in vivo* studies come with limitations, including high costs and ethical considerations (Pastorino, Prearo, and Barceló 2024; Vashishat et al. 2024). Although clinical trials in humans are essential for confirming prebiotic effects and health benefits, they are often limited by ethical constraints and challenges in sampling from different sections of the gastrointestinal system. This restricts the ability of researchers to thoroughly investigate the mechanisms of microbial nutrient degradation, and the specific pathways involved (Li and Zhang, 2022).

Ex vivo models provide a valuable alternative, utilizing tissues and organs extracted from animals to simulate prebiotic interactions under controlled laboratory conditions. These models address some ethical concerns associated with live animal studies and offer insights into gut motility, microbial activity, and prebiotic metabolism within different sections of the large intestine. *Ex vivo* models allow for targeted examination of microbial dynamics and SCFA (short-chain fatty acid) production across sections of the gut, making them particularly useful for studying localized prebiotic effects in different intestinal regions (Costa et al. 2024). *Ex vivo* models are valuable for controlled studies but require careful interpretation due to limitations, such as the lack of systemic interactions found in a whole organism.

This thesis focuses on prebiotic utilization and its impact on gut bacteria, utilizing both *in vitro* and *ex vivo* models. *In vitro* studies employed BALB/c mice fecal inoculum to evaluate the fermentation dynamics of prebiotics with various chain lengths, including XOS, XY, inulin (INU), and combinations such as XOS+XY and INU+XY. The kinetics of prebiotic utilization revealed distinct differences in how these prebiotics were metabolized over 48 h.

The *ex vivo* model, developed as a novel aspect of this thesis, utilized sections of the large intestine from BALB/c mice to examine the prebiotic effects of XY, XOS, and

their combination (XOS+XY mix). These prebiotics, dissolved in basal medium (BM) without a carbon source, were loaded into the cecum, proximal colon (PC), and distal colon (DC) of the mice. After 3 h of incubation, the tissue contents were collected and analyzed for residual XOS and XY, organic acid concentrations, and changes in *Bifidobacteria* and *Bacteroides* levels. *The ex vivo* model enabled the evaluation of prebiotic utilization and its effects on specific bacterial populations in the cecum and colon, along with the production of SCFAs and other acids. The separate loading of XOS, XY, and the XOS+XY mix into the cecum, PC, and DC provided insights into their differential utilization by resident microflora.

CHAPTER 2

LITERATURE REVIEW

2.1. Intestinal Microbiota

Intestinal microbiota plays an essential role in numerous metabolic, physiological, nutritional, and immunological processes, and the composition of the microbiota influences human health (Power et al. 2014). The microbial content of the gastrointestinal tract (GIT) alters throughout its length, ranging from a small diversity and low numbers of microbes in the stomach to a wide diversity and high numbers in the large intestine (Figure 2.1) (Rawi et al. 2021). In adults, feces-derived populations have been estimated to consist of 10^{13} to 10^{14} microorganisms (Li et al. 2020). The most common bacteria in the colonic microbial community members are Firmicutes (~65%), Bacteroidetes (~25%), Actinobacteria (~5%), and Proteobacteria (~8%) (Sasso et al. 2023).

Among the dominant bacterial groups in the gut are the phyla Bacteroidetes and Firmicutes, which together constitute the majority of the microbiota (Evans et al. 2014; Yoo et al. 2024). Bacteroidetes are known for their ability to break down complex carbohydrates and polysaccharides, releasing fermentation products that support host health. Firmicutes, on the other hand, are equally crucial, as many members of this phylum are involved in the production of short-chain fatty acid (SCFA) like butyrate, which is an important energy source for colonic cells and has anti-inflammatory properties (Bin Zhu et al. 2021).

The Clostridium Clusters XIV and IV are significant groups in the phylum Firmicutes, known for their roles in the human gut microbiota. Clostridium Cluster XIV consists of a diverse array of genera, including Clostridium, Eubacterium, Ruminococcus, Coprococcus, Dorea, Lachnospira, Roseburia, and Butyrivibrio. These genera contribute to various metabolic activities in the gut. Clostridium Cluster IV, another important group in the gut ecosystem, includes species from the Clostridium, Eubacterium, Ruminococcus, and Anaerofilum genera. Clusters XIV and IV constitute approximately 10–40% of the total bacterial population in the gut microbiota (Lopetuso et al. 2013; Guo

et al. 2020). They are particularly notable for their ability to produce a wide array of enzymes that degrade complex polysaccharides and oligosaccharides that are otherwise indigestible by the host. Clostridium Clusters XIV and IV are essential contributors to the gut microbiota's functional diversity and metabolic capacity, influencing host physiology and health outcomes (Ghosh and Pramanik, 2021; Portune et al., 2016).

In addition to Bacteroidetes and Firmicutes, several minor bacterial phyla contribute to gut microbial diversity, including Actinobacteria, Proteobacteria, and Verrucomicrobia. Members of the Actinobacteria phylum, such as *Bifidobacterium* species, are considered beneficial for gut health due to their involvement in carbohydrate fermentation and SCFA production (Senghor et al. 2018; Pushpanathan et al. 2019; Grigor'eva 2020). Proteobacteria, though less abundant, are often highly metabolically versatile, but an overgrowth of certain Proteobacteria members can be associated with dysbiosis and inflammation (Vester-Andersen et al. 2019; Shin, Whon, and Bae 2015). Meanwhile, Verrucomicrobia, which includes the well-known species *Akkermansia muciniphila*, has been linked to improved gut barrier integrity and metabolic health (Pérez-Monter et al. 2022; Aggarwal, Sunder, and Verma 2022).

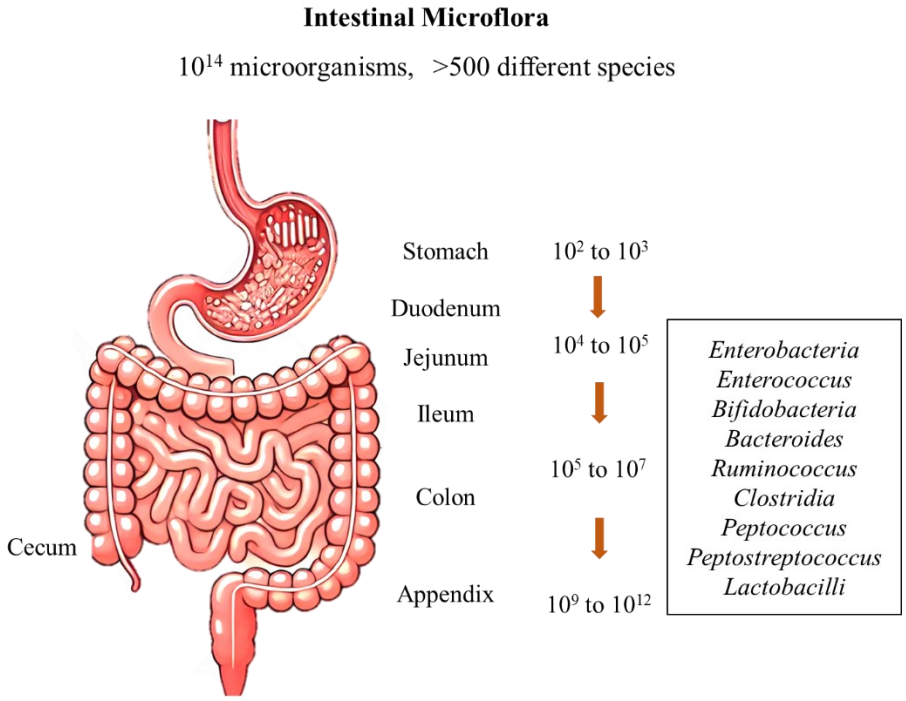

Figure 2. 1. Variations in microbial numbers across the length of the gastrointestinal tract (Source: Rawi et al., 2021).

The balance and diversity in these bacterial groups are essential for maintaining a healthy gut environment. They work in concert to ferment dietary fibers and prebiotics, producing metabolites that promote gut health and support systemic metabolic functions (Fusco et al. 2023; Koh et al. 2016). Disruptions in this bacterial ecosystem, known as dysbiosis, have been linked to a range of health issues, including inflammatory bowel disease, obesity, and metabolic disorders.

The colon is around 1.5 m long, and it is the distal part of the GIT. The colon is itself subdivided into three segments according to nutrient availability and bacterial activity: the proximal, transverse, and distal colons, each one with different and unique physiological attributes (Figure 2.2) (Bass and Wershil, 2016). Colon pH values are different from the upper gastrointestinal tract, and they also vary among the different sections of the large intestine. In the proximal colon, pH values can range from 5.2 to 5.9 (Abuhelwa et al. 2017). These slightly lower pH values are due to the large number of microorganisms inhabiting the colon, which ferment carbohydrates to generate SCFA (Blaak et al. 2020). However, during transit through the transverse and distal colons, these fatty acids are absorbed and H_2CO_3 secretion is promoted, changing the environment more alkaline (Becker and Seidler 2024). At the transverse part of the colon pH can reach approximately 5.8-7.4 and the distal part of the colon can reach pH values of 6.3-7.7 (Patel, Bhadoria, and Patani 2022).

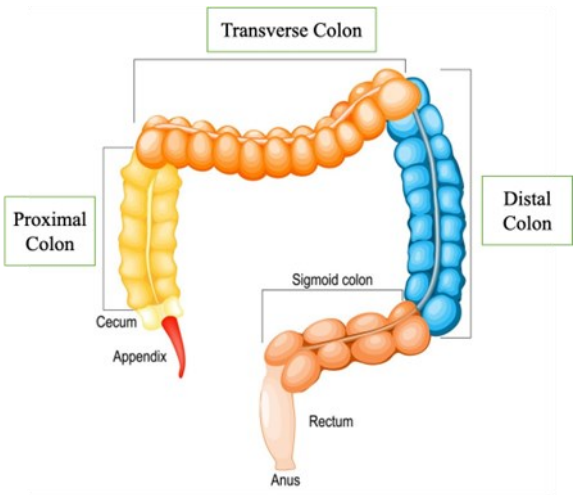

Figure 2. 2. Parts of the human large intestine, cecum, proximal colon, transverse colon, and distal colon (Source: Bass and Wershil, 2016).

Following the fermentation of carbohydrates in the gut, SCFAs are produced as key metabolic end products. The most prevalent SCFAs in the large intestine are acetate, propionate, and butyrate, which account for 90-95% of total SCFAs (Figure 2.3.). Typically, their proportions are approximately 60% for acetate, 25% for propionate, and 15% for butyrate (Jue Wang et al. 2019; Fusco et al. 2023; Bedu-Ferrari et al. 2022; Den Besten et al. 2013). Other SCFAs, such as iso-butyrate, valerate, and iso-valerate, are present at significantly lower concentrations. These SCFAs can be detected in feces, reflecting the extent of microbial fermentation occurring in the colon (Fei et al. 2020; Lange, Proczko-Stepaniak, and Mika 2023; Rawi et al. 2021). SCFAs are well-recognized for their beneficial roles in maintaining health, particularly in the gut environment.

Among the SCFAs, butyrate is noted for its diverse health-promoting effects, which include inhibiting pathogenic growth through pH modulation, serving as an energy source for colonocytes, and enhancing mineral and ion absorption (Fernández et al. 2016). Additionally, butyrate and propionate have immunomodulatory functions; they can stimulate the differentiation of T-regulatory cells, thereby contributing to the regulation of inflammation and immune tolerance (Louis et al. 2004). Propionate also exhibits metabolic benefits, such as promoting satiety and reducing serum cholesterol levels (Psichas et al. 2015). Acetate, the most abundant SCFA in the gastrointestinal tract, enters the circulation and is utilized as an energy source by the host, supplying approximately 10% of the body's daily energy requirements (Table 2.1) (Den Besten et al. 2013; Silva, Bernardi, and Frozza 2020).

SCFAs are crucial for the maintenance of colonic health and host metabolism. They contribute to the defense mechanisms of the gut barrier, exhibit anti-inflammatory properties, and enhance insulin sensitivity, which collectively helps to maintain metabolic homeostasis (Ashaolu and Ashaolu, 2021; Den Besten et al., 2013; Stein et al., 2000). Moreover, the capacity of different dietary fibers to stimulate SCFA production varies, as β-glucan, XY, guar gum, XOS, FOS, GOS, and inulin have been shown to increase butyrate levels, whereas arabinoxylan and guar gum are known to elevate propionate production (Poeker et al. 2018). The various functions of SCFAs are detailed in Table 2.1, highlighting their roles in maintaining intestinal health, supporting anaerobic metabolic processes, and enhancing nutrient absorption.

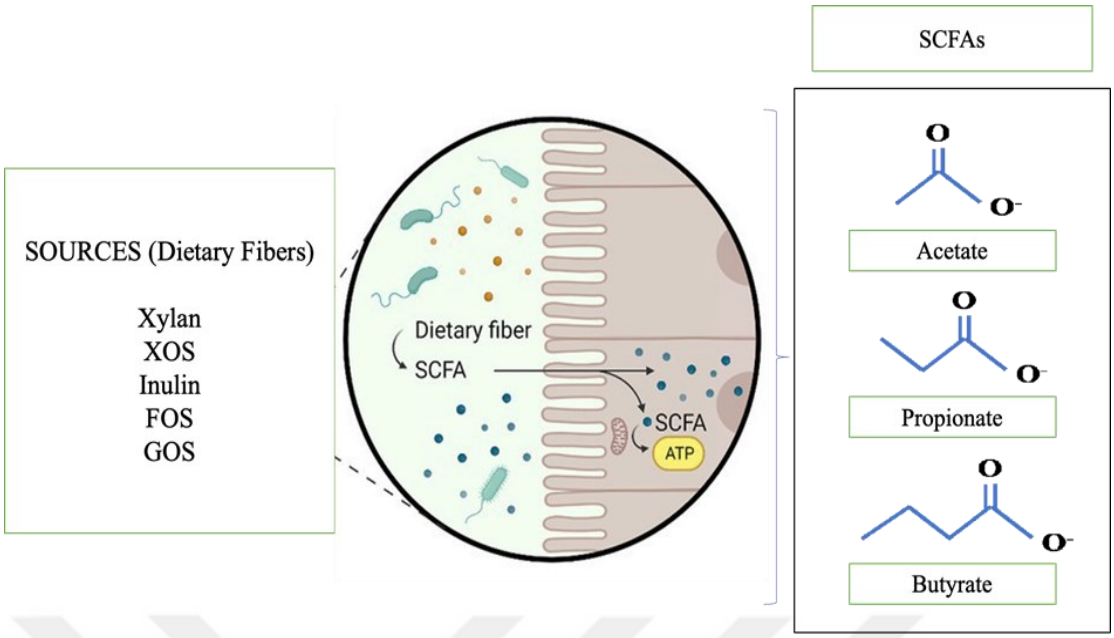

Figure 2. 3. The three most abundant SCFA. Acetate, Propionate, and Butyrate.

The rate at which short-chain fatty acids (SCFAs) are produced is largely dependent on the type and availability of fermentable substrates in the diet, such as dietary fibers, resistant starches, and oligosaccharides (Fusco et al. 2023; Bedu-Ferrari et al. 2022). The distribution and concentration of SCFAs in the gut are shaped by several factors, including the diversity and metabolic activity of the gut microbiota, the molecular structure and solubility of dietary fibers, and the proportion of undigested food components reaching the colon (Shortt et al. 2018; Tannock and Liu 2020). In the bloodstream, the cycle of SCFAs is rapid, resulting in relatively low but variable concentrations, with acetate, propionate, and butyrate measured at approximately 29, 4, and 0.3 mmol/kg, respectively. Each SCFA exhibits distinct metabolic fates: acetate serves as a substrate for lipogenesis and energy metabolism, propionate is involved in gluconeogenesis and cholesterol synthesis regulation, and butyrate acts as a key energy source for colonocytes while also exerting anti-inflammatory and immunomodulatory effects. The metabolic functions and health benefits associated with SCFAs emphasize their importance in nutritional science and therapeutic strategies promoting gut health (Caetano and Castelucci, 2022; Chen et al., 2018).

Table 2. 1. The functions of short-chain fatty acids.

SCFAs	Functions	References
Acetate **CH_3-COO^-**	Energy source for colon epithelial cells, enhancing mineral absorption lipid synthesis, protein acetylation, anti-inflammatory effects, Increases colonic blood flow and oxygen uptake cholesterol synthesis, activity against *E. coli*	(Ashaolu and Ashaolu, 2021; Biswas et al., 2022; Bose et al., 2019; Gong et al., 2022; Hung et al., 2022; Martino et al., 2022; Pomare et al., 1985; Scheppach, 1994; Zheng et al., 2021)
Butyrate **CH_3-CH_2-CH_2-COO^-**	Immunomodulation, amelioration of ulcerative colitis symptoms, upregulation of MUC2 gene expression in mucin production, apoptotic mechanism induction, anti-tumors, therapeutic effects on cancer, distal ulcerative colitis Crohn's disease	(Fernández et al. 2016; Scott et al. 2014; Rawi et al. 2021; Song et al. 2020; Ding et al. 2018; Falony et al. 2006; Bin Zhu et al. 2021; Berger et al. 2021)
Propionate **CH_3-CH_2-COO^-**	Boosts lipid metabolism, stops liver cholesterol, anti-cancer properties, lower lipogenesis, lower serum cholesterol levels, lower carcinogenesis contributing to gluconeogenesis in the liver, inhibition of histone deacetylases	(Hosseini et al. 2011; Fernández et al. 2016; Psichas et al. 2015; Moro Cantu-Jungles et al. 2019; He et al. 2020; Carlson et al. 2017; Chambers et al. 2015; Medina et al. 2021; Reichardt et al. 2014; Langfeld et al. 2021; Bindels et al. 2012; Kircher et al. 2022)
All SCFA	Improve gut health main substrates of colonocytes inhibition of cancerous cell proliferation controlling intestinal homoeostasis regulating pro-inflammatory cytokines	(Ashaolu and Ashaolu, 2021; Bedu-Ferrari et al., 2022; Carlson et al., 2017; Den Besten et al., 2013; Fusco et al., 2023; He et al., 2020; Koh et al., 2016; Lange et al., 2023; Rawi et al., 2021; Silva et al., 2020; Stein et al., 2000; Tawfick et al., 2022; Trompette et al., 2018)

2.2. Plant Cell Wall Polysaccharides on Intestinal Microbiota

Polysaccharides found in plant cell walls (PCWs) are a notable polymeric molecule class of long chains of monosaccharide units linked by glycosidic bonds. Common in nature, these polysaccharides constitute the structural elements of plant cell walls (Miguez et al. 2023; Siemińska-Kuczer, Szymańska-Chargot, and Zdunek 2022). Due to their complex structure, PCW polysaccharides resist digestion by human gastrointestinal enzymes, thereby reaching the large intestine where the gut microbiota partially or entirely ferments them (Yang et al. 2024; Song et al. 2021).

Contrary to simpler carbohydrates, polysaccharides are fermented by the intestinal microbiota and produce a variety of metabolites that are beneficial for the host's health rather than being easily broken down by human digestive enzymes (Cockburn and Koropatkin, 2016; Shang et al., 2018). The reason for this selective degradation is that only specific gut bacterial species have the enzymatic capacity to degrade these complex carbohydrate structures into smaller components, like oligosaccharides, which can be utilized in further metabolic processes (Figure 2.4). The incomplete digestion of these polysaccharides provides a continuous source of fermentable substrates for the gut bacteria, facilitating the production of SCFAs and other health-promoting metabolites (Meldrum and Yakubov, 2024; Wang et al., 2024).

Research examining the relationship between diet and gut microbiota in animal models has demonstrated that dietary composition significantly influences the microbial population in the colon. Specifically, dietary changes have been linked to shifts in the relative abundance of major bacterial phyla, including *Bacteroides*, *Firmicutes*, and *Prevotella* (Scott et al. 2013; Bibbo et al. 2016; Ross et al. 2024). *Bacteroides* species, which are gram-negative and prominent members of the colonic microbial community, play a key role due to their ability to degrade a wide range of polysaccharides (Déjean et al. 2020; Cheng et al. 2022). Certain species within this genus, such as *Bacteroides ovatus*, *Bacteroides thetaiotaomicron*, and *Bacteroides cellulosilyticus*, exhibit versatility in polysaccharide utilization, breaking down substrates such as XY, pectin, galactomannan, arabinogalactan, xyloglucan, β-glucan, and glucomannan (McKee et al. 2021; Fultz et al. 2021). However, the fact that no single bacterial species can degrade each dietary polysaccharide emphasizes how collaborative gut microbial fermentation requires (Lemmens et al. 2021; Zeybek, Rastall, and Buyukkileci 2020). In contrast,

gram-positive *Firmicutes* generally specialize in the metabolism of a narrower range of polysaccharides, which can lead to distinct patterns of SCFA production (Riva et al. 2023; Grigor'eva 2020). The ability of these bacterial groups to metabolize dietary polysaccharides is a crucial factor in shaping the overall composition and functional capacity of the gut microbiota (Sagbasan et al. 2024).

The influence of diet on the gut microbiota composition extends beyond the types of polysaccharides consumed, as the physical and chemical characteristics of the dietary fibers, such as molecular weight and degree of branching, also play a significant role (Guillon and Champ, 2000; Tang et al., 2024; Tungland & Meyer, 2002). These factors determine the accessibility of polysaccharides to microbial enzymes and influence the rate of SCFA production. Consequently, the specific types of SCFAs produced, and their relative proportions, are shaped by both the dietary intake and the resident microbiota, which dynamically interact to modulate the metabolic environment within the gut. Understanding these interactions is crucial for designing dietary interventions aimed at promoting gut health and mitigating metabolic and inflammatory diseases.

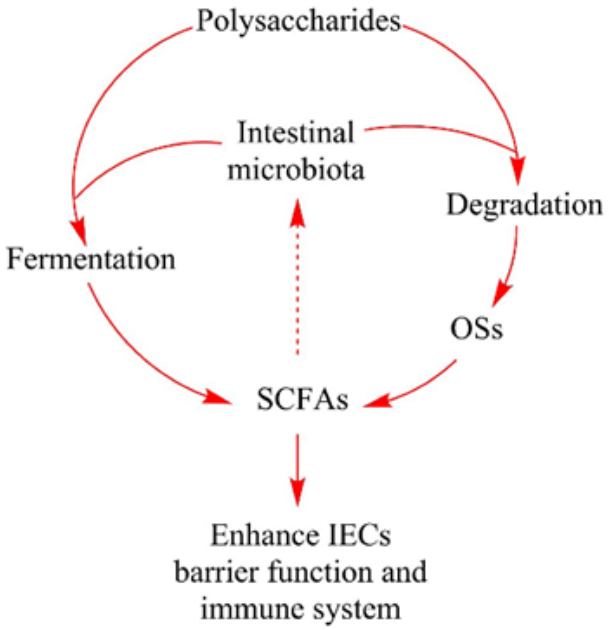

Figure 2. 4. The role of natural polysaccharides in intestinal fermentation (Source: Zhang et al., 2018).

2.3. Lignocellulosic Biomass

Lignocellulosic biomass is the most abundant form of biomass, playing a crucial role in the structural integrity of plants. This abundance makes it widely accessible for various applications (Ashokkumar et al. 2022; Wang et al. 2021). Common sources of lignocellulosic feedstock include agricultural residues, such as wheat straw, sugarcane bagasse, and corn cobs. Figure 2.5 illustrates the three primary components of lignocellulosic biomass: cellulose, which constitutes 35-50%; hemicellulose, making up 20-35%; and lignin, accounting for 10-25% (Srivastava et al. 2019; Raj Kumar, Singh, and Singh 2008). Additionally, lignocellulosic biomass contains minor quantities of pectin, protein, extractives, and ash (Zoghi et al. 2021). Utilizing lignocellulosic biomass as a raw material in sustainable biorefineries has attracted considerable interest for its potential to decrease reliance on fossil fuels, reduce greenhouse gas emissions, and support a circular bioeconomy (Velvizhi et al. 2022).

The increasing volume of organic waste generated from industrial and agricultural activities presents both a significant environmental challenge and a potential opportunity for sustainable waste management. If not managed properly, organic waste can contribute to severe ecological issues, including greenhouse gas emissions, environmental pollution, and public health risks. However, the implementation of a biorefinery approach provides an innovative solution by enabling the efficient recycling of lignocellulosic biomass as a feedstock for bio-based products. (Ganguly and Chakraborty, 2020; Hajam et al., 2023; Nayak and Mishra, 2024). Through the production of valuable commodities such as biofuels, biochemicals, and bioenergy, this strategy not only mitigates the adverse effects associated with waste accumulation but also supports the transition toward a circular and sustainable bioeconomy. By integrating advanced bioconversion technologies, biorefineries contribute to resource efficiency, energy security, and environmental sustainability, thereby playing a crucial role in addressing global waste management challenges (Kumari et al. 2024).

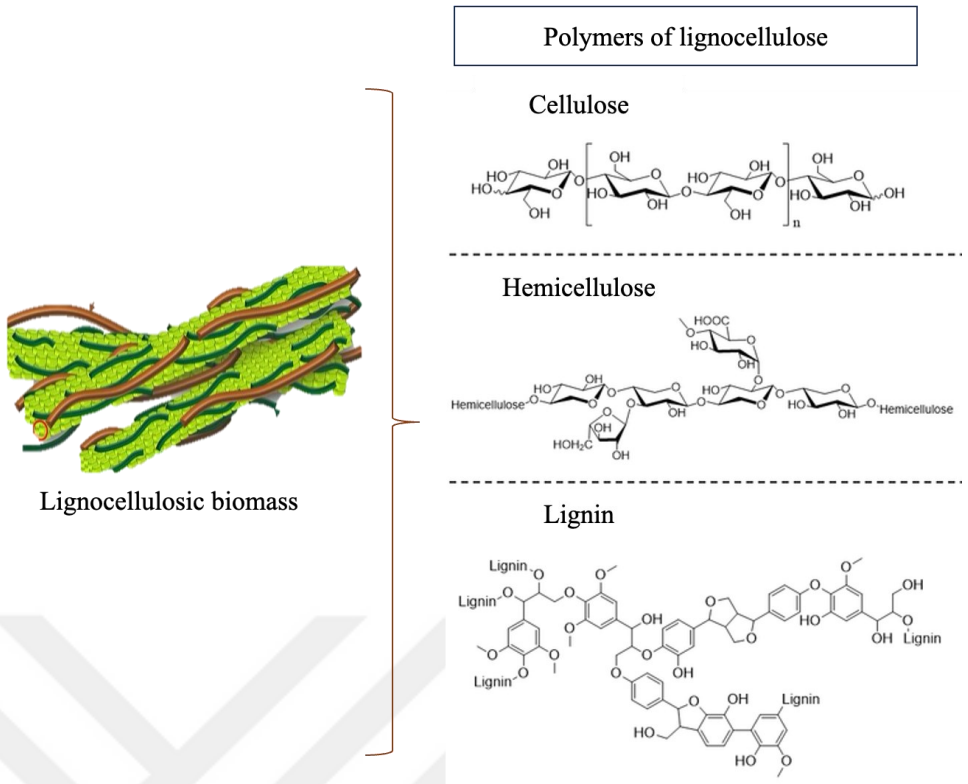

Figure 2. 5. Chemical structure of lignocellulosic biomass.

Cellulose and hemicellulose, the main constituents of lignocellulosic biomass, are polysaccharides that hydrolyze to produce fermentable sugar monomers such as xylose and glucose. These sugars are necessary components for many biotechnological processes, such as the generation of biofuels like bioethanol and biobutanol, biogas, and biochemicals by anaerobic digestion and fermentation (Basera, Chakraborty, and Sharma 2024). However, the protective function of lignin and the complex interlinking of its structural components make lignocellulosic biomass naturally resistant, which makes it difficult to hydrolyze polysaccharides into monomeric sugars (Jahangeer et al. 2024). Therefore, to overcome the lignocellulosic biomass's natural resistance and promote the release of fermentable sugars, effective pretreatment techniques are crucial. The purpose of pretreatment procedures is to increase cellulose accessibility, break down hemicellulose, and alter the structure of lignin in order to improve the overall efficiency of enzymatic hydrolysis (Sun et al. 2016; Alvira et al. 2010). The cellulose, hemicellulose, and lignin content of main lignocellulosic biomass are summarized in Table 2.2.

The conversion of lignocellulosic biomass into biofuels and biochemicals is a multi-step process involving pretreatment, enzymatic hydrolysis, fermentation, and product recovery (Wagle et al. 2022; Poornima et al. 2024). The efficiency of these processes is largely dependent on the initial transformation of polysaccharides into fermentable sugars, which determines the overall yield and productivity of the bioconversion pathway. Therefore, optimizing pretreatment strategies is critical for enhancing the efficiency and cost-effectiveness of lignocellulosic biorefineries (Zhang, Han, and Dong 2021).

Table 2. 2. Lignocellulosic biomass composition as cellulose, hemicellulose, and lignin content.

