

Universidade do Minho
Escola de Ciências

Andreia Rafaela Sá Fernandes

Biological production and characterization of xylooligosaccharides obtained from agro-residues

outubro de 2023

Biological production and characterization of xylooligosaccharides obtained from agro-residues

Andreia Fernandes

UMinho | 2023

Universidade do Minho
Escola de Ciências

Andreia Rafaela Sá Fernandes

Biological production and characterization of xylooligosaccharides obtained from agro-residues

Dissertação de Mestrado
Mestrado em Bioquímica Aplicada

Trabalho desenvolvido sob a orientação da
Doutora Cláudia Amorim
e do
Doutor João Carlos Marcos

outubro de 2023

DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS

Este é um trabalho académico que pode ser utilizado por terceiros desde que respeitadas as regras e boas práticas internacionalmente aceites, no que concerne aos direitos de autor e direitos conexos.

Assim, o presente trabalho pode ser utilizado nos termos previstos na licença abaixo indicada.

Caso o utilizador necessite de permissão para poder fazer um uso do trabalho em condições não previstas no licenciamento indicado, deverá contactar o autor, através do RepositóriUM da Universidade do Minho.



Atribuição-NãoComercial-SemDerivações CC BY-NC-ND

https://creativecommons.org/licenses/by-nc-nd/4.0/

AGRADECIMENTOS

A dissertação de mestrado representa o culminar de um ciclo e, o início de uma fase nova e desafiante. Assim, não poderia terminar esta etapa sem um sincero agradecimento a todos os que de forma direta ou indireta marcaram positivamente o meu percurso pessoal e profissional.

Primeiramente, gostaria de agradecer aos meus orientadores Doutora Cláudia Amorim e Professor Doutor João Carlos Marcos pela oportunidade de desenvolver este tema. Em particular, agradeço à Doutora Cláudia por todos os ensinamentos, pelos conselhos partilhados aos mais diversos níveis, pela paciência e carinho, pela disponibilidade, motivação e rigor com que me brindou e, que foram essenciais no meu percurso. Gostaria de agradecer também à Professora Doutora Lígia Rodrigues pelo acompanhamento exímio, apoio e profissionalismo.

A todas as pessoas do laboratório que me receberam de forma excelente e me acompanharam durante este ano. Em especial à Equipa dos Pré-bióticos que me ajudaram e acolheram com todo o carinho. À Ana Machado, Carolina, Rita, Henrique, Mariana, Raquel e Ana Cordeiro que me acompanharam de perto, tendo vivido comigo todas as tristezas e alegrias. Obrigada pelo carinho, pela saídas, conversas e almoços, pelo apoio e gargalhadas que partilhamos. Agradeço também à Bruna Basto, que me ajudou e presenteou com a simpatia que lhe é característica.

Ana Cordeiro, mereces um parágrafo só teu, não existem palavras suficientes para te agradecer não só por todo o acompanhamento e auxílio a nível profissional, mas também pela boa amizade, paciência, cafés, conselhos e, conversas malucas. Obrigada por me teres enriquecido tanto neste último ano, permitindo-me crescer enquanto pessoa. Sem ti não teria sido o mesmo, obrigada!

À minha família, obrigada pelo apoio pois sem eles nada disto seria possível. Bia, a minha irmã e colega de quarto nestes últimos anos, obrigada pelas maluquices, pelas noites de karaoke no quarto acompanhadas de coreografias improvisadas, por me ouvires e apoiares em todos os momentos. Aos meus colegas de mestrado que me acompanharam durante este projeto, obrigada pelo companheirismo quer nos momentos de descontração, quer nos trabalho. Aos meus amigos Gonçalo, Isabel, Maria, Axel, Catarina, Rui, Simão, Carolina e Diogo obrigada por toda a dinâmica que deram à minha vida desde momentos épicos aos de perda de cognição, obrigada pelo carinho, paciência, ajuda, humor e aconselhamento durantes estes últimos 5 anos. À Inês que mesmo longe esteve sempre presente em todos os momentos, e me motivou à superação dos mais diversos obstáculos. Obrigada por tudo! Ao Lúcio Silva que causou uma primeira impressão digna de um Óscar ao cair de uma cadeira, mas que provou que as amizades mais aleatórias podem ser das que mais nos acrescentam, obrigada por toda a partilha durante este ano (e, obrigada pela cuvette). Terão sempre um lugar especial na minha vida.

STATEMENT OF INTEGRITY

I hereby declare having conducted this academic work with integrity. I confirm that I have not used plagiarism or any form of undue use of information or falsification of results along the process leading to its elaboration. I further declare that I have fully acknowledged the Code of Ethical Conduct of the University of Minho.

RESUMO: Produção biológica e caraterização de xilooligossacáridos obtidos através de agro-resíduos

Os pré-bióticos são compostos interessantes capazes de modular a microbiota intestinal, através da indução do crescimento ou da atividade de bactérias benéficas no trato gastrointestinal, enquanto as patogénicas são inibidas. Vários hidratos de carbono têm sido considerados pré-bióticos, incluindo os xilooligossacáridos (XOS). Estes são os únicos pré-bióticos que podem ser produzidos a partir de biomassa lignocelulósica. De facto, os XOS podem ser produzidos a partir de resíduos agrícolas, o que é encorajador para as indústrias de ingredientes alimentares, uma vez que estas matérias-primas são baratas, abundantes e renováveis por natureza. Em particular, a agroindústria do café gera milhões de toneladas de resíduos sólidos por ano em todo o mundo, nomeadamente a película de café (CSS).

Neste trabalho, foi avaliado o potencial de produção microbiana de XOS a partir de CSS e pellets de CSS (CP), utilizando fermentação direta e um *Bacillus subtilis* recombinante, que contém o gene da xilanase (*xyn2*) de *Trichoderma reesei* (*B. subtilis_xyn2).*

O CP apresentou o maior potencial para a produção de XOS através da fermentação direta com esterilização por autoclave. Após a otimização do processo, o maior rendimento em açúcares redutores (Y_{RS}) foi 63 ± 3 mg. g^{-1} às 8 h, 45 °C, pH 7.0 e 10 $g.L^{-1}$ de açúcares. XOS com o grau de polimerização (DP) superior a 6 foram obtidos.

Estudos *in vitro* com inóculos fecais humanos foram realizados para avaliar e comparar o potencial efeito pré-biótico da oligofrutose comercial (CO) e dos XOS produzido através da fermentação direta de CP. A suplementação com CO (10 $g.L^{-1}$) e mistura de XOS (10 $g.L^{-1}$) aumentou a produção de ácidos gordos de cadeia curta (SCFAs) comparativamente ao branco. A oligofrutose comercial levou à maior produção de lactato (28.0 ± 0.1 mM), acetato (28 ± 1 mM) e propionato (8.80 ± 0.08 mM), enquanto o XOS levou à produção máxima de n-butirato (5.20 ± 0.05 mM). A mistura de XOS também resultou na maior produção de CO_2 (5.20 ± 0.05 mM). O aumento considerável da produção de CO_2 e SCFAs, aliado à redução da concentração de amónio e do pH, indica que os XOS aqui obtidos têm potenciais propriedades funcionais para a saúde humana.

Os resultados obtidos provam que a fermentação direta é uma estratégia promissora e vantajosa para a produção de XOS através da CP. Este trabalho fornece informações importantes para novas abordagens de integração de bioprocessos utilizando agro-resíduos para reduzir os custos de produção e está indubitavelmente em consonância com o conceito de economia circular.

Palavras-chave: Xilooligossacáridos; Prebióticos; Xilanase; *B. subtilis_xyn2*; Fermentação direta

ABSTRACT: Biological production and characterization of xylooligosaccharides obtained from agro-residues

Prebiotics are interesting compounds able to modulate the gut microbiota by inducing the growth or activity of beneficial bacteria in the gastrointestinal tract, while the pathogenic ones are inhibited. Several carbohydrates have been considered prebiotics including xylooligosaccharides (XOS). XOS are the only prebiotics that can be produced from lignocellulosic biomass. Indeed, XOS can be produced from agro-residues, which is encouraging to the food ingredient industries, as these raw materials are inexpensive, abundant, and renewable in nature. In particular, the coffee agro-industry generates million tons of solid residues yearly worldwide, namely coffee silver skin (CSS).

In this work, the potential microbial production of XOS from CSS and CSS pellets (CP) was evaluated, using direct fermentation and a recombinant *Bacillus subtilis* harboring the xylanase gene (*xyn2*) from *Trichoderma reesei* (*B. subtilis_xyn2).*

CP presented the highest potential for XOS production by direct fermentation with autoclave sterilization. After process optimization, the highest reducing sugars yield (Y_{RS}) achieved was 63 ± 3 mg. g^{-1} at 8 h, 45 °C, pH 7.0 and 10 $g.L^{-1}$ of sugars. XOS with the degree of polymerization (DP) over 6 were obtained.

In vitro studies using human fecal inocula were performed to evaluate and compare the potential prebiotic effect of commercial oligofrutose (CO) and the XOS mixture produced through direct fermentation of CP. The supplementation with CO (10 $g.L^{-1}$) and XOS mixture (10 $g.L^{-1}$) increased the production of short-chain fatty acids when compared with the blank. Commercial oligofrutose led to the highest production of lactate (28.0 ± 0.1 mM), acetate (28 ± 1 mM) and propionate (8.80 ± 0.08 mM), while XOS led to the maximum production of n-butyrate (3.29 ± 0.04 mM). The XOS mixture also resulted in the highest production of CO_2 (5.20 ± 0.05 mM). The considerable increase in the production of CO_2 and SCFAs, allied to the reduction in ammonium concentration and pH, indicates that the XOS herein obtained have potential functional properties for human health.

The results obtained prove that direct fermentation is a promising and advantageous strategy for XOS production from CP. This work provides important information for new approaches to integrating bioprocesses using agro-residues to reduce production costs and it is undoubtably aligned with the concept of circular economy.

Keywords: Xylooligosaccharides; Prebiotics; Xylanase; *B. subtilis_xyn2*; Direct fermentation

SCIENTIFIC OUTPUTS

CONFERENCE ABSTRACTS AND POSTERS:

Andreia Fernandes, Ana Cordeiro, Lígia R. Rodrigues, Cláudia Amorim; "Valorization of coffee agro-industry residues for prebiotic production by one-pot fermentation"; Biotechnology for a circular bioeconomy, Merelbeke, Bélgica, 2023; Poster; Conferência Internacional.

TABLE OF CONTENTS

DIREITOS DE AUTOR E CONDIÇÕES DE UTILIZAÇÃO DO TRABALHO POR TERCEIROS ii
AGRADECIMENTOS iii
STATEMENT OF INTEGRITY iv
SCIENTIFIC OUTPUTS 7
LIST OF ABBREVIATIONS 10
LIST OF FIGURES 12
LIST OF TABLES 15
LIST OF SYMBOLS 16
1. INTRODUCTION 17
1.1. Prebiotics 18
1.2. Gut microbiota 20
1.2.1 Analysis and composition of human gut microbiota 20
1.2.2. Human gut microbiota through life stages 21
1.2.3. Modulation of human gut microbiota 22
1.2.4. Models for analyzing the prebiotic effect in human gut microbiota 25
1.3. Xylooligosaccharides 26
1.3.1. Lignocellulosic agro-residues as raw materials 27
1.4. Xylooligosaccharides production 28
1.5. Prebiotic effect of xylooligosaccharides 31
1.5.1. Xylooligosaccharides action on obesity 32
1.5.2. Effects of xylooligosaccharides on diabetes 33
2. EXPERIMENTAL 34
2.1. Materials 34
2.2. Chemical characterization of the residues 35
2.3. Microorganism and pre-inoculum 36
2.4. Direct fermentation conditions 36
2.5. Enzymatic hydrolysis 36
2.6. Optimization of the direct fermentation process 37
2.7. Sugar analysis 37
2.7.1. Reducing sugars 37
2.7.2. Thin-Layer Chromatography 37
2.7.3. High Performance Liquid Chromatography 38
2.8. Xylooligosaccharides production in bioreactor 38
2.9. Prebiotic effect 39
2.9.1. Faecal inoculum 39
2.9.2. Cultured *in vitro* fermentations of oligosaccharides using gut microbiota 39

2.9.3. Technical analysis 40
2.9.4. Evaluation of the microbiota 40
2.9.5. Statistical analysis 40
3. RESULTS AND DISCUSSION 41
3.1. Chemical characterization of the agro-residues 41
3.2. Direct fermentation for the agro-residue selection 42
3.2.1. Direct fermentation *versus* Enzymatic hydrolysis 44
3.3. Influence of the sterilization method 46
3.4. Optimization of the direct fermentation 48
3.4.1. Substrate concentration 48
3.4.2. pH 50
3.4.3. Temperature 51
3.4.4. Qualitative characterization of the XOS mixture produced under optimal conditions 53
3.5. Production of xylooligosaccharides at bioreactor scale 55
3.6. Prebiotic potential 57
3.6.1. Production of lactate and short-chain fatty acids 57
3.6.2. pH change and ammonium production 60
3.6.3. Gas production 61
4. CONCLUSION 62
5. FUTURE PERSPECTIVES 64
REFERENCES 66
APPENDIX 80
1. Direct fermentation for agro-residue selection 80
1.1. Direct fermentation versus enzymatic hydrolysis 81

LIST OF ABBREVIATIONS

abs	Absorbance
ADMO	Algal derived marine oligosaccharides
ASL	Acid-soluble lignin
B. ovatus	*Bacteroides ovatus*
B. subtilis_xyn2	Genetically modified *Bacillus subtilis* 3610 to express *xyn2* from *Trichoderma reesei*
BSG	Brewer's spent grains
C. difficile	*Clostridioides difficile*
CO	Commercial oligofrutose
CP	Coffee silver skin pellets
CP_A	Coffee silver skin pellets sterilized by autoclave
CP_UV	Coffee silver skin pellets sterilized by UV
CP_enz	Coffee silver skin pellets through enzymatic hydrolysis
CSS	Coffee silver skin
DF	Dilution factor
DM	*Diabetes mellitus*
DMT1	*Diabetes mellitus* type 1
DMT2	*Diabetes mellitus* type 2
DNA	Deoxyribonucleic acid
DNS	3,5-dinitrosalicylic acid method

DP	Degree of Polymerization
Escherichia coli	*E. coli*
FOS	Fructo-oligosaccharides
GC-MS	Gas Chromatography-Mass Spectrometry
GI	Gastrointestinal
GLP-1	Glucagon-like peptide-1
GMO	Genetically modified microorganism
GOS	Galactooligosaccharides
HMF	Hydroxymethylfurfural
HMO	Human milk oligosaccharides
HPLC	High-Performance Liquid Chromatography
IBD	Inflammatory Bowel Disease
IMO	Isomaltooligosaccharides
ISAPP	International Scientific Association for Probiotics and Prebiotics
MACs	Microbiota-accessible carbohydrates
MOS	Malto-oligosaccharides
MUC2	Secretory protein found in the human gut
NDOs	Non-digestible oligosaccharides
OD	Optical density
pAOS	Pectin-derived acidic oligosaccharides
PBS	Phosphate-buffered saline

RNA	Ribonucleic acid
rpm	Rotation *per* minute
rRNA	Ribosomal ribonucleic acid
RS	Reducing sugars
SCFA	Short Chain Fatty Acid
SD	Standard deviation
SF	Solid that resulted after filtration
SOS	Soybean oligosaccharides
TLC	Thin-Layer Chromatography
vvm	volume flow per unit of liquid volume per minute
XOS	Xylooligosaccharides
Xyl	Xylose
xyn2	Xylanase gene from *Trichoderma reesei*

LIST OF FIGURES

Figure 1- Positive effects (primary and secondary) of prebiotic intake (Taken from Swanson et al., 2020).

Figure 2- Human gut microbiota establishment and modification in the early stage of life (Taken from Turroni F. et al., 2022).

Figure 3- Relative abundance of key phyla of the human microbiota composition in different stages of life (Babies breast- and formula-fed, baby solid food, toddler antibiotic treatment, toddler healthy or malnourished, adult, elderly, and centenarian healthy and adult obese) (Taken from Ottman et al., 2012).

Figure 4- General structure of xylooligosaccharides (Taken from Almeida et al., 2021).

Figure 5- Composition of lignocellulosic agro-residues: Cellulose, hemicellulose, and lignin (Taken from Sánchez, 2009).

Figure 6- Sugar production yield (Y_{RS}) obtained from direct fermentation using 20 $g.L^{-1}$ of coffee silver skin (CSS) and coffee silver skin pellet (CP) by *Bacillus subtilis_xyn2*. The fermentation assays were performed at 45 °C and 150 rpm with CSS and CP in 2% (w/v) Vogel media pH 7.0. Results are the average of two independent fermentations and duplicate analysis of each sample ± standard deviation.

Figure 7- Thin-layer chromatography (TLC) of the supernatants obtained from the direct fermentation of coffee silver skin (CSS) and coffee silver skin pellet (CP) by *Bacillus subtilis_xyn2*. Samples from the initial and optimal time (0 and 8 h, respectively). A mixture containing 2 $g.L^{-1}$ of xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5) and xylohexaose (X6) was used as standard (S). A mixture of 1 $g.L^{-1}$ of glucose (Glu) was also used as standard. The fermentation assay was performed at 45 °C and 150 rpm with 20 $g.L^{-1}$ of CP in 2% (w/v) Vogel media pH 7.0.

Figure 8- Chromatograms obtained through the quantification of monosaccharides by HPLC for the samples of coffee silver skin (CSS) resulting from direct fermentation using *Bacillus subtilis_xyn2* (CSS at 20 $g.L^{-1}$, pH 7.0, 45 °C and 150 rpm). Green corresponds to the XOS standard at 1.5 g. L^{-1}, blue and red correspond to the CSS samples at 0 and 8 h, respectively.

Figure 9- Chromatograms obtained through the quantification of oligosaccharides by HPLC for the samples of coffee silver skin pellet (CP) resulting from direct fermentation using *Bacillus subtilis_xyn2* (CP at 20 $g.L^{-1}$, pH 7.0, 45 °C and 150 rpm). Green corresponds to the XOS standard at 1.5 g. L^{-1}, blue and red correspond to the CP samples at 0 and 8 h, respectively.

Figure 10- Reducing sugars production yield (Y_{RS}) obtained for *Bacillus subtilis_xyn2* using coffee silver skin pellet (CP) sterilized by autoclave at 20 $g.L^{-1}$, 45 °C, pH 7.0, 150 rpm, through enzymatic hydrolysis and direct fermentation (CP_enz and CP, respectively). Results are the average of two independent fermentations and duplicate analysis of each sample ± standard deviation.

Figure 11- Thin-layer chromatography (TLC) of the supernatants obtained from the enzymatic hydrolysis of coffee silver skin (CP) by *Bacillus subtilis_xyn2*. A mixture containing 2$g.L^{-1}$ of xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5) and xylohexaose (X6) was used as standard (S). The fermentation assay was performed at 45 °C and 150 rpm with CP in 2% (w/v) Vogel media pH 7.0 and sterilized by autoclave.

Figure 12- Sugar production yield (Y_{RS}) obtained from direct fermentation of coffee silver skin pellet (CP) by *Bacillus subtilis_xyn2* using different sterilization methods: autoclave (CP_A) and UV treatment (CP_UV). The control in the absence of microorganism was performed under the same conditions of autoclave sterilization (Control_A). The fermentation assay was performed at 45 °C and 150 rpm with 20 $g.L^{-1}$ of CP in 2% (w/v) Vogel media, pH 7.0. Results represent the average of Y_{RS} for the different assays ± SD.

Figure 13- Sugar production yield (Y_{RS}) obtained from enzymatic hydrolysis of coffee silver skin pellet (CP) by *Bacillus subtilis_xyn2* using different sterilization methods: autoclave (CP_enz_A) and UV treatment (CP_enz_UV). The fermentation assay was performed at 45 °C and 150 rpm with 20 $g.L^{-1}$ of CP in 2% (w/v) Vogel media, pH 7.0. Results represent the average of Y_{RS} for the different assays ± SD.

Figure 14- Reducing sugars production yield (Y_{RS}) obtained for *Bacillus subtilis_xyn 2* by direct fermentation using different coffee silver skin pellet (CP) concentrations (5, 10, 20, 40 e 60 $g.L^{-1}$) in 2% (w/v) Vogel media at pH 7.0, 45 °C and 150 rpm. Results represent the average of Y_{RS} of two independent assays ± standard deviation.

Figure 15- Reducing sugars production yield (Y_{RS}) obtained for *Bacillus subtilis_xyn2* through direct fermentation of coffee silver skin pellet (CP) at 10 $g.L^{-1}$ using different pH values (5.0, 6.0, 7.0 and 8.0). The fermentation assay was performed at 45 °C and 150 rpm with 10 $g.L^{-1}$ of CP in 2% (w/v) Vogel media. Results represent the average of Y_{RS} of two independent assays ± standard deviation.

Figure 16- Reducing sugars production yield (Y_{RS}) obtained for *Bacillus subtilis_xyn2* using different temperatures (30 °C, 37 °C, 45 °C and 50 °C) at a CP concentration of 10 $g.L^{-1}$, pH 7.0 and 150 rpm. Results represent the average of two independent assays ± standard deviation.

Figure 17- Thin layer chromatography (TLC) of the supernatants obtained from the direct fermentation of coffee silver skin pellet (CP) by *Bacillus subtilis_xyn2* in a concentration of 10 $g.L^{-1}$, pH 7.0, 45 °C. **A-** Mobile phase composed of butanol: acetic acid: water (2: 1: 1), sample from the time points 0 h, 8 h (optimal time) and 12 h, and a mixture containing 2 $g.L^{-1}$ of xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5) and xylohexaose (X6) was used as standard (S). **B-** Mobile phase composed of propanol: acetic acid: water (2: 1: 1), sample from the time points 0 h, 8 h (optimal time), and a mixture containing 1 $g.L^{-1}$ of xylose (X1) was used as standard (S).

Figure 18- Optical density at 600 nm profile obtained for direct fermentation, using *Bacillus subtilis_xyn2,* of coffee silver skin pellet (CP) at flask scale and 3.5 L bioreactor scale with (2 vvm) and 3.7 L bioreactor scale without aeration. The fermentations were run using 10 $g.L^{-1}$ of CP, pH 7.0 and 45 °C. Results represent the average of two independent assays ± standard deviation.

Figure 19- Reducing sugars production yield (Y_{RS}) obtained for direct fermentation of coffee silver skin pellet (CP) using *Bacillus subtilis_xyn2* at flask scale and 3.5 L bioreactor scale with (2 vvm) and 3.7 L bioreactor scale without aeration. The fermentations were run using 10 $g.L^{-1}$ of CP, pH 7.0 and 45 °C. Results represent the average of two independent assays ± standard deviation.

Figure 20- Quantification of the lactate and short chain fatty acids (SCFAs), namely acetate, propionate, butyrate and valerate after 48 h of fecal inocula growth from the mix of faecal human inocula in the absence of prebiotics (blank) or in a medium enriched with a prebiotic solution of oligofructose (CO) or XOS mixture at 10 $g.L^{-1}$. Results are the average of two independent fermentations ± standard deviation.

Figure 21- Production of H_2, CO_2 and CH_4 by human fecal inocula at 24 and 48 h in the absence of prebiotics (blank) or enriched with a prebiotic solution of Commercial oligofrutose (CO) or XOS mixture at 10 $g.L^{-1}$. Results are the average of two independent fermentations and duplicate analysis of each sample ± standard deviation.

LIST OF TABLES

Table 1- Conventional processes for xylooligosaccharides production.

Table 2- Chemical characterization of coffee silver skin (CSS), coffee silver skin pellet (CP) and brewers' spent grain (BSG), in dry weight (% (w/w)). Results in form of mean ± SD.

Table 3- Reducing sugars production yield (Y_{RS}) at the optimal time obtained for *Bacillus subtilis_xyn2* by direct fermentation using different coffee silver skin pellet (CP) concentrations (5, 10, 20, 40 e 60 $g.L^{-1}$) in 2% (w/v) Vogel media at pH 7.0, 45 °C and 150 rpm. Results represent the average of Y_{RS} of two independent assays ± standard deviation. Values followed with different letters have statistically significant differences ($p \leq 0.05$) according to one-way ANOVA.

Table 4- Reducing sugars production yield (Y_{RS}) obtained at the optimal time for *Bacillus subtilis_xyn2* through direct fermentation of coffee silver skin pellet (CP) at 10 $g.L^{-1}$ using different pH values (5.0, 6.0, 7.0 and 8.0). The fermentation assay was performed at 45 °C and 150 rpm with 10 g.L-1of CP in 2% (w/v) Vogel media. Results represent the average of YRS of two independent assays ± standard deviation. Values followed with different letters have statistically significant differences ($p \leq 0.05$) according to one-way ANOVA.

Table 5- Reducing sugars production yield (Y_{RS}) at the optimal time obtained for *Bacillus subtilis_xyn2* using different temperatures (30 °C, 37 °C, 45 °C and 50 °C) at a CP concentration of 10 $g.L^{-1}$, pH 7.0 and 150 rpm. Results represent the average of two independent assays ± standard deviation. Values followed with different letters have statistically significant differences ($p \leq 0.05$) according to one-way ANOVA.

Table 6- Concentration of free xylose ($g.L^{-1}$) obtained by direct fermentation of 10 $g.L^{-1}$ CP using *Bacillus subtilis_xyn2*, under the optimal conditions (pH 7.0, 45 °C and 150 rpm) throughout 8 h of fermentation (optimal time). Results represent the average of two independent fermentations ± standard deviation.

Table 7- Evaluation of the pH value after 0, 24 and 48 h of fecal inocula growth from the mixture of the four donors in the absence of prebiotics (Blank) or in a medium enriched with a commercial prebiotic solution of oligofrutose (CO) or XOS mixture (10 $g.L^{-1}$). Results are the average of two independent fermentations and duplicate analysis of each sample ± standard deviation. Values followed with different letters have statistically significant differences ($p \leq 0.05$) according to one-way ANOVA. Lowercase letters represent the analysis between different pH values over time, uppercase letters represent the analysis between different prebiotics tested at 48 h.

Table 8- Evaluation of the ammonium concentration ($g.L^{-1}$) after 48 h of fecal inocula growth from the mixture of the four donors in the absence of prebiotics (Blank) or in a medium enriched with a commercial prebiotic solution of oligofrutose (CO) or XOS mixture (10 $g.L^{-1}$). Results are the average of two independent fermentations and duplicate analysis of each sample ± standard deviation. Values followed with different letters have statistically significant differences ($p \leq 0.05$) according to one-way ANOVA.

LIST OF SYMBOLS

RS	total reducing sugars	mg
$\mathbf{Y_{RS}}$	sugar production yield	$mg.g^{-1}$
$\mathbf{Y_{RSinitial}}$	initial sugar production yield	$mg.g^{-1}$
$\mathbf{Y_{RSmax}}$	maximum sugar production yield	$mg.g^{-1}$
$\mathbf{Y_{RSmin}}$	minimum sugar production yield	$mg.g^{-1}$
$\mathbf{Y_{xyl}}$	free xylose yield	$mg.g^{-1}$

1. INTRODUCTION

The concern about the food products that we include in our diet has been growing. Consumers are increasingly seeking information regarding food quality, sustainability, and safety (Al-Swidi et al., 2014; Kushwah et al., 2019; Waqas et al., 2019). The search for a healthier diet has instigated the growth of the organic food industry exponentially (10% to 30%) (Rana et al., 2017).

In particular, consumers have shown a preference for natural bioactive compounds over modern therapeutic agents designed for health and well-being (Samanta et al., 2015; Forbes, 2018). Thus, due to their beneficial effects on human and animal health and their diverse physiological functions, including immune modulation, glucose homeostasis and selective growth of beneficial gut microbiota, prebiotics have been the focus of the current study of bioactive molecules (Samanta et al., 2015; Gibson et al., 2017).

As XOS have the health benefits described above, they are considered by International Scientific Association for Probiotics and Prebiotics (ISAPP) as a prebiotic oligosaccharide (Gibson et al., 2017), since, in 2017, Gibson et al., reported their resistance to gastric digestion and their ability to stimulate beneficial bacterial microbiota.

XOS have characteristics that increase their potential application at the industrial level and, consequently, at the economic level. These include their stability at a high range of temperatures (up to 100 °C) and pH (2.5 to 8.0) and sweetening potential (Finegold et al., 2014). In fact, the XOS market has an annual growth rate of approximately 4.1% and is expected to reach 130 million dollars by 2025 (Brenelli et al., 2022). The introduction of XOS as dietetic sweeteners to be incorporated into functional foods for diabetic patients is an example of how they can be applied in the market (Finegold et al., 2014).

Furthermore, XOS are composed by chains of xylose residues (whose precursor is xylan) and can be produced from lignocellulosic biomass (which are xylan-rich products), such as agro-residues (Gonçalves et al., 2022). Since lignocellulosic biomass represents the most abundantly available agro-residue in the world (Carrillo et al., 2018; Poletto et al., 2020), and is a resource highly renewable and recoverable (Debjani et al., 2021) the use of XOS for development of food products could contribute to the development of a more sustainable and efficient economy (Chapla et al., 2012). Additionally, the sustainable use of the xylan content present in XOS to produce value-added compounds provides

environmental benefits as it supports the management of billions of tonnes of residues produced per year (Kumar & Satyanarayana, 2015).

XOS can be produced from raw lignocellulosic agro-residue through conventional methods, physicochemical or/and enzymatic methods, (Carrillo et al., 2018; Kumar et al., 2015; Carvalho et al., 2013). Furthermore, XOS can also be produced by direct fermentation. The advantages and disadvantages of the different production methodologies will be discussed in section 4.3.

This project aims to produce XOS by direct fermentation of industrial agro-residues (CSS and CP) using *B. subtilis* containing xylanase gene *xyn2* from *Trichoderma reesei* and to evaluate their prebiotic potential.

1.1. Prebiotics

The increasing interest in prebiotics by the society in general instigated the need for a consensual definition of these compounds. Thus, ISAPP Consensus Statement most recent definition claims that a prebiotic is a "substrate that is selectively utilized by host microorganisms conferring a health benefit" (Swanson et al., 2020).

The main purpose of prebiotics is to stimulate the growth and activity of beneficial gut bacteria that have the ability to metabolise prebiotics to release fermentation by-products such as short-chain fatty acids (SCFAs). These influence the cellular integrity of the gastrointestinal (GI) tract, assisting in glucose homeostasis and modulating immune function (Koh et al., 2016). The mechanism of action of SCFAs allows a decrease of both phenolic and toxic nitrogen compounds (e.g., amines) and the activity of undesirable bacterial enzymes, hence inhibiting the growth of pathogenic bacteria and consequently occurs a reduction in intestinal and faecal pH (Slavin, 2013).

Recent studies reported that using prebiotics as dietary components alleviate symptoms of pathologies related to the immune system, cancer metastasis, type 2 diabetes, and obesity (Swanson et al., 2020). In **Figure 1**, the positive effects (primary and secondary) of prebiotic intake are schematized.

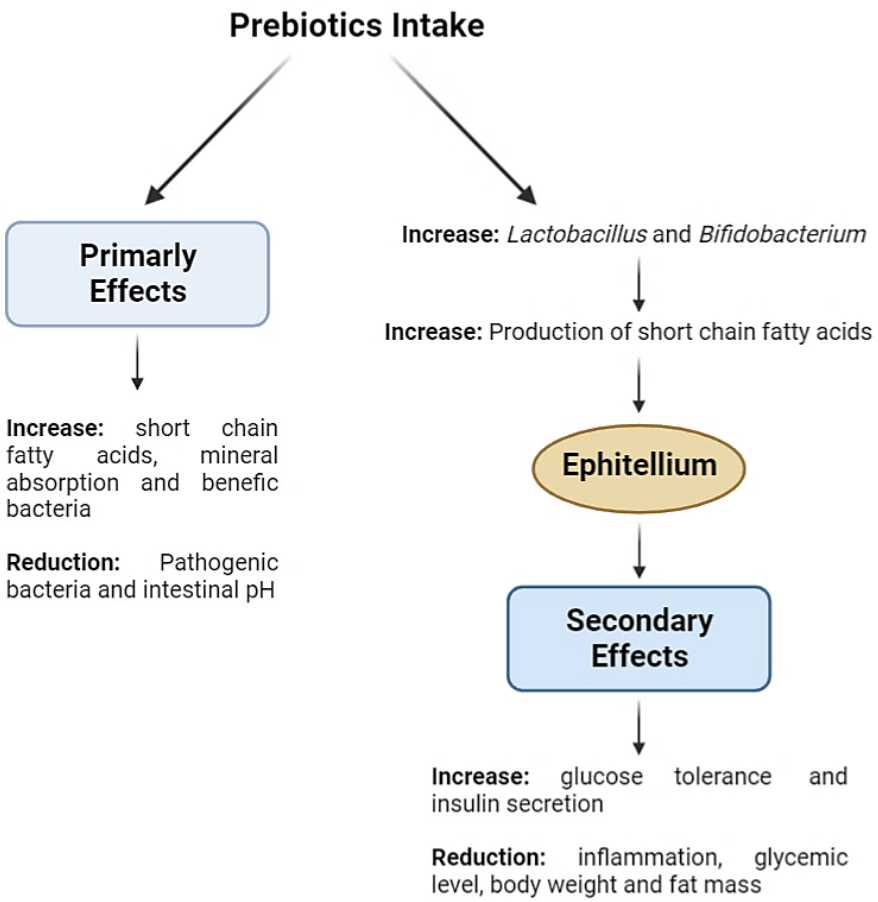

Figure 1- Positive effects (primary and secondary) of prebiotic intake (Taken from Swanson et al., 2020).

Some of the most widely studied prebiotics are non-digestible oligosaccharides (NDOs) can be distinguished on the basis of the type of monomeric sugars that constitute them, being classified as follows: a) fructo-oligosaccharides (FOS), derived from sucrose; b) malto-oligosaccharides (MOS), isomaltooligosaccharides (IMO), trehalose and cyclodextrins, a starch-related oligosaccharide; c) galactooligosaccharides (GOS) and lacto-sucrose, a lactose-related oligosaccharides e, d) others-oligosaccharides, including XOS, algal derived marine oligosaccharides (ADMO) such as carrageenan oligosaccharides (Zilong et al., 2022), pectin-derived acidic oligosaccharides (pAOS), human milk oligosaccharides (HMO), and soybean oligosaccharides (SOS) (Costa et al., 2012; Crittenden et al., 1996; Prapulla et al., 2000; Sangwan et al., 2011).

XOS are a remarkable potential as food ingredients when compared to other prebiotics (AIDP Inc., 2017), due to their price competitiveness, pH stability and heat (Courtin et al., 2009), organoleptic properties (Montané et al., 2006) and multidimensional effects on human health and livestock (Aachary et al., 2015).

1.2. Gut microbiota

The gut microbiota is colonised by a dynamic and complex population of microorganisms (Thursbyet al., 2017), whose composition differs between individuals. These can be categorised as pathogenic, neutral, or beneficial to the host. This microbial population is essentially anaerobic, and it is found in the large intestine (Collins et al., 2016), playing an essential role in human health as they interact with a range of immune and neural cells (Malard et al., 2020).

The physiological functions of the gut microbiota provide multiple benefits to the host such as epithelium formation or strengthening of gut integrity (Natividad et al., 2013), protection against pathogens (den Besten et al., 2013), regulation of host immunity (Bäumler et al., 2016) and energy harvesting (Gensollen et al., 2016). However, if the composition of the microbiota is altered there is a possibility that these mechanisms are destabilised which is referred to as dysbiosis.

As mentioned in subsection 1.1., prebiotics act selectively on beneficial bacteria, stimulating the growth of e.g. *lactobacilli* and *bifidobacteria* (Gibson et al., 2017). The following sections detail the composition and evolution of the human gut microbiota through life stages, the main factors contributing for its modulation, including prebiotic intake and, models for analysing the prebiotic effect in human gut microbiota.

1.2.1 Analysis and composition of human gut microbiota

The ability to explore the extent of the gut microbiota has been considerably developed due to the emergence of culture-independent approaches (e.g., low cost and high-throughput sequencing methods). Since the 16S ribosomal RNA (16S rRNA) gene is common to all *bacteria* and archaea and contains nine regions with high variability (V1-V9), its sequencing is the most widely used procedure (Poretsky et al., 2014; Mizrahi-Man et al., 2013) as it facilitates the distinction of different species.

In contrast to previous techniques (which focused on sequencing the entire 16S rRNA gene), 16S rRNA gene sequencing is currently focused on analysing shorter sub-regions of the gene in greater depth (Mizrahi-Man et al., 2013). The use of shorter read lengths may lead to errors (Poretsky et al., 2014). Note that the whole genome shotgun metagenomics method provides more reliable analyses of the diversity and constitution of the microbiota due to the high sensitivity of the technique resulting from its higher resolution (Poretsky et al., 2014).

The cross-linking of data resulting from these techniques identified species isolated in humans, most of which (about 94 %) belong to the phyla *Proteobacteria*, *Firmicutes*, *Actinobacteria* and *Bacteroidetes*. These are strictly anaerobic and are found in mucosal regions such as the GI tract and oral cavity (Hugon et al., 2015). Since the gut microbiota is poor in species diversity, it is recognised as having a high degree of functional redundancy when compared to microbial communities present in other body locations (Li et al., 2014; Schluter et al., 2012; Pérez-Cobas et al., 2013; Moya et al., 2016)

Through the analysis of a study conducted in different countries, it was also possible to conclude that the gut microbiota is moulded by environmental factors such as diet and possibly by host genetics as country-specific microbial patterns were identified (Li et al., 2014).

1.2.2. Human gut microbiota through life stages

After delivery, the GI tract is colonised, through events such as illness, antibiotic treatments and dietary changes that cause significant alterations in the gut microbiota (Rodriguez et al., 2015; Koenig et al., 2011). Notably, the mode of delivery also influences the microbial composition of infants. Thus, babies born by vaginal delivery have a high abundance of *lactobacilli* during the first days of life (reflecting the high amount of them in the vaginal flora (Avershina et al., 2014; Aagaard et al., 2012). Moreover, the microbiota of babies born by caesarean section is mainly composed of facultative anaerobic species, such as *Clostridium*. The colonisation of the genus *Bacteroides* is delayed and almost non-existent (Jakobsson et al., 2014).

Regarding faecal microbiota, when comparing infants born by the vaginal delivery and by caesarean section, it is found that the similarity of the faecal microbiota of infants to their mothers is about 1.8% higher in infants born by the vaginal delivery (Bäckhed et al., 2015). In early development, the gut microbiota is poor, being dominated mainly by the phyla *Actinobacteria* and *Proteobacteria* (Rodriguez et al., 2015; Bäckhed, 2011). By 2 years of age, the infant gut microbiota resembles the adult microbiota due to changes in composition, diversity, and functional capabilities (Rodriguez et al., 2015; Koenig et al., 2011).

Thus, due to the role of SCFAs acting as immune and metabolic mediators, it has been proposed that their decrease may contribute to the inflammatory ageing process in the gut of older people (Biagi et al., 2013). Accordingly, it is possible to state that the intestinal microbiota is gradually moulded during a human being's lifetime by external factors, such as the mode of delivery, which influence the composition

and diversity of the intestinal microbiota and the production of SCFAs. Consequently, influences the subject's health status in childhood as well as in the future (Turroni et al., 2022). In **Figure 2**, is represented the human gut microbiota establishment and modification in the early stage of life.

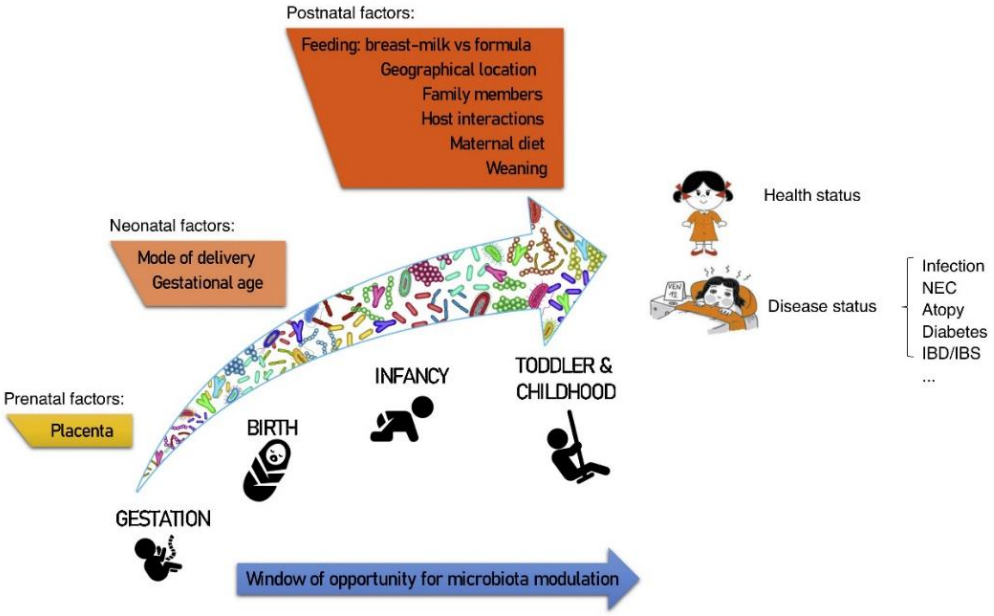

Figure 2- Human gut microbiota establishment and modification in the early stage of life (Taken from Turroni F. et al., 2022).

1.2.3. Modulation of human gut microbiota

The gut microbiota can be modulated by various factors, such as diet (Donaldson et al., 2015), the intestinal mucus (Arike et al., 2016) and its glycosylation (Arpaia et al., 2013; Furusawa et al., 2013; Zarepour et al., 2013), availability of microbiota-accessible carbohydrates (MACs), bile acids (Ridlon et al., 2014; Staley et al., 2017) and prebiotics (Thursbyet al., 2017). Some of these factors, specifically diet, MACs and mucus, establish symbiotic interactions aiming to maintain the balance of the gut microbiota.

Recent studies suggest that extreme diets based on either animal or plant sources cause substantial changes in the human gut microbiota (David et al., 2013). Consequently, feeding methods can also impact the abundance of certain bacterial groups in the gut microbiota of infants. For instance, human milk contains fucosylated oligosaccharides that benefit specific bacterial species, such as *Bifidobacterium longum* and various *Bacteroides* species, enabling effective competition against pathogenic bacteria like *Escherichia coli* (*E. coli*) and *Clostridium perfringens* (Yu et al., 2013; Marcobal et al., 2011).

Regarding the intestinal mucus, it plays a crucial role as a source of carbohydrates for the gut microbiota (Arike et al., 2016). Intestinal mucus contains mucin (MUC2), a highly glycosylated protein, secreted by goblet cells (Ouwerkerk et al., 2013), forming layers of mucus with the outer layer's arrangement offering a unique niche for distinct bacterial populations with different proliferation and resource utilization patterns (Li et al., 2015). The glycosylation of mucus and MUC2 is essential for shaping the gut microbiota and selecting bacterial species more beneficial for the host's health (Arpaia et al., 2013; Furusawa et al., 2013; Zarepour et al., 2013). It is worth noting that carbohydrate degradation products such as lactate and succinate are not accumulated in high concentrations within the human colon of healthy adults, as they serve as substrate for other bacteria, including SCFAs-producing bacteria (namely propionate and butyrate) (Louis et al., 2016). Then, the degradation process can occur in the extracellular environment (e.g., inulin digestion by *Bacteroides ovatus* (*B. ovatus*)) (Rakoff-Nahoum et al., 2016), and this is evident in the external layer of the intestinal mucus, where mucin-degrading bacteria provide mono- or oligosaccharides to other bacteria that lack specialised mucolytic capacity (establishing symbiotic interactions) (Li et al., 2015). On the other hand, many pathogens such as *Salmonella Typhimurium* or *Clostridium difficile* do not possess sialidase (glycosidic bond cleaving enzyme) depending on other organisms present in the gut microbiota to provide carbon sources (Juge et al., 2016). *Ruminococcus gnavus* strains possess the intramolecular trans-sialylation enzyme, which may act in the adaptation of intestinal commensal bacteria to the intestinal mucosal environment, especially in inflammatory bowel diseases (IBD) that are rich in short, sialylated glycan mucins (Crost et al., 2013; Crost et al., 2016); Larsson et al., 2011).

Since, as mentioned above, mucus is the main source of carbohydrates for the intestinal microbiota, it will consequently influence the availability of MACs. It was reported that a diet low in MACs results in a decrease in SCFA production, which plays an essential role in the host's health, for example, through anti-inflammatory mechanisms (Sonnenburg et al., 2014). This type of diet may also lead to a decrease in the thickness of the mucus in the distal colon, allowing closer proximity between microbes and the epithelium, leading to the expression of inflammatory markers (Earle et al., 2015). Recent studies with gnotobiotic mice have shown that certain microbial species can be used to restore growth deficiencies caused by the microbiota of malnourished children, opening therapeutic possibilities to combat the effects of malnutrition (Blanton et al., 2016; Charbonneau et al., 2016; Schwarzer et al., 2016).

Moreover, the complex interaction between bile acids and the gut microbiota is critical for maintaining host health and the balance of the GI system. They act on the dynamics of the bacterial community in the large and small intestines (Ridlon et al., 2014; Staley et al., 2017). Primary bile acids (such as taurocholic acid) have the ability to facilitate the recovery of the microbiota after antibiotic- or toxin-induced dysbiosis by signalling feedback to gut bacteria (Browne et al., 2016). Additionally, the reduced concentration of these acids in the gut enables the expansion of pro-inflammatory microbial taxa, highlighting their significance in regulating inflammation and modulating the composition of the GI microbiota (Kakiyama et al., 2013). Recent studies underline the role of bile acids in the formation of the gut microbiota, influencing the diversity and interactions between different bacterial species.

Lastly, as mentioned in section 1.1., the main purpose of any prebiotic is to stimulate the growth and activity of beneficial gut bacteria, which metabolise them to release fermentation by-products such as SCFAs (for example butyrate, acetate and propionate). SCFA's mechanism of action results in a reduction of toxic nitrogen compounds (e.g. amines) and the activity of undesirable bacterial enzymes, effectively inhibiting the growth of pathogenic bacteria (Slavin, 2013). It has been reported that the inclusion of prebiotics in the diet mitigates the symptoms of pathologies related to the immune system, ageing and type 2 diabetes (Swanson et al., 2020).

Thus, understanding the interaction between the gut microbiota and the various factors that influence it may lead to therapeutic approaches targeting reduction of the negative effects on human health (Kakiyama et al., 2013) caused either by dietary deficiencies (Blanton et al., 2016; Charbonneau et al., 2016; Schwarzer et al., 2016), or at the immunological diseases level. In **Figure 3**, an overview of the relative abundance of key phyla of the human microbiota composition in different stages of life. Measured by either 16S RNA or metagenomic approaches (Deoxyribonucleic acid, DNA). Data from: Babies breast- and formula-fed (Schwartz et al., 2012), baby solid food (Koenig et al., 2011), toddler antibiotic treatment (Koenig et al., 2011), toddler healthy or malnourished (Monira et al., 2011), adult, elderly, and centenarian healthy (Biagi et al., 2010), and adult obese (Zhang et al., 2009).

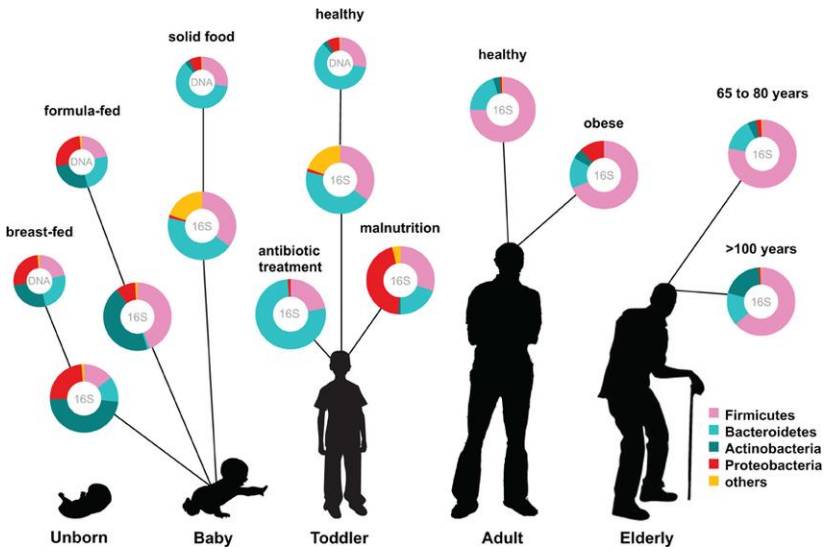

Figure 3- Relative abundance of key phyla of the human microbiota composition in different stages of life (Babies breast- and formula-fed, baby solid food, toddler antibiotic treatment, toddler healthy or malnourished, adult, elderly, and centenarian healthy and adult obese) (Taken from Ottman et al., 2012).

1.2.4. Models for analyzing the prebiotic effect in human gut microbiota

The prebiotic effect can be assessed using *in vitro* or *in vivo* models. *In vivo* models, despite being considered standard models, have disadvantages such as high time and resource consumption and the high degree of variation in microbiota composition between different individuals. Consequently, a large number of individuals is required to address this inter-individual variability in microbiota composition. In addition, the effect of modulation approaches is mainly assessed based on the faecal microbiome (end of the colonic fermentation process) preventing the study of the preceding growth dynamics (Falony et al., 2018).

In this way, *in vitro* models emerge as interesting alternatives that overcome the disadvantages of the previous models. Thus, *in vitro* fermentations have been explored over the years (Venema and van den Abbeele, 2013; Vrancken et al., 2019). These are semi-representative models whose primary aim is to accurately replicate *in vivo* models (fermentation and colonic ecology) using pure cultures of selected bacteria (Falony et al., 2019).

The complexity of *in vitro* models is assessed according to their productivity. However, these models have limitations in the optimal control of the pH process (as SCFAs formed during the fermentation process are not absorbed by these models) and metabolite accumulation (Poppe et al.,

2023). To overcome the pH control barrier and make the assessment of prebiotics more reliable, the pH should be controlled during fermentation to ensure that it closely resembles the pH of the distal colon. Regarding SCFAs, they are easily absorbed by *in vivo* models making their quantification difficult (Carlson et al., 2017).

Thus, *in vitro* models have proven to be the most suitable for studying fermentation kinetics in the colon, with the added benefit of being ethically unbiased (Venema & van den Abbeele, 2013).

1.3. Xylooligosaccharides

XOS, whose general chemical structure is represented in **Figure 4**, are oligomers composed by chains of xylose residues, linked through β (1→4)-xylosidic bonds, with a range of DP from 2 to 10 (Ávila et al., 2020). According to the DP, they can be mainly classified based on their complexity as xylobiose (2 monomers), xylotriose (3 monomers), xylotetrose (4 monomers), xylopentose (5 monomers), xylohexose (6 nomomers) and so on. However, the main chain of xylose can be branched with several side substituents, namely acetyl groups, arabinose, glucuronic acids, and galactose residues (Coelho et al., 2014).

These structural characteristics allow XOS to exhibit prebiotic effects in lower amounts, when compared to other compounds. As a result, the industry is actively focused on developing approaches to improve the effectiveness of these processes and to meet the demand for these compounds. Recent advances have been made towards greener and more efficient processes, by applying process integration strategies to produce XOS from costless lignocellulosic agro-residues and using genetic engineering to create microorganisms that convert these residues to XOS (Amorim et al., 2019).

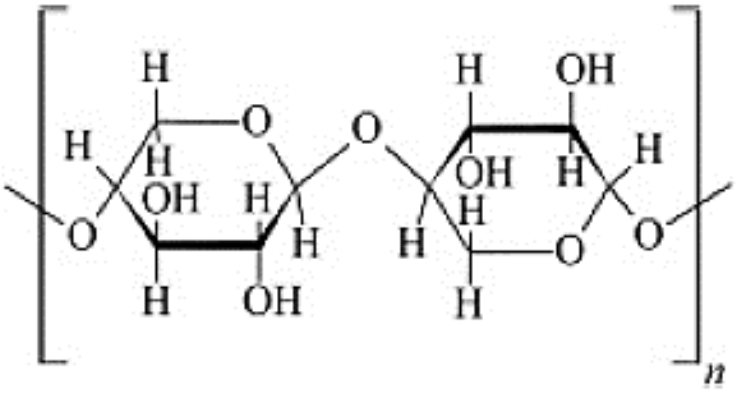

Figure 4- General structure of xylooligosaccharides (Taken from Almeida et al., 2021).

1.3.1. Lignocellulosic agro-residues as raw materials

Lignocellulosic biomass, such as agro-residues, are xylan-rich products that can be used for XOS production (Gonçalves et al., 2022). Lignocellulosic agro-residues are mainly composed by cellulose, hemicellulose and lignin: cellulose is composed by a chain of glucose molecules with hydrogen bonds between different layers of the polysaccharides forming a crystalline conformation; hemicellulose mainly constituted by xylan is the key target for XOS production; and lignin confers structure to the plant, being composed by three major phenolic components, namely p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol (Dutta et al., 2014). In **Figure 5** it is schematized the composition of lignocellulosic agro-residues.