Lignocellulosic biomass	**Composition (% dry weight)**			**References**
Agricultural Residues	Cellulose	Hemicellulose	Lignin	
Almond Shell	50.7	28.9	20.4	(Demirbaş 2002)
Corncob	45	35	15	(Prasad, Singh, and Joshi 2007)
Corn straw	40.6	22.3	18	(Shaheen et al. 2022)
Hazelnut Shell	26.8	30.4	42.9	(Surek and Buyukkileci, 2017)
Oat Straw	37.6	23.3	12.9	(Aqsha et al. 2017)
Pinewood	38.8	23.6	20.4	(Nanda et al. 2013)
Sugarcane bagasse	43.1	35.3	11.4	(Garcìa-Pèrez, Chaala, and Roy 2002)
Sunflower Shell	48.4	34.6	17	(Demirbaş 2002)
Walnut Shell	25.6	22.1	52.3	(Demirbaş 2002)
Wheat Straw	35.9	23.9	19.3	(Kaparaju, Serrano, and Angelidaki 2009)

2.4. Prebiotics

The expression "prebiotic" describes food components that are resistant to intestinal and pancreatic enzymes, allowing them to pass into the colon and be fermented

by the gut microbiota. This fermentation process stimulates the selective proliferation and activity of several health-promoting bacterial populations, particularly *Bifidobacteria* and *Lactobacilli* (Davani-Davari et al. 2019). Prebiotics are essential considering their potential to improve gut health and wellbeing in addition to their capacity to promote the growth of beneficial bacteria.

Typically, prebiotic compounds are characterized as non-digestible and soluble carbohydrates. However, recent studies have suggested that dietary phytochemicals may also exhibit prebiotic properties (Gotteland et al. 2020). This broadening of the definition highlights the potential for a wider array of food components to influence gut microbiota composition and function.

According to Gibson et al. (2004), a substance qualifies as a prebiotic if it meets the following criteria:

1. Resistance to Gastric Acidity and Enzymatic Hydrolysis: Prebiotics must withstand the stomach environment, which includes resisting mammalian enzyme breakdown. Furthermore, they should not be absorbed in the digestive system.
2. Fermentability by Intestinal Microflora: The ability of prebiotics to be fermented by the intestinal microbiota is essential, as this process produces metabolites that exert beneficial effects on gut health.
3. Selective Stimulation of Health-Associated Bacteria: Prebiotics must selectively enhance the growth and/or activity of intestinal bacteria linked to health and well-being, thereby contributing to a balanced gut microbiome.

Oligomeric prebiotics, which include oligosaccharides, are classified based on their molecular size or degree of polymerization. Several oligosaccharides have demonstrated prebiotic activity, including FOS, GOS, XOS, isomaltooligosaccharides, and soybean oligosaccharides. These compounds have been shown to selectively stimulate the growth and activity of beneficial gut microorganisms, thereby contributing to improved host health (Chavan et al., 2023; Ooi, 2021; Yang and Xu, 2018).

Polymeric prebiotics, such as inulin, are characterized by their high molecular weight and extensive degree of polymerization. These polysaccharides reach the colon largely intact, where partial fermentation by resident microbiota selectively promotes the growth and activity of beneficial bacterial genera, supporting gut homeostasis and overall health (Hughes et al., 2022a; Kelly, 2008). This partial fermentation contributes to the establishment of a favorable microbial environment by providing a competitive advantage to saccharolytic bacteria along the large intestine (Moens and De Vuyst, 2017). The

fermentation kinetics of polymeric prebiotics influence the spatial distribution of microbial activity along the colon, thereby potentially modulating regional microbial composition and metabolic profiles (Macfarlane and Macfarlane, 2011).

Prebiotics change the composition of the gut bacterial population; that is, they increase in *Lactobacillus* and *Bifidobacterium* species. Fermentation of these prebiotics by intestinal bacteria results in the formation of SCFA such as acetate, propionate, butyrate, and lactate (Markowiak-Kopeć and Śliżewska, 2020; Soldi et al., 2019). These SCFAs reduce pH, serve as an electron sink for anaerobic respiration in the intestine, and enhance mineral bioavailability (Broekaert et al., 2011; Singh et al., 2015).It also reduces intestinal infection, suppresses the onset of colon cancer, and improves intestinal health (Zhu et al. 2013; Zeng et al. 2019).

2.5. Lignocellulosic Biomass as Prebiotics

Prebiotics derived from lignocellulosic biomass have emerged as a promising area of research due to their potential to enhance gut health while also contributing to the sustainable utilization of agricultural and forestry waste (Verma, Kaushik, and Sirohi 2024; Saini et al. 2022). Lignocellulosic biomass, primarily composed of cellulose, hemicellulose, and lignin, serves as an abundant source of natural polysaccharides that can be transformed into prebiotic oligo- and polysaccharides through various pretreatments and enzymatic saccharifications (Awasthi et al. 2022; Basera, Chakraborty, and Sharma 2024).

Hemicellulose-derived polysaccharides and oligosaccharides, such as XY and XOS, demonstrate a variety of health-promoting properties that render them promising candidates for prebiotic applications (Kango et al. 2022). While XOS has been extensively researched for their prebiotic potential, XY itself stands out as a particularly promising prebiotic due to its polymeric structure.

By-products from agricultural wastes, such as corn stover, wheat straw, and sugarcane bagasse, are rich in hemicellulose and cellulose and can be processed to extract oligosaccharides. Lignocellulosic materials from wood, including bark and sawdust, can also serve as substrates for the production of prebiotic oligosaccharides (Antoniêto et al. 2022; Kaustubh Chandrakant Khaire, Moholkar, and Goyal 2021). Research has indicated

that enzymatic treatment of wood biomass can lead to the formation of prebiotic XOS, enhancing the growth of gut microbiota and improving overall gut health (Cho et al. 2020).

The prebiotic potential of oligosaccharides derived from lignocellulosic biomass is primarily attributed to their ability to modulate gut microbiota composition and activity. Upon fermentation by gut bacteria, these oligosaccharides produce SCFAs, which have numerous health benefits, including:

- Regulating Gut pH: SCFAs help to lower intestinal pH, creating an environment that inhibits the growth of pathogenic bacteria (Ma et al., 2022).
- Enhancing Gut Barrier Function: SCFAs, particularly butyrate, play a crucial role in maintaining the integrity of the intestinal epithelium and supporting mucosal health (Parada Venegas et al. 2019; Plöger et al. 2012).
- Modulating Immune Response: The fermentation of prebiotic oligosaccharides can stimulate the immune system, promoting the production of anti-inflammatory cytokines and enhancing host defense mechanisms (Costa et al., 2022; Yahfoufi et al., 2018).

Utilizing lignocellulosic biomass for prebiotic production aligns with the principles of sustainable agriculture and waste management. This approach not only contributes to the circular economy but also promotes health benefits associated with prebiotic consumption.

In conclusion, prebiotics derived from lignocellulosic biomass represent a sustainable and health-promoting option. The ongoing research into the extraction, characterization, and application of these oligo- and polysaccharides is critical for advancing our understanding of their potential to improve gut health and overall well-being.

2.6. Xylan

XY is the second most abundant PCW polysaccharide next to cellulose and one of the major constituents (25–35%) of lignocellulosic materials (Wierzbicki et al. 2019; Gigli-Bisceglia, Engelsdorf, and Hamann 2020). The most potential sources of XY

comprise many agricultural wastes such as straw, sorghum, corn stalks and cobs, and hulls and husks from starch production, as well as forest and pulping waste products from hardwoods and softwoods (Ebringerová and Heinze, 2000; Sarrouh et al., 2014). Cereal grains are fundamental components of dietary fiber consumed by humans and have high XY content (Bernstein et al. 2013). Depending on their origin, XY is found in several variations, but with all having in common a β-(1,4)-D-xylose backbone. It may be substituted at the 2′-OH or 3′-OH with other molecules such as acetyl groups, 4-O-methyl glucuronyl groups, or arabinose (Goussougli et al. 2021; Liu et al. 2019). The chemical structure of xylan is shown in Figure 2.6 (Oliveira et al., 2019).

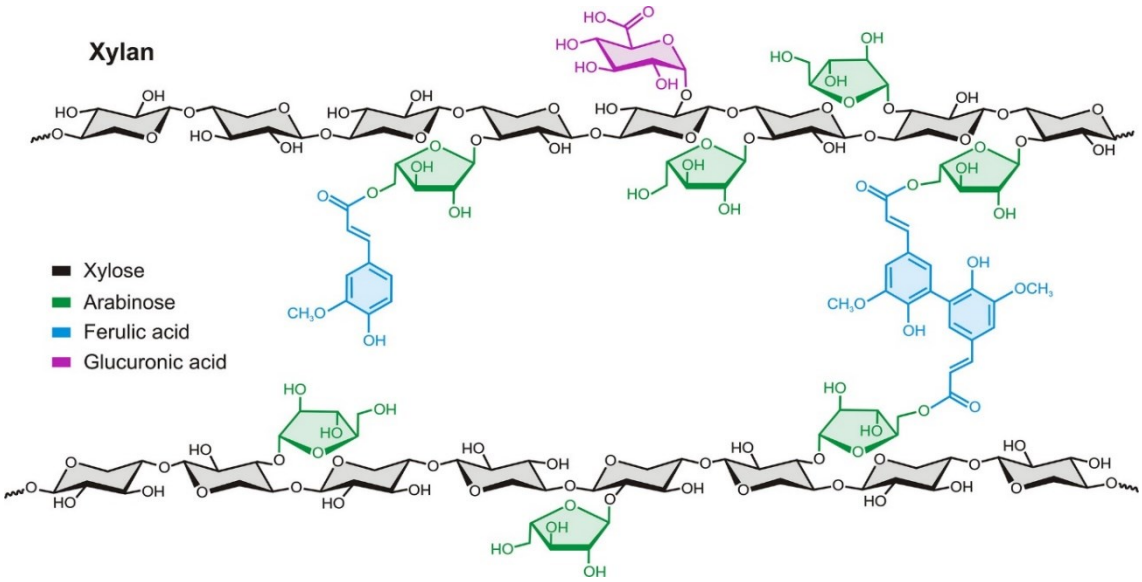

Figure 2. 6. The generalized structure of XY branched with arabinose, ferulic acid, and glucuronic acid (Source: Oliviara et al, 2019).

XY can be classified based on their degree of substitution and the types of side groups present. This classification is crucial for understanding their structural and functional properties, as outlined by Silva et al., (2012):

1. Homo-XY: These are linear polysaccharides that primarily consist of xylose units. They are commonly found in certain types of seaweed and play significant roles in their structural integrity.
2. Glucurono-XY: These XY are characterized by partial acetylation and are predominantly found in hardwood species. The specific properties and

structures of glucurono-XY can vary based on the treatment methods applied during extraction and processing.

3. (Arabino)glucurono-XY: Typical softwood species, these XY incorporate arabinose and glucuronic acid, contributing to their structural diversity and functional roles.
4. Arabino-XY: Frequently located in the starchy endosperm and outer layers of cereal grains, arabino-XY consists of both arabinose and xylose units. They are significant contributors to the dietary fiber content of grains.
5. (Glucurono)arabino-XY: This form is particularly common in the lignified tissues of grass and cereals, where they provide structural support and enhance the plant's resilience.
6. Hetero-XY: These XY are heavily substituted with various mono- or oligosaccharides and are found in cereal bran, seeds, and gum exudates. Their complex structure allows them to interact with other components in the cell wall, influencing the properties of the biomass.

Understanding these classifications helps in the utilization of XY in various applications, including food, pharmaceuticals, and biotechnological processes (Silva et al., 2012; Khaire et al., 2022; Liu et al., 2019; Motta et al., 2013)

XY is an abundant substrate supporting microbial fermentation in the digestive tracts of ruminants as well as humans (Dodd, Mackie, and Cann 2011). Despite the widespread occurrence of XY and its potential health benefits, there is limited information available in the literature regarding the safety of extracted XY when used as a dietary supplement. To address this gap, a study by Qin et al., (2022) was conducted to evaluate the potential toxic effects of XY. The findings indicated that XY is practically nontoxic for human consumption, whether as a food ingredient or dietary supplement. Based on their assessment, the estimated safe dose for humans was determined to be 98 mg per kilogram of body weight per day. For an average adult weighing 70 kg, this corresponds to a daily intake of approximately 6.86 grams, applying a 100-fold safety factor to ensure a margin of safety (Qin et al. 2022). XY and arabinoxylan (AX) proliferated *Bifidobacteria* in *in vitro* gut models conducted using human stool samples (Yang et al., 2021). XY degradation has been reported in ruminants quite well, while some human intestinal bacteria have been investigated for their ability to produce XY-polymer degrading enzymes (Kaur et al., 2019; Moreira and Filho, 2016). XY comprises a variety of chemical linkages, and thus its degradation requires a number of various enzymatic

activities (Biely et al., 2016). XY degradation has not been directly measured in humans, however, from studies of total hemicellulose degradation, it is approximative to be between 51% and 72% (Dodd, Mackie, and Cann 2011). Studies suggest that XY degradation is an important process that contributes to the maintenance of microbial communities in human colonic ecosystems. Yang et al. (2019) have studied turbot (Scophthalmus maximus L.) for the effects of dietary XY on intestinal barrier function and bacteria community. They found that diets containing 1.25% and 5% XY altered the composition of intestinal bacteria positively. Hughes et al. (2007) have studied three AX fractions from wheat on the human fecal microflora. After investigation, they found that total cell numbers increased significantly, and the fermentation of AX was associated with a proliferation of the *Bifidobacteria*, *Lactobacilli*, and *Eubacteria* groups.

Some beneficial species such as *Bifidobacteria* and *Lactobacillus*, except for a few species, cannot directly degrade XY (Rivière et al. 2014; Hopkins et al. 2003). It is known that the carbohydrate mechanism of bacteria in this population differs from species to species and even from strains. Among the bacteria in the human colon, *Bifidobacteria* spp. are the first to colonize the human gastrointestinal tract and reach their highest proportion in the colon (up to 90% of the total colon microbiota in breast-fed infants) during the first 12 months of life. This abundance significantly reduces over time to <5% in adults and reduces even more in the elderly (Rivière et al. 2016). *Bacteroidetes* represent a key xylanolytic group in the human colonic microbiota. Although *Bifidobacteria* species cannot directly metabolize XY, they can utilize XOS. Consequently, the bifidogenic effect of XY may rely on its initial degradation by *Bacteroides* species, which release oligosaccharides through XY hydrolysis that *Bifidobacterium* can then metabolize (Zeybek, Rastall, and Buyukkileci 2020). Supporting this, *Ruminococcus intestinalis* and *Bacteroides* species were able to co-cultivate on XY in a mixed culture, indicating a cooperative relationship (Leth et al. 2018).

Figure 2.7. illustrates the role of dietary polysaccharides, specifically XY, in the gut and its impact on digestive health. It highlights the following key processes:

- Dietary Polysaccharides Intake (XY): The intake of XY, a dietary fiber, is shown to influence gut health by serving as a substrate for microbial fermentation in the colon.
- SCFAs Production: The microbial fermentation of XY leads to the production of SCFAs, including acetate, propionate, and butyrate. These

SCFAs are important metabolic byproducts that have various beneficial effects on gut health.

- Absorption and Effects on the Gut Microbiota: The figure suggests that SCFAs produced in the colon are absorbed and play a role in modulating the composition of the gut microbiota. This process also contributes to the fermentation activity of the bacteria present.

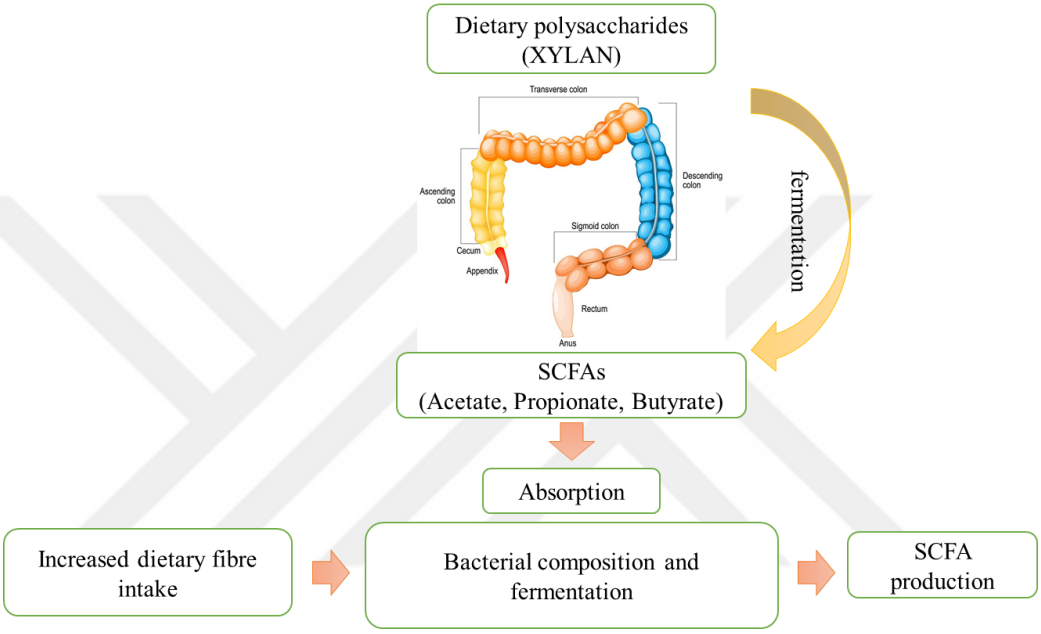

Figure 2. 7. Schematic representation of complex polysaccharides in the large intestine.

2.7. Degradation of Xylan in the Large Intestine

The degradation of XY in the gastrointestinal tract is facilitated by the gut microbiota, which produces xylanolytic enzymes that break down XY into various prebiotic metabolites (Linares-Pastén et al. 2021; Fernandez-Julia, Munoz-Munoz, and van Sinderen 2021). This process requires coordinated action of several specific enzymes to convert XY into XOS and free xylose. Key enzymes include α-L-arabinofuranosidase, α-D-glucuronidase, acetylxylan esterase, and ferulic acid esterase, each of which releases side chains from the XY backbone (Barroca 2021; Razeq 2017).

Endo-1,4-xylanase and β-xylosidase work synergistically to degrade the XY backbone: endo-β-1,4-xylanase hydrolyzes internal β-(1,4) linkages, producing shorter XOS. In contrast, β-xylosidase removes individual xylose units from the non-reducing ends of these oligosaccharides. The β-D-xylosidases, a diverse group of enzymes from various enzyme families, play a crucial role in this process, contributing to the breakdown of XY into absorbable components (Ávila et al. 2020; Delgado-Garcia et al. 2025).

The side chains of XY, including L-arabinofuranose (L-Ara), D-glucuronic acid (D-GlcA), and O-4 methyl D-glucuronic acid (O-4 Me D-GlcA), increase the solubility of XY and offer some protection against enzymatic breakdown of the XY backbone. Modifications such as adding ferulic acid to L-Ara side chains connect XY to cellulose fibers, providing mechanical stability in plant tissues. These structural modifications not only impact the solubility and degradation rates of XY but also influence its functional role as a prebiotic substrate in the gut microbiota (Moreira and Filho, 2016; Zhong et al., 2024).

2.8. Xylooligosaccharides

In oligomeric prebiotics, XOS consists of hydrolysis of XY which is the main subject of the thesis. XOS are a novel prebiotic class that can work against several gastrointestinal disorders (Samanta et al. 2015). XOS are reported to be present naturally in honey, bamboo shoot, fruits, and vegetables. XOS are hydrolysis product of xylan and consist of xylose units through β-(1-4)-xylosidic linkages; xylobiose (2 monomers), xylotriose (3 monomers), xylotetrose (4 monomers), xylopentose (5 monomers), xylohexose (6 monomers) and so on (Silva et al., 2024; Rahmati et al., 2022). The XOS molecular formula is $C_{5n}H_{8n+2}O_{4n+1}$; where, n:2 to 6 (Chelliah et al. 2024).

XOS have recently gained attention for their health-promoting potential, particularly in the area of gastrointestinal health (Lecerf et al. 2012; Jana, Kango, and Pletschke 2021). As non-digestible carbohydrates, XOS reaches the colon intact, where they selectively stimulate the growth and activity of beneficial gut microorganisms (Palaniappan, Antony, and Emmambux 2021). This characteristic is consistent with prebiotics' description as substrates that improve the function of host-associated microbes to provide health advantages (Fibers et al. 2019). In addition to supporting healthy gut

flora, XOS has been shown to lower blood cholesterol, improve mineral absorption, and boost immunity (Yan et al. 2022; Abasubong et al. 2022; Ding et al. 2018). Consuming XOS has also been associated with improved antioxidant capacity, decreased blood glucose levels, and potential anticancer effects (Gupta et al. 2018; Wu et al. 2020; Batsalova et al. 2022).

XOS exhibit a range of biological functions that support overall health, including anti-inflammatory, antibacterial, and antioxidative properties, as well as reducing the activity of harmful bacterial enzymes in the gut (Chakraborty and Bhowal, 2024; Vieira et al., 2020). The fermentation of XOS by beneficial bacteria generates SCFAs, which improve intestinal integrity, support glucose balance, modulate immune responses, and create a less hospitable environment for pathogenic organisms through pH reduction (Palaniappan, Antony, and Emmambux 2021). This fermentation also impacts energy intake by influencing satiety-regulating hormones (Reinehr and Roth, 2015).

Traditionally, prebiotics like inulin and FOS have been favored; however, XOS are emerging as a valuable alternative due to their effectiveness and adaptability in food applications (Echegaray et al. 2023; Lin et al. 2024). Unlike other prebiotics, XOS resists digestion in the upper gastrointestinal tract and has a notable impact on beneficial gut bacteria (Poolsawat et al. 2021; Rao et al. 2024; Van T Pham et al. 2021). Studies in both animals and humans have demonstrated that XOS consumption increases *Bifidobacterium* populations, enhances fecal moisture, and reduces intestinal pH, highlighting its value in promoting gut health and broadening its application in nutraceutical and functional food products (Chavan et al. 2023; Valladares-Diestra et al. 2023; Amir 2021). With its many health advantages, especially for gut health and metabolic function, XOS is a remarkable prebiotic that is an excellent option for dietary interventions meant to enhance general well-being (Aachary and Prapulla, 2011). The studies conducted with XOS-administered doses ranging from 0.4 to 8 g per day reported a statistically significant increase in the abundance of *Bifidobacteria*, highlighting the prebiotic effect of XOS on beneficial gut microbiota (Lyu et al. 2020; Riva et al. 2023; Deng et al. 2023; Lecerf et al. 2012).

2.9. Prebiotic Test Models

Test tubes inoculated with fecal samples in prebiotic testing are frequently used in *in vitro* models because of their convenience, affordability, and simplicity of experimental setup (Likotrafiti et al., 2014; Pham and Mohajeri, 2018). These models provide a straightforward approach to evaluating the fermentative activity of gut microbiota in response to various prebiotics, allowing for the controlled manipulation of experimental conditions (Wiese et al. 2024).

Prebiotic research using *in vitro* models has made it possible to study the metabolism of various prebiotic substrates by the gut microbiota, including oligosaccharides such as XOS, FOS, and GOS, as well as polysaccharides like XY and inulin, and resistant starches (Fehlbaum et al., 2018; Gong and Yang, 2012; Petrova and Petrov, 2017). Beneficial bacteria, mainly *Bifidobacterium* and *Lactobacillus* species, utilize these substrates as energy sources, which may cause the composition of the microbial community to change in preference for a profile that is more beneficial to health. According to *in vitro* research, prebiotic fermentation usually produces SCFAs, such as acetate, propionate, and butyrate, which have been linked to positive outcomes as improved gut barrier function, reduced inflammation, and increased colonic motility (Falck et al. 2013; Moura et al. 2007).

The fermentation kinetics of various prebiotics can be thoroughly examined by *in vitro* models. To simulate different conditions in the gastrointestinal tract, factors such as temperature, pH, substrate concentration, and microbial inoculum can be altered. Advanced *in vitro* models, such as the SHIME (simulator of the human intestinal microbial ecosystem) and TIM (TNO gastrointestinal model) have further refined the simulation of the digestive environment (Fois et al., 2019; Wang et al., 2024; Žukauskaitė et al., 2024). These systems include multiple compartments that represent different sections of the gastrointestinal tract, allowing for the sequential breakdown and fermentation of prebiotics as they pass through simulated regions of the stomach, small intestine, cecum, and colon. Such models are equipped to regulate pH, temperature, and anaerobic conditions, as well as incorporate physiological fluids like gastric juice, bile, and pancreatic enzymes. These features enable the investigation of how different prebiotics are metabolized along the entire digestive tract, providing a more

comprehensive understanding of their functional impact (Gościniak et al., 2022; Pham and Mohajeri, 2018; Singh et al., 2022).

Despite the numerous advantages of *in vitro* models, including high throughput and precise control over experimental conditions, they have certain limitations (Horvath et al. 2016; Astashkina, Mann, and Grainger 2012). The simplified conditions of *in vitro* setups do not fully replicate the complex environment of the human gastrointestinal tract (Lefebvre et al. 2015; Dupont et al. 2019). These models cannot account for critical factors such as immune responses, the effects of the mucosal barrier, and the intricate interactions between the host and the microbiota. Furthermore, there can be differences between the various microbial communities seen in various parts of the human gut and the microbial diversity of fecal inoculum used *in vitro*. Such differences can lead to variations between *in vitro* findings and outcomes observed *in vivo*, potentially limiting the generalizability of the results. Although these limitations, *in vitro* prebiotic studies are crucial in early research stages because they provide important insights that guide the design of more complex in vivo studies and clinical trials (Van den Abbeele et al. 2023; Cunningham et al. 2021; Hutkins et al. 2024). When used alongside other models, such as ex vivo and in vivo systems, *in vitro* models enhance our understanding of how prebiotics influence gut health and microbial ecology.

In vivo studies using animal models are essential for enhancing our understanding of the biological processes and mechanisms associated with prebiotic consumption, especially when examining these effects within the context of a whole organism (Saulnier et al. 2009; Sanders et al. 2019). Such models provide a valuable opportunity to investigate how prebiotics interact with and influence the host's physiology, offering insights into their potential health benefits. These studies are particularly important for evaluating systemic effects that cannot be fully captured through *in vitro* experiments, as they allow for the observation of complex interactions between prebiotics, gut microbiota, and host tissues in a living system (Vashishat et al. 2024; Nguyen et al. 2015).

Rodents, particularly mice and rats, are the most frequently used models due to their extensively characterized gut microbiota and gastrointestinal physiology, which share certain similarities with human GI functions (Vandamme 2015; Steimer 2011). These characteristics make rodents suitable for studying the general effects of prebiotics on gut health and metabolism. However, the exclusive use of rodent models may not always accurately reflect the diversity of responses observed in other species or in humans, necessitating the inclusion of other animal models in research. Alternative

animal models, such as pigs, fish, rabbits, dogs, chickens, and cats, have been employed to investigate specific aspects of prebiotic activity, as each species provides unique insights into prebiotic dynamics (Anadón et al., 2019; Van Loo and Vancraeynest, 2008). For example, pigs are often used because their digestive anatomy and physiology are more similar to humans compared to rodents, while fish models can offer insights into aquatic species' responses to prebiotics and their impact on gut health (Collinder et al. 2003; Sciascia, Daş, and Metges 2016; Roura et al. 2016). Exploring these different models helps researchers understand species-specific differences in prebiotic metabolism and their effects on host health, which is important for translating findings into clinical applications (Mendes-Soares and Chia, 2017; Sonnenburg et al., 2006).

Animal models can be used to observe the systemic changes caused by prebiotic supplementation, such as alterations in gut microbiota composition and their effects on the host's physiological functions. These studies are valuable for evaluating the potential broader impacts of prebiotics beyond localized gastrointestinal effects, offering insights into how these substances may affect overall health. However, using *in vivo* models presents several challenges, including high costs, complex logistical issues, and ethical concerns related to animal testing (Deng et al. 2023). Furthermore, while *in vivo* research provides a comprehensive understanding of prebiotic activity, it often lacks the precision needed to explore specific organ-level mechanisms or localized effects within the GI tract (Yata 2024; Donkers, van der Vaart, and Van de Steeg 2023). Therefore, to fully understand the complex roles of prebiotics, complementary research approaches are often necessary (Vashishat et al. 2024).

Human clinical studies provide critical insights into the effects of prebiotics on host health and the composition of the gut microbiota (Martinez, Bedani, and Saad 2015). Unlike *in vitro* or animal model research, these studies account for individual variability in physiological responses to prebiotic treatments, offering a more accurate depiction of human health.

However, practical and ethical limitations often hinder the ability to monitor dynamic changes in the gut microbiota during clinical investigations (Ma et al., 2018; Quigley, 2017). Invasive sampling procedures, such as frequent biopsies or colonoscopies, result in significant risks and discomfort for participants, making them unsuitable for routine monitoring (Von Wagner et al. 2009; Harlid, Gunter, and Van Guelpen 2021). Consequently, sampling is often limited, restricting the ability to fully understand region-specific microbial fermentation processes and nutrient absorption,

which can vary considerably between the proximal and distal regions of the colon (Martinez, Bedani, and Saad 2015; Biesiekierski et al., n.d.).

An alternative approach to studying the effects of prebiotics is to use *ex vivo* models, which involve tissues and organs removed from animals for examination (Pearce et al. 2018). These models offer a way to address ethical concerns linked to animal research while providing greater control over experimental conditions. *Ex vivo* setups can simulate physiological processes occurring in specific regions of the large intestine, such as gut motility and microbial fermentation of prebiotics, allowing researchers to explore these activities in a controlled environment (Mottawea et al., 2020; Tsilingiri and Rescigno, 2012).