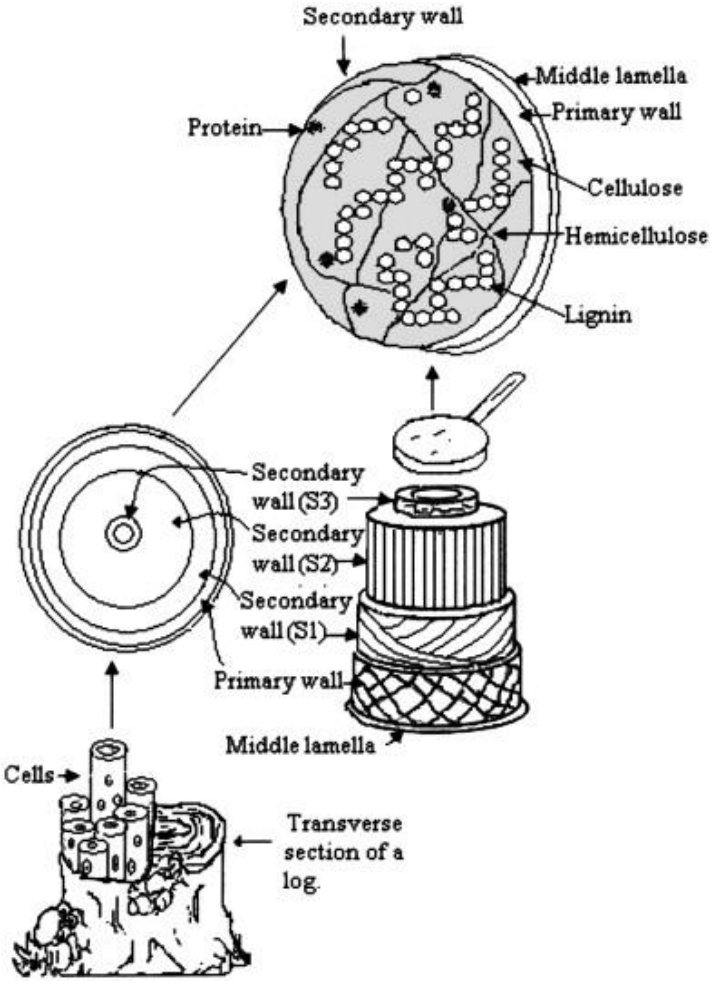

Figure 5- Composition of lignocellulosic agro-residues: Cellulose, hemicellulose, and lignin (Taken from Sánchez, 2009).

The interest in the exploitation of agro-residues has increased substantially, as they are the most abundant renewable resource on earth (Chapla et al., 2012; Kumar et al., 2015). The use of agro-residues that do not compete for food supply is an economically advantageous strategy to produce value-added compounds (Somerville et al., 2010). These may reduce the cost of a microbial fermentation process, leading to a reduction of the production medium costs, which account for most of the upstream cost of a fermentation process (Lotfy, 2007).

In addition, the use of agro-residues also entails environmental benefits, since it helps the management of billions of tons of agro-residue generated annually (Kumar et al., 2015), thus representing an asset for all authorities involved (Samanta et al., 2015). However, the use of agro-residues also presents meaningful challenges, such as variable composition, seasonality, and spoilage risk during storage (Batidzirai et al., 2016).

Additionally, depending on the chemical composition, the suitability of the residues may differ for XOS production. Residues with higher amounts of xylan and low amounts of lignin are better options. Thus, we selected coffee silver skin (CSS) for this project since it is a xylan-rich agro-residue that is produced in high quantities by the coffee industry and when compared with other compounds exhibits prebiotic effects at lower amounts, making them competitively priced prebiotics (Amorim et al., 2019), and CSS pellet (CP), which is a concentrated form of CSS.

1.4. Xylooligosaccharides production

Regarding XOS production methods, they can be obtained from raw lignocellulosic agro-residues through conventional processes, which are the most widely used (Kumar et al., 2015; Carvalho et al., 2013). These can involve one step (physicochemical technique: autohydrolysis and chemical hydrolysis) or two steps (enzymatic and the combination of physicochemical and enzymatic methods (Carrillo et al., 2018). On the other hand, XOS can also be produced by an innovative one-step methodology, namely direct fermentation. **Table 1** lists some advantages and disadvantages of each of these processes.

Table 1- Conventional processes for xylooligosaccharides production

Process		Advantages	Disadvantages
Physicochemical	**Auto hydrolytic**	Generate XOS with a wide range of polymerization (DP > 6) (Kaprelyants et al., 2017; Surek et al., 2017) Generally, one step techniques (Carrillo et al., 2018)	Contamination by undesired by products, including toxic compounds such as furfural and hydroxymethylfurfural (HMF). High downstream costs (Bian et al., 2014). Requires the use of noxious chemicals and consequently specific and more robust equipment.
	Chemical		
Enzymatic hydrolysis		Generate mostly low DP XOS (from DP 2 to 6) (Carvalho et al., 2015; Kaprelyants et al., 2017; Sukri et al., 2018), which present higher sweetening power. More environment-friendly, operating at milder conditions of pressure and temperature without the use of noxious chemicals (Amorim et al., 2019). Xylanolytic enzymes presents high efficiency and specificity, allowing a higher control over DP and a reduced production of undesired xylose and other by-products is obtained (Akpinar et al., 2007). Reproducibility and high yield when using extracted xylan (Charalampopoulos et al., 2012)	The reaction time required is typically longer (Akpinar et al., 2010) The cost of enzyme production, purchase or recovery and recycling (Akpinar et al., 2010) Previous extraction of xylan required in order to have a high yield (Carvalho et al., 2013)

	Does not require special equipment, high temperatures and pressure to operate, as well as produce any undesirable by-products in contrast to autohydrolysis (Akpinar et al., 2009; Samanta et al., 2015)	
Direct Fermentation	Innovative due to not having to buy/produce enzymes, reducing process costs (Amorim et al., 2018) Allows to minimize the amount of free xylose, simplifying the downstream process (Urmann et al., 2010). Therefore, is faster process (Amorim et al., 2018)	Few reports focus on this strategy (Amorim et al., 2018)

Since xylan is mostly present as a xylan-lignin matrix, XOS are generally produced by a combination of methods involving a two-step approach (Carvalho et al., 2013). The first step includes pretreatment of the lignocellulosic biomass to remove lignin and cellulose, increasing the concentration of xylan, or the xylan extraction itself by solubilization, which may be further purified by precipitation. For that purpose, the most common methods are auto-hydrolytic, chemical, or enzymatic. Particularly, pretreatments with alkaline solutions are largely used and considered one of the most efficient in terms of xylan recovery without degradation. The second step consists in the xylan hydrolysis by xylanolytic enzymes (Chapla et al., 2012; Rico et al., 2018), such as endo-1,4-β-xylanases, and endo-1,3-β- xylanases.

The economic viability of XOS production from lignocellulosic agro-residues may be compromised by the low yields associated to the pretreatment step and by the cost of producing or purchasing commercially available xylanases (Reddy et al., 2016a). Hence, recent advances towards more efficient processes have been reported, for instance the use of raw lignocellulosic agro-residues without pretreatment and integration process strategies.

As a way of countering the obstacles imposed by the processes described above, XOS can also be produced by a promising technique: direct fermentation (Amorim et al., 2019). This technique is innovative due to not having to buy enzymes and being simpler and cheaper (and therefore faster), when

compared with the conventional techniques. However, few reports focus on this approach (Amorim et al., 2018). It is anticipated that synthetic biology tools will be increasingly used to create super-microorganisms that will function as integrative cellular (bio)factories capable of directly converting raw lignocellulosic biomass into XOS. Moreover, the use of genetically modified microorganisms (GMOS) may be hampered by regulatory issues, especially in Europe (Amorim et al., 2018).

XOS produced by direct fermentation of xylan suggest that these oligosaccharides have potential to be used as a prebiotic treatment for IBD, since they appear to be highly selective towards butyrate-producing bacteria (Wang et al., 2014). IBD active patients present increased proportions of *Bifidobacterium* and *Lactobacillus* on their intestinal microbiota and reduced butyrate-producing bacteria (Wang et al., 2014).

1.5. Prebiotic effect of xylooligosaccharides

Prebiotic oligosaccharides must be resistant to digestion in the upper gastrointestinal tract, should be able to reach the large intestine intact, in order to modulate the intestinal microbiota (Gibson et al., 2017). The stability of the XOS produced for the digestive system (as the gastric acidity and digestive enzymes) was tested under an *in vitro* digestion process. From this it was concluded that XOS are resistant to hydrolysis throughout the simulated severe conditions of the gastrointestinal system (they maintained their complex structure, without being degraded), implying that they could safely reach the large intestine intact.

As such, XOS are selectively fermented by the gut microbiota in the large intestine (Gibson et al., 2017; Amorim et al., 2019). The gut microbiota uses these oligosaccharides to assist in its growth and consequent production of SCFAs (acetate, propionate, and butyrate), gases (primarily H_2 and CO_2), lactate and other compounds (Topping & Clifton, 2001). SCFAs, and other compounds such as lactate, are key metabolites for gut health, influencing other sites distant from the gut (Gibson et al., 2017). Lactate is a precursor of different SCFAs, such as propionate and butyrate, which are widely considered to promote the prebiotic effect (Flint et al., 2015). SCFAs are reported to be beneficial modulators of metabolic activity, namely energy gain, intestinal homeostasis, the immune system, blood lipids, appetite and renal physiology (Roberfroid et al., 2010; Pluznick, 2016; Boets, et al. 2016).

To date, XOS have been the main focus of several studies due to their various beneficial effects on health (Aachary, Gobinath, Srinivasan, & Prapulla, 2015). *In vivo* studies using XOS have been reported for animals, specifically fish (Abasubong et al., 2018), pigs (Wang et al., 2017) and dogs (Gao et al.,

2017). As for the human gut microbiota, the health benefits provided by XOS have been recently reported in preclinical studies in chronic kidney disease (Yang et al., 2018), functional constipation (Fu et al., 2019), obesity (Fei et al., 2020), diabetes (Khat-udomkiri et al., 2020). Previous studies have also shown that XOS act beneficially in reversing changes in the human gut microbiota during the development of diabetes (Yang et al., 2015). In the sections below (1.5.1. and 1.5.2.) the action of XOS on obesity and diabetes is described.

In addition, due to their recommended lower dose (1.4 - 2.8 g/day) (Finegold et al., 2014), XOS are considered highly competitive in terms of price in comparison to other prebiotics (Amorim et al., 2019). Nevertheless, the potential prebiotic effect of XOS is influenced by their degree of polymerization (DP) as well as their degree of substitution (Sajib et al., 2018).

The prebiotic potential of oligosaccharides can be assessed through *in vitro* models using fecal inocula. These models are more suitable than using pure microorganisms due to the high complexity of the human intestinal microbiota. Furthermore, *in vivo* studies are more time-consuming and expensive, making them an inadequate approach for prebiotic selection (Amorim et al., 2018).

In this work, we conducted *in vitro* studies using human fecal inocula (section 2.9.) to evaluate and compare the prebiotic effect of commercial oligofructose and XOS previously produced by direct fermentation. We proved that the addition of both compounds to the fermentation media increased the production of SCFAs and gases, namely CO_2 and H_2, confirming their prebiotic potential. Our results suggest that the inclusion of XOS as a supplement in the diet may have a beneficial effect on human health.

1.5.1. Xylooligosaccharides action on obesity

The rate of obesity is increasing worldwide and, consequently, the anti-obesity properties of XOS have been the focus of the scientific community in recent years. Human and animal studies have reported that XOS use effective mechanisms in regulating body weight and adipose tissue, such as increasing gene expression of adipogenesis markers and inhibiting fat deposition, resulting in the regulation of food intake and body lipids and changes in the composition of gut microbiota (Lim et al., 2016; 2018; Zhang et al., 2018; Saville et al., 2018; Long et al., 2019; De Freitas et al., 2019).

The impact of XOS on the gut microbiome influences energy extraction from the diet. The gut microbiota is mainly composed of three phyla: *Actinobacteria* (*Bifidobacteria*), *Firmicutes* (*Ruminococcus,*

Clostridium, *Lactobacillus* and *Eubacteria*) and *Bacteroidetes* (*Porphyromonas*, *Prevotella* and *Bacteroides*) (Azad et al., 2018). *Firmicutes* and *Actinobacteria* hold most genes linked to obesity (Turnbaugh et al., 2009; Hansen et al., 2013).

Promoting the growth of *Bifidobacterium* also has a crucial role in inhibiting the development of obesity (Zhang et al., 2020). Several experiments suggest that XOS mitigate obesity-related phyla such as *Firmicutes* and the *Firmicutes/Bacteroidetes* ratio; and increase *Bacteroidetes, Bifidobacterium*, the *Lachnospiraceae* NK4A136 group and *Roseburia* (Long et al., 2019; Zhang et al., 2020) due to increased SCFA production (Faryar et al., 2015; Gobinath et al., 2017; Jagtap et al., 2017), which promote the release of gut hormones (such as glucagon-like peptide-1 (GLP-1)), influencing lipid metabolism (Canfora et al., 2015; Zhao et al., 2017) and leading to a protective action against obesity. Conversely, the decrease in food intake due to XOS can be attributed to the enhanced release of circulating leptin from adipocytes that promotes satiety.

XOS also suppressed fat accumulation, adipose tissue weight and fat mass in the liver by regulating the expression of genes associated with adipogenesis and lipogenesis (Lim et al., 2018; Long et al., 2019), reducing the amount of ceramide and diacylglycerol in plasma (Boini et al., 2018; Zhang et al., 2020). Thus, XOS can be used as a dietary supplement in the treatment of obesity.

1.5.2. Effects of xylooligosaccharides on diabetes

Diabetes mellitus (DM, also known as diabetes) is a characterised metabolic imbalance caused by inadequate insulin secretion (type 1 DM, or DMT1) or insulin action (type 2 DM or DMT2), resulting in elevated plasma glucose levels. It is currently a critical global health problem, being a major risk factor for multiple diseases ranging from cardiovascular and respiratory to hypertension and can even lead to mortality (Lee et al., 2016).

The application of XOS in the treatment of diabetes has been analysed in several diabetic models, revealing that XOS attenuates peripheral insulin resistance in obese insulin-resistant mice via the gut-brain pathway (Chunchai et al., 2018). Additionally, consumption of XOS for 3 months (with DP ranging from 2 to 4) alleviated glucose intolerance in mice fed a high-fat diet. This reduction in intolerance is a result of the ability of XOS to modify gut flora and increase the amount of ceramide (Zhang et al., 2020).

To evaluate the antidiabetic effect of xylobiose (the main disaccharide of XOS), normal mice and diabetic mice (the most used model for DMT2) were also used for six weeks. It was observed that the administration of xylobiose suppressed the improvement of hyperglycaemia in mice with diabetes and

regulated plasma glucose (Lim et al., 2016). In obese mice, the antidiabetic effect of xylobiose increased insulin levels (Lim et al., 2018).

Administration of a monosaccharide derived from XOS (D-xylose) also increased blood glucose and insulin levels in obese mice, attenuating diabetes. This process occurred through the mechanism of suppression of saccharase activity (Lim et al., 2015; 2016; 2018). This mechanism was also observable in human models (Bae et al., 2011), suggesting that the antidiabetic action of XOS was provided to its monosaccharides.

A dosage of 2 mg/d of XOS was administered in humans to evaluate the efficacy of XOS in plasma glucose management. After 2 months, changes in gut microbiota and insulin levels were observed in pre-diabetic individuals (Yang et al., 2015). In addition, XOS consumption by individuals with DMT2 decreased glucose and regulated abnormal lipid metabolism (Zhu et al., 2019). XOS were also reported to decrease impaired intestinal paracellular permeability (an essential function in several diseases, including diabetes). On the other hand, in skeletal muscle, glucose uptake increased (Khat-udomkiri et al., 2019). Based on the results described, we can conclude that XOS may be useful as natural agents in the regulation of diabetes.

2. EXPERIMENTAL

2.1. Materials

All chemicals, media and media components were obtained from Sigma-Aldrich Chemical Ltd. unless otherwise specified. CP and CSS were supplied by Delta S.A. (Portugal). These residues were grinded with a benchtop mixer, dried overnight at 60° C and stored at room temperature until use (Amorim et al., 2019). The relative humidity content of the residues was <5% (w/w), being determined with a humidity analyser MAC 50/1/NH device (Amorim et al., 2019). To acquire a deeper understanding of the composition of this residue, we proceeded to the chemical characterization of the residues. The standard XOS (composed of xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5) and xylohexaose (X6)), and the commercial enzymatic preparation of endo-1,4-β-xylanase M3 from *Trichoderma longibrachiatum* were purchased from Megazyme (Ireland), (Amorim et al., 2019).

2.2. Chemical characterization of the residues

CSS and CP were chemically characterized according to NREL (National Renewable Energy Laboratory) protocols (NREL/TP-510-42618-42622- 4218) for biomass composition analysis. The method used included acid hydrolysis with 72% (w/w) sulfuric acid of each of the residues without the extractives, quantification of the sugars using High Performance Liquid Chromatography (HPLC), detailed in section 5.7.3., and compounds contained in the hydrolysate. The acid insoluble lignin was quantified by gravimetry (Amorim et al., 2019).

A Soxtec 8000 FOSS apparatus was used to determine the quantity of extractives using 80% (v/v) ethanol as solvent. The hemicellulose and cellulose fractions were determined by analysing the data obtained through HPLC. Protein quantification was performed through the Keldahl method to determine the percentage of nitrogen (N_2) present in the sample, which has three main tasks: Digestion, Distillation and Titration (Cadavez V., 2022). The amount of cellulose and hemicellulose was determined using HPLC data (Amorim et al., 2019). As for the percentage of structural ash was calculated as the ratio between the weight of ash and the dry sample weight (consisting of the subtraction between the mass of the residue and the average humidity of the residue), whose value is subsequently multiplied by 100. The total lignin amount was calculated as the sum between the soluble and acid insoluble lignin fractions. As for the percentage of acid-insoluble lignin content, it equals value of the ratio between the weight of insoluble lignin (consisting in the mass of the solid that resulted from filtration (SF) excluding the ash content present in the structure (Amorim et al., 2019)), then divided by 100. Lastly, the acid-soluble lignin (ASL) was quantified spectrophotometrically at a wavelength of 205 nanometres (nm) and at a dilution of 1:30. Subsequently, it was calculated according to the equation 1:

$$\mathrm{w}_{\mathrm{ASL}} = \left[\frac{\mathrm{average_{abs}} * \mathrm{w_{hydrolysed}} * \mathrm{DF}}{\mathrm{w_{dry\ sample(g)}}}\right] * [100 - (\%\ \mathrm{extractives} * 100)] \quad (1)$$

where w_{ASL} is the weight of ASL, the $average_{abs}$ and the $w_{hydrolysed}$ correspond to the media of the absorbances (abs) previously measured spectrophotometrically and the weight of hydrolysed, respectively. The DF represents the dilution factor.

2.3. Microorganism and pre-inoculum

B. subtilis 3610 clone 2, previously designed by Amorim et al., (2018), containing a secretion tag endogenous to *B. subtilis* (> tr|A0A0S2II12| Uniprot) coupled to xylanase *xyn2* from *T. reesei* (X69574.1 Genbank NCBI) at N-terminus site was used in this work. Stock cultures were cultivated on -80 °C glycerol LB Agar plates (Oxoid, City, Country) and stored at ambient temperature in 0.2% (w/v) semi-solid media suspensions (Oxoid), and then propagated at 25 °C in 4% (w/v) PDA Petri dishes (Biolife®) (Amorim et al., 2019).

To prepare the pre-inoculum, a colony of *B. subtilis* 3610 clone 2 was grown on a plate with LB Agar medium overnight at 37 °C and 0 rpm (no shaking), was collected in 2 mL of LB medium (Difco, New Jersey, USA). Next, the cells were grown at 40 °C and 250 rpm for approximately 2 h until they reached an optic density at 600 nm ($OD_{600\ nm}$) around 1.0. This initial culture was diluted to an initial OD of about 0.020 in fermentation medium.

2.4. Direct fermentation conditions

To assess the potential of CP and CSS as alternative substrates for XOS production by direct fermentation, 250 mL Erlenmeyer flasks were filled with 25 mL of 2% (v/v) Vogel medium (Vogel, 1956) at pH 7.0 with 20 $g.L^{-1}$ of substrate. The solid-liquid mixture was sterilized at 121 °C for 15 min. Cells were then grown at 45 °C and 150 rpm during 30 h. The agro-residue with higher performance was selected for further experiments.

To analyse the impact of the sterilization step on the direct fermentation process, a comparative assay between autoclave- and UV-sterilization was performed. The CSS and CP-liquid mixture sterilization by autoclave was performed as previously described. The UV sterilization consisted in submitting the agro-residue (CP) at a UV lamp placed at 30 cm and during 1 h, before its addition to the filter-sterilized (0.2 μm membrane) Vogel pH 7.0 at sterile conditions (Amorim et al., 2019).

The media sterilized by the two methodologies was then fermented at 45 °C and 150 rpm during 30 h.

2.5. Enzymatic hydrolysis

To evaluate the potential for XOS production by enzymatic hydrolysis of CSS and CP a commercial endo-1,4-β-xylanase from *Trichoderma longibrachiatum* was used at a concentration of 1 $U.mL^{-1}$ to hydrolyse 20 $g.L^{-1}$ of each residue in 0.1 M sodium acetate buffer, at pH 4.5, 40 °C, and 150 rpm. The

conditions were stablished based on the Megazyme protocol for this enzyme. The residue-buffer mixture was sterilized by autoclave and UV as previously described section 5. 4..

For all the fermentation assays, liquid samples were taken at 0, 4, 8, 12, 24, and 30 h, centrifuged (6000 rpm for 6 min), and the supernatant was collected for sugar analysis, as detailed in section 2. 7.

2.6. Optimization of the direct fermentation process

The previously described fermentation process (section 3.4.) was optimized variable by variable, namely: substrate concentration (5, 10, 20, 40 and 60 $g.L^{-1}$), pH (5.0; 6.0; 7.0 and 8.0) and temperature (30, 37, 45 and 50 °C). Samples of the supernatant were collected, centrifuged, for further sugar analysis as described in section 2.7. 1.. The optimal process variables were selected, and the sugars mixture produced under optimal conditions were further characterized as described in section 5.7. 3..

2.7. Sugar analysis

2.7.1. Reducing sugars

The dinitrosalicylic acid method, DNS, (Miller, 1959) is a qualitative screening technique that has been used to quantify total reducing sugars, RS (mg), using the xylose calibration curve. Subsequently, the yield of reducing sugars (Y_{RS}, $mg.g^{-1}$) was determined by the ratio of the concentration of RS to the mass of xylan (g) present in the agro-residue used in the fermentation.

2.7.2. Thin-Layer Chromatography

The Thin-Layer Chromatography (TLC) technique was used to perform the qualitative analysis of the composition of XOS (Amorim et al., 2018) obtained through CSS and CP, using plates with a silica matrix (DCAlufolien Kieselgel 60, Merck) for this purpose. The supernatant samples were applied to the TLC plates, with a volume of 17 μL, and the standard used was pure XOS (X2 - X6, 2 $g.L^{-1}$), in a volume of 2 μL. Thus, they were subjected to an ascending chromatography using an mobile phase consisting of butanol, acetic acid, and water (in a ratio of 10:5:5 v/v/v) (Amorim et al., 2019). Then, a staining solution containing 1% (w/v) diphenylamine and 1% (v/v) aniline in acetone was applied to detect the bands. Finally, heating was performed at 105 °C for 10 min (Dutta, S. et al., 2014).

To identify and compare the different components present in the mixtures, the ratio between the distance travelled by the sample and the distance travelled by the standard along the TLC plate was used, and this is called the retention factor (Amorim et al., 2019).

2.7.3. High Performance Liquid Chromatography

The XOS produced under optimal conditions were quantified by HPLC before and after acid hydrolysis to determine both the amount of free xylose and the amount of xylose present in the oligosaccharides. Note that the DP of XOS was predicted by TLC and HPLC (Amorim et al., 2019).

Thus, two different HPLC methods were used to analyse monosaccharides or oligosaccharides. An HPLC equipped with a Knauer-RI detector (Agilent Technologies, USA) and an Aminex HPX 87H column (300mm x 7.8; Biorad, USA) was used to quantify monosaccharides. The sample volume was eluted using sulfuric acid at 5 mM as mobile phase at a temperature of 60 °C and, at a flow rate of 0.9 $mL.min^{-1}$. Furfural and HMF concentrations were also determined by HPLC using a Knauer-UV detector (Amorim et al., 2019).

For the oligosaccharides, an HPLC (JASCO, Japan) equipped with an ELSD detector (SEDERE, Sedex 85, France) and a HILICpak VG-50 4E column was used. A mixture of acetonitrile, methanol, and water 75:20:5 (v/v/v) as mobile phase was utilised at a flow rate of 0.9 $mL.min^{-1}$, temperature 40 °C and the volume injected was 20 µL (Amorim et al., 2019), for 80 min. For the identification of the DP of the oligosaccharides produced, pure XOS (X2-X6) were used as standards.

2.8. Xylooligosaccharides production in bioreactor

The XOS production in the bioreactor, without air injection, was performed in the bioreactor 3.7 L (RALF PLUS SOLO, Bioengineering, Switzerland), containing 0.5 L of culture medium (Cordeiro et al., 2019). A pre-inoculum was prepared as described in section 2. 3.. The pre-inoculum, the culture medium and the bioreactor were autoclaved at 121 °C, during 30 min. The pre-inoculum was added to the bioreactor under aseptic conditions. The fermentation was performed at controlled temperature of 45 °C, agitation of 200 rpm (executed by two Rushton-type impellors) and pH 7.0 (Basto et al., 2019; Cordeiro et al. 2019).

Additionally, another fermentation assay was conducted under the same conditions but with an aeration rate of approximately 2 vvm (volume flow per unit of liquid volume per minute), in a bioreactor Bioengineering RALF 3.5 L containing, 1.5 L of culture medium. 2 mL of samples of the supernatant were collected at 0 h, 3 h, 6 h, 9 h, 12 h, 24 h, 30 h, centrifuged (5000 rpm during 5 min), of which 1 mL was used for further sugar analysis as described previously in subsection 2.7. 1.. and the other 1 mL of sample was resuspended in 1 mL H_2O. Finally, the absorbance (abs) of the resuspended sample was measured at $DO_{600\,nm}$.

2.9. Prebiotic effect

2.9.1. Faecal inoculum

Faecal samples were obtained from four healthy human volunteers (2 female and 2 male donors), with no known metabolic or GI diseases and who had not taken any antibiotics, pre- or probiotic supplements for 3 months prior to the study. Male and female donors, age ranging from 24 to 28 years, were non-smokers and consumed a non-specific Mediterranean diet. The faecal samples were mixed in order to obtain a single pre-inoculum. Samples were collected on site, diluted 1/10 (w/w) in anaerobic (100% N_2) phosphate buffered saline (PBS, 0.1 M, pH 7.0) and kept at 4 °C overnight before inoculation (Amorim et al., 2020).

2.9.2. Cultured *in vitro* fermentations of oligosaccharides using gut microbiota

Fermentations were performed at 37 °C for 48 h in serum bottles. The bottles were filled with 40 mL of growth medium at pH 7.0 (peptonised water 2 $g.L^{-1}$, yeast extract 2 $g.L^{-1}$, NaCl 0.1 $g.L^{-1}$, K_2HPO_4 40 $mg.L^{-1}$, $MgSO_4$. 7 H_2O 0.01 $g.L^{-1}$, $CaCl_2$.6 H_2O 0.01 $g.L^{-1}$, $NaHCO_3$ 2 $g.L^{-1}$, Tween 80 14.8 $mL.L^{-1}$, haemin 5 $mg.L^{-1}$, vitamin K1 74.1 $\mu l.L^{-1}$, cysteine HCl 0.5 $g.L^{-1}$, bile salts 0.5 $g.L^{-1}$, Na_2S.9 H_2O 0.8 mM and resazurin 1 $mg.L^{-1}$). XOS or oligofructose (Orafti®, BENEO, Germany) solutions were added when necessary to a final concentration of 10 $g.L^{-1}$. Apart from filtered and sterilised solutions of vitamin k1 and oligosaccharides, the medium was sterilized by autoclaving and inoculated with 4.4 mL of faecal inoculum. The anaerobic conditions were achieved by pressurizing the upper space of the bottles with nitrogen to 170 KPa.

Liquid samples were taken at different time points (0, 24 and 48 h), centrifuged at 4000 x g for 10 min and the supernatant was used for HPLC analysis (section 5.8.3.). Free space gas samples were used to assess gas production (section 5.8.3.). The pH was measured at all time points and the fermentation broth was removed from the bottles, centrifuged, washed, resuspended in PBS (0.1M pH 7.0) and stored at -20 °C for DNA extraction and subsequent sequence analysis (section 5.8.4.). Fermentations were performed using a blank without prebiotics added as a negative control, and in duplicate.

2.9.3. Technical analysis

The production of lactate and SCFAs (acetate, propionate, and butyrate) was evaluated according to Amorim et al., 2018), using an HPLC (SEDERE, Sedex 85, France) fitted with an ELSD detector and a Prevail Carbohydrate column 188 (250 mm x 4.6 mm; Grace, USA). 40 μL volume of sugar sample was eluted using a mobile phase composed of H_2SO_4 5nM at a flow rate of 0.7 $mL.min^{-1}$, and a temperature of 60 °C. Ammonium was measured using an enzymatic kit (LCK 302) from Hach Lange (Düsseldorf, Germany). Gas samples were analyzed by gas chromatography (GC) using a Bruker Scion 456-GC (Billerica, MA, USA) equipped with two columns: a BR-QPLOT column (30 m length, 0.53 mm internal diameter; film thickness, 20 lm) and a Molsieve packed column (13 9 80/100, 2 m length, 2.1 mm internal diameter). The Molsieve column was used to measure H_2, CO_2 and CH_4, and argon was used as carrier gas at a flow rate of 30 $mL.min^{-1}$; temperatures in the injector, column and detectors were 100, 35 and 130 °C respectively (Amorim et al., 2018), and with a thermal conductivity detector.

2.9.4. Evaluation of the microbiota

Total DNA from faecal fermentations at 48 h and faecal inocula was extracted from liquid samples using the FastDNA SPIN soil kit (MP Biomedicals, Solon, OH), according to the manufacturer's instructions. High-throughput sequencing (16S rRNA gene) using Illumina MiSeq technology was performed on RTL Genesis, and the detailed description of the procedure is previously described (Salvador et al., 2019).

2.9.5. Statistical analysis

Differences between SCFA concentrations at different fermentation times (0, 24 and 48 h) were checked for significance by paired t-test, assuming normal distribution, equal variances and considering both sides of distribution. One-way ANOVA were used for comparison of SCFAs concentrations between the substrates (XOS and oligofructose). The differences were considered significant when p-value was <0.05.

3. RESULTS AND DISCUSSION

3.1. Chemical characterization of the agro-residues

Lignocellulosic agro-residues are mainly composed by cellulose, hemicellulose, and lignin that influences the residue recalcitrance (Dutta et al., 2014). Therefore, agro-residues with higher amounts of xylan (derived from hemicellulose) and lower amounts of lignin are better options for XOS production.

In this sense, coffee sliver skin (CSS) and CSS pellets (CP) were chemically characterized by acid hydrolysis as described in section 1. 2.. Table 2 presents the composition of CSS and CP in terms of protein, hemicellulose (namely xylan, arabinan and acetyl groups), cellulose, lignin (acid soluble and insoluble), extractives and ashes, in dry weight (% (w/w)).

Table 2 – Chemical characterization of coffee silver skin (CSS), coffee silver skin pellet (CP) and brewers' spent grain (BSG), in dry weight (% (w/w)). Results in form of mean ± SD.

Component	**% (w/w)**		
	CSS	**CP**	**BSG***
Proteins	15.8 ± 0.4	18.6 ± 0.3	19.2 ± 0.5
Hemicellulose	15.5 ± 0.3	17.6 ± 0.6	16.5 ± 0.7
Xylan	6.7 ± 0.1	9.4 ± 0.4	10.3 ± 0.2
Arabinan	—	0.7 ± 0.1	5.1 ± 0.3
Acetyl groups	8.8 ± 0.2	7.4 ± 0.1	1.1 ± 0.2
Cellulose	18.9 ± 0.1	18.9 ± 0.4	17.5 ± 0.3
Acid soluble lignin	4.2 ± 0.6	4.85 ± 0.05	4.7 ± 0.3
Insoluble lignin	18.1 ± 0.3	20.1 ± 0.3	22.3 ± 0.6
Extractives	19.73 ± 0.04	17.58 ± 0.08	16.9 ± 0.4
Ashes	2.35 ± 0.04	2.2 ± 0.8	4.54 ± 0.04

***The results regarding the composition of BSG were obtained from a previous study, using acid hydrolysis, performed by Amorim et al. (2018).

The results herein obtained for the chemical composition of CSS are similar to the ones reported by Mussatto et al.(2011b) and Ballesteros et al. (2014). Namely, these authors reported a range of values compatible with the results of Table 2: protein 18.69 ± 0.10 % (w/w), hemicellulose 16.68 ± 1.30 % (w/w) of which 7.61 ± 0.02 % (w/w) corresponds to xylan, acid soluble lignin 7.61 ± 0.16 % (w/w), insoluble lignin 20.97 ± 0.43 % (w/w) and ashes 5.36 ± 0.20 % (w/w). Some differences could be attributed to the extraction process and variety of coffee beans used (Ballesteros et al., 2014).

As expected, CSS and CP present relatively similar composition. However, the content of protein, hemicellulose and insoluble lignin is slightly higher in CP. This difference may result from the heating and high compression pressure (300 MPa) required in the pelletizing process (Pua et al., 2020; Setter et al., 2020).

When compared to the chemical composition reported by Amorim et al. (2019) for brewer's spent grains (BSG), CSS and CP show similar composition in hemicellulose, cellulose, and lignin (Table 2). BSG is a major by-product of the brewing industry that was reported to be an efficient alternative substrate for XOS production by direct fermentation (Amorim et al., 2019). Thus, based on the chemical composition, it is anticipated that CSS and CP may also show potential for XOS production.

3.2. Direct fermentation for the agro-residue selection

A screening assay with 20 $g.L^{-1}$ of CSS and CP was performed to evaluate their potential for XOS production by direct fermentation using *B. subtilis_xyn2* (as detailed in sections 2.3. and 2.4.). Prior to inoculation, the agro-residues in 2% (w/v) Vogel medium were sterilized by autoclave (weight losses < 5% (w/w)). This process possibly contributed to enhance the access of the microorganism enzymes to the hemicellulose and to release some free sugars important for the biomass growth. The fermentation process was monitored by DNS to obtain a qualitative analysis of the potential for XOS production through the amount of the produced reducing sugars, and TLC to obtain a qualitative analysis of the hydrolytic products composition and HPLC.

Figure 6 shows the reducing sugar production yields (Y_{RS}) obtained for CSS and CP.

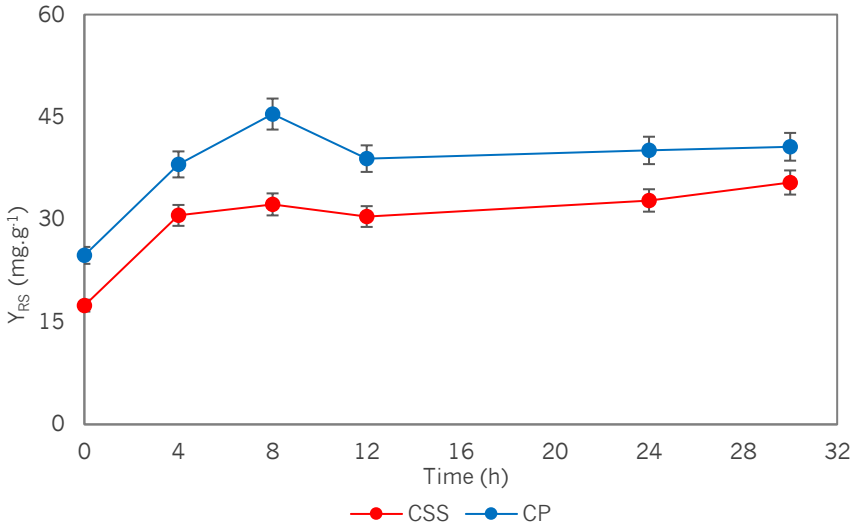

Figure 6- Sugar production yield (Y_{RS}) obtained from direct fermentation using 20 g.L^{-1} of coffee silver skin (CSS) and coffee silver skin pellet (CP) by *Bacillus subtilis_xyn2*. The fermentation assays were performed at 45 °C and 150 rpm with CSS and CP in 2% (w/v) Vogel media pH 7.0. Results are the average of two independent fermentations and duplicate analysis of each sample ± standard deviation.

The degree of polymerization (DP) of the obtained XOS were analyzed by TLC attending the number and position of the observed bands. Figure 7 shows the results from the TLC performed on samples of both agro-residues at the initial and optimal time (0 and 8 h, respectively). The mobile phase used was composed of butanol: acetic acid: water in the ratio 2: 1: 1.

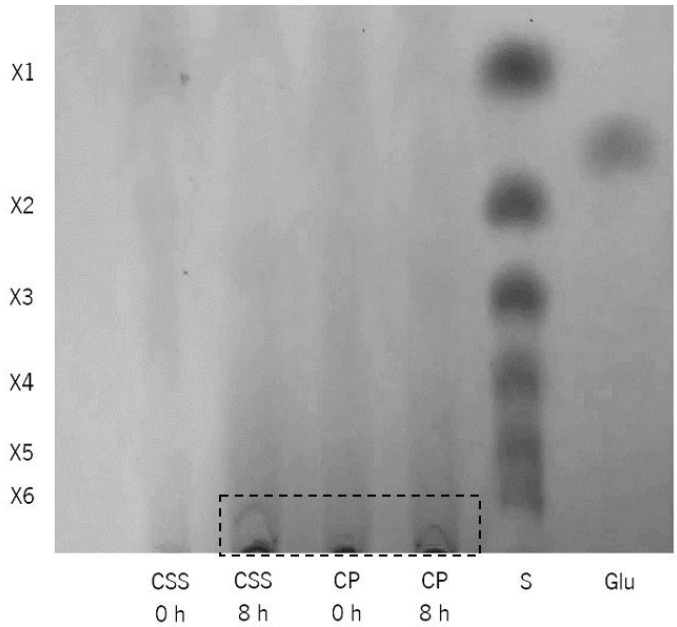

Figure 7– Thin-layer chromatography (TLC) of the supernatants obtained from the direct fermentation of coffee silver skin (CSS) and coffee silver skin pellet (CP) by *Bacillus subtilis_xyn2*. Samples from the initial and optimal time (0 and 8 h, respectively). A mixture containing 2 g.L^{-1} of xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5) and xylohexaose (X6) was used as standard (S). A mixture of 1 g.L^{-1} of glucose (Glu) was also used as standard. The fermentation assay was performed at 45 °C and 150 rpm with 20 g.L^{-1} of CP in 2% (w/v) Vogel media pH 7.0.

Figure 7 shows the absence of bands at the initial time and, the formation of one band with a DP of 6 and over 6 for CSS and CP (respectively), at 8 h. These results corroborate the qualitative analysis of the potential for XOS production through DNS. Thus, a further quantitative analysis by HPLC, was performed as described in section 2. 7. 3. for both agro-residues. We observed the hydrolysis of more complex structures into simpler ones at 8 h for both agro-residues (Figures 8 and 9, Appendix, section 1), which are align with the results obtained from the previous techniques performed. However, it should be noted that the quantification of oligosaccharides and monosaccharides were inconclusive for CSS and CP samples, accordingly.

Thus, it is suggested that CP presents higher potential for XOS production. Therefore, it was selected as a substrate for further studies.

3.2.1. Direct fermentation *versus* Enzymatic hydrolysis

In order to compare the effectiveness of direct fermentation with the use of commercial enzymes, an enzymatic hydrolysis test was performed with 1 U.mL-1 of commercial xylanase from *T. longibrachiatum* incubated with 20 $g.L^{-1}$ of CP in 0.1 M of sodium acetate buffer, pH 4.5 and 45 °C sterilized by autoclave. As described in section 2. 5., CP in sodium acetate buffer was sterilized before incubation with the enzyme. This comparison was established to select the most effective process for XOS production using CP. Figure 10 shows the Y_{RS} profiles for XOS production through direct fermentation (CP) and enzymatic hydrolysis (CP_enz). The Y_{RS} profiles obtained by enzymatic hydrolysis were significantly different from those obtained for direct fermentation (Figure 10). For both approaches, after 8 h of fermentation the highest Y_{RS} (Y_{RSmax}) value seems to be achieved. In addition, Y_{RSmax} obtained for the enzymatic hydrolysis was 18.5 ± 0.2 $mg.g^{-1}$, which is approximately half of the Y_{RSmax} value obtained through direct fermentation (which equals 45 ± 5 $mg.g^{-1}$). Thus, this suggests that the approach used affects the XOS production process yield.

Previous studies, reported by Amorim et al. (2018), showed that the enzymatic hydrolysis of raw BSG (20 $g.L^{-1}$) using 0.8 $U.mL^{-1}$ of a commercial xylanase from *T. longibrachiatum* during 72 h of fermentation obtained a Y_{RSmax} (after 12 h) of, approximately, 3 times higher comparing to the results obtained in the current work for 1 $U.mL^{-1}$ of commercial xylanase, although the time needed to achieve the Y_{RSmax} (designated production time) was also (8 h).

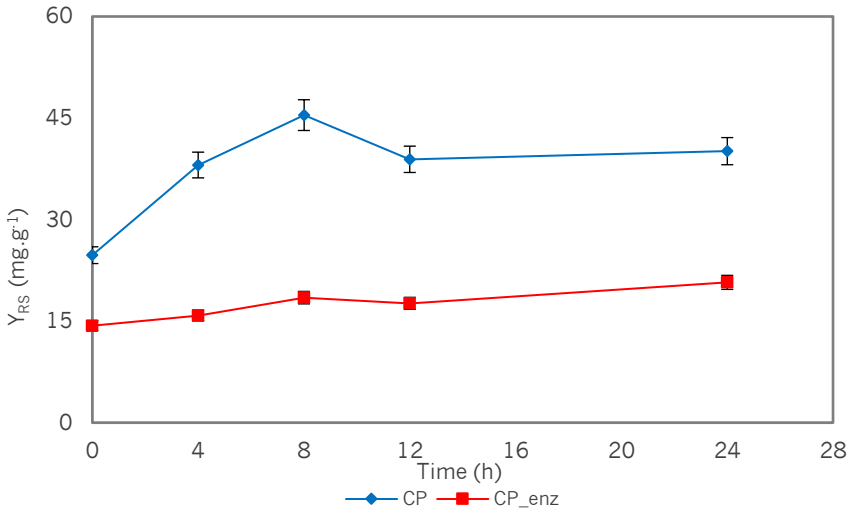

Figure 10- Reducing sugars production yield (Y_{RS}) obtained for *Bacillus subtilis_xyn2* using coffee silver skin pellet (CP) sterilized by autoclave at 20 g.L^{-1}, 45 °C, pH 7.0, 150 rpm, through enzymatic hydrolysis and direct fermentation (CP_enz and CP, respectively). Results are the average of two independent fermentations and duplicate analysis of each sample ± standard deviation.

Furthermore, a qualitative analysis by TLC was conducted with samples from enzymatic hydrolysis at the initial and optimal time (0 h and 8 h, respectively) (Figure 11). The results suggested the presence of xylose (DP of 1) and xylobiose (DP of 2) at both sampling times, which is also noticeable in the Y_{RS} profile of CP_enz since the yield showed minimal variation. When comparing to the previous results obtained through direct fermentation (Figure 7), where occurs the formation of structures with a DP over 6, enzymatic hydrolysis seems to produce simpler structures. These differences are mainly associated with the bacterial metabolism behavior, since, as expected, the consumption of monosaccharides (simpler sugars) present in the media by the microorganism was not observed in the enzymatic hydrolysis.

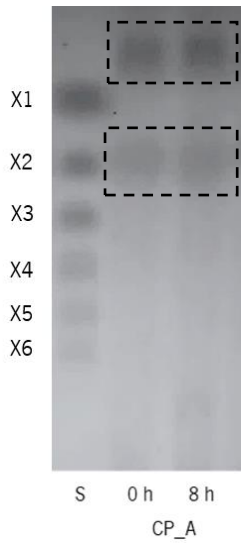

Figure 11 – Thin-layer chromatography (TLC) of the supernatants obtained from the enzymatic hydrolysis of coffee silver skin (CP) by *Bacillus subtilis_xyn2*. A mixture containing 2g.L^{-1} of xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5) and xylohexaose (X6) was used as standard (S). The fermentation assay was performed at 45 °C and 150 rpm with CP in 2% (w/v) Vogel media pH 7.0 and sterilized by autoclave.

On the other hand, the Y_{RSmax} associated to direct fermentation obtained in this work was higher (Y_{RSmax} = 45 ± 5 mg.g^{-1} after 8 h) than those presented by Amorim et al. (2018) (Y_{RSmax} = 39.5 ± 0.9 mg.g^{-1} after 72 h) under optimal conditions (20 g.L^{-1} of BSG, 30 °C and pH 7.0).

In summary, given all the reasons above, and considering the advantages of direct fermentation over enzymatic hydrolysis, such as being an innovative technique due to not having to buy/produce enzymes, reduced process costs and simplification of downstream process (Amorim et al., (2018); Urmann et al., (2010), we concluded that using direct fermentation of CP by *B. subtilis_xyn2* for XOS production is the most suitable approach.

3.3. Influence of the sterilization method

The media was prepared by adding CP at 20 g.L^{-1} to the culture medium followed by a sterilization at 121 °C, 1 atm during 15 min. Although similar to the thermal pretreatment of lignocellulosic biomass (namely liquid hot water and steam explosion), this sterilization operates under milder conditions generally requiring 160-240 °C and 1-30 atm for a few seconds to a few minutes (Behera, Arora, Nandhagopal, & Kumar, 2014).

For a better understanding of the role of sterilization in the production of XOS from CP by *B. subtili_xyn2* through direct fermentation, 2 different conditions were studied, namely the use of CP sterilized by UV treatment and autoclave (as described in section 2.4.), during 30 h of fermentation (Figure 12). A control sterilized by autoclave was performed in parallel, under the same conditions, but without inoculum (Control_A). Each condition was carried out in duplicate.

As expected, autoclave sterilization led to the release of sugars into the fermentation medium (Amorim et al., 2018), which may have stimulated biomass growth, resulting in the highest Y_{RSmax} (42.12 ± 0.02 mg. g^{-1}) achieved at 8 h. It was found that, even without this initial amount of sugars in the medium, the bacteria can grow using CP as the only carbon source (UV-sterilized CP) while taking the same period to reach the highest yield (at 8 h) (Figure 12). Interestingly, the difference between Y_{RSmax} and Y_{RSmin} is comparable for UV-sterilized CP (16.3 ± 0.5 mg. g^{-1}) and autoclave-sterilized CP (21 ± 1 mg.g^{-1}), thus suggesting that the microorganism's metabolism may function similarly under both sterilization processes.

The control without inoculum, showed stable values of reducing sugars during the 30 h of fermentation, which proves that XOS are produced exclusively by *B. subtilis_xyn2*. Therefore, it seems clear that a sterilization process will be crucial in XOS production. On the other hand, the profile curve of the control and UV sterilization suggests that the incubation process itself contributes to the release of sugars into the medium (0-4 h).

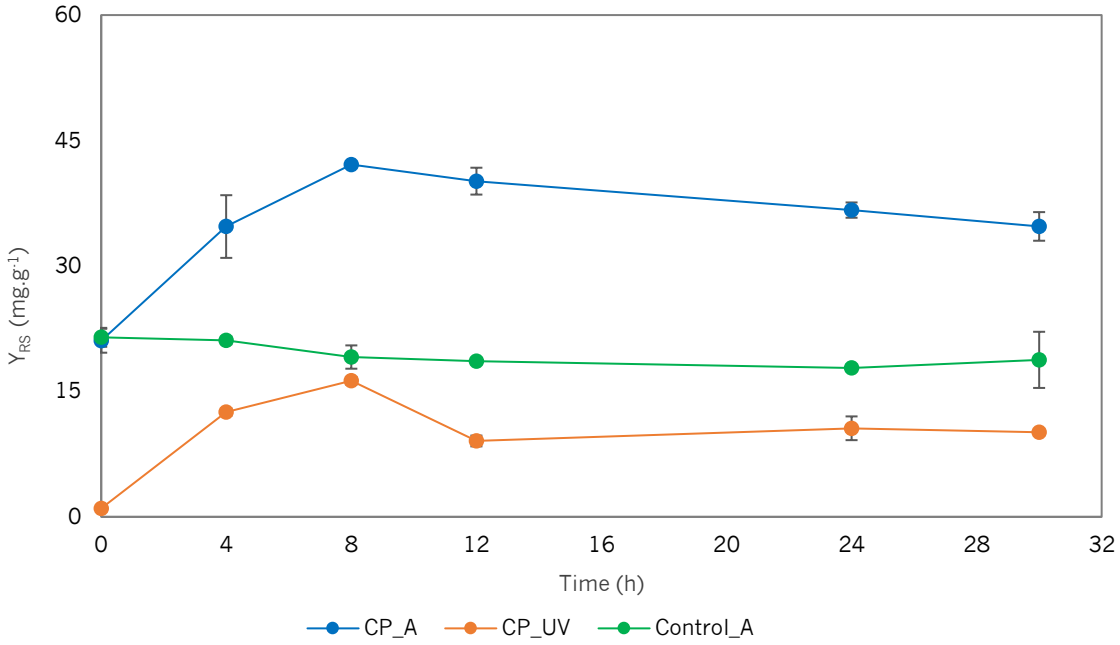

Figure 12- Sugar production yield (Y_{RS}) obtained from direct fermentation of coffee silver skin pellet (CP) by *Bacillus subtilis_xyn2* using different sterilization methods: autoclave (CP_A) and UV treatment (CP_UV). The control in the absence of microorganism was performed under the same conditions of autoclave sterilization (Control_A). The fermentation assay was performed at 45 °C and 150 rpm with 20 g.L^{-1} of CP in 2% (w/v) Vogel media, pH 7.0. Results represent the average of Y_{RS} for the different assays ± SD.

Additionally, the effect of the sterilization method was also studied through the enzymatic hydrolysis of CP (described in section 2.5.) in the same conditions previously detailed in this section. By analyzing the Y_{RS} profile obtained from this assay (Figure 13, Appendix), we observe that the Y_{RSmax} (18.5 ± 0.2 mg. g^{-1}) was achieved at 8 h through autoclave sterilization. Interestingly, the UV-sterilized CP presented sugars in the initial fermentation media ($Y_{RSinitial}$ of 6 ± 1 mg.g^{-1}). The difference between Y_{RSmax} and Y_{RSmin} is also comparable for UV-sterilized CP (12.0 ± 0.3 mg.g^{-1}) and autoclave-sterilized CP (14.33 ± 0.03 mg.g^{-1}). Finally, based on the results obtained in this assay we concluded that direct fermentation with autoclave sterilization is the most efficient approach for XOS production. Therefore, this technique was applied in the next assays.

3.4. Optimization of the direct fermentation

The production of XOS by direct fermentation of CP using *B. subtilis_xyn2* was optimized variable by variable at a flask scale. Particularly, substrate concentration, pH and temperature, were studied individually to evaluate their effect on XOS production as described in section 2.6. Each fermentation condition was tested in duplicate.

3.4.1. Substrate concentration

The effect of the substrate concentration on XOS production through direct fermentation by *B. subtilis_xyn2* was studied using different amounts of CP in 2% (w/v) Vogel medium at pH 7.0, 45 °C and 150 rpm (Figure 14). Concentrations of CP of 5, 10, 20, 40 and 60 g.L^{-1} were tested for process optimization.

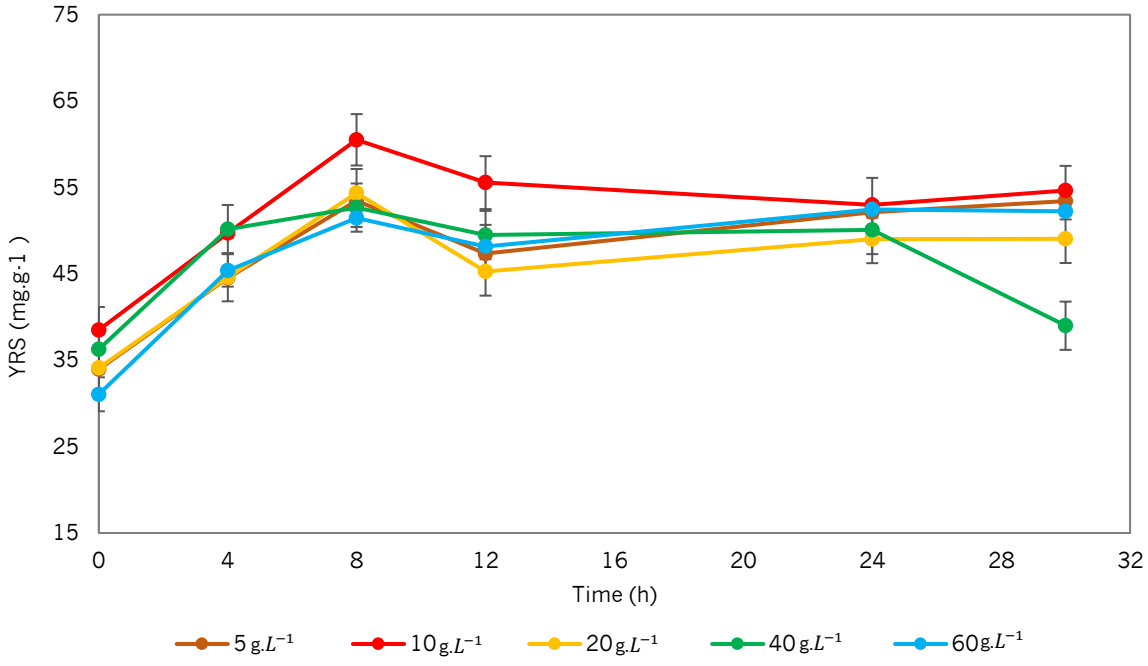

Figure 14- Reducing sugars production yield (Y_{RS}) obtained for *Bacillus subtilis_xyn2* by direct fermentation using different coffee silver skin pellet (CP) concentrations (5, 10, 20, 40 e 60 g.L^{-1}) in 2% (w/v) Vogel media at pH 7.0, 45 °C and 150 rpm. Results represent the average of Y_{RS} of two independent assays ± standard deviation.