Ex vivo models of the gastrointestinal tract, particularly for studying the large intestine, are especially promising for prebiotic research. These models allow researchers to closely replicate critical physiological processes like microbial fermentation and gut motility (Park et al. 2017). By isolating the colon and recreating conditions specific to the large intestine, *ex vivo* setups facilitate targeted experimentation on prebiotic effects, making it possible to observe how prebiotics influence the gut microbiome and metabolite production in a localized environment (Yata 2024). The control offered by *ex vivo* approaches enables researchers to adjust specific variables, such as temperature, nutrient concentration, and oxygen levels, to closely mimic the intestinal environment while also observing responses in real time (Verhoeckx et al. 2015). Consequently, *ex vivo* models serve as a complementary method for prebiotic investigation, helping researchers gain mechanistic insights that are challenging to achieve through other methods.

Tables 2.3 summarize the results of clinical trials, *in vitro* models, and in vivo studies involving XOS, XY, INU, and mixes of some prebiotics detailing their impacts on the microbiome and various clinical biomarkers.

Table 2. 3. Summary of Clinical Trials, In Vitro Models, and In Vivo Studies Involving XOS, XY, INU, and Combinations of Various Prebiotics.

Organism	**Prebiotic Formula**	**Endpoints**	**References**
Human *(Clinical)*	8 g XOS per day	Increased *Bifidobacteria.* Decreased IL-10 production Decreased 10% risk of coronary artery disease Increased HDL levels	(Childs et al. 2014)
Human *(Clinical)*	95% of pure XOS, 4 g per day	Increased *Bifidobacteria* Increased in fecal moisture and fecal pH	(Chung et al. 2007)
Human *(Clinical)*	70% of pure XOS, 1.4 and 2.8 g per day	Increased *Bifidobacteria*	(Finegold et al. 2014)
Human *(Clinical)*	1.4 and 2.8 g XOS per day	Increased *Bifidobacteria* and lactic acid concentration Decreased triglycerides, cholesterol levels	(Na and Kim, 2007)
Human *(Clinical)*	1 g XOS + 3 g inulin per day	Increased *Bifidobacteria* Increased butyrate Decreased cresol	(Lecerf et al. 2012)
Human *(Clinical)*	70% of pure XOS, 2.8 g per day	Decreased *Firmicutes* No effect on blood glucose	(Yang et al., 2015)
Human *(Clinical)*	arabinoxylan-oligosaccharide, 0, 2.2, 4.8 g per day	Increasing fecal *Bifidobacteria* Increases postprandial ferulic acid concentrations	(Maki et al. 2012)
Human *(Clinical)*	Inulin in chocolate, 5, 7.5 g per day	Increased 3- and 4-fold *Actinobacteria*, *Bifidobacteria* Decreased *Lachnobacterium*, *Ruminococcus*, *Desulfovibrio*	(Holscher et al. 2015)
Human *(Clinical)*	FOS+inulin mix, LDF: Low dietary fiber, 8 g per day HDF: High dietary fiber, 16 g per day	LDF: Increased *Bifidobacteria*, *Lactobacillus* Decreased *Ruminococcaceae* HDF: Increased *Bifidobacteria* and *Ruminococcaceae.* Decreased *Faecalibacterium*, *Coprococcus*, *Dorea,* and *Ruminococcus* (*Lachnospiraceae* family)	(Healey et al. 2018)
Human *(Clinical)*	Inulin, 13-15 g per day	Increased *Bifidobacteria*	(Blædel et al. 2016)
Human *(Clinical)*	Short chain-inulin + Long chain-inulin mix	Increased *Bifidobacteria* and *Lactobacillus*	(Soldi et al. 2019)

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Table 2.3 (cont.)

Organism	Prebiotic Formula	Endpoints	References
Human (*in vitro*)	Wheat bran (74% of the dietary fiber comprises xylan and XOS)	Increased the relative abundance of *Dorea*, *Bilophila*, and *Sulfurovum*	(Chen et al., 2020)
Human *(in vitro)*	Arabinoxylan (Fractions: average molecular masses of 354, 278, and 66 kDa)	Increased in proliferation of the *Bifidobacteria*, *Lactobacilli*, and *Eubacteria* groups	(Wang et al., 2020)
Human *(in vitro)*	Xylan	*R. intestinalis* and the *Bacteroides* competitor co-grew in a mixed culture on xylan and dominated *R. intestinalis* on the preferred transport substrate X4 *R. intestinalis* grew rapidly on soluble xylans Boosted distinct taxa in the gut microbiome	(Leth et al. 2018)
Human *(in vitro)*	Arabinoxylan	Among XOS, X3 and X4 were best utilized by *Bifidobacteria*	(Ejby et al. 2013)
Human *(in vitro)*	Arabinoxylan	Arabinoxylan not fermented by *Lactobacilli*, *Enterococci*, *Escherichia coli*, *Clostridium perfringens* Increased *Bifidobacterium longum*	(Crittenden et al. 2002)
Human *(in vitro)*	Xylan	*Roseburia intestinalis* colonized the substrates more efficiently than *Bact. xylanisolvens* *Roseburia and Bacteroides display very high xylanolytic activity*	(Mirande et al. 2010)
Human *(in vitro)*	Arabinoxylan	Increased *Lactobacilli* and *Bifidobacteria* No effect on *Escherichia coli*	(Van Laere et al. 2000)

(cont. on the next page)

Table 2.3 (cont.)

Organism	Prebiotic Formula	Endpoints	References
Fish *(in vivo)*	1% XOS per day (supplemented in daily diet)	Improved growth performance and glycolipid metabolism	(Chen et al., 2022)
Broiler Chickens *(in vivo)*	0.005% or 0.01% of XOS (supplemented in daily diet)	Enhanced production of SCFA through increased cecal fermentation	(Singh et al., 2021)
Broiler Chickens *(in vivo)*	150 mg/kg XOS (supplemented in daily diet)	Increased villus height of duodenum, jejunum, and ileum Reduced fecal ammonia release	(Li et al., 2022)
Pigs *(in vivo)*	100, 250, and 500 mg XOS (supplemented in daily diet)	Reduced pathogenic bacteria and enhanced beneficial bacteria (*Firmicutes* and *Lactobacillus*) Increased concentrations of total SCFAs in the intestine	(Pan et al. 2019)
Mice *(in vivo)*	5% and 15% of Inulin (supplemented in daily diet)	Reduced 50% of colonic tumor load Changed the ratio of *Bacteroidetes/Firmicutes*	(Moen et al. 2016)
Mice *(in vivo)*	10% of XOS (supplemented in daily diet)	Increased *Bifidobacteria* throughout the intestine Reduced expression of interleukin 1b (Il1b) and interferon g (Ifng) in blood Decreased systemic inflammation Increased SCFA concentrations in the gut	(Hansen et al. 2013)
Pigs *(in vivo)*	resistant starch, arabinoxylan, β-glucan	Most of the carbohydrates are fermented in the proximal part, allowing protein fermentation in the distal part Fermentation of xylan produces butyrate primarily in small and large intestine	(Tiwari, Singh, and Jha 2019)
Guinea pigs *(in vivo)*	10% XOS, GOS, inulin, apple pectin	Improved the resistance of guinea pigs to *L. monocytogenes* in XOS and GOS groups Decreased the resistance in inulin and apple pectin groups	(Ebersbach et al. 2010)

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Table 2.3 (cont.)

Organism	**Prebiotic Formula**	**Endpoints**	**References**
Fish (Juvenile turbot) *(in vivo)*	0%, 1.25% and 5% of xylan (supplemented in daily diet)	Increased the gene expression of transforming growth factor β and tight junction protein Tricellulin, and decreased the expression of pro-inflammatory cytokine Il-1β in 1.25% xylan Increased the expression of Il-1β and Muc-2, and decreased the gene expression of tight junction protein Claudin-3 in 5% xylan Increased the abundance of *Clostridium*, *Escherichia coli,* and *Prevotella copri* associated with intestinal disease in 5% xylan	(Yang et al., 2019)
Mice *(in vivo)*	FOS, GOS 0.8, 4, and 8 g/kg per day	Decreased ratio of *Firmicutes* to *Bacteroidetes* Increased abundances of *Bifidobacterium*, *Bacteroides*, *Rikenella*, *Lactobacillus*, and *Clostridium* Decreased abundances of *Oscillospira*, *Coprococcus*, and *Dorea* at the genus level	(Wang et al., 2017)
Mice *(in vivo)*	2% and 7% of XOS (supplemented in daily diet)	Decreased in visceral fat depots, the concentration of inflammatory cytokine MCP-1, and abundance of the *Firmicutes* and *Bacteroidetes* were observed with 7% XOS supplementation No change in blood or liver lipids in both XOS doses Increased SCFA production in the cecum Decreased adiposity through markers of adipogenesis and fat synthesis and induced changes in intestinal microbial composition	(Long et al. 2019)

2.10. Functional Properties of Hydrocolloids Used in Food Applications

Hydrocolloids are essential in food formulation due to their unique physicochemical properties that enable the modification of texture, stabilization, and structural enhancement across a wide range of food products (Sahraeian, Rashidinejad, and Niakousari 2023). Recognized by the European Food Safety Authority (EFSA) and the U.S. Food and Drug Administration (FDA), hydrocolloids are widely employed for their functional versatility and significant contributions to food quality (Viebke, Al-Assaf, and Phillips 2014).

Hydrocolloids play multiple roles in food systems, primarily due to their high water-binding capacity, essential for enhancing texture and retaining moisture in products ranging from sauces and dressings to baked goods (Nishinari et al., 2018). Through their thickening and gelling properties, hydrocolloids contribute to the structural integrity and stability of food products, allowing formulators to achieve the desired consistency in items such as fruit fillings, confectioneries, and dairy-based desserts (Agudelo et al., 2014). Additionally, hydrocolloids serve as fat replacers in products like chocolates and cream-based fillings, creating lower-fat alternatives while preserving texture and sensory appeal (Dias, Alvarenga, and Sousa, 2015).

Moreover, hydrocolloids act as effective emulsifying agents in dairy-based items such as ice cream, yogurt, and beverages, where they stabilize fat-water mixtures, enhance texture, and prevent phase separation, thus maintaining product quality and consumer appeal (Nishinari et al., 2018). In bakery applications, they function as bulking agents, contributing to product volume, improving mouthfeel, and extending shelf life by reducing staling effects (Espert et al., 2019). These multifunctional properties emphasize the crucial role of hydrocolloids in delivering desirable texture, stability, and sensory qualities in a variety of food products.

Table 2.4 provides an overview of the applications of various hydrocolloids in filling food systems, including desserts, puddings, sauces, salad dressings, and jams. XY has potential as a food hydrocolloid due to its complex structure; however, there is currently no research investigating its specific hydrocolloid properties.

Table 2. 4. Summary of functional properties of different hydrocolloids.

Hydrocolloids	Functional properties	Food application	References
Inulin	Fat Replacer	dairy products, desserts	(Yousefi and Jafari 2019; Beccard et al. 2019; Esmaeilnejad Moghadam et al. 2019)
	Gelling and Thickening	jams, jellies, soups, sauces	
	Texture Modifier	yogurt, ice cream	
Guar gum	Water Binding Capacity	beverages, soups, sauces, salad dressings	(Nasrollahzadeh et al. 2021; Himashree, Sengar, and Sunil 2022; Glicksman 2020)
	Thickening Agent	sauces, dairy products	
	Emulsifying	mayonnaise, salad dressings, ice cream, and margarine	
Xanthan gum	Gelling Agent	jams, jellies	(Doublier, Garnier, and Cuvelier 2017; Kongjaroen et al. 2022; Paquet et al. 2014)
	Viscosity Modifier	beverages and sauces	
	Stabilizer	fruit juices, beverages, and suspension-based food products like salad dressings and ketchup	
Starch	Water Absorption and Binding Capacity	sauces, gravies, soups, and beverages	(Dickinson 2018; Marta, Cahyana, and Djali 2020; C. Sun and Fang 2021)
	Emulsifying Agent	salad dressings, mayonnaise, and creams	
	Fat Replacer	low-fat cakes, pastries, and salad dressings	
Pectin	Thermal stabiliser and gelling agent	jams, jellies, marmalades	(Cong et al. 2022; Sharma et al. 2017; Yang et al. 2021)
	Texturizing Agent	yogurt, ice cream	
	Thickening Agent	sauces, dressings, and beverages	

CHAPTER 3

IN VITRO UTILIZATION OF PREBIOTICS BY MICE FECAL MICROBIOTA

3.1. Introduction

The human colon is densely populated by a diverse community of over one trillion microorganisms, which are increasingly recognized as key regulators of human health through their interactions with dietary macronutrients (Bedu-Ferrari et al. 2022). The gut microbiota performs a range of essential physiological functions, including maintaining the integrity of the gut barrier, fermenting indigestible nutrients that escape digestion in the small intestine, and converting these nutrients into metabolites that are readily absorbed by the body (Waclawiková et al. 2022; Wos-Oxley et al. 2012). These activities contribute to the host's energy balance and nutritional status. The microbiota also actively engages with the immune system, modulating immune responses to protect against infections (Waclawiková et al. 2022; Xu et al. 2020; Wan et al. 2020). It suppresses the growth of pathogenic organisms, helping maintain a balanced microbial ecosystem in the gut (Zhao et al. 2017; Mendis, Martens, and Simsek 2018; Bedu-Ferrari et al. 2022). Gut microbiota significantly influences human health and disease through these complex and interlinked activities.

Daily dietary intake significantly influences the composition and function of the gut microbiota, with prebiotics playing a key role in modulating microbial communities. Prebiotics, which are non-digestible food components that promote the growth and activity of beneficial gut bacteria, can profoundly impact gut health and metabolic function (Rawi et al. 2021; Bhatia et al. 2024). Understanding the relationship between diet, including prebiotics, and the gut microbiota offers a promising approach for preventing various chronic diseases, including obesity, type 2 diabetes, cardiovascular diseases, and colorectal cancer (Fernández et al. 2016; Nguyen et al. 2015; Biswas et al. 2022). By targeting microbiota through dietary strategies such as prebiotic

supplementation, it may be possible to improve health outcomes and reduce the risk of diet-related conditions.

Different models, including *in vitro* systems (batch models, multistage continuous fermentation models, artificial digestive systems, and intestine-on-a-chip), *in vivo* studies (using animals such as mice, rats, pigs, dogs, cats, zebrafish, flies, and worms), *ex vivo* techniques, and human clinical trials have been extensively utilized to investigate the interactions between dietary macronutrients and gut microbiota (Chen et al, 2006; Deng et al., 2023; Nguyen et al., 2015; Vashishat et al., 2024). While human trials provide insights under realistic physiological conditions, they are limited in studying the mechanisms of microbial nutrient degradation in the colon parts due to ethical considerations.

In vitro models provide a cost-effective, easy-to-setup approach that is particularly useful for investigating a large number of samples (Emilia et al. 2024; Fibers et al. 2019). These models allow for precise control of gut physiological parameters, such as pH, temperature, retention time, medium composition, and anaerobic conditions, simulating specific gut environments (Wos-Oxley et al. 2012; Swearengen 2018; Waclawiková et al. 2022). They also enable the monitoring of metabolite production, following prebiotic treatments. *In vitro* setups typically involve incubating fecal samples at 37°C for a limited duration of 24–48 hours (Li et al., 2020; Poeker et al., 2018; Verhoeckx et al., 2015). This short incubation period is necessary due to the microbiota's rapid progression to the stationary phase, which occurs as a result of nutrient depletion and the accumulation of inhibitory metabolites.

Prebiotics with varying chain lengths exhibit different consumption rates, and it is hypothesized that XY is consumed more slowly due to its polymeric structure. *In vitro* tests were performed to compare the consumption rates of various prebiotics, focusing on the ease of sample collection at different time points. The primary aim of this part of the study was to investigate the utilization kinetics of XY-based prebiotics and to evaluate their potential for the slow utilization hypothesis. Specifically, the study investigated comparing the fermentation kinetics of XY with XOS and INU. Furthermore, the together administration of oligomeric and polymeric prebiotics was examined to elucidate the kinetics associated with prebiotics of varying chain lengths and to provide a comprehensive understanding of their utilization and fermentation dynamics. To test these hypotheses, the study employed fecal inoculum derived from BALB/c mice, enabling the

analysis of XY-based prebiotic utilization, microbial population dynamics, and resultant metabolite production.

3.2. Materials

XY was extracted from corncob (CC) as described below. XOS (95% purity) was provided kindly by Longlive Biotechnology (Shandong, China). It was composed of 39.3% xylobiose (X2), 31.7% xylotriose (X3), and 23.3% xylotetraose (X4). XOS standards for HPLC were from Megazyme (Ireland). HPLC chromatograms of the Longlive XOS and XOS standards are provided in Appendix B. INU was purchased from Fibrelle (Türkiye). The High Pure PCR Template Preparation Kit (Roche, Switzerland) was utilized for DNA extraction from tissue samples. The FastStart Essential DNA Green Master (Roche, Switzerland) was employed for quantitative PCR (qPCR) analysis. All other chemicals were of analytical grade and purchased from Merck (Darmstadt, Germany).

3.3. Methods

The methods given below were performed to utilize five different prebiotic groups in *in vitro* fecal inoculum.

3.3.1. Xylan Extraction from Corncob

XY was extracted by alkali treatment of the CC obtained from the Aegean Agricultural Research Institute of the Ministry of Agriculture and Forestry (Türkiye). Ground CC was mixed with 15% NaOH at a solid-to-liquid ratio of 1:10. The suspension was kept in the autoclave at 121 °C for 1 h. The solids were removed by filtration and the liquid was neutralized with glacial acetic acid. XY was precipitated with two volumes of ice-cold ethanol. The precipitate was separated by filtration and dried in a forced hot air

oven at 40 ºC until we have a constant weight. The pellets were weighed, ground in a grinder-mixer, and stored at room temperature in air-tight bags.

3.3.2. Sample Collection and Preparation of Fecal Inoculum

Fresh fecal samples were collected from six BALB/c mice, aged 4 to 6 weeks, to ensure consistency and diversity of microbiota. The collected feces were pooled immediately and thoroughly mixed to minimize individual variations. Subsequently, the pooled fecal samples were diluted at a ratio of 1:10 (w/v) with carbohydrate-free basal medium (BM) and homogenized into a slurry for 15 minutes to achieve uniform microbial dispersion. The BM was formulated with peptone water 2 g/L, yeast extract 2 g/L, NaCl 0.1 g/L, K_2HPO_4 0.04 g/L, KH_2PO_4 0.04 g/L, $MgSO_4$ 0.01 g/L, $CaCl_2$ $6H_2O$ 0.01 g/L, $NaHCO_3$ 2 g/L, cysteine HCl 2.5 g/L, bile salts 0.5 g/L, tween 80 2 g/L, 1 mL/L of hemin solution (50 mg/mL), 10 mL/L of vitamin K, and 4 mL resazurin 0.001 g/L according to Salazar et al. (2009). The final concentration of the inoculum was standardized to 10% (w/v) for use in fermentation experiments.

The institutional ethics committee at the Laboratory Animal Production, Care, Application, and Research Center, IZTECH, approved all mice experimental protocols.

3.3.3. Utilization of Model Prebiotics in *in vitro* Cultivation

The experiment was designed to assess the microbial and metabolic impact of five different prebiotic groups compared to a control group. The prebiotics tested included:

- Xylooligosaccharides (XOS)
- Xylan (XY)
- Combination of XOS and XY (XOS+XY mix)
- Inulin (INU)
- Combination of INU and XY (INU+XY mix)
- Control (basal medium only)

For the fermentation assays, each prebiotic substrate was dissolved separately in BM at a final concentration of 10 g/L. This concentration was selected based on prior studies, aiming to simulate a dietary dose relevant to prebiotic intake and to ensure observable fermentation effects in the incubation period (Cruz-Guerrero et al., 2014; Silva et al., 2024; Grimoud et al., 2010).

Each test tube containing a different prebiotic solution was inoculated with 10% of the prepared fecal slurry (6 mL), creating a mixture that would allow for the fermentation of the prebiotic substrate by the gut microbiota. The liquid surface was covered with paraffin (1 mL) to prevent atmospheric diffusion. All treatments were conducted in 4 parallels. The test tubes were inoculated with the fecal slurry (10%) and incubated at 37°C for 48 h. Various parameters were measured regularly to assess microbial dynamics and metabolic outcomes comprehensively. This included cell concentration using the optical density at 600 nm (OD_{600}) method, pH, utilization of model prebiotics, SCFA production, and microbiome composition analysis by qPCR (Figure 3.1).

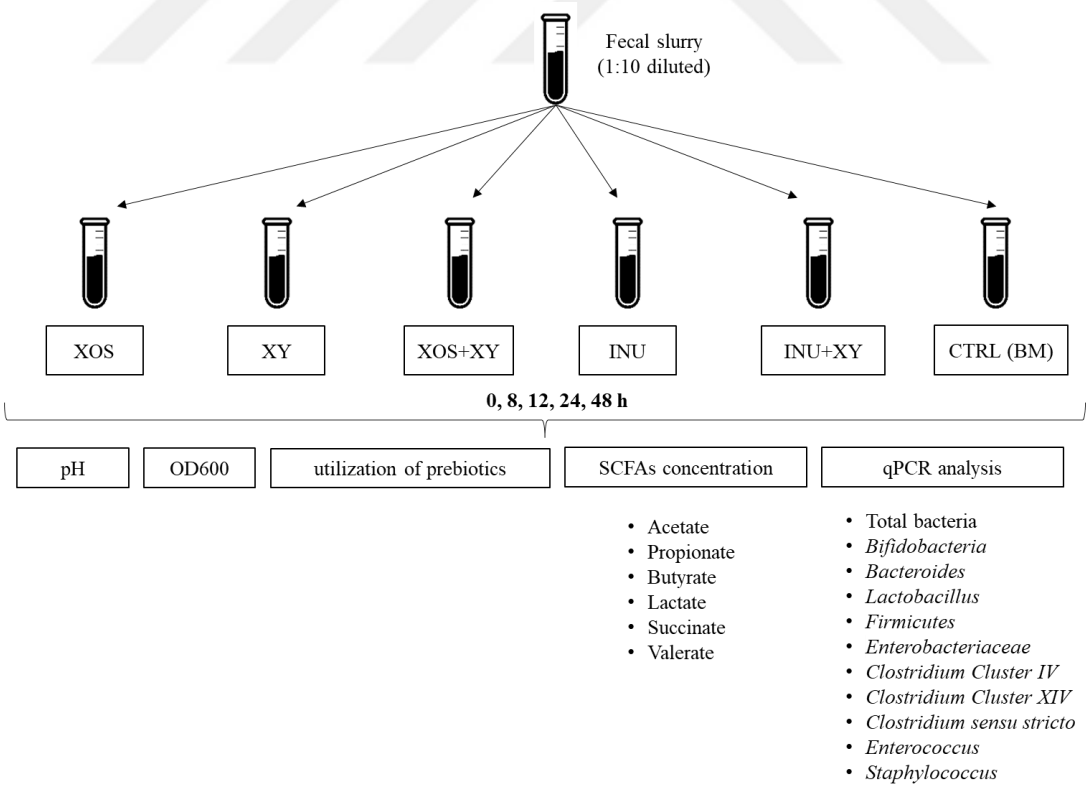


Figure 3. 1. Overview of *in vitro* fecal culture experimental design in the presence of oligomeric, polymeric, oligomeric, and polymeric together prebiotics.

This experimental design allowed for a comprehensive evaluation of the impact of each prebiotic on microbial growth, substrate utilization, and metabolite production, thereby providing insights into the differential efficacy of the tested treatments.

3.3.3.1. Determination of Growth

The growth curve was drawn to assess microbial growth quantitatively by measuring the turbidity of the fecal inoculum at OD_{600}. A spectrophotometer (PG instruments T80 UV/VIS, UK) was utilized for this purpose, with samples taken from the culture at regular intervals (0, 8, 12, 24, 48 h). Before each measurement, the cuvettes were washed thoroughly to prevent contamination or residue buildup that could impact the accuracy of the readings. At each sampling point, 1 mL of broth was withdrawn aseptically. The sample was then homogenized by gentle mixing to ensure even cell suspension before loading into the cuvette. Each reading was performed in triplicate and the mean value was reported.

3.3.3.2. pH Measurement

The pH was monitored to evaluate the acidification resulting from microbial fermentation. As microbial metabolism progresses, fermentation by-products cause pH shifts that reflect microbial metabolic activity. A pH meter (HANNA Instruments, USA) was used for all measurements. Before each session, the pH meter was calibrated using standard buffer solutions (pH 4.0, 7.0, and 10.0) to ensure measurement accuracy. The probe was cleaned between measurements by rinsing with 70% EtOH and then sterilized deionized water to avoid cross-contamination. pH values were measured by submerging the fecal inoculum at the same intervals as OD_{600}. Measurements were performed in triplicate and the average value was reported.

3.3.3.3. Determination of SCFAs and Organic Acids

The concentrations of SCFAs and other organic acids were determined to assess microbial fermentation efficiency and substrate utilization. At regular intervals, samples from each treatment were collected to measure SCFAs (acetate, propionate, butyrate, and valerate), lactate and succinate concentrations. All samples were analyzed using HPLC, with measurements performed in four parallels to ensure accuracy and reproducibility. Before analysis, samples were diluted 10-fold with ultra-pure water. A calibration curve was generated using standard solutions at defined concentrations to quantify SCFA levels. At each time point, aliquots of the culture were collected and centrifuged at 8,000 g for 20 min to remove cells and particulates. The supernatant was filtered through Sartorius syringe membrane filters with a pore size of 0.45 μm to ensure clarity before injection into the HPLC system.

HPLC Conditions for SCFA Analysis:

HPLC System: Thermo Fisher Scientific

Column: BIORAD Aminex HPX-87H (300 × 7.8 mm) + guard column

Mobile Phase: 5 mM H_2SO_4

Injection Volume: 20 μL

Flow Rate: 0.6 mL min^{-1}

Column Temperature: 60°C

Detector: UV at 210 nm

The optimized HPLC settings ensured precise separation and quantification of SCFAs, providing insights into the metabolic outcomes associated with different prebiotic substrates. The data obtained from these measurements were used to evaluate the efficiency of fermentation and the ability of the microbial community to metabolize the prebiotic treatments.

3.3.4. DNA Extraction

35 mg of fecal material was added to a nuclease-free 1.5 mL microcentrifuge tube to initiate the sample lysis and DNA binding process. Following this, 200 μL of Lysis Buffer and 40 μL of Proteinase K were introduced to the tube. The mixture was

thoroughly mixed to ensure complete homogenization and then incubated at 55°C for one hour to facilitate the complete digestion of the fecal material.

After the initial digestion, 200 µL of Binding Buffer was added to the sample, and the mixture was mixed immediately before incubating at 70°C for 10 min. This incubation step is critical for enhancing the binding of nucleic acids to the filter material in subsequent steps. Following the incubation, 100 µL of isopropanol was incorporated into the mixture to promote the precipitation of nucleic acids. The entire solution was then mixed thoroughly to ensure uniform distribution.

To separate the nucleic acids, a High-Purity Filter Tube was inserted into a Collection Tube, and the prepared sample was carefully pipetted into the upper reservoir of the Filter Tube. Subsequently, the complete assembly was placed into a centrifuge at 8,000 × g for 1 minute, which facilitated the effective separation of nucleic acids from the lysate.

The washing steps were performed according to the manufacturer's instructions, consistent with standard protocols for DNA extraction. Following the washing procedure, a prewarmed Elution Buffer at 70°C was utilized to isolate nucleic acids, allowing for the efficient recovery of purified DNA.

3.3.5. Sequencing and Analysis of the Bacterial 16S rRNA Gene

Quantitative PCR (qPCR) amplifications were conducted using the FastStart Essential DNA Green Master (Roche, Basel, Switzerland). Each reaction contained 10 µM of each primer and 50 ng of template DNA in a final volume of 20 µl. Primer sequences, designed to target both phylum and genus taxonomic levels of the 16S rRNA gene, are detailed in Tables 3.1 and 3.2. Primer design was performed using the Primer-BLAST program in the NCBI database, which employs the BLAST algorithm to verify primer specificity. Amplifications were carried out on a LightCycler® 96 thermocycler (Roche, Basel, Switzerland). Thermocycling parameters included an initial pre-incubation at 95 °C for 2 min, followed by 45 cycles of a three-step program: denaturation at 95 °C for 15 sec, annealing at 50–55 °C for 20 sec, and extension at 72 °C for 30 sec. A melt curve was included in each run to confirm the amplification of a single product.

Table 3. 1. Primer sequences for phylum-level detection in the gut microbiota.

Target Organism(s)	Primer Sequence
Firmicutes	F: TGAAACTAAAGGAATTGACG R: ACCATGCACCACCTGTC
Actinobacteria	F: TGTAGCGGTGGAATGCGC R: AATTAAGCCACATGCTCCGCT
Bacteroidetes	F: AAGGTCCCCCACATTGGAA R: CTGCTGCCTCCCGTAGGA
Proteobacteria	F: TCGTCAGCTCGTGTYGTGA R: CGTAAGGGCCATGATG

Table 3. 2. Primer sequences for genus-level detection of bacterial groups and species in the gut microbiota.

Target Organism(s)	Primer Sequence
Total bacteria	F: AGACACGGTCCAGACTCCTAC R: TTTACGGCGTGGACTACCAG
Bifidobacteria	F: CTCCTGGAAACGGGTGG R: GGTGTTCTTCCCGATATCTACA
Bacteroides	F: GAGAGGAAGGTCCCCCAC R: CGCTACTTGGCTGGTTCAG
Lactobacillus	F: GGAATCTTCCACAATGGACG R: CGCTTTACGCCCAATAAATCCGG
Streptococcus	F: CACTATGCTCAGAATACA R: CGAACAGCATTGATGATGTTA
Romboutsia	F: TGACATCCTTTTGACCTCTC R: GCCTCACGACTTGGCTG
E. coli	F: GGTAACGTTTCTACCGCAGAGTTG R: CAGGGTTGGTACACTGTCATTACG
Clostridium Cluster IV	F: GCACAAGCAGTGGAGT R: CTTCCTCCGTTTTGTCAA
Clostridium Cluster XIV	F: CGGTACCTGACTAAGAAGC R: AGTTTYATTCTTGCGAACG
Clostridium sensu stricto	F: TACCHRAGGAGGAAGCCAC R: GTTCTTCCTAATCTCTACGCAT
Enterococcus	F: CCCATCAGAAGGGGATAACACTT R: CCCATCAGAAGGGGATAACACTT
Staphylococcus	F: ACGGTCTTGCTGTCACTTATA R: TACACATATGTTCTTCCCTAATAA

qPCR data was used to calculate ΔΔCt value, which was developed by Livak & Schmittgen, (2001) and measures the fold change of bacteria. Melting curve program analysis using the Roche LightCycler R 480 System was utilized to verify that qPCR operations were successful (Wang et al., 2023). The $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ methods of data analysis were used (Livak and Schmittgen 2001). The measurement of total bacteria served as the reference and target bacteria were taken into consideration (Navidshad, Liang, and Jahromi 2012). The following calculations were used to calculate the relative abundance of bacteria, which was expressed as log2 transformed fold change values.

Relative abundance of target bacteria species concerning the abundance of total bacteria as illustrated in Eq. (2.1):

$$2^{-\Delta Ct} = 2^{-(\text{Ct of target bacteria} - \text{Ct of total bacteria})} \quad \text{Eq.2.1}$$

Fold change of relative abundance of target bacteria compared to the control group (BM without a carbohydrate) as illustrated in Eq. (2.2):

$$2^{-\Delta\Delta Ct} = 2^{-[(\text{Ct of target bacteria} - \text{Ct of total bacteria}) - (\text{Ct of target bacteria} - \text{Ct of total bacteria) control}]} \quad \text{Eq. 2.2}$$

3.3.6. Analytical Methods

The following analytical methods determined moisture content, structural carbohydrates, lignin, and ash in various prebiotic groups.

3.3.6.1. Moisture Content

The moisture content of the biomass was determined by drying a 1 g sample at 105°C in the oven overnight by NREL/TP-510-42621 method (Hames et al. 2008). Samples were stored in the desiccator before taking the weights. Equation 2.3 was used for calculating the moisture content.

$$\text{Moisture \%} = 100 - \frac{\text{Loss in moisture (g)}}{\text{Initial sample weight (g)}} x100 \quad \text{Eq. 2.3}$$

Loss in moisture = Initial sample weight (g) – final sample weight (g)
Initial sample weight = Wet (original) sample weight (g) before drying
Final sample weight = Sample weight (g) after drying

3.3.6.2. Structural Carbohydrate, Lignin, and Ash Analysis

The amounts of cellulose, hemicellulose, and lignin in the alkali extract were measured following the NREL/TP-510-42618 method (Sluiter et al. 2008). Following overnight drying at 40°C, the CC samples (0.3 g) were treated with 3 mL of 72% (w/w) H_2SO_4 for 60 min at room temperature and then diluted to 4% H_2SO_4 by adding 84 mL water. The mixture was hydrolyzed at 121°C for 1 h in an autoclave. The pH was then adjusted to 5-6 by $CaCO_3$. Glucose, xylose, and arabinose concentrations measured in HPLC were used to calculate cellulose and hemicellulose content, applying anhydrous correction factors of 0.90 for hexoses and 0.88 for pentoses. For acid-insoluble lignin (AIL) analysis, the solid remaining after the acid hydrolysis was separated by vacuum filtration through porcelain filter crucibles and dried at 105 °C. The weight of the solid was reported as lignin after subtracting ash content. Ash was determined gravimetrically after burning at 575 °C for 3 h in a furnace (Carbolite, UK). All results were reported on a dry-weight basis.

Carbohydrates in the samples were measured in HPLC (Thermo Ultimate 3000, USA). Monosaccharides (glucose, xylose, fructose, and arabinose), and XOS (X2, X3, and X4) were detected using Rezex RPM column (Phenomenex, USA) at 80 °C with ultrapure water as the mobile phase at 0.6 mL/min at intervals. Glucan, xylan, arabinan, acetyl, glucuronic acid, acid insoluble lignin percentages were calculated using equations 2.4, 2.5, 2.6, 2.7, 2.8, and 2.9, respectively. Samples were centrifuged and filtered through 0.45 µm pore size membrane filters (Sartorius) before analysis in HPLC. Standard solutions of analytes at known concentrations were used to generate calibration curves. Carbohydrates were detected using the refractive index.

$$\text{Glucan \%} = \frac{\text{glucose concentration (g/L)} \times 0.087 \times 100 \times 0.90}{\text{sample weight (dry basis)}} \quad \text{Eq.2.4}$$

$$\text{Xylan \%} = \frac{\text{xylose concentration (g/L)} \times 0.087 \times 100 \times 0.88}{\text{sample weight (dry basis)}} \quad \text{Eq.2.5}$$

$$\text{Arabinan \%} = \frac{\text{arabinose concentration (g/L)} \times 0.087 \times 100 \times 0.88}{\text{sample weight (dry basis)}} \quad \text{Eq.2.6}$$

$$\text{Acetyl \%} = \frac{\text{acetic acid concentration (g/L)} \times 0.087 \times 100 \times 0.72}{\text{sample weight (dry basis)}} \quad \text{Eq.2.7}$$

$$\text{Glucuronic acid \%} = \frac{\text{glucuronic acid concentration (g/L)} \times 0.087 \times 100 \times 0.82}{\text{sample weight (dry basis)}} \quad \text{Eq.2.8}$$

$$\text{AIL \%} = \frac{\text{(the solid remainig after acid hydrolysis (g) - ash content after burning (g)) x100}}{\text{sample weight (dry basis)}} \quad \text{Eq.2.9}$$

3.3.7. Statistical Analysis

All tests were carried out in four parallels, and the average results were reported as mean value ± standard deviation. The data were analyzed by using variance analysis (one way-ANOVA) and Tukey test ($p \leq 0.05$) using Minitab (ver.18.1, Minitab Inc., United Kingdom).

3.4. Results and Discussion

The detailed results and discussion are presented in the following sections, encompassing a comprehensive analysis of key aspects related to the study. This includes the utilization of model prebiotics in *in vitro* cultivation, highlighting their fermentation dynamics and metabolic fate within the simulated gut environment, along with microbial interactions influencing these processes. Additionally, the production of SCFAs and other organic acids is examined, providing insights into the metabolic activity of gut microbiota in response to prebiotic supplementation and its potential implications for host health. Furthermore, the impact of prebiotics on microbial diversity within the in vitro fecal culture is explored, focusing on shifts in microbial composition, potential enrichment of beneficial bacterial taxa, and overall modulation of the gut microbiome. These sections collectively offer a thorough evaluation of the role of prebiotics in shaping gut microbial ecology and metabolic outputs, contributing to a deeper understanding of their functional benefits and relevance in gut health maintenance. This comprehensive evaluation provides critical insights into the potential of prebiotics as modulators of gut microbiota, with implications for both fundamental research and applied nutritional strategies.

3.4.1. Utilization of Model prebiotics in *in vitro* Cultivation

Initial amounts for each substrate (XOS, XY, and INU) were around 10 mg/mL. XOS exhibited rapid utilization in 8 h, followed by a slower utilization phase for the remainder of the 48 h. The complex microbiome utilized the more readily fermentable xylobiose (X2) and xylotriose (X3), resulting in rapid microbial growth and decreased pH due to the production of acidic metabolites. The residual XOS was negligible, indicating near-complete metabolism of the substrate. This utilization emphasized efficient XOS metabolism, particularly during the early phase of the *in vitro* culture. *In vitro* study using human fecal inoculum has shown that XOS with 2-10 degrees of polymerization were metabolized in 24 h, although complete fermentation may require up to 80 h (Kabel et al. 2002). Similarly, a study on piglet intestines revealed that XOS with varying chain lengths were extensively fermented by the ileal, cecal, and colonic microbiota, with shorter-chain XOS fermenting more rapidly compared to their longer-chain oligosaccharides (Moura et al., 2008).

A marked reduction was observed in XY in 8 h, similar to XOS utilization. However, the rate of substrate decline was smaller compared to XOS, suggesting that XY requires initial enzymatic degradation by bacteria into simpler oligosaccharides or monosaccharides before it can be efficiently utilized. This step in the metabolic process likely contributes to the slower overall rate of consumption observed for XY. The need for bacterial enzymes, such as xylanases, to break down the complex polysaccharide structure of XY into smaller, more accessible sugars may limit the speed at which the substrate can be metabolized, especially compared to XOS, which is already present in a more readily available form. At the end of the 48-h period, a higher amount of XY remained compared to XOS, indicating that the system did not achieve complete utilization of this substrate under the conditions of the in vitro culture. This incomplete degradation may result from slow fermentation associated with the polymeric structure of XY.

As XY degraded, there was a corresponding release of X2 and X3 into the medium. The concentration of X2 began to rise almost immediately, reaching a stable level of around 8 h and then remaining relatively constant in 48 h. This XY utilization kinetic suggested that X2 is produced rapidly as a hydrolysis product but was either slowly utilized by the microbial community or accumulated due to limited degradation

beyond this point. Similarly, X3 showed a gradual increase in concentration, reaching a stable level between 8 and 24 h.

The accumulation of X2 and X3 following the breakdown of XY indicated a sequential degradation pathway where XY was first broken into smaller XOS, then served as potential substrates for further microbial metabolism. This sequential breakdown and partial accumulation of XOS highlighted the complexity of XY degradation in the gut microbiome, where different bacterial species may contribute to different steps in the breakdown process. Some species may specialize in initial XY hydrolysis, producing X2 and X3, while others may be required to metabolize these oligosaccharides further.

The combined XOS+XY mix started with an initial total carbohydrate concentration of approximately 17 mg/mL. Over 48 h, XOS was completely utilized, while XY was only partially consumed. By the end of the incubation period, a higher total substrate utilization was observed in the XOS+XY mix compared to XOS or XY alone, attributed to the increased availability of substrates. In the INU+XY mix, both INU and XY were utilized at comparable rates. Substrate consumption in the INU+XY mix progressed steadily over the 48-h period; however, a significant portion of the substrates remained unconsumed at the end of the incubation. Notably, consumption rates slowed after 8 h, which can be attributed to the requirement for polymeric carbohydrates to degrade before being utilized. Additionally, the decrease in pH in the *in vitro* culture and the associated metabolic slowdown might be linearly correlated.

INU amounts displayed a slower decline over 48 h compared to other substrates like XOS or XY. In the first 8 h, the reduction in INU levels was gradual, suggesting that the system metabolized this substrate at a slower rate. By the end of the 48 h, a significant amount of inulin remained. The slower degradation of INU could be attributed to limitations in the availability or activity of INU-specific enzymes, which are necessary for breaking down this polysaccharide into simpler sugars (Afinjuomo et al. 2021).

Monitoring the OD_{600} at 0, 4, 8, 12, 24, and 48 h allowed the observe the dynamics of bacterial growth in the presence of prebiotic substrates. The control was a BM without any substrate (Ctrl). Bacterial growth displayed distinct rates based on the substrate (Figure 3.2B). The *in vitro* fecal cultures with XOS showed the highest growth in the 48-h incubation period. The OD_{600} readings at each time point showed a rapid and sustained increase in cell density, indicating that XOS was highly effective in promoting bacterial proliferation. After 48 h, the OD_{600} reached 1.3, the highest value among all the substrates that were evaluated. XOS offers easily metabolized oligosaccharides. Fast growth,

particularly during the first 24 h, may reflect the bacteria's ability to rapidly utilize the XOS, resulting in accelerated growth during the exponential phase. Compared to other substrates, utilizing XOS in *in vitro* fecal cultures consistently outperformed both individual and combined substrates, highlighting its superior efficacy in enhancing bacterial proliferation.

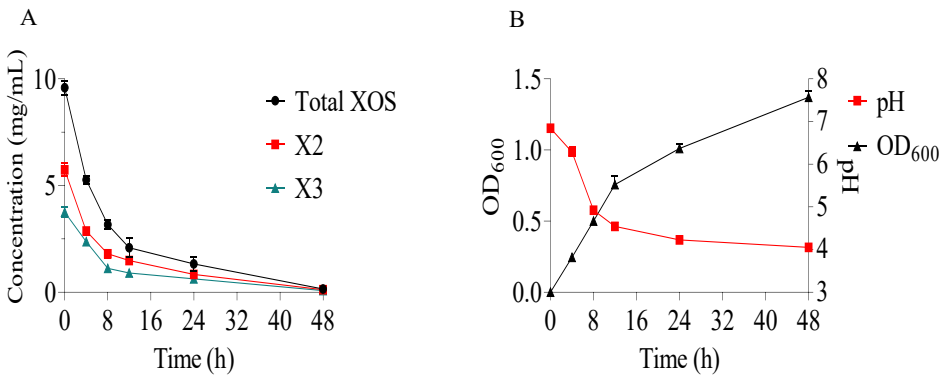
The fecal bacterial growth in the presence of XY was similar to XOS, though at a slightly lower level. By 48 h, the OD_{600} value reached approximately 1.1, indicating that XY supports bacterial growth but not as effectively as XOS. This difference is likely due to the structural complexity of XY compared to XOS. The slower bacterial growth with XY may be due to the requirement for more specialized enzymes to degrade the polysaccharide into oligo- and monosaccharides. These enzymes take longer to produce or may not work as quickly as the enzymes that degrade XOS, which consist of shorter, simpler oligosaccharides. In addition, the rate at which XY is broken down may limit the bacteria's ability to grow rapidly (Salyers et al., 1982). The bacteria must break down both the main XY chain and its side chains, which can be a slow process. As a result, bacterial cultures using XY showed lower OD_{600} values than those with XOS, suggesting that XY is a less readily available carbon source. Despite this, XY still supported significant bacterial growth, demonstrating that it can be utilized, though less efficiently than XOS.

XOS+XY mix followed an intermediate utilization pattern, with OD_{600} values reaching around 1.2 at the end of the 48-h incubation period. This growth rate was higher than that of XY alone, but slightly lower than the growth seen with XOS as a single substrate. The potential for an additive effect occurs when both substrates contribute separately to bacterial growth. XOS, as shorter-chain oligosaccharides, were rapidly utilized, with half of the substrate consumed in the first 4 h and complete utilization achieved by the end of the incubation period. On the other hand, XY, a more complex polysaccharide, reached a stable level of half utilization at 24 h. This can be attributed to its requirement for a more extended breakdown process, which likely slows its utilization. When both substrates are available, the bacteria may metabolize XOS first due to its simpler structure, leading to rapid initial growth, while XY continues to provide a source of energy over a longer period with slower fermentation. This combination ensures that bacterial growth can be sustained for a longer duration. The findings indicate that, while not as effective as XOS alone, the XOS+XY mix supported bacterial multiplication. This is especially crucial for applications where a variety of carbohydrate sources are

frequently available and their interactions can significantly affect microbial activity, such as gut microbiota research or industrial operations.

The pH changes observed during the fermentation of model prebiotics in the *in vitro* mice fecal inoculum indicated active substrate consumption by bacteria. The initial pH of all tubes was 6.8-7.1 and steadily decreased over time. After 24 hours, the most significant pH reduction—from 6.87 to 4.2—was recorded in the XOS, reflecting enhanced bacterial metabolism.

The utilization kinetics of model prebiotics over a 48-h incubation period are presented in Figure 3.2A. Among the tested prebiotics, XOS exhibited the highest utilization efficiency by the fecal microbiota, with an 82.66% consumption rate recorded in the first 24 h. This rapid and extensive degradation can be attributed to the oligomeric nature of XOS, which is composed of short-chain XOS units that are readily accessible to gut bacteria for fermentation. The efficient breakdown of XOS facilitated a favorable microbial environment, supporting bacterial proliferation and metabolic activity, as evidenced by the high consumption rate observed. In contrast, XY was utilized at a significantly slower rate due to its complex polymeric structure and higher molecular weight. The intricate architecture of XY necessitates additional enzymatic hydrolysis steps before it can be fermented by gut microbes, thereby delaying its degradation and metabolic conversion. This discrepancy in utilization rates underscores the crucial role of prebiotic molecular structure in influencing fermentability, microbial accessibility, and metabolic efficiency within the gut ecosystem. Understanding these structural-functional relationships is essential for optimizing prebiotic formulations to modulate gut microbiota and enhance beneficial metabolic outcomes selectively.



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Cont. of Figure 3.2

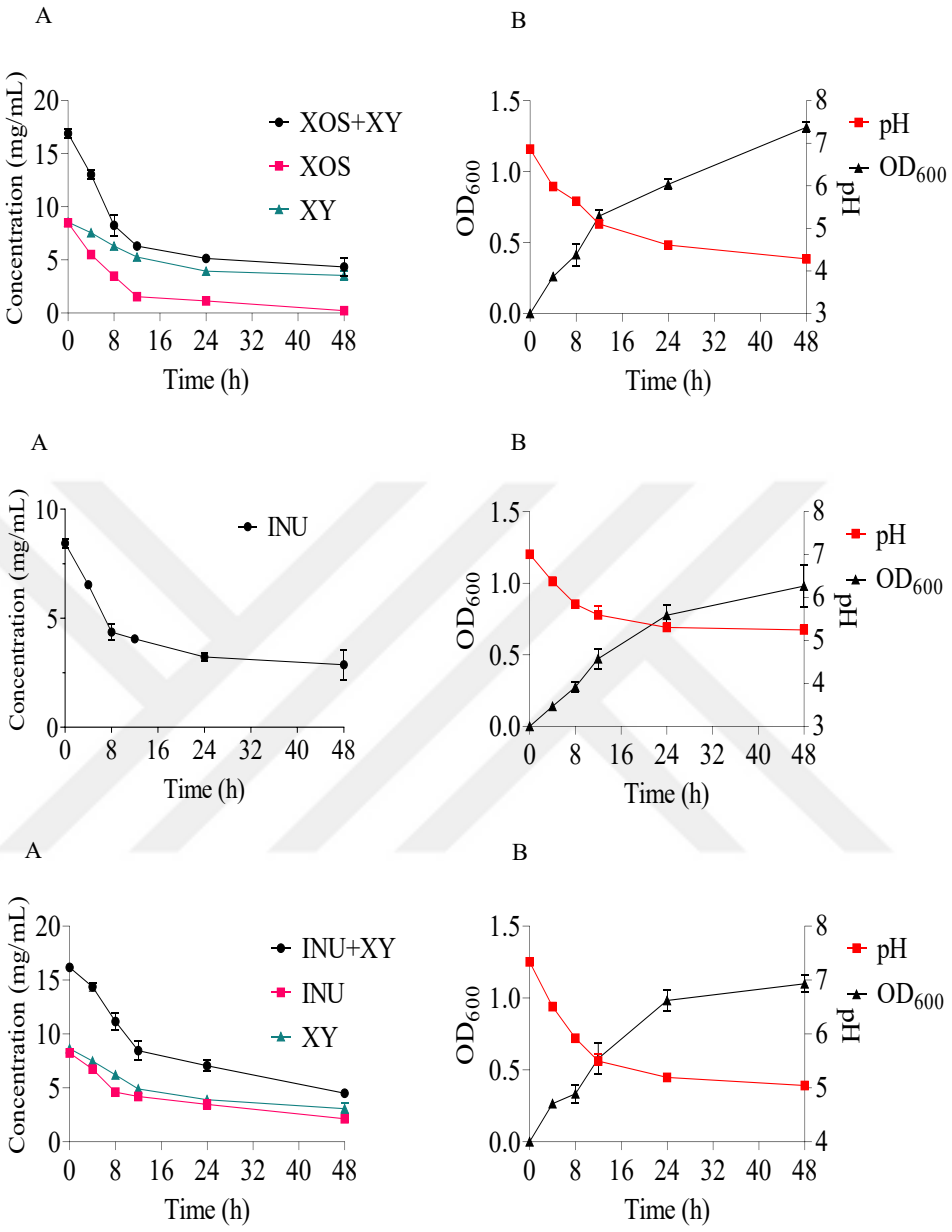

Figure 3. 2. A. Utilization kinetics in *in vitro* fecal inoculum on utilization of XOS (1), XY (2), XOS+XY (3), INU (4), INU+XY (5) in 0, 4, 8, 12, 24, and 48 h. B. Growth curve and pH in *in vitro* fecal inoculum on utilization of XOS, XY, XOS+XY, INU, INU+XY in 0, 4, 8, 12, 24, and 48 h (mean of n = 4 replicates), error bars indicate s.d. (Statistical analyses were performed using one-way ANOVA).

3.4.2. SCFAs and Other Acids

Prebiotic fermentation by intestinal bacteria generates SCFAs, including acetate, propionate, butyrate, lactate, succinate, and valerate (Markowiak-Kopeć & Śliżewska, 2020; Soldi et al., 2019). These SCFAs help lower intestinal pH, serve as electron sinks during anaerobic respiration, and enhance mineral bioavailability (Saha et al., 2021). Among these, acetate, propionate, and butyrate are the most prevalent SCFAs. Butyrate serves as the primary energy source for colonocytes, while acetate and propionate are transported to the liver via the portal vein (Guilloteau et al., 2010). In the liver, propionate supports gluconeogenesis, whereas acetate contributes to cholesterol synthesis and lipogenesis. Additionally, acetate is taken up by muscle and adipose tissues (Moffett et al., 2020).

The production dynamics of key microbial metabolites, including acetate, propionate, butyrate, lactate, succinate, and valerate, were systematically evaluated in fecal inoculum supplemented with various prebiotic substrates over a time course of 0, 8, 12, 24, and 48 hours of incubation (Figure 3.3). The experimental groups included XOS, XY, a combination of XOS and XY, INU, a mixture of INU and XY, and a BM serving as the control group. This comprehensive analysis aimed to assess how different prebiotics, and their combinations influence the metabolic activity of gut microbiota, as reflected in the production profiles of these fermentation-derived acids. By tracking the temporal changes in SCFAs and other organic acids, this study provides insights into the differential fermentation patterns associated with each prebiotic substrate. The presence and relative abundance of these metabolites serve as important indicators of microbial activity, substrate utilization efficiency, and potential shifts in gut microbial composition. Understanding these metabolic responses is critical for evaluating the functional benefits of different prebiotics and their impact on gut health.

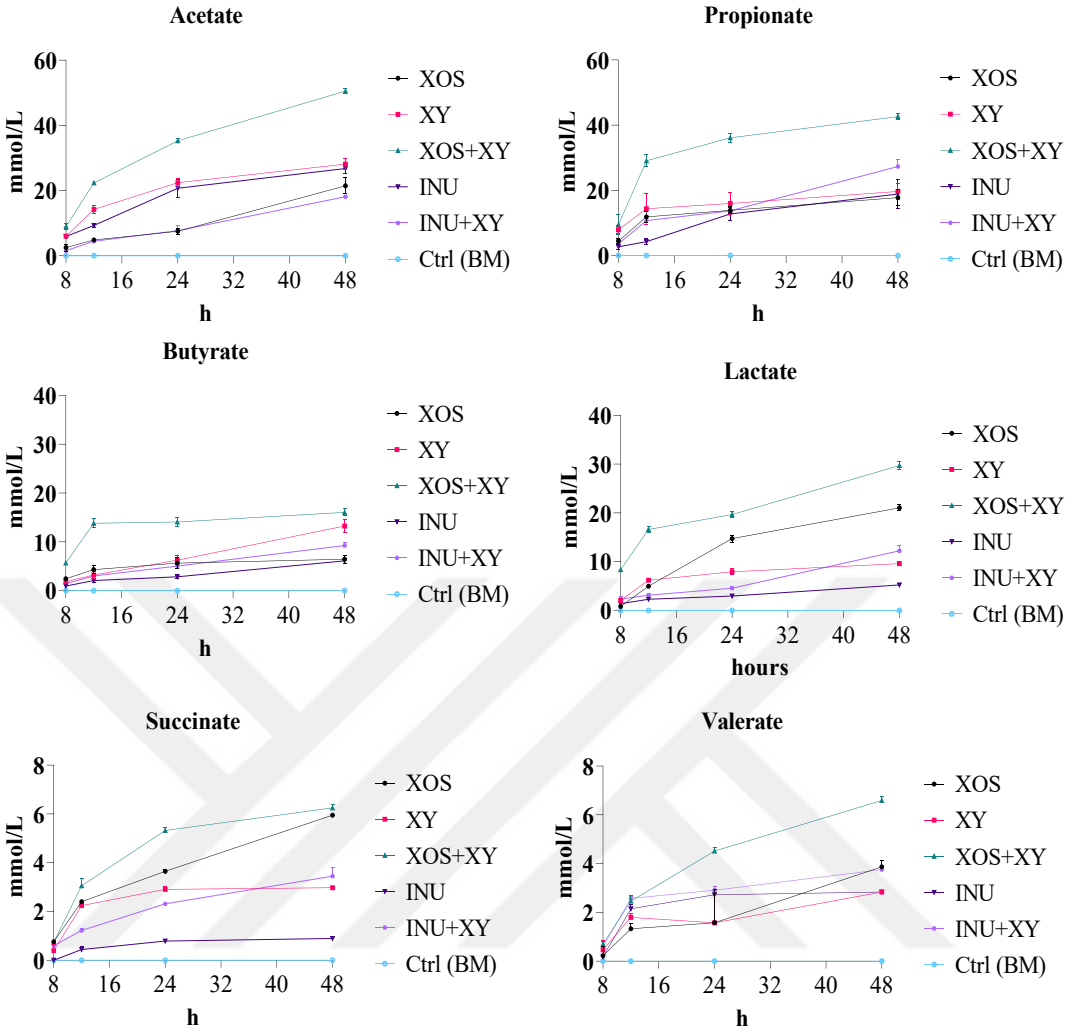

Figure 3. 3. Acetate, propionate, butyrate, lactate, succinate, and valerate concentrations in *in vitro* fecal inoculum on utilization of XOS, XY, XOS+XY, INU, INU+XY in 0, 8, 12, 24, and 48 hours (mean of n = 4 replicates).

Acetate concentrations increased across all treatments over 48 h as was also observed previously in pigs (Wang et al., 2021). When utilized individually, the test tubes containing XY, XOS, and INU produced moderate levels of acetate, measuring 25, 24, and 17 mmol/L, respectively. Following the test tube including XY, the INU+XY mix produced 18 mmol/L of acetate. In contrast, the control group with only BM showed minimal acetate synthesis, highlighting the essential role of the substrates in enhancing the formation of SCFAs.

Propionate levels also increased for all substrate treatments, with the XOS+XY mix showing the highest concentration at 48 h, similar to the trend observed for acetate.

INU+XY, XY, INU, and XOS exhibited moderate propionate production, reaching approximately 25 mmol/L by the end of the incubation. As with acetate, the control group showed negligible changes in propionate concentration.

Butyrate concentrations increased steadily over 48 h across all substrate treatments. The XOS+XY mix achieved the highest butyrate levels, around 17 mmol/L, indicating enhanced butyrate production when both substrates were present together. XY followed closely, suggesting its effectiveness in promoting butyrate formation. XOS and INU individually showed lower butyrate production compared to their mixture, while the INU+XY mix also resulted in relatively higher levels compared to the single substrates. The control group had no detectable butyrate production throughout the incubation.

Lactate production varied across the treatments, with the XOS+XY mix showing a rapid increase in lactate levels, peaking at around 28 mmol/L by 48 hours. XOS alone also resulted in high lactate concentrations, suggesting that these substrates favored lactate-producing bacteria. In contrast, the other treatments, including INU, XY, and INU+XY, showed relatively lower lactate levels, with more gradual increases over time. The control group showed no lactate production, confirming that the substrate types significantly influenced lactate formation.

Succinate and valerate productions were generally lower compared to other acids, but an increase was observed for all substrate treatments, with the highest levels observed in the XOS+XY mix, reaching approximately 6 mmol/L. XOS alone also showed significant succinate and valerate production, suggesting that these substrates could promote acid accumulation. XY and INU+XY mix showed moderate succinate production, while inulin had the lowest succinate and valerate concentrations among the tested substrates. The control group had no succinate and valerate concentrations.

3.4.3. Effect of Prebiotics on Microbial Diversity in *in vitro* Fecal Culture

In the study, the impact of different substrates—XOS, XY, XOS + XY mix, INU, and INU + XY mix—on bacterial composition in an *in vitro* culture inoculated with mice feces were assessed in 0, 4, 8, and 12 h (Figure 3.4). Each point is represented by a bar, with colors indicating the relative abundance of different bacterial groups.

At the phylum level, Firmicutes dominate the microbial community across all time points. This finding is consistent with previous studies that identified Firmicutes as the predominant phylum in the animal gut, playing a critical role in nutrient metabolism and energy uptake (Singh et al., 2013). Bacteroidetes, the second most abundant phylum, exhibited a marked increase in the XY and XOS+XY groups compared to XOS treatments. This increase can be ascribed to XY supplementation selectively promoting the growth of Bacteroidetes, a phylum known for its ability to degrade complex carbohydrates, including XY, into fermentable sugars (Flint et al., 2012). Actinobacteria levels were significantly increased in all prebiotic groups. Proteobacteria, which include several opportunistic pathogens, are often associated with dysbiosis and inflammation when their abundance is elevated (González-Solé et al., 2022). The ability of prebiotic groups to maintain Proteobacteria at consistently low levels may reflect a shift in the gut microbial ecosystem toward a more balanced and health-promoting state.