The Y_{RSmax} was achieved after 8 h of fermentation for all CP concentrations tested. Furthermore, we observed that the initial Y_{RS} (0 h) values for all substrate concentrations show differences. These may be related with the autoclave sterilization process since during the sugar extraction, higher substrate concentrations may present a higher release of reducing sugars amounts but lower Y_{RS} due to media saturation (Amorim et al., 2018). The sensitivity limit of the DNS method may also explain the higher error associated to the Y_{RS} value at 0 h (Amorim et al., 2019). Interestingly, except for 10 $g.L^{-1}$ which lead to the highest value (60.5 ± 3.0 mg. g^{-1}, p-value<0.0001), the Y_{RSmax} was similar for all concentrations (53 ± 2, 54 ± 1, 53 ± 5 and 51 ± 1 $mg.g^{-1}$ for 5, 20, 40 and 60 $g.L^{-1}$, respectively, all at 8 h). The Y_{RS} obtained at the optimal time, followed with the statistically differences for the different CP concentrations are presented in table 3. Furthermore, these concentrations of substrate (5, 20, 40 and 60 $g.L^{-1}$), resulted in the lowest Y_{RSmax} values. This may be due to the increased viscosity and density of the fermentation medium (Moteshafi et al., 2016; Figueiredo et al., 2017), as a result of substrate inhibition or even aeration difficulties (Aachary & Prapulla, 2009; Helianti et al., 2016). In this sense and attending the sensitivity limit of DNS technique, the optimal concentration of CP selected was 10 $g.L^{-1}$. The following optimization studies on the effect of pH and temperature were performed using this concentration.

Table 3- Reducing sugars production yield (Y_{RS}) at the optimal time obtained for *Bacillus subtilis_xyn2* by direct fermentation using different coffee silver skin pellet (CP) concentrations (5, 10, 20, 40 e 60 $g.L^{-1}$) in 2% (w/v) Vogel media at pH 7.0, 45 °C and 150 rpm. Results represent the average of Y_{RS} of two independent assays ± standard deviation. Values followed with different letters have statistically significant differences ($p \leq 0.05$) according to one-way ANOVA.

Concentration ($g.L^{-1}$)	**Y_{RS} ($mg.g^{-1}$)**	**Time (h)**
5	53 ± 2^{a}	8
10	61 ± 3^{b}	
20	54 ± 1^{a}	
40	53 ± 5^{a}	
60	51 ± 1^{a}	

3.4.2. pH

Extracellular pH is an environmental factor affecting the growth of bacteria and enzyme production (Häkkinen et al., 2015). To study the pH effect on the Y_{RS}, 10 g.L^{-1} of CP were fermented by *B. subtilis_xyn2* at 45 °C using different pH from 5.0 up to 8.0 (Figure 15). This pH range was selected considering the balance between the most favorable pH values for sugar extraction and those ideal for bacteria growth and xylanase production (Amorim et al., 2019). The most suitable pH for the process was found to be pH 7.0, providing a Y_{RSmax} of 63 ± 3 mg.g^{-1} after 8 h of fermentation. Interestingly, the Y_{RS} curve profiles corresponding to acidic pH values (pH 5.0 and 6.0) lead to the lowest values of Y_{RSmax} (38 ± 3 mg.g^{-1} at 8 h and 51.3 ± 0.8 mg.g^{-1} at 4 h, respectively), while at pH 8.0 the Y_{RSmax} of 50 ± 2 mg.g^{-1} was achieved at 24 h. In table 4, we observe the Y_{RS} obtained at the optimal time of each pH value, followed with the statistically differences.

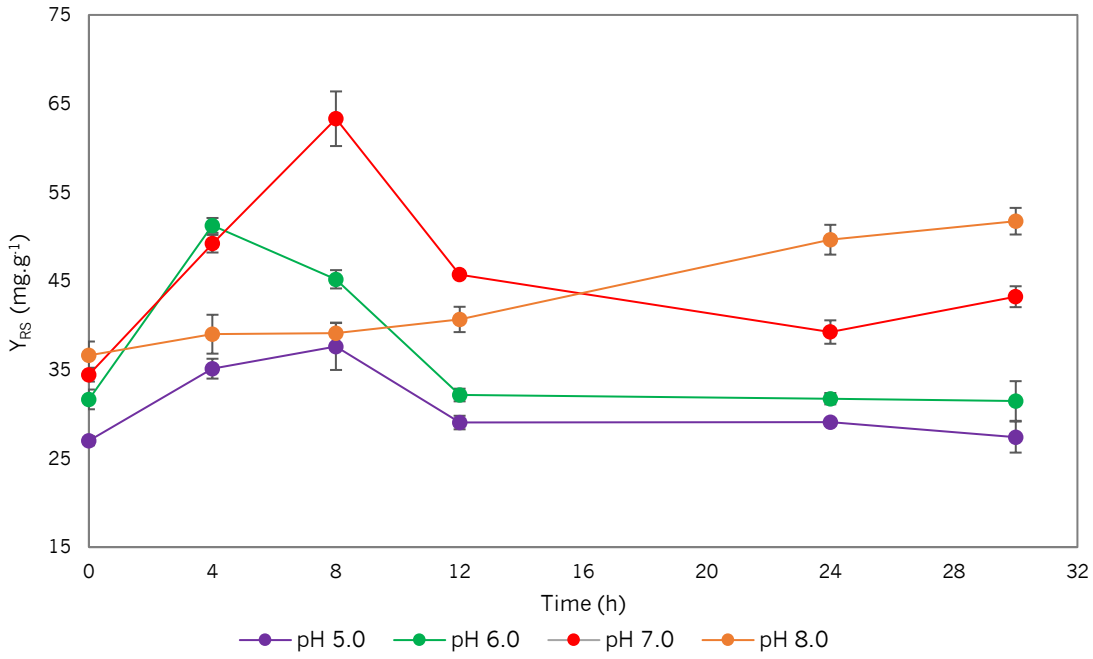

Figure 15- Reducing sugars production yield (Y_{RS}) obtained for *Bacillus subtilis_xyn2* through direct fermentation of coffee silver skin pellet (CP) at 10 g.L^{-1} using different pH values (5.0, 6.0, 7.0 and 8.0). The fermentation assay was performed at 45 °C and 150 rpm with 10 g.L^{-1} of CP in 2% (w/v) Vogel media. Results represent the average of Y_{RS} of two independent assays ± standard deviation.

Table 4- Reducing sugars production yield (Y_{RS}) obtained at the optimal time for *Bacillus subtilis_xyn2* through direct fermentation of coffee silver skin pellet (CP) at 10 $g.L^{-1}$ using different pH values (5.0, 6.0, 7.0 and 8.0). The fermentation assay was performed at 45 °C and 150 rpm with 10 $g.L^{-1}$ of CP in 2% (w/v) Vogel media. Results represent the average of Y_{RS} of two independent assays ± standard deviation. Values followed with different letters have statistically significant differences ($p \leq 0.05$) according to one-way ANOVA.

pH	Y_{RS} ($mg.g^{-1}$)	Time (h)
5	38 ± 3^{a}	8
6	51 ± 1^{b}	4
7	63 ± 3^{c}	8
8	50 ± 2^{b}	24

Previous studies have reported that higher pH values (alkaline conditions) may improve the effectiveness of enzyme activity against the carbohydrates present in the lignocellulosic mass (Merino & Cherry, 2007). On the other hand, acidic pH values (namely pH 5.0-5.5) are reported to be the optimal condition for *B. subtilis_xyn2* xylanase activity. Ho & Heng (2015) and, Kallel et al. (2015), reported that a pH near 7.0 as the most appropriate pH for *B. subtilis* xylanase production using different residues. Thus, considering the presented results and the most suitable pH for the effectiveness of enzymatic activity and for xylanase production by *B. subtilis,* a pH 7.0 was selected for further optimization assays.

3.4.3. Temperature

In order to study the effect of temperature on the Y_{RS}, 10 $g.L^{-1}$ of CP were fermented by *B. subtilis_xyn2* at optimum pH (7.0) and using a temperature range from 30 °C up to 50 °C (Figure 16).

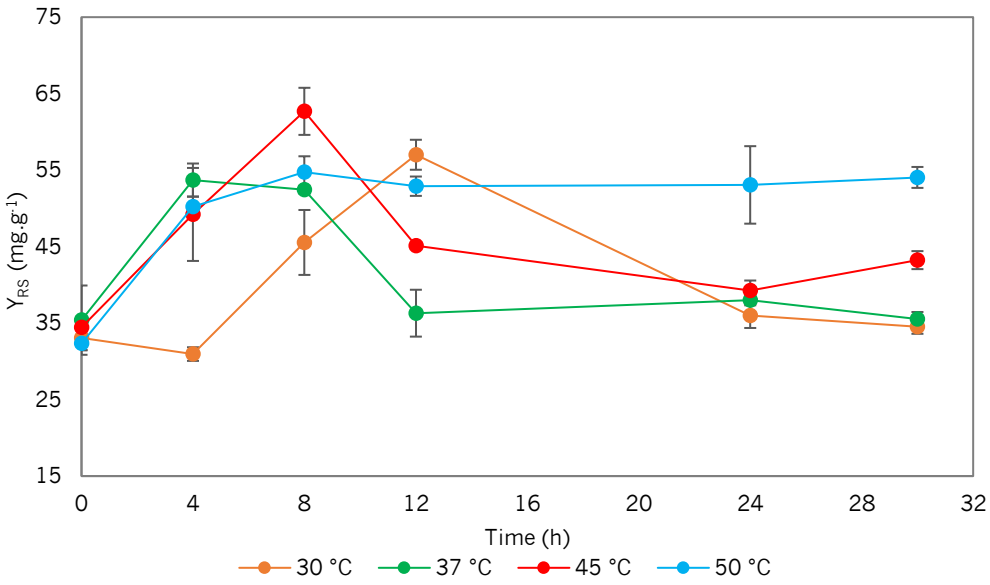

Figure 16- Reducing sugars production yield (Y_{RS}) obtained for *Bacillus subtilis_xyn2* using different temperatures (30 °C, 37 °C, 45 °C and 50 °C) at a CP concentration of 10 g.L^{-1}, pH 7.0 and 150 rpm. Results represent the average of two independent assays ± standard deviation.

The most suitable temperature found was 45 °C leading to a Y_{RS} of 63 ± 3 mg.g^{-1} after 8 h of fermentation (Figure 16). It seems that at this temperature an optimum synergy is reached between the optimum growth temperature for *B. subtilis* (rage of 30-37 °C, (Korsten & Cook, 1996)) and optimum temperature for xylanase production (around 35-37 °C, (Helianti et al., 2016; Irfan et al., 2018)). The Y_{RS} obtained at the optimal time, followed with the statistically differences for the different temperature values are presented in table 5.

Table 5- Reducing sugars production yield (Y_{RS}) at the optimal time obtained for *Bacillus subtilis_xyn2* using different temperatures (30 °C, 37 °C, 45 °C and 50 °C) at a CP concentration of 10 g.L^{-1}, pH 7.0 and 150 rpm. Results represent the average of two independent assays ± standard deviation. Values followed with different letters have statistically significant differences ($p \leq 0.05$) according to one-way ANOVA.

Temperature (°C)	Y_{RS} (mg.g^{-1})	Time (h)
30	57 ± 2 ^{a}	12
37	54 ± 2 ^{a}	4
45	63 ± 3 ^{b}	8
50	55 ± 2 ^{a}	8

At 50 °C it is observed a decrease of the Y_{RSmax} value while at 30 °C it is observed an increase of the optimal time (12 h) possibly due to the negative effect of these temperature on the microorganism growth (Irfan et al., 2018). Irfan et al. (2018) observed a decrease on xylanase activity at temperatures over 50 °C for *B. subtilis* in solid-state fermentation. Therefore, attending the results presented in section 3.4, the most suitable conditions to produce XOS using direct fermentation of CP by *B. subtilis_xyn2* are 10 $g.L^{-1}$ of CP, pH 7.0 and 45 °C.

3.4.4. Qualitative characterization of the XOS mixture produced under optimal conditions

The degree of polymerization (DP) of the produced XOS under the optimal conditions (10 $g.L^{-1}$ of CP, pH 7.0 and 45 °C, section 2.6.) were qualitatively analyzed based on the number and position of the bands observed in the TLC. The TLC was previously optimized in terms of amount of sample, mobile phase and running time (data not shown). Figure 17 shows the optimized TLCs performed on samples of CP at 0, 8 and 12 h and the comparison of the results obtained from using mobile phases with different compositions, namely butanol: acetic acid: water (Figure 17, A) and propanol: acetic acid: water (Figure 17, B) both in the ratio 2: 1: 1.

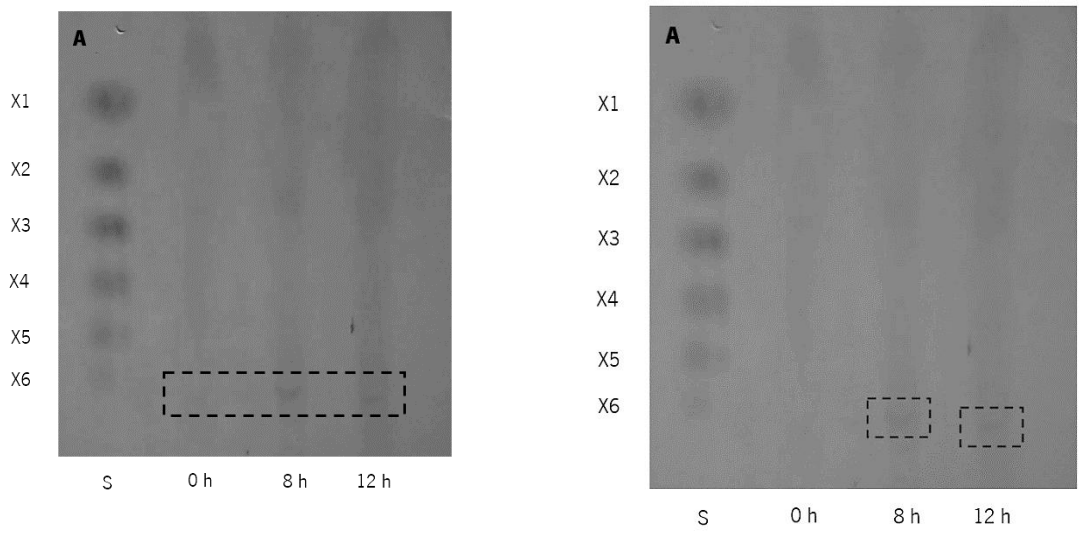

Figure 17- Thin layer chromatography (TLC) of the supernatants obtained from the direct fermentation of coffee silver skin pellet (CP) by *Bacillus subtilis_xyn2* in a concentration of 10 $g.L^{-1}$, pH 7.0, 45 °C. **A-** Mobile phase composed of butanol: acetic acid: water (2: 1: 1), sample from the time points 0 h, 8 h (optimal time) and 12 h, and a mixture containing 2 $g.L^{-1}$ of xylose (X1), xylobiose (X2), xylotriose (X3), xylotetraose (X4), xylopentaose (X5) and xylohexaose (X6) was used as standard (S). **B-** Mobile phase composed of propanol: acetic acid: water (2: 1: 1), sample from the time points 0 h, 8 h (optimal time), and a mixture containing 1 $g.L^{-1}$ of xylose (X1) was used as standard (S).

According to Figure 17, A, *B. subtilis_xyn2* are able to produce oligosaccharides with DP > 6. However, a band with DP ranging from 1 - 2 can be observed at the initial time (0 h) and 2 bands can be observed at the optimal time (8 h), when using the mobile phase composed of propanol: acetic acid: water (Figure 17, B) which possibly indicates oligosaccharides with DP from 2 up to 3. Thus, suggesting the formation of a more complex structure after 8 h of fermentation. The substitution of butanol for propanol in the mobile phase seems to improve the band separation, suggesting that the use of a mobile phase composed of constituents with greater polarity and, consequently, higher solubility in water may favor oligosaccharides with simpler structures. The retention factor of TLC bands corresponding to CP fermentation products may differ slightly from the XOS standard used, since the oligosaccharides produced in this work may have a different composition to the standards.

Furthermore, the fermentation supernatant containing the XOS produced under the optimal conditions was quantified to evaluate the fraction of free xylose. Table 3 shows the concentration free xylose present in the supernatant obtained throughout 8 h of fermentation (optimal time).

Table 6- Concentration of free xylose ($g.L^{-1}$) obtained by direct fermentation of 10 $g.L^{-1}$ of coffee silver skin pellet (CP) using *Bacillus subtilis_xyn2*, under the optimal conditions (pH 7.0, 45 °C and 150 rpm) throughout 8 h of fermentation (optimal time). Results represent the average of two independent fermentations ± standard deviation.

Time (h)	Free xylose concentration ($g.L^{-1}$)	Y_{RS} ($mg.g^{-1}$)
0	1.83	34.4 ± 0.8
4	0.30	49.2 ± 1.0
8	0.18	63 ± 3

As expected, after 8 h of fermentation we observe that the minor amount of free xylose in the fermentation medium decreases (Urmann et al., 2010) in, approximately, ten times (1.823 $g.L^{-1}$ at 0 h and 0.18 g. L^{-1} at 8 h). In contrast, the Y_{RS} from 0 to 8 h two-fold (34.4 ± 0.8 $mg.g^{-1}$ at 0 h and 63 ± 3 $mg.g^{-1}$ at 8 h).

This is a competitive result comparing with previous studies reported for XOS production by direct fermentation of 20 $g.L^{-1}$ of BSG (45 °C, pH 7.0 and 2% (w/v) Vogel) using *B. subtilis_xyn2* whose Y_{RSmax}

achieved was, approximately, 35.11 mg.g^{-1} at 12 h of fermentation (Amorim et al., 2019). Thus, suggesting that direct fermentation of 10 g.L^{-1} of CP using *B. subtilis_xyn2* in the same fermentation conditions is a more cost-effective process for XOS production.

3.5. Production of xylooligosaccharides at bioreactor scale

To date, the reported studies on XOS production by direct fermentation of agro-residues have been conducted at flask scale. However, this small-scale system has some limitations, including the difficulty in controlling aeration conditions and pH, parameters that have an influence on cell growth (Härtig & Jahn, 2012), especially in aerobic and facultative anaerobic microorganisms such as *B. subtilis*.

Thus, a 3.7 L bioreactor (without aeration) and a 3.5 L bioreactor (with aeration) were used for XOS production from CP by direct fermentation using *B. subtilis_xyn2* as described in section 2.4. Briefly, the fermentations were run during 30 h using the process conditions previously optimized for the flask-scale (section 3.4.), namely 10 g.L^{-1} of CP in Vogel 2% (w/v), at pH 7.0 and 45 °C, and with (2 vvm) or without aeration at 200 rpm. Figure 18 shows the average cell growth profiles based on OD_{600nm} results for the fermentations performed at bioreactor scale with and without aeration and at flask scale as control.

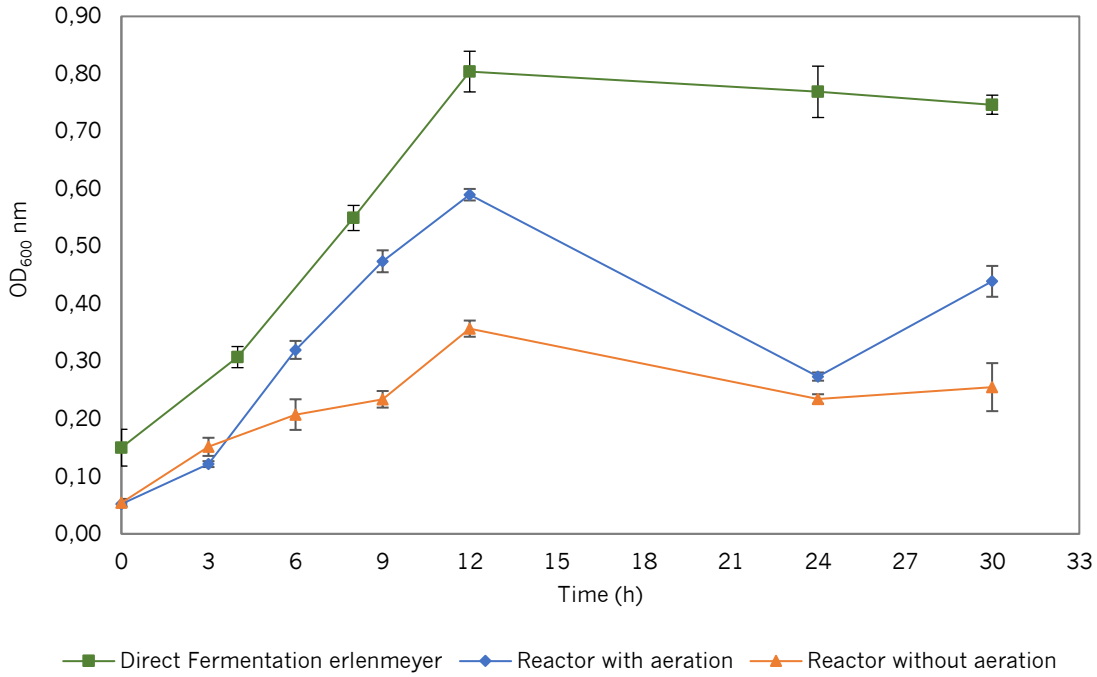

Figure 18- Optical density at 600 nm profile obtained for direct fermentation, using *Bacillus subtilis_xyn2,* of coffee silver skin pellet (CP) at flask scale and 3.5 L bioreactor scale with (2 vvm) and 3.7 L bioreactor scale without aeration. The fermentations were run using 10 g.L^{-1} of CP, pH 7.0 and 45 °C. Results represent the average of two independent assays ± standard deviation.

At the initial time we can observe that at flask scale we optical density (OD) is, three times higher than in the fermentations performed in the bioreactor scale. By analyzing the profile for the bioreactor without aeration we can observe that until 9 h of fermentation *B. subtilis_xyn2* exhibits growth difficulties. Comparing the $OD_{600\,nm}$ curves with and without aeration at 12 h it is possible to observe a 2-fold increase of the $OD_{600\,nm}$ when air is injected (from 0.36 ± 0.01 to 0.59 ± 0.01). Indeed, *B. subtilis* is a facultative anaerobic bacterium, which changes its metabolism depending on the availability of oxygen (O_2) present. Therefore, we can infer that on a large scale and without oxygen injection, the availability of O_2 is not sufficient for the microorganism growth (Nakano & Mario Hulett, 1997; André et al., 2021). Figure 19 shows the Y_{RS} profile of the fermentations with and without aeration and at flask scale as control, performed at bioreactor scale.

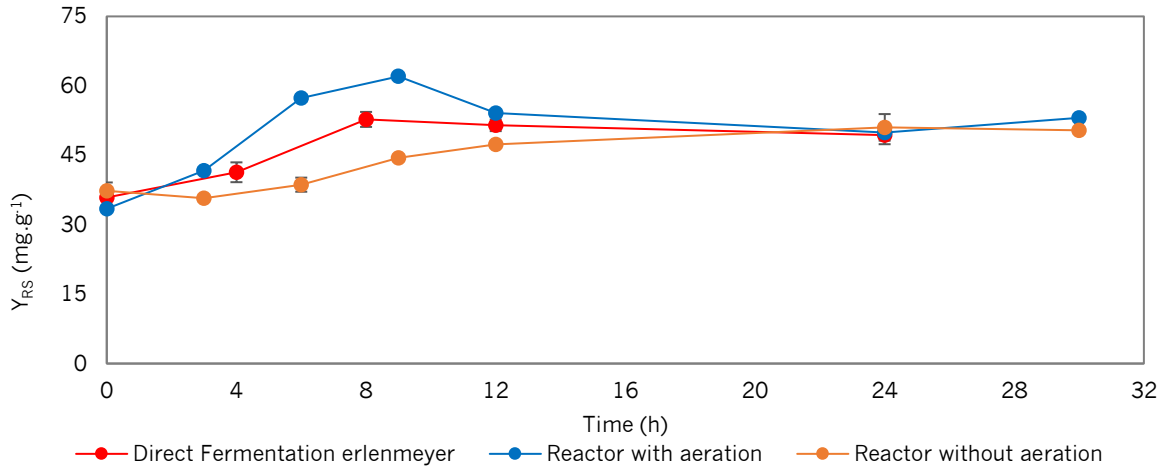

Figure 19- Reducing sugars production yield (Y_{RS}) obtained for direct fermentation of coffee silver skin pellet (CP) using *Bacillus subtilis_xyn2* at flask scale and 3.5 L bioreactor scale with (2 vvm) and 3.7 L bioreactor scale without aeration. The fermentations were run using 10 g.L^{-1} of CP, pH 7.0 and 45 °C. Results represent the average of two independent assays ± standard deviation.

The Y_{RSmax} for fermentation with and without aeration was obtained at 9 h (60.2 ± 0.3 and 44.4 ± 0.4 mg.g^{-1}, respectively). As for the fermentation in Erlenmeyer flasks, a Y_{RSmax} of 53 ± 2 mg.g^{-1} was obtained at 8 h. Comparing the results from Figures 18 and 19 it is suggested that the production of XOS is growth-associated, and the direct fermentation of CP requires a precise amount of biomass to allow a high production and a slow degradation of XOS, leading to their accumulation in a specific time.

This work reinforces the significant impact of the aeration on the metabolism of the microorganism and, consequently, on the process productivity. In order to effectively scale up the production of XOS by *B. subtilis_xyn2*, further studies are needed involving the optimization of certain operating parameters such as the amount of O_2 injected and agitation.

3.6. Prebiotic potential

In vitro experiments were conducted as described in section 2.9. with a mixture of human fecal inocula from 4 healthy donors to assess the prebiotic potential of the XOS mixture produced by direct fermentation of CP under optimal conditions previously determined (section 3.6.): pH 7.0, 37 °C and 10 $g.L^{-1}$. The fermentation was stopped at the optimal time (8 h), and the supernatant containing a sugar mixture rich in XOS was lyophilized and used to assess its prebiotic potential. Commercial oligofructose (CO) was used as positive control for comparison purposes, since it is a widely studied prebiotic (Paone et al., 2022). The prebiotic potential of XOS and oligofructose was assessed through the production of lactate and SCFAs (section 3.6.1.), pH variation and ammonium production (section 3.6.2.) and gas production (section 3.6.3.).

3.6.1. Production of lactate and short-chain fatty acids

The total production of lactate and the main SCFAs (≥ 95%) generated (namely acetate, propionate, n-butyrate and n-valerate) in the human colon is a consequence of multiple bacterial metabolic pathways (Gibson et al., 2017). Figure 20 shows the quantification of the lactate and SCFAs, namely acetate, propionate, butyrate and valerate after 24 and 48 h of fermentation of the mixture of faecal inocula from the four donors.