At the genus level, a gradual increase in beneficial bacteria in the XOS group, particularly *Bifidobacteria*, *Lactobacillus*, *Bacteroides*, and *Clostridium Cluster* XIV, was observed throughout the incubation period, suggesting these bacteria can utilize XOS as a substrate. *Bifidobacteria* showed a particularly strong response, increasing from an initial abundance of 2% to 19%, highlighting the prebiotic potential of XOS to selectively enrich this beneficial bacterial group. This increase is notable because *Bifidobacteria* is associated with various health benefits, such as improved gut barrier function and immune modulation, which contribute to overall gut health (Alessandri et al., 2019). In addition to the growth of *Bifidobacteria*, a significant increase in *Lactobacillus* was observed, rising from 0% to 11% by the end of the experiment. This shift aligned with other studies showing that XOS supports the proliferation of *Lactobacillus* species, known for their roles in immune modulation and inhibition of pathogenic bacteria (Chakraborty et al., 2024; Guo et al., 2023). The selective stimulation of *Bifidobacteria* and *Lactobacillus* spp. emphasized the potential of XOS as a targeted prebiotic oligomer.

Moreover, a reduction in the relative abundance of potentially pathogenic bacteria, including *Enterococcus*, *Staphylococcus*, and *Clostridium sensu stricto*, was observed over time in the XOS group. This suggested that XOS may help suppress these bacteria, likely by enhancing competition from beneficial microorganisms. A slight reduction in *E. coli* levels indicated that XOS may contribute to a healthier microbial balance by reducing undesirable bacteria.

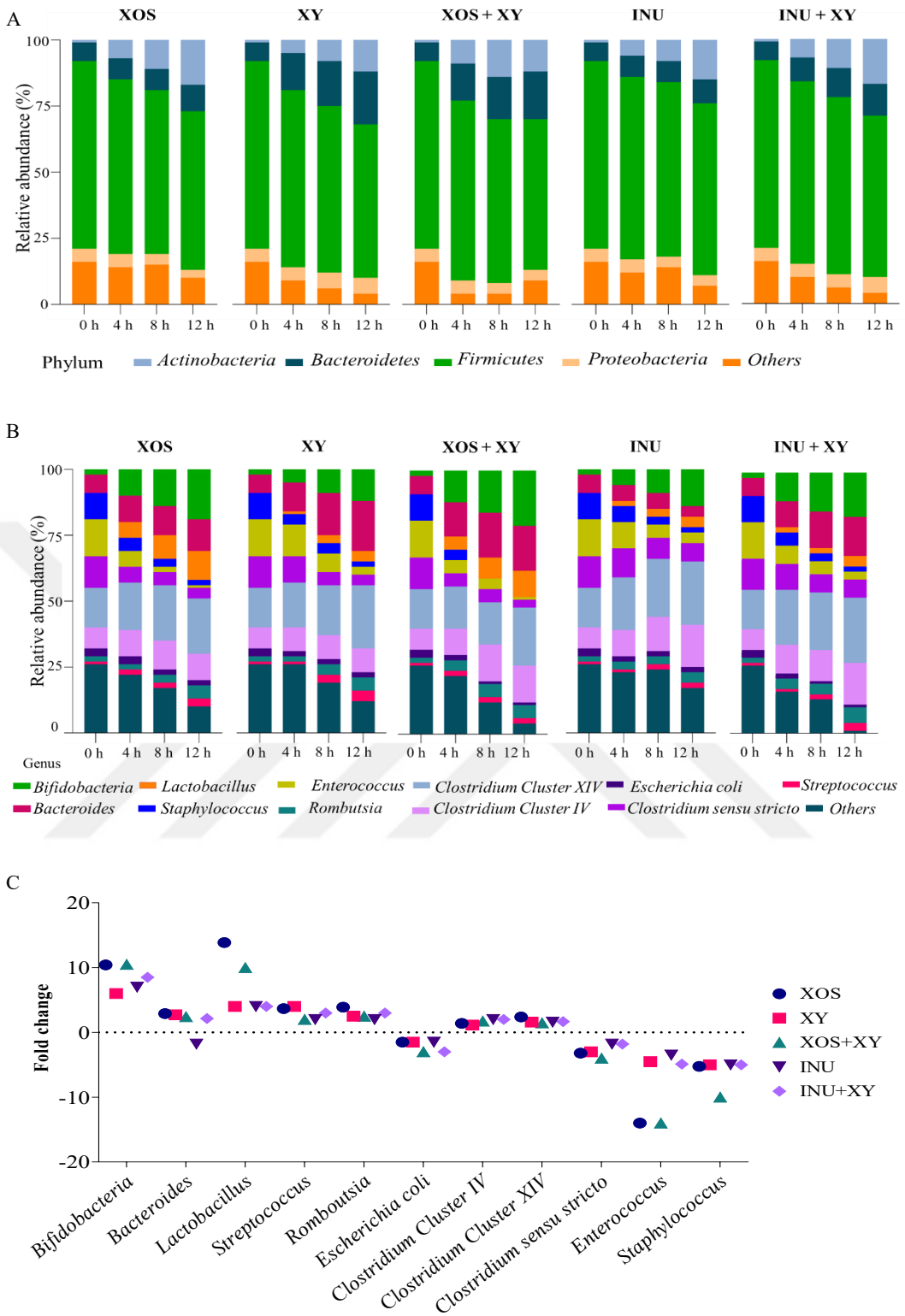

Figure 3. 4. Spatial distribution of microbial community in *in vitro* fecal culture on utilization of XOS, XY, XOS+XY, INU, INU+XY. A. The mean relative abundance of the main phylum in fecal bacteria in 0, 4, 8, and 12 h. B. The mean relative abundance of the top 11 genera in the fecal culture 0, 4, 8, and 12 h. C. The fold change of fecal bacteria in 12 h.

Studies on XOS reported that it consistently promoted beneficial bacteria, particularly *Bifidobacteria* spp., in both animal models and some human trials. While human studies show mixed results, XOS generally supported beneficial bacteria selectively and showed a bifidogenic effect, including enhanced gut barrier integrity, immune support, and SCFA production (Slavin 2013). Similarly observed that a dose of 2 g/day of XOS over 8 weeks led to modest increases in *Bifidobacteria*, along with moderate increases in *Akkermansia muciniphila* and *Lactobacillus*, both associated with improved gut health (Lim et al. 2018). Another study conducted a randomized, double-blind trial where participants received either 1.4 g or 2.8 g of XOS daily for 6 weeks. The higher dose led to a significant increase in *Bifidobacteria* spp. in some individuals, with responses varying based on baseline microbiota composition, suggesting that factors like dose, duration, and individual microbiota composition influence XOS's efficacy (Maki et al. 2012). XOS supplementation at doses of 1.4 g/day and 2.8 g/day for 10 weeks has been shown to increase specific beneficial bacteria, such as *Faecalibacterium prausnitzii* and *Akkermansia*, without significantly altering overall microbial diversity. Plate culture methods revealed higher levels of *Bifidobacteria* and *Bacteroides fragilis* which increased with a 2.8 g/day XOS intake, suggesting dose-dependent effects on microbial populations (Finegold et al. 2014).

Research has also shown that XOS positively affected certain *Lactobacillus* species. Specifically, *Lactobacillus acidophilus* and *Lactobacillus casei* can effectively ferment XOS, selectively stimulating their growth in the gut. The proliferation of *Lactobacillus* spp. may play a role in immune modulation and pathogen inhibition. Additionally, studies on *Lactobacillus paracasei* and *Lactococcus lactis* suggested that they utilize XOS to produce beneficial metabolites, such as lactic acid and other acids, which are essential for lowering colonic pH and inhibiting pathogens (Watanabe et al. 2021). Shin et al. (2015) also reported that XOS supplementation modestly increased *Lactobacillus* spp. These results suggest that XOS may influence various beneficial bacteria in the gut with enhanced microbial diversity.

In conclusion, XOS demonstrates potential as a targeted prebiotic, selectively promoting the growth of beneficial bacteria while suppressing potentially harmful bacteria. This selective enhancement supports gut health by enriching beneficial bacteria, such as *Bifidobacterium* and *Lactobacillus*, which are associated with immune benefits, SCFA production, and pathogen inhibition. These findings reinforced the prebiotic

potential of XOS, showing its ability to contribute to a healthier, more balanced gut microbiota.

The *in vitro* culture inoculated with mice feces with XY as a carbon source led to significant changes in the relative abundances of specific bacterial taxa in 12 h. Notably, there was an increase in the relative abundances of *Bacteroides*, *Bifidobacteria*, and *Clostridium* Cluster XIV, while taxa such as *Staphylococcus*, *Romboutsia*, *Enterococcus*, and *Clostridium sensu stricto* exhibited a decline.

At the early stage of incubation (4 h), an increase in *Clostridium* Cluster XIV suggested that these taxa were directly engaging with XY, likely initiating its degradation. Members of the *Clostridium* genus are well-known for their carbohydrate-active enzyme (CAZyme) systems, which include xylanases and other enzymes capable of degrading complex plant polysaccharides such as XY into smaller, more accessible oligosaccharides (Li et al., 2023). This early enzymatic activity likely generated intermediate fermentation products, such as XOS which could be further utilized by other bacteria in the community.

The levels of *Bacteroides* significantly increased throughout the incubation period. Research has indicated that *Bacteroides* species, common inhabitants of the human gut, possess specialized systems for XY degradation. *Bacteroides* spp. include extracellular xylanases that break down the polysaccharide into XOS, which can subsequently serve as a nutrient source for other beneficial microorganisms, such as *Bifidobacteria* (Zafar and Saier Jr 2021). The consistent increase in *Bacteroides* highlights its role as a primary degrader of XY, likely releasing products that promote the growth of secondary fermenters (Pereira et al. 2021). Previous studies on XY utilization in pig intestines have reported increases in *Bacteroides* and *Bifidobacterium*, suggesting that XY promotes the growth of these bacterial populations in a manner that is dependent on both the dose and structural characteristics of the XY (Wang et al., 2021). A clinical study in humans using different fractions of XY (with average molecular masses of 354, 278, and 66 kDa) demonstrated an increase in the proliferation of *Bifidobacteria*, *Lactobacilli*, and *Eubacteria* groups (Manini, 2015).

Lactobacillus and *Streptococcus* at low relative abundances during the early phase suggest that these genera may be beginning to utilize byproducts from the initial XY degradation. Although *Lactobacillus* and *Streptococcus* are not primary XY degraders, their metabolic profiles suggest an ability to metabolize XOS and xylose released through the initial action of *Clostridium* species (Gulsan et al. 2022). These genera may thus function as secondary fermenters within this microbial ecosystem, indicating an early

establishment of cross-feeding interactions within the microbial community (Mu et al. 2022).

At the end of the 12-h incubation period, *Bacteroides* and *Clostridium* Cluster XIV emerged as the dominant taxa, indicating their proficiency in utilizing XY. The predominance of these taxa implies a highly efficient system for XY utilization, with *Bacteroides* and *Clostridium* Cluster XIV acting as primary degraders and possibly contributing to the production of beneficial metabolites (Linares-Pastén et al. 2021; Despres et al. 2016).

The bacterial composition of the gut microbiome in response to XOS+XY mix supplementation was analyzed in 12 h. *Bifidobacteria* showed a consistently high abundance across all time points, indicating efficient utilization of XOS and XY as substrates. This aligns with the well-known prebiotic effect of these compounds in stimulating *Bifidobacteria* growth (Calvete-Torre et al., 2023; Khangwal et al., 2022; Singh et al., 2015). *Bacteroides* spp. also displayed a significant presence, suggesting its role in polysaccharide breakdown (Fultz et al. 2021). As known degraders of complex carbohydrates, *Bacteroides* species have been linked to fiber-rich diets, and their abundance in this study is consistent with reports demonstrating their ability to thrive on polysaccharides. *Lactobacillus* maintained a moderate abundance, suggesting a supportive role in fermenting oligosaccharides and contributing to the overall fermentation process.

Other genera, including *Staphylococcus*, *Enterococcus*, and *E. coli*, exhibited relatively low abundances, suggesting that the XOS+XY mix affected potentially harmful bacteria. Similar studies indicated that certain prebiotics could promote a microbial balance favoring beneficial taxa, thereby enhancing gut health without encouraging pathogen growth (Anadón et al. 2019; Sarsan et al. 2024). Meanwhile, *Clostridium* Clusters XIV and IV showed a gradual increase over time, supporting their role in secondary fermentation processes. *Romboutsia* and *Streptococcus* were present at moderate levels, possibly benefiting from metabolic byproducts produced by primary fermenters like *Bifidobacteria* and *Bacteroides*.

In the INU group, a gradual increase in gut microbial populations, particularly *Bifidobacterium* and *Clostridium* Clusters IV and XIV, was observed throughout the incubation period. This suggests that these bacterial groups can utilize INU as a substrate. Notably, *Bifidobacterium* exhibited a marked response, increasing from an initial abundance of 2% to 14%, highlighting the prebiotic potential of INU in selectively

enriching beneficial bacteria. Similarly, significant increases in the abundance of *Clostridium* Cluster IV and Cluster XIV were observed, rising from 8% to 16% and 15% to 24% respectively. In contrast, reductions in populations of *Bacteroides*, *Enterococcus*, *Staphylococcus*, and *Clostridium sensu stricto* were noted. These findings provide strong evidence for the selective promotion of beneficial bacteria, such as *Bifidobacterium*, by INU, while concurrently reducing populations of potentially harmful bacteria.

The *in vitro* fecal culture examination of INU was conducted to compare its effects with the prebiotics XOS and XY. For both XOS and INU, the abundance of *Bifidobacteria* spp. remained relatively high across all time points, suggesting that both prebiotics support *Bifidobacteria* growth. *Bacteroides* spp. were more abundant in the XOS samples compared to INU, while *Lactobacillus* showed higher abundance at earlier time points for both XOS and INU. Some *Lactobacillus* species, such as *Lactobacillus paracasei* and *Lactobacillus plantarum*, can utilize INU, though it's less favored compared to XOS. INU fermentation by *Lactobacillus* is slower and generally produces fewer SCFAs to compare XOS (Giani et al., 2022; Gonçalves et al., 2023b; Rastall et al., 2022). Figure 3.4 demonstrated that, although both prebiotics support certain beneficial bacteria, their impact on microbiome composition varies between XOS and INU.

The *in vitro* fecal culture analysis comparing the effects of INU and XY on microbiome composition revealed distinct patterns of bacterial growth over time. Both prebiotics consistently supported a high relative abundance of *Bifidobacteria* spp., indicating their effectiveness in promoting this beneficial genus. However, notable differences were observed between the two treatments. *Bacteroides* spp. were significantly more abundant in XY-treated fecal cultures than in those treated with INU, suggesting that *Bacteroides* may play a crucial role in the breakdown and utilization of XY as a carbon source. This higher abundance indicated that *Bacteroides* possess metabolic capabilities for fermenting XY, which could contribute to their competitive advantage and persistence in XY cultures. *Lactobacillus* showed higher levels initially for both prebiotics but decreased over time, especially INU. *Clostridium* clusters (XIV and IV) had substantial initial levels in both treatments, with a more pronounced decline in INU-treated samples. *Enterococcus* was higher in XY cultures initially, whereas it remained low and stable in INU. Overall, these results highlighted that, while both INU and XY foster beneficial bacteria, they have distinct impacts on the microbiome composition over time.

The utilization of the INU + XY mix in fecal culture demonstrated changes in the relative abundances of various bacterial taxa, expressed as percentages of the total microbial community. *Bifidobacteria* spp., *Bacteroides* spp., and *Clostridium Clusters IV* and *XIV* showed a gradual increase in the 12 h, emerging as dominant genera compared to other groups. *Lactobacillus* maintained a moderate and stable abundance throughout the experiment, indicating a consistent presence without significant fluctuations. Conversely, potentially harmful genera such as *Staphylococcus*, *Enterococcus*, and *E. coli* decreased in abundance in response to the INU + XY mix. These findings suggest that the INU + XY mix may exhibit prebiotic properties, supporting a healthier gut environment by fostering the growth of beneficial bacterial populations.

3.5. Conclusions

In vitro models represent a valuable, cost-effective approach for investigating the prebiotic effects on gut microbiota composition and metabolic outputs, allowing for the examination of multiple experimental conditions with ease of control and precision. These models permit the regulation of physiological parameters such as temperature, nutrient composition, and anaerobic conditions, thereby mimicking specific gastrointestinal environments. *In vitro* setups also enable detailed analysis of metabolite production and microbial dynamics in response to prebiotic substrates, providing crucial insights into gut microbial metabolism. Typically, these studies involve incubating fecal inoculum in short time intervals, as microbial communities rapidly progress to the stationary phase. This controlled approach facilitates the observation of microbial shifts and metabolite production under defined conditions, establishing that it provides preliminary data for understanding the initial responses of gut microbiota to prebiotic interventions.

The findings from this in vitro study provided valuable insight into the XY-based prebiotic effects on gut microbial composition and SCFA production over time. The substrates—XOS, XY, INU, and their combinations with XY—were observed to selectively enrich beneficial bacterial populations while concurrently reducing potentially pathogenic taxa. INU was selected as a well-known prebiotic to compare XY-based prebiotics. Notably, XOS exhibited a significant capacity to increase the relative

abundance of beneficial bacteria, including *Bifidobacteria* and *Lactobacillus*, which are known to support immune modulation, inhibit pathogenic bacteria, and contribute to overall gut health. This selective enhancement emphasized the potential of XOS as a targeted prebiotic capable of promoting a balanced and health-promoting microbiota by fostering beneficial taxa and reducing the presence of pathogenic bacteria, such as *Enterococcus* and *Clostridium sensu stricto*.

Similarly, XY influenced the bacterial community by significantly enhancing *Bacteroides*, *Bifidobacteria*, and *Clostridium* Cluster XIV, suggesting that XY can serve as a substrate for these bacteria. The early increase in *Clostridium* Cluster XIV further indicated that members of this group engage in the degradation of XY to XOS, which supports the growth of other beneficial microorganisms. INU also showed a favorable effect on microbiota by selectively increasing the abundance of *Bifidobacterium* and *Clostridium* Clusters IV and XIV. The selective enrichment of beneficial bacteria by INU supported its utility as a well-known prebiotic for gut health.

SCFA production was markedly influenced by substrate type and combination. The XOS+XY mix led to the highest SCFA levels, with a sharp increase to around 220 mmol/L at 48 h, indicating a potential synergistic effect that may be due to the greater availability of substrates. Acetate was the most abundant metabolite in all prebiotic groups. Individually, XY, XOS, and INU produced moderate acetate levels, with XY generating the highest acetate concentration among single substrates. These SCFAs, particularly acetate, are known to support host health by serving as an energy source for colonic cells and contributing to the regulation of immune responses.

Overall, *in vitro* fecal inoculum tests with XOS, XY, INU, and their mixtures promoted a beneficial gut microbial balance and SCFA production, with each substrate showing beneficial effects on specific bacterial taxa. Detailed examination of the effects of XY, particularly in combination with XOS, was essential for understanding their potential synergistic impact on gut microbiome composition and health in an *in vitro* fecal culture model.

CHAPTER 4

AN EX VIVO MODEL FOR EVALUATION OF PREBIOTIC ACTIVITY OF XYLAN AND XYLOOLIGOSACCHARIDES

4.1. Introduction

The intestinal microbiota plays a significant role in metabolic, physiological, nutritional, and immunological mechanisms. Prebiotics provide health benefits by selectively stimulating the growth of beneficial species in the large intestine (Gibson and Roberfroid 1995). Plant-derived oligo- and polysaccharides not digestible in the gastrointestinal tract serve as prebiotics. Some members of the intestinal microbiota multiply by metabolizing these carbohydrates, localizing them, and showing physiological effects (Gong and Yang, 2012). A primary outcome is the microbial synthesis of short-chain fatty acids (SCFAs) (Carlson et al. 2017; Jayamanohar et al. 2019). SCFAs are first absorbed by the cells of the large intestine and then enter systemic circulation. The most common SCFAs, such as acetate, propionate, and butyrate are slightly acidic and account for 90-95% of SCFAs in the colon (Wang et al., 2019).

Low-molecular-weight carbohydrates, such as FOS, GOS, and XOS, are rapidly fermented by the gut microbiota providing an immediate boost to the growth of beneficial bacteria but preventing them from reaching further parts of the colon, such as the distal colon (DC) (Bhatia et al. 2024; Ravindra Kumar, Næss, and Sørensen 2024). Longer-chain carbohydrates like inulin are catabolized at a slower rate, which may allow them to progress further into the DC (Sheng, Ji, and Zhang 2023). This slower fermentation process can prolong the stimulation of beneficial bacteria, potentially providing sustained health benefits throughout the large intestine.

XY and its hydrolysis product, XOS, are recognized for their prebiotic properties. XY is widely available in nature as a constituent of the plant cell walls so agricultural wastes are considered sustainable and cost-effective feedstock for the production of XY-

based prebiotics (Gonçalves et al., 2023). XY main chain is composed of xylose residues linked by β-(1,4) bonds, with side chains such as acetyl groups, (4-O-methyl) glucuronyl groups, or arabinose at 2'-OH or 3'-OH. XY may be further substituted with ferulic or p-coumaric acid residues. XY and XOS are stable in the upper gut conditions as well as harsh food production conditions such as low pH and high temperature and utilized in the colon by some health-beneficial species (La Rosa et al. 2019; Rashid and Sohail 2021; Samanta et al. 2015). *Bacteroides* and *Roseburia* are the primary XY-degrading genus in the colonic microbiota, through the production of extracellular xylanases. While few *Bifidobacterium* species can metabolize XOS, they are typically unable to degrade XY. However, the oligosaccharides produced from XY degradation by *Bacteroides* may become available for *Bifidobacterium* species through cross-feeding interactions, facilitating the metabolism of XY and demonstrating bifidogenic effects indirectly (Falony et al. 2006; Zeybek, Rastall, and Buyukkileci 2020). Co-culturing *Bifidobacterium animalis* subsp. *lactis* with *Bacteroides ovatus* and *Bacteroides xylanisolvens* revealed the cooperative interactions between these genera in utilizing XY (Zeybek, Rastall, and Buyukkileci 2020).

In vitro models, animal studies, and clinical trials have been conducted to elucidate the effect of prebiotics on microbiota and host health. Studies on prebiotic activity frequently employ test tubes inoculated with fecal material, owing to their simplicity and ease of setup. Single or multiple reactor types are dynamic *in vitro* human digestive system simulator models. Advanced versions such as SHIME (Simulator of Human Intestinal Microbial Ecosystem) and TIM (TNO gastrointestinal model) systems have been developed, incorporating additional components such as stomach, pancreatic, and bile fluids, and mucus (Lemmens et al. 2021; Verhoeckx et al. 2015). Although many models have been designed to simulate microbiota activity, these models cannot fully represent some of the gastrointestinal tract's complex physicochemical and physiological properties including microorganism adhesion, colonization, and localization (Bajury et al. 2018). *In vivo* studies in rodents (mice and rats), pigs, fish, rabbits, dogs, cats, or other animals are essential for understanding biological processes within the context of a whole organism; however, they are costly and do not allow the examination of organ-specific mechanisms (Pastorino, Prearo, and Barceló 2024; Vashishat et al. 2024). Similarly, clinical trials are constrained by ethical limitations that hinder the dynamic monitoring of gut microbiota and the collection of samples from various colon sections, thus limiting their capacity to elucidate the mechanisms underlying microbial nutrient degradation (Li

and Zhang, 2022). Alternatively, the tissues and organs removed from the animals can be utilized *ex vivo* to study the effects of prebiotics in more detail. *Ex vivo* models provide more control over experimental conditions, reduce ethical concerns, and comprehensively evaluate gut motility-regulatory elements, including those in large intestine segments of prebiotic utilization (Costa et al., 2024). We hypothesized that an *ex vivo* model could be developed to test the activity of prebiotic carbohydrates and address some of the limitations of other models. In the thesis, the model was employed using mice colon to investigate the utilization of prebiotics with different chain lengths, such as XOS, XY, and their combination (XOS+XY mix) and their effects on the abundance of selected bacteria in the cecum and colon sections, as well as the synthesis of SCFAs and other acids.

4.2. Materials

XY was extracted from CC as described below. XOS with 95% purity was generously provided by Longlive Biotechnology (Shandong, China), consisting of 39.3% xylobiose (X2), 31.7% xylotriose (X3), and 23.3% xylotetraose (X4). XOS standards (X1-X6) for HPLC analysis were obtained from Megazyme (Ireland). The High Pure PCR Template Preparation Kit (Roche, Switzerland) was used for DNA extraction from tissue samples, and the FastStart Essential DNA Green Master (Roche, Switzerland) was utilized for quantitative PCR (qPCR) analysis. All other chemicals were of analytical grade and sourced from Merck (Darmstadt, Germany).

4.3. Methods

The following experimental methods were used to assess prebiotic activity in an *ex vivo* model based on mice's large intestines. In this model, the effects of the natural polymer xylan (XY) and its hydrolysis product, xylooligosaccharides (XOS), were evaluated. XY and XOS were loaded separately into the separated cecum, proximal colon, and distal colon to examine their utilization by the colonized microflora, as well as the production of short-chain fatty acids. Additionally, levels of *Bifidobacterium* and

Bacteroides were measured by microbial changes in response to these substrates in the *ex vivo* model. The graphical abstract is shown in Figure 4.1.

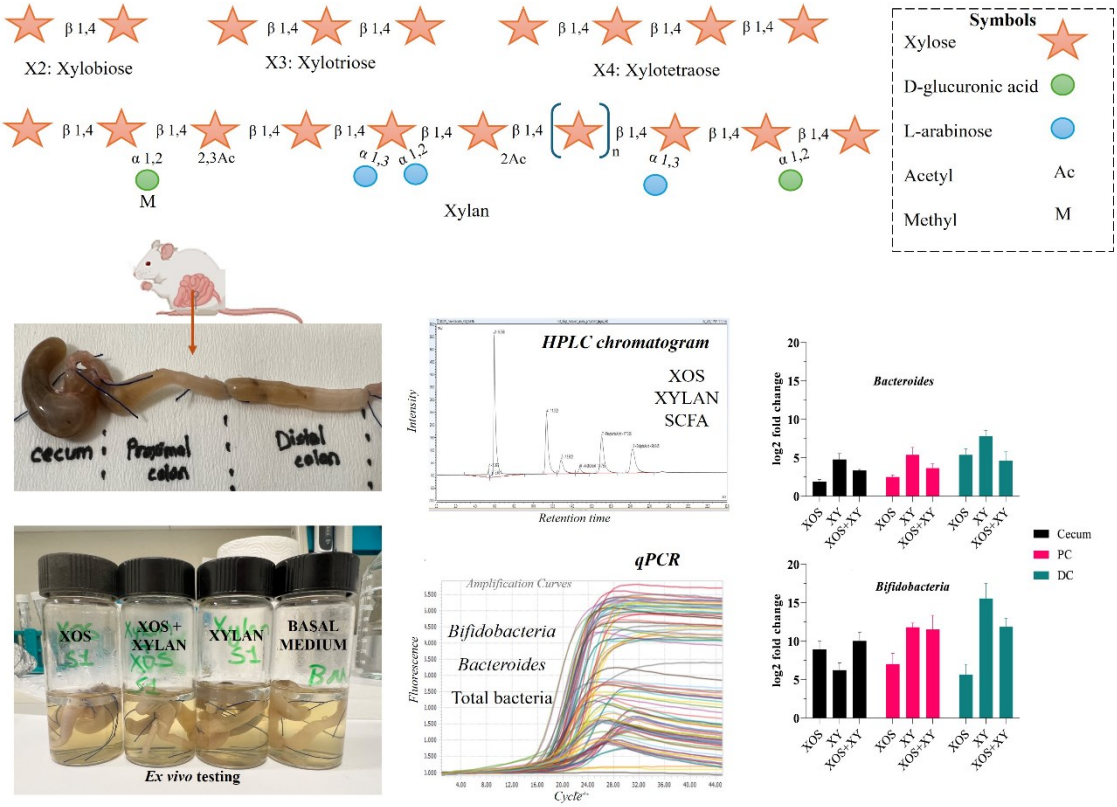

Figure 4. 1. Graphical abstract of the study of *ex vivo* model of different prebiotics.

4.3.1. Xylan Extraction from Corncob

XY was extracted by alkali treatment of the CC obtained from the Aegean Agricultural Research Institute of the Ministry of Agriculture and Forestry (Türkiye). Ground CC was mixed with 15% NaOH at a solid-to-liquid ratio of 1:10. The suspension was kept in the autoclave at 121 °C for 1 h. The solids were removed by filtration and the liquid was neutralized with glacial acetic acid. XY was precipitated with two volumes of ice-cold ethanol. The precipitate was separated by filtration and dried in a forced hot air oven at 40 °C until constant weight. The pellets were weighed, ground in a grinder-mixer, and stored at room temperature in air-tight bags.

4.3.2. Microbial Growth and Cell Concentration

Bifidobacterium animalis subsp. lactis (DSM-10140) was activated from a stock culture maintained at -80°C. The stock culture was inoculated into 1% Reinforced Clostridial Medium (RCM; pH 6.8–7.0), incubated overnight at 37°C, and subsequently enumerated.

Before inoculation, the medium was thoroughly deoxygenated to establish an anaerobic environment essential for the optimal growth of *Bifidobacterium animalis* subsp. *lactis*. To achieve complete oxygen removal, the Hungate method was employed, a well-established technique that ensures stringent anaerobic conditions by displacing oxygen with an inert gas. An anaerobic culture system (Anoxomat, MART Microbiology, USA) was also utilized to create and maintain anaerobic conditions in solid media. The Anoxomat system operates rapidly and automatically, removing oxygen within an anaerobic jar, thereby generating a stable and reproducible anaerobic atmosphere. This controlled environment is critical for successfully cultivating strictly anaerobic bacteria, minimizing oxidative stress and ensuring consistent experimental conditions, which is particularly important for studies investigating microbial metabolism and functional interactions under physiologically relevant conditions (Figure 4.2).

Figure 4. 2. Anoxomat System.

4.3.3. *Ex vivo* Model Set Up

Balb/c mice at 4-6 weeks of age, regardless of gender, were sacrificed with carbon dioxide gas after 16 h of fasting. The large intestine was dissected from the cecum to the anus and removed from the mice. After dissection, the mice's cecum and colon were gently washed with sterile PBS to remove fecal matter. Tissue samples were divided into three distinct segments: 1. cecum, 2. proximal colon (PC, first half of the colon), and 3. distal colon (DC, second half of the colon) (Figure 4.3). Each section was examined for the basal level of the *Bifidobacteria*, *Bacteroides*, and total bacteria amount.

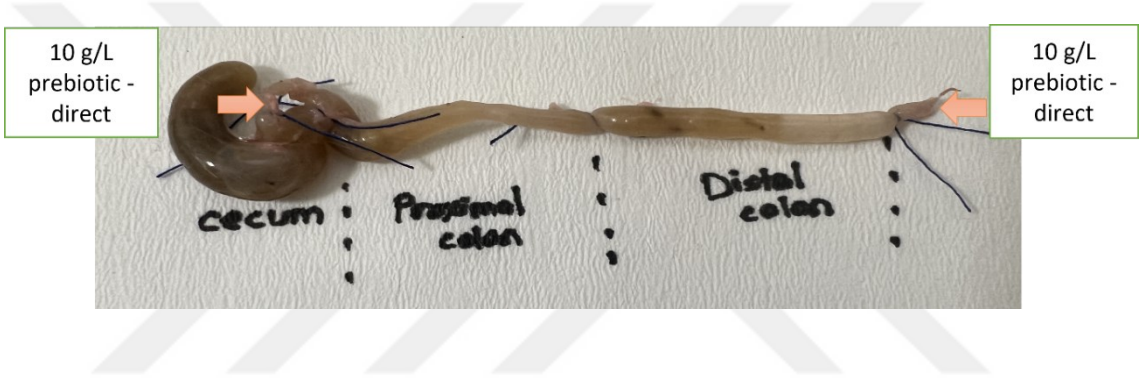

Figure 4. 3. Mice cecum and colon with model prebiotics.

XOS, XY, and XOS+XY mix were dissolved in Basal medium (BM) without any carbon source, each at a concentration of 10 g/L. Prebiotic solutions were loaded to the mice's cecum, PC, and DC, and the tissues were submerged in BM in 10 ml glass test tubes and the liquid surface was covered with paraffin to minimize air diffusion. The contents in the tissues were collected after 3 h of incubation and analyzed for remaining XOS and XY, organic acid concentrations, and fold changes in *Bifidobacteria* and *Bacteroides*. The BM used to submerge the tissues was also analyzed for XOS, XY, and acids.

All mice experimental protocols were approved by the institutional ethics committee at the Laboratory Animal Production, Care, Application, and Research Center, IZTECH.

4.3.4. Extraction of DNA from Tissues

DNA from the cecum and colon tissues of mice was extracted using the High Pure PCR Template Preparation Kit (Roche, Switzerland), following the manufacturer's instructions. Briefly, 35-40 mg of each tissue sample was mixed with tissue-lysis buffer. To ensure thorough homogenization and tissue disruption, sonication was applied for 30 seconds, as vortexing alone was insufficient. Once the tissues were fully fragmented and a homogeneous mixture was obtained, Proteinase K was added, and the samples were incubated at 55°C for 1 h, or until the tissues were completely digested. Following digestion, a binding buffer was added, and the mixture was incubated at 70°C for 10 min. The subsequent washing steps were performed according to the kit protocol. DNA concentration was measured using a Nanodrop spectrophotometer (Thermo Fisher Scientific), ensuring accurate quantification of the extracted DNA.

4.3.5. Quantification of Key Bacteria

qPCR amplifications were performed with 10 µM of each primer and 50 ng template DNA in a final reaction volume of 20 µl. Runs were performed using a LightCycler® 96 thermocycler (Roche, Basel, Switzerland) to assess the abundance of key bacterial phyla—Firmicutes, Bacteroidetes, Actinobacteria, and Proteobacteria—and specific genera, including *Bifidobacterium*, *Bacteroides*, *Lactobacillus*, and the total bacterial population. The cycling conditions were optimized to ensure high specificity and efficiency of amplification, minimizing non-specific products and maximizing reproducibility across replicates.

The V3-V4 region of the bacterial 16S rRNA gene was amplified using FastStart Essential DNA Green Master (Roche, Basel, Switzerland) and primers (g-Bifid-F (5'-CTC CTG GAA ACG GGT GG-3') /g-Bifid-R (5'-GGT GTT CTT CCC GAT ATC TAC A-3')) for total amount of *Bifidobacteria*. For total *Bacteroides* primers were used (AllBac29-F (5'-*GAG AGG AAG GTC CCC CAC*-3')/ *AllBac-412-R (5'-CGC TAC TTG GCT GGT TCA G-3*')); for total *Lactobacillus* primers (Lact-16S-F (5’-GGA ATC TTC CAC AAT GGA CG-3’) /Lact-16S-R (5’-CGC TTT ACG CCC AAT AAA TCC GG-3’)) with thermocycling parameters including a pre-incubation of 2 min at 95 °C, followed by

a three-step amplification program of 45 cycles consisting of a denaturation, annealing and extension step set at 95 °C for 15 s, 55 °C for 20 s and 72 °C for 30 s, respectively. In addition, the following primers were used for the total number of bacteria: F (5'-AGA CAC GGT CCA GAC TCC TAC-3'/ R (5'-TTT ACG GCG TGG ACT ACC AG-3') with thermocycling parameters including a pre-incubation of 2 min at 95 °C, followed by a three-step amplification program of 45 cycles set at 95 °C for 15 s, 50 °C for 20 s and 72 °C for 30 s, respectively (Junick & Blaut, 2012). A melt curve was included in each run to confirm the amplification of a single product. Positive control and no-template (negative) control were added for all runs.

qPCR data was used to calculate ΔΔCt, developed by Livak & Schmittgen (2001), which was a measure of the fold change of bacteria. Melting curve program analysis using the Roche LightCycler R 480 System was utilized to verify that qPCR operations were successful (Z. Wang et al., 2023). The $2^{-\Delta Ct}$ and $2^{-\Delta\Delta Ct}$ methods of data analysis were used (Livak & Schmittgen, 2001). The measurement of total bacteria served as the reference and target bacteria were taken into consideration (Navidshad et al., 2012). The following calculations were used to calculate the relative abundance of bacteria, which was expressed as log2 transformed fold change values.

Relative abundance of target bacteria species concerning the abundance of total bacteria as illustrated in Eq. (1):

$$2^{-\Delta Ct} = 2^{-(\text{Ct of target bacteria} - \text{Ct of total bacteria})} \quad (1)$$

Fold change of relative abundance of target bacteria compared to the control group (BM without a carbohydrate) as illustrated in Eq. (2):

$$2^{-\Delta\Delta Ct} = 2^{-[(\text{Ct of target bacteria} - \text{Ct of total bacteria}) - (\text{Ct of target bacteria} - \text{Ct of total bacteria})\ \text{control}]} \quad (2)$$

4.3.6. Analytical Methods

The following analytical methods determined structural carbohydrates, lignin, and ash, as well as organic acids in prebiotic groups, ensuring accurate quantification and characterization of these components. Standardized protocols were employed to enhance precision and reproducibility, facilitating reliable comparisons across different prebiotic samples.

4.3.6.1. Structural Carbohydrate and Lignin Analysis

The amounts of cellulose, hemicellulose, and lignin in the alkali extract were measured following the NREL/TP-510-42618 method (Sluiter et al., 2008). Following overnight drying at 40°C, the CC samples (0.3 g) were treated with 3 mL of 72% (w/w) H_2SO_4 for 60 min at room temperature and then diluted to 4% H_2SO_4 by adding 84 mL water. The mixture was hydrolyzed at 121°C for 1 h in an autoclave. The pH was then adjusted to 5-6 by $CaCO_3$. Glucose, xylose, and arabinose concentrations measured in HPLC were used to calculate cellulose and hemicellulose content, applying anhydrous correction factors of 0.90 for hexoses and 0.88 for pentoses. For lignin analysis, the solid remaining after the acid hydrolysis was separated by vacuum filtration through porcelain filter crucibles and dried at 105 °C. The weight of the solid was reported as lignin after subtracting ash content. Ash was determined gravimetrically after burning at 575 °C for 3 h in a furnace (Carbolite, Derbyshire, UK). All results were reported on a dry-weight basis.

4.3.6.2. Carbohydrate and Organic Acid Analysis

Carbohydrates and organic acids in the samples were measured in HPLC (Thermo Fisher Scientific Dionex, Sunnyvale, CA, USA). Monosaccharides (glucose, xylose, and arabinose), and XOS (X2, X3, and X4) were detected using Rezex RPM column (Phenomenex, Torrance, CA, USA) at 80 °C with ultrapure water as the mobile phase at 0.6 mL/min. SCFAs (acetate, propionate, butyrate, valerate, and isovalerate) and lactate and succinate were detected using a Aminex HPX-87H column (Biorad, Hercules, CA, USA) at 60 °C, eluted with 5 mM H_2SO_4 at 0.6 mL/min. Samples were centrifuged and filtered through 0.45 µm pore size membrane filters (Sartorius, Göttingen, Germany) before analysis in HPLC. Standard solutions of analytes at known concentrations were used to generate calibration curves. Carbohydrates and acids were detected using refractive index and UV detectors, respectively.

4.3.6.3. Molecular Weight Determination

The molecular weight of the extracted XY was measured using the intrinsic viscosity method in a capillary viscometer (SI Analytics, Mainz, Germany) equipped with an Ostwald capillary tube (Abdel-Azim et al., 1998). The viscometer was immersed in a water bath maintained at 25° C. The sample was loaded into the viscometer capillary and the efflux time, representing the time required for the sample to pass through the capillary, was recorded. The molecular weight was calculated using the intrinsic viscosity from the Mark-Houwink relationship, $Mw = ([\eta]/k)1/\alpha$, where $k = 0.00347$ and $\alpha = 0.98$ (Carvajal-Millan et al., 2005).

4.3.7. Statistical Analysis

All tests were carried out in four parallels, and the average results were reported as mean value ± standard deviation. The data were analyzed by using variance analysis (one way-ANOVA) and Tukey test ($p \leq 0.05$) using Minitab (ver.18.1, Minitab Inc., United Kingdom).

4.4. Results and Discussion

The detailed results and discussion are given in the following sections including characterization of corncob extract, utilization of XOS and xylan in *ex vivo* mice cecum and colon, and effect of XOS and xylan on levels of key bacteria**.**

4.4.1. Characterization of Corncob Extract

Alkali extraction of CC resulted in solid biomass containing 73.4% XY, as shown in Table 1. The molar ratio of xylose to arabinose was 12.5, while the ratios of xylose to acetyl and xylose to glucuronic acid were approximately 8 and 33, respectively. The

HPLC chromatogram of the extract, illustrating the composition, was provided in the supplementary material. During the extraction, cellulose remained largely within the CC matrix, whereas lignin co-extracted alongside XY. The molecular weight of the extracted XY was estimated at 46.41 kDa, determined through intrinsic viscosity measurements, which provide a reliable assessment of polymer size in solution.

Table 4. 1. Composition of the corncob alkali extract (g/100 g dry extract).

Component	Fraction (%)
Cellulose	1.58±0.8
Xylan	73.6±1.7
As anhydrous:	
Xylose	63.4±1.1
Arabinose	5.31±0.3
Acetyl	2.16±0.7
Glucuronic acid	2.71±0.4
Lignin	18.2±2.2
Ash	1.32±0.6
pH (in %1 solution)	6.93±0.2

4.4.2. Utilization of Xylooligosaccharides and Xylan in *ex vivo* Mice Cecum and Colon

In this thesis, the lower gastrointestinal tract of mice, comprising the cecum and colon, was utilized to assess the activity of selected prebiotics. After removing the tissues, the tissues were used in *ex vivo* tests, which allowed the evaluation of prebiotic activity in the microorganisms' natural environment. The density of the organisms varies significantly along different sections of the large intestine, which could influence the utilization patterns and rates of carbohydrate metabolism, thereby affecting prebiotic activity (Piccioni et al. 2023). Using the *ex vivo* model, each section, such as the cecum, PC, and DC, could be investigated separately. This approach enabled the examination of XOS and XY utilization, SCFA production, and the proliferation of critical bacterial

genera in different sections of the lower gut. Unlike in vitro models, which typically rely on fecal inoculum to represent the gut microbiota, the ex vivo model of mice gut preserves the distribution and density of microorganisms, offering deeper insights into how these influence prebiotic activity across different sections of the large intestine (Waclawiková et al. 2022). However, caution should be exercised when extrapolating findings from mice-based models to humans and other animals. The differences may influence the prebiotic activity in size, length, transit time, microflora composition, and biochemistry of the lower gut of mice and humans (Nguyen et al. 2015; Wos-Oxley et al. 2012).

In the *ex vivo* model, the utilization of XOS, XY, and XOS+XY mix in different sections of the mice's lower gastrointestinal tract was followed by measuring xylose, X2, X3, and XY after 3 h of incubation (Table 2). The cecum was filled with 500 µl, while each section of the colon was filled with 250 µl of prebiotic solutions. This setup allowed for the administration of 5 mg of prebiotic into the cecum and 2.5 mg into PC and DC.

In XOS-loaded samples, no XOS was detected after 3h; that is, XOS was utilized completely. In the case of XY, 3.6 mg/ml of the initial 10 mg/ml remained in the cecum, corresponding to 3.2 mg XY utilization in 3 h. Small amounts of X2 and X3 (0.06-0.10 mg/ml) were also detected. In PC and DC, most of the administered XY (2.5 mg) were utilized, and XY, X2, and X3 concentrations were also low (Table 4.2).

When the XOS and XY were loaded together (XOS+XY mix) into the cecum, a substantial amount of XY (6.13 mg/mL) was detected in 3 h of incubation, whereas XOS concentration was much less. A total of 69% of the carbohydrates were metabolized in the cecum in this period. In PC and DC, both XOS and XY were utilized at the same rate except that the remaining X2 concentration was comparably higher in DC.

The *ex vivo* model developed in this work was utilized for prebiotic activity testing of XOS and XY, with a specific focus on investigating the impact of carbohydrate chain length on microbial utilization and fermentation processes. This model provided an effective means to simulate the gastrointestinal environment, allowing for a detailed assessment of how the different structural characteristics of XOS and XY influence their metabolism by gut microbiota. In the *ex vivo* experiments, both XOS and XY were readily utilized by the fecal microbiota across the cecum, PC, and DC of mice, suggesting that all sections of the large intestine contained microbial communities capable of efficiently catabolizing these prebiotics. The results highlight that the gut microbiota is versatile, with diverse microbial populations capable of degrading both oligomeric and polymeric carbohydrates, albeit potentially at different rates. The difference in chain length between

XOS and XY may contribute to variations in microbial fermentation patterns, as the simpler XOS oligosaccharides are more easily accessible to microbes compared to the more complex and larger molecular weight XY polysaccharides, which require additional enzymatic processing.

Table 4. 2. Utilization of XOS, XY, and XOS+XY mix in mice cecum, PC, and DC in the ex vivo model in 3 h.

	Feed* (mg)	**Concentration (mg/ml)**				**Utilization (mg)**
		Xylose	***Xylobiose***	***Xylotriose***	***Xylan***	
Cecum	XOS (5)	0.00±0.00	0.00±0.00	0.00±0.00	--	5
	XY (5)	0.06±0.02	0.07±0.01	0.10±0.04	3.6±0.14	3.2
	XOS (5) + XY (5)	0.31±0.16	1.28±0.60	0.14±0.02	6.13±0.74	6.9
PC	XOS (2.5)	0.04±0.01	0.00±0.00	0.00±0.00	--	2.5
	XY (2.5)	0.04±0.01	0.17±0.08	0.06±0.06	0.82±0.61	2.3
	XOS (2.5) +XY (2.5)	0.47±0.05	0.34±0.21	0.23±0.30	0.43±0.64	4.9
DC	XOS (2.5)	0.00±0.00	0.00±0.00	0.00±0.00	--	2.5
	XY (2.5)	0.14±0.09	0.70±0.40	0.29±0.05	1.33±0.29	2.2
	XOS (2.5) +XY (2.5)	0.25±0.04	1.63±0.07	0.31±0.08	1.01±0.30	4.7

* XOS and XY concentrations of feed were 10 mg/mL. Data are presented as the mean ± standard deviation from four measurements.

The results demonstrated that the majority of the SCFAs synthesized within the tissues were subsequently secreted into the BM, in which the tissues were suspended. This suggests an active transport or diffusion mechanism facilitating SCFA release into the surrounding medium. To quantify SCFA production comprehensively, the concentrations detected in both the external environment and the luminal material were converted into mass units. These values were then aggregated and reported as a total sum

to provide a more accurate representation of SCFA distribution and overall production (Figure 4.4). This approach ensures a standardized comparison across different experimental conditions and allows for a more precise evaluation of SCFA secretion dynamics.

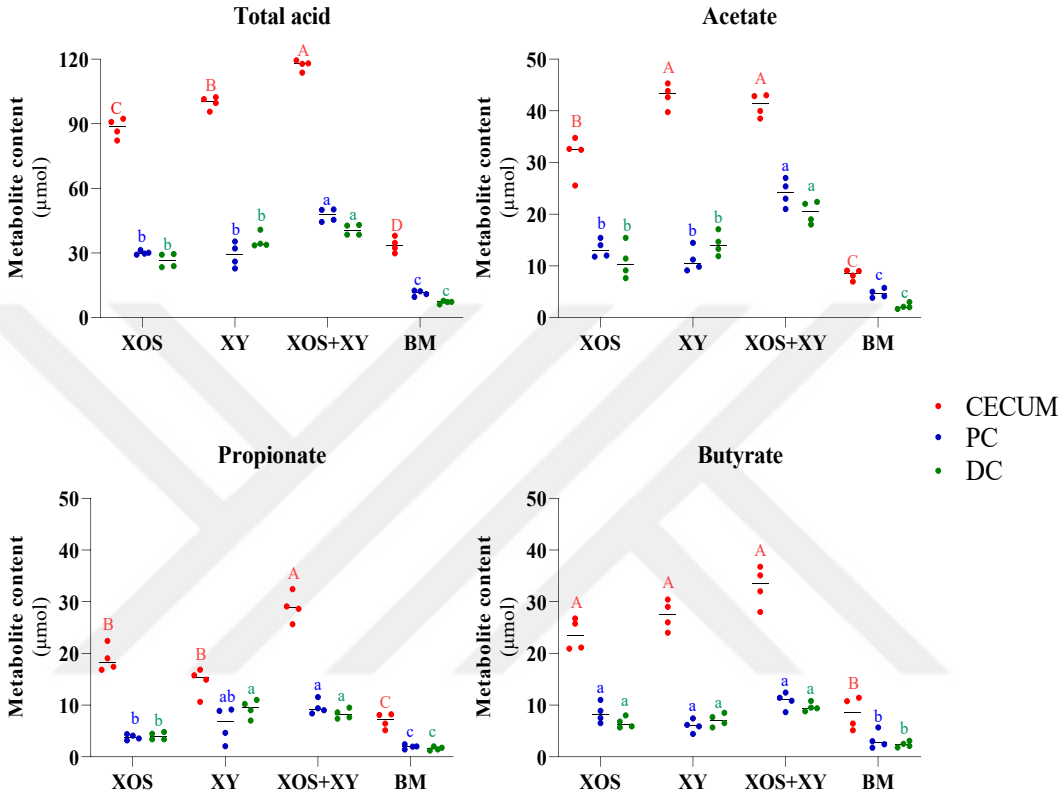

Figure 4. 4. SCFA levels of the mice's cecum, PC, and DC in 3 h incubation with XOS, XY, and XOS+XY mix, and the negative control (BM without carbohydrate source). Total acid is the sum of acetate, propionate, butyrate, valerate, isovalerate, lactate, and succinate levels. Data from each parallel experiment are represented with a dot and averages are represented by dashes. Statistical analyses were performed using one-way ANOVA. Different capital letters indicate statistically significant differences in mice cecum, while different lowercase letters indicate statistically significant differences in the colon among the XOS, XY, and XOS+XY treatments ($P < 0.05$).

Compared to the control (BM), all prebiotic treatments significantly increased acetate, propionate, butyrate, total SCFAs (including valerate and isovalerate), and lactate and succinate (Fig. 4.5). Cecal SCFA production was high, and it gradually decreased throughout the colon ($P < 0.05$). Similar SCFAs were produced in the PC and DC, but

both were significantly less than those in the cecum ($P < 0.05$). Across all incubations, acetate levels were the highest, followed by butyrate and propionate. The XY and XOS+XY mix treatments resulted in acetate levels exceeding 40 µmol. The XOS and XY treatments showed a similar increase in butyrate and propionate levels in the cecum and colon sections.

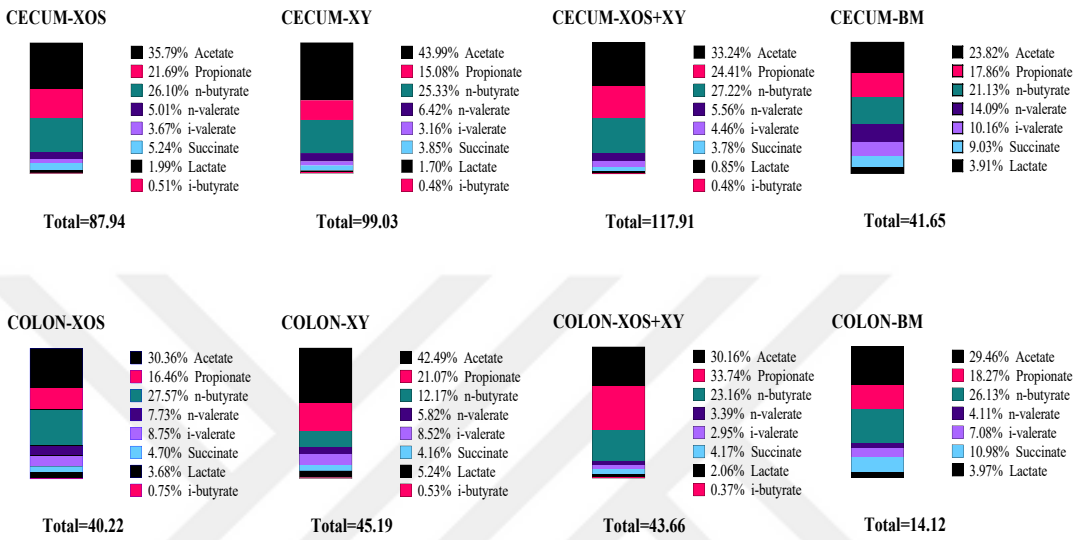

Figure 4. 5. Total acid (acetate, propionate, n-butyrate, i-butyrate, n-valerate, i-valerate, lactate, and succinate) levels of the mice's cecum and colon in 3 h of incubation with XOS, XY, and XOS+XY mix, and the negative control (BM without carbohydrate source).

4.4.3. Effect of Xylooligosaccharides and Xylan on Levels of Key Bacteria

The cecum, PC, and DC microbial community composition at the selected phyla and genus levels demonstrated notable differences among the treatments (XOS, XY, and XOS+XY). Across all gut regions, Firmicutes consistently represented the most abundant phylum, followed by Bacteroidetes, Actinobacteria, and Proteobacteria (Fig. 4.6A). In the cecum, the relative abundance of Firmicutes was the highest in all treatment groups, with minor variations observed. The abundance of Bacteroidetes was notably higher in the XY and XOS+XY treatments. Actinobacteria levels were elevated in the XOS and XOS+XY

groups compared to other treatments. In the DC, the XY treatment resulted in a significant increase in the relative abundance of Actinobacteria. Proteobacteria remained at consistently low levels across all groups.

The *Bifidobacteria* and *Lactobacillus* genera were quantified because of their strong association with prebiotic effects and health benefits (Flach et al., 2018). Both are capable of utilizing XOS as a substrate, leading to the production of SCFAs. *Bacteroides* species were included for their role in the breakdown of complex polysaccharides including XY, producing enzymes that release fermentable substrates supporting cross-feeding interactions. After 3 h incubation with prebiotics, *Bifidobacterium*, *Bacteroides,* and *Lactobacillus* levels in all three sections increased notably compared to the control group (BM without carbohydrates) (Fig. 4.6B). The largest fold changes in *Bacteroides* levels were observed in the DC. XY was the most effective substrate on *Bacteroides* in all three sections with fold changes between 4.8 and 7.8. The highest fold change was observed with the XY in the DC, while the lowest was in the cecum with the XOS. The substrates affected *Bifidobacterium* levels more with higher fold changes (up to 15.6) than that of *Bacteroides* (Fig. 4.6B). In the cecum, comparable fold changes were observed with XOS and mix, while the fold change value was lower with XY. The effect of XY was pronounced more in the later sections. The same was also valid for the mix. Conversely, the effect of XOS on *Bifidobacterium* species was the most evident in the cecum. *Lactobacillus* abundance was highest in XOS treatment, significantly surpassing the levels observed in the XY and mix groups. The combined XOS+XY treatment provided a modest but consistent benefit across all regions, particularly in the PC and DC.

XOS utilization was faster than XY, which could be ascribed to the simpler structure of XOS so that they were readily accessible to the gut microbiota. Polysaccharides cannot be transported into the cells; therefore, they must be broken down enzymatically into smaller units (Riva et al. 2023). XY catabolism is facilitated by a diverse community of bacteria equipped with specialized enzymes (Biely, Šuchová, and Puchart 2023; Malhotra and Chapadgaonkar 2018). In the gut, the Gram-negative *Bacteroides* species (*B. ovatus*, *B. xylanisolvens*, and *B. fragilis*) and Gram-positive *Roseburia* species (*R. intestinalis* from the *Roseburia/Eubacterium rectale* group) are major xylanolytic bacteria that efficiently break down XY into fermentable sugars (Méndez-Líter et al. 2023; Shrivastava et al. 2020). Slow and incomplete degradation of XY in the cecum may enable the remaining XY and its degradation products to reach the distal part of the colon. Despite the absence of XY in the Western-style diet of mice, it

was obvious that microorganisms capable of metabolizing XY were present even in DC. This suggests that the prebiotic effect can be extended throughout the colon with sufficient XY intake. Long-chain fructans were also shown to be degraded partially in the cecum and PC in animal studies, exhibiting prebiotic effects along the gut (Horasan Sagbasan et al. 2024; Hughes et al. 2022; Xu et al. 2020). Benítez-Páez et al, (2016) demonstrated that the combination of FOS and inulin reaches the DC in mice more effectively compared to FOS alone, altering the gut microbiota composition significantly. Conversely, the findings of this thesis revealed that XY metabolism was not affected by the presence of XOS when administrated in a mixture.

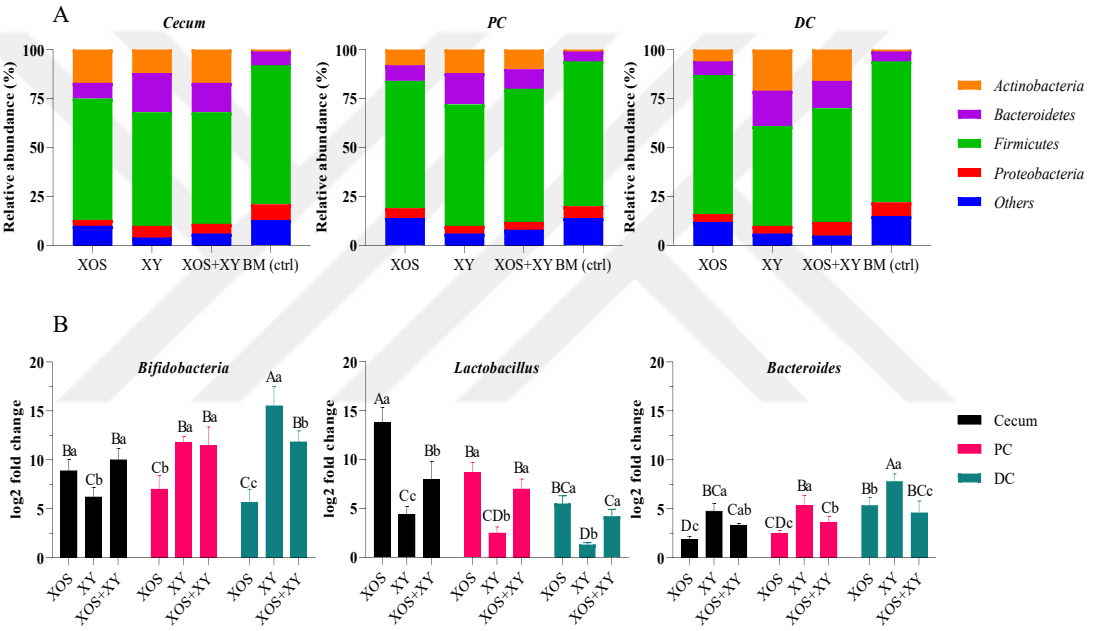

Figure 4. 6. A. The mean relative abundance of four phyla in the cecum, proximal colon (PC), and distal colon (DC) after 3 h of incubation with XOS, XY, and XOS+XY mix. B. Fold change of relative abundance of *Bifidobacteria*, *Bacteroides*, and *Lactobacillus* compared to the control group (BM with no carbohydrate source) in cecum, PC, and DC after 3 h of incubation with XOS, XY, and XOS+XY mix. The results are an average of four replicates. Error bars show standard deviation. Different capital letters indicate statistically significant differences among the prebiotic treatments in all sections. Lowercase letters indicate statistically significant differences among prebiotic treatments within a single section ($P < 0.05$).