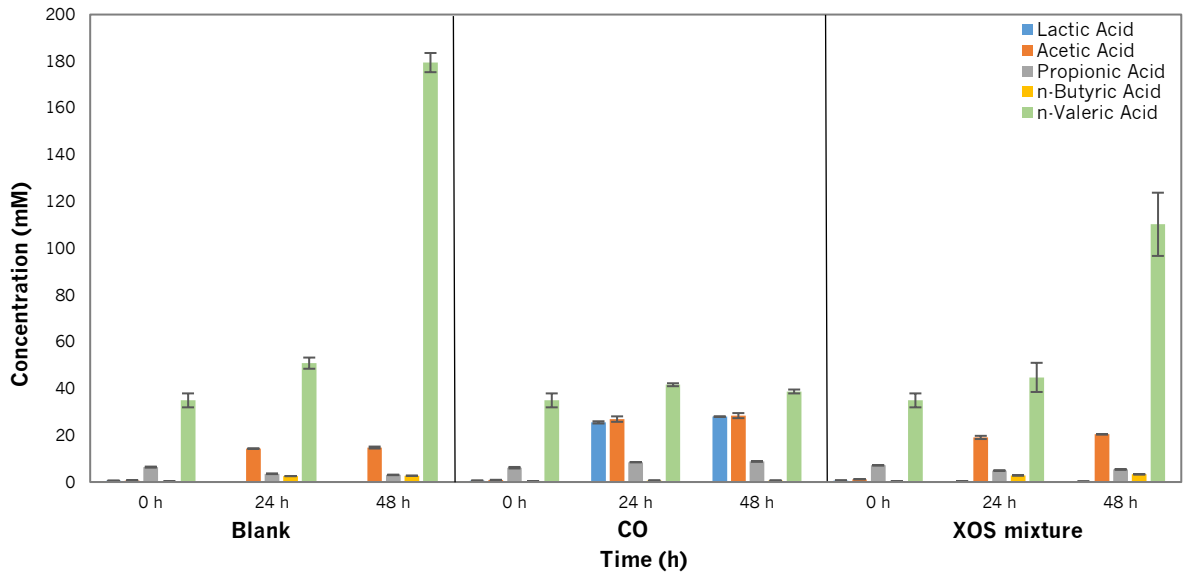

Figure 20- Quantification of the lactate and short chain fatty acids (SCFAs), namely acetate, propionate, butyrate and valerate after 48 h of fecal inocula growth from the mix of faecal human inocula in the absence of prebiotics (blank) or in a medium enriched with a prebiotic solution of oligofructose (CO) or XOS mixture at 10 $g.L^{-1}$. Results are the average of two independent fermentations ± standard deviation.

After 24 h the negative control (blank) presented an increase in the concentration of acetate (from 0.65 ± 0.01 to 14.3 ± 0.1 mM), butyrate (from 0.23 ± 0.01 to 2.52 ± 0.02 mM) and valerate (from 35 ± 1 mM), while there was a total reduction in the amount of lactate and the amount of propionate decreased by half (from 6.4 ± 0.2 to 3.4 ± 0.1 mM). After 48h, the concentration of acetate e butyrate increased slightly (to 14.8 ± 0.4 mM and 2.69 ± 0.06 mM, respectively), and the amount of propionate decreased to 3.0 ± 0.1 mM. The concentration of lactate remained zero.

Furthermore, the supplementation with 10 $g.L^{-1}$ of CO substantially increased the concentration of lactate and SCFAs (specially, acetate, propionate and valerate) after 24 h of fermentation, as expected. However, the concentration of valerate and propionate decreased after 48 h. Particularly, after 24 h, the concentration of lactate was approximately 44-fold higher than the initial time, acetate increased in about 34 times, propionate concentration slightly increased (1.4 times higher), the amount of butyrate approximately duplicated, while the concentration of valerate was 1.2-fold higher. Furthermore, after 48 h, lactate increased 1.1 times, acetate and propionate presented an increase of 1.03-fold, butyrate and valerate decreased 0.99 and 0.93 times, (respectively). Braga et al. (2022) reported similar results with increased production of lactate and acetate resulting from 10 g. L^{-1} of CO supplementation.

The addition of the XOS mixture (CP) also led to an increase in the concentration of SCFAs (acetate, butyrate and valerate) and a slightly decrease in the concentration of lactate and propionate. Namely, after 24 h, the concentration of acetate, butyrate and valerate increased 16, 10 and 1.3 times (respectively). As for the lactate and propionate concentrations it was observed a decrease of 0.5 and 0.7 times (respectively). Moreover, after 48 h, SCFAs (acetate, butyrate, valerate and propionate) presented an increase in concentration. Particularly, acetate and propionate concentrations increased in about 1.1 times, the amount of butyrate increased 1.2 times and valerate concentration duplicated, approximately. On the other hand, the amount of lactate presented a slight reduction of 0.7 times (to 0.20 ± 0.02 mM). These results are in line with previous reports by other authors who have also evaluated the prebiotic effect of commercial XOS using human faecal inocula (Carlson et al., 2017; Buruiana et al., 2017; Sajib et al., 2018).

When comparing to the negative control, CO presented significatively higher concentrations of acetate and SCFAs from 24 to 48 h ($p < 0.05$). As for the XOS mixture, we observed an increase in the SCFAs concentration, and the amount of lactate reduced significantly ($p < 0.05$) from 24 to 48 h. Additionally, when compared to the positive control (CO), XOS mixture presented a reduction statistically significant ($p = 0.0022$) in the lactate concentration from 24 to 48 h. Regarding the amount of SCFAs, in

the same period, it's also observed statistically significative differences ($p < 0.05$). Nevertheless, it is important to emphasize that the XOS used in this work were not purified after their production and, therefore, if purified XOS had been used, we might have achieved better performance. While the total amount of SCFAs produced indicates the prebiotic potential of the substrates tested, their production ratio is also important for estimating the potential health benefits that could be linked to the consumption of these substrates (Gibson et al., 2017).

On both substrates (CO and XOS mixture), acetate was the main SCFA with the higher concentration increase after 24 h of fermentation (27 ± 1 and 19.1 ± 0.7 mM, accordingly), while propionate was the second most produced (8.480 ± 0.008 and 4.9 ± 0.1 mM, respectively). Buruiana et al. (2017) and Carlson et al. (2017) also reported that acetate was the most abundant SCFA when XOS from corn stover and commercial XOS, respectively, were used as substrates for *in vitro* fermentation with human faecal microbiota. Acetate is reported to be beneficial for the prevention of colorectal cancer (Oliveira et al., 2015; Ferro et al., 2016; Casanova et al. 2018), while propionate has a major role in the inhibition of cholesterol synthesis and the deposition of adipose tissue and has been suggested as a dietary factor to suppress appetite and reduce obesity (Arora et al. 2011).

Curiously, XOS supplementation resulted in the highest concentration of butyrate (3.29 ± 0.04 mM) at 24 h. Butyrate has been reported as an essential metabolite for maintaining intestinal homeostasis and general health, having various beneficial effects, namely being associated with the prevention and suppression of colorectal cancer, and also operating at an extra-intestinal level (Gonçalves & Martel, 2013; Berni Canani et al, 2011). In contrast, the addition of oligofructose led to a significant increase in the amount of lactate (28 ± 0.1 mM) and a reduced production of butyrate. However, considering that lactate is a precursor of SCFAs, an increase in fermentation time could result in a reduction in lactate and a higher accumulation of SCFAs.

Thus, we can infer that the amount of lactate and SCFA seems to depend mostly on the addition of prebiotics (Figure 20). However, this observation needs further confirmation through tests with a larger sample size to obtain a more representative insight into the effects of different prebiotics, including individuals from different age groups, diet habits and health backgrounds. Additionally, the combination of different prebiotics may favor fermentation in large parts of the colon enhancing the prebiotic effect (Sajib et al., 2018).

3.6.2. pH change and ammonium production

The main health-promoting effects of prebiotic oligosaccharides are associated with the production of SCFA and the subsequent reduction in pH which promotes a decrease of pathogenic microbiota and an increase within the population of beneficial bacteria (Koh et al., 2016; Slavin, 2013). Table 4 shows the pH values obtained after 0, 24 and 48 h and table 5 shows the quantification of the ammonium concentration at 48 h of fermentation.

Table 7- Evaluation of the pH value after 0, 24 and 48 h of fecal inocula growth from the mixture of the four donors in the absence of prebiotics (Blank) or in a medium enriched with a commercial prebiotic solution of oligofrutose (CO) or XOS mixture (10 $g.L^{-1}$). Results are the average of two independent fermentations and duplicate analysis of each sample ± standard deviation. Values followed with different letters have statistically significant differences ($p \leq 0.05$) according to one-way ANOVA. Lowercase letters represent the analysis between different pH values over time, uppercase letters represent the analysis between different prebiotics tested at 48 h.

Time (h)	pH		
	Blank	**CO**	**XOS mixture**
0	7.58 ± 0.04^{aA}	7.55 ± 0.01^{aA}	7.53 ± 0.01^{aA}
24	6.87 ± 0.02^{bA}	3.93 ± 0.01^{bB}	6.96 ± 0.01^{bC}
48	6.85 ± 0.02^{bA}	3.90 ± 0.01^{bB}	6.97 ± 0.04^{bC}

Table 8- Evaluation of the ammonium concentration ($g.L^{-1}$) after 48 h of fecal inocula growth from the mixture of the four donors in the absence of prebiotics (Blank) or in a medium enriched with a commercial prebiotic solution of oligofrutose (CO) or XOS mixture (10 $g.L^{-1}$). Results are the average of two independent fermentations and duplicate analysis of each sample ± standard deviation. Values followed with different letters have statistically significant differences ($p \leq 0.05$) according to one-way ANOVA.

Sample	Ammonium ($mg.L^{-1}$)
Blank	170 ± 13^{a}
CO	50 ± 1^{b}
XOS mixture	158 ± 5^{a}

The addition of prebiotics resulted in a decrease in pH and ammonium values (Tables 4 and 5). In particular the supplementation with CO resulted in the highest decrease of ammonium content compared with the blank (3-fold). This reduction may be due to the higher degree of purity of CO when compared to the XOS mixture used. Ammonium is produced in the GI tract and absorbed through the intestinal mucosa, however, when present in high quantities, it can contribute to liver dysfunction (Parod, 2014). Accordingly, animal studies have reported that elevated levels of ammonium can damage the colonic mucus layer (Mutuyemungu et al., 2023), making the GI tract more susceptible to pathogens. The reduction in pH and ammonium concentration, suggest a potential prebiotic effect of the produced XOS mixture.

3.6.3. Gas production

The fermentation of oligosaccharides by fecal inocula also influences the amount of gases produced. This gas production can be quantified since *in vitro* models do not absorb the gases produced during the process (Poppe et al., 2023). Figure 21 shows the production of hydrogen (H_2), carbon dioxide (CO_2) and methane (CH_4) after 24 and 48 h of fermentation.

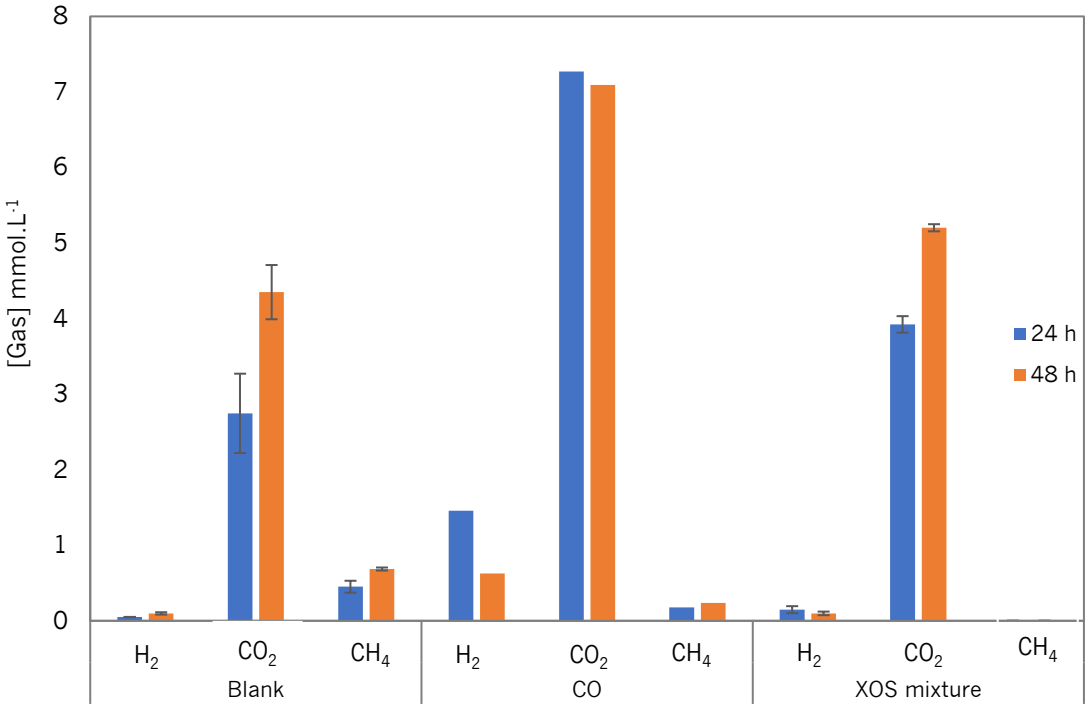

Figure 21- Production of H_2, CO_2 and CH_4 by human fecal inocula at 24 and 48 h in the absence of prebiotics (blank) or enriched with a prebiotic solution of Commercial oligofrutose (CO) or XOS mixture at 10 $g.L^{-1}$. Results are the average of two independent fermentations and duplicate analysis of each sample ± standard deviation.

As expected, larger volumes of CO_2 and H_2 were obtained from CO and XOS mixture when compared to the blank. These are the major gases produced in the gut microbiota by beneficial bacteria (Ghoddusi et al., 2007; Topping & Clifton, 2001). Thus, it is important to link the gas production results herein obtained with the modulation of the gut microbiota. Amorim et al. (2018) reported the same trend in gas production using *in vitro* models with human fecal inoculum and XOS mixture from brewer's spent grains (BSG). Methane was not detected in the mixture of human fecal inocula samples where the XOS mixture was added. Indeed, only some people harbor methanogenic bacteria that utilizes H_2 as electron donors to reduce CO_2 to CH_4 (Scanlan et al., 2008). However, CH_4 production is considered a potential biomarker for diagnosing symptoms and disorders of the gastrointestinal tract. High CH_4 levels are reported to be involved in decreased intestinal motility and constipation, especially in Inflammatory Bowel Disease (IBD) patients with constipation (Chatterjee et al., 2007, Kunkel et al., 2011, Pauff and Miller, 2012).

Overall, the CO_2 production increased in the cultures supplemented with CO and XOS mixture. The maximum production of CO_2 was 6.2 mmol. L^{-1} at 48 h for samples supplemented with CO and 5.2 ± 0.1 mmol. L^{-1} at 48 h for samples containing the XOS mixture. Since high gas production may has adverse effects such as flatulence, distension, and abdominal pain (Mutuyemungu et al., 2023), which constitute a clinical barrier to prebiotic use (Cummings et al., 2001), it is crucial to evaluate through *in vivo* models the recommended daily dose of prebiotic consumption.

Therefore, and considering the results obtained through SCFAs analysis, pH and ammonium analysis, we conclude that the XOS produced through the direct fermentation of CP using *B. subitlis_xyn2* exert positive prebiotic effects on the intestinal microbiota.

4. CONCLUSION

The main purpose of the present thesis was to produce XOS through direct fermentation of agro-residues, namely coffee silver skin (CSS) and coffee silver skin pellet (CP). This method has many advantages such as the simplification and cost reduction, which are attractive goals from the industrial point of view due the sustainability provided. The agro-residue, the process and the sterilization method presenting the highest potential for XOS production were selected for optimization and scale-up studies. Furthermore, through the direct fermentation of a commercial oligofructose (CO) and CP using *B. subtilis_xyn2* (under optimal conditions) was also studied the prebiotic potential of the XOS obtained.

The main conclusions of this thesis are outlined below:

- The chemical characterization through acid hydrolysis of the different agro-residues (CSS and CP), without the extractives, with 72% (w/w) sulfuric acid revealed that, as expected, CP and CSS presented similar compositions. When compared with previous studies that characterized brewer's spent grains (BSG) we observed that the content of hemicellulose, cellulose and lignin were similar. Therefore, since BSG was previously reported to be an efficient alternative substrate for XOS production by direct fermentation, we concluded that CSS and CP may also show potential for XOS production.

- Comparing direct fermentation using *B. subtilis_xyn2* to the use of a commercial xylanase from *T. longibrachiatum*, and considering factors such as the cost of purchasing commercial enzymes, the decrease of production time (8 h), the higher yield values, the equivalence of DP obtained and the potentially decrease of downstream costs, it was possible to confirm that direct fermentation is an advantageous approach to produce XOS using CP.

- The influence of the sterilization method (autoclave and UV treatment) in the production of XOS from 20 $g.L^{-1}$ of CP by *B. subtili_xyn2* through direct fermentation and enzymatic hydrolysis showed a higher yield of reducing sugars with the direct fermentation with autoclave sterilization (42.12 ± 0.02 mg. g^{-1}).

- The direct fermentation of CP using *B. subtilis_xyn2* showed a significant yield increase when the concentration of 10 $g.L^{-1}$ de CP were used. The used of 5, 20, 40 and 60 $g.L^{-1}$ showed a significant yield reduction. This effect could be due to several factors, namely substrate inhibition, mass transfer and aeration issues.

- Optimization of process variables, namely substrate concentration, pH and temperature revealed that the best conditions for direct fermentation using *B. subtilis_xyn2* were 10 $g.L^{-1}$ of CP, pH 7.0 and 45 °C. The amount of free xylose quantified under the optimal conditions showed a decrease in the concentration of free xylose, while the Y_{RSmax} through 8 h of fermentation (optimal time) increased. Lastly, the XOS produced presented a degree of polymerization (DP) over 6.

- The XOS production process in bioreactor by direct fermentation of CP using *B. subtilis_xyn2* (under the optimal conditions) reinforced the impact of the aeration on the metabolism of the microorganism and, consequently, on the process productivity. Furthermore, the optimization of certain operating parameters such as the quantity of O_2 injected and agitation are needed to effectively produced XOS at this scale.

- The prebiotic potential of the XOS mixture produced by direct fermentation of CP under optimal conditions (pH 7.0, 37 °C and 10 $g.L^{-1}$) was evaluated through *in vitro* experiments using a mixture of human fecal inocula from 4 healthy donors. SCFAs generation, pH variation and gas production suggest the potential prebiotic properties of the XOS produced.

- As expected, the fermentation of the XOS mixture by human fecal inocula resulted mainly in the production of acetate, followed by propionate and butyrate (accordingly with reported studies using commercial lactulose and XOS produced by direct fermentation of beechwood xylan), which are important metabolites linked to several health benefits.

- Commercial oligofructose (CO) led to the highest CO_2 production. The XOS mixture used in this assay was not purified. Therefore, future *in vitro* studies should be performed considering that purifying the XOS mixture could enhance the prebiotic effect.

5. FUTURE PERSPECTIVES

Throughout the development of this thesis, many questions emerged that require further investigation. Future approaches to the production of XOS and their potential application are detailed as follows:

- Since the results obtained for XOS production by direct fermentation of agro-residues were promising, it would be advantageous (a) optimize the fermentation process in the bioreactor for the agro-residues used in this work, i.e., optimize the agitation rate and the aeration; b) to evaluate the potential of new agro-residues with favorable chemical composition for XOS production, i.e. containing high hemicellulose/lignin ratios and improve their potential using strategies such as using direct fermentation; optimizing the substrate concentration and the agitation rate and optimize the fermentation process.

- High temperatures were proven advantageous for the direct fermentation of CP, since they improve the accessibility of xylanases to the xylan-lignin matrix, releasing free sugars from CP. As a result, they reduce the medium's viscosity and substrate inhibition. Hence, it would be interesting to use thermophilic xylanase-producing microorganisms.

- Given the new definition of prebiotic mentioned in this thesis and the necessity of more accurate instruments to fully evaluate the effect of prebiotic substances on the modulation of the gut microbiota, *in vitro* tests should be monitored by (a) Metabolomic evaluations, employing potentially reliable techniques such as nuclear magnetic resonance (NMR) or mass spectrometry (MS), to determine the metabolic profile of prebiotic responses and, subsequently, to identify the functionality of the microbiota; (b) high-throughput sequencing analysis, incorporating metagenomics to highlight alterations in the gut microbiota.

- The prebiotic effect of the XOS mixture obtained from CP should be evaluated in greater depth, considering the prospect of market application. To this end, a more complex *in vitro* model should be used, to enable the limitations presented by the model used in this study, including pH control during fermentation. In addition, a larger and more diverse sample selection should be tested to provide a representative insight into the potential effects of each prebiotic on different individuals, including individuals from various age groups, diet patterns and health backgrounds.

- Toxicological data from the XOS mixture obtained using CP must be collected from mammalian cell lines to obtain the approval of regulatory agencies for the collection of experimental data on human beings.

- Another point of interest is the development of a practical food application incorporating XOS into a food matrix, to produce a highly nutritious product with a low sugar content while promoting health benefits.

REFERENCES

Aachary, A. A., & Prapulla, S. G. (2009). Value addition to corncob: Production and characterization of xylooligosaccharides from alkali pretreated lignin-saccharide complex using Aspergillus oryzae MTCC 5154. Bioresource Technology, 100, 991–995.

Aachary, A. A., & Prapulla, S. G. (2011). Xylooligosaccharides (XOS) as an emerging prebiotic: Microbial synthesis, utilization, structural characterization, bioactive properties, and applications. *Comprehensive Reviews in Food Science and Food Safety, 10*, 2–16. https://doi.org/10.1111/j.1541-4337.2010.00135

Aachary, A. A., Gobinath, D., Srinivasan, K., & Prapulla, S. G. (2015). Protective effect of xylooligosaccharides from corncob on 1,2-dimethylhydrazine induced colon cancer in rats. Bioactive Carbohydrates and Dietary Fibre, 5, 146–152.

Aagaard, K., Riehle, K., Ma, J., Segata, N., Mistretta, T.-A., Coarfa, C. et al. (2012) A metagenomic approach to characterization of the vaginal microbiome signature in pregnancy. PLoS ONE 7, e36466 doi: 10.1371/journal.pone.0036466

Abasubong, K. P., Liu, W.-B., Zhang, D.-D., Yuan, X.-Y., Xia, S.-L., Xu, C., & Li, X.-F. (2018). Fishmeal replacement by rice protein concentrate with xylooligosaccharides supplement benefits the growth performance, antioxidant capability and immune responses against Aeromonas hydrophila in blunt snout bream (Megalobrama amblycephala). Fish and Shellfish Immunology, 78, 177–186.

Ahmad, R. (2019). Better health with plants plant oligosaccharides: Trends and future. *Proceeding of the International Conference on Food and Agriculture, 2* (1), 601–607.

Akpinar, O., Ak, O., Kavas, A., Bakir, U., Yilmaz, L., 2007. Enzymatic production of xylooligosaccharides from cotton stalks. J. Agric. Food Chem. 55, 5544–5551. https://doi.org/10.1021/jf063580d.

Akpinar, O., Erdogan, K., & Bostanci, S. (2009a). Production of xylooligosaccharides by controlled acid hydrolysis of lignocellullosic materials. Carbohydrate Research, 344, 660–666.

Akpinar, O., Erdogan, K., Bakir, U., Yilmaz, L., 2010. Comparison of acid and enzymatic hydrolysis of tobacco stalk xylan for preparation of xylooligosaccharides. LWT - Food Sci. Technol. 43, 119–125. https://doi.org/ 10.1016/j.lwt.2009.06.025.

Al-Swidi, A., Huque, S. M. R., Hafeez, M. H., & Shariff, M. N. M. (2014). The role of subjective norms in theory of planned behavior in the context of organic food consumption. *British Food Journal*, *116*, 1561–1580.

Alvarez, C., González, A., Alonso, J. L., S´aez, F., Negro, M. J., & Gull´on, B. (2020). Xylooligosaccharides from steam-exploded barley straw: Structural features and assessment of bifidogenic properties. *Food and Bioproducts Processing, 124*, 131–142. https://doi.org/10.1016/j.fbp.2020.08

Amorim C, Silvério SC, Cardoso BB, Alves JI, Pereira MA, Rodrigues LR. *In vitro* assessment of prebiotic properties of xylooligosaccharides produced by *Bacillus subtilis* 3610. Carbohydr Polym. 2020 Feb 1; 229:115460. doi: 10.1016/j.carbpol.2019.115460

Amorim C, Silvério SC, Cardoso BB, Alves JI, Pereira MA, Rodrigues LR. From lignocellulosic residues to market: Production and commercial potential of xylooligosaccharides, Biotechnology Advances, Volume 37, Issue 7, 2019, 107397, ISSN 0734-9750, doi: 10.1016/j.biotechadv.2019.05.003.

Amorim C, Silvério SC, Silva SP, Coelho E, Coimbra MA, Prather KLJ, Rodrigues LR. Single-step production of arabino-xylooligosaccharides by recombinant *Bacillus subtilis* 3610 cultivated in brewers' spent grain. Carbohydr Polym. 2018 Nov 1; 199:546-554. doi: 10.1016/j.carbpol.2018.07.017.

Amorim, C. C., Rodrigues, L. R., & Prather, K. J. (2018). Development of a bioprocess for the production of xylooligosaccharides [PhD thesis, University of Minho]. https://hdl.handle.net/1822/60591

Amorim, C., Silvério, S. C., & Rodrigues, L. R. (2019). One-step process for producing prebiotic arabino-xylooligosaccharides from brewer's spent grain employing *Trichoderma* species. Food Chemistry, 270, 86–94.

Amorim, C., Silvério, S. C., Gonçales, R. F. S., Pinheiro, A. C., Silva, S. P., Coelho, E., et al. (2019). Downscale fermentation for xylooligosaccharides production by recombinant *Bacillus subtilis* 3610. Carbohydrate Polymers, 205, 176–183.

André AC, Debande L, Marteyn BS. The selective advantage of facultative anaerobes relies on their unique ability to cope with changing oxygen levels during infection. Cell Microbiol. 2021 Aug;23(8):e13338. doi: 10.1111/cmi.13338. Epub 2021 Apr 20. PMID: 33813807

Arantes, A. L., Alves, J. I., Alfons, J. M., Alves, M. M., & Sousa, D. Z. (2018). Enrichment of syngas-converting communities from a multi-orifice baffled bioreactor. Microbial Biotechnology, 11, 639–646.

Arike, L. and Hansson, G.C. (2016) The densely O-glycosylated MUC2 mucin protects the intestine and provides food for the commensal bacteria. J. Mol. Biol

Arora, T., Sharma, R., & Fros, G. (2011). Propionate, Anti-obesity and satiety enhancing factor? Appetite, 56, 511-515.

Arpaia, N., Campbell, C., Fan, X., Dikiy, S., van der Veeken, J., deRoos, P. et al. (2013) Metabolites produced by commensal bacteria promote peripheral regulatory T-cell generation. Nature. 504, 451–455 doi:10.1038/nature12726

Avershina, E., Storrø, O., Øien, T., Johnsen, R., Pope, P., Rudi, K. (2014) Major faecal microbiota shifts in composition and diversity with age in a geographically restricted cohort of mothers and their children. FEMS Microbiol. Ecol. 87, 280–290 doi:10.1111/1574-6941.12223

Ávila, P.F., Martins, M., de Almeida Costa, F.A., Goldbeck, R., 2020. Xylooligosaccharides production by commercial enzyme mixture from agricultural wastes and their prebiotic and antioxidant potential. Bioact. Carbohyd. Diet. Fibr. 24, 100234.

Azad, M., Kalam, A., Sarker, M., Li, T. and Yin, J. 2018. Probiotic species in the modulation of gut microbiota: an overview. BioMed Research International 2018: article ID 9478630.

Bäckhed, F. (2011) Programming of host metabolism by the gut microbiota. Ann. Nutr. Metab. 58(Suppl 2), 44–52 doi:10.1159/000328042

Bäckhed, F., Roswall, J., Peng, Y., Feng, Q., Jia, H., Kovatcheva-Datchary, P. et al. (2015) Dynamics and stabilization of the human gut microbiome during the first year of life. Cell Host Microbe 17, 852 doi: 10.1016/j.chom.2015.05.012

Bae, Y. J., Bak, Y. K., Kim, B., Kim, M. S., Lee, J. H. and Sung, M. K. 2011. Coconut-derived D-xylose affects postprandial glucose and insulin responses in healthy individuals. Nutrition Research and Practice 5(6): 533-539.

Ballesteros, L.F., Teixeira, J.A. & Mussatto, S.I. Chemical, Functional, and Structural Properties of Spent Coffee Grounds and Coffee Silverskin. *Food Bioprocess Technol* 7, 3493–3503 (2014). https://doi.org/10.1007/s11947-014-1349-z

Basto, B. I., Teixeira, Teixeira, J., & Silvério, S. I., Cruz (2019). *Optimization of fungal pigments production through different types of fermentation* [Master's thesis, University of Minho]. https://hdl.handle.net/1822/64899

Batidzirai, B., Valk, M., Wicke, B., Junginger, M., Daioglou, V., Euler, W., Faaij, A.P.C., 2016. Current and future technical, economic and environmental feasibility of maize and wheat residues supply for biomass energy application: illustration for South Africa. Biomass Bioenergy 92, 106–129. https://doi.org/10.1016/j.biombioe.2016.06.010.

Bäumler, A.J. and Sperandio, V. (2016) Interactions between the microbiota and pathogenic bacteria in the gut. Nature 535, 85 93 doi:10.1038/ nature18849

Benítez-Páez, A., Kjølbæk, L., Del Pulgar, E. M. G., Brahe, L. K., Astrup, A., Matysik, S., ... and Sanz, Y. 2019. A multi-omics approach to unraveling the microbiome-mediated effects of arabinoxylan oligosaccharides in overweight humans. mSystems 4(4): article ID e00209-19.

Berni Canani, R., Di Costanzo, M., Leone, L., Pedata, M., Meli, R., & Calignano, A. (2011). Potential beneficial effects of butyrate in intestinal and extraintestinal diseases. World Journal of

Biagi, E., Candela, M., Turroni, S., Garagnani, P., Franceschi, C., Brigidi, P. (2013) Ageing and gut microbes: perspectives for health maintenance and longevity. Pharmacol. Res. 69, 11–20 doi: 10.1016/j.phrs.2012.10.005

Bian, J., Peng, P., Peng, F., Xiao, X., Xu, F., Sun, R.C., 2014. Microwave-assisted acid hydrolysis to produce xylooligosaccharides from sugarcane bagasse hemicelluloses. Food Chem. 156, 7–13. https://doi.org/10.1016/j.foodchem.2014.01.112.

Blanton, L.V., Charbonneau, M.R., Salih, T., Barratt, M.J., Venkatesh, S., Ilkaveya, O. et al. (2016) Gut bacteria that prevent growth impairments transmitted by microbiota from malnourished children. Science 351, aad3311 doi:10.1126/science. aad3311

Boets, E. et al. (2017). Systemic availability and metabolism of colonic-derived short-chain fatty acids in healthy subjects: a stable isotope study. Journal of Physiology, 595, 541–555.
Boini, K. M., Koka, S., Xia, M., Ritter, J. K., Gehr, T. W. and Li, P. L. 2018. Sphingolipids in obesity and related complications. Frontiers in Bioscience 22: 96-116.

Braga, A., Gomes, D., Amorim, C., Silvério, S. C., Alves, J., Rainha, J., Cardoso, B. B., Rodrigues, J. L., & Rodrigues, L. R. (2022). One-step production of a novel prebiotic mixture using Zymomonas mobilis ZM4. *Biochemical Engineering Journal*, *183*, 108443. https://doi.org/10.1016/j.bej.2022.108443

Brenelli, L.B., Bhatia, R., Djajadi, D.T., Thygesen, L.G., Rabelo, S.C., Leak, D.J., Franco, T.T., Gallagher, J.A., 2022. Xylo-oligosaccharides, fermentable sugars, and bioenergy production from sugarcane straw using steam explosion pretreatment at pilot-scale. Bioresour. Technol. 357, 127093. https://doi.org/10.1016/ j.biortech.2022.127093

Browne, H.P., Forster, S.C., Anonye, B.O., Kumar, N., Neville, B.A., Stares, M.D. et al. (2016) Culturing of 'unculturable' human microbiota reveals novel taxa and extensive sporulation. Nature 533, 543–546 doi:10.1038/nature17645

Buruiana, C. T., Gómez, B., Vizireanu, C., & Garrote, G. (2017). Manufacture and evaluation of xylooligosaccharides from corn stover as emerging prebiotic candidates for human health. LWT-Food Science and Technology, 70, 449-459.

Cadavez, V. (22 de 11 de 2022). Determinação do Teor de Azoto em Alimentos para Animais. p. 6.

Canfora, E. E., Jocken, J. W. and Blaak, E. E. 2015. Short-chain fatty acids in control of body weight and insulin sensitivity. Nature Reviews Endocrinology 11(10): 577-591.

Carlson, J. L., Erickson, J. M., Hess, J. M., Gould, T. J., Slavin, J. L. (2017). Prebiotic dietary fiber and gut health: Comparing the *in vitr*o fermentations of beta-glucan, inulin and xylooligosaccharide. Nutrients, 9, 1361.

Carrillo, I., Mendonça, R. T., Ago, M., & Rojas, O. J. (2018). Comparative study of cellulosic components isolated from different Eucalyptus species. *Cellulose, 25* (2), 1011–1029. https://doi.org/10.1007/s10570-018-1653-2

Carvalho, A.F.A., Neto, P.O., de Almeida, P.Z., da Silva, J.B., Escaramboni, B., Pastore, G.M., 2015. Screening of xylanolytic Aspergillus fumigatus for prebiotic xylooligosaccharides production using bagasse. Food Technol. Biotechnol. 53, 428–435. https://doi.org/10.17113/ftb.53.04.15.4160.

Carvalho, A.F.A., Neto, P.O., Silva, D.F., Pastore, G.M., 2013. Xylo-oligosaccharides from lignocellulosic materials: chemical structure, health benefits and production by chemical and enzymatic hydrolysis. Food Res. Int. 51, 75–85. https://doi.org/10.1016/j.foodres.2012.11.021.

Casanova, M.R., Azevedo-Silva, J., Rodrigues, L. R., & Preto, A. (2018). Colorectal cancer cells increase the production of short chain fatty acids by Propionibacterium freudenreichii impacting on cancer cells survival. Frontiers in Nutrition, 5, 44.

Chapla, D., Pandit, P., Shah, A., 2012. Production of xylooligosaccharides from corncob xylan by fungal xylanase and their utilization by probiotics. Bioresour. Technol. 115, 215–221. https://doi.org/10.1016/j.biortech.2011.10.083.

Charalampopoulos, D., & Rastall, R. A. (2012). Prebiotics in foods. *Current Opinion in Biotechnology, 23*, 187–191. https://doi.org/10.1016/j.copbio.2011.12.028

Charbonneau, M.R., O'Donnell, D., Blanton, L.V., Totten, S.M., Davis, J.C.C., Barratt, M.J. et al. (2016) Sialylated milk oligosaccharides promote microbiota-Dependent growth in models of infant undernutrition. Cell 164, 859–871 doi: 10.1016/j.cell.2016.01.024

Chatterjee, S., Park, S., Low, K., Kong, Y., & Pimentel, M. (2007). The degree of breath methane production in IBS correlates with the severity of constipation. *Official journal of the American College of Gastroenterology | ACG*, *102*(4), 837-841.

Chunchai, T., Thunapong, W., Yasom, S., Wanchai, K., Eaimworawuthikul, S., Metzler, G., ... and Chattipakorn, S. C. 2018. Decreased microglial activation through gut-brain axis by prebiotics, probiotics, or synbiotics effectively restored cognitive function in obese-insulin resistant rats. Journal of Neuroinflammation 15(1): article no. 11.

Coelho, E., Rocha, M. A. M., Saraiva, J. A., & Coimbra, M. A. (2014). Microwave superheated water and dilute alkali extraction of brewers' spent grain arabinoxylans and arabinoxylo-oligosaccharides. Carbohydrate Polymers, 99, 415–422.

Collins, S.; Reid, G. Distant site effects of ingested prebiotics. Nutrients 2016, 8, 523.

Cordeiro, A. C., Pereira, Braga, A., & Belo, I. (2019). *Selection of yarrowia lipolytica strains with potential for aromatic alcohols production* [Master's thesis, University of Minho]. https://hdl.handle.net/1822/64698

Crost, E.H. et al. (2016) The mucin-degradation strategy of *ruminococcus gnavus*: The importance of intramolecular trans-sialidases. Gut Microbes 1–11

Crost, E.H., Tailford, L.E., Le Gall, G., Fons, M., Henrissat, B., Juge, N. et al. (2013) Utilization of mucin glycans by the human Gut symbiont ruminococcus gnavus Is strain-Dependent. PLoS One 8, e76341 doi: 10.1371/journal.pone.0076341

Cummings, J. H., Macfarlane, G. T., & Englyst, H. N. (2001). Prebiotic digestion and fermentation. The American Journal of Clinical Nutrition. 73, 415s-420s.

David, L.A., Maurice, C.F., Carmody, R.N., Gootenberg, D.B., Button, J.E., Wolfe, B.E. et al. (2013) Diet rapidly and reproducibly alters the human gut microbiome. Nature 505, 559–563 doi:10.1038/nature12820

De Freitas, C., Carmona, E. and Brienzo, M. 2019. Xylooligosaccharides production process from lignocellulosic biomass and bioactive effects. Bioactive Carbohydrates and Dietary Fibre 18: article ID 100184.

Debjani Ghosh, Anil B. Vir, Gil Garnier, Antonio F. Patti, Joanne Tanner, Continuous flow production of xylooligosaccharides by enzymatic hydrolysis, Chemical Engineering Science, Volume 244, 2021, 116789, ISSN 0009-2509, https://doi.org/10.1016/j.ces.2021.116789.

den Besten, G., van Eunen, K., Groen, A.K., Venema, K., Reijngoud, D.-J., Bakker, B.M. (2013) The role of short-chain fatty acids in the interplay between diet, gut microbiota, and host energy metabolism. J. Lipid Res. 54, 2325–2340 doi:10.1194/jlr. R036012

Donaldson, G.P., Lee, S.M. and Mazmanian, S.K. (2015) Gut biogeography of the bacterial microbiota. Nat. Rev. Microbiol. 14, 20–32 doi:10.1038/nrmicro3552

Dutta, S., Wu, K.C.-W., 2014. Enzymatic breakdown of biomass: enzyme active sites, immobilization, and biofuel production. Green Chem. 16, 4607–4776. https://doi. org/10.1039/C4GC01405G.

Earle, K.A., Billings, G., Sigal, M., Lichtman, J.S., Hansson, G.C., Elias, J.E. et al. (2015) Quantitative imaging of gut microbiota spatial organization. Cell Host Microbe 18, 478–488 doi: 10.1016/j.chom.2015.09.002

Falony, G., Vandeputte, D., Caenepeel, C., Vieira-Silva, S., Daryoush, T., Vermeire, S., et al. (2019). The human microbiome in health and disease: hype or hope. Acta Clin. Belgica 74, 53–64. doi: 10.1080/17843286.2019.1583782

Falony, G., Vieira-Silva, S., and Raes, J. (2018). Richness and ecosystem development across faecal snapshots of the gut microbiota. Nat. Microbiol. 3, 526–528. doi: 10.1038/ s41564-018-0143-5

Faryar, R., Linares-Pastén, J. A., Immerzeel, P., Mamo, G., Andersson, M., Stålbrand, H. and Karlsson, E. N. 2015. Production of prebiotic xylooligosaccharides from alkaline extracted wheat straw using the K80R-variant of a thermostable alkali-tolerant xylanase. Food and Bioproducts Processing 93: 1-10.

Ferro, S., Azevedo-Silva, J., Casal, M., Côrte-Real, M., Baltazar, F., & Preto, A. (2016). Characterization of acetate transport in colorectal cancer cells and potential therapeutic implications. Oncotarget, 7, 70639-70653.

Figueiredo, F. C., Carvalho, A. F. A., Brienzo, M., Campioni, T. S., & Oliva-Neto, P. (2017). Chemical input reduction in the arabinoxylan and lignocellulose alkaline extraction and xylooligosaccharides production. Bioresource Technology, 228, 164–170.

Finegold, S. M., Li, Z., Summanen, P. H., Downes, J., Thames, G., Corbett, K., Dowd, S., Krak, M., & Heber, D. (2014). Xylooligosaccharide increases bifidobacteria but not *lactobacilli* in human gut microbiota. *Food & Function, 5*(3), 436. https://doi.org/10.1039/c3fo60348b

Flint, H. J., Duncan, S. H., Scott, K. P., & Louis P. (2015). Conference on 'Diet, gut microbiology and human health' Symposium 3: Diet and gut metabolism: linking microbiota to beneficial products of fermentation Links between diet, gut microbiota composition and gut metabolism, Proceedings of the Nutrition Society. 74, 13–22.

Flint, H.J.; Scott, K.P.; Louis, P.; Duncan, S.H. The role of the gut microbiota in nutrition and health. Nat. Rev. Gastroenterol. Hepatol. 2012, 9, 577–589.

Forbes (February 18th, 2015) Consumers Want Healthy Foods-And Will Pay More For Them accessed on 10/10/2018 https://www.forbes.com/sites/nancygagliardi/2015/02/18/consumers-want-healthy-foods-and-will-pay-more-for-them/#24de393275c5.

Francesca Turroni, Christian Milani, Marco Ventura, Douwe van Sinderen, The human gut microbiota during the initial stages of life: insights from bifidobacteria, Current Opinion in Biotechnology, Volume 73, 2022, Pages 81-87, ISSN 0958-1669, doi: 10.1016/j.copbio.2021.07.012.

Furusawa, Y., Obata, Y., Fukuda, S., Endo, T.A., Nakato, G., Takahashi, D. et al. (2013) Commensal microbe-derived butyrate induces the differentiation of colonic regulatory T cells. Nature. 504, 446–450 doi:10.1038/nature12721

Gao, Y., Wang, Y., Li, Y., Han, R., Li, C., Xiao, L., Cho, S., Ma, Y., Fang, C., & Lee, A. W. (2017). Repeated sub-chronic oral toxicity study of xylooligosaccharides (XOS) in dogs. Regulatory Toxicology and Pharmacology, 86, 379-385. generation and prebiotic effects of some carbohydrates and their mixtures. Anaerobe, 13, 193-199. Gastroenterology, 17, 1519–1528.

Gensollen, T., Iyer, S.S., Kasper, D.L., Blumberg, R.S. (2016) How colonization by microbiota in early life shapes the immune system. Science 352, 539–544 doi:10.1126/science. aad9378

Ghoddusi HB, Grandison MA, Grandison AS, Tuohy KM. *In vitro* study on gas generation and prebiotic effects of some carbohydrates and their mixtures. Anaerobe. 2007 Oct-Dec;13(5-6):193-9. doi: 10.1016/j.anaerobe.2007.06.002. Epub 2007 Jun 14. PMID: 17669667.

Gibson, G. R., Hutkins, R., Sanders, M. E., Prescott, S. L., Reimer, R. A., Salminen, S. J., Scott, K., Stanton, C., Swanson, K. S., Cani, P. D., Verbeke, K., & Reid, G. (2017). The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. Nature Reviews Gastroenterology & Hepatology, 14, 491–502.

Gobinath, R., Shanmugavelua, K. and Yang, K. L. 2017. Production of prebiotic-xylooligosaccharides from alkali pretreated mahogany and mango wood sawdust by using purified xylanase of *Clostridium* strain BOH3. Carbohydrate Polymer 167: 158-166.

Gonçalves, D.A., Teixeira, J.A., & Nobre, C. (2022). In situ enzymatic synthesis of prebiotics to improve food functionality. Value-Addition in Food Products and Processing Through Enzyme Technology, 253-267, https://doi.org/10.1016/B978-0-323-89929-1.00026-3.

Gonçalves, P., Martel, F. (2013) Butyrate and colorectal cancer: the role of butyrate transport. Current Drug Metabolism, 14, 994-1008.

Hansen, C. H. F., Frøkiær, H., Christensen, A. G., Bergström, A., Licht, T. R., Hansen, A. K. and Metzdorff, S. B. 2013. Dietary xylooligosaccharide downregulates IFN-γ and the low-grade inflammatory cytokine IL-1β systemically in mice. The Journal of Nutrition 143(4): 533-540.

Härtig, E., & Jahn, D. (2012). *Chapter Five - Regulation of the Anaerobic Metabolism in Bacillus subtilis* (61st ed., pp. 195-216). Advances in Microbial Physiology. https://doi.org/10.1016/B978-0-12-394423-8.00005-6

Heinen, P. R., Pereira, M. G., Rechia, C. G. V., Almeida, P. Z., Monteiro, L. M. O., Pasin, T. M., et al. (2017). Immobilized endo-xylanase of Aspergillus tamarii Kita: An interesting biological tool for production of xylooligosaccharides at high temperatures. Process Biochemistry, 53, 145–152.

Helianti, I., Ulfah, M., Nurhayati, N., Suhendar, D., Finalissari, A. K., & Wardani, A. K. (2016). Production of xylanase by recombinant *Bacillus subtilis* DB104 cultivated in agroindustrial waste medium. HAYATI Journal of Biosciences, 23, 125–131.

Ho, A.L., Kosik, O., Lovegrove, A., Charalampopoulos, D., Rastall, R.A., 2018. *In vitro* fermentability of xylo-oligosaccharide and xylo-polysaccharide fractions with different molecular weights by human faecal bacteria. Carbohydr Polym 179, 50–58. https://doi.org/10.1016/j.carbpol.2017.08.077.

Ho, L., & Heng, K. L. (2015). Xylanase production by *Bacillus subtilis* in cost-effective medium using soybean hull as part of medium composition under Submerged Fermentation (SmF) and Solid-State Fermentation (SsF). Journal of Biodiversity, Bioprospecting and Development, 2, 1–13.

Hugon, P., Dufour, J.-C., Colson, P., Fournier, P.-E., Sallah, K., Raoult, D. (2015) A comprehensive repertoire of prokaryotic species identified in human beings. Lancet Infect. Dis. 15, 1211–1219 doi:10.1016/S1473-3099(15)00293-5

Illanes, A., Guerrero, C., Vera, C., Wilson, L., Conejeros, R., & Scott, F. (2016). *Lactose-derived prebiotics*. Elsevier. https://doi.org/10.1016/C2014-0-03393-X.

Irfan, M., Asghar, U., Nadeem, M., Nelofer, R., & Syed, Q. (2018). Optimization of process parameters for xylanase production by *Bacillus* sp. in submerged fermentation. Journal of Radiation Research and Applied Sciences, 9, 139–147.

Jagtap, S., Deshmukh, R. A., Menon, S., & Das, S. (2017). Xylooligosaccharides production by crude microbial enzymes from agricultural waste without prior treatment and their potential application as nutraceuticals. *Bioresource Technology, 245*, 283–288. https://doi.org/10.1016/j.biortech.2017.08.174

Jakobsson, H.E., Abrahamsson, T.R., Jenmalm, M.C., Harris, K., Quince, C., Jernberg, C. et al. (2014) Decreased gut microbiota diversity, delayed *Bacteroidetes* colonisation and reduced Th1 responses in infants delivered by caesarean section. Gut 63, 559–566 doi:10.1136/gutjnl-2012-303249

Juge, N., Tailford, L. and Owen, C.D. (2016) Sialidases from gut bacteria: a mini review. Biochem Soc Trans 44, 166–175 doi:10.1042/BST20150226

Kakiyama, G., Pandak, W.M., Gillevet, P.M., Hylemon, P.B., Heuman, D.M., Daita, K. et al. (2013) Modulation of the fecal bile acid profile by gut microbiota in cirrhosis. J Hepatol 58, 949–955 doi: 10.1016/j.jhep.2013.01.003

Kallel, F., Driss, D., Bouaziz, F., Neifer, M., Ghorbel, R., & Chaabounia, S. E. (2014). Production of xylooligosaccharides from garlic straw xylan by purified xylanase from *Bacillus mojavensis* UEB-FK and their *in vitro* evaluation as prebiotics. Food and Bioproducts Processing, 94, 536–546.

Kaprelyants, L., Zhurlova, O., Shpyrko, T., Pozhitkova, L., 2017. Xylooligosaccharides from agricultural by-products: characterisation, production and physiological effects. Food Sci. Technol. 11, 25–34. https://doi.org/10.15673/fst.v11i3.606.

Khat-udomkiri, N., Toejing, P., Sirilun, S., Chaiyasut, C. and Lailerd, N. 2019. Antihyperglycemic effect of rice husk derived xylooligosaccharides in high-fat diet and low-dose streptozotocin-induced type 2 diabetic rat model. Food Science and Nutrition 8(1): 428-444.

Koenig, J.E., Spor, A., Scalfone, N., Fricker, A.D., Stombaugh, J., Knight, R. et al. (2011) Succession of microbial consortia in the developing infant gut microbiome. Proc. Natl. Acad. Sci. U.S.A. 108(Suppl 1), 4578–4585 doi:10.1073/pnas.1000081107

Koh, A., De Vadder, F., Kovatcheva-Datchary, P., & B¨ackhed, F. (2016). From dietary fiber to host physiology: Short-chain fatty acids as key bacterial metabolites. *Cell, 165*, 1332–1345. https://doi.org/10.1016/j.cell.2016.05.041

Korsten, L., & Cook, N. (1996). Optimizing culturing conditions for *Bacillus subtilis*. South African Avocado Growers' Association Yearbook, 19, 54–58.

Kumar, V., Satyanarayana, T., 2015. Generation of xylooligosaccharides from microwave irradiated agroresidues using recombinant thermo-alkali-stable endoxylanase of the polyextremophilic bacterium *Bacillus halodurans* expressed in Pichia pastoris. Bioresour. Technol. 179, 382–389. https://doi.org/10.1016/j.biortech.2014.12.049.

Kunkel, D., Basseri, R. J., Makhani, M. D., Chong, K., Chang, C., & Pimentel, M. (2011). Methane on breath testing is associated with constipation: a systematic review and meta-analysis. *Digestive diseases and sciences*, *56*, 1612-1618.

Kushwah, S., Dhir, A., & Sagar, M. (2019). Understanding consumer resistance to the consumption of organic food. A study of ethical consumption, purchasing, and choice behaviour. *Food Quality and Preference*, *77*, 1–14.

Larsson, J.M.H., Karlsson, H., Crespo, J.G., Johansson, M.E.V., Eklund, L., Sjövall, H. et al. (2011) Altered o-glycosylation profile of MUC2 mucin occurs in active ulcerative colitis and is associated with increased inflammation. Inflamm Bowel Dis 17, 2299–2307 doi:10.1002/ibd.21625

Lee, J.-E., Min, S. H., Lee, D.-H., Oh, T. J., Kim, K. M., Moon, J. H., ... and Lim, S. 2016. Comprehensive assessment of lipoprotein subfraction profiles according to glucose metabolism status, and association with insulin resistance in subjects with early-stage impaired glucose metabolism. International Journal of Cardiology 225: 327-331.

Li, H., Limenitakis, J.P., Fuhrer, T., Geuking, M.B., Lawson, M.A., Wyss, M. et al. (2015) The outer mucus layer hosts a distinct intestinal microbial niche. Nat. Commun. 6, 8292 doi:10.1038/ncomms9292

Li, J., Jia, H., Cai, X., Zhong, H., Feng, Q., Sunagawa, S. et al. (2014) An integrated catalog of reference genes in the human gut microbiome. Nat. Biotechnol. 32, 834–841 doi:10.1038/nbt.2942

Lim, E., Lim, J. Y., Shin, J. H., Seok, P. R., Jung, S., Yoo, S. H. and Kim, Y. 2015. D-Xylose suppresses adipogenesis and regulates lipid metabolism genes in high-fat diet-induced obese mice. Nutrition Research 35(7): 626-636.

Lim, E., Lim, J., Kim, E., Kim, Y.-S., Shin, J.-H., Seok, P. and Kim, Y. 2016. Xylobiose, an alternative sweetener, ameliorates diabetes-related metabolic changes by regulating hepatic lipogenesis and miR-122a/33a in db/db mice. Nutrients 8(12): article no. 791.

Lim, S., Kim, E., Shin, J.-H., Seok, P., Jung, S., Yoo, S.-H. and Kim, Y. 2018. Xylobiose prevents high-fat diet induced mice obesity by suppressing mesenteric fat deposition and metabolic dysregulation. Molecules 23(3): article no. 705.

Long, J., Yang, J., Henning, S. M., Woo, S. L., Hsu, M., Chan, B., ... and Li, Z. 2019. Xylooligosaccharide supplementation decreases visceral fat accumulation and modulates cecum microbiome in mice. Journal of Functional Foods 52: 138-146.

Lotfy, W.A., 2007. The utilization of beet molasses as a novel carbon source for cephalosporin C production by Acremonium chrysogenum: optimization of process parameters through statistical experimental designs. Bioresour. Technol. 98, 3491–3498. https://doi.org/10.1016/j.biortech.2006.11.035.

Louis, P. and Flint, H.J. (2016) Formation of propionate and butyrate by the human colonic microbiota. Environ Microbiol

Malard, F.; Dore, J.; Gaugler, B.; Mohty, M. Introduction to host microbiome symbiosis in health and disease. Mucosal Immunol. 2020, 14, 547–554.

Marcobal, A., Barboza, M., Sonnenburg, E.D., Pudlo, N., Martens, E.C., Desai, P. et al. (2011) *Bacteroides* in the infant gut consume milk oligosaccharides via mucus-utilization pathways. Cell Host Microbe 10, 507–514 doi: 10.1016/j.chom.2011.10.007

Merino, S.T., Cherry, J. (2007). Progress and Challenges in Enzyme Development for Biomass Utilization. In: Olsson, L. (eds) Biofuels. Advances in Biochemical Engineering/Biotechnology, vol 108. Springer, Berlin, Heidelberg. https://doi.org/10.1007/10_2007_066.