The relative changes in the abundance of *Bacteroides* and *Bifidobacterium* in lower gut sections were calculated using qPCR data upon XOS and XY administration. XOS, XY, and their mix resulted in notable fold-changes in *Bacteroides* across all three sections. The fold change values were higher with XY, which could easily be ascribed to the requirement of *Bacteroides* for XY utilization. The major XY degrading species should have proliferated to support the growth of XY as the sole carbon source (Tiwari et al. 2020). Lower fold changes in *Bacteroides* were observed with XOS, as a broader range of gut microorganisms, predominantly from the phyla *Firmicutes*, *Bacteroidetes*, *Proteobacteria*, *Campylobacterota*, and *Actinobacteriota*, can utilize this oligomer (Deng et al. 2023; Moro Cantu-Jungles et al. 2019). The effects of both XOS and XY were pronounced more in DC. It was difficult to interpret this observation, but it shows that both prebiotics could improve the microflora of DC for their utilization and enhance the prebiotic effect towards the end of the large intestine, as well as the preceding sections. The impact of the XOS+XY mix on the fold change of *Bacteroides* was less potent than XY. The presence of XOS may have attenuated the requirement for *Bacteroides* to provide a carbon source for the microorganisms, some of which could survive on XOS without XY degradation.

The bifidogenic effect of XOS and XY was observed across all sections, with *Bifidobacterium* species showing higher fold change values compared to *Bacteroides*. The effects of XOS, XY, and XOS+XY mix varied by region. XOS and the mix led to higher fold changes in the cecum, whereas XY and the mix were more effective in the PC and DC. The rapid and substantial utilization of XOS in the cecum could promote the proliferation of *Bifidobacterium* species, where XY utilization was slower. It was interesting to observe that the effect was opposite in PC and DC, such that the bifidogenic effect of XY was more profound than that of XOS, based on the fold change values. A possible explanation could be the strong synergy between *Bifidobacterium* and XY-degrading *Bacteroides* species (Zeybek et al., 2020), which might have promoted *Bifidobacterium* more than the other species on XY as the sole carbon source. That could have resulted in higher fold changes in *Bifidobacterium* with XY in the PC and DC, compared to XOS, which can be utilized by a wider range of species.

Total SCFA levels were significantly higher in the cecum compared to the PC and DC, highlighting distinct regional variations in microbial fermentation activity. This observation aligns with the well-established role of the cecum as a primary site for carbohydrate fermentation and SCFA production due to its high microbial density and

prolonged substrate retention time. Among the detected SCFAs, acetate was the most abundant across all incubations, followed by butyrate and propionate, with relatively consistent concentrations observed across the cecal and colonic sections. This distribution pattern reflects the metabolic preferences of gut microbiota and the differential utilization of substrates within specific intestinal regions. Furthermore, the SCFA output in response to supplementation with XOS and XY demonstrated the prebiotic potential of these substrates, as their fermentation resulted in increased SCFA production throughout the intestinal sections they reached. These findings underscore the capacity of XOS and XY to modulate microbial metabolism and enhance SCFA generation, which may contribute to their beneficial effects on gut health.

4.5. Conclusions

The *ex vivo* model of mice's lower gut developed in this study allowed for the investigation of the prebiotic activity in a close-to-real environment containing the colonized microflora. Moreover, it was possible to test the prebiotics on the different sections of the lower gut separately, so that the effect of microbial diversity among the sections of the gut could be reflected. These represent the primary benefits of the *ex vivo* model over the widely used *in vitro* models. This model was used in this study to compare the prebiotic effects of XY and XOS in the cecum, PC, and DC of mice by following utilization rates, SCFA production, and changes in the abundance of key genera. Both carbohydrates were utilized effectively along the cecum and colon by the resident microflora, promoting a bifidogenic effect. Slower utilization of XY can be considered a key property in prolonging the prebiotic effect towards the end of the colon. *Ex vivo* models may offer important insights into prebiotic activity prior to preclinical research, despite the notable physiological differences among mice, humans, and other animals.

CHAPTER 5

TECHNO-FUNCTIONAL PROPERTIES OF ALKALI EXTRACTED XYLAN

5.1. Introduction

Hydrocolloids, as functional ingredients, are integral to food science due to their diverse physicochemical properties that significantly impact texture, stability, and sensory characteristics across a variety of food products. Their primary function stems from their ability to interact with water molecules, enabling high water-binding capacities that enhance moisture retention, improve product consistency, and contribute to a desirable mouthfeel. This property is particularly valuable in products like sauces, dressings, and baked goods, where moisture and texture retention are essential for maintaining quality under diverse processing and storage conditions (Nishinari et al., 2018).

The thickening and gelling capabilities of hydrocolloids play a central role in food structure, allowing formulators to control viscosity, create gel matrices, and stabilize textures in complex food systems. These gelling and thickening functions are particularly beneficial in foods requiring specific structural profiles, such as jams, fruit fillings, and dairy-based desserts, where hydrocolloids form stable gels that retain consistency and firmness even under thermal and mechanical stresses (Agudelo et al., 2014). Additionally, hydrocolloids can serve as fat replacers in formulations, notably in confectioneries and cream-based fillings. Their ability to mimic the mouthfeel of fats enables the production of low-fat alternatives without compromising the sensory experience, aligning with consumer demand for healthier (Dias, Alvarenga, and Sousa, 2015).

Beyond textural applications, hydrocolloids also possess emulsifying properties, making them critical in systems where oil-in-water emulsions are necessary. In dairy-based products like ice cream, yogurt, and beverages, hydrocolloids contribute to emulsion stability, prevent phase separation, and enhance the product's overall texture. These functionalities ensure a uniform distribution of ingredients and maintain product

integrity during processing, storage, and consumption (Nishinari et al., 2018). Furthermore, in bakery products, hydrocolloids act as bulking agents, enhancing volume, improving crumb structure, and extending shelf life by reducing stalling and moisture loss (Espert et al., 2019).

The multifunctionality of hydrocolloids emphasizes their essential role in the development of food products that meet the standards of quality, stability, and consumer acceptability. Hydrocolloids continue to be a focal point in food research and innovation, allowing for the optimization of both functional properties and sensory attributes in a wide range of food formulations.

5.2. Materials

XY was extracted from CC with different alkali solutions as described below. All chemicals were of analytical grade and sourced from Merck (Darmstadt, Germany).

5.3. Methods

The following methods were applied to characterize the XY extracts obtained from CC and to assess their techno-functional properties.

5.3.1. Xylan Extraction of Different Alkali Solutions from Corncob

XY extraction from CC, sourced from the Aegean Agricultural Research Institute of the Ministry of Agriculture and Forestry, was achieved through alkali treatment. Initially, CC was processed to isolate the XY-rich fraction using sodium hydroxide solutions at varying concentrations (5%, 10%, and 15%). A solid-to-liquid ratio of 1:10. Approximately 40 g of powdered CC were treated with alkali in an autoclave at 121°C and 15 psi for 1 hour to enhance XY solubilization.

Following extraction, the solution was acidified to pH 5.0 using glacial acetic acid, and XY was precipitated with ice-cold ethanol. The precipitated XY was then dried in a forced-air oven at 45°C until reaching a constant weight. Once dried, the XY pellets were weighed, ground into a fine powder, and stored at room temperature for further analysis. For consistency, all experimental data and results were reported based on the dry weight of the samples, ensuring reliability throughout the study.

5.3.2. Chemical, Physicochemical, and Physical Analyses of Corncob Extracts

The extract's moisture content was determined by drying in an oven (Memmert, Germany) at 105 °C overnight according to the National Renewable Energy Laboratory (NREL)/TP-510-42621 method. The measurements were performed in triplicates. The dried extract was milled in a laboratory-type plant grinder to a particle size of less than 2 mm and stored at room temperature until analysis.

HPLC was used to determine the concentrations of glucose and xylose in the extracts, allowing for the quantification of cellulose and XY content following extraction. The dry samples were dissolved in water, and the mixture was then treated with 4% H_2SO_4. In an autoclave, the mixture was hydrolyzed for 1 hour at 121°C. The pH was then adjusted to 5-6 by adding $CaCO_3$. Finally, materials were centrifuged and filtered through 0.45 µm pore size membrane filters (Sartorius) before being analyzed in HPLC. Calibration curves were obtained using standard solutions at certain concentrations.

A comprehensive colorimetric analysis was conducted using a colorimeter (CR-200, Minolta, Osaka, Japan) to evaluate the color properties of the prepared extracts. Before analysis, the device was carefully calibrated to ensure measurement accuracy. The color parameters measured included L* (lightness/brightness), a* (representing the red-green axis, with +a* indicating red and -a* indicating green), and b* (representing the yellow-blue axis, with +b* indicating yellow and -b* indicating blue). These measurements were performed for extracts prepared at concentrations of 1%, 3%, and 5%, allowing for a systematic assessment of color variations across different extract concentrations. This approach provides valuable insights into the impact of extract

concentration on color properties, which is critical for applications where color stability and visual appeal are important quality attributes.

5.3.3. Techno-functional Properties of Corncob Extracts

The following techno-functional properties of CC extracts include foaming capacity and stability, emulsifying capacity and stability, and water and oil holding capacity.

5.3.3.1. Foaming Capacity and Foam Stability

The foaming capacity (FC) and stability (FS) of the XY samples were evaluated following the method of Aydemir & Yemenicioğlu (2013). FC was determined in a 25 mL conical tube, 20 ml of extracts (1-3-5%), homogenized with UltraTurrax at 23,000 rpm for 1 min, expressed as the percentage ratio between the foam formed and the volume initial of solution (% v/v). FC was expressed after 5 minutes of rest. FS was evaluated by recording the foam volume at 30 and 180 minutes after foam formation.

5.3.3.2. Emulsifying Capacity and Emulsion Stability

The emulsifying activity (EA) and emulsion stability (ES) of the XY samples were measured using a modified method based on Raji et al. (2017). This approach evaluated the ability of the extracts to form and stabilize emulsions, an important functional property for applications in food systems.

To assess EA, different concentrations of XY extract solutions (1%, 3%, and 5%) were prepared. Five milliliters of each extract concentration were combined with 5 mL of vegetable oil and homogenized using an UltraTurrax homogenizer at 16,000 rpm for 2 minutes to form a stable emulsion. From this mixture, 10 mL of the resulting emulsion was transferred to a conical tube, and the volume of the emulsified layer was measured.

EA was calculated as the percentage ratio of the emulsion layer volume to the total emulsion volume, expressed as % v/v. This value indicated the emulsifying potential of the XY extracts at different concentrations.

ES was evaluated by storing the emulsion samples in conical tubes and measuring the remaining emulsion layer at intervals of 30 minutes, 1 day, and 7 days. ES was calculated as the percentage of the remaining emulsion layer relative to the initial emulsion volume (% v/v). This metric provided insight into the stability of the emulsions over time, indicating the potential for long-term use in formulations. High ES values suggest that the emulsion retains its structure, which is essential for maintaining texture and uniformity in food products over storage.

5.3.3.3. Water and Oil Holding Capacity

The Water Holding Capacity (WHC) of the extract was assessed by adding 1.5 mL of distilled water to 0.5 g of the sample. The mixture was homogenized using a vortex for 90 seconds to ensure even distribution, then incubated at 30°C for 3 hours to allow for maximum water absorption. Following incubation, unbound water was separated from the sample by centrifuging at 6000 g for 20 minutes. WHC was calculated as the grams of water retained per gram of sample (w/w), providing a measure of the extract's ability to retain moisture.

The Oil Holding Capacity (OHC) was measured similarly, with 1.5 g of oil added to 0.5 g of the sample. After vortexing for 90 seconds to promote uniform mixing, the mixture was incubated at 30°C for 3 hours to enable oil absorption. Excess oil was then separated by centrifuging at 6000 g for 20 minutes. OHC was expressed as grams of oil retained per gram of sample (w/w), indicating the extract’s capacity to retain oils.

Both WHC and OHC are critical for evaluating the functional properties of food additives and ingredients, as they affect the texture, stability, and mouthfeel of food products. These properties provide insights into the potential applications of the extract in food formulations, particularly in products where moisture retention or oil binding is desirable.

5.3.4. Antioxidant Activity

The antioxidant activity of XY extracts (5%, 10%, and 15%) was evaluated using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method. This method determined the radical-scavenging capacity of the extracts by measuring their ability to neutralize DPPH free radicals.

XY solutions were prepared at nine different concentrations: 5.0, 4.17, 3.33, 2.5, 1.67, 1.25, 0.83, 0.5, and 0.17 mg/mL. Each concentration was tested for its % inhibition of DPPH radical activity, and the Efficient Concentration 50 (EC50) value was calculated. EC50 represents the concentration required to inhibit 50% of DPPH radical activity, providing a quantitative measure of antioxidant potential.

5.3.5. Statistical Analysis

Each analysis was performed three times to ensure the reliability and accuracy of the results. The outcomes of these analyses were reported as the mean value ± standard deviation (SD). The data were analyzed by using variance analysis (one way-ANOVA) and the Tukey test using Minitab software (ver.18.1, Minitab Inc., United Kingdom) which is a powerful tool for carrying out intricate statistical computations and producing thorough data visualizations. The significance level was set at $p \leq 0.05$ for all statistical tests.

5.4. Results and Discussion

The detailed results and discussion are given in the following sections, including composition, color, techno-functional properties, and antioxidant activities of different alkali-extracted corncob.

5.4.1. Composition of Different Alkali-Extracted Corncob

In the raw CC, cellulose, XY, and lignin represent approximately 39.5%, 28.3%, and 5.5% of the composition, respectively. However, as the alkali concentration increased, there was a marked reduction in cellulose content, dropping to minimal levels in all extracts (5%, 10%, and 15%). Conversely, the proportion of XY showed a significant increase, with the highest concentration observed in the 15% extract, reaching close to 62%. Lignin content also displayed a slight increase across the extracts but remained relatively stable compared to XY. Figure 5.1 illustrates the compositional changes in cellulose, XY, and lignin content in raw CC and its extracts at varying concentrations (5%, 10%, and 15%).

This trend suggested that the extraction process selectively enhances XY concentration while significantly decreasing cellulose, with lignin content remaining less affected. The substantial increase in XY content with higher alkali extraction highlighted the potential for optimizing extraction conditions to selectively isolate XY from CC biomass, which may be beneficial for applications that require high XY purity.

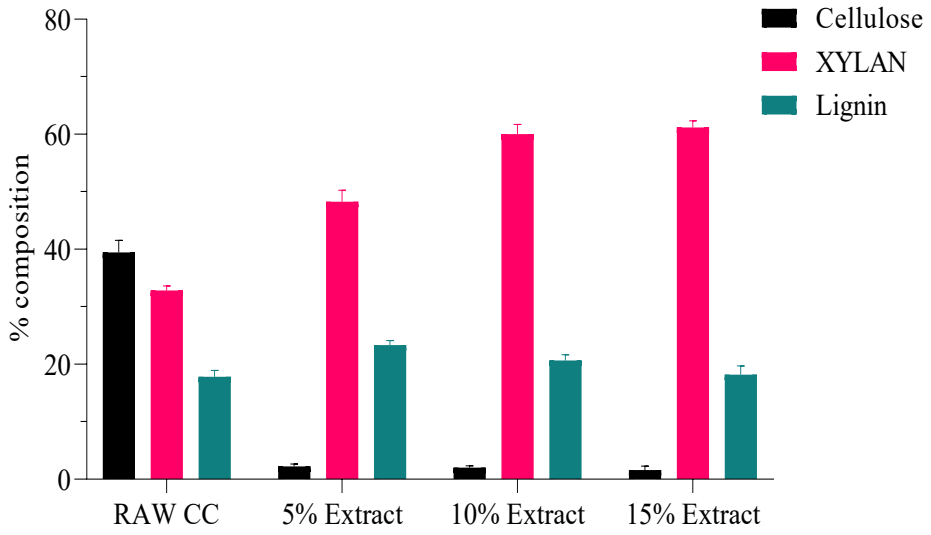

Figure 5. 1. Compositional changes in cellulose, XY, and lignin content in raw CC and its extracts at varying concentrations (5%, 10%, and 15%).

5.4.2. Color of Alkali-Extracted Corncob

The color analysis indicated that alkali extraction significantly influenced the color characteristics of the XY extracts (Table 5.1). The reduction in L* values suggested a decrease in light following extraction, indicating that the treatment may have altered the visual appearance of the extracts. Additionally, variations in the a* and b* values reflected changes in the red and yellowness components, respectively. Notably, higher alkali concentrations (10% and 15%) produced more intense red and yellow hues compared to the 5% extract, which may be attributed to structural modifications in the XY matrix resulting from the alkali treatment. These modifications likely impacted color-bearing molecular groups within the extracts, thereby altering their overall color properties.

Table 5. 1. Color values of XY extracts.

Characteristics	RAW CC	5% Extract	10% Extract	15% Extract
L*	60.93	30.95	38.08	37.43
a*	7.68	6.29	7.05	10.07
b*	24.22	15.17	19.81	27.52

5.4.3. Techno-functional Properties of Alkali-extracted Corncob

The FC and FS after 30 min and 180 min of XY extracts were assessed across varying extract concentrations (5%, 10%, and 15%) and solution concentrations (1%, 3%, and 5%) (Figure 5.2). In the initial measurement, the foaming volume increased with both extract and solution concentration. Specifically, the 5% solutions consistently showed the highest foaming volume, followed by the 3% level, and then the 1% level directly related to XY concentrations.

After 30 min, the foaming volume decreased across all extract concentrations, although the trend of higher foaming volumes with increasing solution concentration remains consistent. After 180 min, a further decrease in foaming volume was observed across all concentrations and levels, highlighting a continued loss of foam stability over

time. However, the 5% solution concentration maintained the highest foaming volume compared to 3% and 1% across each extract concentration, with the highest values seen at 15% extract. Results emphasized higher XY levels were directly associated with greater FC and slower reductions in FS.

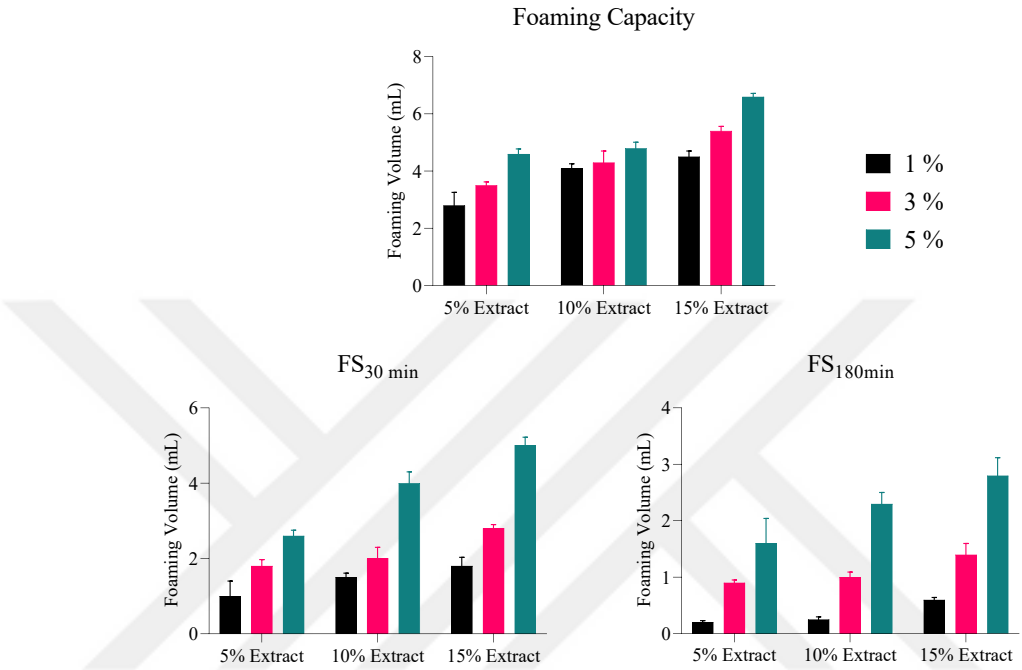

Figure 5. 2. Foaming Capacity and stability after 30 min and 180 min of XY extracts.

The EC and ES of XY extracts were assessed across varying extract concentrations (5%, 10%, and 15%) and solution concentrations (1%, 3%, and 5%) (Figure 5.3). The EC, represented by the emulsion volume (mL), demonstrated a trend of increasing emulsifying ability as the XY extract concentration increased from 5% to 15%. At each extract concentration, higher solution percentages (3% and 5%) exhibited slightly increased emulsion volumes compared to the 1% solution, although the variation between these is minimal. The highest EC was observed with the 15% XY extract at 5% solution, reaching 10 mL, suggesting that higher extract and solution concentrations enhance emulsification potential.

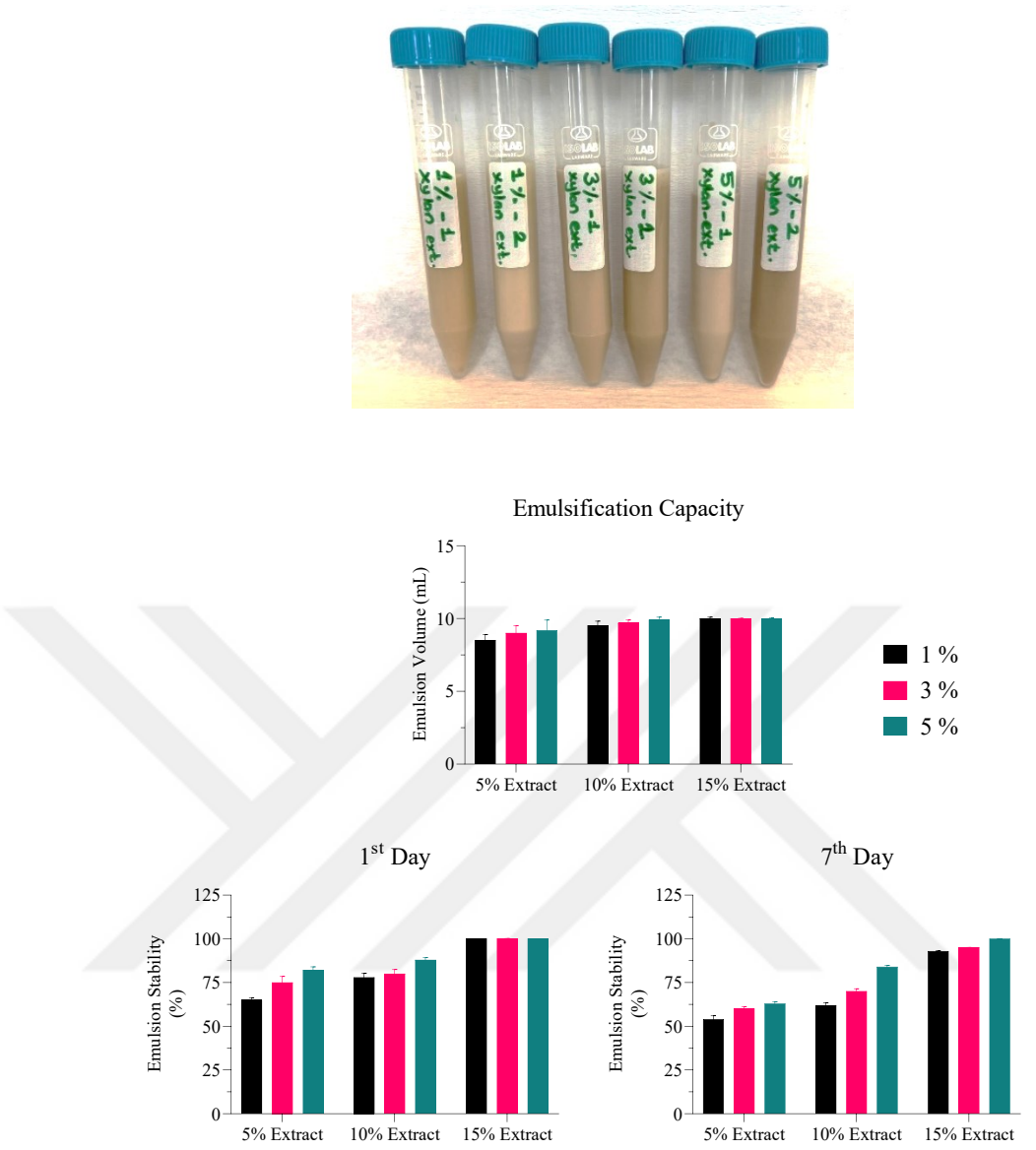

Figure 5. 3. A. Photographic images of XY extracts as emulsion formation, B. Emulsion capacity and stability of XY extracts.

On the first day, ES was expressed as a percentage of the emulsion volume retained relative to the initial emulsion volume. ES increased with both extract and solution concentrations and showed significant improvement from 5% to 15% extract concentrations. The highest ES was observed in the 15% extract at 5% solution, with values of 100%. Figure 5.3.B indicates that ES is positively influenced by higher XY extract concentrations, supporting 15% XY extracts for maximum stability.

ES, measured on the seventh day, revealed that higher XY extract concentrations provided sustained stability over time. Similar to the first day, the 15% extract concentration showed superior stability across all solution levels. At the 5% solution, the 15% extract maintained 100% stability, indicating no phase separation or breakdown. Lower extract concentrations (5%) showed reduced stability over time, especially in the 1% solution, with the lowest ES values across all extracts. Overall, the 15% extract concentration in the 5% solution consistently yielded the highest ES and ES across both time points, making it the optimal combination for applications requiring long-lasting and robust emulsions.

The increase in WHC and OHC with higher XY concentrations suggested that the structural modifications in raw CC induced by the alkali treatment enhance its hydrocolloid properties (Table 5.2). Hydrocolloids are known for their ability to retain water and oil due to their polysaccharide structures, which contain numerous hydrophilic groups (Pirsa and Hafezi 2023). Alkaline treatment can cause partial depolymerization and increase the exposure of hydroxyl groups, thereby enhancing the ability of XY to interact with water and oil molecules.

Table 5. 2. The water and oil holding capacity of XY extracts.

	WHC	**OHC**
RAW CC	1.38	1.48
XY Extract (5% alkali)	2.02	3.6
XY Extract (10% alkali)	2.48	6.48
XY Extract (15% alkali)	2.88	6.71

Studies on similar hydrocolloids indicated that structural changes in the polysaccharide backbone and the formation of a more porous network contribute to improved WHC and OHC (Xiaoning Li et al. 2024). Enhanced hydration and oil absorption make XY extracts promising for applications in food, cosmetics, and pharmaceuticals, where such properties are valuable for texture, moisture retention, and stability.

5.4.4. Antioxidant Activity

Oxidative stress-induced damage has greatly heightened interest in antioxidants (Pisoschi and Pop 2015). Among the commonly used methods for evaluating the antioxidant effects of nutrients is the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay (Fuso et al. 2023).

In this study, the antioxidant activities of XY extracts obtained from CC through alkali solutions (5%, 10%, and 15% alkali extracts) were evaluated using the DPPH radical scavenging assay. This method involves measuring the ability of the extracts to neutralize the stable DPPH radical, resulting in a decrease in absorbance at 517 nm, which reflects the scavenging efficiency of the tested compounds.