Miller, G. L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical Chemistry, 31, 426–428.

Mitmesser, S., & Combs, M. (2017). Prebiotics: Inulin and other oligosaccharides. In *The microbiota in gastrointestinal pathophysiology* (pp. 201–208). Academic Press. https:// doi.org/10.1016/B978-0-12-804024-9.00023-9.

Mizrahi-Man, O., Davenport, E.R., Gilad, Y., and White, B.A. (2013) Taxonomic classification of bacterial 16S rRNA genes using short sequencing reads: evaluation of effective study designs. PLoS ONE 8, e53608 doi: 10.1371/journal.pone.0053608

Morowvat, M.H.; Nezafat, N.; Ghasemi, Y.; Zare, M.H.; Mohkam, M. Probiotic potential of five *lactobacillus* strains isolated from traditional persian yoghurt in fars province, iran: Viewing through the window of phylogenetics. Biosci. Biotechnol. Res. Asia 2015, 12, 1265–1272.

Moteshafi, H., Hashemi, M., Mousavi, S. M., & Mousivand, M. (2016). Characterization of produced xylanase by *Bacillus subtilis* D3d newly isolated from apricot phyllosphere and its potential in pre-digestion of BSG. Journal of Industrial and Engineering Chemistry, 37, 251–260.

Moura, P., Barata, R., Carvalheiro, F., Gírio, F., Loureiro-Dias, M.C., Esteves, M.P., 2007. *In vitro* fermentation of xylooligosaccharides from corn cobs autohydrolysis by *Bifidobacterium* and 18 *Lactobacillus* strains. LWT Food Sci. Technol. 40, 963–972. https://doi.org/10.1016/j.lwt.2006.07.013.

Moya, A. and Ferrer, M. (2016) Functional Redundancy-Induced stability of Gut microbiota subjected to disturbance. Trends Microbiol. 24, 402–413 doi: 10.1016/j.tim.2016.02.002.

Mussatto, S. I., Carneiro, L. M., Silva, J. P. A., Roberto, I. C., & Teixeira, J. A. (2011b). A study on chemical constituents and sugars extraction from spent coffee grounds. *Carbohydrate Polymers, 83*(2), 368–374.

Mutuyemungu, E., Singh, M., Liu, S., & Rose, D. J. (2023). Intestinal gas production by the gut microbiota: A review. *Journal of Functional Foods*, *100*. https://doi.org/10.1016/j.jff.2022.105367

Nakano, M., & Mario Hulett, F. (1997). Adaptation of *Bacillus subtilis* to oxygen limitation. *FEMS Microbiology Letters*, *157*(1), 1-7. https://doi.org/10.1111/j.1574-6968.1997.tb12744.x

Natividad, J.M.M. and Verdu, E.F. (2013) Modulation of intestinal barrier by intestinal microbiota: Pathological and therapeutic implications. Pharmacol. Res. 69, 42–51 doi: 10.1016/j.phrs.2012.10.007

Okazaki, M., Fujikawa, S., Matsumoto, N., 1990. Effect of xylooligosaccharide on the growth of bifidobacteria. Bifidobact. Microflora. 9, 77–86. https://doi.org/10. 12938/bifidus1982.9.2_77.

Oliveira, C. S. F., Pereira, H., Alves, S., Castro, L., Baltazar, F., Chaves, S. R., Côrte-Real, M., & Preto A. (2015). Cathepsin D protects colorectal cancer cells from acetate-induced apoptosis through autophagy-independent degradation of damaged mitochondria. Cell Death and Disease, 6, e1788.

Ottman, Noora & Smidt, Hauke & De Vos, Willem & Belzer, Clara. (2012). The function of our microbiota: Who is out there and what do they do? Frontiers in cellular and infection microbiology. 2. 104. 10.3389/fcimb.2012.00104.

Ouwerkerk, J.P., de Vos, W.M., Belzer, B. (2013) Glycobiome: Bacteria and mucus at the epithelial interface. Best Pract. Res. Clin. Gastroenterol. 27, 25–38 doi: 10.1016/j.bpg.2013.03.001

Paone, P., Suriano, F., Jian, C., Korpela, K., Delzenne, N. M., Van Hul, M., Salonen, A., & Cani, P. D. (2022). *Prebiotic oligofructose protects against high-fat diet-induced obesity by changing the gut microbiota, intestinal mucus production, glycosylation and secretion* (14th ed.). Gut Microbes. https://doi.org/10.1080/19490976.2022.2152307

Parod, R. J. (2014). *Encyclopedia of Toxicology: Ammonium* (3rd ed., pp. 206-208). Biomedical Sciences. https://doi.org/10.1016/B978-0-12-386454-3.00468-1

Pauff, S. M., & Miller, S. C. (2012). Methanogenic flora is associated with altered colonic transit but not stool characteristics in constipation without IBS. *Bone*, *78*(2), 711-716.

Pérez-Cobas, A.E., Gosalbes, M.J., Friedrichs, A., Knecht, H., Artacho, A., Eismann, K. et al. (2013) Gut microbiota disturbance during antibiotic therapy: a multi-omic approach. Gut 62, 1591–1601 doi:10.1136/gutjnl-2012-303184

Pham, V. T., Calatayud, M., Rotsaert, C., Seifert, N., Richard, N., Van den Abbeele, P., Marzorati, M., et al. (2021). Antioxidant Vitamins and Prebiotic FOS and XOS Differentially Shift Microbiota Composition and Function and Improve Intestinal Epithelial Barrier *In Vitro*. *Nutrients*, *13*(4), 1125. MDPI AG. Retrieved from http://dx.doi.org/10.3390/nu13041125.

Poletto, P., Pereira, G. N., Monteiro, C. R. M., Pereira, M. A. F., Bordignon, S. E., & de Oliveira, D. (2020). Xylooligosaccharides: Transforming the lignocellulosic biomasses into valuable 5-carbon sugar prebiotics. *Process Biochemistry, 91*, 352–363. https://doi.org/10.1016/j.procbio.2020.01.005.

Poppe, J., Silva, V. S., Raes, J., Verbeke K., Falony, G. (24 de julho de 2023). Sec. Systems Microbiology: Systematic optimization of fermentation conditions for *in vitro* fermentations with fecal inocula, volume 14, doi: 10.3389/fmicb.2023.1198903

Poretsky, R., Rodriguez-R, L.M., Luo, C., Tsementzi, D., Konstantinidis, K.T., Rodriguez-Valera, F. (2014) Strengths and limitations of 16S rRNA gene amplicon sequencing in revealing temporal microbial community dynamics. PLoS ONE 9, e93827 doi: 10.1371/journal.pone.0093827

Pua, F., Subari, M. S., Ean, L., & Krishnan, S. G. (2020). Characterization of biomass fuel pellets made from Malaysia tea waste and oil palm empty fruit bunch. *Materials Today: Proceedings*, *31*, 187-190. https://doi.org/10.1016/j.matpr.2020.02.218.

Rakoff-Nahoum, S., Foster, K.R. and Comstock, L.E. (2016) The evolution of cooperation within the gut microbiota. Nature 533, 255–259 doi:10.1038/ nature17626

Rana, J., & Paul, J. (2017). Consumer behavior and purchase intention for organic food: A review and research agenda. *Journal of Retailing and Consumer Services*, *38*, 157–165.

Ridlon, J.M., Kang, D.J., Hylemon, P.B., Bajaj, J.S. (2014) Bile acids and the gut microbiome. Curr Opin Gastroenterol 30, 332–338 doi:10.1097/MOG.0000000000000057

Roberfroid, M., Gibson, G. R., Hoyles, L., McCartney, A. L., Rastall, R., Rowland, I., Wolvers, D., Watzl, B., Szajewska, H., Stahl, B., Guarner, F., Respondek, F., Whelan, K., Coxam, V., Davicco, M. J., Léotoing, L., Wittrant, Y., Delzenne, N. M., Cani, P. D., Neyrinck, A. M., & Meheust, A. (2010). Prebiotic effects: metabolic and health benefits. British Journal of Nutrition, 104 (Suppl. 2), S1–S63.

Roberto de Paula do Nascimento, M. R. (2021). Chapter 2 - Emerging Prebiotics: Nutritional and Technological Considerations. pp. 13-46.

Rodriguez, J.M. et al. (2015) The composition of the gut microbiota throughout life, with an emphasis on early life. Microb. Ecol. Health Dis. 26, 26050.

Sajib, M., Falck, P., Sardari, R. R. R., Mathew, S., Grey, C., Karlsson, E. N., & Adlercreutz, P. (2018). Valorization of Brewer's spent grain to prebiotic oligosaccharide: Production, xylanase catalyzed hydrolysis, in-vitro evaluation with probiotic strains and in a batch human fecal fermentation model. Journal of Biotechnology, 268, 61-70.

Salminen, S. (2004) Influence of mode of delivery on gut microbiota composition in seven-year-old children. Gut 53, 1388–1389 doi:10.1136/gut.2004.041640

Salvador, A. F., Cavaleiro, A. J., Paulo, A. M. S., Silva, S. A., Guedes, A. P., Pereira, M. A., et al. (2019). Inhibition studies with 2-bromoethanesulfonate reveal a novel syntrophic relationship in anaerobic oleate degradation. Applied and Environmental Microbiology, 85, e01733–18.

Samanta, A.K., Jayapal, N., Jayaram, C., Roy, S., Kolte, A.P., Senani, S., Sridhar, M., 2015. Xylooligosaccharides as prebiotics from agricultural by-products: production and applications. Bioact. Carbohydr. Diet. Fibre. 5, 62–71. https://doi.org/10.1016/j. bcdf.2014.12.003.

Saville, B. A. and Saville, S. 2018. Xylooligosaccharides and arabinoxylanoligosaccharides and their application as prebiotics. Applied Food Biotechnology 5(3): 121-130.

Scanlan, P.D., Shanahan, F. & Marchesi, J.R. Human methanogen diversity and incidence in healthy and diseased colonic groups using *mcrA* gene analysis. *BMC Microbiol* 8, 79 (2008). https://doi.org/10.1186/1471-2180-8-79

Schluter, J., Foster, K.R., Ellner, S.P. (2012) The evolution of mutualism in gut microbiota via host epithelial selection. PLoS Biol. 10, e1001424 doi:10. 1371/journal.pbio.1001424

Schwarzer, M., Makki, K., Storelli, G., Machuca-Gayet, I., Srutkova, D., Hermanova, P. et al. (2016) *Lactobacillus plantarum* strain maintains growth of infant mice during chronic undernutrition. Science 351, 854–857 doi:10.1126/science. aad8588

Setter, C., Borges, F., Cardoso, C., Mendes, R., & Oliveira, T. (2020). Energy quality of pellets produced from coffee residue: Characterization of the products obtained via slow pyrolysis. *Industrial Crops and Products*, *154*, 112731. https://doi.org/10.1016/j.indcrop.2020.112731

Shokri, D.; Khorasgani, M.R.; Mohkam, M.; Fatemi, S.M.; Ghasemi, Y.; Taheri-Kafrani, A. The inhibition effect of lactobacilli against growth and biofilm formation of pseudomonas aeruginosa. Probiot. Antimicrob. Proteins 2018, 10, 34–42.

Slavin, J. (2013). Fiber and prebiotics: Mechanisms and health benefits. *Nutrients, 5*, 1417–1435. https://doi.org/10.3390/nu5041417

Somerville, C., Youngs, H., Taylor, C., Davis, S.C., Long, S.P., 2010. Feedstocks for lignocellulosic biofuels. Science 329, 790–792. https://doi.org/10.1126/science. 1189268.

Sonnenburg, E.D. and Sonnenburg, J.L. (2014) Starving our microbial self: the deleterious consequences of a diet deficient in microbiota-accessible carbohydrates. Cell Metab. 20, 779–786 doi: 10.1016/j.cmet.2014.07.003

Staley, C., Weingarden, A.R., Khoruts, A., Sadowsky, M.J. (2017) Interaction of gut microbiota with bile acid metabolism and its influence on disease states. Appl Microbiol Biotechnol 101, 47–64 doi:10.1007/s00253-016-8006-6

Sukri, S.S.M., Sakinah, A.M.M., 2018. Production of high commercial value xylooligosaccharides from Meranti wood sawdust using immobilised xylanase. Appl. Biochem. Biotechnol. 184, 278290. https://doi.org/10.1007/s12010-017-2542-0.

Surek, E., & Buyukkileci, A. O. (2017). Production of xylooligosaccharides by autohydrolysis of hazelnut (*Corylus avellana* L.) shell. *Carbohydrate Polymers, 174*, 565–571. https://doi.org/10.1016/j.carbpol.2017.06.109

Swanson, K. S., Gibson, G. R., Hutkins, R., Reimer, R. A., Reid, G., Verbeke, K., Scott, K. P., Holscher, H. D., Azad, M. B., Delzenne, N. M., & Sanders, M. E. (2020). The international scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of synbiotics. Nature Reviews Gastroenterology and Hepatology, 17, 687–701. https:// doi.org/10.1038/s41575-020-0344-2

Thursby, E.; Juge, N. Introduction to the human gut microbiota. Biochem. J. 2017, 474, 1823–1836.

Topping, D. L., & Clifton, P. M. (2001). Short-chain fatty acids and human colonic function: roles of resistant starch and nonstarch polysaccharides. Physiological Reviews, 81, 1031–1064.
Turnbaugh, P. J., Hamady, M., Yatsunenko, T., Cantarel, B. L., Duncan, A., Ley, R. E., ... and Gordon, J. I. 2009. A core gut microbiome in obese and lean twins. Nature 457: 480-484.

Turroni, F.; Ventura, M.; Buttó, L.F.; Duranti, S.; O'Toole, P.W.; Motherway, M.O.C.; van Sinderen, D. Molecular dialogue between the human gut microbiota and the host: A *lactobacillus* and *bifidobacterium* perspective. Cell. Mol. Life Sci. 2014, 71, 183–203.

Urmann, M., Graalfs, H., Joehnck, M., Jacob, L. R., & Frech, C. (2010). Cation-exchange chromatography of monoclonal antibodies: characterization of a novel stationary phase designed for production-scale purification. MAbs, 2, 395–404.

Venema, K., & van den Abbeele, P. (2013). Experimental models of the gut microbiome. Bes Practice and Research Clinical Gastroenterology, 27, 115-126. doi: 10.1016/j. bpg.2013.03.002

Vrancken, G., Gregory, A. C., Huys, G. R. B., Faust, K., and Raes, J. (2019). Synthetic ecology of the human gut microbiota. Nat. Rev. Microbiol. 17, 754–763. doi: 10.1038/ s41579-019-0264-8

Wang, S., Zhang, P. Kong, X., Xie, S., Li, Q., Li, Z., & Zhou, Z. (2017). Delicate changes of bioapatite mineral in pig femur with addition of dietary xylooligosaccharide: Evidences from Raman spectroscopy and ICP. Animal Science Journal, 88,1820-1826.

Wang, W., Chen, L., Zhou, R., Wang, X., Song, L., Huang, S., et al. (2014). Increased proportions of *Bifidobacterium* and the *Lactobacillus* group and loss of butyrate-producing bacteria in Inflammatory Bowel. Journal of Clinical Microbiology, 52, 398–406.

Waqas, A., & Hong, C. (2019). *Study on consumer behaviour and food safety of organic products in Pakistan* [Paper presentation]. The E3S Web of Conferences.

Yang J., Summanen, P. H., Henning, S. M., Hsu, M., Lam, H., Huang, J., Tseng, C.-H., Dowd S. E., Finegold, S. M., Heber, D., & Li, Z. (2015). Xylooligosaccharide supplementation alters gut bacteria in both healthy and prediabetic adults: a pilot study. Frontiers in Physiology, 6, 216.

Yu, Z.T., Chen, C., Kling, D.E., Liu, B., McCoy, J.M., Merighi, M. et al. (2013) The principal fucosylated oligosaccharides of human milk exhibit prebiotic properties on cultured infant microbiota. Glycobiology 23, 169–177 doi:10.1093/glycob/cws138

Zarepour, M., Bhullar, K., Montero, M., Ma, C., Huang, T., Velcich, A. et al. (2013) The mucin MUC2 limits pathogen burdens and epithelial barrier dysfunction during salmonella enterica serovar typhimurium colitis. Infect Immun. 81, 3672–3683 doi:10.1128/IAI.00854-13.

Zhang, B., Hao, G., Cao, H., Tang, H., Zhang, Y. and Deng, S. 2018. The cryoprotectant effect of xylooligosaccharides on denaturation of peeled shrimp (*Litopenaeus vannamei*) protein during frozen storage. Food Hydrocolloids 77: 228-237.

Zhang, C., Abdo, A. A. A., Kaddour, B., Wu, Q., Xin, L., Li, X., Teng, C., et al., 2020. Xylan-oligosaccharides ameliorate high fat diet induced obesity and glucose intolerance and modulate plasma lipid profile and gut microbiota in mice. Journal of Functional Foods 64: article ID 103622.

Zhao, Y., Liu, J., Hao, W., Zhu, H., Liang, N., He, Z., ... and Chen, Z.-Y. 2017. Structure-specific effects of short-chain fatty acids on plasma cholesterol concentration in male Syrian hamsters. Journal of Agricultural and Food Chemistry 65(50): 10984-10992.

Zhou, Z.; Zhang, Y.; Zheng, P.; Chen, X.; Yang, Y. Starch structure modulates metabolic activity and gut microbiota profile. Anaerobe 2013, 24, 71–78. [CrossRef] [PubMed]

Zhu, D., Yan, Q., Liu, J., Wu, X. and Jiang, Z. 2019. Can functional oligosaccharides reduce the risk of diabetes mellitus? The FASEB Journal 33(11): 11655-11667.

Zilong Guo, Yanshang Wei, Yu Zhang, Yinxiao Xu, Ling Zheng, Benwei Zhu, Zhong Yao, Carrageenan oligosaccharides: A comprehensive review of preparation, isolation, purification, structure, biological activities and applications, Algal Research, Volume 61, 2022, 102593, ISSN 2211-9264, https://doi.org/10.1016/j.algal.2021.102593.

APPENDIX

This section includes all the additional results not shown in the section of Results and Discussion. The appendix results show chromatograms obtained from HPLC. The samples used were result of the direct fermentation of coffee silver skin (CSS) and coffee silver skin pellet (CP) at 20 $g.L^{-1}$, pH 7.0, 45 °C using *B. subtilis_xyn2* at the initial time and the optimal time (8 h). Additionally, the comparation between sugar production yield (Y_{RS}) obtained from enzymatic hydrolysis of CP by *B. subtilis_xyn2* using different sterilization methods: autoclave (CP_enz_A) and UV treatment (CP_enz_UV), under 45 °C, pH 7.0, 20 $g.L^{-1}$ of CP in 2 % (w/v) Vogel media and 150 rpm.

1. Direct fermentation for agro-residue selection

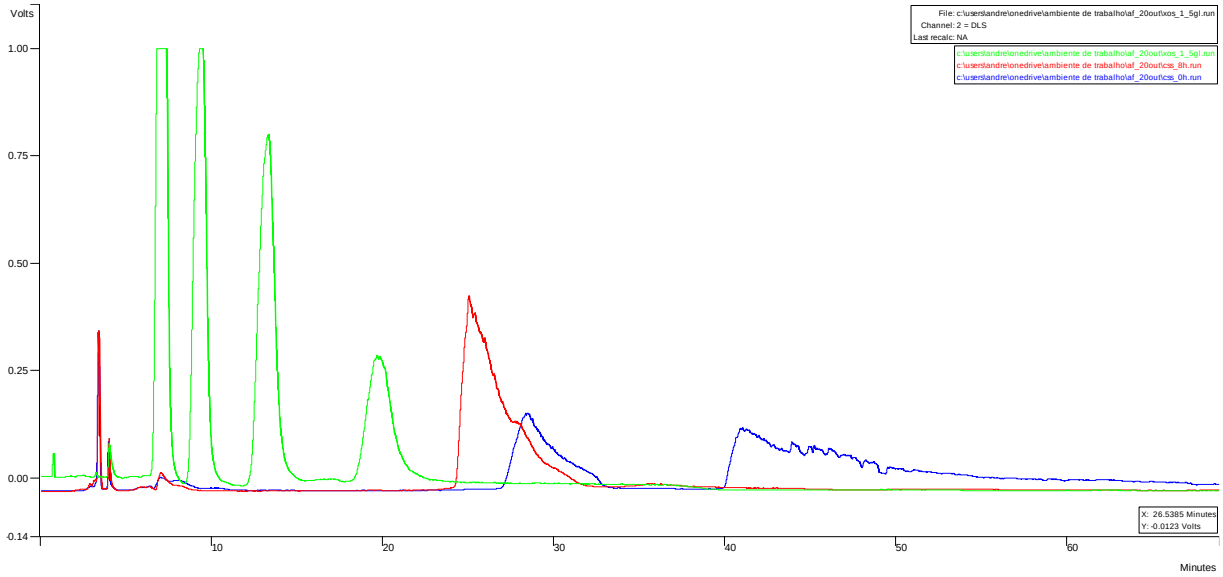

Figure 8- Chromatograms obtained through the quantification of monosaccharides by HPLC for the samples of coffee silver skin (CSS) resulting from direct fermentation using *Bacillus subtilis_xyn2* (CSS at 20 $g.L^{-1}$, pH 7.0, 45 °C and 150 rpm). Green corresponds to the XOS standard at 1.5 g. L^{-1}, blue and red correspond to the CSS samples at 0 and 8 h, respectively.

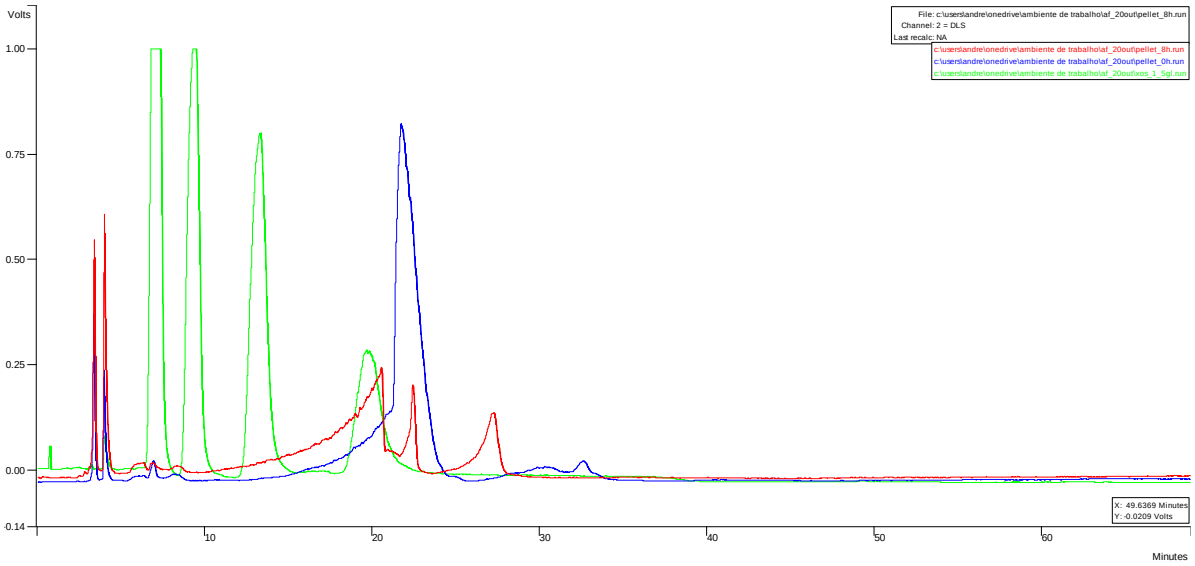

Figure 9- Chromatograms obtained through the quantification of oligosaccharides by HPLC for the samples of coffee silver skin pellet (CP) resulting from direct fermentation using *Bacillus subtilis_xyn2* (CP at 20 g.L^{-1}, pH 7.0, 45 °C and 150 rpm). Green corresponds to the XOS standard at 1.5 g. L^{-1}, blue and red correspond to the CP samples at 0 and 8 h, respectively.

1.1. Direct fermentation versus enzymatic hydrolysis

1.1.1. Sugar analysis

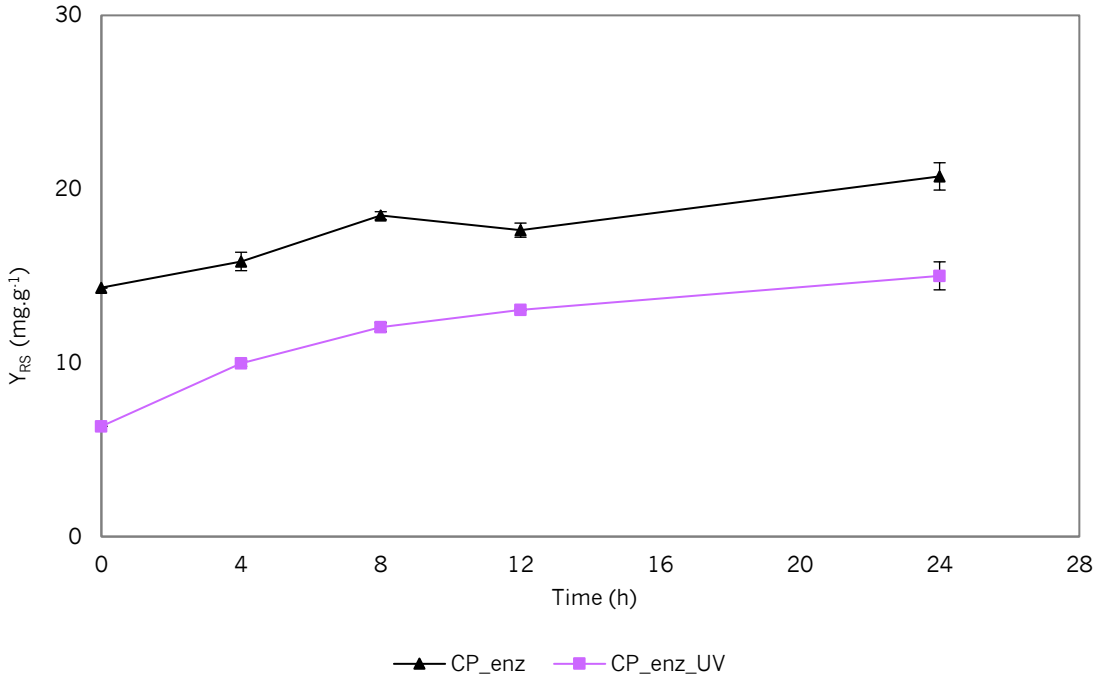

Figure 13- Sugar production yield (Y_{RS}) obtained from enzymatic hydrolysis of coffee silver skin pellet (CP) by *Bacillus subtilis_xyn2* using different sterilization methods: autoclave (CP_enz_A) and UV treatment (CP_enz_UV). The fermentation assay was performed at 45 °C and 150 rpm with 20 g.L^{-1} of CP in 2% (w/v) Vogel media, pH 7.0. Results represent the average of Y_{RS} for the different assays ± SD.