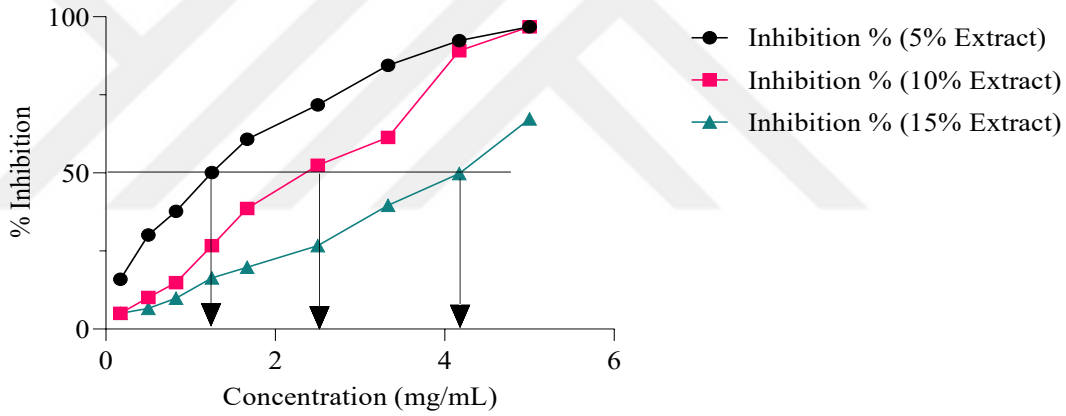

Figure 5. 4. Antioxidant activity of XY extracts

The XY extracts were tested at nine concentrations: 5.00, 4.17, 3.33, 2.50, 1.67, 1.25, 0.83, 0.50, and 0.17 mg/mL. The percentage inhibition of DPPH radical scavenging activity was calculated, and the Effective Concentration 50 (EC50) values were determined from Figure 5.4. The results demonstrated a concentration-dependent antioxidant activity across all XY extracts, with EC50 values of 1.25 mg/mL, 2.40 mg/mL, and 4.17 mg/mL for the 5%, 10%, and 15% alkali extracts, respectively. This indicates that as the concentration of the XY extracts increased, the antioxidant activity also increased, showing their potential as natural radical scavengers.

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APPENDICES

APPENDIX A

STANDARD CURVES USED IN THE ANALYSES

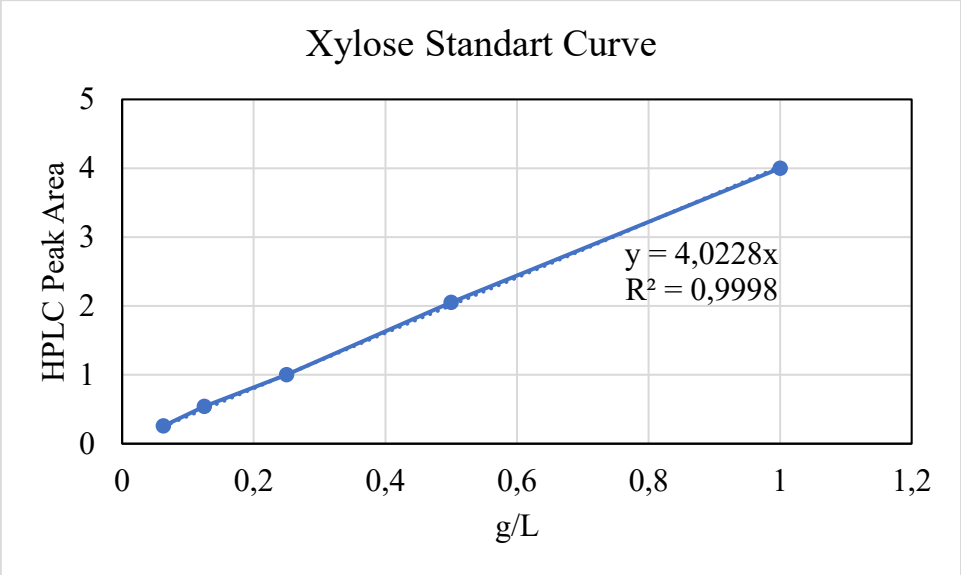

Figure A.1. Xylose standard curve.

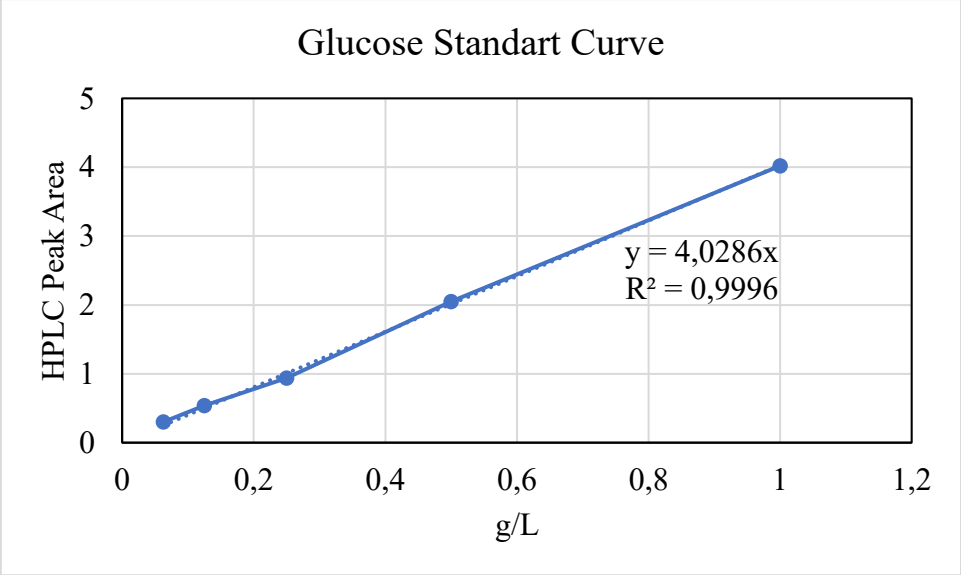

Figure A.2. Glucose standard curve.

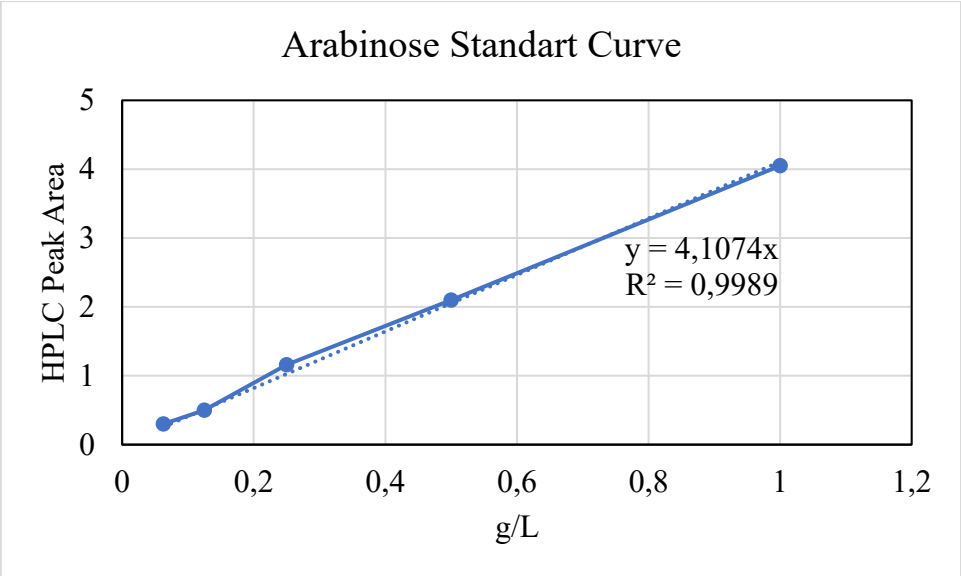

Figure A.3. Arabinose standard curve.

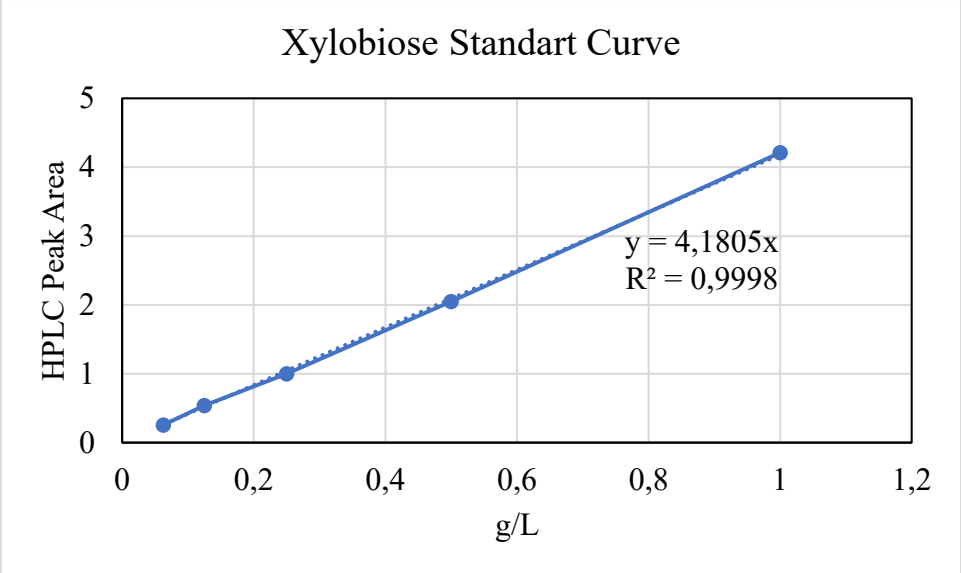

Figure A.4. Xylobiose (X2) standard curve.

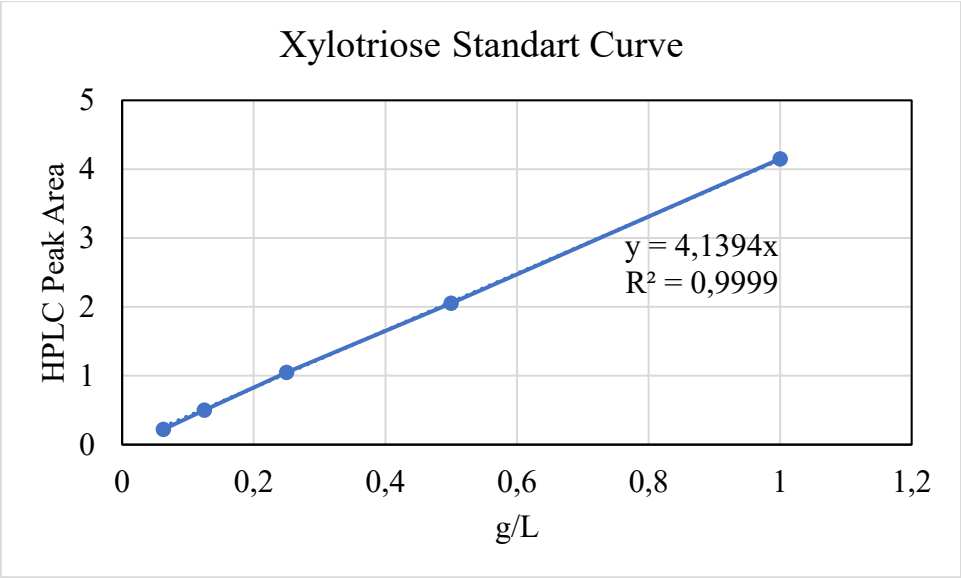

Figure A.5. Xylotriose (X3) standard curve.

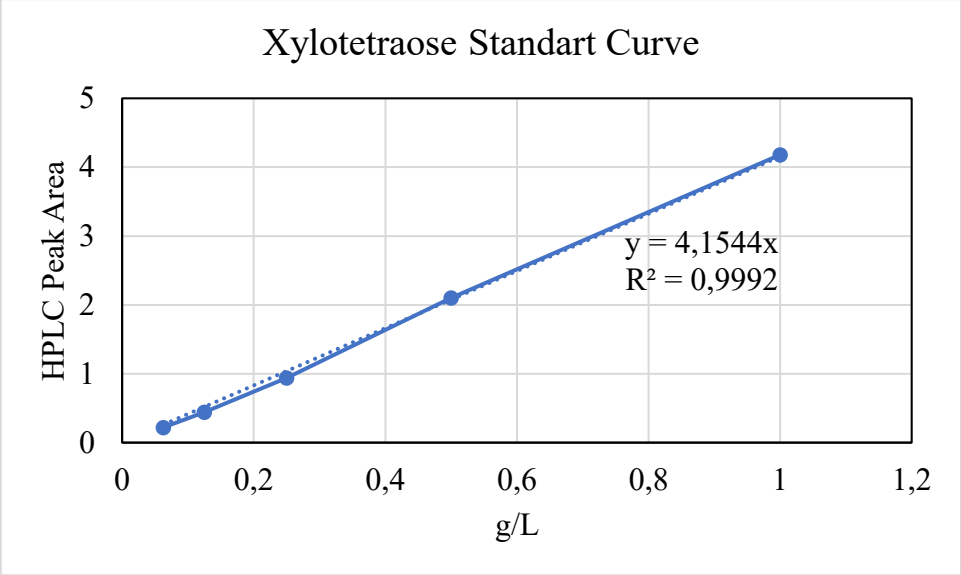

Figure A.6. Xylotetraose (X4) standard curve.

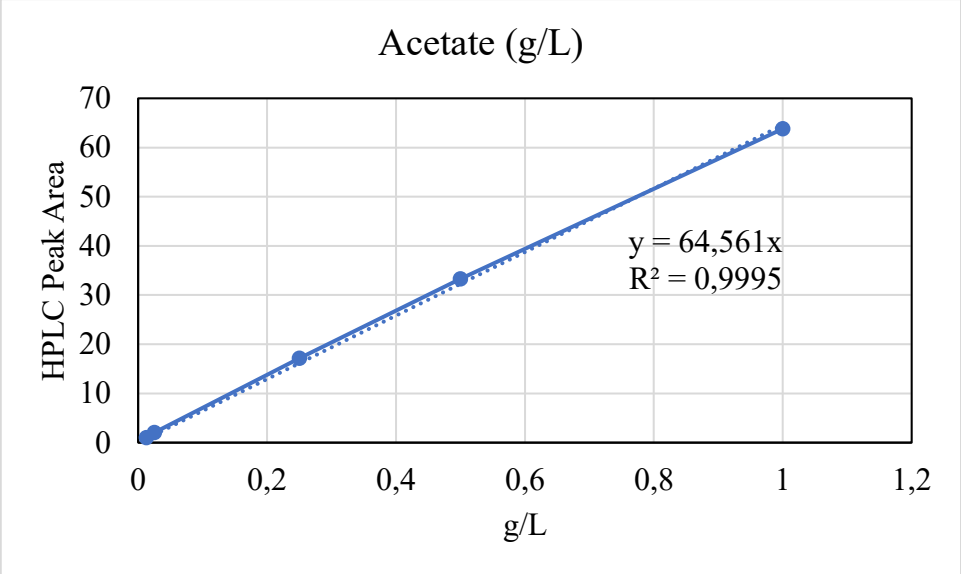

Figure A.7. Acetate standard curve.

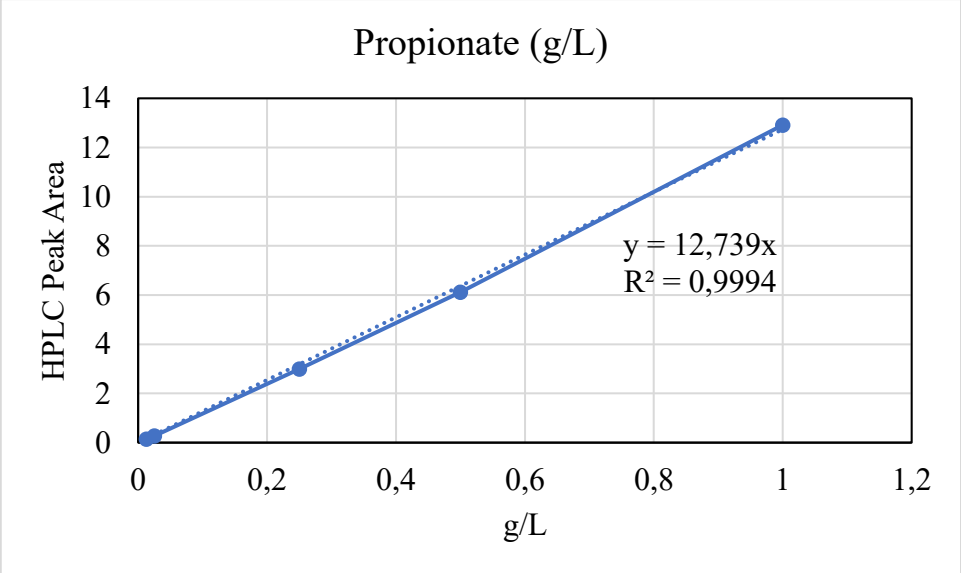

Figure A.8. Propionate standard curve.

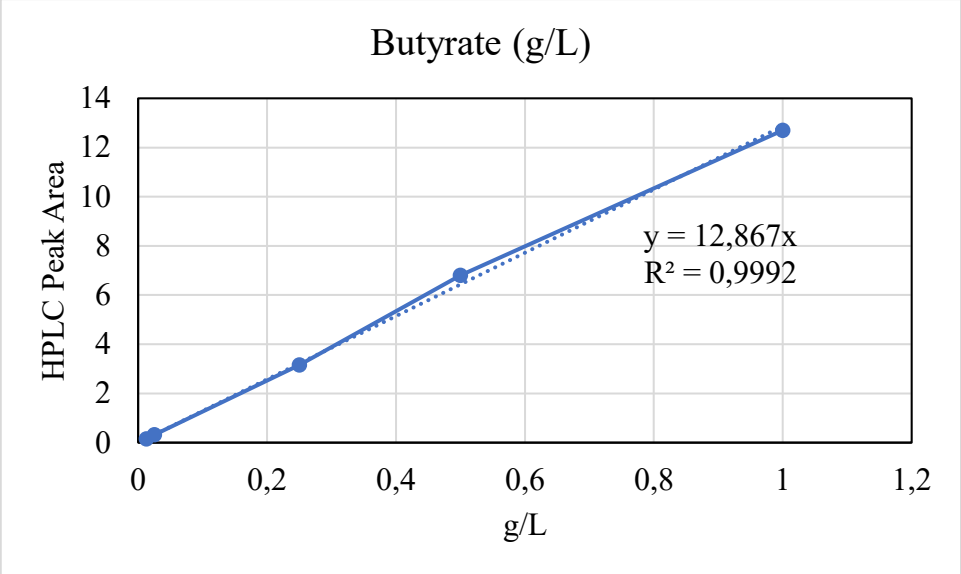

Figure A.9. Butyrate standard curve.

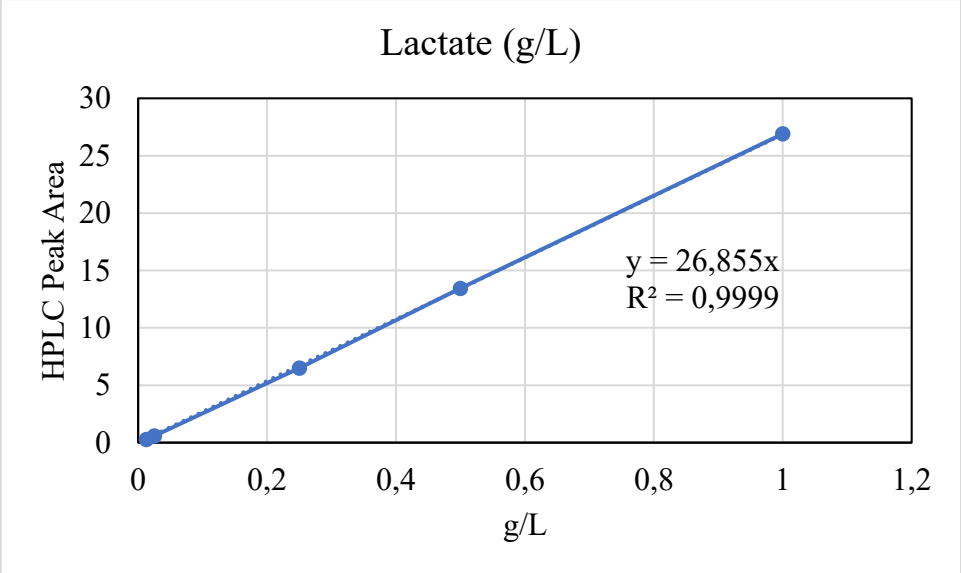

Figure A.10. Lactate standard curve.

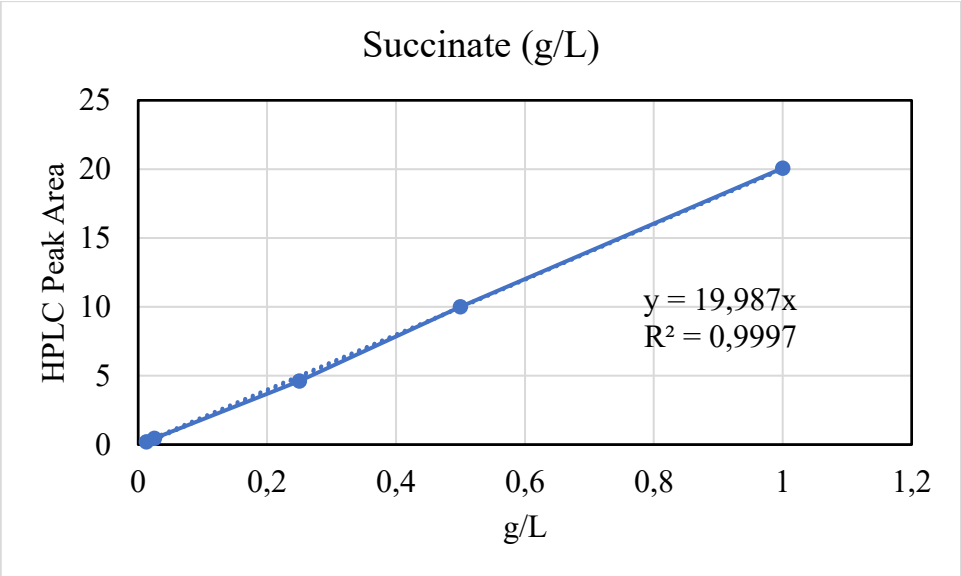

Figure A.11. Succinate standard curve.

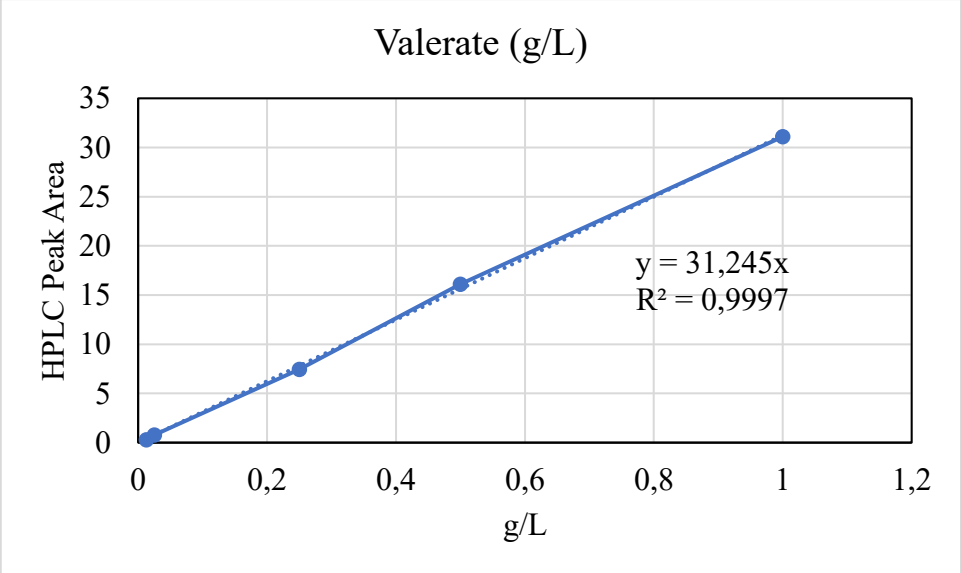

Figure A.12. Valerate standard curve.

APPENDIX B

HPLC CHROMATOGRAMS

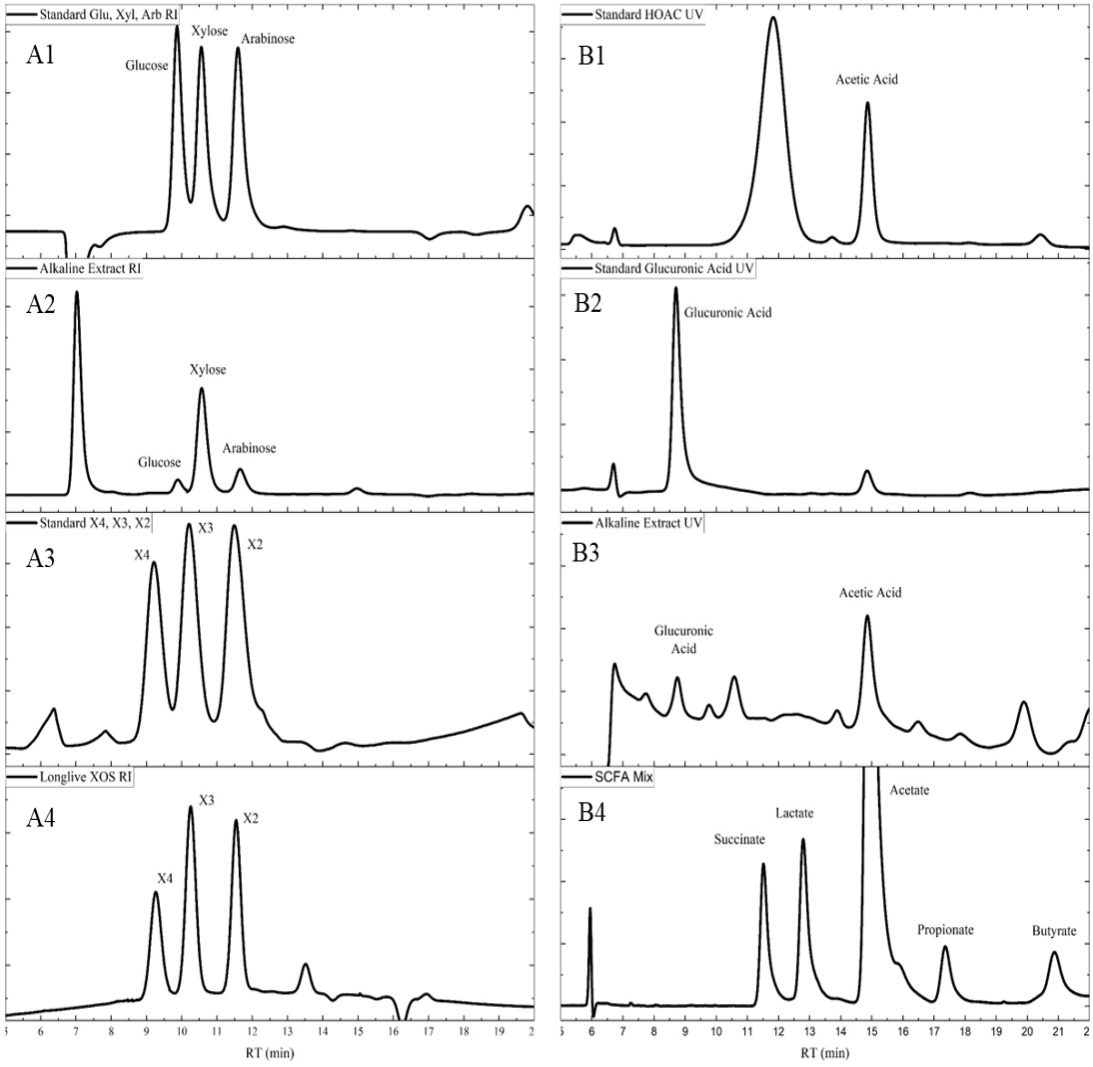

Figure B.1. HPLC chromatograms depicting multiple standards and extracts analyzed using refractive index and UV detectors. Panels A1 to A4 display chromatograms detected with a refractive index detector: (A1) glucose, xylose, and arabinose standards; (A2) alkali extract; (A3) XOS standards; and (A4) Longlive XOS. Panels B1 to B4 show chromatograms using a UV detector: (B1) acetic acid standard; (B2) glucuronic acid standard; (B3) alkali extract; and (B4) standards for succinate, lactate, acetate, propionate, and butyrate.

VITA

Kevser SABANCI

Education:

2017-2024: Ph.D. Department of Food Engineering, Izmir Institute of Technology, İzmir, Turkey
Thesis title: Investigations on The Prebiotic Activity of Xylan and Xylooligosaccharides Using In Vitro Mouse Fecal Culture and Ex Vivo Mouse Colon Model,
Supervisor: Prof. Dr. Ali Oğuz BÜYÜKKİLECİ

2013-2016: M.Sc. Department of Food Engineering, Izmir Institute of Technology, İzmir, Turkey
Thesis title: Ethanol Production from Hazelnut Industry Wastes,
Supervisor: Prof. Dr. Ali Oğuz BÜYÜKKİLECİ

2007-2013: B.Sc. Department of Food Engineering, Ege University, İzmir, Turkey.

Projects:

TUBITAK – 1002 (2023): Ex vivo mice colon use for prebiotic tests, **Kevser Sabancı,** Ali Oğuz Büyükkileci, Şükrü Güleç.

IZTECH – BAP (2023): Investigation of the effect of polysaccharides on cecal microbiota, **Kevser Sabancı,** Ali Oğuz Büyükkileci.

IZTECH – BAP (2021): Determination of *Bifidobacteria* distribution in mice colon, **Kevser Sabancı,** Ali Oğuz Büyükkileci.

Publications:

Surek, E., **Sabanci, K.,** & Buyukkileci, A. O. (2024). The potential of walnut shells for production of oligosaccharides by liquid hot water treatment. *Biomass Conversion and Biorefinery*, *14*(19): 24151-24163.

Sabanci, K., & Buyukkileci, A. O. (2018). "Comparison of liquid hot water, very dilute acid and alkali treatments for enhancing enzymatic digestibility of hazelnut tree pruning residues." *Bioresource Technology* 261: 158-165.